Characterization of cell wall bound β-glucosidase from *Penicillium purpurogenum*

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Abstract

The β-glucosidase from a fungus Penicillium purpurogenum was purified to homogeneity in a 3-step procedure involving ammonium sulphate precipitation, DEAE cellulose chromatography, Sephadex G-100 chromatography. The enzyme has a molecular weight of 132kDa. The pH optimum was found to be 5.5 and it was fairly stable in pH range from 3 to 7. The temperature optimum was 50°C. The purified β-glucosidase was effectively active on para-nitrophenyl-β-D-glucopyranoside (β-PNPG) and cellobiose with K_m values of 0.72 and 4.16mM and V_{max} values of 444.99 x 10^2 and 11.41 x 10^2 µmol/min per mg protein, respectively. The enzyme activity was inhibited by Ag^{3+}, Hg^{2+}, Iodoacetate, N-bromosuccinimide (NBS) and stimulated in presence of Co^{2+}, while ethylene diamine tetra acetic acid (EDTA) showed no inhibition. The use of ethanol show inhibition at higher concentration whereas stimulation was observed at lower concentrations on enzyme activity.

Keywords: β-glucosidase, *Penicillium purpurogenum*, purification

Introduction

β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolytic cleavage of β-glycosidic linkage between two glycone residues or that between glucose and an alkyl or aryl aglycone. The enzyme constitutes a major group among glycoside hydrolases that was isolated from members of all three domains (Eucarya, Archaea, and Bacteria) of living organisms. β-glucosidases play key roles in a variety of fundamental physiological and biotechnological processes depending on the nature and diversity of the glycone or aglycone moiety of their substrates. β-glucosidases now a days are gaining importance due to their use in beverage quality enhancement [1]. The enzyme being a member of cellulase complex has also been used in cellulose bioconversion. Cellulose is the most abundant and an important renewable energy resource [2,3].

A procedure for purification of β-glucosidase isolated from *Penicillium purpurogenum* and its physical, chemical and enzymatic properties have been reported here. Some of the typical characteristics of β-glucosidase were compared with that from other sources.

Materials and Methods

Maintenance and cultivation of the organism

P. purpurogenum isolated in our laboratory and identified by IMT, MTCC, Chandigarh, India was used. The fungus was maintained on potato dextrose agar (PDA) slants with periodic subculturing at every 2 months. The cultures were stored at 4°C. *P. purpurogenum* was cultivated in Czapek Dox (CD) medium used by Patil and Shastri (1981) [4].

Chemicals

Ammonium sulphate, DEAE Cellulose, p-Nitrophenyl β-D-glucopyranoside, p-Nitrophenyl β-D-galactopyranoside, p-Nitrophenyl β-D-Xylopyranoside, p-Nitrophenyl β-D-Mannopyranoside, p-Nitrophenyl α – D-glucopyranoside, 4-Methylumbelliferyl β-D-glucopyranoside, Cellobiose, Gentiobiose, Laminariobiose, Maltose, Soporose, Trehalose, EDTA, β-mercaptoethanol, DEPC, DTT, Iodoacetate, NBS, PCMB, Pyridoxal phosphate were from SRL, Mumbai. Sephadex G-100 from Sigma chemicals, USA, Protein markers (PMWH) of higher range were purchased from Bangalore Genei.

All other chemicals were of analytical grade.

Preparation of crude enzyme

Twenty five grams of biomass was crushed mechanically under cold condition in 50 ml buffer (0.1M sodium acetate pH 5.5). The cell lysate was then centrifuged at 5000*g* for 20 min. The pellet was subjected to the treatment of 1% Tween 20 solution prepared in same buffer for 2 h at 30^{o}C and centrifuged at 5,000*g* for 20 min. The supernatant was collected and used as crude cell wall bound enzyme.

Enzyme purification

Ammonium sulphate precipitation: The crude fraction was treated with ammonium sulphate to 80% saturation, the precipitate was collected by centrifugation at 12,000*g* for 15 min. The precipitate was suspended in 10ml buffer (0.1M sodium acetate pH 5.5) and the suspension was dialyzed against same buffer (2 L) for 18 h. All the procedures were carried out at 4^{o}C.

DEAE Cellulose chromatography: The above enzyme concentrate was applied to a DEAE cellulose column (1 X 50 cm) preequiliberated with buffer (0.1M sodium acetate, pH 5.5). The column was washed with the same buffer containing sodium chloride. A liner NaCl gradient (0.05 to 0.25 M) was then applied at a flow rate of 2 ml/minutes, and fraction of 5 ml each were collected. Absorbance of column effluent was monitored at 280nm (A_{280}).

