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PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR B-XYLOSIDASE PRODUCED BY *ASPERGILLUS NIGER* B03

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Abstract

β-Xylosidase is a major enzyme of the xylanolytic complex, cleaving xylose from the non-reducing end of xylobiose and short chain xylo-oligomers. As a result of its action, the inhibitory effect of the resulting xylo-olimers on xylanase is removed, which is important for the achievement of complete enzymatic hydrolysis of xylan.

The research was carried out with β-xylosidase, produced in a submerged cultivation of *Aspergillus niger* B 03, provided by Biovet AD. The enzyme was purified by subsequent ultrafiltration and gel chromatography on Sephadex G 75 and Sephadex G100. Ultrafiltration was performed in an Amicon ultrafiltration unit through a 10 kDa molecular weight cut-off polysulfone membrane, and the chromatographic isolation was performed on fast protein liquid chromatography (FPLC), Pharmacia Biotech. β-xylosidase purification was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5 % resolving gel.

An extracellular β-xylosidase produced by *Aspergillus niger* B 03 was isolated by column gel chromatography with Sephadex G 75 and Sephadex G 100 to a homogeneous form, according to SDS-PAGE. The enzyme had a molecular weight of 90 500 Da. The optimal pH and temperature for the enzyme action were determined to be pH 3.50 and 70 ^{0}C, respectively. The enzyme showed significant thermal stability at 50 ^{0}C. The kinetic parameters of β-xylosidase were determined with p-nitophenyl-β-D-xylopyranoside as a substrate, the Km value was 0.35 mM and Vmax was 3.03 μmol/(min. mL). The presence of 10 mM Mn^{2+} significantly activated the enzyme, β-xylosidase activity was increased by about 1.5 fold. It was found that xylose was a competitive inhibitor of the isolated β-xylosidase and Ki value was determined to be 1.86 mM.

The purification and the determination of the main biochemical characteristics of β-xylosidase allowed the proper application of the enzyme for enzymatic hydrolysis of xylan along with xylanase. As a result the degree of xylan hydrolysis was significantly increased, and reached 70%.

***Key words**: β-Xylosidase, Aspergillus niger, Purification, Enzymatic hydrolysis of xylan.*

1. Introduction

Hemicellulose accounts for 30 - 35% of the dry weight of the plants. Xylan is the major heteropolysaccharide involved in the hemicellulose composition. It contains a homopolymeric chain composed of β-D-xylopyranose residues linked by β-1,4 glycosidic bonds. Several branches of glucuronic acid, α-L-arabinofuranose, acetic and ferulic acid are linked along the homopolymeric chain [1]. Because of the high heterogeneity of xylan macromolecules, their enzymatic hydrolysis is catalyzed by the synergistic action of a large number of enzymes with hydrolytic activity [2].

Xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) play a key role in the enzymatic hydrolysis of xylan. Xylanase is an endo-enzyme that catalyzes the hydrolysis of xylan to lower-molecular weight xyloligosaccharides and β-xylosidase catalyses hydrolysis of xylobiose and short chain xylo-oligomers by cleaving xylose from their non-reducing end [3, 4].

β-Xylosidase is a major enzyme of the xylanolytic complex. As a result of its action, the inhibitory effect of the resulting xylo-olimers on xylanase is removed, which is important for the achievement of complete enzymatic hydrolysis of xylan [5].

Xylanolytic enzymes in combination with cellulases are widely used in various areas of food industry and bioconversion of plant wastes to fermentable sugars. Hydrolysis products of xylan can be used as food substrates for the microbial production of biomass, ethanol and other organic compounds [2].

For these applications, a great number of the xylanolytic enzymes are produced by fungal and bacterial strains such as *Aspergillus niger*, *Trichoderma reesei*, and *Bacillus* spp. [6].

Generally, filamentous fungi produce multiple isoforms of β-xylosidases. These enzymes have various physico-chemical properties, structures, substrate specificity and productivity [7]. Fungal β-xylosidases typically exhibit molecular weight from 90 kDa to 210 kDa and optimal temperature of around 60 ^{0}C.

