Purification and characterization of an acetyl xylan esterase produced by *Streptomyces lividans*

Claude DUPONT, Nicole DAIGNEAULT, François SHARECK, Rolf MOROSOLI and Dieter KLUEPFEL*

Centre de Recherche en Microbiologie Appliquée, Institut Armand-Frappier, Université du Québec, Laval-des-Rapides, C.P. 100, Québec, Canada

The acetyl xylan esterase cloned homologously from *Streptomyces lividans* [Shareck, Biely, Morosoli and Kluepfel (1995) Gene **153**, 105–109] was purified from culture filtrate of the overproducing strain *S. lividans* IAF43. The secreted enzyme had a molecular mass of 34 kDa and a pI of 9.0. Under the assay conditions with chemically acetylated birchwood xylan the kinetic constants of the enzyme were: specific activity, 715 units/mg, K_m 7.94 mg/ml and V_{max} 1977 units/mg. Optimal enzyme activity was obtained at 70 °C and pH 7.5. Hydrolysis assays with different acetylated substrates showed that the enzyme is specific for deacetylating the *O*-acetyl group of polysaccharides and is devoid of *N*-deacetylation activity. Sequential hydrolysis shows that its action is essential for the complete degradation of acetylated xylan by the xylanases of *S. lividans*.

INTRODUCTION

Acetyl xylan esterases [EC 3.1.1.6] are enzymes that hydrolyse specifically the ester linkages of the acetyl groups in positions 2 and/or 3 of the xylose moieties of natural xylan. Such acetyl ester groups are commonly found in hardwoods, cereals and other annual plants, but are generally absent from soft wood. Together with xylanases, β-xylosidases, α-arabinofuranosidases and α-methylglucuronidases, the acetyl xylan esterase is part of the xylanolytic enzyme system that is capable of the complete hydrolysis of xylan [1]. Biely et al. [2] were the first to report the presence of esterases in the fungal cellulolytic system of *Schizophyllum commune* and showed the high specificity of the enzyme for acetylated xylan. Furthermore, the same group of workers reported the synergistic action of acetyl xylan esterases with xylanases [3]. Since then a number of enzymes with similar activities, produced from both eukaryotes and prokaryotes, have been reviewed by Christov and Prior [4].

Recently, analysis of the DNA region flanking the *xln*B locus [the gene coding for xylanase B (XlnB)] of *Streptomyces lividans* revealed a nucleotide sequence that encoded an acetyl xylan esterase (AxeA) that was located downstream from *xln*B in the DNA insert of plasmid pIAF42 [5]. The amino acid sequence of AxeA contained apparent catalytic and substrate-binding domains separated by a glycine-rich linker region. The substrate-binding region of AxeA showed a high degree of similarity to the corresponding domain of *S. lividans* XlnB and both are located in the C-termini of the genes. A computer search with the amino acid sequence of AxeA of *S. lividans* did not reveal any similarity to the published esterases nor with the acetyl xylan esterases XynC from *Caldocellum saccharolyticum* [6] or XylD of *Pseudomonas fluorescens* [7]. However, striking similarities were found to putative NodB domains in xylanases XylA, XylD and XylE that had been reported for *Cellvibrio mixtus* [8], *Cellulomonas fimi* [9] and *Pseudomonas fluorescens* [10] respectively. In addition, significant similarities were found to NodB from *Rhizobium meliloti* [11] and the chitin deacetylase of *Mucor rouxii* [12].

Here we report the purification and biochemical characterization of the AxeA produced by *S. lividans*.

EXPERIMENTAL

Organisms

Streptomyces lividans IAF10-164 *msi*K$^-$, a xylanase- and cellulase-negative mutant, served as host strain for the cloning experiments [13]. By using established methods of molecular biology [14], protoplasts of this strain were transformed with plasmid pIAF43 [5], which was obtained by subcloning the gene encoding AxeA (*axeA*) on a *Pst*I fragment from plasmid pIAF42 containing *xlnB–axeA* [15]. Deletion of the 2.5 kb *Bgl*II fragment in pIAF42 distal from *xlnB* produced plasmid pIAF44, which was transformed into *S. lividans* 10-164.

