



Title	Characterization of an α -glucosidase, HdAgl, from the digestive fluid of <i>Haliotis discus hannai</i>
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1 Characterization of an α -glucosidase, HdAgl, from the digestive fluid of

2 *Haliotis discus hannai*

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25 **Abstract**

26 Previously, we isolated two α -amylase isozymes, HdAmy58 and HdAmy82, from
27 the digestive fluid of the Pacific abalone *Haliotis discus hannai* (Kumagai et al, 2013,
28 *Comp. Biochem. Physiol.*, B 164, 80-88). These enzymes degraded starch producing
29 maltooligosaccharides but not glucose. However, the digestive fluid itself could produce
30 glucose from starch, indicating that the digestive fluid contains α -glucosidase-like
31 enzymes together with the α -amylases. Thus, in the present study, we isolated this
32 α -glucosidase-like enzyme from the digestive fluid and characterized it to some extent.
33 Isolation of this enzyme was carried out by ammonium sulfate fractionation followed by
34 conventional column chromatographies and FPLC. The purified enzyme showed an
35 apparent molecular mass of 97 kDa on SDS-PAGE, and optimal temperature and pH of
36 45°C and 3.8–5.5, respectively. This enzyme could degrade various sizes of
37 maltooligosaccharides into glucose and released glucose from starch producing no
38 appreciable intermediate oligosaccharides. We concluded that this enzyme is an
39 α -glucosidase (EC 3.2.1.20) exolitically acting on polymer substrate and named HdAgl.
40 HdAgl efficiently degraded maltose but hardly degraded *p*-nitrophenyl
41 α -D-glucopyranoside (α -*p*NPG) and isomaltose. This enzyme was regarded as a
42 maltase-like α -glucosidase that preferably degrades maltose but scarcely aryl glucosides.
43 When starch was used as a substrate, HdAgl converted approximately 40% (w/w) of the
44 starch to glucose. If an abalone α -amylase HdAmy58 was added to the reaction
45 mixture, the glucose yield increased to 60% (w/w). These results suggested that both
46 HdAgl and HdAmy58 play important roles for the production of glucose from dietary
47 starch in the digestive fluid. The amino-acid sequence of 887 residues for HdAgl was
48 deduced by the cDNA method. This sequence showed 41–46% amino-acid identities to
49 those of mammalian and avian α -glucosidases belonging to
50 glycoside-hydrolase-family31.

51

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

53

54 1. Introduction

55

56 Starch is a storage glucan comprising α -1,4-linked D-glucose main chains and
57 α -1,6-linked branched chains. This polysaccharide is used as carbon and energy sources
58 in various organisms including animals, plants and microorganisms. These organisms
59 degrade starch with starch-degrading enzymes, e.g., α -amylase (EC 3.2.1.1),
60 α -glucosidase (EC 3.2.1.20) and glucoamylase (EC 3.2.1.3) to obtain glucose (Thoma et
61 al., 1971). Generally, α -amylase hydrolyzes the internal α -1,4-glycoside linkages of
62 starch producing maltooligosaccharides. α -Glucosidase and glucoamylase degrade
63 maltooligosaccharides and starch producing glucose, i.e., the former enzyme produces
64 α -glucose and the latter produces β -glucose (Chiba, 1997). In mammals, dietary starch
65 is degraded to maltooligosaccharides and limited dextrin by salivary and pancreatic
66 α -amylases. Then, these degradation products are degraded by intestinal
67 maltase-glucoamylase and sucrase-isomaltase and converted to blood glucose (Gray,
68 1992). On the other hand, the starch metabolic mechanism has not been so well
69 understood in marine invertebrates that ingest seaweeds. Recently, herbivorous
70 gastropod such as abalone was shown to possess α -amylases which may play a role for
71 the degradation of algal starches (Nikapitiya et al., 2009). We also isolated two
72 α -amylase isozymes, HdAmy58 and HdAmy82, from the digestive fluid of the Pacific
73 abalone *Haliotis discus hannai* (Kumagai et al., 2013). These enzymes degraded starch
74 producing maltooligosaccharide; however, they could not produce glucose. We recently
75 noticed that the digestive fluid of abalone showed high glucose-producing activity. This

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

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

85

86 **2. Materials and methods**

87

88 *2.1. Materials*

89

90 The Pacific abalone *H. discus hannai* was obtained from a local market in
91 Hakodate, Hokkaido Prefecture, Japan. Corn starch, oyster glycogen,
92 maltooligosaccharides (maltose–maltohexaose), and sucrose were purchased from Wako
93 Pure Chemicals Industries Ltd. (Osaka, Japan). *p*-Nitrophenyl α -D-glucopyranoside
94 (α -*p*NPG) and agarose were purchased from Seikagaku Kogyo (Tokyo, Japan) and
95 TaKaRa (Tokyo, Japan), respectively. Hydroxyapatite (Fast Flow Type) was purchased
96 from Wako Pure Chemicals Industries Ltd., TOYOPEARL Phenyl-650M and
97 TOYOPEARL CM-650M from TOSOH Co. (Tokyo, Japan), and Mono-S 5/50GL from
98 GE Healthcare UK Ltd. (Little Chalfont, Bucking Hamshire, England). Oligotex-dT(30),
99 TaKaRa *Taq* DNA polymerase, 5'-Full RACE and 3'-Full RACE kits, and restriction

100 endonucleases were purchased from TaKaRa. DynaExpress TA PCR Cloning kit
101 (including pTAC-1 vector) was from BioDynamics Laboratory Inc. (Tokyo, Japan).
102 Other reagents were purchased from Wako Pure Chemicals Industries Ltd. α -Amylase
103 (HdAmy58) from *H.discus hannai* was prepared by the method as described previously
104 (Kumagai et al., 2013).

