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Purification and Characterization of β-Mannosidases from White Rot Fungus *Phlebia radiata*

MONIKA PRENDECKA*, AGNIESZKA BUCZYŃSKA and JERZY ROGALSKI

Department of Biochemistry, Maria Curie-Skłodowska University, Lublin, Poland

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Abstract

A β-mannosidase was purified from *Phlebia radiata* grown in a medium containing wheat bran or galactomannan as a carbon source. Maximal activity was observed at pH 5.5 and at 50°C. Highly purified isoforms of β-mannosidase (GM-1, GM-2) isolated from media containing galactomanan and (OT-1) media with wheat bran were obtained by means of column chromatography on Q-Sepharose, SP-Sepharose and chromatofocusing on Polybuffer Exchanger PBE-94.

K e y w o r d s: biodegradation , isozymes, β-mannosidase, *Phlebia radiata,* purification

Introduction

Hemicelluloses are the second most abundant polysaccharides in nature. Mannan is one of the components of wood hemicelluloses, sometimes representing 14–20% of dry weight. The mannose units in some mannans are substituted to varying degrees with galactose, mannose, glucose and *O*-acetyl groups (Puls and Schuseil, 1993). The complete enzymatic degradation of these branched heteroglycans into monosaccharides involves activity of endo-1,4-β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), α-galactosidase (EC 3.2.1.22.) and β-glucosidase (EC 3.2.1.21).

β-Mannosidase is an exoglycosidase which catalyzes the hydrolysis of terminal nonreducing mannose residues in mannans. It is essential for total hydrolysis of plant polysaccharides and may have application in pulp and paper industry in order to enhance the traditional chemical delignification (Suurnäkki *et al.*, 1997). It is also important in the enzymatic saccharification of hemicellulose to monomeric sugars for further conversion to chemical and fuels (Ademark *et al.*, 1999). The use of β-mannosidase and related enzymes in the synthesis of oligosaccharides for medical and other purposes is also interesting (Kobata, 1993).

Relatively few microbial β-mannosidases have been purified and characterized, such as enzyme from *Aspergillus niger* (Elbein *et al.*, 1999; Ademark *et al.*, 1977), but they have been reported to occur in plants and animal tissues (Dey, 1978). The white rot fungus *Phlebia radiata* known as an efficient degrader of lignin (Hatakka and Uusi-Rauva, 1983) was also characterized as a hemicellulolytic enzyme producer (Rogalski *et al.*, 1993).

The purpose of this work was to isolate, purify and characterize β-mannosidase from culture filtrates of *P. radiata*.

Experimental

Materials and Methods

Organism and culture conditions. The inoculum from *Phlebia radiata* Fr. 79 (ATCC 64658) isolated in the Departament of Microbiology of Helsinki, Finland (Hatakka and Uusi-Rauva, 1983), was maintained on 2% (wt/vol) malt agar slants. The procedure of the inoculum preparation was performed in accordance with (Hatakka and Uusi-Rauva, 1983). The mycelial mats were collected and homogenized in a Waring blender. After inoculation with 4% (vol/vol) of the homogenates, 100-ml conical flasks, each containing 10 ml of ADMS-LN medium supplemented with 1% (wt/vol) galactomannan (LBG, Fluka, Buchs,

* Corresponding author: M. Prendecka, Department of Biochemistry, Maria Curie-Skłodowska University, M. Curie-Skłodowskiej 3, 20-033 Lublin, Poland; phone: +48 81 5375521; fax: +48 81 5375761; **e-mail: mprend@biotop.umcs.lublin.pl**

Germany) or wheat bran (Helsingin Mylly Oy, Helsinki, Finland) as a carbon source, were incubated in stationary at 28°C.

Enzyme assay. β-Mannosidase activity was measured in accordance with (Kurakake and Komaki, 2001) using 1 mM pNPM as a substrate. p-Nitrophenol was used as a standard.

Assays. The protein content in the examined enzyme preparation was determined by means of the Bradford method (Bradford, 1976). The saccharide content in protein molecules was determined as in (Dubois *et al.*, 1956). The kinetic constants (K_m and V_{max}) were calculated on IBM PC computer running the Wilmann 4 (1985; M.S.U., MN) software. The molecular weight determination was made using gel permeation chromatography on Protein-Pak 300 SW column connected to the Shimadzu HPLC system composed of LC-9A Chromatography pump, the SPD-M6A diode array detector and the 7125 model sampling valve (Rheodyne, Barkeley, USA) with 100 µl loop. The mobile phase 0.1 M acetate buffer (pH 5.0) was run at the flow rate 0.5 ml/min. The column was calibrated by the linear least square method using the Quattro Pro software (Borland, USA) on IBM PC using the MS II Gel-filtration Standard Set (Serva, Heidelberg, Germany).

