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α-Mannosidase from *Trichoderma reesei* Participates in the Postsecretory Deglycosylation of Glycoproteins

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The 160 kDa α-mannosidase (E.C. 3.2.1.24) isolated from culture filtrate of *Trichoderma reesei* has wide aglycon specificity but cleaves the $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 3$ mannosidic bonds with higher rate than $\alpha 1 \rightarrow 6$ bond and slowly hydrolyses yeast mannan and 1,6-α-mannan. The specific activity of the enzyme and rate constant in the reaction with p-nitrophenyl-α-D-mannopyranoside were 0.15 U/mg and 1.62×10^{-4} μM/min/μg, respectively, at optimal pH 6.5. We have found that *in vitro* enzyme is able to cleave off 30% of total α-mannopyranosyl residues from *N*- and *O*-linked glycans of secreted glycoproteins. The activity of the α-mannosidase toward glycoproteins *in vivo* was studied comparing the structures of *O*- and *N*-linked glycans of glycoproteins isolated from the cultures growing with and without 1-deoxymannojirimycin, an inhibitor of α-mannosidases. Difference in structures of these glycans may be explained by postsecretory deglycosylation catalysed by the α-mannosidase. © 1998 Academic Press

α-Mannosidases (α-D-mannoside mannohydrolase, E.C. 3.2.1.24) were found in all organisms from bacteria to human (1-9). Some α-mannosidases have a high specificity towards terminal mannosidic residues of glycans (10,11) and participate in mannose trimming reactions (endoplasmatic reticulum mannosidase, Golgi mannosidases IA, IB and IC). A deficiency of α-mannosidase in degradatory system of higher eucariotic cells causes the lethal decease, the mannosidosis (12-14).

1,2-α-Mannosidases which cleave side chains of yeast mannan were isolated from *Bacillus* sp. (15), *Aspergillus orizae* (16), *Aspergillus saitoi* (17). Secreted 1,2-1,6-α-mannosidase from *Cellulomonas* sp. (18) and 1,2-1,3-α-mannosidase from *Arthrobacter* (19) were found to hydrolyse α-mannan. The distinction of the *Trichoderma* α-mannosidase is that it hydrolyses the yeast mannan at very low rate.

Another role played by α-mannosidases is the processing of secreted glycoproteins like those from *A. awamori* (20). The most enzymes of *T. reesei* are mannose-containing glycoproteins (21, 22) and may be affected by own mannosidase after secretion. The present work has deal with the properties of the α-mannosidase from *T. reesei* and with its possible effect on the secreted fungal glycoproteins.

MATERIALS AND METHODS

Chemicals. p-Nitrophenyl α-D-mannopyranoside (PNPM), α-mannosidase from jack bean, yeast mannan, 1-deoxymannojirimycin (1-DM), hydrazinemethyl 2-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (α-D-Man-(1 → 2)-α-D-Man-1 → OMe); 3-*O*-α-D-mannopyranosyl-D-mannopyranose (α-D-Man-(1 → 3)-D-Man); methyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (α-D-Man-(1 → 6)-α-D-Man-1 → OMe); $NaBH_3CN$, methyl α-D-mannopyranoside (Man*p* → OMe), p-nitrophenyl-α/β-D-glycosides, bovine serum albumin (BSA) were purchased from Sigma, swainsonine was from Boehringer Mannheim. Novozym 234 was from Nova-Nordisk. (^{14}C)mannose (0.05 mCi/mM) was from Izotov. methyl 3,6-di-*O*-α-D-mannopyranosyl-D-mannopyranose (α-D-Manp-(1 → 3)-, α-D-Man-(1 → 6)α-D-Man-1 → OMe) was kindly provided by Dr. L. Bakinovsky, Institute of Organic Chemistry, Moscow. p-Nitrophenyl 2-*O*-α-D-mannopyranosyl-D-mannopyranose (Np-Man*p*-Man*p*) was prepared by transglycosylation of Np-Man*p* with α-mannosidase from jack bean as described (23) and by the following reversed phase HPLC on a Octadecyl Si100 column (Serva) using H_2O as initial buffer and acetonitrile as eluent

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Abbreviations used: PNPM: p-nitrophenyl α-D-mannopyranoside; Np-Man*p*-Man*p*: p-nitrophenyl 2-*O*-α-D-mannopyranosyl-D-mannopyranose; Man*p* → OMe: methyl α-D-mannopyranoside; 1-DM: 1-deoxymannojirimycin; PHMB: p-hydroxymercuribenzoic acid sodium salt; α-D-Man-(1 → 2)-α-D-Man-1 → OMe: methyl 2-*O*-α-D-mannopyranosyl-α-D-mannopyranoside; α-D-Man-(1 → 3)-D-Man: 3-*O*-α-D-mannopyranosyl-D-mannopyranose; α-D-Man-(1 → 6)-α-D-Man-1 → OMe: methyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside; α-D-Manp-(1 → 3)-, α-D-Man-(1 → 6)α-D-Man-1 → OMe: methyl 3,6-di-*O*-α-D-mannopyranosyl-D-mannopyranose; AMC: 7-amino-4-methylcoumarin.

