Enzymatic modification and characterization of xanthan

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Enzymatic modification and characterization of xanthan

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ABSTRACT

In this thesis an enzymatic approach for the modification and characterization of xanthans was introduced. Complete backbone degradation of xanthan by cellulases was obtained independent on the molar composition of a xanthan sample. It was shown that only xanthan segments that occurred in a disordered xanthan conformation were susceptible to enzymatic backbone degradation. HILIC-ELSD-MS analysis revealed the presence of six different xanthan repeating units (RUs). All RUs consisted of the same pentasaccharide structure, with different acetyl and pyruvate substitution patterns. Interestingly the presence of an acetyl group at the *O*-6 position of the outer mannose unit was shown. Analysis of 5 xanthan samples showed that 5–19% of all acetyl groups present are positioned on the outer mannose. Furthermore, the relative abundance of the RUs present in xanthan samples can vary, even when their molar compositions are the same.

Analysis of the transitional behavior of xanthan based on the enzymatic release of the six types of RUs showed that the acetyl groups on the outer mannose, and not on the inner mannose, as was previously reported, are responsible for the stabilization of xanthans conformation. It was proposed that acetylation of the outer mannose also determines the functional properties of a xanthan solution. Furthermore, it was postulated that 1) The RUs that are either acetylated on the outer mannose units or solely acetylated on the inner mannose units are block wise distributed over the xanthan molecule. 2) Pyruvylated RUs and unsubstituted RUs are randomly distributed.

Screening for xanthan modifying enzymes resulted in the discovery of the first two acetyl esterases being active towards xanthan. AXE3, a xylan acetyl esterase produced by *Myceliophthora thermophila C1*, showed to be specific for the removal of the acetyl groups at the inner mannose unit and was only active towards the disordered xanthan conformation. YesY, a pectin acetyl esterase produced by *Bacillus subtilis* strain 168, specifically removed the acetyl groups at the outer mannose units and its activity is not influenced by xanthan's conformation.

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Chapter 1

General introduction

PROJECT OUTLINE

Polysaccharides are commonly used in food and medical application due to their physico-chemical properties and/or biological functionalities. Polysaccharides can be extracted from plants and aquatic sources or produced through fungal or bacterial fermentation.[1] The properties of polysaccharides often depend on their chemical structure.[2] Altering the chemical structure could, therefore, result in the optimization of the biopolymers functionality. Industrial modifications of polysaccharide structures are mainly made chemically.[3] However, the use of enzymes instead of chemicals would reduce the environmental impact of the process and possibly the energy input.[3] Furthermore, the use of enzymes has the advantage that modifications may be highly specific, and could thus yield in the production of tailored polymers with novel properties. To date, however, there is a lack in suitable enzymes to enable the enzymatic modification and optimization of polysaccharides.

The research described in this thesis was carried out within the FP7 EU-project 'Novel **poly**saccharide **mod**ifying **e**nzymes to optimize the potential of hydrocolloids for food and medical applications' (PolyModE). The general aim of this EU-project was to develop new techniques for the analysis of the chemical structure of polysaccharides. Furthermore, the project aimed for the production and characterization of enzymes that specifically alter the polysaccharide structure in order to optimize both the production as well as the industrial application of these polysaccharides. Xanthan was one of the hydrocolloids studied within the EU-project and is the focus in the PhD research presented.

XANTHAN APPLICATIONS

Xanthan is a negatively charged exocellular heteropolysaccharide that is produced by *Xanthomonas spp.,*[4, 5] with a molecular mass ranging from 1–7*10^6 Dalton.[6, 7] Xanthan is produced by aerobic batch fermentation and is isolated through ethanol precipitation. Removal of the bacterial cells prior to precipitation results in a white end product.[5, 8]

When dispersed in aqueous solvents, xanthan exhibit a weak gel-like behavior,[4] resulting in highly viscous solutions. Upon shear the gel is disrupted, which results in a decrease in viscosity. The initial viscosity can be recovered easily by the removal of the shear and is rather stable over a wide range of ionic strength, pH and polymer concentration.[5, 9] As xanthan is soluble in both cold and hot water and due to its pseudoplastic behavior already obtained at low xanthan concentrations, xanthan is suitable for many industrial applications (Table 1.1.).[5, 10]

Table 1.1. Industrial application of xanthan

Industry	Function	Possible applications
Oil recovery	Reducer of water mobility	Improve pumpability
Personal care	Stabilizer	Lotions , tooth pastes
Food	Stabilizer and rheology modifier	
Dairy		Milkshakes, whipped cream; custards and puddings; yoghurts; water gels; ice creams; sorbets
Bakery		Pastry fillings; gluten free baking
Meat and fish		Canned food; spreads; frozen and ready-to-eat foods
Condiments		Mayonnaise; salad dressings; sauces; soups; jams
Beverages		Fruit juices; powdered beverages

As the functional properties of xanthan are stable over a wide temperature range,[10, 11] applications in food are mainly within the field of stabilization of the textural characteristics of products, which are exposed to either cold or heat. Furthermore, the enhanced gelling properties obtained through interactions of xanthan with galactomannans are used for: the preparation of gelled or thickened foods; the control of particle sedimentation in juices and drinks; and the prevention of crystal formation in frozen products such as ice creams.[5]

THE XANTHAN STRUCTURE

Xanthan is an extracellular polysaccharide and its structure is believed to be repetitive (Figure 1.1). It consists of a cellulose-like β-1,4-glucan backbone with an α-D-mannose-(2→1)-β-D-glucuronic acid-(4→1)-β-D-mannose side chain on the *O*-3 position of every second glucose unit.[12, 13] Approximately 90% of all inner mannose units is acetylated at the *O*-6 position and about 50% of the outer mannose units carry a 4,6 linked pyruvate ketal.[12, 14-16] The exact degree of substitution of xanthan samples is known to vary depending on the fermentation conditions[8, 17] and the *Xanthomonas* strain used for the xanthan production.[14, 15] The biosynthesis of xanthan in *Xanthomonas campestris* has been extensively studied.[18-23] A cluster of 12 '*gum*-genes' has been identified, which encodes for the enzymes involved in the biosynthesis, the polymerization and the secretion of xanthan. An overview of the metabolic pathway is given in Figure 1.2. The pentasaccharide repeating unit is assembled by sequential addition of individual monosaccharide residues. Each monosaccharide addition is catalyzed by a specific glycosyltransferase (GT).

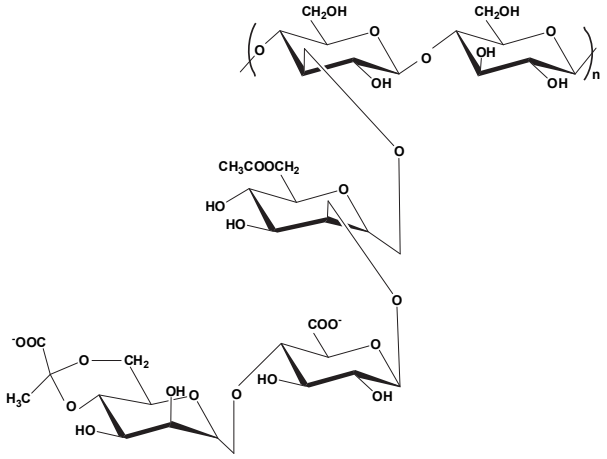

Figure 1.1. The xanthan repeating unit as identified by Jansson et al.[12]

The mannose residues of the repeating unit are substituted by distinct enzymes: Acetyltransferase-I (AT-I) catalyzes the acetylation of the inner mannose and ketal pyruvate transferase (KPT) catalyzes the pyruvylation of the outer mannose. Unexpectedly, a gene was found that encodes for a third decoration enzyme, acetyltransferase-II (AT-II) that catalyzes the acetylation of the outer mannose. Finally, the synthesized substituted repeating unit is added to the growing polymer, which is subsequently secreted.

The presence of a '*gum*-gene' that encodes for the production of an enzyme that is involved in the acetylation of the outer mannose unit indicates that variations in the xanthan structure as proposed by Jansson et al.[12] are likely to occur. Such variations could be controlled by specific mutations in the *Xanthomonas* genome, as there is one specific '*gum*-gene' for the expression of each enzyme involved in the biosynthesis.[18, 20, 23] Several mutant strains have been produced in order to control the biosynthesis of xanthan.[23] Based on the molecular composition of the xanthans obtained, the xanthans were hypothesized to vary in the length and substitution pattern of their side chains. However, detailed structural analysis of these xanthans to confirm the hypothesized structures has never been conducted. Therefore, the exact modifications induced by the mutations are uncertain. Moreover, genetic modifications may go hand in hand with unintentional effects, as the modification at one level can cause unfavorable effects at other parts of the biosynthesis as well. This may results in e.g. an altered molecular mass and changes on molecular level.[24] Controlled modification of the xanthan structure using mutant strains is, therefore, not fully possible.

The biosynthesis of xanthan can also be influenced by changing the fermentation conditions and thereby stimulate or suppress certain enzymes involved in the biosyn-

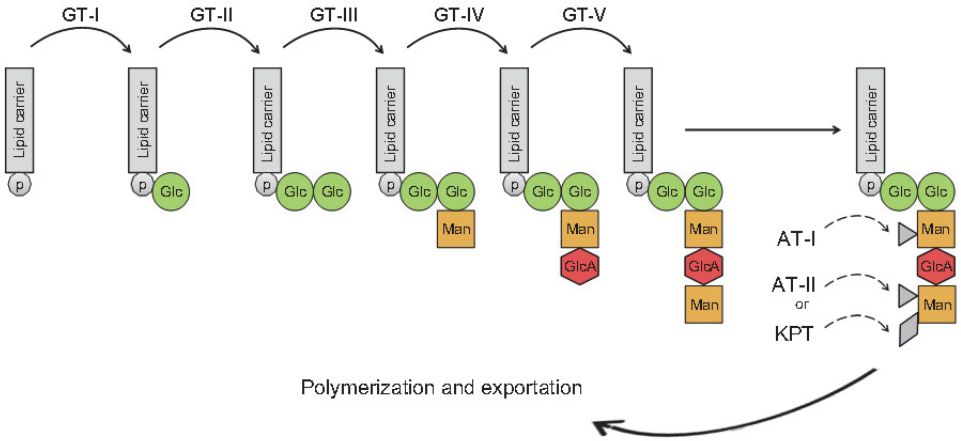

Figure 1.2. The biosynthesis of xanthan in *Xanthomonas campestris* and the enzymes involved as proposed by Katzen et al.[18] p = lipid; GT = glucosyltransferase; AT = acetyltransferase; KPT = ketal pyruvate transferase; Glc = glucose; Man = mannose; GlcA = glucuronic acid; triangle = acetyl groups; diamond: pyruvate group. Dashed arrows indicate that the substitution level may vary.

thesis. A deficiency of oxygen during the fermentation is known to decrease the pyruvate content.[17, 25] In contrast, a deficiency of nitrogen during the fermentation increases the pyruvate content.[8] Furthermore, it is known that the presence or absence of citric acid and magnesium in the growth media respectively increase and decrease the pyruvate levels in the xanthan produced.[26-28] The effect of fermentation conditions on the length of the xanthan side chain or on the level of acetylation have not been studied.

Studies on the xanthan biosynthesis clearly show that variations in the xanthan structure can occur and that not only the *level* of acetylation and pyruvylation, but also the *position* of these substituents can vary based on the production conditions. Apart from the methylation analysis performed by Jansson et al.[12] only one other study focused on the actual analytical characterization of the xanthan structure. In this study the structure was described using glycosidic linkage analysis.[29] and it was confirmed that the outer mannose can be acetylated on the *O-6* position. The ratio between the acetyl groups on the inner and outer mannose units was also determined for the entire polymer. However, structural analysis on the repeating unit (RU) level and the exact distribution of different xanthan side chains over the xanthan backbone has not been studied yet.

CONFORMATION AND PROPERTIES OF XANTHAN IN SOLUTION

Due to the polyelectrolyte nature of xanthan molecules, xanthan is very soluble in both cold and hot water. Xanthan solutions exhibit a pseudoplastic, shear thinning behavior, which depends on temperature, salt concentration, pH and xanthan concentration.[30-32] This unique solution behavior is associated with the conformational behavior of xanthan in solution (Figure 1.3.).
Native xanthan, as produced by *Xanthomonas campestris,* exists in a double-stranded helical conformation with an order-disorder transition upon changes in temperature and/or ionic strength.[33-35] The 'renaturing of xanthan', a process induced by cooling or by an increase in salt concentration, results in the recovering of an ordered conformation. Renatured xanthan, however, does not exhibit exactly the same conformation and rheological properties as the native xanthan.[7, 35-38] The order-disorder transition of the native xanthan can thus be considered as non-reversible. Renatured xanthan also exists in a helical conformation and has an order-disorder transition, that is, in contrast to native xanthan, reversible.[30, 31, 39] A wide variety of physical and physicochemical techniques have been used to study the exact conformation of renatured xanthan. However, the nature of this conformation remains controversial and several models have been proposed (Figure 1.3c.). Some studies proposed a double or multiple stranded double helical structure.[40-42] Other studies showed evidence for a single stranded double helix, in which intramolecular interactions results in the formation of hairpin loops.[7, 32, 43, 44] Morris and co-workers[31] argued that renatured xanthan has a single stranded helix that is stabilized by the alignment of side chains along the backbone, as was confirmed by others.[32, 39]
Although the unique solution properties of xanthans are believed to be strongly related to the order-disorder transition, this transition alone is not sufficient to explain the total rheological behavior of a xanthan solution.[9, 45]

Two models have been proposed to explain the weak gel-like behavior of xanthan. Both models propose the formation of a network of xanthan molecules, which is easily disrupted upon shear, causing xanthans pseudoplastic behavior.[35, 45] The nature of the intermolecular interactions involved in the network formation, however, is uncertain. The model proposed by Ross-Murphy[45] believes that a network is formed through the side-by-side alignment of ordered xanthan molecules. This network is stabilized through non-covalent interactions (Figure 1.3h.). Considering that a xanthan helix can partly dissociate at multiple positions within a helix (Figure 1.3f.), variations in this model are proposed in which the ordered parts of one xanthan molecule align with ordered parts of multiple other xanthan molecules (Figure 1.3g.).[32, 46]

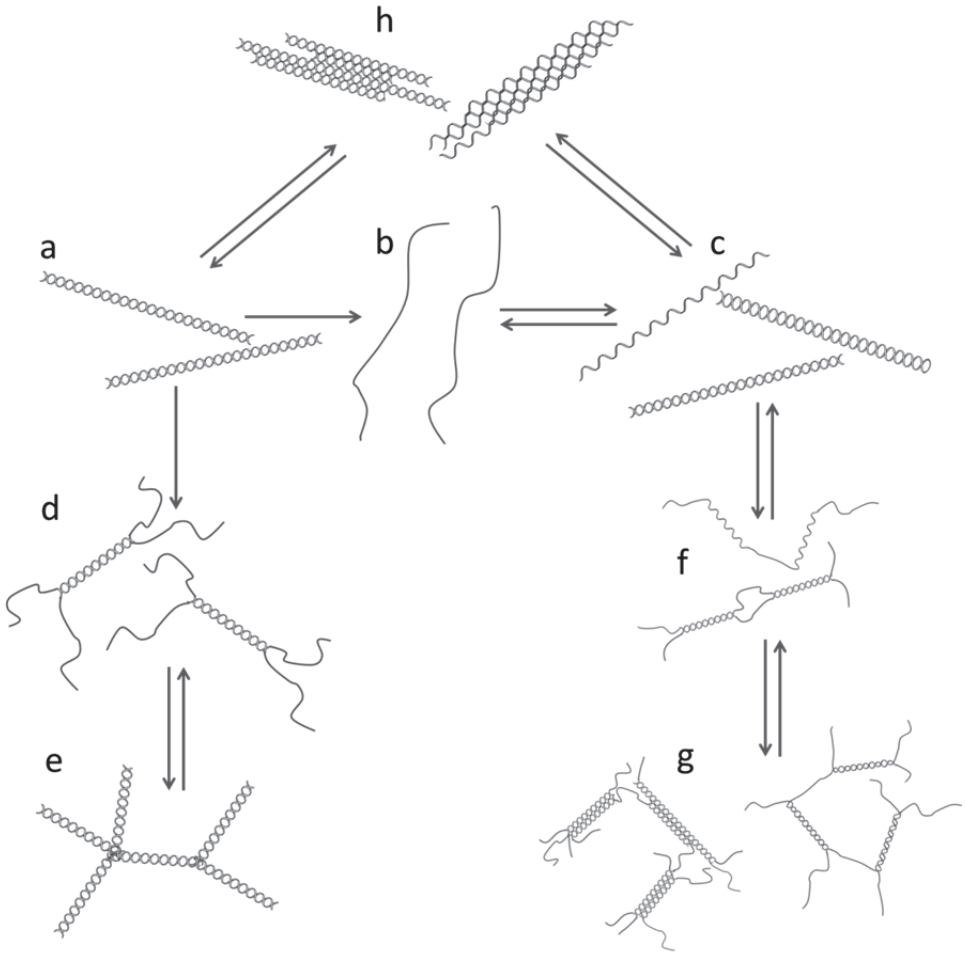

Figure 1.3. Models proposed for the conformational behavior of xanthan in solution. a) native ordered double stranded double helix; b) disordered xanthan strains; c) proposed conformations for renatured xanthan; d) partially dissociated native strains;[41] e) network formation by the association of disordered xanthan segments;[35] f) partially dissociated renatured strains; g) network formation by stacking of ordered xanthan segments;[32, 46] h) network formation by stacking of completely ordered xanthan molecules.[45]

The other model elaborates on the dissociation behavior as described by Liu et al.,[41] in which double stranded xanthan gradually unfolds from the end points of the helix (Figure 1.3d.). Based on this dissociation behavior, it has been argued[35] that the dissociated parts of one helix interact with dissociated parts of one or more other double stranded helices upon renaturing, leading to the formation of a network of double stranded helices (Figure 1.3e.).

Models describing xanthan-galactomannan interactions

One of the main characteristic of xanthan used in industry is the ability to form strong gels with gluco- and galactomannans (GM). The interaction between xanthan and GMs is believed to strongly depend on the conformation of xanthan. However, as the conformation of xanthan is poorly understood, the exact nature of these interactions also remains controversial. Three different models for the interaction have been proposed. An overview of these models is given in Figure 1.4.
The Unilever model, shows that only ordered xanthan structures interact with GM.[31, 47] According to this model the mannan backbone has to be available for interaction, as interactions only occur with smooth mannan regions or with mannan regions which are substituted on every other mannose unit.[48] In contrast, the Norwich model indicates that only disordered xanthan fragments can interact with the backbone of GM.[49] A third model, the Silsoe model,[50] suggests that the strong xanthan-GM gels are a result of homotypic (xanthan-xanthan) and heterotypic interactions (xanthan-galactomannan) with heterotypic junction zones formed by disordered xanthan fragments.

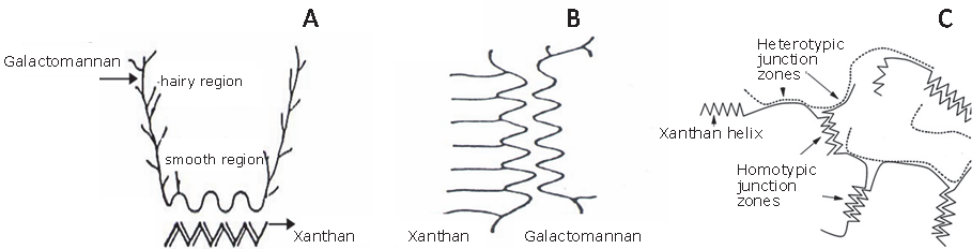

Figure 1.4. Proposed models for the xanthan-galactomannan interactions. A) Unilever model, B) Norwich model, C) Silsoe model.

Influence of the primary xanthan structure on xanthans solution properties

Although multiple models for xanthans solution properties exist, studies are consistent on the factors influencing these properties. The conformational behavior is influenced by solvent conditions, where the disordered conformation is favored with increasing temperature, decreasing ionic strength and/or increasing pH.[30, 31, 51] Furthermore, the primary xanthan structure influences the transitional behavior, where the presence of acetyl groups stabilizes the ordered structure[11, 52, 53] and the presence of pyruvate groups results in a more flexible, disordered conformation.[54, 55]

The influence of the primary structure on the rheological behavior of xanthan has also been studied extensively. The removal of acetyl group give rise to xanthan solutions with increased viscosity and a lower sensitivity to changes in pH.[11, 23] Furthermore, stronger interactions gels are observed using acetyl free xanthan instead of normal xanthan.[56] In contrast, the removal of pyruvate groups reduces the viscosity of a xanthan solution, increases the sensitivity to changes in pH and decreases the influence of salts on the viscosity.[54, 57] The removal of pyruvate groups did not show a clear effect on the interactions with galactomannans.[56] Studies on the influence of the length of the side chain on xanthan solution properties showed that the removal of the outer mannose decreased the viscosity of a xanthan solution and that weaker interaction gels are obtained.[23, 56, 58] The additional removal of the glucuronic acid resulted in an increased viscosity.[23, 58]
As described previously, the xanthan conformation is believed to be important for xanthans solution properties. However the conformation is significantly influenced by xanthans primary structure. Observed influences of the primary structure on solution properties could, therefore, origin from the induced changes in the xanthan conformation.

ACCEPTED CHEMICAL XANTHAN STRUCTURE DOES NOT EXPLAIN PHYSICAL OBSERVATIONS

Generally, it is assumed that the structure proposed by Jansson et al. (Figure 1.1.) is the ideal xanthan structure with only minor variations in the degree of substitution. However, the variations observed in acetyl and pyruvate level, together with information on xanthans biosynthesis clearly point to possible variations in side chain decoration. If this indeed is the case, varieties in the distribution of xanthan substituents may be one of the reasons that the wide variety in models for xanthans solution behavior still does not fully explain xanthans functionality. The hypothesis that different xanthan repeating units exist is strengthened by the observation that the functionality of other carbohydrates strongly depends on the distribution of substituents and/or side chains.[2] Unambiguous information on the xanthan structure is thus needed to help understanding the structure-function relationship of xanthan better, however a good analytical approach to study the xanthan structure is lacking.
Several methods are described in literature to study the chemical structure of polysaccharides. The total sugar composition can be determined after complete chemical hydrolysis of carbohydrates. However, no structural information on polymer level can be obtained from such analysis. Analysis on polymeric structures can be conducted using NMR. A drawback is that the analysis of viscous samples is difficult

and usually leads to broad not well resolved signals.[59] Another approach is to study diagnostic oligosaccharides released upon hydrolysis, in order to reveal the chemical structure. Xanthan oligosaccharides can be obtained through chemical depolymerization, oxidative-reductive depolymerization or sonication.[6, 60, 61] However, random modifications in the side chains can be apparent using such depolymerization techniques. Enzymatic depolymerization of the xanthan backbone as investigated by Rinaudo & Milas[62] is, therefore, more suitable for the production of diagnostic xanthan oligosaccharides. An overview of xanthan degrading and modifying enzymes will be given below.

XANTHANASES

Enzymatic backbone degradation

Xanthan modifying or degrading enzymes can be obtained from xanthan degrading microorganisms, when cultivated in a growth media with xanthan as carbon source.[63-66] Several studies used mixed or purified cultures for the production of xanthanases.[63-65, 67-70] Most of the cultures that could degrade xanthan were rich in *Bacillus spp.* Generally, these cultures produced a *mixture* of enzymes that synergistically degrade the xanthan polymer. The analysis of the degradation products showed that side chain modifications are necessary prior to backbone degradation, as will be further discussed later. The oligosaccharides produced are, therefore, not fully representative for the complete xanthan polymer.

Other studies focused on the degradation of the xanthan backbone using *pure* cellulases and showed that fungal cellulases can partly degrade xanthan. This can only be done under aqueous conditions in which xanthan appears in a partly disordered conformation.[62, 71-73] The xanthan degradation in most studies was monitored by the decrease in viscosity only, and did not focus on the elucidation of the primary xanthan structure.[62, 73] However, some studies followed the degradation by gel permeation chromatography.[68, 71] It was shown that high molecular weight products, rich in substituents, remained at the end point of the enzymatic hydrolysis.[71, 72] It was argued that substituents in the xanthan side chains might hinder the cellulases and prevent the complete hydrolysis of the xanthan backbone into oligosaccharides. As xanthan was only partially hydrolyzed, the oligosaccharides produced are considered to be not representative for the complete xanthan polymer. In order to accurately compare the chemical structure of different xanthans, an enzyme system is necessary which is capable of complete backbone hydrolysis independent on the substituents present.

Enzymatic modification of the xanthan side chains

A combination of specific side chain modifications followed by structural analysis of the modified xanthan could be a good approach for the further elucidation of the xanthan structure. Several techniques to modify the xanthan structure are known. Various chemical treatments can be used for the production of pyruvate and/or acetyl-free xanthan or to produce the xanthan polytetramer in which the outer mannose unit has been removed.[53, 74-76] Xanthans with varying structures can also be obtained by altering the xanthan biosynthesis, as described previously. However, none of these methods results in a controlled modification of the xanthan side chain, which is necessary for the further elucidation of the xanthan structure. Enzymatic modification of the xanthan side chains could be a superior method, as it provides a controlled removal of target groups.

Xanthan lyases

To date xanthan lyases are the only well characterized xanthan modifying enzymes, which are active towards the xanthan polymer. They act exolytically on the xanthan side chain and liberate the outer mannose through β-elimination. In that perspective xanthan lyases are rather peculiar as most other polysaccharide lyases endolytically cleave the glycosidic backbone of a polysaccharide.[77, 78] Based on the amino acid sequence, xanthan lyases are classified into CAZy polysaccharide lyase family 8,[79-81] which contains lyases that are active towards hyaluronate and chondroitin as well (www.cazy.org). Xanthan lyases can be divided into two classes: 1) pyruvate specific lyases, which need the presence of pyruvate groups to be active and 2) xanthan lyases which are active independent on the degree of acetylation and pyruvylation.[24, 82] Several studies have described lyases belonging to the first class,[64, 77, 83, 84] of which two have been purified and characterized.[79, 85] No type 2 lyase has been purified so far. The exact substrate specificity of type 2 lyases, therefore, remains uncertain.
As the characterized xanthan lyases are specific for the removal of pyruvylated outer mannose units, these enzymes could be used for the elucidation of the distribution pattern of the pyruvate groups along the backbone.

Putative xanthan side chain modifying enzymes

Although no xanthan modifying enzymes, other than xanthan lyases, have been fully characterized, some studies reported on the expression of other putative xanthan modifying enzymes by micro-organisms, when cultivated in a growth media with xanthan as carbon source.

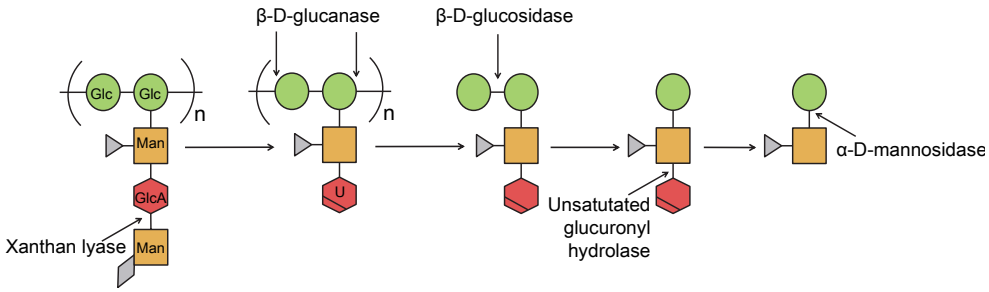

Figure 1.5. Complete enzymatic degradation of xanthan by *Bacillus sp.* strain GL1 as described by Nankai et al.[63] Glc = glucose; Man = mannose; GlcA = glucuronic acid; U = unsaturated glucuronic acid triangle = acetyl groups; diamond: pyruvate group.

