Rheological characteristics of pectin gelation in sugar-acid systems: Insight into structure formation and gel properties

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Kurzfassung

Das pflanzliche Zellwandpolysaccharid Pektin ist ein Lebensmittelinhaltsstoff mit hoher Funktionalität und guter Verbraucherakzeptanz. Es wird in vielen Bereichen der Lebensmittelindustrie, vor allem traditionell als Geliermittel, eingesetzt. In Zucker-Säure-Systemen, wie den klassischen Konfitüren, bildet Pektin Gele aus. Dies beruht auf zwei unterschiedlichen Mechanismen, der abkühlungsinduzierten und der ionotropen Gelierung, die sowohl separat als auch in Kombination ablaufen können. Die Strukturbildung von Pektin hängt von einer Vielzahl interner und externer Faktoren ab, wie z. B. der Pektinart, den molekularen Eigenschaften, dem pH-Wert und dem Calciumgehalt im Gelsystem sowie den Abkühlungsbedingungen. Trotz jahrzehntelanger Forschung auf diesem Gebiet ist der Einfluss dieser Faktoren und ihrer Wechselwirkungen in Bezug auf die Geliereigenschaften des Pektins noch immer nicht vollständig aufgeklärt.

Ziel dieser Arbeit war es, den Einfluss ausgewählter interner und externer Faktoren systematisch zu untersuchen und ihre Bedeutung für den Gelierprozess und die Eigenschaften der fertigen Gele aufzuklären. Dazu wurde zuerst eine Methode entwickelt, anhand derer charakteristische Temperaturen des Strukturbildungsprozesses mittels rheologischer Oszillationsmessungen abgeleitet werden können. Diese Parameter bilden den typischen Gelierprozess ab und erlauben den Vergleich der Strukturbildungskinetik unabhängig von Pektintyp und molekularen Eigenschaften. In verschiedenen Einzelstudien wurde diese neue Methode angewendet und die Gelbildung von insgesamt 15 kommerziellen hoch- und niederveresterten (auch amidierten) Pektinproben detailliert untersucht.

Die Untersuchung der Gelierung von hochverestertem Pektin zeigte, dass die Kinetik der Gelierung sowohl vom pH-Wert im Gelsystem, als auch von der Abkühlrate, beeinflusst wird. Während der Gelierung bewirken einerseits unterschiedliche pH-Werte eine direkte Veränderung des Dissoziationsgrades der freien Carboxylgruppen und der damit verbundenen Anzahl an Wasserstoffbrückenbindungen zwischen diesen funktionellen Gruppen. Andererseits ist der Beginn der Gelierung in dem diese Wasserstoffbrücken-bindungen verstärkt ausgebildet werden, unmittelbar von der Abkühlrate abhängig. Mithilfe der entwickelten Methodik konnte der Übergang von anfangs auftretenden hydrophoben Wechselwirkungen zu Wasserstoffbrückenbindungen (Gelierung) detailliert abgebildet und entsprechende Strukturbildungsgeschwindigkeiten abgeleitet werden.

Die Strukturausbildung der untersuchten niederveresterten Pektinproben wurde zwar ebenfalls von pH-Wert und Abkühlrate beeinflusst, jedoch in geringerem Maße. Hierbei spielt die ionotrope Gelierung über Calciumbrückenbindungen zwischen dissoziierten Carboxyl-gruppen eine wesentlich größere Rolle. Die Ausbildung der Bindungen in diesen Haftzonen war weniger temperaturabhängig, als die der hydrophoben Wechselwirkungen und der Wasserstoffbrückenbindungen. Allerdings zeigte die Änderung der Calciumionen-konzentration einen deutlichen Einfluss auf den Strukturierungsprozess. Auch die

Strukturbildung von niederveresterten Pektinen ließ sich in unterschiedliche Phasen einteilen, die in den Kurven der Strukturbildungsgeschwindigkeit identifiziert werden konnten.

Beim direkten Vergleich der Strukturausbildung eines niederveresterten nicht amidierten und eines sehr ähnlichen amidierten Pektins wurde deutlich, dass zusätzliche Wasserstoffbrückenbindungen zwischen den Amidgruppen die Gelierung unterstützten. Die Strukturausbildung des amidierten Pektins war deshalb weniger abhängig vom pH-Wert und der Calciumionenkonzentration.

Zusätzlich zu den Einzelstudien der kommerziellen Pektinproben wurde eine weitere Studie durchgeführt, in der 12 zusätzliche Proben selbst im Labor hergestellt wurden. In dieser wurde der Einfluss der Verteilung der freien Carboxylgruppen entlang der Pektinhauptkette auf die Gelierung untersucht, sowohl von hochveresterten, als auch von niederveresterten Pektinproben. Dazu wurden die molekularen Eigenschaften eines hochveresterten Pektins mit drei verschiedenen Methoden modifiziert. Das Ausgangspektin wurde sowohl sauer, als auch mit mikrobieller und pflanzlicher Pektinmethylesterase, entestert. Die modifizierten Proben (3 Gruppen mit je 4 Pektinproben unterschiedlichen Veresterungsgrades) unterschieden sich in der Verteilung der Carboxylgruppen, entweder blockweise oder zufällig. Die Gelierung und die Geleigenschaften dieser Proben variierten bei allen Veresterungsgraden sowohl bei der abkühlungsinduzierten als auch bei der ionotropen Gelierung. Pektinproben mit einer zufälligen Verteilung (sauer und mikrobiell modifiziert) zeigten ähnliche Strukturbildungs- und Geleigenschaften, die sich von denen der Pektinproben mit einer blockweisen Verteilung deutlich unterschieden.

Die im Rahmen dieser Arbeit entwickelte Methode erlaubte erstmals den direkten Vergleich der Gelierprozesse von Pektinen verschiedener Veresterungsgrade, bei denen unterschiedliche Mechanismen dominierten. Im Ergebnis zeigte sich, dass der Strukturbildungsprozess stark von den untersuchten Faktoren und ihren Wechselwirkungen abhängt. Bereits geringe Abweichungen von der Standardgelzusammensetzung oder den Gelierbedingungen beeinflussten sowohl die Strukturbildung als auch die Eigenschaften der gebildeten Gele signifikant. Die Ergebnisse, die mit dieser neuen Methode gewonnen wurden, erlauben einen tieferen Einblick in die Gelbildungsmechanismen und die Wechselwirkungen zwischen molekularen Eigenschaften der Pektine, der Zusammensetzung des Gelsystems und den Abläufen bei der Herstellung von Pektingelen.

Abstract

The plant cell wall polysaccharide pectin is a food ingredient with high functionality and good consumer acceptance. It is used in a wide range of food systems and traditionally applied as gelling agent, but it has also relevant properties beside gelation. Pectin forms gels in a sugar-acid environment, like classical jam, by cold-set and ionotropic gelation, acting separately as well as in combination. Structure formation of pectin is strongly determined by several internal and external factors, such as pectin type, molecular characteristics like degree of methoxylation and pattern of free carboxyl groups, or environmental conditions, like pH and calcium ions in the gel system as well as cooling conditions during gelation. Despite of many decades of research, their single effects and interactions are not completely understood. Therefore, this thesis was focused on a systematic examination of some of these factors, in order to clarify their relevance for the pectin gelling process and the final gel properties.

A new method for the investigation of pectin gelation was introduced. Characteristic temperatures of the structuring process were calculated from the structuring velocity curve, derived from rheological oscillation measurements. They reflected the typical kinetic of the gelation process. This method allowed the comparison of pectin gelation, independent on pectin type and molecular parameters, and it was a prerequisite for the broad examination of the impact of internal and external factors. The gelation of 15 commercial citrus pectin samples, both high- and low-methoxylated, partly amidated, was examined in several single studies.

An investigation of the structuring process of high-methoxylated pectin revealed, that pH as well as cooling conditions affected the gelation kinetics. The pH determined the degree of dissociation and, thus, the number of the formed hydrogen bond, and the cooling rate was crucial for the dominating type of junction zones in the course of the gelling process. The threshold of the change from dominating hydrophobic to hydrogen bond was reflected in the structuring velocity curves.

The gelation of low-methoxylated pectin was affected by additional factors and to a different extent, since beside a certain cold-set gelation, the ionotropic gelation is the dominant mechanism. It is less temperature-dependent than cold-set gelation, because of additional formed calcium bridges, which are independent on temperature. The structuring process was affected by added acid (pH) and calcium ions. The structure formation was divided into different phases with varying dominating mechanisms, which were reflected in the structuring velocity curves. Comparing the structure formation kinetics of the low-methoxylated pectin with and without amid groups, amidation supported the gelation due to additional hydrogen bonds formed between the amide groups it was found that additional hydrogen bonds were formed between the amide groups. Thus, structure formation of amidated pectin was less dependent on pH and added calcium ions than that of non-amidated, since the amide group supported the dominating ionotropic gelation.

Beside the studies of the commercial pectins, 12 special samples have been prepared in laboratory-scale for a study, that investigated the effect of the pattern of free carboxyl groups on the gelation kinetic of high- and low-methoxylated pectins. The molecular characteristics of one commercial high-methoxylated pectin were modified by three different methods, using acid, microbial or plant pectinmethylesterases. The modified pectin samples of the obtained 3 groups had a comparable degree of methoxylation but differed in their pattern of free carboxyl groups (block-wise or random). Their gelling kinetics as well as gel properties varied during cold-set as well as ionotropic gelation. Independent of the modification method and degree of methoxylation, pectin samples with a random distribution showed similar structure formation kinetics and properties of the resulting gels, that differed from those of pectin samples with a blockwise distribution.

The newly developed method for the first time allowed the direct comparison of the gelation of pectins with any degree of methoxylation and with varying gelling mechanisms. The presented work demonstrates the high impact of selected factors on the gelation process. Even moderate deviations from the standard gel composition or gelation procedure significantly affect the structure formation and the properties of the final gels after cooling. The results, obtained with the newly developed method, provide deeper insights into the interactions between molecular properties, gel composition and procedures of pectin gelation.

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List of abbreviations and symbols

a-/A	acidic treated sample
CST	critical structuring temperature
DA	degree of amidation
DM	degree of methoxylation
dG´/dt	structuring velocity
f-/F	fPME treated pectin
fPME	PME of fungal origin
G´	storage modulus
G´´	loss modulus
GalA	galacturonic acid
GC	galacturonan content
GP	gel point, gel point temperature
HMP	high-methoxylated pectin
IST	initial structuring temperature
IV	intrinsic viscosity [η]
LMAP	low-methoxylated amidated pectin
LMP	low-methoxylated pectin
MW	molecular weight
OP	original pectin
p-/P	pPME treated sample
PG	polygalacturonases
PME	pectin methylesterases
pPME	PME of plant origin
R-value	stoichiometric ratio of calcium ions and free carboxyl groups
SAG	sugar-acid gel
SDR	structure development rate
tanδ	loss factor at end of measurement

Research motives

Pectin represents a class of heterogeneous plant polysaccharides which is applied in the food industry primarily because of their gelling properties for jams and jellies. Residues from the production of citrus or apple juice represent the major source for pectin production. The composition of these raw materials varies due to climate and geographical growing conditions, harvest time and processing of the fruit, even if they are of the same botanical origin. Extraction conditions, such as temperature, time and pH (usually strong acidic conditions) are also important (May, 2000). All these factors result in pectin differing in molecular characteristics, like molecular weight, content of galacturonic acid (GalA), amount and distribution of methoxylated groups and other substituents over the GalA backbone or number and composition of neutral sugar side chains in rhamnose-rich regions (Schols & Voragen, 1996).

It is general consensus that the main molecular characteristics to predict the gelling behavior of pectin is the degree of methoxylation (DM). In general, two principal mechanisms of pectin gelation are known. Pectin with higher DM (normally > 50%) forms gels by cold-set gelation via hydrophobic interactions between methoxyl groups and hydrogen bonds between different hydrophilic groups of the pectin molecule. This type of gelation requires an environment containing sugar (typically above 55%) and acid (pH < 3.5). Pectin with low DM (< 50%) additionally forms bonds via calcium bridges between free carboxyl groups by ionotropic gelation. This mechanism requires less or no sugar and acid (possible also at pH > 3.5) but a certain amount of calcium ions and a certain length of blocks of free carboxyl groups (Burey, Bhandari, Howes, & Gidley, 2008; Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010; Lopes da Silva, Gonçalves, & Rao, 1995; Thakur, Singh, Handa, & Rao, 1997).

Different factors determine structure formation and properties of pectin gels (Endreß & Christensen, 2009; Rolin, Chrestensen, Hansen, Staunstrup, & Sørensen, 2009; Yapo & Gnakri, 2015). The complex gelling processes and gel properties of pectin systems as well as the influence of various factors have been examined by a variety of methods, including a wide range of rheology-based procedures. The influencing factors on pectin gelation can be categorized into internal and external factors. Internal factors affecting a system are those related to pectin molecular parameter as well as the solution or gel composition. Technological matters and experimental equipment are external factors. Due to the differences in internal and external factors, the comparability of the results from different studies is limited.

In case of external factors, the main influence results from inconsistent parameters in rheological experiments: Several research groups investigated the viscoelastic properties of pectin at different cooling or heating conditions as well as further rheological conditions

(e.g. frequency and strain) (Agoub, Giannouli, & Morris, 2009; Almrhag et al., 2012; Dahme, 1992; Evageliou, Richardson, & Morris, 2000a; Fu & Rao, 2001; Iglesias & Lozano, 2004; Ngouémazong, Nkemamin, et al., 2012; Rao, Van Buren, & Cooley, 1993; Sousa, Nielsen, Armagan, Larsen, & Sørensen, 2015). This also prevents a comparison of these results regardless of the internal factors mentioned in the following. Although the influence of the cooling rate on the structure formation of pectin sugar gels is generally accepted, the detailed effects have not yet been systematically investigated.

In relation to the structuring properties, however, most pectin studies only consider the influence of one or two internal factors, such as pectin type, pectin concentration, co-solute, ion concentration, ion type or acidic and alkaline media for adjusting the pH value (Cameron, Luzio, Goodner, & Williams, 2008; Evageliou, Richardson, & Morris, 2000c; Fraeye, Duvetter, et al., 2010; Gigli, Garnier, & Piazza, 2009; Löfgren, Guillotin, Evenbratt, Schols, & Hermansson, 2005; Löfgren & Hermansson, 2007; Lopes da Silva & Rao, 2006; Ngouémazong, Nkemamin, et al., 2012; Rao & Cooley, 1993, 1994; Rosenbohm, Lundt, Christensen, & Young, 2003; Sousa et al., 2015; Ström, Schuster, & Goh, 2014; Tsoga, Richardson, & Morris, 2004; Tsoga et al., 2004). Regarding the molecular characteristics of pectin, it should be considered that the DM is an average value and does not reflect heterogeneity of pectin molecules such as the molecular chain length and the distribution of methoxylated groups over the pectin backbone. As a consequence, it has been repeatedly shown that various pectins with similar molecular characteristics may differ markedly in their gelling behavior or did not show the expected gelation properties (Fraeye et al., 2009; Kim et al., 2013; Ngouémazong, Kabuye, et al., 2012; O'Brien, Philp, & Morris, 2009; Sousa et al., 2015; Yapo, Robert, Etienne, Wathelet, & Paquot, 2007). Furthermore, the structuring properties of pectin, especially LMP, are often only considered in pectin dispersions. NaOH or a buffer, e.g. sodium citrate or citrate phosphate buffer, are used as solvent (Cardoso, Coimbra, & Lopes da Silva, 2003; Dobies, Kozak, & Jurga, 2004; Fraeye et al., 2009; Gilsenan, Richardson, & Morris, 2000; Iglesias & Lozano, 2004; Kim & Wicker, 2009; Ngouémazong, Tengweh, et al., 2012; Ström et al., 2007; Vincent & Williams, 2009; Yapo & Koffi, 2013) and additionally calcium solution has to be added to induce a gelation. On the one hand these systems do not reflect a complex food system and on the other hand the additional variation on the experimental setting as well as setup (external factors) does not enables a direct comparison of these results. Although the influence of varying internal and external factors on the structure formation of pectin sugar gels is generally accepted, but detailed effects have not yet been systematically investigated.

In addition, analytical description of the structuring process is frequently limited to determination of individual parameters. The most common parameters used for investigating the phase transitions in gelling or melting gel systems are the gel point (GP), gel setting time or temperature and melting point, melting time and temperature, respectively. Rheological measurements give the most reliable data for the examination of sol–gel-transitions. The GP, experimentally determined by oscillation rheology, is often described as crossover of storage

modulus (G´, elastic characteristics) and loss modulus (G´´, viscous characteristics), with loss factor (tanδ) is G´´/G´ = 1 (Arenaz & Lozano, 1998; Audebrand, Kolb, & Axelos, 2006; Gigli et al., 2009; Gilsenan et al., 2000; Holst, Kjøniksen, Bu, Sande, & Nyström, 2006; Löfgren, Walkenström, & Hermansson, 2002; Lootens et al., 2003; Slavov et al., 2009). Though strictly the cross-over of G´ and G´´ might be defined as gel point only when it is independent of frequency (Holst et al., 2006), the point was found to be partly a function of frequency (Lopes da Silva & Gonçalves, 1994; Lopes da Silva et al., 1995; Lopes da Silva & Rao, 2007; Rao et al., 1993; Winter & Chambon, 1986). Sometimes the measured cross-over might be close to but not identical with the real gel point and therefore is named also as "apparent gel point" (Lopes da Silva & Gonçalves, 1994; Lopes da Silva et al., 1995). Lopes da Silva and Rao (2007) further showed that the G´–G´´-crossover depends not only on the oscillation frequency but also on the analytical range of a conventional rheometer, for instance on the ability to detect viscoelastic behavior in samples with low pectin concentration. The GP determined this way might be close to the real sol–gel-transition temperature (Lopes da Silva & Rao, 2007) and can still be applied for the characterization of pectin gelation.

However, the GP is a single point and reflects only a small part of the complex gelation process of pectin. Different structuring phases occur, depending on changes of the dominating type of interactions during gelation (Fu & Rao, 2001; Lopes da Silva & Rao, 2007; Oakenfull & Scott, 1984; Thakur et al., 1997; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). Thus, the transition from liquid (dominating viscous characteristics) to solid (dominating elastic characteristics) is rather a phase than a single point. For these reasons, the determination of the GP is often insufficient for describing the complex structuring process. In some cases, even no transition from sol to gel can be determined, since pre-gelation (dominant elastic properties) occurred already before or at the beginning of the measurement (Evageliou et al., 2000c; Gigli et al., 2009; Iglesias & Lozano, 2004; Kastner et al., 2014; Picout, Richardson, & Morris, 2000). A wide variety of experiments have been carried out to find another method for the gel point definition: esearchers from CP Kelco determined the gelling temperature via conductivity (Bøttger, Christensen, & Stapelfeldt, 2008), and Dobies, Kozak, and Jurga (2004) applied NMR measurements. Oakenfull and Scott (1984) and O'Brien, Philp, and Morris (2009) used relatively simple visual tests. Dahme (1992) and Neidhart, Hannak and Gierschner (2003) defined a strong decrease of tanδ as an indicator for the gel formation. Grosso and Rao (1998) and Fu and Rao (2001) studied the kinetic of pectin gels and defined the structure development rate in order to describe precisely the moment, a single point, at which the formation of junction zones started. However, none of these methods describe a temperature range for the transition from dominating viscous to dominating elastic characteristics.

In summary, pectin forms gels in two different ways, by cold-set and ionotropic gelation, which may act separately as well as in combination. It is often difficult to evaluate and compare these types of gelation. The gelling process is determined by many factors such as

molecular structure and gel composition (e.g. pH-value, pectin concentration, type and quantity of co-solutes and ions), but also processing time and temperature. Over the past decades, the gelling properties of pectin have been intensively investigated. Due to incomplete analytical characterization of the materials or the structuring process and the selection of model systems not representing a complex food matrix, it is still not possible to unravel the importance of individual factors and their interplay in complex food systems. Therefore, the present study focused on a systematic examination of the impact of major factors, both external and internal, on the pectin gelling process and the final gel properties in a sugar-acid environment using a reliable method:

- In order to be able to compare the results within and between the individual studies, it is necessary to introduce a method to evaluate the gelation kinetics, especially the sol-gel transition. It is hypothesized that it is possible to develop such a method for investigating the structuring process of any pectin, and to analyze characteristic parameters of this process using the first derivation of the storage modulus from oscillation rheology measurements.
- Depending on the degree of methoxylation, varying gelling mechanisms dominate cold-set gelation (gelation of high-methoxylated pectin) and ionotropic gelation (additional gelation mechanism of low-methoxylated pectin). Moreover, both types of gelation require different conditions, such as concentration and type of soluble solids or ions as well as pH. Nevertheless, a comparison of the different structure formation processes should be possible using the method developed.
- Hydrogen bonds are one of the three typical types of molecular bonds and interaction forming junction zones involved in the gelation of all pectin. They require a low pH value in order to reduce the dissociation of the carboxyl groups and, thus, the repulsion between the pectin chains. Applying the newly developed method, it shall be displayed to which extent a moderate increase of pH by reducing the added amount of acid and the resulting dissociation of free carboxyl groups will delay the cold set gelation of high methoxylated pectin but accelerate the ionotropic gelation of low methoxylated pectin.
- In general, molecular mobility is high at high temperature. During cooling, the mobility is decreasing, and the formation of junction zones is favored by a closer contact of the macromolecules. It is hypothesized that pectin structure formation kinetics and gel structure vary in dependence on the cooling rate. It is assumed that slow cooling will result in longer junction zones than a rapid cooling, and that the length of the junction zones will determine the gel properties. Applying the newly developed method, it shall be investigated to which the cooling rate determines the gelation, the dominating gelation mechanism and the final gel properties.
- During gelation of low-methoxylated pectin, additional calcium bridges ensure that ionotropic gelation is less temperature-dependent than cold-set gelation. The newly developed method shall be used to investigate to which extent a moderate increase in

the calcium content will accelerate the initial structure formation of low methoxylated pectin in sugar-acid environment.

- The structuring process of amidated low-methoxylated pectin is supported by additional hydrogen bonds including amide groups. These bonds are expected to reduce the influence of acid concentration and calcium content on structure formation compared to low-methoxylated pectin without amide groups.
- Structure formation in a sugar-acid environment depends both on the degree of methoxylation and on the pattern of free carboxyl groups, block-wise or random. It is expected that the influence of the pattern will occur within a certain range of the degree of methoxylation. A minimum number of free carboxyl groups is required to cause differences in the structuring process, but below a certain degree of methoxylation the effect of the pattern of free carboxyl groups decreases significantly.

This thesis is based on the following publications, which has been published and listed in the Annex:

(A1) *New parameters for the examination of the pectin gelation process*. Kastner, Einhorn-Stoll, & Senge (2012), Gums and Stabilisers for the Food Industry 16, 191-197, RSC Publishing, Cambridge, http://dx.doi.org/10.1039/9781849734554-00191 (Annex (A1), page 79).

(A2) *Comparison of molecular parameters, material properties and gelling behaviour of commercial citrus pectins.* Einhorn-Stoll, Kastner, & Senge (2012), Gums and Stabilisers for the Food Industry 16, 199-206, RSC Publishing, Cambridge, http://dx.doi.org/10.1039/9781849734554-00199 (Annex (A2), page 87).

(A3) *Structure formation in sugar containing pectin gels – Influence of Ca^{2+} on the gelation of low-methoxylated pectin at acidic pH.* Kastner, Einhorn-Stoll, & Senge (2012), Food Hydrocolloids, 27, 42-49, https://doi.org/10.1016/j.foodhyd.2011.09.001 (Annex (A3), page 97).

(A4) *Structure formation in sugar containing pectin gels – Influence of tartaric acid content (pH) and cooling rate on the gelation of high-methoxylated pectin*. Kastner, Kern, Wilde, Berthold, Einhorn-Stoll, & Drusch (2014), Food Chemistry, 144, 44-49, https://doi.org/10.1016/j.foodchem.2013.06.127 (Annex (A4), page 107).

(A5) *Structure formation in sugar containing pectin gels – Influence of gel composition and cooling rate on the gelation of non-amidated and amidated low-methoxylated pectin*. Kastner, Einhorn-Stoll, & Drusch (2017), Food Hydrocolloids, 73, 13-20, https://doi.org/10.1016/j.foodhyd.2017.06.023 (Annex (A5), page 115).

(A6) *Influence of enzymatic and acidic demethoxylation on structure formation in sugar containing citrus pectin gels.* Kastner, Einhorn-Stoll, & Drusch (2019), Food Hydrocolloids, 89, 207-215, https://doi.org/10.1016/j.foodhyd.2018.10.031 (Annex (A6), page 125).

Theoretical background

Pectin was first discovered and described in 1825 by the French scientist Henry Braconnot. The polysaccharide is a main component of practically all higher plant tissues. It is present in the middle lamella and in the primary cell wall (Van Buren, 1991).

Pectin is a heterogeneous, complex polysaccharide and contains at least 17 types of monosaccharides (Ridley, O'Neill, & Mohnen, 2001; Vincken et al., 2003). It mainly consists of a high amount of α-(1,4)-linked D-galacturonic acid residues (GalA) named homogalacturonan (HG). In addition, sections of highly substituted rhamnogalacturonan (RG I) are present, consisting of alternating rhamnose and galacturonic acid residues carrying side chains of mainly arabinose and galactose (De Vries, Rombouts, Voragen, & Pilnik, 1982; Schols & Voragen, 1996). Another structural element, present to a much lesser extent, is rhamnogalacturonan II (RG II), the complex and highly branched galacturonan region, can be composed of at least 12 different monosaccharide residues (O'Neill, Ishii, Albersheim, & Darvill, 2004).

Different pectin structural models are considered nowadays: the traditional "smooth and hairy region model" (Fig. 1) (Coenen, Bakx, Verhoef, Schols, & Voragen, 2007; De Vries et al., 1982; Voragen et al., 1995), the "side chain model" (Vincken et al., 2003), the "combined side chain – hairy region model" (Schols, Coenen, & Voragen, 2009) and a "new hypothetical model" (Yapo, 2011). The "smooth and hairy region model" (Fig. 1) suggest a pectin backbone of a linear homogalacturonan (HG) section (smooth region), branched with RG I sections (hairy regions) that contain neutral sugar side chains. In the "side chain model", HG is present as side chains of the RG I backbone and the "combined side chain – hairy region model", HG is available both as a backbone alternating with RG I and as a side chain of RG I regions. The "new hypothetical model", is a model extension of the "combined side chain – hairy region model" and additionally contains RG II at HG side chains.

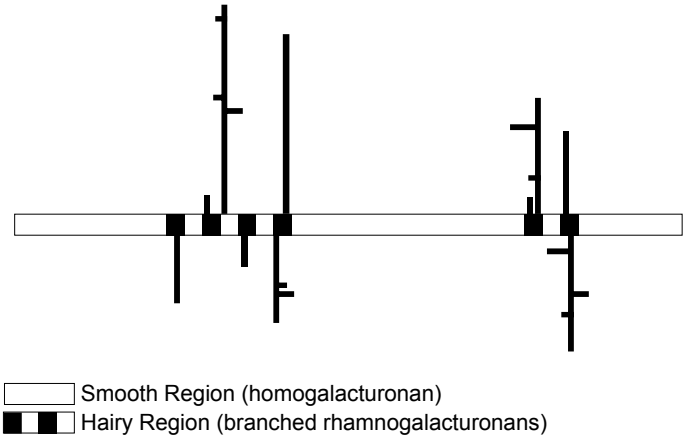

Fig. 1 Schematic overview of the smooth and hairy regions of pectin, according to Schols, Huisman, Bakx and Voragen (2003).

The molecular pectin structure varies in dependence on the substituents at C-6 of GalA in the HG regions (Fig. 2). The GalA residues can be free or methoxylated as well as amidated (Voragen, Coenen, Verhoef, & Schols, 2009). At C-2 or C-3 position, the GalA can also be acetylated, as known in sugar beet or sunflower pectin (May, 1990; Rolin, 2002).

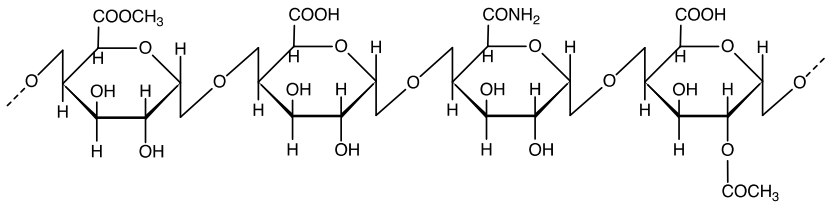

Fig. 2 Homogalacturonan region of pectin constituted of α-1,4-linked D-Galacturonic acid residues with different substituents: methoxylated group, free carboxyl group, amide group, acetyl group.

The major sources of industrial pectin are citrus peel, preferably from lemons or limes, as well as apple pomace, the dried residues from the juicing process (May, 2000; Rinaudo, 1996; Rolin, 2002; Willats, Knox, & Mikkelsen, 2006). In the last decades, pectin has also been extracted from a wide variety of other fruit and vegetable sources such as mango, guava, pomegranate, passion fruit, strawberry, thimbleberry, pineapple, carambola, tamarind, sunflower, sugar beet, broccoli, onion, carrot, tomato, potato, olive, hop, butternut and cacoa pod (Abid, Yaich, Hidouri, Attia, & Ayadi, 2018; Buchholt, Christensen, Fallesen, Ralet, & Thibault, 2004; Cardoso et al., 2003; Coelho et al., 2018; Fissore, Rojas, Gerschenson, & Williams, 2013; Hodgson & Kerr, 1991; Houben, Jolie, Fraeye, Van Loey, & Hendrickx, 2011; Iglesias & Lozano, 2004; Kaya, Sousa, Crépeau, Sørensen, & Ralet, 2014; Kyomugasho, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015; Lima, Paiva, Andrade, & Paixão, 2010; Oosterveld, Voragen, & Schols, 2002; Pereira et al., 2016; Vriesmann & Petkowicz, 2013; Vriesmann, Silveira, & Petkowicz, 2010; Yapo, Lerouge, Thibault, & Ralet, 2007). Commercial citrus and apple pectin differ significantly in molecular composition compared to alternative sources such as sugar beet and sunflower, as summarized below in Table 1.

Table 1 Typical molecular characteristics of pectin from various sources (Buchholt et al., 2004 [(1)]; Iglesias & Lozano, 2004 [(2)]; Müller-Maatsch et al., 2016 [(3)]; Thibault & Ralet, 2003 [(4)]).

	Citrus peel [3, 4]	Apple pomace [3, 4]	Sugar beet [1, 3]	Sun flower [2, 3]
Galacturonic acid	75-85%	> 65%	55-60%	46-75%
Degree of methoxylation	75-80%	75%	62%	> 45%
Degree of acetylation	< 5%	< 5%	30%	10-14%
Neutral sugar	< 15%	< 15%	< 20%	< 5%

Pectin has to be extracted from its native source by different procedures. The extraction from plants requires two main steps: aqueous extraction from the plant material as well as purification and isolation of the extracted pectin from the solution (Fig. 3) (Dominiak et al., 2014; Joye & Luzio, 2000).

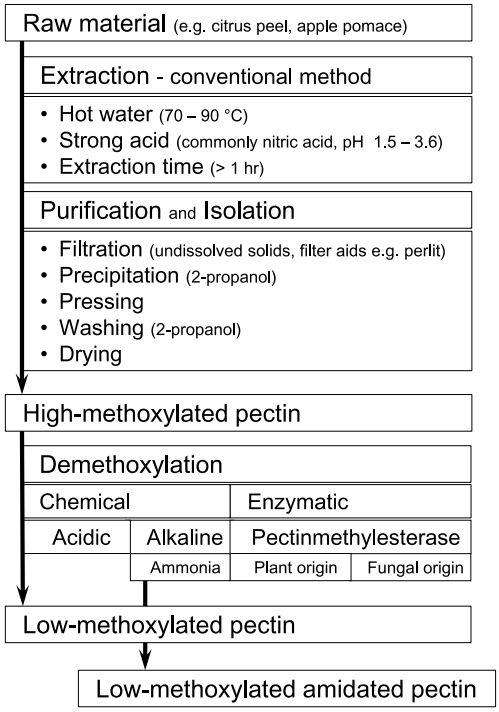

Fig. 3 Industrial pectin extraction process and modification methods.

The aqueous extraction step has the highest variability in its process and is crucial for the pectin properties. To separate pectin from the other plant polymers, extraction is traditionally performed in hot mineral acid (e.g. nitric acid, hydrochloric acid, sulfuric acid). This step reduces neutral sugar content, among other things, so that the extracted product mainly consists of a linear homogalacturonan domain. Typically, the extracted pectin from citrus and apple has a high degree of methoxylation. Its functionality, especially its gelling property, is strongly linked to its molecular structure. To this purpose, the structure of pectin can be modified regarding e.g. degree of methoxylation, pattern of free carboxyl groups and number of amide groups. Chemical or enzymatic methods allow this selective modification. These processes result in release of methyl groups, but pectin backbone may also depolymerize as a secondary reaction (Ralet, Dronnet, Buchholt, & Thibault, 2001; Vincent & Williams, 2009). Under acidic or alkaline conditions, deesterification is generally random. Enzymes such as pectinmethylesterases (PME), extracted from fungal or plant origin, induce different distributions of free carboxyl groups. Similar to chemical deesterification, fungal PME leads to a random distribution. In contrast, plant PME demethoxylate in a blocky pattern (Catoire,

Pierron, Morvan, du Penhoat, & Goldberg, 1998; Duvetter et al., 2009; Fraeye, Colle, et al., 2010; Glahn & Rolin, 1996; Rosenbohm et al., 2003; Savary, Hotchkiss, & Cameron, 2002; Thibault & Ralet, 2003). Chemical demethoxylation of HMP in presence of ammonium ions results in low-methoxylated amidated pectin (LMAP) where around 15-18% of the carboxyl groups are in the amide form (Lopes da Silva & Rao, 2006). Amidation is normally carried out in an aqueous alcohol slurry at low temperature (May, 2000).

The degree of methoxylation (DM) is the main parameter for classifying pectin (Fig. 4). Pectin containing methoxyl groups at 50% or more of the GalA residues are classified as high-methoxylated pectin (HMP).

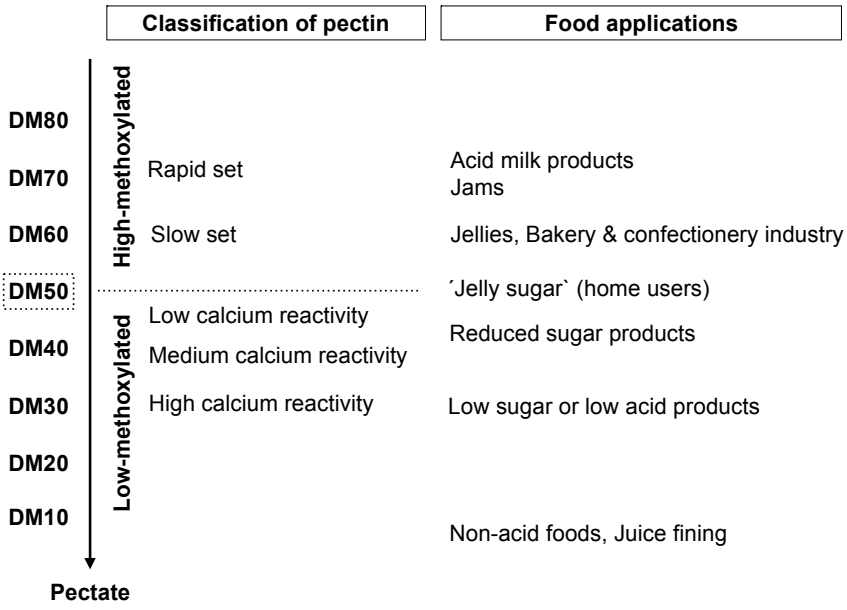

Fig. 4 Classification and application of pectin, depending on its degree of methoxylation (May, 2000).

Depending on their gelation (setting) time, commercial HMP can be further divided into rapid set and slow set (Fig. 4). The low-methoxylated pectin (LMP) are produced by demethoxylation of HMP until less than 50% of the GalA residues are methoxylated. The typical DM of HMP for commercial use is about 60-77%. These HMPs are used in jams, jellies, fruit preparations, bakery fruit fillings and glazes, as well as in acidified milk products. The typical DM of LMP is about 25-40%. They are commonly applied in low sugar products, fruit-based yoghurts, acidified milk drinks, ice cream and in the juice industry (May, 1990; Thibault & Ralet, 2003; Voragen et al., 1995). The additional amide group plays a positive role in LMAP gelation: less calcium is required for gelation and gels show more elastic and transparent properties than those made from LMP. Additionally, gelation is possible in a broader pH range. Therefore, LMAP is suitable for a broader range of applications than HMP or LMP (May, 2000).

Structure formation during gelation

The structure formation of pectin is rather complex and its principles have been broadly investigated and described in the literature in the last decades (Axelos et al., 1996; Burey et al., 2008; Cardoso et al., 2003; Christiaens et al., 2016; Evageliou et al., 2000c; Fraeye, Duvetter, et al., 2010; Garnier, Axelos, & Thibault, 1991; Holst et al., 2006; Löfgren et al., 2005; Lopes da Silva & Rao, 2007; Ngouémazong, Kabuye, et al., 2012; Oakenfull & Scott, 1984; Rees, 1982; Rolin, 2002; Ström et al., 2007; Thakur et al., 1997; Thibault & Ralet, 2003; Tsoga et al., 2004; Vincent & Williams, 2009; Voragen et al., 1995; Yapo & Gnakri, 2015). In general, pectin molecules form a three-dimensional network via specific intermolecular bonds in junction zones in the smooth regions. A high surplus of water can be immobilized in the resulting intermolecular voids.

Hydrophobic interactions, hydrogen bonds and ionic interactions are the three typical bonds or interactions forming junction zones and are thus involved in the gelation of pectin. Depending on the DM and the environmental conditions, they are more or less involved in the gelling process (Fig. 5).

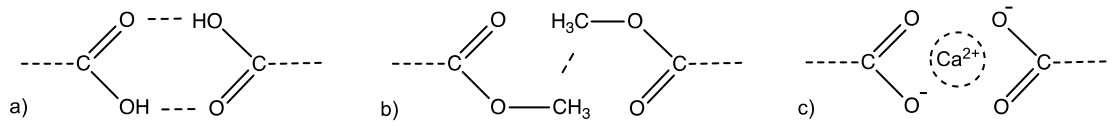

Fig. 5 Schematic illustration of the gelation mechanisms of pectin chains. a: Hydrogen bonds between undissociated carboxyl groups. b: Hydrophobic interactions between methoxyl ester groups. c: Random ionic interactions (crosslinks) between dissociated carboxyl groups. Calcium bridges at subsequent free dissociated carboxyl groups can form egg-boxes.

Independent of DM, hydrophobic interactions are formed between the methyl ester groups immediately at the start of the cooling process and induce the gelation (Oakenfull & Scott, 1984). These interactions have a rather low energy and limited working range of about 2 nm (Walstra, 2002, Chapter 3) and they become weaker with decreasing temperature. Instead, upon further cooling at lower temperatures hydrogen bonds start to form by hydrophilic interactions between undissociated carboxyl groups of the galacturonic acid and/or hydroxyl groups of carboxyl, hydroxyl or amide groups (Oakenfull & Fenwick, 1977; Oakenfull & Scott, 1984). These bonds are also of low energy and with 0.2 nm their working range is even smaller than that of the hydrophobic interactions. Therefore, the pectin molecules have to come in close contact in order to form a gel network. This can be achieved by a high soluble solid concentration (> 50%) since the resulting reduced water activity allows the approach of pectin chains (Evageliou, Richardson, & Morris, 2000b; Thakur et al., 1997). A low pH additionally reduces the dissociation of the carboxyl groups and, as a consequence, suppresses the electrostatic repulsion between pectin molecules, in turn promoting the formation of hydrogen bonds. The influence of the hydrogen bonds gains more importance upon temperature reduction and supports inter-chain association during network formation. Hydrophobic interactions and hydrogen bonds are involved in forming junction zones during

gelation of all types of pectin and are typical for the cold-set gelation (Burey et al., 2008) of HMP.

