Structural environment of an essential cysteine residue of xylanase from *Chainia* sp. (NCL 82.5.1)*

Mala RAO, Suvama KHADILKAR, Kavita R. BANDIVADEKAR and Vasanti DESHPANDE†

Division of Biochemical Sciences, National Chemical Laboratory, Pune 411008, India

N-(2,4-Dinitroanilino)maleimide (DAM) reacts covalently with the thiol group of the xylanase from *Chainia* leading to complete inactivation in a manner similar to *N*-ethylmaleimide, but provides a reporter group at the active site of the enzyme. Increasing amounts of xylan offered enhanced protection against inactivation of the xylanase by DAM. Xylan (5 mg) showed complete protection, providing evidence for the presence of cysteine at the substrate-binding site of the enzyme. Kinetics of chemical modification of the xylanase by DAM indicated the involvement of 1 mol of cysteine residue per mol of enzyme, as reported earlier [Deshpande, Hinge and Rao (1990) *Biochim. Biophys. Acta* 1041, 172–177]. The second-order rate constant for the reaction of DAM with the enzyme was

$3.61 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$. The purified xylanase was alkylated with DAM and digested with pepsin. The peptides were separated by gel filtration. The specific modified cysteinyl peptide was further purified by reverse-phase HPLC. The active-site peptide was located visually by its predominant yellow colour and characterized by a higher A_{340} to A_{210} ratio. The modified active-site peptide has the sequence: Glu-Thr-Phe-Xaa-Asp. The sequence of the peptide was distinctly different from that of cysteinyl peptide derived from a xylanase from a thermotolerant *Streptomyces* species, but showed the presence of a conserved aspartic acid residue consistent with the catalytic regions of other glucanases.

INTRODUCTION

Cellulase-free xylanases (1,4- β -D-xylan xylanohydrolase, EC 3.2.1.8) have received increased attention in recent years due to their environmentally friendly nature and their application in the treatment of hemicellulosic pulp to improve the quality of paper. Conversion of xylan into xylose by xylanases is a key step in the fermentation of hemicellulose into ethanol for effective utilization of lignocellulosic wastes. *Chainia* sp. (NCL 82.5.1), a sclerotial actinomycete, produces several commercially important enzymes such as xylanases [1] and substrate-specific intra- and extracellular glucose/xylose isomerases [2]. The low-molecular-mass xylanase (xylanase II) from *Chainia* has been studied in greater detail [3–5] with respect to its structure–activity relationship. The stability of the enzyme at higher pH and temperature are desirable for its substitution as a partial delignification agent. Recombinant DNA technology provides a reliable approach to changing the properties of the enzyme using site-specific mutagenesis. The knowledge of amino acids involved at or near the active site of an enzyme and identification of a gene coding for the protein are prerequisites for manipulating the protein by site-directed mutagenesis. The gene for the xylanase from *Chainia* has been cloned and expressed in *Escherichia coli* [6,7]. However, the expression of the recombinant xylanase was 2000 times lower than the wild-type xylanase, probably due to the lack of recognition of *Chainia* expression signals in *E. coli* and/or bias for the codon usage. The participation of tryptophan [3,5,8] and carboxyl groups [9,10] in the active site of xylanase, cellulase [11] and lysozyme [12], which are functionally related, has been observed. However, requirement of cysteine as an essential amino acid has been reported only by us in the cases of xylanases from *Chainia* and *Streptomyces* T7 [3,8]. The objective of the present work has been to isolate and sequence the peptide containing the functional cysteine group of the xylanase from *Chainia* and compare it with the catalytic regions of other xylanases. We have used *N*-(2,4-

dinitroanilino)maleimide (DAM), a chromophoric derivative of *N*-ethylmaleimide (NEM), to label the peptide containing the reactive cysteine.

MATERIALS AND METHODS

Materials

DAM (Figure 1) was synthesized according to Clark-Walker and Robinson [13]. Xylan (oat spelt), pepsin and 3,5-dinitrosalicylic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-25 and G-50 were obtained from Pharmacia (Uppsala, Sweden). All other chemicals used were of analytical grade.

Source of enzyme

Chainia (NCL 82.5.1), a sclerotial actinomycete, was isolated at the National Chemical Laboratory, Pune, from a soil sample from Rajasthan. The enzyme was produced by growing the organism on a medium containing wheat bran (5%) and yeast extract (1%) on a shaker (220 rev./min) at 28 °C for 72 h.

