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Substrate Specificities of α-*N*-Acetylgalactosaminidases I and II from Squid Liver

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The substrate specificities of the squid liver α-*N*-acetylgalactosaminidases I and II were studied with natural compounds containing α-*N*-acetylgalactosaminyl or other glycosyl terminals as substrates. Both α-*N*-acetylgalactosaminidases I and II hydrolyzed terminal α-*N*-acetyl-galactosaminyl linkages of the natural compounds investigated; asialo bovine submaxillary mucin, Forssman glycolipid, human ovarian cyst A-glycoprotein and blood group A-type ghosts. On the other hand, the oligosaccharides containing α-galactosyl terminals, ceramide trihexoside and human ovarian cyst B-glycoprotein, were hydrolyzed by α-*N*-acetylgalactosaminidase I but not by α-*N*-acetylgalactosaminidase II. The milk oligosaccharides with other glycosyl terminals were not hydrolyzed by either enzyme. Application of α-*N*-acetylgalactosaminidase from squid liver together with other glycosidases was effective in structural studies of Forssman glycolipid.

Keywords——squid liver; α-*N*-acetylgalactosaminidase; asialo bovine submaxillary mucin; Forssman glycolipid; blood group A-active substance; α-*N*-acetylgalactosaminidase substrate specificity

α-*N*-Acetylgalactosaminyl moieties are known to be the common constituents of various glycoconjugates including Forssman glycolipid, blood group A-active substances and bovine submaxillary mucin. For investigation of the structures and functions of these glycoconjugates, it is convenient to use α-*N*-acetylgalactosaminidase which catalyzes the hydrolysis of α-*N*-acetylgalactosaminyl terminals. In particular, the enzyme is of value for this purpose if it has a broad aglycone specificity toward natural substances and is sufficiently free from contamination with other glycosidases.[1] Previously we have highly purified two α-*N*-acetylgalactosaminidases (I and II) from squid liver.[2] Some glycosidases show low activities toward natural substrates in spite of high activity toward artificial substrates. Moreover, the contaminant enzymes in those glycosidase preparations sometimes show high activities toward natural substrates. Therefore, it is very important to know their specificities for natural substrates. In the present study, we describe the substrate specificities of the two α-*N*-acetylgalactosaminidases from squid liver toward the natural substances containing α-*N*-acetylgalactosaminyl terminals and those containing other glycosyl terminals. We also describe the application of α-*N*-acetylgalactosaminidase I for structural analysis of Forssman hapten glycolipid.

Experimental

Materials——The squid liver α-*N*-acetylgalactosaminidases I and II were purified as previously descrived.[2] *p*-Nitrophenyl (PNP)-α-D-*N*-acetylgalactosaminide was purchased from Aldrich Co. (Milwaukee, U.S.A.). Sodium taurocholate and sodium taurodeoxycholate were from Calbiochem.-Behring Co. (La Jolla, U.S.A.). Triton X-100, *Ulex europeus* lectin and glucosylceramide were from Sigma Co. (St. Louis, U.S.A.). Lacto *N*-tetraose, lacto-*N*-neotetraose and fucosyl lactose were purified from fresh human milk.[3] *N*-Acetylglucosaminyl lactose was prepared from lacto *N*-tetraose by enzymatic hydrolysis with β-galactosidase. Ascidian β-*N*-acetylhexosaminidase and water

melon α-galactosidase were prepared according to the reported methods,[4,5] and jack bean β-galactosidase was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Anti-A and -B blood grouping sera (human) were from Ortho Diagnostics (London, U.K.). Forssman glycolipid and ^{3}H-labeled Forssman glycolipid, which was labeled at the terminal galactosamine moiety, were kindly supplied by Dr. A. Makita, the University of Hokkaido, ceramide trihexoside (CTH) by Dr. Y. Suzuki, the University of Shizuoka, and human ovarian cyst A- and B-glycoproteins by Dr. M. Endo, the University of Hirosaki. Bovine submaxillary mucin (major type) was isolated by the method described by Tettamanti and Pigman.[6] Asialo bovine submaxillary mucin (ABSM) was obtained by acid hydrolysis of the isolated mucin. Blood group A-type ghosts were prepared by the procedure described by Dodge *et al.*[7] All other chemicals obtained from commercial sources were of the highest grade available.

