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The carbohydrate moiety of α-galactosidase from *Trichoderma reesei*

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α-Galactosidase from *Trichoderma reesei* is a glycoprotein that contains O- and N-linked carbohydrate chains. There are 6 O-linked glycans per protein molecule that are linked to serine and threonine and can be released by β-elimination. Among these are monomers: D-glucose, D-mannose, and D-galactose; dimers: α1-6 D-mannopyranosyl-α-D-glycopyranoside and α1-6 D-glucopyranosyl-α-D-galactopyranoside and one trimer: α-D-glucopyranosyl-α1-2 D-mannopyranosyl-α1-6 D-galactopyranoside. N-linked glycans are of the mannose-rich type and may be released by treating the protein with Endo-β-*N*-acetyl glycosaminidase F or by hydrozinolysis. The enzyme was deglycosylated with Endo-β-*N*-acetyl glycosaminidase F as well as with a number of exoglycosidases that partially remove the terminal residues of O-linked glycans. The effect of enzymatic deglycosylation on the properties of α-galactosidase has been considered. The effects of tunicamycin and 2-deoxyglucose on the secretion and glycosylation of the enzyme during culture growth have been analysed. The presence of two glycoforms of α-glactosidase differing in the number of N-linked carbohydrate chains and the microheterogeneity of the carbohydrate moiety of the enzyme are described.

***Keywords*: glycoprotein, α-glactosidase, carbohydrate moiety, O-linked glycans, N-linked glycans, hydrazinolysis, glycoforms, AMC-O-linked glycan derivations**

Introduction

α-Galactosidase (melibiase) (EC 3.2.1.22) is an exoglycosidase, removing terminal galactose residues from the galactopolysaccharides and carbohydrate moiety of glycoproteins. α-Galactosidases have been isolated from a number of sources including plants [1, 2], fungi and yeast [3, 4]. α-Galactosidase considered in the present work was isolated from the culture liquid of the fungus *Trichoderma reesei* [5] that belongs to deuteromycetous fungal genus of the class Hyphomycetes. Similarly to other fungal α-galactosidases from *Aspergillus niger* [6] and from *Aspergillus awamori* [7], α-galactosidase from *T. reesei* is also a glycoprotein [8].

Despite extensive studies of enzymes from mycelial fungi, they have been insufficiently studied as glycoproteins. For example, from a variety of secretory glycosidase from *Trichoderma* species, the structure of the carbohydrate moiety has only been determined for cellobiohydrolase from *T. reesei* [9]. The role of glycosylation in these enzymes has been considered for a few proteins from the genus *Aspergillus*: glucoamylase from *A. awamori* [10, 11], glucoseoxidase from *A. niger* [12], acid carboxypeptidase from *A. saitoi* [13], and several others. These studies, however, are far from being based on a detailed structural characterization of the carbohydrate moiety for the glycoproteins under consideration. The microheterogeneity of the carbohydrate moiety has been extensively studied for a great number of glycoproteins from various sources [14], but not for proteins and enzymes from lower eucaryotes.

In the present work we consider some structural features of the carbohydrate moiety of α-galactosidase from *T. reesei* and compare the properties of the native and deglycosylated proteins with the aim of determining the role played by glycosylation.

Materials and methods

Materials

[^{14}C] D-Mannose (0.05 mCi mmol^{-1}), [^{3}H]$NaBH_4$ (0.04 mCi mmol^{-1}), and [^{3}H] D-glucosamine (0.05 mCi mmol^{-1}) were purchased from Izotop, Russia. Aminomethylcoumarin, mannan from yeast, 1-deoxymannojirimycin, deoxynojirimycin, 2-aminopyridine, hydrazine, $NaBH_4$, $NaBH_3CN$, 2-deoxyglucose, p-nitrophenyl-α-D-galactopyranoside (PNPG), sodium dodecylsulphate (SDS)

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and other salts and carbohydrates, were purchased from Sigma.

Enzymes: Pronase E was purchased from Serva: Novozym 234 was purchased from Nova-Nordisk, α-Galactosidase from *T. reesei* was isolated in accordance with a procedure reported [15]; α-mannosidase was purchased from Sigma; β-galactosidase, α- and β-glucosidases, β-mannosidase, were purchased from Bio-Chemis, Russia, Endo-β-*N*-acetyl glucosaminidase F from *Flavobacterium meningosepticum* (Endo F) was purchased from Boehringer-Mannheim.

Mannooligosaccharides were isolated from yeast mannan after partial acetolysis as described [16].

Enzymatic assays

α-Galactosidase activity was evaluated using PNPG as substrate at 3 mM concentration [17]. The unit of enzyme activity was defined as the amount of the enzyme that hydrolysed 1 μmol of substrate per min at 37 °C.

The K_M and k_{cat} values were determined by the method of initial rates in hydrolysis of natural and synthetic substrates. Linearization was carried out by the Lineweaver-Burk method. The inhibition constants were measured following the Dixon method by varying the concentration of the substrate (PNPG) in the reaction mixture.

The pH stability of the native and deglycosylated α-galactosidase were determined using 50 mM of phosphate-citrate (pH 2.2–4.0), acetate (pH 4.0–5.6), or phosphate (pH 6.0–8.0) buffers containing 0.1 M NaCl.

Analytical methods

The protein quantity was measured by the Lowry procedure [18] with BSA as standard. In addition, the concentration of pure α-galactosidase solution was determined using the extinction coefficient $E_{278} = 1.86\ ml\ mg^{-1}\ cm$ [8].

