Biochimica et Biophysica Acta, 662 (1981) 22–29
Elsevier/North-Holland Biomedical Press

BBA 69404

PURIFICATION AND CHARACTERIZATION OF TWO FORMS OF EXTRACELLULAR β-GLUCOSIDASE FROM JUTE PATHOGENIC FUNGUS *MACROPHOMINA PHASEOLINA*

SAGAR C. SAHA, ARUNIK SANYAL, RAMENDRA K. KUNDU, SYAMALIMA DUBE * and DIPAK K. DUBE *

University College of Science, Department of Biochemistry, 35, Ballygunge Circular Road, Calcutta 700 019 (India)

(Received April 28th, 1981)

Key words: β-Glucosidase; Nojirimycin; Phytopathogen; Thermostability; (Jute)

Two forms of β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from the culture filtrate of *Macrophomina phaseolina* were separated and partially purified by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography (DE-52) and gel filtration. The final preparation was purified 103-fold and 88-fold for β-glucosidase-I and β-glucosidase-II, respectively. Polyacrylamide gel electrophoresis of the purified enzymes imparted a single band at pH 8.3. The two forms differ from each other with respect to molecular weight (323 600 for β-glucosidase I and 220 000 for β-glucosidase II)pH optima, temperature optima, electrophoretic mobility and substrate specificity. The two forms of β-glucosidase may also be differentiated by inhibition experiments using inhibitors like glucono-δ-lactone and nojirimycin. Of the two inhibitors tested nojirimycin is more potent for β-glucosidase-I than that for β-glucosidase-II. The energy of activation for the two enzymes is also different (12.02 kcal/mol for glucosidase I and 10.0 kcal/mol for glucosidase II).

Introduction

In order to understand the involvement of extracellular hydrolytic enzymes in cellulose degradation, studies on extracellular β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) of the phytopathogenic fungus *M. phaseolina* were undertaken [1–4]. In this communication, we now report the presence of two forms of extracellular β-glucosidase activity produced by the fungus *M. phaseolina*. The two forms of β-glucosidase have been partially purified and characterized.

Materials and Methods

The fungus *M. phaseolina* was grown in the synthetic medium according to the method of Capellini and Peterson [5]. For the enzyme production, the fungus was grown for 12 days at 30°C in a medium containing (%, w/v) 1.5 carboxymethyl cellulose/0.1 NH_4NO_3/0.05 $MgSO_4 \cdot 7\,H_2O$/0.1 KH_2PO_4/0.1 yeast extract at pH 5.0; *M. phaseolina* was a kind gift from Jute Agricultural Research Institute, Barrackpore, India. CM-cellulose and cellobiose were purchased from Loba-chemie Indo-Austranal; Sephadex (G-100, G-150 and G-200) are products of Pharmacia Fine Chemicals (Sweden). Substrates like *o*-nitrophenyl-β-D-glucoside, salicin, amydalin, β-methyl-D-glucoside, glucose oxidase, peroxidase, glucono-δ-lactone and DEAE-cellulose (DE-52) were obtained from Sigma Chemical Co., St. Louis, MO, poly(ethylene glycol) 6000 was from B.D.H. (U.K.). Ovalbumin (45 000) and bovine serum albumin were from Serva Feinbiochemica, Heidelberg, F.R.G. Nojirimycin was a generous gift from Shigeharu, Inouye, Japan. Other chemicals used are of analar grade.

Enzyme assays. β-Glucosidase activity was estimated by measuring the release of *o*-nitrophenol from

* Present address: International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya.

0005-2744/81/0000–0000/$02.50

the substrate *o*-nitrophenyl-β-D-glucopyranoside [6]. The assay mixture in a total volume of 1.0 ml contained 0.5 ml of 0.05 M citrate buffer (pH 4.8)/0.025 ml *o*-nitrophenyl-β-D-glucoside (0.04)/0.1 ml culture filtrate as enzyme source. The incubation was carried out for 30 min at 55°C for glucosidase I and at 65°C for glucosidase II. The reaction was stopped by the addition of 3.0 ml 0.1 M NaOH. The colour thus developed was estimated at 410 nm in a Bausch and Lomb (Spectronic 20). One unit of enzyme activity is defined as the liberation of 0.025 μmol *p*-nitrophenol per 30 min at 55 or 65°C.

