Carbohydrate Polymers 111 (2014) 222–229

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

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

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ARTICLE INFO

Article history:
Received 29 October 2013
Received in revised form 11 April 2014
Accepted 15 April 2014
Available online 26 April 2014

Keywords:
Enzymatic deacetylation
Xanthan conformation
AXE3
Position of acetyl groups

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 48 h 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 favouring the disordered conformation. AXE3-deacetylated xanthan was hydrolyzed using cellulases and analyzed for its repeating units using UPLC–HILIC–ELSD/ESI–MS. This 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, 57% of all acetyl groups, representing 70% of all acetyl groups on the inner mannose units, were hydrolyzed.

1. Introduction

The bacterial polysaccharide xanthan is a polymer having a (1 → 4) linked β-D-glucopyranosyl backbone, with a β-D-mannopyranosyl-(1 → 4)-β-D-glucopyranosyluronic acid-(1 → 2)-α-D-mannopyranosyl side chain substituted to every other glucopyranosyl residue at the *O*-3 position (Fig. 1) (Jansson, Kenne, & Lindberg, 1975). Approximately 85% of all inner Man*p* residues are 6-*O*-acetylated and 50–70% of all terminal Man*p* residues are substituted with a pyruvic acid ketal. Additionally, about 5–25% of all terminal Man*p* residues are substituted with an acetyl group at the *O*-6 position (Kool, Gruppen, Sworn, & Schols, 2013a; Stankowski, Mueller, & Zeller, 1993).

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 (Morrison, Clark, Talashek, & Yuan, 2004; Shatwell, Sutherland, Ross-Murphy, & Dea, 1990b, Shatwell, Sutherland, Ross-Murphy, & Dea, 1990a, 1991). 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 (Shatwell, Sutherland, & Ross-Murphy, 1990a; Shatwell et al., 1990b). How the position of acetyl influences the xanthan functionality, however, is 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 (Bradshaw, Nisbet, Kerr, & Sutherland, 1983; Pinto, Furlan, & Vendruscolo, 2011). However, such a process randomly removes acetyl groups and backbone degradation might be apparent. Another method to control the degree of acetylation in xanthan is the use of specific *Xanthomonas* strains and/or fermentation conditions for the xanthan production (Flores Candia & Deckwer, 1999; Hassler & Doherty, 1990). 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 (Sandford et al., 1977). 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 the

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http://dx.doi.org/10.1016/j.carbpol.2014.04.064
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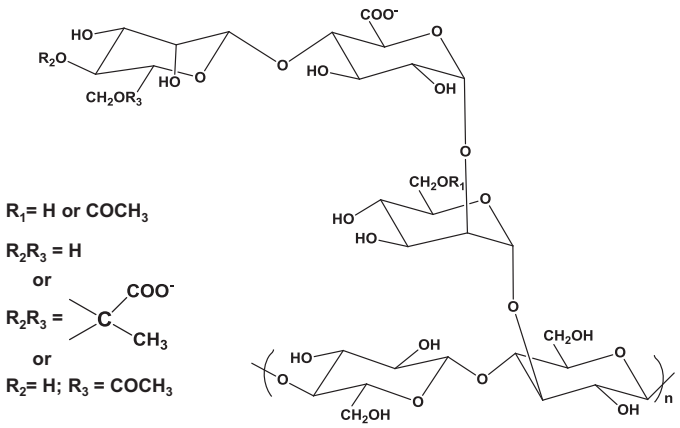


Fig. 1. The xanthan repeating unit.

