ELSEVIER

FEMS Microbiology Letters 145 (1996) 457–461

FEMS MICROBIOLOGY LETTERS

Purification and characterization of xylanase from alkali-tolerant *Aspergillus fischeri* Fxn1

K. Chandra Raj, T.S. Chandra *

Department of Chemistry, Indian Institute of Technology, Madras 600 036, India

Received 11 September 1996; revised 24 October 1996; accepted 28 October 1996

Abstract

Alkali-tolerant *Aspergillus fischeri* Fxn1 produced two extracellular xylanases. The major xylanase (M_r 31 000) was purified to electrophoretic homogeneity by ammonium sulfate precipitation, anion exchange chromatography and preparatory PAGE. Xylose was the major hydrolysis product from oat spelt and birch wood xylans. It was completely free of cellulolytic activities. The optimum pH and temperature were 6.0 and 60°C, respectively. pH stability ranged from 5 to 9.5 and the $t_{1/2}$ at 50°C was 490 min. It had a K_m of 4.88 mg ml^{-1} and a V_{max} of 588 μmol min^{-1} mg^{-1}. The activity was inhibited (95%) by $AlCl_3$ (10 mM). This enzyme appears to be novel and will be useful for studies on the mechanism of hydrolysis of xylan by xylanolytic enzymes.

Keywords: *Aspergillus fischeri*; Alkali tolerance; Xylanase; Purification

1. Introduction

Hemicelluloses are the second most abundant plant polysaccharide in nature. Xylans which are β-1,4-linked xylose polymers substituted by acetyl, α-D-glucuronyl, and α-L-arabinofuranosyl groups form the predominant fraction in hemicellulose followed by arabinans, galactans, glucans and mannans. To hydrolyze xylan completely, a consortium of enzymes called xylanolytic enzymes are required. Endo-xylanases have been investigated extensively compared to other xylanases as seen from many reviews [1].

Among the various applications of xylanases, the major one is in the removal of hemicellulose components in pulp and improvement of the paper qualities [2]. To be effective in applications in paper industries, xylanases should retain activity under alkaline conditions and be devoid of cellulases. Such xylanases were reported from alkalophilic bacteria and actinomycetes [3]. Although two alkali-tolerant fungi *Cephalosporium* sp. [4] and *Aspergillus fischeri* Fxn1 [5] have been reported to produce xylanases active under alkaline conditions, none has been purified and characterized. The present study is the first report on the purification and characterization of xylanase from an alkali-tolerant fungus.

* Corrresponding author. Tel.: +91 (44) 2351365, ext. 3113; Fax: +91 (44) 235-0305; E-mail: chem2@iitm.ernet.in, chem3@iitm.ernet.in

PII S0378-1097(96)00452-1

2. Materials and methods

2.1. Organism and xylanase production

A. fischeri Fxn1 isolated in our laboratory was used for production of enzyme. Solid-state cultures in 500-ml Erlenmeyer flasks were carried out using wheat bran (10 g) with 10 ml mineral base medium [5] and yeast extract (0.1%) at pH 9.0 and 30°C. Spores from 4-day-old potato dextrose agar slants were inoculated, and after 60 h of incubation the enzyme was extracted with 50 ml of 0.05 M potassium phosphate buffer (pH 6). The supernatant was used as the crude enzyme.

2.2. Enzyme assays

Xylanase was assayed using birch wood xylan [5]. Reducing sugars released were measured by the 3,5-dinitrosalicylic acid method. One unit of xylanase is defined as the amount of enzyme that liberated 1 μmol of xylose equivalent per min. β-D-Xylosidase was assayed according to Poutanen and Puls [6]. Acetyl esterase was assayed similarly to β-xylosidase using *p*-nitrophenyl acetate as substrate. Cellulase was assayed according to the IUPAC method. Protein was estimated using Folin-Ciocalteu phenol reagent.

2.3. Purification

After $(NH_4)_2SO_4$ (60% w/v) precipitation of the supernatant, the precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 8.0 (PPB), dialyzed and loaded (25 ml) onto a DEAE-cellulose column (1.5×10.0 cm) pre-equilibrated with PPB. Fractions (3 ml) were collected (0.5 ml min^{-1}) by elution with PPB in a continuous NaCl gradient (0–0.3 M). The pooled xylanase fractions (4–14) were lyophilized to 8 ml, dialyzed and loaded onto a DEAE-Sephadex A50 column (1.0×10.0 cm) pre-equilibrated with PPB. Fractions (3 ml) were collected (0.3 ml min^{-1}) by elution with the same buffer in a continuous NaCl gradient (0–0.5 M). The xylanase fractions (12–30) were pooled, lyophilized and applied to preparatory tube gel electrophoresis.

