α-N-Acetylgalactosaminidase from the Limpet, Patella vulgata*

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α-N-Acetylgalactosaminidase, devoid of β-N-acetylhexosaminidase, has been purified 860-fold from the limpet, Patella vulgata. The final preparation gives one protein band on disc gel electrophoresis, sodium dodecyl sulfate-disc gel electrophoresis, and disc gel isoelectrofocusing. By Sephadex G-200 filtration, the molecular weight of this enzyme is 200,000 at pH 4.2 and is 45,000 at pH 7.0. The enzyme is active at pH 4.2 but inactive at pH 7.0. These results suggest that limpet α-N-acetylgalactosaminidase exists as an active oligomer in the acidic pH and an inactive monomer in the neutral or alkaline pH. In spite of its electrophoretic purity, the final enzyme preparation contains about 0.1% β-galactosidase and 6.7% α-galactosidase activity. Although the β-galactosidase activity in the final preparation is regarded as a contaminant, the α-galactosidase activity may be due to the intrinsic activity of the enzyme for the following reasons: both α-N-acetylgalactosaminidase and α-galactosidase activities are inactivated to the same degree upon heat and pH inactivation; both enzymes exhibit identical dissociation and association behavior as a function of pH; both activities are inhibited by galactose and N-acetylgalactosamine.

The physical properties of this enzyme are: pH optimum, pH 3.8; isoelectric point, pH 5.5; $K_a$ for p-nitrophenyl α-N-acetylgalactosaminide, 0.6 ms; $K_m$ for Forsmann hapten glycolipid, 0.036 ms. This enzyme liberates the N-acetylgalactosamine unit from Forsmann hapten glycolipid, blood group A active glycoprotein, and bovine submaxillary glycoprotein, and blood group A active glycoproteins.

The preparation of α-N-acetylgalactosaminidase entirely free of β-N-acetylhexosaminidase activity provides a valuable tool for the structural analysis of complex carbohydrates, since α-N-acetylgalactosaminyl units are common constituents of both glycoproteins and glycolipids. Although α-N-acetylgalactosaminidase has been partially purified from beef and pig liver (1), human liver (2), beef spleen (3), earthworm (4), snail (5), the gastropod Turbo coruscus (6), and bacteria (7), all of these sources are also rich in β-N-acetylhexosaminidase activity. Because of this, it is extremely difficult to avoid the presence of some contaminating β-N-acetylhexosaminidase in the final purified preparations (6). Moreover, the α-N-acetylgalactosaminidases isolated from the above-mentioned sources have not been examined extensively for their specificities toward different naturally occurring glycoconjugates. We found that limpet acetone powder commercially available as a source for crude β-glucuronidase is rich in α-N-acetylgalactosaminidase which can hydrolyze α-N-acetylgalactosaminyl units from both glycoproteins and glycosphingolipids. Furthermore, we observed that the β-N-acetylhexosaminidase present in limpet acetone powder barely hydrolyzes the β-N-acetylgalactosaminyl unit in Forsmann hapten and globoside, although this enzyme is very active against both p-nitrophenyl β-N-acetylgalactosaminide and p-nitrophenyl β-N-acetylgalactosaminide. Herewith, we report a simple procedure for the isolation of limpet α-N-acetylgalactosaminidase in electrophoretically homogeneous form and its general properties. We have also demonstrated the use of this enzyme for the structural analysis of various glycoconjugates, including Forsmann hapten, bovine submaxillary glycoprotein, and blood group A active glycoproteins and glycolipids.

EXPERIMENTAL PROCEDURES

Materials - Limpet acetone powder (Patella vulgata) was purchased from Sigma; p-nitrophenyl α-N-acetylgalactosaminide from Pierce Chemical Co.; galactose oxidase, from Worthington Biochemical Co.; β-N-acetylhexosaminidase (8), β-galactosidase (9), and α-galactosidase (10) were isolated from jack bean and fig in our laboratory. Forsmann hapten was isolated from dog intestine (11). The following compounds were generous gifts: bovine submaxillary glycoprotein, Dr. W. Pigmann, New York Medical College; pig submaxillary mucin, Dr. D. Aminoff, University of Michigan; ovariain cyst glycoprotein, Dr. T. Kristiansen, University of Uppsala; blood group A active glycoprotein from oyster, Dr. A. Ōgamo, Kitasato University, Tokyo, Japan; O-glycosidically linked glycopeptides from fe-tuin, Dr. E. A. Davidson, Pennsylvania State University. All other chemicals used were obtained from commercial sources and were of the highest grade. Unless otherwise indicated all carbohydrates referred to in this paper are of D configuration.

