PURIFICATION AND CHARACTERIZATION OF α-GLUCOSIDASE FROM MOSS *HYOPHILLA NYMANIANA* (FLEISH.) MENZEL

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ABSTRACT

Objective: The present study was undertaken to extract and purify α-glucosidase N-linked glycosylation enzyme from moss *Hyophilla nymaniana* (Fleish.) Menzel.

Methods: Frozen protonemal cells were taken for crude enzyme extraction, and the enzyme α-glucosidase was purified from the prepared crude enzyme extract by ammonium sulfate $(NH_4)_2SO_4$ precipitation, gel filtration and finally on diethylaminoethyl sephadex column chromatography.

Results: The final purification step of the enzyme resulted in 35 fold purification with a recovery of 4%. A single protein band of 72±5 kilodalton was seen on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The physiochemical characterization of the enzyme reveled the enzyme had a wide pH stability range 4-7 with optimum pH 5 while the temperature stability study revealed the enzyme was stable up to 60°C while the optimum temperature of the purified enzyme was 45°C. The enzyme was strongly inhibited by Hg^{2+} and Ag^{2+} at 1 mM concentration while Mg^{2+}, K^+, and Na^+ ions enhanced the enzyme activity at the same concentration. The kinetic study of the enzyme showed K_m and V_{max} of the enzyme 5.2 mM/ml and 8.6 U/ml, respectively.

Conclusion: The wide pH and temperature stability range show its suitability toward industrial application.

Keywords: α-glucosidase, *Hyophilla nymaniana* (Fleish.) Menzel. gel filtration, Diethylaminoethyl sephadex column chromatography.

INTRODUCTION

α-Glucosidases (EC 3.2.1.20) is an exoglycosidase that catalyze the release of α-D-glucose from the non-reducing end of substrates. Beside α-glucosidases, oligo-1, 6-glucosidase, and sucrase-isomaltase are also categorized as α-glucosidases. This α-glucosidases is ubiquitously present in nature and are classified into three categories. Type I glucosidase prefers aryl glucosides and sucrose, more efficiently than maltose, Type II prefers maltose and isomaltose and has low activity toward aryl glycosides while Type III has the same specificity as Type II but also attack starch [1-3]. In plants, α-glucosidase have many important functions. Apparently, the main function of these enzymes consists in the hydrolysis of oligosaccharides produced from starch to yield glucose [4] which serves as an energy source for the developing plant. Apart from that plant α-glucosidase could initiate the degradation of natural starch granules in pea chloroplasts and barley seeds in the absence of α-amylases [5]. Furthermore, two α-glucosidases I and II isolated from mung bean have been reported to play a key role in the biosynthetic processing of asparagine-linked oligosaccharides [6,7]. Besides these facts, a large number of α-glucosidase inhibitors isolated from the plant are being used as anti-diabetic drugs for diabetes mellitus Type II [8]. Furthermore, α-glucosidase deficiency is directly related to rare multisystem genetic disorder Pompe disease that is characterized by impaired or deficiency of the lysosomal α-glucosidase. Apart from this, plant α-glucosidase is extensively used in biotechnology and have important applications in both the food and the pharmaceutical industries. So, in view of the above several attempts have been made to purify α-glucosidase to homogeneity from different plants such as rice [9], buckwheat [10], sugar beet [11], spinach [12], and pea [13] to know its possible role in these plants. Although, the role of this enzyme in the lower plant is still not clear and only a few attempts have been made untill date to purify this α-glucosidase from these plants such as mosses. Therefore, the present paper aims to report for the first time purification to homogeneity and determination of few characteristics of the α-glucosidase enzyme from the moss *Hyophilla nymaniana* (Fleish). Menzel.

METHODS

Plant material and chemicals used

Frozen protonema cells of the moss *H. nyamiana* were taken for crude enzyme extraction. The substrate *p*-nitrophenyl-α-D-glucopyranoside was obtained from Sigma-Aldrich. Sephadex G-200 and diethylaminoethyl (DEAE) cellulose were obtained from Sigma-Aldrich. Ready to use protein molecular weight marker mixture for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was procured from Himedia.