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in the gradient-elution mode (0-90%). 1,6-α-Mannan was obtained from yeast mannan by treatment with α1,2-1,3-mannosidase from *Oerskovia* as described (2).

Microorganism growth. For isolation of α-mannosidase, the culture of *T. reesei* was grown for 72 h at 30°C in the culture flasks with shaking (150 rpm) in a medium containing 0.2% $\hat{E}H_2PO_4$, 0.15% $NaNO_3$, 0.15% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 1% peptone, 0.5% yeast extract, 0.1% mannose.

To obtain fraction of glycoproteins containing (^{14}C)-labelled mannose, the culture was grown under same conditions for 48 h, then mycelium was collected by filtration and inoculated into the same medium containing 8 μCi/ml of (^{14}C)-mannose (24). After cultivation for 8 h, the culture filtrate was used for isolation of total protein fraction. For the fungus growth with 1-DM, the mycelium filtrated from the 48 h culture was inoculated into the same medium containing 20 μg/ml of 1-DM, then the culture was grown for 16 h, and the culture filtrate was used for isolation of a total protein fraction.

For a control of the culture growth, the mass of mycelium was determined by weighting after washing and drying.

For a control of the α-mannosidase secretion in the presence of 1-DM, the sample of the culture filtrate was desalted with a Sephadex G-50 column, and the α-mannosidase activity was measured.

Protoplasts. Cells from 50 ml of *T. reesei* culture were centrifuged (3000g, 40 min), washed twice by 50 mM Tris/HCl, pH 7.2, and then incubated with Novozym 234 (5 mg/ml) in 15 ml of the same buffer with 0.7 M KCl for 2 h at 37°C . Obtained protoplasts were washed twice by 40 ml of the same buffer, centrifuged (2000g, 65 min) and destroyed by adding the same buffer without KCl. Activity of α-mannosidase was measured in all fractions during this procedure.

Analytical methods. The protein quantity was measured by the Lowry procedure with BSA as standard (25). SDS/PAGE was carried out by the Laemmli method (26). Radioactivity was measured using liquid scintillation counter BETAMAN 1206 (LKB). Products of mannooligosaccharides hydrolysis witn α-mannosidase were analysed as alditol acetates by GLC/MS (Incos-50 mass spectrometer, Finnigan MAT) (27) on a DB-5 column with a temperature gradient of 180-250°C (1.5°C/min) and, alternatively, by TLC on Kieselgel 60 plates (Merck) in butanol:ethanol:water (4:2:1).

Enzyme assays. α-Mannosidase activity toward PNPM was measured in 50 mM Tris/HCl buffer, pH 7.2 as described (28). In the hydrolysis of yeast mannan, mannooligosacharides and mannose containing glycoproteins, releasing reducing sugar was detyermined by method of Somogy-Nelson (29). Alternatively, quantuties of the liberated mannose were measured by GLC-MS as corresponding alditol acetate. One unit of the α-mannosidase activity was defined as the amount of the enzyme that hydrolysed 1 μM of PNPM per min at 37°C, pH 7.2. The inhibition constants were determined varying the concentration of inhibitor in the reaction mixture (Dixon method) (30). The K_m and k_{cat} values were found by the method of initial rates with the Lineweaver-Burk linearization. Activities of other glycosidases were determined using appropriate PNP-glycosides as described (31).

