Impact of HPLT Treatments on Micellar Caseins and Whey Proteins

vorgelegt von
Dipl.-Ing.
Daniel Baier
aus Berlin

von der Fakultät III - Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades

Doktor der Ingenieurwissenschaften
- Dr.-Ing -

genehmigte Dissertation

Promotionsausschuss
Vorsitzende: Prof. Dr. Dipl.-Ing. habil. Cornelia Rauh
Gutachter: Prof. Dr. Dipl.-Ing. Dietrich Knorr
Gutachter: Dr. Christophe Schmitt
Gutachter: Prof. Dr. Alain le Bail

Tag der wissenschaftlichen Aussprache: 11. Juli 2014

Berlin 2014
D 83

Abstract

High pressure - low temperature treatments (HPLT) combine the effects of high pressures with low temperature and freezing effects. The aim of this study is to investigate the impact of HPLT on major milk protein fractions and to identify the potential of this technology for modification of these frequently used proteins.

Pressure assisted freezing (PAF, -35 °C) and pressure shift freezing (PSF, -15 °C) were applied from 100-600 MPa to identify the most promising process parameters for protein modification. High pressure treatments at room temperature (HP) were performed as reference values for the impact of pressure. Milk protein dispersions (whey protein isolate - WPI; micellar casein - MC; 80:20 MC:WPI and 20:80 MC:WPI weight based mixtures) were treated at pH 7.0 and pH 5.8 at a concentration of 2 % (w/w). Colloidal stability, structural changes and modification in techno-functional properties were used as an indicator for protein modification on different molecular levels.

HPLT induced specific fibrous flocks in samples with 80 and 100 % MC. These flocks showed a shear thickening effect and represent a promising HPLT specific functional property. Especially PSF treatments induced new secondary structure motifs (β-sheet) from random coils in WPI containing samples. Those structuring effects could not be observed for HP treatments. These changes can be influenced by altering the sample composition or the treatment pH. Furthermore, slight increases in foam stability were detected especially for pure WPI solutions after HPLT as well as after HP treatments, while emulsification properties remained almost unchanged.

The results of the present study indicate a specific modification of milk proteins via HPLT treatments which is promising for new food applications.

Kurzfassung

Hochdruck-Tieftemperaturbehandlungen (HPLT) vereinen die Wirkungen hoher Drücke mit denen niedriger Temperaturen und Gefriereffekten. Ziel dieser Untersuchungen ist es den Einfluss von HPLT auf die Hauptproteinfraktionen der Milch zu ermitteln und das Potential dieser Technologie für die Modifizierung dieser häufig eingesetzten Proteine zu identifizieren.

Druckunterstütztes Gefrieren (PAF, -35 °C) und Druckwechselgefrieren (PSF, -15 °C) wurden bei Drücken von 100-600 MPa angewendet, um die vielversprechendsten Prozessparameter zu identifizieren. Hochdruckversuche bei Raumtemperatur (HP) wurden als Referenzversuche für den Einfluss des Druckes durchgeführt. Milchproteindispersionen (Molkenproteinisolat - WPI; mizelläres Casein - MC und 80:20 MC:WPI und 20:80 MC:WPI gewichtsbasierte Mischungen) wurden bei pH 7.0 und pH 5.8 und einer Konzentration von 2 % (w/w) behandelt. Die kolloidale Stabilität, strukturelle Änderungen und Modifizierungen der techno-funktionellen Eigenschaften wurden als Indikator für Proteinmodifikationen auf verschiedenen molekularen Ebenen verwendet.

Es wurden fibrilläre Flocken durch HPLT in Proben erzeugt, welche 80 oder 100 % MC enthielten. Diese Flocken zeigten scherverdickendes Verhalten und stellen eine aussichtsreiche HPLT-spezifische funktionelle Besonderheit dar. Neue Sekundärstrukturmotive (β-Faltblatt) konnten in WPI-haltigen Proben aus ungeordneten Strukturen, insbesondere durch PSF, erzeugt werden. Solche Strukturierungseffekte konnten bei HP-Behandlungen nicht festgestellt werden. Diese Änderungen können durch Variation der Probenzusammensetzung oder des Behandlungs-pH-Wertes beeinflusst werden. Weiterhin wurde insbesondere für reine WPI-Lösungen ein geringer Anstieg der Schaumstabilität nach HPLT- und HP-Behandlungen festgestellt, während die Emulgiereigenschaften weitestgehend unbeeinflusst blieben.

Die Ergebnisse der vorliegenden Untersuchungen zeigen die spezifische Modifikation von Milchproteinen durch HPLT-Behandlung auf, welche vielversprechend für neue Lebensmittelanwendungen ist.

Acknowledgements

This work would not have been possible without the support of a couple of people to whom I would like to express my grateful thanks.

First of all, I would like to thank Prof. Dr. Dipl.-Ing. Dietrich Knorr for giving me the chance to work in the field of HPLT processing, for his tremendous support in all scientific and administrative cases, and for his guidance throughout the whole period of my dissertation.

Very special thanks go to Dr. Christophe Schmitt for supporting me in every concern, for sharing his extensive knowledge on analytic and proteins, for the kind and fruitful working atmosphere within our project and last but not least, for his endless appreciation for all administrative problems.

A further special thanks goes to Prof. Alain le Bail for bringing in his tremendous knowledge on HPLT processing of proteins as a member of my thesis committee.

Furthermore, I would like to thank Prof. Dr. Ing. habil. Cornelia Rauh for being the chair of my defense committee and for giving me much room to realize my ideas.

This theses would also not have been possible without the financial support of the Nestlé Research Center in Lausanne and the Product Technology Center Beauvais. Thank you for trusting in the potential of an emerging technology. A special thanks goes to Dr. Max Puaud, Sigrid Chadjichristos and Lucille Lepagnol for fruitful discussions during the project meetings and for taking care of the industrial relevance. I also want to express my grateful thanks to Doreen Schiller, Benedict Purschke and Martha Maaßen who significantly contributed to my thesis.

I am looking back to a couple of years with many interesting research matters but also with a lot of fun within the Department of Food Biotechnology and Food Process Engineering. Therefore, I have to express my gratitude to all my colleagues who made our Department to the best working place on earth! Special thanks goes to Sophie and Amy Uhlig for their administrative support and to Irene Hemmerich, Stefan Boguslawski and Martin Bunzeit for their technical assistance.

I would also like to take the chance to thank Dr.-Ing. Alexander Mathys and Dr.-Ing. Kai Reineke for many scientific discussions and for keeping me a part of the spores group although I went over to the proteins. Furthermore, I would like to thank Dr.-Ing. Marcus Volkert for keeping in touch after leaving the university and still being a contact person for every concern.

A particular and indescribable thanks goes to Anne Heckelmann for her love, her endless patience in all but especially in difficult situations and last but not least for giving me the chance to start a small family. I also have to thank her for contributing to my thesis in all working fields, for giving me scientific input, for helping me to develop analytical methods and perform experiments, for discussing all the scientific and administrative matters and for proof reading the document. I hope that I will be able to return your efforts somehow.

I would also like to thank my brother, Thomas Baier, for proof reading the thesis with a complete different point of view.

Ich wäre nicht, was ich heute bin, wenn ich nicht mein Leben lang auf die Unterstützung meiner Eltern hätte zählen können. Ich danke euch für all eure Bemühungen mir eine herausragende Ausbildung zugute kommen zu lassen und für euer Verständnis in allen Situationen meines Lebens von ganzem Herzen.

Table of Contents

List of Figures

List of Tables

List of Annexes

List of Abbreviations and Symbols

Abbreviation	Translation
HP	High pressure
HPLT	High pressure - low temperature
CD	Circular dichroism
Cn	Casein
CSIC	Consejo Superior de Investigaciones Cientificas
ENITIAA	Ecole Nationale d'Ingénieurs des Techniques des Industries Agro-Alimentaires
ICE	Labeling of ice crystal structures
KULeuven	Katholieke Universiteit Leuven
Lac	Lactalbumin
Lg	Lactoglobulin
MC	Micellar casein
PAF	Pressure assisted freezing
PAT	Pressure assisted thawing
PIF	Pressure induced freezing
PIT	Pressure induced thawing
PSF	Pressure shift freezing
SbC	Sub-zero cooling
TUB	Technische Universität Berlin (Berlin Institute of Technology)
UM2	Université Montpellier 2
UNIPRESS	Institute of High Pressure Physics of the Polish Academy of Sciences
URL	Unilever Research Laboratories
US	United States
VTT	VTT Biotechnology (Valtion teknillinen tutkimuskeskus)
WPI	Whey protein isolate

Symbol	Translation	SI·unit
C_p	Heat capacity	$kJ \cdot kg^{-1} \cdot K^{-1}$
d	Diameter	m
G	Gibbs (free) energy	J
g	Gravitational acceleration	$m \cdot s^{-2}$
i	Index of a component	-
l	Index for liquid state	-
n	Total amount of a substance	mol
p	Pressure	MPa
S	Entropy	$J \cdot kg^{-1}$
s	Index for solid State	-
T	Temperature	°C or K
U	Internal energy	J
V	Volume	m^3
v	Velocity	$m \cdot s^{-1}$
α	Thermal expansion	K^{-1}
β	Compressibility factor	MPa^{-1}
η	Dynamic viscosity	Pa·s
μ	Chemical potential	$J \cdot mol^{-1}$
ρ	Density	$kg \cdot m^{-3}$

1. Introduction and Literature Review

The application of high isostatic pressure on food systems is of increasing interest since the last two decades. However, the main interest in the field of food technology is still the preservation by use of high pressure at ambient or elevated temperature. These high pressure processes can increase the shelf life of food by inactivation of for instance pathogenic or spoilage causing microorganisms and quality affecting enzymes. The number of implemented industrial high pressure units is still growing and reached a value of 222 in the middle of 2013 (see Figure 1). Furthermore, it can be seen from Figure 1 that more than half of the units are implemented in America while a quarter of the units is installed in Europe. The main application fields for high pressure preservation are vegetable and meat products. Guacamole is one of the best known high pressure preserved products but also wet salads or vegetable ready-to-eat meals are available on the market. However, in the case of vegetables high pressure is often used to inactivate enzymes to preserve the freshness of a product. This is also the aim when beverages like smoothies are high

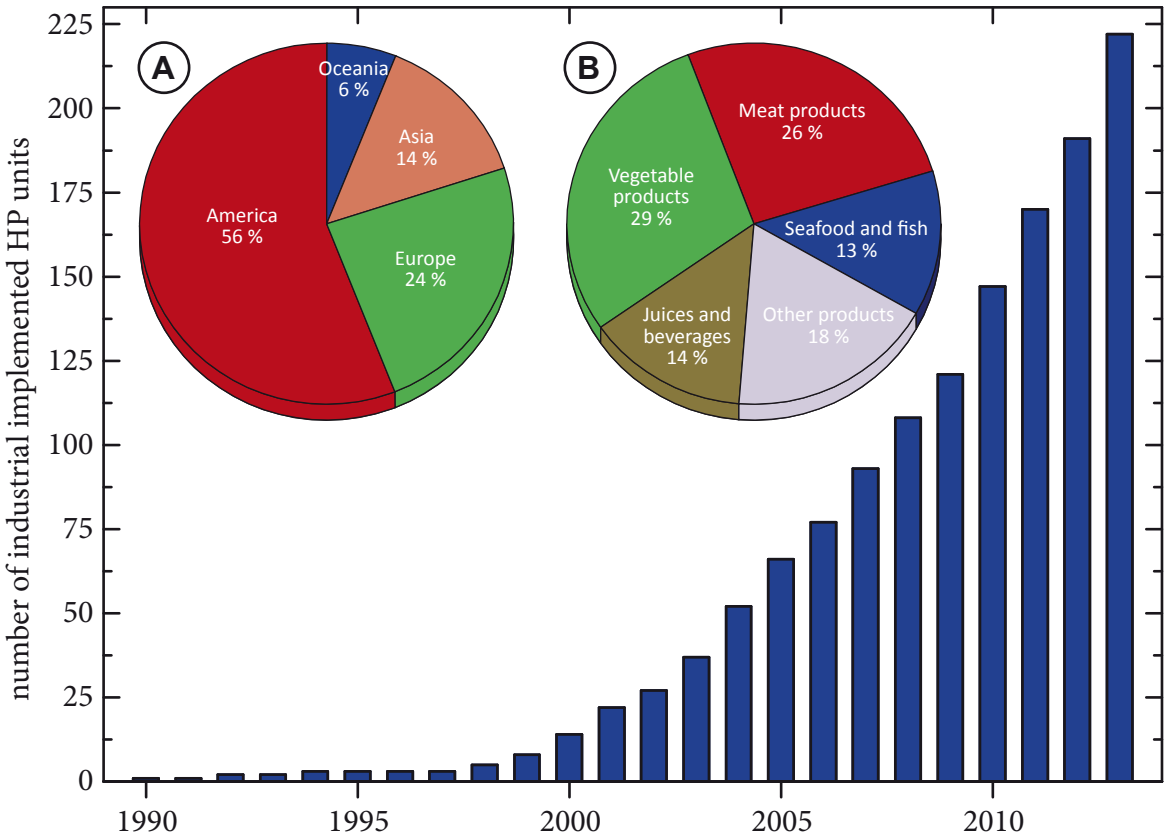


Figure 1: Number of industrial implemented high pressure units (not included: 15 dismantled units). A: Global distribution of implemented high pressure units. B: Spread of industrial applications of implemented high pressure units (Data kindly provided by C. Tonello Samson, Hiperbaric S.A., 2013).

pressure treated. In the case of meat preservation the main target is the inactivation of pathogenic microorganisms. Especially, species of *Listeria* which have a zero tolerance in the US are known to be pressure sensitive and, thus pressure enables the production of for instance *Listeria*-free sliced smoked (raw) ham. Nevertheless, high pressure is already used to modify food properties. There are several known applications like shucking of oysters or lobster meat extraction. Especially, the shucking of oysters can be seen as a protein influencing process as it opens the oyster or other mussels by relaxing a muscle. A more obvious protein influencing application is the usage of high pressure in cheese production to reduce the time for rennet coagulation. However, high pressure will stay a niche technology until industrial demands for continuous equipment can be fulfilled and clear statements about maintenance charges and especially about life time of vessels are available. Nevertheless, high pressure offers a third variable beside time and temperature which enables new process structures and thus, the option of creating products with new textural and functional properties. The investigation of pressure effects on proteins is also a concern of marine biology as microorganisms were found to reproduce in the deepest point of the oceans – the Mariana trench – where the hydrostatic pressure is 110 MPa (see Figure 2). However, pressure is also relevant for continental sub-surfaces. In general the pressure increase can be estimated by 10 MPa per km for hydrostatic pressure, ~15 MPa per km in sediments and ~28 MPa per km in continental and oceanic rocks (Hantschel & Kauerauf, 2009). However, these environments represent the largest habitats for microbial life (Edwards et al., 2012). It is often supposed that microorganisms are able to adopt to extreme conditions and thus, enables them to occupy most sub-oceanic and sub-continental regions (Picard & Daniel, 2013). It is obvious that this adoption has to include protein modifications for instance to create pressure resistant enzymes and to preserve the function of carrier proteins. However, a link between the genetically caused modification of proteins in microorganisms and a pressure induced protein modification cannot be found due to the different origins. Nevertheless, the influence of the amino acid composition and the suggested existence of pressure resistant and sensitive amino acids on protein behavior under pressure are valid for both, microbial proteins and other proteins, for instance, food proteins.

Since the work of Bridgman (1912) it is well known that the solid phase of water, generally called ice, possesses different structures in dependence on the applied pressure. The combination of high pressure and subzero temperatures (high pressure - low temperature – HPLT) enables different

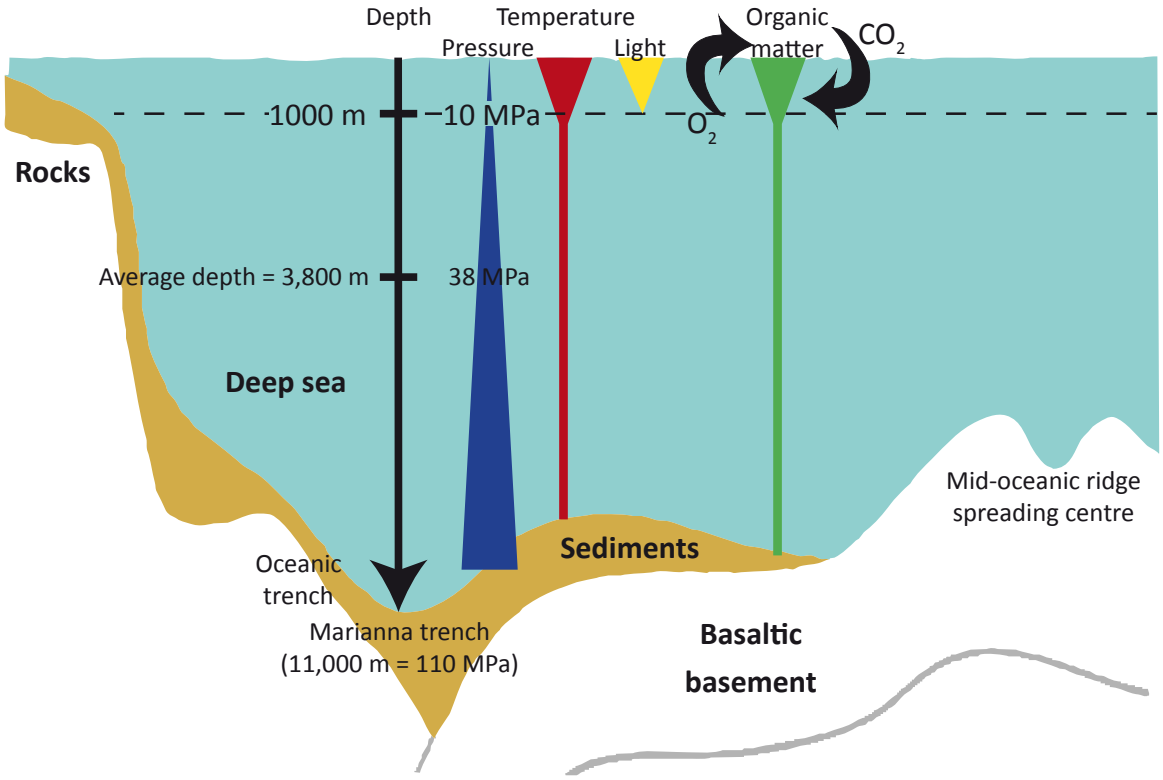
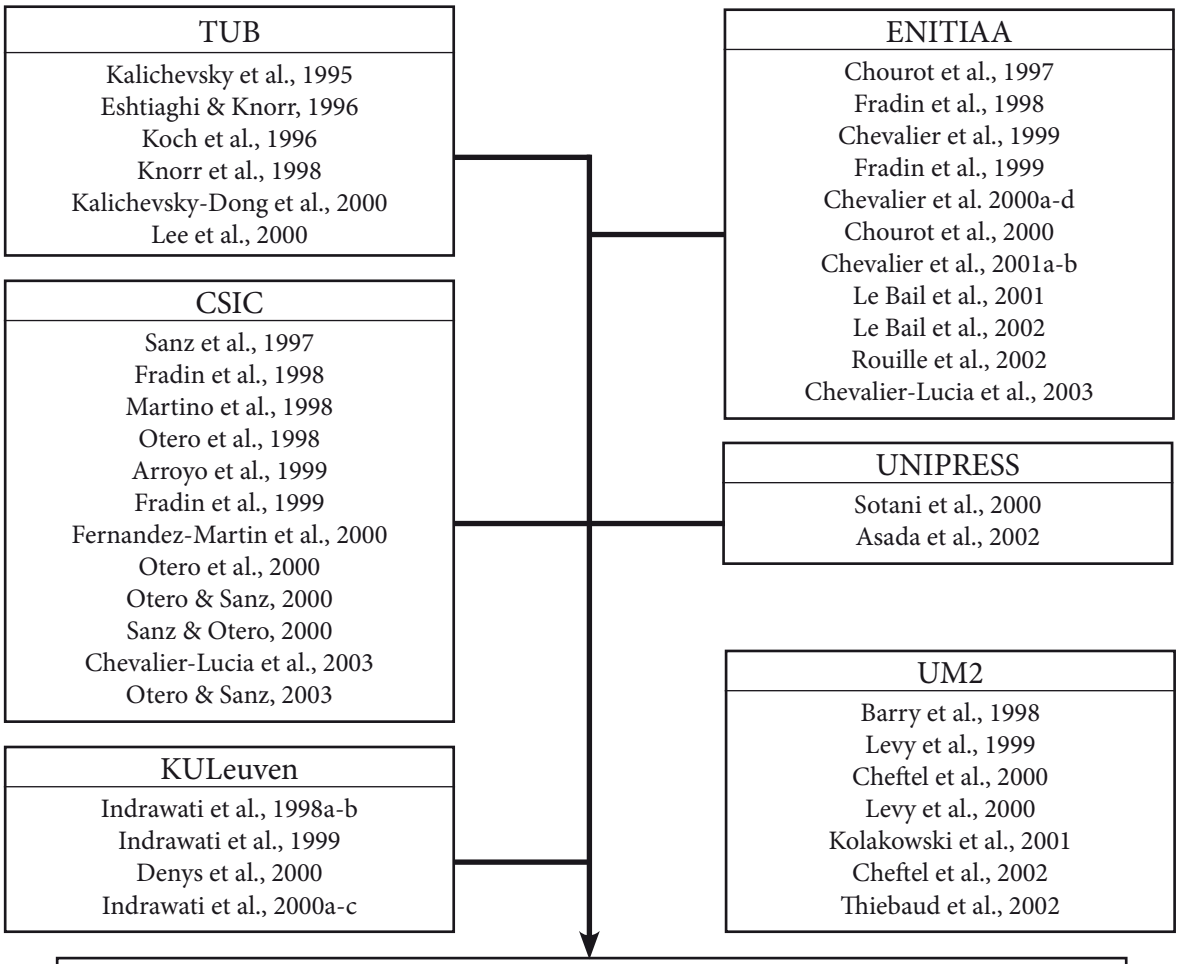


Figure 2: Illustration of pressure, temperature light and organic matter changes in deep sea regions (re-drawn from Picard & Daniel, 2013).

opportunities for phase transitions or treatments below zero degree Celsius. In conclusion, the combination of high pressures and subzero temperatures offers a wide range of process options. The amount of available data on HPLT induced changes in structure and function of relevant food proteins is still quite low. This fact can mainly traced back to a lack of HPLT units with an adequate vessel volume. A HPLT unit with a vessel volume of about 300 mL was constructed within present project of this thesis. The aim was to point out the effects of HPLT treatments on the structure and functionality of the most frequently used food proteins – the bovine milk proteins. High pressure treatments at room temperature were used as reference treatments to point out the differences between common high pressure treatments and HPLT treatments. Finally, conclusions on application fields for HPLT treatments of bovine milk proteins are drawn. The first chapter of this thesis highlights the basics of high pressure effects on water-ice-phase transitions as a general basis for HPLT treatments. It gives a general overview on modifications of proteins with regards to structural and functional properties of bovine milk proteins.

1.1 History and Development of HPLT Focused Research in European Food Science and Technology

Inspired by the publication of Haas et al. (1972) the Department of Food Biotechnology and Food Process Engineering (Berlin Institute of Technology, TUB) started in cooperation with Unilever Research to screen possible applications for HPLT processes in food science and technology. Kalichevsky et al. (1995) summarized the existing publications and reported possible applications based on the mentioned cooperation. In consequence, an increased research activity in several European research institutions could be observed. Figure 3 shows an overview of research activities in the late 1990s up to the beginning of the 21st century in Europe. A consortium of seven institutions including the TUB (Germany), VTT Biotechnology (VTT, Finland), Consejo Superior de Investigaciones Cientificas (CSIC, Spain), Katholieke Universiteit Leuven (KULeuven, Belgium), Ecole Nationale d'Ingénieurs des Techniques des Industries Agro-Alimentaires (ENITIAA, France), Unilever (URL, Netherlands) and High Pressure Research Center (UNIPRESS, Poland) was formed and the first international HPLT project was started. The project was entitled "Low temperature-pressure processing of foods: Safety and quality aspects, process parameters and consumer acceptance" (SAFE ICE). It is obvious from Figure 3 that a huge number of publications was created from this project which still build a base for current research. Beside the consortium one institution should be mentioned which was also working in the field of HPLT treatments with special focus on proteins - Université Montpellier 2 (UM2, France). This institution was more focused on the investigation of pure protein systems while the SAFE ICE consortium concentrated on process development and effects on real food systems. Most of the institutes were still active in the field of HPLT after the SAFE ICE project, but however, the research activities decreased. Current research activities are shown in Figure 4. Nevertheless, a commercial frozen dessert was HPLT treated at the TUB in a trial and showed surprising sensorial properties. This finding led to the first cooperation of TUB with Nestlé PTC Beauvais and ended up in a patent and the first publication on this topic (Puaud et al., 2007-2013; Volkert et al., 2012). Both project partners decided that the special effects of HPLT treatments on sugar rich dairy based frozen food foams and emulsions should be investigated more detailed and that the responsible components have to be identified. Therefore, it was decided to take micellar casein and whey protein isolate as the most promising techno-



SAFE ICE	
TUB	Luscher et al., 2004; Luscher & Knorr, 2004; 2004; Schlüter et al., 2004; Urrutia Benet et al., 2004; Luscher et al., 2005; Shen et al., 2005; Benet et al., 2006; Urrutia-Benet et al., 2007; Urrutia et al., 2007
CSCI	Molina-Garcia et al., 2004; Prestamo et al., 2004; Abascal et al., 2005; Guignon et al., 2005; Prestamo et al., 2005; Sanz, 2005; Fernandez et al., 2006a; Fernandez et al., 2006b; Guignon et al., 2006; Otero et al., 2006; Otero & Sanz, 2006; Sanz et al., 2006; Fernandez et al., 2007; Otero et al., 2007; Urrutia et al., 2007
KULeuven	Van Buggenhout et al., 2004; Van Buggenhout et al., 2005a; Van Buggenhout et al., 2005b; Van Buggenhout et al., 2006a; Van Buggenhout et al., 2006b; Van Buggenhout et al., 2006c; Araya et al., 2007; Castro et al., 2007; Van Buggenhout et al., 2007
ENITIAA	Zhu et al., 2004a; Zhu et al., 2004b; Zhu et al., 2004c; Zhu et al., 2004d; Alizadeh et al., 2005; Sequeira-Munoz et al., 2005; Zhu et al., 2005a; Zhu et al., 2005b; Ramaswamy et al., 2006; Zhu et al., 2006a; Zhu et al., 2006b; Alizadeh et al., 2007; Otero et al., 2007; Tironi et al., 2007
VTT	Lille & Autio, 2007
URL	Industrial partner
UNIPRESS	Industrial partner

Figure 3: Consortium and contributors for the European project SAFE ICE and additional European institutes with HPLT research activities. Publications are related to the affiliation of the primary author if more than one affiliation is engaged.

TUB	Puaud et al., 2007-2013; Volkert et al., 2012; Eichhorn et al., 2013
CSCI	Fernandez et al., 2008; Guignon et al., 2008; Otero et al., 2012; Vaudagna et al., 2012
KULeuven	Van Buggenhout et al., 2008
ENITIAA	Ousegui et al., 2008; Alizadeh et al., 2009; Tironi et al., 2010
UM2	Lille & Autio, 2007

Figure 4: Current research in the field of HPLT treatments of biomaterial and food related samples. Publications are related to the affiliation of the primary author if more than one affiliation is engaged.

functional ingredients and perform a systematic research on HPLT induced effects on those two protein fractions within the present study. This development shows that research in the field of HPLT applications in food science and technology followed a top-down approach which started with complex food systems and led to the development of systematic research to fully understand the effects of HPLT and to identify its opportunities. The present study contributes to this aim by using a bottom-up approach. The identification of process-structure-function relationships is used as a base for proposing specific application fields in the food industry.

1.2 Impact of High Isostatic Pressure on Water-Ice-Phase Transitions

The main component of protein solutions and dispersions is water. It is the most abundant molecule on Earth and plays a major role in human life. Water is also the major component of most liquid and fresh foods and thus, basically influences the impact of processes on treated food. Therefore, its properties in the pressure-temperature landscape have to be taken into account for the present study. Although water is one of the best studied molecules, there is still a lot of research in physics, chemistry and also in food science where water is in focus. Despite the simple structure of the water molecule, containing one oxygen and two hydrogen atoms, it exhibits some anomalies which gain high scientific interest. The two best known anomalies are the increasing density of water during cooling and the expansion while freezing under atmospheric pressure. Water is also the main component of fresh food products and plays a major role in food technology. It acts for instance as a solvent for important ingredients, as an important partner for chemical reactions, heat and pressure transmitting media, and last but not least, as the most important food in its pure form. However, regarding the shelf life of foods and transportation and storage costs water also possesses negative attributes as it enables microbial

spoilage and is often the main contributor to the volume and the mass of fresh foods. Processes like drying allow removing water and thus, extending the shelf life and reduce the transport and storage costs. However, removing water often causes some undesired changes in the food matrix and an immobilization of the active water (displayed by the water activity a_W) by freezing or adding water binding substances like salts or sugars might influence the product as well. It is obvious that freezing should represent the least influencing method for the immobilization of water. Nevertheless, the expansion of water during freezing and freeze concentration effects could cause some undesired changes like cell destruction or diffusion of high-value compounds into intercellular regions. Freezing in combination with applying high isostatic pressure offers some new processes which are able to avoid undesired changes during freezing and furthermore enables the usage of pressure induced microbial inactivation or protein modification. However, the field of protein modification is not studied well until today mainly due to the limited availability of adequate equipment. The investigation of water under high pressures already started in the beginning of the 20th century when Bridgman developed the first phase diagram of water (Bridgman, 1912). Especially the astronomic research led to an intensive study on water under extreme pressure and temperature conditions. Current research reported that at least 13 crystalline ice formations (including two modification of ICE I – hexagonal ICE I*h* and the metastable cubic form ICE I c) are existent (Zheligovskaya & Malenkov, 2006; Malenkov, 2009). Beside the crystalline forms also amorphous forms and so called "clathrates" can be found (Marboeuf et al., 2012). The basics of influences of high pressure on the freezing behavior of water are highlighted within the following subchapters.

1.2.1 Thermodynamic Basics of Phase Transitions

First of all, some characteristic factors are needed. The compressibility factor β is given by:

$$\beta = \left(\frac{\partial V}{\partial p}\right)_T \tag{1.1}$$

where V denotes the Volume, p ist the pressure and T denotes the temperature. The thermal expansion factor is given by:

$$\alpha = \left(\frac{\partial V}{\partial T}\right)_p = -\left(\frac{\Delta S}{\Delta p}\right)_T \tag{1.2}$$

and the heat capacity can be displayed as:

$$C_p = T \cdot \left(\frac{\partial S}{\partial T}\right)_p \tag{1.3}$$

In a system where pressure, temperature and the total amount of substances (n) are the independent variables the free energy is given by the Gibbs function (1.4):

$$G = U + pV - TS \tag{1.4}$$

where G is the Gibbs energie, U is the internal energy, V is the volume and S the entropy. According to the first and second law of thermodynamics an infinitesimal change of the internal energy is given by:

$$\mathrm{d}U = T\mathrm{d}S - p\mathrm{d}V + \sum_i \mu_i \mathrm{d}n_i \tag{1.5}$$

with μ as the chemical potential of the component i. Combining equation (1.4) and (1.5), changes in the Gibbs energy can be written as:

$$\mathrm{d}G = -S\mathrm{d}T + V\mathrm{d}p + \sum_i \mu_i \mathrm{d}n_i \tag{1.6}$$

For a phase in thermal, hydrostatic and diffusive equilibrium it follows:

$$\mathrm{d}G = 0 \tag{1.7}$$

For a pure substance like water ($\sum_i \mu_i \mathrm{d}n_i = 0$) equation (1.6) combined with equation (1.7) gives:

$$0 = -S\mathrm{d}T + V\mathrm{d}p \tag{1.8}$$

For a water-ice phase transition where the two phases have to be in equilibrium equation (1.8) is identical for both phases:

$$-S_l\mathrm{d}T + V_l\mathrm{d}p = -S_s\mathrm{d}T + V_s\mathrm{d}p \tag{1.9}$$

where index l denotes the liquid phase and index s the solid phase. Rearrangement of equation (1.9) leads to:

$$\frac{\mathrm{d}T}{\mathrm{d}p} = \frac{\Delta_{ls}V}{\Delta_{ls}S} = \frac{T\,\Delta_{ls}V}{\Delta_{ls}H} \tag{1.10}$$

where dT/dp denotes the slope angle of the phase boundary. Equation (1.10) is the

Clausius-Clapeyron equation and can be used to basically describe phase transitions of water. Unfortunately, the Clausius-Clapeyron equation can only be used for first order phase transitions which are only the case for phase transitions of liquid water to the ice formations ICE I*h* to ICE VII. For these cases the first derivatives of the free energy are given by the entropy $S = -(\partial G/\partial T)_p$ and the molar volume $V = (\partial G/\partial P)_T$. Both are changing step wise and the latent heat is either released or absorbed. The other phase transition processes for the formation of low-temperature crystal-ordered ices are of second order. Consequently, the first derivatives of the free energy remain discontinuous and the second order derivatives undergo disintegration (Dunaeva et al., 2010). These are given by the heat capacity $C_p = -T(\partial^2 G/\partial T^2)_p$, the compressibility $\beta_T = -1/V \cdot (\partial^2 G/\partial p^2)_T$ and the volume expansion $\alpha = -1/V \cdot (\partial^2 G/\partial T \cdot \partial p)$. Dunaeva et al. (2010) took the 10 known triple points from the phase diagram of water (see Table 1) as fixed values and approximated the phase transition lines by treating the published data with the following equation:

$$T(p) = a + b \cdot P + c \cdot \ln(p) + \frac{d}{p} + e \cdot \sqrt{p} \tag{1.11}$$

Table 1: Triple points of water in the p-T landscape

Phase transition	T [K]	p [MPa]	Source
L–I*h*–III	251.165	208.566	IAPWS, 2008
L–III–V	256.16	350.1	"
L–V–VI	273.31	632.4	"
L–VI–VII	355.0	2216	"
I*h*–II–III	238.5	213	Abascal et al., 2005
II–III–V	248.9	344	"
II–V–VI	209.8	608	Tari et al., 2000
VI–VII–VIII	278.16	2120	Song et al. 2003
VII–VIII–X	100.0	62000	Yurtseven, Salihoglu, 1998
L–VII–X	~1500	~40000	Schwager, Boehler, 2008
	1040.0	35000	Lin et al., 2005
	1000	47000	Goncharov et al., 2005

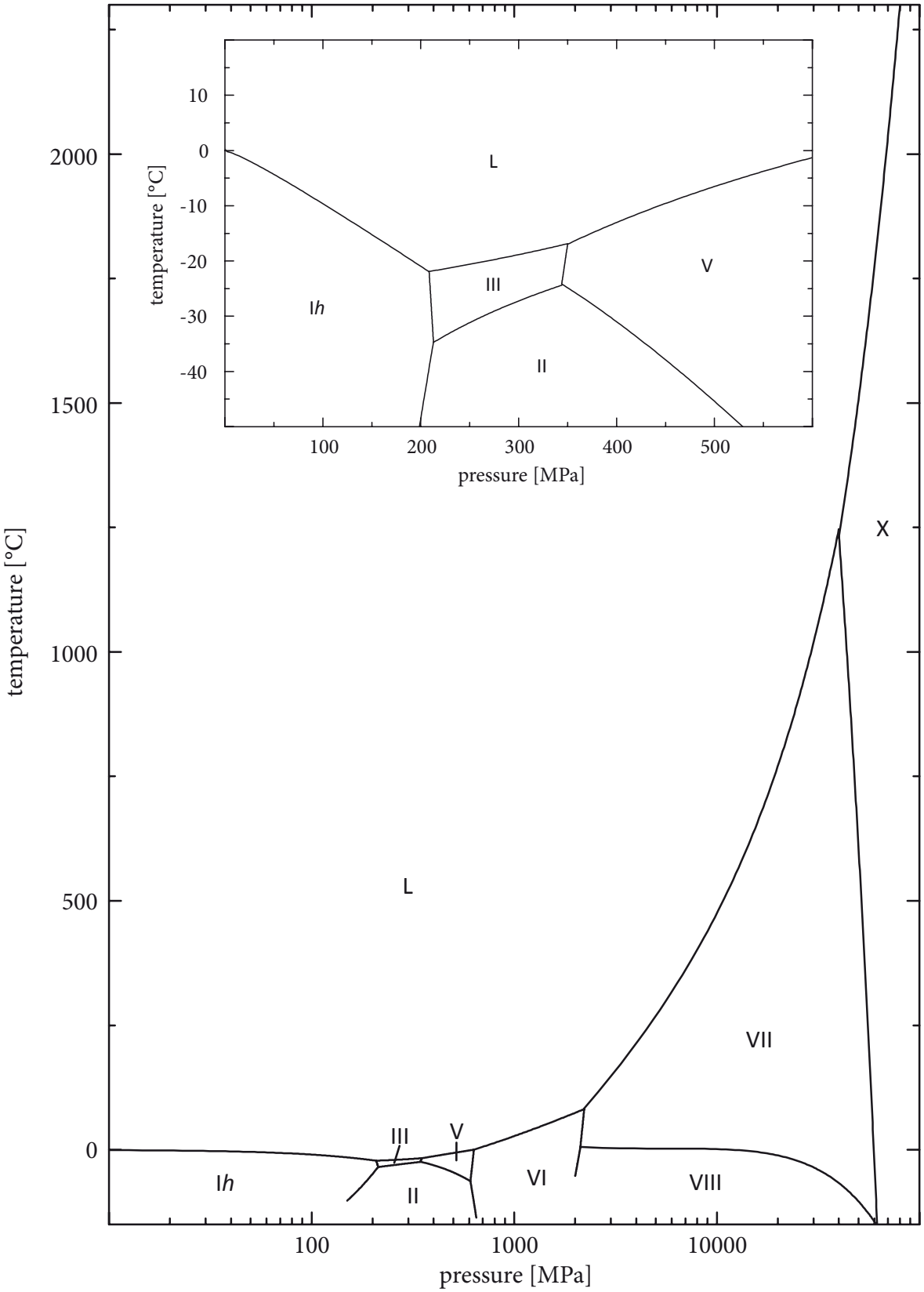


Figure 5: Phase diagram of water with its different ice modifications.

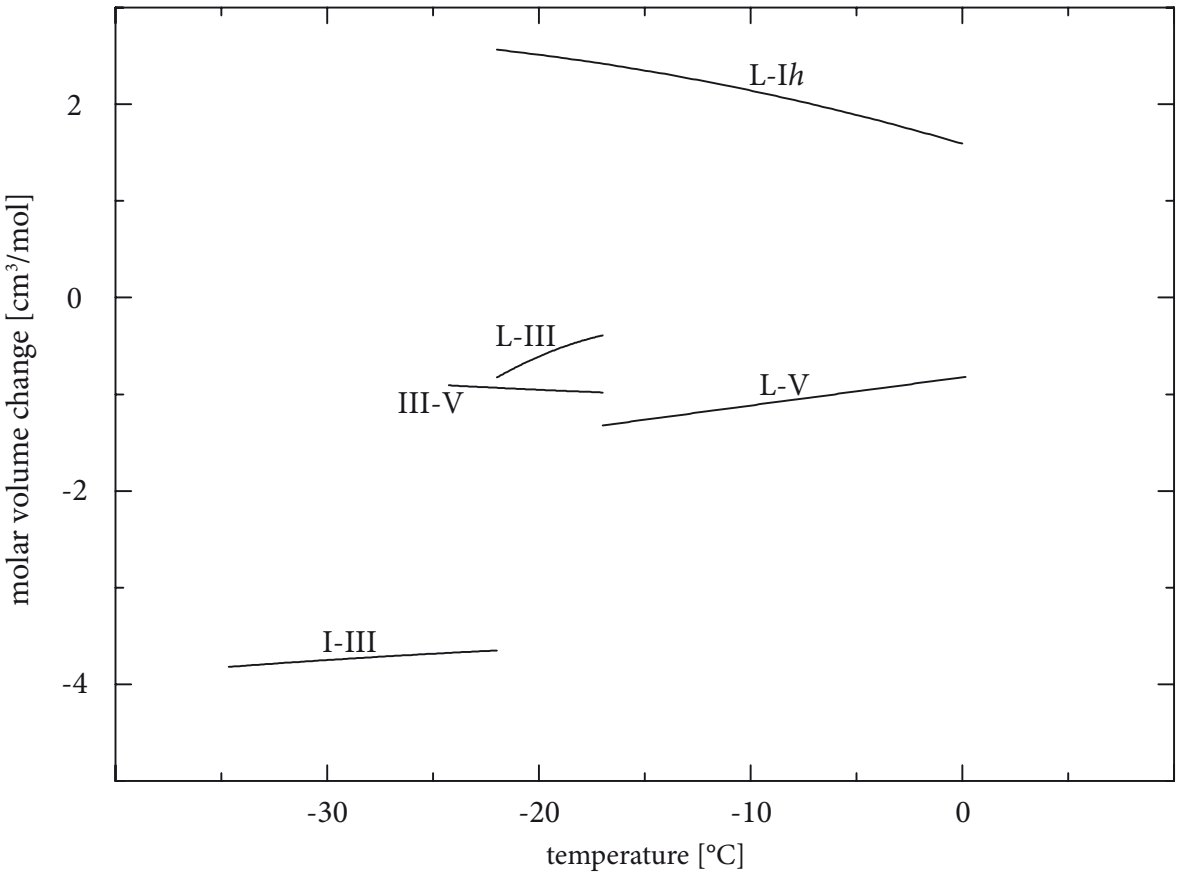


Figure 6: Change in molar volume during selected phase transitions of water in dependence of the phase transition temperature.

where the coefficients a to e are given in Annex 1. Calculating the p-T coordinates leads to the phase transition lines which are presented in Figure 5 – the phase diagram of water. However, the changes of the molar volume during the phase transitions are not well explained until today although they are used for many thermodynamic calculations. There are four models known to describe the connection between pressure, volume and temperature (Dunaeva et al., 2010): The Birch-Murnaghan equation, the Vinet equation, fundamental equations based on multiparametric functionals of the Helmholtz and Gibbs free energies, and semiempirical equations of state. By using experimental data the molar volume changes can be approximated by the following equation (Dunaeva et al., 2010):

$$\Delta V = c + b \cdot T + a \cdot T^2 \tag{1.12}$$

where the coefficients a to c are given in Annex 2. Calculation of the change in molar volume leads to the results shown in Figure 6. It is obvious that ICE I*h* is the only ice formation which possesses a positive volume change and, thus a lower density than water. This effect will be

discussed in detail in the following chapter because it can be used within high pressure – low temperature (HPLT) processing. The relevant ice modifications within the HPLT domain are I*h*, III and V. These ice formations differ in the structure of their crystals. ICE I*h* is formed in the pressure range from atmospheric to ~210 MPa and possesses a hexagonal crystal structure with only small deviation from the ideal hexagonal symmetry. The crystal structure belongs to the space group 194 ($P6_3/mmc$) and one unit cell contains 4 water molecules. The crystal structure of ICE I*h* is shown in Figure 7A. ICE I*h* borders on ICE III (triple point between liquid water, ICE I*h* and ICE III at -21.985 °C and 209.9 MPa; triple point between ICE I*h*, ICE III and ICE II at -34.7 °C and 212.9 MPa). ICE III forms tetragonal ice crystals which belong to the space group 224 (P41212). ICE III borders as mentioned above to ICE I but also to ICE V (triple point with liquid water at -16.986 °C and 350.1 MPa; triple point with ICE II at -24.3 °C and 344.3 MPa). It is obvious that ICE III is only stable in a quite small pressure-temperature range. The crystal structure of ICE III can be also seen in Figure 7B. ICE V forms monoclinic crystals of the space group 15 (C2/c). It borders to ICE III and II (see above) and to ICE VI (triple point with liquid water at 0.16 °C and 632.4 MPa; triple point with ICE II and ICE VI at ~-55 °C and ~620 MPa). Its crystal structure can be seen in Figure 7C. Other crystal ice forms and the amorphous ice forms are not of technical interest in the HPLT domain and the reader is referred to review articles where those are discussed in detail (Zheligovskaya & Malenkov, 2006; Malenkov, 2009; Dunaeva et al., 2010).

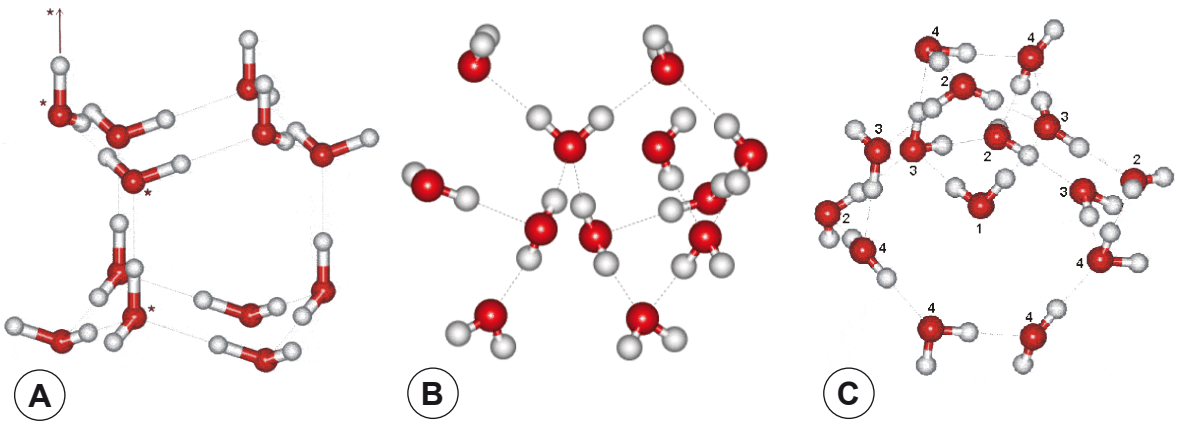


Figure 7: Crystal structures of relevant ice modifications. A: Ice I*h*, B: Ice III, C: Ice V (Chaplin, C., http://www1.lsbu.ac.uk/water/, accessed: 2014/01/19).

1.2.2 High Pressure – Low Temperature Process Options

Figure 8 shows the relevant part of the phase diagram of water for high pressure – low temperature (HPLT) treatments. The process options in the subzero temperature domain of the phase diagram will be named according to the nomenclature of Urrutia Benet et al. (2004). Generally, the HPLT processes can be divided into two subclasses, where one includes processes with phase transitions and the other processes without phase transitions. The only member of the first subclass is subzero cooling (SbC) under high pressure without ice nucleation. This process is given in Figure 8 by the course A-B-C-D. The SbC process is suitable for storage of freeze-sensitive products below their freezing point to preserve them against microbial or enzymatic spoilage without inducing damages due to freezing. Within the other subclass three options of phase transition can be found: Freezing, thawing and solid-solid phase transitions. Obviously, solid-solid phase transitions cannot be achieved from atmospheric conditions without a freezing step, but they are included into many freezing or thawing process where the course of pressure

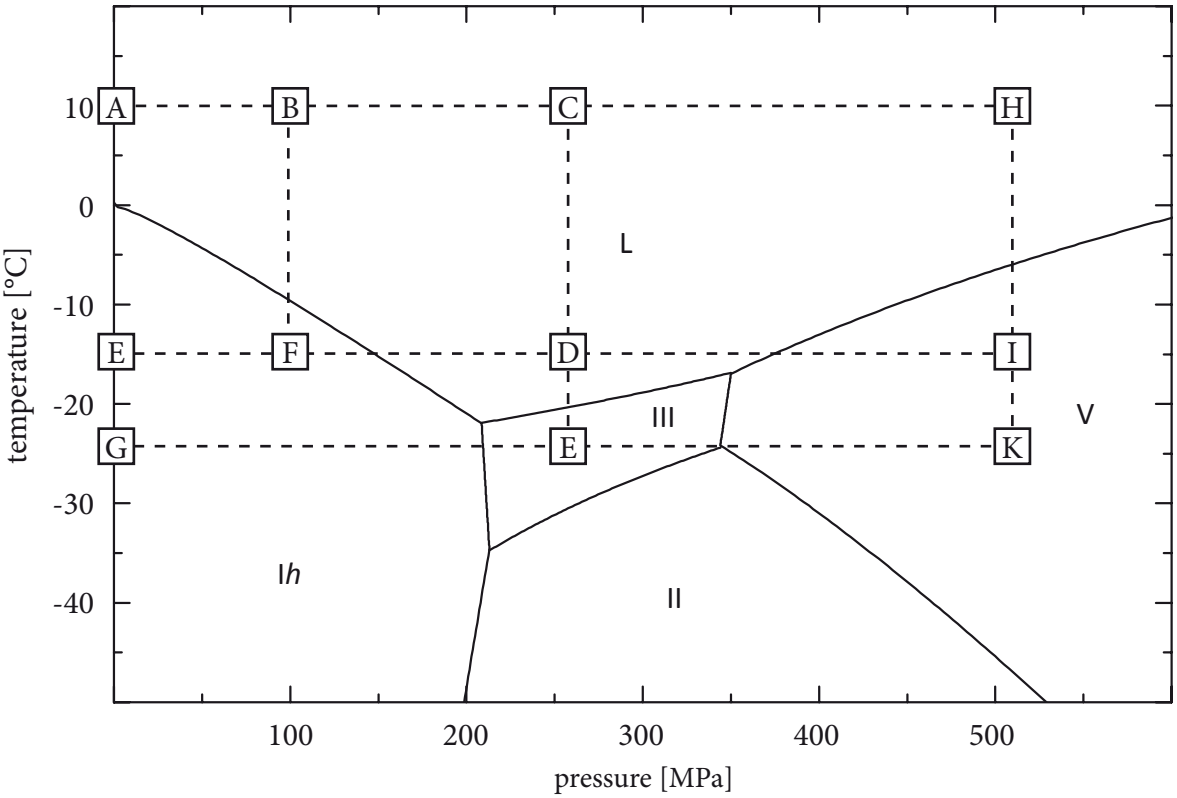


Figure 8: HPLT process options in the p-T landscape. A-B-C-D: Sub-zero cooling (SbC), E-F-B-A: Pressure assisted thawing (PAT), E-F-D-C-B-A: Pressure induced thawing (PIT), A-B-C-D-E-G and A-B-C-H-I-K-E-G: Pressure assisted freezing (PAF), A-B-C-D-I: Pressure induced freezing (PIF), A-B-C-D-F-E: Pressure shift freezing (PSF).

and temperature passes the solid-solid phase transition lines. It is shown in Figure 8 that three possibilities for HPLT thawing processes are existent. Pressure assisted thawing (PAT) describes a thawing process at constant pressure using the temperature difference between the sample and the surroundings as the driving force. This process can be also used to thaw samples which are stored under pressure in higher ice modifications. Furthermore, a recrystallization from atmospheric ICE I to higher ice modifications by a pressure increase and a subsequent thawing by increasing the temperature belongs to the PAT processes as well. The course E-F-B-A within Figure 8 shows an exemplary pathway of a PAT process. Pressure induced thawing (PIT) is used as a terminus for a process where thawing of the sample is induced by an increase in pressure. However, this is only feasible for thawing samples frozen in ICE I and a subsequent heating is necessary to avoid freezing of the sample during decompression. It should be mentioned that the last part is somehow pressure assisted but in difference to a PAT process the pressure is used as the driving force for melting in the case of PIT. An exemplary PIT process is shown in Figure 8 by the course E-F-D-C-B-A. Pressure shift thawing (PST) is only possible to thaw samples from higher

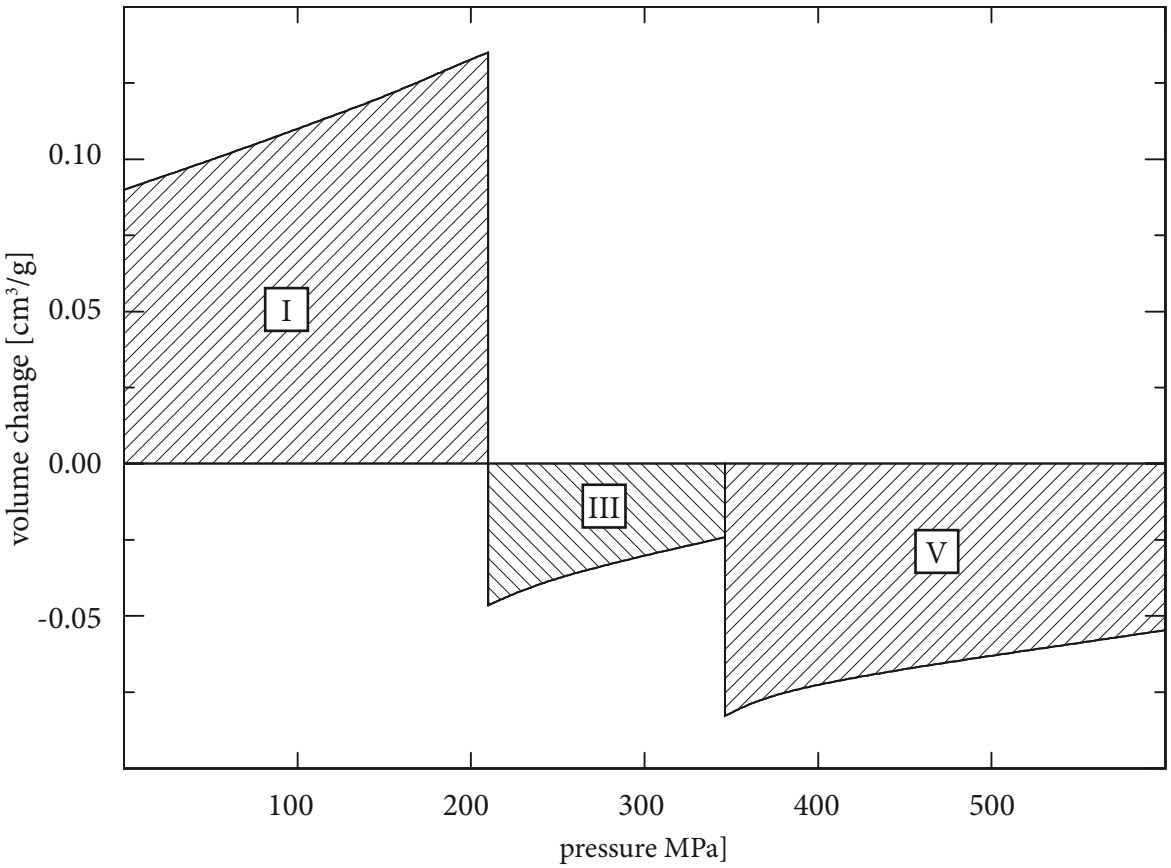


Figure 9: Volume change during water-ice phase transitions for the HPLT relevant pressure range. Data obtained from Bridgman (1912), Fletcher (1970).

ice modifications. Starting for instance in ICE V a pressure release will lead to a temperature shift to the corresponding melting temperature and the course of the temperature will follow the phase transition line of ICE V, III and finally ICE I. However, the freezing processes are more important for the present study. Analogous processes like for thawing are also existent for HPLT freezing processes. Pressure assisted freezing (PAF) offers the possibility of freezing to higher ice modifications. Samples are frozen at an almost constant pressure due to a decrease in temperature caused by external cooling. Samples can be frozen to ICE III and ICE V within the shown p-T-domain. An exemplary process of freezing to ICE III (A-B-C-D-E-G, PAF III) and to ICE V (A-B-C-H-I-K-E-G, PAF V) is shown in Figure 8. It should be mentioned that it is also possible to reach a form of metastable ICE I within the thermodynamic stable region of ICE III. This was shown by Schlüter et al. (2004) for freezing potato cylinders in the range of 210 to 240 MPa. Furthermore, it should be noticed that a recrystallization to ICE I will occur during the decompression (as indicated by the pathways of PAF III and PAF V). Figure 9 shows the volume changes between the three ice modifications. It can be seen that the recrystallization to ICE I will induce a large volume change which could induce mechanical stress to the sample. Another possibility to obtain higher ice formations is pressure induced freezing (PIF). Samples are undercooled under pressure without freezing and the phase transition line is passed by a further increase of the pressure. In consequence, the three ice formations III, V and VI can be obtained. The course A-B-C-D-I in Figure 8 shows exemplary a PIF process to ICE V. A last but very important process option is pressure shift freezing (PSF). It is shown by the course A-B-C-D-F-E in Figure 8 that the pressure release is used to nucleate the sample. However, it is only possible to reach ICE I due to the positive slope of the phase transition lines of the other ice modifications. In comparison to other freezing processes PSF induces an instantaneous nucleation and a homogeneous crystal size distribution all over the sample. Phase transitions which are induced by pressure changes are of special interest because pressure changes can be realized much faster and more homogeneous in comparison to temperature changes (Urrutia Benet et al., 2004). Possible applications for the presented HPLT process options will be given in a subsequent chapter.