Chromatography and chromatofocusing. Columns for ion-exchange chromatography (2×20 cm; packed with Q-Sepharose fast flow equilibrated with 50 mM Tris-HCl buffer, pH 7.5 and packed with SP-Sepharose fast flow equilibrated with 20 mM acetate buffer, pH 4.1) and chromatofocusing (1×30 cm; packed with Polybuffer Exchanger PBE-94; eluted in the pH range 7.4–3.5) were attached to the BIO-RAD Econo System.

Results and Discussion

Dynamics of β-mannosidase secretion. The first step in the examination was to determine the optimal growth condition of *P. radiata* strain for the production of β-mannosidase. *P. radiata* strain was grown on ADMS-LN medium containing LBG or wheat bran as the carbon source. The extracellular activities were monitored for 14 days (Fig. 1). The maximum β-mannosidase activities on the media with wheat bran were measured on the 11th day; on the media containing LBG they increased gradually on the 4th and 5th day and then declined. The protein content in post-culture medium with LGB was twice as in the case when wheat bran was used as a carbon source.

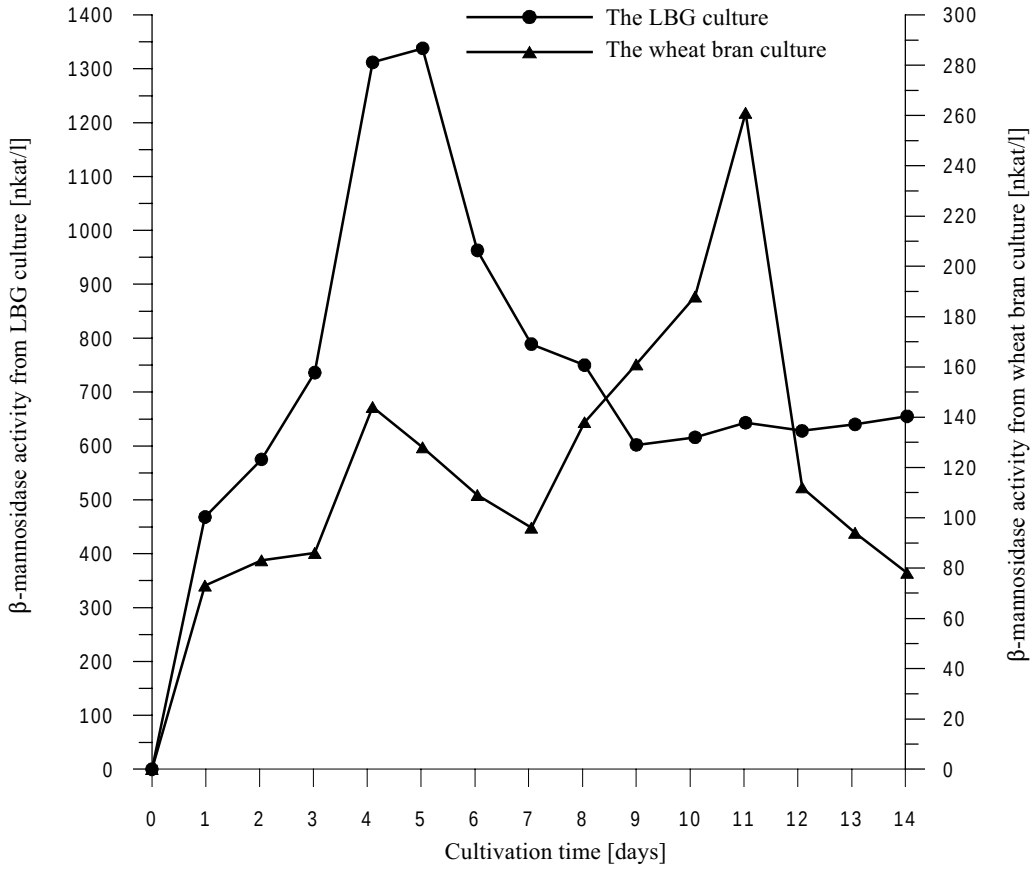

Fig. 1. Production of β-mannosidase by *Phlebia radiata* on ADMS-LN medium containing wheat bran (▲) or galactomannan (●) as sole carbon source.

