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Separation of enzymes from polyenzyme mixture used in medicine and pharmacy

II. Purification and characterization of extracellular β-glycosidases with high transglycosylation activities from *Aspergillus oryzae*

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Three extracellular β-glycosidases with different substrate specificities have been isolated from *Aspergillus oryzae* (Luizym®) and purified to electrophoretic homogeneity by molecular-sieve and ion-exchange chromatographic methods. The enzymes were characterized as monomeric glycoproteins with an estimated molecular mass of 95 kDa by SDS-PAGE and 92 kDa by gel-permeation chromatography on Superose 12 HR 10/30. β-glycosidase I (pH_{opt} 4.8; T_{opt} 40 °C, pI 4.5) was able to catalyze the hydrolysis of aryl-β-galactopyranosides (o- and p-), where as β-glycosidase II and III were found to be active towards aryl-β-gluco- and xylopyranosides. The specific chemical modifications of different amino acid residues showed that tryptophyl and carboxyl residues play an important role for the enzyme activity. The isolated β-glycosidases exhibited high levels of transglycosylation activities and were used for the production of tri- and tetrasaccharides from lactose and whey permeate.

1. Introduction

Glycosidases catalyze the hydrolysis of glycosidic linkages in disaccharides, aryl- and alkylglycosides and have potential applications for sequence determination of oligosaccharides [1, 2]. In addition to the hydrolytic activity, some β-glycosidases display transglycosylation activities and are able to catalyze the transfer of carbohydrate moieties to hydroxyl groups of various compounds [3]. This important property extends the biotechnological applications of these enzymes in the production of some biologically active oligosaccharides and other compounds [4]. Much work has been devoted to the purification and characterization of β-galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23), enzymes with different functions, widespread in numerous microorganisms, plant and animal tissues [5–7]. Prenosil et al. [8] proposed the general reaction scheme for the enzymatic production of galactooligosaccharides (Gal-OS) by transgalactosylation. Some Gal-OS, such as trimers and larger oligomers of saccharides (the enzymatic transgalactosylation products from lactose), were found to be useful bifidus factors [9, 10]. Extensive studies revealed that oligosaccharides can reach the lower digestive tract without being absorbed and can be utilized by intestinal *Bifidobacteria* as an energy source [10]. Recently, these oligosaccharides have become of interest for human health (e. g. maintenance of the normal intestinal balance, reduction of cholesterol levels, improvement of lactose tolerance, synthesis of B-complex vitamins and etc.) [9].

In this paper, we present the purification of three β-glycosidases and the biochemical characterization of a specific aryl-β-galactosidase from the commercial enzyme preparation Luizym®. The purified enzyme has high transglycosylation activity that can be applied for the production of oligosaccharides from lactose and whey permeate.

2. Investigations, results and discussion

2.1. Purification and characterization of β-glycosidases

Among the biological sources, the fungus *Aspergillus oryzae* is regarded as a good producer of numerous glycoside hydrolase activities [11]. β-Glycosidase activities were purified by a simple four-step chromatographic procedure summarized in Table 1. Three different β-glycosidase activities were found out in the final step – ion-exchange rechromatography on Mono Q HR 5/5. They were tentatively named β-glycosidase I, β-glycosidase II and β-glycosidase III, according to their mobility on native PAGE (Fig. 1A – lane 2). β-Glycosidase I was purified to homogeneity 14.72 fold with a recovery of 8.9% and had high transglycosylation activity. The purified enzyme showed a single protein band on SDS-PAGE when stained with silver nitrate (Fig. 1B) and on IEF-PAGE with a pI of 4.5 (Fig. 1C). The relative molecular mass of the enzyme estimated by gel filtration on a Superose 12 HR 10/30 column and calculated from the relative mobility of standard proteins on SDS/PAGE was 92 kDa and 95 kDa, respectively. On the basis of these results, the purified β-glycosidase I was considered to be a monomeric protein.

