Substrate specificity and mode of action of acetylxylan esterase from *Streptomyces lividans*

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Abstract The substrate specificity of purified acetylxylan esterase (AcXE) from *Streptomyces lividans* was investigated on partially and fully acetylated methyl glycopyranosides. The enzyme exhibited deacetylation regioselectivity on model compounds which provided insights pertaining to its function in acetylxylan degradation. The enzyme catalyzed double deacetylation of methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside and methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside at positions 2 and 3. Two methyl xylopyranoside diacetates, which had a free hydroxyl group at position 2 or 3, i.e. the derivatives that most closely mimic monoacetylated xylopyranosyl residues in acetylxylan, were deacetylated 1 to 2 orders of magnitude faster than methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside and methyl 2,3-di-*O*-acetyl-β-D-xylopyranoside. These observations explain the double deacetylation. The second acetyl group is released immediately after the first one is removed from the fully acetylated methyl β-D-xylo- and -glucopyranoside. The results suggest that in acetylxylan degradation the enzyme rapidly deacetylates monoacetylated xylopyranosyl residues, but attacks doubly acetylated residues much more slowly. Evidence is also presented that the *St. lividans* enzyme could be the first real substrate-specific AcXE.

Key words: Acetylxylan esterase; Substrate specificity; Acetylated methyl glycoside; Action mode; *Streptomyces lividans*

1. Introduction

Acetylxylan esterases (AcXEs) are microbial enzymes that liberate acetic acid from partially acetylated 4-*O*-methyl-D-glucuronoxylan, the main hardwood hemicellulose [1–3]. Although a considerable number of these enzymes have been found in various microbial cellulolytic and hemicellulolytic systems [4], little is known about their mode of action and substrate specificity. Our recent investigation pertaining to the action of AcXE from *Schizophyllum commune* on partially and fully acetylated methyl glycosides [5] revealed that the pattern of deacetylation of the substrates is compatible with the function of the enzyme in hemicellulose degradation. Specifically, in the glycosides, the *S. commune* enzyme most rapidly deacetylated the 3 position and then the 2 position, to give 2,4-di- and 2- and 4-monoacetyl derivatives from Me-β-Xyl*p*. Likewise, 2,4,6-tri-*O*-Ac-Me-β-Glc*p* and 4,6-di-*O*-Ac-Me-β-Glc*p* were the principal products from 2,3,4,6-tetra-*O*-Ac-Me-β-Glc*p*. The enzyme also catalyzed double 2,3-deacetylation of 2,3,4,6-tetra-*O*-Ac-Me-β-Man*p* in a highly selective way. The ability of the AcXE from *S. commune* to deacetylate the mannopyranoside and acetylgalactomannan suggested that the enzyme may not be an AcXE but rather a more general polysaccharide or carbohydrate deacetylase.

In the present work we report results of a similar study regarding the AcXE from *Streptomyces lividans*. Several properties of this esterase differed from those of the *S. commune* enzyme. In contrast to the *S. commune* enzyme, AcXE of *St. lividans* shows no activity on non-carbohydrate esters, and its molecule contains an amino acid sequence which appears to be identical with the xylan-binding domain encountered in an *St. lividans* endo-β-1,4-xylanase [6,7].

2. Materials and methods

2.1. Enzyme

The investigated AcXE is produced by a genetically modified strain of *St. lividans* IAF43, which overproduces the esterase together with xylanase B [6]. The AcXE was purified from xylose-spent culture medium as described by Dupont et al. [7].

2.2. Enzyme assay

Activity of AcXE was determined on acetylxylan by measuring the release of acetic acid [7]. One unit is defined as the amount of enzyme needed to release 1 μmol of acetic acid in 1 min.

2.3. Carbohydrates

Fully acetylated methyl glycopyranosides were obtained as described [5]. Diacetates (2,3-, 2,4- and 3,4-) of methyl β-D-xylopyranoside were generous gifts from Dr. P. Kovac (National Institutes of Health, Bethesda, MD, USA), Dr. J. Hirsch (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia) and Dr. A. Fernandes-Mayoralas (Instituto de Quimica Organica General, CSIC, Madrid, Spain).