Gelation of LMP is additionally governed by an ionotropic gelation. Calcium ion bridges are formed between dissociated carboxyl groups via ionic interactions (Fig. 6) at pH above 3.5 (Burey et al., 2008; Fraeye, Duvetter, et al., 2010). They start to form immediately after gel preparation and are much stronger than hydrophobic and hydrogen bonds and with about 20 nm their working range is rather long (Walstra, 2002, Chapter 3). Therefore, pectin gels with combined or dominating ionic junction zones require less soluble solids than HMP gels can also be formed in sugar-free systems.

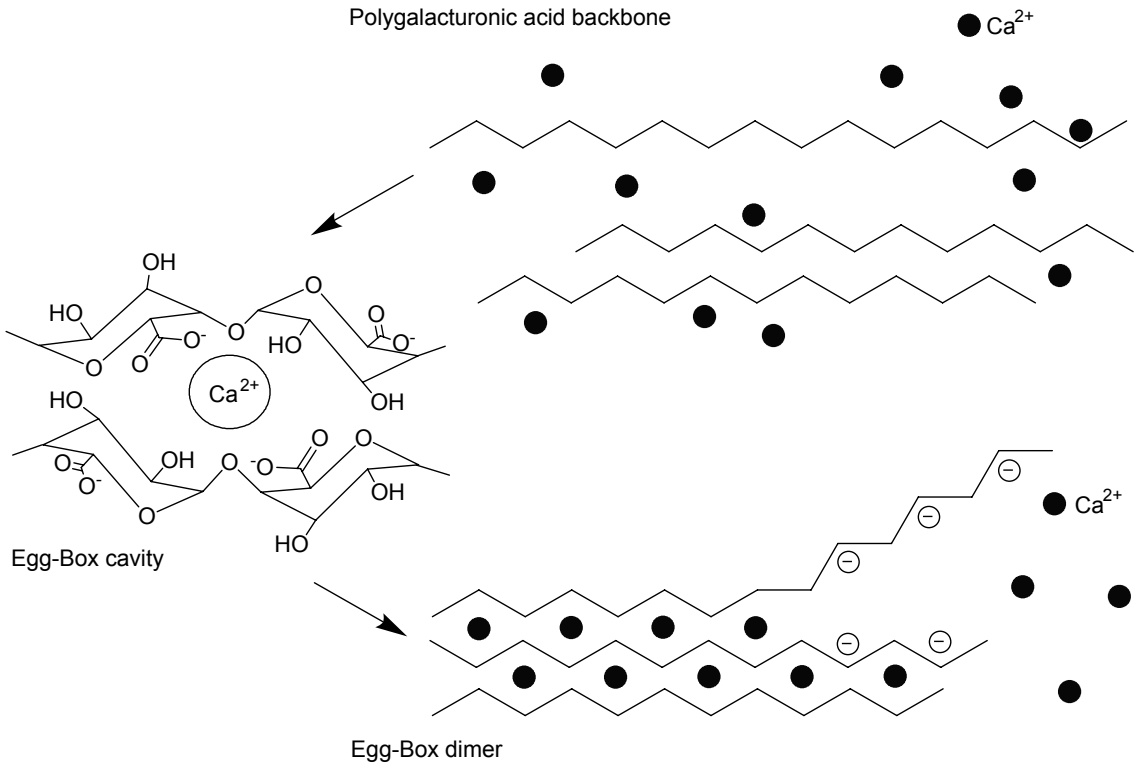

Fig. 6 Schematic representation of the gelation of low-methoxylated pectin, in presence of calcium ions; egg-box-model according to Morris, Powell, Gidley, & Rees, 1982.

Ionic interactions require a certain number (block) of about 6 to 20 subsequent dissociated carboxyl groups (Fraeye, Duvetter, et al., 2010; Liners, Thibault, & Cutsem, 1992; Luzio & Cameron, 2008; Powell, Morris, Gidley, & Rees, 1982; Vincent & Williams, 2009) in order to form so called “egg-boxes” (Fig. 6). Short “egg-box” junction zones are formed between two pectin chains at high temperature by addition of calcium. Lowering of the temperature, highly cooperative helix compounds were formed by a conformational transition. This process is induced / accelerated by charge neutralization, lower mobility and subsequent helix aggregation of pectin chains. Thus, after initial dimer formation also inter-dimer interactions

and crosslinking of pectin molecules occur by associations of two- or threefold helices due to hydrophobic interactions and hydrogen bonds (Cárdenas, Goycoolea, & Rinaudo, 2008; Cardoso et al., 2003; Gilsenan et al., 2000). The ionotropic gelation occurs in pectin solutions without heating (Ström & Williams, 2003; Vincent & Williams, 2009) and may be performed as an isothermal titration at room temperature (Fang et al., 2008). It has been reported that ionic interactions formed at higher temperature tend to be initially less stable but that their stability will increase during cooling (Cárdenas et al., 2008; Garnier, Axelos, & Thibault, 1993).

The formation of junction zones during LMAP gelation is still not completely understood. The distribution of amide and methoxyl groups in LMAP remains unclear, it is assumed that the structure is rather heterogeneous (Guillotin, Van Loey, Boulenguer, Schols, & Voragen, 2007). It seems that all described types of bonds and interactions contribute to this process and that LMAP gels are additionally stabilized by hydrogen bonds involving the amide group (Alonso-Mougán, Meijide, Jover, Rodríguez-Núñez, & Vázquez-Tato, 2002; Black & Smit, 1972; Löfgren, Guillotin, & Hermansson, 2006).

Determination and comparison of gelling properties – influencing factors

The gel properties of pectin have been investigated for many decades. Doesburg & Grevers (1960) reported investigations of gel-setting time of HMP in a sugar-acid environment at around 1930. The percentage of sagging of a gel cone under its own weight was established as an empiric method by the IFT Committee (1959), based on the work of Cox & Higby (1944). It is known as SAG-method and is still applied in the pectin producing industry in order to standardize pectin for commercial use and to evaluate the strength of pectin gel. The standard composition of gels in this method depends on the pectin type (Table 2): HMP gel, with dominant cold-set gelation, is formed from a solution containing about 65% soluble solids (mainly sucrose) and at pH around 2.3 (adjusted by adding 48.8% w/v tartaric acid). A gel for ionotropic gelation (LMP and LMAP) additionally requires a reduced total solid content of 31%, a pH around 3.0 (adjusted by adding 54.3% w/v citric acid) and the addition of calcium ions. These two sugar-acid model gel formulations are common compositions and simulate a conventional jam (May, 2000; Rolin, 2002).

Table 2 Properties of standard sugar-acid gel (HMP) and sugar-calcium gel (LMP and LMAP).

	Sugar-acid gel	Sugar-calcium gel
pH	2.3	3.0
Total solid	65%	31%
Pectin content	0.27%	0.67%
Calcium ions ($CaCl_2$)	–	0.62 mM/100g gel

Other new methods were stablished, providing more information on gel properties and gelation time or temperature. Endreß et al. (1996) developed the Pectinometer-method, which is based on the method of Lüers & Lochmüller (1927) and examines the breaking strength. Oakenfull & Scott (1984) as well as O'Brien, Philp, & Morris (2009) used relatively simple visual tests, and Rao, Cooley, Walter, & Downing (1989) introduced instrumental texture profile parameters for the investigation of setting temperature and time. Pectin producer CP Kelco determined the gelling temperature via conductivity measurements (Bøttger et al., 2008). Nowadays, researchers and pectin manufacturers characterize the pectin structuring and gel properties by fundamental rheological measurements. Small amplitude oscillatory rheological measurements are used for studying structure as well as network development of different food gels (Doublier et al., 1992). The stored energy (storage modulus, G´ (Pa)) and the dissipated energy (loss modulus, G´´ (Pa)) of a sample are determined in these rheological test during a sinusoidal strain cycle (Clark & Ross-Murphy, 1987; Doublier, Launay, & Cuvelier, 1992). Both, the elastic and the viscous characteristics are measured and, as a result, the viscoelastic properties are evaluated. Other rheological properties, such as the complex viscosity (G*) and the loss factor (tanδ) are calculated from the moduli. Using G´ and G´´ data as a function of frequency (ω), it is possible to define important parameters affecting the structure formation, such as gelling temperature or time (Rao & Cooley, 1993).

Several factors related to solution or gel composition, technological settings and molecular characteristics determine pectin gelation and gel properties. This section summarizes major influencing factors with impact on pectin structure formation. Some of these factors are studied in the presented thesis (Annex A1-A6):

- ***pH of gel composition****:* The typical cold-set gelation in sugar-acid environment requires a low pH. This low pH reduces the dissociation of the carboxyl groups. As a consequence, the electrostatic repulsion between pectin molecules is suppressed and hydrogen bonds are formed between the non-dissociated carboxyl groups and secondary hydroxyl groups (Agoub et al., 2009; Lopes da Silva & Rao, 2007; Oakenfull & Fenwick, 1977; Oakenfull & Scott, 1984; Thakur et al., 1997; Voragen et al., 1995). At lower pH, less carboxylic groups dissociate. The undissociated groups are able to form hydrogen bonds, especially at local ''high-acid spots''. This might cause pre-gelation and microgel formation. Ross-Murphy (1984) described such structures as ''incomplete gels''. Typical ionotropic gelation above pH 4.5 is relatively independent of pH (Lootens et al., 2003). Below pH 4.5, the pectin charge density decreases and the affinity for calcium ions decreases. Additional hydrogen bonds between undissociated carboxyl groups compensate the reduced number of ionic junction zones during structure formation (Cardoso et al., 2003; Lootens et al., 2003). At lower pH, especially below pH 3.5, LMP can form also gels even in absence of calcium ions. Results of different studies revealed that with decreasing pH and dissociation of carboxyl groups a transition from a two-fold to a three-fold helix

conformation occurs and is supported by hydrogen bonds (Gilsenan et al., 2000; Kjoniksen, Hiorth, Roots, & Nystrom, 2003; Lootens et al., 2003). Nevertheless, at low pH, the calcium ions have a reinforcing effect on LMP gels (Lootens et al., 2003).

- ***Temperature**:* The cooling conditions should be defined in order to achieve an optimum gel structure (Cardoso et al., 2003; Dahme, 1992; Garnier et al., 1993; Rao et al., 1993). However, there is no specification for "optimum", structure formation and gel properties depend on the application. In jam production, for example, the gelling temperature of the added pectin should correspond to the respective filling temperature. Pre-gelation as a result of a too high gelling temperature leads to air inclusion, heterogeneous gel structure and increased syneresis. Too low gelling temperature, in contrast, causes fruit separation and softer gels (May, 2000). It is therefore important to define and control the structure formation as well as the gel properties of pectin.
- ***Calcium ions**:* The most important divalent cations in pectin gelation are calcium ions. Their impact on pectin gelation depends on the stoichiometric ratio (*R*-value) between calcium ions and dissociated free carboxyl groups among two single pectin chains. It is calculated as $R = 2[Ca^{2+}]/[COO^-]$ (Axelos & Kolb, 1990; Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2006; Cárdenas et al., 2008; Garnier et al., 1993; Ngouémazong, Nkemamin, et al., 2012; Ström et al., 2007). At a theoretical saturation threshold of the *R*-value ($R = 1$), every calcium ion in the gel is bound to two dissociated carboxyl groups. This threshold is affected on the one hand by the degree of dissociation of the carboxyl groups and, thus, by the pH in the gel system. At the pK_a of GalA at pH about 3.5 (Ralet et al., 2001), 50% of the carboxyl groups are dissociated. On the other hand, the binding of calcium to pectin chains also depends on the distribution of the free carboxyl groups along the backbone (block-wise or random). Ionic interactions require a certain number (blocks) of about 6 - 20 subsequent dissociated carboxyl groups (Fraeye, Duvetter, et al., 2010; Liners et al., 1992; Luzio & Cameron, 2008; Powell et al., 1982; Vincent & Williams, 2009) in order to form egg-box junction zones. Vincent and Williams (2009) therefore suggested a modified R_{eff}, in which only dissociated carboxyl groups in blocks are considered. However, the calculation of their exact number requires, however, detailed knowledge of the pectin molecular structure. Calcium ions may also interact with single randomly distributed dissociated carboxyl groups. In case these groups are oriented to the outside of the egg-boxes, larger dimer aggregates and even an extended network are formed (Braccini & Pérez, 2001; Fraeye, Colle, et al., 2010; Fraeye et al., 2009). In contrast, excess calcium ions, located in the gap between galacturonic acid molecules and interacting with other C-atoms than C-6 (Siew, Williams, & Young, 2005), might course a certain electrostatic repulsion. The number of unspecific or random calcium crosslinks will increase with higher calcium ion content. When the calcium content becomes too high, precipitation and/or syneresis will occur and the gel strength decreases (Fraeye et al., 2010; Grosso & Rao, 1998). Other divalent cations (e.g. Mg^{2+}, Zn^{2+}, Ba^{2+},

Cu^{2+}, Fe^{2+}) have a comparable impact on pectin gelation, but the type and concentration of the ions determine the structure formation and gel properties (Axelos et al., 1996; Huynh, Chambin, du Poset, & Assifaoui, 2018; Kyomugasho et al., 2016; Mierczyńska, Cybulska, Sołowiej, & Zdunek, 2015).

- ***Amidation**:* Amidated pectin require less calcium ions for ionotropic gelation due to the lower amount of dissociated carboxyl groups and additional hydrogen bonds formed between the amide groups (Alonso-Mougán et al., 2002; Capel et al., 2006; Löfgren et al., 2006; Lootens et al., 2003). Gels prepared from amidated pectin are stronger, especially at low pH, and less sensitive to syneresis (Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2005; Racape, Thibault, Reitsma, & Pilnik, 1989; Thakur et al., 1997; Thibault & Ralet, 2003).
- ***Degree of methoxylation and pattern of free carboxyl groups**:* Several studies focused on the impact of the pattern of free carboxyl groups or substituents along the pectin backbone on structure formation of pectin (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Fraeye et al., 2009; Löfgren et al., 2005; Ngouémazong, Tengweh, et al., 2012; Ström et al., 2007). It is common knowledge that demethoxylation by acid or alkali as well as a by most fungal pectinmethylesterases (fPME) results in a random distribution, whereas demethoxylation by PME of plant origin (pPME) produces a more blockwise distribution of the free carboxyl groups. The degree of methoxylation and the distribution of the carboxyl groups in a more random or more block-wise pattern determine the gelling process, in particular ionotropic gelation (Fraeye, Colle, et al., 2010; Fraeye et al., 2009; Löfgren et al., 2005; Lutz, Aserin, Wicker, & Garti, 2009; Ngouémazong, Tengweh, et al., 2012; Yapo & Koffi, 2013). Pectin with a dominating block-wise distribution is able to gel at lower calcium concentrations than pectin with a more random distribution of free carboxyl groups. Gels of pectin with block-wise distribution were found to become more cross-linked and elastic properties increased (Fraeye, Colle, et al., 2010; Löfgren et al., 2005).

There are other factors, including e.g. pectin molecular characteristics and gel composition, which influence gelation significantly but have not been tested in this thesis:

- ***Acetylation**:* Some pectin types, in particular sugar beet pectin, contain a high amount of acetyl substituents and have poor gelling properties (Alba, Laws, & Kontogiorgos, 2015; Oosterveld, Beldman, Searle-van Leeuwen, & Voragen, 2000; Ralet, Crépeau, Buchholt, & Thibault, 2003). During ionotropic gelation, acetyl groups significantly reduce gel strength as well as gel formation ability (Renard & Jarvis, 1999; Vriesmann & Petkowicz, 2013). They act as spacers and inhibit the close contact between pectin macromolecules.
- ***Branching**:* A high amount of neutral sugar side chains in the rhamnogalacturonan may affect the gel formation (Hwang & Kokini, 1992; Ngouémazong, Kabuye, et al., 2012; Oosterveld et al., 2000; Schmelter, Wientjes, Vreeker, & Klaffke, 2002). For example,

debranching of pectin caused lower gel strength, lower elastic properties and a “weaker” gel structure in rheological characterizations of calcium gels. The reduced gel strength is attributed to a reduction of polymer chain entanglements, mainly due to the reduction in side chain entanglements in debranched pectin solutions (Ngouémazong, Kabuye, et al., 2012).

- ***Chain length**:* Several studies revealed that pectin gelation was affected by a reduction of the molecular weight of pectin (Capel et al., 2005; Kim, Yoo, Kim, Park, & Yoo, 2008; Luzio & Cameron, 2008; Ngouémazong, Kabuye, et al., 2012; Powell et al., 1982). Strong depolymerisation results in short pectin chains, which are not able to form an extended network and prevent gelation (Capel et al., 2005). This was confirmed by results of our group which were no subject of this thesis and are presented elsewhere (Kastner, Einhorn-Stoll, & Drusch, 2018).
- ***Monovalent cations**:* Monovalent cations, in particular sodium and potassium, cause gelation of LMP or HMP with block-wise pattern or free carboxyl group also in the absence of calcium ions (Ström et al., 2014; Wehr, Menzies, & Blamey, 2004; Yoo et al., 2009; Yoo, Fishman, Savary, & Hotchkiss, 2003). The gel characteristics depends on the type and concentration of the monovalent ions. A combination of charge neutralization and ionic strength effects is assumed to be responsible for this type of gelation (Ström et al., 2014).
- ***Pectin content**:* In general, gel strength increases significantly with pectin concentration (Fraeye et al., 2009; Fu & Rao, 2001; Han et al., 2017; Lopes da Silva & Rao, 2007). At a low pectin concentration, ionic bonds are more frequently formed within a single chain, and such intramolecular bonds do not contribute to gelatin. With increasing pectin concentration, intermolecular interactions become dominating and more / longer junction zones are formed (Cardoso et al., 2003; Jarvis & Apperley, 1995).
- ***Soluble solids**:* In general, soluble solids such as sucrose reduce the pectin-solvent interaction and promote contact between pectin chains as well as the formation of junction zones (Evageliou et al., 2000c; Fu & Rao, 2001; Grosso, Bobbio, & Airoldi, 2000; Tsoga et al., 2004). Structure formation and gel properties depend not only on the sugar concentration but also on the type of sugar and the pH value. For example, adding sucrose increases the elastic properties of pectin gels more than adding glucose (Grosso & Rao, 1998). The mechanisms of the influence of sugar is not completely understood (Fraeye et al., 2009).

Results and discussion

1 Examination of the gelling process and determination of typical structuring temperatures[1]

The commonly used gel point (GP) was defined as intercept of storage modulus (G´) and loss modulus (G´´) in a rheological oscillation measurement (Fig. 7a, bottom). In some special cases such as pre-gelation, however, G´ is higher than G´´ already from the start of the rheological measurements, or the curves are more or less parallel during a longer cooling period without clear intercept (Fig. 7a, top). Therefore, additional reliable parameters were introduced in order to compare and evaluate the structure formation and gelation kinetics of any pectin.

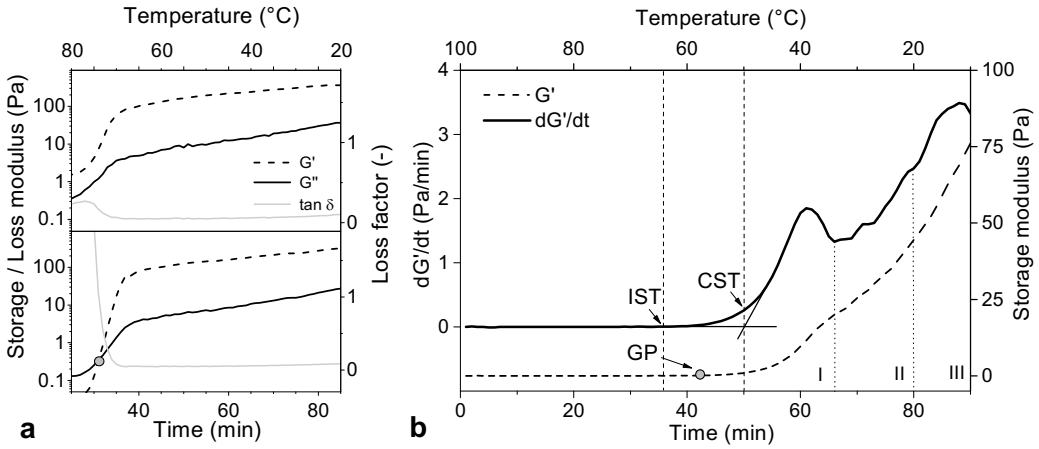

Fig. 7 (a) Oscillation measurements of high-methoxylated pectin during cooling (1 K/min), the storage modulus (G´; —); loss modulus (G´´; —), loss factor (tanδ; —, thin line). Top: without gel point (GP; tanδ = G´´/G´ ≠ 1), bottom: with GP (•; tanδ = G´´/G´ = 1). (b) Typical curve of the structure formation of pectin (in this case low-methoxylated pectin) during cooling (1 K/min); full curve = dG´/dt, dashed curve = G´; dashed vertical lines = IST and CST; dotted vertical lines = possible phases I, II, III. The GP is marked as (•) on the G´ curve (Kastner, Einhorn-Stoll, & Senge, 2012b; Kastner et al., 2014).

The first derivation of G´ as function of time, the structuring velocity dG´/dt, was used for a better description of the gelling kinetic (Grosso & Rao, 1998) (Fig. 7b). This method was modified in the presented work by defining new parameters for structure formation from the smoothed structuring velocity curve (dG´/dt), the initial structuring temperature (IST) and the critical structuring temperature (CST) (Fig. 7b). The IST is defined as the temperature at which dG´/dt differs from 0 for the first time, it indicates the start of structure formation in a system with still dominating liquid-like (viscous) character (G´ < G´´). The CST is defined as

[1] Parts of the section were published as Kastner et al. 2012a, 2012b, 2014 and Einhorn-Stoll et al. 2012 (Annex (A1)-(A4)).

the extrapolated temperature of the first strong increase of structuring velocity curve and reports the first acceleration in structure formation during transition to a more solid-like (elastic) system (G´ > G´´) (Fig. 7b).

All pectin samples, high- and low-methoxylated, which have been investigated (Annex (A1) and (A2), pages 79 and 87) with the introduced method showed continuous structure formation during the cooling process, either by a steady development of G´ or by a change of structuring velocity curve. The method was successfully applied also for samples with pre-gelation and no detectable GP (Fig. 7a, top).

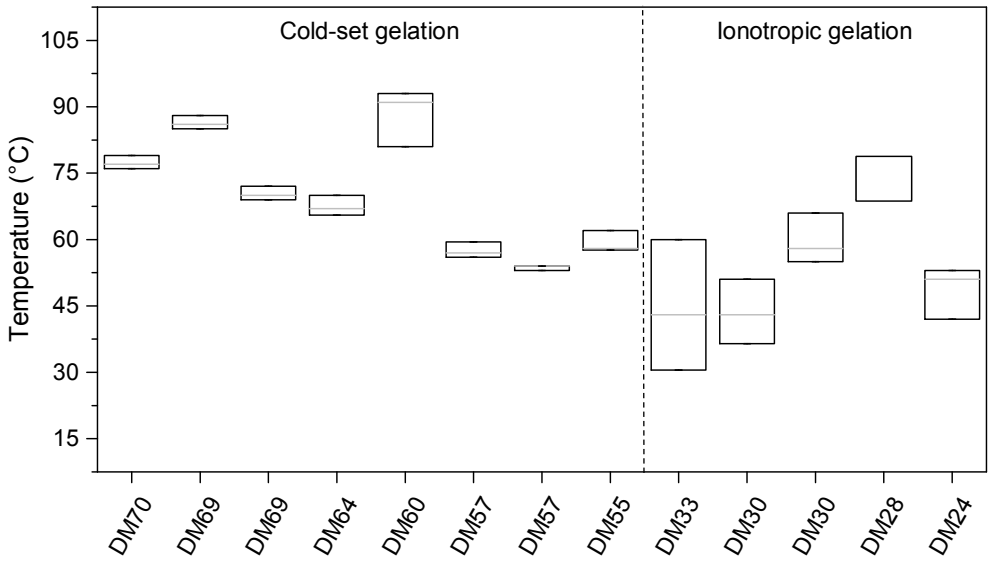

Fig. 8 The average values of initial structuring temperature (IST), critical structuring temperature (CST) and gel point temperature (GP) of commercial pectin samples with different degree of methoxylation (DM) (analyzed in Kastner et al. (2012a) and Einhorn-Stoll et al. (2012)). The boxes include the IST (highest line), CST (lowest line) and GP (grey line in the box).

The difference between IST and CST varied and the classical GP marked only one single point in this range (Fig. 8). IST was higher than or equal to GP (if the latter was regularly determined) in all tested standard sugar-acid or sugar-calcium gels (Fig. 8), and the CST was found mostly below the GP. The structuring process was obviously strongly accelerated after a certain critical number of junction zones were formed in an increasingly solid-like system (G´ > G´´). Sol-gel-transition is more a process in a certain temperature range than a single point like the GP, depending on gel composition and on temperature. The IST during a typical cold-set gelation indicated the first detectable structure formation by hydrophobic interactions. Almost all structuring temperatures of investigated HMP and in part LMP samples were above 50 °C (Fig. 8), the typical range of hydrophobic interactions. Further cooling promoted formation of more hydrophobic interactions and the system became more elastic. This was indicated by the CST. Hydrogen bonds started to form below 50 °C, which is reflected in another increase of the curve (Fig. 7b). In case of ionotropic gelation, the gelation was comparable but additional calcium bridges supported the junction zone

formation over the whole cooling range, beginning from high temperatures.

The shape of the structuring velocity curves (e.g. DM70 and DM30 in Fig. 9b and c, respectively) as well as the temperature range of structure formation (e.g. DM69, DM57, DM30 in Fig. 8) is characteristic for any single pectin and may vary also for pectin samples of similar DM.

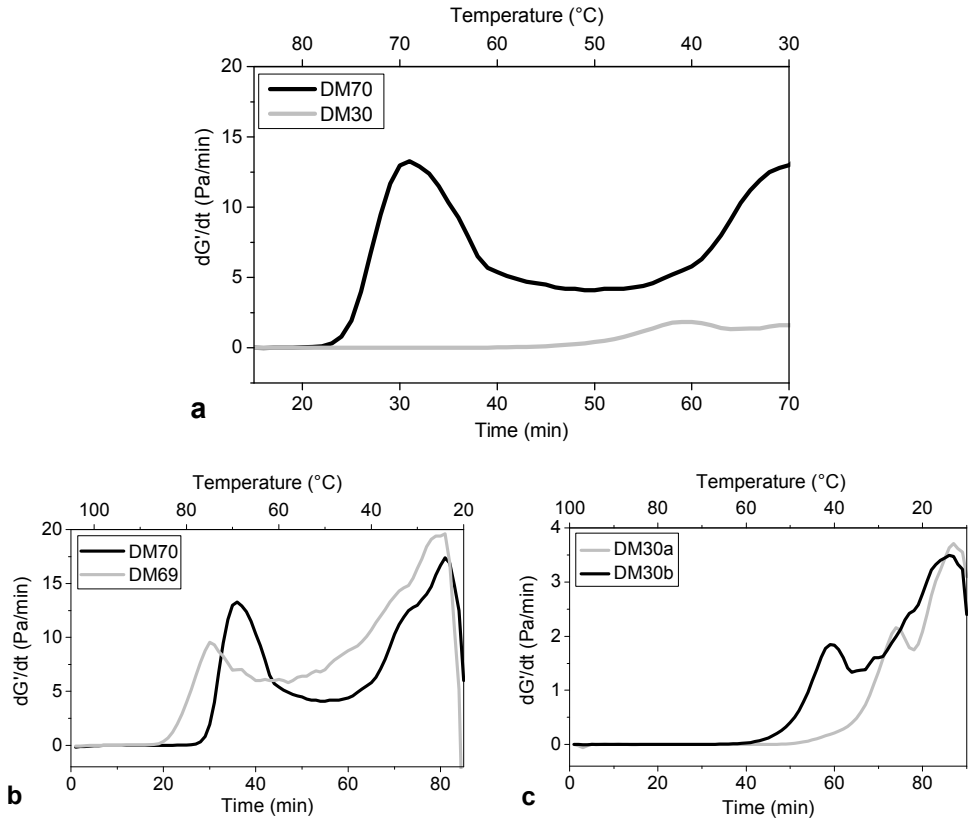

Fig. 9 Comparisons of the structure formation (dG´/dt) of pectin during cooling (1 K/min) in dependence on degree of methoxylation: (a) comparison of an HMP (DM70) and a LMP (DM30) from the same company (shown from 85 to 30 °C); (b) comparison of two HMP from different companies with similar DM (cooled from 105 to 20 °C); (c) comparison of two LMP from different companies with similar DM (cooled from 100 to 10 °C) (Kastner et al., 2012a).

Fig. 9 shows structuring velocity curves (dG´/dt) of typical LMP and HMP gels from the presented study. The shape and level of the curves varied. The levels of the LMP curves were generally lower than those of HMP curves (Fig. 9a), this was explained by the decreasing solid content from 65% for HMP to only 31% for LMP. The varying shapes of dG´/dt curves of HMP and LMP probably resulted from differences in gelation type (cold-set gelation for HMP and ionotropic gelation for LMP) and from different interactions and bonds dominating in dependence on temperature during the structuring processes. Different shapes of curves of pectin samples with similar DM were identified in both processes (Fig. 9b, c). Increase and decrease in the curves represent typical phases of the structuring process as known form literature: The gelation of HMP, a typical cold-set gelation in sugar-acid

environment, is considered to be a two-step process with two types of interactions. Hydrophobic interactions between the methoxyl groups of the GalA at temperature dominate in phase I above 50 °C, supported by the high concentration of sugar (around 65%) that reduces the pectin-solvent interaction and promotes the hydrophobic interactions (Fig. 7b, Fig. 9b). Phase II is characterized by weakening of the hydrophobic interactions below 50 °C and increased formation of hydrogen bonds between non-dissociated carboxyl groups as well as hydroxyl groups. Both are supported by the low pH (2.3), which reduces the dissociation of the carboxyl groups and the electrostatic repulsion between pectin molecules (Fig. 7b). The temperature range around 50 °C is known as the typical threshold between the two phases of cold-set gelation (Alonso-Mougán et al., 2002; Evageliou et al., 2000c; Joesten & Schaad, 1974; Oakenfull, 1984; Oakenfull & Fenwick, 1977). Comparable tendencies were found in LMP gelation (Fig. 7b, Fig. 9c). However, additional junction zones via calcium bridges were formed by ionotropic gelation already at high temperature in the first phase of gelation (Grant, Morris, Rees, Smith, & Thom, 1973). They are independent on temperature and are built during the complete gelation process. Similar to HMP gels, hydrophobic interactions were promoted by sugar, the impact of hydrogen bonds increased during cooling and inter-chain or inter-dimer associations occurred (phase II). Random electrostatic interactions of calcium ions with single dissociated carboxyl groups of neighbored pectin molecules (calcium crosslinking) additionally promoted the structuring process during final cooling (phase III). However, ionotropic gelation in the applied LMP gel systems depended on the number of dissociated free carboxyl groups. At pH around 3 (below the pK_a = 3.5) this number should be relatively low. As a consequence, the formation of the typical egg-box junction zones should be limited and more interactions between undissociated carboxyl groups via hydrogen bonds would be formed instead.

The presented study revealed that alterations in the structuring velocity curves of pectin samples (Fig. 9b) indicated changing structuring mechanisms during gelation (Fig. 10). Fig. 10 is a summary of all interactions contributing to pectin gelation during cooling:

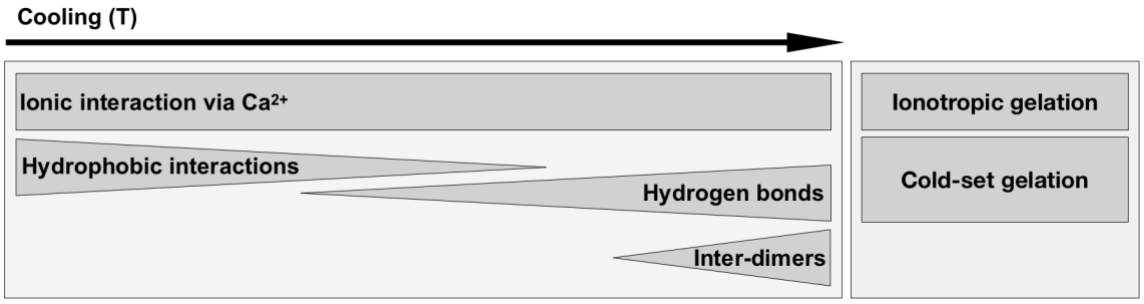

Fig. 10 Schematic illustration of structure mechanisms during ionotropic gelation and cold-set gelation, in dependence on temperature (T).

The decrease of the initial hydrophobic interactions is compensated by an increasing number of hydrogen bonds. Later on, dimer associates and inter-dimer aggregates might form within

the pectin gel in the final phase of gelation (Fig. 10). In general, the steady increase of G´ indicated the continuous structure formation during the entire cooling process (Fig. 7b).

IST and CST mark the temperature range of initial and accelerated gelation and directly reflect the impact of DM, in this study. They support the understanding of the gelling process of pectin in dependence on different factors (e.g. molecular characteristics) which affect the structure formation of pectin by supporting or inhibiting the interactions of pectin molecules in the growing junction zones during sol-gel transition.

Cold-set and ionotropic gelation differ in gel composition and the dominating gelation mechanisms, and the two gelation types are not directly comparable. Using structuring velocity curves, it was, however, possible to compare tendencies and phases of their structure formation kinetics in order to understand the impact of individual factors on the two gelation mechanisms.

2 Examination of the pH influence by varying the acid content[2]

Subject of this section is the influence of varying pH values on gelation of two pectin types, one HMP with DM of 69.8%, GC of 74.3% and intrinsic viscosity of 554 cm^3/g, and one LMP, with DM of 30.2%, GC of 81.5% and intrinsic viscosity of 336 cm^3/g. The gel composition was varied in comparison to the standard gels (Table 2, page 29). Variations of the acid concentration (48.8% w/v tartaric acid) in HMP gels resulted in pH values between 2.0 and 2.5, whereas the pH of standard HMP gel was 2.2 (Annex (A4), page 107). pH values of LMP gels were varied from 2.9 to 4.0, using different amounts of 54.3% w/v citric acid, whereas the pH of standard sugar-calcium gel was 3.1 (Annex (A5), page 115). In the next section HMP will be discussed first, then LMP and finally a complete comparison of HMP and LMP.

Comparing the structuring temperatures of the HMP series, the maximum difference of IST and CST, respectively, were only about 3 to 4 K. Lowering the pH resulted in a decrease of IST from 79.6 to 75.3 °C, CST from 77.2 to 74.3 °C and GP from 76.5 to 73.0 °C. The structuring velocity curves, however, varied considerably (Fig. 11a). At the lowest acid content (pH 2.5) structure formation was slow, probably because the majority of free carboxylic groups dissociated at low pH and were not available for formation of hydrogen bonds. Moreover, intermolecular electrostatic repulsion between dissociated carboxyl groups additionally inhibited structure formation. Gels with moderate addition of acid (pH 2.4, 2.2 and 2.1), showed the highest structuring velocities and similar curves (Fig. 11a). These acid contents allowed a rapid undisturbed gel formation. In gels with the highest acid content (pH 2.0), the structure formation was delayed again. Less carboxylic groups dissociated, and many hydrogen bonds were formed, in particular in local spots. They contributed to pre-gelation and microgel formation and induced a heterogeneous gel structure.

[2] Parts of the section were published as Kastner et al. 2014 and 2017 (Annex (A4) and (A5)).

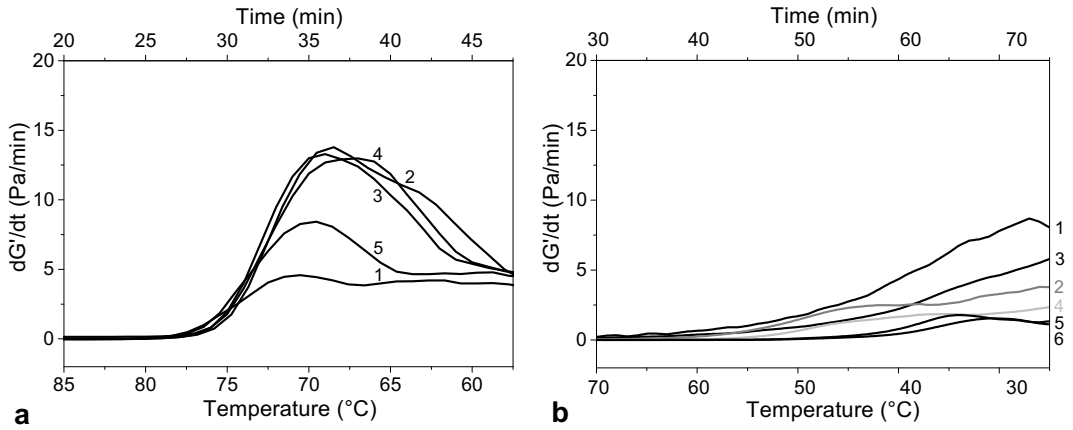

Fig. 11 Influence of acid concentration of (a) sugar-acid gel (HMP) and (b) sugar-calcium gel (LMP) on structuring velocity (dG´/dt). (a) tartaric acid in mM/kg gel: (1) 9.6 = pH 2.5, (2) 15.9 = pH 2.4, (3) 22.3 = pH 2.2, (4) 28.7 = pH 2.1, (5) 35.1 = pH 2.0; (b) citric acid in mM/kg gel: (1) 4.7 = pH 4.0, (2) 11.0 = pH 3.6, (3) 17.3 = pH 3.3, (4) 23.6 = pH 3.1, (5) 29.8 = pH 3.0, (6) 36.1 = pH 2.9 (Kastner, Einhorn-Stoll, & Drusch, 2017; Kastner et al., 2014).

The viscoelastic properties of the cooled final HMP gels differed considerably with varying acid content (Fig. 12a). The elastic properties dominated in all gels (tanδ < 1, Fig. 12a). The gels of intermediate pH 2.4 to 2.1 were similar, strong and comparable to those of other HMP samples as investigated in Section 1 (page 35). The higher the pH, the weaker and more viscous became the gels because of the limited number of hydrogen bonds. The heterogenous gels at the lowest pH were weak due to pre-gelation and microgel formation.

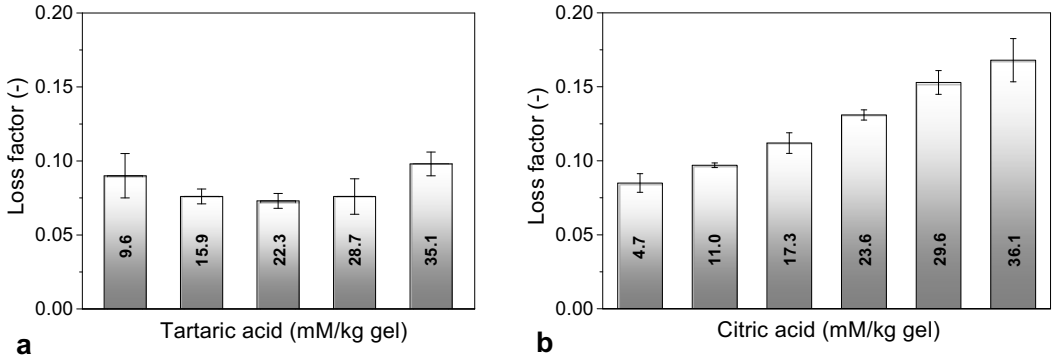

Fig. 12 Influence of acid concentration of (a) sugar-acid gel (HMP) and (b) sugar-calcium gel (LMP) on loss factor (tanδ) at end of cooling (10 °C). (a) tartaric acid in mM/kg gel: (1) 9.6 = pH 2.5, (2) 15.9 = pH 2.4, (3) 22.3 = pH 2.2, (4) 28.7 = pH 2.1, (5) 35.1 = pH 2.0; (b) citric acid in mM/kg gel: (1) 4.7 = pH 4.0, (2) 11.0 = pH 3.6, (3) 17.3 = pH 3.3, (4) 23.6 = pH 3.1, (5) 29.8 = pH 3.0, (6) 36.1 = pH 2.9 (Kastner et al., 2017, 2014).

