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Purification and characterization of α-galactosidase from a thermophilic fungus *Thermomyces lanuginosus*

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Abstract

An extracellular α-galactosidase was purified to electrophoretic homogeneity from a locust bean gum-spent culture fluid of a mannanolytic strain of the thermophilic fungus *Thermomyces lanuginosus*. Molecular mass of the enzyme is 57 kDa. The pure enzyme which has a glycoprotein nature, afforded several forms on IEF, indicating its microheterogeneity. Isoelectric point of the major form was 5.2. Enzyme is the most active against aryl α-D-galactosides but efficiently hydrolyzed α-glycosidically linked non-reducing terminal galactopyranosyl residues occurring in natural substrates such as melibiose, raffinose, stachyose, and fragments of galactomannan. In addition, the enzyme is able to catalyze efficient degalactosylation of polymeric galactomannans leading to precipitation of the polymers. Stereochemical course of hydrolysis of two substrates, 4-nitrophenyl α-galactopyranoside and galactosyl[1]mannotriose, followed by ^{1}H NMR spectroscopy, pointed out the α-anomer of D-galactose was the primary product of hydrolysis from which the β-anomer was formed by mutarotation. Hence the enzyme is a retaining glycosyl hydrolase. In accord with its retaining character the enzyme catalyzed transgalactosylation from 4-nitrophenyl α-galactopyranoside as a glycosyl donor. Amino acid sequence alignment of N-terminal and two internal sequences suggested that the enzyme is a member of family 27 of glycosyl hydrolases.

Keywords: α-Galactosidase; Retaining glycosyl hydrolase; Galactomannan; Thermophilic fungus; *Thermomyces lanuginosus*

1. Introduction

Besides glycoproteins and glycolipids α-linked galactosyl residues are found in nature in two major forms: (i) as single unit branches of β-1,4-mannans and β-1,4-glucomannans which are, after cellulose, the most abundant polysaccharides in softwood, and (ii) as a part of short oligosaccharides melibiose (α-galactosyl-1,6-glucose), raffinose (α-galactosyl-1,6-sucrose) and stachyose (α-galactosyl-1,6-raffinose) accompanying sucrose in sugar beet and soy.

The enzymatic degradation of the above-mentioned saccharides involves, among other enzymes, the action of α-galactosidase(s) liberating free galactose from the non-reducing end of the substrates. Hence, the systematic name of this glycosidase is α-galactoside galactohydrolase (EC 3.2.1.22). According to their substrate specificities α-galactosidases can be divided into two groups [1]. The first group contains α-galactosidases active only on oligosaccharides with low degree of polymerization, for example melibiose, raffinose, stachyose and short fragments of galacto(gluco)mannans. These enzymes are usually very active on artificial substrates like aryl α-galactosides. The second group of α-galactosidases consists of enzymes active on polymeric substrates. However, similarly to the enzymes of the first group, they attack short oligosaccharides, mainly fragments of degraded polymers, as well as artificial α-galactosides.

The most important industrial application of α-galactosidases is presently in the sugar-making industry ([2,3] and references therein). Small amounts of raffinose and/or stachyose negatively affect crystallization of sucrose. They can be easily eliminated and converted to sucrose by an α-galactosidase treatment. It is important that the mode of

Abbreviations: NPGal, 4-nitrophenyl α-D-galactopyranoside; $GalMan_3$, β-D-mannopyranosyl-1,4-β-D-mannopyranosyl-1,4-(6-*O*-α-D-galactopyranosyl)-D-mannopyranose; LBG, locust bean gum; GG, guar gum

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action and stability of α-galactosidases are met with industrial demands as these enzyme properties reduce expenses for cooling and re-heating. The potential use of α-galactosidases for the processing of food products from soybean has also been demonstrated [4,5]. Presence of raffinose family sugars in soybean and other leguminous plant seeds may induce gastric distress in humans due to microbial flora of the lower intestinal tract that metabolizes these sugars with extensive gas production. Another possible application of α-galactosidase is seen in pulp and paper industry, where galactosidases could enhance the bleaching effect of endo-β-1,4-mannanases on softwood Kraft pulp [6]. One of the looked-for properties of industrially important α-galactosidases is their thermal stability.

At the present time, an increased interest in α-galactosidases can be seen in human medicine. Several α-galactosidases are able to cleave off the terminal α-1,3-linked D-galactosyl residue from the blood B type cell surface glycoprotein, thus causing B→O blood type conversion [7]. Moreover, those α-galactosidases, that are able to hydrolyze terminal glyco(sphingo)lipid α-galactosyl residue, might be used for treatment of Fabry disease [8–10], an X-chromosome-linked recessive lysosomal storage disorder. The disease is caused by a deficiency of the lysosomal α-galactosidase A, resulting in a progressive accumulation of glycosphingolipids, predominantly globotriaosylceramide, throughout the human body [11].

In the previous paper [12] we found the strain *Thermomyces lanuginosus* IMI 158749 to be an efficient producer of endo-β-1,4-mannanase and α-galactosidase. This capability was very rare among the strains of this species, which have a reputation of the best naturally occurring β-xylanase producers [12,13]. In this paper we report isolation and characterization of an extracellular α-galactosidase from this thermophilic fungus.

2. Materials and methods

2.1. Materials

Locust bean gum (LBG), guar gum (GG), 4-nitrophenyl glycosides of α-galactose, β-mannose, α-L-arabinopyranose and α-L-arabinofuranose were from Sigma (St. Louis, MO, USA), 4-nitrophenyl glycosides of α-glucose and α-xylose from Koch-Light Laboratories (Coinbrook, Bucks, UK), and those of β-xylose, β-glucose, β-galactose, α-mannose from Lachema (Brno, Czech Republic). Melibiose was donated by Production Department, Institute of Chemistry (Bratislava, Slovakia), and both raffinose and stachyose were purchased from Fluka (Steinheim, Germany). Galactosyl[1]mannotriose (β-mannosyl-1,4-β-mannosyl-1,4-(6-*O*-α-galactosyl)-mannose) and β-1,4-mannotriose were obtained from Megazyme (Wicklow, Ireland). Chromatography carriers, DEAE Sepharose and Phenyl Sepharose, were purchased from Pharmacia (Uppsala, Sweden).

2.2. Microorganism and growth conditions

The strain of thermophilic fungus *Thermomyces lanuginosus* IMI 158749 originated from International Mycological Institute, Kew, Surrey, UK.