Sephadex G-100 chromatography: The dialyzed enzyme concentrate of the above fraction was applied on to a column (1 X 50 cm) of the Sephadex G-100 preequilibrated with above mentioned buffer and the column was eluted with the same buffer. Fractions of 4 ml each were collected. Absorbance of each fraction was also measured at 280nm.

Analytical methods

Determination of β-glucosidase activity: β-glucosidase activity was determined by the method of Riou *et al*., (1998) [5] using β-PNPG as a substrate. One unit of β-glucosidase activity was considered as that amount of enzyme, which liberated 1 µmol of *p*- nitrophenol per minute under experimental conditions.

The activity on cellobiose was determined by the method of Workman and Day (1982) [6]. The amount of glucose produced from cellobiose hydrolysis was determined by the method of Miller *et al.,* (1959) [7]. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of glucose under experimental conditions.

Protein measurement: Proteins were measured by the method of Lowry *et al.,* (1951) [8] using BSA as a standard protein. The concentration of protein during purification studies was calculated from the absorbance at 280nm.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE): After Sephadex G-100 chromatography the fractions showing highest specific activity were subjected to SDS-PAGE. The electrophoresis was carried out using 7.5% polyacrylamide gel in Tris glycine buffer (pH 8.3) containing 1% SDS [9]. A constant current of 1.5 mA per Sq. cm of slab was applied for 4 to 6 h at 28^{o}C. Fifty µg of denatured enzyme was subjected to each well for electrophoresis. The gel was stained with bromophenol blue to get protein bands.

Activity staining: Activity staining was performed by incubating the native gel with 2mg % 4-MUG (4-Methylumbelliferyl β-glucopyranoside) for 15 min at 37^{o}C, the released methylumbelliferone was observed under UV light and photographed. This method was a modification of the method used by Sprey and Lambert (1983) [10].

Characterization of purified enzyme

Effect of pH on purified enzyme activity and stability: Optimum pH for β-glucosidase activity was determined by assaying the β-glucosidase activity from pH 3 to 7 using sodium citrate (0.1M, pH 3 to 3.5) and sodium acetate (0.1M, pH 4 to 7) buffers. The activity was determined as described earlier.

The stability of the enzyme was determined by incubating the enzyme in presence of buffers of different pH range (3 to 7) for 2 h before determination of activity as described earlier.

Effect of temperature on purified enzyme activity and stability: The enzyme activity was measured at different temperatures ranging from 30 to 65°C.

The stability of enzyme was determined by exposure of the enzyme to different temperatures for 2 h before the activity was determined as described earlier.

Effect of inhibitors and chelators on enzyme activity: The effect of various enzyme inhibitors (1 & 10mM) such as diethylpyrocarbonate (DEPC), iodoacetate, dithiothreitol (DTT), β-mercaptoethanol, parachloromercuribenzoate (pCMB), N-bromosuccinimide (NBS), pyridoxal phosphate and ethylene diamine tetra acetic acid (EDTA) were determined by preincubation with the enzyme solution for 30 min at 50°C before the addition of substrate. The relative enzyme activity was measured.

Effect of metal ions and alcohol on enzyme activity: Effect of metal ions (e.g. Ag^{3+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+} and Zn^{2+} [1 & 10mM]) was investigated by exposure of enzyme to these metallic ions in the reaction mixture. In the same way ethanol [15, 20 and 90% (vol/vol)] was also added in reaction mixture and the enzyme activity was determined.

Substrate specificity & kinetic properties: Relative activity of the enzyme towards various substrates (5 mM) was determined. The kinetic parameters like K_m and V_{max} were determined for PNPG and cellobiose in concentration range of 0.31 to 5 mM using Lineweaver-burk plot [11].

Results and Discussion

A summary of the purification of the β-glucosidase from *Penicillium purpurogenum* is given in (**Table 1)**. The enzyme was purified to 31 fold with 23% yield. In Sephadex G-100 chromatography a single peak (Fraction No. 12 to 20) was obtained. The enzyme was found to be homogenous on SDS-PAGE and the molecular weight of β-glucosidase was found to be 132kDa **(Figure 1,2,3)**. The reported molecular weight for purified β-glucosidases from other sources ranges from 39.8kDa to 480kDa [12-14].

The optimum pH for the β-glucosidase was found to be 5.5. The study shows that the enzyme was fairly stable at low pH. The optimum temperature for enzyme activity was recorded to be 50^{o}C. The enzyme shows stability in Temperature range of 30 to 65^{o}C **(Figure 4,5)**. The β-glucosidase from *P. purpurogenum* was, however, similar to other fungal β-glucosidases having similar pH and temperature optima (50^{o}C, pH 5.5), as well as pH stability and thermal stability [15-17].

