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# Purification and Some Properties of β-Mannosidase, β-*N*-Acetylglucosaminidase, and β-Galactosidase from Apple Snails (*Pomacea canaliculata*)

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β-Mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase were purified from the viscera of apple snails (*Pomacea canaliculata*) using ammonium sulfate precipitation, hydrophobic chromatography on Butyl Toyopearl 650M, gel filtration on Sephacryl S-300HR, affinity chromatography on fucosyl-Sepharose 4B, and Q-Sepharose column chromatography. Molecular sizes of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase were estimated to be approximately 90 kDa, 54 kDa, and 65 kDa by SDS-polyacrylamide gel electrophoresis and to be 450 kDa, 145 kDa, and 66 kDa by gel filtration, respectively. The isoelectric points of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase were to be 4.3, 4.5, and 4.9, respectively. The optimum pHs of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase toward corresponding *p*-nitrophenyl(NP)-β-D-glycosides were 5.0, 3.5–6.0, and 3.5–6.0, respectively. The β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase were stable at pHs of 5.0–8.0, 3.5–9.0, and 3.5–9.0, respectively. The Km values of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase toward respective *p*NP-glycoside at pH 4.0 were calculated as 5.68 mM, 39.5 mM, and 0.725 mM, respectively.

## INTRODUCTION

The exoglycosidases of Mollusc, which are well known as abundant resources of glycosidases, act on substrates with broad aglycon specificity and are useful for tools of saccharide engineering (Muramatsu 1966, Muramatsu and Egami 1967, and Sugahara *et al.* 1972). In preceding paper (Hirata *et al.* 1996), purification of β-xylosidase and α-fucosidase from the crude extract prepared from viscera of apple snails (*Pomacea canaliculata*) was reported and it was found that the crude extract was abundant in other glycosidase activities such as β-mannosidase, α-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase. We, therefore, have attempted a simultaneous purification of other exoglycosidases from the crude extract and purified β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase. Purification and characterization of the three enzymes will be described.

## MATERIALS AND METHODS

Apple snails were captured in a rice field in a suburb of Fukuoka City in July and their viscera were frozen at –20 °C until use. *p*-nitrophenyl(NP)-glycosides were offered by Seikagaku Kogyo Co. or obtained from Sigma Chemical Co. Butyl Toyopearl 650M was purchased from Tosoh Co. and Sephacryl S-300HR, Q-Sepharose, and Q-Sepharose HP

were from Pharmasia LKB Biotechnology. Fucosyl-Sepharose 4B was prepared by the method of Teichberg *et al.* (1988).

*Enzyme assay.* Glycosidase activity was measured in 0.33 M NaCl–0.133 M sodium acetate buffer, pH 4.0, using *p*NP-glycosides as substrates (Hirata *et al.* 1996). One unit of enzyme activity was defined as the amount of the enzyme releasing 1 $\mu$mol of *p*-nitrophenol per min at 37 °C.

