Carbohydrate Polymers 97 (2013) 368–375

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

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ARTICLE INFO

Article history:
Received 12 March 2013
Received in revised form 16 May 2013
Accepted 20 May 2013
Available online 25 May 2013

Keywords:
Renatured xanthan
Conformation
Cellulases
Degree of substitution
HPSEC
Circular dichroism

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.

1. Introduction

Xanthan is an extracellular polysaccharide that is secreted by the microorganism *Xanthomonas campestris* (Jeanes, Pittsley, & Senti, 1961), with a molecular mass ranging from 1 to 7 × 10^6 Da (Milas, Reed, & Printz, 1996; Milas, Rinaudo, & Tinland, 1986). 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 (Fig. 1) (Jansson, Kenne, & Lindberg, 1975). 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 (Cadmus et al., 1976; Orentas, Sloneker, & Jeanes, 1963; Sutherland, 1981). No information is available regarding the distribution pattern of these substituents.

In solution, native xanthan, as produced by *X. campestris*, exists in a double-stranded helical conformation with an order–disorder transition upon changes in temperature and/or ionic strength (Bezemer, Ubbink, Kooker de, Kuil, & Leyte, 1993; Liu & Norisuye, 1988a; Matsuda, Biyajima, & Sato, 2009). 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 (Callet, Milas, & Rinaudo, 1987; Capron, Brigand, & Muller, 1998; Matsuda et al., 2009; Milas et al., 1996; Oviatt & Brant, 1994). 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 (Holzwarth, 1976; Milas & Rinaudo, 1979; Morris, Rees, & Young, 1977).

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 (Callet et al., 1987; Dentini, Crescenzi, & Blasi, 1984; Rinaudo, 2004; Shatwell, Sutherland, Dea, & Ross-Murphy, 1990). Because both the fermentation conditions and the *Xanthomonas* strain used during the production of xanthan influence the primary structure of xanthan, differences in conformational behaviour may be present between different batches of xanthan. Therefore, the primary structure of

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http://dx.doi.org/10.1016/j.carbpol.2013.05.045

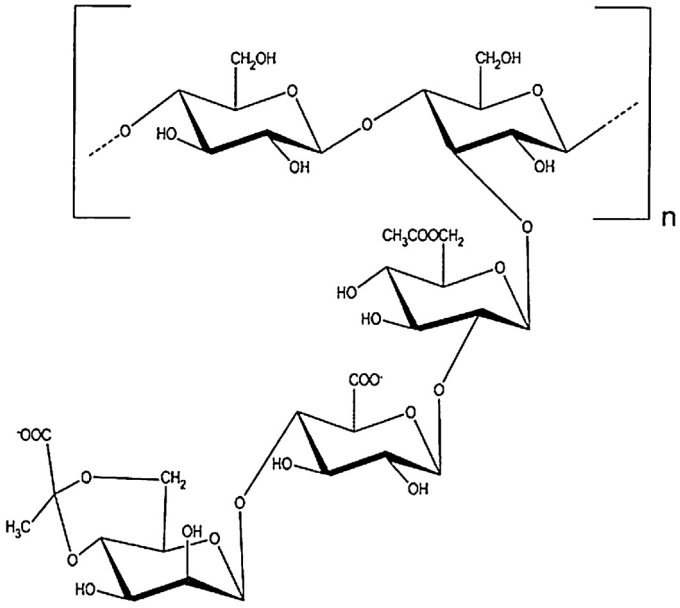

Fig. 1. The ideal repeating unit of xanthan as reported by Jansson et al. (1975).

xanthan is of importance when the transitional behaviour 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 (Cheetham & Mashimba, 1991; Christensen & Smidsrød, 1996; Rinaudo & Milas, 1980; Sutherland, 1984). 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 (Cheetham & Mashimba, 1991; Sutherland, 1984). Because differences in the primary structure also cause differences in the order–disorder behaviour, it was also hypothesized that differences in conformation, due to differences in the primary structure, result in enzyme resistant xanthan strands (Rinaudo & Milas, 1980; Sutherland, 1984). 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.

2. Materials and methods

2.1. 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 (Bradshaw, Nisbet, Kerr, & Sutherland, 1983). 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 mL^{-1} xanthan solution was mixed with Amberlite IR-120-H^+ ion-exchange material (BDH, Poole Dorset, UK) for 30 min at room temperature. The ion exchange material was removed from the xanthan solution by centrifugation (5000 × *g*, 15 min, 20 °C) (Rinaudo & Milas, 1978). 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".

