Characterization of an α-glucosidase, HdAgl, from the digestive fluid of *Haliotis discus hannai*

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Abstract

Previously, we isolated two α-amylase isozymes, HdAmy58 and HdAmy82, from the digestive fluid of the Pacific abalone *Haliotis discus hannai* (Kumagai et al, 2013, *Comp. Biochem. Physiol*., B 164, 80-88). These enzymes degraded starch producing maltooligosaccharides but not glucose. However, the digestive fluid itself could produce glucose from starch, indicating that the digestive fluid contains α-glucosidase-like enzymes together with the α-amylases. Thus, in the present study, we isolated this α-glucosidase-like enzyme from the digestive fluid and characterized it to some extent. Isolation of this enzyme was carried out by ammonium sulfate fractionation followed by conventional column chromatographies and FPLC. The purified enzyme showed an apparent molecular mass of 97 kDa on SDS-PAGE, and optimal temperature and pH of 45^{o}C and 3.8–5.5, respectively. This enzyme could degrade various sizes of maltooligosaccharides into glucose and released glucose from starch producing no appreciable intermediate oligosaccharides. We concluded that this enzyme is an α-glucosidase (EC 3.2.1.20) exolitically acting on polymer substrate and named HdAgl. HdAgl efficiently degraded maltose but hardly degraded *p*-nitrophenyl α–D-glucopyranoside (α-*p*NPG) and isomaltose. This enzyme was regarded as a maltase-like α-glucosidase that preferably degrades maltose but scarcely aryl glucosides. When starch was used as a substrate, HdAgl converted approximately 40% (w/w) of the starch to glucose. If an abalone α-amlylase HdAmy58 was added to the reaction mixture, the glucose yield increased to 60% (w/w). These results suggested that both HdAgl and HdAmy58 play important roles for the production of glucose from dietary starch in the digestive fluid. The amino-acid sequence of 887 residues for HdAgl was deduced by the cDNA method. This sequence showed 41–46% amino-acid identities to those of mammalian and avian α-glucosidases belonging to glycoside-hydrolase-family31.

Key words: gastropod, abalone, *Haliotis*; α-glucosidase; cDNA cloning; GHF31.

1. Introduction

Starch is a storage glucan comprising α-1,4-linked D-glucose main chains and α-1,6-linked branched chains. This polysaccharide is used as carbon and energy sources in various organisms including animals, plants and microorganisms. These organisms degrade starch with starch-degrading enzymes, e.g., α-amylase (EC 3.2.1.1), α-glucosidase (EC 3.2.1.20) and glucoamylase (EC 3.2.1.3) to obtain glucose (Thoma et al., 1971). Generally, α-amylase hydrolyzes the internal α-1,4-glycoside linkages of starch producing maltooligosaccharides. α-Glucosidase and glucoamylase degrade maltooligosaccharides and starch producing glucose, i.e., the former enzyme produces α-glucose and the latter produces β-glucose (Chiba, 1997). In mammals, dietary starch is degraded to maltooligosaccharides and limited dextrin by salivary and pancreatic α-amylases. Then, these degradation products are degraded by intestinal maltase-glucoamylase and sucrase-isomaltase and converted to blood glucose (Gray, 1992). On the other hand, the starch metabolic mechanism has not been so well understood in marine invertebrates that ingest seaweeds. Recently, herbivorous gastropod such as abalone was shown to possess α-amylases which may play a role for the degradation of algal starches (Nikapitiya et al., 2009). We also isolated two α-amylase isozymes, HdAmy58 and HdAmy82, from the digestive fluid of the Pacific abalone *Haliotis discus hannai* (Kumagai et al., 2013). These enzymes degraded starch producing maltooligosaccharide; however, they could not produce glucose. We recently noticed that the digestive fluid of abalone showed high glucose-producing activity. This

led us to consider that α-glucosidase-like enzyme was contained in the digestive fluid, and engaged in the degradation of starch together with α-amylases. To date, an α-glucosidase from marine mollusk was investigated with a sea hare *Aplysia fasciata* (Andreotti et al., 2006); however, no information for abalone α-glucosidases is currently available to the authors' knowledge.

In the present study, we isolated the α-glucosidase-like enzyme form the digestive fluid of *H. discus hannai* and characterized its basic properties and primary structure. Accordingly, we identified this enzyme as a maltase-like α-glucosidase belonging to the glycosid-hydrolase-family31.

2. Materials and methods

2.1. Materials

The Pacific abalone *H. discus hannai* was obtained from a local market in Hakodate, Hokkaido Prefecture, Japan. Corn starch, oyster glycogen, maltooligosaccharides (maltose–maltohexaose), and sucrose were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). *p*-Nitrophenyl α-D-glucopyranoside (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 TOSOH Co. (Tokyo, Japan), and Mono-S 5/50GL from GE Healthcare UK Ltd. (Little Chalfont, Bucking Hamshire, England). Oligotex-dT(30), TaKaRa *Taq* DNA polymerase, 5'-Full RACE and 3'-Full RACE kits, and restriction

endonucleases were purchased from TaKaRa. 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. α-Amylase (HdAmy58) from *H.discus hannai* was prepared by the method as described previously (Kumagai et al., 2013).

2.2. Assay for α-glucosidase activity

According to our preliminary experiments, the digestive fluid showed low activity toward α-*p*NPG, but high glucose-producing activity toward starch and maltooligosaccharides. Therefore, we examined the α-glucosidase-like enzyme activity by assaying the glucose released from starch. A reaction mixture containing 0.2% (w/v) starch and 10 mM sodium phosphate buffer (pH 6.0), where the starch had been solubilized by heating at 100°C for 10 min, was pre-incubated at 30°C for 5 min, and then 50 μL of enzyme solution (0.01–0.05 units) was added to 950 μL of the reaction mixture, incubated at 30 °C for 30 min, and heated at 100°C for 3 min to terminate the reaction. The glucose released by the reaction was determined with a Glucose CII test kit WAKO (Wako Pure Chemicals Industries Ltd.). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1.0 μmol glucose per min. Substrate specificity of the enzyme was examined by the use of following substrates: maltooligosaccharides (maltose–maltotriose; G2–G6), sucrose, isomaltose, trehalose, starch, glycogen, and α-*p*NPG. Substrate concentration for oligosaccharides was 5 mg/ml, while the concentrations for glycogen and α-*p*NPG were 2 mg/ml and 2.5 mM, respectively. Degradation of α-*p*NPG was detected by measuring absorbance at 410 nm

after the reaction was terminated by the addition of 0.1 M sodium carbonate in a final concentration of 0.067 M. *p*-Nitrophenol released was determined with the molar extinction coefficient 1.81×10^5 $M^{-1} \cdot cm^{-1}$. One unit of *p*NPG-degrading activity was defined as the amount of enzyme that released 1.0 μmol *p*-nitrophenol per min.

