Purification and Enzymatic Properties of β -*N*-Acetylhexosaminidase from Common Octopus Liver

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The liver of common octopus *Octopus vulgaris* contained extremely high activity of β -*N*-acetylhexosaminidase which is useful for structural and functional studies of complex carbohydrates. A simple procedure has been devised for the isolation of β -*N*-acetylhexosaminidase from the liver in an electrophoretically homogeneous form. The procedure involved heat treatment at 68°C for 20 min, Sephadex G-200 gel filtration, and CM-Sephadex C-50 chromatography. The purified enzyme was sufficiently free from other glycosidases and proteases. The molecular weight of the enzyme was estimated to be 147,000 by Sephadex G-200 gel filtration. The enzyme exhibited the optimum pH at pH 5.0 and was stable between pH 4 and 8. For *p*-nitrophenyl β -*N*-acetylglucosaminide, *K*_m was 0.63 mM and *V*_{max} was 192 μ mol/min·mg.

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 complex carbohydrates such as glycoproteins and glycolipids,^{1,2)} since these biologically important compounds contain β -*N*-acetylglucosaminyl units as their major constituents. 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⁾ and ascidian.¹⁹⁾ In the utilization of glycosidase for the structural and functional studies, the enzyme must be sufficiently free from contamination by other glycosidases and proteases. 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 found that common octopus liver offers a very good source for the isolation of β -*N*-acetylhexosaminidase since it contains extremely high activity of the enzyme. In this paper, we describe a simple procedure for the purification of β -*N*-acetylhexosaminidase from common octopus liver and some properties of the purified enzyme.

Material and Methods

Acetone Powder of Common Octopus Liver

The liver of common octopus *Octopus vulgaris* was obtained from a fish store. Fresh liver was homogenized with its 5 volumes of acetone and filtered. The residue on the filter was washed with acetone repeatedly and dried under reduced pressure.

Enzyme Assays

Activities of β -*N*-acetylhexosaminidase and other glycosidases were assayed at 37°C by using the corresponding *p*-nitrophenyl glycoside as substrate. The enzyme solution (50 μ l) was added to 0.3 ml of 2 mM *p*-nitrophenyl glycoside dissolved in 0.05 M sodium citrate buffer, pH 4.6. After incubation for a preset time, 3.0 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 400 nm. One unit of enzyme was defined as the amount of enzyme which hydrolyzes 1 μ mol of *p*-nitrophenyl glycoside/min under the conditions described above. The specific activity of the enzyme was expressed as unit per mg of protein, as determined by the method of Lowry *et al.*²⁰⁾ with crystalline bovine serum albumin as the standard.

Analytical Methods

Polyacrylamide disc gel electrophoresis was

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performed according to the procedure described by Davis²¹⁾ using a Tris/glycine buffer, pH 8.3. 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). The molecular weight of common octopus β -*N*-acetylhexosaminidase was determined by Sephadex G-200 gel filtration according to the procedure of Andrews.²²⁾ Protease activity was examined by using Azocoll as substrate.²³⁾

Results

Glycosidase Activities in Common Octopus Liver

One gram of the acetone powder of common octopus liver was homogenized with 20 ml of 0.05 M sodium citrate buffer, pH 4.6, and centrifuged. Various glycosidase activities in the supernatant obtained were assayed. Table 1 shows typical data of glycosidase activities found in the liver. Comparatively high activities of glycosidases were found in the liver. Particularly, β -*N*-acetylhexosaminidase activity is extremely high. This suggests that common octopus liver is a quite suitable source for the isolation of β -*N*-acetylhexosaminidase.

Table 1. Glycosidase activities in acetone powder of octopus liver

Glycosidase	Units/g of acetone powder
β - <i>N</i> -Acetylhexosaminidase [EC 3.2.1.30]	150
α -Mannosidase [EC 3.2.1.24]	14
α - <i>N</i> -Acetylglactosaminidase [EC 3.2.1.49]	11
β -Galactosidase [EC 3.2.1.23]	8.5
β -Mannosidase [EC 3.2.1.25]	4.0
α -Fucosidase [EC 2.2.1.51]	2.5
α -Galactosidase [EC 3.2.1.22]	1.5