105

106 2.2. Assay for α -glucosidase activity

107

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

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

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

134

135 2.3. Purification of α -glucosidase-like enzyme from abalone

136

137 The α -glucosidase-like enzyme, named HdAgl in the present study, was purified
138 from the digestive fluid of abalone *H. discus hannai* as follows: 10 abalones (an average
139 shell size, 10 x 6 cm; an average weight, 80 g) were dissected with a scalpel and the
140 adductor muscles were removed. The digestive fluid was then collected from the
141 stomach lumen by aspiration using a Pasteur pipette. By this procedure, approximately
142 20 mL of the digestive fluid was obtained from the 10 abalones. The digestive fluid was
143 mixed with 40 mL of 10 mM sodium phosphate (pH 6.0) and centrifuged at $10,000 \times g$
144 for 10 min. The supernatant (crude enzyme) was then subjected to ammonium sulfate
145 fractionation and the fraction precipitated between 20 and 60% saturation of ammonium
146 sulfate was collected by centrifugation at $10,000 \times g$ for 10 min. The precipitates were
147 dissolved in 10 mM sodium phosphate buffer (pH 6.0) containing 40%-saturated

148 ammonium sulfate and subjected to a TOYOPEARL Phenyl-650M column (2.5×19.0
149 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted stepwisely
150 with 40%, 30%, 20%, 10%, and 0%-saturated ammonium sulfate in 10 mM sodium
151 phosphate buffer (pH 6.0) (Fig. 1A). α -Glucosidase activity was detected in the
152 fractions eluted with 20%-saturated ammonium sulfate. These fractions (fraction
153 numbers 59–65) were pooled and dialyzed against 10 mM sodium phosphate buffer (pH
154 6.0), and applied to a TOYOPEARL CM-650M column (2.5×23.3 cm) pre-equilibrated
155 with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0–0.3
156 M NaCl (Fig. 1B). In this chromatography, α -glucosidase activity was detected in the
157 fractions eluted at around 0.15 M NaCl (fraction numbers 63–68). These fractions were
158 dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and applied to a
159 hydroxyapatite column (1.2×16.5 cm) pre-equilibrated with the same buffer. The
160 adsorbed proteins were eluted with a linear gradient of 0.01–0.3 M sodium phosphate
161 buffer (pH 6.0) and α -glucosidase activity was detected in the fractions eluted at around
162 0.05 M sodium phosphate buffer (fraction numbers 58–66) (Fig. 1C). The active
163 fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0)
164 and concentrated to approximately 5 mL by ultrafiltration with VIVASPIN 20 (Sartorius
165 AG, Goettingen, Germany). The concentrate was subjected to a Mono-S 5/50GL column
166 pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0) and the proteins
167 adsorbed to the column were eluted with a linear gradient of 0–0.15 M NaCl. In this
168 chromatography, an α -glucosidase-like enzyme (HdAgl) with the approximate
169 molecular mass of 97 kDa was eluted at around 0.05 M NaCl (Fig. 1D and 2). By the
170 above procedure, HdAgl was purified 35.4-fold from crude enzyme at a yield of 0.3%
171 with the specific activity 9.34 U/mg (Table 1).

172

173 *2.4. Protein concentration*

174

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

177

178 *2.5. SDS-PAGE*

179

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.

187

188 *2.6. Thin-layer chromatography*

189

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°C for
195 10 min.

196

197 *2.7. Amino-acid sequence analysis*

198

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.

207

208 *2.8. cDNA cloning of HdAgl*

209

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

220 indicated that this enzyme belongs to GHF31. For the PCR, a successive incubation at
221 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s was repeated 30 cycles in 20 µL of
222 reaction mixture containing 50 mM KCl, 15 mM Tris-HCl (pH 8.1), 0.2 mM each of
223 dATP, dTTP, dGTP, and dCTP, 2.5 mM MgCl₂, and 10 pmol primers, 20 ng
224 hepatopancreas cDNA, and 0.5 U of TaKaRa *Taq* DNA polymerase. The amplified
225 cDNAs were cloned with a DynaExpress TA PCR Cloning kit and pTAC-1 vector
226 (BioDynamics Laboratory Inc.). cDNAs encoding 5'- and 3'-regions of the HdAgl
227 cDNA were amplified with 5'-Full RACE and 3'-Full RACE kits (TaKaRa). Homology
228 search for deduced amino-acid sequences was performed using the BLAST tool
229 provided by National Center for Biotechnology Information
230 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

