マダイ肝臓からのβ-N-アセチルヘキソサミニダーゼの精製とその性質

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Purification and Characterization of β-*N*-Acetylhexosaminidase from the Liver of Red Sea Bream

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β-*N*-Acetylhexosaminidase was purified to an electrophoretically homogeneous form from the liver of red sea bream *Pagrus major* and the properties of the purified enzyme were studied. The molecular weight of the enzyme was estimated to be 150,000 by gel filtration. The enzyme exhibited its maximum activity at pH 4.0, 4.5 and 5.0 toward the substrates *p*-nitrophenyl β-*N*-acetylglucosaminide, chitobiose and bovine submaxillary glycoprotein, respectively. The *Km* and *Vmax* of the enzyme for *p*-nitrophenyl β-*N*-acetylglucosaminide were 0.42 mM and 235 μmol/min·mg. The enzyme liberated approximately 100% of the *N*-acetylglucosamine occurring in bovine submaxillary glycoprotein as a minor carbohydrate component, revealing that the *N*-acetylglucosamine existed at the nonreducing end of the carbohydrate moieties.

β-*N*-Acetylhexosaminidase (EC 3.2.1.30) which catalyzes the hydrolysis of the β-*N*-acetylglucosaminyl and β-*N*-acetylgalactosaminyl linkages is widely distributed among living organisms. The enzyme is very useful for the structural and functional studies of glycoconjugates such as glycoproteins and glycolipids,[1,2] since these biologically important compounds contain β-*N*-acetylglucosaminyl units as the major constituent. To date, many β-*N*-acetylhexosaminidases have been isolated from mammalian tissues,[3-7] plants,[8-11] bacteria,[12-16] fungi,[17] top shell,[18] ascidian[19] and octopus.[20] In the utilization of a glycosidase for the structural and functional studies, the enzyme must be sufficiently free from contamination by other glycosidases. In spite of remarkable development of methods for enzyme purification, it is still quite difficult to isolate homogeneous β-*N*-acetylhexosaminidase entirely free from other glycosidases.

We have found that the liver of red sea bream *Pagrus major* may be advantageous source for the isolation of β-*N*-acetylhexosaminidase since it contains a large amount of the enzyme.[21] In this paper, we report the purification of β-*N*-acetylhexosaminidase from red sea bream liver and the properties of the purified enzyme.

Material and Methods

Materials

p-Nitrophenyl β-D-*N*-acetylglucosaminide and *p*-nitrophenyl α-D-*N*-acetylgalactosaminide were purchased from Koch-Light Laboratories, Ltd. The other *p*-nitrophenyl glycosides were from Nakarai Chemicals, Ltd. Sephacryl S-200 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals. Ultrogel AcA 34 was from LKB, Ltd. Hydroxyapatite was from Seikagaku Kogyo Co., Ltd. The livers were taken from fresh red sea bream *P. major* and treated with acetone.[21] The resultant acetone powder was stored in a freezer until use. Bovine submaxillary glycoprotein was isolated from bovine submaxillary glands according to the procedure described for the preparation of ovine submaxillary glycoprotein,[22] involving precipitation with cetyltrimethyl ammonium bromide, Sepharose CL-4B gel filtration and DEAE-cellulose chromatography. The carbohydrate composition of the glycoprotein obtained (μg/mg of lyophilized glycoprotein) was determined by gas chromatography[23] (neutral and amino sugars) and the thiobarbituric acid method[24] (sialic acid): sialic acid (as *N*-acetylneurmainic acid), 124; galactosamine, 132; glucosamine, 30; galactose,

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16; fucose, 5. Chitobiose was prepared from chitin (powder, Nakarai Chemicals, Ltd.) essentially according to the procedure described by Mizuochi.[25]

Enzyme Assays

Glycosidase activities were assayed at 37°C by using the corresponding *p*-nitropheyl glycoside as the substrate. The enzyme solution (10–50 μl) was added to 0.3 m*l* of 2 mM *p*-nitrophenyl glycoside dissolved in 0.05 M sodium citrate buffer, pH 4.6. After incubation for a preset time, 3 m*l* of 0.2 M sodium borate buffer, pH 9.8, was added to stop the reaction and the absorbance of the resultant solution was measured at 400 nm. One unit of enzyme was defined as the amount of enzyme which hydrolyzes 1 μmol of *p*-nitrophenyl glycoside per min under the conditions described above.