A decrease of pH from 4.0 to 2.9 in LMP gels reduced the number of dissociated carboxyl groups, which are potential binding sites for calcium ions, and inhibited ionotropic gelation. In contrast, electrostatic repulsion between pectin molecules decreased at lower pH, favored association and aggregation of pectin chains by intermolecular hydrogen bonds, and

additionally stabilized these gel systems in the absence of calcium. The structuring temperatures of LMP decreased significantly with decreasing pH, in case of IST from 74.3 to 51.8 °C and of CST from 53.5 to 40.0 °C. A clear GP was found only at pH < 3.1 (57.2 to 46.8 °C). The temperatures decreased due to the lower number of dissociated carboxyl groups, necessary for the rapid formation of ionic junction zones via calcium bridges at high temperature, and the gelling process was delayed (Fig. 11b). Limited hydrophobic interactions (only 30% of the carboxyl groups were methoxylated) were not sufficient to induce sufficient gelation at temperatures > 50 °C. The structure formation of LMP at low pH was mainly due to hydrogen bonds, but they were formed later and at lower temperatures. The structuring velocity curves decreased with decreasing pH (Fig. 11b). Obviously, the increasing number of hydrogen bonds between non-dissociated carboxyl groups was not able to compensate the lack of calcium bridges. The same effect was found for the viscoelastic gel properties; with decreasing pH, LMP gels became more viscous and softer, by still dominating elastic properties, the gel structure was more brittle (Fig. 12b).

In summary, the variation in added acid resulted in pH of 2.0 to 2.5 for HMP gels and 2.9 to 4.0 for LMP gels, respectively. The structure formation and the viscoelastic properties were affected even by small deviations from the pH value in the standard gel formulation. The impact on HMP and LMP gels differed. In HMP gels at pH between 2.1 and 2.4 structure formation and gel properties were similar and the start of gelation did not change when the pH value increased or decreased. The later structure formation, however, was influenced, what was reflected in the viscoelastic properties. They were pronounced, although elastic properties still dominated the system, the gels became more viscous. Both higher and lower acid concentrations inhibited cold-set gelation in different ways and altered the gel structure. In case of LMP, the increase in pH accelerated ionotropic gelation systematically and the gels became softer, more homogeneous and less brittle. The investigation of ionotropic gelation at different pH revealed that a reduced number of dissociated carboxyl groups by decreasing pH continually delayed pectin gelation and affected the gel properties. The increased formation of hydrogen bonds was not sufficient to compensate the reduced number of ionic junction zones for ionotropic gelation and therefore resulted in an increase of viscous properties of the gels.

3 Examination of the influence of cooling conditions[3]

In this section, the influence of the cooling rate systematically examined using the previously chosen pectin samples: HMP, with DM 69.8%, GC 74.3% and an intrinsic viscosity of 554 cm^3/g and LMP, with DM 30.2%, GC 81.5% and an intrinsic viscosity of 336 cm^3/g.

The cooling rates during gelation of pectin vary depending on the surrounding conditions and the pectin content (Dahme, 1992). To relate the influence of cooling on structure formation in the selected sugar-acid environment, in a first experiment a standard gel of HMP in jam jars were cooled at room temperature (Annex (A4), page 107). The temperature decrease as well as the cooling gradient were recorded. The average cooling rate was 0.16 to 0.45 K/min, determined in the temperature range of the phase transition from liquid to solid between 85 and 50 °C, of the used HMP. Considering these results, the structure formation for standard gels of HMP (Annex (A4), page 107) and LMP (Annex (A5), page 115) were investigated at cooling rates from 0.25 to 2.00 K/min.

The individual results are presented separately below, first for HMP and later for LMP. The influence of the cooling rate on cold-set and ionotropic gelation is finally compared.

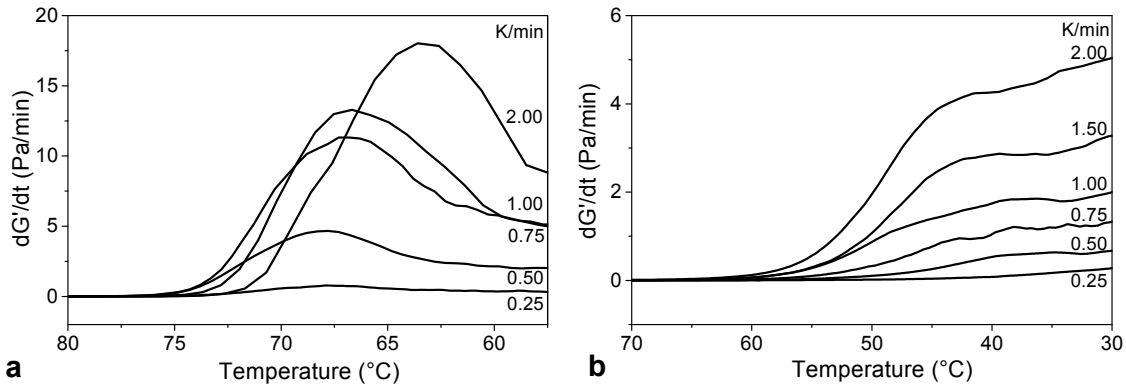

Fig. 13 Influence of cooling rate of (a) sugar-acid gel (HMP) and (b) sugar-calcium gel (LMP) on structuring velocity (dG´/dt) (Kastner et al., 2017, 2014).

[3] Parts of the section were published as Kastner et al. 2014 and 2017 (Annex (A4) and (A5)).

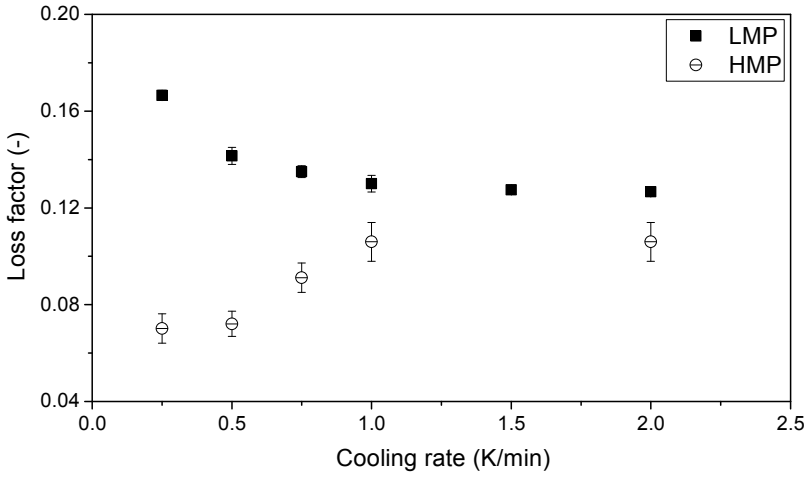

Fig. 14 Influence of cooling rate of sugar-acid gel (HMP) and sugar-calcium gel (LMP) on loss factor (tanδ) at end of measurement (10 °C).

The structuring temperatures (IST, CST, GP) of the HMP gels decreased mostly not significantly with increasing cooling rates from 0.25 to 2.00 K/min (Annex (A4), page 107). They varied for IST from 83.6 to 73.0 °C, for CST between 75.3 and 71.4 °C and for GP from 77.4 to 71.0 °C. The structuring velocity curves of the HMP gels, however, strongly increased (Fig. 13a) at higher cooling rate. Considering also the viscoelastic gel properties after cooling (Fig. 14), the tested gels results can be divided into three groups:

- Low cooling rates of 0.25 and 0.50 K/min: The structure formation (Fig. 13a) of these gels started earlier (higher IST), and the level of structuring velocity curves was lower than in all other gels. It seems that there was sufficient time for an optimum arrangement of pectin molecules to form many strong intermolecular interactions and long junction zones for gelation. As indicated by low values of the loss factor ($\tan\delta < 1$), the final gels were more elastic and less viscous than all others (Fig. 14).
- Medium cooling rate of 0.75 K/min: The maximum structuring velocity of the gels was higher than those of the two lower rates and more similar to those of the third group (Fig. 13a). The structure formation started, however, earlier and the IST was more comparable to those of lower cooling rates. The final gel was an intermediate, too (Fig. 14). It differed clearly from the slowly cooled gels but only slightly from the rapidly cooled.
- High cooling rates of 1.0 K/min or 2.0 K/min: The structuring velocity in these gels had the highest level (Fig. 13a), but the structure formation started at low IST. Independent on temperature, a certain time was necessary to form junction zones and for the optimum interaction of pectin chains. The final gels were the least elastic and most viscous and the gel structure was less homogeneous compared to gels prepared at slow cooling rates, although elastic properties dominated the system ($\tan\delta < 1$, Fig. 14). A possible explanation is, that in the early stage of structure formation less molecular arrangement

occurred and shorter junction zones or even local microgels were formed (Fig. 15). As a result, the structure of the three-dimensional network was less homogeneous. It is not clear, whether the structure formation was already complete after the end of the measurement. Some authors examined pectin gels after the end of the cooling process by continuing rheological measurements and found an aging effect, sometimes referred as annealing (Evageliou et al., 2000b; Fu & Rao, 2001; Lopes da Silva & Gonçalves, 1994). This effect may be caused by a transition of shorter to longer junction zones (Fig. 15), as described for gelatin gelation by Ziegler and Foegeding (1990).

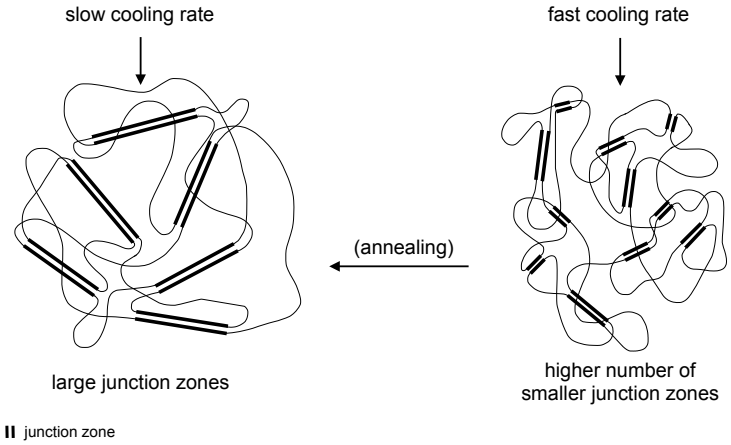

Fig. 15 Formation of pectin junction zones during cooling (Kastner et al., 2014).

Depending on cooling rate, the IST of LMP gels varied between 59.5 and 65.8 °C and the CST differed between 43.4 and 54.1 °C. The GP for all cooling rates was between IST and CST (Annex (A5), page 115). The structuring velocity curves of the LMP gels showed a systematic increase with increasing cooling rate (Fig. 13b). In case of the lowest rate of 0.25 K/min, it seems that the energy content of the system was rather high during the long time at high temperature, and the small number of hydrophobic interactions (at the low DM) was not sufficient for a substantial gel formation. The formation of hydrogen bonds was delayed, too. The distance between pectin molecules was large and the ionic bonds were not completely stable, yet (Cárdenas et al., 2008; Garnier et al., 1993). Moreover, calcium ions might be delocalized between the pectin molecules instead of binding closely in bridges and forming a strong gel. The latter effect might be comparable to the counter ion condensation as described by Siew et al. (2005). A faster cooling (e.g. 2 K/min) supported early formation of calcium bridges, stabilized by the more rapid formation of hydrogen bonds. With increasing cooling rate, the loss factor significantly decreased (but was below 1, Fig. 14), indicating that the final gel structure depends on the cooling rate, too. Thus, the influence of the cooling rate on junction zone length and the resulting final gel structure, as described for gels with hydrogen bonds such as gelatin (Ziegler & Foegeding, 1990) and the above described cold-set gelation (Fig. 15), could not be confirmed for ionotropic gelation.

In summary, the influence of cooling on the structuring velocity curves was similar in both cold-set and ionotropic gelation, only their level differed. The cooling rate in all pectin gels had a moderate influence on the structuring temperatures IST and CST but clearly affected the structuring velocity and the properties of the final gels. The most important difference with respect to the cooling rate is the difference in the final gel properties. Gels behaved completely opposite with increasing tanδ for HMP gels and decreasing tanδ for LMP gels. The additional junction zones formed via calcium bridges in the LMP altogether reduced the influence of the cooling rate on the pectin gelation. These results agree with results above (Section 1, page 35), which described differences in the gelling mechanisms of HMP and LMP in sugar-acid gel formulations caused by the additional occurrence of ionic interactions and the temperature dependence of the specific gelation mechanisms (Fig. 10, page 38). Comparing to HMP, rapid cooling promoted early structure formation during cold-set gelation and a less elastic structure of resulting gels occurred due to a rapid formation of shorter junction zones. In contrast, at slow cooling a retarded formation of longer junction zones took place and the final gel structure was more compact and elastic.

4 Examination of the influence of calcium ion content on ionotropic gelation[4]

This investigation varied the amount of calcium ions in the standard LMP gel. The influence of calcium ions depends on the stoichiometric ratio between calcium ions and the dissociated free carboxyl groups, calculated as *R*-value = 2[Ca^{2+}]/[COO^-] (Axelos & Kolb, 1990). The standard LMP gel has a standard *R*-value of 0.7, which was varied in both directions, down to 0.46 and up to 0.94. The used LMP sample was the same as in Section 2 (page 40) and Section 3 (page 43).

IST, CST and GP increased with higher calcium content (Fig. 16a). IST and CST developed in a nearly parallel way with IST > CST and a distance of about 10 K. The GP changed differently: At low calcium concentration (*R*-value = 0.46) it was found about 25 K below IST and also 15 K below CST, but at high calcium content (*R*-value > 0.82) GP was higher than IST (Fig. 16a).

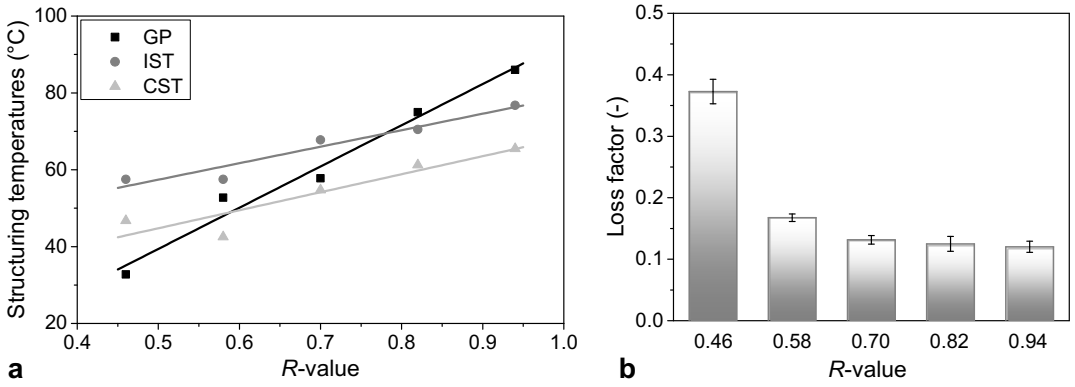

Fig. 16 Comparison of (a) structuring temperatures (GP, IST and CST) and (b) gel properties (loss factor) in dependence on calcium content (*R*-value). GP = gel point temperature; IST = initial structuring temperature; CST = critical structuring temperature (Kastner et al., 2012b).

The structuring velocity increased with higher calcium content, and the shape of the curves varied (Fig. 17). Two to three phases of structure formation were detected during gelation of LMP with different calcium content. They probably reflected the different mechanisms during ionotropic gelation, as described in Section 1 and Fig. 10 (page 38). Depending on the calcium content, the starting and final temperatures of the phases varied. The first phase might be ascribed to the rapid formation of ionic junction zones via calcium bridges at high temperature, combined with a small number of random crosslinks. This phase was clearly detected in gels with *R*-value above 0.58 and shifted to higher temperature with increasing calcium content. The second phase probably indicated the action of hydrogen bonds at lower temperatures, whereas the third phase was dominated by inter-dimer interactions. In gels

[4] Parts of the section were published as Kastner et al. 2012b (Annex (A3)).

with the least calcium content (R = 0.46) only two phases were found. Their structure formation started at lower temperatures by a combined influence of ionic and hydrogen bonds and the first and second phases were united. Also, at the highest tested calcium content no clear difference between first and second phase, but this time ionic interactions dominated the complete gelling process with.

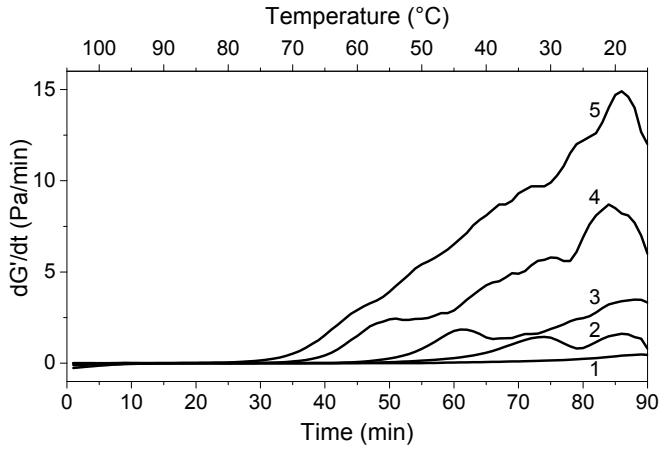

Fig. 17 Influence of calcium concentration on structuring velocity (dG´/dt) with *R*-value: (1) *R* = 0.46, (2) *R* = 0.58, (3) *R* = 0.70, (4) *R* = 0.82, (5) *R* = 0.94 (Kastner et al., 2012b).

The calcium content affected not only the gelation process but also the final gel structure (Fig. 16b). Systems with lowest calcium content gelled mainly by non-ionic interactions and the gels were weak with dominating elastic properties. Gels with a higher amount of calcium formed more ionic interactions, were more stable and elastic. The highest concentration of calcium produced gels with a heterogeneous structure, resulting from pre-gelation and microgel formation, they were more brittle.

In summary, the tested LMP gels prepared at pH 3 and with around 31% sucrose, required at least a small amount of calcium in ionic junction zones for successful gelation. Hydrophobic interactions, hydrogen bonds and other mechanisms alone were not sufficient, even at the promoting sugar content. The structuring temperatures increased (up to 20 K) with increasing calcium content and confirmed the importance of the ions for LMP gelation. All types of calcium bridges obviously increased the structuring velocity during cooling and supported the formation of viscoelastic gels with dominating elastic properties. Above a certain calcium content, pre-gelation might take place during or immediately after gel preparation. The properties of the final gels confirmed the varying gel structures, resulting from different structuring mechanisms in dependence on the calcium content.

5 Examination of the influence of amide group during ionotropic gelation[5]

A direct comparison of the structuring processes of a non-amidated and an amidated LMP, prepared with comparable calcium contents in relation to free carboxyl groups (0.7 for the LMP and 0.73 for the LMAP) and under identical conditions of acid content and cooling rate was the subject of this study. The non-amidated LMP sample was the same as in the previous study (Section 4, page 47). Table 3 shows the molecular characteristics of LMP and LMAP.

Table 3 Comparison of LMP and LMAP gel with similar *R*-value. Degree of methoxylation (DM), galacturonic acid content (GC), degree of amidation (DA), intrinsic viscosity ([η]), stoichiometric ratio of calcium ions and free carboxyl groups (*R*-value), initial structuring temperature (IST), critical structuring temperature (CST), gel point temperature (GP), loss factor as determined after the end of the gelation (tanδ) (Kastner et al., 2017).

	DM (%)	GC (%)	DA (%)	[η] (cm^3/g)	*R*-value	IST (°C)	CST (°C)	GP (°C)	tanδ
LMP	30.2	81.5	-	336	0.70	65.8	53.1	57.2	0.131
LMAP	32.2	68.4	19.1	450	0.73	65.8	60.3	64.4	0.079

The initial gelling process of both samples was similar (comparable IST), but the acceleration of structure formation, as characterized by CST and GP, started significantly earlier in the LMAP gel than in that of LMP (Table 3). The structuring velocity curves of non-amidated and amidated pectin (Fig. 18) and the final gel properties differed. The LMP gel was more viscous ('soft') and the LMAP gel more elastic (brittle) (Table 3).

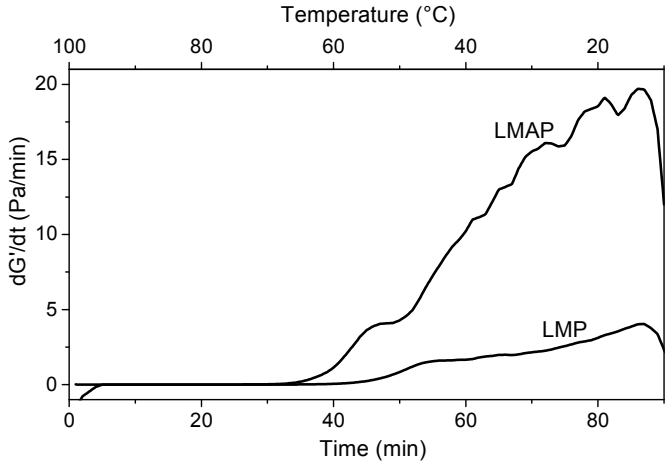

Fig. 18 Comparison of structuring velocity curves (dG´/dt) of LMP and LMAP sugar-calcium gels with similar *R* (0.70, 0.73, respectively) under the same conditions (Kastner et al., 2017).

The direct comparison revealed differences in the gelling mechanisms of non-amidated and amidated pectin, in particular due to the additional hydrogen bonds formed by the amide

[5] Parts of the section were published as Kastner et al. 2017 (Annex (A5)).

group in LMAP. The impact of these bonds and their dependence on acid concentration (pH), as well as the effect of the calcium content, on gelation of LMAP will be discussed in detail.

Acid content

Similar to the study of Section 2 (page 40), variations of the citric acid concentration in LMAP gels resulted in pH values between 3.0 and 4.0 and the *R*-value was 1.09 (Annex (A5), page 115).

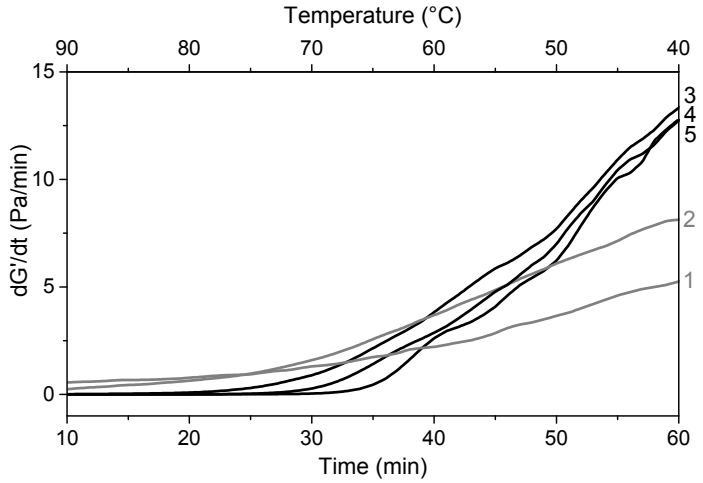

Fig. 19 Influence of acid concentration on structuring velocity (dG´/dt) of sugar-calcium gelation of LMAP. Citric acid in mM/kg gel: (1) 4.7 = pH 4.0, (2) 11.0 = pH 3.5, (3) 17.3 = pH 3.3, (4) 23.6 = pH 3.1, (5) 29.8 = pH 3.0 (Kastner et al., 2017).

The results can be divided into two groups according to the structuring temperatures, structuring velocity curves and viscoelastic properties of the gels. In general, the structuring temperatures decreased significantly with decreasing pH, and a clear GP was found only at pH above 3.1. In the first group, at pH 4.0 and 3.5, the structuring process started at higher temperatures compared to the second group, (IST were 89.2 and 89.6 °C and CST were 72.4 and 73.7 °C, respectively), and the structuring velocity curves had a lower level (Fig. 19). At pH 3.3 and below, the structure formation started later with IST of 78.1 to 70.0 °C and CST of 68.0 to 58.9 °C. Comparing the final gel properties, at higher pH the gels were more viscous than that at lower pH, even though the elastic properties still dominated the system.

As described previously for non-amidated LMP (Section 4, page 47), also in LMAP the higher number of hydrogen bonds between non-dissociated carboxyl groups at lower pH did not compensate the lack of calcium bridges. In contrast to LMP, the steep increases of the structuring velocity curves of LMAP (number 3 to 5, Fig. 19) underlined the supportive effect of the higher number of hydrogen bonds formed due to the presence of the amide groups. These bonds, on the one hand supported as well as accelerated the gelling process, and on the other hand they compensated to a certain extent the lack of ionic interactions at lower temperature.

Calcium ion content

Similar amounts of calcium ions like in the study on non-amidated LMP (Section 4, page 47) were added to the gels of LMAP. The *R*-values were between 0.72 and 1.45. The *R*-values in the LMAP gels were higher than in LMP gels (between 0.46 and 0.94), despite of a similar amount of added calcium and comparable DM of the two samples, since the presence of amidated groups reduced the number of free dissociated carboxyl groups of LMAP.

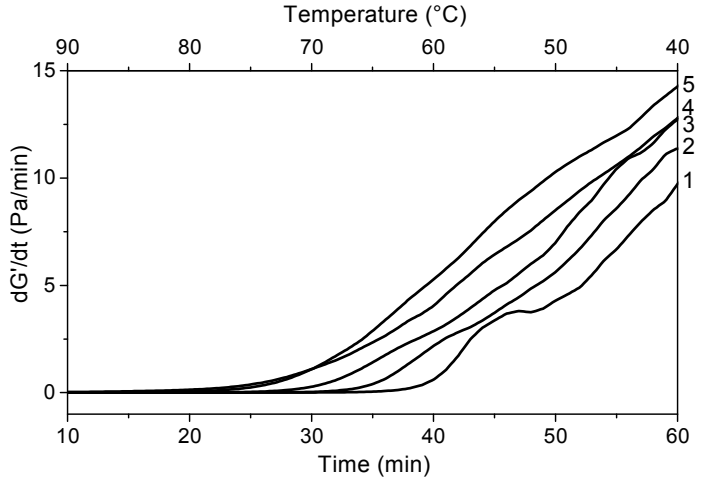

Fig. 20 Influence of calcium content on structuring velocity (dG´/dt) of LMAP with *R*-value: (1) *R* = 0.73, (2) *R* = 0.90, (3) *R* = 1.09, (4) *R* = 1.28, (5) *R* = 1.45.

The shape of the structuring velocity curves of LMAP was similar for all *R*-values (Fig. 20), but the start of the gelling process differed. Below 60 °C, the structuring velocity curves increased parallel over a range of 30 K (Fig. 20) and achieved a similar level, followed by a subsequent non-linear structure development (not shown). The structuring temperatures IST and CST significantly increased with increasing calcium content. The IST varied in a broader range (64.3 to 81.8 °C) than the CST (60.3 to 69.4 °C). The GP differed between IST and CST. At *R*-value = 1.45 no GP was detectable (Annex (A5), page 115). With increasing *R*-value, the IST raised faster than the CST. It is assumed, that a high calcium content in all gels induced a more rapid initial structure formation by immediately forming calcium bridges between blocks of dissociated free carboxyl groups (ionotropic gelation). The main structuring process, as indicated by the CST, was not accelerated to the same extent. It seems reasonable that, as a consequence of a surplus of calcium ions and the low share of dissociated carboxyl groups in blocks, calcium ions did not only form bridges but also bound to only one pectin chain and induced an electrostatic repulsion of chains by positive charges. This effect might disturb the gel stabilization by hydrogen bonds between undissociated carboxyl as well as amide groups (cold set-gelation). These bonds have a small working range and were not formed due to the distance between neighbored charged parts of pectin molecules. The gel properties of LMAP also reflected this difference by an increase of the

viscous characteristics with increasing calcium content (tanδ: 0.08 to 0.10) and indicated a rather inhomogeneous structure (Annex (A5), page 115).

Summarizing the influence of additional amide groups in dependence on pH (3.0 and 4.0) and calcium concentration (*R*-value between 0.73 and 1.45) by comparing the impact of increasing calcium content on the gelation of non-amidated LMP and amidated LMP of similar DM (Section 4, page 47), considerable differences were found. LMAP required less calcium for starting the structuring process. The lower number of dissociated free carboxyl groups in amidated pectin were earlier saturated with calcium ions, reducing the number of ionic interactions. The formation of additional hydrogen bonds involving amide groups, however, probably promoted the structuring process. An increase of pH supported the number of potential binding sites for calcium ions and, thus, accelerated gelation in both pectin gels of LMP and LMAP. The impact of pH on the gelation of LMAP was lower, because the additional hydrogen bonds including amide group in these gels seemed to be less dependent on pH than hydrogen bonds between carboxyl groups. Though the comparison was influenced by the different stoichiometric ratio, also a direct comparison of the gelation of LMP and LMAP with similar *R*-values in the range of 0.70 to 0.94 confirmed the results.

6 Examination of the influence of pattern of free carboxyl groups along pectin backbone on cold-set and ionotropic gelation[6]

One of the first studies of this work (Section 1, page 35) (Annex (A1), page 79) compared the structure formation of groups of commercial pectin samples, HMP as well as LMP. The molecular characteristics within the groups were similar, their structure formation differed considerably. A parallel study, testing the impact of molecular characteristics of these commercial pectin samples on their gelling and material properties (Annex (A2), page 87), found only limited correlation. A possible explanation might be a different distribution of the free carboxyl groups along the galacturonic acid backbone. It is generally known that, beside the degree of methoxylation, also this distribution has an impact on the material and functional properties of pectin.

In a systematic study (Annex (A6), page 125) one commercial HMP (named OP, Table 4) was demethoxylated by two enzymatic treatments with fungal pectin methyl esterase (fPME: f-pectin; F) or plant PME (pPME: p-pectin; P) as well as by a chemical treatment with acid (a-pectin; A). The three resulting types of pectin, each with four ranges of DM (average DM of 62%, 57%, 50%, 41%, respectively), had different distributions of free carboxyl groups along the backbone (random for f-pectin and a-pectin or block-wise for p-pectin). The two HMP groups were named as DM62 and DM57, and the two LMP groups as DM50 and DM41.

Table 4 Molecular characteristics of reference sample (OP) and modified samples in dependence on the method of demethoxylation. Degree of methoxylation (DM), galacturonic acid content (GC), intrinsic viscosity ([η]), sodium ion content (Na^+) (Kastner et al., 2019).

	Sample	DM (%)	GC (%)	[η] (cm^3/g)	R^2	Na^+ (mg/g)
Reference	OP	68.0 ± 0.9	83.1 ± 1.5	538	0.997	10.5 ± 0.0
pPME	P61	60.9 ± 0.5	84.7 ± 1.3	528	0.996	16.6 ± 0.5
	P57	56.8 ± 0.6	86.5 ± 0.8	503	0.998	21.4 ± 0.1
	P51	51.4 ± 0.6	82.1 ± 0.4	320	0.994	20.2 ± 0.0
	P40	40.4 ± 0.6	84.9 ± 1.1	239	0.980	25.1 ± 0.1
fPME	F62	61.9 ± 0.3	88.5 ± 1.0	531	0.998	11.0 ± 0.1
	F56	56.1 ± 0.6	94.2 ± 2.0	530	0.997	12.6 ± 0.1
	F49	49.0 ± 0.2	82.3 ± 0.4	309	1.000	20.8 ± 0.1
	F42	41.9 ± 0.5	78.0 ± 0.1	298	0.957	24.3 ± 0.4
Acidic	A62	62.0 ± 0.1	90.0 ± 0.6	470	0.990	0.7 ± 0.0
	A57	57.3 ± 0.3	93.9 ± 1.2	453	0.994	0.5 ± 0.0
	A50	49.7 ± 0.7	88.3 ± 0.4	421	0.998	0.0 ± 0.0
	A42	41.5 ± 0.7	81.7 ± 1.0	414	0.992	0.1 ± 0.0

The block-wise or random distribution of free carboxyl groups in the HMP was tested by their calcium sensitivity. The value of the pPME treated samples was about 100x higher (P61 =

[6] Parts of the section were published as Kastner et al. 2019 (Annex (A6)).

427.2 and P45 = 509.0 mPas, respectively) than that of the corresponding f-pectin and a-pectin samples, (4 to 6 mPas). The type of distribution was also assumed for the corresponding LMP, even if the blocks of free carboxyl groups increased with decreasing DM.

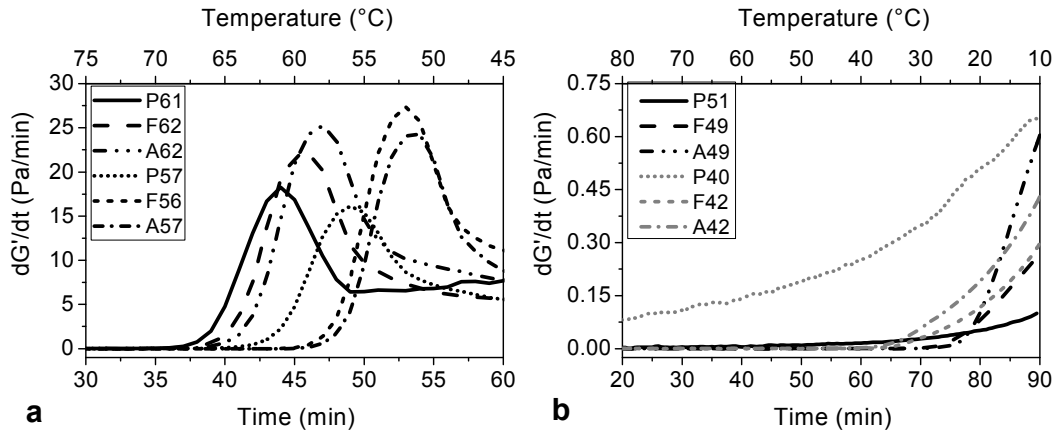

Fig. 21 Influence of pectin demethoxylation method on the structuring velocities (dG´/dt) during cooling at 1 K/min. Sugar-acid gels of DM62 and DM57 samples (HMP) during 75 to 45 °C in (a) and sugar-calcium gels of DM50 and DM42 samples (LMP) during 80 to 10 °C in (b) (Kastner et al., 2019).

The structuring velocity curves are shown in Fig. 21a. The start of the gelling process (IST) of the sugar-acid gels of HMP samples differed significantly (Fig. 22a). At DM62, the order of nearly all structuring temperatures (IST, CST, GP) was p-pectin > f-pectin > a-pectin samples (Fig. 22a). At DM57, the structuring temperatures for p-pectin were significantly higher than those for f-pectin and a-pectin, which were similar in IST, CST and GP.

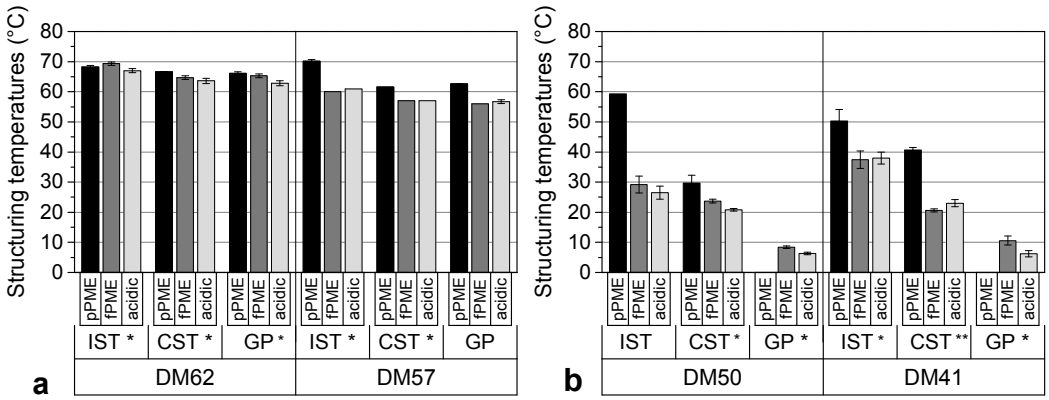

Fig. 22 Influence of pectin demethoxylation method on the structuring temperatures. Sugar-acid gels of DM62 and DM57 samples (HMP) in (a) as well as sugar-calcium gels of DM50 and DM42 samples (LMP) in (b). Structuring temperatures: IST = initial structuring temperature, CST = critical structuring temperature and GP = gel point. Significant differences: * for P < 0.05 and ** for P < 0.01 (Kastner et al., 2019).

In contrast to the sugar-acid gels, the structuring temperatures of the sugar-calcium gels of LMP increased with decreasing DM (Fig. 22b). No sol-gel transition was detected for the p-pectin samples. At the start of rheological measurements, the gels underwent pre-gelation with elastic dominating over viscous properties (data not shown).

The viscoelastic properties of the final gels after cooling (tanδ) were similar for the two HMP groups (DM62 and DM57) (Fig. 23a). The elastic properties dominated in all HMP gels, but they were lower for the DM57 samples than for the DM62 pectin. This indicated a more elastic gel structure of the DM57 samples.

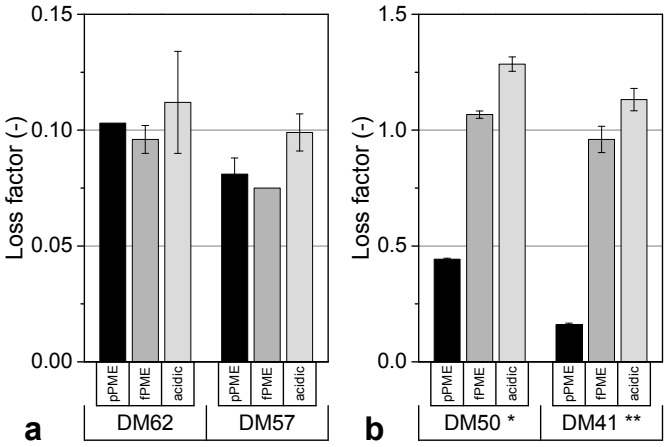

Fig. 23 Gel properties of pectin gels determined at the end of gelation (10 °C) in dependence on the demethoxylation method. Loss factor (tanδ) of sugar-acid gels of DM62 and DM57 samples (HMP) in (a) as well as loss factor of sugar-calcium gels of DM50 and DM42 samples (LMP) in (b). Significant differences: * for P < 0.05 and ** for P < 0.01 (Kastner et al., 2019).

The gel properties of the final LMP samples differed significantly (Fig. 23b), with the tanδ values of p-pectin << f-pectin < a-pectin at both DM41 and DM50. The values at DM41 were lower than at DM50. For the p-pectin LMP samples, tanδ values were below 1, indicating a gel structure with dominating elastic properties. Comparing the f-pectin and a-pectin samples at 10 °C, the f-pectin samples had lower values of tanδ. However, the tanδ was above 1.0 for both groups, as typical for dominating viscous properties dominated. After additional cooling to 5 °C, the tanδ decreased below 1.0, but the values of the f-pectin (0.826 for F49, 0.805 for F42) were still lower than those of the a-pectin samples (0.914 for A50 and 0.959 for A42).

The expected influence of the demethoxylation method and the resulting distribution of free carboxyl groups on the gelling properties of the modified samples was confirmed. However, the results depended also on two other factors, sodium content and molecular weight, which changed in dependence of the applied modification method (Table 4).

With increasing demethoxylation, the intrinsic viscosity within the three pectin groups (p, f, a) decreased differently. Comparing the intrinsic viscosity of the pectin samples within a group, at DM well above 50% the a-pectin samples were more depolymerized (≥ 50 cm^3/g) than the

enzymatic treated, and at DM around and below 50 the enzymatically treated samples were more depolymerized (≥ 100 cm^3/g) than the acidic treated (Table 4).

All modifications altered the sodium content of the pectin (Table 4), it was highest in the p-pectin group (16.6 – 25.1 mg/g), lower in the f-pectin samples (11.0 – 24.3 mg/g) and it was close to zero in the a-pectin samples (0.7 – 0.1 mg/g). Comparing only the enzymatically treated samples, the HMP of p-pectin (16.6 and 21.4 mg/g) had a higher sodium content than the f-pectin samples (11.0 and 12.6 mg/g); whereas for the LMP, the sodium contents of the corresponding p-pectin and f-pectin samples were similar.

Molecular weight and sodium content are therefore included in the following discussion on the impact of the distribution of free carboxyl groups on pectin gelation. Since the methods of gel preparation (sugar-acid gels for the HMP and sugar-calcium gels for the LMP) and the viscoelastic properties of the resulting gels differed strongly, the two gel types will be discussed separately.

Sugar-acid gels

The small differences in the structuring temperatures between the pectin samples at DM62 (Fig. 21a and Fig. 22a) were attributed to differences in free carboxyl group distribution (block-wise or random) and/or to the sodium ion content. Despite the high calcium sensitivity of p-pectin, the block-wise pattern had a small effect on gelation. The free carboxyl groups in some blocks possibly bound sodium ions, and, as a result, the number of free carboxyl groups was insufficient for additional ionotropic gelation. Thus, differences in structuring temperatures probably were the result of differences in the sodium ion content.

