Comparative Study on General Properties of Alginate Lyases from Some Marine Gastropod Mollusks

Running title: Alginate Lyases from Marine Gastropod

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ABSTRACT: Alginate lyase (EC 4.2.2.3) is an enzyme that splits glycosyl linkages of alginate chain via β-elimination producing unsaturated oligoalginates. This enzyme is widely distributed in herbivorous marine mollusks, brown algae, and marine and soil bacteria. In the present study, we determined the general properties and partial amino-acid sequences of alginate lyases from three Archeogastropoda, i.e., *Haliotis discus hannai*, *H. iris*, and *Omphalius rusticus*, and one Mesogastropoda, i.e., *Littorina brevicula*, in order to enrich the information about functional and structural diversity in gastropod alginate lyases. The alginate lyases were extracted from hepatopancreas of these animals and purified by ammonium sulfate fractionation followed by conventional column chromatography. Single alginate lyases with molecular masses of approximately 28 kDa, 34 kDa, and 34 kDa were isolated from *H. discus, H. iris,* and *O. rusticus*, respectively. While three alginate lyases with molecular masses of 35 kDa, 32 kDa, and 28 kDa were isolated from *L. brevicula*. These enzymes were identified as poly(M) lyase (EC 4.2.2.3) since they preferably degraded poly(M)-rich substrate. Western blot analysis using an antiserum raised against *H. discus* enzyme suggested that *H. iris*, and *O. rusticus* enzymes shared similar primary/higher order structure with *H. discus* enzyme, but the *L. brevicula* enzymes did not. *H. discus, H. iris,* and *O. rusticus* enzymes were classified to polysaccharide-lyase family-14 by the analysis of partial amino-acid sequences, while the *L. brevicula* enzymes were not.

KEY WORDS: **alginate lyase, gastropod, mollusks, polysaccharide-lyase family, amino-acid sequence**

INTRODUCTION

Alginate is an acidic heteropolysaccharide consisting of β-D-mannuronic acid (M) and α-L-guluronic acid (G), which are arranged as homopolymeric poly(M) and poly(G) blocks and heteropolymeric poly(MG) block.[1-3] Alginate exists as an intercellular structural material of brown algae and a constituent of biofilms of certain bacteria.[1-4] Since the solution of sodium alginate exhibits high viscosity and the calcium salt forms elastic gel, alginate has been used as a wide range of food and industrial materials.[2,5] Recently, enzymatically degraded alginate was found to exhibit certain biological activities, e.g., promotion of root growth in higher plants,[6-8] acceleration of a growth rate of *Bifidobacterium* sp.,[9] and induction of production of cytotoxic cytokines in human mononuclear cells,[10,11] suppression of IgE,[12] and antihypertensive effects.[13-14] Accordingly, alginate-degrading enzyme i.e., alginate lyase, as well as alginate oligosaccharides is attracting attentions of researchers in the fields of food and pharmaceutical industries.

Alginate lyase degrades alginate by a β-elimination mechanism to produce alginate oligosaccharides forming a double bond between the C4 and C5 carbons at the non-reducing terminus of the oligosaccharides.[2-4] This enzyme is distributed over herbivorous marine mollusks,[15-23] brown algae,[24,25] marine and soil bacteria,[4,26-30] and chlorella virus.[31] As for molluscan enzymes, poly(M) lyase (EC 4.2.2.3) has been isolated from abalone (*Haliotis. rufescens*,[15] *H. corrugate*,[15] *H. tuberculata*,[20] *H. discus hannai*[22,23]), turban shell (*Turbo cornutus*[18]), sea hare (*Dolabella auricular*[16]), and small marine snail (*Littorina* sp.[17]), and the lyase activity has been detected in sea hare (*Aplysia depilans* and *A. californica*)[19] and bivalves (*Choromytilus meridionalis*, *Perna perna*, *Spisula solidissima*).[32] These molluscan alginate lyases appear to play roles for

degradation of alginate in their dietary algae and facilitate the assimilation of intracellular nutrients of the algae. In addition, the degradation products of alginate also appeared to be utilized by the mollusks as a carbon source. Namely, it was recently reported that alginate oligosaccharides were incorporated to hepatopancreas of turban shell.[33] Further, we have noticed that the substantial amounts of alginate oligosaccharides were accumulated in hepatopancreas of abalone satiated with *Laminaria* sp., and these oligosaccharides were consumed by fasting for a few days (unpublished results). The metabolic pathways of alginate oligosaccharides in mollusks have remained obscure.

Among the molluscan alginate lyases, abalone and turban shell enzymes are the best characterized ones. For example, endolytic and exolytic alginate lyases, HdAly and HdAlex, respectively, have been isolated from digestive fluid of the pacific abalone *H. discus hannai*,[22,23] while two endolytic enzymes, SP1 and SP1 have been isolated from hepatopancreas of turban shell *T. cornutus*.[18,21] Primary structures of HdAly and HdAlex were deduced by the cDNA method,[22,23] while that of SP2 was determined by the protein method.[21] These abalone and turban shell enzymes are classified as members of polysaccharide-lyase family-14 (PL-14) among 18 PL families on the basis of hydrophobic cluster analysis of primary structure (http://www.cazy.org/). Compared with abalone and turban shell enzymes, other molluscan alginate lyases have not been so well characterized and no primary structure data is available. Therefore, other than abalone and turban shell enzymes at present we cannot classify the molluscan alginate lyases vastly i.e., which PL families other molluscan alginate lyases belongs to. In order to improve current information about the molluscan alginate lyases, it is important to study many different molluscan enzymes comparatively.

In the present study, we compared basic properties and partial amino-acid sequences of alginate lyases from *H. discus hannai*, *H. iris*, and *Omphalius rusticus* (Archeogastropoda), and from *L. brevicula* (Mesogastropoda), in order to obtain information about the functional and structural diversity of alginate lyases in marine gastropod mollusks.