Some researchers isolated fungal and bacterial β-xylosidases that are dimers and trimers and have a molecular weight above 100 kDa. Terrasank *et al.*, [8], isolated β-xylosidase from *Penicillium janczewskii*, which was a dimer with molecular weight of 200 kDa. The enzyme showed significant activity in the pH range of 3 - 5 with pH optimum at 5, and at temperatures of 70 - 80 ^{0}C with thermal optimum at 75 ^{0}C. Khisti and Gokhale [9] isolated β-xylosidase from *Aspergillus niger* NCIM 1207 with a molecular weight of 336 kDa, which was a trimer. Monomeric β-xylosidase was purified from *Trichoderma sp.* SY, the enzyme had a molecular weight of about 80 kDa and exhibited maximal activity at pH 5.0 and 55 ^{0}C [10].

Therefore, the discovery of β-xylosidases with specific characteristics is of great interest.

The present study reports the purification and biochemical characterization of β-xylosidase produced by *Aspergillus niger* B03. The fungus is a good producer of xylanolytic enzymes and relevant studies related to the endoxylanase production have been published by the authors [11, 12]. The isolation and characterization of concomitant β-xylosidase will allow the proper application of the xylanolytic enzyme complex from *Aspergillus niger* B03 for enzymatic hydrolysis of xylan.

2. Materials and Methods

2.1 Microbial strain

The research was carried out with a strain of *Aspergillus niger* B 03, provided by Biovet AD from where.

2.2 Biosynthesis of β-xylosidase

The submerged cultivation was carried out in 500 mL flasks containing 50 mL of nutrient medium with composition (g/L): corn cobs - 24, wheat bran - 16, malt sprout - 6, urea - 2.6, $(NH_4)_2HPO_4$ - 2.6. pH was adjusted to 6.7 - 6.8, and the flasks were sterilized at 121 ^{0}C for 30 min. The medium was inoculated with 10% vegetative inoculum and the strain was cultivated on a shaker at 180 min^{-1} and 28 ^{0}C for 64 h.

2.3 Ultrafiltration

The ultrafiltration was carried out in an Amicon ultrafiltration unit through a 10 kDa molecular weight cut-off polysulfone membrane.

2.4 Gel chromatography

Gel chromatography was performed on Fast protein liquid chromatography (FPLC), Pharmacia Biotech. Purification was carried out in two steps, sequentially using Sephadex G 75 and Sephadex G 100. Initially, in the column filled with Sephadex G75 a sample of 26.6 mg total protein was applied. Fractions exhibiting β-xylosidase activity were pooled and concentrated by ultrafiltration, and a sample containing 1.05 mg of protein was loaded in a column packed with Sephadex G100. Elution was performed with 0.05 M NaCl at 19.8 mL/h. Fractions of 7 mL were collected.

2.5 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5 % resolving gel by the method of Laemli [13]. The results were used for monitoring of β-xylosidase purification and for determination of molecular weight. The molecular weight of the enzyme was estimated using the following protein calibration kit: ovotransferrin (78,000 Da), albumin (66,250 Da), ovalbumin (45,000 Da), carbonic anhydrase (30,000 Da), myoglobin (17,200 Da), and cytochrome c (12,300 Da).

2.6 Characterization of β-xylosidase

The effect of pH on β-xylosidase activity was studied in citrate-phosphate buffer in the range of pH 3 - 8.5 at 50 ^{0}C.

The effect of temperature on the enzyme activity was investigated in the range of 30 - 80 ^{0}C at pH 5.0.

Stability of β-xylosidase was tested at pH 5 and at temperatures of 50, 60 and 70 ^{0}C.

Kinetic parameters of β-xylosidase catalysed enzyme reaction Km and Vmax were determined using the Lineweaver-Burk graphic method. p-Nitrophenol-β-D-xylopyranoside was used as substrate at a concentration of 0.18, 0.36, 0.55, 0.73, 0.91 mM. The enzyme reaction was carried out at pH 5 and 50 ^{0}C.