Estimation of protein. Proteins were determined by the method of Lowry et al. [7] using bovine serum albumin as standard or by the absorbance at 280 nm.

Polyacrylamide gel electrophoresis. Gel electrophoresis wsa carried out at pH 8.3 by the method of Davis [8] on 7.5% acrylamide gels with bromophenol blue as a tracking dye. A current of 3 mA/gel was applied. Protein was stained with Coomassie blue.

To locatd the β-glucosidase activity the gels were incubated with 0.02 M *o*-nitrophenyl-β-D-glucoside in 0.05 M citrate buffer (pH 4.8) for 30 min at 55°C. The position of the β-glucosdase was visualised as a yellow transparent band after addition of 4 ml 0.1 M $NaHCO_3$.

β-Glucosidase mediated hydrolysis kinetics. The kinetics of the β-glucosidase catalyzed hydrolysis of substrates like cellobiose, *o*-nitrophenyl-β-D-glucoside, salicin, amydalin and β-methyl-D-glucoside were measured by the method analogous to discontinuous glycostat assay [14].

Purification of β-glucosidase. (i) $(NH_4)_2SO_4$ fractionation: - The entire operation was carried out at 4–6°C. To the culture filtrate solid $(NH_4)_2SO_4$ was slowly added with constant stirring up to 80% saturation. The whole mixture was kept at 4°C for 2 h and centrifuged at 10 000 × *g* for 30 min in a refrigerated centrifuge. The sediment was dissolved in 10 mM Tris-HCl buffer (pH 7.0) and is hereafter referred to as the $(NH_4)_2SO_4$ fraction (0–80%).

(ii) Gel filtration: - An aliquot of the '$(NH_4)_2SO_4$ fractionation' (containing 14 000 units β-glucosidase activity) was applied to a Sephadex G-100 column (100 × 1 cm) previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.0). 50 fractions each containing 2.0-ml were collected in an automatic fraction collector (Fractometer 200 - Buchler, U.S.A.). β-Glucosidase activity was determined with a 50-μl aliquot from each fraction according to the method of Mitchell et al. [6]. As two different peaks of β-glucosidase appeared in the eluted fractions of Sephadex column the first peak was designated as β-glucosidase I and the second as β-glucosidase II (Fig. 1).

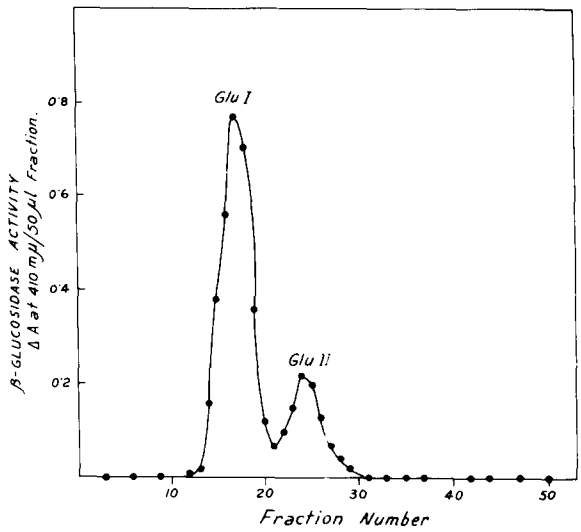

Fig. 1. Gel filtration of $(NH_4)_2SO_4$ fraction of culture filtrate through Sephadex G-100 column. β-glucoside activity (●——●).

(iii) β-Glucosidase I: The active pooled fraction of β-glucosidase I obtained from the Sephadex G-100 column was applied to a DE-52 column (16 × 0.5 cm) previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.0). After loading the column was washed with 200 ml of 0.01 M Tris-HCl buffer (pH 7.0)/0.05 M KCl. The enzyme was eluted with 100 ml Tris-HCl buffer having a linear gradient of KCl (0.05–0.25 M).

