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Instructions for use

Catalytically Important Amino-Acid Residues of Abalone Alginate Lyase HdAly

Assessed by Site-directed Mutagenesis

Abbreviated title: Catalytically important residues of abalone alginate lyase

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## 1. Introduction

Alginate is an anionic heteropolysaccharide comprised of β-D-mannuroate (M) and α-L-guluronate (G) [1-3]. These uronate units are arranged as a poly(M)-block, a poly(G)-block, and an alternating or random poly(MG)-block. Alginate is found in inter-cellular matrices of brown algae and in biofilms of certain bacteria [1-4]. Alginate extracted from brown algae is used in a variety of industrial and food materials because of its unique physical properties i.e., the sodium salt solution exhibits high viscosity and the calcium salt forms elastic gel [2,5]. Alginate oligosaccharides have also attracted scientific interest because they exhibit various biological activities, i.e., promoting root growth in higher plants [6-8], increasing a growth rate of Bifidobacterium sp. [9], and inducing production of cytotoxic cytokines in human mononuclear cells [10]. Alginate oligosaccharides have been produced by enzymatic degradation using the alginate-degrading enzyme, alginate lyase.

Alginate lyase splits the glycoside linkages of alginate chains via β-elimination to produce oligosaccharides possessing an unsaturated sugar, 4-deoxy-L-erythro-hex-4-enopyranosyluronate, at the new non-reducing terminus [2-4]. Alginate lyases are found in brown algae [11,12], marine mollusks [13-17], *Chlorella* virus [18] and various soil and marine bacteria [4,19-21], and have been classified as poly(M) lyases (EC 4.2.2.3) and poly(G) lyases (EC 4.2.2.11), which preferentially degrade the poly(M)-block and poly(G)-block, respectively. Recently, bifunctional alginate lyases which act on both poly(M) and poly(G) blocks have also been isolated [20,22]. Alginate lyases from herbivorous marine mollusks such as abalone and turban shell are poly(M) lyases. Most alginate lyases are endolytic although a few exolytic enzymes have also been isolated from *Sphingomonas* sp. [23] and abalone [17]. Alginate

lyases are useful not only for production of alginate oligosaccharides but also for production of protoplasts from brown algae because alginate is a major constituent of the cell wall and intercellular matrices in this organism [14,24-27].

Currently, polysaccharide lyases are classified in 18 families (PL-1-18) on the basis of hydrophobic-cluster analysis of primary structure (http://www.cazy.org/). Alginate lyases are grouped among seven families, i.e., PL-5, -6, -7, -14, -15, -17, and -18. Three-dimensional structures of PL-5 (*Sphingomonas* sp. A1-III) [28,29], PL-7 (*Corynebacterium* sp. ALY-1) [30,31], and PL-18 enzymes (*Alteromonas* sp. 272 alginate lyase) (Motoshima et al., Protein Data Bank: 1J1T) have been solved and the structural motifs of the former two were named $\alpha 6/\alpha 5$ barrel and $\beta$-sandwich (or $\beta$-jelly roll), respectively. Tyr, His, Asn (Gln), and Arg were found to be arranged in almost identical spacial configuration in the catalytic sites of the PL-5 and -7 enzymes [30] and were suggested to participate in catalysis.

Structure/function analysis of molluscan alginate lyases has not kept pace with the advances made on the bacterial enzymes. We previously reported on enzymatic properties and primary structures of endolytic and exolytic alginate lyase, HdAly and HdAlex, from the pacific abalone *Haliotis discus hannai* [16,17]. While HdAly and HdAlex share 67% amino-acid identity, their alginate-degrading properties were found to differ significantly. HdAly splits internal glycosidic linkages of alginate chains to produce unsaturated tri- and disaccharide as major end products, while HdAlex splits the second glycosidic linkage from the reducing terminus of the alginate chain producing only unsaturated disaccharide. Although the two enzymes were found to have extensive $\beta$-structure, amino-acid residues required for catalytic action have not been identified. We have been attempting to express recombinant HdAly in sufficient amount

to enable determination of structure/function relationships in the abalone alginate lyases. We previously reported that most recombinant HdAly produced with the pET-3a-*Escherichia coli* expression system (Novagen, WI, USA) was found in inclusion bodies even when the growth temperature was lowered to 19°C [16]. While subsequent production of HdAlex in the TOPO expression system (Invitrogen, Carlsbad, CA, USA) yielded active enzyme in the soluble fraction [17], yields were modest. In addition to these systems, we also attempted to produce HdAly using various *E. coli* systems employing various soluble tags and cold inducible promoters; however, we have not succeeded yet to obtain enough amounts of soluble recombinants for biochemical studies.

Recently, we developed a novel yeast expression system suitable for recombinant proteins that tend to form inclusion bodies in *E. coli* expression systems (Sahara and Ohgiya, 2008; published elsewhere). This system consists of an expression vector carrying a cold-inducible promoter from yeast and a host *Saccharomyces cerevisiae,* and recombinant proteins are inducibly expressed at ≤10°C. In the present study, we show the production of recombinant HdAly with this system and investigate catalytically important amino-acid residues of HdAly by site-directed mutagenesis.