1.3 Proteins in the Pressure-Temperature-Landscape

The following chapter points out the basics of proteins and their response to pressure treatments with focus on milk proteins. The basics of protein functionality are highlighted and opportunities for modifications will be shown. Finally, the basics of HPLT induced changes in proteins are given.

1.3.1 General Basics of Proteins

Generally, protein structure can be divided into primary, secondary, tertiary and quaternary structure. The primary structure is built of 20 L-α-amino acids, varying in number and order, which are linked by peptide bonds like displayed in Figure 10. Thus, the primary structure of a protein is also often called the amino acid sequence and is significantly involved in the overall protein structure. Different secondary structure motifs like α-helix and β-sheet are formed depending on the amino acids and their sequence. The number and the alignment of these motifs are called the secondary structure. The secondary structure is stabilized by hydrogen bonds. Pauling et al. (1951) were the first who identified the a-helix as an important secondary structure motif. 3.6 amino acids form one turn of the α-helix which is stabilized by a hydrogen

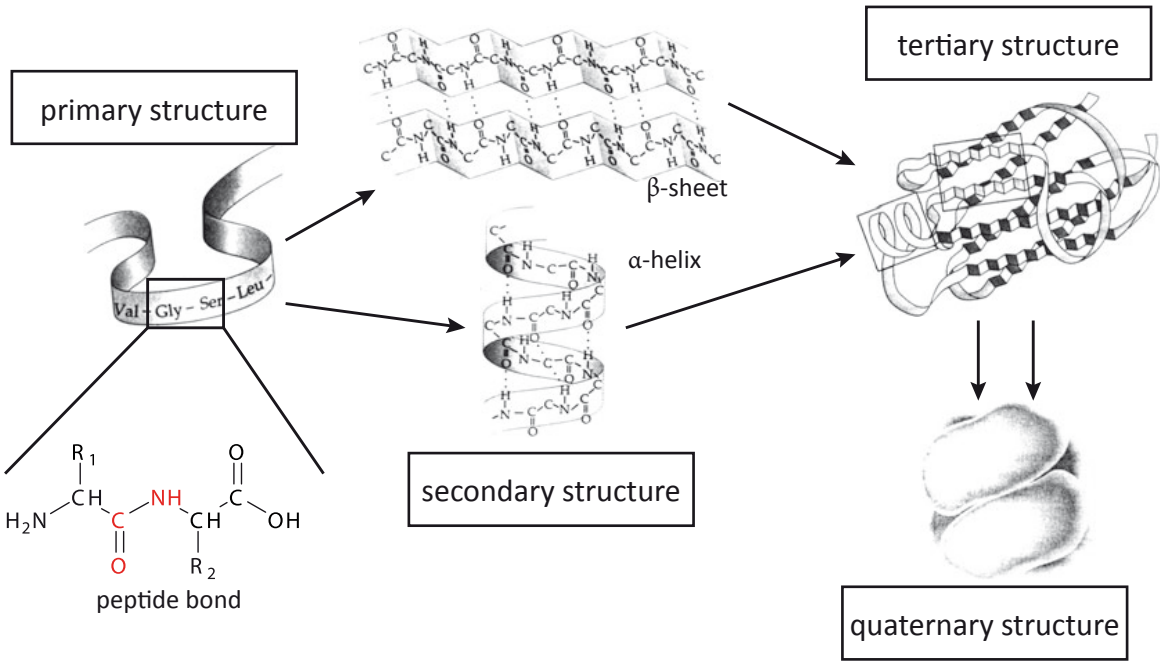


Figure 10: Structural levels of protein conformation (Rehner & Daniel, 2010; redrawn with modifications).

bond involving 13 atoms between the oxygen and the hydrogen of this bond. The oxygen is a carbonyl oxygen of the peptide bond and the hydrogen belongs to the nitrogen of another peptide bond. The β-sheet structure was also first postulated by Pauling et al. (1951). Two kinds of β-sheet structures are existent – the parallel and the antiparallel β-sheet. The peptide chains are orientated in the same direction (from the N-terminus to the C-terminus) in case of the parallel β-sheet structure. For the antiparallel β-sheet the chains are orientated reversed. This structure is also stabilized by hydrogen bonds. Again, the carbonyl oxygen and the hydrogen of the peptide nitrogen are used to build these hydrogen bonds. In addition to the two mentioned and most important secondary structure motifs β-turns and β-bulges are existent. A β-turn is a tight turn of the polypeptide chain to form a loop (for instance in antiparallel β-sheet structures). A β-bulge represents a motif which often occurs as an irregularity in antiparallel β-sheet structures. The three-dimensional structure of a single polypeptide chain – which consequently means the way of folding – is called the tertiary structure. Folding of protein molecules reduces the surface which is available for contact with a solvent and thus, increases the protein stability. Different intermolecular interactions are used to stabilize the tertiary structure. Depending on the amino acid residues there are disulfide bonds, hydrophobic and electrostatic interactions as well as hydrogen bonds possible. Finally, the interactions of protein molecules to form for instance dimeric or oligomeric molecules lead to the formation of the quaternary structure. The quaternary structure is stabilized by electrostatic and hydrophobic interactions.

According to Messens et al. (1997) "*the functional properties of food proteins are those physicochemical properties that affect the behaviour of proteins in food systems during preparation, processing, storage or consumption*". This statement already highlights – beside the scientific complexity – the industrial importance for a broad research in this field. First of all, proteins were often provided in dry forms such as concentrates or isolates. The first step in processing is consequently a solvation or hydration process which points out the high importance of protein solubility. A high solubility, especially a high water solubility, should therefore be preserved during processing as it is often necessary for application. On the other hand, a high water solubility generally implicates a low water binding capacity and thus, diminishes the application as for instance a thickener. A protein will go into solution until a maximum concentration is reached and an equilibrium between solubilized and not solubilized protein molecules arises. The solubility of proteins generally depends on the extrinsic factors pH value, ionic strength, polarity of the solvent and

the temperature. Beside their solubility or water binding properties the ability to bind fat or to interact with other hydrophobic substances is often of interest for food applications. This is especially the case when an emulsion should be stabilized by a protein. An emulsion is a mixture containing two phases which cannot be dissolved into each other. It is formed by dispersing one phase (dispersed phase) in the other phase (continuous phase). This system is thermodynamic instable due to the interfacial tension between both phases. Consequently, the thermodynamic stable form is reached when the boundary surface is minimal which is the case for two separated phases. Proteins are often suitable to be used as an emulsifier due to their amphiphilic structure. They possess hydrophilic as well as hydrophobic parts and thus, are able to reduce the interfacial tension between the two phases. The protein molecules first have to reach the interface and afterwards have to rearrange at the phase boundary (Dalgleish, 1997). Concluding, small and flexible molecules will be faster in occupying the phase boundary (Grunden et al., 1974) while proteins with slower adsorption tend to cause higher long term stabilities. However, an emulsion can never become a thermodynamic stable system – it can only obtain kinetic stability. The difference in density between the two phases will always lead to creaming of the dispersed phase according to the law of Stokes:

$$v = \frac{d^2 \cdot g \cdot \Delta\rho}{18 \cdot \eta} \qquad (1.13).$$

Where v represents the velocity of creaming, d is the diameter of the droplet, g is the gravitation, $\Delta\rho$ is the difference in density between the two phases and η represents the dynamic viscosity of the continuous phase. It is obvious, that a smaller droplet size or a higher viscosity of the continuous phase will lead to a decreased creaming of the oil droplets. Especially the high influence of the dynamic viscosity clearly depicts the temperature sensitivity of emulsions. Generally, similar considerations as above can be made for dispersing air in a liquid phase during the creation of foams. It was suggested over a long time that especially for foams an unfolding step of the protein after adsorption is necessary (Damodaran, 1994). However, recent investigations showed that the structural changes of β-Lg at the phase boundary are minimal (Wierenga & Gruppen, 2010). A difference to emulsions is the importance of creating viscoelastic films between the air bubbles to avoid drainage and thus, thinning of lamellas. Interactions between proteins are necessary for both systems – emulsions and foams. However, a repulsion of adsorbed protein molecules at different oil droplets will increase the stability while attractive forces will enhance

flocculation which is the first step of coalescence. In the case of foams, attractive forces will create more stable lamellas while repulsion will induce thicker lamellas with higher drainage.

1.3.2 Bovine Milk Proteins

Bovine milk proteins are the scientifically best investigated proteins which could be certainly drawn back to their high nutritional value and their outstanding techno-functional properties. Bovine milk contains about 3.5 % of protein which can basically be divided into two fractions – the caseins and the whey proteins. The main difference between caseins and whey proteins is their solubility at pH 4.6. Whey proteins stay soluble while caseins precipitate at this pH value. Another major difference between whey proteins and caseins is their heat stability. Caseins are stable against heat for 24 h at 100 °C or for up to 20 min at 140 °C while whey proteins are completely denatured at 90 °C for 10 min (Fox, 1992). Coagulation of caseins at higher treatment intensities might be the consequence of a thermally induced pH shift or interactions with the denatured whey proteins (Singh & Flanagan, 2006). In general the conformational structure of milk proteins is well examined due to the possibility of an easy isolation from the raw material (Cheftel et al., 1992). The general protein composition of bovine milk is given

Table 2: Characteristics of the major proteins in bovine milk (Ng-Kwai-Hang, K. F. & Fuquay, J. W., 2011)

Protein	Concentration [g L^{-1}]	Molecular mass [a] [kDa])	Genetic variants detected	Isoelectric point [a,b]
α_{S1}-Casein	10	23.164	A,**B**,C,D,E,F,G,H	4.44 – 4.76
α_{S2}-Casein	2.6	25.388	**A**,B,C,D	
β-Casein	9.3	23.983	A^1,$\mathbf{A}^2$,A^3,B,C,D,E,F,G	4.83 – 5.07
κ-Casein	3.3	19.038	**A**,B,C,E,F^S,F^IG^S,G^E,H,I,J	5.45 – 5.77
β-lactoglobulin	3.2	18.277	A,**B**,C,D,E,F,H,I,J	5.13
α-lactalbumin	1.2	14.175	A,**B**,C	4.2 – 4.5
Serum albumin	0.4	66.267		4.7 – 4.9
Immunoglobulin	0.8	143.000 – 1,030. 000		

[a] Molecular mass, isoelectric point are for the genetic variants in bold
[b] Farell, J. R. et al., (2004)

in Table 2. Both, whey proteins and caseins are heterogeneous and significantly differ in their cysteine content, their proline content and the amount of phosphate. Further diverseness arises from the occurrence of genetic polymorphism (Singh & Flanagan, 2006).

The caseins represent about 80 % of the bovine milk protein. They can be divided into three sub groups – α-, β- and κ-casein. The α-caseins (α-Cn) are furthermore subdivided into α_{S1}- and α_{S2}-Cn. A sometimes mentioned γ-casein is the result of a limited proteolysis of β-casein (Singh & Flanagan, 2006). Differences in their structural and functional properties can be mainly traced back to differences in the amino acid composition and distribution (see Table 3). Under native conditions caseins exist in a micellar structure composed of 94 % protein and 6 % small ions. Their main function is to deliver calcium and phosphate to the neonate in a soluble form (Farrell & Thompson, 1988). The calcium binding properties are directly related to the phosphoserine residues in the respective casein molecule. Binding of calcium ions reduces the negative net charge of the molecules and thereby the electrostatic repulsion. As a consequence isolated α- and β-casein precipitate in the presence of calcium ions near a neutral pH (Hoagland et al., 2001).

κ-casein (κ-Cn) possesses only one phosphorylated serine residue and binds little to calcium. Therefore it remains soluble at all calcium concentrations and even stabilizes other casein fractions against precipitation and formation of large aggregates (Rollema, 1992). Furthermore, κ-Cn is the only casein fraction that is glycosylated containing up to four tri- or tetra-saccharide moieties. Its stabilizing properties rise with increasing carbohydrate content (Takeuchi et al., 1985). Due to a lack of adequate crystallization and characterization methods caseins were often considered as random coil proteins. However, they possess some secondary and an unordered tertiary structure, which is less stable and more flexible compared to typical globular proteins (Swaisgood, 1992). κ-Cn is with 10-20 % α-helices, 20-30 % β-structure and 15-25 % β-turns the most structured amongst the casein fractions. A highly hydrophobic n-terminal with positive net charge and a polar c-terminal with negative net charge donate it a highly amphipathic character. Both domains are connected by a positive β-strand peptide sensitive to chymosin degradation. Cleavage of κ-Cn into a hydrophobic and a hydrophilic region destabilizes the micelle and leads to curd formation during cheese manufacturing. The tertiary structure probably consists of antiparallel and parallel β-sheets, a β-α-β-motif for the hydrophobic domain and a β-turn-β-strand-β-turn conformation for the joining peptide sequence (Singh & Flanagan, 2006).

Table 3: Amino acid composition of the casein fractions of bovine milk (Farrell, J. R. et al., 2004)

	Amino acid	α_{S1}-casein	α_{S2}-casein	β-casein	κ-casein
Positively charged	Arginine	6	6	4	5
	Histidine	5	3	5	3
	Lysine	14	24	11	9
Negatively charged	Aspartic acid	7	4	4	4
	Glutamic acid	25	24	19	12
	Serino phosphate	8	11	5	1
Polar, uncharged	Serine	8	6	11	12
	Threonine	5	15	9	15
	Asparagine	8	14	5	8
	Glutamine	14	16	19	14
	Cysteine	0	2	0	2
	Proline	17	10	35	20
Hydrophobic	Alanine	9	8	5	14
	Valine	11	14	19	11
	Isoleucine	11	11	10	12
	Leucine	17	13	22	8
	Methionine	5	4	6	2
	Glycine	9	2	5	2
Aromatic	Phenylala-nine	8	6	9	4
	Tyrosine	10	12	4	9
	Tryptophan	2	2	1	1

[a] Mercier et al (1971)
[b] Farrell; JR et al., (2004;)

α_{S1}-Cn can be divided in a major and a minor component differing in one phosphoserine residue. The reported levels of α-helices and β-sheets are ranging from 12-22 % and 0-17 %,

respectively depending on the analytical method applied (Creamer et al., 1981; Byler & Farrell, 1989). Anionic clusters and three hydrophobic regions in the amino acid chain are responsible for a distinct hydrophobic and a highly charged polar domain. Both domains exhibit high flexibility (Swaisgood, 1992). These structural properties are related to the strong dependency on extrinsic factors, like pH-value and ionic strength (Farrell et al., 2004). α_{S2}-Cn represents 10 % or less of the micellar casein (MC), but provides at least 20 % of the phosphoserine in the micelle. Hence, it shows the highest calcium sensitivity and has to be stabilized by equimolar amounts of κ-Cn (Toma & Nakai, 1973; Snoeren et al., 1977; Davies & Law, 1983; Stewart et al., 1987). α_{S2}-Cn is more structured than α_{S1}-Cn due to 40 % fewer proline residues and the formation of intra- and intermolecular disulfide bonds (Swaisgood, 1992). CD spectra indicate 24 % of α-helices, 30 % β-structure and 24 % turns (Hoagland et al., 2001). The presence of three anionic clusters makes it the most hydrophilic amongst the caseins. Primary structure analysis suggests a negatively charged, polar N-terminal with two anionic clusters, followed by a hydrophobic domain between residues 90 to 120, another anionic cluster in a polar domain and a hydrophobic, positively charged c-terminal ranging from amino acid 160 to 207 (Swaisgood, 1992; Ng-Kwai-Hang & Fuquay, 2011).

β-casein (β-Cn) contains more proline residues than the other caseins and possesses no cysteine residues. Six forms differing in the number of 0-5 phosphorylated serine residues are available. Theoretical considerations suggest that the secondary structure is composed of 10% α-helix, 17 % β- and 70 % unordered structure, probably due to the high proline content. β-Cn is the most hydrophobic fraction with a polar N-terminal representing 10 % of the chain length but possessing more than 30 % of the charged groups and two large hydrophobic regions representing 75 % of the molecule (Singh & Flanagan, 2006).

As not all hydrophobic domains of the caseins can be shielded from water contact they tend to self-association. In dependency on pH and ionic strength of the surrounding media α_{S1}-Cn forms dimers, tetramers and hexamers (Payens & van Markwijk, 1963; Ho & Waugh, 1965; Payens & Schmidt, 1965; Schmidt & van Markwijk, 1968; Swaisgood & Timashef, 1968; Schmidt, 1970b, 1970a; Padiernos et al., 2009). α_{S2}-Cn associates less extensively than the α_{S1} form, which can be traced back to a higher electrostatic repulsion of the charged clusters. Increasing ionic strength favors association but above 0.2 M the degree of association declines again. Within SDS PAGE, α_{S2}-Cn was present as monomer and dimer. Under reducing conditions

only the monomeric form was found, indicating that association is a consequence of disulfide-bridge formation (Hoagland et al., 2001). The self-association of β-Cn is a monomer-polymer equilibrium based on hydrophobic interactions (Farrell et al., 2004). The polymerization degree is strongly temperature-dependent. Only monomers can be found at temperatures from 0-4 °C and the hydrodynamic behavior of the molecule resembles a random coil with high solvation (Tanford, 1961; Payens & van Markwijk, 1963). When temperature raises a rapid self-association to large polymers can be observed. Both cysteine residues of the κ-Cn molecule are located in the loops and turns of the hydrophobic domain and are therefore accessible to form polymers by disulfide crosslinking (Swaisgood et al., 1964). The majority of κ-Cn is located at the micelle surface having at least one κ-Cn around to form stable disulfide-linked polymers with (Carroll & Farrell, 1983). Different models exist to describe the micelle structure out of which the sub-unit model is the most popular one. Submicelles having diameters of 10-15 nm possess a hydrophobic core surrounded by a hydrophilic coat. The polar moieties of κ-Cn are concentrated at one area; the remaining surface is occupied by the hydrophilic parts of the other casein fractions. Submicelles aggregate to colloidal particles ranging from 80 to 300 nm by calcium phosphate interaction. Submicelles with high κ-Cn content are exposed to the micelle surface whereas those with low or no κ-Cn content have to be buried in the interior to form stable colloids (Schmidt, 1982; Home, 2006). Consequently, the size of a micelle is inversely proportional to its overall κ-Cn content.

The whey proteins represent about 20 % of the bovine milk protein. They possess a great number of individual proteins and thus, are even more heterogeneous than the caseins. Nevertheless 95 % are composed of four major components β-lactoglobulin (β-Lg), α-lactalbumin (β-Lac), bovine serum albumin and immunoglobulin. They are all typical globular proteins possessing high levels of secondary, tertiary and, in most cases, quaternary structure. The amino acid composition of the whey proteins is shown in Table 4.

β-Lg is the most important whey protein fraction and represents approximately 50 % of the non-casein protein. Nine genetic variants of β-Lg are known with A and B as the most common. β-Lg A and B only differ in position 64 and 118 of the primary chain (Singh & Flanagan, 2006). Their proportion is varying in milk from different cows and in relation to the overall casein content (Aschaffenburg & Drewry, 1957). Secondary structure analysis revealed 16 % α-helices, 58 % β-sheet and 25 % unordered structure (Clark & Smith, 1989). They are folded to nine

anti-parallel β-strands, of which eight are involved in a β-barrel (Papiz et al., 1986; Brownlow et al., 1997). β-Lg can bind to hydrophobic and amphipathic molecules at different binding sites and thereby stimulates lipase activity. Retinol can be bound in an internal cavity and fatty acids in a hydrophobic cleft on the molecule's surface (Sawyer et al., 1998). The native β-Lg exists as a dimer. Self-association depends on temperature, pH, ionic strength and protein concentration. Below pH 3.5 a monomeric form is available. Between pH 3.5 and 5.2 the molecules reversibly

Table 4: Amino acid composition of the whey protein fractions of bovine milk (Farrell, J. R. et al., 2004)

	Amino acid	β-lactoglobulin	α-lactalbumin
Positively charged	Arginine	3	1
	Histidine	2	3
	Lysine	15	124
Negatively charged	Aspartic acid	10	12
	Glutamic acid	16	7
	Serino phosphate		0
Polar uncharged	Serine	7	7
	Threonine	8	7
	Asparagine	5	13
	Glutamine	9	7
	Cysteine	5	8
	Proline	8	2
Hydrophobic	Alanine	15	3
	Valine	9	6
	Isoleucine	10	8
	Leucine	22	13
	Methionine	4	1
	Glycine	4	6
Aromatic	Phenylalanine	4	4
	Tyrosine	4	4
	Tryptophan	2	4

associate to tetramers and octamers. Above pH 7.5 and at increased temperatures the dimer dissociates and starts to unfold (Singh & Flanagan, 2006). The protein structure possesses two disulfide bridges and one free thiol group. The latter is buried in the structural interior protected by an α-helix. If secondary and tertiary structures are disrupted by external influences, the thiol group is exposed and may participate in disulfide exchange or crosslinking (Boland, 2011). Heating also disrupts the β-sheet structure and enhances hydrophobic interactions which may contribute to aggregation and gel formation. The β-structures mostly recover during cooling, but usually the tertiary structure remains altered (Bhattacharjee et al., 2005).

Approximately 20 % of the whey protein is composed of tryptophan-rich α-Lac (Singh & Flanagan, 2006). It is involved into the biosynthesis of lactose in the Golgi apparatus of the mammary gland (Ebner et al., 1966). 24 % α-helices, 41 % β-sheet and 35 % random structures are observed via CD spectra (Clark & Smith, 1989). The overall structure of α-Lac is similar to those of hen egg-white lysozyme. Structurally α-Lac and hen egg-white lysozyme are homologous and can evolutionarily be traced back to a common precursor (Acharya et al., 1990). A helical α-domain and a rather unordered smaller β-domain are separated by a cleft region, which builds the active site in the lysozyme. In α-Lac enzymatic activity is blocked by the absence of certain amino acids and a sterical blockade by Tyr103 (Warme et al., 1974). The α-region is rather unpolar and contributes to the tendency to aggregate at low or high pH-values (Kronman & Andreotti, 1964; Kronman et al., 1964). The β-domain is highly variable and loop structures can be observed instead of helices when pH or temperature are altered (Harata & Muraki, 1992). α-Lac is a metalloprotein, naturally binding to calcium in a helix-turn-helix motif between the two domains. The cation is bound close to the molecule's surface coordinated by seven oxygen atoms (two and three donated by protein's carbonyl and carboxyl groups and further two by water molecules) to a pentagonal bipyramid. Calcium also contributes to the protein's stability (Hiraoka et al., 1980; Harata & Muraki, 1992; Pike et al., 1996).

In addition there are some minor protein fractions. Immunoglobulins are typically composed of two heavy and two light chains linked by disulfide bridges. They are extremely heat labile, which makes processing of higher immunoglobulin concentration, for example in colostrum, rather difficult. Bovine serum albumin exhibits a high molecular weight, a high number of disulfide bonds and an ability to bind components with low molecular weight. Both proteins contribute to the health insurance of the neonate (Boland, 2011).

Milk proteins are often used as functional ingredients in foods. Their functional properties are influenced by intrinsic and extrinsic factors like it is the case for every protein. However, the intrinsic factors like size and surface hydrophobicity can be influenced by extrinsic factors like pH value, ionic strength or temperature (Singh & Flanagan, 2006). The bovine milk proteins possess unique intrinsic properties and are available in different forms which can be furthermore modified by physical, chemical or enzymatic processes (Singh & Flanagan, 2006). The solubility of the whey proteins is excellent over the entire pH range while the caseins are completely insoluble in the region of their isoelectric point (4.0-5.0). The MC show an U-shaped pH-solubility profile and their solubility in the critical pH range may be improved by enzymatic hydrolyses (Flanagan & FitzGerald, 2002). This effect can be traced back to a reduction of the molecular weight and the exposure of polar groups. The whey proteins are able to bind water in the range of 0.3-0.6 g per gram protein mainly due to surface interactions with amino acids. In contrast, MC are able to bind up to 4 g water per gram protein due to the void structure of the micelles (Carr et al., 2003; Mulvihill & Ennis, 2003). MC offer a high surface hydrophobicity and a well-balanced distribution of hydrophilic and hydrophobic domains. They possess a high conformational flexibility and thus, they are suitable to interact with oil-water interfaces. The whey proteins also adsorb fast at oil-water interfaces and are supposed to be able to re-orientate. Consequently, both milk protein fractions have the ability to stabilize emulsions. However, whey protein stabilized emulsions are supposed to be slightly less stable in comparison to emulsions stabilized by MC under the same conditions. Yet, emulsions stabilized with pure β-Lg may have an increased stability due to the formation of intermolecular disulfide bonds (Singh & Flanagan, 2006). Caseinates generally create high foam expansions and give emulsions with higher droplet coverage in comparison with whey protein concentrates but stability of both foams and emulsions is less. Heating of whey proteins and enzymatic hydrolyses of caseinates improve the interfacial stabilization properties, but too extensive heating or hydrolyses give contrary results (Flanagan & FitzGerald, 2002). However, acid and rennet coagulated caseinates are insoluble in water and therefore only applicable for fat or water binding or to give an additional nutritive value to foods (Rollema & Muir, 2009). Sodium, potassium or ammonium caseinates in contrast possess high water solubility at neutral pH values. Sodium caseinates solutions are highly viscous at concentrations about 15 % and display pseudoplastic behavior above this concentration (Singh & Flanagan, 2006; Rollema & Muir, 2009). The handling of solutions at higher concentrations

(20 %) is difficult even at higher temperature due to an increasing shear depending behavior with increasing concentration (Rollema & Muir, 2009). The viscosity is reported to be the lowest at pH 7 and depends on the calcium concentration. The milk proteins can furthermore form rigid, heat-induced irreversible gels which hold water and fat and are useful for structural support (Singh, 2003). Gelation of whey protein occurs between 80-100 °C depending on the purity and concentration (usually above 8 %) and for calcium caseinates reversible gelation occurs for concentrations above 15 % and temperatures between 50-60 °C (Singh & Flanagan, 2006).

1.3.3 Basics of Pressure Sensitivity of Proteins

A pressure increase will favor every reaction with a negative reaction volume according to the principle of Le Chattelier. The volume of a protein can be divided into three contributors: the volume of its atoms, the volume of internal cavities and its solvation volume containing negative parts from the hydration of peptide bonds and amino acid side chains (Kauzmann, 1959; Richards, 1977; Masson, 1992). Surprisingly, Bridgman was also the first who reported an effect of pressure on proteins (Bridgman, 1914). He showed that the coagulation of egg-white albumen is possible at pressures from 300-1200 MPa. Interestingly, he also mentioned the freezing of water to ICE VI at 1200 MPa and 20 °C, but there was no effect of this additional impact. Furthermore, he compared treatments at 0 °C and 20 °C and reported a more distinct coagulation for treatments at 0 °C. These findings might already have been an indicator for the results of Hawley (1971) and Brandts et al. (1970) who found an elliptical curve for the denaturation of proteins (chymotrypsinogen and ribonuclease A). It seems obvious that pressure and temperature cannot be regarded independently. Hawley suggested that a protein can only exist in two defined phases: native or denatured. According to this assumption the denaturation process can be regarded as a phase transition process and the derivation from chapter 1.1.1 can be used as it was also done by Hawley. Consequently, the difference in free energy of both states is given by:

$$\Delta G = G_{denatured} - G_{native} \tag{1.14}$$

Combining equation (1.14) with equation (1.6) for a pure substance gives:

$$\mathrm{d}\Delta G = -\Delta S \mathrm{d}T + \Delta V \mathrm{d}p \tag{1.15}$$

where the Δ denotes the differences between denatured and native state. An integration of equation (1.15) for a chosen reference point T_0 and p_0 with a second-order approximation gives (Smeller, 2002; Meersman et al., 2006a; Meersman et al., 2006b):

$$\begin{aligned}\Delta G = {} & \Delta G_0 - \Delta S_0 \cdot (T - T_0) + \Delta C_p \cdot \left[(T - T_0) - T \cdot \ln\left(\frac{T}{T_0}\right)\right] \\ & + \Delta V_0 \cdot (p - p_0) - \frac{\Delta\beta}{2} \cdot (p - p_0)^2 + \Delta\alpha \cdot (T - T_0) \cdot (p - p_0)\end{aligned} \tag{1.16}.$$

It is important to mention that the exemplary phase boundary which is presented in Figure 11 has only its elliptical shape for the following conditions (Smeller, 2002):

$$\Delta\alpha^2 > \frac{\Delta C_p \cdot \Delta\beta}{T_0} \tag{1.17}.$$

These kinds of phase diagrams can only be drawn for the denaturation or unfolding of a single protein. However, proteins are rarely existent as single chains and thus, interactions have to be taken into account when talking about pressure induced protein denaturation. First of all, the structure of a protein influences its pressure sensitivity. The primary structure and thus, the available amino acids influence the higher structure of a protein. Unfortunately, a prediction of, for instance, secondary structure motifs from the primary structure is not possible until today. However, the primary structure of a protein is supposed to be pressure insensitive up to 1,000-1,500 MPa due to the fact that the reaction volume of bond exchanges and changes in bond angels are nearly zero (Van Eldik et al., 1989; Mozhaev et al., 1996; Hendrickx et al., 1998). Van Eldik et al. (1989) showed a stabilization for hydrogen bonds for a model system which can be traced back to a smaller inter-atomic distance in the hydrogen-bonded atoms (Mozhaev et al., 1996). However, exchanges between existent hydrogen bonds are possible (Van Eldik et al., 1989) and thus, changes in the secondary structures by high pressures are possible. There are shifts between α-helix and β-sheet structures reported (Wong & Heremans, 1988) but no clear direction of changes (increase or decrease) could be pointed out and the reversibility of these changes seems to depend on e.g. decompression rate and the extend of changes in those structures (Carrier et al., 1990). It is generally accepted that applying high pressure leads to the unfolding of proteins which means changing their tertiary structure. There are different

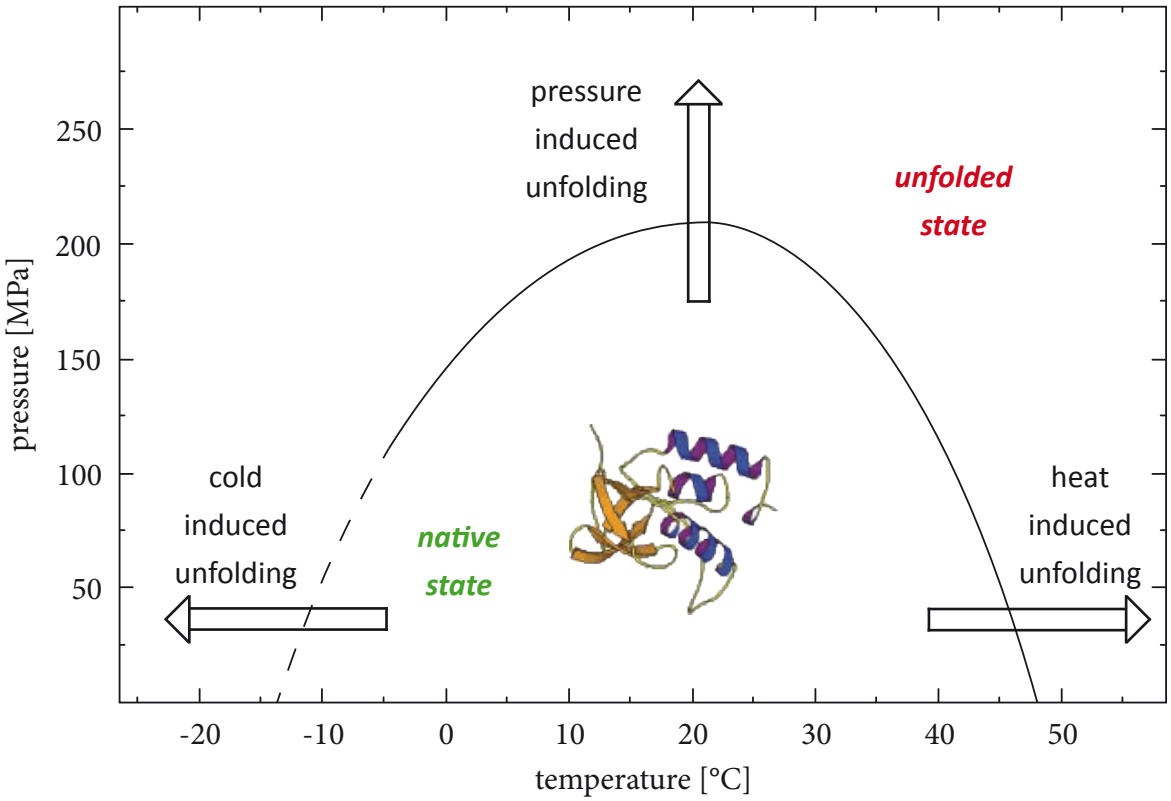


Figure 11: Different possibilities of protein denaturation / unfolding in the p-T domain. Phase boundary (ΔG=0) for staphylococcal nuclease (Daniel et al., 2006; redrawn with modifications)

molecular reasons for the sensitivity of the tertiary structure. The tertiary structure is mainly stabilized by hydrophobic and electrostatic interactions which both are pressure sensitive due to their large negative reaction volumes for disruption (Van Eldik et al., 1989; Mozhaev et al., 1996). Furthermore, the negative reaction volume of unfolding is in the range of tens to hundreds of mL·mol^{-1} (Silva & Weber, 1993; Mozhaev et al., 1996) mainly due to an imperfect packing with the existence of cavities (Richards, 1977; Roche et al., 2012a; Roche et al., 2012b). The fact that oligomeric proteins are mainly stabilized by pressure sensitive electrostatic and hydrophobic interactions clearly explains the distinct pressure sensitivity of the quaternary structure. This leads to a dissociation of oligomeric proteins at quite low pressures of 50-200 MPa (Mozhaev et al., 1996). Schade et al. (1980) reported a large negative reaction volume (-500 mL·mol^{-1}) for the dissociation of oligomeric proteins which clearly depicts the pressure induced dissociation.

It was already in 1899 when Hite showed that high pressure influences, amongst other things, the appearance of milk (Hite, 1899). Since this report there were several studies which investigate pressure effects on milk. Today it is known that high pressure influences the viscosity and the turbidity of milk and promotes the release of calcium which all are related to changes in milk

proteins (Cheftel & Dumay, 1996). Due to the structural differences between WPI and MC also pressure influences on those two fractions are different. The changes in turbidity can be traced back to a change of MC. Several authors reported a change in the micelle size due to pressure treatments. Schmidt & Buchheim (1970) measured a decrease in the micelle size after high pressure treatments by the use of electron microscopy. However, beside the generally reported dissociation of the micelles also the formation of larger aggregates is reported depending on the chosen pressure, temperature and time conditions, and the sample composition. Needs et al. (2000) showed an increase in the micelle size (up to 9 %) after treatment of raw skim milk at 200 MPa, but detected a strong decrease of micelle size (up to 50 %) for higher pressures (250-600MPa). Gaucheron et al. (1997) reported a reduced micelle size for a 250 MPa treatment at 4 °C but an increased micelle size for the same pressure at 40 °C. However, the authors furthermore stated a temperature independent decrease of the micelle size for pressures above 450 MPa. Other authors also reported a temperature independent reduction of the micelle size at higher pressures for skim milk (Huppertz et al., 2004a; Anema et al., 2005). In a pressure range of 200-300 MPa a pH and temperature dependent increase of the micelle size was reported (Huppertz et al., 2004a; Anema et al., 2005). Huppertz & De Kruif (2006, 2007) suggested a mechanism for disruption of casein micelles. The authors proposed that pressure induced solubilization of micellar calcium phosphate leads to a disruption of casein micelles in an early stage of pressure treatments due to electrostriction effects. However, the fact that hydrophobic interactions are the main force in micellization clearly depicts the pressure sensitivity of MC. Other authors suggested furthermore an effect of re-association of micelles upon pressure release (Orlien et al., 2006; Orlien et al., 2010). It is also reported that the fractions of MC dissociate in the order β>κ>α (Lopez-Fandino et al., 1998). Arias et al. (2000) reported a decreased solubilization of MC at pH 5.5 in comparison to pH 7.0. The different results for measuring the micelle sizes clearly depicts the importance of an accurate characterization of the treatment parameters as well as the sample conditions and point out the complexity of investigating pressure effects on MC.
The whey proteins β-Lg and α-Lac differ significantly in their pressure resistance (Messens et al., 1997). This is mainly traced back to the existence of the free thiol group in β-Lg and the higher number of intramolecular disulfide bonds in α-Lac. Johnston et al. (1992) were one of the first who showed a decreased non-casein nitrogen in the milk serum after high pressure treatments and concluded a whey protein denaturation from these findings. It is reported that

a pressure of at least 100 MPa is necessary to denature β-Lg while pressures up to 400 MPa induce a denaturation of about 70-80 % of β-Lg (Lopez-Fandino et al., 1996; Lopez-Fandino & Olano, 1998; Arias et al., 2000; Garcia-Risco et al., 2000; Scollard et al., 2000; Lanciotti et al., 2004; Hinrichs & Rademacher, 2005). However, α-Lac seems to be pressure resistant up to pressures between 400 MPa (Huppertz et al., 2004b) and 500 MPa (Lopez-Fandino et al., 1996; Gaucheron et al., 1997; Lopez-Fandino et al., 1998; Hinrichs & Rademacher, 2005). Furthermore, the pH value during the treatment significantly influences the denaturation of especially β-Lg. It is reported that denaturation at more acidic pH values is less pronounced in comparison to neutral pH (Dufour et al., 1994; Arias et al., 2000). The enhanced denaturation is traced back to an enhanced reactivity of the free thiol group at neutral pH which leads to thiol-disulfide exchanges and thus, to an irreversible unfolding (Cheftel & Dumay, 1996). Hinrichs et al. (1996) found a reaction rate of 2.5 for denaturation of β-Lg and thus, showed a concentration dependent denaturation. Consequently, higher concentrations lead to an increased denaturation in comparison to lower concentration of β-Lg. It is also reported that pressure and temperature have a synergistic effect on the denaturation of β-Lg. Treatments at 300 MPa and 50-60 °C led to the same denaturation (almost 100 %) as treatments at 400 MPa and 40-60 °C (Lopez-Fandino & Olano, 1998; Garcia-Risco et al., 2000) while treatments at 300 MPa and 4 °C led to a decreased denaturation in comparison to treatments at 20 °C (Gaucheron et al., 1997). Furthermore, a strong dependence on the dissolving media is reported. Funtenberger et al. (1995) found the most pronounced denaturation of β-Lg at pH 7 when dissolved in pressure stable bis-Tris buffers while the denaturation was less pronounced in water or phosphate buffer. It is also reported that pressure induces interactions between caseins and whey proteins. Especially, interactions between β-Lg and κ-Cn were found by several authors (Lopez-Fandino et al., 1997; Hinrichs & Rademacher, 2004; Huppertz et al., 2004b; Zobrist et al., 2005). These interactions could lead to an increased denaturation of whey proteins for instance in skim milk (Mazri et al., 2012). Most studies identify the denaturation of whey proteins by detecting a decrease in non-casein nitrogen in the serum phase (solubility at pH 4.6). However, a few studies also investigated structural changes. Hayakawa et al. (1996) showed a reduction in α-helices of β-Lg of about 90 % after a pressure treatment at 1,000 MPa. The authors traced this finding back to a pressure induced breakdown of weak hydrogen bonds and van der Waals forces. Other authors (Wong & Heremans, 1988; Carrier et al., 1990) also reported a shift between α-helices and

β-sheets due to pressure treatments. Another possibility of structural changes seems to be the formation of non-native α-helices from native β-sheet structures (Yang et al., 2001). Belloque et al. (2000) found that pressure treatments at 100 MPa lead to partial unfolding of β-Lg while the core of the molecule remains structured. Furthermore, they reported flexibility of the entire molecule for pressures between 300 and 400 MPa. It was also found that the core of β-Lg A becomes more easily flexible in comparison to β-Lg B. Huppertz et al. (2004b) suggested a three step mechanism for the pressure induced denaturation of β-Lg and α-Lac in milk by combining research results. In a first step β-Lg is unfolded (Kuwata et al., 2001) and the free thiol group is exposed (Tanaka et al., 1996; Moller et al., 1998; Stapelfeldt & Skibsted, 1999). Afterwards, the free thiol group can interact with κ-Cn, α-Lac or β-Lg through thiol-disulfide exchange. Finally, during pressure release unfolded but non-interacting β-Lg and α-Lac refold to an almost native state (Belloque et al., 2000; Ikeuchi et al., 2001).

Changes in functional behavior of milk proteins are of high scientific interest besides studying structural changes. The pressure induced coagulation of egg-white protein (Bridgman, 1914) already indicated that pressure influences functional properties of proteins. Many studies were performed on the rennet coagulation of milk proteins and the influence on cheese making properties (Ohmiya et al., 1987; Desobry-Banon et al., 1994; Lopez-Fandino et al., 1997; Brooker et al., 1998; Lopez-Fandino & Olano, 1998; Pandey et al., 2000; Buffa et al., 2001; Huppertz et al., 2005; Zobrist et al., 2005; Keim et al., 2006; Shaker et al., 2008; Bakopanos et al., 2010). Also the pressure induced gelation properties under different conditions were investigated quite detailed (Zasypkin et al., 1996; Kanno et al., 1998; Ipsen et al., 2000; Abbasi & Dickinson, 2001, 2002; Briscoe et al., 2002a; Briscoe et al., 2002b; Kanno & Mu, 2002; Anema, 2008; He & Ruan, 2009; Anema, 2010; Venir et al., 2010). However, only a few reports on high pressure induced changes in the foaming and emulsification properties are existent. The group of Dickinson did a lot of studies on pressure effects on β-Lg stabilized emulsions in the late 1990th. They found that pressure treatments (up to 800 MPa) of β-Lg or whey protein concentrate solutions lead to the formation of emulsions with higher droplet sizes in comparison to emulsions from native β-Lg (Galazka et al., 1995; Galazka et al., 1996). Furthermore, they showed that a treatment of the whole emulsion at 800 MPa for 60 min leads to a less pronounced destabilization in comparison to a thermal treatment at 65 °C for 5 min (Dickinson & James, 1998). However, high pressure was found to induce flocculation in treated emulsions leading to

an overall increased droplet size (Galazka et al., 1995; Galazka et al., 1996; Dickinson & James, 1998).

Concluding, high pressure induced changes in β-Lg seem not to influence the behavior of the molecules at the interfacial layer in stabilized emulsions (Galazka et al., 1996; Dickinson & James, 1998; Lopez-Fandino, 2006) but influences the behavior during the creation of the emulsion. Similar findings were also made for soy protein isolates (Wang et al., 2008; Li et al., 2011). Dumay et al. (1996) showed that pressure treatments of β-Lg stabilized emulsions at 450 MPa lead to an increase in viscosity when treated at 25 °C in comparison to treatments at 10 °C while treatments at 40 °C lead to gelled emulsions. Other authors treated whey protein isolate (WPI) and concentrate (WPC) solutions at higher concentrations (10 % w/w) and reported decreased emulsification activity and stability (Kresic et al., 2006). Huiping et al. (2011) also reported decreased emulsification properties for pressure treated WPI. However, high pressure seems to have different influences on the single fractions of whey protein. Octavio Rodiles-Lopez et al. (2008) showed that pressure treatment of α-Lac leads to improved emulsification properties. Furthermore, other authors reported decreased emulsion stability for walnut protein (Qin et al., 2012; Qin et al., 2013) indicating that pressure generally influences emulsification properties of proteins. The same authors also reported influences on foaming capacity and foam stability of pressure treated walnut proteins. They found that both parameters slightly increase after pressure treatments. Kresic et al. (2006) also reported an increased foamability for pressure treated WPI but decreased foamability for treated whey protein concentrate. However, the foam stability was increased for both. Nevertheless, other authors (Lim et al., 2008) found an increased overrun for pressure treatments up to 300 MPa for 15 min for whey protein concentrate and an increased foam stability up to 600 MPa. In contrast, Li et al. (2011) found that for pressure treated soy protein isolate the foamability increased with increasing pressure up to 600 MPa while the foam stability decreased. The different findings for same proteins or oppositional behavior of other globular proteins indicate the complexity of pressure induced changes in protein functionality and thus, clearly depicts the need for a deeper research in this field. Still, pressure effects on the functional behavior of caseins or mixtures of whey proteins with caseins like in skim milk cannot be found until today except for the influence on cheese making properties.

1.3.4 HPLT Induced Changes in Proteins

In comparion to the findings on the impact of pressure presented in the previous chapter the amount of available data regarding HPLT effects on proteins is still quite low. It is difficult to divide the possible effects of HPLT treatments. HPLT treatments combine the effects of high pressure and low temperature with another impact – the nucleation of water. It is known that freezing can cause protein denaturation for instance by freeze concentration effects (Franks, 1995) or by interactions with ice crystals (Strambini & Gabellieri, 1996). It is also reported that some enzymes lose their functionality during freezing or storage in frozen state (Tamiya et al., 1985; Seguro et al., 1989; Privalov, 1990) which indicates a protein denaturation (Carpenter et al., 1993; Prestrelski et al., 1993). In conclusion, the different ways of nucleation and the application of different pressure-temperature conditions represent a complex field of research regarding denaturation of proteins. Kolakowski et al. (2001) reported a different structural influence of HPLT treatments in comparison to HP treatments at ambient or elevated temperature on β-Lg. The authors pointed out that pressure treatment at 25 °C (300 MPa) lead to β-Lg aggregation while treatments at lower temperature (+4, -2 or -16 °C) did not cause aggregation. They also found that pressure shift frozen β-Lg retained 93 % of its native structure. The authors concluded that low temperatures protect β-Lg against pressure induced aggregation at pH 7.0. It was also pointed out that pressure induced dissociation of β-Lg dimers (Valente-Mesquita et al., 1998) is more pronounced at low temperatures (Kolakowski et al., 2001). Kolakowski et al. (2001) showed furthermore a marked blue shift in the 4th derivate spectra indication a significantly exposure of tryptophan and tyrosine residues while cooling the samples at 200 MPa to -15 °C. Furthermore, they showed that the exposure of tryptophan and tyrosine residues was also more pronounced for treatments at 300 MPa and 4 °C in comparison to 20 °C in Bis-TRIS buffer and concluded that cold-denatured states of β-Lg are reversible and/or lead to less aggregation after pressure release. Unfortunately, investigations in the sub-zero temperature domain for pressure effects on casein micelles cannot be found until today. Regnault et al. (2004) compared the impact of pressure treatments of skim milk at 20 °C and 9 °C and found that a lower temperature leads to a more pronounced decrease in micelle size and a higher decrease in turbidity. The findings of the present study also indicate that changes of structural and functional properties of WPI and MC induced by HPLT treatments are different in comparison to common HP treatments.

1.4 Pressure and Temperature as a Tool for Specific Protein Modification

Apparently, based on the knowledge about the denaturation of proteins which was reviewed in the previous chapters, the mechanisms of pressure and temperature induced changes in proteins have to be different. Pressure supports reactions with a negative reaction volume and, due to the same principle temperature increases will support endothermic reactions. However, pressure and temperature cannot be regarded as separated parameters as it is obvious from the Gibbs equation. Proteins offer several possibilities of inter- and intramolecular interactions due to their complex structure. Consequently, the combination of a certain pressure and temperature will induce a specific modification of a protein. The application of high temperature can lead to the cleavage of covalent bonds and thus, can modify the primary structure of proteins. However, covalent bonds are supposed to be pressure stable up to 1000-1500 MPa (Mozhaev et al., 1996). Panick et al. (2003) showed that pressure treatments of *Staphylococcus* nuclease always led to a decrease of α-helical and β-sheet in favor of random coil structures but a kind of plateau was reached at 300 MPa. In contrast temperature treatments of 70 °C led to a complete degradation of all secondary structure motifs while further pressure increase did not cause increasing structure degradation. Other authors from this working group showed that for *Staphylococcus* nuclease a temperature induced denaturation (Gibbs free energy becomes zero) can be found at -13.6 °C and 48 °C (Seemann et al., 2001). In conclusion, *Staphylococcus* nuclease was used to create a phase diagram including cold, heat and pressure denaturation (Panick et al., 2003). It could be shown that the nucleation to ICE V did not influence the pressure induced degradation of secondary structure. However, the authors clearly depict two different denatured states of *Staphylococcus* nuclease. Temperature and pressure induced denaturation generate different changes in secondary structure which points out the different denaturation processes of both technologies. Furthermore, a clear formation of new ordered structures could be shown in the case of ubiquitin by other authors (Herberhold & Winter, 2002). It was also shown quite early that pressure seems to stabilize proteins against temperature denaturation (Gekko & Hasegawa, 1989) which is clearly related to the location and orientation of the elliptical phase boundary. Considine et al. (2007a) reviewed former studies of heat and high pressure effects on milk proteins and pointed out some significant differences of the denaturation via both technologies. Panick et al. (1999) showed a temperature induced aggregation accompanied by an increase in

intermolecular β-sheet content of β-Lg above 60 °C while pressure treatments of about 130 MPa led to a decrease in β-sheet structures, unfolding and aggregation. Considine et al. (2007b) proposed a model for the pressure and temperature induced denaturation. They showed that temperatures above 75 °C induce a wide range of disulfide-linked β-Lg polymers and stable hydrophobic adducts. In contrast, pressure treatments between 150 and 200 MPa mainly generated non-native β-Lg monomers and polymers were found at higher pressures but without the presence of hydrophobic adducts. They concluded that the absence of hydrophobic adducts is due to the weakening of hydrophobic interactions under high pressure. Also differences between pressure and temperature effects on α-Lac were found. While high temperatures can induce large disulfide linked dimers and larger aggregates (Lyster, 1970) those aggregates were found to be absent after pressure treatments (Patel et al., 2005). Patel et al. (2005) furthermore showed that the loss in solubility is β-Lg>BSA>α-Lac for pressure treatments while it is BSA>β-Lg~α-Lac for thermal treatments (Havea et al., 1998). However, protein aggregation in this case is reported to be quite similar between pressure and temperature – but as a main difference the rupture of covalent bonds during high temperature treatments which does not occur under high pressures should be pointed out. There are also differences between pressure and temperature effects on casein micelles reported. Anema & Li (2003a, 2003b) showed a marked increase in micelle size when skim milk was heat treated at pH 6.5 while a decrease was shown at higher pH (>6.7). In contrast pressure treatments of 300 MPa at neutral pH were found to induce a strong decrease in micelle size (Gaucheron et al., 1997; Anema et al., 2005). However, due to the large amount of possibilities to combine pressure and temperature and their thermodynamic linkage, it is difficult to draw general conclusions on the different effects but the large amount of combinations offer the opportunity of specific protein modifications especially in protein mixtures. Different protein fractions can be affected if their phase ellipses are not identical and consequently pressure and temperature can be used as a specific tool for protein modification.

