Impact of an Enzymatic Hydrolysis on the Functional Properties of Globular Proteins

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Kurzfassung

Lipophile, bioaktive Substanzen, wie z. B. Lipide mit einem hohen Gehalt an Omega-3-Fettsäuren, haben einen positiven Einfluss auf die menschliche Gesundheit, allerdings sind sie auch anfällig für Autoxidation, welches ihre Verwendung limitiert. Folglich werden Mikroverkapselungstechniken zur Stabilisierung dieser Inhaltsstoffe benötigt, welche im Folgenden ihren Einsatz in Lebensmitteln ermöglichen. Die Mikroverkapselung mittels Sprühtrocknung von Emulsionen findet in diesem Zusammenhang häufig Anwendung, allerdings werden hierfür grenzflächenaktive Verkapselungsmaterialien benötigt. Während des Emulgier- und Sprühtrocknungsprozesses stabiliseren diese Emulgatoren die Emulsionstropfen gegen Deformation in Form von Scherung und Dilatation. Des Weiteren ist die chemische Stabilisierung des oxidationsempfindlichen Lipids während der Prozesse und der anschließenden Lagerung essentiell, welches im Idealfall ebenfalls von dem grenzflächenaktiven Emulgator erfüllt wird.

In der vorliegenden Studie erfolgte die Mikroverkapselung von Fisch- und Rapsöl mittels der Sprühtrocknung von Emulsionen, welche mit β-Laktoglobulin (β-LG), Erbsenprotein (PPI) oder enzymatischen Hydrolysaten dieser Proteine stabilisert wurden. Zur Hydrolyse wurden zwei Endopeptidasen mit unterschiedlicher Spezifität in ihrem Spaltungsverhalten eingesetzt, welches die Funtionalität der Hydrolysate stark beeinflusste. Bovines Trypsin zeigte im Gegensatz zu Alcalase ein höheres Potenzial Peptidmischungen mit verbesserten funktionellen Eigenschaften zu erzeugen. Die strukturelle Integrität der Grenzflächenfilme wurde durch die enzymatische Hydrolyse nicht negativ beeinflusst. Zusätzlich zeigten die Hydrolysatfilme eine erhöhte Widerstandskraft gegen Deformation während der Grenzflächenrheologieexperimente (in Form von Scherung und Dilatation). Die Verbesserung der funktionellen Eigenschaften war stärker ausgeprägt für PPI, da β-LG im unmodifizierten Zustand bereits eine deutlich höhere Funtionalität aufwies. Die elektrostatische Anlagerung von enzymatisch modifiziertem Pektin an β-LG und β-LG-Hydrolysate hergestellt mit Trypsin beeinflusste die Grenzflächenadsorption der Proteine und Hydrolysate stark und hing maßgeblich vom Modifikationsgrad des Pektin und dem Hydrolysegrad des β-LG ab. Des Weiteren resultierte aus der Anwesenheit von Pektin eine erhöhte physikalische Stabilität der Grenzflächenfilme während der grenzflächenrheologischen Experimente, der Emulsionsherstellung und der Sprühtrocknung.

Zusätzlich konnte die chemische Stabilität der mikroverkapselten Öle durch enzymatische Hydrolyse im Vergleich zum unmodifizierten Substrat deutlich erhöht werden. Wie stark die Lipidoxidation verzögert wurde, hing stark von dem verwendeten Enzym und dem Hydrolysegrad ab. Neben der Erhöhung der physikalischen Stabilität des Öls

konnte durch die Anwesenheit von Pektin auch die Lipidoxidation in mikroverkapseltem Fischöl verzögert werden.

Die Ergebnisse der vorliegenden Studie zeigen deutlich das Potential von enzymatischen Hydrolysaten, hergestellt aus globulären Proteinen, Lipide sowohl physikalisch als auch chemisch zu stabilisieren.

Abstract

Lipophilic bioactive compounds sensitive to autooxidation, like lipids rich in omega-3 fatty acids, provide beneficial health effects but exhibit limited applicability in their pure form. Accordingly, microencapsulation techniques are needed to transform these bioactives into stable ingredients and enable their incorporation into food matrices for fortification. Microencapsulation by spray-drying of emulsions is frequently used in this regard, however interfacially-active wall materials are needed to stabilise the interface of the emulsion droplets against shear and dilatational deformation during the emulsification and spray-drying process. Furthermore, chemical stabilisation of the sensitive lipid is crucial during the processes and subsequent storage, which is ideally also provided by the interfacially-active wall material.

In the present study, fish oil and rapeseed oil were microencapsulated by spray-drying of emulsions stabilised by β-lactoglobulin (β-LG), pea proteins (PPI) or with enzymatic hydrolysates thereof. Two endopeptidases with different cleaving specificity were used for hydrolysis, which strongly affected the functionality of the hydrolysates. In contrast to alcalase, bovine trypsin proved to be more suitable to produce peptide mixtures with improved functional properties, respectively. Enzymatic hydrolysis did not negatively affect the structural integrity of the interfacial layers during the spray-drying process. In addition, hydrolysate layers exhibited increased resilience to deformation during interfacial rheology experiments (under shear and dilatation). The improvement of the functional properties due to enzymatic hydrolysis was more distinct for PPI compared to β-LG, since unmodified β-LG already possessed considerably better funtionality. Electrostatic attachment of enzymatically modified pectin to β-LG and its tryptic hydrolysates affected their interfacial adsorption, which strongly depended on the molecular characteristics of pectin and the degree of hydrolysis of β-LG. Furthermore, pectin reinforced the physical stability of the interfacial layers during interfacial rheology, emulsification and spray-drying.

Furthermore, the chemical stability of the microencapsulated lipophilic compounds sensitive to autoxidation was clearly increased by all protein hydrolysates compared to the unmodified substrates. However, the extent of the retardation of lipid oxidation was strongly affected by the specificity of the enzyme and the degree of hydrolysis. Besides the increase in the physical stability of the lipids, the presence of pectin in the formulation also slowed down lipid oxidation of the microencapsulated fish oil.

The results of the present study clearly indicate the potential of hydrolysates from globular proteins to both physically and chemically stabilise lipids.

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List of Abbreviations and Symbols

Abbreviation	Translation
AA	amino acid
α-L	acidic subunit of legumin
α-LA	α-lactalbumin
Ala	alanine
APPH	pea protein hydrolysate produced by alcalase
Asp	aspartic acid
a/w	air/water
β-L	basic subunit of legumin
β-LG	β-lactoglobulin
BSA	bovine serum albumin
BHA	butylated hydroxyanisole
CCD	charge-coupled device
Cys	cystein
DE	dextrose equivalent
DH	degree of hydrolysis
DH3(A) / DH6(A	hydrolysate of β-lactoglobulin in a degree of hydrolysis of 3 / 6 % produced with alcalase
DH3(T) / DH6(T)	hydrolysate of β-lactoglobulin in a degree of hydrolysis of 3 / 6 % produced with trypsin
DH6	hydrolysate of β-lactoglobulin in a degree of hydrolysis of 6 % produced with trypsin
DHA	docosahexaenoic acid
DM	degree of methoxylation
DTT	dithiothreitol
EU	European Union
EPA	eicosapentaenoic acid
FTIR	fourier transform infrared spectroscopy
GC	galacturonic acid content
GMP	caseinoglycomacropeptide
HMP	high-methoxylated pectin
Ile	isoleucine
LCPUFA	long-chain polyunsaturated fatty acid
LMP	low-methoxylated pectin
LVE	linear viscoelastic regime
MCT	medium chain triglyceride
ME	microencapsulation efficiency
Met	methionine
MW	molecular weight
ORAC	oxygen radical absorbance capacity
Phe	phenylalanine
PPI	pea protein isolate

Abbreviation	Translation
PPH	pea protein hydrolysate
ROS	reactive oxygen species
SEC	size exclusion chromatography
Ser	serine
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TPPH	pea protein hydrolysate produced by trypsin
Trp	tryptophan
Tyr	tyrosine
o/w	oil/water
UV	ultraviolet
WPC	whey protein concentrate
WPH	whey protein hydrolysate
WPI	whey protein isolate
XPS	X-ray photoelectron spectroscopy

Symbol	Translation	Unit
A	interfacial area	μm^2
A_0	unperturbed interfacial area	μm^2
$\Delta A/A_0$	interfacial area amplitude	μm^2
$\Delta\sigma/\sigma_0$	interfacial tension amplitude	mN/m
δ	phase shift	-
γ	deformation	%
E	Gibbs modulus	mN/m
E^*	complex dilatational modulus	mN/m
E'	dilatational storage modulus	mN/m
E''	dilatational loss modulus	mN/m
G^*_i	complex interfacial shear modulus	mN/m
G'	interfacial storage modulus	mN/m
G''	interfacial loss modulus	mN/m
h_{tot}	total number of peptide bonds	meqv/g of protein
η	bulk viscosity	Pa*s
η^*_i	complex interfacial shear viscosity	Pa*s*m
σ	interfacial tension	mN/m
σ_0	unperturbed interfacial tension	mN/m
ϕ	phase angle	°
p_L	Laplace pressure	Pa
M	molar concentration	mol/l
M	torque	N*m
Ω	angular disc velocity	rad/s
R	disc radius	mm
t	time	s
ω	frequency	Hz

1 Motivation and Objectives

Since the beginning of the 21st century, there is a growing demand for bioactive food compounds. The role of food is shifting from its basic function of the satisfaction of hunger and the supply of essential nutrients to additionally providing beneficial effects to health, wellness and to prevent diseases (Đorđević et al., 2015; Fang & Bhandari, 2012). In this regard, lipids with a high content of long-chain polyunsaturated fatty acids (LCPUFAs) like fish oil, have received increased attention in the last two decades, due to their beneficial health effects (Taneja & Singh, 2012). The intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the main LCPUFAs in fish oils, is generally unsufficient in Western Europe (Bauch, Lindtner, Mensink, & Niemann, 2006) and accordingly the fortification of foods with these compounds is highly recommended (Kim & Mendis, 2006).

Since these bioactives, including LCPUFAs, are often prone to instability due to degradation or deterioration during the manufacturing process, storage, distribution and consumption, their application in foods is challenging (Taneja & Singh, 2012). Therefore, a lot of research has focused on the development of microencapsulation and controlled release systems to overcome instability issues, mask undesireable properties and enable targeted delivery or release of these components (Dias, Ferreira, & Barreiro, 2015). Among the available techniques for microencapsulation of sensitive lipophilic compounds, spray-drying of emulsions is most popular (Drusch, Regier, & Bruhn, 2012), since it is economical, continuous, low-cost and yields high-quality microcapsules by using widely available equipment (Gouin, 2004). Due to the rapid dehydration of the atomised feed emulsion droplets during drying, the lipid is embedded finely dispersed in a carbohydrate-based glassy matrix (Drusch, Regier, et al., 2012), which slows down oxygen permeation and lipid oxidation, respectively (Orlien, Risbo, Rantanen, & Skibsted, 2006). Since the drying of the atomised droplets takes place at their surface and the maximum temperature at the droplet surface is equal to the wet bulb temperature, which is usually in the order of 50 °C (Elversson & Millqvist-Fureby, 2005; Zuidam & Shimoni, 2010), the encapsulant is only exposed to a mild heat treatment of 50-80 °C (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007).

The availability of suitable wall materials for microencapsulation by spray-drying is limited to gums, proteins, polysaccharides, mono-, di- and oligosaccharides and cellulose derivates (Drusch, Regier, et al., 2012). The choice of the most applicable substance refers to its costs and its physico-chemical properties, i.e. solubility, molecular weight, glass transition temperature, crystallinity, diffusibility, film-forming and emulsifying properties (Gharsallaoui et al., 2007). Since no wall material meets all requirements for a wall material, usually a combination of substances is used to attain all es-

sential properties (Shahidi & Han, 1993). Carbohydrates as the bulk constituent of the wall material have proven to be suitable for the encapsulation of lipids (Serfert et al., 2013, 2014; Young, Sarda, & Rosenberg, 1993b). In this regard, the use of corn syrup solids with a DE of 38 provided a good balance between the formation of dense, uniform glassy matrices (Hogan, McNamee, O'Riordan, & O'Sullivan, 2001), since a decrease in molecular weight of the carbohydrate decreases oxygen permeation through the glassy matrix (Gharsallaoui, Saurel, Chambin, & Voilley, 2012; Subramaniam et al., 2013). However, further decrease of the molecular weight also decreases the glass transition temperature to critical values for maintaining capsule stability during storage (Roos, 2010).

Apart from the carbohydrate source as reviewed by Drusch et al. (2012), the composition of the oil/water-interface, its thickness and strength, the presence of antioxidants at the interface, metal-chelating ability, and oxygen- and radical-scavenging ability play a key role in the stabilisation of the lipid, in liquid and spray-dried emulsions. Emulsifiers who form highly viscoelastic interfacial layers and possess a high surface activity are essential to maintain structural integrity of the emulsion troughout the process as reviewed by Jayasundera, Adhikari, Aldred, & Ghandi (2009). The main whey protein fraction, β-lactoglobulin (β-LG), forms highly viscoelastic interfacial layers, due to a high packing density and strong intermolecular interactions (Dickinson, 2001). However, the interfacial activity, i.e. the decrease in interfacial tension due to adsorption of a protein, is lower for β-LG compared to low molecular weight surfactants and smaller proteins (Dickinson, 1992). Since a high interfacial activity is essential during emulsification (Walstra, 1993) and spray-drying (Drusch, Hamann, Berger, Serfert, & Schwarz, 2012), a modification of the molecular structure is needed to increase the interfacial activity of β-LG, e.g. by enzymatic hydrolysis (Panyam & Kilara, 1996). An enzymatic hydrolysis of β-LG results in a decrease of the molecular weight and frequently in exposure of the hydrophobic amino acids from the molecules interior, thereby increasing the interfacial acitivity (Davis, Doucet, & Foegeding, 2005; Ipsen et al., 2001; Perez, Carrera Sánchez, Rodríguez Patino, Rubiolo, & Santiago, 2012). Besides the degree of hydrolysis (DH), the milieu conditions during hydrolysis, the composition and pretreatment of the substrate and the specificity of the enzyme used, essentially affects the functionality of the hydrolysate (Gauthier & Pouliot, 2003; Ipsen et al., 2001). However, also the degree of hydrolysis must be limited to maintain or even increase the strength of the viscoelastic layers after adsorption of the peptides to the interface (Turgeon, Gauthier, Molle, & Leonil, 1992; van der Ven, Gruppen, de Bont, & Voragen, 2001). These findings were attributed to the need of a certain peptide size to enable distinct regions of hydrophilic and hydrophobic amino acids and the availability of sufficient possibilities for intermolecular interactions (Foegeding, Davis, Doucet, & McGuffey, 2002; Singh & Dalgleish, 1998).

Milk proteins compared to plant proteins in general exhibit a lower molecular weight and higher flexibility, generating faster diffusion and adsorption to the interface during emulsification in comparison to those derived from plant sources (Benjamin, Silcock, Beauchamp, Buettner, & Everett, 2014; Can Karaca, Low, & Nickerson, 2015). Plant proteins, e.g. from pea origin, exhibit a compact globular structure with low flexibility and low suface hydrophobicity caused by inclusion of the hydrophobic patches in the molecules interior, which limits their ability to unfold and to form strong viscoelastic layers (Can Karaca et al., 2015; Gueguen, 1989; Jiang, Zhu, Liu, & Xiong, 2014). However, it was reported, that enzymatic hydrolysis of pea proteins may also improve their emulsifying properties (Barac et al., 2011, 2012; Humiski & Aluko, 2007).

Another approach to reinforce interfacial layers of β-LG or pea protein, is the electrostatic attachment of an oppositely charged biopolymer (Dickinson, 2011b; Ganzevles, Fokkink, van Vliet, Cohen Stuart, & de Jongh, 2008; Ganzevles, van Vliet, Cohen Stuart, & de Jongh, 2007; Gharsallaoui, Yamauchi, Chambin, Cases, & Saurel, 2010; Gharsallaoui, Saurel, et al., 2010; Serfert et al., 2013). Non-interfacially-active pectin may be enzymatically modified to different degrees of methoxylation and degrees of blockiness by the use of pectinmethylesterases with different specificity (Ralet, Dronnet, Buchholt, & Thibault, 2001; Ralet & Thibault, 2002; Wagoner, Vardhanabhuti, & Foegeding, 2016). The modified pectins exhibit a varying binding affinity and strength of the complexes formed with the protein, due to amount and distribution of the free carboxyl groups within the galacturonic acid backbone of the molecule, respectively (Ganzevles et al., 2008; Ganzevles, van Vliet, et al., 2007; Wagoner et al., 2016).

Furthermore, it was hypothesised, that interfacially-active antioxidants are most effective in the stabilisation of lipids in oil-in-water emulsions, since they are located at the interface, where oxidation reactions are promoted (McClements & Decker, 2000). Accordingly, an emulsifier is needed, which can stabilise the oil/water-interface during emulsification, atomisation and drying, resulting in an efficient encapsulation of the oil (Rusli, Sanguansri, & Augustin, 2006) and additionally protect the lipid against oxidation throughout the process and during subsequent storage of the dried capsules.

Besides the possibility to increase the interfacial properties by limited enzymatic hydrolysis of globular proteins, the modification may also involve improvement of the antioxidant properties in liquid hydrolysate-stabilised emulsions for β-LG (Elias et al., 2006; Elias, Kellerby, & Decker, 2008; Blanca Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005) and pea proteins (Humiski & Aluko, 2007; Pownall, Udenigwe, & Aluko, 2010; Zhang, Xiong, Chen, & Zhou, 2013). The increase in antioxidant performance was attributed to the improved accessibility of the involved amino acids, chelation of prooxidative transition metals, free radical scavenging, the inactivation of reactive oxygen species and the reduction of hydroperoxides (Conway,

Gauthier, & Pouliot, 2013; Elias et al., 2008; Humiski & Aluko, 2007; Pownall et al., 2010; Zhang et al., 2013). The use of commercial whey protein hydrolysates for the microencapsulation of sensitive lipophilic compounds by spray-drying emulsions has been reported in two studies so far, however due to the instrumental setup or the peptide profile of the hydrolysate, only a poor microencapsulation effiency was achieved (Ramakrishnan et al., 2013; Ramakrishnan, Ferrando, Aceña-Muñoz, De Lamo-Castellví, & Güell, 2012). The application of pea protein hydrolysates as wall materials in these systems has not been reported so far.

The aim of this work is to prove that enzymatically modified globular proteins exhibit improved functional properties with respect to the physico-chemical stabilisation of liquid emulsions and spray-dried emulsions for the microencapsulation of sensitive lipophilic compounds.

To accomplish this, the following objectives are investigated in the present study:

1) Controlled enzymatic hydrolysis of β-lactoglobulin can improve the stability of lipids sensitive to autoxidation during the microencapsulation process by spray-drying of emulsions and subsequent storage of the microencapsulated oil
2) Electrostatic attachment of pectin to the protein- or peptide-based interfacial layers further enhances a) physical and b) chemical stabilisation of these systems
3) The adsorption kinetics and interfacial rheology of the interfacial layers involved in the systems mentioned before are determined by the molecular characteristics of the protein, peptides and pectins
4) The concept of a controlled enzymatic modification for improving protein functionality can be transferred to pea protein as an alternative model protein from plant origin with respect to a) physical and b) chemical stabilisation of lipids

2 Literature Review

Globular proteins from animal and plant origin exhibit great nutritional and functional importance, accordingly they are used in many different applications in the food industry (Gharsallaoui, Yamauchi, et al., 2010; Blanca Hernández-Ledesma et al., 2005; van der Ven, Gruppen, de Bont, & Voragen, 2002). Animal proteins, especially those from milk, have been extensively studied for their use in foods matrices. In contrast to caseins, who are transformed into cheese by precipitation, the whey proteins are a by-product of this process, accumulating as a dilute nutrient stream (Kilara & Vaghela, 2004). Up to 1950, whey was disposed or used for animal feed, however, since then advances in science and technology have added value to whey proteins as an ingredient in the food and pharmaceutical industry. Whey proteins exhibit a higher biological value than egg protein, since they contain high amounts of essential amino acids (Smithers, 2008) and the functional properties enable their use in the formation and stabilisation of emulsions and foams (Kinsella & Whitehead, 1989). Additionally whey is widely available, since in 2008 the world production accounted for 160 million tons, with a growth rate of > 2 % per year, accompanying the cheese production (Smithers, 2008).

However, a trend has emerged towards the replacement of animal-based proteins with those from plant origin in foods, e.g. due to consumers shifting to vegetarian and vegan diets, which may also result from animal diseases like bovine spongiform encephalopathy (Can Karaca et al., 2015; Nesterenko, Alric, Silvestre, & Durrieu, 2013). Soy is the plant protein studied by far the most, however pea proteins also present a suitable alterantive due to their high nutritive value, low price, non-allergenic character, wide abundance and their functional properties (Can Karaca et al., 2015; Gharsallaoui, Cases, Chambin, & Saurel, 2009). Additionally the European Union attempts to reduce the economic dependence from the soyproducing countries and therefore promotes the production of grain legumes like pea inside the EU (Le Gall, Guéguen, Séve, & Quillien, 2005).

2.1 Structure of Globular Proteins and their Modification by Enzymatic Hydrolysis

The functional properties of whey proteins are dominated by their main fraction, β-lactoglobulin (β-LG), due to its high content and specific physicochemical properties (McClements, 2016). The β-LG monomer is composed of 162 amino acids with a molecular weight (MW) of 18.4 kDa and has its isoelectric point at pH 5.2 (Walstra, Wouters, & Geurts, 2006). Depending on the pH, β-LG arranges as a monomer ($3.0 < pH > 8.0$), dimer (pH 5.3 - 7.9) or octamer (pH 3.1 - 5.1) (Kilara & Vaghela, 2004). The molecule exhibts a globular structure stabilised by hydrophobic interactions in the core and two disulphide bonds (Kilara & Vaghela, 2004). Additionally β-LG has one free sulfhydryl group natively incorporated in the interior, which is exposed during unfolding (Sava, Van der Plancken, Claeys, & Hendrickx, 2005). The secondary structure of β-LG is composed of nine strands of β-sheet, whereas eight of them are arranged as β-barrel with a hydrophobic core and a single α-helix is attached to the molecules surface (Kilara & Vaghela, 2004). During adsorption, β-LG rearranges its molecular structure and forms intermolecular disulfide bonds, whereas some of the native structure is preserved (Dickinson, 1992). Accordingly, a decrease of intramolecular and an increase in intermolecular β-sheet structure was observed via FTIR for β-LG after adsorption to an oil/water-interface (Fang & Dalgleish, 1997).

The physico-chemical properties of the pea proteins differ from those of the whey proteins, as characteristic for pulse proteins, they possess a compact globular structure, stabilised by disulphide bonds, hydrophobic and Van-der-Waals interactions (Jiang et al., 2014). Commercial pea protein isolate contains two main fractions, the albumins and globulins, amounting to 25 % and 65-80 % of the total protein, respectively. The albumins are subdivided into three fractions of 26, 6 and 3 kDa (Le Gall et al., 2005). In contrast, the globulins are further subclassified into legumin, vicilin and convicilin, exhibiting a molecular weight of 350-400, 170 and 280 kDa, each composed of several subunits (Gatehouse, Croy, Morton, Tyler, & Boulter, 1981; Gueguen, 1989). Convicilin is often referred to the third fraction of the globulins, although it has also been classified as a variant of vicilin (O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004). However, usually legumin and vicilin are considered as the main fractions of pea protein. Both exhibit quarterny structure composed of three polypeptides linked to a trimer by hydrogen bonds, whereas legumin shows two of these trimers linked to a hexamer (Gueguen, Chevalier & Schaeffer, 1988; Tandang-Silvas et al., 2010). The subunits of legumin contain disulfide-linked α- and β-chains, whereas the hydrophilic α-chains are located on the surface and the hydrophobic β-chains are buried in the molecules interior (Karaca, Low, & Nickerson, 2011). The albumins contribute 50 % to the sulphurous amino acids in pea protein isolate, compared to the globulins

containing only low amounts, vicilin is even unable to form disulfide bonds due to the lack of cysteine residues (Barac et al., 2010; Boulter & Croy, 1997).

The molecular structure of proteins can be modified by enzymatic hydrolysis, which affects the functional properties of the resulting peptide mixtures, respectively. Many factors contribute to the outcome of the hydrolysis process, i.e. the degree of hydrolysis, milieu conditions, purity and pre-treatment of the substrate, enzyme specificity and enzyme/substrate-ratio and the method of termination of the enzymatic activity (Davis et al., 2005; Gauthier & Pouliot, 2003; Ipsen et al., 2001). Since peptide bonds within the substrate are cleaved during enzymatic hydrolysis, the modification yields peptides with lower molecular weight, loss or alteration of the native structure and enhanced interaction of the peptides with themselves and the environment (Kilara & Panyam, 2003). In an overview study of 11 commercially available proteases for the hydrolysis of whey protein and casein the impact of the enzyme specificity was emphasised (van der Ven et al., 2002). The authors characterised the molecular weight distribution of the hydrolysates using gel electrophoresis (SDS-PAGE) and and size exclusion chromatography (SEC) and due to DH and enzyme specificity, the peptide distribution and the functionality strongly differed. Accordingly, it becomes apparent, that the monitoring of the DH is essential to control the progress of hydrolysis and to enable the termination of the enzymatic activity at the favoured DH. The pH-stat method of Adler-Nissen (1986) is the most frequently used method for DH supervision (Davis et al., 2005; Doucet, Otter, Gauthier, & Foegeding, 2003; Fernández, Suárez, Zhu, FitzGerald, & Riera, 2013; Perez, Sanchez Carrera, Rodriguez Patino, Rubiolo, & Santiago, 2012; Severin & Xia, 2006). The cleavage of peptide bonds during hydrolysis results in free carboxyl and amino groups, existing in their dissociated or protonated state depending on the milieu conditions (Adler-Nissen, 1986). Under alkaline conditions, the amount of dissociated carboxyl groups predominates and the amount of peptide bonds cleaved is proportional to the liberated H^+-ions, depending on the degree of protonation of the amino groups. Accordingly, the calculation of DH, i.e. the percentage of peptide bonds cleaved, is enabled by monitoring the base consumption to maintain a constant pH (Adler-Nissen, 1986). A large variety of commercial enzyme preparations is available, containing endo-, and exopeptidases, however for the creation of peptides with good functional properties only the use endopeptidases is reasonable (López-Barrios, Gutiérrez-Uribe, & Serna-Saldívar, 2014). Among the endopeptidases, the serin protease trypsin is frequently used due to its high specificity, exclusively cleaving peptide bonds C-terminal to arginine and lysine residues within the amino acid sequence (Olsen, Ong, & Mann, 2004). This enables the cleavage of 17 peptide bonds within the β-LG molecule (Cheison, Brand, Leeb, & Kulozik, 2011) and accordingly, the prediction of the resulting peptides is possible when using trypsin. Another frequently used enzyme is the commercial endopeptidases preparation alcalase, which is produced by *Bacillus licheniformis* and contains mostly Subtilisin Carlsberg. However, the specificity of this

protease is clearly lower compared to trypsin, accordingly the possible cleavage sites within the substrate molecules strongly increases and renders the prediction of possible peptides resulting from hydrolysis impossible (Doucet, Gauthier, Otter, & Foegeding, 2003). Since trypsin and alcalase strongly differ in their specificity to cleave certain peptide bonds, the hydrolysates produced with these enzymes possess varying functionality (Caessens, Visser, Gruppen, & Voragen, 1999; Davis et al., 2005; Humiski & Aluko, 2007; Kim et al., 2007).

2.2 Interfacial Adsorption Kinectics and Interfacial Rheology of Globular Proteins and Their Hydrolysates

Globular proteins like those from pea or whey have been extensively used in the production and stabilisation of disperse systems, i.e. foams and emulsions. Before an emulsion droplet can be broken down into smaller droplets, its deformation is needed by overcoming the Laplace pressure (p_L) at the interface, i.e. the pressure difference between the convex and concave side, given by $p_L = 2\ \sigma / r$, where σ is the interfacial tension and r is the radius of the droplet (Walstra, 1993). Thus, an emulsifier, e.g. a protein, is needed to rapidly adsorb to the newly created oil/water-interface during emulsification and to reduce the interfacial tension, which facilitates droplet breakup, since thereby the amount of energy needed for droplet disintegration also decreases (Walstra, 1993). Furthermore it is crucial for an effective emulsifier to strongly bind to the interface after adsorption to protect the newly created droplets against immediate coalescence and flocculation (Dickinson, 2009). Proteins adsorb spontaneously to oil/water- and air/water-interfaces and the main thermodynamic driving force of this process is the removal of the non-polar molecule parts from their unfavourable aqueous environment in the bulk solution (Dickinson, 1992, 2011b). The other driving force for adsorption is the unfolding of the protein at the interface, which affects the balance of the protein-protein and protein-water interactions (Dickinson, 2011b). As recently reviewed by Dickinson (2011), the adsorption process of proteins to an interface consists of four stages: (I) The biopolymer diffuses from the bulk to the interface, which is affected by bulk concentration and size of the molecules. (II) Usually, an energy barrier controls the actual adsorption step, due to the interactions of the protein with the interface. Modifications of the molecules physico-chemical properties, e.g. by increasing its suface hydrophobicity (Wierenga, Meinders, Egmond, Voragen, & de Jongh, 2003), enables regulation of the magnitude of this barrier (Dickinson, 2011b). (III) After adsorption, the protein usually changes its conformation (Zhai et al., 2010), i.e. a globular protein unfolds, resulting in an exposure of the initially inaccessible hydrophobic amino acid residues from the molecules interior (Tcholakova, Denkov, Sidzhakova, & Campbell, 2006). Furthermore, the protein rearranges these non-polar groups towards the oil phase, whereas the hydrophilic groups are in contact with the aqueous phase, accordingly the contact area of oil and water molecules, the interfacial tension and the surface free energy are reduced (McClements, 2016). Globular proteins undergo irreversible unfolding (also surface denaturation) during the adsorption process (Dickinson, 2011b), however its extent is usually limited due to high bulk protein contents resulting in high rates of adsorption (Wierenga, Egmond, Voragen, & de Jongh, 2006). Due to the conformational changes of the protein, a steric stabilising layer is formed, which protects the interface against coalescence (Cases, Rampini, & Cayot, 2005). (IV) In the last stage, a densely packed, concentrated protein layer develops, which may exhibit glass- or gel-like structure (Dickinson, 2011b). The interfacial layer is reinforced by intermolecular

crosslinking by hydrogen bonding, hydrophobic and covalent bonding and electrostatic interactions between adjacent molecules (Bos & van Vliet, 2001; Mitropoulos, Mütze, & Fischer, 2014), additionally, structural defects in the film may be recovered (Dickinson, 2011b).

After formation of the interfacial layer, its resilience during emulsification and the subsequent colloidal stability of the dispersed system under physical stress, is attributed to its mechanical properties, i.e. its interfacial rheology in the form of dilatation and shear (Erni, Windhab, & Fischer, 2011; Murray, 2011). So far, no direct correlation of the interfacial rheology of an adsorbed biopolymer interfacial layer to its emulsifying and foaming properties and the subsequent stability of these systems was reported, although the significance of the interfacial rheology concerning this matter has often been highlighted (Benjamins, Lyklema, & Lucassen-Reynders, 2006; Erni et al., 2011; Freer, Yim, Fuller, & Radke, 2004; Krägel & Derkatch, 2010; Krägel, 2014; Maldonado-Valderrama, Miller, Fainerman, Wilde, & Morris, 2010; Murray, Xu, & Dickinson, 2009; Murray, 2011; Xu, Dickinson, & Murray, 2008). Thus, the link between the deformation of the interfacial layer and the forces involved is studied as a function of time (Bos & van Vliet, 2001). Properties of interfacial layers subjected to dilatation (compression/expansion) were related to the stability during emulsification, since they represent the relaxation processes within the interfacial layer and diffusional exchange of protein with the bulk phase (Benjamins et al., 2006; Dickinson, 2011b). During the processing, dispensing and the consumption of food emulsions these deformations are likely to occur (Murray et al., 2009). On the contrary, interfacial shear rheology is sensitive to the magnitude of the lateral intermolecular interactions between the biopolymers within the layer, thereby the monitoring of structure and network formation is enabled, which has been associated with the long-term stability of emulsions (Benjamins et al., 2006; Dickinson, 2011b; Krägel & Derkatch, 2010; Krägel, 2014). The interpretation of the results arising from these two kinds of deformation should be performed together, since both affect interfaces under common process conditions (Freer et al., 2004; Maldonado-Valderrama et al., 2010). The viscoelastic moduli in dilatational rheology originate from the interfacial tension response resulting from variation of the interfacial area, e.g. by changing the volume of a droplet of surfactant solution, when using a pendant drop tensiometer, and thereby compressing and expanding the interfacial layer (Dickinson, 2011b). In interfacial shear rheology experiments the interfacial film is submitted to deformations by movement of a measuring probe, e.g. a biconical disk geometry, to the interface at constant area and the resulting torque enables to calculate the interfacial shear moduli (Dickinson, 2011b; Krägel & Derkatch, 2010).

With respect to the interfacial activity of β-LG, i.e. the decrease in interfacial tension due to adsorption of the protein (Dickinson, 1992), it is lower for β-LG compared to other proteins originating from milk, e.g. β-casein (Cases et al., 2005). The authors ascribed this to the more flexible random coil structure of β-casein, which enables the pro-

tein to diffuse, adsorb, reorient and reorganise its comfomation faster than rather rigid β-LG (Lopez & Dufour, 2001). Additionally, flexible molecules like casein have a higher content of non-polar groups, which enables them to decrease the interfacial tension more effectively (Dickinson, 2011b). Caseinoglycomacropeptide (GMP), a polypeptide derived from κ-casein by the action of chymosin during cheesemaking, exhibited a higher surface activity than β-LG, due to a lower MW and the flexible random coil structure, respectively (Martinez, Carrera Sanchez, Rodriguez Patino, & Pilosof, 2009). The impact of molecular size, flexibility and stability with respect to the interfa-cial activity of β-LG, α-lactalbumin (α-LA) and bovine serum albumin (BSA) was investigated by Suttiprasit, Krisdhasima, & McGuire (1992). Since α-LA possesses the lowest MW, accompanied with the highest flexibility, it shows the highest interfacial activity. In contrast, the adsorption rate of BSA is clearly the lowest of all proteins, which is essentially attributed to the high molecular weight. β-LG exhibits half the size of BSA, but has a higher MW and less flexibility than α-LA, which result in an interfacial activity of the three proteins in the order α-LA > β-LG > BSA (Suttiprasit et al., 1992). When comparing the interfacial activity of proteins from milk (caseinate and whey protein isolate) and soy (β-conglycinin and glycinin), those from milk diffused faster to the a/w-interface (Rodriguez Nino, Carrera Sanchez, Ruiz-Henestrosa, & Rodriguez Patino, 2005). Additionally, caseinate always reached the interface faster compared to the other proteins, which was attributed to the difference in MW of the biopolymers.