MATERIALS AND METHODS

Materials

The pacific abalone *H. discus hannai* was obtained from a local market in Hakodate, Hokkaido prefecture, Japan. The blackfoot abalone *H. iris* was kindly supplied by Central Research Laboratory of Nippon Suisan Kaisha Ltd. Small marine gastropods, *O. rusticus* and *L. brevicula* were collected from tidal zone in the shore of Hakodate. Sodium alginate (*Macrocystis pyrifera* origin) was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Poly(M) block, poly(MG) block, and poly(G) blocks were prepared by the method of Gacesa and Wusteman.[34] TOYOPEARL CM-650M was purchased from Tosoh Co. (Tokyo, Japan). Other chemicals were from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Preparation of gastropod alginate lyases

Alginate lyase from *H. discus hannai*, HdAly, was isolated as reported previously.[22] Alginate lyases from *H. iris*, *O. rusticus*, and *L. brevicula*, named HiAly, OrAly, and LbAly, respectively, in the present study, were isolated as following methods. HiAly

was extracted from the minced hepatopancreas (approx. 100 g) of *H. iris* with 300 ml of 10 mM sodium phosphate buffer (pH 7.0) for 30 min. The extract was centrifuged at 10,000 x g for 10 min, and the supernatant was subjected to ammonium sulfate fractionation. Proteins precipitated between 70 and 90% saturation of ammonium sulfate was collected by centrifugation and dissolved in and dialyzed against 10 mM sodium phosphate buffer (pH 7.0). The dialysate was then subjected to TOYOPEARL CM-650M column (2.0 x 20 cm) pre-equilibrated with the same buffer and the proteins adsorbed to the column were eluted with a linear gradient from 0 to 0.3 M NaCl in 10 mM sodium phosphate buffer (pH 7.0). HiAly showing a single band with 34 kDa on SDS-PAGE was eluted at around 0.25 M NaCl. Protein and activity yields from the initial extract of HiAly were 1.12 mg and 1.24%, respectively. OrAly was extracted from the minced hepatopancreas (approx. 50 g) with 150 ml of sodium phosphate buffer (pH 7.0) and subjected to ammonium sulfate fractionation. Proteins precipitated between 60 and 90% saturation of ammonium sulfate were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and subjected to TOYOPEARL CM-650M column (2.0 x 20 cm) equilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient from 0 to 0.3 M NaCl in the same buffer. OrAly was eluted at around 0.12 M NaCl from the column; however, it contained small amounts of 40 – 70-kDa proteins. Thus, OrAly was further purified by hydroxyapatite column chromatography. Namely, the OrAly fraction was applied to a hydroxyapatite column (1.4 x 22 cm) pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.0) and the adsorbed proteins were eluted with a linear gradient from 0.01 to 0.3 M potassium phosphate (pH 7.0). OrAly showing a single band of approximately 34 kDa on SDS-PAGE was eluted at around 0.2 M potassium phosphate buffer (pH 7.0). Protein and activity yields from

the initial extract of OrAly were 0.51 mg and 5.4%, respectively. LbAly was extracted from the minced hepatopancreas (approx. 30 g) with 10 mM sodium phosphate buffer (pH 7.0) and subjected to ammonium sulfate fractionation. LbAly precipitated between 70 and 100% saturation of ammonium sulfate was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and then subjected to a TOYOPEARL CM-650M column (1.6 x 14 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0 to 0.3 M NaCl. In this chromatography, alginate lyases were eluted at three positions, namely, at around 0.08, 0.1, and 0.16M NaCl. Molecular masses of the enzymes included in the 0.08, 0.1, and 0.16M NaCl fractions were estimated as 35 kDa, 32 kDa, and 28 kDa, respectively. Thus, we named these enzymes LbAly35, LbAly32, and LbAly28, respectively. These enzymes were further purified by hydroxyapatite column chromatography to remove trace amounts of contaminated proteins similarly to the case of OrAly. Protein and activity yields of LbAly 35, LbAly32, and LbAly28 were 0.22 mg and 1.0%, 0.18 mg and 0.8%, and 0.1 mg and 0.5%, respectively. SDS-PAGE of these purified alginate lyases are shown in Fig. 1.

Assay for lyase activity

Alginate lyase activity was assayed at 30^{o}C in a reaction mixture containing 10 mM sodium phosphate buffer (pH 7.0) and 0.12% (w/v) sodium alginate, poly(M), poly(MG) or poly(G) substrate, and 0.01 – 0.05 mg/ml enzyme. Degradation of substrates was monitored by measuring the absorbance at 235 nm with a spectrophotometer HITACHI Model U-3010 (Tokyo, Japan) equipped by a thermal controlling apparatus SP-12R (TAITEC, Tokyo, Japan). One unit of alginate lyase was defined as the amount of enzyme that increases Abs235nm to 0.01 for 1 min. Optimal

temperature of enzyme was determined by measuring the activity at 10 – 70°C in 10 mM sodium phosphate buffer (pH 7.0). Thermal stability of enzyme was assessed by measuring the activity remaining after the heat treatment at 15 – 55°C for 20 min. pH dependence of the activity was determined in a reaction mixture containing 50 mM sodium phosphate buffer adjusted to pH 4.5 – 10.5. pH stability of enzyme was assessed as follows. The enzyme was dissolved in 50 mM sodium phosphate buffer adjusted to pH 3.0 – 11.0 and incubated at 30°C for 15 min. Then, 0.05 ml of the enzyme solution was added to 0.25 ml of ice-cold 100 mM sodium phosphate buffer (pH 7.0) and 0.1 ml of the mixture was subjected to activity assay under standard conditions (30°C and pH 7.0) as described above.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 0.1% (w/v) SDS-10% (w/v) polyacrylamide slab gel by the method of Porzio and Pearson.[35] After the electrophoresis, the gel was stained with 0.2% (w/v) Coomassie Brilliant Blue R-250, 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.

