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Conformation and microenvironment of the active site of a low molecular weight 1,4-β-D-glucan glucanohydrolase from an alkalothermophilic *Thermomonospora* sp.: Involvement of lysine and cysteine residues ☆

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Abstract

Conformation and microenvironment at the active site of 1,4-β-D-glucan glucanohydrolase was probed with fluorescent chemo-affinity labeling using *o*-phthalaldehyde. OPTA has been known to form a fluorescent isoindole derivative by cross-linking the proximal thiol and amino groups of cysteine and lysine. Modification of lysine of the enzyme by TNBS and of cysteine residue by PHMB abolished the ability of the enzyme to form an isoindole derivative with OPTA. Kinetic analysis of the TNBS and PHMB-modified enzyme suggested the presence of essential lysine and cysteine residues, respectively, at the active site of the enzyme. The substrate protection of the enzyme with carboxymethylcellulose (CMC) confirmed the involvement of lysine and cysteine residues in the active site of the enzyme. Multiple sequence alignment of peptides obtained by tryptic digestion of the enzyme showed cysteine is one of the conserved amino acids corroborating the chemical modification studies.

Keywords: 1,4-β-D-Glucan glucanohydrolase; OPTA; *Thermomonospora* sp.; Active site microenvironment; TNBS; PHMB; Lysine; Cysteine

Cellulolytic and hemicellulolytic enzymes have raised enormous interest in the past decade in view of their ecological significance and biotechnological applications [1,2]. In a recent report from this laboratory we have shown that the purified 1,4-β-D-glucan glucanohydrolase (TSC) with molecular weight of 14.2 kDa from *Thermomonospora* sp. has a single active site for the substrates carboxymethylcellulose (CMC) and xylan [3]. TSC has been crystallized and the crystals diffract to better than 2.3 Å resolution [4].

Structure–function relationships are one of the central issues in the investigation of biological macromolecules. The conformational integrity of an enzyme is essential for its activity. Investigations involving chemical modification of an enzyme can potentially yield insights into structure–function relationships. In the present paper, we have attempted to correlate the loss in catalytic activity to the conformational changes occurring in TSC. The role of essential lysine and cysteine at the active site of the enzyme has been assessed by kinetic analysis of the TNBS and PHMB-modified enzyme. This is the first report of presence of lysine in the active site of cellulase. The nature of the active site has been assessed by using chemo-affinity labeling wherein *o*-phthalaldehyde, which has an absolute specificity for NH_2 and SH groups, was used. The conformation and microenvironment has also been assessed. Data presented here suggest for the time that cysteine and lysine residues are present in the active site of TSC by fluorescent labeling and group specific chemical modification.

Materials and methods

Production and purification of TSC. TSC was produced and purified according to the earlier report [3]. The carboxymethyl cellulase activity

☆ *Abbreviations:* kDa, kilo dalton; CMC, carboxymethyl cellulose; OPTA, *o*-phthalaldehyde; TNBS, 2,4,6-trinitrobenzenesulfonic acid; PHMB, *p*-hydroxy-mercury benzoic acid; HPLC, high performance liquid chromatography.

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and protein were determined according to Miller and Bradford [5,6], respectively.

Modification of TSC with o-phthalaldehyde. Enzyme sample (5 μg) was incubated with 1 mM OPTA for 10 min at 25 °C. The formation of enzyme-isoindole derivative was followed spectrophotometrically by monitoring the increase in fluorescence at 420 nm with excitation wavelength fixed at 338 nm. The aliquots were removed at intervals to check the residual carboxymethyl cellulase activity of the enzyme [7].

Modification of TSC with TNBS. TSC (10 μg) was incubated with varying concentrations of 2,4,6-trinitrobenzenesulfonic acid (4–10 mM) in water, in the presence of 1 ml of 4% sodium bicarbonate in the reaction volume of 3 ml at 37 °C in the dark. Aliquots were withdrawn at suitable time intervals to check the residual activity of enzyme. One milliliter of 10% SDS solution was added to solubilize the protein upon addition of 0.5 ml of 1 M HCl. The absorbance of solution at 340 nm was read against blank containing water instead of protein solution. An extinction coefficient of $1.4 \times 10^4\ M^{-1}cm^{-1}$ was used to calculate the number of amino groups [8]. The degree of inactivation in presence of different amounts of CMC was also determined. Enzyme samples (10 μg each) were incubated with different concentrations of CMC for 10 min before addition of inhibitors. Control tubes with only enzyme; only inhibitor and inhibitor/substrate were incubated under identical conditions.

Inactivation kinetics of TSC by PHMB. TSC (20 μg) was incubated with varying concentrations of *p*-hydroxy-mercury benzoic acid (PHMB) in 50 mM phosphate buffer, pH 7, at 25 °C and assayed for carboxymethyl cellulase activity at different time intervals. Control tubes having only enzyme or only inhibitor or inhibitor and substrate were incubated under identical conditions. Substrate protection studies were performed by incubating the enzyme with the substrate CMC for 10 min prior to the addition of the modifier.