The pure β-glycosidase I was most active between pH 4.6–5.0. This slightly acidic pH optimum of the en-

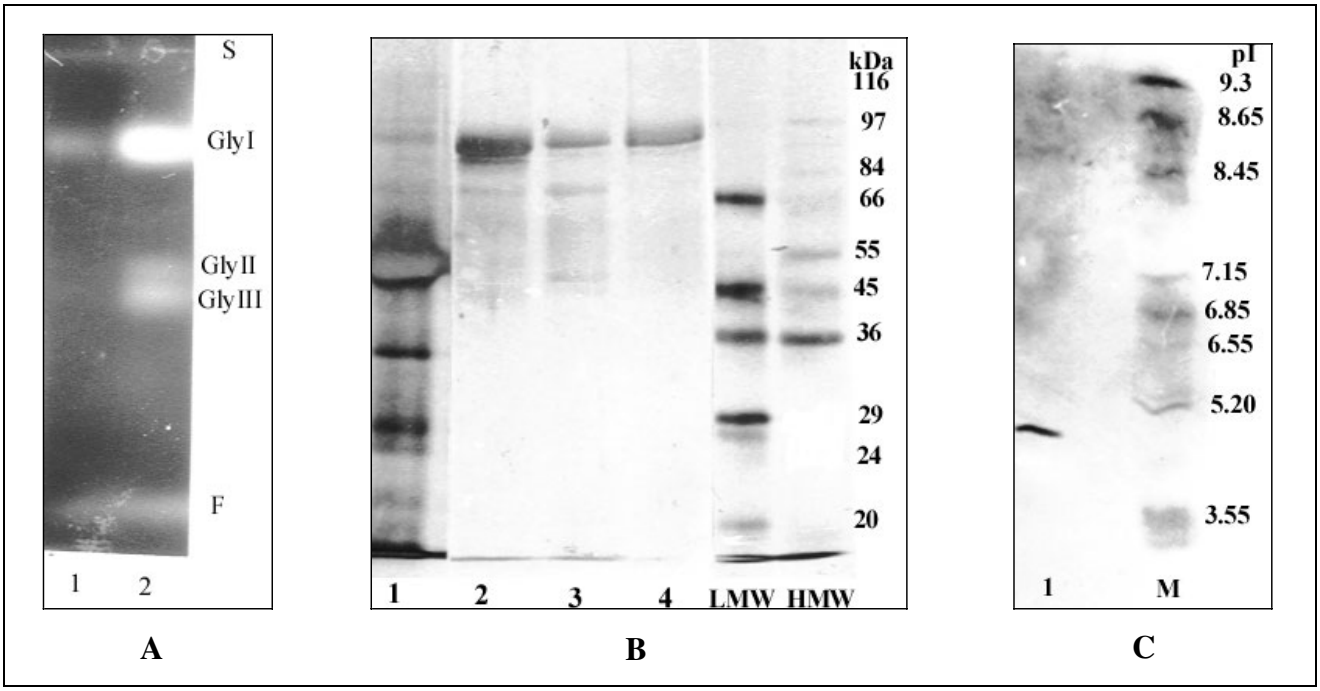

Fig. 1: PAGE of the purified *A. oryzae* β-glycosidase I: (A) native-PAGE visualized with MeUmb-β-D-Gal (lane 1 – purified β-glycosidase I; lane 2 – β-glycosidase active fraction before rechromatography on Mono Q HR 5/5); (B) SDS-PAGE (lane 1 – crude enzyme; lane 2 – β-glycosidase active fraction after Mono Q HR 5/5; lane 3 – β-glycosidase active fraction after Superose 12 HR 10/30; lane 4 – purified β-glycosidase I); (C) IEF-PAGE (lane 1 – purified β-glycosidase I, M – standard proteins)

zyme (4.8) fits into the values characteristic for numerous glycoside hydrolases from fungi [4, 12] and corresponds to the values determined for β-glycosidases from *Trichoderma reesei* [13], *Termitomyces clypeatus* [14], *Aspergillus tamarii* [15] and *Phanerochaete chrysosporium* [16]. The enzyme was stable in the pH range of 4.5–7.0 (48 h at 4 °C) and retained 100% of activity at pH 5.0, 81% of activity at pH 4.0 and 60% of activity at pH 3.5.