2.2. 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 (Ruiter, Schols, Voragen, & Rombouts, 1992). 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) (Bradshaw et al., 1983).

2.3. Circular dichroism

The ellipticities (in mDeg) of 2 mg mL^{-1} xanthan solutions were monitored at 219 nm from 10 to 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 s, 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 Eq. (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 (Greenfield, 2006).

$$\alpha = 1 - \frac{\theta_t - \theta_U}{\theta_F - \theta_U} \quad (1)$$

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.

2.4. Enzymatic hydrolysis

Cellulases in the experimental enzyme preparation C1-G1 from *Myceliophthora thermophila C1* (Dyadic Netherlands, Wageningen, The Netherlands) (Kühnel, Schols, & Gruppen, 2011) 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

Table 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

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 (Lever, 1972). 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%.

2.5. High performance size exclusion chromatography (HPSEC)

HPSEC was performed on a Dionex 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} (Milas et al., 1986). 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 to 34 min.

3. Results and discussion

3.1. 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 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 (Fig. 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 (Hassler & Doherty, 1990; Sutherland, 1984).

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.

3.2. Order–disorder transitions

The transitional behaviour 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 (Fig. 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 (Morris et al., 1977; Shatwell, Sutherland, & Ross-Murphy, 1990). 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.

In contrast to the other xanthans studied, the transitional behaviour 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 behaviour of PFX in the measured temperature range (Holzwarth, 1976; Morris et al., 1977; Shatwell, Sutherland, Dea, et al., 1990). As shown in Fig. 2C, lowering the ionic strength, by the conversion of PFX into PFX-H^+, does influence the conformational behaviour. Due to the removal of the counter ions of the glucuronic acid units, negative charges are induced in the xanthan side chains. Thereby the

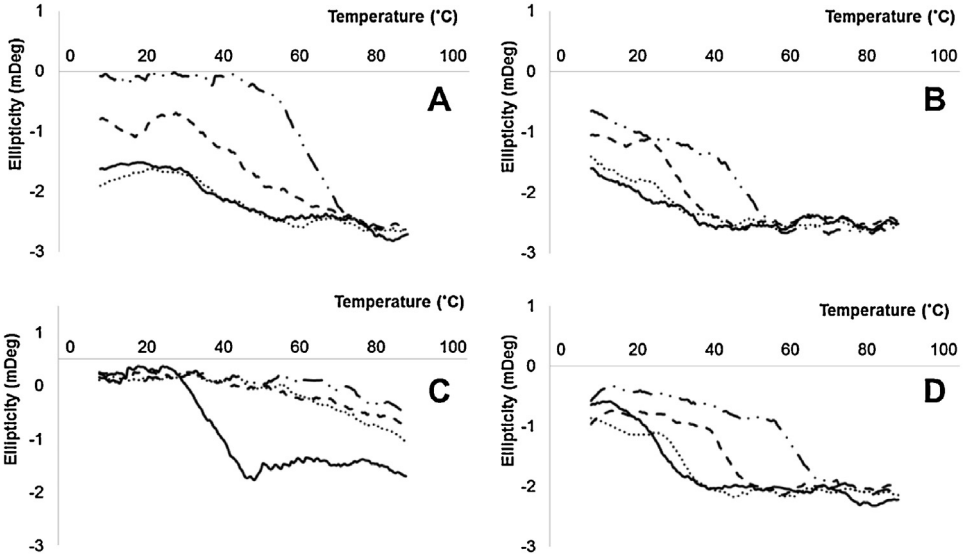

Fig. 2. Transition profiles of xanthan in: the H^+-form in deionized water (—); the Na^+-form in deionized water (····); the Na^+-form in 2 mM NaCl (– –); and the Na^+ form in 10 mM NaCl (–···–). (A) Unmodified xanthan; (B) acetyl free xanthan; (C) pyruvate free xanthan; (D) acetyl and pyruvate free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

Table 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.

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 Eq. (1). The normalized fitted graphs are depicted in Fig. 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.

3.3. 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 Fig. 3 several temperature and salt conditions were selected in which the conformation of xanthan ranges from $\alpha = 0$ to $\alpha = 1$. An overview of all conditions is given in Table 2.