β-Glucosidase II: The same procedure was carried out with β-glucosidase II except for the elution of β-glucosidase II the KCl gradient was 0.2–0.6 M.

(iv) Gel filtration of glucosidase I and glucosidase II on Sephadex G-150: - The active fractions of glucosidase I and glucosidase II from two different DE-52 columns were pooled separately and dialyzed against 5 l 0.01 M Tris-HCl buffer (pH 7.0) for 12 h with several changes. After exhaustive dialysis, each fraction was concentrated by dialysing against poly(ethylene glycol) 6000. Finally each of the concentrated enzyme fractions was charged onto separate

Sephadex G-150 column (85 × 1 cm). The eluted fraction was used for further studies.

Results and Discussion

Purification of β-glucosidase

The overall summary of purification of β-glucosidase I and β-glucosidase II from the culture filtrate of *M. phaseolina* towards homogeneity is shown (Table I).

(i) $(NH_4)_2SO_4$ fractionation. Upon addition of $(NH_4)_2SO_4$ in the culture filtrate there was about 28% loss in enzyme activity. There was no appreciable change in enzyme activity when the $(NH_4)_2SO_4$ fraction was heated at 50°C for 30 min.

(ii) Gel filtration. The $(NH_4)_2SO_4$ fraction was then charged onto Sephadex G-100. The first pool in the fractions 15–20 was designated as glucosidase I and fractions 22–28 as glucosidase II. It is evident from Table I that there is about 5-fold purification for glucosidase I and 2.6-fold purification for β-glucosidase II.

(iii) DE-52 column chromatography. The two pooled fractions obtained from Sephadex G-100 column were separately charged on two different DE-52 columns. The recoveries of enzyme activity for glucosidase I and glucosidase II in this step are 82% and 62%, respectively. The specific activity was increased by 51-fold for glucosidase I and 44-fold for glucosidase II (Fig. 2A and B). Higher ionic strength is required for the elution of glucosidase II activity from the DE-52 column.

(iv) Sephadex G-150 column chromatography. The pooled active fractions from DE-52 column were separately charged onto Sephadex G-150 column. The recoveries in this step were 87% for glucosidase I and 92% for glucosidase II, respectively. The fold of purification of glucosidase I was about 103 and that for glucosidase II was 8.

(v) Protein pattern of the purified enzyme on

TABLE I

CHART FOR THE PURIFICATION OF β-GLUCOSIDASE I AND β-GLUCOSIDASE II FROM THE CULTURE FILTRATE OF *M. PHASEOLINA* GROWN IN CM-CELLULOSE MEDIUM

Glu-I is β-glucosidase I and Glu-II is β-glucosidase II.

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)
Culture filtrate	60.5	27 500 [a]	454.54	1
0–80% $(NH_4)_2SO_4$ fractionation	55	20 000 [a]	363.63	0.8
Heated $(NH_4)_2SO_4$ fraction at 50°C for 30 min	40	20 000 [a]	500	1.10
Sephadex G-100 gel filtration				
Charging material	28	14 000 [a]		4.92
Recovery of Glu-I	3	7 720	2 240	
Recovery of Glu-II	3.4	4 080 [b]	1 200	2.64
DE-52 chromatography				
Charging material for Glu-I	1.25	2 800 [a]		
Recovery for Glu-I	0.99	2 310 [a]	23 333	51.34
Charging material for Glu-II	3.0	3 600 [b]		
Recovery for Glu-II	0.112	2 250 [b]	20 000	44.1
Sephadex G-150 gel filtration				
Charging material for Glu-I	0.80	2 200 [a]		
Recovery for Glu-I	0.409	1 910 [a]	46 669	102.68
Charging material for Glu-II	0.1	2 200 [a]		
Recovery for Glu-II	0.0505	2 020 [a]	40 000	88.02

[a] One unit is defined as the liberation of 0.025 μmol *p*-nitrophenol/30 min at 55°C.
[b] One unit is defined as the liberation of 0.025 μmol *p*-nitrophenol/30 min at 65°C and at optimum pH.