The optimum temperature of the purified β-glycosidase I was found to be at 40 °C. The purified enzyme was stable for 24 h at 35 °C and retained 80% of activity at 40 °C for 3 h. At 50 °C β-glycosidase I lost half of its activity for 1 h and 90% of activity for 6 h. Although the purified enzyme displayed maximal activity at 60 °C it was unstable at this temperature and lost its activity for 2 h.

2.2. Substrate specificity and kinetic analysis

The relative rates of hydrolysis of various substrates by the purified enzymes (β-glycosidases I, II and III) were studied and the results are presented in Table 2. All the purified enzymes were able to catalyze effectively the hydrolysis of oNPGal (*o*-nitrophenyl-β-D-galactopyranoside)

Table 1: Purification of β-glycosidase I from *Aspergillus oryzae*

Purification steps	Volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification (fold)	Recovery (%)
Initial crude enzyme	45.0	22954.0	28.14	816.14	1	100
Acrylex P-100	14.4	19566.7	16.03	2769.71	1.49	85.24
Mono Q HR	2.6	11440.0	3.29	3477.29	4.29	49.83
Superose 12	2.2	7971.5	1.41	5561.50	6.81	34.72
Mono Q HR	1.5	2037.1	0.17	12053.73	14.72	8.9

Table 2: Substrate specificity of purified β-glycosidases

Substrate	β-Glycosidase I		β-Glycosidase II		β-Glycosidase III	
	Enzyme activity (IU)	Specific activity (IU/mg protein)	Enzyme activity (IU)	Specific activity (IU/mg protein)	Enzyme activity (IU)	Specific activity (IU/mg protein)
oNP-β-Gal	1358	12018.2	1256	6337.03	1300	1565.98
pNP-β-Gal	2282	20194.6	1901	9591.32	2103	2533.27
oNP-β-Glu	–	–	6012	30332.9	3852	4640.12
pNP-β-Glu	–	–	3499	17653.8	1941	2338.13
oNP-β-Xyl	18.5	163.71	1557	7855.7	1032	1243.15
pNP-β-Xyl	93.3	825.6	107.9	544.4	154.1	185.63
pNP-α-Gal	–	–	–	–	–	–
pNP-β-Cell	–	–	426.1	2149.8	198.4	238.99

and pNPGal (*p*-nitrophenyl-β-D-galactopyranoside). The purified β-glycosidase I had no activity on lactose, cellobiose, stachyose, melibiose and sucrose (data not shown). It also had no activity on *p*-nitrophenyl-α-galactopyranoside, indicating its specificity to the β-glycosidic linkage. β-Glycosidase II and β-glycosidase III catalyzed not only the hydrolysis of the β-D-galactopyranoside, but also the hydrolysis of β-D-glucopyranoside and β-D-xylopyranoside linkages, similar to many other β-glycosidases [12, 17]. On the basis of substrate specificity, β-glycosidase I might be characterized as strictly specific aryl-β-galactosidase.

The apparent K_m and V for oNPGal of β-glycosidase I were 2.5 mM and 1828.15 IU · mg^{-1}, respectively, according to Lineweaver-Burk plots.

Substrate inhibition of the enzyme was also observed at p- and oNPGal concentrations above 10 mM (data not shown).

The purified β-glycosidase I was completely inhibited by 5 mM Fe^{2+} and about 45% of its activity was inhibited by 2 mM Fe^{3+} and 2 mM Ag^{+}. On the other hand, Ca^{2+}, Co^{2+}, Zn^{2+}, Sn^{2+}, Mg^{2+}, Li^{2+}, Cu^{2+} had little or no inhibitory effect on the β-glycosidase activity. Some of the chemical reagents – EDTA, SDS, pHMB (p-chloromercuribenzoate) – did not affect the activity, also. Group specific reagents as CMC (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide hydrochloride) and NBS (*N*-bromosuccinimide) strongly inhibited the β-glycosidases. 55% of the initial β-glycosidase I activity were inhibited by 100 mM CMC, whereas only 7.5 μmol of NBS caused 100% inhibition. The substrate protection against NBS and CMC demonstrated that carboxylic and tryptophyl groups are important for the activity. 20 mM iodoacetamide inhibited 30% of the initial β-glycosidase I activity, but this reagent is not strictly specific for the sulfhydryl groups and it is difficult to make conclusions for the participation of the Cys in the catalytic process, what's more, pHMB did not affect the activity.