Enzyme Assay with PNP-Derivatives and Protein Determination——The enzyme activities with PNP-derivatives as substrates were determined by the procedure described previously.[2] One unit of enzyme was defined as the amount of enzyme hydrolyzing 1 μmol of PNP-derivatives per min under the conditions described previously.[2] Protein concentrations were determined by the method of Lowry *et al.*[8] using bovine serum albumin as a standard.

Hydrolysis of ABSM——The assay mixture contained 40 μg of ABSM (11.2 μg as *N*-acetylgalactosamine) and 0.017 unit of the enzyme in 0.1 ml of 0.05 M sodium citrate buffer, pH 4.0 (for α-*N*-acetylgalactosaminidase I) or 4.5 (for α-*N*-acetylgalactosaminidase II). After incubation for an appropriate time at 37 °C, the reaction was terminated by the addition of 0.1 ml of 0.8 M borate buffer, pH 9.1. The amount of *N*-acetylgalactosamine liberated was determined by means of the Morgan–Elson reaction.[9]

Hydrolysis of Forssman Glycolipid——Enzymatic hydrolysis of the terminal α-*N*-acetylgalactosaminyl linkage of Forssman glycolipid was performed according to the procedure described by Suzuki and Suzuki.[10] Briefly, the reaction mixture contained 25 μg (8950 dpm) of ^{3}H-labeled Forssman glycolipid (specific activity of 5.38×10^5 dpm/mmol), 0.05 unit of the enzyme and 0.2 mg of sodium taurodeoxycholate in 0.2 ml of 0.05 M sodium citrate buffer, pH 4.0. After incubation at 37 °C for an appropriate time, the reaction was stopped by the addition of 2.5 ml of chloroform–methanol (2 : 1, v/v). After 0.05 ml of unlabeled *N*-acetylgalactosamine solution (1 mg/ml) and 0.25 ml of water were added, the reaction mixture was partitioned. The upper phase was transferred into a vial containing 10 ml of aqueous counting scintillant ACS II (Amersham Co., Arlington Heights, U.S.A.) and counted with an Aloka LSC-700 scintillation counter (Aloka Co., Tokyo, Japan).

Hydrolysis of Human Ovarian Cyst A- and B-Glycoproteins and Blood Group A-Type Ghosts——Hydrolysis of human ovarian cyst glycoproteins and blood group A-type ghosts was determined by means of the hemagglutination inhibition test according to the method described previously.[5] Human ovarian cyst A-glycoprotein (400 μg) was incubated at 37 °C with 0.16 unit of the enzyme in 0.6 ml of 0.05 M sodium citrate buffer, pH 4.0 (for α-*N*-acetylgalactosaminidase I) or 4.5 (for α-*N*-acetylgalactosaminidase II). In the case of blood group A-type ghosts, 480 μl of the suspension (0.9 mg protein/ml) was incubated with 0.36 unit of the enzyme in 0.6 ml of 0.05 M sodium citrate buffer, pH 3.2 (for α-*N*-acetylgalactosaminidase I) or 4.5 (for α-*N*-acetylgalactosaminidase II). Aliquots (25 μl) of the reaction mixture were taken at appropriate time intervals and tested for hemagglutination inhibition activities with blood group A-active erythrocytes and anti-A serum. To determine H-activity, the hemagglutination inhibition test was performed with H-active erythrocytes and anti-H reagent (*Ulex europeus* lectin). In the case of human ovarian cyst B-glycoprotein, 133 μg of the glycoprotein was incubated at 37 °C with 0.24 unit of the enzyme in 0.2 ml of 0.05 M sodium citrate buffer, pH 4.0 (for α-*N*-acetylgalactosaminidase I) or 4.5 (for α-*N*-acetylgalactosaminidase II). Aliquots (25 μl) of the reaction mixture were taken at appropriate time intervals and tested for hemagglutination inhibition activities with blood group B-active erythrocytes and anti-B serum.

Hydrolysis of Oligosaccharides——Ten micrograms of the oligosaccharides (0.14—0.20 mM) were incubated with 0.02 unit of the enzyme in 0.1 ml of 0.05 M sodium citrate buffer (pH 4.0) at 37 °C for 20 h. The reaction was stopped by heating the mixture in a boiling water bath for 3 min and the released sugars were analyzed on Avicel thin layer plates (Merck, Darmstadt, F.R.G.) as previously described.[11] When oligosaccharides of the raffinose series were hydrolyzed, 1.4 mM oligosaccharides were incubated with 0.02 unit of the enzyme in 0.1 ml of 0.05 M sodium citrate buffer (pH 4.0) at 37 °C for 20 h. The free galactose liberated was determined according to the procedure described by Finch *et al.*[12]

Hydrolysis of CTH——CTH (10 μg) was incubated with 0.02 unit of the enzyme in 0.1 ml of 0.05 M sodium citrate buffer (pH 4.0) containing 0.1 mg of sodium taurodeoxycholate at 37 °C for 18 h. The reaction was stopped by the addition of 4 volumes of chloroform–methanol (2 : 1, v/v). After partitioning, the lower layer was dried and applied to a Silica gel G thin layer plate (Merck, Darmstadt, F.R.G.). The plate was developed with chloroform–methanol–water (65 : 35 : 8, v/v/v) and visualized with orcinol reagent.