The fungus was maintained on 2% agar slants containing 0.125% Bactopeptone, 0.125% yeast autolysate and 0.3% glucose (w/v). The fungus was let to sporulate at room temperature. The spores were aseptically transferred to a 100 ml Erlenmeyer flask containing 30 ml sterile cultivation medium composed of (g/l): yeast autolysate, 14.3; $(NH_4)_2SO_4$, 2.1; $MgSO_4 \cdot 7H_2O$, 3.0; $CaCl_2 \cdot 2H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 10.0; the main carbon source – glucose or LBG, 20.0. The strain was cultivated on glucose medium on a rotary shaker at 45°C and 150 rpm for 4–5 days after which the fungus created lumps 2–3 mm in diameter. Then 1.25 ml of culture was aseptically taken and transferred to a 1 l cultivation flask containing 250 ml of the production medium with LBG as carbon source. After 75 h growth at 45°C and 150 rpm, biomass was separated with filtration through a cloth and extracellular medium was then filtered through bacteriological filter unit Nalgene 120-0020 (Sybron/Nalge, Rochester, NY, USA).

2.3. Purification of α-galactosidase

A galactomannan-spent cell-free culture fluid was concentrated with an Amicon ultrafiltration cell, model 8400 (W.R. Grace, Danvers, MA, USA) using a 10 kDa cut-off membrane. The concentrate was applied to DEAE–Sepharose fast flow column (2.5×25 cm) equilibrated with 50 mM sodium phosphate buffer (pH 6.0) containing 0.02% (w/v) NaN_3 (buffer A). Elution was done at the flow rate 33 ml/h and 11 ml (20 min) fractions were collected. Adsorbed proteins were liberated with linear increasing gradient of NaCl (0–0.5 M) in buffer A. α-Galactosidase active fractions were pooled and concentrated and desalted by ultrafiltration on an Amicon ultrafiltration cell model 8050 (W.R. Grace) using 10 kDa cut-off membrane.

Concentrated sample after DEAE Sepharose chromatography was applied to Phenyl Sepharose column (0.9×9 cm) equilibrated with 1.3 M $(NH_4)_2SO_4$ in buffer A. Elution was done at the flow rate 17 ml/h and 1.9 ml (6 min 40 s) fractions were collected. Adsorbed proteins were liberated from the carrier with linear decreasing gradient 1.3–0 M $(NH_4)_2SO_4$ in buffer A. α-Galactosidase active fractions were pooled and concentrated and desalted by ultrafiltration.

2.4. Enzyme assay

α-Galactosidase was quantitatively assayed using 1 mM

4-nitrophenyl α-D-galactopyranoside in 50 mM sodium acetate buffer (pH 4.5) at 50°C. Liberated 4-nitrophenol was determined spectrophotometrically at 410 nm after addition of 2 volumes of saturated solution of sodium tetraborate. One unit of enzyme activity is defined as amount of enzyme liberating 1 μmol of 4-nitrophenol in 1 min under assay conditions, and corresponds to 16.7 nkat.

The presence of α-galactosidase activity in chromatographic fractions was followed by cup-plate method on 2% agar with built-in 0.5 mM solution of 4-methylumbelliferyl α-D-galactopyranoside (Sigma) in 50 mM sodium acetate buffer (pH 4.5). The fluorescence of released 4-methylumbelliferone was observed directly without alkalization after illumination with ultraviolet light (366 nm).

Since the purified α-galactosidase was found at high dilutions (~30 μg/ml and lower) unstable in the absence of substrates, in experiments aiming to determine the pH and temperature optima, the reaction mixtures containing the enzyme in the absence of substrates were supplied with 0.5 mg/ml BSA (Reanal, Budapest, Hungary).

The presence of endo-β-mannanase activity in chromatographic fractions was monitored using 0.5% (w/v) solution of Ostazin Brilliant Red-locust bean gum [14], in 50 mM sodium phosphate buffer (pH 6.0) as the substrate. To 250 μl of this solution preheated to 50°C, 5 μl of the fractions were added. After 5 min, the reaction was stopped by addition of 500 μl of ethanol. After 30 min equilibration at ambient temperature, the precipitated polymer was centrifuged and the amount of dyed fragments in supernatant was quantified spectrophotometrically at 550 nm (absorption maximum of the dye). Endo-β-mannanase activity is expressed as absorbance at 550 nm (A_{550}).

2.5. Electrophoretic methods

Electrophoresis was carried out according to Laemmli [15] in 1 mm thick 10% polyacrylamide slab gels at 10°C and constant current 20 mA. Proteins were visualized with Coomassie Brilliant blue R 250 (Sigma) and molecular masses were determined according to mobilities of molecular mass protein standards (Sigma). Glycoprotein character of the enzyme was established directly in the separation gels with periodic acid-Schiff's reagent staining method [16].

Native electrophoresis was done similarly with the exceptions that sample was not boiled and loading and electrophoretic buffers did not contain sodium dodecyl sulfate (SDS). When the electrophoretic run was completed, separation gel was re-buffered with 0.2 M sodium phosphate buffer (pH 6.0; twice for 5 min). The gel was then brought into contact with 1 mM solution of 4-methylumbelliferyl α-galactopyranoside in 50 mM sodium phosphate buffer (pH 6.0). In this less acidic pH (compared to the optimum pH 4.5) liberation of fluorescing aglycon could be followed visually under UV light without alkalization, which further increases the intensity of fluorescence.

Isoelectric focusing (IEF) was carried out in 5.5% polyacrylamide gels with ampholyte pH gradient 3–7 (Serva). Proteins and α-galactosidase activity were visualized as described above.

2.6. Amino acid sequencing

Purified α-galactosidase did not contain any barrier to partial amino acid sequencing. The N-terminal as well as the internal sequences (prepared by the proteolytic digestion with trypsin followed by HPLC separation of the protein fragments) of a purified α-galactosidase were determined by automated Edman degradation on a cLC PROCISE (PE-Biosystems, Foster City, CA, USA) protein sequencer. Degradation products (phenylthiohydantoin-amino acid derivatives) were separated by a reverse-phase HPLC system (Perkin–Elmer) and identified by comparison of retention time with phenylthiohydantoin-amino acid standards (Perkin–Elmer).

Sequences homologous with that were determined for *T. lanuginosus* α-galactosidase were found and compared by BLASTP search program [17] of the Non-redundant Peptide Sequence Database at the National Center for Biotechnology Information via world wide web interface (http://pbil.univ-lyon1.fr/BLAST/).

2.7. pH and temperature optima

Influence of pH on the enzyme activity was determined in the pH range 2.1–10.0 in the following 50-mM buffers containing 0.5 mg/ml BSA: sodium citrate (2.1–4.5), sodium acetate (3.5–5.5), sodium phosphate (5.3–8.0), Tris–HCl (7.5–9.0) and glycine–NaOH (8.5–10.0). Enzyme (11.4 mU) was incubated at 50°C for 7 min in the presence of 1 mM 4-nitrophenyl α-D-galactopyranoside dissolved in the buffers. Influence of temperature on enzyme activity was followed in 50 mM sodium acetate buffer (pH 4.5) in a temperature range 30–80°C using 1 mM 4-nitrophenyl α-D-galactopyranoside.