3.3.1. 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 incubation 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 lowering the temperature to 40 °C. So, both the enzyme activity as well as the xanthan conformation depends on temperature. To be able to solely determine the influence of the conformation on the cellulase activity, the effect of a reduced temperature, and thereby a reduced 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 Fig. 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.

3.3.2. 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 3 gives an overview of: (1) the type of xanthan, (2) the ionic strength and temperature of the xanthan

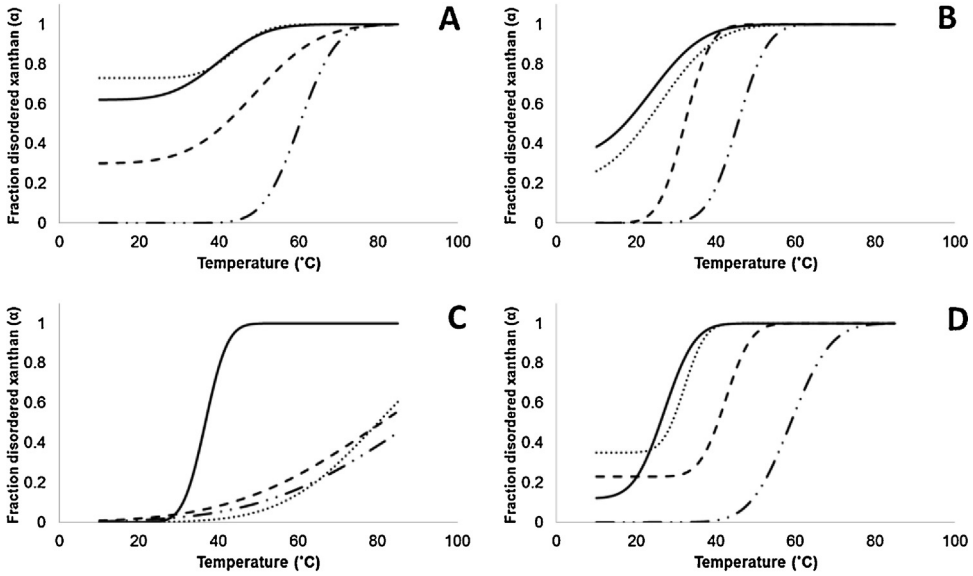

Fig. 3. Fraction of disordered xanthan as function of temperature (°C) in: the H^+-form in deionized water (—); the Na^+ form in deionized water (····); the Na^+ form in 2 mM NaCl (– –); and the Na^+ form in 10 mM NaCl (–···–). (A) Unmodified xanthan; (B) acetyl free xanthan; (C) pyruvate free xanthan; (D) acetyl and pyruvate free xanthan.

