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Process Biochemistry 40 (2005) 2339–2343

PROCESS BIOCHEMISTRY

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Separation and identification of endoxylanases from *Bacillus subtilis* and their actions on wheat bran insoluble dietary fibre

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Received 8 December 2003; received in revised form 16 September 2004; accepted 22 September 2004

Abstract

A novel and convenient method based on native polyacrylamide gel electrophoresis (PAGE) and homogenization extraction was used for the purification of xylanase from crude enzymes. Two xylanases were purified by this method from the crude enzyme preparation from the selected strain of *Bacillus subtilis*. Subsequent analysis with thin layer chromatography and high pressure liquid chromatography (HPLC) indicated that these two xylanases were endo-acting enzymes, designated xyl I and xyl II. Both enzymes showed their activities in the pH range from 5.0 to 9.0 at 50 °C and had similar optimum activities at pH 7.0 and at 50 °C. Mn^{2+} ions enhanced their xylanolytic activities to 2.7-fold whereas Fe^{3+} completely inhibited them. The action of endoxylanase xyl II on wheat bran insoluble dietary fibre was also studied. The hydrolysis products were shown to contain feruloyl oligosaccharides by paper chromatography.

Keywords: *Bacillus subtilis*; Electrophoresis; Endoxylanase; Purification; Wheat bran

1. Introduction

Wheat bran is produced worldwide in enormous quantities as an important by-product of the cereal industry. The outer tissues of the wheat kernel imply that the wheat bran consists mainly of cell wall polysaccharides, among which xylans represent 40% of dry matter [1]. Xylans consist of a linear backbone of β-(1 → 4) linked D-xylopyranosyl residues containing individual α-L-arabinofuranosyl residues attached through O-2 and/or O-3 [2]. A feature of some xylans is the existence of feruloyl residues that are esterified with L-arabinofuranose [3]. Feruloyl groups can form cross-linkages between polysaccharide chains by peroxidase-catalysed dimerization or between lignin and polysaccharides via ether-ester bonds [4].

Due to their structural complexity several specific enzymes are required for the complete depolymerization of wheat bran xylans. Among the xylanolytic systems, endoxylanases (endo-1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) are the crucial enzyme components [5,6]. Most xylanases from bacteria and fungi are produced as a mixture of different hydrolytic enzymes [7,8]. The classical procedures of xylanases purification, for example, precipitation, ion exchange, affinity and gel exclusion chromatography methods need a number of purification steps and considerable time [9]. In this research, an enzyme purification procedure using native polyacrylamide gel electrophoresis (PAGE) combined with homogenization extraction was described. Two different endoxylanases were successfully purified from a crude enzyme preparation derived from a selected strain of *Bacillus subtilis*. To our knowledge, this approach has not been previously reported for xylanases purification. In addition, some properties of the two endoxylanases and their actions on wheat bran insoluble dietary fibre are presented in this paper.

2. Materials and methods

2.1. Materials

Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1 from *Bacillus licheniformis*, 120 KNU/g), the protease Alcalase

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doi:10.1016/j.procbio.2004.09.019

2.4 L FG (EC 3.4.21.62, from *B. licheniformis*, 2.4 AU/g), and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were from Novo Nordisk, Bagsvaerd, Denmark. Amberlite XAD-2 was obtained from Rohm and Haas Company (Philadelphia, U.S.A.). Birchwood xylan and chemicals for electrophoresis were purchased from Sigma Company. Other chemicals were all of analytical grade.

Wheat bran was obtained after flour milling. The bran was milled and passed through a 0.5 mm sieve.

Crude enzyme preparation from a selected strain of *B. subtilis* was kindly supplied by Dr. Tao Liu of the Sunhy Biology Company (Wuhan, the People's Republic of China). Four grams of crude enzyme were dissolved in 100 mL of acetate buffer (0.05 M, pH 5.0). The precipitate was removed by centrifugation (20,000 × *g*, 20 min), whereas the resulting supernatant was used as the crude enzyme solution.

