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Purification and Characterization of Two Thermostable Acetyl Xylan Esterases from *Thermoanaerobacterium* sp. Strain JW/SL-YS485

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Two acetyl esterases (EC 3.1.1.6) were purified to gel electrophoretic homogeneity from *Thermoanaerobacterium* sp. strain JW/SL-YS485, an anaerobic, thermophilic endospore former which is able to utilize various substituted xylans for growth. Both enzymes released acetic acid from chemically acetylated larch xylan. Acetyl xylan esterases I and II had molecular masses of 195 and 106 kDa, respectively, with subunits of 32 kDa (esterase I) and 26 kDa (esterase II). The isoelectric points were 4.2 and 4.3, respectively. As determined by a 2-min assay with 4-methylumbelliferyl acetate as the substrate, the optimal activity of acetyl xylan esterases I and II occurred at pH 7.0 and 80°C and at pH 7.5 and 84°C, respectively. K_m values of 0.45 and 0.52 mM 4-methylumbelliferyl acetate were observed for acetyl xylan esterases I and II, respectively. At pH 7.0, the temperatures for the 1-h half-lives for acetyl xylan esterases I and II were 75° and slightly above 100°C, respectively.

Plant cell walls contain up to 7% O-bound acetyl groups by dry weight. About 60 to 70% of xylose residues in hardwood xylans are esterified with acetic acid, and acetyl xylans also exist in annual plants. Efficient and complete degradation of acetyl xylans requires the cooperation of an esterase as well as other xylanolytic enzymes (2, 6, 10).

Acetyl xylan esterases (EC 3.1.1.6) have been measured in several xylanolytic or cellulolytic fungi (4, 12, 13, 22, 24, 25) and in some bacteria including *Pseudomonas fluorescens* (8), *Butyrivibrio fibrisolvens* (9), *Streptomyces* spp. (11), *Thermoanaerobacterium saccharolyticum* (15), *Caldocellum saccharolyticum* (16), *Thermomonospora fusca* (1, 17), and *Fibrobacter succinogenes* (18, 19). Only a few of these enzymes have been purified, including the enzymes from *Trichoderma reesei* (26), *Aspergillus oryzae* (27), *F. succinogenes* (18, 19), and *Thermomonospora fusca* (1).

Xylanolytic enzymes for industrial utilization of hemicellulosic material must exhibit high degrees of stability and activity. Here we report the purification and characterization of two thermostable acetyl xylan esterases from *Thermoanaerobacterium* sp. strain JW/SL-YS485, an anaerobic, thermophilic endospore former that uses various substituted xylans for growth. One of the acetyl xylan esterases has a particularly high degree of thermostability and high levels of activity on acetylated xylan.

Thermoanaerobacterium sp. strain JW/SL-YS485 is a recently isolated thermophilic anaerobic bacterium that grows in a pH range of 3.85 to 6.35 and a temperature range of 30 to 66°C (15a). From this organism, two β-xylosidases, an α-glucuronidase, and a cell-associated endoxylanase have been purified and characterized (24a). We now report the purification and characterization of two thermostable acetyl xylan esterases from this organism.

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MATERIALS AND METHODS

Organism and medium. *Thermoanaerobacterium* sp. strain JW/SL-YS485 was recently isolated by S. Liu of this laboratory from a hot spring in Yellowstone National Park, Wyo. It was grown under an atmosphere of O_2-free N_2 at 60°C in 0.1% yeast extract, 0.3% birch xylan, 0.1% urea, 0.018% $MgCl_2 \cdot 6H_2O$, 0.174% K_2HPO_4, 0.408% phthalic acid (monopotassium salt), 0.05% vitamin solution (7), 0.5% mineral solution (7), 0.025% $Na_2S \cdot 9H_2O$, and 0.025% cysteine $HCl \cdot H_2O$. Cells used for enzyme purification were grown in a 100-liter fermentor under anaerobic conditions at a constant pH of 6.0, with the automatic additions of either 0.5 M NaOH or 0.1 M HCl.