Purification of β -*N*-Acetylhexosaminidase from Common Octopus Liver

1. Step 1. Extraction and ammonium sulfate precipitation—All operations of the enzyme purification were carried out at about 5°C. A 10-g portion of the acetone powder of common octopus liver was homogenized in 200 ml of 0.05 M sodium citrate buffer, pH 4.6. The homogenate was centrifuged at $15,000 \times g$ for 30 min. To the clear extract, solid ammonium sulfate was added to 35% saturation. After standing for 3 h, the precipitate was removed by centrifugation and

more ammonium sulfate was added to the supernatant to obtain 75% saturation. After standing overnight, the precipitate collected by centrifugation was dissolved in a minimum volume of distilled water and dialyzed exhaustively against water. The dialyzed solution was centrifuged to obtain a clear solution. Although 30% of β -*N*-acetylhexosaminidase activity was lost, about 40% of proteins was removed by the dialysis.

2. Step 2. Heat treatment—To determine the proper heating condition, small portions of the enzyme solution obtained at the end of Step 1 were heated at 68°C for various lengths of time and the residual activities were assayed. The result is shown in Fig. 1. On heating at 68°C for 20 min, about 50% of β -*N*-acetylhexosaminidase activity was lost. On the other hand, the heating inactivated almost all of β -galactosidase which could not be separated from β -*N*-acetylhexosaminidase by the following Sephadex G-200 gel filtration and denatured a fair amount of proteins. On the basis of the result of this preliminary experiment, the enzyme solution was heated at 68°C for 20 min and centrifuged to remove the denatured proteins.

Fig. 1. Thermal inactivation of glycosidases in crude enzyme solution by heating at 68°C. Experimental details are described in the text. ●, β -*N*-acetylhexosaminidase; ○, α -mannosidase; △, β -galactosidase; ▲, α -*N*-acetylglactosaminidase.

3. Step 3. Sephadex G-200 gel filtration—The enzyme solution obtained at Step 2 was subjected to Sephadex G-200 gel filtration. The elution profile is shown in Fig. 2. β -*N*-Acetylhexosaminidase was separated sufficiently from α -mannosidase which was major contaminating glycosidase. The fractions containing β -*N*-

Fig. 2. Sephadex G-200 gel filtration of the enzyme solution obtained at Step 2. The enzyme solution (15 ml) containing 0.3 g of protein was applied to a Sephadex G-200 column (4.5×65 cm) previously equilibrated with 0.05 M sodium citrate buffer, pH 5.0. The column was eluted with the same buffer at a flow rate of 20 ml/h and 10-ml fractions were collected. ---, absorbance at 280 nm; ●, β -N-acetylhexosaminidase; ○, α -mannosidase.

Fig. 3. CM-Sephadex C-50 chromatography of the β -N-acetylhexosaminidase preparation obtained from Step 3. The enzyme solution (10 ml) containing 32 mg of protein was applied to a CM-Sephadex C-50 column (2.2×21 cm) previously equilibrated with 0.025 M sodium citrate buffer, pH 5.0. The column was eluted at a flow rate of 30 ml/h with the same buffer (a), followed by elution with 0.05 M sodium citrate buffer, pH 5.4 (b), and then eluted at a flow rate of 15 ml/h with a sodium chloride gradient in 0.05 M sodium citrate buffer, pH 5.8 (c). Fractions (10 ml) were collected. ---, absorbance at 280 nm; ●, β -N-acetylhexosaminidase.

Table 2. Purification of β -N-acetylhexosaminidase from 10 g of octopus liver acetone powder

Procedures	Total units	Total protein mg	Specific activity units/mg	Recovery %
Crude extract	1500	2310	0.649	100
35–75% $(\text{NH}_4)_2\text{SO}_4$ ppt	1420	1160	1.22	94.7
Dialysis against H_2O	994	662	1.50	66.3
Heat treatment	490	310	1.58	32.7
Sephadex G-200	443	36.0	12.3	29.5
CM-Sephadex C-50	292	2.2	133	19.5

acetylhexosaminidase activity as shown by horizontal bar were pooled and the enzyme was precipitated by reverse dialysis against saturated ammonium sulfate solution. The precipitate collected by centrifugation was dissolved in 3 ml of

