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Citation	Biochimie, 93(10), 1720-1730 https://doi.org/10.1016/j.biochi.2011.06.004
Issue Date	2011-10
Doc URL	http://hdl.handle.net/2115/47072
Type	article (author version)
File Information	AkAly30 (HUSCAP).pdf



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1 **cDNA cloning of an alginate lyase from a marine gastropod *Aplysia kurodai* and assessment of**
2 **catalytically important residues of this enzyme**

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28 **Abstract**

29 Herbivorous marine gastropods such as abalone and sea hare ingest brown algae as a major diet
30 and degrade the dietary alginate with alginate lyase (EC 4.2.2.3) in their digestive fluid. To date alginate
31 lyases from Haliotidae species such as abalone have been well characterized and the primary structure
32 analyses have classified abalone enzymes into polysaccharide-lyase-family 14 (PL-14). However, other
33 gastropod enzymes have not been so well investigated and only partial amino-acid sequences are currently
34 available. To improve the knowledge for primary structure and catalytic residues of gastropod alginate
35 lyases, we cloned the cDNA encoding an alginate lyase, AkAly30, from an Aplysiidae species *Aplysia*
36 *kurodai* and assessed its catalytically important residues by site-directed mutagenesis. Alginate lyase cDNA
37 fragments were amplified by PCR followed by 5'- and 3'-RACE from *A. kurodai* hepatopancreas cDNA.
38 The finally cloned cDNA comprised 1,313 bp which encoded an amino-acid sequence of 295 residues of
39 AkAly30. The deduced sequence comprised an initiation methionine, a putative signal peptide for secretion
40 (18 residues), a propeptide-like region (9 residues), and a mature AkAly30 domain (267 residues) which
41 showed ~40% amino-acid identity with abalone alginate lyases. An *Escherichia coli* BL21(DE3)-pCold I
42 expression system for recombinant AkAly30 (recAkAly30) was constructed and site-directed mutagenesis
43 was performed to assess catalytically important amino-acid residues which had been suggested in abalone
44 and *Chlorella* virus PL-14 enzymes. Replacements of K99, S126, R128, Y140 and Y142 of recAkAly30 by
45 Ala and/or Phe greatly decreased its activity as in the case of abalone and/or *Chlorella* virus enzymes.
46 Whereas, H213 that was essential for *Chlorella* virus enzyme to exhibit the activity at pH 10.0 was
47 originally replaced by N120 in AkAly30. The reverse replacement of N120 by His in recAkAly30
48 increased the activity at pH 10.0 from 8 U/mg to 93 U/mg; however, the activity level at pH 7.0, i.e., 774.8
49 U/mg, was still much higher than that at pH 10.0. This indicates that N120 is not directly related to the pH
50 dependence of AkAly30 unlike H213 of vAL-1.

51

52 *Keywords:* *Aplysia*, alginate lyase, cDNA cloning, polysaccharide-lyase family 14, site-directed
53 mutagenesis

54

55 1. Introduction

56

57 Seaweeds play important ecological roles in the communities of various marine organisms and
58 provide a suitable habitat environment for them. Moreover, seaweeds are the staple diets for herbivorous
59 marine invertebrates such as sea urchin, abalone and sea hare. These marine invertebrates preferably ingest
60 seaweeds' fronds and digest seaweeds' polysaccharides, i.e., alginate, cellulose, mannan, and laminarin,
61 with appropriate polysaccharide-degrading enzymes, i.e., cellulase, alginate lyase, mannanase, laminarinase
62 in their digestive tracts [1-11]. Among the seaweeds' polysaccharides, alginate from large-size brown algae,
63 i.e., Laminariales and Sargassaceae, is one of the most abundant carbohydrates in the marine environment.
64 This polysaccharide is synthesized as a structural constituent of cell wall and intracellular matrices in
65 brown algae and a structural component of biofilms in certain bacteria [12,13]. In the alginate polymer, two
66 kinds of monouronic acid, i.e., β -D-mannuronic acid (M) and its C5 epimer α -L-guluronic acid (G), are
67 1,4-linked to form G-block, M-block and MG-block [14]. Gastropod alginate lyases preferentially degrade
68 1,4-linkage of M-block *via* β -elimination mechanism and thus regarded as poly(M) lyase (EC 4.2.2.3). The
69 degradation products of alginate produced by the gastropod enzymes are usually tri- and disaccharides
70 possessing an unsaturated sugar, 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid, at the non-reducing
71 end [2,3,11,15,16]. These oligosaccharides are considered to be assimilated as carbon and energy sources
72 by the gastropods through their own metabolic systems or through the fermentative reaction of intestinal
73 bacteria [17, 18]. Overall metabolic pathway for the alginate in gastropods still remains to be elucidated.

74 To date gastropod alginate lyases have been investigated mainly on Haliotidae species, e.g.,
75 *Haliotis rufescens* and *Haliotis corrugate* [19], *Haliotis tuberculata* [20,21], *Haliotis discus hannai* [2,3],
76 and *Haliotis iris* [6]. Besides abalone enzymes, isolation and characterization have been achieved in the
77 enzymes from turban shell *Turbo cornutus* [16], small marine snail *Littorina* sp. [22], *Omphalius rusticus*
78 and *Littorina brevicula* [6], and sea hare *Dolabella auricula* [23] and *Aplysia kurodai* [11]. Although the
79 general properties of the gastropod enzymes have been repeatedly investigated, primary structure analysis
80 and structure/function study on gastropod alginate lyases have not been advanced compared with bacterial
81 enzymes [24-31].

82 Entire amino-acid sequences of gastropod alginate lyases were reported only in three enzymes,
83 i.e., the endolytic enzyme HdAly and exolytic enzyme HdAlex from abalone *H. discus hannai* [2,3], and an
84 endolytic enzyme SP2 from turban-shell *T. cornutus* [32]. The sequences of the abalone enzymes were
85 analyzed by the cDNA method, while that of the turban-shell enzyme was determined by the protein
86 method. Hydrophobic cluster analyses have classified the abalone enzymes under
87 polysaccharide-lyase-family 14 (PL-14) (<http://www.cazy.org>). Recently, partial amino-acid sequences of
88 small snail and sea hare alginate lyases were determined and they were suggested to be the members of
89 PL-14 [6,11]. These studies led us to consider that PL-14-type alginate lyases are widely distributed in
90 herbivorous gastropods. Besides the gastropods, some bacteria and fungi have also been shown to possess
91 genes encoding PL-14 enzymes, although the gene products have not been isolated and characterized
92 [33-36]. To clarify the range of distribution of PL-14-type alginate lyases in gastropods, primary structure
93 analysis for alginate lyases should be carried out in as many species as possible. Other than Haliotidae,
94 Aplysiidae species like *Aplysia kurodai* appeared to be a good source for alginate lyase. This animal is a
95 typical herbivorous gastropod and possesses various polysaccharide-degrading enzymes [9-11]. Recently,
96 partial amino-acid sequences of alginate lyases, AkAly28 and AkAly33, from *A. kurodai* were determined;
97 however, entire amino-acid sequences of *Aplysia* enzymes have not been determined yet.

98 Beside the gastropod alginate lyases, *Chlorella* virus enzymes, which cleave the glycoside linkage
99 of structural polysaccharides (not alginate but contain glucuronate residues) of *Chlorella* cell wall via
100 β -elimination, are known as another prominent member of PL-14 [37, 38]. Recently, crystal structure of
101 *Chlorella* virus vAL-1 was solved [38] and the catalytic domain of this enzyme was revealed to have a
102 β -jelly roll fold with a deep active cleft. Further, amino-acid residues responsible for the catalytic function
103 of vAL-1 were investigated by site-directed mutagenesis and some amino-acid residues responsible for the
104 catalytic action were shown to be located in β -strands A3-A6 and loops L1-L2 which surround the active
105 cleft of this enzyme. Interestingly, the primary structure of these strand and loop regions are fairly well
106 conserved in abalone alginate lyases [38]. This fact strongly suggests that the higher order structure and the
107 catalytic residues of abalone enzymes are also similar to those of *Chlorella* virus enzyme. Gastropod and
108 *Chlorella* virus seem to be in a phylogenically great distance. Thus, comparative study on primary

109 structures between gastropod and *Chlorella* virus enzymes may also provide us novel knowledge regarding
110 the origin and molecular evolution of PL-14 enzymes.

111 In the present study, to enrich the information about the primary structure of gastropod alginate
112 lyases, we cloned a cDNA encoding an alginate lyase isozyme, AkAly30, from a common sea hare *A.*
113 *kurodai*. Further we investigated amino-acid residues responsible for the activity of AkAly30 by
114 site-directed mutagenesis considering the results obtained in *Chlorella* virus vAL-1.

115

116 **2. Materials and methods**

117

118 *2.1. Materials*

119

120 TOYOPEARL CM-650M was purchased from Toyo soda (Tokyo, Japan). Sodium alginate
121 (*Macrocystis pyrifera* origin) was from Sigma–Aldrich (St. Louis, MO, USA). Poly(M)-rich, poly(G)-rich,
122 and random(MG) substrates were prepared from the alginate by the method of Gacesa and Wusteman [39].
123 Mannuronate and guluronate contents in the substrates were estimated by the method of Morris et al. [40].
124 The mannuronate content in the alginate was 60%, while those in the poly(M)-rich and the random(MG)
125 substrates were 86% and 64%, respectively. The guluronate content in the poly(G)-rich substrate was 99%.
126 The TA PCR cloning kit (pTAC-1) was purchased from Biodynamics (Tokyo, Japan). Oligotex-dT(30),
127 AmpliTaq Gold PCR Master Mix from ABI, 5'- and 3'-Full RACE kits, cold shock expression vector pCold
128 I, Site-directed Mutagenesis System Mutan™ -Express Km, restriction endonucleases and T4 DNA ligase
129 were purchased from TaKaRa (Tokyo, Japan). Ni-NTA resin was purchased from Invitrogen (Carlsbad, CA,
130 USA). Other reagents used were from Wako Pure Chemicals Industries Ltd. (Osaka, Japan).