2.3. Transglycosylation activity

In order to optimize the conditions of transglycosylation reaction, some parameters were studied. Different optimal pH values for hydrolysis and transglycosylation reactions were observed in the case of *E. coli* β-galactosidase [18] and acid β-galactosidase from *Achatina achatina* [19]. In our case, a pH optimum of 5.5 for the transglycosylation reaction was obtained (pH 4.8 for the hydrolysis reaction), giving the possibility to control the reaction equilibrium. Investigating the effect of lactose concentration on the oligosaccharide (OS) production, maximum OS were obtained when 15% lactose was used in the reaction mixture. The amount of OS formed was determined by HPLC analysis (Fig. 2A) and the results indicated that the β-glycosidase from *Aspergillus oryzae* catalyzed the transgalactosylation reaction. During the initial 5 h of incubation, the lactose was converted to glucose, galactose and OS. An increasing in trisaccharide content compared to the disaccharide was observed after 2 h of incubation. For periods of incubation longer than 18 h an increase in tetrasaccharide content was established. The concentration of OS reached a maximum by 24 h and thereafter gradually decreased (Fig. 2B). Under optimal conditions for the transglycosylaton reaction (pH 5.5, temperature of 40 °C, 15% lactose), a tetrasaccharide and a new disaccharide ($R_f = 14.5$), different from the lactose ($R_f = 13.5$) were produced (Fig. 2A).

With whey permeate (10%, w/v) as a substrate in the reaction mixture, the β-glycosidase I from *A. oryzae* produced trisaccharides that might be determined as galactosyllactose. The results from TLC analysis (Fig. 3) suggest a transfer of galactose to lactose, because there is no galactose as a product of lactose hydrolysis. On the contrary, using β-glycosidase from *Sporotrichum thermophile* [20] in the same reaction mixture, the trisacchatides produced might be determined as glucosyllactose, due to the lack of glucose at 22 h of incubation as a product of lactose hydrolysis.

The trisaccharides were separated from the accompanying monosaccharides (glucose and galactose as hydrolysis products) and lactose by molecular-sieve chromatography on Bio Gel P-2 column, equilibrated and eluted with distilled water at a flow rate of 16 ml/h.