Proteins from animal origin in comparison to those derived from plant sources, in general exhibit a lower molecular weight and higher flexibility, generating faster diffusion and adsorption to the interface during emulsification (Can Karaca et al., 2015). Accordingly, the interfacial activity of plant proteins is clearly lower, which limits their ability of fast stabilisation of the newly created droplets and the prevention of (re-)coalescence (Can Karaca et al., 2015; Damodaran, 2005). Furthermore, plant proteins exhibit a compact globular structure with low flexibility and low surface hydrophobicity caused by inclusion of the hydrophobic patches in the molecules interior, which limits their ability to unfold and to form strong viscoelastic layers (Can Karaca et al., 2015; Gueguen, 1989; Jiang et al., 2014). However, the interfacial activity of legumin and vicilin was higher compared to BSA at the dodecane/water- and air/water-interface (Dagorn-Scaviner, Gueguen, & Lefebvre, 1986, 1987). Vicilin diffused and adsorbed faster to the interface, which was attributed to the lower molecular weight and higher molecular flexibility. Although the pea globulins exhibited a stronger interfacial tension decrease, BSA was able to faster diffuse and adsorb to the interface. When vicilin and legumin were both present in the aqueous phase, vicilin dominated the interface, even at low vicilin/legumin-ratios (Dagorn-Scaviner et al., 1986, 1987). In another study, the equilibrium interfacial tension of plant and milk proteins at the oil/water-interface was investigated (Amine, Dreher, Helgason, & Tadros, 2014). Although the pea proteins yielded

interfacial tension results comparable to Na-caseinate, the absolute values are too low for protein based interfacial layers, which indicates some interfacially-active fatty acids from the MCT oil decreased the interfacial tension even further (Bahtz et al., 2009).

β-LG formed stronger viscoelastic layers during dilatational rheology experiments at the oil/water-interface compared to casein, which was attributed to a high packing density and strong protein-protein-interactions within the interfacial layer compared to the loose packing of casein molecules with weaker intermolecular interactions (Cases et al., 2005). Similar observations were reported for β-LG and β-casein (Ridout, Mackie, & Wilde, 2004). The formation of highly viscoelastic β-LG interfacial layers is attributed to the formation of intermolecular disulphide bonds (Dickinson & Matsumura, 1991), which subsequently enables the formation of other intermolecular interactions (hydrogen bonds, hydrophobic associations, electrostatic interactions) (Croguennec, Renault, Bouhallab, & Pezennec, 2006). In a comparative study of soy protein isolate (SPI) and whey protein isolate (WPI), WPI exhibited higher dilatational moduli, which was ascribed to a more condensed structure within the interfacial layer compared to SPI (Rodriguez Nino et al., 2005). In addition, β-LG exhibited also clearly higher interfacial moduli during interfacial shear rheology experiments compared to β-casein, indicating the formation of weaker network by β-casein (Ridout et al., 2004). This is already observed at the beginning of the experiment, which reveals, that β-LG quickly forms densely packed layers with strong intermolecular interactions in contrast to β-casein (Erni et al., 2011). The behaviour of β-LG under interfacial shear deformation has been studied by far less than its dilatational rheology. Rühs, Affolter, Windhab, & Fischer (2013) investigated the interfacial shear behaviour of β-LG under pH-shifting conditions from pH 2-6. The interfacial shear moduli were low below pH 3 and the elastic shear modulus steadily increased, when the pH was increased and the authors attributed this to to the increment of the attractive forces between the molecules. In comparison to β-casein, β-LG exhibited increased interfacial shear moduli and this has been ascribed to a weaker network of β-casein as described above under dilatational deformation (Ridout et al., 2004), since interfacial shear rheology is very sensitive to the lateral molecular interactions in the layer (Krägel, 2014).

The dilatational moduli of pea protein were comparable to those of sodium caseinate and potato protein at pH 7 and 10 at various protein contents studied, however at very low protein contents they clearly increased (Amine et al., 2014). However, since the authors used unpurified MCT oil for the experiments, a contribution of interfacially-active free fatty acids to the interfacial response to compression and expansion cannot be ruled out. The dilatational moduli of pea protein isolate (PPI) at pH 2.4 were stronger in comparison to pH 7.0, since the globulin subunits of PPI are dissociated under acidic conditions enabling the formation of denser and stronger interfacial networks (Gharsallaoui et al., 2009).

It has been highlighted, that by a slight modification of globular proteins, e.g. by enzymatic hydrolysis, their interfacial activity and interfacial properties can be enhanced (Jung, Gunes, & Mezzenga, 2010). However, an enzymatic hydrolysis may result in an improvement of the interfacial activity and interfacial rheology of globular proteins, but it can also be detrimental. In general, smaller peptides diffuse and adsorb faster to the interface, however a minimum peptide size auf 2 kDa is needed to possess distinct hydrophilic and hydrophobic patches and a sufficient number of network points, which enable the formation of strong viscoelastic layers to stabilise the interface (Davis et al., 2005; Gauthier & Pouliot, 2003; van der Ven et al., 2001). It has been reported, that a limited enzymatic hydrolysis of β-LG using chymotrypsin increased its interfacial activity due to an increase in the exposed hydrophobic areas (Perez, Sanchez Carrera, et al., 2012). Similar results have been reported by Davis et al. (2005) and the degree of the improved interfacial activity of β-LG hydrolysates strongly depended on the enzyme used for modification. Several authors investigated the interfacial activity of isolated fractions of β-LG hydrolysates obtained by an ultrafiltration process (Turgeon, Gauthier, & Paquin, 1991), however usually commercial hydrolysate-based ingredients are crude unfractionated products (Davis et al., 2005). The interfacial activity of commercial whey protein hydrolysates was increased compared to unmodified whey protein isolate (Drusch, Hamann, et al., 2012; Tamm, Sauer, Scampicchio, & Drusch, 2012). The authors concluded, that molecular weight profile is a crucial factor affecting the interfacial activity, besides the surface hydrophobicity, the hydrodynamic radius, the number of ionisable groups and the state of aggregation. Some proteases used for enzymatic hydrolysis yield peptides with a strong tendency to aggregation (Otte et al., 1997), which causes a decreased interfacial activity by the decrease in surface hydrophobicity (Davis et al., 2005), which is the main driving force for interfacial adsorption (Dickinson, 1992). The interfacial rheology (shear and dilatation) of whey protein hydrolysates has only been investigated by few authors (Davis et al., 2005; Ipsen et al., 2001; Perez, Carrera Sánchez, et al., 2012; Perez, Sanchez Carrera, et al., 2012; Tamm et al., 2012). A low degree of hydrolysis was found favourable, to maintain the interfacial properties of the whey proteins. This may explain the decreased interfacial shear moduli of all whey protein hydrolysates in comparison to the unmodified protein, since the lowest DH produced was 19 % (Ipsen et al., 2001). However it was hypothesised, that a very slight hydrolysis may yield peptides with a simpler molecular structure, which enables the better transmission of interfacial stresses due to compression and expansion and thus explain the increased interfacial moduli for the hydrolysates in comparison to the substrate (Davis et al., 2005). Pea protein hydrolysates have not been characterised with respect to their interfacial activity and interfacial rheology, however studies for proteins from plant origin suggest, that an enzymatic hydrolysis of pea proteins may improve their interfacial properties (Martinez, Carrera Sanchez, Ruiz-Henestrosa, Rodriguez Patino, & Pilosof, 2007; Rodriguez Patino et al., 2007).

The interfacial activity and rheology of proteins may be strongly affected by the addition of polysaccharides (Dickinson, 2011b). For β-LG and non-interfacially-active pectin, a pH below the isoelectric point of the protein (positive net charge) and above the pK_a of the carboxyl groups on the galacturonic backbone of pectin (negative net charge) is needed to enable the formation of soluble complexes between the proteins and polysaccharides (Gancz, Alexander, & Corredig, 2005; Ganzevles, Zinoviadou, van Vliet, Cohen Stuart, & de Jongh, 2006; Wagoner et al., 2016). Accordingly, the successful electrostatic attachment of the polysaccharide to the protein is conventionally indicated by a reversal of electrical charge as detected by electrophoretic mobility measurements, i.e. by the ζ-potential (Dickinson, 2011b). Depending on whether the adsorption of the pectin is simultaneous to the protein or the protein adsorbs before the pectin is added to the bulk phase, the adsorption kinetics and the rheological properties of the interfacial layer strongly differ (Dickinson, 2011b). The presence of pectin in the bulk delayed interfacial adsorption and thus decreased the interfacial activity of β-LG as a function of the binding affinity of the biopolymers towards each other determined by the overall charge of the pectin, which is given by the amount of free carboxyl groups within the galacturonic acid backbone of pectin (Ganzevles, van Vliet, et al., 2007). It was shown, that a high-methoxylated pectin (HMP, low overall charge) forms weaker complexes with β-LG in the bulk, which enables the formation of dense protein interfacial layers with higher interfacial dilatational moduli (Ganzevles, van Vliet, et al., 2007). In contrast, low-methoxylated pectin (LMP, high overall charge) strongly binds to β-LG in the bulk and accordingly the interfacial adsorption was clearly delayed due to the increase in the hydrodynamic radius. Furthermore the interfacial layers exhibited lower dilatational moduli compared to pure β-LG and β-LG/HMP-fomulations (Ganzevles, van Vliet, et al., 2007). In addition the interfacial shear moduli of β-LG increased due to the addition of pectin and the authors ascribed this to a denser packing of the protein molecules induced by the oppositely charged pectin reducing the electrostatic repulsion between the protein molecules (Ganzevles et al., 2006). Besides the overall charge (degree of methoxylation) of the pectin, the local charge density (also degree of blockiness) also determines the binding affinity of the pectin to the protein, which can be modified by chemical or enzymatic modification (Einhorn-Stoll, Kastner, Hecht, Zimathies, & Drusch, 2015; Meyer-Hansen, Nielsen, & Rolin, 2009; Ralet et al., 2001; Ralet & Thibault, 2002; Wagoner et al., 2016).

2.3 Globular Proteins and their Hydrolysates as Wall Material in Microencapsulation by Spray-Drying

The spray-drying of emulsions for the microencapsulation of bioactive compounds sensitive to autoxidation is a frequently applied technique (Drusch, Regier, et al., 2012). Spray-drying belongs to the class of the physical techniques for encapsulation, like extrusion, which are based on the incorporation of the bioactive in finely dispersed form in a glassy matrix, usually containing carbohydrates as bulk constituent (Kunz, Krückeberg, & Weißbrodt, 2003). Food ingredients, which are usually microencapsulated by spray-drying are mainly flavours, probiotics, polyphenols and lipids (Fang & Bhandari, 2012; Gharsallaoui et al., 2007). The process of spray-drying is composed of four stages: preparation of the dispersion or emulsion; homogenisation of the dispersion; atomisation of the feed emulsion; and dehydration of the atomised droplets (Shahidi & Han, 1993). When developing new formulations for this process, the wall material used has to be carefully selected (Drusch, Regier, et al., 2012), i.e. it should ideally possess (Shahidi & Han, 1993): good rheological properties at high concentration and easy manipulation during the process, low viscosity in solution at high solids content, the ability to disperse or emulsify the bioactive while not reacting with it, the property to seal and hold the bioactive throughout the process and storage and protect it against environmental conditions. Furthermore the wall material should be soluble in solvents used in the food industry, completely release those upon dehydration and must be food-grade. However, to achieve the desired properties of the wall material, usually a combination of substances is used, since no material meets all requirements (Shahidi & Han, 1993). The availability of suitable wall materials for microencapsulation by spray-drying is limited to gums, proteins, polysaccharides, mono-, di- and oligosaccharides and cellulose derivates (Drusch, Regier, et al., 2012). A frequently used wall material combination for spray-drying is a blend of whey proteins (as interfacially-active agents) and carbohydrates (as bulk matrix constituent) (Bae & Lee, 2008; Berendsen, Güell, & Ferrando, 2015; Carneiro, Tonon, Grosso, & Hubinger, 2013; Choi, Ryu, Kwak, & Ko, 2010; Fäldt & Bergenståhl, 1996). However, the ability whey protein concentrate (WPC) to encapsulate soybean oil was rather low compared to sodium caseinate (Fäldt & Bergenståhl, 1996). Similar findings were reported, when WPC was used as the sole wall material to encapsulate soya oil (Hogan, McNamee, O'Riordan, & O'Sullivan, 2001). A high homogenisation pressure during production of the feed emulsion and 4 homogenisation passes strongly increased the microencapsulation performance of whey protein isolate (WPI) and WPC, although they were still lower compared to sodium caseinate (Keogh & O'Kennedy, 1999). Since whey proteins form interfacial layers with a high viscoelasticity, they should exhibit good suitability for the stabilisation of oil droplets during emulsification, atomisation and drying (Cases et al., 2005; Ridout et al., 2004; Rouimi, Schorsch, Valentini, & Vaslin, 2005), which is important to yield feed emulsion being stable against coalescence and creaming before spray-drying (Drusch, Berg, et al., 2009).

A high viscoelasticity of the interfacial layer is crucial during these processes, since it displays its resilience against deformation (Murray, 2011) and accordingly enables the integrity of the layer to be maintained during the spray-drying process (Rusli et al., 2006). A low viscoelasticity may lead the to destruction of the layer due to the high shear stress affecting the droplets during spray-drying (Mahmoudi, Gaillard, Boué, Axelos, & Riaublanc, 2010), which results in the leakage of the encapsulant, a decrease in the microencapsulation efficiency (Soottitantawat, Yoshii, Furuta, Ohkawara, & Linko, 2003) and most likely in a decreased oxidative stability of the oil, respectively. The success of the microencapsulation process can be determined by comparing the oil droplet size distribution of the emulsions before and after the spray-drying process, in addition to the extractable oil content of the resulting microcapsules, which yields the microencapsulation efficiency (Drusch & Berg, 2008; Westergaard, 2004). Besides a high viscoelasticity of the interfacial layers formed by the emulsifier, the latter should exhibit a high interfacial activity. This is important to enable the formation of small droplets in feed emulsions (Walstra, 1993) and accumulation of excess emulsifier at the surface of the drying droplets, which forms a protective coating at the particle surface and further increases the microencapsulation efficiency (Elversson & Millqvist-Fureby, 2006) and reduces the stickiness in sugar- and acid-rich powders (Jayasundera et al., 2009). Since the interfacial activity of the whey proteins and the main fraction β-lactoglobulin (β-LG) is lower compared to e.g. caseins or α-lactalbumin (α-LA) (Cases et al., 2005; Suttiprasit et al., 1992), an increase in their interfacial acitivity is needed, which may be achieved by limited enzymatic hydrolysis (Davis et al., 2005).

However, an enzymatic hydrolysis not necessarily improves the emulsifying and emulsion stabilising properties of the hydrolysates. In a comparative study using 11 commercial enzymes, the emulsion forming ability and emulsion stability decreased for all hydrolysates (van der Ven et al., 2001). Similar results were reported for a commercial whey protein hydrolysate with a degree of hydrolysis of 27 %, which is rather high (Agboola, Singh, Munro, Dalgleish, & Singh, 1998) and a higher content of high molecular weight peptides was found at the oil/water-interface compared to the bulk phase. Since low molecular weight peptides lack secondary and tertiary structure, their ability to provide steric stabilisation and strong interfacial layers is limited (Agboola et al., 1998). A minimum peptide size of 2 kDa was reported to be essential for good emulsifying and emulsion stabilising properties due to distinct hydrophilic and hydrophobic patches and a sufficient amount of network points to enable the formation of strong viscoelastic layers (Davis et al., 2005; Gauthier & Pouliot, 2003; van der Ven et al., 2001). However, when the degree of hydrolysis was limited, the emulsifying and emulsion stabilising properties of whey protein hydrolysates were improved (Caessens et al., 1999). Since a controlled enzymatic hydrolysis can increase the strength of the viscoelastic layers at the interface (see chapter 2.2), the use of these ingredients may also improve the physical stability of emulsions during the microencapsulation by spray-drying.

Due to the high susceptibility of lipids sensitive to autoxidation, stabilisation of the oil during the entire process is essential to minimise the extent of lipid oxidation, since the off-flavours resulting from the deterioration of the oil may lead to rejection of the product by the consumer (Siefarth, Serfert, Drusch, & Buettner, 2013). Accordingly, antioxidants are needed to protect the lipid against degradation and it was reported the most effective antioxidants are located at the interface, where lipid oxidation is initiated and oxidation reactions are promoted (McClements & Decker, 2000). Thus, emulsifiers used for the physical stabilisation of the feed emulsions during emulsification and spray-drying, which additionally possess antioxidant properties, are the ideal choice of wall material for the microencapsulation of lipids prone to autoxidation, since they also chemically stabilise the oil, acting as ingredients with dual functionality (Adjonu, Doran, Torley, & Agboola, 2014). It has been reported that WPI-stabilised emulsions containing corn oil exhibited a higher stability to lipid oxidation compared to those stabilised by soy protein isolate (Hu, McClements, & Decker, 2003b). However, casein-stabilised algal oil emulsions showed less hydroperoxide formation compared to WPI-stabilised formulations (Djordjevic, McClements, & Decker, 2006). Furthermore, β-LG showed the strongest retardation of lipid oxidation in emulsified salmon oil compared to WPI, α-LA and sweet whey (Hu, McClements, & Decker, 2003a). Since the bulk of antioxidant amino acids in β-LG are buried in the globular structure, an enzymatic hydrolysis can enhance their accessibility and thus enhance the antioxidant activity of the protein (Elias et al., 2008; Blanca Hernández-Ledesma et al., 2005). The potential of enzymatic hydrolysates of β-LG and WPI to stabilise sensitive lipids against autoxidation in liquid emulsions has been reported by many authors (Conway et al., 2013; Dryáková, Pihlanto, Marnila, Ůrda, & Korhonen, 2010; Elias et al., 2006, 2008; Kamau & Lu, 2011; Lin, Tian, Li, Cao, & Jiang, 2012; Peña-Ramos, Xiong, & Arteaga, 2004; Peng, Kong, Xia, & Liu, 2010; Peng, Xiong, & Kong, 2009; Zhang, Wu, Ling, & Lu, 2013). However, only in two studies so far the application of whey protein hydrolysates to stabilise spray-dried microencapsulated lipids prone to autoxidation was reported (Ramakrishnan et al., 2013, 2012). The experimental setup used in this work yielded microcapsules with a poor microencapsulation efficiency, which does not reflect the potential of these ingredients for microencapsulation.

A limited enzymatic hydrolysis of pea protein also improved the emulsifying and emulsion stabilising properties in comparison to the unmodified protein (Barac et al., 2011, 2012). Furthermore, the antioxidant properties of pea proteins were enhanced by enzymatic hydrolysis (Humiski & Aluko, 2007; López-Barrios et al., 2014; Pownall et al., 2010; Pownall, Udenigwe, & Aluko, 2011; X. Zhang et al., 2013). However, hydrolysed pea proteins have not been used for the microencapsulation lipids sensitive to autoxidation, despite their high suitability in this regard.

Another method to increase the physical and chemical stability of these lipophilic compounds during processing and storage is the addition of non-interfacially-active pectin

to the formulation. When the milieu conditions of the aqueous phase are adjusted correctly, complexes between the protein and the pectin can reinforce the interfacial layers (see chapter 2.2). Due to the formation of thicker interfacial layers (Katsuda, McClements, Miglioranza, & Decker, 2008) and the iron-chelating ability of pectin (Chen, McClements, & Decker, 2010), the oxidative stability of lipids in liquid emulsions was enhanced. Furthermore, an increase in the oxidative stability of spray-dried fish oil emulsions microencapsulated by β-LG/pectin-layers was also reported (Serfert et al., 2013).

3 Manuscript 1: Whey protein hydrolysates reduce autoxidation in microencapsulated long chain polyunsaturated fatty acids

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3.1 Abstract

The aim of the present study was to investigate the ability of partially hydrolysed whey proteins to microencapsulate fish oil. Microcapsules were produced by spray-drying emulsions, composed of fish oil, glucose syrup (DE38) and β-lactoglobulin or hydrolysates thereof prepared using bovine trypsin or alcalase. Hydrolysis did not negatively affect encapsulation performance during spray-drying and microencapsulation efficiency was very high for all samples (99±0.5 %). However, enzymatic hydrolysis resulted in reduced formation of lipid hydroperoxides for all hydrolysate-stabilised microcapsules, except for the hydrolysate produced by alcalase (3% degree of hydrolysis). Hydrolysis by trypsin resulted in a more narrow molecular weight peptide profile and an increased accessibility of antioxidant amino acids.

3.2 Introduction

The microencapsulation of food ingredients has been a constantly growing area of interest over the past number of decades. Sensitive, unstable compounds can be stabilised by entrapment inside a protective carbohydrate-based glassy matrix. Spray-drying, spray granulation or extrusion of emulsions are well established encapsulation methods.

A number of studies has been carried out over the last decade to identify the key factors for the structure formation, physical stability and functionality of microcapsules (Drusch, Regier, et al., 2012). Apart from the carbohydrate (Hogan, McNamee, O'Riordan, et al., 2001; Subramaniam et al., 2013), which is the bulk constituent of the capsule matrix, the type of emulsifier also has a significant impact on the physical structure of the microcapsules and their protective performance. The functional properties for the process of encapsulation must be considered. Small oil droplet size in feed emulsions was found to correlate with lower surface oil on microcapsules (Danviriyakul, McClements, Decker, Nawar, & Chinachoti, 2002; Soottitantawat et al., 2003). Furthermore, structural integrity of the interfacial layer must be maintained during processing in order to minimize non-encapsulated core material. On the other hand, antioxidant activity of the emulsifier may increase the protective performance, since the emulsifier is located at the site where lipid oxidation is initiated (McClements & Decker, 2000).

Milk proteins are frequently used for the microencapsulation of oil, due to their amphiphilic character resulting in good interfacial activity and emulsifying properties. Besides their use in stabilising emulsions, whey proteins have been used for the microencapsulation of lipophilic food ingredients (Fäldt & Bergenståhl, 1996; Hogan, McNamee, O'Riordan, et al., 2001; Partanen et al., 2008).

The microencapsulation efficiency of anhydrous milk fat by whey protein isolate was found poor, but could be significantly increased using lactose as additional wall material (Young, Sarda, & Rosenberg, 1993a). The ability of whey protein concentrate to encapsulate soybean oil was found rather low compared to sodium caseinate (Fäldt & Bergenståhl, 1996), despite efficiently stabilising emulsions (Hogan, McNamee, O'Riordan, et al., 2001). This might be attributed to their globular and rigid structure, their high molecular weight and the hydrophobic groups being buried in the interior of the molecule and thus limiting its functionality (Gauthier & Pouliot, 2003) as pronounced hydrophilic and hydrophobic regions are crucial for a good emulsifier (Turgeon et al., 1992). In contrast, smaller molecules possess higher rates of diffusion, thus a higher surface activity and less protein is needed to cover interfaces of the same size (Adjonu et al., 2014).

One possibility to alter the functional properties of milk proteins, e.g. whey proteins, is to perform enzymatic hydrolysis. To produce whey protein hydrolysates (WPH) with superior emulsification properties generally a low degree of hydrolysis is favourable. The functional properties of peptide mixtures arising from enzymatic hydrolysis depend on many factors, e.g. the type of enzyme used and the degree of hydrolysis (DH) achieved (Ipsen et al., 2001). An enzymatic hydrolysis of whey proteins results in a reduction of molecular weight and can effect the exposure of hydrophobic groups from the interior of the molecules (Tavano, 2013), hence increase the surface activity (Davis et al., 2005; Drusch, Hamann, et al., 2012; Tamm et al., 2012) and the area coverable compared to the native protein (O'Regan & Mulvihill, 2010) thereby increasing the stability against recoalescence (Walstra, 2003) and the viscoelasticity of interfacial films (Perez, Sanchez Carrera, et al., 2012; Tamm et al., 2012).

Apart from the ability of whey proteins to stabilise dispersed systems, they also possess bioactive properties, e.g. antioxidant activity, which can be enhanced by limited enzymatic hydrolysis (Elias et al., 2006, 2008; Blanca Hernández-Ledesma et al., 2005). This increase was attributed to an improved accessibility of antioxidative amino acids and the improved chelation of prooxidative transition metals, especially iron (Conway et al., 2013). In addition to chelation the antioxidative behaviour is exhibited by the scavenging of free radicals, the inactivation of reactive oxygen species (ROS) and the reduction of hydroperoxides (Elias et al., 2008). In native β-LG, many of the antioxidative amino acids like Cys, Trp and Met are buried in the interior of the molecule and hence poorly accessible for radicals (Elias, McClements, & Decker, 2005). Accordingly the authors propose an exposure of these areas by denaturation or limited hydrolysis to increase the antioxidative efficiency without loosing its capacity to chelate prooxidants like iron or copper. The antioxidant activity was ascribed to the free sulfhydryls due to faster oxidation than α-tocopherol (Tong, Sasaki, McClements, & Decker, 2000) or other amino acids (Elias et al., 2005) and high radical scavenging and iron-binding values as well as a significant oxidation of Met and Tyr in various peptides (Elias et al., 2006). As whey proteins and their hydrolysates possess antioxidant and emulsifying activity,

they have already been used to stabilise oil/water-emulsions (Djordjevic et al., 2006; Elias et al., 2006, 2005; Tong et al., 2000) acting as ingredients with dual functionality (Adjonu et al., 2014). Currently, there are only two studies so far using whey protein hydrolysates to produce microcapsules containing oxidation-prone ingredients like oil rich in polyunsaturated fatty acids (Ramakrishnan et al., 2013, 2012). However, due to the experimental setup a low microencapsulation efficiency of 50 % or below was reported and therefore these studies may not reflect the potential of whey protein hydrolysates in microencapsulation.

Aim of the present study was to investigate the ability of β-lactoglobulin and hydrolysates thereof to microencapsulate oil rich in polyunsaturated fatty acids in a glassy carbohydrate-based matrix by spray-drying. As hydrolysates have already shown to exhibit excellent antioxidative and emulsifying properties in o/w-emulsions, it is hypothesised they can act more efficiently as emulsifiers and antioxidants during the production and storage of corresponding microcapsules compared with those stabilised by the unmodified substrate. Characterisation of the proteins was performed by analysis of their molecular weight profile (SEC and SDS-PAGE), spray-dried carrier matrix particles and microcapsules were analysed regarding surface composition (XPS). Additionally the extractable oil content of the microcapsules was determined and lipid oxidation was monitored by development of the hydroperoxide content.

3.3 Materials and methods

3.3.1 Materials

Refined fish oil (Omevital 18/12 TG Gold) was purchased from BASF Personal Care and Nutrition GmbH, Illertissen, Germany. The oil contained 14 % docosahexaenoic acid and 21 % eicosapentaenoic acid. β-lactoglobulin (β-LG, Davisco Foods International Inc., Le Sueur, USA) was used as emulsifier and enzymatically modified. For enzymatic hydrolysis trypsin (from bovine pancreas, cat# T8003, EC: 3.4.21.4, 12238 BAEE units / mg protein) and a serine protease (alcalase 2.4L, cat# P4860, EC: 3.4.21.62, 2.59 AU/g protein) both purchased from Sigma Aldrich, Taufkirchen, Germany, were used. Glucose syrup (DE38, C*Dry 01934) was kindly provided from Cargill Deutschland GmbH, Krefeld, Germany. All chemicals were of reagent grade.

3.3.2 Enzymatic hydrolysis of β-LG

Solutions containing 7 wt% β-LG were prepared with distilled water the day before hydrolysis and stirred for at least 12 hours to ensure complete rehydration of the protein. 1 wt% Trypsin was dissolved in 1 mM HCl and alcalase was diluted 1:50 (w/w) prior to addition to the substrate solution. Hydrolysis for both enzymes was conducted at 45 °C and pH 8.0 using the pH-stat-method of Adler-Nissen (Adler-Nissen, 1986) and a h_{tot}-value of 7.2 meqv/g of protein for the total number of peptide bonds in the protein substrate (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010). The degree of hydrolysis (DH) was calculated according to (Adler-Nissen, 1986) as follows:

$$\mathrm{DH} = \frac{B \cdot N_B \cdot 100\%}{\alpha \cdot m_P \cdot h_{tot}}$$

where B = base consumption, N_b = normality of the base, α = average degree of dissociation of the α-NH groups (0.863), m_P = mass of protein, h_{tot} = total number of peptide bonds in the substrate (meqv/g protein). The substrate solution was allowed to equilibrate before addition of the enzyme preparation, the pH was adjusted to 8.0 with 0.1 M NaOH using an autotitrator (Titrando 902) controlled with tiamo software (both Metrohm AG, Filderstadt, Germany). Subsequently the enzyme preparation was added at an enzyme/substrate-ratio of 1:800 (DH6) or 1:1600 (DH3) for trypsin and a diluted enzyme preparation/substrate-ratio of 1:400 for alcalase, both with respect to the protein content of the substrate solution. Monitoring and control of pH was carried out dosing 0.1 M NaOH with the autotitrator mentioned before. The whole experimental setup was similar to that described elsewhere (Cheison et al., 2010). Once the DH was reached (i.e. 3.0 or 6.0 %), as determined by NaOH consumption, the reaction mixture was heated to 75 °C for 30 min, which successfully terminated all enzymatic activity. Samples for the determination of the molecular weight profile were taken after hydrolysis and frozen until analysis. Emulsion preparation and microencapsulation of the fish oil were carried out straight after hydrolysis. Throughout the manuscript the hydrolysates will be referred to as DH3(T) / DH6(T) for those produced with trypsin and DH3(A) / DH6(A) for those produced using alcalase, with the numbers indicating the degree of hydrolysis of the protein. β-LG(3) or β-LG(6) correspond to the substrate used in each data set.

3.3.3 Molecular weight distribution of β-LG and its hydrolysates

Tris-Tricine SDS-PAGE

To characterise the impact of the enzymatic treatment on the peptide composition of the hydrolysates, a tris-tricine SDS-PAGE under reducing conditions was conducted as already published (Schägger & Von Jagow, 1987) using slight modifications. This SDS-PAGE system is especially suitable to detect peptides smaller 10 kDa (Schägger & Von Jagow, 1987), which is expected to be the bulk in the analysed hydrolysates. Samples were diluted with tris-tricine sample buffer (Bio-Rad Laboratories GmbH, München, Germany), spiked with 1 M DL-dithiothreitol (DTT) solution (Sigma) as reducing agent and heated to 90 °C for 5 min. In the next step the samples were loaded on a 16.5 % tris-tricine gel (cat# 345-0065) and separated using tris-tricine running buffer (cat# 161-0744), both Bio-Rad, performing under running conditions as advised by the manufacturer. Gels were fixed in aqueous methanol/acetic acid (40/10 %), stained with 0.025 % Coomassie brilliant blue G250 (Serva Electrophoresis GmbH, Heidelberg, Germany) in 10 % acetic acid and decolourised in 10 % acetic acid. The molecular weight (MW) marker used was a commercial mixture of seven polypeptides (cat#MWSDS17S (Sigma Aldrich, Taufkirchen, Germany)), covering the range between 2.5 and 17 kDa.

Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) was carried out using a TSKgel G2000SW column (7.5mm ID × 60cm, particle size 10μm, Tosh Biosciences LLC, King of Prussia, PA, USA) with a TSKgel CW guard column ((7.5mm ID × 60cm, particle size 10μm). An isocratic elution of 30 % acetonitrile containing 0.1 % TFA (v/v) was used at 1.0 mL/min. Elution was monitored with UV absorbance at 214 nm. The samples were diluted in water and 20 µL of 1 g/L protein/peptide solutions were injected onto the columns. Bovine serum albumin (66.7kDa), β-lactoglobulin (18.4kDa), α-lactalbumin (14.2kDa), cytochrome C (12.0kDa), bacitracin (1,422Da), Leu-Trp-Met-Arg (605Da) and Asp-Glu (262Da) were used as molecular weight standards (all Sigma Aldrich, Dublin, Ireland). Samples and standards were pre-filtered through 0.22 µm low protein binding membrane filters (Sartorius Stedim, Surrey, UK) prior to application to the column. All solvents were filtered under vacuum through 0.45 µm high velocity filters (Millipore Ltd., Durham, UK).

3.3.4 Emulsion preparation

All water-soluble ingredients were dissolved in distilled water. A coarse emulsion was prepared by shear homogenisation. Subsequently the emulsion was further homogenised using a high-pressure homogeniser (Panda 2K; Niro Soavi Deutschland, Lübeck, Germany) at a pressure range of 250/50 bar and applying 2 passes. The final emulsion contained 2.2 wt% unmodified or hydrolysed β-LG, 14.5 wt% fish oil and 29.5 wt% glucose syrup summing up to a dry matter content of 46.2 wt%.

3.3.5 Spray-drying of carrier matrix particles and emulsions for the microencapsulation of fish oil

Microencapsulation of the fish oil was performed by spray-drying the emulsions on a pilot plant spray-dryer (Mobile Minor 2000, Niro A/S, Copenhagen, Denmark) at 180/70 °C inlet/outlet temperature and 4 bar with rotary atomization (22.000 rpm). For the analysis of the surface accumulation of β-LG and its hydrolysates on the surface of matrix particles, protein solutions without fish oil were prepared and spray-dried under the same conditions as mentioned above. The ratio of protein : glucose syrup was kept constant as in the microcapsules with 0.08.

3.3.6 Determination of the oil droplet size distribution of fresh and reconstituted spray-dried emulsions

Oil droplet sizes (ODS) before and after spray-drying (i.e. reconstituted in the instrument using destilled water) were analysed by laser diffraction based on mie scattering using a LA-950 (Horiba Jobin Yvon GmbH, Unterhaching, Germany). Within the instrument the emulsions were adjusted to optimum transmission before measurement. The results of the volume distribution are presented as the 10th, 50th and 90th percentile of the oil droplets and the width of the distribution is expressed as the span value. All results shown were obtained from at least two replicates using a refractive index of 1.45 and 1.33 for fish oil and pure water, respectively. These values have been determined experimentally in an earlier study from our research group.