2.4. Enzymic deacetylations

Reactions were performed in homogeneous solutions containing 0.1 M sodium phosphate buffer (pH 6.0) at 40°C. Concentrations of substrates were as follows: methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside, 14.5 mM; methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside, 10.4 mM; methyl 2,3-, 2,4- and 3,4-di-*O*-acetyl-β-D-xylopyranosides, all 15 mM. After the addition of prewarmed enzyme solution, aliquots were taken periodically and subjected to product analysis.

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Abbreviations: AcXE, acetylxylan esterase; 2,3,4-*O*-Ac-Me-β-Xyl*p*, methyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside; 2,3,4,6-tetra-*O*-Ac-β-Glc*p*, methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside; 2,3,4,6-tetra-*O*-Ac-Me-β-Man*p*, methyl 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranoside

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2.5. Analysis of reaction mixtures and identification of products

Enzymic deacetylations of methyl *O*-acetyl-D-glycopyranosides were followed by GLC after conversion of reaction products to their trimethylsilyl ethers [5]. Reaction mixtures were also analyzed by TLC on silica gel 60 (Merck) in ethyl acetate-benzene-2-propanol (2:1:0.1, v/v). The sugars were visualized with *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent [8]. Deacetylation products from various methyl glycopyranosides were identified by GLC-MS and NMR spectroscopy as described earlier [5].

3. Results

3.1. Action of AcXE on methyl per-O-acetyl-D-glycosides

At enzyme concentrations (0.1–1 U/ml) sufficient to observe rapid deacetylation of beechwood acetylxylan (precipitation of deacetylated polysaccharide), the *St. lividans* AcXE appears to be almost inactive on fully acetylated methyl glycopyranosides. Unusually high enzyme concentrations (50–100 U/ml) were required to observe a relatively fast deacetylation of 2,3,4-tri-*O*-Ac-Me-β-Xyl*p*, the compound structurally closely related to doubly acetylated xylopyranosyl residues in acetylxylan. It was necessary to keep the rate of enzymic deacetylation high enough to circumvent the effect of spontaneous migration of acetyl groups in partially deacetylated products [5]. As shown in Fig. 1, AcXE catalyzed conversion of 2,3,4-tri-*O*-Ac-Me-β-Xyl*p*, essentially in one step, to 4-*O*-Ac-Me-β-Xyl*p* and in a very high yield (~70%). Theoretical intermediates of the double deacetylation, 2,4- or 3,4-di-*O*-Ac-β-Xyl*p*, were not observed in the reaction mixture. The only detected diacetate was 2,3-di-*O*-Ac-Me-β-Xyl*p*, and this was produced in a low concentration. Apparently, the removal of the acetyl group from the 4-position resulted in the formation of a diacetate which was also a poor enzyme substrate and, therefore, persisted in the reaction mixture. Similarly, further conversion of 4-*O*-Ac-Me-β-Xyl*p* to a completely deacetylated product is very slow (Fig. 1) indicating that AcXE can only weakly hydrolyze the acetyl group in position 4.

The AcXE exhibited essentially identical regioselectivity to that above towards 2,3,4,6-tetra-*O*-Ac-Me-β-Glc*p*. The enzyme removed two acetyl groups simultaneously from positions 2 and 3, to give 4,6-di-*O*-Ac-Me-β-Glc*p* in 90–95% yield.

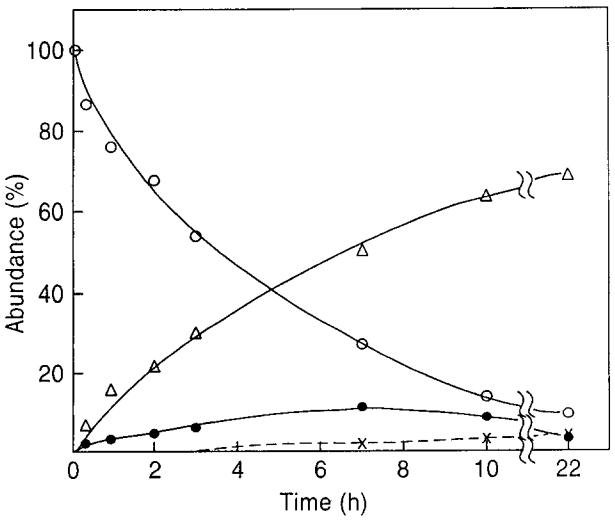

Fig. 1. Time course of 2,3,4-tri-*O*-Ac-Me-β-Xyl*p* (14.5 mM) deacetylation by AcXE from *St. lividans* (75 U/ml) as evaluated by gas chromatography of trimethylsilyl ethers. (○) 2,3,4-Tri-*O*-Ac-Me-β-Xyl*p*; (●) 2,3-di-*O*-Ac-Me-β-Xyl*p*; (△) 4-*O*-Ac-Me-β-Xyl*p*; (×) fully deacetylated substrate.