Protein purification. The micellium from 10 L of the culture was discharged after centrifugation (3000g, 40 min) and supernatant was concentrated 30-fold and transferred to 50 mM Tris/HCl buffer, pH 7.2 (buffer A) using hollow fibers. The resulting mixture was loaded on a DEAE-TOYPEARL 650M column (2 cm $\times$ 20 cm) equilibrated with buffer A, and proteins were eluted with a linear gradient (0 – 0.5 M) of NaCl in buffer A. Fractions with α-mannosidase activity were collected, concentrated to 6 ml on Amicon PM30 membrane, and loaded on a Sephacryl S-300 column (2 cm $\times$ 140 cm) equilibrated with buffer A and eluted with the same buffer. α-Mannosidase fraction obtained was loaded on a Mono Q HR5/5 column (Pharmacia) equilibrated with buffer A, and protein was eluted with a linear gradient of 0 – 0.5 M of NaCl in buffer A. The purified enzyme solution was saturated with glycerol (50% v/v) and stored at –20°C.

To obtain fraction of the (^{14}C) -mannose labelled glycoproteins, 100 ml of culture filtrate was desalted by gel-filtration on a Sephadex G-50 column equilibrated with buffer A, then loaded on a DEAE TOYPEARL 650M column (0.5 cm $\times$ 5 cm) equilibrated with the same buffer, and eluted with 1 M NaCl in buffer A. The fraction was dialyzed against water, lyophilized and used as a substrate for the α-mannosidase. The same method was used to purify the glycoprotein fraction from cultures grown with and without 1-DM. 2mM PHMB was added in all buffers to inhibit the α-mannosidase during purification. A fraction of intracellular glycoproteins was isolated from the protoplasts prepared from 100 ml of fungal culture. The resulting liquid was centrifuged (3000 g, 40 min) and precipitate was discharged. Supernatant was dialized twice against 2 L of the 2 mM PHMB solution in buffer A. The fraction obtained was loaded on a DEAE-Toyopearl 650M column (23 cm $\times$ 2.5 cm) equilibrated with buffer A and eluted by 1 M NaCl in the same buffer. The protein fraction obtained was dialized against water, liophilized, and used for analysis of *O*- and *N*-linked glycans.

Pronase digestion. (^{14}C)-Mannose labelled glycoproteins (500 μg) were treated by 20 μg of Pronase E (E.C. 3.4.24.31) in 20 mM sodium phosphate buffer, pH 7.7 at 37°C for 54 h. Reaction was terminated by boiling and the pronase digest was treated with the α-mannosidase.

Activity of the α-mannosidase toward glycans of glycoproteins. α-Mannosidase activity toward the (^{14}C)-mannose labelled glycoproteins was measured in 20 mM sodium phosphate buffer, pH 6.7 at 31°C. Samples of the reaction mixture after definite time intervals were acidified to pH 2 with 5 M HCl and passed through a Dowex-50 (H^+ form) column. Eluted fraction of (^{14}C)-mannose was dried, and radioactivity was measured. The enzymatic activity was defined as the ratio of radioactivity liberated during reaction to total radioactivity in the sample. To estimate the activities toward *O*- and *N*-linked glycans separately, the reaction was terminated by heating (100°C, 5 min), then the samples were dialized against water, and *O*-linked glycans were liberated by β-elimination as described (27). The samples were applied on a Dowex-50 (H^+ form) column and fraction of liberated *O*-linked glycans was eluted with water. The level of *O*-deglycosylation was determined as a decreasing of radioactivity in the liberated *O*-linked glycans of the glycoproteins treated by the α-mannosidase. The glycoproteins containig *N*-linked glycans were eluted from Dowex-50 with 2 M HCl. The level of *N*-deglycosylation was determined in the same manner as for the *O*-linked sugar chains.

Analysis of O-linked glycans. Five mg of the glycoprotein fraction, prepared as described above, was suspended in 200 ml of anhydrous hydrazine and kept under argon in a sealed ampule at 60°C for 6 h, then hydrazine was evaporated at reduced pressure. Qualitative analysis of the released glycans was performed by TLC on Kieselgel 60 plates (Merck) in the system ethanol:butanol:water (2:2:1).

Derivatization of the *O*-linked sugar chains into 4-methylcoumarin-glycamines (AMC-sugars) was carried out as described (32). AMC-sugars obtained were analysed by HPLC on a Lichrosorb TSK-NH_2 column in 80% acetonitrile/ 20% 50 mM sodium acetate buffer, pH 4.1, v/v with detection at 360 nm.

Analysis of N-linked glycans. Derivatization of the *N*-linked sugar chains into 4-methylcoumarin glycamines (AMC-sugars) was carried out as for *O*-linked glycans. The analysis of these AMC-sugars was carried out by HPLC on a Lichrosphere ODS column (Merck) with a linear gradient (0 to 90%) of acetonitrile in H_2O. The retentions for AMC-maltooligosaccharides of various lengths were preliminary determined (32).

