Essential carboxy groups in xylanase A

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An endo-1,4- β -xylanase of *Schizophyllum commune* was purified to homogeneity through a modified procedure employing DEAE-Sepharose CL-6B and gel-filtration chromatography on Sephadex G-50. The role of carboxy groups in the catalytic mechanism was delineated through chemical modification studies. The water-soluble carbodi-imide 1-(4-azonia-4,4-dimethylpentyl)-3-ethylcarbodi-imide iodide (EAC) inactivated the xylanase rapidly and completely in a pseudo-first-order process. Other carbodi-imides and Woodward's Reagent K were less effective in decreasing enzymic activity. Significant protection of the enzyme against EAC inactivation was provided by a mixture of neutral xylo-oligomers. The pH-dependence of the EAC inactivation revealed the presence of a critical ionizable group with a pK_a value of 6.6 in the active site of the xylanase. Treatment of the enzyme with diethyl pyrocarbonate resulted in modification of all three histidine residues in the enzyme with 100% retention of original enzymic activity. Titration of the enzyme with 5,5-dithiobis-(2-nitrobenzoic acid) and treatment with iodoacetamide and *p*-chloromercuribenzoate indicated the absence of free/reactive thiol groups. Reaction of the xylanase with tetranitromethane did not result in a significant activity loss as a result of modification of tyrosine residues.

INTRODUCTION

Hemicellulose, a complex macromolecule found in close association with cellulose in plant material, is composed largely of the highly variable polysaccharide xylan, a β -1,4-linked polymer of xylosyl residues with branched substituents (Timmell, 1964). Bacteria and fungi capable of degrading xylan synthesize xylanolytic systems of enzymes that act synergistically to achieve its hydrolysis (Dekker & Richards, 1976). Predominant enzymes within these systems are the endo-1,4- β -xylanases (endo-1,4- β -D-xylan xylanohydrolase, EC 3.2.1.8), which randomly attack internal xylosidic linkages on the xylan backbone. Despite the intense interest in the conversion of cellulosic and hemicellulosic biomass into fermentable products by using these enzymes, and thereby in their vast industrial potential, little information exists as to their mechanism of catalysis. Tavobilov *et al.* (1981) have presented kinetic data implying the presence of an ionizable group of pK_a 4.5 in the active site of an *Aspergillus niger* endoxylanase, and suggested that it may be a carboxy group. Tryptophan and histidine have been shown to play catalytic roles in a *Streptomyces* endoxylanase (Keskar *et al.*, 1989). Analogous endohydrolases, the cellulases, have been investigated more intensively (Hurst *et al.*, 1977; Yaguchi *et al.*, 1983; Paice *et al.*, 1984; Clarke & Yaguchi, 1984; Høj *et al.*, 1989), and roles for carboxy groups in catalysis by these enzymes have been proposed. These data, as well as sequence homology studies (Yaguchi *et al.*, 1983; Paice *et al.*, 1984; Morosoli *et al.*, 1986; Moranelli *et al.*, 1986), have supported the proposal (Banks & Vernon, 1963) that all glycosidases share a common mechanism with hen egg-white lysozyme.

The endo-1,4- β -xylanase of the basidiomycete *Schizophyllum commune*, designated xylanase A, has been previously isolated and purified, and is an enzyme of exceptionally high activity (Váradi *et al.*, 1971; Paice *et al.*, 1978). The pH optimum of xylanase A activity has been determined to be around 5.0 and the pH-activity profile is bell-shaped (Paice *et al.*, 1978). These observations imply the presence of two ionizable groups essential for catalysis by the enzyme, one with an acidic pK_a and a second

with a pK_a value closer to neutrality. Chemical modification, kinetic and stoichiometric experiments on xylanase A described in the present study provide the first conclusive evidence for the involvement of carboxy groups in the mechanism of catalysis of an endo-1,4- β -xylanase, supporting the analogy of a lysozyme-type catalytic mechanism among this family of enzymes.