Preparation of antiserum against HdAly

An antiserum to the abalone alginate lyase HdAly was raised as follows. HdAly purified by TOYOPEARL CM-650M column chromatography was subjected to SDS-PAGE using a 2 mm-thick slab gel and briefly stained with Coomassie Brilliant Blue R-250. Gel portion containing HdAly was excised from the gel with a scalpel and homogenized

with 50 mM sodium phosphate buffer (pH 7.5) using glass homogenizer. The homogenate was dialyzed overnight against the same buffer and centrifuged at 10,000 x g for 10 min. The HdAly in the supernatant was lyophilized and stored at -20^{o}C until use. This procedure was repeated several times to obtain approx. 0.2 mg of the gel-purified HdAly. Then, the HdAly was dissolved in 0.2 ml of 0.12 M NaCl – 10 mM sodium phosphate buffer (pH 7.0) and emulsified with an equal volume of Freunds' complete adjuvant (Wako Pure Chemical Industries, Ltd.). The emulsion (0.1 ml) was injected to the backside of a male rabbit (New Zealand White) for three times at 2 weeks intervals with the same dosage. After confirmation of antibody production (on day 7 after the last injection), cardiac blood was obtained from the rabbit and solidified at 37^{o}C for 1 h and cooled to 4^{o}C. The serum was collected by centrifugation at 10,000 x g for 10 min and stored in aliquots at -80^{o}C until use.

Western blot analysis

Western blot analysis was performed according to the method of Towbin et al.[36] Alginate lyase was subjected to SDS-PAGE, and then transferred to a nitrocellulose membrane (Tosoh Co.) using a Horize-Blott semi-dry blotter (ATTO, Tokyo, Japan) at 120 mA for 120 min. Rabbit anti-HdAly antiserum generated in the present study was used as the primary antibody and horseradish-peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) was used as the secondary antibody. The secondary antibody on the blot was detected by color development with 0.6% (w/v) 4-chloro-1-naphtol and 0.3% (v/v) hydrogen peroxide in 20% (v/v) methanol-0.05 M Tris-HCl (pH 7.5). MagicMark XP Western Protein Standard (Invitrogen, Carlsbad, CA, USA) was used as a molecular mass marker for the blot.

Determination of partial amino-acid sequences

The N-terminal amino-acid sequences of alginate lyases were determined with the samples electrically blotted to a poly(vinylidene difluoride) (PVDF) membrane after SDS-PAGE using a protein sequencer Procise 492 (Applied Biosystems, Foster City, CA, USA). For the analysis of internal amino-acid sequences of the alginate lyase, proteolytic fragments were prepared by the digestion with 1/100 (w/w) of lysylendopeptidase at 37°C for 2 h. The fragments were transferred to a PVDF membrane after SDS-PADE and several well separated fragments on the membrane were subjected to the sequencer. When matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for the analysis of amino acid sequences, the target band on SDS-PAGE was excised from the gel and subjected to in-gel tryptic digestion with Montage In-Gel Digest Kit (Millipore, Waltham, MA, USA) according to the manufacturer's protocol. Peptide fragments were extracted from the gel with 50% (v/v) acetonitrile-5% (v/v) trifluoroacetic acid and desalted with ZipTip (Millipore, Bedford, MA, USA), and then subjected to a MALDI TOF-MS (ABI 4700 proteomics analyzer, Applied Biosystems). The amino-acid sequences of the fragments were determined by MS/MS mode with DeNovo Explorer software (Applied Biosystems). Homology searches of the amino-acid sequences to databases were performed with the FASTA and BLAST programs (http://fasta.ddbj.nig.ac.jp/top-j.html, http://blast.ddbj.nig.ac.jp/top-j.html) provided by DNA Data Bank of Japan.

Protein concentration

Protein concentration was determined by the Biuret[37] or Lowry[38] method using bovine

serum albumin fraction V as a standard protein.

RESULTS

Determination of general properties of the alginate lyases

Alginate lyases, HdAly, HiAly, and OrAly, isolated from *H. discus hannai*, *H. iris*, and *O. rusticus*, showed the molecular masses of 28 kDa, 34 kDa, and 34 kDa, respectively (Fig. 1). While, three alginate lyases from *L. brevicula* showed the molecular masses of 35 kDa, 32 kDa and 28 kDa. Accordingly, we named them LbAly35, LbAly32, and LbAly28, respectively. In the present study, we used LbAly35 for the determination of enzyme properties as a representative of *Littorina* enzymes, since the yield of LbAly35 was the highest. Overall properties of the *Littorina* enzymes have been confirmed to be similar to each other.

In case of substrate preferences of the enzymes, all alginate lyases tested in the present study preferably degraded poly(M) block (Fig. 2). Specific activities of HdAly, HiAly, OrAly, and LbAly35 toward poly(M) block were 9080, 4620, 2040, and 4320 U/mg, respectively. These alginate lyases also could degrade poly(MG) block but hardly poly(G) block. Accordingly, these enzymes were regarded as poly(M) lyases (EC 4.2.2.3). But slight differences among the enzymes were also demonstrated. Namely, HdAly, HiAly, and OrAly degraded native alginate in a similar rate to poly(MG) block, while, LbAly degraded it at a higher rate than poly (MG) block. Such type of substrate preference of LbAly35 was also shown in LbAly32 and LbAly28 (data not shown). This substrate preference may be one of the characteristic properties of *Littorina* enzymes. HiAly, OrAly, and LbAly35 were regarded as endolytic alginate lyases like HdAly and

SP2 as these enzymes rapidly decreased viscosity of alginate substrate in the initial phase of the reaction (data not shown).