2.8. pH and temperature stabilities

Stability of α-galactosidase was followed in the pH range 2.1–10 in 75 mM buffers containing 0.5 mg/ml BSA. The buffers were the same as in the study of the pH and temperature optima. After 2 h and 5 h incubation at 50°C, aliquots were taken and immediately assayed for residual α-galactosidase activity.

Thermal stability of the enzyme was followed at various temperatures in the range 50–75°C. The enzyme was incubated in 90 mM sodium acetate buffer (pH 4.5) containing 0.5 mg/ml BSA. At time intervals aliquots were withdrawn and immediately cooled in ice-cold water. Residual activities, determined in both cases at 50°C on 1 mM

4-nitrophenyl α-galactoside in 50 mM sodium acetate buffer (pH 4.5), are expressed as percentage of activity of zero-time control of untreated enzyme.

2.9. Hydrolysis of galactose-containing oligosaccharides

To 60 μl of 1% solution (w/v) of melibiose, raffinose or stachyose in 50 mM sodium acetate buffer (pH 4.5), 2 μl of appropriately diluted α-galactosidase solution (66.6 mU) was added. Mixtures were incubated at 40°C and at various time intervals aliquots were taken and analyzed by TLC on silica gel sheets (Merck, Darmstadt, Germany). Melibiose and products of its hydrolysis were followed in *n*-butanol/ethanol/water (10:8:7, by volume). Hydrolysis of raffinose and stachyose was followed in *n*-propanol/ethyl acetate/water (6:1:3, by volume). Sugars were detected by spraying the dried chromatograms with 1% orcinol (w/v) ethanol containing 10% sulfuric acid (v/v).

2.10. Determination of kinetic parameters

These were determined in 50 mM sodium acetate buffer (pH 4.5) at 50°C. Hydrolysis of 4-nitrophenyl α-D-galactopyranoside was quantified on the basis of released 4-nitrophenol similarly as in the standard enzyme assay. Melibiose and raffinose hydrolysis was quantified by determination of released reducing sugars (galactose), determined with Somogyi–Nelson reagent [18]. Different reducing equivalents of mono- and oligosaccharides in the assay were taken into consideration.

2.11. Transglycosylation

A 200 mM solution of 4-nitrophenyl α-D-galactopyranoside in 50 mM sodium acetate buffer (pH 4.5) was incubated with 0.2 U of the purified α-galactosidase (1 μl) at 40°C and at time intervals aliquots were taken. The reaction was stopped by 5 min heating at 100°C (boiling in water bath) and after cooling reaction products were analyzed by TLC on silica gel (*n*-butanol/ethanol/water 10:8:7, by volume) or by analytical HPLC at room temperature on Lichrospher 100 NH2 5 μm (4×250 mm) column (Watrex, Prague, Czech Republic) connected with WellChrom K-2300 refractor index detector (Knauer, Berlin, Germany). 4-Nitrophenol and 4-nitrophenyl glycosides were also followed in the column eluate by UV-detector DVW-10 (D-Star Instruments, Manassas, VA, USA) operating at wavelength 335 nm. Regardless of the detection mode, isocratic elution with the system acetonitrile:water (70:30, v/v) was used at a flow rate 0.5 ml/min.

2.12. Stereochemistry of hydrolysis of glycosidic linkage

4-Nitrophenyl α-D-galactopyranoside (10 mM) and galactosyl[1]mannotriose (10 mM) were used as substrates of α-galactosidase. The reactions were performed in 20 mM deuterized sodium acetate buffer (pD 5.0). The amount of enzyme that would guarantee rapid complete hydrolysis of the substrate was found in preliminary experiments based upon TLC examination of the reaction mixtures. After the appropriate enzyme concentrations had been established, 500 μl of 10 mM solution of the substrate was mixed with 50 μl of the enzyme solution in D_2O. ^1H NMR spectra of the reaction mixtures were recorded at 50°C on a Bruker AM spectrometer operating at 300 MHz. NMR spectra of expected hydrolysis products were obtained for comparative purposes under the same conditions. Chemical shifts are referred to acetone as standard.

2.13. Degalactosylation of polymeric substrates

Cloudy opalescent solutions (0.2%; w/v, 10 ml) of locust bean gum and guar gum in 50 mM sodium acetate buffer

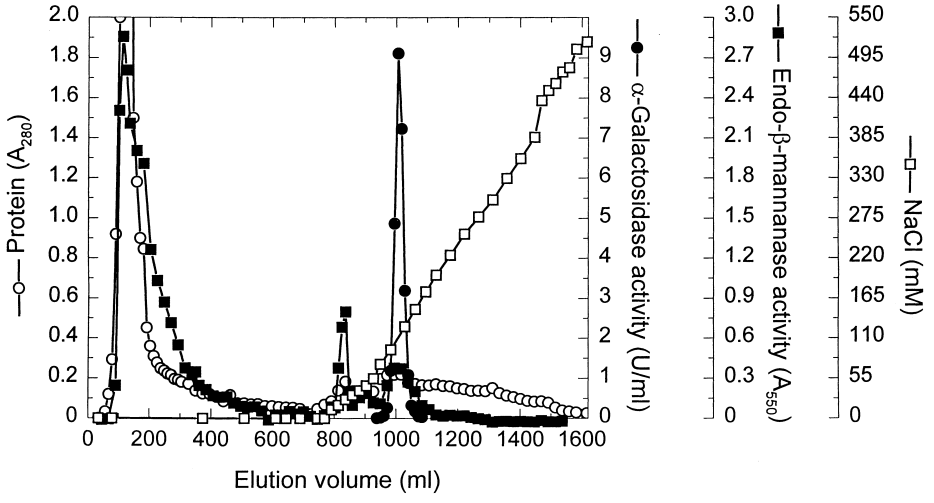

Fig. 1. Anion exchange chromatography of concentrated culture medium of *Thermomyces lanuginosus* grown on locust bean gum on a DEAE Sepharose column. Fractions were tested for protein (○), α-galactosidase (●) and endo-β-mannanase (■) activities, and for NaCl concentration (□).