Enzyme assay. An enzyme unit was defined as the amount of enzyme that produced 1 μmol of product per min, and specific activity was expressed as units of activity per milligram of protein. For routine assays, acetyl esterase activity was determined by measuring the amount of 4-methylumbelliferone released from 4-methylumbelliferyl acetate. Sodium phosphate buffer (pH 7.0; 100 μl of 100 mM) and 280 μl of H_2O were preincubated at 70°C for 12 min. Ten microliters of enzyme solution was added to the buffer, and the reaction was initiated within 1 min by adding 10 μl of 100 mM 4-methylumbelliferyl acetate in dimethyl sulfoxide. After 2 to 10 min, the reaction was stopped by adding 600 μl of 50 mM citric acid, and A_{354} was determined.

For the determination of substrate specificity, the following substrates were tested: acetylated (larch) xylan (about 33% acetyl group, a gift from Scott Borneman), cellulose acetate (about 40% acetyl group), glucose pentaacetate, triacetin, tri-*O*-acetyl galactal, xylose tetraacetate, and 4-methylumbelliferyl butyrate (Sigma, St. Louis, Mo.). Enzyme activities against 4-methylumbelliferyl butyrate were determined as described for 4-methylumbelliferyl acetate. For other substrates, an amount containing 0.1 mg of the acetyl moiety was used in a 400-μl reaction mixture; reactions were stopped by adding 600 μl of ice cold water (no activity was observed at 25°C). The liberated acetic acid was quantified with an enzymatic analysis kit from Boehringer Mannheim (catalog no. 148261), used according to the instructions of the supplier.

The protein concentration of the purified enzyme was estimated by its A_{280} by using bovine serum albumin as the standard. In cell extracts and partially purified preparations, the protein was estimated by the dye-binding method (5) by using bovine serum albumin as the reference standard.

Enzyme purification. Bacterial cells were resuspended in 50 mM phosphate buffer (pH 7.0) and disrupted with a French press at 1.25×10^5 kPa. Cell extract was obtained by centrifugation at 4°C and 100,000 × *g* for 60 min with an L8-M ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). All purification steps were performed at room temperature in the presence of 0.02% (wt/vol) sodium azide to prevent microbial growth. Chromatography media and prepacked columns were purchased from Pharmacia Biotech, Inc. (Piscataway, N.J.).

(i) Ion-exchange chromatography on DEAE-Sepharose. Cell extract was loaded onto a DEAE-Sepharose FF column (2.6 by 56 cm; Pharmacia Biotech, Inc.) which had been previously equilibrated with 25 mM bis-Tris propane {1,3-bis[tris(hydroxymethyl)-methylamino]propane} buffer (pH 7.0). Proteins

were eluted with a NaCl gradient (0 to 1 M) in 1,120 ml of bis-Tris propane buffer at a flow rate of 4 ml/min. Fractions of 8 ml were collected.

(ii) Hydrophobic interaction chromatography. The esterase active proteins were eluted in two peaks from the DEAE-Sepharose column. The proteins giving each peak of activity were pooled separately and mixed with a 4 M $(NH_4)_2SO_4$ solution to give a final $(NH_4)_2SO_4$ concentration of 1.2 M. These mixtures were chromatographed separately using a phenyl-Sepharose CL-4B column (1 by 18 cm), which had been previously equilibrated with bis-Tris propane buffer containing 1.2 M $(NH_4)_2SO_4$. Proteins were eluted with a 180-ml gradient of $(NH_4)_2SO_4$ (from 1.2 to 0 M) and ethylene glycol (0 to 10%, vol/vol) in bis-Tris propane buffer at a flow rate of 0.75 ml/min. Fractions of 3 ml were collected.

