Available online at www.sciencedirect.com
ScienceDirect
Enzyme and Microbial Technology 41 (2007) 440–446
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Probing the active site environment of alkaliphilic family 11 xylanase from *Penicillium citrinum*: Evidence of essential histidine residue at the active site

Tanmay Dutta, Rupam Sahoo, Sougata Sinha Ray, Arindam Bhattacharjee, Rajib Sengupta, Sanjay Ghosh *

Department of Biochemistry, University College of Science, Calcutta University, 35, Ballygunge Circular Road, Kolkata 700019, West Bengal, India

Received 26 December 2006; received in revised form 23 March 2007; accepted 26 March 2007

Abstract

Alkaliphilic xylanases are not only important for their biotechnological applications, but also for their implications in protein structure–function. Here, we present for the first time the presence of single active site histidine residue in the microenvironment of GH-family 11 alkaliphilic xylanase from extremophilic fungus *Penicillium citrinum* MTCC 6489 using chemical modifications. The kinetic studies showed a time dependent inactivation of xylanase by OPTA or DEPC resulting in a pseudo-first-order kinetics with a second-order rate constant of 49.8 and 5.08 min^{-1} M^{-1}, respectively. The difference spectrum of DEPC modified versus native protein exhibit an absorbance maximum at 244 nm characteristic of the formation of *N*-carbethoxyhistidine, which is completely reversed by neutralized hydroxylamine implying the presence of histidine residue. Moreover, the rate of inactivation shows pH dependence with an inflection point at 6.2. CD studies reveal no significant change in the DEPC modified xylanase conformation. Substrate dependent protection (0.5% xylan) from DEPC inactivation phenomenon conclusively proves the presence of histidine residue in the active site. To explore the presence of tryptophan in the active site xylanase is modified with NBS, which reveals its position in close proximity to active site, but not involved in catalysis.

E.C. number: 3.2.1.8

Keywords: GH-family 11 xylanase; *Penicillium citrinum*; Active site histidine; Extremophilic fungus; Substrate protection; Isoindole fluorescence

1. Introduction

Cellulase-free xylanases (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8) from extremophiles are gaining importance due to their biotechnological applications in paper and pulp industries and as useful model systems for structure–function studies [1,2]. Xylanases act on β-1,4-linked xylopyranosyl residues of the xylan backbone and in conjunction with cellulases convert the cellulosic biomass to sugars [3]. A number of family 10 xylanases with higher pH optima have been isolated from various bacteria and fungi [4–6]. Family 10 and 11 xylanases have been reported from a number of *Bacillus* sp., with a pH optimum of pH 8–10 [1,5,7–9]. The pH activity profiles of enzymes are highly dependent on the pK_a of the catalytic residues which are themselves dependent on the local environment and hence on the nature of the amino acids in the vicinity of the catalytic residues [1]. Many of the conserved amino acids of xylanases are believed to be structurally important for confirming the correct folding and packing [10].

In family 11 xylanases, two glutamate residues have been implicated in the catalytic mechanism. These two carboxylic acid residues suitably located in the active site are involved in the formation of the intermediate; one acts as a general acid catalyst by protonating the substrate, while the second performs a nucleophilic attack, which results in the departure of the leaving group and the formation of the α-glycosyl enzyme intermediate [5].

Chemical modification is one of the versatile tools to delineate requirement for catalytic activity of an enzyme. The identity

Abbreviations: GH, glycosyl hydrolase; DEPC, diethylpyrocarbonate; OPTA, *o*-phthalaldehyde; TNBS, 2,4,6-trinitrobenzenesulfonic acid; PHMB, *p*-hydroxymercuribenzoic acid; NBS, *N*-bromosuccinamide

* Corresponding author. Tel.: +91 33 2461 5445; fax: +91 33 2476 4419.
E-mail addresses: ghoshs71@hotmail.com, sgbioc@caluniv.ac.in (S. Ghosh).

0141-0229/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2007.03.012

of the functional amino acid(s) in the active site environment in alkaliphilic xylanase has therefore been explored by kinetic and chemical modification studies using diethylpyrocarbonate, *o*-phthalaldehyde and *N*-bromosuccinamide [11–14]. Diethylpyrocarbonate is specific for histidine at neutral pH. Presence of tryptophan residue in the microenvironment of xylanases could be identified by using tryptophan specific chemical modifier *N*-bromosuccinamide [15,16].

Extremophilic *Penicillium citrinum* secrets a low molecular weight novel xylanase (22 kDa) with a p*I* of 3.6 and belongs to the family G/11 according to the numerical classification of glycosyl hydrolase [17]. The enzyme exhibited stability and activity over a wide pH range of 4–10 with a pH optimum of 8.5. Despite the importance of extremophilic xylanases in biotechnological application no reports are documented deciphering the active sites and the microenvironment of fungal alkaliphilic xylanases. Hence, the characterization of active site residues of family 11 fungal alkaliphilic xylanase from *P. citrinum* would be the first report in that list.

In this paper, we present evidence by chemical modification studies that a xylanase loses its catalytic activity when a single histidine residue is modified with the histidine selective reagent DEPC.

