Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 1335-1342

AN α-MANNOSIDASE PURIFIED FROM *ASPERGILLUS SAITOI* IS SPECIFIC FOR α1,2 LINKAGES

by

Katsuko Yamashita, Eiji Ichishima*,
Masanori Arai* and Akira Kobata

*From the Department of Biochemistry, Kobe University School of Medicine, Ikuta-ku, Kobe 650 Japan and *Laboratory of Enzymology and Microbial Chemistry, Tokyo Nōkō University, Fuchu, Tokyo 183 Japan*

Received August 27,1980

SUMMARY: The substrate specificity of an α-mannosidase purified from *Aspergillus saitoi* was studied in detail. This enzyme hydrolyzes yeast mannan partially but does not act on p-nitrophenyl α-mannopyranoside. Survey of the action of the enzyme on various oligosaccharides liberated from glycoproteins indicated that the enzyme hydrolyzes Manα1→2Man linkage but not Manα1→3Man and Manα1→6 Man linkages at all. All Manα1→2 residues in intact bovine pancreatic ribonuclease B were removed completely by incubation with the α-mannosidase.

Ever since the pioneering work of Ashwell and Morell on the clearance mechanism of serum glycoproteins by liver parenchymal cells in rabbit (1), there has accumulated much evidence showing the important role of the carbohydrate moieties of glycoproteins in various cellular recognition phenomena. Some of the evidence indicated that mannosyl residues of asparagine-linked sugar chains are involved as a part of recognition signals. For instance, fibroblasts in culture recognize 6-phosphomannosyl residue of the carbohydrate moieties of various acid hydrolases and selectively endocytose them (2-6). The uptake of these lysosomal enzymes infused into the anesthesized animal was also observed (7). In this case, the uptake occurs mainly in Kupffer cells of liver and in macrophages mediated by a cell surface receptor specific for mannosyl and *N*-acetylglucosaminyl residues (8-11). For the elucidation of the mechanism of these interesting phenomena, structural study of the signal sugar chains

0006-291X/80/191335-08$01.00/0

is essential. α-Mannosidases with some restricted aglycon specificities might become invaluable tools for such studies. This paper describes about the substrate specificity of the α-mannosidase recently purified from *Aspergillus saitoi* by Ichishima et al (12).

MATERIALS AND METHODS

Chemicals and Oligosaccharides -- Manα1→3Manβ1→4GlcNAc$_{OT}$[1], Manα1→2Manα1→3Manβ1→4GlcNAc$_{OT}$ and Man$_9$·GlcNAc$_{OT}$ (for structure, see Chart I) were purified from urine of a mannosidosis patient (13). Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man$_5$·GlcNAc·GlcNAc$_{OT}$), (Manα1→2)·Man$_5$·GlcNAc·GlcNAc$_{OT}$, (Manα1→2)$_2$·Man$_5$·GlcNAc·GlcNAc$_{OT}$, and (Manα1→2)$_3$·Man$_5$·GlcNAc·GlcNAc$_{OT}$ were obtained from bovine pancreatic ribonuclease B by hydrazinolysis method (14). Man$_5$·GlcNAc$_{OT}$, Man$_6$·GlcNAc$_{OT}$ and Man$_7$·GlcNAc$_{OT}$ (for structures, see Chart I) were prepared from ovalbumin glycopeptides V, IV (15) and III (16) according to the cited references. Manα1→6Manβ1→4XylNAc$_{OT}$ was obtained by periodate oxidation of Man$_5$·GlcNAc$_{OT}$ as reported previously (13). NaB[^{3}H]$_4$ (242 mCi/mmol) was purchased from New England Nuclear, Boston, Mass., and bovine pancreatic ribonuclease B from Sigma Chemical Co. Ltd.

Enzymes -- α-Mannosidase was purified from culture filtrate of *Aspergillus saitoi* according to the method described previously (12). The enzyme gave a single band in polyacrylamide disc gel electrophoresis when stained by Coomassie blue, and essentially free from protease activity. The molecular weight of enzyme, as calculated by gel filtration mobility was 51,000.