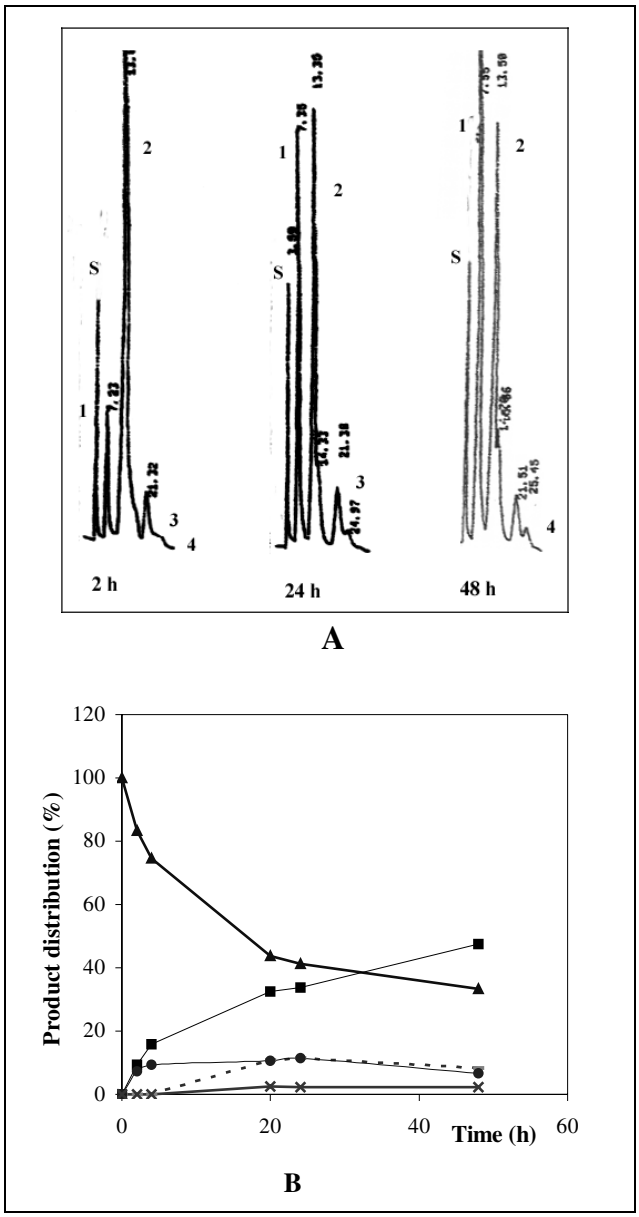

Fig. 2: A) HPLC chromatography of the hydrolysis products after incubating lactose (15%) with *A. oryzae* β-glycosidase I: 1-monosaccharides; 2-lactose; 3-trisaccharides; 4-tetrasaccharides. B) Time course of Gal-OS formation by *A. oryzae* β-glycosidase I. Product distributions were calculated from peak areas (HPLC analyses) and expressed as a percentage from the total sugar content: ▲-lactose, ■ – monosaccharides, ● – trisaccharides, - - - disaccharides, × – tetrasaccharides

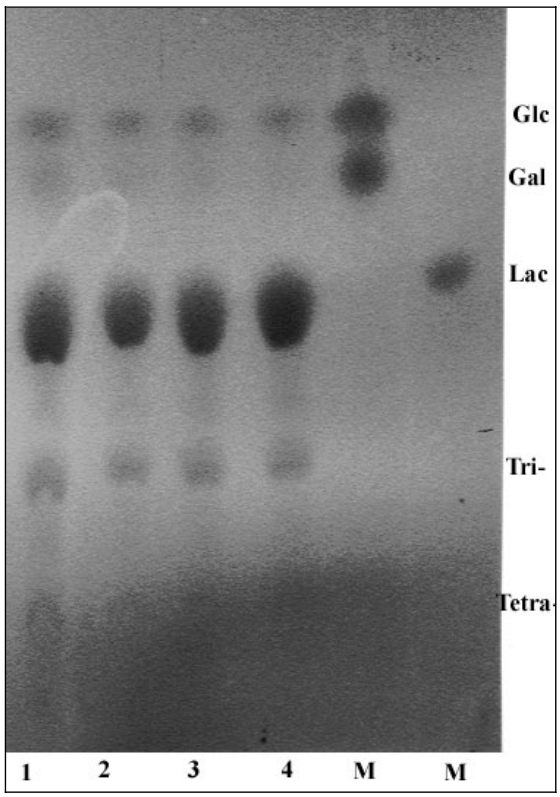

Fig. 3: TLC analyses of products formed from whey permeate (10%) during incubation with *A. oryzae* β-glycosidase I: Glc-glucose; Gal-galactose; Lac-lactose; Tri-trisaccharides; Tetra-tetrasaccharides; 1 – 4 h; 2 – 7 h; 3 – 20 h; 4 – 24 h; M – sugar standards

The obtained pure trisaccharides were tested (by the Hungarian partner of the contract-IC15-CT96-1000) and the results showed that they were good growth factors for the intestinal *Bifidobacteria*.

3. Experimental

3.1. Enzyme

The commercially available polyenzyme product Luizym® from *A. oryzae* was provided by Luitpold-Pharma GmbH (Munich, Germany).