1.5 HPLT Treatments – Not Only a Simple Pressure-Temperature Combination

As mentioned above, HPLT treatments combine effects of cold temperature, high pressure and nucleation of water. Consequently, several additional influences like freeze concentration effects and mechanical stress have to be taken into account. It is reported for different fields of food technology that HPLT processes have special effects on products, product compounds or microorganisms. It was already shown in the 1960s that shifts between ICE III and ICE I led to higher inactivation in comparison to freezing to ICE I or pressure treatments without freezing (Edebo & Heden, 1960; Hedén, 1964). These findings were also confirmed more recently for *Listeria inoccua* (Luscher et al., 2004) and vegetative cells of *Bacillus subtilis* (Shen et al., 2009). Choi et al. (2008) reported that *Escherichia coli* and *Listeria monocytogenes* can be significantly inactivated by pressure shift freezing in milk while no significant inactivation could be detected for *Staphylococcus aureus*. These results indicate that there is also a biodiversity regarding pressure shift freezing like it is already known for high pressure treatments. Kalichevsky et al. (1995) suggested possible applications for HPLT, for instance for gentle freezing processes. Haas et al. (1972) showed that pressure assisted freezing to ICE I (0.3-10 MPa) with a subsequent air or freeze drying led to a reduced cell damage for several vegetables and meat in comparison to conventional freezing. Kanda et al. (1992) and Fuchigami & Teramoto (1997) found that pressure shift freezing of tofu preserves the structure of the product while air frozen tofu was deformed and showed drip loss after thawing. Pressure shift freezing of mango and peaches was also found to cause less cell cracking in comparison to air-blast freezing at -40 °C (Otero et al., 2000). These findings were confirmed for carrots (Fuchigami et al., 1997; Van Buggenhout et al., 2007), potatoes (Koch et al., 1996; Schlüter et al., 2004; Urrutia-Benet et al., 2007; Zhiyi et al., 2012) and eggplants (Otero et al., 1998). Urrutia, et al. (2007) showed in European project "SAFE ICE" via microscopic online observations the effects of HPLT treatments on plant, fish and meat tissues, starch granules and gels. Figure 12 exemplarily shows the effect of a PAF treatment and subsequent recrystalization on apple tissue. It can be seen that especially the recrystalization to ICE I induces extensive cell disruption which can be traced back to the large volume change (see 1.2.1). It was also shown by another working group that pressure assisted thawing preserves quality of for instance surimi (Takai et al., 1991) or beef (Deuchi & Hayashi, 1991; Makita, 1992). Other authors found that pressure shift freezing of salmons caused less

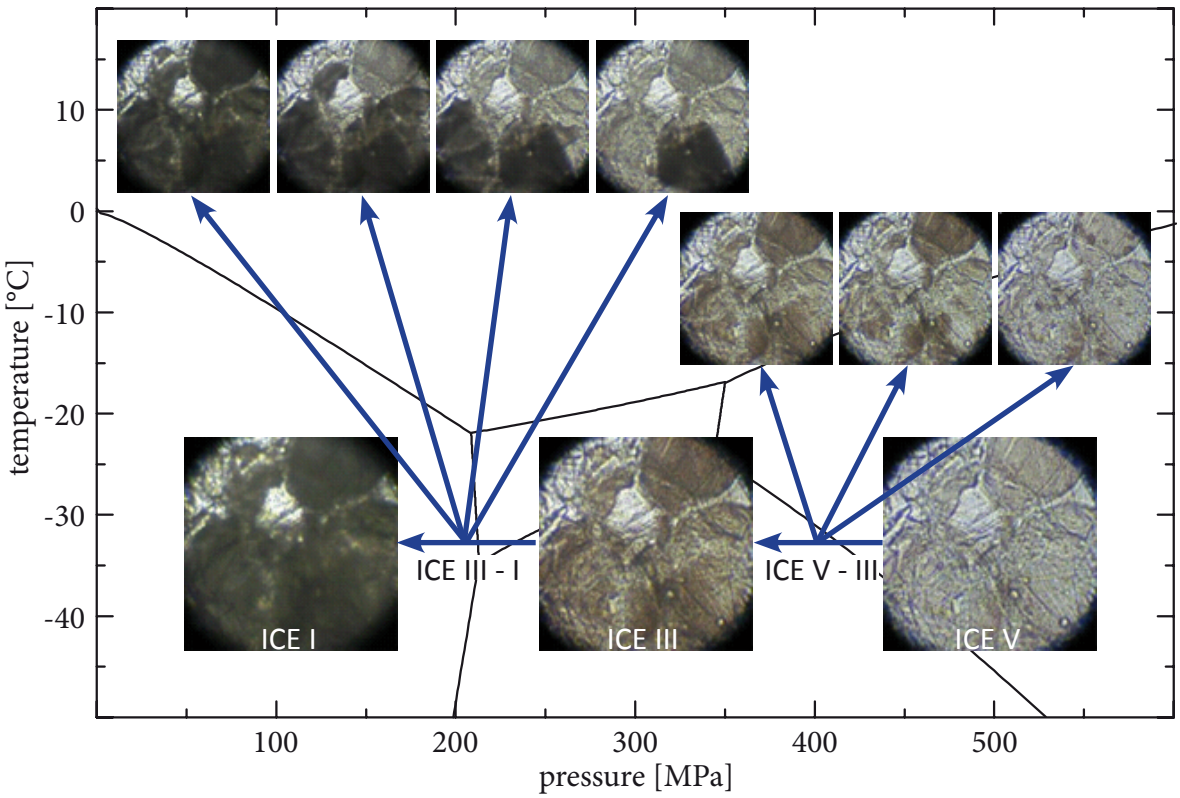


Figure 12: Microscopic online pictures form apple tissue frozen in ICE V with recrystalization to ICE III and ICE I (redrawn from Urrutia et al., 2007)

cell damage in comparison to air blast freezing (Alizadeh et al., 2005; Alizadeh et al., 2007). Recently, Vaudagna et al. (2012) showed that solid-solid phase transitions of frozen cured beef carpaccio caused less color and textural changes but increased expressible water in comparison to unfrozen pressure treated samples. However, it is also mentioned that HPLT treatments can cause protein denaturation and high color changes (Massaux et al., 1999; Chevalier et al., 2000; Fernandez-Martin et al., 2000) and thus, beneficial freezing or thawing parameters for muscle food still have to be identified (Cheftel et al., 2000; Cheftel et al., 2002). Johnston (2000) investigated the influence of pressure shift freezing and pressure assisted thawing (according to the used nomenclature) on rheological properties of cheddar and mozzarella cheese. The author found that pressure shift freezing and pressure assisted thawing avoid most of the changes which occur due to freezing and thawing at atmospheric pressure but cheddar cheeses were different to untreated ones. Treated cheeses seem to be more elastic in comparison to the untreated in both cases. However, same changes occurred for the mozzarella cheese but those cheeses were not affected by conventional freezing. Barry et al. (1998) showed that pressure shift freezing lead to a better preservation of the microscopic structure of a heat induced β-Lg gel in comparison

to freezing at atmospheric pressure. These results were also confirmed for agar, gelatin, starch, ovalbumin and β-Lg gels by other authors (Kalichevsky-Dong et al., 2000). Yet, these authors found that although the structure was preserved quite well for all gels the strength was decreased for agar and gelatin gels and increased for starch and protein gels. Lille & Autio (2007) showed that pressure shift freezing leads to the formation of smaller pores with a homogeneous distribution in starch gels compared to freezing in still air or in liquid. Furthermore, it was shown by other authors (Levy et al., 1999, 2000; Thiebaud et al., 2002) that pressure shift freezing of oil-water emulsions induce irregular shaped ice crystals or clusters of crystals which were homogeneously distributed all over the sample. The authors concluded that initially created ice crystals aggregate during the growth and formed the mentioned clusters. Otero et al. (2012) investigated the potential of pressure shift freezing for freeze concentration of orange juice. The authors showed that the concentration increases and the ice crystal size decreases with increasing pressure and decreasing temperature. Furthermore, the possibility of subzero storage in the liquid state to avoid damages was already mentioned about 35 years ago (Charm et al., 1977). It was suggested by the authors that subzero storage under pressure could be an economic alternative for frozen storage of food. Volkert et al. (2012) investigated the influence of HPLT treatments on sugar rich dairy based frozen food emulsions and foams. The authors showed that pressure assisted freezing of aerated foams leads to smaller ice crystals and an increased smoothness and mouth coating in comparison to conventional frozen samples. Recently pressure shift freezing was also proposed as an alternative for the creation of decellularized scaffolds (Eichhorn, et al.). The examples mentioned above depict the complexity of the field of HPLT treatments and points out the broad variety of possible applications. However, there is still a high need for research on these topics to understand the mechanisms of detected effects and to identify the most proper process conditions.

2. Results and Publications

The following chapter includes the publications and additional results for connecting the experimental parts. The development of the experimental plan is included in the connecting paragraphs and supported by further experimental data.

Previous studies showed a specific HPLT effect on dairy based foams and emulsions (Volkert et al., 2012). It is reported that PSF treated samples possessed a reduced average ice crystal size with a shift of the whole distribution to smaller diameters. HPLT treated samples showed a reduced overrun, whereas especially samples after solid-solid phase transitions (ICE I – ICE III – ICE I) were found to contain a highly decreased amount of air. However, it was also shown that HPLT treated samples had an increased smoothness and mouth coating which clearly indicates the potential of HPLT treatments to generate specifically modified protein based samples. Based on the findings of the mentioned study it was proposed that, beside the reduction in ice crystal size, especially a modification of the milk proteins should be responsible for the changed sensorial properties. The aim of the present study is to identify HPLT induced changes in whey proteins and micellar caseins in simple water based dispersions. Modifications are investigated in different scales – on molecular, macroscopic and functional level.

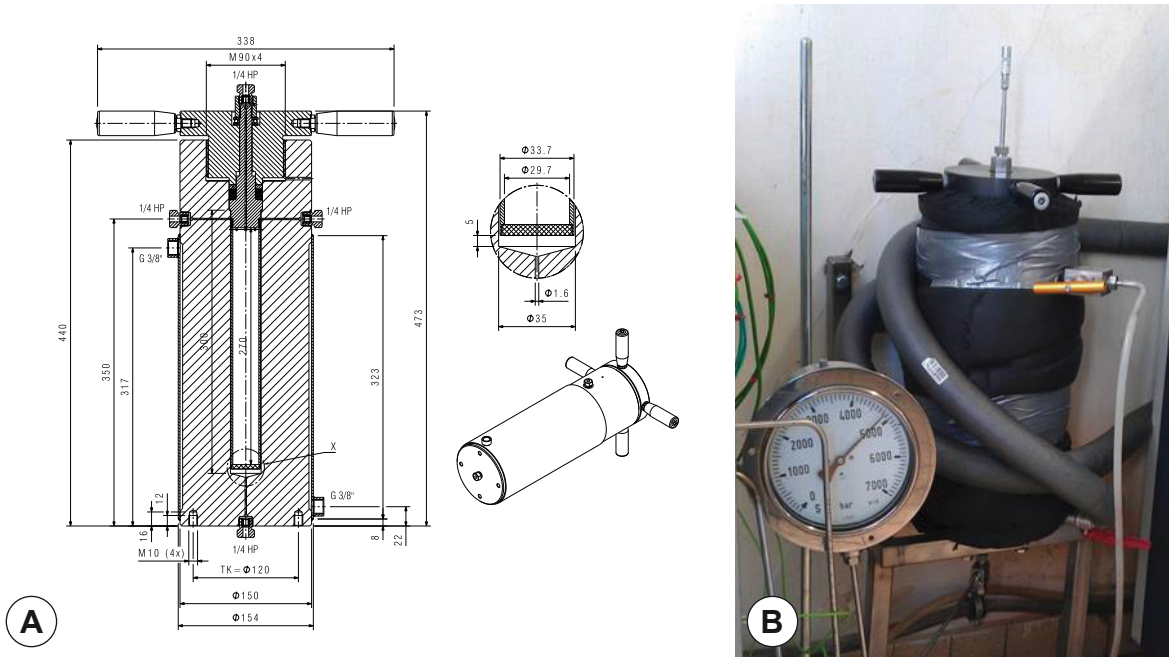


Figure 13: A: Design drawing of the HPLT vessel (SITEC). B: Photograph of the HPLT Vessel.

The aim of the first experimental part is to screen the pressure range between 100 and 600 MPa to identify the most effective treatment conditions. A new HPLT unit for using pressures up to 600 MPa in a temperature range from -50 to 100 °C was built. Figure 13 shows the HPLT unit and the design drawing of the vessel. The vessel is equipped with several access options to implement thermocouples or to connect high pressure tubes. The construction enables online pressure and temperature measurements.

Samples are treated at 2 % (w/w) protein concentration in deionized water to avoid gelation during the treatments. Dispersions are either treated at pH 7.0 with intend to have similar conditions like in bovine milk or at pH 5.8 to reduce the electrostatic repulsion of the proteins by getting closer to their isoelectric point. The pH is adjusted by the addition of 1 M HCl or NaOH. It has been decided not to treat the proteins in a buffer to prevent additional ionic effects on the protein modification. Changes in the solubility of WPI and MC fractions at sample pH (7.0 or 5.8) or at pH 4.6 are taken as an indicator for protein modification. The results of the first experimental section of this project are summarized in the following chapter.

2.1 Effect of High Pressure - Low Temperature Processing on Composition and Colloidal Stability of Casein Micelles and Whey Proteins

2.1.1 Manuscript

Authors: Daniel Baier, Christophe Schmitt and Dietrich Knorr

Revised version accepted by International Dairy Journal

Effect of high pressure - low temperature processing on composition and colloidal stability of casein micelles and whey proteins

Daniel Baier[1], Christophe Schmitt[2] and Dietrich Knorr[1]

[1] TU Berlin, Dept. of Food Biotechn. and Food Process Eng., Berlin, Germany

[2] Nestlé Research Center, Department. of Food Science and Technolgy., CH-1000 Lausanne 26, Switzerland

1 Abstract

The aim of this study was to identify the impact of high pressure treatments in the subzero temperature domain (HPLT – high pressure - low temperature) on milk protein fractions. Single whey protein solutions, micellar casein dispersions and two mixtures (micellar caseins:whey proteins weight mixing ratios 80:20 and 20:80) were treated at a concentration of 2 % (w/w) and at two different pH values (7.0 and 5.8). Pressures varying from 100 to 600 MPa were applied at three different temperatures (-15 °C, -35 °C and ambient temperature) to identify the effect of freezing (pressure assisted freezing, pressure shift freezing) on colloidal stability of proteins. Changes in the composition of the protein fractions were studied by identifying the soluble fractions at pH 7.0 and pH 4.6 via quantitative reversed-phase HLPC (RP-HPLC). Furthermore, the absorbance of the samples was measured and the particle size distributions of the soluble protein fractions were determined by dynamic light scattering.

The whey protein fractions could only be affected by HPLT treatments at pH 7.0 if caseins were present in the samples (mixtures). However, HPLT treatments at pH 5.8 could affect the whey protein fractions also without the presence of high amounts of caseins but effects in milk-like mixtures were decreased. The β-lactoglobulin

fractions formed specific aggregates containing native protein when frozen to higher ice formations at pH 5.8. The casein fractions formed on the one hand large aggregates (flocks) and on the other hand the solubility (non-sedimented particles after centrifugation) was increased by the creation of smaller micelles. All pressure treatments led to a decrease in light absorbance. However, the formation of flocks could be observed only for HPLT treated samples, which leads to the conclusion that HPLT treatments can induce a special structure in milk protein based samples. Treatments at pH 5.8 decreased the effects of treatments on casein fractions. Furthermore, a slow decompression rate increased the HPLT effects on the whey proteins while caseins were not affected. An influence of the released calcium form casein micelles on the denaturation of whey protein fractions could not be found for HPLT treatments.

2 Introduction

High pressure processing was mainly seen as an alternative pasteurization or sterilization method over a long period of time. However, the high potential of using high pressure to modify food ingredients or to produce new kinds of sensorial properties came into the focus of food science and product development. The majority of published studies deal with the effect of high pressure applied at room temperature or in some cases at elevated temperatures (Messens, VanCamp, & Huyghebaert, 1997). Although milk is not a commercially available pressure treated product the first scientific report on pressure treatments of food dealt with the inactivation of microorganisms in milk (Hite, 1899). However, high pressure treatments and research in this field was longtime focused on microbial inactivation and the application of high pressure as an alternative preservation method. Bridgman (1914) was the first who reported the potential of high pressure to denature proteins by showing the pressure induced coagulation of egg white protein. Since the 1990s also the

investigations of pressure effects on bovine milk proteins increased (Huppertz, Fox, de Kruif, & Kelly, 2006). Most of these studies were performed at room temperature or elevated temperatures. Regarding the elliptical shape of the phase diagram of proteins (Smeller, 2002) it is obvious that beside a cold denaturation also the combination of low temperatures and high pressures can lead to a modification of proteins. According to the Chatelier's principle pressure will promote every reaction which has a negative reaction volume (e.g. unfolding of globular proteins). However, in the subzero domain of the phase diagram of water an additional factor has to be taken into account – the nucleation of water. Bridgman (1912) already showed that

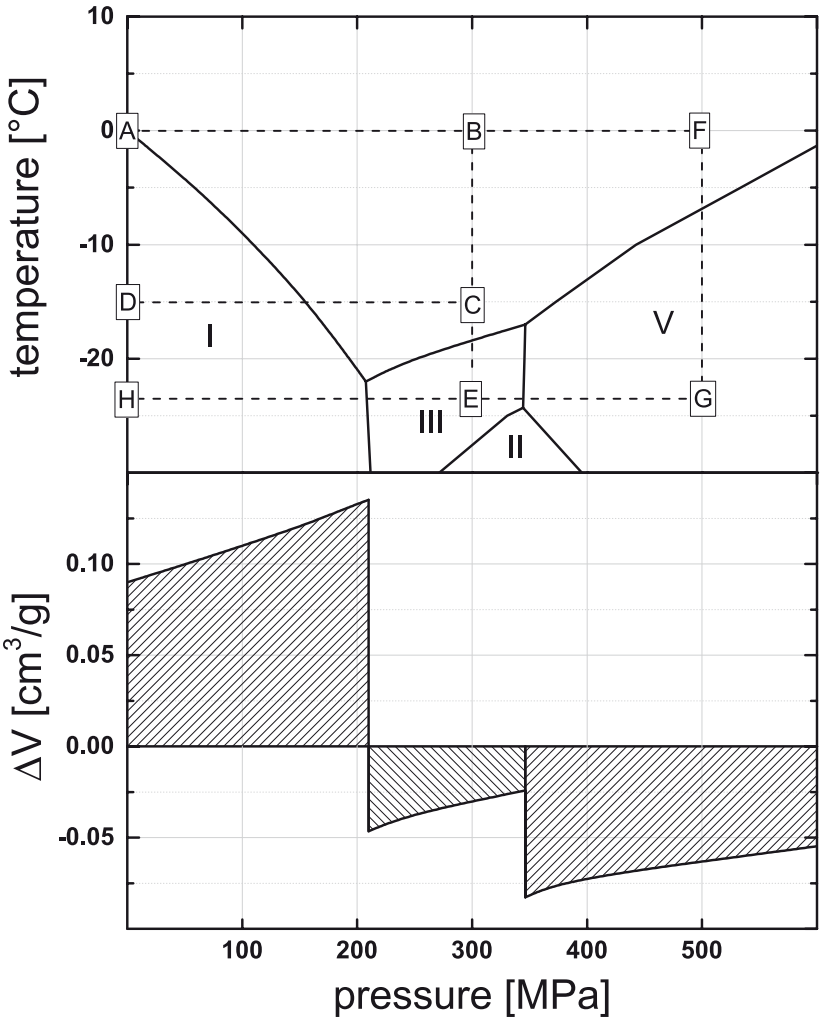


Figure 1: Top: Phase diagram of water with HPLT process options – A-B-C-D: Pressure shift freezing; A-B-E-H and A-F-G-H: Pressure assisted freezing to ICE III and V. Bottom: Volume changes during phase transitions. Data obtained from Bridgman (1912), Fletcher (1970).

pressure has an impact on the nucleation of water and developed the first phase diagram of water. The combination of high pressure and subzero temperatures enables a couple of new process options a complete overview is given by Urrutia Benet et al. (2004). Two different process options were used within this study – pressure assisted freezing (PAF) and pressure shift freezing (PSF). According to the given terminology (Urrutia Benet, et al., 2004) pressure assisted freezing means that an unfrozen sample is frozen at (constant) high pressure and the temperature gradient between the cooling medium and the sample is the driving force for the freezing process. This process allows freezing of water to three different ice-modifications (ICE I, III and V) within the relevant pressure range (up to 600 MPa). These ice modifications differ in their crystal structure and consequently in their density (see Figure 1). ICE I (frozen water at atmospheric pressure) is the only ice modification with a lower density as liquid water, while ICE III has the highest and ICE VI has the second highest density among the three. In conclusion, this fact denotes that a recrystallization to ICE I during the decompression leads to a high volume change and, thus, to an additional mechanical stress (see Figure 1). Pressure shift freezing in contrast means that the sample is cooled down under pressure without nucleation and the phase transition to ICE I occurs during the decompression. This instantaneous nucleation leads to a homogeneous size distribution of very small ice crystals. Several authors reported an enhanced inactivation of microorganisms pressure treated in the subzero temperature domain (Hashizume, Kimura, & Hayashi, 1995; Hayakawa, Ueno, Kawamura, Kato, & Hayashi, 1998; Luscher, Balasa, Frohling, Ananta, & Knorr, 2004; Moussa, Perrier-Cornet, & Gervais, 2007; Perrier-Cornet, Tapin, Gaeta, & Gervais, 2005; Picart, Dumay, Guiraud, & Cheftel, 2004, 2005). An overview on the applications of HPLT processes can be found in the review of Cheftel, Thiebaud, & Dumay (2002). However, only a few investigations were made for HPLT treatments of milk proteins. Kolakowski, Dumay, & Cheftel (2001) showed that unfolding of β-lactoglobulin is reduced for pressure treatments

at -2 °C in comparison to treatments at 25 °C. The authors also reported only a slight denaturation (97 % of native structure) for pressure shift freezing at -16 °C and 300 MPa. Volkert, Puaud, Wille, & Knorr (2012) suggested that changes in the protein conformation could be responsible for changed sensorial properties in HPLT treated sugar rich dairy based frozen food foams.

The protein fractions of milk can generally be divided into two fractions – the whey proteins and the micellar caseins (Fox, 1992). The caseins represent about 80 % of the bovine milk protein and they are organized in micelles and sub-micelles which are insoluble at pH 4.6. This micellar structure is known to be pressure sensitive and dissociation and re-aggregation of these micelles is reported by several authors (Anema, Lowe, & Stockmann, 2005; Gaucheron, et al., 1997; Huppertz & De Kruif, 2006; Huppertz, Fox, et al., 2006; Huppertz, Kelly, & Fox, 2006; Huppertz & de Kruif, 2007; Knudsen & Skibsted, 2010; Needs, Stenning, Gill, Ferragut, & Rich, 2000; Orlien, Knudsen, Colon, & Skibsted, 2006; Orlien, Boserup, & Olsen, 2010; Schmidt & Buchheim, 1970; Shibauchi, Yamamoto, & Sagara, 1992). Schmidt & Buchheim (1970) already identified a decrease in the size of casein micelles after HP treatments. The results which were reported until today show partly different effects of HP treatments on the size of casein micelles. The effect of HP on the size of micelles depends strongly on the applied pressure, the treatment temperature, the treatment time and on the sample composition (raw milk, reconstituted skim milk). An increase in the average micelle size as well as a decrease could be achieved by controlling these parameters. Furthermore, an increase of caseins in the soluble phase of milk is reported for HP treatments (Garcia-Risco, Olano, Ramos, & Lopez-Fandino, 2000; Huppertz, Fox, & Kelly, 2004b; Lopez-Fandino, De La Fuente, Ramos, & Olano, 1998). The caseins consist mainly of α-, β- and κ-casein. It was found that in bovine milk the dissociation from micelles is in the order β>κ>α (Lopez-Fandino, et al., 1998).

The native globular whey proteins are soluble at pH 4.6 and consist of three major

protein fractions. The largest fraction (~56 %) represent the β-lactoglobulin (β-Lg) with the two sub-fractions β-Lg A and B. β-Lg is known to be more pressure sensitive in comparison to the second major protein fraction – the α-lactalbumin (α-Lac, ~20 %) (Messens, et al., 1997). The different pressure sensitivity refers to the different content in disulfide bonds and free sulfhydryl groups. The α-Lac molecule contains no free sulfhydryl groups and four disulfide bonds, while both β-Lg types contain one free and reactive sulfhydryl group and two disulfide bonds. Several studies (Arias, Lopez-Fandino, & Olano, 2000; Hinrichs & Rademacher, 2005; Lopez-Fandino, Carrascosa, & Olano, 1996; Lopez-Fandino & Olano, 1998) reported that pressures above 100 MPa are necessary to induce denaturation of β-Lg and pressures up to 400 MPa result in a denaturation of 70-80 %. It is also reported that treatment temperature is a very important factor regarding the denaturation of β-Lg. Gaucheron, et al. (1997) reported a decreased denaturation for treatments at 4 °C in comparison with 20 °C. In comparison, α-Lac seems to be resistant up to pressures between 400 MPa (Huppertz, Fox, & Kelly, 2004a) and 500 MPa (Gaucheron, et al., 1997; Hinrichs & Rademacher, 2005; Lopez-Fandino, et al., 1996; Lopez-Fandino & Olano, 1998). Furthermore, interactions between caseins and whey proteins, especially β-Lg and κ-Cn, were found by several authors (Hinrichs & Rademacher, 2004; Huppertz, Fox, & Kelly, 2004c; Lopez-Fandino, Ramos, & Olano, 1997; Zobrist, Huppertz, Uniacke, Fox, & Kelly, 2005). Therefore, increased denaturation of whey proteins were reported in e.g. skimmed milk in comparison to WPI solutions (see e.g. Mazri, Sanchez, Ramos, Calvo, & Perez, 2012). Bravo, Felipe, Lopez-Fandino, & Molina (2013) also reported a dependence of the denaturation of WPI on the scale of the equipment while the dissociation of casein micelles was independent (effects were supposed to be due to different temperature distributions). It was also reported that the pressure release rate influences the denaturation of milk proteins (Bravo, Molina, & Lopez-Fandino, 2012; Merel-Rausch, Kulozik, & Hinrichs, 2007). Detailed overviews on HP effects on milk proteins are given in several review articles

(Chawla, Patil, & Singh, 2011; Cheftel & Dumay, 1996; Considine, Patel, Anema, Singh, & Creamer, 2007; Huppertz, Kelly, & Fox, 2002; Huppertz, Fox, et al., 2006; Huppertz, Smiddy, Upadhyay, & Kelly, 2006).

The aim of this study is to point out basic effects of different HPLT process options on milk protein fractions and to compare them with effects of pressure treatments at room temperature to evaluate the potential of HPLT treatments for protein modification.

3 Material and Methods

3.1 Material

Whey protein isolate powder (WPI) was obtained from Fonterra (WPI 895, Fonterra, Auckland, New Zealand). This WPI is obtained by ion exchange and ultrafiltration of sweet whey. The protein content of the powder was 92.63 % (w/w), furthermore it contained 0.18 % (w/w) fat, 5.87 % (w/w) moisture and 1.6 % (w/w) ash. Micellar casein powder (MC) in an almost native state was obtained from the Hungarian Dairy Research Institute (MPI-85 MC, Hungarian Dairy Research Institute, Mosonmagyaróvár, Hungary). These micelles were manufactured by microfiltration and ultrafiltration of skimmed milk. The powder contained 85.1 % (w/w) protein, 1.5 % (w/w) fat, 4.9 % (w/w) water and 7.5 % (w/w) ash.

3.2 Methods

3.2.1 Sample preparation

The WPI solutions were prepared by diluting a specific amount of powder in deionized water and stirring it for 1 h at room temperature. The MC dispersions were prepared by giving a specific amount of powder to preheated deionized water (50 °C), stirring it for 1 h and gently homogenizing it in a high pressure homogenizer (ElmusiFlex-C5, Avestin, Inc., Ottawa, Canada) at a maximum pressure of 30 MPa.

Protein dispersions were prepared on a w/w ratio and pH values were either 7.0 (native) or set to 5.8 using HCl and NaOH (1 M, Merck KGaA, Darmstadt, Germany). The samples were double packed in PE pouches to strictly avoid a penetration of the pressure transmitting medium (PTM). All samples were freshly prepared and kept at 4 °C until analyses.

3.2.2 HPLT treatments

The HPLT treatments were conducted in an experimental HPLT unit containing a high pressure vessel with 265 mL volume (Sitec Sieber AG, Zurich, Switzerland) connected to an air driven high pressure pump (DS XHW-1373 Haskel, CA, USA). The vessel is equipped with a heating-cooling jacket and tempering was realized with a cryostat (Ultra-Kryomat RUK 50-D, Lauda, Germany). An 80 % (v/v) ethanol water mixture was used as tempering medium as well as pressure transmitting medium (PTM, freezing point below -59 °C). Two type K thermocouples enabled temperature measurements of the PTM at the bottom of the vessel and inside of a sample at the top of the vessel. The pressure was measured with a pressure transducer (Intersonde HP28, Watford, England). The samples were thawed at room temperature before further preparations or analytics.

3.2.3 HPLC based quantification of changes in protein fractions

A centrifugation step for 30 min at 10,000 g was applied to obtain a supernatant containing the soluble fraction. Furthermore, the acid soluble fraction was determined by lowering the pH to 4.6 and a subsequent centrifugation step as described above. A HPLC method according to Bordin et al. (2001) was applied to detect changes in the compositions of the protein fractions (WPI/MC) and to quantify losses due to aggregation / flocculation. A C4 column (Jupiter 5u, C4, 300A, 150 x 2.0 mm, Phenomenex Inc., CA, USA) was used in a Dionex UltiMate 3000 System with a diode array detector (DAD-3000), a LPG-3400SD pump and an auto sampler (Dionex Corp., Sunnyvale, CA, USA). The temperature of the column was kept at 40 °C. A

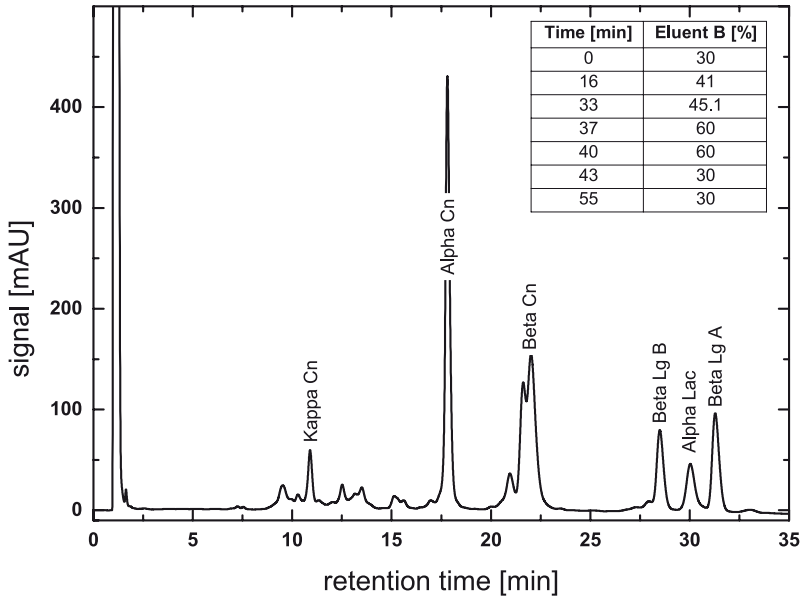


Figure 2: Sample chromatogram of a casein / whey protein dispersion (pH 7.0, 2 % protein, w/w, 80 % casein:20 % whey protein). Table: Gradient of eluent B (0.17 % TFA in acetonitrile).

0.2 % (v/v) solution of trifluoro acetic acid (TFA) in water was used as eluent A and 0.17 % (v/v) TFA in acetonitrile was the composition of eluent B. The gradient and a sample chromatogram are shown in Figure 2. The flow was 0.5 mL/min and the pressure was about 90 bar. The injected sample volume was 20 µL (if not differently indicated). The signal was detected at 214 nm and pure protein standards (Sigma-Aldrich St. Louis, MO, USA) were used for identification. The protein samples were prepared as follows. Samples were diluted 1:5 (v/v) with a Guanidine-HCl-solution. 100 mL of Guanidine-HCl-solution contained 183.8 mg sodium citrate, 385.6 mg DTT and 71.65 g guanidine. Samples were heated to 60°C after diluting with the Guanidine-HCl-solution and cooled down to room temperature. The samples were filtered through a 0.45 µm filter after a further 1:4 (v/v) dilution step with deionized water. All samples were prepared for the HPLC analyses immediately after the treatments to avoid further formation or disruption of aggregates.

3.2.4 Particle size distribution / Turbidity measurements

The turbidity of the samples was measured at 600 nm wavelength with a UV/VIS spectrophotometer via recording of the sample absorbance (Lambda 25, Perkin Elmer, Waltham, MA, USA). Samples were diluted with deionized water to achieve absorbance values below 1. Measurements were performed in disposable semi-micro polystyrene cuvettes (VWR International bvba, Leuven, Belgium). All measurements were performed in triplicates.

The changes in particle size distribution of the soluble phase in samples containing casein were measured by using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments GmbH, Herrenberg, Germany). The measurements were performed at 25 °C (5 min equilibration time) in triplicates. The scattered light was collected in an angle of 173°. All measurements were conducted in disposable semi-micro polystyrene cuvettes (VWR International bvba, Leuven, Belgium). Measurements were performed within 1 h after treatments.

3.2.5 Determination of Ca2+-ion release

The release of calcium from pressure treated MC samples was determined by using a calcium sensitive electrode (perfectION™ comb Ca Combination Electrode, Mettler-Toledo GmbH, Gießen, Germany). The system was freshly calibrated before the measurements with a diluted calcium standard solution (CA STANDARD 1000 MG/L, Mettler-Toledo GmbH, Gießen, Germany). All measurements were performed in triplicates at room temperature and immediately after the treatments.

4 Results and discussion

4.1 Influence of sample composition

Single protein dispersions and mixtures were treated to identify interactions between protein fractions and, thus, draw conclusions on the impact of the sample

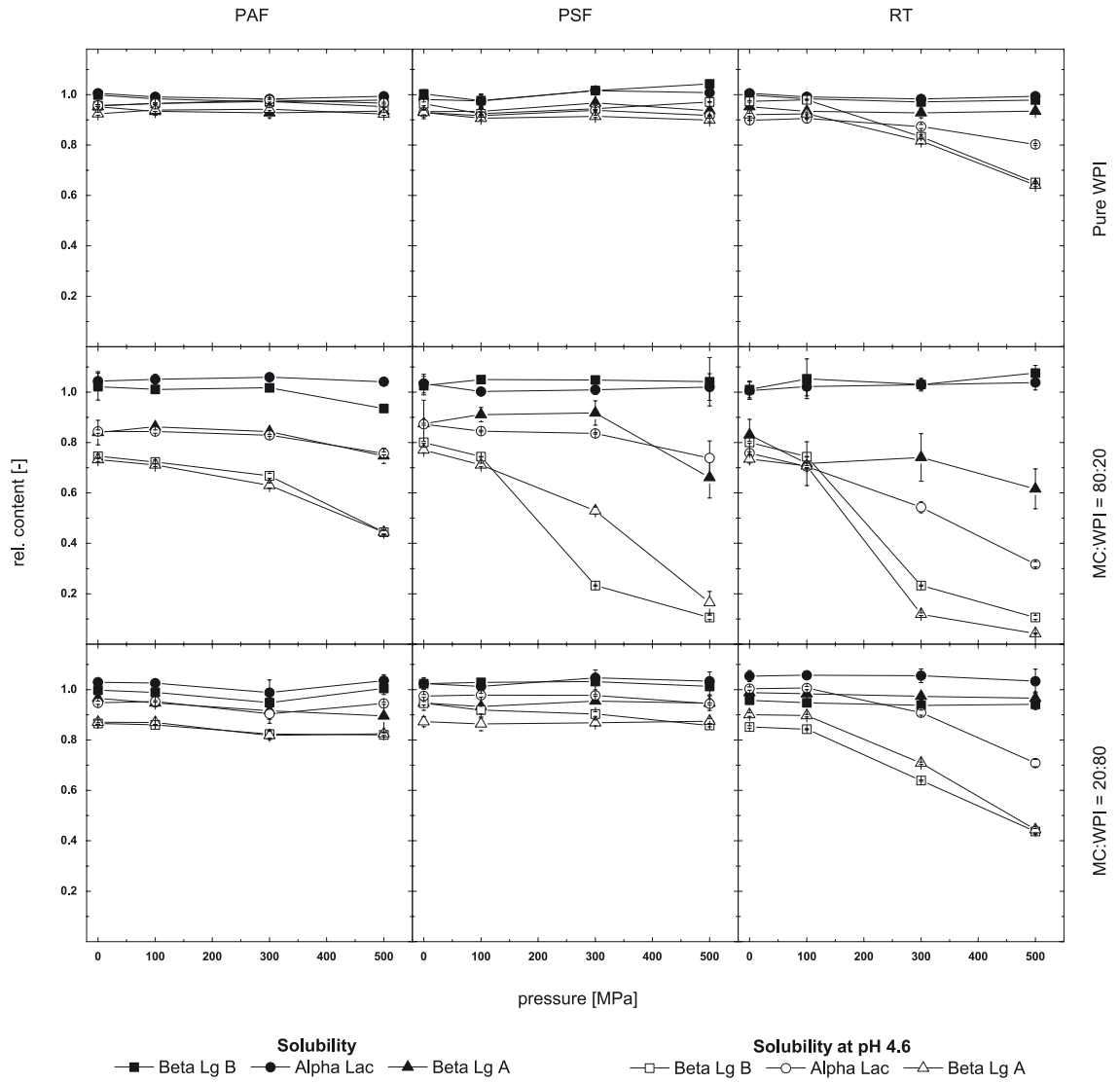


Figure 3: Relative content of WPI fractions after centrifugation (soluble fraction) at native sample pH-value (pH 7) and at pH 4.6 for different treatments. Contents are normalized by the protein content of the respective untreated sample (without any centrifugation step).

composition. Figure 3 shows the results for the WPI fractions of samples treated at pH 7.0 at different pressures, temperatures and sample compositions. A decrease in the solubility at pH 4.6 could be detected for pure WPI solutions treated at pressures above 100 MPa at room temperature. However, HPLT treatments (PAF and PSF) could not affect WPI fractions in a pure solution at pH 7.0. A similar effect was reported by Kolakowski et al. (2001) who could not found a pressure induced formation of β-Lg aggregates for treatments at low temperatures (4 °C, -2 °C) and only a very

small unfolding for a PSF treatment at -20 °C. The low temperature seems to inhibit pressure induced aggregation of the WPI fractions or lower the aggregation rate to very small values so that it occurred out of the experimental window. The results obtained at room temperature are in accordance with other reports from literature (Huppertz, et al., 2002; Huppertz, et al., 2004c) where a denaturation of β-Lg up to 90 % at pressures above 400 MPa in milk was shown and a minimum pressure of above 100 MPa was stated for denaturation. Treatments in the subzero temperature domain only affected the WPI in the presence of MC. The largest changes in protein composition were observed for treatments where MC was the main protein fraction (MC:WPI = 80:20). This effect leads to the assumption that the presence of MC promotes the denaturation of WPI and a minimum amount is necessary to observe this effect. Mazri, et al. (2012) reported a higher denaturation of whey proteins for pressure treatments of reconstituted skimmed milk or whey in comparison to WPI solutions which were pressure treated at 20 °C. This effect can be traced back to an interaction which occurs especially between the two β-Lg fractions and the κ-Cn (Hinrichs & Rademacher, 2004; Huppertz, et al., 2004a; Lopez-Fandino, et al., 1997; Zobrist, et al., 2005). A decrease of soluble β-Lg fractions was only observed for treatments of 80:20 mixtures. This decrease was the highest for PSF treated samples and the largest effect was observed for β-Lg A. This leads to the assumption that interactions between caseins and β-Lg need a certain amount of caseins.

A higher resistance of α-Lac, which is known for treatments at room or elevated temperature, could also be found for HPLT treatments. This higher resistance can be explained by the different structure of β-Lg and α-Lac (Gaucheron, et al., 1997; Hinrichs, Rademacher, & Kessler, 1996; Lopez-Fandino, et al., 1996; Messens, et al., 1997). The higher amount of intramolecular disulphide bonds and the absence of a free sulfhydryl group are suggested to be responsible for the higher baroresistance of α-Lac. Furthermore, pressures above 100 MPa seem to be necessary for a specific denaturation of WPI fractions which is in accordance with the findings of

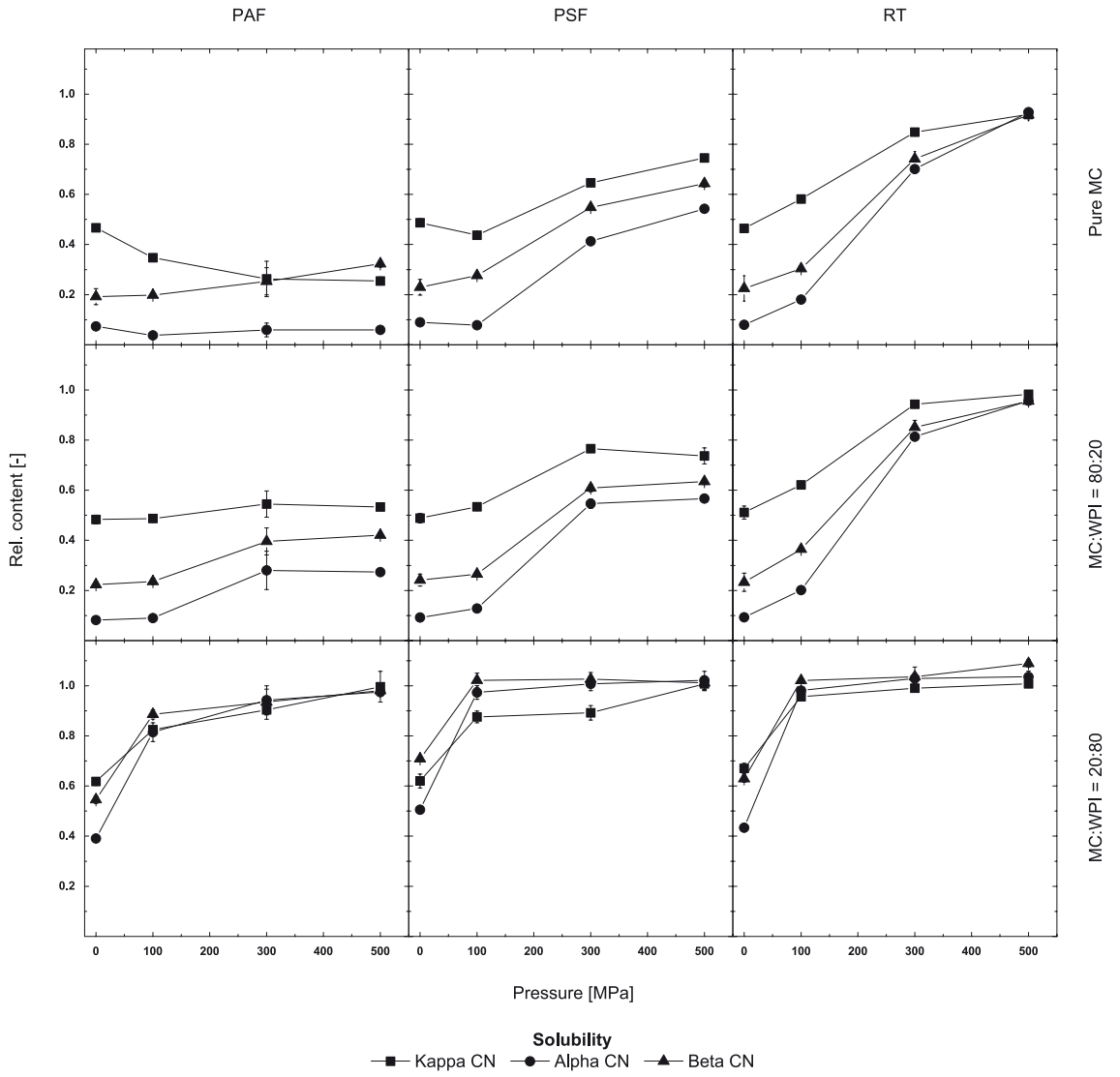


Figure 4: Relative content of MC fractions after centrifugation (soluble fraction) at native sample pH-value (pH 7) for different treatments. Contents are normalized by the protein content of the respective untreated sample (without any centrifugation step).

other authors for treatments at room temperature (Arias, et al., 2000; Hinrichs & Rademacher, 2005; Lopez-Fandino, et al., 1996; Lopez-Fandino & Olano, 1998). However, the highest denaturation was found for both β-Lg fractions at a weight ratio of MC:WPI = 80:20 at room temperature and for PSF at the maximum pressure of 600 MPa (about 90 % of insoluble protein at pH 4.6). In comparison the highest denaturation of both β-Lg fractions in a pure WPI solution was about 35 % when treated at room temperature. Interestingly, the denaturation of the β-Lg fractions was higher for PSF treated milk-like mixtures than for PAF treated. The decompression

Table 1: Results of the particle size measurement of the soluble phase of a 2 % (w/w) MC:WPI mixture (80:20). Pressure treatments were performed at 500 MPa

Sample	z-average [nm]	Polydispersity index (PDI) [-]
Untreated	113.880 ± 0.507	0.216 ± 0.013
PSF	75.108 ± 1.429	0.222 ± 0.006
PAF	88.104 ± 0.363	0.194 ± 0.006
RT	97.785 ± 0.521	0.089 ± 0.017

seems to have a higher impact than slow freezing under pressure in combination with a freeze concentration effect. The maximum denaturation for α-Lac was also found for a treatment of a 80:20 mixture at room temperature (about 70 % at 500 MPa) leading to the assumption that α-Lac is more stable at low temperatures, too.

Kolakowski et al. (2001) reported that pressure treatments in the subzero domain lead to different denatured protein states in comparison with treatments at room temperature. This fact leads to the assumption that the denaturation mechanism could also be different. Kolakowski et al (2001) reported furthermore about the difficulty in differentiating pressure, temperature and mechanical effects for denaturation via HPLT treatments.

Treatments of micellar casein dispersions led to an increase in solubility of all three MC fractions when samples were not frozen under pressure (see Figure 4). In relationship with the decrease in turbidity this finding leads to the conclusion that the size of the micelles changed. Other authors reported also a pressure dependent change in the size distribution of MC (Anema, et al., 2005; Huppertz, et al., 2004b; Orlien, et al., 2006). The authors reported that depending on the pressure, temperature and time conditions a dissociation and a re-association of casein micelles occurs. Figure 5 shows exemplary the particle size distribution of the soluble phase of an 80:20 mixture of Cn and WPI. It can be seen that pressure treatments led to a shift of the distribution to smaller particles. Interestingly, treatments at room temperature led to a reduction in the broadness of the distribution (reduction in PDI, see Table 1). The z-average is shifted to smaller particle size for all treatment conditions. The

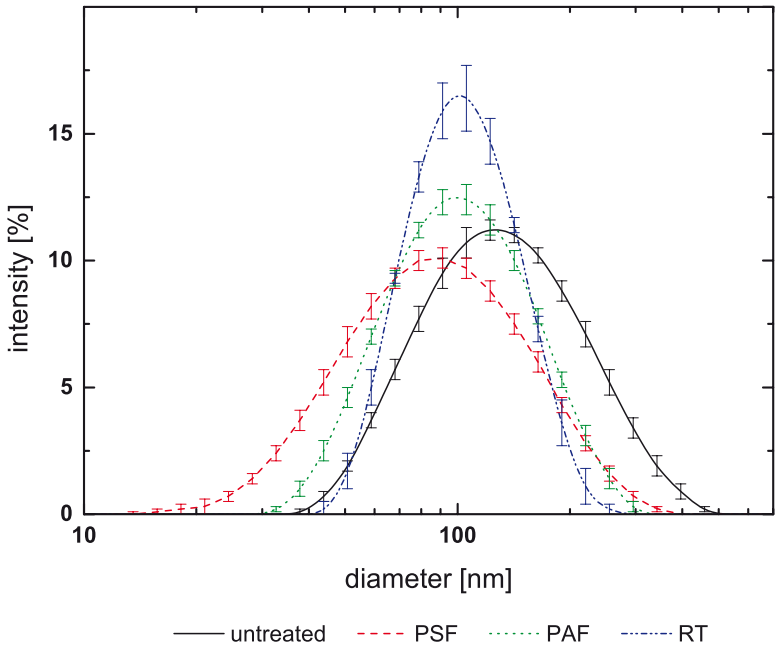


Figure 5: Particle size distribution of the soluble phase of a 2 % (w/w) MC:WPI mixture (80:20). Pressure treatments were performed at 500 MPa.

smallest value was found for the PSF treatment. Consequently, the way of freezing seems to influence the re-aggregation of micelles leading to a change in the particle size distribution of the soluble phase.

HPLT treated MC samples always contained large flocks which could be removed by centrifugation. Otherwise, Figure 4 shows a drastic increase in the solubility of MC fractions after pressure treatments at room temperature and PSF treatments. This increase is most pronounced for treatments at room temperature. Furthermore, the increase for PSF treated samples is higher in comparison to PAF treated samples. In conclusion, it can be assumed that freezing under pressure (PAF) promotes the re-association of larger micelles (for pure MC dispersion a decrease in the soluble part was found for Kappa Cn and Alpha Cn). This effect suggests that the impact of freeze concentration on the forming of large aggregates is more pronounced during the pressure dwell (PAF) than during the decompression (PSF). The different behavior of the three MC fractions for PAF treatments indicates that the formation of flocks with a different composition could be possible. Treatments of MC in the

presence of WPI had only a significant impact if the WPI was the major protein component of the mixture, i.e. 20:80 ratio. In conclusion, it can be assumed that an increased amount of WPI led to an increased solubilization of all three MC fractions. The results for the 80:20 mixture are almost similar to the results of treated pure MC dispersions except for the PAF treatments where a slight increase in solubility was found. Treatments of the reversed mixture (MC:WPI = 20:80) led to an almost complete solubility of all three MC fractions. These findings lead to the assumption that the presence of a high amount of WPI retards the re-association of the micelles.

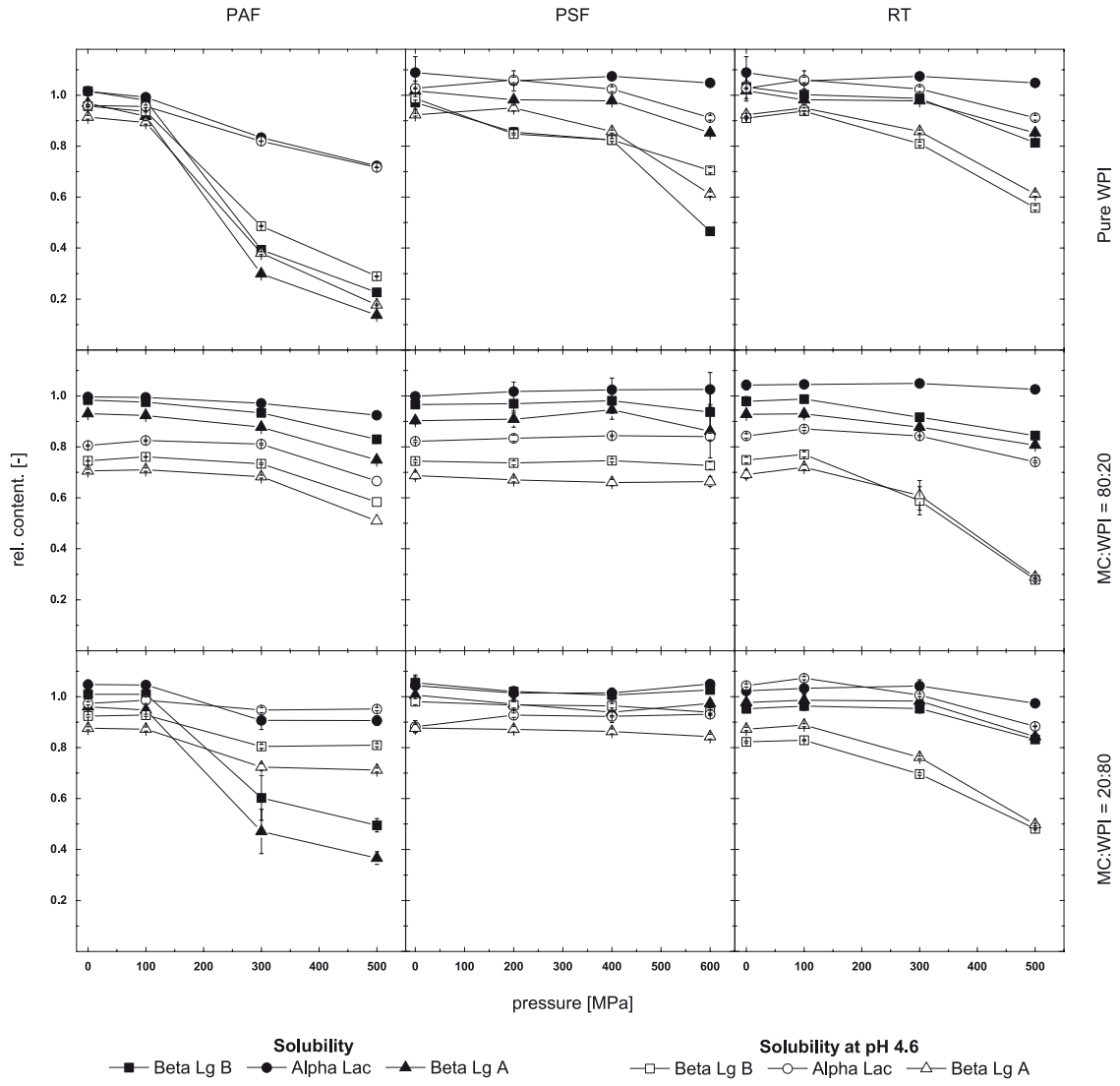


Figure 6: Relative content of WPI fractions after centrifugation (soluble fraction) at native sample pH-value (pH 5.8) and at pH 4.6 for different treatments. Contents are normalized by the protein content of the respective untreated sample (without any centrifugation step).