Radioactive Labeling of Forsmann Hapten Glycolipid - The trit-
ium labeling of the terminal N-acetylgalactosamine of Forssman hapten glycolipid was done by our modification of the galactose oxidase and NaB\textsubscript{3}H\textsubscript{4} reduction procedure as described by Radin (12). We have found that the use of sodium taurodeoxycholate as detergent increased considerably the ability of the enzyme to oxidize the terminal N-acetylgalactosamine moiety in this glycolipid. Ten milligrams of Forssman hapten was dissolved in 4 ml of 0.05 M sodium phosphate buffer, pH 7.0, containing 20 mg of sodium taurodeoxycholate. To this solution, 0.5 ml of galactose oxidase solution (1 mg/ml of 0.05 M phosphate buffer, pH 7.0) was added and the mixture incubated at 37°. After 4 h, an additional 0.5 ml of galactose oxidase was added; then the mixture was incubated overnight. The oxidized Forssman hapten was extracted into the lower layer obtained after the addition of 4 volumes of chloroform/methanol (2:1 v/v). After drying, the oxidized Forssman hapten was dissolved in a mixture of 0.2 ml of H\textsubscript{2}O and 1.0 ml of tetrahydrofuran. Ten microliters of NaB\textsubscript{3}H\textsubscript{4} solution (100 mCi/0.1 ml of 0.1 N NaOH) was added and the mixture left standing at room temperature overnight. Then, 0.1 ml of NaB\textsubscript{3}H\textsubscript{4} (50 mg/ml) was added and the sample let stand for 2 h and the excess NaB\textsubscript{3}H\textsubscript{4} was destroyed with 0.5 ml of 10 N acetic acid. After the tetrahydrofuran was removed in a flash evaporator, the sample was dialyzed against water. The tritiated Forssman hapten glycolipid was then purified by preparative thin layer chromatography.

**Enzyme Assays—** Activities of \(\alpha\)-N-acetylgalactosaminidase and other glycosidases were routinely assayed at 37° by using the appropriate \(p\)-nitrophenyl glycoside as substrate. The enzyme solution (1 to 50 \(\mu\)l) was added to 0.5 ml of 2 \(\mu\)M \(p\)-nitrophenyl glycoside dissolved in 0.5 M sodium citrate buffer, pH 4.0. After incubation for a preset time, 3.5 ml 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 490 nm. One unit of enzyme was defined as the amount of enzyme which hydrolyzes 1 \(\mu\)mol of \(p\)-nitrophenyl glycoside/min under the conditions described above. The specific activity of the enzyme was expressed as units per mg of protein, as determined by the method of Lowry et al. (13) with crystalline bovine serum albumin as the standard.

For using labeled Forssman glycolipid as substrate, 80 \(\mu\)g of the glycolipid (about 10,000 cpm) was incubated at 37° in 0.5 ml of 0.05 M sodium citrate buffer, pH 4.0, containing 0.1% of sodium taurodeoxycholate and the chosen amount of enzyme. The reaction was stopped by heating the incubation mixture in a bath of boiling water for 3 min and the liberated radioactive sugar was determined by dialysis methods as previously described (14).

The endo-\(\alpha\)-N-acetylgalactosaminidase activity was assayed by procedure described by Bhavananden et al. (15).