131

132 *2.2. Isolation of an alginate lyase AkAly30*

133

134 The common sea hare, *Aplysia kurodai* (body length, ~16 cm; body weight, ~415 g) was
135 collected in the coast of Hakodate, Hokkaido, Japan in July 2009. Approximately 38 mL of digestive fluid

136 was harvested from 5 animals by cautiously squeezing their stomachs after dissection. The fluid was
137 dialyzed against 2 mM sodium phosphate buffer (pH 7.0) and centrifuged at 12,000×g for 15 min to
138 remove insoluble materials. The supernatant was used as a crude enzyme for further purification. The crude
139 enzyme (382 mg total protein) was subjected to ammonium sulfate fractionation, and the protein
140 precipitated between 60 and 90% saturation of ammonium sulfate was collected by centrifugation at
141 12,000×g for 15 min. The precipitates were dissolved in and dialyzed against 10 mM sodium phosphate
142 buffer (pH 7.0) and centrifuged at 12,000×g for 15 min to remove insoluble materials. The supernatant was
143 applied to a column of TOYOPEARL CM-650M (2×22 cm) pre-equilibrated with 10 mM sodium
144 phosphate buffer (pH 7.0). The adsorbed proteins were eluted with a linear gradient of 0-0.3 M NaCl (total
145 volume 600 mL) in 10 mM sodium phosphate (pH 7.0) at a flow rate of 15 mL/min, and eluent was
146 collected as 7-mL fractions. Alginate lyases were separately eluted as four peaks at around 0.13-0.23 M
147 NaCl. The former three peak fractions contained plural alginate lyases, e.g., AkAly28 and AkAly33 which
148 we previously reported [11], whereas the last peak fractions contained only 30-kDa protein with the highest
149 alginate lyase activity among the four peak fractions. In the present study, we focused on this enzyme as a
150 new *Aplysia* alginate lyase isozyme and named AkAly30. By the above purification procedure, AkAly30
151 was purified 97.58-fold at a yield of 7.15% and the specific activity 5,796.4 U/mg (Table 1).

152

153 2.3. Assay for alginate lyase activity

154

155 Alginate lyase activity was determined in 1 mL of reaction mixture containing 0.15% (w/v)
156 alginate, 0.15 M NaCl, 10 mM sodium phosphate buffer (pH 6.0), and an appropriate amount of enzyme at
157 30 °C. The progress of the enzyme reaction was monitored by measuring the absorbance at 235 nm with a
158 Model 3010 spectrophotometer (HITACHI, Tokyo, Japan) equipped by a temperature-control device
159 SP-12R (TAITEC, Tokyo, Japan). One unit (U) of alginate lyase was defined as the amount of enzyme that
160 increases absorbance at 235nm to 0.01 for 1 min. Temperature dependence was measured at 15–65 °C in 10
161 mM sodium phosphate buffer (pH 6.0). Thermal stability was assessed by measuring the activity remaining
162 after the heat treatment of the enzyme at 15–55 °C for 20 min. pH dependence of the enzyme was

163 determined at 30 °C in reaction mixtures adjusted to pH 3.0–10.0 with 50 mM sodium phosphate buffer. pH
164 stability was assessed by measuring the activity at pH 6.0 after the incubation of enzyme in 50 mM sodium
165 phosphate buffer (pH 3.0-10.0) at 40 °C for 30 min. Substrate specificity was determined with reaction
166 mixtures containing 0.15% sodium alginate, poly(M)-rich, random(MG), or poly(G)-rich substrates at pH
167 6.0 and 30 °C. Average values for triplicate measurements were used for all activity assays.

168

169 *2.4. Thin-layer chromatography*

170

171 To analyze the degradation products of alginate produced by AkAly30, 1.0% poly(M)-rich
172 substrate in 0.1 M NaCl and 10 mM sodium phosphate buffer (pH 6.0) was degraded with an appropriate
173 amount of AkAly30 at 30 °C for 12 h. An aliquot (10 µL) of the reaction mixture was withdrawn at
174 appropriate time and heated at 95 °C for 2 min to inactivate the enzyme. Then, 2 µL of reaction mixture
175 was subjected to TLC-60 plate (Merck KGaA, Darmstadt, Germany) and developed with 1-butanol–acetic
176 acid–water (2:1:1, v/v). The sugars separated on the plate were visualized by heating the plate at 110 °C for
177 5 min after spraying with 10% (v/v) sulfuric acid in ethanol. Unsaturated oligosaccharides and α -keto acid
178 (4-deoxy-5-keto uronic acid) on the TLC plate were detected with 6% thiobarbituric acid (TBA) [41].
179 Standard unsaturated oligosaccharides were prepared as described previously [2, 3].

180

181 *2.5. SDS-PAGE*

182

183 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with
184 0.1% (w/v) SDS-10% (w/v) polyacrylamide slab gel according to the method of Porzio and Pearson [42].
185 After the electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v)
186 methanol-10% (v/v) acetic acid, and the background of the gel was destained with 5% (v/v) methanol–7%
187 (v/v) acetic acid. Protein Marker, Broad Range (New England BioLabs, Ipswich, MA, USA) was used as a
188 molecular mass marker.

189

190 2.6. *Protein determination*

191

192 Protein concentration was determined by the biuret method [43] or the method of Lowry et al.
193 [44] using bovine serum albumin fraction V as a standard protein.

194

195 2.7. *Determination of N-terminal and internal amino-acid sequences*

196

197 The N-terminal amino-acid sequence of AkAly30 was determined with an ABI Procise 492
198 protein sequencer (Applied Biosystems, Foster city, CA, USA). Internal amino-acid sequences of AkAly30
199 were determined with in-gel-digested tryptic fragments and a matrix-assisted laser desorption
200 ionization-time of flight mass spectrometer (MALDI-TOF MS) (Proteomics Analyzer 4700, Applied
201 Biosystems). Determination of the amino-acid sequences was carried out with DeNovo Explorer software.
202 The lysylendopeptidyl fragments separated by reverse phase HPLC equipped with a Mightysil Rp-18 (4.6
203 mm×150 mm) column were also subjected to MALDI-TOF MS and protein sequencer. Homology searches
204 for the amino-acid sequences on the public databases were performed with the FASTA and BLAST
205 programs (<http://fasta.ddbj.nig.ac.jp/top-j.html>, <http://blast.ddbj.nig.ac.jp/top-j.html>) provided by DNA
206 Data Bank of Japan.

207

208 2.8. *cDNA cloning and nucleotide sequence analysis*

209

210 Total RNA of *A. kurodai* was extracted from 1 g of the hepatopancreas by the guanidinium
211 thiocyanate–phenol method [45]. mRNA was selected from the total RNA with Oligotex-dT(30) according
212 to the manufacturer's protocol. Hepatopancreas cDNA was synthesized from the mRNA with a TaKaRa
213 cDNA synthesis kit using random hexanucleotide primers. cDNAs encoding the AkAly30 were amplified
214 from the hepatopancreas cDNA by polymerase chain reaction (PCR) using degenerated primers synthesized
215 on the basis of partial amino-acid sequences of AkAly30. PCR was performed with AmpliTaq Gold DNA
216 polymerase in 20 µL of reaction mixture containing 50 mM KCl, 15 mM Tris–HCl (pH 8.0), 0.2 mM each

217 of dATP, dTTP, dGTP, and dCTP, 2.5 mM MgCl₂, and 5 pmol/μL primers, 1 ng/μL template DNA, and 0.5
218 units AmpliTaq Gold DNA polymerase. A successive reaction at 94 °C for 20 s, 55 °C for 20 s and 72 °C for
219 45 s was repeated for 30 cycles with Thermal Cycler Dice mini (TaKaRa, Tokyo, Japan). cDNAs derived
220 from 5'- and 3'-terminal regions of mRNA were amplified with 5'- and 3'-Full RACE kits (TaKaRa),
221 respectively. The size of cDNA was estimated by 1.2% agarose-gel electrophoresis. The PCR products
222 were cloned with a TA PCR cloning kit (pTAC-1) (Invitrogen, CA, USA), and nucleotide sequences of the
223 cDNAs were analyzed with a BigDye-terminator Cycle sequencing kit (Applied Biosystems) and an ABI
224 3130 xl Genetic Analyzer (Applied Biosystems).

225

226 2.9. Production of recombinant AkAly30

227

228 The restriction sites, *NdeI* and *BamHI*, were introduced to the 5'- and 3'-termini of the AkAly30
229 cDNA, respectively, by PCR with the primers including each restriction sites. The amplified cDNA was
230 digested with *NdeI* and *BamHI* and ligated between *NdeI* and *BamHI* restriction sites of pCold I expression
231 plasmid (Novagen, WI, USA) which had been digested with the appropriate restriction enzymes. *E. coli*
232 BL21(DE3) (Novagen, WI, USA) was transformed with the pCold I-AkAly30-cDNA and cultured
233 overnight in 250 mL of 2×YT medium containing 50 μg/mL ampicillin at 37 °C. After the cold shock at 15
234 °C for 1 h, recombinant AkAly30 was expressed by the induction with 1 mM isopropyl
235 1-thio-β-D-galactoside. In fourteen hours of the induction, cells were harvested by centrifugation at
236 8,000×g for 10 min and suspended with 50 mL of 50 mM sodium phosphate (pH 8.0), 0.5 M NaCl, 1%
237 TritonX-100, 10 mM imidazole (pH 8.0) and 0.01 mg/mL lysozyme. The cells were then sonicated 6-10
238 times for 30 s with each 1 min interval on ice. The cell lysate was then centrifuged at 10,000×g for 10 min
239 and the supernatant was mixed with 0.2 mL of Ni-NTA resin (Invitrogen) and left at 4 °C for 1 h with
240 occasionally mixing. The resin was transferred to a small plastic column (0.5 x 2.5 cm) and unadsorbed
241 materials were washed out with 20 mL of 40 mM imidazole buffer (pH 8.0). Then, adsorbed proteins were
242 eluted from the resin with 150 mM imidazole buffer (pH 8.0) and collected as 0.5-mL fractions. The
243 fractions showing alginate lyase activity were pooled. The thus purified recombinant AkAly30 was

244 dialyzed against 10 mM sodium phosphate (pH 6.0) and stored at 4 °C until use.

