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Purification and Characterization of an Acetyl Esterase from *Aspergillus niger*

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ABSTRACT

Optimized acetyl esterase enzyme production conditions using *Aspergillus niger* ATCC 10864 in 14-L fermentation jars were determined to be 33°C, 1.5 vvm aeration, and 300 rpm agitation without pH control. The acetyl esterase was purified by precipitation in 60–80% saturation in ammonium sulfate. The pellet was applied directly to a Pharmacia high-load Phenyl Sepharose column for hydrophobic interaction chromatography and purified to homogeneity in two steps.

Stability and kinetic characteristics of the acetyl esterase were determined over a pH range of 4.0–7.5 and from 4 to 45°C. At temperatures >25°C, stability was superior at pH values <5.0. The temperature activity optimum was 35°C, and the pH optimum was 7.0. The V_{max} was determined to be 46,700 U/mg protein, and the K_m was 0.023*M* *p*-nitrophenyl acetate at pH 6.5 in 0.2*M* phosphate buffer at 35°C. The mol wt of the enzyme was 35,000 dalton by size-exclusion chromatography and SDS gel electrophoresis. The N-terminal amino acid sequence and the glycosylation composition were also determined.

Index Entries: Acetyl esterase; acetyl xylan esterase; *Aspergillus niger;* hemicellulose.

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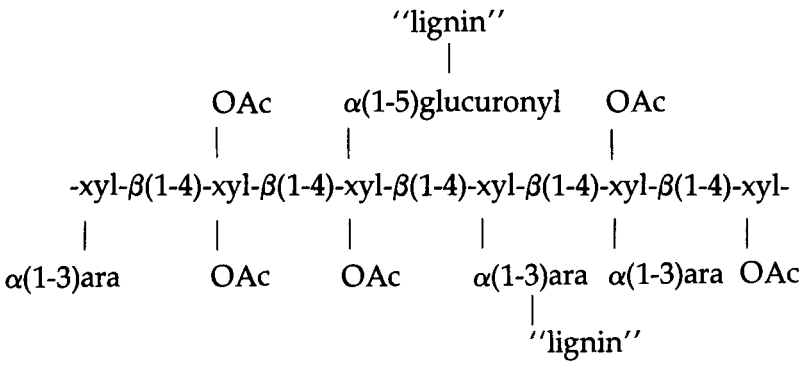
INTRODUCTION

Xylan, the major hemicellulose component of many plants, occurs naturally in a partially acetylated form. Lignin is also covalently associated with hemicellulose through α-(1,3)-L-arabinose and α-(1,5)-4-O-methyl-D-glucuronyl ester linkages to the β-1,4-linked-D-xylose backbone (*1*). It has been suggested that these substitutions of xylan are one of the main resistances of plant cell-wall degradation.



Complete xylan degradation requires a combination of enzymes capable of hydrolyzing each component. One of these is acetyl xylan esterase (AXE) (E.C.31.1.6), which removes acetyl groups from the xylan backbone (*2–4*). Esterases that deacetylate acetylated xylan are present in cellulolytic systems of several fungi, streptomycetes, plants, and animals (*2*). Esterases present in fungal systems are found to exhibit much higher activity against acetylated xylan than any esterase of nonfungal origin (*4*). Esterases that are not active against acetylated xylan have also been detected from fungi (*5*).

Mitchell et al. found the degree of acetylation of xylan to be inversely related to the rate of enzymatic hydrolysis of the hemicellulose (*6*). Furthermore, Overend and Johnson found that lignin-carbohydrate complexes (LCC) from *Populus deltroides* all had *O*-acetyl residues (*7*). In studies to characterize three distinct LCC isolated from steam-exploded poplar, AXE isolated from *Schizopyllum commune* and xylanase were necessary for removal of any monosaccharide from any of the LCC fragments that were studied. Poutanen et al. showed the AXE, a feruloyl esterase, and an acetyl esterase were necessary enzymes involved in the hydrolysis of arabinoxylans from wheat (*8*). *A. oryzae* reportedly was a good source of these esterase enzymes. This article involves studies of the production of AXE from *Aspergillus niger* and characterization of acetyl esterases not active against acetylated xylan.

