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Purification and Characterization of α-L-Fucosidase from the Liver of Seahare, *Aplysia kurodai*

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An α-L-fucosidase [EC 3.2.1.51] was isolated from liver of the marine gastropod, *Aplysia kurodai.* The enzyme was purified by procedures involving extraction, ammonium sulfate precipitation, and chromatographies on diethylaminoethyl (DEAE)-Sephadex, hydroxylapatite and Sepharose 6B. The final preparation was sufficiently free from other glycosidase activities and gave a single protein band on disc gel electrophoresis. The molecular weight of the enzyme was estimated to be 330000 by Sephadex G-200 column chromatography. The enzyme has two optimal pH values, one at pH 3.0 and the other at pH 6.4, but it was stable only in the pH range from 4.5 to 6.0. The enzyme released fucose not only from *p*-nitrophenyl α-L-fucoside but also from human milk oligosaccharides.

Keywords——α-L-fucosidase; seahare liver; *Aplysia kurodai*; enzyme purification; enzyme properties; two optimal pHs

α-L-Fucosidase [EC 3.2.1.51] is very useful for structural analysis and for the elucidation of the biological function of complex carbohydrates, since α-linked fucosyl units are common constituents of biologically important compounds such as glycoproteins and glycolipids.[1] In particular, the enzyme is extremely valuable for this purpose if it is sufficiently free from contamination by other glycosidases and has a broad aglycon specificity.

α-L-Fucosidase has been partially purified from mammalian tissues,[2] invertebrates,[3] bacteria[1,4] and plants.[5] Although the aglycon specificity of the enzyme isolated from these sources is generally narrow, the enzyme from marine gastropods exhibits broad aglycon specificity.[6] During work on the isolation of glycosidases,[7] we found that the liver of a marine gastropod, *Aplysia kurodai*, is rich in α-L-fucosidase. In this paper, we describe a simple procedure for the isolation of this α-L-fucosidase free from activities of other glycosidases, and we report some properties of the purified enzyme.

Materials and Methods

Materials——*p*-Nitrophenyl (PNP)-α-L-fucoside was purchased from Nakarai Chemical Co. (Osaka, Japan), PNP-α- and β-D-xylosides and PNP-α-D-*N*-acetylgalactosaminide were from Koch-Light laboratories (Colnbrook, U.K.), PNP-α-D-*N*-acetylglucosaminide was from Aldrich Chemical Co. (Milwaukee, U.S.A.), and PNP-α- and β-D-galactosides, PNP-α- and β-D-glucosides, PNP-α-D-mannoside, PNP-β-L-fucoside, PNP-β-D-*N*-acetylgalactosaminide and PNP-β-D-*N*-acetylglucosaminide were from Sigma Chemical Co. (St. Louis, U.S.A.). DEAE-Sephadex A-50 and Sepharose 6B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Lacto-*N*-fucopentaose I and a mixture of lacto-*N*-fucopentaoses II and III were prepared from human milk as described by Kobata.[8] All other chemicals were obtained from commercial sources and were of the highest grade available. The livers were taken from fresh seahare and were kept frozen at −20 °C until use. In some experiments, the tissue was homogenized and extracted without freezing.

Enzyme Assay——α-L-Fucosidase and other glycosidase activities were determined by a modification of the procedures described previously[7b] using *p*-nitrophenyl glycosides as substrates. The enzyme solution (10 μl) was added to 0.1 ml of 2 mM *p*-nitrophenyl glycoside dissolved in 0.05 M sodium citrate buffer (pH 4.0 or 6.3). After incubation of the mixture for an appropriate period at 37 °C, 0.7 ml of 0.2 M sodium borate buffer (pH 9.8) was added

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and the p-nitrophenol liberated was determined from the absorption at 400 nm. One unit of enzyme was defined as the amount of enzyme which hydrolyzed 1 μmol of p-nitrophenyl glycoside per minute under the conditions described above. The specific activity was expressed as units per mg of protein.