245

246 *2.10. Site-directed mutagenesis*

247

248 Site-directed mutagenesis of AkAly30 was carried out using a Site-directed Mutagenesis System
249 MutanTM-Express Km (TaKaRa) and pCold I-AkAly30-cDNA as a template. Mutations in the cDNA were
250 confirmed by nucleotide sequencing. Expression and purification of the mutant AkAly30 were performed
251 by the same method as for recAkAly30.

252

253 *2.11. Prediction of three-dimensional structure of AkAly30*

254

255 Three-dimensional structure of AkAly30 was predicted by the SWISS-MODEL software
256 (<http://swissmodel.expasy.org/>) using the structure data for *Chlorella* virus PL-14 enzyme vAL-1 (PDB ID:
257 3GNE, chain A) as a template.

258

259 **3. Results**

260

261 *3.1. cDNA encoding AkAly30*

262

263 The N-terminal amino-acid sequence of 28 residues of AkAly30 was determined as
264 ATTVWSLSSVPHSSHVSTILGHFKPIYH by the protein sequencer. This sequence showed 7%, 14%, 11%,
265 68%, and 57% amino-acid identity with the corresponding sequences of abalone HdAly [2], abalone
266 HdAlex [3], turban shell SP2 [32], sea hare AkAly28 and AkAly33 [11], respectively. The amino-acid
267 sequences of tryptic and lysylendopeptidyl fragments of AkAly30 are summarized in Table 2. The sequence
268 of a tryptic fragment T-1, GMFFSTFFGGSEK, shared 77% amino-acid identity with the 216-228 residues
269 of abalone HdAly and HdAlex and turban shell SP2. This region is known to be highly conserved among
270 PL-14 enzymes [3, 46]. On the other hand, a lysylendopeptidyl fragment L-2, LPGLFGGEN, showed 67%

271 and 78% identities to the 96-104 residues of HdAlex, and SP2 and HdAly, respectively. Whereas the
272 sequence of L-3, YDVYFENFGFGIGGK, showed 69% identity to the 80-95 residues of both HdAly and
273 HdAlex and 53% identity to the 80-95 residues of SP2. The sequence of another lysylendopeptidyl
274 fragment L-1, WHSISEEVHINTVGK, showed 47% and 53% identity to the 170-184 residues of HdAly
275 and SP2, respectively. Such sequence similarity of AkAly30 to other known PL-14 enzymes indicates that
276 AkAly30 also belongs to PL-14.

277 cDNA encoding entire amino-acid sequence of AkAly30 was amplified by PCR as follows. First,
278 cDNA-1, the cDNA encoding internal amino-acid sequence of AkAly30, was amplified by PCR with the
279 degenerated primers, AkAly30Fw and AkAly30Rv, designed on the basis of N-terminal and internal
280 amino-acid sequences (Table 3). By the nucleotide sequence analysis, the cDNA-1 was revealed to consist
281 of 270 bp encoding the 90 amino-acid residues (Fig. 1). Next, 3'- and 5'-RACE PCRs were performed with
282 specific primers shown in Table 3, and cDNA-3RACE (830 bp) and cDNA-5RACE (408 bp) were
283 amplified, respectively (Fig. 1). By overlapping the nucleotide sequences of cDNA-5RACE, cDNA-1 and
284 cDNA-3RACE, in this order, the nucleotide sequence of total 1,313 bp including the complete translational
285 region for AkAly30 was determined (Fig. 1 and Fig. 2) (the sequence is available from the DNA Data Bank
286 of Japan with the accession number AB610185). The reliability of this sequence was confirmed with
287 cDNA-Full (1,043 bp) that was newly amplified with a specific primer-pair, Full5F- Full3R (Table 3, Fig.
288 1). The translational initiation codon ATG was found in the nucleotide positions from 178 to 180 and the
289 termination codon TAG was found from 1,063 to 1,065 (Fig. 2). Accordingly, the amino-acid sequence of
290 295 residues was deduced from the translational region of 885 bp. In the 3'-terminal region of the cDNA, a
291 putative polyadenylation signal sequence, AATAAA, and a poly (A)⁺ tail were found. The N-terminal
292 region of 18 residues except for the initiation Met, i.e., ARVVKWVFFALFVAICNA, was predicted as the
293 signal peptide for secretion by the method of von Heijne [47] and the following region of 9 residues,
294 EETEERSKR, was regarded as a propeptide-like region of this enzyme since this region was absent in the
295 purified AkAly30 protein. Accordingly, the mature AkAly30 was considered to consist of 267 residues with
296 the calculated molecular mass of 29,722 Da which is well consistent with the molecular mass, 30 kDa,
297 estimated by SDS-PAGE. All the amino acid sequences of T-1, L-1, L-2 and L-3 fragments (Table 2) are

298 seen in the deduced sequence (Fig. 2). Thus, the cDNA was concluded to be of AkAly30.

299 The deduced amino-acid sequence of AkAly30 was compared with those of several PL-14
300 enzymes (Fig. 3). The amino-acid sequence of AkAly30 shared 39%, 42%, and 21% identities with those of
301 abalone HdAlex [3], abalone HdAly [2] and *Chlorella* virus vAL-1 [38], respectively. In Fig. 3, locations of
302 β -strands in the three-dimensional structure of vAL-1 [38] are indicated with boxes. β -strands A4-A6
303 configure the active cleft of vAL-1 and contain catalytically important residues, i.e., K99, S126, R128,
304 Y140, and Y142, which protrude toward the cleft [38] (see Fig. 8). One of the cysteine pairs, i.e.,
305 C106-C115 in HdAly and HdAlex, which was suggested to form a disulfide linkage in turban-shell SP2
306 [32], were conserved as C115-C124 in AkAly30. On the other hand, another cysteine pair, C145-C150 in
307 HdAly and HdAlex, which was reported to be important in catalytic action of SP2 [32], was replaced by
308 R154-A159 in AkAly30. N105 that has been suggested as a carbohydrate-chain anchoring residue in SP2
309 [32] was conserved in HdAly and HdAlex; however, it was replaced by K114 in AkAly30. These
310 characteristics in primary structure of AkAly30 suggest that the *Aplysia* alginate lyase has been much
311 deviated from other gastropod enzymes.

312

313 3.2. Production of recombinant AkAly30

314

315 Recombinant AkAly30 (recAkAly30) was produced with the cold-inducible *E. coli* expression
316 system as described under “Materials and Methods”. Thus, cDNA-EX coding for the mature AkAly30
317 region was amplified from the cDNA-Full by the PCR with the forward primer, ExFw including an *Nde*I
318 site whose inner ATG sequence is applicable as a translational initiation codon in pCold I, and the reverse
319 primer, ExRv including a *Bam*HI site (Table 4). The amplified cDNA-EX was ligated to pCold I vector and
320 introduced to a host strain *E. coli* BL21 (DE3). By this manipulation, recAkAly30 was produced as a fusion
321 protein possessing a hexahistidine (HHHHHH-) tag at the N-terminus. From the cell lysate of 250 mL
322 culture, 0.6 mg of recAkAly30 was purified by the Ni-NTA affinity chromatography (Table 5). The
323 recAkAly30 showed a single band on SDS-PAGE with an apparent molecular mass of 30 kDa although a
324 trace amount of an unidentified protein with ~63 kDa was seen (Fig. 4). We used the recAkAly30 without

325 further purification since the amount of the contaminated protein seemed negligible in alginate-lyase assay.

326 General properties of recAkAly30 were investigated by comparing with native AkAly30
327 (natAkAly30) (Fig. 5). The optimal temperature and pH were shown at around 55 °C and pH 6.0 in both
328 recAkAly30 and natAkAly30 (Fig. 5A and C). Temperatures that caused a 50% inactivation during
329 20-min incubation were 48 °C and 46 °C for recAkAly30 and natAkAly30, respectively (Fig. 5B). Both
330 enzymes showed considerably high pH stability, i.e., their activities did not significantly decreased at pH
331 4.5-9.0, e.g., retained more than 80% of the original activity, by the incubation at 40 °C for 30 min (Fig.
332 5D). Substrate preference of natAkAly30 was slightly different from that of recAkAly30 (Fig. 5E and F).
333 Namely, both enzymes exhibited the highest activity toward poly(M)-rich substrate as reported with other
334 gastropods' poly(M) lyases; however, the activity toward sodium alginate was appreciably different
335 between two enzymes. Thus, natAkAly30 could degrade sodium alginate as efficiently as poly(M)-rich
336 substrate whereas recAkAly30 could not (Fig. 5E and F). This may due to the subtle difference in the
337 higher order structure or folding between natAkAly30 and recAkAly30. Namely, recAkAly30 was
338 produced in the *E. coli* system in the absence of the propeptide-like region which may affect the correct
339 folding of the associated proteins.