231

232 **3. Results**

233

234 *3.1. Enzymatic properties of HdAgl*

235

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

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

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

253 HdAgl preferably degraded smaller substrates like maltose and maltotriose than
254 larger substrates (Table 2). Namely, the activities toward maltotetraose–maltohexaose
255 were approximately 75% of that for maltose (Table 2). HdAgl could degrade
256 polysaccharides such as starch and glycogen showing relative activities of 36.5% and
257 13.5%, respectively. On the other hand, HdAgl scarcely degraded aryl α -glucoside and
258 isomaltose (an α -1,6-linked disaccharide), i.e., it showed only 0.6% and 2.7% relative
259 activities, respectively. HdAgl could not degrade sucrose and trehalose. On the basis of
260 these results, we concluded that HdAgl is a maltase-like α -glucosidase.

261

262 3.2. *Glucose release from starch in the coexistence of HdAgl and HdAmy58*

263

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

268 the digestive fluid along with the α -amylases. Thus, we investigated the effects of
269 coexistence of HdAgl and HdAmy58 on the degradation rate of starch. As shown in Fig.
270 5, when starch was degraded by HdAmy58 alone, glucose was hardly produced as
271 reported previously (Kumagai et al., 2013). Whereas, approximately 40% (w/w) of
272 starch was converted to glucose by HdAgl in an 8-h reaction. On the other hand, when
273 starch was degraded in the coexistence of two enzymes, the glucose yield increased to
274 60%. This improvement may be due to the increase in the number of substrate sites for
275 HdAgl by the endolytic action of HdAmy58. Although relative amount for
276 α -glucosidase and α -amylase in the digestive fluid has not been determined yet, above
277 results suggest that both HdAgl and HdAmy58 participate in the glucose production in
278 the digestive fluid.

279

280 *3.3. Primary structure analysis for HdAgl*

281

282 The N-terminal amino-acid sequence of HdAgl was determined by the protein
283 sequencer as DSSQXHLKGEHRSDXYPET- (Underlined residues showed weak peak
284 signals in the sequencing, but later confirmed with cDNAs as they are; two Xs were not
285 identified, but later revealed as Cys with cDNAs). The N-terminal sequences of two
286 lysylendopeptidyl fragments of HdAgl were determined as
287 STNSVLFDASLAPLIFSDQM- (P1) and TADGSAPIVGEVWPGKTVFP- (P2). These
288 sequences showed 60% and 65% amino-acid identities to the 232nd–251st residues and
289 480th–499th residues of human maltase-glucoamylase (MGAM) that belongs to GHF31
290 (Nichols et al., 1998), respectively. On the basis of these partial amino-acid sequences,
291 we synthesized the degenerated forward primer Fw (from P1) and the reverse primers

292 Rv (from P2) (Table 3). Besides these primer, a reverse primer CatR was also
293 synthesized on the basis of the conserved signature sequence of GHF31 enzymes, i.e.,
294 (L/I/M)WIDMNE (Frandsen and Svensson., 1998) since the partial amino-acid
295 sequences of HdAgl showed similarity to the sequences of GHF31 enzymes. cDNAs
296 encoding HdAgl were then amplified by the nested PCR using these primers from the
297 abalone hepatopancreas cDNA. As a result, a cDNA with approximately 0.75 kbp
298 (Agl-cDNA1) was successfully amplified. The Agl-cDNA1 comprised 756 bp that
299 encoded an amino-acid sequence of 252 residues. Then, a series of specific primers for
300 3'-RACE and 5'-RACE were synthesized on the basis of the nucleotide sequence of
301 Agl-cDNA1 (Table 3). By using these primers, Agl-3RACE-cDNA (1463 bp) covering
302 the 3'-terminal region and Agl-5RACE-cDNA (643 bp) covering the 5'-terminal region
303 were amplified by 3'-RACE and 5'-RACE, respectively. By overlapping the nucleotide
304 sequences of Agl-5RACE-cDNA, Agl-cDNA1, and Agl-3RACE-cDNA in this order, a
305 nucleotide sequence of 2759 bp encoding the amino-acid sequence of 887 residues for
306 HdAgl was determined. The reliability of this sequence was confirmed with
307 AglFull-cDNA, which was newly amplified by PCR with a specific primer pair, FullFw
308 and FullRv (Table 3, Fig. 6). This nucleotide sequence and the following deduced
309 amino-acid sequence are available from DNA Data Bank of Japan with the accession
310 number AB820091.

311 The N-terminal amino-acid sequence of HdAgl, DSSQXHLKGEHRSDXYPET-
312 (Xs; not identified residues) determined by the protein sequencer, was shown in the
313 deduced sequence as DSSQCHLKGEHRSDCYPET. Thus, the N-terminus of mature
314 HdAgl protein was found to be the 43rd Asp and accordingly this enzyme was
315 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 FGVHGNGTGRVFKRDQ, 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.

335

336 **4. Discussion**

337

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

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

349

350 *4.1. Enzymatic properties of HdAgl*

351

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

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

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

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

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

374

375 4.2. Primary structure of HdAgl

376

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

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

391

392 4.3. Physiological roles of HdAgl for the digestion of algal starch

393

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

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

412

413 **Acknowledgements**

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415 Marine Science Bases in Tohoku) of the Ministry of Education, Culture, Sports, Science
416 and Technology, Japan.