4.2 Influence of lowering pH

Lowering the pH to 5.8 increases the attraction of the WPI molecules by decreasing their net charge. It is shown in Figure 6 that the denaturation of the WPI fractions in samples with high amounts of WPI (WPI solution, MC:WPI=20:80 mixture) is enhanced especially for HPLT treatments. Otherwise, a decreased denaturation was observed for treatments of milk-like mixtures. Arias, et al (2000) also reported a decreased denaturation of β-Lg in milk pressure treated at room temperature and pH 5.5. A possible explanation is that interactions between WPI fractions occurred, leading to the formation of insoluble aggregates. Either the presence of caseins retards the formation of theses aggregates or a minimum concentration of WPI is necessary to provide a certain amount of reaction partners. A specific effect was found for WPI samples frozen to higher ice modifications (ICE III, V). These samples contained large flocks after the HPLT treatment which is in agreement with a decrease of the soluble protein content. It is noticeable that the amount of denatured β-Lg A and B (soluble at pH 4.6) is decreased in a smaller extent in comparison to the soluble amount which leads to the assumption that the created flocks contain native protein. However, these specific flocks were only formed when samples were frozen to higher ice modifications. Similar flocks were formed when mixtures with a high WPI content were PAF treated suggesting that a high amount of reaction partners is necessary or the presence of caseins retards the formation of these aggregates.

Table 2: Absorbance at 600 nm for MC containing PAF treated samples at different pH values

Sample	Absorbance [-]							
	PAF pH 7.0				PAF pH 5.8			
	Untreated	100 MPa	300 MPa	500 MPa	Untreated	100 MPa	300 MPa	500 MPa
MC	18.48 ± 0.08	21.70 ± 0.50	9.09 ± 1.72	7.31 ± 1.56	16.34 ± 0.07	17.16 ± 0.91	5.13 ± 1.62	6.50 ± 2.12
MC:WPI = 80:20	15.97 ± 0.23	16.19 ± 0.36	7.66 ± 0.13	5.68 ± 0.23	13.73 ± 0.44	14.77 ± 0.20	10.28 ± 2.13	5.74 ± 1.19
MC:WPI = 20:80	1.66 ± 0.01	0.54 ± 0.08	0.21 ± 0.01	0.21 ± 0.00	1.42 ± 0.02	0.88 ± 0.03	14.09 ± 0.38	11.14 ± 0.52

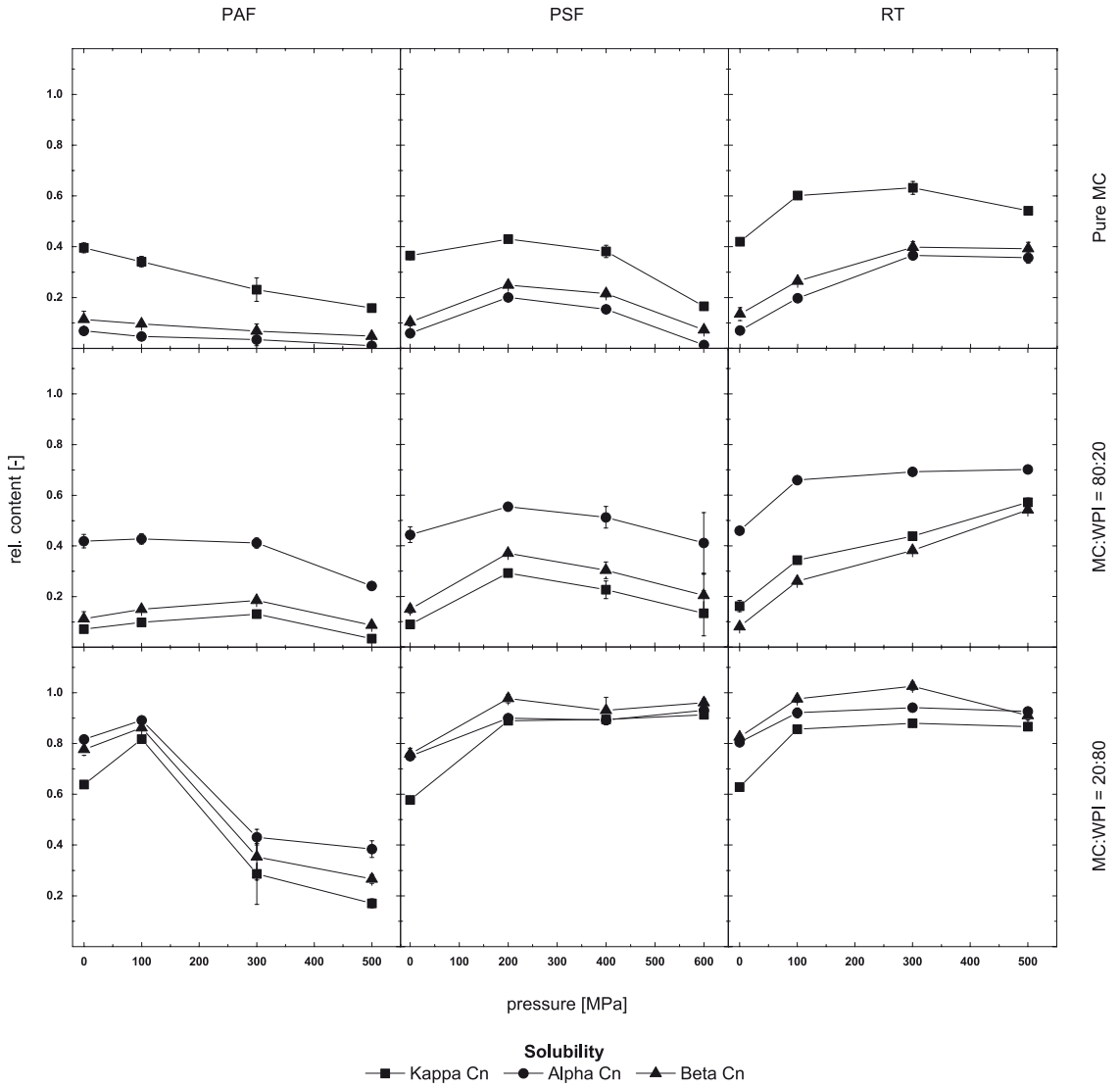


Figure 7: Relative content of MC fractions after centrifugation (soluble fraction) at native sample pH-value (pH 5.8) for different treatments. Contents are normalized by the protein content of the respective untreated sample (without any centrifugation step).

The influence of lowering the pH to 5.8 on the MC fractions is shown in Figure 7. The effects on all three MC fractions are less pronounced in comparison with treatments at pH 7.0 when MC is the main protein component of the dispersions (pure dispersion, 80:20 mixture). The results for pressure treatments of 80:20 mixtures are in agreement with the findings of Arias et al. (2000) who reported a higher solubilization of MC fractions for treatments at pH 7.0 in comparison with pH 5.5. PAF treatments of mixtures with a high amount of WPI (MC:WPI=80:20) with freezing to higher ice formations led to a strong decrease of the solubility of all three

MC fractions. This leads to the assumption that increased interactions between WPI and MC fractions occurred which led to the formation of insoluble aggregates.

Table 2 shows the changes for the absorbance at 600 nm for PAF treated MC samples and mixtures. It is obvious that a PAF treatment at pH 7.0 led to a decrease of the absorbance which could be attributed to a decrease in the micelle size (Anema, Lee, Schrader, & Buchheim, 1997; Schrader, Buchheim, & Morr, 1997). The absorbance values are a cumulative contribution of the disaggregated micelles and the newly formed large flocks. It can be seen that a PAF treatment at pH 7.0 led to a decrease in absorbance with increasing pressure for all MC containing samples. This leads to the assumption that the influence of the flocks had a smaller impact on the absorption. The same conclusion could be drawn for samples treated at pH 5.8 except a mixture where the WPI was the main protein component. The β-Lg fractions were able to form settleable aggregates at pH 5.8 and it seems that these aggregates have a major impact on the absorption of the samples.

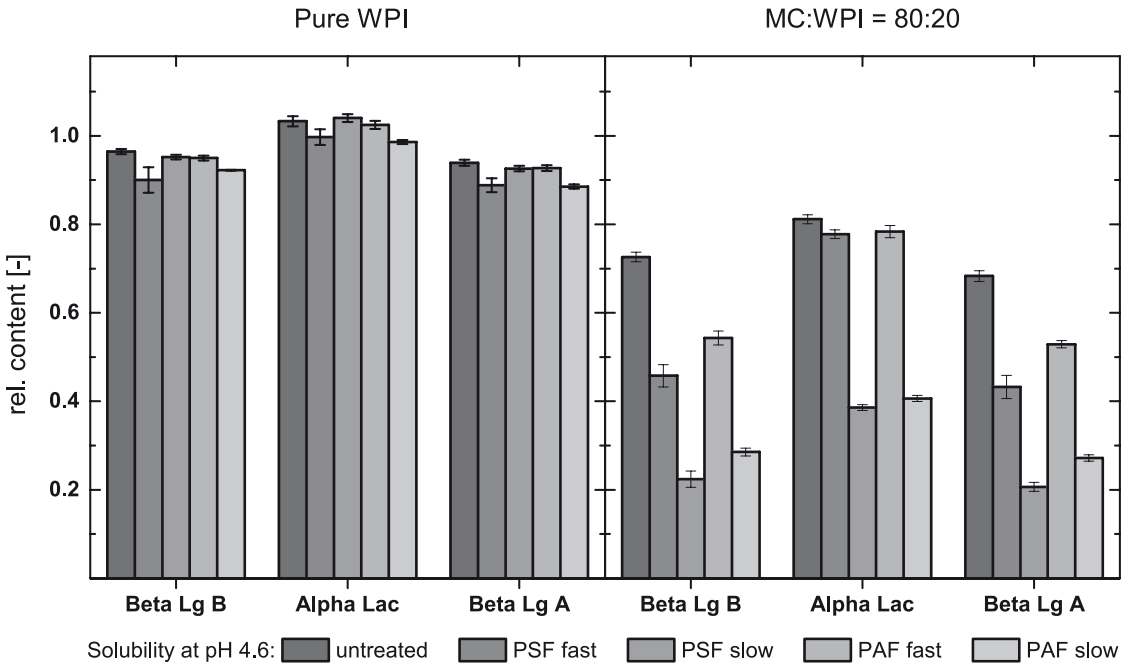


Figure 8: Relative content of WPI fractions after centrifugation at pH 4.6 (soluble fraction) for different HPLT treatments and different decompression rates (fast: 200 MPa / s; slow: 2 MPa / s). Contents are normalized by the protein content of the respective untreated sample (without any centrifugation step).

4.3 Influence of decompression rate

The decompression rate influences the time available for all sample constituents to adapt to the thermodynamic conditions (pressure and temperature changes). Several reports in literature state that there is an influence of the decompression rate on the pressure induced changes in MC functionality (Fertsch, Mueller, & Hinrichs, 2003; Merel-Rausch, Duma, & Hinrichs, 2006; Merel-Rausch, et al., 2007). In addition to the rate of pressure and temperature changes during decompression the nucleation behavior has to be taken into account for HPLT treatments. It is reported that the temperature follows partially the freezing line for a slow decompression (Levy, Dumay, Kolodziejczyk, & Cheftel, 1999; Picart, et al., 2004; Thiebaud, Dumay, & Cheftel, 2002) while fast decompression leads to a supercooling (which depends on the pressure and temperature conditions) with a subsequent instantaneous shift to the freezing temperature (Otero & Sanz, 2006). Figure 8 and Figure 9 show the influence of the decompression rate for PSF and PAF treatments of single protein

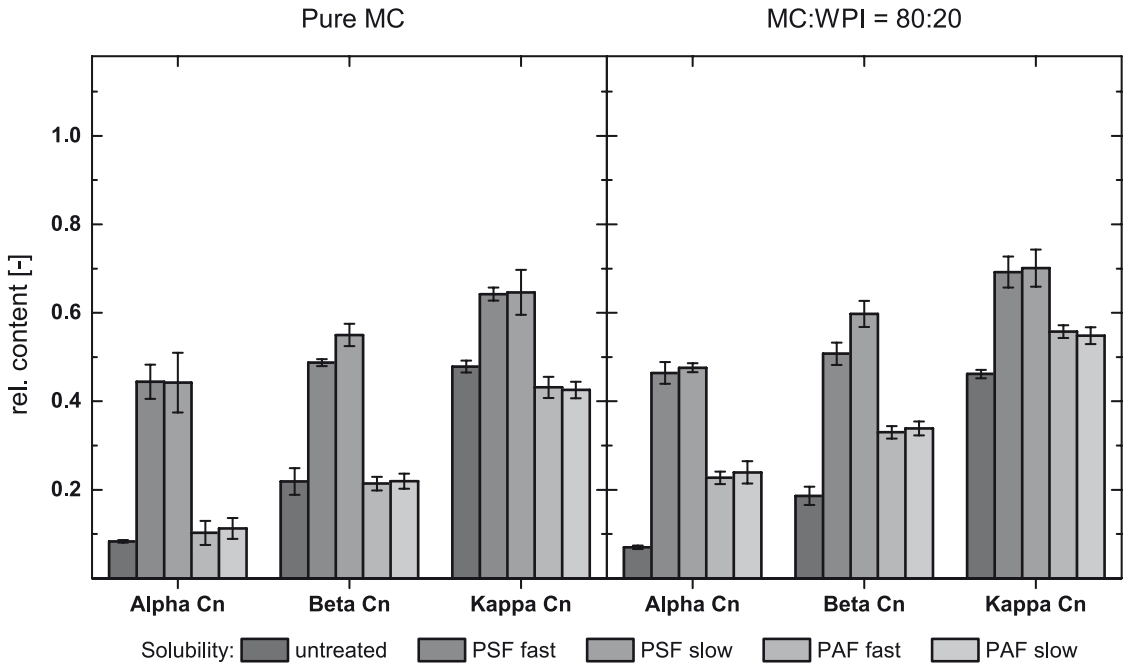


Figure 9: Relative content of MC fractions after centrifugation at sample pH (pH 7.0, soluble fraction) for different HPLT treatments and different decompression rates (fast: 200 MPa / s; slow: 2 MPa / s). Contents are normalized by the protein content of the respective untreated sample (without any centrifugation step).

dispersions and for an 80:20 mixture. The impact on the MC was limited for both the single MC and 80:20 mixture. Only the β-Cn fraction is affected by a change of the decompression rate for PSF treatments (about 7-10 %). This leads to the assumption that the decompression rate has no influence on the solubilization of the MC fractions for HPLT treatments. However, a possible influence on the insoluble fraction (flocks, aggregates and re-associated micelles) could not be excluded from the data and has to be investigated. Otherwise, the WPI fractions are highly affected by the decompression rate, when treated in a 80:20 protein dispersion. A slow decompression led to a higher denaturation for all the three WPI fractions. Interestingly, the highest effect was found for the most pressure resistant fraction (α-Lac; about 40 % difference). It can be supposed that an interaction between the MC fractions and the WPI fractions occurred which is dependent on the reaction time. Interestingly, this effect seems to be independent from the kind of HPLT treatment as the extent of changes between slow and fast decompression is similar for PAF and PSF treated samples. This leads to the assumption that especially the time or speed of crystallization to ICE I has an impact on the denaturation of WPI fractions. The different behavior of the β-Lg and the α-Lac fractions offers the potential of HPLT treatments to induce different modifications in milk protein samples by changing the decompression rate.

4.4 Influence of Ca-ion release

The results of the pressure induced calcium release are presented in Figure 10. The maximum calcium concentration of 125 mg/L was detected for a treatment at 500 MPa at room temperature. This concentration was used to identify the impact of released calcium on the denaturation / aggregation of the WPI fractions (added as calcium chloride). Huppertz, et al. (2004a) reported a decrease of WPI denaturation after the removal of calcium from milk samples before pressure treatments. It is obvious from Figure 10 that the presence of free calcium in the surrounding media does not promote the denaturation / aggregation of WPI in pure solutions for the

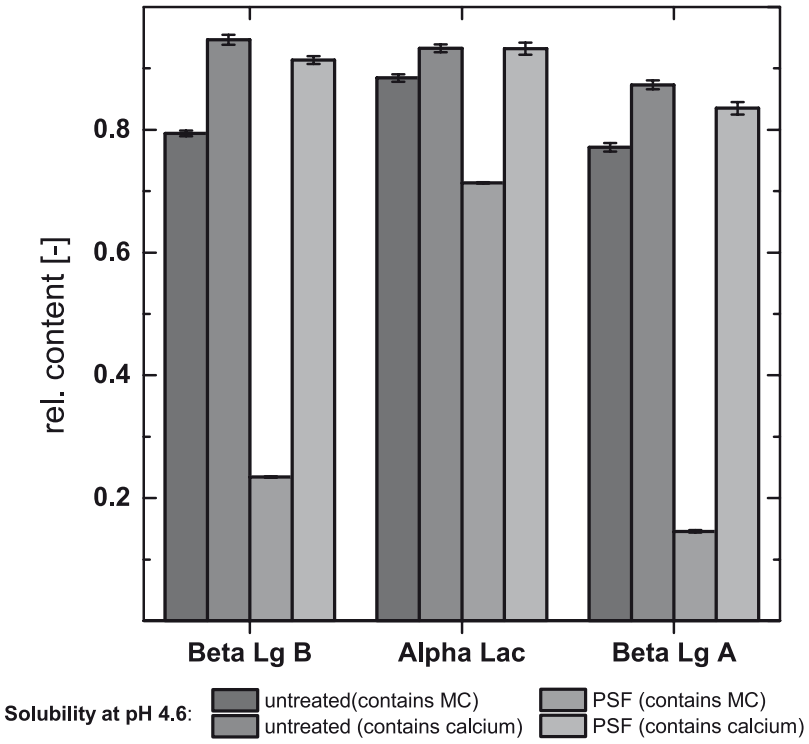


Figure 10: Relative content of WPI fractions after centrifugation at pH 4.6 for PSF treatments at 300 MPa in the presence of 80 % MC or 125 mg/L calcium chloride. Contents are normalized by the protein content of the respective untreated sample (without any centrifugation step).

used calcium concentration under HPLT conditions. In conclusion, the presence of MC seems to be necessary to affect WPI fractions in the subzero temperature domain for applied pressures and temperatures. This leads to the assumption that interactions between the MC and the WPI fractions have to take place to denature the WPI fractions under HPLT conditions. Further investigations have to be performed to identify the kind of interactions involved.

5 Conclusions

The present study shows that HPLT can induce changes in milk protein dispersions which are different from those induced by pressure treatments at room temperature. The formation of aggregates and large flocks indicates changes in the protein-protein interactions and further investigations on e.g. changes in the protein structure should

be performed for HPLT treatments. The higher baroresistance of α-Lac in comparison to the β-Lg fractions could be also found in the HPLT domain. A clear dependence on the sample composition was shown for all kind of pressure treatments with the highest effect on the WPI fractions in the presence of a large amount of MC (80:20 mixture). However, the highest effect on the solubility of the MC fractions could be observed in the presence of a high WPI amount (MC:WPI=20:80). The MC fractions formed large flocks after HPLT treatments although the solubility could be increased at once. Furthermore, the denaturation of WPI fractions could be increased for samples with a large amount of WPI by decreasing the pH to 5.8 but not for milk-like mixtures. The β-Lg fractions formed specific aggregates which contained native protein when frozen to higher ice formations at pH 5.8 in pure solutions or mixtures with a high amount of WPI. A clear influence of the decompression rate on the denaturation of all WPI fractions was found while the solubilization of MC fractions was unchanged. However, it could be also shown that the release of calcium from MC was not responsible for the enhanced denaturation of WPI in the presence of MC. The formation of aggregates with different protein compositions points out the high potential of HPLT treatments as a tool for tailor made protein modifications.

6 References

Anema, S. G., Lee, S. K., Schrader, K., & Buchheim, W. (1997). Effect of pH on the turbidity of pressure-treated calcium caseinate suspensions and skim milk. Milchwissenschaft-Milk Science International, 52, 141-146.

Anema, S. G., Lowe, E. K., & Stockmann, R. (2005). Particle size changes and casein solubilisation in high-pressure-treated skim milk. Food Hydrocolloids, 19, 257-267.

Arias, M., Lopez-Fandino, R., & Olano, A. (2000). Influence of pH on the effects of high pressure on milk proteins. Milchwissenschaft-Milk Science International, 55, 191-194.

Bordin, G., Raposo, F. C., de la Calle, B., & Rodriguez, A. R. (2001). Identification and quantification of major bovine milk proteins by liquid chromatography. Journal of Chromatography A, 928, 63-76.

Bravo, F. I., Molina, E., & Lopez-Fandino, R. (2012). Effect of the high-pressure-

release phase on the protein composition of the soluble milk fraction. Journal of Dairy Science, 95, 6293-6299.

Bravo, F. I., Felipe, X., Lopez-Fandino, R., & Molina, E. (2013). High-pressure treatment of milk in industrial and pilot-scale equipments: effect of the treatment conditions on the protein distribution in different milk fractions. European Food Research and Technology, 236, 499-506.

Bridgman, P. W. (1912). Water under pressure. Proceedings of the American Academy of Arts and Sciences, 48, 450-558.

Bridgman, P. W. (1914). The coagulation of albumen by pressure. Journal of Biological Chemistry, 19, 511-512.

Chawla, R., Patil, G. R., & Singh, A. K. (2011). High hydrostatic pressure technology in dairy processing: a review. Journal of Food Science and Technology-Mysore, 48, 260-268.

Cheftel, J. C., & Dumay, E. (1996). Effects of high pressure on dairy proteins: A review. In High Pressure Bioscience and Biotechnology (Vol. 13, pp. 299-308).

Cheftel, J. C., Thiebaud, M., & Dumay, E. (2002). Pressure-assisted freezing and thawing of foods: A review of recent studies. High Pressure Research, 22, 601-611.

Considine, T., Patel, H. A., Anema, S. G., Singh, H., & Creamer, L. K. (2007). Interactions of milk proteins during heat and high hydrostatic pressure treatments - A review. Innovative Food Science & Emerging Technologies, 8, 1-23.

Fertsch, B., Mueller, M., & Hinrichs, J. (2003). Firmness of pressure-induced casein and whey protein gels modulated by holding time and rate of pressure release. Innovative Food Science & Emerging Technologies, 4, 143-150.

Fletcher, N. H. (1970). Other forms of ice. In The Chemical Physics of Ice: Cambridge University Press.

Fox, P. F. (1992). Advanced Dairy Chemistry - 1: Proteins. London: Blackie Academic & Professional.

Garcia-Risco, M. R., Olano, A., Ramos, M., & Lopez-Fandino, R. (2000). Micellar changes induced by high pressure. Influence in the proteolytic activity and organoleptic properties of milk. Journal of Dairy Science, 83, 2184-2189.

Gaucheron, F., Famelart, M. H., Mariette, F., Raulot, K., Michel, F., & LeGraet, Y. (1997). Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk. Food Chemistry, 59, 439-447.

Hashizume, C., Kimura, K., & Hayashi, R. (1995). Kinetic-analysis of yeast inactivation by high-pressure treatment at low-temperatures. Bioscience Biotechnology and Biochemistry, 59, 1455-1458.

Hayakawa, K., Ueno, Y., Kawamura, S., Kato, T., & Hayashi, R. (1998). Microorganism inactivation using high-pressure generation in sealed vessels under sub-zero temperature. Applied Microbiology and Biotechnology, 50, 415-418.

Hinrichs, J., Rademacher, B., & Kessler, H. G. (1996). Reaction kinetics of pressure-induced denaturation of whey proteins. Milchwissenschaft-Milk Science International, 51, 504-509.

Hinrichs, J., & Rademacher, B. (2004). High pressure thermal denaturation kinetics of whey proteins. Journal of Dairy Research, 71, 480-488.

Hinrichs, J., & Rademacher, B. (2005). Kinetics of combined thermal and pressure-induced whey protein denaturation in bovine skim milk. International Dairy Journal, 15, 315-323.

Hite, B. H. (1899). The Effect of Pressure in the Preservation of Milk - A Preliminary Report. West Virginia Agricultural and Forestry Experiment Station Bulletin, 58, 15-35.

Huppertz, T., Kelly, A. L., & Fox, P. F. (2002). Effects of high pressure on constituents and properties of milk. International Dairy Journal, 12, 561-572.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004a). High pressure-induced denaturation of alpha-lactalbumin and beta-lactoglobulin in bovine milk and whey: a possible mechanism. Journal of Dairy Research, 71, 489-495.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004b). Dissociation of caseins in high pressure-treated bovine milk. International Dairy Journal, 14, 675-680.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004c). High pressure treatment of bovine milk: effects on casein micelles and whey proteins. Journal of Dairy Research, 71, 97-106.

Huppertz, T., & De Kruif, C. G. (2006). Disruption and reassociation of casein micelles under high pressure: Influence of milk serum composition and casein micelle concentration. Journal of Agricultural and Food Chemistry, 54, 5903-5909.

Huppertz, T., Fox, P. F., de Kruif, K. G., & Kelly, A. L. (2006). High pressure-induced changes in bovine milk proteins: A review. Biochimica Et Biophysica Acta-Proteins and Proteomics, 1764, 593-598.

Huppertz, T., Kelly, A. L., & Fox, P. F. (2006). High pressure-induced changes in ovine milk. 2. Effects on casein micelles and whey proteins. Milchwissenschaft-Milk Science International, 61, 394-397.

Huppertz, T., Smiddy, M. A., Upadhyay, V. K., & Kelly, A. L. (2006). High-pressure-induced changes in bovine milk: a review. International Journal of Dairy Technology, 59, 58-66.

Huppertz, T., & de Kruif, C. G. (2007). Disruption and reassociation of casein micelles during high pressure treatment: influence of whey proteins. Journal of Dairy Research, 74, 194-197.

Knudsen, J. C., & Skibsted, L. H. (2010). High pressure effects on the structure of casein micelles in milk as studied by cryo-transmission electron microscopy. Food Chemistry, 119, 202-208.

Kolakowski, P., Dumay, E., & Cheftel, J. C. (2001). Effects of high pressure and low temperature on beta-lactoglobulin unfolding and aggregation. Food Hydrocolloids, 15, 215-232.

Levy, J., Dumay, E., Kolodziejczyk, E., & Cheftel, J. C. (1999). Freezing kinetics of a model oil-in-water emulsion under high pressure or by pressure release. Impact on ice crystals and oil droplets. Food Science and Technology-Lebensmittel-Wissenschaft & Technologie, 32, 396-405.

Lopez-Fandino, R., Carrascosa, A. V., & Olano, A. (1996). The effects of high pressure on whey protein denaturation and cheese-making properties of raw milk. Journal of Dairy Science, 79, 929-936.

Lopez-Fandino, R., Ramos, M., & Olano, A. (1997). Rennet coagulation of milk subjected to high pressures. Journal of Agricultural and Food Chemistry, 45, 3233-3237.

Lopez-Fandino, R., De La Fuente, M. A., Ramos, M., & Olano, B. (1998). Distribution of minerals and proteins between the soluble and colloidal phases of pressurized milks from different species. Journal of Dairy Research, 65, 69-78.

Lopez-Fandino, R., & Olano, A. (1998). Effects of high pressures combined with moderate temperatures on the rennet coagulation properties of milk. International Dairy Journal, 8, 623-627.

Luscher, C., Balasa, A., Frohling, A., Ananta, E., & Knorr, D. (2004). Effect of high-pressure-induced ice I-to-Ice III phase transitions on inactivation of Listeria innocua in frozen suspension. Applied and Environmental Microbiology, 70, 4021-4029.

Mazri, C., Sanchez, L., Ramos, S. J., Calvo, M., & Perez, M. D. (2012). Effect of high-pressure treatment on denaturation of bovine beta-lactoglobulin and alpha-lactalbumin. European Food Research and Technology, 234, 813-819.

Merel-Rausch, E., Duma, I. P., & Hinrichs, J. (2006). Pressure-induced modification of casein micelles - Influence of pressure built-up rate, pressure level, release rate and temperature on viscosity and particle size. Milchwissenschaft-Milk Science International, 61, 255-259.

Merel-Rausch, E., Kulozik, U., & Hinrichs, J. (2007). Influence of pressure release rate and protein concentration on the formation of pressure-induced casein structures. Journal of Dairy Research, 74, 283-289.

Messens, W., VanCamp, J., & Huyghebaert, A. (1997). The use of high pressure to modify the functionality of food proteins. Trends in Food Science & Technology, 8, 107-112.

Moussa, M., Perrier-Cornet, J.-M., & Gervais, P. (2007). Damage in Escherichia coli cells treated with a combination of high hydrostatic pressure and subzero temperature. Applied and Environmental Microbiology, 73, 6508-6518.

Needs, E. C., Stenning, R. A., Gill, A. L., Ferragut, V., & Rich, G. T. (2000). High-pressure treatment of milk: effects on casein micelle structure and on enzymic coagulation. Journal of Dairy Research, 67, 31-42.

Orlien, V., Knudsen, J. C., Colon, M., & Skibsted, L. H. (2006). Dynamics of casein micelles in skim milk during and after high pressure treatment. Food Chemistry, 98, 513-521.

Orlien, V., Boserup, L., & Olsen, K. (2010). Casein micelle dissociation in skim milk during high-pressure treatment: Effects of pressure, pH, and temperature. Journal of Dairy Science, 93, 12-18.

Otero, L., & Sanz, P. D. (2006). High-pressure-shift freezing: Main factors implied in the phase transition time. Journal of Food Engineering, 72, 354-363.

Perrier-Cornet, J. M., Tapin, S., Gaeta, S., & Gervais, P. (2005). High-pressure inactivation of Saccharomyces cerevisiae and Lactobacillus plantarum at subzero temperatures. Journal of Biotechnology, 115, 405-412.

Picart, L., Dumay, E., Guiraud, J. P., & Cheftel, J. C. (2004). Microbial inactivation by pressure-shift freezing: effects on smoked salmon mince inoculated with Pseudomonas fluorescens, Micrococcus luteus and Listeria innocua. Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology, 37, 227-238.

Picart, L., Dumay, E., Guiraud, J. P., & Cheftel, J. C. (2005). Combined high pressure-sub-zero temperature processing of smoked salmon mince: phase transition phenomena and inactivation of Listeria innocua. Journal of Food Engineering, 68, 43-56.

Schmidt, D. G., & Buchheim, W. (1970). Electron microscope investigation of the substructure of casein micelles in cows' milk. Milchwissenschaft, 25, 596-600.

Schrader, K., Buchheim, W., & Morr, C. V. (1997). High pressure effects on the colloidal calcium phosphate and the structural integrity of micellar casein in milk .1. High pressure dissolution of colloidal calcium phosphate in heated milk systems. Nahrung-Food, 41, 133-138.

Shibauchi, Y., Yamamoto, H., & Sagara, Y. (1992). Conformational change of casein micelles by high pressure treatment. In C. Balny, R. Hayashi, K. Heremans & P. Masson (Eds.), High Pressure and Biotechnology. Paris: John Libbey.

Smeller, L. (2002). Pressure-temperature phase diagrams of biomolecules. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology, 1595, 11-29.

Thiebaud, M., Dumay, E. M., & Cheftel, J. C. (2002). Pressure-shift freezing of o/w emulsions: influence of fructose and sodium alginate on undercooling, nucleation, freezing kinetics and ice crystal size distribution. Food Hydrocolloids, 16, 527-545.

Urrutia Benet, G., Schlüter, O., & Knorr, D. (2004). High pressure-low temperature processing. Suggested definitions and terminology. Innovative Food Science & Emerging Technologies, 5, 413-427.

Volkert, M., Puaud, M., Wille, H.-J., & Knorr, D. (2012). Effects of High Pressure-Low Temperature treatment on freezing behavior, sensorial properties and air cell distribution in sugar rich dairy based frozen food foam and emulsions. Innovative Food Science & Emerging Technologies, 13, 75-85.

Zobrist, M. R., Huppertz, T., Uniacke, T., Fox, P. F., & Kelly, A. L. (2005). High-pressure-induced changes in the rennet coagulation properties of bovine milk. International Dairy Journal, 15, 655-662.

2.1.2 Supplemental Results and Conclusions for Experimental Plan

It was shown in the first experimental series that HPLT can affect whey proteins and micellar caseins in a different way compared to high pressure treatments at room temperature. Especially, the following specific HPLT effects were observed:

- Whey protein fractions can only be affected at pH 7.0 in the presence of micellar casein,
- Large flocks are formed in casein containing samples due to HPLT treatments,
- All micellar casein fractions are solubilized at pH 7.0 due to HPLT treatments, diverse effects at pH 5.8,
- Insoluble β-Lg aggregates involving native protein are formed due to freezing to higher ice modifications (PAF) at pH 5.8,
- Decompression rate only influences whey protein fractions and not micellar casein fractions but presence of casein is necessary to affect whey proteins,
- Denaturation of whey proteins in the presence of micellar casein is not related to release of calcium from micelles.

It is proposed that the increasing solubilization effect of the MC fractions with increasing amount of WPI can be traced back to the presence of whey proteins which may act as a barrier for re-aggregation of the casein fractions. However, another possible explanation could be that the solubilization of the casein fractions increases with decreasing MC concentration. Additional experiments with the responding concentrations of MC (2 %, 1.6 % and 0.4 % w/w) were performed to verify this hypothesis. Figure 14 shows the results for treatments of the pure MC dispersions with the respective concentrations belonging to 2 % pure dispersion (2 % w/w), 2 % MC:WPI=80:20 mixture (1.6 % w/w) and MC:WPI=20:80 (0.4 % w/w). It is obvious that a full solubilization of one of the MC fractions was not possible at the tested concentrations. The solubilization effects for the three concentrations are similar, and differences are almost in the range of deviations. These findings support the thesis from the above manuscript that the presence of WPI influences the pressure induced solubilization of casein fractions during HPLT treatments.

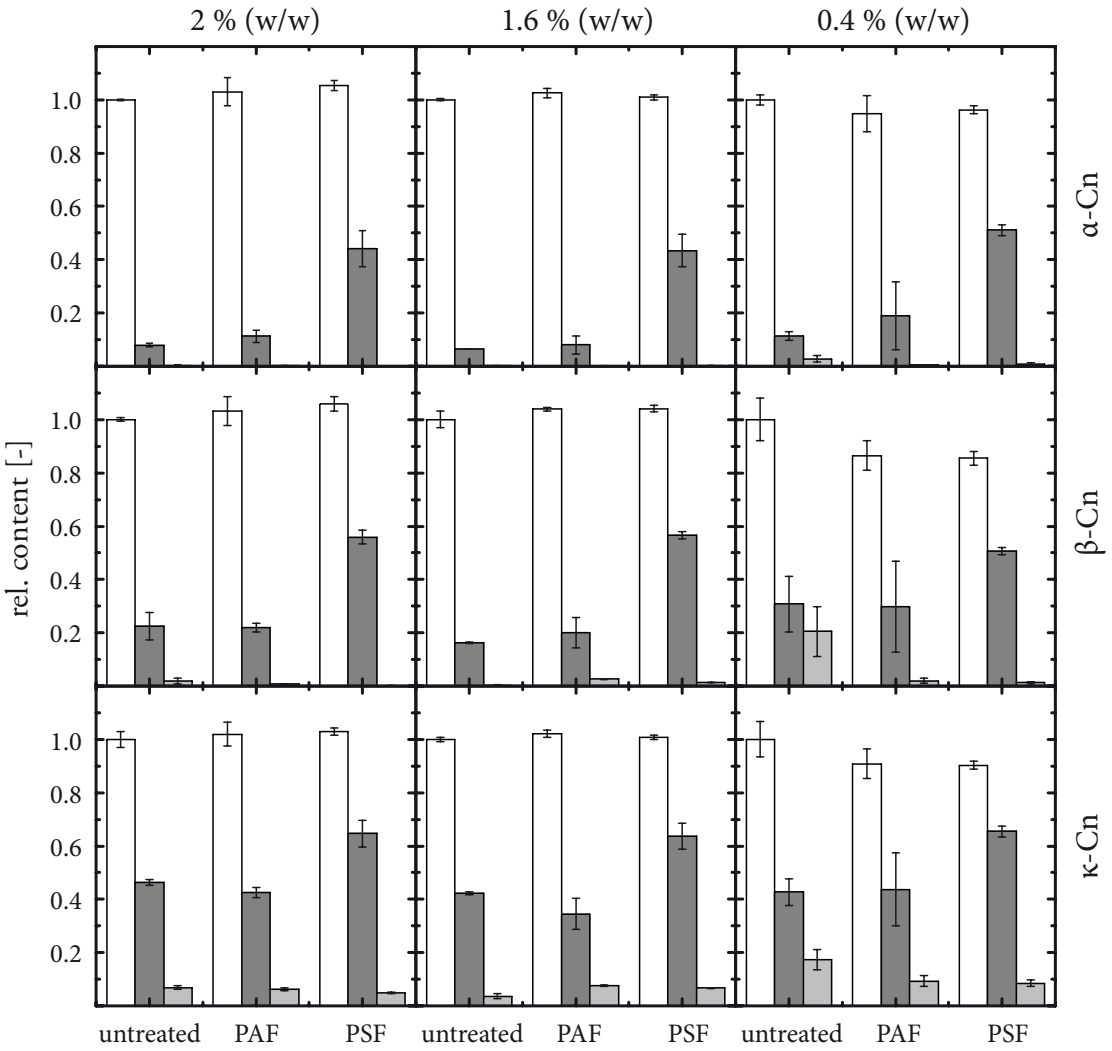


Figure 14: Relative content of MC fractions after centrifugation (soluble fraction) at native sample pH-value (pH 7) for different treatments. Contents are normalized by the protein content of the respective untreated sample (without any centrifugation step).

The same experiments were also performed for the WPI solutions. The results are shown in Figure 15. As expected the reduced amount of WPI in the solutions did not affect the denaturation. Concluding, for both fractions – MC and WPI – the presence of the other fraction promotes the HPLT induced effects. These findings support the thesis that interactions between both fractions occur during HPLT treatments.

It was found that the effect on all fractions increased with increasing pressure and that especially freezing to higher ice modifications caused specific effects. Within the experimental plan it was decided to investigate the changes in the molecular structure of the proteins and to perform further analytics on the formed flocks. The following experiments were performed at 500 MPa

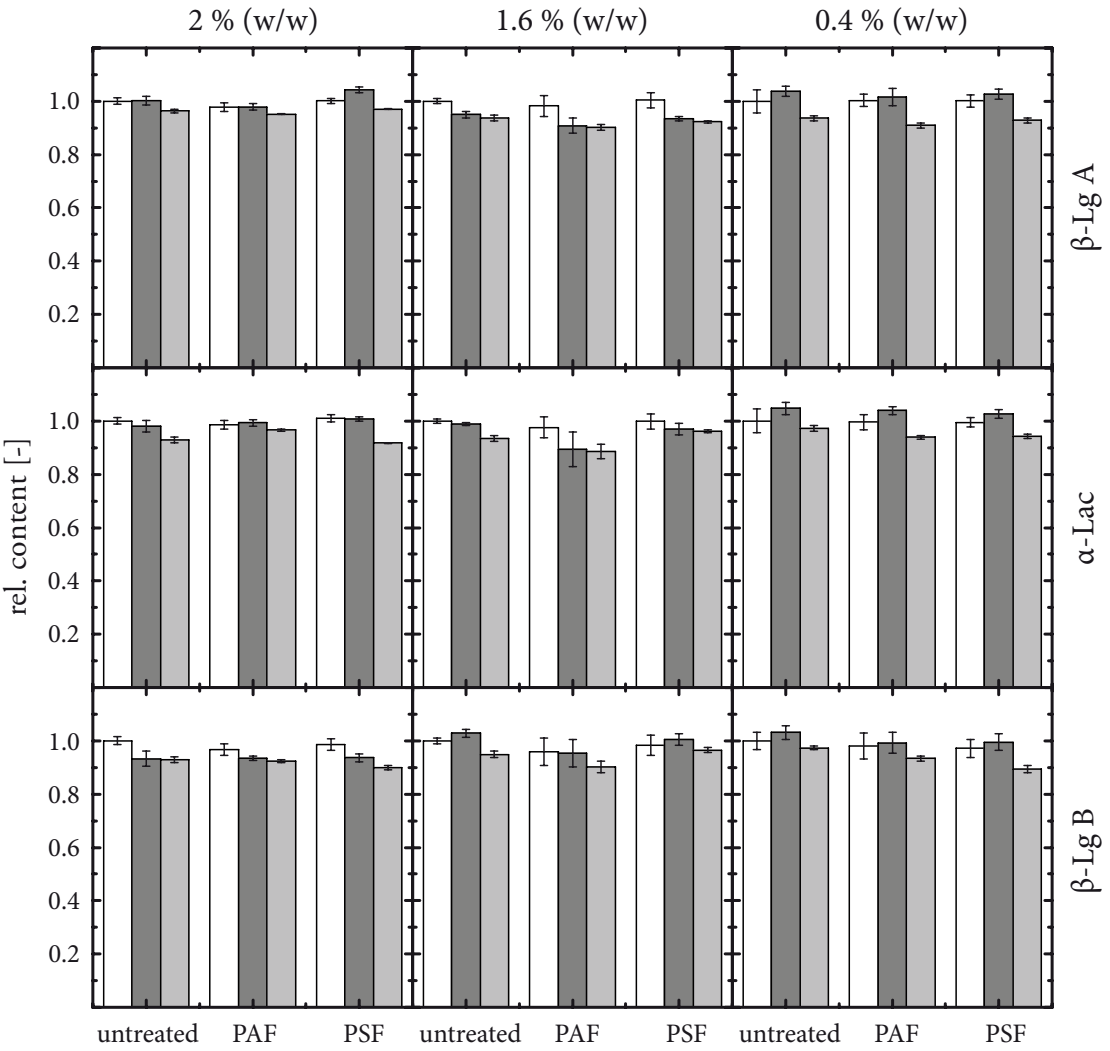


Figure 15: Relative content of WPI fractions after centrifugation (soluble fraction) at native sample pH-value (pH 7) and at pH 4.6 for different treatments. Contents are normalized by the protein content of the respective untreated sample (without any centrifugation step).

to achieve the most pronounced effects. Measurements of secondary and tertiary structure via circular dichroism, changes in surface hydrophobicity and exposure of free thiol groups were chosen as meaningful investigations for structural changes. The results of the mentioned structural analyses are presented in the following chapter.

2.2 High Pressure – Low Temperature Treatment Induced Structural Changes in Micellar Caseins and Whey Proteins

2.2.1 Manuscript

Authors: Daniel Baier, Benedict Purschke, Christophe Schmitt, Harshadrai M. Rawel and Dietrich Knorr

Submitted to Food Chemistry

High pressure – low temperature treatment induced structural changes in micellar caseins and whey proteins

Daniel Baier[1], Benedict Purschke[1], Christophe Schmitt[2], Harshadrai M. Rawel[3] and Dietrich Knorr[1]

[1] TU Berlin, Dept. of Food Biotechn. and Food Process Eng., Berlin, Germany

[2] Nestlé Research Center, Department. of Food Science and Technology, CH-1000 Lausanne 26, Switzerland

[3] Department of Food Chemistry, Institute of Nutritional Science, University of Potsdam

1 Abstract

Structural changes in micellar caseins and whey proteins due to high pressure – low temperature treatments were investigated and compared to changes caused by high pressure treatments at room temperature. Single whey protein isolate (WPI) solutions, single micellar casein (MC) dispersions and mixtures with a weight mixing ratio of MC:WPI = 80:20 or 20:80 (w/w) were treated at a pressure of 500 MPa for 20 min at room temperature, -15 °C (pressure shift freezing) or -35 °C (pressure assisted freezing). Samples were treated at pH 7.0 and pH 5.8. Surface hydrophobicity, accessible thiol groups, near and far UV circular dichroism, flow particle image analyses and stability against several chaotropic agents were used to characterize structural changes in proteins as well as the structure of the formed aggregates. Surface hydrophobicity and accessible thiol groups remained almost unchanged after HPLT treatments while HP treatments at room temperature caused an unfolding of WPI leading to an increased surface hydrophobicity and an exposure of thiol groups. Specific changes in the secondary structure indicated by an increase

in β-sheets were found for HPLT treatments while the tertiary structure remains unchanged. Large flocks stabilized by hydrophobic and hydrogen interactions were formed in casein containing samples due to HPLT treatments. Depending on the pH and the applied HPLT treatment (pressure assisted freezing - PAF or pressure shift freezing - PSF) these interactions differed from the interactions in native micelles. PSF treatments at pH 5.8 led to thermal sensitive MC fractions, which became insoluble after heating to 70 °C while PAF treatment in the presence of WPI induced hydrogen interactions. The findings of this study point out the potential of HPLT treatments to obtain specific protein modifications.

2 Introduction

Until today there is only a limited number of publications on high pressure – low temperature (HPLT) processing of food ingredients although the phase diagram of water in the relevant pressure and temperature domain is already known since the beginning of the last century (Bridgman, 1912). HPLT processes allow on the one hand freezing of water to higher ice modifications (pressure assisted freezing – PAF) and on the other hand instantaneous freezing to atmospheric ice by decompression at subzero temperature (pressure shift freezing – PSF). Please refer to Urrutia Benet, Schlüter, & Knorr (2004) for a detailed overview on the HPLT process options and their nomenclature. It is known that proteins possess a phase diagram with an elliptical shape (Hawley, 1971; Smeller, 2002) which depicts the option to reach a denatured state by temperature changes (heat or cold denaturation) or by pressure changes (pressure denaturation). It is obvious from the phase diagram of water (see Figure 1) that the subzero temperature domain includes three ice modifications in the relevant pressure range. Figure 1 shows also that only ICE I has a lower density than liquid water. Therefore, HPLT processes enable beside a cold or a pressure induced denaturation also a mechanical stress during the recrystallization process while

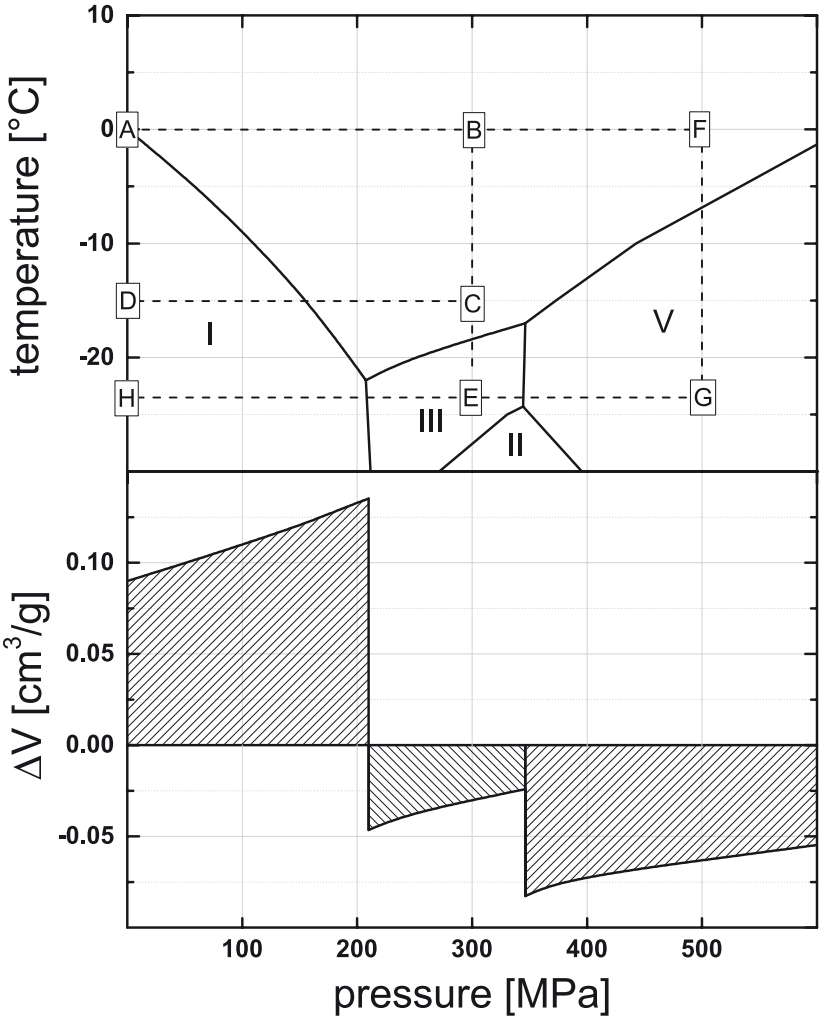


Figure 1: Top: Phase diagram of water with HPLT process options – A-B-C-D: Pressure shift freezing; A-B-E-H and A-F-G-H: Pressure assisted freezing to ICE III and V. Bottom: Volume changes during phase transitions. Data obtained from Bridgman (1912), Fletcher (1970).

decompression to ambient pressure. In general, HPLT processes provide a wide range of combinations of pressure, temperature and mechanical based changes, which could influence the protein structures. Pressure induced changes are based on the Chatelier's Principle and, thus, promote reactions with a negative reaction volume, like unfolding of globular proteins. Interactions between molecules can be divided into electrostatic interactions, hydrogen bonds, hydrophobic interactions and covalent bonds. Effect of pressure differs regarding the respective reaction volumes ΔV of these interactions. Masson (1992) highlighted that the volume of

a protein is the sum of three contributions: the volume of its constitutive atoms, the volume of internal cavities and its solvation volume containing negative parts from the hydration of peptide bonds and amino acid side chains (Kauzmann, 1959; Richards, 1977; Zamyatnin, 1972). In consequence, electrostatic interactions and hydrophobic interactions are the main targets of high pressure because of their positive ΔV (10 to 20 mL.mol-1) on formation (Masson, 1992). Hence, hydrogen bonds and especially covalent bonds are almost pressure insensitive due to very small reaction volumes (+1 to -3 mL.mol-1). Schade (1980) reported a very large negative volume change (-500 mL.mol-1) for the dissociation of oligomeric proteins and, thus, conclude that the quaternary structure of a protein is very pressure sensitive. Furthermore, the tertiary structure is also affected by high pressures. Gekko & Noguchi (1979) and (Gekko & Hasegawa (1989) suggested an incomplete pressure induced unfolding of proteins. Regarding the secondary structure a shift in the amount of α-helices and β-sheets is reported by Wong & Heremans (1988) and Heremans & Wong (1985). However, the direction of changes (decrease or increase) and the reversibility seems to depend on e.g. the compression rate and the extend of changes in secondary structure (Carrier, Mantsch, & Wong, 1990). Another factor for pressure sensitivity is for instance an imperfect packing of the protein molecule (Richards, 1977). All the mentioned structural changes are accompanied by large hydration changes which are the major reason for a volume decrease and, thus, are associated with dissociation and unfolding of proteins (Masson, 1992). It is reported that pressures of 100 MPa lead to a partial unfolding of β-lactoglobulin (β-Lg), which represents the largest fraction of the whey proteins, whereas the core of the molecule retains its structured elements (Belloque, Lopez-Fandino, & Smith, 2000). These authors reported flexibility for the entire structure of β-Lg for pressures between 300 and 400 MPa. β-Lg is composed of β-Lg A and B and the authors found that the core of β-Lg A becomes more easily flexible in comparison to β-Lg B. Yang, Dunker, Powers, Clark, & Swanson (2001) observed

a conversion of β-sheets to non-native α-helices for β-Lg treated at 600 MPa at 50 °C. Several authors reported a dissociation of casein micelles under pressure and related this effect to a destabilization of hydrophobic interactions (Gaucheron, et al., 1997; Huppertz, Fox, & Kelly, 2004). This effect was also reported by Baier, Schmitt, & Knorr (submitted) for HPLT treatments. A comparison of online measurements of size distribution and of size distribution after decompression suggests that the final size distribution after decompression is composed of pressure induced dissociated micelles and re-associated particles which were formed during decompression (Anema, 2008; Huppertz & De Kruif, 2006; Huppertz, Kelly, & de Kruif, 2006; Orlien, Knudsen, Colon, & Skibsted, 2006). The aim of this work was to point out structural changes in major milk protein fractions induced by HPLT treatments and compare them to changes via high pressure treatments at room temperature. The specificity of HPLT treatments in comparison to common HP treatments should be pointed out and the potential of HPLT treatments should emphasized.

3 Material and Methods

3.1 Material

Whey protein isolate powder (WPI) was obtained from Fonterra (WPI 895, Fonterra, Auckland, New Zealand). This WPI is obtained by ion exchange and ultrafiltration of sweet whey. The protein content of the powder was (Nx6.38) 92.63 % (w/w), furthermore it contained 0.18 % (w/w) fat, 5.87 % (w/w) moisture and 1.6 % (w/w) ash. Micellar casein powder (MC) in an almost native state was obtained from the Hungarian Dairy Research Institute (MPI-85 MC, Hungarian Dairy Research Institute, Mosonmagyaróvár, Hungary). These micelles were manufactured by microfiltration and ultrafiltration of skimmed milk. The powder contained 85.1 % (w/w) protein (Nx6.38), 1.5 % (w/w) fat, 4.9 % (w/w) water and 7.5 % (w/w) ash.

3.2 Methods

3.2.1 Sample preparation

The WPI solutions were prepared by diluting a specific amount of powder in deionized water and stirring it for 1 h at room temperature. The MC dispersions were prepared by giving a specific amount of powder to preheated deionized water (50 °C), stirring it for 1 h and gently homogenizing it in a high pressure homogenizer (ElmusiFlex-C5, Avestin, Inc., Ottava, Canada) at a maximum pressure of 30 MPa. Protein dispersions were prepared on a w/w ratio and pH values were either 7.0 (native) or set to 5.8 by use of 1 M HCl and NaOH (Merck KGaA, Darmstadt, Germany). The samples were double packed in polyethylene (PE) pouches to strictly avoid a penetration of the PTM (pressure transmitting medium). All samples were freshly prepared and kept at 4 °C until analyses.

3.2.2 HPLT treatments

The HPLT treatments were conducted in an experimental HPLT unit containing a high pressure vessel with 265 mL volume (Sitec Sieber AG, Zurich, Switzerland) connected to a DS XHW-1373 air driven high pressure pump (Haskel, CA, USA). The vessel is equipped with a heating-cooling jacket and tempering was realized with a cryostat (Ultra-Kryomat RUK 50-D, Lauda, Germany). An 80 % (v/v) ethanol-water mixture was used as tempering medium as well as pressure transmitting medium (PTM, freezing point below -59 °C). Two type K thermocouples enabled temperature measurements of the PTM at the bottom of the vessel and inside of a sample at the top of the vessel. The pressure was measured with a pressure transducer (Intersonde HP28, Watford, England). The samples were thawed at room temperature before further preparations or analysis.

