Purification and evaluation for effects of temperature on extracellular xylanase activity from *Aspergillus oryzae* DSM 1863

1. Introduction

Xylan is a major ingredient of the hemicellulose complex. To hydrolyze xylan, the cooperative action of several enzymes of different functions is necessary. The enzyme involved in the degradation of main polysaccharide chain are endo-1,4-β-D-xylanase (EC 3.2.1.8) randomly cleaving the xylan backbone, β-D-xylosidase (EC3.2.1.37) cleaving xylose monomers from the non-reducing end of xylooligosaccharides. The removal of side groups is catalysed by α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylosidases (EC 3.2.1.172), ferulic acid esterases (EC 3.1.1.73) and p-coumaric acid esterases (EC 3.1.1.-) (Collins et al., 2005).

Endoxylanase plays a key role in xylan hydrolysis and has applications in various industries including pulp (Zhao et al., 2006; Ayyachamy and Vatsala, 2007), bread making (Jiang et al., 2005; Romanowska et al., 2006), brewery, beverage (Polizeli et al., 2005), xylitol, feed enzyme (Khandeparker and Numan, 2008). Xylanase is used for pulp industry as it supports the bleaching process, reduce the toxic chemicals (chlorine) used to bleach lignin contained in the paper. Other important applications of xylanase are to make the bread fine and soft and extend the storage time; to purify fruit juice, wine and beer; and to form xylitol glucose used in confectionary industry. In the breeding, xylanase expedites the digestion process of food containing xylan and at the same time helps to reduce the viscosity in the digestive system and follow by many positive effects such as improved food absorption, improved microorganism of the intestine in the advantageous direction, reduced digestion disorder and drier excrement (Bernier et al., 1983).

A variety of microbes, including bacteria, yeast and filamentous fungi, has been reported to produce xylanases, in which the most potent producers are fungi (Haltrich et al., 1996). A number of xylanases have been purified from a wide variety of microbes such as *Bacillus* strains (Bernier et al., 1983; Panbangred et al., 1985; Ayyachamy and...
Vatsala, 2007) and Aspergillus strains (Fernandez-Espin et al., 1994; Fialho and Carmona, 2004; Krisana et al., 2005; Lu et al., 2008; Nair et al., 2008). However, xylanases are produced mainly by Aspergillus and Trichoderma sp. on an industrial scale. The fungus Aspergillus oryzae has been used in the tradition food industry of Japan for over 1000 years. As A. oryzae has been long used in food production, it is considered as a safe fungus and certificated by World Health Organization (MaChida, 2002). A. oryzae is able to secrete a large amount of enzyme in the environment. This property has been used in the commercial production for recombinant enzyme and natural enzyme of A. oryzae. Many types of recombinant protein are generated in submerged culture medium; however, in solid fermenting medium, A. oryzae as well as other types of fungus, is able to generate a large amount of enzyme which is higher than that generated in the submerged culture medium. In Japan, many types of commercial enzyme have been produced by solid fermentation as well as by conventional fermentation (Tsuchiya et al., 1994).

Here, we purified and characterized of xylanase from A. oryzae DSM1863. We also identified the optimum temperature and thermostable of the purified xylanase. The biochemical characteristics suggested that the xylanase has a potential application, including use as a feed enzyme.

2. Materials and methods

2.1 Strain and culture conditions

The Aspergillus oryzae DSM1863 purchased from the German Microorganism Collection Center (DSMZ) was grown on the liquid medium containing 2 g/l NaNO₃; 1 g/L KH₂PO₄; 0.5 g/L MgSO₄; 0.5 g/L KCl; 10 g/L soybean powder; 40 g/L corn cobs; pH 7.0. The inoculated flasks were incubated for 4 days at 30°C on a rotary shaker at 200 rpm.

2.2 Chemicals

Birch wood xylan was purchased from Biochemika; 3,5-dinitrosalicylic acid (DNS) from Fluka. Sephadex G200 and DEAE-cellulose were supplied by Pharmacia Co. (GE Healthcare). SDS was supplied Sigma Aldrich Co. (St, Louis, USA). Agar, soybean powder and corn cobs were from Biotech (Vietnam). NaHPO₄; Na₂HPO₄; NaNO₃ and MgSO₄ were from BioBasic Inc (Ontario, Canada). All other chemicals were of analytical grade unless otherwise stated.

2.3 Method

2.3.1. Xylanase purification

The culture was centrifuged for 10 min, at 8000 xg. Four ml of the crude enzyme extract (114.3 units) was applied to a Sephadex G-200 column (2.6 x 6 cm) pre-equilibrated with 50 mM potassium phosphate buffer pH 7.5 at a flow rate of 25 ml/h then washed with the same buffer. The eluate was collected with 18 ml per fraction. A highly active xylanase pool of 5 ml through Sephadex G-200 column was further applied to a DEAE-cellulose ion exchange chromatography pre-equilibrated with 50 mM Tris HCl buffer pH 8.0 containing 50 mM NaCl (buffer A), then washed with the same buffer. The protein was eluted with 50 mM Tris HCl buffer pH 8.0 containing 1000 mM NaCl (buffer B) at a flow rate of 20 ml/h until OD₂₈₀nm < 0.010. The eluate was collected with 7.5 ml per fraction. The fractions containing highly active xylanase activity were pooled and used as purified enzyme for characterization. All purification steps were carried out at 4°C, unless otherwise specified.