MATERIALS AND METHODS

Materials

Concanavalin A-Sepharose, *p*-chloromercuribenzoate, 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide hydrochloride (CMC), diethyl pyrocarbonate, 5,5'-dithiobis-(2-nitrobenzoic acid), 1-[3-(diethylamino)propyl]-3-ethylcarbodi-imide (EDC), iodoacetamide, oat spelts xylan, Woodward's Reagent K (2-ethyl-5-phenylisoxazolium-3-sulphonate) (WRK), Mes, Tris, Mops, 4-hydroxybiphenyl, D-xylose, methylamine and tetranitromethane were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Iodomethane was provided by Anachemia Chemicals (Toronto, Ontario, Canada), and $Na_2B_4O_7$ was obtained from BDH Chemicals (Toronto, Ontario, Canada). Sephadex G-50 (superfine grade) and DEAE-Sepharose CL-6B were products of Pharmacia-LKB (Montreal, Quebec, Canada). Bio-Rad Laboratories (Richmond, CA, U.S.A.) supplied Bio-Gel P-60, Bio-Gel P-6-DG, AG-1 X8 (100–200 mesh; Cl^- form), acrylamide, SDS, molecular-mass markers, *NNN'*-tetramethylethylenediamine and methylenebisacrylamide. [3H]Methyl iodide was a product of Amersham Canada (Oakville, Ontario, Canada). 1-(4-Azonia-4,4-dimethylpentyl)-3-ethylcarbodi-imide iodide (EAC) was synthesized from the free base of EDC and iodomethane by the method of Sheehan *et al.* (1961) (m.p. 93.5–94.5 °C) and stored in a vacuum desiccator. All other chemicals used were purchased from Fisher Scientific (Toronto, Ontario, Canada) and were of analytical grade.

Analytical methods

Xylanase activity was routinely measured by incubating 50 μ l of an enzyme solution with 300 μ l of 0.5% oat spelts xylan

Abbreviations used: CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide hydrochloride; EDC, 1-[3-(diethylamino)propyl]-3-ethylcarbodi-imide; EAC, 1-(4-azonia-4,4-dimethylpentyl)-3-ethylcarbodi-imide iodide; WRK, Woodward's Reagent K (2-ethyl-5-phenylisoxazolium-3-sulphonate); d.p., degree of polymerization.

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(soluble fraction, in 200 mM-sodium acetate buffer, pH 5.0) for 10–20 min at 20 °C. The concentration of reducing sugar was then determined by the 3,5-dinitrosalicylic acid method of Miller (1959). Concentrations of xylanase were determined by amino acid analysis with a Beckman System Gold amino acid analyser. Protein samples to be analysed were hydrolysed with 200 μ l of 5.7 M-HCl in sealed evacuated tubes at 110 °C for 22 h. SDS/PAGE was carried out as described by Laemmli (1970), and protein bands were detected with Coomassie Brilliant Blue dye. Radioactivity measurements were made on a Packard Tri-Carb 2000 scintillation counter with Ecolume scintillation fluid (ICN, Montreal, Quebec, Canada). H.p.l.c. analysis of soluble xylo-oligosaccharides was performed on a Millipore Waters model 510 apparatus with an Aminex HPX 42A column (Bio-Rad Laboratories) eluted with distilled water at 0.6 ml/min. Detection of carbohydrates was achieved by using an Erma Optical Works model ERC-7510 refractive-index detector. Assays for glucuronic acid were performed according to the method of Blumenkrantz & Asboe-Hansen (1973), with the exception that 4-hydroxybiphenyl was used instead of 3-hydroxybiphenyl. Total carbohydrate was assayed according to the method of Dubois *et al.* (1956). U.v.- and visible-absorption measurements were made with either a Varian model 2290 or a Beckman DU-8 recording spectrophotometer.

Growth of organism and enzyme production

S. commune strain Delmar (A.T.C.C. 38548) and a freeze-dried ethanol precipitate of a 9-day culture of *S. commune* were kindly provided by M. Paice (Pulp and Paper Research Institute Canada, Pointe Claire, Quebec, Canada). *S. commune* cultures were maintained at 4 °C on either plates or slants of potato/dextrose/agar (Difco Laboratories, Detroit, MI, U.S.A.). Xylanase was produced by using the medium and conditions as described by Jurasek & Paice (1988). All cultures for xylanase production were 150 ml of medium in 500 ml Erlenmeyer flasks, inoculated with a 1 cm piece of agar from an actively growing 4-day plate culture of *S. commune* and incubated for 5 days at 30 °C. Cultures were shaken at 250 rev./min on a rotary shaker.