2.2. Native polyacrylamide gel electrophoresis and protein preparation

Native PAGE was performed in the absence of sodium dodecyl sulphate (SDS) according to the method of Davis [10] with 4% stacking and 10% separating gels. The sample buffer did not contain SDS or β-mercaptoethanol. The electrophoresis buffer was a 0.05 M Tris/0.384 M glycine system (pH 8.3). The separating gel was run at a constant current of 20 mA for about 3 h.

Once electrophoresis was complete, two longitudinal strips were cut out from the sides of the gel with a razor blade and proteins were stained with Coommassie Brilliant Blue G-250 [11]. The stained side strips were lined up along the edges of the unstained gel and then each band corresponding to the stained proteins was cut out with a razor blade [12]. Each slice was subsequently washed three times for 5 min with 0.25 M Tris–HCl buffer (pH 6.7, 2 mL) in a 25 mL flask, followed by three rinses with distilled water for 5 min. The residual water was removed with a pipette and the gel slices were chopped into pieces of 2–5 mm and an appropriate volume of 0.05 M acetate buffer at pH 5.0 (the ratio of buffer volume to gel piece volume was 2:1) was added. The sample was homogenized discontinuously at 10,000 rpm for 3 min with a homogenizer (high speed controllable homogenizer, FSH-2 Model). The gels were removed from the sample by centrifugation (20,000 × *g*, 20 min) and the resulting supernatant solution was used as purified protein. The process described was repeated several times to obtain sufficient purified proteins for subsequent studies.

2.3. Enzymes assays

Xylanase activity was routinely assayed in a reaction mixture (2 mL) containing boiled birchwood xylan (1%, w/v), 0.05 M acetate buffer, pH 5.0 and appropriate diluted enzyme solution. The reducing sugar produced in the reaction mixture at 50 °C was measured by a dinitrosalicylic acid method with xylose as standard [13].

2.4. Analysis of xylanase reaction product

The xylanase reaction products were preliminarily analyzed by thin layer chromatography on silica gel plates using *n*-butanol/pyridine/water (6:4:3) as the solvent system. Sugars were stained with an orcinol–sulphuric acid spray. The products were further analyzed by high pressure liquid chromatography (HPLC) using a Sugar-Pak^{TM}1 column (300 mm × 6.5 mm i.d.). The column was maintained at 85 °C. A sample volume of 10 μL was run at a flow rate of 0.5 mL/min. The detector signal was electronically monitored with a Waters 2401 refractive index detector.

2.5. Preparation of wheat bran insoluble dietary fibre

Wheat bran (10 g) was autoclaved for 45 min at 121 °C in order to destroy endogenous enzymatic activities [14] and subsequently swollen at 60 °C for 16 h in water (300 mL) with continuous stirring. α-Amylase (0.75 mL) was then added. Beakers were heated in a boiling water bath for 40 min and shaken gently every 5 min. The pH was adjusted to 7.5 with 0.275 M NaOH, and samples were incubated with protease (0.3 mL) at 60 °C for 30 min with continuous agitation. After the pH had been adjusted to 4.5 with 0.325 M HCl, amyloglucosidase (0.35 mL) was added and the mixture was incubated at 60 °C for 30 min with continuous agitation. The suspension was centrifuged (20,000 × *g*, 10 min). The residue was stirred in hot distilled water, washed repeatedly by decantation with large volumes of hot water, and then washed with cold distilled water until no cloudiness was evident. Finally, it was washed twice with hot distilled water, 95% (v/v) ethanol and acetone successively and then dried at 40 °C overnight in a vacuum oven to give wheat bran insoluble dietary fibre [15].

2.6. Enzymatic degradation of wheat bran insoluble dietary fibre

One gram of wheat bran insoluble dietary fibre was incubated in 40 mL of endoxylanase and 0.05 M acetate buffer (pH 5.0) at 50 °C in the dark for 60 h with constant stirring. After heat inactivation of the enzyme (100 °C, 10 min), wheat bran insoluble dietary fibre hydrolysate was obtained by centrifugation (20,000 × *g*, 20 min) and passed through a 0.45 μm filter.

