

Purification and Characterization of Sea Squirt α -N-Acetylgalactosaminidase

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Sea squirt α -N-acetylgalactosaminidase was purified to homogeneity. Its molecular weight was estimated to be approximately 160,000 by gel filtration and 40,000 by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition. The chromatographic and electrophoretic behaviors indicated that the enzyme was composed of four subunits. The optimum pH of the enzyme reaction was about 4.0 at 37°C, while the enzyme was stable in the range of pH 5.0 to 6.0 during 4 h preincubation at 37°C. Although the enzyme (0.1 unit) was stable at 0°C for 30 min in the presence of 7.5 mM metal ions (Al^{3+} , Ba^{2+} , Ca^{2+} , K^{+} , Mn^{2+} , Pb^{2+} , Sr^{2+} , and Zn^{2+}), almost 40% of the enzyme activity was lost in the presence of Cu^{2+} , Hg^{2+} , monoiodoacetic acid, and EDTA. The enzyme hydrolyzed aryl N-acetyl- α -D-galactosaminide as well as $\text{GalNAc}\alpha 1\rightarrow 4\text{GalNAc}\alpha 1\rightarrow$ _n 4GalNAc-p-aminobenzoic acid ethyl ester (ABEE) ($n=1-4$), but $\text{GalNAc}\alpha 1\rightarrow 4\text{GalNAc-ABEE}$ only scarcely. Furthermore, an allergenic pentasaccharitol ABEE derivative, $\text{GalNAc}\alpha 1\rightarrow 2\text{Fuc}\alpha 1\rightarrow 3(\text{GalNAc}\beta 1\rightarrow 4)\text{GlcNAc}\beta 1\rightarrow 2(3\text{-acetoamido-3-deoxy})\text{L-threose-ABEE}$, the minimum structural unit for the sea squirt allergenicity was hydrolyzed to 95 mol% for 72 h incubation with the enzyme. The enzyme could be utilized as a powerful tool for the structural analyses of the carbohydrate epitopes of the sea squirt allergen molecules.

[**Key words:** α -N-acetylgalactosaminidase, sea squirt, glycosidase, purification, characterization]

Sea squirt (*Styela plicata*) allergy is a typical type I hypersensitivity of allergic reaction. Oyster shuckers sensitized to sea squirt antigens develop severe allergic symptoms when they inhale sprays of body fluid expelled through the exhalant orifice of sea squirts adherent on oyster shells (1–4). The IgE epitopes responsible for the allergic attack in the antigenic glycoprotein molecules in the body fluid have been predicted to reside in unusual saccharide chain moieties with common multiantennary structures containing an N-acetyl-galactosamine (GalNAc) residue at the respective nonreducing terminals, which provoke skin reaction and conjunctival congestion in asthmatic patients (5–7). Several glycosidases with relatively high activities were found to be present in the body fluid and internal organs (8–10). Modifications of the allergenic saccharide chains by the enzymes might render the structural analyses complicated. Demonstration of the specificities of these purified enzymes would be useful for structural analyses of the IgE epitopes. Alpha-N-acetylgalactosaminidase (α -GalNAcase) has been regarded as an important enzyme for the structural analyses, because the removal of a nonreducing terminal GalNAc residue from an allergenic bisectational pentasaccharide molecule led to the abolition of its allergenicity (7). Thus, we purified α -GalNAcase capable of hydrolyzing the allergenic saccharide chains and characterized its enzymatic properties.

Alpha-GalNAcase activity was assayed by measuring the release of p-nitrophenol (PNP) from PNP-N-acetyl- α -D-galactosaminide (PNP- α -GalNAc). The assay solutions contained 10 mM PNP- α -GalNAc, 0.1 M citrate buffer, at pH 4.0, and an appropriate amount of the enzyme. Incubation was carried out 10 min at 37°C. One unit of α -GalNAcase is defined as the amount of enzyme that releases one μmol of PNP per minute under the

above conditions. Sea squirt α -GalNAcase was purified according to the following procedure. Acetone powders prepared from the internal organs (20 g) were suspended in 500 ml of 1% NaCl for 1 h at room temperature. To the supernatant solution, ammonium sulfate was added up to 70% saturation. The precipitate was dialyzed against 50 mM acetate buffer, at pH 5.0 and the dialysate was applied to a DEAE-Toyopearl 650S column (4 $\times$ 20 cm, Tosoh, Tokyo) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of acetate buffer from 50 to 500 mM. Alpha-GalNAcase was co-eluted with β -N-acetylhexosaminidase and a part of β -galactosidase. The active fractions were concentrated by ultrafiltration using a YM-30 membrane (Amicon, Beverly, MA, USA) and chromatographed on a column of Ultrogel AcA 44 (4.4 $\times$ 100 cm, LKB, Bromma, Sweden) with 0.1 M acetate buffer at pH 5.0. The α -GalNAcase fraction having the main activity was free from β -N-acetylhexosaminidase and β -galactosidase in this step. The active fractions were pooled and applied to a column of chromatofocusing gel PBE 94 (1 $\times$ 15 cm, Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 25 mM piperazine-HCl buffer at pH 5.5 and eluted with 40-times diluted Pharmalyte-HCl, at pH 3.0. The α -GalNAcase was eluted around pH 4. The active fractions were pooled. The enzyme purification procedures were repeated 10 times. The pooled active fractions were concentrated to approximately 1 ml and then dialyzed against 0.2 M acetate buffer at pH 5.0, and stored at -20°C with 30% glycerol.

