Purification and some characteristics of the acetylxylan esterase from *Schizophyllum commune*

Nora HALGAŠOVÁ, Eva KUTEJOVÁ and Jozef TIMKO*

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 842 51 Bratislava, Slovak Republic

Acetylxylan esterase from *Schizophyllum commune* was purified using ion-exchange and hydrophobic chromatography. The enzyme has a molecular mass of 31 kDa, as determined by SDS/PAGE, or 18 kDa, according to gel filtration. Glycosylation of the enzyme was not detected. Acetylxylan esterase is relatively stable under laboratory conditions; it retains full activity at pH 6.2–8.5 upon incubation at 25 °C for 7 h, but loses nearly the whole activity upon incubation at 60 °C for 30 min. The pH optimum of the enzyme activity is 7.7 and its temperature optimum lies between 30 and 45 °C. Ca^{2+} and Co^{2+} inhibit markedly the activity of acetylxylan esterase at a concentration of 10 mM, as do Mn^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} at a concentration of 1 mM.

INTRODUCTION

Acetylxylan esterases liberate acetic acid from acetylated xylan, which occurs as a major hemicellulose component of hardwood trees and annual plants. These enzymes also belong to the less known components of xylanolytic systems of many micro-organisms. A few acetylxylan esterases have been fully purified (Poutanen and Sundberg, 1988; McDermid et al., 1990; Bachmann and McCarthy, 1991; Sundberg and Poutanen, 1991; Tenkanen et al., 1991; Castanares et al., 1992), some others have been partially purified and characterized (Lee et al., 1987; Johnson et al., 1988a,b; Poutanen et al., 1990). A genomic DNA fragment from the extreme thermophile *Caldocellum saccharolyticum* coding for this enzyme has been cloned in *Escherichia coli* (Lüthi et al., 1990). Acetylxylan esterases are enzymes of different substrate specificities. In some micro-organisms, several enzymes with acetylxylan esterase activity are present, suggesting that every enzyme has a unique function and that different enzymes cleave different linkages. Therefore, the study of the function and effect of acetylxylan esterases in the degradation of hemicellulose is far from being accomplished.

The presence of acetylxylan esterases in fungal cellulolytic systems has been reported by Biely et al. (1985, 1987). These enzymes have been characterized as being relatively more active against acetylxylan than esterases from other sources and are produced in appreciable levels by the fungi (Biely et al., 1985, 1988b). The optimal conditions for production of acetylxylan esterase by *S. commune* and *Trichoderma reesei* and some aspects of its regulatory control have been presented by Biely et al. (1988a). Some new methods, which have been used in the study of partially purified acetylxylan esterase of *S. commune*, have been described by the same authors (Biely et al., 1988b).

As described above, the fungus *S. commune* serves as an important source of acetylxylan esterase activity. Moreover, this acetylxylan esterase seems to be not accompanied by major amounts of other esterases, i.e. the esterase system of *S. commune* belongs to the less complex ones. Therefore, it could be useful for the study of the mechanism of enzymic degradation of the hemicellulose. In this paper, the procedure for purification of the acetylxylan esterase from *S. commune* and some of the properties of the enzyme are described. In addition, a newly developed, simple, and inexpensive method for the assay of acetylxylan esterase activity is reported.

MATERIALS AND METHODS

Materials

Fully acetylated beechwood acetylxylan (degree of acetylation 2.0, i.e. 2 mol of acetyl groups per mol of anhydroxylose units) used for the acetylxylan esterase assay, and beechwood xylan used as a substrate for endo-1,4-β-xylanase were gifts from Dr. A. Ebringerová and Dr. P. Biely (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic). Partially acetylated larchwood acetylxylan, used for the comparative study of acetylxylan esterase activity, was prepared as described by Mitchell et al. (1990). The reaction conditions were chosen so that the degree of acetylation was about 1.2. All other chemicals were standard commercial products from Lachema (Brno, Czech Republic), Serva (Heidelberg, Germany), Sigma Chemical Co. (St Louis, Missouri, U.S.A.), ICN Biochemicals (Cleveland, Ohio, U.S.A.), Beta Lab (Surrey, U.K.) and Whatman Biochemicals Ltd. (Maidstone, U.K.).