Electrophoresis was carried out by the Laemmli method.

CD spectra were recorded with a Jobin Yvon Mark 3 CD-spectrometer at protein concentrations of 0.2–1 mg ml^{-1}.

Comparative amino-acid analysis of two forms of α-galactosidase, the native α-galactosidase and α-galactosidase after β-elimination was performed with a Biotronik amino-acid analyser after hydrolysis in 5.7 M HCl and 10 mM phenol at 110 °C for 24 and for 48 h.

Terminal amino acid analysis was performed by an Applied Biosystems (ABi) 473A gas-liquid-solid-phase sequencer.

Enzymatic deglycosylation of α-galactosidase

The native α-galactosidase was treated with Endo F in a 75 mM sodium phosphate buffer, pH 5.8, for 28 h at 37 °C. 0.2 U of Endo F was added to 160 mg of α-galactosidase. Treatment with α-mannosidase was done under the following conditions: 0.02 M sodium acetate buffer, pH 4.5, 0.1 mM $ZnCl_2$, 0.1% β-mercaptoethanol, 2–3 U of α-mannosidase per mg of protein. Treatment with α-glycosidase or β-galactosidase was done in the same buffer with 2–3 U of each enzyme per mg of α-galactosidase.

Deglycosylation of denaturated α-galactosidase was performed under the same conditions and ratios of the glycoprotein to glycosidases.

Denaturation of α-galactosidase

Acid denaturation of α-galactosidase prior to the further deglycosylation was done as follows: the enzyme was dissolved in water to a concentration of 40 mg ml^{-1}, the solution was acidified with 1 M HCl to pH ≈ 0.7 and incubated for 24 h at 37 °C. Denaturation with 0.1% SDS was performed in 75 mM phosphate buffer, pH 5.8, in a boiling water bath for 5 min.

Proteolytical digestion of α-galactosidase was obtained as follows: the acid denaturated α-galactosidase was incubated with Pronase E in 20 mM Tris-HCl buffer, pH 7.5 at 37 ° for 24 h; α-galactosidase to Pronase E ratio was 50 : 1 (w/w).

Monosaccharide composition analysis

An analysis of neutral sugars was performed by GLC-MS, using arabinose as standard. The protein was hydrolysed in 2 N HCl at 100 °C for 3 h, the released saccharides were reduced by $NaBH_4$, acetylated and characterized as alditol acetates [19]. An Incos-50 (Finnigan MAT) mass spectrometer with a DB-5 column (temperature gradient 80–200 °C, 1.5 °C min^{-1}) was used. Galactosamines were determined with an amino-acid analyser after 16 h hydrolysis of the protein in 4 N HCl at 100 °C [20].

Composition analysis of O-linked glycans was performed after their release from the glycoprotein by β-elimination described below. Then the O-linked glycans were hydrolysed in 2 N HCl at 100 °C and characterized as alditol acetates by GLC-MS.

Composition analysis of N-linked glycans was performed after release from the protein by hydrozinolysis as described below, and was studied in a similar way to O-linked glycans.

Alkaline β-elimination

The O-linked glycans were released by alkaline β-elimination as follows. A 20 mg portion of the protein was incubated in 3 ml of 0.1 M NaOH and 2 M $NaBH_4$ at 30 °C for 48 h, treated in accordance with [21] and the structure of the O-linked carbohydrate chains was determined.

β-Elimination of O-linked glycans was done in the presence of [^{3}H] $NaBH_4$ at concentration 0.04 mCi ml^{-1}.

Hydrazinolysis and pyridylamination of N-linked carbohydrate chains

A 12–24 mg portion of α-galactosidase was incubated in 800 μl of hydrazine at 100 °C under argon in a sealed ampoule. After the reaction was complete, hydrazine was

evaporated in a rotary evaporator. For reacetylation, 5 ml of 9.8% aqueous $NaHCO_3$ and 200 ml of acetyl chloride were added to the protein once and again after 5 min, the mixture was kept for 30 min at room temperature and applied to 2 ml column of Dowex-50 in the H^+ form. The column was washed with five volumes of water, and eluates were pooled and lyophilized.

The pyridylamination of the N-linked carbohydrate chains was carried out according to the method of Hase *et al.* [22]. N-linked glycans (10–100 μg) were dissolved in 500 μl of a 2-aminopyridine solution prepared by mixing 212 μg of 2-aminopyridine, 220 μl of HCl and 400 μl of H_2O and then kept at 100 °C for 13 min. After 30 μl of a reducing reagent was added (prepared by mixing 100 μg of $NaBH_3CN$, 200 μl of the 2-aminopyridine solution, and 250 μl of water immediately before use), the reaction mixture was incubated at 90 °C for 15 h for reductive amination. The reaction mixture was filtrated on a Bio-Gel P4 column (5×150 mm). Fractions containing N-linked (PA-N-linked) glycans were collected and lyophilized. PA-N-linked glycans prepurified by gel filtration were applied to the ODS 120T column (Pharmacia). Chromatography was carried out with a linear gradient of 0–100% acetonitrile, 0.1% TFA, starting buffer − 0.1% TFA in water. The spectrophotometrical detection at 224 nm as well as fluorometrical monitoring with excitation and emission wavelengths of 320 and 400 nm, respectively, was used to collect carbohydrate-containing fractions.

Analytical gel-filtration of N-linked glycans was done using a Bio-Gel P4 column (180×0.5 cm) in NH_4HCO_3 buffer, pH 8.3. The Bio-Gel P4 column was calibrated by pyridylamino derivatives (PA) of dextran hydrolysate [23], PA of $Man_6GlcNAc_2$ isolated from α-amylase from A. oryzae [24] and PA-N-carbohydrates from human α-acid protein [25].