2.4. Electrophoresis and activity staining

Proteins were analyzed by slab PAGE (12% gel) using Coomassie Blue R250. Preparatory PAGE was carried out in tube gels (1.2×6.0 cm; 12% gel) and the proteins were trapped using a dialysis membrane. The xylanase active fractions (8–12) were pooled, dialyzed against PPB (pH 6.0) and stored at 0°C. Activity staining (zymogram) was performed using 2% (w/v) agar gel containing 0.5% (w/v) birch wood xylan and Congo Red staining [7].

2.5. Characterization

The relative molecular mass of xylanase was deter-

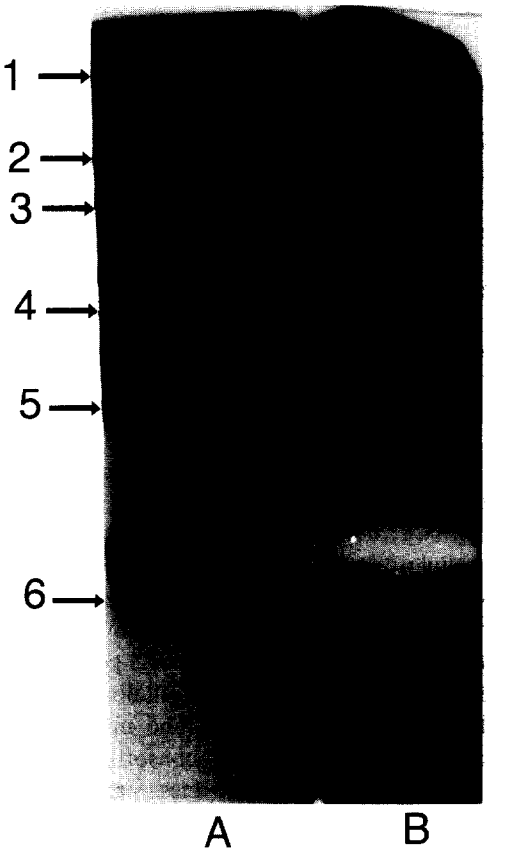

Fig. 1. (A) SDS-PAGE analysis of purified xylanase. Left lane: (1) myosin (205 kDa), (2) β-galactosidase (116 kDa), (3) bovine albumin (66 kDa), (4) egg albumin (45 kDa), (5) carbonic anhydrase (29 kDa). Right Lane: pure xylanase. (B) Activity staining of pure xylanase.

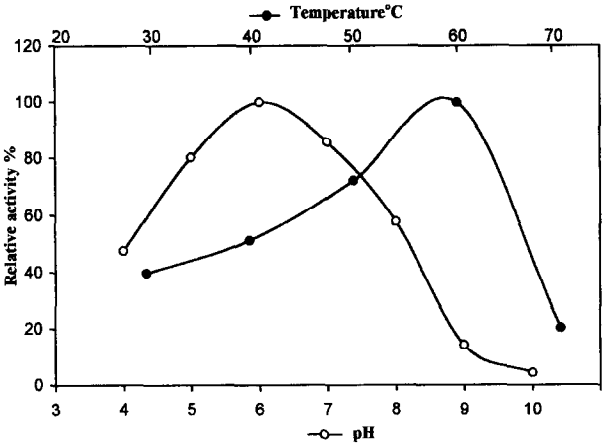

Fig. 2. Effect of pH and temperature on activity of xylanase of *Aspergillus fischeri* Fxn1.

mined by SDS-PAGE. The pH stability was determined by incubating the enzyme in various buffers for 24 h at 5°C and thermal stability was assessed from 30 to 70°C at pH 6. K_m and V_{max} for birch xylan were determined (pH 6.0 and 30°C) from the Lineweaver-Burk plot.

