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Regioselective deacetylation of cellulose acetates by acetyl xylan esterases of different CE-families

Clemens Altaner [a], Bodo Saake [a], Maija Tenkanen [b], Jaime Eyzaguirre [c], Craig B. Faulds [d], Peter Biely [e], Liisa Viikari [f], Matti Siika-aho [f], Jürgen Puls [a,*]

[a] *Institute of Wood Chemistry, Federal Research Centre of Forestry and Forest Products, Leuschnerstr. 91, D-21002 Hamburg, Germany*
[b] *Department of Applied Chemistry and Microbiology, University of Helsinki, FIN-00014 Helsinki, Finland*
[c] *Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile and Universidad Nacional Andrés Bello, Santiago, Chile*
[d] *Food Materials Science Division, Institute of Food Research, Colney, Norwich NR4 7UA, UK*
[e] *Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia*
[f] *VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Espoo, Finland*

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Abstract

Cellulose acetate (CA) was found to be a substrate of several acetyl xylan esterases (AXE). Eight AXE from different carbohydrate esterase (CE) families were tested on their activity against CA with a degree of substitution of 0.7 and 1.4. The classification of the AXEs into CE families according to their structure by hydrophobic cluster analysis followed clearly their activity against CA. Within the same CE family similar, and between the CE families different deacetylation behaviours could be observed. Furthermore, each esterase family showed a distinct regioselective mode of action. The CE 1 family enzymes regioselectively cleaved the substituents in C2- and C3-position, while CE 5 family enzymes only cleaved the acetyl groups in C2-position. CE 4 family enzymes seemed to interact only with the substituents in C3-position. Evidence was found that the deacetylation reaction of the CE 1 family enzymes proceeded faster in C2- than in C3-position of CA. The enzymes were able to cleave acetyl groups from fully substituted anhydroglucose units.

Keywords: Cellulose acetate; Acetyl xylan esterase; Regioselective deacetylation; CE families; ^{13}C-NMR-spectroscopy

1. Introduction

Regioselectively substituted polysaccharide derivatives are difficult to prepare by chemical methods due to a nearly similar reactivity of the hydroxyl groups within the anhydroglucose units (AGU). Physical and chemical properties of cellulose derivatives are strongly influenced by the amount and distribution of the substituents (Kamide and Saito, 1994). Up to now chemical methods were applied to yield regioselectively substituted cellulose derivatives (Klemm et al.,

* Corresponding author. Tel.: +49-40-73962-514; fax: +49-40-73962-502.
E-mail address: jpuls@holz.uni-hamburg.de (J. Puls).

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1997). The general requirement of multiple synthesis steps and a usually low overall yield are serious disadvantages of these chemical methods. For cellulose esters and especially for cellulose acetates (CA), treatment with acetyl esterases may offer an attractive alternative. Enzymatic catalysis by lipases for the manufacture of regioselectively acetylated mono- and disaccharides is well known (Shaw and Klibanov, 1987) and was recently reviewed by La Ferla (2002). Different regioselective modes of action in respect to per-O-acetylated methylglycosides are also reported for acetyl xylan esterases (AXE) (Biely et al., 1996a,b, 1997). Tenkanen (1998) has shown that microorganisms produce multiple acetyl esterases, which are specialized on substrates of different chain length. An enzyme-catalysed deacetylation of CA was first reported by Gu et al. (1993), who found a reduction of the degree of substitution (DS) of CA from DS 2.5 to 2.2 and 1.7 to 1.3 after 4 days of incubation. *Neisseria sicca* was the first isolated organism, where the culture filtrate showed a deacetylation effect on CA (Sakai et al., 1996). Deacetylation was also shown to have a positive effect on the fragmentation of CA chains by endoglucanases (Saake et al., 1996). The regioselectivity of acetyl esterase action associated with endoglucanase against CA was demonstrated by Altaner et al. (2001). However, besides deacetylation, the polymeric CAs were concomitantly degraded into oligomers due to the association of the esterases with endoglucanases. The properties of an acetyl esterase from *Neisseria sicca* were first reported by Moriyoshi et al. (1999). However, the mode of action was not studied in detail. We have subsequently demonstrated the possibility of enzyme-aided production of regioselectively 6-O-acetylated polymeric CA by an isolated AXE from *Aspergillus niger* (Altaner et al., 2003).