The number of N-linked chains in A and B forms of α-galactosidase was found by hydrazinolysis as described above with the subsequent reduction of the released N-linked glycans by [^{3}H]$NaBH_4$ (0.04 mCi ml^{-1}) using lactose as standard.

Hydrazinolysis of O-linked oligosaccharides

A 5 mg portion of native α-galactosidase was suspended in 600 μl of anhydrous hydrazine and kept under argon in a sealed ampoule at 60 °C for 6 h. Then hydrazine was removed by evaporation in rotary evaporator. The sample was dissolved in 200 μl of water, acidified with 5 M HCl to pH 3–4, and applied to the 2 ml column packed with Dowex 50 in H^+ form. The column was washed with five volumes of water. Carbohydrate-containing fractions were pooled and lyophilized. Qualitative analysis of the released carbohydrates was performed by thin-layer chromatography (TLC) on Kieselgel 60 TLC plates (Merck) in the following proportions ethanol : butanol : water, 2 : 2 : 1.

Derivatization of the O-linked oligosaccharides into *N*-(4-methylcoumarin-7-yl)glycamines (AMC-oligosaccharides)

Lyophilized O-linked glycans were dissolved in 200 μl of a 1% solution of acetic acid in ethanol and mixed with 2 mg of AMC dissolved in 200 μl of the same solution. After incubation for 2 h at 50 °C, 5 mg of $NaBH_3CN$ dissolved in 50 μl of methanol was added and the reaction mixture was kept under argon in a sealed ampoule for 18–20 h at 40 °C. The reaction was stopped by adding concentrated acetic acid to pH 2–2.5, the reaction mixture was lyophilized and borates were removed by evaporation from methanol on a rotary evaporator (five times adding 1.5 ml of methanol). To remove the unreacted AMC, the sample was subjected to TLC on a Kieselgel plate (Merck) in the system methanol : chloroform, 1 : 3. After TLC, AMC-oligosaccharides were eluted from the plate with a 50% aqueous solution of acetonitrile, lyophilized, and used for further analysis.

Analysis of AMC-oligosaccharides

To perform a qualitative analysis of AMC-oligosaccharides, we used TLC on a Kieselgel 60 plate in the following proportions: butanol : acetic acid : water, 3 : 2 : 1. For quantitative analysis HPLC was carried out on a Lichrosorb TSK-NH_2 column in a system containing 80% acetonitrile in 50 mM of Na-acetate buffer, pH 4.1. Collected fractions were analysed with a Hitachi spectrofluorimeter with excitation and emission wavelengths of 365 and 440 nm, respectively.

The exoglycosidase digestion of AMC-O-oligosaccharides was done by incubation of $100 \div 300$ nmol of each carbohydrate with $0.05 \div 0.1$ U of the appropriate exoglycosidase at 37 °C for 6 h. The resulting mixtures were analysed by HPLC as described.

Analysis of the O-linked glycans by GLC-MS

Alditols released after *β*-elimination were methylated by the Hakomori procedure [26] and then subjected to GLC-MS analysis by an Incos-50 Finnigan MAT mass spectrometer [10, 27] on a DB-5 column (temperature gradient 80–200 °C at 1.5 °C min^{-1}). Alternatively, alditols of O-linked glycans were acetylated [10] and subjected to GLC-MS analysis.

Acetolysis/hydrolysis of methyl esters of O-linked glycans was performed according to [28]. The partially methylated alditols obtained were analysed by GLC-MS [27, 28]. The *O*-acetylated partially *O*-methylated carbohydrates were identified by their typical retention time and MS-spectra [10, 28].

Cultivation with inhibitors

The culture of *T. reesei* was grown at 30 ± 1 °C in a liquid medium with constant stirring. The culture medium contained KH_2PO_4, 1 g l^{-1}, $NaNO_3$, 1.5 g l^{-1}, $(NH_4)_2SO_4$, 1.5 g l^{-1}; $MgSO_4 \times 7H_2O$, 0.5 g l^{-1} wheat bran, 20 g l^{-1}. Experiments on the effect of 2-deoxyglucose and

tunicamycin on secretion were carried out according to the following procedure. The *T. reesei* culture was preincubated for 48 h, the mycelium was separated by centrifugation and carried over into a medium containing an inhibitor and the corresponding labelled monosaccharide. The culture was grown for 8 h (Tunicamycin was preliminarily dissolved in 0.2 M NaOH. Independent experiments were carried out to check whether the introduction of such concentrations of an alkali had any effect on the culture growth and secretion of α-galactosidase.). The amounts of labelled monosaccharides introduced into the medium were D-mannose: 8 μCi ml^{-1}; *N*-acetylglucosamine: 32 μCi ml^{-1}. The amount of mycelium was determined by weighing after it was centrifuged, washed and dried.

The cultivation of *T. reesei* in the presence of 1-deoxymannonajirimicyn or deoxynajirimicyn was performed in the same medium at 30 °C with the addition of the inhibitor to a concentration of 20 ÷ 25 μg ml^{-1} before seeding. Growth time was 72 h.

Intracellular α-galactosidase

One hundred ml of the culture was grown as described above for 48 h. Mycelium was centrifuged and washed twice with 100 ml of 50 mM Tris–HCl, pH 7.2. Collected mycelium was resuspended in 4 ml of the same buffer with 2 mg ml^{-1} of Novozym 234 and incubated with constant stirring at 20 °C for 5 h. The resulting mixture was centrifuged (20 000 × **g**) and the supernatant was then used for the isolation of the intracellular α-galactosidase [15].