classification of carbohydrate hydrolases and carbohydrate lyases (Davies et al., 2005). 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 (Biely, 2012; Tenkanen, Eyzaguirre, Isoniemi, Faulds, & Biely, 2003). 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 (Kool et al., 2013b; Rinaudo & Milas, 1980; Sutherland, 1984). In solution xanthan can adapt an ordered helical conformation or a random disordered conformation (Bezemer, Ubbink, Kooker de, Kuil, & Leyte, 1993; Matsuda, Biyajima, & Sato, 2009). 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 (Milas & Rinaudo, 1979; Morris, Rees, & Young, 1977). This alignment of side chains when xanthan appears in an ordered conformation and/or the stacking of ordered structures into a network is assumed to make xanthan resistant against enzymatic modifications (Rinaudo & Milas, 1980; Sutherland, 1984). Recently, it was proven that only xanthan molecules that appear in a disordered conformation are susceptible to enzymatic backbone degradation by cellulases (Kool et al., 2013b). 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 te 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 the deacetylation of the inner and/or outer mannose units.

2. Materials and methods

2.1. 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 (Kool et al., 2013a). A detailed overview of the chemical characterization is given in Table 1. Acetylated xylooligosaccharides were obtained and characterized as described by Koutaniemi et al., 2013. Sugar beet pectin (SBP6230) (Buchholt, Christensen, Fallesen, Ralet, & Thibault, 2004) was obtained from DuPont (Brabrand, Denmark). Chitin and chitosan oligosaccharides (degree of polymerization of 2–6) were purchased from Seikagaku Corp. (Tokyo, Japan).

2.2. 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 (Kool et al., 2013b; Morris et al., 1977). The fraction of disordered conformation (α) was, therefore, estimated using Eq. (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.

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

θ_F was determined in 10 mM NaCl solution at 20 °C and θ_U was determined in Millipore water 70 °C.

2.3. Carbohydrate acetyl esterases

An overview of the carbohydrate acetyl esterases tested, including their origin and their known substrate specificities is given in Table 2. AXE2 and AXE3, both produced by *Myceliophthora thermophila* C1 (formerly termed *Chrysosporium lucknowense* C1 (Visser et al., 2011)), were produced and purified as described elsewhere (Hinz et al., 2009; Pouvreau et al., 2011). AnAXE and RG-04 were extracted and purified from *Aspergillus* sp. preparations as described by Kormelink, Lefebvre, Strozyk, and Voragen (1993) and Searle-van Leeuwen, Broek, Schols, Beldman, and Voragen (1992), respectively. The coding sequences of PdaA, PdaB, 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 *Escherichia coli* Rosetta 2(DE3)(pLysSRARE2) (Novagen) using auto-induction medium (Studier, 2005). Enzyme purification was done by affinity chromatography using a 1-ml Strep-Tactin Superflow Plus Column (Qiagen, Hilden, Germany) as described elsewhere Remoroza et al. (2014).

2.4. 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 (pH 5.6), 10 mM NaCl solution (pH 5.5) and 50 mM sodium citrate buffer (pH 6.0). The acetic acid release was determined using a Megazyme acetic

Table 1
Chemical characterization of xanthan including the molecular composition and the ratio of the RUs present.

	Glc:Man:GlcA Molar ratio	Acetyl content (w/w%)	Pyruvate content (w/w%)	RU-I	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

Table 2
Characteristics of the carbohydrate acetyl esterases used in this study, including abbreviation, 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 et al. (2011), Hinz et al. (2009)
	AXE3	*Myceliophthora thermophila* C1	HQ324257	CE-1	Pouvreau et al. (2011), Hinz et al. (2009)
	AnAXE	*Aspergillus niger*	Unknown	CE-1	Kormelink et al. (1993)
Polysaccharide deacetylase (putative chitin deacetylase)	PdaA	*Bacillus licheniformis* DSM 13	AAU39762.1	CE-4	Own unpublished data
	PdaB	*Bacillus licheniformis* DSM 13	AAU39149.1	CE-4	Own unpublished data
Pectin acetyl esterase	PAE2	*Pectobacterium atrosepticum* SCRI1043	CAG75311	Unknown	Own unpublished data
	PAE4	*Bacillus licheniformis* DSM 13	AAU42913	CE-12	Remoroza et al. (2014)
	RG-04	*Aspergillus aculeatus*	Unknown	CE-12	Searle-van Leeuwen et al. (1992)

acid kit (Megazyme, Wicklow, Ireland) and expressed as percentage of the total acetyl content present in 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 (4 °C; 18 h). All incubations were performed in duplicate.