Culture conditions

For the production of the enzymes, *S. lividans* IAF43 and IAF44 were grown as previously described by Kluepfel et al. [16] with xylose as carbon source. Thiostrepton, a gift from Bristol Myers-Squibb (Montréal, Québec, Canada), was added after sterilization as selective marker in quantities of 5 μg/ml. The medium was inoculated with a spore suspension and incubated in Erlenmeyer flasks at 34 °C for 30–40 h on a shaker rotating at 240 rev./min; 6 % (v/v) of this pre-culture served as the inoculum for enzyme production in the same medium agitated for 69 h under the same conditions.

Enzyme recovery

The fermentation broth was first filtered through glass wool, then centrifuged at 10000 ***g*** for 30 min at 4 °C and the enzyme precipitated from the supernatant with 3 vol. of 95 % ethanol. The precipitate was recovered by centrifugation at 15000 ***g*** for 45 min at 4 °C. The sediment, after being rinsed with acetone and being dried under vacuum, constituted the crude enzyme preparation.

Abbreviations used: AxeA, acetyl xylan esterase; *axeA*, gene coding for AxeA; HEWL, hen-egg-white lysozyme; XlnA, XlnB, XlnC, xylanase A, B, C; *xln*B, gene coding for XlnB.

* To whom correspondence should be addressed.

Enzyme purification

The crude enzyme preparation of AxeA was dissolved and dialysed against 10 mM Tris/HCl buffer, pH 8.5, and treated in batches with an anion-exchange resin (Accell Plus® quaternary methylamine; Waters, St-Laurent, Québec, Canada) for 30 min at 4 °C with slight agitation. The resin was removed by filtration on a Büchner funnel and the filtrate dialysed against 20 mM Mes/NaOH buffer, pH 6.0. The dialysate was then adsorbed on a cation-exchange HR15 CM-Protein Pak® AP- semi-preparatory HPLC column (20 cm × 2 cm; Waters) pre-equilibrated with Mes/NaOH buffer. Elution was performed with a linear gradient from 0 to 1.0 M NaCl and was monitored with a UV recorder set at 280 nm. Fractions were collected; the active fractions were pooled and then concentrated by ultrafiltration in an Amicon cell (Amicon, Danvers, MA, U.S.A.) with a YM 3 membrane. Glycerol was added to a final concentration of 5% (v/v) and the solution stored at −70 °C. XlnB from strain IAF44 was purified as described previously [16].

Enzyme substrates

Birchwood, oat spelt xylan, chitin, *N*-acetylglucosamine, glucose penta-acetate, xylose tetra-acetate, 4-methylumbelliferyl acetate and *p*-nitrophenyl acetate were purchased from Sigma (St. Louis, MO, U.S.A.). Birchwood and oat spelt xylan were acetylated by the method described by Johnson et al. [17]. For this purpose the xylan was dissolved in DMSO at 55 °C and solid potassium borate was added to a final concentration of 0.8%. Acetic anhydride, preheated to 60 °C, was added slowly to the mixture and incubated at room temperature for 2 h. DMSO and excess acetic acid were removed by extensive dialysis for 5 days under running tap water, followed by two 24 h dialysis steps against Milli-Q water. The dialysate was centrifuged and the supernatant containing the acetylated xylan was freeze-dried. The degree of acetylation was determined by measuring the acetic acid content, after alkaline hydrolysis for 16 h, as described below.

Analytical methods

Enzyme assays

The specific activity of the AxeA was determined by the method of Johnson et al. [17]. In an Eppendorf tube, 500 μl of a 1% (w/v) solution of acetylated xylan in 100 mM sodium phosphate, pH 6.0, was preheated to 50 °C in a water bath for 10 min, after which 100 ng of enzyme was added. After 10 min of incubation at 50 °C, the reaction was stopped by the addition of 100 μl of 0.33 M H_2SO_4. The sample was centrifuged for 2 min at 13000 ***g*** in an Eppendorf Microfuge at room temperature and the released acetic acid was measured in the supernatant.

Synthetic substrates (4-methylumbelliferyl acetate and *p*-nitrophenyl acetate) were dissolved in *N*,*N*-dimethylformamide before being made up to a final concentration of 2 mM with 100 mM phosphate buffer, pH 6.0. The assay was performed as indicated above and the reaction stopped by the addition of 100 μl of 1 M sodium bicarbonate, pH 11.0. The release of chromogenic compounds was determined at 700 nm for the 4-methylumbelliferyl acetate and 405 nm for the *p*-nitrophenyl acetate assays respectively.

