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Enzymatic properties and primary structures of two α -amylase isozymes from the
pacific abalone *Haliotis discus hannai*

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Abstract

Two α -amylase (EC 3.2.1.1) isozymes, HdAmy58 and HdAmy82, with approximate molecular masses of 58 kDa and 82 kDa, respectively, were isolated from the digestive fluid of the pacific abalone *Haliotis discus hannai*. Optimal temperatures and pHs for HdAmy58 and HdAmy82 were at 30°C and 6.7, and 30°C and 6.1, respectively. Both enzymes similarly degraded starch, glycogen, and maltooligosaccharides larger than maltotriose producing maltose and maltotriose as the major degradation products. However, the activity toward maltotetraose was appreciably higher in HdAmy82 than HdAmy58. cDNAs encoding HdAmy58 and HdAmy82 were cloned and the amino-acid sequences of 511 and 694 residues for HdAmy58 and HdAmy82, respectively, were deduced. The putative catalytic domains of HdAmy58 and HdAmy82 were located in the 17-511th and 19-500th amino-acid regions, respectively, and they showed approximately 50% amino-acid identity to each other. These sequences also showed 62 – 99% amino-acid identity to the catalytic domains of known α -amylases that belong to glycoside-hydrolase-family 13. The difference in the molecular masses between HdAmy58 and HdAmy82 was ascribed to the extension of approximately 190 residues in the C-terminus of HdAmy82. This extended region showed 41 – 63% amino-acid identity with the ancillary domains of several α -amylases previously reported.

Key words: abalone, *Haliotis*; α -amylase; cDNA cloning; GHF-13

1. Introduction

α -Amylase (EC 3.2.1.1) is the enzyme that hydrolyses internal α -1,4-glycoside linkages of α -glucans such as starch and glycogen. This enzyme has been used for the degradation and saccharification of starch in the fields of food, bakery, paper, sugar and starch industries (Monfort et al., 1996; Pandey et al., 2000; Van der Maarel et al., 2002; Gupta et al., 2003). α -Amylases distribute over bacteria, fungi, plants, invertebrates, and higher animals and many of these enzymes belong to glycoside-hydrolase-family 13 (GHF13) (Jensen and Olsen 1992; Henry et al., 1993; Iefuji et al., 1996; Le Moine et al., 1997; Koyama et al., 2000; Saxena et al., 2007; Tripathi et al., 2007; Hmidet et al., 2008; Elarbi et al., 2009; Nikapitiya et al., 2009). This enzyme plays a crucial role in the degradation of storage α -glucan providing maltooligosaccharides and glucose, the most important carbon and energy sources in many organisms.

The pacific abalone *Haliotis discus hannai* is an herbivorous gastropod that feeds on seaweeds and assimilates seaweeds' polysaccharides, e.g., alginate, cellulose, mannan and laminarin, as carbohydrate nutrients. The feeding behavior of abalone has long been investigated from the view point of aquaculture (Mai et al., 1995; Takami et al., 1998) and enzymatic properties of polysaccharide-degrading enzymes of abalone have also been investigated to enrich the knowledge for the digestion mechanisms of seaweeds' polysaccharides by abalone (Shimizu et al., 2003; Suzuki et al., 2003; Suzuki et al., 2006; Ootsuka et al., 2006; Kumagai and Ojima 2009). Such information seems to be indispensable to understand how marine mollusks acquire carbohydrate nutrients from seaweeds.

Polysaccharide-degrading enzymes prominent in the digestive fluid of abalone are

alginate lyase (Shimizu et al., 2003; Suzuki et al., 2006), cellulase (Suzuki et al., 2003), mannanase (Ootsuka et al., 2006), β -1,3-glucanase (Kumagai and Ojima 2009), and α -amylase (Nikapitiya et al., 2009). Among these enzymes, α -amylase is considered to play an important role for the digestion of storage α -glucan contained in red and green seaweeds. To date, basic properties of an α -amylase from the disk abalone *Haliotis discus discus* were investigated by using the recombinant enzyme (Nikapitiya et al., 2009). However, properties of native α -amylase from *H. discus discus* has not been investigated as in cases of other polysaccharide-degrading enzymes from the pacific abalone *Haliotis discus hannai* (Shimizu et al., 2003; Suzuki et al., 2003; Suzuki et al., 2006; Ootsuka et al., 2006; Kumagai and Ojima 2009).

Under these circumstances, we recently noticed that two α -amylase isozymes with the approximate molecular masses of 58 kDa and 82 kDa were contained in the digestive fluid of *H. discus hannai*. The 58-kDa α -amylase was considered as a similar enzyme as that previously investigated with *H. discus discus* (Nikapitiya et al., 2009). However the 82-kDa enzyme appeared to be an unidentified enzyme. Therefore, in the present study, we isolated the two α -amylases from the digestive fluid of *H. discus hannai*, and compared their general properties. Further, we cloned the cDNAs encoding each enzyme and investigated the difference in the primary structures between the two enzymes.

2. Materials and methods

2.1. Materials

The pacific abalone, *H. discus hannai*, was obtained from a local market in Hakodate, Hokkaido Prefecture, Japan. Soluble starch (corn origin), glycogen (oyster origin), maltooligosaccharides (maltose – maltohexaose), pullulan, and sucrose were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). α -*p*-Nitrophenylglucoside (α -*p*NPG) and agarose were purchased from Seikagaku Kogyo (Tokyo, Japan) and TaKaRa (Tokyo, Japan), respectively. Hydroxyapatite (Fast Flow Type) was purchased from Wako Pure Chemicals Industries Ltd., TOYOPEARL Phenyl-650M and TOYOPEARL CM-650M from Toyo Soda Mfg. Co. (Tokyo, Japan), and Mono-S 5/50GL from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England). Oligotex-dT(30), TaKaRa *Taq* DNA polymerase, 5'-Full RACE and 3'-Full RACE kits, and restriction endonucleases were purchased from TaKaRa (Tokyo, Japan). DynaExpress TA PCR Cloning kit (including pTAC-1 vector) was from BioDynamics Laboratory Inc. (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemicals Industries Ltd.

2.2. Assay for α -amylase activity

α -Amylase activity was assayed with a substrate solution containing 10 mM sodium phosphate buffer (pH 6.0) and 0.2% (w/v) soluble starch which had been dissolved by heating at 100°C for 10 min. The reaction was initiated by the addition of 50 μ L of enzyme solution (0.01-0.05 units) to 950 μ L of the substrate solution. Reducing sugars produced by the reaction were determined by the method of Park and Johnson (1949). One unit (U) of α -amylase was defined as the amount of enzyme that produces reducing sugars equivalent to 1.0 μ mol glucose/min. Temperature dependence of α -amylase was

assayed at 10 – 60°C. pH dependence of α -amylase was measured at 30°C with the reaction mixtures adjusted to pH 4.5 – 8.0 with 10 mM sodium phosphate buffer.