Consequently, the β-mannosidase purification was carried out from *P. radiata* cultures grown on 11th and 5th day. The collected data allowed for the comparison of a stimulatory effect of different carbon sources (LBG or wheat bran) on the secretion of β-mannosidase. Wheat bran is rich in hemicelluloses containing both xylans and mannans (Smith and Hatley, 1983), whereas galactomannan is a mannooligosaccharide in which the mannose-to-galactose unit ratio is 4:1 (Sachslehner *et al*., 1998). In both carbon sources numerous β-mannoside linkages which are hydrolyzed by β-mannosidase are present. In comparison with the activity of mannosidase from wheat bran cultures the enzyme from the LBG cultures manifested higher activity. This indicates that the LBG is a better inductor of mannosidase production than wheat bran in *P. radiata.* Similar observations were described by Sachslehner *et al.*, (1997) who noticed that mannans and mannooligosaccharides are better inductors of mannosidase synthesis in *Sclerotium rolfsii* than D-galactose, D-mannose, raffinose, melibiose, lactose, cellobiose, cellulose or xylan. According to Kremincky and Biely (1997) the level of β-mannosidase in *Aureobasidium pullulans* was 10- to 100-fold higher on the galactomannan than on the any other carbon sources. Also, *Cellulomonas fimi* secreted β-mannosidase only during growth on mannose or mannan (Stoll *et al*., 1999).

Effect of pH and temperature. The subsequent goal was to determine of temperature and pH influence on the β-mannosidase activity. The effect of temperature was investigated over the range of 4–70°C. The highest activity of mannosidase isolated from both cultures occurred at 50°C (Fig. 2A). The temperature optimum of other fungal mannosidases is slightly higher – between 60–70°C for the enzyme from *Aspergillus awamori* (Kurakake and Komaki, 2001), and 70°C for *A. niger* (Ademark *et al*., 1999).

A much wider bracket of temperature optimum can be observed for bacterial mannosidases. β-mannosidase from *Bacillus sp.* was observed to have the optimum of temperature in 25°C (Waino and Ingvorsen, 1999). On the other hand, enzymes from bacterial thermophilic strains required almost 100°C *e.g. Thermotoga neapolitana* (87°C) (Duffaund *et al*., 1997) or *Pyrococcus furiosus* (105°C) (Bauer *et al*., 1996).

The optimum pH for the β-mannosidase from *P. radiata* isolated from both cultures was 5.5 (Fig. 2B). Other fungal β-mannosidases have slightly lower pH optima – 5.0 for *A. niger* (Ademark *et al*., 1999) and *A. awamori* (Kurakake and Komaki, 2001) and 3.5 for *Trichoderma reesei* (Kulminskaya *et al*., 1999). The optimum pH is fairly varied for other organisms. It is 7.0 for *Bacillus* sp. (Waino and Ingvorsen, 1999), 7.4 for *P. furiosus* (Bauer *et al*., 1996) and 3.3 for *Helix aspersa* (Charrier and Rouland, 2001).

Purification of extracellular β-mannosidase. The results representing purification are shown in Tables I and II and Figures 3–5. After the ion exchange chromatography on Q-Sepharose, several protein fractions were observed with β-mannosidase activity corresponding to the peak fractions eluted in 0.18 M NaCl (in the case of purification from the media with wheat

Table I
Purification of *Phlebia radiata* β-mannosidase from post-culture liquid containing 1% wheat bran as sole carbon source

Preparation	Total protein (mg)	Specific activity (nkat/mg)	Purification (-fold)	Yield (%)
Supernatant 21 000×g	34.78	511.80	1.00	100.00
Q-Sepharose	5.29	3152.69	6.16	93.67
SP-Sepharose	1.67	9033.27	17.65	84.91
PBE 94 OT-1 (pI 4.65)	0.21	62209.29	121.55	73.44

Table II
Purification of *Phlebia radiata* β-mannosidase from post-culture liquid containing 1% galactomannan as sole carbon source.

