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Purification and Characterization of Amylases from Small Abalone (*Sulculus diversicolor aquatilis*)

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Amylases II-1 and II-2 with molecular weights of 55.7 and 65 kDa, respectively, were purified to electrophoretical homogeneity from small abalone (*Sulculus diversicolor aquatilis*) by ammonium sulfate fractionation, Sepharose CL-6B, CM-Sepharose CL-6B, and Sephacryl S-100 chromatographs. They had optimal temperatures of 45 and 50 °C and an optimal pH of 6.0. The purified amylases were stable at pH 5.0−8.0 and 6.0−8.0, respectively. They were completely or partially inhibited by Hg^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, iodoacetamide, phenylmethanesulfonyl fluoride, and *N*-ethylmaleimide, suggesting the existence of cysteine at their active sites. Digestion tests against various polysaccharides suggested that the purified amylases II-1 and II-2 are neoamylases which can hydrolyze both α-1,4 and α-1,6 glucosidic bonds. Amylase II-2 might be an *exo*- and II-1 an *endo*-/*exo*-amylase.

KEYWORDS: Amylase; abalone; characteristics of amylase; purification of amylase

INTRODUCTION

Wild-type small abalone is frequently found in the tidal area of oceans and utilizes algae and plankton as feed. However, the aquatic farmers along the northeastern coast of Taiwan usually use *Gracilaria tenuistiplitate*, which is rich in polysaccharides, agar-agar, to feed the cultured small abalone (*1*). According to Duckworth and Yaphe (*2*), the agar was a complex polysaccharide with α-1,3 and *β*-1,4 linkages of galactose. It was divided into three groups. They are neutral agarose with 1,4-linkage of 3,6-anhydro-α-L-galactose and 1,3-linkage of *β*-D-galactose, pyruvated agarose with little sulfation in which 5% D-galactose and 2% 3,6-anhydro-α-L-galactose are replaced by 4,6-*O*-(1-carboxyethyldiene)-D-galactose and galactose sulfate, respectively, and sulfated galactan with little or even no 3,6-anhydro-α-L-galactose and 4,6-*O*-(1-carboxyethylidene)-D-galactose.

Amylase, a digesting enzyme of polysaccharides, is widely distributed in animals, plants, molds, yeasts, and bacteria. According to the hydrolytic types and end products of amylose, it is divided into three types: α-amylase that can hydrolyze the glucan 1,4-α-glucoside of amylose and produce glucose, oligomaltose, and dextrin; *β*-amylase that can hydrolyze glucan 1,4-α-glucoside of amylose with a nonreducing end and produce maltose; and *γ*-amylase that can hydrolyze glucan 1,4-α-glucoside of amylose with a nonreducing end and produce glucose, maltose, maltotriose, and oligomaltose (*3*). In food industries, amylase is frequently employed to produce buns, breads, and cakes in baking and dextrin, maltose, beer, alcohol, miso (a fermented rice or bean), and cheese in fermentation (*4*−*8*). In biological function, it plays an important role in carbohydrate digestion and metabolism in plants and animals (*9*).

According to Vera et al. (*10*), agar-agar could be hydrolyzed at the *β*-1,4 linkage by *β*-agarase and then further by *β*-neoagarotetraose hydrolase and α-neoagarobiose hydrolase from *Pseudoalteromonas atlantica* ATCC 19292 to produce neoagaro-oligosaccharides, oligomers, neoagarobiose, D-galactose, and 3,6-anhydro-L-galactose. The small abalone is fed with *G. tenuistiplitate*, which is rich in agar-agar. Accordingly, the type of amylase found and how the agar-agar is digested in the small abalone digestive organ are of interest to many scientists. To investigate the type of carbohydrate hydrolytic enzymes in small abalone, the amylase was purified from the viscera and characterized.

MATERIALS AND METHODS

Materials. Small abalone (*Sulculus diversicolor aquatilis*) was purchased from an aquatic farm in northern Taiwan. They were transported to the laboratory and stored at −20 °C.

Extraction of Crude Enzyme. The viscera of small abalone were homogenized with 3 volumes of chilled 10 mM Tris−maleate buffer containing 5 mM $CaCl_2$ (pH 8, extraction buffer). After 30 min of centrifugation at 12000*g*, the supernatant was dialyzed against the same buffer overnight and then subjected to ammonium sulfate fractionation.

Ammonium Sulfate Fractionation. Solid ammonium sulfate was gently added to the crude enzyme with stirring. The precipitates at 20−60% saturation were collected by 30 min of centrifugation at 5000*g* and dissolved in a minimal volume of extraction buffer. The resulting sample was then dialyzed against extraction buffer for 24 h and used as crude enzyme solution. All procedures were performed at 4 °C.

Sepharose CL-6B Column Chromatography. The crude enzyme solution was chromatographed on a Sepharose CL-6B column (2.6 ×

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95 cm) with 10 mM Tris−maleate buffer (pH 7) as elution buffer. The flow rate was 0.7 mL/min, and fractions of 5.0 mL were collected.

CM-Sepharose CL-6B Column Chromatography. Fractions with amylolytic activity from Sepharose CL-6B were collected and concentrated by ultrafiltration (cutoff = 1000 Da; Amicon ultrafiltration, YM10). The resulting sample was chromatographed on a CM-Sepharose CL-6B column (1.6 × 30 cm), equilibrated with 66 mM KH_2PO_4/Na_2HPO_4 buffer (pH 6.0). It was washed with the same buffer and eluted by a linear gradient of 0−0.5 M NaCl in the same buffer at a flow rate of 0.5 mL/min. Fractions of 5.0 mL were collected and concentrated as above.

