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A model for cleavage of *O*-glycosidic bonds in glycoproteins

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The present work investigated the possibility of cleavage of α-linkages between mannose or galactose and serine/threonine residues by α-mannosidase and α-galactosidase. The study was carried out initially with model synthetic compounds imitating the *O*-glycosidic bond in glycoproteins, and further with glucoamylase. It was shown that α-mannosidase and α-galactosidase can hydrolyse these linkages after proteolytic digestion of glucosamylase.

Keywords: *O*-glycoprotein, glucoamylase, α-mannosidase, α-galactosidase

Introduction

One type of *O*-glycosidic bond in glycoproteins is α-mannosidic or α-galactosidic linkages by which oligosaccharides are attached to serine or threonine residues. These linkages are found in yeast mannans [1], worm collagens [2, 3], cell wall proteins of *Cryptococcus laurentii* [4] and a number of enzymes secreted by *Aspergillos niger* [5], *Asp. oryzae* [6] and *Penicillium melinii* [7]. Unlike the *N*-glycoproteins, such *O*-glycoproteins have not been reported thus far as having any endoglucanases capable of hydrolysing the mannosidic or galactosidic linkages to serine or threonine. The present work examines this possibility on glucosamylase from *Asp. awamori* × 100/D27, an *O*-glycoprotein, carrying about 40 short carbohydrate chains with mannose linked to serine or threonine [8]. However, such a large number of sugars (as compared with that on *N*-glycoproteins) and their dense distribution on the polypeptide chain [9] hamper investigation directly on the protein. In particular, preparation of short mannose peptides linked either to serine or threonine is extremely difficult. Therefore, the study was carried out initially with the following model compounds synthesized so that their structure would correspond closely to that of glucoamylase: *N*-*t*-butyloxycarbonyl-*O*-α-D-mannopyranosyl-L-serylglycine-*N*′-methylamide (α-Man-Ser-Pep) and *N*-t-butyloxycarbonyl-*O*-α-D-mannopyranosyl-L-threonylglycine-*N*′-methylamide (α-Man-Thr-Pep). By using these models, we determined and compared kinetic data for the hydrolysis of Man-Ser and Man-Thr linkages.

Materials and methods

Materials

Mannose and galactose were purchased from Serva (Germany), [^{14}C]mannose (0.05 mCi mmol^{-1}) and [^{14}C]-galactose (0.04 mCi mmol^{-1}) from Izotop (Russia).

Enzymes

α-Mannosidase from *Canavalia* (jack bean) (Sigma, USA) was used in the study after additional purification as follows. Commercial α-mannosidase was dialysed against 0.02M sodium acetate buffer containing 0.1% by vol β-mercaptoethanol and 0.1 mM $ZnCl_2$ for 12 h and then subjected to chromatography on a Mono S FPLS column (Pharmacia-LKB, FPLC system, Sweden) using 0.02M sodium acetate, pH 4.1, as starting buffer and 0.5M NaCl as eluent. Fractions carrying the main α-mannosidase activity were collected and transferred in a buffer composed of 2.5M $(NH_4)_2SO_4$, 0.1M sodium acetate, 1 mM $ZnCl_2$ and 0.1% by vol β-mercaptoethanol, to be used for further experiments.

α-Mannosidase from ginseng was purified as described in [10], α-galactosidase from *Asp. awamori* × 100/D27 as described in [11], β-mannosidase and α-glucosidase from the same source were prepared as described in [12], glucoamylase, also from the same source, by the method in [13]. Pronase E, proteinase K, and trypsin (Sigma, USA)

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were used in the study. Glycoamylase with metabolically radiolabelled sugars was prepared according to the procedure in [8].

