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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	https://hdl.handle.net/2115/47072
Type	journal article
File Information	AkAly30 (HUSCAP).pdf

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

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Abstract

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

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Isolation of an alginate lyase AkAly30

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

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

2.3. Assay for alginate lyase activity

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

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

2.4. Thin-layer chromatography

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

2.5. SDS-PAGE

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

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*

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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

of dATP, dTTP, dGTP, and dCTP, 2.5 mM $MgCl_2$, and 5 pmol/μL primers, 1 ng/μL template DNA, and 0.5 units AmpliTaq Gold DNA polymerase. A successive reaction at 94 ^{o}C for 20 s, 55 ^{o}C for 20 s and 72 ^{o}C for 45 s was repeated for 30 cycles with Thermal Cycler Dice mini (TaKaRa, Tokyo, Japan). cDNAs derived from 5'- and 3'-terminal regions of mRNA were amplified with 5'- and 3'-Full RACE kits (TaKaRa), respectively. The size of cDNA was estimated by 1.2% agarose-gel electrophoresis. The PCR products were cloned with a TA PCR cloning kit (pTAC-1) (Invitrogen, CA, USA), and nucleotide sequences of the cDNAs were analyzed with a BigDye-terminator Cycle sequencing kit (Applied Biosystems) and an ABI 3130 xl Genetic Analyzer (Applied Biosystems).

2.9. Production of recombinant AkAly30

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

dialyzed against 10 mM sodium phosphate (pH 6.0) and stored at 4 ^{o}C until use.

2.10. Site-directed mutagenesis

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

2.11. Prediction of three-dimensional structure of AkAly30

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

3. Results

3.1. cDNA encoding AkAly30

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

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

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

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

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

3.2. Production of recombinant AkAly30

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

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

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

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

3.3. Assessment of catalytically important residues of AkAly30

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

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

3.4. Prediction of three-dimensional structure of AkAly30

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

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

4. Discussion

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

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

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

In *Chlorella* virus enzyme vAL-1, the active site was shown to span K197-Y235 which involves three β-strands (A4, A5, and A6) [38]. These segments organize the active cleft in the three-dimensional structure of this enzyme. Among the amino-acid residues extruded toward the active cleft, K197, S219, R221, Y233 and Y235 play key roles for the catalytic action and/or substrate binding of this enzyme [38]. These residues appeared to be conserved as K99, S126, R128, Y140 and Y142 in AkAly30, and replacements of these residues by Ala and/or Phe indeed greatly decreased the activity of AkAly30. Among these residues, K99 and R128, which correspond to K95 and R119 of abalone HdAly, have been shown to be crucially important with the site-directed mutants of HdAly produced with the cold-inducible yeast expression system [46]. Namely, replacement of K95 and R119 in HdAly by Ala greatly reduced the activity [46]. The other three catalytically important residues in vAL-1, i.e., S219, Y233 and Y235, were found to be conserved as S126, Y140 and Y142 for AkAy30. The replacement of these residues by Ala and/or Phe also greatly decreased the activity of AkAly30. These facts indicate that the catalytically important residues are commonly conserved in *Chlorella* virus and gastropod enzymes.

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

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

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

Acknowledgements

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

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Legends to figures

Fig. 1. Schematic structure of AkAly30-cDNA.

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

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

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

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

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

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 ^{o}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 ^{o}C
648 for 20 min. (C) pH dependence was measured at 30 ^{o}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 ^{o}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.
655
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 ^{o}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

disaccharide; ΔM2, unsaturated trisaccharide; ΔM3, unsaturated tetrasaccharide; ΔM4, unsaturated pentasaccharide; ΔM5, unsaturated hexasaccharide.

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

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

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

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

691 Table 1. Summary for the purification of AkAly30.

692

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 [a]Crude enzyme after the dialysis against 10 mM sodium phosphate (pH 7.0).

694 [b]Fraction precipitated between 60 and 90% saturation of ammonium sulfate.

695 [c]AkAly30 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 [a]T-1 tryptic fragment; L-1 – L3, Lysylendopeptidyl fragments.

