Pergamon

PII: S0031-9422(98)00359-8

Phytochemistry Vol. 49, No. 7, pp. 1867–1874, 1998

Printed in Great Britain
0031-9422/98/$ - see front matter

ESSENTIAL CARBOXYL RESIDUES IN THE ACTIVE SITE OF A XYLANASE FROM *TRICHODERMA LONGIBRACHIATUM*

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(*Received in revised form* 2 *April* 1998)

Key Word Index—*Trichoderma longibrachiatum*; Deuteromycotina; chemical modification; xylanase; carboxyl groups.

Abstract—Essential carboxyl residues were identified in the active site of an endo-β-1,4-xylanase isolated from *Trichoderma longibrachiatum*. Tryptophan residues were implicated in catalysis but were not essential for activity. Tyrosine residues were not involved in catalysis. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Hemicellulases potentially have many industrial applications. In the baking industry they may play a role in depolymerising wheat flour arabinoxylans, causing changes in the rheological properties of dough [1]. Other areas of application include the pulp and paper industry, where their use may replace harmful chloride-containing chemicals [2], as well as in fruit juice manufacture and in the production of animal feed with increased digestibility [3]. Xylanases have been purified from many species of bacteria and fungi. The bacterial xylanases characterised to date comprise several domains joined by linker sequences. Fungal enzymes, however, appear to be comprised of little more than a catalytic domain [4]. This results in a wide range of M_r values being reported for xylanases [4, 5]. A pattern can be observed, where enzymes fall into two groups: low-M_r (11,000–22,000 Da), high p*I* (8.3–10.0); and high-M_r (43,000–50,000), low p*I* (3.6–4.5); exceptions do, however, occur [5]. The enzymic hydrolysis of carbohydrates by xylanases is proposed to operate via a double displacement reaction, involving an acid catalyst and a nucleophile, with retention of anomeric configuration [6].

Various amino acids are essential for activity in xylanases from several species. A particularly well characterised fungal endoxylanase is xylanase A from *Schizophyllum commune* and carboxyl groups have proved to be essential for activity using chemical modification techniques [7]. Glu 87 was specifically identified as the nucleophile in the reaction by chemical modification followed by isolation of modified peptides which were then sequenced [8]. Carboxyl groups were implicated in xylanase from several species including *Trichoderma reesei* xylanase II [9], by sequence analysis. Chemical modification studies indicated an essential carboxyl group in an alkalothermophilic *Bacillus* species [10]. *Bacillus circulans* xylanase was studied by X-ray crystallography and by site-directed mutagenesis [11]. This study identified Glu 78 as the nucleophile and Glu 172 as the acid-base catalyst. Tyrosine residues are conserved in many xylanases and are essential for catalytic activity in xylanase A of *S. commune* [12]. Tryptophan residues are also important in the xynC xylanase from *Fibrobacter succinogenes* [13] and in the xylanases of several *Bacillus* species [14, 15].

RESULTS AND DISCUSSION

Chemical modification of carboxyl groups

The participation of carboxyl groups in xylanase enzyme activity was investigated using two water soluble carbodi-imides, which specifically modify carboxyl groups at pH 6.0. Incubation of enzyme solution with both reagents resulted in a loss of enzyme activity. 1-Ethyl-3-[3-(dimethyl amino) pro-