Temperature dependence of α-glucosidase was assayed at 10–60^{o}C using maltose as a substrate. pH dependence of α-glucosidase was measured at 30^{o}C with the reaction mixtures with different pH values; pH 2.0–2.9 adjusted with 10 mM glycine-HCl buffer, pH 2.9–6.0 adjusted with 10 mM sodium citrate buffer, and pH 6.0–8.0 adjusted with 10 mM sodium phosphate buffer. All assays were triplicated and the average data were adopted as representatives.

2.3. Purification of α-glucosidase-like enzyme from abalone

The α-glucosidase-like enzyme, named HdAgl in the present study, was purified from the digestive fluid of abalone *H. discus hannai* as follows: 10 abalones (an average shell size, 10 x 6 cm; an average weight, 80 g) were dissected with a scalpel and the adductor muscles were removed. The digestive fluid was then collected from the stomach lumen by aspiration using a Pasteur pipette. By this procedure, approximately 20 mL of the digestive fluid was obtained from the 10 abalones. The digestive fluid was mixed with 40 mL of 10 mM sodium phosphate (pH 6.0) and centrifuged at 10,000 × g for 10 min. The supernatant (crude enzyme) was then subjected to ammonium sulfate fractionation and the fraction precipitated between 20 and 60% saturation of ammonium sulfate was collected by centrifugation at 10,000 × g for 10 min. The precipitates were dissolved in 10 mM sodium phosphate buffer (pH 6.0) containing 40%-saturated

ammonium sulfate and subjected to a TOYOPEARL Phenyl-650M column (2.5×19.0 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted stepwisely with 40%, 30%, 20%, 10%, and 0%-saturated ammonium sulfate in 10 mM sodium phosphate buffer (pH 6.0) (Fig. 1A). α-Glucosidase activity was detected in the fractions eluted with 20%-saturated ammonium sulfate. These fractions (fraction numbers 59–65) were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0), and applied to a TOYOPEARL CM-650M column (2.5×23.3 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-glucosidase activity was detected in the fractions eluted at around 0.15 M NaCl (fraction numbers 63–68). These fractions were dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and applied to a hydroxyapatite column (1.2×16.5 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0.01–0.3 M sodium phosphate buffer (pH 6.0) and α-glucosidase activity was detected in the fractions eluted at around 0.05 M sodium phosphate buffer (fraction numbers 58–66) (Fig. 1C). The active fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and concentrated to approximately 5 mL by ultrafiltration with VIVASPIN 20 (Sartorius AG, Goettingen, Germany). The concentrate was subjected to a Mono-S 5/50GL column pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0) and the proteins adsorbed to the column were eluted with a linear gradient of 0–0.15 M NaCl. In this chromatography, an α-glucosidase-like enzyme (HdAgl) with the approximate molecular mass of 97 kDa was eluted at around 0.05 M NaCl (Fig. 1D and 2). By the above procedure, HdAgl was purified 35.4-fold from crude enzyme at a yield of 0.3% with the specific activity 9.34 U/mg (Table 1).

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173 *2.4. Protein concentration*

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

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178 *2.5. SDS-PAGE*

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180 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was
181 carried out with 0.1% (w/v) SDS–10% (w/v) polyacrylamide gel according to the
182 method of Porzio and Pearson (1977). After the electrophoresis, the gel was stained with
183 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol–10% (v/v) acetic
184 acid, and destained with 5% (v/v) methanol–7% (v/v) acetic acid. Protein Marker, Broad
185 Range (New England BioLabs, Ipswich, MA, USA) was used as a molecular mass
186 marker.

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188 *2.6. Thin-layer chromatography*

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190 Degradation products of substrates produced by HdAgl were analyzed by
191 thin-layer chromatography (TLC). The products (approximately 10 μg) were spotted on
192 a TLC-60 plate (Merck, Darmstadt, Germany) and developed with a solvent of
193 n-butanol–acetic acid–water (2:1:1 (v:v:v)). The sugars developed on the plate were
194 stained by spraying 10% (v/v) sulfuric acid in ethanol followed by heating at 130^{o}C for
195 10 min.

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197 *2.7. Amino-acid sequence analysis*

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199 The N-terminal amino-acid sequence of α-glucosidase was determined with an
200 ABI Procise 492 sequencer (Applied Biosystems, Foster city, CA USA). The purified
201 protein was dialyzed against 20% acetonitrile–0.1% trifluoroacetic acid, adsorbed to
202 glass filter, and then subjected to the protein sequencer. For the determination of internal
203 amino-acid sequences, the enzyme was digested with 0.4% lysylendopeptidase at 37°C
204 for 20 min, subjected to SDS-PAGE, and electrically transferred to a polyvinylidene
205 difluoride membrane. Several fragments well separated on the membrane were
206 subjected to the sequencer.

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208 *2.8. cDNA cloning of HdAgl*

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210 Total RNA was extracted from the hepatopancreas of abalone by the guanidinium
211 thiocyanate-phenol method (Chomczynski and Sacchi, 1987). mRNA was selected from
212 the total RNA with an Oligo-dT(30) kit (TaKaRa) according to the manufacturers'
213 protocol. cDNA was synthesized from the mRNA with a cDNA synthesis kit (TaKaRa)
214 using random oligonucleotide primers. Degenerated primers for amplification of HdAgl
215 cDNAs were synthesized on the basis of its partial amino-acid sequences determined by
216 protein sequencer. Besides these primers, a primer was synthesized on the basis of the
217 highly conserved signature sequence among glycosyl-hydrolase-family31 (GHF31)
218 α-glucosidases, maltase-glucoamylases and sucrase-isomaltases (Frandsen and
219 Svensson., 1998; Nichols et al., 1998), since partial amino-acid sequences of HdAgl

indicated that this enzyme belongs to GHF31. For the PCR, a successive incubation at 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s was repeated 30 cycles in 20 μL 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 $MgCl_2$, and 10 pmol primers, 20 ng hepatopancreas cDNA, and 0.5 U of TaKaRa *Taq* DNA polymerase. The amplified cDNAs were cloned with a DynaExpress TA PCR Cloning kit and pTAC-1 vector (BioDynamics Laboratory Inc.). cDNAs encoding 5'- and 3'-regions of the HdAgl cDNA were amplified with 5'-Full RACE and 3'-Full RACE kits (TaKaRa). Homology search for deduced amino-acid sequences was performed using the BLAST tool provided by National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