**Analytical Methods—** The free \(N\)-acetylgalactosamine liberated by the enzyme was determined by the Morgan-Elson reaction (16). Polyacrylamide disc gel electrophoresis was performed according to the procedure described by Davis (17) using a Tris/glycine buffer, pH 8.3. Gels were stained for protein with 0.1% Amido black in methanol/acetic acid/water (5:1.5, v/v/v) and destained with the same solvent without dye. The molecular weight of \(\alpha\)-N-acetylgalactosaminidase was estimated by sodium dodecyl sulfate disc gel electrophoresis (18) and by Sephadex G-200 gel filtration (19) using either 0.05 M sodium phosphate buffer, pH 7.0, or 0.05 M sodium citrate buffer, pH 4.2. Thin layer chromatography of glycolipid was performed with Silica Gel G (Merek) and developed by the following solvent system: chloroform/methanol/water (60:30:6 (v/v/v)).

For examining the destruction of blood group A activity, 5 \(\mu\)l (0.18 unit) of \(\alpha\)-N-acetylgalactosaminidase was incubated at 37° overnight with 20 \(\mu\)g of blood group A active substance dissolved in 95 \(\mu\)l of 0.05 M sodium citrate buffer, pH 4.0, containing 0.9% NaCl. After heating in a boiling water bath for 3 min to stop the reaction, an aliquot of this solution was subsequently used for hemagglutination inhibition test. The hemagglutination inhibition test was carried out by using Cooke microtiter (20); 12.5 \(\mu\)l of antisera was added to 12.5 \(\mu\)l of a 2-fold serial dilution of blood group A substances. After incubating at 37° for 1 h, 25 \(\mu\)l of 0.6% suspension of erythrocytes (in 0.15 M NaCl) was then added. The mixture was further incubated at 37° for 1 h and the agglutination was examined by visual inspection.

**RESULTS**

**Purification of \(\alpha\)-N-Acetylglactosaminidase from Limpet Acetone Powder**

Unless otherwise indicated, all operations of the enzyme isolation were carried out at 5°.

**Step 1: Extraction and Ammonium Sulfate Precipitation—** A 20-g portion of limpet acetone powder was homogenized in 1,000 ml of distilled water. The suspension was centrifuged at 17,000 \(\times\) g for 30 min and the residue was discarded. To the extract, 0.2 volume of 0.3 M sodium citrate buffer, pH 4.2, was added. After standing overnight it was centrifuged to obtain about 1,200 ml of clear extract, to which solid (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was added to 20\% saturation. After standing for 4 h, the precipitated protein which contained \(\alpha\)-N-acetylgalactosaminidase was collected by centrifugation and dissolved in 20 ml of 0.05 M sodium citrate buffer, pH 4.2, because of its enhanced stability in this buffer.

**Step 2: Gel Filtration on Sephadex G-200 at pH 7.0—** A 10 ml portion of the enzyme solution obtained at Step 1 was dialyzed thoroughly against 0.05 M sodium phosphate buffer, pH 7.0, before applying to a Sephadex G-200 column (5 \(\times\) 80 cm) which had been previously equilibrated with the same buffer. The column was eluted with the same buffer at 22 ml/h.

The elution profile is shown in Fig. 1A. It should be noted that at pH 7.0 \(\alpha\)-N-acetylgalactosaminidase was eluted after \(\beta\)-galactosidase and separated from \(\beta\)-hexosaminidase. The later fractions containing \(\alpha\)-N-acetylgalactosaminidase activity and minimal \(\beta\)-galactosidase activity were pooled, as indicated by the horizontal bar, and precipitated by adding solid (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4},
to 70% saturation. The precipitate was dissolved in 7 ml of 0.05 M sodium citrate buffer, pH 4.2.

Step 3: Gel Filtration on Sephadex G-200 at pH 4.2—The sample obtained at the end of Step 2 was applied to a Sephadex G-200 column (2 × 55 cm) which had been equilibrated with 0.05 M sodium citrate buffer, pH 4.2, and was eluted with the same buffer. α-N-Acetylgalactosaminidase was eluted together with the first small protein peak and separated from a large protein peak containing α-galactosidase activity which at pH 4.2 was eluted after the α-N-acetylgalactosaminidase (Fig 1A). Those fractions containing α-N-acetylgalactosaminidase activity as shown by the horizontal bar were pooled and precipitated by reverse dialysis against saturated (NH₄)₂SO₄ solution. The precipitate was dissolved in 1.0 ml of 0.05 M sodium phosphate buffer, pH 7.0, and applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm) which had been equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, and dialyzed exhaustively against the same buffer. The remaining 10 ml portion of the enzyme solution obtained at Step 1 was subjected to the Sephadex columns in the same manner as illustrated in Steps 2 and 3.