The kinetic parameters were determined by using acetylated birchwood xylan at concentrations ranging from 0.2 to 5.0% (w/v) to which the enzyme solution was added. At intervals, 100 μl of the reaction mixture was transferred into a tube containing 20 μl of 0.33 M H_2SO_4 and placed immediately on ice.

The thermostability of the enzyme was estimated by incubating enzyme solutions (100 μl, 10 μg/ml) at different temperatures for 15 min. Afterwards the solutions were frozen quickly in an acetone/solid CO_2 bath and kept at −78 °C until the remaining activity was determined by the assay described above.

Acetic acid quantification

The released acetic acid was quantified by HPLC with an Aminex HPX-87H column (7.8 mm × 300 mm; Bio-Rad, Mississauga, Ontario, Canada) with a mobile phase of 0.033 M H_2SO_4 at 45 °C and a flow rate of 0.5 ml/min. The acetic acid was detected with a differential refractometer, Waters model 410. The data were collected and processed with a Shimadzu C-R4AX integrator (Fisher-Scientific).

Enzyme activities

All activities are expressed in international units, where 1 unit is defined as the amount of enzyme needed to release 1 μmol of acetic acid in 1 min. All results reported are averages of at least three independent experiments.

Studies on the enzyme synergy

The effect of sequential hydrolysis of acetylated and non-acetylated xylan was studied with AxeA, xylanase A (XlnA) and xylanase B (XlnB). Substrate solutions at a final concentration of 1% (w/v) in 100 mM sodium citrate buffer, pH 6.0, were incubated for 48 h at 50 °C with each enzyme at a concentration of 1 unit/ml. After the first incubation, the second enzyme was added (1 unit/ml) and the incubation continued for another 48 h. The reactions were stopped by acidification with H_2SO_4 at a final concentration of 0.66 M. Then the samples were analysed for their xylo-oligosaccharide content.

Analysis of xylo-oligosaccharides

The products resulting from the enzymic hydrolysis of acetylated and non-acetylated xylan were analysed by HPLC. To remove any residual solids the samples were centrifuged for 2 min at maximum speed (13000 ***g***) in an Eppendorf Microfuge. The supernatants were filtered through 0.45 μm pore-size Acro® disc filters (Gelman Science, MI, U.S.A.). These filtrates were analysed by injecting 20 μl on to an Aminex HPX-42A HPLC column (7.8 mm × 300 mm; Bio-Rad) heated to 80 °C and using MilliQ water as the mobile phase at a flow rate of 0.5 ml/min. The xylo-oligosaccharides were detected with a differential refractometer, Waters model 410.

Substrate-binding studies

Insoluble oat spelt xylan and Avicel were used as substrates. In an Eppendorf tube, 50 mg of the substrate was suspended in 100 mM sodium phosphate buffer, pH 7.0, containing 50 mM NaCl and mixed with 5 units of AxeA at 4 °C (final volume 500 μl). At intervals the insoluble substrate was separated by centrifugation. The supernatant was recovered and assayed for esterase activity as described above. In some experiments 50 mg of the insoluble substrates was pre-treated by mixing with 100 μg of hen-egg-white lysozyme (HEWL), incubated for 30 min at room temperature, washed three times with the sodium phosphate buffer and used immediately for the binding assay.

Protein

The protein content of enzyme preparations was determined by the method of Lowry et al. [18] with BSA (Bio-Rad) as standard.

Determination of isoelectric point

Analytical isoelectric focusing was performed on PhastGel® containing Pharmalyte carrier ampholytes in the pH range 3–10 with the PhastSystem®. The gels were silver-stained after the run (Pharmacia-LKB Biotechnologies, Uppsala, Sweden; Separation and Development Technique Files No. 100 and 210).

Determination of molecular mass

The molecular mass and homogeneity of the purified AxeA were verified by SDS/PAGE [12.5% (w/v) gel] as described by Laemmli [19]. The sample was treated with 10 mM dithiothreitol at 37 °C for 15 min in 80 mM Tris/HCl, pH 7.8, followed by alkylation with 20 mM iodoacetamide at 70 °C for 15 min before loading on the gel.