MATERIALS AND METHODS

Preparation of Media

The basal medium consisted of 3.0 g/L $NaNO_3$, 1.0 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 0.1 g/L $FeSO_4 \cdot 7H_2O$, 0.2% Tween 80, 5.0 mL/L trace element solution, and 2.5 mL/L biotin solution (*6*). For inoculum, the basal medium was supplemented with 10.0 g/L sucrose and 1.0 g/L bactopeptone. Solid medium for Petri dishes contained, in addition, 2% agar.

For enzyme production, the bactopeptone was reduced to 0.1 g/L. Combinations of carbon sources that were investigated included 10.0 g/L of sucrose, 10.0 g/L of Solka-floc SW-40 (Fiber Sales and Development, Urban, OH), 10.0 g/L oat spelt xylan (Sigma Chemical Co., St. Louis, MO), or 10.0 g/L acetylated oat spelt xylan. Chemically acetylated oat spelt xylan was prepared according to Mitchell et al. (*6*).

Growth of *Aspergillus niger* to Produce AXE and Acetyl Esterase

Inoculum preparation involved sterilization of 200 mL of medium in 500-mL baffled shake flasks and inoculation with a loop of spores from a Petri dish of *Aspergillus niger* ATCC 10864. Enzyme production conditions were optimized using the Simplex method of parameter optimization (*9*) in 10 L of medium inoculated with log-phase mycelia in 14-L fermentation vessels. The medium combination that was found most effective in producing AXE activity contained: 1% Solka Floc, 1% oat spelt xylan, 1% sucrose 0.1% Bactopeptone, and mineral salts. Other optimum conditions included 33°C temperature, 15 L/min aeration, 300 rpm agitation, no pH control, and 48 h of time.

Assays for AXE and Acetyl Esterase Activities

The enzyme production characteristics were developed on the basis of the following assay for AXE. The AXE activity was measured using chemically acetylated xylan as substrate (*6*). A 1% (w/v) solution of the substrate in 0.4*M* phosphate buffer at pH 6.5 was used. The reaction mixture contained a 2:1 volume ratio of substrate solution and enzyme preparation. Incubation was for 1 h at 30°C. Following centrifugation of samples in a microfuge for 10 min, the supernatant was filtered through 0.45-μm nitrocellulose filters for HPLC analysis. The released acetic acid from the reaction was detected using a Bio-Rad (Hercules, CA) HPX-87H column maintained at 43°C. Samples were eluted with 0.02*N* H_2SO_4 at a flow rate of 0.6 mL/min. The resulting peaks for acetic acid were detected with a Waters R401 refractometer after a retention time of 16.0 min.

The enzyme purification characteristics were developed on the basis of the following assay for acetyl esterase. Acetyl esterase was assayed after dialysis using 0.0031*M* *p*-nitrophenyl acetate in 0.2*M* phosphate buffer at pH 6.5 and 30°C. A solution of 0.05 g of *p*-nitrophenyl acetate (Sigma N-8130) was dissolved in 30 mL ethanol. The above solution was added dropwise into 60 mL 0.2*M* phosphate buffer, pH 6.5. The absorption at A_{410} of this solution was approx 0.1. All blanks and samples were assayed by adding 0.3 mL of each to 3.0 mL of the substrate solution. Absorption at 410 nm was read at 20-s intervals over a 2-min period. The concentration of *p*-nitrophenol that is released by the enzymatic reaction was determined from Beer's Law using an extinction coefficient = 0.142 mL/μmol cm.

Stability of Acetyl Esterase

The stability of acetyl esterase was measured over the pH range of 4.0–7.5 in 0.2*M* phosphate buffers. After incubation of the 60–80% ammonium sulfate fraction at 4, 25, and 37°C for 0, 7, and 14 d, activity was assayed at pH 6.5 and 30°C using *p*-nitrophenyl acetate as substrate.