The similarities between carbohydrate active esterases according to their structure were studied by hydrophobic cluster and amino acid sequence analysis (Coutinho and Henrissat, 2002). Within the currently 13 classified carbohydrate esterase (CE) families AXEs are found in CE families 1–7. For this group of esterases the classification according to their structure was verified by their activity against polymeric xylan and glucomannan as well as their mode of action on per-O-acetylated methylsaccharides (Tenkanen et al., 2003). In a comparative experiment, we have incubated enzymes of the CE families 1, 4 and 5 as well as two unclassified AXEs with CA. Among the esterases used in this study the 3D-structures have been solved so far only for the CE 5 family enzymes. These were the AXE from *Trichoderma reesei* (Hakulinen et al., 2000) and the AXE II from *Penicillium purpurogenum* (Ghosh et al., 1999, 2001). Furthermore, respective data are also available from two feruloyl esterases (FAE) of *Clostridium thermocellum* (Schubot et al., 2001; Prates et al., 2001), which have been classified into CE 1 family. All these enzymes exhibit a three layer (α/β/α)-sandwich fold with a classical Ser-Asp-His catalytic triad as active site.

In this work the mode of action of eight AXEs belonging into three CE families against CA was studied with special reference for the production of regioselectively substituted CAs.

2. Materials and methods

2.1. Material

All enzymes used in this study have been purified to homogeneity by the groups mentioned in Table 1. Among them were three CE 1 family enzymes, FAE from *Aspergillus oryzae*, AXE I from *Penicillium purpurogenum* and AXE from *Schizophyllum commune*. The CE 4 family AXE was from *Streptomyces lividans*. The AXE II from *Penicillium purpurogenum* and the AXE from *Trichoderma reesei* belong to the CE 5 family. Two unclassified enzymes, FAE A from *Aspergillus niger* and the acetylglucomannan esterase (AGME) from *Aspergillus oryzae* were also included in the investigation (Table 1). CA DS 0.7 was a gift of Hoechst-Celanese (Charlotte, NC/USA) and CA DS 1.4 was obtained from Rhodia-Acetow (Freiburg, Germany). Their DS and substituent distribution within the AGU were determined by ^{13}C-NMR-spectroscopy (Buchanan et al., 1991; Kowsaka et al., 1988). The percentage of acetyl groups located in C2-, C3- and C6-position

Table 1
Specification of acetyl esterases, used in this study

Enzyme	Organism	CE family	Mw (kDa)	PI	pH-optimum	Reference
FAE	*Aspergillus oryzae*	1	30	3.6	4.5–6.0	Tenkanen et al. (1991, 2003)
AXE I	*Penicillium purpurogenum*	1	48	7.5	5.3	Egana et al. (1996) and Gordillo and Eyzaguirre (2002)
AXE	*Schizophyllum commune*	1	31	3.4	7.7	Halgasova et al. (1994) and Biely et al. (1996b)
AXE	*Streptomyces lividans*	4	34	9.0	6.0–7.5	Shareck et al. (1995) and Dupont et al. (1996)
AXE II	*Penicillium purpurogenum*	5	23	7.8	5.5–6.0	Egana et al. (1996) and Gutierrez et al. (1998)
AXE	*Trichoderma reesei*	5	34	6.8, 7.0	5.0–6.0	Sundberg and Poutanen (1991) and Margolles-Clark et al. (1996)
FAE A	*Aspergillus niger*	Unclass.	30	3.3	5.0	Faulds and Williamson (1994) and de Vries et al. (1997)
AGME	*Aspergillus oryzae*	Unclass.	36	4.6	5.0–5.5	Tenkanen et al. (1995, 2003)

was 22:45:33 for CA DS 0.7 and 36:31:33 for CA DS 1.4, respectively.