PHMB titration of TSC. TSC (7×10^{-5} M) in 50 mM phosphate buffer, pH 7.0, was titrated with 10 μl PHMB (1 mM) and the progress of the reaction was monitored spectrophotometrically at 250 nm. Simultaneously aliquots of the reaction mixture were withdrawn to assay the residual activity. The number of Cys residues modified was determined by the method of Boyer [9].

Circular dichroism measurements. Circular dichroism spectra were recorded in a Jasco-J715 spectrophotometer at ambient temperature using a cell of 1 mm path length. Replicate scans were obtained at 0.1 nm resolution, 0.1 nm bandwidth, and a scan speed of 50 nm/min. Spectra were average of six scans with the baseline subtracted spanning from 260 to 190 nm in 0.1 nm increments. The CD spectra of the native, TNBS (10 mM), and PHMB (1mM) modified TSC (25 μg/ml) were recorded in 10 mM sodium phosphate buffer, pH 7.

Multiple sequence alignment of TSC. The amino acid sequencing of the six peptides obtained by tryptic digestion was carried out. The peptide ASHMDQTYYLCSDVNFV with 17 amino acid residues was used for sequence alignment with the members of conserved domain family of cellulose binding domains of different enzymes. The multiple sequence alignments were constructed using CLUSTAL W software.

Results and discussion

Formation of isoindole derivative at the active site

Conformation and microenvironment at the active site of TSC was probed with fluorescent chemo-affinity labeling using *o*-phthalaldehyde as the chemical initiator. *o*-Phthalaldehyde is bifunctional reagent that forms isoindole derivative due to its reaction with SH and NH_2 groups of lysine and cysteine residues [10].

o-Phthalaldehyde reacted with TSC yielding a stable fluorescent derivative, which exhibited an absorbance characteristic of isoindole (420 nm) when excited at 338 nm with the complete loss of activity of the enzyme to hydrolyze both CMC and xylan (Fig. 1). Complete inactivation by *o*-phthalaldehyde might be due to the formation of isoindole derivative by cross-linking the proximal thiol and amino groups located at or near the active site of the enzyme. The substrates (CMC) protect the enzyme from inactivation by OPTA. Moreover, there was a linear relationship between fluorescence increase and enzyme inactivation.

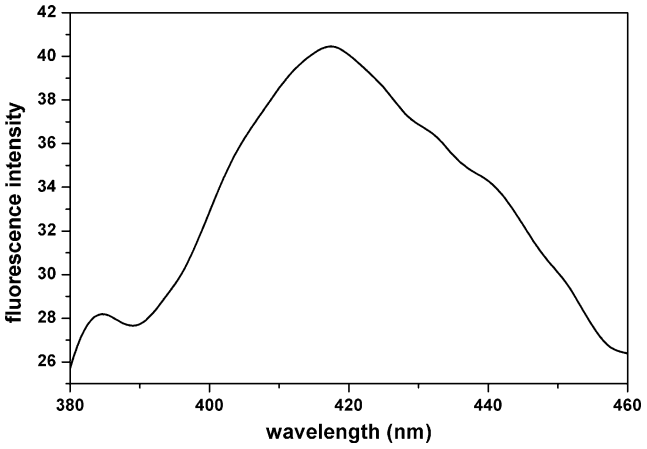

Fig. 1. Isoindole fluorescence of TSC-modified by OPTA. Enzyme sample (5 μg) was incubated with 1 mM OPTA for 10 min at 25 °C. The isoindole fluorescence was monitored at λ_{excit} 338 nm and λ_{emiss} 420 nm.

Inactivation kinetics of TNBS-modified enzyme

Incubation of TSC with different concentrations of TNBS resulted in a time and concentration dependent loss of enzyme activity as shown in (Fig. 2). The reaction followed pseudo first order kinetics. The pseudo first order

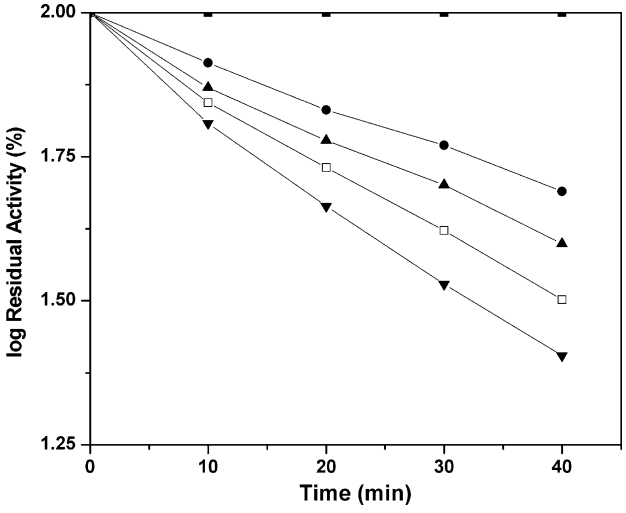

Fig. 2. Kinetics of inactivation of TSC by TNBS. Pseudo first order plots for the inactivation of TSC by TNBS. Enzyme was incubated with 4 mM (●), 6 mM (▲), 8 mM (□), 10 mM (▼), and control (■) at 37 °C. Aliquots were removed at indicated time intervals.