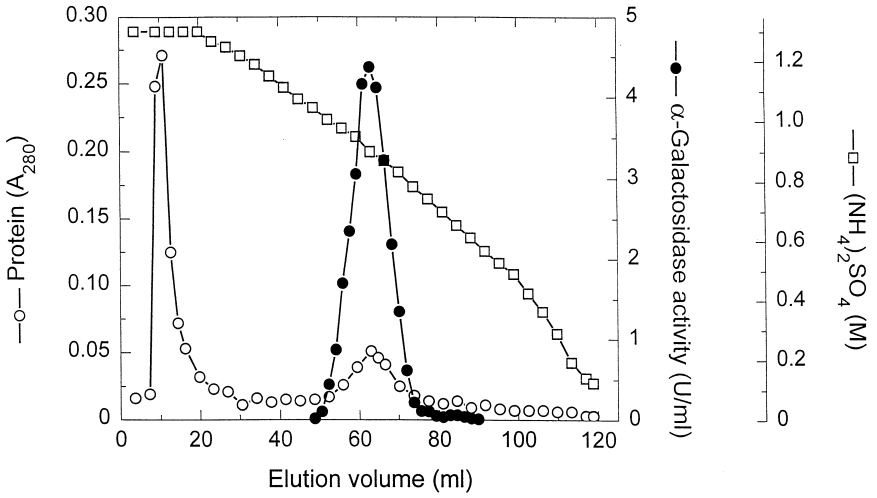

Fig. 2. Hydrophobic interaction chromatography of α-galactosidase active fractions, obtained after DEAE Sepharose chromatography, on a Phenyl Sepharose column. Fractions were tested for protein (○) and α-galactosidase activity (●), and for ammonium sulfate concentration (□).

(pH 4.5) were prepared after two 15 min heatings at 100°C under stirring, followed with centrifugation (1 min, $3000 \times g$) in order to remove insoluble particles. Then 0.82 U (100 μl) of α-galactosidase was added. Mixtures were incubated at 40°C under occasional stirring. Released galactose was determined with Somogyi–Nelson reagent [18] and is expressed as a percentage of anhydrogalactose present originally in the galactomannan, assuming galactose-to-mannose ratio is 23:77 and 38:62 for locust bean gum and guar gum, respectively [19].

3. Results and discussion

3.1. Production of the enzyme

During growth of *Thermomyces lanuginosus* IMI 158749 on 2% locust bean gum, the fungus produced high levels of extracellular α-galactosidase and endo-β-1,4-mannanase [12]. Time course of appearance of both enzyme activities was almost identical and both activities reached their maxima after 72–75 h after which they started to decline. In a typical experiment α-galactosidase activity reached about 0.8 U/ml.

3.2. Purification of α-galactosidase

Concentrated culture fluid was chromatographed on a column of DEAE Sepharose. Elution profile of protein and enzyme activities is shown in Fig. 1. Most of the protein was not trapped on the column. α-Galactosidase was adsorbed on the column and was eluted after application of a linear NaCl gradient as a sharp single peak at 110 mM salt concentration. Although the peak gave a single band on SDS polyacrylamide gel electrophoresis (PAGE), it still contained detectable endo-β-mannanase activity. Final purification of α-galactosidase was achieved by hydrophobic interaction chromatography on a Phenyl Sepharose column (Fig. 2). Major UV-light adsorbing material eluted with equilibration buffer. α-Galactosidase was liberated with decreasing ammonium sulfate gradient and eluted as a single protein peak at 0.9 M ammonium sulfate concentration.

The purification procedure is summarized in Table 1. By combination of anion exchange and hydrophobic interaction chromatographies α-galactosidase of *T. lanuginosus* was purified 1600 times with the overall yield 17.8%.

3.3. Physico-chemical properties

α-Galactosidase was found homogenous as judged from SDS PAGE (Fig. 3A). However, single protein band was rather diffused indicating some heterogeneity. Its molecular mass was estimated to be 57 kDa. Similarly, a single and somewhat diffuse band was observed after glycoprotein staining (data not shown) which may explain the ob-

Table 1
Summary of purification of α-galactosidase from *Thermomyces lanuginosus*

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Degree of purification
Crude medium	1500	6106.5	1020	0.168	100	1
DEAE Sepharose chromatography	6.5	10.2	433	42.4	42.5	254.1
Phenyl Sepharose chromatography	2.2	0.682	181.8	266.6	17.8	1596

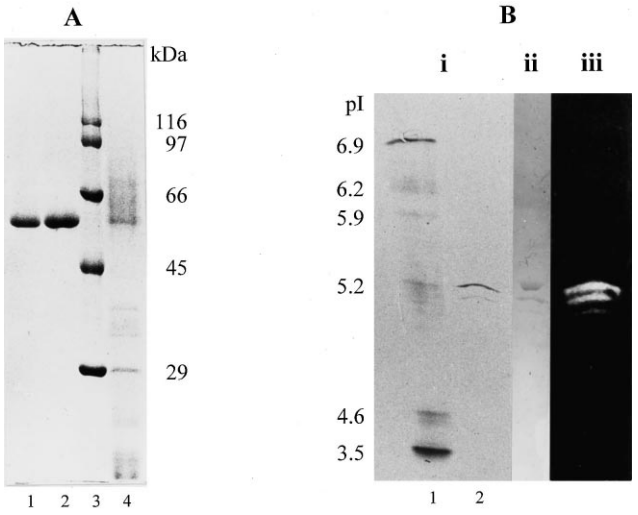

Fig. 3. SDS–PAGE and IEF of *Thermomyces lanuginosus* α-galactosidase. (A) SDS–PAGE monitoring of the purification of α-galactosidase. Lane 1, α-galactosidase active fractions obtained after DEAE Sepharose chromatography; lane 2, purified α-galactosidase obtained after Phenyl Sepharose chromatography; lane 3, molecular mass standards; lane 4, concentrated culture filtrate. Proteins were detected with Coomassie Brilliant blue R-250. (B) IEF of purified α-galactosidase. (i) Detection of proteins, as above: lane 1, standards with indicated p*I* values; lane 2, purified α-galactosidase; (ii) detection of glycoproteins with periodic acid-Schiff's reagent; (iii) activity stain with 4-methylumbelliferyl α-galactoside.

served protein heterogeneity. On IEF the enzyme gave one major and several minor protein bands (Fig. 3B(i)). All of them showed glycoprotein nature (Fig. 3B(ii)) and α-galactosidase activity on 4-methylumbelliferyl α-D-galactopyranoside (Fig. 3B(iii)). The p*I* value of the major form appears to be 5.2.

Molecular mass 57 kDa and isoelectric point 5.2 are within the intervals of the values (50–80 kDa and p*I* 4.0–8.5) reported for other fungal α-galactosidases [20–27].

3.4. Effect of pH and temperature on enzyme activity and stability

The effect of pH and temperature on enzyme activity and stability is shown in Fig. 4. The enzyme is most active at pH 4.5–5.0 and 65–70°C. At 50°C the enzyme is stable at wide pH range 3.0–7.5. It is interesting that stability of the enzyme at slightly alkaline conditions in pH interval 7.5–8.5 is much higher in Tris–HCl buffer than in sodium phosphate buffer. At pH 4.5 the enzyme is fully stable for 6 h at temperatures up to 60°C, but at temperatures above 70°C it is rapidly inactivated. The half-life of the enzyme at 65°C is 3 h.