Figure 1 Structure of DAM

Abbreviations used: DAM, *N*-(2,4-dinitroanilino)maleimide; NEM, *N*-ethylmaleimide; TFA, trifluoroacetic acid.

* NCL communication no. 6193.

† To whom correspondence should be addressed.

Purification of enzyme

The enzyme was purified according to the procedure described by Bastawde [14] with slight modifications. All steps were carried out at 4 °C unless otherwise stated. The culture filtrate was obtained by centrifugation of the fermentation broth at 8000 *g* for 20 min. The supernatant (7890 units, 9850 mg) was concentrated by the addition of 3 vol. of chilled ethanol. The precipitate was recovered by centrifugation at 10000 *g* for 25 min, dried under vacuum and was dissolved in 20 ml of 10 mM phosphate buffer, pH 7.0. The undissolved solid particles were removed by centrifugation, and the clear supernatant (7570 units, 3788 mg) was treated with DEAE-cellulose equilibrated with 10 mM phosphate buffer, pH 7.0. The slurry was filtered and the filtrate containing enzyme was collected. The cake was washed with 10 ml of buffer to recover the enzyme. The pooled filtrate (40 ml, 2705 units, 344 mg) was concentrated under vacuum, and used for further purification by gel filtration using a Sephadex G-50 column. A 1 ml portion of this enzyme preparation was applied to the column (1.5 cm × 100 cm) equilibrated with 10 mM acetate buffer, pH 5.8. The fractions were collected at the rate of 2 ml/min and estimated for xylanase activity. The active fractions of low-molecular-mass xylanase were pooled (902 units, 22 mg) and further concentrated by drying in a vacuum desiccator.

Enzyme assay

A sample (2 g) of xylan was suspended in 100 ml of 50 mM sodium acetate buffer (pH 5.7) and stirred for 12–16 h. The insoluble fraction was removed by centrifugation and the soluble fraction was used for the xylanase assay. The xylanase was assayed by incubating 0.5 ml of appropriately diluted enzyme with 0.5 ml of xylan (1 %) at 50 °C for 30 min [15]. The reducing sugar was estimated by the 3,5-dinitrosalicylic acid method using D-xylose as a standard [16]. One unit of xylanase was defined as the amount of enzyme that produces 1 μ mol of xylose/min from xylan. Protein was determined by the method of Lowry et al. [17].

Kinetics of inactivation of xylanase by DAM

The purified xylanase (5 μ g) was incubated with different concentrations of DAM in 50 mM sodium acetate buffer, pH 5.8, in a total volume of 2.5 ml at 25 °C. Aliquots of 0.5 ml were removed periodically and the residual activity was determined by the addition of 0.5 ml of xylan (1 %) followed by incubation at 50 °C for 30 min. The pseudo-first-order rate constant (*k*) was determined from the slope of the plot of log(residual activity) against time of reaction. The second-order rate constant was calculated from the slope of the plot of pseudo-first-order rate constant against concentration of inhibitor [18]. Controls containing enzyme or DAM alone were simultaneously run. DAM was dissolved in methylcellosolve. The amount of methylcellosolve carried in the reaction mixture had no effect on the enzyme activity.

Substrate protection against inactivation by DAM

The xylanase (5 μ g) was incubated with different amounts of xylan (1–5 mg) for 10 min at 4 °C. A 10 μ l aliquot of DAM (500 μ M) was added and the reaction mixture was incubated at 25 °C for 15 min in a total volume of 250 μ l. The xylanase activity was estimated by adding xylan to a final concentration of 5 mg per reaction mixture. Parallel controls for enzyme activity for various amounts of xylan in the absence of DAM were run.

Modification of xylanase with DAM

The enzyme (20 mg) was treated with 5 ml of DAM (50 mM) for 3 h at 25 °C in a total volume of 10 ml in 50 mM sodium acetate buffer, pH 5.0. The modified enzyme was precipitated with 3 vols. of ethanol at 0 °C. The precipitated protein was recovered by centrifugation at 10000 *g* for 30 min and was dried under vacuum. The activity and the absorption spectra of the native and modified enzyme were monitored.

Proteolysis of DAM-treated enzyme

The yellow precipitate of DAM-treated enzyme was dissolved in 2.5 ml of 10 mM HCl and digested with 400 μ g of pepsin at a protein to proteinase ratio of 10:1 (w/w) at 37 °C for 24 h at pH 2.0.

