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One-Step Purification and Characterization of a Low Molecular Weight Xylanase from *Aspergillus terreus* NRRL 1960

Aytac KOCABAS[1,2] Didem SUTAY KOCABAS[3] Ufuk BAKIR BOLUKBASI[3]*

[1] Department of Biotechnology, Middle East Technical University, 06531 Ankara, TURKEY

[2] Department of Chemical Engineering, Ege University, 35100 Izmir, TURKEY

[3] Department of Chemical Engineering, Middle East Technical University, 06531 Ankara, TURKEY

*Corresponding Author
e-mail: ubakir@metu.edu.tr

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Abstract

The aim of study was to investigate purification and characterization of xylanase produced by industrially important strain of *Aspergillus terreus*. The xylanase was purified by one-step hydrophobic interaction chromatography technique 19-fold with 61% yield. Molecular weight and isoelectric point of the enzyme were determined as 19 kDa and pH 9.0, respectively. The enzyme was found to be unglycosylated. Kinetic experiments at 50°C and pH 7.0 resulted in apparent K_m and V_{max} values of 2.5±0.05 mg xylan/ml and 50.2±0.4 IU/µg protein, respectively. According to its biochemical properties, the enzyme was found to be a member of family-11 xylanase group. Due to its low molecular weight, the enzyme could be advantageous for industrial applications.

Keywords: *Aspergillus terreus*; xylanase; hydrophobic interaction chromatography; enzyme characterization

INTRODUCTION

Xylanases belong to a group of glycosidic enzymes (*o*-glycoside hydrolases, EC 3.2.1.x) which are responsible for the endohydrolysis of 1,4-β-D-xylosidic linkage in xylan [1]. Their official name given in International Union of Biochemistry and Molecular Biology Enzyme Nomenclature Database is endo-1,4-β-xylanase with the classification number of EC 3.2.1.8. Commonly used synonyms for xylanases are xylanase; endo-(1,4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; β-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo-1,4-β-xylanase and β-D-xylanase. Xylanases have been classified into two groups based on their amino acid sequences and structural homologies; family-10 (formerly family-F) and family-11 (formerly family-G). Xylanases of family-10 have high molecular weight (>30 kDa) with low pI values, whereas family-11 xylanases have low molecular weight (<30 kDa) with high pI values [1-3]. In terms of protein structure, the most conserved region of a family is the catalytic domain and regarding the catalytic properties, family-10 xylanases exhibit greater catalytic versatility or lower substrate specificity than family-11 xylanases [4]. Microorganisms generally produce more than one type of xylanase in multiple forms as a result of differential mRNA processing, partial proteolysis or differences in the degree of amidation and glycosylation. Therefore they differ in substrate binding site and in the number of unsubstituted xylose units in polysaccharide backbone [5].

The substrate of xylanases is xylan which is a complex polysaccharide involving a backbone of β-1,4-glycosidic bond linked xylose residues [6]. Xylans are hemicelluloses found in the cell wall and in the middle lamella of plant cells, representing up to 30–35% of the total dry weight [6,7]. Hemicelluloses are the second most abundant natural polysaccharide on the earth. They cover a range of non-cellulose polysaccharides made up of monosaccharide units such as D-xylose, D-mannose, D-glucose, L-arabinose, D-galactose, D-glucuronic acid and D-galacturonic acid, in different proportions. Hemicelluloses generally consist of two or more types of sugar, therefore they are not a well defined polymer group [7]. Since xylans, the main building block of hemicelluloses, are heterogeneous compounds and have a complex chemical nature, a xylanolytic enzyme system is required for their complete breakdown. The xylanolytic enzyme system is made up by the cooperative action of different hydrolytic enzymes such as β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and p-coumaric acid) esterase [6,8].