Table 1. Purification of β-glucosidase from *P. purpurogenum.*

Step	Total Protein (mg)	Total activity (U)	Sp act (U/mg of protein) X10^2	Purification (fold)	Recovery (%)
Crude extract	120	86350	7.19	1	100
Ammonium sulphate precipitation	12	66210	55.17	7.67	76.67
DEAE Cellulose Chromatography	2.9	21425	73.87	10.26	24.81
Sephadex G-100 Chromatography	0.88	19574	22.24	30.91	22.66

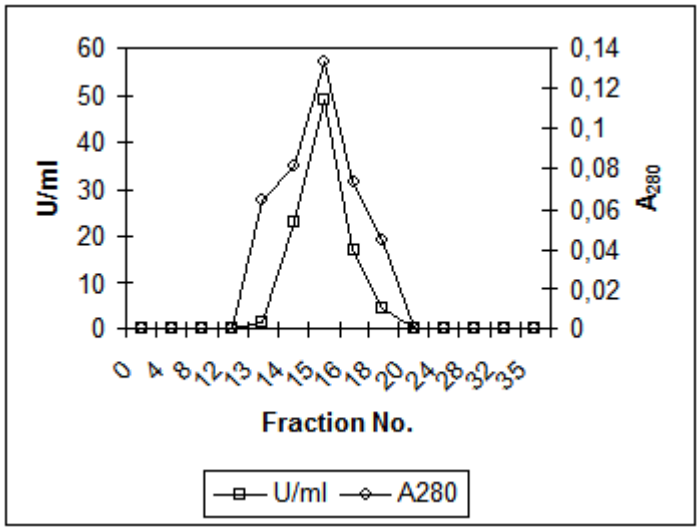

Figure 1. Purification of β-glucosidase from *Penicillium purpurogenum* by Sephadex G-100 chromatography.

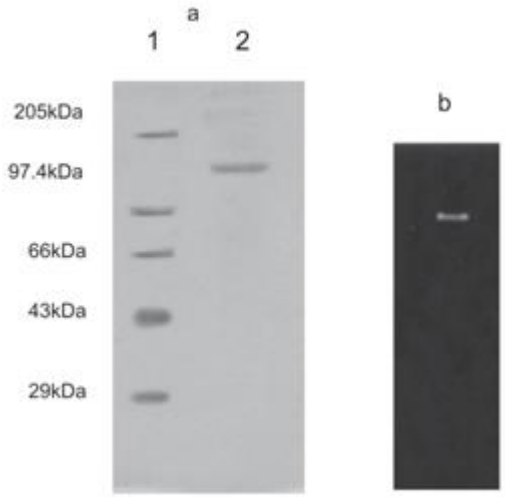

Figure 2. a) SDS-PAGE of β-glucosidase from *Penicillium purpurogenum*. Lane 1 contains M_r standards (myosin, phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase) and lane 2 contain 50 µg of β-glucosidase from final step. b) Purified β-glucosidase from *Penicillium purpurogenum* developed with fluorogenic substrate 4-MUG.

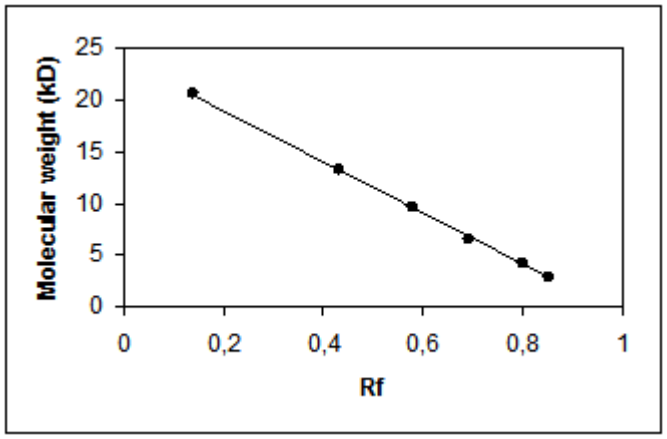

Figure 3. Determination of molecular weight of enzyme protein by SDS-PAGE. The pure protein standards are myosin (205 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