The complete degradation of xanthan by *Bacillus sp*. strain GL1 and the enzymes involved have been described by Nankai et al.[63] (Figure 1.5.). A pyruvate specific xanthan lyase and a β-D-glucanase are produced extracellular. Removal of the terminal mannose through β-elimination showed to be necessary before the backbone can be hydrolyzed by β-D-glucanase.[63, 66] The tetramers obtained are brought intracellular to be further degraded into monosaccharides. Incubation of the xanthan polymer with the intracellular enzyme fraction showed that none of these side chain modifying enzymes are active towards the xanthan polymer.[66, 86]

Another study reported on the secretion of several xanthan modifying enzymes during the growth of *Paenibacillus alginolyticus* XL1 on xanthan media (Figure 1.6.).[24] Analysis of the molecular weight distribution showed that high molecular weight (HMW) material remained in the xanthan media after growth. This HMW material had limited solubility and showed interaction with Congo red, which interacts with β-glucans, indicating that the cellulosic backbone was still intact. The enzymes were, therefore, concluded to be active towards the intact xanthan polymer. As the complete culture broth showed lyase activity towards both intact xanthan as well as pyruvate-free xanthan, it was hypothesized that next to the purified pyruvate-specific lyase[84], a second (type 2) lyase was present. Furthermore, a deacetylase and an uronic acid releasing enzyme were secreted by *P. alginolyticus* XL1. Both enzymes were partly purified. The uronic acid releasing enzyme was only active towards lyase-treated xanthan and is, therefore, similar to the unsaturated glucuronyl hydrolase produced by *Bacillus sp.* strain GL1.[86] However, this enzyme is produced extracellular and does not require backbone degradation to be active. The deacetylase was not further characterized. Hence, no enzymes are readily available for the structural analysis and modification of xanthan.

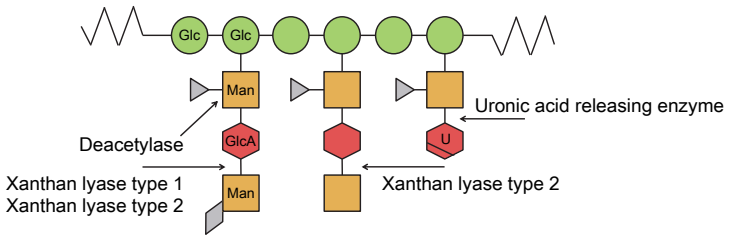

Figure 1.6. Xanthan modifying enzymes produced by *Paenibacillus alginolyticus* XL1 as proposed by Ruijssenaars.[24] Glc = glucose; Man = mannose; GlcA = glucuronic acid; U = unsaturated glucuronic acid triangle = acetyl groups; diamond: pyruvate group.

AIM AND OUTLINE OF THE THESIS

The substitution pattern of several polysaccharides showed to be important for their functional properties. The wide variety in models to describe the functional properties of xanthan was therefore, hypothesized to be the result of a more complex chemical xanthan structure than generally assumed, especially regarding the substitution pattern of the substituents. The aim of the research described in this thesis was, therefore, to develop an enzymatic method to characterize and compare the primary structure of different xanthans. Furthermore, enzymes that solely modify the xanthan side chain were searched for to enable the controlled modification of xanthan.

In **chapter 2** the influence of both the primary as well as the secondary structure of xanthan on the enzymatic degradation of the backbone by cellulases was investigated. The structure of oligosaccharides obtained from different xanthan samples after enzymatic hydrolysis of the backbone were elucidated using HPAEC and LC-ELSD-MS^n and are discussed in **chapter 3**. In **chapter 4** the correlation between the transitional behavior of xanthan and the position of substituents within the xanthan side chain is discussed, based on the chemical structure of diagnostic oligosaccharides released by cellulases at different levels of disordered conformation. From the correlations observed, information on the distribution pattern of the substituents over the xanthan backbone was obtained as well. **Chapters 5** and **6** report the characterization of two different acetyl esterases, from *Myceliophthora thermophila C1* and *Bacillus subtilis* strain 168, which partly deacetylate xanthan in a different manner. **Chapter 7** discusses the most important findings of this study and their impact for future research on the structure-function relationship of xanthan.

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Chapter 2

The influence of the primary and secondary xanthan structure on the enzymatic hydrolysis of the xanthan backbone

ABSTRACT

Differently modified xanthans, varying in degree of acetylation and/or pyruvylation were incubated with the experimental cellulase mixture C1-G1 from *Myceliophthora thermophila C1*. The ionic strength and/or temperature of the xanthan solutions were varied, to obtain different xanthan conformations. The exact conformation at the selected incubation conditions was determined by circular dichroism. The xanthan degradation was analyzed by size exclusion chromatography. It was shown that at a fixed xanthan conformation, the backbone degradation by cellulases is equal for each type of xanthan. Complete backbone degradation is only obtained at a fully disordered conformation, indicating that only the secondary xanthan structure influences the final degree of hydrolysis by cellulases. It is thereby shown that, independently on the degree of substitution, xanthan can be completely hydrolyzed to oligosaccharides. These oligosaccharides can be used to further investigate the primary structure of different xanthans and to correlate the molecular structure to the xanthan functionalities.

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INTRODUCTION

Xanthan is an extracellular polysaccharide that is secreted by the microorganism *Xanthomonas campestris,*[1] with a molecular mass ranging from 1–7*10^6 Dalton.[2, 3] Xanthan is water-soluble and xanthan solutions exhibit high pseudoplastic flow even at low concentrations. Xanthan has a β-1,4-glucan backbone with on every second glucose unit a (3→1) linked α-D-mannose-(2→1)-β-D-glucuronic acid-(4→1)-β-D-mannose side chain (Figure 2.1.).[4] Depending on the *Xanthomonas* strain and the fermentation conditions used for xanthan production, approximately 90% of the inner mannose units are *O*-6 acetylated, and 30-50% of the terminal mannose groups carry a 4,6-linked pyruvic acid acetal group.[5-7] No information is available regarding the distribution pattern of these substituents.

In solution, native xanthan as produced by *Xanthomonas campestris,* exists in a double-stranded helical conformation with an order-disorder transition upon changes in temperature and/or ionic strength.[8-10] An ordered conformation of xanthan can be recovered by cooling or by increasing the ionic strength in a process called renaturation of xanthan. Renatured xanthan however, does not exhibit the exact same conformation and rheological properties as the native xanthan.[3, 10-13] In this respect the order-disorder transition of the native xanthan can be considered non-reversible. Renatured xanthan also exists in a helical conformation, and has an order-disorder transition, which is, in contrast to native xanthan, reversible.[14-16]

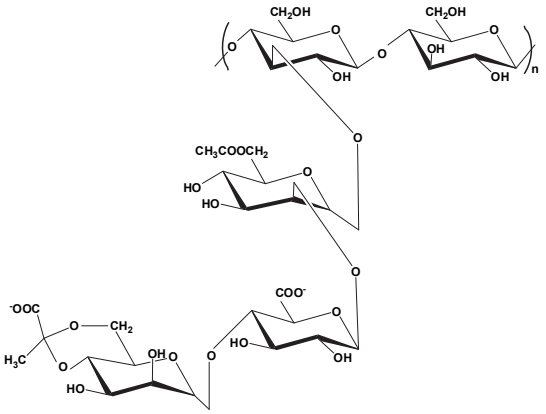

Figure 2.1. The ideal repeating unit of xanthan as reported by Jansson et al.[4]

The order-disorder transitions of both native and renatured xanthan, and thereby the viscosity of a xanthan solution, depends strongly on the molecular composition of xanthan, particularly with respect to the presence of acetyl and/or pyruvate groups. A more ordered, helical conformation is obtained by the removal of pyruvate groups, whereas a more disordered conformation is obtained by the removal of acetyl groups.[12, 17-19] Because both the fermentation conditions and the *Xanthomonas* strain used during the production of xanthan influence the primary structure of xanthan, differences in conformational behavior may be present between different batches of xanthan. Therefore, the primary structure of xanthan is of importance when the transitional behavior is studied. However, until to date no suitable method is available for the comparison of the primary structure of different batches of xanthan. Characterization of diagnostic xanthan oligosaccharides could help reveal the exact xanthan structure. Production of such oligosaccharides by chemical degradation of the xanthan backbone will also cause degradation of the xanthan side chains and is therefore not suitable. An enzyme-based method for the production of xanthan oligosaccharides, that will specifically degrade the xanthan backbone, leaving the side chains intact, would be more useful.

Previous studies have shown that xanthan can be degraded by cellulases under aqueous conditions in which xanthan appears in a disordered conformation.[20-23] However, analysis of the degradation products using gel permeation chromatography showed that high molecular weight products remain. Such high molecular weight fractions have a higher content of pyruvate and acetyl groups than the parental xanthan. Therefore, it has been discussed that the accessibility of the backbone towards enzymatic degradation might be reduced by the presence of these substituents in the side chains.[20, 22] Because differences in the primary structure also cause differences in the order-disorder behavior, it was also hypothesized that differences in conformation, due to differences in the primary structure, result in enzyme resistant xanthan strands.[21, 22] However, no conclusive studies were performed to confirm these assumptions.

To our knowledge, in literature no clear overview is present for the effect of both the primary xanthan structure as well as the secondary xanthan structure on the enzymatic hydrolysis of the xanthan backbone. In this study we therefore analyzed the combined influence of the degree of substitution as well as the xanthan conformation, on the enzymatic degradation of xanthan.

MATERIALS AND METHODS

Xanthan samples

Unmodified, renatured xanthan in Na^+ salt form (RX) was obtained from DuPont (Melle, France). Acetyl free xanthan (AFX) was produced by a saponification treatment of RX with 1 M NaOH (18 h; 4°C). Pyruvate free xanthan (PFX) was produced by heating RX to 100°C in a 5 mM trifluoroacetic acid (TFA) solution for 90 min.[24] Acetyl- and pyruvate-free xanthan (APFX) was produced by a 5 mM TFA treatment followed by saponification. All modified polymers were dialyzed against demineralized water for 24 h and then lyophilized.

In order to analyze the influence of the sodium counter ions on the xanthan conformation, the chemically modified xanthans and the normal xanthan were converted to their H^+-form. A 2 $mg \cdot mL^{-1}$ xanthan solution was mixed with Amberlite IR-120-H^+ ion-exchange material (BDH, Poole Dorset, UK) for 30 minutes at room temperature. The ion exchange material was removed from the xanthan solution by centrifugation (5000 x g, 15 min., 20°C).[25] The supernatant was neutralized using 100 mM NaOH, dialyzed against demineralized water for 24 h, and then lyophilized. The generated xanthans will hereafter be referred to as "H^+" for "proton-form".

Xanthan composition

The constituent monosaccharide compositions of the unmodified xanthan and the chemically modified xanthans, in the sodium-form as well as in the proton-form, were determined by methanolysis.[26] The degree of acetylation of xanthan samples was measured using a Megazyme acetic acid kit (Megazyme, Wicklow, Ireland) after a saponification step with 1 M NaOH (18 h; 4°C). The degree of pyruvylation was measured using a Megazyme pyruvic acid kit (Megazyme) after acid hydrolysis with 1 M TFA (100 min.; 90°C).[24]

Circular Dichroism

The ellipticities (in mDeg) of 2 $mg \cdot mL^{-1}$ xanthan solutions were monitored at 219 nm from 10-85°C using a Jasco J-715 Spectropolarimeter (Jasco Corp., Tokyo, Japan) with a heating rate of 30°C·h^{-1}, a data pitch of 0.5°C, a response time of 1 sec, a sensitivity of 100 mDeg and at a bandwidth of 2 nm. The transition profiles of RX, AFX, PFX and APFX were determined in 0, 2 and 10 mM NaCl solutions, the transition profiles of RX-H^+, AFX-H^+, PFX-H^+ and APFX-H^+ were determined in demineralized water. The

temperature was controlled using a Jasco PTC-348 WI controller. Quartz cuvettes with an optical path of 1 mm were used.
The transition profiles obtained were used to determine the fraction of disordered conformation (α) at a certain ionic strength and temperature using Equation 1 with: θ_t = ellipticity at a given temperature; θ_U = ellipticity of a completely disordered structure and θ_F = ellipticity of a completely ordered structure.[27]

$$\alpha = 1 - (\theta_t - \theta_U)/(\theta_F - \theta_U) \quad (1)$$

The minimum and maximum ellipticities were determined for each type of xanthan, θ_F was determined in 10 mM NaCl solutions at 15°C and θ_U was determined in the H^+-form at 85°C. The obtained curves were normalized by the best-fit parameters.

Enzymatic hydrolysis

Cellulases in the experimental enzyme preparation C1-G1 from *Myceliophthora thermophila C1* (Dyadic Netherlands, Wageningen, The Netherlands)[28] were used to hydrolyze the xanthan backbone at several temperature and salt conditions. Solutions containing 2 mg·mL^{-1} xanthan were prepared in 0, 2 and 10 mM NaCl for RX, AFX, PFX and APFX, and solutions containing 2 mg·mL^{-1} xanthan were prepared in demineralized water for RX-H^+, AFX-H^+, PFX-H^+ and APFX-H^+. The hydrolysis was performed by incubating 1 mL of a xanthan solution with 60 µg protein for 0, 3, 24 or 48 h at 40, 45, 50, 55 or 60°C. The hydrolysis was stopped by rapidly cooling the digests to 6°C. Samples were kept at 6°C until analysis. In order to exclude possible influences of the chosen incubation conditions on the enzyme activity, carboxymethyl cellulose (Sigma-Aldrich, Tseinheim, Germany) was used as reference substrate.
The amount of reducing end sugars was determined using the PAHBAH assay.[29] The degree of hydrolysis (DH) is determined by the increase in reducing end sugars after enzymatic hydrolysis. If every glucose linkage in the backbone is split, a DH of 100% is obtained. Complete hydrolysis of xanthan to its repeating units, would, therefore, correspond to a maximal DH of 50%. Assuming that the maximum degradation of xanthan is obtained when xanthan is hydrolyzed to its repeating unit, a DH of 50% would correspond to a degree of degradation of 100%.

High performance size exclusion chromatography (HPSEC)

HPSEC was performed on an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA). A set of three TSK-Gel columns (Tosoh Bioscience, Tokyo, Japan) was used in series with separation columns G-6000PW, G-3000PW and G-2500PW (7.8 mm × 300 mm). The

column temperature was set at 55°C. The samples (20 µl, 2 mg·mL^{-1}) were eluted with 1% (v/v) ethylene glycol in 0.2–µm-filtered 0.2 M $NaNO_3$ at a flow rate of 0.8 ml·min^{-1}.[2] The eluate was monitored using refractive index detection (Shodex RI 101; Showa Denko K.K., Kawasaki, Japan). Molecular masses were estimated with the help of pullulan molecular-mass standards (Polymer Laboratories, Palo Alto, CA, USA). The xanthan degradation products were divided into three fractions: 1) non-degraded xanthan (R_t = 18-25 min); 2) intermediate degradation products (R_t = 25-31 min) and 3) completely degraded xanthan (R_t = 31-34 min). The relative molecular weight distribution was calculated by dividing the integrated RI peak area of each fraction by the total RI peak area measured from 18-34 minutes.

RESULTS AND DISCUSSION

Xanthan composition

To ensure that no changes were made during the chemical treatments other than the targeted modifications, unmodified xanthan (RX), acetyl free xanthan (AFX), pyruvate free xanthan (PFX) and acetyl- and pyruvate-free xanthan (APFX), both in the Na^+-form and in the H^+-form, were analyzed for their molecular compositions (Table 2.1.) and for their molecular weight distributions.

The ratio glucose:mannose:glucuronic acid is approximately 1.00:0.79:0.47 for all xanthans. This indicates that no changes in the sugar composition occurred during the chemical treatments. Assuming an ideal repeating xanthan structure (Figure 2.1.), a ratio of 1:1:0.5 is expected. Hence, our results indicate that there are some irregularities in the repeating structure of xanthan. These irregularities could be caused by downstream processing or by irregularities in the biosynthesis.[22, 30]

The conversion of xanthan to the H^+-form, removes the remaining acetyl groups of AFX and APFX, as well as some residual pyruvate groups from PFX. Because the removal of acetyl groups and pyruvate groups was intended in AFX/APFX and PFX, respectively, these deviations in substitution are acceptable within our study. No significant changes are observed in the monosaccharide ratios.

To confirm that the chemical treatments did not result in backbone degradation, the molecular weight distributions (M_w) of the generated xanthans were determined using HPSEC (results not shown). No changes in M_w were observed in any of the modified xanthans. Therefore, it was concluded that the treatments used to generate the modified xanthans did not cause relevant changes in the xanthan compositions, except for the removal of pyruvate and/or acetyl groups as intended.

Table 2.1. Molecular composition of normal xanthan and chemically modified xanthan in the Na^+-form and in the H^+-form

Xanthan	Glc:Man:GlcA Molar ratio	Acetyl content w/w%	Pyruvate content w/w%
RX	1.00 : 0.80 : 0.46	5.79	3.70
AFX	1.00 : 0.81 : 0.48	1.31	3.88
PFX	1.00 : 0.79 : 0.49	6.24	0.87
APFX	1.00 : 0.81 : 0.47	1.06	0.60
RX -H^+	1.00 : 0.75 : 0.46	5.87	3.77
AFX -H^+	1.00 : 0.79 : 0.46	0.00	3.98
PFX-H^+	1.00 : 0.76 : 0.48	6.30	0.29
APFX-H^+	1.00 : 0.81 : 0.45	0.00	0.64

Order-disorder transitions

The transitional behavior of the four xanthans, induced by increasing the temperature, was analyzed by circular dichroism at different salt concentrations (0, 2 and 10 mM NaCl). The temperature profiles (Figure 2.2.) show that the mid-point transition temperature (T_m) of xanthan increases with increasing ionic strength; it decreases due to the removal of acetyl groups; and it increases due to the removal of pyruvate groups, as was previously reported.[16, 18] An overview of all T_m observed is given in Table 2.2. When dissolved in demineralized water, removal of both the acetyl and pyruvate groups result is a lower T_m. However, at increasing salt concentrations, this effect on the T_m is not observed. Furthermore, the temperature range of the transition of the modified xanthans is significantly smaller than for RX, probably because the removal of the substituents clearly results in less molecular variability.

Table 2.2. Midpoint-transition temperatures of normal xanthan and chemically modified xanthan at different salt concentrations

Xanthan	Midpoint-transition Temperature		
	Demineralized water	2 mM NaCl	10 mM NaCl
RX	44	49	61
AFX	27	32	47
PFX	80	81	≥ 85
APFX	33	43	59
RX -H^+	40	n.a.[a]	n.a.
AFX -H^+	25	n.a.	n.a.
PFX-H^+	37	n.a.	n.a.
APFX-H^+	28	n.a.	n.a.

[a]: n.a. = not analyzed

In contrast to the other xanthans studied, the transitional behavior of PFX is not significantly affected by an increasing ionic strength in the temperature range measured. In the presence of pyruvate groups, an increase in ionic strength will lower the repulsive forces by shielding the negative charges of the pyruvate groups. This results in a more rigid, helical structure, as is observed for RX. The removal of pyruvate groups, and thereby removal of negative charges, already reduced the repulsive forces in PFX. An increase in ionic strength therefore does not influence the conformational behavior of PFX in the measured temperature range.[14, 16, 31] As shown in Figure 2.2c., lowering the ionic strength, by the conversion of PFX into PFX-H^+, does influence the conformational behavior. Due to the removal of the counter ions of the glucuronic acid units, negative charges are induced in the xanthan side chains. Thereby the electrostatic repulsion between side chains is increased, resulting in a lower T_m.

From the temperature profiles obtained, the fraction disordered conformation (α) at a given temperature and ionic strength was determined using Equation 1. The normalized fitted graphs are depicted in Figure 2.3. These fitted curves can be used to calculate the fraction of disordered conformation at a given temperature and ionic strength. Furthermore, it is possible to determine the solution conditions needed, in order to obtain a certain fraction of disordered conformation. The xanthan conformation during an enzyme incubation can now be controlled by selecting a specific incubation condition. Hence it is now possible to independently study the influence of the xanthan conformation on the enzymatic hydrolysis of xanthan for xanthans having different levels of substitution, but originating from the same xanthan batch.

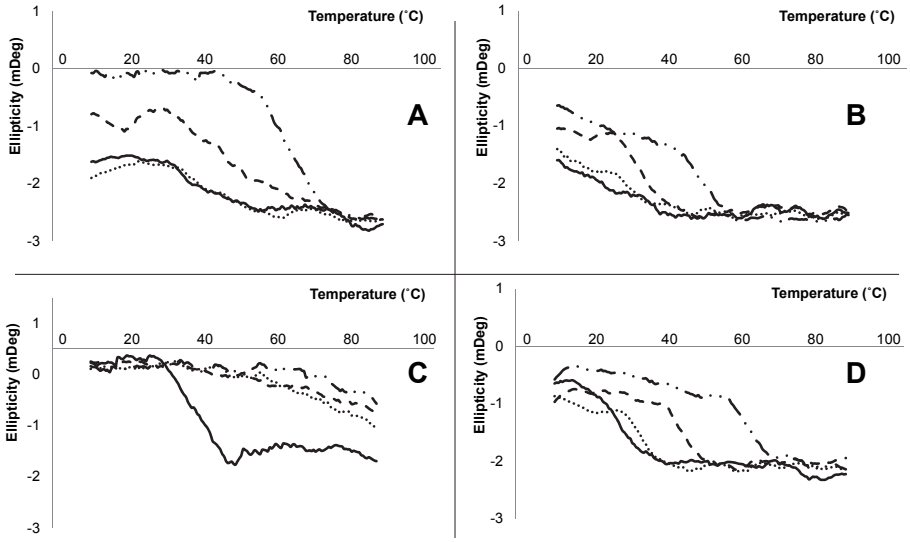

Figure 2.2. Transition profiles of xanthan in: the H^+-form in deionized water (———); the Na^+-form in deionized water (·····); the Na^+-form in 2 mM NaCl (– – –); and the Na^+-form in 10 mM NaCl (—·—). A) unmodified xanthan; B) acetyl free xanthan; C) pyruvate free xanthan; D) acetyl and pyruvate free xanthan.

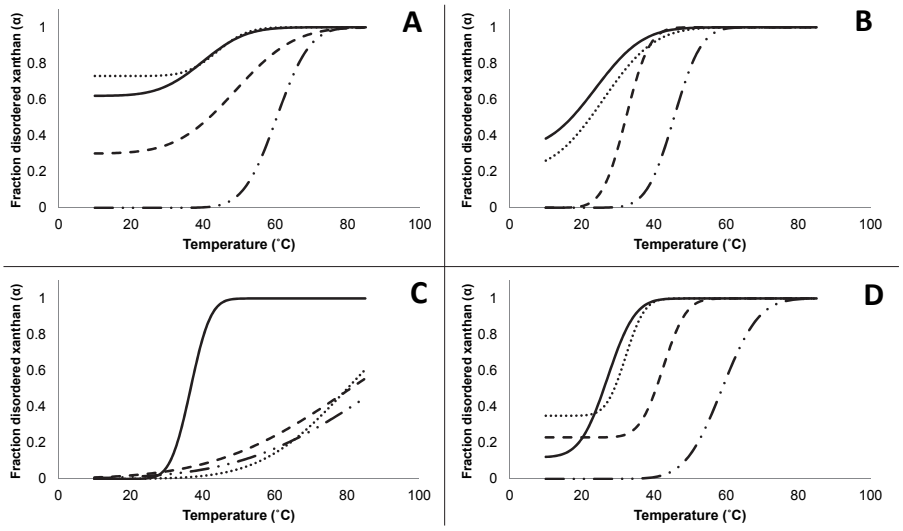

Figure 2.3. Fraction of disordered xanthan as function of temperature (°C) in: the H^+-form in deionized water (———); the Na^+-form in deionized water (·····); the Na^+-form in 2 mM NaCl (– – –); and the Na^+-form in 10 mM NaCl (—·—). A) unmodified xanthan; B) acetyl free xanthan; C) pyruvate free xanthan; D) acetyl and pyruvate free xanthan.

Enzymatic degradation

To determine the influence of the xanthan conformation on the enzymatic hydrolysis of xanthan, the fraction of disordered conformation (α) was varied for different enzyme incubations. Based on Figure 2.3. several temperature and salt conditions were selected in which the conformation of xanthan ranges from α=0 to α=1. An overview of all conditions is given in Table 2.3.

Influence of the incubation conditions on the cellulase activity

The activity of enzymes may also be affected by the ionic strength and temperature of a solution. The influence of the selected inubation conditions on the cellulase activity was, therefore, determined using carboxymethyl cellulose as a model substrate. The cellulase activity was not significantly influenced by the changes in ionic strength; the temperature, however, does affect the cellulase activity. The highest activity was observed at 55°C. This activity is reduced to 72%, by cellulase activity, must be minimized. Therefore, the end point of the enzymatic hydrolysis has been used to determine the influence of the conformation on the enzymatic hydrolysis of xanthan. The end point of the reaction was determined by monitoring the RX degradation by cellulases in time. Because the lowest cellulase activity was detected at 40°C, this temperature was used to verify the end point of the degradation. The molecular weight distributions of RX digests in time are shown in Figure 2.4a. After 3 h of incubation the enzyme digest shows non-degraded high molecular weight material, some intermediate degradation products and completely degraded low molecular weight material. After 24 h of incubation, the intermediate degradation products are further degraded into completely degraded low molecular weight material. However the non-degraded, high molecular weight material remains. This indicates that under these conditions part of the xanthan is resistant to enzymatic hydrolysis. No significant changes in the molecular weight distribution are observed when the incubation was extended for another 24 h. We, therefore, conclude that after 48 h of incubation, the maximal degradation will surely be reached at every incubation condition tested.

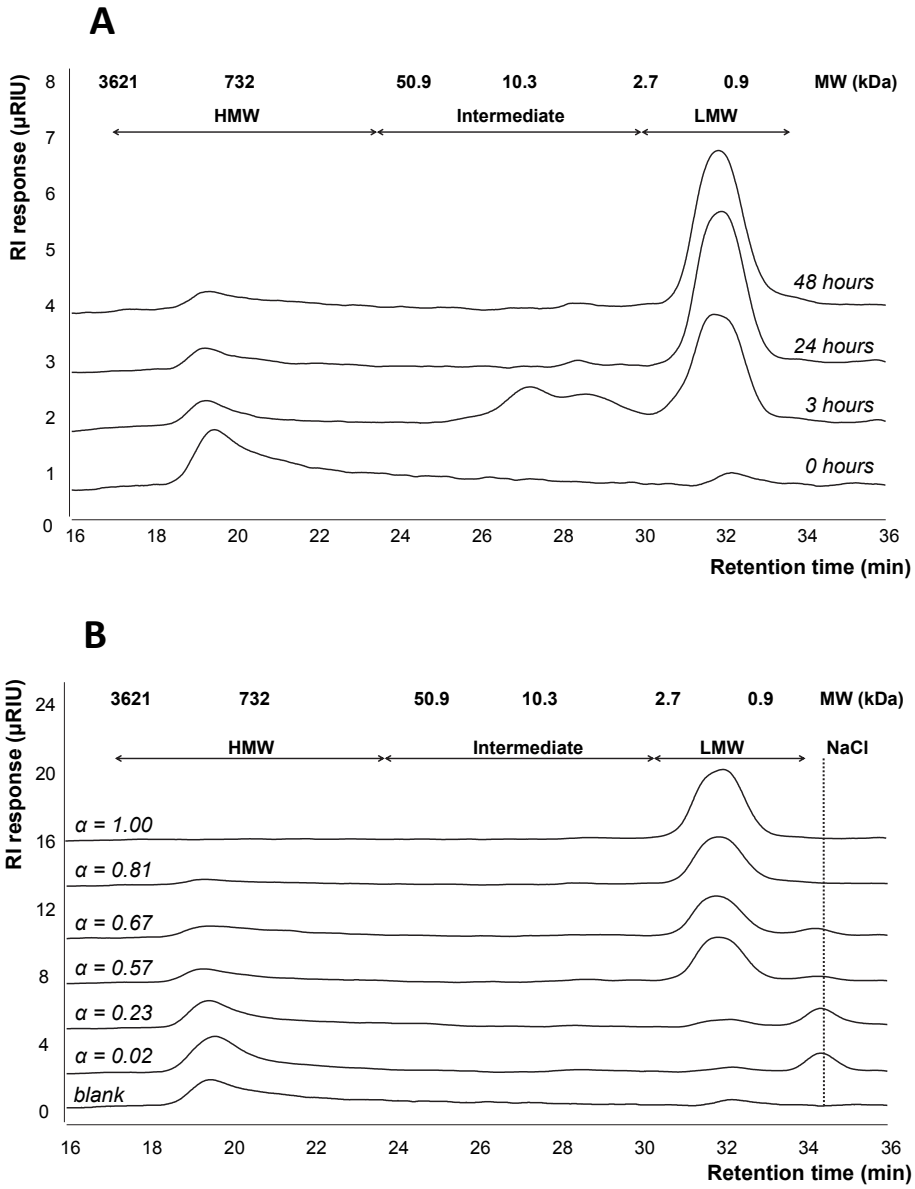

Figure 2.4. HPSEC elution patterns of normal xanthan digests. A: xanthan degradation followed in time at 40˚C in demineralized water. B: 48 h digests of normal xanthan differing in conformation (α = fraction of disordered conformation; blank = untreated xanthan).