The major industrial application area of xylanases is the paper and pulp industry in prebleaching of kraft pulp to minimize the use of harsh chemicals [7]. A growing interest has been raised for xylanases due to their potential in xylooligosaccharides (XOs) production. XOs are sugar oligomers, made up of xylose units and industrially produced from lignocellulosic biomass [9]. Xylanases are also used as food additives to poultry, in wheat flour, for the extraction of coffee, plant oils and starch, in the improvement of nutritional properties of agricultural silage and grain feed, for clarification of fruit juices and wine, degumming of plant fiber sources, release of aroma and anti-oxidant molecules, production of biopharmaceuticals and for bioconversion of lignocelluloses to sugar, ethanol and other useful substances. Due to the huge industrial usage area, identifying and obtaining new xylanases with different specificities and properties became very important [6,7,10].

Xylanases are found in plants, algae, insects, protozoans and microorganisms. Filamentous fungi are known for their high extracellular xylanase production capability, among the microbial xylanase sources [11]. *Aspergillus terreus*, a member

of filamentous fungi family, was subject of several studies, such as medium optimization, cloning, purification, biochemical characterization, improving the bread volume ability and enzymatic deinking capability, for its different strains but not NRRL 1960 strain [11-17].

Sa-Pereira et al. (2003) [18] analyzed the purification strategies used for xylanase purification and reported that microbial xylanases were mainly purified by chromatographic methods; ion-exchange, gel filtration and hydrophobic chromatography. Other methods such as affinity techniques, electro-elution, aqueous two-phase systems and immunopurification were also used but to a lesser extent.

The aim of the present study was to investigate purification, biochemical characterization of the extracellular xylanase from the fungus *A. terreus* NRRL 1960 cultivated in liquid-state culture on birchwood xylan as a carbon source.

MATERIALS AND METHODS

Microorganism, Production Media and Fermentation Conditions

Aspergillus terreus strain NRRL 1960 was kindly provided by Dr. Leo de Graaff (Wageningen University, Netherlands). The microorganism was grown and sporulated on solid potato dextrose agar for 4 days at 30 °C. Stock spore suspensions were prepared in 1% (w/v) peptone solution and 10^7 spores/mL spores were inoculated into the 100 mL liquid preculture containing (in g/L): glucose, 25; corn steep liquor, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 2; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.1.

Enzyme production was carried out in 250 mL flasks in a shaker incubator at 37 °C with 170 rpm shaking rate in a modified medium defined by Cros and Schneider [19] containing (in g/L): birchwood xylan, 2; corn steep liquor, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. The production culture was inoculated with 10% of preculture. The production medium was centrifuged at 10,000 x*g* for 15 min to remove the cell debris and filtered through 0.45 µm-pore-size membrane (Millipore, USA). The supernatant was used as the crude enzyme solution.

Xylanase Purification

Enzyme purification was carried out by hydrophobic interaction chromatography using an ÄKTA Prime FPLC system (Amersham Biosciences, USA). HiLoad 16/10 Phenyl Sepharose High Performance column (20 ml, 1.6 cm x 10 cm) (Amersham Biosciences, USA), previously equilibriated with 100 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl, was operated at a flow rate of 3 ml/min. Elution was performed by applying a linear gradient of 1.0-0.0 M NaCl in the same buffer. Active fractions were collected and homogenity was determined by SDS-PAGE and pure xylanase was used in enzyme characterization studies.

Enzyme and Protein Assays

Xylanase activity was determined by measuring the amount of released reducing sugars from xylan by the 3,5-dinitrosalicylic acid (DNS) method [20]. For the assay, 1% birchwood xylan was dissolved in 50 mM sodium phosphate buffer at pH 7.0 centrifuged at 5000*g* for 20 minutes to clear of the insoluble xylan particles and used as substrate [21]. One unit of xylanase activity (IU) is defined as the amount of enzyme required to produce 1 µmole of xylose equivalents per minute at 50 °C and pH 7 in 50 mM sodium phosphate buffer.

For quantitative protein determination Bradford method [22] was employed using bovine serum albumin (BSA) as a standard.