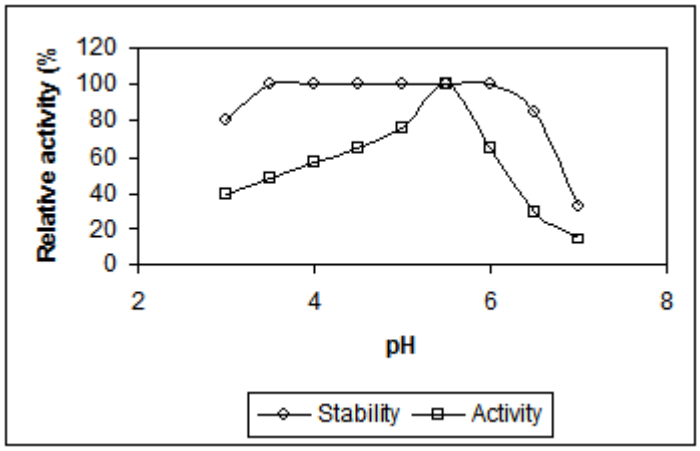

Figure 4. Effect of pH on stability (○) and activity (□) of purified β-glucosidase from *P. purpurogenum*.

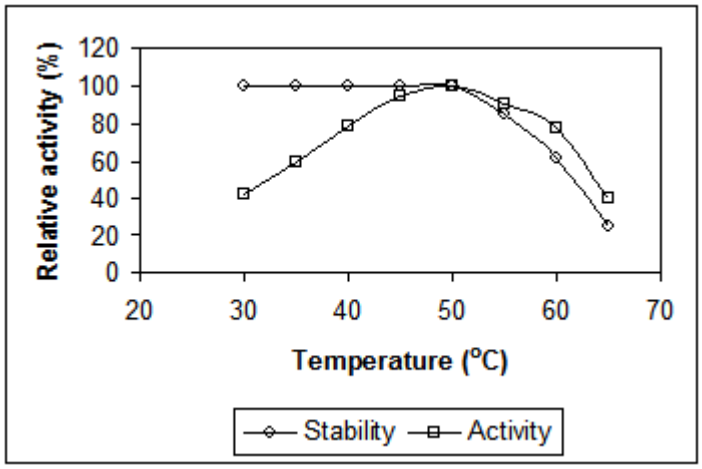

Figure 5. Effect of temperature on stability (○) and activity (□) of purified β-glucosidase from *P. purpurogenum.*

β-glucosidases have been divided into three groups on the basis of substrate specificity: aryl- β-glucosidases (which hydrolyze exclusively aryl β-glucosides), cellobiases (which hydrolyze oligosaccharides only) and β-glucosidases (which show activity on both substrate type and from the most commonly observed group of cellulolytic microorganism). The β-glucosidase from *P. purpurogenum* shown to hydrolyze cellobiose readily. The enzyme shows less activity towards rutin, laminariobiose and soporose and no activity was found towards gentiobiose and maltose respectively. The purified enzyme hydrolyzes p-Nitrophenyl β-D-glucopyranoside and 4-methylumbelliferyl β-D-glucopyranoside, while no activity was reported against p-Nitrophenyl β-D-galactopyranoside, p-Nitrophenyl β-D-Xylopyranoside and p-Nitrophenyl α-D-glucopyranoside **(Table 2)**. This result demonstrates that the purified enzyme belongs to the third group of β-glucosidases. Same results were reported for other β-glucosidases also [18-20].

Table 2. Relative activity of β-glucosidase from *P. purpurogenum* on various substrates.

Substrate (5mM)	Relative activity
p-Nitrophenyl β-D-glucopyranoside (β 1-4)	100
p-Nitrophenyl β-D-galactopyranoside (β 1-4)	0
p-Nitrophenyl β-D-Xylopyranoside (β 1-4)	0
p-Nitrophenyl β-D-Mannopyranoside (β 1-4)	0
p-Nitrophenyl α –D-glucopyranoside (α 1-4)	0
4-Methylumbbelliferyl β-D-glucopyranoside (β 1-4)	65
Cellobiose (β 1-4)	100
Gentiobiose (β 1-6)	0
Laminariobiose (β 1-3)	45
Maltose (α 1-4)	0
Soporose (β 1-2)	32
Trehalose (β 1-4)	75