340 Degradation products of poly(M)-rich substrate produced by recAkAly30 and natAkAly30 were
341 analyzed by TLC (Fig. 6). Sulfuric acid staining indicated that the two enzymes similarly produced tri- and
342 disaccharides as major end products along with a series of intermediary oligosaccharides. TBA staining
343 indicated that the degradation products were unsaturated sugars and small amount of α -keto acid
344 (4-deoxy-5-keto-uronic acid). These results indicate that AkAly30 is an endolytic alginate lyase that
345 degrades the internal glycosyl linkages of alginate polymer *via* β -elimination mechanism.

346

347 3.3. Assessment of catalytically important residues of AkAly30

348

349 Three-dimensional structure of *Chlorella* virus vAL-1 (PDB ID, 3GNE) [38], another prominent
350 member of PL-14, inspired us to examine if the catalytically important residues of the virus enzyme are
351 conserved in gastropod enzymes. Actually, basic amino-acid residues, K197 and R221 of vAL-1 were seen

352 as K99 and R128 in AkAly30. On the other hand, H213 in vAL-1 was not conserved in AkAly30, i.e., H213
353 is replaced by N120 (Fig. 3). At first, to assess the functional importance of K99 and R128 in AkAly30, we
354 replaced these residues with Ala by the site-directed mutagenesis using the mutation primers listed in Table
355 4 and prepared the mutants by the same method as for wild-type recAkAly30 (Fig. 7). As shown in Table 6,
356 activities of the mutants, K99A and R128A, were less than 1/100 of wild-type recAkAly30. On the other
357 hand, a control mutation to K171A, which is not located in the catalytic cleft, hardly affected the activity.
358 These results strongly suggest that the basic amino-acid residues K99 and R128 of AkAly30 corresponding
359 to K197 and R221 of vAL-1 are closely related to the catalytic action of AkAly30, e.g., binding to carboxyl
360 residues of alginate substrate and/or abstraction of a proton from the C5 carbon. On the other hand,
361 replacements of S219, Y233 and Y235 by Ala and/or Phe in vAL-1 were shown to decrease the activity to
362 1.8%, 53% and 82%, respectively of original value [38]. The corresponding mutant of AkAly30, i.e.,
363 S126A, Y140F and Y142F, also showed considerably decreased activity i.e., 16~64% of the wild-type
364 AkAly30 (Table 6). These results indicate that K99, R128, S126, Y140 and Y142 in AkAly30 are closely
365 relating to the catalytic action of this enzyme similarly to the case of K197, R221, S219, Y233 and Y235 in
366 vAL-1. On the other hand, H213 of vAL-1 was reported to be important to exhibit the activity at pH 10.0
367 [38]. Interestingly, this His was originally replaced by N120 in AkAly30. Therefore, we reversely replaced
368 N120 of AkAly30 by His. By this mutation, the activity at pH 10.0 increased from 8 U/mg to 93 U/mg;
369 however, the activity at pH 7.0 was still significantly higher, i.e., 774.8 U/mg, than that at pH 10.0. This
370 indicates that the N120 is not directly relating to the pH dependence of AkAly30 like H213 in vAL-1.

371

372 *3.4. Prediction of three-dimensional structure of AkAly30*

373

374 Three-dimensional structure of AkAly30 was predicted by the homology modeling using the
375 structure data of vAL-1 (PDB ID, 3GNE) as a template. The predicted structure for the region from 40 to
376 227 residues of AkAly30 was compared with the corresponding region from 140 to 317 residues of vAL-1
377 (Fig. 8). The predicted structure indicated that AkAly30 was in a glove-like β -jelly roll fold consisting of
378 two anti-parallel β -sheets (sheets A and B) as in the case of vAL-1. The catalytically important residues,

379 K99, R128, S126, Y140, and Y142 of AkAly30 were located in the active cleft and their side chains were
380 predicted to protrude to inside of the cleft similarly to the case of vAL-1. On the other hand, N120 of
381 AkAly30 was located in the outside of the cleft and appeared to stabilize the vicinal structures by hydrogen
382 bonding. The predicted structure of AkAly30 appeared to well explain the results of site-directed
383 mutagenesis on AkAly30 (see Fig. 3 and Table 6).

384

385 **4. Discussion**

386

387 In the present study, we isolated an alginate lyase isozyme, AkAly30, from the digestive fluid of *A. kurodai*
388 by ammonium sulfate fractionation followed by conventional column chromatography. According to the
389 basic properties of AkAly30 (Figs. 5 and 6), this enzyme was regarded as a typical poly(M) lyase (EC
390 4.2.2.3). Since we had isolated another two alginate lyases, AkAly28 and AkAly33, from the same *Aplysia*
391 species [11], *A. kurodai* appeared to possess at least three different poly(M) lyase isozymes in the digestive
392 fluid. In the present study, we focused on the major isozyme AkAly30 and cloned its cDNA.

393 The deduced amino-acid sequence of AkAly30 comprised a putative signal peptide region of 18
394 residues, a propeptide-like region of 9 residues, and a mature enzyme domain of 267 residues (see Fig. 2).
395 The occurrence of the propeptide-like region in AkAly30 was the first demonstration among gastropods'
396 alginate lyases so far investigated. Namely, this region was not seen in the deduced sequences of abalone
397 HdAly and HdAlex. Generally, propeptide regions of some prokaryotic and eukaryotic proteins are known
398 to act as intramolecular chaperones, which urge correct folding of their associated proteins and/or structural
399 organization, subunit formation, localization, modulation of activity and stability of proteins [48]. However,
400 physiological significance of the propeptide-like region of AkAly30 is currently obscure. The amino-acid
401 sequence of the mature AkAly30 showed ~39% and ~42% identity to those of abalone HdAlex and HdAly,
402 and ~21% to the corresponding region of *Chlorella* virus vAL-1 (Fig. 3). These sequence similarities
403 indicate that AkAly30 is also a member of PL-14. Recently, the PL-14 enzymes were further divided into
404 three subfamilies [49]. The bacterial and eukaryotic PL-14 enzymes reported so far were classified under
405 the subfamily 3, whereas viral PL-14 enzymes were spread over subfamilies 1–3. According to the

406 phylogenetic analysis for the primary structures of PL-14 enzymes, AkAly30 was placed on the clade of
407 subfamily 3 together with other eukaryote PL-14-subfamily-3 enzymes (data not shown). Thus, AkAly30
408 was also regarded as a member of PL-14 subfamily 3.

409 Here it should be noted that the origin of gastropod alginate lyases is still controversial. Namely,
410 it is possible to consider that the gastropod alginate lyases are products of the intestinal bacteria since
411 alginate-lyase producing bacteria were reported to be present in the digestive tracts of Haliotidae
412 gastropods [17, 18]. Indeed, many gastropod enzymes were isolated from hepatopancreas and/or digestive
413 fluid [2, 3, 6, 13, 15, 16, 19–23]; however, these enzymes have not been proved to be the gastropods' gene
414 products. Therefore, to obtain information about the origin of AkAly30, we prepared chromosomal DNA
415 from the hepatopancreas and attempted to amplify the structural gene(s) for AkAly30 from the
416 chromosomal DNA by the PCR using specific primers designed from the AkAly30 cDNA. As a result, a
417 DNA fragment comprising the coding region for C-terminal part of AkAly30 (from I124 to G295) and
418 3'-noncoding region of 56 nucleotides was successfully amplified with the specific primer pair,
419 5'-ATTGGCGGGAAACTGCCTG-3' (forward primer) and 5'-TGAACCAACAAGGACCTCG-3' (reverse
420 primer), which spans the 547th and 1118th nucleotide of AkAly30 cDNA (data not shown but see Fig. 2).
421 On the other hand, the DNA fragments for the N-terminal region of AkAly30 was not amplified with the
422 primer pair, 5'-TCATCATCGTCGTCGAAGAGCC-3' (forward primer; same as Full5F in Table 3) and
423 5'-TGCTGTAGAAACCGGCACC-3' (reverse primer), which spans the 132th and 469th nucleotide of the
424 cDNA. This may imply the occurrence of large size intron(s) in the 5'-terminal region of the structural gene.
425 We are now trying to clone the entire structural gene for AkAly30 from the chromosomal DNA of *A.*
426 *kurodai* to definitely determine the origin of this enzyme.

427 Expression of recAkAly30 with the cold-inducible *E. coli* expression system appeared to be
428 much advantageous compared with the expression of recombinant abalone enzymes in *E. coli*. Namely, we
429 previously produced recombinant abalone alginate lyase HdAly in the *E. coli* system; however, yield was
430 very modest [2,3]. But in this study recAkAly30 was successfully expressed in *E. coli* with a yield
431 sufficient for the determination of enzymatic properties. The superiority of AkAly30 to HdAly in the
432 expression in *E. coli* may be due to the structural stability of *Aplysia* enzyme, e.g., the temperatures that

433 caused 50% inactivation during 20-min incubation were at around 45 °C for AkAly30 and 38 °C for HdAly.
434 The higher thermal stability of AkAly30 may be an advantageous property for the correct folding of the
435 recombinant in *E. coli* cells.

436 In *Chlorella* virus enzyme vAL-1, the active site was shown to span K197-Y235 which involves
437 three β -strands (A4, A5, and A6) [38]. These segments organize the active cleft in the three-dimensional
438 structure of this enzyme. Among the amino-acid residues extruded toward the active cleft, K197, S219,
439 R221, Y233 and Y235 play key roles for the catalytic action and/or substrate binding of this enzyme [38].
440 These residues appeared to be conserved as K99, S126, R128, Y140 and Y142 in AkAly30, and
441 replacements of these residues by Ala and/or Phe indeed greatly decreased the activity of AkAly30. Among
442 these residues, K99 and R128, which correspond to K95 and R119 of abalone HdAly, have been shown to
443 be crucially important with the site-directed mutants of HdAly produced with the cold-inducible yeast
444 expression system [46]. Namely, replacement of K95 and R119 in HdAly by Ala greatly reduced the
445 activity [46]. The other three catalytically important residues in vAL-1, i.e., S219, Y233 and Y235, were
446 found to be conserved as S126, Y140 and Y142 for AkAly30. The replacement of these residues by Ala
447 and/or Phe also greatly decreased the activity of AkAly30. These facts indicate that the catalytically
448 important residues are commonly conserved in *Chlorella* virus and gastropod enzymes.