Organism and growth conditions

Schizophyllum commune (ATCC 38548) was obtained from Dr. P. Biely. The inoculum was grown for 3 days at 30 °C in a 1.5% (w/v) malt extract broth (Desrochers et al., 1981) on a rotatory shaker at 150 cycles/min. Acetylxylan esterase production culture was grown for 10 days in 500 ml Erlenmayer flasks containing 60 ml of a medium optimized for cellulase production (Desrochers et al., 1981) under analogous conditions.

Enzyme purification

Culture supernatant was precipitated by addition of 2.5–3 vol. of cold (−18 °C) ethanol. After overnight settling, the precipitate was recovered by centrifugation at 2000 *g* for 20 min. The crude enzyme preparation was dissolved in 20 mM sodium phosphate buffer, pH 7.0, and clarified by centrifugation for 10 min at 6000 *g*. After having been passed through a bacterial filter, the filtrate was equilibrated with 20 mM sodium phosphate buffer, pH 7.0, by gel filtration on Sephadex G25-coarse (Pharmacia

* To whom correspondence should be addressed.

LKB, Uppsala, Sweden; 29 mm diam. × 200 mm long column) and applied to a 19 mm diam. × 170 mm long column of DEAE-Sephadex A-50 (Pharmacia LKB) in the same buffer. After removal of the unbound material, the column was eluted by a 500 ml 0–1 M linear NaCl gradient. Fractions of 5 ml were collected and assayed for protein and acetylxylan esterase, acetyl esterase and xylanase activities.

The fractions containing the acetylxylan esterase activity were pooled, dialysed against 20 mM sodium phosphate buffer, pH 7.0, and applied to a column (14 mm dia. × 90 mm long) of Q Sepharose Fast Flow (Pharmacia LKB). The column was eluted by 30 ml of 20 mM sodium phosphate buffer, pH 7.0, followed by 180 ml of a 0–1 M linear NaCl gradient. Fractions of 1.5 ml were collected and assayed for protein and acetylxylan esterase and acetyl esterase activities. The active fractions were pooled, dialysed against 1.7 M $(NH_4)_2SO_4$ in 40 mM sodium phosphate buffer, pH 7.0, and applied to the f.p.l.c. phenyl-Superose HR 5/5 column (Pharmacia LKB) equilibrated with the same buffer. After removal of the unbound material, the column was developed with a 30 ml 1.7–0 M linear $(NH_4)_2SO_4$ gradient in 40 mM sodium phosphate buffer, pH 7.0. The active fractions from the last chromatography were used for characterization of the acetylxylan esterase.

Enzyme assays

The acetyl esterase activity was assayed by the method of Johnson et al. (1988a) with some modification. Aliquots (100 μl) of the enzyme diluted in 20 mM sodium phosphate buffer, pH 7.0, were mixed with 800 μl of distilled water, and the enzyme reaction was started by adding 100 μl of 10 mM 4-nitrophenylacetate dissolved in dimethyl sulphoxide. After a 5 min incubation at 25 °C, the A_{420} was determined. One unit of the enzyme activity was defined as the amount of the enzyme that releases 1 μmol of 4-nitrophenol/min under the above assay conditions.

The activity of xylanase was assayed by the modification (Biely and Vršanská, 1988) of the method based on determination of the amount of reducing sugar liberated from xylan (Paleg, 1959).

For the assay of acetylxylan esterase activity, the fully acetylated acetylxylan was used as a substrate. This assay was based on the pH change resulting from the release of acetic acid from acetylxylan. Aliquots (500 μl) of the enzyme diluted in 20 mM sodium phosphate buffer, pH 7.0, were mixed with 4.4 ml of distilled water. The reaction was started by adding 100 μl of 5% (w/v) acetylxylan solution in dimethyl sulphoxide and the initial pH in the reaction mixture was recorded. After a 90 min incubation at 25 °C, the final pH was determined. Concentrations of the released acetic acid were obtained from the calibration curve prepared as follows. The pH of 0–2 mM acetic acid solutions in 2 mM sodium phosphate buffer with the pH previously adjusted to 7.0 was measured. The differences between the pH of 2 mM sodium phosphate buffer and the pH of the same buffer containing an increasing amount of acetic acid were plotted against the corresponding concentrations of acetic acid. One unit of the enzyme activity was defined as the amount of the enzyme that releases 1 μmol of acetic acid/min under the above assay conditions.