Sephacryl S-100 Column Chromatography. The concentrated sample was chromatographed on a Sephacryl S-100 column (2.6 × 95 cm) and eluted with 10 mM Tris−maleate buffer (pH 7) at a flow rate of 0.5 mL/min. Fractions of 5.0 mL were collected and concentrated as above.

Discontinuous Polyacrylamide Gel Electrophoresis (Disc-PAGE). The purity and molecular mass (M_r) of purified amylases were determined by using disc-PAGE with 12.5% polyacrylamide (pH 8.3) and a current of 30 mA (*11*). After electrophoretical running, the gels were stained with Coomassie brilliant blue G-250 according to Hames' method (*12*). Protein ladders with M_r of 10 kDa (Life Technologies, Inc., Gaithersburg, MD) were used as protein marker.

Determination of Protein Concentration. Protein concentration was determined according to Lowry's method (*13*) using bovine serum albumin as standard.

Amylolytic Activity Assay. The amylolytic activity was determined using hydrolyzed potato starch (final concentration = 0.5%) as substrate (*14*). To 1 mL of 1.0% hydrolyzed potato starch was added 1.0 mL of the appropriate concentration of amylases in 10 mM Tris−maleate buffer containing 5 mM $CaCl_2$ (α-, *β*-, and *γ*-amylases or purified amylases II-1 and II-2), and the mixture was incubated at 37.5 °C for 10 min. The enzymatic reaction was then stopped by the addition of 2.0 mL of 4 mM 3,5-dinitrosalicylic acid. After color development had been promoted by heating at 100 °C for 5 min, the reducing sugar reacted with 3,5-dinitrosalicylic acid and formed a red color compound. The resulting samples were chilled in ice water for 2 min and then diluted with 16.0 mL of distilled water. The absorbance at 490 nm (A_{490}) was then measured. One unit of enzyme activity was defined as the amount of amylase that could cause an increase in A_{490} of 1 within 10 min of reaction at 37.5 °C. Specific activity was expressed as units of enzymatic activity per milligram of protein.

Influence of Temperature and pH. *Optimal Temperature.* To 1.0 mL of reaction mixture (0.5% hydrolyzed potato starch in 10 mM Tris−maleate buffer, pH 7.0) was added 1.0 mL of the purified amylases II-1 and II-2, and the mixture was incubated at various temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 °C) for 10 min. The reaction was stopped by the addition of 2.0 mL of 4 mM 3,5-dinitrosalicylic acid. After 5 min of heating at 100 °C, the reaction mixtures were chilled in ice water for 2 min and then diluted with 16.0 mL of distilled water. The absorbance at 490 nm (A_{490}) was then measured for the determination of amylolytic acitivity (*14*).

Thermal Stability. Purified amylases in 10 mM Tris−maleate buffer (pH 7.0) were incubated at various temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 °C) for 30 min. The residual activity was measured according to Bernfeld's method (*14*).

Optimal pH. To 1.0 mL of reaction mixture at various pH values (0.2 M citrate buffer, pH 4.0−6.0; 0.2 M phosphate buffer, pH 6.0−8.0; and 0.2 M bicarbonate buffer, pH 8.0−10.0) was added 1.0 mL of the purified amylases II-1 and II-2, and the mixture was incubated at 37.5 °C for 10 min. The reaction was stopped by the addition of 2.0 mL of 4 mM 3,5-dinitrosalicylic acid. After 5 min of heating at 100 °C, the reaction mixtures were chilled in ice water for 2 min and then diluted with 16.0 mL of distilled water. The absorbance at 490 nm (A_{490}) was then measured for the determination of amylolytic acitivity (*14*).

pH Stability. Purified amylases in buffers at various pH values (0.2 M citrate buffer, pH 4.0−6.0; 0.2 M phosphate buffer, pH 6.0−8.0; and 0.2 M bicarbonate buffer, pH 8.0−10.0) were incubated at 4 °C for 12 h. The residual enzyme activity was determined according to Bernfeld's method (*14*).

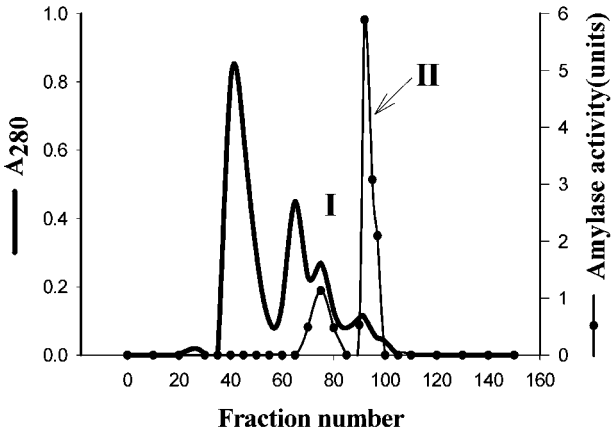

Figure 1. Chromatograph of amylases from small abalone on Sepharose CL-6B. [Sepharose CL-6B column (2.6 × 90 cm) was previously equilibrated with 10 mM Tris–maleate buffer, pH 7; the enzyme was eluted with the same buffer.]