Analytical Methods

Protein was determined by the method of Lowry *et al.*[26] with bovine serum albumin as the standard. Disc polyacrylamide gel electrophoresis was performed at pH 9.5 in 7% gel according to the procedure described by Davis.[27] Gel was stained for protein with 0.25% Coomassie Brilliant Blue R-250 in ethanol/acetic acid/water (9: 2: 9, v/v/v) and destained with ethanol/acetic acid/water (25: 8: 65, v/v/v). *N*-Acetylglucosamine liberated by β-*N*-acetylhexosaminidase from chitobiose and bovine submaxillary glycoprotein was determined by the Morgan-Elson reaction modified by Reissig *et al.*[28] except for using double volume (0.2 m*l*) of 0.8 M potassium borate buffer, pH 9.1.

Results and Discussion

Purification of β-N-Acetylhexosaminidase from Red Sea Bream Liver

1. Step 1. Extraction and ammonium sulfate precipitation—All operations of the enzyme purification were carried out at about 5°C. The acetone powder (10 g) of red sea bream liver was homogenized in 200 m*l* of 0.05 M sodium citrate buffer, pH 4.6. The homogenate was centrifuged at 15,000×g for 30 min. The supernatant was fractionated with solid ammonium sulfate. The precipitate formed at 35–75% ammonium sulfate saturation was collected by centrifugation, dissolved in a minimal volme of 0.025 M sodium phosphate buffer, pH 7.2, and dialyzed against the same buffer. The dialyzate was centrifuged at 15,000×g for 30 min to remove insoluble matter.

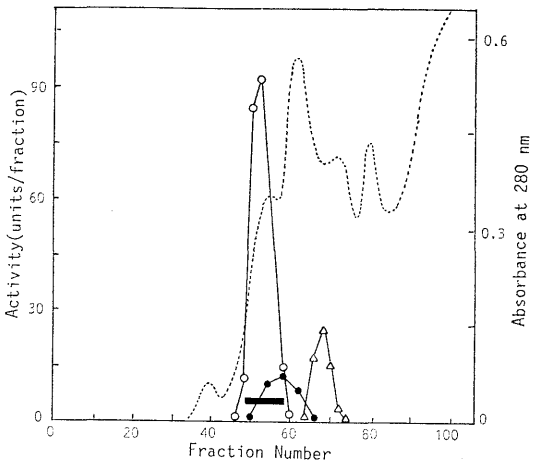

Fig. 1. Sephacryl S-200 Gel Filtration of the Enzyme Solution Obtained at Step 1. The enzyme solution (10 m*l*) was applied to a Sephacryl S-200 column (3.6×94 cm) previously equilibrated with 0.05 M sodium phosphate buffer, pH 7.2, containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 25 m*l*/h and 10-m*l* fractions were collected. ----, absorbance at 280 nm; ○, β-*N*-acetylhexosaminidase activity; ●, β-galactosidase activity; △, α-*N*-acetylgalactosaminidase activity.

2. Step 2. Sephacryl S-200 gel filtration—The enzyme solution from Step 1 was subjected to Sephacryl S-200 gel filtration. The elution profile is shown in Fig. 1. The fractions containing β-*N*-acetylhexosaminidase as shown by a horizontal bar were pooled.

3. Step 3. Heat treatment—The enzyme solution from Step 2 was heated at 55°C for 10 min and then centrifuged at 15,000×g for 30 min to remove denatured proteins. This treatment eliminated almost entirely β-galactosidase although about 50% of the β-*N*-acetylhexosaminidase activity was lost (Fig. 2). The heat treatment was an effective purification step since complete separation of β-*N*-acetylhexosaminidase from β-galactosidase was unattainable by the subsequent chromatography.