Assay of α-glucosidase

For determination of α-glucosidase activity, the incubation mixture contained a reaction volume of 500 μl containing 200 μl of p-nitrophenyl-α-D-glucopyranoside (12.5 mM), 100 μl of enzyme, 200 μl of sodium acetate buffer (50 mM, pH 5), incubated at 45°C. The reaction was terminated after 40 minutes by the addition of 0.2 M Na_2CO_3 buffer. Liberated p-nitrophenol was measured spectrophotometerically at 405 nm. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μM of substrate per minute.

Purification of α-glucosidase

All purification steps were done at 4°C. During purification, PNP-α-Man was mainly used as a substrate for α-glucosidase. Protein concentration was measured by measurements of light absorption at 280 nm with bovine serum albumin as the standard.

Preparation of crude enzyme solution

Frozen protonema cells of *H. nymaniana* cultured in ¼ strength MS medium containing 1.5% sucrose [14] were taken and homogenized in 100 mm phosphate buffer, pH (7.0) containing 10 mM β-mercaptoethanol. The homogenate sample was sonicated at amplitude (35 %) and energy 15,000 Joule with pulse rate 10 seconds on and 10 seconds off for 10 minutes at 4°C and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant obtained was

dialyzed against 50 mM sodium acetate buffer pH 5.2. This extract is the crude enzyme solution.

Ammonium sulfate precipitation

The crude enzyme extract was subjected to $(NH_4)_2SO_4$ precipitation and the precipitate formed with 80% saturation with $(NH_4)_2SO_4$ was dissolved in 20 mM sodium acetate buffer (pH 5.2) and dialyzed against the same buffer. The supernatant obtained by centrifugation at 10,000 g for 30 minutes was used in the next purification step.

Sephadex G-100 gel filtration

An enzyme solution (10 ml) obtained from $(NH_4)_2SO_4$ precipitation was placed on a sephadex G-200 column equilibrated with a 20 mm sodium acetate buffer pH 5.2 and the column was washed with the same buffer. The glucosidase was eluted with the same buffer at a flow rate of 10 ml/hrs and fraction of 2 ml was collected. Fractions containing α-glucosidase activity was examined for α-glucosidase activity and protein content.

DEAE column chromatography

Active fractions eluted from step 3 were loaded onto a 30 cm × 2 cm DEAE cellulose column equilibrated with 20 mM sodium acetate buffer pH 5.2 and the column was washed first with the above buffer. The α-glucosidase activity was eluted with a linear gradient of sodium chloride from 0 to 0.6 M. The most active fractions showing distinct peak were pooled out and concentrated by amicon ultrafiltration device and stored at −20°C.

Physiochemical properties of the enzyme

Molecular weight determination by SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli [15] under reducing conditions in the presence of 2-mercaptoethanol. Proteins were stained with Coomassie Brilliant Blue R-250.

Effect of temperature on enzyme activity and stability

Determination of the optimum temperature for α-glucosidase was performed with 12.5 mM PNP-α-glu in 0.05 M sodium acetate buffer (pH 5) at various temperatures (20-70°C). The thermal stability of the purified enzyme was determined by pre-incubating the enzyme solution in sodium acetate buffer (0.05 M; pH 5) for 1 hr at various temperatures (20-70°C) and then the residual activities were calculated.

Effect of pH on enzyme activity and stability

The optimum pH for α-glucosidase activity was determined with 12.5 mM PNP-α-glu in 0.05M sodium acetate buffer of various pH (2-8) at 45°C for 30 minutes. The effect of pH on enzyme stability was calculated by pre-incubating the enzyme solution in 0.05 M sodium acetate buffer at different pH values (2-8) for 1 hr at 45°C and then the residual activities were determined.

Action of metal ions on α-glucosidase activity

The effect of different metal ions (Fe^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Na^+, K^+ Zn^2, Hg^+, Ag^+) on glucosidase activity was tested with preincubating the enzyme with these metal ions at 2 mm concentration 30 minutes at 37°C and pH 5.

Kinetic parameter

The K_m and V_{max} of α-glucosidase were calculated by linear regression analysis by Lineweaver-Burk plot (double reciprocal plot) using various

concentrations (2.5, 5, 7.5, 10, 12.5, 15 mM) of substrate p-nitrophenyl α-glucopyranoside, and the enzyme activity were measured by standard assay condition.