3.3.7 Analysis of the surface composition of spray-dried carrier matrix particles by X-ray photoelectron spectroscopy (XPS)

XPS provides information on the elemental composition of a surface. For the XPS measurements the spray-dried carrier matrix particles were stuck on a sample holder by an electrically conductive double-sided adhesive carbon based Leit Tab and analysed by an XPS spectrometer (Full-Lab Omicron) using an Al K X-ray source (1486.6 eV). The spectrometer energy scale was calibrated with respect to the C 1s (C-C, C-H) component set at 285 eV. For data fitting CASA XPS software package was used. After appropriate Shirley background removal a Marquardt peak-fitting procedure was applied using Gaussian-Lorentzian curves.

3.3.8 Determination of the extractable oil content as a degree of microencapsulation efficiency

The non-encapsulated fish oil in microcapsules produced by spray-drying of emulsions is mainly located at the particle surface or in droplets located close to the surface (Drusch & Berg, 2008). To evaluate the success of encapsulation of the fish oil, the extractable oil content was analysed (Westergaard, 2004). The method is based on gravimetrical determination of the fat after its extraction with petrol ether. Results are presented as extractable fat with respect to the total calculated fat content of the powder.

3.3.9 Determination of the hydroperoxide content of fish oil in spray-dried and reconstituted emulsions

The stability of the microencapsulated fish oil was observed by analysing the hydroperoxide content over a period of 11 weeks. Samples were stored in the dark in desiccators over a saturated solution of magnesium chloride (33 % relative humidity) at room temperature.

To extract the fish oil from microcapsules a blend of 2-propanol/isooctane was used after redissolving an aliquot of the powder in water. The hydroperoxide content as a measure of lipid oxidation was analysed using a well established method (International Dairy Federation, 1991) for the determination of the peroxide value in anhydrous milk fat with slight modifications (Drusch, Serfert, et al., 2012). The extracted oil was diluted with 2-propanol and incubated at 60 °C for 30 min after adding iron-II-chloride and ammonium thiocyanate solution. Finally the extinction was read at 485 nm. A calibration curve was prepared using iron-III-chloride. Hydroperoxide content was calculated from the extinction:

$$Hydroperoxide\ content\ [mmol/kg\ oil] = \frac{Fe(III)[\mu g]}{m[g] * 55.84}$$

with Fe(III) as the iron content of the sample as calculated from the calibration curve and m as the mass of the oil.

The individual samples were stored in two independent desiccators and analysed in triplicate weekly.

3.3.10 Statistical analysis

The oil droplet size distribution of fresh and reconstituted spray-dried emulsions was analysed using Design Expert 7.0.0 (Stat Ease Inc., Minneapolis, USA). A general factorial design was created using the factors “enzyme type”, “degree of hydrolysis” and “spray-drying” (before and after the process). All significant differences reported are based on a probability value of $p < 0.05$.

3.4 Results and discussion

3.4.1 Molecular weight distribution of β-LG and its hydrolysates analysed by SDS-PAGE and size exclusion chromatography (SEC)

As shown in fig. 3.1, hydrolysis resulted in a significant alteration of the molecular weight distribution for all hydrolysates. The substrate showed the most intensive band for β-LG at around 18 kDa, but also light bands for fractions of higher molecular weight were visible, the smallest most likely were dimers of β-LG and α-LA (Monahan, German, & Kinsella, 1995). Data from the literature indicate that the detected bands can be attributed to fragments of the milk fat globule membrane protein, butyrophilin (Cheison, Leeb, Toro-Sierra, & Kulozik, 2011). Other whey proteins like bovine serum albumin and immunoglobulins are susceptible to alcalase and trypsin hydrolysis.

Hydrolysates produced with trypsin showed intensive bands between 6.2 and 8.2 kDa by SDS-PAGE (fig. 3.1). This can be attributed to a peptide fraction resulting from enzymatic hydrolysis of β-LG with a MW of 8,332 Da and these bands were present in both DH, as it is among the first peptides released during β-LG-hydrolysis using trypsin

(Fernández & Riera, 2013). Trypsin is known to prefer lysine and arginine residues as cleavage sites, which results in 18 scissile bonds within the primary structure of β-LG (Cheison et al., 2010). Additionally, under the conditions chosen, trypsin also shows chymotrypsin-like behaviour, cleaving peptide bonds involving bulky, hydrophobic amino acids (Cheison et al., 2010). According to Fernández & Riera (Fernández & Riera, 2013) the peptides between 6 and 8 kDa can be ex-plained by different stages occurring during hydrolysis: initially peptides from the N- and C-terminal areas of the intact substrate are released during hydrolysis. Due to a relative resistance of the internal molecular core to trypsin digestion (Fernández & Riera, 2013), intermediary peptides (like those between 6 and 8 kDa), resulting from initial hydrolysis, are not cleaved further before most of the intact β-LG is degraded. This hypothesis is supported by several bands found in DH3(T) with a molecular weight of 14 kDa or larger (most likely remaining intact β-LG and intermediary peptides of it).

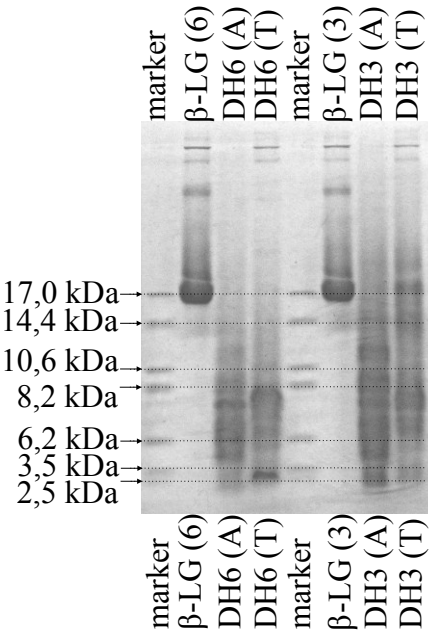

Figure 3.1 SDS–PAGE under reducing conditions of β-lactoglobulin (β-LG) and hydrolysates thereof prepared by trypsin (DH3(T) or DH6(T)) and alcalase (DH3(A) or DH6(A)). Numbers in sample names indicate the degree of hydrolysis.

These bands are not visible in DH6(T) and another intensive band between 2.5 and 3.5 kDa appeared instead which could be attributed to a peptide fraction possessing a MW of 3,255 Da (Fernández & Riera, 2013). At this stage of the hydrolysis intermediary peptides are cleaved to a larger extent. The observations mentioned afore are sup-

ported by the SEC results (see fig. 3.2). The peptides >10 kDa showed a strong decrease from DH 3 to 6, whereas the fractions between 2-5 and 5-10 kDa increased less.

In contrast to trypsin, the alcalase enzyme preparation produced by B. licheniformis, mainly consisting of Subtilisin Carlsberg, shows a lower specificity, with a preference to cleave at aromatic amino acid residues and at the sulfur-containing residue Met (Doucet, Otter, et al., 2003). In addition to the major enzyme, alcalase also contains further proteases of minor amount, (Doucet, Otter, et al., 2003; Svendsen & Breddam, 1992), which results in even more favoured cleavage sites within β-LG. Hydrolysates produced using alcalase exhibited a variety of peptides being smaller than 14.4 kDa, within the range of 3.5 and 8.2 kDa showing a similar intensity for both DH (fig. 3.1).

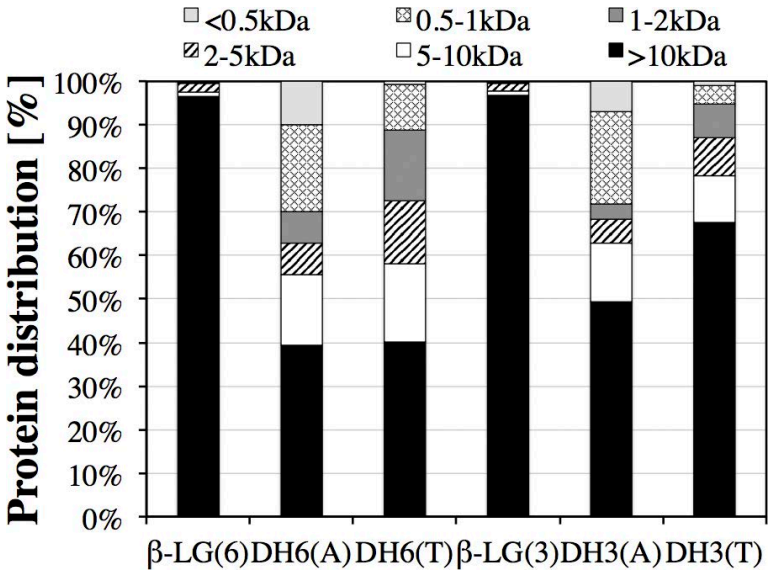

Figure 3.2 Molecular weight distribution of proteins and peptides as analysed by size exclusion chromatography; β-lactoglobulin (β-LG) and hydrolysates thereof prepared by trypsin (DH3(T) or DH6(T)) and alcalase (DH3(A) or DH6(A)). Numbers in sample names indicate the degree of hydrolysis.

Accordingly only a slight increase for the fraction of 2-10 kDa was found with increasing DH in SEC results (see fig. 3.2). Visible bands with higher or lower molecular weight than the afore mentioned diminished from DH3(A) to DH6(A), most likely also due to the relative cleaving resistance as already discussed for hydrolysates produced with trypsin (Fernández & Riera, 2013). The effect could be even more pronounced than for tryptic hydrolysates as indicated by an increased amount of peptides < 2 kDa detected by SEC analysis of the hydrolysates (see fig. 3.2). SEC results indicate that all hydrolysates contained peptides with a MW below the detection limit of the gel and did not appear as bands accordingly as they were not retained by the gel network.

3.4.2 Oil droplet size distribution of fresh and reconstituted spray-dried emulsions

The oil droplet size distribution (ODS) in emulsions before and after spray-drying is shown in tab. 3.1. All proteins efficiently stabilised the oil droplets during emulsification, atomisation and drying with a 90th percentile below 2 μm in all emulsions.

Table 3.1 Oil droplet size distribution of fish oil emulsions (fresh and reconstituted after spray-drying) stabilised with β-lactoglobulin (β-LG) and hydrolysates thereof prepared by different enzymes (alcalase or trypsin)

Sample	Percentile of oil droplet size [µm]			Span
	10th	50th	90th	
Liquid emulsions before spray-drying				
β-LG(3)	0.43 ± 0.01	0.94 ± 0.00	1.54 ± 0.03	1.13
DH3(A)	0.48 ± 0.12	0.98 ± 0.10	1.68 ± 0.09	1.22
DH3(T)	0.41 ± 0.00	0.90 ± 0.01	1.47 ± 0.02	1.17
β-LG(6)	0.56 ± 0.05	1.04 ± 0.03	1.64 ± 0.03	1.03
DH6(A)	0.32 ± 0.02	0.81 ± 0.03	1.58 ± 0.07	1.55
DH6(T)	0.35 ± 0.01	0.83 ± 0.01	1.40 ± 0.01	1.27
Reconstituted emulsions after spray-drying				
β-LG(3)	0.44 ± 0.02	0.94 ± 0.02	1.51 ± 0.03	1.14
DH3(A)	0.54 ± 0.02	1.08 ± 0.04	1.95 ± 0.16	1.30
DH3(T)	0.41 ± 0.00	0.89 ± 0.01	1.46 ± 0.01	1.18
β-LG(6)	0.61 ± 0.02	1.08 ± 0.01	1.71 ± 0.00	1.02
DH6(A)	0.31 ± 0.01	0.80 ± 0.04	1.81 ± 0.06	1.87
DH6(T)	0.39 ± 0.03	0.87 ± 0.03	1.48 ± 0.01	1.25

However, with respect to the stability during atomisation and drying slight changes in the oil droplet size distribution (90th percentile) occurred in emulsions containing β-LG and tryptic hydrolysates (maximum increase: 0.08 µm for DH6(T)), whereas emulsions containing hydrolysates produced by alcalase showed a significant increase in the 90th percentile of the oil droplet size distribution (maximum increase: 0.27 µm for DH3(A)). This could be attributed to the increased amount of peptides < 2 kDa in these hydrolysates. Several authors hypothesised this to be the minimum molecular weight to exhibit good functionality regarding the formation and stabilisation of foams and emulsions (Turgeon et al., 1992; van der Ven et al., 2001) if the peptide additionally possesses pronounced hydrophobic and hydrophilic areas (Turgeon et al., 1992). Thus the higher content of fractions < 2 kDa found in hydrolysates produced with alcalase compared to those made with trypsin and β-LG (see fig. 3.2) might explain the alteration in functionality.

3.4.3 Surface composition of spray-dried carrier matrix particles and microcapsules and microencapsulation efficiency of spray-dried microcapsules

The surface composition of the carrier matrix particles and DH3 microcapsules as analysed by X-ray photoelectron spectroscopy is presented in tab. 3.2. Comparing the DH3 and DH6 data sets shows a noticeable difference in the surface composition. From DH3 to DH6 an increase of the nitrogen content at the surface of the matrix particles can be observed, being more pronounced for hydrolysates produced with trypsin. This can be attributed to the reduction of peptide size resulting in a higher surface activity. As the lifetime of a droplet during spray-drying is very short, the emulsifiers surface adsorption is assumed to be mainly diffusion-controlled (Landström, Alsins, & Bergenståhl, 2000). Thus an increased content of small peptides in the hydrolysates results in higher nitrogen content on the particle surface. Hence, the highest nitrogen content on the surface of all particles containing hydrolysates produced by alcalase can be explained by the highest amount of small peptides in the formulation.

Table 3.2 Properties of spray-dried carrier matrix particles containing β-lactoglobulin (β-LG) or hydrolysates thereof prepared by different enzymes (alcalase or trypsin) and glucose syrup (DE38)

Sample	Surface composition of powder particles			Surface coverage [%]
	C [%]	O [%]	N [%]	
β-LG(3)*	74.8 ± 0.4	22.6 ± 0.3	2.6 ± 0.1	16.6
DH3(A)*	74.9 ± 0.4	22.0 ± 0.3	3.1 ± 0.1	19.8
DH3(T)*	74.4 ± 0.4	22.8 ± 0.3	2.8 ± 0.1	17.9
β-LG(3)	56.5 ± 0.3	37.4 ± 0.3	6.1 ± 0.1	38.9
DH3(A)	58.4 ± 0.3	35.3 ± 0.3	6.3 ± 0.1	40.2
DH3(T)	56.9 ± 0.3	38.7 ± 0.3	4.4 ± 0.1	28.1
β-LG(6)	59.1 ± 0.3	35.0 ± 0.3	5.9 ± 0.1	37.6
DH6(A)	59.0 ± 0.3	34.3 ± 0.3	6.7 ± 0.1	42.7
DH6(T)	60.7 ± 0.3	33.8 ± 0.3	5.5 ± 0.1	35.1

[a]Spray-dried microcapsules containing fish oil and the matrix components mentioned before.

The lowest nitrogen content for all matrix particle surfaces was detected for formulations containing hydrolysates made with trypsin. This could be due to a higher content of peptides with higher MW compared to DH3/DH6(A) matrix particles possibly exhibiting stronger peptide-carbohydrate interactions with the other matrix component (Soltanizadeh et al., 2014). The relatively high nitrogen content at the surface of β-LG stabilised matrix particles could be due to the intact globular structure possessing less possibilities for interaction with the carbohydrates and thus less retention inside the matrix during spray-drying. Surface coverage was not high enough to provide an "internal coating" as it was observed in other studies on the encapsulation of proteins using hy-

droxypropyl methylcellulose for surface modification (Elversson & Millqvist-Fureby, 2006). In contrast it is described in the literature that protein at the particle surface, especially during ageing of the particle, can adversely affect the solubility (Scheidegger et al., 2013). These effects are not likely to be pronounced at the present level of surface accumulation.

In contrast to carrier matrix particles spray-dried microcapsules showed an altered surface composition due to the fish oil being additionally present in the formulation. This resulted in a decrease of nitrogen on the surface. The observed change in surface composition can be attributed to a thin film of fish oil present at the particle surface (Jafari, Assadpoor, Bhandari, & He, 2008). Additionally the peptides are bound at the oil/water-interface in microcapsules compared to matrix particles, resulting in a decrease of protein present at the particle surface for microcapsules.

The extractable oil content of the spray-dried microcapsules ranged between 0.5 and 1.4 % and represents a high microencapsulation efficiency (ME) of 98.7 to 99.5 % for all proteins used as emulsifiers. The high ME could partly be attributed to the use of glucose syrup with a DE of 38, as a positive relationship was found between increasing DE and increased ME (Hogan, McNamee, O'Riordan, et al., 2001). The authors ascribed this to the formation of a less porous, more uniform matrix due to the smaller oligosaccharides. The changes in ODS as described above were not distinct enough to negatively affect ME as described in other studies (Danviriyakul et al., 2002; Soottitantawat et al., 2003).

3.4.4 Lipid oxidation of spray-dried emulsions

In fig. 3.3A lipid oxidation in microcapsules stabilised with DH3(A) and DH3(T) are compared with those stabilised with unmodified β-LG. For the first two weeks of storage the hydroperoxide content of all samples only slightly increased, in the following DH3(T) contents increased slower than β-LG and DH3(A), whereas the latter showed a similar progression. After 11 weeks of storage DH3(A) and β-LG samples exhibited final hydroperoxide contents of 131 ± 10.3 and 113 ± 6.0 mmol/kg oil in contrast to DH3(T) containing only 54.8 ± 7.7 mmol/kg oil.

Various publications in recent years have focussed on the impact of the physical structure of the microcapsules on the rate of lipid oxidation in the encapsulated oil (Bae & Lee, 2008; Drusch & Berg, 2008; Drusch, Regier, et al., 2012; Drusch, Serfert, Scampicchio, Schmidt-Hansberg, & Schwarz, 2007; Serfert, Drusch, Schmidt-Hansberg, Kind, & Schwarz, 2009). Microencapsulation efficiency may affect the rate of lipid oxidation (Drusch & Berg, 2008; Serfert et al., 2013), but was very similar in all samples in the present study. Free volume elements negatively affect lipid oxidation,

since they determine oxygen permeation within the matrix (Drusch, Regier, et al., 2012). Therefore this factor cannot explain the differences in oxidative stability observed in the present study.

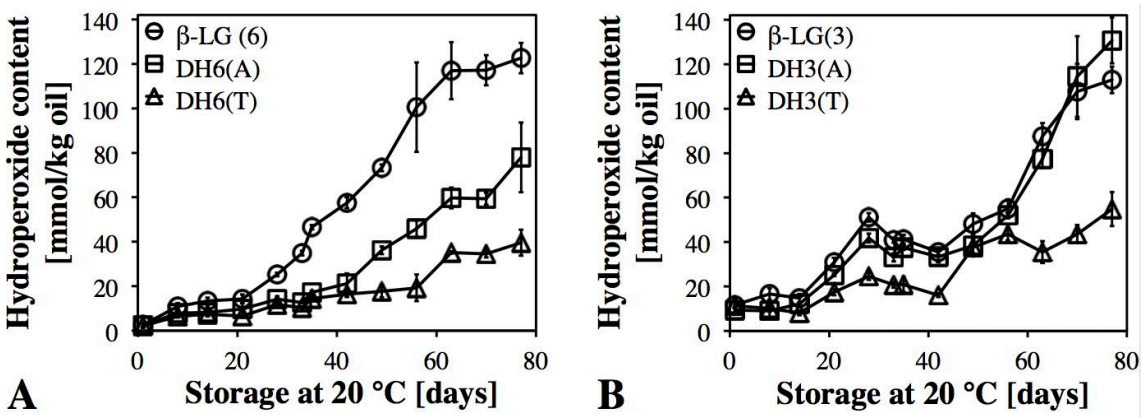

Figure 3.3 Development of hydroperoxide content in fish oil-loaded microcapsules stabilised with β-lactoglobulin (β-LG) or hydrolysates of β-LG prepared with trypsin (T) or alcalase (A), stored at room temperature (and 33%rh). A: unmodified b-LG and samples with a degree of hydrolysis of 6 %, B: unmodified β-LG and samples with a degree of hydrolysis of 3 %.

Another important issue is the chemical stabilisation of the encapsulated oil. It is well known that for the characterisation of the efficiency of antioxidants via in vitro assays several protocols need to be used and to be adapted to the specific food matrix to provide reliable results. Compounds acting as effective antioxidants in liquid emulsions do not necessarily stabilise these emulsions after conversion into microcapsules by spray-drying (Serfert, Drusch, & Schwarz, 2009). Another work from our research group recently did not find a correlation between the antioxidant activity of fish protein hydrolysates tested in vitro using three different assays and the formation of hydroperoxides in spray-dried fish oil emulsions stabilised with these hydrolysates. Accordingly the antioxidant properties of the whey protein hydrolysates by in vitro assays were not determined but their possible mechanisms of action will be discussed in the following section.

β-LG and its hydrolysates were found to possess antioxidative capacity (i.e. scavenging of free radicals) and the capability to chelate prooxidative metals like copper or iron (Elias et al., 2008). In liquid emulsions, hydrolysed whey proteins stabilise emulsified oils rich in polyunsaturated fatty acids (Elias et al., 2006, 2008; Lin et al., 2012). Due to their surface activity and their role as emulsifier the peptides are located at the oil-water interface, where lipid oxidation is initiated (McClements & Decker, 2000). Since the peptides remain at the interface in the dried particle, they also stabilise long chain poly-

unsaturated fatty acids in spray-dried emulsions efficiently (Moreau & Rosenberg, 1996), decomposing lipid hydroperoxides to the non-reactive lipid hydroxides by methionine, scavenge radicals by amino acid oxidation and hindering substrate prooxidant interactions by forming a protective layer around the oil droplets (Elias et al., 2008).

To explain the difference in the oxidative stability between spray-dried emulsions containing protein hydrolysates prepared by different enzymes, a more detailed discussion of the antioxidative properties of resulting peptides is necessary. The major contribution to the antioxidant activity of β-lactoglobulin was ascribed to Trp and Tyr, induced by the capacity of their indolic and phenolic groups to donate hydrogen (Hernández-Ledesma, Recio, & Amigo, 2008). Due to a faster oxidation of free Cys and Trp in β-LG compared to oxidation of the emulsified menhaden oil the authors attributed antioxidant properties to the two amino acids. In contrast, no involvement of Met in free radical scavenging could be found, most likely prevented by the tertiary structure of the protein (Elias et al., 2005). Apart from individual amino acids, peptide fractions of β-LG can also exhibit high antioxidant activity, e.g. Trp-Tyr-Ser- Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile (a product of β-LG hydrolysis) showed a higher ORAC-value than the frequently used synthetic antioxidant BHA (Hernández-Ledesma et al., 2005). Other authors found Tyr20 and Met24, also present in the fraction mentioned above, to be significantly oxidised after 4 days of storage in o/w-emulsions accompanied with a higher peroxyl radical scavenging and iron binding ability of the related β-LG-hydrolysates (Elias et al., 2006). The metal chelation capacity depends on the amount and accessibility of negatively charged amino acid residues to bind to the positively charged metal ions (Elias et al., 2008). As the ability to chelate metal ions depends on pH, it is important to mention that groups being involved in chelation processes by providing electrons (e.g. COOH, CONH and NH2) remain charged at pH above neutral (Zhou et al., 2012).

Since unmodified β-LG and its hydrolysates have an identical amino acid pattern, i.e. the same amount of antioxidant amino acids within the primary structure (Elias et al., 2006), the substrate and the peptide mixtures resulting from hydrolysis primarily differ in the reduction or even complete loss of their tertiary structure (Elias et al., 2008). Accordingly an alteration in the accessibility of antioxidant amino acids, initially being buried in the interior of the molecule, for ROS and metal ions results for the hydrolysates due to their exposure by hydrolysis. This accounts for the free Cys121 (H. C. Liu, Chen, & Mao, 2007) as well as for Trp, Tyr and Met (Hernández-Ledesma et al., 2005) being exposed by enzymatic hydrolysis using trypsin. It is hypothesised that for DH3(T) the enzymatic hydrolysis resulted in an increase of the antioxidant capacity, as the stabilised microcapsules exhibited less than half the hydroperoxide contents as microcapsules based on β-LG and DH3(A) after the entire storage period (see fig. 3.3A).

The course of hydroperoxide formation in fish oil microcapsules stabilised with β-LG hydrolysates with DH6 and unmodified β-LG is presented in fig. 3.3B. After 3 weeks of storage the spray-dried emulsion stabilised with β-LG showed a faster increase in hydroperoxide content compared to samples based on hydrolysed protein, which exhibited similar contents up to 7 weeks of storage. Subsequently the generation of hydroperoxides in sample DH6(A) exhibited a rise in contrast to DH6(T). The final values in hydroperoxide contents after 11 weeks of storage accounted for 122.6 ± 6.8, 78.0 ± 15.7 and 39.6 ± 5.9 mmol/kg oil for β-LG, DH6(A) and DH6(T), respectively. Accordingly it can be assumed a significant amount of antioxidant amino acids are being exposed to the lipid droplets due to an increase in the degree of hydrolysis from DH3(A) to DH6(A).

Comparing the data for emulsions stabilised with protein hydrolysate of DH3(T) and DH6(T) no major difference in the course of lipid oxidation in emulsions stabilised with hydrolysates produced with trypsin occurred. This can be explained by the specific cleavage of trypsin within the β-LG molecule. As less cleavage sites are available compared to alcalase, most likely the bulk of amino acids with antioxidant activity from the interior of the substrate is already exposed at DH3.

In contrast hydrolysates produced by alcalase revealed the most pronounced differences with DH3 showing an almost twofold hydroperoxide content after the entire storage period than DH6. It can be assumed that advanced hydrolysis at DH6 significantly increased the amount of accessible antioxidant amino acids. As alcalase is known to prefer cleavage sites next to Trp, Tyr and Phe (Hernández-Ledesma et al., 2008), peptides with a highly antioxidative character can result from hydrolysis. Unfortunately most of these AA are located in the interior of the unmodified β-LG molecule, being inaccessible for the enzyme. Additionally as a consequence of the high amount of scissile bonds available, the probability of cleaving in the outer molecule areas is higher than in the inner parts. This is supported by the molecular weight profile of DH3(A), possessing peptides over the entire range between 2.5 and 17 kDa (fig. 3.1). As the AA-sequence mentioned before (Hernández-Ledesma et al., 2005) contains 9 possible cleavage sites of alcalase it is most unlikely to be found in hydrolysates produced using this enzyme preparation. Due to the reported aggregation potential of peptides < 2 kDa re-sulting from alcalase hydrolysis (see fig. 3.1) (Doucet, Gauthier, et al., 2003), a masking of the antioxidative sequences can result in increased hydroperoxide contents compared to the other microcapsules stabilised by all other hydrolysates and unmodified β-LG. Due to the advanced hydrolysis in DH6(A) a significantly lower hydroperoxide content could be observed for the corresponding microcapsules, as antioxidative AA-sequence can stabilise the encapsulated oil, despite the aggregating character likely still present at this DH.

3.5 Conclusions

The enzymatic hydrolysis of β-LG affects the stability of fish oil microencapsulated by spray-drying. Based on available literature, we propose that hydrolysis results in an improved accessibility of amino acid residues with antioxidant properties and hence an increased stability of the encapsulated oil. The low degree of hydrolysis applied (up to 6.0 %) did not negatively affect encapsulation performance during spray-drying. Despite limited changes in oil droplet sizes of emulsions stabilised by hydrolysates produced with alcalase, all microcapsules showed a high microencapsulation efficiency of 99 ± 0.5 %. The formation of lipid hydroperoxides was found to be reduced for hydrolysate-stabilised microcapsules except for DH3(A). The significantly reduced lipid oxidation of microencapsulated fish oil stabilised by hydrolysates produced with trypsin was attributed to the enzyme specificity, exhibiting considerably less cleavage options compared to alcalase. Hence this resulted in a narrower molecular weight peptide profile and an increased accessibility of antioxidant amino acids. Future work will expand the experimental approach to other types of dispersed systems.

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4 Manuscript 2: Functional and antioxidant properties of whey protein hydrolysate/pectin complexes in emulsions and spray-dried microcapsules

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4.1 Abstract

The aim of the present study was to investigate the potential of partially hydrolysed whey proteins to microencapsulate fish oil by spray-drying bilayer feed emulsions containing pectin, an oppositely charged biopolymer. Microcapsules were composed of fish oil, β-lactoglobulin (β-LG) or hydrolysates thereof produced by trypsin (DH6) and glucose syrup (DE38) as the matrix component. Pectin was attached to protein single-layer emulsions via electrostatic interactions using the layer-by-layer technique at pH 4.0. All emulsions exhibited good process stability during atomisation and drying as indicated by oil droplet size distribution and accordingly the microencapsulation efficiency was high (≥ 95.2 %) for all samples. The hydroperoxide formation in fish oil microcapsules prepared from single-layer feed emulsions was reduced in the presence of DH6 due to increased accessibility of antioxidant amino acids. The attachment of pectin to protein- or peptide-stabilised single-layer emulsions yielded bilayer microcapsules with increased stability (lower hydroperoxide content during storage). Increased thickness of the interfacial layer and the immobilisation of prooxidative ions by pectin may add to the antioxidative effect of the hydrolysate.

4.2 Introduction

The stabilisation of emulsions by multiple layers of oppositely charged biopolymers has received increasing attention in the last decade. The formation of strong electrostatic complexes between proteins and anionic polysaccharides results from their positive and negative charge below the isoelectric point of the protein (Rodríguez Patino & Pilosof, 2011). Bilayer emulsions prepared by the layer-by-layer technique, which involves consecutive adsorption of a second biopolymer component to oppositely charged primary emulsion droplets, mostly exhibit increased physical stability compared to single layer emulsions (Güzey & McClements, 2006; Serfert et al., 2013).

Several factors affect the stability of lipids prone to oxidation in complex food matrices and the physical and chemical composition of the interface in an o/w emulsion plays a key role in the prevention of autoxidation. β-LG has been used for the stabilisation of emulsions, due to its antioxidant activity (Elias et al., 2006). Partial enzymatic hydrolysis may enhance the antioxidant effect of β-LG, since many antioxidant amino acids are buried within its native globular structure. Besides their stabilisation of sensitive lipophilic ingredients in liquid emulsions (Elias et al., 2006), β-LG hydrolysates also retarded lipid oxidation in spray-dried emulsions (Tamm et al., 2015). Electrostatic attachment of pectin to primary emulsion droplets stabilised with proteins (e.g. β-LG) yields a thicker physical barrier to hinder metal-lipid interactions and furthermore pectin is

known to chelate prooxidative metals (Chen et al., 2010). Accordingly, interfacial complexes of β-LG/pectin may reduce the formation of hydroperoxides in fish oil microencapsulated by spray-drying (Serfert et al., 2013).

Aim of the present study was to investigate the potential of bilayer-stabilised emulsions prepared from partially hydrolysed β-LG and pectin for the microencapsulation of lipophilic bioactive ingredients. β-LG and its hydrolysates were characterised by their peptide distribution (SEC and SDS-PAGE). Physical properties of liquid emulsions (oil droplet size distribution and ζ-potential), emulsion stability during spray-drying and subsequent stability of the encapsulated fish oil were examined. The latter includes microencapsulation efficiency and hydroperoxide formation.

4.3 Material and methods

4.3.1 Materials

Refined fish oil (Omevital 18/12 TG Gold, 12% docosahexaenoic acid, 21% eicosapentaenoic acid) was purchased from BASF Personal Care and Nutrition GmbH, Illertissen, Germany. β-lactoglobulin (β-LG, Davisco Foods International Inc., Le Sueur, USA) was used as cationic emulsifier at pH 4.0 unmodified and enzymatically hydrolysed. Enzymatic hydrolysis was conducted with trypsin (cat#T8003, EC:3.4.21.4, 12238 BAEE units/mg protein; Sigma Aldrich, Taufkirchen, Germany). Three different types of pectin were used as anionic polysaccharides to form an additional interfacial layer around the oil droplets: A low-methoxylated pectin (LMP) and two types of high-methoxylated pectin (HMP1 and HMP2) with varying degree of methoxylation (DM) and galacturonic acid content (GC). LMP, HMP1 and HMP2 exhibited a DM of 33, 72 and 70 % and the GC was 88, 76 and 82 %, respectively. Glucose syrup (C*Dry01934, DE38) was purchased from Cargill Deutschland GmbH, Krefeld, Germany.

4.3.2 Enzymatic hydrolysis of β-LG

Enzymatic hydrolysis of β-LG was performed as described elsewhere (Tamm et al., 2015). A solution containing 7 wt% β-LG was hydrolysed to a degree of hydrolysis (DH) of 6 % (DH6) using the pH-stat method of Adler-Nissen (1986) and an enzyme/substrate-ratio of 1:800.

4.3.3 Molecular weight distribution of β-LG and DH6

The characterisation of the peptide composition of β-LG and DH6 was performed by tris-tricine SDS-PAGE (Schägger, 2006) and size exclusion chromatography (SEC) as described elsewhere (Tamm et al., 2015). SDS-PAGE was performed under reducing conditions using DL-dithiothreitol (Sigma Aldrich). For comparison a standard marker (cat#MWSDS17S (Sigma Aldrich)) was used, containing seven polypeptides between 2.5 and 17.0 kDa. SEC analysis was conducted on a TSKgel G2000SW column (7.5 mm ID × 30 cm, particle size 10 μm, Tosoh Biosciences LLC, USA) in series with a TSKgel CW guard column (7.5 mm ID × 4 cm, particle size 10 μm). An isocratic elution of 30 % acetonitrile containing 0.1 % TFA (v/v) was used at a flow rate of 1.0 mL/min and the elution was monitored with UV absorbance at 214 nm.

4.3.4 Preparation and physical characterisation of single and bilayer emulsions

Emulsions were prepared using the layer-by-layer technique. Coarse emulsions were produced by rotor-stator homogenisation (21,500 rpm, 90 s; T25 basic, IKA, Staufen, Germany) and contained 10 wt% fish oil, 0.5 wt% β-LG or DH6 (in 0.1 M acetate buffer) and 44.5 wt% glucose syrup. After high-pressure homogenisation (500 bar, 3 passes; PandaPlus, GEA Niro Soavi, Parma, Italy), emulsions were diluted with buffered solutions containing either glucose syrup (single layer) or glucose syrup and 0.4 wt% pectin (bilayer), respectively. These emulsions were again homogenised (400 bar, 1 pass) to yield final single layer (0.25 wt% protein, 5 wt% fish oil, 44.5 wt% glucose syrup) and bilayer emulsions (additionally 0.2 wt% pectin).

The oil droplet size of emulsions before and after spray-drying was analysed by laser diffraction (LA-950, Horiba Jobin Yvon GmbH, Bensheim, Germany). Results of the volume distribution are reported as the 90^{th} percentile of the oil droplets, since this parameter is sensitive for shifts towards larger droplets. Measurements were performed in triplicate.