0.025 M sodium citrate buffer, pH 5.0, and dialyzed exhaustively against the same buffer.

4. Step 4. CM-Sephadex C-50 chromatography—The β -N-acetylhexosaminidase solution obtained at Step 3 was subjected to CM-Sephadex

C-50 chromatography. As the elution profile is shown in Fig. 3, the column was first eluted with the same buffer, and then with 0.05 M sodium citrate buffer, pH 5.4. These elutions removed some contaminating proteins. Subsequently, β -*N*-acetylhexosaminidase was eluted with a sodium chloride gradient in 0.05 M sodium citrate buffer, pH 5.8. The fractions containing the enzyme activity as shown by horizontal bar were pooled and concentrated by ultrafiltration with a Diaflo PM-10 membrane. A summary of the specific activity and recovery during the purification procedure is given in Table 2.

Properties of β -*N*-Acetylhexosaminidase

1. Purity—The final enzyme preparation gave one protein band on disc gel electrophoresis, as shown in Fig. 4. To evaluate the possible contamination by other glycosidases, the purified enzyme (0.17 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. No activities of α - and β -galactosidase, α - and β -mannosidase, α - and β -glucosidase, α -fucosidase, α -*N*-acetylgalactosaminidase, and β -glucuronidase were observed. No protease activity was also observed.

2. Molecular weight—The molecular weight of β -*N*-acetylhexosaminidase was estimated to be 147,000 by Sephadex G-200 gel filtration (Fig. 5).

3. pH optimum and pH stability—The effect of pH on the enzyme activity was examined by

Fig. 4. Disc gel electrophoresis of purified β -*N*-acetylhexosaminidase at pH 9.5.

Fig. 5. Estimation of molecular weight of β -*N*-acetylhexosaminidase by Sephadex G-200 gel filtration. Column, 2.4 × 76 cm, Eluent, 0.05 M sodium phosphate buffer, pH 6.8, containing 0.1 M NaCl. 1, ribonuclease A; 2, chymotrypsinogen A; 3, ovalbumin; 4, bovine serum albumin; 5, common octopus β -*N*-acetylhexosaminidase; 6, aldolase; 7, catalase; 8, ferritin.

using *p*-nitrophenyl β -*N*-acetylglucosaminide as substrate in sodium citrate and sodium phosphate buffers (Fig. 6). The enzyme exhibited the maximum activity at pH 5.0 and did not exhibit the activity above pH 8.

Fig. 6. Effect of pH on β -*N*-acetylhexosaminidase activity. ○, sodium citrate buffer; ●, sodium phosphate buffer.

The stability of the enzyme at various pH values was examined. The enzyme was incubated in buffers ranging from pH 2.5 to 9.5 for 4 h at 25°C and then the residual activity was assayed at pH 4.6. Untreated enzyme dissolved in 0.05 M sodium phosphate buffer, pH 6.8, was used as a control and was assumed to have 100% activity. As shown in Fig. 7, the enzyme activity was fairly

Fig. 7. Effect of pH on the stability of β -*N*-acetylhexosaminidase. $\circ$, sodium citrate buffer; $\bullet$, sodium phosphate buffer; Δ , ammonium chloride buffer.

stable between pH 4 and 8.

4. Effect of substrate concentration—The effect of substrate concentration on the velocity of hydrolysis of *p*-nitrophenyl β -*N*-acetylglucosaminide was studied. The initial velocities were determined by the method of Algranati.²⁴ The Michaelis constant (K_m) and the maximum velocity were calculated from the Lineweaver-Burk plots shown in Fig. 8. The K_m value was 0.63 mM and the maximum velocity was 192 μ mol/min $\cdot$ mg.

Fig. 8. Lineweaver-Burk plots for hydrolysis of *p*-nitrophenyl β -*N*-acetylglucosaminide.

5. Effect of inhibitors—The effects of inhibitors on the enzyme activity were examined by incubating the enzyme with various inhibitors for 30 min at 25°C before the addition of *p*-nitrophenyl β -*N*-acetylglucosaminide. As shown in Table 3, the enzyme was strongly inhibited by Ag^+ and Hg^{2+} , and competitively inhibited by *N*-acetylgalactosamine and *N*-acetylglucosamine.