4. Step 4. Ultrogel AcA 34 gel filtration—The enzyme solution obtained after the heat treatment was concentrated and subjected to Ultrogel AcA 34 gel filtration. The elution profile is shown in Fig. 3. The fractions containing β-*N*-acetylhexosaminidase were pooled and concentrated by ultrafiltration with a Diaflo PM-10 membrane.

5. Step 5. DEAE-Sephadex A-50 chromatography—The enzyme solution from Step 4 was

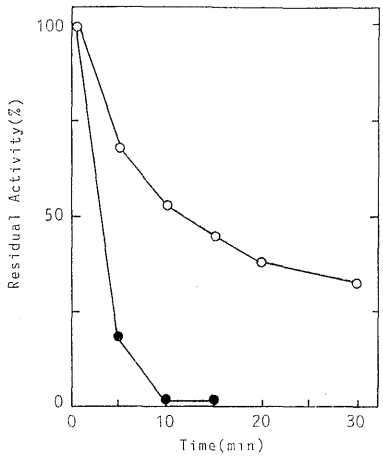

Fig. 2. Thermal Inactivation at 55°C of β-*N*-Acetylhexosaminidase and β-Galactosidase in the Enzyme Solution Obtained at Step 2. Small portions of the enzyme solution were heated at 55°C for various lengths of time and the residual activities were assayed. ○, β-*N*-acetylhexosaminidase activity; ●, β-galactosidase activity.

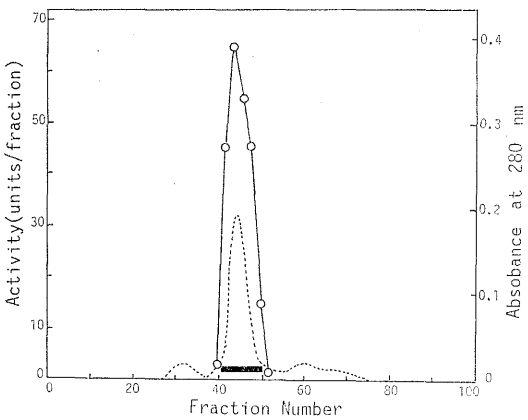

Fig. 3. Ultrogel AcA 34 Gel Filtration of the Enzyme Solution Obtained at Step 3. The enzyme solution was concentrated to 2 m*l* by ultrafiltration with a Diaflo PM-10 membrane and applied to an Ultrogel AcA 34 coumn (2.0×87 cm) previously equilibrated with 0.05 M sodium phosphate buffer, pH 7.2, containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 7 m*l*/h and 3.7-m*l* fractions were collected. ----, absorbance at 280 nm; ○, β-*N*-acetylhexosaminidase activity.

dialyzed against 0.025 M sodium phosphate buffer, pH 7.2, and subjected to DEAE-Sephadex A-50 column chromatography. The column was eluted stepwise with 0.025 M sodium phosphate buffer, pH 7.2, containing 0–0.1 M NaCl. The elution profile is shown in Fig. 4. About 60% of