Analytical Methods——Protein concentration was determined spectrophotometrically at 280 nm or by the method of Lowry *et al.*[10] using bovine serum albumin as the standard. Polyacrylamide disc gel electrophoresis was performed according to the procedure described by Davis[11] using Tris–glycine buffer (pH 8.3). Gels were stained with 0.25% Coomassie brilliant blue G-250 in 12.5% trichloroacetic acid according to the method described by Diezel *et al.*[12] and destained with 5% acetic acid. To check the enzyme activity after electrophoresis, the gel was immediately sliced into 0.3 cm sections. The enzyme activity in gel slices was determined by incubation of each section of gel with 0.5 ml of 2 mM p-nitrophenyl α-L-fucoside dissolved in 0.05 M sodium citrate buffer (pH 6.3). The molecular weight of α-L-fucosidase was estimated by Sephadex G-200 gel filtration using 0.05 M phosphate buffer (pH 7.0) according to the procedure of Andrews.[13] The Sephadex G-200 column (1.6 × 62 cm) was eluted with the buffer at a flow rate of 6 ml per h. Fractions of 1 ml were collected. The elution volume was calculated from the midpoint of the peak. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out according to the procedure described by Laemmli.[14] The following proteins, obtained from Pharmacia Fine Chemicals, were used as standards for molecular weight estimation: chymotrypsinogen A (25000), ovalbumin (43000), bovine serum albumin (67000), aldolase (158000), catalase (232000), and ferritin (440000). The hydrolysis of oligosaccharides by α-L-fucosidase from seahare liver was performed as follows. Each of the oligosaccharides (100 μg) was treated with 0.03 unit of the enzyme in 100 μl of 0.05 M citrate–phosphate buffer (pH 6.3) at 37 °C for 18 h. The reaction was stopped by heating the mixture in a boiling water bath for 3 min and the mixture was deproteinized and desalted by passage through a small column of Dowex 1- × 8 (HCO_3^- form) with Dowex 50- × 8 (H^+ form) stacked on top.[9] The eluate from the column was concentrated and applied on a thin layer plate. Thin layer chromatography was performed with cellulose plates (Merck) and development was done with the following solvent system, ethyl acetate–pyridine–acetic acid–water (5 : 5 : 1 : 3, by volume). The plate was sprayed with alkaline-silver nitrate reagent.[15]

Results

Purification of α-L-Fucosidase

All isolation and purification procedures were carried out at 0—4 °C.

Step 1. Extraction and Ammonium Sulfate Precipitation——Frozen or fresh seahare liver (50 g) was homogenized in 4 volumes (v/w) of distilled water with a Waring blender