Purification of Acetyl Esterase

The 48-h fermentation broth was filtered through cheese cloth, and $(NH_4)_2SO_4$ was added to 60% of saturation. The mixture was centrifuged and the pellet discarded. After adding sufficient solid $(NH_4)_2SO_4$ to bring to 80% of saturation, the suspension was centrifuged and the pellet was saved. The pellet was dissolved in 1*M* $(NH_4)_2SO_4$ and applied to a Pharmacia phenyl-sepharose preparative FPLC column. The proteins were eluted with a descending gradient in $(NH_4)_2SO_4$. The fractions were dialyzed before assay using *p*-nitrophenyl acetate as substrate at pH 6.5 and 30°C. A sample of the phenyl-sepharose fraction with acetyl esterase activity was run on a Pharmacia Mono-Q column to demonstrate purity.

Kinetics of Acetyl Esterase

Following purification of the acetyl esterase, the pH-, temperature-, and substrate-activity profiles were determined at pH 6.5 in a thermostated double-beam spectrophotometer using 0.0031*M* *p*-nitrophenyl acetate as substrate. The assay procedure given above was adapted to accommodate changes necessary to conduct these experiments.

Molecular-Weight Determination of Acetyl Esterase

Analytical size-exclusion chromatography was performed on a Pharmacia Smart Chromatography system using a calibrated Pharmacia Superdex 75 column following procedures outlined by Himmel and Squire (*10*). A 12% homogeneous gel SDS PAGE of the purified acetyl esterase

was conducted using Novex wide-range protein standards from 2.5 to 200 kDa as markers.

N-Terminal Sequence Analysis of Acetyl Esterase

N-terminal sequence analysis was performed by Edman degradation using an Applied Biosystems (Foster City, CA) Gas Phase Sequencer model 470 and an on-line phenylisothiocyanate-amino acid analyzer following methods described by Applied Biosystems. Samples for amino acid analysis were dialyzed against distilled water, and hydrolyzed with constant boiling HCl in evacuated and sealed tubes at 110°C for 24, 48, and 72 h. After hydrolysis, the amino acids were derivitized with (dimethylamino)azobenzene-sulfonyl chloride (DABS) following the procedure of Knecht and Chang (*11*) and analyzed by reverse-phase HPLC. Cysteine was determined as cysteic acid after performic acid oxidation of the enzyme.

Carbohydrate Analysis of Acetyl Esterase

The quantification of carbohydrate in the glycoprotein is based on the amino acid analysis in which 20 μL of the sample (claimed to be 667 μg/mL protein) were analyzed, and the amount was 10.35 μg (equivalent to 581 μg/mL protein, not including Trp and Cys). To determine the amount of neutral and amino sugars, 93.2 μg of protein was analyzed. A 46.6-μg equivalent of hydrolysates was injected. The amounts of galactosamine and glucosamine were compensated for with hydrolysis recovery rates of 80 and 85%, respectively, based on the yields of the amino sugars in bovine fetuin.

RESULTS AND DISCUSSION

Fementation Profile

Fermentation broths obtained from all *A. niger* cultures de-esterified chemically acetylated xylan. However, the amount of activity varied with the carbon source. Cultures containing xylan, cellulose, and sucrose as the carbon sources yielded the most desirable enzyme activity profile with a maximum after 2 d of growth. The combination of xylan and sucrose did not produce much enzyme activity; cellulose could therefore be considered an inducer for the formation of AXE. Sucrose served as a readily available carbon source for cell growth.