2.3. Purification of α -amylases isozymes from abalone

Two α -amylase isozymes were purified from digestive fluid of abalone *H. discus hannai* as follows: 15 abalones (an average shell size, 10 x 6 cm; an average body weight, 80 g; kept alive in a sea-water tank at 15°C by kelp-feeding) were dissected with a scalpel to remove the adductor muscles. The digestive fluid was then collected from the stomach lumen by aspiration with Pasteur pipette. By this procedure, approximately 30 mL of the digestive fluid was obtained from the 15 abalones. The digestive fluid was mixed with 70 mL of 10 mM sodium phosphate (pH 6.0) and centrifuged at $10,000 \times g$ for 10 min to obtain supernatant (crude enzyme). The crude enzyme was then fractionated by ammonium sulfate fractionation and the fraction precipitated between 30 and 60% saturation of ammonium sulfate was collected by centrifugation at $10,000 \times g$ for 10 min. The precipitates were dissolved in 10 mM sodium phosphate buffer (pH 6.0) containing 50%-saturated ammonium sulfate and subjected to a TOYOPEARL Phenyl-650M column (2.5×25 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted stepwisely with 50%, 40%, 30%, 20%, and 0%-saturated ammonium sulfate in 10 mM sodium phosphate buffer (pH 6.0) (Fig. 1A). α -Amylase activity was detected in the fractions eluted with 0%-saturated ammonium sulfate. These fractions (fraction numbers 134-136) were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0), and applied to a TOYOPEARL CM-650M column (2.5×34 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted

with a linear gradient of 0-0.3 M NaCl (Fig. 1B). In this chromatography, α -amylase activity was detected in the fractions eluted at around 0.1 M NaCl (fraction numbers 33-41, designated Fr1) and 0.2 M NaCl (fraction numbers 50-57, designated Fr2). Fr1 was dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and applied to a hydroxyapatite column (1.2×20 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0 – 0.3 M sodium phosphate buffer (pH 6.0) and α -amylase was detected in the fractions eluted at around 0.05 M sodium phosphate buffer (fraction numbers 23-25) (Fig. 1C). The active fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and concentrated to approximately 2 mL by ultrafiltration with VIVASPIN 20 (Sartorius AG, Goettingen, Germany). The concentrates were subjected to a Mono-S 5/50GL column pre-equilibrated with 10 mM sodium phosphate (pH 6.0) and the proteins adsorbed to the column were eluted with a linear gradient of 0 – 0.3 M NaCl. In this chromatography, an α -amylase with approximately 82 kDa was eluted at around 0.05 M NaCl (Fig. 1D and 2). This α -amylase was designated as HdAmy82 after the scientific name of *H. discus hannai* and the molecular mass of 82 kDa. On the other hand, Fr2 was dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and concentrated to approximately 2 mL by ultrafiltration with VIVASPIN 20. The concentrates were then applied to a Mono-S 5/50GL column pre-equilibrated with same buffer and proteins adsorbed to the column were eluted with a linear gradient of 0 – 0.15 M NaCl followed by 0.3 M NaCl. In this chromatography, an α -amylase with approximately 58 kDa was eluted at around 0.13 M NaCl (Fig. 1E and 2). This enzyme was designated HdAmy58. By the above procedures, HdAmy82 was purified 109 fold with the specific activity of 29 U/mg and the yield of 0.9%, while HdAmy58 was purified 150 fold with the specific

activity of 45 U/mg and the yield of 0.2% (Table 1).

2.4. Protein concentration

Protein concentration was determined by the method of Lowry et al., (1951) using bovine serum albumin fraction V as a standard protein.

2.5. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 0.1% (w/v) SDS – 10% (w/v) polyacrylamide gel according to the method of Porzio and Pearson (1977). After the electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol – 10% (v/v) acetic acid, and destained with 5% (v/v) methanol – 7% (v/v) acetic acid. Protein Marker, Broad Range (New England BioLabs, Ipswich, MA, USA) was used as a molecular mass marker.

2.6. Thin-layer chromatography

Degradation products of maltooligosaccharides, starch and glycogen produced by α -amylase were analyzed by thin-layer chromatography (TLC). The products (approximately 10 μ g) were spotted on a TLC-60 plate (Merck, Darmstadt, Germany) and developed with a solvent of n-butanol – acetic acid – water (2:1:1 (v:v:v)). The sugars developed on the plate were stained by spraying 10% (v/v) sulfuric acid in

ethanol followed by heating at 130°C for 10 min.

2.7. N-terminal amino-acid sequence

The N-terminal amino-acid sequence of α -amylase was determined with an ABI Procise 492 sequencer (Applied Biosystems, Foster city, CA, USA) using the samples electrically transferred to the polyvinylidene-difluoride membrane (Applied Biosystems) after SDS-PAGE.

2.8. Cloning of α -amylase cDNAs

Total RNA was extracted from the hepatopancreas of abalone by the guanidinium thiocyanate-phenol method (Chomczynski and Sacchi, 1987). mRNA was selected from the total RNA with an Oligo-dT(30) kit (TaKaRa) according to the manufacturers' protocol. cDNA was synthesized from the mRNA with a cDNA synthesis kit (TaKaRa) using random oligonucleotide primers. cDNAs encoding α -amylase were amplified by the PCR with degenerated primers synthesized on the basis of the N-terminal amino-acid sequence of the α -amylase and the internal amino-acid sequences conserved among α -amylases previously reported. For the amplification of cDNA, a successive reaction at 95°C for 30 s, 50°C for 30 s, and 72°C for 120 s was repeated 30 cycles in 20 μ L of reaction mixture containing 50 mM KCl, 15 mM Tris-HCl (pH 8.1), 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 2.5 mM MgCl₂, and 10 pmol primers, 20 ng hepatopancreas cDNA, and 0.5 U of TaKaRa *Taq* DNA polymerase. The amplified cDNAs were cloned with pTAC-1 vector and the DynaExpress TA PCR Cloning kit

(BioDynamics Laboratory Inc., Tokyo, Japan). cDNAs encoding 5'- and 3'-regions of the α -amylase cDNA were amplified with 5'-Full RACE and 3'-Full RACE kits (TaKaRa) and cloned with pTAC-1 vector as described above. Homology search for deduced amino-acid sequences were performed using the BLAST tool provided by National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

3. Results

3.1. General properties of α -amylase isozymes HdAmy58 and HdAmy82

Optimal temperatures of HdAmy58 and HdAmy82 were similarly observed at around 30°C, while their optimal pHs were observed at 6.7 and 6.1, respectively (Fig. 3). HdAmy58 and HdAmy82 degraded maltooligosaccharides larger than trisaccharide, starch, and glycogen but not degraded pullulan, agarose, laminarin, carboxymethylcellulose, and α -pNPG. These results indicated that both HdAmy58 and HdAmy82 were regarded as a typical α -amylase (EC 3.2.1.1) that specifically degrades α -1,4-glucoside linkages of α -1,4-glucan and maltooligosaccharides larger than trisaccharide.

3.2. Degradation products produced by HdAmy58 and HdAmy82

Degradation products of maltooligosaccharides, starch, and glycogen produced by HdAmy58 and HdAmy82 were analyzed by TLC. As shown in Fig. 4, both enzymes could degrade maltooligosaccharides larger than maltotriose producing mainly maltotriose and maltobiose. However, the rate for the degradation of maltotetraose was

appreciably different between the two enzymes. Thus HdAmy58 modestly degraded maltotetraose producing maltotriose and maltobiose and a part of the original maltotetraose substrate remained undigested even in the 24-h reaction. Whereas HdAmy82 rapidly degraded the tetrasaccharide and the original maltotetraose was almost completely degraded within 5 h (Fig. 4). Such difference in the degradation rates for maltotetraose was also shown in the degradation of maltopentaose and maltohexaose. Namely, the maltotetraose produced from maltopentaose and maltohexaose by HdAmy58 was not readily digested by HdAmy58 while that produced by HdAmy82 was rapidly digested by HdAmy82 (Fig. 4). These results indicate that HdAmy82 can degrade maltotetraose more efficiently than does HdAmy58. On the other hand, both enzymes similarly degraded starch and glycogen producing maltose, maltotriose, and glucose (Fig. 4). This result is consistent with the result that the specific activities of toward starch are comparable between HdAmy58 and HdAmy82, i.e., 45U/mg and 29 U/mg, respectively (see Table 1).