Gel filtration on Sephadex G-25

The peptic digest of DAM-treated xylanase was subjected to gel filtration on a Sephadex G-25 column (25 cm × 25 cm) in water. Fractions (2.5 ml each) were collected at the rate of 30 ml/h and analysed for absorption at 210 and 340 nm. Fractions showing maximum absorption at 210 nm and 340 nm were pooled and processed separately.

Purification of cysteinyl peptide by HPLC

The pooled preparation of yellow fractions from a Sephadex G-25 column was further purified by reverse-phase HPLC on a Vydac 218TP510 semi-preparative column (10 mm × 250 mm; 5 μ m, 3000 Å) with a Beckman system consisting of two model 112 pumps with a model 420 gradient controller. The column was eluted with a linear gradient of acetonitrile in 0.1 % trifluoroacetic acid (TFA) and water in 0.1 % TFA. The eluted peaks were detected with a model 165 variable-wavelength detector and displayed on a Hewlett-Packard model 3390A integrator.

Sequencing of cysteine-containing peptide

Sequence analysis of the HPLC-purified peptide was carried out by automated gas-phase Edman sequencing with an Applied Biosystems model 477A sequencer.

RESULTS

Chemical modification of xylanase by DAM

The low-molecular-mass xylanase (xyl II) from *Chainia* was purified by ethanol precipitation and DEAE-cellulose chromatography followed by gel filtration on Sephadex G-50. The homogeneity of the enzyme was confirmed by SDS/PAGE [19]. A total purification of 9.2-fold over the culture filtrate was obtained with a final enzyme yield of 11 %. The specific activity of the purified enzyme was 41 units/mg. The molecular mass of xylanase from *Chainia* was determined to be 5000–6000 Da by gel filtration and SDS/gel electrophoresis [4,14].

Reaction of the low-molecular-mass xylanase from *Chainia* with *p*-hydroxymercuribenzoate and subsequent kinetic analysis implied the presence and participation of 1 mol of cysteine in the

Figure 2 Kinetics of inactivation of xylanase by DAM

Enzyme (5 μ g) was preincubated at 25 °C with DAM ($\square$, 0 mM; $\circ$, 0.5 mM; ∇ , 1 mM; $\triangle$, 2 mM; $\diamond$, 3 mM). The inset shows the apparent order of reaction with respect to reagent concentration. The logarithms of observed pseudo-first-order rate constants (k) calculated from the main Figure were plotted against the logarithm of the inhibitor concentration.

active site of the enzyme [3]. In the present work, the functional cysteine group was labelled by reaction with DAM, a chromophoric thiol-specific derivative of NEM. The treatment of xylanase with DAM resulted in progressive inhibition of enzyme activity with increasing concentrations of the reagent (Figure 2). A double-logarithmic plot of the observed pseudo-first-order rate constant against reagent concentration yielded a reaction order of 0.95 (Figure 2, inset). The second-order rate constant for the inactivation of the xylanase by DAM was calculated to be $3.61 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$. The DAM-treated enzyme showed complete inhibition of the enzyme activity with a concomitant increase in absorbance at 340 nm (Figure 3) characteristic of DAM-cysteine and corresponded to an incorporation of 0.9 mol of DAM/mol of enzyme.

Evidence for the presence of cysteine in the active site

Figure 4 depicts the results of protection of xylanase by varying amounts of xylan against inactivation by DAM. A progressive protection of inactivation by DAM was observed with increasing amounts of xylan, indicating that xylan and DAM compete for the same binding site, i.e. the cysteine residue in the active site. Xylan (5 mg) offered complete protection against inactivation of the enzyme by DAM. These results confirm that the cysteine is present at or near the substrate-binding region of the enzyme and the thiol modified by DAM is in the active site.

Figure 3 Absorption spectrum of the native and modified enzyme

The enzyme (20 mg) was incubated with 5 ml of DAM (50 mM) in a total volume of 10 ml in 50 mM sodium acetate buffer, pH 5.0, for 3 h at 25 °C and precipitated with ethanol. 'Optical density' $\equiv$ absorbance.

Figure 4 Protection of xylanase by substrate against inactivation by DAM

The xylanase (5 μ g) was incubated with varying amounts of xylan for 10 min at 4 °C prior to incubation with 10 μ l of DAM (500 μ M) at 25 °C for 15 min, in a total volume of 250 μ l. The xylanase activity was assayed by adjusting xylan to a final concentration of 5 mg per reaction mixture.