Formation of AXE activity was studied under a variety of conditions of agitation, aeration, and temperature to establish an optimum. The results of a fermentation using the final set of optimization parameters are given in Fig. 1. Productivity was greatest using conditions of 300 rpm,

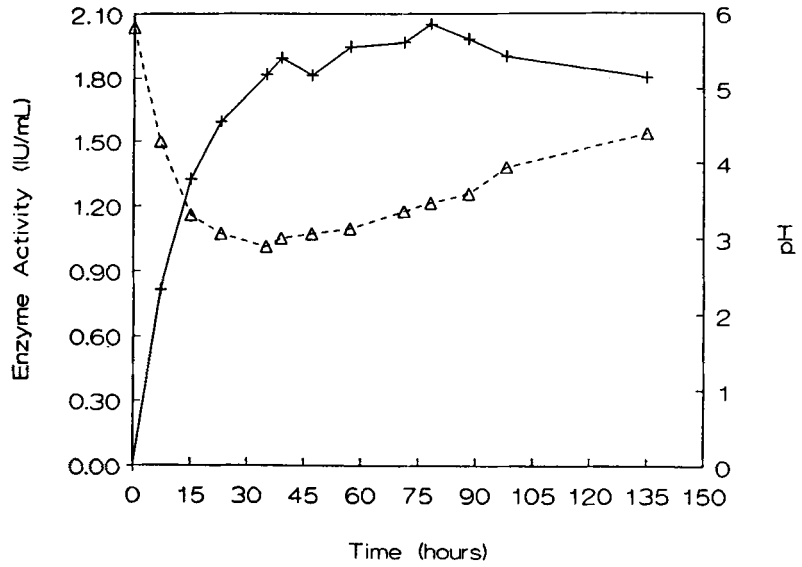


Fig. 1. Profile of pH (+) and acetyl esterase activity (△) vs time of fermentation.

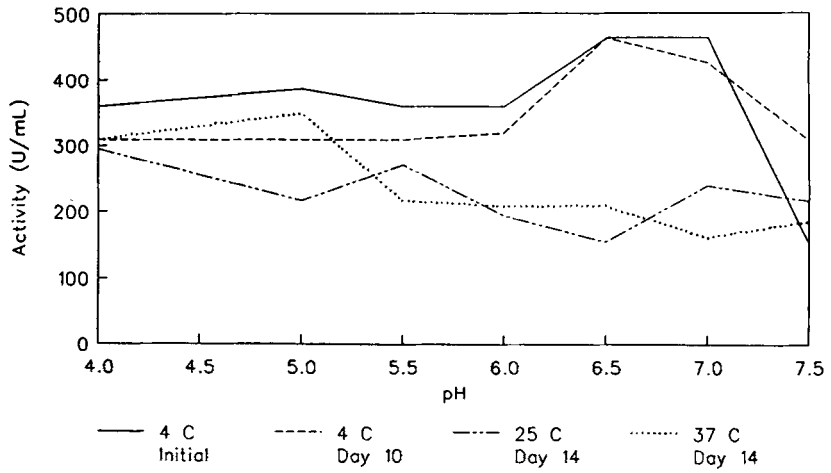


Fig. 2. Stability profile of acetyl esterase as functions of temperature and pH.

1.5 vvm, and 33°C. Enzyme formed only during the first 40 h after inoculation and appeared stable for another 100 h under the given fermentation conditions. Since the pH was not controlled in these fermentations, data in Fig. 1 also show that the AXE activity was stable at pH values between 3 and 4 for over 100 h at 33°C.

The stability of acetyl esterases was measured over a pH range of 4.0–7.5 at three temperatures over a period of 2 wk. The enzyme was assayed at pH 6.5 and 30°C. The data in Fig. 2 show that at temperatures >25°C, stability was superior at pH values <5.0. The enzyme production data presented above support this finding.