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484 **Figure legends**

485

486 **Fig. 1.** Purification of α -glucosidase from the digestive fluid of the abalone. (A)
487 TOYOPEARL Phenyl-650M column chromatography of proteins precipitated between
488 20 and 60% saturation of ammonium sulfate. (B) TOYOPEARL CM-650M column
489 chromatography of the α -glucosidase obtained by TOYOPEARL Phenyl-650M
490 chromatography. (C) Hydroxyapatite column chromatography of the α -glucosidase
491 obtained by TOYOPEARL CM-650M chromatography. (D) Mono-S 5/50GL column
492 chromatography of the α -glucosidase obtained by Hydroxyapatite chromatography.
493 Protein elution and enzyme activity are indicated with open and closed circles,
494 respectively, in (A)–(C). Those are indicated with solid line and shaded boxes,
495 respectively, in (D).

496

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

501

502 **Fig. 3.** Temperature dependence, thermostability, pH dependence, and NaCl dependence
503 of HdAgl. (A) Temperature dependence of HdAgl was examined at 10–60°C in a
504 reaction mixture containing 5mg/ml maltose and 10 mM sodium phosphate buffer (pH
505 6.0). (B) pH dependence of HdAgl was examined at 30°C in the following reaction
506 mixtures adjusted to pH 2.0–2.9 with 10 mM glycine-HCl buffer (\blacktriangle), pH 2.9–6.0 with
507 10 mM sodium citrate buffer (\bullet), and pH 6.0–8.0 with 10 mM sodium phosphate

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

512

513 **Fig. 4.** Thin-layer chromatography for the degradation products of starch and
514 maltooligosaccharides produced by HdAgl. Two mg/mL starch and 5 mg/mL
515 maltooligosaccharides in 10 mM sodium phosphate buffer (pH 6.0) were degraded with
516 0.01 U/mL HdAgl at 30°C. The reaction was terminated at appropriate times by heating
517 at 100°C for 3 min and 1 μL of the reaction mixture was applied to TLC plate. M,
518 oligosaccharide markers comprising G1 and G2–G6. G1, glucose; G2–G6, maltose to
519 maltohexaose.

520

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

525

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

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

535

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

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556 Table 1. Summary for the purification of HdAgl.

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

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

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

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

568 ^d Active fraction obtained by hydroxyapatite chromatography.

569 ^e HdAgl purified by Mono-S chromatography.

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580 Table 2. Substrate specificity of HdAgl

581	Substrates	Relative activity (%)
582	Maltose* ¹	100
583	Maltotriose	99.9
584	Maltotetraose	75.6
585	Maltopentaose	74.1
586	Maltohexose	73.5
587	Isomaltose* ²	2.7
588	α -pNPG	1.6
589	Trehalose* ³	0
590	Sucrose	0
591	Starch	36.5
592	Glycogen	13.5

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

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

605	Names	Sequences ^a
606	Fw	5'-CCNYTNATHHTTYWSNGAYCA-3'
607	Rv	5'-GTYTTNCCNGGCCANACYTC-3'
608	CatR	5'-TCRTTCATRTC DATCCANA-3'
609	3raceF1	5'-GTCCCGTGACAATTCGCTACC -3'
610	3raceF2	5'-CATGGTCAATGAGATGGACTGG -3'
611	3raceF3	5'-GGATCTGCTCCTATAGTTGGGG -3'
612	5raceRT	5'-GTAGAGCATTGCTG-3'
613	5raceF1	5'-ACACGAATATGTACGGCAGC -3'
614	5raceF2	5'-GGTCGGAATAGAACCAAACGG -3'
615	5raceR1	5'-CGTACAAGTTGGTAGTTGGC -3'
616	5raceR2	5'-CACTGAGATCTGTAGCATGTGG -3'
617	FullFw	5'-ACTCCAAGCAACTCCACACC -3'
618	FullRv	5'-CTACCAAGTCATAACGAACGGC-3'

619 ^aD,A/G/T, H,A/C/T, N,A/C/G/T; R,A/G; S,C/G, W,A/T, Y,C/T.

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628 Fig. 1.