Stepwise Hydrolysis of Forssman Glycolipid by Glycosidases——Forssman glycolipid (10 μg) was incubated with α-*N*-acetylgalactosaminidase I (0.03 unit) from squid liver in 0.1 ml of 0.05 M sodium citrate buffer, pH 4.0, containing 0.8 mg of sodium taurocholate at 37 °C for 16 h. The reaction was stopped by heating the reaction mixture in a boiling water bath for 3 min, and then 1 μl of 1 M NaCl solution and 0.3 unit of ascidian β-*N*-acetylhexosaminidase (10 μl) were added to the reaction mixture. After incubation at 37 °C for 96 h, 1 ml of chloroform–methanol (2 : 1, v/v) and 0.15 ml of water were added to the reaction mixture. The resultant lower layer was dried and incubated with water melon α-galactosidase (0.018 unit) in 0.1 ml of 0.05 M sodium citrate buffer, pH

4.0, containing 0.1 mg of sodium taurodeoxycholate at 37 °C for 47 h. The reaction mixture was heated in a boiling water bath for 3 min followed by addition of 0.062 unit of jack bean β-galactosidase. After incubation at 37 °C for 96 h, the reaction was stopped by the addition of 1 ml of chloroform–methanol (2 : 1, v/v). The products from each step were chromatographed on a Silica gel G thin layer plate (Merck, Darmstadt, F.R.G.) in chloroform–methanol–water (65 : 35 : 8, v/v/v). The glycolipid bands were visualized with orcinol reagent.

Results

In our previous study, the two enzymes, α-*N*-acetylgalactosaminidases I and II, were purified from squid liver and characterized.[2] The two final preparations were found to be homogeneous on disc gel electrophoresis. Enzyme II was sufficiently free from other glycosidase activities when PNP-derivatives were used as substrates, whereas enzyme I contained a small amount of α-galactosidase activity (about 7% of α-*N*-acetylgalactosaminidase activity). Enzyme I had a pH optimum of 3.0 (with PNP-α-*N*-acetylgalactosaminide as the substrate) and was heat-stable. Its activity was inhibited by *N*-acetylgalactosamine and galactose. On the other hand, enzyme II had a pH optimum of 4.2 and was heat-labile. *N*-Acetylgalactosamine inhibited the activity of enzyme II but galactose did not. The apparent Michaelis constants (K_m) and maximum velocities (V_{max}) for PNP-α-*N*-acetylgalactosaminide were 3.33 mM and 114 μmol/min/mg protein for enzyme I, and 1.55 mM and 154 μmol/min/mg protein for enzyme II. We describe here the further characterization of these enzymes using natural compounds as substrates.

Hydrolysis of Natural Substrates Containing α-*N*-Acetylgalactosaminyl Linkages

ABSM——Figure 1 shows the time courses for the hydrolysis of ABSM by α-*N*-acetylgalactosaminidases I and II. ABSM was hydrolyzed by the two enzymes at almost the same rate. After incubation for 24 h, 92.5% and 91.9% of α-*N*-acetylgalactosaminyl linkages in ABSM were hydrolyzed by enzymes I and II, respectively. The pH optima of α-*N*-acetylgalactosaminidases I and II were 3.9 and 4.5, respectively. K_m and V_{max} values were calculated from Lineweaver–Burk plots. The K_m and V_{max} values of α-*N*-acetylgalactosaminidases I and II were 2.50 mM and 0.91 μmol/min/mg protein, and 0.71 mM and 1.18 μmol/min/mg protein, respectively.