That the enzyme preparation was free from contamination with β-galactosidase, β-*N*-acetylhexosaminidase, α-mannosidase and α-L-fucosidase was confirmed by incubating following the radioactive oligosaccharides (2 nmole) prepared according to the cited references, with 100 ng enzyme in 40 μl of 0.1 M sodium acetate buffer, pH 5.0 at 37°C for 18 h: Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAc$_{OT}$[2], GlcNAcβ1→3Galβ1→4Glc$_{OT}$ (17), Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (18), Fucα1→6GlcNAc$_{OT}$ (19), Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glc$_{OT}$ (20) and Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glc$_{OT}$ (20), respectively. None of these oligosaccharides was hydrolyzed.

Enzyme Assay -- The standard assay mixture for α-mannosidase activity contained 2 nmole (approximately 3.5 x 10^4 cpm) of radioactive oligosaccharide and either 50 ng (for the determination of substrate specificity) or 25 ng (for kinetic studies) of enzyme in 40 μl of 0.1 M sodium acetate buffer, pH 5.0. The mixture was incubated at 37°C for 18 h, and the reaction was stopped by adding 20 μl of 0.1 N NaOH. The solution was transferred to Whatman No.3MM paper and subjected to paper chromatography using ethylacetate/pyridine/acetic acid/water (5:5:1:3) as a solvent for appropriate length of time. Radioactive components on the chromatogram was located by Packard model 7210 radiochromatogram scanner, and the amount of radioactivity in each peak was determined by liquid scintillation counting.

General Methods -- Radioactivity was determined by Aloka liquid scintillation spectrometer model LSC-700. Radioactivity on paper

---

[1] Subcript OT is used in this paper to indicate NaB^{3}H$_4$ reduced oligosaccharides. All sugars mentioned in this paper are of D configuration except for fucose.

[2] Ohkura, T., Yamashita, K. and Kobata, A. m.s. in preparation.

was determined after incubating paper pieces with 1 ml of water in the counting vials and adding 7 ml of scintillation fluid. Bio-Gel P-4 (smaller than 400 mesh) column chromatography was performed by using a column (2 x 100 cm) equipped with water jacket. During operation, the column was kept at 55°C by circulating warm water in the jacket. Elution was performed with distilled water, and a mixture of glucose oligomers prepared by acid hydrolysis of dextran (19) was used as internal standards. Differential refractometer R-403 (Waters Associates Inc., Framingham, Mass.) was used for monitoring standards eluted from the column, and eluate was fractionated 3.0 ml per tube.

RESULTS

Studies with Small Oligosaccharides -- Although *Asparagillus* α-mannosidase does not act on artifitial substrates such as p-nitrophenyl α-mannopyranoside (12), it can readily convert Manα1→2Manα1→3Manβ1→4GlcNAc$_{OT}$ to Manα1→3Manβ1→4GlcNAc$_{OT}$ (Fig. 1A). On the other hand Manα1→3Manβ1→4GlcNAc$_{OT}$ and Manα1→6Manβ1→4XylNAc$_{OT}$ were completely resistant to enzyme action (Fig. 1B and C). Based on these results, the kinetic properties of *Aspergillus* α-mannosidase were studied by using Manα1→2Manα1→3Manβ1→4GlcNAc$_{OT}$ as a substrate. As shown in Fig. 2A and B, the reaction rate is proportional to protein concentration up to 25 ng of enzyme in the standard condition and is linear for at least 16 h. The activity of the enzyme as a function of pH