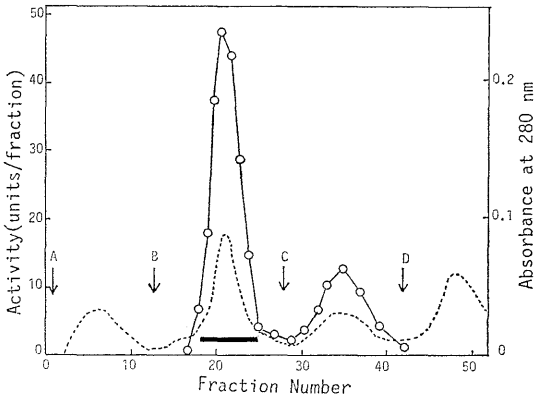

Fig. 4. DEAE-Sephadex A-50 Chromatography of the Enzyme Solution Obtained at Step 4. The enzyme solution (2 m*l*) containing 7 mg of protein in 0.025 M sodium phosphate buffer, pH 7.2, was applied to a DEAE-Sephadex A-50 column (1.6×15 cm) which had been equilibrated with the same buffer. The column was eluted at a flow rate of 10 m*l*/h successively with 0.025 M sodium phosphate buffer, pH 7.2(A), the buffer +0.025 M NaCl(B), the buffer +0.05 M NaCl(C) and the buffer +0.075 M NaCl(D). Fractions (5 m*l*) were collected. ----, absorbance at 280 nm; ○, β-*N*-acetylhexosaminidase activity.

the β-*N*-acetylhexosaminidase activity applied was eluted with the buffer containing 0.025 M NaCl. About 20% of the enzyme activity was subsequently eluted with the buffer containing 0.05 M NaCl. The main peak of the enzyme activity as shown by a horizontal bar was pooled and concentrated by ultrafiltration with a Diaflo YM-10 membrane. As the purity of this enzyme preparation was examined by disc polyacrylamide gel electrophoresis, it gave a mian protein band accompanied by two faint bands. To evaluate the possible contamination by other glycosidases, the enzyme (0.4 unit) was incubated at 37°C for 2 h with each of the following *p*-nitrophenyl glycosides: α- and β-galactopyranosides, α- and β-mannopyranosides, α- and β-glucopyranosides, α-L-fucopyranoside, α-*N*-acetylgalactosaminide and β-glucuronide. The enzyme contained no other glycosidase activities. From this evaluation, the partially purified enzyme was judged to be a practical β-*N*-acetylhexosaminidase preparation utilizable for the structural and functional studies of glycoconjugates.

6. Step 6. Hydroxyapatite chromatography—The enzyme solution from Step 5 was dialyzed against 0.025 M potassium phosphate buffer, pH

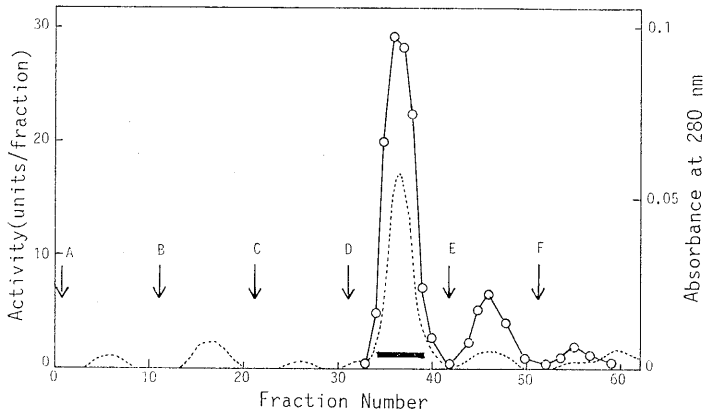

Fig. 5. Hydroxyapatite Chromatography of the β-*N*-Acetylhexosaminidase Solution Obtained at Step 5. The enzyme solution (2 m*l*) containing 2 mg of protein in 0.025 M potassium phosphate buffer, pH 6.9, was applied to a Hydroxyapatite column (1.6×6 cm) which had been equilibrated with the same buffer. The column was eluted at a flow rate of 8 m*l*/h successively with 0.025 M (A), 0.05 M (B), 0.075 M (C), 0.1 M (D), 0.15 (E) and 0.25 M (F) potassium phosphate buffers, pH 6.9. Fractions (3 m*l*) were collected. ----, absorbance at 280 nm; ○, β-*N*-acetylhexosaminidase activity.