715 [b]Residue 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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Table 3. Nucleotide sequences for PCR primers.

Primer name	Sequences[a,b]
PCR	
AkAly30Fw	5′-GTNWSNACNATHYTNGGNCAYTTYAARCCNAT-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′-TCACACCATTTGCCAAGGC-3′

[a]W, adenine or thymine; S, cytosine or guanine; Y, cytosine or thymine; R, adenine or guanine; H, adenine or thymine or cytosine; N, adenine or guanine or cytosine or thymine. [b]Amino-acid sequences used for designing the degenerated primers are in the parentheses.

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

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Primers[a]	Sequences[b]
ExFw	5′-AGATCA**CATATG**GCAACAACGGTGTGG-3′
ExRv	5′-CGTTAAA**GGATCC**AACAAGGACCTCG-3′
K99A-F	5′-GGTATTGGCGGG**GCA**CTGCCTGGTCTT-3′
N120H-F	5′-TCGGGCGGTTCA**CAC**CCCTCCAGTTGC-3′
S126A-F	5′-TCCAGTTGCTTC**GCT**CTCAGACTGATG-3′
R128A-F	5′-TGCTTCTCTCTC**GCA**CTGATGTGGCGC-3′
Y140F-F	5′-GATGGTGAGCTC**TTC**GCTTACATCCC-3′
Y142F-F	5′-GAGCTCTACGCT**TTC**ATCCCTACCAAC-3′
K171A-F	5′-TTGGGCAGAGGA**GCA**TTTCGATTTATG-3′

737 [a]ExFw 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. [b]Mutation sites are indicated with bold letters.

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Table 5. Purification of recAkAly30.

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

[a]Cell lysate was prepared from 250 mL of culture.

[b]recAkAly30 was purified by Ni-NTA affinity chromatography.

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

	pH 7.0		pH 10.0	
Mutant	Specific activity (U/mg)	Relative value (%)	Specific activity (U/mg)	Relative value (%)
Wild-type	2,121	100	8	8.60
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

[a]Not detectable.

Fig. 1

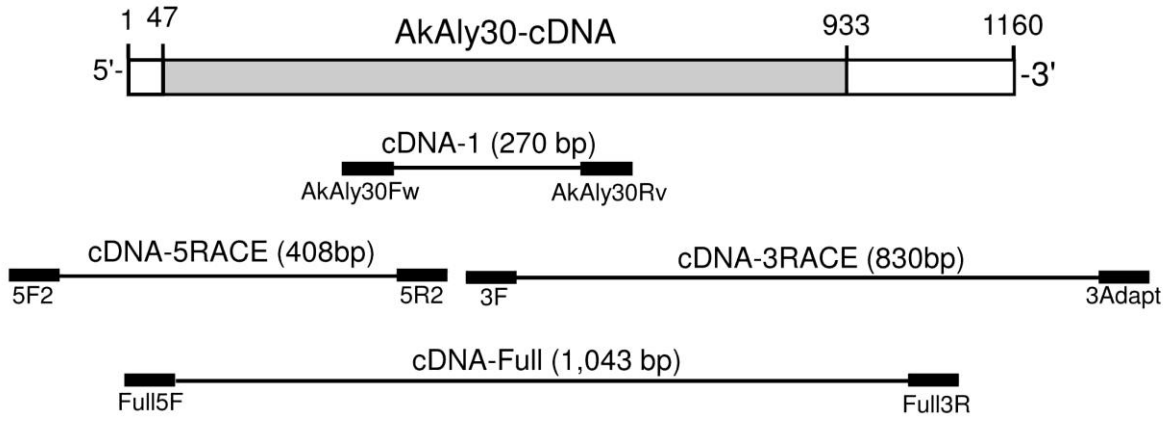


Fig. 2

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TCATCATCGTCGTCAAGAGCCAGATCTGCGGCAGACATCGGCGATCATGGCACGAGTTGTGAAATGGGTCTTCTTTGCA    79
Full5F                                          M  A  R  V  V  K  W  V  F  F  A    11