Protein determination

The protein elution profile after chromatography on the particular columns was estimated by measuring the A_{280} of the samples. Determination of the protein in the samples containing acetylxylan esterase activity, which has been used to calculate the specific activity of the purified protein, was performed by a Coomassie Brilliant Blue assay (Sedmak and Grossberg, 1977) using BSA as a standard.

SDS/PAGE

SDS/PAGE was performed according to Laemmli (1970) in 10% (w/v) polyacrylamide gels. The gels were stained with silver (Oakley et al., 1980). The molecular mass markers were obtained from Serva Heidelberg (Heidelberg, Germany).

Gel filtration on Superose 12

Superose 12 HR 10/30 with a Gel Filtration Calibration Kit – Low Molecular Weight (13700–67000 Da) (Pharmacia LKB, Sweden) were used for determination of the molecular mass of the acetylxylan esterase under standard conditions.

Endoglycosidase H treatment

To remove a possible N-linked carbohydrate, the enzyme was treated with Endoglycosidase H (endo-β-*N*-acetylglucosaminidase H of *Streptomyces plicatus*; Boehringer Mannheim, Germany) as recommended by Trimble and Maley (1984). Two enzyme samples (each containing 1 μg of the enzyme in a total volume of 20 μl) in 50 mM sodium citrate buffer, pH 5.0, and in 50 mM sodium phosphate buffer, pH 6.0, respectively, were boiled for 2 min in the presence of 0.02% (w/v) SDS. After cooling, 0.5 munits of Endoglycosidase H was added and the samples were incubated at 37 °C overnight. The product of Endoglycosidase H treatment was analysed by SDS/PAGE.

Temperature and pH optima and stability

In all of the determinations, the acetyl esterase activity was measured using 4-nitrophenylacetate as a substrate. For estimation of the temperature or pH optimum, the relative activity was determined at several temperatures or pH. To estimate the temperature or pH stability, the residual activity after the incubation of aliquots of the enzyme at different temperatures or pH was measured.

Effects of metal ions

The influence of metal ions on the enzyme was investigated by addition of different metal salts to a final concentration of 0.1, 0.5, 1.0 and 10 mM in the reaction mixture usually used for the determination of the acetyl esterase activity.

RESULTS

The acetylxylan esterase activity assay

The new method for the assay of acetylxylan esterase activity described above enables a simple and relatively fast determination of the enzyme activity in numerous fractions obtained from chromatographic columns. Although less sensitive than the acetyl esterase activity assay with the substrate 4-nitrophenylacetate, it was very useful for monitoring acetylxylan esterase activity during purification. As little as tens of micrograms of acetic acid in the reaction mixture can be determined.

The fully acetylated beechwood acetylxylan was a convenient substrate for the assay of acetylxylan esterase activity. Using the partially acetylated larchwood xylan as the substrate, the acetylxylan esterase activity was reduced by about 25%.