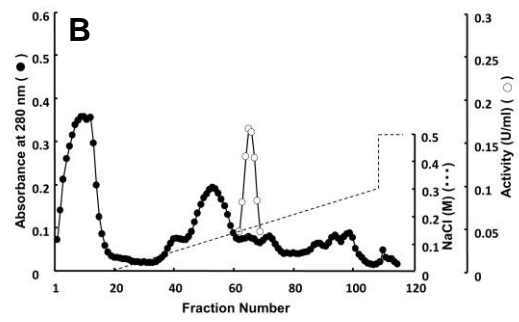
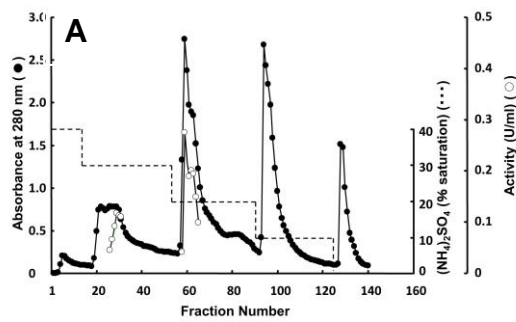
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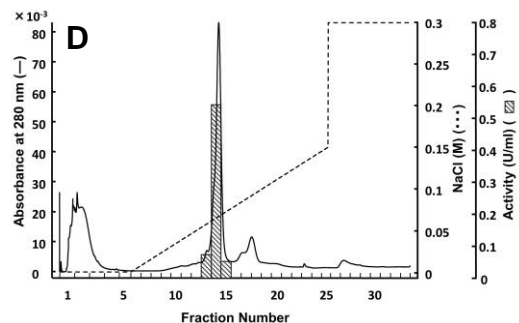
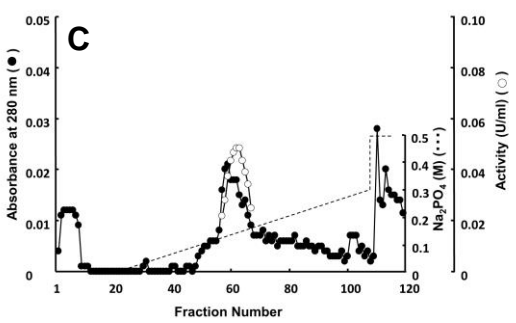
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652 Fig. 2.

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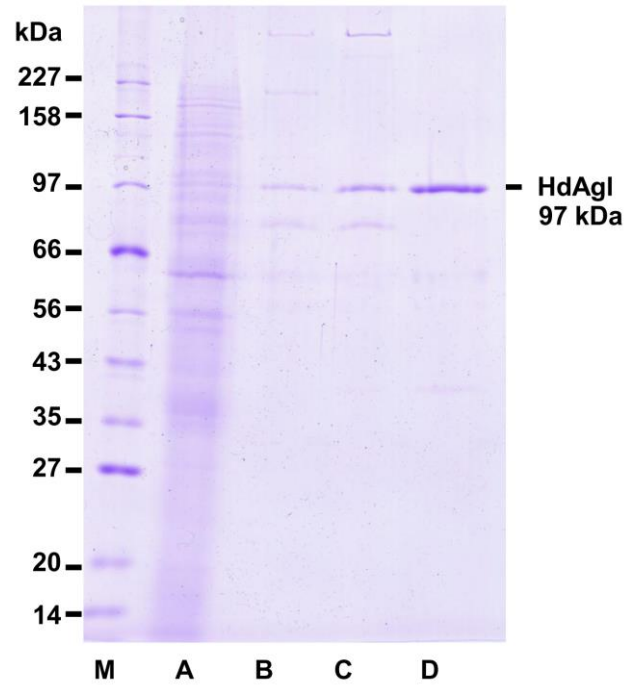
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676 Fig. 3.

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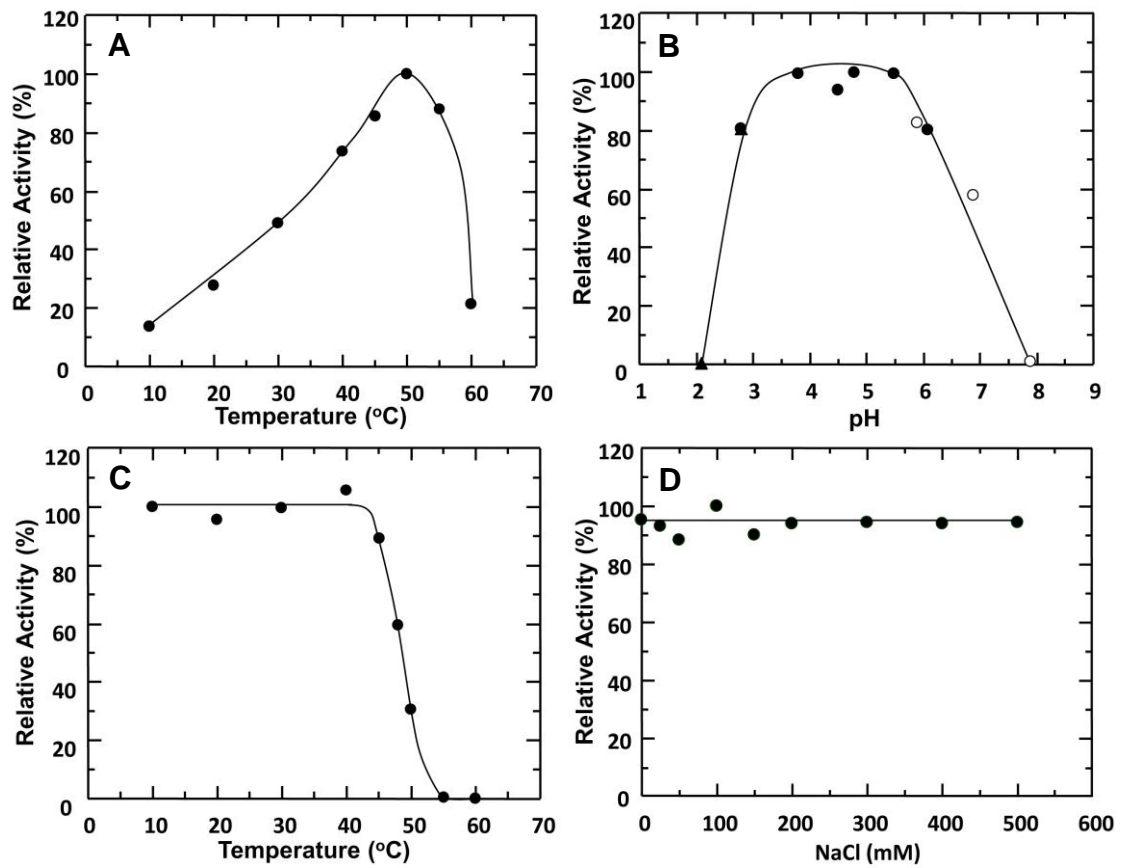
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700 Fig. 4.