3.2.3 Measurement of surface hydrophobicity (ANS fluorescence)

1-anilino-8-naphthalene sulfonate (ANS) was used as hydrophobic probe (Bonomi, Iametti, Pagliarini, & Peri, 1988). ANS concentrations from 30 to 150 µmol/g protein (identified via Biuret method with BSA as standard) were used. A stock solution of ANS (ammonium salt, Sigma LifeScience, St. Louis, Missouri, USA) of 1 mM in a 10 mM phosphate buffer was prepared and diluted to concentrations between 60 and 300 µM. Protein samples were diluted with deionized water to a concentration of approximately 2 g/L. The final concentrations of ANS were reached after mixing the diluted sample with the ANS buffers in a ratio of 1:1. Fluorescence signals were measured by using a Perkin Elmer fluorescence spectrophotometer (650-10S and 150 Xenon Power Supply, Perkin Elmer, Waltham, Massachusetts, USA) with a measurement board (OMB-DAQ 3000, Omega Engineering Inc., Stamford, Connecticut, USA) and a LabView program (LabView Version 8.5.1, National Instruments, Austin, TX, USA) for data collection. Samples were filled into quartz cuvettes with 10 mm path length (101-QS, Hellma Analytics, Mühlheim, Germany), the excitation wavelength was set to 390 nm (2 nm slid width) and the emission wavelength was set to 470 nm (2 nm slid width).

3.2.4 Determination of accessible thiol groups

Changes in the amount of accessible thiol groups were detected by the use of Ellman's reagent (DTNB2- - 5,5'-dithiobis(2-nitrobenzoic acid)) according to Ellmann (1959). A method based on Hoffmann & Hamm (1978) was used. Ellman's reagent contained 0.01 M DNTB2- (Merck, KGaA, Darmstadt, Germany) solved in ethanol (96 %, Merck KGaA, Darmstadt, Germany). Samples were diluted in a 0.2 M Tris-HCl buffer for the determination of fast accessible thiol groups and in a 0.2 M Tris-HCl buffer with 1 % SDS for the determination of total accessible thiol groups. The dilution (v/v) was 1:15 for pure WPI, 1:10 for the MC:WPI=80:20 and 1:3 for the MC:WPI=80:20 mixture due to the different thiol content of the samples and their absorbance (final volume: 1500 µL). Absorbance of the samples was measured

in disposable half micro cuvettes (VWR International bvba, Leuven, Belgium) in a UV/VIS spectrophotometer at 412 nm. The absorbance of the diluted samples at 412 nm was used as a blank. Afterwards, 25 µL of Ellmann's reagent were added to the samples and after mixing a reaction time of 15 min was used before measuring the absorbance at 412 nm. A calibration curve with glutathione was used to quantify the concentration of accessible thiol groups. All samples were analyzed in triplicates.

3.2.5 Analyses of changes in secondary and tertiary structure (CD)

A J-710 spectropolarimeter (Jasco Labor und Datentechnik GmbH, Groß-Umstadt, Germany) was used to measure circular dichroism (CD) spectra of the samples. Spectra for the tertiary structure were measured between 250 and 320 nm (near UV) and for the secondary structure in the range of 190 to 260 nm (far UV). A path length of 10 mm (Cylindrical quartz glass cuvette 121-QS, Hellma GmbH & Co. KG, Müllheim, Germany) was used for tertiary structure measurements and a path length of 1 mm (120-QS, Hellma GmbH & Co. KG, Müllheim, Germany) for secondary structure. The step resolution was set to 1 nm, the scan speed to 50 $nm \cdot min^{-1}$ and the band width to 1 nm. The sensitivity was 10 mdeg for tertiary and 20 mdeg for secondary structure measurements. Samples were diluted with a 50 mM phosphate citrate buffer with the respective sample pH (7.0 or 5.8). The dilution was 1:10 (v/v; 0.2 %) for the tertiary and 1:100 (v/v; 0.02 %) if no other remarks are made. The phosphate citrate buffer was used as blank and measured 4 times. Samples were measured 8 times and spectra were averaged and analyzed by J-700 for Windows Standard Analysis software (Jasco Labor und Datentechnik GmbH, Groß-Umstadt, Germany).

3.2.6 Picture based particle analyses of casein containing flocks and aggregates

A flow particle image analysis (FPIA) was used to characterize large protein flocks and aggregates after HPLT treatments. The applied equipment was a FPIA 3000

(Sysmex Corporation, Kobe, Japan) with a 5x magnification lens. Particles were measured in the low power field (LPF). Samples were directly injected into the sheath fluid (Particle sheath, Sysmex Corporation, Kobe, Japan) in the sample inlet and after an automatic dilution of the device the measurement was performed. The equivalent spherical diameter and the maximum distance were used as size parameters of the samples and the circularity and the aspect ratio for the evaluation of the shape of the particles.

3.2.7 Identification of interactions in casein containing flocks and aggregates

Different reagents were used to identify the interactions between the protein molecules in the formed flocks and aggregates. Reagents were chosen according to Schmitt, et al. (2010) and Liu & Hsieh (2008). The solubility of the flocks and aggregates in phosphate buffer containing different reagents was determined to draw conclusions on the interactions between the protein molecules. Sodium chloride (NaCl) was used to identify electrostatic interactions, dithiothreitol (DTT) was applied to identify interactions over disulfide bonds, urea was used for cleaving hydrogen bonds and sodium dodecyl sulfate (SDS) for identification of hydrophobic interactions. The reagents were dissolved in a 0.02 M phosphate buffer with the respective concentrations given in Table 1. The pH of the buffers was set to the sample pH (7.0 or 5.8) and samples were mixed 1:1 with the respective buffer. A reaction time of 30 min at room temperature with agitation was used for buffers containing NaCl, urea or SDS. For an effective action of DTT it was necessary to

Table 1: Concentrations of reagents used in 0.02 M phosphate buffer for identification of interactions in protein flocks and aggregates

Reagent	Concentration
Sodium chloride (NaCl)	0.5 M
Dithiothreitol (DTT)	0.1 M
Urea	8.0 M
Sodium dodecyl sulfate (SDS)	0.1 M

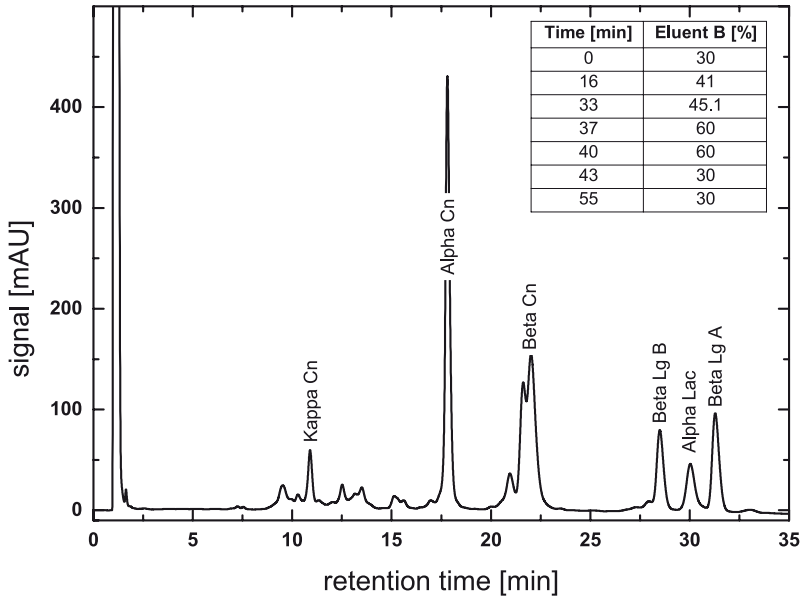


Figure 2: Sample chromatogram of a casein / whey protein dispersion (pH 7.0, 2 % protein, w/w, 80 % casein : 20 % whey protein). Table: Gradient of eluent B (0.17 % TFA in acetonitrile).

heat the buffer-sample mixture to 70°C for 15 min and a subsequent reaction time of 15 min at room temperature was applied as well. Furthermore, a buffer containing all reagents (total) at the above-mentioned concentrations was used to identify synergistic effects of the reagents. These sample-buffer mixtures were prepared as described for the DTT samples. All samples were also diluted in pure phosphate buffer as a control and both preparation steps (with and without heating) were used to identify the impact of heating on the sample. The solubility of all samples was determined by a centrifugation step for 30 min at 10,000 g and an analysis of the supernatants via HPLC. A HPLC method according to Bordin et al. (2001) was applied to detect changes in the compositions of the protein fractions (WPI/MC) and to quantify losses due to aggregation / flocculation. A C4 column (Jupiter 5u, C4, 300A, 150 x 2.0 mm, Phenomenex Inc., CA, USA) was used in a Dionex UltiMate 3000 System with a diode array detector (DAD-3000), a LPG-3400SD pump and an auto sampler (Dionex Corp., Sunnyvale, CA, USA). The temperature of the column was kept at 40 °C. A 0.2 % (v/v) solution of trifluoroacetic acid (TFA) in water was

used as eluent A and 0.17 % (v/v) TFA in acetonitrile was the composition of eluent B. The gradient and a sample chromatogram are shown in Figure 2. The flow was 0.5 mL.min-1 and the corresponding column pressure amounted to about 90 bar. The injected sample volume was 20 µL (if not indicated otherwise). The signal was detected at 214 nm. The protein samples were prepared as follows: Samples were diluted 1:5 (v/v) with a Guanidine-HCl-solution. 100 mL of Guanidine-HCl-solution contained 183.8 mg sodium citrate, 385.6 mg DTT and 71.65 g guanidine. Samples were heated to 60°C after diluting with the Guanidine-HCl-solution and cooled down to room temperature. The samples were filtered through a 0.45 µm filter after a further 1:4 (v/v) dilution step with deionized water. All samples were prepared for the HPLC analyses immediately after the treatments to avoid further formation or disruption of aggregates.

4 Results and discussion

4.1 Changes in surface hydrophobicity of whey proteins

An exemplary set of concentration depending fluorescence signals is given in Figure 3. F_{max} denotes a value for the exposed hydrophobic groups and K_D (ANS concentration for reaching half of F_{max}) can be used as an indicator for the affinity of the protein to bind the dye. The increase in F_{max} can be traced back to an exposure of hydrophobic groups from the core of the globular whey proteins (Dufour, Hoa, & Haertle, 1994; Gaucheron, et al., 1997; Tanaka, Koyasu, Kobayashi, & Kunugi, 1996). Figure 4 shows the changes in F_{max} and K_D for single WPI solutions treated at HPLT or HP conditions. It can be seen that especially the K_D is more influenced by treatments at room temperature in comparison to HPLT treatments. K_D which can be seen as an indicator for the affinity of the proteins to bind the dye molecules is not significantly influenced by HPLT treatments in comparison with an untreated sample. However, the maximum amount of bound dye (represented by F_{max}) is significantly

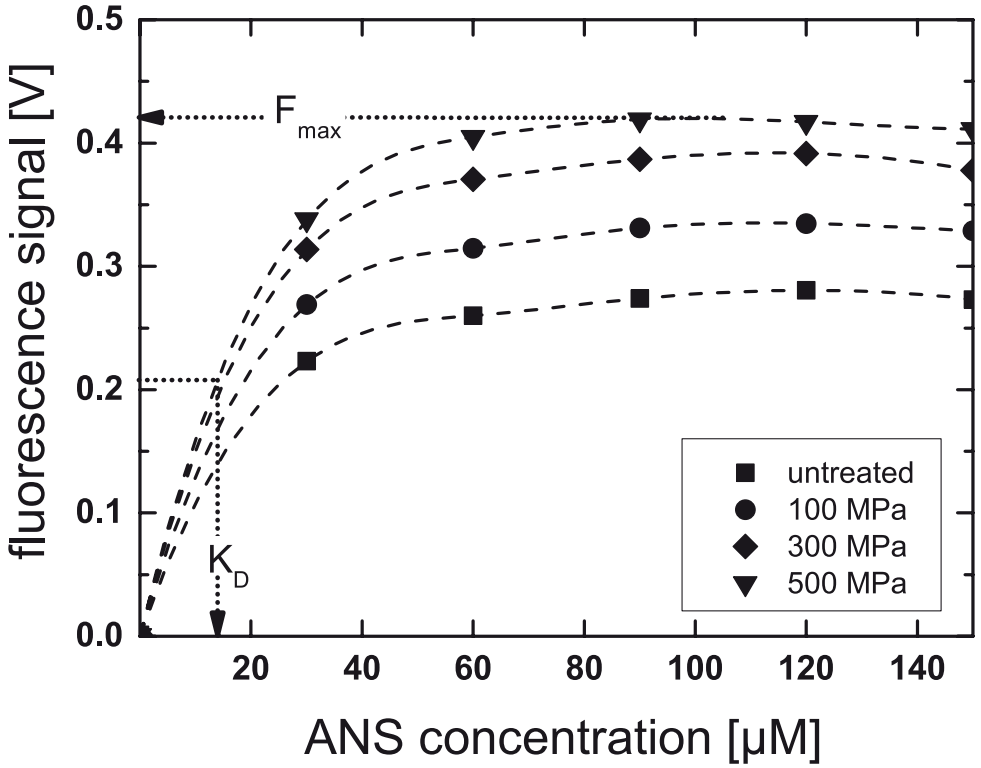


Figure 3: Exemplary course of ANS fluorescence signal and determination of maximum fluorescence (F_{max}) and binding affinity (K_D) for a 2% (w/w) WPI solution.

influenced by both HPLT and HP room temperature treatments. The increase is the highest for a treatment at room temperature at pH 7.0 while HPLT treatments at pH 7.0 have only minor impact on the F_{max}. For treatments at pH 5.8 the behavior is reversed and both HPLT and HP treatments resulted in a similar increase in F_{max}. This finding indicates a different mechanism of influencing the surface hydrophobicity via HPLT or HP treatments. Arias, Lopez-Fandino, & Olano (2000) reported a decreased denaturation of β-Lg in pressure treated milk at pH 5.5 in comparison to treatments at pH 7.0. This result could be also found for changes in F_{max}. Higher effects of HPLT treatments on whey protein fractions at pH 5.8 were also reported in a former study on HPLT effects on milk proteins (Baier, et al., submitted) and could be also found for changes in F_{max} during this study. The effect of freeze concentration during the ice formation can be an explanation for this effect as this is the main difference of HPLT treatments in comparison to HP treatments beside the low temperature. This effect

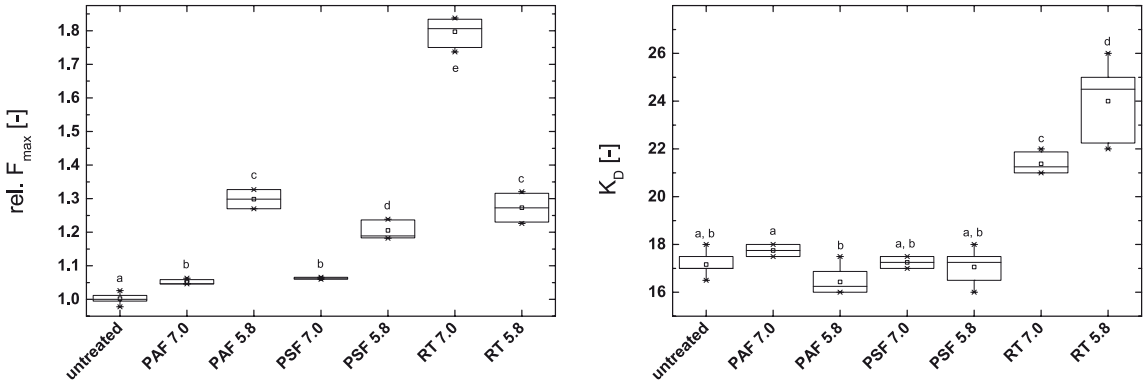


Figure 4: Box plots of changes in relative maximum ANS fluorescence (F_{max} normalized by F_{max} of untreated sample) and binding affinity (K_D) of single WPI solutions treated at 500 MPa and different temperature conditions (RT, PAF, PSF). Boxes with different letters are significantly different at a level of 0.05.

seems to be higher for freezing under pressure to higher ice modifications (PAF) in comparison to freezing during the decompression (PSF). However, the changes in K_D for treatments at room temperature are not correlated to the changes in F_{max}. A distinct increase in K_D, and thus, in the affinity to bind the hydrophobic probe could be detected for samples treated at room temperature (independent of the treatment pH). This finding can be assumed as another indicator for a different denaturation mechanism of HP treatments at room temperature and HPLT treatments as it was also suggested by other authors (Kolakowski, Dumay, & Cheftel, 2001).

4.2 Determination of accessible thiol groups

The changes in accessible thiol groups are presented in Figure 5. It is obvious that HP treatments at room temperature have the highest influence on fast accessible thiol groups and furthermore the total accessible thiol groups are only influenced by these treatments. The amount of fast accessible thiol groups is increased after HP treatments at room temperature at pH 7.0 by a factor of about 5 and by a factor of 3 at pH 5.8. This increase can be traced back to an exposure of free thiol groups which increases the reactivity of the WPI molecules (mainly β-Lg) which is also reported by several other authors (Dufour, et al., 1994; Funtenberger, Dumay, & Cheftel, 1997; Tanaka, et al., 1996). These results indicate again the limited denaturation at pH

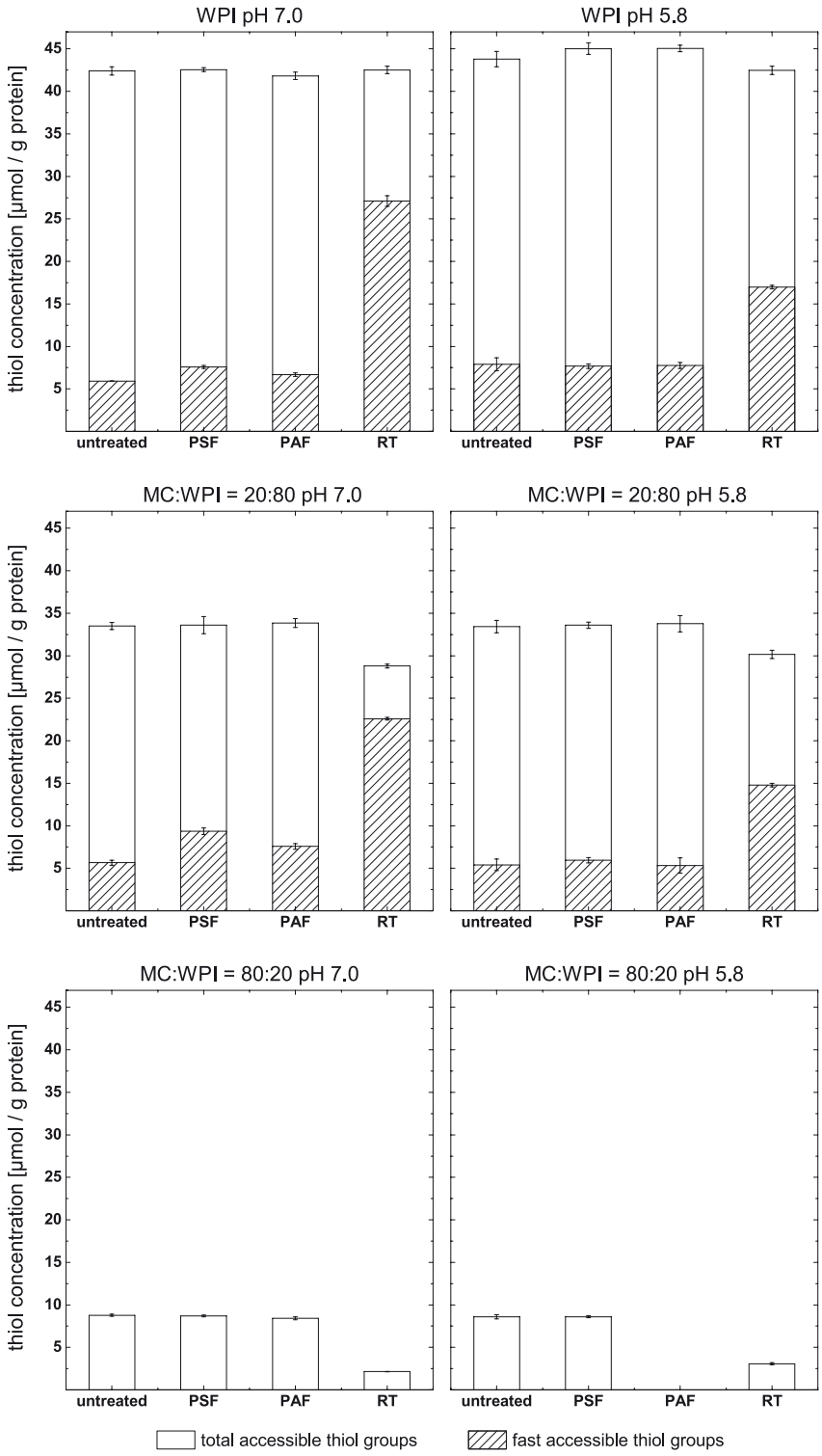


Figure 5: Fast and total accessible thiol groups of different milk protein dispersions treated at 500 MPa and different temperature conditions (RT, PAF, PSF). Fast accessible thiol groups for MC:WPI = 80:20 mixtures could not be determined because of the high amount of flocks in the samples. The accessible thiol groups of PAF treated MC:WPI mixture at pH 5.8 could not be determined due to the stability of the flocks against SDS solubilization.

5.8 and also the reduced effect of HPLT treatments on single WPI solutions (Baier, et al., submitted). HPLT treatments could only markedly affect the amount of fast accessible thiol groups in the presence of MC. This could be also an explanation for the results of a former study (Baier, et al., submitted) where effects on WPI fractions were enhanced in the presence of MC. The decrease in the total accessible thiol groups for HP treatments at room temperature which occurs especially in the presence of MC could be explained by interactions of β-Lg with especially κ-Cn which is also reported by Huppertz, Fox, de Kruif, & Kelly (2006). This effect seems to be more pronounced at neutral pH.

4.3 Changes in secondary and tertiary structure

It can be seen in Figure 6 that the higher influence of HP treatments at room temperature in comparison with HPLT treatments could be also confirmed by determination of the secondary structure. However, changes in the far UV CD signal could be observed for all the treatment conditions. Treatments of single WPI solutions (pH 7.0) at room temperature led to an increase of the peak intensity at 208 nm which corresponds to an increase in α-helix structures. Simultaneously, the peak intensity at 200 nm decreased which denotes a decrease in β-sheet structures. Similar findings of shifts from β-sheet structures to α-helix structures were also reported by Heremans, et al. (1985), Wong, et al (1988) and Aouzelleg, Bull, Price, & Kelly (2004). Furthermore, a pressure induced unfolding for these conditions is indicated by an increase in the peak intensity at 195 nm which is correlated to an increase in random coil structures. In comparison the changes of α-helix structures due to PAF treatments are lower. A slight increase of β-sheet structures (indicated by positive peak at 198 nm) could be observed and a slight increase in random structures is indicated by an increased peak intensity of the negative peak at 193 nm. However, PSF treatments induced a different change in the secondary structure. An increase in the positive peak intensity at 193 nm in combination with a decrease in the negative peak intensity at 193 nm indicates a structuring effect with

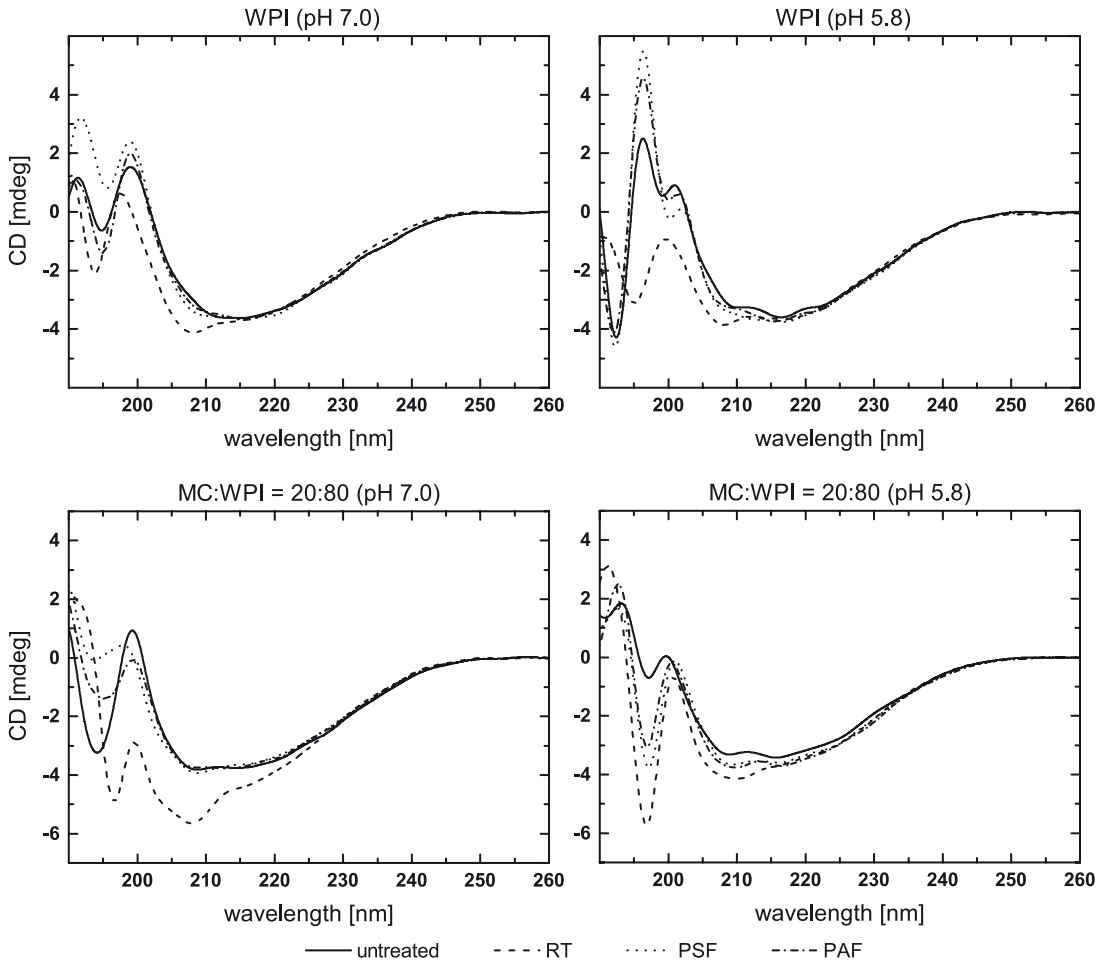


Figure 6: Far UV CD spectra of milk protein dispersions (single WPI solution, MC:WPI = 20:80 mixture) treated at different temperatures (RT, PSF, PAF) and different pH values.

the formation of α-helices from random structures.

Regarding the far UV CD spectra of single WPI solutions at pH 5.8 it is obvious that again a HP treatment at room temperature caused the most distinct changes. An increase in the negative peak intensity at 208 nm indicates again an increase in α-helix structures and the existence of a negative peak at 195 nm refers to an increase of random structures combined with a decrease of β-sheet structures. However, both kinds of HPLT treatments led only to a slight increase in β-sheet structures. Pressure induced changes of secondary structure motifs can be explained by changes in the hydrophobic regions of β-Lg which are responsible for the stabilization of α-helices and β-sheet structures (Considine, Patel, Anema,

Singh, & Creamer, 2007). Interestingly, HPLT treatments seems to prevent on the one hand the pressure induced unfolding maybe due to low temperatures and on the other hand especially PSF treatments seem to cause a structuring effect which can induce specific changes in protein functionality.

Treatments of mixtures of WPI and MC (MC:WPI = 20:80) also changed the far UV CD spectra (see Figure 6). Again HP treatments at room temperature caused the most distinctive changes at both pH values. At pH 7 a strong increase in the CD signal in the wavelength range from 208 to 222 nm indicating an increase in α-helix structures while the peak intensity around 198 nm decreases which can be correlated to a decrease of β-sheet structures. Furthermore, the amount of disordered structures is denoted by a higher negative peak intensity around 193 nm. Due to the almost absence of secondary structure motifs in caseins it is likely that the solubilization of MC fractions due to HP and HPLT treatments (see e.g. Baier, et al., submitted) led also to an increase in the amount of disordered structures after treatments. Samples treated at pH 5.8 showed a less pronounced change in α-helix and β-sheet structures as the CD spectra in the range above 198 nm are quite similar to the untreated sample. The lower effect of treatments at pH 5.8 are in accordance with the work of Arias, et al. (2000) who reported a diminished denaturation of β-Lg in milk at pH 5.5 in comparison to neutral pH. Samples with a high amount of MC (single MC dispersions, MC:WPI = 20:80 mixtures) showed only a small amount of secondary structure motifs (see supplemental material) and only slight changes could be observed.

HPLT treatments at pH 7.0 as well as at pH 5.8 did not alter the near UV CD spectra of single WPI solutions (see Figure 7). However, HP treatments at room temperature induced changes in the tertiary structure at both pH values. The CD signal is lower for HP treated WPI samples (pH 7.0) over the complete near UV range and the peak intensity is lowered as well. This finding could be correlated to a loss of tertiary structure which is also reported by Aouzelleg, et al. (2004). The loss of tertiary structure

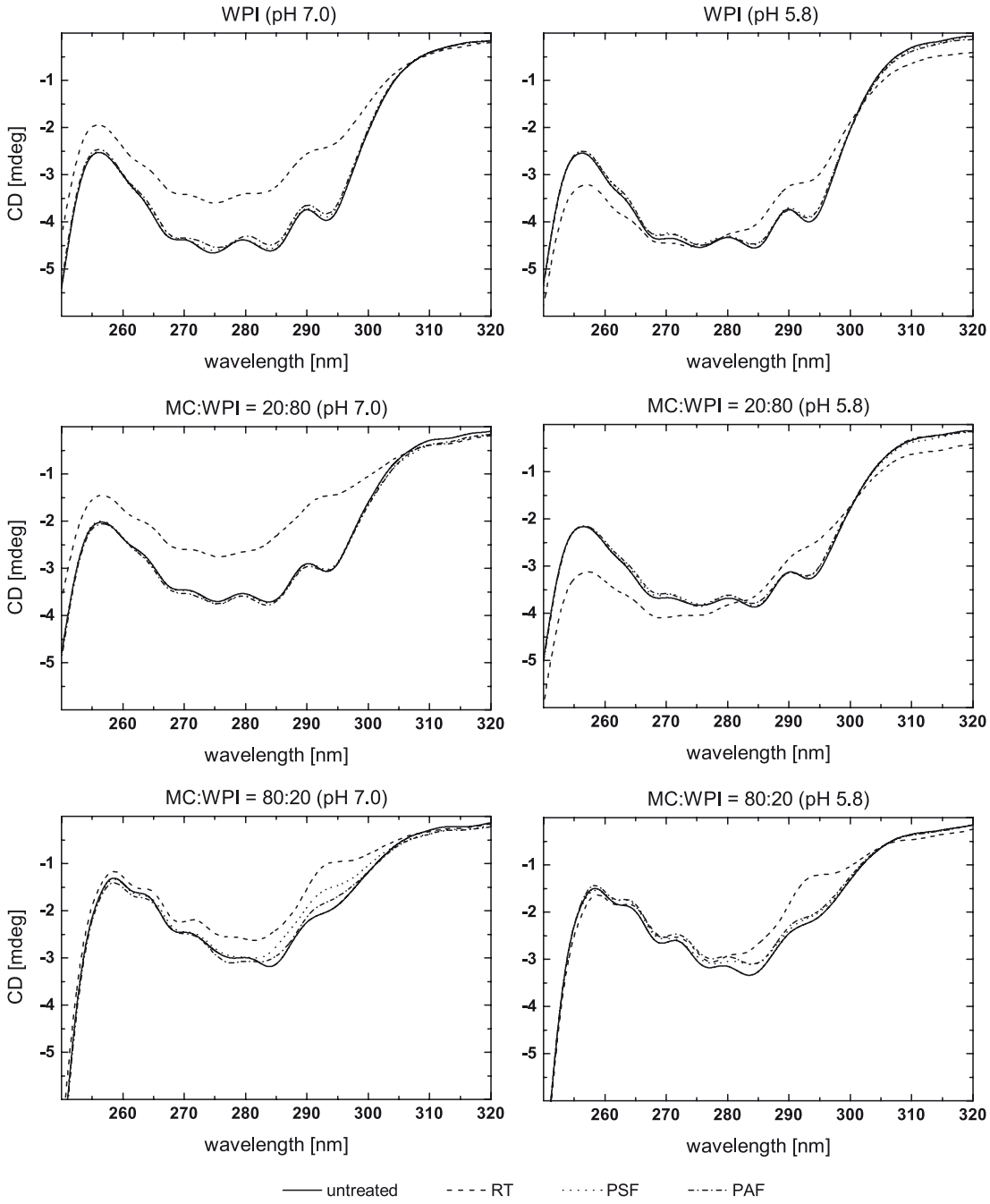


Figure 7: Near UV CD spectra of milk protein dispersions (single WPI solution, MC:WPI = 20:80, MC:WPI = 80:20 mixture) treated at different temperatures (RT, PSF, PAF) and different pH values.

could be traced back to a change in the environment of hydrophobic or polar amino acid residues (Considine, et al., 2007; X. M. Liu, Powers, Swanson, Hill, & Clark, 2005). This assumption is also supported by the strong decrease of tryptophan peak intensity at 295 nm. Again HPLT induced changes could not be observed which is

in agreement with the findings of Gaucheron, et al. (1997) and Kolakowski, Dumay, & Cheftel (2001) who also find a diminished pressure induced denaturation effect at lower temperatures. However, changing the pH of single WPI solutions to 5.8 decreases the effect on the tertiary structure. This effect was also observed for changes in the surface hydrophobicity and also other authors reported a decreased denaturation for β-Lg at lower pH values (pH 5.5, Arias, et al., 2000). This effect may be traced back to the reduced repulsion at pH values closer to the isoelectric point, which could lead to different interactions of the molecules. Furthermore, the intensity of changes in tertiary structure seems to be correlated to the amount of WPI. It can be seen from Figure 7 that with a decreasing amount of WPI (mixtures with MC) the overall effect is diminished. This effect can be allocated to the lower amount of structured elements from caseins which also diminish the overall near UV CD signal. In conclusion, HPLT treatments seem to have some specific effects on the secondary structure of WPI while leaving the tertiary structure unaffected. HP treatments at room temperature caused an unfolding of WPI molecules and a shift from α-helix to β-sheet structures. This study also confirms a diminished effect at lower pH (5.8) which was also found by other authors (Arias, et al., 2000; Baier, et al., submitted).

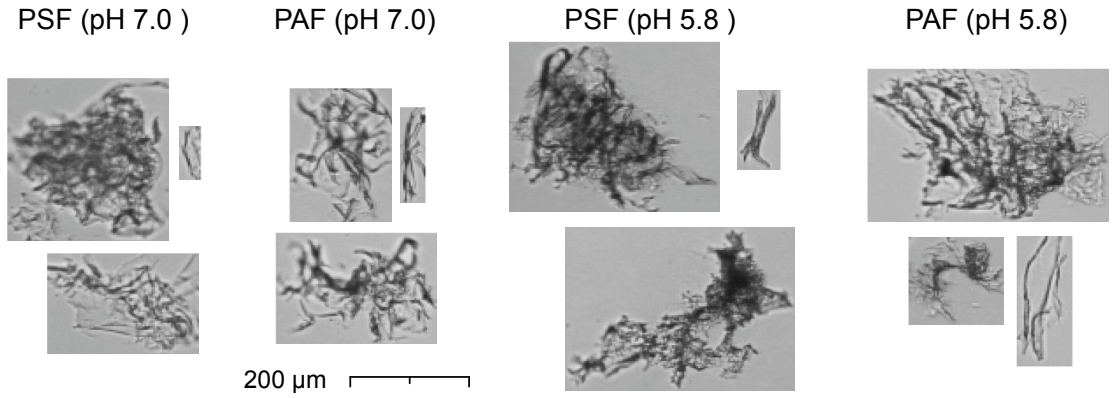


Figure 8: Selected FPIA pictures of some very large flocks in pure MC dispersions after HPLT treatments.

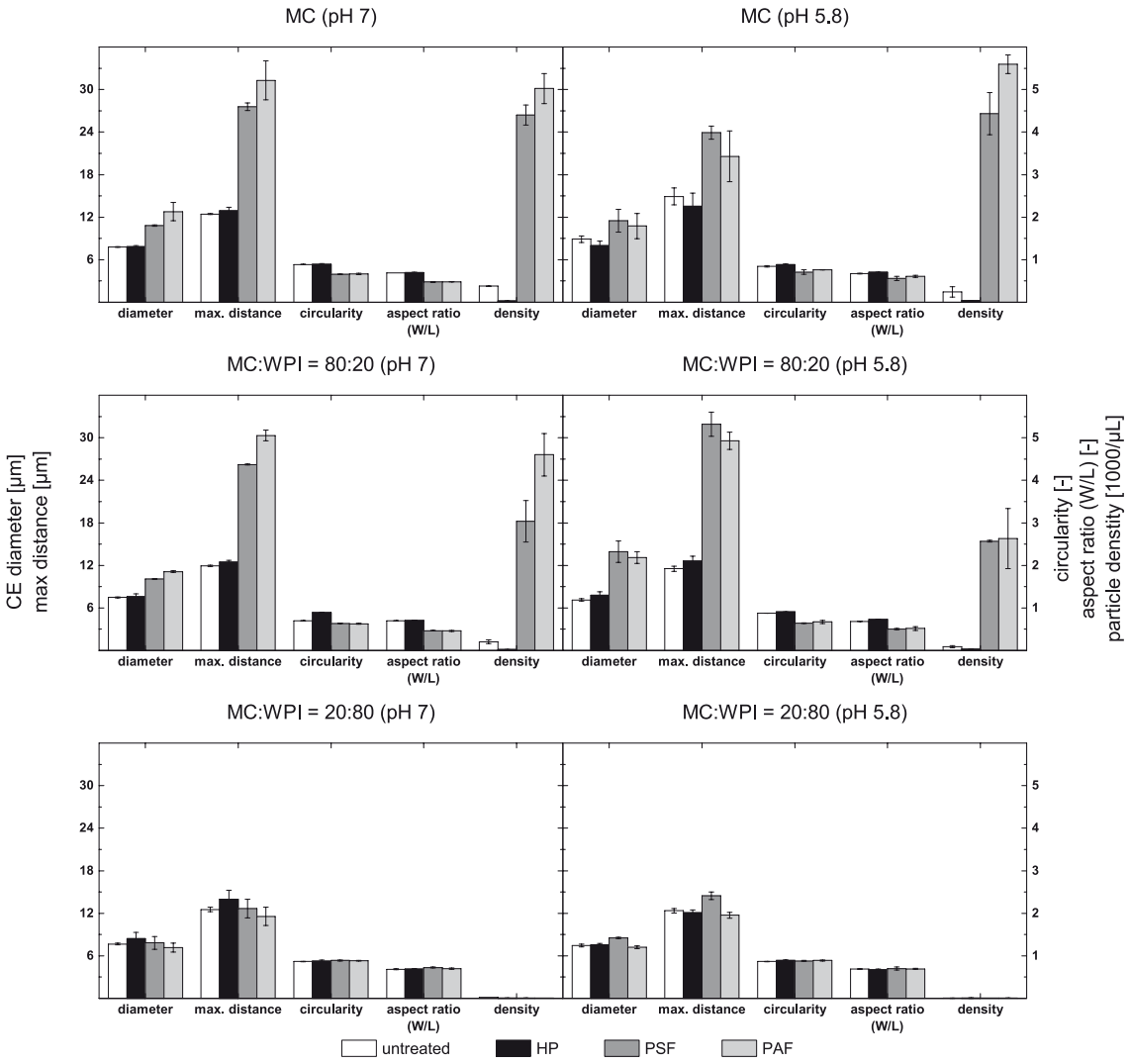


Figure 9: Results of size and shape analyses of MC containing samples after pressure treatments at different temperatures (HP, PSF, PAF) obtained via Flow Particle Image Analysis (FPIA).

4.4 Characterization of casein containing flocks and aggregates

HPLT treated casein containing samples always contained large flocks although the overall solubility of MC fractions is often increased (Baier, et al., submitted). Some exemplary pictures of very large flocks are presented in Figure 8. It can be seen that the flocks are aggregates of long fibers. Both, single fibers and large flocks should change the techno-functional behavior of these samples. Figure 9 shows the results of the size and shape analyses of MC containing samples after treatments. It is obvious that only HPLT treatments caused an increase of the size

displayed by an increased diameter. The formed flocks have an almost elongated shape which is denoted by the strong increase in the maximum distance of about a factor of 2 at pH 7.0 and a decrease in the circularity from about 0.9 to about 0.65. The high increase in the particle density up to 5,000 particles per microliter for single MC dispersions depicts the creation of a large amount of created particles in the measurement range. However, a high amount of MC seems to be necessary to create a measureable amount of large flocks. This fact is indicated by the very low particle density of HPLT treated mixtures with a low amount of MC (MC:WPI = 20:80). PAF

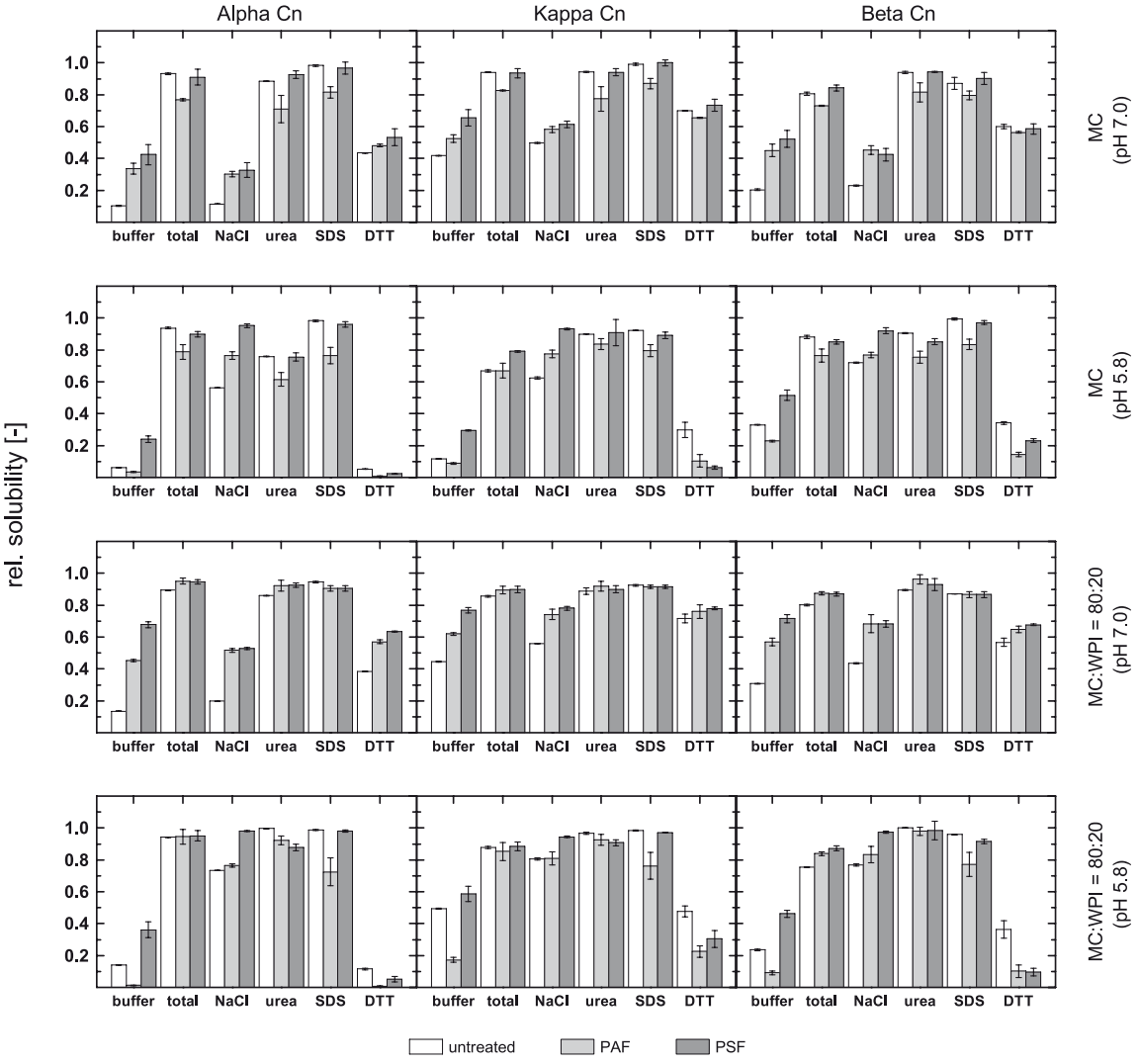


Figure 10: Relative solubility of MC fractions after dissolving in different buffers and centrifugation at 10,000 g for 30 min. Solubilities are normalized by the total amount of the respective fraction in the untreated sample determined via quantitative HPLC (total amount contains both, soluble and insoluble parts of the respective fraction).

treatments seem to induce higher amounts of larger particles especially at neutral pH which could be related to the higher dissociation of native micelles (Baier, et al., submitted) and, thus, to the higher amount of reaction partners. However, lowering the pH to 5.8 again reduces the effect possibly due to a decreased reactivity. The samples which contained flocks were dissolved in buffer systems with different reagents to evaluate the interactions in the created aggregates. Figure 10 shows the relative solubility of the respective MC fractions after dissolving in the mentioned buffers. The solubility in the pure phosphate buffer increased at pH 7.0 for all treatment conditions and at pH 5.8 only for PSF treatments, while it decreases for PAF treatments. The solubilization effect is most pronounced for α-Cn due to its low initial solubility. This is in accordance with previous results (Baier, et al., submitted). It is obvious that urea and SDS caused the highest solubilization in both untreated and treated samples. This fact displays the main interactions in the casein micelles and presumably in most of the flocks which are hydrophobic interactions and hydrogen bonds (Y. Liu & Guo, 2008). It was not possible to reach a full solubilization in single MC dispersions treated at pH 7.0 and 5.8 after PAF treatments. In conclusion, these flocks are stabilized by strong interactions which cannot be separated by the applied detergents. The presence of WPI at neutral pH seems to retard the formation of these interactions as there is no difference between untreated and PAF treated samples. In mixtures at pH 5.8 PAF treated samples cannot be fully solubilized in the presence of SDS but in buffer containing urea. This leads to the assumption that hydrogen bonds formed during PAF treatments prevent complete solubilization after dissociation of hydrophobic interactions. Interestingly, dissolving in the presence of DTT caused some differences in single MC dispersions especially at pH 5.8 although a formation of disulphide bonds (thiol-disulphide exchange) is unlikely due to the absence of the necessary free thiol groups. However, these samples were heated to activate DTT and, thus, a solubilization effect is possible, which was checked by heating a phosphate buffer sample at the same conditions (data not shown).

This solubilization could be confirmed for pH 7.0 but not for pH 5.8. It seems that PSF treated samples contain thermal sensitive proteins which became insoluble in addition to the flocks after heating. At pH 5.8 electrostatic interactions seem to be more important for the solubility of all three MC fractions which can be traced back to a shift in the surface charge of the molecules. Especially for PAF treated samples the solubility is markedly enhanced after dissolving in NaCl buffer in comparison to the untreated sample, which leads to the assumption that electrostatic interactions play a major role in the stability of these flocks.

In conclusion, HPLT treatments caused new interactions depending on the pH and on the kind of HPLT treatments, which enable a formation of specific flocks with presumably new functional properties.

5 Conclusions

The study pointed out that HPLT induced effects differ from effects induced by common HP treatments at room temperature. HPLT treatments induced specific changes in milk protein samples although the changes are often smaller in comparison to HP treatments at room temperature. Both kind of HPLT treatments (PAF and PSF) led to changes in the secondary structure of WPI containing samples while leaving the tertiary structure unchanged. A structuring effect was observed especial for PSF treatments indicated by an increase in β-sheet structures, while HP treatments at room temperature led to an unfolding. The amount of accessible thiol groups was not affected by HPLT treatments as well as the change in surface hydrophobicity was quite small while large changes were observed for treatments at room temperature. However, HPLT treatments enabled the formation of large casein based flocks mainly stabilized by hydrophobic and hydrophilic interactions. PAF treatments of mixtures with a high amount of MC (MC:WPI = 80:20) led to the formation of flocks with different interactions in comparison to native micelles. In

contrast, PSF treatments at pH 5.8 caused thermal sensitive protein fractions which became insoluble after heating to 70 °C. In conclusion, HPLT treatments provide the opportunity to create specific modifications of milk protein samples and, thus, enable changing functional properties and new product applications.

6 Acknowledgements

The authors like to acknowledge Anne Heckelmann for supporting the development of protein analytics and fruitful discussions on protein interactions. Furthermore, the authors like to thank Doreen Schiller for her assistance with the HPLC analytics.

7 References

Anema, S. G. (2008). Effect of milk solids concentration on whey protein denaturation, particle size changes and solubilization of casein in high-pressure-treated skim milk. International Dairy Journal, 18(3), 228-235.

Aouzelleg, A., Bull, L. A., Price, N. C., & Kelly, S. M. (2004). Molecular studies of pressure/temperature-induced structural changes in bovine beta-lactoglobulin. Journal of the Science of Food and Agriculture, 84(5), 398-404.

Arias, M., Lopez-Fandino, R., & Olano, A. (2000). Influence of pH on the effects of high pressure on milk proteins. Milchwissenschaft-Milk Science International, 55(4), 191-194.

Baier, D., Schmitt, C., & Knorr, D. (submitted). Effect of high pressure - low temperature processing on composition and colloidal stability of casein micelles and whey proteins. International Dairy Journal (submitted manuscript).

Belloque, J., Lopez-Fandino, R., & Smith, G. M. (2000). A H-1-NMR study on the effect of high pressures on beta-lactoglobulin. Journal of Agricultural and Food Chemistry, 48(9), 3906-3912.

Bonomi, F., Iametti, S., Pagliarini, E., & Peri, C. (1988). A spetcrofluorimetric approach to the estimation of the surface hydrophobicity modifications in milk proteins upon thermal treatment. Milchwissenschaft, 43, 281-285.

Bordin, G., Raposo, F. C., de la Calle, B., & Rodriguez, A. R. (2001). Identification and quantification of major bovine milk proteins by liquid chromatography. Journal of Chromatography A, 928(1), 63-76.

Bridgman, P. W. (1912). Water under pressure. Proceedings of the American Academy of Arts and Sciences, 48, 450-558.

Carrier, D., Mantsch, H. H., & Wong, P. T. T. (1990). Pressure-induced reversible changes in secondary structure of poly(L-lysine) an IR spectroscopic study. Biopolymers, 29, 837-844.

Considine, T., Patel, H. A., Anema, S. G., Singh, H., & Creamer, L. K. (2007). Interactions of milk proteins during heat and high hydrostatic pressure treatments - A review. Innovative Food Science & Emerging Technologies, 8(1), 1-23.

Dufour, E., Hoa, G. H. B., & Haertle, T. (1994). High-pressure effects on β-lactoglobulin interactions with ligands studied by fluorescence. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology, 1206(2), 166-172.

Ellmann, G. L. (1959). Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics, 82, 70-77.

Funtenberger, S., Dumay, E., & Cheftel, J. C. (1997). High pressure promotes beta-lactoglobulin aggregation through SH/S-S interchange reactions. Journal of Agricultural and Food Chemistry, 45(3), 912-921.

Gaucheron, F., Famelart, M. H., Mariette, F., Raulot, K., Michel, F., & LeGraet, Y. (1997). Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk. Food Chemistry, 59(3), 439-447.

Gekko, K., & Hasegawa, Y. (1989). Effect of temperature on the compressibility of native globular proteins. J. Phys. Chem., 93, 426-429.

Gekko, K., & Noguchi, H. (1979). Compressibility of globular proteins in water at

25 8C. J. Phys. Chem., 83, 2706-2714.

Hawley, S. A. (1971). Reversible pressure-temperature denaturation of chymotrypsinogen. Biochemistry, 10(13), 2436-&.

Heremans, K., & Wong, P. T. T. (1985). Pressure effects on the Raman spectra of proteins: pressure-induced changes in the conformation of lysozyme in aqueous solution. Chem. Phys. Lett., 118, 101-104.

Hoffmann, K., & Hamm, R. (1978). Sulfhydryl and disulfide groups in meats. Advanced Food Research, 24, 1-111.

Huppertz, T., & De Kruif, C. G. (2006). Disruption and reassociation of casein micelles under high pressure: Influence of milk serum composition and casein micelle concentration. Journal of Agricultural and Food Chemistry, 54(16), 5903-5909.

Huppertz, T., Fox, P. F., de Kruif, K. G., & Kelly, A. L. (2006). High pressure-induced changes in bovine milk proteins: A review. Biochimica Et Biophysica Acta-Proteins and Proteomics, 1764(3), 593-598.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004). High pressure treatment of bovine milk: effects on casein micelles and whey proteins. Journal of Dairy Research, 71(1), 97-106.

Huppertz, T., Kelly, A. L., & de Kruif, C. G. (2006). Disruption and reassociation of casein micelles under high pressure. Journal of Dairy Research, 73(3), 294-298.

Kauzmann. (1959). Some factors in the interpretation of protein denaturation. Advances in Protein Chemistry, 14, 1-63.

Kolakowski, P., Dumay, E., & Cheftel, J. C. (2001). Effects of high pressure and low temperature on beta-lactoglobulin unfolding and aggregation. Food Hydrocolloids, 15(3), 215-232.

Liu, K. S., & Hsieh, F. H. (2008). Protein-protein interactions during high-moisture extrusion for fibrous meat analogues and comparison of protein solubility methods using different solvent systems. Journal of Agricultural and Food Chemistry, 56(8), 2681-2687.

Liu, X. M., Powers, J. R., Swanson, B. G., Hill, H. H., & Clark, S. (2005). High hydrostatic pressure affects flavor-binding properties of whey protein concentrate. Journal of Food Science, 70(9), C581-C585.