## 2. Materials and Methods


### 2.1. Materials

Live abalones were purchased from a local market in Hakodate, Hokkaido prefecture, Japan. Abalone alginate lyase, HdAly, was prepared by the method of Shimizu et al. [16]. Thallus of *Laminaria japonica* was collected from the Hakodate

seashore. Cellulase Onozuka R-10 was purchased from Yakult Co. Ltd (Tokyo, Japan). Alginate sodium salt and CelLytic Y were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bacto Trypton, Bacto Yeast Extract, and Yeast Nitrogen Base without Amino Acids were purchased from Difco (Detroit, MI, USA). Competent *E. coli* XL1-Blue and QuickChange Site-Directed Mutagenesis Kit were purchased from Stratagene (Los Angeles, CA, USA). KOD -Plus- DNA polymerase was purchased from TOYOBO (Osaka, Japan). *S. cerevisiae* BY4743 *pep4Δ prb1Δ* (*MAT*a/*MAT*α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0 pep4Δ/pep4Δ prb1Δ/prb1Δ*) was produced by disruption of these protease genes in the parental strain BY4743 according to the protocol provided by Dr. Güldener, et al [32]. Ni-NTA resin was purchased from Invitrogen (Carlsbad, CA, USA). Leucine, histidine, uracil and other chemicals were purchased from Wako Pure Chemical industries (Osaka, Japan).

*2.2. Production of recombinant HdAly using a cold-inducible yeast expression system*

Recombinant HdAly (recHdAly) was expressed using a yeast cold-inducible expression system as follows. The cDNA fragment encoding the catalytic region of HdAly was amplified from the HdAly cDNA (Shimizu et al. [16]; accession No. AB110094, DNA Data Bank of Japan) by PCR with KOD -plus- DNA polymerase using primer pair F1 and R1 (Table 1). This PCR introduced an initiation codon ATG and *Xho* I site to the 5'- and 3'-termini of the cDNA, respectively. The amplified cDNA was digested with *Xho* I and then ligated to *Sma* I- and *Xho* I-digested pLTex321sV5H that we had constructed as a novel expression vector harboring a yeast cold-inducible promoter (Sahara and Ohgiya, published elsewhere; 2008). Proteins could be highly produced at low temperatures and exhibited high solubility. (Fig. 1). According to this

construction of the expression plasmid pLTex-HdAly, a V5 epitope tag and a 6xHis tag

for purification by Ni-NTA affinity chromatography were fused to the C-terminus of

HdAly. The pLTex-HdAly was cloned with *E. coli* XL1-Blue and the insertion of the

HdAly cDNA in the plasmid was confirmed by nucleotide sequencing using an ABI 310

Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). *S. cerevisiae* BY4743

*pep4Δ prb1Δ* was then transformed with the expression plasmid. Yeast transformants

were selected on SD-ura agar (0.67% Yeast Nitrogen Base without Amino Acids/ 2%

glucose/ 2% agar/ 20 μg/ml histidine/ 30 μg/ml leucine). A single colony was picked and

inoculated into 50 ml of SD-ura/leu (0.67% Yeast Nitrogen Base without Amino Acids/

2% glucose/ 200 μg/ml histidine). The yeast was cultured overnight at 30°C and a 7 ml

portion of the culture was transferred to 700 ml of SD-ura/leu. The medium was

incubated at 30°C until the $OD_{600}$ reached 0.75, at which time the culture was

transferred to 4°C in order to induce expression of recHdAly. After 72 h of incubation at

4°C, the cells were harvested by centrifugation at 10,000x*g* for 10 min yielding

approximately 2.5 g (wet weight) of yeast pellet. The pellet was suspended in 6.25 ml of

CelLytic Y (Sigma-Aldrich) and incubated at 4°C overnight with gentle stirring, and

then homogenized at 0°C with glass beads (425-600 μm, Sigma-Aldrich) using a Bead

Beater (BioSpec Products, Inc.,Bartlesville, OK , USA). The 1-min homogenization steps

were repeated 10 times with 1-min intervals on ice. The homogenate was then

centrifuged at 10,000x*g* for 10 min to obtain the supernatant. The recHdAly was

purified from the supernatant fraction by Ni-NTA affinity chromatography as follows.

Two volumes of 100 mM sodium phosphate buffer (pH 7.5) and 1/10 volume of 5 M NaCl

were added to the supernatant to adjust pH and salt concentration. The solution was

subjected to a Ni-NTA affinity column (1 x 3 cm) preequilibrated with 10 mM sodium

phosphate (pH 7.5) and 0.5 M NaCl. Proteins were eluted stepwisely with 20 mM, 40 mM and 200 mM imidazole in the same buffer. SDS-PAGE and enzyme asssy revealed that the fraction eluted with 200 mM imidazole contained recHdAly.

### 2.3. Site-directed mutagenesis

Site-directed mutagenesis of HdAly was achieved using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) and pLTex-HdAly cDNA as a template and the oligonucleotide primers listed in Table 1. Mutations introduced into the cDNA were confirmed by nucleotide sequencing. Mutant proteins were expressed and purified as described for wild-type recHdAly.