The ζ-potential of emulsions was examined using a Zetasizer (Nano-ZS, Malvern Instruments GmbH, Herrenberg, Germany). Emulsions were diluted 200-fold with 0.1 M acetate buffer prior to ζ-potential-measurements and analysed in triplicate.

4.3.5 Spray-drying of single and bilayer emulsions and characterisation of spray-dried microcapsules

Microencapsulation of fish oil was conducted by spray-drying feed emulsions (see section 2.4) on a pilot-scale spray-dryer (Mobile minor, Niro A/S, Copenhagen, Denmark) at 180/70 °C inlet/outlet temperature with a two-fluid nozzle (1.9 bar). For evaluation of the microencapsulation efficiency, the extractable oil content in microcapsules was analysed. After extraction of the non-encapsulated oil using petrol ether, the oil content was determined gravimetrically (Westergaard, 2004).

4.3.6 Lipid oxidation of microencapsulated fish oil

The lipid oxidation of microencapsulated fish oil was monitored by the hydroperoxide formation for 12 weeks. Samples were stored in desiccators in the dark at room temperature in an atmosphere of 33 % relative humidity. Microcapsules were incubated in an aqueous solution containing 0.7 wt% pectinase (Sigma Aldrich, cat#17389) to digest the pectin layer. Subsequently the oil was extracted by a blend of 2-propanol/isooctane and the hydroperoxide content was analysed to quantify the extent of lipid oxidation (International Dairy Federation, 1991) with slight modifications (Tamm et al., 2015). Each determination included by two repetitions of the oil extraction and three analytical replicates of each extraction.

4.4 Results and discussion

4.4.1 Molecular weight distribution of β-LG and DH6

Enzymatic hydrolysis of β-LG using trypsin affected the molecular weight distribution and the results are shown in fig. 4.1. β-LG mainly contains one protein as shown by the most intense band around 18 kDa in the SDS-PAGE gel and the SEC results. DH6 exhibits strong bands between 6.2 and 8.2 kDa and at 2.5 kDa in the SDS-PAGE gel and the SEC results confirm the peptides in DH6 are mostly smaller than 10 kDa.

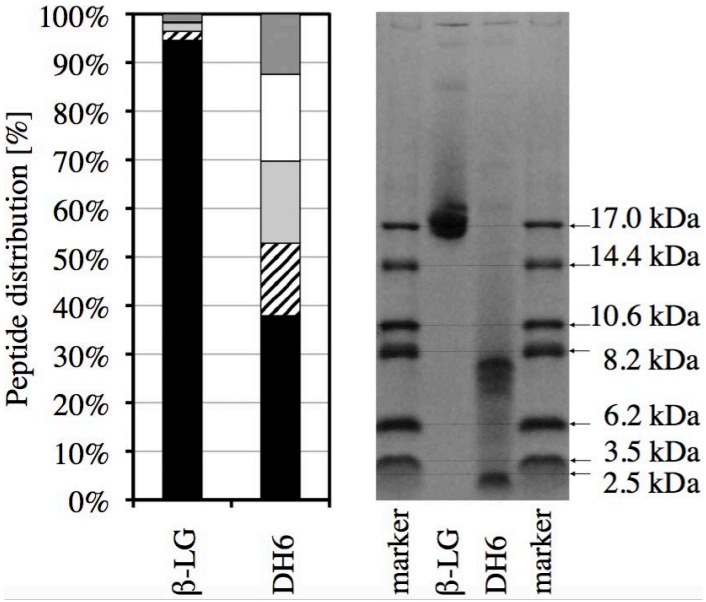

Figure 4.1 Molecular weight characterisation of β-LG and a hydrolysate thereof with a degree of hydrolysis of 6 % (DH6). Left: size exclusion chromatography results of β-LG and DH6; Legend: black: > 10 kDa, black/white: 5 - 10 kDa, light grey: 2 - 5 kDa, white: 1 - 2 kDa, dark grey: < 1 kDa. Right: tris-tricine SDS-PAGE results of β-LG and DH6 under reducing conditions in comparison to marker peptides.

Most notable is the absence of the β-LG band in the SDS-PAGE indicating that the majority of β-LG was hydrolysed by trypsin. SEC results however show that over one third of the peptides in DH6 is larger than 10 kDa. This can be explained by either newly formed disulphide bonds between peptides or the formation of peptide aggregates that cannot be dissociated by the SEC eluent (Le Maux et al., 2013).

4.4.2 Characterisation of single and bilayer emulsions and spray-dried microcapsules

The physicochemical properties of feed emulsions and spray-dried microcapsules are shown in tab. 4.1. All emulsions were physically stable during atomisation and drying as indicated by the 90^{th} percentile of the oil droplet size of feed emulsions and reconstituted emulsions after spray-drying. For β-LG/HMP2-bilayer and DH6-single layer emulsions the oil droplet size decreased due to the shearing forces affecting droplets > 2 µm in the atomising nozzle (Soottitantawat et al., 2003). The ζ-potential of β-LG-single layer emulsions ranged between 25.3 and 28.2 mV, which is in good agreement with the results of Güzey & McClements (2006) for β-LG-single layer corn oil emulsions. However, the ζ-potential of DH6-single layer emulsions decreased compared to β-LG-single layer emulsions, which may be attributed to the exposure of carboxyl and

amino groups from the peptide bonds due to enzymatic hydrolysis. In addition an increased number of amino groups directed towards the aqueous phase and more peptides adsorbed to the interface may affect the droplet charge and cause the increase in ζ-potential. This yields DH6/pectin-bilayer emulsions with a higher ζ-potential compared to β-LG/pectin-bilayer emulsions after charge reversal due to pectin addition. The microencapsulation efficiency was high for all samples and increased due to reinforcement of the interfacial layer by pectin in bilayer emulsions in comparison to spray-dried single layer emulsions prepared with the same protein.

Table 4.1 Physicochemical properties of feed emulsions and spray-dried fish oil microcapsules stabilised by β-LG or a hydrolysate thereof with a degree of hydrolysis of 6 % (DH6) and different types of low- (LMP) or high-(HMP) methoxylated pectin.

Sample	90^{th} percentile of oil droplet size [µm]		ζ-potential [mV]		Microencapsulation efficiency [%]
	fresh	reconstituted	Single layer	Bilayer	
β-LG	0.86 ± 0.15	1.23 ± 0.31	+ 26.1 ± 0.8	-	97.3 ± 0.2
β-LG/LMP	0.70 ± 0.02	0.62 ± 0.01	+ 26.2 ± 1.1	- 24.5 ± 1.5	99.7 ± 0.0
β-LG/HMP1	0.90 ± 0.03	0.72 ± 0.01	+ 27.3 ± 0.9	- 23.8 ± 1.2	99.7 ± 0.1
β-LG/HMP2	2.62 ± 0.49	0.74 ± 0.00	+ 27.0 ± 1.2	- 10.4 ± 0.6	99.5 ± 0.1
DH6	5.01 ± 0.65	2.42 ± 0.17	+ 14.4 ± 0.8	-	95.5 ± 0.3
DH6/LMP	1.33 ± 0.01	1.25 ± 0.05	+ 14.7 ± 0.8	- 13.0 ± 0.4	98.7 ± 0.3
DH6/HMP1	1.46 ± 0.02	1.19 ± 0.02	+ 15.5 ± 0.8	- 13.5 ± 1.0	98.6 ± 0.1
DH6/HMP2	1.48 ± 0.01	1.30 ± 0.03	+ 13.0 ± 1.1	- 11.4 ± 0.9	98.7 ± 0.1

4.4.3 Lipid oxidation of microencapsulated fish oil

The hydroperoxide content in microencapsulated fish oil after spray-drying exhibited only slight differences (0.5 – 3.5 mmol/kg oil). During storage, β-LG-single layer microcapsules showed by far the steepest increase in its hydroperoxide content compared to all other microcapsules, with a final content of 280 mmol/kg oil after 12 weeks. In contrast, the final hydroperoxide contents for DH6-single layer, β-LG/pectin-bilayer and DH6/pectin-bilayer microcapsules were 162, 105 - 121 and 78 - 106 mmol/kg oil, respectively. This clearly indicates, that lipid oxidation in microencapsulated fish oil may be considerably retarded by the use of β-LG partially hydrolysed to a DH of 6 %, as reported recently (Tamm et al., 2015). Increased accessibility of antioxidant amino acids due to enzymatic hydrolysis enhances the effectiveness of the peptides adsorbed to the o/w-interface, where lipid oxidation is initiated and oxidation reactions are promoted (McClements & Decker, 2000). In addition peptides < 1kDa in whey protein hydrolysates may exhibit increased iron chelating activity (O'Loughlin, Kelly, Murray,

FitzGerald, & Brodkorb, 2015). Formation of bilayer feed emulsions further inhibited lipid oxidation in microcapsules. The increased thickness of the interfacial layer in bilayer compared to single layer emulsions provides an additional physical barrier around the oil droplets and may hinder metal-lipid interactions to a greater extent (Katsuda et al., 2008). Bilayer emulsions exhibited a negative ζ-potential, thus the pectin layer would attract positively charged ions like iron, but probably immobilises these prooxidant metal ions, respectively (Chen et al., 2010). The complex formation with pectin is more effective in terms of stabilisation of the encapsulated oil than the antioxidative effect of DH6, since differences in lipid oxidation are less pronounced among β-LG- and DH6-bilayer microcapsules.

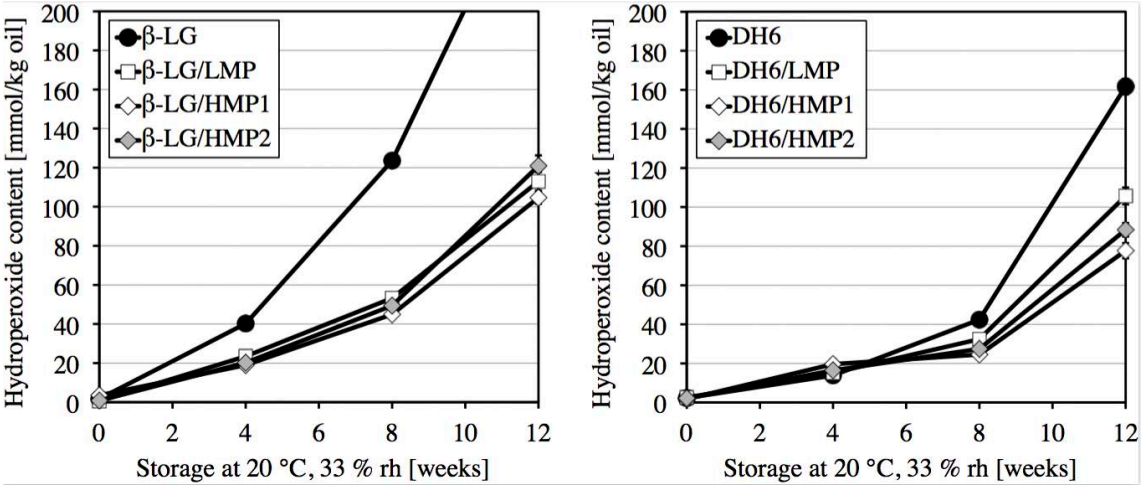

Figure 4.2 Development of the hydroperoxide content in microencapsulated fish oil stabilised by β-LG or a hydrolysate thereof with a degree of hydrolysis of 6 % (DH6) and low-(LMP) or high-(HMP) methoxylated pectin. Samples were stored in the dark at 33 % relative humidity. Left: microcapsules containing β-LG and pectin. Right: microcapsules stabilised by DH6 and pectin.

4.5 Conclusions

Bilayer emulsions containing two oppositely charged biopolymers, pectin and β-LG or DH6, are potential systems to chemically and physically stabilise sensitive lipophilic ingredients. Droplet stability during spray-drying was provided by all single and bilayer systems irrespective of the type of protein or pectin. The microencapsulation efficiency was high for all samples (≥ 95.2 %) and increased slightly for bilayer emulsions. Hydroperoxide formation was retarded in DH6- compared to β-LG-single layer microcapsules due to increased accessibility of antioxidant amino acids. Electrostatic deposition of LMP or HMP to the protein layer in feed emulsions yields fish oil microcapsules

with increased protection to lipid oxidation, most likely due to increased interfacial layer thickness and immobilisation of prooxidant metal ions by pectin.

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5 Manuscript 3: Impact of enzymatic hydrolysis on the interfacial rheology of whey protein/pectin interfacial layers at the oil/water-interface

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5.1 Abstract

Aim of the present study was to investigate the complex formation of β-lactoglobulin (β-LG) hydrolysates and different types of pectin at the oil/water-interface by dynamic interfacial pressure and the properties of the complexes by interfacial rheology (dilatation and shear). The degree of hydrolysis (DH) of β-LG was varied (3 and 6 %) as well as the degree of methoxylation of the pectins, i.e. two low-(LMP, 32 and 35 %) and two high-methoxylated (HMP, 64 %) pectins were used. The pectins exhibited different local charge densities, due to enzyme specificity during demethoxylation. All experiments were performed at a constant biopolymer content (0.01 wt%), pH (4.0) and temperature (22 °C). β-LG/pectin interactions clearly delayed interfacial adsorption, which was more pronounced for LMP with a high local charge density. With increasing DH of β-LG, the interfacial adsorption was accelerated, however β-LG/pectin layers exhibited higher dilatational elastic moduli than hydrolysate/pectin layers. In contrast to dilatational rheology, β-LG formed weaker interfacial layers than its hydrolysates during shear time sweeps, despite the presence of pectin. Pectin and hydrolysates exhibited strong viscoelastic interfacial layers partially more than one order of magnitude higher than β-LG. However, during shear amplitude sweeps all interfacial layers were resistant to increasing strain and the films collapsed within the same region. Therefore, targeted structural modifications using enzymes with high specificity enables the production of β-LG hydrolysates and demethoxylated pectins with tailored functionality with respect to their interfacial rheology characteristics.

5.2 Introduction

Whey proteins are frequently used for the stabilisation of disperse systems, i.e. emulsions or foams. However, their functionality is limited in their native form, due to their globular structure and the inaccessible hydrophobic amino acid residues (Gauthier & Pouliot, 2003). However, the functional properties of globular proteins can be improved by controlled enzymatic hydrolysis. The resulting functionality depends on many factors such as composition and pre-treatment of the substrate, enzyme specificity, milieu conditions during hydrolysis and the degree of hydrolysis achieved (Gauthier & Pouliot, 2003; Ipsen et al., 2001). The enzymatic modification of the molecular structure yields peptide mixtures with lower molecular weight, less secondary and tertiary structure compared to the substrate (Davis et al., 2005). In addition, it may result in exposure of hydrophobic groups and an increasing number of ionisable groups (Panyam & Kilara, 1996). Accordingly, hydrolysed proteins can stabilise a larger interfacial area (O'Regan & Mulvihill, 2010) and their diffusional transport and affinity for adsorption to the in-

terface is accelerated (Ipsen et al., 2001). This increase in interfacial activity at the air/water-interface has been proved by interfacial tensiometry for hydrolysates of whey protein (Althouse, Dinakar, & Kilara, 1995; Drusch, Hamann, et al., 2012; Gauthier, Paquin, Pouliot, & Turgeon, 1993; Tamm et al., 2012) and β-lactoglobulin (β-LG) (Davis et al., 2005; Perez, Sanchez Carrera, et al., 2012; Turgeon et al., 1992), which is the main protein fraction occurring in bovine whey. However, the degree of hydrolysis (DH) has to be controlled carefully to maintain peptide functionality. At a high DH the bulk of peptides has a low molecular weight with an insufficient amount of distinct hydrophilic and hydrophobic patches resulting in decreased functionality (Turgeon et al., 1992). Thus, small peptides are unable to form strong intermolecular interactions at the interface (Singh & Dalgleish, 1998) and to stabilise the latter with a cohesive, viscoelastic layer of high packing density (Dickinson, 2011b). Hydrolysates with a limited DH may form stronger interfacial layers, resisting external deformations more effectively (Davis et al., 2005; Perez, Sanchez Carrera, et al., 2012; Tamm et al., 2012). The interfacial films are formed by hydrogen bonding, hydrophobic and covalent bonding, and electrostatic interactions between adjacent molecules (Bos & van Vliet, 2001; Mitropoulos et al., 2014).

However, the addition of another biopolymer to the solution, e.g. non-surface active pectin, may strengthen the interfacial layers in comparison to pure protein layers due to the formation of electrostatic complexes (Ganzevles, van Vliet, et al., 2007). To enable complexation, the pH of the solution has to be below the isoelectric point of the protein (positive net charge) and above the pK_a of the carboxyl groups on the galacturonic backbone of pectin (negative net charge) (Gancz et al., 2005; Ganzevles et al., 2006; Wagoner et al., 2016). Basically, the protein/polysaccharide-ratio, the biopolymers overall charge and local charge density and the milieu conditions dictate the binding affinity towards each other. Variation of these factors determines whether a complex is formed, its strength or if phase separation occurs (yielding complex coacervates or precipitate) (Ganzevles, van Vliet, et al., 2007; Ganzevles et al., 2006). Accordingly, the characteristics of the complexes may differ greatly with respect to interfacial adsorption and the formation of strong interfacial layers (Ganzevles, Kosters, van Vliet, Cohen Stuart, & de Jongh, 2007; Ganzevles, van Vliet, et al., 2007; Ganzevles et al., 2006). The highest binding affinity of pectin to β-LG was observed for low-methoxylated pectin, which possesses a high content of free carboxyl groups within the galacturonic acid backbone (Ralet et al., 2001). Additionally, pectins with a high local charge density, i.e. a blockwise distribution of the carboxyl groups, form even stronger complexes (Sperber, Cohen Stuart, Schols, Voragen, & Norde, 2009). The binding affinity of the pectin to β-LG decreases, if the degree of methoxylation (DM) increases or the local charge density (also degree of blockiness) decreases, i.e. the free carboxyl groups are more randomly distributed, respectively (Sperber et al., 2009). These specific structural characteristics of pectin can be generated by an enzymatic treatment with different types

of pectinmethylesterase (PME) (Einhorn-Stoll et al., 2015). In its unmodified form, pectin exhibits a DM of 67-73 % (Endreß & Christensen, 2009), however the DM may be reduced by plant- or fungal-derived PME. Enzymatic demethoxylation yields a statistic (plant) or random (fungal) distribution of the free carboxyl groups, as a function of enzyme specificity (Ralet et al., 2001; Ralet & Thibault, 2002). Accordingly, the molecular structure of modified pectin can be tailored by the choice of the enzyme, steering the local charge density, and the DM, adjusting the overall charge (Wagoner et al., 2016).

Interfacial layers stabilised by electrostatic complexes can be engineered either by sequential or simultaneous adsorption (Ganzevles, van Vliet, et al., 2007). Sequential adsorption involves the formation of a protein layer initially and subsequent addition of the polysaccharide to yield a bilayer interface (Dickinson, 2011b). For simultaneous adsorption, a solution containing both protein and polysaccharide is prepared, resulting in mixed interfacial layers. This method may yield more stable emulsions compared to those from sequential adsorption (Jourdain, Leser, Schmitt, Michel, & Dickinson, 2008). However, when emulsions prepared by the sequential technique are homogenised again after addition of the polysaccharide (Ogawa, Decker, & McClements, 2004; Serfert et al., 2013), the layer composition is rather mixed and not strictly composed of an inner protein- and an outer polysaccharide-layer.

Once the interfacial layer has been formed, its resilience during emulsion formation and the subsequent colloidal stability of the dispersed system under physical stresses is attributed to its mechanical properties, i.e. its interfacial rheology in the form of dilatation and shear (Erni et al., 2011; Murray, 2011).

So far, no direct correlation of the interfacial rheology of an adsorbed biopolymer to its emulsifying and foaming properties and the subsequent stability of these systems was reported. Nevertheless, the importance of interfacial rheology in this regard has often been highlighted (Benjamins et al., 2006; Erni et al., 2011; Freer et al., 2004; Krägel & Derkatch, 2010; Krägel, 2014; Maldonado-Valderrama et al., 2010; Murray et al., 2009; Murray, 2011; Xu et al., 2008). In this manner, properties of interfacial layers under dilatation (compression/expansion) are associated with the stability during emulsification, since they are linked to relaxation processes within the interfacial layer and diffusional exchange of protein with the bulk phase (Benjamins et al., 2006; Dickinson, 2011b). These deformations are likely to occur during processing, dispensing and the consumption of food emulsions (Murray et al., 2009). In contrast, interfacial shear rheology is sensitive to the extent of the lateral intermolecular interactions between the biopolymers within the layer, enabling the monitoring of structure and network formation, which has been associated with the long-term stability of emulsions (Benjamins et al., 2006; Dickinson, 2011b; Krägel & Derkatch, 2010; Krägel, 2014). Accordingly, the link between the deformation of the interfacial layer and the forces involved is studied as a function of time (Bos & van Vliet, 2001). Due to the fact that shear and dilatational de-

formation affects interfaces under common process conditions, both should be interpreted together (Freer et al., 2004; Maldonado-Valderrama et al., 2010).

The viscoelastic moduli in dilatational rheology originate from the interfacial tension response resulting from variation of the interfacial area. When using a pendant drop tensiometer, the latter is performed by changing the volume of a droplet of surfactant solution and thereby compressing/expanding the interfacial layer (Dickinson, 2011b). In contrast, in interfacial shear rheology experiments the interfacial film is submitted to deformations by movement of a measuring probe to an interface at constant area and the resulting torque enables to calculate the interfacial shear moduli (Dickinson, 2011b; Krägel & Derkatch, 2010).

A lot of research has focused on the interfacial dilatational rheology of unhydrolysed whey proteins at air/water(a/w)- and oil/water(o/w)-interfaces. However only few studies report the interfacial dilatational rheology of whey protein hydrolysates at the air/water-interface (Davis et al., 2005; Drusch, Hamann, et al., 2012; Perez, Carrera Sánchez, et al., 2012; Perez, Sanchez Carrera, et al., 2012; Tamm et al., 2012) and only in one publication the interfacial shear rheology of whey protein hydrolysates was investigated (Ipsen et al., 2001). The interfacial shear moduli of β-LG interfacial layers at the a/w-interface increased in the presence of pectin (Ganzevles et al., 2006). Additionally, the interfacial dilatational moduli were higher for β-LG, when pectin was present at the a/w-interface (Ganzevles et al., 2006) or at the o/w-interface (Oliveira, von Staszewski, Pizones Ruiz-Henestrosa, Pintado, & Pilosof, 2016; Serfert et al., 2013).

As reviewed by Dickinson (2011a) recently, the structure of multicomponent layers at o/w- and a/w-interfaces deserves increased research attention in the next few years and neither pure whey protein hydrolysates (WPH) nor WPH/pectin-complexes have been characterised in terms of their interfacial rheology at the o/w-interface so far. Furthermore, the results obtained at the a/w-interface may differ from those obtained at the o/w-interface (Ganzevles, van Vliet, et al., 2007; Xu et al., 2008), since the hydrophobic side chains of the adsorbed molecules may penetrate into the oil phase, due to their better solvation in oil in comparison to air, highlighting the importance of this research (Miller et al., 2000; Murray, Ventura, & Lallemant, 1998; Xu et al., 2008).

The aim of the present study was to investigate the adsorption kinetics and the resulting interfacial rheology parameters of tryptic hydrolysates of β-LG and complexes of protein and different types of pectin. The interfacial activity and interfacial dilatational characteristics at the a/w-interface were enhanced for hydrolysed whey proteins in comparison the unmodified substrate, accordingly this effect is also expected to be present at the o/w-interface, despite the different nature of these two interfaces. Furthermore it is hypothesised, the complexation with pectin further improves the interfacial rheology parameters, under shear and dilatation, without a strong reduction in interfacial activity in comparison to the unmodified substrate and pure protein systems. In this context, we

propose that LMP pectin with a high local charge density may yield the strongest interfacial complexes due to the high binding affinity to the proteins. In contrast, HMP and a low local charge density should result in weaker interfacial layers. Thereby valuable insights may be gained with respect to the process stability of the systems under study. A pendant drop tensiometer and an interfacial shear rheometer were used to characterise pure protein layers and protein/pectin-complexes with respect to structure formation, their linear viscoelastic regime and frequency dependence under both deformation modes.

5.3 Material and methods

5.3.1 Materials

β-Lactoglobulin (β-LG, Davisco Foods International Inc., Le Sueur, USA) was used as received and in enzymatically-modified form. For enzymatic hydrolysis trypsin (from bovine pancreas, cat#T8003, EC: 3.4.21.4, 12238 BAEE units/mg protein) was used (Sigma-Aldrich, Taufkirchen, Germany). MCT oil (CremerCoor 60/40 MCT, Cremer Oleo, Germany) was used after removal of interfacially active compounds with an activated magnesium silicate (Florisil 60-100 mesh, VWR International GmbH, Dresden, Germany). Four different pectins enzymatically demethoxylated to varying degree of methoxylation prepared by a plant(p)- and fungal(f)-derived pectinmethylesterase (PME), as published recently (Einhorn-Stoll et al., 2015) were used for complex formation with the protein. The modification yielded two low-methoxylated (LMP) and two high-methoxylated pectins (HMP) with degrees of methoxylation (DM) of 32 % (f32), 35 % (p35) 64 % (p64, f64), respectively. Enzymatic treatment of pectin with plant-based PME results in a blockwise distribution of the free carboxyl groups within the galacturonic acid backbone, however fungal PME cause a statistical distribution of theses groups (Ralet et al., 2001; Ralet & Thibault, 2002).

5.3.2 Enzymatic hydrolysis of β-LG

Enzymatic hydrolysis of β-LG was performed as described elsewhere (Tamm et al., 2015). A solution containing 2 wt% β-LG was hydrolysed to a degree of hydrolysis (DH) of 3 and 6 % (DH3 and DH6) using the pH-stat method of Adler-Nissen (1986) and an enzyme/substrate-ratio of 1:1600 and 1:800, respectively. The DH was calculated from base consumption according to Adler-Nissen (1986) using a h_{tot}-value of

7.2 meqv/g of protein for the total number of peptide bonds in the substrate (Seronei Chelulei Cheison et al., 2010).

5.3.3 Molecular weight distribution of β-LG and hydrolysates thereof

The characterisation of the peptide composition of β-LG its hydrolysates was performed by tris-tricine SDS-PAGE and size exclusion chromatography (SEC) as described elsewhere (Tamm et al., 2015). SDS-PAGE was executed under reducing conditions by spiking samples with 1M DL-dithiothreitol (Sigma Aldrich). For comparison a standard marker (cat#MWSDS17S (Sigma Aldrich)) was used, containing seven polypeptides between 2.5 and 17.0 kDa. SEC analysis was conducted on a TSKgel G2000SW column (7.5 mm ID × 30 cm, particle size 10 μm, Tosoh Biosciences LLC, USA) in series with a TSKgel CW guard column (7.5 mm ID × 4 cm, particle size 10 μm). An isocratic elution of 30 % acetonitrile containing 0.1 % TFA (v/v) was used at a flow rate of 1.0 mL/min and the elution was monitored with UV absorbance at 214 nm.

5.3.4 Interfacial adsorption and dilatational rheology

For the study of interfacial adsorption and dilatational rheology experiments, an automated drop tensiometer (OCA20, Dataphysics GmbH, Germany) equipped with an oscillating drop generator (ODG20, Dataphysics) was used. The experimental setup was described in detail elsewhere (Tamm et al., 2012). Briefly, a droplet of protein- or protein/pectin-solution was created at the tip of a stainless-steel needle into the MCT-oil-phase within a quarz-glass cuvette. The shape of the droplet is captured by a CCD-camera and fitted to the Young-Laplace-equation to calculate the interfacial tension. With constant droplet volume the adsorption kinetics to the interface may be studied. Throughout the manuscript the MCT-oil/water-interface will be called o/w-interface. The adsorption kinetics are reported by the interfacial pressure π, i.e. the difference of the interfacial tension σ of a system free of any protein and σ at the time t.

If the interfacial area is subjected to small sinusoidal perturbations by variations of the droplet volume, the adsorbed interfacial layer is compressed and expanded, respectively (Ravera, Loglio, & Kovalchuk, 2010). The resulting change in interfacial tension can be directly related to the interfacial dilatational viscoelasticity. The elastic modulus E during these oscillations is defined by the Gibbs equation

$$E = \frac{d\sigma}{dA/A} ,$$

with the interfacial tension σ and the total interfacial area A. The external disturbance, i.e. the sinusoidal oscillation of the interfacial area A at a frequency ω, results in the oscillation of the interfacial tension σ at the same frequency delayed by the phase angle ϕ at the time t (Ravera et al., 2010):

$$A = A_0 + \Delta A \sin(\omega t) ,$$

$$\sigma = \sigma_0 + \Delta\sigma \sin(\omega t + \phi) ,$$

where ΔA and $\Delta\sigma$ are the amplitudes of periodic interfacial area and interfacial tension variation and A_0 and σ_0 represent the unperturbed interfacial area and interfacial tension, respectively. The complex viscoelastic modulus $E^* = E' + iE''$ (Rühs, Scheuble, Windhab, & Fischer, 2013) is composed of an elastic part E' (storage modulus) representing the recoverable energy stored in the interface and a viscous part E'' (loss modulus) describing the energy lost through relaxation processes (Cascão Pereira, Théodoly, Blanch, & Radke, 2003). These moduli can be calculated by a Fourier transformation using the equations below (Cascão Pereira et al., 2003; Rühs, Scheuble, et al., 2013):

$$E' = \Delta\sigma \frac{A_0}{\Delta A} \cos\phi ,$$

$$E'' = \Delta\sigma \frac{A_0}{\Delta A} \sin\phi .$$

A droplet (28 µl) of the investigated solution was created inside the cuvette filled with purified MCT-oil. The protein and pectin content was kept constant at 0.01 wt% and the pH was adjusted to 4.0 using 0.01 M hydrochloric acid. After an equilibration time of 14 h the interfacial tension was constant for all samples and subsequently the linear viscoelastic regime was determined. The deformation amplitude was varied (0.7-5.6 % interfacial area) at a frequency of 0.01 Hz, where each oscillation consisted of 8 cycles and before each step the interfacial film was allowed to recover for 20 min. The deformation amplitude applied for the following frequency sweeps was set to 2.8 %, which was well within the linear viscoelastic regime and provided a good balance between a strong instrument signal and a linear interfacial tension response at the applied strain

(Rühs, Affolter, et al., 2013). The frequency sweeps were performed after the amplitude sweeps and the frequency was varied from 0.001-0.1 Hz. Between each frequency step the droplet was allowed to rest for 20 min to regain equilibrium. All pendant drop experiments were performed at a constant temperature of 22 °C.

5.3.5 Interfacial shear rheology

Interfacial shear rheology experiments were performed on a shear rheometer (Physica MCR 301, Anton Paar Germany GmbH, Ostfildern, Germany) equipped with a biconical disk geometry (Erni et al., 2003). When the Boussinesq number is larger than 1, the experimental setup may be considered as an isolated two-dimensional fluid and the viscous contribution of the bulk phase flow to the interfacial viscosity may be neglected (Rühs, Scheuble, Windhab, Mezzenga, & Fischer, 2012). Accordingly, the complex interfacial shear viscosity η^*_i is defined as follows (Erni et al., 2003; Rühs, Affolter, et al., 2013):

$$|\eta_i^*| = \frac{M - \frac{8}{3}R^3(\eta_1+\eta_2)\Omega}{4\pi R^2 \Omega}$$

where Ω and R are the angular velocity and the radius of the disk, respectively. M is the torque and η_1 and η_2 are the viscosities of the two phases. When a sinusoidal oscillation with a deformation $\gamma_s(t) = \gamma_0 \cos(\omega t)$ is applied to the interface, a stress response $\tau(t) = \tau_0 \sin(\omega t+\delta)$ delayed by phase shift δ may be determined (Rühs, Affolter, et al., 2013). The dynamic complex interfacial shear modulus $G^*_i(\omega)$ is then defined by

$$G_i^*(\omega) = \tau_0 e^{i\delta} / \gamma_0 = |G_i^*|(\cos\delta + i\sin\delta) = G_i'(\omega) + iG_i''(\omega)\ ,$$

where $G_i'(\omega)$ is the interfacial storage modulus and $G_1''(\omega)$ is the interfacial loss modulus.

In all interfacial shear experiments a time sweep was applied, followed by a frequency sweep and an amplitude sweep. The time sweep characterised the structure formation of the interfacial layer and the measurement was performed for 9 h with constant deformation ($\gamma_s(t) = 0.1$ %) and frequency ($f = 1$ Hz). Subsequently the interface was left undisturbed for 20 min before the frequency sweep was started ($\gamma_s(t) = 0.1$ %,

$f = 0.01$-1 Hz), with 20 min without perturbation before each frequency step. Finally, the interface was subjected to amplitude sweeps ($\gamma_s(t) = 0.1$ - 100%, $f = 0.3$ Hz) and the interfacial layer was left undisturbed for 20 min before each oscillation. All interfacial shear experiments were performed at a constant temperature of 22 °C.

5.4 Results and discussion

5.4.1 Molecular weight distribution of β-LG and DH6

Enzymatic hydrolysis of β-LG using trypsin resulted in a strong shift in the molecular weight profile as presented in fig. 5.1. The main protein fraction β-LG represents the bulk of peptides in the unhydrolysed sample as displayed by a band of high intensity around 18 kDa in the SDS-PAGE gel and the SEC results. DH3 shows some bands at about 18 kDa, which can be attributed to residual intact β-LG. Additionally, many intermediate peptides can be found between 2.5 and 17 kDa with some bands of higher intensity between 6.2 and 8.2 kDa.