Antibodies and Western immunoblotting

Antibodies were raised against AxeA in New Zealand white rabbits by injecting 57 μg of pure enzyme combined with complete Freund's adjuvant, followed by three additional injections of 115 μg of AxeA in incomplete Freund's adjuvant at intervals of 1, 2 and 7 weeks respectively. Western blotting was performed with anti-AxeA antibodies coupled to ^{125}I-labelled Protein A (Amersham, Oakville, Ontario, Canada) as previously described [20].

RESULTS AND DISCUSSION

Cloning and expression of the *axe*A and *xln*B genes

Plasmid pIAF43 was constructed by cloning the 3.1 kb *Pst*I fragment of pIAF42 [15] into *Escherichia coli* plasmid pTZ18U under the control of the p*lac* promoter (Figure 1). Then pSUR [21], a 2 kb fragment derived from pIJ702 containing an origin of replication for *Streptomyces* and the *tsr* gene (thiostrepton resistance) as selective marker, was inserted in the *Hin*dIII site distal from the *axe*A gene, and this vector was used to transform *S. lividans* strain IAF10-164.

Production and purification of AxeA

The enzyme was produced in shaken flask cultures with minimal medium containing xylose as sole carbon source. Under these conditions the AxeA was over-produced owing to the use of the multicopy vector pIAF43. Optimal enzyme levels were obtained after approx. 69 h of incubation, which corresponded to the depletion of the carbon source. Further incubation did not improve the enzyme yields.

After separation of the mycelium, the crude AxeA was recovered from the culture filtrates by precipitation with ethanol. Absorption on an anionic resin of the redissolved acetone-dried powder removed most of the unwanted proteins, leaving the AxeA mainly in solution. After the buffer had been changed by dialysis to Mes/NaOH, pH 6.0, the enzyme was purified from the remaining proteins by cationic HPLC with a NaCl gradient. Cation exchange chromatography resulted in a major protein peak, eluting at 133 mM NaCl, containing the AxeA activity. Gel chromatography of the pooled active fractions on Sephacryl S-100 HR (Pharmacia) yielded a well-defined single peak (results not shown). In SDS/PAGE analysis of the purified AxeA, treatment with 5% (v/v) 2-mercaptoethanol yielded a doublet (results not shown). This doublet was reduced to a single band by replacing 2-mercaptoethanol by dithiothreitol as reducing agent, followed by alkylation of the protein sample with

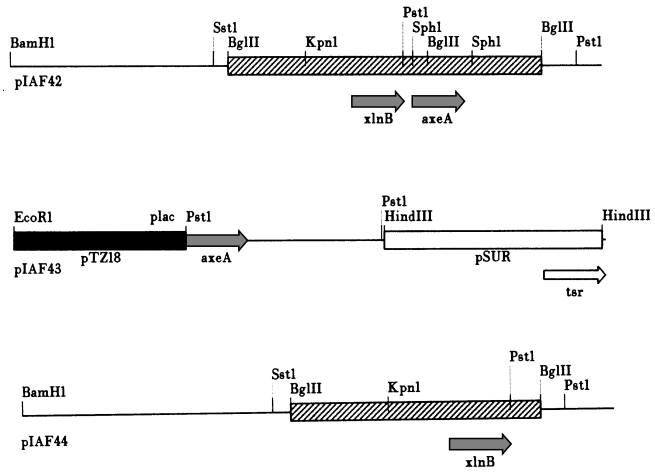

Figure 1 Restriction maps of plasmids pIAF42, pIAF43 and pIAF44

The hatched box represents the 5.5 kb insert in pIAF42 containing the operon consisting of the *xln*B and *axe*A genes, which are indicated by arrows. The black box is the *E. coli* plasmid pTZ18 and the open box is the *S. lividans* pSUR plasmid. The *axe*A gene is proximal to the promoter p*lac*.