Finally, the α -GalNAcase fraction was purified 2056-fold from a crude enzyme extract. Fourteen units of the purified α -GalNAcase with a specific activity of 61.7 units/mg were obtained from 200 g of acetone powder per 8 kg of sea squirt internal organs.

Gel filtration of α -GalNAcase on a Sephadex G-200 (Fig. 1a) column gave a single peak with an apparent

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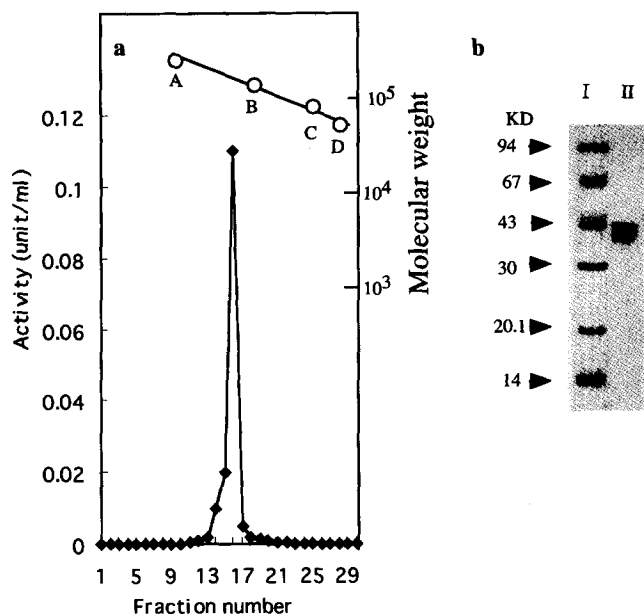


FIG. 1. (a) Gel chromatographic estimation of the molecular weight of α -N-acetylgalactosaminidase on a Sephadex G-200 column (1.6 $\times$ 100 cm). $\blacklozenge$, α -N-Acetylgalactosaminidase; $\circ$, eluted fraction of standard proteins; A, catalase (M_r 240,000); B, sea squirt β -N-acetylhexosaminidase (M_r 132,000); C, BSA (M_r 68,000); D, ovalbumin (M_r 43,000), and (b) SDS-PAGE in 12.5% acrylamide gel. Lane I: phosphorylase 94 KD, bovine serum albumin, 67 KD, ovalbumin 43 KD, carbonic anhydrase 30 KD, soybean trypsin inhibitor 20 KD and α -lactalbumin 14 KD were used as molecular weight standards. Lane II: purified sea squirt α -N-acetyl-galactosaminidase.

molecular weight of approximately 160,000. Only a single protein band was detected at the position around the apparent molecular weight of 40,000 by SDS-PAGE under reducing conditions (Fig. 1b, lane II). Chromatographic and electrophoretic behaviors suggest that the enzyme exists as a tetramer.

Purified α -GalNAcase was very stable at -20°C for 6 months. The optimum pH of the enzyme reaction was about 4.0 at 37°C , while the enzyme was stable in the range of pH 5.0 to 6.0 during 4 h preincubation at 37°C . Although the enzyme (0.1 unit) was stable at 0°C for 30 min in the presence of 7.5 mM of various metals (Al^{3+} , Ba^{2+} , Ca^{2+} , K^+ , Mn^{2+} , Pb^{2+} , Sr^{2+} , and Zn^{2+}), about 40% of the enzyme activity was lost in the presence of Cu^{2+} , Hg^{2+} , monoiodoacetic acid, and EDTA.