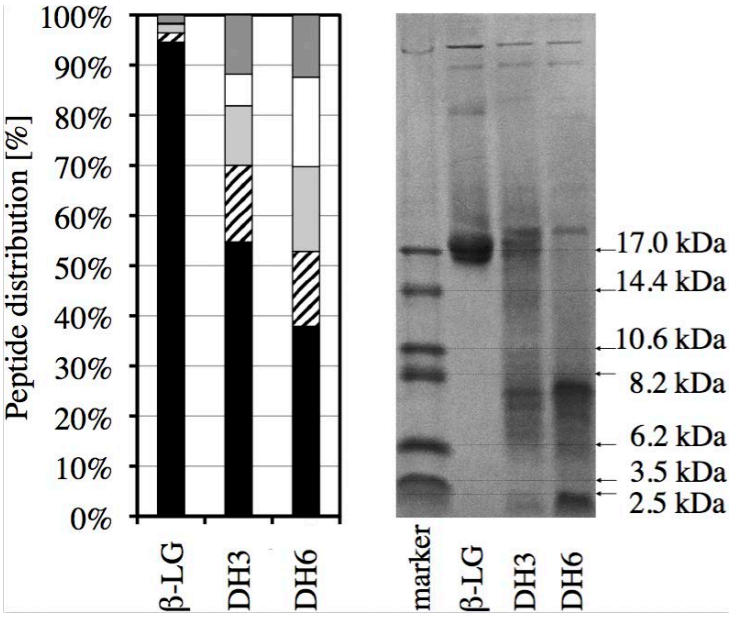

Figure 5.1 Molecular weight characterisation of β-LG and hydrolysates thereof with degree of hydrolysis of 3 % (DH3) and 6 % (DH6). Left: size exclusion chromatography results; Legend: black: > 10 kDa, black/white: 5 - 10 kDa, light grey: 2 - 5 kDa, white: 1 - 2 kDa, dark grey: < 1 kDa. Right: tris-tricine SDS-PAGE results under reducing conditions in comparison to marker peptides.

The peptide distribution determined by SEC confirms that almost the half of all peptides in DH3 is below 10 kDa. Strong bands between 6.2 and 8.2 kDa dominate the SDS-PAGE profile of DH6, in addition to a band at 2.5 kDa. The SEC results for DH6 indicate the bulk of peptides has a molecular weight below 10 kDa. These characteristic agree well with the results published earlier for β-LG, DH3 and DH6 (Tamm et al., 2015), where the molecular weight profiles of proteins and peptides and enzymatic scission of trypsin is reported in more detail.

5.4.2 Adsorption of β-LG, DH3 and DH6 as affected by the presence of pectin

During the interfacial adsorption of proteins the molecules diffuse from the bulk to the interface, adsorb, rearrange their conformational organisation, including the exposure of the hydrophobic parts to the oil phase (Dickinson, 2011b). Thereby a densely packed glass- or gel-like layer with intermolecular crosslinks is formed (Dickinson, 2011b). The adsorption of β-LG, DH3 and DH6 as affected by different types of pectin to the o/w-interface is presented in fig. 5.1 in terms of the interfacial pressure as a function of time. Regarding pure proteins or peptides, DH3 and DH6 exhibit a higher interfacial pressure compared to β-LG, indicating a more efficient packing of the peptides at the interface (Benjamins et al., 2006; Murray, 2011). In addition the hydrolysates exhibited a faster initial increase in interfacial pressure, which may be attributed to a lower molecular weight and the exposure of natively buried hydrophobic residues in the globular structure of β-LG due to hydrolysis. Accordingly, this results in an increase in the surface hydrophobicity of the molecules, both facilitating a more rapid adsorption to the interface (Adjonu et al., 2014; Davis et al., 2005; Perez, Carrera Sánchez, et al., 2012; Turgeon et al., 1992). Similar observations were reported for β-LG hydrolysates (Davis et al., 2005), soy glycinin hydrolysates (Ruiz-Henestrosa, Carrera Sanchez, Pedroche, Millan, & Rodriguez Patino, 2009) and soy protein hydrolysates (Martinez, Carrera Sanchez, Rodriguez Patino, & Pilosof, 2009) at the a/w-interface. Small peptides who diffuse rapidly and adsorb to the interface fast may be desorbed subsequently by larger peptides featuring better interfacial properties (> 2 kDa and distinct hydrophilic and hydrophobic patches) with a stronger affinity for the interface (Foegeding et al., 2002; Turgeon et al., 1991; van der Ven et al., 2001). The enhancement in surface hydrophobicity of the molecules increases their incompatibility with the aqueous phase, which is the driving force for their diffusion to the interface (Rodriguez Patino, Rodriguez Nino, & Carrera Sanchez, 1999a).

If pectin is present in the bulk solution, the interfacial adsorption of β-LG is strongly affected (fig. 5.2A), however, the interfacial pressure increase is delayed for β-LG/LMP- in comparison to β-LG/HMP-complexes. The delay may not be ascribed to bulk viscosi-

ty effects caused by pectin, since the shift in viscosity of the bulk upon pectin addition was negligible (data not shown). Instead this phenomenon results from the lower charge density of HMP yielding in a decreased binding affinity of HMP to β-LG, respectively (Ganzevles, van Vliet, et al., 2007). Thus, LMP possesses a higher binding affinity for β-LG, which causes the formation of strong electrostatic complexes with lower diffusion coefficients due to the increased hydrodynamic radius (Ganzevles, van Vliet, et al., 2007). Apart from the slower diffusion of the complexes, the higher binding affinity of LMP to β-LG limits the release of protein from the complex towards the interface (Ganzevles, Kosters, et al., 2007). Since β-LG-molecules adsorbed to the interface form complexes with pectin which possess a net negative charge (Ganzevles et al., 2006), the latter would repel complexes from the bulk, slow down β-LG-molecules and thus inhibit the formation of a compact and dense protein layer. This effect is most pronounced in β-LG/p35-solutions, since plant-derived PMEs yield modified pectins with a blockwise distribution of carboxyl groups, i.e. a higher local charge density (Wagoner et al., 2016), within the galacturonic acid backbone in contrast to a random distribution caused by fungal PME treatment (Ralet et al., 2001; Ralet & Thibault, 2002). Accordingly, β-LG and p35 form the strongest complexes (Ganzevles, Kosters, et al., 2007; Sperber et al., 2009), resulting in the lowest interfacial pressure of all systems. However, HMP with less binding sites for β-LG due to the lower charge density may dissociate from the protein upon its adsorption to the interface, enabling the formation of a more densely packed protein film (Ganzevles, Kosters, et al., 2007). Since f32 exhibits a similar DM, i.e. amount of free carboxyl groups, interfacial adsorption in β-LG/f32-systems is also delayed within the first 60 min, but due to the random distribution of the carboxyl groups the interfacial pressure after 14 h of adsorption is similar to pure β-LG.

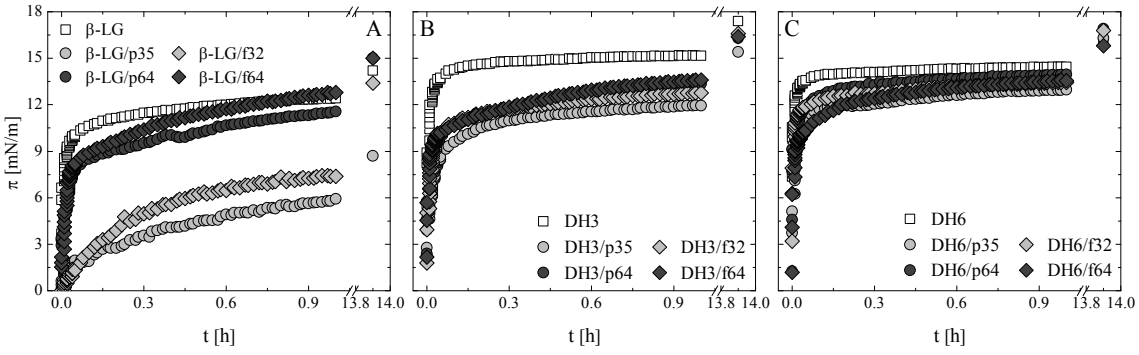

Figure 5.2 Interfacial pressure development as a function of adsorption time for β-LG (A) and hydrolysates thereof with degree of hydrolysis of 3 % (DH3, B) and 6 % (DH6, C) and pectins in different degrees of methoxylation at the MCT-oil/water-interface. Bulk protein and pectin content was 0.01 wt% at pH 4.0. Temperature 22 °C.

With increasing degree of hydrolysis, the presence of pectin delays the interfacial adsorption and film formation of the peptides to a smaller extent, independent of the DM (fig. 5.2B and C). As enzymatic hydrolysis proceeds, the amount of molecules available for complexation increases and thus also more free peptide molecules may be available in the bulk to adsorb to the interface in comparison to β-LG/pectin solutions. It was hypothesised that the number of available protein molecules within the complex and their mobility through the complex also has strong impact on the film formation (Ganzevles, van Vliet, et al., 2007). Additionally, an enzymatic hydrolysis was found to decrease the ζ-potential of the resulting peptide solutions (Liu et al., 2014; Mahmoud, Malone, & Cordle, 1992; Teh, Bekhit, Carne, & Birch, 2016) or peptide-stabilised emulsions (Hu, Ren, Zhao, Cui, & He, 2011; Tamm, Härter, Brodkorb, & Drusch, 2016) in comparison to the unmodified substrate. Thus, the overall positive charge of the hydrolysates decreases with increasing DH, respectively. Accordingly, a lower positive surface charge of the peptides emerging from β-LG hydrolysis yields weaker complexes with pectin, which interfere less in interfacial adsorption and film formation of the former. Less charged molecules enable the formation of interfacial films of higher density, i.e. higher interfacial pressure, by intermolecular crosslinking due to decreased repulsion (Xu et al., 2008) among adsorbing molecules and those in the bulk, thus a lower energy barrier exists for adsorption (Wierenga, Meinders, Egmond, Voragen, & De Jongh, 2005). After 14 h of adsorption all DH3- and DH6-based systems show similar final surface pressures, since rearrangements of the amino acid side chains towards the favoured phase allow the formation of interfacial layers of comparable packing density (Lucassen-Reynders, Benjamins, & Fainerman, 2010; Mitropoulos et al., 2014).

5.4.3 Dilatational amplitude sweeps of β-LG, DH3 and DH6 as affected by the presence of pectin

For the determination of the linear viscoelastic regime, the interfacial films after 14 h of adsorption time were exposed to sinusoidal area oscillations at different amplitudes ($\Delta A/A_0$ = 0.7-5.6 %) with constant frequency (f = 0.01 Hz) according to (Rühs, Affolter, et al., 2013). The results of this experiment are presented in fig. 5.3 in terms of the interfacial moduli E' and E'' as a function of the amplitude $\Delta A/A_0$. When the amplitude is increased too strong, the interfacial structures may be destroyed and the interfacial moduli drop dramatically (Wüstneck, Moser, & Muschiolik, 1999). All pure protein and protein/pectin-solutions were within the linear viscoelastic regime (LVE) for the amplitude range studied, instancing the results of pure β-LG, DH3 and DH6 layers (fig. 5.3A). The loss modulus E'' decreased with increasing degree of hydrolysis for all amplitudes, since the hydrolysates possess a simpler molecular structure and less energy is dissipated by structural rearrangements (Benjamins et al., 2006; Davis et al., 2005).

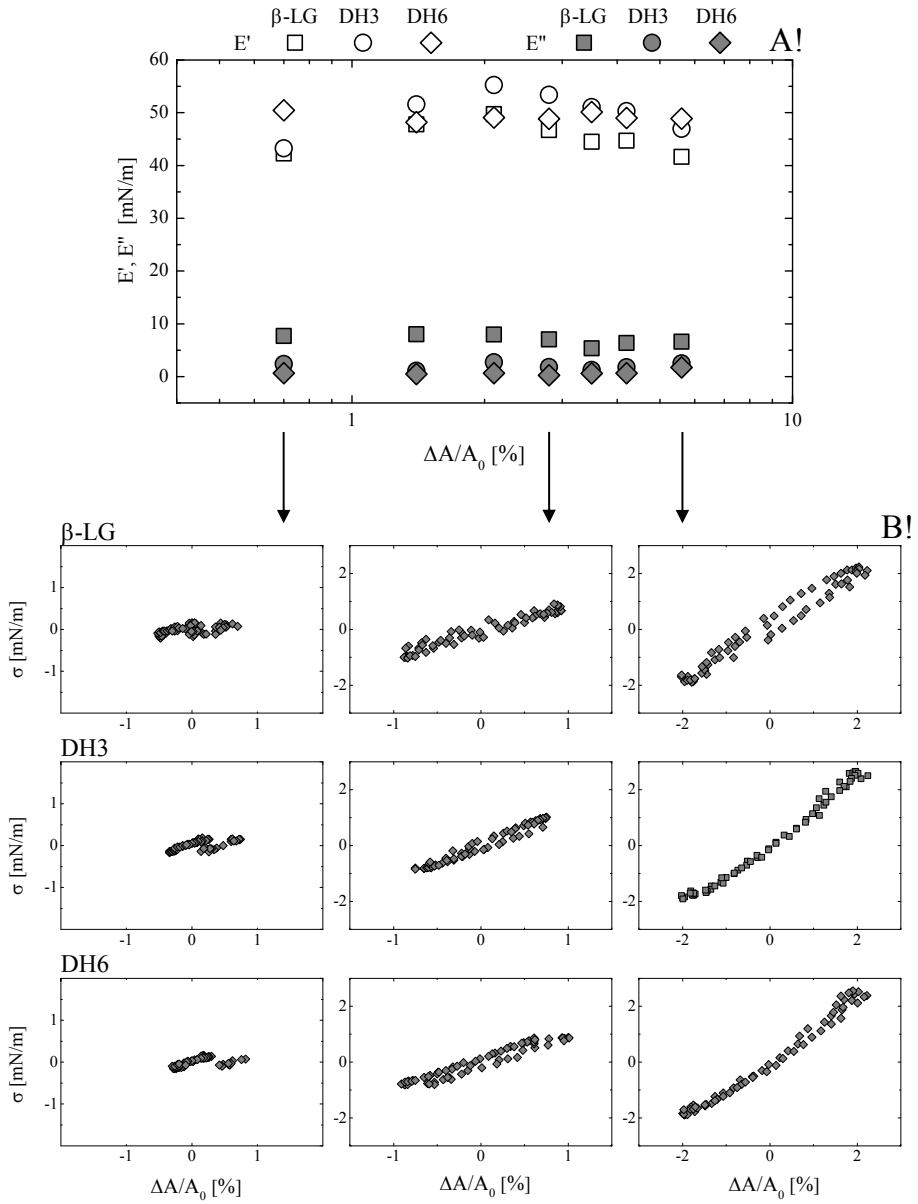

Figure 5.3 Dilatational amplitude sweeps of β-lactoglobulin (β-LG) and hydrolysates thereof with a degree of hydrolysis of 3 % (DH3) and 6 % (DH6). Protein films were adsorbed to the MCT-oil/water-interface for 14 h. A: Dilatational moduli E', E'' during amplitude sweeps (ΔA/A0 = 0.7-5.6 %; f = 0.01 Hz). B: Lissajous plots at different deformation amplitudes (0.7, 2.8 and 5.6 %). Bulk protein content was 0.01 wt% at pH 4.0. Temperature 22 °C.

However, the visualisation of Lissajous plots, i.e. the change in interfacial tension vs. the change in surface area extracted from one single oscillation cycle, provides some additional information about the experimental results (fig. 5.3B). At low amplitudes (0.7 %) the detected signal is too weak and the Lissajous plots show nonlinear scattering. However for 2.8 % a strong, linear signal results from the oscillation for all proteins and peptides. When the amplitude was further increased (5.6 %), the plot for β-LG

shows an elliptic shape with slight distortion, whereas the hydrolysates exhibit almost linear plots with slight distortion at their maximum stress/strain. Accordingly for the subsequent frequency sweeps an amplitude of 2.8 % was used, which provided a good balance between a strong instrument signal and a linear interfacial tension response (Rühs, Affolter, et al., 2013). The chosen amplitude agrees with those applied in other studies (Freer et al., 2004; Rühs, Affolter, et al., 2013).

5.4.4 Dilatational frequency sweeps of β-LG, DH3 and DH6 as affected by the presence of pectin

All interfacial layers studied exhibited a strong viscoelastic character, which may be attributed to a high packing density, as observed in surface pressure evaluation (see chapter 5.3.2), and high intermolecular interactions (Dickinson, 2011b). A quasi-equilibrium drop shape is required for interfacial tension calculation, thus intense perturbation of the interface must be avoided to leave the film intact and to remain within the LVE (Torcello-Gómez, Maldonado-Valderrama, de Vicente, et al., 2011). Therefore, the frequency dependence of the interfacial layers was evaluated by variation of f in the range of 10^{-3} - 10^{-1} Hz, since higher frequencies may result in deviations of the droplet profile from the Laplacian shape at o/w-interfaces, mimicking imaginary interfacial moduli (Leser, Acquistapace, Cagna, Makievski, & Miller, 2005). The results of the dilatational frequency sweeps are presented in fig. 5.4. All interfacial layers were primarily elastic as indicated by a maximum phase angle ϕ of 14 ° for β-LG/f32, ϕ was within the range of 0 - 3 ° for hydrolysate-based layers and 3 - 14 ° for β-LG-based interfacial films (data not shown). This indicates the formation of a gel-like structure with strong protein-protein- or peptide-peptide-interactions within the interfacial layers (Martinez et al., 2009) and more relaxation processes occurring in β-LG-based interfacial films (Benjamins et al., 2006). The dilatational storage modulus E' represents the strength of inter-protein linkages due to conformational rearrangement and the intra-protein rigidity resisting the deformation of the interface (Cascão Pereira et al., 2003). Accordingly β-LG-based interfacial films exhibit the highest E', since β-LG is more rigid compared to the flexible peptides in DH3 and DH6 (Agboola et al., 1998). In contrast, the loss modulus E'' reflects the loss of energy through relaxation processes (Benjamins et al., 2006). An increase in E' accompanied with a decrease in E'' was observed with increasing oscillation frequency for all β-LG/pectin systems except for β-LG/p35. This phenomenon is a characteristic of viscoelastic interfacial layers (Murray et al., 1998; Rodriguez Patino, Rodriguez Nino, & Carrera Sanchez, 1999b) and indicates the timescale of the oscillation is too short at higher frequencies to enable diffusional exchange of proteins or peptides with the bulk phase or relaxation processes within the network (Freer et al., 2004). At the highest frequency (0.1 Hz), ϕ was always zero for hydrolysate-based films, directly reflecting the surface equation of state (Lucassen-Reynders et

al., 2010), in contrast to β-LG-based interfacial films ($\phi \leq 5$ °). For lower frequencies, time effects like rearrangement, adsorption and relaxation processes gain importance and increase the viscous character of the interfacial film (Rühs, Affolter, et al., 2013), since the protein is given more time to adapt to the deformation of the interface (Maldonado-Valderrama et al., 2005). Globular proteins like β-LG exhibit only minor and very slow desorption, once adsorbed to an interface (Fainerman et al., 2006). However peptides resulting from enzymatic hydrolysis of β-LG may be desorbed more easily due to their simpler molecular structure and less lateral binding options (Turgeon et al., 1991).

All hydrolysate/pectin-based layers exhibit constant storage moduli independent from the frequency applied, in contrast to most β-LG/pectin-based interfacial layers. The former may be attributed to faster transport of peptides from the bulk to the interface, nullifying the perturbation of the interfacial tension from its equilibrium generated by the variation of the interfacial area (Shrestha, Matsumoto, Ihara, & Aramaki, 2008). Additionally, the independence of *E'* from the frequency indicates only very slight relaxation processes occur for hydrolysate-based interfacial films (Ganzevles et al., 2006). Alternatively, the relaxation processes may be faster than the timescale of the oscillation due to the simplified molecular structure of the peptides (Davis et al., 2005). In systems containing f32/f64, *E'* is similar for DH3 and DH6, suggesting the interfacial layers form equally strong complexes with pectin due to the random distribution of the carboxyl groups in these pectins (Ganzevles, van Vliet, et al., 2007). The highest overall *E'* was detected for β-LG/p35, showing no frequency dependence. The low DM and high local charge density of p35 may enhance the cohesion between complexes within the adsorbed layer and strong binding of one pectin molecule to multiple β-LG molecules in the interfacial layer (Ganzevles, Kosters, et al., 2007), eventually even preventing relaxation processes to occur.

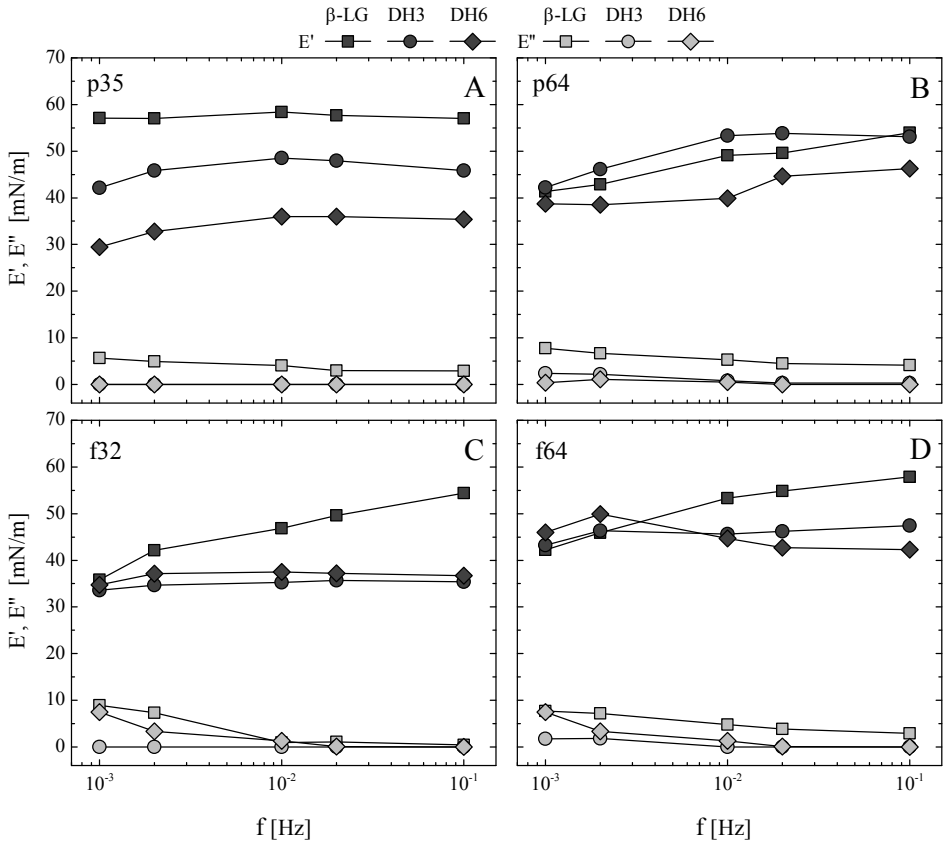

Figure 5.4 Dilatational frequency sweeps of protein/pectin solutions at pH 4.0. Bulk solutions contained 0.01 wt% β-lactoglobulin (β-LG) and hydrolysates thereof with a degree of hydrolysis of 3 % (DH3) and 6 % (DH6) and 0.01 wt% low-/ or high-methoxylated pectin, enzymatically demethoxylated by fungal- (f32/f64) or plant-derived protease (p35/p64). Numbers indicate the degree of methoxylation. Temperature 22 °C.

5.4.5 Shear time sweeps of β-LG, DH3 and DH6 as affected by the presence of pectin

The results from interfacial shear rheology experiments give insight into the mechanical strength of the intermolecular interactions of adsorbed molecules at the interface (Krägel & Derkatch, 2010; Piazza, Dürr-Auster, Gigli, Windhab, & Fischer, 2009). Accordingly, the formed lateral interactions between the molecules, i.e. hydrogen, hydrophobic, covalent bonding and electrostatic interactions result in the experimentally detected interfacial shear moduli (Bos & van Vliet, 2001). To monitor the structure formation of proteins and peptides as affected by pectin, time sweeps were performed for 9 h ($\gamma = 0.1$ %, $f = 1$ Hz). The final interfacial shear moduli after 9 h are presented in tab. 5.1.

Table 5.1 Interfacial shear modulus of protein/pectin solutions after 9 h of adsorption at pH 4.0. Bulk solutions contained 0.01 wt% β-lactoglobulin (β-LG) and hydrolysates thereof with a degree of hydrolysis of 3 % (DH3) and 6 % (DH6) and low-/ or high-methoxylated pectin, enzymatically demethoxylated by fungal- (f32/f64) or plant-derived protease (p35/p64). Numbers indicate the degree of methoxylation. Temperature 22 °C.

Pectin	Protein	Interfacial shear modulus after 9 h $\lvert G_i^* \rvert$ [mN/m]
-	β-LG	6.3
	DH3	67.5
	DH6	21.1
p35	β-LG	2.2
	DH3	103
	DH6	135
p64	β-LG	3.1
	DH3	50.6
	DH6	83.3
f32	β-LG	21.4
	DH3	88.0
	DH6	115
f64	β-LG	14.9
	DH3	40.3
	DH6	83.6

β-LG exhibits the lowest G^* independent of the type or presence of pectin (2.2 - 21.4 mN/m). However G^* increased for pure DH3- and DH6-films and even stronger interfacial layers were formed when pectin was present in the system. The strongest interfacial layers were composed of hydrolysates and LMP, due to the higher binding affinity as discussed in chapter 5.3.3, whereas DH6/p35-systems exhibited the highest overall modulus caused by the higher local charge density of the pectin compared to f32 (Wagoner et al., 2016). It has been reported the shear moduli increase with increasing internal molecular cohesion and structuring of the protein (Bos & van Vliet, 2001). Accordingly the higher shear moduli for hydrolysate-based interfacial layers in the present study may rather be attributed to the lateral intermolecular interactions and the more efficient packing of the molecules at the interface as observed in interfacial pressure development (see fig. 5.2).

In contrast to the dilatational moduli, β-LG shows clearly lower moduli than hydrolysate-based films. However, the range of shear moduli determined (G^* = 2.2-21.4 mN/m) is comparable to those reported for pure β-LG (2 mN/m) and β-LG/LMP (10 mN/m) at pH 4.5 and the same protein and pectin content at the air/water(a/w)-interface (Ganzevles et al., 2006). Additionally, the same research group reported the interfacial shear moduli of complexes of β-LG and the uncharged polysac-

charide pullulan, which was carboxylated to different charge densities at the same pH and biopolymer contents as mentioned above (Ganzevles, Kosters, et al., 2007). The surface shear experiments yielded interfacial moduli of 15 mN/m (pure β-LG) and a maximum increase due to pullulan addition to $G^* = 22$ mN/m. In another study, complexes of soy protein isolate (SPI) and HMP at the a/w-interface exhibited in a slight increase of *G'* at a SPI/HMP-mixing ratio of 1. However, when the ratio was increased to 10, a strong increase of *G'* was observed, which is within the range of *G** for pure β-LG reported in the present study (Piazza et al., 2009).

5.4.6 Shear amplitude and frequency sweeps of β-LG, DH3 and DH6 as affected by the presence of pectin

To investigate the network properties (*G'* and *G''*) as a function of strain, amplitude sweeps were applied to the interfacial layers after the time and frequency sweep as presented in fig. 5.5. For p35/p64-based systems, *G'* for the hydrolysates is at least one order of magnitude higher than films containing β-LG, which is consistent with the final results of the time sweep. Overall, LMP-based films containing hydrolysate exhibit the highest *G'* and DH6 shows the highest *G'* independent of the type of pectin present. Despite the differences observed in the magnitude of the interfacial moduli for the various protein/peptide- and pectin-combinations, the amplitude at which the breakdown of the films occurs ($\gamma \approx 10$ %) is similar for all systems studied. All hydrolysate-based films exhibit a weak strain overshoot (type III), i.e. a pronounced local maximum of *G"*, which is a characteristic of soft glassy materials (Hyun et al., 2011). This maximum has been attributed to interaction processes and binding sites within the layer indicating the balance between destroyed and newly formed bonds (Hyun et al., 2011). The overshoot indicates the network structure is getting weaker already before it breaks down completely due to larger deformation (Torcello-Gómez, Maldonado-Valderrama, Gálvez-Ruiz, et al., 2011). In contrast, β-LG films show shear thinning (type I) upon breakdown of the interfacial layers, i.e. the network segments orient with the flow field and the network will not recover (Hyun et al., 2011). Accordingly, the protein/pectin-networks under study show varying strength in their molecular interactions, as displayed by their different interfacial moduli. However, the strain at which the network gets destroyed is similar for all systems.

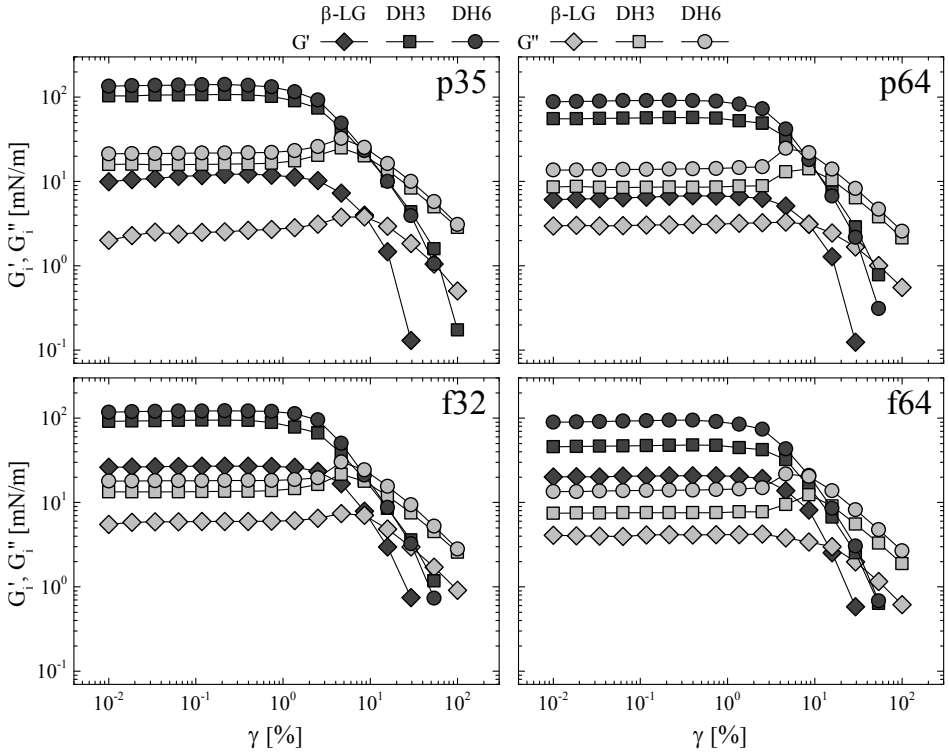

Figure 5.5 Shear amplitude sweeps of β-lactoglobulin (β-LG) and hydrolysates thereof with a degree of hydrolysis of 3 % (DH3) and 6 % (DH6). Protein films were adsorbed to the MCT-oil/water-interface for 9 h. Bulk protein and pectin content was 0.01 wt% at pH 4.0. Temperature 22 °C.

During frequency sweeps of protein/peptide-pectin systems *G'* was always higher than *G''* for all biopolymer combinations and did not exhibit a crossover (Torcello-Gómez, Maldonado-Valderrama, Gálvez-Ruiz, et al., 2011) as already observed for all shear experiments. No frequency dependence was observed for any of the systems, except slighty increasing moduli for β-LG films. The range of *G'* and *G''* was always DH6 > DH3 > β-LG, which is in accordance with the time and amplitude sweeps.

5.5 Conclusions

Partial enzymatic hydrolysis strongly affected the interfacial rheology parameters of β-lactoglobulin in the presence and absence of pectin with different degrees of methoxylation and local charge densities. The combination of interfacial dilatational and shear experiments yields integral information about the resilience of the interfacial layers under deformation. Interfacial adsorption (during pendant drop tensiometry) was delayed by complex formation of β-LG and pectin, however β-LG hydrolysates were less affected. β-LG/pectin-complexes exhibited strong frequency dependence in dilata-

tional rheology in contrast to hydrolysate/pectin-layers. During interfacial shear time sweeps β-LG formed only weak layers in comparison to hydrolysates in the presence of pectin and the interfacial shear moduli were always clearly higher for hydrolysate /pectin-layers compared to β-LG/pectin. Although the hydrolysates formed stronger interfacial layers they could not withstand higher strain during shear amplitude sweeps. Accordingly, by controlled enzymatically modifying the molecular structure of β-LG and pectin, interfacial layers with enhanced interfacial properties may be formed. Future work should focus on including further techniques to characterise the formation and structure of adsorbed mixed interfacial layers, e.g. by neutron reflectivity and time-resolved fluorescence anisotropy (Ganzevles et al., 2008) or brewster angle microscopy, whereas the latter additionally enables the in-situ investigation of the film structure during compression and expansion (Murray et al., 2009).

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6 Manuscript 4: Functional properties of pea protein hydrolysates in emulsions and spray-dried microcapsules

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6.1 Abstract

In the present study the impact of partial enzymatic hydrolysis on the functional properties of pea protein isolate (PPI) was investigated. PPI and pea protein hydrolysates (PPH) with various degrees of hydrolysis (DH) were characterised by molecular weight distribution, interfacial activity and dilatational rheology (pendant drop tensiometry) and emulsion properties (oil droplet size and ζ-potential). Their suitability for the microencapsulation of nutritional oils by spray drying and prevention of hydroperoxide formation during storage was evaluated. Only at very low DH (1 %), could stable emulsions be produced using alcalase PPH as emulsifier, most likely due to enhanced bulk aggregation, however the oil droplet size was increased compared to PPI-stabilised emulsions. In contrast, trypsin PPH-stabilised emulsions exhibited smaller oil droplets and an increased surface charge (ζ-potential) with increasing DH in comparison to PPI-stabilised emulsions. Differences observed were reflected in dilatational rheological experiments. Depending on the enzyme used to produce PPH, the dilatational moduli increased and the phase angle decreased (trypsin), i.e. stronger and more elastic interfacial layers were formed, or vice versa (alcalase). The PPI- and trypsin-derived PPH-containing emulsions were stable during atomisation and drying, resulting in high microencapsulation efficiencies (94.5 95.6 %). Trypsin PPH exhibited a higher potential than PPI to reduce lipid oxidation of rapeseed oil in spray-dried emulsions during storage as demonstrated by hydroperoxide formation. This effect may be attributed to the altered physical proper-ties of the interfacial film as well as the antioxidative effects of the hydrolysed proteins.

6.2 Introduction

The creation and stabilisation of an emulsion essentially requires surfactants like proteins to reduce the interfacial tension between oil and water and to create a gradient in interfacial tension, which facilitates droplet breakup (Walstra, 1993). The rate of lowering the interfacial tension is crucial for the stabilisation of the interface (Damodaran, 2005). A protein should meet the following properties: fast diffusion and adsorption to the newly created interface during homogenisation, unfolding of its molecular structure and the formation of highly viscoelastic films by intermolecular interactions at the interface to stabilise the increased interfacial area against (re)coalescence (Damodaran, 2005). Thus, the functional properties of proteins with respect to their application in dispersed food systems depends on the physical, chemical and conformational properties, i.e. size, shape, amino acid composition and sequence, charge and charge distribution (Rodriguez Nino et al., 2005). As a result of their structural characteristics globular proteins may possess limited emulsifying properties due to their high molecular weight,

compact tertiary structure, inclusion of their hydrophobic groups in the molecules interior and low molecular flexibility (Barac et al., 2011; Davis et al., 2005; Martinez et al., 2007; Ruiz-Henestrosa et al., 2009).