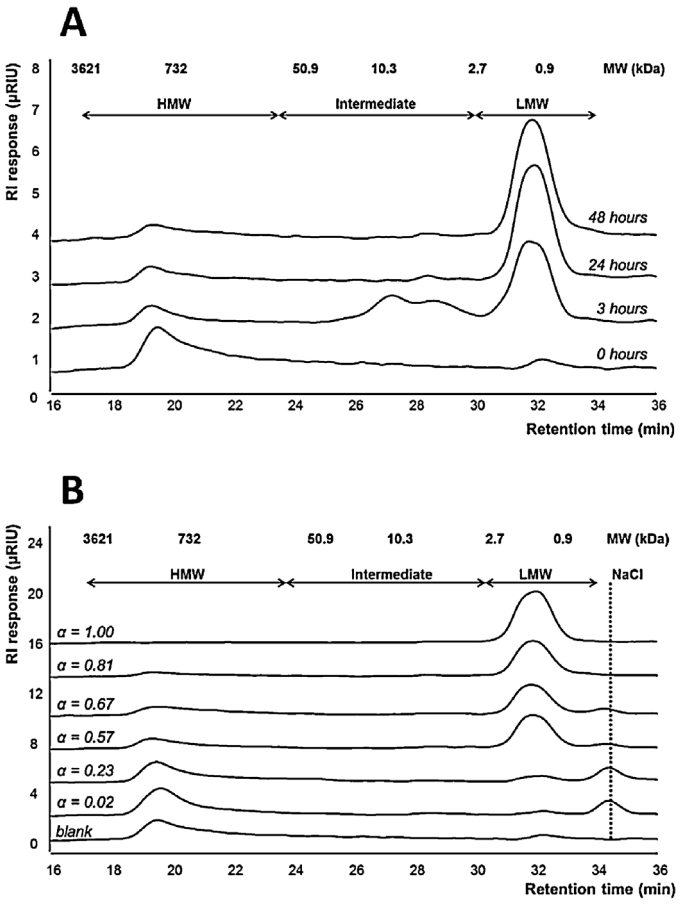

Fig. 4. HPSEC elution patterns of normal xanthan digests. (A) Xanthan degradation followed in time at 40 °C; in demineralized water. (B) 48 h digests of normal xanthan differing in conformation (α = fraction of disordered conformation; blank = untreated xanthan).

solution during enzyme hydrolysis, (3) the corresponding fraction of disordered conformation (α), and (4) the degree of hydrolysis (DH) after 48 h of incubation. Fig. 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 (Rinaudo & Milas, 1980) showed a correlation between the speed of hydrolysis and the xanthan conformation. 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 Fig. 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 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 (Lever, 1972). 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 Fig. 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 Fig. 5B. The elution profiles show that as long as xanthan exists in a completely disordered

Table 3
Degree of hydrolysis obtained after incubation of xanthan with cellulases for 48 h 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 = 100%: all backbone linkages are degraded, based on the increase in reducing end sugars as measured by PAHBAH assay.

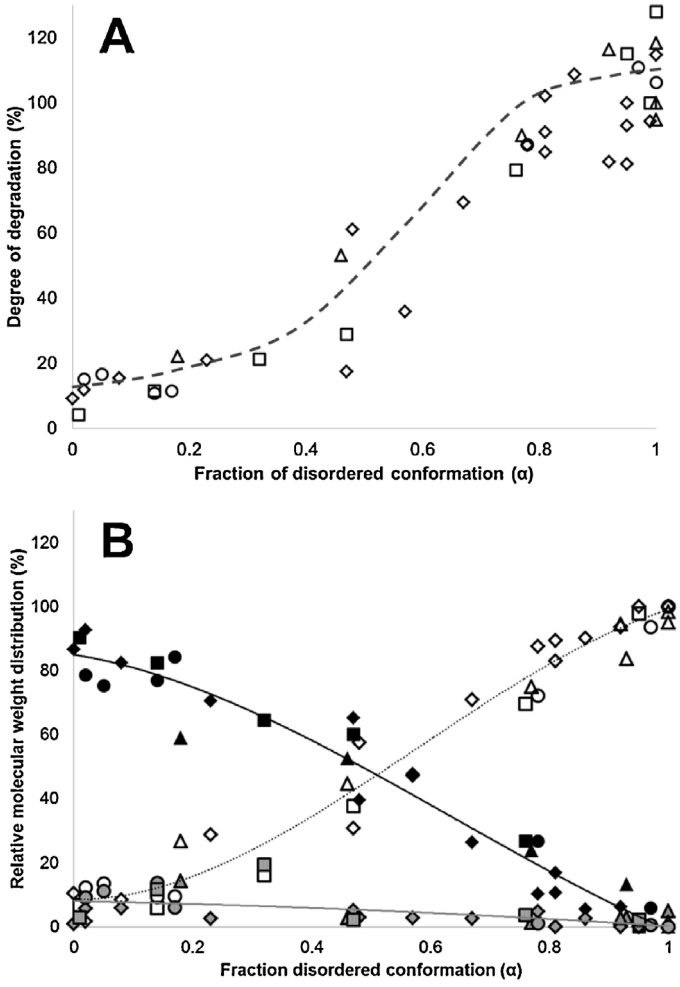

Fig. 5. Correlation between the fraction disordered xanthan (α) and the enzymatic hydrolysis after a 48 h incubation with the experimental enzyme preparation C1-G1 based on: (A) the degree of hydrolysis measured by the increase in reducing end sugars. (B) The molecular weight distribution measured by HPSEC. Diamonds: normal xanthan; triangles: AFX; dots: PFX; squares: APFX. Open symbols: xanthan oligosaccharides; grey symbols: intermediate degradation products; closed symbols: non-degraded xanthan.

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 (Fig. 5A), and the relative abundance of smaller fragments (Fig. 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 (Cheetham & Mashimba, 1991; Sutherland, 1984). 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 (1984).