RESULTS AND DISCUSSION

Purification of α-glucosidase from protonema cells

A protocol was established for the purification of α-glucosidase from moss *H. nymaniana*. The purification of the enzyme was done in three major steps, namely ammonium sulfate precipitation, gel filtration, and ion exchange chromatography. Table 1 summarizes the sequential purification steps of α-glucosidase. The protein in the crude enzyme extract was precipitated with 30-80% $(NH_4)_2SO_4$. After 80% ammonium sulfate saturation, the enzyme suspension was dialyzed using sodium acetate buffer (pH 5) for 24 hrs at 4°C. The dialyzed enzyme solution was subsequently separated via gel filtration chromatography on a Sephadex G-100 column. The elution profile of the α-glucosidase is shown in (Fig. 1). The active fractions were pooled and purified further via anion-exchange chromatography on DEAE-Sephadex column. A sharp, distinctive peak, which fits only one protein band was obtained at a fraction eluted from approximately 0.35-0.45 M NaCl. The result of α-glucosidase activity and the protein profile on DEAE-Sephadex column are shown in (Fig. 2). The enzyme was purified to 35 fold, with a specific activity of 50 U/mg and a total volume yield of 4% relative to the crude enzyme. Although, a 71 fold purification with 17% recovery was obtained for α-glucosidase from rice through $(NH_4)_2SO_4$ precipitation, cation exchange chromatography, and gel filtration column [16].
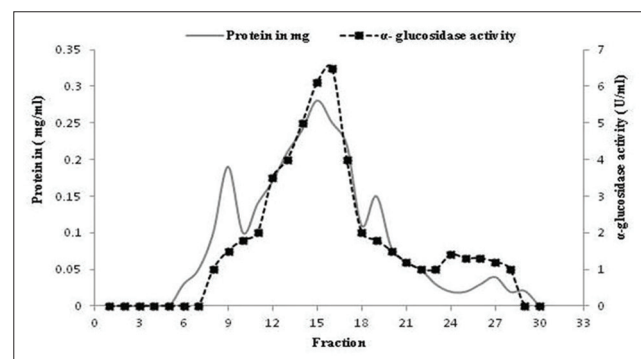

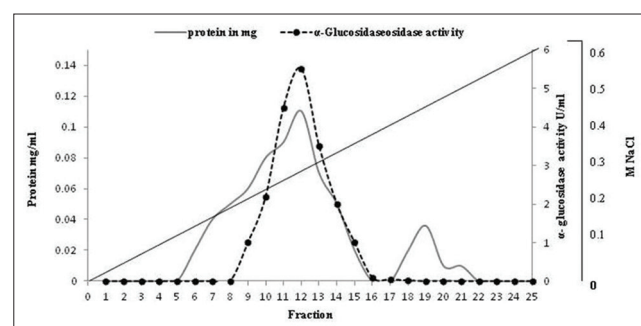**Fig. 1: Elution profile of α-glucosidase on sephadex G-100 column**

Fig. 2: Elution profile of active fractions of α-glucosidase on DEAE-cellulose column

Table 1: Purification steps of α-glucosidase from *H. nymaniana*

Purification steps	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude	350	500	1.43	1	100
Ammonium sulfate precipitation (80%)	24	160	6.67	4.66	34
Gel filtration	2.8	65	23.2	16.22	13
Ion exchange chromatography	0.44	22	50	35	4

H. nymaniana: Hyophilla nymaniana

Characterization of α-glucosidase from *H. nymaniana*

Molecular mass of the purified enzyme
The purified α-glucosidase appeared to be homogeneous according to the criteria of SDS-PAGE, as shown in (Fig. 3). A single band with a molecular mass of 72±5 kDa was seen on SDS gel. The apparent molecular weight of the native enzyme was estimated at approximately 135±5 kDa suggesting two identical subunits of the enzyme. A slightly higher molecular weight of the enzyme was obtained in *Pisum sativum* (110 kDa) [17], *Hordeum vulgare* 92 kDa [18], and in *Vitis vinifera* 100 kDa [19]. However, the purified α-glucosidase from yeast *Torulaspora pretoriensis* exhibited slightly lower molecular weight of 69 kDa on SDS-PAGE [20].