3.3. Primary structure of HdAmy58 and HdAmy82

The N-terminal amino-acid sequences of HdAmy58 and HdAmy82 were determined as SMYSDPHXSS and EFTDPHXNG, respectively, by a protein sequencer (“X” was later revealed to be Cys by the cDNA method). The N-terminal sequence of HdAmy58 showed 90% similarity to the corresponding sequence of the previously reported α -amylase from *H. discus discus* (Nikapitiya et al., 2009; GenBank accession number, ABO26610). On the other hand, the N-terminal sequence of HdAmy82 showed 67% similarity to α -amylase from the common cockle *Cerastoderma edule* (GenBank

accession number, ACA34380). We then synthesized the forward degenerated primer 58Fw1 on the basis of the N-terminal sequence of HdAmy58, and the reverse primer 58Re1 on the basis of the conserved sequences among molluscan α -amylases previously reported (Nikapitiya et al., 2009; GenBank accession number, EF103352, Le moine et al., 1997; GenBank accession number, X99729, Moal et al., 1997; GenBank accession number, AF320668) (Table 2). cDNAs encoding HdAmy58 were amplified by the PCR using these primers from the hepatopancreas cDNA. As a result, a cDNA with approximately 0.9 kbp (58-cDNA) was amplified. The 58-cDNA comprised 920 bp that encoded an amino-acid sequence of 306 residues. Then, a series of specific primers for 3'-RACE and 5'-RACE were synthesized on the basis of the nucleotide sequence of 58-cDNA (Table 2). By using the specific primers, 58-3RACE-cDNA (791 bp) covering the 3'-terminal region and 58-5RACE-cDNA (630 bp) covering the 5'-terminal region were amplified by 3'-RACE and 5'-RACE, respectively. By overlapping the nucleotide sequences of 58-5RACE-cDNA, 58-cDNA, and 58-3RACE-cDNA in this order, a nucleotide sequence of 1648 bp encoding the amino-acid sequence of 551 residues for HdAmy58 was determined. The reliability of this sequence was confirmed with 58Full-cDNA, which was newly amplified by PCR with a specific primer pair, 58FullFw and 58FullRe (Table 2) (Fig. 5).

The cDNAs encoding HdAmy82 were also amplified with the primers listed in Table 2 by the same procedure for the amplification of HdAmy54. By overlapping the nucleotide sequences of 82-5RACE-cDNA (382 bp), 82-cDNA (1264 bp), and 82-3RACE-cDNA (949 bp), the nucleotide sequence of 2197 bp that encoded the amino-acid sequence of 694 residues of HdAmy82 was determined (Fig. 6). The reliability of the overlapped sequence was confirmed with 82Full-cDNA that was newly

amplified with a specific primer pair, 82FullFw and 82FullRe (Table 2). The nucleotide and deduced amino-acid sequences for HdAmy58 and HdAmy82 are available from DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/>) with accession numbers AB748934 and AB748935, respectively.

The N-terminal 15 and 17 residues in the deduced sequences of HdAmy58 and HdAmy82 except for the initiation methionine were regarded as the signal peptide regions, since these regions were absent in native HdAmy58 and HdAmy82 and predicted as a signal peptide for secretion by SignalP 4.0 software (<http://www.cbs.dtu.dk/services/SignalP/>). Thus mature HdAmy58 and HdAmy82 were concluded to consist of 551 and 686 amino-acid residues, respectively.

The 17-511th amino-acid region of HdAmy58 showed 50% similarity to the 19-500th amino-acid region of HdAmy82 (Fig. 7A) and 99-62% similarity to the sequences of α -amylases from several marine mollusks that belong to glycosyl hydrolase family 13 (GHF13) (see the discussion part). This indicated that the N-terminal approximately 495 and 480 residues for HdAmy58 and HdAmy82, respectively, corresponded to the GHF-13-type catalytic domain (Fig. 7B). Accordingly, the C-terminal approximately 190 residues of HdAmy82 was revealed to be the specific region that was absent in HdAmy58. This region was considered as an ancillary domain of HdAmy82 since it showed considerably high sequence similarities to the ancillary domains of several α -amylases previously reported (Da Lage et al., 2004).

4. Discussion

4.1. Enzymatic properties of HdAmy58 and HdAmy82

In the present study, two α -amylases HdAmy58 and HdAmy82 were isolated from the digestive fluid of the pacific abalone *H. discus hannai*. HdAmy58 showed optimal temperature and pH at around 30°C and 6.7, respectively, while HdAmy82 showed at around 30°C and 6.1, respectively. HdAmy58 and HdAmy82 efficiently degraded α -1,4-glucan such as starch and glycogen but not α -pNPG, pullulan, agarose, laminarin, and CMC. Thus HdAmy58 and HdAmy82 were regarded as a typical α -amylase (EC 3.2.1.1) that degrades internal α -1,4-glycoside linkages of α -glucan. Both enzymes could degrade maltooligosaccharides larger than maltotriose producing maltose and maltotriose; however, HdAmy82 degraded maltotetraose more rapidly than did HdAmy58. These results may indicate that HdAmy82 compensates for the low activity of HdAmy54 toward maltotetraose in the digestive fluid. Both HdAmy58 and HdAmy82 were capable of producing maltooligosaccharides; however, they hardly produced glucose. Therefore, abalone should have another enzyme such as exo-type amylase and/or α -glucosidase to completely depolymerize seaweeds' starch. To date, occurrence of α -glucosidase has been shown in sea hare *Aplysia fasciata* (Andreotti et al., 2006); however, general properties and primary structures of other molluscan α -glucosidases have not been so well investigated. Quite recently we found that the digestive fluid (crude enzyme) of the abalone *H. discus hannai* could produce substantial amount of glucose from potato and seaweed starches. This indicated that the crude enzyme contained α -glucosidase along with the α -amylases HdAmy58 and HdAmy82. Generally, α -glucosidase of mammalian animals is known as an intestinal enzyme expressed in epithelial cell membrane (Galand, 1989). If abalone α -glucosidase is produced as free enzyme in the digestive fluid, this enzyme may possess substantially

different structures and functions from those of the mammalian enzymes. We are now attempting to isolate and characterize the abalone α -glucosidase from the digestive fluid.