CTTTTTGTGGCAATCTGCAACGCCGAGGAAACGGAGGAGAGATCAAAAAGAGCAACAACGGTGTGGTCCCTGAGCTCTGTC   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
CCGCACTCCAGTCACGTGAGTACCATCCTCGGCCACTTCAAGCCCATCTACCATGACTGGGGAGACGACTCCATCAGCACC   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

AGTACCAAACACTCCAGCTCCAGAGCGCTAAGGATCTTCTACGAGAAAGGCTCCTATTCCAAAGTTCATGATCACAGAGGT   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

GCCGGTTTCTACAGCAGGCCTAGTGCCATCTCATCCAGTGTTGACGCTATGATCCTCAAGTATGACGTCTACTTCGAGAAC   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
TTTGGCTTCGGTATTGGCGGGAAACTGCCTGGTCTTTTTGGCGGCGAGAATGGTGAAGGCGCTTACAAGTGCTCGGGCGGT   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
TCAAACCCCTCCAGTTGCTTCTCTCTCAGACTGATGTGGCGCAAGGACGGAGATGGTGAGCTCTACGCTTACATCCCTACC   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

AACCAGGAAAGCGGATTTAAAGATCGCGATGACGTCATTGCCCACAGCACTTACGGGCAGTCTTTGGGCAGAGGAAAATTT   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

CGATTTATGAATAACAAGTGGCACTCAATCTCGGAGGAGGTTCACATCAACACCGTGGGCAAAACAGACGGCTGGGTCAAA   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
ATCTGTGTCCAGGCGGAGGGCCATTCCCAGCAATGCTACACGGCCAACCACTTGCGCATGCGCAATACCAACAGCCATCAT   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

CTCCGAGGCATGTTCTTCTCCACCTTCTTCGGGGGCAGTGAGAAAAGTTACGCGGCGCCCAACGACTGTTACTCTTACTTC   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
AAGAACTTCCAGATCTTAACTCCTTCTCACGCAGTTGTTGGTTAGGCTTAAGTGGTTTTACCAAGAAGTAAAAAAAAAACG   970
K  N  F  Q  I  L  T  P  S  H  A  V  V  G  *                                       295

AGGTCCTTGTTGGTTCATTTAACGCACTGTGTGTGTTGTGTGTTTATCGTAAATGCCTTGGCAAATGGTGTGAGCCTTGGC  1051
                                                Full3R
AAATGGTGTGAAATTACATTTTAAAAATTCTTAATTAATTGTTCTTGAAGAAAAGTCATGAAATAAATGGTTTCACAAAAT  1132

TGCAGCATAACCTGAAAAAAAAAAAAAA                                                       1160
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Fig. 3