Forssman Glycolipid——As is generally known, the glycolipid-hydrolyzing activities of some glycosidases are affected by detergents; these include α-galactosidase,[13] β-galactosidase[14] and β-*N*-acetylhexosaminidase.[15] The effects of detergents on the hydrolysis of ^{3}H-labeled Forssman glycolipid by the two squid α-*N*-acetylgalactosaminidases were also investigated using sodium taurodeoxycholate and sodium taurocholate (Fig. 2). For the hydrolysis of Forssman glycolipid by α-*N*-acetylgalactosaminidases I and II, sodium taurodeoxycholate was the most effective at the concentration of 0.1%. On the other hand, sodium taurocholate was effective for α-*N*-acetylgalactosaminidase I but not for α-*N*-

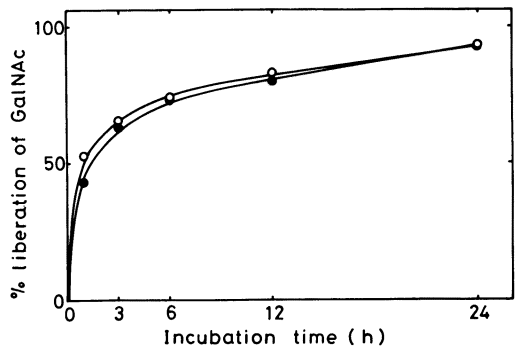

Fig. 1. Liberation of *N*-Acetylgalactosamine from ABSM by the Squid α-*N*-Acetylgalactosaminidases

ABSM (40 μg) was incubated with 0.017 unit of α-*N*-acetylgalactosaminidase I or II in 0.1 ml of 0.05 M sodium citrate buffer, pH 4.0, for various times. The percent liberations of *N*-acetylgalactosamine were calculated against the total *N*-acetylgalactosamine content in ABSM. ○, α-*N*-acetylgalactosaminidase I; ●, α-*N*-acetylgalactosaminidase II.

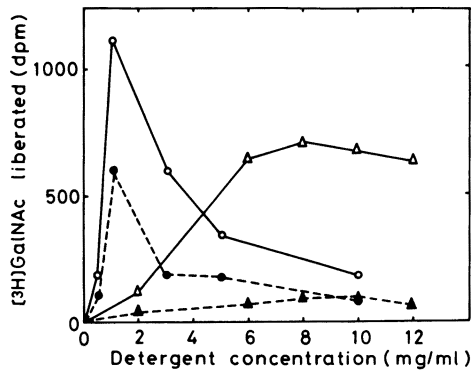

Fig. 2. Effects of the Detergents on the Hydrolysis of Forssman Glycolipid by the Squid α-*N*-Acetylgalactosaminidases

Aliquots of ^{3}H-labeled Forssman glycolipid (10 μg, 3580 dpm) were incubated with α-*N*-acetylgalactosaminidase I (0.012 unit) or α-*N*-acetylgalactosaminidase II (0.033 unit) in the presence of 0.05—1.2 mg of the detergents in 0.1 ml of 0.05 M sodium citrate buffer, pH 4.0, for 20 min. ○, △, α-*N*-acetylgalactosaminidase I; ●, ▲, α-*N*-acetylgalactosaminidase II; ○, ●, sodium taurodeoxycholate; △, ▲, sodium taurocholate.

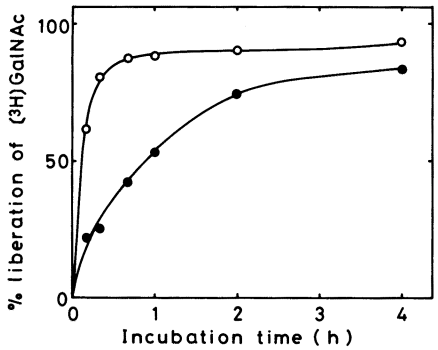

Fig. 3. Liberation of *N*-Acetylgalactosamine from Forssman Glycolipid by the Squid α-*N*-Acetylgalactosaminidases

Aliquots of ^{3}H-labeled Forssman glycolipid (25 μg, 8950 dpm) were incubated with 0.05 unit of α-*N*-acetylgalactosaminidase I or II in the presence of 0.2 mg of sodium taurodeoxycholate in 0.2 ml of 0.05 M sodium citrate buffer, pH 4.0, for various times. The percent liberations of ^{3}H-labeled *N*-acetylgalactosamine were calculated against the total count of ^{3}H-labeled Forssman glycolipid. ○, α-*N*-acetylgalactosaminidase I; ●, α-*N*-acetylgalactosaminidase II.

acetylgalactosaminidase II. No significant hydrolysis was observed in the absence of the detergents or in the presence of Triton X-100. The pH optima for the hydrolysis of Forssman glycolipid by α-*N*-acetylgalactosaminidases I and II in the presence of 0.1% sodium taurodeoxycholate were 3.9 and 4.0, respectively.