Effect of Metal Ions or Inhibitors. Purified amylases in 10 mM Tris−maleate buffer (pH 7.0) and various metals or inhibitors were incubated at 37.5 °C for 30 min. Final concentrations of the metals and inhibitors were 5.0 mM. After 30 min of incubation, the remaining activity was measured according to Bernfeld's method (*14*).

Thin-Layer Chromatography (TLC) Analysis of Hydrolysate. Amylose, amylopectin, glycogen, *G. tenuistiplitate*, or agar-agar was incubated with α-, *β*-, or *γ*-amylases or purified amylases II-1 or II-2 at 37.5 °C for 72 h. The hydrolysis reaction was stopped by heating at 100 °C for 10 min. The final concentration of these polysaccharides was 0.5%. About 5 *μ*L of starch hydrolysates and a mixture of glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), and maltopentaose (G5) were dropped onto the TLC (Merck Co.) and separated by an ethyl acetate/acetic acid/water (2:1:1) system at room temperature for 2 h. After separation, the resulting TLC plates were sprayed with a mixture of 5% sulfonic acid/methanol (1:1) and then heated at 100 °C for 30 min. They were photographed after the brown spots developed.

RESULTS AND DISCUSSION

Purification and Molecular Mass (M_r) of Amylases. The crude amylases were isolated by extraction with 10 mM Tris−maleate buffer and ammonium sulfate fractionation at 20−60% saturation and then eluted through a Sepharose CL-6B chromatograph. Fractions with amylolytic activity (peak I, tubes 65−80; peak II, tubes 85−100) were concentrated (**Figure 1**). Because only 0.02% activity of peak I was obtained at this step (**Table 1**), it was not further purified. However, ∼28% activity of peak II was obtained (**Table 1**) and so was further purified by using a CM-Sepharose CL-6B column. Two amylolytic activity peaks were observed and designated amylases II-1 and II-2 (**Figure 2**). They were further purified to electrophoretical homogeneity by using Sephacryl S-100 (**Figures 3** and **4**). About 793.4- and 454.29-fold purifications were achieved for amylases II-1 and II-2, respectively. The M_r values of the purified amylases II-1 and II-2 were 55.7 and 65.0 kDa, respectively, as estimated by disc-SDS-PAGE.

In general, the M_r values of α-, *β*-, and *γ*-amylases were 42−115, 31.6−215, and 48−86 kDa, respectively (*15*−*21*). The M_r of purified amylase II-1 was similar to that of *Antheraea mylitta* and *Bacillus brevis* α-amylases (58 kDa) (*22*, *23*), tilapia intestine and barley *β*-amylases (56.4 and 56.2 kDa) (*6*, *18*), and *Rhizopus* species *γ*-amylase (58.6 kDa) (*24*), whereas the M_r of amylase II-2 was similar to that of *Bacillus acidocaldarius* and *Streptomyces rimosus* α-amylases (68 and 65.7 kDa) (*25*, *26*), wheat (64 kDa) (*6*), *Bacillus polymya* (67 kDa) (*27*), and

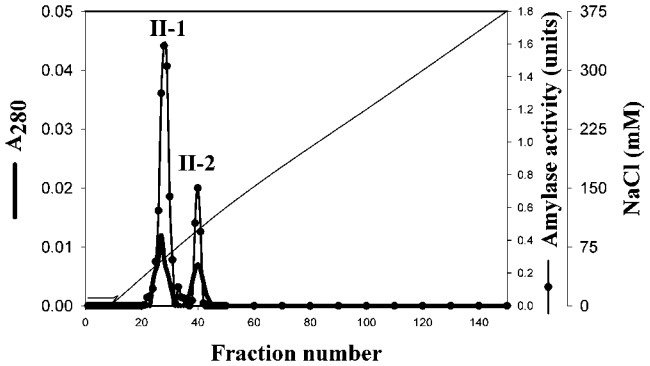

Figure 2. Chromatograph of amylases from small abalone on CM-Sepharose CL-6B. [Amylase peak II from the Sepharose CL-6B column was chromatographed on a CM-Sepharose CL-6B column (1.6 × 30 cm) previously equilibrated with 66 mM KH_2PO_4/Na_2HPO_4 buffer, pH 6.0, and washed with the same buffer; the enzyme was eluted by a linear gradient of 0–0.5 M NaCl.]

Table 1. Summary of the Purification of Amylases from Small Abalone (*S. diversicolor aquatilis*)

procedure	total protein (mg)	total act. (units)	specific act. (units/mg)	recovery (%)	purifn (factor)
crude enzyme	3636.00	6360	1.75	100.00	1.0
ammonium sulfate fractionation (30–70%)	2553.60	4080	1.60	64.15	0.9
Sepharose CL-6B					
peak I	682.40	120	0.18	0.02	0.1
peak II	64.00	1782	27.84	28.02	15.9
CM-Sepharose CL-6B					
peak II-1	0.72	693	961.82	10.90	550.0
peak II-2	0.60	346	576.67	5.44	329.5
Sephacryl S-100					
peak II-1	0.26	361	1388.46	5.68	793.4
peak II-2	0.20	159	795.00	2.50	454.3

B. megaterium β-amylases (67 kDa) (*28*) and *Sclerotium rolfsii* γ-amylase (64 kDa) (*29*).

Substrate Specificity. Both purified amylases II-1 and II-2 could strongly hydrolyze amylose, amylopectin, glycogen, and *G. tenuistiplitate* and moderately hydrolyze agarose, pullulan, agar-agar, cellulose, and alginin, but could not hydrolyze β- and γ-cyclodextrins (**Table 2**). However, unlike amylase II-2, the II-1 could also hydrolyze maltopentaose, maltotetraose, maltotriose, and α-cyclodextrin. It could hydrolyze almost all of the substrates that could be hydrolyzed by α-, β-, and γ-amylases and the substrates with longer carbon chains such as amylose, agar-agar, alginin, cellulose, and pullulan (**Table 2**).