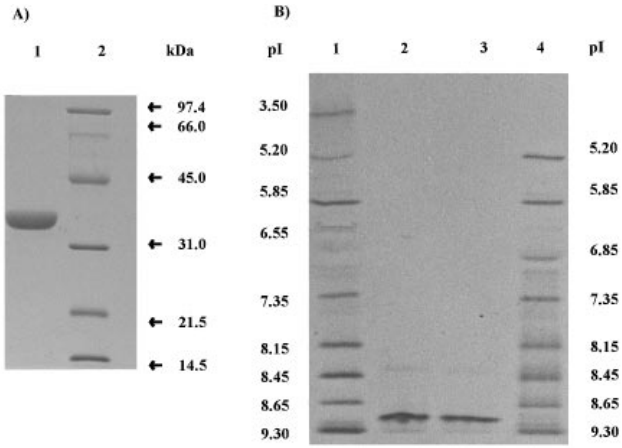

Figure 2 SDS/PAGE [12.5% (w/v) gel] (A) and analytical electrofocusing (B) of purified AxeA

(**A**) Lane 1, 7.8 μg of protein; lane 2, molecular mass markers. (**B**) Lane 1, Pharmacia pI marker proteins; lane 2, 250 ng of protein; lane 3, 125 ng of protein; lane 4, Pharmacia pI marker proteins.

iodoacetamide. This observation indicated that a disulphide bond is not easily accessible to reducing agents. The resistant disulphide bond probably did not involve the two cysteine residues found in the substrate-binding domain of AxeA because the same domain is found in XlnB [5], which does not show the same electrophoretic behaviour [17]. Therefore from the analysis of the primary structure of AxeA we assume that this resistant disulphide bond is located in the core of the catalytic domain involving Cys-3 and Cys-177.

The homogeneity of the enzyme preparation was confirmed by SDS/PAGE (Figure 2), which showed a single band with an estimated molecular mass of 34 kDa. Analytical isoelectric focusing of the enzyme on a pH gradient of 3.0–10 showed a pI of 9.0. The Western blot analysis of the three *S. lividans* xylanases

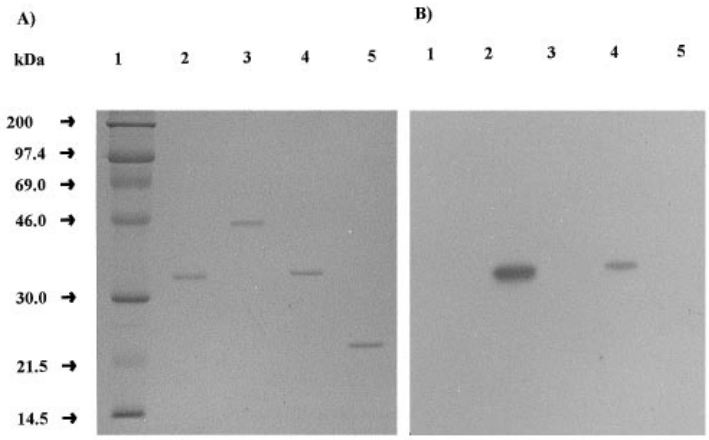

Figure 3 SDS/PAGE [12.5% (w/v) gel] of AxeA, XlnA, XlnB and XlnC (A) and Western electroblotting (B) with immune sera raised against purified AxeA

Lane 1, molecular mass markers; lane 2, AxeA; lane 3, XlnA; lane 4, XlnB; lane 5, XlnC.

Table 1 Binding of AxeA and AxeA2 (the catalytic domain only of AxeA) in the presence of insoluble substrates

Values are the percentage activity of AxeA remaining in the supernatant after incubation of the enzyme with the insoluble substrate.

Avicel					Oat spelt xylan				
	Without HEWL (%)		With HEWL (%)			Without HEWL (%)		With HEWL	
Time (min)	AxeA	AxeA2	AxeA	AxeA2	Time (min)	AxeA	AxeA2	AxeA	AxeA2
0	100	100	100	100	0	100	100	100	100
15	55	78	90	86	0.5	5	82	6	87
60	48	–	90	–	5	8	83	6	85
1440	25	–	87	–	15	–	81	–	87