3.2. Materials

oNPGal, pNPGal and other o/p-nitrophenyl glycosides, Methylumbeliferyl-β-D-glycopyranosides (MeUmb-β-D-Gal, MeUmb-β-D-Glc, MeUmb-β-D-Cel), CMC and Bovine Serum Albumin (BSA fr. V) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA); lactose, cellobiose, sucrose, trehalose, CH_3-β-cellobioside, stachyose, sallicine, L(D)-melibiose, β-D-gentiobiose, α-sophorose were provided from Koch-Light (Colnbrook Bucks, England); Acrylex P-100 was obtained from Reanal (Budapest, Hungary); Bio Gel P-2 was from Bio-Rad Lab., Inc. (USA); NBS, iodoacetamide and pHMB were products of Fluka Chemie AG (Germany); Mono Q HR 5/5, Superose 12 HR 10/30, Protein molecular mass kits and isoelectric focusing kits were purchased from Pharmacia Fine Chemicals Inc. (Uppsala, Sweden). All chemicals were of analytical grade.

3.3. Assay of β-glycosidase activity

β-Glycosidase activities were determined by measuring the rate of hydrolysis of different o/pNPGlycopyranosides [17]. The reaction mixture, containing 0.1 ml of appropriately diluted enzyme and 0.9 ml of 2 mM substrate in 50 mM sodium-acetate buffer (pH 4.7), was incubated at 40 °C for 10 min. The reaction was terminated and color developed by the addition of 0.5 ml of 1M Na_2CO_3. The amount of *o-/p*-nitrophenol liberated was determined spectrophotometrically at 405 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the release of 1 μmol *o-/p*-nitrophenol per min under the assay conditions. The enzyme activity towards nonchromogenic substrates was measured using the standard procedures for determining either glucose [21] or reducing sugars [22, 23].

3.4. Protein determination

Protein was assayed by the method of Lowry [24], using crystalline BSA as a standard or by measuring absorbance at 280 nm for the column effluents.

3.5. Polyacrylamide gel electrophoresis (PAGE) and analytical isoelectrofocusing (IEF)

PAGE was performed in polyacrylamide gels (10% T and 2.65% C) under nondenaturing conditions according to Davis [25]. SDS-PAGE (12.5% T) was performed as described by Laemmli [26]. The proteins were detected by staining with 0.05% Comassie Blue R250 in 30% CH_3OH, 10% CH_3COOH, water (v/v/v) or by silver nitrate staining according to Blum [27]. High molecular mass protein standards (Sigma) were used as markers.
IEF-PAGE was performed using mini IEF gel system with a polyacrylamide gel (5% T and 3.3% C) with ampholytes covering a pH range 3.0–10.0 as a carrier, according to Roberstson et al. [28].

3.6. Enzyme purification

All purifications steps were carried out at 4 °C.
Step 1: The polyenzyme preparation (0.45 g) was dissolved in 0.05 M sodium-acetate buffer, pH 5.0 to 45 ml and was centrifuged for 20 min at 7600 × g. The supernatant was loaded onto an Acrylex P-100 column (XK 2.6/100 cm). The column was equilibrated and eluted with a flow rate of 24 ml/h and 3 ml fractions were collected. β-Galactosidase active fractions were pooled and concentrated by freeze-drying.
Step 2: Freeze-dryed β-glycosidase active fraction (0.035 g) was dissolved in 0.05 M sodium-acetate buffer, pH 5.0 and loaded on to a Mono Q HR 5/5 anion-exchange column, equilibrated with the same buffer. β-Galactosidase activity was eluted by a linear gradient of NaCl (0–0.7 M) in the same buffer at a flow rate of 0.4 ml · min^{-1}. Fractions of 1 ml were collected and those with β-galactosidase activity were loaded on to a Superose 12 HR 10/30 column, equilibrated and eluted with 0.05 M sodium-acetate buffer, pH 4.7, containing 0.15 M NaCl at a flow rate of 0.4 ml · min^{-1}.
Step 3: β-Galactosidase active fractions were pooled and rechromatographed on a Mono Q HR 5/5 anion-exchange column, equilibrated and eluted with 0.05 M sodium-acetate buffer, pH 4.0 with a linear gradient of NaCl (0–0.7 M) at a flow rate of 0.4 ml · min^{-1}.