### 2.4. Determination of enzyme activity

Alginate-degrading activity of recHdAly was measured in 3 ml of reaction mixture containing 0.12% (w/v) sodium alginate, 10 mM sodium phosphate (pH 7.0), and 10-30 μg/ml of recHdAly. Progress of alginate degradation was monitored by measuring an increase in absorbance at 235 nm due to formation of a double bond between C-4 and C-5 at the new reducing terminus of the alginate chain. One unit of alginate lyase was defined as the amount of enzyme that increased $A_{235}$ by 0.01 per min at 30°C and pH 7.0. The optimal reaction temperature for recHdAly was assessed by measuring activity at 15-50°C and pH 7.0, while the optimal pH was assessed by measuring activity at pH 5.5-9.5 at 30°C. Thermal stability of recHdAly was assessed by measuring the activity remaining after heating at 15-45°C for 20 min.

### 2.5. Mass spectrometry

In order to assess the glycosylation of recHdAly, proteolytic fragments of recHdAly were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Thus, the protein band of recHdAly on SDS-PAGE was excised from the gel and washed with 25 mM $NH_4HCO_3$-40% (v/v) ethanol five times. The gel was cut into pieces and dehydrated with 1 ml of acetonitrile and dried in vacuo. Then, 0.1 μg/ml trypsin in 25 mM $NH_4HCO_3$ was added to the gel pieces and incubated at 37$^o$C overnight. Peptide fragments were extracted from the gel pieces with 50% (v/v) acetonitrile, 5% (v/v) trifluoroacetic acid. The extracts were dried in vacuo, dissolved in 5 μl of 50% (v/v) acetonitrile-0.1% (v/v) trifluoroacetic acid, and subjected to an ABI 4700 proteomics analyzer (Applied Biosystems). Amino-acid sequences of the fragments were analyzed in MS/MS mode with DeNovo Explorer software (Applied Biosystems).

*2.6. Production of Laminaria protoplasts with recHdAly*

A *L. japonica* thallus was sliced into small strips (approximately 1 mm in thickness and 10 mm in length) and suspended in 1 ml of an artificial sea water medium containing 0.6 M mannitol as an osmotic stabilizer. To the medium, 20 U/ml of recHdAly and 2% (w/v) cellulase Onozuka R-10 (Yakult Co. Ltd., Tokyo, Japan) were added and the mixture was incubated at 15$^o$C for 2-6 h. Release of protoplasts from the thallus strips was observed using a Nikon TS100 microscope (Nikon, Tokyo, Japan).

*2.7. Circular dichroism of recHdAly*

Circular dichroism of recHdAly was measured at 20$^o$C in 10 mM sodium phosphate (pH 7.0) using a J-600 spectropolarimeter (JASCO, Tokyo, Japan).

*2.8. SDS-PAGE*

SDS-PAGE was carried out as described [33] using 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS. After the electrophoresis, the gel was stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol-5% (v/v) acetic acid and destained with 10% (v/v) acetic acid-5% (v/v) methanol. Densitometric analysis was carried out using Scion Image program (Scion Co., Frederick, MD, USA). Protein Marker, Broad Range (New England BioLabs, Ipswich, MA, USA) was used as a molecular mass marker.

*2.9. Protein concentration*

Protein concentration was determined by the biuret [34] or Lowry [35] method using bovine serum albumin fraction V as a standard protein.

## 3. Results

*3.1. Characteristics of recHdAly*

By the cold-inducible yeast expression system, an appreciable amount of recHdAly was expressed in a soluble form. The yield of recHdAly in the soluble fraction was roughly 10% of total expressed recHdAly according to SDS-PAGE followed by densitometry (Fig. 2). By the Ni-NTA affinity chromatography, approximately 0.3 mg of recHdAly was purified from 700 ml culture (Table 2). The recHdAly protein exhibited a single band on SDS-PAGE with an apparent molecular mass of 28,000 Da (Fig. 2). The optimal temperature and pH of the recHdAly, around 35°C and pH 8, respectively, were similar to those of native HdAly (Figs. 3A and B). On the other hand, temperatures at

which activity decreased to 50% of the original activity by a 20-min treatment at pH 7.0 were 30 and 40°C for the recombinant and native enzymes, respectively (Fig. 3C). Thus, recHdAly seemed to be more labile than native HdAly. The lability of recHdAly may be ascribable to the lack of an N-linked carbohydrate chain that has been suggested to be present at Asn105 of HdAly [16]. Because the recHdAly was expressed in the cytosol fraction in this system, it is unlikely that they are glycosylated. Thus, in order to confirm the lack of carbohydrate chain in recHdAly, tryptic fragments of recHdAly were analyzed by MALDI/TOF MS in comparison with native HdAly (Fig. 4A, B). In the spectrum for recHdAly fragments, a peak with the mass of 1592.6 was detected (Fig. 4A). However, no peak with the same mass was detected in the spectrum for native HdAly (Fig. 4B). Then the amino-acid sequence of the 1592.6 fragment was analyzed by MS/MS mode and it was determined as LPGLFGGWTNCSGGR. This sequence was consistent with the sequence spanning Lue113 and Arg127 of HdAly, and Asn105, the putative glycosylated residue, was located in this sequence. Since the molecular mass of this fragment, 1592.6, was the same as that calculated from the corresponding amino-acid sequence, Asn105 in recHdAly was considered to be unglycosylated. On the other hand, the fragment with the molecular mass 1592.6 was not detected in the mass spectrum of native HdAly (Fig. 4B). This suggests that Asp105 of native HdAly was glycosylated and the glycosylation of Asp105 shifted the peak to outside of this m/z range. The peak corresponding to the glycosylated fragment has not been identified yet. It is also possible to consider that the carbohydrate moiety interrupted the tryptic cleavage  by steric hindrance and repressed the production of the 1592.6 fragment. Since molecular masses of other peaks in MS were similar between recHdAly and native HdAly, glycosylation was considered to occur only in Asn-105.