Both purified amylases had higher hydrolytic ability against glycogen, amylose, and amylopectin than maltopentaose, maltotetraose, and maltotriose (**Table 2**). Because they could hydrolyze only long carbon chain carbohydrates, they were considered not to be α-glucosidases. They could also degrade pullulan with α-1,4 and α-1,6 glycoside linkages (**Table 2**). When their hydrolytic abilities were compared against that of pullulan, the purified amylases II-1 and II-2 had about 7.5 and 1.7% hydrolytic ability, respectively, suggesting they are debranching enzymes. This phenomenon was similar to that of *Aspergillus niger* γ-amylase (*30*) but different from that of hard clam amylase A-II (*31*). The purified amylase II-1 could hydrolyze agarose, amylose, pullulan, glycogen, amylopectin, maltopentaose, maltotetraose, maltotriose, *G. tenuistiplitate*, and

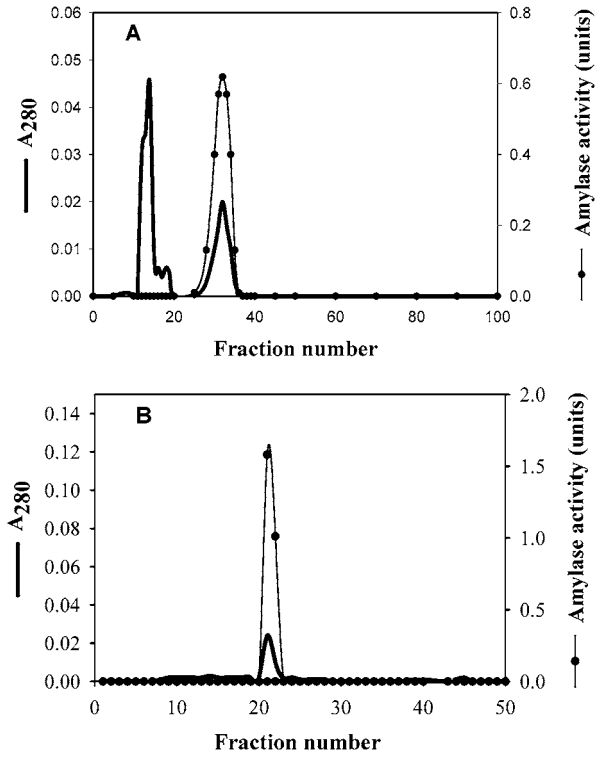

Figure 3. Chromatograph of amylase peaks II-1 and II-2 from small abalone on Sephacryl S-100. [Amylases II-1 (A) and II-2 (B) from small abalone were applied on a Sephacryl S-100 column (2.6 × 90 cm) previously equilibrated with 10 mM Tris–maleate buffer, pH 7.0; the enzymes were eluted with the same buffer.]

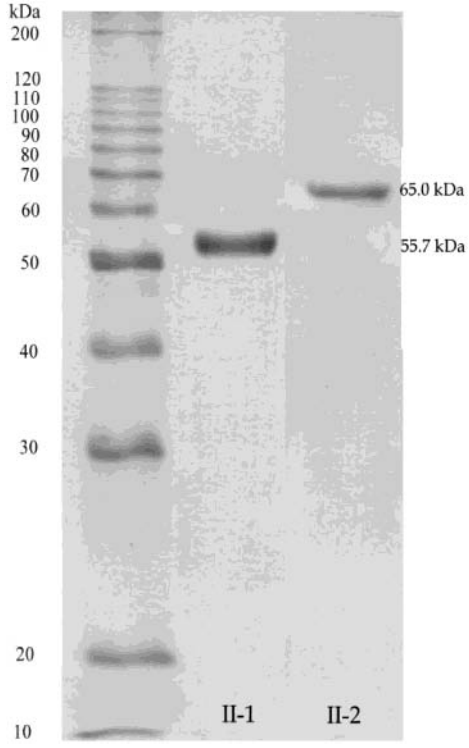

Figure 4. SDS-PAGE profiles of purified amylases II-1 and II-2 (left lane, protein marker; concentration of acrylamide = 12.5%).

agar-agar and was considered to be a γ-amylase-like enzyme. However, it had ~34% hydrolytic activity on agarose, compared with that of agarase (**Table 2**), suggesting it can hydrolyze the α-1,3 or β-1,4 linkage of galactose. The α- or β-1,4 linkage of