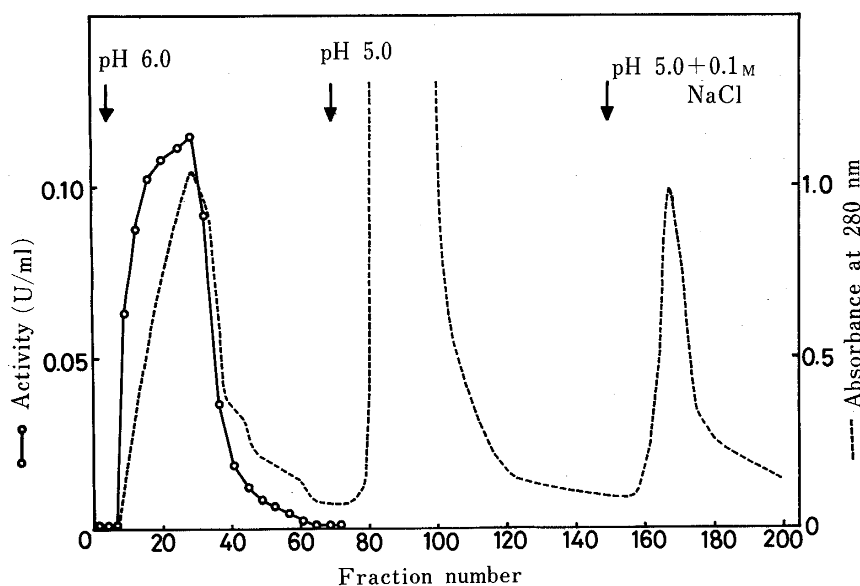

Fig. 1. DEAE-Sephadex A-50 Column Chromatography of the α-L-Fucosidase Preparation Obtained by Ammonium Sulfate Precipitation

The sample (190 mg protein) was applied to a DEAE-Sephadex A-50 column (1.5 × 6 cm) which had been equilibrated with 0.02 M phosphate buffer (pH 6.0). The column was eluted with the same buffer, followed by 0.05 M sodium citrate buffers (pH 5.0 and pH 5.0 containing 0.1 M NaCl), and 20 ml fractions were collected. ----, absorption at 280 nm; ○, α-L-fucosidase activity.

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(twice for 30 s). The homogenate was centrifuged at 30000 × *g* for 20 min and the pellet was discarded. The extract was dialyzed against 20 volumes of 0.05 M sodium citrate buffer (pH 4.5) overnight, and then centrifuged at 25000 × *g* for 20 min to obtain a clear extract. The supernatant solution was brought to 25% saturation with solid ammonium sulfate and allowed to stand for 4 h. After removal of the precipitate by centrifugation (25000 × *g* for 20 min), more ammonium sulfate was added to the supernatant fluid to obtain 75% saturation. After standing overnight, the precipitated protein, which contained α-L-fucosidase, was collected by centrifugation at 10000 × *g* for 30 min and dissolved in 40 ml of 0.05 M sodium citrate buffer (pH 5.0).

Step 2. DEAE-Sephadex Column Chromatography——The enzyme solution obtained at step 1 was dialyzed against 0.02 M phosphate buffer (pH 6.0) and applied to a column of DEAE-Sephadex A-50 which had been equilibrated with the same buffer. The column was first eluted with the same buffer and then with 0.05 M sodium citrate buffer (pH 5.0) followed by 0.05 M sodium citrate buffer (pH 5.0) containing 0.1 M sodium chloride, as shown in Fig. 1. α-L-Fucosidase was eluted with 0.02 M phosphate buffer (pH 6.0). Although the enzyme preparation before the DEAE-Sephadex column step contained 560% β-*N*-acetylhexosaminidase and 550% β-galactosidase activities, the two activities in the α-L-fucosidase fraction obtained from the column were less than 0.9% of α-L-fucosidase activity (Table II). α-L-Fucosidase was effectively separated from β-*N*-acetylhexosaminidase and β-galactosidase by this column chromatography. The fractions containing α-L-fucosidase activity were pooled.

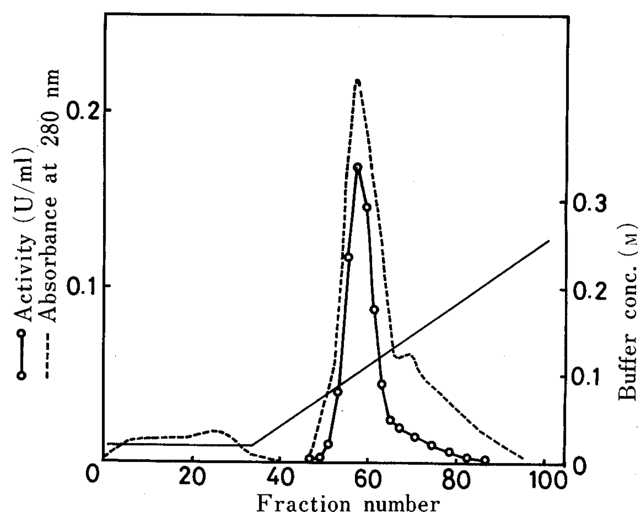

Fig. 2. Hydroxylapatite Column Chromatography of the α-L-Fucosidase Preparation Obtained by DEAE-Sephadex A-50 Column Chromatography