The slightly acidic pH optimum of the enzyme (4.5–5.0) fits into the values characteristic for fungal glycosyl hydrolases. However, the observed temperature optimum (65°C) and temperature stability (up to 60°C) are higher by at least 10°C than the values reported for α-galactosidases from most of microbial sources. This is important from the view of possible industrial applications. However, the thermostability of the enzyme reaches neither that of α-galactosidases from extremophilic marine bacteria *Thermotoga neapolitana* [28] and *Thermotoga maritima* [29] nor that of endo-β-1,4-xylanases produced by other *T. lanuginosus* strains [13,30].

3.5. Amino acid sequencing

Edman degradation of the enzyme gave the single N-terminal amino acid sequence LVRPGNVGKLPALGwN-TwNAFGXDIDAT (tryptophan residues written in lowercase letters were not determined with certainty). This supports the hypothesis that the multiple forms observed on IEF are probably products of the same gene which differ in degree of glycosylation of single polypeptide chain. Other possibilities for the observed heterogeneity of the enzyme are different degree of amidation or slight

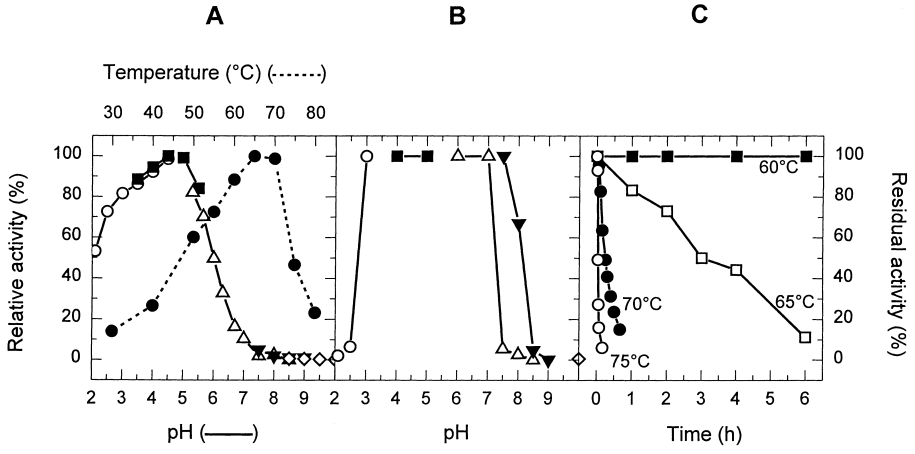

Fig. 4. pH and temperature optimum and stability of *T. lanuginosus* α-galactosidase. (A) (i) pH optimum determined in the following 50 mM buffers: sodium citrate (2.1–4.5) (○), sodium acetate (3.5–5.5) (■), sodium phosphate (5.3–8.5) (△), Tris–HCl (7.5–9.0) (▼), and glycine–NaOH (8.5–10.0) (◇); (ii) temperature optimum (···●···) determined in 50 mM sodium acetate buffer (pH 4.5). (B) Residual enzyme activity after 5 h incubation in buffers described in A. (C) Stability of the enzyme in 50 mM sodium acetate buffer (pH 4.5) at temperatures indicated.

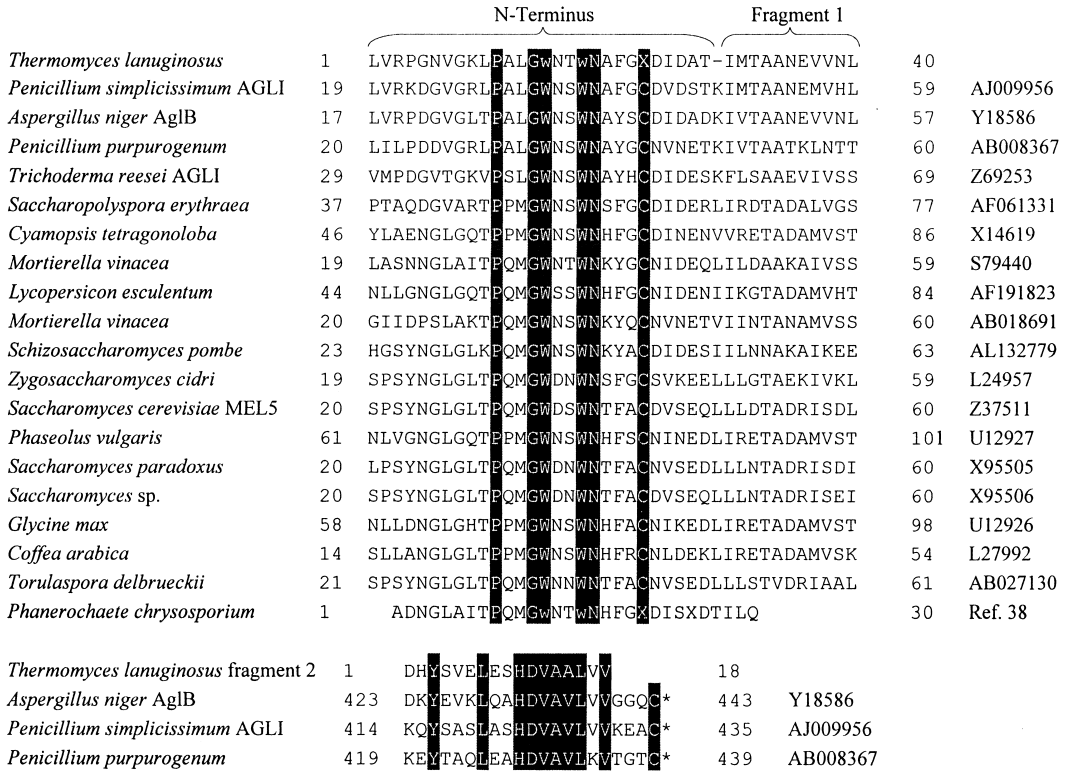

Fig. 5. Sequence alignment of *T. lanuginosus* α-galactosidase with other α-galactosidases. Amino acid residues conserved in all sequences are shown in black boxes. Database accession numbers of homologous α-galactosidases are on the right side. *Polypeptide termination.

post-translational proteolytic modifications of its C-terminus. The absence of methionine on the N-terminus suggests that the enzyme was digested with a signal peptidase prior its secretion.

Both glycosylation and prior-secretion signal peptidase digestion are common among proteins secreted outside the eukaryotic cells. Glycosylation could be responsible for the enhanced thermostability of the enzyme and/or might protect it from attack of proteases.