Table 2. Substrate Specificity of the Purified Amylases II-1 and II-2 from Small Abalone (*S. diversicolor aquatilis*)

		amylase activity[a]					
substrate	concn	II-1	II-2	α-	β-	γ-	agarase
soluble starch	1%	100.0	100.0	100.0	100.0	100.0	0
agarose	0.2%	3.6 (34)[b]	1.5 (14)[b]	0.0	0.0	41.0	100.0
amylose	1%	95.2	72.5	4.5	10.0	4.6	0.0
pullulan	1%	7.5	1.7	0.0	0.0	23.2	4.1
glycogen	1%	56.5	63.9	85.8	77.2	127.8	0.0
amylopectin	1%	69.4	62.8	95.9	40.0	134.1	0.0
maltopentaose	2 mM	51.1	0.0	14.6	21.9	42.9	0.0
maltotetraose	2 mM	5.3	0.0	18.2	23.8	56.1	0.0
maltotriose	2 mM	10.0	0.0	4.5	0.0	27.2	0.0
α-cyclodextrin	5 mM	3.8	0.0	1.6	0.02	2.4	0.0
β-cyclodextrin	5 mM	0.0	0.0	0.0	0.0	2.4	0.0
γ-cyclodextrin	5 mM	0.0	0.0	0.0	0.0	1.4	0.0
G. tenuistiplitate	0.1%	77.2	82.0	0.0	0.0	7.1	0.0
cellulose	0.1%	4.1	17.6	0.0	0.0	0.0	0.5
agar-agar	0.1%	15.2	8.6	0.0	0.0	26.0	1.9
alginin	0.1%	5.3	34.8	0.0	0.0	0.0	0.9

[a] Enzyme activity was determined after 30 min of reaction with various substrates at 37.5 °C and expressed as percentages of the activities as compared with soluble starch. (α-, β-, and γ-amylases were purchased from Sigma Chemical Co.) [b] Activity was expressed as the percentage ratio of the activity to that of agarase against agarose. (Agarase was purchased from Boehringer Mannheim.)

guluronic acid and mannuronic acid in alginin and the β-1,4 linkage of glucose in cellulose could also be hydrolyzed by purified amylases II-1 and II-2 (**Table 2**). These phenomena indicated that the purified amylases might be cellulase-like enzymes. Because amylase II-2 could not hydrolyze the cyclodextrin, it might be an *exo*-amylase, whereas amylase II-1 could slightly hydrolyze α-cyclodextrin and was considered to be both an *endo*- and *exo*-amylase.

Effect of pH and Temperature. The optimal pH values of purified amylases II-1 and II-2 were identical at 6.0, whereas they were stable at pH 5−8 and 6−8, respectively (**Figure 5**). The pH stability was similar to that of α-amylases from *Clostridium butyrium* T-7 (pH 5.5−7.5) (*32*) and *Bacillus brevis* (pH 5−9) (*22*), tilapia β-amylase (pH 5.5−7.5) (*18*), and *Aspergillus niger* γ-amylase (pH 3.0−6.5) (*30*). The optimal pH of amylases seemed to be species-dependent. The copepod α-amylase (pH 6.0) (*33*) and short-necked clam α-amylase (pH 6.2) (*34*) have optimal pH values similar to that of the purified amylases II-1 and II-2 (**Figure 5**). Those of hepatopancreas α-amylase of *Uca minax*, *Uca pugnax*, and *Uca pugilator* (pH 7.3) (*35*), α-amylases from human sliver and pancreas, and dog pancreas (pH 6.9) (*36*), ostrich pancreas α-amylase (pH 7.5) (*37*), chicken pancreas α-amylase (pH 7.5) (*38*), *B. acidocaldarius* α-amylase (pH 3.5) (*25*), and bettle α-amylase (pH 5.2−5.4) (*39*) are different from that of purified amylases in this study. The optimal pH values of γ-amylases from *Coniophora cerebella* (pH 4.0−4.5) (*19*), *Rhizopus* species (pH 4.5−5.0) (*24*), *A. niger* (pH 4.5−5.0) (*30*), and *Aspergillus awamori* (pH 4.5) (*20*) were found to be lower than that of purified amylases.

The optimal temperatures for purified amylases II-1 and II-2 were 45 and 50 °C, respectively (**Figure 6**). Both purified amylases were stable at <40 °C, and the temperatures for 50% inactivation of these two amylases were 50 and 55 °C, respectively (**Figure 6**).

The optimal temperatures for *Streptomyces rimosus* α-amylase (35−40 °C) (*26*), copepod α-amylase (40 °C) (*33*), short-necked clam α-amylase (36 °C) (*34*), *Perna viridis* α-amylase (43 °C) (*40*), bettle α-amylase (36−40 °C), *Penaeus japonicus* α-amylase (40 °C) (*41*), tilapia intestine β-amylase (40 °C) (*18*), and *Bacillus polymya* β-amylase (30 °C) (*27*) were lower than

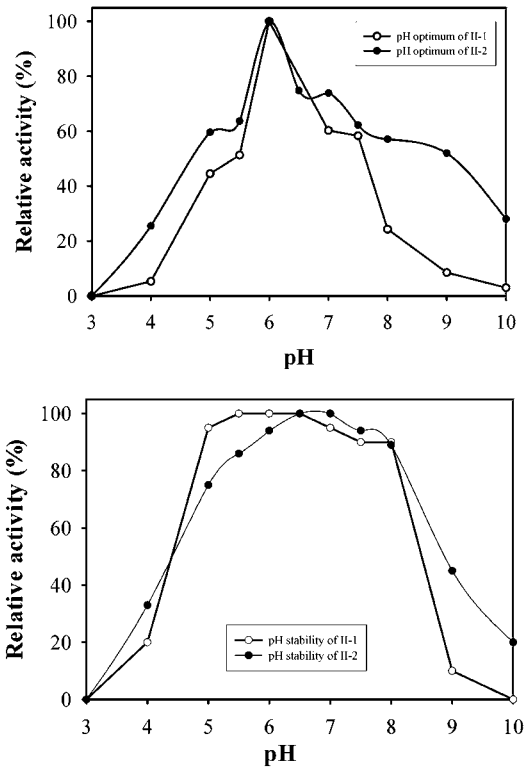

Figure 5. Effect of pH on amylases II-1 and II-2 from the small abalone (top, optimal pH; bottom, pH stability).