*Purification of β-mannosidase, β-N-acetylglucosaminidase, and β-galactosidase.* The purification of the enzymes was done principally by the method described in the preceding paper (Hirata *et al.*, 1996). The 1 kg of viscera was homogenized in 1 M NaCl. After centrifugation , the supernatant (crude extract) was adjusted to pH 4.0 with acetic acid. After the resulting precipitate was removed by centrifugation, the supernatant was saturated with ammonium sulfate to 50%. The precipitate was dissolved in 10 mM sodium acetate buffer, pH 4.0. The resulting supernatant was referred to as the glycosidase-rich solution. To the glycosidase-rich solution, solid NaCl was added to a final concentration of 4 M. After centrifugation, the supernatant solution was put on a Butyl Toyopearl 650 M column (2.5×30 cm) equilibrated with 4 M NaCl-10 mM sodium acetate buffer, pH 4.0. After washing out the unadsorbed proteins, the glycosidases were eluted with 0.2 M NaCl-10 mM sodium acetate buffer, pH 4.0. The glycosidase fraction was concentrated, put on a Sephacryl S-300HR column (3×145 cm) equilibrated with 10 mM sodium acetate buffer, pH 4.0, and the column was developed with the same buffer. In this gel filtration, β-mannosidase and β-*N*-acetylglucosaminidase were eluted in the first fraction (fraction S1) together with α-fucosidase, and β-galactosidase was eluted in the second fraction (fraction S2) together with β-xylosidase and α-fucosidase. Fraction S1 containing β-mannosidase and β-*N*-acetylglucosaminidase and S2 containing β-galactosidase were separately put on a fucosyl Sepharose 4B column (1.5×15 cm) in 0.5 M NaCl-10 mM sodium acetate buffer, pH 4.0, to remove α-fucosidase, a main contaminant in each fraction. The fraction S1' obtained by passing S1 through a fucosyl-Sepharose 4B column as well as the fraction S2' from S2 were separately purified by Q-Sepharose column (1×30 cm) chromatography using a linear gradient of NaCl from 0 to 0.4 M in 10 mM Tris-HCl buffer, pH 7.0. Final purification of each enzyme was achieved by a Q-Sepharose HP column (0.5× 5.0 cm) in a fast protein liquid chromatography (FPLC) apparatus (Pharmasia Fine Chemicals, Uppsala, Sweden) under the same condition as described above.

*Analytical methods.* Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. SDS polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (1970) by using a gel concentration of 10%. The gel was stained for protein with Coomassie brilliant blue R-250 or for glycoprotein with periodic acid-Schiff reagent (PAS) (Zacharius *et al.*, 1969). Molecular size was measured by SDS-PAGE according to the method of Weber *et al.* (1969) and by Sephacryl S-300HR gel filtration using 50 mM Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl according to the procedure of Andrews (1964).

*Isoelectric point.* The isoelectric point of the enzyme was measured using

electrofocusing on a 5% polyacrylamide gel column (2.5×100 mm) containing 2% carrior ampholyte of pH 3.5–10.5 (LKB; Bromma, Sweden) described by Svensson (1962).

*Amino acid and N-terminal sequence analysis.* The enzyme was hyrolyzed with constant-boiling HCl containing 0.05% 2-mercaptoethanol in evacuated sealed tube at 110 °C for 24 h. Amino acid analysis was done with a Hitachi 655-A amino acid analyzer. The N-terminal sequence of the enzyme was determined by the DABITC/PITC double coupling method of Chang *et al.* (1978).

*Sugar content.* Neutral sugar was measured by the phenol-sulfuric acid method of Dubois *et al.* (1956) and the sugar content was expressed by percent as the amount of mannose.

*Effects of pH and temperature on activity and stability.* The optimum pH of the enzyme was determined by incubation with *p*NP-glycosides in the buffers of various pHs (pH 2.5–9.0) at 37 °C for 15 min and the optimal temperature of the enzyme was determined at various temperatures from 30 to 85 °C in the enzyme assay. The pH stability of the enzyme was measured after preincubation in the buffers (pH 2.5–10.5) at 4 °C for 24 h and the thermal stability of the enzyme was measured after incubation in 10 mM sodium acetate buffer, pH 4.0, at various temperatures for 3 min. The residual activities were measured at pH 4.0, 37 °C.

*Action toward natural substrate.* Konjak mannan (100 $\mu$g) was digested with $\beta$-mannosidase (10 mU) for 20 h, Chitin and the oligomers, $(GlcNAc)_{2\text{-}6}$ (100 $\mu$g, respectively) with $\beta$-*N*-acetylglucosaminidase (10 mU) for 20 h, and lactose (100 $\mu$g) with $\beta$-galactosidase (30 mU) for 5h in 100 $\mu$l of 10 mM sodium acetate buffer, pH 4.0, at 37 °C, respectively. Analysis of the sugar produced by glycosidase digestion was done by a ascending thin layer chromatography on Kieselgel 60 (Merck) using n-butanol-acetic acid-water (10:5:1, v/v) as a solvent.