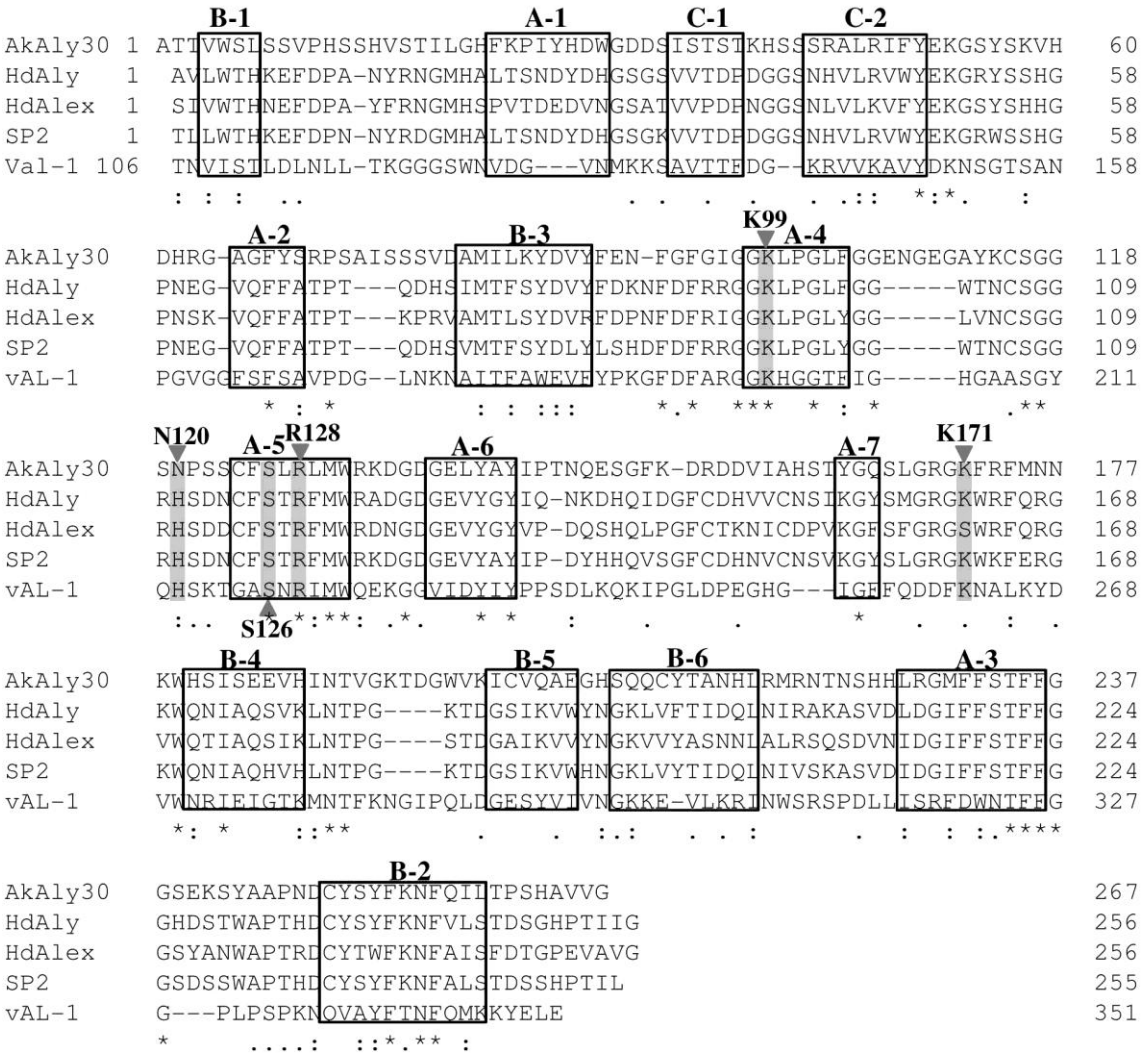

B-1
A-1
C-1
C-2
AkAly30 1 ATTVWSLSSVPHSSHVSTILGHFKPIYHDWGDDSISTSTKHSSSRALRIFYEKGSYSKVH 60
HdAly 1 AVLWTHKEFDPA-NYRNGMHALTSNDYDHGSGSVVTDPDGGSNHVLRVWYEKGRYSSHG 58
HdAlex 1 SIVWTHNEFDPA-YFRNGMHSPVTDEDVNGSATVVPDPNGGSNLVLKVFYEKGSYSHHG 58
SP2 1 TLLWTHKEFDPN-NYRDGMHALTSNDYDHGSGKVVTDPDGGSNHVLRVWYEKGRWSSHG 58
Val-1 106 TNVISTLDLNLL-TKGGGSWNVDG---VNMKKSAVTTPDG--KRVVKAVYDKNSGTSAN 158
A-2
B-3
K99
A-4
AkAly30 DHRG-AGFYSRPSAISSSVDAMILKYDVYFEN-FGFGIGGKLPGLFGGENGEGAYKCSGG 118
HdAly PNEG-VQFFATPT---QDHSIMTFSYDVYFDKNFDFRRGGKLPGLFGG-----WTNCSGG 109
HdAlex PNSK-VQFFATPT---KPRVAMTLSYDVPFDPNFDFRIGGKLPGLYGG-----LVNCSGG 109
SP2 PNEG-VQFFATPT---QDHSVMTFSYDLYLSHDFDFRRGGKLPGLYGG-----WTNCSGG 109
vAL-1 PGVGGFSFSAVPDG--LNKNAITFAWEVFYPKGFDFARGGKHGGTFIG-----HGAASGY 211
N120
A-5
R128
A-6
A-7
K171
AkAly30 SNPSSCFSLRLMWRKDGDGELYAYIPTNQESGFK-DRDDVIAHSIYGQSLGRGKFRFMNN 177
HdAly RHSDNCFSTRFMWRADGDGEVYGYIQ-NKDHQIDGFCDHVVCNSIKGYSMGRGKWRFQRG 168