3.1. Enzymatic properties of HdAgl

Optimal temperature and pH of HdAgl was observed at around 50°C and pH 3.8–5.5, respectively (Fig. 3A and B). The temperature that caused a half inactivation of HdAgl during 30 min incubation was found at around 48°C (Fig. 3C). HdAgl did not require the presence of NaCl for the activity and showed practically no NaCl-concentration dependence (Fig. 3D).

HdAgl released glucose from starch producing no intermediate oligosaccharides (Fig. 4). When maltooligosaccharides were degraded, HdAgl produced glucose and oligosaccharides that were one-glucose unit smaller than the substrates in the previous

steps (Fig .4). These results indicated that HdAgl acted as exolytically toward the substrates cleaving off the terminal glucose residues. According to our preliminary experiments using *p*-nitrophenyl α-D-maltoside (α-*p*NPM) as a substrate, HdAgl was considered to cleave the non-reducing terminal α-1,4-linkage since HdAgl produced α-*p*NPG and glucose from α-*p*NPM in the early stage of reaction (data not shown). It is noteworthy that the oligosaccharides which were one glucose unit larger than the original substrates were produced by HdAgl in 10–60 min reaction (Fig .4). These suggest that HdAgl possesses transglycosylation activity that is known as a characteristic property for retaining enzymes (Chiba, 1997).

HdAgl preferably degraded smaller substrates like maltose and maltotriose than larger substrates (Table 2). Namely, the activities toward maltotetraose–maltohexaose were approximately 75% of that for maltose (Table 2). HdAgl could degrade polysaccharides such as starch and glycogen showing relative activities of 36.5% and 13.5%, respectively. On the other hand, HdAgl scarcely degraded aryl α-glucoside and isomaltose (an α-1,6-linked disaccharide), i.e., it showed only 0.6% and 2.7% relative activities, respectively. HdAgl could not degrade sucrose and trehalose. On the basis of these results, we concluded that HdAgl is a maltase-like α-glucosidase.

3.2. Glucose release from starch in the coexistence of HdAgl and HdAmy58

We previously isolated two α-amylase isozymes, HdAmy58 and HdAmy82, from the digestive fluid of *H. discus hannai* (Kumagai et al., 2013). These enzymes were considered to play roles in the degradation of starch from dietary algae in the digestive fluid. Accordingly, HdAgl was also considered to contribute the starch degradation in

the digestive fluid along with the α-amylases. Thus, we investigated the effects of coexistence of HdAgl and HdAmy58 on the degradation rate of starch. As shown in Fig. 5, when starch was degraded by HdAmy58 alone, glucose was hardly produced as reported previously (Kumagai et al., 2013). Whereas, approximately 40% (w/w) of starch was converted to glucose by HdAgl in an 8-h reaction. On the other hand, when starch was degraded in the coexistence of two enzymes, the glucose yield increased to 60%. This improvement may be due to the increase in the number of substrate sites for HdAgl by the endolytic action of HdAmy58. Although relative amount for α-glucosidase and α-amylase in the digestive fluid has not been determined yet, above results suggest that both HdAgl and HdAmy58 participate in the glucose production in the digestive fluid.

3.3. Primary structure analysis for HdAgl

The N-terminal amino-acid sequence of HdAgl was determined by the protein sequencer as DSSQXHLKGEHRSDXYPET- (Underlined residues showed week peak signals in the sequencing, but later confirmed with cDNAs as they are; two Xs were not identified, but later revealed as Cys with cDNAs). The N-terminal sequences of two lysylendopeptidyl fragments of HdAgl were determined as STNSVLFDASLAPLIFSDQM- (P1) and TADGSAPIVGEVWPGKTVFP- (P2). These sequences showed 60% and 65% amino-acid identities to the 232nd–251st residues and 480th–499th residues of human maltase-glucoamylase (MGAM) that belongs to GHF31 (Nichols et al., 1998), respectively. On the basis of these partial amino-acid sequences, we synthesized the degenerated forward primer Fw (from P1) and the reverse primers

Rv (from P2) (Table 3). Besides these primer, a reverse primer CatR was also synthesized on the basis of the conserved signature sequence of GHF31 enzymes, i.e., (L/I/M)WIDMNE (Frandsen and Svensson., 1998) since the partial amino-acid sequences of HdAgl showed similarity to the sequences of GHF31 enzymes. cDNAs encoding HdAgl were then amplified by the nested PCR using these primers from the abalone hepatopancreas cDNA. As a result, a cDNA with approximately 0.75 kbp (Agl-cDNA1) was successfully amplified. The Agl-cDNA1 comprised 756 bp that encoded an amino-acid sequence of 252 residues. Then, a series of specific primers for 3'-RACE and 5'-RACE were synthesized on the basis of the nucleotide sequence of Agl-cDNA1 (Table 3). By using these primers, Agl-3RACE-cDNA (1463 bp) covering the 3'-terminal region and Agl-5RACE-cDNA (643 bp) covering the 5'-terminal region were amplified by 3'-RACE and 5'-RACE, respectively. By overlapping the nucleotide sequences of Agl-5RACE-cDNA, Agl-cDNA1, and Agl-3RACE-cDNA in this order, a nucleotide sequence of 2759 bp encoding the amino-acid sequence of 887 residues for HdAgl was determined. The reliability of this sequence was confirmed with AglFull-cDNA, which was newly amplified by PCR with a specific primer pair, FullFw and FullRv (Table 3, Fig. 6). This nucleotide sequence and the following deduced amino-acid sequence are available from DNA Data Bank of Japan with the accession number AB820091.