Liu, Y., & Guo, R. (2008). pH-dependent structures and properties of casein micelles. Biophysical Chemistry, 136(2-3), 67-73.

Masson, P. (1992). Pressure denaturation of Proteins. In C. Banly, K. Hayashi, K. Heremans & P. Masson (Eds.), High Pressure and Biotechnology (pp. 89-99).

Orlien, V., Knudsen, J. C., Colon, M., & Skibsted, L. H. (2006). Dynamics of casein micelles in skim milk during and after high pressure treatment. Food Chemistry, 98(3), 513-521.

Richards, F. M. (1977). Areas, volumes, packing and protein structure. Ann. Rev. Biophys. Chem, 20, 321-342.

Schade, B. C., Rudolph, R., Lüdemann, H. D., & Jaenicke, R. (1980). Reversible high pressure dissociation of lactic dehydrogenase from pig muscle. Biochemistry, 19, 1305-1332.

Schmitt, C., Moitzi, C., Bovay, C., Rouvet, M., Bovetto, L., Donato, L., Leser, M. E., Schurtenberger, P., & Stradner, A. (2010). Internal structure and colloidal behaviour of covalent whey protein microgels obtained by heat treatment. Soft Matter, 6(19), 4876-4884.

Smeller, L. (2002). Pressure-temperature phase diagrams of biomolecules. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology, 1595(1-2), 11-29.

Tanaka, N., Koyasu, A., Kobayashi, I., & Kunugi, S. (1996). Pressure-induced change in proteins studied through chemical modifications. International Journal of Biological Macromolecules, 18(4), 275-280.

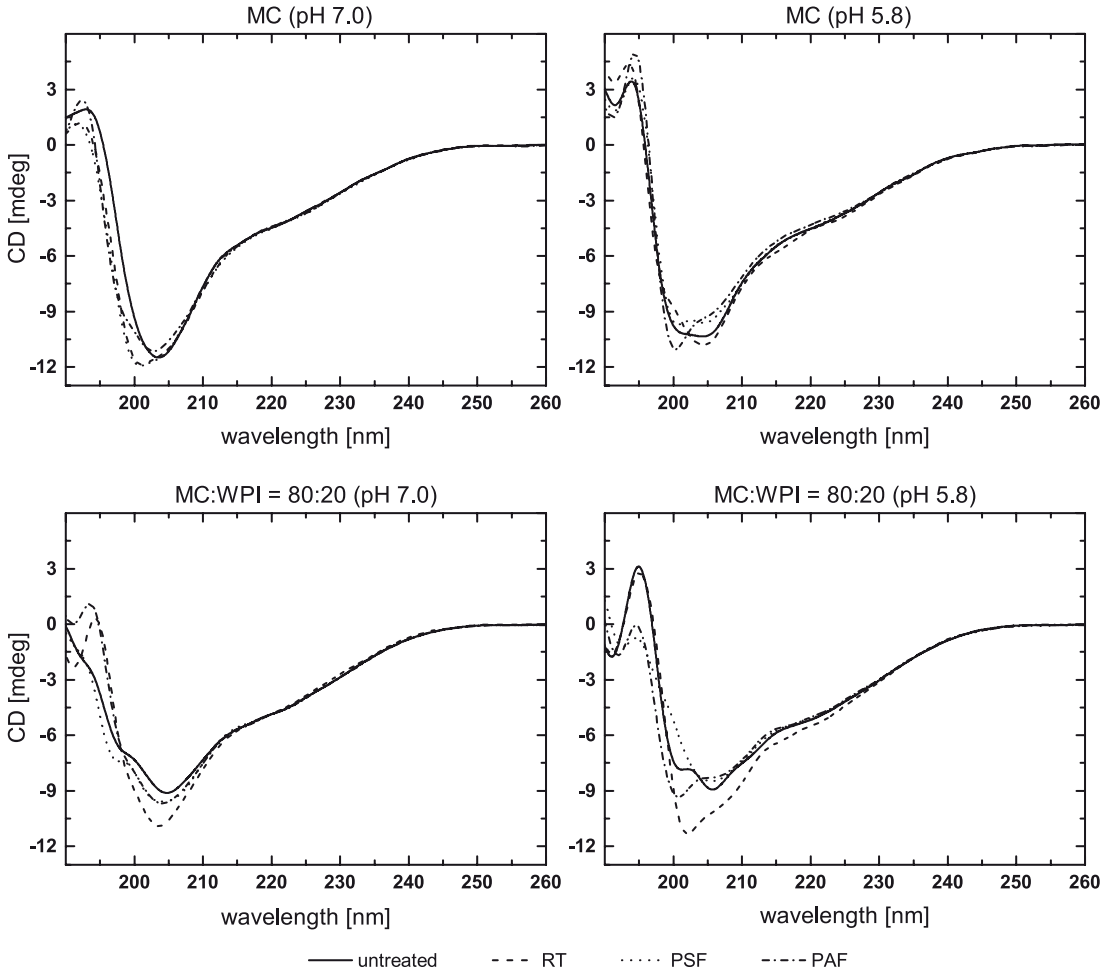
Urrutia Benet, G., Schlüter, O., & Knorr, D. (2004). High pressure-low temperature processing. Suggested definitions and terminology. Innovative Food Science & Emerging Technologies, 5(4), 413-427.

Wong, P. T. T., & Heremans, K. (1988). Pressure effects on protein secondary structure and hydrogen exchange in chymotrypsinogen. Biochim. Biophys. Acta, 956, 1-9.

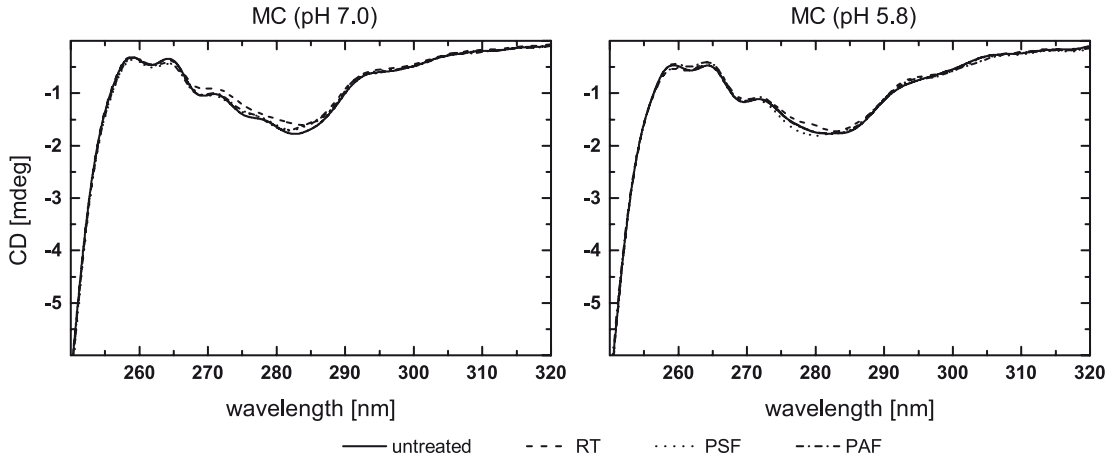
Yang, J., Dunker, A. K., Powers, J. R., Clark, S., & Swanson, B. G. (2001). beta-Lactoglobulin molten globule induced by high pressure. Journal of Agricultural and Food Chemistry, 49(7), 3236-3243.

Zamyatnin. (1972). Protein volume in solution. Pro. Biophys. Mol. Biol., 24, 109-123.

8 Supplemental material



Suppl. Figure 1: Far UV CD spectra of milk protein dispersions (single MC dispersion, MC:WPI = 80:20 mixture) treated at different temperatures (RT, PSF, PAF) and different pH values.



Suppl. Figure 2: Far UV CD spectra of single MC dispersion treated at different temperatures (RT, PSF, PAF) and different pH values.

2.2.2 Supplemental Results and Conclusions for Experimental Plan

The investigations of HPLT induced structural changes in micellar casein and whey proteins showed that HPLT treatments induce different structural changes in comparison to HP treatments at room temperature. Although the extent of changes is often smaller, a few HPLT specific changes were found:

- Large flocks and aggregates in casein based samples contain fibrillar protein structures and networks of fibers,
- Flocks are mainly stabilized by similar interactions as casein micelles,
- HPLT treatments led to changes in the secondary structure without changing tertiary structure of WPI,
- HPLT treatments seem to build up secondary structure motifs from random coil structures.

Additional structural informations were investigated by measuring the zeta potential (ζ) as an indicator for the charge of the molecules. The charge of particles or molecules is an important factor as it is correlated to the stability of a dispersion (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948; Müller, 1996). A lower negative zeta potential (higher absolute value) means a higher repulsion and consequently a higher stability of the dispersion. When particles or molecules are in contact with a polar medium like water, they possess a surface charge due to the dissociation of functional groups on the surface. The zeta potential is highly influenced by the ionic neighborhood of the particle or molecule (Salopek et al., 1992). A Zetasizer Nano ZS (Malvern Instruments Inc., Worcestershire, UK) was used to perform the measurements. Samples were diluted in 0.05 M phosphate citrate buffer to a final concentration of 0.1% (w/v) protein (pH value of buffer was identical to sample pH). Electrophoretic cuvettes (Disposable folded capillary cell, Malvern Instruments Inc., Worcestershire, UK) were used and samples were filled in with special focus on avoiding bubbles. Measurements were performed at a voltage of 40 V depending on the ionic strength of the sample and a temperature of 25°C. All measurements were performed in triplicates whereas all analyses were done again in triplicates (leading to 18 values per treatment in duplicates).

Figure 16 shows the results of the determination of the zeta potential. It can be seen that changes

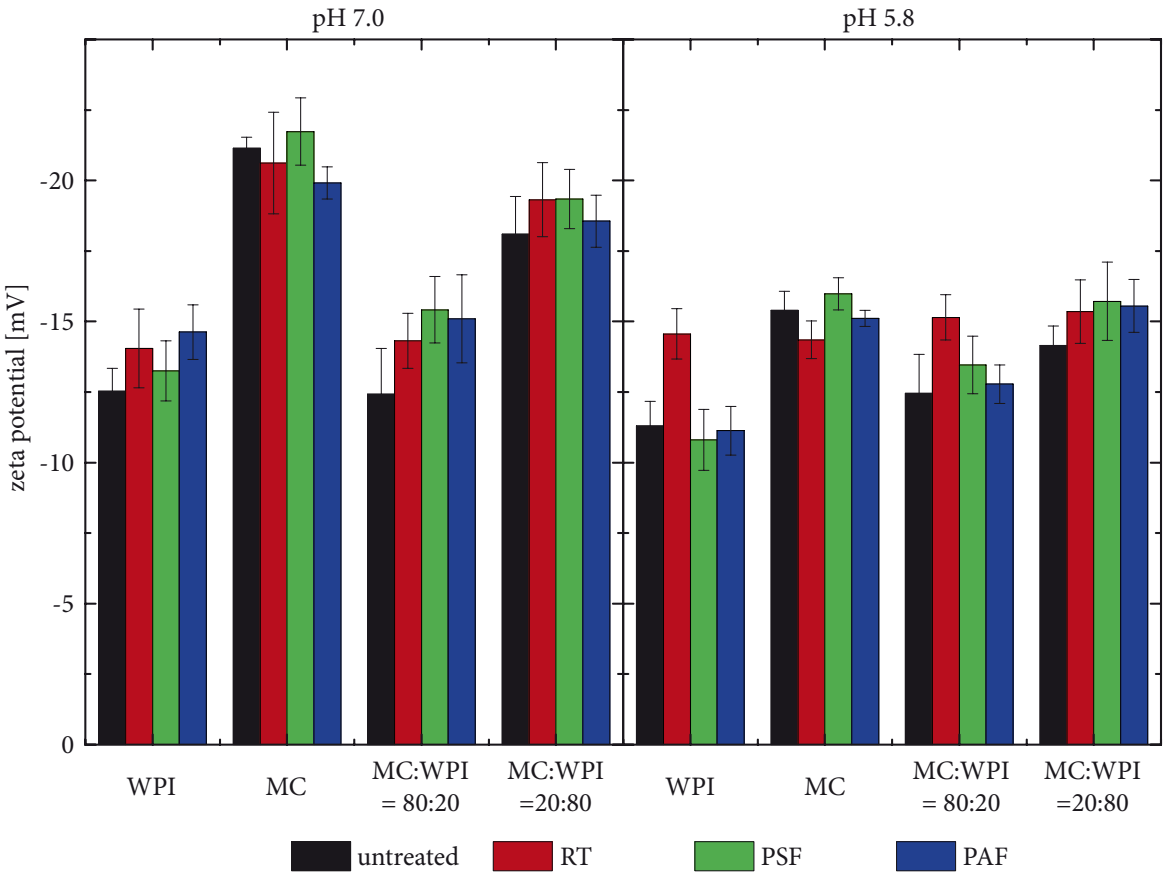


Figure 16: Zeta potential of treated milk protein dispersion with different pH value and different sample composition.

due to treatments are quite low and significant changes are difficult to find because of high deviations. Treatments of samples with a high WPI content at pH 5.8 (pure solution and MC:WPI = 20:80 dispersion) and room temperature induce an increase in the zeta potential. Silva et al. (2013) showed a decreasing foam stability with an increasing zeta potential for casein micelles at different pH values. However, the reported changes of these authors are within their predicted accuracy of the measurement. Other authors (Perrechil & Cunha, 2013) were also not able to show a direct correlation between the stability of emulsions stabilized by sodium caseinate and κ-carragenan and the zeta potential. Yet, Schmitt et al. (2007) showed a decreased zeta potential with decreasing pH (7.0-6.0) for heat induced soluble whey protein aggregates related to an increased liquid foam stability. The zeta potential highly depends on the surrounding media and results are difficult to compare due to the differences in the mechanisms used to influence the zeta potential. A general correlation between the zeta potential and the functional behavior

seems not to be possible due to the complexity of stabilization mechanisms. Nevertheless, the presented structural changes and especially the formation of large flocks may influence the functional properties of the treated milk protein suspensions. Foam and emulsion stability as well as rheological and surface rheological properties will be taken as an indicator for changes in the functional behavior of milk protein based dispersions within the following chapter.

2.3 Changes in Functionality of Whey Protein and Micellar Casein after HPLT Treatments

2.3.1 Manuscript

Authors: Daniel Baier, Christophe Schmitt and Dietrich Knorr

Revised version published in Food Hydrocolloids (Volume 44, February 2015, Pages 416–423)

Changes in functionality of whey protein and micellar casein after high pressure – low temperature treatments

Daniel Baier[1], Christophe Schmitt[2] and Dietrich Knorr[1]

[1] TU Berlin, Dept. of Food Biotechn. and Food Process Eng., Berlin, Germany

[2] Nestlé Research Center, Department of Food Science and Technology, CH-1000 Lausanne 26, Switzerland

1 Abstract

Changes in functional properties of micellar caseins (MC) and whey proteins (WPI) due to high pressure – low temperature (HPLT) treatments were investigated and compared to changes induced via high pressure treatments at room temperature (HP). Single whey protein solutions, micellar casein dispersions and two mixtures (micellar caseins:whey proteins weight mixing ratios 80:20 and 20:80) were treated at a concentration of 2 % (w/w) and at two different pH values (7.0 and 5.8). Oscillating pendant drop and shear experiments were performed to identify changes in the rheological behavior at air/water interface and in bulk, respectively. Foaming and emulsification experiments were conducted to investigate further impacts on the functional behavior. Both, HPLT and HP treatments led to a decreased emulsion stability for emulsions from pure WPI solutions independent on the treatment pH, while the foam stability was increased for these samples. In comparison, the changes for pure MC dispersions were in the same direction but less pronounced. HPLT treatments of MC rich samples always contained large flocks which changed the rheological behavior of these samples from a Newtonian to a shear-thickening behavior. The elastic part of the surface dilatational modulus was increased for a pure WPI solution after HPLT and HP treatments while the viscous part remained

unaffected. However, changes in functional properties highly depended on the sample composition and results for mixtures differ from those for pure dispersions.

2 Introduction

Proteins play a major role as functional ingredients in food as they offer the potential to create and stabilize disperse systems like foams, emulsions and gels. In general functionality can be regarded as 'any property of a food or food ingredient except its nutritional ones that influences its utilization' (Pour-El, 1981). Dispersed systems are thermodynamic instable as their free energy is higher than for the two single phases. In consequence, additives are needed to stabilize the dispersed phase within the continuous phase. Proteins are suitable for this challenge due to their amphiphilic character, and, thus they are widely used in food technology to stabilize foams and emulsions. However, the ability to create and stabilize dispersed food systems strongly depends on the structural properties, extrinsic factors and the process of creation. The creation and stabilization process can be divided into two steps – a diffusion of the protein to the interface and an arrangement at the phase boundary (Dalgleish, 1997). As a third step protein-protein interactions may stabilize especially foams by building a viscoelastic film (Kinsella, 1981). It is obvious that these steps require different molecular properties. Small and flexible molecules are able to be fast at the interface and, thus, help to create a disperse system (Grunden, Vadehra, & Baker, 1974). In contrast, proteins with a slow adsorption tend to cause higher long term stabilities in the case of foams (Kinsella, 1981). However, the structural reasons for the different functional properties of similar proteins are not fully understood until today. It is generally accepted that small changes in the molecular structure like refolding or disulfide exchanges can induce large changes in the functional behavior of proteins. Hence, high pressure treatments which favor reactions with negative reaction volume – provide the opportunity to modify

protein structures (Belloque, Lopez-Fandino, & Smith, 2000; Gaucheron, et al., 1997; Gekko & Hasegawa, 1989; Gekko & Noguchi, 1979; Heremans & Wong, 1985; Huppertz, Fox, & Kelly, 2004a; Wong & Heremans, 1988) and, thus, their functional properties. Former studies showed that high pressure – low temperature (HPLT) treatments induce different structural changes in milk proteins in comparison to high pressure treatments at room temperature (Baier, Purschke, Rawel, Schmitt, & Knorr, submitted; Kolakowski, Dumay, & Cheftel, 2001). These findings indicate the possibility of specific changes in functionality. However, HPLT effects on functionality changes are rarely investigated. Volkert, Puaud, Wille, & Knorr (2012) found changed sensorial properties of frozen dairy foams after HPLT treatments which could indicate a changed protein functionality. Reports on HP effects at room or elevated temperature identified the potential of pressure induced changes of protein functionality (Dumoulin & Hayashi, 1998; Galazka, Dickinson, & Ledward, 2000; Messens, VanCamp, & Huyghebaert, 1997). Pittia, Wilde, Husband, & Clark (1996) reported reduced emulsifying capacity and a decreased foamability of ß-lactoglobulin (ß-Lg) after pressure treatments from 300-900 MPa. However, other authors reported an increased foam stability depending on the treatment pH and the dwell time (Ibanoglu & Karatas, 2001). Galazka, Dickinson, & Ledward (1999) reported an increased droplet size and a faster creaming for a globular plant protein when treated as a pure substance but a slightly increased stability when treated in the presence of polysaccharides. Consequently, changes in functionality depend on the one hand on the exact sample composition and on the other hand on the treatment conditions. In comparison to the mentioned studies HPLT treatments offer two new options for modification beside pressure: cold denaturation (Hawley, 1971; Smeller, 2002) and effects caused by crystallization. The subzero temperature domain of the phase diagram of water enables different freezing processes. According to the nomenclature of Urrutia Benet, Schlüter, & Knorr (2004) pressure assisted freezing (PAF) denotes the cooling of a sample below the freezing line at almost constant

pressure. Regarding the phase diagram of water this process enables the freezing to higher ice modifications (Bridgman, 1912) with different crystal structures and lower density in comparison to the common atmospheric ice (ICE I). Another process option is to induce the nucleation by the pressure released, which is called pressure shift freezing (PSF). The sample is pressurized and undercooled in the liquid state and the crystallization is instantaneously induced when the pressure is released and the freezing line of ICE I is passed. The aim of this study is to identify the potential of HPLT treatments to modify the functionality of milk proteins.

3 Material and Methods

3.1 Material

Whey protein isolate powder (WPI) was obtained from Fonterra (WPI 895, Fonterra, Auckland, New Zealand). This WPI is obtained by ion exchange and ultrafiltration of sweet whey. The protein content of the powder was 92.63 % (w/w), furthermore it contained 0.18 % (w/w) fat, 5.87 % (w/w) moisture and 1.6 % (w/w) ash. Micellar casein powder (MC) in an almost native state was obtained from the Hungarian Dairy Research Institute (MPI-85 MC, Hungarian Dairy Research Institute, Mosonmagyaróvár, Hungary). These micelles were manufactured by microfiltration and ultrafiltration of skimmed milk. The powder contained 85.1 % (w/w) protein, 1.5 % (w/w) fat, 4.9 % (w/w) water and 7.5 % (w/w) ash.

3.2 Methods

3.2.1 Sample preparation

The WPI solutions were prepared by diluting a specific amount of powder in deionized water and stirring it for 1 h at room temperature. The MC dispersions were prepared by giving a specific amount of powder to preheated deionized water

(50 °C), stirring it for 1 h and gently homogenizing it in a high pressure homogenizer (ElmusiFlex-C5, Avestin, Inc., Ottawa, Canada) at a maximum pressure of 30 MPa. Protein dispersions were prepared on a w/w ratio and pH values were either 7.0 (native) or set to 5.8 by the usage of HCl and NaOH (1 M, Merck KGaA, Darmstadt, Germany). The samples were double packed in polyethylene (PE) pouches to strictly avoid a penetration of the PTM. All samples were freshly prepared and kept at 4 °C until analyses.

3.2.2 HPLT treatments

The HPLT treatments were conducted in an experimental HPLT unit containing a high pressure vessel with 265 mL volume (Sitec Sieber AG, Zurich, Switzerland) connected to an air driven high pressure pump (DS XHW-1373 (Haskel, CA, USA). The vessel was equipped with a heating-cooling jacket and tempering was realized with a cryostat (Ultra-Kryomat RUK 50-D, Lauda, Germany). An 80 % (v/v) ethanol water mixture was used as tempering medium as well as pressure transmitting medium (PTM, freezing point below -59 °C). Two type K thermocouples enabled temperature measurements of the PTM at the bottom of the vessel and inside of a sample at the top of the vessel. The pressure was measured with a pressure transducer (Intersonde HP28, Watford, England). The samples were thawed at room temperature before further preparations or analytics.

3.2.3 Rheological analysis of casein based flocks

Viscosity measurements were performed to identify changes in the rheological behavior of samples which contained large flocks. A MCR 301 rotational viscometer with a CC 27 single gap cylinder (Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) was used to analyze the rheological properties. The single gap cylinder had a gap of 1.13 mm and a sample volume of 19.35 mL. Shear experiments were performed at 20 °C with a linear ramp of 60 s up to 500 1/s, a dwell time of 60 s at 500 1/s and a ramp of 60 s to 0 1/s. Shear experiments were performed in

duplicates. The Herschel-Bulkley model was applied to characterize the flow curves and the hysteresis area was determined. The Herschel-Bulkley model is given by the following equation:

$$\tau = \tau_0 + k \cdot \dot{\gamma}^n$$

where τ represents the shear stress, τ_0 is the yield stress, k is the consistency, $\dot{\gamma}$ is the shear rate and the exponent n represents the flow behavior ($n = 1$ → Newtonian fluid, $n < 1$ → shear thinning fluid, $n > 1$ shear thickening fluid). For $\tau_0 = 0$ (no yield stress) the Herschel-Bulkley model becomes the power law model.

3.2.4 Determination of emulsification properties

Protein samples were diluted with a 10 mM phosphate buffer (pH 7.0) to a concentration of 0.4 % (v/v). Commercial sunflower oil (12.5 % v/v) was added to the diluted protein dispersions and pre-homogenized with an Ultra-Turrax T25 (IKA) with a S25 18 G homogenizing tool at 9.500 U/min for 5 min. Pre-homogenized samples were high pressure homogenized in a ElmusiFlex-C5 (Avestin, Inc., Ottawa, Canada) at a homogenization pressure of 30 MPa.

Particle size distributions of fresh emulsions were determined by using a HORIBA LA-950 (Retsch Technology, Haan, Germany). Samples were stabilized by mixing 1:1 with a sodium phosphate buffer containing 5 % (w/v) SDS and analyzed immediately.

Emulsification properties were analyzed by determining the phase separation after centrifugation. 10 mL of each emulsion were centrifuged for 30 min at 4,000 g (5810 R, Eppendorf AG, Hamburg, Germany) at 20 °C and the volumes of cream, free oil and serum were determined and normalized by the total emulsion volume.

3.2.5 Determination of foaming properties

Samples were diluted to 0.05 % (v/v) with 10 mM phosphate buffer (pH 7.0 or pH 5.0) and 20 mL of diluted samples were foamed with a DFA 100 foam analyzer (Krüss GmbH, Hamburg, Germany) to a total height of 180 mm (foaming column: 20 mm

internal diameter). Gas flow was set to 0.15 L/min and an original G4 (10-16 µm pore size) glass frit was used. Decrease of foam and increase of drainage were detected by measuring light transmission and identifying the border between foam and drainage. All samples were foamed at least in duplicates and measurements were performed for 30 min. Foam stability was calculated at 20 min by dividing the actual foam volume by the maximum foam volume after the foaming. Furthermore, the foam density was calculated by dividing actual liquid in the foam (difference of initial liquid and drainage) by the foam volume.

3.2.6 Dilatational rheology

A pendant drop device (OCA20, DataPhysics Instruments GmbH, Filderstadt, Germany) was used to determine the surface rheological properties of whey protein solutions. The investigations of samples containing MC were not possible due to the formation of large flocks. A drop volume of 15 µL in air was used and the drop was allowed to equilibrate for 30 min after creation and the average interfacial tension (IFT) was determined. A volume change of 10 % with a frequency of 0.01 Hz at a controlled temperature of 20 C was applied to get the elastic (E') and the viscous (E'') part of the dilatational modulus. All experiments were performed at least in duplicates.

4 Results and discussion

4.1 Rheological behavior of MC rich samples

Samples with high MC content (single MC, MC:WPI = 80:20 dispersions) always contained large flocks with an average equivalent spherical diameter of about 12 µm after HPLT treatments (see also Baier, Purschke, et al., submitted; Baier, Schmitt, & Knorr, submitted). Shear experiments of all samples were performed and are shown in Figure 1. A clear effect on flow behavior was observed for single MC

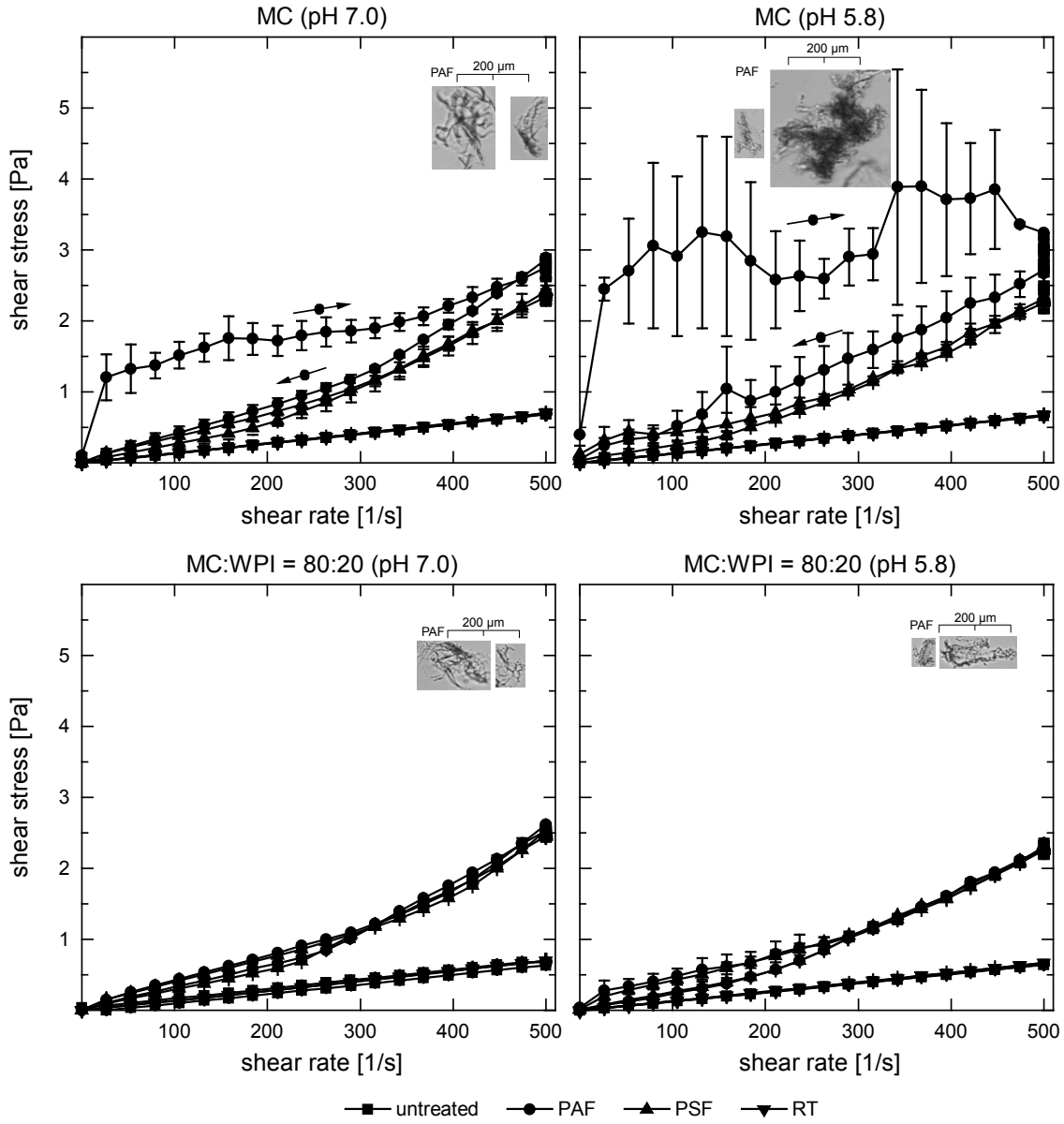


Figure 1: Flow curves for shear experiments in a rotational rheometer with a single gap cylinder at 20 °C (ramp to 500 1/s in 60 s, 60 s at 500 1/s, ramp to 0 1/s in 60 s). Exemplary pictures of large flocks are given within the graphs.

dispersions after PAF treatments. This effect was enhanced at pH 5.8 in comparison to pH 7.0. The large standard deviation values indicate some heterogeneity of the samples which can be due to the presence of flocks which are larger than the rheometer gap that was used. This was also confirmed by the decrease of shear stress at a constant shear rate which denotes a partial disaggregation of these flocks. Consequently, a hysteresis was found for these two samples. Interestingly, PAF and PSF treatments of all samples with a high MC content led to structures

which show a shear thickening effect while untreated and samples treated at room temperature show a Newtonian behavior. This leads to the assumption that HPLT treatments are able to induce new structures in comparison with HP treatments at room temperature. Especially, the pictures of the flocks from pure MC dispersion show a dense structure which indicates the formation of a gel-like network. Within all the pictures of the flocks a large number of single and linked fibrillar structures can be found. The particle size distribution of the flocks is quite broad (Baier, Purschke, et al., submitted) and it seems that especially the largest particles contribute to the rheological behavior. The results of applying the Herschel-Bulkley model to the flow curves are shown in Table 1. It is indicated by the flow behavior index that untreated samples and samples which were pressure treated at room temperature have an index of almost 1 and, thus, behave like Newtonian fluids. Especially, PAF treated samples of pure MC show a distinct hysteresis area and an increased consistency. These findings indicate the formation of network structures which is in accordance with the particle pictures shown in Figure 1. However, the presence of WPI in the sample diminished these effects and smaller hysteresis areas were detected. This finding could indicate that an interaction of MC with WPI is more pronounced than interactions between the MC. Interestingly, samples which were PSF treated at pH 5.8 show decreased consistencies (also found for PAF treated mixtures with MC:WPI ratio of 80:20) but still exhibits a distinct hysteresis area. However, other authors (Saglam, Venema, de Vries, Shi, & van der Linden, 2013; Saglam, Venema, de Vries, & van der Linden, 2014) were able to create microparticle gels from WPI solutions by heating them at high concentration which also showed shear thickening properties. Beliciu & Moraru (2011) could induce a slight shear thickening effect for MC dispersions at concentrations above 12.5 % by heating them to 80 or 90 °C ($n \approx 1.1$). These findings promote the thesis that gel-like particles were formed during HPLT treatments which may be traced back to concentration effects during freezing.

Table 1: Herschel-Bulkley parameter and hysteresis area for MC rich samples after different pressure treatments. Regression of applying the Herschel-Bulkley model to the backward flow curve gave an average R^2 of 0.9774±0.0252

Parameter	pH 7.0							
	MC				MC:WPI = 80:20			
	untreated	RT	PAF	PSF	untreated	RT	PAF	PSF
Consistency k [mPa·s]	1.36 ±0.06	1.27 ±0.08	3.38 ±0.49	0.97 ±0.65	1.27 ±0.01	1.38 ±0.17	2.04 ±0.39	1.31 ±0.31
Flow behavior index n	1.00 ±0.01	1.01 ±0.01	1.05 ±0.02	1.25 ±0.10	1.01 ±0.00	1.00 ±0.02	1.11 ±0.03	1.18 ±0.04
Hysteresis area [Pa·s-1]	-0.22 ±0.07	-0.39 ±0.16	331.82 ±74.06	29.34 ±24.73	-1.10 ±0.17	-0.67 ±0.69	23.79 ±10.25	23.53 ±6.22
Parameter	pH 5.8							
	MC				MC:WPI = 80:20			
	untreated	RT	PAF	PSF	untreated	RT	PAF	PSF
Consistency k [mPa·s]	1.40 ±0.05	1.20 ±0.05	5.40 ±5.16	0.46 ±0.31	1.17 ±0.01	1.14 ±0.08	0.50 ±0.14	0.85 ±0.42
Flow behavior index n	0.99 ±0.01	1.02 ±0.01	1.03 ±0.16	1.37 ±0.13	1.02 ±0.00	1.03 ±0.01	1.34 ±0.04	1.26 ±0.09
Hysteresis area [Pa·s-1]	1.50 ±1.30	-0.24 ±0.49	883.46 ±242.28	56.44 ±27.38	-0.44 ±0.23	-1.50 ±0.11	55.71 ±33.86	41.56 ±15.27

4.2 Determination of emulsification properties

Figure 2 shows the results of the particle size measurements of the fresh emulsions. It can be seen that the median is about 3 µm for all samples and the span is in the majority of cases below 1.5 µm. The differences between the untreated and treated samples are quite low. This is due to the fact that the droplet size of an emulsion primarily depends on the emulsification process if an excess of the emulsifier is present (McClements, 2004).

A clear phase separation between an oil free serum phase and a compact emulsion layer was observed after centrifugation of MC rich samples. Therefore, the amount of serum was taken as an indicator for the emulsion stability. For the interpretation of the data it is assumed that the stability decrease is proportional to the increase in

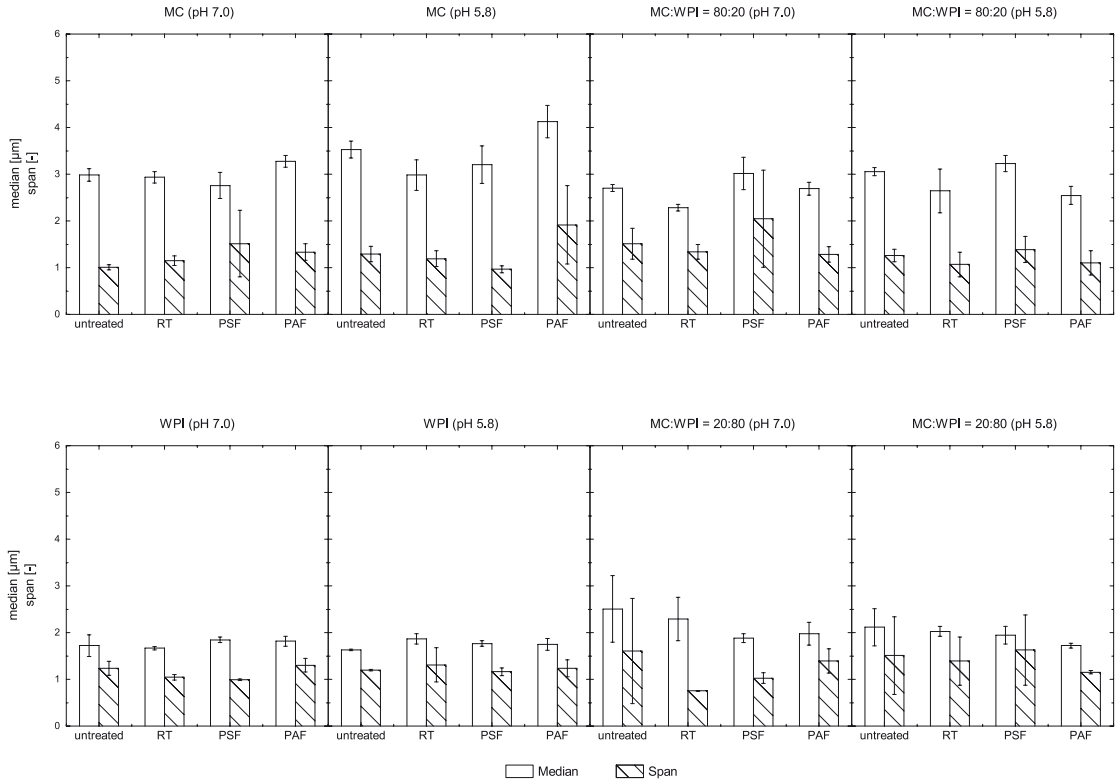


Figure 2: Results of the particle size measurements of fresh prepared emulsions from HPLT and HP treated pure MC or WPI dispersions and mixtures with MC:WPI = 80:20 or 20:80 (v/v) ratio at pH 7.0 or 5.8.

the amount of serum as a smaller emulsion layer is related to an enhanced phase separation. The results for the emulsifications are shown in Figure 3. It can be seen that the differences between the volumes of the serum phases are quite small. However, for samples treated at pH 7 a tendency to a lower stability can be observed. In consequence, a relation between the increased viscosity due to the formation of gel-like flocks and the emulsion stability cannot be found. It should be mentioned that the large flocks which were present after the HPLT treatments did probably not resist the emulsification process which could explain the similarity between samples treated at room temperature and HPLT treated samples. Furthermore, HP and HPLT treatments led to a solubilization of casein fractions and to the formation of smaller micelles (Anema, Lowe, & Stockmann, 2005; Baier, Schmitt, et al., submitted; Huppertz, Kelly, & Fox, 2006; Huppertz, Smiddy, Upadhyay, & Kelly, 2006). This decrease in micelle size could reduce the stabilizing properties of MC due to the

possible formation of thinner interfacial films and less steric repulsion. The results of Baier, Purschke, et al. (submitted) and the rheological investigations from 4.1 indicate that HPLT treatments promote the aggregation of MC. It can be assumed that also HP treatments at room temperature induce the same structural change but aggregation does not occur due to the missing freezing step. Other authors also reported the possibility of forming larger micelles depending on the pressure-temperature range (Anema, et al., 2005; Huppertz, Fox, & Kelly, 2004b; Needs, Stenning, Gill, Ferragut, & Rich, 2000; Regnault, Thiebaud, Dumay, & Cheftel,

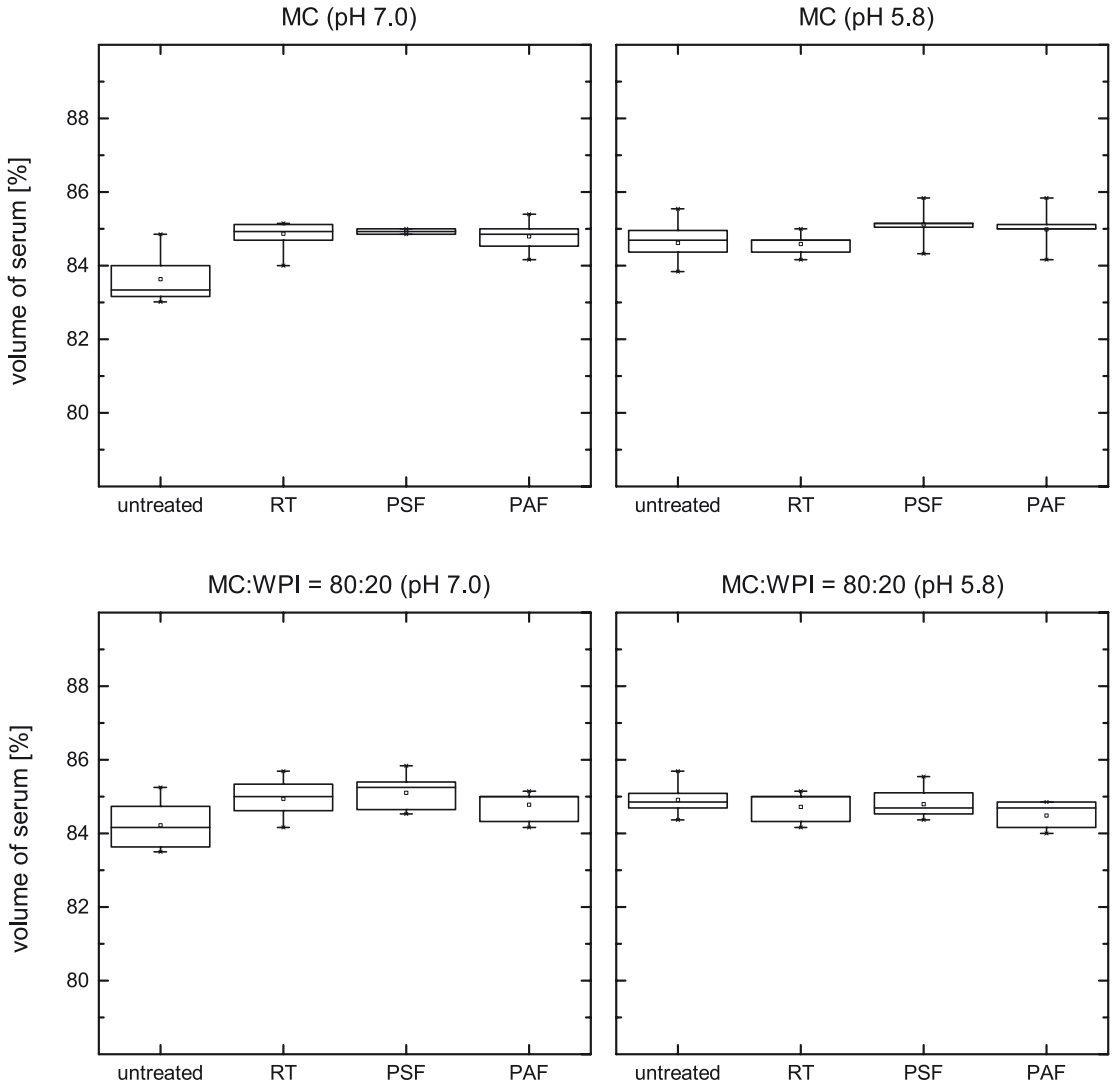


Figure 3: Amount of serum of centrifuged emulsions (0.4 % v/v protein in 0.01 M phosphate buffer, 12.5 % sunflower oil) after centrifugation for 30 min at 4,000 g (20 °C).

2004). Consequently, the attraction between the caseins may be increased after pressure treatments which would lead to a decreased repulsion and, thus, to a decreased emulsion stability.

The identification of a clear serum phase after centrifugation was not possible for samples with a high WPI content due to overall higher emulsion stability in comparison to MC rich samples. In consequence, a higher cream content will be correlated to a lower stability as it is related to an enhanced creaming of oil droplets. The results of the emulsification experiments are shown in Figure 4. It can be seen as a general

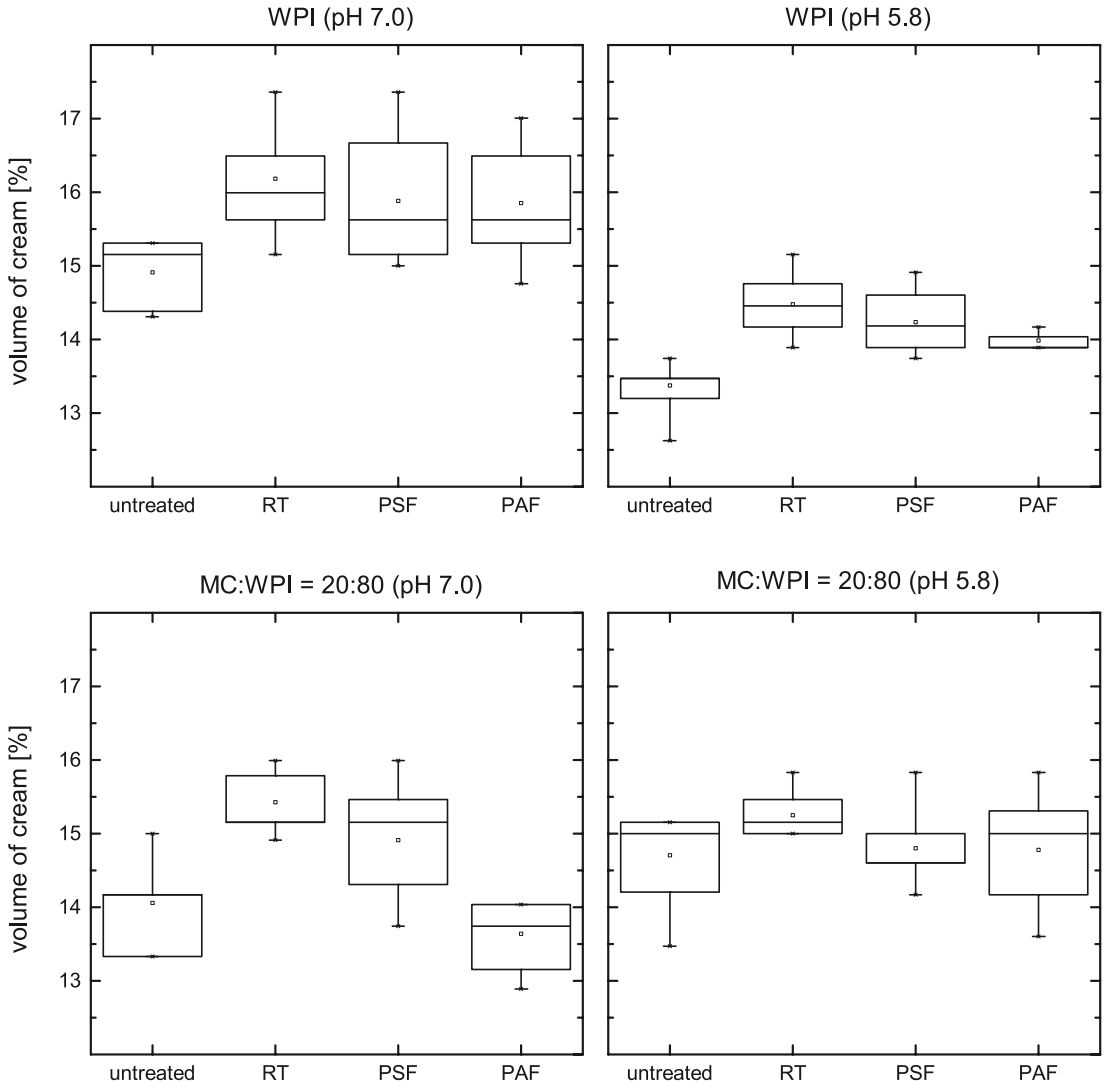


Figure 4: Amount of cream of centrifuged emulsions (0.4 % v/v protein in 0.01 M phosphate buffer, 12.5 % sunflower oil) after centrifugation for 30 min at 4,000 g (20 °C).

trend that in most cases a decreased stability after HP or HPLT treatments was found. This is in accordance with the findings of other authors who reported a lower stability for emulsions created from HP treated protein dispersions (Galazka, Dickinson, & Ledward, 1996; Galazka, Ledward, Dickinson, & Langley, 1995). A lower stability may be caused by sulfide-disulfide exchanges and protein aggregation which would lead to a lower flexibility of the proteins. Furthermore, high pressure treatments lead to an increased surface hydrophobicity of WPI which would enhance the attraction of protein molecules and oil droplets or protein stabilized oil droplets. Hydrophobic interactions play a major role in emulsification as the protein is not unfolded at the interface and, thus, the presence of more hydrophobic patches at the protein surface would not contribute to the stabilization of one single droplet. Furthermore, higher hydrophobic interactions could increase the affinity for flocculation. A higher hydrophobicity would only increase the probability of the protein to adsorb at the interface and thereby influence the formation of an emulsion, not its stabilization (Damodaran, 1994, 2005). It should be mentioned that an excess of protein in this study as stated above led to the fact that the creation of the emulsion was only influenced by the homogenization and not by the protein properties. HPLT effects seem to be higher for treatments of single WPI solutions in comparison to mixtures with a high WPI content. Especially, PAF treatments of mixtures at both pH did not change the creaming. It is reported, that the stabilization of an emulsion is a quite complex process which depends on several extrinsic factors like ionic strength (Damodaran, 2005) or pH value (Dickinson, Murray, & Stainsby, 1988). HPLT treatments and HP treatments at room temperature induce different changes in milk proteins like aggregation, partial unfolding or release of ionic calcium from MC (Baier, Purschke, et al., submitted; Baier, Schmitt, et al., submitted). Consequently, a clear correlation between the treatment and the emulsion stability needs a detailed investigation of all effects which might have an influence.

4.3 Determination of interfacial and foaming properties

Milk proteins are often used for foam formation or stabilization due to their surface active character. Figure 5 shows the results of the determination of surface rheological parameters for a pure WPI solution. Neither HP treatments at room temperature nor PAF treatments seem to influence the interfacial tension of a 2 % WPI solution. The elastic part (E') of the surface dilatational modulus was the only parameter which was influenced by changing the pH and also after HP treatments, especially for treatments at room temperature. This increase was more pronounced for treatments at pH 7.0 which may be traced back to the lower initial value. An increased elasticity can contribute to a higher stress resistance and, thus, to a higher stability of created foams (Bos & van Vliet, 2001; Foegeding, Luck, & Davis, 2006; Wierenga & Gruppen, 2010; Wilde, 2000). It can be seen from Figure 6 that the effect on foam stability is higher for a pure WPI solution in comparison to mixtures. Both, HPLT and HP treatments at room temperature led to an increased foam stability for foams from pure WPI solution. However, the increase is most pronounced for HP treatments at room temperature (+14 % at pH 7.0 and +23.5 % at pH 5.8). Furthermore, it

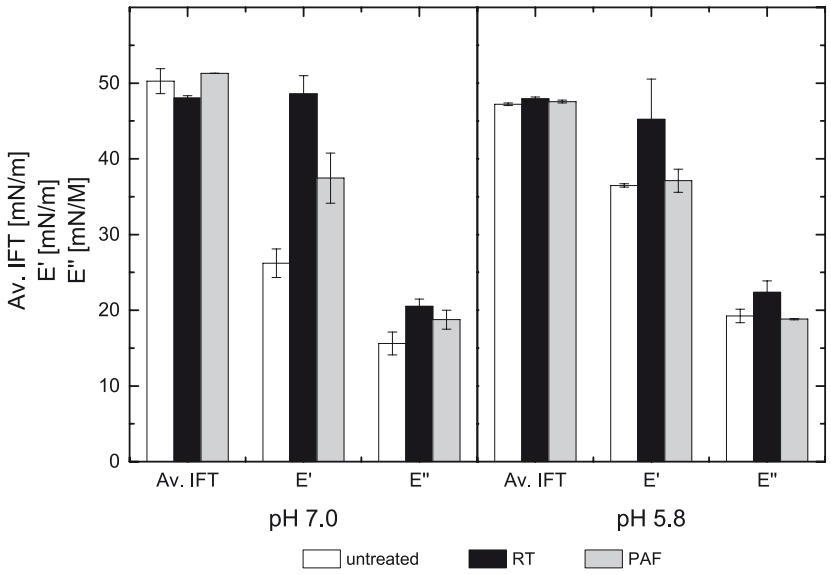


Figure 5: Average interfacial tension, elastic (E') and viscous (E'') part of surface dilatational modulus of pure WPI solutions (2 % w/w) after PAF or HP treatment from oscillating pendant drop analysis at 0.01 Hz.

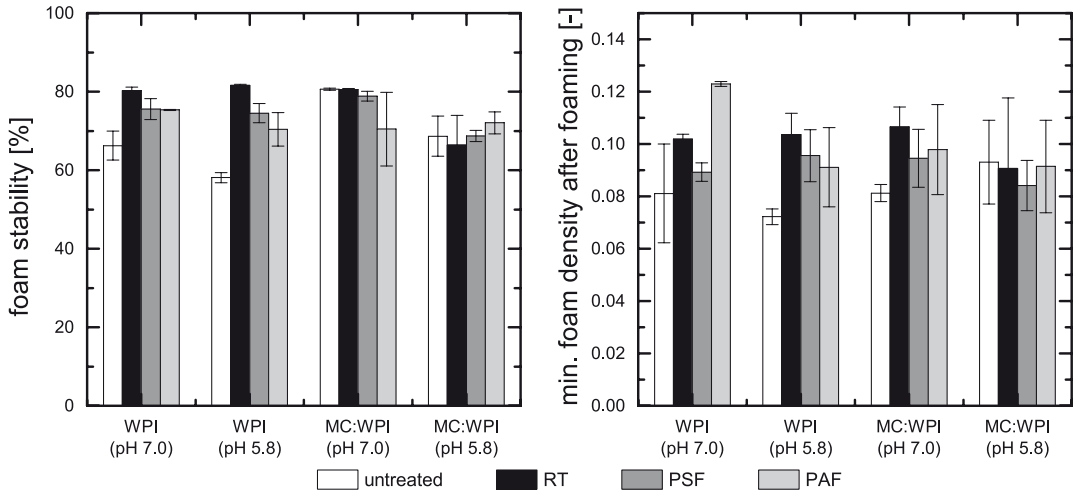


Figure 6: Foam stability and minimal foam density after foaming (amount of liquid in the foam) for foams from pure WPI solutions or MC:WPI = 20:80 (v/v) mixtures after HP or HPLT treatments.

can be seen from Figure 6 that the minimum foam density after the creation of the foam, which is related to the amount of water in the lamellas of the foam, is higher for foams from pressure treated WPI samples (increase of up to 50 % for PAF treatment of pure WPI at pH 7.0). This leads to the assumption that the lamellas are thicker and, thus, the stability is increased. The course of the foam density (data not shown) shows an increasing foam density of the untreated samples during the 20 min of investigation up to 0.2 (MC:WPI = 20:80, pH 5.8). In combination with the lower densities after foaming this indicates a faster drainage for untreated samples. These foams break down without a detectable amount of drainage after the process of foam formation (nearly the complete liquid is drained out direct after the foaming) which indicates a dryer foam for untreated samples. The treated samples seem to be able to hold the water in the lamellas and, thus, produce more wet foams with a higher stability. A higher amount of bound water at the interface could also explain the higher elastic part of the surface dilatational modulus. Nevertheless, a clear relation between the foam stability and the surface dilatational properties cannot be found from the present data.