4.2. Primary structure of HdAmy58 and HdAmy82

Enzymatic properties of HdAmy58 and HdAmy82 were similar to each other except for degradation rates for maltotetraose; however, molecular masses were significantly different between two enzymes. To understand the reason for such great difference in the molecular masses of the two enzymes, we analyzed their primary structures by the cDNA method. The deduced amino-acid sequences of HdAmy58 and HdAmy82 were found to comprise 511 and 694 residues, respectively. The N-terminal regions of approximately 500 residues of HdAmy58 and HdAmy82 showed fairly high similarity (50%) to each other and the C-terminal approximately 190 residues of HdAmy82 was regarded as the extended ancillary domain of this enzyme. Thus, the difference in the molecular masses between HdAmy58 and HdAmy82 was attributed to the occurrence of the C-terminal ancillary domain in HdAmy82. The sequence of HdAmy54 was almost identical with that of an α -amylase from the disk abalone *H. discus discus* (Nikapitiya et al., 2009; GenBank accession number, ABO26610). Between two sequences, only one amino-acid replacement was found in the 433th residue, i.e., Leu in HdAmy54 is replaced by Phe in the disk abalone enzyme. Therefore, HdAmy58 was regarded as the similar enzyme previously reported in the disk abalone (Nikapitiya et al., 2009). The sequence of HdAmy58 showed 41 – 67 % similarities with those of GHF-13-type α -amylases from marine mollusks and bacteria. Thus, this

enzyme was regarded as a member of GHF-13 α -amylases.

On the other hand, HdAmy82 was considered to be an α -amylase that had not been characterized yet. This enzyme possessed an ancillary domain in the C-terminus, which was absent in HdAmy58 (Fig. 7A and B). Some of GHF-13 α -amylases are known to possess the family-20-type carbohydrate-binding modules (CBM) (Da-Lage et al. 2004). Thus, we performed BLAST-searching for the C-terminal of HdAmy82 to examine whether this region corresponds to the CBM. However, this region showed no similarity to any CBMs. Instead, it showed 56%, 51% and 37% similarity to the ancillary domains of α -amylases from *Corbicula fluminea* (GenBank accession number, AAO17927), *Mytilus edulis* (GenBank accession number, ACA34372) and a marine bacterium *Pseudoalteromonas haloplanktis* (GenBank accession number, CCA41481), respectively (Fig. 8). The C-terminal region of α -amylase from *P. haloplanktis* was reported to assist its extracellular secretion (Feller et al., 1998). However, in case of HdAmy82, a putative signal peptide for secretion was located in the N-terminus (Fig. 6). On the other hand, it may be possible to consider that this region promotes the binding to smaller substrates such as maltotetraose since the activity of HdAmy82 toward this substrate was much higher than that of HdAmy54 (Fig. 4). Prediction of secondary structure for the C-terminal region of HdAmy82 with GOR method (Garnier J et al., 1996) indicated that this region was made up of β -sheets, turns, and random coils. This led us to consider that the C-terminal domain of HdAmy82 possesses somewhat similar structural motif to the known CBMs possessing a ' β -sandwich' motif (Boraston AB et al., 2004). To reveal the physiological rolls of the C-terminal ancillary domain of HdAmy82, studies on the recombinant HdAmy82 expressed with and without the C-terminal domain are expected.

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Figure legends

Fig. 1. Purification of α -amylase isozymes from abalone. (A) TOYOPEARL Phenyl-650M column chromatography of proteins precipitated between 30 and 60% saturation of ammonium sulfate. (B) TOYOPEARL CM-650M column chromatography of the α -amylase obtained by TOYOPEARL Phenyl-650M chromatography. (C) Hydroxyapatite column chromatography of the α -amylase fraction 1 (Fr1) obtained by TOYOPEARL CM-650M chromatography. (D) Mono-S 5/50GL column chromatography of Fr1. (E) Mono-S 5/50GL column chromatography of the α -amylase fraction 2 (Fr2) obtained by TOYOPEARL-CM-650M. Protein elution and enzyme activity are indicated with open and closed circles (or shaded boxes), respectively.

Fig. 2. Monitoring of purification of α -amylases from the abalone by SDS-PAGE. Lane 1, marker proteins; lane 2, purified HdAmy58; lane 3, purified HdAmy82.

Fig. 3. Temperature and pH dependences of HdAmy58 and HdAmy82. (A) Temperature dependence of α -amylases was measured at 10 – 60°C in a reaction mixture containing 0.2% starch and 10 mM sodium phosphate buffer (pH 6.0). (B) pH dependence of α -amylase was measured at 30°C in reaction mixtures adjusted to pH 4.5-8.0 with 10 mM sodium phosphate buffer. ●, HdAmy58; ○, HdAmy82. Triplicate measurements were performed and typical results are shown as representative ones.

Fig. 4. Thin-layer chromatography for the degradation products of maltooligosaccharides, starch and glycogen produced by HdAmy58 and HdAmy82. 5

mg/mL maltooligosaccharides, 2 mg/mL starch and 2 mg/mL glycogen in 10 mM sodium phosphate buffer (pH 6.0) were degraded with 0.01 U/mL HdAmy58 and HdAmy82 at 30°C. The reaction was terminated at appropriate times by mixing with an equal volume of 10 % (w/v) trichloroacetic acid and 1 µL of the mixture was applied to TLC plate. M, marker sugars comprising G1 – G6. G1, glucose; G2 – G6, maltose to maltohexaose.

Fig. 5. Nucleotide and deduced amino-acid sequences of HdAmy58 cDNA. The translational initiation codon ATG, the termination codon TAG and putative polyadenylation signal AATAAA are boxed. A putative signal peptide for secretion is indicated by a dotted underline. The amino-acid sequences determined with intact HdAmy58 (N-terminus) are indicated by the underline. The annealing sites of PCR primers (see Table 2) are indicated by arrows over the nucleotide sequence.

Fig. 6. Nucleotide and deduced amino-acid sequences of HdAmy82 cDNA. Indices are used as in Fig. 5. The annealing sites of PCR primers (see Table 3) are indicated by arrows over the nucleotide sequence.

Fig. 7. (A) Alignment for amino-acid sequences of HdAmy58 and HdAmy82. The amino-acid sequences were aligned by Clustal X software. The positions of identical residues and gaps are indicated by asterisk (*) and dash (–), respectively. (B) Schematic representation for the primary structures of HdAmy58 and HdAmy82.

Fig. 8. Alignment for amino-acid sequences of ancillary domains from HdAmy82 and other α -amylases. The amino-acid sequence of ancillary domain of HdAmy82 is aligned with those of α -amylases from *Corbicula fluminea* (GenBank accession number, AAO17927), *Mytilus edulis* (GenBank accession number, ACA34372) and *Pseudoalteromonas haloplanktis* (GenBank accession number, CCA41481). The positions of identical residues and gaps are indicated by asterisk (*) and dash (–), respectively.

Table 1. Summary for the purification of HdAmy58 and HdAmy82.

Samples	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude ^a	945	0.3	251	1	100
Phenyl ^b	157	0.4	82	1.5	33
HdAmy58					
CM ^c	6.4	23	17	77	6.8
Mono-S ^d	0.01	45	0.5	150	0.2
HdAmy82					
CM ^c	15	3.0	44	11	18
Hydroxy ^e	0.9	6.7	6.0	25	2.4
Mono-S ^f	0.08	29	2.3	109	0.9

^a Crude enzyme after the dialysis against 10 mM sodium phosphate (pH 6.0)

^b Active fraction obtained by TOYOPEARL Phenyl-650M chromatography.

^c Active fraction obtained by TOYOPEARL CM-650M chromatography.

^d HdAmy58 purified by Mono-S chromatography.

^e Active fraction obtained by hydroxyapatite chromatography.

^f HdAmy82 purified by Mono-S chromatography.

Table 2. DNA primers used for the amplification of HdAmy58 and HdAmy82 cDNAs.