A, B or C with anti-AxeA antibodies showed cross-reactivity only with XlnB (Figure 3). This was expected because both proteins share a high degree of similarity in their C-terminal substrate-binding domains [5]. The putative function of the C-terminal domain as a specific substrate binding domain for xylan is shown in Table 1. The results indicate a strong preference of the enzyme for binding to xylan. When the enzyme was incubated for 1 h in presence of Avicel, only half of the activity was recovered in the supernatant. However, when Avicel was pre-treated with 100 μg of HEWL to prevent non-specific association, 90 % of the activity remained in solution. This showed that the enzyme adsorbed non-specifically to the cellulosic substrate. When insoluble oat spelt xylan was used, no such difference between the pretreated and untreated substrates was observed. In both cases only 5–8 % of the total activity was recovered in the supernatant. These results confirm the existence of a specific xylan-binding domain similar to that reported recently for the XylD of *C. fimi* [22]. Furthermore, the significant degree of similarity found between the catalytic domains of AxeA and NodB can also be found in XylD, which might indicate that the latter enzyme could also have acetyl xylan esterase activity. This would have to be investigated by using an appropriate substrate with an enzyme lacking xylanase activity. To show that the C-terminal domain of the protein was responsble for the binding of the enzyme to insoluble xylan, fractions obtained from the purification containing only the catalytic domain of the enzyme (AxeA2) were used in the binding assay. As shown in Table 1, the catalytic domain alone does not associate significantly with either Avicel or insoluble oat spelt xylan. This indicated that the strong association with insoluble xylan observed with AxeA was mediated by the C-terminal xylan-binding domain.

Characterization of AxeA

The Michaelis–Menten constants were determined with 100 ng of AxeA and chemically acetylated birchwood xylan at concentrations ranging from 2.0 to 50 mg/ml. Non-linear regression software [23] was used to determine the K_m and V_{max} as 7.94 mg/ml and 1977 units/mg of enzyme respectively. On chemically acetylated birchwood and oat spelt xylans, the specific activities of AxeA were 715 and 890 units/mg respectively. To evaluate the substrate specificity of the AxeA, several other acetylated substrates were investigated. Thus the enzyme de-acetylated both xylose tetra-acetate and glucose penta-acetate with specific activities of 22 and 17 units/mg respectively. The differences in specific activities observed between acetylated mono- and poly-saccharides indicated strongly that the natural substrate of AxeA is the acetyl xylan. Because the xylan was chemically acetylated, it is likely that all free hydroxyl groups (positions 2, 3 and 5) could be randomly modified. However, in our experiments, enzymic hydrolysis could never remove all the acetyl groups from this substrate. This is evidence that the AxeA

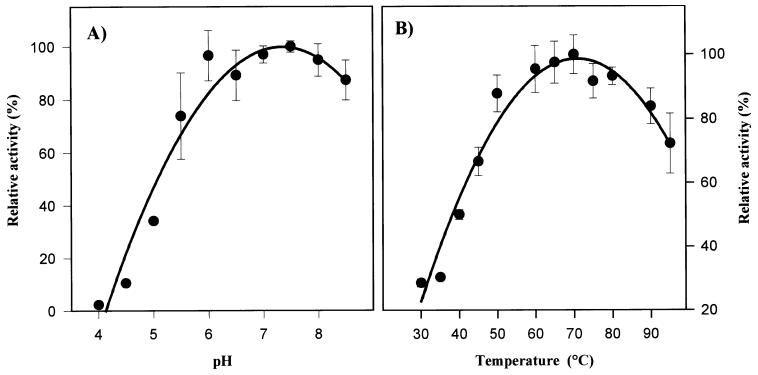

Figure 4 Optimum pH (A) and optimum temperature (B) of AxeA

The highest level of activity determined in each case was set at 100%.

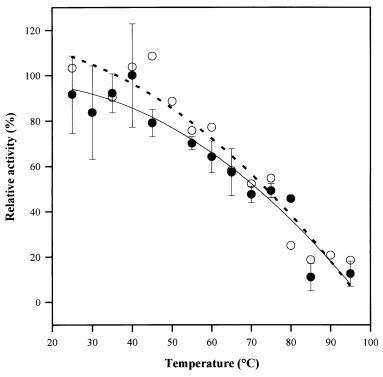

Figure 5 Thermostability of AxeA

Solutions of AxeA were incubated for 15 min at different temperatures and transferred to an acetone/solid CO_2 bath (−78 °C). The enzyme was thawed and the specific activity determined with the assay described in the Experimental section. The highest level of activity determined was set at 100%. ○, Enzyme incubated without xylan; ●, enzyme incubated with 1% birchwood xylan.