Table 2.3. Degree of hydrolysis obtained after incubation of xanthan with cellulases for 48 hours at different fractions of disordered conformation. The xanthan conformation was controlled by varying the temperature and ionic strength

Sample	Temperature (˚C)	NaCl added (mM)	Disordered fraction (α)	DH[a] (%)
RX- H^+	40	0	0.81	41
RX- H^+	50	0	0.95	51
RX- H^+	60	0	0.99	50
RX	40	0	0.81	42
RX	50	0	0.95	50
RX	60	0	1	57
RX	40	2	0.47	9
RX	45	2	0.57	18
RX	50	2	0.67	35
RX	55	2	0.78	43
RX	60	2	0.86	54
RX	40	10	0	5
RX	45	10	0.02	6
RX	50	10	0.08	8
RX	55	10	0.23	10
RX	60	10	0.48	31
AFX	40	0	0.92	53
AFX	60	0	1	43
AFX	40	2	0.93	32
AFX	50	2	1.0	46
AFX	60	2	1.0	46
AFX	40	10	0.18	10
AFX	45	10	0.46	27
AFX	50	10	0.77	45
AFX	60	10	0.99	54
PFX- H^+	40	0	0.78	44
PFX- H^+	45	0	0.97	55
PFX- H^+	50	0	0.99	50
PFX- H^+	60	0	1.0	53
PFX	40	0	0.02	5
PFX	60	0	0.14	8
PFX	40	10	0.05	8
PFX	60	10	0.17	6
APFX- H^+	40	0	0.99	50
APFX- H^+	60	0	1	64
APFX	40	2	0.47	14
APFX	45	2	0.76	40
APFX	50	2	0.95	57
APFX	40	10	0.01	2
APFX	50	10	0.14	6
APFX	55	10	0.32	10

[a]: DH = degree of hydrolysis. DH=100%: all backbone linkages are degraded, based on the increase in reducing end sugars as measured by PAHBAH assay.

Influence of the xanthan conformation on the enzymatic hydrolysis of xanthan

The xanthan conformation during the enzymatic hydrolysis was controlled by selecting different temperatures and ionic strengths for xanthan solutions. Table 2.3. gives an overview of: 1) the type of xanthan; 2) the ionic strength and temperature of the xanthan solution during enzyme hydrolysis; 3) the corresponding fraction of disordered conformation (α); and 4) the degree of hydrolysis (DH) after 48 h of incubation. Figure 2.5a. shows the correlation between the xanthan conformation and the final degree of degradation based on the DH, where, due to a repeating backbone unit with 2 glucose units, a DH of 50% correspond to a degree of degradation of 100%. It is clearly shown that an increase in α leads to a higher degree of degradation at the end point of the reaction. When xanthan exists in a completely ordered conformation, no enzymatic hydrolysis is observed. It is, therefore, concluded that a disordered conformation is necessary for enzymatic degradation. A previous study showed a correlation between the speed of hydrolysis and the xanthan conformation.[21] In that study it was also observed that no enzymatic degradation occurs at a completely ordered conformation. However, the influence of substituents on the enzymatic hydrolysis was not analyzed. From the results shown in Figure 2.5a. it can now be concluded that the degree of substitution does not significantly influence the final degree of degradation, as long as xanthan exists in the same conformation. Furthermore our results show that not only the speed of hydrolysis, but also the final degree of hydrolysis is influenced by the xanthan conformation. When xanthan exists in a completely disordered conformation a maximum DH of ~60% is observed (Table 2.3.). This DH exceeds the maximum theoretical value of 50% assuming that cellulases can hydrolyze xanthan to the repeating units. The assay used to determine the increase in reducing end sugars could give a different response to the xanthan repeating units than towards glucose, as is also the case with different monosaccharides.[29] Therefore, an overestimation in the DH could exist.

Based on the DH, cellulases seem to be able to completely degrade xanthan to its repeating unit when xanthan is present in a completely disordered conformation. To confirm these findings the molecular weight distributions of the xanthan digests were determined by HPSEC. The HPSEC elution patterns of normal unmodified xanthan digests, obtained at different xanthan conformations (α), are shown in Figure 2.4b. To be able to compare the cellulase degradability of all xanthans tested, the relative molecular weight distributions of all digests, obtained at different α, were determined. The results are shown in Figure 2.5b.

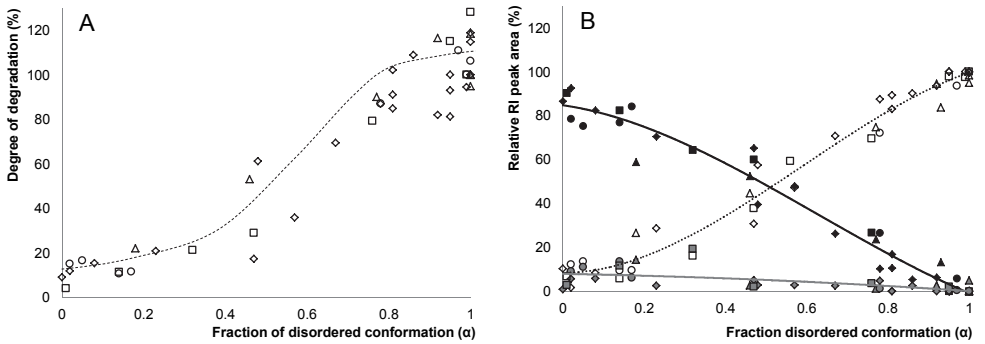

Figure 2.5. Correlation between the fraction disordered xanthan (α) and the enzymatic hydrolysis after a 48 h incubation with the experimental enzyme preparation C1-G1 based on: A) The degree of hydrolysis measured by the increase in reducing end sugars B) The molecular weight distribution measured by HPSEC. Normal xanthan (◆); AFX (▲); PFX (●); APFX (■). Open symbols: xanthan oligosaccharides; grey symbols: intermediate degradation products; closed symbols: non-degraded xanthan.

The elution profiles show that as long as xanthan exists in a completely disordered conformation, xanthan is completely degraded to low molecular weight material. The HPSEC results thereby confirm that the cellulases can completely hydrolyze xanthan to the xanthan repeating units, independently of the degree of substitution. When xanthan is not completely in the disordered conformation, high molecular weight material remains in the enzyme digests. The degree of degradation as measured by the reducing end assay (Figure 2.5a.), and the relative abundance of smaller fragments (Figure 2.5b.) thereby fully match and show a clear correlation between the xanthan degradation and the xanthan conformation. This correlation is similar for each type of xanthan. The final xanthan degradation by cellulases at a given α is, therefore, solely controlled by the xanthan conformation.

In earlier studies it was hypothesized that the accessibility of the backbone towards enzymatic degradation might be reduced by the presence of substituents in the side chains.[20,22] Because the precise conformation of xanthan under the chosen enzyme conditions was not monitored in these studies, we assume that the observed enzyme resistancy in these studies is due to the presence of (partly) ordered xanthan strains, as was also posted as one of the hypothesis by Sutherland.[22]

Considerations on the transitional behavior of renatured xanthan

Figures 2.4b. and 2.5a. show that all enzyme digests obtained with an α ≤0.95, contain enzyme resistant xanthan with the same molecular mass as that of untreated xanthan. This indicates that at a given condition xanthan molecules are either completely degraded to low molecular weight material or completely enzyme resistant. Because

only xanthan molecules in the disordered conformation will be hydrolyzed by cellulases, this indicates that during the order-disorder transition two populations exists: 1) *completely* ordered xanthan molecules and 2) *completely* disordered xanthan molecules. However, studies on the transitional behavior of xanthan report that xanthan gradually dissociates from the outsides of the helices or that due to intramolecular differences, sequences of ordered and disordered conformations exists within a molecule.[32, 33] In that case, enzymatic hydrolysis of these partially dissociated xanthan helices would result in enzyme resistant degradation products with a lower molecular weight than the untreated xanthan. Because this is not observed with HPSEC, it is most likely that the renatured xanthan used in this study does not follow the same transitional behavior as previously described.

Different populations of ordered and disordered conformation, however, might be obtained when high intermolecular variations exist within a xanthan batch.[12, 18] Differences in primary structures within one batch would results in different T_m for each xanthan molecule. At a given condition a certain xanthan molecule could, therefore, completely exist in an ordered conformation whereas another type of xanthan molecule completely exists in a disordered conformation. Although possible, it is unlikely that at all conditions chosen in this study, such a sharp division is obtained in the xanthan conformations, especially when it is assumed that each type of xanthan gradually dissociates as described above. High intermolecular variations, therefore, do not seem to explain the HPSEC results obtained in this study.

An explanation for the high molecular weight material in the enzyme digests could be that renatured xanthan does not exists as single or double stranded helices, but as a multiple stranded network of xanthan helices as was recently reported.[34] Because the size exclusion method used in this study is not able to distinguish between molecular mass values beyond $5.0*10^6$ Dalton. Partial dissociation of xanthan molecules from a multiple stranded network would explain our finding as long as the remaining network has a $M_w \geq 5.0*10^6$ Dalton. Intermolecular differences might control which parts of the network dissociate first, explaining the observed differences in the degree of substitution between the degraded and non-degraded xanthan in previous studies.[22] Another study reported on the side-by-side association of ordered xanthan structures.[32] Alignment of all enzyme resistant ordered xanthan structures into a network, which is larger than the HPSEC detection limit of our method, might therefore also explain the high molecular weight observed in the enzyme digests. Based on our findings we would therefore conclude that ordered xanthan structures do not exist as single or double helices, but as a network of multiple helices.

CONCLUSIONS

We have investigated the influence of the primary and secondary structure of xanthan on the enzymatic hydrolysis of the xanthan backbone. A clear correlation between the secondary structure and the extent of enzymatic degradation of xanthan is observed, where only disordered xanthan structures are hydrolyzed by cellulases. When in the disordered form, no correlation exists between the primary structure of xanthan and the final xanthan degradation by cellulases. By controlling the xanthan conformation it is, therefore, possible to completely degrade different types of xanthan into xanthan oligosaccharides. Further characterization of the oligosaccharides produced from different types of xanthan, enables the comparison of the primary xanthan structures, especially regarding the repeating units present. Consequently, further research into the influence of the distribution of the repeating units on xanthans functionality will be possible.

The presence of non-degraded, high molecular weight xanthan after enzymatic hydrolysis of xanthan which was partly present in a disordered conformation was hypothesized to be caused by the presence of a xanthan network.

ACKNOWLEDGMENTS

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Chapter 3

Comparison of xanthans by the relative abundance of its six constituent repeating units

ABSTRACT

Five xanthans were hydrolyzed to their repeating units using cellulases. Hydrophilic interaction chromatography with online electrospray ionization ion trap mass spectrometry and evaporative light scattering detection was used to analyze the oligomers released. It was concluded that six different pentamer repeating units (RUs) exists within a xanthan sample. The most abundant RU shows acetylation on the inner mannose and pyruvylation on the outer mannose. The second most abundant RU shows acetylation on both the inner and the outer mannose. It becomes clear that more variations in the xanthan structure exist than generally recognized. Comparison of five different xanthan samples revealed that, although the molecular composition of xanthan samples can be exactly the same, the ratio in which the RUs occur can differ significantly. It is, therefore, concluded that xanthan samples should be characterized for both, their molecular composition and the relative abundance of the RUs present.

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INTRODUCTION

Xanthan gum is an exopolysaccharide secreted by *Xanthomonas spp.* The generally accepted xanthan structure consists of a cellulosic backbone with trisaccharide side chains linked to every alternate glucose unit. These side chains consist of mannose–glucuronic acid–mannose units, which are substituted with an acetyl group on the inner mannose and a pyruvic acid ketal on the outer mannose.[1] The exact degree of substitution, however, is known to vary depending on the fermentation conditions[2, 3] and the *Xanthomonas* strain used for xanthan production.[4, 5] The primary structure of the produced xanthan can be controlled by specific mutations in the *Xanthomonas* genome.[6] Six different pentamer repeating units are proposed based on the genotype of the *Xanthomonas* strain used. It was shown that suppression of the gene involved in the pyruvylation of the outer mannose resulted in a higher degree of acetylation. It was, therefore, suggested that xanthan side chains can also be acetylated on the outer mannose. However, the exact position of the acetyl groups was not determined. The six repeating units proposed by Hassler & Doherty[6] were, therefore, only hypothesized and not experimentally determined. Another study also report on double acetylated side chains.[7] In this study sugar linkage analysis was used to determine the position of the second acetyl group. It was shown that acetylation on the *O*-6 position of the outer mannose, as proposed by Hassler & Doherty,[6] indeed occurs in xanthan molecules. About 24% of all outer mannose units are acetylated according to this study. However, the precise structure has not been linked to the individual repeating units and the ratio in which they coexist has not been determined.

To date, no conclusive study has been performed on the exact position of the acetyl groups in the xanthan side chains. The exact structure of the different xanthan repeating units present in one xanthan sample has, therefore, never been determined. In a previous study it is shown that cellulases can completely degrade xanthan to the xanthan repeating units.[8] In the present study, these repeating units will be further characterized and used for quantification of the xanthan repeating units present in different xanthan samples. We thereby introduce a method that enables the unambiguous comparison of different xanthans.

MATERIALS AND METHODS

Xanthan samples

Five types of renatured xanthan were kindly provided by DuPont (Melle, France). The molecular compositions (Table 3.1.) are determined as previously described.[8] All samples have a molar glucose:mannose:glucuronic acid ratio which is close to the expected ratio 2:2:1. The xanthan samples differ in their degree of substitution. Xanthans A and B show similar acetyl and pyruvyl contents of ~5.7% (w/w) and 4.5% (w/w), respectively. This corresponds to 1.15 acetyl and 0.56 pyruvyl groups per side chain, which indicates that the side chains can indeed be multiple acetylated. Xanthans C and D also have a similar composition to one another but have a slightly higher pyruvyl content and a slightly lower acetyl content compared to xanthans A and B. Xanthan E has a higher degree of pyruvylation, corresponding to almost fully pyruvylated side chains.

Enzymatic hydrolysis

Solutions containing 2 mg·ml^{-1} xanthan were prepared in demineralized water and hydrolyzed using cellulases from the experimental enzyme preparation C1-G1 from *Myceliophtora thermophila C1* (Dyadic Netherlands, Wageningen, The Netherlands).[8, 9] The hydrolysis was performed by incubating 1 ml of a xanthan solution with 60 µg protein for 48 h at 60°C. After incubation, the digests were boiled (10 min) and centrifuged (10,000 g; 10 min; 25°C). The supernatants were analyzed by HPSEC, HPAEC and UPLC-ELSD-MS^n.

Table 3.1. Molecular composition of five xanthan samples

Xanthan type	Glc:Man:GlcA Molar ratio	Acetyl content w/w%	Pyruvate content w/w%
Xanthan A	1 : 0.88 : 0.41	5.6	4.4
Xanthan B	1 : 0.91 : 0.43	5.9	4.6
Xanthan C	1 : 0.92 : 0.44	4.9	5.1
Xanthan D	1 : 0.91: 0.43	4.9	5.2
Xanthan E	1 : 0.94 : 0.45	4.8	7.3

High performance size exclusion chromatography (HPSEC)

HPSEC was performed as described previously.[8] Molecular masses were estimated using pullulan molecular-mass standards (Polymer Laboratories, Palo Alto, CA, USA).

High performance anion exchange chromatography (HPAEC)

HPAEC was performed on an ICS5000 HPLC system (Dionex, Sunnyvale, CA, USA), equipped with a CarboPac PA-1 column (2 mm ID x 250 mm; Dionex) in combination with a CarboPac PA guard column (2 mm ID x 25 mm) and an ISC5000 ED PAD-detector (Dionex). The digests were centrifuged (10,000 g; 10 min; 25°C) and 2x diluted before injection onto the column (10 µl). Samples were eluted at a flow rate of 0.3 ml·min^{-1} with the following elution profile of 0.1 M sodium hydroxide (NaOH) and 1 M sodium acetate (NaOAc) in 0.1 M NaOH: 0–10 min, 0 – 50 mM NaOAc in 0.1M NaOH; 10–35 min, 50–400 mM NaOAc in 0.1 M NaOH; 35–40 min, 400–1000 mM NaOAc in 0.1 M NaOH; 40–45 min washing step with 1 M NaOAc in 0.1 M NaOH; 45–60 min, equilibration with 0.1 M NaOH. The glucose released was quantified based on the response factor of standard D-glucose.

Hydrophilic interaction liquid chromatography with evaporative light scattering and mass spectrometry detection (HILIC-ELSD-MS)

Digests were analyzed using UPLC-ELSD-MS^n on a HILIC BEH amide column (Waters Corporation, Milford, MA, USA) as described elsewhere with a modified gradient.[10] The following elution profile was used, with A) 1% (v/v) acetonitrile (ACN) in water; (B) 100% ACN; and (C) 2% (v/v) formic acid in 200 mM ammonium formate solution: 0–1 min, isocratic 15% A, 80% B and 5% C; 1–25 min, linear to 45% A, 50% B and 5% C; 25–30 min linear to 55% A, 40% B and 5% C; 30 – 35 min, isocratic 55% A, 40% B and 5% C; 35–35.1 min linear to 15% A, 80% B and 5% C; 35.1–40 min, isocratic 15% A, 80% B and 5%. The xanthan digests were centrifuged and diluted 1:1 with ACN before injection (5 µl) into the system. The Acquity BEH Amide column was coupled to a splitter (Accurate, Dionex Corporation) directing the eluent to an ELSD and to an ESI-MS^n-detector with a ratio 10:1 respectively. The ELSD micro flow nebulizer (Sedere, France) had a gas pressure of 3.5 bar and a gas flow of 1.75 L·min^{-1}. The drift tube temperature of the ELSD was set to 50°C and the gain to 12. MS-detection was performed in negative mode on a Velos Pro ion trap MS (Thermo Scientific, San Jose, CA, USA) with the ion source voltage set to –4.5 kV, capillary temperature 250°C, sheath gas 30 (arbitrary units), auxiliary gas 12 (arbitrary units). Mass spectra were acquired over the scan range m/z 300–2000. MS^n-collection parameters included

normalized collision energy 35 (arbitrary units), activation Q 0.25 (arbitrary units), activation time 30 ms and isolation width 2 m/z.

RESULTS AND DISCUSSION

Enzymatic hydrolysis of the xanthan backbone

The different xanthans were incubated with cellulases in order to completely degrade the xanthans into their repeating units. In a previous study we showed that cellulases from the C1-G1 preparation from *Myceliophthora thermophila C1* can completely hydrolyze the xanthan backbone when xanthan is present in a completely disordered conformation.[8] To ensure that the different xanthans were indeed completely hydrolyzed at the incubation conditions chosen, HPSEC was used to determine the molecular weight distribution of the xanthan digests (Figure 3.1). After a 48 h. incubation all xanthan samples are indeed completely hydrolyzed to xanthan oligosaccharides. The xanthan degradation products can, therefore, be used for quantification and characterization.

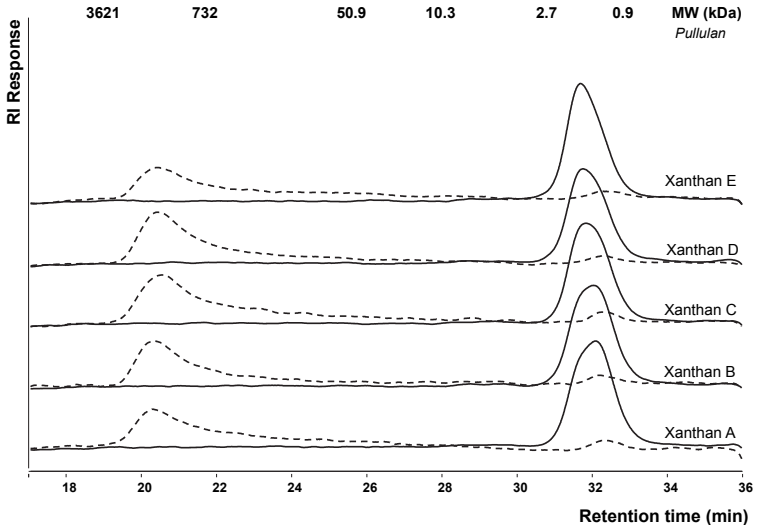

Figure 3.1. HPSEC elution patterns of xanthan digests obtained after 48 h of incubation at 60˚C (solid line), and the corresponding xanthan blanks (dotted line).

Analysis of xanthan degradation products using HPAEC

As HPAEC is a common method for mono- and oligosaccharide analysis,[11] the xanthan digests were analyzed by HPAEC (Figure 3.2). Small amounts of glucose were released (≤5% of all glucose) in all digests, while release of mannose and/or glucuronic acid was not observed. It is, therefore, concluded that the cellulases used do not show any side chain degrading activity. The free glucose could originate from parts of the backbone where the side chain is lacking,[12] enabling the cellulases to release glucose.
Next to the release of glucose, two additional peaks are observed in the HPAEC elution patterns. Because HPAEC is conducted under alkali conditions, acetyl esters originally present will be saponified online. The two peaks will, therefore, represent: 1) the unsubstituted pentamer RU and 2) the pyruvylated pentamer RU. Because side chains carrying a pyruvic acid acetal are more negatively charged than unsubstituted side chains, the pyruvylated RU elutes later, as was confirmed by the analysis of a pyruvate free xanthan (results not shown). No other degradation products are observed in the HPAEC elution pattern of any of the xanthan digests. Therefore, we conclude that under the chosen enzyme conditions, all xanthans are completely degraded to the xanthan RUs, confirming the HPSEC results.

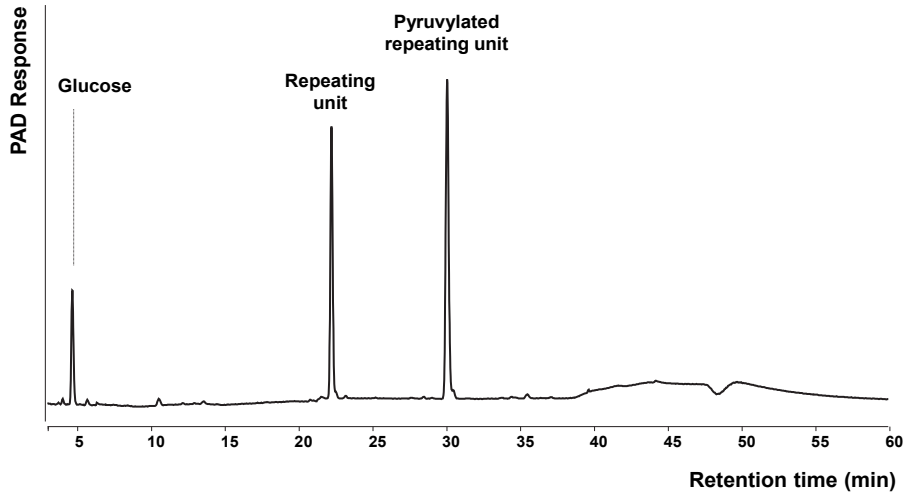

Figure 3.2. HPAEC elution pattern of the 48h xanthan A cellulase digest.

Characterization of xanthan repeating units using LC-MS

No information on the position of acetyl groups can be obtained by HPAEC due to the online saponification of the acetyl esters. The xanthan repeating units (RUs) were, therefore, further characterized using hydrophilic liquid interaction chromatography (HILIC) with online mass spectrometry (MS).

The HILIC elution pattern of the xanthan A digest (Figure 3.3.) shows that the different RUs present in the cellulase digest are well separated. Five different peaks are recognized. The broad peaks are a results of partial α-/β-anomer separation, as was also observed for maltodextrins using the same column.[10] Electrospray ionization ion trap mass spectrometry (ESI-IT-MS^n) was used to identify all five compounds. The dominant peak (peak 3) has a *m/z-value* of 953, corresponding to the negative ion of the RU that is both pyruvylated and acetylated. Figure 3.4 shows the fragmentation pattern of this RU, according to the nomenclature described by Domon & Costello.[13] The most abundant fragments are *m/z* 909, 791 and 703, which were interpreted as follows: *m/z* 909 (953-44, loss of a carboxyl group), 791 (953-162, loss of a glucose unit) and 703 (953-250, loss of pyruvylated mannose). The fragment *m/z* 541 can be annotated as the RU without 2 hexoses and a pyruvyl group. This mass loss can only be explained when one glucose is cleaved simultaneously with the pyruvylated outer mannose. Such double cleavage, from both sides of an oligosaccharides, has been reported previously in the fragmentation pattern of xyloglucan oligomers.[14]

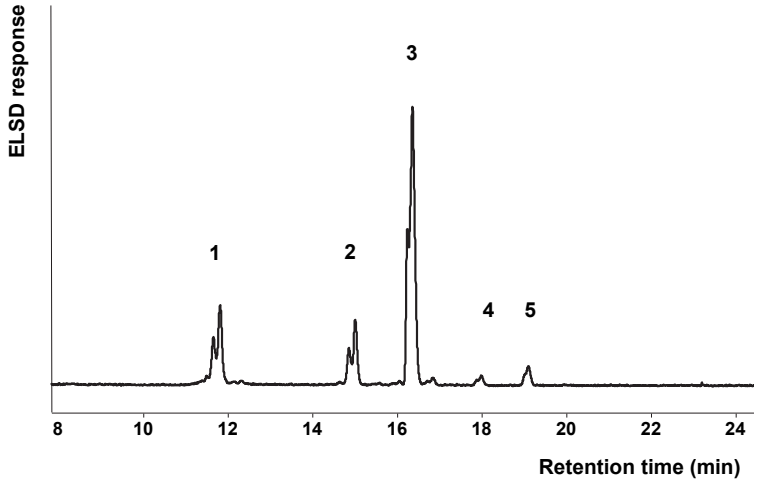

Figure 3.3. HILIC ELSD elution pattern of the 48h xanthan A cellulase digest.

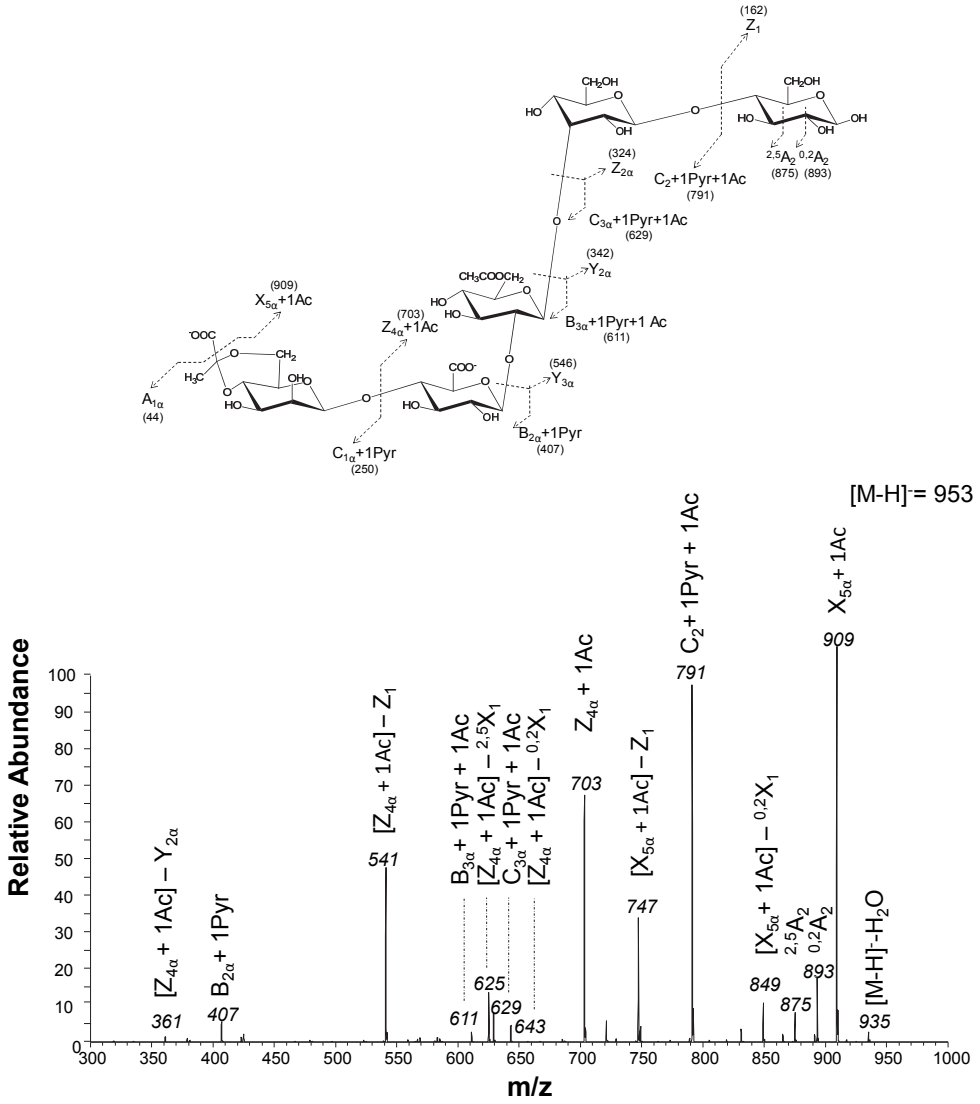

Figure 3.4. MS^2 fragmentation pattern of the acetylated and pyruvylated pentamer repeating unit, eluting at 16.5 min. in Figure 3 and the chemical structure of *m/z* 953 with the observed cleavages according to the nomenclature of Domon & Costello.[13]

Table 3.2 shows the *m/z-values* and the fragmentation patterns of all compounds in the xanthan A digest. Considering the double cleavage observed in the fragmentation pattern of the acetylated and pyruvylated RU, all compounds could be identified as xanthan pentamer RUs differing in substitution pattern. Peak 1 (Figure 3.3.) has a *m/z*

Table 3.2. Fragmentation pattern and identification of the peaks in Figure 3.3 according to the nomenclature of Domon & Costello[13] as depicted in Figure 3.4.