Acetyl esterase AXE3 was further characterized for its activity towards 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, 2, 5 and 10 mM NaCl solutions (pH ~5.6) 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 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 *M. thermophila* C1 (Dyadic Netherlands, Wageningen, The Netherlands) at 60 °C for 48 h (Kool et al., 2013b). The xanthan digests obtained were analyzed for their repeating units using HILIC–ELSD–MS^n as described previously (Kool et al., 2013a).

3. Results and discussion

3.1. 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 putative Pdas (Table 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 (Kool et al., 2013b), none of the xylan AEs released significant amounts of acetic acid from xanthan either. In the absence of salts, AXE3 was able to release approximately 30% of all acetyl groups. Previous experiments (Kool et al., 2013b) showed that under these conditions approximately 80% of all xanthan molecules appear 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 favour 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 (pH 5.5–6.0). At these conditions, none of the enzymes, except AXE3, was active against their known specific substrate, while all enzymes showed the expected activity in a 50 mM sodium citrate buffer, pH 6.0 (data not shown). Except for AXE3, none of the enzymes is, therefore, active under conditions favouring 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 originating from *M. thermophila* C1, showed that the two enzymes, although from different CE families, have similar activity towards acetylated xylan oligosaccharides (Hinz et al., 2009; Pouvreau et al., 2011) However, substrate specificity tests showed that AXE2 has a more specific mode of action than AXE3. More general studies showed that enzymes from CE family 1 are, in contrast to CE family 5, not specific for *O*-2 deacetylation (Altaner et al., 2003). The difference observed between the activities towards xanthan of AXE2 and AXE3, even though they origin from the same fungal host, can probably be explained by differences in their substrate specificity and/or substrate binding.

Other studies, describing acetyl xylan esterases from different CE families, showed that within the CE1 family several enzymes exist which are known to deacetylate (galacto)glucomannans, their oligomers or acetylated mannopyranosyl units at the *O*-2 and/or *O*-3 position (Biely, Côte, Kremnicjý, Weisleder, & Greene, 1996; Tenkanen, 1998; Tenkanen, Puls, Rättö, & Viikari, 1993). Furthermore, Montanier et al. (2009) showed that CE family 2 acetyl

Table 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.

Solvent 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

esterases are active towards both galactoglucomannans and konjac glucamannans, indicating that these enzymes might be able to deacetylate Man*p* residues which are acetylated on the *O*-6 position as well. Topakas et al. (2010) further investigated the specificity of CE family 2 acetyl esterases and proved that these enzymes are dedicated to the *O*-6 deacetylation of aldohexoses. Although most of the acetyl mannan esterases reported in literature only showed activity towards oligomer material, future screening for xanthan acetyl esterases certainly should include these acetyl mannan esterases as xanthan is (*O*-6) acetylated at the Man*p* residues.

3.2. 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 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 (Fig. 2a). 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 units interact with the xanthan backbone. These acetyl groups would, therefore, be positioned at the inside of the xanthan helix (Morris et al., 1977; Pelletier, Viebke, Meadows, & Williams, 2001; Tako & Nakamura, 1984) 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 (Kool et al., 2013a). 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 (Hashimoto, Miki, Tsuchiya, Nankai, & Murata, 1998; Ruijssenaars, de Bont, & Hartmans, 1999), indicating that that the outer mannose is accessible for enzymes when xanthan appears in an ordered conformation. Because AXE3 is only active towards disordered xanthan segments, it is expected that AXE3 is specific for the removal of the acetyl groups from the inner mannose unit.

