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Biochemical characterization of two xylanases from yeast *Pseudozyma hubeiensis* producing only xylooligosaccharides

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ABSTRACT

Two distinct xylanases from *Pseudozyma hubeiensis* NCIM 3574 were purified to homogeneity. The molecular masses of two native xylanases were 33.3 kDa (PhX33) and 20.1 kDa (PhX20). PhX33 is predominant with α-helix and PhX20 contained predominantly β-sheets. Xylanase, PhX33, possesses three tryptophan and one carboxyl residues at the active site. The active site of PhX20 comprises one residue each of tryptophan, carboxyl and histidine. Carboxyl residue is mainly involved in catalysis and tryptophane residues are solely involved in substrate binding. Histidine residue present at the active site of PhX20 appeared to have a role in substrate binding. Both the xylanases produced only xylooligosaccharides (XOS) with degree of polymerization (DP) 3–7 without formation of xylose and xylobiose. These XOS could be used in functional foods or as prebiotics. Lc ms-ms ion search of tryptic digestion of these xylanases revealed that there is no significant homology of peptides with known fungal xylanase sequences which indicate that these xylanases appear to be new.

1. Introduction

Next to cellulose, xylans are the most important renewable hetero-polysaccharides abundantly found in nature. This abundant hemicellulosic component is composed of a backbone of β-1,4-linked xylopyranose units of about 100 in soft wood and 200 in hard woods substituted particularly at 2-OH and 3-OH positions with diverse side groups and also at 1-OH position at the end of xylan chain. Depending upon the plant origin, the xylan backbone was found to be covalently bound to varying degree of acetic acid, arabinose, D-glucuronic acid, 4-*O*-methyl-D-glucuronic acid, ferulic acid and p-cumaric acid. For the hydrolysis of xylan, the synergistic action of several enzymes of different functions is necessary. The main enzyme involved in the fractionation of xylan polysaccharide chain is endo-1,4-β-xylanase (EC 3.2.1.8). Other enzymes such as β-xylosidase (EC 3.2.1.37), α-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72), α-glucuronidase (EC 3.2.1.139), and feruloyl esterase (EC 3.1.1.73) remove side groups in heteroxylans. (Bastawde, 1991; Collins et al., 2005). Most of the fungi and bacteria are known to express functionally diverse multiple forms of xylanases. This multiplicity could be a result of post-translational modification (glycosylation, auto-aggregation or/and proteolytic digestion), genetic redundancy or differential mRNA processing.

The xylanolytic systems are extensively studied in fungi (*Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp., etc.) and bacteria (*Bacillus* sp., *Streptomyces* sp.) but there are very few examples from the yeast (Chavez et al., 2006). Cellulase-free xylanases are generally studied for its application in paper and pulp, food and textile industries. All endo-xylanases reported so far produced oligosaccharides with xylose as end product of xylan hydrolysis. However, none of the endo-xylanases produce smaller (DP around 3–7) xylooligosaccharides (XOS) without the production of xylose as one of the end products of xylan hydrolysis. The importance of such endo-xylanases, which produce smaller xylooligosaccharides, is yet to be defined due to lack of information on the suitable enzymes.

XOS act as the prebiotic, which beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (*Bifidobacterium* and *Lactobacilli*) by suppressing the activity of enteroputrefactive and pathogenic organism and also facilitate the absorption of nutrients. XOS can be used as ingredients of functional food, cosmetics, pharmaceuticals or agricultural products and as a plant growth regulator. In addition to the healthy effects, XOS present interesting physico-chemical properties, they are moderately sweet, and stable over a wide range of pH and temperatures and have organoleptic characteristics suitable for incorporation into foods (Barreteau et al., 2006; Gibson and Roberfroid, 1995; Katapodis et al., 2002). XOS have importance in decreasing the blood lipids, protecting liver functions, decreasing blood pressure, anticancer and regulating blood sugar. It was found that the supplementation of XOS was successful on inhibiting the precancerous lesions, promoting the growth of bifidobacteria and lowering the cecal pH value (Hsu

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et al., 2004; Moure et al., 2006). Therefore, XOS-containing diets are considered to be beneficial in improving gastrointestinal health. Furthermore, XOS seemed to be more efficient than the fructooligosaccharides in dietary supplementation. The preventive effect of XOS against contact hypersensitivity was also investigated in mice (Yoshino et al., 2006).

A new yeast strain isolated in our laboratory was identified as *Pseudozyma hubeiensis*. It produces very high levels of cellulase-free xylanase when grown on xylan-containing media (Bastawde et al., 1994). We report here the purification and characterization of two independent xylanases from a yeast *P. hubeiensis* NCIM 3574. These two xylanases showed differences in their mode of action, amino acids at active sites and their application in xylooligosaccharide production. The new yeast strain isolate, *P. hubeiensis*, is reported here by us for the first time for such kinds of studies from India.

2. Methods

2.1. Chemicals

Xylan (oat-spelts), 3,5-dinitrosalysilic acid, sinapinic acid, DEAE-cellulose, Sephadex-G-50, SDS–PAGE markers, *N*-ethylmaleimide (NEM), iodoacetate, phenylmethylsulfonylfluoride (PMSF), *p*-chloromercuribenzoate (PCMB), diethylpyrocarbonate (DEPC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), 2,4,6-trinitrobenzenesulfonic acid (TNBS), *N*-bromosuccinimide (NBS), *N*-acetylimidazole (NAI), 2,3-butanedione, citraconic anhydride, acetic anhydride, phenylglyoxal, HEPES, and MES were purchased from Sigma–Aldrich Co., St. Louis, USA. TLC plates were obtained from Merck. Ampholytes in the range of 3–10 were procured from Bio-Rad. All the other chemicals used were of analytical grade and of the highest purity and were available locally. Citraconic anhydride and acetic anhydride were prepared in 1,4-dioxane. The stocks of DEP and phenylglyoxal were prepared in acetonitrile and methanol, respectively.