Table 1. Purification of β-*N*-Acetylhexosaminidase from 10 g of Acetone Powder of Red Sea Bream Liver

Steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)
Extract	1260	1920	0.656	100
$(NH_4)_2SO_4$ ppt and dialysis	1010	472	2.14	80
Sephacryl S-200	850	30.0	28.3	67
Heat treatment	433	16.1	26.9	34
Ultrogel AcA 34	378	6.6	57	30
DEAE-Sephadex A-50	228	2.0	110	18
Hydroxyapatite	135	0.95	140	11

6.9, and subjected to hydroxyapatite column chromatography. The column was eluted stepwise with 0.025–0.25 M potassium phosphate buffer, pH 6.9. The elution profile is shown in Fig. 5. Most of the β-*N*-acetylhexosaminidase activity was eluted with 0.1 M buffer. The main peak of the enzyme activity as shown by a horizontal bar was pooled and concentrated by ultrafiltration with a Diaflo YM-10 membrane. The concentrate was used for characterization as the final β-*N*-acetylhexosaminidase preparation.

The overall purification process of the enzyme is summarized in Table 1.

Properties of β-N-Acetylhexosaminidase

1. Purity—The final β-*N*-acetylhexosaminidase preparation gave one protein band on disc polyacrylamide gel electrophoresis (Fig. 6).

2. Molecular weight—The molecular weight of

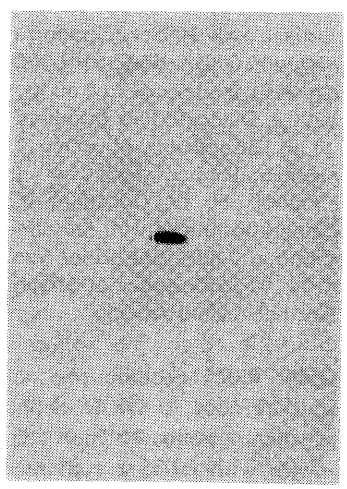

Fig. 6. Disc Polyacrylamide Gel Electrophoresis of Purified β-*N*-Acetylhexosaminidase.

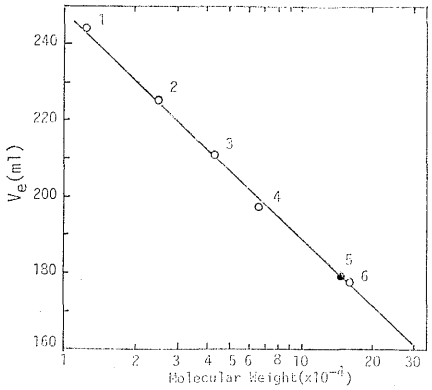

Fig. 7. Estimation of Molecular Weight of β-*N*-Acetylhexosaminidase by Ultrogel AcA 34 Gel Filtration. Column, 2.0×87 cm; Eluent, 0.05 M sodium phosphate buffer, pH 7.2, containing 0.1 M NaCl. 1, cytochrome c; 2, chymotrypsinogen A; 3, ovalbumin; 4, bovine serum albumin; 5, red sea bream β-*N*-acetylhexosaminidase; 6, aldolase.

β-*N*-acetylhexosaminidase was estimated to be 150,000 by gel filtration on an Ultrogel AcA 34 column calibrated with standard proteins of known molecular weight (Fig. 7). This value agrees approximately with the molecular weight of β-*N*-acetylhexosaminidase isolated from common octopus liver.[20]

3. pH optimum—The effect of pH on the β-*N*-actetylhexosaminidase activity was examined by using *p*-nitrophenyl β-*N*-acetylglucosaminide, chitobiose and bovine submaxillary glycoprotein as substrates in sodium citrate-phosphate buffer. The enzyme exhibited its maximum activity toward these substrates at pH 4.0, 4.5 and 5.0, respectively (Fig. 8).