The recHdAly was capable of producing protoplasts from a brown alga as was native HdAly although thermal stability of recHdAly was lower than native HdAly. Namely, recHdAly along with cellulase Onozuka R-10 degraded *L. japonica* thallus releasing protoplasts as did native HdAly (Fig. 5). These results indicate that the overall properties of recHdAly expressed in the yeast system are practically identical to those of native HdAly.

*3.2. Catalytically important residues*

By comparing the primary structure of HdAly with those of other PL-14 enzymes, e.g., turban shell SP2 [15] and *Chlorella* virus CL2 [18], several regions were found to be highly conserved [16,17]. In the conserved regions of HdAly, several basic amino-acid residues, i.e., Arg92, Lys95, Arg110, Arg119, and Lys196, were especially highly conserved (Fig. 6). These facts recalled us to the proposal of Gacesa [3] that basic amino-acid residues participating in catalysis are conservative in polysaccharide lyases. Therefore, in order to assess the functional importance of these basic residues in HdAly, we prepared site-directed mutants by replacing these five residues with Ala, and designated R92A, K95A, R110A, R119A, and K196A. In addition to these mutants, we also prepared a mutant K162A by replacing Lys162 in a central non-conserved region with Ala as a control mutant. As shown in Fig. 7, activity of recHdAly was significantly affected by four of the six mutations, i.e., activity of K95A was completely abolished, while that of R92A, R110A, and R119A decreased to approximately 35% or less of wild-type recHdAly. These decreases in activity were considered not due to the great changes in higher order structure of recHdAly by the mutations. Namely, molecular ellipticity $[\theta]_{218nm}$ of wild-type and mutant recHdAly were approximately $-7 \times 10^{-3}$ deg·

cm²/dmol which was similar to that of native HdAly, -7.5 x 10⁻³ deg・cm²/dmol [16]. Accordingly, the basic amino-acid residues affected to the activity HdAly were suggested to have played important roles in the catalytic action of HdAly, e.g., binding to carboxyl residues of the substrate and/or abstraction of a proton from the C5 carbon. Among these basic residues, K95 was considered to be of central importance because the K95A completely lost the activity. On the other hand, activities of K162A and K196A were comparable to that of wild-type recHdAly suggesting that these residues have no important function in catalysis.


## 4. Discussion

Previous attempts to produce recombinant abalone alginate lyase HdAly in *E. coli* expression systems yielded very modest amounts of protein [16, 17]. In the present study, we could obtain sufficient amounts of soluble recHdAly for biochemical analysis using the cold-inducible yeast expression system. It was reported that solubility of proteins expressed in *E. coli* was considerably improved at low growth temperatures [36,37]. However, we have not succeeded yet to obtain sufficient amount of recombinant HdAly by lowering growth temperature in an *E. coli* system [16] and even with the *E. coli* system employing the cold-inducible pCold vector. Thus, the use of yeast system may present a solution for the soluble expression of HdAly. Furthermore, the cold-induction was preferable for the expression of recHdAly since it was labile compared with native HdAly (Fig. 3C).

Enzymatic properties of recHdAly expressed in the yeast system were nearly the same as those of native HdAly with respect to specific activity, optimal pH, optimal temperature, and protoplast-producing ability from *L. japonica*. On the other hand, the

temperature for half-inactivation of recHdAly, 30°C, was approximately 10 degree lower than that for native HdAly. The instability of recHdAly was considered to be due to the lack of native glycosylation, which was supported by mass spectrum analysis of tryptic fragments from recHdAly. Namely, the fragment including the putative glycosylated residue Asn105 in native HdAly was detected as a non-glycosylated species in the digests of recHdAly; however, the same fragment was not detected in digests of native HdAly. The reason for the lack of putative glycosylated fragment in the mass spectrum of native HdAly fragments was considered to be due to the shift of the glycosylated fragment to out of the m/z range or suppression of formation of this fragment by a steric hindrance from carbohydrate moiety. Anyway, we may consider that the carbohydrate chain helps to stabilize HdAly.