The N-terminal amino-acid sequence of HdAgl, DSSQXHLKGEHRSDXYPET-(Xs; not identified residues) determined by the protein sequencer, was shown in the deduced sequence as DSSQCHLKGEHRSDCYPET. Thus, the N-terminus of mature HdAgl protein was found to be the 43rd Asp and accordingly this enzyme was concluded to comprise 845 residues with the calculated molecular mass of 94821 Da.

316 The 24 residues following the initiation Met in the deduced sequence of HdAgl were
317 predicted as the signal peptide region for secretion by SignalP 4.0 software
318 (http://www.cbs.dtu.dk/services/SignalP/). The sequence of 17 residues,
319 FGVHGNGTGRVFVKRDQ, which locates between the signal peptide region and the
320 mature enzyme domain, was regarded as a propeptide-like region of this enzyme since
321 this region was absent in the native HdAgl.

322 The 43rd–887th amino-acid region of HdAgl showed 46% identity to the sequence
323 of quail acid α-glucosidase I (GAA I) (Kunita et al., 1998), 41–42% identities to those
324 of human lysosomal acid α-glucosidase (GAA) (Hoefsloot et al., 1988), and the
325 N-terminal domain of maltase-glucoamylase (MGAM) and sucrase-isomaltase (SIM)
326 (Chantret et al., 1992). These enzymes belong to GHF31. The sequence known as the
327 signature sequence region1 of GHF31 enzymes, i.e., WIDMNE, was completely
328 conserved in the 476th–481st residues of HdAgl, while the sequence
329 GVDICGFRGDSDEELCTRWLQLGAFYPFMRSHN of HdAgl (604th–636th residues)
330 showed 70 – 76 % identity with the signature sequence region 2 of the above GFH31
331 enzymes (Frandsen and Svensson., 1998; Nichols et al., 1998) (Fig. 6). The catalytic
332 nucleophile residue (Asp478) in the signature region 1 and the acid/base catalytic
333 residue (Asp576) were also conserved in HdAgl. Thus, HdAgl was regarded as a
334 member of GHF31.

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336 **4. Discussion**

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338 The Pacific abalone *H. discus hannai* possesses various kinds of seaweeds'
339 polysaccharide-degrading enzymes such as alginate lyase, mannanase, cellulase and

laminarinase in the digestive fluid (Shimizu et al., 2003; Suzuki et al., 2003; Suzuki et al., 2006; Ootsuka et al., 2006; Kumagai and Ojima 2009). In addition to these enzymes, we recently isolated two α-amylase isozymes, HdAmy82 and HdAmy58, from the digestive fluid of abalone (Kumagai et al., 2013). These enzymes produced maltose–maltotetraose from starch, but did not produce glucose. However, the digestive fluid of abalone was capable of producing glucose from starch. This fact led us to consider that the digestive fluid contains α-glucosidase-like enzyme(s) besides the α-amylases. In the present study we isolated this enzyme, HdAgl, and characterized to some extent.

4.1. Enzymatic properties of HdAgl

The molecular mass of HdAgl was estimated to be 97 kDa by SDS-PAGE. HdAgl showed optimal temperature and pH at around 50^{o}C and 3.8–5.5, respectively.

Since HdAgl directly released glucose from starch, this enzyme was considered to be an α-glucosidase or a glucoamylase. However, HdAgl showed transglycosylation activity which is a characteristic property in retaining-type enzymes like α-glucosidase. While glucoamylase acts in an inverting manner and does not catalyze transglycosylation. Therefore, we regarded HdAgl as an α-glucosidase.

HdAgl hardly degraded α-*p*NPG; however, it most efficiently degraded maltose and maltotriose and showed 30% activity toward starch compared with that to maltose. Accordingly, HdAgl was considered to be a maltase-like α-glucosidase that recognizes the terminal maltoside structure of substrates and hydrolyzed α-1,4-glycoside linkage of the maltose unit (Chiba, 1988). HdAgl appeared to act on the non-reducing terminus of

substrates as reported in other α-glucosidases (Chiba et al., 1979) since this enzyme produced α-*p*NPG and glucose from α-*p*NPM in the early stage of reaction (data not shown).

These properties of HdAgl were appreciably different from those of the α-glucosidase from *Aplysia fasciata* (Andreotti et al., 2006). HdAgl degraded starch and glycogen, while the *Aplysia* enzyme did not. HdAgl hardly degraded α-*p*NPG, while the *Aplysia* enzyme well degraded this substrate. The estimated molecular mass of HdAgl was 97,000, while that of the *Aplysia* enzyme was 69,000. On the basis of these differences, HdAgl was considered to be a different-type α-glucosidase from the *Aplysia* enzyme.

4.2. Primary structure of HdAgl

The amino-acid sequence of HdAgl was deduced by the cDNA method. The sequence comprised 887 residues and the mature enzyme domain (845 residues) showed approximately 40% amino-acid identities to those of mammalian and avian GHF31 enzymes such as lysosomal acid α-glucosidase (GAA), maltase-glucoamylase (MGAM) and sucrase-isomaltase (SIM). These identities indicated that HdAgl was also classified under GHF31. The 24 residues of the deduced sequence of HdAgl after the initiation Met was predicted as the secretion signal peptide region of this enzyme (Fig. 7). This type of signal peptide was also found in the deduced sequence of GAA, but not in those of MGAM and SIM. The latter two enzymes possess a cytoplasm region and a transmembrane region followed by an O-glycosylated stalk which is rich in threonine and serine (Nichols et al., 1998). Thus, HdAgl was found to be similar to GAA rather

than MGAM and SIM with respect to the signal peptide structure. Occurrence of the secretion signal in the deduced sequence of HdAgl is consistent with the fact that HdAgl has been secreted to the digestive fluid as a soluble enzyme.

4.3. Physiological roles of HdAgl for the digestion of algal starch

The glucose production by HdAgl was significantly improved by the coexistence of α-amylase in the reaction mixture (Fig. 5). This improvement seemed to be caused by the increase in the terminal sites of starch chain by the α-amylase action. This means the increase in the number of substrates for HdAgl. Thus, the coexistence of two enzymes was considered to be important for the digestion of dietary starch in the digestive fluid. Indeed, our preliminary experiments revealed that HdAgl (0.03 U/ml) alone produced glucose from the dried frond of *Porphyra yezoensis* (a red seaweed) in a yield of 10 % (w/w of total glucan), while the yield increased to 30% by the coexistence of HdAmy58 (0.03 U/ml) (data not shown).