The inhibitory effect of xylose on β-xylosidase activity was investigated by addition to the reaction mixture of xylose at concentrations of: 0.5, 1, 2, 3.33, 6.67, and

15.00 mM. The enzyme reaction was carried out at pH 5 and 50 °C. An Enzyme kinetic SYGMAPLOT software was used to determine the type of inhibition and Ki value.

The influence of metal ions on β-xylosidase activity was studied at concentrations of 5 and 10 mM at 30 °C for 30 min.

2.7 Enzymatic hydrolysis of xylan

Enzymatic hydrolysis of xylan was studied with β-xylosidase along with multiple endoxylanases Xln-1 and Xln-2, isolated in previous studies [12]. The hydrolysis was performed with 10 mL of 10 mg/mL birchwood xylan, 2 U of β-xylosidase and 12 U endoxylanase activity of enzyme preparation, containing Xln-1 and Xln-2 at pH 5.0 and 40 °C, for up to 360 min. At certain time intervals the reducing sugars released were determined [14], and xylan hydrolysis was calculated.

2.8 Determination of β-xylosidase activity

β-Xylosidase activity was determined according to the method described by Ponpium *et al.*, [15]. 1 mM solution of p-nitrophenol-β-D-xylopyranoside in 0.1 M acetate buffer with pH 5 was used as a substrate. One unit of β-xylosidase activity was defined as the amount of enzyme which released 1 μmol p-nitrophenol for 1 min. at pH 5.0 and 50 °C.

2.9 Determination of protein content

Protein concentration was determined by the method of Lowry, [16].

3. Results and Discussion

As a first step for the purification of β-xylosidase, ultrafiltration through a 10 kDa molecular weight cut-off polysulfone membrane was used. As a result of the ultrafiltration performed, β-xylosidase was purified 1.41 folds at a yield of 66.74% (Table 1). A sample of the resulting ultra-concentrate was subjected to Sephadex G75 separation. The elution profile of β-xylosidase on Sephadex G75 gel chromatography is shown on Figure 1.

The peak of β-xylosidase activity coincided with the first protein peak, and it was eluted immediately after the column void volume, which suggested that the molecular weight of the enzyme was greater than 75 000 Da (Figure 1). As a result of the performed gel chromatography, a 21.62-fold purification degree of the enzyme was achieved at a yield of 58.89% (Table 1).

Purity of the isolated β-xylosidase of the individual purification steps was monitored by SDS-PAGE (Figure 2). In the sample, after the gel chromatography with Sephadex G75, the presence of three protein bands was detected (Figure 2).