Enzyme purification

The enzyme complex was obtained by fractional precipitation of the crude culture filtrate with ethanol at –20 °C as described by Jurasek & Paice (1988). All subsequent procedures were performed at 4 °C unless otherwise specified. The xylanase-rich precipitate obtained was dissolved in 200 mM-sodium acetate buffer, pH 5.0, and freeze-dried. A modified procedure based on the two protocols published for the purification of xylanase A (Váradi *et al.*, 1971; Jurasek & Paice, 1988) was used to purify the enzyme to homogeneity. The crude complex (approx. 0.6 g) was resuspended in 10–12 ml of distilled water and applied to 2.5 cm $\times$ 50 cm column of Sephadex G-50 (ultrafine grade) equilibrated with distilled water to separate brown pigments and salt from the sample. The xylanase-active fractions were freeze-dried, resuspended in 5 ml of 10 mM-ammonium bicarbonate buffer, pH 8.5, and applied to a 2.5 cm $\times$ 50 cm column of DEAE-Sephadex CL-6B, pre-equilibrated with the same buffer. The column was eluted with a linear gradient consisting of 400 ml of the equilibration buffer and 400 ml of 150 mM-pyridine/acetate buffer, pH 5.0. Xylanase eluted at approximately one-third of the linear gradient was freeze-dried, resuspended in 1 ml of 10 mM-ammonium bicarbonate buffer, pH 8.5, and applied to a 1.5 cm $\times$ 100 cm column of Bio-Gel P-60, pre-equilibrated with the same buffer. The xylanase recovered from this column showed only one band at 21 kDa when subjected to SDS/PAGE. In some cases affinity chromatography of partially purified enzyme preparations on concanavalin A–Sephadex was used to remove

unwanted carbohydrate and glycoprotein contaminants, increasing the efficiency of later steps. The modified procedure described here, however, was sufficient for the production of large quantities of homogeneous xylanase (15–20 mg/l of culture). The enzyme was indefinitely stable when stored in solution at –20 °C, and could be kept in dilute solutions at 4 °C for several weeks with no discernible loss of activity.

Xylo-oligomer preparation

Soluble oligosaccharides of xylan devoid of undesirable glucuronic acid substituents were prepared by first hydrolysing 0.5 g of soluble oat spelt xylan in 125 mM-H₂SO₄ for 11 min according to the method of John *et al.* (1982). Acidic xylo-oligomers were then removed by ion-exchange chromatography on AG-1 X8 resin (Cl[–] form) eluted with distilled water. Neutral oligomers, which appear in the void volume, were pooled and freeze-dried (yield approx. 0.35 g). H.p.l.c. analysis revealed that this mixture consists of oligomers ranging from d.p. 1 to d.p. > 8 in the following proportions: d.p. 1–8 and d.p. > 8, 19:15:14:12:10:8:6:4:10. Enzymic degradation of this mixture by xylanase A results in the rapid production of the limit xylo-oligosaccharides xylobiose (d.p. 2), xylotriose (d.p. 3) and xylose. Glucose, xylose, maltose, cellobiose and maltotriose were used as standards for this h.p.l.c. analysis.

Modification by carboxy-group-specific reagents

Various group-specific reagents were used to modify the functional groups in xylanase A. Reagents that specifically modify carboxy groups, the water-soluble carbodi-imides EAC, EDC, CMC and the isoxazolium salt WRK, were screened for inhibitory action by incubating the reagents (50 mM final concentration) with xylanase A (2.4 μ M) in 50 mM-Mes/NaOH buffer, pH 6.0, at 25 °C for 50 min. At the indicated times 10 μ l samples of the reaction mixtures were withdrawn and added to 40 μ l of 100 mM-sodium acetate buffer, pH 5.0, to quench residual reagent. The remaining activity of the diluted enzyme derivatives was determined by the 3,5-dinitrosalicylic acid assay and expressed as a percentage of a control. In a parallel experiment reactions with the enzyme and carboxy-group-modifying reagents were performed in the presence of 25 mM-methylamine as an added nucleophile. The reaction of xylanase A with EAC was further studied by varying EAC concentrations in the reaction mixture in the range 10–50 mM. To assess the ability of the neutral xylo-oligomers to protect xylanase A from modification with EAC, the enzyme (2.4 μ M) was incubated with EAC (20 mM) in the presence of various concentrations of the xylo-oligomer preparation (0.125–0.750 %). The pH-dependence of EAC modification of xylanase A was investigated by incubating 2.4 μ M enzyme with EAC (20 mM) in 50 mM-KCl/HCl buffers, pH 4.1–4.8, 50 mM-Mes/NaOH buffers, pH 5.0–6.6, and 50 mM-Mops/NaOH buffers, pH 7.0–8.1. Reactions in KCl/HCl buffer were monitored periodically to ensure that the pH did not fluctuate during the course of the study. At appropriate time intervals 10 μ l samples of the reaction mixtures were removed and quenched in 40 μ l of 100 mM-sodium acetate buffer, pH 5.0, and assayed for residual activity as described above.