The sample (20 mg protein) was applied to a hydroxylapatite column (1.9 × 12 cm) which had been equilibrated with 0.02 M phosphate buffer (pH 6.0). The column was eluted with the same buffer followed by linearly increasing molarities of the same buffer, and 10 ml fractions were collected. ----, absorption at 280 nm; ○, α-L-fucosidase activity.

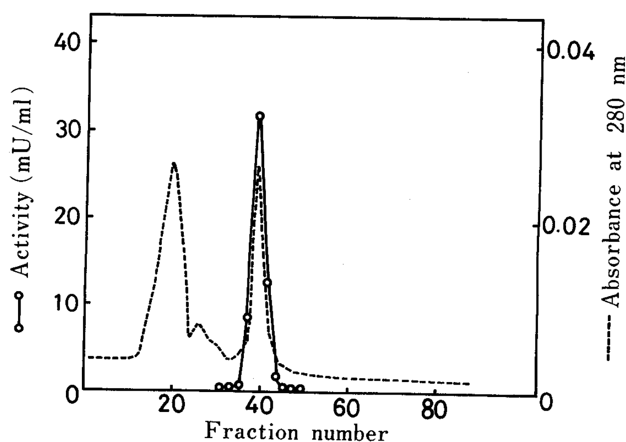

Fig. 3. Sepharose 6B Gel Filtration of the α-L-Fucosidase Preparation Obtained by Hydroxylapatite Column Chromatography

The sample (15 mg protein) was applied to a Sepharose 6B column (1.2 × 90 cm) which had been equilibrated with 0.05 M sodium citrate buffer (pH 5.0). The column was eluted with the same buffer at a flow rate of 5 ml per hour, and 2 ml fractions were collected. ----, absorption at 280 nm; ○, α-L-fucosidase activity.

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TABLE I. Purification of α-L-Fucosidase from the Liver of *Aplysia kurodai*

	Total unit	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)
Crude extract	239.9	4353.4	0.055	100
$(NH_4)_2SO_4$ ppt.	209.5	2955.4	0.074	87.3
DEAE-Sephadex	75.6	208.8	0.36	31.5
Hydroxylapatite	39.6	83.5	0.47	16.5
Sepharose 6B	31.1	11.4	2.73	13.0

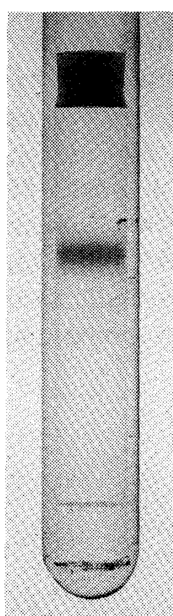

Fig. 4. Disc Gel Electrophoresis of the Seahare Liver α-L-Fucosidase at pH 8.3

The gel was stained with Coomassie brilliant blue G-250.

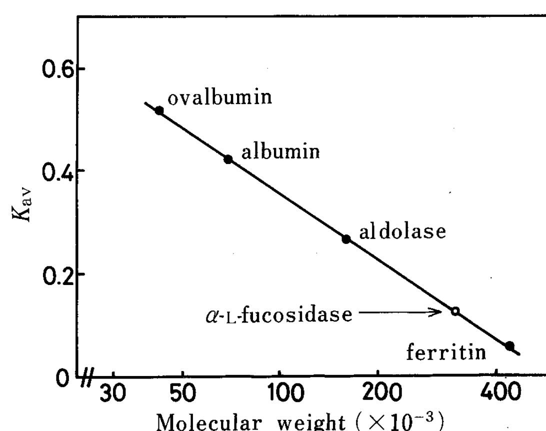

Fig. 5. Estimation of Molecular Weight by Sephadex G-200 Gel Filtration

The experimental conditions are described in the text.