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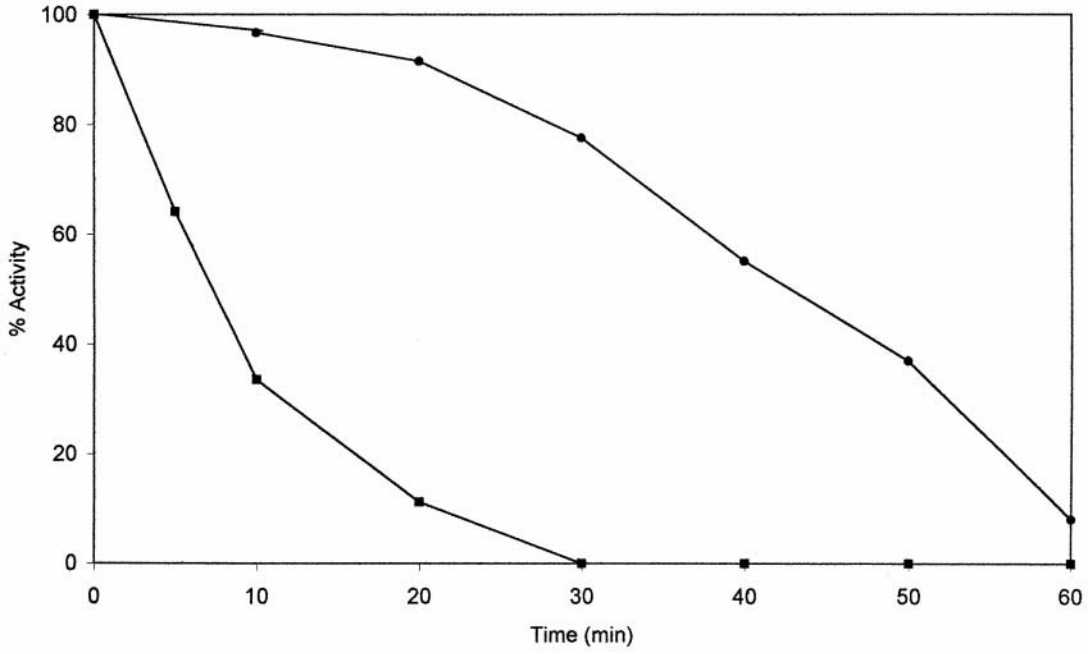

Fig. 1. Inactivation of xylanase by carbodiimides specific for carboxyl groups. Enzyme (0.5 μM) in 0.05 M MES/NaOH buffer, pH 6.0 was incubated with 0.05 M EDCI (1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide methiodide) (●) and 0.05 M EDAC (1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide) (■). Samples (20 μl) were removed from the reaction mixtures at indicated times and quenched in 0.05 M citrate–HCl buffer, pH 4.8, and assayed for residual activity.

pyl] carbodi-imide methiodide (EDCI) was the most effective reagent with enzyme activity decreasing more rapidly and approaching zero after 20 min. Inactivation with 1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide (EDAC) took longer with catalytic activity approaching zero after 80 min (Fig. 1). This had also been reported previously with xylanase A from *S. commune* [7]. A mechanism proposed by Chan *et al.* [16] suggested that a protonated carboxylic side chain is capable of catalysing its own modification by a carbodi-imide generating an *O*-acylisourea product. With EDCI this *O*-acyl product can undergo intramolecular rearrangement and be transformed to the stable *N*-acylurea derivative which is not susceptible to nucleophilic attack by water or added nucleophiles [17]. Coupled with this is the fact that EDCI has a linear structure in solution, which is unable to undergo a stabilising tautomerisation reaction, unlike EDAC, which is mostly present in a cyclic unreactive form [18]. This may explain the enhanced reactivity of EDCI as a modification reagent.

To determine the order of the inactivation reaction, enzyme was incubated with various concentrations of carbodi-imide. Concentrations of EDCI ranging between 2.5–30 mM were chosen. Plots of % initial activity against time showed characteristic inactivation curves (Fig. 2). Pseudo-first order rate constants for the inactivation reaction at each concentration of EDCI were determined from semi-log plots (data not shown). These data can be plotted according to the method of Levy *et al.* [19], which relates the rate constants of the inactivation reactions (k), at various reagent concentrations. A plot of log (k) vs log [carbodi-imide] has a slope equal to the average order of the inactivation reaction with respect to the reagent concentration. Figure 3 shows a Levy plot for various concentrations of EDCI. The data fits a straight line with a slope of 0.91 indicating that the enzyme is inactivated by reaction with at least one molecule of EDCI.

Adding a substrate, to bind to the active site, should shield any essential carboxyl groups from modification leading to a reduced rate of inactivation. The inactivation reactions were, therefore, performed in the presence of soluble xylo-oligomers, prepared from oat spelt xylan. As EDCI rapidly modified the enzyme, it was easier to observe protection effects with EDAC. In a reaction mixture with no substrate added, the enzyme activity was reduced to 50% within 17 min and to an undetectable level by 45 min by EDAC. The presence of substrate prevented this inactivation in a concentration dependent manner. The addition of 0.25% (w/v) soluble oat spelt xylan substrate protected the enzyme with activity remaining above 50% after 60 min incubation (Fig. 4). The highest concentration of the substrate protected the enzyme for longer and this is consistent with previous results [7]. The ability to protect the enzyme from inactivation can be plotted according to the method of Scrutton