RESULTS

Two α-mannosidase forms were isolated from the culture liquid of *T. reesei* (Table 1): the major form with

TABLE 1

Purification of α-Mannosidase from *T. reesei* Enzyme Activity toward PNPM and Protein Concentrations Were Determined as Described in the Materials and Methods Section

Purification step	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	% of total initial activity
Crude extract						
	10000	1880				
major form			11.0	0.0056	1.0	89.4
minor form[a]			1.3	0.0007	1.0	10.6
Ultrafiltration						
	380	990				
major form			7.8	0.0079	1.4	62.6
minor form[a]			0.9	0.0009	1.4	7.42
DEAE TOYOPEARL						
	40	121				
major form			4.8	0.040	7.1	38.4
minor form[a]			0.6	0.005	7.1	4.6
Sephacryl S-300						
major form	47	36.0	3.30	0.09	16.1	26.8
minor form	45	12.6	0.39	0.03	42.9	3.2
MonoQ						
major form	8.5	7.40	1.12	0.15	27.9	8.9
minor form	3.0	0.34	0.11	0.32	457.1	0.1

[a] Calculated from the distribution found after Sephacryl S300 separation.

the specific activity 0.15 U/mg and the minor form with the specific activity 0.32 U/mg. The activity of the major form was estimated to be 90% of the total α-mannosidase activity after cultivation for 72 h. The estimation of molecular mass by SDS PAGE yield 80 ± 5 kDa for the major form and 95 ± 5 kDa for the minor form. Comparison of molecular weights according to analytical gel filtration and SDS PAGE showed that the major form consists of two identical 80 kDa subunits and the minor form of two identical 95 kDa subunits. No other enzymes active toward PNPM, α-mannan and mannooligosacharides were found in culture liquid during purification.

The major form of the α-mannosidase was chosen for determination of enzymatic properties and the action on secreted glycoproteins. Purification of the major form included concentration and desalting on the hollow fibers, ion-exchange chromatography on a DEAE-TOYOPEARL column and gel-filtration on a Sephacryl S-300 column. FPLC on Mono Q column was the final step of purification. The protein inactivated by lyophilization, but it was stable for at least 1 year when kept in 50% glycerol solution at −20°C. Purified α-mannosidase contained less than 0.2 % of admixtured carbohydrate activities that was tested by incubation with the following substrates: p-nitrophenyl α/β-D-glucopyranosides, p-nitrophenyl α/β-D-galactopyranosides, p-nitrophenyl β-mannopyranoside, p-nitrophenyl α/β-D-xylopyranosides, p-nitrophenyl α-L-arabinopyranoside, p-nitrophenyl N-acetyl-α/β-D-galactosaminides, p-nitrophenyl β-D-cellobioside. The α-mannosidase is rather a secreted enzyme. The ratio of the activities in the culture liquid, in the cell walls and inside cells found after destroying of protoplasts was 1:1.2:0.5.

The α-mannosidase pH optimum is closed to 6.5 with half-maximal activity at pH 5.3 and 8.1, the protein is stable in pH range of 5.0 − 7.8. EDTA does not affect the enzymatic activity while Ca^{2+} slightly activates enzyme. The value of binding constant $K_{Ca}^{2+} = 0.168$ mM was found analysing plot of k_{cat} versus (Ca^{2+}). Mercury compounds (PHMB and Hg^{2+}) at comcentrations of 2 mM inactivate enzyme but the activity may be recovered by consequent addition of cystamine or β-mercaptoethanol.

The GLC/MS analysis shown that the α-mannosidase cleaves only mannose residues from yeast mannan, 1,6-α-mannan, Np-Man*p*-Man*p* and from carbohydrate moiety of glycoproteins. Table 2 shows the kinetic parameters of the PNPM hydrolysis. The substrate specificity of the enzyme was studied using mannooligosaccharides and their derivatives (Table 3). The enzyme was shown to cleave the α1 → 2 and α1 → 3 mannosidic bonds with higher velocity than the α1 → 6

TABLE 2

Kinetic Parameters of Reactions Catalyzed by the α-Mannosidase

Substrate	K_m	$k_{cat}, \left(\frac{\mu M}{min \times \mu g}\right) \times 10^7$
PNPM	0.13 mM	1.62×10^3
yeast mannan	8.7 mg/ml	2.3
1,6-α-mannan	9.3 mg/ml	3.1