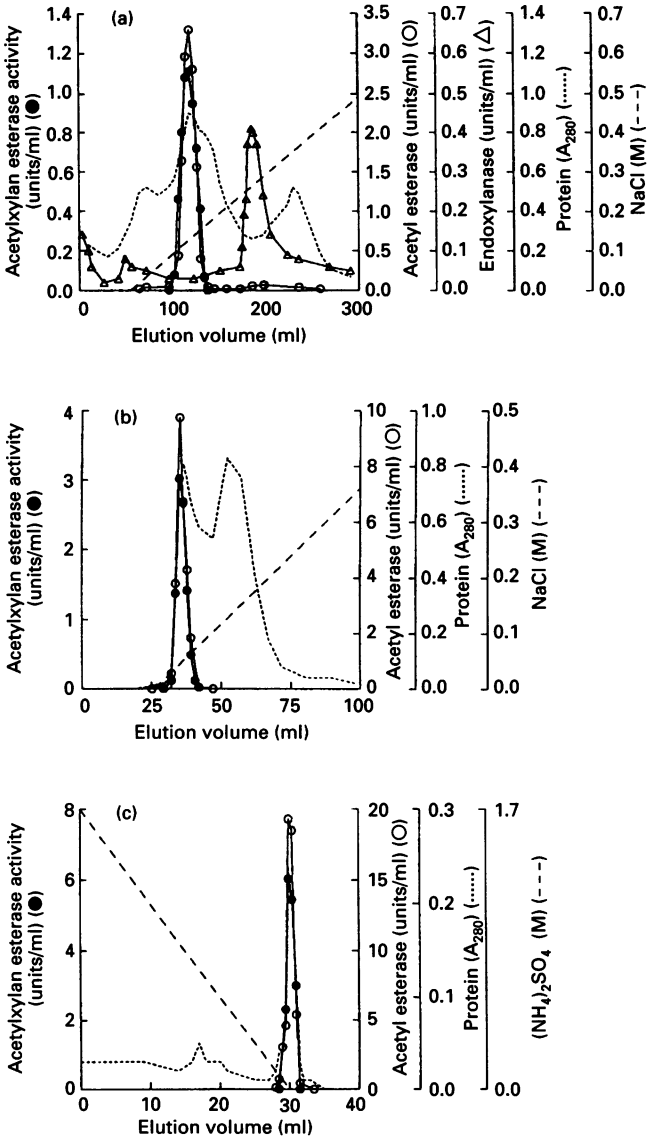

Figure 1 Chromatography of *S. commune* extracellular protein on the particular columns

(**a**) Anion-exchange chromatography on DEAE-Sephadex A 50. (**b**) Anion-exchange chromatography on Q Sepharose Fast Flow. (**c**) Hydrophobic chromatography on phenyl-Superose HR 5/5.

Purification of the acetylxylan esterase

In the first step of the acetylxylan esterase purification, i.e. ethanol precipitation of the culture supernatant, the *S. commune* extracellular protein was easily and conveniently concentrated and the total acetylxylan esterase activity was recovered with an increase in the enzyme specific activity.

The second purification (chromatography on DEAE-Sephadex A-50) resulted in a separation of the acetylxylan esterase activity from the xylanase activity. The latter activity was recovered as several species; the most prominent of them was not bound to the resin. In contrast, all the acetylxylan esterase activity was bound to DEAE-Sephadex and then eluted with 0.13 M NaCl (Figure 1a).

Further purification was carried out on a strong anion-exchanger, Q Sepharose Fast Flow column. Acetylxylan esterase was bound to the exchanger and eluted with 0.05 M NaCl (Figure 1b).

The final purification step was hydrophobic chromatography on the f.p.l.c. phenyl-Superose HR 5/5 column. The enzyme was bound very strongly to the column and was eluted in a single peak at the end of the descendent salt gradient (Figure 1c). The acetylxylan esterase obtained from the phenyl-Superose column was free of detectable xylanase activity. The purification procedure is summarized in Table 1.

Characterization of the acetylxylan esterase

The acetylxylan esterase fraction obtained from the phenyl-Superose column was used for characterization of the enzyme. The enzyme preparation could be frozen at −20 °C without loss of activity for at least 6 months.

The purity of the acetylxylan esterase prepared by the described method was verified by SDS/PAGE. The isolated enzyme gave in the electrophoresis a single protein band with a corresponding molecular mass of 31 kDa (Figure 2). The molecular mass of the enzyme as determined by gel filtration on Superose 12 was 18 kDa.

Treatment of the isolated acetylxylan esterase with Endoglycosidase H did not affect the behaviour of the enzyme during SDS/PAGE, indicating that N-glycosylation of the enzyme does not occur. Accordingly, the enzyme did not bind to concanavalin A–Sepharose. Thus, it appears likely that the enzyme is not glycosylated at all.

The pH optimum of the purified acetylxylan esterase is 7.7, and its temperature optimum lies between 30 and 45 °C (Figures 3a and 3b). At pH 6.2–8.5, the enzyme retained full activity for

Table 1 Purification of the acetylxylan esterase from *S. commune*

A representative isolation is evaluated except for the value designated *, which is calculated from several purification procedures. For the purification, 260 ml of the culture supernatant was used. Abbreviations: AXE, acetylxylan esterase; AE, acetyl esterase.

Purification step	Total protein (mg)	Total activity (units)		Specific activity (units/mg)		Yield (%)		Purification (fold)	
		AXE	AE	AXE	AE	AXE	AE	AXE	AE
Culture supernatant	52.0	31.8*	53.0	0.6	1.0	100	100	1.0	1.0
Ethanol precipitation	23.8	31.0	51.3	1.3	2.2	97.5	96.8	2.2	2.2
Sephadex G 25	22.2	30.6	50.3	1.4	2.3	96.2	94.9	2.3	2.3
DEAE-Sephadex A 50	5.3	18.6	49.3	3.5	9.3	58.5	93.0	5.8	9.3
Q Sepharose Fast Flow	1.5	10.6	29.1	7.1	19.4	33.3	54.9	11.8	19.4
Phenyl-Superose HR 5/5	0.1	2.3	5.9	23.1	59.2	7.2	11.1	38.5	59.2