Effect of temperature and pH
The effect of temperature on purified α-glucosidase was examined from 25°C to 70°C at pH 5. The highest α-glucosidase activity was observed at 45°C (Fig. 4a). The enzyme was unstable and was inactivated at a temperature above 60°C (Fig. 4b). Results were mostly accordance to the purified α-glucosidase obtained from *Oryza sativa* [16] and

Hordeum vulgare [21] having an optimum temperature of 45°C. The effect of pH on α-glucosidase activity was studied by using l2.5 mM-p- nitrophenyl α-D-glucopyranoside in sodium acetate buffer ranging from pH 2.5 to 8.0. The optimum pH of the purified enzyme was 5 (Fig. 4c). The enzyme activity was retained after incubation for 1 hrs at pH values between 4 and 7 (Fig. 4d) but was unstable below pH 3 and above 7. Our results were in accordance with the purified α-glucosidase obtained from sugar beet seeds [21] and green gram seeds [22]. Although, a slightly higher optimum pH 5.7 and 5.5 was obtained for α-glucosidase in barley malt [17]. Furthermore, a similar broad pH range of 4.0-7.0 and 3.5-8.5 was reported in *Oryza sativa* [16] and *Sulfolobus solfataricus* [23].

Effect of metal ions
The activity of α-glucosidase treated with various metal ions were assayed and depicted in (Fig. 5). Among the different metal ions tested below, considerable loss in the activity was observed with Fe^{3+} Hg^{2+} and Ag^+ with only 40%, 30%, and 20% of the residual activity remaining while Cu^{2+} and Co^{2+} slightly inhibited the enzyme activity at the same concentration. However, K^+, Na^+, and Mg^{2+} enhanced glucosidase activity at the same concentration. The metal ions (Ba^{2+}, Co^{2+} Hg^{2+}, Mn^{2+}, and Zn^{2+}), pCMB, and EDTA have been generally reported as an inhibitor for α-D-glucosidase isolated from sugar beet [21] while Ag^+, Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and SDS considerably inhibited the α-glucosidase in *Rhizobium* sp. [24].

K_m and V_{max}
The Michaelis-Menten kinetics of purified α-glucosidase enzyme was estimated by Lineweaver-Burk plot by using various substrate concentrations of *p*-nitrophenyl α-D-glucopyranoside. The K_m and the V_{max} of α-glucosidase were 5.2 mM/ml and 8.6 U/ml, respectively (Fig. 6). However, Michaelis-Menten kinetics of α-glucosidase with other substrate 4-methylumbelliferyl α-D-glucopyranoside were K_m 0.141 μM, V_{max}, 6.79 μmol/minute/mg while with substrate p-nitrophenyl α-D-glucopyranoside were K_m, 0.037 μM; V_{max} 2.92 μmol/minute/mg, respectively [25].