2.3.2. Xylanase activity estimation

Xylanase activity was determined by measuring an increase of concentration of reducing sugars produced by enzymatic hydrolysis of birchwood xylan. A reaction mixture of 100 µl of the crude or purified xylanase containing 0.1 µg total protein was incubated with 400 µl of 0.5% (w/v) birch wood xylan in 20 mM potassium phosphate buffer pH 6.5 at 55°C for 5 min. To arrest the reducing sugar released in the reaction mixture, 1.25 ml of 3.5, dinitrosalicylic acid (DNS) was added. The reduced sugars were determined by measuring the absorbance at 540 nm (Miller, 1959). D-xylene was used as standard. One unit (IU) of xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose per min under the standard assay conditions. All measurements were carried out three times and from these values the average value was taken.

2.3.3. SDS-PAGE and protein concentration

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (Laemmli, 1970) with Bio-Rad equipment. SDS-PAGE was usually performed on gels containing 12.5% (w/v) acrylamide according to the manufacturer’s recommendation. The gels were stained with Coomassie Brilliant Blue R- 250 for protein. Protein was estimated by the method of Bradford with the bovine serum albumin as standard (Bradford, 1976).

2.3.4. Effects of temperature on xylanase activity and thermostability

To investigate the effect of the reaction temperature on the xylanase activity, 100 µl of the purified enzyme (0.1 µg protein) was incubated together with 400 µl of 0.5% (w/v) of birch wood xylan in 20 mM potassium phosphate buffer pH 6.5 at different temperatures 37°C; 40°C; 45°C; 50°C; 55°C; 60°C, and 70°C for 5 min and then the reducing sugars were arrested by addition of 1.25 ml DNS reagent. To study the thermostability of the enzyme, 10µl of the purified xylanase (0.1 µg protein) were incubated at temperatures ranging from 37°C; 50°C; 60°C to 70°C for different intervals of time before using it for assay.

3. Results

3.1. Purification of xylanase

The xylanase production by A. oryzae DSM1863 in the mineral medium was 114.3 U/ml (specific activity of 615.04 U/mg protein) after 72 hours of cultivation. This culture supernatant was applied to Sephadex G-200 and DEAE-cellulose chromatography. The pooled Sephadex G-200 fractions containing high xylanase activity (3768
IU/mg) were applied further to the DEAE-cellulose ion exchange chromatography (Fig. 2). The xylanase was purified with a factor of 11 and a yield of 28%. The purified enzyme gained a specific activity of 6768 IU/mg (Tab. 1) and showed a unique protein band on SDS-PAGE (Fig. 2). The molecular weight of this protein was estimated to be 21.0 kDa.

Table 1. Purification of xylanase from *Aspergillus oryzae* DSM1863

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein (mg/ml)</th>
<th>Total activity U/ml</th>
<th>Specific activity (U/mg)</th>
<th>Purification factor</th>
<th>Yield (%)</th>
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<tr>
<td>Crude enzyme</td>
<td>0.1858</td>
<td>114.3</td>
<td>615.04</td>
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<td>100</td>
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<tr>
<td>Sephadex G200</td>
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<td>78.7</td>
<td>3768</td>
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<tr>
<td>DEAE-Sephadex</td>
<td>0.0126</td>
<td>84.3</td>
<td>6768</td>
<td>11</td>
<td>28</td>
</tr>
</tbody>
</table>

3.2. Optimum temperature and thermostability

The xylanase from *A. oryzae* DSM1863 acted in a large temperature range from 40°C to 70°C. The xylanase activity increased gradually from 82% (744.6 U/mg) at 40°C to the maximum of 100% (903.1 U/mg) at 60°C (Fig. 2) and then decreased gradually to 82% (738.3 U/mg) at 70°C. The temperature treatment from 37°C to 70°C for 1-8 hours showed an obvious temperature effect on the xylanase stability. The xylanase from *A. oryzae* DSM1863 possessed a thermostability in a temperature range 37-50°C.
The residual activity profiles of the xylanase treated at 37°C and 50°C were quite similar (Fig. 3). The residual xylanase activity decreased gradually to 62% (650.6 U/mg) and 74% (775.9 U/mg) for 8 hours of the temperature treatment at 50°C and 37°C, respectively, whereas the xylanase activity lost one half when it was treated just for 1 or 2 hours at 70°C or 60°C, respectively.

The xylanase from various Aspergillus strains had different optimum temperatures however the optimum temperatures ranged from 45°C to 60°C. The optimum temperature was 60°C (XynF1 from A. oryzae (Kitamoto et al., 1999)), 55°C (endo-1,4-β-xylanase B from Aspergillus cf. niger BCC14405) (Krisana et al., 2005), 50°C (xylanase from A. giganteus) (Fialho and Carmona, 2004), the xylanase I and II from A. sydowii SBS 45 (Nair et al., 2008), 45°C (xylanase from A. ficuum AF-98 (Lu et al., 2008)).

4. Conclusions

A xylanase was purified from the culture supernatant of A. oryzae DSM1863 throughout Sephadex G-200 chromatography and DEAE-cellulose ion exchange chromatography. The purified xylanase showed a specific activity of 6768 IU/mg protein and a protein band of about 21 kDa on SDS-PAGE. The enzyme showed the optimum temperature was observed at 60°C. The purified xylanase was stable in the temperature range 37-50°C.

5. Acknowledgement

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6. References


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