The substrate specificity of the purified α -GalNAcase was examined at 37°C for 10 min in a reaction mixture containing 0.1 unit of the enzyme and 10 mM of phenyl α -GalNAc, PNP- α -GalNAc, PNP-N-acetyl- α -D-glucosaminide, PNP-N-acetyl- β -D-glucosaminide, PNP- α -D-glucoside, PNP- β -D-glucoside, PNP- α -D-galactoside, PNP- β -D-galactoside, PNP- α -D-mannoside, PNP- β -D-mannoside, PNP- α -D-fucoside, PNP- β -D-fucoside, PNP- α -D-xyloside, or PNP- β -D-xyloside in 0.1 M citrate buffer, at pH 4.0. Only aryl α -GalNAc substrates were hydrolyzed by the enzyme. The results indicate that the enzyme was free from other glycosidases. The hydrolytic activities (0.1 unit) were also examined against 10 mM ABEE-modified naturally occurring complex saccharides. ABEE-labeled α -1,4-linked GalNAc-oligomers, prepared from hydrolyzates of polygalactosamine PF 102 (Higeta Shoyu Ltd., Choshi) (11), were allowed to react with the α -GalNAcase for 24 h and 72 h under monitoring at 340 nm on HPLC (Table 1). Among the ABEE-tagged α -1,4-linked GalNAc-oligomers, (GalNAc) $_4$ - and (GalNAc) $_3$ -ABEE were preferentially hydrolyzed by 12 h to (GalNAc) $_2$ -ABEE up to 83.2 mol% and 91.5 mol%, respectively. However, only 2.2 mol% of GalNAc-ABEE was produced by hydrolysis of (GalNAc) $_2$ -ABEE at 72 h. Allergic pentasaccharitol-ABEE derivative, GalNAc α 1 $\rightarrow$ 2Fuc α 1 $\rightarrow$ 3(GalNAc β 1 $\rightarrow$ 4)GlcNAc β 1 $\rightarrow$ 2(3-acetoamido-3-deoxy)L-threose-ABEE, the minimum structural unit for the sea squirt allergenicity (7, 12), was easily converted to a corresponding nonallergenic tetra-saccharitol derivative up to 95 mol% after 72 h incubation, but only 12 mol% of Fuc-ABEE was released from

TABLE 1. Substrate specificity of sea squirt α -N-acetylgalactosaminidase for α -1,4-GalNAc-oligomers

Substrate	h	Product mol%					
		GalNAc-ABEE	(GalNAc) $_2$ -ABEE	(GalNAc) $_3$ -ABEE	(GalNAc) $_4$ -ABEE	(GalNAc) $_5$ -ABEE	(GalNAc) $_6$ -ABEE
(GalNAc) $_6$ -ABEE*	0						100
	24		38.3	<0.01	17.9	43.9	<0.01
	72		84.4	<0.01	19.4	3.2	<0.01
(GalNAc) $_5$ -ABEE*	0					100	
	24		30.0	<0.01	2.0	67.9	
	72		89.4	8.0	0.8	1.8	
(GalNAc) $_4$ -ABEE*	0				100		
	24		83.2	9.2	7.6		
	72	0.2	99.7	0.1	<0.01		
(GalNAc) $_3$ -ABEE*	0			100			
	24		91.5	8.5			
	72	2.9	94.7	2.4			
(GalNAc) $_2$ -ABEE*	0		100				
	24		100				
	72	2.2	96.9				

Each substrate (10 nmol) was incubated with 0.1 unit of the purified α -N-acetylgalactosaminidase in 0.1 M citrate buffer, at pH 4.0 and 37°C for 24 and 72 h.

* The abbreviations (GalNAc) $_2$ -ABEE, (GalNAc) $_3$ -ABEE, (GalNAc) $_4$ -ABEE, (GalNAc) $_5$ -ABEE, (GalNAc) $_6$ -ABEE represent the *p*-amino-benzoic acid ethylester derivatives of hydrolyzates of polygalactosamine PF 102 of GalNAc α 1 $\rightarrow$ 4GalNAc, GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc, GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc, GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc, and GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc α 1 $\rightarrow$ 4GalNAc, respectively. The products were analyzed by reversed-phase HPLC on a Wakosil 5C18-20 column (0.4 $\times$ 25-cm, Wako Purechem., Kyoto) at a flow rate of 0.8 ml/min with 50 mM NaH_2PO_4 -acetonitrile (92 : 8, v/v) at 35°C (15) monitored at 340 nm.

GalNAc α 1 $\rightarrow$ 2 Fuc-ABEE under the above conditions. Since the complex carbohydrate epitopes of the sea squirt allergens still remain unknown (6, 13–15), the application of the enzyme for their structural analyses could be a powerful tool.

In most cases the purified α -GalNAcase exhibited concomitant α -galactosidase activity (16–19). The purified sea squirt α -GalNAcase (0.1 unit) exhibited α -galactosidase activity below the limit of detection (less than 0.0006 unit). The substrate specificity of this enzyme was similar to that of squid liver enzyme II (19). The sea squirt α -GalNAcase is a tetramer composed of 40 kDa subunits. The molecular weight of each enzyme subunit was smaller than that of the α -GalNAcases from domestic chicken (49 kDa) (17), limpet *Patella vulgata* (45 kDa) (19) and chicken (44.5 kDa) (20). Furthermore, these other α -GalNAcases varied in subunit structure from a monomer to a tetramer (16–20). Recently, complex types of protein glycoconjugates containing a nonreducing terminal α -GalNAc residue have been reported successively from bovine milk (21), apolipoprotein H (22), halobacterium (23) and Tamm-Horsfall glycoprotein (24). The physiological significance of the presence of a nonreducing terminal α -GalNAc residue has been inquired. The enzyme may also be used for the elucidation of their physiological function.

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