CONCLUSIONS

The present study first time reports the purification and characterization procedure of α-glucosidase from the moss *H. nymaniana*. The purified enzyme exhibited wide pH stability (4-7) and high-temperature stability range up to 65°C showing its suitability toward industrial
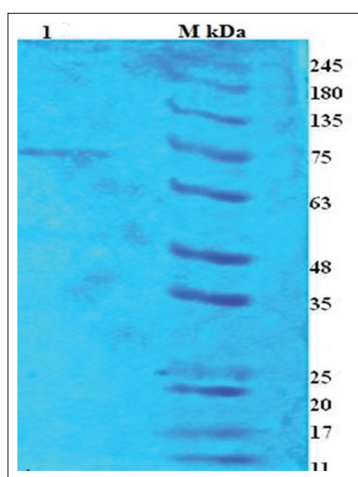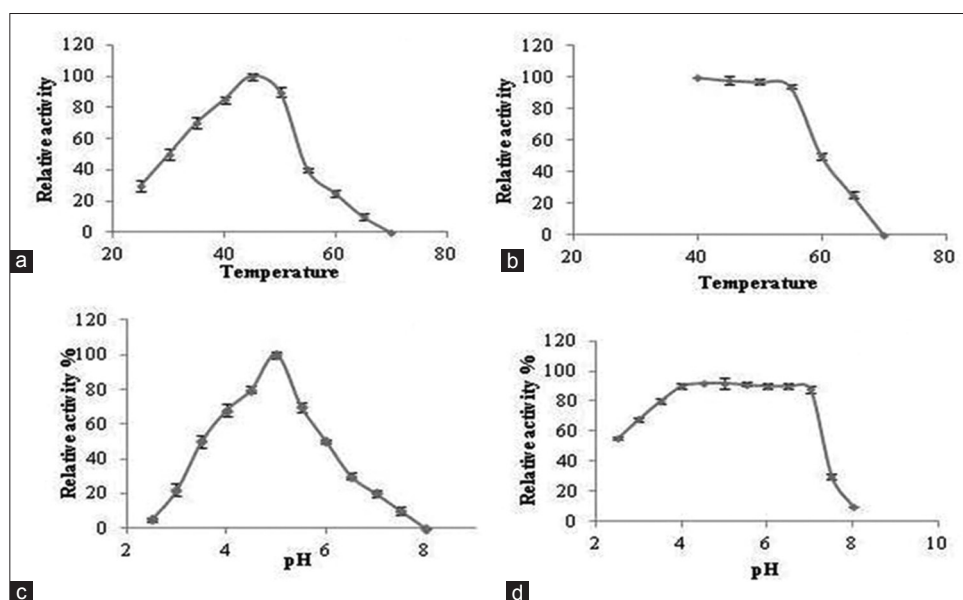

Fig. 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified α-glucosidase from *H. nymaniana* [Lane 1] Purified enzyme after gel filtration and [lane M] molecular weight marker

Fig. 4: Effect of temperature and pH on activity and stability of purified α-glucosidase from *H. nymaniana*. Data shown are averages of three experiments ± standard deviation
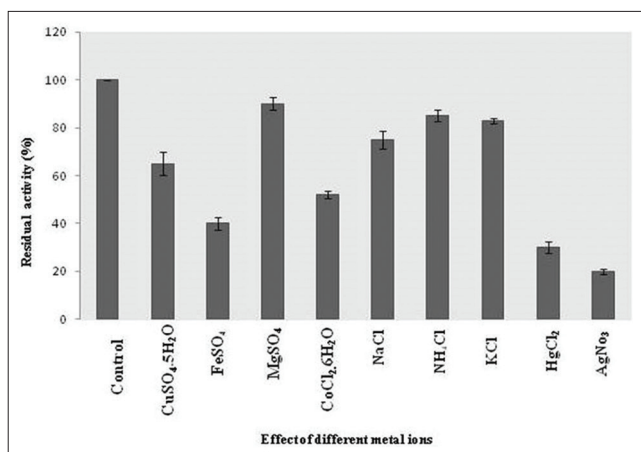
Fig. 5: Effect of different metal ions on α-glucosidase activity data shown are average of three experiments ± standard deviation
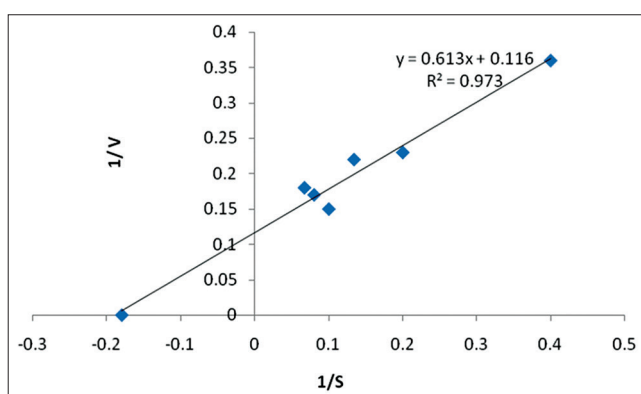

Fig. 6: Lineweaver-Burk plot of purified α-glucosidase against p-nitrophenyl α-D- glucopyranoside

application. The final purification step led to 35 fold purification with 4% of enzyme recovery.

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