3.3. Characterization of the AXE3 activity towards xanthan

3.3.1. Optimal temperature

Fig. 2b shows the temperature profiles of AXE3 towards xanthan in a salt free environment and towards acetylated xylan oligosaccharides at its optimal pH of 7.0 (Pouvreau et al., 2011). Optimal performance of AXE3 towards xanthan was observed in the temperature range 50–55 °C, whereas a broader and lower optimal

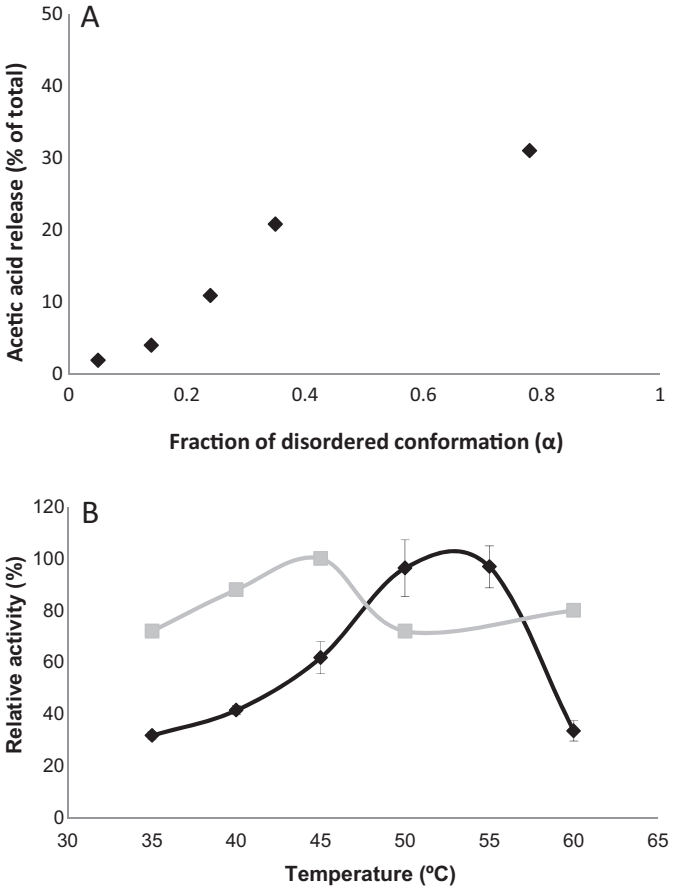


Fig. 2. Optimal conditions for the deacetylation of xanthan by AXE3. (A) Influence of the xanthan conformation (α) on the acetic acid release from xanthan after a 48 h incubation with AXE3 at 40 °C. (B) Influence of the temperature on the AXE3 activity towards xanthan (♦) and acetylated xylan oligosaccharides (■) at their respective optimal incubation conditions. The data for acetylated xylan are adapted from Pouvreau et al. (2011).

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

3.3.2. 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. (2011). The specific activity towards acetylated xylooligosaccharides is 8.3 U mg protein^{-1}. The specific activity towards xanthan is 600× 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.

3.3.3. 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 15%, 35% and 57% of all acetyl groups, respectively. Subsequently, the relative abundance of the repeating units (RUs) present in cellulase digests of the partly deacetylated xanthans was analysed using HILIC–ELSD–MS (Kool et al., 2013a) and compared to the relative

abundance of the RUs present in the cellulase digest of unmodified xanthan (Fig. 3). The observed trend of modification 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 RUs 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 (Kool et al., 2013a), 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 (Kool et al., 2013a). The intensity of these different fragments, was used to determine the ratio in which the two single acetylated RUs were present before and after AXE3 modification (Fig. 4). Before modification, ~10% of all single acetylated RUs are acetylated on the outer mannose, while this