Step 3. Hydroxylapatite Column Chromatography——The pooled fractions containing α-L-fucosidase obtained at step 2 were applied to a column of hydroxylapatite which had been equilibrated with 0.02 M phosphate buffer (pH 6.0). The column was washed with the starting buffer and eluted with linearly increasing molarities of the same buffer. α-L-Fucosidase was eluted as a single peak, as shown in Fig. 2. The fractions containing α-L-fucosidase were pooled and concentrated by ultrafiltration with a Diaflo Membrane PM-30.

Step 4. Sepharose 6B Gel Filtration——The concentrated enzyme preparation obtained from hydroxylapatite column chromatography was dialyzed against 100 volumes of 0.05 M sodium citrate buffer (pH 5.0) and applied to a Sepharose 6B column previously equilibrated with the same buffer. The column was eluted with the same buffer; α-L-fucosidase was eluted in the third protein peak as shown in Fig. 3. The enzyme activity and 280 nm absorption peak were completely coincident. The fractions containing α-L-fucosidase activity were pooled and concentrated by ultrafiltration with a Diaflo Membrane PM-30.

Properties of α-L-Fucosidase

Purity——Table I summarizes the specific activities and recoveries during the purification of the α-L-fucosidase from seahare liver. The final α-L-fucosidase preparation obtained after these purification steps was fairly homogeneous as examined by disc gel electrophoresis

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Table II. Activities of Other Glycosidases in the α-L-Fucosidase Preparations

Name of glycosidase	Relative activity	
	Preparation from DEAE-Sephadex (%)	Preparation from Sepharose 6B (%)
α-Glucosidase	0	0
β-Glucosidase	0.01	0.01
α-Galactosidase	0	0
β-Galactosidase	0.9	0
α-Xylosidase	0	0
β-Xylosidase	0.01	0
α-*N*-Acetylglucosaminidase	0	0
β-*N*-Acetylglucosaminidase	0.01	0.01
α-*N*-Acetylgalactosaminidase	0.01	0.01
β-*N*-Acetylgalactosaminidase	0.1	0
α-Mannosidase	0	0
β-L-Fucosidase	0	0
α-L-Fucosidase	100	100

at pH 8.3 (Fig. 4). Apparent α-L-fucosidase activity was detected in the gel section corresponding to that stained for protein in Fig. 4. When other glycosidase activities in the final preparation were examined, no activities of α- and β-glucosidases, α- and β-galactosidases, α- and β-xylosidases, α- and β-*N*-acetylglucosaminidases, α- and β-*N*-acetylgalactosaminidases, α-mannosidase or β-L-fucosidase were obtained (Table II). Even in the preparation from the DEAE-Sephadex column chromatography step, glycosidase activities other than that of α-L-fucosidase accounted for less than 0.9% of the α-L-fucosidase activity.

Molecular Weight——When the molecular weight of α-L-fucosidase was estimated by gel filtration on Sephadex G-200 at pH 7.0, an apparent molecular weight of 330000 was obtained (Fig. 5). When the enzyme was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, it gave a major protein band at the position corresponding to a molecular weight of 60000 and a minor band with a molecular weight of 29000.

pH Optimum——The effect of pH on the activity of the α-L-fucosidase was determined in citrate–phosphate (McIlvaine) buffer using *p*-nitrophenyl α-L-fucoside as a substrate. As shown in Fig. 6, α-L-fucosidase from seahare liver showed two pH optima, one at pH 3.0 and the other at pH 6.4.

pH and Heat Stability——The stability of the enzyme at various pHs was investigated by placing the enzyme in 0.05 M citrate–phosphate buffer ranging in pH from 2.5 to 7.5 at room temperature for 22 h prior to assay with *p*-nitrophenyl α-L-fucoside at pH 4.0. α-L-Fucosidase was stable in the pH range of 4.5 to 6.0. In order to investigate thermal inactivation of α-L-fucosidase, the enzyme was heated at various temperatures for 5 min in 0.05 M sodium citrate buffer, pH 4.5 or 6.0, and the residual α-L-fucosidase activity was determined. The enzyme was stable at 50 °C but lost 30% of its activity at 60 °C and almost all of the activity at 75 °C in 5 min.