Peak number	RT (min)	$[M-H]^-$ (m/z)	Ion fragments (m/z)	Proposed structure	Structure code
1	11.7 – 12.3	925	**907** ($[M-H]^-$-H_2O), **865** ($[M-H]^-$-$^{0,2}X_1$), **847** ($[M-H]^-$-$^{2,5}X_1$), **763** ($[M-H]^-$-Glc), **703** ($[M-H]^-$-AcMan), **625** ($[M-H]^-$-$^{2,5}X_1$; -AcMan), **541** ($[M-H]^-$-Glc; -AcMan), **379** ($[M-H]^-$-Glc-Glc-AcMan), **361** ($[M-H]^-$-Glc-Glc; -AcMan)		RU-1
2	14.9 – 15.3	883	**823** ($[M-H]^-$-$^{0,2}X_1$), **805** ($M-H]^-$-$^{2,5}X_1$), **721**$[M-H]^-$-Glc), **703**($[M-H]^-$-Man), **643** ($[M-H]^-$-$^{0,2}X_1$; -Man), **625** ($[M-H]^-$-$^{2,5}X_1$; -Man), **541**($[M-H]^-$-Glc; -Man), **361**($[M-H]^-$-Glc-Glc;-Man)		RU-2
			823 ($[M-H]^-$-$^{0,2}X_1$), **805** ($M-H]^-$-$^{2,5}X_1$), **721**$[M-H]^-$-Glc), **661**($[M-H]^-$-AcMan), **601** ($[M-H]^-$-$^{0,2}X_1$; -AcMan), **583** ($[M-H]^-$-$^{2,5}X_1$; -AcMan), **499**($[M-H]^-$-Glc; -AcMan), **379**($[M-H]^-$-Glc-Glc-Man)		RU-3
3	16.3 – 16.7	953	**909** ($[M-H]^-$-CO_2), **893** ($[M-H]^-$-$^{0,2}X_1$), **849** ($M-H]^-$-CO_2; -$^{0,2}X_1$), **791** ($[M-H]^-$-Glc), **747** ($[M-H]^-$Glc; -CO_2), **703** ($[M-H]^-$-PyrMan), **625** ($[M-H]^-$-$^{2,5}X_1$; -PyrMan), **541** ($[M-H]^-$-Glc; -PyrMan), **407** ($[M-H]^-$-Glc-Glc-AcMan), **361** ($[M-H]^-$-Glc-Glc; -PyrMan)		RU-4
4	18.0 – 18.3	841	**781** ($[M-H]^-$-$^{0,2}X_1$), **763** ($M-H]^-$-$^{2,5}X_1$), **679** ($[M-H]^-$-Glc), **661** ($[M-H]^-$-Man), **601** ($[M-H]^-$-$^{0,2}X_1$; -Man), **517** ($[M-H]^-$-Glc-Glc), **499** ($[M-H]^-$-Glc; -Man)		RU-5
5	19.1 – 19.4	911	**867** ($[M-H]^-$-CO_2), **851** ($[M-H]^-$-$^{0,2}X_1$), **807** ($M-H]^-$-CO_2; -$^{0,2}X_1$), **749** ($[M-H]^-$-Glc), **705** ($[M-H]^-$-Glc; -CO_2), **661** ($[M-H]^-$-PyrMan), **601** ($[M-H]^-$-$^{0,2}X_1$; -PyrMan), **583** ($[M-H]^-$-$^{2,5}X_1$; -PyrMan), **569** ($[M-H]^-$-Glc-Glc), **499** ($[M-H]^-$-Glc; -PyrMan), **407** ($[M-H]^-$-Glc-Glc-Man)		RU-6

● glucose; ● mannose; ⊘ glucuronic acid; ○ acetyl groups; ● pyruvic acid ketal

value of 925, which corresponds to the negative ion of a RU that is substituted with two acetyl groups. The MS-fragmentation shows that one acetyl group is positioned on the inner mannose and the other on the outer mannose, as was previously shown by Stankowski et al.[7] using linkage analysis. Peak 2 has a *m/z-value* of 883, which corresponds to the negative ion of a single acetylated RU. Figure 3.5. shows the fragmentation pattern of this RU and the structures of the two possible acetylated RUs. The most abundant fragments are *m/z* 721, 703 and 541, which were interpreted as follows: 721 (883-162, loss of a glucose), 703 (883-180, loss of a mannose unit) and 541 (883-342, loss of a glucose and mannose unit). The loss of unsubstituted mannose is indicative for acetylation on the inner mannose. Fragments *m/z* 661 and 499 were interpreted as follows: *m/z* 661 (883-222, loss of an acetylated mannose unit) and 499 (883-384, loss of an acetylated mannose and a glucose unit). These fragments are thereby specific for acetylation on the outer mannose. The fragmentation pattern of peak 2 is, therefore, indicative for the presence of two different single acetylated repeating units. Based on the relative abundance of the specific *m/z* fragments, it can be concluded that most of the single acetylated RUs are acetylated on the inner mannose (≤85%).

Peaks 4 and 5 in the HILIC elution pattern (Figure 3.3.) can be identified as the unsubstituted xanthan RU and the non-acetylated, but pyruvylated RU respectively. In total six different pentamer RUs are identified. Thereby we confirm that the RUs proposed by Hassler & Doherty[6] are indeed present in xanthan. However, these authors suggested that some RUs are only synthesized after specific mutations in the DNA of *Xanthomonas* and that native *Xanthomonas* strains would only express RUs that are substituted on both the inner and outer mannose. In this study it is now shown that all six repeating units can coexist in one xanthan sample, even when the *Xanthomonas* strain is not genetically modified. Therefore, it is concluded that '*the*' xanthan repeating unit does not exist and that the xanthan structure is much more complex than generally depicted in literature. Whether the presence and abundance of the different RUs are resulting from irregularities in the biosynthesis or originate from the downstream processing of xanthan during production remains uncertain.

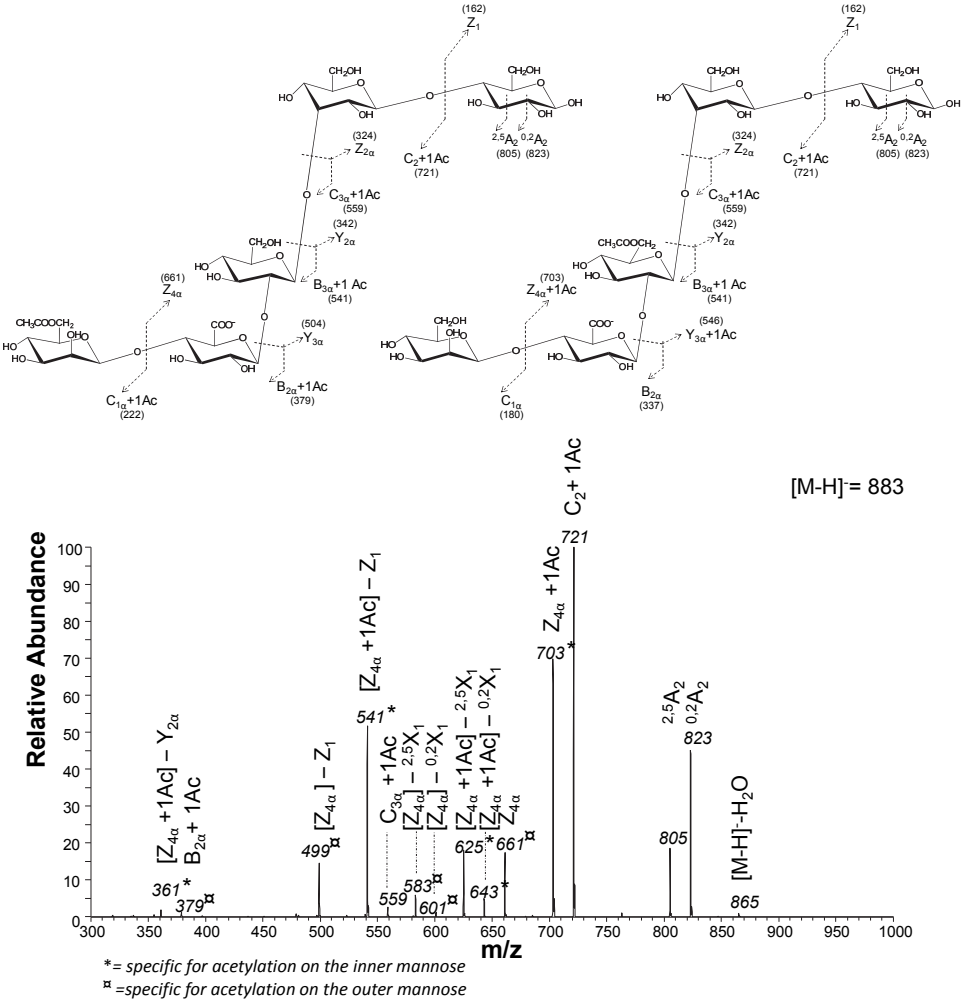

Figure 3.5. MS^2 fragmentation pattern of the single acetylated pentamer repeating unit, eluting at 15.1 min. in Figure 3.3. and the two isomeric chemical structures of *m/z* 883 with the observed cleavages according to the nomenclature of Domon & Costello.[13]

Relative abundance of the xanthan repeating units in different xanthan samples based on UPLC-ELSD response

Now that it is shown that the position of the acetyl group can vary, xanthan samples with the same levels of acetylation and pyruvylation may still differ in the relative abundance of the RUs present. The five xanthan samples were, therefore, also compared based on the RUs present in their cellulase digests.

HILIC elution patterns of the different cellulase digests showed that every type of xanthan contained all six RUs (results not shown). Because no suitable standards were available, the ratio in which the RUs are present in the xanthan samples was determined based on the ELSD-response, as a previous study showed that the ELSD response of different types of oligosaccharides is similar.[15] Because the two isomeric single acetylated RUs elute simultaneously, the MS fragmentation pattern was used to determine the ratio in which these RUs are present.

Table 3.3. gives an overview of the relative abundance of the RUs present in the xanthan samples. The most abundant RU in all xanthan types is RU-4, which is acetylated on the inner mannose and pyruvylated on the outer mannose. Depending on the type of xanthan, the second most abundant repeating unit in xanthan is the double acetylated repeating unit (RU-1), the repeating unit acetylated on the inner mannose (RU-2) or the pyruvylated repeating unit (RU-6). Together the two double substituted side chains typically represent about 79% of all xanthan side chains, independent of the xanthan type. The results, therefore, indicate that independent of the conditions used during the xanthan production, approximately the same amount of double substituted side chains are present.

The influence of the production conditions on the biosynthesis of xanthan and the degree of pyruvylation of the xanthan produced has been described in various studies. A deficiency of nitrogen during the fermentation increases the pyruvate content, whereas a deficiency of oxygen decreases the pyruvate content.[2, 3, 16] Furthermore, it is known that the presence of citric acid in the growth media results in a xanthan with a high pyruvate level[17] and limitations of the amount of magnesium or phosphate in the growth media results in a xanthan with low pyruvate levels.[18] In most of these studies, however, the effect of the fermentation conditions on the level of acetylation was not studied. This study shows that there is no clear correlation between the fermentation conditions and the *total* amount of double substituted side chains synthesized However, a correlation between fermentation conditions and the acetyl:pyruvyl-ratio on the outer mannose is observed. We therefore propose that suppression of the biosynthesis of a pyruvate group on the outer mannose, by selective fermentation conditions, results in higher levels of acetylation on the outer mannose. This is in alignment with Davidson[18] who observed an increase in the total acetyl content with

Table 3.3. Relative abundance (mol%) of the repeating units in different xanthan samples based on the ELSD-response after HILIC separation of 48 h cellulase digests

Xanthan type	RU-1	RU-2	RU-3	RU-4	RU-5	RU-6	Substitution outer mannose (%)	Substitution inner mannose (%)	% of all acetyl groups on outer mannose	Double substituted RUs (%)	Ac:Pyr-ratio on the outer mannose
Xanthan A	19	10	2	62	3	4	87	91	19	81	1:3.2
Xanthan B	17	11	1	61	3	7	86	89	14	78	1:3.8
Xanthan C	4	12	1	75	2	6	86	91	5	79	1:16.2
Xanthan D	10	8	0	64	3	15	89	82	11	74	1:7.9
Xanthan E	5	3	1	73	3	15	94	81	6	78	1:14.7

● glucose; ◍ mannose; ⊘ glucuronic acid; ○ acetyl groups; ● pyruvic acid ketal

decreasing pyruvate content. It also aligns with results found by Hassler & Doherty[6] which showed that blocking the ketalase activity through mutations in gene L, results in the production of xanthan with high acetyl levels. In total 5-19% of all acetyl groups are positioned on the outer mannose, while Stankowski[7] mentioned values of 20-25% for their xanthan based on sugar linkage analysis. No clear correlation is observed between the acetylation of the inner mannose and the substitution on the outer mannose.

Differentiation between 'similar' xanthans based on their repeating units

Comparison of the molecular composition of the different xanthans (Table 3.1.) showed that xanthan C and D are almost identical. However, Table 3.3. illustrates the existence of differences between these xanthans. Xanthan D has twice as much RU-6, which is only substituted with a pyruvate group on the outer mannose, than xanthan C, whereas xanthan C has higher levels of RU-4. Furthermore, xanthan D has higher levels of the double acetylated RU, whereas xanthan C has more single acetylated RUs (RU-2 and RU-3). Thereby, xanthan C has a slightly higher degree of substitution on the inner mannose and a slightly lower degree of substitution on the outer mannose compared to Xanthan D. Cross analysis of the acetyl and pyruvate content of the xanthans (Table 3.1.) corresponds rather well with the acetyl and pyruvate levels calculated from the type and relative abundance of the repeating units. It can, therefore, be concluded that two xanthans similar in molecular composition, may significantly differ in their structure. Establishing these differences in the structure of xanthans, may help to explain differences in functionality between two xanthans with the same molecular composition.

Impact of new findings

The effect of acetyl and pyruvate groups on the functionality of xanthan is widely discussed in literature and conclusions are generally based on the quantitative analysis of the substituents. The influence of the exact distribution of the substituents on the functionality of xanthan, however, has been largely ignored in functionality studies due to the assumption that the xanthan substitution pattern is close to the idealized repeating unit.

Some studies show that acetyl and/or pyruvyl groups do not influence the xanthans functionality.[19, 20] However, most studies show that acetyl groups stabilize the xanthan conformation, reduce the interaction with galactomannans and reduce the xanthan viscosity, while pyruvyl group have the reversed effect.[21-23] The effect of acetyl groups is attributed to increased association of the side chains with the backbone, due to

hydrogen bonding between the xanthan backbone and the acetyl groups on the inner mannose.[24-26]

Our study shows that the substitution pattern within the xanthan side chains is different than generally assumed and varies between xanthan samples. It is therefore likely that the relative abundance of the different RUs needs to be taken into account when studying the effect of the substituents on xanthan's functionality, especially regarding the substitution of the outer mannose unit. Because it is now shown that about 11% of all acetyl groups are positioned on the outer mannose, conclusions on the effect of acetyl groups on xanthans functionality should be reconsidered.

CONCLUSIONS

In this research an analytical method is introduced to characterize and compare xanthan samples. The unambiguous identification and quantification of six different RUs, demonstrate that substitution on the outer mannose unit is much more abundant than generally assumed. In addition to the 66-88% of the outer mannose units that are pyruvylated, 5-21% of the outer mannose units are acetylated. Comparison of different xanthans showed that the ratio in which the six repeating units are present, differs between xanthan samples even when the molecular composition is similar. It is, therefore, concluded that the characterization of xanthan samples should include the relative abundance of the RUs present, as well as the molecular composition of a xanthan sample.

ACKNOWLEDGMENTS

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Chapter 4

The influence of the six constituent xanthan repeating units on the order-disorder transition of xanthan, based on the cellulase degradation of disordered xanthan segments

ABSTRACT

Xanthans occurring in different levels of disordered conformation were enzymatically hydrolyzed to their six pentamer repeating units (RUs). The RUs present in the enzyme digests were analyzed using LC-MS. As only disordered xanthan segments are degraded by cellulases, the influence of the six different RUs on the transitional behavior of xanthan could be studied. The results indicate that especially xanthan segments rich in RUs that are acetylated on the outer mannose unit stabilize the xanthan conformation. Acetylation of the inner mannose did not show to have a stabilizing effect on the xanthan conformation. As the enzymatic release of pyruvylated RUs gradually increased with increasing levels of disordered xanthan segments, it is concluded that the distribution of these RUs is random. On the contrary, xanthan segments rich in single or double acetylated RUs were instantly hydrolyzed indicating a block wise distribution of these RUs.

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INTRODUCTION

Xanthan is a polysaccharide that gives solutions with high pseudoplastic flows, when dissolved in water.[1] This solution property holds over a wide pH and temperature range, making xanthan very suitable as viscosifier and/or stabilizer for the food industry.[2, 3] The stability of a xanthan solution is mainly addressed to the helical conformation of xanthan.[4] The stability of this conformation depends on the temperature and salt concentration of the solvent as well as on the primary xanthan structure.[5-7] Xanthan is a bacterial exo-polysaccharide that consists of a β-(1→4) linked glucose backbone, with a (3→1) linked α-D-mannose-(2→1)-β-D-glucuronic acid-(4→1)-β-D-mannose side chain attached to every other glucose unit (Figure 4.1.).[8] Variations in the primary xanthan structure are mainly due to the substituents present in the side chains. On average 85% of all inner mannose units are acetylated at the *O*-6 position and 50% of all outer mannose units carry a pyruvate group.[9, 10] The presence of acetyl groups is reported to stabilize the helical conformation,[2] while pyruvate groups destabilize the helical conformation.[5, 11] It is generally assumed that only the inner mannose unit can be acetylated and the stabilizing effect of the acetyl groups is directed to hydrogen bonds formed with the xanthan backbone.[12, 13] However, in a recent study we showed that xanthan consists of 6 different repeating units and that depending on the production conditions, 5-20% of all xanthan side chains are acetylated on the outer mannose units.[14]

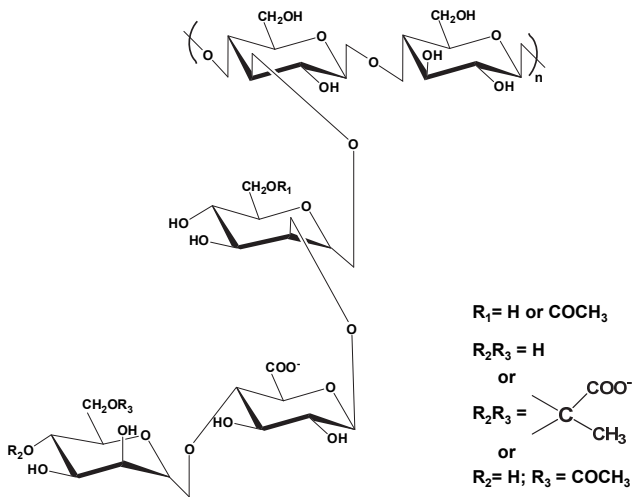


Figure 4.1. The xanthan repeating unit.

Thereby, the question arises whether the substitution of the acetyl group on either the inner or outer mannose is important for its stabilizing effect on the xanthan conformation. It was also shown that the total degree of substitution on the outer mannose is rather constant, while the pyruvate:acetate ratio on the outer mannose depends on the fermentation conditions [14]. It is, therefore, plausible to postulate that the adverse effect of acetyl and pyruvate groups on the stability of the xanthan conformation is due to the type of substitution at the outer mannose unit: acetate or pyruvate. This could indicate that the location of the acetyl group within the side chain indeed is important for the stability of the xanthan conformation. If so, it could be that the results obtained in previous studies, that assume that only the inner mannose unit can be acetylated, were misinterpreted and have not always led to the proper explanation. Hence, the aim of the present study is to better understand the influence of the primary xanthan structure and the acetylation pattern, on the stability of the xanthan conformation.

MATERIALS AND METHODS

Xanthan samples

Three types of renatured xanthan, differing in acetyl and pyruvyl contents, were obtained from DuPont (Melle, France). The molecular composition of the samples and the relative abundance of the repeating units present were measured as described previously.[14] An overview of the xanthan compositions is given in Table 4.1. The molar compositions of xanthans A and B are rather similar. Variations between these xanthans exist in their substitution patterns, where xanthan A has more acetyl groups on the outer mannose than xanthan B. Xanthan C is rich in pyruvate groups and has a relatively low level of acetylation compared to the other two xanthans. This translates into a low degree of acetylation on the outer mannose compared to xanthans A and B.

Table 4.1 Molar composition of different xanthan samples

Xanthan type	Glc:Man:GlcA Molar Ratio	Acetyl content (w/w%)	Pyruvate content (w/w%)	RU-1	RU-2	RU-3	RU-4	RU-5	RU-6	Ac:Pyr-ratio on the outer mannose
Xanthan A	1:0.88:0.41	5.6	4.4	19	11	2	62	2	4	1:3.1
Xanthan B	1:0.91:0.43	5.9	4.6	14	12	1	67	2	4	1:4.7
Xanthan C	1:0.94:0.45	4.8	7.3	4	2	1	77	1	15	1:18.4

● glucose; ◯ mannose; ⊘ glucuronic acid; ○ acetyl groups; ● pyruvic acid ketal

Enzymatic hydrolysis

Solutions containing 2 mg·ml^{-1} xanthan A were prepared in 0, 2 and 10 mM NaCl and solutions containing 2 mg·ml^{-1} xanthan B or xanthan C were prepared in 0 and 10 mM NaCl. Xanthan was hydrolyzed by incubating 1 ml of a xanthan solution with 60 µg protein from the experimental cellulase mixture C1-G1 from *Myceliophthora thermophila* C1 (Dyadic Netherlands, Wageningen, The Netherlands). The cellulase mixture was desalted prior to incubation using Micro Bio-Spin chromatography columns following the company's description (Bio-Rad Laboratories, Hercules, CA, USA).
Incubations were performed at temperatures in the range 30–60°C and continued for 48 h in order to obtain the end point of the enzymatic degradation. After hydrolysis, the digests were cooled to 6°C.

Circular dichroism

The xanthan conformation at the enzyme incubations chosen was determined using circular dichroism. The transition profiles of the three xanthans, in 0, 2 and 10 mM NaCl solutions, were determined as described previously[15] and used to estimate the fraction of disordered xanthan (α) in the different enzyme incubations using Equation 1 with: θ_t = ellipticity at a given temperature; θ_U = ellipticity of a completely disordered structure and θ_F = ellipticity of a completely ordered structure.[16]

$$\alpha = 1 - (\theta_t - \theta_U)/(\theta_F - \theta_U) \quad (1)$$

The minimum and maximum ellipticities were determined for each type of xanthan, θ_F was determined in 10 mM NaCl solutions at 15°C and θ_U was determined in demineralized water at 85°C. The curves obtained were normalized by the best-fit parameters.

High performance size exclusion chromatography (HPSEC)

HPSEC was performed on an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) as described previously.[15] Apparent molecular mass distributions were estimated using pullulan molecular-mass standards (Polymer Laboratories, Palo Alto, CA, USA). Xanthan digests were centrifuged (10,000 g; 10 min; 25˚C) prior to injection.

HILIC-ELSD–ESI-IT-MS^n

Digests were analyzed using UPLC-ELSD-MS^n on a HILIC BEH amide column (Waters Corporation, Milford, MA, USA), which was coupled to an ELSD and an ESI-MS^n-detector as described elsewhere.[14] The xanthan digests were filtered using Amicon-0.5 mL centrifugal filter devices with a 10 kDa cut off (EMD Millipore Corporation, Billerica, MA, USA) to remove any remaining high molecular weight xanthan. The filtrate was diluted 1:1 (v:v) with milliQ water followed by a 1:1 (v:v) dilution with acetonitrile before injection into the system.

The ELSD peak area was used to determine the ratio in which the different xanthan repeating units (RUs) were present in the xanthan digests.[14] The peak areas of the different RUs of fully degraded xanthan, obtained after incubation under conditions in which $\alpha = 1$, were used to determine the maximum ELSD-peak area for each type of RU. As the two single acetylated RU elute simultaneously, the MS-fragmentation pattern was used to distinguish and semi-quantify between the RUs which are solely acetylated at the inner or outer mannose.[14] To determine which part of each specific RU was released at a given xanthan conformation, the ELSD peak areas of each RU present in digests obtained at $\alpha < 1$, are expressed as % of the maximum ESLD peak area of that RU.

RESULTS AND DISCUSSION

Order-disorder transitions

The normalized fitted graphs for the three xanthans, obtained through CD analysis, are shown in Figure 4.2. It can be concluded that xanthan C exhibits the lowest mid-point transition temperature (T_m). This was expected, as xanthan C has the highest pyruvate content, which destabilizes the ordered conformation.[5] Xanthans A and B have lower levels of pyruvylation and higher levels of acetylation compared to xanthan C. Thereby, they have a higher T_m compared to xanthan C. This correlation has been reported before for other xanthans.[5, 17]

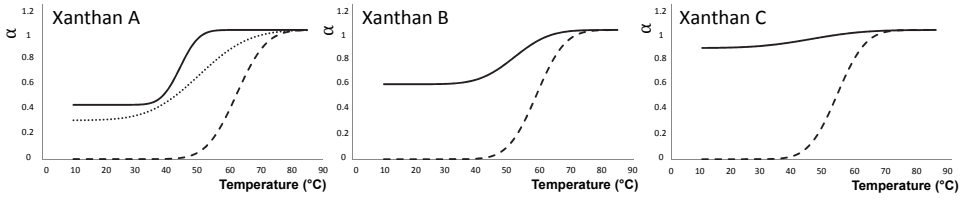


Figure 4.2. Fraction of disordered xanthan as function of temperature (˚C) in: deionized water (——), 2mM NaCl (·····) and 10 mM NaCl (– – – -). α = fraction of disordered conformation.

Although xanthans A and B have a rather similar molecular composition (Table 4.1.), their transitional behavior is different: xanthan B has lower transition temperatures than xanthan A, indicating a less stable ordered conformation.[5, 17] This difference is most likely the result of the differences observed in the substitution patterns of xanthan A and B. As xanthan A has a higher acetyl:pyruvate-ratio on the outer mannose than xanthan B (Table 4.1.), it is hypothesized that acetylation of the outer mannose increases the stability of the helical conformation.

The graphs were further used to determine which incubation conditions should be chosen to ensure a specific fraction of disordered conformation. An overview of all incubation conditions and the corresponding xanthan conformations is given in Table 4.2.