2.2. Microorganism and production media

The yeast strain was isolated from decaying sandal wood (Bastawde et al., 1994) and deposited at National Collection of Industrial Microorganism (NCIM) Resource Centre, National Chemical Laboratory, Pune 411008, India with accession no. 3574 (NCYC 3431). This yeast strain was first isolated in our laboratory in 1990 from decaying sandal wood and identified by NCYC as *P. hubeiensis* in 2008 using 26S rDNA D1/D2 sequencing and standard chemotaxonomic tests. The strain is maintained on MGYP agar slopes or in broth. MGYP medium contained 0.3% malt extract, 1.0% glucose, 0.3% yeast extract, 0.5% bactopeptone and 2% agar. The medium used for xylanase production contained 0.05% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.2% KH_2PO_4, 0.1% yeast extract, 0.5% bactopeptone, 2% xylan; prior to sterilization, the initial pH of the medium was adjusted to 5.5.

2.3. Xylanase production and purification

Xylanase production was performed in 500-ml Erlenmeyer flasks with 150 ml of the production medium. The flasks were inoculated with 5% inoculum prepared in MGYP liquid medium and incubated at 28 °C on a rotary shaker (170 rpm). The cell growth was harvested after 48 h by centrifugation (7000*g*, 10 min) and the supernatant was used as a crude enzyme preparation. The broth was concentrated in rotavapour at 40 °C under vacuum. The concentrated enzyme sample (5 ml) was loaded on DEAE-cellulose column, previously equilibrated with phosphate buffer pH 7.0 (50 mM). The same buffer was passed through the column after loading the concentrated enzyme, and the fractions were collected. The active fractions were pooled together and the pooled fraction was concentrated in rotavapour under vacuum at 40 °C. The concentrated sample from DEAE-cellulose column (2 ml) was loaded on Sephadex G-50 column, which was pre-equilibrated with 10 mM phosphate buffer, pH 7.0, and the fractions (1 ml/10 min/tube) were collected. The fractions were analyzed for enzyme activity and protein and two distinct xylanase peaks were identified which were designated as PhX20 and PhX33.

2.4. Enzyme assay

The xylanase activity was estimated as reported earlier (Bastawde et al., 1994). The total assay mixture of 1 ml consisted of 0.5 ml of xylan solution (1%) prepared in respective buffer and 0.5 ml suitably diluted enzyme. The assay mixture was incubated at 60 °C for 30 min and the liberated reducing sugars were analyzed by the dinitrosalicylic acid (DNS) method (Fischer and Stein, 1961). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar equivalents per min.

2.5. Enzyme characterization

The molecular mass of xylanases was determined by 10% SDS–PAGE. The molecular mass of native enzymes was also confirmed by MALDI-ToF mass spectrometry Voyager DE-STR (Applied Biosystems) system equipped with a 337-nm nitrogen laser. The matrix was prepared in deionised water containing sinapinic acid (10 mg/ml), 50% acetonitrile and 0.1% TFA. Xylanases were mixed with matrix (1:1) and 2 μl of the preparation was spotted on plate, dried at room temperature and analyzed.

The optimum pH of both the enzymes (PhX20 and PhX33) was determined by estimating activities at 60 °C in 50 mM citrate buffer at various pH values (3.0–6.5). The optimum temperature of both the xylanases was determined by measuring the enzyme activity at various temperatures (40–75 °C) in 50 mM of citrate buffer, pH 4.0 for PhX20 and at pH 5.5 for PhX33. The pH stability was studied by incubating the enzymes in 50 mM buffer systems with pH ranging from 2.0 to 9.0 (glycine–HCl, pH 2.0–3.5; citrate buffer, pH 4.0–6.0; Tris–HCl, pH 6.5–9.0) at 30 °C. The residual activity was then assayed under standard assay conditions. The temperature stability was determined by incubating the enzymes in 50 mM Tris–HCl citrate buffer (pH 7.0) at 60 °C and 70 °C followed by measuring the residual activity under standard assay conditions. The effect of heavy metals on enzyme activity was studied by determining the activity in the presence of 10 mM concentration of respective metal salts. The protein was measured by Lowry method (Lowry et al., 1951) using bovine serum albumin as standard.

The iso-electric focusing polyacrylamide gel electrophoresis (IEF–PAGE) was performed in a vertical gel apparatus using ampholytes (Bio-Rad) of the pH range 3–10. Approximately 20 ug of the purified protein was applied to the gel and focused at 200–400 V for a period of 4–5 h. Protein bands were visualized by silver staining (0.2% w/v). The corresponding unstained gel was soaked in 1 ml of KCl (10 mM) for 30 min and its pH was checked. Glycoprotein content of the purified enzyme preparation was determined by the phenol sulfuric acid method.

The K_m and K_{cat} values of the native and modified enzymes were determined under standard assay conditions using 0.5–3.0 mg of soluble xylan. The constant values were calculated by fitting data to linear regression using Lineweaver–Burk plot.

The pH effect on K_m and K_{cat} values of both xylanase was determined by varying the substrate concentration in the range of