Characterization and sequencing of an active-site cysteine-containing peptide from the xylanase of a thermotolerant *Streptomyces*

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The kinetics of chemical modification of the xylanase from a thermotolerant *Streptomyces* T₇ indicated the involvement of 1 mol of cysteine residue/mol of enzyme [Keskar, Srinivasan & Deshpande (1989) *Biochem. J.* **261**, 49–55]. The chromophoric reagent *N*-(2,4-dinitroanilino)maleimide (DAM) reacts covalently with thiol groups of xylanase with complete inactivation. Protection against inactivation was provided by the substrate (xylan). The purified xylanase that had been modified with DAM was digested with pepsin and the peptides were purified by gel filtration followed by peptide mapping. The active-site peptide was distinguished from the other thiol-containing peptides by comparison of the peptides generated by labelling the enzyme in the presence and in the absence of the substrate. The peptide mapping of the modified enzyme in the absence of xylan showed three yellow peptides, whereas in the presence of xylan only two yellow peptides were detected. The active-site peptide protected by the substrate failed to form the complex with DAM. The modified active-site peptide was isolated and sequenced. Gas-phase sequencing provided the following sequence: Ser-Val-Ile-Met-Xaa-Ile-Asp-His-Ile-Arg-Phe. This is the first report on the isolation and sequencing of the active-site peptide from a xylanase. The comparison of reactive cysteine-containing peptide sequence with the catalytic regions of other glucanases revealed the presence of a conserved aspartic acid residue.

INTRODUCTION

Xylanase (1,4- β -D-xylanohydrolase, EC 3.2.1.8) catalyses the random hydrolysis of xylosidic bonds in xylan to xylo-oligosaccharides and xylose, which can be used as food and fuel. Xylan is the major component of hemicelluloses, which represent one-third of the polysaccharide content of renewable plant biomass. In Nature, xylanases have a major function of softening cell walls to allow cell growth, differentiation and abscission (Whitaker, 1971). Commercially, cellulase-free xylanases have a potential application in the paper and pulp industry for selective hydrolysis of hemicelluloses (Jurasek & Paice, 1986). Bioconversion of xylan into useful products will improve the overall economics of the processing of lignocellulosic biomass (Biely, 1985; Wang *et al.*, 1988). Xylanases have been identified in a wide range of organisms, and appear to be quite diverse in nature (McCarthy, 1987; Ball & McCarthy, 1989). Among prokaryotes, actinomycetes have been shown to be highly efficient in the degradation of biomass, but few enzyme systems have been investigated (Ishaque & Kluepfel, 1981; Vanzyl, 1985). In spite of the biotechnological importance of xylanases, limited information regarding their mechanism of action is available. Large active centres containing several subsites appear to be characteristic of fungal xylanases (Biely *et al.*, 1981; Vrsanska *et al.*, 1982; Meagher *et al.*, 1988). However, there are few data shedding light on the enzyme–substrate interaction of xylanases. On the other hand, the catalytic mechanism of lysozyme and cellulases, which are functionally related to xylanases, has been delineated in detail (Imoto *et al.*, 1972; Yaguchi *et al.*, 1983). The commercial and biological importance of xylanase and its potential relationship to lysozyme and cellulases make it an excellent candidate for active-site studies.

Information regarding the sequence of amino acid residues involved at or near the active site of the enzyme is necessary for

the functional characterization of the protein and is a prerequisite for its manipulation by site-directed mutagenesis, which can lead to the synthesis of a new protein with desirable properties. Although the protein sequences involved in the catalytic domains of cellulases and xylanases have been compared (West *et al.*, 1989), the sequences around the functional residues have not been hitherto determined. We have earlier reported the isolation of a high-xylanase-producing thermotolerant *Streptomyces* T₇ and shown the involvement of tryptophan and cysteine residues at the active site of the xylanase (Keskar *et al.*, 1989). As a first step towards an understanding of structure–function relationship of xylanases, the present paper reports the isolation and sequencing of the reactive cysteine-containing peptide from the xylanase of *Streptomyces* T₇ with the use of the chromophoric thiol-specific reagent *N*-(2,4-dinitroanilino)maleimide (DAM). The active-site sequence of the xylanase is compared with the catalytic regions of other glucanases.