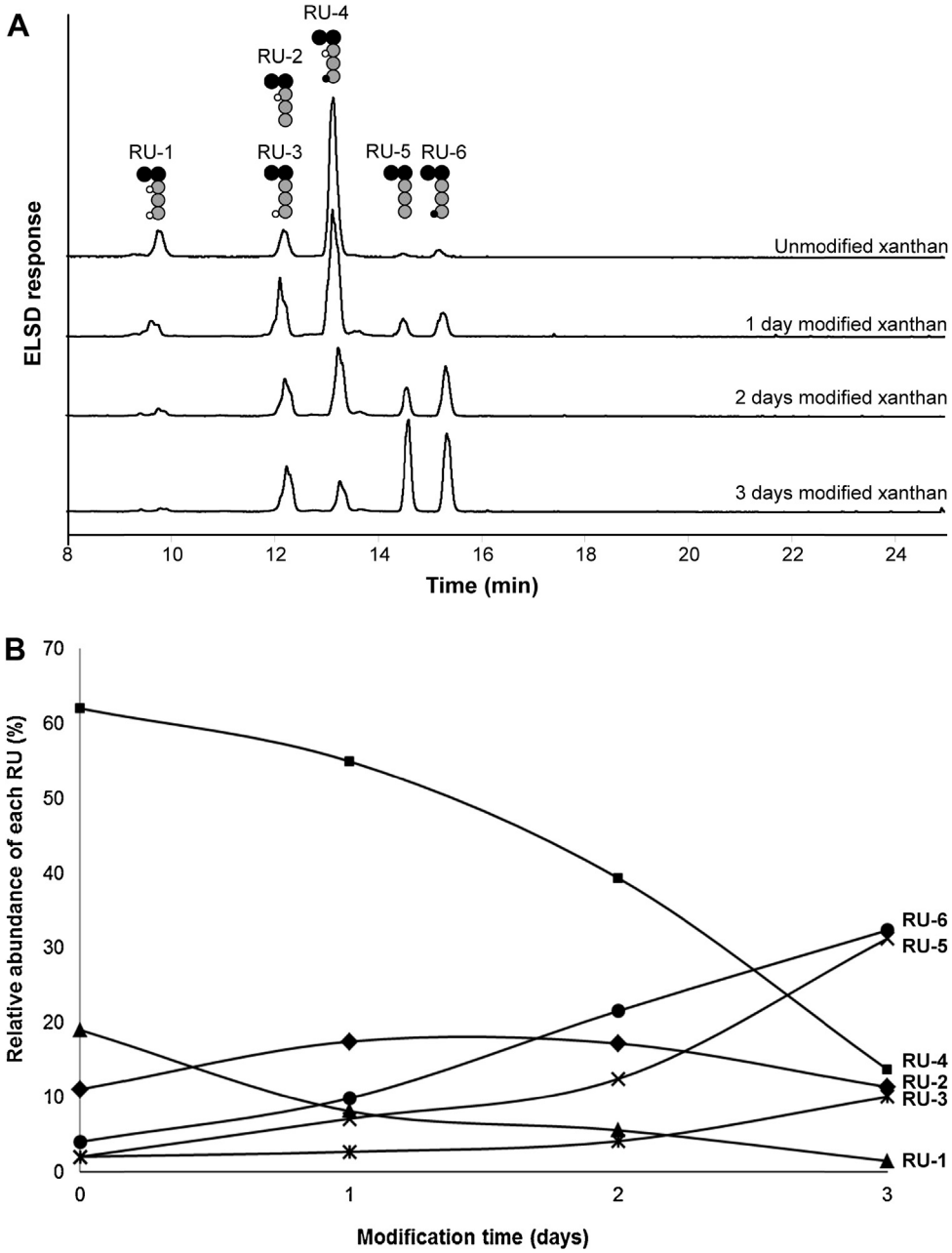
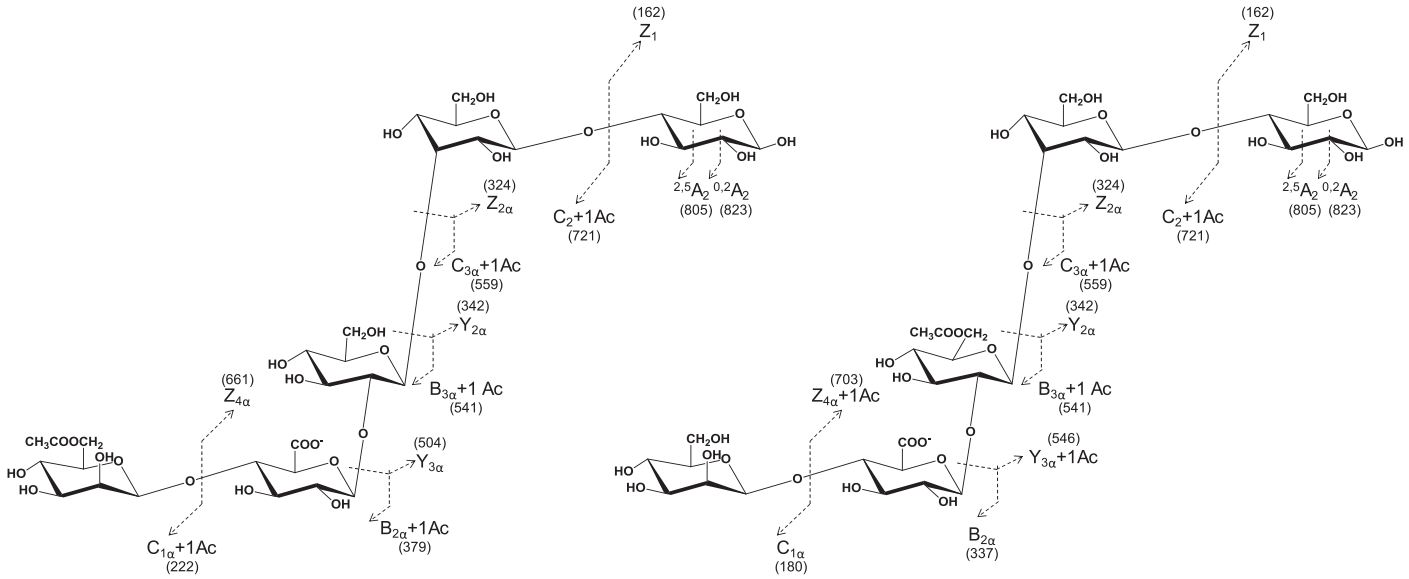