Two internal amino acid sequences have also been determined. They were identified as IMTAANEVVNLG (fragment 1) and DHYSVELESHDVAALVVG (fragment 2). Although the enzyme has a glycoprotein nature, none of the determined sequences contains any potential *N*-glycosylation site. Comparison of these sequences with amino acid sequences of other α-galactosidases is given in Fig. 5. Both N-terminal and fragment 1 as well as fragment 2 sequences were found highly homologous with the sequences of α-galactosidases from two *Penicillium* and one *Aspergillus niger* species whose complete amino acid sequences were derived from gene sequences. In these three enzymes, sequences homologous to fragment 1 directly follow their N-terminal sequences. Therefore fragment 1 sequence of *T. lanuginosus* α-galactosidase is assumed to be also a continuation of the determined N-terminal sequence. In this form, i.e., as an extended N-terminal sequence, the sequences are aligned in Fig. 5, upper part. The alignment suggests that the two tryptophan residues determined with uncertainty are really tryptophans. Similarly, the unidentified residue X is apparently cysteine since this is conserved in all homologous sequences. While for the extended N-terminal of *T. lanuginosus* α-galactosidase 21 homologous sequences were found, the sequence of fragment 2 shows homology with sequences of three α-galactosidases only: *A. niger* AglB (67% identity, 83% homology), *Penicillium simplicissimum* AGLI (61% identity, 67% homology) and *Penicillium purpurogenum* (50% identity, 72% homology). The high similarity with α-galactosidases presented in Fig. 5, all of which are classified as glycosyl hydrolase family 27 members

Table 2
Kinetic parameters of *Thermomyces lanuginosus* α-galactosidase for three substrates

Substrate	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
4-Nitrophenyl α-D-galactopyranoside	0.5	52.4	49.8	97.8
Melibiose (α-galactosyl-1,6-glucose)	2.4	2.4	2.3	0.9
Raffinose (α-galactosyl-1,6-sucrose)	11.3	34.1	32.4	2.9

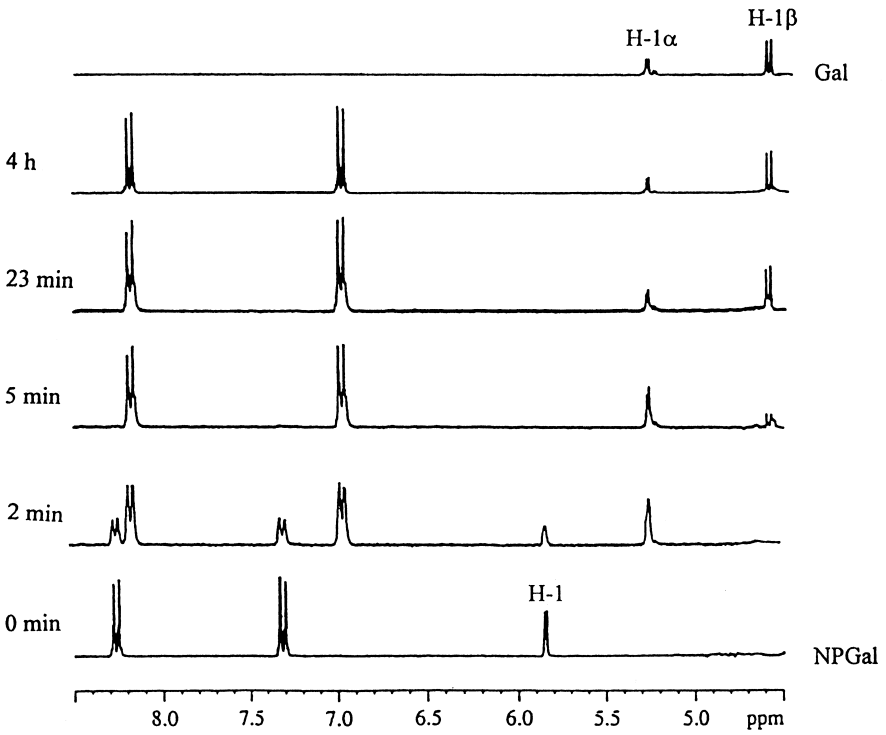

Fig. 6. Hydrolysis of 4-nitrophenyl α-D-galactopyranoside with *T. lanuginosus* α-galactosidase monitored by ^{1}H NMR spectroscopy. The spectra show the proton resonances of free and bound 4-nitrophenol (6.8–8.3 ppm) and anomeric regions of the spectra of the substrate and released D-galactose (4.5–5.9 ppm) at times indicated. The top spectrum shows resonances of anomeric hydrogens of D-galactose at anomeric equilibrium.

(http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf_27.html) suggests that α-galactosidase from *T. lanuginosus* also belongs to family 27 of glycosyl hydrolases [31,32].

3.6. Catalytic properties

T. lanuginosus α-galactosidase did not attack at an appreciable rate the following 4-nitrophenyl glycosides: β-galactoside, α- and β-glucoside, α- and β-mannoside, α- and β-xyloside, α-L-arabinopyranoside and α-L-arabinofuranoside but was very active on artificial substrates - 4-nitrophenyl and 4-methylumbelliferyl α-galactopyranosides. The enzyme was also active on naturally occurring α-linked galactose-containing oligosaccharides melibiose, raffinose and stachyose. Melibiose and raffinose are hydrolyzed more rapidly than stachyose, which undergoes a double degalactosylation. The rate-limiting step is the liberation of the first galactosyl moiety in stachyose since the resulting raffinose is degalactosylated to sucrose much faster and is therefore hardly observed among the products. For these reasons stachyose is not included in the Table 2 showing the kinetic parameters of hydrolysis of 4-nitrophenyl α-galactopyranoside, melibiose and raffinose. The best substrate appears to be the aryl glycoside for which the enzyme shows the highest V_{max} and the lowest K_m values. Both oligosaccharides are hydrolyzed by about two orders slower and, in agreement with the results of TLC examination, with approximately with the same efficiency.

Activities of α-galactosidases on aryl α-D-galactosides as well as melibiose, raffinose and stachyose are exhibited by almost all α-galactosidases. Other naturally occurring substrates which may be used to distinguish catalytic properties of α-galactosidases are fragments of galactomannan. As will be described later, *T. lanuginosus* α-galactosidase is able to hydrolyze galactosyl^{1}mannotriose where galactose is attached to the reducing end mannose residue. According to this, *T. lanuginosus* α-galactosidase substrate specificity is most closely related to *P. simplicissimum* AGLI [27,33]. Other fungal α-galactosidases showed somewhat different specificity on galactomannooligosaccharides [20,34,35]. Unfortunately, only some α-galactosidases have been tested for activity against various galactomannooligosaccharides.