In the present study, we confirmed that the abalone digestive fluid contained maltase-like α-glucosidase along with α-amylases. This strongly suggests that both of these enzymes play important roles for glucose-production in the digestive fluid. On the other hand, intestinal membrane-binding type α-glucosidase such as MGAM plays important roles to provide glucose in higher animals like human. To enrich information about the variation in the starch-degrading systems among animal species, it seems important to comparatively study the properties of various starch-degrading enzymes from different species. In this context, we are now attempting to characterize the membrane-binding type α-glucosidases of abalone.

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Figure legends

Fig. 1. Purification of α-glucosidase from the digestive fluid of the abalone. (A) TOYOPEARL Phenyl-650M column chromatography of proteins precipitated between 20 and 60% saturation of ammonium sulfate. (B) TOYOPEARL CM-650M column chromatography of the α-glucosidase obtained by TOYOPEARL Phenyl-650M chromatography. (C) Hydroxyapatite column chromatography of the α-glucosidase obtained by TOYOPEARL CM-650M chromatography. (D) Mono-S 5/50GL column chromatography of the α-glucosidase obtained by Hydroxyapatite chromatography. Protein elution and enzyme activity are indicated with open and closed circles, respectively, in (A)–(C). Those are indicated with solid line and shaded boxes, respectively, in (D).

Fig. 2. Monitoring of purification of α-glucosidase from the abalone by SDS-PAGE. M, marker proteins; lanes A-D, active fractions obtained by TOYOPEARL Phenyl-650M chromatography, TOYOPEARL CM-650M chromatography, Hydroxyapatite chromatography, and Mono-S 5/50GL chromatography (HdAgl), respectively.

Fig. 3. Temperature dependence, thermostability, pH dependence, and NaCl dependence of HdAgl. (A) Temperature dependence of HdAgl was examined at 10–60^{o}C in a reaction mixture containing 5mg/ml maltose and 10 mM sodium phosphate buffer (pH 6.0). (B) pH dependence of HdAgl was examined at 30^{o}C in the following reaction mixtures adjusted to pH 2.0–2.9 with 10 mM glycine-HCl buffer (▲), pH 2.9–6.0 with 10 mM sodium citrate buffer (●), and pH 6.0–8.0 with 10 mM sodium phosphate

buffer(○). (C) Thermostability of HdAgl was examined by measuring the remaining activity after heat treatment at 10–60°C for 20 min. (D) NaCl dependence of HdAgl was examined with reaction mixtures containing 5mg/ml maltose, 10 mM sodium phosphate buffer (pH 6.0), and 0–500 mM NaCl.

Fig. 4. Thin-layer chromatography for the degradation products of starch and maltooligosaccharides produced by HdAgl. Two mg/mL starch and 5 mg/mL maltooligosaccharides in 10 mM sodium phosphate buffer (pH 6.0) were degraded with 0.01 U/mL HdAgl at 30^{o}C. The reaction was terminated at appropriate times by heating at 100^{o}C for 3 min and 1 μL of the reaction mixture was applied to TLC plate. M, oligosaccharide markers comprising G1 and G2–G6. G1, glucose; G2–G6, maltose to maltohexaose.

Fig. 5. Improvement of degradation of starch by coexistence of HdAgl and HdAmy58. Two mg/mL starch in 10 mM sodium phosphate buffer (pH 6.0) were degraded in the presence of 0.028 U/mL HdAgl alone (○), 0.028 U/mL HdAmy58 alone (△), and coexistence of 0.028 U/ml HdAgl and HdAmy58 (●) at 30^{o}C.

Fig. 6. Nucleotide and deduced amino-acid sequences of cDNA encoding HdAgl. The translational initiation codon ATG, the termination codon TAG and the putative polyadenylation signal AATAAA are boxed. The putative signal peptide for secretion is indicated by a dotted underline. The amino-acid sequences determined with intact HdAgl (N-terminus) and peptide fragments obtained by lysylendopeptidase digestion are indicated by the underline and double underline. The catalytic amino acid residues

in GHF31 are marked with bold letters and boxes. The signature sequences of GHF31 are shaded. The annealing sites of PCR primers (see Table 3) are indicated by arrows over the nucleotide sequence.

Fig. 7. Differences in N-terminal regions of primary structures among HdAgl, Human GAA, and Human MGAM.

Table 1. Summary for the purification of HdAgl.

Samples	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude[a]	913.	0.28	256	1	100
Phenyl[b]	59.3.	0.33	20	1.2	7.8
CM[c]	1.66	3.7	6.1.	13.3.	2.4
Hydroxy[e]	0.49	5.1	2.5	18.1	1.0
Mono-S[f]	0.08	9.3	0.76	35.4.	0.3

[a] Crude enzyme after the dialysis against 10 mM sodium phosphate buffer (pH 6.0).

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

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

[d] Active fraction obtained by hydroxyapatite chromatography.

[e] HdAgl purified by Mono-S chromatography.

Table 2. Substrate specifity of HdAgl

Substrates	Relative activity (%)
Maltose*[1]	100
Maltotriose	99.9
Maltotetraose	75.6
Maltopentaose	74.1
Maltohexose	73.5
Isomaltose*[2]	2.7
α -*p*NPG	1.6
Trehalose*[3]	0
Sucrose	0
Starch	36.5
Glycogen	13.5

*[1-3] One unit of α-glucosidase was defined as the amount of enzyme that released 2.0 μmol glucose per min since one split of substrate would produce two glucose. Relative activity 100% corresponded to 25.7 U/mg.

Table 3. DNA primers used for the amplification of HdAgl cDNAs.

Names	Sequences[a]
Fw	5’-CCNYTNATHTTYWSNGAYCA-3’
Rv	5’-GTYTTNCCNGGCCANACYTC-3’
CatR	5’-TCRTTCATRTCDATCCANA-3’
3raceF1	5’-GTCCCGTGACAATTCGCTACC -3’
3raceF2	5’-CATGGTCAATGAGATGGACTGG -3’
3raceF3	5’-GGATCTGCTCCTATAGTTGGGG -3’
5raceRT	5’-GTAGAGCATTGCTG-3’
5raceF1	5’-ACACGAATATGTACGGCAGC -3’
5raceF2	5’-GGTCGGAATAGAACCAAACGG -3’
5raceR1	5’-CGTACAAGTTGGTAGTTGGC -3’
5raceR2	5’-CACTGAGATCTGTAGCATGTGG -3’
FullFw	5’-ACTCCAAGCAACTCCACACC -3’
FullRv	5’-CTACCAAGTCATAACGAACGGC-3’

[a]D,A/G/T, H,A/C/T, N,A/C/G/T; R,A/G; S,C/G, W,A/T, Y,C/T.