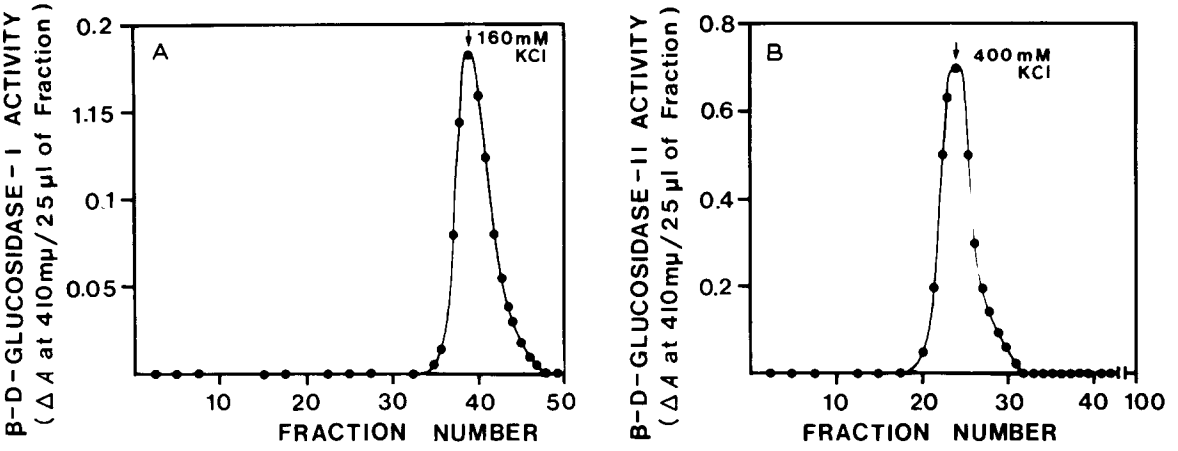

Fig. 2. Cellulose DE-52 column chromatography of (A) β-glucosidase I and (B) β-glucosidase II obtained from two different pooled fractions from the Sephadex G-100 column.

polyacrylamide gel. The protein band of purified β-glucosidase I and β-glucosidase II are shown in Fig. 3. It is to be noted that the electrophoretic mobility of β-glucosidase I on polyacrylamide gel is lower than that of β-glucosidase II [2].

General properties of purified enzymes glucosidase I and glucosidase II

The condition of the assay of the enzymes was in accordance with the fact that the liberation of *o*-nitrophenol was linear with respect to both time and enzyme concentration.

(a) pH optimum. The optimum pH for β-glucosidase I is 5.5, whereas for glucosidase II it is comparatively broader lying between 4.8 and 6.

(b) Temperature optima. The optimum temperature for β-glucosidase I activity is 55°C while that for β-glucosidase II is 65°C.

(c) Determination of molecular weight. The molecular weights of β-glucosidase I and β-glucosidase II were determined as 323 600 and 213 800, respectively, by Sephadex G-150 and G-200 column chromatography using standards such as albumin, ovalbumin, alkaline phosphates and catalase.

(d) Heat inactivation. Thermostability of β-glucosidase I and β-glucosidase II was analysed by preincuba-

Fig. 3. Polyacrylamide gel electrophoretic pattern of proteins of β-glucosidase I and β-glucosidase II: A: protein band of β-glucosidase I, B: protein band of β-glucosidase II.