Optimal temperature and thermal stability of the alginate lyases were determined (Fig. 3a and b). The optimal temperature for HiAly was the lowest, i.e., at 35^{o}C, whereas the optimal temperature for both HdAly and OrAly was moderate, i.e. 45^{o}C. LbAly35 showed the highest optimal temperature at 50^{o}C. The thermal stability of the enzymes was then assessed by measuring the activity remaining after 20-min incubation at 15 – 55^{o}C. Temperatures at which activity decreased to 50% of the original activity for 20 min incubation were 38, 41, 43, and 50^{o}C for HiAly, OrAly, HdAly, and LbAly35, respectively (Fig. 3b). This indicated that the thermal stability of LbAly35 was roughly 10 degrees higher than the other enzymes. Then, optimal pH of the enzymes was determined (Fig. 4a). HiAly, HdAly, and OrAly showed the highest activity at around pH 8.0 – 8.5, while LbAly35 showed at pH 7.5, which is 0.5 – 1.0 pH unit lower than those of other enzymes. pH stability of the enzymes was assessed by measuring the activity remaining after 15-min incubation at various pHs and 30^{o}C. As shown in Fig. 4b, the pH ranges at which 90% or more activity of the enzymes remained were at pH 6 - 9 for HdAly, HiAly, and OrAly, while pH 3 – 11 for LbAly35. Thus, LbAly35 showed considerably high stability in a broad pH range compared with the other molluscan enzymes.

Western-blot analysis with anti-HdAly antiserum

According to the substrate preference and thermal and pH stabilities, LbAly35 appeared to possess somewhat different properties from the other molluscan enzymes. The differences in enzyme properties may be in part due to the differences in the primary

and/or higher order structure of the enzyme. Therefore, we examined occurrence of the structural differences between LbAly35 and other molluscan alginate lyases by Western-blot analysis using rabbit anti-HdAly antiserum. As shown in Fig. 5, the antiserum cross reacted with HiAly and OrAly; however, it showed practically no reactivity to the *Littorina* enzymes, LbAly35, LbAly 32, and LbAly 28. Accordingly, HiAly and OrAly were considered to share similar primary/higher order structure with HdAly, while LbAly35, LbAly32, and LbAly28 were not.

Determination of partial amino-acid sequences of the alginate lyases

Western blot analysis indicated that substantial structural differences existed between HdAly and *Littorina* enzymes (LbAlys). Then, we determined N-terminal amino-acid sequences of LbAlys along with those of HiAly and OrAly, and compared them with the sequences of HdAly and SP2 which belong to PL-14. As shown Fig. 6, N-terminal amino-acid sequences of HiAly and OrAly showed high similarity to those of HdAly and SP2, while those of LbAlys showed similarity less than 15%. FASTA and BLASTA searches indicated that the N-terminal amino-acid sequences of LbAlys showed practically no similarity to any sequences currently deposited to the protein and nucleic-acid databases. In order to obtain information about the overall characteristics in primary structure, we digested the HdAly, HiAly, OrAly, and LbAly35 with trypsin and subjected to MALDI-TOF-MS. As shown in Fig 7 a-d, several peptide peaks with similar molecular masses were detected among the MS of HdAly, HiAly, and OrAly. Then, we focused on several fragments with the molecular masses around 800 – 1,500 Da and subjected to the analysis of primary structure with MS/MS mode. Accordingly, the amino-acid sequences of two fragments from HiAly with molecular masses of

1340.74 Da and 1443.72 Da were determined as IVFTIDHLNIR and RGEWQNIAQSVR, respectively. These sequences showed high similarity to the sequences of 1331.75-Da and 1073.57-Da fragments from HdAly, i.e., LVFTIDQLNIR and WQNIAQSVK, respectively. These sequences correspond to the regions of 214 - 224 residues and 187 – 205 residues in the primary structure of HdAly determined previously.[22] On the other hand, amino-acid sequences of 1340.73-Da and 1101.56-Da fragments from OrAly were determined as WQNIAQSVR and IVFTIDHLNIR, respectively. These were also similar to the sequences of HdAly, LVFTIDQLNIR and WQNIAQSVK, respectively. On the other hand, there appeared no fragment showing similar molecular mass to the HdAly fragments in MS of LbAly35, especially around a mass range of 800 – 1,500 Da. Thus, we selected some smaller fragments of LbAly35 and determined their amino-acid sequences by MS/MS mode. However, no fragment showing appreciable similarity to the primary structure of HdAly was found. For example, amino-acid sequences, TISSGIFR and IPGIWGGAMK, determined with 880.43-Da and 1029.53-Da fragments from LbAly35, respectively, showed no similarity to any regions in the primary structure of HdAly. These sequences from LbAly35 also showed no similarity to any sequences currently deposited in the databases. Although the above analyses were limited to partial amino-acid sequences, we consider that HiAly and OrAly are classified to PL-14 as in case with HdAly and SP2, while not the *Littorina* enzymes.