3.4. Considerations on the transitional behaviour of renatured xanthan

Figs. 4B and 5B show that all enzyme digests obtained with an $\alpha \leq 0.95$, 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 behaviour 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 (Liu & Norisuye, 1988b; Norton, Goodall, Frangou, Morris, & Rees, 1984), 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 behaviour as previously described.

Different populations of ordered and disordered conformation, however, might be obtained when high intermolecular variations exist within a xanthan batch (Callet et al., 1987; Shatwell, Sutherland, & Ross-Murphy, 1990). 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 (Gulrez, Al-Assaf, Fang, Phillips, & Gunning, 2012). Because the size exclusion method used in this study is not able to distinguish between molecular mass values beyond 5.0×10^6 Da. Partial dissociation of xanthan molecules from a multiple stranded network would explain our finding as long as the remaining network has a $M_w \geq 5.0 \times 10^6$ Da. 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 (Sutherland, 1984). Another study reported on the side-by-side association of ordered xanthan structures (Norton et al., 1984). 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.

4. 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.

Acknowledgment

This research was supported by the European Community within a consortium PolyModE KBBE-2007-3-3-07 and is gratefully acknowledged.

References

Bezemer, L., Ubbink, J. B., Kooker de, J. A., Kuil, M. E., & Leyte, J. C. (1993). On the conformational transitions of native xanthan. *Macromolecules*, *26*, 6436–6446.

Bradshaw, I. J., Nisbet, B. A., Kerr, M. H., & Sutherland, I. W. (1983). Modified xanthan – Its preparation and viscosity. *Carbohydrate Polymers*, *3*(1), 23–38.

Cadmus, M. C., Rogovin, S. P., Burton, K. A., Pittsley, J. E., Knutson, C. A., & Jeanes, A. (1976). Colonial variation in *Xanthomonas campestris* NRRL B-1459 and charaterization of the polysaccharide from a variant strain. *Canadian Journal of Microbiology*, *22*(7), 942–948.

Callet, F., Milas, M., & Rinaudo, M. (1987). Influence of acetyl and pyruvate content on rheologyical properties of xanthan in dilute solution. *International Journal of Biological Macromolecules*, *9*(5), 291–293.

Capron, I., Brigand, G., & Muller, G. (1998). Thermal denaturation and renaturation of a fermentation broth of xanthan: Rheological consequences. *International Journal of Biological Macromolecules*, *23*, 215–225.

Cheetham, N. W. H., & Mashimba, E. N. M. (1991). Characterisation of some enzymatic-hydrolysis products of xanthan. *Carbohydrate Polymers*, *15*(2), 195–206.

Christensen, B. E., & Smidsrød, O. (1996). Dependence of the content of substituted (cellulosic)regions in prehydrolysed xanthans on the rate of hydrolysis by *Trichoderma reesei* endoglucanase. *International Journal of Biological Macromolecules*, *18*(1–2), 93–99.

Dentini, M., Crescenzi, V., & Blasi, D. (1984). Conformational properties of xanthan derivatives in dilute aqueous solution. *International Journal of Biological Macromolecules*, *6*(2), 93–98.

Greenfield, N. J. (2006). Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. *Nature protocols*, *1*(6), 2527–2535.

Gulrez, S. K. H., Al-Assaf, S., Fang, Y., Phillips, G. O., & Gunning, A. P. (2012). Revisiting the conformation of xanthan and the effect of industrially relevant treatments. *Carbohydrate Polymers*, *90*(3), 1235–1243.

Hassler, R. A., & Doherty, D. H. (1990). Genetic engineering of polysaccharide structure: Production of variants of xanthan gum in *Xanthomonas campestris*. *Biotechnology Progress*, *6*(3), 182–187.

Holzwarth, G. (1976). Conformation of the extracellular polysaccharide of xanthomonas campestris. *Biochemistry*, *15*(19), 4333–4339.

Jansson, P. E., Kenne, L., & Lindberg, B. (1975). Structure of extracellular polysaccharide from *Xanthomonas campestris*. *Carbohydrate Research*, *45*(DEC), 275L 282.

Jeanes, A., Pittsley, J. E., & Senti, F. R. (1961). Polysaccharide B-1459: A new hydrocolloid polyelectrolyte produced from glucose by bacterial fermentation. *Journal of Applied Polymer Science*, *17*, 519–526.