Name	Sequence ^a
HdAmy58	
58Fw1	5'-TCNATGTAYAGYGARCCNCA-3'
58Re1	5'-CCNCCNCCNTGNCCNCG-3'
58Fw2	5'-TGGCTCAAGTCTTCAGGAAG-3'
5race58RT	5'-CCAGCCATAGTC-3'
5race58F1	5'-ACCATATGGCTACTGTGGAG-3'
5race58F2	5'-CCAATGAGAACCGTGTAGTCAC-3'
5race58R1	5'-TTTCGCAATGTCCGACCGTC-3'
5race58R2	5'-GGGTGATGGCACTCCTACC-3'
58FullFw	5'-TGTTTAGCGTTCCTGCTGCC-3'
58FullRe	5'-AGATTACAGCTCCTGACC-3'
HdAmy82	
82Fw1	5'-GARTTYACNGAYCCNCA-3'
82Re1	5'-GGCTGGCAGCCCTGTTCGG-3'
82Fw2	5'-TCATGGCGGCGGCGGAGG-3'
5race82RT	5'-TCTGAATTGCGC-3'
5race82F1	5'-AACAGGAACGGGCAGGTAGC-3'
5race82F2	5'-GTACCATACAGGAGAGAGC-3'
5race82R1	5'-GATCCTCATTACCGCACCTG-3'
5race82R2	5'-CCTGTACAGCACAGTAACC-3'
82FullFw	5'-CGTCTGCCATGATTCCTACCTGG-3'
82FullRe	5'-CACGTTTCGCACGTCTCCATG-3'

^aN,A/C/G/T; R,A/G; Y, C/T.

Fig. 1.

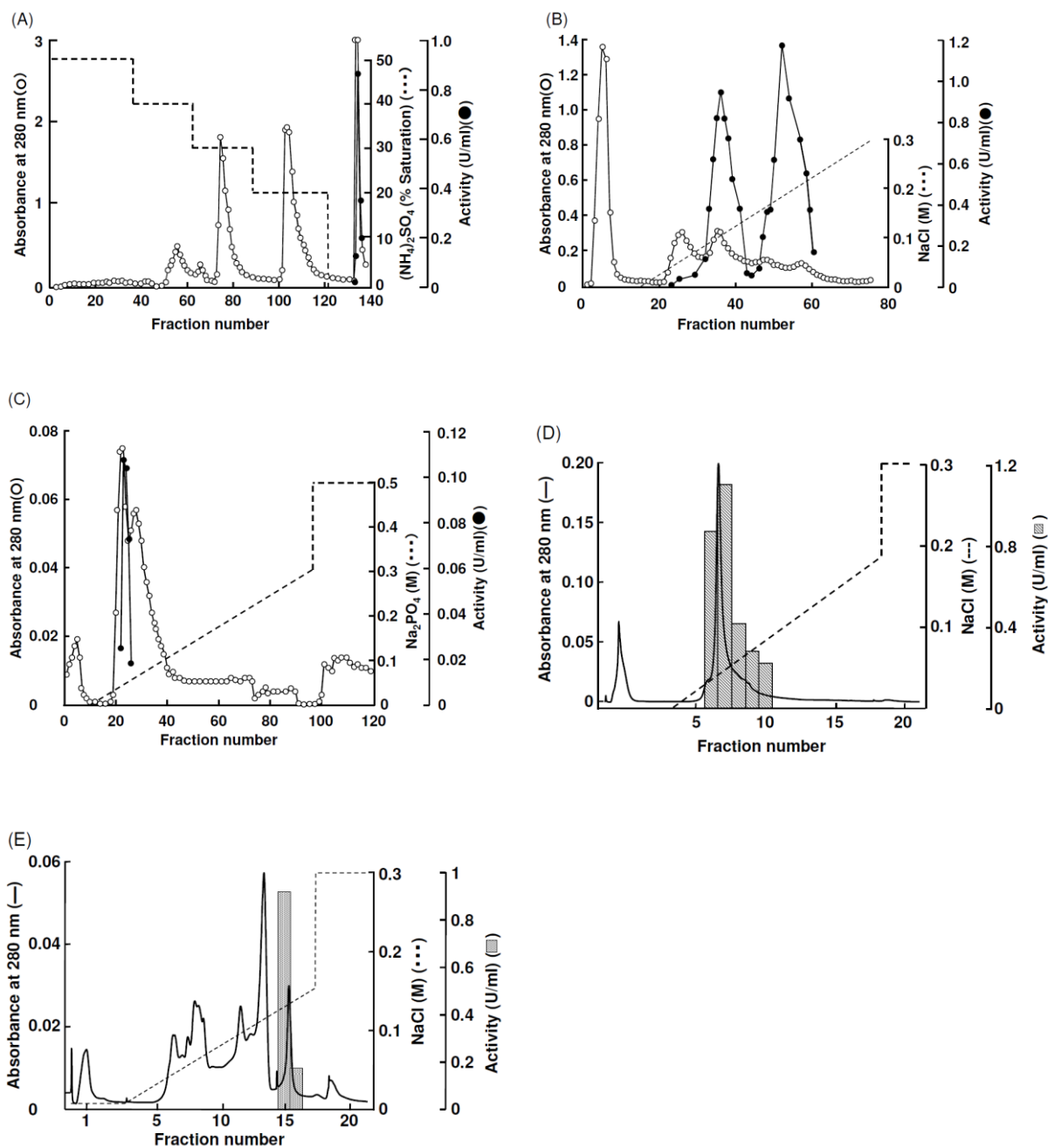


Fig. 2.

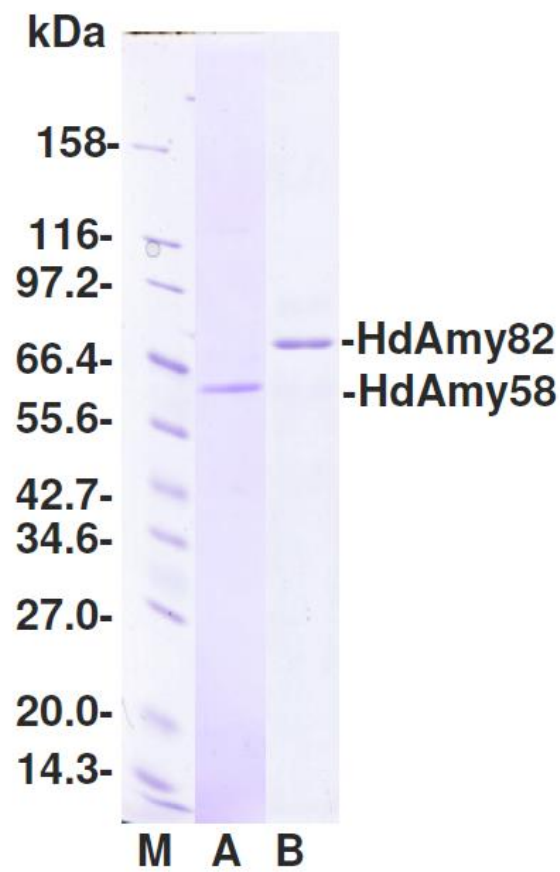


Fig. 3.