Enzyme Kinetics——The effect of substrate concentration on the initial velocity was determined at 37 °C using 0.05 M sodium citrate buffer (pH 6.3), with *p*-nitrophenyl α-L-fucoside as a substrate. The apparent Michaelis constant and maximum velocity, calculated from a Lineweaver–Burk plot were 0.328 mM and 1.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of protein, respectively. The enzyme was competitively inhibited by L-fucose.

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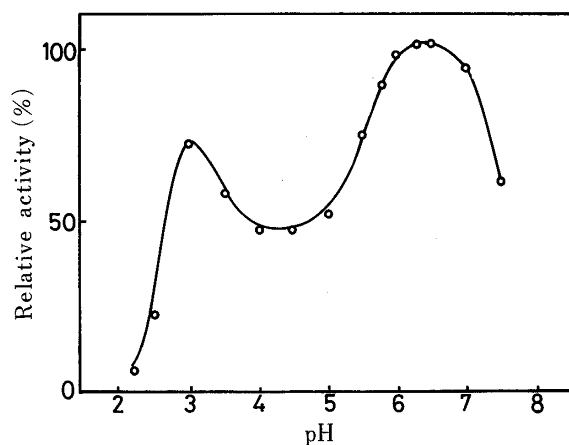

Fig. 6. Effect of pH on α-L-Fucosidase Activity

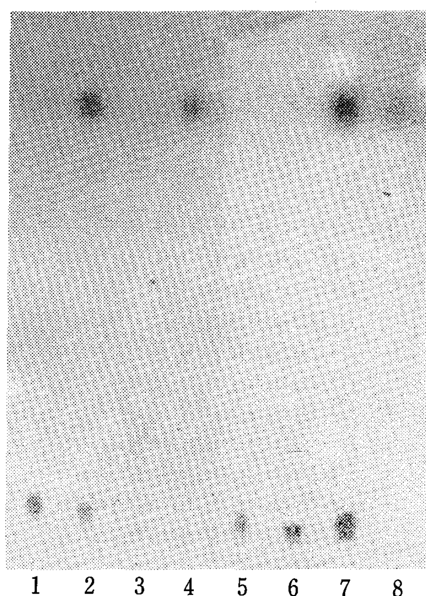

Fig. 7. Hydrolysis of Milk Oligosaccharides by the Seahare Liver α-L-Fucosidase

The incubation conditions and the procedure for thin layer chromatography are described in the text. 1, lacto-*N*-fucopentaose I without enzyme; 2, lacto-*N*-fucopentaose I with enzyme; 6, lacto-*N*-fucopentaose II/III without enzyme; 7, lacto-*N*-fucopentaose II/III with enzyme; 3 and 5, enzyme only; 4 and 8, standard L-fucose.

TABLE III. Effect of Various Inhibitors on the Activity of α-L-Fucosidase

Inhibitors	Inhibitor concentration (mM)	Relative activity (%)
$HgCl_2$	1.0	11.9
$AgNO_3$	1.0	86.3
	10.0	21.6
$MgCl_2$	1.0	89.9
	10.0	34.0
$MnCl_2$	1.0	90.2
	10.0	42.3
$CaCl_2$	1.0	93.6
	10.0	40.0
KCl	1.0	93.8
	10.0	32.3
KBr	1.0	97.9
	10.0	43.3
NaCl	10.0	87.0
$ZnSO_4$	10.0	116.7
EDTA	1.0	93.7
PCMB	5.0	5.3

Inhibitors——The effect of various inhibitors on the enzyme activity was investigated. As shown in Table III, α-L-fucosidase was markedly inhibited by *p*-chloromercuribenzoate and Hg^{2+}. The enzyme was also inhibited by Mg^{2+}, Mn^{2+}, Ca^{2+}, and K^+ at 10 mM but not 1 mM.