In the DM57 group, the p-pectin samples had a more block-wise distribution of the free carboxyl groups and the structure formation started at significantly higher temperatures than that of the pectin samples with random distribution (Fig. 22a). The higher block-wise pattern might support ionotropic gelation and/or the formation of longer regions of hydrogen bonding due to longer blocks (Fraeye, Colle, et al., 2010; Luzio & Cameron, 2008; Ström et al., 2007; Willats et al., 2001). A supportive ionotropic gelation was assumed due to a native calcium content of the OP (1.49 mg/g). The other possible explanation, the formation of longer hydrogen bonding regions in blocks of free carboxyl groups, was not reasonable at 60 – 70 °C the start temperature of the gelation of p-pectin.

The structuring temperatures of the HMP with randomly distributed free carboxyl groups (fPME and acidic treatment), were different at DM62 and were similar at DM57 (Fig. 22a). However, a difference in the start of gelation (IST), corresponding to differences in pectin sodium content (12 mg/g for a-pectin vs. 0 mg/g for f-pectin), was found at both DM. These sodium ions partly shielded the dissociated carboxyl groups, reduced repulsion between pectin chains and accelerated the structure formation for the f-pectin samples.

The DM of the pectin samples had an impact on the viscoelastic character of the gels cooled to 10 °C. The gels were stronger (more elastic than viscous) at lower DM (Fig. 23a). This

was explained by the higher number of free carboxyl groups at lower DM, which were able to form more hydrogen bonds. The influence of blocks of free carboxyl groups was not significant for sugar-acid gels.

The gel properties of the DM62 samples differed visibly but not significantly (Fig. 23a). Though an order of a-pectin > p-pectin > f-pectin was detected for tanδ, which became more pronounced for the DM57 samples, the overall differences were small, especially in comparison to the LMP (Fig. 23b). An impact of the sodium ions was possible. They might have bound to some free carboxyl groups in the enzymatically treated samples and reduced the number of hydrogen bonds. Moreover, an influence of different molecular weights was probable.

Sugar-calcium gels

Structure formation and gel properties after cooling of the different pectin types differed strongly also for LMP gels (Fig. 21b and Fig. 22b). In the DM50 group, the gelling process of the p-pectin with a block-wise distribution of free carboxyl group started at about 60 °C, was 30 K before that of the a-pectin and f-pectin due to additional ionotropic gelation of the block-wise distributed carboxyl groups. Despite the added calcium ions in the a-pectin and f-pectin samples, ionotropic gelation was still limited at DM50 due to their randomly distributed free carboxyl groups. The course of the structure formation of the three pectin types also differed. The f-pectin and a-pectin gels showed a steeper increase in gelation velocity below 30 °C, because hydrogen bond formation dominated in this temperature region.

The structure formation of the DM41 group was delayed in comparison to the DM50 group for the p-pectin but accelerated for the other two samples, and the differences between the three types generally decreased. On the one hand, the rapid formation of hydrophobic interactions was further reduced by the decreasing DM. On the other hand, the total demethoxylation was so high, that also the f-pectin and a-pectin now contained longer blocks of free carboxyl groups and were able to undergo more ionotropic gelation.

Discussing the properties of cooled LMP gels (Fig. 23b), it has to be considered that the measurements were made at 10 °C. However, only after cooling to 5 °C gel points have been found and only for f-pectin and a-pectin samples, whereas the p-pectin samples showed no gel point due to pre-gelation. Nevertheless, the final p-pectin gels were the most elastic of all the tested samples, since the longer junction zones formed in blocks of free carboxyl groups by hydrogen bonds and / or calcium bridges, strongly affected the gel properties. The results agreed with those of Fraeye et al. (2009), Löfgren et al. (2005), Ngouémazong, Tengweh, et al. (2012) and Rolin (2002).

Comparing the properties of f-pectin and a-pectin samples after cooling to 10 °C (Fig. 23b), the a-pectin formed significantly more viscous and less elastic structures than the f-pectin. However, since the gel points of these samples were mainly found below 10 °C, gelation was not complete at the test temperature. After cooling to 5 °C (below the GP), the tanδ of the f-pectin again were below those of the a-pectin samples. The lower gel points for the a-pectin

samples also explained, why these gels tended to be more viscous. The f-pectin and a-pectin samples differed in their content of sodium ions and intrinsic viscosity, and the impact of these two parameters on pectin gelation in general is not sufficiently known and requires further investigation.

Summarizing the block-wise or random distribution of free carboxyl groups affected the structure formation in sugar containing gels. The gelation of all pPME-treated pectin samples (containing more blocks of free carboxyl groups) started earlier than that of pectin samples with a random distribution due to the additionally possible ionotropic gelation. The differences increased with decreasing DM as a result of the growing number and length of the blocks of free carboxyl group. The properties of all final HMP gels were similar and independent of blockiness, but the LMP gels of p-pectin samples were stronger and more elastic than those of a- and f-pectin samples.

Though treatments with fPME as well as acid both resulted in pectin samples with a random distribution of free carboxyl groups, they differed in their sodium content and intrinsic viscosity and, as a result, in their structure formation. The gelation of the f-pectin samples started earlier than that of the a-pectin since sodium ions in the f-pectin reduced the electrostatic repulsion between pectin and accelerated the formation of intermolecular junction zones. The final gels of the f-pectin samples were less viscous and more elastic than those of the a-pectin.

The results of this study showed the impact of the pattern of free carboxyl groups, depending on the demethoxylation method, on structure formation and viscoelastic gel properties. At similar DM, gelation of pectin samples with a block-wise pattern of free carboxyl groups started at higher temperatures than of those with a more random pattern. The differences became stronger with decreasing DM as a result of the growing number and length of the blocks of free carboxyl groups. No critical value threshold of DM, below which this effect vanished, was found in the investigated DM range. A general additional impact of sodium ions and molecular weight on gelation and viscoelastic properties of the gels was assumed, but not convincingly verified, and requires further investigations.

Concluding remarks and outlook

Pectin has been extensively investigated over the last decades in order to improve the understanding of interactions between its molecular and functional properties.

A newly developed method for the investigation of structure formation allows the determination of characteristic parameters, the initial and critical structuring temperature, of any pectin gelation. This method is a fast screening test, not only suitable for pectin gelation but also for investigations of many other structuring processes in systems with components from different origin.

Using this method, the investigation of different influencing factors gave the following results:

- An optimum range of pH was found for the structure formation as well as the final gel properties of high-methoxylated pectin at intermediate acid concentration. At higher pH, the number of non-dissociated carboxyl groups decreased and reduced the chance for the formation of hydrogen bonds. At lower pH, the gel structure became inhomogeneous due to pre-gelation or microgel formation. In case of low-methoxylated and partly also low-methoxylated amidated pectin, an increase of pH supported the number of potential binding sites for calcium ions and, thus, accelerated gelation. Gels of low-methoxylated pectin became more elastic as well as brittle. The impact of pH on the gelation of low-methoxylated amidated pectin was lower, because the formation of additional hydrogen bonds including amide group in these gels seemed to be less dependent on pH than hydrogen bonds between carboxyl groups.
- As expected, the impact of the cooling conditions on low-methoxylated pectin gels differed considerably from that on high-methoxylated pectin gels. Since the additionally formed calcium bridges during ionotropic gelation of low-methoxylated pectin were nearly independent on temperature, they supported the structuring process in a higher temperature range and reduced the effect of the cooling rate on low-methoxylated pectin gelation.
- Investigations of the influence of calcium ions on the structuring process of low-methoxylated pectin generally confirmed the crucial role of these ions in the gelling process. A higher calcium content in all gels induced a more rapid initial structure formation by immediately developing calcium bridges between blocks of dissociated free carboxyl groups. Above a certain calcium content, however, pre-gelation took place and resulted in an inhomogeneous or brittle gel structure.
- Comparing a non-amidated and an amidated low-methoxylated pectin of similar degree of methoxylation with respect to the impact of the calcium concentration on structure formation, significant differences were found. On the one hand, the lower number of dissociated free carboxyl groups in the amidated pectin were earlier saturated with

calcium ions what reduced the number of ionic interactions. On the other hand, the formation of additional hydrogen bonds involving amide groups promoted the structuring process.

- At similar degree of methoxylation, gelation of pectin with a more block-wise distribution of free carboxyl groups started earlier than that of pectin with a more random distribution. The differences became stronger with decreasing degree of methoxylation as a result of the increased number and length of the blocks of free carboxyl group. No critical value of degree of methoxylation was found, below which this effect might have vanished. Further investigations at degree of methoxylation below 40% will be necessary for a final answer.

The presented thesis allows a broader and more systematic insight into pectin gelation and the direct comparison of structuring processes based on different mechanism by using the newly defined structuring parameters. The results support a better understanding of the structuring process and resulting gel properties in sugar-acid systems, considering the influence of selected internal and external factors. They shall support the choice of optimum pectin and conditions for specific applications.

In contrast to other studies, which often applied more or less theoretical model gel systems and different investigation methods, the impact of various factors on pectin gelation in a practically relevant sugar-acid gel was studied using only one single method. In addition to the confirmation of literature data, new results were revealed and the effect of the tested factors on the different gelation mechanisms was shown: Even a small variation of pH-value or calcium content had significant effects, both in cold-set and ionotropic gelation. The examinations on the effect of the cooling conditions revealed that ionotropic gelation started already at high temperatures and dominated the complete structure formation in presence of calcium ions. Furthermore, a strong influence of the amide group on the gelation in sugar-acid system was found by comparing two nearly identical low-methoxylated pectin sample, one amidated and one non-amidated. Though they had the same degree of methoxylation and similar R-value (calcium concentration in relation to the number free carboxyl groups), their structure formation differed due to additional hydrogen bonds formed including the amide groups. This was shown for the first time. Not only the pattern of the free carboxyl groups along the pectin backbone, as referred in the literature, but also the sample preparation and resulting changes in molecular properties (molecular weight and sodium ion content) were crucial factors and determined the pectin gelation. Furthermore, phases of the structuring process of the investigated pectin samples were identified in the structure velocity curves, indicating the different dominating mechanisms.

A ranking of the individual factors with respect to their importance for the gelling process and the properties of pectin gels is up to now not possible. Each of them has its own special impact on the different types of pectin gelation. Moreover, the combination of single factors affects the various types of pectin gelation in different ways.

Examination of the pectin structuring process should be complemented by imaging methods such as atomic force microscopy, chemical force microscopy, transmission electron microscopy or scanning electron cryomicroscopy, especially with regard to the phases during ionotropic and cold-set gelation. These methods have proved to give additional valuable information on gel structure.

In addition to the tested internal and external factors pH-value, calcium content, amide group, pattern of free carboxyl group and cooling rate, other parameters with impact on pectin gelation should be investigated in more detail using the newly developed method. Beside the degree of methoxylation and pattern of free carboxyl groups, the molecular weight has an important effect on pectin gelation. During extraction and modification, both demethoxylation and depolymerisation occur parallel. Up to now it was not possible to determine the individual contribution of degree of methoxylation and molecular weight, respectively, on pectin gelation, this should be studied in detail. Future work should also cover the impact of sodium ions on the pectin structuring process. Sodium hydroxide is often used during alkaline and enzymatic modification in order to keep the pH constant. Some of the sodium ions bind so strongly to the pectin molecules, that they are found even after intensive purification of modified pectin samples. It is assumed that monovalent ions delay the structuring process of modified pectin by binding to dissociated carboxyl groups and reducing ionotropic gelation. Another subject, worth deeper examination, is the effect of acetyl groups. It is generally known that these groups affect gelation, but the mechanism is still not clear. Pectin is a heterogeneous and complex biopolymer with extraordinary broad functionality beside gelation. Some effects are not completely understood, in particular with respect to details of the molecular structure. The subjects named above refer mainly to the homogalacturonan region and to the impact of its alterations on pectin functionality. However, also the effect of the branched rhamnogalacturonan regions and in particular the neutral sugar side chains on gelation require further study.

The newly developed method is well suitable to determine characteristic structuring temperatures in order to characterize the gelling properties for quality management, in particular for pectin suppliers. It will be helpful to evaluate pectin structure formation also in other fields of application beside classical jams, e.g. for food preservation by active or edible packaging. Some of these problems may require a modified gelling system with respect to sugar content, pH or added ions, and for these studies the knowledge of the impact of the single factors will be crucial. Any results of further systematic investigations of pectin gelation, using the newly developed method, will considerably contribute to an increasing understanding of the structure-function-relationship of pectin, and they will support the development of pectin applications also in other fields beside food products, such as medicine, pharmacy, cosmetics or environmental protection.

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Annex

(A1) New parameters for the examination of the pectin gelation process

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NEW PARAMETERS FOR THE EXAMINATION OF THE PECTIN GELATION PROCESS

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1 INTRODUCTION

Industrial pectins are mainly obtained from by-products of the citrus or apple juice industry by acidic extraction. They are used as gelling or thickening agents in order to improve the texture of food products. Pectins are branched polysaccharides with a backbone of galacturonic acid and neutral sugar side chains, present in the plant cell walls. The galacturonic acid molecules are partly esterified with methanol. Materials with more than 50 % methoxylated carboxyl groups (degree of methylation DM > 50 %) are named as high-methoxylated pectins (HMP) and those with less than 50 % are low-methoxylated (LMP). The typical DM for commercial use is about 60 to 77 % for HMP and about 25 to 40 % for LMP.[1] The DM is crucial for the complex pectin gelation process. HMP form gels in the presence of at least 55 % soluble solids (mostly about 65 % sugar) and at low pH (< 3.5).[2,3] Their gelling mechanism is a combination of hydrophobic interactions between methoxyl groups, favored by higher temperature, and hydrogen bonds between undissociated carboxyl groups, dominating at lower temperature.[4] The LMP network formation is less dependent on pH and soluble solids than the HMP gelation, it is promoted by the presence of Ca^{2+}, forming intermolecular ionic junction zones between smooth regions of neighbored chains (egg-box model).[1,5]

Fundamental knowledge of the material and structuring properties of pectins, especially of their gelling behavior, is of crucial importance for their application in foods as well as for the configuration of technological processes. The gelation temperature of pectins depends not only on their botanical source, manufacturing conditions and molecular and material properties but also on the gel preparation procedure and cooling conditions. There are many experimental studies of the complex pectin gelation process as transition from sol to gel as well as of the "gel point", the temperature at which the material properties change from liquid to solid. Sometimes, however, it can be difficult to define a clear gel point because the pectin gelation process is rather complex.

Mechanical properties of final pectin sugar model gels have been investigated for instance using empirical tests, based on gel breaking, or the °SAG-method.[6,7] They are applied in the pectin industry in order to standardize pectins for commercial use. Nowadays mainly fundamental rheological measurements are applied for gel examination. They allow the

investigation of the structure formation during the gelling process with determination of setting time and temperature (gel point) as well as the final gel properties.
In oscillation measurements, widely used for the examination of the pectin structuring process, the gel point was frequently defined as cross-over of storage modulus G´ and loss modulus G´´ ($\tan\delta = G''/G' = 1$). This method was developed initially for chemical gelation.[8] In most food products, however, physical gelation via junction zones occurs and sometimes no clear gel point can be determined for pectin gels, for instance because of pre-gelation.[9,10,11] Therefore, a modified method examined the point of intersection as a function of frequency,[3,8] where the gel point as $\tan\delta = 1$ might be defined only in case it is independent on the frequency.[11] But also this method is not always exact and, therefore, sometimes the described point is named as "apparent gel point".[3] Other indicators of the gel formation were described by a strong decrease of $\tan\delta$[12,13] or using the structure development rate SDR = dG´/dt during cooling[14]. Nevertheless, the gel point determination is not completely clear, yet.
The objective of this study is it, therefore, to investigate the structuring process of commercial pectin gels using additional new gelling parameters that allow a detailed examination of influencing factors such as botanical origin, preparation conditions, molecular and material properties, gel preparation (pectin concentration, pH, soluble solid, divalent cations) or experimental parameters like cooling rate on the gelling process.

2 MATERIALS AND METHODS

2.1 Materials

High or low methylated non-standardized commercial citrus pectins were obtained from three pectin companies. For data protection reasons an anonymous pectin declaration 2A, 3B, etc. had to be made. Gelling parameters and the DM of the samples are given in Table 3, further molecular characteristics and the relating determination methods are presented in a parallel paper.[15]

2.2 Gel preparation

The pectin-sucrose-gel composition and preparation was based on the USA-sag method[6,7] (Table 2). In contrast to HMP gel preparation, the LMP gelation requires Ca^{2+} and a different pH (Table 1). All experiments were made at least in duplicate for each pectin gel.
2.2.1 HMP sucrose gel preparation: Dry pectin powder was dissolved in demineralised water. While stirring, the mixture was heated quickly to boiling. The sucrose was added and the HMP-sucrose mass was reduced to a defined value. Afterwards, the pH of the mass was reduced degraded by addition of tartaric acid solution.
2.2.2 LMP sucrose gel preparation: Demineralised water, citric acid solution and sodium citrate solution were mixed in a steel pot and the dry pectin powder was added while stirring. The mixture was heated quickly to boiling, sucrose was added and the mixture was boiled again. The calcium chloride dehydrate solution was added and while stirring the LMP-sucrose mass was reduced to a defined value. The detailed information of the ingredients of the two gels is given in Table 1.

Table 1 *Preparation of gel samples with pectins of different DM*

Ingredients	***HMP gel***	***LMP gel***
Pectin content	2.75 g	6.00 g
Demineralized water	430.00 g	637.50 g
Sucrose	647.25 g	264.00 g
48.8 % tartaric acid solution	7.00 ml	-
54.3 % citric acid solution	-	7.50 ml
6 % sodium citrate solution	-	15.00 ml
2.205 % calcium chloride dehydrate solution	-	37.50 ml
Reduced to	***1015 g***	***900 g***
Total pectin concentration	***0.27 %***	***0.67 %***

2.2 Rheological measurements

Oscillation measurements of pectin-sugar model gels were carried out on a Physica MCR 301 (Anton Paar). The applied geometry was a double gap rotational cylinder CC27/P1 with peltier cylinder temperature system TEZ 150P. After gel preparation, about 15 ml of the sample was transferred immediately into the pre-heated rheometer (105 °C) and cooled to 20 °C (HMP) or 10 °C (LMP) with a cooling rate of 1 K/min. The sample was coated with silicone oil and the cylinder was closed with a special lid in order to avoid evaporation. The dynamic rheological parameters storage modulus G´ and loss modulus G´´ as well as the loss factor tanδ = G´´/G´ of the pectin-sugar model systems were recorded during cooling (temperature sweep) at a frequency of 1 Hz and deformation amplitude of 0.001.

2.3 Ridgelimeter (USA-sag) method

The USA-sag method was implemented by the IFT committee for pectin standardisation. This method is rather empirical but frequently used for routine or quality tests in the pectin industry, yet.
The rest of the hot pectin-sugar solution, prepared as described above, was filled into three special glasses and stored at 25 °C for 24 h before measurement. For an accurate result, the average gel properties of the three gel cones had to be within the limits shown in Table 2.
A Lab850 pH-meter with a special penetration electrode from Schott Instruments was used for the determination of the pH in the gels after Ridgelimeter measurements. Moreover, the soluble solid SS was determined in the gel using a refractometer (Schmidt and Haensch).

Table 2 *Conditions for the different pectin gels*

Ingredients	***HMP gel***	***LMP gel***
pH	2.2 - 2.4	2.8 - 3.2
SS	64.5 - 65.5 %	30 - 32 %
Gel strength	19.5 - 27.0 %sag	

2.4 Examination of structure formation

Three characteristic gelling temperatures were calculated: The (apparent) *gel point temperature (GP)* as cross-over of G´ and G´´, the *initial structuring temperature (IST*) and *critical structuring temperature (CST)* as shown in Figure 1 from the first derivation of the storage modulus (dG´/dt = structure formation velocity) using Origin 8.1 software. The IST is defined as the temperature at which the structure formation velocity was different from 0 for the first time. The CST is the extrapolated temperature for the first strong increase of the structure formation velocity. In contrast to the GP, these two structuring temperatures could be detected for any pectin we have ever investigated. Moreover, from the structure formation velocity curve often different structuring phases could be identified.
The details of the new defined parameters are shown in Figure 1.

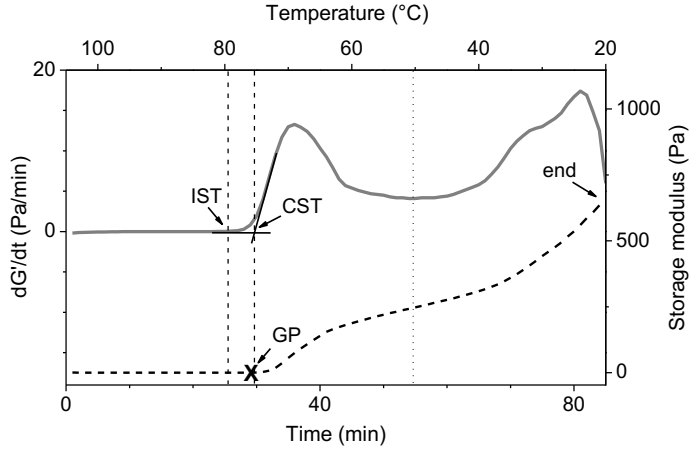

Figure 1 *Evaluation of the structure formation during cooling of an HMP-gel (sample 3A). Full line = dG´/dt; dotted line = storage modulus G´; IST = initial structuring temperature; CST = critical structuring temperature; end = end level at 20 °C; GP = gel point = tanδ = 1.*

3 RESULTS AND DISCUSSION

3.1 Structure formation temperatures

The rheological studies were made using different but constant preparation conditions for HMP and LMP, respectively, (Table 1) and allowed the comparison of the gelling processes and gel properties. Sometimes, the rheological measurements gave no clear setting point as intercept of G´ and G´´, causing uncertainty for identifying the exact gelling temperature. Especially in these cases, the new structuring parameters IST and CST allowed a better description and comparison of the structure formation process.
The structure formation velocity curve dG´/dt characterised not only the gelling kinetic but also different phases in the gelation process by alterations of the structure formation velocity.

Table 3 *Characteristics and gelling properties of the tested pectin samples. DM = degree of methoxylation, GP = gelling point, IST = initial structuring temperature, CST = critical structuring temperature, G'_{end} = final storage modulus, $\tan\delta_{end}$ = final loss factor.*

			Parameters of the gelation process				
Source	*Sample*	*DM*	*GP*	*IST*	*CST*	G'_{end}	$\tan\delta_{end}$
		%	*°C*	*°C*	*°C*	*Pa*	
1	**1B**	59.6	91	93	81	1077	0.078
	1C	24.2	51	53	42	91	0.151
2	**2A**	68.9	86	88	85	815	0.068
	2B	55.1	58	62	58	639	0.054
	2C	30.1	43	51	37	81	0.200
3	**3A**	69.8	77	79	76	587	0.061
	3B	57.1	57	60	56	877	0.051
	3C	63.6	67	70	66	788	0.057
	3D	32.8	43	60	31	44	0.166
	3E	30.2	58	66	55	128	0.128
	3F	27.7	-	79	69	293	0.120
	3G	69.0	70	72	69	296	0.090
	3H	56.5	54	54	53	348	0.087

3.2 Gelling process

A higher DM, in general, leaded to a faster start of the incipient structuring process at higher gelation temperatures (Table 3 and Figure 2a). This is well-known and crucial for the pectin application.

In order to investigate the influence of manufacturing conditions on the gelling process, two HMP (2A and 3A) and two LMP (2C and 3E), respectively, with similar DM but from different companies were compared. Differences in the start of the structuring process and structuring velocity were observed (Figure 2b and 2c, Table 3). As shown in Figure 2b, there was an earlier increase in dG´/dt of the 2A gel, with structure formation temperatures GP, IST and CST about 9 K higher than in the 3A gel. The same was found for the two LMP with a difference > 15 K. This means that the gelling properties of pectins were strongly dependent on their processing conditions and that the DM is one indicator but not the only parameter for evaluating the gelling properties.

An increase or decrease of structure formation velocity dG´/dt can indicate different gelling process phases. It seems that single structuring mechanisms (hydrogen bonds beside hydrophobic interaction for HMP gelation, egg-box junction zones via calcium bridges and hydrogen bonds for LMP) occur in typical temperature ranges of the gelling process. It was found that, even if the IST for two tested HMP or two LMP varied, the typical structuring phases during cooling can behave comparable. Also using the SDR theory[14], two phases of the gelation process have been already described in past publications. The publication of further results in this field with other influencing parameters is in preparation.

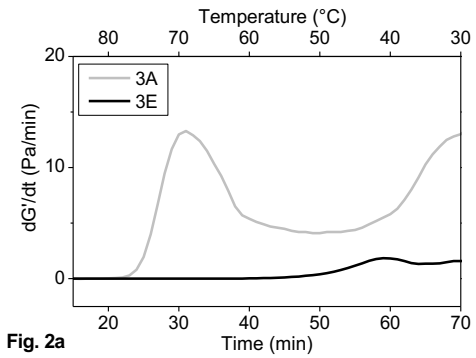

Fig. 2a

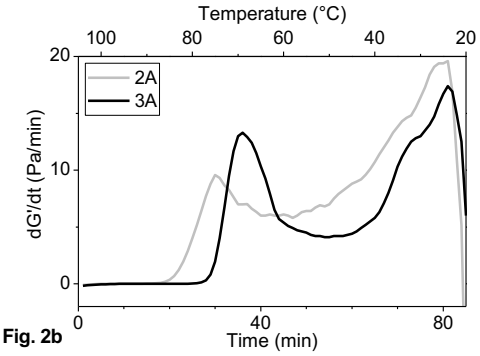

Fig. 2b

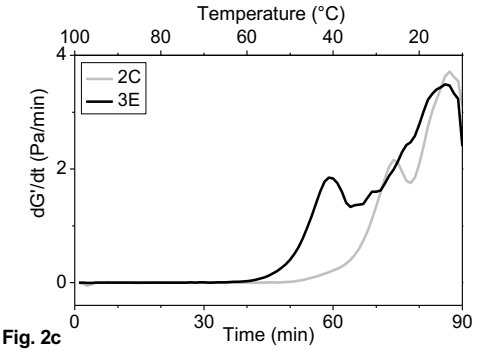

Fig. 2c

Figure 2 *Structure developing velocity dG´/dt during cooling in dependence on degree of methoxylation.*
a: comparison of a HMP and a LMP from the same company (shown from 85 to 30 °C); b: comparison of two HMP from different companies with similar DM (cooled from 105 to 20 °C); c: comparison of two LMP from different companies with similar DM (cooled from 100 to 10 °C).

The relationship between CST and GP of the tested gels is shown in Figure 3. From this significant correlation can be concluded that the newly defined structuring temperatures CST and the nearly CST - parallel IST are a complementary way to evaluate the incipient gelling process, especially for gels without a clear G´-G´´ cross-over. The influence of the manufacturing conditions on the setting behavior of pectins could be sufficiently examined by IST and CST.

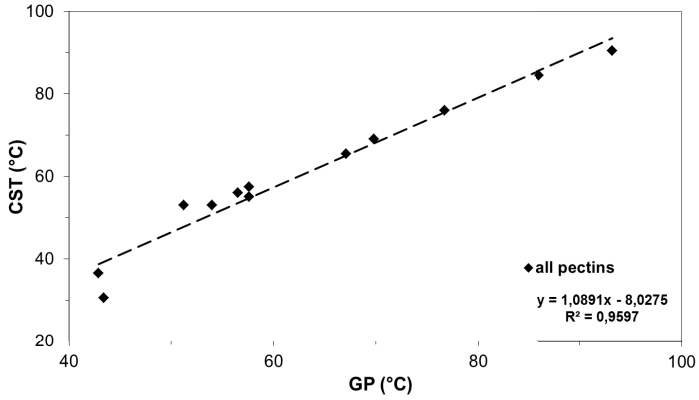

Figure 3 *Correlation of structure formation temperatures GP and CST for all pectin sucrose gels.*

4 CONCLUSIONS

The analysis of the chemical structure of the examined pectins from different companies showed the complexity of the polymer properties and their influence on the pectin gel characteristics, depending on the pectin manufacturing process. The newly defined initial structuring temperature IST and critical structuring temperature CST were, beside the GP, suitable parameters in order to describe the incipient gel structuring process for both types of pectins. Even samples with no clear GP could be evaluated this way. Therefore, IST and CST have proved to be valuable complementary parameters and can help to understand the structure formation processes more detailed. They can be used for optimizing the pectin production process as well as the pectin application in food products.

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(A2) Comparison of molecular parameters, material properties and gelling behaviour of commercial citrus pectins

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COMPARISON OF MOLECULAR PARAMETERS; MATERIAL PROPERTIES AND GELLING BEHAVIOUR OF COMMERCIAL CITRUS PECTINS

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1 INTRODUCTION

Pectins are important gelling and thickening agents for the food industry. They are extracted mainly from citrus fruits and apples but also from different other plant materials such as sunflower, sugar beet or other sources. The industrial production has a long tradition and the main steps like extraction or precipitation are well-known.[1] Nevertheless, the parameters and details of the treatments, such as temperatures, pH or drying procedure, can vary considerably between different pectin producing companies and even within one company. Moreover, the pectin sources are biological materials with seasonal and local variations, and it is necessary but not always completely possible for the pectin producers to adapt the technology to the raw material.

Pectin molecules are long mainly galacturonic acid backbones with side chains of neutral sugars. The galacturonic acid molecules are partly methoxylated and the pectins are divided into high-methoxylated (HMP) or low-methoxylated (LMP) with degree of methoxylation (DM) above or below 50 %, respectively.[1,2,3] LMP are mostly made from HMP by chemical demethoxylation procedures with acidic or alkaline conditions;[1] the resulting pectins are used for different food products.

In previous experiments, pectin modifications such as demethoxylation and amidation were made from HM-pectin of one company in laboratory scale. It was found that the material properties and thermal degradation behaviour of the resulting LMP varied considerably in dependence on their molecular parameters and preparation conditions.[4,5] Laboratory preparation and industrial production differ, however, not only with respect to the raw material properties and amount of processed material but also in the applied equipment and resulting technological conditions. The question is, whether results of model pectins can be transferred to industrially produced materials from different companies. Therefore, commercial citrus pectins from three different companies were examined in detail and compared with respect to their molecular and material properties and especially their gelling behaviour. These parameters are relevant for practical pectin application and their interactions and inter-dependencies can give valuable information for pectin producing as well as using companies.

2 MATERIALS AND METHODS

2.1 Materials

All samples were commercial pectins, kindly provided from three pectin companies. For data protection reasons they are named with 1, 2 and 3, and the pectins are labelled with 1A, 1B, etc. The detailed examined parameters are given in Table 1.

2.2 Methods

The molecular parameters galacturonan content GC, degree of methoxylation DM, intrinsic viscosity IV and the material properties colour and dissolution time as well as thermal degradation properties were determined as described previously.[5,6] The gelling behaviour was tested by oscillation measurements with temperature sweep as described in a parallel paper.[7] The particle size of the dry powder was determined using a Horiba particle sizer and the electron scanning micrographs were made by a specialised laboratory in the university.

3 RESULTS AND DISCUSSION

3.1 General screening and comparison of high and low methoxylated pectins

3.1.1 Molecular and material properties

First impressions of differences between both pectin groups give the ***electron scanning micrographs*** in Figure 1. The HMP particles had a visibly rougher surface and were more porous than the according LMP. Methoxylation in industrial scale is often made in an acidic environment[1] where the majority of free carboxylic groups are undissociated. The pectin macromolecules show low electrostatic repulsion and are able to form many strong inter- and intramolecular hydrogen bonds. The result is a compact structure that is trapped during drying in a partly crystalline state and can negatively influence the dissolution properties. The main problem in the hydration and dispersion of pectin powder is to limit the "fish-eye effect". Some of the tested pectins were really difficult to dissolve (Table 1) and confirmed this experience. It took mostly more than 20 min and sometimes even more than 1 h to dissolve 100 mg pectin in 50 ml distilled water, and the smoother surface and a partly crystalline state of LMP particles (in comparison to HMP) additionally delayed the necessary hydration process.

Another general result of the demethoxylation procedure was the smaller ***chain length*** of the pectins. The intrinsic viscosity (IV) of the tested LMP was significantly lower than that of the according HMP (Table 1). The reason is that any chemical demethoxylation, independent on acidic or alkaline conditions, does not only cleave the ester bonds but also, to a certain extent, the glycosidic linkages in the galacturonic acid backbone.

The third general difference between HMP and LMP was the ***colour***, in particular the b-value (Table 1). LMP of company 1 and 2 hade a significantly higher +b-value (yellow) than the according HMP, in case of company 3 this effect, however, was not clear. It seems that more unsaturated uronides developed during demethoxylation in company 1 and 2 which were able to form brown-coloured reaction products whereas in company 3 this was partly prevented. These differences between the companies were confirmed by the ***galacturonan content*** (GC = purity). Intensive chemical treatment can cause not only browning unsaturated uronides but also removes neutral polysaccharides and impurities

and, thus, increases the GC. The difference in GC between HMP and LMP of company 3 were smaller than in case of company 1 or 2 (Table 1).
The differences in ***particle size and size distribution*** between HMP and LMP (Table 1) depended neither clearly on the DM nor on the pectin company, but probably mainly on the milling equipment and conditions. The particle size should be, however, not completely neglected by the pectin producers because of its influence on dissolution and application as discussed above.

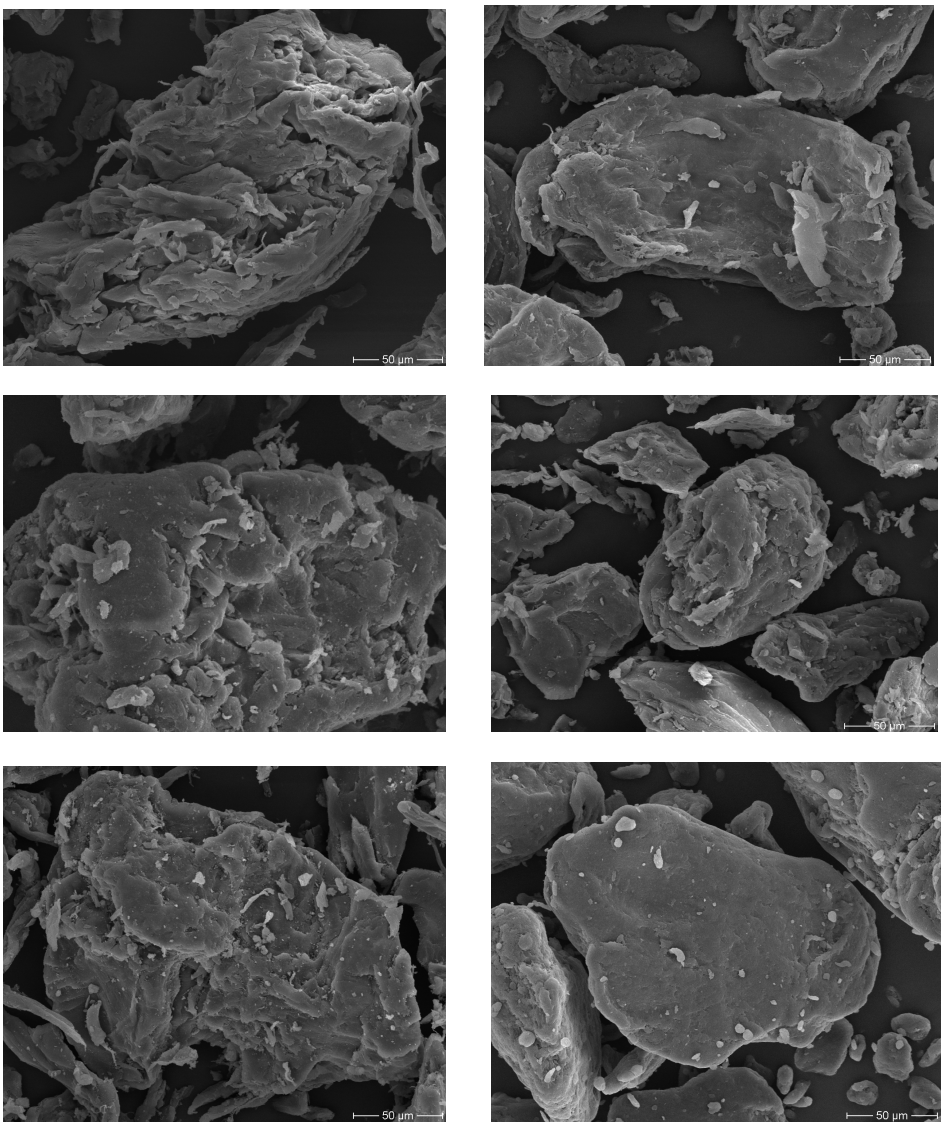

Figure 1 *Electron scanning microscopy of HMP (left side) and LMP (right side) of the three different companies. First line = company 1, second line = company 2, third line = company 3.*

Table 1: *Molecular parameters, material and gelling properties of the tested pectins*
GC=galacturonan content, DM degree of methoxylation, IV=intrinsic viscosity, E_{500}=absorption at 500 nm, A_o=particle surface, T_{pDSC}=peak temperature in DSC signal, T_{on} and T_{pDTG}= extrapolated onset- and peak temperature in DTG signal, ΔT=peak width, v_{max}=maximum degradation velocity, GP=gelling point, IST=initial structuring temperature, CST=critical structuring temperature, G'_{end}=final storage modulus, tan δ_{end}= final loss factor.

		molecular parameters			colour			dissolution			particle analysis		thermal degradation					gelation				
source	sample	GC	DM	IV	L	a	b	time	E_{500}	pH	median	A_O	T_{pDSC}	T_{onDTG}	T_{pDTG}	ΔT	v_{max}	GP	IST	CST	G'_{end}	tan δ_{end}
		%	%	cm^3/g				min			µm		°C	°C	°C	K	%/min	°C	°C	°C	Pa	
1	1A	89.3	60.9	639	88.6	1.1	12.9	15	0.048	4.04	145	607	240.5	220.4	237.8	27.6	19.9					
	1B	85.5	59.6	598	89.7	1.1	10.9	>60	0.045	3.62	178	437	239.4	220.6	236.1	25.8	22.2	90.5	93.2	81.0	1077	0.078
	1C	93.7	24.2	318	90.0	1.5	16.9	30	0.022	3.52	110	905	238.4	217.3	233.8	28.5	17.3	51.2	53.0	42.0	91	0.151
2	2A	81.6	68.9	647	86.4	2.0	12.2	20	0.131	3.42	101	1118	244.0	220.5	240.3	35.6	16.3	86.0	88.0	84.5	815	0.068
	2B	87.7	55.1	492	87.7	1.6	11.4	30	0.166	3.45	96	1154	245.0	220.4	240.7	34.5	16.2	57.6	61.5	57.5	639	0.054
	2C	91.5	30.1	358	81.2	3.4	24.0	30	0.442	3.18	82	1244	246.7	221.0	241.0	33.5	15.8	42.9	51.0	36.5	81	0.200
3	3A	80.9	69.8	554	89.9	1.4	11.0	25	0.056	3.48	101	1114	249.6	231.7	246.1	24.3	22.2	76.7	79.0	76.0	587	0.061
	3B	83.4	57.1	576	88.4	1.7	12.9	>60	0.054	3.56	81	1570	247.9	228.7	243.9	25.7	20.7	56.5	59.5	56.0	877	0.051
	3C	81.5	63.6	608	88.5	1.8	14.0	>60	0.028	3.55	98	1113	249.6	231.1	245.8	24.7	21.4	67.1	70.0	65.5	788	0.057
	3D	84.8	32.8	363	87.7	1.1	13.0	>60	0.022	5.24	118	1127	230.9	213.3	228.4	54.0	17.2	43.4	59.5	30.5	44	0.166
	3E	81.5	30.2	336	84.0	2.7	20.1	>60	0.039	4.91	149	523	234.1	217.0	231.6	50.4	17.8	57.6	66.0	55.0	128	0.128
	3F	78.5	27.7	327	86.9	1.5	15.0	45	0.040	5.24	103	1295	233.9	216.1	231.4	46.9	15.6		78.5	69.0	293	0.120
	3G	82.3	69.0	518	84.0	2.8	15.6	45	0.040	3.64	112	956	248.2	229.6	244.4	25.1	22.0	69.8	72.0	69.0	296	0.090
	3H	87.0	56.5	529	88.2	1.8	12.9	>60	0.065	3.66	89	1129	246.2	226.0	242.3	27.0	20.3	54.0	54.0	53.0	348	0.087

3.1.2 Thermal analysis

A further method for a quick evaluation of pectin is the thermal analysis as combination of differential scanning calorimetry DSC and thermogravimetry TG. The DSC signals give information on ***transition and degradation enthalpies***. The TG and its first derivation DTG allow insight into ***thermal stability*** by the extrapolated temperatures $T_{on\ DTG}$ and into ***homogeneity*** by the degradation time, measured as peak width ΔT, both from the DTG signal. The values are given in Table 1 and some typical signals in Figure 2.