2.6. Analysis of hydrolytic products

To 10 ml of 1% (w/v) xylan (oat spelts or birch) at pH 6.0, 2.0 ml of xylanase (100 IU ml^{-1}) was added and incubated at 50°C. When hydrolysis reached equilibrium, the hydrolysate was lyophilized to powder, redissolved in 50% (v/v) methanol (300 μl), applied (10.0 μl) onto thin-layer chromatographic plates and developed with a solution of *n*-butanol:acetic acid:water (2:1:1, v/v) [7]. The sugars were visualized using concentrated H_2SO_4:95% ethanol (1:1, v/v) reagent and heating at 150°C for 20 min.

3. Results and discussion

3.1. Purification

Preliminary activity staining showed two xylanases in the culture broth. Ammonium sulfate precipitation followed by DEAE-cellulose column chromatography removed the coloring matter. DEAE-Sephadex A50 column chromatography resulted in one

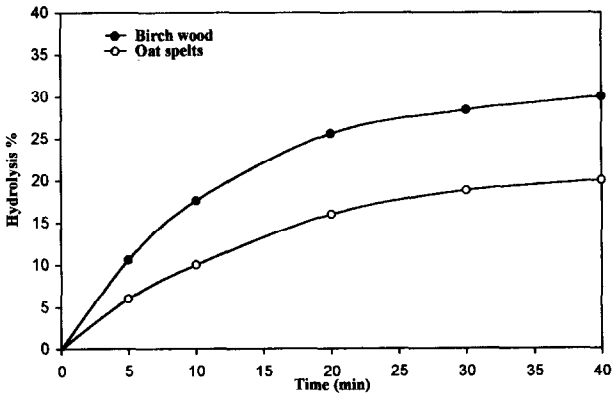

Fig. 3. Hydrolysis of xylan by xylanase of *Aspergillus fischeri* Fxn1 at pH 6.0 and 50°C. Hydrolysis (%)=[reducing sugars released]×100/[initial total sugars].

xylanase peak which showed two protein bands by SDS-PAGE. These were further separated by preparatory PAGE. The resulting xylanase fraction with 25% yield showed 11.5-fold purity, electrophoretic homogeneity and a single band on activity staining (Fig. 1).

3.2. Characterization

The approximate M_r was 31 000, and those of *Aspergillus* sp. xylanases were also reported to be from 13 000 to 50 000 [8,9].

The K_m was 4.8 mg ml^{-1} which was low compared to the reported values of 2–17 mg ml^{-1} [10] showing high affinity for xylan and the V_{max} was 588 μmol min^{-1} mg^{-1}. It had no cellulase activity which is a desirable feature for potential application in pulp and paper industries. β-Xylosidase and esterase were not detected.

Al^{3+} inhibited activity of xylanase (retained 5% of original) similar to that from *A. niger* [8], Co^{2+}(110%) stimulated activity as in *A. nidulans* xylanase [9] and K^+, Na^+, Ca^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+} Mg^{2+} and EDTA had no effect. Hg^{2+} (70%) and Fe^{3+}(70%) showed less effect, although most xylanases are inhibited by Hg^{2+} [11].

3.3. Effect of pH and temperature

The optimum pH of the xylanase was 6.0, although it retained over 50% of maximum activity at pH 8.0 (Fig. 2). The optimum pH for other fungal xylanases are also in the range 4.0–7.0 [11]. pH stability ranged from 5.5 to 9.5 with retention of more than 85% of the activity. The optimum temperature was 60°C (Fig. 2) while most other fungal xylanases are active from 40 to 55°C [11]. The activation energy was 27.4 kJ mol^{-1} which was less than the reported values of 30–68.2 kJ mol^{-1} [9]. Although the half-lives for inactivation at 50 and 55°C were 490 and 60 min at the optimum temperature of 60°C, the enzyme became inactivated rapidly ($t_{1/2}$ 8 min) suggesting that thermal stability was enhanced by the substrate as reported previously [12]. The enthalpy of activation for thermal denaturation was 318 kJ mol^{-1} which was higher than that of other xylanases [13].

3.4. Hydrolysis pattern

The degree of hydrolysis of birch wood xylan (30%) was higher than that of oat spelt xylan (20%) (Fig. 3). Oat spelt xylan with 75% xylose, 10%arabinose and 15% glucose (Sigma catalogue) is more substituted [14] compared to birch wood xylan which has >90% xylose. TLC analysis showed xylose as the product in the hydrolysate independent of the type of xylan and higher oligosaccharides were not detected. These data suggest that the mode of action of xylanase is endwise (exo) and that oat spelt xylan may have more substituted xylose residues on the ends which limit the xylanase action. A xylanase from *Aeromonas caviae* [7] with a similar mode of action has been reported which releases xylobiose as the major hydrolysis product.