Assay of enzymatic activities of exoglycosidases

Routinely, a glycopeptide containing radioactively labelled sugar was incubated with a 0.001–0.005 U of respective glycosidase activity at 37 °C in 1.2 ml buffer. Incubation of α-mannosidase was done in 0.02M sodium acetate buffer containing 0.1 mM $ZnCl_2$ and 0.1% by vol β-mercaptoethanol, at pH 4.5; α-galactosidase and β-mannosidase were incubated in 0.02M sodium acetate buffer, at pH 4.5 and 3.5, respectively. At different times, samples (100 µl) were collected and digestions were stopped by heating to 100 °C for 2 min. Then the exoglucosidase-released sugars were separated from glycopeptide by HPLC on a Lichrosorb RP 8 (Pharmacia-LKB) column (4 mm × 250 mm, 10 µm). Resolution was achieved using gradient elution with H_2O as a starting buffer and acetonitrile as eluent, at a flow rate of 0.5 ml min^{-1}. The 1 ml fractions were collected and monitored by scintillation counting. As an alternative procedure, free sugar liberated through hydrolysis was measured by the method in [14].

Proteolytic digestion of glucoamylase

Proteolytic treatment was carried out using native glucoamylase. The protein (1–1.5 mg) was diluted in 0.8 ml 0.02M Tris-HCl buffer, pH 7.5, and exposed to digestion by protease for 20 h at 30 °C. The amount of a respective protease was 0.5% that of glucoamylase. The reaction was stopped by heating in a boiling water bath for 10 min. After the hydrolysate was cooled, the pH was adjusted to 4.3–4.4 by adding 200 µl of 1.2M sodium acetate, pH 4.3. The sample (without additional purification) was then treated by exoglycosidases. The peptides obtained from proteolytic digests were analysed by GPLC on a Sephadex G-50 Super Fine column (0.5 cm × 100 cm) in 0.1M NH_4HCO_3 to determine their molecular weights. As standards, peptide mol. wr kits from Serva (Germany) were used. The rate of proteolytic hydrolysis of glucoamylase was calculated from integrated areas of gel filtration chromatographic peaks.

Denaturation of glucoamylase

Denaturation of glucoamylase was carried out by the following procedure. 2 mg protein and 1 mg dithiothreitol were dissolved in 0.3 ml 0.1M Tris-HCl buffer, pH 7.6, containing 5 mM EDTA and 7M guanidine-HCl. After 3 h incubation, 100 mg iodoacetic acid were added and after 1 h the reaction was stopped by adding an excess of β-mercaptoethanol. The protein was then dialysed against water.

Enzyme deglycosylation of glucoamylase

Proteolytic hydrolysate of glucoamylase metabolically radiolabelled with [^{14}C]mannose and [^{3}H]galactose was exposed to digestion by exoglycosidases. Briefly, to 1.5 mg of the sample were added 1.5 U α-galactosidase and 1.5 U α-galactosidase activities at 31 °C. Then the sample, pre-treated with both glycosidases, was supplemented with 5 U of α-mannosidase activity at 31 °C.

The extent of deglycosylation upon treatment with α-mannosidase and α-galactosidase was determined by the ratio of radioactivity liberated through hydrolysis to total radioactivity in the protein. The measurement was done as follows: after several incubations of varied duration, samples of the reaction mixture were collected, acidified to pH 2 with 5M HCl and then chromatographed on a Dowex-50 (H^+ form) column. Radioactivity was determined by scintillation counting. Deglycosylation by α-glucosidase was evaluated by measuring the amount of liberated glucose using the glucose oxidase method [15]. Deglycosylation-induced changes in the number of *O*-sugars were determined as outlined in [16].

Synthesis of glycopeptides

NMR spectra were recorded on a Bruker AC-200 spectrometer (Germany). Optical rotations were determined with a Perkin-Elmer 241 MC polarimeter, for compounds I, II, VII, VIII, IX, X in H_2O and for compounds III, IV, V, VI in $CHCl_3$, at 25 °C.