Preparation	Total protein (mg)	Specific activity (nkat/mg)	Purification (-fold)	Yield (%)
Supernatant 21 000×g	50.17	1 163.82	1.00	100.00
Q-Sepharose	6.23	8 519.16	7.32	90.84
SP-Sepharose GM-1	1.11	17 887.91	15.37	34.17
PBE 94 GM-1 (pI 4.80)	0.11	135 945.81	116.81	26.36
SP-Sepharose GM-2	1.19	24 603.15	21.14	50.19
PBE 94 GM-2 (pI 3.80)	0.15	173 607.03	149.17	44.07

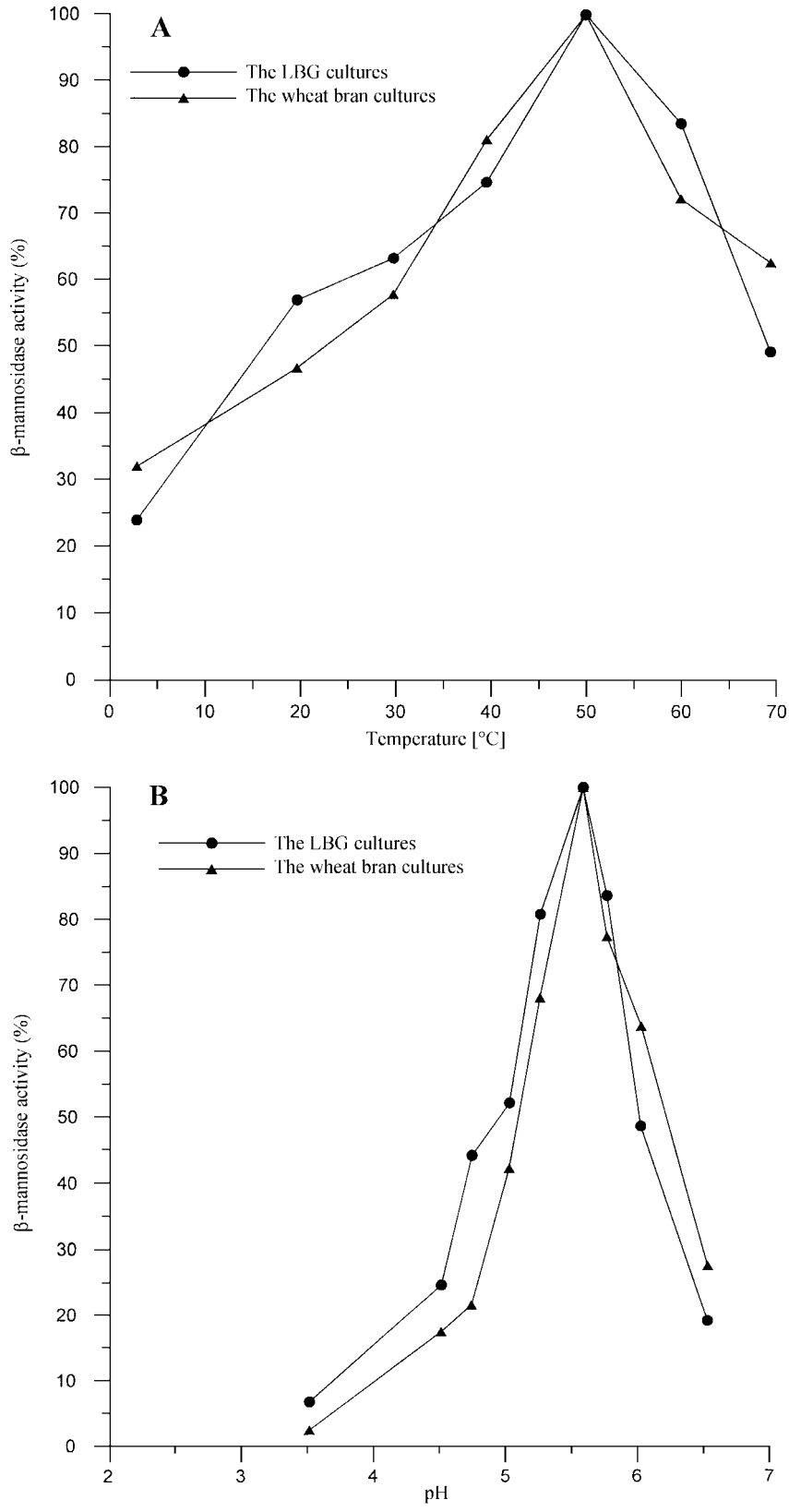

Fig. 2. Effect of temperature(A) and pH (B) on the activity of β-mannosidase isolated from 5th or 11th day of *Phlebia radiata* growth on galactomannan (▲) or wheat bran (●).