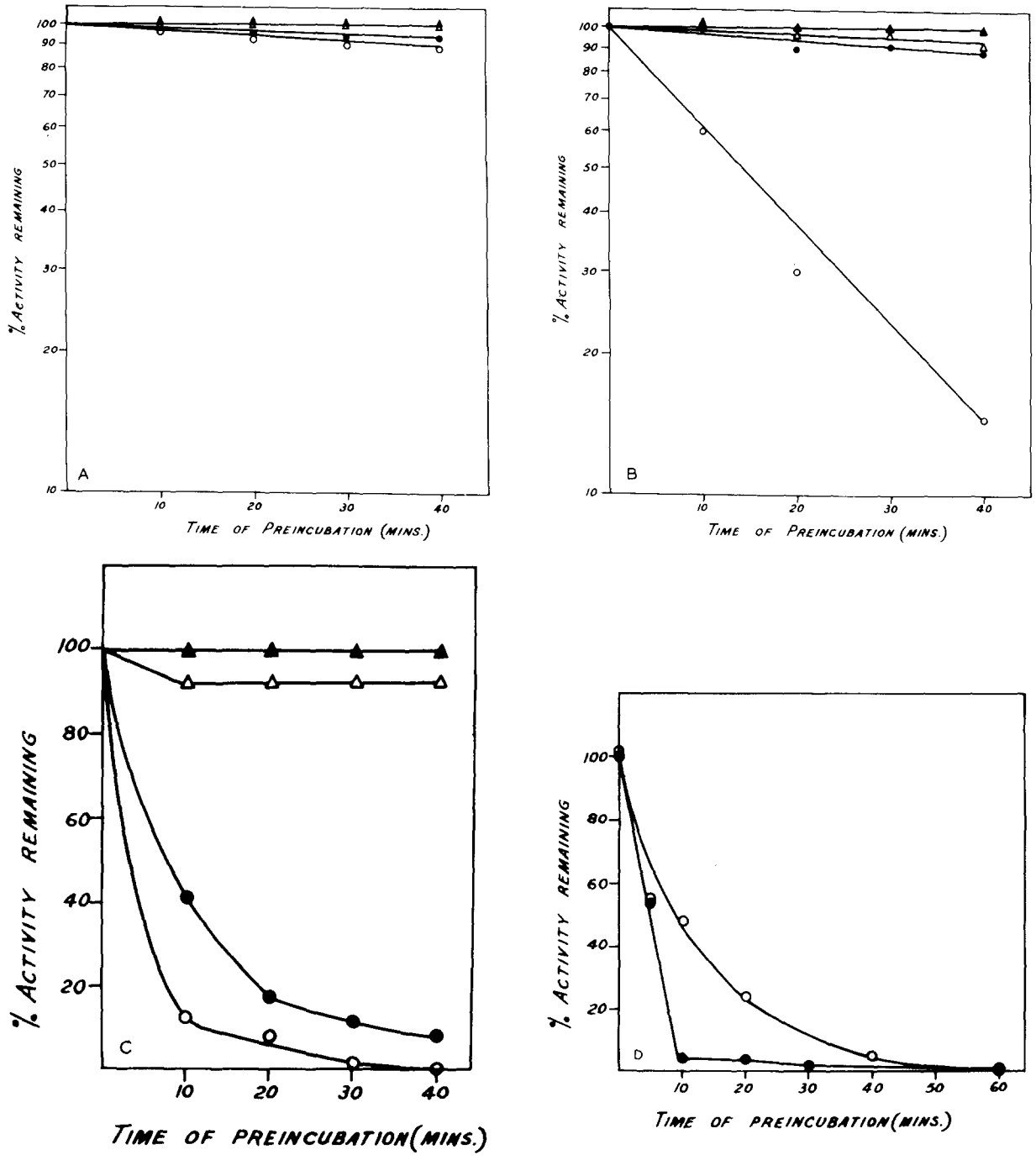

Fig. 4. Thermal denaturation of β-glucosidase I and β-glucosidase II isolated from culture filtrate of *M. phaseolina* grown in CM-cellulose medium. 10 units of each β-glucosidase I/II were diluted in buffer so that the mixture contained 1.0 mg/ml bodine serum albumin/10 mM Tris-HCl (pH 7.0)/10 mM sodium citrate buffer (pH 5.0) preincubation was carried out at the indicated temperature. At the time indicated aliquots (0.15 ml) were removed and assayed for activity at 55°C. Initial activity corresponds to 100%. A. At 50°C in two different pH: ●——●, β-glucosidase I, pH 5.0; ○——○, β-glucosidase I, pH 7.0; ▲——▲, β-glucosidase II, pH 5.0; △——△, β-glucosidase II, pH 7.0. B. At 60°C in two different pH: ●——●, β-glucosidase I, pH 5.0; ○——○, β-glucosidase II, pH 7.0; ▲——▲, β-glucosidase II, pH 5.0; △——△, β-glucosidase II, pH 7.0. C. At 65°C in two different pH: ●——●, β-glucosidase I, pH 5.0; ○——○, β-glucosidase I, pH 7.0; ▲——▲, β-glucosidase II, pH 5.0; △——△, β-glucosidase II, pH 7.0. D. At 70°C at pH 5.0 ●——●, β-glucosidase I; ○——○, β-glucosidase II.