Dichloromethane was distilled over P_2O_5. Methanol (Aldrich) was used without additional purification. Triethylamine was distilled over KOH. Column chromatography was performed on silica gel L 100/160 (Chemapol, Czechoslovakia), TLC was carried out on precoated plates of silica gel 60 (Merck, Germany) using the following eluents: toluene:acetone, 1:2 by vol (A); 2:1 by vol (B); 4:1 by vol (C); acetonitrile:water, 9:1 by vol (D); 1-butanol: acetic acid:water, 3:1:1 by vol (E).

N-*t-Butyloxycarbonyl-*L*-serylglycine-*N′*-methylamide* (*I*). To the solution of glycine-*N*′-methylamide hydrochloride (0.66 g, 5.5 mmol) and triethylamine (0.76 g, 5.6 mmol) in dimethylformamide (10 ml) was added dropwise a solution of *N*-t-butyloxycarbonyl-L-serine pentafluorophenyl ether (1.9 g, 5 mmol) in acetonitrile (15 ml) at 10 °C. After stirring at room temperature for 2 h, the mixture was diluted with ethyl acetate (90 ml). The precipitate of triethylamine hydrochloride was filtered, the solvent was removed by distillation under reduced pressure (below 40 °C) and the oil obtained was purified by column chromatography using eluent A. Fractions containing the final product were combined and evaporated to a volume of 100 ml. The precipitate obtained after overnight storage at room temperature was collected and dried; yield 0.87 g (63%). $[\alpha]_D$ −7.1°. TLC R_F 0.51 (D); R_F 0.21 (A). ^{1}H NMR (^2H_2O): δ 1.45 (s, 9H, $3CH_3$); 2.75 (s, 3H, CH_3); 3.86 (d, 3H, βCH_2-Ser, $J_{\alpha,\beta}$ = 4.9 Hz); 3.93 (s, 2H, αCH_2-Gly); 4.18 (t, 1H, αCH-Ser, $J_{\alpha,\beta}$ = 4.9 Hz).

N-*t*-*Butyloxycarbonyl*-L-*threonylglycine*-N′-*methylamide* (*II*). This compound was synthesized and purified by the procedure described for compound I. The yield was 40%, $[\alpha]_D$ −18.5°. TLC R_F 0.55 (D), R_F 0.23 (A). ^{1}H NMR (DMSO-d_6) δ: 1.06 (d, 3H, CH_3-Thr); 1.41 (s, 9H, $3CH_3$); 2.58 (d, 3H, NCH_3); 3.67 (t, 2H, αCH_2-Gly); 3.90 (m, 2H, α,βCH-Thr); 6.48 (d, 1H, NH-Thr), 7.65 (q, 1H, NHMe); 8.16 (t, 1H, NH-Gly).

N-*t*-*Butyloxycarbonyl*-O-(*2,3,4,6-tetra*-O-*acetyl*-α-D-*manno*-*pyranosyl*)-L-*serylglycine*-N′-*methylamide* (*III*). To a mixture of compound I (138 mg, 0.50 mmol), silver triflate (141 mg, 0.55 mmol), tetramethylurea (0.66 µl, 0.55 mmol) and molecular sieves (3 Å, 1 g) in dichloromethane (3 ml) was added dropwise with stirring 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide [17] (235 mg, 0.55 mmol) in dichloromethane (1 ml). The mixture was kept in darkness at room temperature for 48 h. The molecular sieves were filtered off and the filtrate, diluted with dichloromethane, was successively washed with water, sodium hydrogen carbonate solution and water, then dried (sodium sulfate), and evaporated to an oil which was purified by column chromatography using eluent (B). The product-containing fractions were collected, combined and evaporated. The resultant clear oil was dried *in vacuo*. Yield 11 mg (36%), $[\alpha]_D$ +15.4°, R_F 0.45 (A).

N-*t*-*Butyloxycarbonyl*-O-(*2,3,4,6-tetra*-O-*acetyl*-α-D-*manno*-*pyranosyl*)-L*threonylglycine*-N′-*methylamide* (*IV*). This was synthesized and purified by the same procedure described for compound III. Yield 30%, $[\alpha]_D$ +8.8°, TLC R_F 0.5 (A).