Characterization of the Xylanase

SDS-PAGE and Isoelectric Focusing

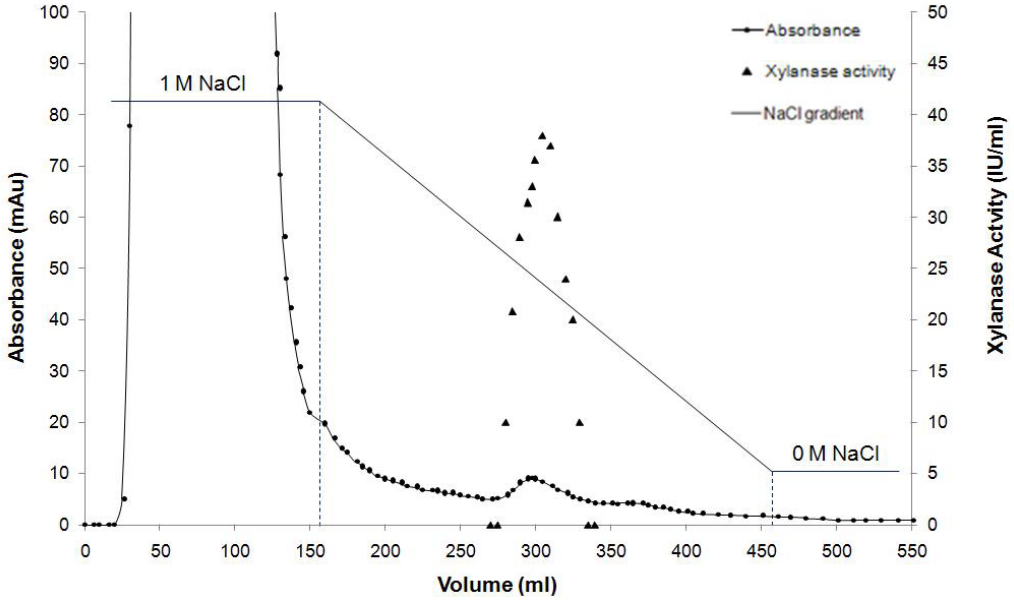
The homogeneity and molecular mass of xylanase were determined in the presence of sodium dodecyl sulphate (SDS-PAGE) according to the method of Laemmli (1970) [23] using 4% stacking and 12% separating gels with Mini-Protean II Dual Slab cell system (BioRad, USA). PageRuler™ Plus Prestained Protein Ladder molecular weight markers (Fermentas, Canada) were in the range of 10-250 kDa. Gel run was performed at constant voltage at 50 V for 15 min, then at 100 V for 1 h. Gels were stained using silver staining method according to Blum et al. (1987) [24] for molecular weight determination. The gel was also stained using glycoprotein detection kit (Sigma-Aldrich, Germany) to predict the possible sugar moieties of xylanase. Peroxidase with 18% carbohydrate content and BSA were used as positive and negative controls, respectively. Isoelectric point of the protein was determined on an IEF gel containing ampholytes in a pH range of 3.5-10.0 (Sigma-Aldrich, Germany) in an Amersham Multiphor II Electrophoresis System (Amersham Biosciences, USA) in METU Molecular Biology and Biotechnology Research and Development Center. The sample and the IEF markers, in a range of 3.5-9.3 (Amersham Biosciences, USA) were run at 100 V for 15 min, at 200 V for 15 min, and at 450 V for 4 h. After that the gel was stained with Coomassie blue.

Zymogram Analysis

Zymogram analysis was performed according to the method described by Ratanakhanokchai and coworkers [25] with some modifications. Crude enzyme sample was incubated in boiling water for only 5 seconds since renaturation of the enzyme was not possible when boiled for 5 min due to its thermo-versatile nature. Then, the enzyme was electrophoresed on an SDS-polyacrylamide gel containing 0.1% birchwood xylan. After