(iii) Ion-exchange chromatography on DEAE-Sephacel. The active fractions from the phenyl-Sepharose CL-4B column were dialyzed against 2 liters of 25 mM sodium citrate buffer (pH 6.4) overnight and applied onto a DEAE-Sephacel column (0.5 by 9 cm) equilibrated to pH 6.4 with 25 mM sodium citrate buffer (pH 6.4). Proteins were eluted with a linear pH gradient of 25 mM sodium citrate buffer from pH 6.4 to 3. The flow rate was 0.4 ml/min. After elution, the fractions with activity were pooled in dialysis tubing and concentrated 20-fold by using polyethylene glycol (21).

(iv) Gel filtration. The concentrated enzyme samples (0.2 ml) from the DEAE-Sephacel step were further purified by fast protein liquid chromatography with a prepacked Superose 6 column (10/30). Bis-Tris propane buffer containing 0.2 M NaCl was used for protein elution at 0.4 ml/min.

Determination of molecular mass and pI. The molecular masses of the purified enzymes were determined by gel filtration and gel electrophoresis. The gel filtration molecular weight markers were MW-GF-200 (Sigma). Sodium dodecyl sulfate (SDS) (12%) and 4 to 30% native gradient polyacrylamide gels were used for the analysis of purified enzymes. Gels were cast by using the Mini-PROTEAN II Multi-Casting Chamber (Bio-Rad, Richmond, Calif.), according to the supplier's instructions. Gel electrophoresis was performed with the Mini-PROTEAN II Dual Slab Cell (Bio-Rad). The gradient gel was electrophoresed at 160 V and 4°C for 16 h with a buffer of 90 mM Tris, 80 mM boric acid, and 2.5 mM Na_2-EDTA. Isoelectric focusing was carried out by using the PhastSystem (Pharmacia Biotech Inc.) in the pH range of 3 to 9 (PhastGel IEF 3-9). Electrophoresis calibration kits from Pharmacia Biotech Inc. were used to determine the markers. Proteins in polyacrylamide gels were stained with Coomassie blue (R 250).

NH_2-terminal sequence. The NH_2-terminal sequence was determined by automated Edman degradation (protein sequencer model 470A or 477A; Applied Biosystems, Inc., Foster City, Calif.) at the Office of the Vice President for Research, University of Georgia (Athens).

Chemicals. All chemicals were purchased from Sigma, unless otherwise indicated.

RESULTS

Two acetyl esterases in cell extracts of strain YS485. The majority (>75%) of the esterase activity was cell associated, and only a small amount was observed in the culture supernatant. The acetyl esterase activity was induced by various xylans. With 4-methylumbelliferyl acetate as the substrate, specific activities between 0.12 and 0.25 μmol/min/mg of protein were obtained when the organism was grown on arabinose, cellobiose, galactose, glucose, mannose, or xylose; however, a specific activity greater than 0.8 μmol/min/mg of protein was observed when xylan (beechwood, birchwood, larchwood, or oat spelts) was provided as the carbon source for growth. The highest level of activity was observed in the late exponential growth phase, and up to 64% of the total activity was cell associated. In cell extract, a specific acetyl esterase activity of 0.9 μmol/min/mg of protein was determined with 4-methylumbelliferyl acetate as the substrate. The activity was separated into two distinct peaks during ion-exchange chromatography on DEAE-Sepharose (Fig. 1). The acetyl esterase in the first peak was named esterase I, and that in the second peak was named esterase II.

Purification of esterases I and II. The enzyme purification is summarized in Table 1. After gel filtration, both enzymes were purified to electrophoretic homogeneity. Only one band was obtained for each enzyme when 2.5 μg of protein was loaded on 1.0-mm native gradient gel or 1 μg was used on PhastGel IEF 3-9.

Substrate specificity. The purified enzymes exhibited acetyl xylan esterase activity. They liberated acetic acid from acetylated xylan and acetylated sugars as well as butyric acid from 4-methylumbelliferyl butyrate. Whereas esterase I exhibited higher levels of activity than esterase II when saturating concentrations of acetylated xylose were used, esterase II exhibited higher levels of activity than esterase I when acetylated xylan was used as the substrate (Table 2).