bran as a carbon source) (Fig. 3A) and in 0.32 M NaCl (LBG – as a carbon source, Fig. 3B).This step resulted in the nearly 7-fold purification with recovery of 94 and 91. Active fractions were pooled and put on a SP-Sepharose column (Fig. 4). The absorbed materials were eluted by linear gradient of NaCl. In the case of purification from the media with wheat bran the protein

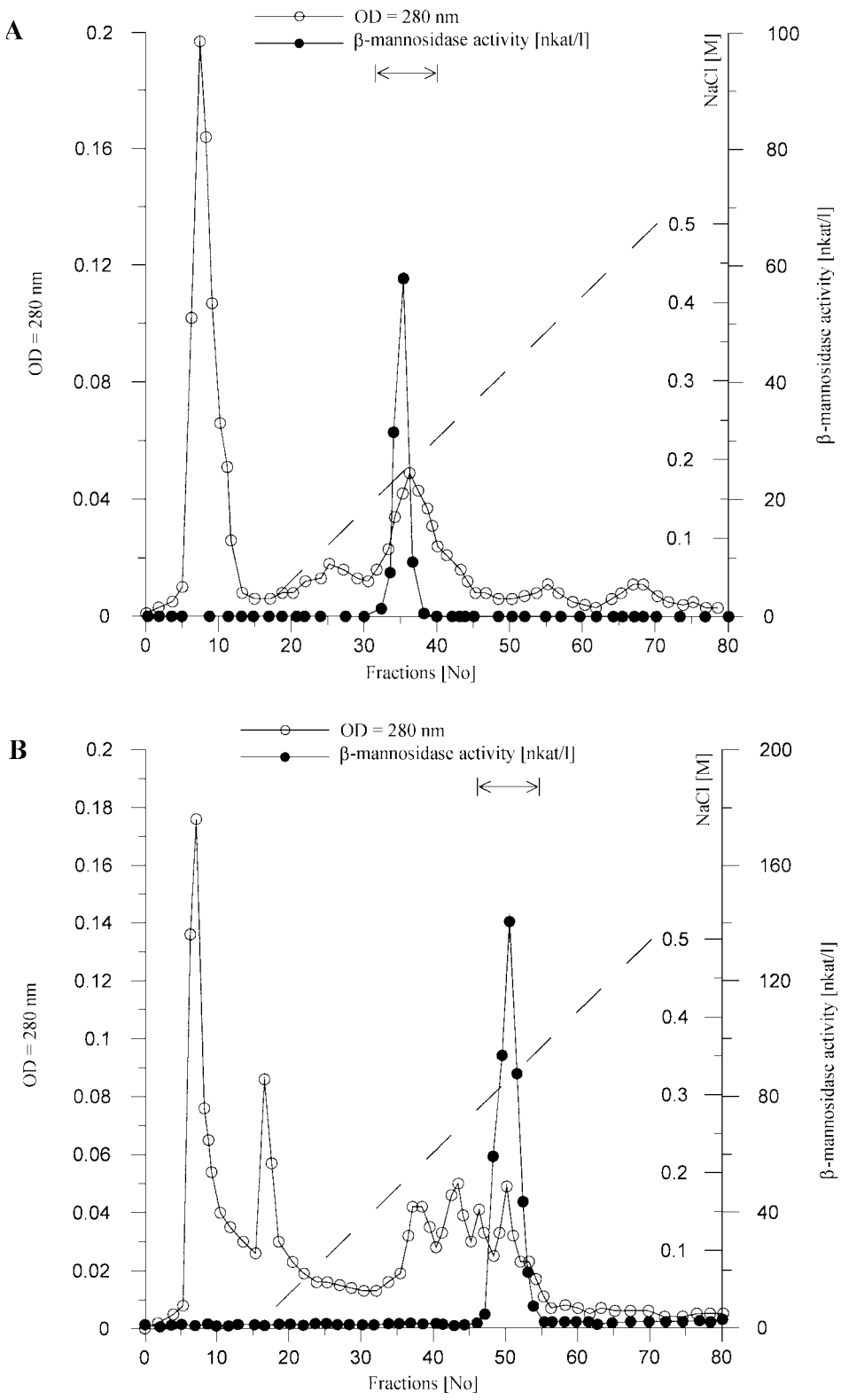

Fig. 3. The ion-exchange chromatography on Q-Sepharose (fast flow) of crude β-mannosidase from *Phlebia radiata* post culture liquid containing wheat bran (A) and galactomannan (B) as the carbon source