TABLE 3

Specificity of the α-Mannosidase in Hydrolysis of Mannooligosaccharides

Substrate	Mannose released, $\left(\frac{\mu M}{min \times ml \times U}\right) \times 10^{-10}$
Man*p* → OMe	4.3
α-D-Man(α1 → 2)-D-Man	4.9
α-D-Man(α1 → 6)-α-DMan-1 → OMe	0.3
α-D-Man(1 → 3)-D-Man	2.0
α-D-Manp-(1 → 3)-,α-D-Man-(1 → 6)α-D-Man-1 → OMe	2.5

bond. The α-mannosidase has a wide aglycon specificity cleaving oligomannosaccharides, PNPM and Man*p* → OMe. The reaction is competitively inhibited by the product, D-mannose (K_I = 31 mM). 1-DM also competitively inhibits the enzyme with K_I = 0.2 mM.

Activity of the α-mannosidase toward the mannose containing secretory glycoproteins of *T.reesei* was studied *in vitro*. The fraction of glycoproteins was isolated from the culture filtrate of the fungi after 8 h growth with (^{14}C) -mannose to minimize the influence of the α-mannosidase. The fraction was then purified and treated with the α-mannosidase at the ratio of the α-mannosidase to total protein (0.0075 U per mg of glycoproteins) which was achieved after 48 h of the culture growth. About 30% of the total (^{14}C)-mannose was released by α-mannosidase after the 8 h incubation at these conditions. It was not possible to increase the quantity of the mannose released either by increasing the reaction time or the enzyme quantity. The quantity of the mannose released increased when the glycoproteins were treated preliminary by Pronase E (Fig. 1). The pH optimum of activity toward glycoproteins coinsides with those for activity toward PNPM. The enzyme activity toward glycans decreases to about 30% of maximum in the acid medium during the culture growth *in vivo*.

The process of the *in vitro* mannose liberating separately from *N*- and *O*-linked glycans is shown in Fig. 2. These data suggest that preliminary *N*-deglycosylation promotes more complete *O*-deglycosylation.

The activity of α-mannosidase toward glycoproteins *in vivo* was studied comparing the structures of *O*- and *N*-linked glycans of glycoproteins isolated from the cultures growing with and without 1-DM. It was found firstly that 1-DM does not affect the α-mannosidase se-

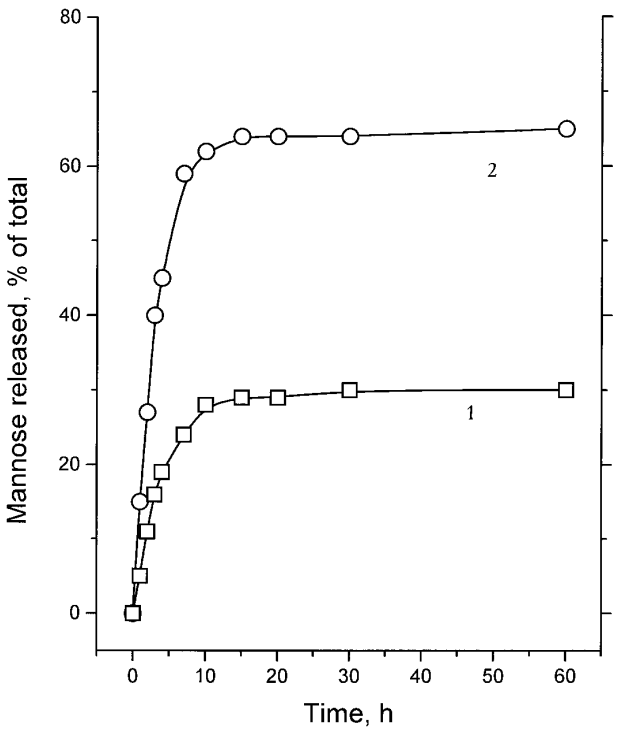

FIG. 1. Releasing of mannose by α-mannosidase: 1 - from the native fungal glycoproteins; 2 - from the glycoproteins treated by Pronase E; Incubation was carried out in 10 mM potassium phosphate buffer, pH 6.7, 37°C, 7.5 × 10^{-3} U of α-mannosidase per mg of glycoproteins. Aliquotes of reaction mixture were seized after definite time intervals and quantity of mannose was estimated by measurements of radioactivity as described in Materials and methods section.