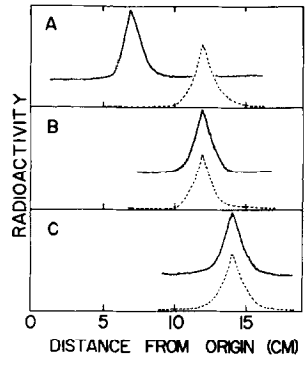


Fig. 1. Chromatographic analysis of the product of *Aspergillus* α-mannosidase digestion. The reaction mixture was applied to a sheet of Whatman No.3MM paper and developed for 24 h with ethylacetate-pyridine-acetic acid-water (5:5:1:3). The results of radiochromatogram scanning obtained before (solid line) and after (dotted line) enzymatic digestion of Manα1→2Manα1→3Manβ1→4GlcNAc$_{OT}$, Manα1→3Manβ1→4GlcNAc$_{OT}$ and Manα1→6Manβ1→4XylNAc$_{OT}$ are shown in A, B and C, respectively.

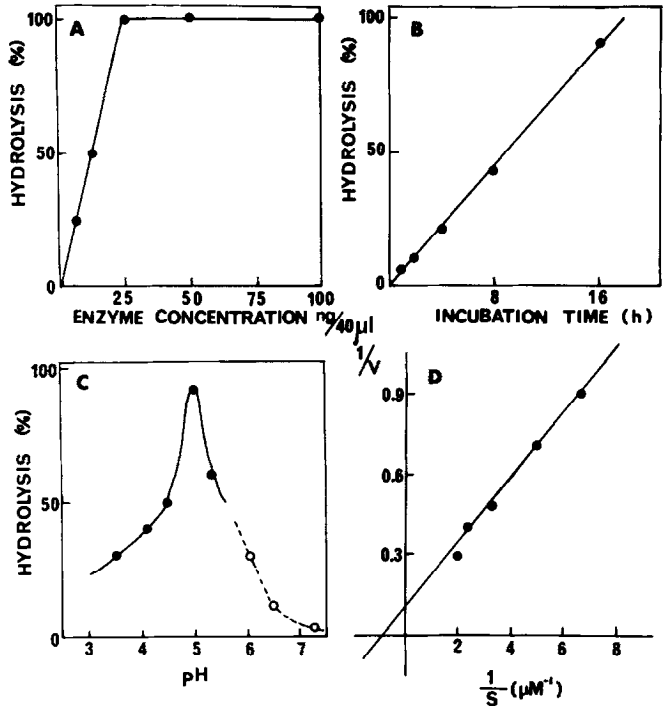


Fig. 2. Kinetic data of *Aspergillus* α-mannosidase obtained by using Manα1→2Manα1→3Manβ1→4GlcNAc$_{OT}$ as a substrate. A, effect of enzyme concentration on the reaction rate. The conditions of incubation are the same as those descirbed under "Enzyme Assay", expect that protein concentration was varied as indicated. B, effect of incubation time on the reaction rate. The conditions of incubation are the same as those described under "Enzyme Assay", expect that the time of incubation was varied as indicated. C, pH optimum of the *Aspergillus* α-mannosidase. The reactions were run as descirbed under "Enzyme Assay' except for 16 h incubation with different buffer, at 0.1 M as indicated; ●——● , sodium acetate buffer; O····O , sodium phosphate buffer. D, effect of substrate concentration on the hydrolysis rate. The conditions of incubation are the same as those described under "Enzyme Assay", except that the time of incubation was 3 h and the concentration of substrate was varied.

is shown in Fig. 2C. The optimum pH is approximately 5.0. Since the enzyme is quite heat stable, reactions were stopped by changing the pH of reaction media to alkaline, at which point enzyme activity is almost negligible. The double reciprocal plot of the substrate concentration and the reaction rate is shown in Fig. 2D. From the data, the Km and Vmax values for the tetrasaccharide were calculated as 0.8 mM and 10 µmol/min/mg enzyme, respectively.