Stoichiometry of EAC inactivation

A 1 ml solution of xylanase A (96 μ M) in 50 mM-Mes/NaOH buffer, pH 6.0, was treated with 50 mM-[³H]EAC for 230 min, when the concentration of [³H]EAC was increased by 10 mM to lower enzyme activity to zero. The reaction was quenched at 320 min with 100 μ l of 1 M-sodium acetate buffer, pH 5.0, and the contents of the reaction vial were applied to a 1 cm $\times$ 45 cm column of Bio-Gel P-6-DG, pre-equilibrated with 10 mM-ammonium acetate buffer, pH 8.5, to remove unbound [³H]EAC.

Fractions (1.5 ml) eluted from this column were counted for radioactivity, and those fractions that contained protein were pooled and freeze-dried. A 150 μ l sample of one fraction from the leading edge of the protein peak was dried, hydrolysed for 22 h as described above and subjected to amino acid analysis.

To quantify the number of carboxy groups modified by EAC with competitive inhibitors present, 500 μ l of a 48 μ M solution of xylanase A in 50 mM-Mes/NaOH buffer, pH 6.0, in the presence of 1.0% neutral xylo-oligomers and 60 mM-[3 H]EAC for 70 min, and then the reaction was stopped with the addition of 75 μ l of 1 M-sodium acetate buffer, pH 5.0. Chromatography of the EAC/xylanase mixture on Bio-Gel P-6-DG and protein analysis were performed as described above.

Modification of tyrosine, cysteine and histidine residues

Nitration of tyrosine residues was performed by using tetranitromethane as described by Sokolovsky *et al.* (1966). Portions (20 μ l) of a 20 mM stock solution of tetranitromethane in 95% (v/v) ethanol were added to 0.8 ml samples of xylanase A (48 μ M) in 100 mM-Tris/HCl buffer, pH 8.0, in a cuvette and incubated at 25 $^{\circ}$ C for 300 min. The number of modified tyrosine residues was estimated spectrophotometrically by using the ϵ_{428} value of 4100 M $^{-1}$ ·cm $^{-1}$ for 3-nitrotyrosine (Sokolovsky *et al.*, 1966). At appropriate intervals a 10 μ l sample of the reaction mixture was removed, diluted in 40 μ l of 100 mM-sodium acetate buffer, pH 5.0, and assayed for activity as described above. Control solutions were identical except for the omission of tetranitromethane.

Histidine residues in xylanase A were converted into *N*-ethoxycarbonyl derivatives by treatment of the enzyme with diethyl pyrocarbonate. A 0.8 ml sample of xylanase A (24 μ M) in 50 mM-Mes/NaOH buffer, pH 6.0, was placed in a quartz cuvette, and 40 μ l of a 0.5 M solution of diethyl pyrocarbonate (final concentration 25 mM) in 95% (v/v) ethanol was added at zero time. The conversion of histidine residues was continuously monitored by following the increase in absorbance at 242 nm. The number of modified histidine residues was calculated by using the ϵ_{242} value of 3.2×10^3 M $^{-1}$ ·cm $^{-1}$ for the *N*-ethoxycarbamoylhistidine derivative (Mühlrad *et al.*, 1969). Samples (10 μ l) were removed periodically, diluted in 40 μ l of 100 mM-sodium acetate buffer, pH 5.0, and assayed for activity as described above.