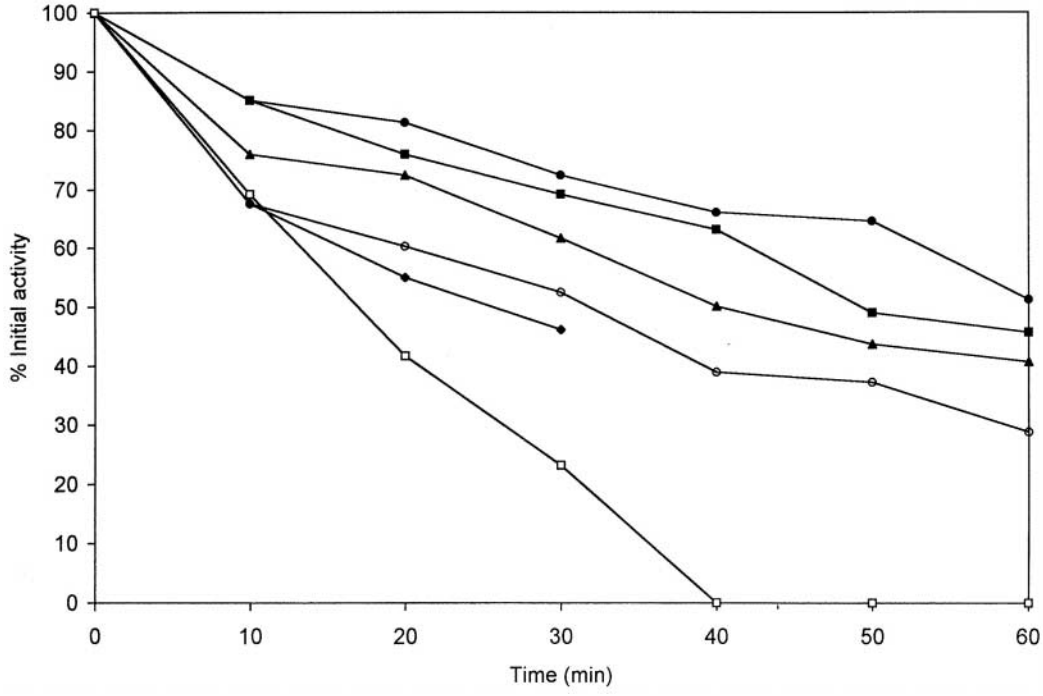

Fig. 2. Inactivation of xylanase with various concentrations of EDCI (1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide methiodide). Enzyme (0.5 μM) in 0.05 M MES/NaOH buffer, pH 6.0, was treated with EDCI. Final EDCI concentrations were 2.5 mM (●), 5.0 mM (■), 7.5 mM (▲), 10 mM (○), 20 mM (◆) and 30 mM (□). Samples (20 μl) were removed at indicated times and quenched in 0.05 M citrate–HCl buffer, pH 4.8, and assayed for residual activity.

and Utter [20]. This relates the concentration of substrate with the ratio of the rate constants obtained in the presence and absence of substrate (Fig. 5). The slope obtained can be extrapolated to the origin giving a ratio of zero, indicating that the enzyme-substrate complex cannot be inactivated