4. pH stability—The stability of the purified enzyme at various pH values was examined. The enzyme was incubated in buffers ranging from pH 3.0 to 8.5 for 2 h at 25°C and then the residual activity was assayed under the standard condition. The β-*N*-acetylhexosaminidase activity was stable in the range of pH 6.5–8 and unstable below pH 6 (Fig. 9). The enzyme exhibits its maximum activity around pH 5 toward natural substrates (Fig. 8). It is therefore advisable to use the enzyme around pH 5 in its application to the structural study of glycoconjugates although it is not stable at this pH. Fortunately, the enzyme activity was remarkably protected at pH 5 by the addition of bovine serum albumin and bovine submaxillary glycoprotein (Fig. 10).

5. Effect of substrate concentration—The effect

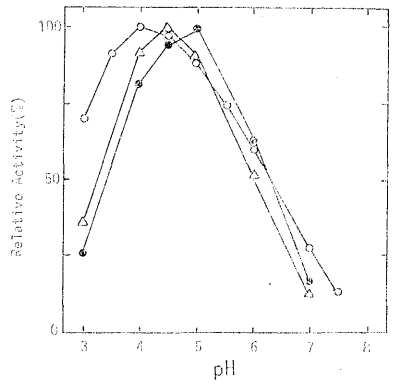

Fig. 8. Effect of pH on the β-*N*-Acetylhexosaminidase Activity toward *p*-Nitrophenyl β-*N*-Acetylglucosaminide, Chitobiose and Bovine Submaxillary Glycoprotein. For chitobiose, the incubation mixtures contained 0.1 μmol of chitobiose and 0.7 unit of the enzyme in 0.5 m*l* of 0.025 M sodium citrate-phosphate buffers, pH 3–7. For bovine submaxillary glycoprotein, the mixtures contained 1 mg of the glycoprotein and 2 units of the enzyme in 0.5 m*l* of the buffers. After incubation at 30°C for 2 h, the liberated *N*-acetylglucosamine was determined by the Morgan-Elson reaction. ○, *p*-nitrophenyl β-*N*-acetylglucosaminide; △, chitobiose; ●, bovine submaxillary glycoprotein.

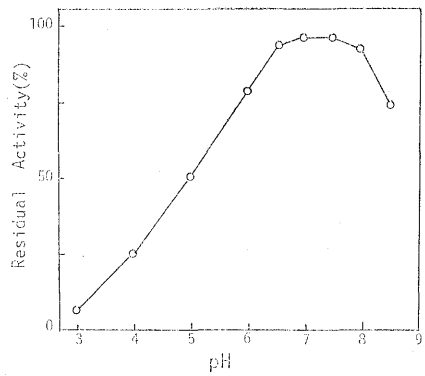

Fig. 9. Effect of pH on the Stability of β-*N*-Acetylhexosaminidase. pH 3–6, sodium citrate buffer; pH 6.5–8.5, sodium phosphate buffer.

of substrate concentration on the reaction rate of the purified β-*N*-acetylhexosaminidase was examined at 37°C using 0.05 M sodium citrate buffer, pH 4.6 The inital velocities were measured by the method of Algranati.[29] The *Km* value and maximum velocity for *p*-nitrophenyl β-*N*-acetylglucosaminide were determined from the Lineweaver-Burk plots to be 0.421 mM and 235 μmol/min·mg, respectively (Fig. 11).

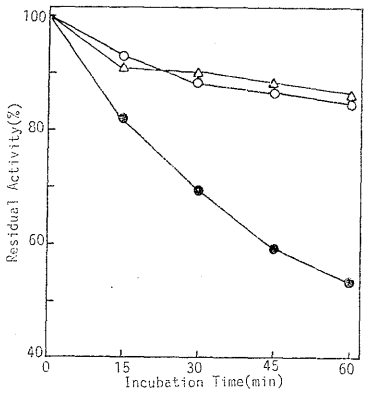

Fig. 10. Effects of Bovine Serum Albumin and Bovine Submaxillary Glycoprotein on the Stability of β-*N*-Acetylhexosaminidase. The enzyme (0.05 unit) was incubated at 37°C in 0.15 m*l* of 0.05 M sodium citrate buffer, pH 5.0, in the presence and absence of 0.1 mg of bovine serum albumin or 0.2 mg of bovine submaxillary glycoprotein. After a preset time, the residual activity was assayed. ●, enzyme alone; ○, enzyme +bovine serum albumin; △, enzyme +bovine submaxillary glycoprotein.