An overwhelming amount of literature is available for whey protein stabilised emulsions. Legume proteins present a serious option to replace widely used proteins from animal origin, in food and pharmaceutical applications (Aberkane, Roudaut, & Saurel, 2014; Karaca et al., 2011). Soy protein is the legume protein studied far most in the last decades (O'Kane et al., 2004). However in comparison pea proteins possess the advantages of being non-allergenic, of high nutritive value (Gharsallaoui et al., 2012), and exhibit similar emulsifying properties (Aluko, Mofolasayo, & Watts, 2009). Pea protein has been used as emulsifier in liquid emulsions (Aluko et al., 2009; Amine et al., 2014; Barac et al., 2010, 2011, 2012; Dagorn-Scaviner et al., 1987; Gharsallaoui et al., 2009, 2012; Gharsallaoui, Yamauchi, et al., 2010; Humiski & Aluko, 2007; Karaca et al., 2011), and as emulsifier in spray-dried emulsions for the microencapsulation of oil (Aberkane et al., 2014; Gharsallaoui et al., 2012).

Due to the complex molecular structure it must be hypothesised that the emulsifying properties of pea proteins are not fully exploited in the native state. A limited enzymatic hydrolysis is a suitable technique to improve the functional properties of globular proteins (Davis et al., 2005; Ipsen et al., 2001; Martinez et al., 2009; Minones Conde & Rodriguez Patino, 2005; Perez, Sanchez Carrera, et al., 2012; Ruiz-Henestrosa et al., 2007, 2009; van der Ven et al., 2001), however they were also found to be impaired in some studies (Martinez et al., 2009, 2007; Minones Conde & Rodriguez Patino, 2005; van der Ven et al., 2001). Generally, an enzymatic hydrolysis leads to a reduction in molecular weight, facilitating the coverage of a larger interfacial area in comparison to the unmodified protein (O'Regan & Mulvihill, 2010). Hydrolysis may result in the exposure of hydrophobic patches from the interior of the globular molecule (Damodaran & Paraf, 1997) and thus increases the interfacial activity (Minones Conde & Rodriguez Patino, 2007) initiated by a higher rate of diffusion (Ruiz-Henestrosa et al., 2007). Additionally, the molecular flexibility may be increased, enabling a more rapid unfolding and the formation of intermolecular interactions between adsorbed molecules (Perez, Sanchez Carrera, et al., 2012). The functional properties of peptides created by enzymatic hydrolysis depend on a multitude of factors, e.g. the specificity of the enzyme used or the degree of hydrolysis (Ipsen et al., 2001). Generally, with respect to the functional properties in emulsions, a low degree of hydrolysis (1-10 %) is favourable (O'Regan & Mulvihill, 2010). Apart from the evaluation of the oil droplet size distribution and ζ-potential to characterise protein-stabilised emulsions, the interfacial activity of proteins and peptides is often studied, i.e. the decrease in interfacial tension due to protein adsorption (Minones Conde, Escobar, Pedroche Jimenez, Rodriguez, & Rodriguez Patino, 2005). A decreased interfacial tension lowers the energy input required to create the emulsion and thus facilitates the creation of smaller droplets (Bos &

van Vliet, 2001). Furthermore the interfacial dilatational rheology of proteins has been the objective of many investigations since compression/ expansion experiments are linked to emulsion stability during emulsification (Benjamins et al., 2006).

In addition to their functional properties proteins also exhibit antioxidant properties in oil/water-emulsions (Adjonu et al., 2014; Berton-Carabin, Ropers, & Genot, 2014). These properties include the chelation of metals, free radical scavenging, binding of secondary lipid oxidation products and/or the formation of a physical barrier protecting the lipid phase (Berton-Carabin et al., 2014). Apart from extensively studied proteins, e.g. milk proteins (Serfert et al., 2014; Tamm et al., 2015), pea proteins also exhibit antioxidant properties which were improved due to enzymatic hydrolysis (Pownall et al., 2010; Zhang et al., 2013). Recently published results on hydrolysed β-lactoglobulin indicate that the antioxidant properties of peptides does also improve the oxidative stability in oils microencapsulated by spray-drying (Tamm et al., 2015), but no such approach has been reported for pea protein hydrolysates.

Aim of the present study was to investigate the impact of a limited enzymatic hydrolysis on the physicochemical properties at the oil/water-interface, encapsulation properties and antioxidant effects of pea proteins in spray-dried emulsions. The application of two proteases with varying cleaving specificity allows the generation of peptide distributions strongly differing in their functional properties. The proteins and hydrolysates were characterised by their molecular weight distribution (SEC and SDS-PAGE) and their interfacial dilatational rheology (pendant drop tensiometry). Emulsifying properties and subsequent stability of emulsions stabilised by pea protein isolate (PPI) and pea protein hydrolysates (PPH) were analysed regarding oil droplet size and ζ-potential. Rapeseed oil (as a reference for an oil with a high proportion of unsaturated fatty acids) was microencapsulated by spray-drying of emulsions stabilised by PPI or PPH and the microencapsulation efficiency and lipid oxidation in terms of hydroperoxide formation were evaluated from microcapsules.

6.3 Materials and methods

Pea protein isolate (PPI, Pisane F9, Cosucra, Warcoing, Belgium, 85 % protein as is) was kindly provided by Georg Breuer GmbH (Königstein, Germany) and stored at 4 °C. For enzymatic hydrolysis trypsin (from bovine pancreas, cat#T8003, EC: 3.4.21.4, 12238 BAEE units/mg protein) and a serine protease (alcalase 2.4L, cat# P4860, EC: 3.4.21.62, 2.59 AU/g protein) both purchased from Sigma–Aldrich (Taufkirchen, Germany) were used. Glucose syrup (DE38, C*Dry 01934) was purchased from Cargill (Krefeld, Germany). Commercial rapeseed oil was purchased at local supermarket. All chemicals were of reagent grade.

6.3.1 Enzymatic hydrolysis of PPI and molecular weight distribution of PPI and PPH

Enzymatic hydrolysis of pea protein isolate was conducted as described elsewhere (Tamm et al., 2015). In the following the pea protein hydrolysates (PPH) will be abbreviated by DHX(Y), with X being the degree of hydrolysis and Y indicating the enzyme used for modification, i.e. trypsin (T) or alcalase (A). Solutions containing 5 wt% PPI were hydrolysed to a degree of hydrolysis (DH) of 1, 2, 4, 6 or 8 % using the pH-Stat method of Adler-Nissen (1986) at pH 8.0. The DH was calculated using a h_{tot}-value of 8.41 meqv/g of protein (Owusu-Apenten, 2002) for the total number of peptide bonds in the protein substrate. The enzyme/substrate-ratio (w/w) was varied for each enzyme and DH, for hydrolysis using trypsin the following ratios have been used: DH1 (1:6500), DH2 (1:2000), DH4 (1:800), DH6 (1:600) and DH8 (1:200). Accordingly for hydrolysis via alcalase these ratios were applied: DH1 (1:700), DH2 (1:400), DH4 (1:300), DH6 (1:200) and DH8 (1:100). The inactivation of the enzyme was conducted by heating the reaction mixture to 75 °C for 30 min when the required DH was obtained. Samples for the determination of the molecular weight distribution were taken after hydrolysis and frozen until further analysis. Emulsions were prepared using freshly prepared hydrolysed samples.

Tris-tricine SDS-PAGE

The characterisation of the peptide composition of PPI and the PPH resulting from enzymatic hydrolysis was performed by SDS-PAGE under reducing and non-reducing conditions (Laemmli, 1970). The method used described in details elsewhere (Tamm et al., 2015). For reducing conditions samples were additionally spiked with 1 M DL-dithiothreitol (DTT, Sigma Aldrich). After treating the samples at 90 °C for 5min they were loaded on a 16.5 % tris-tricine gel (cat#345-0065) and separated using tris-tricine running buffer (cat# 161-0744), both Bio-Rad, performing under running conditions as advised by the manufacturer. The molecular weight (MW) markers used were commercial mixtures of polypeptides: marker 1 (cat#MWSDS17S; Sigma Aldrich, 2.5-17 kDa) and marker 2 (cat#26632; Thermo Scientific, 3.4-100 kDa).

Size exclusion chromatography (SEC)

SEC was carried out using two TSKgel G2000SW column in series (7.5 mm ID × 30 cm, particle size 10 μm, Tosh Biosciences LLC, USA) with a TSKgel CW guard column (7.5 mm ID × 4 cm, particle size 10 μm). Isocratic elution of 30 % acetonitrile containing 0.1 % TFA (v/v) was used at a flow rate of 1.0 mL/min. Elution was monitored with UV absorbance at 214 nm. The samples were diluted in water and 20 μL of 1 g/L protein/peptide solutions were injected onto the columns. Bovine serum albumin (66.7 kDa), β-lactoglobulin (18.4 kDa), α-lactalbumin (14.2 kDa), cytochrome C (12.0 kDa), bacitracin (1,422 Da), Leu-Trp-Met-Arg (605 Da) and Asp-Glu (262 Da) were used as molecular weight standards (all Sigma-Aldrich, Dublin, Ireland). The relative size distribution was estimated by integrating the relevant area under the chromatogram. Samples and standards were pre-filtered through 0.22 μm low protein binding membrane filters (Sartorius Stedim, UK) prior to application to the column. All solvents were filtered under vacuum through 0.45 μm high velocity filters (Millipore Ltd., UK).

6.3.2 Interfacial rheology of PPI and PPH

For the characterisation of the interfacial properties at the MCT oil/water(o/w)-interface an automated drop tensiometer (OCA20, Dataphysics GmbH, Germany) equipped with an oscillation drop generator (ODG20, also Dataphysics) was used and the experimental setup was described elsewhere (Tamm et al., 2012). MCT oil (CremerCoor 60/40 MCT, Cremer Oleo, Germany) was used after purification with an activated magnesium silicate (Florisil 60-100 mesh, VWR, Germany) to remove interfacially active compounds. PPI and PPH solutions were prepared with 0.5 wt% protein in distilled water and adjusted to pH 8.0. A syringe containing the protein solution was connected to the ODG and a drop of the solution was created at the tip of a needle (diameter 1.65 mm) into a cuvette of purified MCT oil. After interfacial aging for 30 min, the droplet was forced into sinusoidal oscillations, i.e. compression and expansion of the interfacial film, by variation of the drop volume at an amplitude of $\Delta A/A = 4$ % and a constant frequency of 0.1 Hz. Accordingly the time-dependent viscoelastic dilatational properties were determined at an interfacial age of 30 min. The deformation amplitude was well within the linear viscoelastic regime. Each determination consisted of 10 cycles. The variation of the droplet volume and the interfacial area accordingly results in a change of the interfacial tension, which may be estimated from the shape of the droplet via the Young-Laplace equation (Benjamins et al., 2006). The interfacial dilatational modulus E* and the phase angle ϕ may be determined from the small change in interfacial area (A) generating a change in interfacial tension (σ) as discussed in detail elsewhere (Lucassen & Van Den Tempel, 1972b). All interfacial experiments were conducted at a constant temperature of 22 °C. All experiments consisted of two replicates, except PPI and

DH4(T) were done with 4 replicates to calculate the standard deviation for all samples using the highest coefficient of variation from the 4-fold repetition.

6.3.3 Preparation of PPI- and PPH-emulsion and physical characterisation

All powders were dissolved in distilled water prior to emulsification. First, a coarse emulsion was prepared by shear homogenisation at 21.500 rpm for 90 s (Ultra-Turrax T25 basic, IKA, Germany). Subsequently the emulsion was further homogenised using a high-pressure homogeniser (Panda Plus, Niro Soavi, Germany) at 250/50 bar, applying 2 passes.

In a preliminary test emulsions stabilised with 2 wt% PPI, DH4(T) or DH4(A) and 10 wt% rapeseed oil were prepared at pH 8.0. Subsequently the oil droplet size distribution (ODSD) was determined after 24 h using laser diffraction (LA-950, Horiba Jobin Yvon GmbH, Germany). ODSD results of the volume distribution are reported as 10^{th}, 50^{th} and 90^{th} percentiles of the oil droplets and the emulsions were diluted to optimum transmission inside the instruments measuring cell. Measurements were conducted at least in triplicate.

Subsequently emulsions were prepared using 2 wt% PPI, DH1(A), DH2(A), DH1(T), DH2(T), DH4(T) and DH6(T) 10 wt% rapeseed oil at pH 8.0 and the ODSD was determined after 0 and 24 h. Additionally the ζ-potential of emulsions stabilised by PPI and hydrolysates produced by trypsin (TPPH) was detected by electrophoretic light scattering (Zetasizer Nano-ZS, Malvern Instruments GmbH, Germany), respectively. For ζ-potential measurements the emulsions were diluted 200-fold before analysis. The emulsions were characterised as described above the first set of emulsions. A refractive index of 1.47 was used for ODSD and ζ-potential measurements (*Food Chemicals Codex*, 2004). Each Emulsion was prepared three times and ODSD and ζ-potential was determined at least in triplicate.

6.3.4 Preparation of PPI- and PPH-emulsions for the microencapsulation of rapeseed oil by spray-drying and characterisation of feed emulsions and microcapsules

Feed emulsions for subsequent spray-drying were prepared as outlined in chapter 2.3 using 2 wt% PPI, DH2(T), DH4(T) or DH6(T), 10 wt% rapeseed oil and 33 wt% of glucose syrup DE38 to achieve a dry matter of 45 wt%. The pH was adjusted to 8.0 by adding 0.1 M NaOH. Spray-drying was carried out on a Mobile minor (Niro A/S, Copenha-

gen, Denmark) at 180/70 °C inlet/outlet temperature and a two-fluid nozzle operating at 1.9 bar.

ODSD of fresh and reconstituted emulsions was analysed according to chapter 2.3 to evaluate the impact of the spray-drying process. In addition the extractable oil content was determined as an extent of the microencapsulation efficiency. The method is based on a gravimetrical determination of the non-encapsulated oil accessible by extraction with petrol ether (Westergaard, 2004), which is essentially situated on and close to the surface of microcapsules (Drusch & Berg, 2008).

6.3.5 Determination of the hydroperoxide content in microencapsulated rapeseed oil

To evaluate the stability of encapsulated rapeseed oil, the hydroperoxide content was monitored for 30 weeks. Samples were stored in desiccators at room temperature in the dark and the relative humidity was adjusted to 33 % using a saturated solution of magnesium chloride.

After oil extraction from the microcapsules via a blend of 2-propanol/isooctane/water the hydroperoxide value as a degree of lipid oxidation was determined employing a well-established method with slight modifications (International Dairy Federation, 1991). For further details of the method please refer to (Morales-Medina, Tamm, Guadix, Guadix, & Drusch, 2016). All analyses were conducted by two independent oil extractions and at least three replicates from each extraction.

6.3.6 Statistical analysis

Results from the oil droplet size distribution and ζ-potential of PPI- and TPPH-stabilised emulsions were analysed using Design expert 7.0.0 (Stat Ease Inc., Minneapolis, USA). A general factorial design was created using the factors ‘degree of hydrolysis’ and ‘age of emulsions’. Additionally Grubb’s Test was used to eliminate outliers in hydroperoxide results. All significant differences reported are based on a probability value of $p < 0.05$.

6.4 Results and discussion

6.4.1 Molecular weight distribution of PPI and PPH

Generally, pea proteins can be subdivided into the main fractions globulins (50-60 %) and albumins (15-25 %) (Djoullah, Djemaoune, Husson, & Saurel, 2015). The salt-soluble globulin fraction is composed of the legumin-type (11S) and vicilin-type (7S) proteins. Legumin is a hexameric protein with a molecular weight of 360-400 kDa in its associated form, consisting of monomers composed of disulphide-linked acidic (40 kDa) and basic (20 kDa) subunits (Croy, Gatehouse, Evans, & Boulter, 1980a). The basic, hydrophobic subunit is buried in the interior, whereas the acidic, hydrophilic subunit is located in the outer part of the associated macromolecule (Braudo et al., 2006). In contrast, vicilin exists as a trimer of 160-200 kDa, essentially composed of 50 kDa- and 30-35 kDa-subunits (Croy, Gatehouse, Evans, & Boulter, 1980b). The third major globulin fraction in pea proteins, convicilin (7S), consists of 71 kDa-subunits and possesses a molecular weight of 290 kDa in its native form (Croy, Gatehouse, Tyler, & Boulter, 1980). The albumin fraction is rich in sulphurous amino acids and contains the major component PA2 (26 kDa) and the minor PA1 fraction composed of two subunits (3 & 6 kDa) (Le Gall et al., 2005).

The impact of enzymatic hydrolysis using either trypsin or alcalase on the molecular weight distribution of pea protein isolate is shown in fig. 6.1. A significant shift in the peptide composition was observed for all hydrolysates, compared to the unmodified substrate, independent of the enzyme used. The PPI essentially exhibits peptides larger than 30 kDa under reducing and non-reducing conditions, however also peptides of lower molecular weight (MW) are visible. The addition of the reducing agent DTT results in the cleavage of inter- and intramolecular disulphide bonds (Lane, 1978), accordingly the intensified bands at 40 and 20 kDa under reducing conditions may be attributed to the dissociated acidic (α-L) and basic polypeptides (β-L) of the legumin subunit (60 kDa) of unmodified PPI, respectively (Gueguen et al., 1988). The trimeric vicilin (160-200 kDa) consists of subunits of different MW displayed primarily at ~50 kDa and post-translationally cleaved fragments of this polypeptide with MW of 33, 30, 19, 16, 13.5 or 12.5 kDa, according bands could be found in the PPI peptide profile (Boulter & Croy, 1997; Casey, 1982; Croy, Gatehouse, Evans, et al., 1980a, 1980b; Croy, Gatehouse, Tyler, et al., 1980; Gueguen et al., 1988; Le Gall et al., 2005; O'Kane et al., 2004). Another band is found at 71 kDa, displaying the subunit of the third globular fraction convicilin (210 - 280 kDa) (Barac et al., 2010; Croy, Gatehouse, Tyler, et al., 1980; O'Kane et al., 2004). Several bands could be detected between 71 and 50 kDa, which might be attributed to proteolytic intermediates arising from the convicilin subunits, since similar observations have been published for PPI (O'Kane et al., 2004) and soybean β-conglycinin (Qi, Wilson, & Tan-Wilson, 1992). These intermediates origi-

nate from a protease present in the seeds and 1 or 2 kDa peptides result from the cleavage. This might explain the relatively high amount of peptides < 2 kDa found in unmodified PPI as shown by SEC analysis (see fig. 6.2). These peptides are too small to be retained in the gel network during SDS-PAGE and thus cannot be analysed using this technique.

The major component (70 %) of the pea albumin fraction 2 (PA2) may be attributed to its dimeric form with a band at 52 kDa and the monomer at 26 kDa under non-reducing and reducing conditions, respectively (Gruen, Guthrie, & Blagrove, 1987). A minor albumin fraction (Lipoxygenase) is visible around 95 kDa (Djoullah et al., 2015). Additionally pea albumin 1 (PA1) is composed of two subunits (3 and 6 kDa), but most likely its content in the PPI preparation is too low to be visualised by SDS-PAGE (Le Gall et al., 2005), however some peptides of this size were detected via SEC (fig. 6.2). The band intensity of PPI increases under reducing conditions, as the associated subunits of globulins with high MW dissociate by the addition of DTT. In contrast under non-reducing conditions they are unable to enter the resolving gel due to their size and remain in the stacking gel (Benjamin et al., 2014).

The impact of the enzymatic treatment of PPI is discussed in detail only for the globulin fractions (legumin and vicilin), since the albumins were found to possess a higher susceptibility to trypsin than the globulins (Bhatty, 1988) and intact albumins could not be detected in the their unmodified form at DH2(T) any more. According to the manufacturer the protein fractions globulins/albumins are present at a ratio of 85/15 in the PPI preparation.

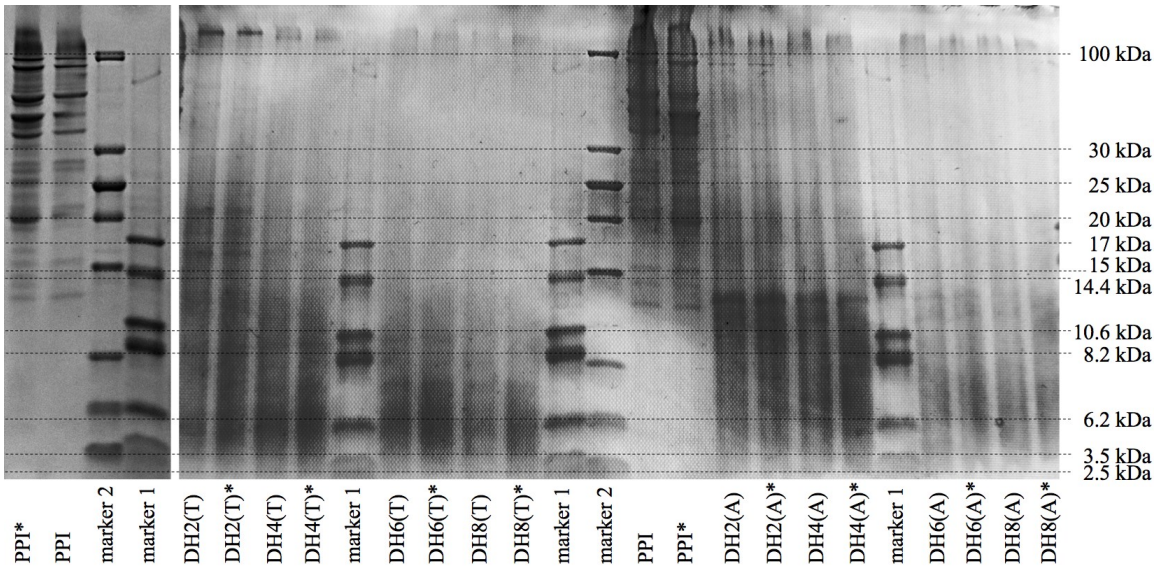

Figure 6.1 Tris-tricine SDS-PAGE of pea protein isolate (PPI) and hydrolysates thereof produced using trypsin (T) or alcalase (A) under reducing (*) and non-reducing conditions. Numbers in sample names indicates the degree of hydrolysis (DH).

The cleavage sites of trypsin in the amino acid sequence result in 71 possible peptides for the legumin subunit (UniProt: P02857) and 54 / 57 peptides for the vicilin subunits (UniProt: P13918 / Q43626), with trypsin exclusively cleaving C-terminal to Lys and Arg (Olsen et al., 2004). The examination of hydrolysates produced using trypsin (TPPH) shows a strong reduction of the MW, with the bulk of peptides ≤ 20kDa for DH2 (fig. 6.1). Additionally, bands of MW > 100 kDa, i.e. associated globulins (Benjamin et al., 2014), are observed for all TPPH with decreasing intensity for increasing DH. The maximum peptide size decreases with increasing DH, which is in good agreement with the findings of Karamać, Amarowicz, Kostyra, & Sijtsma (1998), who essentially detected peptides < 30 kDa (DH2), < 17 kDa (DH6) and < 12 kDa (DH7). This findings are supported by the SEC results for the TPPH (fig 6.2), since only 18 and 8 % of peptides > 20 kDa could be detected for DH2 and DH6, respectively. The enzymatic degradation of legumin by trypsin starts at the disordered C-terminal segment of the hydrophilic α-L chain of the 60 kDa subunit, due to a high content of Arg and Lys and its exposed position on the outside of the associated macromolecule (Braudo et al., 2006). Since the hydrophobic β-L chain is directed towards the molecules interior and thus hardly accessible for the enzyme (Plumb, Carr, Newby, & Lambert, 1989; Schwenke et al., 2001), the enzyme first degrades the intermediate α-L polypeptide, resulting in dissociation of the hexameric molecule and subsequent cleavage of the β-L polypeptides (Braudo et al., 2006; Plumb et al., 1989; Szymkiewicz & Jędrychowski, 2005). These findings support the results, as for DH2(T) only a slight band at 20 kDa is observed (fig. 6.1), i.e. remaining β-L polypeptides, and the bulk of α-L polypeptides is already cleaved into smaller peptide fractions with the exception of associated globulins > 100 kDa. The trypsin hydrolysis of vicilin was found to initially cleave the ~50 kDa subunit into the peptides also resulting from post-translational proteolysis as mentioned above (J. A. Gatehouse, Lycett, Croy, & Boulter, 1982). Some peptides of the 16 kDa fraction might have remained intact at DH2(T) and DH4(T), but none of the other bands is still visible and the bulk of peptides has a MW < 15 kDa. No literature is available about the enzymatic degradation of convicilin, but only a minor amount of unmodified convicilin was detected via SDS-PAGE at DH2(T) and disappeared for increasing DH. In contrast to the results of Le Gall et al. (2005) no resistance to trypsin hydrolysis was observed for any of the PPI fractions. The results from SEC analysis (fig. 6.2) reveal some more details of the trypsin cleavage behaviour. From PPI to DH4(T) primarily peptides > 2kDa result from hydrolysis and the content of peptides > 20 kDa remains almost constant from DH2 to DH4, i.e. essentially peptides with a MW of 2 - 20 kDa arise from hydrolysis up to DH4. Accordingly some β-L, vicilin subunits and associated globulins are still present at these DH. As the DH increases further from 4 to 6 % for TPPH only minor amounts of peptides > 20 kDa are present (8 %) and also the amount of peptides < 2 kDa increases (58 %).

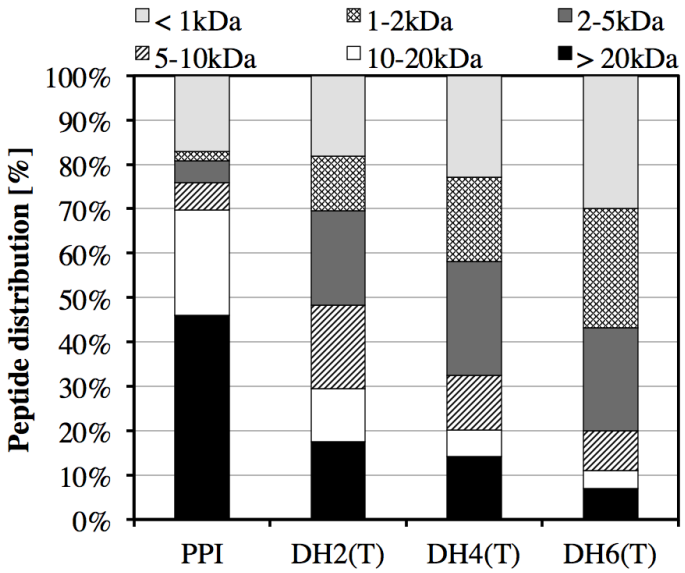

Figure 6.2 Molecular weight distribution of pea protein isolate (PPI) and their trypsin- hydrolysates as analysed by size exclusion chromatography (SEC). Numbers in sample names indicates the degree of hydrolysis.

The specificity of alcalase with respect to the cleavage of peptide bonds is significantly lower compared to trypsin (Doucet, Otter, et al., 2003) and accordingly prediction of peptides resulting from hydrolysis of PPI using alcalase (APPH) is complex. The SDS-PAGE results of APPH also exhibited bands of associated globulins > 100 kDa with decreasing intensity for increasing DH (fig. 6.1), as already observed for TPPH. The bulk of peptides is < 15 kDa at DH2(A), i.e. only minor amounts of intact globulin and albumin subunits were present at this DH. The vicilin fractions were found to be more resistant to alcalase than the legumin fractions and convicilin (fastest degradation), as demonstrated by immunoblotting (Szymkiewicz & Jędrychowski, 2005; Szymkiewicz, Wróblewska, & Jêdrychowski, 2003). Accordingly, intense bands at 13.5 and 12.5 kDa for DH2(A) and DH4(A) might be assigned to the polypeptide fragments of ~50 kDa subunits of vicilin (Gatehouse et al., 1982), as mentioned above. However, as the DH increases further to 6 and 8 % the bulk of peptides is < 8 kDa, with a large amount of peptides most likely being < 2 kDa and were not retained by the pores of the gel, accordingly. Due to the exposure of the α-L chain on the molecule's exterior (Braudo et al., 2006), the hydrolysis of legumin most likely will be initiated in this region. As Subtilisin Carlsberg, the main peptidase of alcalase, has a low specificity, it is known to produce hydrolysates with high contents of peptides < 1 kDa (Spellman, Kenny, O'Cuinn, & FitzGerald, 2005).

6.4.2 Interfacial rheology of PPI and PPH

At present there is no study available reporting the interfacial activity and interfacial dilatational rheology of pea protein hydrolysates neither at the air/water- nor at the oil/water-interface. The interfacial activity, i.e. the ability to adsorb to o/w-interfaces and decrease the interfacial tension (Minones Conde et al., 2005), is an important property of proteins during emulsification as thereby droplet breakup is facilitated and stabilisation of the newly created interface of the small droplets is enabled (Walstra, 1993). PPI and PPH solutions at the MCT oil/water-interface did not show differences with respect to their interfacial activity for the protein content and solution composition studied. All PPI/PPH solutions exhibited an interfacial tension of about 11±0.5 mN/m after 30 min of drop age (data not shown). Vicilin exhibited a higher interfacial activity compared to legumin at the dodecane/water-interface due to the lower size and higher flexibility, as reviewed by (Dagorn-Scaviner et al., 1987). Since a relatively high protein content was used in the present study an excess of protein is available for interfacial stabilisation superimposing the differences between PPI and PPH samples.

In contrast to the interfacial activity, distinct differences could be detected for the interfacial dilatational rheology parameters derived from compression/expansion experiments of the protein and peptide-stabilised films. The viscoelastic dilatational modulus E* of PPI (11.6 mN/m) and TPPH (10.3-13.0 mN/m) varied only slightly in contrast to E* for APPH films ranging between 7.3 and 8.0 mN/m , i.e. the total resistance of APPH films to deformation (elastic and viscous) was lower compared to those of PPI and TPPH (Ruiz-Henestrosa et al., 2007). The degree of E* provides information about how fast interfacial tension gradients are compensated during perturbation of the interface and how fast uniformity is restored (Lucassen & Van Den Tempel, 1972a). The viscoelastic character of the films is also reflected in the storage (E') and loss modulus (E''), with E' representing the recoverable energy stored in the interface (elastic) and E'' constituting the loss of energy via relaxation processes (viscous), respectively (Benjamins et al., 2006). All samples exhibit viscoelastic behaviour, whereas for PPI and TPPH samples the elastic contribution (E') to E* is clearly larger than the viscous part (E''). In contrast for APPH films the difference between E' and E'' is definitely lower. The phase angle ϕ clearly underlines the differences between the samples, with 0 ° and 90 ° representing a perfectly elastic and viscous interfacial film, respectively: at DH2 and DH4 for TPPH ϕ decreases (24.4 and 25.2 °) compared to PPI (34.4 °) and it increases again with increasing DH, whereas its value for DH8 (32.2 °) is close to PPI. Accordingly the character of the adsorbed films is more elastic for DH2 and DH4 films compared to PPI. In contrast to TPPH, ϕ increase for APPH films (37.5-38.5 °) in comparison to PPI independent of DH, indicating a shift to more viscous films.

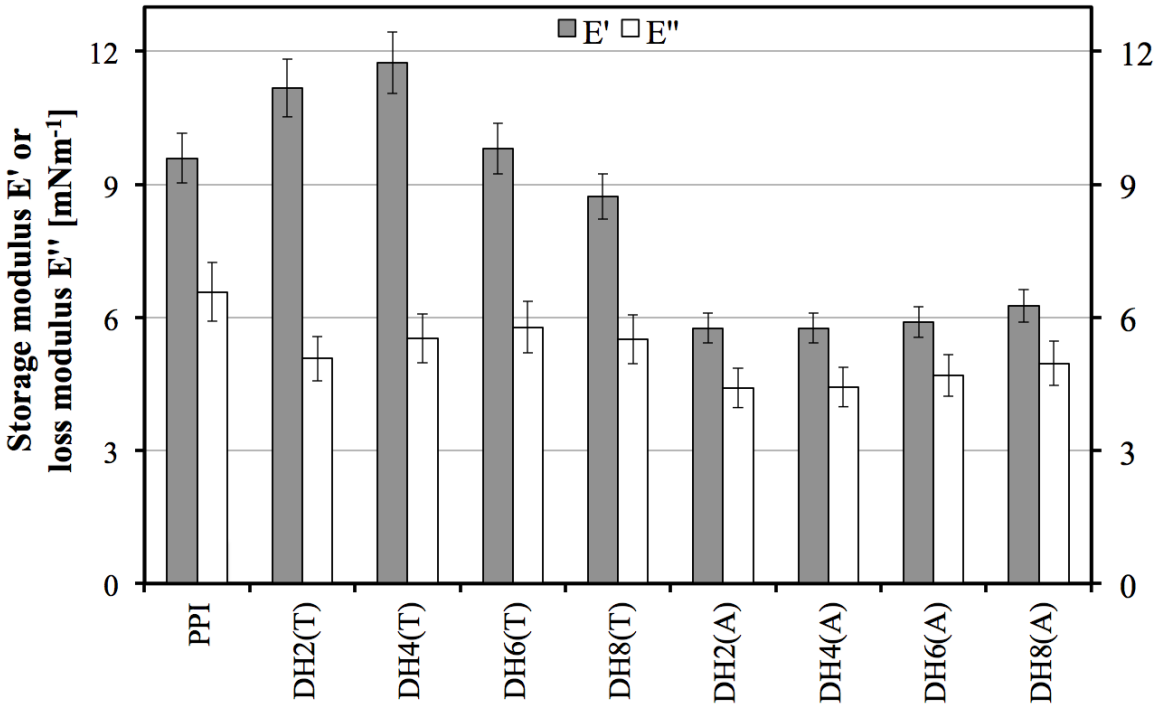

Figure 6.3 Interfacial rheological parameters of pea protein isolate (PPI) and hydrolysates thereof produced using trypsin (T) or alcalase (A) at the MCT oil/water interface. Numbers in sample names indicates the degree of hydrolysis; drop volume 28 ml; oscillation frequency 0.1 Hz; amplitude DA/A ¼ 4%; pH 8.0; protein content 0.5 wt%; temperature 22 °C.