Studies with High Mannose Type Oligosaccharides and with An Intact Glycoprotein -- The results obtained by studies with small

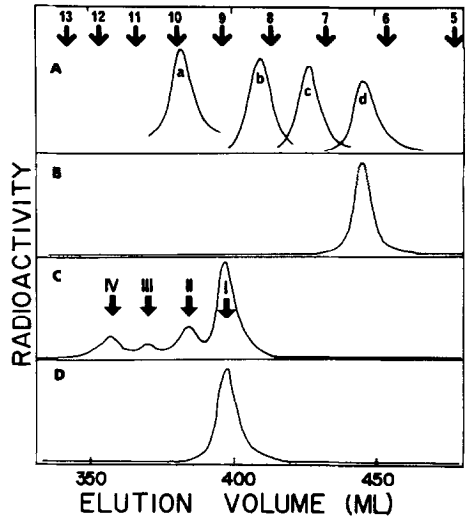
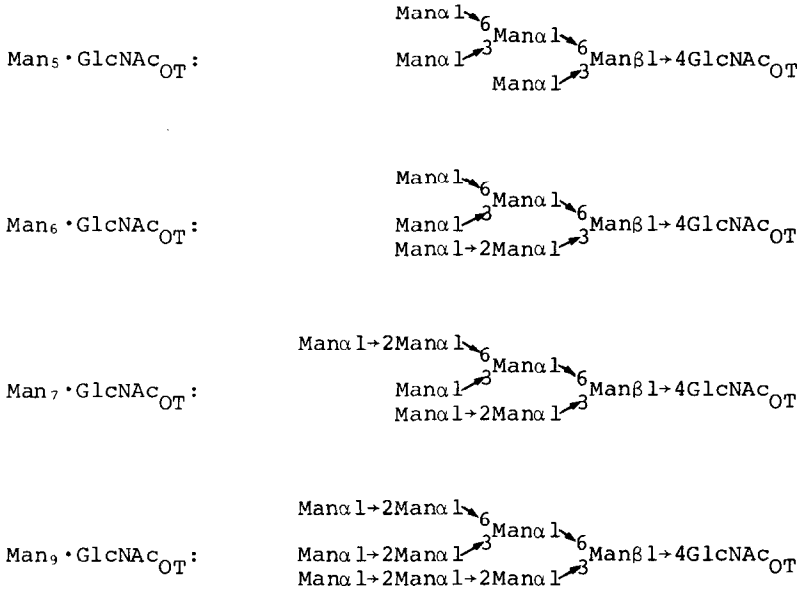


Fig. 3. Hydrolysis of high mannose type sugar chains by *Aspergillus* α-mannosidase. The radioactive sugars were subjected to Bio-Gel P-4 column chromatography, and the radioactivity in each tube (3.0 ml/tube) was determined by liquid scintillation spectrometer. Arrows at the top of the figure indicate the eluting positions of glucose oligomers (numbers indicate the glucose units), and the arrows in (C) indicate the positions of standard oligosaccharides: I, $Man_5 \cdot GlcNAc \cdot GlcNAc_{OT}$; II, $(Man\alpha1\rightarrow2) \cdot Man_5 \cdot GlcNAc \cdot GlcNAc_{OT}$; III, $(Man\alpha1\rightarrow2)_2 \cdot Man_5 \cdot GlcNAc \cdot GlcNAc_{OT}$; IV, $(Man\alpha1\rightarrow2)_3 \cdot Man_5 \cdot GlcNAc \cdot GlcNAc_{OT}$. A, elution patterns of $Man_9 \cdot GlcNAc_{OT}$ (a), $Man_7 \cdot GlcNAc_{OT}$ (b), $Man_6 \cdot GlcNAc_{OT}$ (c) and $Man_5 \cdot GlcNAc_{OT}$ (d); B, $Man_9 \cdot GlcNAc_{OT}$, $Man_7 \cdot GlcNAc_{OT}$, $Man_6 \cdot GlcNAc_{OT}$ and $Man_5 \cdot GlcNAc_{OT}$ incubated with *Aspergillus* α-mannosidase under the conditions described under "Enzyme Assay"; C, the radioactive oligosaccharide fraction obtained by hydrazinolysis of bovine ribonuclease B. The condition of hydrazinolysis is described in the previous paper (14); D, the radioactive oligosaccharide fraction obtained by hydrazinolysis of bovine ribonuclease B incubated with *Aspergillus* α-mannosidase. Five milligrams of bovine ribonuclease B was incubated with 250 ng of the α-mannosidase in 50 μl of 0.1 N sodium acetate, pH 5.0 for 18 h. The reaction mixture was dialyzed by passing through a collodion bag, and lyophilized. The residue was then subjected to hydrazinolysis. Released oligosaccharides were *N*-acetylated and converted to radioactive oligosaccharide by reduction with $NaB[^3H]_4$.