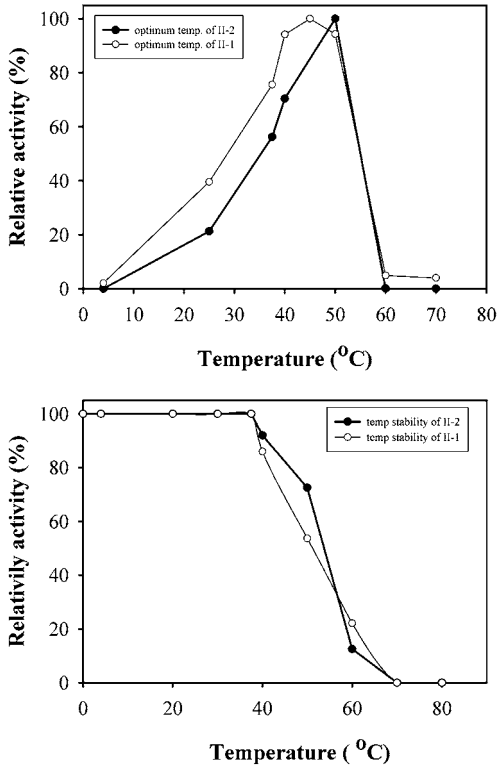

Figure 6. Effect of temperature on amylases II-1 and II-2 from the small abalone (top, optimal temperature; bottom, thermal stability).

those of purified amylases in this study. They were almost similar to that of hard clam amylase-AII (50 °C) (*31*), *Bacillus* sp. No. 195 α-amylase (45 °C) (*42*), kiwi α-amylase (50 °C) (*43*), *Bacillus polymyxa* No. 72 β-amylase (45 °C) (*27*), *Bacillus* var. *mycoides* β-amylase (50 °C) (*8*), and soybean, sweet potato, wheat, and barley β-amylase (45, 45, 45, and 50 °C, respec-

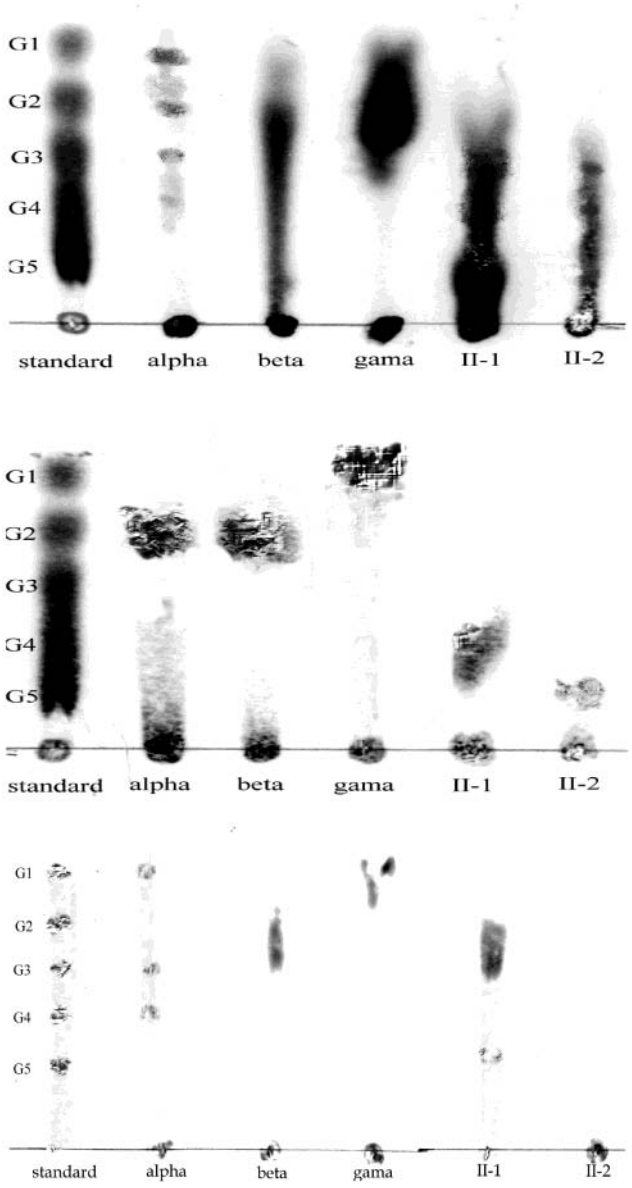

Figure 7. Thin-layer chromatogram pattern of digested amylose (top), amylopectin (middle), and glycogen (bottom) with various amylases and purified amylases II-1 and II-2. [Digestion conditions, 37.5 °C for 72 h; mobile phase, ethyl acetate/acetic acid/water (2:1:1); developed with 5% sulfuric acid/methanol (v/v); standard, G1 (glucose), G2 (maltose), G3 (maltotriose), G4 (maltotetraose), G5 (maltopentaose). Alpha, α-amylase from porcine pancrease; beta, β-amylase from sweet potato; gama, γ-amylase from *A. niger*; II-1, II-2, purified amylases.]