3.7. Stereochemistry of hydrolysis

Changes in the ^{1}H NMR signals of anomeric region during the hydrolysis of 4-nitrophenyl α-D-galactopyranoside (NPGal) and galactosyl^{1}mannotriose ($GalMan_3$) are shown in Figs. 6 and 7. Both substrates were completely degraded within 5 min. The process of hydrolysis was considerably faster than the spontaneous mutarotation. Liberation of 4-nitrophenol is reflected in the shift of resonances of aromatic hydrogen atoms to lower ppm values simultaneously with disappearance of the anomeric hydrogen signal of NPGal (doublet at 5.84 ppm, $J_{1,2}$ 3.43 Hz). It is replaced with doublet at 5.27 ppm ($J_{1,2}$ 3.42 Hz), which

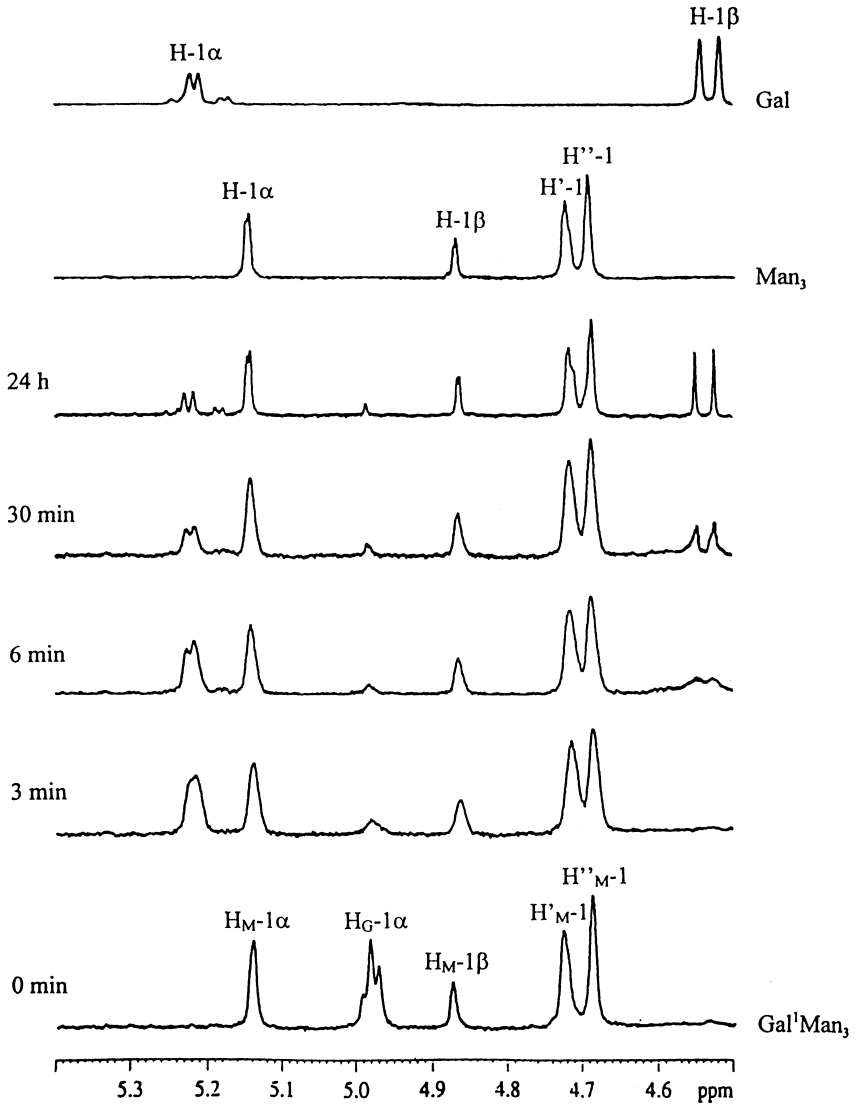

Fig. 7. Hydrolysis of galactosyl[1]mannotriose with *T. lanuginosus* α-galactosidase monitored by ^{1}H NMR spectroscopy. The spectra show resonances of anomeric hydrogens of the substrate ($GalMan_3$) and the products formed at times indicated. The top spectra show the resonances of anomeric hydrogens of D-galactose (Gal) and mannotriose (Man_3) at anomeric equilibria.

is assigned to H-1 of α-galactopyranose [36]. α-Anomer of galactopyranose slowly undergoes mutarotation and at equilibrium the β-anomer of galactopyranose (doublet at 4.59 ppm, $J_{1,2}$ 7.80 Hz) predominates (ratio 1.9:1 in favor of the β-anomer). Both pyranose anomers are to a small extent isomerized to corresponding furanose forms, probably due to a higher temperature (50°C) at which the experiment was performed. Doublets at 5.22 and 5.30 ppm are assigned to anomeric protons of α-galactofuranose and β-galactofuranose, respectively [36]. The latter is partially overlapped with resonances of α-galactopyranose.

In the case of $GalMan_3$, the anomeric proton signals of α-galactose is split by interaction not only with H-2 atom of galactose but also with two hydrogen atoms on C-6 atom of the neighboring mannosyl residue, resulting most probably in a pair of unresolved overlapping triplets in the interval 4.97–5.00 ppm. Anomeric hydrogen signals of mannosyl residues of mannotriose and $GalMan_3$ were assigned according to Harjunpää et al. [37]. Similar to the time course of NPGal hydrolysis, the primary hydrolytic product of $GalMan_3$ is α-galactopyranose, which undergoes mutarotation to β-galactopyranose. At equilibrium, the spectra show again weak resonances of both galactofuranose forms.

On the basis of the liberation of α-galactopyranose as the primary product of hydrolysis of both artificial substrate 4-nitrophenyl α-galactopyranoside and naturally occurring substrate galactosyl[1]mannotriose, we conclude that *T. lanuginosus* α-galactosidase is a retaining glycosyl hydrolase. The retaining character was recently reported for *Phanerochaete chrysosporium* α-galactosidase, which was also suggested to be a member of family 27 of glycosyl hydrolases [38]. This also supports classification of *T. lanuginosus* α-galactosidase to family 27 of glycosyl hydro-

lases. Moreover, considering the postulation of Henrissat and Bairoch [31,32] that glycosyl hydrolases grouped into the same family share a common catalytic mechanism, all family 27 classified enzymes are expected to be retaining glycosyl hydrolases.