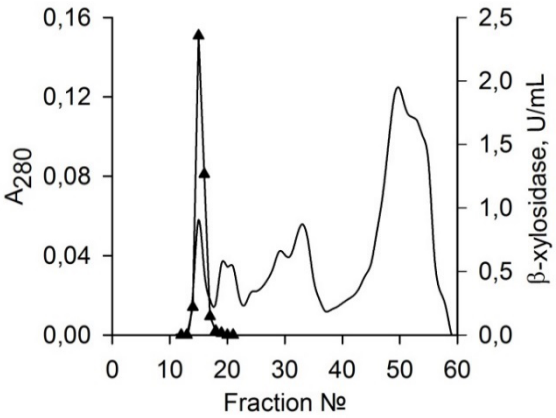

Figure 1. Elution profile of β-xylosidase by Sephadex G75 separation

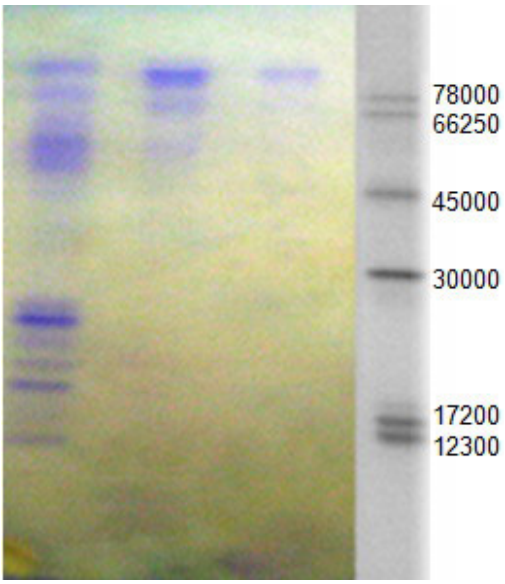

Figure 2. SDS-PAGE of β-xylosidase purification (from left to right): First column - ultra-concentrate; Second column - after Sephadex G75; Third column - after Sephadex G100; Fourth column - protein molecular weight kit

Table 1. Isolation and purification of β-xylosidase

Step	Total activity, U	Total protein, mg	Specific activity, U/mg	Purification fold	Yield, %
Crude enzyme	46.36	76	0.61	1	100
Ultraconcentrate	30.94	35.88	0.86	1.41	66.74
Sephadex G75	27.30	2.07	13.19	21.62	58.89
Sephadex G100	9.90	0.69	14.34	23.51	21.35

Subsequent isolation step for β-xylosidase was performed with Sephadex G100 gel chromatography (Figure 3). The achieved purification degree of the enzyme was 23.51 folds at a yield of 21.35% (Table 1). According to SDS-PAGE (Figure 2), the isolated β-xylosidase was purified to a homogeneous form with the molecular weight of the enzyme being 90 500 Da.

Main biochemical characteristics of the purified β-xylosidase were identified. The optimal pH of the enzyme was pH 3.5 (Figure 4). At pH values of 3 - 5.5 β-xylosidase exhibited over 80% of its activity, and at pH 8.5 the enzyme did not display activity.

Optimal temperature for enzyme action of β-xylosidase was 70 ^{0}C (Figure 5). Above this temperature a sharp decrease in β-xylosidase activity was observed. The high thermal optimum of β-xylosidase allows the technological process of xylan hydrolysis to be performed at high temperatures, which is beneficial against microbial contamination.

Thermal stability of the isolated β-xylosidase is presented in Figure 6. The enzyme showed high stability at 50 ^{0}C, retaining over 80% of its activity for 120 min. With the increase in the temperature, the enzyme stability decreased, and at about 60 ^{0}C about 50% of the activity was retained for 60 minutes, and at 70 ^{0}C the relative activity was only 30%.