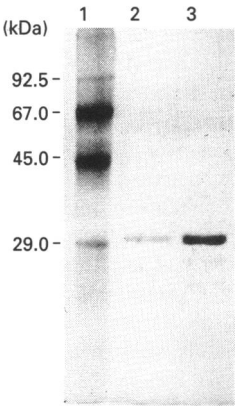

Figure 2 SDS/PAGE of purified acetylxylan esterase

Lane 1, high-molecular-mass standards; lanes 2 and 3, acetylxylan esterase preparation after phenyl-Superose HR 5/5 chromatography at two different loadings: 0.4 μg (2), and 1.3 μg (3). The gel was stained with silver.

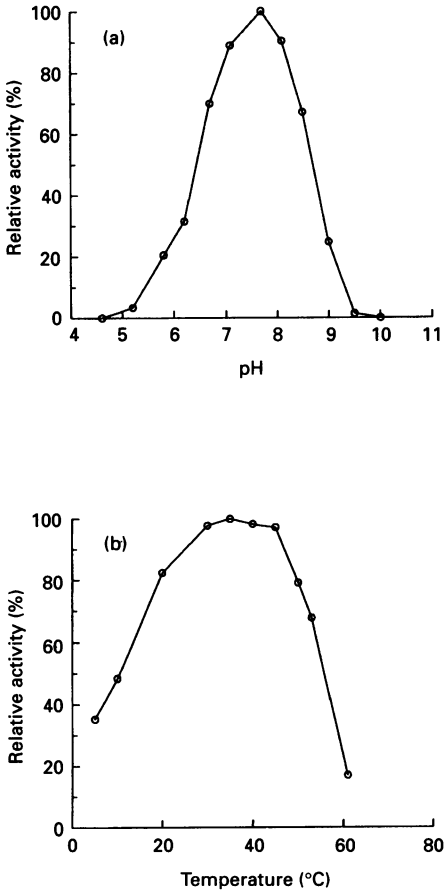

Figure 3 Effect of (a) pH and (b) temperature on the activity of the purified acetylxylan esterase

(**a**) Enzyme activities (expressed as percentage of highest value recorded) were measured in universal Britton–Robinson buffer I, pH 4.1–10.2 (0.04 M H_3PO_4, 0.04 M CH_3COOH, 0.04 M H_3BO_3, pH adjusted by addition of 0.2 M NaOH) at 25 °C. (**b**) The acetyl esterase activity assay was performed as described in the Materials and methods section at 5–61 °C. The concentrations of the enzyme in the incubation mixture were 0.02 unit/ml for both (**a**) and (**b**).

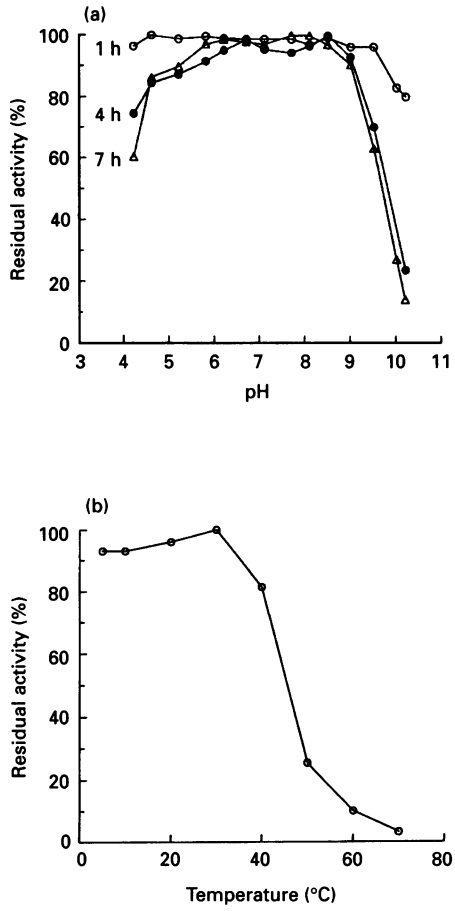

Figure 4 Effect of (a) pH and (b) temperature on the stability of the acetylxylan esterase

(**a**) The residual enzyme activity (expressed as percentage of activity before incubation) was determined after incubation of aliquots of the enzyme in Britton–Robinson buffer I, pH 4.1–10.2, at 25 °C for 1 (○), 4 (●) and 7 h (△), after readjustment of the pH. (**b**) The residual enzyme activity (expressed as percentage of activity before incubation) was determined after incubation of aliquots of enzyme in 20 mM sodium phosphate buffer, pH 7.0, at 5–70 °C for 30 min. The concentrations of the enzyme in the incubation mixture were 0.02 unit/ml for both (**a**) and (**b**).