Table 1
Purification of Acetyl Esterase from *A. niger*

Fraction	Activity U/mL	Protein mg/mL	Specific activity U/mg	Purification factor
Broth	1.91	N.D.		
60% Pellet	32.54	0.69	47.16	
80% Pellet	395.07	0.50	790.14	1.00
Phenyl-Seph.	3603.52	0.64	5630.50	7.64

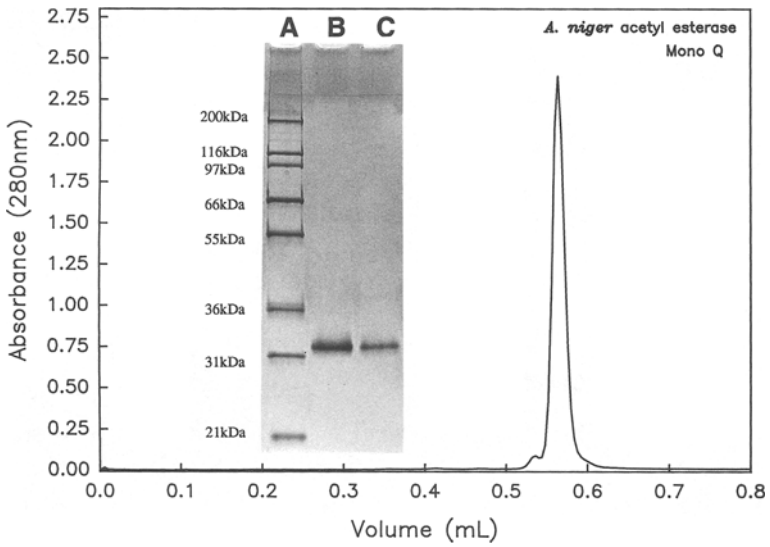


Fig. 3. Samples of acetyl esterase from Phenyl-Sepharose column run on Mono-Q column and denaturing gel electrophoresis (inset) to demonstrate enzyme purity. Lanes (a), and (b), and (c) were loaded with 20 μL of Novex MK12 mol-wt standards, 20 μg acetyl esterase, and 5 μg acetyl esterase, respectively.

Purification of Acetyl Esterase

Purification of acetyl esterase from the *A. niger* fermentation broths was accomplished in two steps—ammonium sulfate precipitation and hydrophobic interaction chromatography. Data in Table 1 show a 7.6-fold improvement in specific activity as a result of the phenyl-sepharose chromatography. A reliable protein concentration in the fermentation broth could not be developed; therefore, overall purification cannot be accessed.

A Pharmacia Mono-Q anion-exchange resin chromatogram, shown in Fig. 3, demonstrated virtually pure protein with acetyl esterase activity. The ratio of the 0.56 peak area to total peak area by the UV detector was 1.000. A Pharmacia Superdex gel filtration chromatography profile of the

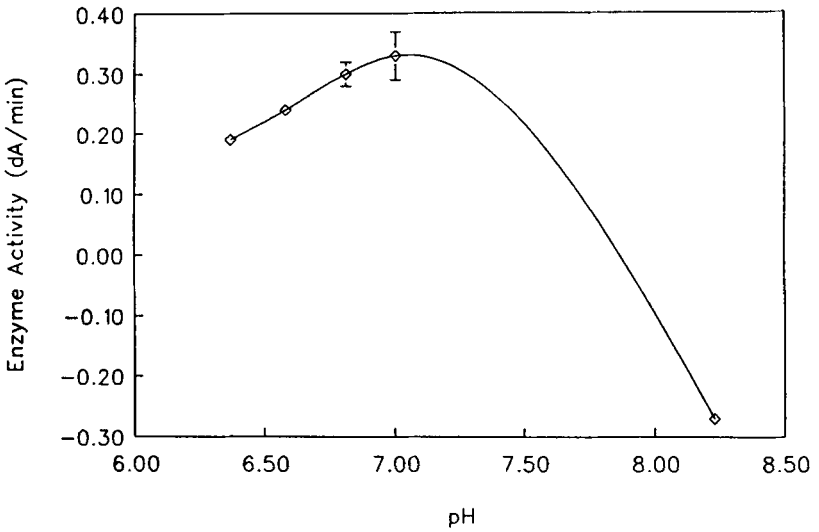


Fig. 4. Activity of acetyl esterase as a function of pH.

same material (not shown) had an acetyl esterase peak area to total peak area of 0.999. Denaturing gel electrophoresis (inset to Fig. 3) showed no contaminating bands, even in the overloaded lanes in which 20 μL of protein solution were applied.