DISCUSSION

General properties of the molluscan alginate lyases

In the present study, we isolated alginate lyases, HdAly, HiAly, and OrAly, from *H. discus hannai*, *H. iris*, and *O. rusticus*, respectively, while three alginate lyases, LbAly35, LbAly32, and LbAly28, from *L. brevicula*. The occurrence of heterogeneous alginate lyases in *Littorina* sp. was previously reported, and an enzyme with the molecular mass of 40 kDa, named alginate lyase VI, was isolated.[17] LbAly35 may correspond to the alginate lyase IV because of the similar molecular mass. HdAly, HiAly, OrAly, and LbAly35 preferably degraded poly(M) block but not poly(G) block, thus they were regarded as poly(M) lyases (EC 4.2.2.3). Slight differences between LbAly35 and the other enzymes were shown in the degradation rate for native alginate substrate. Namely, LbAly35 degraded native alginate in a higher rate than poly(MG) block. Although the reason for this characteristic action of LbAly35 to alginate is obscure, this may reflect the differences in the splitting site of alginate by *Littorina* and *Haliotis* enzymes. Namely, it has been reported that the *Littorina* alginate lyase IV split M/M but *Haliotis* enzyme split M/G, M/M, or G/M ("/" represents the splitting site).[17] Precise analysis for the substrate specificity using oligosaccharides with known structures is essential to clarify the reason for the difference in substrate preference between *Haliotis* and *Littorina* enzymes. Different properties of LbAly35 from the other enzymes were also seen in pH dependences and temperature and pH stabilities. For example, optimal pH of LbAly35 was at pH 7.4, which was 0.5 – 1.0 pH unit more acidic side than those of HdAly, HiAly, and OrAly. The temperature that caused a half inactivation during 30-min incubation was observed at around 50^{o}C in LbAly35, while those were at around 40^{o}C in other enzymes. Further, LbAly35 was stable in a wider pH range compared with the other enzymes, i.e., LbAly35 showed practically no decrease in activity upon incubation at pH 3 – 11 for 15 min, while other enzymes greatly

inactivated at pH below 5 and above 9. These results strongly suggest that LbAly35 is structurally more stable than the other molluscan enzymes. The high stability of LbAly35 might be related to the molecular adaptation of enzyme to habitat temperature of *L. brevicula*. Namely, *L. brevicula* inhabits in a tidal zone where the habitat temperature greatly changes, e.g., from 15 to 40^{o}C in a day due to the exposure to direct sunshine as well as the ebb and flow of the tide. On the other hand, *Haliotis* and *Omphalius* inhabit under the tidal zone where habitat temperature appeared to be modestly changes around 10 – 15^{o}C.

Structural diversity in the alginate lyases

According to Western-blot analysis HiAly and OrAly were found to show high cross reactivity with rabbit anti-HdAly antiserum. On the other hand, LbAly35, LbAly32, and LbAly28 showed practically no cross-reactivity with the antiserum. These led us to consider that HiAly and OrAly share similar primary/higher order structure to HdAly, while LbAly35, LbAly32, and LbAly28 do not. Then, we determined partial amino-acid sequences of these enzymes and confirmed that HiAly and OrAly possess the homologous sequences with those of HdAly and SP2 that belong to PL-14. These results suggested that HiAly and OrAly were also classified to PL-14. It is noteworthy that partial amino-acid sequences of LbAly35 showed practically no similarity to the sequence of HdAly and to any sequences deposited in the protein and nucleic acid databases. This may indicate that the *Littorina* alginate lyase is classified to a novel polysaccharide-lyase family whose primary structure has not determined yet. The diversity of primary structures between *Littorina* and *Haliotis* enzymes may be relating to the phylogenetic divergence in gastropod. Namely, *Haliotis*, *Turbo*, and *Omphalius*

belong to Archeogastropoda, while *Littorina* belongs to Mesogastropoda. This also suggests that the molluscan PL-14 enzyme is specifically distributed in Archeogastropoda among gastropods. In order to explore the phylogenetic relationship and process of molecular evolution for molluscan alginate lyases, it seems necessary to compare genetic structures and primary structures of alginate lyases from different orders. Analysis of complete amino-acid sequence of LbAly35 is now underway.

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Figure captions

Fig. 1 SDS-PAGE for the alginate lyases.

Mk, molecular mass markers. HdAly, *H. discus hannai* enzyme; HiAly, *H. iris* enzyme; OrAly, *O. rusticus* enzyme; LbAly35, LbAly32 and LbAly 28, *L. brevicula* enzymes.

Fig. 2 Substrate preference of the alginate lyases. Activities of HdAly (a), HiAly (b), OrAly (c) and LbAly35 (d) were measured with the sodium alginate（○）, M-block（●）, MG-block（△）or G-block（▲）in a concentration of 0.12% (w/v). Alginate lyase activity was determined by monitoring the increase in absorbance at 235 nm due to the degradation of alginate via β-elimination.

Fig. 3 Temperature dependence and thermal stability of the alginate lyases. (a) Activity was measured at various temperatures in a reaction mixture containing 0.12% (w/v) sodium alginate, 10 mM sodium phosphate buffer (pH 7.0), and 1 U/ml enzyme. (b) Enzyme was incubated in 10 mM sodium phosphate buffer (pH 7.0) at indicated temperatures for 20 min and then remaining activity was assayed at 30^{o}C in the same mixture. ○, HdAly; ●, HiAly; △, OrAly; and ▲, LbAly35.

Fig. 4 pH dependence and pH stability of the alginate lyases. (a) Activity was measured at 30^{o}C in a reaction mixture containing 10 mM sodium phosphate buffer adjusted to various pHs. (b) Enzyme was incubated at 30^{o}C at various pHs for 15 min and the remaining activity was measured at 30^{o}C and pH 7.0. ○, HdAly; ●, HiAly; △, OrAly; and ▲, LbAly35.

Fig. 5 Western blot analysis for the alginate lyases. The same samples as in Fig 1 were used for SDS-PAGE followed by the Western blot analysis. Anti-HdAly antiserum and horseradish-peroxidase-conjugated goat anti-rabbit IgG were used as the primary and the secondary antibody, respectively. MagicMark XP Western Protein Standard was used for the molecular mass markers (Mk).

Fig. 6 Comparison of N-terminal amino-acid sequences of the alginate lyases. The N-terminal amino acid sequences of HiAly, OrAly and LbAlys were aligned with those of HdAly[22] and SP2[21]. Id.(%) indicates the sequence identity with HdAly. Both HiAly and OrAly showed 90.5% and 85% identity with those of HdAly and SP2, respectively. While the sequences of LbAly35, LbAly32 and LbAly28 showed the identity less than 15% with the sequences of HdAly and SP2.