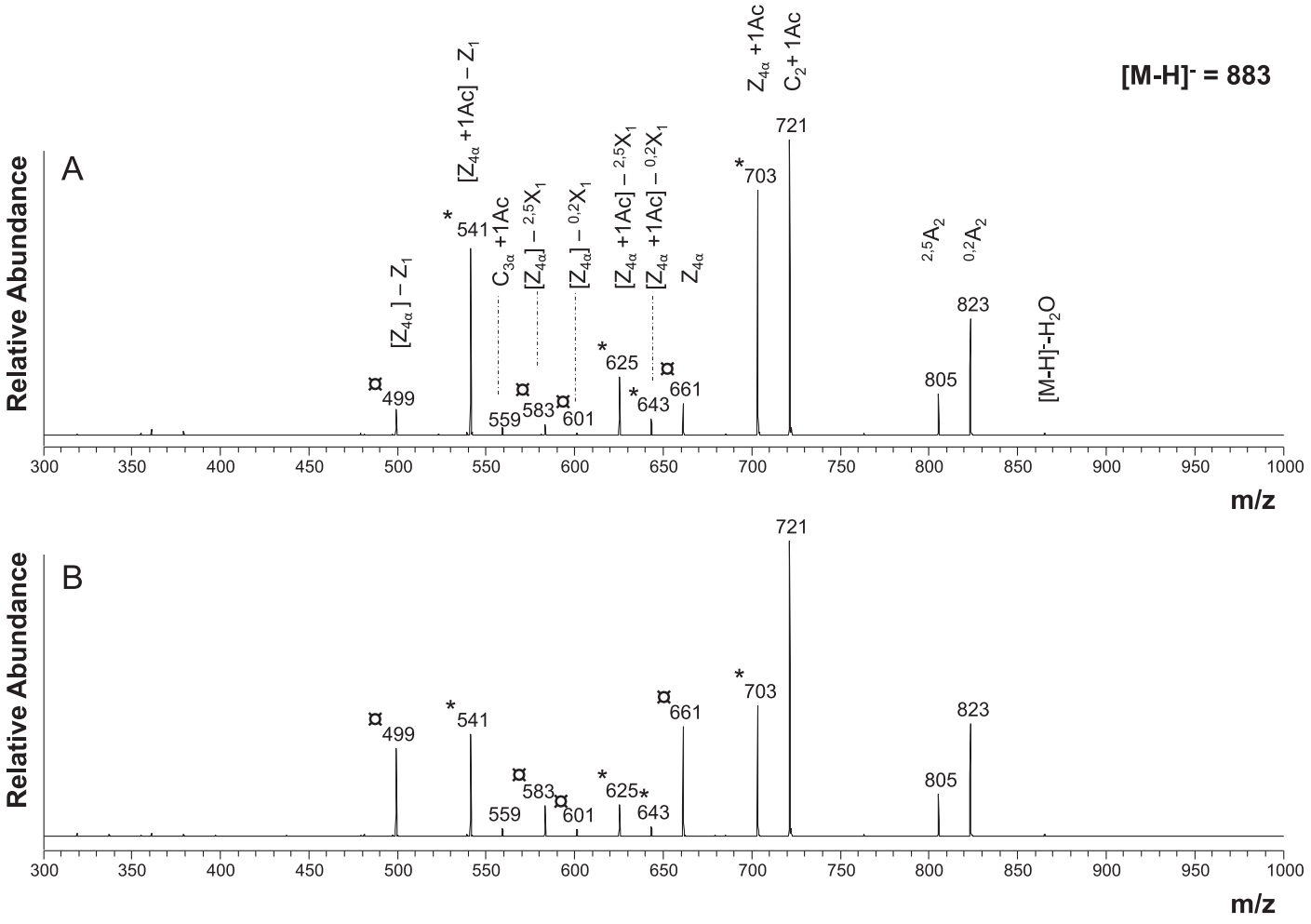