oligosaccharides suggested that *Aspergillus* α-mannosidase hydrolyzes only Manα1→2Man linkage. For further confirmation of this specificity, action of the enzyme on a series of high mannose type oligosaccharides was investigated. As summarized in Fig. 3A and B, $Man_5 \cdot GlcNAc_{OT}$, which does not contain any Manα1→2Man linkage, was completely resistant to the enzyme action. In contrast, $Man_6 \cdot GlcNAc_{OT}$, $Man_7 \cdot GlcNAc_{OT}$ and $Man_9 \cdot GlcNAc_{OT}$ were all converted to $Man_5 \cdot GlcNAc_{OT}$. This result indicated that all Manα1→2Man linkages in the three

Chart I. The structures of high mannose type oligosaccharides used as substrates.



oligosaccharides (for structures, see Chart I) were removed by the enzyme action.

In order to find out if the enzyme can remove Manα1→2 residue from intact glycoprotein, the oligosaccharide patterns of bovine ribonuclease B before and after incubation with *Aspergillus* α-mannosidase were studied comparatively. As reported in the previous paper (14), bovine pancreatic ribonuclease B contains four different asparagine-linked sugar chains: $Man_5 \cdot GlcNAc \cdot GlcNAc$, $(Man\alpha 1\rightarrow 2) \cdot Man_5 \cdot GlcNAc \cdot GlcNAc$, $(Man\alpha 1\rightarrow 2)_2 \cdot Man_5 \cdot GlcNAc \cdot GlcNAc$ and $(Man\alpha 1\rightarrow 2)_3 \cdot Man_5 \cdot GlcNAc \cdot GlcNAc$, in which $Man_5 \cdot GlcNAc \cdot GlcNAc$ represents $Man\alpha 1\rightarrow 6(Man\alpha 1\rightarrow 3)Man\alpha 1\rightarrow 6(Man\alpha 1\rightarrow 3)Man\beta 1\rightarrow 4GlcNAc\beta 1\rightarrow 4GlcNAc$. These four oligosaccharides were qualitatively released from the ribonuclease by hydrazinolysis and separated on Bio-Gel P-4 column (Fig. 3C). When the ribonuclease was incubated first with *Aspergillus* α-mannosidase and then subjected to the same treatment after dialysis, only a single oligosaccharide

component corresponding to $Man_5 \cdot GlcNAc \cdot GlcNAc_{OT}$ was obtained (Fig. 3D). These results indicated that *Aspergillus* α-mannosidase can remove all Manα1→2 residues from intact bovine ribonuclease B.

DISCUSSION

Swaminathan et al (21) reported that an α-mannosidase fraction from *Aspergillus niger* shows relatively high specificity for Manα1→2Man linkage, although it can cleave Manα1→3Man and Manα1→6Man linkages at the rate 1/100 of that of Manα1→2Man linkage. The enzyme from *Aspergillus saitoi* seems to be more highly specific for Manα1→2Man linkage, and can be used as a reliable reagent to detect Manα1→2Man linkage in the sugar chain and to remove only Manα1→2 residue from sugar chains. Since the high mannose type sugar chains of mammalian glycoproteins have such structures as $(Man\alpha1\rightarrow2)_{0\sim4} \cdot Man_5 \cdot GlcNAc \cdot GlcNAc$, the enzyme can be used as an useful tool for their identification. The heat stability of the enzyme is worthy to note because α-mannosidases are generally very heat labile. Swaminathan et al reported that the activity of enzyme from *Aspergillus niger* sharply declines at temperature higher than 45°C (21). The *Aspergillus* enzyme retained 81% of its activity after heating at 60°C for 10 minutes. The stability of the α-mannosidase from *Aspergillus saitoi* is one of useful characteristic, because prolonged incubation might be necessary in study of some intact glycoproteins. Since the enzyme can act on intact glycoprotein as well as oligosaccharides, it should be a valuable tool to elucidate the physiological role of sugar chains in glycoproteins.