Kühnel, S., Schols, H., & Gruppen, H. (2011). *Aiming for the complete utilization of sugar-beet pulp: Examination of the effects of mild acid and hydrothermal pretreatment followed by enzymatic digestion*, *4*(1), 1–14.

Lever, M. (1972). A new reaction for colorimetric ceterminationof carbohydrates. *Analytical Biochemistry*, *47*, 273–279.

Liu, W., & Norisuye, T. (1988a). Order–disorder conformation change of xanthan in 0.01 M aqueous sodium-chloride – Dimensional behaviour. *Biopolymers*, *27*(10), 1641–1654.

Liu, W., & Norisuye, T. (1988b). Thermally induced conformation change of xanthan – Interpretation of viscosity behaviour in 0.01 M Aqueous sodium-chloride. *International Journal of Biological Macromolecules*, *10*(1), 44–50.

Matsuda, Y., Biyajima, Y., & Sato, T. (2009). Thermal denaturation, renaturation, and aggregation of a double-helical polysaccharide xanthan in aqueous solution. *Polymer Journal*, *41*(7), 526–532.

Milas, M., Reed, W. F., & Printz, S. (1996). Conformations and flexibility of native and re-natured xanthan in aqueous solutions. *International Journal of Biological Macromolecules*, *18*(3), 211–221.

Milas, M., & Rinaudo, M. (1979). Conformational investigation on the bacterial polysaccharide xanthan. *Carbohydrate Research*, *76*, 189–196.

Milas, M., Rinaudo, M., & Tinland, B. (1986). Comparative depolymerisation of xanthan gum by ultrasonic and enzymatic treatments – Rheological and structural properties. *Carbohydrate Polymers*, *6*(2), 95–107.

Morris, E. R., Rees, D. A., & Young, G. (1977). Order disorder transition for a bacterial polysaccharide in solution. A role for polysaccharide conformation in recognition between Xanthomonas pathogen and its plant host. *Journal of Molecular Biology*, *110*(1), 1–16.

Norton, I. T., Goodall, D. M., Frangou, S. A., Morris, E. R., & Rees, D. A. (1984). Mechanism and dynamics of conformational ordering in xanthan polysaccharide. *Journal of Molecular Biology*, *175*(3), 371–394.

Orentas, D. G., Sloneker, J. H., & Jeanes, A. (1963). Pyruvic acid content and constituent sugar of exocellular polysaccharides from different species of the genus *Xanthomonas*. *Canadian Journal of Microbiology*, *9*(3), 427–430.

Oviatt H.W., Jr., & Brant, D. A. (1994). Viscoelastic behavior of thermally treated aqueous xanthan solutions in the semidilute concentration regime. *Macromolecules*, *27*(9), 2402–2408.

Rinaudo, M. (2004). Role of substituents on the properties of some polysaccharides. *Biomacromolecules*, *5*(4), 1155–1165.

Rinaudo, M., & Milas, M. (1978). Polyelectrolyte behaviour of a bacterial polysaccharide from *Xanthomonas campestris*: Comparison with carboxymethylcellulose. *Biopolymers*, *17*, 2663–2678.

Rinaudo, M., & Milas, M. (1980). Enzymic-hydrolysis of the bacterial polysaccharide xanthan by cellulase. *International Journal of Biological Macromolecules*, *2*(1), 45–48.

Ruiter, A. d., Schols, H. A., Voragen, A. G. J., & Rombouts, F. F. (1992). Carbohydrate analysis of watersoluble uronic acid-containing polysaccharides with the high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Analytical Biochemistry*, *207*, 176–185.

Shatwell, K. P., Sutherland, I. W., Dea, I. C. M., & Ross-Murphy, S. B. (1990). The influence of acetyl and pyruvate substituents on the helix-coil transition behaviour of xanthan. *Carbohydrate Research*, *206*(1), 87–103.

Shatwell, K. P., Sutherland, I. W., & Ross-Murphy, S. B. (1990). Influence of acetyl and pyruvate substituents on the solution properties of xanthan polysaccharide. *International Journal of Biological Macromolecules*, *12*(2), 71–78.

Sutherland, I. W. (1981). Xanthomonas polysaccharides – Improved methods for their comparison. *Carbohydrate Polymers*, *1*(2), 107–115.

Sutherland, I. W. (1984). Hydrolysis of unordered xanthan in solution by fungal cellulases. *Carbohydrate Research*, *131*(1), 93–104.