Table 4.2. Enzyme incubation conditions and the corresponding fraction of xanthan present in a disordered conformation (α)

Xanthan	Salt concentration (mM NaCl)	Temperature (°C)	Fraction of disordered conformation (α)
Xanthan A	10	30	0
		40	0
		50	0,05
		60	0,38
	0	30	0,42
		40	0,52
		50	0,93
		60	1
Xanthan B	10	30	0
		40	0
		50	0,11
		60	0,55
	0	30	0,58[a]
		40	0,62
		50	0,75
		60	1
Xanthan C	10	30	0
		40	0,03
		50	0,32
		60	0,82
	0	30	0,87[a]
		40	0,91
		50	0,95[a]
		60	1

[a]: HILIC-ESLD results are not further shown, as results were equal to the results of other digests of the same xanthan obtained at a similar α.

Molecular weight distribution of xanthan degradation products

For the present study it is assumed that, when xanthan appears in a partly disordered conformation, cellulases will completely degrade the disordered parts to the xanthan repeating units (RUs). The ordered parts remain as non-degraded high molecular weight material.[15] To determine whether this assumption indeed applies to the 3 xanthans used in this study, all xanthan digests were analyzed for the molecular weight distribution of the degradation products present. No intermediate degradation products were observed in the elution profiles obtained (data not shown) confirming the assumption made: disordered parts are completely degraded to RUs and ordered parts remain as high molecular weight material. The low molecular weight fractions of the digests obtained at various α can thus be used to study the influence of different primary xanthan structures on the dissociation behavior of xanthan.

Influence of the constituent repeating units on the xanthan conformation as monitored by cellulase fingerprinting of disordered xanthan segments

As only disordered xanthan segments are susceptible to enzymatic backbone degradation, degradation products present in a xanthan digest must have been part of a disordered xanthan segment. Analysis of the degradation products in xanthan digests obtained at given α will, therefore, enable the correlation between the transitional behavior of xanthan and its primary structure on RU level instead of molar level.

Contribution of the differently substituted repeating units to the stability of the xanthan conformation

Figure 4.3. shows the appearance of the individual RUs from 3 different xanthans obtained at various levels of disordered conformation and the dependence of their abundance on the type of xanthan. Clear differences in relative abundance of the RUs at different conformations could be recognized for the 3 xanthans. To enable the correlation between the xanthan conformation and the primary xanthan structure, the presence of each individual RU in the xanthan digests obtained at $\alpha \leq 0.95$ was expressed as percentage of the total amount of that RU present in xanthan (Figure 4.4.).

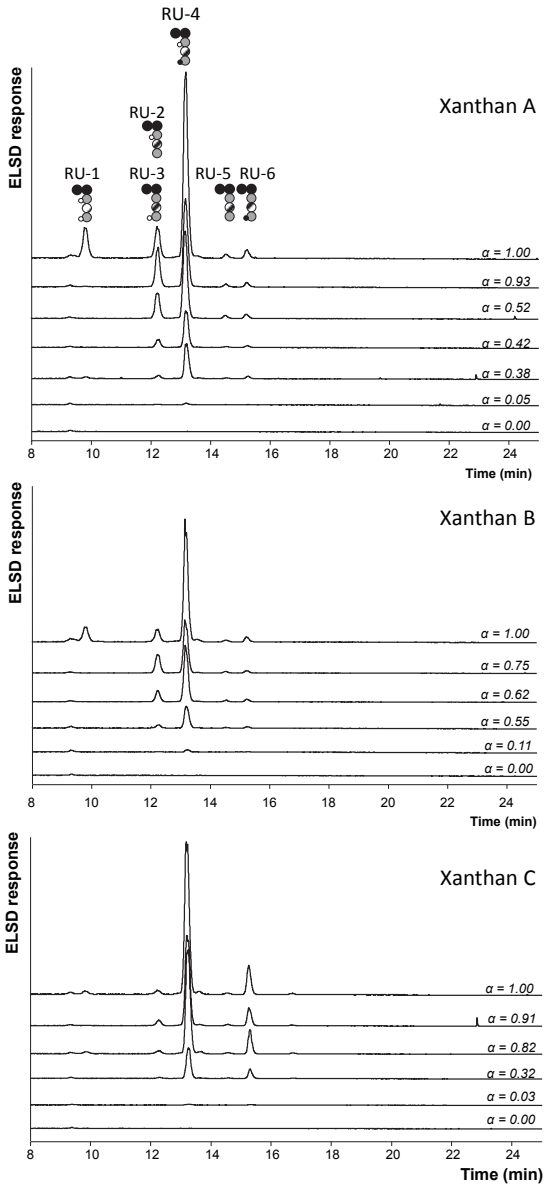
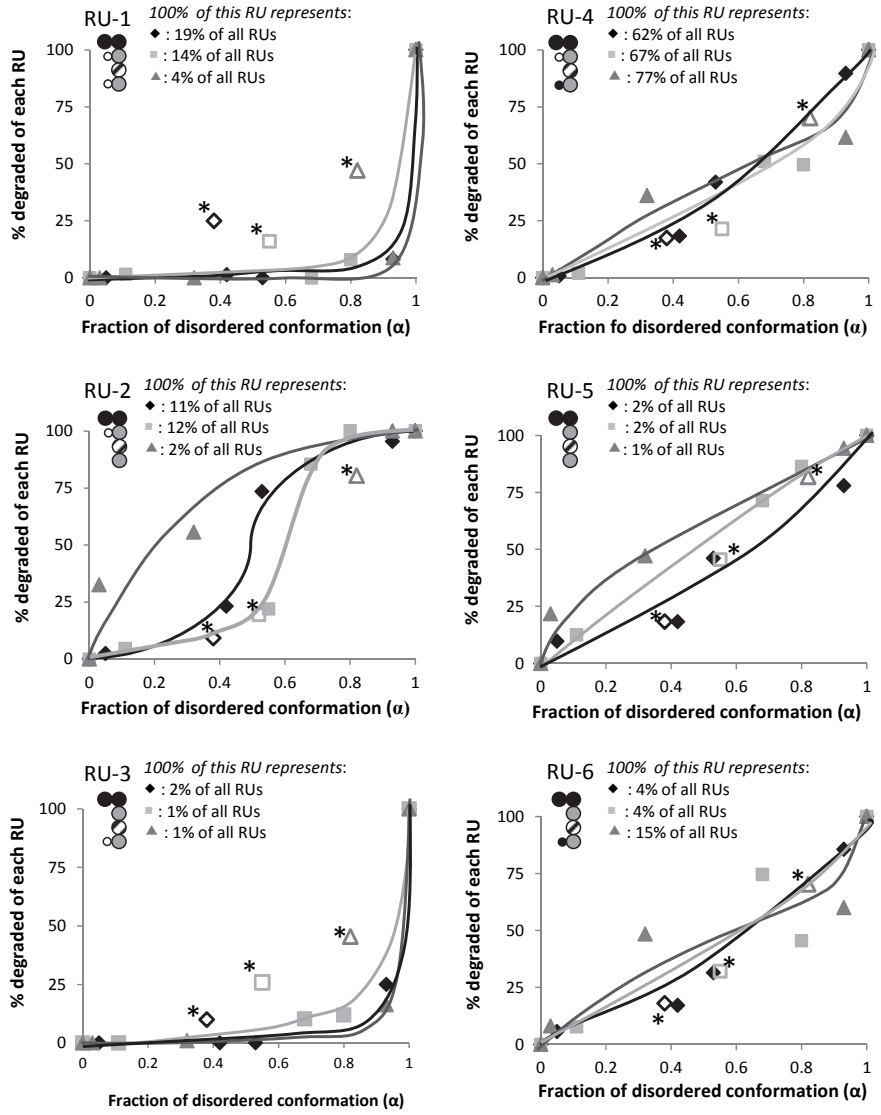


Figure 4.3. HILIC-ELSD elution profiles of xanthan digests obtained after a 48 h incubation with cellulases at different fractions of disordered conformation. Glucose ● ; mannose ● ; glucuronic acid ● ; acetyl group ○ ; pyruvic acid acetal ● .

The results suggest 3 different possible correlations between the xanthan conformation and the enzymatic release of a RU. An exponential trend is observed for the enzymatic release of double acetylated RU (RU-1) and the RU which is solely acetylated on the outer mannose (RU-3) when $\alpha \geq 0.8$. Digests obtained after incubation at 60˚C are an exception to this trend, which will be discussed later on. A Sigmoidal trend is observed for the enzymatic release of the RU that is solely acetylated on the inner mannose (RU-2). The xanthan conformation range in which this trend is observed differs with the type of xanthan: xanthan A $0.3 \leq \alpha \leq 0.6$; xanthan B $0.5 \leq \alpha \leq 0.65$ and xanthan C $0.0 \leq \alpha \leq 0.4$. Almost maximal enzymatic release of RU-2 is obtained already at $\alpha=0.7$ for all 3 xanthans. The remaining three RUs show a rather linear correlation between the xanthan conformation and their enzymatic release throughout the range $\alpha=0$-1. Because pyruvylated RUs, with (RU-4) and without (RU-6) acetylation at the inner mannose, show the same linear correlation between the xanthan conformation and their enzymatic release, the results indicate that acetylation on the inner mannose does not have a significant impact on xanthan's transitional behavior. Segments rich in acetyl groups on the outer mannose (RU-1 and RU-3), however, have a strong stabilizing effect on the ordered xanthan conformation as they are not present in chain segments unfolding easily. Previous studies,[12, 13] that assumed that only the inner mannose can be acetylated, concluded that acetyl groups on the inner mannose are responsible for xanthan's conformation stabilization. In contrast, we now conclude that the acetyl groups at the outer mannose stabilize the xanthan conformation. The substitutions *at the outer mannose* could, therefore, be key in the xanthan conformation and thus in xanthan's physical properties. As the total degree of substitution on the outer mannose is rather constant between xanthan samples, this could indicate that the acetyl:pyruvate-ratio on the outer mannose determines the transitional behavior of a xanthan sample. As xanthan A contains more RU-1 and RU-3 than xanthan B (21% and 15% respectively), these results are in line with the higher transition temperatures observed for xanthan A compared to xanthan B (Figure 4.2.).

Our results do not necessarily conflict with a previous study reporting that the position of the acetyl groups on either the inner or outer mannose did not influence xanthan's functionality.[18] The xanthans used in that study were produced by mutant strains and were either solely acetylated on the inner mannose or solely acetylated on the outer mannose. The disadvantage of the use of such xanthans is that they lack the pyruvate groups. Their solution behavior may, therefore, not be representative for standard xanthan, as used in our study.



* = Data from digests which were obtained after incubation at 60°C and are excluded from the trend line

Figure 4.4. Degradation pattern of the each xanthan repeating unit, expressed as % of the maximum release of that repeating unit, as function of the xanthan conformation based on HILIC ELSD peak area. Different lines represent the degradation pattern of the RUs in: Xanthan A (◆); xanthan B (■) and xanthan C (▲). glucose ● ; mannose ◍ ; glucuronic acid ◪ ; acetyl group ○; pyruvic acid acetal ● .

Proposed distribution pattern of the xanthan repeating units

The trends observed for the correlation between the enzymatic release of the six types of RU and the xanthan conformation, provides tentative information on the distribution of the different RUs over the xanthan backbone.

RU-4 constitutes at least 60% of all RUs present in the xanthan samples. The linear trend observed for the enzymatic release of this RU, indicating a rather random distribution of this RU, is not surprising. RU-5 and RU-6, each responsible for only ~5% of all side chains, also show a linear trend for their enzymatic release and are thus randomly distributed as well, following the distribution of RU-4.

RU-1 and RU-3, both acetylated on the outer mannose, have exponential trends for their enzymatic release and RU-2, which is solely acetylated on the inner mannose, exhibits a sigmoidal trend (Figure 4.4.). Based on the sudden release of these RUs at a certain xanthan conformation, it is proposed that these RUs are distributed in a semi block wise manner. This could indicate that a minimal abundance of these RUs in a xanthan segment already controls the unfolding behavior of that specific segment. Segments enriched in RU-1 and RU-3 only unfold when $\alpha \geq 0.8$ and segments enriched in RU-2 all unfold at $\alpha \leq 0.6$. The linear correlation between the release of RUs 4-6 and the xanthan conformation could thus be explained by a random distribution of these RUs over xanthan segments which are either enriched with RU-1 and RU-3, unfolding at high levels of disordered conformation *or* enriched with RU-2, unfolding at low levels of disordered conformation. A more precise description of the distribution of the different RUs cannot be given on the basis of the current research. Further research on larger xanthan oligosaccharides, consisting of several RUs, should reveal the exact distribution of the different RUs along the xanthan backbone.

Interactions stabilizing the xanthan conformation – Effect of the solvent conditions on the release of individual repeating units.

It is known that different types of molecular interactions are differently influenced by ionic strength and/or temperature: an increase in ionic strength especially reduces the electrostatic repulsion forces through shielding of the negative charges; an increase in temperature reduces electrostatic interactions and reduces hydrogen bonding up to a certain temperature.[19, 20] By studying the influence of the solvent conditions on the unfolding behavior of different xanthan segments it is, therefore, possible to determine which interactions are involved in the stabilization of the xanthan conformation. Xanthan A was used for this. Based on the transition profiles (Figure 4.2a.), solution conditions were chosen in which the xanthan A conformation was constant (at $\alpha \sim 0.50$ and ~ 0.80), but in which the ionic strength and temperature

of the xanthan solutions differed. The relative abundance of the RUs present in the cellulase digests obtained after incubation at the chosen conditions was compared. The results are shown in Table 4.3. The two digests obtained at elevated temperature (2mM NaCl 55˚C; 10 mM NaCl 60˚C) were relatively rich in RUs carrying an acetyl group on the outer mannose. The digests obtained at low temperatures in demineralized water did not contain such RUs, but were relatively rich in pyruvylated RUs. Due to the absence of salts in these digests, the negative charges of the pyruvate groups are not shielded. The relatively high abundance of pyruvylated RUs in these digests can thus be explained by increased electrostatic repulsion, making these RUs available for cellulase degradation. As segments rich in outer mannose acetylated RUs only unfolded at elevated solution temperatures, these type of RUs most likely stabilize the xanthan conformation through the formation of hydrogen bonds. Changes in temperature did not influence the dissociation behavior of segments that are rich in RUs that are solely acetylated on the inner mannose. It is, therefore, concluded that the acetyl groups on the outer mannose units, and not on the inner mannose, are involved in intra- and/or intermolecular interactions through hydrogen bonding.

Table 4.3 Influence of the solvent conditions on the relative abundance of the repeating units released during the enzymatic xanthan degradation at fixed xanthan conformations

Incubation condition	Conformation (α)	Total degradation (%)	RU-1	RU-2	RU-3	RU-4	RU-5	RU-6
10 mM NaCl; 60°C	0.48	34	16	16	2	59	3	4
Millipore; 40°C	0.52	37	0	18	0	71	5	6
2 mM NaCl; 55°C	0.78	87	6	25	3	56	5	5
Millipore; 45°C	0.81	85	0	22	0	70	3	5

● glucose; ◍ mannose; ▨ glucuronic acid; ○ acetyl groups; ● pyruvic acid ketal

CONCLUSIONS

This study has shown, in contrast to previous reports, that especially those RUs that are acetylated on the outer mannose unit are involved in the stabilization of the ordered xanthan conformation by hydrogen bonding. Acetylation of the inner mannose does not have a significant effect on the conformation stability. Based on the correlation between the α and the enzymatic release of individual RUs it is postulated that pyruvylated RUs are randomly distributed over the xanthan backbone and that double and single acetylated RUs are organized in a more or less block wise manner. Furthermore, the results point out that it is possible to control and direct the *exact* transitional behavior of a xanthan molecule by controlling the solvent conditions.

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Chapter 5

Characterization of an acetyl esterase from *Myceliophthora thermophila C1* able to deacetylate xanthan

ABSTRACT

Screening of eight carbohydrate acetyl esterases for their activity towards xanthan resulted in the recognition of one active esterase. AXE3, a CAZy family CE1 acetyl xylan esterase originating from *Myceliophthora thermophila* C1, removed 31% of all acetyl groups present in xanthan after a 48h incubation. AXE3 activity towards xanthan was only observed when xanthan molecules were in the disordered conformation. Optimal performance towards xanthan was observed at 53°C in the complete absence of salt, a condition favoring the disordered conformation. AXE3-deacetylated xanthan was hydrolyzed using cellulases and analyzed for its repeating units using HILIC-ELSD-MS^n. It showed that AXE3 specifically removes the acetyl groups positioned on the inner mannose and that acetyl groups positioned on the outer mannose are not removed at all. After a prolonged incubation at optimal conditions, 60% of all the acetyl groups, representing 75% of all acetyl groups on the inner mannose units, were hydrolyzed.

Submitted as: Marijn M. Kool; Henk A. Schols; Martin Wagenknecht; Sandra W.A. Hinz; Bruno M. Moerschbacher and Harry Gruppen. ***Submitted for publication***

INTRODUCTION

The bacterial polysaccharide xanthan is a polymer having a β-1,4-glucan backbone, carrying a glycosidically linked α-D-mannose-(2→1)-β-D-glucuronic acid-(4→1)-β-D-mannose side chain on the *O*-3-position of every second glucose unit (Figure 5.1.).[1] Approximately 85% of all inner mannose units are acetylated at the *O*-6 position and 50-70% of all terminal mannose units are substituted with a pyruvic acid ketal. Additionally, about 5-25% of all terminal mannose units are substituted with an acetyl group at the *O*-6 position.[2, 3]

The acetyl and pyruvate groups of xanthan are known to have a large influence on the viscosity of xanthan solutions and their stability towards the addition of salts, changes in temperature, and variations in solvent acidity.[4-6] Lowering the degree of xanthan acetylation results in improved viscosity and stability of xanthan solutions as well as in improved interactions of xanthan with galactomannans.[4, 7] How the position of acetyl influences the xanthan functionality, however, remains unknown. Targeted removal of specific acetyl groups from the xanthan side chains would thus be useful to further explore the functionality of xanthan. To date, acetyl groups are removed using an alkali treatment.[8, 9] However, such a process randomly removes acetyl groups and backbone degradation might be apparent.

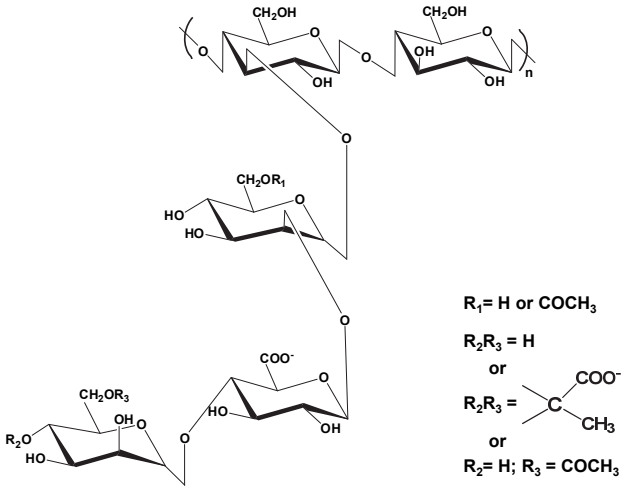

Figure 5.1. The xanthan repeating unit.

Another method to control the acetyl levels in xanthan is the use of specific *Xanthomonas* strains and/or fermentation conditions for the xanthan production.[10, 11] Altering the fermentation conditions of xanthan, however, also influences the pyruvate levels in the xanthan produced, which are also known to be of great importance for xanthans functionality.[12] Therefore, both methods described are not applicable for the specific removal of acetyl groups. Targeted modification using enzymes that specifically remove acetyl groups from xanthan would be more useful. However, such enzymes have not been described to date. Because no xanthan acetyl esterases are known, targeted database mining for xanthan acetyl esterases is not possible. Nevertheless, due to the similarity in the mode of action of different carbohydrate acetyl esterases, the classification of carbohydrate acetyl esterases is far less specific compared to classification of the carbohydrate hydrolases and carbohydrate lyases.[13] The exact activities of carbohydrate acetyl esterases are, therefore, much less predictable compared to other carbohydrate-active enzymes. Many carbohydrate acetyl esterases, especially from CAZy families CE1, CE3 and CE6, are known to have an a-specific binding site.[14, 15] Hence, searching for xanthan acetyl esterases could be done by testing known carbohydrate acetyl esterases for their activity towards xanthan. However, such a screening for xanthan modifying enzymes is not fully straight forward. Several studies on the enzymatic degradation of the xanthan backbone by cellulases have shown that the conformation of xanthan in solution is critical for enzymatic degradation.[16-18] In solution xanthan can adapt an ordered helical conformation or a random disordered conformation.[19, 20] Although the exact nature of the helical conformation is still under debate, it is believed that the xanthan side chains are aligned with the xanthan backbone.[21, 22] This alignment of side chains when xanthan appears in an ordered conformation and/or the stacking of order structures into a network is assumed to make xanthan resistant against enzymatic modifications.[17, 18] Recently, it was proven that only xanthan molecules that appear in a disordered conformation are susceptible to enzymatic backbone degradation by cellulases.[16] When screening carbohydrate acetyl esterase for possible side activities towards xanthan, the xanthan conformation should thus be taken into account.

In this study several carbohydrate acetyl esterases were screened for their activity towards xanthan appearing in an ordered or in a partly disordered conformation. The temperature optimum of the active enzyme was determined and the influence of the xanthan conformation on the enzyme activity was studied in detail. Structural analysis of the enzymatically modified xanthan was performed to determine the specificity of the active enzyme with respect to acetylation of the inner or outer mannose unit.

MATERIALS AND METHODS

Chemicals and substrates

All chemicals used were, if not mentioned otherwise, of analytical grade. The xanthan used was obtained by DuPont (Melle, France) and characterized as described previously.[3] A detailed overview of the chemical characterization is given in Table 5.1. Acetylated xylooligosaccharides were obtained and characterized as described by Koutaniemi et al.[23] Sugar beet pectin (SBP6230)[24] was obtained from DuPont (Brabrand, Denmark). Chitin and chitosan oligosaccharides (degree of polymerization of 2–6) were purchased from Seikagaku Corp. (Tokyo, Japan).

Circular Dichroism

Far-UV CD spectra of 2 mg·mL^{-1} xanthan in 0, 1, 2, 5 and 10 mM NaCl solutions were measured at 20, 40 and 70°C using a Jasco-J-715 spectropolarimeter (JASCO, Tokyo, Japan). A quartz cuvette with an optical path of 1 mm was used. The temperature was regulated using a PTC-348 WI controller (JASCO). In the 190-300 nm wavelength region (0.2 nm resolution) 10 scans were accumulated with a scan rate of 100 nm·min^{-1} and a time constant of 0.125 s. The final spectra are the average of these scans. Prior to the spectral analysis the wavelength scans were corrected for the buffer background signal.

Previous research has shown that the decrease in ellipticity (θ) at 219 nm correlates almost linearly with the fraction of xanthan present in the disordered conformation.[16, 21] The fraction of disordered conformation (α) was, therefore, estimated using Equation 1 with: θ_s= ellipticity of the sample at 219 nm; θ_U = ellipticity of a completely disordered structure at 219 nm and θ_F = ellipticity of a completely ordered structure at 219 nm.

$$\alpha = (\theta_s - \theta_F) / (\theta_U - \theta_F) \quad (1)$$

The ellipticity of θ_F was determined in 10 mM NaCl solutions at 20°C and the ellipticity of θ_U was determined in Millipore water 70°C.

Table 5.1 Chemical characterization of xanthan

	Glc:Man:GlcA Molar Ratio	Acetyl content (w/w%)	Pyruvate content (w/w%)	RU-1	RU-2	RU-3	RU-4	RU-5	RU-6
Xanthan	1 : 0.88 : 0.41	5.6	4.4	19	11	2	62	2	4

● glucose; ◍ mannose; ⊘ glucuronic acid; ○ acetyl groups; ● pyruvic acid ketal

Carbohydrate acetyl esterases

An overview of the carbohydrate acetyl esterases tested, their origin and their known substrate specificities is given in Table 5.2. AXE2 and AXE3,[25, 26] both produced by *Myceliophthora thermophila* C1 (formerly termed *Chrysosporium lucknowense* C1[27]), were obtained from Dyadic (Wageningen, The Netherlands). AnAXE and RG-04 were extracted from *Aspergillus spp*. preparations as described by Kormelink[28] and Searle-van Leeuwen,[29] respectively. The coding sequences of CDA-II, CDA-III, PAE2 and PAE4 were cloned in pET22b-StrepIIc, a pET-22b(+) (Novagen, Merck KGaA, Darmstadt, Germany) derivative that additionally contains a sequence coding for the StrepII affinity tag, and heterologously expressed in *E. coli* Rosetta 2(DE3)(pLysSRARE2) (Novagen) using auto-induction medium.[30] Enzyme purification was done by affinity chromatography using a 1 ml Strep-Tactin Superflow Plus Column (Qiagen, Hilden, Germany) as described elsewhere.[31]

Enzyme assays

All enzymes were desalted prior to testing their activity towards xanthan using Micro Bio-Spin chromatography columns following the company's description (Bio-Rad Laboratories, Hercules, CA, USA).

The carbohydrate acetyl esterases were screened for their activity towards xanthan by incubating 1 mL of a 2 mg·mL^{-1} xanthan solution with 8–35 µg enzyme at 40°C for 48 h. The enzyme activity was tested in Millipore water, 10 mM NaCl solutions and 50 mM sodium citrate buffer (pH 6.0). The acetic acid release was determined using a Megazyme acetic acid kit (Megazyme, Wicklow, Ireland) and expressed as percentage of the total acetyl content present in the parental xanthan. The company's protocol was downscaled to microtiter plate scale to enable medium throughput analysis. The total acetyl content was determined by the analysis of the acetic acid released after a saponification step with 1 M NaOH (18 h; 4˚C). All incubations were performed in duplicate.

Table 5.2. Characterization of the carbohydrate acetyl esterases used in this study, including abbreviations, origin, Gene bank accession number, CAZy family and references

Known activity	Abbreviation	Origin	GeneBank Accession number	CAZy family	Reference
Acetyl xylan esterase	AXE2	*Myceliophthora thermophila* C1	HQ324256	CE-5	Pouvreau (2011); Hinz (2009)
	AXE3	*Myceliophthora thermophila* C1	HQ324257	CE-1	Pouvreau (2011); Hinz (2009)
	AnAXE	*Aspergillus niger*	unknown	CE-1	Kormelink (1993)
Chitin deacetylase	CDA-II	*Bacillus licheniformis* DSM 13	AAU39149	CE-4	Not available
	CDA-III	*Bacillus licheniformis* DSM 13	AAU39762	CE-4	Not available
Pectin acetyl esterase	PAE2	*Pectobacterium atrosepticum* SCRI1043	CAG75311	Unknown	Not available
	PAE4	*Bacillus licheniformis* DSM 13	AAU42913	CE-12	Remoroza (2013)
	RG-04	*Aspergillus aculeatus*	unknown	CE-12	Searle-van Leeuwen (1992)

Acetyl esterase AXE3 was further characterized for its action on xanthan. The precise influence of the xanthan conformation on the enzyme activity was determined by incubating 2 mg·mL^{-1} xanthan in Millipore water, 1 mM, 2 mM, 5 mM and 10 mM NaCl solution with 25 µg of protein at 40°C for 48 h. The temperature optimum was determined in a salt free environment within the temperature range 35–60°C after a 24 h incubation. The specific activity of AXE3 towards the xanthan was determined after a 24 h incubation at 55˚C in a salt free environment. A 24 h incubation for xanthan was chosen as the reaction still is in the linear range and shorter incubation times would not release sufficient amounts of acetic acid to accurately determine the specific activity. All incubations were performed in four replicates.
The mode of action of AXE3 towards xanthan was determined by structural analysis of unmodified and enzymatically deacetylated xanthan. Solutions of the latter were dialyzed against Millipore water and lyophilized. The obtained xanthan was redissolved in Millipore water (2 mg·mL^{-1}) and incubated with the cellulase preparation C1-G1 from *Myceliophthora thermophila* C1 (Dyadic Netherlands, Wageningen, The Netherlands) at 60°C for 48 h.[16] The xanthan digests obtained were analyzed for their repeating units using HILIC-ELSD-ESI-IT-MS^n as described previously.[3]

RESULTS AND DISCUSSION

Screening for xanthan acetyl esterase activity

All acetyl esterases (AEs) available in our laboratories were tested for their activity towards xanthan. The enzyme activities of the AEs were tested at different solvent conditions in order to analyze the activity towards different xanthan conformations.
None of the pectin and chitin AEs (Table 5.2.) was able to release acetic acid from xanthan at all incubation conditions tested (data not shown). In the presence of salts, when xanthan appears in a completely ordered conformation,[16] none of xylan AEs released significant amounts of acetic acid from xanthan either. In the absence of salts, AXE3 (CE1 family) was able to release approximately 30% of all acetyl groups. Previous experiments[16] showed that under these conditions approximately 80% of all xanthan molecules appears in a disordered conformation. It is, therefore, likely to assume that AXE3 can only deacetylate xanthan that is in a disordered conformation, probably because the acetyl groups are only accessible in that conformation. Consequently, enzymatic deacetylation of xanthan might only be possible by those AEs that are active under conditions that favor the disordered conformation. Hence, the activity of all AEs towards their model substrate (acetylated sugar beet pectin, acetylated xylan oligosaccharides or chitin/chitosan oligosaccharides) was tested in a

salt free solution at 40˚C. None of the enzymes, except AXE3, was active against their known specific substrate at this condition, while all enzymes showed the expected activity in the presence of salt (data not shown). Except for AXE3, none of the enzymes is, therefore, active under conditions favoring the disordered conformation, which could explain why only AXE3 was found to be active towards xanthan.
Previous research towards AXE2 (CE5 family) and AXE3 (CE1 family) both from *M. thermophila* C1, showed that both enzymes, although from different CE families, showed similar activity towards acetylated xylan oligosaccharides.[25, 26] However, substrate specificity tests showed that AXE2 has a more specific mode of action. More general studies including acetyl xylan esterases from different CE families showed that enzymes from CE family 1, to which AXE3 belongs, have a lower specificity for xylans than acetyl esterases from other CE families.[14] The difference observed between the activities towards xanthan of AXE2 and AXE3, even though they origin from the same bacterial host, can probably be explained by differences in their substrate specificity and/or substrate binding.