Step 4: Chromatography on DEAE-Sephadex A-50 Column—The two enzyme solutions obtained from Step 3 were mixed and applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm) previously equilibrated with 0.05 M sodium phosphate buffer, pH 7.0. The column was first washed with the same buffer. Minor contaminants, α-fucosidase and β-hexosaminidase, were eluted by 0.05 M sodium citrate buffer, pH 6.0; subsequently α-N-acetylgalactosaminidase was eluted by 0.05 M sodium citrate buffer, pH 6.0, containing 0.05 M NaCl as shown in Fig. 2A. The α-N-acetylgalactosaminidase fractions were pooled and dialyzed against 0.05 M sodium citrate buffer, pH 4.2, to restore the stability of the enzyme, and then concentrated by reverse dialysis against Ficoll.

Step 5: Chromatography on CM-Sephadex C-50 Column—The enzyme solution (6 ml) obtained from Step 4 was dialyzed against 0.05 M sodium acetate buffer, pH 4.6, and applied to a column (1.3 × 25 cm) of CM-Sephadex C-50 which had been equilibrated with the same buffer. The column was eluted with the same buffer to wash off the proteins not absorbed by the column and then with 0.05 M sodium acetate buffer, pH 5.0 and then 5.6, as shown in Fig. 2B. Most of the α-N acetylgalactosaminidase was eluted by this buffer at pH 5.0. The enzyme fractions were pooled, dialyzed against 0.05 M sodium citrate buffer, pH 4.2, and concentrated by reverse dialysis against Ficoll.

Step 6: Gel Filtration on Sephadex G-200—The fraction (1.7 ml) obtained from Step 5 was applied to a Sephadex G-200 column (1.4 × 100 cm) previously equilibrated with 0.05 M sodium citrate buffer, pH 4.2. The column was eluted with the same buffer; α-N-acetylgalactosaminidase was eluted in the second protein peak as shown in Fig. 3. The enzyme activity and the 280 nm absorption peaks were completely overlapping and symmetrical. The fractions containing α-N-acetylgalactosaminidase were pooled and concentrated by reverse dialysis against Sephadex G-200 powder. A summary of the specific activity and the recovery during the purification procedure is given in Table I.

General Properties of α-N-Acetylgalactosaminidase

Purity—The final enzyme preparation gives one protein band on disc gel electrophoresis at pH 8.3 (Fig. 4), sodium dodecyl sulfate disc gel electrophoresis and also on disc gel isoelectrofocusing. The protein band on disc gel electrophoresis and disc gel isoelectrofocusing corresponds to the enzyme activity which hydrolyzes p-nitrophenyl α-N-acetylgalactosaminide. For evaluating the possible cross-contamination by

![Fig. 2](http://www.jbc.org/)

**Fig. 2. A**, DEAE-Sephadex A-50 chromatography of α-N-acetylgalactosaminidase preparation obtained from Step 3. The enzyme solution (5 ml) containing 20 mg of protein was applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm) which had been equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, 2 ml per fraction was collected. **B**, CM-Sephadex C-50 chromatography of α-N-acetylgalactosaminidase preparation obtained from Step 4. The enzyme solution (6 ml) containing 6 mg of protein was applied to a CM-Sephadex C-50 column (1.3 × 25 cm) which had been equilibrated with 0.05 M sodium acetate buffer, pH 4.5, 2 ml/fraction was collected. **C**, α-N-acetylgalactosaminidase.