2.2. Methods

2.2.1. Acetic acid quantification

Liberated acetic acid was determined after centrifugation with the Boehringer acetic acid test combination (Boehringer, Mannheim/Germany).

2.2.2. Protein content

The protein content of enzyme solutions was determined with the modified Lowry Protein Assay (Pierce, Rockford, Illinois/USA) and bovine serum albumin (BSA) as standard.

2.2.3. Acetyl esterase activity

Ninety microliters of a 0.2% CA DS 0.7 solution or CA DS 1.4 suspension were incubated with 10 μl enzyme solution at 40 °C in a 0.2 M Na-phosphate/citric acid buffer at pH 5. Amount of protein and hydrolysis time used were as indicated later in the text. The reaction was stopped by boiling for 5 min.

2.2.4. Enzymatic degradation of CAs for NMR measurements

Fifty milliliters of a 0.2% CA solution or suspension were incubated with the indicated enzyme dosage for 70 h at 40 °C in distilled water and mixed with a magnetic stirrer. Boiling for 5 min terminated the reaction. The solutions were freeze-dried and subsequently dissolved in DMSO-d_6 as a 10% solution for NMR characterisation.

2.2.5. NMR-spectroscopy

A 400 MHz spectrometer (Varian–Mercury) with a 5 mm probe was used at 60 °C. One hundred milligrams of the samples were dissolved in approximately 0.7 ml deuterated DMSO-d_6. The central DMSO-d_6 signal at 39.43 ppm was used as reference.

Quantitative ^{13}C-NMR-spectroscopy of the acetylated substrates was measured under 'inverse gated decoupling' (IGATED) with a pulse angle of 45° and a relaxation delay of 3.5 s.

Due to the long measuring times and small sample amounts the ^{13}C-NMR measurements on the esterase treated CA samples were conducted under standard conditions with broad band decoupling. This procedure only allowed a qualification without quantitative evaluation.

3. Results

3.1. Deacetylation of CA by acetyl esterases dependent on the CE families

Clear differences between the CE families regarding to their activity on CA were observed. The CE 1 family enzymes were the most active (Table 2). Deacetylation proceeded fast already at low enzyme concentrations. An enzyme dosage of 1.7 μg mg^{-1} CA DS 0.7 cleaved about 35–45% of the acetyl substituents within 4 h. A 10-fold increase of the enzyme dosage resulted in a minor increase in deacetylation up to 45–50%, indicating that already all easily accessible substituents have been cleaved off. Some of the remaining substituents were deacetylated in a much slower reaction. A prolonged incubation for 102 h indicated that at most approximately 60% of the substituents were accessible for the CE 1 family enzymes. CE 1 family enzymes also were the only ones showing a detectable activity against CA DS 1.4 after 4 h of incubation, applying an enzyme dosage of 16.7 μg mg^{-1} CA.

Deacetylation of CA DS 0.7 by CE 5 family enzymes proceeded slowly even at high enzyme concentrations, reaching a maximum deacetylation of 23–36%.

The same amount of acetyl groups (31%) were accessible to the CE 4 family enzyme. However, the reaction velocity was faster in the beginning, compared to CE 5 family enzymes.

After 4 h incubation the deacetylation of the unclassified FAE A from *A. niger* was comparable to that of the CE 4 family enzyme. However, after prolonged incubation (102 h) a much higher deacetylation (50% compared to 31%) was achieved, indicating more accessible substituents for the FAE A from *A. niger*.

The unclassified AGME from *A. oryzae* was almost inactive against CA DS 0.7 and liberated only 10% of the substituents after 102 h.

Obviously enzymes within the same CE family show a similar substrate specificity, while clear differences between the CE families were observed. The deacetylation behaviour of CAs corresponded perfectly with the classification of the enzymes into the CE families on the basis of their structure by hydrophobic cluster analysis (http://afmb.cnrs-mrs.fr/ ~ cazy/CAZY/index.html).