Influence of the xanthan conformation on the acetic acid release by AXE3

To be able to determine the influence of the xanthan conformation on the AXE3 activity in more detail, the conformation of xanthan at 40˚C in solutions with different NaCl concentrations was analyzed using circular dichroism (CD). The measured ellipticity (θ) at 219 nm and the corresponding fraction of disordered conformation (α) are given in Table 5.3. As the exact xanthan conformation is now known for the different incubation conditions, it is possible to correlate α to the ability of AXE3 to release acetic acid from xanthan (Figure 5.2). With an increasing α, an increase in the acetic acid release is observed, indicating that AXE3 is indeed only active towards disordered xanthan fragments. Several studies on the xanthan conformation proposed that the acetyl groups positioned on the inner mannose unit show interaction with the xanthan backbone.

Table 5.3. The ellipticity at 219 nm (θ_{219}) and the corresponding fraction of disordered conformation (α) of xanthan (2 mg·mL^{-1}) under different solvent conditions derived from far-UV CD spectra

Sample condition	θ_{219}	α
Millipore water 70˚C	-3.89	1.00
Millipore water 40˚C	-3.02	0.78
1 mM NaCl 40˚C	-1.37	0.35
2 mM NaCl 40˚C	-0.93	0.24
5 mM NaCl 40˚C	-0.55	0.14
10 mM NaCl 40˚C	-0.20	0.05
10 mM NaCl 20˚C	0.00	0.00

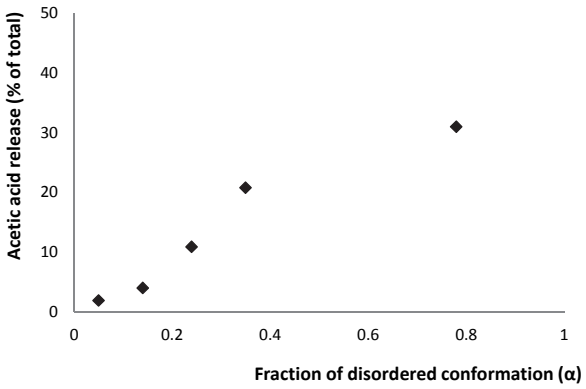

Figure 5.2. Influence of the xanthan conformation (α) on the acetic acid release from xanthan after a 48 h incubation with AXE3 at 40˚C.

These acetyl groups would, therefore, be positioned at the inside of the xanthan helix[21, 32, 33] and would thus not be accessible for acetyl esterases when xanthan is in the ordered conformation.

In a recent study we showed that, depending on the xanthan production process, approximately 20% of all acetyl groups can be positioned on the outer mannose.[3] Whether these acetyl groups also fold to the inside of the helical conformation is unknown. However, it is known that xanthan lyases are able to remove the outer mannose in the presence of salts,[34, 35] indicating that the outer mannose is accessible for enzymes when xanthan appears in an ordered conformation. Because AXE3 is only active towards disordered xanthan fragments, it is expected that AXE3 is specific for the removal of the acetyl groups from the inner mannose unit.

Characterization of the AXE3 activity towards xanthan

Temperature optimum

Figure 5.3. shows the temperature optima of AXE3 towards xanthan in a salt free environment and towards acetylated xylan oligosaccharides at its optimal pH of 7.0.[26] AXE3 has a clear temperature optimum for xanthan deacetylation between 50-55˚C, whereas a broader and lower temperature optimum (35-45˚C) was observed for the deacetylation of xylan oligosaccharides at pH 7.0. With increasing temperature, the xanthan conformation changes to a more disordered structure, which is necessary for AXE3 to be active.

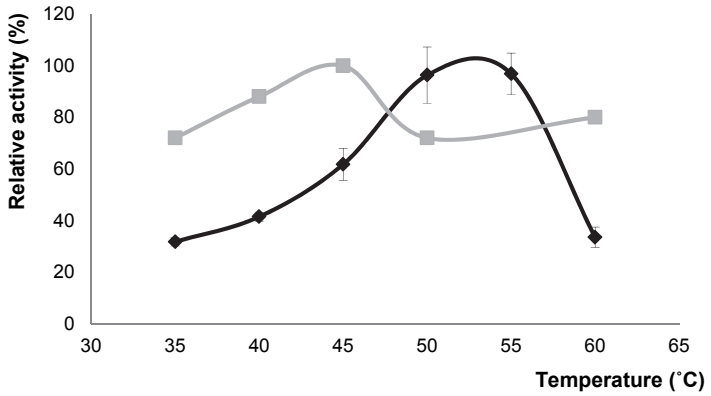

Figure 5.3. Temperature optimum of AXE3 towards xanthan (◆) and acetylated xylan oligosaccharides (■) at their optimal conditions. The data for acetylated xylan are based on Pouvreau et al.[26]

The higher temperature optimum observed for the AXE3 deacetylation of xanthan, compared to the deacetylation of xylan might, therefore, be the result of both increased substrate accessibility and the enzyme inactivation at elevated temperatures.

Specific activity

The specific activity of AXE3 towards xanthan was determined at its optimal conditions and compared to the specific activity towards xylan as determined by Pouvreau et al.[26] The specific activity towards acetylated xylooligosaccharides is 8.3 U·mg protein^{-1}. The specific activity towards xanthan is 600x lower: 13 mU·mg protein^{-1}. It is, therefore, concluded that although the enzyme can remove acetyl groups from xanthan, the annotation of the enzyme being an acetyl xylan esterase is fully correct.

Characterization of the enzymatically modified xanthan

Xanthan was partly deacetylated by incubating xanthan at 55˚C with AXE3 for 1, 2 and 3 days, resulting in the release of 17%, 47% and 58% of all acetyl groups, respectively. Subsequently, the relative abundance of the repeating units (RUs) present in cellulase digests of the partly deacetylated xanthans was analyzed using HILIC-ELSD-MS^2 and compared to the relative abundance of the RUs present in the cellulase digest of unmodified xanthan (Figure 5.4.). Quantification of the exact modifications introduced by AXE3, based on the ELSD response, remains difficult due to a lack of proper standards.

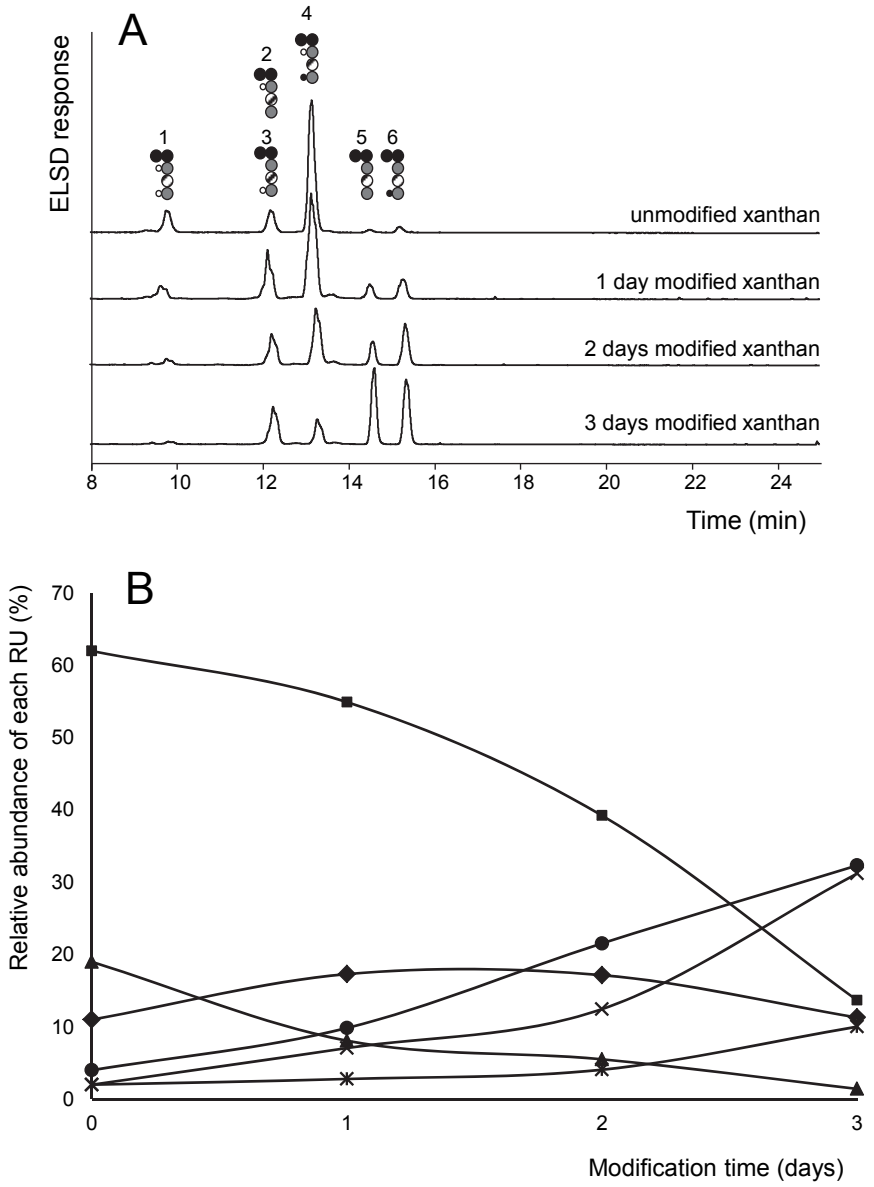

Figure 5.4. AXE3 modification of xanthan followed in time. A) HILIC-ELSD elution profiles of cellulase digests of unmodified xanthan and xanthan modified with AXE3 for 1 day, 2 days or 3 days. Glucose ●; mannose ◍; glucuronic acid ◴ ; acetyl groups ○ ; pyruvic acid ketal ● . B) Relative abundance of the 6 xanthan repeating units present in cellulase digests of xanthan at different levels of AXE3 modification. RU-1 (▲); RU-2 (◆); RU-3 (*); RU-4 (■); RU-5 (✕); RU-6 (●)

The observed trend of modification, however, is absolutely clear: After a one day treatment with AXE3, the double acetylated RUs and the acetylated+pyruvylated RUs decreased slightly in their abundance. The amount of single acetylated RUs, unsubstituted RUs and pyruvylated RUs slightly increased. Extending the AXE3 treatment resulted in a further increase of the unsubstituted RUs and pyruvylated RUs, while all the other RUs decrease in their abundance. After a 3 day treatment with AXE3 almost no double acetylated RUs or acetylated+pyruvylated RUs are left in the AXE3 deacetylated xanthan. However, single acetylated RUs remain present. As AXE3 is hypothesized to be specific for the deacetylation of the inner mannose, the double acetylated repeating unit would be converted into single acetylated RUs that are acetylated on the outer mannose. Simultaneously, single acetylated RUs that are acetylated on the inner mannose are converted into unsubstituted RUs. As the two single acetylated RUs elute simultaneously from the HILIC column,[3] no clear decrease in the total amount of single acetylated RUs would be observed in the HILIC-ELSD profile. Nevertheless, changes in the ratio between the two single acetylated RUs, induced by the AXE3 modification, can be determined using the MS-fragmentation pattern, as inner mannose acetylated RUs and outer mannose acetylated RUs have a different set of diagnostic fragment ions.[3] The intensity of these different fragments, was used to determine the ratio in which the two single acetylated were present before and after AXE3 modification (Figure 5.5.). Before modification, ~10% of all single acetylated RUs are acetylated on the outer mannose, while this value increased to ~50% after a 3 day modification. This indicates that the RUs that are acetylated on the outer mannose accumulate during AXE3 deacetylation. It is, therefore, concluded that AXE3 is highly specific for the removal of acetyl groups on the inner mannose unit and that the acetyl groups on the outer mannose are not hydrolyzed. Characterization of the unmodified xanthan structure showed that ~80% of all acetyl groups are substituted to the inner mannose. After a 3 day incubation ~60% of all acetyl groups are removed by AXE3, which corresponds to the removal of ~75% of all acetyl groups positioned on the inner mannose unit. Whether complete removal of the acetyl groups on the inner mannose is possible remains uncertain, as the incubation time was not further extended in this research.

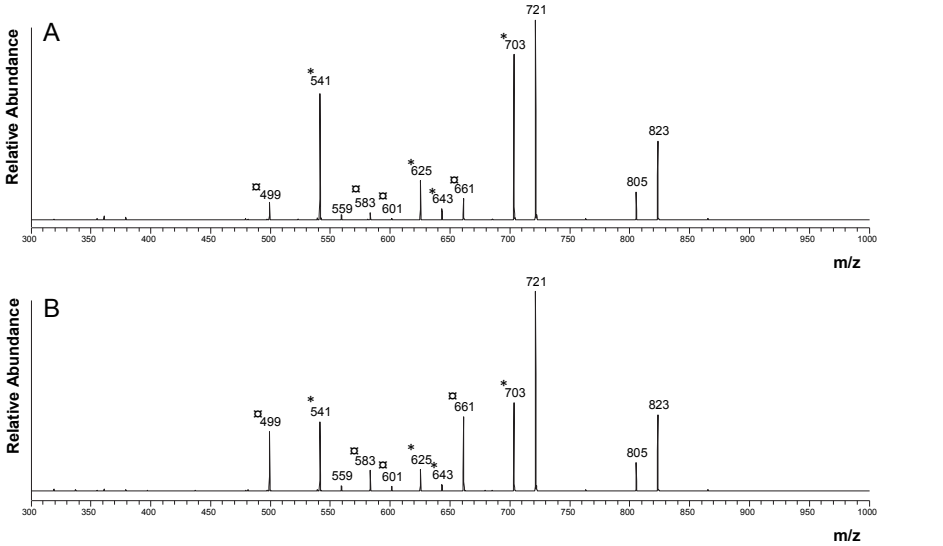

Figure 5.5. MS^2-fragmentation pattern of the single acetylated repeating unit (eluting at 12.5 min in Figure 5.4a.) present in the cellulases digests of A) unmodified xanthan and B) 3 days AXE3 modified xanthan.

CONCLUSIONS

Acetyl xylan esterase 3 (AXE3) a CE family 1 esterase originating from *Myceliophthora thermophila* C1, is capable of removing 60% of all acetyl groups is xanthan. Enzyme activity was only observed when xanthan is present in the disordered conformation. Although the specific activity of AXE3 towards xanthan is very low, it is the first acetyl esterase reported that is active towards xanthan. Structural characterization of the AXE3 modified xanthan showed that AXE3 is specific for the removal of the acetyl groups positioned on the inner mannose unit, although after a 3 day incubation not all acetyl groups were removed. As the xanthan conformation showed to be important for the enzymatic deacetylation of xanthan, it is concluded that screening for potential xanthan AEs should include incubation conditions that support both the disordered as well as the ordered xanthan conformation.

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Chapter 6

Characterization of an acetyl esterase from *Bacillus subtilis* strain 168 able to deacetylate the outer mannose of xanthan

ABSTRACT

YesY, a pectin acetyl esterase originating from *Bacillus subtilis* strain 168, removed 22% of all acetyl groups present in xanthan after a 24h incubation. YesY activity towards xanthan was mainly observed in the presence of salts, when xanthan molecules appear in the ordered conformation. Optimal performance towards xanthan was observed at pH 6.3 and 55°C. YesY deacetylated xanthan was hydrolyzed using cellulases and the digest was analyzed for its repeating units present. It was shown that YesY is specific for the removal of the acetyl groups positioned on the outer mannose and that acetyl groups positioned on the inner mannose are not removed at all. After extended incubation, all acetyl groups on the outer mannose residues were hydrolyzed.

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To be submitted

INTRODUCTION

Xanthan, the exopolysaccharide produced by *Xanthomonas spp*., is widely used in industry as rheology modifier and food stabilizer.[1, 2] In solution, xanthan adapts a secondary ordered conformation,[3, 4] making the viscosity of xanthan solutions rather stable over a wide pH and temperature range and towards addition of salts.[2, 5] The stability of this secondary conformation towards the addition of salt and towards changes in temperature is strongly affected by the primary xanthan structure.[6-8] Xanthan has a β-1,4-linked glucan backbone with trisaccharide side chains linked to every other glucose unit (Figure 6.1.). The side chains consist of (3→1) linked α-D-mannose-(2→1)-β-D-glucuronic acid-(4→1)-β-D-mannose units.[9] The inner mannose unit is mostly acetylated and the outer mannose can be pyruvylated (~65%), acetylated (~15%) or unsubstituted (~20%).[10] Removal of pyruvate groups results in solutions with a lower but more stable viscosity.[11, 12] The removal of acetyl groups results in stronger interactions with galactomannans and gives rise to xanthan solutions with increased viscosity.[13, 14] Furthermore, the viscosity stability of the solution, especially at low pH, increases significantly due to deacetylation.[8] Consequently, the production of xanthan with controlled acetyl levels could be useful to broaden xanthans applications in industry.

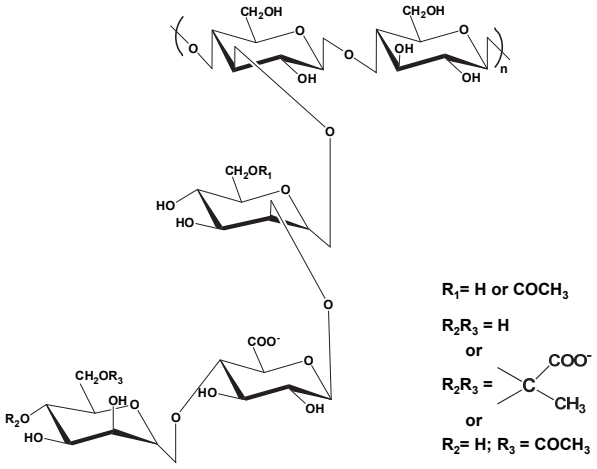


Figure 6.1. The xanthan repeating unit.

Acetyl groups can easily be removed from xanthan by saponification.[15] However, this process is random and the removal of pyruvate groups and degradation of the xanthan backbone can also occur, which will result in a loss in viscosity. A more controlled method to influence the acetyl levels in xanthan is the production of xanthan using mutant strains.[14] A disadvantage of the use of mutant strains to control the acetyl levels in xanthan is that partial removal of the acetyl groups is not possible.[14] Furthermore, mutations in one part of the *Xanthomonas* genome can affect other aspects of the xanthan biosynthesis as well, e.g. degree of pyruvylation, molecular weight.[16] Complete control of the xanthan structure produced is, therefore, not possible. Additionally the use of xanthans produced by genetically modified strains is prohibited in Europe, that limits the possible application of the non-acetylated xanthans produced (Regulation (EC) No 1830/2003).
Enzymatic removal of the acetyl groups could be a better method for the production of xanthans with low levels of acetylation. However, to date only one enzyme has been described, that can partly deacetylate xanthan.[17] This enzyme exclusively removes the acetyl groups from the inner mannose unit and is only active towards xanthan in the absence of salt, when xanthan appears in the random disordered xanthan conformation. Recently, a pectin acetyl esterase from *Bacillus subtilis* strain 168 was described, which was found to have a side activity towards xanthan.[18] In the present study this new enzyme is further characterized for its activity towards xanthan.

MATERIALS AND METHODS

Chemicals and substrates

All chemicals used were, if not mentioned otherwise, of analytical grade. The xanthan used was kindly provided by DuPont (Melle, France) and characterized.[10] A detailed overview of the chemical characterization is given in Table 6.1.

Table 6.1. Chemical characterization of xanthan

	Glc:Man:GlcA Molar Ratio	Acetyl content (w/w%)	Pyruvate content (w/w%)	RU-1	RU-2	RU-3	RU-4	RU-5	RU-6
Xanthan	1 : 0.88 : 0.41	5.6	4.4	19	11	2	62	2	4

● glucose; ◯ mannose; ⊘ glucuronic acid; ○ acetyl groups; ● pyruvic acid ketal

Enzyme assays

The coding sequence of YesY[19] was cloned in pET22b-StrepIIc (Novagen, Merck, KGaA, Darmstadt, Germany), and heterologously expressed in *E. coli* Rosetta 2(DE3)(pLysSRARE2) (Novagen). Enzyme purification was done as described elsewhere.[20]

Enzyme incubations were initially performed at two xanthan concentrations to rule out the effect of a high solution viscosity on the enzyme activity at higher substrate concentrations. Xanthan solutions, 2 mg·mL^{-1} and 5 mg·mL^{-1}, were incubated with 4 µg YesY·mg xanthan^{-1}. Incubations were performed in 50 mM citric acid buffer pH 6 for 24 h at 40°C.

The effect of the enzyme to substrate ratio on the acetic acid release by YesY was determined by incubation of 2 mg·mL^{-1} xanthan solutions in 50 mM sodium citrate buffer pH 6.0, with enzyme concentrations ranging from 5.4 µg to 81 µg YesY·mg xanthan^{-1}

The influence of the xanthan conformation on the enzyme activity was determined by incubating 2 mg·mL^{-1} xanthan in Millipore water, 1 mM, 2 mM, 5 mM and 10 mM NaCl solutions with 54 µg YesY·mg xanthan^{-1} at 40˚C for 24 h. The pH of the solutions was ~5.6. As control, the influence of all the solution conditions on the YesY activity towards pectin was also investigated.

The temperature optimum was determined in 50 mM sodium citrate buffer pH 6.0 within the temperature range 30-80°C after a 6 h incubation of 2 mg·mL^{-1} xanthan solutions with 54 µg YesY·mg xanthan^{-1}. The pH optimum was determined in 50 mM McIlvain buffers with pHs 3–8 after a 6 h incubation of 2 mg·mL^{-1} xanthan solutions with 54 µg YesY·mg xanthan^{-1} at 40˚C.

The acetic acid release was determined using a Megazyme acetic acid kit (Megazyme, Wicklow, Ireland) and expressed as percentage of the total acetyl content in the parental xanthan. The company's protocol was downscaled to microtiter scale. The total acetyl content was determined by the analysis of the acetic acid release after a saponification step with 1 M NaOH (18 h; 4°C). All enzyme incubations were performed in duplicates.

Determination of the relative abundance of the six constituent RUs in YesY modified xanthan

The mode of action of YesY towards xanthan was determined by the structural analysis of unmodified and enzyme-treated xanthan. Enzymatically deacetylated xanthan solution was dialyzed against demineralized water for 24 h and lyophilized. The xanthan obtained was redissolved in Millipore water (2 mg·mL^{-1}) and incubated with the experimental cellulases preparation C1-G1 from *Myceliophthora thermophila C1* (Dyadic Netherlands, Wageningen, The Netherlands) at 60°C for 48 h.[21] The xanthan digests obtained were analyzed for their repeating units (RUs) using HILIC-ELSD-ESI-IT-MS^n as described elsewhere.[10]

RESULTS AND DISCUSSION

In a previous study the expression and purification of the pectin acetyl esterase YesY was described.[18] Substrate specificity tests towards various acetylated polysaccharide substrates showed that YesY was also active towards xanthan (Table 6.2.). Although the specific activity towards xanthan was ~40 times lower compared to the activity towards pectin, this side activity is very interesting as only one other enzyme has been described to date that can remove acetyl groups from xanthan.[17] In this study the YesY activity towards xanthan was further investigated.

Table 6.2. Acetic acid release after a 10 min, 2 h or 24 h incubation of various substrates (5 mg·mL^{-1}) with YesY in 50mm sodium citrate buffer pH 6.0 taken from Wagenknecht et al.[18]

Substrate	Acetic acid release (µg·mL^{-1})		
	10 min	*2h*	*24h*
Xanthan	n.d.	4	20
Konjac gluco mannan	n.d.	0	2
Chitin	n.d.	n.d.	n.d.
56% reacetylated chitosan	n.d.	n.d.	n.d.
Xylan oligosachharides	n.d.	3	49
Pectin	28	64	90
pNP-acetate	n.d.	n.d.	n.a.

n.d. = not detected
n.a. = not analyzed

Influence of the xanthan concentration on the YesY activity

The specific activity of YesY towards xanthan as determined by Wagenknecht et al.[18] was determined on 5 mg·mL^{-1} substrate solutions. In order to determine if the high viscosity of a 5 mg/ml xanthan solution hinders the enzyme, the activity of YesY was also determined towards a lower xanthan concentration. The enzyme to substrate ratio was kept constant compared to the previous study. After a 24 h incubation, in which the reaction is in the linear range, 8.28 µg acetic acid (6.9% of all acetyl groups present) was released. This equals a specific activity of 11.9 mU·mg protein^{-1}, which is similar to the specific activity found previously (11.6 mU·mg protein^{-1}). It was, therefore, concluded that the viscosity of xanthan solutions with concentrations up to 5 mg·mL^{-1} xanthan does not influence the YesY activity.
It was decided to use 2 mg·mL^{-1} xanthan solutions for further experiments.

Influence of the enzyme:substrate ratio on the acetic acid release by YesY

Determination of the specific activity showed that, independent of the substrate concentration used, only ~7% of all acetic acid groups is released from xanthan after 24 h of incubation. Extending the incubation time did not result in the release of more acetic acid. Due to the repetitive structure of xanthan, it is not expected that neighboring side chains within the xanthan molecule hinder YesY in its activity towards xanthan.

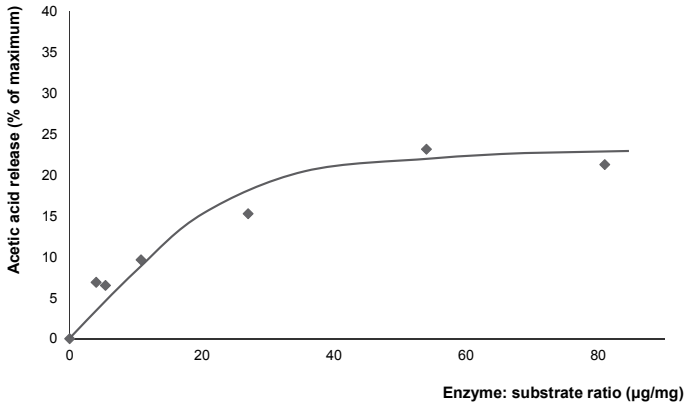


Figure 6.2. Influence of the enzyme:substrate-ratio on the acetic acid release by YesY after 24 h of incubation at 40°C, pH 6.

The low acetyl release could be due to inactivation or inhibition of the enzyme during the incubation. In order to test this hypothesis the influence of the E:S-ratio on the enzyme activity was determined. The results are shown in Figure 6.2. With increasing E:S-ratio, an increase in the acetic acid release is observed, indicating that the enzyme was indeed hindered in its activity at low E:S-ratio. Increasing the enzyme concentration to values higher than 54 µg enzyme·mg xanthan^{-1} does not result in a further increase in acetic acid release. After 24 h of incubation a maximum of 22% of all acetyl groups was released from xanthan.
It was decided to use an E:S-ratio 54 µg enzyme·mg xanthan^{-1} for further experiments.