Preliminary experiments showed that samples with high MC content exhibit the best foam stability in a 10 mM phosphate buffer at pH 7.0. Figure 7 shows the results

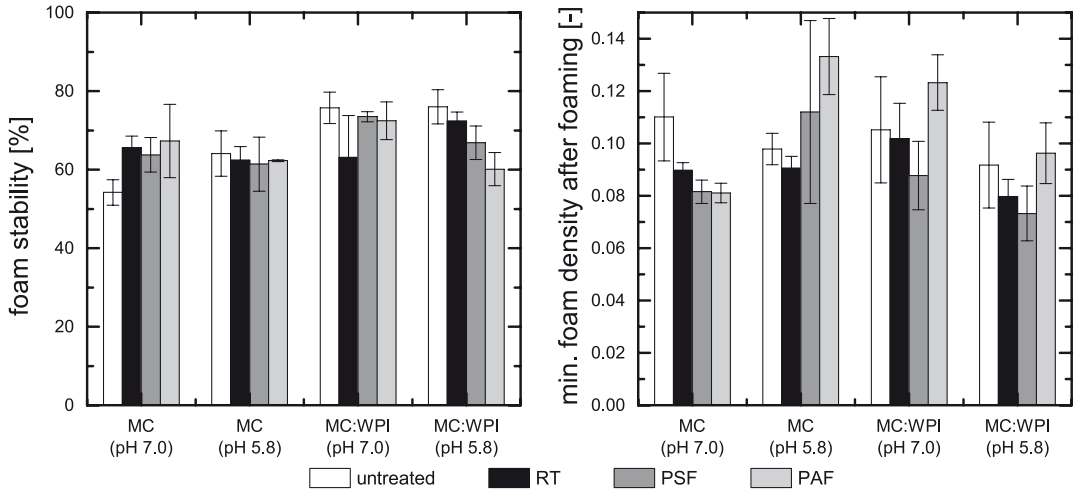


Figure 7: Foam stability and minimal foam density after foaming (amount of liquid in the foam) for foams from pure MC solutions or MC:WPI = 80:20 (v/v) mixtures after HP or HPLT treatments.

of the foaming experiments. It can be seen that the deviations especially for the HPLT treated samples are quite high. This can be traced back to the formation of large flocks and, thus, the inhomogeneity of these samples. Again, it seems that the changes in the rheological properties due to the formation of large flocks are not directly correlated to a change in the functional properties as it was also mentioned for emulsions above. However, a slight increase in stability could be observed only for foams from a pure MC dispersion treated at pH 7 (increase of about 10 %) while treatments of mixtures with a high MC content led to the formation of foams with a lower stability (higher effect for HPLT treatments at pH 5.8; up to 16 %). A clear tendency for the foam density could not be observed. These findings point out the complex mechanism of the stabilization of disperse systems.

5 Conclusions

HPLT and HP treatments at room temperature were able to induce modifications in the functional behavior of micellar caseins and whey proteins. Emulsions and foams created from treated pure WPI solutions showed the highest changes in stability. The emulsion stability was decreased while an increase of the foam stability was

detected. However, the increase in foam stability was most pronounced for HP treatments at room temperature. Changes in the functionality of pure MC dispersions were only observed when samples were treated at pH 7.0. The large flocks which were induced in samples with a high amount of MC highly influenced the rheological behavior of these samples. The shear flow changed from a Newtonian to a dilatant behavior indicating new application possibilities for this special modification. The oscillating pendant drop experiments showed an increased elastic part of the surface dilatational modulus for treated WPI solutions, whereupon HP treatments at room temperature led to the highest increase. However, treatments of mixtures led to different results which indicate the high influence of interactions between MC and WPI. Further investigations on the influence of other food ingredients on the induced modifications should be performed to get a deeper insight in the complex process-structure-function relationship of milk proteins.

6 Acknowledgements

The authors like to acknowledge Anne Heckelmann for supporting the development of protein analytics and fruitful discussions during the interpretation of the data. Furthermore, the authors like to thank Martha Maaßen for her assistance during the emulsification and foaming experiments. The authors also would like to acknowledge Stephan Drusch, Frederic Tamm and Adrian Kape for providing the pendant drop device and their assistance during the measurements.

7 References

Anema, S. G., Lowe, E. K., & Stockmann, R. (2005). Particle size changes and casein solubilisation in high-pressure-treated skim milk. Food Hydrocolloids, 19(2), 257-267.

Baier, D., Purschke, B., Rawel, H., Schmitt, C., & Knorr, D. (2013). High pressure – low temperature treatment induced structural changes in micellar caseins and whey proteins. Food Chemistry (submitted manuscript).

Baier, D., Schmitt, C., & Knorr, D. (2013). Effect of high pressure - low temperature processing on composition and colloidal stability of casein micelles and whey proteins. International Dairy Journal (submitted manuscript).

Beliciu, C. M., & Moraru, C. I. (2011). The effect of protein concentration and heat treatment temperature on micellar casein-soy protein mixtures. Food Hydrocolloids, 25(6), 1448-1460.

Belloque, J., Lopez-Fandino, R., & Smith, G. M. (2000). A H-1-NMR study on the effect of high pressures on beta-lactoglobulin. Journal of Agricultural and Food Chemistry, 48(9), 3906-3912.

Bos, M. A., & van Vliet, T. (2001). Interfacial rheological properties of adsorbed protein layers and surfactants: a review. Advances in Colloid and Interface Science, 91(3), 437-471.

Bridgman, P. W. (1912). Water under pressure. Proceedings of the American Academy of Arts and Sciences, 48, 450-558.

Dalgleish, D. G. (1997). Food emulsions stabilized by proteins. Current Opinion in Colloid & Interface Science, 2(6), 573-577.

Damodaran, S. (1994). Structure-function relationship of food proteins. In N. S. Hettiarachchy & G. R. Ziegler (Eds.), Protein functionality in food systems. New York: Marcel Dekker, Inc.

Damodaran, S. (2005). Protein stabilization of emulsions and foams. Journal of Food Science, 70(3), R54-R66.

Dickinson, E., Murray, B. S., & Stainsby, G. (1988). Coalescence Stability of Emulsion-Sized Droplets at a Planar Oil-Water Interface and the Relationship to Protein Film Surface Rheology. Journal of the Chemical Society-Faraday Transactions I, 84, 871-883.

Dumoulin, M., & Hayashi, R. (1998). High Pressure, a Unique Tool for Food Texturization Food Science and Technology International, Tokyo, 4(2), 99-113.

Foegeding, E. A., Luck, P. J., & Davis, J. P. (2006). Factors determining the physical properties of protein foams. Food Hydrocolloids, 20(2-3), 284-292.

Galazka, V. B., Dickinson, E., & Ledward, D. A. (1996). Effect of high pressure on the emulsifying behaviour of beta-lactoglobulin. Food Hydrocolloids, 10(2), 213-219.

Galazka, V. B., Dickinson, E., & Ledward, D. A. (1999). Emulsifying behaviour of 11S globulin Vicia faba in mixtures with sulphated polysaccharides: comparison of thermal and high-pressure treatments. Food Hydrocolloids, 13(5), 425-435.

Galazka, V. B., Dickinson, E., & Ledward, D. A. (2000). Influence of high pressure processing on protein solutions and emulsions. Current Opinion in Colloid & Interface Science, 5(3-4), 182-187.

Galazka, V. B., Ledward, D. A., Dickinson, E., & Langley, K. R. (1995). High pressure effects on emulsifying behavior of whey protein concentrate. Journal of Food Science, 60(6), 1341-1343.

Gaucheron, F., Famelart, M. H., Mariette, F., Raulot, K., Michel, F., & LeGraet, Y. (1997). Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk. Food Chemistry, 59(3), 439-447.

Gekko, K., & Hasegawa, Y. (1989). Effect of temperature on the compressibility of native globular proteins. J. Phys. Chem., 93, 426-429.

Gekko, K., & Noguchi, H. (1979). Compressibility of globular proteins in water at

25 8C. J. Phys. Chem., 83, 2706-2714.

Grunden, L. P., Vadehra, D. V., & Baker, R. C. (1974). Effects of proteolytic enzymes on the functionality of chicken egg albumen. Journal of Food Science, 39(4), 841-843.

Hawley, S. A. (1971). Reversible pressure-temperature denaturation of chymotrypsinogen. Biochemistry, 10(13), 2436-&.

Heremans, K., & Wong, P. T. T. (1985). Pressure effects on the Raman spectra of proteins: pressure-induced changes in the conformation of lysozyme in aqueous solution. Chem. Phys. Lett., 118, 101-104.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004a). High pressure-induced denaturation of alpha-lactalbumin and beta-lactoglobulin in bovine milk and whey: a possible mechanism. Journal of Dairy Research, 71(4), 489-495.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004b). High pressure treatment of bovine milk: effects on casein micelles and whey proteins. Journal of Dairy Research, 71(1), 97-106.

Huppertz, T., Kelly, A. L., & Fox, P. F. (2006). High pressure-induced changes in ovine milk. 2. Effects on casein micelles and whey proteins. Milchwissenschaft-Milk Science International, 61(4), 394-397.

Huppertz, T., Smiddy, M. A., Upadhyay, V. K., & Kelly, A. L. (2006). High-pressure-induced changes in bovine milk: a review. International Journal of Dairy Technology, 59(2), 58-66.

Ibanoglu, E., & Karatas, S. (2001). High pressure effect on foaming behaviour of whey protein isolate. Journal of Food Engineering, 47(1), 31-36.

Kinsella, J. E. (1981). Functional-Properties of Proteins - Possible Relationships between Structure and Function in Foams. Food Chemistry, 7(4), 273-288.

Kolakowski, P., Dumay, E., & Cheftel, J. C. (2001). Effects of high pressure and low temperature on beta-lactoglobulin unfolding and aggregation. Food Hydrocolloids, 15(3), 215-232.

McClements, D. J. (2004). Protein-stabilized emulsions. Current Opinion in Colloid & Interface Science, 9(5), 305-313.

Messens, W., VanCamp, J., & Huyghebaert, A. (1997). The use of high pressure to modify the functionality of food proteins. Trends in Food Science & Technology, 8(4), 107-112.

Needs, E. C., Stenning, R. A., Gill, A. L., Ferragut, V., & Rich, G. T. (2000). High-pressure treatment of milk: effects on casein micelle structure and on enzymic coagulation. Journal of Dairy Research, 67(1), 31-42.

Pittia, P., Wilde, P. J., Husband, F., & Clark, D. C. (1996). Functional and structural properties of beta-lactoglobulin as affected by high pressure treatment. Journal of Food Science, 61(6), 1123-1128.

Pour-El. (1981). Protein functionality: Classification, definition and methodology. In J. P. Cherry (Ed.), Protein functionality in foods.

Regnault, S., Thiebaud, M., Dumay, E., & Cheftel, J. C. (2004). Pressurisation of raw skim milk and of a dispersion of phosphocaseinate at 9 degrees C or 20 degrees C: effects on casein micelle size distribution. International Dairy Journal, 14(1), 55-68.

Saglam, D., Venema, P., de Vries, R., Shi, J., & van der Linden, E. (2013). Concentrated whey protein particle dispersions: Heat stability and rheological properties. Food Hydrocolloids, 30(1), 100-109.

Saglam, D., Venema, P., de Vries, R., & van der Linden, E. (2014). Exceptional heat stability of high protein content dispersions containing whey protein particles. Food Hydrocolloids, 34(1), 68-77.

Smeller, L. (2002). Pressure-temperature phase diagrams of biomolecules. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology, 1595(1-2), 11-29.

Urrutia Benet, G., Schlüter, O., & Knorr, D. (2004). High pressure-low temperature processing. Suggested definitions and terminology. Innovative Food Science & Emerging Technologies, 5(4), 413-427.

Volkert, M., Puaud, M., Wille, H.-J., & Knorr, D. (2012). Effects of High Pressure-Low Temperature treatment on freezing behavior, sensorial properties and air cell distribution in sugar rich dairy based frozen food foam and emulsions. Innovative Food Science & Emerging Technologies, 13, 75-85.

Wierenga, P. A., & Gruppen, H. (2010). New views on foams from protein solutions. Current Opinion in Colloid & Interface Science, 15(5), 365-373.

Wilde, P. J. (2000). Interfaces: their role in foam and emulsion behaviour. Current Opinion in Colloid & Interface Science, 5(3-4), 176-181.

Wong, P. T. T., & Heremans, K. (1988). Pressure effects on protein secondary structure and hydrogen exchange in chymotrypsinogen. Biochim. Biophys. Acta, 956, 1-9.

2.3.2 Supplemental Results and Conclusions for Experimental Plan

The results which were shown in the previous chapter indicate that HPLT treatments can influence the functional behavior of whey protein and micellar casein. It could be shown that:

- Casein-based flocks and aggregates show shear thickening behavior,
- Foam stability is increased for treated pure whey proteins and pure micellar caseins at pH 7.0 but decreased for mixtures,
- Emulsion stability is only slightly affected by the tested parameters, a tendency to lower stabilities after treatments can be assumed.

Within the manuscript, the increased foam stability of whey protein solutions was drawn back to the changed surface rheological properties. Furthermore, the bubble size or size distribution may be changed and picture analyses were used to figure out these changes. Figure 17 shows some exemplary box plots for the bubble size distribution. However, a clear relation between the bubble size and the foam stability was only found for pure WPI solutions. A clear reduction

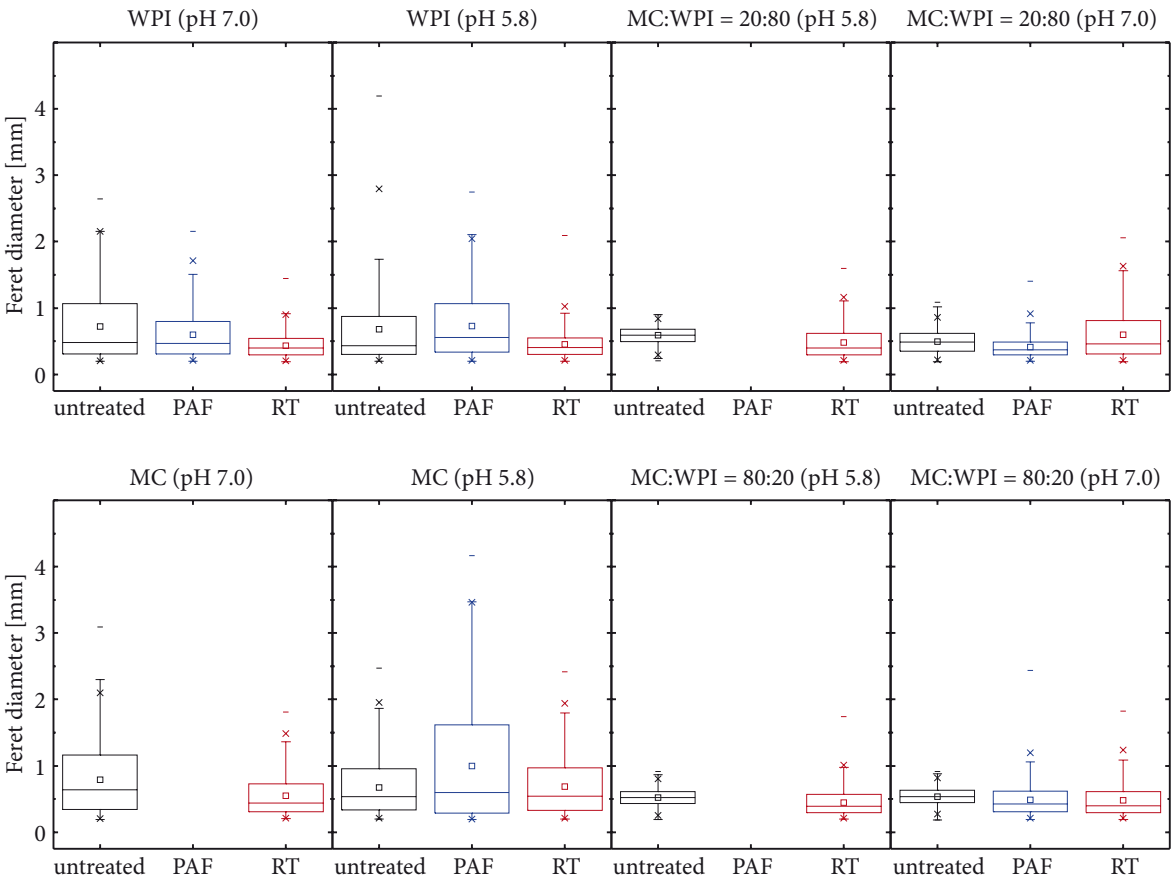


Figure 17: Box plots of bubble sizes of foams from different milk protein dispersions foamed after treatments.

in the bubble size was not found, but the width of the distribution is reduced. A reduction in the distribution of the bubble size with a shift to smaller sizes increases the stability of the foam. This effect can be traced back to a lower disproportionation due to smaller differences in the Laplace pressure of the bubbles. A tendency of a smaller bubble size distribution correlated to a higher foam stability could also be found for pure micellar casein dispersions at pH 7.0. The mixtures show a small size distribution even for the untreated samples and changes after treatments are quite low. This is in line with the findings for the foam stability where changes were also less pronounced in comparison to treatments of pure protein dispersions. However, bubble size distributions were measured by taking photographs of the column and using picture analyses. Advanced investigation of bubble size and distribution may be helpful to get distinct results. Nevertheless, diverse results about a relation between bubble size distribution and foam stability are shown by other authors. Kamath et al. (2008) found that foams from pasteurized homogenized whole milk show a lower stability but smaller bubble sizes in comparison with foams from pasteurized skim milk which were more stable but show larger bubble size. The results of these authors show also that the shifts in bubble size are different for both systems. Foams from skim milk show a shift to larger bubble sizes over their life time while foams from whole milk show a shift to smaller bubble sizes. The authors suggested different collapse mechanisms of both foams: Bubbles in foams from skim milk are proposed to coalescence more frequently while bubbles in foams from whole milk are supposed to underlie disproportionation. This disproportionation could increase in the number smaller bubbles and the authors suggest a rupture of the larger bubbles which would in summary lead to a shift of the bubble size to smaller bubble sizes. Other authors (Ruiz-Henestrosa et al., 2014) recently showed a clear correlation between smaller bubble sizes and an increased foam stability for mixtures of β-lactoglobulin and soy globulins. Concluding, the mechanism of stabilization seems to have the major impact on foam stability and is not necessarily correlated to the bubble size and size distribution. This may also explain the varying findings for different milk protein dispersions during the present study.

3. Conclusions and Perspective

The following chapter summarizes the major findings of the present study, draws conclusions and gives an outlook for future perspectives. Possible models for mechanisms of HPLT induced modification of whey protein and micellar casein are given. Finally, needs for further investigations are pointed out.

3.1 Conclusions and Possible Mechanisms for HPLT Induced Modification of Whey Protein and Micellar Casein

Although it is already known that HPLT treatments affect milk proteins in a different way compared to common HP treatments (Kolakowski et al., 2001; Dumay et al., 2006), systematic studies are still missing. Within this study, investigations of colloidal stability (see 2.1) and structural changes (see 2.2) of whey protein (WPI) and micellar casein (MC) showed elementary differences between HPLT treated samples and samples treated at room temperature. However, the presence of the other protein fraction – MC or WPI – clearly influences the induced changes within the regarded fraction. These findings lead to the conclusion that both fractions interact with each other during HPLT treatments as well as during treatments at room temperature. Nevertheless, interactions of both fractions differ between both treatment options. It was shown that all WPI fractions are not affected by HPLT treatments up to 600 MPa at pH 7.0. This result is supported by the findings, that HPLT treatments did not alter the tertiary structure of WPI. However, changes in secondary structure were found but did not affect colloidal stability. In contrast, HP treatments at room temperature changed both – colloidal stability and tertiary structure of WPI. As a general trend, it can be concluded that the pressure effect on WPI at the tested concentration is increasing with an increased amount of MC. This effect is known for HP treatments at room temperature for the comparison of WPI and skim or whole milk (Mazri et al., 2012) but is shown first for HPLT treatments in the present study. The different results of HPLT treatments and HP treatments at room temperature indicate different mechanisms in

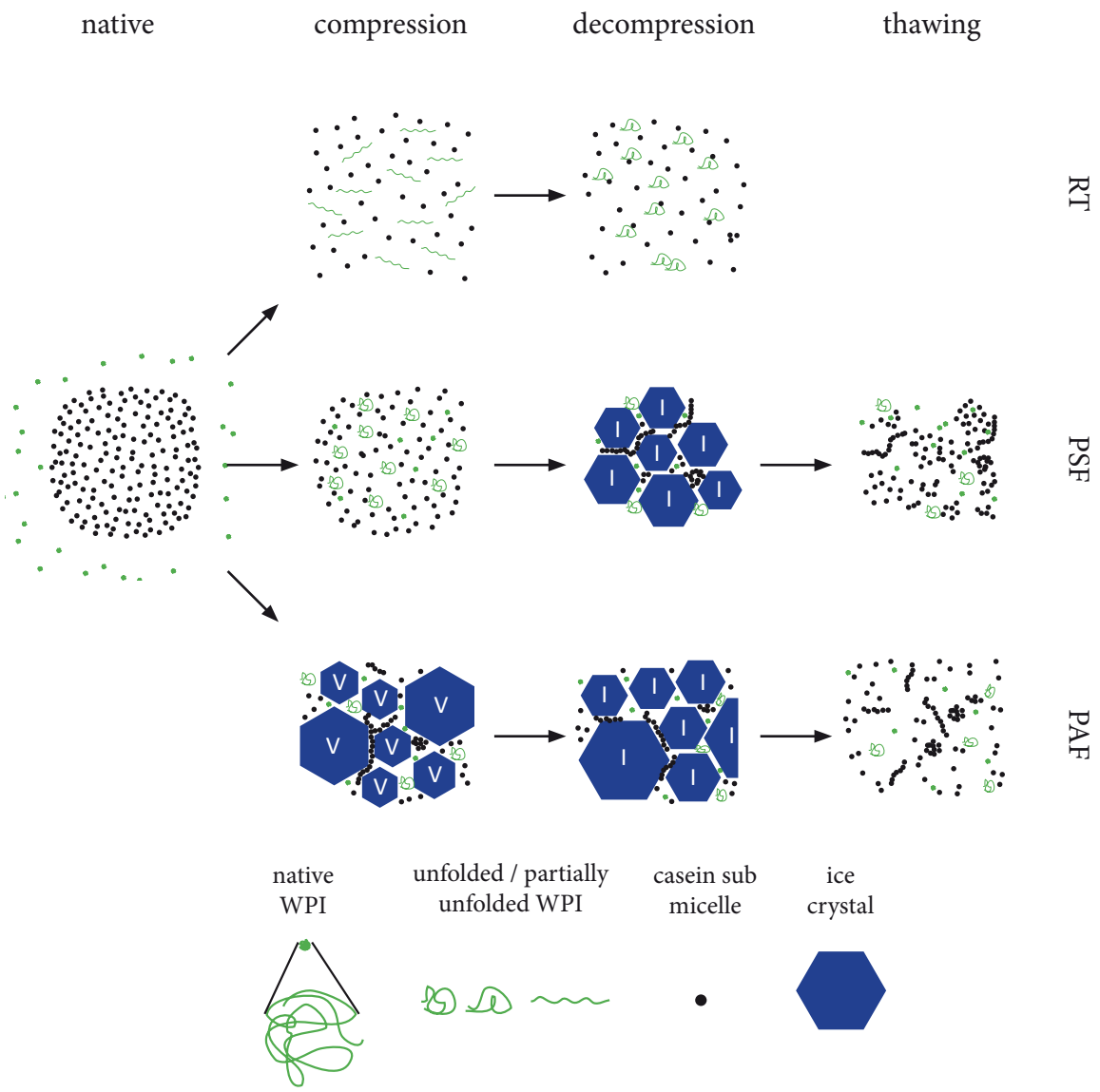


Figure 18: Suggested mechanisms for HPLT induced modification of whey protein and micellar casein during the different processing steps. Proportions of protein fractions and ice crystals do not refer to real conditions.

protein modification for both kinds of processes. It was also shown that solubilization of MC fractions highly depends on the presence of WPI and at pH 7.0 an almost full solubilization of all MC fractions is possible in the presence of 80 % WPI (see 2.1). However, MC fractions are able to form large aggregates and flocks during HPLT treatments which possess a high volume but seem to have a low protein density. Consequently, mechanisms for HPLT induced changes in milk protein fractions can by suggested. Figure 18 shows possible mechanisms for HPLT treatments of milk protein dispersions at pH 7.0. As a first step a partial unfolding of WPI is suggested and a dissociation of casein micelles takes place. This step is independent of

the kind of treatment but the extent may differ depending on the temperature. The extent of changes influences the reversibility of the modifications and thus, changes in tertiary structure during HPLT treatments (PAF and PSF) are reversible although some secondary structure motifs are changed. Especially, the high increases in random coil structure after HP treatments at room temperature influences the folding of the WPI and thus, decreases the overall extent of tertiary structure. Furthermore, a decrease in accessible thiol groups after HP treatments at room temperature indicates the formation of new disulfide links (see 2.2). The formation of ice crystals during HPLT treatments affects proteins in two different ways. On the one hand, protein molecules can adsorb at the surface of the crystals and thus, be modified (Strambini & Gabellieri, 1996). On the other hand, freeze concentration may enhance interactions between proteins (Franks, 1995). Hence, PAF and PSF treatments may cause different effects because of different time spans that are available for interactions with ice crystals or different freezing rates. Freezing to higher ice modifications with re-crystallization during decompression (PAF) which is connected to large volume expansion of the ice crystals, may furthermore compress or disrupt formed aggregates. Consequently, structural changes are affected by several factors during HPLT processing.

A change of the pH to 5.8 alters the extent of protein modification and thus, the remaining changes after decompression and thawing. Changes in particle size of the soluble fraction and decrease of turbidity are smaller or reversed and large casein flocks are built in a higher extent. These aggregates show a shear thickening effect and hence, possess interesting new functional properties (see 2.3). WPI are able to form special aggregates which contain native protein when frozen at pH 5.8 in pure solution (or in a MC:WPI = 20:80 mixture) to higher ice formations (not included in mechanism). However, these aggregates do not affect the bulk rheology like casein flocks. Furthermore, the reduction in total accessible thiol groups is less pronounced in the mixture at pH 5.8 which can be traced back to a lower reactivity of the thiol group (Cheftel & Dumay, 1996). Secondary structure investigations showed that HPLT treatments – especially PSF treatments induce new structures (β-sheet and α-helix) from random coil structure (see 2.2). However, the tertiary structure is again not affected. This effect can be regarded as a main

difference between HPLT treatments and HP treatments at room temperature. It could be also an explanation for the less pronounced changes in foam and emulsion stability for HPLT treated samples (see 2.3). As a general trend HPLT and HP treatments of pure WPI or MC dispersions increase foam stability and decrease emulsion stability. Effects are most pronounced for HP treatments at room temperature. Changes are found to be more pronounced for WPI at pH 5.8 and for MC at pH 7.0. These findings can be explained by changes in surface hydrophobicity and surface rheology. An increased surface hydrophobicity supports hydrophobic interactions which lead to higher elasticity (elastic part of surface dilatational modulus). On the one hand this leads to an increased foam stability due to higher interactions in the lamellas and on the other hand increased attractions will support flocculation and thus, decrease emulsion stability. However, treatments of mixtures of WPI and MC showed less pronounced effects. The relatively small changes in foam and emulsion stability may be traced back to the chosen conditions during functionality analyses. Changes in concentrations, pH value or ionic strength could be helpful to get more distinct changes for general conclusions. Finally, this is the first time that the creation of casein based flocks is observed. Those flocks can be created at a quite low protein concentration and build large volume particles with a low protein content. These properties can be helpful for using them as structuring ingredient (e.g. thickening agent). The formation of flocks may depend on the concentration and the creation of flocks from WPI could also be possible at higher protein concentrations. This thesis is supported by the different concentration of MC and WPI that are necessary to form a high pressure induced gel, which is about 4 % for MC dispersions and about 20 % for WPI solutions (data not shown). However, the present study identified some process-structure-function relationships for HPLT treatments of milk proteins and clearly points out the high potential of this emerging technology for protein modification. Nevertheless, the broad field of protein modification and process parameters of HPLT treatments still leave a high amount of further questions. Some possible approaches for further research in this field are suggested in the following chapter.

3.2 Future Perspective

The present study points out some basic effects of HPLT treatments on structural and functional changes of micellar casein and whey protein. The potential of this technology to modify milk proteins and create specific structure is shown and basic mechanisms are suggested. Nevertheless, further research is needed to get a deeper insight in the complex mechanisms of HPLT induced protein modification. Concerning the findings of the present study, the following steps are suggested:

- Investigation of interactions of milk proteins during HPLT treatments with other food ingredients like sugar or salt,
- Investigation of influence of protein concentration on HPLT induced protein modification,
- Testing of other protein systems for their potential to form fibrillar protein flocks (e.g. caseinates, plant proteins),
- Testing of other proteins and ingredients for potential interactions with caseins (inclusion into flocks)
- Detailed investigation of possible applications for HPLT induced flocks (e.g. thickening agent, creation of new textural properties),
- Online measurements of protein aggregation to identify the most important processing steps and optimal processing parameters (e.g. by microscopic cells, structural investigations via diamond anvil cells),
- Development of controlling systems for a targeted triggering of nucleation under pressure
- Development of continuous HPLT units for industrial use.

By performing the above mentioned steps, the following goals can be reached:

- Creation of models for reaction mechanisms for a complete understanding of HPLT induced protein modification,
- Highlighting of the high potential of HPLT treatments to generate specific protein structures with new functional properties,
- Optimizing HPLT processes for economic industrial implementation.

However, it is obvious that performing all the steps and the achievement of all the mentioned goals needs systematic research in the fields of process engineering, protein chemistry and basic food science. Nevertheless, previous studies and the systematic investigations of the present study clearly point out the high potential of HPLT treatments for specific protein modification and thus, substantiate the need for further research to endorse HPLT treatments as a tool for modification of functional food ingredients.

4. References

Abascal, J. L. F., Sanz, E., Fernandez, R. G., et al. (2005). A potential model for the study of ices and amorphous water: TIP4P/Ice. Journal of Chemical Physics, 122(23).

Abbasi, S. & Dickinson, E. (2001). Influence of sugars on high-pressure induced gelation of skim milk dispersions. Food Hydrocolloids, 15(3), 315-319.

Abbasi, S. & Dickinson, E. (2002). Influence of high-pressure treatment on gelation of skim milk powder plus low methoxyl pectin dispersions. High Pressure Research, 22(3-4), 643-647.

Acharya, K. R., Stuart, D. I., Phillips, D. C., et al. (1990). A critical-evaluation of the predicted and X-ray structures of alpha-lactalbumin. Journal of Protein Chemistry, 9(5), 549-563.

Alizadeh, E., Chapleau, N., Hayert, M., et al. (2005). Freezing and microstructure of fish: Example of Salmon (Salmo salar) using conventional and pressure shift freezing. In Proceedings of the Intrafood - Innovation in Traditional food, Valencia.

Alizadeh, E., Chapleau, N., de lamballerie, M., et al. (2007). Effect of different freezing processes on the microstructure of Atlantic salmon (Salmo salar) fillets. Innovative Food Science & Emerging Technologies, 8(4), 493-499.

Alizadeh, E., Chapleau, N., de-Lamballerie, M., et al. (2009). Impact of Freezing Process on Salt Diffusivity of Seafood: Application to Salmon (Salmo salar) Using Conventional and Pressure Shift Freezing. Food and Bioprocess Technology, 2(3), 257-262.

Anema, S. G. & Li, Y. M. (2003a). Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. Journal of Dairy Research, 70(1), 73-83.

Anema, S. G. & Li, Y. M. (2003b). Effect of pH on the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. Journal of Agricultural and Food Chemistry, 51(6), 1640-1646.

Anema, S. G., Lowe, E. K. & Stockmann, R. (2005). Particle size changes and casein solubilisation in high-pressure-treated skim milk. Food Hydrocolloids, 19(2), 257-267.

Anema, S. G. (2008). Heat and/or high-pressure treatment of skim milk: changes to the casein micelle size, whey proteins and the acid gelation properties of the milk. International Journal of Dairy Technology, 61(3), 245-252.

Anema, S. G. (2010). Effect of pH at pressure treatment on the acid gelation of skim milk. Innovative Food Science & Emerging Technologies, 11(2), 265-273.

Araya, X. I. T., Hendrickx, M., Verlinden, B. E., et al. (2007). Understanding texture changes of high pressure processed fresh carrots: A microstructural and biochemical approach. Journal of Food Engineering, 80(3), 873-884.

Arias, M., Lopez-Fandino, R. & Olano, A. (2000). Influence of pH on the effects of high pressure on milk proteins. Milchwissenschaft-Milk Science International, 55(4), 191-194.

Arroyo, G., Sanz, P. D. & Prestamo, G. (1999). Response to high-pressure, low-temperature treatment in vegetables: determination of survival rates of microbial populations using flow cytometry and detection of peroxidase activity using confocal microscopy. Journal of Applied Microbiology, 86(3), 544-556.

Asada, S., Sotani, T., Arabas, J., et al. (2002). Density of water at subzero temperature under high pressure: measurements and correlation. Journal of Physics-Condensed Matter, 14(44), 11447-11452.

Aschaffenburg, R. & Drewry, J. (1957). Genetics of the beta-lactoglobulins of cows milk. Nature, 180(4582), 376-378.

Bakopanos, C., Moatsou, G., Kandarakis, I., et al. (2010). Effect of high pressure treatment at various temperatures on the rennet clotting behavior of bovine and ovine milk. Milchwissenschaft-Milk Science International, 65(3), 266-269.

Barry, H., Dumay, E. & Cheftel, J. C. (1998). Influence of pressure-assisted freezing on the structure, hydration and mechanical properties of a protein gel. In N. S. Isaacs: High pressure food science, bioscience and chemistry (343-353). Royal Society of Chemistry, London.

Belloque, J., Lopez-Fandino, R. & Smith, G. M. (2000). A H-1-NMR study on the effect of high pressures on beta-lactoglobulin. Journal of Agricultural and Food Chemistry, 48(9), 3906-3912.

Benet, G. U., Chapleau, N., Lille, M., et al. (2006). Quality related aspects of high pressure low temperature processed whole potatoes. Innovative Food Science & Emerging Technologies, 7(1-2), 32-39.

Bhattacharjee, C., Saha, S., Biswas, A., et al. (2005). Structural changes of beta-lactoglobulin during thermal unfolding and refolding - An FT-IR and circular dichroism study. Protein Journal, 24(1), 27-35.

Boland, M. (2011). Whey proteins. In G. O. Phillips & P. A. Williams: Handbook of food proteins. Woodhead Publishing, Cambridge.

Brandts, J. F., Oliveira, R. J. & Westort, C. (1970). Thermodynamics of protein denaturation - effect of pressure on denaturation of ribonuclease-A. Biochemistry, 9(4), 1038-1047.

Bridgman, P. W. (1912). Water under pressure. Proceedings of the American Academy of Arts and Sciences, 48, 450-558.

Bridgman, P. W. (1914). The coagulation of albumen by pressure. J. Biol. Chem., 19, 511-512.

Briscoe, B., Luckham, P. F. & Staeritz, K. U. (2002a). Pressure induced changes in the gelation of milk protein concentrates. In: Trends in High Pressure Bioscience and Biotechnology, Proceedings (445-452).

Briscoe, B. J., Luckham, P. F. & Staeritz, K. U. (2002b). Gelation of milk protein concentrates induced by moderate hydrostatic pressures. High Pressure Research, 22(3-4), 633-637.

Brooker, B. E., Ferragut, V., Gill, A. L., et al. (1998). Properties of rennet gels formed from high pressure treated milk. In: Fresh Novel Foods by High Pressure (55-59).

Brownlow, S., Cabral, J. H. M., Cooper, R., et al. (1997). Bovine beta-lactoglobulin at 1.8 angstrom resolution - Still an enigmatic lipocalin. Structure, 5(4), 481-495.

Buffa, M., Trujillo, A. J. & Guamis, B. (2001). Rennet coagulation properties of raw, pasteurised and high pressure-treated goat milk. Milchwissenschaft-Milk Science International, 56(5), 243-246.

Byler, D. M. & Farrell, H. M. (1989). Infrared spectroscopic evidence for calcium-ion interaction with carboxylate groups of casein. Journal of Dairy Science, 72(7), 1719-1723.

Carpenter, J. F., Prestrelski, S. J. & Arakawa, T. (1993). Separation of freezing-induced and drying-induced denaturation of lyophilized proteins using stress-specific stabilization.1. Enzyme-activity and calorimetric studies. Archives of Biochemistry and Biophysics, 303(2), 456-464.

Carr, A. J., Southward, C. R. & Creamer, L. K. (2003). Protein hydration and viscosity of dairy fluids. In P. F. Fox & P. L. H. McSweeney: Advanced Dairy Chemistry – 1: Proteins (1289–1323). Kluwer Academic / Plenum Publishers, New York.

Carrier, D., Mantsch, H. H. & Wong, P. T. T. (1990). Pressure-induced reversible changes in secondary structure of poly(L-lysine) an IR spectroscopic study. Biopolymers, 29, 837-844.

Carroll, R. J. & Farrell, H. M. (1983). Immunological approach to location of k-casein in the casein micelle by electron-microscopy. Journal of Dairy Science, 66(4), 679-686.

Castro, S. M., Van Loey, A., Saraiva, J. A., et al. (2007). Effect of temperature, pressure and calcium soaking pre-treatments and pressure shift freezing on the texture and texture evolution of frozen green bell peppers (Capsicum annuum). European Food Research and Technology, 226(1-2), 33-43.

Chaplin, M. (2014). Water structure and science. http://www1.lsbu.ac.uk/water/, Accessed: 2014.

Charm, S. E., Longmaid, H. E. & Carver, J. (1977). Simple system for extending refrigerated, nonfrozen preservation of biological-material using pressure. Cryobiology, 14(5), 625-636.

Cheftel, J.-C., Cuq, J.-L. & Lorient, D. (1992). Die wichtigsten Proteinsysteme in Lebensmitteln - Milchproteine. In: Lebensmittelproteine: Biochemie, Funktionelle Eigenschaften, Ernährungsphysiologie, Chemische Modifizierung. Behr's Verlag GmbH & Co, Hamburg.

Cheftel, J. C. & Dumay, E. (1996). Effects of high pressure on dairy proteins: A review. In: High Pressure Bioscience and Biotechnology (299-308).

Cheftel, J. C., Levy, J. & Dumay, E. (2000). Pressure-assisted freezing and thawing: Principles and potential applications. Food Reviews International, 16(4), 453-483.

Cheftel, J. C., Thiebaud, M. & Dumay, E. (2002). Pressure-assisted freezing and thawing of foods: A review of recent studies. High Pressure Research, 22(3-4), 601-611.

Chevalier-Lucia, D., Le Bail, A., Ghoul, M., et al. (2003). High pressure calorimetry at sub-zero temperature: evaluation of the latent heat and frozen water ratio of gelatin gels. Innovative Food Science & Emerging Technologies, 4(4), 361-366.

Chevalier, D., Le Bail, A., Chourot, J. M., et al. (1999). High pressure thawing of fish (whiting): Influence of the process parameters on drip losses. Food Science and Technology-Lebensmittel-Wissenschaft & Technologie, 32(1), 25-31.

Chevalier, D., Le Bail, A. & Ghoul, M. (2000a). Freezing and ice crystals formed in a cylindrical food model: part I. Freezing at atmospheric pressure. Journal of Food Engineering, 46(4), 277-285.

Chevalier, D., Le Bail, A. & Ghoul, M. (2000b). Freezing and ice crystals formed in a cylindrical food model: part II. Comparison between freezing at atmospheric pressure and pressure-shift freezing. Journal of Food Engineering, 46(4), 287-293.

Chevalier, D., Sentissi, M., Havet, M., et al. (2000c). Comparison of air-blast and pressure shift freezing on Norway lobster quality. Journal of Food Science, 65(2), 329-333.

Chevalier, D., Sequeira-Munoz, A., Le Bail, A., et al. (2000d). Effect of pressure shift freezing, air-blast freezing and storage on some biochemical and physical properties of turbot (Scophthalmus maximus). Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology, 33(8), 570-577.

Chevalier, D., Le Bail, A. & Ghoul, M. (2001a). Evaluation of the ice ratio formed during quasi-adiabatic pressure shift freezing. High Pressure Research, 21(5), 227-235.

Chevalier, D., Le Bail, A. & Ghoul, M. (2001b). Effects of high pressure treatment (100-200 MPa) at low temperature on turbot (Scophthalmus maximus) muscle. Food Research International, 34(5), 425-429.

Choi, M. J., Min, S. G. & Hong, G. P. (2008). Effect of high pressure shift freezing process on microbial inactivation in dairy model food system. International Journal of Food Engineering, 4(5).

Chourot, J. M., Boillereaux, L., Havet, M., et al. (1997). Numerical modeling of high pressure thawing: Application to water thawing. Journal of Food Engineering, 34(1), 63-75.

Chourot, J. M., Le Bail, A. & Chevalier, D. (2000). Phase diagram of aqueous solution at high pressure and low temperature. High Pressure Research, 19(1-6), 581-589.

Clark, D. C. & Smith, L. J. (1989). Influence of alcohol-containing spreading solvents on the secondary structure of proteins - a circular-dichroism investigation. Journal of Agricultural and Food Chemistry, 37(3), 627-633.

Considine, T., Patel, H. A., Anema, S. G., et al. (2007a). Interactions of milk proteins during heat and high hydrostatic pressure treatments - A review. Innovative Food Science & Emerging Technologies, 8(1), 1-23.

Considine, T., Patel, H. A., Singh, H., et al. (2007b). Influence of binding conjugated linoleic acid and myristic acid on the heat- and high-pressure-induced unfolding and aggregation of beta-lactoglobulin B. Food Chemistry, 102(4), 1270-1280.

Creamer, L. K., Richardson, T. & Parry, D. A. D. (1981). Secondary structure of bovine alpha-S1-casein and beta-casein in solution. Archives of Biochemistry and Biophysics, 211(2), 689-696.

Dalgleish, D. G. (1997). Food emulsions stabilized by proteins. Current Opinion in Colloid & Interface Science, 2(6), 573-577.

Damodaran, S. (1994). Structure-function relationship of food proteins. In N. S. Hettiarachchy & G. R. Ziegler: Protein functionality in food systems. Marcel Dekker, Inc., New York.

Daniel, I., Oger, P. & Winter, R. (2006). Origins of life and biochemistry under high-pressure conditions. Chemical Society Reviews, 35(10), 858-875.

Davies, D. T. & Law, A. J. R. (1983). Variation in the protein-composition of bovine casein micelles and serum casein in relation to micellar size and milk temperature. Journal of Dairy Research, 50(1), 67-75.

Denys, S., Van Loey, A. M. & Hendrickx, M. E. (2000). Modeling conductive heat transfer during high-pressure thawing processes: Determination of latent heat as a function of pressure. Biotechnology Progress, 16(3), 447-455.

Derjaguin, B. V. & Landau, L. (1941). Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. Acta Phys. Chim. URSS, 14, 633-662.

Desobry-Banon, S., Richard, F. & Hardy, J. (1994). Study of acid and rennet coagulation of high pressurized milk. Journal of Dairy Science, 77(11), 3267-3274.

Deuchi, T. & Hayashi, R. (1991). Pressure-application to thawing of frozen foods and to preservation under subzero temperature. In R. Hayashi: High Pressure Science for Food (101-110). San-Ei Publishing, Kyoto, Japan.

Dickinson, E. & James, J. D. (1998). Rheology and flocculation of high-pressure-treated beta-lactoglobulin-stabilized emulsions: Comparison with thermal treatment. Journal of Agricultural and Food Chemistry, 46(7), 2565-2571.

Dufour, E., Hoa, G. H. B. & Haertle, T. (1994). High-pressure effects on β-lactoglobulin interactions with ligands studied by fluorescence. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology, 1206(2), 166-172.

Dumay, E., Lambert, C., Funtenberger, S., et al. (1996). Effects of high pressure on the physico-chemical characteristics of dairy creams and model oil/water emulsions. Food Science and Technology-Lebensmittel-Wissenschaft & Technologie, 29(7), 606-625.

Dumay, E., Picart, L., Regnault, S., et al. (2006). High pressure-low temperature processing of food proteins. Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics, 1764(3), 599-618.

Dunaeva, A. N., Antsyshkin, D. V. & Kuskov, O. L. (2010). Phase diagram of H2O: Thermodynamic functions of the phase transitions of high-pressure ices. Solar System Research, 44(3), 202-222.

Ebner, K. E., Denton, W. L. & Brodbeck, U. (1966). Substitution of alpha-lactalbumin for B protein of lactose synthetase. Biochemical and Biophysical Research Communications, 24(2), 232-236.

Edebo, L. & Heden, C. G. (1960). Disruption of frozen bacteria as a consequence of changes in the crystal structure of ice. Journal of Biochemical and Microbiological Technology and Engineering, 2(1), 113-120.

Edwards, K. J., Becker, K. & Colwell, F. (2012). The deep, dark energy biosphere: Intraterrestrial life on earth. Annual Review of Earth and Planetary Sciences, Vol 40, 40, 551-568.

Eichhorn, S., Baier, D., Horst, D., et al. (2013). Pressure shift freezing as potential alternative for generation of decellularized scaffolds. International Journal of Biomaterials, Article ID 693793.

Eshtiaghi, M. N. & Knorr, D. (1996). High hydrostatic pressure thawing for the processing of fruit preparations from frozen strawberries. Food Biotechnology, 10(2), 143-148.

Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., et al. (2004). Nomenclature of the proteins of cows' milk - Sixth revision. Journal of Dairy Science, 87(6), 1641-1674.

Farrell, H. M., Jr. & Thompson, M. P. (1988). Calcium binding proteins. In M. P. Thompson: Caseins as Calcium Binding Proteins. CRC Press, Boca, Ratan, Florida.

Fernandez-Martin, F., Otero, L., Solas, M. T., et al. (2000). Protein denaturation and structural damage during high-pressure-shift freezing of porcine and bovine muscle. Journal of Food Science, 65(6), 1002-1008.

Fernandez, P. P., Otero, L., Guignon, B., et al. (2006a). High-pressure shift freezing versus high-pressure assisted freezing: Effects on the microstructure of a food model. Food Hydrocolloids, 20(4), 510-522.

Fernandez, P. P., Prestamo, G., Otero, L., et al. (2006b). Assessment of cell damage in high-pressure-shift frozen broccoli: comparison with market samples. European Food Research and Technology, 224(1), 101-107.

Fernandez, P. P., Sanz, P. D., Molina-Garcia, A. D., et al. (2007). Conventional freezing plus high pressure-low temperature treatment: Physical properties, microbial quality and storage stability of beef meat. Meat Science, 77(4), 616-625.

Fernandez, P. P., Otero, L., Martino, M. M., et al. (2008). High-pressure shift freezing: recrystallization during storage. European Food Research and Technology, 227(5), 1367-1377.

Flanagan, J. & FitzGerald, R. J. (2002). Functionality of Bacillus proteinase hydrolysates of sodium caseinate. International Dairy Journal, 12(9), 737-748.

Fletcher, N. H. (1970). Other forms of ice. In: The Chemical Physics of Ice. Cambridge University Press.

Fox, P. F. (1992). Advanced Dairy Chemistry - 1: Proteins. Blackie Academic & Professional, London.

Fradin, J. F., Le Bail, A., Sanz, P. D., et al. (1998). Note. Behaviour of packaging materials during high pressure thawing. Food Science and Technology International, 4(6), 419-424.

Fradin, J. F., Le Bail, A., Sanz, P. D., et al. (1999). Behaviour of packaging materials during high pressure thawing (vol 4, pg 419, 1998). Food Science and Technology International, 5(3), U1-U1.

Franks, F. (1995). Protein destabilization at low-temperatures. Advances in Protein Chemistry, Vol 46, 46, 105-139.

Fuchigami, M., Kato, N. & Teramoto, A. (1997). High-pressure-freezing effects on textural quality of carrots. Journal of Food Science, 62(4), 804-808.

Fuchigami, M. & Teramoto, A. (1997). Structural and textural changes in kinu-tofu due to high-pressure-freezing. Journal of Food Science, 62(4), 828-837.

Funtenberger, S., Dumay, E. & Cheftel, J. C. (1995). Pressure-induced aggregation of beta-lactoglobulin in pH 7.0 buffers. Food Science and Technology-Lebensmittel-Wissenschaft & Technologie, 28(4), 410-418.

Galazka, V. B., Ledward, D. A., Dickinson, E., et al. (1995). High pressure effects on emulsifying behavior of whey protein concentrate. Journal of Food Science, 60(6), 1341-1343.

Galazka, V. B., Dickinson, E. & Ledward, D. A. (1996). Effect of high pressure on the emulsifying behaviour of beta-lactoglobulin. Food Hydrocolloids, 10(2), 213-219.

Garcia-Risco, M. R., Olano, A., Ramos, M., et al. (2000). Micellar changes induced by high pressure. Influence in the proteolytic activity and organoleptic properties of milk. Journal of Dairy Science, 83(10), 2184-2189.

Gaucheron, F., Famelart, M. H., Mariette, F., et al. (1997). Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk. Food Chemistry, 59(3), 439-447.

Gekko, K. & Hasegawa, Y. (1989). Effect of temperature on the compressibility of native globular proteins. J. Phys. Chem., 93, 426-429.

Goncharov, A. F., Goldman, N., Fried, L. E., et al. (2005). Dynamic ionization of water under extreme conditions. Physical Review Letters, 94(12).

Grunden, L. P., Vadehra, D. V. & Baker, R. C. (1974). Effects of proteolytic enzymes on the functionality of chicken egg albumen. Journal of Food Science, 39(4), 841-843.

Guignon, B., Otero, L., Molina-Garcia, A. D., et al. (2005). Liquid water ice I phase diagrams under high pressure: Sodium chloride and sucrose models for food systems. Biotechnology Progress, 21(2), 439-445.

Guignon, B., Ramos, A. M., Infante, J. A., et al. (2006). Determining thermal parameters in the cooling of a small-scale high-pressure freezing vessel. International Journal of Refrigeration-Revue Internationale Du Froid, 29(7), 1152-1159.

Guignon, B., Torrecilla, J. S., Otero, L., et al. (2008). The initial freezing temperature of foods at high pressure. Critical Reviews in Food Science and Nutrition, 48(4), 328-340.

Haas, G. J., Prescott, H. E. & D'Intino, J. (1972). Pressure freezing-air drying: A new technique to reduce deterioration in drying tissue. Journal of Food Science, 37(3), 430-433.

Hantschel, T. & Kauerauf, A. (2009). Pore pressure, compaction and tectonics. In: Fundamentals of Basin and Petroleum Systems Modeling (31-101). Springer Berlin Heidelberg.

Harata, K. & Muraki, M. (1992). X-ray structural evidence for a local helix-loop transition in alpha-lactalbumin. Journal of Biological Chemistry, 267(3), 1419-1421.

Havea, P., Singh, H., Creamer, L. K., et al. (1998). Electrophoretic characterization of the protein products formed during heat treatment of whey protein concentrate solutions. Journal of Dairy Research, 65(1), 79-91.

Hawley, S. A. (1971). Reversible pressure-temperature denaturation of chymotrypsinogen. Biochemistry, 10(13), 2436-2442.

Hayakawa, I., Linko, Y. Y. & Linko, P. (1996). Mechanism of high pressure denaturation of proteins. Food Science and Technology-Lebensmittel-Wissenschaft & Technologie, 29(8), 756-762.

He, J.-S. & Ruan, K. (2009). Kinetics of phase separation during pressure-induced gelation of a whey protein isolate. Food Hydrocolloids, 23(7), 1729-1733.

Hedén, C.-G. (1964). Effects of hydrostatic pressure on microbial systems. Bacteriology Reviews, 28(1), 14-29.

Hendrickx, M., Ludikhuyze, L., Van den Broeck, I., et al. (1998). Effects of high pressure on enzymes related to food quality. Trends in Food Science & Technology, 9(5), 197-203.

Herberhold, H. & Winter, R. (2002). Temperature- and pressure-induced unfolding and refolding of ubiquitin: A static and kinetic Fourier transform infrared spectroscopy study. Biochemistry, 41(7), 2396-2401.

Hinrichs, J., Rademacher, B. & Kessler, H. G. (1996). Reaction kinetics of pressure-induced denaturation of whey proteins. Milchwissenschaft-Milk Science International, 51(9), 504-509.

Hinrichs, J. & Rademacher, B. (2004). High pressure thermal denaturation kinetics of whey proteins. Journal of Dairy Research, 71(4), 480-488.

Hinrichs, J. & Rademacher, B. (2005). Kinetics of combined thermal and pressure-induced whey protein denaturation in bovine skim milk. International Dairy Journal, 15(4), 315-323.

Hiraoka, Y., Segawa, T., Kuwajima, K., et al. (1980). Alpha-lactalbumin - a calcium metalloprotein. Biochemical and Biophysical Research Communications, 95(3), 1098-1104.