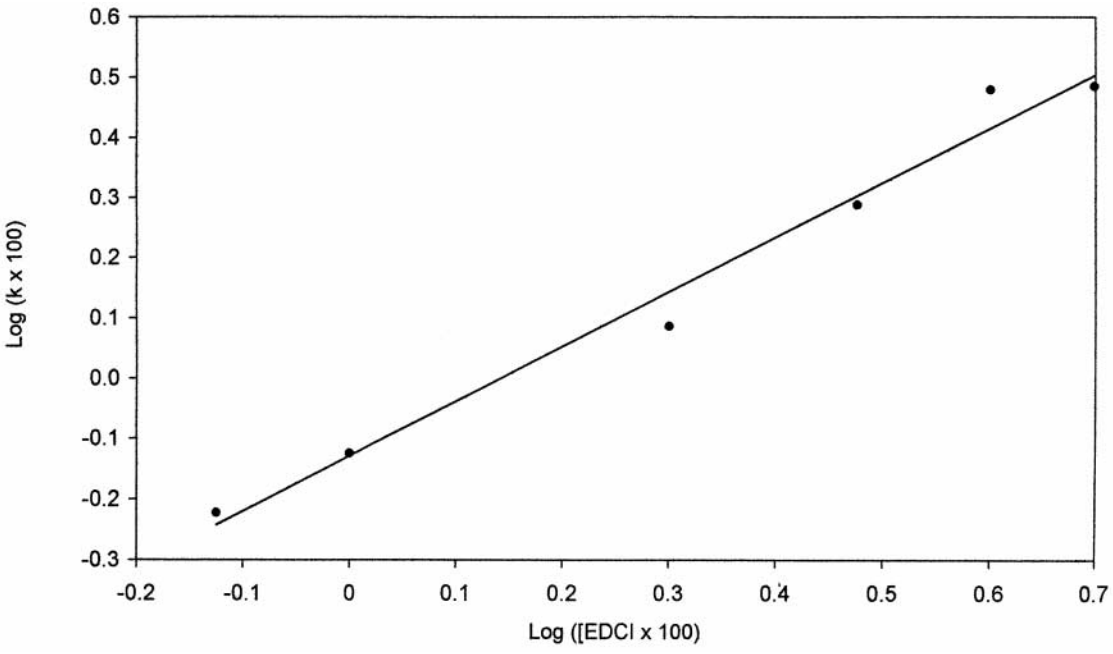

Fig. 3. Rate of inactivation of xylanase with various concentrations of EDCI (1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide methiodide). The rate of inactivation of xylanase with respect to EDCI concentration was plotted according to the method of Levy *et al.* [19].

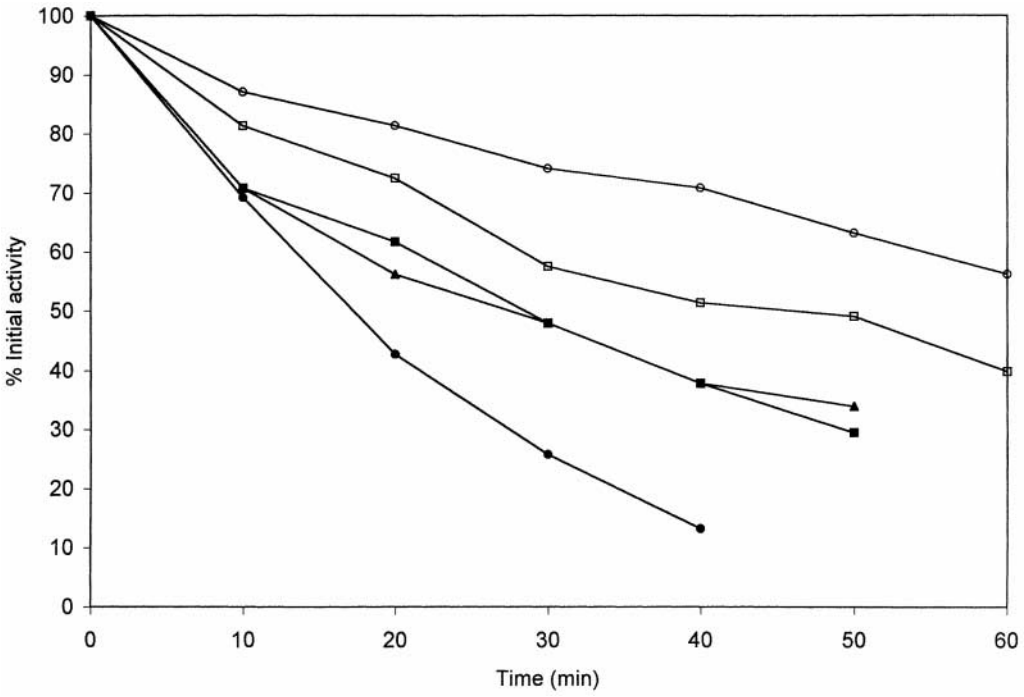

Fig. 4. Protection by soluble xylan solution of xylanase from modification by EDAC (1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide). Enzyme (0.5 μM) in 0.05 M MES/NaOH buffer pH 6.0 was treated with 0.1 M EDAC in the absence (●) and in the presence of 0.05% (■), 0.10% (▲), 0.125% (□) and 0.25% (○) (w/v) of soluble oat spelts xylan solution. At time intervals aliquots (20 μl) were removed and quenched in 50 mM citrate–HCl buffer, pH 4.8 and assayed for enzyme activity.

with modifying reagent. This suggests that the acidic amino acids which are necessary for enzyme activity are located at the active site.