tion at 50, 60, 65 and 70°C at pH 5.0 and 7.0 and subsequent assays for β-glucosidase activity at their respective optimum temperatures. Preincubation for 30 min for both glucosidase I and II resulted in no significant loss of enzyme activity both at pH 5.0 and 7.0 (Fig. 4A). However, due to preincubation at 60°C particularly at pH 7.0, glucosidase I shows a rapid loss of activity.

On the other hand, there is no such loss in activity in the case of glucosidase II at the aforementioned pH (Fig. 4B). Similar differential thermal stability was observed when the enzymes were preincubated at 65°C (Fig. 4C). β-Glucosidase II was found to be stable both at pH 5.0 and 7.0 even after 40 min of preincubation. At 70°C both glucosidase I and II are inactivated rapidly; however, the rate of inactivation is a little faster in the case of glucosidase I even at pH 5.0 (Fig. 4D).

(e) Effect of some metal ions and chemical agents. The effects of different metal cations, chelating agents on the activity of glucosidase I and II are shown in Table II. Of the metal ions tested Hg^{2+} and Mo^{6+} are the most potent inhibitors of both glucosidase I and glucosidase II. Sodium azide, thiomerosal, *N*-ethylmaleimide and EDTA have pratically no inhibitory effect upon the activity of the enzyme. Among the electrolytes tested, NaCl and KCl have no inhibitory effects on either of the enzyme forms. *p*-Chloromercuribenzoate, the well known SH blocking agent, was found to inhibit both forms of the enzyme. No inhibition was detected with NaN_3, thiomerosal, NaCl, KCl or EDTA.

(f) K_m of various substrates for β-glucosidase I and β-glucosidase II. Michaelis-Menten kinetics were determined for the β-glucosidase catalyzed hydrolysis of cellobiose using different substrate concentrations at pH 4.8. Michaelis-Menten kinetics for glucosidase-catalyzed hydrolysis for different substrates were carried out at optimum temperatures (55°C for β-glucosidase I and 65°C for β-glucosidase II). The rate was proportional to glucosidase concentration. The values of K_m for cellobiose are 1.6 and 0.9 mM for β-glucosidase I and β-glucosidase II, respectively. Similarly the K_m values for *o*-nitrophenyl-β-D-glucoside are 1.25 and 0.125 mM for β-glucosidase I and β-glucosidase II (Table III), respectively. The K_m values for other substrates e.g., salicin, methyl-β-D-glycoside and amydalin for both β-glucosidase I and β-glucosidase II are given in Table III. It is to be noted that K_m values for all the substrates except *o*-nitrophenyl-β-D-glucoside are lower in the case of glucosidase II. The results in Table III show the K_m of *o*-nitrophenyl-β-D-glucoside

TABLE II

THE EFFECTS OF METAL IONS, SOME CHEMICAL AGENTS AND EDTA ON THE ACTIVITY OF β-GLUCOSIDASE I AND β-GLUCOSIDASE II

Activity is expressed as a percentage of activity level in absence of metal ions. NEM, *N*-ethylmaleimide and PCMB, *p*-chloromercuribenzoate.