Results

O-glycosylation and carbohydrate composition

The carbohydrate composition of α-galactosidase includes mannose, glucose, galactose, and *N*-acetylglucosamine in the molar proportions Man:Glc:Gal:Glc:NAc = 10:1:0.5:1 according to GLC-MS data obtained for neutral sugars and data of amino-acid analysis for *N*-acetyl glucosamine. α-Galactosidase is a glycoprotein with a mixed type of glycosylation. Alkaline β-elimination in the presence of [^{3}H]$NaBH_4$ released 6 O-linked carbohydrate chains per molecule. Similar results (6 O-carbohydrate chains) were obtained by subjecting to comparative amino-acid analysis the native α-galactosidase and the α-galactosidase after β-elimination. Four serine residues are transformed into alanine, and two threonine residues into γ-aminobutyric acid and glycine upon β-elimination (Table 1). The composition of intact O-linked carbohydrates, analysed by GLC-MS after acid hydrolysis, was Man : Glc : Gal = 5 : 8 : 15.

Glycoforms of α-galactosidase

α-Galactosidase isolated and used in the present work can be separated by ion-exchange HPLC on a TSK-SP-5PW

Table 1. Number of amino acid residues per mole of α-galactosidase (A and B forms) before and after reductive alkaline β-elimination.

Amino acid	*Composition (mol/mol of enzyme)*			
	Form A		*Form B*	
	before beta-elimination	*after beta-elimination*	*before beta-elimination*	*after beta-elimination*
Asx	62	62	61	62
Thr	29	27	29	27
Ser	30	26	30	26
Glx	27	27	27	27
Pro	25	24	25	25
Gly	43	44	43	44
Ala	46	50	46	50
Cys	7	7	7	7
Val	21	21	21	21
Ile	15	16	15	15
Leu	36	36	36	36
Tyr	16	16	16	16
Phe	12	12	12	12
Lys	11	12	12	12
His	10	10	10	10
Arg	15	15	15	15
Trp	NA	NA	NA	NA
Met	5	5	5	5
Gamma-aminobutyric acid	–	1	–	2

column into two forms, termed form A and form B (Figure 1). These forms cannot be separated by SDS-PAGE and analytical gel filtration nor by reverse phase HPLC. The amino-acid compositions of forms A and B are identical (Table 1) and also the N-terminal sequences of amino acids for forms A and B: **A M P D G T T G**. However, there is a significant difference in carbohydrate composition between forms A and B, both in the monomer composition and in the number of N-carbohydrate chains α-galactosidase molecule (Tables 2, 3).

These data suggest that α-galactosidase exists in the culture liquid in at least two glycoforms. Probably the heterogeneity of the carbohydrate moiety allows isolation of two glycoforms by ion-exchange chromatography. It should be noted that despite the difference in carbohydrate compositions and in behaviour in ion-exchange HPLC between forms A and B, they show no difference in CD spectra. It is also worth noting that forms A and B exhibit identical kinetic characteristics, K_M and k_{cat} (Table 4), and have the same pH and thermal stability.

N-linked glycans

The presence and number of N-linked glycans were determined by treating the proteolytic digest of α-galactosidase by endo F with subsequent reduction of released N-linked glycans in the presence of $[^3H]NaBH_4$ and by hydrazinolysis, followed by reduction in the presence of $[^3H]$ $NaBH_4$. Data obtained by both methods coincide. Intact N-linked glycans contained only mannose as a neutral sugar.

In Figure 2a the separation is shown of PA-N-linked glycans of α-galactosidase (form A) into two fractions by gel filtration on a Bio-Gel P4. These fractions were each subjected to reverse phase HPLC, yielding eight subfractions (Figure 2b). Analytical gel-filtration on Bio-Gel P4 was applied in order to determine the molecular mass of an

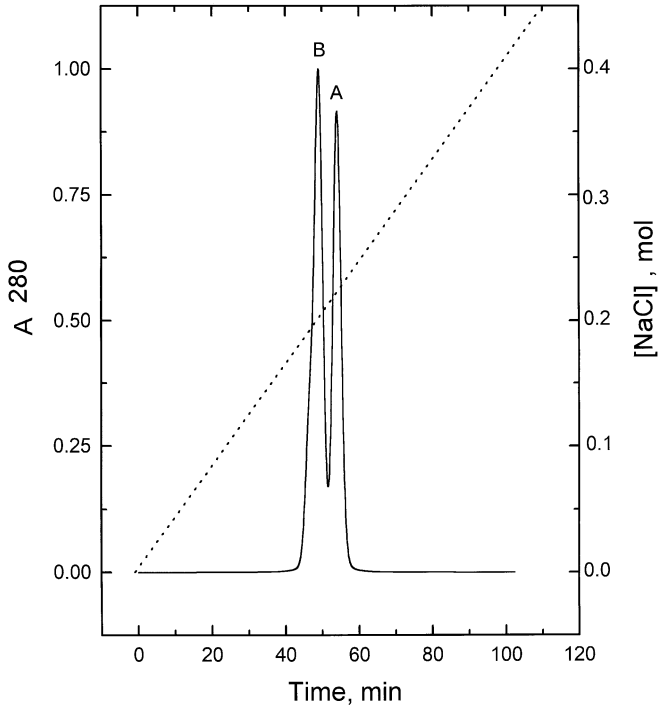

Figure 1. Separations of two *α*-galactosidase forms (A and B) on a TSK SP 5 PW column in 50 mM sodium acetate buffer. Elution was performed with a gradient of NaCl (0–0.5 M) as indicated. The elution of proteins was detected by absorbance at 280 nm. Two fractions (A and B) contained *α*-galactosidase activity were collected.