3.2. Mode of action of acetyl esterases against CA

In Table 3 the deacetylation values of the samples prepared for NMR-spectroscopy are given. The maximum deacetylation values (Table 2) were not reached for all samples. Because of requirements of the NMR experiments the enzymatic deacetylation of the NMR-samples had to be performed in water instead of buffered solutions. Especially for esterases with alkaline pH-optima (Table 1) this experimental set-up resulted in decreasing activity with increasing deacetylation. In some of the experiments the available amount of enzyme was limited. It seems not very likely that the slightly different experimental conditions resulted in a changed mode of action. However, it cannot be ruled out completely.

The difference between the esterases, grouped into the particular CE families, can also be demonstrated in the regioselectivity of their action, revealed by ^{13}C-NMR-spectroscopy.

The CE 1 family esterases exclusively deacetylated CA in the C2- and C3-positions, resulting in a regioselectively C6-substituted CA (Fig. 1a). This result perfectly confirmed the maximum deacetylation value of approximately 60% (Table 2). The deacetylation of the NMR-sample by AXE from *S. commune* only reached 27% (Table 3) and the C2- and C3-signals were not completely removed. A further deacetylation in these positions under more favourite conditions can be postulated, if the results of Table 2 (59% deacetylation) are considered. The C2-signals are much more reduced than the C3-signals. The incomplete deacetylation of this sample allowed us to assume, that the deacetylation of the C2-position seemed to be faster compared to the one in C3-position. The signals of the substituents in C3-position were the best resolved, and the 3-mono, 2,3-di, 3,6-di and 2,3,6-tri AGUs could be distinguished according to the assignment of Buchanan et al. (1991) (see Fig. 1d). Because the signals of the 2,3-di and 2,3,6-tri AGUs vanish compared to the reference, the CE 1 family enzymes were able to attack substituents in fully acetylated AGUs. However,

Table 2
Activity of isolated AEs against CA DS 0.7 and 1.4

Enzyme	Organism	CE family	Enzyme per CA DS 0.7 1.7 μ mg^{-1}	Enzyme per CA DS 0.7 16.7 μ mg^{-1}	Enzyme per CA DS 0.7 16.7 μ mg^{-1}	Enzyme per CA DS 1.4 16.7 μ mg^{-1}
			(4 h)	(4 h)	(102 h)	(4 h)
			Cleaved acetyl groups (%)			
FAE	*Aspergillus oryzae*	1	40	46	59	9
AXE I	*Penicillium purpurogenum*	1	35	51	64	11
AXE	*Schizophyllum commune*	1	45	49	59	8
AXE	*Streptomyces lividans*	4	9	22	31	1
AXE II	*Penicillium purpurogenum*	5	2	9	36	1
AXE	*Trichoderma reesei*	5	1	2	23	0
FAE A	*Aspergillus niger*	Unclass.	5	26	50	1
AGME	*Aspergillus oryzae*	Unclass.	0	–	10[a]	0[a]

[a] Due to dilution of the enzyme solution 0.15% instead of 0.18% CA solution or suspension.

Table 3
Reaction conditions and deacetylation of samples prepared for ^{13}C-NMR-spectroscopy

Enzyme	Organism	CE family	Substrate CA DS	Enzyme per CA (μg mg^{-1})	Deacetylation rate (%)	Mode of action
FAE	*Aspergillus oryzae*	1	0.7	1.7	58	C2 and C3
FAE	*Aspergillus oryzae*	1	1.4	1.7	30	C2 and C3
AXE	*Schizophyllum commune*	1	0.7	1.7	27	C2 and C3
AXE	*Streptomyces lividans*	4	0.7	0.4	16	C3
AXE II	*Penicillium purpurogenum*	5	0.7	16.7	22	C2
FAE A	*Aspergillus niger*	Unclass.	0.7	1.7	29	C2 and C3
AGME	*Aspergillus oryzae*	Unclass.	0.7	1.7	2	–

the resistance of CA with a DS higher than 2.5 against acetyl esterase attack (Saake et al., 1996) and the reduction in the deacetylation reaction with increasing DS (Altaner et al., 2001) implied that unsubstituted or low substituted neighbouring AGUs were required for the enzyme action. This mode of action was also found for an AXE, isolated from a commercial *A. niger* enzyme preparation (Altaner et al., 2003).