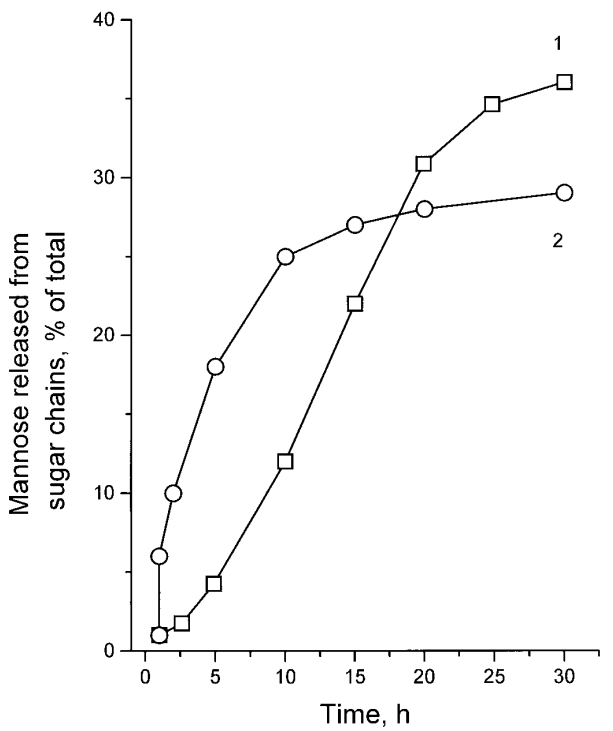

FIG. 2. Releasing of mannose by α-mannosidase: 1 - from the *O*-linked glycans of fungal glycoproteins; 2 - from the *N*-linked glycans of fungal glycoproteins Incubation was carried out in 10 mM potassium phosphate buffer, pH 6.7, 37°C, 8.0 × 10^{-3} U of the α-mannosidase per mg of glycoproteins. Quantity of mannose released was estimated by measurements of radioactivity as described in Materials and methods section.

cretion and the culture growth at the concentration less than 250 μg/ml. When 1-DM was added, the structures of *O*- and *N*-linked glycans in the glycoprotein fraction were changed. The ratio of disaccharides to monosaccharides increased for *O*-linked glycans in glycoproteins obtained from the culture grown with 1-DM (Fig.3). The similar effect was observed for *N*-linked glycans. A quantity of long sugar chains increased that was judged from an increase in the retention time during reverse phase HPLC of the AMC-derivatives (Fig. 4).

The intracellular glycoproteins were isolated from protoplasts, O- and *N*-linked glycans were separated in the form of AMC-sugars as described above. Comparison of *O*- and *N*-linked glycans of the intracellular glycoproteins of the fungi grown with and without 1-DM reveals no difference in the sugar chain length. This fact suggests that the α-mannosidase affects only the glycoproteins secreted.

DISCUSSION

α-Mannosidase from *T.reesei* differs from other bacterial and fungal mannosidases mainly regarding activity toward the yeast mannan. The α-mannosidases from *A. saitoi* (17), *A. oryzae* (16) and *Penicillum citrinum* (3) are active toward native α-mannan. Bacterial α-mannosidases hydrolyzing Np-Man*p* also hydrolyze $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ bonds in yeast mannan (18). α-Mannosidase from *T. reesei* cleaves $\alpha 1 \rightarrow 2$, $\alpha 1 \rightarrow 3$, and $\alpha 1 \rightarrow 6$ mannosidic bonds in various manno-oligosaccharides (Table 3), but its activity towards α-mannan is incredibly low (Table 2). 1,2-α-Mannosidase from *A. niger* was reported to reveal the similar behavior, being active toward $\alpha 1 \rightarrow 2$-mannooligosacharides but not toward yeast mannan (33). Thus, in contrast with other secretory bacterial and fungal mannosidases, the *Trichoderma* α-mannosidase can not be used for the degradation of the yeast cell walls.

The minor α-mannosidase found in the culture filtrate of *T. reesei* has similar general properties. It hydrolyzes yeast mannan with k_{CAT} of the same as the major form, and PNPM with a k_{cat} of 0.9×10^{-7} μM/min/μg . This isoenzyme was shown to be also inhibited by PHMB and Hg^{2+}. Both α-mannosidase isoforms have similar pH-profiles of activity. Multiplicity of α-mannosidases in yeast was explained previously as a result of proteolysis of subunits (34). Two isoforms of 1,2-α-mannosidase having similar kinetic parameters were isolated from *Penicillum citrinum* (3).