MATERIALS AND METHODS

Materials

BSA, xylan (oat spelts), 3,5-dinitrosalicylic acid, standard amino acid mixture and pepsin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden). DAM was synthesized according to the procedure described by Clark-Walker & Robinson (1961). All the other chemicals used were of analytical grade.

Source of enzyme

The xylanase from *Streptomyces* T₇ (N.C.I.M. 34; National Collection of Industrial Microorganisms, Pune, India) was produced and purified by ethanol precipitation and DEAE-

Abbreviation used: DAM, *N*-(2,4-dinitroanilino)maleimide.

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cellulose chromatography followed by gel filtration on Sephadex G-50. The purity was confirmed by SDS/PAGE and analytical isoelectric focusing (Keskar *et al.*, 1989).

Enzyme assays

Xylan (2 g) was suspended in 100 ml of 50 mM-sodium acetate buffer, pH 5.0, and was stirred for 12–16 h. The insoluble fraction (about 50%) was removed by centrifugation and the soluble fraction was used for xylanase assay. Xylanase was assayed by incubating 0.5 ml of appropriately diluted enzyme with 0.5 ml of 1% xylan at 60 °C for 30 min (Mandels & Weber, 1969). The reducing sugar formed was determined by the 3,5-dinitrosalicylic acid method, with D-xylose as a standard (Miller, 1959). One unit of xylanase was defined as the amount of enzyme that produces 1 μ mol of xylose/min from xylan under the given assay conditions. Protein was determined by the method of Lowry *et al.* (1951), with BSA as a standard.

Amino acid composition

The amino acid composition of the protein was determined by the method of Spackman *et al.* (1958). Protein (1 mg) was hydrolysed for 24 h and 48 h at 110 °C with 6 M-HCl in tubes that had been sealed under vacuum. Total cysteine content in the protein was also determined by performic acid oxidation (Hirs, 1967). Reduction of disulphide linkages with NaBH₄ in 8 M-urea and subsequent titration with 5,5'-dithiobis-(2-nitrobenzoic acid) was performed according to the method of Cavallini *et al.* (1966). The tryptophan and tyrosine contents were determined by the method of Goodwin & Morton (1946).

Reaction of DAM with xylanase

The purified xylanase (2 μ g) was incubated with various concentrations of DAM (dissolved in methylCellosolve) in 50 mM-sodium acetate buffer, pH 5.0, in a reaction volume of 0.05 or 0.5 ml at 25 °C for 10 min. Then 0.5 ml of 1% (w/v) xylan was added and the residual activity was determined by the usual assay for xylanase. Control tubes containing the enzyme alone and enzyme plus DAM were also run under identical conditions. The corresponding amounts of methylCellosolve had no effect on the enzyme activity.

Protection against inactivation by substrate

The xylanase (2 μ g) was incubated with different amounts of xylan (0.12–1.2 mg) before the addition of 10 μ l of 50 mM-DAM to give a total volume of 50 μ l. The xylanase activity was assayed by adding xylan to a final concentration of 5 mg per reaction mixture.

Isolation and purification of cysteine-containing peptides

Alkylation of xylanase with DAM. A 40 ml portion of 50 mM-DAM was added to 10 mg of purified enzyme in a total volume of 50 ml in 50 mM-sodium acetate buffer, pH 5.0. The reaction was allowed to proceed for 2.5 h at 25 °C. The xylanase activity was assayed before and after alkylation. DAM-treated enzyme was precipitated with 3 vol. of chilled ethanol. The precipitate was recovered by centrifugation at 9000 g for 30 min and dried under vacuum. The absorption spectra of the native and DAM-treated xylanase were recorded.

Proteolysis of DAM-treated xylanase. The yellow pellet was dissolved in 1.5 ml of 10 mM-HCl and the pH was adjusted to 2.0. Proteolytic digestion was carried out at 37 °C for 24 h at pH 2.0 with pepsin at a protein/proteinase ratio of 10:1 (w/w).

Sephadex G-25 chromatography. For the purification of peptides, the peptic digest of DAM-treated xylanase was applied to a Sephadex G-25 column (3 cm $\times$ 26 cm) equilibrated with water. Fractions were collected at the rate of 36 ml/h and

analysed for absorption at 280 nm and 340 nm. Fractions showing maximum absorption at 280 nm (peak I) and 340 nm (peak II) were pooled separately. Peak II was further concentrated by freeze-drying.