The time courses for the hydrolysis of ^{3}H-labeled Forssman glycolipid by α-*N*-acetylgalactosaminidases I and II are shown in Fig. 3. Both enzymes cleaved the terminal α-*N*-acetylgalactosaminyl linkage of Forssman glycolipid, although the rate of hydrolysis by α-*N*-acetylgalactosaminidase II was slower than that of hydrolysis by α-*N*-acetylgalactosaminidase I. Enzyme I hydrolyzed 93.8% of Forssman glycolipid after 4 h of incubation. Enzyme II hydrolyzed the lipid at a rate a little slower than that of enzyme I, and hydrolyzed it almost completely (96.1%) during prolonged incubation (4 h) with the addition of further enzyme.

The effects of the substrate concentration on the enzyme activity were investigated under the optimal conditions, *i.e.*, in the presence of 0.1% sodium taurodeoxycholate at pH 4.0 and 37 °C. The K_m and V_{max} values, calculated from Lineweaver–Burk plots, were 0.030 mM and 0.50 μmol/min/mg protein for α-*N*-acetylgalactosaminidase I, and 0.038 mM and 0.30 μmol/min/mg protein for α-*N*-acetylgalactosaminidase II.

Blood Group A-Active Substances——Blood group A-active substances, such as human ovarian cyst A-glycoprotein and blood group A-type ghosts, have terminal α-*N*-acetylgalactosaminyl linkages in their molecules. It has been reported that blood group A-activity is destroyed by the cleavage of terminal α-*N*-acetylgalactosamine, and H-activity is increased simultaneously.[16] The cleavage of the terminal α-*N*-acetylgalactosamine by the squid enzymes was monitored by means of the hemagglutination inhibition test as shown in Fig. 4. The blood group A-activity of human ovarian cyst A-glycoprotein decreased during incubation with these two enzymes. Avicel thin layer chromatography showed the liberation of *N*-acetylgalactosamine alone in the incubation mixture (data not shown). In contrast, there was an increase of H-activity, which was assayed by using the hemagglutination inhibition test with H-active erythrocytes and anti-H reagent (data not shown). After 18 h of incubation,

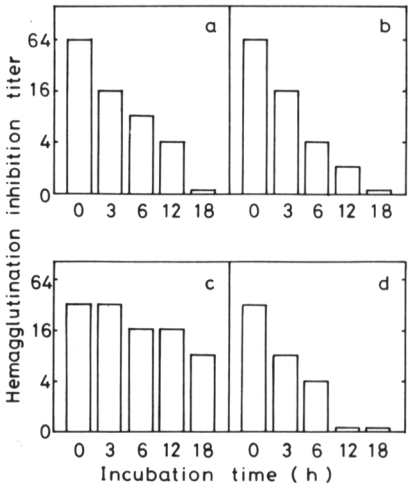

Fig. 4. Abolition of Blood Group A-Activity of Ovarian Cyst A-Glycoprotein and Blood Group A-Ghosts by the Squid α-*N*-Acetylgalactosaminidases

Human ovarian cyst A-glycoprotein (400 μg) was incubated with 0.16 unit of α-*N*-acetylgalactosaminidase I (a) or II (b) in 0.6 ml of sodium citrate buffer, pH 4.0 (a) or 4.5 (b). Blood group A-type ghosts (432 μg) were incubated with 0.36 unit of α-*N*-acetylgalactosaminidase I (c) or II (d) in 0.6 ml of 0.05 M sodium citrate buffer, pH 3.2 (c) or 4.5 (d). Aliquots (25 μl) of the reaction mixture were taken at various time intervals and tested for hemagglutination inhibition activities.