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Sephadex G-200 filtration of α-N-acetylgalactosaminidase preparation obtained at Step 5. The enzyme solution (1.7 ml) containing 3.6 mg of protein was applied to a Sephadex G-200 column (1.4 × 100 cm) which had been previously equilibrated with 0.05 M sodium citrate buffer, pH 4.2. The column was eluted with the same buffer at a flow rate of 10 ml/h, 2.0 ml/fraction were collected.
other glycosidases, 0.08 unit of the final preparation of α-N-acetylgalactosaminidase was incubated separately with each of the following p-nitrophenyl glycosides for 22 h: α- and β-galactopyranosides, α- and β-mannopyranosides, α-L-fucopyranoside, α- and β-N-acetylgalactosaminides, and β-glucuronidase.

No activity of α- and β-mannosidase, α-fucosidase, α-N-acetylgalactosaminidase, β-glucuronidase, β-N-acetyhexosaminidase, and endo-α-N-acetylgalactosaminidase was observed. However, this preparation contained about 0.1% of β-galactosidase activity and 6.7% of α-galactosidase activity. This latter activity may be due to the intrinsic specificity of α-N-acetylgalactosaminidase. In order to investigate this possibility, the enzyme was subjected to heat and pH inactivation. The results of this study revealed that these two enzyme activities were decreased to the same extent by these two treatments.

**Molecular Weight**—The molecular weight of α-N-acetylgalactosaminidase, estimated from its chromatographic mobility on Sephadex G-200, is about 45,000 at pH 7.0, 0.05 M sodium phosphate buffer, and is about 200,000 at pH 4.2, 0.05 M sodium citrate buffer. When the sample was examined by disc gel electrophoresis in the presence of sodium dodecyl sulfate according to the procedure described by Weber and Osborn (18), the molecular weight is about 50,000. This study shows a possible tetramer structure for α-N-acetylgalactosaminidase. The existence of tetramer for bovine α-N-acetylgalactosaminidase has also been described (21).

**Optimum pH and Isoelectric Point**—With both p-nitrophenyl α-N-acetylgalactosaminidase and Forssman hapten glycolipid as substrates, optimal activity of this enzyme is at pH 3.8 with citrate phosphate buffer. The isoelectric point of this enzyme is pH 5.5, determined by disc gel isoelectrofocusing using the ampholytes with the pH distributed between pH 3 to 6 or 5 to 8.

**pH Stability and Heat Stability**—The stability of α-N-acetylgalactosaminidase at various pH values was studied by placing the enzyme in 0.05 M citrate phosphate buffer ranging from pH 2.0 to 10.0 for 4 h. The enzyme was then diluted 10-fold in distilled water and assayed at pH 4.0. The enzyme was stable at pH 4.0 for 1 h and was inactivated at pH 10.0.

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in pH from 2.8 to 8.0 at room temperature for 20 h prior to assay with p-nitrophenyl α-N-acetylgalactosaminide at pH 4.0. The enzyme is stable over the pH range of 3.6 to 6.8, but rapidly loses activity below 3.4 or above 7.2. At pH 8.0, it retained only 1% of its activity. The enzyme is stable at 37°C for 5 h in 0.05 M sodium citrate buffer, pH 4.2; however, it loses about 50% of its activity when standing at 40°C for 30 min and 70% at 45°C for 10 min. After heating the enzyme at 40°C for 30 min, and if then cooled at 0°C for 5 to 10 min before assaying the activity, 80% of its original activity can be detected. This indicates that a high temperature may cause the dissociation of this enzyme, and that cooling reassociates its subunits.

Inhibitors – The effect of metal ions and several sugar derivatives on α-N-acetylgalactosaminidase activity was investigated by preincubating the enzyme with the inhibitor for 20 min at 37°C before the addition of p-nitrophenyl α-N-acetylgalactosaminide. The results are summarized in Table II. Of the various metal ions tested, Ag⁺ and Hg²⁺ were potent inhibitors. On the other hand, Ca²⁺ and Ba²⁺ somewhat increased the activity. Zn²⁺, Mg²⁺, Mn²⁺, and Cu²⁺ do not affect the enzyme activity. N-Acetylglucosamine did not show any inhibitory action, while galactose and N-acetylgalactosamine exerted some inhibitory effect.