449 The amino-acid residues, H213, R221, Y233, and Y235 of *Chlorella* virus vAL-1 were reported
450 to be responsible for the activity in alkaline pH range [38]. Most of these residues were conserved in
451 gastropod enzymes (Fig. 3). However, H213 of vAL-1 was considered to be replaced by N120 in AkAly30.
452 This may cause somewhat different pH dependent properties of AkAly30, and actually the optimal pH of
453 AkAly30 was observed at pH 6.0 unlike other gastropod enzymes which usually show optimal pH at
454 around 8.0 [2, 3, 6]. Therefore, we reversely replaced N120 of AkAly30 by His; however, the optimal pH
455 of this enzyme was practically unchanged, i.e., the activity level at pH 7.0 still remained much higher than
456 that at pH 10.0 (Table 6). Thus, the amino-acid residues which determine the optimal pH seemed to be
457 different between vAL-1 and AkAly30.

458 In the present study, we found that AkAly30 is a new member of PL-14-subfamily-3 alginate
459 lyases. AkAly30 was considered to be a superior material for protein-engineering studies on gastropod

460 alginate lyases since this enzyme was expressed in the *E. coli* expression system with a sufficient amount
461 for the determination of enzymatic properties. Further, we confirmed that the catalytically important
462 amino-acid residues are highly conserved between *Chlorella* virus enzyme and gastropod enzymes. The
463 structural similarity between *Chlorella* virus and gastropod enzymes suggest the occurrence of a common
464 ancestral gene for these lyases. Further studies on distribution of PL-14-type alginate lyase genes and
465 phylogenetic analysis for the primary structures of alginate lyases will provide information about the
466 molecular evolution process of PL-14 enzymes.

467

468 **Acknowledgements**

469 This study was supported in part by the Regional Innovation Cluster Program (Global type) and a
470 Grant-in Aid for Scientific Research (No. 19380117) of the Ministry of Education, Culture, Sports, Science
471 and Technology, Japan.

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610 **Legends to figures**

611

612 Fig. 1. Schematic structure of AkAly30-cDNA.

613 Shaded and open boxes indicate coding and non-coding regions of AkAly30-cDNA, respectively. The
614 numbers in the top of the figure indicate the nucleotide positions. Relative positions for cDNA-1,
615 cDNA-5RACE, cDNA-3RACE, and cDNA-Full are indicated with thin lines along with the positions of
616 PCR primers indicated with bold lines.

617

618 Fig. 2. The nucleotide and deduced amino-acid sequences of the AkAly30.

619 Residue numbers both for nucleotide and amino-acid are indicated to the right of each row. The
620 translational initiation codon ATG, termination codon TAG, and a putative polyadenylation signal
621 AATAAA are boxed. A putative signal peptide is indicated by a dotted underline. The amino-acid
622 sequences determined with intact AkAly30 (N-terminus) and peptide fragments (T1, L-1 - L-3) are
623 indicated with solid lines under the amino-acid sequence. The positions of 5F2, 3Adapt, Full5F and Full3R
624 primers are indicated with arrows under the nucleotide sequence. The sequence data are available from the
625 DNA Data Bank of Japan with an accession number, AB610185.

626

627 Fig. 3. Comparison of amino-acid sequence of AkAly30 with those of other PL-14 enzymes.

628 The amino-acid sequence of AkAly30 (DDBJ accession number, AB610185) was aligned with those of
629 abalone HdAly (DDBJ accession number, AB110094) and HdAlex (DDBJ accession number, AB234872)
630 and *Chlorella* virus vAL-1 (DDBJ accession number, AB044791). Identical, highly conservative, and
631 conservative residues among sequences are indicated by asterick (*), colon (:), dot (.), respectively. The
632 amino-acid residues replaced by Ala (or Phe) and His are indicated by shadowing. Regions corresponding
633 to β -sheets A (β -strands, A1-A7), B (β -strands, B1-B6), and C (β -strands, C1 and C2) in the three
634 dimensional structure of vAL-1 [38] are boxed.

635

636 Fig. 4. Analysis for expression and purification of recAkAly30 by SDS-PAGE.

637 *E. coli* cells expressing recAkAly30 were sonicated in lysis buffer and recAkAly30 was purified from the
638 supernatant of the cell lysate by Ni-NTA affinity chromatography as described in the text. Mk, molecular
639 mass markers; Bef, cells before IPTG induction; Aft, cells after IPTG induction; Soni, sonicated cells after
640 IPTG induction; Sup, supernatant of the IPTG-induced cell lysate; Ppt, precipitates of the IPTG-induced
641 cell lysate; Pass, the passed through fraction of the supernatant of the IPTG-induced cell lysate from
642 Ni-NTA column; R, recAkAly30 purified with Ni-NTA column.

643

644 Fig. 5. General properties of natAkAly30 and recAkAly30.

645 (A) Temperature dependence of natAkAly30 (●) and recAkAly30 (○) were measured at 15-65 °C in a
646 reaction mixture containing 0.15% sodium alginate, 0.15 M NaCl and 10 mM sodium phosphate (pH 6.0).
647 (B) Thermal stability was assessed by measuring the activity remaining after the heat-treatment at 15-55 °C
648 for 20 min. (C) pH dependence was measured at 30 °C in reaction mixtures adjusted to pH 3-10 with 50
649 mM sodium phosphate buffer. (D) pH stability was assessed by measuring the activity remaining after
650 30-min incubation at various pHs and 40 °C. (E) Substrate preference of natAkAly30 was determined in a
651 reaction mixture containing either the sodium alginate (○), poly(M)-rich substrate (●), poly(MG)-rich
652 substrate (▲) or poly(G)-rich substrate (Δ) in a concentration of 0.15% (w/v). Degradation of substrates
653 was monitored by measuring the increase in absorbance at 235 nm. (F) Substrate preference of recAkAly30
654 was determined as in the case of natAkAly30.

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656 Fig. 6. Thin-layer chromatography for degradation products of poly(M)-rich substrates by natAkAly30 and
657 recAkAly30.

658 Poly(M)-rich substrate (1.0% (w/v)) in 10 mM sodium phosphate buffer (pH 6.0) containing 0.1 M NaCl
659 was degraded by natAkAly30 (A and B, left) and recAkAly30 (A and B, right) at 30 °C for 12 h. Aliquots
660 of reaction products (each 2 μL) were applied to TLC-60 plate and developed with 1-butanol–acetic
661 acid–water (2:1:1, v:v:v). (A) Total sugars separated on the plate were visualized by spraying with sulfuric
662 acid in ethanol. (B) Unsaturated sugars and α-keto acid were detected by TBA staining. M, marker
663 oligosaccharides; α-ka, α-keto acid (an open chain form of 4-deoxy-5-keto-uronic acid); ΔM, unsaturated

664 disaccharide; Δ M2, unsaturated trisaccharide; Δ M3, unsaturated tetrasaccharide; Δ M4, unsaturated
665 pentasaccharide; Δ M5, unsaturated hexasaccharide.

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667 Fig. 7. SDS-PAGE for the purified AkAly30 mutants.

668 Mk, molecular mass markers; Lane 1, wild-type AkAly30; Lane 2, K99A mutant; Lane 3, N120H mutant,
669 Lane 4, S126A mutant; Lane 5, R128A mutant; Lane 6, Y140F mutant; Lane 7, Y142F mutant and Lane 8,
670 K171A mutant.

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672 Fig. 8. Predicted three-dimensional structure of AkAly30.

673 (A) The predicted three-dimensional structure of AkAly30. (B) The crystal structure of vAL-1 (PDB ID:
674 3GNE, chainA) corresponding to the region for the predicted structure of AkAly30. The β -strands, A4, A5,
675 and A6, in β -sheet A which forms the active cleft and contains catalytically important residues are indicated
676 with yellow, red and blue ribbons, respectively. The structures were drawn using RasWin Ver. 2.7.4.2
677 (<http://www.openrasmol.org/OpenrasMol.html>).

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691 Table 1. Summary for the purification of AkAly30.

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Samples	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude ^a	382	59.4	22,696	1	100
AS ^b	46.5	219.2	10,191	3.69	44.90
TOYOPEARL CM-650M ^c	0.28	5,796.4	1,623	97.58	7.15

693 ^aCrude enzyme after the dialysis against 10 mM sodium phosphate (pH 7.0).

694 ^bFraction precipitated between 60 and 90% saturation of ammonium sulfate.

695 ^cAkAly30 purified by TOYOPEARL CM-650M chromatography.

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712 Table 2. Partial amino-acid sequences of AkAly30.

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Peptide ^a	Sequences	Similarity to other enzymes ^b
T-1	GMFFSTFFGGSEK	HdAly (residues 216-228, 77%) HdAlex (residues 216-228, 77%) SP2 (residues 216-228, 77%)
L-1	WHSISEEVHINTVGK	HdAly (residues 170-184, 47%) SP2 (residues 170-184, 53%)
L-2	LPGLFGGEN	HdAly (residues 96-104, 78%) HdAlex (residues 96-104, 67%) SP2 (residues 96-104, 67%)
L-3	YDVYFENFGFGIGGK	HdAly (residues 80-95, 69%) HdAlex (residues 80-95, 69%) SP2 (residues 80-95, 53%)

714 ^aT-1 tryptic fragment; L-1 – L3, Lysylendopeptidyl fragments.