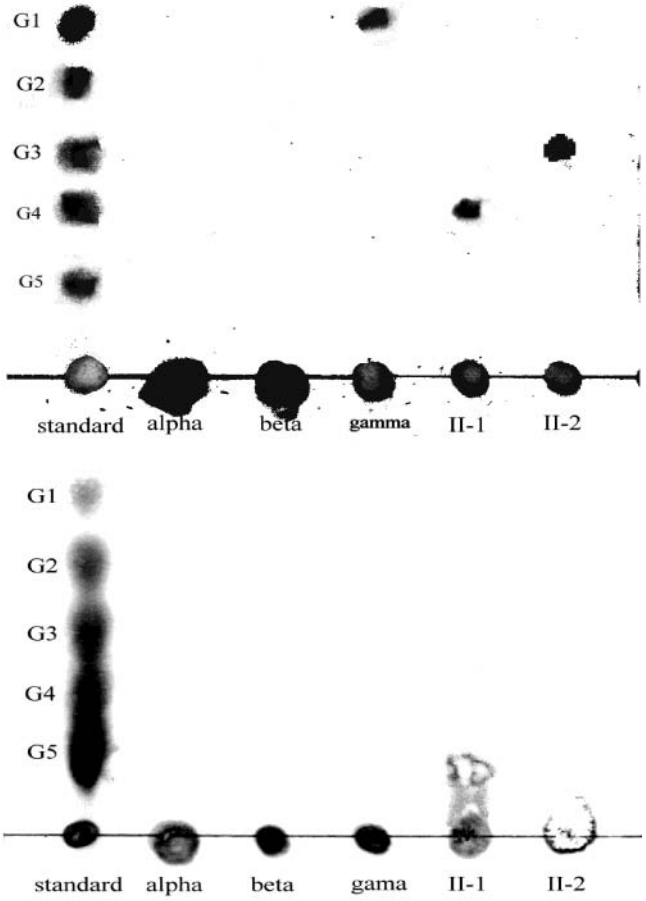

Figure 8. Thin-layer chromatogram pattern of digested *G. tenuistiplitate* (top) and agar-agar (bottom), *G. tenuistiplitate* and agar-agar with various amylases, and purified amylases II-1 and II-2 (refer to the legend of **Figure 7**).

tively) (*6*) but lower than those of *Bacillus lentus* α-amylase (70 °C) (*16*), *Bacillus licheniformis* α-amylase (76 °C) (*44*), *Bacillus acidocaldarius* α-amylase (75 °C) (*25*), *Clostridium* sp. β-amylase (75 °C) (*5*), *Penicillum oxalicum* γ-amylase (55−60 °C), and *A. awamori* γ-amylase (60 °C) (*20*, *21*).

The thermostabilities of the purified amylases II-1 and II-2 were similar to those of mesophilic living bettle α-amylase (stable at <45 °C) (*39*), *Clostridium butyrium* T-7 α-amylase (at <40 °C) (*32*), *Bacillus* sp. No. 195 α-amylase (at <45 °C) (*42*), *Alteromonas haloplanctis* A-23 α-amylase (at <50 °C) (*45*), and *A. awamori* γ-amylase (at <50 °C) (*21*). However, they were lower than those of *B. lentus* α-amylase (at <85 °C) (*16*), *B. acidocaldarius* α-amylase (at <80 °C) (*25*), copepod α-amylase (at <60 °C) (*33*), *B. licheniformis* α-amylase (at <60 °C) (*44*), *Bacillus circulans* α-amylase (at <60 °C) (*46*), and *Coniophora cerebella* γ-amylase (at <60 °C) (*19*).

TLC Analysis of Hydrolysate. As indicated in **Figure 7**, the hydrolysates of amylopectin by amylases II-1 and II-2 were found to be maltotetraose and maltopentaose, respectively, whereas those of amylose were the mixture of maltotriose, maltotetraose, maltopentaose, and maltohexaose. The products of glycogen hydrolyzed by amylase II-1 were also the mixture of maltose, maltotriose, maltotetraose, and maltopentaose. However, no low glucomaltose was found on the hydrolysates of glycogen and *G. tenuistiplitate* degraded by amylase II-2 (**Figures 7** and **8**). According to **Figure 8**, the hydrolysates of *G. tenuistiplitate* and agar-agar degraded by amylase II-1 were maltopentaose and maltotetraose, respectively. Although the hydrolysate of agar-agar by amylase II-2 was maltotriose, no low glucomaltose was observed on that of *G. tenuistiplitate* hydrolyzed by II-2 (**Figure 8**).