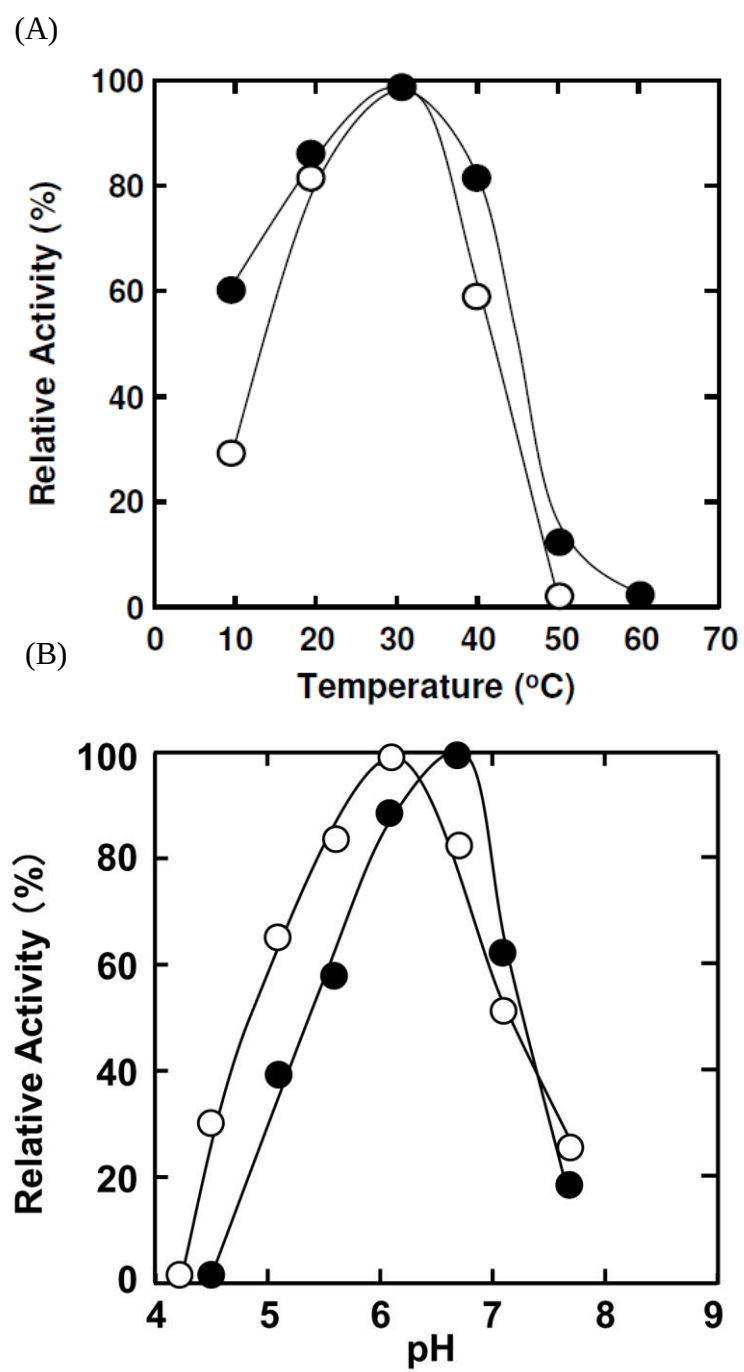


Fig. 4.

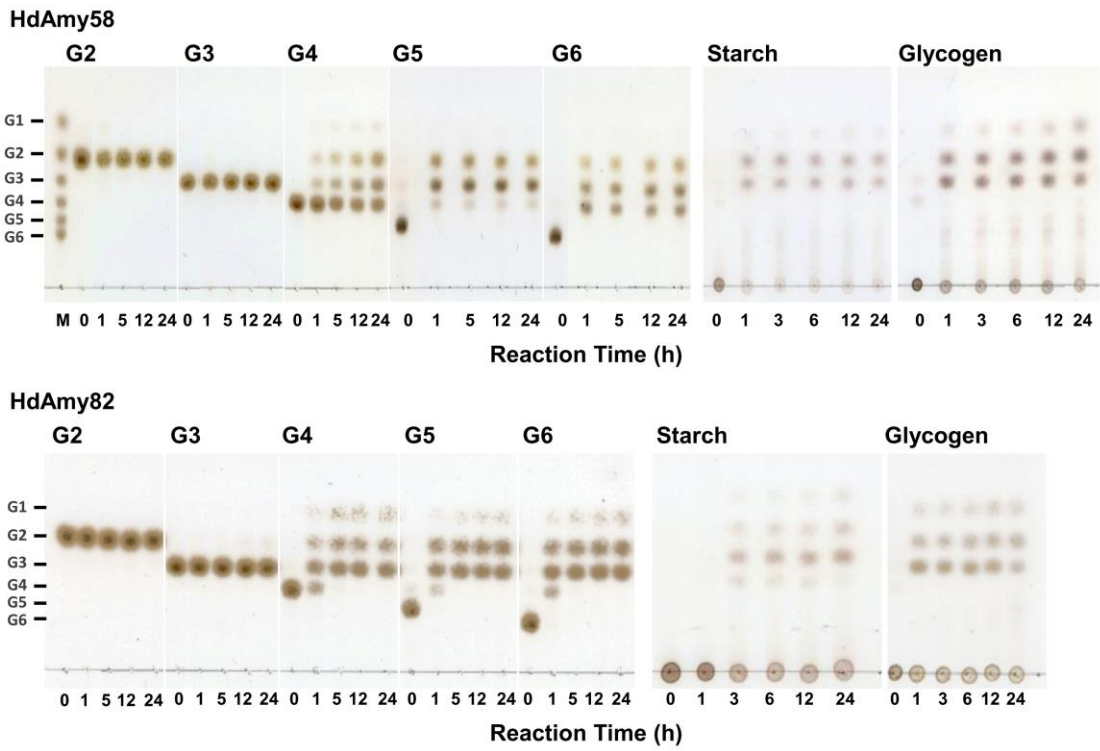


Fig. 5.