715 ^bResidue numbers for similar sequence regions in abalone HdAly, HdAlex and turban-shell SP2, and their
716 amino-acid identities (%) are shown in the parentheses.

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723 Table 3. Nucleotide sequences for PCR primers.

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Primer name	Sequences ^{a,b}
PCR	
AkAly30Fw	5'-GTNWSNACNATHYTNGGNCAYT TYAARCCNAT-3' (VSTILGHFKPI)
AkAly30Rv	5'-NCCNCCRAANARNCCNGGNA-3' (LPGLFGG)
3'-RACE	
3F	5'-GACTCCATCAGCACCAGTAC-3'
3Adapt	5'-CTGATCTAGAGGTACCGGATCC-3'
5'-RACE	
5F2	5'-TGAAGGCGCTTACAAGTGC-3'
5R2	5'-ATCCTTAGCGCTCTGGAGC-3'
Confirmation	
Full5F	5'-TCATCATCGTCGTCAAGAGCC-3'
Full3R	5'-TCACACCATTGCCAAGGC-3'

725 ^aW, adenine or thymine; S, cytosine or guanine; Y, cytosine or thymine; R, adenine or guanine; H, adenine
726 or thymine or cytosine; N, adenine or guanine or cytosine or thymine. ^bAmino-acid sequences used for
727 designing the degenerated primers are in the parentheses.

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735 Table 4. Primers used for subcloning and mutagenesis of AkAly30-cDNA.

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Primers ^a	Sequences ^b
ExFw	5' -AGATCACAT ATGGCAACAACGGTGTGG -3'
ExRv	5' -CGTTAA AGGATCCAACAAGGACCTCG -3'
K99A-F	5' -GGTATTGGCGGG GCACTGCCTGGTCTT -3'
N120H-F	5' -TCGGGCGGTT CACACCCCTCCAGTTGC -3'
S126A-F	5' -TCCAGTTGCTT CGCTCTCAGACTGATG -3'
R128A-F	5' -TGCTTCTCTCT CGCACTGATGTGGCGC -3'
Y140F-F	5' -GATGGTGAGCT CTTCGCTTACATCCC -3'
Y142F-F	5' -GAGCTCTACGCT TTTCATCCCTACCAAC -3'
K171A-F	5' -TTGGGCAGAG GAGCATTTCGATTTATG -3'

737 ^aExFw and ExRv, forward and reverse primers including NdeI and BamHI restriction sites (bold) used for
738 amplification of AkAly30 expression cDNA; K99A-F, forward primer used for mutagenesis of Lys99 to
739 Ala, for example. ^bMutation sites are indicated with bold letters.

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750 Table 5. Purification of recAkAly30.

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Preparation	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell lysate ^a	84	5,376	64	100	1
recAkAly30 ^b	0.6	1,260	2,100	23.4	33

752 ^aCell lysate was prepared from 250 mL of culture.

753 ^brecAkAly30 was purified by Ni-NTA affinity chromatography.

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773 Table 6. Specific activity of wild-type and mutant AkAly30.

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Mutant	pH 7.0		pH 10.0	
	Specific activity (U/mg)	Relative value (%)	Specific activity (U/mg)	Relative value (%)
	Wild-type	2,121	100	8
K99A	14	0.66	ND ^a	0
N120H	774.8	36.53	93	100
S126A	347.2	16.37	ND	0
R128A	12.8	0.60	ND	0
Y140F	810.9	38.23	2	2.15
Y142F	1350.4	63.67	5	5.38
K171A	1763.6	83.15	6	6.45

775 ^aNot detectable.

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Fig. 1

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Fig. 2

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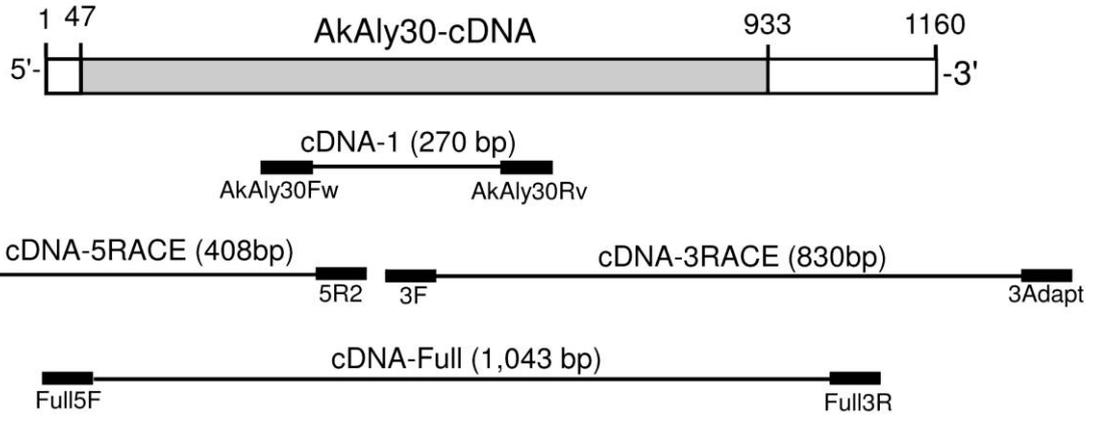
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TCATCATCGTCGTC AAGAGCCAGATCTGCGGCAGACATCGGCGATC ATGGCACGAGTTGTGAAATGGGTCTTCTTTGCA 79
 Full5F → M A R V V K W V F F A 11

CTTTTGTGGCAATCTGCAACGCCGAGGAAACGGAGGAGAGATCAAAAAGAGCAACAACGGTGTGGTCCCTGAGCTCTGTC 160
 L F V A I C N A E E T E E R S K R A T T V W S L S S V 38

N-terminus
 CCGCACTCCAGTACGTCAGTACCATCTCGGCCACTTCAAGCCCATCTACCATGACTGGGAGACGACTCCATCAGCACC 241
 P H S S H V S T I L G H F K P I Y H D W G D D S I S T 65

AGTACCAAACACTCCAGCTCCAGAGCGCTAAGGATCTTCTACGAGAAAGGCTCTATTCCAAAGTTCATGATCACAGAGGT 322
 S T K H S S S R A L R I F Y E K G S Y S K V H D H R G 92

GCCGTTTCTACAGCAGGCTAGTGCCATCTCATCCAGTGTGACGCTATGATCCTCAAGTATGACGTCTACTTCGAGAAC 403
 A G F Y S R P S A I S S S V D A M I L K Y D V Y F E N 119

L-3
 TTTGGCTTCGGTATTGGCGGAAACTGCCTGGTCTTTTTGGCGGCAGAGAATGGTGAAGGCGCTTACAAGTGCCTGGGCGGT 484
 F G F G I G G K L P G L F G G E N G E G A Y K C S G G 146

L-3 L-2
 TCAAACCCCTCAGTTGCTTCTCTCTCAGACTGATGTGGCGCAAGGACGGAGATGGTGAAGTCTACGCTTACATCCCTACC 565
 S N P S S C F S L R L M W R K D G D G E L Y A Y I P T 173

AACCAGGAAAGCGGATTTAAAGATCGCGATGACGTCATTGCCACAGCACTTACGGGCAGTCTTTGGGCAGAGGAAAATTT 646
 N Q E S G F K D R D D V I A H S T Y G Q S L G R G K F 200

CGATTTATGAATAACAAGTGGCACTCAATCTCGGAGGAGGTTACATCAACACCGTGGGCAAAACAGACGGTGGGTCAAA 727
 R F M N N K W H S I S E E V H I N T V G K T D G W V K 227

L-1
 ATCTGTGTCCAGGCGGAGGCCATTCCAGCAATGCTACACGGCAACCACTTGGCATGGCAATACCAACAGCCATCAT 808
 I C V Q A E G H S Q Q C Y T A N H L R M R N T N S H H 254

CTCCGAGGCATGTTCTTCTCCACCTTCTTCGGGGCAGTGAGAAAAGTTACGCGGCGCCAACGACTGTTACTCTTACTTC 889
 L R G M F F S T F F G G S E K S Y A A P N D C Y S Y F 281

T-1
 AAGAACTTCCAGATCTTAACTCCTTCTCACGAGTTGTTGGT TAGCTTAAGTGGTTTTACCAAGAAGTAAAAAAAACG 970
 K N F Q I L T P S H A V V G * 295