ACKNOWLEDGEMENT - The authors wish to thank to Miss J. Fujii for her expert secretarial assistance. This work has been supported in part by research grants from the Scientific Research Funds (1979-1980) of the Ministry of Education, Science and Culture of Japan and of the Cancer Division, Public Health Bureau, the Ministry of Health and Welfare, Japan.

REFERENCES

1. Ashwell, G., and Morell, A. G. (1974) Adv. Enzymol., 41, 99-128
2. Kaplan, A., Achord, D. T., and Sly, W. S. (1977) Proc. Natl. Acad. Sci. U.S., 74, 2026-2030
3. Sando, G. N., and Neufeld, E. F. (1977) Cell, 12, 619-627
4. Kaplan, A., Fischer, D., and Sly, W. S. (1978) J. Biol. Chem., 253, 647-650
5. Natowicz, M. R., Chi, M. M. Y., Lowry, O. H., and Sly, W. S. (1979) Proc. Natl. Acad. Sci. U.S., 76, 4322-4326
6. Hasilik, A., and Neufeld, E. F. (1980) J. Biol. Chem., 255, 4946-4950
7. Stahl, P., Siz, H., Rodman, J. S., Schlesinger, P., Tulsiani, D. R. P., and Touster, O. (1976) Proc. Natl. Acad. Sci. U.S., 73, 4045-4049
8. Achord, D., Brot, F., and Sly. W. S. (1977) Biochem. Biophys. Res. Commun., 77, 409-415
9. Stahl, P. D., Rodman, J. S., Miller, M. J., and Schlesinger, P. H. (1978) Proc. Natl. Acad. Sci. U.S., 75, 1399-1403
10. Kawasaki, T., Etoh, P., and Yamashina, I. (1978) Biochem. Biophys. Res. Commun., 81, 1018-1024
11. Schlesinger, P. H., Doebber, T. W., Mandell, B. F., White, R., DeSchryver, C., Rodman, J. S., Miller, M. J., and Stahl, P. (1978) Biochem. J., 176, 103-109
12. Ichishima, E., Arai, M., and Kumagai, H. (1980) Seikagaku, 52, in press
13. Yamashita, K., Tachibana, Y., Mihara, K., Okada, S., Yabuuchi, H., and Kobata, A. (1980) J. Biol. Chem., 255, 5126-5133
14. Liang, C. -J., Yamashita, K., and Kobata, A. (1980) J. Biochem. (Tokyo), 88, 51-58
15. Tai, T., Yamashita, K., Ogata, A. -M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y., and Kobata, A. (1975) J. Biol. Chem., 250, 8569-8575
16. Tai, T., Yamashita, K., Ito, S., and Kobata, A. (1977) J. Biol. Chem., 252, 6687-6694
17. Kobata, A., and Ginsburg, V. (1972) J. Biol. Chem., 247, 1525-1529
18. Liang, C. -J., Yamashita, K., Muellenberg, C. G., Shichi, H., and Kobata, A. (1979) J. Biol. Chem., 254, 6414-6418
19. Nishigaki, M., Yamashita, K., Matsuda, I., Arashima, S., and Kobata, A. (1978) J. Biochem. (Tokyo), 84, 823-834.
20. Nishigaki, M., Muramatsu, T., Kobata, A., and Maeyama, K. (1974) J. Biochem. (Tokyo), 75, 509-517
21. Swaminathan, N., Matta, K. L., and Bahl, O. P. (1972) J. Biol. Chem., 247, 1775-1779