Studies reporting the interfacial dilatational characteristics of pea protein films at the oil/water-interface are scarce. Amine, Dreher, Helgason, & Tadros (2014) reported interfacial properties of PPI compared to potato protein and Na-caseinate, however differences in pH and surface pressure do not allow comparison to the results of the present study. The dilatational rheology parameters of PPI at different pH were reported by Gharsallaoui et al. (2009, 2010), but the authors investigated the properties of PPI after long-term adsorption of the protein to the o/w-interface. It was hypothesised that in early stages of adsorption primarily vicilin adsorbs due to its higher surface activity compared to legumin (Dagorn-Scaviner et al., 1986) but is replaced by legumin during surface aging (Gharsallaoui et al., 2009).

The finding that a low degree of hydrolysis increased the viscoelastic properties of TPPH up to a DH of 4 % is in agreement with results reported in the past for other globular proteins like whey (Damodaran & Paraf, 1997; Kilara & Panyam, 2003; Perez, Sanchez Carrera, et al., 2012), sunflower protein (Minones Conde & Rodriguez Patino, 2005), soy glycinin (Ruiz-Henestrosa et al., 2009) and soy protein isolate (Martinez et al., 2009, 2007). The reduction in molecular weight accompanied by the exposure of the hydrophobic regions of the molecules (Gueguen, 1989; Kuipers, Alting, & Gruppen,

2007; Minones Conde & Rodriguez Patino, 2007; Perez, Sanchez Carrera, et al., 2012; Ruiz-Henestrosa et al., 2007) and the increased flexibility of the peptides resulting from enzymatic hydrolysis (Barac et al., 2011, 2012; Martinez et al., 2009) enables improved accumulation at the interface by protein-protein-interactions (Barac et al., 2012). This may be explained by the formation of more dense interfacial layers (Ravera, Ferrari, Santini, & Liggieri, 2005; Rodriguez Nino et al., 2005) due to the increased homogeneity of the peptide distribution (Gharsallaoui et al., 2009; Perez, Sanchez Carrera, et al., 2012) and the higher availability of binding sites for intermolecular interactions (Perez, Sanchez Carrera, et al., 2012), i.e. non-covalent intermolecular interactions and covalent intermolecular disulphide crosslinks (Bos & van Vliet, 2001). Thus this facilitates stronger viscoelastic networks at the interface due to an increased amount of interactions between adsorbed segments (Perez, Sanchez Carrera, et al., 2012). Since the peptides possess simpler molecular structure they might be able to better transmit interfacial stresses during oscillation and less energy is dissipated by rearrangement processes within the adsorbed layer compared to PPI (Davis et al., 2005). As the DH increases further to 6 and 8 %, the molecular weight of the peptides decreases and accordingly fewer possibilities are available between the peptides for the formation of a coherent interfacial network (Ipsen et al., 2001). In addition more peptides remain in the bulk phase instead of adsorbing to the interface (Lam & Nickerson, 2013; Minones Conde & Rodriguez Patino, 2007) due to increased peptide-peptide and protein-peptide interactions and decreased peptide-oil interactions (Creusot, Gruppen, Vankoningsveld, Dekruif, & Voragen, 2006). Hence the viscoelastic modulus decreases and the phase angle increases.

The interfacial properties of APPH were poor in comparison to PPI and TPPH. This may be explained by enhanced aggregation between the peptides, since the commercial enzyme preparation used (alcalase 2.4L) is known to contain a glutamyl endopeptidase responsible for peptide aggregation as observed for whey protein hydrolysates (Creusot et al., 2006; Doucet, Gauthier, et al., 2003; Doucet, Otter, et al., 2003; Spellman et al., 2005). The authors identified small molecular weight peptides (< 2 kDa) to be responsible for the aggregate formation by hydrophobic interactions (Doucet, Gauthier, et al., 2003; Doucet, Otter, et al., 2003) and the peptides were even able to aggregate with unhydrolysed protein (Creusot et al., 2006). Since alcalase contains a low specificity with respect to its cleavage behaviour it is very likely small molecular weight peptides with a strong affinity for aggregation also resulted from PPI hydrolysis using alcalase. Accordingly increased aggregation in the bulk phase may limit the adsorption to the interface as competition for the hydrophobic binding sites of APPH peptides occurs between these two processes (Middelberg, Radke, & Blanch, 2000; Zhu & Damodaran, 1994) and may explain the lower E*, E', E'' and the high ϕ compared to PPI and TPPH peptides.

6.4.3 Physical characterisation of PPI- and PPH-emulsions

With 5.48 ± 0.72 µm (90th perentile) PPI-stabilised emulsions exhibited a larger oil droplet size compared to PPH-stabilised emulsions, which was unaffected by 24 h of storage in all percentiles (tab. 1). The oil droplet size after homogenisation and physical stability of the peptide-stabilised emulsions was affected by DH and type of enzyme used. No stable emulsions could be produced using DH4(A) as emulsifier and emulsions showed phase separation immediately after homogenisation. Accordingly for the examination of the emulsifying properties of APPH a lower DH of 1 and 2 %, was chosen. Emulsification with pea protein hydrolysate with DH1(A) resulted in stable emulsions, however the oil droplet size (ODS) was increased compared to PPI and increased further during storage. At DH2 the ODS was strongly increased, i.e. 32.09 ± 1.30 µm for the 90th percentile, and the emulsion showed complete phase separation after 24 h. This may be explained by the strong tendency for aggregation for hydrolysates produced by alcalase due to the known glutamyl endopeptidase activity in alcalase as already discussed in chapter 6.3.2 (Creusot et al., 2006). Since aggregation limits the interfacially-active material available for stabilisation of the newly created interface during homogenisation (Middelberg et al., 2000; Zhu & Damodaran, 1994) this may explain why APPH possessed poor emulsifying properties compared to PPI. Additionally the dilatational moduli of APPH were rather low and thus no interfacial layer efficiently restoring interfacial tension gradients and reducing recoalescence during emulsification may have been created (Lucassen & Van Den Tempel, 1972a).

Table 6.1 Oil droplet size distribution of rapeseed oil-in-water emulsions stabilised by pea protein isolate (PPI) and hydrolysates thereof produced using trypsin (T) or alcalase (A). Numbers in sample names indicates the degree of hydrolysis. Refractive index: 1.47; pH 8.0; protein content 2 wt%; oil content 10 wt%.

Sample	Age of emulsions [h]	Percentile of oil droplets [µm]		
		D_{10}	D_{50}	D_{90}
PPI	0	1.99 ± 0.34	3.29 ± 0.50	5.48 ± 0.72
	24	2.02 ± 0.14	3.33 ± 0.18	5.49 ± 0.23
DH1(A)	0	0.33 ± 0.12	3.79 ± 0.18	7.13 ± 0.64
	24	1.03 ± 1.25	4.69 ± 0.49	8.32 ± 0.36
DH2(A)	0	0.52 ± 0.21	12.49 ± 2.70	32.09 ± 1.30
	24	-	-	-
DH1(T)	0	2.02 ± 0.14	3.31 ± 0.13	5.52 ± 0.21
	24	2.08 ± 0.12	3.41 ± 0.16	5.53 ± 0.18
DH2(T)	0	1.38 ± 0.12	2.36 ± 0.23	4.22 ± 0.36
	24	2.02 ± 0.07	3.44 ± 0.10	5.91 ± 0.18
DH4(T)	0	1.11 ± 0.01	1.58 ± 0.06	2.59 ± 0.16
	24	1.38 ± 0.08	2.34 ± 0.18	3.91 ± 0.34
DH6(T)	0	1.08 ± 0.00	1.47 ± 0.01	2.39 ± 0.02
	24	1.18 ± 0.03	1.78 ± 0.10	2.86 ± 0.19

In contrast, TPPH-stabilised emulsions at DH1 exhibited an ODSD comparable to PPI-stabilised emulsions in all percentiles with no instabilities after 24 h. As the DH was further increased, the ODS decreased in all percentiles down to the smallest droplet size for the highest DH at 6 %. The enzymatic hydrolysis results in a decrease in molecular weight, an exposure of the hydrophobic segments from the core of the molecules (Gueguen, 1989; Zhang et al., 2013) and increased molecular flexibility (Ducel, Richard, Popineau, & Boury, 2004) enabling increased interfacial activity of the peptides. In addition the peptides possess less secondary and tertiary structure (Davis et al., 2005) and are able to cover a larger interfacial area compared to the unmodified proteins (O'Regan & Mulvihill, 2010). The molecular weight distribution for TPPH is more homogenous compared to APPH and shows a lower content of low molecular weight peptides due to the enzyme specificity, allowing the formation of more dense interfacial films with higher viscoelasticity for TPPH (Krause, Wüstneck, Seifert, & Schwenke, 1998; Perez, Sanchez Carrera, et al., 2012; Rodriguez Nino et al., 2005; Ruiz-Henestrosa et al., 2007) and all TPPH effectively stabilised the newly created oil droplets during and after homogenisation (van der Ven et al., 2001).

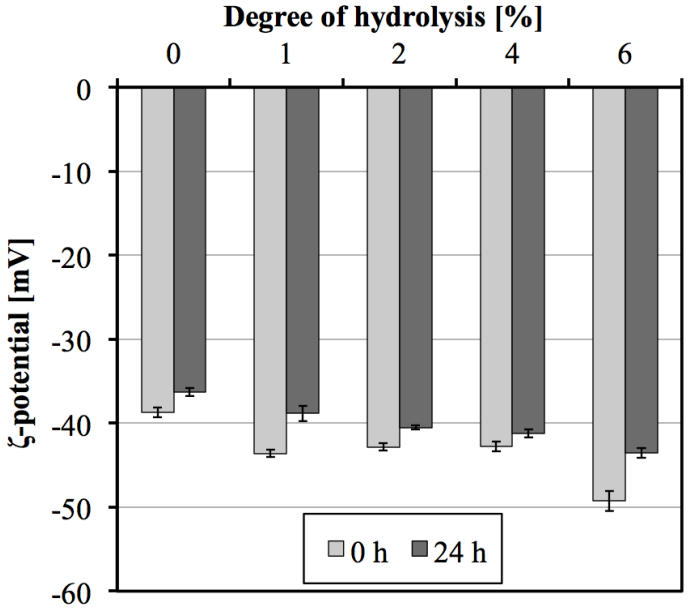

Figure 6.4 ζ-Potential of rapeseed oil-in-water emulsions stabilised with pea protein isolate and hydrolysates produced using trypsin as a function of the degree of hydrolysis. Emulsions contained 2 wt% protein, 10 wt% rapeseed oil and were adjusted to pH 8.0.

The ζ-potential for PPI- and TPPH-stabilised emulsions is presented in fig. 6.4. All emulsions were adjusted to pH 8.0 and accordingly the droplets carry a net negative charge at a pH > pI of all protein fractions (Karaca et al., 2011). With increasing DH a trend of slightly decreasing ζ-potential could be observed, which may be attributed to

the liberation of carboxylic groups due to enzymatic hydrolysis being dissociated above the pI and thus increasing the net negative charge of the peptides, as already reported for casein hydrolysates (Mahmoud et al., 1992). Additionally the exposure of charged domains within hydrophobic segments from the core of several globular proteins was found to result in an increased surface hydrophobicity accompanied by an increased absolute ζ-potential, providing enhanced stability against flocculation (Delahaije, Wierenga, van Nieuwenhuijzen, Giuseppin, & Gruppen, 2013). As trypsin hydrolysis of PPI results in increased hydrophobicity due to exposure of hydrophobic segments natively buried in the interior of the molecules (Barac et al., 2012; Pownall et al., 2010) this may additionally decrease the ζ-potential of TPPH with increasing DH and consequently increase droplet stability after 24 h as shown in tab. 1. However, an increase of the ζ-potential was observed after 24 h of storage, most likely initiated by rearrangement processes of the hydrophobic segments.

6.4.4 Microencapsulation of PPI- and PPH-stabilised rapeseed oil by spray-drying

The impact of the spray-drying process on the oil droplet size distribution of rapeseed oil-emulsions is shown in tab. 6.2. The ODS of liquid feed emulsions differed from the results presented in chapter 6.3.3, i.e. the ODS increased for PPI in all percentiles, and slightly decreased for PPH in all percentiles independent of DH. The observed effect may be attributed by the increased viscosity of the continuous phase due to the addition of glucose syrup in feed emulsions. As unmodified PPI molecules possess a significantly higher MW compared to TPPH, the transport of molecules towards the oil/water-interface may be hindered to a greater extent. These explanations are supported by the findings of Guzey, McClements, & Weiss (2003), who observed a strong decrease of the diffusion coefficient of bovine serum albumin (BSA) at the air/water-interface at pH 7 due to the addition of up to 40 wt% of glucose. Similar observations were reported for soy protein isolate (Ruiz-Henestrosa, Carrera Sanchez, & Rodriguez Patino, 2008) and ovalbumin (Antipova, Semenova, & Belyakova, 1999) in the presence of sucrose. The decrease of the surface activity was not only ascribed to the increase in solution viscosity but also to an increase in protein hydrophilicity due to a layer of sugar molecules binding around the protein via carboxyl groups of the proteins and hydroxyl groups of the sugars (Guzey et al., 2003). An increase of the hydrophobic interactions in the core of globular proteins by to the addition of sugars (McClements, 2002) may further stabilise their globular structure in solution and thus decrease the overall free energy due to the proteins adsorption to an interface, which is the thermodynamic driving force initiating this process (Guzey et al., 2003). However, the increase in viscosity may be the dominating factor affecting the shift in droplet size due to the addition of glucose syrup in this study.

All emulsions were stable during the spray-drying process and the ODS even slightly decreased in all percentiles for PPI, DH2(T) and DH4(T) emulsions as a result of the shearing forces affecting the droplets in the atomising nozzle, as already reported (Soottitantawat et al., 2003). The authors observed a decrease in oil droplet size only for droplets > 2 µm, whereas smaller droplets were not affected by atomisation, which agrees with the findings of the present study.

Table 6.2 Oil droplet size distribution of rapeseed oil-in-water emulsions (liquid and reconstituted after spray-drying) stabilised with pea protein isolate (PPI) or hydrolysates thereof produced using trypsin (DHX(T)) and microencapsulation efficiency of spray-dried microcapsules. Numbers in sample names indicates the degree of hydrolysis. Liquid emulsions contained 2 wt% protein, 10 wt% rapeseed oil, 33 wt% glucose syrup DE38 and were adjusted to pH 8.0.

Sample	Percentile of oil droplets [µm]			Microencapsulation efficiency [%]
	D_{10}	D_{50}	D_{90}	
Liquid emulsions before spray-drying				
PPI	2.60 ± 1.76	5.64 ± 1.12	9.85 ± 1.44	-
DH2(T)	0.51 ± 0.03	1.49 ± 0.26	2.80 ± 0.58	-
DH4(T)	0.66 ± 0.03	1.24 ± 0.07	2.06 ± 0.16	-
DH6(T)	0.40 ± 0.05	1.00 ± 0.05	1.76 ± 0.06	-
Reconstituted emulsions after spray-drying				
PPI	1.47 ± 1.39	4.26 ± 1.12	7.83 ± 1.47	94.47 ± 0.12
DH2(T)	0.51 ± 0.02	1.36 ± 0.18	2.56 ± 0.40	95.42 ± 0.09
DH4(T)	0.53 ± 0.02	1.14 ± 0.04	1.99 ± 0.05	95.35 ± 0.10
DH6(T)	0.36 ± 0.03	0.99 ± 0.04	1.98 ± 0.05	95.60 ± 0.10

The microencapsulation efficiency (ME) was high for all samples, varying between 94.5 % for PPI-microcapsules and 95.4 – 95.6 % for TPPH-stabilised microcapsules and is in good agreement with previously reported results from our research group for whey protein isolate (Serfert et al., 2014), whey protein hydrolysates (Tamm et al., 2015), fish protein hydrolysates (Morales-Medina et al., 2016) and n-OSA starch (Serfert, Drusch, Schmidt-Hansberg, et al., 2009). Since larger droplets are more prone to disruption during atomisation and may cause higher amounts of surface oil on the powder surface (Aberkane et al., 2014), the slightly lower ME for PPI might be attributed to the higher ODS compared to TPPH. The high ME results from the choice of the type of glucose syrup with a dextrose equivalent of 38, since this degree of modification yields less porous, more uniform glassy matrices upon drying in comparison to low DE products, efficiently entrapping the encapsulant (Hogan, McNamee, O'Riordan, et al., 2001).

6.4.5 Lipid oxidation of rapeseed oil in spray-dried emulsions stabilised by PPI and PPH

Due to the poor physical stability of the emulsions prepared with APPH and the large oil droplet size, spray-drying was only performed with emulsions containing PPI and TPPH. Fresh rapeseed oil used for the preparation of microcapsules exhibited no detectable hydroperoxide content. However the microcapsules produced by spray-drying contained up to 2.6 ± 0.6 mmol/kg oil after emulsification, atomisation and drying. Oxygen inclusion during premix emulsification, enhanced distribution of oxygen, pro-oxidants and lipid oxidation products and the increasing interfacial area throughout homogenisation may result in the deterioration of the oil as well as lipid oxidation during the atomisation and drying step (Serfert, Drusch, & Schwarz, 2009). During the first six weeks of storage only a slight increase of the hydroperoxide content was observed for all samples, whereas from 12 weeks upwards the PPI-stabilised microcapsules showed a more distinct increase in comparison to TPPH-stabilised samples (fig. 6.5). After 30 weeks of storage PPI-microcapsules exhibit the highest level of hydroperoxides (24.7 mmol/kg oil), compared to TPPH-microcapsules exhibiting considerably less (10.4 – 15.5 mmol/kg oil), with decreasing contents with increasing DH of the hydrolysates. Since the only variation in microcapsule composition is the type of emulsifier used, i.e. PPI or TPPH in various DH, the observed differences in hydroperoxide formation must be ascribed to the functional and antioxidant properties of the proteins.

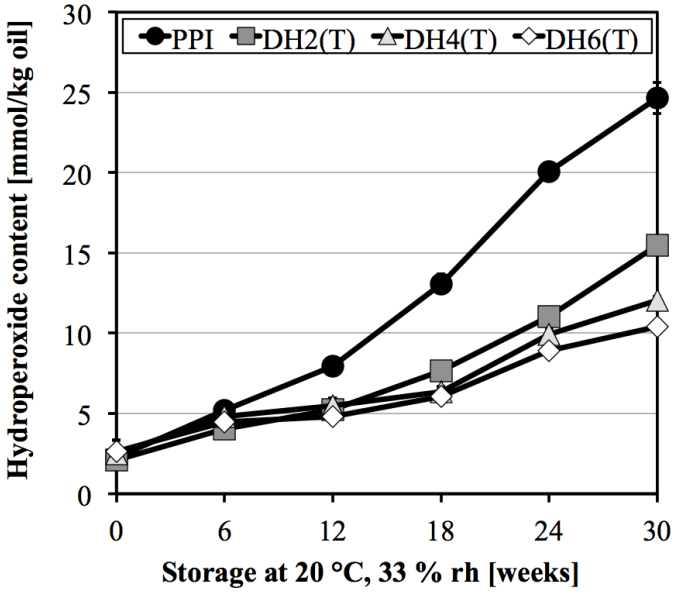

Figure 6.5 Development of hydroperoxide content of microencapsulated rapeseed oil stabilised with pea protein isolate (PPI) and hydrolysates thereof produced using trypsin (DHX(T)). Samples were stored in the dark. Numbers (X) in sample names indicates the degree of hydrolysis of the pea protein hydrolysate used.

Lipid oxidation is initiated at the o/w-interface and thus the most effective antioxidants are amphiphilic molecules like proteins being adsorbed at the interface (McClements & Decker, 2000). Proteins and peptides remain at the interface during and after spray-drying, accordingly they also stabilise the oil within the glassy matrix of microcapsules during storage. To some extent pea proteins already exhibit antioxidant properties in their native form, although in unmodified PPI the bulk of antioxidative amino acids is inaccessible due to association and molecular tertiary structure. An enzymatic hydrolysis may expose these amino acids and enable antioxidative activity of the segments. However, an enzymatic hydrolysis may result in an increase or decrease of the antioxidative activity as determined by in vitro assays. Although high amounts of the amino acids (AA) with the highest antioxidant capacity, i.e. the sulphur-containing AA Cys and Met and Trp (Humiski & Aluko, 2007; Zhang et al., 2013) are lacking in pea proteins, they contain comparably high amounts of hydrophobic and aromatic AA, like Pro, Val, Leu, Ile and Phe, which also possess antioxidant activity (Pownall et al., 2010). Although theses AA constitute less than 10 % of the storage protein in seeds, the albumin fraction provides 50 % of the sulphur AA (Boulter & Croy, 1997), possesses a higher susceptibility to trypsin hydrolysis and thus is most likely already cleaved at the early stages of hydrolysis (Bhatty, 1988), exposing the highly antioxidant AA Cys and Met. The DPPH radical scavenging activity of PPI hydrolysates produced using trypsin was found poor compared to other enzymes and results from other studies (Humiski & Aluko, 2007). Zhang et al., (2013) reported a significant inhibition of 2-thiobarbituric acid reactive substances (TBARS) in a liposomal model system stabilised using TPPH compared to the unmodified PPI. The authors ascribed the reduced TBARS to the stabilisation of radicals, to the binding of pro-oxidative Fe^{2+} and to the formation of a physical TPPH peptide barrier around liposome particles to separate them from oxidation initiators. Besides the TPPH, the authors identified several peptides resulting from PPI hydrolysis using Flavourzyme and Protamex and a MW of 1-2 kDa (9-17 AA residues), primarily composed of Leu, Lys, Glu, Gln, Val, Pro and hydrophobic AA at the N-terminus, strongly inhibiting TBARS formation. Since the content of peptides with a MW of 1-2 kDa increases for proceeding DH in TPPH, i.e. from 20 % for PPI up to 67 % for TDH(6), it is most likely TPPH contained more antioxidant AA with advancing enzymatic degradation. Furthermore several peptides with essentially hydrophobic AA at the N-terminus, i.e. Phe, Ile, Val, were identified resulting from vicilin hydrolysis using trypsin (Gatehouse et al., 1982). Additionally, it was hypothesised the orientation of negatively charged side chains of TPPH towards the aqueous phase increased the liposome surface charge and protected the liposomes against cationic pro-oxidants and anionic radicals, like superoxides (Zhang et al., 2013). The aforementioned observations may explain the increased oxidative stability of microencapsulated rapeseed oil stabilised by hydrolysates of PPI produced using trypsin.

The ODSD of the reconstituted emulsions showed, that the interfacial area of oil in PPI microcapsules was by far smaller compared to TPPH microcapsules (see tab. 6.2). Generally, a higher interfacial area resulting from a decrease in droplet size is expected to favour the possible contact between oil phase and pro-oxidants (e.g. free radicals, metals) or oxygen, accordingly several authors reported an increase in lipid oxidation due to a decrease in droplet size, as reviewed by Berton-Carabin, Ropers, & Genot (2014). However, lipid oxidation in linoleic acid emulsions stabilised with whey protein isolate (WPI) or sodium caseinate (SC) was decreased when the oil droplet size decreased and the authors ascribed this effect to more protein bound per g of oil per droplet und thus more protein interacts with lipids enhancing its antioxidative effects (Ries, Ye, Haisman, & Singh, 2010). In the present study the hydroperoxide content decreased as the oil droplet size of the emulsions decreased, accompanied with an increase in DH. In addition, an increase of DH was reported to enhance packaging and condensation of β-lactoglobulin hydrolysates at the interface (Perez, Sanchez Carrera, et al., 2012) most likely due to a more homogenous peptide size (Gharsallaoui et al., 2009) allowing more intermolecular interactions between the adsorbed peptides (Perez, Sanchez Carrera, et al., 2012). Thus the formation of an improved physical barrier protecting the oil droplets may be facilitated with increasing DH. Accordingly in summary, TPPH films at the interface provided an improved physical barrier and enhanced antioxidant properties with increasing DH and counterbalanced the effect of a higher interfacial area facilitating oxidation (Berton-Carabin et al., 2014).

6.5 Conclusions

The functional properties of enzymatic hydrolysates of pea protein isolate were strongly affected by the degree of hydrolysis and the type of enzyme used. The low specificity of alcalase and most likely an increased tendency for aggregation in the bulk negatively affected the functional performance of peptides produced by this enzyme. In contrast trypsin proved to be a suitable enzyme to create hydrolysates from PPI possessing superior physicochemical and antioxidant properties in comparison to PPI. Apart from the formation of interfacial films possessing enhanced viscoelasticity, adsorbed TPPH peptides facilitated a higher surface charge of emulsion droplets and the coverage of a larger interfacial area, especially when increasing the continuous phase viscosity. In addition, TPPH peptides exhibited good encapsulation performance during spray-drying and limited lipid oxidation of microencapsulated rapeseed oil during storage. Future studies should focus on the isolation of specific peptides involved in structure formation at oil/water-interfaces to gain deeper insight in stabilising mechanisms and the pea protein fractions involved in these processes.

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7 General discussion

7.1 Impact of Enzymatic Hydrolysis on the Molecular Characterstics of β-Lactoglobulin and Pea Proteins

In the present study, controlled enzymatic hydrolysis of β-lactoglobulin (β-LG) and pea proteins was conducted by the pH-stat method (Adler-Nissen, 1986) using bovine trypsin and alcalase. The effect on the molecular weight profile of the hydrolysates was evaluated by tris-tricine SDS-PAGE, due to its superior resolution between 5 and 20 kDa (Schägger & Von Jagow, 1987) and size exclusion chromatography (SEC) (O'Loughlin et al., 2013). The modification resulted in a significant shift in the molecular weight distribution for all hydrolysates (see fig. 3.1, 3.2, 4.1, 5.1, 6.1 and 6.2). Since two endopeptidases with different specificity for preferably cleaved peptide bonds were used, the peptide distributions of the hydrolysates strongly varied. Trypsin possesses a higher specificity, since it only cleaves peptide bonds C-terminal to Lys and Arg (Olsen et al., 2004), and accordingly the peptides resulting from enzymatic attack are easier to predict. The high specificity yields for both proteins peptide mixtures with a narrow peptide profile (fig. 3.1 and 6.1) and a low content of low molecular weight peptides (< 2 kDa), which are known to exhibit poor functionaliy as emulsifiers (Turgeon et al., 1992). Due to the specificity of trypsin, 18 scissile bonds are available within the β-LG molecule (Seronei Chelulei Cheison et al., 2010) in comparison to 71 and 54/57 available bonds to tryptic attack for the main protein fractions from pea protein isolate (PPI), legumin (UniProt: P02857) and vicilin (UniProt: P13918/Q43626), respectively. It has been reported, the internal core of β-LG possesses a relative resistance to trypsin cleavage, which results in many relatively large intermediary peptides at the initial stages of hydrolysis (Fernández & Riera, 2013). Accordingly, the peptide bonds on the surface of the molecule, being easier accessible to the enzyme, are cleaved first before the resulting intermediary peptides are further degraded. Similar observations have been reported for enzymatic hydrolysis of pea proteins. The tryptic attack starts on easily accessible molecular patches like a disordered segment on the legumin macromolecule, which subsequently leads to the loss of the hexameric molecular structure and the exposure of the hydrophobic patches to trypsin cleavage (Braudo et al., 2006). This clearly shows the challenging prediction of peptides resulting from enzymatic action for an enzyme with high specificity like trypsin, especially for substrates like pea proteins exhibiting a high molecular weight and many potential cleavage sites. The peptide profiles and the process of enzymatic degradation by trypsin are discussed in more detail in chapter 3.4.1 and 6.4.1 for β-LG and PPI, respectively.

In comparison, the peptide profiles of hydrolysates from β-LG and PPI produced with alcalase contained fragments with a larger variety of molecular weight (fig. 3.1 and 6.1), compared to those produced by trypsin. Due to the low specificity, also a higher amount of low molecular weight peptides (< 2 kDa) was present in the peptide mixtures (fig. 3.2). The detection limit of the SDS-PAGE gels used (fig. 3.1 and 6.1) is 2 kDa, accordingly these small peptides were not retained by the gel network and do not appear in the peptide profiles. Accordingly, the determination of these peptides is only possible by SEC (fig. 3.2). It turns out, that due the lower specificity of Subtilisin Carlsberg the predicition of the resulting peptides is much more complex for this enzyme. In addition, the commercial preparation alcalase also contains minor amounts of other proteases, yielding even more possible cleavage sites in the molecular structure of the substrates (Doucet, Otter, et al., 2003; Svendsen & Breddam, 1992). Since a glutamyl endopeptidase content is present in the alcalase preparation, small hydrophobic peptides with a strong tendency to aggregation are genereated due to enzymatic attack, resulting in in-creased peptide-peptide and protein-peptide interactions in the bulk (Creusot, 2006; Doucet, Otter, et al., 2003; Spellman et al., 2005).

In summary, controlling the enzymatic hydrolysis of β-LG and PPI using trypsin is facilitated due to the high enzyme specificity, yielding peptide mixtures with a narrow molecular weight profile. In contrast, the low specificity of the commercial protease preparation alcalase produces broad molecular weight profiles with a high probability of containing peptides with strong tendency to aggregate, which may limit the functionality of these hydrolysates.

7.2 Physical Stability of Oil/Water-Interfaces

In the present study, oil/water-interfaces were physically stabilised using:

- globular proteins from two different sources (β-LG and pea protein)
- enzymatic hydrolysates of these proteins produced by two endopeptidases
- mixed biopolymer systems containing non-surface-active pectin and proteins or hydrolysates

The proteins and hydrolysates were characterised related to their interfacial adsorption and the interfacial rheology of their layers (under shear and dilatation). In addition, they were used for the formation of feed emulsions, which were subsequently spray-dried to microencapsulate the emulsified oil. With respect to the bilayer formation of pectin and proteins or hydrolysates, the charge reversal of the droplet surface was monitored by their ζ-potential. The process stability of the emulsions was monitored by the oil droplet size distribution of liquid and reconstituted emulsions and by the microencapsulation efficiency of the resulting capsules.

7.2.1 Physical Stability of Oil/Water-Interfaces Containing Proteins and Hydrolysates

Unmodified β-LG successfully adsorbed to the oil/water-interface, increased the interfacial pressure and thus exhibited interfacial activity, respectively (annex A1). However, due to enzymatic hydrolysis, the interfacial activity increased (annex A1), i.e. the peptides increased the interfacial pressure faster and more effectively (Dickinson, 1992). After their formation, the layers were subjected to dilatational and shear deformation to evaluate their stability to physical stresses. The elastic moduli of the interfacial layers during dilatational amplitude (annex A4) and frequency sweeps (annex A2) increased due to enzymatic hydrolysis. However, the increase in the complex and elastic interfacial shear moduli caused by the enzymatic modification of β-LG was more pronounced for trypsin hydrolysates and only slight for alcalase hydrolysates (annex A3 and A4). Despite this increase in the viscoelastic response due to shear deformation, the hydrolysate-stabilised interfacial layers did not withstand higher strain compared to the unmodified protein. The results mentioned before were determined at pH 8.0 to compare them to the emulsification and spray-drying experiments in manuscript 1 (chapter 3). Additionally, the experiments were conducted at pH 4 (see chapter 5), however these results obtained at pH 4.0 show the same trend and thus they will not be further discussed here (see chapter 5).

During emulsification, atomisation and drying, β-LG and its hydrolysates demonstrated good droplet stabilisation. This was reflected in a similar oil droplet size in all percentiles in liquid feed emulsions and reconstituted emulsions after the spray-drying process

(tab. 3.1). The microencapsulation efficiency was high for all samples (99.0 ± 0.5 %) (chapter 3.4.3). One exception from these results was observed when the protein content of the feed emulsions was strongly reduced. As a consequence, the tryptic hydrolysate of β-LG with a degree of hydrolysis of 6 % (DH6) exhibited larger feed emulsion droplets compared to β-LG (tab. 4.1). In addition, the droplet size decreased during atomisation and drying and yielded a lower microencapsulation efficiency compared to β-LG microcapsules.

Enzymatic hydrolysis of β-LG increased its interfacial activity independent from the enzyme used, which can be attributed to faster adsorption kinetics resulting from an increase in surface hydrophobicity and a decrease in molecular weight (Davis et al., 2005; Jung et al., 2010), accompanying a decrease of the kinetic barrier to adsorption developed by native proteins (de Jongh & Wierenga, 2006; Sengupta, Razumovsky, & Damodaran, 1999). The increase in hydrophobicity also enhances the incompatibility of the molecules with the aqueous phase, which is regarded as the main driving force for the interfacial adsorption of proteins and peptides, since it enables the removal of the non-polar molecule parts from the aqueous bulk phase (Dickinson, 1992, 2011b). The hydrolysates increased the interfacial pressure more effectively, which indicates a higher packing density at the interface (Murray, 2011). Despite the differences in interfacial activity, the oil droplet size of the feed emulsions was similar for β-LG- and hydrolysate-stabilised systems. The rate of adsorption is not a factor under turbulent homogenising conditions at a protein content of 1-3 %, but rather the rate at which the interfacial tension is decreased by the emulsifier (Damodaran, 2005). Thus the differences in surface pressure increase may not have been distinct enough to affect the droplet size of the emulsions. Accordingly, the unmodified protein and its hydrolysates exhibit similar efficiency to stabilise the newly created droplet interfaces during emulsification at this protein and hydrolysate content. However, when the hydrolysate content was reduced from 2 wt% (tab. 3.1) to 0.25 wt% (tab. 4.1), DH6-stabilised emulsions exhibited an increased oil droplet size (from 1.4 µm to 5.0 µm in the 90^{th} percentile). This may be attributed to a deficiency of peptides with good emulsifiying properties at this hydrolysate content to effectively stabilise the interfacial layers against recoalescence during emulsification due to poor steric effects (caused by a DH of 6 %) and an insufficient surface charge of the droplets to enable effective repulsion (as observed by a low ζ-potential (tab. 4.1)) (Adjonu et al., 2014; Dalgleish, 1997; Damodaran, 2005; Tirok, Scherze, & Muschiolik, 2001). However, it is most likely, that these deficiencies were compensated by the use of higher hydrolysate contents in the bulk. Due to their size, those larger oil droplets were affected by the shearing forces within the atomising nozzle in the spray-drying process, which caused a reduction in the oil droplet size in all percentiles (Soottitantawat et al., 2003). Accordingly, the microencapsulation efficiency for these microcapsules was clearly lower compared to all other samples, which may be explained by the leakage of oil due to the shearing caused by the nozzle, as observed for

the microencapsulation of flavour by spray-drying (Soottitantawat et al., 2003). The viscoelastic moduli of DH6 were higher than β-LG under shear and dilatational deformation indicating stronger interfacial layers, which is an important factor to counteract coalescence (Damodaran, 2005) Accordingly, the hypothesis is supported, that a hydrolysate content of 0.25 wt% was insufficient to stabilise the amount of oil present in the formulation. In addition, it was observed, that the strength of the viscoelastic moduli increased when increasing the protein content in the bulk, which would increase the stability to droplet coalescence, (Mitropoulos et al., 2014).