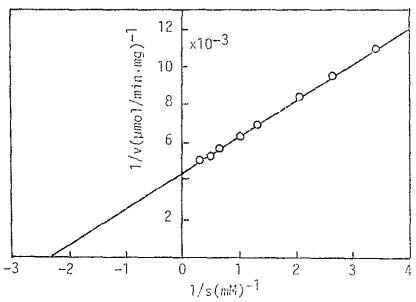

Fig. 11. Lineweaver-Burk Plots for Hydrolysis of *p*-Nitrophenyl β-*N*-Acetylglucosaminide.

6. Liberation of *N*-acetylglucosamine from bovine submaxillary glycoprotein by β-*N*-acetylhexosaminidase—Bovine submaxillary glycoprotein contains small amounts of glucosamine, galactose and fucose in addition to the major sugar components, sialic acid and galactosamine.[30,31] Since our preparation also contained 3.0% glucosamine, the glycoprotein was exhaustively digested by the purified enzyme. After 20 h, approximately 100% of the *N*-acetylglucosamine present in the glycoprotein was liberated and no further liberation was observed for the next 24 h (Fig. 12). This result indicated that all the *N*-acetylglucosamine exists at the nonreducing end of the carbohydrate chain of the glycoprotein.

The liver of red sea bream is readily available.

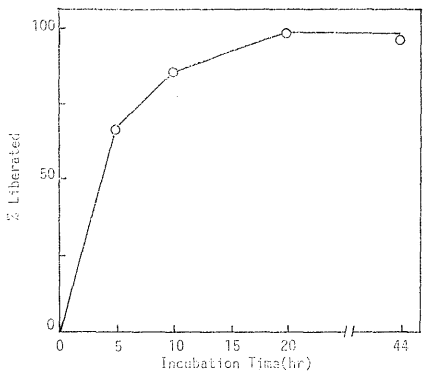

Fig. 12. Liberation of *N*-Acetylglucosamine from Bovine Submaxillary Glycoprotein by β-*N*-Acetylhexosaminidase. A mixture (2 m*l*) containing 2.7 mg of bovine submaxillary glycoprotein and 4 units of the enzyme in 0.025 M sodium citrate buffer, pH 5.0, was incubated at 30°C in the presence of toluene. After 5, 10, 20 and 44 h, 0.5-m*l* portions of the incubation mixture were used for determination of the liberated *N*-acetylglucosamine. Two units of the enzyme in 0.1 m*l* of 0.025 M sodium citrate buffer, pH 5.0, was added to the mixture at 5, 10 and 20 h.

Moreover, the liver not only contains a large amount of β-*N*-acetylhexosaminidase, but also is rich in α-*N*-acetylgalactosaminidase which has not been favored with the suitable source so far. We have established a simple purification procedure for α-*N*-acetylgalactosaminidase from red sea bream liver, which is featured by an application of the change in molecular size dependent on pH and also demonstrated that the enzyme acts effectively on glycoproteins and glycolipids (manuscript in preparation). It should be noted that sufficient separation of β-*N*-acetylhexosaminidase from α-*N*-acetylgalactosaminidase on gel filtration is attainable only around pH 7.2 (Fig. 1). α-*N*-Acetylgalactosaminidase behaves around this pH as a monomer with a molecular weight of 40,000[22] while the enzyme behaves around pH 4.2 as an oligomer with the similar molecular weight to β-*N*-acetylhexosaminidase.

Thus, enough amonuts of both β-*N*-acetylhexosaminidase and α-*N*-acetylgalactosaminidase, which are valuable for the glycoconjugate research, may be at the same time isolated from 10–20 g of the acetone powder of red sea bream liver.

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