Effect of Substrate Concentration – The effect of substrate concentrations on the reaction rate of α-N-acetylgalactosaminidase was measured at 37°C using 0.05 M sodium citrate buffer, pH 4.0. The apparent Michaelis constant (Kₘ) for each substrate was determined from the Lineweaver-Burk plots to be 0.6 mM for p-nitrophenyl α-N-acetylgalactosaminide and 0.036 mM for Forssman hapten glycolipid.

Application of α-N-Acetylgalactosaminidase for Structural Studies of Various Glycoconjugates

Hydrolysis of Forssman Hapten Glycolipid – α-N-Acetylgalactosaminidase cleaves the terminal N-acetylgalactosamine efficiently from tritium-labeled Forssman hapten glycolipid. This cleavage was checked by measuring the released radioactive N-acetylgalactosamine and also by thin layer chromatography of the produced globoside. The sequencing of Forssman hapten glycolipid by individual glycosidases is illustrated graphically in Fig. 5. Forssman hapten glycolipid was treated sequentially as follows: first with α-N-acetylgalactosaminidase from limpet α-N-acetylgalactosaminidase; thereafter, the liberated α-N-acetylgalactosamine was preincubated with p-nitrophenyl α-N-acetylgalactosaminide at pH 4.0. The liberated α-N-acetylgalactosamine was then measured.

Application of α-N-Acetylgalactosaminidase for Structural Studies of Various Glycoconjugates

Degradation of blood group A activity by α-N-acetylgalactosaminidase as followed by hemagglutination inhibition tests

The assay conditions are described in the text.

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⁎ PSM, porcine submaxillary mucin; +, inhibit the agglutination; -, not inhibit the agglutination; α-GalNAcase, α-N-acetylgalactosaminidase.
dase; second with \( \beta \)-N-acetyhexosaminidase; third with \( \alpha \)-galactosidase; and finally with \( \beta \)-galactosidase. When Forssman hapten glycolipid was incubated with the mixture of \( \alpha \)- and \( \beta \)-galactosidases and \( \beta \)-hexosaminidase, without \( \alpha \)-N-acetylglucosaminidase, no conversion of the original glycolipid was observed. These results show clearly that the anomic sequential arrangement of sugar units in Forssman hapten glycolipid is \( \text{GalNAc} \rightarrow \text{GalNAc}\beta \rightarrow \text{Gala} \rightarrow \text{Gal}\beta \rightarrow \text{Glc} \rightarrow \text{Cer} \).

Hydrolysis of Bovine Submaxillary Glycoprotein—Asialo bovine submaxillary glycoprotein was incubated with \( \alpha \)-N-acetylgalactosaminidase in 0.05 M sodium citrate buffer, pH 4.0, and the resultant free hexosamine was determined by the Morgan-Elson Reaction (16). As shown in Fig. 6, 100% of the total \( \alpha \)-galactosaminidase was liberated from asialo bovine submaxillary glycoprotein by this enzyme in 6 h. In the same sample, incubated with \( \beta \)-N-acetyhexosaminidase, no hexosamine was liberated.

Hydrolysis of Blood Group A Active Substances—Several blood group A active glycoproteins and glycolipids obtained from various sources were subjected to hydrolysis by \( \alpha \)-N-acetylgalactosaminidase and the hemagglutination inhibition activity of the resultant samples was examined (Table III). The compounds tested were blood group A active glycoproteins from pig submaxillary mucin and from human ovarian cyst; the glycolipid was from dog intestine. Hemagglutination inhibiting activities of all three of these blood group A substances were reduced or destroyed by \( \alpha \)-N-acetylgalactosaminidase.

**DISCUSSION**

By the simple procedure described above, we have isolated a highly purified preparation of \( \alpha \)-N-acetylgalactosaminidase from a convenient source, commercially available limpet acetone powder. The major feature of this preparative method is the application of our observation that the molecular weight of this enzyme is about 200,000 at pH 4.2 and is 45,000 at pH 7.0. As shown in Fig. 1A, at pH 7.0, this enzyme is eluted from a Sephadex G-200 column after most of the other glycosidases, whereas at pH 4.2, it is eluted from the same column prior to most of the other glycosidases (Fig. 1B). By this simple procedure, we were able to eliminate most of the contaminating glycosidases.