peak showing β-mannosidase activity was eluted in 0.2–0.25 M NaCl (OT-1), while in the case of enzyme from the media with LBG there were two protein peaks including β-mannosidase activity eluted in 0.17–0.22 M NaCl (GM-1) and 0.5M NaCl (GM-2). This step resulted in the nearly 20-fold purification of the enzymes with the recovery of 85, 34 and 50. The main active fractions were pooled accordingly and further fractionated by chromatofocusing on Polybuffer exchanger PBE-94. The chromatographic patterns are

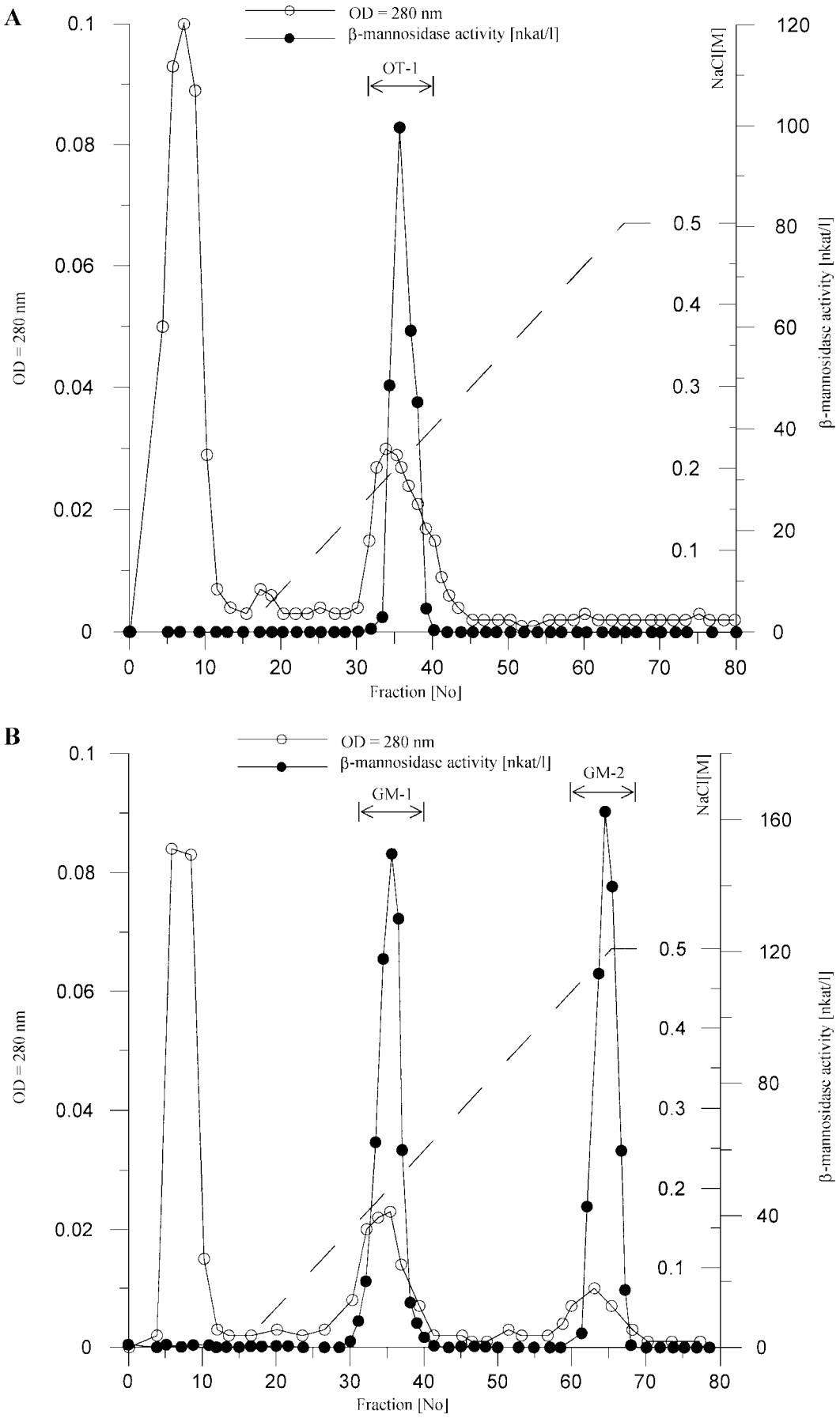

Fig. 4. Chromatography on SP-Sepharose fast flow of partially purified post Q-Sepharose *P. radiata* β-mannosidase from medium containing wheat bran (A) and galactomannan (B).

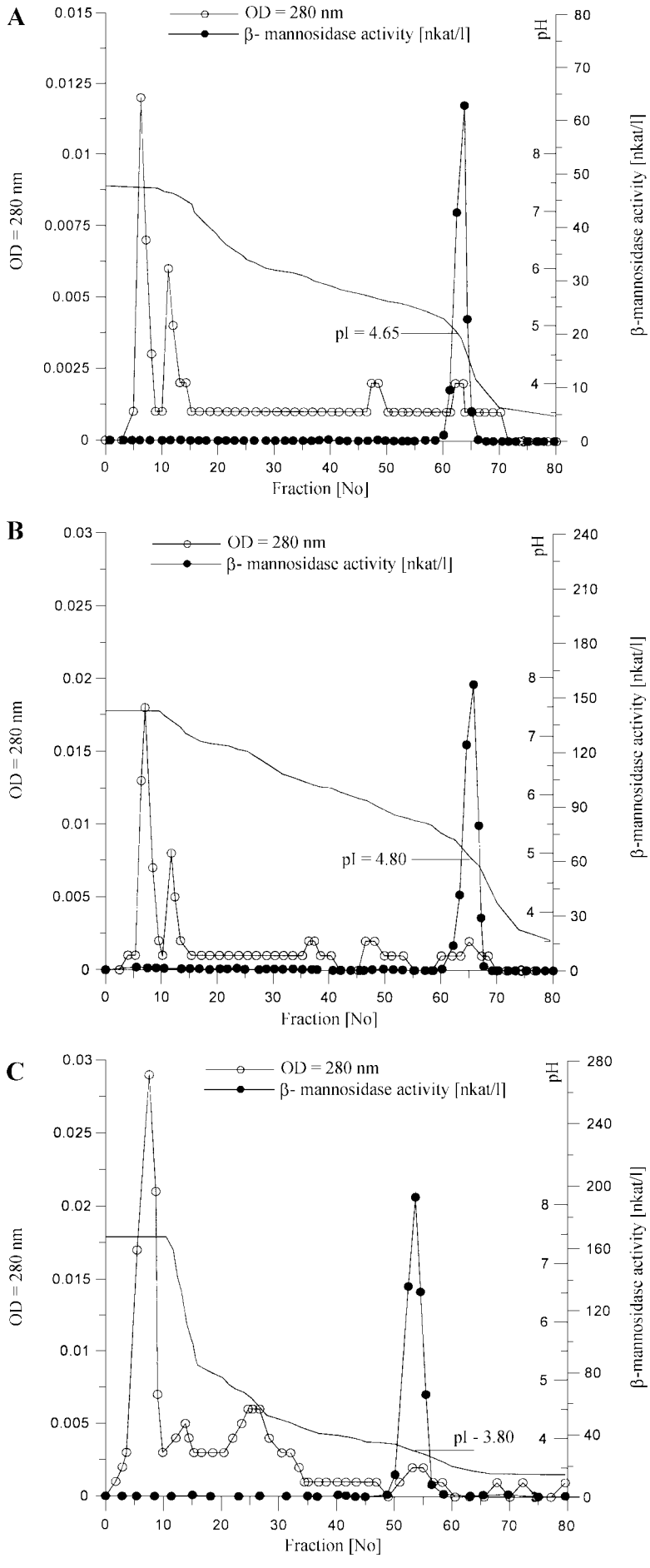

Fig. 5. Chromatofocusing pattern for (OT-1) fraction of SP-Sepharose-purified β-mannosidase from medium containing wheat bran (A); fraction (GM-1) (B) and fraction (GM-2) (C) from medium containing galactomannan.

