Heat-stability and primary structure of the major alginate lyase isozyme LbAly35 from *Littorina brevicula*

Running title: Primary structure of *Littorina* alginate lyase

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Abstract

Previously we isolated the major alginate lyase isozyme LbAly35 from a marine snail *Littorina brevicula* and showed that this enzyme was significantly heat-stable in a broad pH range compared with other molluscan alginate lyases (Hata et al., Fish. Sci. (2009) 75:755-763). LbAly35 showed practically no similarity to other molluscan alginate lyases in the N-terminal amino-acid sequence of 20 residues and no cross-reactivity with anti-abalone alginate lyase antiserum. These led us to consider that the primary structure of LbAly35 is considerably deviated from other molluscan enzymes. Thus, in the present study, we first compared the thermal stability of LbAly35 with an abalone alginate lyase, HdAly, and found that the first order inactivation rate constants for LbAly35 at 40^{o}C and 45^{o}C were 1/20 and 1/45 of those for HdAly, respectively. Then, we cloned cDNAs encoding LbAly35 and characterized its deduced amino-acid sequence comparing with those of other molluscan alginate lyases. The cDNAs were amplified by PCR and 5′- and 3′-RACE PCRs from the *L. brevicula* hepatopancreas cDNA using degenerated primers synthesized on the basis of partial amino-acid sequences of LbAly35. The cDNA covering entire translational region of LbAly35 comprised 1,093 bp and encoded an amino-acid sequence of 296 residues. The amino-acid sequence consisted of an initiation methionine, a putative signal peptide for secretion (22 residues), a propeptide-like region (10 residues), and a mature LbAly35 domain of 263 residues. Although the N-terminal region of LbAly35 was significantly deviated from those of other molluscan alginate lyases, the catalytic domain of LbAly35 showed ~45% identity to other molluscan enzymes which had been classified under polysaccharide-lyase-family-14 (PL-14). In addition, the amino-acid residues crucially important for the catalytic actions of PL-14 enzymes were also conserved in LbAly35. Accordingly, LbAly35 was regarded as a member of PL-14 as other molluscan alginate lyases despite of the significant deviation of its N-terminal region.

Keywords: Alginate lyase; LbAly35; *Littorina brevicula*; amino-acid sequence; cDNA cloning

Introduction

Alginate is an acidic heteropolyuronic acid found in cell wall and intracellular matrices of brown seaweeds (Phaeophyta) and also in biofilms of certain bacteria [1–4]. Alginate comprises 1-4-linked β-D-mannuronate (M) and α-L-guluronate (G) which constitute homopolymeric poly-M and poly-G blocks and heteropolymeric poly-MG block in alginate chain [1, 3]. Since alginate solution exhibits high viscosity and calcium salt forms elastic gel, this polysaccharide has been widely used as viscosifiers and gelling agents in various industrial fields such as food and beverage, paper and printing, and pharmaceutical industries. Degradation products of alginates are also known to be useful materials since they exhibits variety of biofunctions such as promotion of growth of *Bifidobacterium* sp. [5], acceleration of growth of plant roots [6, 7], stimulation of human keratinocytes [8], emulsifying fish myofibrillar protein [9], enhancing penicillin production of *Penicillium chrysogenum* [10]. In addition, low-molecular-weight derivatives have also been shown to cause production of cytotoxic cytokine in human mononuclear cells [11] and prevention of cardiovascular and cerebrovascular diseases through antioxidation [12, 13]. In this context, alginate-degrading enzymes, i.e., alginate lyase, have been attracting attentions of researchers working on the relating subjects.

Alginate lyase (poly (M) lyase (EC 4.2.2.3) and poly (G) lyase (EC 4.2.2.11)) is a group of enzymes that catalyze cleavage of 4-O-linked glycosidic linkages of alginate chain through β-elimination forming unsaturated uronic acid at the non-reducing terminus of resulted oligosaccharides [14]. This enzyme has been found in brown algae [15, 16], marine and soil bacteria [4, 17–21], herbivorous marine mollusks [22–30], and *Chlorella* virus [31]. According to the database for carbohydrate-active enzymes (CAZy, http://www.cazy.org), these alginate lyases have been classified under seven families, i.e., polysaccharide-lyase-family (PL) 5, 6, 7, 14, 15, 17, and 18. Among these family enzymes, the PL-7 alginate lyase is most widely distributed in marine and soil bacteria and has been extensively investigated to date [18–21]. On the other hand, distribution of PL-14 enzyme is relatively restricted in specific organisms and its major producers are herbivorous marine mollusks such as abalone and sea hare [26, 27, 29, 30]. Compared with bacterial alginate lyases, molluscan alginate lyases had not been so well investigated; however, recently general properties of molluscan enzymes have been gradually accumulating. Namely, abalone alginate lyases were isolated from *Haliotis rufescens* and *Haliotis corrugate* [32], *Haliotis tuberculata* [33], *Haliotis discus hannai* [26, 27], and *Haliotis iris* [28]. Alginate lyases from other molluscs have been isolated from a turban-shell *Turbo cornutus* [24], small marine snail *Littorina* sp. [23], *Omphalius rusticus* and *Littorina brevicula* [28], and sea hare *Dolabella auricular* [22], and *Aplysia kurodai* [29, 30]. Although the general biochemical properties of the molluscan alginate lyases have been repeatedly investigated, primary structures of molluscan alginate lyases have been analyzed only in a few species.