## RESULTS AND DISCUSSION

*Purification of $\beta$-mannosidase, $\beta$-N-acetylglucosaminidase, and $\beta$-galactosidase*

$\beta$-Mannosidase, $\beta$-*N*-acetylglucosaminidase, and $\beta$-galactosidase were separated from other proteins and colored materials in Butyl Toyopearl 650 M column chromatography and they were eluted in the same fractions with 0.2 M NaCl together with other glycosidases. The fractions were pooled and filtered on a Sephacryl S-300HR gel column (Fig. 1). By this gel filtration, these glycosidases were found to be distributed into two fractions, S1 containing $\beta$-mannosidase, $\beta$-*N*-acetylglucosaminidase, and $\alpha$-fucosidase and S2 containing $\beta$-galactosidase and $\beta$-xylosidase. The each fractions, S1 and S2, was passed through a fucosyl-Sepharose 4B column to remove $\alpha$-fucosidase contaminated. The resulting nonadsorbed fractions of S1' from S1 and S2' from S2 were obtained. $\beta$-Mannosidase and $\beta$-*N*-acetylglucosaminidase were purified from fraction S1' by Q-Sepharose column chromatography. $\beta$-Galactosidase was purified from S2'. Finally $\beta$-mannosidase, $\beta$-*N*-acetylglucosaminidase, and $\beta$-galactosidase were separately obtained

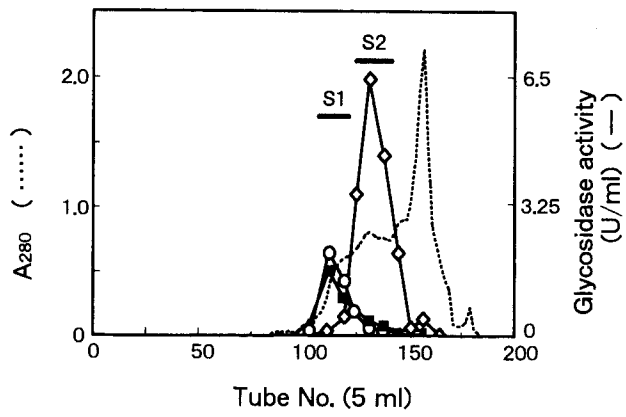


**Fig. 1.** Gel Filtration on a Sephacryl S-300HR of Glycosidase fraction.

Glycosidase fraction obtained from Buty Toyopearl column chromatography was filtered on a Sephacryl S-300HR column (2.5×145 cm) equilibrated with 10 mM sodium acetate buffer, pH 4.0. The fractions indicated by bars were pooled as S1 and S2.
■, β-mannosidase; ○, β-*N*-acetylglucosaminidase; ◇, β-galactosidase.

**Table 1.** Purifications of β-Mannosidase, β-*N*-Acetylglucosaminidase, and β-Galactosidase from the Viscera of *P. canaliculata*.

| Fractions | Total protein (mg)[a] | Total activity (U) | Specific activity (U/mg) | Recovery (%) |
|---|---|---|---|---|
| β-mannosidase | | | | |
| Glycosidase-rich Soln. | 7280[b] | 606 | 0.083 | 100 |
| Butyl Toyopearl 650 M | 4700 | 243 | 0.051 | 40 |
| Sephacryl S-300HR | 309 | 240 | 0.77 | 39.6 |
| Q-Sepharose | 15 | 210 | 14.0 | 34.6 |
| β-*N*-acetylglucosaminidase | | | | |
| Glycosidase-rich Soln. | 7280[b] | 395 | 0.054 | 100 |
| Butyl Toyopearl 650 M | 4700 | 350 | 0.074 | 88.6 |
| Sephacryl S-300HR | 309 | 350 | 1.13 | 88.6 |
| Q-Sepharose | 22 | 96.8 | 4.4 | 24.5 |
| β-galactosidase | | | | |
| Glycosidase-rich Soln. | 7280[b] | 4023 | 0.55 | 100 |
| Butyl Toyopearl 650 M | 4700 | 2209 | 0.47 | 54.9 |
| Sephacryl S-300HR | 841 | 953 | 1.13 | 23.6 |
| Q-Sepharose | 7.2 | 86.4 | 12.0 | 2.1 |

a Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.
b The crude enzyme solution was prepared from 1 kg of the viscera and contained a large amount of colored materials.

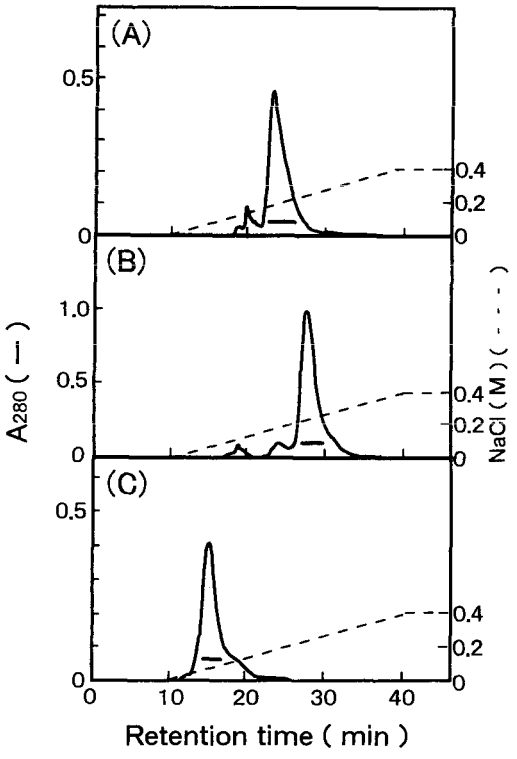


**Fig. 2.** Q-Sepharose HP Column FPLC-Elution Profiles of β-Mannosidase (A), β-*N*-Acetylglucosaminidase (B), and β-Galactosidase (C) Fractions.

The respective glycosidase fractions obtained by Q-Sepharose column chromatographies were put on a Q-Sepharose HP column (0.5×5 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.0, and eluted by a linear gradient of NaCl from 0 to 0.4 M in the same buffer at a flow rate of 0.5 ml/min. Each fraction, indicated by the bar, was pooled and used for their characterizations as the purified enzymes.

by rechromatography of Q-Sepharose HP column FPLC (Fig. 2). Each active fractions indicated by bars in Fig. 2A–C gave one band on SDS-PAGE. Each of the enzyme preparations had negligible amount of contaminating activities, β-mannosidase, β-*N*-acetylglucosaminidase, β-galactosidase, β-xylosidase, α-fucosidase, or β-*N*-acetyl-galactosaminidase except for its inherent activity.

The results of purification of three enzymes are shown in Table I. The yields of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase from 1 kg of the visera were 15, 22, and 7.2 mg, respectively. This purification procedure described above was

found very effective in obtaining several glycosidases since β-xylosidase (18 mg) and α-fucosidase (130 mg) had been purified from the same glycosidase-rich fraction.

**Molecular size and isolelctric point**

Molecular sizes of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase were estimated to be approximately 90 kDa, 54 kDa, and 65 kDa by SDS-PAGE (Fig. 3A), and to be 450 kDa, 145 kDa, and 66 kDa by gel filtration (Fig. 3B), respectively, indicating that β-galactosidase have monomeric while β-mannosidase and β-*N*-acetylglucosaminidase are oligomeric structures. The subunit size of the purified β-mannosidase is similar to that (90 kDa) of human serum and urine β-mannosidases (Percheron *et al.* 1992). The size of the purified β-galactosidase is similar to a monomer form (65 kDa) of human β-galactosidase (Hubbes 1992).