The enzyme was titrated with 5,5'-dithiobis-(2-nitrobenzoic acid) according to the method of Ellman (1959) to assess the number of free thiol groups present. A 0.8 ml sample of xylanase A (24 μ M) in 8 M-urea/50 mM-Tris buffer, pH 8.0, was added to a quartz cuvette with 5,5'-dithiobis-(2-nitrobenzoic acid) (final concentration 1.1 mM). The change in absorbance at 412 nm was continuously monitored at 25 $^{\circ}$ C for 50 min, and the number of cysteine residues was estimated by using the ϵ_{412} value of 1.36×10^4 M $^{-1}$ ·cm $^{-1}$ for the release of 5-mercapto-2-nitrobenzoic acid. In addition, solutions of xylanase A (2.4 μ M) in 10 mM-Tris/HCl buffer, pH 8.0, were treated with *p*-hydroxymercuribenzoate (1.5 mM) and iodoacetamide (20 mM), reagents that preferentially modify thiol groups. Reaction mixtures were incubated at 25 $^{\circ}$ C for 50 min, and 10 μ l samples were periodically removed, diluted in 40 μ l of 100 mM-sodium acetate buffer, pH 5.0, and assayed as described above. Reactions with iodoacetamide were also carried out in 50 mM-Mes/NaOH buffer, pH 6.0, under otherwise identical conditions.

RESULTS

Reagents with restricted amino acid-specificity were used to assess the importance of certain functional groups for the activity of xylanase A. The carboxy-group-specific modifying agents

Fig. 1. Inactivation of xylanase A by chemical reagents specific for carboxy groups

Enzyme (2.4 μ M) in 50 mM-Mes/NaOH buffer, pH 6.0, was incubated at 25 $^{\circ}$ C with 50 mM-WRK (▲), 50 mM-CMC (△), 50 mM-EDC (●) and 50 mM-EAC (○). Samples (10 μ l) were removed from the reaction mixtures at the indicated times, quenched in 100 mM-sodium acetate buffer, pH 5.0, and assayed for residual activity.

EAC, EDC, CMC and WRK varied markedly in their ability to inhibit xylanase A under the conditions employed (Fig. 1). The enzyme was most rapidly inactivated by EAC in the absence of an added nucleophile, and retained only 10% activity after 20 min incubation with 50 mM reagent. Inactivation rates were considerably lower in the cases of CMC, EDC and WRK (16%, 12% and 7.6% respectively, relative to inactivation by EAC), and the inclusion of methylamine (25 mM) as an added nucleophile had no significant effect on these rates. However, the xylanase retained less than 1% catalytic activity after prolonged incubation (≥ 240 min) with each of these reagents under the conditions employed.

Semi-logarithmic plots of residual activity as a function of time of inactivation for various concentrations of EAC were linear, indicating that in all cases the inactivation process obeys pseudo-first-order kinetics (Fig. 2). Analysis of the order of

Fig. 2. Inactivation of xylanase A with various concentrations of EAC

Enzyme (2.4 μ M) in 50 mM-Mes/NaOH buffer, pH 6.0, was treated with EAC at 25 $^{\circ}$ C. Final EAC concentrations were 50 mM (□), 40 mM (▲), 30 mM (△), 20 mM (●) and 10 mM (○). Reactions were assayed for residual activity as indicated in Fig. 1 legend.

Fig. 3. Protection by xylo-oligomers of xylanase A from modification by EAC

Enzyme ($2.4 \mu\text{M}$) in 50 mM-Mes/NaOH buffer, pH 6.0, was treated with EAC (20 mM) in the absence ($\circ$) and in the presence of 0.125 % ($\bullet$), 0.250 % ($\triangle$), 0.500 % ($\blacktriangle$) and 0.750 % ($\square$) neutral xylo-oligomers. Residual activity of quenched samples was determined as indicated in Fig. 1 legend. Inset: the apparent pseudo-first-order rate constants obtained in the presence (k'_p) and in the absence (k'_a) of xylo-oligomers (X_n) were plotted according to the method of Scrutton & Utter (1965).

reaction by the method of Levy *et al.* (1963) yielded a slope of 1.1 (result not shown), indicating that an average of at least one molecule of inhibitor binds one molecule of xylanase for inactivation.

To determine if the carboxy groups modified by EAC are located in the active site, modification with EAC was carried out in the presence of neutral xylo-oligosaccharides. The acid hydrolysis of oat spelt's xylan and subsequent removal of oligomers substituted with glucuronic acid eliminated the possibility of the EAC interacting with the ligand. This procedure also served to increase the ability of the xylanase to bind the ligand by removing interfering branched substituents present on xylan. In a reaction with 20 mM-EAC at pH 6.0 with no protective ligand present, the enzyme was inactivated to the extent of 50 % within 20 min, with only 21 % activity remaining after 50 min. The presence of xylo-oligomers (0.125–0.750 %) prevented this rapid inactivation in a concentration-dependent manner (Fig. 3). During the course of the experiments the xylo-oligosaccharides were enzymically degraded to xylobiose and xylotriose, suggesting that these limit xylo-oligomers provided protection to one or more essential carboxy groups. Protection versus xylo-oligomer concentration was plotted according to the method of Scrutton & Utter (1965) (Fig. 3 inset) and extrapolated to the origin, indicating that the enzyme-substrate complex cannot be inactivated by EAC. Confirmation that EAC does not interact with the xylo-oligomers was obtained by first incubating EAC with the ligands, then comparing the inactivation rate with that of a control EAC solution when allowed to react with xylanase A. These rates were identical.