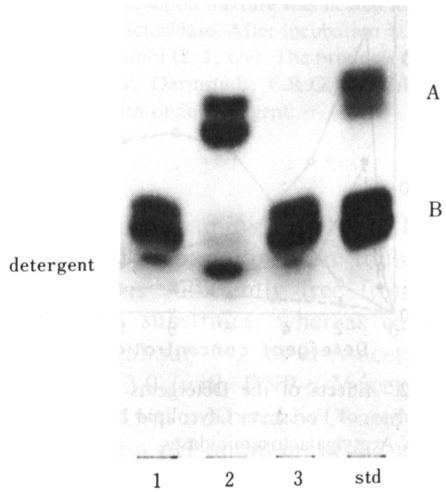

Fig. 5. Hydrolysis of CTH by the Squid α-*N*-Acetylgalactosaminidases

CTH (10 μg) was incubated with 0.02 unit of α-*N*-acetylgalactosaminidase I or II in 0.1 ml of 0.05 M sodium citrate buffer (pH 4.0) containing 0.1 mg of sodium taurodeoxycholate at 37 °C for 18 h. The products were chromatographed on a Silica gel G thin layer plate, and visualized by using the orcinol reagent. 1, CTH after incubation without enzyme; 2, the products of CTH after incubation with α-*N*-acetylgalactosaminidase I; 3, the products of CTH after incubation with α-*N*-acetylgalactosaminidase II; std, standard glycolipids; A, lactosylceramide; B, CTH.

blood group A-activity was abolished by both enzymes (Fig. 4a and b). In the case of blood group A-type ghosts, α-*N*-acetylgalactosaminidase II completely abolished their A-activities after 12 h as shown in Fig. 4d, whereas α-*N*-acetylgalactosaminidase I did not act well on blood group A-type ghosts and the complete abolition of their A-activities was not observed even after 18 h of incubation (Fig. 4c). The optimal pHs of α-*N*-acetylgalactosaminidases I and II were 4.0 and 4.5 for the hydrolysis of ovarian cyst A-glycoprotein, and 3.2 and 4.5 for the blood group A-type ghosts, respectively.

Hydrolysis of Natural Compounds Containing α-Galactosyl Terminals

As described previously, α-*N*-acetylgalactosaminidase I contained about 7% α-galactosidase, whereas α-*N*-acetylgalactosaminidase II contained practically no α-galactosidase activity toward artificial substrates. Therefore, it is important to investigate the hydrolysis of terminal α-galactosyl linkages in natural compounds by α-*N*-acetylgalactosaminidases I and II.

α-Methyl Galactoside and Oligosaccharides of the Raffinose Series——As shown in Table I, α-methyl galactoside and oligosaccharides of the raffinose series were hydrolyzed by α-*N*-acetylgalactosaminidase I and the liberation of galactose was observed. In contrast, α-*N*-acetylgalactosaminidase II did not appreciably hydrolyze these compounds.

CTH——The hydrolysis of CTH, which contains a terminal α-galactosyl linkage, by α-*N*-acetylgalactosaminidases I and II was investigated. As shown in Fig. 5, CTH was completely hydrolyzed by α-*N*-acetylgalactosaminidase I but was not hydrolyzed by α-*N*-acetylgalactosaminidase II even after 18 h of incubation.

Human Ovarian Cyst B-Glycoprotein——Human ovarian cyst B-glycoprotein, which

TABLE I. Hydrolysis of Oligosaccharides by the Squid α-*N*-Acetylgalactosaminidases I and II

Oligosaccharide	Concentration (mM)	Enzyme (units)	Incubation time (h)	Hydrolysis (%)[a]	
				Enzyme I	Enzyme II
Melibiose	1.4	0.02	60	41.1	0.3
Raffinose	1.4	0.02	60	10.3	0.5
Stachyose	1.4	0.02	60	3.9	0.4
α-Methyl galactoside	1.4	0.02	60	13.5	1.2

a) The amounts of galactose liberated were determined according to the procedure described by Finch *et al.*[12]

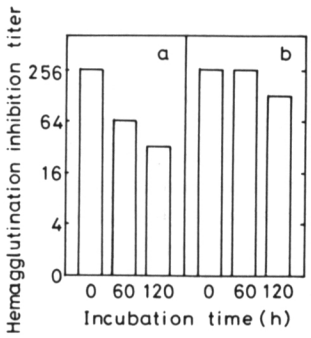

Fig. 6. Abolition of Blood Group B-Activity of Human Ovarian Cyst B-Glycoprotein by the Squid α-*N*-Acetylgalactosaminidases

Human ovarian cyst B-glycoprotein (133 μg) was incubated with 0.24 unit of α-*N*-acetylgalactosaminidase I (a) or II (b) in 0.2 ml of 0.05 M sodium citrate buffer, pH 4.0 (a) or 4.5 (b). Aliquots (25 μl) of the reaction mixture were taken at appropriate time intervals and tested for hemagglutination inhibition activities.