Metals	Relative activity β-glucosidase I		Relative activity β-glucosidase II	
	+ Metal [a]	Metal + EDTA [b]	Metal [a]	Metal + EDTA [b]
Mn^{2+}	110	105	102	105
Ca^{2+}	92.86	88.1	100	100
Fe^{3+}	100	110	102	105
Hg^{2+}	23.8	59.8	25	84.4
Mo^{6+}	3.38	85.7	31.25	87.4
Mg^{2+}	100	100	100	102
Pb^{2+}	92.85	92.85	93.75	96.88
Cu^{2+}	92.85	95.24	96.88	93.75
Co^{2+}	107.14	90.48	94.0	96.0
Zn^{2+}	85.24	95.72	100	102
NEM	100	105	100	102
PCMB	30.5	68.0	28.5	78.5

[a] Enzymes were preincubated with metal ion at 37°C for 30 min.

[b] Enzymes were preincubated with metal ion and EDTA.

TABLE III

K_m OF VARIOUS SUBSTRATES FOR β-GLUCOSIDASE I AND β-GLUCOSUDASE II

Substrate	K_m (mM)		V (10^{-6} mol s)	
	Glu-I	Glu-II	Glu-I	Glu-II
Cellobiose	1.66	0.9	0.333	0.11
Salicin	0.5	0.25	0.055	0.051
O-Nitrophenyl β-D-glucoside	1.25	0.125	0.285	0.118
Methyl-β-D-glucoside	15.38	11.76	0.125	0.066
Amydalin	0.285	0.113	0.154	0.077

for β-glucosidase I. Though β-glucosidases from *Sporotrichum pulverulentum, Trichodoma viride, Botryodiplodia theobromae Pat* and *Aspergillus niger* are being able to attack both *o*-nitrophenyl-β-D-glucosidase and cellobiose, the K_m for *o*-nitrophenyl-β-D-glucoside is lower than for cellobiose in each case [10].

(g) Inhibition of β-glucosidase I and β-glucosidase II activities by glucono-δ-lactone and noijirimycin. The type of inhibition of glucono-δ-lactone and the inhibition constant were determined from initial velocity measurements at various concentrations of substrate (0.2–1.6 mM for β-glucosidase I and 0.2–0.5 mM for β-glucosidase II) and at three different inhibitor concentrations.

The results were plotted according to Lineweaver and Burk (data not shown). The common intercept on 1/V axis indicates that the inhibition of the enzyme by glucono-δ-lactone is competitive. The K_i values obtained from the secondary plot are about 17 μM for β-glucosidase I and 30 μM for β-glucosidase II (Fig. 5A).

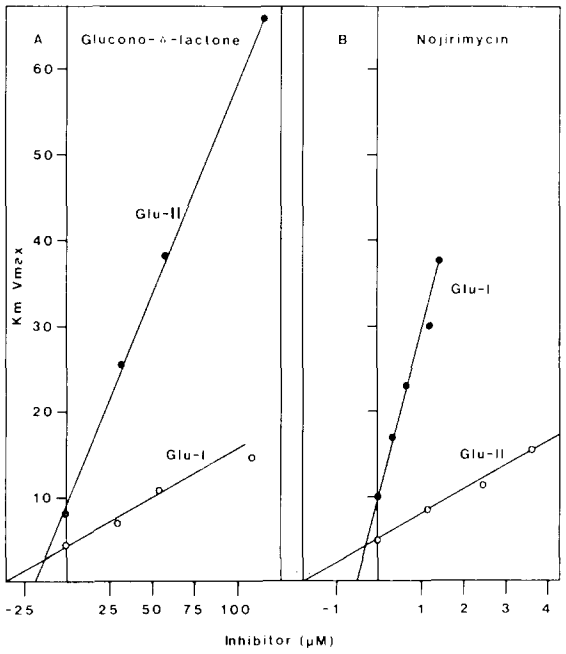

Fig. 5. Determination of inhibition constant for glucosidase I and II. A, For glucono-δ-lactone; V and K_m were determined at different concentrations of inhibitor, e.g., 0, 28, 56 and 112 μM. B. For nojirimycin V and K_m were determined at various concentrations of inhibitor, e.g., 0, 0.312, 0.625 and 1.25 μM.