3.7. Effect of pH and temperature on the enzyme activity and stability

The effect of pH on the enzyme activity was determined by performing assays at 40 °C in the pH range 3.5–7.0 in the following 0.05 M buffers: sodium-acetate (pH 3.5–5.6) and sodium-citrate-phosphate buffer (pH 5.6–7.0). The temperature dependence was determined in a temperature range 0 °C–60 °C.
pH-Stability of β-galactosidase was determined in the pH range 3.5–7.0. The enzyme was incubated in the above mentioned 0.05 M buffers at 40 °C for different time intervals (0–48 h). Aliquots were taken and immediately assayed for a residual β-galactosidase activity.
Thermal stability was investigated by measuring the remaining activity after enzyme incubation in standard buffer solutions (0.05 M sodium-acetate, pH 4.0) at different temperatures (4 °C–60 °C) for 10 h.

3.8. Kinetic study

Kinetic parameters of the purified β-galactosidase (β-glycosidase I) were determined by varying o-NPGal concentration (0.1 mM–20 mM) into the reaction mixture and assaying hydrolysis activity as described previously. The K_m and V_{max} values were calculated from the Lineweaver-Burk plots. The substrate specificity was determined by measuring the release of *o-/p*-nitrophenol from different *o-/p*-nitrophenyl glycosides.

3.9. Effect of metal ions and group specific reagents

The effect of some metal ions and reagents on the enzyme activity was monitored by preincubating the purified enzyme with the individual reagent (2 mM) in 0.05 M sodium-acetate buffer (pH 4.0) at 22 °C for 10 min. The activity was then measured at optimal conditions and expressed as a percentage of a control carried out in the absence of metal ions or reagents.
CMC was used to modify the carboxyl groups [29] by incubating the reagent (40–100 mM) with the purified β-galactosidase in 0.05 M Mes/NaOH buffer, pH 6.0 at 22 °C for 60 min. The residual activity of the enzyme derivatives was determined (at different time intervals) by the method mentioned above and expressed as a percentage of a control.
The oxidation of tryptophyl residues by NBS was carried out in a reaction mixture, which consisted of purified β-galactosidase in 0.05 M sodium-acetate buffer, pH 5.0 and NBS (1 μM–100 μM) at 22 °C [30]. Aliquots of the reaction mixture were withdrawn to assay the residual activity.
Jodacedamide was used to modify the sulfhydryl groups by incubating the reagent (20 mM) with the purified enzyme in 10 mM Tris. HCl buffer, pH 8.0.

3.10. Transglycosylation activity and product analyses

The reaction mixtures containing different concentrations of lactose (10%–30%), 50 mM sodium phosphate buffer (pH 5.5) and enzyme (3260 IU–9780 IU) in a total volume of 20 ml were incubated at 40 °C. The aliquots were withdrawn at different time intervals during the period of 48 h and the amount of Gal-OS formed was determined. The hydrolysis of lactose was determined by measuring of the glucose using the glucose oxydase-peroxidase method [21]. The total sugars were analyzed by phenol-sulfuric acid method [31]. Reducing sugars were determined by DNS method of Miller [32].

TLC analyses were performed on Silica gel 60 (alluminium sheets 25×25 cm, E. Merck, Darmstadt, Germany) and developed by the ascending method with n-propanol : water : acetic acid (7 : 2 : 1,v/v/v). Transglycosylation products were identified by HPLC (Waters-Millipore) using a μB ondapak column (3.9×300 cm) with acetonitrile : water (80 : 20, v/v) as a mobile phase at a flow rate of 1 ml min^{-1}. Elution was monitored by differential refractometer (R 401, Millipore, USA).

For the preparative purification of Gal-OS, a 5 ml reaction sample containing 600 mg of total sugars was applied to a Bio Gel P-2 column (1.6×100 cm) and eluted with distilled water at a flow rate of 16 ml h^{-1}. Fractions (3 ml) were collected, analyzed for total sugars by phenol-sulfuric acid method, and concentrated by vacuum evaporation.

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