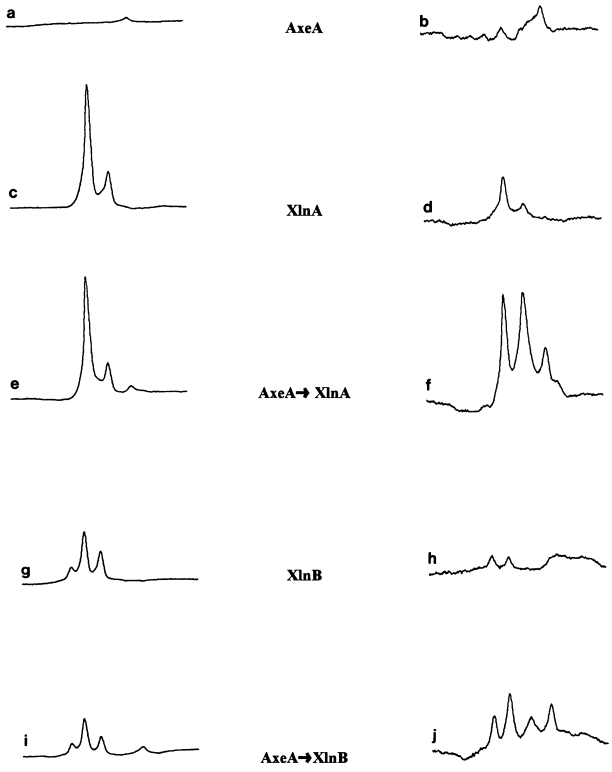

Figure 6 Synergism between AxeA and xylanases of *S. lividans*

HPLC profiles of sequential hydrolysis of birchwood xylan (left-hand column) and chemically acetylated xylan (right-hand column). Each chromatogram was corrected for non-specific release of material by subtraction of a control chromatogram. (**a**, **b**) Substrates incubated with AxeA; (**c**, **d**) substrates incubated with XlnA; (**e**, **f**) substrates incubated with AxeA with the addition of XlnA after 48 h; (**g**, **h**) substrates incubated with XlnB; (**i**, **j**) substrates incubated with AxeA with the addition of XlnB after 48 h.

is specific for the removal of *O*-acetyl groups on specific positions on the xylose moiety.

Although AxeA shows a significant degree of similarity in the N-terminal region to the chitin deacetylase from *Mucor rouxii* [12] it does not hydrolyse N-acetylated polysaccharides such as chitin or *N*-acetylglucosamine; neither are synthetic (aryl) acetates such as *p*-nitrophenyl acetate or methylumbelliferyl acetate hydrolysed under the conditions used. Only when incubated for up to 16 h could activity, which was less than 0.5 unit/mg of enzyme, be observed.

The enzyme was active over a broad pH range with 80% of its relative activity found between pH 5.5 and 8.5 with an optimum at pH 7.5 (Figure 4A). The temperature optimum of the AxeA was 70 °C, but the enzyme retained 75% of its activity at 95 °C (Figure 4B). Determination of the remaining activity after incubation of the enzyme for 15 min at different temperatures indicated that, under these conditions, the enzyme had a T_{50} of 72 °C, the T_{50} value being the temperature at which 50% activity remains after incubation (Figure 5). Incubation with 1% (w/v) birchwood xylan did not affect the T_{50} value, indicating that the substrate has no protective effect on the enzyme.

Synergism of AxeA with *S. lividans* xylanases

Studies on synergism were performed with AxeA, XlnA and XlnB. XlnC was omitted because this enzyme, belonging to family 11 of xylanases, had been shown to be indistinguishable from XlnB [24]. Incubation of both acetylated and non-acetylated birchwood xylan with AxeA did not liberate any xylo-oligosaccharides (Figures 6a and 6b). Moreover, after xylanase

treatment, the resulting xylo-oligosaccharides were not hydrolysed further by AxeA (results not shown), which confirmed the absence of any xylanolytic activity of AxeA. Hydrolysis of non-acetylated xylan with XlnA and XlnB showed complete hydrolysis (Figures 6c and 6g), whereas acetylated xylan was fully hydrolysed by the *S. lividans* xylanases only when pretreated with AxeA (Figures 6f and 6j). This clearly demonstrates the necessity of removing the acetic acid moieties from the acetylated xylan before hydrolysis of the substrate by the xylanases. Incubations with both enzymes (AxeA and XlnA or XlnB) together did not yield any significant difference from the pattern resulting from the samples treated sequentially (results not shown).