A pH-dependence of EAC inactivation of xylanase A was observed, with maximum inactivation occurring between pH 5.5 and 6.0 (Fig. 4). This Dixon plot (Dixon & Webb, 1979) of $\log k_{app}$ versus pH shows the presence of critical ionizable groups with pK_a values of 5.2 and 6.6, conceivably the protonated form of either aspartic acid or glutamic acid residues.

Fig. 4. Effect of pH on the pseudo-first-order rate constants (k_{app}) for the inactivation of xylanase A by EAC

Enzyme ($2.4 \mu\text{M}$) and EAC (20 mM) were incubated at 25 °C for 1 h in 50 mM-KCl/HCl buffers, pH 4.1–4.8 buffers ($\square$), 50 mM-Mes/NaOH buffers, pH 5.0–6.6 ($\bullet$) and 50 mM-Mops/NaOH buffers, pH 7.0–8.1 ($\triangle$). Values for k_{app} were determined from the linear plots of $\log(\text{residual activity})$ versus time.

SDS/PAGE of enzyme samples before and after incubation with EAC showed bands of identical mobility, proving that inactivation of the xylanase was not a result of cross-linking or aggregation of enzyme molecules.

The extent of modification of xylanase by EAC at pH 6.0 was assessed after gel filtration of reaction mixtures on Bio-Gel P-6-DG. With the use of [^3H]EAC to facilitate quantification, 3.9 ± 0.35 equivalents of EAC were found to bind to the xylanase for complete inactivation of the enzyme. When xylo-oligomers were included in the reaction to protect the active site from [^3H]EAC action, it was calculated that 2.9 ± 0.36 amino acid residues were modified, with only a 15 % loss of enzyme activity. This suggests that one essential residue is protected by the substrate from modification by EAC.

The results obtained with EAC alone do not preclude the possible existence of a catalytically essential cysteine or tyrosine residue, since phenol and thiol groups may also be modified by carbodi-imides under slightly acidic conditions (Carraway & Koshland, 1968). Indeed, several bacterial xylan-degrading enzymes, including the β -D-xylosidase of *Bacillus pumilus* (Kerstens-Hilderson *et al.*, 1984) and a *Streptomyces* xylanase (Kesar *et al.*, 1989), have been shown to contain essential thiol groups and/or histidine residues. Incubation of the enzyme for an extended period of time (360 min) with a 20-fold molar excess of the highly specific tyrosine-nitrating reagent tetranitromethane resulted in the modification of one tyrosine residue and the concomitant loss of 16 % of initial activity, indicating that tyrosine residues do not play an essential catalytic role in xylanase A activity.

Titration of the enzyme with 5,5'-dithiobis-(2-nitrobenzoic acid) (1.1 mM) in 8 M-urea at pH 8.0 over an extended time period did not result in the release of 5-mercapto-2-nitrobenzoic acid, suggesting that no free thiol groups exist in the xylanase A molecule. The lack of a reactive thiol group in the intact enzyme was confirmed by reaction with the specific cysteine-residue modifiers iodoacetimide (at pH 6 and 8) and *p*-chloromercuribenzoate (at pH 8) at 20 mM, neither of which had any perceptible effect on xylanase activity after 60 min incubation.

Diethyl pyrocarbonate (25 mM) was incubated with the xylanase A at pH 6.0, and was found to modify all three of the histidine residues present in the protein (Fig. 5). This modification did not result in any loss of enzymic activity during the 60 min course of the experiment.

Fig. 5. Relationship between the number of modified histidine residues/molecule and enzymic activity upon treatment of xylanase A with diethyl pyrocarbonate

Xylanase A (24 μ M) in 50 mM-Mes/NaOH buffer, pH 6.0, was treated with diethyl pyrocarbonate (25 mM) at 25 °C for 60 min. The number of modified histidine residues/molecule (●) was estimated spectrophotometrically by using the ϵ_{242} value of $3.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Residual activity of reaction mixture (○) was determined and expressed as a percentage of an appropriate control.