AGGTCCTTGTGGTTCAITTAACGCACTGTGTGTGTTGTGTTTATCGTAAATGCCTTGGCAAATGGTGTGAGCCTTGGC 1051
 Full3R ← AATAAA

AAATGGTGTGAAATTACATTTTAAAAATTCTTAATTAATTGTTCTTGAAGAAAAGTCATGA AATAAATGGTTTCACAAAAT 1132

TGCAGCATAACCTGAAAAAAAAAAAAA 1160

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Fig. 3

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817 AkAly30 1 ATT**B-1**VWSLSSVPHSSHVSTILGH**A-1**FKPIYHDWGDD**C-1**ISTST**C-2**KHSSSRALRIFYEKGSYSKVH 60
 HdAly 1 AVLWTHKEFDPA-NYRNGMHALTSNDYDHGSSGVVTD**C-1**DGGSNHVLRVWY**C-2**EKGRYSSHG 58
 HdAlex 1 SI**B-1**VWTHNEFDPA-YFRNGMHS**A-1**PVTD**C-1**EDVNGSATVVPD**C-2**NGGSNLVLKVFY**C-2**EKGSYSHHG 58
 SP2 1 TILWTHKEFDPN-NYRDGMHALTSNDYDHGSGKV**C-1**VTD**C-2**DGGSNHVLRVWY**C-2**EKGRWSSHG 58
 Val-1 106 TNV**B-1**ISTLDLNL-LTKGGGSW**A-1**VDG---V**C-1**NMKKS**C-2**AVT**C-2**FDG--**C-2**KRVVKAVYDKNSGTSAN 158
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820 AkAly30 DHRG**A-2**-AGFYSRPSA**B-3**ISSV**A-4**DAMILKYDVYFEN-FGF**K99**GIC**A-4**GKLPGLFGGENEGAYKCSGG 118
 HdAly PNEG**A-2**-VQFFATPT---QDHS**B-3**IMTFSYDVYFDKN**A-4**DFR**A-4**GGKLPGLFGG-----WTNCSSGG 109
 HdAlex PNSK**A-2**-VQFFATPT---KPRV**B-3**AMTLSYDV**A-4**FDPN**A-4**DFR**A-4**GGKLPGLYGG-----LVNCSSGG 109
 SP2 PNEG**A-2**-VQFFATPT---QDHS**B-3**VMTFSYDLYLSHDF**A-4**DFR**A-4**GGKLPGLYGG-----WTNCSSGG 109
 vAL-1 PGV**A-2**GG**A-2**FSFS**B-3**VDPD**A-4**--LNK**A-4**NAITFAWE**A-4**VYPKGFDFAR**A-4**GKHGG**A-4**TEIG-----HGAASGY 211
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823 AkAly30 SNPSS**N120**CFSLRLM**A-5**WRKDG**R128**IGELY**A-6**YAIPTNQESGFK-DRDDVIAHST**A-7**YGG**K171**SLGRGKFRFMNN 177
 HdAly R**A-5**HS**A-5**DNC**A-6**CFSTR**A-6**FMWRAD**A-6**IG**A-6**EVYGYIQ-NKDHQIDGFCDHVVCNSIKGY**A-7**SMGRGKWR**A-7**FQ**A-7**RG 168
 HdAlex R**A-5**SD**A-5**DC**A-6**CFSTR**A-6**FMWRD**A-6**NGD**A-6**IG**A-6**EVYGYVP-DQSHQLPGFCTKNICDPV**A-7**KGF**A-7**SFGRGKWR**A-7**FQ**A-7**RG 168
 SP2 R**A-5**SD**A-5**DC**A-6**CFSTR**A-6**FMWRK**A-6**DG**A-6**IG**A-6**EVY**A-6**YAI**A-6**P-DYHHQVSGFCDHNV**A-7**CNSV**A-7**KGY**A-7**SLGRGK**A-7**W**A-7**K**A-7**FER**A-7**G 168
 vAL-1 Q**A-5**HS**A-5**KT**A-6**GA**A-6**SN**A-6**R**A-6**IM**A-6**W**A-6**Q**A-6**E**A-6**KG**A-6**GV**A-6**IDY**A-6**IM**A-6**PP**A-6**SD**A-6**L**A-6**K**A-6**Q**A-6**K**A-6**IP**A-6**GLD**A-6**PE**A-6**GHG---**A-7**L**A-7**GF**A-7**F**A-7**Q**A-7**DD**A-7**FK**A-7**N**A-7**AL**A-7**K**A-7**Y**A-7**D 268
 : . . S**A-5**126 * : * : . * . * * : . . * . . : .

826 AkAly30 KW**B-4**HSISEE**B-5**VE**B-6**INTV**A-3**GKT**B-5**DG**B-6**W**B-6**V**A-3**K**B-6**IC**B-6**V**B-6**Q**B-6**AE**B-6**GH**A-3**S**B-6**Q**B-6**Q**B-6**C**B-6**Y**B-6**T**B-6**AN**B-6**HL**B-6**RM**B-6**R**B-6**NT**B-6**NS**B-6**SH**B-6**HL**B-6**RG**B-6**MF**B-6**FF**B-6**ST**B-6**FF**B-6**G 237
 HdAly KW**B-4**Q**B-4**NI**B-4**A**B-4**Q**B-4**S**B-4**V**B-4**K**B-4**L**B-4**NT**B-4**PG----K**B-5**TD**B-5**GS**B-5**IK**B-5**V**B-5**W**B-5**Y**B-5**NG**B-5**KL**B-5**V**B-5**FT**B-5**ID**B-5**Q**B-5**L**B-5**NI**B-5**RA**B-5**K**B-5**AS**B-5**VD**B-5**LD**B-5**G**B-5**IF**B-5**FF**B-5**ST**B-5**FF**B-5**G 224
 HdAlex VW**B-4**Q**B-4**T**B-4**IA**B-4**Q**B-4**S**B-4**IK**B-4**L**B-4**NT**B-4**PG----S**B-5**TD**B-5**GA**B-5**IK**B-5**V**B-5**V**B-5**Y**B-5**NG**B-5**K**B-5**V**B-5**Y**B-5**AS**B-5**NN**B-5**IAL**B-5**RS**B-5**Q**B-5**SD**B-5**V**B-5**NI**B-5**D**B-5**G**B-5**IF**B-5**FF**B-5**ST**B-5**FF**B-5**G 224
 SP2 KW**B-4**Q**B-4**NI**B-4**A**B-4**Q**B-4**H**B-4**V**B-4**L**B-4**NT**B-4**PG----K**B-5**TD**B-5**GS**B-5**IK**B-5**V**B-5**W**B-5**H**B-5**NG**B-5**KL**B-5**V**B-5**Y**B-5**T**B-5**ID**B-5**Q**B-5**L**B-5**NI**B-5**V**B-5**SK**B-5**AS**B-5**VD**B-5**ID**B-5**G**B-5**IF**B-5**FF**B-5**ST**B-5**FF**B-5**G 224
 vAL-1 V**B-4**W**B-4**NR**B-4**IE**B-4**IG**B-4**TR**B-4**IM**B-4**NT**B-4**FK**B-4**NG**B-4**IP**B-4**QL**B-4**D**B-4**GES**B-4**Y**B-4**V**B-4**TV**B-4**NG**B-4**KE**B-4**-**B-6**VL**B-6**K**B-6**R**B-6**IN**B-6**WS**B-6**RS**B-6**P**B-6**DL**B-6**LL**B-6**IS**B-6**RF**B-6**D**B-6**W**B-6**NT**B-6**FF**B-6**G 327
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829 AkAly30 GSEKSYA**B-2**APND**B-2**CYSY**B-2**FK**B-2**N**B-2**F**B-2**Q**B-2**IL**B-2**TPSHAVVG 267
 HdAly GHDSTWAP**B-2**TH**B-2**CYSY**B-2**FK**B-2**N**B-2**F**B-2**V**B-2**L**B-2**STDSGHPTIIG 256
 HdAlex GSYANWAP**B-2**TR**B-2**CY**B-2**T**B-2**W**B-2**FK**B-2**N**B-2**F**B-2**A**B-2**IS**B-2**FD**B-2**T**B-2**G**B-2**PE**B-2**V**B-2**AV**B-2**G 256
 SP2 GSDSSWAP**B-2**TH**B-2**CYSY**B-2**FK**B-2**N**B-2**F**B-2**AL**B-2**STDSSHP**B-2**T**B-2**IL 255
 vAL-1 G---PLPSPKN**B-2**OV**B-2**AY**B-2**FT**B-2**N**B-2**F**B-2**OM**B-2**K**B-2**Y**B-2**E**B-2**L 351
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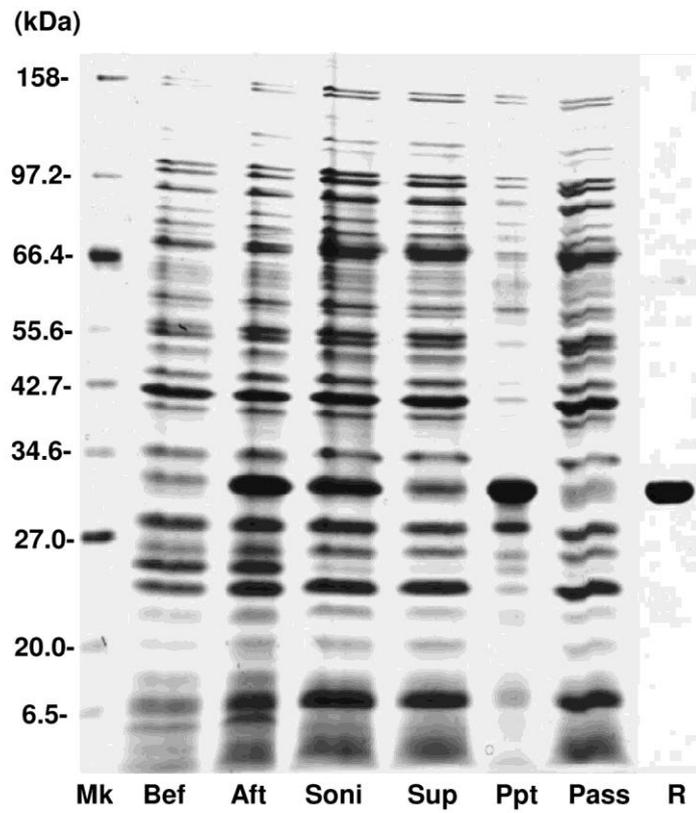
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Fig. 4