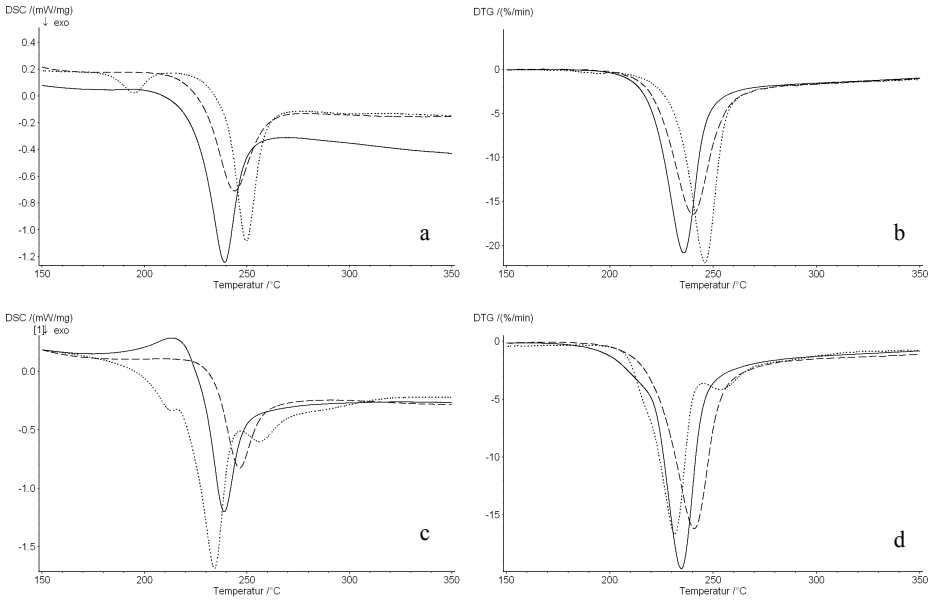

Figure 2 *DSC signal (a,c) and DTG signals (b,d) of HMP (a,b) and LMP (c,d).*
—— = company 1, ---- = company 2,•••• = company 3.

Comparing HMP and LMP, the main differences are clearly visible: The two HMP of company 1 and 3 were more similar (DTG peak form) and homogenous (peak width) than the according LMP. In case of company 2 these differences were smaller.
Comparing the DSC signals of the three HMP in detail, the pectins of company 1 and 2 were similar and had only one exothermic degradation peak. The HMP of company 3, however, had another smaller exothermic peak before the main fraction degradation and also a small weight loss in the according DTG signal; that means another component beside the normal pectin in this sample was degraded. The HMP of company 2 seemed to be the least homogenous; the peak width in DTG was about 10 K higher than that of the two others. Nevertheless, the DTG signals of the 3 HMP were partly similar.
Comparing the three LMP, the differences were even clearer. LMP of company 1 had an endothermic pre-peak with only small weight loss; it might result mainly from a conformation transition as discussed elsewhere.[5] The LMP of company 3 had the most shoulders and peaks in DSC as well as DTG signals and the broadest peaks, it was the least homogenous.
The DSC and DTG signals of the three companies were representative (= found for several single samples). They were a kind of a "fingerprint" and allowed an easy detection of potential differences in the pectin production process which were discussed in the analysis

of molecular parameters. Unfortunately, the processing details of the single materials were more or less secret and it was not possible to confirm the assumptions about differences in technology. Nevertheless, it is generally possible to use the thermal analysis for a quick screening in quality control for detection of changes in quality and processing.

3.1.3 Gelling properties

The structuring process parameters and the gel properties of the tested pectins were not completely comparable because of different gel compositions and gelation mechanisms. Nevertheless, s***tructuring temperatures*** of HMP were higher than those of LMP with one exception in company 3. The ***final values of G´*** (solid-like properties) were higher for almost all HMP than for the according LMP and those of ***tan δ*** (brittleness) were definitely higher for the LMP.

3.2 Comparison of single pectins with similar degree of methoxylation

The degree of methoxylation is often used as a key parameter for pectin application. The following examples show, however, that pectins with comparable molecular parameters (especially nearly identical DM) of different companies and even from one company but different production periods were far from similar in their material properties and gelling behaviour. The examples were the HMP 2A, 3A and 3G with DM about 69 % and the LMP 2C and 3E with DM 30 %, for the detailed values see Table 1.
Beside the general effects of demethoxylation as discussed above, there were several specific differences. The first can be seen from the ***thermal analysis*** as discussed in 3.1.2 above. In particular, the HMP of company 2 was less thermal stable and less homogenous than the according samples from company 3. In case of the LMP, the tendencies were just opposite in thermal stability but the same with respect to homogeneity. These were the first indicators of different processing parameters and resulting properties.
The ***intrinsic viscosities*** were higher for the pectins from company 2, what allowed the conclusion that this company prepared their pectins under conditions (especially pH and T), which caused less cleaving of bonds in the backbone. Another difference was the high ***galacturonan content*** (purity) of the LMP 2C, most of neutral sugars and impurities were removed during demethoxylation. Such an effect was found also for company 1 and it is known from laboratory scale pectin modifications, too, where it was found especially after acid treatments.
The most interesting differences with high practical relevance were found for the ***structuring process*** and the ***gel properties***. In case of HMP, the structuring temperatures varied not only between the two companies for about 10 K but also for about 7 K between 3A and 3G from one company but different years (Figure 3). Also the end level of the storage modulus G'_{end} and of the loss factor tan δ_{end} differed not only between the two companies but also within one company. In case of the LMP, the gelpoint could not be determined for all samples, the structuring temperatures were, however, clearly higher for company 3. The LMP-gel of company 3 was more solid (higher G´) and brittle (lower tan δ) than that of company 2.

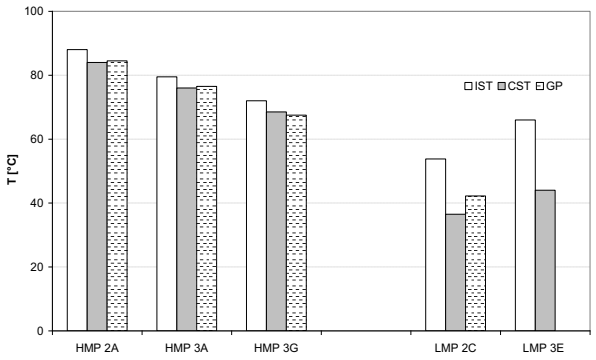

Figure 3 *Structuring temperatures of HMP and LMP.*
IST = initial structuring temperature, CST = critical structuring temperature, GP = gelpoint.

3.3 Statistical analysis

The statistical tests for interdependencies of molecular parameters, material properties and structuring were made by comparing either all pectins together, or the groups of HMP and LMP as well as 8 pectins from one company separately (Tab.2). As can be clearly seen, DM was the key parameter for many other properties. It had not only the significant well-known impact on the gelpoint but also on intrinsic viscosity (molecular weight) as discussed above. The interaction of DM and thermal stability was found only for the pectins of one company, this confirmed the results of a previous examination of model pectins.[4] Influences of pectin purity (GC) and molecular weight (IV) were found mainly in the LMP-group and are probably indirectly influenced by the DM.

Table 2 *Statistical analysis of the interactions of molecular parameters and material and structuring properties.*
XXX = R > 0.9, XX = R > 0.8. For the single parameters see Table 1.

	all pectins n=32	all HMP n=20	all LMP n=12	one company n=18
DM - IV	xxx			xxx
DM - GP	xx	xxx		xxx
DM - IST		xx		
DM - CST		xxx	xxx	
DM - Tp_{DTG}				xxx
GC - GP	xx			
GC - IST			xx	
GC - Tp_{DTG}			xx	
IV - GP	xx			
IV - IST			xxx	
IV - CST			xxx	
IV - Tp_{DTG}				xxx
GP - IST	xx	xxx		
GP - CST	xxx	xxx		xxx

The correlations of the classical gelpoint and the new structuring parameters IST and CST are of special interest. It should be considered, however, that a gelpoint could be determined not for all LMP. The correlation of gelpoint and CST was significant for all categories except LMP; that supports the application of CST as complementation to or instead of the gelpoint and is highly important, in particular for gelation processes without clear gelpoints like sometimes were found in LMP.

4 CONCLUSIONS

The presented results confirmed the general influence of processing parameters on the pectin quality and application for a collection of commercial pectins from different companies, that were found before for model pectins made from one original sample. These parameters - such as pH, demethoxylation temperature and drying conditions - influenced the molecular weight, colour, purity, thermal stability and homogeneity of the materials. Moreover, they determined the state (amorphous or crystalline) and material properties (surface quality and porosity) of the pectin powder particles, which are crucial for the application properties such as dissolution and gelation.
The degree of methoxylation is a key factor for many tested pectin properties, especially in the gelation process. But it is not the only factor what was revealed by a comparison of pectins with similar DM.
The thermal analysis proved to be a helpful and rapid screening method for pectin characterisation. Differences, found in DSC and TG, were confirmed by analysis of molecular parameters.
Any pectin producing company should try to promote the favourable material properties, such as rough porous particles and an amorphous state with easily cleavable inter- and intramolecular interactions, in order to produce pectins with excellent application properties.

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(A3) Structure formation in sugar containing pectin gels – Influence of Ca^{2+} on the gelation of low-methoxylated pectin at acidic pH

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Structure formation in sugar containing pectin gels – Influence of Ca^{2+} on the gelation of low-methoxylated pectin at acidic pH

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ABSTRACT

A new method for the examination of the pectin gelation process is presented as a complementation of the most common determination of the gelling point (cross-over of G' and G'') from oscillation measurements. It is based on the first derivation dG'/dt from oscillation measurements (named as structuring velocity), and defines an initial as well as a critical structuring temperature. These allow an exact determination of the start of structure formation and description of the structuring process also in gels with pre-gelation that showed no clear GP. Moreover, phases and mechanisms of gelation can be identified and structure developing rates can be calculated.

The application of this method on the gelation of low-methoxylated pectin at pH 3 and 30% saccharose with different contents of Ca^{2+} was tested. The results show differences as well as similarities between the GP and the newly defined structuring parameters that could be partly explained by varying structuring mechanisms at different Ca-content. The initial structuring process started probably with ionic interactions (egg-box junction zones and random crosslinks) via Ca-bridges as well as hydrophobic interactions at temperatures $\geq 60\,^{\circ}C$, it was nearly completed around 40 °C. Hydrophilic interactions (below 50 °C) and inter-dimer aggregations (below 25 °C) perhaps dominated the gelation during further cooling. In dependence on the Ca-content, two to three phases could be identified during the structuring process. The properties of the gels after cooling were tested by oscillation measurements as well as the USA-sag method. With increasing calcium content the elastic behaviour of the gels increased but they became also more and more brittle.

1. Introduction

Pectins are typical gelling agents, traditionally applied in jam and jelly, but used also in other food products such as soft drinks and milk products. The knowledge of the structuring properties and, in particular, the gelling temperature of the pectins is of essential technological importance. The exact determination of this sol–gel transition temperature, i.e. the "gel point" at which the material properties change from more liquid – like to more solid – like, therefore, has been studied for several decades.

Rheological methods, especially oscillation measurements, are often applied and frequently the cross-over of G' and G'' with $\tan\delta = G''/G' = 1$ is defined as gel point (Arenaz & Lozano, 1998; Audebrand, Kolb, & Axelos, 2006; Gigli, Garnier, & Piazza, 2009; Gilsenan, Richardson, & Morris, 2000; Löfgren, Walkenström, & Hermansson, 2002; Lootens et al., 2003; Slavov et al., 2009; Stang-Holst, Kjöniksen, Bu, Sande, & Nyström, 2006). The method has been developed by Winter and Chambon (1986) initially for chemical gelation. In food products, however, mostly physical gelation via junction zones occurs. Moreover, the point of intersection partly was found to be a function of frequency (Lopes da Silva, 1994; Lopes da Silva, Goncalves, & Rao, 1995; Lopes da Silva & Rao, 2006; Rao, van Buren, & Cooley, 1993; Winter & Chambon, 1986). Strictly, the cross-over of G' and G'' might be defined as gel point only when it is independent on frequency (Stang-Holst et al., 2006). Sometimes it might be close to but not identical with the real gel point and therefore is named also "apparent gel point" (Lopes da Silva, 1994; Lopes da Silva et al., 1995).

Several attempts have been made to find another method for the gel point definition: researchers from CP Kelco determined the gelling temperature via conductivity (Böttger, Christensen, & Stapelfeldt, 2008), and Dobies, Kozak, and Jurga (2004) used NMR measurements. Oakenfull and Scott (1984) and O'Brien, Philp, and Morris (2009) used relatively simple visual tests. Dahme (1992) and Neidhart, Hannak, and Gierschner (1996, 2003) defined a strong decrease of $\tan\delta$ as an indicator for the gel formation.

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Grosso and Rao (1998) and Fu and Rao (2001) studied the structuring kinetic of pectin gels and defined the structure development rate SDR = dG′/dt in order to describe precisely the moment, at which the formation of junction zones began. The problem of the gel point determination is, however, not completely solved, yet.

Citrus and apple pectins, isolated from by-products of the fruit juice industry, are the most common pectin types (Rolin, 2002). Their gelling properties vary in dependence on material and environmental factors (Lopes da Silva et al., 2006). Among the material parameters, the number of methoxylated carboxyl groups (degree of methoxylation DM) and their distribution in the polygalacturonic acid backbone (degree of blockiness DB) are very important (Fraeye, Colle, et al., 2010; Fraeye et al., 2009). Materials with DM > 50% are named as high-methoxylated pectins (HMP) and those with DM < 50% as low-methoxylated (LMP). The typical DM for commercial use is about 60–77% for HMP and about 25–40% for LMP (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995).

The pectin gelation processes are rather complex (Cardoso, Coimbra, & Lopes da Silva, 2003). The most important environmental factors are pH, Ca^{2+} and soluble solids (e.g. sugar). They have different and partly opposite effects on the gelling process of different pectin types. High-methoxylated pectins (HMP) form gels in the presence of >55% soluble solids (mostly about 65% sugar) and at pH < 3.5 (Lopes da Silva, 1995; Rolin, 2002; Thakur, Sing, & Handa, 1997). Their gelling mechanism is explained as a combination of hydrophobic interactions, favoured by higher temperature, and hydrogen bonds between undissociated carboxyl groups, dominating at lower temperature (Oakenfull & Fenwick, 1977; Oakenfull & Scott, 1984). Low-methoxylated pectins (LMP) gel in the presence of Ca^{2+}, forming intermolecular ionic junction zones between smooth regions of neighboured chains (Braccini & Perez, 2001; Morris, Powell, Gidley, & Rees, 1982; Thakur et al., 1997; Voragen et al., 1995). Several studies investigated the special influence of Ca^{2+} and/or pH on the gelling process of LMP in a watery system with no or only small amounts of sugar (Audebrand et al., 2006; Braccini & Perez, 2001; Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2005, 2006; Cardenas, Goycoolea, & Rinaudo, 2008; Cardoso et al., 2003; Dobies et al., 2004; Fang et al., 2008; Fraeye et al., 2009; Garnier, Axelos, & Thibault, 1993; Gilsenan et al., 2000; Lootens et al., 2003; Ngouimazong, Kabuye, et al., 2011; Ngouimazong, Tengweh, et al., 2011; Ralet, Dronnet, Buchholt, & Thibault, 2001; Ström et al., 2007; Thibault & Rinaudo, 1986). Some authors also tested the influence of higher sugar content (Fu & Rao, 2001; Grosso & Rao, 1998; Löfgren, Guillotin, & Hermansson, 2006; Löfgren et al., 2002). A current review (Fraeye, Duvetter, Doungla, van Loey, & Hendricks, 2010) gives a good summery of the role of calcium ions in pectins. LMP gelation is favoured by higher pH than that of HMP (above the pectin pK_a 3.5) because the electrostatic interactions via Ca-bridges require a certain number of dissociated carboxyl groups (Fraeye, Colle, et al., 2010; Fraeye et al., 2009; Fraeye, Duvetter, et al., 2010; Thakur et al., 1997).

In principal, three possible types of junction zones can be formed by LMP: hydrophobic interactions between methoxyl ester groups, hydrophilic interactions between undissociated carboxyl groups and/or hydroxyl groups via hydrogen bonds as well as ionic interactions between dissociated carboxyl groups via Ca-bridges (Fig. 1). The latter require a minimum number of 6–14 consecutive dissociated carboxyl groups in order to form the typical egg-box structure (Fraeye, Duvetter, et al., 2010). In case the total number of such groups is rather low (below the pK_a of pectin), these typical junction zones can be limited. Instead, Ca^{2+} could interact with single dissociated carboxyl groups in an undissociated neighbourhood, forming monocomplexes by charge reversal on a single chain or random (unspecific) crosslinking between

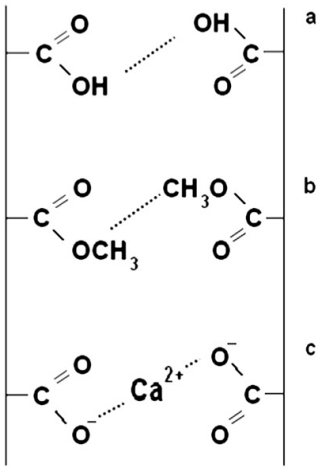

Fig. 1. Structure formation mechanisms in pectin gelation. a: Hydrogen bonds between undissociated carboxyl groups, b: hydrophobic interactions, c: random ionic interactions (crosslinks) between dissociated carboxyl groups. Ca-bridges at subsequent free dissociated carboxyl groups can form egg-box junction zones as known from many references (e.g. Braccini et al., 2001).

separate chains (Fang et al., 2008; Siew & Willams, 2005). Though these complexes are no typical Ca^{2+}-junction zones, they additionally reduce the electrostatic repulsion, possibly create even electrostatic attraction and, in any case, promote a closer contact of the pectin molecules and support gel structure formation. Similar effects are described also by Cardoso et al. (2003), Fraeye et al. (2009) and Ngouimazong, Tengweh, et al. (2011).

Electrostatic repulsion between pectin molecules is low at pH < pK_a because of a high share of undissociated carboxyl groups. This favours association and aggregation of pectin chains by intermolecular hydrogen bonds and additionally stabilises these systems in the absence of calcium (Cardoso et al., 2003; Gilsenan et al., 2000). Moreover, Cardenas et al. (2008) assume that, after initial dimer formation, also inter-dimer interactions and cross-linking of pectin molecules occur by associations of threefold helices with contributions of hydrophobic interactions and hydrogen bonds.

In contrast to HMP, high sugar content is not essential for LMP gelation but could support it by binding water and promoting close contact of neighboured molecules.

Altogether, the typical calcium-mediated LMP gelation can be seen as a two-step process: After initial dimerisation by strong electrostatic interchain associations via calcium ions with contributions of hydrophobic interactions at high temperature and hydrogen bonds at lower temperature, follows a subsequent aggregation of dimers that additionally increases gel strength (Cardenas et al., 2008; Lopes da Silva & Rao, 2006).

The aim of the presented paper is (I) to present a new method for the characterisation of the pectin gel structuring process by using the first derivation of the storage modulus dG'/dt from oscillation measurements and (II) to apply this method for the investigation of calcium influence on gelation of LMP at pH 3.0 and in the presence of 30% saccharose.

2. Materials and methods

The gel composition and preparation is based on a method that is applied in the pectin industry for testing the gelling properties by

the Ridgelimeter (USA-sag method, IFT Committee, 1959) according to Cox and Higby (1944). The quantities were slightly modified in order to get the necessary amount of pectin solution. All experiments were made four times, the control tests without calcium in duplicate.

2.1. Materials

The pectin was a commercial low-methoxylated non-standardized citrus pectin with 81.5% galacturonic acid content, DM 30.2% and intrinsic viscosity = 336 cm^3/g. Citric acid, tri-sodium citrate dehydrate and calcium chloride dehydrate were of analytical grade (Sigma–Aldrich), saccharose was of food quality from a local supermarket.

2.2. Methods

2.2.1. Gel preparation

637.5 g demineralised water, 7.5 ml 54.3% w/v citric acid solution and 15 ml 6% w/v sodium citrate solution were mixed in a steel pot and 6 g dry pectin powder, mixed with about 40 g saccharose, was added while stirring. The suspension was heated quickly until boiling, 224 g saccharose was added in 3 portions and the solution was boiled again. Afterwards, the required amount of 2.205% w/v calcium chloride dehydrate solution (25/31/37.5/44/50 ml, respectively) was added and while further boiling and stirring the total mass was reduced to 900 g. The whole process should take no more than about 5 min.

The stochiometric ratio between calcium and carboxyl content $R = 2Ca^{2+}/COOH$ was 0.46/0.58/0.70/0.82 and 0.94, respectively. This means 0.42/0.52/0.62/0.73 and 0.83 mM $CaCl_2$ per 100 g gel. The regular amount of $CaCl_2$ in the standard procedure is 37.5 ml (0.62 mM/100 g gel, R 0.70); variations were made in both directions.

2.2.2. Rheological measurements

The applied rheometer was a Physica MCR 301 (Anton Paar, Germany). Oscillation measurements (temperature sweep) of storage modulus G' and loss modulus G'' were made using a double gap rotational cylinder CC27/P1 with Peltier cylinder temperature system TEZ 150P. Samples were transferred onto the pre-heated rheometer (100 °C) and cooled to 10 °C with a cooling rate of 1 K/min. The sample was coated with silicone oil and the cylinder was closed with a special lid in order to avoid evaporation. Dynamic rheological parameter (G' and G'') were recorded during cooling at a frequency of 1 Hz and a deformation amplitude of γ 0.001.

2.2.3. Ridgelimeter (USA-sag method)

This method is rather empirical but frequently used for routine tests in the pectin industry, yet. The hot pectin solution was filled into three special glasses which were stored at 25 °C for 24 h before measurement. The single gels were removed carefully from the glasses and transferred on a plate. The percentage of sagging of the gel cone under its own weight within 2 min is measured. From this value the °SAG can be calculated.

2.2.4. pH

The pH was determined in the gel after Ridgelimeter measurement using a Lab850 pH-meter (Schott Instruments) and a special penetration electrode.

2.2.5. Examination of structure formation

From the G' data, the first derivation was calculated and smoothed using Origin 8.1 software. Two characteristic temperatures were determined from this first derivation as shown in Fig. 2.

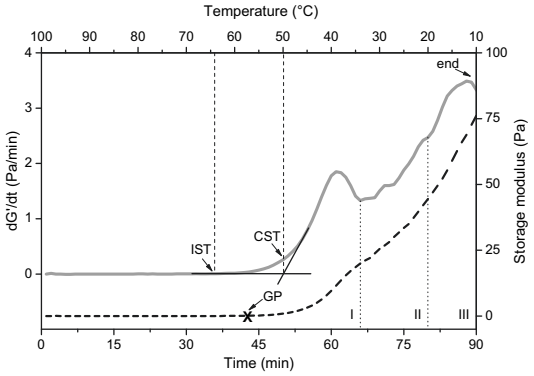

Fig. 2. Evaluation of the first derivation dG'/dt in a gel with 0.62 mM $CaCl_2$. Full curve = dG'/dt, dotted curve = G'; vertical lines give IST and CST (---) and the start of structuring phases (• • • •); IST = initial structuring temperature and CST = critical structuring temperature; end = end level at 10 °C. The GP is marked as X on the G' curve.

The *initial structuring temperature IST* is the temperature at which the value dG'/dt was different from 0 for the first time and the *critical structuring temperature CST* is the extrapolated temperature of the first strong increase of dG'/dt.

The *average structure developing rates* SDR_a was calculated from differences of storage moduli during cooling time for the total gelling process:

$$SDR_a = \frac{G'_{end} - G'_{IST}}{t_{end} - t_{IST}}$$

G'_{IST} and t_{IST} are parameters at the initial structuring temperature IST and G'_{end} and t_{end} are the final values at 10 °C.

3. Results and discussion

3.1. Structure formation parameters

Rheological tests of the gelling behaviour of pectins sometimes give no clear (apparent) "gel point" (GP) as a cross-over of G' and G''. Either G' may be higher than G'' already from the start of the rheological measurements, or the curves are more or less parallel during a longer cooling period without a clear intercept. The difficulties of studying pectin gelation in general are discussed by Lopes da Silva and Rao (2006) and those of LMP by Fraeye, Colle, et al. (2010) and Fraeye, Duvetter, et al. (2010). Therefore, an additional method might be helpful in order to describe the structure formation process.

The first derivation dG'/dt was used already for the description of the gelling kinetic of pectins and calculation of the structure developing rate SDR (Cardoso et al., 2003; Fu & Rao, 2001; Grosso & Rao, 1998). dG'/dt can be seen as *structuring velocity* and changes of this velocity are indicators for the start of structuring process as well as for further alterations (phases) during cooling. After smoothing the first derivation of the G' curve the new parameters *initial structuring temperature IST* and the *critical structuring temperature CRT* (Fig. 2) were determined. The IST is an indicator for the start of structure formation and the CRT for a first acceleration in structure formation. These two temperatures could be found for any pectin we have ever studied, not only in the experiments described in this paper. Therefore, this method seems to be a good

alternative or complement for the classical "gel point", defined as cross-over of G' and G''.

3.2. Application of the structure formation parameters temperature on the gelation of LMP

The characteristic temperatures defined above shall be applied for the discussion of the gelation of LMP at pH about 3 and with 30% saccharose in the presence of different amounts of Ca^{2+}.

Typically, gelation of LMP is dominated initially at high temperature by formation of egg-box junction zones via calcium-bridges and hydrophobic interactions. During further cooling, the influence of hydrogen bonds should increase, supported by inter-chain inter-dimer associations. Random electrostatic interactions of Ca^{2+} with single dissociated carboxyl groups of pectin chains (calcium crosslinking) could promote the structuring process.

In case of the applied pectin system, some divergences from this typical behaviour were expected: the number of methoxyl groups was rather low at 30% DM but should not be ignored; the high starting temperature of the measurements (100 °C) is favourable for hydrophobic interactions. The free carboxyl groups were assumed to be randomly distributed as it is typical for most commercial pectins after chemical demethoxylation (Fraeye, Duvetter, et al., 2010; Ngouimazong, Tengweh, et al., 2011). The number of dissociated free carboxyl groups should be relatively low at pH about 3 (below the pK_a 3.5). Therefore, it was expected that the formation of typical egg-box junction zones would be limited and more interactions between undissociated carboxyl groups via hydrogen bonds would be formed instead. The number of random (unspecific) calcium crosslinking via single dissociated carboxyl groups should increase with rising Ca^{2+}. The high sugar content in the gels could additionally promote the interchain interactions as it is known from HMP gelation.

As can be seen from Fig. 3, the gel point temperature as well as the initial and the critical structuring temperatures increased at higher calcium content. This confirmed the crucial role of Ca^{2+} for the gelling of LMP (e.g. Cardenas et al., 2008; Cardoso et al., 2003; Fraeye, Colle, et al., 2010; Fraeye et al., 2009; Fraeye, Duvetter, et al., 2010; Grosso & Rao, 1998; Lootens et al., 2003). IST and CST developed in a nearly parallel way; IST was always about 10 K higher than CST. The gel point temperature GP behaved different: at low calcium concentration (0.42 mM, $R = 0.46$) it was found about 25 K below the IST and also CST, and at high calcium content (>0.73 mM, $R > 0.82$) GP was above IST. It should be considered, however, that at high calcium content the GP could not be determined for all samples (Table 1) and the values were therefore rather vague. This will be discussed in detail below.

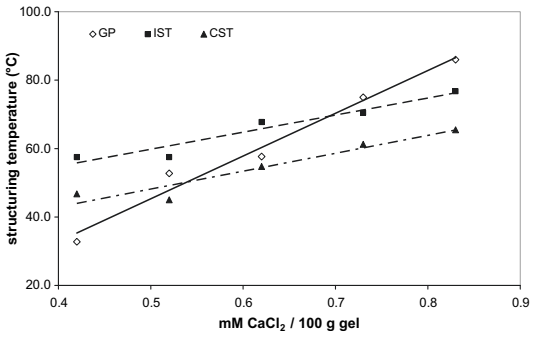

Fig. 3. Comparison of the structuring temperatures in dependence on calcium content. GP = gel point = intersection of G' and G'' (—); IST = initial structuring temperature (– – –); CST = critical structuring temperature (– - –).

The detailed discussion of the gelling process with respect to possible structuring mechanisms at different Ca-contents is illustrated by Figs. 2, 4 and 5a–d. Figs. 2 and 5 are single measurement curves, the data of IST and CST in the text are medium values of four repeated measurements. For reproducibility see Fig. 4 and Table 1. Some of the structuring velocity curves allowed the hypothesis of a two- or three-phase gelling process (Fig. 4). Two phases of gelation are also described by Fu and Rao (2001), the according temperature ranges and activation energies for the first (70–50 °C) and second phase (50 and 20 °C) varied in different pectins.

(i) Mixtures without any calcium did not gel at all but started a kind of "structure formation" below 20 °C (Table 1). A possible explanation give Ngouimazong, Tengweh, et al. (2011) who suggest a gel-like characteristic in concentrated LMP systems in the absence of calcium and without junction zone formation. Even a high share of sugar, that should allow a gelation of LMP also under these conditions as described by Gilsenan et al. (2000) and Cardoso (2003), had no real gel-promoting effect.

(ii) A low number of Ca^{2+} (0.42 mM/100 g, R 0.46) were already sufficient to initiate a certain gel formation as can be seen in comparison to (i). A continuous structuring process started at IST 57 °C (Fig. 5a) probably with a small number of ionic egg-box and/or crosslinking via Ca^{2+} as well as by hydrophobic interactions. They were, however, not strong enough for a real gelation. With further cooling increasing formation of hydrogen bonds started (Cardoso et al., 2003; Gilsenan et al., 2000) and the structuring process was accelerated despite of the decreasing influence of hydrophobic interactions. This is indicated by the CST 47 °C. Additionally, dimer associations could become more important with decreasing temperature. A low GP of 32 °C below CST confirmed the transition from the liquid-like to a dominating solid-like system.

(iii) At higher Ca^{2+} concentration (0.52 mM/100 g, R 0.58) IST and CST were comparable to (ii) but this time the GP at 53 °C was found already shortly after IST 57 °C (Fig. 5b). Two clear phases could be defined: phase 1 started from IST, probably with formation of egg-box and other ionic interactions as explained by Cardoso et al. (2003), Siew and Williams (2005) and Fraeye et al. (2009) as well as hydrophobic junction zone. It was strongly supported by hydrogen bonds (especially below CST 42 °C) as well as by high sugar content and accelerated with increasing contact between pectin chains during cooling. The second phase started rather late at about 20 °C and could be possibly ascribed mainly to increasing dimer associations and inter-dimer aggregations.

(iv) The structuring process of gels containing 0.62 mM $CaCl_2$/100 g (R 0.7) began earlier than in (iii) at IST 67 °C (Fig. 3), the higher calcium content obviously accelerated the structure formation considerably by formation of more ionic interactions. The GP 58 °C was found again between IST and CST (55 °C), but on a higher level than in (iii). This time even three phases could be identified in the structuring process. It was assumed that ionic junction zones via Ca-bridges together with hydrophobic interactions dominated the first phase, and that hydrogen bonds became a supporting force in the second. This second phase seemed to be partly comparable to the first one of (iii). The transition from phase 1 to 2 was near 40 °C. Dimer interactions and inter-dimer aggregations could be ascribed to a third phase below 20 °C, comparable to phase 2 of the gels in (iii).

Table 1
Data of structure formation. $CaCl_2$ is given as mM content in the final gel mass of 900 g as well as R = stoichiometric ratio Ca^{2+}/COO^-; GP = gel point = intersection of G' and G''; tan δ_{end} = loss factor at 10 °C; IST = initial structuring temperature; CST = critical structuring temperature; M = mean value.

	$CaCl_2$		Gel properties		G' and G''				dG'/dt	
	mM/100 g gel	R	pH	°SAG	G' 10 °C (Pa)	G'' 10 °C (Pa)	tan δ_{end}	GP (°C)	IST (°C)	CST (°C)
1	0	0	3.26		0.05	0.5	10.0		19	18
2	0	0	3.34		0.05	0.51	10.2		21	21
M	*0*	*0*	*3.3*		*0.05*	*0.51*	*10.1*		*20*	*19.5*
1	0.42	0.46	3.13		6	2	0.415	30	57	46
2	0.42	0.46	3.17		8	3	0.333	34	56	47
3	0.42	0.46	3.18		7	3	0.374	33	59	47
4	0.42	0.46	3.18		7	3	0.369	34	58	47
M	*0.42*	*0.46*	*3.17*		*7*	*2.75*	*0.373*	*32.8*	*57.5*	*46.8*
1	0.52	0.58	3.12	13.07	28	5	0.172	55	56	50
2	0.52	0.58	3.12	30.11	35	5	0.159	50	60	42
3	0.52	0.58	3.21	24.50	33	5	0.162	51	58	40
4	0.52	0.58	3.15	42.26	25	4	0.177	55	56	38
M	*0.52*	*0.58*	*3.15*	*27.49*	*30.25*	*4.75*	*0.168*	*52.8*	*57.5*	*42.5*
1	0.62	0.70	3.13	100.68	73	11	0.138	58	71	50
2	0.62	0.70	3.03	101.34	81	12	0.133	57	64	54
3	0.62	0.70	3.10	100.84	97	13	0.128	58	67	57
4	0.62	0.70	3.11	95.50	105	12	0.127	58	69	58
M	*0.62*	*0.7*	*3.09*	*99.59*	*89*	*12*	*0.132*	*57.8*	*67.8*	*54.8*
1	0.73	0.82	3.07	127.38	219	26	0.121	75	70	61
2	0.73	0.82	3.06	127.79	216	29	0.134		74	63
3	0.73	0.82	3.07	142.24	213	26	0.118		70	61
4	0.73	0.82	3.08	138.96	206	25	0.127		68	60
M	*0.73*	*0.82*	*3.07*	*134.09*	*213.5*	*26.5*	*0.125*	*75*	*70.5*	*61.3*
1	0.83	0.94	3.07	146.82	348	40	0.121	80	76	65
2	0.83	0.94	3.09	150.96	397	48	0.121		78	66
3	0.83	0.94	3.06	156.37	395	47	0.119	92	80	65
4	0.83	0.94	3.06	150.50	345	42	0.120		73	66
M	*0.83*	*0.94*	*3.07*	*151.16*	*371.25*	*44.3*	*0.120*	*86*	*76.8*	*65.5*

(v) With further increasing calcium content of 0.73 mM/100 g (R 0.82), the determination of the gel point was possible only for one of four samples and the resulting value was, therefore, rather vague. In contrast, IST and CST could be determined without any problems but this time below GP (Fig. 5c). It seems that partly pre-gelation (formation of micro-gel particles) happened already during the preparation process (no GP found by temperature sweep tests) or immediately after preparation in the starting phase of the measurements (GP > IST), though the pectin mixtures seemed to be homogenous, yet. A similar effect was described by Morris (2009). He defined a "weak gel structure", formed by some egg-box junction zones rapidly after gel preparation and a "true gel structure", developing on cooling by other mechanisms. The micro-gels of the "weak gel structures" seemed to be irregular solid particles that caused an "apparent gel point" but a real network was formed later during further cooling. The structuring process of the tested pectin system could be divided into three phases again, the first below IST 70 °C with acceleration at CST 61 °C, the second beginning at around 40 °C and the third below 22 °C. The structuring processes in these phases were assumed to be comparable to those explained in (iv).

(vi) At the highest calcium content (0.83 mM/100 g gel, R 0.94) less clear structuring phases could be defined and the GP, found already at 86 °C, was as vague as in (v). The "true" gelling process (phase 1) started at IST 77 °C and was accelerated at about 65 °C (CST). The high number of Ca^{2+} probably was able to increase the structuring velocity by forming more ionic interactions that were dominating the whole gelling process. Some small peaks or shoulders below 45 °C could be perhaps ascribed to the supporting effect of hydrogen bonds but formed no single second phase. The clear peak below 20 °C, resulting from increasing dimer interactions, corresponds again to phase 3.

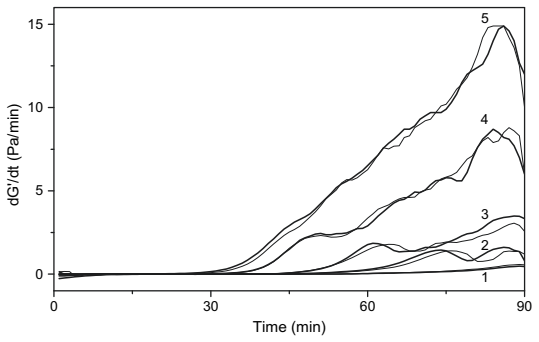

Fig. 4. Structure developing velocity dG'/dt in dependence on calcium content in 100 g gel: 1: 0.42 mM, 2: 0.52 mM, 3: 0.62 mM, 4: 0.73 mM, 5: 0.83 mM.

Altogether, during gelation of LMP with different Ca-content two to three phases were found with differing starting and final temperatures. The first phase could be ascribed to ionic egg-box and random crosslinks together with hydrophobic interactions; it was clearly detected in gels with more than 0.52 mM $CaCl_2$/100 g and shifted to higher temperature with increasing Ca^{2+}. The second phase (in gels with less Ca^{2+} also found as first one) perhaps indicated the contribution of hydrogen bonds and the third phase

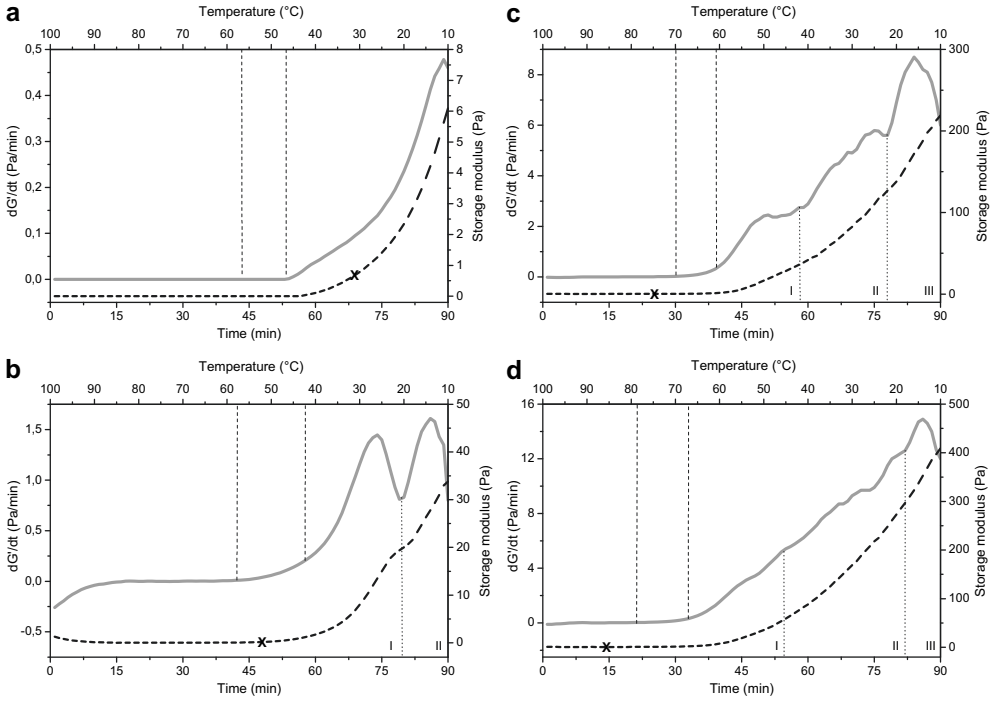

Fig. 5. Diagrams of gels with different calcium content in 100 g gel: a: 0.42 mM, b: 0.52 mM, c: 0.73 mM, d: 0.83 mM; full curve = dG'/dt, dotted curve = G'; vertical lines give IST and CST (----) and the start of structuring phases (• • • •); X on the G' curves marks the GP. For 0.62 mM/100 g gel see Fig. 2.