Table 2 The effects of metal ions on the activity of acetylxylan esterase from *S. commune*

Enzyme activity is given as percentage of enzyme activity determined under the same assay conditions but in the absence of metal ion. The concentration of the enzyme in the incubation mixture was 0.02 unit/ml. n.d., enzyme activity not measured.

Concentration of metal ion (mM)	Enzyme activity (%)					
	Ca^{2+}	Co^{2+}	Mn^{2+}	Zn^{2+}	Cu^{2+}	Fe^{2+}
0.1	n.d.	96	99	90	83	82
0.5	n.d.	87	73	61	50	29
1	99	62	15	12	0	4
10	30	14	n.d.	n.d.	n.d.	n.d.

at least 7 h (Figure 4a), but it lost nearly all the activity after incubation for 30 min at 60 °C (Figure 4b).

Examination of the effect of metal ions on the acetylxylan esterase activity revealed the following features. All the metal

ions examined showed an inhibitory effect on the enzyme activity. Both Cu^{2+} and Fe^{2+} at a concentration of 0.1 mM inhibited the enzyme activity by about 17%. If Co^{2+}, Mn^{2+} or Zn^{2+} were used at the same concentration, the retained activity would be > 90%. Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} (1 mM) inhibited the enzyme by 85–100%. Ca^{2+} and Co^{2+} (10 mM) were necessary to inhibit markedly the activity of the enzyme. The effects of metal ions on the acetylxylan esterase activity are summarized in Table 2.

DISCUSSION

To cultivate *S. commune*, we used a medium optimized for cellulase production. The medium contained 1% (w/v) cellulose (Desrochers et al., 1981), which was found to be a very good inducer of acetylxylan esterase production (Biely et al., 1988a).

As reported by Biely et al. (1987, 1988a,b), the major acetyl esterase form in *S. commune* was found to be identical with that of acetylxylan esterase and it was detectable using both 4-nitrophenylacetate and acetylxylan as substrates. In another paper, activation effect of xylanase on the activity of acetylxylan esterase from *S. commune* has been described (Biely et al., 1986). Most probably, this effect is not manifested if the esterase activity is measured using the acetyl esterase substrate, 4-nitrophenylacetate. During the entire acetylxylan esterase purification, the peaks of acetylxylan esterase activity coincided with those of acetylesterase, indicating that the single enzyme is probably responsible for the two activities. After separation on the DEAE-Sephadex column, however, an unequal relative increase of the acetylxylan esterase and acetyl esterase specific activity was observed. One explanation could be that the separation of the xylanase from the acetylxylan esterase on the DEAE-Sephadex column removes the activation effect of xylanase upon the acetylxylan esterase activity.

This paper presents a new method for the determination of the acetylxylan esterase activity. The method is based on measuring pH changes that result from the release of acetic acid during enzymic catalysis. This technique has several advantages over the earlier described procedures employing h.p.l.c. or enzymic determination of the acetic acid, although it is less sensitive. It is simple, inexpensive, and relatively fast. The sensitivity of the method is approx. 10^4 times lower than that of the h.p.l.c. method. The measurements are highly reproducible except when the enzyme activity is assayed in culture supernatant or in the presence of metal ions. Therefore, the values of the total activity in the culture supernatant are only approximate. Obviously, the method mentioned cannot be used for determination of the enzyme pH optimum or pH stability.

The molecular mass of the acetylxylan esterase was 31 kDa, as determined by SDS/PAGE, and 18 kDa according to gel filtration. Discrepancies of nearly the same magnitude have been reported for acetylxylan esterases from *T. reesei* (Sundberg and Poutanen, 1991) and esterase from *A. oryzae* (Tenkanen et al., 1991). The difference could be explained as the result of retarded migration of the enzyme in gel chromatography as a result of some interaction with the gel matrix. Similar behaviour of some other xylanolytic enzymes has been observed (Faulds and Williamson, 1991; Gilbert et al., 1992).