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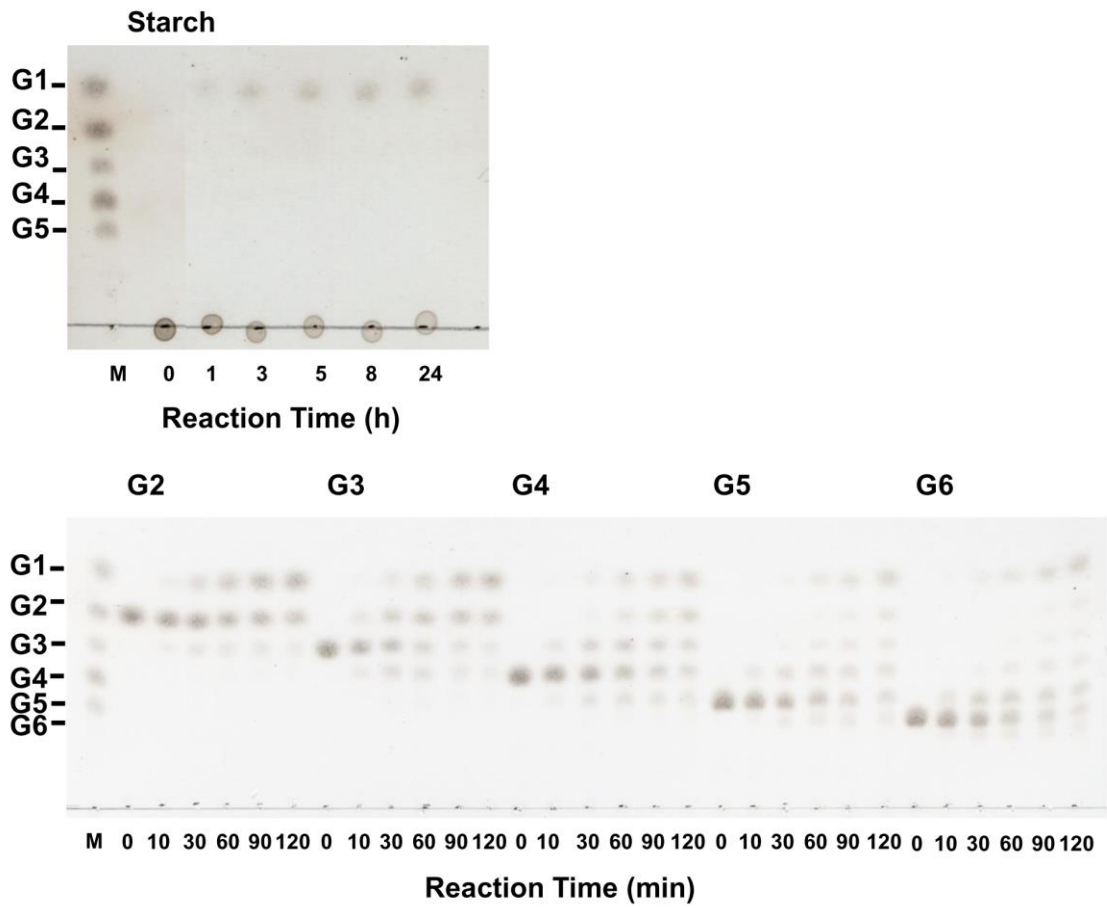
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724 Fig. 5