N-*t*-*Butyloxycarbonyl*-O-(*4,6-di*-O-*acetyl*-*2,3*-O-*carbonyl*-β-D-*mannopyranosyl*)-L-*serylglycine*-N′-*methylamide* (*V*). This was synthesized exactly as compound III from 4,6-di-*O*-acetyl-2,3-*O*-carbonyl-α-D-mannopyranosyl bromide [18]. The reaction mixture was stored overnight at room temperature and the product was isolated and purified as for compound III. Yield 61% $[\alpha]_D$ +9.1°, TLC R_F 0.27 (A). ^{1}H NMR (DMSO-d_6): δ 1.41 (s, 9H, $3CH_3$); 2.02 (s, 3H, CH_3-Ac); 2.09 (s, 3H, CH_3-Ac); 2.58 (d, 3H, NCH_3); 3.66 (g, 2H, CH_2-Gly); 4.13 (m, 1H, αCH-Ser); 5.25 (d, 1H, H-1, $J_{1.2}$ = 3.0 Hz); 7.25 (d, 1H, NH-Ser); 7.71 (m, 1h, NHMe); 8.24 (t, 1H, NH-Gly).

N-*t*-*Butyloxycarbonyl*-O-*2,3,4,6-tetra*-O-*benzyl*-α-D-*galacto*-*pyranosyl*)-L-*seryl*-*glycine*-N′-*methylamide* (*VI*). This was synthesized in one step by *in situ* activation of ethyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-galactopyranoside with bromine in the presence of peptide as the glycosyl acceptor by the method reported in [19]. A solution of ethyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-galactopyranoside (105 mg; 0.18 mmol), peptide I (42 mg; 0.15 mmol) and tetramethylurea (36 µl, 0.36 mmol) in dichloromethane (3 ml) was stirred for 2 h in the presence of molecular sieves (3 Å, 0.5 g) at room temperature. The bromine (8.5 µl, 0.15 mmol) was added dropwise and stirring continued for 20 min. More tetramethylurea (18 µl, 0.18 mmol) and silver triflate (115 mg, 0.45 mmol) were then added and the reaction mixture was kept in the dark at room temperature for 5 h. The molecular sieves and silver bromide were filtered off, the filtrate diluted with chloroform was washed with cold sodium hydrogen carbonate solution and water, dried and evaporated to an oil which was then purified by column chromatography using eluent C: yield 77 mg (54%), $[\alpha]_D$ +26.1°, R_F 0.66 (B).

Compounds III, IV, V were deacetylated with a 5% solution of triethylamine in methanol at room temperature overnight. The solvent was removed by evaporation and purified by HPLC on a Whatman Partosil 10 ODS3 C-8 column with a gradient (0–100%) of acetonitrile in water. Fractions containing the final product were lyophilized.

Methylamide N-*t*-*butyloxycarbonyl*-O-α-D-*mannopyranosyl*-L-*serylglycine*-N′-*methylamide* (*VII*). This was prepared from compound III; yield 93%, $[\alpha]_D$ +12.8°, R_F 0.5 (E). ^{1}H NMR (DMSO-d_6) δ: 1.48 (s, 9H, $3CH_3$): 2.58 (d, 3H, NCH_3); 4.14 (m, 1H, αCH-Ser); 4.72 (d, 1H, $J_{1.2}$ = 3.5 Hz, H-1); 7.12 (d, 1H, NH-Ser); 7.60 (q, 1H, NHMe); 8.27 (t, 1HgG, NH-Gly). The only ^{13}C NMR signal of relevance was 102.017 m.g., $J_{C_1H_1}$ = 170.6 Hz in the anomeric carbon resonances area, corresponding to the α-anomer [20, 21].