Hydrolysis of Oligosaccharides——Hydrolysis of oligosaccharides from human milk by the seahare enzyme was investigated. As shown in Fig. 7, lacto-*N*-fucopentaose I was

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hydrolyzed by this enzyme, and released free fucose was detected on a thin layer plate. The enzyme also cleaved fucose from the mixture of lacto-*N*-fucopentaoses II and III.

Discussion

We have isolated and purified an α-L-fucosidase from the liver of seahare by the simple procedure described above. The final preparation of α-L-fucosidase gave a single protein band on disc gel electrophoresis and was entirely free from all other glycosidase activities tested. Among the purification processes, DEAE-Sephadex column chromatography was very effective in eliminating the contaminating glycosidases. All other glycosidase activities detected in the partially purified preparation from the DEAE-Sephadex column amounted to less than 0.9% of the α-L-fucosidase activity.

Although α-L-fucosidase has been partially purified from several marine gastropods,[3] the molecular weight of the enzyme was not elucidated. In the present study, the molecular weight of the enzyme from the liver of *Aplysia kurodai* was estimated to be 330000 by Sephadex G-200 column chromatography. Based upon sodium dodecyl sulfate electrophoresis under denaturing conditions, the α-L-fucosidase contains a major subunit of molecular weight 60000 and a minor one of molecular weight 29000, but the major protein accounted for 95% of the total. The minor protein may reflect minor degradation of the major protein during the sodium dodecyl sulfate treatment. Thus, the enzyme seems to be composed of equal molecular-weight subunits. Some glycoproteins are known to behave anomalously in SDS-containing gels.[16] The apparent subunit molecular weight of 60000 may not be correct because α-L-fucosidase from seahare liver is presumed to be a glycoprotein on the basis of its affinity for concanavalin A-Sepharose (data not shown). Estimation of the molecular weight of the subunit with more certainty is necessary.

The pattern of α-L-fucosidase activity at different pHs showed a bimodal profile (Fig. 6). However, it seems unlikely that two types of α-L-fucosidase with different dependence of the activity on pH exist in the final preparation of the enzyme because of its electrophoretic purity. The biphasic pattern may be due to a change in the state of ionization of the components of the system as the pH changes. Recently, an α-L-fucosidase purified from *Octopus vulgaris* was shown to have two pH optima.[3e]

D'Aniello *et al.* have shown that α-L-fucosidase from marine gastropod is not inhibited by divalent cations at concentrations up to 16 mM.[3e] In contrast, α-L-fucosidase from seahare liver was markedly inhibited by Mg^{2+}, Ca^{2+}, Mn^{2+}, and K^{+} at 10 mM but not at 1 mM. The enzyme activity seems to depend on the ionic strength of the medium.

α-L-Fucosidase from the seahare was able to hydrolyze milk oligosaccharides. The enzyme seems to hydrolyze Fucα1→2Gal linkage in oligosaccharides, because the enzyme almost completely hydrolyzed lacto-*N*-fucopentaose I. The enzyme also may be able to hydrolyze Fucα1→3GlcNAc and/or Fucα1→4GlcNAc linkages. These results suggest broad aglycon specificity of α-L-fucosidase from seahare liver for natural substrates.

Exoglycosidases are useful for elucidating the structure and function of glycoconjugates.[17] Because of the ease of removal of contaminating glycosidases and the broad aglycon specificity, this enzyme may be valuable for structural and functional studies of fucose-containing glycoconjugates.

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