Hite, B. H. (1899). The effect of pressure in the preservation of milk - A preliminary report. West Virginia Agricultural and Forestry Experiment Station Bulletin, 58, 15-35.

Ho, C. & Waugh, D. F. (1965). Interactions of bovine alphas-casein with small Ions. Journal of the American Chemical Society, 87(1), 110-117.

Hoagland, P. D., Unruh, J. J., Wickham, E. D., et al. (2001). Secondary structure of bovine alpha(S2)-casein: Theoretical and experimental approaches. Journal of Dairy Science, 84(9), 1944-1949.

Home, D. S. (2006). Casein micelle structure: Models and muddles. Current Opinion in Colloid & Interface Science, 11(2-3), 148-153.

Huiping, L., Yonghua, W. & Yiwen, X. (2011). The effect of ultra-high pressure on the functional property and conformation of whey protein isolated. Advanced Materials Research, 201-203, 1008-1014.

Huppertz, T., Fox, P. F. & Kelly, A. L. (2004a). High pressure treatment of bovine milk: effects on casein micelles and whey proteins. Journal of Dairy Research, 71(1), 97-106.

Huppertz, T., Fox, P. F. & Kelly, A. L. (2004b). High pressure-induced denaturation of alpha-lactalbumin and beta-lactoglobulin in bovine milk and whey: a possible mechanism. Journal of Dairy Research, 71(4), 489-495.

Huppertz, T., Hinz, K., Zobrist, M. R., et al. (2005). Effects of high pressure treatment on the rennet coagulation and cheese-making properties of heated milk. Innovative Food Science & Emerging Technologies, 6(3), 279-285.

Huppertz, T. & De Kruif, C. G. (2006). Disruption and reassociation of casein micelles under high pressure: Influence of milk serum composition and casein micelle concentration. Journal of Agricultural and Food Chemistry, 54(16), 5903-5909.

Huppertz, T. & De Kruif, C. G. (2007). Disruption and reassociation of casein micelles during high pressure treatment: influence of whey proteins. Journal of Dairy Research, 74(2), 194-197.

IAPWS (2008). Revised release on the pressure along the melting and sublimation curves of ordinary water substance. http://www.iapws.org/relguide/meltsub.pdf, Accessed: 2008.

Ikeuchi, Y., Nakagawa, K., Endo, T., et al. (2001). Pressure-induced denaturation of monomer beta-lactoglobulin is partially irreversible: Comparison of monomer form (highly acidic pH) with dimer form (neutral pH). Journal of Agricultural and Food Chemistry, 49(8), 4052-4059.

Indrawati, Van Loey, A., Denys, S., et al. (1998a). Enzyme sensitivity towards high pressure at low temperature. Food Biotechnology, 12(3), 263-277.

Indrawati, Van Loey, A., Ludikhuyze, L., et al. (1998b). Isobaric isothermal inactivation of lipoxygenase in crude green beans extract (Phaseolus vulgaris L.) at subzero and elevated temperature. Fresh Novel Foods by High Pressure, 186, 182-182.

Indrawati, Van Loey, A. M., Ludikhuyze, L. R., et al. (2000a). Kinetics of pressure inactivation at subzero and elevated temperature of lipoxygenase in crude green bean (Phaseolus vulgaris L.) extract. Biotechnology Progress, 16(1), 109-115.

Indrawati, I., Van Loey, A. M., Ludikhuyze, L. R., et al. (1999). Soybean lipoxygenase inactivation by pressure at subzero and elevated temperatures. Journal of Agricultural and Food Chemistry, 47(6), 2468-74.

Indrawati, I., Ludikhuyze, L. R., Van Loey, A. M., et al. (2000b). Lipoxygenase inactivation in green beans (Phaseolus vulgaris L.) due to high pressure treatment at subzero and elevated temperatures. Journal of Agricultural and Food Chemistry, 48(5), 1850-1859.

Indrawati, I., Van Loey, A. M., Ludikhuyze, L. R., et al. (2000c). Kinetics of pressure inactivation at subzero and elevated temperature of lipoxygenase in crude green bean (Phaseolus vulgaris L.) extract. Biotechnology Progress, 16(1), 109-15.

Ipsen, R., Otte, J., Dominguez, E., et al. (2000). Gelation of whey protein induced by proteolysis or high pressure treatment. Australian Journal of Dairy Technology, 55(2), 49-52.

Johnston, D. E., Austin, B. A. & Murphy, R. J. (1992). Effects of high hydrostatic-pressure on milk. Milchwissenschaft-Milk Science International, 47(12), 760-763.

Johnston, D. E. (2000). The effects of freezing at high pressure on the rheology of Cheddar and Mozzarella cheeses. Milchwissenschaft-Milk Science International, 55(10), 559-562.

Kalichevsky-Dong, M. T., Ablett, S., Lillford, P. J., et al. (2000). Effects of pressure-shift freezing and conventional freezing on model food gels. International Journal of Food Science and Technology, 35(2), 163-172.

Kalichevsky, M. T., Knorr, D. & Lillford, P. J. (1995). Potential food applications of high-pressure effects on ice-water transitions. Trends in Food Science & Technology, 6(8), 253-259.

Kamath, S., Huppertz, T., Houlihan, A. V., et al. (2008). The influence of temperature on the foaming of milk. International Dairy Journal, 18(10-11), 994-1002.

Kanda, Y., Aoki, M. & Kosugi, T. (1992). Freezing of tofu (soybean curd) by pressure shift freezing and its structure - (Studies on pressure shift freezing 1). Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi, 39(7), 608-614.

Kanno, C., Mu, T. H., Hagiwara, T., et al. (1998). Gel formation from industrial milk whey proteins under hydrostatic pressure: Effect of hydrostatic pressure and protein concentration. Journal of Agricultural and Food Chemistry, 46(2), 417-424.

Kanno, C. & Mu, T. H. (2002). Gel formation of individual milk whey proteins under hydrostatic pressure. In: Trends in High Pressure Bioscience and Biotechnology, Proceedings (453-460).

Kauzmann (1959). Some factors in the interpretation of protein denaturation. Adv. Prot. Chem., 14, 1-63.

Keim, S., Kulozik, U. & Hinrichs, J. (2006). Texture and stabilizing bonds in pressure-induced, heat-induced and rennet-induced milk protein gels. Milchwissenschaft-Milk Science International, 61(4), 363-366.

Knorr, D., Schlueter, O. & Heinz, V. (1998). Impact of high hydrostatic pressure on phase transitions of foods. Food Technology, 52(9), 42-45.

Koch, H., Seyderhelm, I., Wille, P., et al. (1996). Pressure-shift freezing and its influence on texture, colour, microstructure and rehydration behaviour of potato cubes. Nahrung-Food, 40(3), 125-131.

Kolakowski, P., Dumay, E. & Cheftel, J. C. (2001). Effects of high pressure and low temperature on beta-lactoglobulin unfolding and aggregation. Food Hydrocolloids, 15(3), 215-232.

Kresic, G., Lelas, V., Herceg, Z., et al. (2006). Effects of high pressure on functionality of whey protein concentrate and whey protein isolate. Lait, 86(4), 303-315.

Kronman, M. J. & Andreotti, R. E. (1964). Inter- and intramolecular interactions of α-lactalbumin. I. The apparent heterogeneity at acid pH. Biochemistry, 3(8), 1145-1151.

Kronman, M. J., Vitols, R. & Andreotti, R. (1964). Inter- and intramolecular interactions of α-lactalbumin. II. Aggregation reactions at acid pH. Biochemistry, 3(8), 1152-1160.

Kuwata, K., Li, H., Yamada, H., et al. (2001). High pressure NMR reveals a variety of fluctuating conformers in beta-lactoglobulin. Journal of Molecular Biology, 305(5), 1073-1083.

Lanciotti, R., Chaves-Lopez, C., Patrignani, F., et al. (2004). Effects of milk treatment with dynamic high pressure on microbial populations, and lipolytic and proteolytic profiles of Crescenza cheese. International Journal of Dairy Technology, 57(1), 19-25.

Le Bail, A., Chevalier, D., Chourot, J. M., et al. (2001). High pressure calorimetry. Comparison of two systems (differential vs. single cell). Application to the phase change of water under pressure. Journal of Thermal Analysis and Calorimetry, 66(1), 243-253.

LeBail, A., Chevalier, D., Mussa, D. M., et al. (2002). High pressure freezing and thawing of foods: a review. International Journal of Refrigeration-Revue Internationale Du Froid, 25(5), 504-513.

Lee, D. U., Schluter, O., Heinz, V., et al. (2000). High pressure treatment of liquid whole egg and advantages of low temperature application. High Pressure Research, 19(1-6), 521-526.

Levy, J., Dumay, E., Kolodziejczyk, E., et al. (1999). Freezing kinetics of a model oil-in-water emulsion under high pressure or by pressure release. Impact on ice crystals and oil droplets. Food Science and Technology-Lebensmittel-Wissenschaft & Technologie, 32(7), 396-405.

Levy, J., Dumay, E., Kolodziejczyk, E., et al. (2000). Kinetics of freezing of an oil-in-water emulsion by pressure release. Impact on ice crystals and oil droplets. High Pressure Research, 19(1-6), 573-579.

Li, H. J., Zhu, K. X., Zhou, H. M., et al. (2011). Effects of high hydrostatic pressure on some functional and nutritional properties of soy protein isolate for infant formula. Journal of Agricultural and Food Chemistry, 59(22), 12028-12036.

Lille, M. & Autio, K. (2007). Microstructure of high-pressure vs. atmospheric frozen starch gels. Innovative Food Science & Emerging Technologies, 8(1), 117-126.

Lim, S. Y., Swanson, B. G. & Clark, S. (2008). High hydrostatic pressure modification of whey protein concentrate for improved functional properties. Journal of Dairy Science, 91(4), 1299-1307.

Lin, J. F., Gregoryanz, E., Struzhkin, V. V., et al. (2005). Melting behavior of H2O at high pressures and temperatures. Geophysical Research Letters, 32(11).

Lopez-Fandino, R., Carrascosa, A. V. & Olano, A. (1996). The effects of high pressure on whey protein denaturation and cheese-making properties of raw milk. Journal of Dairy Science, 79(6), 929-936.

Lopez-Fandino, R., Ramos, M. & Olano, A. (1997). Rennet coagulation of milk subjected to high pressures. Journal of Agricultural and Food Chemistry, 45(8), 3233-3237.

Lopez-Fandino, R., De La Fuente, M. A., Ramos, M., et al. (1998). Distribution of minerals and proteins between the soluble and colloidal phases of pressurized milks from different species. Journal of Dairy Research, 65(1), 69-78.

Lopez-Fandino, R. & Olano, A. (1998). Effects of high pressures combined with moderate temperatures on the rennet coagulation properties of milk. International Dairy Journal, 8(7), 623-627.

Lopez-Fandino, R. (2006). Functional improvement of milk whey proteins induced by high hydrostatic pressure. Critical Reviews in Food Science and Nutrition, 46(4), 351-363.

Luscher, C., Balasa, A., Frohling, A., et al. (2004). Effect of high-pressure-induced ice I-to-Ice III phase transitions on inactivation of Listeria innocua in frozen suspension. Applied and Environmental Microbiology, 70(7), 4021-4029.

Luscher, C. & Knorr, D. (2004). Inactivation of bacteria on frozen foods by high-pressure-induced ice crystal phase transitions. Chemie Ingenieur Technik, 76(12), 1819-1822.

Luscher, C., Schluter, O. & Knorr, D. (2005). High pressure-low temperature processing of foods: impact on cell membranes, texture, color and visual appearance of potato tissue. Innovative Food Science & Emerging Technologies, 6(1), 59-71.

Lyster, R. L. J. (1970). Denaturation of Alpha-Lactalbumin and Beta-Lactoglobulin in Heated Milk. Journal of Dairy Research, 37(2), 233-243.

Makita, T. (1992). Application of high-pressure and thermophysical properties of water to biotechnology. Fluid Phase Equilibria, 76, 87-95.

Malenkov, G. (2009). Liquid water and ices: understanding the structure and physical properties. Journal of Physics-Condensed Matter, 21(28).

Marboeuf, U., Schmitt, B., Petit, J. M., et al. (2012). A cometary nucleus model taking into account all phase changes of water ice: amorphous, crystalline, and clathrate. Astronomy & Astrophysics, 542.

Martino, M. N., Otero, L., Sanz, P. D., et al. (1998). Size and location of ice crystals in pork frozen by high-pressure-assisted freezing as compared to classical methods. Meat Science, 50(3), 303-313.

Massaux, C., Béra, F., Steyer, B., et al. (1999). High hydrostatic pressure effects on freezing and thawing processes of pork meat. In H. Ludwig: Advances in High Pressure Bioscience and Biotechnology (497-500). Springer Berlin, Heidelberg.

Masson, P. (1992). Pressure denaturation of Proteins. In C. Banly, K. Hayashi, K. Heremans & P. Masson: High Pressure and Biotechnology (89-99).

Mazri, C., Sanchez, L., Ramos, S. J., et al. (2012). Effect of high-pressure treatment on denaturation of bovine beta-lactoglobulin and alpha-lactalbumin. European Food Research and Technology, 234(5), 813-819.

Meersman, F., Dobson, C. M. & Heremans, K. (2006a). Protein unfolding, amyloid fibril formation and configurational energy landscapes under high pressure conditions. Chemical Society Reviews, 35(10), 908-917.

Meersman, F., Smeller, L. & Heremans, K. (2006b). Protein stability and dynamics in the pressure-temperature plane. Biochimica Et Biophysica Acta-Proteins and Proteomics, 1764(3), 346-354.

Messens, W., VanCamp, J. & Huyghebaert, A. (1997). The use of high pressure to modify the functionality of food proteins. Trends in Food Science & Technology, 8(4), 107-112.

Molina-Garcia, A. D., Otero, L., Martino, M. N., et al. (2004). Ice VI freezing of meat: supercooling and ultrastructural studies. Meat Science, 66(3), 709-718.

Moller, R. E., Stapelfeldt, H. & Skibsted, L. H. (1998). Thiol reactivity in pressure-unfolded beta-lactoglobulin. Antioxidative properties and thermal refolding. Journal of Agricultural and Food Chemistry, 46(2), 425-430.

Mozhaev, V. V., Heremans, K., Frank, J., et al. (1996). High pressure effects on protein structure and function. Proteins-Structure Function and Genetics, 24(1), 81-91.

Müller, R. H. (1996). Zetapotential und Partikelladung in der Laborpraxis – Einführung in die Theorie, praktische Meßdurchführung, Dateninterpretation. Wissenschaftliche Verlagsgesellschaft, Stuttgart.

Mulvihill, D. M. & Ennis, M. P. (2003). Functional milk proteins: production and utilization. In P. F. Fox & P. L. H. McSweeney: Advanced Dairy Chemistry – 1: Proteins (1175–1228). Kluwer Academic / Plenum Publishers, New York.

Needs, E. C., Stenning, R. A., Gill, A. L., et al. (2000). High-pressure treatment of milk: effects on casein micelle structure and on enzymic coagulation. Journal of Dairy Research, 67(1), 31-42.

Ng-Kwai-Hang, K. F. & Fuquay, J. W. (2011). Milk Proteins | Heterogeneity, Fractionation, and Isolation. In J. W. Fuquay: Encyclopedia of Dairy Sciences (Second Edition) (751-764). Academic Press, San Diego.

Octavio Rodiles-Lopez, J., Eugenia Jaramillo-Flores, M., Gutierrez-Lopez, G. F., et al. (2008). Effect of high hydrostatic pressure on bovine alpha-lactalbumin functional properties. Journal of Food Engineering, 87(3), 363-370.

Ohmiya, K., Fukami, K., Shimizu, S., et al. (1987). Milk curdling by rennet under high pressure. Journal of Food Science, 52(1), 84-87.

Orlien, V., Knudsen, J. C., Colon, M., et al. (2006). Dynamics of casein micelles in skim milk during and after high pressure treatment. Food Chemistry, 98(3), 513-521.

Orlien, V., Boserup, L. & Olsen, K. (2010). Casein micelle dissociation in skim milk during high-pressure treatment: Effects of pressure, pH, and temperature. Journal of Dairy Science, 93(1), 12-18.

Otero, L., Solas, M. T., Sanz, P. D., et al. (1998). Contrasting effects of high-pressure-assisted freezing and conventional air-freezing on eggplant tissue microstructure. Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food Research and Technology, 206(5), 338-342.

Otero, L., Martino, M., Zaritzky, N., et al. (2000). Preservation of microstructure in peach and mango during high-pressure-shift freezing. Journal of Food Science, 65(3), 466-470.

Otero, L. & Sanz, P. D. (2000). High-pressure shift freezing. Part 1. Amount of ice instantaneously formed in the process. Biotechnology Progress, 16(6), 1030-1036.

Otero, L. & Sanz, P. D. (2003). High pressure-assisted and high pressure-induced thawing: Two different processes. Journal of Food Science, 68(8), 2523-2528.

Otero, L., Ousegui, A., Guignon, B., et al. (2006). Evaluation of the thermophysical properties of tylose gel under pressure in the phase change domain. Food Hydrocolloids, 20(4), 449-460.

Otero, L. & Sanz, P. D. (2006). High-pressure-shift freezing: Main factors implied in the phase transition time. Journal of Food Engineering, 72(4), 354-363.

Otero, L., Ousegui, A., Benet, G. U., et al. (2007). Modelling industrial scale high-pressure-low-temperature processes. Journal of Food Engineering, 83(2), 136-141.

Otero, L., Sanz, P., Guignon, B., et al. (2012). Pressure-shift nucleation: A potential tool for freeze concentration of fluid foods. Innovative Food Science & Emerging Technologies, 13, 86-99.

Ousegui, A., LeBail, A. & Havet, M. (2008). Numerical modeling of a high pressure thawing process of a biomaterial. Aiche Journal, 54(2), 544-553.

Padiernos, C. A., Lim, S. Y., Swanson, B. G., et al. (2009). High hydrostatic pressure modification of whey protein concentrate for use in low-fat whipping cream improves foaming properties. Journal of Dairy Science, 92(7), 3049-3056.

Pandey, P. K., Ramaswamy, H. S. & St-Gelais, D. (2000). Water-holding capacity and gel strength of rennet curd as affected by high-pressure treatment of milk. Food Research International, 33(8), 655-663.

Panick, G., Malessa, R. & Winter, R. (1999). Differences between the pressure- and temperature-induced denaturation and aggregation of beta-lactoglobulin A, B, and AB monitored by FT-IR spectroscopy and small-angle X-ray scattering. Biochemistry, 38(20), 6512-6519.

Panick, G., Herberhold, H., Sun, Z., et al. (2003). Heat, cold and pressure induced denaturation of proteins. Spectroscopy-an International Journal, 17(2-3), 367-376.

Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., et al. (1986). The structure of beta-lactoglobulin and its similarity to plasma retinol-binding protein. Nature, 324(6095), 383-385.

Patel, H. A., Singh, H., Havea, P., et al. (2005). Pressure-induced unfolding and aggregation of the proteins in whey protein concentrate solutions. Journal of Agricultural and Food Chemistry, 53(24), 9590-9601.

Pauling, L., Corey, R. B. & Branson, H. R. (1951). The structure of proteins: Two hydrogen-bonded helical configurations of the polypeptide chain. Proceedings of the National Academy of Sciences, 37(4), 205-211.

Payens, T. A. J. & van Markwijk, B. W. (1963). Some features of association of beta-casein. Biochimica Et Biophysica Acta, 71(3), 517-530.

Payens, T. A. J. & Schmidt, D. G. (1965). Thermodynamic parameters of association of alphas-casein C. Biochimica Et Biophysica Acta, 109(1), 214-222.

Perrechil, F. A. & Cunha, R. L. (2013). Stabilization of multilayered emulsions by sodium caseinate and kappa-carrageenan. Food Hydrocolloids, 30(2), 606-613.

Picard, A. & Daniel, I. (2013). Pressure as an environmental parameter for microbial life - A review. Biophysical Chemistry, 183, 30-41.

Picart, L., Dumay, E., Guiraud, J. P., et al. (2004). Microbial inactivation by pressure-shift freezing: effects on smoked salmon mince inoculated with Pseudomonas fluorescens, Micrococcus luteus and Listeria innocua. Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology, 37(2), 227-238.

Picart, L., Dumay, E., Guiraud, J. P., et al. (2005). Combined high pressure-sub-zero temperature processing of smoked salmon mince: phase transition phenomena and inactivation of Listeria innocua. Journal of Food Engineering, 68(1), 43-56.

Pike, A. C. W., Brew, K. & Acharya, K. R. (1996). Crystal structures of guinea-pig, goat and bovine alpha-lactalbumin highlight the enhanced conformational flexibility of regions that are significant for its action in lactose synthase. Structure, 4(6), 691-703.

Prestamo, G., Palomares, L. & Sanz, P. (2004). Broccoli (Brasica oleracea) treated under pressure-shift freezing process. European Food Research and Technology, 219(6), 598-604.

Prestamo, G., Palomares, L. & Sanz, P. (2005). Frozen foods treated by pressure shift freezing: Proteins and enzymes. Journal of Food Science, 70(1), S22-S27.

Prestrelski, S. J., Arakawa, T. & Carpenter, J. F. (1993). Separation of freezing-induced and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. 2. Structural studies using infrared-spectroscopy. Archives of Biochemistry and Biophysics, 303(2), 465-473.

Privalov, P. L. (1990). Cold denaturation of protein. Critical Reviews in Biochemistry and Molecular Biology, 25(4), 281-306.

Puaud, M., Wille, H. E., Knorr, D., et al. (2007-2013). Aerated frozen dessert preparation, e.g. low fat and/or low sugar ice cream, involves pressurizing ingredient mix together with food grade gas, e.g. carbon dioxide. patent, (WO2007128826-A1 WOEP054471 09 May 2007 EP1854364-A1 EP113695 09 May 2006 EP2020865-A1 EP728923 09 May 2007 AU2007247118-A1 AU247118 09 May 2007 CA2650831-A1 CA2650831 09 May 2007 MX2008014164-A1 MX014164 05 Nov 2008 US2009202701-A1 US299761 05 Nov 2008 BR200711450-A2 BR011450 09 May 2007 US8182853-B2 US299761 05 Nov 2008 MX301997-B MX014164 05 Nov 2008 AU2007247118-B2 AU247118 09 May 2007).

Qin, Z., Guo, X., Lin, Y., et al. (2012). Effects of high hydrostatic pressure on physicochemical and functional properties of walnut (Juglans regia L.) protein isolate. Journal of the Science of Food and Agriculture, 93(5), 1105-1111.

Qin, Z. H., Guo, X. F., Lin, Y., et al. (2013). Effects of high hydrostatic pressure on physicochemical and functional properties of walnut (Juglans regia L.) protein isolate. Journal of the Science of Food and Agriculture, 93(5), 1105-1111.

Ramaswamy, H. S., Zhu, S. & Le Bail, A. (2006). High-pressure differential scanning calorimetry: Comparison of pressure-dependent phase transition in food materials. Journal of Food Engineering, 75(2), 215-22.

Regnault, S., Thiebaud, M., Dumay, E., et al. (2004). Pressurisation of raw skim milk and of a dispersion of phosphocaseinate at 9 degrees C or 20 degrees C: effects on casein micelle size distribution. International Dairy Journal, 14(1), 55-68.

Rehner, G. & Daniel, H. (2010). Biochemie der Ernährung. Spektrum Akademischer Verlag, Heidelberg.

Richards, F. M. (1977). Areas, volumes, packing and protein structure. Ann. Rev. Biophys. Chem, 20, 321-342.

Roche, J., Caro, J. A., Norberto, D., et al. (2012a). Cavities in the hydrophobic core govern pressure unfolding of proteins. Biophysical Journal, 102(3), 218a.

Roche, J., Caro, J. A., Norberto, D. R., et al. (2012b). Cavities determine the pressure unfolding of proteins. Proceedings of the National Academy of Sciences of the United States of America, 109(18), 6945-6950.

Rollema, H. S. (1992). Casein association and micelle formation. In P. F. Fox: Advanced Dairy Chemsitry - 1: Proteins (111-140). Elsevier, London.

Rollema, H. S. & Muir, D. D. (2009). Casein and related products. In A. Y. Tamime: Dairy Powders and Concentrated Products. John Wiley & Sons, Ltd., Chichester, United Kingdom.

Rouille, J., Lebail, A., Ramaswamy, H. S., et al. (2002). High pressure thawing of fish and shellfish. Journal of Food Engineering, 53(1), 83-88.

Ruiz-Henestrosa, V. M. P., Martinez, M. J., Sanchez, C. C., et al. (2014). Mixed soy globulins and beta-lactoglobulin systems behaviour in aqueous solutions and at the air-water interface. Food Hydrocolloids, 35, 106-114.

Salopek, B., Krasic, D. & Filipovic, S. (1992). Measurement and application of zeta potential. Mining-Geological-Petroleum Engineering Bulletin, 4(1), 147-151.

Sanz, P. D., Otero, L., deElvira, C., et al. (1997). Freezing processes in high-pressure domains. International Journal of Refrigeration-Revue Internationale Du Froid, 20(5), 301-307.

Sanz, P. D. & Otero, L. (2000). High-pressure shift freezing. Part 2. Modeling of freezing times for a finite cylindrical model. Biotechnology Progress, 16(6), 1037-1043.

Sanz, P. D. (2005). Freezing and thawing of foods under pressure. Novel Food Processing Technologies, 141, 233-260.

Sanz, P. D., Guignon, B., Ramos, A. M., et al. (2006). Determining thermal parameters in the cooling of a small-scale high-pressure freezing vessel. International Journal of Refrigeration, 29(7), 1152-9.

Sawyer, L., Brownlow, S., Polikarpov, I., et al. (1998). beta-lactoglobulin: Structural studies, biological clues. International Dairy Journal, 8(2), 65-72.

Schade, B. C., Rudolph, R., Lüdemann, H. D., et al. (1980). Reversible high pressure dissociation of lactic dehydrogenase from pig muscle. Biochemistry, 19, 1305-1332.

Schluter, O., Benet, G. U., Heinz, V., et al. (2004). Metastable states of water and ice during pressure-supported freezing of potato tissue. Biotechnology Progress, 20(3), 799-810.

Schlüter, O., Benet, G. U., Heinz, V., et al. (2004). Metastable states of water and ice during pressure-supported freezing of potato tissue. Biotechnology Progress, 20(3), 799-810.

Schmidt, D. G. & van Markwijk, B. W. (1968). Further studies on associating subunit of alphaS1-casein. Biochimica Et Biophysica Acta, 154(3), 613-614.

Schmidt, D. G. (1970a). Differences between association of genetic variants B, C and D of alphaS1-casein. Biochimica Et Biophysica Acta, 221(1), 140-142.

Schmidt, D. G. (1970b). Association of alphaS1-casein-B at pH 6.6. Biochimica Et Biophysica Acta, 207(1), 130-138.

Schmidt, D. G. & Buchheim, W. (1970). Electron microscope investigation of the substructure of casein micelles in cows' milk. Milchwissenschaft, 25(10), 596-600.

Schmidt, D. G. (1982). Association of caseins and casein micelle structure. In P. F. Fox: Developments in Dairy Chemistry - 1 (61-86). Applied Science Publishers, London.

Schmitt, C., Bovay, C., Rouvet, M., et al. (2007). Whey protein soluble aggregates from heating with NaCl: Physicochemical, interfacial, and foaming properties. Langmuir, 23(8), 4155-4166.

Schwager, B. & Boehler, R. (2008). H2O: another ice phase and its melting curve. High Pressure Research, 28(3), 431-433.

Scollard, P. G., Beresford, T. P., Needs, E. C., et al. (2000). Plasmin activity, beta-lactoglobulin denaturation and proteolysis in high pressure treated milk. International Dairy Journal, 10(12), 835-841.

Seemann, H., Winter, R. & Royer, C. A. (2001). Volume, expansivity and isothermal compressibility changes associated with temperature and pressure unfolding of staphylococcal nuclease. Journal of Molecular Biology, 307(4), 1091-1102.

Seguro, K., Tamiya, T., Tsuchiya, T., et al. (1989). Effect of chemical modifications on freeze denaturation of lactate-dehydrogenase. Cryobiology, 26(2), 154-161.

Sequeira-Munoz, A., Chevalier, D., Simpson, B. K., et al. (2005). Effect of pressure-shift freezing versus air-blast freezing of carp (Cyprinus carpio) fillets: A storage study. Journal of Food Biochemistry, 29(5), 504-516.

Shaker, R. R., Osaili, T. M., Clark, S., et al. (2008). Rheological properties of rennet-induced milk gels as affected by high pressure treatment at different temperatures. Journal of Food Agriculture & Environment, 6(2), 86-91.

Shen, T., Benet, G. U., Brul, S., et al. (2005). Influence of high-pressure-low-temperature treatment on the inactivation of Bacillus subtilis cells. Innovative Food Science & Emerging Technologies, 6(3), 271-278.

Shen, T., Bos, A. P. & Brul, S. (2009). Assessing freeze-thaw and high pressure low temperature induced damage to Bacillus subtilis cells with flow cytometry. Innovative Food Science & Emerging Technologies, 10(1), 9-15.

Silva, J. L. & Weber, G. (1993). Pressure Stability of Proteins. Annual Review of Physical Chemistry, 44, 89-113.

Silva, N. N., Piot, M., de Carvalho, A. F., et al. (2013). pH-induced demineralization of casein micelles modifies their physico-chemical and foaming properties. Food Hydrocolloids, 32(2), 322-330.

Singh, H. (2003). Milk proteins: Functional Properties. In H. Roginski, J. W. Fuquay & P. F. Fox: Encyclopedia of Dairy Sciences (1976–1982). Academic Press, London.

Singh, H. & Flanagan, J. (2006). Milkproteins. In Y. H. Hui: Handbook of Food Science, Technology and Engineering. Taylor & Francis Group, Boca Raton.

Smeller, L. (2002). Pressure-temperature phase diagrams of biomolecules. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology, 1595(1-2), 11-29.

Snoeren, T. H. M., Vanderspek, C. A. & Payens, T. A. J. (1977). Preparation of k-casein and minor alphaS-casein by electrostatic affinity chromatography. Biochimica Et Biophysica Acta, 490(1), 255-259.

Song, M., Yamawaki, H., Fujihisa, H., et al. (2003). Infrared investigation on ice VIII and the phase diagram of dense ices. Physical Review B, 68(1).

Sotani, T., Arabas, J., Kubota, H., et al. (2000). Volumetric behaviour of water under high pressure at subzero temperature. High Temperatures-High Pressures, 32(4), 433-440.

Stapelfeldt, H. & Skibsted, L. H. (1999). Pressure denaturation and aggregation of beta-lactoglobulin studied by intrinsic fluorescence depolarization, Rayleigh scattering, radiationless energy transfer and hydrophobic fluoroprobing. Journal of Dairy Research, 66(4), 545-558.

Stewart, A. F., Bonsing, J., Beattie, C. W., et al. (1987). Complete nucleotide-sequences of bovine alpha$_{S2}$-casein and beta-casein C DNAs - Comparisons with related sequences in other species. Molecular Biology and Evolution, 4(3), 231-241.

Strambini, G. B. & Gabellieri, E. (1996). Proteins in frozen solutions: Evidence of ice-induced partial unfolding. Biophysical Journal, 70(2), 971-976.

Swaisgood, H. E., Brunner, J. R. & Lillevik, H. A. (1964). Physical parameters of k-casein from cows milk. Biochemistry, 3(11), 1616-1623.

Swaisgood, H. E. & Timashef, S. N. (1968). Association of alphaS-casein c in alkaline pH range. Archives of Biochemistry and Biophysics, 125(1), 344-361.

Swaisgood, H. E. (1992). Chemistry of the caseins. In P. F. Fox: Advanced Dairy Chemistry - 1: proteins (63-110). Elsevier, London.

Takai, R., Kozhima, T. T. & Suzuki, T. (1991). Low temperature thawing by using high-pressure. In Proceedings of the XVII Intl. Congress of Refrigeration, Montreal, Québec.

Takeuchi, M., Tsuda, E., Yoshikawa, M., et al. (1985). Fractionation and characterization of 9-subcomponents of bovine k-casein-A. Agricultural and Biological Chemistry, 49(8), 2269-2276.

Tamiya, T., Okahashi, N., Sakuma, R., et al. (1985). Freeze denaturation of enzymes and its prevention with additives. Cryobiology, 22(5), 446-456.

Tanaka, N., Tsurui, Y., Kobayashi, I., et al. (1996). Modification of the single unpaired sulfhydryl group of beta-lactoglobulin under high pressure and the role of intermolecular S-S exchange in the pressure denaturation [Single SH of beta-lactoglobulin and pressure denaturation]. International Journal of Biological Macromolecules, 19(1), 63-68.

Tanford, C. (1961). Physical chemistry of macromolecules. Wiley.

Tari, O., Yurtseven, H. & Salihoglu, S. (2000). Calculation of a phase diagram for the ice II-V-VI transitions. Calphad-Computer Coupling of Phase Diagrams and Thermochemistry, 24(4), 475-482.

Thiebaud, M., Dumay, E. M. & Cheftel, J. C. (2002). Pressure-shift freezing of o/w emulsions: influence of fructose and sodium alginate on undercooling, nucleation, freezing kinetics and ice crystal size distribution. Food Hydrocolloids, 16(6), 527-545.

Tironi, V., Lebail, A. & De Ilamballerie, M. (2007). Effects of pressure-shift freezing and pressure-assisted thawing on sea bass (Dicentrarchus labrax) quality. Journal of Food Science, 72(7), C381-C387.

Tironi, V., de Lamballerie, M. & Le Bail, A. (2010). Quality changes during the frozen storage of sea bass (Dicentrarchus labrax) muscle after pressure shift freezing and pressure assisted thawing. Innovative Food Science & Emerging Technologies, 11(4), 565-573.

Toma, S. J. & Nakai, S. (1973). Calcium sensitivity and molecular-weight of casein-alphaS5. Journal of Dairy Science, 56(12), 1559-1562.

Urrutia-Benet, G., Balogh, T., Schneider, J., et al. (2007). Metastable phases during high-pressure-low-temperature processing of potatoes and their impact on quality-related parameters. Journal of Food Engineering, 78(2), 375-389.

Urrutia Benet, G., Schlüter, O. & Knorr, D. (2004). High pressure-low temperature processing. Suggested definitions and terminology. Innovative Food Science & Emerging Technologies, 5(4), 413-427.

Urrutia, G., Arabas, J., Autio, K., et al. (2007). SAFE ICE: Low-temperature pressure processing of foods: Safety and quality aspects, process parameters and consumer acceptance. Journal of Food Engineering, 83(2), 293-315.

Valente-Mesquita, V. L., Botelho, M. M. & Ferreira, S. T. (1998). Pressure-induced subunit dissociation and unfolding of dimeric beta-lactoglobulin. Biophysical Journal, 75(1), 471-476.

Van Buggenhout, S., Tran Thanh, T., Sila, D., et al. (2004). Influence of pectin conversions combined with high pressure shift freezing on the texture of frozen carrots. Communications in agricultural and applied biological sciences, 69(2), 289-92.

Van Buggenhout, S., Maes, V., Messagie, I., et al. (2005a). Enzymatic firming of high-pressure frozen strawberry halves by means of exogenous pectinmethylesterase. Communications in agricultural and applied biological sciences, 70(2), 259-62.

Van Buggenhout, S., Messagie, I., Van Loey, A., et al. (2005b). Influence of low-temperature blanching combined with high-pressure shift freezing on the texture of frozen carrots. Journal of Food Science, 70(4), S304-S308.

Van Buggenhout, S., Lille, M., Messagie, I., et al. (2006a). Impact of pretreatment and freezing conditions on the microstructure of frozen carrots: Quantification and relation to texture loss. European Food Research and Technology, 222(5-6), 543-553.

Van Buggenhout, S., Messagie, I., Maes, V., et al. (2006b). Minimizing texture loss of frozen strawberries: effect of infusion with pectinmethylesterase and calcium combined with different freezing conditions and effect of subsequent storage/thawing conditions. European Food Research and Technology, 223(3), 395-404.

Van Buggenhout, S., Messagie, I., Van der Plancken, I., et al. (2006c). Influence of high-pressure-low-temperature treatments on fruit and vegetable quality related enzymes. European Food Research and Technology, 223(4), 475-485.

Van Buggenhout, S., Grauwet, T., Van Loey, A., et al. (2007). Effect of high-pressure induced ice I/ice III-transition on the texture and microstructure of fresh and pretreated carrots and strawberries. Food Research International, 40(10), 1276-1285.

Van Buggenhout, S., Grauwet, T., Van Loey, A., et al. (2008). Structure/processing relation of vacuum infused strawberry tissue frozen under different conditions. European Food Research and Technology, 226(3), 437-448.

Van Eldik, R., Asano, T. & Lenoble, W. J. (1989). Activation and reaction volumes in solution. 2. Chemical Reviews, 89(3), 549-688.

Vaudagna, S. R., Gonzalez, C. B., Guignon, B., et al. (2012). The effects of high hydrostatic pressure at subzero temperature on the quality of ready-to-eat cured beef carpaccio. Meat Science, 92(4), 575-581.

Venir, E., Marchesini, G., Biasutti, M., et al. (2010). Dynamic high pressure-induced gelation in milk protein model systems. Journal of Dairy Science, 93(2), 483-494.

Verwey, E. J. W. & Overbeek, J. T. G. (1948). Theory of the stability of lyophobic colloids. Elsevier Publishing Company, Inc., New York.

Volkert, M., Puaud, M., Wille, H.-J., et al. (2012). Effects of High Pressure-Low Temperature treatment on freezing behavior, sensorial properties and air cell distribution in sugar rich dairy based frozen food foam and emulsions. Innovative Food Science & Emerging Technologies, 13, 75-85.

Wang, X. S., Tang, C. H., Li, B. S., et al. (2008). Effects of high-pressure treatment on some physicochemical and functional properties of soy protein isolates. Food Hydrocolloids, 22(4), 560-567.

Warme, P. K., Momany, F. A., Rumball, S. V., et al. (1974). Computation of structures of homologous proteins - Alpha-lactalbumin from lysozyme. Biochemistry, 13(4), 768-782.

Wierenga, P. A. & Gruppen, H. (2010). New views on foams from protein solutions. Current Opinion in Colloid & Interface Science, 15(5), 365-373.

Wong, P. T. T. & Heremans, K. (1988). Pressure effects on protein secondary structure and hydrogen exchange in chymotrypsinogen. Biochim. Biophys. Acta, 956, 1-9.

Yang, J., Dunker, A. K., Powers, J. R., et al. (2001). beta-Lactoglobulin molten globule induced by high pressure. Journal of Agricultural and Food Chemistry, 49(7), 3236-3243.

Yurtseven, H. & Salihoglu, S. (1998). A phase diagram for the ice VI-VII-VIII transitions. Modern Physics Letters B, 12(8), 271-279.

Zasypkin, D. V., Dumay, E. & Cheftel, J. C. (1996). Pressure- and heat-induced gelation of mixed beta-lactoglobulin/xanthan solutions. Food Hydrocolloids, 10(2), 203-211.

Zheligovskaya, E. A. & Malenkov, G. G. (2006). Crystalline water ices. Russ. Chem. Rev., 75(1), 64-85.

Zhiyi, L., Shuhua, C., Fengxia, L., et al. (2012). Effects of high-pressure low-temperature freezing&thawing process on potato qualities. Advanced Materials Research, 554-556, 1521-5.

Zhu, S., Bulut, S., Le Bail, A., et al. (2004a). High-pressure differential scanning calorimetry (DSC): Equipment and technique validation using water-ice phase-transition data. Journal of Food Process Engineering, 27(5), 359-376.

Zhu, S., Le Bail, A., Ramaswamy, H. S., et al. (2004b). Characterization of ice crystals in pork muscle formed by pressure-shift freezing as compared with classical freezing methods. Journal of Food Science, 69(4), E190-E197.

Zhu, S., Ramaswamy, H. S. & Le Bail, A. (2004c). High-pressure differential scanning calorimetry: Evaluation of phase transition in pork muscle at high pressures. Journal of Food Process Engineering, 27(5), 377-391.

Zhu, S., Ramaswamy, H. S. & Le Bail, A. (2005a). High-pressure calorimetric evaluation of ice crystal ratio formed by rapid depressurization during pressure-shift freezing of water and pork muscle. Food Research International, 38(2), 193-201.

Zhu, S., Le Bail, A. & Ramaswamy, H. S. (2006a). High-pressure differential scanning calorimetry: Comparison of pressure-dependent phase transition in food materials. Journal of Food Engineering, 75(2), 215-222.

Zhu, S., Ramaswamy, H. S. & Le Bail, A. (2006b). Calorimetry and pressure-shift freezing of different food products. Food Science and Technology International, 12(3), 205-214.

Zhu, S. M., Le Bail, A., Chapleau, N., et al. (2004d). Pressure shift freezing of pork muscle: Effect on color, drip loss, texture, and protein stability. Biotechnology Progress, 20(3), 939-945.

Zhu, S. M., Ramaswamy, H. S. & Le Bail, A. (2005b). Ice-crystal formation in gelatin gel during pressure shift versus conventional freezing. Journal of Food Engineering, 66(1), 69-76.

Zobrist, M. R., Huppertz, T., Uniacke, T., et al. (2005). High-pressure-induced changes in the rennet coagulation properties of bovine milk. International Dairy Journal, 15(6-9), 655-662.

5. Annex

Annex 1: Coefficients for Eqn. 1.11

Phase transition	T-p range		Coefficients					
	T [K]	p [bar]	a	b	c	d	e	
L-I	251.165–273.1	0-2085.66	273.0159	–0.0132	–0.1577	0	0.1516	
L-III	251.165–256.16	2085.66-3501	10.277	0.0265	50.1624	0.5868	–4.3288	
L-V	256.16–273.31	3501-6324	5.0321	–0.0004	30.9482	1.0018	0	
L-VI	273.31–355.0	6324–22160	4.2804	–0.0013	21.8756	1.0018	1.0785	
L-VII	355.0–~1500	22160–~400000	–1355.42	0.0018	167.0609	–0.6633	0	
L-X	~1500–~2500	~400000–~900000	0.2524	0.0019	0.2795	0.5	1.1675	
I-II	200.0–238.5	1500–2130	0.2569	0.1026	0.2931	0.5	0.3789	
I-III	238.5–251.165	2085.66–2130	18.9177	–0.3447	124.452	0.5709	0	
II-III	238.5–248.9	2130–3440	6.8396	0.0007	33.13	0.5	–0.5162	
II-V	209.8–248.9	3440–6080	6.6088	–0.028	36.2934	0.0086	0.73583	
II-VI	200.0–209.8	6080–6500	1.0231	–0.3688	6.2306	0.501	0.7367	
III-V	248.9–256.16	3440–3501	0.7846	0.1647	–0.5408	0.9999	–5.3526	
V-VI	209.8-273.31	6080–6324	0.1181	0.4754	–5.8193	0.9997	–33.7273	
VI-VII	278.16-355.0	21200–22160	–47.8507	0	–389.006	0.9932	28.8539	
VI-VIII	200.0-278.16	20000–21200	–8.2483	0.0518	–81.5196	0.9991	0	
VII-VIII	100.0-278.16	21200–620000	696.858	–0.0009	-52.7565	1.4191	0.8696	
VII-X	100.0-~1500	~400000–620000	28.3439	–0.007	331.927	1.0001	0	
VIII-X	0-100	620000–635500	10	–0.0128	9.9998	10	9.9873	

Annex 2: Coefficients for Eqn. 1.12

Phase transition	T range [K]	Coefficients		
		a	b	c
Ih–L	251.165–273.15	47.859	–0.426	$8.968 \cdot 10^{-4}$
III–L	251.165–256.16	489.668	–3.769	$7.257 \cdot 10^{-3}$
V–L	256.16–273.31	10.511	–0.042	$2.394 \cdot 10^{-5}$
VI–L	273.31–355.0	10.136	–0.048	$6.058 \cdot 10^{-5}$
VII–L	355.0–~1500	3.369	$-4.657 \cdot 10^{-3}$	$1.506 \cdot 10^{-6}$
Ih–II	200.0–238.5	–4.928	$4.183 \cdot 10^{-3}$	$-2.239 \cdot 10^{-7}$
Ih–III	238.5–251.165	–20.794	0.126	$-2.299 \cdot 10^{-4}$
II–III	238.5–248.9	–28.095	0.229	$-4.635 \cdot 10^{-4}$
II–V	209.8–248.9	0.948	$-8.532 \cdot 10^{-3}$	$8.87 \cdot 10^{-6}$
III–V	248.9–256.16	16.242	–0.126	$2.294 \cdot 10^{-4}$
V–VI	209.8–273.31	–0.683	$7.064 \cdot 10^{-5}$	$-3.383 \cdot 10^{-7}$
VI–VII	278.16–355.0	–0.63	$-1.717 \cdot 10^{-3}$	$9.807 \cdot 10^{-7}$

Daniel Baier • Sanddornweg 1 • 16548 Glienicke
Phone: +49 33056 / 780218 • Mobile: +49 179 / 3948829 • Mail: daniel.baier@tu-berlin.de

Curriculum Vitae and List of Publications

Personal Details:

Name:	Baier, Daniel
Date / place of birth:	04. July, 1983 / Berlin
Family status:	Married
Nationality:	German

Education:

Sep. 1990 – Jun. 1996:	Jules-Verne-Schule in Berlin (Primary school)
Aug. 1996 – Jun. 2003:	Max-Reihnhardt-Oberschule in Berlin (Grammar school) Degree: Abitur (A-Levels)
Oct. 2004 – May 2010:	Studies in Food Technology at Technische Universität Berlin Degree: Dipl.-Ing. (grade A)

Military / Alternative Civilian Service:

Aug. 2003 – May 2004:	Civilian service at "Volkssolidarität" (food on wheels) in Berlin

Work Experiences:

Aug. 2001 – Jun. 2007:	"Hallo Pizza", Berlin, Germany: Preparation and delivery of food
Jul. 2007 – Mar. 2008:	Student assistant at the Department of Chemical & Process Engineering, Technische Universität Berlin: Teaching experience, preparation of demonstration experiments

Daniel Baier • Sanddornweg 1 • 16548 Glienicke
Phone: +49 33056 / 780218 • Mobile: +49 179 / 3948829 • Mail: daniel.baier@tu-berlin.de

Oct. 2008 – Dec. 2009:	Student assistant at the Department of Food Process Engineering and Food Biotechnology, Technische Universität Berlin: BMBF-project "Optische Methoden zur Reinigungs- und Kontaminationskontrolle" (optical methods for cleaning and contamination validation)
Jan. 2010 – Mar. 2010:	Internship at "Leibniz Institut für Agrartechnik Potsdam-Bornim e.V.": Investigation of effects of high pressure treatments on fresh vegetable products

Minor Thesis:

Subject: "Untersuchung der druckinduzierten Keimung und Inaktivierung bakterieller Endosporen mit durchflusszytometrischen Methoden" (Investigation of pressure induced germination and inactivation of bacterial endospores with flow cytometric methods)
Supervision: Prof. Dr. Dipl.-Ing. Dietrich Knorr, Dr. Dipl.-Ing. Alexander Mathys

Diploma Thesis:

Subject: "Comparison of fluorescence based methods for rapid detection of the pressure induced germination of bacterial spores"
Supervision: Prof. Dr. Dipl.-Ing. Dietrich Knorr, Dr. Dipl.-Ing. Alexander Mathys, Dipl.-Ing. Kai Reineke

Daniel Baier • Sanddornweg 1 • 16548 Glienicke
Phone: +49 33056 / 780218 • Mobile: +49 179 / 3948829 • Mail: daniel.baier@tu-berlin.de

Publications / Presentations:

Peer reviewed publications:

2010 **Baier, D. , K. Reineke, I. Doehner, A. Mathys & D. Knorr:**
Fluorescence-based methods for the detection of pressure-induced spore germination and inactivation, *High Pressure Research*, First published on: 03 November 2010 (iFirst).

2012 **Reineke, K., I. Doehner, K. Schlumbach, D. Baier, A. Mathys & D. Knorr:**
The different pathways of spore germination and inactivation in dependence of pressure and temperature. *Innovative Food Science & Emerging Technologies*, 01/2012; 13:31-41.

2013 **Reineke, K., N. Ellinger, D. Berger, D. Baier, A. Mathys, P. Setlow & D. Knorr:**
Structural analysis of high pressure treated Bacillus subtilis spores. *Innovative Food Science & Emerging Technologies*, 01/2013; 17:43-53.

Eichhorn, S., D. Baier, D. Horst, U. Schreiber, H. Lahm, R. Lange & M. Krane:
Pressure shift freezing as potential alternative for generation of decellularized scaffolds. International *Journal of Biomaterials*, 01/2013; 2013:693793.

Reineke K., K. Schlumbach, D. Baier, A. Mathys & D. Knorr:
The release of dipicolinic acid - The rate-limiting step of Bacillus endospore inactivation during the high pressure thermal sterilization process. *International Journal of Food Microbiology*, 01/2013; 162(1):55-63.

2014

Baier, D., C. Schmitt & D. Knorr:
Changes in functionality of whey protein and micellar casein after high pressure – low temperature treatments. *Food Hydrocolloids.* 01/2015; 44:416-423.

Presentations:

Mar. 2009:

Baier, D., A. Mathys & D. Knorr:
Durchflusszytometrische Bestimmung des physiologischen Zustandes bakterieller Endosporen. Poster presentation at *ProcessNet technical committee meeting "Lebensmittelverfahrenstechnik 2009*" in Lausanne, Switzerland.

Oct. 2009:

Baier, D., A. Mathys & D. Knorr:
Untersuchungen druckinduzierter Keimung und Inaktivierung bakterieller Endosporen mit Hilfe durchflusszytometrischer Methoden. Poster presentation at *GDL-Kongress "Lebensmitteltechnologie 2009"* in Lemgo, Germany.

Nov. 2009:

Baier, D., A. Mathys & D. Knorr:
Flow cytometric discrimination of different physiological states of bacterial endospores and vegetative cells. Poster presentation at *"EFFoST Conference 2009"* in Budapest, Hungary.

Daniel Baier • Sanddornweg 1 • 16548 Glienicke
Phone: +49 33056 / 780218 • Mobile: +49 179 / 3948829 • Mail: daniel.baier@tu-berlin.de

Mar. 2010: **Baier, D., K. Reineke, A. Mathys & D. Knorr:**
Fluoreszenzbasierte Detektion der Keimung bakterieller Endosporen während der Hochdruckbehandlung. Poster presentation at *ProcessNet technical committee meeting "Lebensmittelverfahrenstechnik 2010*" in Karlsruhe, Germany.

Jul. 2010: **Baier, D., A. Mathys & D. Knorr:**
Application of flow cytometry to distinguish between different physiological states of bacterial endospores and vegetative cells after high pressure treatments. Poster presentation at *IFT "Annual Meeting 2010"* (Nonthermal Processing Division), Chicago, IL, USA.

Jul. 2010: **Baier, D., K. Reineke, I. Doehner, A. Mathys & D. Knorr:**
Fluorescence based methods for the detection of pressure induced spore germination and inactivation. Presentation at *"48th EHPRG International Conference"*, Uppsala, Sweden.

Aug. 2010: **Baier, D., K. Reineke, I. Doehner, A. Mathys & D. Knorr:**
Comparison of fluorescence based methods for rapid detection of the pressure induced germination of bacterial spores. Presentation at "*6th International Conference on High Pressure Bioscience and Biotechnology (HPBB2010)"*, Freising, Germany.

May 2011 **Baier, D., A. Mathys & D. Knorr:**
Identification of different physiological states of bacterial spores and distinction from vegetative cells after high pressure treatments via flow cytometry. Poster presentation at *"ICEF 11 Meeting"*, Athens, Greece.

Jun. 2011 **Baier, D., K. Reineke, I. Doehner, A. Mathys & D. Knorr:**
Pressure induced spore-germination – fast detection via fluorescence based methods. Poster presentation at *IFT "Annual Meating 2011"* (Nonthermal Processing Division), New Orleans, LA, USA.

Oct. 2011 **Baier, D., K. Kummer, K. Reineke, A. Mathys & D. Knorr:**
Rapid Detection of Pressure Induced Germination of Bacterial Spores and Distinction from Vegetative Cells via Flow Cytometry. Presentation at *"EFFoST Annual Meeting 11"*, Berlin, Germany.

Sep. 2012 **Baier, D., K. Kummer, K. Reineke, A. Mathys & D. Knorr:**
Detection of pressure induced germination of bacterial spores – Challenges and need for fast and accurate methods. Presentation at *"49th EHPRG International Conference"*, Thessaloniki, Greece.

Baier, D., A. Singer, A. Heckelmann & D. Knorr:
Inactivation of relevant fruit enzymes – Structure-function-relationship. Presentation at "49th EHPRG International Conference", Thessaloniki, Greece.

Daniel Baier • Sanddornweg 1 • 16548 Glienicke
Phone: +49 33056 / 780218 • Mobile: +49 179 / 3948829 • Mail: daniel.baier@tu-berlin.de

Nov. 2012	**Baier, D., K. Kummer, K. Reineke, A. Mathys & D. Knorr:** Fast and accurate detection of pressure induced germination of bacterial spores – advantages and limits of applying fluorescence dyes for different measurement techniques. Presentation at *"EFFoST Annual Meeting 12"*, Montpellier, France.
Feb. 2013	**Baier, D., K. Naudorf, K. Reineke, A. Mathys & D. Knorr:** Druckinduzierte Keimung bakterieller Sporen – Screening-Methoden zur schnellen und exakten Messung. Presentation at P*rocessNet technical committee meeting „Lebensmittel-verfahrenstechnik"*, Quakenbrück, Germany.

Language Skills:

German:	Native language
English:	Fluent
French:	Basic skills

Other Interests:

Music:	Playing the guitar
Sports:	Table tennis
	Bicycling

Berlin, 28/11/2014

Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die Dissertation selbständig verfasst habe. Alle benutzten Hilfsmittel und Quellen sind aufgeführt.

Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Veröffentlichungen von irgendwelchen Teilen der vorliegenden Dissertation sind von mir, wie in der vorstehenden Publikationsliste aufgeführt, vorgenommen worden.

Berlin, 02.06.2014

Daniel Baier