The site-directed mutagenesis for HdAly allowed us to investigate the conserved basic amino-acid residues relating to the catalytic action. Amino-acid residues comprising the catalytic site of the PL-5 and PL-7 enzymes have been identified by structural studies [28,30,31]. Although the structural motifs of PL-5 and PL-7 enzymes are completely different, i.e., $\alpha5/\alpha6$ barrel and glove-like $\beta$-sandwich (or $\beta$-jelly roll), respectively, the spacial configuration of catalytic residues, Tyr, His, Asn (or Gln), and Arg are remarkably similar between the two enzymes. Accordingly, it was suggested that both PL-5 and PL-7 enzymes have evolved through convergent evolution [30]. A general enzymatic mechanism of alginate lyase has been proposed by Gacesa [3]. The mechanism includes neutralization of an alginate carboxyl group and abstraction of the C5 proton by a concerted action of some basic amino-acid residues in the catalytic site of alginate lyase. Recently, we proposed that there were highly conserved regions among the PL-14 enzymes including the molluscan alginate lyases [16,17]. In these conserved

regions, several basic amino-acid residues that could conceivably participate in catalysis were found. In the present study, we focused on Arg92, Lys95, Arg110, Arg119, and Lys196, and prepared site-directed mutants in order to assess their contributions to catalytic action. Replacement of Lys95 by Ala caused complete inactivation of recHdAly, while replacement of Arg92, Arg110, and Arg119 caused 65% or more inactivation. Therefore, the region spanning Arg92 and Arg119 was considered to be closely related to the catalytic action of HdAly. The Arg92-Arg119 region is located in the N-terminal half of the molecule which shows relatively high homology with other PL-14 enzymes. The region spanning Phe88 and Arg123 including the Arg92-Arg119 region appeared to be most highly conserved in the N-terminal region (see Fig. 6, underlined). This strongly suggests that the Phe88-Arg123 region participate in the formation of the catalytic site of HdAly. In order to confirm this, we are now investigating the functional importance of the other conserved amino-acid residues in this region using various site-directed mutants.

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**Figure legends**


Fig. 1. Schematic figure for the expression plasmid pLTex-HdAly.


Fig. 2. Monitoring of preparation of recHdAly by SDS-PAGE. Yeast cells were homogenized with CelLytic Y and Bead Beater and recHdAly was purified from the supernatant of cell lysate by Ni-NTA affinity chromatography as described in the text. Mk, protein mass markers; Nat, native HdAly; Co, cell lysate of yeast carrying no plasmid; Ex, cell lysate of yeast expressing recHdAly; Ppt, precipitate of cell lysate of yeast expressing recHdAly; Sup, supernatant of cell lysate of yeast expressing recHdAly; Rec, purified recHdAly.


Fig. 3. Effects of pH and temperature on the enzymatic activities of native and recombinant HdAly. (A) Activities were measured at 30$^o$C in reaction mixtures adjusted to various pH values with 50 mM sodium phosphate containing 0.12% sodium alginate, and 1 U/ml of either native HdAly (●) or recHdAly (○). (B) Activities were measured at various temperatures in reaction mixtures containing 0.12% sodium alginate, 10 mM sodium phosphate (pH 7.0), and 1 U/ml of either native HdAly (●) or recHdAly (○). (C) Native HdAly (●) and recHdAly (○) were incubated in 10 mM sodium phosphate (pH 7.0) at various temperatures for 20 min, after which remaining enzymatic activities were assayed at 30$^o$C.


Fig. 4. Mass spectrometry of recHdAly fragments. (A) MALDI/TOF-MS spectrum of the tryptic fragments of recHdAly. Labeled peaks correspond to the matched peptides

from HdAly sequence [16]. The residual numbers, amino-acid sequences, and molecular masses are indicated above the peaks. The putative glycosylated residue, Asn105, is seen in the sequence for the fragment with the molecular mass of 1592.6. (B) Spectrum of the tryptic fragments of native HdAly. The fragment corresponding to the LPGLFGGWTNCSGGR peptide in the recHdAly fragments is absent. This suggests that Asn105 is glycosylated in native HdAly.

Fig. 5. Production of protoplasts from *Laminaria* thallus by the digestion with HdAly. Strips of *Laminaria japonica* thallus were digested with either native HdAly (2.5 U/ml) (A) or recHdAly (2.5 U/ml) (B) together with 2% (w/v) cellulase Onozuka R-10 (Yakult Co.) in an artificial seawater medium containing 0.6 M mannitol.

Fig. 6. Comparison of amino-acid sequences of HdAly and PL-14 enzymes. The amino-acid sequence of HdAly (GenBank accession number AB110094) was aligned with those of turban shell SP2 [15] and *Chlorella* virus CL2 [18]. Identical, highly conservative, and conservative residues among sequences are indicated by asterisk (*), colon (:), dot (.), respectively. Conserved regions are boxed. The basic amino-acid residues replaced with Ala are indicated by shadowing. Mutations are indicated above the sequences like "R92A". The conserved region spanning Phe88 and Arg123 is underlined (see "Discussion").

Fig. 7. Enzymatic activities of HdAly mutants. (A) SDS-PAGE of the mutants. Mk, molecular mass marker protein. WT, wild-type HdAly. (B) Alginate lyase activities of the mutants expressed as relative values. Activity was measured at 30°C in a

reaction mixture containing 0.12% (w/v) sodium alginate, 10 mM sodium phosphate (pH 7.0), and 0.01 mg/ml of the mutated enzymes. Complete inactivation was observed in K95A, and significantly decreased activities were observed in R92A, R110A, and R119A, indicated with black bars.