The α-mannosidases involved in trimming of glycans was shown to be, as a rule, highly specific. The enzyme from *S. cerevisiae* removes mannose from $Man_9GlcNAc$ providing a single isomer of $Man_8GlcNAc$ (4). The processing α-mannosidase from calf liver cleaves three of

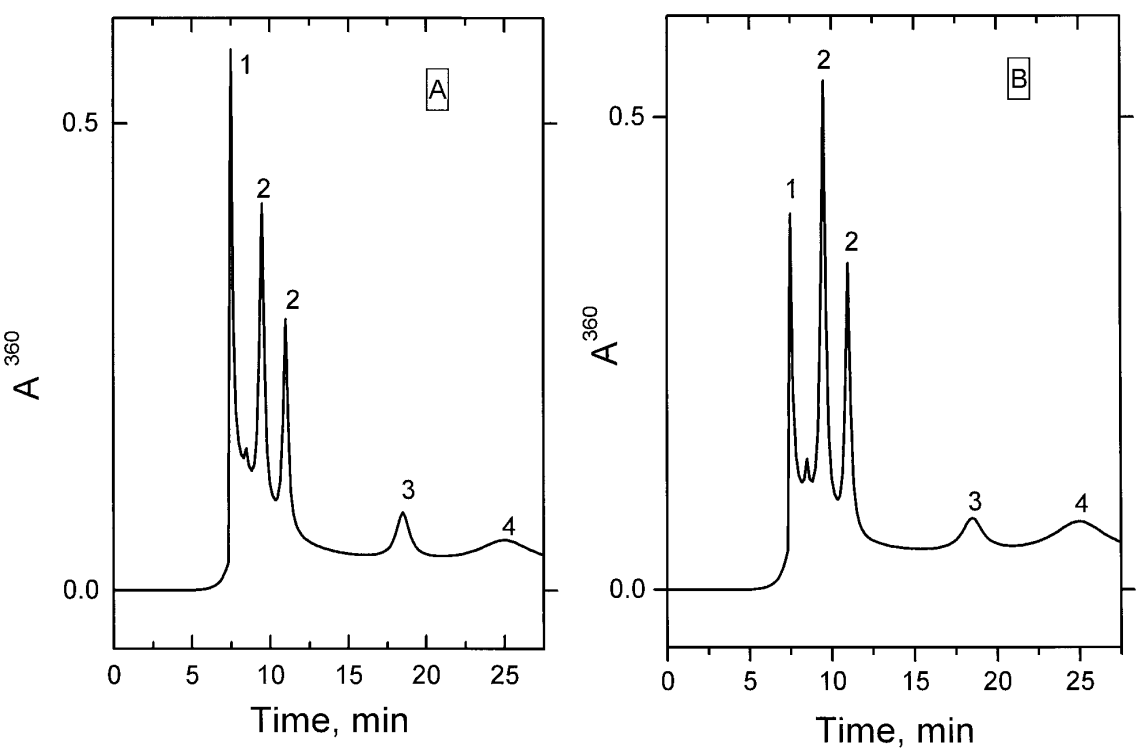

FIG. 3. Separation of AMC-derivatives of *O*-linked glycans isolated from cultures grown without 1-DM (A) and with 1-DM (B) by HPLC on the Lichrosorb TSK-NH_2 column. Peaks were identified by TLC on a Kieselgel 60 plate in butanol:acetic acid:water (3:2:1) as: 1- AMC-monosaccharides; 2- AMC-disaccharides; 3- AMC-trisaccharides, 4- AMC-tetrasaccharides.