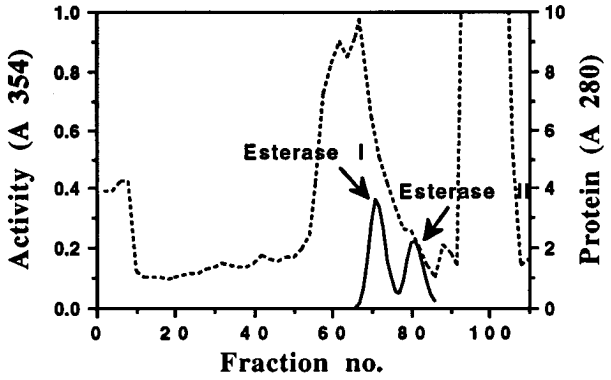

FIG. 1. Ion-exchange chromatography of acetyl xylan esterases I and II on DEAE-Sepharose. – – –, A_{280}; ——, activity with 4-methylumbelliferyl acetate as the substrate as indicated by A_{354} of the enzyme assays.

Molecular masses, pIs, and sequences. Esterases I and II exhibited molecular masses of 170 and 110 kDa, respectively, on a 4 to 30% native gradient polyacrylamide gel. Each enzyme was composed of one type of subunit with molecular masses of 32 and 26 kDa on SDS-polyacrylamide gels (Fig. 2). The molecular masses estimated by gel filtration were 195 kDa for esterase I and 106 kDa for esterase II. The data suggested that esterase I was a hexamer and that esterase II was a tetramer. Isoelectric focusing by using the PhastSystem showed that esterase I had a pI at pH 4.2 and that esterase II had a pI at pH 4.3. The NH_2-terminal sequences obtained from the purified enzymes were GLFXM MXWLQ KLREY TGT for acetyl xylan esterase I and MLLKD GDVVL FQGYX YTY for acetyl xylan esterase II. Only the second of 18 amino acid residues was the same for both enzymes.

Kinetic properties. As determined by an initial 2-min assay with 4-methylumbelliferyl acetate as the substrate, the highest activities for acetyl xylan esterases I and II were at pH 7.0 and 80°C and at pH 7.5 and 84°C, respectively (Fig. 3a). The Arrhenius energies (E_a) of esterases I and II were 29 and 27 kJ/mol, respectively (Fig. 4). At pH 7.0 and 70°C, esterases I and II had K_ms of 0.45 and 0.56 mM, respectively, with 4-methylumbelliferyl acetate as the substrate; K_{cat} values were 14,690 μmol/min/μmol for esterase I and 11,236 μmol/min/μmol for esterase II.

Stability of enzymes. At 70°C, esterase I was stable in a relatively narrow pH range around 7.0, whereas esterase II did not lose activity after 3 h of incubation in a pH range from 6 to 8 (Fig. 3b). Both enzymes were thermostable (Fig. 5). The temperature for the 1-h-half-life time of esterase I was about 75°C (Fig. 5a). For esterase II, the temperature for the 1-h-half-life time was slightly above 100°C (Fig. 5b); the enzyme, sealed in glass micropipettes, lost 75% activity within 5 min at 104°C (data not shown).

DISCUSSION

Since its importance was recognized 7 years ago (2, 4), acetyl xylan esterase has attracted increasing attention (12, 22, 25). To seek enzymes with high degrees of activity and for hemicellulose degradation under thermophilic conditions (14, 24,

TABLE 1. Purification of acetyl xylan esterases I and II

Step	Acetyl xylan esterase I			Acetyl xylan esterase II		
	Total protein (mg)	Total activity (μmol/min)	Sp act[a] (U/mg)	Total protein (mg)	Total activity (μmol/min)	Sp act (U/mg)
Cell extract[b]	1,068.8	<942	<0.9	3,326.4[c]	<2,993	<0.9
DEAE-Sepharose	138.5	485	3.5	233.2	723.7	3.1
Phenyl-Sepharose	43.9	316	7.2	21.8	408.1	18.7
DEAE-Sephacel	6.8	223	33.0	9.3	258.7	27.8
Gel filtration	3.5	216	62.0	0.8	67.3	82.0