N-*t*-*Butyloxycarbonyl*-O-α-D-*mannopyranosyl*-L-*threonyl*-*glycine*-N′-*methylamide* (*VIII*). This was obtained compound IV and gave a yield of 87%. $[\alpha]_D$ +5.6°, TLC R_F 0.52 (E).

N-*t*-*Butyloxycarbonyl*-O-β-D-*mannopyranosyl*-L-*serylglycine*-N′-*methylamide* (*IX*). This was obtained from compound V, with a yield of 87%, $[\alpha]_D$ +7.5°, R_F 0.5 (E). The ^{1}H NMR (DMSO-d_6) spectrum was identical to that of compound VII, except for the signal with parameters 4.69 (d, 1H, $J_{1.2}$ = 2.8 Hz, H-1). In the ^{13}C NMR spectrum, the only relevant signal was 101.055 m.g., $J_{C_1H_1}$ = 162 Hz present in the anomeric carbon resonance area, which can be assigned to the β-anomer [20, 21].

Methylamide N-*t*-*butyloxycarbonyl*-O-α-D-*galactopyranosyl*-L-*serylglycine*-N′-*methylamide* (*X*). To a 1% solution of tetra-*O*-benzyl-α-D-galactopyranoside VI in ethanol was added an equal amount of palladium (10%) on activated charcoal and the compound was hydrogenolysed in a hydrogen atmosphere at a pressure of 4.5 bar at room temperature overnight. The catalyst was removed by centrifugation, the solution was evaporated, and the residue was purified by HPLC as above. $[\alpha]_D$ +22.5°. ^{1}H NMR (DMSO-d_6) δ: 1.40 (s, 9H, $3CH_3$); 2.58 (d, 3H, NCH_3); 3.67 (d, 2H, CH_2-Gly); 4.12 (m, 1H, αCH_2-Ser); 4.68 (d, 1H, $J_{1.2}$ = 2.3 Hz, H-1); 7.09 (d, 1H, NH-Ser); 7.58 (q, 1H, NH-Me); 8.23 (t, 1H, NH-Gly).

Results and discussion

Enzymatic hydrolysis of Man-Ser/Thr linkages was examined initially on the synthetic compounds α-Man-Ser-Pep and α-Man-Thr-Pep that structurally resemble *O*-glycoproteins with this type of bond.

Glycosylation of mannose-containing glycoproteins was carried out using respective acylglycosyl bromides in the presence of silver triflate and 1,1,3,3-tetramethylurea. Another condensing agent, HgCN, lowered the yield by 40% compared with silver triflate.

The model compounds appeared to be the substrates for the already characterized α-mannosidases from jack bean and ginseng. The Linweaver–Burk plots for the hydrolysis of α-Man-Ser-Pep and α-Man-Thr-Pep by α-mannosidase from *Canavalia* are shown in Fig. 1 and K_M and V_{max} values for the above α-mannosidases are summarized in Table 1. As can be seen, the kinetic properties of the two

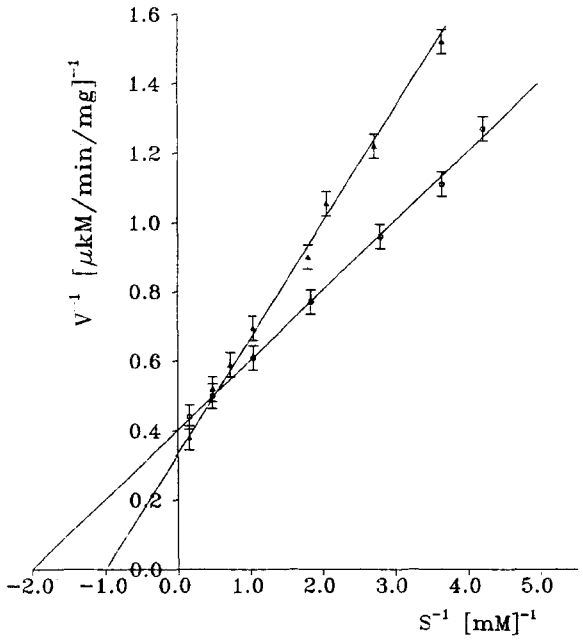

Figure 1. Lineweaver–Burk plot of Jack bean α-mannosidase for ○, α-Man-Ser-Pep; and △, α-Man-Thr-Pep.