(in 2-phase processes the second one) might be dominated by inter-dimer interactions. At the highest tested Ca-content, the ionic interactions seem to dominate the whole gelling process with no clear difference between phases 1 and 2. Fig. 6 shows a possible model of the structuring process.

3.3. Gel structure after cooling

The question is, whether the calcium content influenced not only the gelation process but also the final gel structure. This will be discussed considering different gel parameters.

The final values of G' and G'' after cooling at 10 °C as well as the Ridgelimeter tests gave information about different gel properties. All three parameters strongly correlated with the increasing Ca-content (Table 1 and Figs. 7 and 8).

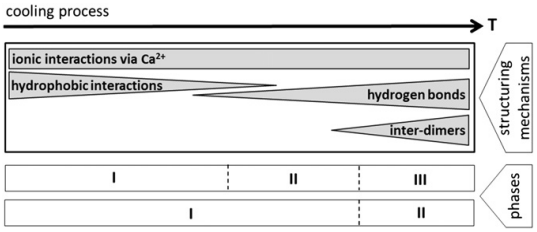

Fig. 6. Model scheme of the structuring process of the tested low-methoxylated pectin system during cooling for a 2 phases or 3 phases process.

The °SAG value is characteristic for the ability of a gel to keep its shape under its own weight (gel form stability). The samples with no Ca^{2+} did not really gel and could not be measured. Those with low content (0.42 mM) were rather weak and deformed quickly within the 2 min measuring time, it was impossible to get results by this method. The gels became stiffer and less sagging at higher Ca-content and, moreover, more and more brittle. The increase of °SAG was not linear and, altogether, moderate (the highest value was about six times higher than the smallest (Fig. 7)).

The storage modulus at the end of cooling (G'_{end}) characterised the elastic material properties of the samples. These increased with higher content of Ca^{2+} (maximum increase factor > 50, Fig. 8),

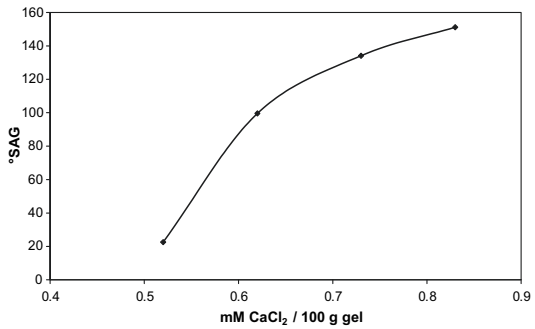

Fig. 7. Influence of the calcium content on the gel form stability (°SAG) in the Ridgelimeter method.

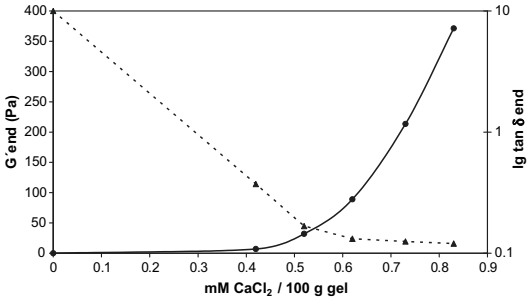

Fig. 8. Influence of the calcium content on the storage modulus G' (full line) and the loss factor tan δ_{end} (dotted line) at the end of the cooling phase at 10 °C.

which made the gels more stable and elastic. That was found also by Ngouimazong, Tengweh, et al. (2011) for gels without sugar.

The influence of the loss modulus G'' can be found in the loss factor tan $\delta = G''/G'$ (Fig. 8). Though G'' also increased with higher Ca^{2+} (factor about 15), the differences were not as high as in case of G' and thus G' dominated. After complete cooling, tan δ_{end} was the highest in mixtures without any calcium (about 10, Table 1), confirming their dominating liquid-like viscous material properties. With increasing Ca^{2+}, tan δ_{end} decreased and above 0.62 mM it was nearly constant. These gels were more solid-like and elastic but also increasingly brittle as found already during the Ridgelimeter tests. A comparable effect of the calcium concentration on gel properties was found also by Cardenas et al. (2008), Fraeye, Colle, et al. (2010) and Ngouimazong, Tengweh, et al. (2011).

The varying properties of the final gels confirmed the assumption of varying gel structures, resulting from different structuring mechanisms in dependence on the calcium content. Systems with low Ca-content, gelled mainly by non-ionic interactions, were very weak and deformable. Gels with a higher (optimum) amount of Ca^{2}, formed by a combination of ionic and other interactions were formstable and rather elastic. Gels with the highest concentration of Ca^{2+}, dominated by ionic structuring mechanisms and showing pre-gelation, were brittle and susceptible to mechanical destruction.

3.4. Structure developing rate

The average structure developing rates SDR_a was calculated for the total gelation process (Fig. 9). It increased strongly and nonlinear with the calcium contents of the gels and confirmed the well known role of Ca^{2+} for the gelation of low-methoxylated pectins.

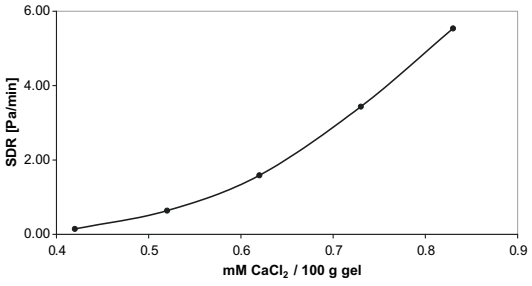

Fig. 9. Influence of the calcium content on the structure developing rate during the whole structure formation (SDR_a).

4. Conclusions

(i) The application of the first derivation dG'/dt, the structuring velocity curve, supports the understanding of the gelation process. The suggested new parameters initial structuring temperature IST and critical structuring temperature CST do not describe the same event in pectin gelation like the widely used cross-over of G' and G'' (gel point GP). The IST was higher than the GP as long as the latter could be determined regularly. In these cases, IST can be seen as the beginning of the structuring process in a system with dominating liquid-like character. The CST of the tested gels was found mainly below the GP. It seems that the structuring process was strongly accelerated after a certain critical number of junction zones had been formed in an increasingly solid-like material. The classical GP, however, seemed to be not always an indicator of real structure formation. It might also result from pre-gelation and the formation of irregular solid particles in micro-gels.

The two new parameters have proved to be good indicators for the start and the further development of structure formation in low-methoxylated pectin gels at varying calcium concentrations. All samples could be evaluated, also those with no clear GP. IST and CST are not seen as complete substitutes of the GP, but they give valuable complementary information and, in case of no clear GP, they are an alternative method for the examination of structure formation. Moreover, the application of the dG'/dt curve allowed the identification of single phases of the gelation, detected by increasing and decreasing structuring velocities. The calculation of structure developing rates, which was made only for the total gelation process, could be applied possibly also to single phases.

(ii) The tested LMP gels at pH 3 and with 30% saccharose required at least a small amount of calcium in ionic junction zones for successful gelation; hydrophobic interactions, hydrogen bonds and other mechanisms alone have been proved to be not sufficient, even at the promoting high sugar content. The first phase of the structuring process started at temperatures of ≥ 60 °C by formation of egg-box junction zones and random crosslinks via Ca-bridges. It was supported by hydrophobic interactions and seemed to be nearly completed at about 40 °C. The second and third phase of the structuring process until the end at 10 °C were probably dominated by hydrophilic interactions (assumed below 50 °C) and dimer aggregations (below 25 °C), respectively. The transition of the GP to higher temperature with increasing Ca-content clearly confirmed the importance of the ions for the gelation process. All types of Ca-bridges obviously increased the gelation velocity during cooling considerably and supported the formation of stable elastic gels. Above a certain calcium content, pre-gelation could take place during or immediately after gel preparation that changed the gel structure. These gels were very elastic but became more and more brittle. The properties of the final gels confirmed the varying gel structures, resulting from different structuring mechanisms in dependence on the calcium content.

(iii) The presented interpretations of the rheological parameters and their relation to structuring mechanisms are partly assumptions, yet, and have to be confirmed by further experiments using additional methods and pectin types. The application of the first derivation dG'/dt and the calculated new initial and critical structuring temperatures on the gelation of low-methoxylated pectins is, however, a first step into

the examination of their general importance for the pectin gelation process.

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(A4) Structure formation in sugar containing pectin gels – Influence of tartaric acid content (pH) and cooling on the gelation of high-methoxylated pectin

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Structure formation in sugar containing pectin gels – Influence of tartaric acid content (pH) and cooling rate on the gelation of high-methoxylated pectin

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ABSTRACT

The aim of the study was the application of a recently published method, using structuring parameters calculated from dG′/dt, for the characterisation of the pectin sugar acid gelation process. The influence of cooling rate and pH on structure formation of HM pectin gels containing 65 wt.% sucrose were investigated. The results show that the structure formation process as well as the properties of the final gels strongly depended on both parameters. With increasing cooling rates from 0.5 to 1.0 K/min the initial structuring temperature slightly decreased and the maximum structuring velocity increased. The lower the cooling rates, the firmer and more elastic were the final gels. With increasing acid content (decreasing pH from 2.5–2.0) the initial structuring temperatures were nearly constant. The final gel properties varied visibly but not systematically. Gels with the lowest and highest pH were less elastic and weaker compared to those with medium acid concentrations.

1. Introduction

Pectins are branched polysaccharides composed of partially methoxylated polygalacturonic acid. Pectins, isolated from the cell walls of higher plants, are important gelling or thickening agents for the food industry. They are commonly applied in jams and jellies, but they are also used e.g. as stabilisers in acidified milk drinks or as thickeners to improve the viscosity and texture of oil-in-water emulsions. The process of structure formation during gelation is rather complex but its principles are well established and described in the literature (Fraeye, Duvetter, Doungla, van Loey, & Hendrickx, 2010). It is generally known that gels are formed and water can be immobilised when junction zones in the smooth regions of pectin molecules form a three-dimensional network via specific intermolecular bonds. Depending on the degree of methoxylation (DM), two main gelation mechanisms are possible. Pectins with a DM above 50% (high-methoxylated, HM) form gels in the presence of saccharides (typically sucrose 55–75 wt.%) in acidic environment at pH 2.5–3.5. Their gelling mechanism is a combination of hydrophobic interactions and hydrogen bonds. The low-methoxylated pectin (DM < 50%) network formation is less dependent on pH and soluble solids than the HM pectin gelation. It is promoted by the presence of Ca^{2+}, forming intermolecular ionic junction zones between dissociated carboxyl groups (Fraeye et al., 2010; Thakur, Singh, & Handa, 1997; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995).

The most common parameters used for investigating the phase transitions in gelling or melting gel systems are the gel point (GP), gel setting time or temperature, melting point, melting time and temperature, respectively. Rheological measurements give the most common and reliable data for the examination of such sol–gel-transitions. The experimental detection of the GP is often described as crossover of storage modulus (G′) and loss modulus (G″) at a certain frequency (Gigli, Garnier, & Piazza, 2009; Iglesias & Lozano, 2004; Stang-Holst, Kjöniksen, Bu, Sande & Nyström, 2006). Lopes da Silva and Rao (2007), however, showed the limitations of this method. The G′–G″-crossover depends on the oscillation frequency as well as on the analytical range of a conventional rheometer, for instance in case of the detection of viscoelastic behavior in samples with low concentration. Nevertheless, the GP determined this way might be close to the real sol–gel-transition temperature (Lopes da Silva & Rao, 2007). Another method to evaluate pectin systems using oscillation measurements is the structuring development rate (SDR), calculated as dG′/dt (Rao & Cooley, 1993). The research group of the present study suggested additional structuring parameters (Kastner, Einhorn-Stoll, & Senge, 2012a, 2012b). The initial structuring temperature (IST) is defined as the temperature at which dG′/dt is different from 0 for the first time, and the critical structuring temperature (CST) is the extrapolated temperature of the first strong increase of dG′/dt.

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The structure formation during pectin gelation is an important factor in the production of many food products. In jams with large pieces of fruit, it is important to have rapid setting pectins, to ensure that the structure formation is fast and, thus, to ensure that the fruit pieces are evenly distributed in the product. In the production of clear gels, it is important to have no air bubbles, what can be achieved through the use of slower set pectins as well as through prevention of pre-gelation (May, 2000).

The typical sugar acid gel (SAG) formation of HM pectin is considered to be a two-step process with two types of interactions: The high sugar concentration reduces the pectin-solvent interaction and promotes hydrophobic interactions between the methoxyl groups of the polygalacturonic acid. These interactions dominate at higher temperature. The low pH reduces the dissociation of the carboxyl groups. As a consequence, the electrostatic repulsion between pectin molecules is suppressed and hydrogen bonds can be formed between the non-dissociated carboxyl groups and secondary hydroxyl groups. This mechanism dominates at low temperature (Lopes da Silva & Rao, 2007; Oakenfull & Fenwick, 1977; Oakenfull & Scott, 1984; Thakur et al., 1997; Voragen et al., 1995). As a consequence, the most important factors for the HM pectin structuring process and gel properties are intrinsic variables of the pectin molecules (DM, distribution of the ester groups along the pectin backbone, molecular weight, neutral sugar side chains and charge density) as well as extrinsic factors (pH, ionic strength, soluble solids, pectin amount and temperature).

In addition to the pectin type and buffer system, the cooling rate affects the elastic properties of pectin structure formation. Therefore Rao and Cooley (1993) concluded that the cooling conditions should be controlled to achieve an optimal gel structure. Several rheological studies therefore investigated the viscoelastic properties of pectin systems at different cooling or heating conditions in order to examine the transition from viscous fluids (sol) to elastic solids (gel) or vice versa. Moreover, different pectin, sugar and buffer concentrations of the pectin systems were applied in previous publications. Dahme (1992) investigated the influence of different cooling rates and concluded that the cooling rate of the pectin gel process should be less than 1 K/min to ensure that the structure formation can be detected uniformly without disturbances. Usually, a cooling rate of 1 K/min was used for the investigation of the structuring properties of pectin systems (Agoub, Giannouli, & Morris, 2009; Almrhag et al., 2012; Evageliou, Richardson, & Morris, 2000; Löfgren & Hermansson, 2007). However, in other studies structure formation of pectin gels was investigated using high cooling rates of 3 K/min (Iglesias & Lozano, 2004; Löfgren, Walkenström, & Hermansson, 2002) or low cooling rates of 0.5 K/min or below (Fu & Rao, 2001; Ngouémazong et al., 2012; Rao & Cooley, 1993).

Though the influence of the cooling rate on structure formation of pectin sugar gels is generally accepted, the detailed effects were not systematically examined, yet. Furthermore, also the influence of pH on the kinetics of structure formation of HM pectin sugar acid gels has not been investigated by rheological methods in detail, yet. Therefore the objective of the present study was, to characterise this structure formation of HM pectin sugar acid gels in dependence on (I) different cooling conditions and (II) varying pH by using various tartaric acid concentrations. The structuring process of the gels will be examined by measuring not only the classical gel point but also the recently published structuring parameters initial and critical structuring temperature IST and CST (Kastner et al., 2012a, 2012b) as calculated from rheological measurements.

2. Materials and methods

Two series of experiments were carried out to evaluate the influence of the cooling conditions and of pH on the HM SAG structuring process. In the first series, standard HM SAG formulations were cooled under different cooling rates (0.25, 0.50, 0.75, 1.00, and 2.00 K/min). In the second experimental series, HM SAG compositions of varying tartaric acid concentrations (3 mL: 9.6 mM/kg gel, 5 mL: 15.9 mM/kg gel, 7 mL: 22.3 mM/kg gel, 9 mL: 28.7 mM/kg gel, and 11 mL: 35.1 mM/kg gel) were investigated at a cooling rate of 1.00 K/min.

2.1. Materials

A commercial available high-methoxylated non-standardised citrus pectin with 74.3% galacturonan content, a DM of 69.8% and an intrinsic viscosity of 554 cm^3/g was used for all experiments. The tartaric acid was of analytical grade. Sucrose was food grade and purchased locally.

2.2. Methods

2.2.1. Preparation of the pectin sugar acid gels

The gel composition and preparation was based on the empiric method of the IFT Committee (1959). The concentration of tartaric acid was varied from 9.6 to 35.1 mM/kg gel. In all experiments the total concentration of solids was held constant at 65 wt.%, including the 0.27 wt.% pectin.

The standard procedure for gel preparation was as described below: 2.75 g pectin (0.27 wt.%) and 40 g sucrose were dissolved in 430 g demineralised water by stirring. The suspension was heated until boiling, 607.3 g sucrose was added in 3 portions under continuous stirring and the solution was boiled again. While further boiling and stirring, the total mass was reduced to 1020 g. Afterwards, 7 mL (22.3 mM/kg gel) of 48.8% w/v tartaric acid solution was added in the first experimental series. In the second series the tartaric acid was varied (expected pH range: 2.5 to 2.0): 3 mL (9.6 mM/kg gel), 5 mL (15.9 mM/kg gel), 7 mL (22.3 mM/kg gel), 9 mL (28.7 mM/kg gel), and 11 mL (35.1 mM/kg gel). The whole gel preparation took no longer than 5 min. The respective SAG solutions were poured into jam jars or the preheated rheometer, as described in Section 2.2.2 and Section 2.2.3. The final properties of the SAG solution were within the limits of total solids 64.5–65.5 wt.%. The total solid was determined by an automatic refractometer (Schmidt and Haensch, Germany).

2.2.2. Determination of a suitable range for the cooling rate

Cooling tests were performed in order to determine cooling rates of SAG with relevance for industrial applications and, thus, to define parameters for the subsequent rheological measurements. The hot SAG solution was filled into jars (200 mL) similar to those used in the food industry for jam production. The lids were closed and the samples cooled down in a bundle at room conditions. The temperatures during cooling and the cooling gradients were recorded in different areas of the bundle and different areas of the individual jam jars. For this, the glasses were arranged in 3 layers, each with nine jars. Moreover, one single jar was investigated in the same way. All measurements in the bundle as well as in the single jar were repeated five times.

2.2.3. Rheological measurements

The viscoelastic behavior of SAG during cooling was assessed by small deformation oscillation measurements using a rheometer Physica MCR 301 (Anton Paar, Germany) with a profiled rotational cylinder CC27/P1 (diameter 26.66 mm, length 40.01 mm) and Peltier cylinder temperature system TEZ 150P. Hot SAG solutions (Section 2.2.1) were transferred onto the pre-heated rheometer (105 °C) and the free surface of the samples was coated with silicone oil, the cylinder was closed with a special lid in order to minimise evaporation and cooled to 20 °C. The cooling rate was varied in the first experimental series (0.25, 0.50, 0.75, 1.00,

2.00 K/min). In the second experimental series the cooling rate was kept constant at 1 K/min. The dynamic rheological parameters (G′ and G″) were recorded during cooling at a frequency of 1 Hz and a strain of 10^{-3}. All SAG were prepared at least three times for each cooling rate as well as for each acid concentration.

From the rheological measurements three temperatures to characterise the pectin structuring process were calculated. The classical GP was defined as cross-over of G′ and G″ with $\tan\delta = G''/G' = 1$. IST and CST were determined as previously described (Kastner et al., 2012a, 2012b) from the structuring velocity, calculated as the first derivation of G′ (dG′/dt), using the OriginPro 8.6 software (OriginLab Corporation, USA).

2.2.4. pH measurements

After the rheological measurements the pH was determined in the cooled gel (20 °C) using a Lab850 pH-meter (Schott Instruments) and a special penetration electrode (BlueLine 14pH, Schott Instruments).

2.2.5. Statistical analysis

Analysis of variance was carried out using OriginPro 8.6 software. The Holm-Bonferroni test was used to determine statistically significant differences ($p < 0.05$).

3. Results and discussion

The average cooling rate of the single jar without external cooling was 0.45 K/min, determined in the temperature range of 85–50 °C, in which the phase transition from liquid to solid took place. Cooling rates of the jars in the bundle differed from 0.16 to 0.44 K/min in the same temperature range. In detail, the jars in the middle of the bundle showed an average cooling rate of 0.16 as well as 0.20 K/min and the glasses on the edge of the bundle had a cooling rate of 0.41 to 0.44 K/min. Considering these results, cooling rates for the rheological experiments were selected within this range and above this range in order to determine the effect of cooling rate on structuring process. The structure formation for standard HM SAG in presence of 65 wt.% sucrose was investigated at different cooling rates from 0.25 to 2.00 K/min. The structure formation for HM SAG with varying tartaric acid concentration from 9.6 to 35.1 mM/kg gel was investigated at a constant cooling rate of 1 K/min.

3.1. Structuring process as observed for a standard HM SAG

The rheological measurements allowed the characterisation of structure formation with respect to the immobilisation of water, the determination of the sol–gel transition and the quality of the final gel (Fig. 1a).

Sometimes in samples cooled at a high cooling rate (2 K/min) or in samples with high tartaric acid content (>28.7 mM/kg gel), no clear GP could be measured in replicate measurements (Fig. 1a). A similar observation has been described in previous studies, even at low pectin concentration (Evageliou et al., 2000; Gigli, Garnier, & Piazza, 2009; Iglesias & Lozano, 2004; Picout, Richardson, & Morris, 2000). In contrast, for all samples it was possible to determine the sol–gel transition range reliably by calculating IST and CST from the first derivation of the storage modulus (Fig. 1b, Table 1, Table 2).

Additionally, often different structuring phases as described by Oakenfull and Fenwick (1977), Oakenfull and Scott (1984), Voragen et al. (1995), Thakur et al. (1997), Fu and Rao (2001), Lopes da Silva and Rao (2007), and Kastner et al. (2012b) could be identified from the structuring velocity curve (Fig. 1b). The phases of structure formation of standard HM SAG, containing 22.3 mM tartaric acid/kg gel and cooled with 1 K/min, are shown in Fig. 1b. The rise in storage modulus as well as structuring velocity was low from 105 to 82 °C, nearly no structure formation occurred. With further cooling and after passing the GP (sol–gel transition), the storage modulus increased slowly but continuously and the structuring velocity rose rapidly until 68 °C. The initial structure formation was rapid and strong. Below this temperature, a further increase of G′ but a decrease of the structuring velocity was observed until about 50 °C. The gel further solidified at nearly constant structuring velocity. It is known that during the whole gelation process the hydrophobic interactions are weakened (Oakenfull & Fenwick, 1977) and the hydrogen bonds are strengthened (Joesten & Schaad, 1974) with decreasing temperature as described by Alonso-Mougán, Meijide, Jover, Rodríguez-Núñez, and Vázquez-Tato (2002), Evageliou et al. (2000), and Oakenfull and Scott (1984). Probably, the phases of dG′/dt indicate these changes of the structuring mechanisms during structure formation from hydrophobic interactions at higher temperatures to hydrogen bonds at lower temperatures. It might be assumed that during gelation the loss of hydrophobic interactions is compensated by an increasing number of hydrogen bonds. Additionally, later on dimer association and inter-dimer

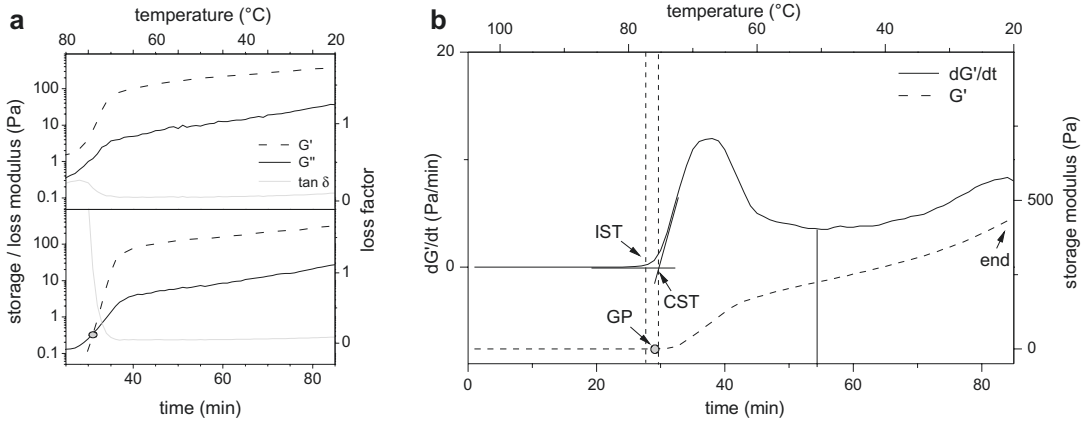

Fig. 1. (a) Oscillation measurements of a HM SAG with acid content of 35.1 mM/kg gel during cooling (1 K/min), G' (——, bold line); G'' (- - -), tanδ (—, thin line). Top: without gel point GP, bottom: with GP (●). (b) Typical curve of the structure formation of HM SAG with standard formulation (22.3 mM/kg gel) during cooling (1 K/min); full curve = dG'/dt, dotted curve = G'; dotted vertical lines give IST and CST and the start of structuring phases; end: end level at 20 °C. The GP is marked as ● on the G' curve.

Table 1
Structuring temperatures in dependence on cooling rate (experimental series 1).

Cooling rate (K/min)	IST (°C)	CST (°C)	GP (°C)
0.25	81.6 ± 2.6	75.3 ± 2.7	77.4 ± 4.0
0.50	83.6 ± 0.3	74.3 ± 0.5	76.6 ± 0.3
0.75	79.6 ± 0.2	75.1 ± 0.8	74.6 ± 1.3
1.00	78.0 ± 2.4	75.3 ± 0.8	75.3 ± 0.7
2.00	73.0 ± 1.1	71.4 ± 0.9	71.0 ± 1.1

Table 2
Structuring temperatures in dependence on tartaric acid concentration (experimental series 2).

Tartaric acid (mM/kg gel)	IST (°C)	CST (°C)	GP (°C)
9.6	79.6 ± 2.1	77.2 ± 1.0	76.5 ± 0.5
15.9	79.3 ± 3.1	76.6 ± 0.9	77.0 ± 1.3
22.3	78.9 ± 2.5	75.9 ± 1.2	76.3 ± 1.8
28.7	75.5 ± 2.1	74.9 ± 1.4	74.7 ± 0.6
35.1	75.3 ± 1.7	74.3 ± 1.3	73.0 ± 0.0

aggregation within the pectin gel can take place. In any case, a steady increase of G′ indicates continuous structure formation during the whole cooling process.

3.2. Effect of the cooling rate on structure formation and gel properties

The structuring temperatures GP (77.4 to 71.0 °C), IST (81.6 to 73.0 °C), and CST (75.3 to 71.4 °C) showed a slight but mostly not significant decrease with increasing cooling rates from 0.25 to 2.00 K/min (Table 1). Only the IST of the gels with the highest (2.00 K/min) and the lowest (0.25 and 0.50 K/min) cooling rates significantly differed.

The structuring velocity, however, strongly increased (Fig. 2a). These results can be divided into three different groups:

At low cooling rates of 0.25, 0.50 K/min the structure formation of these gels started earlier (higher IST, Table 1) than structure formation in all other gels. It seems that there was sufficient time for an optimum arrangement of pectin molecules to form many strong intermolecular interactions and long junction zones for gelation (Fig. 3). As indicated by low values of the loss factor, after cooling the corresponding gels were more elastic and less viscous than all others (Fig. 2b). At a cooling rate of 0.75 K/min, the maximum structuring velocity of the gels was higher than those of the two lower rates and more similar to those of the third group. The structure formation started, however, earlier and the IST was more comparable to those of lower cooling rates. The final gel was an intermediate one, too. It differs clearly from the slowly cooled gels but only slightly from the rapidly cooled. In gels prepared at high cooling rates of 1.0 K/min or 2.0 K/min the process of structure formation started at lower IST. Probably, independent on temperature a certain time was necessary for the formation of junction zones and for the optimum interaction of pectin chains. The final gels were the least elastic and most viscous and showed a less homogeneous gel structure compared to gels prepared at slow cooling rates. A possible reason is that in the early stage of structure formation less molecular arrangement occurs, and more shorter junction zones or even a certain share of local microgels are formed (Fig. 3), which cause a less homogeneous structure of the SAG three-dimensional network. It is not clear whether the structure formation was already complete after the end of the measurement. Some authors examined pectin gels after the end of the cooling process by continuing rheological measurements and found an aging effect, sometimes referred to annealing (Evageliou et al., 2000; Fu & Rao, 2001; Lopes da Silva & Gonçalves, 1994). This effect may be caused by a transition of shorter to longer junction zones (Fig. 3) as described for gelatin gelation by Ziegler and Foegeding (1990).

3.3. Effect of tartaric acid content on structure formation and gel properties

Recent atomic force microscopy studies of Fishman and Cooke (2009) have shown that the gel structure was affected by pH and that the distribution of pectin strands and the spaces between strands were relevant factors for evaluation of gel properties like gel strength. For gel formation of HM pectins a low pH is required in order to reduce the dissociation of carboxyl groups and, thus, to reduce electrostatic repulsion. The non-dissociated carboxyl groups can form hydrogen bonds with each other or with hydroxyl groups (Thibault & Ralet, 2003).

In the present study all parameters used for the characterisation of structure formation during the gelation process were nearly independent of the acid concentration that was 9.6 mM/kg gel (pH 2.52 ± 0.03), 15.9 mM/kg gel (pH 2.40 ± 0.03), 22.3 mM/kg gel (pH 2.18 ± 0.04), 28.7 mM/kg gel (pH 2.07 ± 0.12), and 35.1 mM/kg gel (pH 2.03 ± 0.08), respectively. All gels showed rather similar

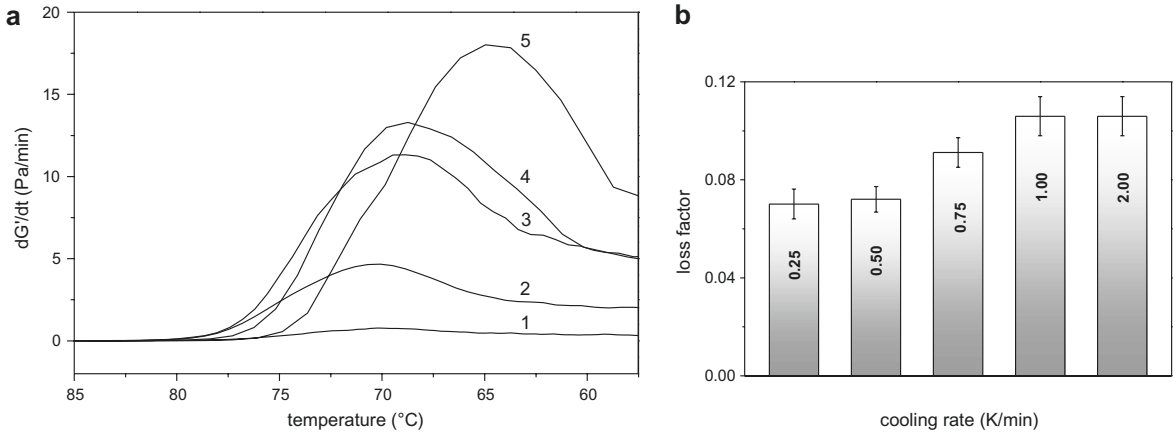

Fig. 2. Influence of cooling rate of standard HM SAG on (a) structuring velocity (dG'/dt): 1: 0.25 K/min, 2: 0.50 K/min, 3: 0.75 K/min, 4: 1.00 K/min, 5: 2.00 K/min; (b) tanδ at the end of the cooling phase at 20 °C.

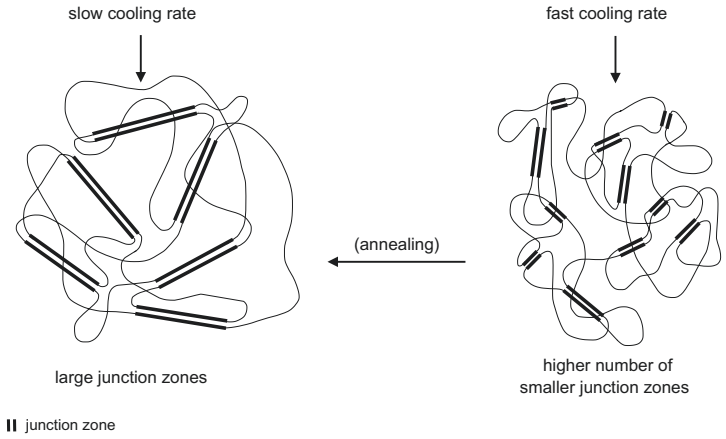

Fig. 3. Formation of pectin junction zones during cooling.

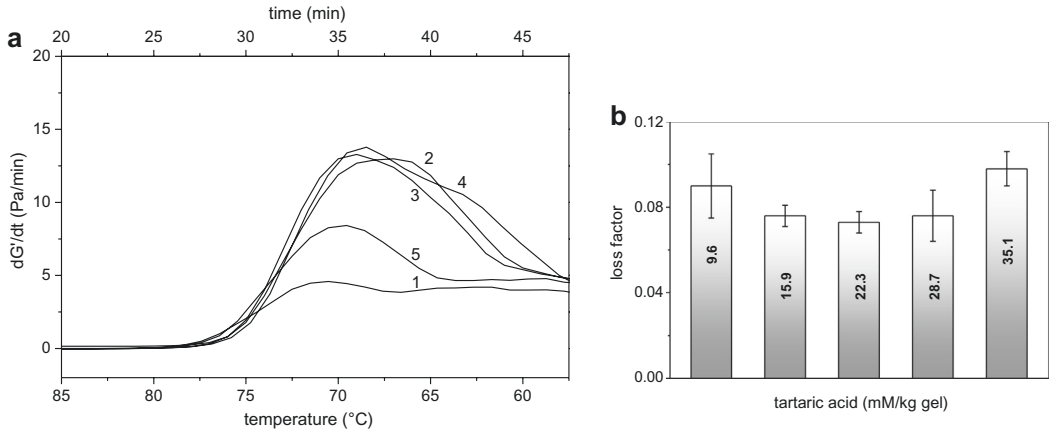

Fig. 4. Influence of acid concentration of HM SAG during cooling (1 K/min) on (a) structuring velocity (dG'/dt): 1: 9.6 mM/kg gel, 2: 15.9 mM/kg gel, 3: 22.3 mM/kg gel, 4: 28.7 mM/kg gel, 5: 35.1 mM/kg gel; (b) tanδ at the end of the cooling phase at 20 °C.

structuring temperatures (IST, CST, and GP). The differences from the lowest to the highest concentration of tartaric acid were only about 3 K for each temperature (Table 2). The curves of structuring velocity however, varied to a great extent. At the lowest acid content (9.6 mM/kg gel) structure formation occurred very slowly, probably because at low pH the majority of free carboxylic groups are dissociated and not available for formation of hydrogen bonds (Agoub et al., 2009). Moreover, intermolecular electrostatic repulsion between dissociated carboxyl groups can inhibit structure formation (Evageliou et al., 2000). Gels with moderate addition of tartaric acid (15.9, 22.3, and 28.7 mM/kg gel) showed the highest structuring velocity, their curves were similar (Fig. 4a). These acid contents allowed a rapid undisturbed gel formation. In the gels with the highest acid content (35.1 mM/kg gel) the structure formation was rather slowly, too. In these gels less carboxylic groups were dissociated. The undissociated groups were able to form hydrogen bonds, especially at local "high-acid spots", and might contribute to pre-gelation and microgel formation (Fig. 4a). Ross-Murphy (1984) described such structures as "incomplete gels".

The properties of the final gels with varying acid content differed considerably, too (Fig. 4b). The reasons are similar to those explained above: The gels of intermediate pH were strong, similar to each other and comparable to those of other HM pectins in previous works (Kastner et al., 2012a). At high pH the gels were weaker and more viscous because of the limited number of hydrogen bonds. The "incomplete gels" at the lowest pH were weaker, too because of pre-gelation and microgel formation. A similar effect was discussed for the influence of the amount of calcium ions on the gelation of LM pectins (Kastner et al., 2012b) where a minimum concentration of calcium was required for gelation and a high amount caused microgels, too.

4. Conclusions

The initial structuring temperature IST and critical structuring temperature CST were suitable parameters in order to describe the incipient structure formation for HM sugar acid gels. Even samples with no clear GP, the traditional parameter for characterisation of structure formation, could be evaluated this way. Therefore, IST and CST have proved to be valuable additional parameters for the characterisation of structure formation and can help to understand the structure formation processes in more detail. Structuring phases could be identified from the shapes of the structuring velocity curves during the gelation process of the

HM pectins. It is assumed that they indicate changes of the dominating type of interactions during structure formation.

Structure formation as well as the properties of the final gels strongly depended on the cooling rate. The cooling rate affected both, the structure formation and the SAG structure after cooling. At higher cooling rates, structure formation started earlier and a weaker structure of resulting gels occurred due to a rapid formation of shorter junction zones. In contrast, at low cooling rates a retarded formation of longer junction zones takes place and the final SAG structure is more compact and elastic.

The investigation of the influence of the acid content gave an intermediate optimum range for the structure formation and the final SAG structure at 15.9–28.7 mM/kg gel. Lower as well as higher tartaric acid concentrations were critical and showed a lower structuring velocity and weaker final gels.

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(A5) Structure Formation in sugar containing pectin gels – Influence of composition and cooling rate on the gelation of non-amidated and amidated low-methoxylated pectin

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pH

ABSTRACT

Gel structure formation and gel properties of low-methoxyl pectin (LMP) and low-methoxyl amidated pectin (LMAP) with similar degree of methoxylation have been investigated by oscillatory rheological measurements. The gelling process was examined in a sugar-acid environment matching the conditions in jams and jellies. Factors studied included cooling rate, calcium content and pH. Parameters derived from the rheological measurements comprised the gel point, structuring velocity, initial and critical structuring temperature, average structuring developing rate and loss factor ($\tan\delta_{end}$).

The influence of the cooling rate on the gelling process of LMP was moderate and the influence on the final gel properties was significant, $\tan\delta_{end}$ decreased with increasing cooling rate. The calcium content significantly affected the structuring process of LMAP and the final gel properties. At high calcium content, the gelling process started at a higher temperature but the resulting gels were less strong. The pH had a significant but partly opposite effect on the gelation of LMP as well as LMAP. The differences in gelation behavior between LMP and LMAP can be explained by the lower number of available blocks of free carboxyl groups in LMAP as well as by the formation of additional hydrogen bonds through the amide groups.

1. Introduction

Pectin is a polysaccharide and extracted from plant cell walls. It is used in the food industry for its gelling, thickening, stabilizing, and emulsifying properties. Variations in pectin structure enable this broad range of commercial applications. Depending on the degree of methoxylated carboxyl groups (degree of methoxylation, DM) pectins are traditionally classified as high-methoxylated pectins (HMP) with DM > 50% and low-methoxylated pectins (LMP) with DM < 50%. Chemical de-esterification of HMP in presence of ammonium ions results in low-methoxylated amidated pectin (LMAP).

For understanding of the gelling process of pectin it is helpful to review the types of junction zones in pectin gels. A limited number of hydrophobic interactions may be formed between the methyl ester groups immediately at the beginning of the cooling process and induce the gelation (Oakenfull & Scott, 1984). These interactions have a rather low energy, a limited working range of about 2 nm (Walstra, 2002, Chapter 3) and become weaker with decreasing temperature. Upon further cooling, at lower temperatures hydrophilic interactions between undissociated carboxyl groups of the galacturonic acid and/or hydroxyl groups of carboxyl, hydroxyl or amide groups can develop via hydrogen bonds (Oakenfull & Fenwick, 1977; Oakenfull & Scott, 1984). These bonds are also of low energy and with 0.2 nm their working range is even smaller than that of the hydrophobic interactions. That means that the pectin molecules have to come in close contact in order to form a gel network. This can be achieved by a high soluble solid concentration (>50%) since the resulting reduced water activity allows the approach of pectin chains (Evageliou, Richardson, & Morris, 2000; Kastner et al., 2014; Thakur, Singh, Handa, & Rao, 1997). The influence of the hydrogen bonds gains more importance upon temperature reduction and supports inter-chain association during network formation. These two types of junction zone formation occur during gelation in all types of pectin and are typical for a cold-set gelation (Burey, Bhandari, Howes, & Gidley, 2008). However, gelation of LMP is governed by ionic interactions between dissociated carboxyl groups, typically via calcium ion bridges (Thibault &

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Ralet, 2003) in an ionotropic gelation (Burey et al., 2008). Calcium bridges represent a third type of junction zone formation and start to form immediately after gel preparation. They are much stronger than hydrophobic and hydrogen bonds and with about 20 nm their working range is rather long (Walstra, 2002, Chapter 3). Pectin gels with combined or dominating ionic junction zones require less soluble solids than HMP gels based on hydrophobic or hydrogen bonds, and can be formed also in sugar-free systems. The ionotropic gelation can take place in pectin solutions without heating (Ström & Williams, 2003; Vincent & Williams, 2009) and can be performed also as isothermal titration at room temperature (Fang et al., 2008). It seems to be possible, however, that ionic interactions formed at higher temperature tend to be initially not stable but their stability will increase during cooling (Cárdenas, Goycoolea, & Rinaudo, 2008; Garnier, Axelos, & Thibault, 1993). In LMAP, junction zones are additionally stabilized by hydrogen bonds involving the amide group (Alonso-Mougán, Meijide, Jover, Rodrı;guez-Núñez, & Vázquez-Tato, 2002; Black & Smit, 1972; Löfgren, Guillotin, & Hermansson, 2006).