GTCATTGTAGCAACGTCTGCAATGATTCTACCTGGTGTGTTAGCGTTCTGCTGCCAACGGCCCTAGCGTGGATGTACAGTGATCCCCACTGTTTCAAGTGGTAGGAGTGCATCACCCAC 120
 M I P T W C L A F L L P T A L A S M Y S D P H C S S G R S A I T H 33
 CTGTTTGGAGTGGACGTGGTGGACATTGGCAAGAATGTGAGAGGTTCTTGGACCATATGGCTACTGTGGAGTTCAGATATCTCTCCCAATGAGAACCGTGTAGTCACTAACCCAAAG 240
 L F E W T W S D I A K E C E R F L G P Y G Y C G V Q I S P P N E N R V V T N P R 73
 CGGCCATGGTGGGAGAGGTACACGCTGTCACTACAACTTCAGACGAGGAGCGGCTCAGAGCAGGACCTGAAGGACATGGTGTCCAGGTGCAACAAGGCCGAGTCAGAATATATGCG 360
 R P W W E R Y Q P V S Y K L Q T R S G S E Q D L K D M V S R C N K A G V R I Y A 113
 GACACTGTGATTAATCACATACCGGTGTTGGAGGTACGGTCAAGGACAGGCTGGATCGTGTACGACGCCAACGCTGAAGTACCCAGGCTTCCATACGGCCCAACCGACTTCAAC 480
 D T V I N H M T G V G G T G Q G T A G S S Y D A N Q L K Y P G V P Y G P T D F N 153
 GACAACTCCAACCTGCCACACTGGCAGATGCAAAATTCATACTACAATAACCCGGAAGAGGTGAGGAAGTCCCGTTGGTGGGTTGGCGGATCTGAAGGCCGCAAGGATTACGTGAGG 600
 D N S N C H T G D M Q I H N Y N N P E E V R N C R L V G L A D L K A G K D Y V R 193
 AGCGAGATTGAGGGTTACCTTAACCACTGGTGGAGATTGGATTGCTGGATTGAGAGTGGAGCTGCCAAACATATGTGGCCAGGGGACCTGACTGCTATATTGGGAGTGTAAAGAT 720
 S E I E G Y L N H L V D I G I A G F R V D A A K H M W P G D L T A I F G S V K N 233
 CTCGGTCTGACGTGTTTGGGGTGGCAAGAGCCTTTCGTCTCCAGGAGGTAAATCGACATGGTGGTGGAGCGGATCAAGGGAGACCGAGTACCTTGGCAGTGGCAGGGTCACCAACTTC 840
 L R S D V F G G G K K P F V F Q E V I D M G G E P I K G D Q Y L G S G R V T N F 273
 ATTTTGGTCCCAAGTTGGCTCAAGTCTTCAGGAAGCAAAAGCCATGAAGTATTTATCCAAGTGGGATCAGCTTGGGGATGTTGAATCCGACGATTCTGTCTTCATCATGAAC 960
 I F G A K L A Q V F R K Q N A M K Y L S N W G S A W G M L K S D D S V V F I D N 313
 CATGACAATCAGCGTGGTATGGCGGCGGCGAGGTGTTCTCACCCACTTTGAGCCCGTCTTATAAGTGGCTACAGCCTTCATGCTGGCCCATCCATATGTTTCCCGAGGGTGATG 1080
 H D N Q R G H G G G G G V L T H F E P R P Y K L A T A F M L A H P Y G F P R V M 353
 AGCTCATATAACTCAACCAAGCAACACTGACCAAGGACCCCTCAGAACGGCGCATGAGTACTAAGCCGGTAACCATCAATGGTATGGTCTGTGGTAACGGCTGGAGTGTGAGCAC 1200
 S S Y N F N Q A N T D Q G P P Q N G D M S T K P V T I N G M V C G N G W T C E H 393
 CGATGGCGCAGATGTACAACATGGTTGCCCTCAGGAACATAGCGGTTACTCCGGTTATCAAAATTGGTGGTCAAGGCTCAGACTATCAGATAGCATTTTCCAGAGGAACCAAGGCCCTT 1320
 R W R Q M Y N M V A F R N I A G Y S G L S N W W S G S D Y Q I A F S R G N K A L 433
 ATTGGCTCAACCTCGAGGATATGACCTTTCCAAATCCGTAAACATGGACTCCGTCTGGAAGCTATTGCGATGTCAATTCTGGCAACCTCGAGAAGCGCGTGTACTGGCAACACA 1440
 I A F N L E G Y D L S K S L N T G L P S G S Y C D V I S G N L E N G G C T G K T 473
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 I D V D G S G H A S I H I S S S D K D P M V A I H V G A K K G S S P K R V G * 511
 CTGTAAATTGGTCAGGAGCTGTAATCTTACATGGAGACGTGCAACGTGATAAATCTGTTATTTATACCAAAAAAAAAAAAAAAAAA 1648

Fig. 6

TGGGAGTCAATGTTGGGCGAGTATGGGATTGTCTCAGCTCTGCTGGTCTGTGCGGCGAGGTTACGGATCCCCTGCAATGGGAAGCAGGTATAGTGCACCTGTTTGG 120
 M W A Q Y G I V S A L L V L S A S A E F T D P H C N G K Q V I V H L F E 36
 TGGAGGTGGCCAGACATCSCCTTGGAGTGTGAAGATACCTGTCTAAGAAAGTTACTGTGCTGTACAGGTATCCCACCAACGAGCAGTGGTGGTACGGAAGGACTACCCCGTCT 240
 W R W P D I A L E C E R Y L S K K G Y C A V Q V S P P N E H V V V T K D Y P R P 76
 TGGTGGGAGGTGTACCAGCCAGTCAGTTACAACCTACCAAGCAGGTGCGGTAAATGAGGATCAGTTCAAGGACATGGTTCCAGGTGCAAGAAAGTTGGGTCAGGGTTATTGTTGATGG 360
 W W E V Y Q P V S Y K L T S R C G N E D Q F K D M V S R C K K V G V R V I V D G 116
 GTTATAATCATATGGCCGGCTGGGAGATCTGGAACAGGAACGGCAGGTAGCAGTCTCAACTCCGACAACCTGGACTTCCCTGGTGTACCATACAGGAGAGAGCATTTCATGACCC 480
 V I N H M A G L G R S G T G T A G S S F N S D N L D F P G V P Y R R E H F N D R 156
 AGCAGTGTCCATCTGTGACGTAACBTCAACAATATGGCATCCCAATATGTGAGAACTGCTATCTGTTGGCTCACTGATTGAATCAAGGCGTCAAGTACGTGAGGACAAG 600
 S T C P S G D G N V N N Y G D P N N V R N C Y L V G L T D L N Q G V E Y V R D K 196
 ATCTCTGCTACTTCAATAACCTCATTGATATCGAGTGTCTGTTTTAGATTGACGCTGCCAACACATGTGCGCTGCTGACATCAGCGCAATTCAGAAAGCGGTGAAGGATCTCCCT 720
 I S G Y F N N L I D I G V S G F R I D A A K H M W P A D I S A I Q K R V K D L P 236
 GAGGAGGTAAAGCTTCTGGGCCATGAGGTGATTGACGAGATGATGGCGGATTAAGTGGGAGATACACTTCTTAGGCTACGCTCACTGAGATGAGATATTGTCAAAAATAGCT 840
 E G G K A F W A H E V I D Q N D G A I K V G E Y T S L G Y V T E M R Y C Q K I A 276
 CAAGGTGTACACAACCTTTGGAGGCTTGGCAATGTGCTGACTATGGCTGGGGCATGACACCTTACGTAACGCCCTTAGTCTTTGTTGATAATCAGGACATCAGAGGTCATGGTGGT 960
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 GATGCTGGAGCGCTGACAAAGATGTTGATATCAATGGAGATGGCTCTGTGGTAATGGCTGGGTTTGTGAGCAGAGTGGGCCCCCATGCGCAACATGGTGGCTTCAGGAACGCTGTG 1200
 D A G P P D K D V S I N G D G S C G N G W V C E H R W A P I A N M V A F R N A V 396
 GCTGGCACTGGCATCGAACCTGTTGATAGTGGGACGTGCTGCGCTTTGCCAGAGTTAACAAGGCTTCTTTGCGATGGCGAAGCAAGGAAATCTGGATCAAACTTCCGACAGGG 1320
 A G T G I E H N F D S G D V V A F A R G N K G F F A M A K Q G N L D Q T F R T G 436
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 L P A G E Y C D I I H D C Q Q K V T V D G S G N A H V H I S N N E E P I V A I I 476
 GTTGTGTCCCACTGGCAGTACAGGAGCGGAAACACTGGAGAGGAACTCAAGCCCGCTTACCTCAGGCCCTGTTGTGACACCCACTATGGAAGTGTAGTTGGAACACACAGTG 1560
 V G G P T G S T G G G N T G G G T Q A P V T S G P V V T P D Y G S G S W K H T V 516
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 I L V Q R Q T V P G Q D L F I R G G I D H A H M T G C S S S V S G N P C A I P I 556
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 R H R D L G S G P H F S K Y N A W S A G D N Y L D W Y G A E P G Q G S S A T G T 596
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 P A L W T T N N A G R P E Y S K Y N T F G D H Y W M V D I D M D C S K T K D G F 636
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 F E F K S V V N G G W E G N V A G G S C S G A G A A T P P Y S S K N H F G R C G 676
 TACCTCAATGTCTCCACTGGGCGAGCGCAGCTGTTCTATCACACAGCTTACCTAAGCCAGTGTGTGTAAGGATGGTGAACATATGAGAAGATGATTGGATTGAATTATATGA 2160
 Y L N V F H W G S G S C S I T Q L T * 694
 AATAAGCCCTATAAAAAAGTCAAAAAAAAAAAAAA 2197