DISCUSSION

The nature of the pH-activity profile for xylanase A (Paice *et al.*, 1978) implicates the participation of both an acidic amino acid residue and possibly a histidine residue in its mechanism of catalysis. Alternatively, a second acidic residue may be located in a hydrophobic environment, resulting in an increase of its pK_a , as exemplified by hen egg-white lysozyme (Chipman & Sharon, 1969). The data given in the present paper provide evidence for the presence of only catalytically essential carboxy groups in the active site of the *S. commune* xylanase, while eliminating the possible involvement of histidine, tyrosine or cysteine residues in catalysis by the enzyme.

The water-soluble carbodi-imide EAC in the absence of added nucleophile was shown to inhibit xylanase A activity very rapidly and completely in comparison with EDC, CMC or WRK, and was therefore chosen for subsequent chemical modification studies. The superior capacity of EAC as a carboxy-group-modifying reagent relative to other compounds has been documented in the study of yeast enolase (George & Borders, 1979), bovine thrombin (Chan *et al.*, 1988), *A. niger* glucoamylase (Svensson *et al.*, 1988) and the *S. commune* endoglucanase I (Clarke & Yaguchi, 1985). This enhanced reactivity of EAC has been attributed to both its stability in the reactive linear tautomeric form (Tenforde *et al.*, 1972) and the rapid rearrangement of initial *O*-acyl adducts to stable *N*-acyl derivatives (Timkovich, 1977).

Protection studies employing xylo-oligomers and [^3H]EAC demonstrated that only one active-site carboxy group per molecule is modified for inactivation. This observation is not surprising in view of the experimental conditions used, and does not preclude the existence of a second catalytically essential carboxylate group in the active centre of the enzyme. At pH 6.0, only a limited number of glutamic acid and aspartic acid residues would remain protonated. This would restrict the extent of EAC reaction, since according to a recently published mechanism for carbodi-imide modification of proteins the protonation of

carboxy groups is required. It is postulated that these groups serve to both protonate the carbodi-imide nitrogen atom and subsequently provide the attacking nucleophilic carboxylate anion (Chan *et al.*, 1988).

Taken together with the above observations, the pK_a of 6.6 obtained from the Dixon plot of EAC inactivation (Fig. 4) can be ascribed to an essential carboxy group with an elevated pK_a in the active centre of xylanase A. This residue conceivably acts as an acid catalyst in the mechanism of action of the enzyme. At present, it is unclear what the acidic limb of the Dixon plot of EAC inactivation represents. Theoretically, the increase in H^+ ion concentration should enhance inactivation rates, as observed in similar studies with other enzymes, including *S. commune* cellulase (Clarke & Yaguchi, 1985), *A. niger* glucoamylase (Svensson *et al.*, 1988) and *Bacillus amyloliquefaciens* α -amylase (Kochhar & Dua, 1984). However, bell-shaped pH-dependence curves have also been reported for the carbodi-imide inactivation of an *A. niger* cellulase (Hurst *et al.*, 1977) and an *Aspergillus saitoi* glucoamylase (Inokuchi *et al.*, 1981). It is possible that this pH-dependence reflects acid-induced conformational changes in the enzymes, thereby hindering the access of carbodi-imides to the confined regions of active centres. Thus, although essential carboxy groups may become protonated under conditions of lower pH, and hence more susceptible to reaction with carbodi-imides, the slight conformational alterations would block subsequent modifications.

Evidence has accumulated for a lysozyme-type mechanism among the β -1,4-glycan hydrolases, particularly within the cellulase system of enzymes (Paice & Jurasek, 1979; Yaguchi *et al.*, 1983; Paice *et al.*, 1984; Clarke & Yaguchi, 1985; Morosoli *et al.*, 1986; Moranelli *et al.*, 1986). Subtle differences in active-site geometry, precise positioning of catalytic amino acid residues relative to binding sites and other structural variation among the endohydrolases (Høj *et al.*, 1989) underscore the necessity for more widespread study of structure-function relationships in these enzymes if the generality of a hen egg-white lysozyme mechanism of catalysis is to be established. Such information with regard to the potentially valuable endoxylanases is especially lacking. Further studies are required to delineate the identity of residues responsible for catalysis and substrate binding in this enzyme.

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