[a] Determined with 4-methylumbelliferyl acetate as the substrate in 25 mM bis-Tris propane buffer (pH 6.4) at 70°C.
[b] Specific activity in the cell extract, 0.9 (U/mg protein), was the sum of activities of acetyl xylan esterases I and II. Total activity for esterases I and II was calculated on the basis of acetyl xylan esterases I and II. Total activity for esterases I and II was calculated on the basis of the ratio obtained after separation on DEAE-Sepharose.
[c] Since the preparation contained less acetyl xylan esterase II than I (Fig. 1), more than three times more crude extract was used for the purification of esterase II.

28), we further purified and characterized the acetyl xylan esterases from *Thermoanaerobacterium* sp. strain JW/SL-YS485, a thermophilic anaerobe. The (acetyl xylan) esterase II from this organism exhibited a higher level of specific activity and higher degree of stability than those reported for acetyl xylan esterases from other organisms.

Because of the instability of *p*-nitrophenyl acetate and α-naphthyl acetate, it is difficult to determine acetyl esterase activity on these substrates at pH values above 6.5 or 7.0 (16, 20). Using 4-methylumbelliferyl acetate, which is more stable, we obtained a pH optimum of 7.5 and an optimal temperature (2-min assay) of 84°C for esterase II. These values were slightly lower for esterase I. The optimum temperature and thermostability of esterase II are the highest among the acetyl xylan esterases characterized to date (4, 11, 13, 16).

Only one K_m, 2.7 mM (α-naphthyl acetate) for acetyl xylan esterase from *F. succinogenes*, has been reported (18, 19). In our work, esterases I and II showed K_m values of 0.45 and 0.52 mM, respectively, when 4-methylumbelliferyl acetate was used as the substrate.

The specific activities of purified acetyl xylan esterases have been determined with various substrates. With xylose tetraacetate as the substrate, the acetyl xylan esterases from *Trichoderma reesei* have specific activities of 42 and 43 μmol/min/mg of protein (700 and 720 μkat/g) (26); esterases I and II (this study) exhibit specific activities of 738 and 521 μmol/min/mg protein, respectively. When acetyl xylan is used as the substrate, acetyl xylan esterases from *Thermomonospora fusca* and *F. succinogenes* have specific activities of 0.06 and 8.63 μmol/min/mg, respectively (1, 18, 19); we have obtained specific activities of 5.2 and 12.4 μmol/min/mg protein for esterases I and II, respectively. Acetyl xylooligomers were not tested because purified acetyl xylooligomers were not available. Esterases I and II from the *Thermoanaerobacterium* sp. (this work) are oligomers, while all other purified acetyl xylan esterases are apparently monomeric enzymes (1, 18, 19, 26, 27). No other NH_2-terminal sequences of acetyl xylan esterase have been reported to date.

Streptomyces species contain more than one deacetylating enzyme, but the enzymes have not been purified (11). The fractionation of culture filtrates by gel electrophoresis or ion-exchange chromatography indicated the presence of multiple acetyl xylan esterases in *Trichoderma reesei* and *Trichoderma viridi* (3). However, the two enzymes isolated from *Trichoderma reesei* have been found to be very similar in their properties (26). In addition to acetyl xylan esterases, *Trichoderma reesei* also contains an acetyl esterase acting on xylooligomers but not on polymeric substrates (23). In contrast, both enzymes from *Thermoanaerobacterium* sp. strain JW/SL-YS485 described here deacetylate acetylated xylan. Hence, they are regarded as true acetyl xylan esterases. Furthermore, the purified enzymes are distinctly different from each other in molecular size, NH_2-terminal sequence, kinetic properties, and thermostability.