Table 3. Effect of various inhibitors on activity of β -*N*-acetylhexosaminidase

Inhibitors	Inhibitor concentration mM	Relative activity %
AgNO_3	1.0	0
HgCl_2	1.0	0
CaCl_2	10	100
$\text{Zn}(\text{AcO})_2$	10	100
MgSO_4	10	97
MnCl_2	10	95
CuSO_4	10	87
Glucose	10	100
Galactose	10	100
<i>N</i> -Acetylglucosamine	10	92
<i>N</i> -Acetylgalactosamine	10	80

6. Effect of sodium chloride—The effect of sodium chloride on the enzyme activity was examined since β -*N*-acetylhexosaminidase from the liver of marine top shell was remarkably activated by sodium chloride.¹⁸ As shown in Fig. 9, only 5% activation by sodium chloride was observed at the concentration of 1 M.

Fig. 9. Effect of sodium chloride concentration on β -*N*-acetylhexosaminidase activity.

7. Stability during storage—The stability of β -*N*-acetylhexosaminidase activity depended on the protein concentration; the enzyme activity was unstable in the diluted enzyme solution. Therefore, the purified enzyme was concentrated to a minimum volume by ultrafiltration. The concentrated enzyme solution in 0.05 M sodium citrate buffer, pH 5.0, was kept in an ice bath without loss of its activity for at least 3 weeks. On the other hand, the enzyme activity was unstable on freezing and thawing the concentrated solution. For long storage, sucrose was added to the concentrated enzyme solution to obtain a concentration of 1%, followed by lyophilization. During lyophilization under this condition, no appreciable loss of the enzyme activity was observed. The

lyophilized enzyme may be kept in a freezer for several years.

Discussion

Electrophoretically homogeneous β -*N*-acetylhexosaminidase has been isolated from common octopus liver by the simple procedure. The advantage in the use of common octopus liver as a source of the enzyme is that it contains extremely high β -*N*-acetylhexosaminidase activity and that it is readily available. Usually, a fairly large sized common octopus has about 50 g of liver which contains about 1,500 units of β -*N*-acetylhexosaminidase activity. One may be able to isolate a sufficient amount of the enzyme from 50 g of the liver by the present purification procedure.

The acetone powder of the liver gave a clear enzyme extract to facilitate the subsequent purification procedure. Moreover, the total activity of the enzyme in the acetone powder is preserved for several years when the powder is kept in a freezer. The treatment of the fresh liver with acetone decreased about 10% of β -*N*-acetylhexosaminidase activity.

The extractability of the enzyme from common octopus liver increased more than 20% after moderate autolysis of the liver, suggesting that most of the enzyme occur in the lysosomes of the liver.

Although the heating of the crude enzyme solution at 68°C for 20 min decreased about 50% of β -*N*-acetylhexosaminidase activity, it eliminated almost all of β -galactosidase and α -*N*-acetylgalactosaminidase activities (Fig. 1). It was very difficult in this sample to separate β -*N*-acetylhexosaminidase from β -galactosidase by conventional chromatographies. Therefore, the heat treatment was the effective step for the elimination of β -galactosidase. α -Mannosidase was rather stable on the heating. However, it was sufficiently separated from β -*N*-acetylhexosaminidase by Sephadex G-200 gel filtration (Fig. 2). It should be noted that the purified β -*N*-acetylhexosaminidase was almost inactivated when it was heated at 68°C for 20 min (data not shown), although the crude one was rather thermostable.

Muramatsu reported that β -*N*-acetylhexosaminidase,¹⁸⁾ α -mannosidase,^{25,26)} and β -mannosidase²⁵⁾ from marine top shells were remarkably activated by sodium chloride. However, practically no effect of sodium chloride on the common octopus β -*N*-acetylhexosaminidase ac-

tivity was observed (Fig. 9).

Many β -*N*-acetylhexosaminidases have been isolated from various sources, and thus far, all which have been studied are active in cleaving the terminal β -*N*-acetylglucosamine present in complex carbohydrates. Particularly, β -*N*-acetylhexosaminidases from marine animals have a broad aglycon specificity.²⁷⁾ Accordingly, the common octopus β -*N*-acetylhexosaminidase, which can be isolated by the simple purification procedure from the readily available source, may be valuable for structural and functional studies on various complex carbohydrates.

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