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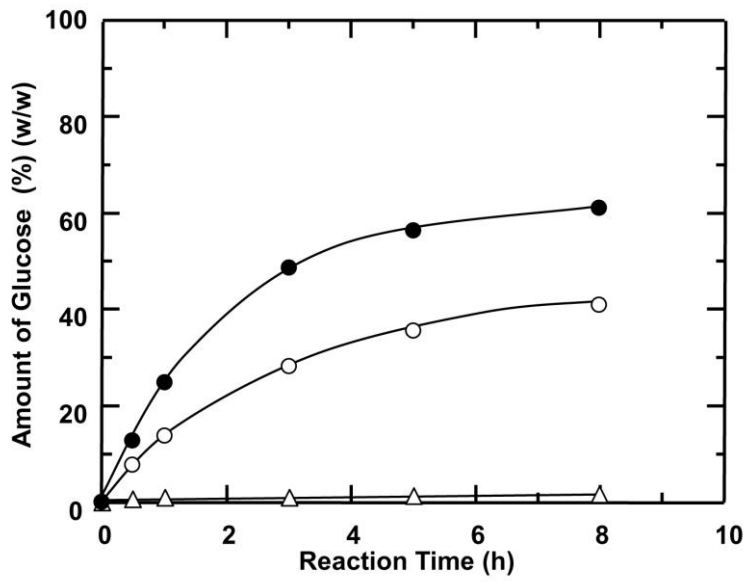
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748 Fig. 6

749 ^{FullF1}
GGATCACTCCAAAGCACTCCACACATGTGGCGGCCCCAGAGACTTGGCGTGCATGAAGCTGTGATAGCGGCACTGGTGTACAGGCTGGGTGCACGGGTTTGGTGTCCATGGTAAACGG 120
M W R P Q R L G V H E A V I A A L V L Q A W V H G F G V H G N G 32

750 CACCGGGCGTGTTCGTAAGCGGTGACCAGGACTCATCCAGTGTCACTCAAAGGCGAACACAGGAGTGATTTACCCAGAGACAGGCGTCACAGAGGCTGCCTTTTGTACAGTGG 240
T G R V F V K R D Q D S S Q C H L K G E H R S D C Y P E T G V T E A A C L S R G 72

751 TTGCTGCTGGCAGACTGCAACAGTGAAGGGGCCCTACTGTTTACCCGCCCCGGGAAGACTACTCTGTACAGAAAGTAATAAACAAATGGCAGGTCGGTGTGTGACAAGAAGCCA 360
C C W Q T S N V K G A P Y C F Y P P G E D Y S V Q K V I N N G R S V L L T R S Q 112

752 ACATACCACCTGGCCATAAGACGTATGACGTATGACGCTACAGGCTGACGTGTGCGAGGAGACAAGCAGCAGACTCCGATTTAAAATATACGACCCAAACAACAAGCGCTATGAAGTTCCTCTACC 480
H T H W P N D V M D L Q A D V V E E T S S R L R F K I Y D P N N K R Y E V P L P 152

753 GCCTCAACAGGCACTCGGTTCTTCGCAAAACTGATCTCTCTACCCATCGACCAACCCCTTCGGCCTCACCCTCACCAGGAGTCAACAAATAGTGTCTGTGACGCGGAGTCT 600
L N K A S G S S Q Q T D Y S F T I D H N P F G L T V T R K S T N S V L F D A S L 192

754 ^{Fw} CGCTCCTCTGATCTTCTCCGACAGATGCTACAGATCTCAGTGGGGTCCCACTACCAACTTGTACGGCATCGGAGAAACAGGAAGCCTTCCGGATCAACTGGAGAAGGGTCTCTGG 720
A P L I F S D Q M L Q I S V G V P T T N L Y G I G E N R K P F R I Q L E K G P G 232

755 CTATCCCTATGGGCTCATGATATCATCCCGGAGATCAACAGAAATATGACGGCAGCCACCCATTTGTGGTCCGAATAGAACCACCAACCGCGACGATTTGGGATATTCTCATTAACAG 840
Y S L W A H D I I P E I N T N M Y G S H P F V V G I E P N G D A F G I F L I N S 272

756 CAATGCTCTAGTATCGACTTATTCACACGATCCCGTGACAACTTCGCTACCGGCCCTTGGGGAACTACTGGACTTCTACGTTTCACTGGCCCTACGCGGATGACGTCATCGATCA 960
N A L R I D C M S I P T S P V T I R Y R A L N G I L D F Y V F T G P T P D K N I I G 312

757 GTACTGGACTTTGATGGACGCCACCACTTCCGCCACTACTGGTCGATAGGATACCATCTGATAGTGGGGATACGGCGGAACAAGCGGATGAACCAACATACAGAGATGAGGAA 1080
Y W T L I G Q P P L P P Y W S L G Y H L S R W G Y G G T S G M N A T I Q R M R N 352

758 CAAACAGATGCCATTTGACACCATCTGGAACGACATCGACTACATGGTCAATGAGATGGACTGACCTACGACCACACGGTGTATGGTCAGCTGCCTGACCTTGTAAAGATATACATAG 1200
K Q M P F D T I W N D I D Y M V N E M D W T Y D H T V Y G Q L P D L V K D I H S 392

759 TCATGGGAGAAATACGTCATGATCTTGGATCCCGCATCAGCAACACCCAGCGAAGGTCAGTATGCCCCGATGACGACGCGATAACAGATGATATCTTCGTAAGACTGCCGACGG 1320
H G E K Y V M I L D P G I S N T Q P K G Q Y A P Y D D G I T D D I F V K T A D G 432