An attempt to microsequence the acetylxylan esterase has revealed that the N-terminus of the enzyme is blocked.

The activity of acetyl esterase was reported to decrease with increase in the degree of acetylation of acetylxylan used as a substrate (Mitchell et al., 1990). The acetyl esterase activity of a commercial *T. reesei* cellulase preparation almost ceased when the degree of acetylation of the substrate was higher than 1.4. However, the acetylxylan esterase of *S. commune* degrades very rapidly the fully acetylated acetylxylan even in the absence of xylanase. This is a new phenomenon which has not been described previously and which seems likely to have interesting practical implications.

This work was supported by a research grant (2/999 136) from the Grant Agency of Slovak Academy of Sciences. We thank Dr. P. Biely for critical reading of the manuscript.

REFERENCES

Bachmann, S. L. and McCarthy, A. J. (1991) Appl. Environ. Microbiol. **57**, 2121–2130
Biely, P. and Vršanská, M. (1988) Methods Enzymol. **160**, 638–648
Biely, P., Puls, J. and Schneider, H. (1985) FEBS Lett. **186**, 80–84
Biely, P., MacKenzie, C. R., Puls, J. and Schneider, H. (1986) Bio./Technology **4**, 731–733
Biely, P., MacKenzie, C. R., Schneider, H. and Puls, J. (1987) in Wood and Cellulosics: The Role of Fungal Acetyl Xylan Esterases in the Degradation of Acetyl Xylan by Fungal Xylanases (Kennedy, J. F., Phillips, G. O. and Williams, P. A., eds.), pp. 283–289, Ellis Horwood, Chichester
Biely, P., MacKenzie, C. R. and Schneider, H. (1988a) Can. J. Microbiol. **34**, 767–772
Biely, P., MacKenzie, C. R., and Schneider, H. (1988b) Methods Enzymol. **160**, 700–707
Castanares, A., McCrae, S. I. and Wood, T. M. (1992) Enzyme Microb. Technol. **14**, 875–884
Desrochers, M., Jurasek, L. and Paice, M. G. (1981) Dev. Ind. Microbiol. **22**, 679–684
Faulds, C. B. and Williamson, G. (1991) J. Gen. Microbiol. **137**, 2339–2345
Gilbert, M., Breuil, C., Yaguchi, M. and Saddler, J. N. (1992) Appl. Biochem. Biotechnol. **34/35**, 247–259
Johnson, K. G., Fontana, J. D. and MacKenzie, C. R. (1988a) Methods Enzymol. **160**, 551–560
Johnson, K. G., Harrison, B. A., Schneider, H., MacKenzie, C. R. and Fontana, J. D. (1988b) Enzyme Microb. Technol. **10**, 403–409
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lee, H., To, R. J. B., Latta, R. K., Biely, P. and Schneider, H. (1987) Appl. Environ. Microbiol. **53**, 2831–2834
Lüthi, E., Jasmat, N. B. and Bergquist, P. L. (1990) Appl. Microbiol. Biotechnol. **34**, 214–219
McDermid, K. P., Forsberg, C. W. and MacKenzie, C. R. (1990) Appl. Environ. Microbiol. **56**, 3805–3810
Mitchell, D. J., Grohmann, K. and Himmel, M. E. (1990) J. Wood Chem. Technol. **10**, 111–121
Oakley, B. R., Kirsch, D. R. and Morris, N. R. (1980) Anal. Biochem. **105**, 361–363
Paleg, L. G. (1959) Anal. Chem. **31**, 1092–1094
Poutanen, K. and Sundberg, M. (1988) Appl. Microbiol. Biotechnol. **28**, 419–424
Poutanen, K., Sundberg, M., Korte, H. and Puls, J. (1990) Appl. Microbiol. Biotechnol. **33**, 506–510
Sedmak, J. J. and Grossberg, S. E. (1977) Anal. Biochem. **79**, 544–552
Sundberg, M. and Poutanen, K. (1991) Biotechnol. Appl. Biochem. **13**, 1–11
Tenkanen, M., Schuseil, J., Puls, J. and Poutanen, K. (1991) J. Biotechnol. **18**, 69–84
Trimble, R. B. and Maley, F. (1984) Anal. Biochem. **141**, 515–522

Received 13 July 1993/5 October 1993; accepted 12 October 1993