The unclassified FAE A *A. niger* seemed to show the same regioselectivity as CE 1 enzymes, cleaving substituents in C2- and C3-position and leaving the C6-substituents attached (Fig. 1b). In the NMR experiment deacetylation of this sample (29%, Table 3) did not reach the value, obtained under optimal conditions (50%, Table 2). However, the maximal deacetylation value of 50% implies that the enzyme will never cleave all of the substituents in C2- and C3-position.

The CE 5 family enzyme AXE II *P. purpurogenum* deacetylated CA only in the C2-position, leaving the C3- and C6-acetyl groups attached (Fig. 1c). This was also consistent with the maximal deacetylation value of 36% (Table 2). The enzyme attacked substituents in C2-position regardless of the fact, whether the AGU was tri-, di- or mono-substituted. This can also be assumed, because it seemed that the 2,3,6-tri signal was reduced while the 3,6-di signal was increased. The ability of the CE 5 family AXE *T. reesei* to cleave substituents from double-acetylated residues of acetylated xylan was also suggested by Hakulinen et al. (2000).

The NMR-sample of the CE 4 family AXE *S. lividans* was not deacetylated to its maximum of approximately one third (Table 2). Only 16% of the substituents were cleaved off (Table 3) in this experiment. Changes in the ^{13}C-NMR-spectrum could only be observed for the signals of the C3-position (Fig. 1d). In this deacetylation stage exclusively the signal of the 3-mono substituted AGUs were reduced, while the C3-signals of the 2,3-di-, 3,6-di- and 2,3,6-trisubstituted AGUs persisted. It can be concluded from these results that CE 4 family enzymes only interact with substituents located in C3-position.

The low deacetylation of the unclassified AGME *A. oryzae* sample prevented to draw any conclusions on its mode of action.

The CE 1 family enzyme FAE from *A. oryzae* was also tested on its regioselective mode of action against CA DS 1.4 (Fig. 2). As expected deacetylation only occurred at C2- and C3-position. Compared to the CA DS 0.7 the amount of remaining substituents at these two positions was higher, most likely due to more highly substituted regions along the polymer chain.

4. Discussion

The deacetylation of polymeric CA by the AXEs differed from the deacetylation of per-O-acetylated glucose (Biely et al., 1996a,b, 1997; Tenkanen et al., 2003). Deacetylation never occurred at C6-position, but differences were observed at C2- and C3-positions. Deacetylation of 2,3,4,6-tetra-*O*-Ac-