Influence of the xanthan conformation on the YesY activity

Recently, we showed that the enzymatic removal of acetyl groups from xanthan by AXE3 strongly depends on the secondary xanthan structure.[17] It was, therefore, investigated if the activity of YesY towards xanthan is also influenced by the xanthan conformation. The exact fraction of disordered xanthan (α) in solution can be controlled by selecting specific solvent conditions and can be determined using circular dichroism. The exact xanthan conformations obtained in the solution condition chosen in this study were previously determined.[17] An overview of these solution conditions, the corresponding xanthan conformation and the acetic acid release by YesY after a 24 h incubation is given in Table 6.3. Enzyme incubations performed in the complete absence of salt, resulted in a clear decrease in the acetic acid release. The presence of only 1 mM NaCl already showed to double the acetic acid release compared to an incubation in Millipore water. A further increase of the salt concentration to 10 mM NaCl did not result in a further increase in acetic acid release. The clear decrease in enzyme activity in the absence of salts indicates that counter ions are necessary for enzyme stabilization. No significant difference in the acetic acid

Table 6.3. Incubation conditions, the corresponding ellipticity (θ) and fraction of disordered conformation (α) (taken from Kool et al.[17]) and the acetic acid release from xanthan after a 24 h incubation at the different incubation conditions

Sample condition	θ_{219}	α	Acetic acid release (% of maximum release)
Millipore water 40˚C	-3.02	0.78	8
1 mM NaCl 40˚C	-1.37	0.35	15
2 mM NaCl 40˚C	-0.93	0.24	13
5 mM NaCl 40˚C	-0.55	0.14	17
10 mM NaCl 40˚C	-0.20	0.05	14
50 mM CA buffer 40 ˚C	n.a.	n.a.	22

n.a. = not analysed

release was observed within the digests obtained at various salt concentrations, and thus within the digests obtained at different xanthan conformations. It is, therefore, concluded that the YesY activity, in contrast to the AXE3 activity, does not dependent on the xanthan conformation. As the acetyl groups on the inner mannose are believed to be directed to the inside of the ordered helical xanthan structure,[17, 22] the YesY activity towards xanthan is hypothesized to be specific for the removal of the acetyl groups on the outer mannose.

Temperature optimum of YesY

The temperature profile of YesY was determined after a 6 h incubation in the temperature range 30-80˚C. A pH of 6.0 was chosen as it showed to be the optimal pH (see below). The results (Figure 6.3a.) indicate a temperature optimum between 50-60˚C. A clear drop in activity was observed when the temperature was further increased, indicating enzyme inactivation at temperatures above 65˚C for 6 h. The temperature optimum towards pectin determined after a 10 minute incubation,[18] was higher compared to the optimum observed towards xanthan (Figure 6.3a.). Also, the clear drop in activity that was observed for xanthan was not observed in the temperature profile of YesY towards pectin.

pH optimum of YesY

The pH optimum of YesY was determined after a 6 h incubation in the pH range 3.5 – 8 at 40˚C and compared to the pH profile of YesY towards pectin. Figure 6.3b. shows a clear pH optimum towards xanthan at pH 6.3. Two pH optima were observed for YesY towards pectin: one equal to the pH optimum towards xanthan at pH ~6.3 and the other, with a much higher specific activity at pH-values ≥7.5.[18] Because correction for autohydrolysis was necessary for xanthan digests obtained at pH≥7.5, the enzyme activity towards xanthan at high pH values could not be determined accurately.

Characterization of the enzymatically modified xanthan

YesY is especially active towards xanthan in the presence of salts, and thereby towards the ordered xanthan conformation. In a previous study we showed that the acetyl groups on the inner mannose was not accessible for AXE3 deacetylation when xanthan appears in an ordered conformation.[17] It is, therefore, hypothesized that YesY is specific for the deacetylation of acetyl groups positioned at the outer mannose unit. In order to analyze the specificity of YesY, YesY modified xanthan was degraded to its repeating units (RUs) using a cellulase preparation. Subsequently, the relative

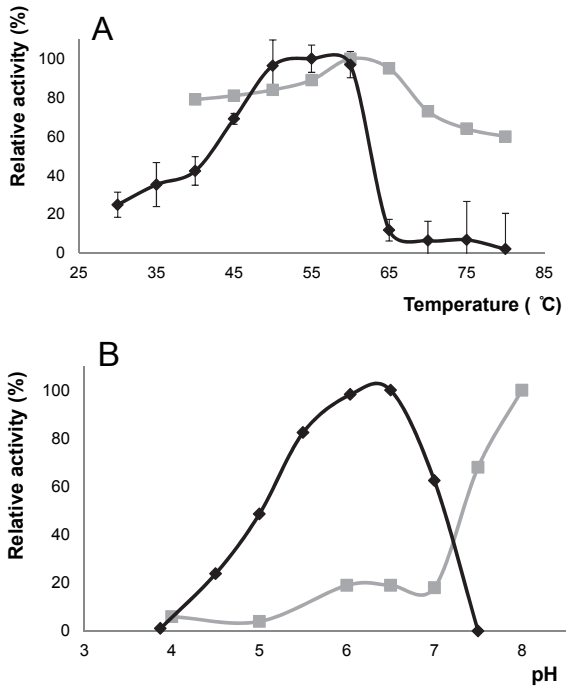


Figure 6.3. A: Temperature optimum of YesY towards xanthan obtained after a 6 h incubation at pH 6. B: pH optimum of YesY towards xanthan obtained after a 6h incubation at 40°C. Xanthan (◆); pectin (■) (data taken from Wagenknecht et al.[18])

abundance of the repeating units (RUs) present in cellulase digests of the YesY deacetylated xanthan was analyzed using HILIC-ELSD-MS[10] and compared to the relative abundance of the RUs present in the cellulase digest of unmodified xanthan (Figure 6.4.). As observed in earlier studies, five different peaks are present in the elution profile of unmodified xanthan.[10] Co-elution of the two single acetylated RUs was confirmed by MS-fragmentation as described previously.[10] After a 24 h incubation with YesY, no double acetylated RUs (peak 1) were detected in the modified xanthan, the amount of single acetylated RUs (peak 2) increased, and no changes in the abundance occurred for the other RUs. As no decrease in the amount of pyruvylated and acetylated RU (peak 3), or increase in unsubstituted (peak 4) and/or pyruvylated (peak 5) RUs was observed, it is concluded that YesY is indeed specific for the removal of acetyl groups from the outer mannose. The increase in the abundance of single acetylated RUs, is thus the result of the conversion of double acetylated RUs into RUs which are solely acetylated on the inner mannose. To determine if RUs that are solely acetylated on the outer are also deacetylated by YesY, or that acetylation at the inner mannose is necessary for YesY deacetylation, the remaining single acetylated RUs

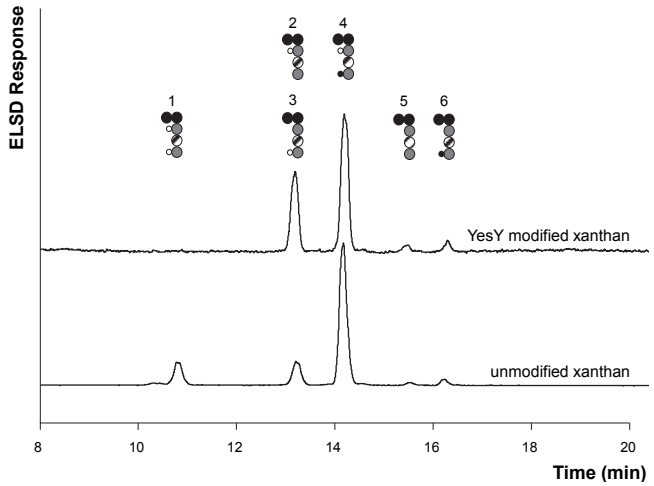
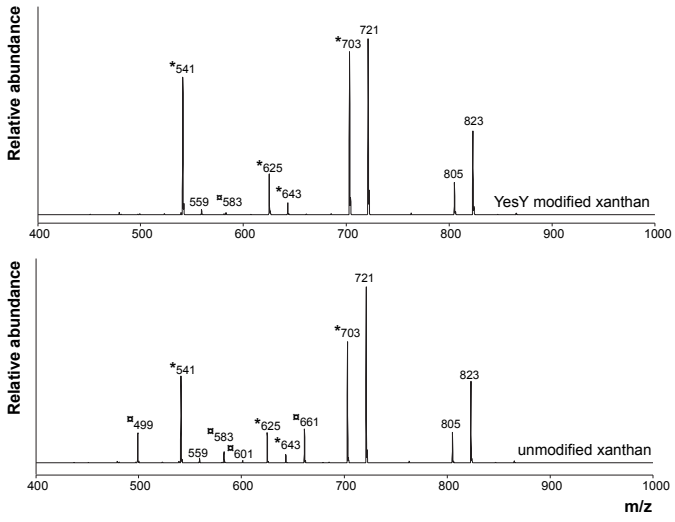


Figure 6.4. HILIC-ELSD elution profiles of cellulase digests of unmodified xanthan and xanthan modified with YesY for 24 h. Glucose ● ; mannose ● ; glucuronic acid ◐ ; acetyl groups ○ ; pyruvic acid ketal • .



¤ Fragments specific for the acetylation on the outer mannose
* Fragments specific for the acetylation of the inner mannose

Figure 6.5. MS^2-fragmentation pattern of the single acetylated repeating unit (eluting at 13.2 min in Figure 4.4.) of unmodified and YesY modified xanthan

were further characterized. Changes in the abundance of the two single acetylated RUs, induced by the YesY modification, can be determined using the MS-fragmentation pattern since inner and outer mannose acetylated RUs have a different set of diagnostic fragment ions.[10] The intensity of these fragment ions in the fragmentation pattern of the single acetylated RU before and after YesY modification are shown in Figure 6.5. Before modification ~25% of all single acetylated RUs were acetylated on the outer mannose. After YesY modification 100% of the RUs were acetylated at the inner mannose indicating that no RUs remain which are acetylated on the outer mannose. Acetyl groups at the outer mannose unit can thus be hydrolyzed by YesY, independent on the substitution pattern of the inner mannose.

YesY also showed to be active towards xanthan when xanthan appears in a partly disordered conformation (Table 6.3.). At such conformations the acetyl groups at the inner mannose are better accessible to enzymatic hydrolysis. The YesY specificity towards partly disordered xanthan and completely ordered xanthan could thus be different. The YesY specificity towards partly disordered xanthan was, therefore, investigated by the structural analysis of xanthan, which was modified with YesY in a 2 mM NaCl solution, where α=0.24 (results not shown). A similar HILIC-elution profile was obtained compared to the elution profile obtained for xanthan modified with YesY at a completely ordered conformation. It was, therefore, concluded that YesY is specific for the deacetylation of the outer mannose unit independent on the xanthan conformation.

CONCLUSIONS

YesY, a pectin acetyl esterase originating from *Bacillus subtilis* strain 168 is capable in removing 22% of all acetyl groups in xanthan. Optimal activity was obtained at 55°C, in the presence of salts at pH 6.3. YesY is specific for the removal of the acetyl groups positioned on the outer mannose unit, and its activity is independent on the acetylation on the inner mannose, all acetyl groups on the outer mannose can be removed. Thereby the first acetyl esterase is described that specifically deacetylates the outer mannose units of xanthan, and which can be used for the production of xanthans with altered and possibly improved functionalities.

ACKNOWLEDGEMENTS

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Chapter 7

General discussion

Multiple models exist in literature to describe the physical properties of a xanthan solution. Although studies on the biosynthesis of xanthan show that variations in the xanthan structure may occur, studies dealing with the physical properties of xanthan generally assume that the xanthan structure is rather repetitive with one single repeating unit (RU). This repeating unit is acetylated at the inner mannose and pyruvylated on the outer mannose (Figure 7.1; RU-4). However, variations in the degree of substitutions are known to occur, and in general only 90% of the inner mannose units are believed to be acetylated and 50% of the outer mannose units are believed to be pyruvylated. Starting this study, we hypothesized that xanthans primary structure is more complex than a polymer of this type of RU. As a consequence, the conflicting models proposed in literature were hypothesized to find their origin in differences between the primary structures of the xanthans used. As no evidence for such differences was available at the start of this project, the main aim of this research was to characterize the xanthan primary structure in more detail than was usually done. According to the approach chosen, diagnostic xanthan oligosaccharides, preferable of intact RUs, should be produced as a first step to achieve this aim. To prevent random degradation or modification of the xanthan primary structure and consequently of the oligomers produced, it was chosen to use enzymes for the production of such oligosaccharides. Subsequently, the produced oligosaccharides needed to be characterized and quantified in order to compare the primary structure of multiple xanthan samples. Furthermore, enzymes that specifically modify the side chains in order to study the distribution pattern of the acetyl and/or pyruvate groups within the molecule were searched for.

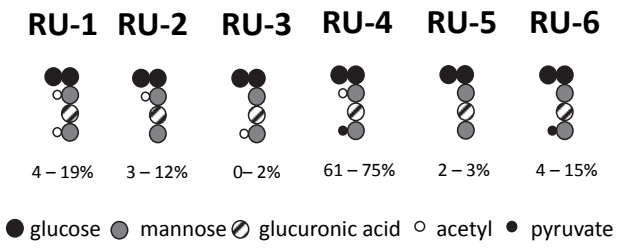

Figure 7.1. The six xanthan repeating units as revealed by HILIC-ESI-MS. The range of their relative abundance within 5 different xanthan samples, as determined in chapter 3 is given below each structure.

PRODUCTION AND CHARACTERIZATION OF XANTHAN REPEATING UNITS

Previous studies on the enzymatic backbone degradation of xanthan showed that xanthan can only be degraded by cellulases when incubations are performed in the absence of salts.[1-3] However, high molecular weight material rich in substituents remained at the end of the enzymatic hydrolysis.[2, 3] This led to the hypothesis[2, 3] that xanthan is only susceptible to cellulase degradation when xanthan appears in the disordered conformation *or* that the substituents in the side chains hinder complete enzymatic xanthan degradation. A conclusive study to test either one of the hypotheses was never conducted. With the research presented in chapter 2, we can now conclude that only the xanthan conformation influences the enzymatic backbone degradation of xanthan. The remaining high molecular weight material observed in other studies, therefore, is the result of the presence of ordered xanthan segments during the enzymatic hydrolysis.

Because the cellulase preparation used completely degraded xanthan to its constituent RUs, without undesired additional modification of the xanthan side chains, it was possible to determine the xanthan structure on RU level. Structure analysis of the RUs released after enzymatic backbone degradation by the cellulases showed that six different xanthan RUs are present in a xanthan sample (Figure 7.1.). This led to the conclusion that the repetitive structure of one single type of RU as a model for xanthan is incorrect.[4] The xanthans analyzed (in total 5 different xanthans in chapters 3 and 4) in this study all consist of the same 6 RUs, which shared the same pentasaccharide structure, while having different acetyl and pyruvate substitution patterns. The existence of truncated side chains in unmodified xanthans, as hypothesized by Sutherland[2] was not found although explicitly searched for.
The most interesting variation observed within the six RUs, is the substitution of the outer mannose with an acetyl group. Although this variation was hypothesized based on xanthan biosynthesis studies,[5-7] conclusive evidence was never provided prior to the start of the present study. The possibility of this substitution has, therefore, always been neglected when studies on the structure-function relationship of xanthan were performed. The influence of the acetyl groups on the functionality of xanthan has always been based on the total level of acetylation. As it is now proven that 5-20% of the outer mannose units can be acetylated, covering 5-19% of all acetyl groups within a xanthan sample (chapter 3), we suggest that previous outspoken conclusions made on structure function-relationship of xanthan, especially regarding the influence of acetylation, should be reconsidered.

As it was found that the primary xanthan structure does not hinder the backbone degradation of xanthan by cellulases (chapter 2), it was also possible to compare the structures of various xanthan samples. Quantification of the RUs present in the different xanthan cellulase digests enabled the comparison on the RU level (chapter 3). The major conclusion drawn from this comparison is that the relative abundance of the RUs between xanthan samples can vary, even when their molecular compositions are the same.

In chapter 4 was shown that particularly those segments enriched in RUs that are acetylated on the outer mannose stabilize the xanthan conformation through the formation of hydrogen bonds. In contrast to previous studies, which assumed that only the inner mannose unit is acetylated, it was shown that acetylation of the inner mannose does not significantly contribute to the stability of the ordered xanthan conformation. In conclusion: the position of the acetyl group within the xanthan side chain determines the influence of the acetyl groups on xanthan's transitional behavior. The relative abundance of the different RUs, therefore, determines the conformational behavior of a xanthan sample and not the molar composition. Xanthan samples with equal molar compositions can, therefore, still vary in their conformational behavior and thus in their solution properties.

DISTRIBUTION OF THE DIFFERENT REPEATING UNITS OVER THE XANTHAN BACKBONE

The correlation between the enzymatic release of individual types of RUs and the fraction of disordered conformation showed that the distribution pattern of the differently substituted RUs is non-regular (chapter 4). It was proposed that pyruvylated RUs are randomly distributed in the xanthan samples studied. In contrast, the single and double acetylated RUs are proposed to be distributed in a block wise manner. Based on this distribution pattern, a tentative model is proposed for the transitional behavior of differently substituted xanthan segments (Figure 7.2.). According to this model, segments rich in outer mannose acetylated RUs dissociate to a random disordered conformation only at the end of the xanthan transition. Segments rich in RUs which are solely acetylated on the inner mannose dissociate rather easily into a disordered conformation. Furthermore, it is hypothesized that the intermolecular interactions involved within a xanthan network are induced by the acetyl groups on the outer mannose. The junction zones of a xanthan network would thus be rich in outer mannose acetylated RUs.

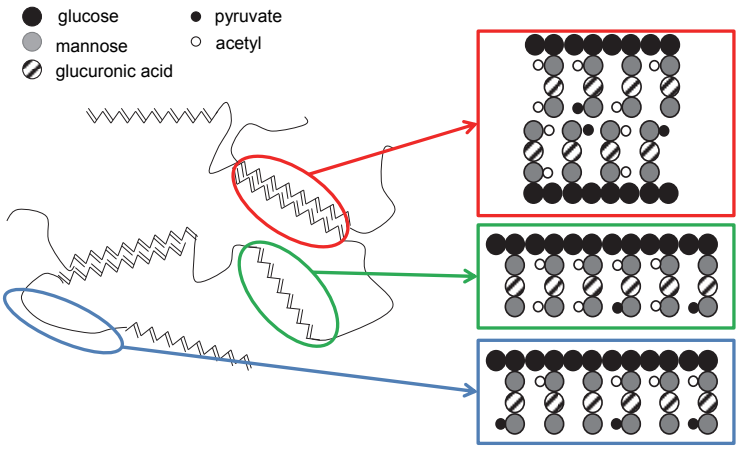

Figure 7.2. Proposed conformational behavior of xanthan when 50% appears in a disordered conformation.[8]

The proposed distribution pattern of the RUs is based on the release of single RUs and is thus based on indirect evidence. Expanding the cellulase fingerprinting method in order to obtain xanthan oligosaccharides larger than 1 RU is, therefore, important in view of understanding the structure-function relationship of xanthan.

Structural analysis of xanthan fragments larger than 1 RUs

To obtain xanthan oligosaccharides larger than 1 RU (RU_n), xanthan A (chapter 4) present in a completely disordered conformation (60°C; Millipore water) was incubated with the cellulases for different time intervals (unpublished results). Analysis of the digests obtained with HPAEC (according to chapter 3) showed that the single RUs, from now on denoted ' RU_1', were dominant in the digests (Figure 7.3.). Other structures were released as well, which were proposed to be RU_n structures based on the HPAEC elution patterns observed for other mono- and glucooligosaccharides. The highest variety in these RU_n structures was obtained after 2 h of incubation.

The 2 h digest was further analyzed by HILIC-ELSD-MS (according to chapter 3) to identify the different RU_n structures present. Similar to the HPAEC profiles, the RU_1 structures dominated the elution profile (results not shown). Quantification based on ELSD peak area indicated that 74% of the xanthan molecule was released as RU_1. Based on the quantification of the individual types of RU_1 obtained, it was estimated which percentage of each type of RU was present as RU_n. An overview is given in Table 7.1. The outer mannose acetylated RUs (RU-1 and RU-3) are mainly present as RU_n. More than 70% of these RUs are present as larger xanthan fragments, which represent

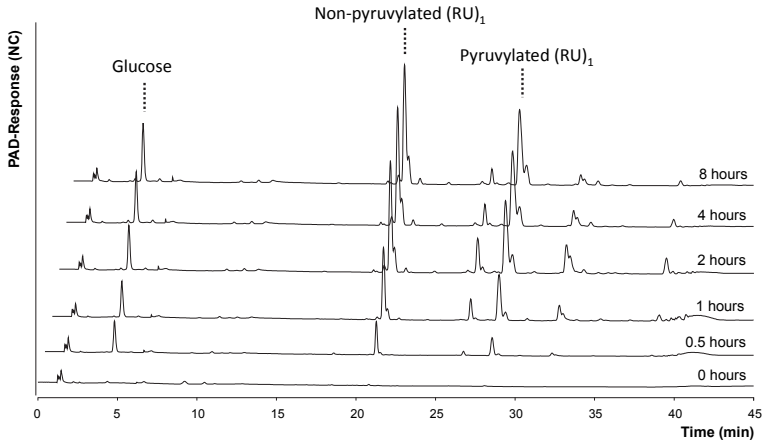

Figure 7.3. HPAEC elution pattern of xanthan digests obtained after incubation of xanthan with cellulases in Millipore water at 60°C for different time intervals. Annotation of peaks as described in chapter 3.

26% of the total xanthan digest. These results, thereby, confirm the conclusion made in chapter 4 that outer mannose acetylated RUs are distributed in a rather block wise manner. Similarly, these results confirm that the RU that is solely acetylated on the inner mannose is also distributed in a block wise manner, as only 6% of this RU is present as RU_n structures, and thus in 26% of the xanthan structure. Furthermore, the conclusion that RU-4 is distributed randomly is confirmed as 23% of this RU is present in 26% of the molecule.

Table 7.1. Appearance of the six different xanthan RUs in a 2 h cellulase digest (Millipore; 60°C) as RU_1 or as/in RU_n

Type of RU[a]	Present as RU_1 (% of total)	Present as RU_n (% of total)
Total xanthan sample	74	26
RU-1	28	72
RU-2	94	6
RU-3	26	74
RU-4	77	23
RU-5	85	15
RU-6	60	40

[a]= The molecular structures of RU -1-6 are given in Figure 7.1.

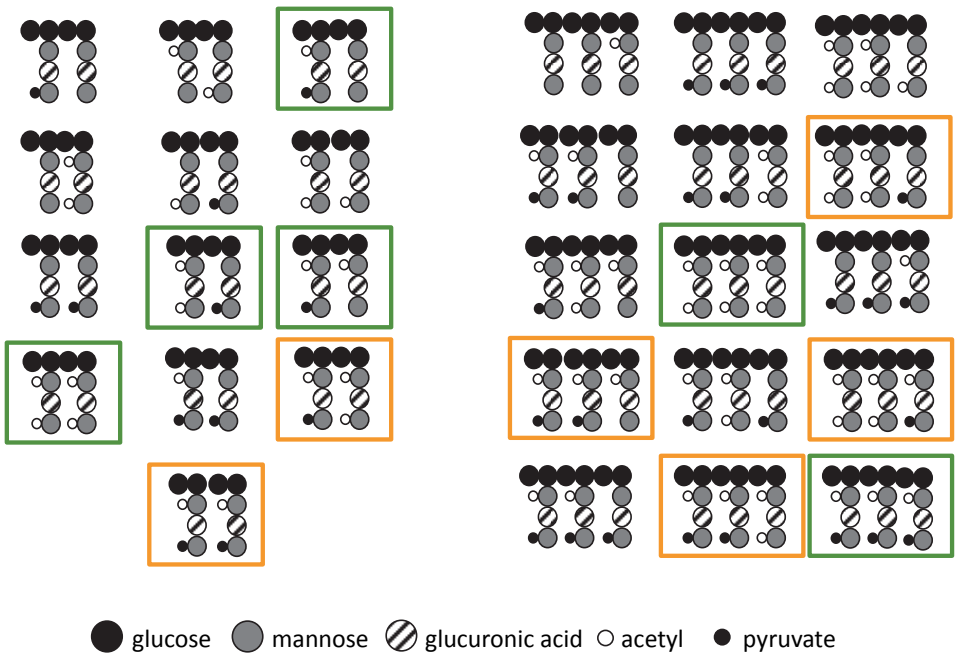

Figure 7.4. Structures of xanthan oligosaccharides present in a 2 hr. xanthan digest as determined by HILIC-MS^n. The oligosaccharides consisting of only 1 repeating unit are excluded. orange box = most abundant; green box second most abundant

In order to verify that the RU_n structures obtained are indeed enriched in RU-1 and RU-3, effort was directed in obtaining good MS-spectra of the RU_n structures. The RU_n structures present in the 2hr digest were, therefore, separated from the RU_1 by size exclusion chromatography, using three Superdex 30 columns in series and ammonium formate buffer (250 mM) elution.[9] Three different fractions were obtained, which were analyzed by UPLC-MS^n. One of the fractions obtained indeed contained the RU_1 structures, another fraction was rich in RU_2 structures and the third fraction contained RU_3 structures. Larger xanthan oligosaccharides (XaOS) were not observed. All RU_2 and RU_3 structures found are presented in Figure 7.4.

In total, 21 different structures are possible for RU_2 and 35 for RU_3 when the order of the individual RUs within each XaOS is not considered. However, only 28 different structures were detected by MS-analysis. Although quantification of the different XaOS observed was difficult, an estimation on the most abundant structures present was made based on the ELSD elution profiles. These abundant structures detected were indeed enriched with side chains acetylated on the outer mannose, as 7 out of the 12 dominant structures contained at least 1 RU acetylated on the outer mannose unit. In

total 12 out of the 30 individual RUs within the dominant structures was acetylated on te outer mannose. As xanthan appeared in a complete disordered conformation during the production of the RU_n, this indicates that the RUs which are not acetylated at the outer mannose are preferentially hydrolyzed by the cellulases used. Structures rich in pyruvylated side chains were also detected in the dimer and trimer fractions of the digest. This was expected as these RUs are randomly distributed and represent ~65% of all side chains within the studied xanthan. Therefore, approximately 65% of all side chains present in the XaOS should be pyruvylated, which was indeed the case (10 out of 12 of the dominant structures; or 15 out of the 30 individual RUs). In conclusion, the distribution pattern proposed in chapter 4 was confirmed by structural analysis of larger XaOS.

The results presented only provide a first indication on the RU distribution pattern of xanthan, since fragments of 2 or 3 RUs are not sufficiently long to make conclusive statements on the RU distribution pattern. Especially when considering the fact that 76% of all RUs were released as individual RUs. As the cellulases used in this research seem to prefer the release of single xanthan RUs (Figure 7.3.), screening for other cellulases that prefer the release of larger XaOS could be useful for further studies towards the distribution pattern of the six RUs.

The xanthan structure revisited

In chapters 2-4 it was shown that xanthan has more variation in its primary structure than was generally assumed. Especially the observation that the outer mannose can be acetylated showed to be important, as this type of substitution is concluded to be important for the conformational behavior of xanthan. Furthermore, combined with the data presented above, assumptions on the distribution pattern of the different xanthan RUs can be made. Based on the research presented in this thesis, we now propose a new model for the xanthan structure (Figure 7.5.). In this model the xanthan structure is divided into segments, which are either rich in outer mannose acetylated RUs (RU-1 and RU-3) or rich in RUs which are solely substituted with an acetyl group on the inner mannose (RU-2). The remaining RUs 4, 5 and 6 are proposed to be randomly divided over these different segments. It should be considered that the relative abundance of the RUs differs for different xanthan samples (chapter 3) and consequently the precise distribution will vary. The validity of this postulated structure should be tested in future research. The use of specific side chain modifying enzymes will be of help to achieve this.