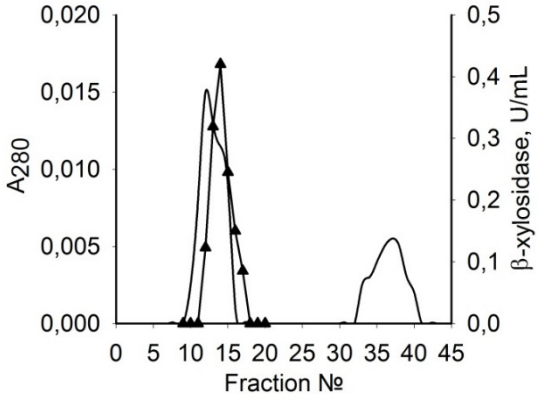

Figure 3. Elution profile of β-xylosidase by Sephadex G100 separation

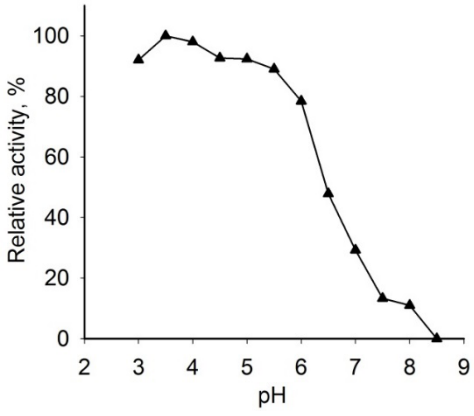

Figure 4. Effect of pH on β-xylosidase activity

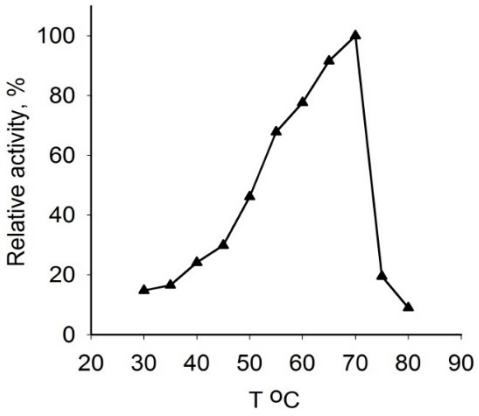

Figure 5. Influence of temperature on β-xylosidase activity

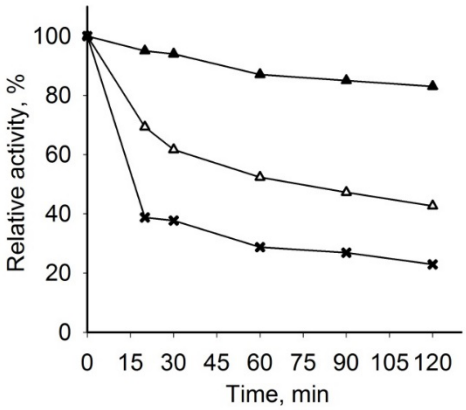

Figure 6. Thermal stability of β-xylosidase: (▲) 50 °C; (△) 60 °C; (×) 70 °C

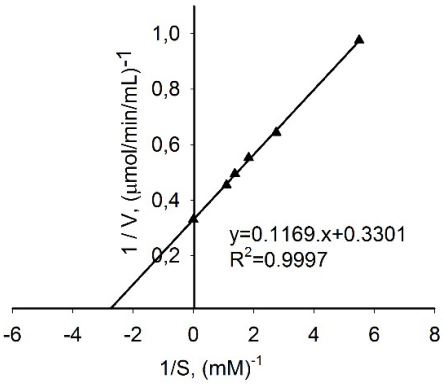

Figure 7. Lineweaver-Burk plot for determination of kinetic parameters Km and Vmax of β-xylosidase

Km and Vmax kinetic parameters of β-xylosidase were determined when p-nitrophenol-β-D-xylopyranoside was used as a substrate. Km value was 0.35 mM and Vmax was 3.03 µmol/min/mL (Figure 7).

Many authors indicated that β-xylosidase was competitively inhibited in the presence of xylopyranose [4, 5]. Using an enzyme kinetic software, competitive inhibition of the enzyme by xylose was established with the inhibition constant Ki being 1.86 mM. The results obtained confirmed the finding of other authors that β-xylosidase was inhibited by xylose and other

monosaccharides. The inhibition constant Ki varied between 2.3 - 650 mM for enzymes isolated from different microbial strains [17].

The isolated β xylosidase from *Aspergilus niger* B03 had similar kinetic characteristics (Km = 0.35 mM and Ki = 1.86 mM) with xylan-induced β-xylosidase produced by *Aspergillus versicolor* (Km = 0.19 mM and Ki = 2.0 mM) [5].

Effect of some metal ions on β-xylosidase activity is shown in Table 2.

Table 2. Effect of metal ions on β-xylosidase activity

Salt	Relative activity, %	
	Concentration, mM	
	5	10
None	100	100
$CoCl_2$	84.04	80.79
$CuCl_2$	43.02	41.71
$ZnSO_4$	56.69	55.39
$Pb(NO_3)_2$	23.8	22.5
$AgNO_3$	0	0
$CuSO_4$	72	59.3
$MgSO_4$	87.3	79.81
$FeSO_4$	86.65	89.58
NaCl	89.91	72.65
$CaCl_2$	86.98	82.74
$MnCl_2$	**118.7**	**153.44**
$MnSO_4$	**126.38**	**163.5**

With the exception of Mn^{2+}, all others ions led to decrease in β-xylosidase activity. The most pronounced suppressive action exhibited Ag^{+}, Pb^{2+} and Cu^{2+}. Mn^{2+} ions in the form of $MnSO_4$ and $MnCl_2$ significantly activated β-xylosidase, and at concentration of 10 mM Mn^{2+} the relative activity reached about 160%. The results obtained differed from those obtained from Saha, [4], the author reported that the activity of β-xylosidases from *Fusarium proliferatum* was not affected by the presence of metal ions.