The pH dependence of the inactivation was examined by performing the reaction over a range of pH values (Fig. 6). A reaction mixture of 0.5 μM

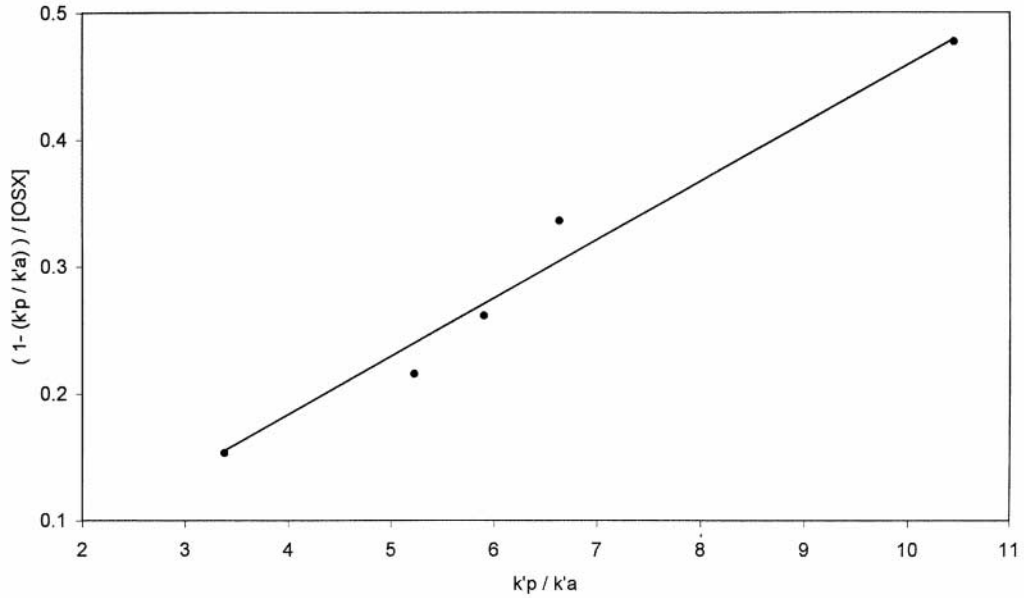

Fig. 5. Effect of soluble xylan solution on pseudo-first-order rate constants for the inactivation of xylanase by EDAC (1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide). The apparent pseudo-first-order rate constants obtained in the presence ($k'p$) and absence ($k'a$) of soluble oat spelts xylan ([OSX]) were plotted according to the method of Scrutton and Utter [20].