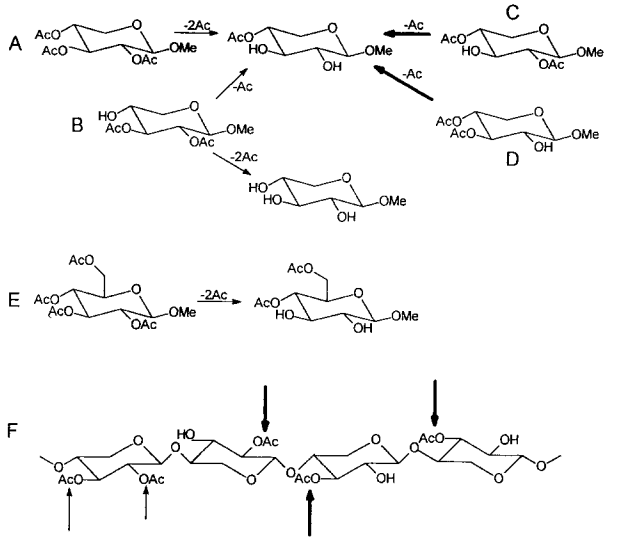

Fig. 2. Main deacetylation reactions catalyzed by AcXE from *St. lividans*. Substrates: (A) 2,3,4-tri-*O*-Ac-Me-β-Xyl*p*; (B) 2,3-di-*O*-Ac-Me-β-Xyl*p*; (C) 2,4-di-*O*-Ac-Me-β-Xyl*p*; (D) 3,4-di-*O*-Ac-Me-β-Xyl*p*; (E) 2,3,4,6-tetra-*O*-Ac-Me-β-Glc*p*; (F) hypothetical fragment of acetylxylan. Thin arrows denote slow deacetylations; thick arrows fast deacetylations.

AcXE was also tested on several other per-*O*-acetylated methyl glycopyranosides. The enzyme deacetylated 2,3,4,6-tetra-*O*-Ac-Me-α-Glc*p*, 2,3,4,6-tetra-*O*-Ac-Me-α-Gal*p* and 2,3,4,6-tetra-*O*-Ac-Me-β-Gal*p*, but at slower rates than observed for 2,3,4,6-tetra-*O*-Ac-Me-β-Glc*p*. In all cases, the major products were the corresponding 4,6-di-*O*-Ac derivatives. Minor products were 2,6-di-*O*-Ac derivatives. Surprisingly, under identical conditions, the enzyme showed negligible activity on 2,3,4,6-tetra-*O*-Ac-Me-α- and -β-Man*p*.

3.2. Action on diacetates of Me-β-Xylp

The observation that 2,3,4-tri-*O*-Ac-Me-β-Xyl*p* and 2,3,4,6-tetra-*O*-Ac-Me-β-Glc*p* were deacetylated directly to 4-*O*-Ac-Me-β-Xyl*p* and 4,6-di-*O*-Ac-Me-β-Glc*p*, suggested two possibilities. Those were: (i) the enzyme either deacetylated the two positions simultaneously; or (ii) slowly hydrolyzed the first acetyl group and then very rapidly removed the second acetyl group. The latter hypothesis was substantiated by experiments in which 2,3,4-tri-*O*-Ac-Me-β-Xyl*p* and all three possible Me-β-Xyl*p* diacetates were used as substrates. 2,3,4-tri-*O*-Ac-Me-β-Xyl*p* was converted almost exclusively into 4-*O*-Ac-Me-β-Xyl*p*. The 4-acetate was also the sole product of the AcXE action on 2,4-di-*O*-Ac-Me-β-Xyl*p* and 3,4-di-*O*-Ac-Me-β-Xyl*p*. Thus, the enzyme rapidly deacetylated positions 2 and 3. When these positions are acetylated and position 4 is underivatized, as it is in 2,3-di-*O*-Ac-Me-β-Xyl*p*, the major deacetylation product was Me-β-Xyl*p*. 4-*O*-Ac-Me-β-Xyl*p* was generated as a minor product, possibly as a result of spontaneous acetyl migration from position 3 to position 4. Two substrates, 2,3,4-tri-*O*-Ac-Me-β-Xyl*p* and 2,3-di-*O*-Ac-Me-β-Xyl*p*, were poorly hydrolyzed in comparison to 2,4- and 3,4-di-*O*-Ac-Me-β-Xyl*p*. Table 1 presents the initial rates of their deacetylation, as well as the major initial products. The rates of deacetylation were compared with the rates of spon-