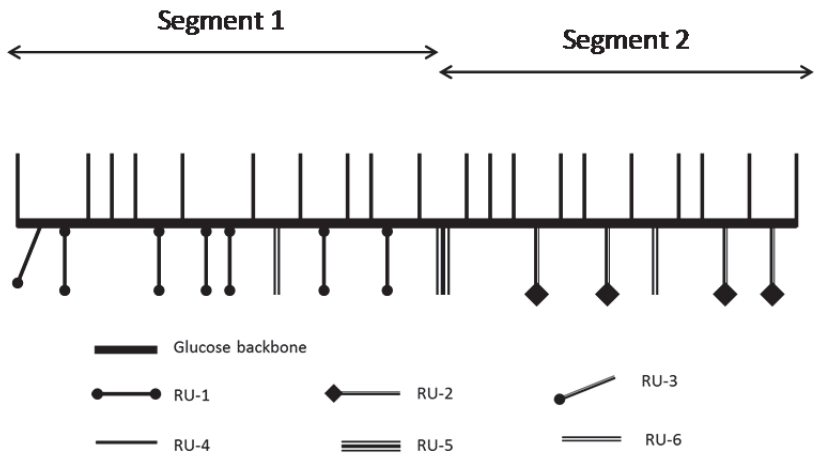

Figure 7.5. Tentative structure of xanthan as reconstructed from cellulase fingerprinting. The molecular structures of RU 1-6 are given in Figure 7.1.

XANTHAN SIDE CHAIN MODIFYING ENZYMES

Modification of the xanthan side chains, as extension to the introduced cellulase fingerprinting method, would be useful to further elucidate the distribution pattern of the six RUs. As a first step to achieve this, enzymes were searched for which specifically modify the xanthan side chains.

Xanthan acetyl esterases

In chapters 5 and 6 the first two acetyl esterases able to act on xanthan ever mentioned in literature were recognized, described and characterized for their action towards xanthan. One of the enzymes, YesY, specifically removes acetyl groups at the outer mannose unit and is capable of removing 100% of its target within a 24 h incubation. The other enzyme, AXE3, is specific for the removal of acetyl groups positioned at the inner mannose units and releases 75% of these targets after a 3 day incubation. Complete enzymatic removal of the acetyl groups on the inner mannose was not obtained using AXE3, which was not understood. Further screening for xanthan acetyl esterases that enable complete xanthan deacetylation would thus be useful. It was shown that the acetyl groups at the inner mannose are only susceptible for enzymatic removal when xanthan is in a disordered conformation Furthermore, most enzymes need counter ions for their stability. Future screening for putative

xanthan acetyl esterases should, therefore, be performed in solution conditions that favor both, the disordered as well as the ordered xanthan conformation, in order not to overlook any acetyl esterase activity. Further research towards the mode of action of the two presented enzymes, by structural analysis of larger XaOS after cellulase degradation, should be considered as well. Such studies will reveal whether the enzymes act randomly over the backbone or if they show a single chain-multiple attack action, which could help to further determine the distribution pattern of the acetyl groups over the xanthan molecule.

In chapter 4 it was discussed that the position of the acetyl group within the xanthan side chain is important for the functional properties of xanthan. Due to the focus of this thesis, no rheological experiments were performed to confirm this assumption. Future, analysis of the rheology of xanthan, which was either modified with YesY or with AXE3, would reveal the exact correlation between the position of the acetyl groups within the xanthan side chain and the solution properties of xanthan. Furthermore, the enzymes could be used for the production of xanthans with novel rheological properties. As the specific activity of both acetyl esterases is rather low (YesY=11.9 mU·mg protein^{-1} and AXE3=13 mU·mg protein^{-1}), further research towards xanthan acetyl esterases with a higher specific activity is useful, especially for the large scale production of modified xanthans with altered functional properties.

Pyruvate specific xanthan lyases

Several xanthan lyases are known that specifically remove the pyruvylated mannose from the xanthan side chain.[10, 11] In addition to the research described in the previous chapters, an effort was directed towards the production of such lyases and the further use of these enzymes for structure elucidation. Two xanthan lyases from *Bacillus* sp. strain GL1 (BAB21059.1) and *Paenibacillus alginolyticus* XL-1 (AAG24953.1) were successfully cloned, expressed and purified as described elsewhere.[12] They will be referred to as XalA_GL1 and XalA_XL1, respectively.
To verify the specificity towards pyruvylated outer mannose units, the produced lyases were incubated with 'normal' xanthan (xanthan A in chapter 4), pyruvate free xanthan (PFX in chapter 2) and highly pyruvylated xanthan (xanthan C in chapter 4). The elution profiles of the digests after incubation with Xal_GL1 are shown in Figure 7.6. Similar elution patterns were obtained for XalA_XL1 digests. These results indicate that both enzymes were active towards xanthan independent on the level of pyruvylation. Furthermore, both enzymes released mannose and pyruvylated mannose as revealed by HPAEC analysis (Figure 7.6). The presence of pyruvylated

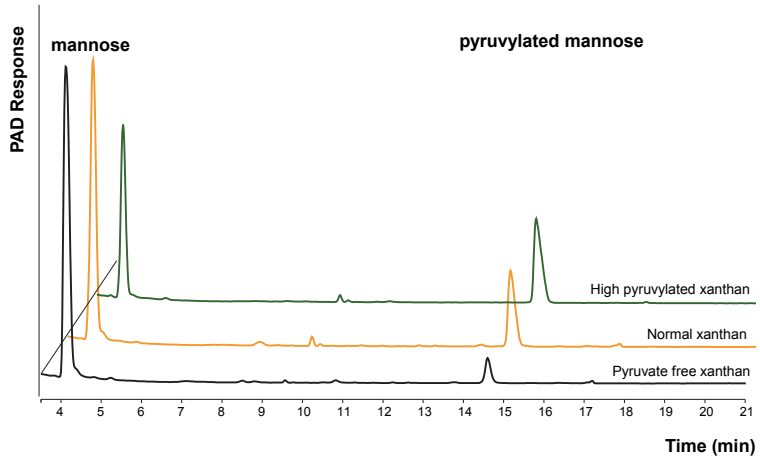

Figure 7.6. HPAEC elution profiles of xanthans differing in pyruvate content after a 24h incubation Xanthan lyase Xal_GL1.

mannose in the digests was verified by the detection of mannose and pyruvic acid in a molar ratio 1:1 after acid hydrolysis of the degradation products. Analysis of the modified xanthan by HILIC-ELSD-MS after degradation of the backbone by cellulases, confirmed that all different xanthan side chains are modified by the lyases, independent on the presence of a substituent on the outer mannose. Both lyases are thus active towards pyruvylated, acetylated and unsubstituted outer mannose units. The lyases produced are thereby different from the lyases described before,[10, 13] even though the amino acid sequence of the enzymes were exactly the same.
The xanthan conformation showed to be important for both enzymatic backbone degradation (chapter 2-4) and side chain modification (chapter 5) of xanthan. The observed difference in activity between the cloned lyases and the lyases, however, did not originate from differences in the xanthan conformation, as similar lyase activities were observed towards xanthan in a fully ordered or disordered conformation.

In conclusion, the lyases produced in this study really have a different specificity compared to the lyases described before, even though the amino acid sequence is exactly the same. Although the difference in activity is not fully understood, they might origin from the expression system used for the enzyme production, as we cloned the lyases in an *E. coli* strain and the original lyases were produced by *Bacillus* spp. As the cloned lyases were not specific for any type of substitution, they cannot be used for enzymatic xanthan fingerprinting. Further research towards specific xanthan lyases is, therefore, necessary.

Microbial sources for other xanthan modifying enzymes

As described in chapter 2, *P. alginolyticus* XL1 can produce a variety of xanthan side chain modifying enzymes.[14] Therefore, *P. alginolyticus* strain (DSMZ_5050) was cultured as described elsewhere.[10] The crude enzyme extract was analyzed for the enzymes present, by the characterization of degradation products present in a xanthan digests obtained after incubation with this extract (results not shown). The production of several xanthan modifying enzymes by *P. alginolyticus* was indeed confirmed including the production of: xanthan lyases; a xanthan acetyl esterase and a pyruvate removing enzyme (unpublished results). As, the analysis of the digest by HPAEC only showed the presence of mannose and pyruvylated mannose, and not of oligosaccharides structures, it was concluded that all enzymes are active towards the xanthan polymer and are thus potential xanthan modifying enzymes.

Next to the cultivation of *P. alginolyticus* on xanthan medium, xanthan was also fermented by microbiota from the human gut as the human gut flora showed to be a good 'producer' for several carbohydrate modifying and degrading enzymes.[15] Characterization, using HPSEC, HPAEC and Maldi-Tof MS (results not shown), of the degradation products present in a xanthan digests obtained after incubation with the supernatant of a 10 day fermentation liquid revealed the presence of several enzymes: an acetyl esterase, a backbone degrading enzyme and an enzyme that releases dimers of β-mannose-(1→4)-β-D-glucuronic acid (unpublished results). In order to determine whether the side chain modifying enzymes were active prior to backbone degradation, the enzyme action of the enzyme cocktail was monitored in time. It was found that backbone degradation occurs prior to the removal of the glucuronic acid-mannose dimers. The acetic acid was released directly from the polymer. The enzyme incubations were conducted in 50 mM sodium citrate buffer, pH 5.5. A disordered xanthan conformation is, thus, not required for this acetyl esterase to be active. As more than 40% of all acetyl groups were released by the enzyme cocktail, part of the acetyl groups removed must have been originated from the inner mannose. The acetyl esterase produced by the human gut flora is, therefore, different from the two enzymes characterized in chapters 5 and 6.

Despite the problems in expressing the pyruvate specific lyase from *P. alginolyticus*, both microbial sources described show to be good potential sources for the production of novel xanthan modifying enzymes. However, no further attempts were made towards the purification of the enzymes detected in the culture broths.

IMPACT OF OUR FINDINGS ON UNDERSTANDING THE STRUCTURE-FUNCTION RELATIONSHIP OF XANTHAN

Using the approach introduced in the presented study, a more detailed correlation between the xanthan substituents and xanthan's functionality can be obtained compared to previous studies. Because no rheological analysis is performed in this research, our findings regarding the primary xanthan structure were correlated to published results on xanthan's functionality.

Xanthan conformation

In chapter 4, it was concluded that the position of the acetyl groups within the xanthan repeating units is important for the stability of the xanthan conformation. Substituents on the outer mannose are, therefore, proposed to be most important for xanthans solution behavior. Although the acetylation of the outer mannose has never been considered when studying the conformation of xanthans, the influence of the length of the xanthan side chains has been discussed.[16-18] It was concluded that xanthan lacking the outer mannose, also called the 'polytetramer', conducts a helical conformation *in the excess of salts*, which is similar to that of normal xanthan.[16] *Under aqueous conditions* most of the polytetramer already appeared in a disordered conformation at 25°C.[17] These results thereby confirm that the stability of the xanthan conformation depends on the outer mannose.

Viscosity of xanthan solutions

The influence of the primary structure on xanthans rheology has been studied more extensively than its influence on the xanthan conformation. Nevertheless, the viscosity of xanthan solutions is believed to depend on the xanthan conformation.[19] As especially the substituents on the outer mannose unit determine xanthans conformation (Chapter 4), we expect that this also applies to the viscosity of xanthan. Acetylation of the outer mannose is, therefore, hypothesized to reduce the viscosity of a xanthan solution to a larger extent than the acetylation of the inner mannose.
The influence of the total degree of acetylation on the viscosity of a xanthan solution has mostly been studied by the aspecific removal of acetyl groups, which resulted in an increase of the viscosity.[20, 21] The influence of the acetyl position on the viscosity, however, was only studied once.[7] In that study it was concluded that the position of the acetyl groups within the xanthan molecule is not important for the viscosity. This was based on the comparison of the viscosity of xanthans produced by mutant strains.

These xanthans were either solely acetylated on the inner mannose *or* on the outer mannose and were *not* pyruvylated at all.[7] The influence of the pyruvate groups and/or intramolecular interactions on xanthan's viscosity was thus neglected. As discussed in chapter 4, the distribution of the six RUs in a xanthan segment is important for the final transitional behavior of that segment. The use of mutant strains to study the influence of the primary structure on xanthan's viscosity might, therefore, not be representative for standard xanthans. This is especially plausible as the production of xanthans by mutant strains may also result in changes other than at the level of acetylation, e.g. the molecular weight and the level of pyruvylation.[14] Furthermore, the viscosity of the xanthans produced by mutant strains was determined before xanthan was exposed to a heat treatment. The first heat treatment is known to change the xanthan conformation and xanthan's functionality.[22-24] The solution behavior of the xanthan produced by mutant strains could thus differ significantly to that of standard xanthans as a result of different sample treatments. Indications on the structure-function relationship obtained by xanthans from mutant strains are, therefore, difficult to translate to standard xanthan produced by wild-type *Xanthomonas spp.* We, therefore, conclude that the approach introduced in this thesis enables a more precise correlation between the primary xanthan structure and the viscosity of a xanthan solution, than the approaches used till to date.

Xanthan-galactomannan interactions

Studies on the influence of xanthan acetylation on the interaction with galactomannans showed that acetyl free xanthan forms stronger interaction gels than normal xanthan.[18, 25, 26] This improved interaction is believed to result from an increased chain flexibility of xanthan molecules upon deacetylation.[20] As acetylation at the outer mannose is most important for the stability of the xanthan conformation, we suggest that especially the acetyl groups at the outer mannose hinder the interactions with galactomannans.

Shatwell et al.[18] used xanthan produced by mutant strains to study the influence of xanthan acetylation on the xanthan-galactomannan interactions. One of the xanthan samples produced was particularly rich in acetyl groups (7.7% w/w), pointing to acetylation at both the inner and the outer mannose. Alkaline removal of the acetyl groups increased the gel strength of this particular xanthan with a factor four, while the gel strength of the standard xanthan only slightly increased.[18] Taken into account precautions about the use of mutant strains, based on these results it can be speculated that acetylation at the outer mannose indeed reduced gel strengths to a larger extent than acetylation at the inner mannose.

FUTURE PERSPECTIVES

In conclusion, this thesis shows that the primary xanthan structure is more complex than was generally assumed. Hence, characterization of xanthan samples based on molar composition is insufficient. Although no physical studies were performed in this study, it is concluded that the position of the acetyl groups is important for xanthans functionality. Conclusions on the influence of the primary xanthan structure on xanthans functionality made previously should, therefore, be reconsidered. Future work on the xanthan functionality should characterize xanthan samples on RU level and not solely on molar composition. Additionally, the two acetyl esterases described in this research could help to reveal the exact influence of the position of the acetyl group on xanthan functional properties.

Although a method is introduced for a more detailed structure characterization, analytical challenges remain as the distribution pattern of different substituents along the backbone of xanthan has not yet been established completely. Expanding the enzymatic analytical toolbox introduced in this research is, therefore, necessary. The availability of other backbone degrading enzymes, which release XaOS larger than RU_3 could help studying the distribution pattern. Furthermore, other side chain modifying enzymes, e.g. an enzyme that specifically removes the pyruvate group or acetyl esterases with a high specific activity, would be useful. In these future studies, the conformation during enzyme incubations has to be considered.

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Summary

SUMMARY

Xanthan is widely used as rheology modifier and emulsifier in food industry. However, the influence of the primary and secondary structure on, and the exact interactions involved in the rheology properties are not fully understood.

In **chapter 1** an overview is given of different models proposed in literature that explain xanthans functional properties. The exact influence of the secondary xanthan conformation on xanthan's functional properties remains uncertain, however the secondary conformation is believed to be an important factor for xanthan's solution properties. The xanthan primary structure is also known to influence xanthans functional properties, however the primary structure affects the xanthan secondary conformation as well. It was, therefore, hypothesized that the different models presented in literature that explain xanthan's functionality, origin from differences in the primary structure of the xanthan samples used. However, till to date no good analytical method is available to study xanthan's primary structure in detail. Hence the aim of this thesis was to develop an enzyme based method to study xanthan's primary structure, to enable a better understanding into the structure-function relationship of xanthan.

To be able to compare the primary structure of multiple xanthans, diagnostic xanthan oligosaccharides are necessary. In this research an enzymatic approach was used to produce such oligosaccharides. As the enzyme activity might depend on both the primary and the secondary xanthan structure, the influence of both these structures on the enzymatic degradation of xanthan was studied (**chapter 2**). It was shown that the xanthan backbone can be degraded by cellulases from *Myceliophthora thermophila* C1 independent on the primary xanthan structure. However only disordered xanthan segments can be degraded. Undegraded ordered xanthan structures remain present as high molecular weight material, indicating that ordered xanthan structures are present in a network of molecules.

As xanthan can be enzymatically degraded independent on the primary structure, the primary structure of different xanthans can be compared. In **chapter 3** five xanthans were enzymatically degraded to their repeating units (RUs). The type and relative abundance of the different RUs were determined using UPLC-HILIC-ELSD-MS^n. It was shown that six different RUs exist within a xanthan molecule. Surprisingly the outer mannose could be unsubstituted, pyruvylated or acetylated, indicating that the primary structure is more complex than generally assumed. Comparison of xanthan samples on RUs level showed that although the molecular composition of two samples

is exactly the same, the relative abundance of the RUs in these samples could still be different.

As only disordered xanthan segments are susceptible to enzymatic degradation, the influence of the six different RUs on the transitional behavior of xanthan could be studied (**chapter 4**). It was concluded that especially the acetyl groups on the outer mannose stabilize the helical xanthan conformation. The acetyl groups on the inner mannose did not show to have a significant effect on the stability of the xanthan conformation. The position of the acetyl group within the xanthan side chain could, therefore, be important for xanthan's functionality as well. As the enzymatic release of pyruvylated RUs increased linearly with the fraction of disordered conformation, it was concluded that the pyruvylated RUs are randomly distribution over the xanthan molecules and that the distribution of the different RUs over the xanthan molecule could be important for the xanthan's transitional behavior.

In **chapter 5** several carbohydrate acetyl esterases were tested for their activity towards xanthan. AXE3, an acetyl xylan esterase produced by *Myceliophthora thermophila* C1, can remove ~60% of all acetyl groups within the xanthan molecule. However, the enzyme is only active towards disordered xanthan segments. Analysis of the RUs present in the modified xanthan indicated that AXE3 is specific for the removal of the acetyl groups on the inner mannose.

In **chapter 6** YesY, a pectin acetyl esterase from *Bacillus subtilis* strain 168, was characterized for its activity towards xanthan. In contrast to AXE3, this enzymes is active towards the ordered xanthan conformation. Analysis of the RUs present in the modified xanthan indicated that YesY is specific for the removal of the acetyl groups on the outer mannose. Thereby two complementary acetyl esterases are described, which can be further used for the structure elucidation of xanthan and/or the production of novel xanthans with improved functionality.

Chapter 7 provides an overview of the information described in previous chapters, in order to address important points for further studies on the structure-function relationship of xanthan. In addition, possible strategies are discussed to improve the 'toolbox' for the structural analysis of xanthan. The characterization of xanthan oligosaccharides consisting of 2 or 3 RUs, showed that RUs that are acetylated on the outer mannose units are distributed in a rather block wise manner. Furthermore, other potential sources for xanthan modifying enzymes were introduced in chapter 7, to enable the continuation of structure elucidation of xanthans

Samenvatting

SAMENVATTING

De polysacharide xanthaan wordt in de levensmiddelenindustrie veelvuldig gebruikt als stabilisator, emulgator en als reologie controleur. De exacte interacties betrokken bij de specifieke eigenschappen van een xanthaanoplossing en de invloed van de primaire en secundaire xanthaan structuur op deze eigenschappen zijn tot op heden echter onbekend.

Hoofdstuk 1 geeft een overzicht van de bestaande modellen om de functionele eigenschappen van xanthaan te verklaren. Het wordt algemeen aangenomen dat zowel de primaire als de secundaire xanthaan structuur de functionele eigenschappen van een xanthaanoplossing sterk beïnvloeden. De precieze invloed van met name de secundaire blijft echter onzeker, aangezien de primaire structuur ook bepalend is voor de secundaire xanthaan structuur. In het begin van dit onderzoek is daarom de hypothese gesteld dat de in literatuur beschreven verschillende en soms tegenstrijdige modellen om de functionaliteit van xanthaan te verklaren hun oorsprong vinden in verschillen in de primaire, ofwel chemische structuur van de gebruikte xanthaan monsters. Tot op heden is er geen goede methode beschikbaar om de chemische structuur van xanthaan in detail te onderzoeken. Het doel van dit onderzoek was daarom om een geschikte methode te ontwikkelen om de chemische structuur van xanthaan te onderzoeken, zodat de structuur-functie relatie van xanthaan in de toekomst beter begrepen kan worden.

Om de chemische structuur van verschillende xanthaan monsters met elkaar te kunnen vergelijken, zijn diagnostische xanthaan oligosachariden nodig. In dit onderzoek is gekozen voor een enzymatische afbraak van xanthaan om zulke oligosachariden te produceren. Echter, de enzym activiteit zou ook afhankelijk kunnen zijn van de secundaire xanthaan structuur, ofwel xanthaan vouwing. De invloed van zowel de chemische structuur als de xanthaan vouwing op de enzymatische afbraak van xanthaan is daarom eerst onderzocht. In **hoofdstuk 2** laten we zien dat cellulases van *Myceliophthora thermophila* C1 de xanthaan backbone kunnen afbreken onafhankelijk van de chemische structuur van een xanthaan monster. De xanthaan vouwing beïnvloed de enzymatische afbraak aanzienlijk, waarin alleen xanthaan segmenten afgebroken worden die aanwezig zijn in een open ontvouwen structuur. De onafgebroken xanthaan segmenten blijven aanwezig in een geordende gevouwen structuur en hebben een hoog molecuul gewicht. Dit toont aan dat geordende gevouwen xanthaan segmenten een netwerk vormen.

De chemische structuur van xanthaan beïnvloedt de enzymatische afbreekbaarheid van xanthaan niet. De chemische structuur van verschillende xanthaan monsters kan

daarom met elkaar worden vergeleken. In **hoofdstuk 3** wordt beschreven hoe 5 verschillende xanthaan monsters enzymatisch afgebroken worden tot hun herhalingseenheid (HE). Om het type en de relatieve hoeveelheid van de verschillende HEn aanwezig te onderzoeken, is gebruik gemaakt van UPLC-HILIC-ELSD-MS^n. Wij konden concluderen dat een xanthaan molecuul is opgebouwd uit 6 verschillende HEn. De acetylering van de buitenste mannose was verrassend en toont aan dat de chemische structuur van xanthaan complexer is dan algemeen wordt aangenomen. Het vergelijken van xanthaan monsters op basis van de relatieve hoeveelheid van de 6 HEn aanwezig, toonde aan dat xanthaan monsters met exact dezelfde moleculaire samenstelling toch een andere samenstelling in HEn kunnen hebben.

Alleen die xanthaan segmenten die aanwezig zijn in de ontvouwen ongeordende structuur kunnen afgebroken worden door cellulases. Het is daardoor mogelijk om de invloed van de 6 verschillende HEn op de stabiliteit van de gevouwen geordende xanthaan structuur te onderzoeken (**hoofdstuk 4**). Wij hebben aangetoond dat met name de acetylgroepen aan de buitenste mannose de gevouwen xanthaan structuur stabiliseren. Acetylgroepen op de binnenste mannose hadden geen duidelijk effect op de xanthaan vouwing. Hieruit blijkt dat de positie van de acetylgroepen in een xanthaan molecuul bepalend is voor de secundaire xanthaan structuur. De positie van de acetylgroepen is dus van wezenlijk belang voor de functionele eigenschappen van een xanthaan monster. HEn met een pyruvaat groep bleken zich geleidelijk te ontvouwen, wat aantoont dat deze HEn willekeurig verdeeld zijn over een xanthaan molecuul.

In **hoofdstuk 5** werden verschillende koolhydraat acetyl esterases getest voor hun activiteit op xanthaan. AXE3, een acetyl xylaan esterase geproduceerd door *Myceliophthora thermophila* C1, verwijderde ongeveer 60% van alle aanwezige acetylgroepen in xanthaan. Enzym activiteit was echter alleen mogelijk op ongevouwen ongeordende xanthaan segmenten. Karakterisering van de chemische structuur van enzymatisch gemodificeerd xanthaan bewees dat AXE3 specifiek is voor het verwijderen van de acetylgroepen die gepositioneerd zijn aan de binnenste mannose.

Hoofdstuk 6 beschrijft de gedetailleerde karakterisering van de enzym activiteit van pectine acetyl esterase YesY, geproduceerd door *Bacillus subtilis* ras 168, op xanthaan. In tegenstelling tot AXE3, is dit enzym actief op gevouwen xanthaan segmenten. Analyse van de chemische structuur van enzymatisch gemodificeerd xanthaan toonde aan dat YesY specifiek is voor het verwijderen van de acetylgroepen die gepositioneerd aan de buitenste mannose. Gedurende dit onderzoek hebben we dus de eerste 2 enzymen gevonden en gekarakteriseerd die de acetylgroepen in xanthaan

kunnen verwijderen. Deze twee complementerende enzymen kunnen in vervolg onderzoeken gebruikt worden voor de opheldering van de chemische xanthaan structuur en/of de productie van xanthanen met verbeterde functionele eigenschappen.

Tot slot wordt in **Hoofdstuk 7** een overzicht gegeven van de behaalde resultaten, en worden de consequenties van deze resultaten voor verder onderzoek naar de structuur-functie relatie van xanthaan bediscussieert. Verder worden er aanvullende mogelijkheden gegeven om de chemische structuur van xanthaan in de toekomst in meer detail te karakteriseren. Voorlopige resultaten tonen aan dat HEn met een acetylgroep op de buitenste mannose in blok formaties georganiseerd zijn. Daarnaast geeft hoofdstuk 7 een overzicht van potentiële bronnen voor andere xanthaan modificerende enzymen, die bij kunnen dragen aan de verdere opheldering van de xanthaan structuur.

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About the author

CURRICULUM VITAE

Marijn M. Kool was born on January 16th 1984 in Oosterhout, The Netherlands. After graduating from high school (Mgr. Frencken College, Oosterhout) in 2002, she started her studies Food Technology at Wageningen University in 2002. Her BSc degree was completed with a thesis on the acidification of liquid coffee at the Laboratory of Food Chemistry. Her MSc degree in Food Technology, with a specialization in Product Functionality, was completed with a thesis on the isolation and characterization of a potential antimicrobial compound from fermented soybeans at the Laboratories of Food Microbiology and Food Chemistry. Marijn spent the last 5 months of her study in Palmerston North, New Zealand, where she studied the main ellagitannins present in boysenberries at the New Zealand Institute for Plant and Food Research.

After graduating in June 2008, she worked as a researcher at the Laboratory of Food Chemistry for 6 months, before starting the work described in this PhD thesis in May 2009. Marijn is now temporarily employed as post-doc researcher at the Laboratory of Food Chemistry of Wageningen University.

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Kool, M.M.; Comeskey, D.J.; Cooney, J.M.; McGhie, T.K. Structural identification of the main ellagitannins of a boysenberry (*Rubus loganbaccus* x *baileyanus* Britt.) extract by LC-ESI-MS/MS, MALDI-TOF-MS and NMR spectroscopy. Food Chemistry, **2010**, 119 (4), 1535-1543.

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PATENTS

Kool, M.M.; Schols, H.A., Moerschbacher, B.M.; Wagenknecht M.; Means and methods for producing deacetylated xanthan. Europe patent application number: 13003212.1, filed: 24th of June **2013**.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Courses

- Summer Course Glycosciences (VLAG), Wageningen, The Netherlands, 2010†
- Advanced Food Analysis (VLAG), Wageningen, The Netherlands, 2010†
- Chromeleon course (Dionex), Heeze, The Netherlands, 2011
- Food and Biorefinery Enzymology (VLAG), Wageningen, The Netherlands, 2011†

Conferences

- 8th Carbohydrate engineering meeting (Institute of protein biochemistry), Ischia, Italy, 2009
- EPNOE meeting (VLAG), Wageningen, The Netherlands, 2011†
- 12th International Hydrocolloid Conference (Whistler Centre for Carbohydrate research), West-Lafayette (IN), USA, 2012†
- Gums and Stabilizers for Food Industry (Food Hydrocolloids Trust), Wrexham, UK, 2013‡

General courses

- VLAG PhD introduction week (VLAG), 2009
- Project and time management (WGS), 2009
- Techniques for writing and presenting a scientific paper (WGS), 2010
- Scientific writing (VLAG), 2012
- Career Perspectives (WGS), 2012

Additional activities

- Preparation PhD research proposal
- Food Chemistry study trip to Ghent, Belgium, 2009
- PhD trip FCH to Switzerland/Italy, 2010‡
- PhD trip FCH to Singapore/Malaysia, 2012‡
- PolyModE project meetings 2009/2013
- BSc/MSc thesis student presentations and colloquia, 2009/2013
- PhD presentations Food Chemistry, 2009/2013

† Poster; ‡ Poster and oral presentation
VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences
WGS; Wageningen Graduate School
FCH: Food Chemistry

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