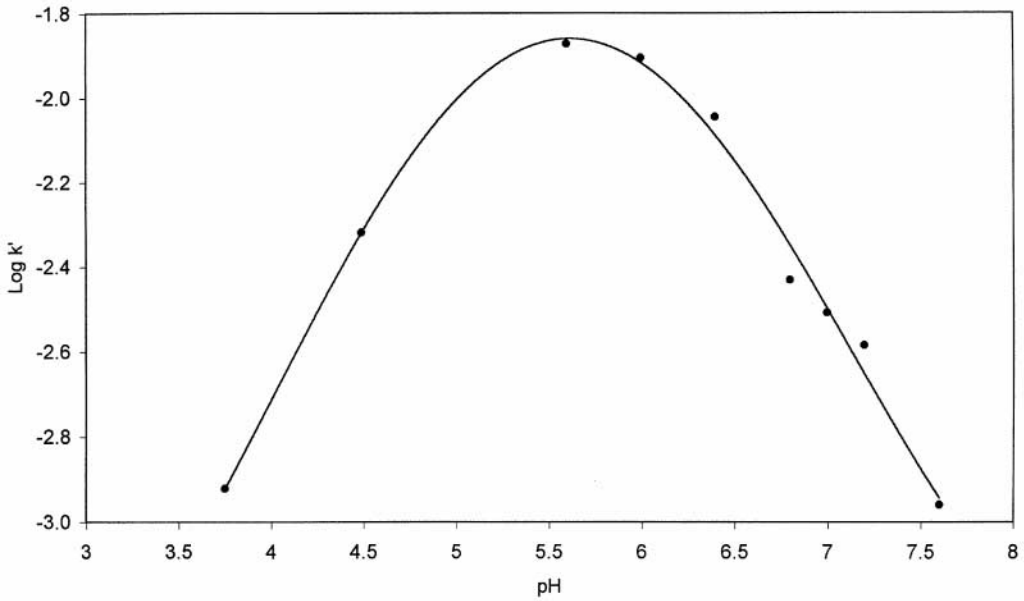

Fig. 6. Effect of pH on the pseudo-first-order rate constants (k') for the inactivation of xylanase by EDCI (1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide methiodide). Enzyme (0.5 μM) was incubated with EDCI (30 mM) at 25°C for 1 h over a range of pH. Buffers chosen were 0.05 M citrate–HCl, pH 3.5–5.5, 0.05 M MES/NaOH, pH 5.5–6.5 and 0.05 M MOPS/NaOH, pH 6.8–8.0. Values for k' were determined from linear plots of log enzyme activity vs time.

enzyme and 30 mM EDCI was incubated for 1 h at 25°. The Dixon plot [21] of log k vs pH was bell shaped with maximum inactivation occurring between pH 5.0 and 6.5, and indicated the presence of two ionisable groups with pK_a values of 5.1 and 6.2. Bray and Clarke [7] reported similar results for xylanase A of *S. commune*, with two ionisable groups of 5.2 and 6.6; in addition they have also reported the presence of one essential glutamate residue in the active site [8]. The pH profile of a xylanase from an alkalothermophilic *Bacillus* species showed apparent pK values of 5.2 and 6.4 for the free enzyme and 4.9 and 6.9 for an enzyme-substrate complex [10]. Davoodi *et al.* [22] report an active site glutamic acid residue with a high pK_a of 6.8 in a *Bacillus circulans* xylanase due to electrostatic interactions with another adjacent glutamic acid.

Chemical modification of tyrosine residues

Two modifying agents were used to examine the involvement of tyrosine residues, *N*-acetyl imidazole (NAI) and tetra-nitromethane (TNM). NAI converts a tyrosyl residue into an *O*-acetyl-tyrosine derivative and TNM nitrates tyrosine. Incubation of NAI with enzyme resulted in a loss of activity to 50%. A 6 μM enzyme solution was incubated with 20 mM and 40 mM concentrations of TNM, the rise in absorbance at 428 nm was measured and the enzyme activity assayed. The initial A_{428} was 0.025 which increased to 0.072 and 0.121 with the addition of 20 and 40 mM TNM respectively. Using ε_{428} of 4100 $M^{-1}\,cm^{-1}$ for 3-nitrotyrosine this equates with the modification of 3 and 5 tyrosine residues respectively. During the incubation however, the enzyme activity was unaffected. These data suggest that tyrosine residues are not essential for activity. NAI is polar and preferentially modifies exposed surface tyrosine residues and may therefore not modify the essential residues buried within the enzyme to abolish activity completely. The modification of exposed tyrosine residues may cause steric hinderance or conformational changes that impede the approach of substrate. Tyrosine residues are conserved in many xylanases and are essential for catalytic activity in xylanase A of *S. commune* [12].

Chemical modification of tryptophan

N-Bromo succinimide (NBS) was used to investigate whether any catalytically essential tryptophan residues reside in the enzyme active site. Tryptophan, which contains an indole ring absorbing strongly at a wavelength of 280 nm, is converted by NBS to oxindole with a resultant decrease in absorbance [23]. The absorbance decrease observed upon treatment of enzyme with NBS indicated the modification of one tryptophan residue. Samples were removed every time an aliquot of NBS was added and assayed for catalytic activity. There was no significant decrease in enzyme activity, after the addition of an NBS concentration 40 times greater than enzyme concentration. This suggests that the tryptophan residue is not essential for the activity of the enzyme. However, Bandivadekar and Deshpande [24] investigated a low-M_r weight xylanase from *Chainia* species which contained three

tryptophan residues one of which proved to be essential for activity, as shown by fluorimetric analysis. Tryptophan residues have also been found to be important in the xynC xylanase from *Fibrobacter succinogenes* [13] and in the xylanases of several *Bacillus* species [14, 15].