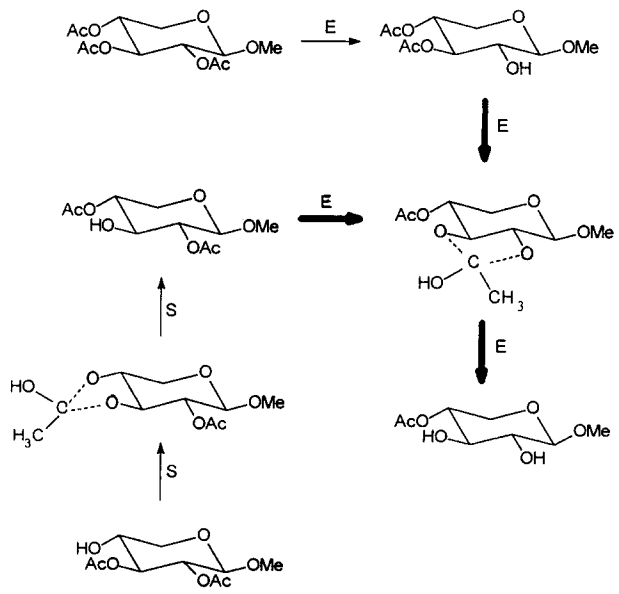

Fig. 3. Partial steps proposed for the deacetylation mechanism of methyl β-D-xylopyranosides. Thin arrows indicate slow steps; thick arrows fast steps. *E* represents an enzyme-catalyzed reaction or transformation; *S* represents a spontaneous transformation.

taneous transformation of the diacetates (measured without enzyme under identical experimental conditions [5]). At the utilized enzyme concentration, the observed deacetylation rates greatly exceeded the spontaneous transformation rates of diacetates. Therefore, with the exception of the formation of 4-acetate from 2,3-diacetate, the results were not significantly influenced by spontaneous acetyl group migration. An important observation, confirming the high regioselectivity of the *St. lividans* AcXE was that 2,4-diacetate was deacetylated approx. 100-fold more rapidly, and 3,4-diacetate approx. 40-fold more rapidly than triacetate and 2,3-diacetate.

4. Discussion

The *St. lividans* AcXE displays a different regioselectivity for deacetylation of methyl glycosides in comparison to lipases used for carbohydrate deacetylation [9–13]. The deacetylation regioselectivity for positions 2 and 3 is complementary to the regioselectivity of most other deacetylating enzymes, which mainly release acetyl groups from position 4 in xylopyranosides [13] and position 6 in hexopyranosides [14]. It is interesting to compare the catalytic properties of AcXE from *St. lividans* with those of AcXE from *S. commune* which was the first enzyme of its type examined for its action on acetylated methyl glycopyranosides [5]. The *St. lividans* enzyme does not hydrolyze aryl acetates, such as 4-nitrophenyl- or 4-methylumbelliferylacetate, whereas the *S. commune* enzyme does. *St. lividans* AcXE is more specific for deacetylation of carbohydrates, and particularly of acetylxylan, perhaps due to the presence of an apparent xylan-binding domain identical to one found in a *St. lividans* endo-β-1,4-xylanases [6,7]. As shown here, *St. lividans* AcXE also differs from the *S. commune* enzyme in its action on acetylated carbohydrates.