Table 1. Purification of xylanase from *Aspergillus terreus* NRRL 1960

Purification Step	Volume (ml)	Protein (µg/mL)	Total Protein (µg)	Activity (IU/mL)	Total Activity (IU)	Specific Activity (IU/µg)	Purification Fold	Yield (%)
Culture supernatant	75	14.0	1052.6	28.1	2108	2	1	100
Hydrophobic interaction chromatography	44	0.8	35.2	29.4	1294	37	19	61

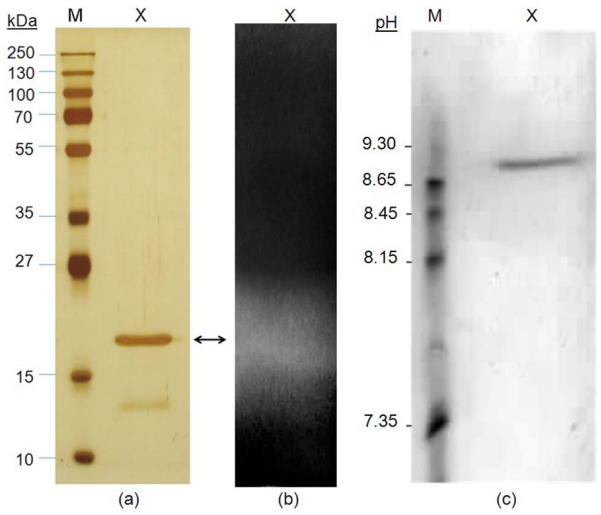


Kocabas Fig1. *A. terreus* NRRL 1960 xylanase purification by hydrophobic interaction chromatography at 3 ml/min flow rate, 100 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl, elution with the same buffer without NaCl

electrophoresis, the gel was incubated in isopropanol (25% v/v) for 30 min in a 100 mM sodium phosphate buffer at pH 7.0 to remove the SDS and renature the enzyme in the gel. Then, the gel was washed two times in the same buffer without isopropanol for 30 min at 40°C. The gel was soaked in 0.1% Congo red solution for 15 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. After soaking the gel into 0.5% acetic acid solution, the background turned to dark blue and clear band indicating the xylanase activity was identified.

Kinetic Studies

The enzyme obeys Michaelis-Menten kinetics and the K_m and V_{max} values were determined from Lineweaver-Burk plot [26] at different birchwood xylan concentrations ranging from 0.2 to 4.0% (w/v) under standard assay conditions.

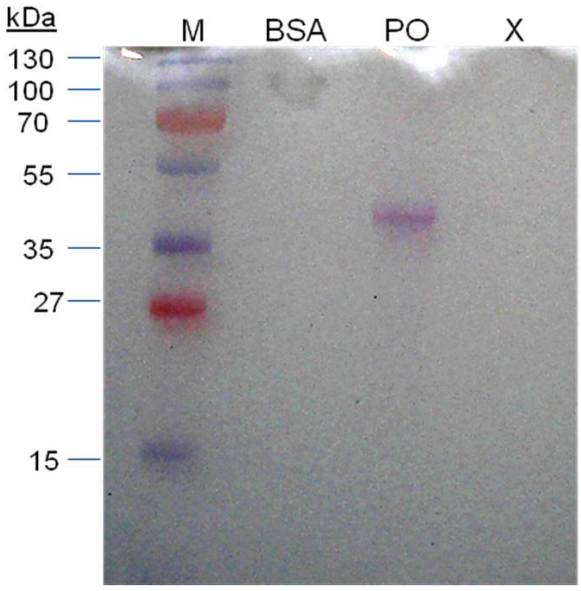


Kocabas Fig2. (a) SDS-PAGE (b) Zymogram (c) Isoelectric focusing analysis of *A. terreus* NRRL 1960 purified xylanase. Lanes; M: Marker, X: Purified xylanase