Table III
Physico-chemical and kinetic data of purified isosymes of *Phlebia radiata* β-mannosidase

Enzyme	Molecular weight (kDa)	Isoelectric point (pI)	Carbohydrate content (%)	K_m (mM)	V_{max} (μM/min/mg)
GM-1	104.60 ± 3.89	4.80 ± 0.16	13.87 ± 1.41	0.29 ± 0.07	42.46 ± 3.76
GM-2	89.50 ± 4.11	3.80 ± 0.21	10.72 ± 1.68	1.78 ± 0.09	48.52 ± 1.13
OT-1	100.30 ± 2.71	4.65 ± 0.14	15.23 ± 2.49	0.34 ± 0.06	38.15 ± 2.07

shown in Figure 5. In each of the three cases β-mannosidase activity was concentrated in one narrow peak. The isoelectric points were estimated to be 4.65 (OT-1), 4.80 (GM-1) and 3.80 (GM-2). All these forms were purified approximately 150-fold with the activity yield of 26–73% (Table I and II). The purified forms of β-mannosidase showed high levels of specific activity (about 174 000 nkat/mg protein), which is almost 350 times higher than that reported for the highly purified enzyme from *A. niger* (Ademark *et al.*, 1999), and over 168 times higher than that from *Penaeus japonicus* (Chuang and Yang, 1991). The levels of purification achieved previously isolated for different β-mannosidases ranged from 53 (Ademark *et al*., 1999) to 250 (Sasaki *et al*., 1999) and 720 (Chuang and Yang, 1991). The physicochemical and kinetic constants are shown in Table III. Homogeneity of the β-mannosidase fractions was examined by size-exclusion high-performance liquid chromatography where the final purified enzymatic preparations appear as a single chromatographic peak. The isoelectric point of GM-1 covers the value obtained for *T. reesei* (Kulminskaya *et al.,* 1999). Previously described β-mannosidases have different isoelectric points in acidic pH- for *A. niger* it is 5.0 (Ademark *et al*., 1999), 4.5 for *Sclerotium rolfsi* (Gübitz *et al*., 1996), and 4.3 for *Pomacea candiculata* (Hirata *et al*., 1998).

The molecular weights of the purified enzymes are similar while their values vary: 100.3 kDa (OT-1); 104.6 kDa (GM-1) and 89.5 kDa (GM-2). The molecular weights of other β-mannosidases are different and vary at 58 kDa for *S. rolfsi* (Gübitz *et al*., 1996); between 105 kDa (Kulminskaya *et al.*, 1999) to 120 kDa (Elbein *et al*., 1977) for *T. reesei*, and between 135 kDa (Boquelet *et al*., 1978) and 180 kDa (Ademark *et al*., 1999) for *A. niger*.

The method of Dubois (1956) made it possible to determine the carbohydrate content in our forms of -mannosidase. All the above enzymes contain the carbohydrate content in their structure: 15.23% in OT-1; 13.87% in GM-1, and 10.72% in GM-2. This indicates that β-mannosidase from *P. radiata* is a glicoproteine. Similar carbohydrate content (17%) was described for *A. niger* (Ademark *et al.*, 1999).

The last goal was to determine the kinetic constants of purified enzymes. GM-1 and OT-1 (0.29 and 0.34 mM) had higher affinity for *p*-nitrophenylmannopyranoside in *P. radiata* than GM-2 (1.78 mM). The earlier reported *A. niger* (Ademark *et al*., 1999) β-mannosidase has a similar affinity for pNPM (0.30 mM) as β-mannosidase from *Trichospora cutanoun* (0.25 mM) (Oda and Tonomura, 1996). Higher values of 0.70 mM for *P. furiosus* (Bauer *et al.*, 1996), 0.80 mM for *A. awamori* (Neustroev *et al*., 1991), or 0.46 mM (Boquelet *et al*., 1978) and 2 mM (Elbein *et al*., 1977) – similar to GM-2 K_m – were reported earlier for *A. niger*. β-Mannosidase from *T. reesei* (Kulminskaya *et al.*, 1999) had the highest affinity for pNPM (0.12 mM) of all reported.

In summary, the β-mannosidase derived from *P. radiata* has general properties similar to those shown by others, making it a potentially viable enzyme for biotechnological purposes.

Acknowledgements

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