Peptide mapping. Separation of peak II was obtained by a two-dimensional combination of electrophoresis and chromatography (Ingram, 1958) except that solvent system pyridine/butan-1-ol/acetic acid/water (10:15:3:12, by vol.) was used for paper chromatography.

Location of reactive peptide

A 5 mg portion of xylanase was mixed with 150 ml of 3% (w/v) xylan and the mixture was allowed to stand for 1 h at 25 °C. Then 20 ml of 50 mM-DAM was added and the mixture was incubated at 25 °C for 10 min. A 50 μ l portion was removed and assayed for xylanase activity. The mixture was concentrated to 55 ml by freeze-drying and then precipitated with chilled ethanol (165 ml). The precipitate recovered after centrifugation was dried and digested with pepsin. The purification and separation of yellow peptides was carried out by gel filtration followed by peptide mapping.

Isolation of reactive peptide

The treatment of xylanase (10 mg) with DAM and subsequent peptide mapping was carried out in the absence of substrate. The active peptide was isolated by extracting the corresponding region of the chromatogram with 10% (v/v) acetic acid. The extract was concentrated by freeze-drying. The peptide mapping of the peak II was repeated five times to obtain a sufficient quantity of reactive peptide.

Sequencing of the cysteine-containing peptide

Sequence analysis was performed by automated gas-phase Edman sequencing with an Applied Biosystems model 477A sequencer.

RESULTS

Table 1 shows the amino acid composition of the xylanase from *Streptomyces T.*. In order to ensure complete hydrolysis the digestion of the protein was carried out for 24 h and 48 h, but there was no difference in the data obtained for the two periods of digestion. Hence the data for 24 h have been given. Previous work has shown the presence of 3 mol of cysteine residues/mol of the xylanase. Amino acid analysis of the performic acid-oxidized sample of the enzyme (Table 1) showed the presence of 5 mol of cysteine/mol determined as cysteic acid. Thiol-group determination of the reduced enzyme also showed the presence of 5 mol of thiol groups/mol, indicating that the enzyme contained three thiol groups and one disulphide linkage per molecule of enzyme. Chemical modification of xylanase with *p*-hydroxymercuribenzoate and subsequent kinetic analysis showed the involvement of 1 mol of cysteine residue/mol of the protein (Keskar *et al.*, 1989).

DAM, a yellow derivative of *N*-ethylmaleimide, reacts with cysteine as shown in Scheme 1 (Clark-Walker & Robinson, 1961). The reaction of the xylanase with DAM showed complete inhibition of activity at 1 or 10 mM concentration when the reaction was performed in a volume of 0.5 or 0.05 ml respectively (Fig. 1), suggesting that the molar ratio of the enzyme to the inhibitor rather than the concentration of the inhibitor was important for the inactivation. The alkylated enzyme shows an absorption peak at 340 nm characteristic of the DAM–cysteine adduct in addition to the peak at 260 nm (Fig. 2). As judged by the size of the peak at 340 nm, the stoichiometry of incorporation of DAM is calculated to be 2.7 mol of DAM/mol of enzyme.

Table 1. Amino acid composition of xylanase from *Streptomyces T₇*

Cysteine was determined as cysteic acid after performic acid oxidation. Values of tyrosine and tryptophan are as determined by the method of Goodwin & Morton (1946). The calculations were based on an M_r of 20000 for the enzyme.

Amino acid	Composition (mol/mol of enzyme)
Asx	20
Thr	10
Ser	11
Glx	31
Pro	4
Gly	28
Ala	5
Met	4
Ile	3
Leu	2
Val	5
Tyr	5
Phe	2
Lys	5
His	37
Arg	2
Trp	5
Cys	5
	184

Scheme 1. Reaction of cysteine with DAM

Isolation and purification of cysteine-containing peptides

The peptic digest of DAM-treated xylanase was fractionated on Sephadex G-25 (Fig. 3). The fractions showing a higher A_{340}/A_{280} ratio (peak II), which showed a distinct yellow colour indicating the presence of cysteine-containing peptides, were pooled. Further purification of peak II was achieved by paper electrophoresis followed by chromatography. The peptide map of DAM-treated enzyme was identical with that of the native enzyme except that a faint yellow spot was visible at the solvent

Fig. 1. Inactivation of xylanase with DAM

Enzyme (2 μg) was incubated with 0.2–1.2 mM-DAM in 0.5 ml (○) or with 2–12 mM-DAM in 0.05 ml (●) in 50 mM-sodium acetate buffer, pH 5.0, at 25 °C for 10 min.