Although this xylanase released xylose from xylan it was not acting on *p*-nitrophenyl-β-D-xyloside, indicating specificity towards xylan or higher xylo-oligosaccharides. However, systematic studies with specific xylo-oligosaccharides are necessary to reveal the actual mechanism of action. As there are no reports on the isolation or characterization of such xylanases from alkali-tolerant fungi, this enzyme appears to be novel and will be useful in understanding the mechanism of hydrolysis of xylan by xylanolytic enzymes. Further systematic kinetic studies on the molecular mechanism of its catalysis are being carried out.

Acknowledgments

We thank ICAR, New Delhi, India for financial support of this work.

References

[1] Coughlan, M.P. and Hazlewood, G.P. (1993) β-1,4-D-Xylan degrading enzyme systems: Biochemistry, molecular biology and applications. Biotechnol. Appl. Biochem. 17, 259–289.
[2] Roberts, J.C., Mc Carthy A.J., Flynn N.J. and Broda, P. (1990) Modification of paper properties by the treatment of pulp with *Saccharomonospora viridis* xylanase. Enzyme. Microb. Technol. 12, 210–213.
[3] Srinivasan, M.C. and Rele, M.V. (1995) Cellulase-free xyla-

nases from microorganisms and their applications to pulp and paper biotechnology: an overview. Ind. J. Microbiol. 35, 93–101.

[4] Bansod, S.M., Dutta-Choudary, M., Srinivasan, M.C. and Rele, M.V. (1993) Xylanase active at high pH from an alkali-tolerant *Cephalosporium species*. Biotechnol. Lett. 15, 965–970.

[5] Chandra Raj, K and Chandra, T.S. (1995) A cellulase-free xylanase from alkali-tolerant *Aspergillus fischeri* Fxn1. Biotechnol. Lett. 17, 309–314.

[6] Poutanen, K. and Puls, J. (1988) Characteristics of *Trichoderma reesei* β-xylosidase and its use in the hydrolysis of solubilised xylans. Appl. Microbiol. Biotechnol. 28, 425–432.

[7] Kubata, B.K., Suzuki, J., Horitsu, H., Kawai, K. and Takamizawa, K. (1994) Purification and characterization of *Aeromonas caviae* ME-1 xylanase V which produces exclusively xylobiose from xylan. Appl. Environ. Microbiol. 60, 531–535.

[8] Frederick, M.M., Chih-Han Kiang, Frederick, J.R. and Reilly, P.J. (1985) Purification and characterization of Endoglucanases from *Aspergillus niger*. I. Two isoenzymes active on xylan backbones near branch points. Biotechnol. Bioeng. 27, 525–532.

[9] Fernandez-Espinar, M.T, Pinaga, F., Sanz, P., Ramon, D. and Valles, S. (1993) Purification and characterization of a neutral endoxylanase from *Aspergillus nidulans*. FEMS Microbiol. Lett. 113, 223–228.

[10] Koyama, H., Ujiie, M., Tanaguchi, H. and Sasaki, T.C. (1990) Purification and some properties of xylan-hydrolyzing enzyme from *Robillarda* sp. Y-20. Enzyme. Microb. Technol. 12, 218–224.

[11] Wong, K.K.Y. and Saddler N.J. (1992) *Trichoderma* xylanases, their properties and applications. Crit. Rev. Biotechnol. 12, 413–435.

[12] Kavita, R., Bandivadekar and Deshpande, V.V. (1994) Enhanced stability of cellulase-free xylanase from *Chainia* sp. (N.C.L 82.5.1). Biotechnol. Lett. 16, 179–182.

[13] Arase, A., Yoma, T., Urabe, I., Hata, Y., Katsube, Y. and Okada, H. (1993) Stabilization of xylanase by random mutagenesis. FEBS Lett. 316, 123–127.

[14] Dekker, R.F.H. and Richards, G.N. (1975) Purification, properties and mode of action of hemicellulase I by *Ceratocystis paradoxa*. Carbohydr. Res. 39, 97–114.