Table 1. Kinetic properties of carbohydrases in hydrolysis of model glycopeptides.

Enzyme	*Glycopeptide*	K_M	V_{max}
		mM	*μmol min*$^{-1}$ *mg*$^{-1}$
α-Mannosidase	α-Man-Ser-Pep	0.5	2.5
from jack bean	α-Man-Thr-Pep	1	3
α-Mannosidase	α-Man-Ser-Pep	0.5	2.7
from ginseng	α-Man-Thr-Pep	0.9	2.9
α-Galactosidase	α-Gal-Ser-Pep	2	6.6
β-Mannosidase	β-Man-Ser-Pep	27	0.05

mannosidases for the above substrates are similar, but the specificity is different, notably for a number of mannobioses of different α-linkage configurations [10]. By comparison, the kinetic parameters of two similar α-mannosidases from *Asp. niger*, for the hydrolysis of mannobioses are as follows: K_M and V_{max} values of α-mannosidase for α-(1-4)-mannobiose are 8 mM and 12.3 μmol min^{-1} mg^{-1}, respectively; those of α-(1-2)-mannosidase for α-(1-2)-mannobiose are 2 mM and 1.3 μmol min^{-1} mg^{-1}, respectively [22, 23]. Interestingly, these data scarcely differ from kinetics seen for the hydrolysis of model gycopeptides. Somewhat higher V_{max} values for the α-Man-Thr peptide are probably due to additional steric interferences from the CH_3-group in threonine.

α-Mannosidase was also tested for hydrolytic activity towards *N*-t-butyloxycarbonyl-*O*-β-D-mannopyranosyl-L-serylglycine-*N'*-methylamide (β-Man-Ser-Pep). However, no such activity was found. β-Mannosidase from *Asp. awamori* × 100/D 27 also failed to cleave the α-mannosidic bond in the respective glycopeptides and the hydrolysis of β-Man-Ser-Pep proceeded at a considerably lower rate (Table 1). It is noteworthy that the presence of β-mannosidic linkages with serines or threonine on *O*-glycoproteins has not been reported.

This study was carried out with glucoamylase. In this enzyme the carbohydrates are present as 12 single D-mannose residues, 20–22 disaccharides composed of 2-*O*-D-mannopyranosyl-D-mannoses, three trisaccharides which are D-mannopyranosyl-α-(1-6)-[D-glycopyranosyl-α-(1-3)]-D-mannopyranose, and three trisaccharides which are D-mannopyranosyl-α-(1-6)-[½*d*-galactopyranosyl-α-(1-3)]-D-mannopyranose [8]. As shown in this work, treatment of the native glucoamylase with α-mannosidase results in the removal of 24–26% of total mannose, with only terminal mannose cleaved from the di- and trisaccharides. In this case, cleavage of the Man-Ser/Thr bond does not occur. A higher concentration of mannosidase or longer incubation fail to induce a higher level of deglycosylation [8]. Most likely, conformational changes induced by partial deglycosylation do not affect the Man-Ser/Thr bonds which remain resistant to cleavage. Only in the denaturated protein (see the Materials and methods section) 20–25% of sites become susceptible to hydrolysis. It should be noted that similar results were obtained with native cellobiohydrolase from *Trichoderma viride*, showing the same glycosylation type as glucoamylase. Treatment by α-mannosidase removes 4.6 of 24.6 mannosyl residues, and it is impossible to achieve a greater extent of deglycosylation [24].