Isolation and purification of the cysteine-containing peptide

In order to locate the reactive cysteine-containing peptide, the DAM-treated enzyme was hydrolysed with pepsin. The mixture

Figure 5 Gel filtration on Sephadex G-25 of peptic peptides of modified xylanase

DAM-treated enzyme (4 mg) was digested with pepsin (400 μ g) at 37 °C for 24 h at pH 2 and the digest was subjected to gel filtration on Sephadex G-25. Fractions (2.5 ml) were collected and monitored for absorption at 210 nm (○) and 340 nm (×).

Figure 6 Purification of the cysteinyl peptide by reverse-phase HPLC

The pooled yellow fraction from a Sephadex G-25 column was subjected to reverse-phase HPLC according to the conditions described in the Materials and methods section.

of peptides was subjected to gel filtration on a Sephadex G-25 column. (Figure 5). The fractions showing distinct yellow colour and higher A_{340}/A_{210} ratios were pooled and lyophilized. Further purification of the yellow peptide was achieved by its separation on HPLC (Figure 6). The peptide containing the DAM-cysteine (peak A) was separated from the unreacted DAM (peak B).

Sequencing of the cysteine-containing peptide

Analysis of the cysteinyl peptide (Figure 6, peak A) yielded the sequence Glu-Thr-Phe-Xaa-Asp. The Xaa represents a modified cysteine residue which is unable to form a phenylthiohydantoin derivative.

DISCUSSION

The xylanase from *Chainia* belongs to the G family of xylanases which encompasses the low-molecular-mass basic xylanases, most of which have been isolated from *Bacillus* sp. [20]. Xylanases are functionally related to cellulases and lysozyme. Tryptophan and carboxylic acids have been shown to be involved in the catalytic mechanism of xylanases, cellulases and lysozyme. However, involvement of cysteine residues in the active site of xylanases appears to be restricted only to actinomycete xylanases [3,8]. The xylanase from *Cellulomonas fimi* has been shown to contain distinct cellulose- and xylan-binding domains [21]. The protein sequences of the catalytic domains of cellulases and xylanases have been compared [22]. However, the sequences around the functional residues have not been delineated. As a step towards the understanding of the structure–function relationship of xylanases, the primary objective of the present work was to decipher the structural environment of the functional cysteinyl residue in the xylanase from *Chainia* and compare it with that of xylanase from *Streptomyces*. NEM is a potent modifier for cysteine residues. However, it is of little value as a marker of such groups, as it forms colourless derivatives. The strategy used in the present work was to use DAM, the chromophoric derivative of NEM, as a reporter for isolating the peptide containing the cysteine group. The inability of DAM to inactivate the enzyme in the presence of xylan provides evidence that the modified thiol is in the active site. The taxonomic status of *Chainia* is controversial. While chemical and genetic data indicate a close relationship between the genus *Chainia* and *Streptomyces*, the development of vegetative mycelium into sclerotic granules and the presence of 2,3-diaminopropenoic acid in the cell wall are the main distinguishing features of *Chainia*. Our results reveal that although both the cysteinyl peptides contain phenylalanine and aspartic acid residues, the sequence of amino acids around the functional cysteine of xylanase from the thermotolerant *Streptomyces* T-7 [23] is distinctly different from that around the reactive cysteine of xylanase from *Chainia*. Comparison of the partial amino acid sequence of the xylanase from *Chainia* with the catalytic regions of xylanases from alkalophilic *Bacillus* sp. [24], *Clostridium thermocellum* [25] and *Cryptococcus albidus* [26,27] revealed a homologous region containing one aspartic and two glycine residues as the highly conserved residues. The active-site cysteinyl peptide from *Chainia* shows the presence of a conserved aspartic acid residue, but lacks the universal glycine residues. Whether the essential tryptophan residue of the xylanase from *Chainia* lies in the neighbourhood of the cysteine or is distributed distantly on the polypeptide chain and constitutes a substrate-binding pocket on folding of the protein would await collection of sequencing data for peptide containing the essential tryptophan residue. Nevertheless, the knowledge of amino acid sequence around the reactive cysteine

will be useful for deciphering the structure–function relationship of the xylanase using site-specific mutagenesis.

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