Unmodified β-LG forms highly viscoelastic layers at the oil/water-interface, resulting from a high packing density and strong intermolecular interactions (Dickinson, 2001; Roth, Murray, & Dickinson, 2000; Rühs, Scheuble, et al., 2013). This was also observed in the present study under shear and dilatational deformation, however, the viscoelastic moduli increased further, when hydrolysed β-LG was used (annex A1-3). This increase may be ascribed to the higher packing density of the peptide molecules in comparison to the globular β-LG, as already indicated by the higher surface pressure of the hydrolysates (Benjamins et al., 2006; Lucassen-Reynders et al., 2010; Mitropoulos et al., 2014; Perez, Sanchez Carrera, et al., 2012). In the present study, the DH was limited to a maximum of 6 %, since hydrolysates of higher DH displayed decreased interfacial activity and viscoelasticity in preliminary experiments due to their low molecular weight (data not shown). This can be explained by an increased tendency to aggregation of small peptides, especially those produced by alcalase (Creusot, 2006), which causes limited adsorption, since the adsorption and aggregation processes compete for the peptides' hydrophobic contacts (Davis et al., 2005). Furthermore, low molecular weight peptides do not possess distinct hydrophilic and hydrophobic patches to enable the formation of a coherent interfacial layer due to fewer possibilities for interactions (Foegeding et al., 2002; Ipsen et al., 2001; Singh & Dalgleish, 1998). However, when the DH of the hydrolysates is limited and thus the bulk of peptides exhibits a sufficient molecular weight, the interfacial layers might be able to better transmit interfacial stresses, due to the simpler molecular structure and less possible rearrangement processes, which results in increased interfacial moduli with a more elastic character (Davis et al., 2005).

In contrast to β-LG, PPI exhibited a similar interfacial activity compared to its hydrolysates (see chapter 6.4.2). For tryptic hydrolysates with a low DH (2-4 %), the elastic dilatational modulus increased and the viscous modulus decreased, however when the DH was further increased, the moduli were similar to PPI (fig. 6.3). Hydrolysates produced by alcalase generally possessed clearly lower moduli compared to PPI and trypsin hydrolysates, which were constant for DH 2-8 %.

Since emulsions stabilised with PPI-hydrolysates produced with alcalase (APPH) showed an increase in the oil droplet size (50^{th} and 90^{th} percentile) in comparison to PPI

(tab. 6.1), APPH were not used for the microencapsulation experiment. In contrast to β-LG and its hydrolysates, the oil droplet size in feed emulsions exibits a distinct decrease in all percentiles with increasing DH of PPI-hydrolysates produced by trypsin (TPPH) (tab. 6.2). The spray-drying process only affected the oil droplet size of PPI-emulsions, which was reduced in reconstituted emulsions and yielded a high microencapsulation efficiency for PPI (94.5 %) and TPPH (95.4-95.6 %, tab. 6.2).

The enzymatic hydrolysis of PPI did not affect its interfacial activity. It has been shown, that the protein content in solution has a high impact on the diffusional transport of the proteins to the interface (lag phase) and the rate of lowering the interfacial tension (Drusch, Hamann, et al., 2012; Tamm et al., 2012). Accordingly, since a comparatively high protein content was used for the experiment (0.5 wt%), this might have superimposed possible differences in the interfacial activity, which can be expected due to the shift in molecular weight (fig. 6.1 and 6.2) caused by enzymatic hydrolysis. In contrast to β-LG, the choice of the enzyme used for hydrolysis of PPI yielded peptides with varying emulsifying properties of TPPH and APPH. Emulsions containing APPH generally exhibited an increased oil droplet size in all percentiles compared to PPI- and TPPH-emulsions and only at a DH of 1 % emulsion stability for 24 h could be achieved (tab. 6.1). When the DH of APPH was increased to 2 %, a sixfold increase of the oil droplet size occurred (90^{th} percentile) compared to PPI-emulsions. According to this, emulsions immediately separated into two phases after homogenisation, when the DH was further increased. The dilatational moduli of APPH were clearly lower compared to the other samples, which decreased the protection against recoalescence during emulsification (Damodaran, 2005). In addition, hydrolysates produced by alcalase are known to contain peptides with high aggregation potential (Creusot, 2006; Doucet, Otter, et al., 2003; Spellman et al., 2005), which limits their interfacial properties and may explain their poor emulsifiying and interfacial dilatational properties of APPH (Davis et al., 2005). Due to the increased droplet size of PPI-stabilised feed emulsions, the droplets were affected by the shearing forces within the atomising nozzle as discussed above for the tryptic β-LG-hydrolysate with DH6 and a low protein content. The shearing effect of the nozzle generated a decrease in oil droplet size and a lower micro-encapsulation efficiency for PPI- compared to TPPH-stabilised emulsions (Soottitantawat et al., 2003). When the DH of TPPH increased, the oil droplet size of the feed emulsions decreased and was not affected by atomisation and drying. The molecular weight distribution for TPPH was more homogenous compared to APPH due to the enzyme specificity, allowing the formation of more dense interfacial films with higher viscoelasticity for TPPH, which enabled an increased stability to droplet coalescence (Damodaran, 2005; Krause et al., 1998; Perez, Sanchez Carrera, et al., 2012; Rodriguez Nino et al., 2005; Ruiz-Henestrosa et al., 2007). It was hypothesised in chapter 1 (hypothesis 4a), that an enzymatic hydrolysis of pea proteins can improve the func-tionality of the protein with respect to the physical stability of lipids during emulsification and spray-drying.

This hypothesis has proved to be true by the decrease of the oil droplet size in all percentiles in hydrolysate- compared to PPI-stabilised feed emulsions and reconstituted emulsions after spray-drying. Additionally, the microencapsulation efficiency was slighty increased in hydrolysate-stabilised microcapsules (see manuscript 4).

In summary, both β-LG and PPI successfully stabilised oil/water-interfaces. However, since β-LG molecules are smaller, more flexible and possess a higher surface hydrophobicity (Can Karaca et al., 2015; Gueguen, 1989; Jiang et al., 2014), their transport and adsorption to the interface during emulsification is facilitated (Damodaran, 2005). Accordingly, β-LG and its hydrolysates (independent from the enzyme used) produced stable emulsions, being unaffected by atomisation and drying. PPI-emulsions contained larger oil droplets, showing instability during spray-drying, however tryptic hydrolysis of PPI strongly improved the emulsifiying properties and process stability. In contrast, alcalase produced PPI hydrolysates with poor functionality. With respect to interfacial rheology, hydrolysis improved the functionality of β-LG and PPI, except for APPH. The enhancement in functionality with respect to the physical stabilisation of oil/water-interfaces due to enzymatic hydrolysis was more pronounced for PPI compared to β-LG, since β-LG already exhibited considerably better functionality in its unmodified form.

7.2.2 Physical Stability of Oil/Water-Interfaces Containing Proteins and Hydrolysates as Affected by Pectin

The interfacial activity of β-LG and its hydrolysates was affected by pectin, which strongly depended on the DH of the hydrolysate as well as the degree of methoxylation (DM) and the local charge density of the pectin (fig. 5.2). When the DH of the hydrolysates increased, their interfacial activity was less influenced compared to unmodified β-LG, independent of the type of pectin. For β-LG the interfacial adsorption was delayed due to the presence of low-methoxylated pectin (LMP), being most pronounced for LMP with a high local charge density. However, high-methoxylated pectin (HMP) only slightly influenced the adsorption of β-LG. During dilatational rheology experiments and in the presence of pectin, β-LG systems exhibited frequency dependence in contrast to the hydrolysates and all biopolymer systems provided good stabilisation of the interfacial layers (fig. 5.4). In contrast, in interfacial shear rheology experiments, β-LG/pectin formed clearly weaker layers than hydrolysate/pectin, although all interfacial layers were destabilised at the same strain during amplitude sweeps (fig. 5.5).

Furthermore, the impact of pectin on the process stability of protein layers during the microencapsulation of oil has been investigated. In addition to β-LG, a tryptic hydrolysate with a DH of 6 % (DH6) was applied as emulsifier in this experiment, due to its superior interfacial activity and interfacial rheology parameters (chapter 5). β-LG-based emulsions exhibited slightly lower oil droplet sizes (90^{th} percentile) compared to DH6-based formulations, accompanied by a higher microencapsulation efficiency (tab. 4.1). All emulsions exhibited good process stability during atomisation and drying, except β-LG/HMP2-bilayer- and DH6-single-layer emulsions. The microencapsulation efficiency of the single-layer emulsions were clearly lower compared to the bilayer-emulsions stabilised with the same protein/hydrolysate, respectively.

The pronounced delay in the interfacial adsorption of β-LG in the presence of LMP (fig. 5.2) may be ascribed to the higher binding affinitiy of β-LG and LMP due to the higher amount of free carboxyl groups within the galacturonic acid backbone of LMP (Ganzevles, van Vliet, et al., 2007). Accordingly, stronger complexes were formed compared to β-LG/HMP, which resulted in an increase in the hydrodynamic radius and lower coefficients of diffusion for those complexes (Ganzevles, van Vliet, et al., 2007). However, after 14 h of adsorption time the surface pressure of β-LG/pectin-systems was similar, with the exception of systems containing β-LG and LMP, which was enzymatically modified by a plant-derived pectinmethylesterase (p35), yielding pectins with a high local charge due to the blockwise distribution of the carboxyl groups (Wagoner et al., 2016). Accordingly the binding affinity of this pectin and β-LG is enhanced, which results in a clearly lower surface pressure of this system. Since hydrolysates contain more molecules than their substrates and hydrolysates were found to possess a decreased ζ-potential with increasing DH (Liu et al., 2014; Mahmoud et al., 1992; Teh

et al., 2016), yielding weaker interactions between pectin and hydrolysates, the interfacial adsorption of β-LG hydrolysates is less affected by pectin. During dilatational frequency sweeps, β-LG and its hydrolysates exhibited similar dilatational moduli in the absence of pectin (data not shown). However, when pectin was present in the system, β-LG layers displayed strong frequency dependence and higher elastic moduli, especially at higher frequencies (fig. 5.4). In contrast, the moduli of hydrolysate-based layers were independent from the frequency applied. Relaxation processes occurring in β-LG-layers at lower frequencies can explain this distinction, however at higher frequencies these processes are too slow to take place within the timescale of the oscillation (Freer et al., 2004). Since the hydrolysates possess a more simple molecular structure, relaxation processes may occur very fast or not at all and additionally, faster diffusion of peptides from the bulk to the interface may quickly nullify the interfacial tension increase caused by the increase in interfacial area during oscillation (Davis et al., 2005; Ganzevles et al., 2006; Shrestha et al., 2008). In contrast, during interfacial shear experiments, layers composed of hydrolysates and LMP exhibited the strongest moduli, indicating a higher mechanical strength of the intermolecular interactions between the adsorbed molecules (Krägel & Derkatch, 2010; Piazza et al., 2009). This can be attributed to a more efficient packing of the molecules at the interface, as already observed by the differences in interfacial pressure (fig. 5.2). Although β-LG hydrolysate/LMP layers stabilised the oil/water-interface most effectively under shear deformation, all interfacial layers exhibited a breakdown at the same amplitude (fig. 5.5).

However, when the interfacial properties of mixed β-LG or hydrolysates and pectin are linked to the microencapsulation performance, it has to be considered, that the pectins used in both experiments were not the same. All pectins used in interfacial experiments were produced by enzymatic demethoxylation of a commercial HMP (via plant- or fungal-derived PME) as described recently (Einhorn-Stoll et al., 2015). In contrast, the pectins used for the microencapsulation experiments were commercial products, accordingly the method of modification is not neccesarily comparable. In fact, most commercial LMPs are produced by chemical demethoxylation (Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010; Ngouémazong et al., 2012), which generates a random distribution of the free carboxyl groups, i.e. low local charge density (Wagoner et al., 2016). Accordingly, the LMP used for microencapsulation (chapter 4) is best comparable to f32 (chapter 5), with respect to DM and a low local charge density caused by the random cleavage of the fungal pectinmethylesterase (PME) used for modification (Ralet et al., 2001; Ralet & Thibault, 2002). Furthermore, since the HMPs used for microencapsulation are also commercial products, which usually possess a low local charge density (or degree of blockiness), due to the acidic extraction conditions (Meyer-Hansen et al., 2009) and exhibit a high DM, they are best compared to f64 (chapter 5). The feed emulsions for microencapsulation of the oil were produced by the layer-by-layer technique and since the protein- or hydrolysate-layer is already formed when pectin is added

to the bulk, the interfacial activity of β-LG and DH6 is not affected by pectin addition. When emulsions are prepared by the layer-by-layer technique and homogenised again after addition of the polysaccharide, as performed in the present study, (Ogawa et al., 2004; Serfert et al., 2013), the layer composition is rather mixed and not strictly composed of an inner protein- and an outer polysaccharide-layer. However, since all pectin-stabilised interfacial layers exhibited good process stability as observed by a constant oil droplet size (90^{th} percentile) in liquid and spray-dried emulsions, no link to the interfacial rheology parameters can be established. Certainly, the oil droplet size decreased due to pectin addition, which can be ascribed to the formation of thicker interfacial layers and a more effective steric stabilisation, which retards flocculation and coalescence, respectively (Damodaran, 2005; Dickinson, 2011b). Furthermore, the pectins compensated the insufficient stabilisation observed in DH6-single layer emulsions (see chapter 7.2.1), potentially by the binding of one pectin with multiple peptides adsorbed to the interface and thus proving stronger cohesion within the adsorbed layer (Ganzevles, Kosters, et al., 2007). It was hypothesised in chapter 1, (hypothesis 2a), that the electrostatic attachment of pectin further enhances the physical stabilisation of protein- or peptide-based interfacial layers, which has proved to be true as discussed above. In addition it was hypothesised (hypothesis 3), that the adsorption kinetics and interfacial rheology of these interfacial layers are determined by the molecular characteristics of the biopolymers, which has also proved to be true as discussed in chapter 7.2.1 and 7.2.2.

In summary, pectins with different molecular characteristics strongly affected the interfacial activity of β-LG, but the impact decreased with increasing DH. Protein- or hydrolysate-layers were also reinforced by pectin addition during interfacial rheology, however the breakdown during shear amplitude sweeps was similar for all formulations. With respect to microencapsulation of oil, bilayer emulsions exhibited a lower oil droplet size and a higher microencapsulation efficiency compared to single layer emulsions. Accordingly, interfacial layers can be engineered by targeted enzymatic modification of β-LG and pectins, due to the selection of enzymes with high specificity.

7.3 Chemical Stability of Sensitive Lipophilic Compounds in Spray-Dried Emulsions

The chemical stability of long chain polyunsaturated fatty acids (LCPUFAs) microencapsulated by spray-drying of emulsions was successfully enhanced in the present study, due to the use of enzymatically hydrolysed β-LG (chapter 3) or hydrolysed PPI (chapter 6) as emulsifiers and by the electrostatic attachment of non-interfacially-active pectin to these protein- and peptide-stabilised interfacial layers (chapter 4).

During a storage period of 11 weeks, lipid oxidation in microencapsulated fish oil, which is rich in the LCPUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), was clearly delayed by the use of enzymatically-modified β-LG (fig. 3.3). However, the DH and enzyme specificity strongly affected the degree of lipid oxidation. In this regard, hydrolysates produced by trypsin were most effectively, independent of the DH. In contrast, for hydrolysates produced by alcalase, a DH of 6 % was needed to effectively slow down autoxidation. In was hypothesised in chapter 1 (hypothesis 1), that a controlled enzymatic hydrolysis of β-LG can improve the stability of lipids sensitive to autoxidation during the microencapsulation process by spray-drying of emulsions and subsequent storage of the microencapsulated oil. This has been proved to be true as presented above and is discussed in detail in manuscript 1. Furthermore it was hypothesised in chapter 1 (hypothesis 4b), that the concept of a controlled enzymatic modification for improving protein functionality, with respect to the chemical stabilisation of lipids, could be transferred to pea protein as an alternative globular model protein from plant origin. The proof of this hypothesis has been presented in manuscript 4. In accordance with the findings for hydrolysed β-LG, an enzymatic hydrolysis also improved the antioxidant properties of PPI during microencapsulation by spray-drying and storage of encapsulated rapeseed oil. Due to the poor functionality of APPH (cf. 7.2.1), only TPPH were used for the microencapsulation experiments and the degree of lipid oxidation of the microencapsulated oil decreased with increasing DH of TPPH during 30 weeks of storage (fig. 6.5). Besides the enhancement of the physical stability of β-LG- and β-LG-hydrolysate-stabilised emulsions, it was hypothesised in chapter 1 (hypothesis 2b), that electrostatic attachment of pectin to these interfacial layers also improves the chemical stability of the microencapsulated oil. This has been demonstrated in manuscript 2, by the strong inhibition of lipid oxidation in fish oil microcapsules stabilised by non-interfacially-active pectin and β-LG or DH6 (fig. 4.2) during 12 weeks of storage. The impact of pectin was even more pronounced than for the hydrolysate retarding autoxidation of fish oil most effectively in spray-dried single-layer emulsions (DH6) (fig. 3.3 and fig. 4.2).

In the present study, the bulk matrix component (glucose syrup with a dextrose equivalent of 38) was kept constant to enable focusing on the chemical stability of microencapsulated LCPUFAs by proteins, hydrolysates and pectins. Additionally, the spray-

drying conditions were also held constant for all experiments and thus it is assumed, that the properties of the glassy carbohydrate-based matrix were comparable in between the experiments. Accordingly, other factors, apart from the protective effect of the proteins, hydrolysates and pectins like the contribution of free volume elements due to excess protein in the formulation (Drusch, Rätzke, et al., 2009; Drusch, Serfert, et al., 2012; Serfert et al., 2013), will be neglected in this section.

In general, interfacially-active antioxidants are most effective in the stabilisation of lipids prone to oxidation in oil/water-emulsions, since they are located at the interface, where lipid oxidation is iniated and oxidation reactions are promoted (McClements & Decker, 2000). During the spray-drying process, the proteins and peptides remain at the interface due to their interfacial activity and thus they also stabilise the microencapsulated oil in the dried state. The increased oxidative stability of the sensitive lipids microencapsulated by β-LG- or PPI-hydrolysates may be attributed to the increased accessibility of antioxidant amino acids or amino acid sequence being natively buried within the globular structure of β-LG (Conway et al., 2013; Elias et al., 2005) or PPI fractions (Zhang et al., 2013). This hypothesis is supported by the fact, that the substrate and the hydrolysate contain the same amount of antioxidant amino acids in the primary structure and accordingly, the substrate and peptides primarily differ in their tertiary structure (Elias et al., 2006, 2008). Various explanations have been stated for the antioxidant properties of proteins and hydrolysates, e.g. the decomposition of lipid hydroper-oxides, free radical scavenging by amino acid oxidation, the physical hindering of prooxidant-lipid interactions (Conway et al., 2013; Elias et al., 2008, 2005; Humiski & Aluko, 2007; Zhang et al., 2013), the donation of hydrogen (Moreau & Rosenberg, 1996) and the chelation of prooxidant metal ions like iron or copper (Conway et al., 2013; Elias et al., 2006). With respect to β-LG and its hydrolysates produced by trypsin or alcalase, the specificity of the enzyme and the DH determinated the antioxidant char-acter of the peptide mixtures. Due to the limited number of cleavage sites of trypsin within the amino acid sequence of β-LG, at a DH of 3 % the bulk of antioxidant amino acids was already exposed and an increase of the DH to 6 % only slightly enhanced the inhibition of lipid oxidation of the microencapsulated fish oil (fig. 3.3). In contrast, for β-LG hydrolysates produced by alcalase a DH of 3 % did not increase the stability of the microencapsulated fish oil. However, an increase of the DH to 6 % resulted in clear-ly lower hydroperoxide contents compared to β-LG after 11 weeks of storage, but the trypsin hydrolysates were more effective in this regard. This development may be explained by the low specificity of alcalase, resulting in cleavage in the outer molecular parts of β-LG and not in the interior of the globular structure, which contains the bulk of the antioxidant amino acids (Elias et al., 2008). At DH of 6 %, the globular structure of the peptides was further degraded, exposing more antioxidant amino acids, which results in a lower degree of lipid oxidation. The possible peptide sequences arising from the enzymatic hydrolysis of β-LG and their antioxidant properties are discussed in more

detail in chapter 3.4. Similar results have been published recently for fish oil microencapsulated by spray-drying using fish protein hydrolysates (Morales-Medina et al., 2016). In a similar manner to the enhancement of the antioxidant properties of β-LG due to enzymatic hydrolysis, the enzymatic modification of PPI also resulted in an increase of its antioxidant performance. When the DH of the TPPH increased the hydroperoxide content of the microencapsulated rapeseed oil decreased, which may be ascribed to the increased accessibility of the antioxidant amino acids in TPPH. This is discussed in more detail in chapter 6.4.5. In addition, when pectin was electrostatically attached to primary protein- or hydrolysate-based interfacial layers, lipid oxidation in microencapsulated fish oil was strongly slowed down, due to the metal chelating activity of pectin (Chen et al., 2010) and the formation of a thicker interfacial barrier, which physically hindered prooxidant-lipid interactions (Katsuda et al., 2008).

In summary, enzymatic hydrolysis of β-LG and PPI improved the chemical stability of lipids sensitive to autoxidation during microencapsulation by spray-drying and subsequent storage of the microencapsulated lipids. The degree of lipid oxidation strongly depended on the accessibility of the antioxidant amino acids as affected by the DH and the specificity of the enzyme. The electrostatic attachment of non-interfacially-active pectin to protein- or hydrolysate-stabilised interfacial layers further improved the stability of the microencapsulated lipids due to increased thickness of the interfacial layers and metal chelating activity of the pectin.

7.4 Concluding Remarks and Outlook

The results of the present study emphasise the improved functional properties of enzymatically hydrolysed β-LG and PPI with respect to their role in the physical stability of emulsions during emulsification, in the microencapsulation by spray-drying and in the chemical stability during subsequent storage of the encapsulated lipids sensitive to autoxidation. However, the degree of hydrolysis and the enzyme used for modification have to be carefully selected to yield peptide mixtures with improved functionality. Although the potential of protein hydrolysates to stabilise LCPUFAs in liquid emulsions has been proved in the past, their use in spray-dried emulsions has not been reported so far.

The electrostatic attachment of non-interfacially-active pectin to protein- or hydrolysate-based interfacial layers presents a promising way to engineer networks with enhanced physical stability as observed in the process stability of emulsions during spray-drying and in interfacial rheology experiments in the present study. Especially enzymatic modification of the pectin enabled the steering of the binding affinity towards the other proteins and hydrolysates and thus its stability under physical stress due to processing, respectively. Furthermore, pectin also increased the chemical stability of lipids prone to autoxidation during spray-drying and subsequent storage of the emulsions.

From the commercial point of view there are still some questions remaining: the known bitter taste of whey protein hydrolysates may limit the acceptance of the hydrolysates in food matrices, however, this is expected to be masked by the carbohydrate component of the encapsulating matrix (Yang et al., 2012). Despite the easy scale-up of the microencapsulation process by spray-drying from the pilot to the industrial scale (Ré, 1998), the process on the industrial level still needs further adjustment, e.g. due to the longer residence time of the drying particles in larger spray-dryers, which affects chemical and physical stability of the product (Langrish, 2007). Accordingly, the application of these biopolymers as wall materials for the microencapsulation of sensitive lipophilic compounds by spray-drying, as investigated in the present study, has to be seriously considered. One possible application is the incorporation of the microencapsulated LCPUFAs in powdered infant formulas, which are usually fortified with these fatty acids to match the composition of the mothers' milk (Innis, 2007). In this regard, the advantage of being non-allergenic highlights the use of pea protein hydrolysate-based formulations compared to those containing milk proteins (Gharsallaoui et al., 2012).

However, from the scientific point of view there are also some questions remaining: can the enzymatically-hydrolysed proteins be applied for the stabilisation of other food systems, e.g. foams or gels? Furthermore, the combination of PPH and pectin may improve the physical and chemical stability of microencapsulated lipids as already reported for PPI/pectin-combinations (Aberkane et al., 2014; Gharsallaoui, Saurel, et al., 2010). In

addition, since pectin dominated the antioxidant protection of the microencapsulated lipid independent from the degree of modification of β-LG, this phenomenon needs to be analysed in more detail. In this regard, the structural properties of the adsorbed interfacial layers could be characterised further by specular neutron reflectivity to determine the layer thickness and density (Ganzevles et al., 2008) and to quantify the amount of adsorbed biopolymer by ellipsometry (Dickinson, 2011b). In addition, the structural integrity of the layers during compression and expansion experiments can be visualised by brewster angle microscopy and simultaneous use of a Wilhelmi plate apparatus enables the determination of the dilatational moduli of the interfacial layers (Murray et al., 2009).

Besides, the antioxidant mechanisms of the proteins and hydrolysates need to be investigated in more detail. In this regard, the characterisation of the radical scavenging ability of the proteins and hydrolysates may be performed by the use of electron spin resonance (Rohn & Kroh, 2005) to prove their antioxidant activity. This method enables the quantification of the velocity of degradation of synthetic radicals (antioxidative potential) and the amount of radicals, which are degraded within a certain timescale by a defined amount of antioxidant (antioxidative capacity) (Rohn & Kroh, 2005). Subsequently to radical scavenging, which causes oxidation if the antioxidant amino acids, their regeneration is an important factor to recover their antioxidant activity, which can be achieved by methionine sulfoxide reductase in the case of methionine (Elias et al., 2008; Weissbach, Resnick, & Brot, 2005). However, the possible regeneration mechanisms of oxidised amino acids in food systems have not been investigated yet.

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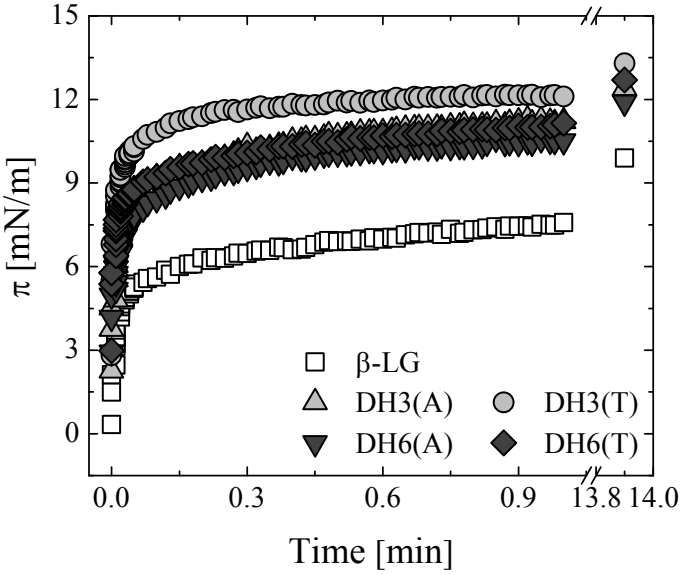
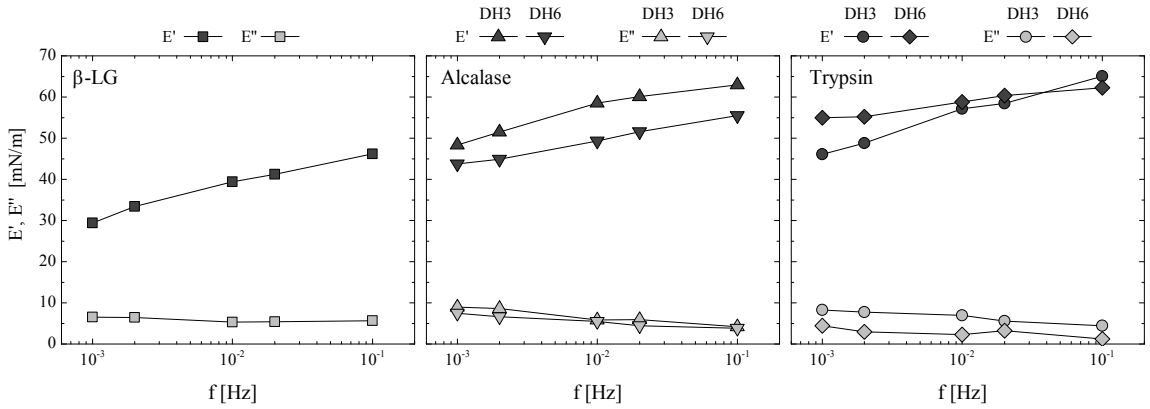
9 Annex



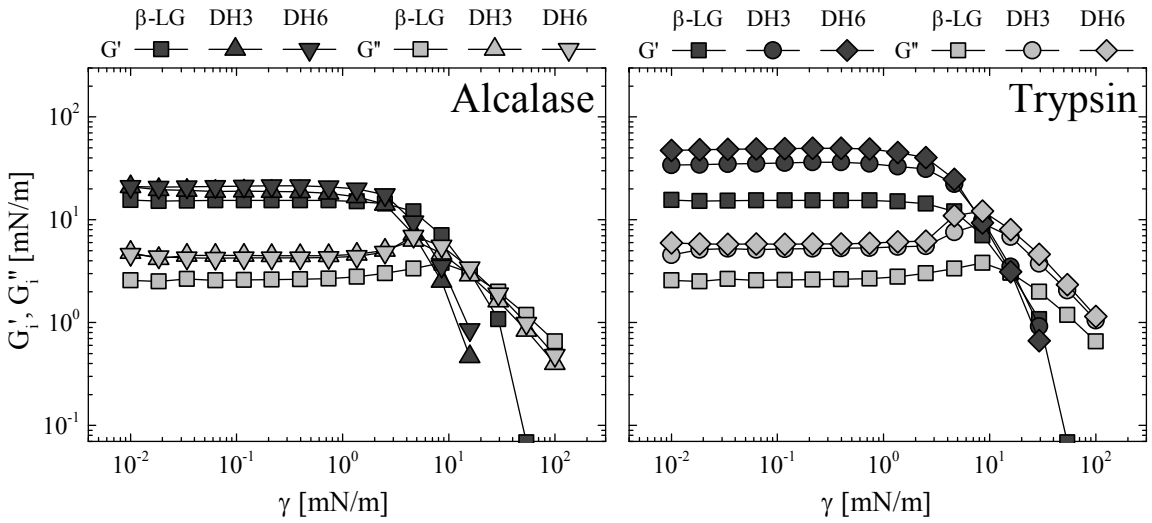
A 1 Interfacial pressure development as a function of adsorption time for β-LG and hydrolysates thereof with degree of hydrolysis of 3 % (DH3) and 6 % (DH6) and produced by two different enzymes (alcalase (A) and trypsin (T)) at the MCT-oil/water-interface. Bulk protein and pectin content was 0.01 wt% at pH 8.0. Temperature 22 °C.



A2 Dilatational frequency sweeps of β-LG and hydrolysates thereof with degree of hydrolysis of 3 % (DH3) and 6 % (DH6) and produced by two different enzymes (alcalase (A) and trypsin (T)) at the MCT-oil/water-interface. Bulk protein and content was 0.01 wt% at pH 8.0. Temperature 22 °C.

A3 Interfacial shear modulus of protein/pectin solutions after 9 h of adsorption at pH 8.0. Bulk solutions contained 0.01 wt% β-lactoglobulin (β-LG) and hydrolysates thereof with a degree of hydrolysis of 3 % (DH3) and 6 % (DH6) and produced by two different enzymes (alcalase (A) and trypsin (T)). Temperature 22 °C.

Protein	Interfacial shear modulus after 9 h $\|G_i^*\|$ [mN/m]
β-LG	11.5
DH3(A)	17.3
DH6(A)	14.9
DH3(T)	25.1
DH6(T)	39.9



A4 Shear amplitude sweeps of β-lactoglobulin (β-LG) and hydrolysates thereof with a degree of hydrolysis of 3 % (DH3) and 6 % (DH6)) and produced by two different enzymes (alcalase (A) and trypsin (T)). Protein films were adsorbed to the MCT-oil/water-interface for 9 h. Bulk protein content was 0.01 wt% at pH 8.0. Temperature 22 °C.

Curriculum vitae

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04/2010 – 03/2011	Project work at Beuth University of Applied Sciences, Berlin, subject *„Vorhersage des Aufschäumverhaltens bei Lebensmittelschäumen mittels dynamischer Tropfenkonturanalyse“*
10/2005 – 09/2009	Studies in Food Technology, Beuth University of Applied Sciences, Berlin, degree Bachelor of Science, subject of Master thesis: *„Vitamin E - Bestimmung in Milch mittels HPLC“*
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Publications, Oral Presentations & Posters

Peer-reviewed Publications

F. Tamm, S. Drusch (2017): *Impact of enzymatic hydrolysis on the interfacial rheology of whey protein/pectin interfacial layers at the oil/water-interface.* Food Hydrocolloids, 63, 8–18. *10.1016/j.foodhyd.2016.08.013*

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F. Tamm, G. Sauer, M. Scampicchio, S. Drusch (2012): *Pendant drop tensiometry for the evaluation of the foaming properties of milk- derived proteins,* Food Hydrocolloids, 27(2), 371–377. *10.1016/j.foodhyd.2011.10.013*

ORAL PRESENTATIONS

M. Najmabadi, F. Tamm, M. Klaiber, Y. Baroud, S. Drusch, S. Simon: *Real-time determination of interfacial tension from the shape of a pendant drop based on embedded image processing.* Oral Presentation ILASS – Europe, 25th European Conference on Liquid Atomization and Spray Systems, Chania, Greece.

POSTER PRESENTATIONS

F. Tamm, K. Gies, S. Diekmann, Y. Serfert, T. Strunskus, A. Brodkorb, S. Drusch (2015): *Impact of enzymatic hydrolysis of β-lactoglobulin on lipid oxidation of microencapsulated fish oil,* Poster Presentation International Whey Conference, Rotterdam.

F. Tamm, K. Gies, S. Diekmann, Y. Serfert, S. Drusch (2013): *Influence of a limited enzymatic hydrolysis on the functional properties of β-lactoglobulin for microencapsulation,* Poster Presentation International BRG Conference on Bioencapsulation, Berlin.

S. Diekmann, **F. Tamm**, S. Drusch (2012): *Auswirkungen einer enzymatischen Hydrolyse auf die Grenzflächenaktivität und -rheologie von β-Lactoglobulin,* Poster Presentation Jahrestagung der Gesellschaft der Deutschen Lebensmitteltechnologen e.V., Dresden.