Table 1 Primers used for subcloning and mutagenesis of HdAly-cDNA.

| Primers | Sequences |
|---------|-----------|
| F1 | 5'-ATGGCGGTATTGTGGACACAT-3' |
| R1 | 5'-GGGGCTCGAGTCCTATGATGGTAGGG-3' |
| R92A-F | 5'-CTTTGACTTCAGA<u>AGCC</u>GGAGGAAAATTACC-3' |
| K95A-F | 5'-GACGCGGAGGA<u>GCA</u>TTACCGGGACTG-3' |
| R110A-F | 5'-GCTCTGGTGGA<u>GCG</u>CACTCGGACAAC-3' |
| R119A-F | 5'-GCTTCTCGACC<u>GCG</u>TTCATGTGGAGG-3' |
| K196A-F | 5'-GGTATAATGGT<u>GCG</u>CTGGTTTTCAC-3' |
| K162A-F | 5'-GGGACGTGGT<u>GCG</u>TGGAGGTTCC-3' |

F1 and R1, forward and reverse primers used for amplifying HdAly cDNA; R92A-F, forward primer used for mutagenesis of Arg92 to Ala, for example. Mutation sites are indicated by underlining.

Table 2. Purification of recombinant HdAly.

| Preparation | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Cell lysate[a] | 260 | 1680 | 6.45 | 100 | 1 |
| recHdAly[b] | 0.305 | 362 | 1190 | 21.5 | 184 |

[a]Cell lysate was prepared from 700 ml of culture.

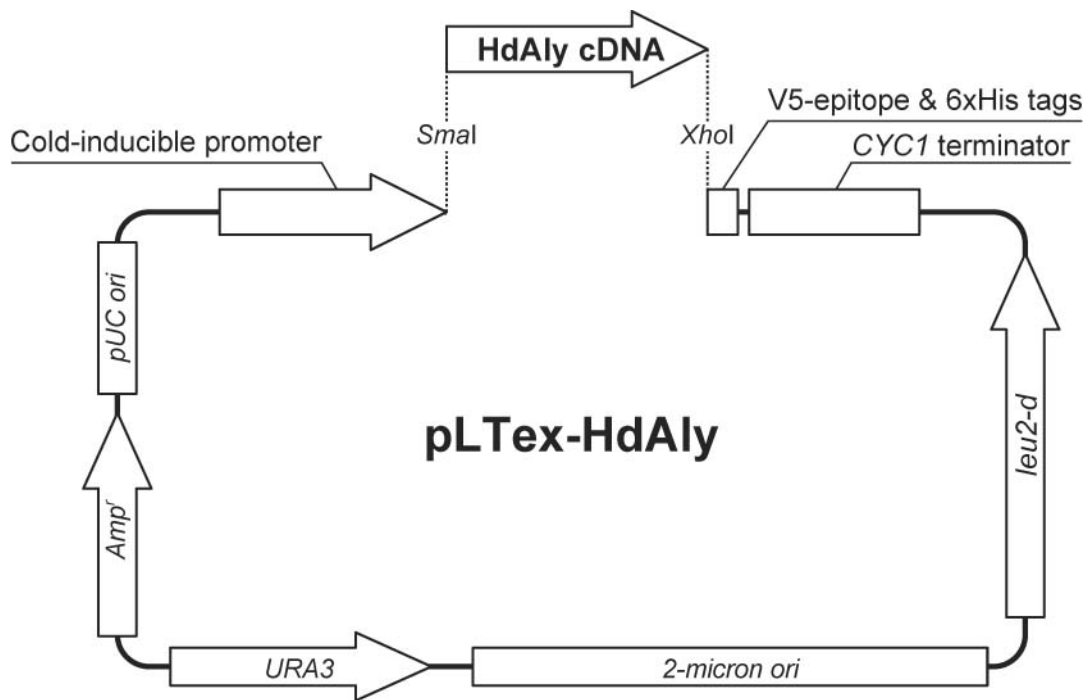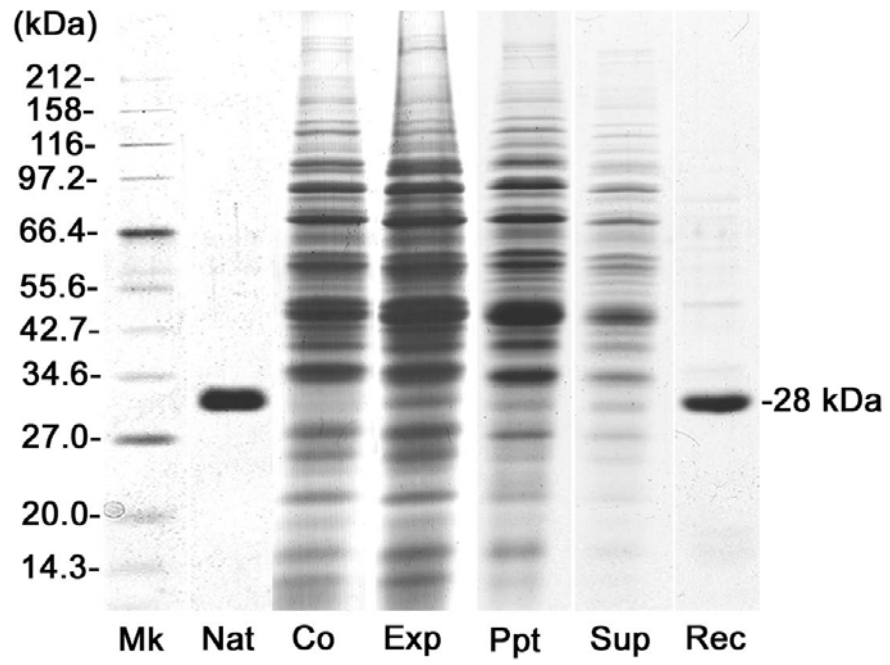[b]recHdAly was purified by Ni-NTA affinity chromatography.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.