At this time, it is not possible to definitely determine the positioning of the two enzymes with regard to intracellular or

TABLE 2. Substrate specificity of purified esterases[a]

Substrate	Acetyl xylan esterase (U/mg)	
	I	II
Acetylated xylan	5.2	12.4
Cellulose acetate	0	0
Methylumbelliferyl acetate (100 mM)	82	101
Methylumbelliferyl butyrate (100 mM)	1.7	3.5
Glucose pentaacetate	424	425
Triacetin	505	835
Tri-*O*-acetyl galactal	640	534
Xylose tetraacetate	738	521

[a] The assay mixture (0.4 ml) contained 25 mM sodium phosphate buffer (pH 7.0), 0.156 μg of esterase I or 0.09 μg of esterase II, and the substrate, as indicated in Materials and Methods. The assay mixture was incubated at 70°C for 2 min, and then the reaction was stopped by chilling the mixture in an ice bath. The released acetate was determined by a coupled enzyme assay (Boehringer Mannheim, catalog no. 148261).

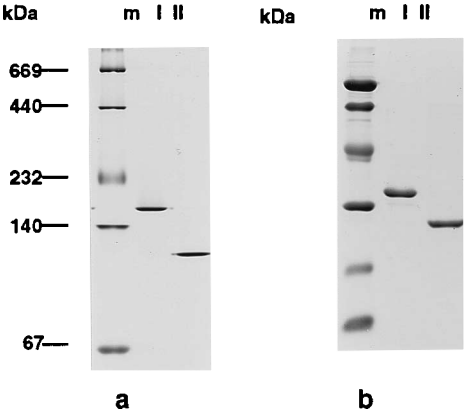

FIG. 2. Polyacrylamide gel electrophoresis analysis of purified enzymes. (a) Nondenatured enzymes analyzed with 4 to 30% gradient native gel; (b) denatured enzymes analyzed with SDS–12% polyacrylamide gel. m, molecular masses of standard proteins (indicated on the left); I and II, acetyl xylan esterases I and II, respectively.

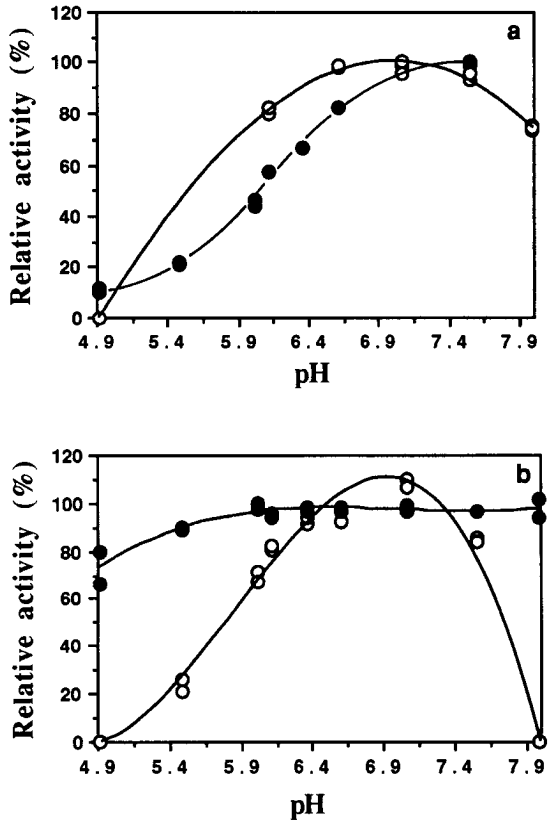

FIG. 3. Effects of pH on acetyl xylan esterases in 25 mM sodium phthalate (pH 4.9 to 6.4) and sodium phosphate (pH 6 to 8) buffer. (a) Determination of pH optimum. The activities of acetyl xylan esterases I (○) and II (●) were obtained by using the 2-min assay procedure as described in Materials and Methods, except for the indicated buffers. The highest level of activity determined for each enzyme was set to 100% (b) pH stabilities. Esterase I (1.07 μg) or II (0.64 μg) (in 6 μl) was added to 384 μl of buffers with different pH values (pH determined at 70°C), and the enzymes were incubated for 1 h (esterase I) or 3 h (esterase II) at 70°C. Then, 10 μl of 100 mM 4-methylumbelliferyl acetate was added to the mixture for determination of the remaining activities of acetyl xylan esterases I (○) and II (●). The activity at each pH value without preincubation of the enzymes represents 100% activity.