Fig. 1.

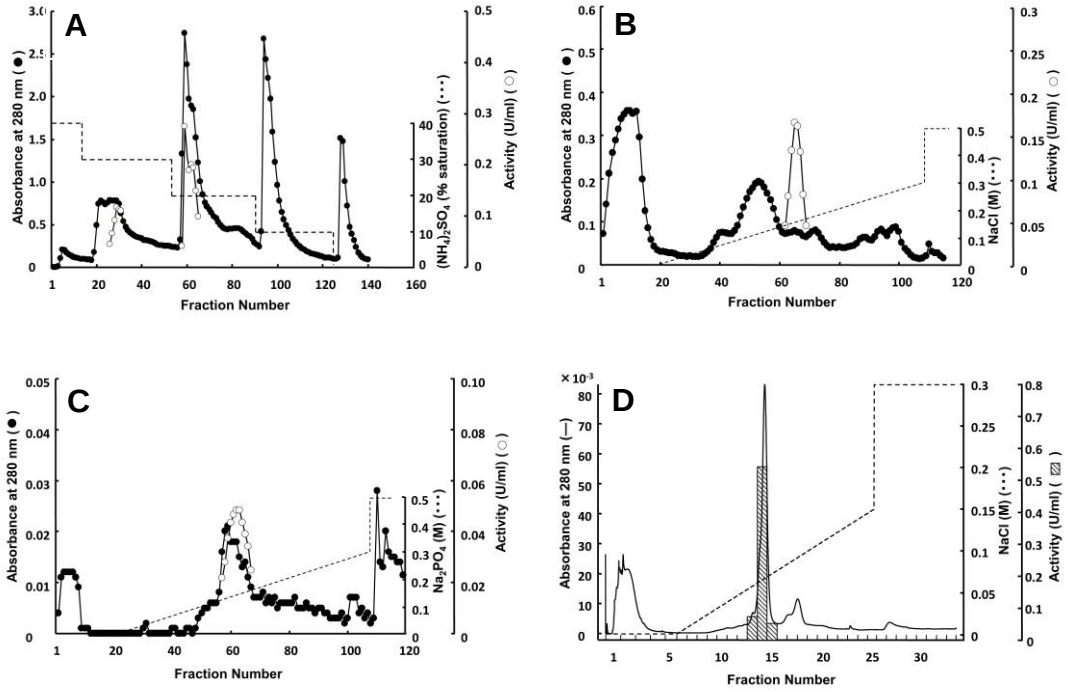

A
Absorbance at 280 nm (●)
(NH4)2SO4 (% saturation) (···)
Activity (U/ml) (○)
Fraction Number
B
Absorbance at 280 nm (●)
NaCl (M) (···)
Activity (U/ml) (○)
Fraction Number
C
Absorbance at 280 nm (●)
Na2PO4 (M) (···)
Activity (U/ml) (○)
Fraction Number
D
×10-3
Absorbance at 280 nm (—)
NaCl (M) (···)
Activity (U/ml) (▨)
Fraction Number

Fig. 2.

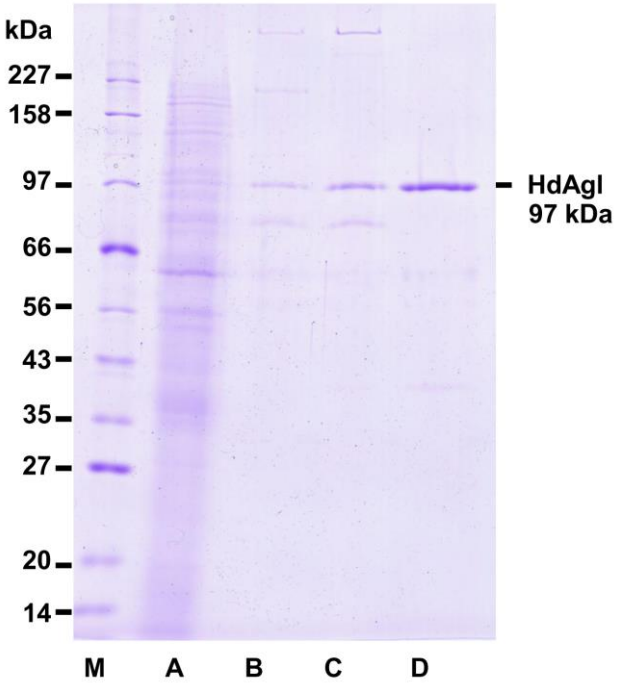


Fig. 3.