Table 2. Number of partially methylated alditol acetates of O-linked glycans per mol of *α*-galactosidase.

Methylated alditol acetate	*Form A*	*Form B*
1,5Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol	0.6	2.2
1,5Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol	1.8	1.1
1,5Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	3.6	2.7
1,5,6Tri-*O*-acetyl-2,3,4-tetra-*O*-methylglucitol	0.5	1.4
1,5,6Tri-*O*-acetyl-2,3,4-*O*-methylgalactitol	2.9	2.0
1,3,5Tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol	0.5	0.5

Table 3. O- and N-linked carbohydrate chains of *α*-galactosidase (A and B forms).

Carbohydrate chains	*Quantity (mol/mol of enzyme)*	
	Form A	*Form B*
Man(1 → *O*)Ser/Thr	0.1	0.8
Glc(1 → *O*)Ser/Thr	0.7	0.7
Gal(1 → *O*)Ser/Thr	1.8	1.1
Man(1*α* → 6)Glc(1 → *O*)Ser/Thr	0.5	1.4
Glc(1*α* → 6)Gal(1 → *O*)Ser/Thr	2.4	1.5
Glc(1*α*→6)Man(1*α*→3)Gal(1→*O*)Ser/Thr	0.5	0.5
N-linked carbohydrate chains	3	5

Table 4. Kinetic parameters of reactions catalysed by *α*-galactosidase.

	K_M (mM)	k_{cat} (μmol min μg)$^{-1}$
Native *α*-galactosidase (A and B forms)	0.050	5.1×10^{-2}
α-Galactosidase treated by endo F (form A)	0.025	1.1×10^{-2}
α-Galactosidase treated by endo F and exoglycosidases(form A)	0.013	0.6×10^{-2}

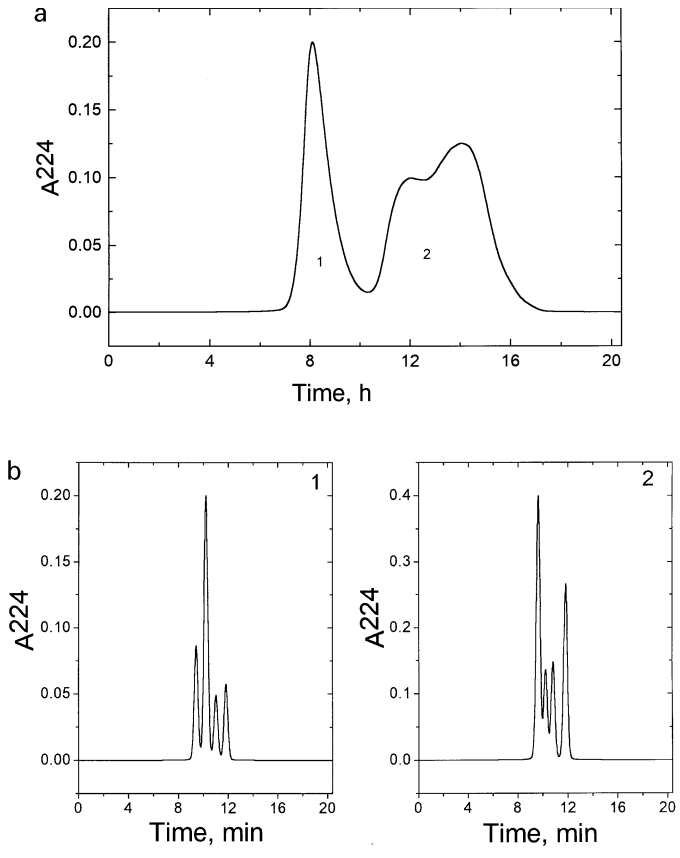

Figure 2. Separation of PA-N-linked glycans of *α*-galactosidase (form A). (a) Gel-permeation chromatography of Bio-Gel P4 column in 0.1 M NH_4HCO_3, pH 8.3. The elution was detected by absorbance at 224 nm. (b) Reverse phase chromatography of the obtained fractions on an ODS-120T column. Elution was performed with a linear gradient of 0–100% acetonitrile, 0.1% TFA, starting buffer–water, 0.1% TFA. The spectrophotometrical detection at 224 nm as well as fluorometrical monitoring with excitation and emission wavelengths of 320 and 400 nm respectively was used to detect carbohydrate-contained fractions.

individual glycan. This procedure was repeated for the B-form of the protein. It was found that the A-form contains four $Man_7ClcNAc_2$, two $Man_6ClcNAc_2$ and two $Man_5ClcNAc_2$ chains, and the B-form five $Man_7ClcNAc_2$, one $Man_6ClcNAc_2$ and five $Man_5ClcNAc_2$ chains. The individual glycans differ in the number of mannose units. The presence of a greater number of individual PA-oligosaccharides than glycosylation sites in the α-galactosidase points to the occurrence of microheterogeneity.