The deacetylation of 2,3,4-tri-*O*-Ac-Me-β-Xyl*p* and 2,3,4,6-tetra-*O*-Ac-Me-β-Glc*p* by both enzymes ultimately results in the same products. However, there are significant differences in individual steps leading to the double deacetylation. Whereas *S. commune* AcXE deacetylates the 3 position first and only then the 2 position, *St. lividans* AcXE appears to deacetylate the two positions almost simultaneously. This generates high yields of 4-*O*-Ac-*O*-Ac-Me-β-Xyl*p* and 4,6-di-*O*-Ac-Me-β-Glc*p* from the corresponding fully acetylated glycosides, without significant accumulation of intermediates (Fig. 2). Experiments with Me-β-Xyl*p* diacetates provided data which allow straightforward interpretation of the catalytic reaction mechanism (Fig. 2). The AcXE from *St. lividans* shows unusually high preference for deacetylation of the 2 position when the 3 position is not acetylated. Conversely, the 3-acetyl group is removed easily when position 2 is not acetylated. Derivatives in which both these adjacent positions are acetylated, 2,3,4-tri-*O*-Ac-Me-β-Xyl*p* and 2,3-di-*O*-Ac-Me-β-Xyl, are found to be very poor substrates in comparison with the other two diacetates. This explains why the enzyme shows such a low affinity towards fully acetylated methyl glycosides in comparison with its outstanding performance on acetylxylan. The data also suggest that *St. lividans* AcXE rapidly deacetylates monoacetylated xylopyranosyl residues of the polysaccharide, but almost ignores the doubly acetylated residues (Fig. 2). In contrast to the *S. commune* AcXE, the *St. lividans* enzyme has great difficulty in removing the first acetyl group from fully acetylated glycosides. However, for substrates in which only one of the positions 2 and 3 is acetylated, deacetylation of position 2 or 3 takes place rapidly. It is difficult to imagine that the enzyme has an equal ability to deacetylate positions 2 and 3. We hypothesize that for deacetylation of these two positions, under conditions where the adjacent hydroxyl group is non-esterified, the enzyme uses the same reaction mechanism. One may envision a mechanism involving an enzyme-catalyzed formation of a five-membered transition state (Fig. 3), from which the acetyl group is rapidly released. Such intermediates are believed to be involved in the

Table 1
Enzymic and spontaneous transformation of Me-β-Xyl tri- and di-*O*-acetates

Substrate	Deacetylation by *St. lividans* AcXE			Spontaneous transformation	
	Initial rate		Major products	Rate (mM min^{-1})	Products (4 h) (2,3-:2,4-:3,4-)
	mM min^{-1}	mM min^{-1} U^{-1} ml			
2,3,4-Tri-Ac	0.53	0.0071	4-Ac	not applicable	
2,3-Di-Ac	0.50	0.0066	de-Ac:4-Ac- (ratio starting at ~1:0.7)	0.0057	1:0.1:0.4
2,4-Di-Ac	1.07	0.67	4-Ac	0.022	0.15:1:0.7
3,4-Di-Ac	0.37	0.23	4-Ac	0.018	0.07:0.5:1

Substrates, 15 mM; concentration of AcXE of *St. lividans* 75 U/ml for 2,3,4-tri-*O*-Ac and 2,3-di-*O*-Ac, and 1.6 U/ml for 2,4-di-*O*-Ac and 3,4-di-*O*-Ac.

spontaneous migration of acetyl groups along the glycopyranoid ring [15]. A similar spontaneous step may be responsible for the generation of 4-*O*-Ac-Me-β-Xyl*p* during AcXE treatment of 2,3-di-*O*-Ac-Me-β-Xyl*p* (Fig. 3).

The high specificity of the *St. lividans* esterase for acetylxylan is underlined by the lack of its appreciable action on 2,3,4,6-tetra-*O*-Ac-Me-β-Man*p* and on partially chemically acetylated galactomannan (unpublished results). Once the equatorial 2-*O*-acetyl group in glucopyranosides and probably also in xylopyranosides becomes axial, the enzyme cannot act. This implies that *St. lividans* AcXE will not be able to deacetylate mannopyranosyl residues in partially acetylated galactoglucomannan or glucomannan. This is in contrast to the catalytic properties of AcXE from *S. commune*, which shows a high regioselectivity for a successive double deacetylation of 2,3,4,6-tetra-*O*-Ac-Me-β-Man*p* to give almost exclusively 4,6-di-*O*-Ac-Me-β-Man*p* [5]. Thus, the substrate binding sites of *St. lividans* AcXE and *S. commune* AcXE must be considerably different.

Consequently, of these two enzymes only the *St. lividans* AcXE appears to be the real substrate-specific esterase. The question remains as to whether the *St. lividans* AcXE will be capable of attacking 3-*O*-acetyl derivatives of mannopyranosides with an unsubstituted 2-position. The five-membered intermediate (Fig. 3) could be formed regardless of the orientation of the C-2 hydroxyl group [16].

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