Chemical Characterization of Acetyl Esterase

The mol wt of the *A. niger* acetyl esterase was determined by size-exclusion chromatography on the Pharmacia Superdex column to be 35,000 dalton. The migration of the acetyl esterase on the SDS polyacrylamide gel electrophoresis corresponded to that of marker proteins of a mol wt of 32,000 dalton.

The N-terminal amino acid sequence determined for the *A. niger* acetyl esterase was SGSLQGITDFGDNPTGVGMYIYVPNNLASNPGIVV-HHY. A computer search of the EMBL, GenBank, Swiss-Prot protein sequence data bases showed no significant homologies to this sequence.

The carbohydrate analysis of the *A. niger* acetyl esterase is given below in terms of *p*mol carbohydrate moiety/μg protein.

GalN	GlcN	Gal	Glc	Man
8.89	59.15	18.83	26.09	441.07

Kinetic Characterization of Acetyl Esterase

The relationship between acetyl esterase activity and pH was measured in a thermostated double-beam spectrophotometer at 35°C, using 0.0031*M* *p*-nitrophenyl acetate as substrate. The optimum pH was 7.0, as seen in Fig. 4. At pH values >7.0, the substrate became unstable and autohydrolyzed.

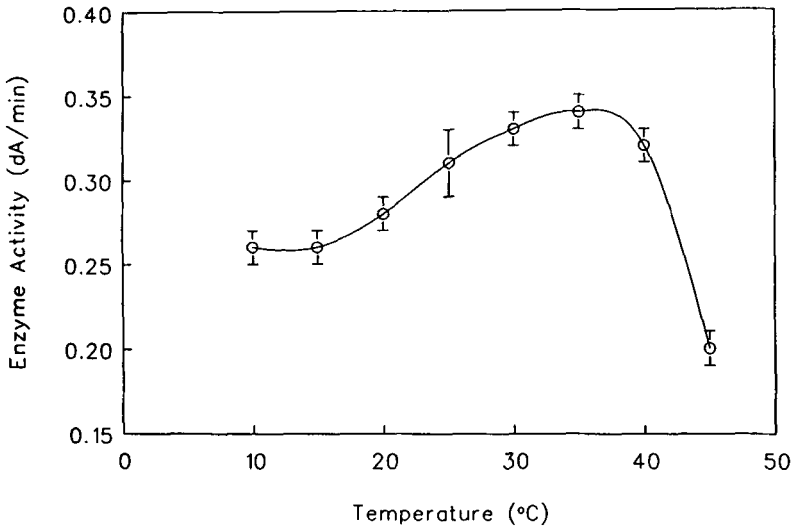


Fig. 5. Activity of acetyl esterase as a function of temperature.

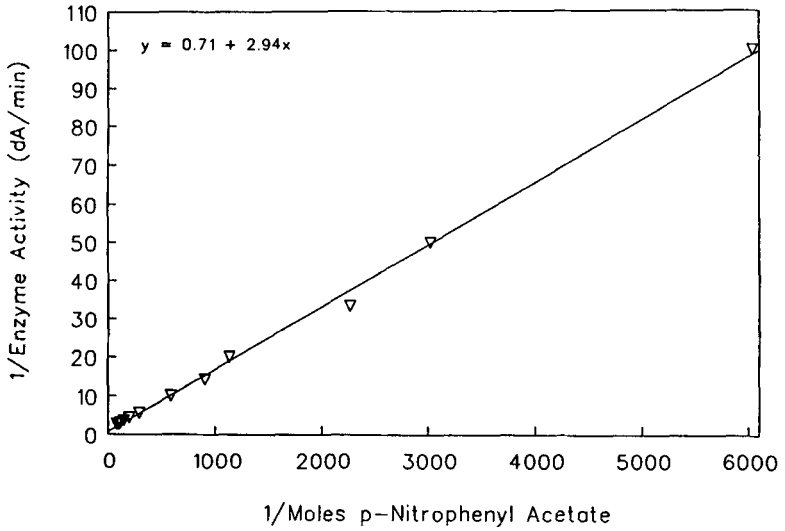


Fig. 6. Activity of acetyl esterase as a function of substrate concentration.