760 ^{ceF3} ATCTGCTCTATAGTGGGGAGGTGGCGGGGAAGACGGTATTCACAGATTTCACTACCCCTAAGGCTGAAGCATGGTGGCAGAAACACGCTCAGATCATGCAGCTCAACTTCCATT 1440
S A P I V G E V W P G K T V F P D F T H P K A E A W W Q K H A Q I M H A Q L P F 472

761 TGATGGCACTGGATTGACATGAACGAGCCCTCCAACTCAAGGACGGTCTGTGACAGGATGTGGCAACACTCTCTGGAAACCCCGCTATACACCACCCCTGAATGGAGCAGCGT 1560
D G I W I M N E P S N F K D G S V T G C G N N S L E N P P Y T P P L N G S S V 512

762 CATAAGAAGCGTGTGATGTCATCCAGCTCCTACGGAGGACTCCACTACAACCTGCATAATCTGTACGGCCACTTCGAGGGCAAGCAACATACAATGTTCTAAAAATATCATCGG 1680
I Q K T L D C M S S T S Y G G L H Y N L H N L Y G H F E G K A T Y N V L K N I I G 552

763 AAAGAGCCATTTGTTCTATCTCGCTACTTTCCGCGGAAGCGGAAATACCTGCACACTGGGAAGGAGACAACCTTGTGACTGGTCAGACTTGTACTACTTATACCAGAGTTCT 1800
K R P F V L S R S T F A G S G N Y V A H W E G N F A D W S D L Y Y S I P E V L 592

764 CAGTTTCAACATGTTCCGATCCCTTCCACCGCGTGGACATATGGGTTTCAGAGGCGACTCAGACGAGGAACCTGTACAAGATGGCTACAGTGGGCGCTTACCCCTTTCATGAG 1920
S F N M F G I P F T G V D I C G F R G D S D E E L C T R W L Q L G A F Y P F M R 632

765 GTCTCAACAGAAATGTGGCGCGGACAAAGATCCCGCAGCGATGCGATTTAGTTCGCTGCGCATGACCGGAACCGGAAGCCCTGAGGCTCCGTTACCGCTTCTCCCTTCTCTGTA 2040
S H N Q N V A P D K D P A A M R F S S A A H D R N R E A L R L R Y R L L P F L Y 672

766 CTCTCTATGTCAGGAGGAGGCGAGTGGCCAGGCCCTCATCTTCCAGTACCCGACTGATTCGCGCGCTACAGCATAGACAGACAGTTCCTGTGGGGAGACTCGCTGCTCATAAGTCC 2160
S L M S R R R A V A R P L I F Q Y P T D S A A Y S I D R Q F L W G D S L L I S P 712

767 TGTGTTGACAGGGGGAACAGACAGTGAACGCTATTTTCTAAAGACAGTGGTACGATTTCTCACGGGGCGGAAGTGTCCAAAACAGGCAATGGTCACTATCAGTGCACCGCT 2280
V L D R G N R T V N A Y F P K D T W Y D F F T G A E V S K T G Q W S V I S A P L 752

768 GGACAAGATCAATGCCACTCAGAGGAGGCGGTTCCCGACACAGGTGCCCGATGTACCACAGAGAAGAGCGCACAAATGACTTCGCTTGGTGGCGTCTGCGGGCTCTAG 2400
D K I N V H L R G G S V V P T Q V P D V T T E K S R H N D F G L V V A S S G S R 792

769 GACAGCGCAAGGCTTCTGTATGGGACGACGAGAGACCTTAGATGCACCGTTTAAACAATCAAAATCAATCTGGACGGGAGCGTCTGACGCTCCACTGCAAGTCCAGCACTACGC 2520
T A Q G F L Y W D D G E T L D A P F N N I Q F N L D G E R L T S T V K S S N Y A 832

770 CACCACAATGACGTTAGGAGCATCAACGTGACGTTGGCCACGCGCTTCAACTGTGACAGTCAACGGTATGGGCGTCGCACACTTCTACGACCATCACACAAAGGCTTCTTCGGT 2640
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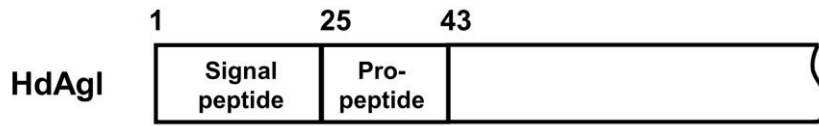
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T R L K V D L L K P F V M T W * 887

772 Fig. 7

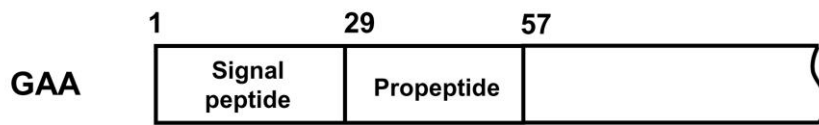
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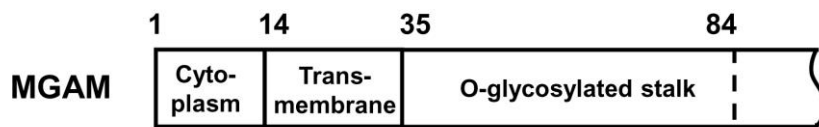


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