Fig. 7 Mass spectrometry for tryptic fragments of the alginate lyases. Mass spectrograms for the tryptic fragments of HdAly, HiAly, OrAly, and LbAly35 are shown in figures (a), (b), (c) and (d), respectively. Molecular masses and amino-acid sequences of the peak fragments labeled by arrows are shown on right sides of the arrows. Identical amino-acid residues among HdAly, HiAly, and OrAly fragments are underlined. Numbers indicated by superscript on the HdAly fragments in (a) correspond to the residue numbers in the complete primary structure of HdAly[22].

Fig. 1

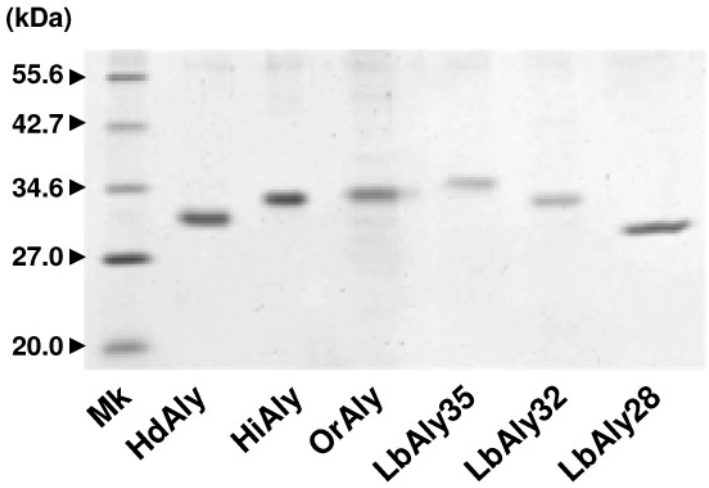

(kDa)
55.6
42.7
34.6
27.0
20.0
Mk
HdAly
HiAly
OrAly
LbAly35
LbAly32
LbAly28

Fig. 2 a - d

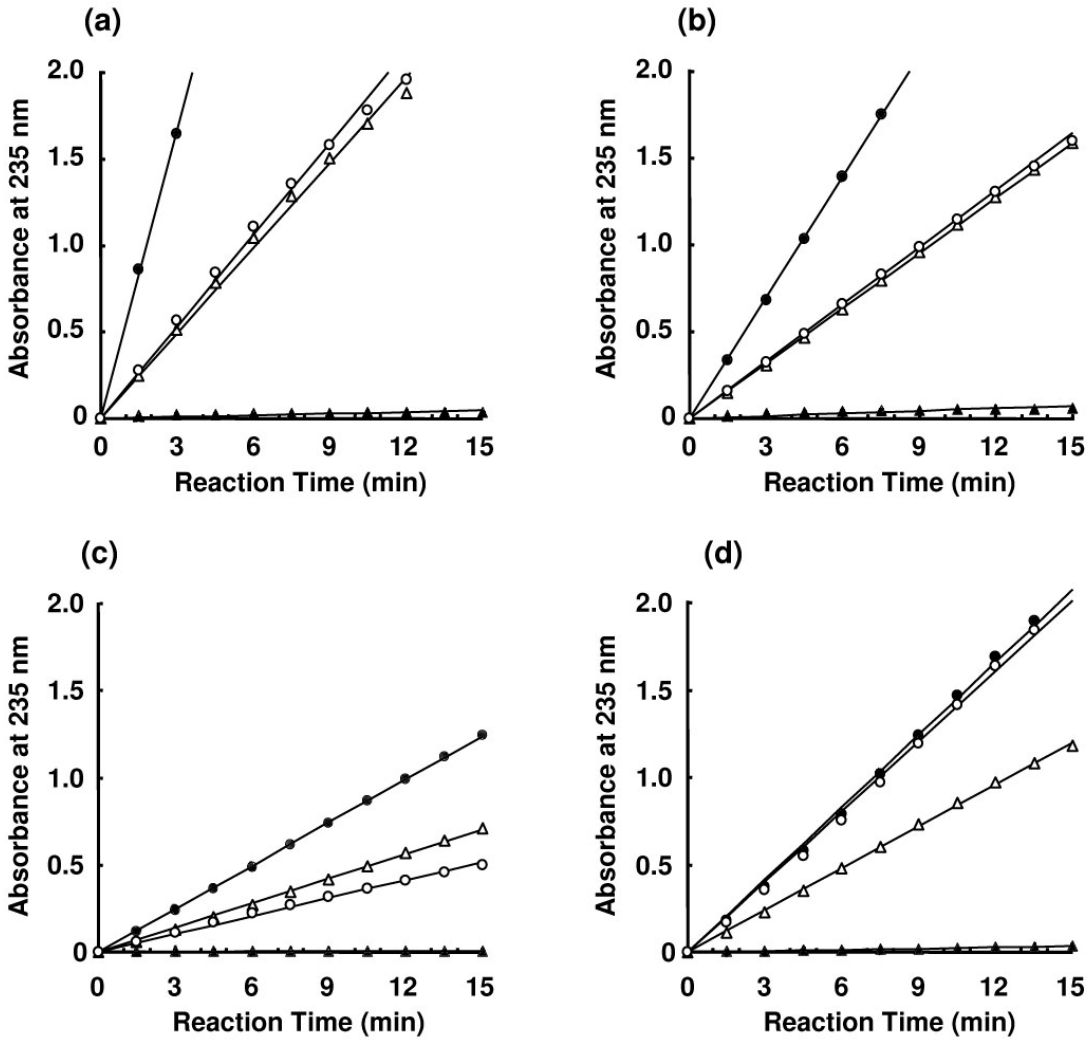

(a)
(b)
(c)
(d)
Absorbance at 235 nm
Reaction Time (min)
0
0.5
1.0
1.5
2.0
0
3
6
9
12
15