2. Materials and methods

2.1. Materials

Birchwood xylan, dinitrosalicylic acid, diethylpyrocarbonate (DEPC), *o*-phthalaldehyde (OPTA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), *p*-hydroxymercuribenzoic acid (PHMB), *N*-ethylmaleimide, *N*-bromosuccinamide (NBS) and hydroxylamine were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used in this work were of analytical grade.

2.2. Microorganisms and growth conditions

P. citrinum (MTCC-6489) is an alkali tolerant fungus. It was isolated from soil from the Dhapa situated near Kolkata, India. *P. citrinum* was cultivated in solid-state fermentation. The enzyme was extracted from solid matrix and was purified as described previously [17]. The purity of the enzyme was confirmed by SDS-PAGE and gel filtration chromatography [17].

2.3. Purification of xylanase

The *P. citrinum* was grown at 30 °C for 96 h for the production of xylanase. The enzyme was purified to homogeneity from the extracellular culture filtrate by 0–80% ammonium sulfate precipitation (w/v), followed by phenyl sepharose affinity chromatography. Native molecular weight was determined on gel filtration chromatography using Amersham Pharmacia Biotech Superdex-200 HR size-exclusion column [17].

2.4. Xylanase assay

The xylanase assay was carried out by incubating 0.3 mL of appropriately diluted enzyme in 0.05 M phosphate buffer, pH 7.0, with 0.3 mL of 1% birch wood xylan (w/v) in a final volume of 0.6 mL, at 50 °C for 30 min. The released reducing sugar was determined by the dinitrosalicylic acid method using D-xylose as standard [17]. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose equivalent per min from xylan under assay conditions. Protein concentration was determined according to the method of Bradford [18] using bovine serum albumin as standard.

2.5. Kinetic inactivation and isoindole formation by OPTA

Fresh OPTA solution was made in methanol for each experiment. The modification was carried out by incubating 2.5 μM of xylanase in 0.05 M potassium phosphate, pH 7.0, with varying concentrations of OPTA, at 25 ± 2 °C. Methanol had no effect on the activity of the enzyme and was always less than 2 % (v/v). At different time intervals, an aliquot was withdrawn from the reaction mixture and the residual activity was measured after stopping the reaction by adding 5 μL of 0.01 M cysteine. The formation of xylanase–isoindole derivative was followed spectrofluorimetrically by monitoring the increase in fluorescence with the excitation wavelength fixed at 338 nm [13].

2.6. Reaction of xylanase with DEPC, PHMB and *N*-ethylmaleimide

Xylanase (2.5 μM) in potassium phosphate buffer, 0.05 M, at pH 7.0 was incubated with varying concentrations of DEPC (0.002–0.01 M) at 25 ± 2 °C. Freshly prepared DEPC in absolute ethanol was used; samples were removed periodically at different time intervals and the reaction was arrested by the addition of 50 mL of 0.01 M imidazole buffer, pH 7.5. The residual activity of the diluted enzyme derivative was determined under standard assay conditions and expressed as a percentage of the control. The exact concentration of the stock solution was calculated from the increase in the absorbance at 230 nm when an aliquot of the DEPC solution was added to a solution of 0.01 M imidazole in 0.05 M potassium phosphate buffer, pH 7.5, using an extinction coefficient of 3200 M^{-1} cm^{-1} [11,12]. For pK_a, determinations, xylanase was incubated with DEPC at various pH values (pH 5.5–8). The modification reaction is specific for an unprotonated histidine residue between pH 5.5 and 7.5 [11]. The stoichiometry of the formation of *N*-carbethoxyhistidine residues was calculated from the increase in absorbance at 244 nm using the extinction coefficient of 3200 M^{-1} cm^{-1}. The reaction was initiated by the addition of DEPC and was terminated when the maximum absorbance at 244 nm had been attained.

Xylanase (2.5 μM) was incubated with different concentrations of PHMB (0.01–0.05 M) in 0.05 M potassium phosphate buffer, pH 7 at 25 ± 2 °C. Samples were removed at different time intervals and assayed for residual xylanase activity. Control tubes having only enzyme or only inhibitor were incubated under identical conditions. Similar experiments were performed in the presence of *N*-ethylmaleimide.

2.7. Substrate protection against inactivation by DEPC

The xylanase (5 μg) was incubated with different amounts of xylan (1–5 mg) for 10 min at 4 °C. A 10 μL aliquot of DEPC (0.01 M) was added and the reaction mixture was incubated at 25 ± 2 °C for 15 min in a total volume of 250 μL. 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 DEPC were run.

2.8. Fluorescence measurements

Fluorescence measurements were performed with a Hitachi F3010 automatic recording spectrofluorimeter with an excitation and emission bandwidth of 4 mm in a quartz cuvette. An excitation wavelength of 295 nm was used. The effect of NBS on the activity and fluorescence of xylanase was determined after incubation of enzyme (2.5 μM) with different aliquots of NBS at 25 ± 2 °C for 15 min.

2.9. Circular dichroism spectroscopy

CD spectra were recorded in a Jasco-J715 spectropolarimeter 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^{-1}. Spectra were averages of six scans with the baseline subtracted spanning from 250 to 195 nm in 0.1 nm increments. The CD spectra of the native and DEPC (0.01 M) modified xylanase (25 μg mL^{-1}) were recorded in 0.05 M potassium phosphate buffer (pH 7.0).