Fig. 2. Absorption spectra of native and DAM-treated enzyme

Enzyme (10 mg) was incubated with 40 ml of 50 mM-DAM in 50 mM-sodium acetate buffer, pH 5.0, in a total volume of 50 ml at 25 °C for 2.5 h. The treated enzyme was precipitated with ethanol as described in the text.

front. Reaction of cysteine residues with DAM is known to increase the mobility of the resulting peptides (Clark-Walker & Robinson, 1961). The peptide map of peak II showed only three dark-yellow peptides. Absence of ninhydrin-positive peptides indicated that yellow cysteine-containing peptides were separated from the rest of the peptides during gel filtration.

Location of the reactive peptide

To ascertain the position of the reactive cysteine-containing peptide, among the three yellow peptides, the property of the active site to bind substrate and thereby be protected against inactivation by the cysteine-specific reagent was utilized. Fig. 4 shows the protection of the xylanase by substrate against inactivation by DAM. Complete protection was obtained by as little as 1 mg of xylan, indicating the specificity of the reaction. When the modification of the xylanase was carried out in the presence of xylan, the peptide containing the reactive cysteine residue appeared colourless, in contrast to the other yellow cysteine-containing peptides. The comparison between the peptide maps obtained in the absence and in the presence of xylan shows that the peptide containing the reactive cysteine residue failed to indicate the characteristic yellow colour in the presence

Fig. 3. Sephadex G-25 chromatography of peptic digest of DAM-treated xylanase

Fractions (5 ml) were collected. Absorbance at 280 nm ($\bigcirc$) and 340 nm ($\bullet$) was recorded.

Fig. 4. Protection of xylanase by substrate against inactivation by DAM

Xylan (0.1–1.2 mg) was added to xylanase (2 μ g) before the incubation with 10 μ l of 50 mM-DAM for 10 min at 25 °C in a total volume of 50 μ l. The xylanase activity was assayed by adding xylan to make the final concentration of 5 mg per reaction mixture.

of xylan. The R_f value of the reactive peptide was the highest among the three cysteine-containing peptides.

Isolation and sequencing of the reactive cysteine-containing peptide

After locating the position of the reactive cysteine-containing peptide, a similar experiment was carried out in the absence of xylan and the active peptide was isolated. Analysis of the modified peptide yielded the sequence Ser-Val-Ile-Met-Xaa-Ile-Asp-His-Ile-Arg-Phe. The Xaa at position 5 indicates the absence of a phenylthiohydantoin derivative and the presence of a modified cysteine residue.

DISCUSSION

Despite the wealth of biochemical and physiological data available on xylanases, the detailed molecular mechanism by which the catalysis occurs is not known. Our results represent the first approach of discerning the structural environment of a

functional amino acid residue for xylanases. We have used DAM, a chromogenic derivative of *N*-ethylmaleimide, to label the three thiol groups of the xylanase. The strategy used for distinguishing the active-site peptide from other thiol-containing peptides was to protect the active-site cysteine residue selectively by the substrate (xylan). Differential peptide mapping between the peptic digests of xylanase treated with DAM in the presence and in the absence of xylan allowed the specific isolation of the reactive cysteine-containing peptide. The sequencing of the peptide shows a predominance of hydrophobic amino acids.

Earlier it was suggested that all glycosidases may follow the same mechanistic pathway as lysozyme (Simpson *et al.*, 1980). The involvement of tryptophan (Keskar *et al.*, 1989; Deshpande *et al.*, 1990) and carboxy groups (Bray & Clarke, 1990) in the binding of substrate to xylanase, lysozyme (Yaguchi *et al.*, 1983) and cellulase (Hurst *et al.*, 1977; Clarke, 1987) suggests a structural relationship between these enzymes. The active-site peptide sequence of xylanase from *Streptomyces T₇* contains an aspartic acid residue, in consonance with its conservation in other endoxylanases and cellobiohydrolases, but lacks the universal Asp-Gly-Yaa-Gly sequence found in the other glucanases (Bastawde *et al.*, 1991). Whether the presence of cysteine as the essential functional residue is typical of actinomycetes xylanases will await further accumulation of data on such sequences around the active site of xylanases.

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