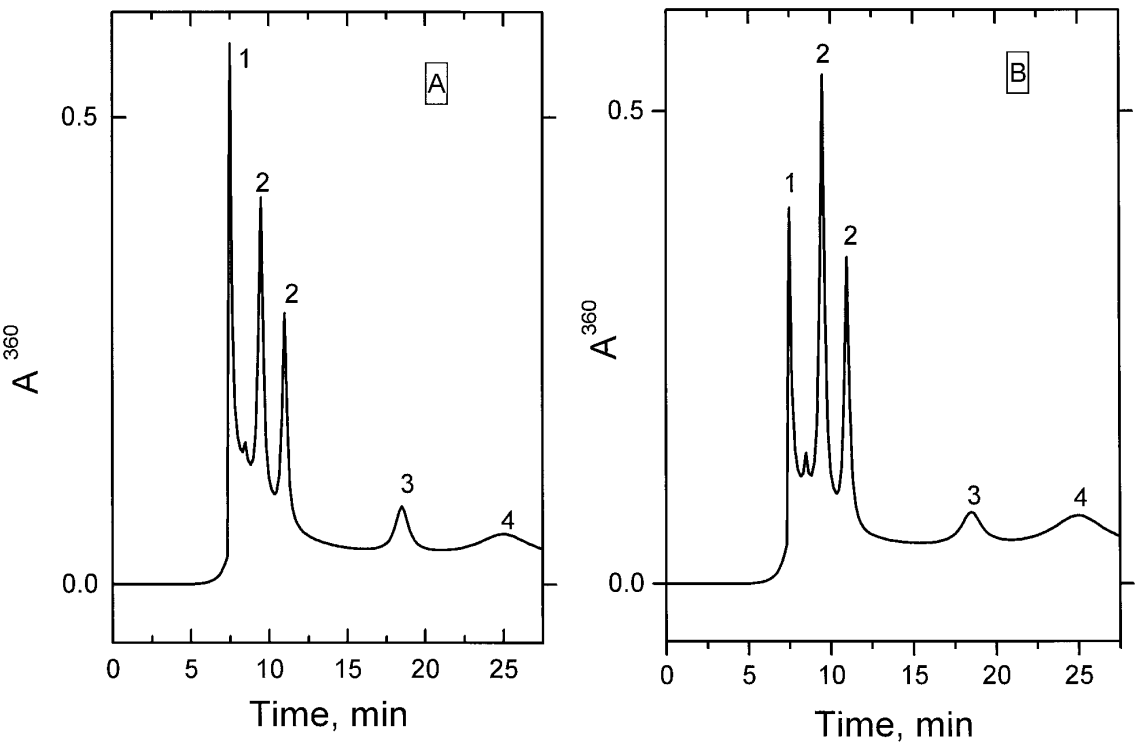

FIG. 4. Separation of AMC-derivatives of *N*-linked glycans isolated from cultures grown without 1-DM (A) and with 1-DM (B) on the Lichrosphere ODS column (250 × 4 mm) with a gradient (0 to 90%) of acetonitrile in water, detection at 360 nM.

the four $\alpha 1 \rightarrow 2$ linked mannose residues from $Man_9(GlcNAc)_2$ (10). The endo-α-mannosidase from rat liver involved in processing of *N*-linked glycans also revealed high specificity (35). In contrast, the enzyme from *T. reesei* cleaved off about 30% of the total mannose from *O*- and *N*-linked glycans of glycoproteins secreted (Fig. 1). Such a high level of deglycosylation may be explained by a broad specificity. We suggest that the enzyme is able to remove the $\alpha 1 \rightarrow 2$-, $\alpha 1 \rightarrow 3$- and $\alpha 1 \rightarrow 6$ linked mannose residues from *N*- and *O*-linked glycans of the secreted glycoproteins. The level of *O*- and *N*-deglycosylation increases to 70% after a preliminary proteolitic treatment of the total fraction of glycoproteins. These facts suggest that a level of deglycosylation is determined by steric factors rather than enzyme specificity. The α-mannosidase considered is similar by its mode of action to the broad specificity 1,2-1,3-1,6-α-mannosidase from rat liver (36) and to the rat kidney cytosolic α-mannosidases involved in mannosidosis (37).

1-DM inhibits deglycosylation of glycoproteins in the culture liquid (Fig. 3,4) but does not affect the carbohydrate structures of intracellular glycoproteins. Therefore, we may suppose that α-mannosidase considered catalyzes particularly postsecretory deglycosylation.

The similar process was reported for the removal of sialic acid from CHO-produced glycoproteins in batch culture where levels of desialidation depended only on the quantity of the sialidase activity in the culture. α-Fucosidase and β-galactosidase activities have also been characterized in CHO sell supernatants. One suggested that these enzymes participate in the partial degradation of glycoproteins secreted (38).

The biological role of the postscretory α-mannosidase deglycosylation is still unclear. We reported previously that multiple forms of glucoamylase from fungus *A.awamori* arise under action of the fungal protease (39). Such multiplicity is a basis for the correct functioning of the fungal amylolitic complex (40). Cleavage of the terminal mannose residues from *O*-linked glycans of glucoamylase by α-mannosidase makes limited proteolysis possible. An addition in a growing culture of 1-DM prevents the deglycosylation and the following proteolysis and causes the appearance of single glucoamylase isoform (27). The limited proteolysis was suggested to cause appearance of isoforms of cellobiohydrolase from *T. reesei* (41). The partial deglycosylation with the α-mannosidase may influence on these processes. Another possibility is an involving of the α-mannosidase in the utilization of mannose-rich glycoproteins as a source of amino acids for a fungal growth.

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