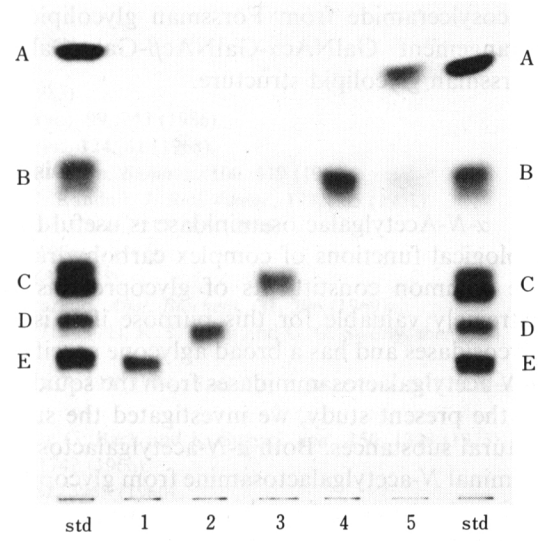

Fig. 7. Stepwise Hydrolysis of Forssman Glycolipid by Glycosidases

The experimental conditions are given in the text. 1, Forssman glycolipid after incubation without enzyme; 2, the products of Forssman glycolipid after incubation with α-*N*-acetylgalactosaminidase I (0.03 unit); 3, 2+β-*N*-acetylhexosaminidase (0.3 unit); 4, 3+α-galactosidase (0.018 unit); 5, 4+β-galactosidase (0.062 unit); std, standard glycolipids; A, glucosylceramide; B, lactosylceramide; C, CTH; D, globoside; E, Forssman glycolipid.

contains terminal α-galactosyl linkages, has been reported to lose its blood group B-activity on cleavage of the terminal α-galactose.[17] The cleavage of terminal α-galactose by the two enzymes was monitored by means of the hemagglutination inhibition test. As shown in Fig. 6, abolition of blood group B-activity was observed for α-*N*-acetylgalactosaminidase I but not for α-*N*-acetylgalactosaminidase II during 60 h of incubation. A slight loss was observed in the case of enzyme II after 120 h. This may be due to the very low level of α-galactosidase activity contained in the enzyme preparation (0.2% of α-*N*-acetylgalactosaminidase activity with PNP-derivatives). Release of only galactose was observed on Avicel thin layer chromatography of the incubation mixture of enzyme I after 120 h of incubation (data not shown).

Hydrolysis of Oligosaccharides Containing Other Glycosyl Terminals

The oligosaccharides which contain glycosyl terminals other than α-*N*-acetylgalactosaminyl and α-galactosyl terminals were incubated with α-*N*-acetylgalactosaminidase

I or II, and the free sugars produced were analyzed by Avicel thin layer chromatography. Neither of the two enzymes hydrolyzed lacto *N*-tetraose or lacto *N*-neotetraose (containing a β-galactosyl terminal), *N*-acetylglucosaminyl lactose (containing a β-*N*-acetylglucosaminyl terminal) or fucosyl lactose (containing an α-L-fucosyl terminal) under the conditions described in Experimental.

Application of α-*N*-Acetylgalactosaminidase I for Structural Study of Glycolipid

α-*N*-Acetylgalactosaminidase I was applied in a structural investigation of Forssman glycolipid together with ascidian β-*N*-acetylhexosaminidase and water melon α-galactosidase, which were previously characterized by us,[4,5] and jack bean β-galactosidase. The products were analyzed by silica gel thin layer chromatography as shown in Fig. 7. The chromatogram is consistent with the successive formation of globoside, CTH, lactosylceramide and glucosylceramide from Forssman glycolipid, and therefore with the anomeric sequential arrangement GalNAcα-GalNAcβ-Galα-Galβ-Glcβ-ceramide of the sugar units in the Forssman glycolipid structure.

Discussion

α-*N*-Acetylgalactosaminidase is useful for structural analysis and for elucidation of the biological functions of complex carbohydrates, since α-linked *N*-acetylgalactosaminyl units are common constituents of glycoproteins and glycolipids. In particular, the enzyme is extremely valuable for this purpose if it is sufficiently free from contamination by other glycosidases and has a broad aglycone specificity. Previously, we have highly purified the two α-*N*-acetylgalactosaminidases from the squid liver and described their enzymatic properties.[2] In the present study, we investigated the substrate specificities of the two enzymes toward natural substances. Both α-*N*-acetylgalactosaminidases I and II from squid liver cleaved the terminal *N*-acetylgalactosamine from glycoproteins and glycolipids, including blood group A-active glycoprotein from human ovarian cyst, asialo bovine submaxillary glycoprotein, blood group A-type ghosts, and Forssman glycolipid. α-*N*-Acetylgalactosaminidases I and II seem to have broad aglycone specificities for natural substrates, although the rates of hydrolysis by the two enzymes were slightly different.