The isolated β-xylosidase from *Aspergilus niger* B03 was applied for enzymatic hydrolysis of xylan along with multiple endoxylanases Xln-1 and Xln-2, isolated previously from the same strain [12]. The results are presented in Figure 8.

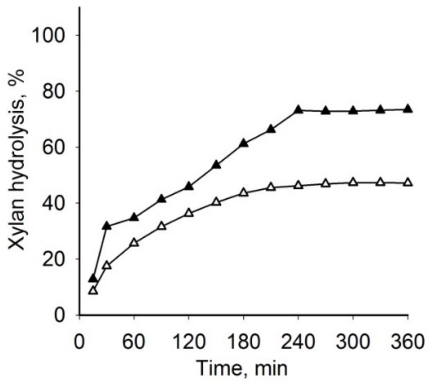

Figure 8. Enzymatic hydrolysis of xylan by: (△) endoxylanase (Xln-1 and Xln-2), (▲) endoxylanase (Xln-1 and Xln-2) and β-xylosidase

As a result of the catalytic action of β-xylosidase the degree of xylan hydrolysis was significantly increased, reaching about 70%. Many authors reported that xylosidase hydrolyzes the low molecular weight xylooligosaccharides, formed after endoxylase action, thus eliminating their inhibiting effect on endoxylanase. Isolated β-xylosidase exhibits suitable properties for carrying out enzymatic xylan hydrolysis, which is a prerequisite for its practical application.

Table 3 presents a comparative study of β-xylosidases from various *Aspergillus* species.

Table 3 Comparison of β-xylosidases from *Aspergillus* species

Fungus	Optimum temperature, °C	Optimum pH	Activation from metal ions	Km, mM	Ki	Molecular weight, Da	Reference
Aspergillus niger	70	3 - 5	Mn^{2+}	0.35	1.86	90,500	In this work
Aspergillus versicolor	45	5.5	Ca^{2+}, Ba^{2+}, Mg^{2+}, Co^{2+} Zn^{2+}, Mn^{2+}	0.19	2	100,000	[5]
Aspergillus fumigatus	70	4.5 - 7	-	-	-	72,500	[3]
Aspergillus carbonarius	60	4	-	0.2	1.9	100,000	[18]
Aspergillus phoenicis	75	4 - 4.5	Ca^{2+}, Mg^{2+}, Mn^{2+}	2.36	-	132,000	[19]
Aspergillus niger **NCIM 1207**	65 - 75	4.5	Fe^{2+}, Fe^{3+}	-	-	336,000	[9]
Aspergillus ochraceus	70	3 - 5.5	Mn^{2+}, Mg^{2+}	0.66		137,000	[20]

4. Conclusions

- A β-xylosidase enzyme produced by *Aspergillus niger* B 03 was isolated by gel chromatography to a homogeneous form, according to SDS-PAGE. The enzyme had a molecular weight of 90,500 Da. The optimal pH and temperature of the enzyme were determined to be pH 3.5 - 5 and 70 ^{0}C, respectively. Isolated β-xylosidase exhibitted significant stability at 50 ^{0}C. Kinetic parameters of the isolated enzyme when *p*-nitrophenol-β-D-xylopyranoside was used as a substrate were Km = 0.35 mM and Vmax = 3.03 μmol/min/mL.

- Xylose was found to be a competitive inhibitor of β-xylosidase, with an inhibition constant Ki = 1.86 mM. The presence of 10 mM Mn^{2+} ions significantly activated the enzyme.

- The purification and the determination of the main biochemical characteristics of β-xylosidase allowed the proper application of the enzyme for enzymatic hydrolysis of xylan along with xylanase. As a result the degree of xylan hydrolysis was significantly increased, and reached 70%.

5. References

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