Isoelectric points of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase were approximately 4.3, 4.5, and 4.9, respectively.

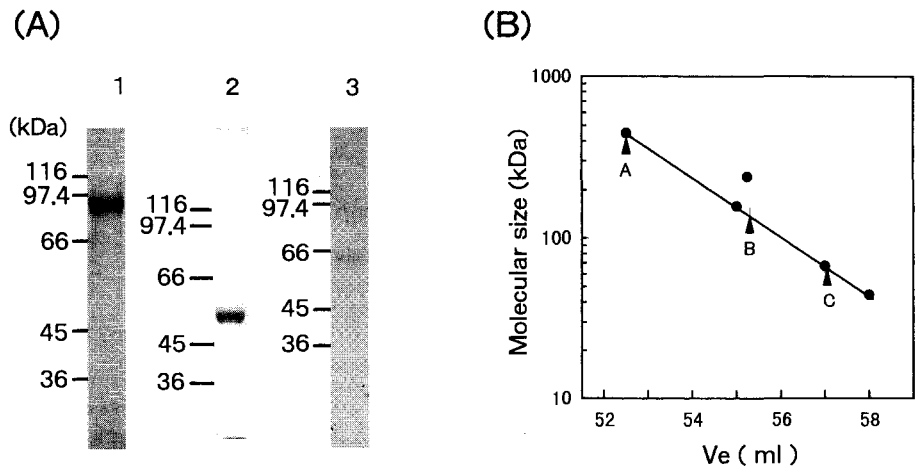


**Fig. 3.** Measurement of Molecular Sizes of the Purified Enzymes by SDS-PAGE (A) and Sephacryl S-300HR Gel Filtration (B).

(A) The measurement of molecular size by SDS-PAGE was done in the presence of 2-mercaptoethanol using β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and glyceraldehyde-3-phosphate dehydrogenase (36 kDa) as standard proteins. 1, β-mannosidase; 2, β-*N*-acetylglucosaminidase; 3, β-galactosidase. (B) Measurement of the apparent molecular size by gel filtration was done on a Sephacryl S-300HR column (1.0×76 cm) in 0.2 M NaCl-50 mM Tris-HCl buffer, pH 7.5, using 1, ferritin (450 kDa); 2, catalase (240 kDa); 3, aldolase (158 kDa); 4, bovine serum albumin (68 kDa); 5, ovalbumin (45 kDa); and 6, chymotrypsinogen A (25 kDa) as standard proteins. A, β-mannosidase; B, β-*N*-acetylglucosaminidase; C, β-galactosidase.

**Chemical composition**

Amino acid compositions of the purified β-mannosidase, β-galactosidase, and β-*N*-acetylglucosaminidase are shown in Table Ⅱ. Their bands on SDS-PAGE were positive with PAS-staining indicating that they are glycoproteins. The sugar contents of β-mannosidase, β-galactosidase, and β-*N*-acetylglucosaminidase were approximately 2.9, 2.8, and 4.0%, respectively. The N-terminal sequences of β-mannosidase and β-*N*-acetylglucosaminidase were I/L-D-S-G-I/L- and V-E-P-P-, respectively, but that of β-galctosidase was not detected.