Structure of O-linked glycans

The O-linked glycans were split from α-galactosidase by alkaline treatment. Products of *β*-elimination were identified by GLC-MS analysis as methylated alditol acetates. Results of analysis of partially methylated alditol acetates of O-linked glycans are presented in Table 2. The structures of O-linked glycans are presented in Table 3. The presence of only α-bonds in glycans of the glycoprotein was indicated by the complete deglycosylation of the proteolytic digest by only the α-exoglycosidases. Alternatively, the structures of the O-linked glycans were determined by hydrazinolysis which released carbohydrate chains with the subsequent derivatization of free O-linked glycans into AMC derivatives. Experiments with $[^3H]NaBH_4$ have shown that by making use of hydrazinolysis appropriate for releasing O-linked oligosaccharides of the mannose type, hydrazinolysis removed more than 80% of O-linked glycans. Chromatographic separation of AMC derivatives of O-linked glycans by HPLC is illustrated in Figure 3. Structures of O-linked glycans were also tested by treatment with exoglycosidases. One advantage of the method is the determination of the presence of trisaccharides when compared with GLC-MS.

Properties of deglycosylated α-galactosidase

Deglycosylation of the native α-galactosidase with Endo F removes about 1–2 N-linked glycans from both A and B forms (Figure 4). Increasing incubation time above 28 h does not lead to any additional release of N-glycans. Subsequent treatment of the partially deglycosylated α-galactosidase with exoglycosidases (α-mannosidase and

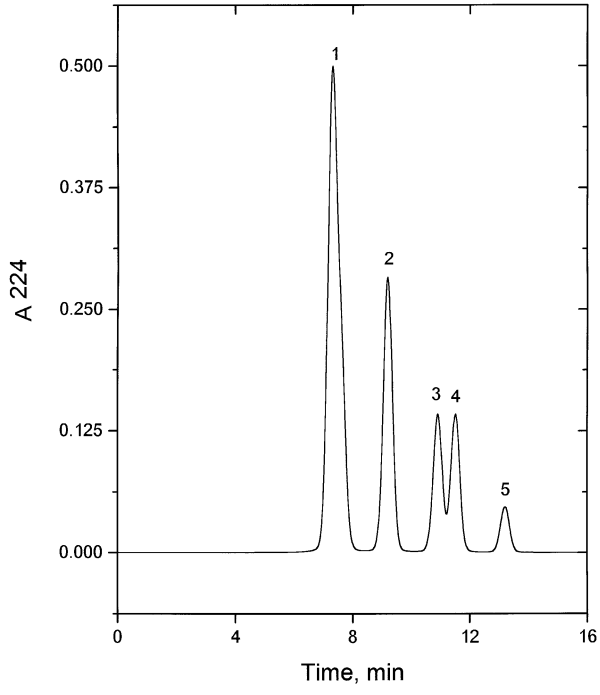

Figure 3. Separation of the AMC-O-linked glycans on a Lichrosorb TSK NH_3 column. Isocratic elution by a mixture of acetonitril: 50 mM sodium acetate, pH 4.2, (4 : 1) has been used. The elution was detected by absorbance at 224 nm and by following spectrofluorimetric monitoring of factions with excitation and emission wavelength of 365 and 440 nm respectively. 1 AMC, 2 AMC-monosaccharides, 3 and 4, AMC-disaccharides, 5 AMC-trisaccharides.

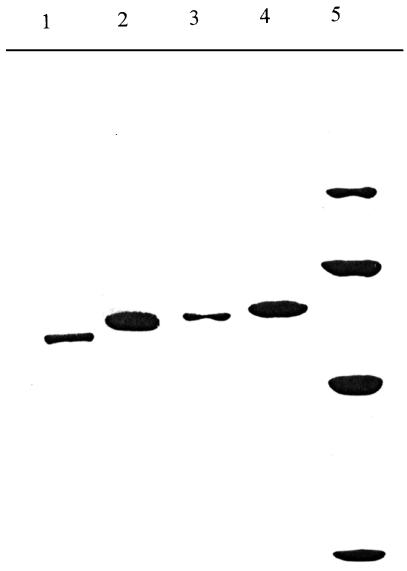

Figure 4. SDS/PAGE electrophoresis of the native and deglycosylated *α*-galactosidase (form B). Lane 1, *α*-galactosidase treated by endo F and exoglycosidases after denaturation; Lane 2, *α*-galactosidase treated by endo F and exoglycosidases; Lane 3, *α*-galactosidase treated by endo F; Lane 4, native *α*-galactosidase; Lane 5, protein standards: phosphorylase A (98 kDa), BSA (67 kDa), ovalbumin (45 kDa), *α*-chymotrypsin (25 kDa).

α-glucosidase) enables the removal of about 30% of mannose and about 20% of glucose from O-linked glycans.

Deglycosylation strongly affects the properties of α-galactosidase. For example, the removal of only 1–2 N-linked glycans considerably diminishes the thermal and pH stability of the enzyme (Figures 5, 6). The following partial removal of O-linked glycans has an even more dramatic effect on the pH and thermal stability. The K_M and k_{cat} values for PNPG hydrolysis are changed (Table 4), which presumably suggests that the spatial structure of the active centre of the enzyme suffers a substantial change in partial deglycosylation. Interestingly, such a significant change in stability and kinetic characteristics of the enzyme is accompanied, according to CD data, by only slight conformational changes (Figure 7).

The most complete deglycosylation was achieved by a treatment of denatured α-galactosidase with Endo F and exoglycosidases together. Prior acid denaturation of α-galactosidase enables the release of three N-linked glycans, and prior denaturation with SDS the release of 4 N-linked glycans (Figure 4).