Structure formation and properties of pectin gels strongly depend on intrinsic and extrinsic factors (Endress & Christensen, 2009; Rolin, Chrestensen, Hansen, Staunstrup, & Sørensen, 2009; Yapo & Gnakri, 2015). Intrinsic factors affecting a system are those related to the composition, e.g. type of pectin and concentration, agent used for pH adjustment, presence of divalent cations or co-solutes like sugar. Important extrinsic (technological) factors are e.g. heating and cooling conditions.

The influence of calcium ions depends on the stoichiometric ratio between calcium ions and the dissociated free carboxyl groups. This ratio is calculated as $R = 2[Ca^{2+}]/[COO^{-}]$ (Axelos & Kolb, 1990; Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2006; Cárdenas et al., 2008; Garnier et al., 1993; Ström et al., 2007). A theoretical saturation threshold of the R exists at which every calcium ion in the gel is bound to two dissociated carboxyl group. This threshold is affected on one hand by the degree of dissociation of the carboxyl groups and, thus, by the pH in the gel system. The pK_a of pectin is about 3.5 (Ralet, Dronnet, Buchholt, & Thibault, 2001), at this pH 50% of the carboxyl groups are dissociated. On the other hand the binding of calcium to pectin chains also depends on the distribution of these groups (block-wise or random). Ionic interactions require a certain number (blocks) of about 6–14 subsequent dissociated carboxyl groups (Liners, Thibault, & Cutsem, 1992; Luzio & Cameron, 2008; Powell, Morris, Gidley, & Rees, 1982) in order to form junction zones named as “egg-boxes”. Vincent and Williams (2009) therefore suggested a modified R_{eff}, in which only dissociated carboxyl groups in blocks are considered. The calculation of their exact number requires, however, detailed knowledge of the pectin molecular structure. Single randomly distributed dissociated carboxyl groups may also interact with calcium ions. In case they are oriented to the outside of the egg-boxes, they could form larger dimer aggregates and even an extended network (Braccini & Pérez, 2001; Fraeye et al., 2009, 2010). Moreover, excess calcium ions may be located in the gap between galacturonic acid molecules and interact with other C-atoms than C6 (Siew, Williams, & Young, 2005), they might course a certain electrostatic repulsion. The number of rather unspecific or random calcium crosslinks will increase with increasing calcium ion content.

The degree of methoxylation and the distribution of the carboxyl groups in a more random or more block-wise way can influence the gelling process especially with respect to the ionotropic gelation (Fraeye et al., 2009; Ngouémazong et al., 2012). According to Fraye et al. (2009, 2010) pectin with a dominating block-wise distribution of the dissociated carboxyl groups is able to gel at a lower calcium concentration (R) than pectin with a more random distribution of free carboxyl groups. At higher calcium content ($R > 1$) pectin gels were found to become more cross-linked and elastic and a plateau of the storage modulus (G') was reached only at very high calcium contents with R up to 5.0. Additional crosslinks might result from random interactions of calcium ions with single carboxyl groups as described above. When the calcium content becomes too high, precipitation and/or syneresis can occur and the gel strength may be reduced (Fraeye et al., 2010; Grosso & Rao, 1998). Gels prepared from amidated pectins were found to be less sensitive to syneresis (Thakur et al., 1997; Thibault & Ralet, 2003) than those of non-amidated pectins.

The complex gelling process and gel properties of pectin systems as well as the influence of different intrinsic and extrinsic factors on the gelation have been successfully examined by a variety of methods including a wide range of rheology-based methods. In the majority of studies, however, only one or two intrinsic factors have been varied, like e.g. pectin concentration, co-solutes, ion concentration, type of ions or acid and alkaline media to adjust the pH (Evageliou et al., 2000; Fraeye et al., 2010; Gigli, Garnier, & Piazza, 2009; Guillotin, Van Kampen, Boulenguer, Schols, & Voragen, 2006; Lopes da Silva & Gonçalves, 1994; Löfgren & Hermansson, 2007; Löfgren et al., 2006; Rao & Cooley, 1993; Sousa, Nielsen, Armagan, Larsen, & Sørensen, 2015; Ström et al., 2007; Tsoga, Richardson, & Morris, 2004). As a consequence the results of some of these studies are hardly comparable because of differences in the intrinsic factors of the model system as well as extrinsic factors in the experimental setup, methodology or measuring equipment. In addition, often only one parameter, the classical gel point (GP) defined as crossover of storage modulus G' and loss modulus G'' at a certain frequency, was reported (Gigli et al., 2009; Holst, Kjøniksen, Bu, Sande, & Nyström, 2006; Iglesias & Lozano, 2004). In recent years, the rheological characterization of a gelling process was significantly improved by using new parameters like the initial structuring temperature (IST), defined as the temperature at which the first derivation of G' as a function of time (dG'/dt) differed from zero for the first time, and the critical structuring temperature (CST) as the extrapolated temperature of the first strong increase of dG'/dt (Kastner, Einhorn-Stoll, & Senge, 2012a, 2012b; Einhorn-Stoll, Kastner, & Senge, 2012; Einhorn-Stoll, Kastner, Hecht, Zimathies, & Drusch, 2015; Kastner et al., 2014). This method allows the evaluation of the gelling kinetics and the final gel properties and, thus, gives information about systems without clear gel point. These new parameters are now generally accepted and have been used by several other groups (Garrido, Lozano, & Genovese, 2015; Sousa et al., 2015; Wang, Hua, Yang, Kang, & Zhang, 2014).

For these reasons, in the last years the overall aim of our group was the investigation of the pectin gelling process and the final gel properties in a broad study, covering all major factors such as pectin type, content of calcium ions, pH and cooling rate. Several results have already been achieved and published and complementary examinations are the subject of the present study. The kinetics of structure formation of HMP gels, a typical cold-set gelation, as well as the properties of the final gels were investigated before at varying cooling rates and pH (Kastner et al., 2014). A high cooling rate promoted early structure formation and resulted in a less elastic gel compared to a low cooling rate. Varying the pH by differences in the acid concentration showed that an optimum range for the structure formation as well as for the final gel properties at intermediate acid concentration exists. In the present study the influence of the cooling rate on the gelation of LMP will be investigated because it is assumed that here the temperature-independent ionic interactions (ionotropic gelation) will dominate the gelation process and reduce the influence of the cooling rate (cold-set gelation). Moreover, the influence of pH on the

gelation of LMP and LMAP will be examined, because it is assumed that a reduced number of dissociated carboxyl groups will considerably alter the gelling kinetics. On the one hand, a reduced number of dissociated carboxyl groups limits the ionic junction zones but on the other hand it supports the formation of hydrogen bonds. The impact of the calcium concentration on LMP gelation was studied already in non-amidated LMP gels (Kastner et al., 2012b). It was found that high calcium content caused gelling at higher temperature, a more rapid process and resulted in more elastic and also partly brittle gels. The present study will complete this research complex by analyzing the impact of the calcium concentration also on LMAP. Differences are expected because in LMAP the number of available blocks of free carboxyl groups at a similar DM is lower than in LMP and the role of hydrogen bonds is more pronounced because of the amide groups in the LMAP.

2. Materials and methods

2.1. Materials

Two commercially available citrus pectins were used in the present study. The low-methoxylated pectin with DM 30% and the low-methoxyl amidated pectin with DM 32% and DA 19% have already been characterized in previous studies (Einhorn-Stoll, Kastner, & Drusch, 2014a; Einhorn-Stoll, Prinz, & Drusch, 2014b; Einhorn-Stoll et al., 2015; Kastner et al., 2012b). Citric acid, trisodium citrate dihydrate and calcium chloride dihydrate were of analytical grade (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Sucrose was food grade and purchased locally.

2.2. Methods

2.2.1. Examination of the structure formation process and final gel structure

The gelation of LMP and LMAP was investigated in a sugar-acid environment. The standard gel composition and preparation for LMP gels was the same as in Kastner et al. (2012b): A solution of 637.5 g demineralized water, 264 g sucrose, 7.5 mL 54.3% w/v citric acid solution, 15 mL 6% w/v sodium citrate solution, 37.5 mL 2.205% w/v calcium chloride dihydrate solution and 6 g pectin were reduced by boiling to a total mass of 900 g. This standard gel composition was varied depending on the aim of the specific investigation, but the total content of solids was kept constant for all systems at 30 wt %. All gels were prepared at least in triplicate.

In order to investigate the gelling process of LMP gels, three different aspects were considered. (I) For the variation of the cooling conditions, standard LMP gels were cooled with different rates of 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00 K/min, comparable to the recent investigation of HMP (Kastner et al., 2014). (II) The influence of calcium on the structuring process of LMAP was studied, under the same conditions like in the study of LMP (Kastner et al., 2012b). Different amounts of 2.205% w/v calcium chloride dihydrate solution were added: 25 mL (4.2 mM/kg gel), 31 mL (5.2 mM/kg gel), 37.5 mL (6.2 mM/kg gel), 44 mL (7.3 mM/kg gel), and 50 mL (8.3 mM/kg gel). The resulting stoichiometric ratio between calcium and free carboxyl content $R = 2[Ca^{2+}]/[COO^{-}]$ was 0.73/0.90/1.09/1.28 and 1.45, respectively. The regular amount of $CaCl_2$ in the standard procedure was 37.5 mL (6.2 mM/kg gel, R = 1.09); variations were made in both directions. (III) The pH influence was tested for both LMP types by adding different amounts of 54.3% w/v citric acid solution. For the LMP, the pH range was from 5.0 to 2.9 with the following volumes of citric acid solution: 1.5 mL (4.7 mM/kg gel), 3.5 mL (11.0 mM/kg gel), 5.5 mL (17.3 mM/kg gel), 7.5 mL (23.6 mM/kg gel), 9.5 mL (29.8 mM/kg gel), and 11.5 mL (36.1 mM/kg gel). The regular amount of citric acid in the standard LMP gel was 7.5 mL (23.6 mM/kg gel); variations were made in both directions. The stoichiometric ratio of calcium ions in the LMP gels was R = 0.7. For the LMAP, the pH range was from 5.3 to 3.0 with the following added volumes of citric acid solution: 1.5 mL (4.7 mM/kg gel), 3.5 mL (11.0 mM/kg gel), 5.5 mL (17.3 mM/kg gel), 7.5 mL (23.6 mM/kg gel), and 9.5 mL (29.8 mM/kg gel). The stoichiometric ratio of calcium ions was R = 1.09. The standard cooling rate for all gels of both pectins was held constant at 1 K/min.

The viscoelastic behavior of the samples during cooling was characterized by small deformation oscillation measurements using a rheometer Physica MCR 301 (Anton Paar, Ostfildern, Germany) equipped with a profiled rotational cylinder CC27/P1 (diameter 26.66 mm, length 40.01 mm) and Peltier cylinder temperature system TEZ 150 P. Samples were transferred onto the preheated rheometer (100 °C) and cooled to 10 °C by a standard cooling rate of 1 K/min or in variation from 0.25 to 2 K/min, respectively. The dynamic rheological parameters, storage modulus (G′) and loss modulus (G″), were recorded during cooling at constant frequency of 1 Hz and strain of 10^{-3} as previously described (Kastner et al., 2012b).

The final structure of the gels was characterized by the loss factor at 10 °C ($\tan\delta_{end}$). The pH was determined in the cooled gels using a Lab850 pH-meter (Schott Instruments) and a special penetration electrode (BlueLine 14 pH, Schott Instruments).

2.2.2. Determination of structuring temperatures and average structure developing rate

Different parameters were calculated from the measurements of the temperature sweeps in order to describe the gelling process: The classical gel point (GP) was defined as cross-over of G′ and G″ with $\tan\delta = G''/G' = 1$. Initial structuring temperature (IST) and critical structuring temperature (CST) were determined from the structuring velocity (dG′/dt), the first derivation of G′ as function of time using OriginPro9.1 software (OriginLab Corp. Northampton, USA) (Kastner et al., 2012a, 2012b, 2014). The structuring velocity curves were mean curves of at least 3 separate measurements. For the overall structuring process the average structure developing rate (SDR_a) was calculated (Kastner et al., 2012b):

$$SDR_a = \frac{G'_{end} - G'_{IST}}{t_{end} - t_{IST}}$$

2.2.3. Statistical analysis

Analysis of variance with a Kruskal-Wallis test (KWANOVA) was carried out in order to examine the significance of effects of cooling rate, calcium concentration (R) and acid concentration (pH) on structuring temperatures (IST, CST, GP), $\tan\delta_{end}$ and SDR_a. All tests were performed with OriginPro9.1 software (OriginLab Corp. Northampton, USA) at a significance level of 0.05 (95% confidence interval).

3. Results and discussion

3.1. Effect of the cooling rate on structure formation and gel properties for LMP

Standard LMP gels were cooled at different cooling rate ranging from 0.25 to 2.00 K/min. It was assumed that the formation of junction zones during gelation of LMP was less dependent of temperature than in case of HMP gelation (Kastner et al., 2014).

The structuring velocity curve (dG′/dt) of the LMP gels showed a systematic increase with increasing cooling rate (Fig. 1). In case of

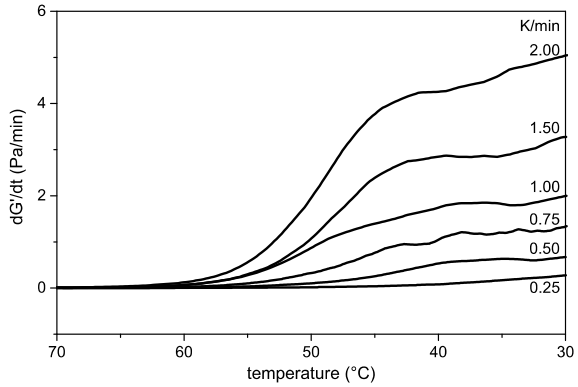

Fig. 1. Influence of the cooling rate (K/min) of standard LMP gel on the structuring velocity (dG'/dt).

the lowest cooling rate of 0.25 K/min it seems that the energy content of the system was rather high during the long time keeping at high temperature and the small number of hydrophobic interactions (at the low DM) was not sufficient for a substantial gel formation. The formation of hydrogen bonds was delayed, too. The distance between pectin molecules was large and the ionic bonds were not completely stable, yet (Cárdenas et al., 2008; Garnier et al., 1993). Moreover, calcium ions might be delocalized between the pectin molecules instead of binding closely in bridges and forming a strong gel. The latter effect might be comparable to the counter ion condensation as described by Siew et al. (2005). It can be, in general, assumed that a fast cooling (e.g. 2 K/min) supports early formation of calcium bridges, stabilized by the more rapid formation of hydrogen bonds. The IST varied between 59.5 and 65.8 °C (Table 1), the CST differed between 43.4 and 53.6 °C. The GP significantly differed and was between IST and CST for all cooling rates (Table 1). The SDR_a of the non-amidated LMP gels was generally low (Table 1). It slightly, but significantly increased with increasing cooling rate because the strong reduction of cooling time dominated the moderate increase of G′.

The final LMP gels were relatively viscous (soft or liquid-like), their $\tan\delta_{end}$ was rather high (Table 1). With increasing cooling rate, the $\tan\delta_{end}$ significantly but only slightly decreased, indicating that the final gel structure depended only weakly on the cooling rate. Thus, the influence of the cooling rate on junction zone length and the resulting final gel structure, as described for gels with hydrogen bonds such as gelatin (Ziegler & Foegeding, 1990) and HMP gels (Kastner et al., 2014), could not be confirmed for the gelation of LMP via calcium bridges.

Comparing the influence of the cooling rate on the structuring behavior of LMP with that of HMP gels (Kastner et al., 2014), similarities as well as differences are found. The cooling rate in all pectin gels had a moderate influence on the structuring temperatures IST and CST but clearly increased the structuring velocity and affected the properties of the final gels. The cooling rate impact on LMP gels differed strongly from that of the HMP. The structuring temperature IST and CST of LMP gels were generally lower than those of HMP gels but also their structuring velocity was much lower. The most important difference with respect to the cooling rate is the difference in the final gel properties as indicated by $\tan\delta_{end}$. Gels behaved completely opposite with increasing $\tan\delta_{end}$ for HMP gels and decreasing $\tan\delta_{end}$ for LMP gels. The additional junction zones formed via calcium bridges in the LMP altogether reduced the influence of the cooling rate on the pectin gelation. These results confirm the postulated differences in the gelling mechanisms of HMP and LMP in sugar acid gel formulations caused by the additional occurrence of ionic interactions.

3.2. Effect of calcium concentration on structure formation and gel properties for LMAP

The amount of $CaCl_2$ added to the gels was chosen based on the examination of the impact of calcium on gel formation of non-amidated LMP (Kastner et al., 2012b). The amount of $CaCl_2$ in the standard gel was varied in both directions, so that a stoichiometric ratio between 0.72 and 1.45 were reached. The R-values in the LMAP gels were higher than those in the corresponding LMP gels at the same calcium content. Despite of a similar DM (about 30%) of one LMAP and LMP, the presence of amidated carboxyl groups reduced the number of free dissociated carboxyl groups of LMAP and, thus, ionotropic gelation. Moreover, it was expected that even at similar R-values the results would differ because of the more pronounced role of hydrogen bonds of the amide groups in the cold-set gelation of LMAP.

The structuring velocity curves were similar in shape for all R (data not shown), only the start of the gelling process differed. Below 60 °C, the structuring velocities increased parallel over a range of 30 K and achieved a similar level, followed by a subsequent non-linear structure development. The structuring temperatures IST and CST significantly increased with increasing calcium content (Table 2). This is in agreement with Lootens et al. (2003). The IST varied in a relatively broad range ($\Delta T = 17.5$ K) compared to the CST, which varied in a smaller range ($\Delta T = 9.9$ K). The GP varied by 14.4 K between the samples, at R = 1.45 no GP was detectable. With increasing R, the IST raised faster than the CST. It is assumed that a high calcium content in all gels induced a more rapid initial structure formation by immediately forming calcium bridges between blocks of dissociated free carboxyl groups (ionotropic gelation). The main structuring process as indicated by the CST, however, was not accelerated to the same extent. It seems likely that as a consequence of a surplus of calcium ions and the low share

Table 1
Gelling parameters of standard LMP gel in dependence on the cooling rate. IST = initial structuring temperature, CST = critical structuring temperature, GP = gel point, $\tan\delta_{end}$ = loss factor as determined after the end of the gelation, SDR_a = average structure developing rate. *P* indicates significant differences: * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

Cooling rate (K/min)	IST (°C)	CST (°C)	GP (°C)	$\tan\delta_{end}$	SDR_a (Pa/min)
0.25	59.5	43.4	56.6	0.167	0.2
0.50	58.1	49.5	52.8	0.142	0.6
0.75	61.2	51.3	60.8	0.135	1.1
1.00	65.8	53.1	57.2	0.131	1.6
1.50	62.4	54.1	57.9	0.128	2.9
2.00	61.3	53.6	53.6	0.127	3.9
P	*		**	**	**

Table 2
Gelling parameters of LMAP gel in dependence on the concentration of calcium. R = stoichiometric ratio of calcium ions, IST = initial structuring temperature, CST = critical structuring temperature, GP = gel point, $\tan\delta_{end}$ = loss factor as determined after the end of the gelation, SDR_a = average structure developing rate. *P* indicates significant differences: * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

R	IST (°C)	CST (°C)	GP (°C)	$\tan\delta_{end}$	SDR_a (Pa/min)
0.73	64.3	60.3	64.4	0.079	10.5
0.90	68.2	64.9	68.7	0.085	10.4
1.09	75.4	68.1	74.8	0.089	10.1
1.28	79.6	70.2	78.8	0.098	10.4
1.45	81.8	69.4	–	0.101	9.7
P	***	***	*	***	

of dissociated carboxyl groups in blocks calcium ions did not only form bridges, but rather bound to one pectin chain and induced an electrostatic repulsion by their positive charge. The latter inhibits the gel stabilization by hydrogen bonds between undissociated carboxyl as well as amide groups (cold set-gelation), which have a much smaller working range and could not crosslink due to the distance between neighbored charged parts of the pectin molecules.

The SDR_a was rather high and nearly constant, independent on R (Table 2). The loss factor ($tan\delta_{end}$) of the final LMAP gels was rather low in comparison to the corresponding standard LMP gel cooled at 1 K/min (Table 1). The SDR_a slightly, but significantly increased at higher R (Table 2). The corresponding gels were more solid-like, as it was also described by (Lootens et al., 2003), and showed an increased brittleness. It must be assumed that at the highest calcium concentration local micro-gels as described in Kastner et al. (2014), have been formed rapidly before the start of the measurement, because no clear gel point could be determined for these gels.

The presented results of the effect of calcium ions on the structuring process of LMAP as well as those of the previous publication concerning the effect on non-amidated LMP (Kastner et al., 2012b) generally confirm the crucial role of calcium ions in the gelling process of low-methoxylated pectin. Comparing the influence of increasing calcium content on the gelation of non-amidated LMP with amidated LMP of a similar DM, however, considerable differences were found. LMAP required less calcium for starting the structuring process. An increase in calcium ions accelerated structure formation only to a certain extent, because the number of free carboxyl groups with block-wise distribution was smaller. After saturation of these groups with calcium ions, additional calcium may cause repulsion of pectin chains and delay the initial gelling step. The formation of hydrogen bonds of the amide groups, however, considerably contributed to the cold-set gelation at lower temperature and altered the kinetics of the structuring process as well as the properties of the final gels. It has to be kept in mind that the comparison is influenced by the different stoichiometric ratio. However, also a direct comparison of previously reported data on LMP gels (Kastner et al., 2012b) and the LMAP gels of the present study with similar R in the range of 0.7–0.9 confirmed the differences described above.

3.3. *Effect of acid content (pH ≤ 4) on structure formation and gel properties for LMP and LMAP*

Variation of the concentration of acid in LMP and LMAP gels results in pH-values that varied around 3.5, the pK_a of pectin (Table 3). As outlined in 3.2, addition of 6.25 mM $CaCl_2$/kg gel meant a different stoichiometric ratio for the two pectin types. R amounted to 0.7 in the LMP gels and to 1.09 for the LMAP gels, respectively. Through variation of the pH it is possible to examine, how a reduction of dissociated carboxyl groups and pectin-calcium interactions on the one hand, and a possible increase of hydrogen bonds between undissociated carboxyl groups on the other hand affects gelation.

For LMP as well as LMAP, IST and CST decreased significantly with decreasing pH. A clear GP was only found at pH ≤ 3.1 (Table 3). With decreasing pH, the structuring temperatures decreased because of a reduction in the number of dissociated carboxyl groups with block-wise distribution. The latter are necessary for the rapid formation of ionic junction zones via calcium bridges at high temperature and decreased with increasing acid content. Thus, a reduced number of calcium bridges delayed the gelling process (Fig. 2a and b). Limited hydrophobic interactions (only about 30% of the carboxyl groups were methoxylated) were not sufficient for a quick effective gelation at temperatures > 50 °C. The structure formation at low pH is mainly due to the action of hydrogen bonds, but this process starts later at lower temperatures.

The different level and shape of the structuring velocity curves of the LMP and LMAP gels (Fig. 2) confirm the differences in gelation behavior of the two pectin types. For LMP, the structuring velocity decreased with decreasing pH. Obviously, the increasing number of hydrogen bonds between the non-dissociated carboxyl groups could not compensate the lack of calcium bridges. In case of LMAP, the steep increases of the curves (number 3 to 5) underline the supportive effect of the higher number of hydrogen bonds formed due to the presence of the amide groups. These groups considerably accelerated the gelling process and more than compensated the lack of ionic interactions at lower temperature.

Comparing the SDR_a of the LMP and LMAP gelation, the differences were even more evident (Table 3). For LMP, values were rather low with a maximum at the highest pH of 4.0. In contrast, in case of LMAP the SDR_a at pH 4.0 was at its minimum, but increased strongly down to pH 3.3 and formed a plateau at lower pH. Also the comparison of the final gel properties ($tan\delta_{end}$) of the LMP and LMAP showed that the samples behaved differently with decreasing pH (Table 3). LMP gels became more viscous (liquid-like) and the gel structure was less compact. In contrast, LMAP gels became more elastic (solid-like) and the gel structure was in general more compact, independent from the pH. These differences confirmed the impact of the decreasing number of calcium bridges and the positive effect of the hydrogen bonds as discussed above.

The presented results are in agreement with literature data. The pectin backbone, the polygalacturonate chain, may undergo a conformational transition with a shift in pH. By lowering the temperature at pH below the pK_a, a more compact three-fold helix can be formed by minimizing electrostatic repulsion (Gilsenan, Richardson, & Morris, 2000). Therefore, more carboxyl groups can act as donors in hydrogen bonds. Löfgren et al., (2006) also demonstrated that the pH affects the structuring process and gel properties of pectin. The microstructure of pectin gels at pH 3 and

Table 3
Gelling parameters of LMP and LMAP gels in dependence on the concentration of acid. IST = initial structuring temperature, CST = critical structuring temperature, GP = gel point, $tan\delta_{end}$ = loss factor as determined after the end of the gelation, SDR_a = average structure developing rate. *P* indicates significant differences: * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

Citric acid (mM/kg gel)	LMP						LMAP					
	pH	IST (°C)	CST (°C)	GP (°C)	$tan\delta_{end}$	SDR_a (Pa/min)	pH	IST (°C)	CST (°C)	GP (°C)	$tan\delta_{end}$	SDR_a (Pa/min)
4.7	4.0	74.3	53.5	–	0.085	3.9	4.0	89.6	72.4	–	0.105	5.0
11.0	3.6	72.0	53.8	–	0.097	3.7	3.5	88.2	73.7	–	0.107	6.5
17.3	3.3	72.7	57.6	–	0.112	3.0	3.3	78.1	68.0	–	0.089	10.9
23.6	3.1	65.8	53.1	57.2	0.131	1.6	3.1	75.4	68.1	74.8	0.089	10.7
29.8	3.0	52.8	42.4	47.5	0.153	1.6	3.0	70.0	58.9	67.7	0.088	10.8
36.1	2.9	51.8	40.0	46.8	0.168	1.4	–	–	–	–	–	–
P		**	*		**	*		**	**		*	*

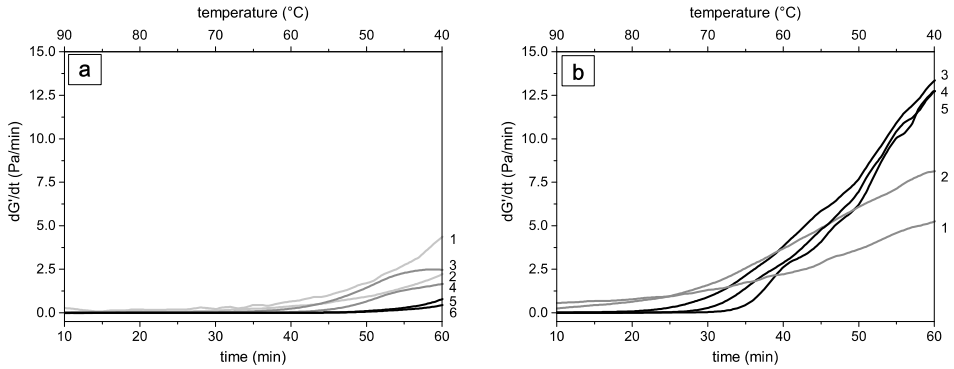

Fig. 2. Influence of the acid concentration of (a) LMP gels (R = 0.70) and (b) LMAP gels (R = 1.09) on the structuring velocity (dG'/dt); 1: pH 4.0, 2: pH 3.6 (LMP) or 3.5 (LMAP), 3: pH 3.3, 4: pH 3.1, 5: pH 3.0, 6: pH 2.9.

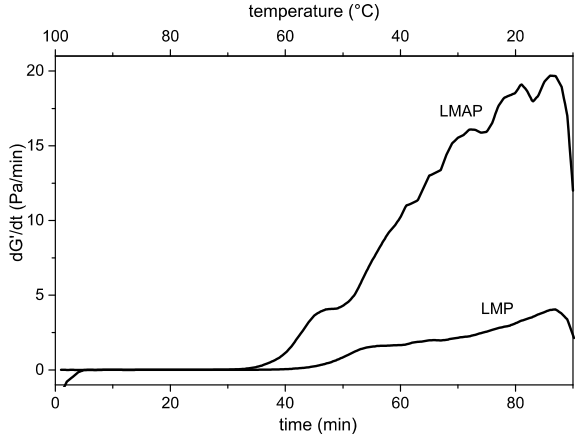

Fig. 3. Comparison of the structuring velocity curves (dG'/dt) of two LMP and LMAP gels with similar R (0.70, 0.73, respectively) under the same conditions.

30% sucrose was open and highly aggregated, compared to gels formed at pH 7 with a denser and more entangled structure. The authors stated that this difference resulted from a lower charge density and more compact conformational structure of the pectin molecules at pH 3. For amidated pectin they reported, that the structure formation at pH 3 was the highest of all tested gels and that the amide groups additionally strengthened the gel network by forming more hydrogen bonds.

Comparing the influence of pH on the gelation of LMP and LMAP with that on HMP (Kastner et al., 2014), a general decrease of the structuring temperatures with decreasing pH was found for all pectins. The effect was, however, much stronger for the two LMP samples. Once more, the gelling mechanisms were crucial for the understanding. Whereas in case of HMP a decreasing pH led to an increase in non-dissociated carboxyl groups and thus increased the chance for the formation of hydrogen bonds in the cold-set gelation, in case of LMP (and partly also the LMAP) it reduced the number of potential binding sites for calcium ions and, thus, inhibited the ionotropic gelation. Comparing the final gel properties ($tan\delta_{end}$), the effect of pH was strongest for LMP (strong increase) and lowest for LMAP (weak decrease). Obviously, the gelation of the LMAP was more affected by the formation of hydrogen bonds including the amide group, which seemed to be less pH dependent than those of the carboxyl groups.

3.4. Direct comparison of the structuring process of a LMP and a LMAP gelation

Finally, a direct comparison of the structuring processes of a non-amidated and an amidated LMP, prepared with comparable calcium content (0.7 for the LMP and 0.73 for the LMAP) and under identical conditions of pH and cooling rate, might illustrate the principal differences between the structure formations of the two pectin types (Fig. 3, Table 4).

The structure formation velocities of non-amidated and amidated pectin differed significantly. The initial gelling process of LMP and LMAP was similar (comparable IST) but the real structure formation of the LMAP gel, as characterized by the GP temperature and the CST, started significantly earlier than that of LMP. Also the SDR_a and the final gel structure ($tan\delta_{end}$) differed considerably. The SDR_a of the LMAP was more than five times as high as that of the LMP, the LMP gel was much more viscous ('soft') and the LMAP gels much more elastic (brittle).

Thus, the direct comparison of the two gels clearly confirms the differences in the gelling mechanisms of non-amidated and amidated pectins as discussed above and, especially, the role of the amide group hydrogen bonds on the gelation of amidated LMP.

Table 4
Comparison of LMP and LMAP gels with similar R. DM = degree of methoxylation, GC = galacturonan content, DA = degree of amidation, R = stoichiometric ratio of calcium ions, IST = initial structuring temperature, CST = critical structuring temperature, GP = gel point, $tan\delta_{end}$ = loss factor as determined after the end of the gelation, SDR_a = average structure developing rate. *P* indicates significant differences between LMP and LMAP: * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

	DM (%)	GC (%)	DA (%)	R	IST (°C)	CST (°C)	GP (°C)	$tan\delta_{end}$	SDR_a (Pa/min)
LMP	30	82	–	0.70	65.8	53.1	57.2	0.131	1.6
LMAP	32	68	19	0.73	65.8	60.3	64.4	0.079	10.5

4. Conclusions

The general aim of our work on structure formation of pectin gels was a detailed investigation of the influence of different parameters on the gelation kinetic of high-methoxylated, low-methoxylated and amidated pectins and on the different types of junction zones between pectin chains (hydrophobic interactions, hydrogen bonds and calcium-bridges).

As expected, the impact of the cooling conditions on LMP gels differed considerably from that on HMP gels. The formation of calcium bridges, which have a low sensitivity to temperature, reduced the effect of the cooling rate on LMP gelation. A delay of structure formation upon decrease of the pH was found for all types of pectin. In case of HMP (Kastner et al., 2014) it led to an increase in non-dissociated carboxyl groups and, thus, increased the chance for the formation of hydrogen bonds. For LMP and partly also LMAP, a decrease of pH reduced the number of potential binding sites for calcium ions and, thus, inhibited the gelation. Moreover, the gelation of LMAP was considerably influenced by the formation of hydrogen bonds including the amide group, which seemed to be less dependent on pH than hydrogen bonds between the carboxyl groups. Comparing a non-amidated and an amidated LMP, differences in the structure formation were also found with respect to the calcium concentration. On the one hand, fast saturation of the low number of dissociated free carboxyl groups in LMAP with calcium ions may result in a reduced number of ionic interactions. On the other hand, the formation of hydrogen bonds involving the amide groups in LMAP may considerably promote the structuring process. A LMAP gel seemed to be more 'stable' than a non-amidated LMP gel prepared at the same calcium ratio R.

In summary, the results presented confirm the assumptions on the differences in HMP, LMP and LMAP gelation and proof the principal differences in the structure formation of pectin with varying DM in dependence on the gelling conditions. This knowledge is essential because pectin is prepared from biological raw materials with varying structure and, thus, properties. Pectin suppliers adjust their products to specific requirements by adapting the method of extraction and subsequent modification. The type of demethoxylation of the commercial LMP applied in the presented work was not defined in detail. We assume, however, that also the type of the pectin demethoxylation (acidic, enzymatic) may have a certain effect on the gelling process of pectin. Therefore, the impact of different pectin demethoxylation methods on the structuring process will be the subject of our further work.

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(A6) Influence of enzymatic and acidic demethoxylation on structure formation in sugar containing citrus pectin gels

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Influence of enzymatic and acidic demethoxylation on structure formation in sugar containing citrus pectin gels

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ABSTRACT

Aim of the present study was to investigate the impact of different demethoxylation methods and the co-occuring side effects on the molecular properties and structure formation in pectin gels.

A high-methoxylated citrus pectin (HMP) was demethoxylated using either hydrochloric acid or pectin methylesterases of plant (pPME) or fungal (fPME) origin. pPME treatment causes a more block-wise distribution of free carboxyl groups, fPME or acidic treatment a random distribution. Twelve pectin samples with four different degrees of methoxylation (DM) between 62% and 41% were prepared. The gelation process was studied by oscillatory measurements.

In pectin samples from pPME treatment structure formation started at higher temperature and the final gels were more elastic in comparison to pectin from the two other modifications. The impact of the block-wise distribution of the free carboxyl groups became more evident with decreasing DM. The gelling process of pectin samples with random distribution was similar independent of DM.Side effects of all demethoxylation reactions were an altered sodium ion content (high in enzymatically treated pectin, close to zero in acidic treated) and a decrease of the molecular weight with increasing degree of demethoxylation. These side effects additionally altered the gelation process and the final gel properties in different ways.

1. Introduction

Pectin has a broad range of application in the food industry as thickener, gelling agent and stabilizer due to the ability of network formation under various environmental conditions. Pectin is extracted commercially from different botanical sources using acids (May, 1990; Rolin, 2002). Raw material as well as extraction conditions determine the pectin quality. After extraction, commercial pectin is often amidated by alkaline or demethoxylated by enzymatic procedures. Differences in the processing conditions alter the chemical characteristic of the pectin (see “Theoretical background” below) and, thus, affect the structure formation of their gels. Demethoxylation is one of the main modification processes. It is performed by chemical or enzymatic methods, which have different side effects. For instance, acidic treatment removes neutral sugar side chains as well as sodium ions from the pectin molecule, whereas enzymatic modification using pectin methylesterase does not affect neutral sugars but adds sodium ions by using NaOH to keep pH constant. These ions bind strongly to the newly formed free carboxyl groups (Einhorn-Stoll, Kastner, Urbisch, Kroh, & Drusch, 2019) and influence some pectin properties.

Pectin gelation has been widely investigated (i.e. Christiaens, Van Buggenhout, et al., 2016; Lopes da Silva & Rao, 2007; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995; Yapo & Gnakri, 2015). It is challenging to compare the results of the studies due to differing botanical origin and varying content of the pectin, model gel composition, gel cooling conditions or measured structuring parameters. Some authors prepared their pectin samples under defined conditions, whereas others used commercial pectin without knowing or considering the production conditions. The impact of preparation and modification conditions on pectin gels in a sugar-acid environment has not been investigated in detail up to now. Previous studies of our group (Kastner, Einhorn-Stoll, & Drusch, 2017; Kastner, Einhorn-Stoll, & Senge, 2012a,b; Kastner et al., 2014) have shown that citrus pectin samples with similar chemical characteristics, such as degree of methoxylation (DM) and molecular weight, differed in their gelling process and final gel properties. This was found for both HMP and LMP gels. The results were, however, inconsistent, what was attributed to the different origins and unknown processing conditions of the tested commercial pectin samples.

The presented study is the first systematic investigation of the impact of acidic and enzymatic demethoxylation methods on the structuring process of the modified pectin samples in high-sugar gel systems. Groups of pectin samples with similar degree of methoxylation but

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varying distribution of free carboxyl groups were prepared from a commercial citrus pectin under defined conditions. The structuring process and the final gels were studied at different levels of DM by small deformation oscillatory rheological tests. It was expected that the varying pattern of free carboxyl groups, resulting from the different methods, would have the dominating impact on the pectin gelation. Moreover, an additional influence of side effects of the demethoxylation process, in particular depolymerisation and increase or decrease of the content of sodium ions, on pectin gelation was assumed and examined in detail.

1.1. Theoretical background

Pectin is a complex polysaccharide and major component of the cell-wall of plants. Depending on botanical origin, development stage of the plant tissue as well as extraction and modification methods, the chemical characteristic of pectin differs strongly. The pectin polymer contains a high share of α-(1,4)-linked D-galacturonic acid residues (GalA). They form linear homogalacturonan sections and, together with rhamnose, branched rhamno-galacturonan sections (Voragen, Coenen, Verhoef, & Schols, 2009). Moreover, the GalA residues at C-6 position can be methylesterified or amidated. At C-2 or C-3 position, the GalA may be acetylated as e.g. in sugar beet pectin (Ridley, O'Neill, & Mohnen, 2001; Willats et al., 2001).

Depending on the degree of methoxylation (DM), pectin is classified as high-methoxyl pectin (HMP, 50% or above of carboxyl groups esterified) and low-methoxyl pectin (LMP, less than 50% of carboxyl groups esterified) (Lopes da Silva & Rao, 2007). The non-esterified (free) carboxyl groups are distributed along the pectin backbone either randomly or block-wise. The degree of blockiness (DB) describes this distribution pattern. It indicates the ratio of non-esterified mono-, di- and trimers of GalA, cleaved by endopolygalacturonase, to the total amount of non-esterified GalA. Daas, Arisz, Schols, De Ruiter and oragen (1998) introduced the DB, and the impact on pectin gelation was investigated and discussed by several researchers (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Limberg et al., 2000b; Ngouémazong, Tengweh, et al., 2012). The determination of the DB is a complex procedure. Therefore, Glahn and Rolin (1996) suggested the screening method of testing the calcium sensitivity (CS), providing information on calcium reactivity of pectin and, indirectly, on the pattern of free carboxyl groups. Dissolved pectin with a mainly block-wise pattern has a higher ability to interact via calcium bridges, therefore the viscosity of this solution is higher than that of pectin with a random pattern.