TABLE IV

K_i OF GLUCONO-δ-LACTONE AND NOJIRIMYCIN FOR β-GLUCOSIDASE I AND β-GLUCOSIDASE II USING *O*-NITROPHENYL-β-D-GLUCOSIDE AS SUBSTRATE

	Glucono-δ-lactone		Nojirimycin	
	K_i	K_m/K_i	K_i	K_m/K_i
β-Glucosidase I	17.0	26.47	0.5	1 320
β-Glucosidase II	30.0	6.0	1.6	156.25

The type of inhibition of nojirimycin and the inhibition constant was determined from initial velocity measurement at various concentrations of substrate (0.4–2.0 mM for β-glucosidase I and 0.2–0.5 mM for β-glucosidase II) and at three different inhibitor concentrations (0.125, 0.625 and 1.25 μM). The results were plotted according to Lineweaver and Burk (data not shown). The common intercept on 1/v axis indicates that the inhibition of enzyme by nojirimycin is competitive. The K_i value obtained from the secondary plot is about 0.5 μM for β-glucosidase I and 1.6 μM for β-glucosidase II (Fig. 5B).

Inhibitors like nojirimycin and glucono-δ-lactone are frequently used for characterization of β-glucosidase [11–12]. The values of K_m/K_i for β-glucosidase I as well as β-glucosidase II isolated from the culture filtrate of *Macrophomina phaseolina* for glucono-δ-lactone pertains to 26.47 and 6.0, respectively, using *o*-nitrophenyl-β-D-glucoside as substrate. When nojirimycin is used as the inhibitor the K_m/K_i values for the β-glucosidase II were found to be 1320 and 156, respectively (Table IV). Deshpande et al. [10] reported the K_m values for enzyme A and B of 1,4 β-glucosidase while studying the enzymes isolated from *S. pulverulentum.* The authors reported the value of K_m/K_i of free enzymes for glucono-δ-lactone approx. 2500 for enzyme B and 13 000 for enzyme A with cellobiose as substrate. Our results strongly suggest that both glucono-δ-lactone and nojirimycin are more potent inhibitors for β-glucosidase I so far as the inhibitory potency is concerned. Nojirimycin is a more potent inhibitor than glucono-δ-lactone even for β-glucosidase I.

(h) The energy of activation. At the pH of maximum activity (pH 5.0) the rate V was determined as a function of temperature over the range 30–50°C for

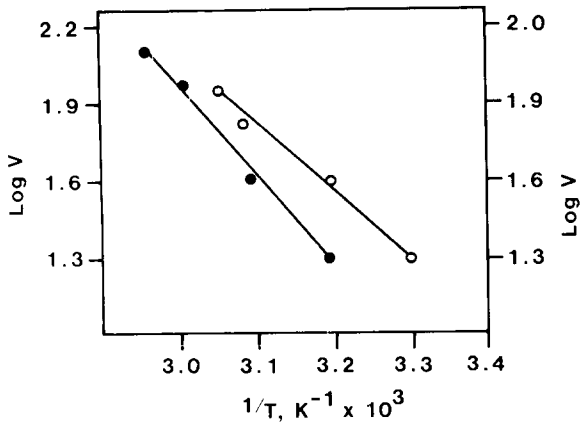

Fig. 6. Energy of activation of glucosidase I (●———●) and glucosidase II (○———○).

β-glucosidase I and 40–65°C for β-glucosidase II using cellobiose as substrate (Fig. 6). The energy of activation is 12.02 kcal/mol for β-glucosidase I and 10.0 kcal/mol for β-glucosidase II. The energy of activation for *Trichoderma viride* cellulose catalyzed hydrolysis of a cellotetrose and amorphous cellulose has been found to be 6.5 and 5.1 kcal/mol, respectively [13].

It may be concluded that β-glucosidase I and II are two different enzymes although β-glucosidase I is inducible in nature and glucosidase II is rather constitutive as is evidenced by the fact that is also appears in the culture filtrate of non-cellolosic source (unpublished data). Our unpublished results indicate that β-glucosidase I is absent in the culture of filtrate of *M. phaseolina* when grown in non-cellulose media.

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