α-*N*-Acetylgalactosaminidase has been purified from various sources,[1] but almost all the purified enzyme preparations showed α-galactosidase activity. α-*N*-Acetylgalactosaminidase I from squid liver also contained 7% α-galactosidase activity toward PNP-α-galactoside as a substrate, while α-*N*-acetylgalactosaminidase II was free from α-galactosidase activity. Not only PNP-α-galactoside, but also ceramide trihexoside, human ovarian cyst B-glycoprotein, α-methyl galactoside, and oligosaccharides of the raffinose series which contain α-linked terminal galactose in their structures, were hydrolyzed by α-*N*-acetylgalactosaminidase I. On the other hand, α-*N*-acetylgalactosaminidase II did not cleave the terminal galactose from these natural compounds under the conditions described here. As described previously,[2] the existence of α-*N*-acetylgalactosaminidase without α-galactosidase activity was confirmed in the present study.

The kinetic data showed that both α-*N*-acetylgalactosaminidases I and II have extremely low K_m values for Forssman glycolipid, compared with PNP-α-*N*-acetylgalactosaminide and asialo bovine submaxillary mucin. This indicates that both α-*N*-acetylgalactosaminidases I and II from squid liver have high affinities for Forssman glycolipid. The two enzymes may be available for structural analysis of other glycolipids.

α-*N*-Acetylgalactosaminidases from squid liver showed broad aglycon specificities for natural substances. As shown in Fig. 7, enzyme I can be used in combination with β-*N*-acetylhexosaminidase, α- and β-galactosidases to convert Forssman glycolipid sequentially

into globoside, trihexosyl-, lactosyl-, and glucosylceramide. Because of their broad aglycone specificities, α-*N*-acetylgalactosaminidases from squid liver will be very useful for elucidating the structures and functions of glycoconjugates. In particular, enzyme II, which is free from α-galactosidase activity, should be of value for this purpose.

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References

1) Y.-T. Li and S.-C. Li, "The Glycoconjugates," Vol. 1, ed. by M. I. Horowitz and W. Pigman, Academic Press, Inc., New York, 1977, pp. 52—67; A. Kobata, *Anal. Biochem.*, **100**, 1 (1979); Y. Uda, S.-C. Li and Y.-T. Li, *J. Biol. Chem.*, **252**, 5194 (1977).
2) T. Itoh and Y. Uda, *J. Biochem.* (Tokyo), **95**, 959 (1984).
3) A. Kobata, *Methods Enzymol.*, **28**, 262 (1972).
4) Y. Uda and T. Itoh, *J. Biochem.* (Tokyo), **93**, 847 (1983).
5) T. Itoh, Y. Uda and H. Nakagawa, *J. Biochem.* (Tokyo), **99**, 243 (1986).
6) G. Tettamanti and W. Pigman, *Arch. Biochem. Biophys.*, **124**, 41 (1968).
7) J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).
8) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9) J. L. Reissig, J. L. Strominger and L. F. Leloir, *J. Biol. Chem.*, **217**, 959 (1955).
10) Y. Suzuki and K. Suzuki, *J. Biol. Chem.*, **249**, 2098 (1974).
11) M. Hiraiwa and Y. Uda, *Chem. Pharm. Bull.*, **33**, 1563 (1985).
12) P. R. Finch, R. Yuen, H. Schachter and M. A. Moscarello, *Anal. Biochem.*, **31**, 296 (1969).
13) S. Hakomori and B. Siddiqui, *J. Biol. Chem.*, **246**, 2271 (1971); K. J. Dean and C. C. Sweely, *ibid.*, **254**, 9994 (1979).
14) T. Miyatake and K. Suzuki, *Biochim. Biophys. Acta*, **337**, 333 (1974); S.-C. Li, Y. Mazzotta, S.-F. Chien and Y.-T. Li, *J. Biol. Chem.*, **250**, 6786 (1975).
15) S.-C. Li and Y.-T. Li, *J. Biol. Chem.*, **254**, 5153 (1970); G. Back and K. Suzuki, *ibid.*, **250**. 1328 (1975).
16) H. Tuppy and W. L. Staudenbauer, *Biochemistry*, **5**, 1742 (1966).
17) M. L. Zarnitz and E. A. Kabat, *J. Am. Chem. Soc.*, **82**, 3953 (1960).