2.7. Fractionation of feruloyl oligosaccharides

The enzymatic hydrolyzate was applied to a column (30 cm × 2.5 cm) of Amberlite XAD-2 (previously washed with 95% ethanol and then water). Elution was carried out with 2 column volumes of distilled water, 3 column volumes

of 50% (v/v) methanol/water and 2 column volumes of methanol. The fraction eluted by methanol/water was concentrated to 5 mL. The Feruloyl oligosaccharides in the concentrated elution were analyzed by paper chromatography, which was performed on Whatman No. 1 paper by the descending method with *n*-butanol/acetic acid/water (12:3:5) as the mobile phase [16]. The separated feruloyl oligosaccharides were located by UV radiation (before and after exposure to NH_3) [17], and stained with an oxalate/aniline reagent [18] (2 volumes of 2% aniline in ethanol and 3 volumes of 2.5% oxalic acid).

3. Results and discussion

3.1. Native PAGE and protein preparation

Xylanases are typically purified using two or more chromatographic steps [19]. The approach based on separation by native PAGE and purification by homogenization extraction was used in order to simplify the purification of xylanases excreted by *Bacillus*. The effect of different electrophoretic conditions on separation of crude enzyme was studied. The ionic strength and pH of the separating gel buffer were the main factors influencing native PAGE. For the crude xylanases, a gel concentration of 10%, 0.375 M Tris–HCl (pH 8.9) as separation buffer and 0.05 M Tris/0.384 M glycine (pH 8.3) as running buffer provided a satisfactory separation effect. As shown in Fig. 1, five clear protein bands were obtained after native PAGE for the crude enzyme solution, and named as bands 1, 2, 3, 4 and 5 from low to high molecular weight. After running another gel, the five proteins were purified by homogenization extraction according to the method described in Section 2. The protein purification procedure using native polyacrylamide gel electrophoresis (PAGE) combined with homogenization extraction allowed considerable time saving.

3.2. Identification of endoxylanase

3.2.1. Xylanolytic activity assays for proteins

The five purified proteins (0.2 mL) were incubated with 1% birchwood xylan at 50 °C and pH 5.0 for 30 min, respectively, and the reducing sugars produced were determined spectrophotometrically at 550 nm. A higher absorbance indicated a high level of reducing sugar produced and consequently, a high enzyme activity. Of the five protein bands, bands 4 and 5 showed enzyme activity towards xylan, which demonstrated that these bands were well xylanases. Thus, it can be seen that the biological activity of the proteins was preserved after gel electrophoresis for the reason that no dissociating agent which affects protein charge was present in the native polyacrylamide gel electrophoresis method. Therefore, native polyacrylamide gel electrophoresis is an effective method to separate proteins with biological activity.

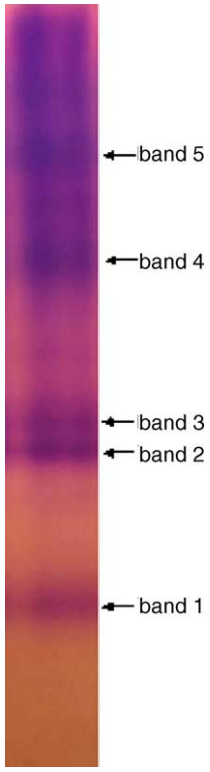

Fig. 1. Native PAGE of xylanases from *Bacillus subtilis*. The crude enzyme solution (0.1 mL) was electrophoresed at pH 8.3 on a 10% acrylamide gel and stained with Coommassie Brilliant Blue G-250. Bands 1, 2, 3, 4 and 5 were the different proteins from *Bacillus subtilis*.

3.2.2. Xylanase degradation pattern

The mode of action of xylanases was identified by thin layer chromatography of hydrolysates of birchwood xylan incubated with purified bands 4 and 5, respectively. The hydrolysis reaction was done at 50 °C and pH 5.0 for 24 h. Both bands 4 and 5 degraded xylan at random, the end products released were xylobiose, xylotriose, xylotetraose and higher xylooligosaccharides without xylose (Fig. not shown). The hydrolysate of band 5 was analyzed by HPLC (Fig. 2), and identical result was obtained with thin layer chromatography. The results indicated that the purified bands 4 and 5 were of typical endoxylanases, and defined as xyl I and xyl II.