**Table 2.** Amino Acid Compositions of Purified Enzymes

| Amino acid | mole % and (Nearest integar)[a] | | |
|---|---|---|---|
| | β-mannosidase | β-*N*-acetylglucosaminidase | β-galactosiodase |
| Asx | 17.1 (136) | 12.7 (61) | 12.2 (70) |
| Thr | 3.4 (27) | 5.6 (27) | 7.0 (40) |
| Ser | 6.9 (55) | 9.2 (44) | 6.5 (37) |
| Glx | 9.0 (72) | 14.1 (68) | 8.3 (48) |
| Pro | 6.5 (51) | 4.1 (20) | 7.5 (43) |
| Gly | 9.0 (72) | 10.0 (48) | 7.9 (45) |
| Ala | 8.8 (70) | 8.6 (41) | 8.7 (50) |
| Val | 4.9 (39) | 7.4 (36) | 7.8 (45) |
| Cys | N. D. | N. D. | N. D. |
| Met | 2.6 (21) | 1.3 (6) | 1.5 (9) |
| Ile | 4.5 (35) | 3.8 (18) | 4.6 (26) |
| Leu | 9.0 (72) | 7.7 (37) | 9.8 (56) |
| Tyr | 4.8 (38) | 4.5 (22) | 4.5 (26) |
| Phe | 4.7 (37) | 3.8 (18) | 4.3 (25) |
| Lys | 3.9 (31) | 2.0 (10) | 4.3 (25) |
| His | 0.6 (5) | 1.3 (6) | 1.8 (10) |
| Arg | 4.2 (34) | 3.7 (8) | 3.3 (9) |
| Trp | N. D. | N. D. | N. D. |
| | (795) | (480) | (574) |
| CHO(w/w) | 2.9% | 2.8% | 4.0% |

a, The nearest intergers of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosiodase were calculated assuming the molecular weight to be 87, 52, and 62 kDa, respectively.

N. D., not determined.

**Effects of pH and temperature**

The optimum pHs of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase toward *p*NP-β-D-glycosides were at around 5.0, 3.5–6.0, and 3.5–6.0, (Fig. 4) and they retained almost full activities at pH regions of 5.0–8.0, 3.5–9.0, and 3.5–9.0 for 24 h at 4 °C, respectively. The optimal temperatures of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase were at 45, 55, and 50 °C, respectively. β-*N*-Acetylglucosaminidase

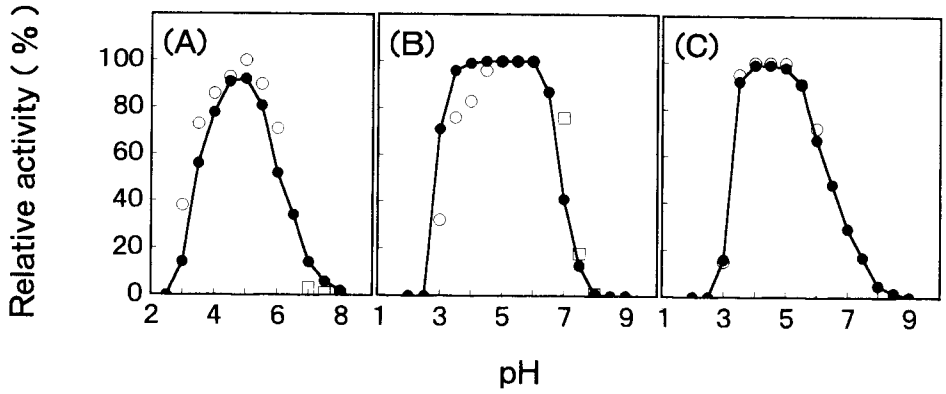


**Fig. 4.** Effects of pHs on Glycosidase Activies

Glycosidase activity was measured in 0.133 M buffer. The activity was expressed as a percent of the maximal activity.
(A) β-mannosidase; (B), β-*N*-acetylglucosaminidase; (C), β-galactosidase;
○, sodium acetate; ●, citrate-phosphate; □,Tris-HCl.