Fig. 3 a & b

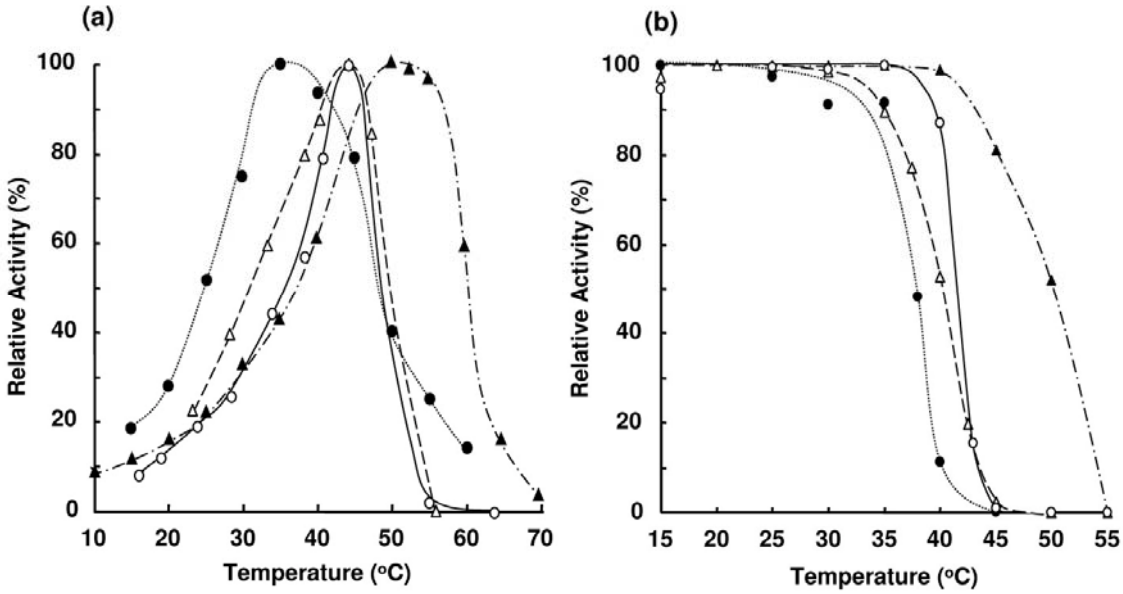

(a)
Relative Activity (%)
100
80
60
40
20
0
10
20
30
40
50
60
70
Temperature (°C)
(b)
Relative Activity (%)
100
80
60
40
20
0
15
20
25
30
35
40
45
50
55
Temperature (°C)

Fig. 4 a & b

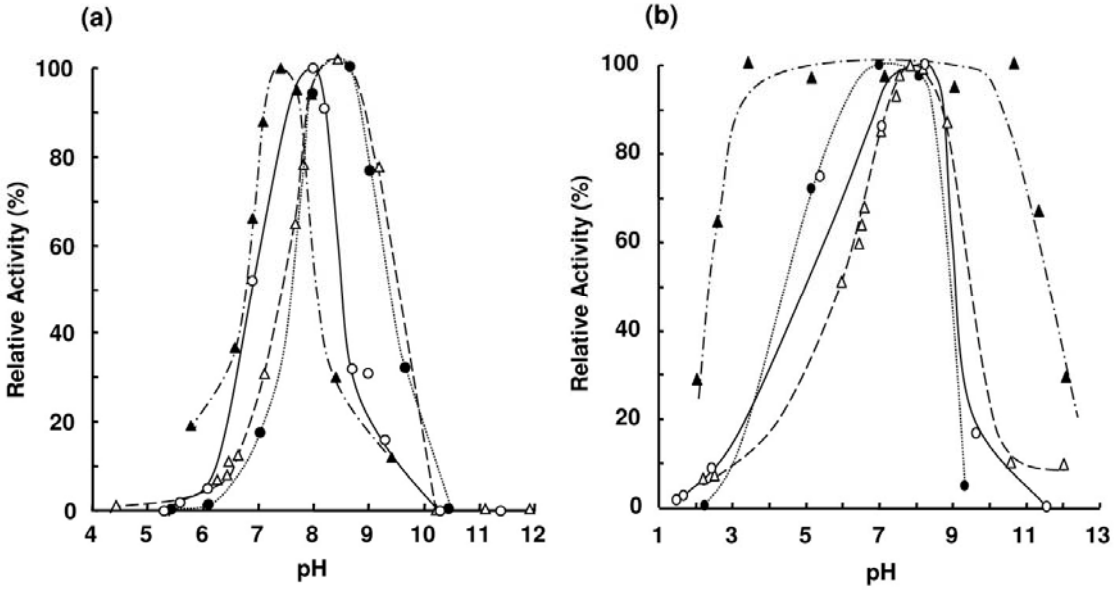

(a)
Relative Activity (%)
100
80
60
40
20
0
4
5
6
7
8
9
10
11
12
pH
(b)
Relative Activity (%)
100
80
60
40
20
0
1
3
5
7
9
11
13
pH