HdAlex RHSDDCFSTRFMWRDNGDGEVYGYVP-DQSHQLPGFCTKNICDPVKGFSFGRGSWRFQRG 168
SP2 RHSDNCFSTRFMWRKDGDGEVYAYIP-DYHHQVSGFCDHNVCNSVKGYSLGRGKWKFERG 168
vAL-1 QHSKTGASNRIMWQEKGGVIDYIYPPSDLKQKIPGLDPEGHG---IGFFQDDFKNALKYD 268
S126
B-4
B-5
B-6
A-3
AkAly30 KWHSISEEVHINTVGKTDGWVKICVQAEGHSQQCYTANHLRMRNTNSHHLRGMFFSTFFG 237
HdAly KWQNIAQSVKLNTPG----KTDGSIKVWYNGKLVFTIDQLNIRAKASVDLDGIFFSTFFG 224
HdAlex VWQTIAQSIKLNTPG----STDGAIKVVYNGKVVYASNNLALRSQSDVNIDGIFFSTFFG 224
SP2 KWQNIAQHVHLNTPG----KTDGSIKVWHNGKLVYTIDQLNIVSKASVDIDGIFFSTFFG 224
vAL-1 VWNRIEIGTKMNTFKNGIPQLDGESYVIVNGKKE-VLKRINWSRSPDLLISRFDWNTFFG 327
B-2
AkAly30 GSEKSYAAPNDCYSYFKNFQILTPSHAVVG 267
HdAly GHDSTWAPTHDCYSYFKNFVLSTDSGHPTIIG 256
HdAlex GSYANWAPTRDCYTWFKNFAISFDTGPEVAVG 256
SP2 GSDSSWAPTHDCYSYFKNFALSTDSSHPTIL 255
vAL-1 G---PLPSPKNQVAYFTNFQMKKYELE 351

Fig. 4

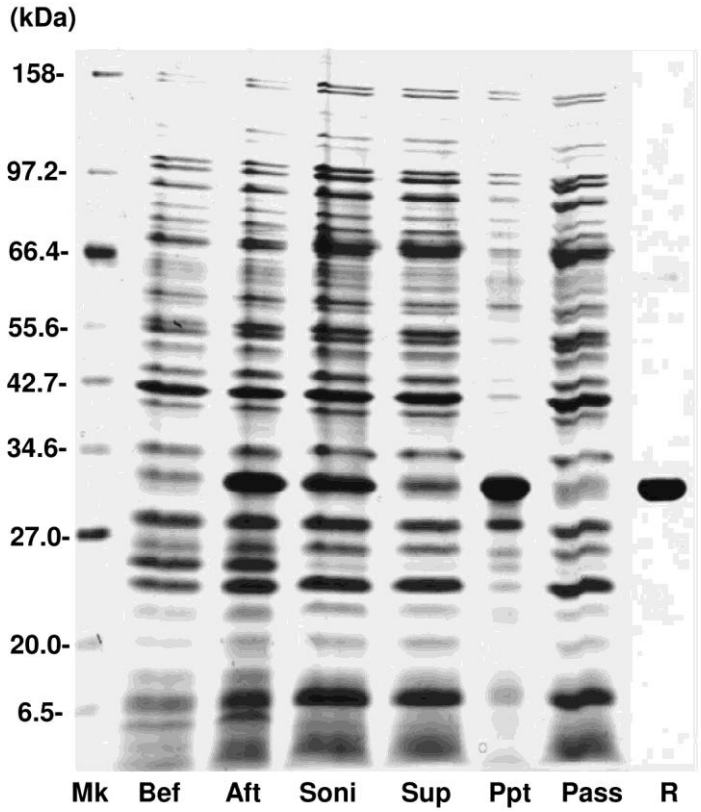

(kDa)
158-
97.2-
66.4-
55.6-
42.7-
34.6-
27.0-
20.0-
6.5-
Mk
Bef
Aft
Soni
Sup
Ppt
Pass
R