Fig. 3. AXE3 modification of xanthan followed in time (A) HILIC–ELSD elution profiles of cellulase digests of unmodified xanthan and xanthan modified with AXE3 for 1, 2 or 3 days. Glucose ●; mannose ◍ ; glucuronic acid ⊘ ; acetyl group ○; pyruvic acid acetal •. (B) Relative abundance of the 6 xanthan repeating units present in cellulases digests of xanthan at different levels of AXE 3 modification. *Acetylated + pyruvylated RU* (■); *single inner mannose acetylated RU* (♦); *double acetylated RU* (▲); *pyruvylated RU* (●); *unsubstituted RU* (×); *single outer mannose acetylated RU* (∗).





* = specific for acetylation on the inner mannose

¤ = specific for acetylation on the outer mannose

Fig. 4. MS^2-fragmentation pattern of the single acetylated repeating unit (eluting at 12.5 min in (A) present in the cellulases digests of (A) unmodified xanthan and (B) 3 days AXE3-modified xanthan.

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 57% of all acetyl groups are removed by AXE3, which corresponds to the removal of ~70% 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.

4. Conclusions

Acetyl xylan esterase 3 (AXE3) a CE family 1 esterase originating from *M. thermophila* C1, is capable of removing ~57% of all acetyl groups in 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.

Acknowledgements

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

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