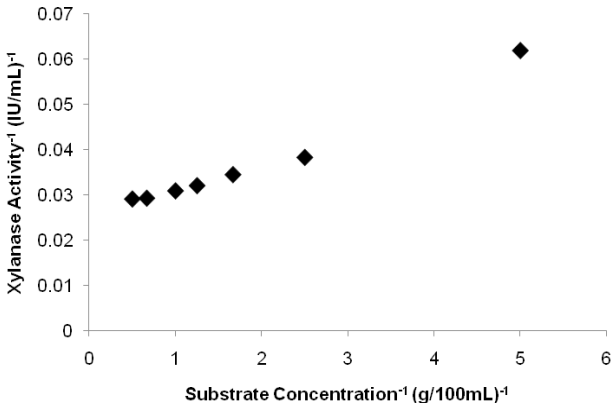
RESULTS AND DISCUSSION

Purification of Xylanase

A. terreus NRRL 1960 xylanase was purified using one-step hydrophobic interaction chromatography (Fig1) with a purification fold of 19 and recovery yield of 61% (Table 1) and used in further biochemical characterization studies. In xylanase purification studies, generally two to five chromatographic purification steps (including concentration) were used and recovery yields range from 0.2 to 78% [18]. Achieving a recovery yield of 61% and purification fold of 19 for *A. terreus* NRRL 1960 xylanase by one-step purification method is a very promising result for scale-up studies.



Kocabas Fig3. Glycosylation analysis of *A. terreus* NRRL 1960 purified xylanase



Kocabas Fig4. Lineweaver-Burk plot of effect of substrate concentration on *A. terreus* NRRL 1960 purified xylanase

Molecular Weight and Isoelectric Point Determinations

Molecular weight of xylanase was determined as 19 kDa by SDS-PAGE (Fig2a) which was confirmed by activity staining zymogram analysis (Fig2b). The isoelectric point (pI) of xylanase was determined as 9.0 (Fig2c).

Biochemical properties of xylanases are commonly based on the bacterial and fungal studies, which showed that microbial xylanases are generally single subunit proteins with molecular masses in the range of 8-145 kDa [10]. According to the published information on three-dimensional structures, most microbial xylanases are single-subunit proteins [19], in consistency with our results. In addition, Wong and coworkers [2] reported a conserved relationship between molecular weights and pI values of xylanases by investigating the conservation through the evolutionary processes of xylanases purified from *Aspergillus*, *Bacillus*, *Clostridium*, *Streptomyces*, and *Trichoderma* species. Two groups are proposed: with a low molecular weight (<30 kDa) and basic pI (family-11), and with a high molecular weight (>30 kDa) and acidic pI (family-10), with some exceptions [2-4]. Despite this relation is not very common in fungi, low molecular mass basic xylanases are mostly identified [10]. Similarly, according to our results, *A. terreus* NRRL 1960 xylanase is a low molecular weight (19 kDa) enzyme with basic pI value (pH 9.0), hence a member of the family-11. Having a low molecular weight could make the enzyme advantageous for industrial applications.

Glycosylation Studies

Possible sugar moieties of xylanase were analyzed using glycoprotein detection kit (Sigma-Germany). As shown in Figure 3, the positive control, peroxidase showed purple band as the result of its 18% carbohydrate content. No purple band was detected for BSA, the negative control, and for xylanase. *A. terreus* NRRL 1960 xylanase appeared as an enzyme without carbohydrate content, hence unglycosylated (Fig3).

Kinetic Studies

Initial reaction rates of birchwood xylan hydrolysis by *A. terreus* NRRL 1960 purified xylanase were determined at different substrate concentrations ranging from 0.2 to 4.0% (w/v) and Michaelis-Menten kinetics was observed. K_m and V_{max} values were determined from Lineweaver-Burk plots 2.5±0.05 mg xylan/ml and 50.2±0.4 IU/µg protein, respectively (Fig4).

CONCLUSIONS

Xylanase was produced as a part of current studies in our laboratory by *Aspergillus terreus* NRRL 1960. The enzyme was purified by one-step hydrophobic interaction chromatography *c*. 19-fold with 61% yield. Reaching a high purity level by only one-step chromatographic process makes the enzyme production system advantageous for industrial application. In addition, purified xylanase could be favorable for industry due to its low molecular weight.

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