3.8. Glycosyl transfer reactions

In consonance with its retaining character, the enzyme catalyzed at high concentration of 4-nitrophenyl α-galactopyranoside, in addition to its hydrolysis to galactose and 4-nitrophenol, also glycosyl transfer reactions. Fig. 8 shows the products formed at 200 mM substrate concentration analyzed by HPLC and TLC. The enzyme was found to transfer galactosyl residues to another substrate molecule leading to 4-nitrophenyl glycosides of galactooligosaccharides detectable both refractometrically and spectrophotometrically. These compounds were generated in about 7% yield calculated on the basis of the starting substrate. Glycosyl transfer occurs in a small extent also to free galactose (approx. 4% as calculated on the starting substrate) giving rise to compound showing elution time and thin layer chromatographic mobility of a disaccharide. The anomeric character of the newly synthesized glycosidic linkage in the transfer products was shown to be α-galactosidic both enzymatically and by NMR spectroscopy. The position of the linkage has not been established in this work.

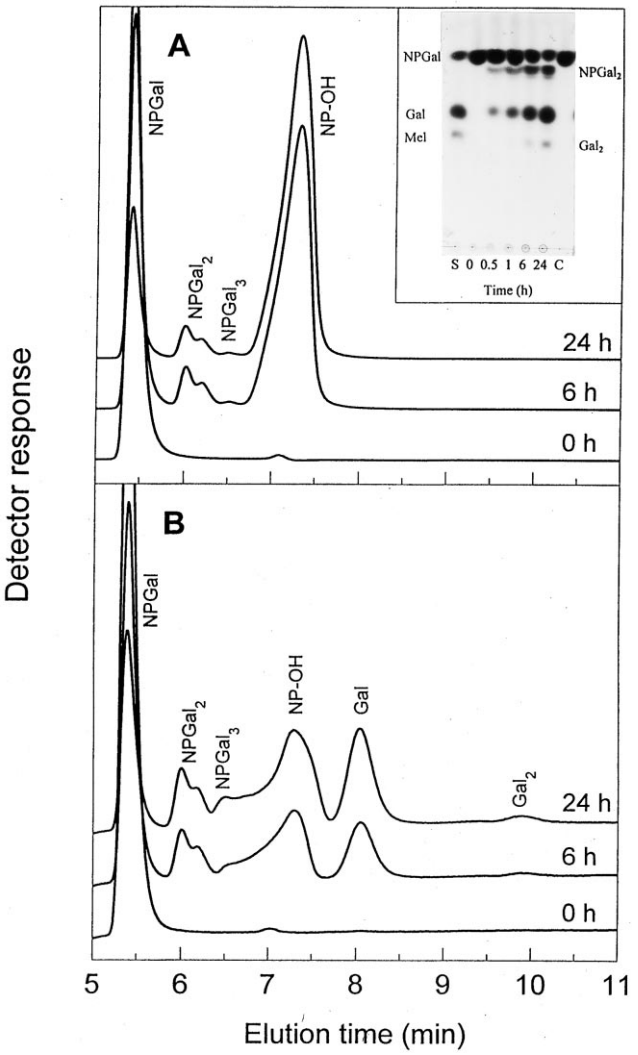

Fig. 8. Transgalactosylation reactions catalyzed by *Thermomyces lanuginosus* α-galactosidase with 4-nitrophenyl α-D-galactopyranoside as the substrate. Reactions were carried out at 200 mM substrate concentration in 50 mM sodium acetate buffer (pH 4.5) at 40°C, and products analyzed by HPLC using spectrophotometric (A) and refractometric detection (B) or by TLC (inset). NPGal, 4-nitrophenyl α-D-galactopyranoside; $NPGal_2$, 4-nitrophenyl α-D-galactobioside; $NPGal_3$, 4-nitrophenyl α-D-galactotrioside; NP-OH, 4-nitrophenol; Gal, D-galactose; Gal_2, α-D-galactosyl-D-galactose; Mel, melibiose; S, standards; C, enzyme-free control.

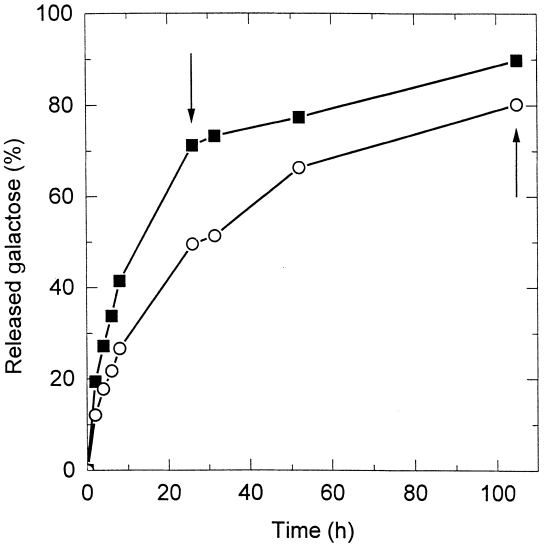

Fig. 9. Degalactosylation of galactomannans with *T. lanuginosus* α-galactosidase. Time course of liberation of D-galactose from locust bean gum (■) and guar gum (○). The reaction was carried out at 0.2% polysaccharide concentration in 50 mM sodium acetate buffer (pH 4.5) at 40°C. Arrows indicate the time of the first sign of precipitate formation.

3.9. Degalactosylation of polymeric galactomannans

During treatment of both locust bean gum and guar gum with α-galactosidase, galactose was the only product of hydrolysis. Time course of galactose liberation is shown in Fig. 9. In the later stages of degalactosylation of both polymeric galactomannans precipitation of the polymers was observed. At the same enzyme concentration, LBG was precipitated sooner than GG probably due to its lower galactose content. Precipitation of LBG and GG was observed when galactose content in the polysaccharide decreased to approximately 7% and 9.5%, respectively. This is in agreement with McCleary and Matheson [39], who observed precipitation below 12% galactose content.

The ability of *T. lanuginosus* α-galactosidase to debranch polymeric legume seed galactomannans is quite rare among microbial α-galactosidases. This feature is

common to α-galactosidases from *P. simplicissimum* AGLI, *P. ochrochloron* and *A. niger*. [25,27,33,40]. The degree of degalactosylation of polymeric galactomannans by *T. lanuginosus* enzyme was found to be the highest among microbial α-galactosidases and approaches that by enzymes from plants, e.g., lucerne and guar [19,41]. Similar to the plant α-galactosidases, the action of *T. lanuginosus* enzyme on galactomannans led to polymer precipitation. This ability also supports classification of *T. lanuginosus* α-galactosidase to family 27 of glycosyl hydrolases since the guar α-galactosidase has been classified to this family. The efficient degalactosylation of galactomannans with a high galactose:mannose ratio may be used for modification of gelling and rheological properties of the polysaccharides. As regards industrial potential of the enzyme, it would be important to identify the enzyme segment(s) responsible for activity on polysaccharides.

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