The optimum temperature for acetyl esterase was found to be 37°C. As seen in Fig. 5, the enzyme was unstable at higher temperatures.

The V_{max} was determined to be 46,700 U/mg protein, and the K_m was 0.023*M* *p*-nitrophenyl acetate at pH 6.5 in 0.2*M* phosphate buffer at 35°C. The Lineweaver-Burk plot of the data is shown in Fig. 6.

The acetyl esterase enzyme that has been characterized has been found to be different than acetyl-xylan esterases. When the purified enzyme was assayed using chemically acetylated oat spelt xylan as substrate, no evidence could be found for acetate in the incubation mixture.

CONCLUSIONS

The optimum fermentation parameters for AXE were established using an HPLC analysis of acetate hydrolyzed from acetylated xylan. The purification of AXE was the intent of this investigation. However, the ease of assay with the chromophoric substrate, relative to HPLC analysis of acetate led to the characterization of acetyl esterase instead of AXE.

The purification and characterization of an AXE from *A. niger* DS16813 had been reported by Kormelink et al. (*12*), which had a mol wt of 30,450 dalton, a pH optimum of 5.5–6.0, a temperature optimum of 50°C, and was stable at 45°C for at least 8 h. The enzyme characterization described in this article resulted in a mol wt of 35,000 dalton, a pH optimum of 7.0, and a temperature optimum of 35°C. These were clearly different enzyme activities from the same fungal species.

The stability of the acetyl esterase described here exhibited declining stability at 37°C over a 14-d period at the optimum pH for activity. Acetyl esterases from *A. awamori* and *Trichoderma reesei* were stable at 60–75°C, respectively, when assayed using α-naphthyl acetate as a substrate (*13,14*). The latter acetyl esterases also exhibited distinct specificity toward acetylated xylans, but none toward acetylated pectins.

ACKNOWLEDGMENTS

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REFERENCES

1. Chesson, A., Gordon, A. H., and Lomax, J. A. (1983), *J. Sci. Food Agric.* **34,** 1330–1340.
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. Biely, P., MacKenzie, C. R., and Schneider, H. (1988), *Can. J. Microbiol.* **34,** 767–772.
5. Johnson, K. G., MacKenzie, C. R., and Fontana, J. D. (1988), *Methods in Enzymol.* **160(68),** 551–560.
6. Mitchell, D. J., Grohmann, K., Himmel, M. E., Dale, B. E., and Schroeder, H. A. (1990), *J. Wood Chem. Technol.* **10,** 111–121.
7. Overend, R. P. and Johnson, K. G. (1991), in *Enzymes and Biomass Conversion,* Latham, G. F. and Himmel, M. E. eds., ACS Symposium Series 460, Washington, DC, pp. 270–287.

8. Poutanen, K., Tankanen, M., Korte, H., and Puls, J. (1991), in *Enzymes in Biomass Conversion,* Latham, G. F. and Himmel, M. E., eds., ACS Symposium Series 460, Washington, DC, pp. 426–436.
9. Hendrix, C. D. (1980), *Chemtech* **10,** 488–497.
10. Himmel, M. E. and Squire, P. G. (1981), *Int. J. Peptide Protein Res.* **17,** 365.
11. Knecht, R. and Chang, J.-Y. (1986), *Anal. Chem.* **58,** 2375–2379.
12. Kormelink, F. J. M., Lefebvre, B., Strozyk, F., and Voragen, A. G. J. (1993), *J. Biotechnol.* **27,** 267–282.
13. Sundburg, M., Poutanen, K., Markkanen, P., and Linko, M. (1990), *Biotechnol. Appl. Biochem.* **12,** 670–680.
14. Sundburg, M. and Poutanen, K. (1991), *Biotechnol. Appl. Biochem.* **13,** 1–11.