Effect of tunicamycin and 2-deoxyglucose on the secretion of α-galactosidase

The mycelium wet weight and the α-galactosidase content of the culture filtrates were determined at intervals during growth of mycelia. Addition of tunicamycine (to a final concentration 10 μg ml^{-1}) and 2-deoxyglucose (to a final concentration 50 μg ml^{-1}) to the culture medium at the beginning of the growth had little effect on the growth of the fungus. By using labelled mannose and *N*-acetyl glucosamine, it was found that at a concentration of 5–10 μg ml^{-1} *N*-glycosylation is inhibited by 70%. The use of 2-deoxyglucose inhibits *O*-glycosylation by 30–40%. However, changes in α-galactosidase content of the culture liquid were not observed either for tunicamycine or for 2-deoxyglucose.

Discussion

Mixed glycosylation resulting in the presence of both N- and O-linked oligosaccharide chains, established for α-galactosidase from *T. reesei*, is typical of glycoproteins from fungi and yeast [10, 11]. Besides the usual type of O-linked glycans (Table 3), α-galactosidase from *T. reesei* includes one more type of O-glycosyl bond, α-Gal-O-Ser/Thr, previously found in worm collagen [29]. O-linked glycans in glycoproteins from fungi and yeast are represented by short oligosaccharide chains composed of no more than four α-monosaccharide units. Only mannose-containing short O-linked glycans are characteristic of O-glycoproteins from yeast [30]. O-linked glycans in glycoproteins

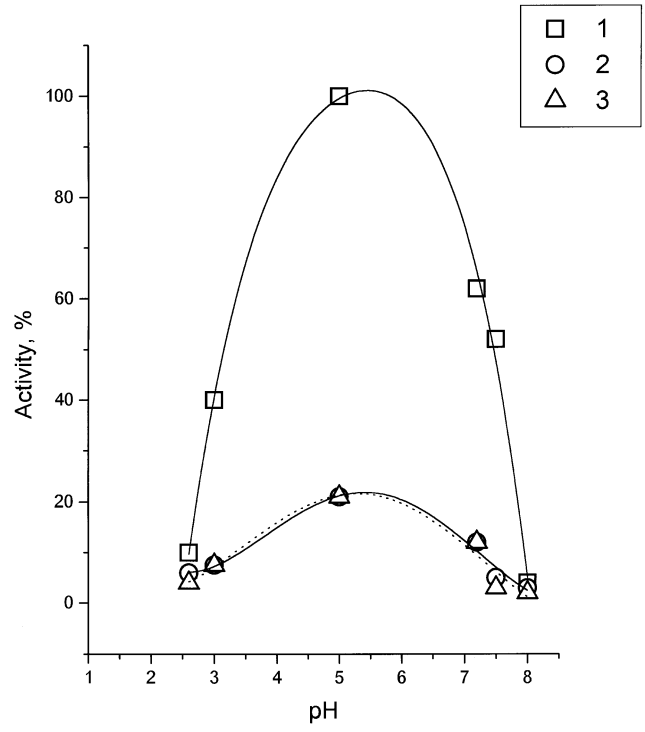

Figure 5. pH-stability of native and deglycosylated *α*-galactosidase. The pH stability of the native and deglycosylated proteins was determined by incubation in 50 mM of phosphate-citrate (pH 2.2–4.0), acetate (pH 4.0–5.6), or phosphate (pH 6.0–8.0) buffers containing 0.1 M NaCl at 37 °C for 24 h followed by enzymatic assay. 1, native *α*-galactosidase; 2, *α*-galactosidase treated by endo F; 3, *α*-galactosidase treated by endo F and exoglycosidases.

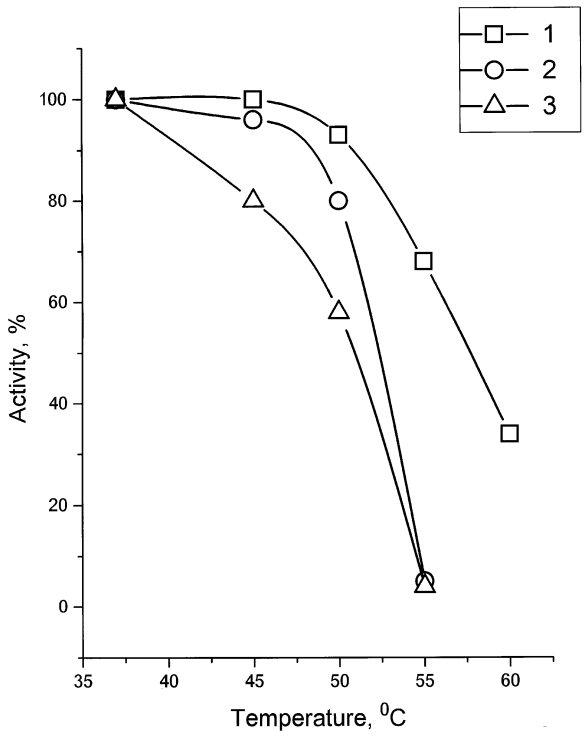

Figure 6. Thermal stability of native and deglycosylated *α*-galactosidase. Measurements of activity were performed after incubation of *α*-galactosidase at a given temperature for 10 min in a sodium phosphate buffer, pH 6.8.