Fig. 5

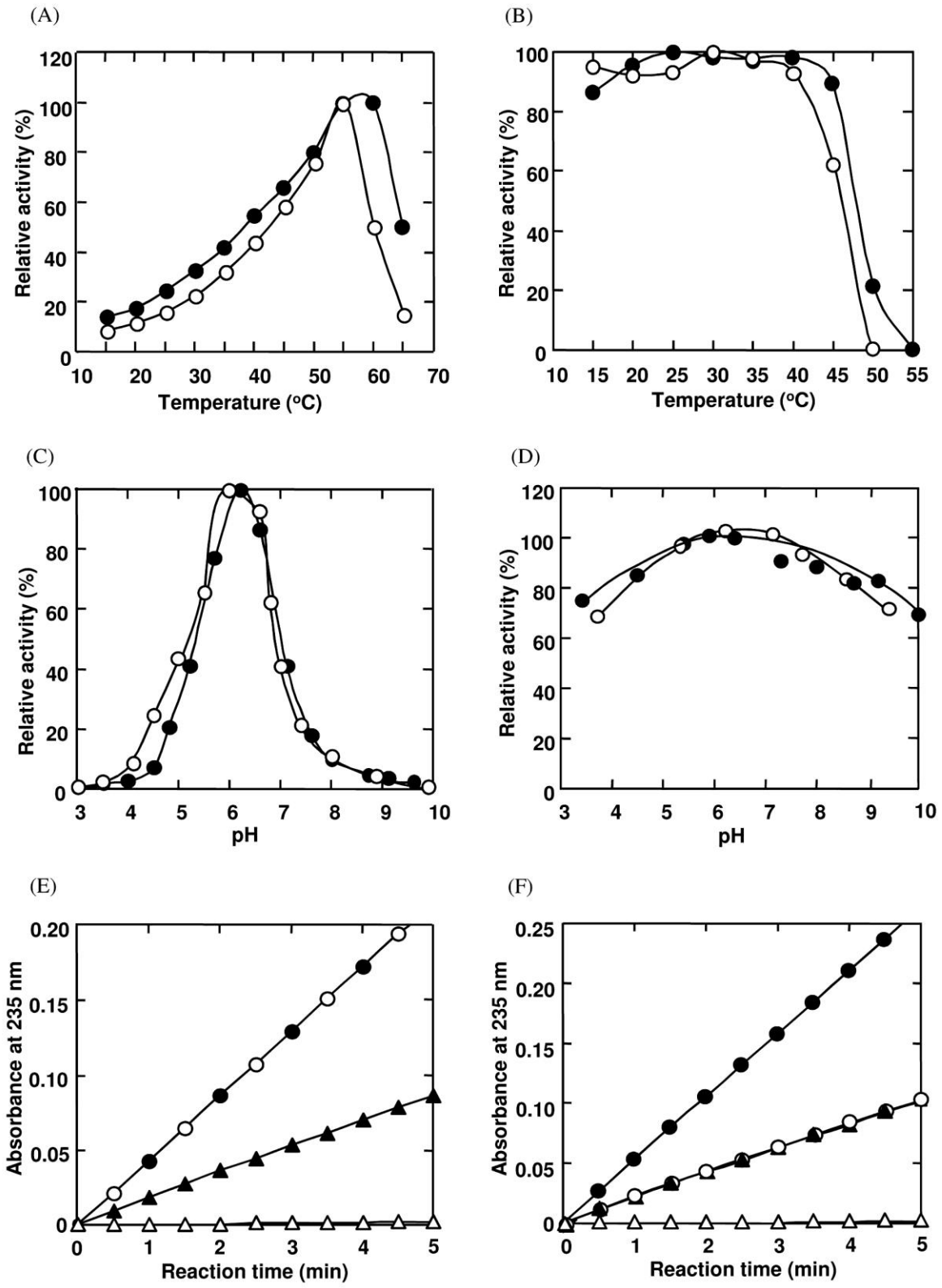

(A)
Relative activity (%)
Temperature (°C)
(B)
Relative activity (%)
Temperature (°C)
(C)
Relative activity (%)
pH
(D)
Relative activity (%)
pH
(E)
Absorbance at 235 nm
Reaction time (min)