These studies show that AxeA is specific to O-acetylated polysaccharides as substrate and demonstrate the importance of AxeA for the complete degradation of hemicellulose. By removing the acetyl group from xylan, this enzyme allows accessibility of the substrate to xylanases. Because AxeA and XlnB are organized as an operon on the chromosome of *S. lividans*, the separation of each will allow the further investigation of the role and function of these enzymes in the degradation pathway of xylan and the assessment of the importance of each for the growth and survival of *S. lividans* in its natural habitat.

We thank Dr. Peter Biely for a critical reading of the manuscript. This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and by the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR).

REFERENCES

1 Biely, P. (1985) Trends Biotechnol. **3**, 286–290
2 Biely, P., Puls, J. and Schneider, H. (1985) FEBS Lett. **186**, 80–84
3 Biely, P., MacKenzie, C. R., Puls, J. and Schneider, H. (1986) Bio/Technology **4**, 731–733
4 Christov, L. P. and Prior, B. A. (1993) Enzyme Microb. Technol. **15**, 460–475
5 Shareck, F., Biely, P., Morosoli, R. and Kluepfel, D. (1995) Gene **153**, 105–109
6 Lüthi, E., Love, D. R., McAnulty, J., Wallace, C., Caughey, P. A., Saul, D. and Bergquist, P. L. (1990) Appl. Environ. Microbiol. **56**, 1017–1024
7 Ferreira, L. M. A., Wood, T. M., Williamson, G., Faulds, C., Hazlewood, G. P., Black, G. W. and Gilbert, H. J. (1993) Biochem. J. **294**, 349–355
8 Hazlewood, G. P. and Gilbert, H. J. (1993) in Hemicellulose and Hemicellulases (Coughlan, M. P. and Hazlewood, G. P., eds.), pp. 103–126, Portland Press, London
9 Millward-Sadler, S. J., Poole, D. M., Henrissat, B., Hazlewood, G. P., Clarke, J. H. and Gilbert, H. J. (1994) Mol. Microbiol. **11**, 375–382
10 Millward-Sadler, S. J., Davidson, K., Haxlewood, G. P., Black, G. W., Gilbert, H. J. and Clarke, J. H. (1995) Biochem. J. **312**, 39–48
11 Egelhoff, T. T., Fisher, R. F., Jacobs, T. W., Mulligan, J. T. and Long, S. R. (1985) DNA **4**, 241–248
12 Kafetzopoulos, D., Thireos, G., Vournakis, J. and Bouriotis, V. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8005–8008
13 Hurtubise, Y., Shareck, F., Kluepfel, D. and Morosoli, R. (1995) Mol. Microbiol. **17**, 367–377
14 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15 Vats-Mehta, S., Bouvrette, P., Shareck, F., Morosoli, R. and Kluepfel, D. (1990) Gene **86**, 119–122
16 Kluepfel, D., Vats-Mehta, S., Aumont, F., Shareck, F. and Morosoli, R. (1990) Biochem. J. **267**, 45–50
17 Johnson, K. G., Fontana, J. D. and MacKenzie, C. R. (1988) Methods Enzymol. **160**, 551–560
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
19 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
20 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
21 Paradis, F. W., Morosoli, R., Shareck, F., Dupont, C. and Kluepfel, D. (1996) Appl. Microbiol. Biotechnol. **45**, 646–651
22 Black, G. W., Hazlewood, G. P., Millward-Sadler, S. J., Laurie, J. I. and Gilbert, H. J. (1995) Biochem. J. **307**, 191–195
23 Leatherbarrow, P. J. (1987) Enzfitter, A Non-linear Regression Data Analysis Program for IBM PC, Elsevier, Amsterdam
24 Biely, P., Kluepfel, D., Morosoli, R. and Shareck, F. (1993) Biochim. Biophys. Acta **1162**, 246–254

Received 29 January 1996/22 May 1996; accepted 7 June 1996