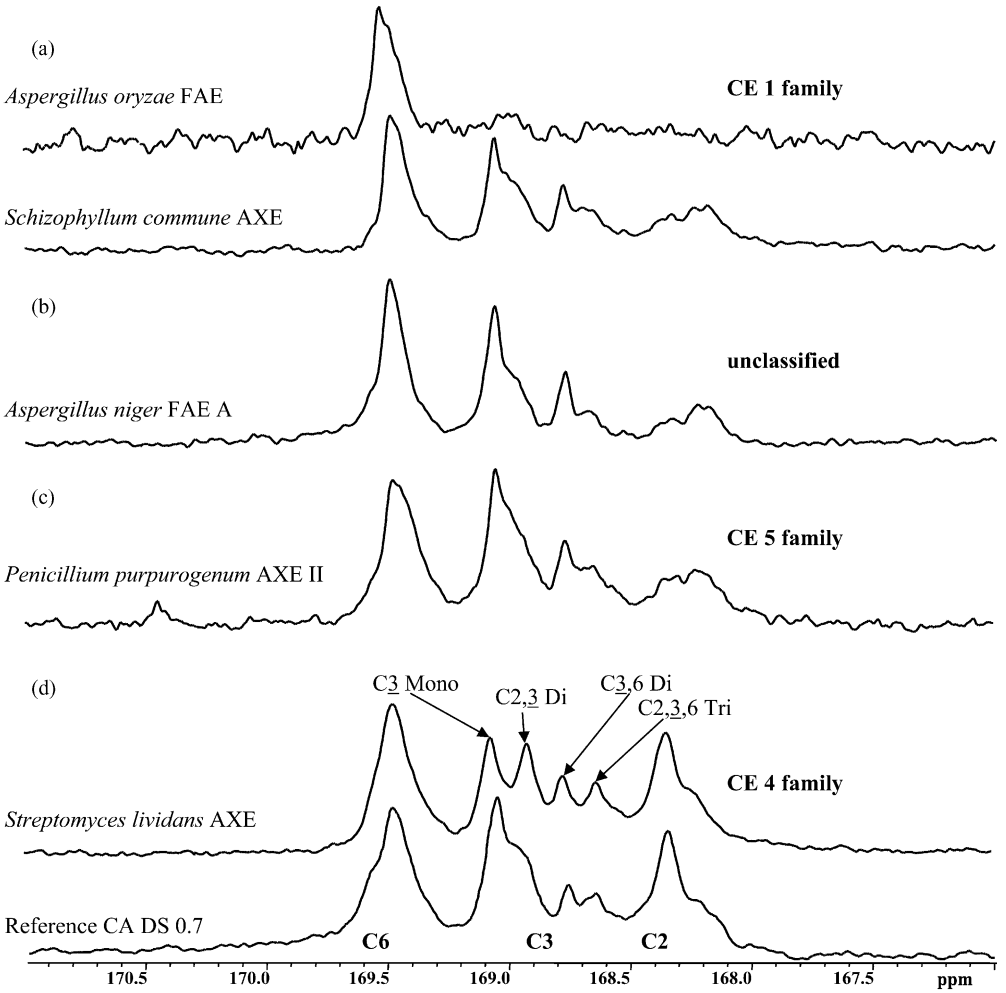

Fig. 1. ^{13}C-NMR spectra of the carbonyl signals of CA DS 0.7 incubated with acetyl esterases (assignment: Buchanan et al. (1991)). (a) CE 1 family enzymes FAE *A. oryzae* and AXE *S. commune* (b) unclassified FAE A *A. niger* (c) CE 5 family enzyme AXE II *P. purpurogenum* (d) CE 4 family enzyme AXE *S. lividans*.

Me-Glc*p* by CE 4 and 5 family esterases proceeded almost simultaneously at C2- and C3-position, resulting in a doubly deacetylated product. In contrast polymeric CA was only deacetylated in one position, C2- for CE 5 and C3- for CE 4 family enzymes. Possibly these differences are due to the fact that some enzymes could bind the monomeric substrates in reverse modes (Hakulinen et al., 2000), in which the acetyl group, placed in the active site, is located in C2- or in C3-position in the other binding mode. The polymeric substrates apparently orient the acetylated glucopyranosyl residues more precisely into the binding site, so that deacetylation occurred in only one mode.

CE 1 family esterases cleaved first the substituents, located in C3-position from 2,3,4,6-tetra-*O*-Ac-Me-Glc*p*, followed by a much slower deacetylation of the substituents in C2- or C4-position. Again deacetylation of 2,3,4,6-tetra-*O*-Ac-Me-Glc*p* was not consistent with the mode of action on polymeric CA. The NMR-spectrum of CA DS 0.7 deacetylated by the CE 1 family esterase AXE *S. commune*, suggested a preferential cleavage