Enzyme kinetics

K_m values were obtained for unmodified, carboxyl-modified and tyrosyl-modified enzymes (data not shown). The K_m value obtained for the unmodified enzyme was 0.7 mg/ml of soluble oats spelts xylan. The carboxyl modified enzyme was reduced to 55% activity and the K_m was determined to be 12.02 mg/ml soluble oats spelts xylan. The tyrosyl modified enzyme was reduced to 40% activity after prolonged incubation and the K_m was determined to be 38.2 mg ml^{-1} soluble oats spelts xylan. If tyrosyl residues are used for the binding of carbohydrates then an increase in K_m would be expected. The increase in K_m for the tyrosyl modified enzyme was approximately 5 times that for the carboxyl modified. The increase in K_m for the carboxyl modified enzyme is probably due to the decreased value of k_{cat} in the macroscopic constant, K_m.

It is of particular interest that tyrosine and tryptophan residues were not found to be essential in this study. These residues are frequently found in the binding site of carbohydrate-binding proteins [25] and have been found in this capacity in other xylanases. It would appear from accumulating evidence that whilst the catalytic mechanism of the xylanases follows the hen egg white lysozyme paradigm, the residues involved in substrate binding vary from enzyme to enzyme.

EXPERIMENTAL

Analytical methods

Xylanase activity was measured routinely by incubating a suitably diluted aliquot of enzyme soln with an equal vol of 1% (w/v) soluble oat spelt xylan soln. This was prepared by dissolving oat spelt xylan in 0.05 M MES/NaOH buffer, pH 6.0, for 2 h. This was then centrifuged (1500 g) in a bench top centrifuge for 5 min to remove insoluble material. The supernatant was the soluble oat spelts xylan fraction. This reaction mixture was incubated for 30 min at 55° [26]. The concentration of reducing sugars formed was determined using the method of Somogyi [27]. Activity was expressed as μmol of reducing sugar released per min per ml of undiluted enzyme (units ml^{-1}) using xylose as standard.

Growth of organism and enzyme production

Trichoderma longibrachiatum was an unnumbered wild type strain isolated from paper sludge, a gift from Dr Nakas of the College of Environmental Science and Forestry, SUNY. *T. longibrachiatum* was maintained on potato dextrose agar slopes at 4° and spores were produced by culturing the fungus on agar plates containing Vogel salts at 25° [28]. Spores were removed into sterile water and filtered through sterile miracloth (Calbiochem) to remove mycelia and agar. A spore concentration of 10^5 spores per ml was used to inoculate 500 ml Erlenmeyer flasks containing 100 ml of Vogel medium and 1% (w/v) α-cellulose, pH 7.0 according to the method of Royer and Nakas [26]. The flasks were shaken at 28° for 5 days at 150 rpm. The supernatant after centrifugation was the crude enzyme preparation.

Enzyme purification

All procedures were performed at 4°. The contents of the shake flasks were centrifuged at 12,000*g* for 20 min and protein precipitated from the crude supernatant using ammonium sulphate (20–80% saturation). The xylanase-rich precipitate was dissolved in 0.1 M sodium acetate buffer, pH 5.0, and was applied to a Bio-Gel P-60 (fine) gel filtration column (2.5 × 100 cm) equilibrated with the same buffer with a flow rate of 15 ml h^{-1}. Fractions (5 ml) were collected, then assayed for enzyme activity and those with activity were pooled and freeze dried. Several fractions contained xylanase activity. However, only fraction III showed a single band of M_r 24 kDa when subjected to SDS–PAGE and further analysis by MALDI-TOF estimated the molecular weight to be 23,776 Da. This fraction was used for the modification studies.

Modification by carboxyl group specific reagents

Water-soluble carbodi-imides specifically modify carboxyl groups and 1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide (EDAC) and 1-ethyl-3-[3-(dimethyl amino) propyl] carbodi-imide methiodide (EDCI) were obtained from Sigma (Poole, Dorset) and their inhibitory action was investigated by incubating the reagent (various final concentrations) with lyophilised enzyme in 0.05 M MES/NaOH, pH 6.0 at 20°C for approximately 60 min. At appropriate intervals 50 μl aliquots were removed and diluted with 200 μl of 0.05 M citrate–HCl buffer, pH 4.8. The enzyme activity was measured as described above.