3.3. Enzyme characterization

3.3.1. Effect of temperature and pH on purified endoxylanases xyl I and xyl II activities

The optimum temperature of purified endoxylanases was determined by incubating the enzymes for 30 min at different temperatures ranging from 30 to 80 °C, with 1% xylan in 0.05 M acetate buffer at pH 5.0. As shown in Fig. 3, endoxylanases xyl I and xyl II were optimally active at 50 °C. With the continuous rise in temperature, the relative activities were reduced drastically. Finally, the two enzymes were almost inactivated at 80 °C.

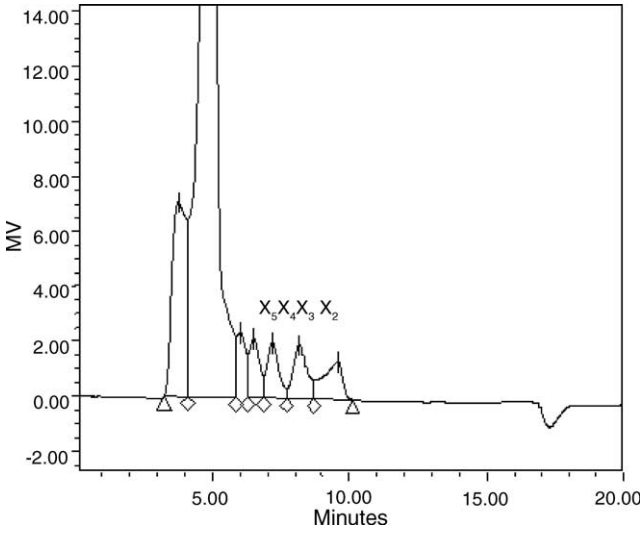

Fig. 2. HPLC of the birchwood xylan hydrolysate by purified xylanase band 5 (X_2: xylobiose; X_3: xylotriose; X_4: xylotetraose; X_5: xylopentaose). The purified xylanase band 5 was incubated in 0.05 M acetate buffer (pH 5.0) containing 1% birchwood xylan for 24 h at 50 °C.

The pH profile of purified endoxylanases was evaluated by incubating the enzymes for 30 min in the presence of appropriate buffers: 0.05 M sodium acetate buffer (pH 4.0–5.0), 0.05 M KH_2PO_4–NaOH buffer (pH 6.0–8.0) and 0.05 M glycine–NaOH (pH 9.0–10.0) at 50 °C. The activity of each sample was then quantified at room temperature by the dinitrosalicylic acid method. The pH profiles were presented in Fig. 4 and showed an optimum at pH 7.0 for purified endoxylanases xyl I and xyl II. The xyl I exhibited excellent activity at pH 6.0 (92%) and pH 8.0 (95%). The xyl II exhibited high activity at pH values ranging from 6.0 to 9.0 with only 6% relative activity at pH 4.0 and 7% activity at pH 10.0. A rapid decrease in activity was observed above 9.0 due to enzyme denaturation.

3.3.2. Effect of metal ions and reagents on enzyme activity

Xylanolytic activity in the presence of different metal ions, a chelating agent (EDTA) and a protein disulphide

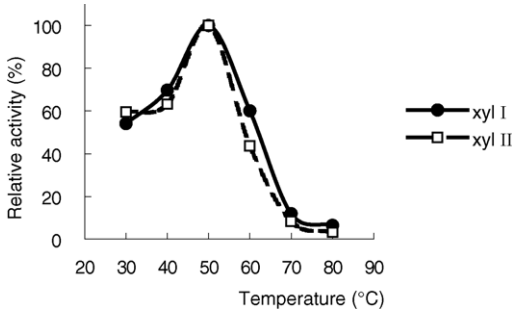

Fig. 3. Effect of temperature on the activities of purified endoxylanases xyl I (●) and xyl II (□) from *Bacillus subtilis*. The enzyme solution in acetate buffer (0.05 M, pH 5.0) was incubated for 30 min at various temperatures, and then the residual enzyme activities were assayed. Enzyme used, 0.2 mL.