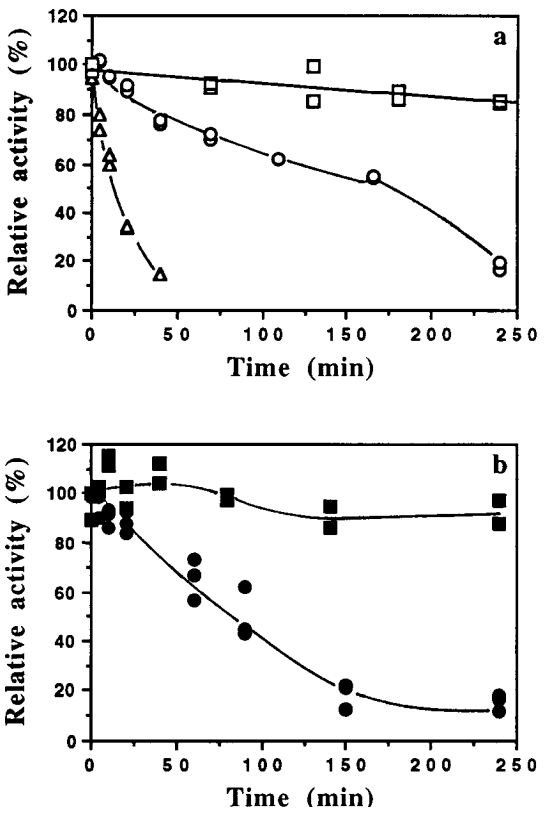

FIG. 5. Thermostabilities of purified esterases. (a) Acetylxylan esterase I (1.4 μg) (in 8 μl of buffer) was added to 382 μl of preincubated 25 mM sodium phosphate buffer (pH 7.0) in 1.5-ml Eppendorf centrifuge tubes, and the enzyme solutions were incubated at 72°C (□), 74.5°C (○), or 77°C (△) for various times in the absence of substrate. The remaining activity was determined by adding 10 μl of 100 mM 4-methylumbelliferyl acetate and continuing incubation at the same temperature for an additional 2 min. The activity of the enzyme at time zero (no preincubation) was used as 100%. (b) Acetyl xylan esterase II (0.86 μg) (in 8 μl of buffer) was incubated as described for acetyl xylan esterase I, but at 88.5°C (■) or in boiling water (●); the remaining activity was assayed at 80°C.

cell-associated extracellular location. On the basis of the determined properties of the two enzymes, however, it is possible that the function of esterase II is to cleave extracellular acetyl groups from the polymeric xylan, while the function of acetylase I is to remove acetyl groups intracellularly from small acetylated xylose oligomers. If this is the case, acetylase I could more specifically be called an acetyl (oligo)xylose esterase. We intend to prove this concept after cloning and sequencing the gene for both enzymes and obtaining mutants without one or the other acetyl xylan esterase.

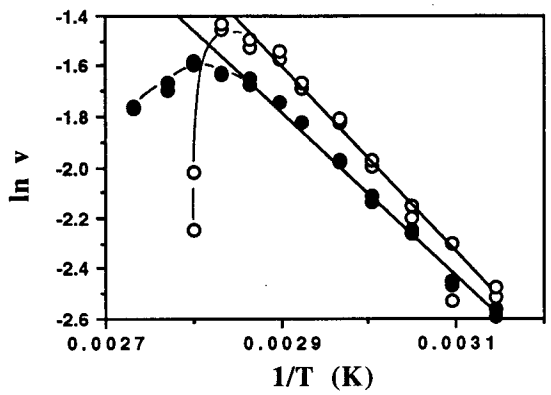

FIG. 4. Arrhenius plot of temperature dependence. Initial 2-min assay procedure activities of acetyl xylan esterases I (○) and II (●) were determined with 4-methylumbelliferyl acetate as substrate.

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