Fig. 7

(A)

HdAmy58	MIPTW-CLAFLLPTALASMYSDPHCSSGRSAITHLFEWTWSDIAKECERFLGPYGYCGVQISPPNENRVVTNPR-RPWERYQPVSYKLQTRSGSEQDLK	98
HdAmy82	MWAQYGIVSALLVLSASAEFTDPHCN-GKQVIVHLFEWRWPDIALECERYLSKKGCAVQVSPNEHVVTKDYPWPWEVYQPVSYKLTSRCGNEDQFK	99
	* ** *** * * * * * * * * * * * * * * * * * *	
HdAmy58	DMVSRCKAGVRIYADTVINHMTGVGGTGGGTAGSSYDANQLKYPGPYPGPTDFNDNSNCHTGMQIHNNPPEEVRNCRVLGLADLKAGKDYVRSEIEG	198
HdAmy82	DMVSRCKKVGVRIVDGVINHMAGLGRSGGTAGSSFNLDLDFGVPYRREHFNDRSTCPSGDGNVNNYGDPNVNRNCLVGLTDLNQGVEYVRDKISG	199
	***** * *	
HdAmy58	YLNHLVDIGIAGFRVDAAKHMPGDLTAIFGSVKNLRSDFVGGGKKPFVQEVIDMGGEPKIGDQYLGSGRVTNIFGAKLAQVFRKQNAWKYLSNWGSA	298
HdAmy82	YFNNLIDIGVSGFRIDAACHMWPADISAIQKRVKDLPEGGKAFWAHEVIDQNDGAIKVGGEYTSLGVTMRYCQKIAQGVHNFGELEGNVVDYG	292
	* *	
HdAmy58	WGMLKSDDSVVFDNHDNRGHGGGGVLTIFEPRPYKLATAFMLAHPYGFPRVMSSYNFQANTDQGPQNGDMSTKPVTING-MVCGNGWTCHEHRWQ	397
HdAmy82	WGMTPSANALVFVDNHDNRGHGGGGNLITHKTPRDYKMAQAFTLAYNYGFVRVMSSYFFGDD-SDAGPPD-KDVSINGDGSNGWVCEHRWAP	385
	*** * * * * * * * * * * * * * * * * * * * *	
HdAmy58	MYNMVAFRNIAGYSLSNWWSGSDYQIAFSRGNKALIAFNLEGYDLKSLNTGLPSGSYCDVISGNLENGGCTGKTIDVDGSGHASIHSSSKDPMVAI	497
HdAmy82	IANMVAFRNAVAGTGIEHWFDSDG-VVAFARGNKGFFAMAKQG-NLDTFRTGLPAGEYCDIHD-CQQKVTVDGSGNAHVHISNNE-EPIVAI	475
	***** * * * * * * * * * * * * * * * * * * *	
HdAmy58	HVGAKKGSS-----PKRVG	511
HdAmy82	IVGGPTGSGGGNTGGGTQAPVTSGPVPTPDYSGSGWKHTVILVQRQTVPGQDLFIRGGIDHAHMTGCSSSVSGNPCAIPIRHRDLGSGPHFSKYNAWSA	575
	** ** * *	
HdAmy82	GDNYLDWYGAEPGGSSATGTPALWTTNAGRPEYSKYNTFGDHYWMVDIDMDCSKTKDGFEEFKSVVNGGWEKNVAGGSCSGAGAATPPYSSKNHFGRC	675
HdAmy82	GYLNVFHWGSGSCSITQLT	694

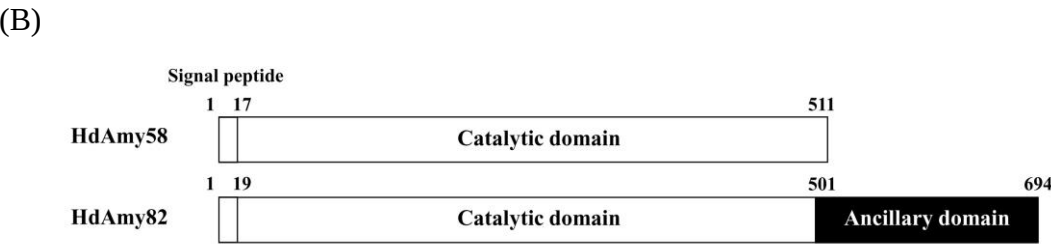


Fig. 8

HdAmy82	PVVTDPYG—SGSWKHTVILVQRQTVPGQDLFIRGGIDHAHM—TGCSSSVSGNPCAIPIRHRDLGSGPHFSKYNAWSA	575
<i>C. fluminea</i>	PLPTAPAG—WSRTVVLMERQTSVGQDLFIRGGLDHKTH—TGCTSDAATSACSIPIRVRPLGTGSHYDRYNAWSA	576
<i>M. edulis</i>	GIVSPTNGVPKGFHRTIILFEGRTAPGQDLFIRGGIDEGHR—QGQHTAASSACAIPKSHDLGSSDHFAYNAWRQ	538
<i>P. haloplanktis</i>	—————ESDWQRTVIFINAQTQSGQDMFIRGGIDHAYANANLGRNCQTSNFECAMPIRHNNLKN—VTTSPWKA	545
	* * *** ***** * * ** *	
HdAmy82	GDNYLDWYGAEPGQG—SSATGTPALWTTN—NAGRPEYSKYNTFGDHYWMVDIDMDCSKTKDGFEEF	639
<i>C. fluminea</i>	HDDHLDWYGAEANQGRYNGAMAQGTALWTTN—SAGQAGHSDLNTYGGHYWLVDVMDCKTDNGWFEL	644
<i>M. edulis</i>	GDSYLDWYGAEPGQGDYHGQTPQGTPAVWTTN—QPGKPGYNELNSYGEHFWLVDIEMDCDKTENGWFEV	606
<i>P. haloplanktis</i>	NDNYLDWYGIENGQS—SEAEGSATDWTNNVWPAGWGAECTVNTDGFVTLNIIWGEHYWMLDVDMDCKAVNGWFEL	621
	* ***** * * ***** * * * * * * * *	
HdAmy82	KSVVNG—GWEGNVAGGSCSGAGAATPPYSSKNHFGRCGYLNVFHWGSGSCSITQLT	694
<i>C. fluminea</i>	KAVVNN—EWEGNVAGTCTGSGAGAPTAQTGNHWARCGMMNVFHFNSASCEIKSLP	699
<i>M. edulis</i>	KAVINS—NWESNVPGNSCH—YGNVPYRSQNHWARCGMKNVLHFSAVNCEISEIPTS	660
<i>P. haloplanktis</i>	KAFIKNGQGWETAIADN—ATPYTSTNHMAQCGKINKFEFNNSGVVIRSF	669
	* ** * * * *	