(F)
Absorbance at 235 nm
Reaction time (min)

Fig. 6

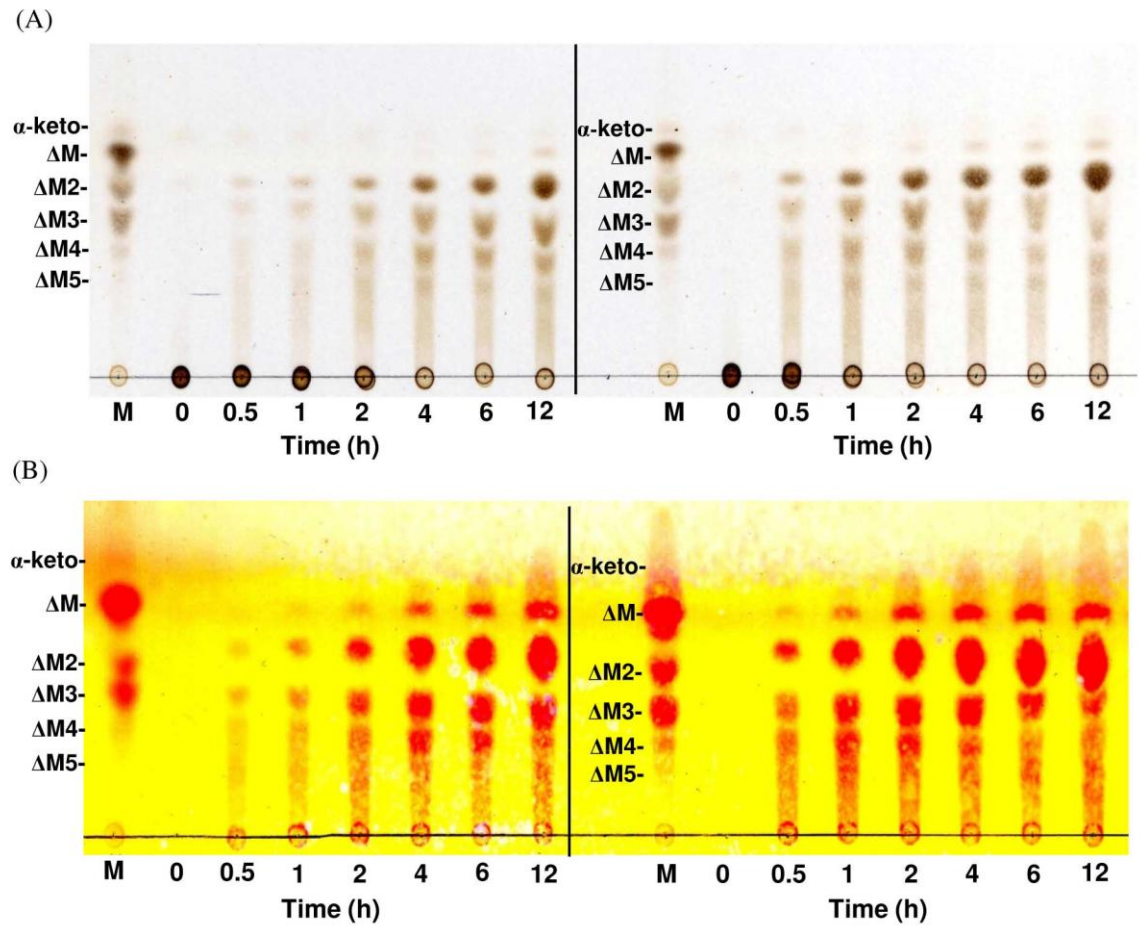

(A)
α-keto-
ΔM-
ΔM2-
ΔM3-
ΔM4-
ΔM5-
M 0 0.5 1 2 4 6 12
Time (h)
(B)
α-keto-
ΔM-
ΔM2-
ΔM3-
ΔM4-
ΔM5-
M 0 0.5 1 2 4 6 12
Time (h)