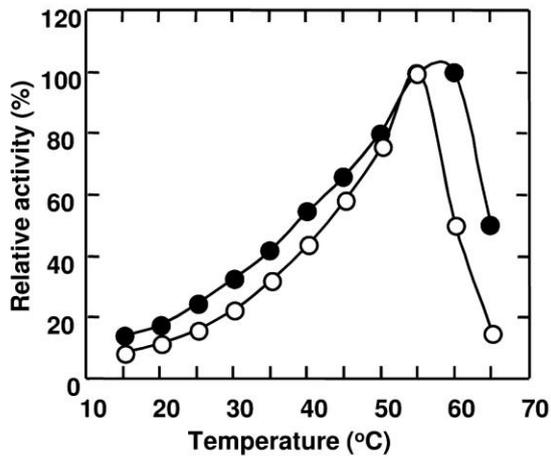
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Fig. 5

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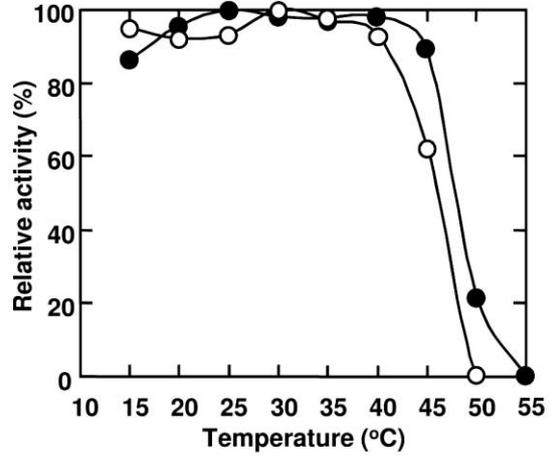
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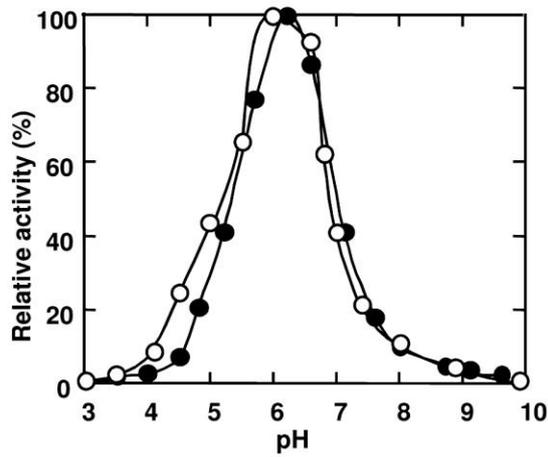
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(C)



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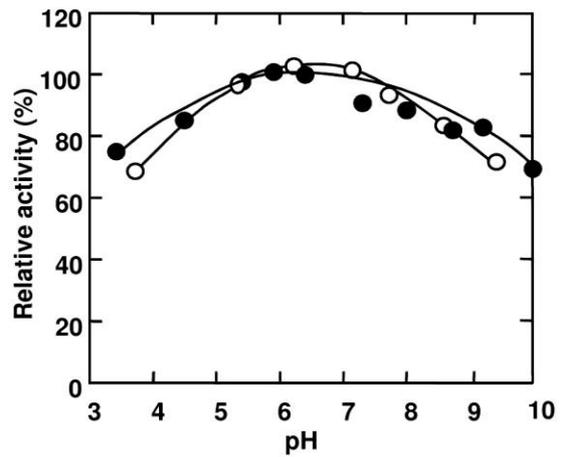
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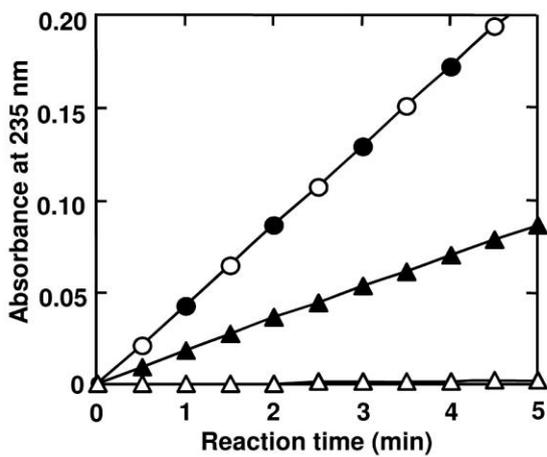
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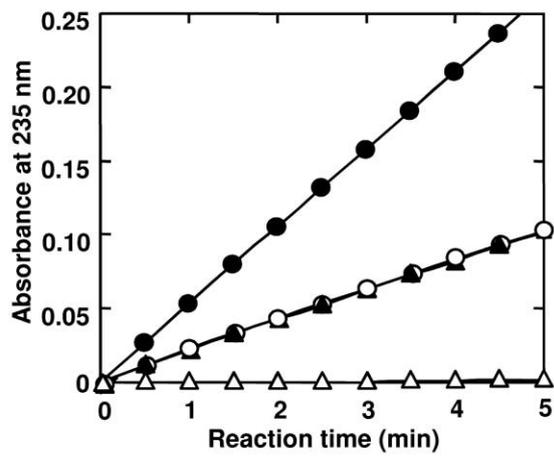
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897 Fig. 6

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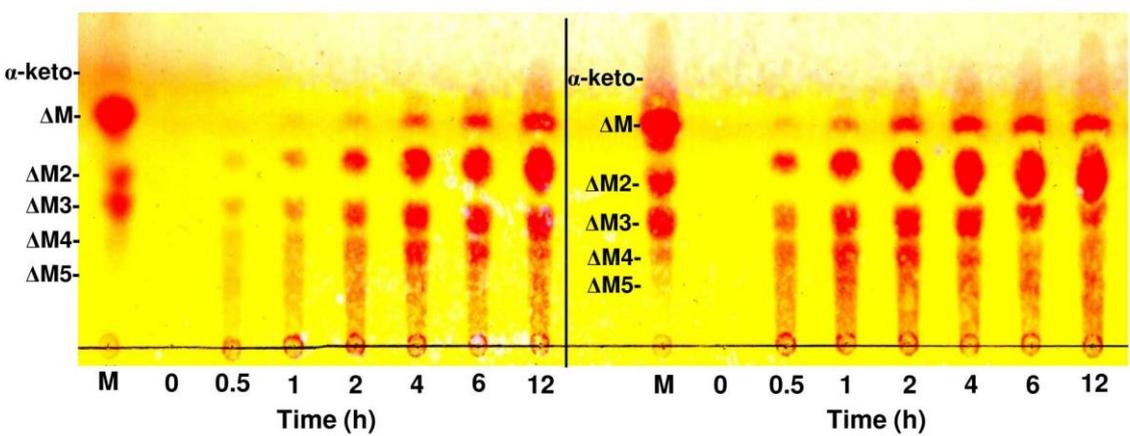
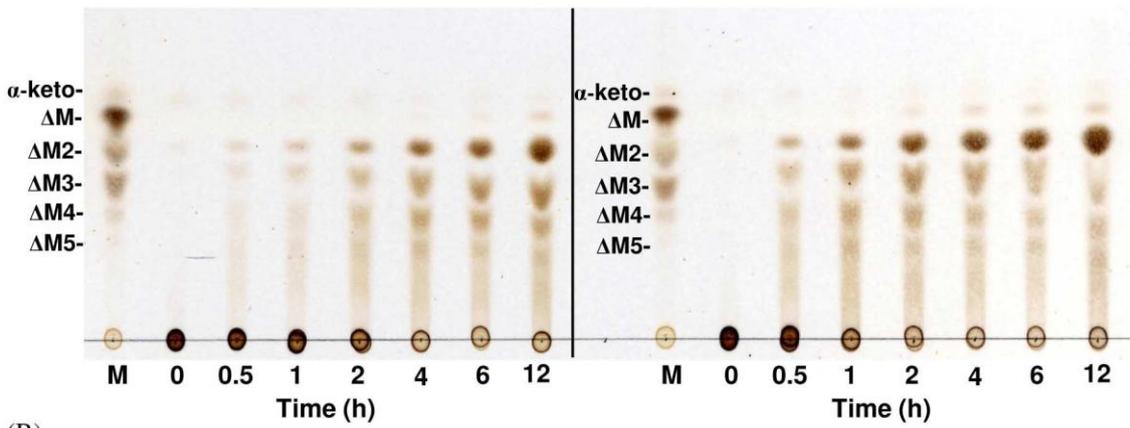
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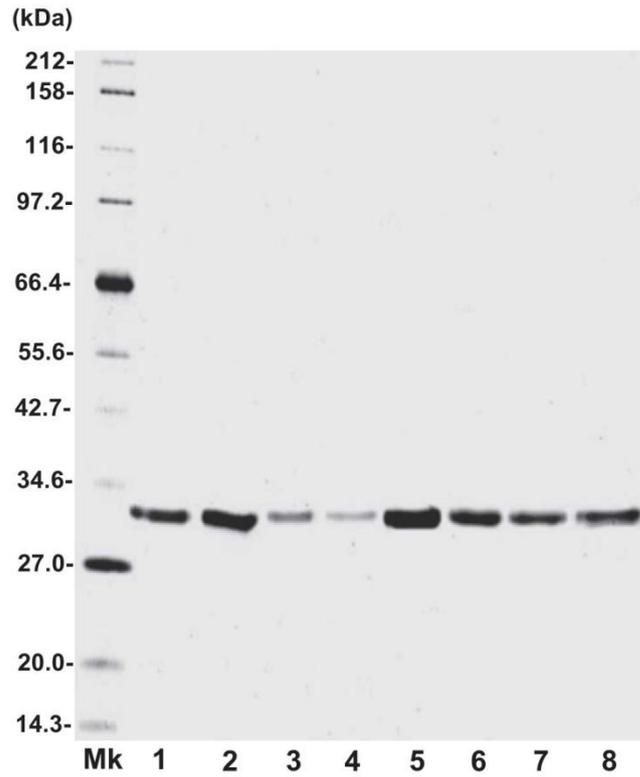
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923 **Fig.7**

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950 Fig. 8

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