The purification procedure outlined in Table I, enabled us to recover 25% of the enzyme with an 860 fold increase in its specific activity. The final preparation is electrophoretically homogeneous by disc gel electrophoresis, sodium dodecyl sulfate-dick gel electrophoresis, and disc gel isoelectrofocusing. In spite of its electrophoretic purity, the final preparation contains about 0.1% of \( \beta \)-galactosidase activity which we regard as a contaminant. However, the 6.7% of \( \alpha \)-galactosidase activity shown in the final preparation may be the intrinsic activity of the limpet \( \alpha \)-N-acetylgalactosaminidase for the following reasons. Both enzyme activities, \( \alpha \)-N-acetylgalactosaminidase and \( \alpha \)-galactosidase, were reduced to the same degree by heat and pH inactivation. Both enzymes had the identical association and dissociation behavior as a function of pH. Both activities behaved identically during Sephadex G-200 chromatography at pH 7.0 and pH 4.2. Both activities also behaved identically when the sample was chromatographed on a DEAE-Sephadex A-50 column. Furthermore, both activities were inhibited by galactose as well as by \( \beta \)-acetylgalactosamine (cf. Table III). It should be pointed out that limpet extract contains two \( \alpha \)-galactosidase activities. By Sephadex G-200 filtration at pH 4.0, one of them is eluted together with \( \alpha \)-N-acetylgalactosaminidase and the other overlapped with \( \beta \)-galactosidase peak. The latter does not have \( \alpha \)-N-acetylgalactosaminidase activity. The close structural resemblance between galactose and \( \alpha \)-acetylgalactosamine makes it conceivable that the same enzyme may hydrolyze both \( \alpha \)-galactosides and \( \alpha \)-N-acetylgalactosaminides. However, we recognize the possibility that \( \alpha \)-galactosidase may be a separate contaminating enzyme in our \( \alpha \)-N-acetylgalactosaminidase preparation. In conjunction with this observation, Dean et al. (22) recently reported that the R-form of pure \( \alpha \)-galactosidase isolated from human liver also hydrolyzed \( \alpha \)-nitrophenyl \( \alpha \)-N-acetylgalactosaminide and Forssman hapten glycolipid.

As shown in Fig. 1, the molecular weight of this enzyme is about 200,000 at pH 4.2 and is 45,000 at pH 7.0 from Sephadex G-200 column chromatography. When \( \alpha \)-N-acetylgalactosaminidase obtained from the pH 7.0 column was reapplied to the pH 4.2 column, the enzyme was eluted in an earlier fraction than that of the pH 7.0 column. On the other hand, the enzyme fraction obtained from the pH 4.2 column would move to the position of lower molecular weight in the pH 7.0 column. This strongly suggests that \( \alpha \)-N-acetylgalactosaminidase dissociates at pH 7.0 and associates at pH 4.2. This association-dissociation procedure is reversible. Wang and Weissman (21) have shown that \( \alpha \)-N-acetylgalactosaminidase from bovine liver dissociated at low enzyme concentration and high temperature, although they did not check the pH dependency. \( \beta \)-Galactosidase from mouse liver has been shown to reversibly dissociate at alkaline pH to subunits of smaller molecular weight (23).

This enzyme cleaves the terminal \( \alpha \)-acetylgalactosamine from a number of glycoproteins and glycolipids, including blood group A active glycoproteins from human ovarian cyst, oyster viscera (24), and from porcine submaxillary mucin, blood group A active glycolipid from dog intestine, asialo bovine submaxillary glycoproteins, and Forssman hapten. As shown in Fig. 5, this enzyme can be used in combination with \( \beta \)-N-acetylgalactosaminidase, \( \alpha \)- and \( \beta \)-galactosidases to convert Forssman hapten sequentially into globoside, trisaccharide-1, lactosyl-1, and glucosylceramide. Because of its broad specificity, this enzyme will be extremely valuable for the structural analysis of glycoconjugates.

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