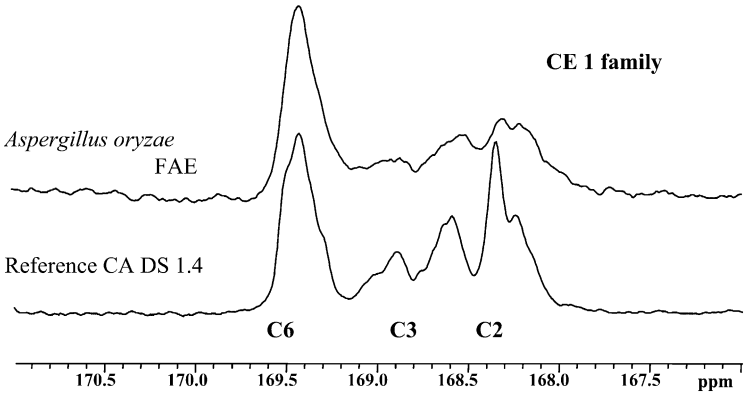

Fig. 2. ^{13}C-NMR spectra of the carbonyl signals of CA DS 1.4 incubated with the CE 1 family enzyme FAE *A. oryzae*.

from C2-position. Therefore, it seemed that the substrate specificity of the esterases cannot be predicted from the action on acetylated monosaccharides. The fact that the CE 1 family esterases were the most active on CA supported the previous conclusion that the esterases in this family appeared most versatile and may thus have a rather open active site in which different esterified polymers may be bound (Tenkanen et al., 2003). Acetyl group migration within the polymeric CA can be excluded at this point, because the NMR-samples of the different CE-families showed different substituent distributions, although they have been incubated under the same conditions.

Mannose differs from glucose in the axially oriented hydroxyl group in C2-position. The *O*-acetyl groups in native pine glucomannan (DS 0.36) are rather evenly distributed on C2- and C3-position of the mannose residues (Lindberg et al., 1973). Neither of the CE 5 family enzymes was able to cleave substituents from acetylated glucomannan (Tenkanen et al., 2003). The enzyme's specialization for the C2-position in polymeric substrates was perhaps the reason for its inability to deacetylate polymeric acetylated glucomannan. However, the 3D structures of the CE 5 family enzymes revealed rather tight active sites, in which xylans seem to fit (Hakulinen et al., 2000; Ghosh et al., 2001). Some amino acid residues which most probably are involved in the substrate recognition of AXE II from *P. purpurogenum* are located in flexible regions of the molecule (Ghosh et al., 2001). They appear to be flexible enough not only to accommodate xylan chains, but also CA molecules possessing the additional hydroxymethyl group at the C6-position.

Surprisingly, the CE 4 family enzyme seemed only to interact with acetyl groups in C3-position although it was reported that it neither was able to cleave any substituents from polymeric acetylated glucomannan nor the monomeric 2,3,4,6-tetra-*O*-Ac-Me-Man*p* (Biely et al., 1996a). This led us to the speculation that for this enzyme an equatorial orientation in C2-position is essential for binding the substrate into the active site, because only glucose and xylose residues are deacetylated. The results also support the idea that the physiological role of the AXEs is to operate on polymeric substrates and, that at least some of them may have a substrate binding site accomodating more than one sugar residue of the main chain. The low action of AGME from *A. oryzae* on CA was surprising as it is able to deacetylate native xylan in addition to glucomannan (Tenkanen et al., 2003).

5. Conclusion

The action of AXEs against CA followed clearly the classification of esterases into different CE families. Clear differences between the enzymes of the single CE families 1, 4 and 5 as well as the unclassified proteins could be observed, while the enzymes within the same CE families interacted in

the same way with CA. Furthermore, the obtained results suggested that the two unclassified enzymes examined in this study are members of other than the already known CE families. The data confirm the results of the previous work on acetylated xylan and glucomannan (Tenkanen et al., 2003).

Due to the different regioselective modes of action found for esterases of different CE families, they can be used for the production of regioselectively substituted CA. With the help of CE 1 and 5 family esterases, regioselectively 6-O-acetylated and 3,6-O-acetylated CA, respectively, could be prepared, while CA can be regioselectively modified in C3-position with the CE 4 family enzyme.

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