Inhibition studies give an insight into the nature of an enzyme, its cofactor requirements and the active center [21]. Amongst the inhibitors N- bromosuccinimide (NBS) was found to strongly inhibit the enzyme activity. Iodoacetate shows weak inhibition, whereas other inhibitors show no change in activity. The inhibition of purified enzyme by NBS indicates the role of tryptophan in the active site of the enzyme. The EDTA was found to be ineffective against enzyme and shows no inhibition. The enzyme was inhibited in presence of $Hg^{2+}$and ethanol (90%) while enhanced activity was recorded in presence of Co^{2+} and at lower concentrations of ethanol **(Table 3)**. The NBS was also reported as enzyme inhibitor by Riou *et al.* and Lin *et al.* for β-glucosidase from *A. oryzae* and *T. lanuginous*-SSBP respectively [5,22]. Iodoacetate was reported to inhibit slightly the β-glucosidase from *C. papyrosolvens* (Sharmila *et al.* 1998) [23]. Chen *et al.* (1994) reported Ag^{2+} and Hg^{2+} to inhibit the enzyme activity, while Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} were reported to enhance β-glucosidase from *Orpinomyces* sp. strain PC-2.[18] Effect of alcohol was also studied by Perry *et al.* (2001) showing enhancement in activity at lower alcohol concentration and about 50% inhibition at higher alcohol concentration for β-glucosidase of *T. aurantiacus* [24].

Table 3. Effect of metal ions, chelators, inhibitors and ethanol on activity of purified β-glucosidase from *P. purpurogenum.*

Cations or reagent	Relative activity (1mM)	Relative activity (10mM)
Control	100	100
$AgNO_3$	**70**	**64**

$CaCl_2.2H_2o$	94	92
$CoCl_2.6H_2o$	**130**	**128**
$CuSO_4.5H_2o$	90	90
$FeSO_4.7H_2o$	95	94
$HgCl_2$	**30**	**10**
KCl	99	98
$MgSO_4.7H_2o$	87	84
$MnCl_2.4H_2o$	90	87
NaCl	97	94
$ZnSO_4.7H_2o$	89	87
EDTA	99	97
β-mercaptoethanol	100	98
DEPC	90	85
DTT	87	85
Iodoacetate	**75**	**72**
NBS	**20**	**10**
PCMB	88	85
Pyridoxal phosphate	80	79
Ethanol (15%)	**110**	
Ethanol (20%)	**105**	
Ethanol (90%)	**45**	

The reaction kinetics of the purified enzyme was determined from Lineweaver-Burk plot under optimum condition (10min, pH 5.5, 50^{o}C). The enzyme had apparent K_m values of 0.71 and 4.16mM and V_{max} values of 44499.83 and 1141.92 µmol/min per mg protein for PNPG and cellobiose, respectively **(Table 4, Figure 6)**. The higher catalytic efficiency (V_{max} / K_m) value for PNPG compared to that of cellobiose indicated that the PNPG was the preferred substrate for purified β-glucosidase **(Table 4)**. Affinity of the β-glucosidase for PNPG was considerably higher than those reported for β-glucosidase of *P. etchellsii* [25], *C. sake* [26], *C. peltata* [3], *D. vanrijiae* [27] and *C. cacaoi* [28].

Table 4. Kinetic parameters of β-glucosidase from *Penicillium purpurogenum*.

Substrate	K_m (mM)	V_{max} (µmol/min per mg protein)	V_{max} / K_m
p-Nitrophenyl β-D-glucopyranoside	0.71	44499.81	62675.79
Cellobiose	4.16	1141.92	274.5

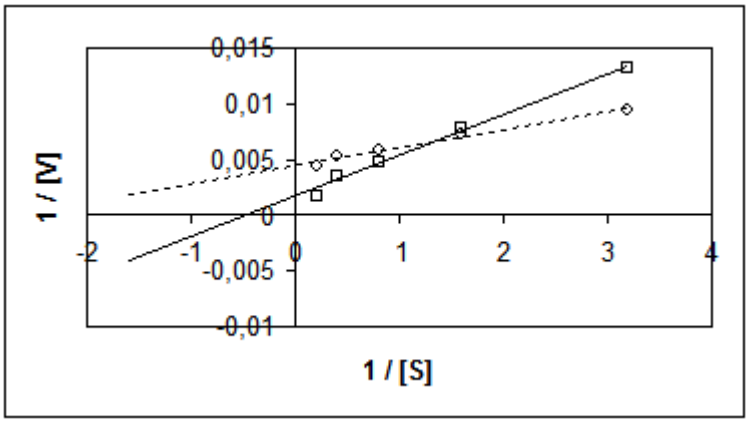

Figure 6. Lineweaver-Burk plot of β-glucosidase activity with PNPG (---○---) and Cellobiose (---□---) as substrates.

Conclusion

The β-glucosidase isolated from *P. purpurogenum* belongs to the third group of β-glucosidases. It is stable at low pH and high temperature. These properties indicate that the enzyme can be exploited commercially.

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