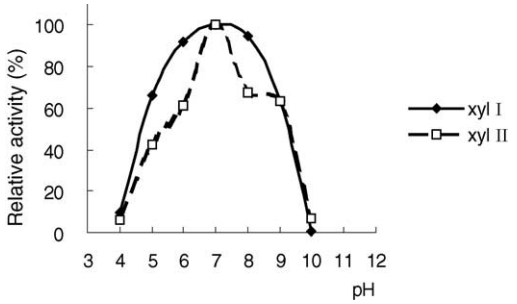

Fig. 4. Effect of pH on the activities of purified endoxylanases xyl I (◆) and xyl II (□) from *Bacillus subtilis*. The enzyme solution in appropriate buffer (0.05 M) was incubated for 30 min at 50 °C, and then the residual enzyme activities were assayed. Enzyme used, 0.2 mL. The following buffers were used: acetate (pH 4.0–5.0), KH_2PO_4–NaOH (pH 6.0–8.0) and glycine–NaOH (pH 9.0–10.0).

reducing agent (β-mercaptoethanol), in a final concentration of 0.005 M, was measured by the dinitrosalicylic acid method. Controls were done with additive but in the absence of enzyme. As shown in Table 1, the activities of purified endoxylanases xyl I and xyl II were partially inactivated by $Zn^{2+}$and K^+, strongly inactivated by Cu^{2+}, completely inhibited by the Fe^{3+} ions. Xyl II activity was reduced drastically in the presence of NH_4^+ ions. Mn^{2+} ions stimulated strongly their xylanolytic activities to 2.7-fold. The enzyme activities of purified endoxylanases xyl I and xyl II were also stimulated in the presence of the protein disulphide reducing reagent, β-mercaptoethanol. The activity of endoxylanase xyl II was inactivated more strongly than that of Xyl I by EDTA. Ca^{2+} ions had no influence on their activities.

3.4. Action on wheat bran insoluble dietary fibre

Wheat bran insoluble dietary fibre was incubated with purified endoxylanase xyl II and the enzymatic hydrolysate was applied to a column of Amberlite XAD-2, which is a

Table 1
Influence of chemical agents on the activities of purified endoxylanases xyl I and xyl II

Chemical agents	Relative activity (%) at 0.005 M additive concentration	
	xyl I	xyl II
Control	100	100
KCl	78.9	54.5
$CaCl_2$	97.7	97.8
$MnCl_2$	278.9	257.0
NH_4Cl	63.8	40.0
$ZnSO_4$	75.7	77.3
$Fe_2(SO_4)_3$	0	0
$CuSO_4$	13.3	36.8
EDTA	68.8	37.2
β-Mercaptoethanol	108.3	107.9

Fig. 5. Paper chromatography of hydrolysis products of wheat bran insoluble dietary fibre incubated with purified endoxylanase xyl II. The sugars were stained with an oxalate/aniline reagent.

polymeric adsorbent binding aromatic compounds [20]. The fraction eluted by methanol/water was subjected to paper chromatography. These separated compounds fluoresced blue in UV radiation and their colour changed green on exposure to NH_3, indicating they were feruloyl oligosaccharides [21]. When these feruloyl oligosaccharides were stained with an oxalate/aniline reagent, at least four reddish spots were seen (Fig. 5). This further demonstrated that the purified endoxylanase xyl II cleaved the backbone of xylan at random and the esterified ferulic acid did not limit degradation of xylan in wheat bran insoluble dietary fibre by the endoxylanase.

4. Conclusion

The described method for purification of xylanases from the selected strain of *B. subtilis* using native polyacrylamide gel electrophoresis combined with homogenization extraction was efficient and rapid. Two different purified endoxylanases, Xyl I and xyl II, had similar optimum temperature and pH. Mn^{2+} ions enhanced their activities to 2.7-fold whereas Fe^{3+} completely inhibited them. Both enzymes showed a mode of action on birchwood xylan, releasing xylooliogosaccharides without xylose. In addition, the purified endoxylanase xyl II was able to hydrolyze wheat bran insoluble dietary fibre, producing feruloyl oligosaccharides.

Acknowledgement

The authors thank Dr. Tao Liu (Sunhy Biology Company Wuhan, China) for his helpful discussions.

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