The agarase, like glycosyl hydrolase, has two hydrolysis types. One is β-agarase, which hydrolyzes the β-D-1,4-linkage of D-galactose and 3,6-anhydro-L-galactose in agarose and releases neoagaro-oligosaccharides with a reducing end of D-galactose and a nonreducing end of 3,6-anhydro-L-galactose. The other one is α-agarase, hydrolyzing the α-L-1,3-linkage of agarose to produce oligosaccharides with agarobiose (*10*, *47*, *48*). Because the main carbohydrate of *G. tenuistiplitate* and agar-agar is agarose, the purified amylase II-1 could hydrolyze both *G. tenuistiplitate* and agar-agar, and II-2 could hydrolyze *G. tenuistiplitate* (**Figure 8**). This phenomenon suggested that the purified amylases were agarase-like enzymes.

Table 3. Effect of the Metal Ions on the Purified Amylases II-1 and II-2 from Small Abalone (*S. diversicolor aquatilis*)

		amylase activity[a]				
metal	concn (mM)	II-1	II-2	α-	β-	γ-
none		100.0	100.0	100.0	100.0	100.0
LiCl	5	95.6	96.9	104.4	99.8	99.9
NaCl	5	100.0	98.0	104.4	99.6	99.9
KCl	5	112.8	97.7	104.4	95.7	99.9
AgCl	5	116.2	71.4	126.6	146.6	130.3
$MgCl_2$	5	114.2	218.1	104.1	98.9	99.9
$CaCl_2$	5	136.6	100.0	100.0	94.4	99.9
$BaCl_2$	5	98.0	100.4	104.4	99.4	103.3
$ZnCl_2$	5	0.0	7.0	6.1	98.2	116.2
$CdCl_2$	5	3.6	4.6	4.7	6.3	11.1
$HgCl_2$	5	0.0	0.0	0.0	8.0	3.8
$CuCl_2$	5	0.0	0.0	0.0	101.3	130.3
$MnCl_2$	5	316.1	132.8	116.8	129.4	122.4
$FeCl_2$	5	28.4	34.6	104.4	120.6	130.4
$CoCl_2$	5	43.2	37.1	116.8	108.6	122.4
$NiCl_2$	5	18.3	0.0	7.6	8.1	116.2
$FeCl_3$	5	23.5	58.5	2.8	127.5	111.7
$AlCl_3$	5	26.3	0.0	85.3	97.8	141.7

[a] After 30 min of incubation with various metal ions at 37.5 °C, the residual activities of amylases were determined and expressed as percentages of the activities as compared with that without metals.

Table 4. Effect of Inhibitors on the Purified Amylases II-1 and II-2 from Small Abalone (*S. diversicolor aquatilis*)

		amylase activity[a] (%)				
inhibitor[b]	concn (mM)	II-1	II-2	α-	β-	γ-
none		100.0	100.0	100.0	100.0	100.0
NEM	5	91.5	75.9	100.0	100.0	93.5
urea	5	82.4	72.4	97.0	91.7	86.8
EDTA	5	28.9	14.1	51.0	100.0	100.0
IAA	5	72.1	42.4	100.0	42.7	85.7
PMSF	5	42.5	28.0	100.0	97.0	100.0

[a] Enzyme activity was determined after 30 min of reaction with various substrates at 37.5 °C and expressed as percentages the activities as compared with soluble starch. [b] NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; IAA, iodoacetamide.

Metal Ion Effects. As indicated in **Table 3**, Ca^{2+}, Mg^{2+}, Mn^{2+}, K^+, and Ag^+ activated the purified amylases II-1, whereas Mg^{2+} and Mn^{2+} activated II-2. However, Mn^{2+} strongly enhanced the amylases II-1 and II-2 to 316.1 and 132.8%, respectively. Although Ag^+ activated amylase II-1, it moderately inhibited amylase II-2. Hg^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, and Al^{3+} could completely, whereas Co^{2+} and Fe^{3+} partially inhibited the purified amylases II-1 and II-2, which was similar to *Clostridium butyricum* and *Thermoactinomyces vulgaris* (*32*, *34*, *49*). Mg^{2+} could activate short-necked clam α-amylase (*34*) and amylases II-1 and II-2 (**Table 3**) and inhibit *T. vulgaris* and *C. butyricum* T-7 α-amylase (*32*). However, Hg^{2+}, Zn^{2+}, and Cu^{2+} could inhibit amylases II-1 and II-2 and α-amylases of short-necked clam, *T. vulgaris*, and *C. butyricum* T-7 (*32*, *34*, *49*). The purified amylases II-1 and II-2 could be completely inhibited by Hg^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+}, suggesting the existence of cysteine on their active sites.

Inhibitor Effects. As indicated in **Table 4**, *N*-ethylmaleimide (NEM) and urea could partially inhibit the activity of purified amylase II-2 and slightly inhibit II-1 and *A. niger* γ-amylase activities. Ethylenediaminetetraacetic acid (EDTA) and phenylmethanesulfonyl fluoride (PMSF) could inhibit the purified amylases II-1 and II-2, whereas iodoacetamide (IAA) could highly inhibit the II-2 and sweet potato β-amylase activities and partially inhibit the II-1 and *A. niger* γ-amylase (**Table 4**). According to the inhibitor study, purified amylases II-1 and II-2 seemed to be different from α-, β-, and γ-amylases. As we know, PMSF can react with serine, cysteine, and histidine residues and change the conformation of proteins, which consequently cause the enzyme activity to be lost (*50*). NEM and IAA can react with an −SH group and form irreversible interaction (*50*, *51*). The purified amylases II-1 and II-2 could be partially inhibited by IAA, PMSF, and NEM. This result further confirmed the existence of cysteine at their active sites.

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