(A)

(B)

27
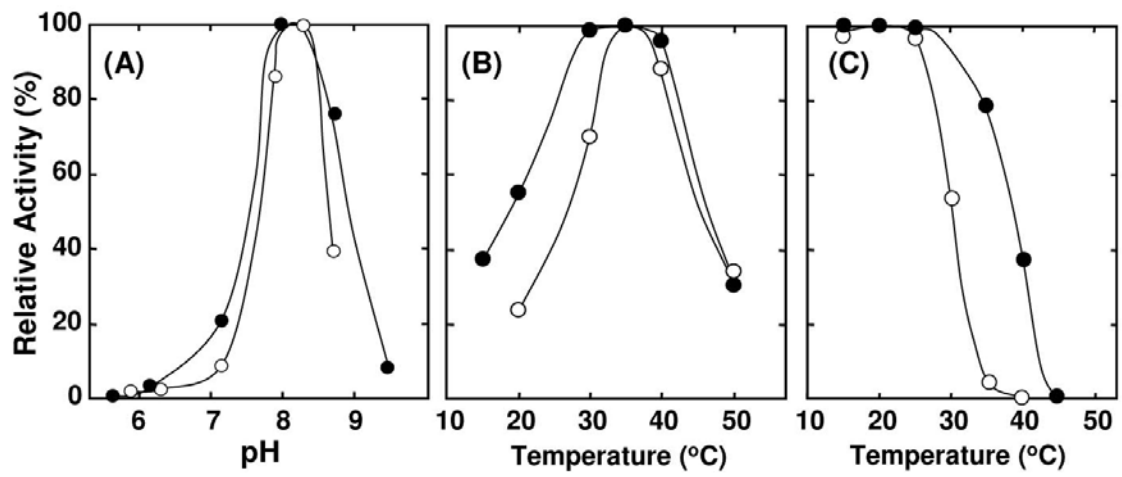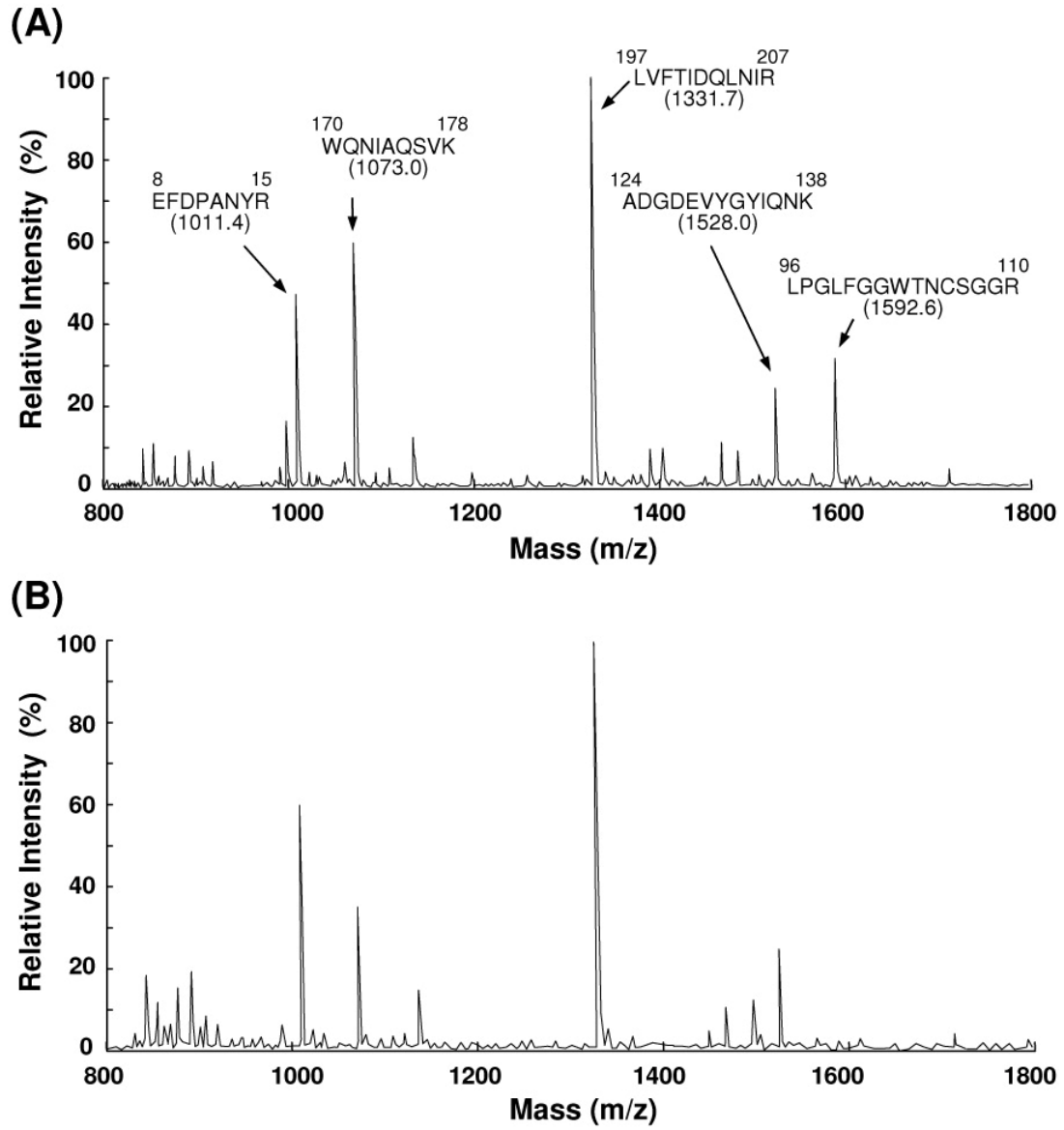
Fig. 5.
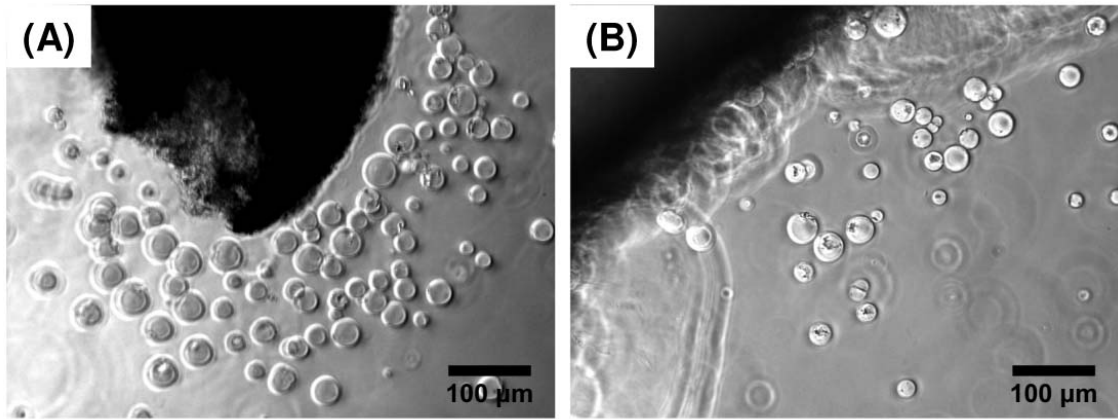
Fig. 6.

```
                                                                    R92 K95
HdAly     1   AVLWTHKEFD PANYRNGMHA LTSNDYDHGS GSVVTDPDGG SNHVLRVWYE    50
SP2       1   TLLWTHKEFD PNNYRDGMHA LTSNDYDHGS GKVVTDPDGG SNHVLRVWYE    50
CL2      87   TKNTNVISTL DLNLLTKGGG SWNVDGVNMK KSAVTTFDG- -KRVVKAVYD   135
                  *          *        **  **     *::   *:

HdAly         KGRYSSHGPN EG-VQFFATP TQ-DHSIMTF SYDVYFDKNF DFRRGGKLPG    98
SP2           KGRWSSHGPN EG-VQFFATP TQ-DHSVMTF SYDLYLSHDF DFRRGGKLPG    98
CL2           KNSGTSANPG VGGFSFSAVP DGLNKNAITF AWEVFYPKGF DFARGGKHGG   185
              *   :*  *    *    * * *   : : :**  ::: ::: * ** ****  *

                        R110          R119
HdAly         LFGGWTNCSG GRHSDNCFST RFMWRADGDG EVYGY----I QNKDHQIDGF   144
SP2           LYGGWTNCSG GRHSDNCFST RFMWRKDGDG EVYAY----I PDYHHQVSGF   144
CL2           TFIGHGAASG YRHSKYGASN RIMWQEKGGV IDYIYPPSDL KQKIPGLDPE   235