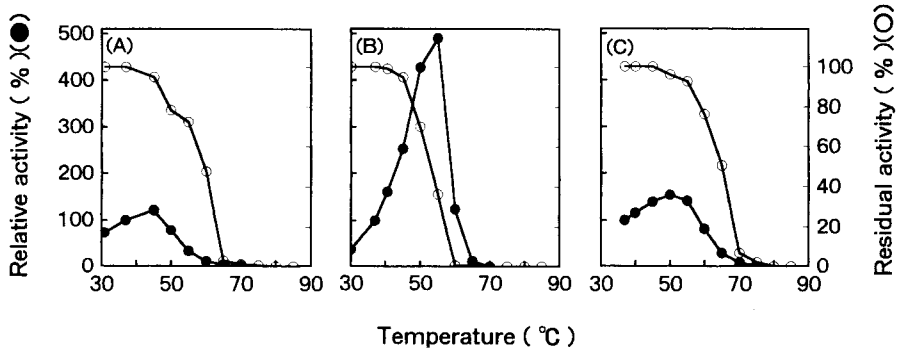


**Fig. 5.** Effects of Temperatures on Glycosidase Activities and Stabilities.

The activity was mesured at various temperatures for 15 min and was expressed as a percent of that at 37 °C. The residual activity was measured after preincubation at various temperatures for 3 min in 10mM sodium acetate buffer, pH 4.0 and was expressed as a percent of the activity at 37 °C. (A) β-mannosidase; (B), β-*N*-acetylglucosaminidase; (C), β-galactosidase.

showed higher sensitivity toward temperature than the others. After heating for 3 min, β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase were stable until at about 45 °C, 45 °C, and 50 °C, respectively (Fig. 5).

**Effects of various reagents on activity**

The effects of various reagents on the activities of the enzymes were examined by incubating the enzymes in 10 mM sodium acetate buffer, pH 4.0, with the reagents for 30 min at room temperature and then assaying the residual activities. One mM of metal ions ($Cu^{2+}$, $Mn^{2+}$, $Mg^{2+}$, $Zn^{2+}$, and $Ca^{2+}$), EDTA, and dithiothreitol did not have much effect on the activities of three enzymes. On the other hand, 1 mM *p*-chloromercuribenzoic acid inhibited the activities of β-*N*-acetylglucosaminidase and β-galactosidase by 97% and 88%, respectively, after incubation in 10 mM sodium acetate buffer, pH 4.0, for 30 min at room temperature, but did not affect on that of β-mannosidase. The activities of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase from *Turbo cornutus* are inhibited by $Zn^{2+}$ and $Cu^{2+}$, EDTA, and $Cu^{2+}$, respectively (Muramatsu and Egami 1967, and Yamada *et al.* 1973).

**Estimations of Km and Vmax**

Km value of the enzyme toward *p*NP-glycoside was calculated by Lineweaver-Burk plot (Lineweaver *et al.* 1934). The Km values of β-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase were estimated to be 5.68 mM (Vmax= 151 μ mol/min/mg), 35.5 mM (Vmax= 305 μ mol/min/mg), and 0.353 mM (Vmax= 21.4 μmol/min/mg), respectively. These enzymatic properties are similar to those of *Charonia lampas* and *T. cornutus* (Muramatsu 1966, Muramatsu and Egami 1967, and Yamada *et al.* 1973).

**Enzyme acticvity toward natural substrate**

β-Mannosidase released mannose residue from konjak mannan having β-(1→4) linkage. β-*N*-acetylglucosaminidase hydrolyzed β-1→4 linkaged $(GlcNAc)_{2-6}$ completely to *N*-acetylglucosamine but acted slightly on chitin. β-Galactosidase hydrolyzed lactose completely. These results showed that three enzymes are able to liberate each β1→4 linkaged terminal resudue from these sugar chains. β-Mannosidase and β-*N*-acetylglucosaminidase could act on the respective insoluble polysaccharide. The mulluscans β-mannosidase and β-*N*-acetylglucosaminidase for *Achatina flica, T.cornutus, etc.* have been reported to possess broad aglycon specificity but not reported about the action toward polysaccharides. The β-galactosidase activity toward lactose is same character as the β-galactosidases of *T. cornutus, etc.* which are named as lactase.

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