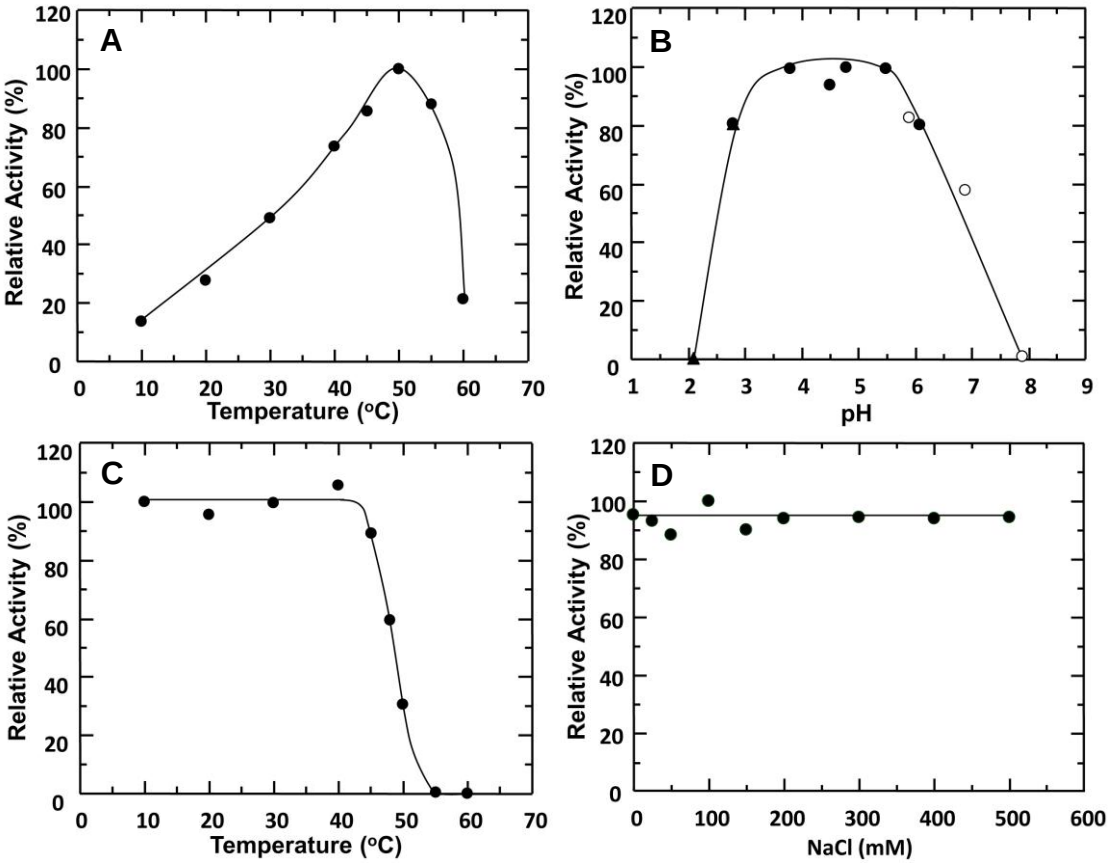

A
Relative Activity (%)
Temperature (°C)
B
Relative Activity (%)
pH
C
Relative Activity (%)
Temperature (°C)
D
Relative Activity (%)
NaCl (mM)

Fig. 4.

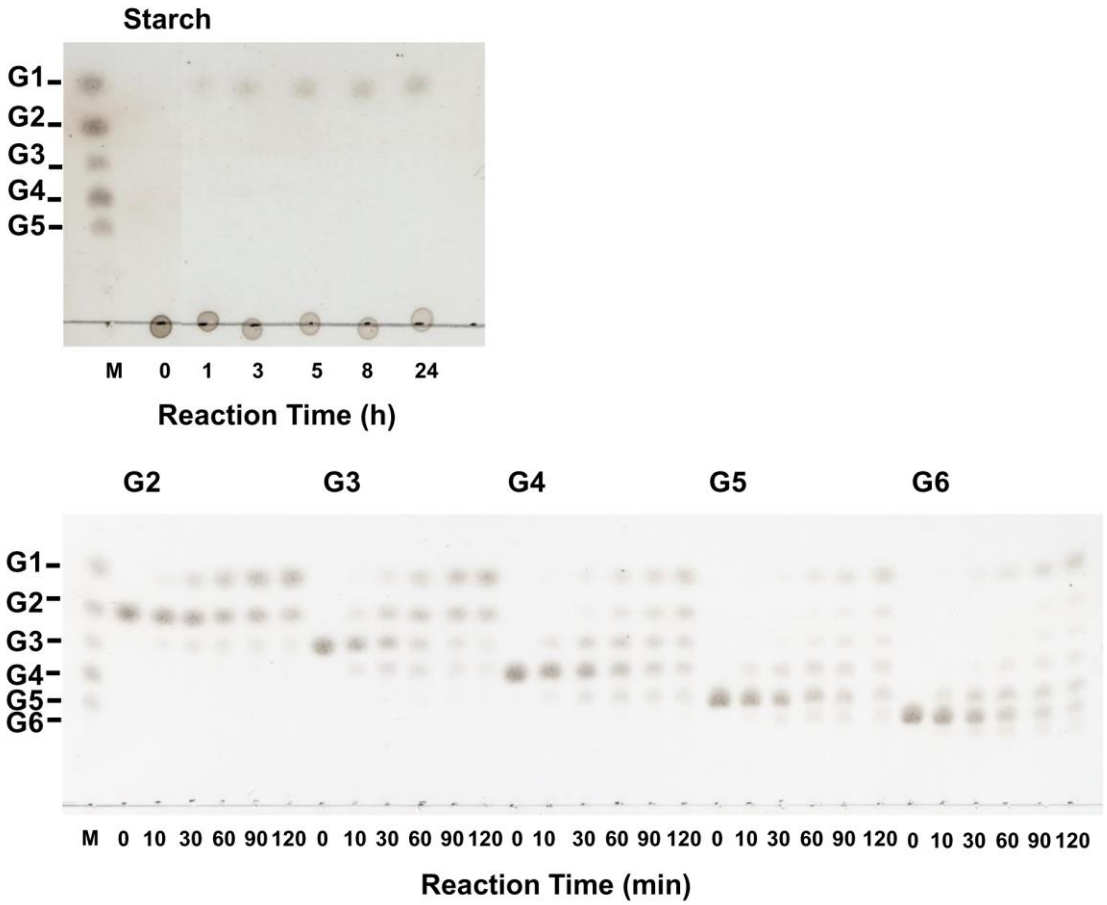

Starch
G1
G2
G3
G4
G5
M 0 1 3 5 8 24
Reaction Time (h)
G2 G3 G4 G5 G6
G1
G2
G3
G4
G5
G6
M 0 10 30 60 90 120 0 10 30 60 90 120 0 10 30 60 90 120 0 10 30 60 90 120 0 10 30 60 90 120
Reaction Time (min)