              :  *      **  ***    *  * **:    *        * *    :        :

                              K162
HdAly         CDHVVCNSIK GYSMGRGKWR FQRGKWQNIA QSVKLNT--- -PGKTDGSIK   190
SP2           CDHNVCNSVK GYSLGRGKWK FERGKWQNIA QHVHLNT--- -PGKTDGSIK   190
CL2           GQGI------ GFFQDDFKNA LKYDVWNRIE IGTKMNTFKN GIPQLDGESY   279
                 *:        *       :    * *      :** *     : **

                K196
HdAly         VWYNGKLVFT IDQLNIRAKA SVDLDGIFFS TFFGGHDSTW APTHDCYSYF   240
SP2           VWHNGKLVY' IDQLNIVSKA SVDIDGIFFS TFFGGSDSSW APTHDCYSYF   240
CL2           VIVNGKKE-' LKGINWSRSP ELLISRFCWN TFFGG---PL PSPKNQVAYF   325
              *   ***    :  :*       :         :: *****         :  :**


HdAly         KNFVLSTDSG HPTIIG                                        256
SP2           KNFALSTDSS HPTIL                                         255
CL2           TNFQMKKYE                                               334
                 **
```

Fig. 7.

**(A)**



Mk  WT  R92A  K95A  R110A  R119A  K162A  K196A

**(B)**