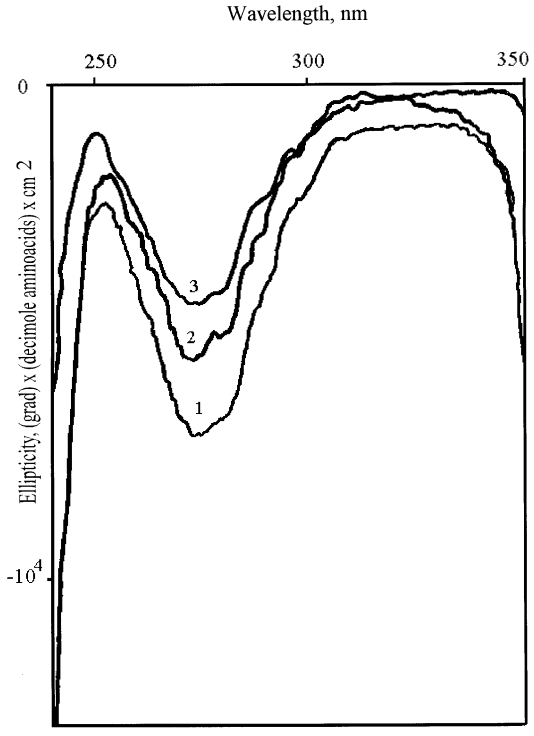

Figure 7. Molecular ellipticity Θ of native and deglycosylated *α*-galactosidase. 1, native *α*-galactosidase; 2, *α*-galactosidase treated by endo F; 3, *α*-galactosidase treated by endo F and exoglycosidases.

from fungi have a somewhat different structure. For example, O-linked glycans in glucosamylases from *A. awamori* [10] and *A. niger* [27] also include, along with O-mannosyl and O-dimannosyl residues, di- and trisaccharides containing terminal glucose and galactose. In cellobiohydrolase from *T. reesei*, an O-glucose has been found to be directly linked to the polypeptide chain [9].

N-Linked oligosaccharides of glycoproteins from low eucryotes belong, as a rule, to high-mannose N-linked chains. All of them are composed of different numbers of mannosyl residues in α-configuration, added to Man-*β*-1-4-$(GlcNAc)_2$. The N-linked glycans of the α-galactosidase under consideration belong to this type of N-linked glycans. Note that the only exception yet known is *β*-galactosidase from *A. oryzae* whose N-linked glycans contain glucose and galactose along with mannose [31].

Hydrolysis and subsequent pyridylamination of N-linked glycans is a convenient method of their release from the protein and derivatization, making possible reliable detection of N-linked carbohydrate chains in nM concentrations. Alkaline *β*-elimination, used as a method for releasing O-linked glycans from the polypeptide chain hinders the introduction of any fluorescent label therein. Hydrazinolysis has been used for obtaining unreduced O-linked glycans and releasing *N*-acetyl galactosamin-containing O-linked glycans [32]. Hydrazinolysis of short O-linked glycans, used by us for the first time as a method for studying their structure, shows that nearly quantitative cleavage of short O-linked carbohydrates from the protein molecule is possible. This makes the use of hydrazinolysis for analysing short O-linked glycans of glycoproteins from fungi and yeast fairly promising.

The phenomenon of microheterogeneity of the carbohydrate component of glycoproteins has been reported for a vast majority of glycoproteins from many sources [14]. Previously, we have found microheterogeneity in O-linked carbohydrate chains of glycoproteins from *A. awamori*, resulting from trimming (partial deglycosylation by exoglycosidases) and differentiation of the transferase activity during culture growth [33]. In the case of α-galactosidase from *T. reesei*, the presence of at least two glycoforms of the enzyme cannot be related to postsecretory or intercellular trimming. The addition of a glycosidase inhibitor (1-deoxymannonojirimycin or 1-deoxynojirimycin) has no effect on the formation of glycoforms. The intracellular α-galactosidase form obtained by dissecting the mycelium is also represented by two glycoforms according to chromatographic data. Note that for a *T. reesei* culture grown for a long time the number of glycoforms of α-galactosidase that could be separated by chromatography on a TSK SP-5W column increased to three to four. The biological significance of the existence of at least two glycoforms of α-galactosidase remains unclear and requires additional

investigation. Data in Table 4 contain the noninteger number of O-linked glycans of one or another form that confirms the existence of microheterogeneity in the two chromatographically separable glycoforms.

For the α-galactosidase under study, both O- and N-linked glycans play an important part in the formation of the protein structure, being necessary for the maintenance of its nativity. Even the partial deglycosylation of a protein molecule causes a reduction in its pH and thermal stability, whereas profound deglycosylation leads to irreversible denaturation. Such an effect on *O*- and *N*-glycosylation on the structure and functions of glycoproteins has been reported for several fungal glycoproteins: glucoamylase from *A. awamori* [10], acid carboxypeptidase from *A. saitoe* [13], and glucoamylase from *A. niger* [11]. However, cleavage of 30% of the glycans from the *A. niger* glucose oxidase has no effect on its stability and enzymatic properties [12]. Note that complete deglycosylation of a protein molecule by enzymatic methods is hampered by conformational hindrances in the native spatial structure. This effect exists in glycoproteins of this glycosylation type [34].

The relationship between glycosylation and secretion of α-galactosidase was studied *in vivo*, with tunicamycin used as inhibitor of *N*-glycosylation [35, 36], and with 2-deoxyglucose as a nonspecific inhibitor of *O*-glycosylation. The mechanism of inhibition by 2-deoxyglucose is associated with glucose-6-phosphate/mannose-6-phosphate interconversion [37]. As indicated in Results, decreasing the *N*- or *O*-glycosylation level does not inhibit secretion. Note that data on the effect of glycosylation on secretion reported by several authors for lower eucaryotic cells are controversial and variable for each particular protein [38–40]. Therefore, the relationship between the types of glycosylation and secretion is specific for each particular case. This problem will be studied in detail for secretory enzymes from *Trichoderma*..

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