Fig.7

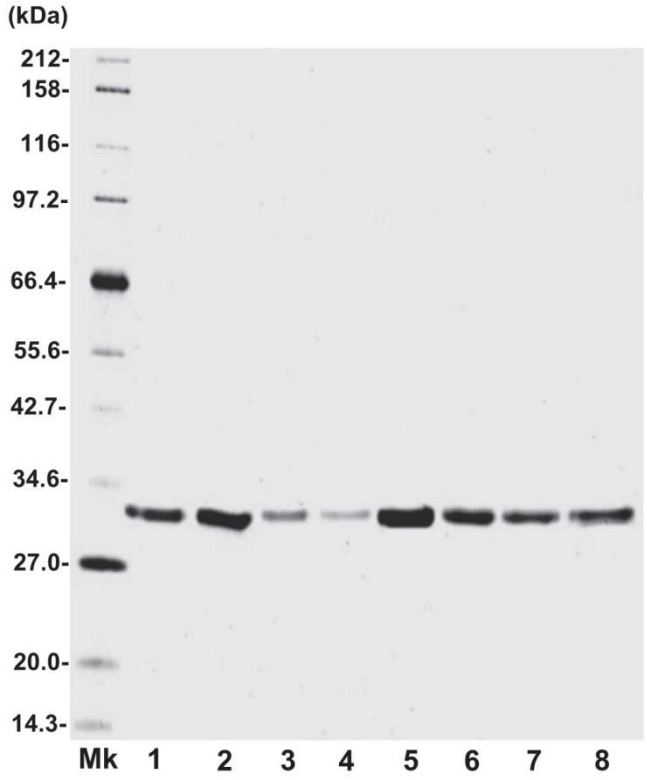
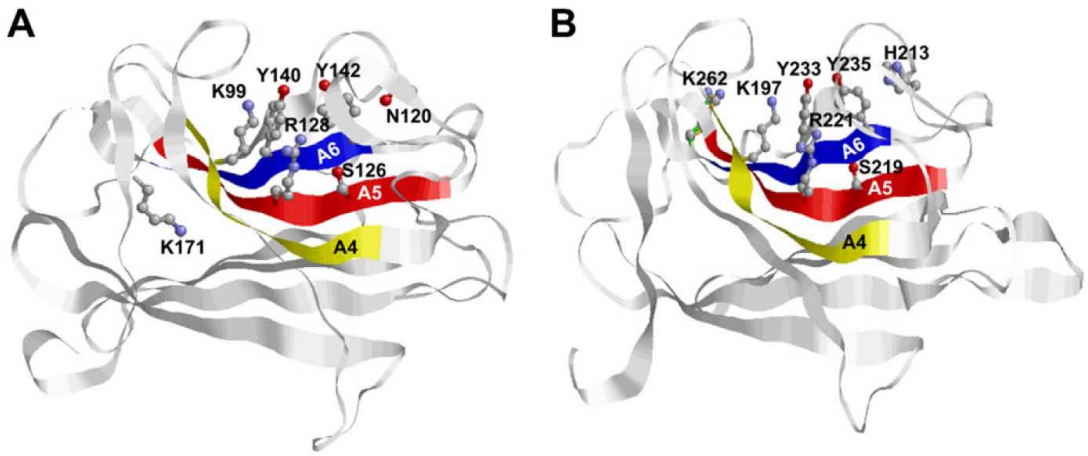


Fig. 8


A
K99
Y140
Y142
N120
R128
A6
S126
A5
K171
A4
B
K262
K197
Y233
Y235
H213
R221
A6
S219
A5
A4