The reaction with EDCI and EDAC was further studied by varying the concentrations of EDCI (2.5–30 mM) and EDAC (25–125 mM). To assess the ability of a soluble oat spelt xylan fraction to protect against EDCI modification, the enzyme was incubated with EDCI in the presence of various concentrations of a soluble oat spelt xylan preparation. The pH dependence of EDCI modification was examined by incubating 50 μM enzyme with EDCI (30 mM) over a range of pH. Buffers chosen

were 0.05 M citrate–HCl pH 3.5–5.0, 0.05 M MES/NaOH pH 5.5–6.5 and 0.05 M MOPS/NaOH pH 6.8–8.0. At time intervals 50 μl aliquots were removed and diluted in 200 μl of 50 mM citrate–HCl buffer, pH 4.8 and assayed for catalytic activity.

Chemical modification of tyrosine

N-Acetyl imidazole (NAI) was employed to modify tyrosine residues into *O*-acetyl-tyrosine derivatives as described by Bray and Clarke [12]. A 600 μl aliquot of a 14 μM enzyme solution in 10 mM potassium phosphate buffer was placed in a microcentrifuge tube and dry NAI was added to give a final concentration of 60 mM and incubated at 20° for 120 min. At time intervals 10 μl aliquots were removed and diluted into 50 μl of 100 mM sodium acetate buffer, pH 5.0, and assayed for enzyme activity.

The tyrosine modifying reagent tetranitromethane (TNM) was also used to nitrate tyrosine residues as described by Sokolovsky *et al*. [29]. A 700 μl aliquot of a 6 μM enzyme sol in 100 mM Tris–HCl buffer, pH 8.0, was placed in a cuvette with 17.5 μl of a stock sol of TNM (20–40 mM) and incubated at 25° for 200 min. The number of modified tyrosine residues was estimated spectrophotometrically using the ε_{428} value of 4100 M^{-1} cm^{-1} for 3-nitrotyrosine. At certain time intervals, 10 μl aliquots of reaction mixture were removed, diluted into 50 μl of 100 mM sodium acetate buffer, pH 5.0, and assayed for residual enzyme activity as described.

Chemical modification of tryptophan

The tryptophan modifying reagent *N*-bromosuccinimide (NBS) was used to assess the possible role of tryptophan residues in enzyme activity. NBS oxidises the 3-methylindole group of tryptophan to 3-methyloxindole. A 1 ml volume of a 14 μM enzyme sol in 25 mM sodium acetate buffer, pH 4.8, was titrated with 20 μl aliquots of 0.5 mM NBS (freshly made up in the same buffer). At each titration point a 20 μl aliquot was removed and quenched in 80 μl of 15 mM L-tryptophan in 50 mM sodium acetate buffer, pH 6.0. The number of tryptophan residues oxidised was calculated using the normal absorbance methods of Spande and Witcop [23].

Enzyme kinetics

K_m and V_{max} were determined experimentally for unmodified and carboxyl and tyrosyl modified enzyme sols. A 1 ml sol of enzyme (2.3 μg ml^{-1}) was incubated at 55° with various concentrations (0.25–30 mg ml^{-1}) of soluble oat spelts xylan solution in 0.1 M sodium acetate buffer, pH 5.0. At time intervals 200 μl were removed and assayed for reducing sugar concentration using a modification of the Somogyi–Nelson method. Reagent I was modified by the addition of 2.5 mg of xylose per 100 ml. The initial velocity of the reaction was determined by linear regression from the linear portion of the slope of the graph of reducing sugar formation against time. Hanes plots were used to determine K_m and V_{max}, and the *y*-axis intercept was determined by linear regression.

The carboxyl groups in a 1 ml sol of enzyme (32 μg ml^{-1}) were modified using EDAC as described above until the activity was approximately 50%. The modified enzyme (1 ml) was incubated at 55° with 1 ml of various concentrations (2–40 mg ml^{-1}) of soluble oats spelt xylan in 0.1 M sodium acetate buffer, pH 5.0, and at time intervals 200 μl was removed and assayed for reducing sugar concentration. Modification of tyrosyl groups was performed as described above by the addition of 2.7 mg dry NAI to 1 ml of enzyme (42 μg ml^{-1}) in 10 mM potassium phosphate buffer, pH 6.5, until the activity was approximately 50%. The modified enzyme (1 ml) was incubated at 55° with 1 ml of various concentrations (5–30 mg ml^{-1}) of soluble oats spelt xylan in 0.1 M sodium acetate buffer, pH 5.0, and at time intervals 200 μl was removed and assayed for reducing sugar concentration.

Acknowledgements—This work was funded by the Biotechnology and Biological Sciences Research Council, with additional support from RHM Research and Engineering Ltd.

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