Fig. 5

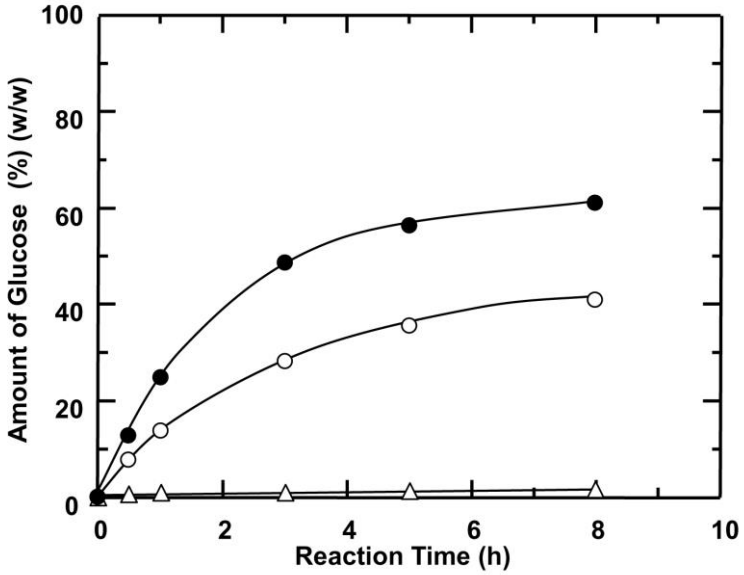
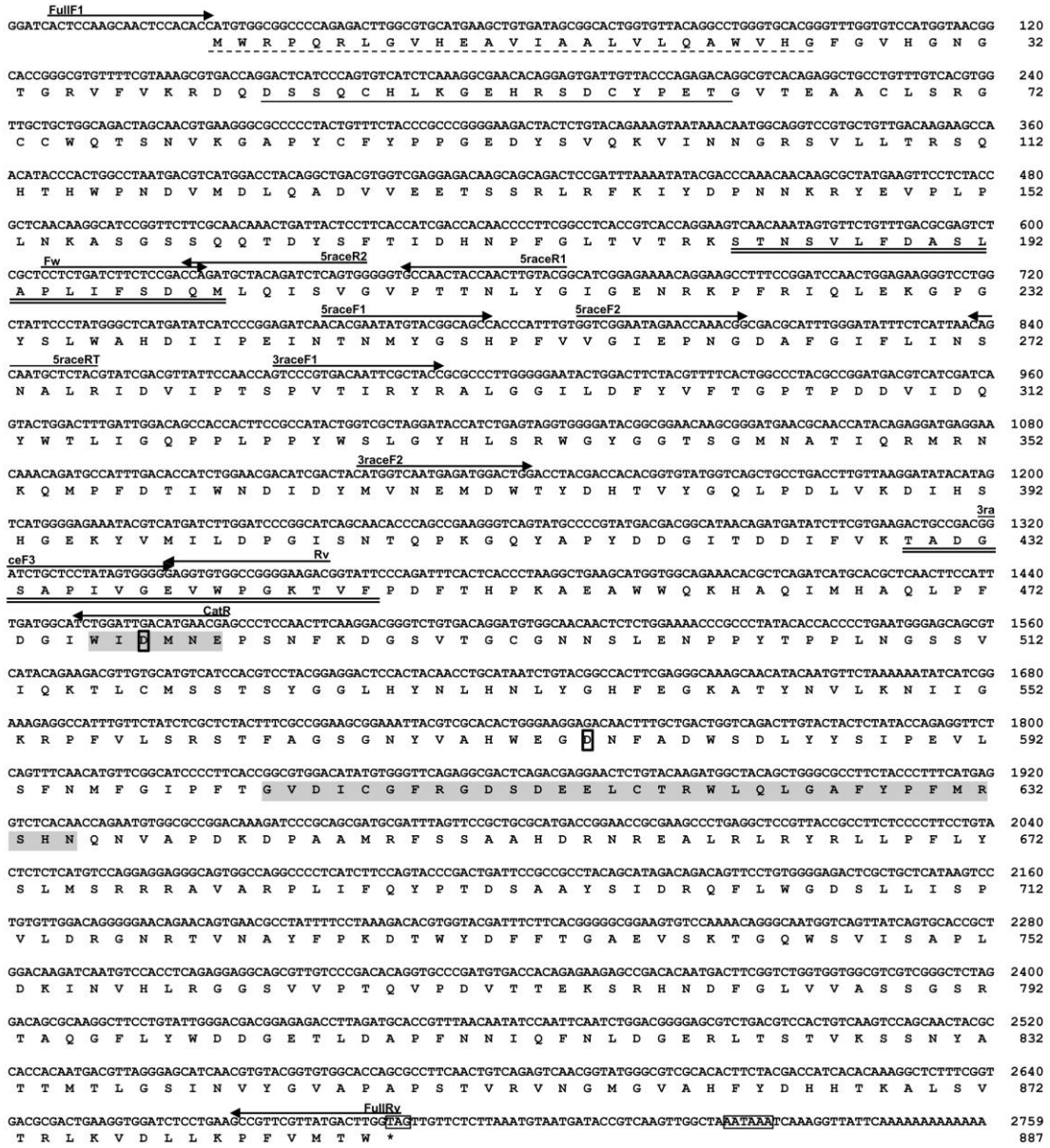


Fig. 6


FullF1
5raceR2
5raceR1
Fw
5raceF1
5raceF2
5raceRT
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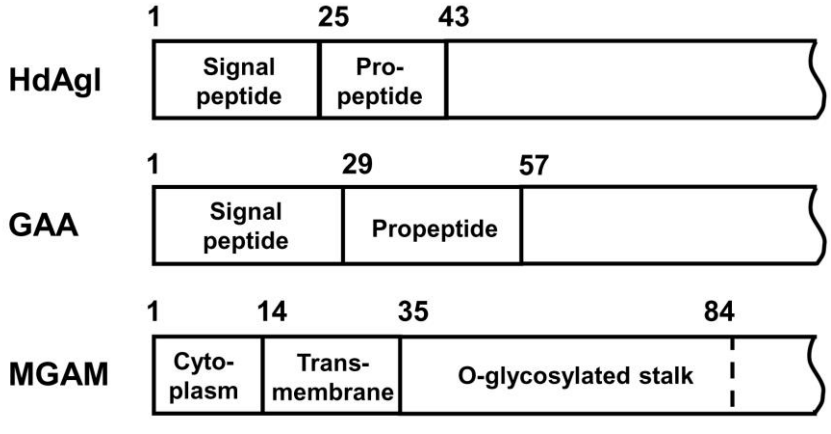
3ra
ceF3
Rv
CatR
FullRv

Fig. 7


1
25
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HdAgl
Signal peptide
Pro-peptide
1
29
57
GAA
Signal peptide
Propeptide
1
14
35
84
MGAM
Cyto-plasm
Trans-membrane
O-glycosylated stalk