Pectin with a defined DM and pattern of free carboxyl groups along the backbone is designed by modification procedures using chemical treatment or pectin methylesterase (PME). Depending on the origin of the PME, different patterns of free carboxyl groups are achieved, a more block-wise pattern with PME of plant origin, or a more random pattern using PME of fungal origin (Fraeye, Colle, et al., 2010; Limberg et al., 2000a; Ralet, Crépeau, Buchholt, & Thibault, 2003). Any acidic or alkaline chemical demethoxylation of pectin initially results in a random distribution of the free carboxyl groups (Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010; Thibault & Ralet, 2003). With decreasing DM, however, also chemically modified pectins form blocks of free carboxyl groups. These blocks are necessary for the formation of stable egg-box junction zones with calcium, requiring at least blocks of eight to fourteen consecutive free carboxyl groups (Powell, Morris, Gidley, & Rees, 1982; Liners, Thibault, & Cutsem, 1992; Luzio & Cameron, 2008), would not be possible in these LMP.

DM and pattern of free carboxyl groups strongly determine the functional properties of pectin, and even small differences in the molecular structure of modified pectin has a strong effect. The most important property of pectin, the ability to form gels, is additionally influenced by environmental factors such as added ions (especially calcium), pH (degree of dissociation of the free carboxyl groups) and added sugars. The general impact of these factors on pectin gelation mechanisms is described in several references (Burey, Bhandari, Howes, & Gidley, 2008; Fraeye et al., 2010; Jensen, Rolin, & Ipsen, 2010; Lopes da Silva, Gonçalves, & Rao, 1995; Thakur, Singh, Handa, & Rao, 1997), as follows:

- Pectin with high DM and low DB (mainly random distribution of the free carboxyl groups) forms gels via junction zones by hydrophobic interactions and hydrogen bonds (cold-set gelation) in an environment containing sugar and acid at pH < 3.5 (pK_a of pectin).
- Pectin with low DM forms additional bonds via calcium bridges by ionotropic gelation due to the partly block-wise distribution of the free carboxyl groups. This mechanism requires none or less sugar and acid and is possible also at pH > 3.5.
- Pectin with high DM and high DB (partly block-wise distribution of the free carboxyl groups) is to a certain extent able to undergo ionotropic gelation, too. It forms gels at pH > 3.5 and is applied in acidified dairy drinks with pH up to 4.5.

Detailed investigations of the impact of the pattern of free carboxyl groups on pectin gelation gave varying results. For instance, Rolin and Vries (1990) showed that HMP with a block-wise distribution started to gel at higher temperatures than HMP with a more random distribution. Löfgren, Guillotin, Evenbratt, Schols, and Hermansson (2005) obtained the shortest gelation time for pectin samples with high DB without adding calcium ions. They also reported, that two gels containing 60% sucrose and pectin with varying pattern of free carboxyl groups had completely different properties when calcium was added, and that the pH was changed. Ström et al. (2007) showed, that pectin gel properties did not necessarily correlate with DB. Ngouémazong, Jolie, et al. (2012) investigated the stiffness of pectin gels and concluded, that during ionotropic gelation junction zones of pectin with rather random pattern might be shorter but their number was higher, compared to a pectin with more block-wise pattern at same DM.

In summary it becomes evident, that the impact of DM and pattern of free carboxyl groups on pectin gelation and gel properties has been widely examined, but the results are not consistent and only partly comparable.

2. Materials and methods

2.1. Pectin samples

High methoxylated citrus pectin (not standardized) with a DM of 68% (named as OP68) was obtained from CP Kelco (Lille Skensved, Denmark). It was modified enzymatically by fungal PME (f-pectin) and plant PME (p-pectin), as well as chemically by acidic de-esterification (a-pectin). Each modification type was used to prepare four levels of demethoxylation (approximately 62%, 57%, 50% and 41% DM), resulting in 12 different pectin samples. The physio-chemical properties of the 57% and 41% DM pectin samples were characterized already in a previous study (Einhorn-Stoll, Kastner, Hecht, Zimathies, & Drusch, 2015). All chemicals used were of analytical grade.

2.2. Enzymes

The two enzymes used in the study were fungal PME (fPME, Fructozym Flot from *Aspergillus niger*, Erbslöh, Geisenheim, Germany) and plant PME (pPME) from orange peel, prepared in the laboratory according to the methods of Arbaisah, Asbi, Junainah, and Jamilah (1997) and Kim, Teng, and Wicker (2005). Oranges were purchased from the local supermarket.

2.3. Demethoxylation of the pectin

The procedure for enzymatic demethoxylation was based on the

methods of Williams, Foster, and Schols (2003) and Limberg et al. (2000a), and was performed by the pH-stat-method using a Titrando with 50-mL dosing unit (902 Titrando and 800 Dosino; Deutsche METROHM GmbH & Co. KG, Filderstadt, Germany). The pH of the pectin solution (1 wt %) was kept constant with 0.25 M NaOH at the optimum pH of the PME (7.4 for pPME and 4.4 for fPME, respectively). To stop the process at the intended DM and to inactivate the enzyme, the pH of the solution was decreased to 3.2 and the solution was then heated at 90 °C for 10 min.

Chemical demethoxylation was performed by acidic modification at room temperature. The OP68 (1 wt%) was dissolved in 0.5 M or 2 M hydrochloric acid solution for HMP and LMP samples, respectively, as described by Einhorn-Stoll, Glasenapp, and Kunzek (1996). After demethoxylation, all pectin samples were precipitated by adding the fourfold volume of 95 vol% ethanol. The precipitate was washed at least five times with 95 vol% ethanol, coarsely ground, dried at 50 °C for at least 3 h and milled (ZM1 with 250 µm sieve, Retsch, Haan, Germany). The samples were stored at -10 °C until further application.

2.4. Analytical characterization of model pectin samples

Degree of methoxylation and galacturonic acid content (GC) were analyzed photometrically. The chromotropic method of Bäuerle, Otterbach, Gierschner and Baumann (1977) was used for DM and the hydroxydiphenyl method of Blumenkrantz and Asboe-Hansen (1973) for the determination of the free uronic acids.

The intrinsic viscosity ([η]) of pectin solutions was analyzed using a LOVIS rolling ball viscometer (LOVIS 2000M; Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with a 1.59 mm capillary and a steel ball (d = 1.5 mm). Pectin samples were solubilized for 24 h in sodium oxalate buffer (0.15 M sodium chloride and 0.005 M sodium oxalate, pH was adjusted to 6.0 using 0.1 M sodium chloride) at concentrations of 0.2, 0.1, 0.05, 0.025, 0.0125% (w/v). Pectin solutions and solvent were investigated at 20 °C and at an angle of 50°. The density of pectin solutions and solvent were measured using a density meter DMA38 (Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) at 20 °C. The relative (η_{rel}) and the specific (η_{sp}) viscosities were calculated from the following relations (Harding, 1997; Morris, Foster, & Harding, 2002):

$$\frac{\eta}{\eta_0} = \left(\frac{t}{t_0}\right)\left(\frac{\rho}{\rho_0}\right) = \eta_{rel} \quad (1)$$

where η is the viscosity, t is the efflux time and ρ is the density of the pectin solution as well as η_0 is the viscosity of the solvent, t_0 and ρ_0 are the corresponding efflux time and density, respectively. The specific viscosity is defined as:

$$\eta_{sp} = \eta_{rel} - 1 \quad (2)$$

Extrapolating the specific viscosity to zero concentration, the intrinsic viscosity was obtained (Chou & Kokini, 1987):

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} \quad (3)$$

where c is the concentration of the pectin solution. The [η] is related to the molecular weight by an empirical relationship, the Mark-Houwink equation (Houwink, 1940). The molecular weight (M) of pectin samples was determined by:

$$[\eta] = kM^{\alpha} \quad (4)$$

using values for the coefficients: α (0.79) and k (2.16×10^{-2}), which are suitable for pectin (Berth, Anger, & Linow, 1977; Schmelter, Wientjes, Vreeker, & Klaffke, 2002).

The molecular weight distribution of the pectin was determined by gel permeation chromatography (GPC) using the parameters and instruments as described by Wegener, Kaufmann and Kroh (2017). The calibration function was as follows: elution time 15 min ≈ 380 kDa, 17 min ≈ 100 kDa, 19 min ≈ 12 kDa and 21 min ≈ 0.3 kDa (Einhorn-Stoll et al., 2019).

The ash content was determined in a muffle furnace at 525 °C. The ash was used to measure the calcium content of the OP86 and sodium content of all pectin samples with a flame photometer (Jenway PFP7, Jenway, Staffordshire, USA) as described by Vetter and Kunzek (2003).

The calcium sensitivity of HMP was determined according to Glahn and Rolin (1996). The measurements were carried out using a Brookfield rotational viscometer, type DV-II + Pro LV (AMETEK GmbH - BU Brookfield, Lorch, Germany) at 20°C using the spindle V72 at 40 rpm. The calcium sensitivity was calculated for a pectin sample as the difference in viscosity between the sample with ($\eta_{Ca^{2+}}$) and without calcium (η_0):

$$CS = \eta_{Ca^{2+}} - \eta_0 \quad (5)$$

2.5. Rheological properties and structuring parameters

Gels were prepared in triplicate, using the HMP method (sugar-acid environment) for the pectin samples with 62% and 57% DM and the LMP method (sugar-calcium environment) for the samples with 50% and 41% DM. The method for HMP gels was described in detail in Kastner et al. (2014): 2.75 g pectin (0.27 wt%) was dissolved in 430 g demineralized water and 647.3 g sucrose was added. The total mass was reduced to 1020 g by boiling. Afterwards, 7 mL of 48.8% w/v tartaric acid solution was added. The final solutions had a pH of 2.2 and were within 64.5–65.5 wt% total solids.

The method for LMP gels was described before (Kastner et al., 2012a,b): 6 g pectin (0.67 wt%) and 264 g sucrose were dissolved in 637.5 g demineralized water, 7.5 mL 54.3% w/v citric acid solution, 15 mL 6% w/v sodium citrate solution and 37.5 mL 2.205% w/v $CaCl_2$ solution. The solution was reduced by boiling to a final mass of 900 g. The final solutions had a pH of 2.8 and a total solids content of approximately 32 wt%. The total solids contents for HMP and LMP gels were determined by an automatic refractometer (Schmidt and Haensch, Berlin, Germany).

The viscoelastic behavior of the samples during cooling was characterized by small deformation, oscillation measurements using a rheometer (Physica MCR 301, Anton Paar, Ostfildern, Germany) equipped with a profiled rotational cylinder (CC27/P1, diameter 26.66 mm, length 40.01 mm) and a Peltier cylinder temperature system (TEZ 150P). Samples were transferred into the pre-heated rheometer (105 °C) and cooled to 10 °C at a standard cooling rate of 1 K/min. The dynamic rheological parameters storage modulus (G′) and loss modulus (G″) were recorded during cooling at constant frequency of 1 Hz and strain of 10^{-3} as previously described (Kastner et al., 2012a,b; 2014). The final structure of the gels was characterized by the loss factor ($\tan\delta_{end}$) after cooling at 10 °C.

To describe the gelling process, the following structure parameters were calculated: the classical gel point (GP) was determined as the cross-over of G′ and G″ ($\tan\delta = G''/G' = 1$), and the initial structuring temperature (IST) and the critical structuring temperature (CST) were calculated from the structuring velocity curve (dG′/dt) using OriginPro9.1 software (OriginLab Corp., Northampton, USA). IST is the temperature at which the value dG′/dt differed from zero for the first time, and CST is the extrapolated temperature of the first strong increase of dG′/dt (Kastner et al., 2012a,b). The presented structuring velocity curves were the average of at least 3 measurements.

2.6. Statistical analysis

In order to determine the differences of the individual samples in relation to the DM, a one factorial ANOVA followed by a post hoc test (Tukey) was applied, after a normality test (Shapiro-Wilk). To analyze the effect of pectin modification on structuring temperatures and final

Table 1
Molecular parameters of reference sample and modified samples in dependence on the method of demethoxylation (pPME, fPME and acidic treated pectin samples). Degree of methoxylation (DM), galacturonic acid content (GC), intrinsic viscosity ([η]) with the fit of the curve, molecular weight (MW) and sodium ion content (Na^+).

	Sample	DM*(%)	GC (%)	[η]	MW	Na^+
				(cm^3/g)	(kDa)	(mg/g)
Reference	OP68	68.0^{a} ± 0.9	83.1 ± 1.5	538 ± 10	367.3	10.5 ± 0.0
pPME	P61	60.9^{b} ± 0.5	84.7 ± 1.3	528 ± 8	358.6	16.6 ± 0.5
	P57	56.8^{c} ± 0.6	86.5 ± 0.8	503 ± 5	337.3	21.4 ± 0.1
	P51	51.4^{d} ± 0.6	82.1 ± 0.4	320 ± 6	190.3	20.2 ± 0.0
	P40	40.4^{f} ± 0.6	84.9 ± 1.1	239 ± 8	131.5	25.1 ± 0.1
fPME	F62	61.9^{b} ± 0.3	88.5 ± 1.0	531 ± 9	361.2	11.0 ± 0.1
	F56	56.1^{c} ± 0.6	94.2 ± 2.0	530 ± 7	360.4	12.6 ± 0.1
	F49	49.0^{e} ± 0.2	82.3 ± 0.4	309 ± 9	182.0	20.8 ± 0.1
	F42	41.9^{f} ± 0.5	78.0 ± 0.1	298 ± 8	173.9	24.3 ± 0.4
Acidic	A62	62.0^{b} ± 0.1	90.0 ± 0.6	470 ± 6	309.5	0.7 ± 0.0
	A57	57.3^{c} ± 0.3	93.9 ± 1.2	453 ± 7	295.4	0.5 ± 0.0
	A50	$49.7^{d,e}$ ± 0.7	88.3 ± 0.4	421 ± 3	269.3	0.0 ± 0.0
	A42	41.5^{f} ± 0.7	81.7 ± 1.0	414 ± 4	263.6	0.1 ± 0.0

*Different letters denote significant differences between individual samples as found by one factorial ANOVA followed by post hoc test (Tukey).

gel properties, the non-parametric Kruskal-Wallis test was used. All tests were performed with OriginPro9.1 software (OriginLab Corp., Northampton, USA) at a significance level of 0.05 (95% confidence interval).

3. Results

3.1. Pectin characterization

Three types of modified citrus pectin, differing in the DM as well as in the distribution of free carboxyl groups along the backbone, have been produced by enzymatic and chemical treatments. The molecular parameters of all samples are shown in Table 1. Demethoxylation of commercial high-methoxylated citrus pectin (OP68, DM of 68%) by pPME (p-pectin), fPME (f-pectin) and acidic (a-pectin) treatment resulted in samples with an average DM of 62%, 57%, 50%, 41%, respectively. Therefore, the groups were named for HMP as DM62 and DM57, as well as for LMP as DM50 and DM41. The DM of the pectin samples within the single groups in general differed not significantly, despite of the samples of DM50: The DM of P51 and F49 differed significantly, but those of P51 and A50 as well as those of F49 and A50 were not significantly different (Table 1).

Results in Table 1 show, how the intrinsic viscosity decreased with increasing demethoxylation. The intrinsic viscosity at the lowest DM (DM41) was reduced to about 44% (239 cm^3/g) of the value of OP68 (538 cm^3/g) for p-pectin, to 55% (298 cm^3/g) for f-pectin and to 67% (414 cm^3/g) for a-pectin (Table 1). The intrinsic viscosity of enzymatically and acidic treated samples differed at most DM. At DM well above 50%, the a-pectin samples were more depolymerized (A57 = 453 cm^3/g) than the enzymatically treated (P57 = 503 cm^3/g and F56 = 530 cm^3/g). However, at DM around and below 50%, the enzymatically treated samples were more depolymerized (e.g. P51 = 320 cm^3/g and F49 = 309 cm^3/g) than the acid treated samples (e.g. A50 = 421 cm^3/g). These results of intrinsic viscosity are supported by GPC data (Fig. 1). The samples with lower DM (DM41) showed a broader molecular weight (MW) distribution (elution time 12–17 min) than samples with DM57 (elution time 12–16 min) around an MW of 380 kDa (elution time 15 min). With further demethoxylation, the main peak decreased, and a slight additional peak was formed with a MW of about 12 kDa (elution time 19 min) for the DM41 group.

Apart from demethoxylation and depolymerisation, all modifications altered the sodium content of the pectin samples: It became higher in the p-pectin (16.6–25.1 mg/g) and f-pectin samples (11.0–24.3 mg/g) than in OP68 (10.5 mg/g), but it was close to zero in the a-pectin samples (0.7–0.1 mg/g) (Table 1). Comparing the enzymatically treated samples, the pPME treated HMP samples (16.6 and 21.4 mg/g) had a higher sodium content than the fPME treated (11.0 and 12.6 mg/g), whereas for the LMP the sodium contents of the corresponding p- and f-pectin samples were similar (Table 1).

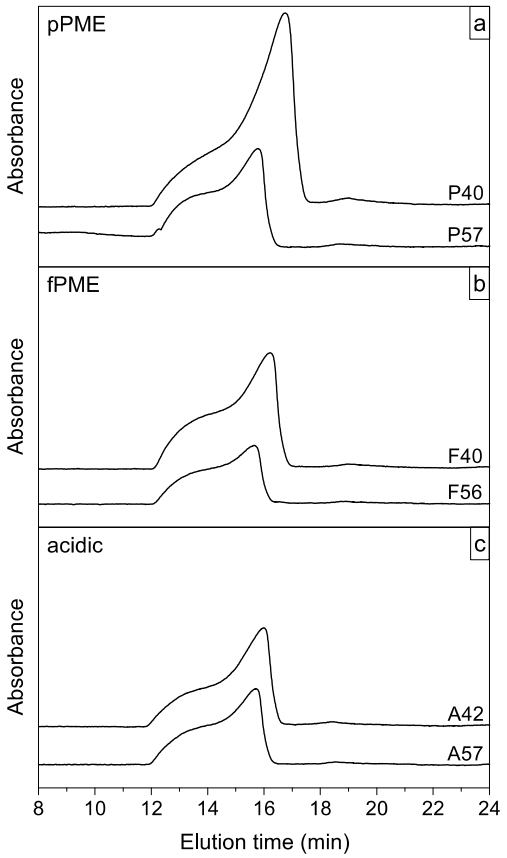

Fig. 1. GPC images of modified pectin samples, treated by (a) plant PME, (b) fungal PME or (c) acid. HMP group with average DM of 57% (P57, F56 and A57) as well as LMP group with average DM of 41% (P40, F40, A42).

The different modification methods determined the more block-wise

Table 2
Comparison of calcium sensitivity (CS) of high-methoxylated samples, treated by pPME (P), fPME (F) and with acid (A).

	Sample	CS (mPas)
DM62	P61	427.2
	F62	4.0
	A62	5.9
DM57	P57	509.0
	F56	4.0
	A57	5.2

or random pattern of free carboxyl groups of the HMP samples, as reflected by the calcium sensitivity (Table 2). The values of the p-pectin samples DM62 and DM57 were about 100x higher (427.2 and 509.0 mPas, respectively) than the corresponding values of f-pectin and a-pectin samples (4–6 mPas).

3.2. Rheological characterization of the pectin gelation

3.2.1. Structure formation and structuring temperatures

The structuring velocity (dG'/dt) of the HMP samples is shown in Fig. 2a. The start of the gelling process (IST) of these samples differed significantly. At DM62, the order of nearly all structuring temperatures (IST, CST and GP) was p-pectin > f-pectin > a-pectin samples (Fig. 3a). At DM57, the structuring temperatures for p-pectin were considerably higher than those of f-pectin and a-pectin, which had similar IST, CST and GP.

In general, the structuring velocity of all gels increased during cooling, and was higher for sugar-acid gels of HMP than for calcium gels of LMP (Fig. 2b). The structure formation of the DM50 samples started later and at lower temperature than that of the DM41 samples. The latter, however, showed a slower rate of increase in structuring velocity.

The structuring temperature of the calcium gels increased with decreasing DM (Fig. 3b). The calcium gels were cooled down to 5 °C, because up to 10 °C no GP of the LMP samples with a random free carboxyl group distribution were identified. Thus, the determined gel points were 8 °C for F49, 11 °C for F42, 6 °C for A50 as well as for A42. No sol-gel transition was detected for the p-pectin samples; at the beginning of rheological measurements, the gels underwent pre-gelation with elastic properties dominating over viscous properties (data not shown).

3.2.2. Gel properties after cooling

The viscoelastic properties of the pectin gels after cooling (determined at 10 °C) were characterized by the $\tan\delta_{end}$. In general, the higher the $\tan\delta_{end}$ value, the more the viscous properties dominate over the elastic gel properties. The $\tan\delta_{end}$ values were similar in the two HMP groups (Fig. 4a). The $\tan\delta_{end}$ value of the DM57 samples was slightly lower than that of the DM62 samples; this indicates that the gel structure of the DM57 samples is more elastic.

The gels of the LMP samples differed significantly at the end of the cooling process (Fig. 4b), with $\tan\delta_{end}$ values of p-pectin ≪ f-pectin < a-pectin at both DM41 and DM50. The DM41 values were slightly lower than those at DM50 (Fig. 4b). For the pPME treated LMP samples, $\tan\delta_{end}$ values were below 1, elastic properties dominated, and a gel structure was formed. Comparing the f- and a-pectin samples at 10 °C, the f-pectin had the lower $\tan\delta_{end}$ value, however, the $\tan\delta_{end}$ was above 1.0 for both, indicating dominant viscous properties. After additional cooling to 5 °C, the $\tan\delta_{end}$ decreased below 1.0, but the values for the f-pectin samples (0.826 for F49, 0.805 for F42) were still lower than those of the a-pectin samples (0.914 for A50 and 0.959 for A42).

4. Discussion

4.1. Comparison of molecular parameters of the demethoxylated pectin samples

The varying intrinsic viscosity and the change in the molecular weight of the modified pectin samples result from different effects. Acidic demethoxylation of pectin was accompanied by a certain depolymerisation. Depolymerisation by backbone hydrolysis as side reaction was found also by Diaz, Anthon, and Barrett (2007) as well as Fraeye et al. (2010). This effect was limited and increased slowly with decreasing DM, since the demethoxylation was performed at room temperature using moderate concentrations of HCl. The depolymerisation of the p-pectin samples probably was caused by side reactions of depolymerizing enzymes and by β-elimination. Commercial PME often contain small amounts of depolymerizing enzymes such as polygalacturonase (Benen, Vincken, & van Alebeek, 2002; Christiaens, Van Buggenhout et al., 2016). A possible side activity in the self-prepared (non-aseptic) PME was confirmed by electrophoresis patterns (not shown). For instance, bands at 65 kDa were detected, outside of the typical range for pPME between 25 and 54 kDa (Benen, Alebeek, Voragen, & Visser, 2002). Polygalacturonases vary in molecular mass between 30 and 75 kDa (Benen & Visser, 2002b) and pectate and pectin lyases between 22.8 and 82.3 kDa (Benen & Visser, 2002a), they might

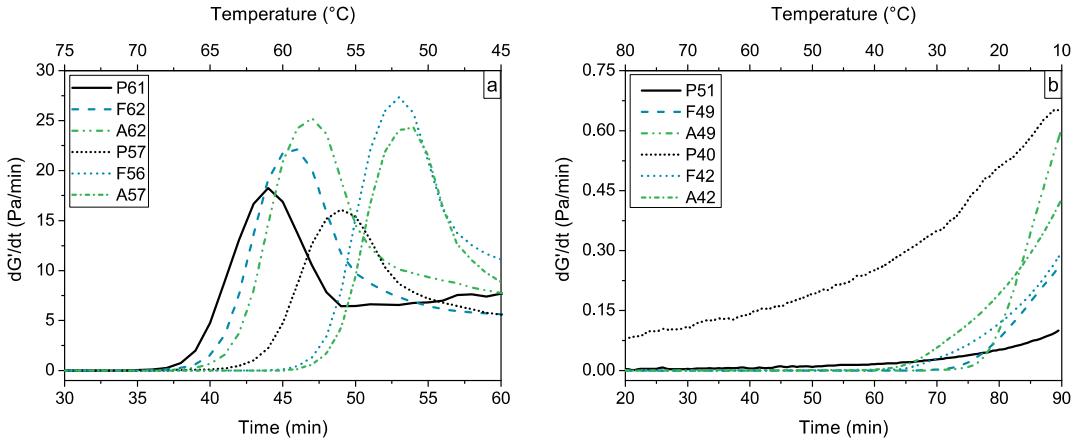

Fig. 2. Influence of pectin demethoxylation method on the structuring velocities (dG'/dt, mean curves) during cooling at 1 K/min. Sugar-acid gels of DM62 and DM57 samples (HMP) during 75 to 45 °C in (a) and sugar-calcium gels of DM50 and DM42 samples (LMP) during 80 to 10 °C in (b).

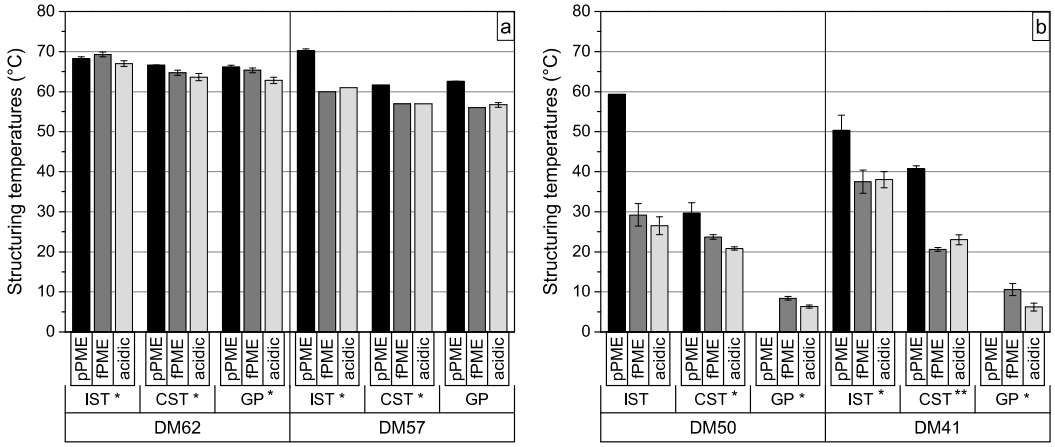

Fig. 3. Influence of pectin demethoxylation method on the structuring temperatures. Sugar-acid gels of DM62 and DM57 samples (HMP) in (a) as well as sugar-calcium gels of DM50 and DM42 samples (LMP) in (b). Structuring temperatures: IST = initial structuring temperature, CST = critical structuring temperature and GP = gel point. Significant differences: * for $P < 0.05$ and ** for $P < 0.01$.

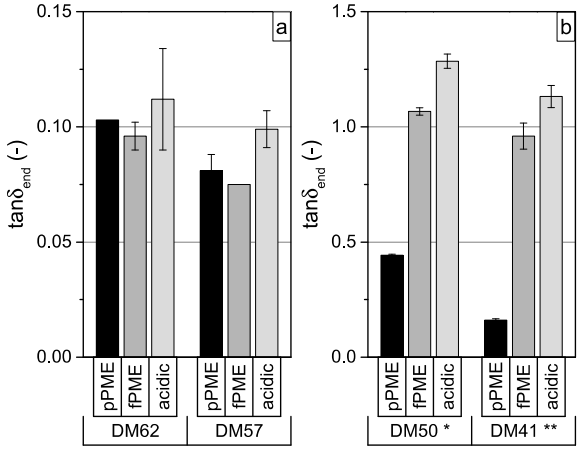

Fig. 4. Gel properties of pectin gels determined at the end of gelation (10 °C) in dependence on the demethoxylation method. Loss factor ($\tan\delta_{end}$) of sugar-acid gels of DM62 and DM57 samples (HMP) in (a) as well as loss factor of sugar-calcium gels of DM50 and DM42 samples (LMP) in (b). Significant differences: * for $P < 0.05$ and ** for $P < 0.01$.

have formed the bands at 65 kDa. In general, depolymerizing enzymes were not found in young developed sweet oranges except of the abscission zone of the fruit (Burns, Lewandowski, Nairn, & Brown, 2002), and should not be contained in the pPME extract. However, a fungal infection of orange fruits, for instance by *Botrytis cinerea* (Lionetti et al., 2017), was possible during transport and storage. *B. cinerea* genome indicates a large array of depolymerizing enzymes such as polygalacturonase or pectin lyase (Blanco-Ulate et al., 2014) and the enzymes might have been co-extracted from the fruits. In case of the p-pectin samples, beside the effect of depolymerizing enzymes also β-elimination was possible. This reaction is favored at higher temperatures and at a pH above 8, but was detected also at pH 6 (Diaz et al., 2007). Thus, β-elimination during the pPME-treatment at pH 7.4 and 30 °C was possible. This reaction, as well as enzymatic depolymerisation, increased with reaction time. As a result, the intrinsic viscosity and molecular weight of p-pectin of group DM41 (P40) was the lowest (Table 1) and the MW distribution the broadest (Fig. 1) of all modified pectin samples.

The differences in the sodium content resulted from the modification procedures. For the a-pectin samples, the sodium ions contained in the commercial pectin were widely removed during demethoxylation (Table 1). For the enzymatically demethoxylated pectin samples, the increased sodium content resulted from NaOH used to keep pH constant during the pH-stat method. The number of free carboxyl groups increased during demethoxylation, and some of these groups dissociated and bound sodium ions. Due to the higher pH (7.4 > 4.4), treatment with pPME required more NaOH than demethoxylation with fPME and resulted in a higher concentration of sodium ions in HMP samples (DM61 and DM57). The higher the start pH, the more NaOH was necessary to adjust the initial pH and to keep it constant during modification. However, the difference in the sodium content of the p-pectin and f-pectin samples vanished with decreasing DM, and the content in the LMP samples became similar. Though more sodium ions were added for preparing samples of p-pectin in comparison to f-pectin during the longer reactions, above a certain threshold the ions were in surplus and were removed during further processing. The residual ions were strongly bound, and their content was independent on the amount added before.

Calcium sensitivity, used in order to characterize the distribution of free carboxyl groups after demethoxylation (Dominiak et al., 2014; Glahn & Rolin, 1996; Limberg et al., 2000a), was high for the p-pectin samples and confirmed the desired block-wise distribution. Calcium sensitivity increased with decreasing DM due to increased block formation of free carboxyl groups with demethoxylation. The calcium sensitivity was low and nearly independent of DM for the HMP after fPME and acidic treatment. The values confirmed a random distribution of the free carboxyl groups for these samples.

4.2. Comparison of structure formation and gel properties

The method of gel preparation (sugar-acid gels for the HMP and calcium gels for the LMP) as well as the viscoelastic properties of the resulting gels differed, therefore the two types of gels will be discussed separately.

4.2.1. Gelation of sugar-acid gels

Generally, HMP form gels in the presence of sugar and acid (pH < 3.5). These conditions promote the structure formation by reducing the distance between pectin molecules. The low pH decreases the pectin dissociation and the electrostatic repulsion of the negatively charged carboxyl groups. Sodium ions might have a comparable effect,

they shield the dissociated carboxyl groups and reduce the electrostatic repulsion (Einhorn-Stoll et al., 2015; Ström & Goh, 2013; Ström, Schuster, & Goh, 2014). Initially, at a temperature > 50 °C, hydrophobic interactions between ester groups dominate. They are reduced during cooling, and are replaced by hydrogen bonds between carboxyl and hydroxyl groups (Oakenfull & Fenwick, 1977). These two interaction types should be independent of the number of free carboxyl group. However, HMP with a partly block-wise distribution of free carboxyl groups (i.e. pectin prepared by pPME) also undergoes ionotropic gelation immediately after the start of cooling (Christiaens, Van Buggenhout, et al., 2016; Fraeye, Colle, et al., 2010; Löfgren et al., 2005; Ngouémazong, Jolie, et al., 2012), and in these cases the DM becomes important.

In the presented investigation, the difference of the structuring temperature of the samples at DM62 was small (Figs. 2a and 3a). It was attributed to differences in free carboxyl group distribution (block-wise or random) and/or to the sodium ion content, which was in P61 > F62 > A62. Despite the high calcium sensitivity of p-pectin samples (Table 2), their more block-wise distribution had only a small effect on gelation. The free carboxyl groups in some blocks of the P61 possibly were shielded by sodium ions via counterion condensation (Celus et al., 2017; Irani, Owen, Mercadante, & Williams, 2017; Siew, Williams, & Young, 2005) and, as a result, they were insufficient for additional ionotropic gelation.

In the DM57 group, the structure formation of the p-pectin samples started at significantly higher temperatures than that of the pectin samples with a statistical distribution (Figs. 2a and 3a). The higher number and/or longer blocks of free carboxyl groups supported ionotropic gelation as well as the formation of longer regions of hydrogen bonding (Fraeye, Colle, et al., 2010; Luzio & Cameron, 2008; Ström et al., 2007; Willats et al., 2001). The necessary calcium ions for the supportive ionotropic gelation were contained in the OP86 (1.49 mg/g). The other possible explanation, the formation of longer hydrogen bonding regions in blocks of free carboxyl groups, is not reasonable at 60–70 °C when the gelation of p-pectin started.

For the two HMP with more randomly distributed free carboxyl groups (fPME and acidic treatment), the structuring temperatures differed slightly at DM62 and were similar at DM57 (Fig. 3a). However, a difference in the start of gelation (IST), corresponding to a difference in pectin sodium content (12 mg/g for f-pectin vs. 0 mg/g for a-pectin samples), was found at both DM. The sodium ions partly shielded the dissociated carboxyl groups, reduced repulsion between pectin chains and allowed an earlier structure formation for the f-pectin samples. Schmelter, Vreeker and Klaffke (2001) observed a similar effect during gelation. In addition, they found, that inactivated fungal PME had an influence on gelation by accelerating structure formation and strengthening the final gels compared to pectin without PME-protein.

The DM of the pectin samples had an impact on the viscoelastic character of the final gels after cooling to 10 °C (Fig. 4a). They were slightly stronger (more elastic than viscous) at lower DM. This is explained by the higher number of free carboxyl groups at lower DM, resulting in more hydrogen bonds. The influence of blocks of free carboxyl groups was insignificant for sugar-acid gels.

The viscoelastic properties of the DM62 samples differed visibly but not significantly. An order of a-pectin > p-pectin > f-pectin was detected for $\tan\delta_{end}$ and became more pronounced for the DM57 samples, however, the total differences were small. This was possibly an influence of different molecular weights, which were significantly lower for the a-pectin than for the p- and f-pectin (Table 1).

4.2.2. Gelation of sugar-calcium gels

The gelation mechanism of sugar-calcium gels differs from that of sugar-acid gels. The formation of hydrophobic interactions above 50 °C is reduced because there are fewer ester groups. Thus, gelation starts later but is accelerated during cooling by increased hydrogen bond formation below 50 °C. The additional ionotropic gelation via calcium bridges starts immediately with cooling and is stronger in LMP samples than in most HMP because of the higher number of subsequent free carboxyl groups (Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2006; Fraeye, Colle, et al., 2010; Lootens et al., 2003; Ngouémazong, Jolie, et al., 2012).

As expected for the presented work, the structure formation and the gel properties after cooling of the three pectin types differed strongly also for LMP gels in dependence on the pattern of free carboxyl groups. In the DM50 group, the gelling process of the p-pectin samples with a block-wise distribution began at about 60 °C. This was 30 K above the temperature of the according a- and f-pectin samples (Figs. 2b and 3b), since the block-wise distribution allowed additional rapid ionotropic gelation. Despite of the added calcium ions, ionotropic gelation was still limited in the a- and f-pectin samples at DM50, due to their still mostly randomly distributed free carboxyl groups. The course of the structure formation of the three pectin types also differed (Figs. 2b and 3b): The f- and a-pectin gels showed a steeper increase in gelation velocity below 30 °C, since hydrogen bond formation was dominating in this temperature region.

The structure formation of the DM41 group was delayed in comparison to the DM50 group for the p-pectin but accelerated for the other two samples, and the difference between the three types generally decreased. On the one hand, the rapid formation of hydrophobic interactions was further reduced by the decreasing DM. On the other hand, the total demethoxylation was so high, that also the f-pectin and a-pectin now were able to form longer blocks of free carboxyl groups and to undergo more ionotropic gelation.

Discussing the properties of cooled LMP gels, it has to be considered, that these measurements generally were made at 10 °C. Gel points were found for f- and a-pectin samples, however, only during cooling to 5 °C, and the p-pectin samples showed no gel point at all due to pre-gelation. Nevertheless, the final p-pectin gels at 10 °C were the most elastic of all tested samples (Fig. 4b). The higher length and number of junction zones, formed in blocks of free carboxyl groups of the pPME treated samples by calcium bridges, strongly affected their gel properties. The results agree with those of Fraeye et al. (2009), Löfgren et al. (2005), Ngouémazong, Tengweh, et al. (2012) and Rolin (2002). Comparing the properties of the f- and a-pectin gels, a-pectin samples formed a significantly more viscous and less elastic structure than f-pectin samples. However, since the gel points of these samples were mainly found below 10 °C, gelation was not completed at the final measurement temperature. After cooling to 5 °C (below the GP), the $\tan\delta_{end}$ values of the f-pectin gels again were lower than those of the a-pectin samples. The lower gel point temperatures for the a-pectin gels also explained the higher viscosity of these gels. The results confirm an influence of sodium ions and possibly also of inactivated fPME for LMP, as described by Schmelter et al. (2001). The f- and a-pectin samples varied, however, not only in their content of sodium ions and in the presence of inactivated fPME, they differed additionally in intrinsic viscosity and molecular weight. The influence of these parameters on pectin gelation in general is not sufficiently known and requires further investigation.

4.3. Final remarks

The results of the study are summarized as follows: (1) The block-wise or random distribution of free carboxyl groups affected the velocity of structure formation in sugar containing pectin gels. Samples demethoxylated by pPME contained longer blocks of free carboxyl groups, and their gelation started earlier than that of samples demethoxylated by fPME or acid with a more random distribution. The differences increased with decreasing DM, since more free carboxyl groups in the pPME samples formed longer blocks, which supported ionotropic gelation. The final HMP gels had a similar viscoelasticity that was mostly independent of the distribution of the free carboxyl groups and, thus, on the method of demethoxylation. In contrast, the LMP gels of the p-pectin samples were stronger and more elastic than

those of the f- and a-pectin samples, thus the demethoxylation method had an impact on the gel structure. (2) The treatments with fPME and acid both resulted in pectin with a random distribution of free carboxyl groups, but the pectin samples showed differences in sodium content and intrinsic viscosity as well as in the presence of inactivated PME. The gelation of f-pectin samples started earlier than that of a-pectin samples, since sodium ions reduced the electrostatic repulsion between pectin molecules in the f-pectin samples and accelerated the formation of intermolecular junction zones. The final gels of f-pectin samples were less viscous and more elastic than those of a-pectin samples.

As a result of the presented work, a general influence of the method of demethoxylation on pectin gelation and gel properties was found. It is based on variations in the pattern of free carboxyl groups and, additionally, on differences of the sodium ion content and the molecular weight.

5. Conclusions

The present work focused on the effect of type and extent of the demethoxylation of pectin on its gelation process and on the properties of the final gel. Pectin from different modification methods varied in the pattern of the free carboxyl groups as well as in the sodium ion content and the molecular weight, and these differences affected the pectin gelation.

As expected, the pattern of the free carboxyl groups was the dominating factor for the gelation kinetic at any DM. It was, however, less important for the final gel properties, in particular at high DM. Beside this main factor, also the side effects of the demethoxylation reactions, molecular weight reduction, sodium ion content and possibly also the content of inactivated enzymes, had an impact on the gelation kinetic as well as on all final gel properties. The resulting properties were crucial in particular for the different structure formation and gel properties of the two groups of pectin with statistical distribution of free carboxyl groups.

The presented results show in detail the impact of the pectin modification type on the gelation process and the final gel properties in a broad range of DM in sugar-acid as well as sugar-calcium gel systems. They should be considered, when choosing the optimum pectin for a special application.

The impact of molecular weight and sodium ions on pectin gelation at different DM is still not completely understood and requires further investigation. In particular, the independent effects of the individual factors are the subject of the ongoing work.

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List of abbreviations

a-/A	acidic treated sample
CS	calcium sensitivity
CST	critical structuring temperature
DB	degree of blockiness
dG′/dt	structuring velocity
DM	degree of methoxylation
f-/F	fPME treated sample
fPME	PME of fungal origin
G′	storage modulus
G″	loss modulus
GalA	galacturonic acid
GC	galacturonic acid content
GP	gel point temperature
GPC	gel permeation chromatography
HMP	high-methoxylated pectin
IST	initial structuring temperature
LMP	low-methoxylated pectin
MW	molecular weight
p-/P	pPME treated sample
PME	pectin methylesterase
pPME	PME of plant origin
OP	original pectin
$\tan\delta_{end}$	loss factor at end of measurement
[η]	intrinsic viscosity

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