Of particular interest with respect to naturally occurring processes seems to be proteolytic treatment of the native protein and subsequent complete deglycosylation of the resulting glycopeptides by exoglycosidases. We attempted to elucidate whether partial proteolytic degradation led to the complete cleavage of α-Man-Ser-Pep. For this, native glucoamylase was treated with trypsin, pronase or proteinase K. Proteolysis gave glycopeptides with masses of

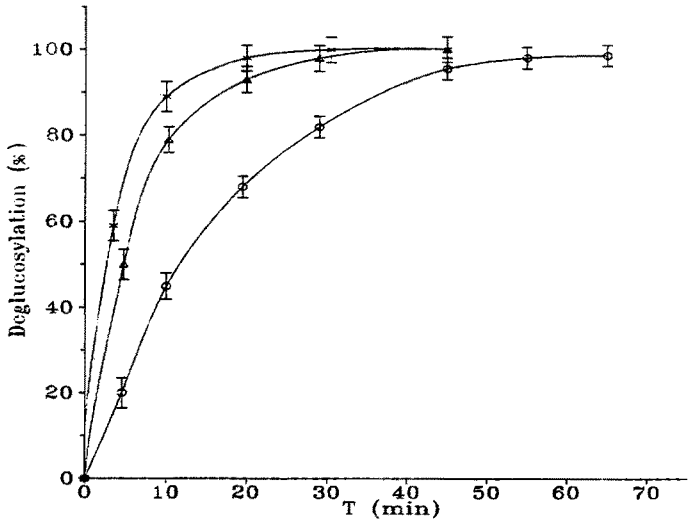

Figure 2. Rate curves for the deglycosylation of glucoamylase glycopeptides by △, α-galactosidase; ⁎, α-glucosidase; and ○, α-mannosidase.

about 12–10 kDa in the case of trypsin and not less than 8 kDa in the case of proteinase K and Pronase.

It was found that sequential digestion of glycopeptides first by α-galactosidase and α-glucosidase and then by α-mannosidase causes complete deglycosylation (Fig. 2). α-Galactosidase and α-glucosidase treatments results in the removal of the terminal glucose and galactose from trisaccharides, rendering certain residues (mannose) susceptible to sequential digestion. As expected, the rates of hydrolysis of mannose residues were almost the same for α-mannosidases from jack bean and ginseng.

Table 1 gives kinetic data for the hydrolysis of another synthetic compound studied in this work, *N*-t-butyloxycarbonyl-*O*-α-D-mannopyranosyl-L-serylglycine-*N'*-methylamide (α-Gal-Ser-Pep). From these data it is evident that α-Gal-Ser-Pep is a substrate for α-galactosidase from *Asp. awamori*. The K_M and V_{max} values for its hydrolysis are similar to those observed for the hydrolysis of α-Man-Ser-Pep. It is possible, therefore, that the same mechanism as described above for glucoamylase may be valid for the hydrolysis by α-galactosidase of Gal-Ser/Thr linkages in glycoproteins.

Overall, these results lead to a conclusion that *O*-glycoproteins after proteolytic degradation can be completely cleaved by sequential digestions by exoglycosidases which, at the final stage, remove sugars directly from a polypeptide chain. The primary removal of terminal sugars in oligosaccharides (partial deglycosylation) is possibly of importance as judged by the 8–10-fold increased proteolytic rate. It is clear, however, that for complete deglycosylation (cleavage) of *O*-linkages between mannose and serine/threonine proteolysis is an essential requirement.

The proposed mechanism is not perhaps the only possibility. For example, the work in [25] described the α-endomannosidase located in the Golgi apparatus of rabbit spleen cells which is involved in glycoprotein processing. This means that this and similar enzymes may have hydrolytic ability for *O*-mannosidic linkages. Further, a β-endoxylanase has been reported which cleaves the Xyl-Ser linkage in the corresponding *O*-glycoproteins without prior proteolytic degradation [26]. Therefore, it is possible that there may be other similar endo enzymes capable of cleaving the type of linkage examined here.

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