Fig. 5

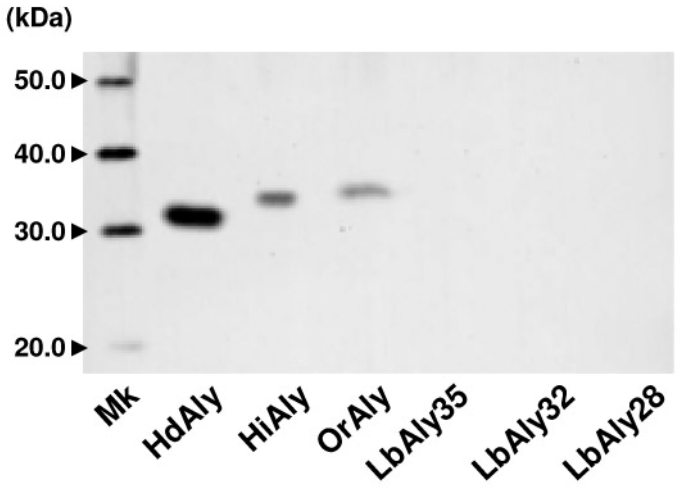
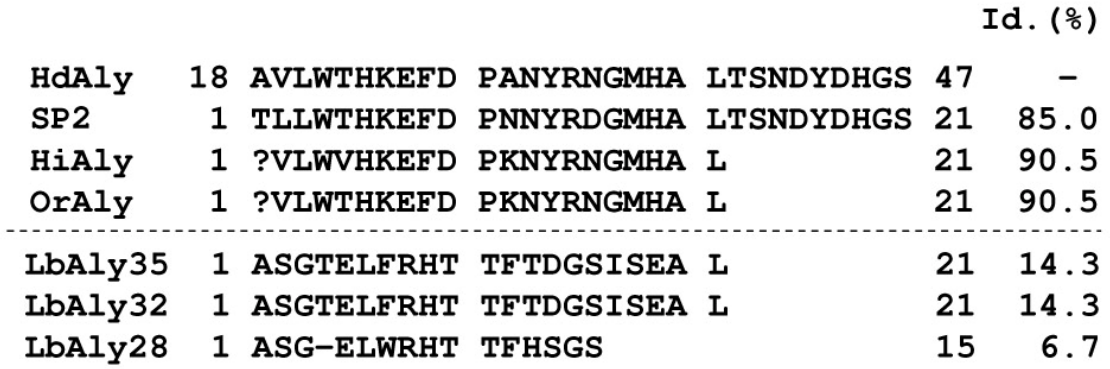
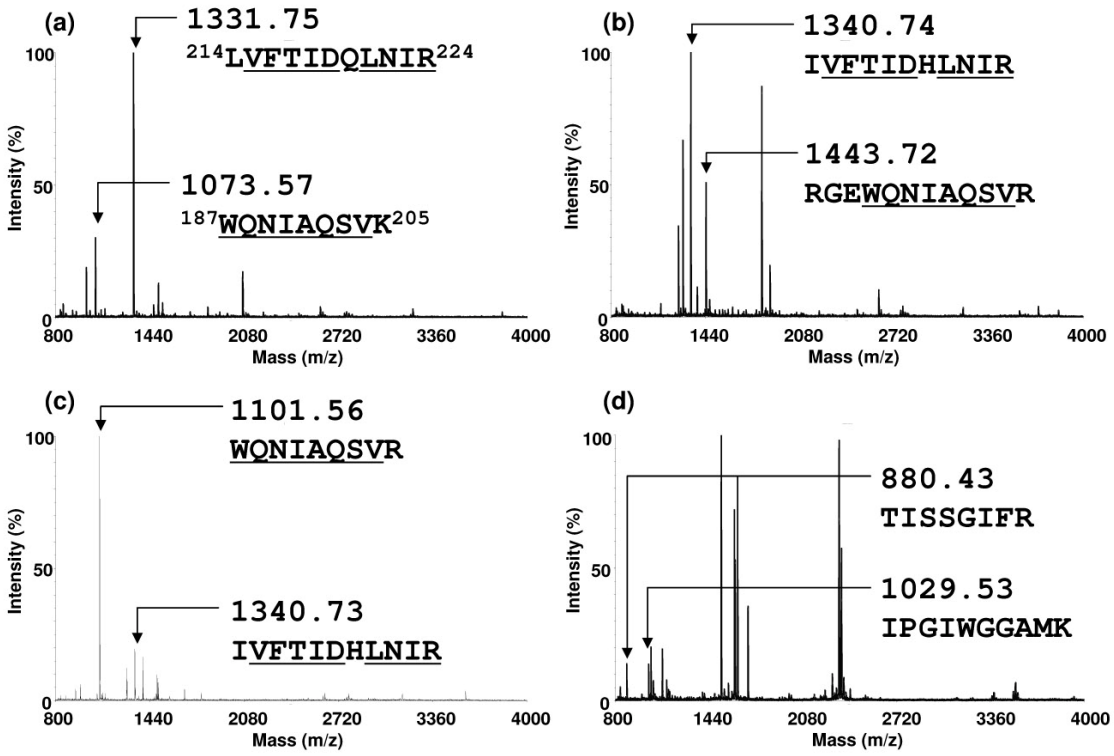

(kDa)
50.0
40.0
30.0
20.0
Mk
HdAly
HiAly
OrAly
LbAly35
LbAly32
LbAly28

Fig. 6



				Id.(%)
HdAly	18	AVLWTHKEFD PANYRNGMHA LTSNDYDHGS	47	-
SP2	1	TLLWTHKEFD PNNYRDGMHA LTSNDYDHGS	21	85.0
HiAly	1	?VLWVHKEFD PKNYRNGMHA L	21	90.5
OrAly	1	?VLWTHKEFD PKNYRNGMHA L	21	90.5
LbAly35	1	ASGTELFRHT TFTDGSISEA L	21	14.3
LbAly32	1	ASGTELFRHT TFTDGSISEA L	21	14.3
LbAly28	1	ASG-ELWRHT TFHSGS	15	6.7

Fig. 7 a - d


(a)
1331.75
214LVFTIDQLNIR224
1073.57
187WQNIAQSVK205
(b)
1340.74
IVFTIDHLNIR
1443.72
RGEWQNIAQSVR
(c)
1101.56
WQNIAQSVR
1340.73
IVFTIDHLNIR
(d)
880.43
TISSGIFR
1029.53
IPGIWGGAMK
Intensity (%)
Mass (m/z)
100
50
0
800
1440
2080
2720
3360
4000