To date, complete primary structures of molluscan alginate lyases have been reported in four species, i.e., endolytic and exolytic alginate lyases HdAly [26] and HdAlex [27] from abalone, SP2 from turban-shell *T. cornutus* [25] and AkAly30 from *A. kurodai* [30]. Hydrophobic cluster analyses (http://www.cazy.org) classified these molluscan alginate lyases under PL-14. Besides the molluscan alginate lyases, *Chlorella* virus enzyme vAL-1 was also classified under PL-14 [34]. Three dimensional structure of vAL-1 [34] solved by the X-ray diffraction method has provided important information for us to understand structure-function relationship of PL-14 molluscan alginate lyases [30]. The amino-acid residues crucially important for the catalytic action of vAL-1 were completely conserved in the putative β-strands and loops of abalone HdAly and sea hare AkAly30 [30, 35]. Such comparative studies between molluscan alginate lyases and *Chlorella* virus enzyme have enriched information about molecular diversity and/or resemblance of PL-14 enzymes.

To investigate the functional and structural diversity of alginate lyases in marine gastropod mollusks, we previously compared the basic properties and partial amino acid sequences of alginate lyases from three Archeogastropoda, *H. discus hannai*, *H. iris*, and *O. rusticus* and one Mesogastropoda, i.e., *L. brevicula* [28]. The major alginate lyase isozyme LbAly35 *of L. brevicula* was identified as a poly(M) lyase (EC 4.2.2.3) like other molluscan enzymes. However, LbAly35 showed a characteristic property, i.e., significantly high heat-stability in a broad pH range, unlike other molluscan enzymes. In addition, the N-terminal 20 amino-acid residues of LbAly35 showed less than 14% identities to those of other molluscan PL-14 alginate lyases. Further, LbAly35 showed no cross-reactivity with antiserum raised against an abalone alginate lyase, HdAly, in western blot analysis [28]. These facts suggested that LbAly35 possesses a primary/higher-order structure considerably different from other molluscan PL-14 enzymes.

Therefore, in the present study, we first confirmed that the heat-stability of LbAly35 is significantly higher than that of abalone HdAly. Then we determined the primary structure of LbAly35 by the cDNA method and compared it with those of other molluscan alginate lyases to characterize the primary structure of LbAly35.

Materials and methods

Materials

Living *L. brevicula* (approximately 300 animals) were collected from the shore of Hakodate, Hokkaido, Japan, in June 2009. The animals were dissected and the ~30 g of hepatopancreas was homogenized with 10 mM sodium phosphate buffer (pH 7.0) and centrifuged at 12,000×g for 15 min. The supernatant was used as crude enzyme. LbAly35 was purified from the crude enzyme by the method described in the previous report [28]. Total RNA was extracted from ~3 g of hepatopancreas by the guanidinium thiocyanate-phenol method [36], and mRNA was selected from the total RNA with Oligotex-dt(30) (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. Abalone alginate lyase HdAly was isolated from the digestive fluid of *H. discus hannai* as reported previously [26]. Sodium alginate (*Macrocystis pyrifera* origin) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The pCR-TOPO2.1 TA cloning kit was from Invitrogen (CA, USA). 5′- and 3′-Full RACE kits were from TaKaRa. AmpliTaq Gold PCR Master Mix was purchased from Applied Biosystems (Foster city, CA, USA). Other reagents were of analytical grade from Wako Pure Chemicals Industries Ltd. (Osaka, Japan).

Assay for alginate lyase activity

Alginate lyase activity was assayed in 1 mL of reaction mixture containing 0.12% (w/v) sodium alginate, 50 mM sodium phosphate buffer (pH 7.0), and an appropriate amount of enzyme at 30^{o}C. Degradation of the substrate was monitored by measuring $Abs_{235\ nm}$ 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 $Abs_{235\ nm}$ to 0.01 for 1 min. To assess the thermal stability of enzyme, LbAly35 and HdAly were incubated at 40^{o}C and 45^{o}C for various time intervals and the activity remaining after the incubation was measured under the standard conditions described above. Denaturation constants for LbAly35 and HdAly were estimated as the first order rate constants.

Partial amino-acid sequence analysis

The N-terminal amino-acid sequence of LbAly35 was determined with an ABI Procise 492 protein sequencer (Applied Biosystems). Internal amino-acid sequence of LbAly35 was determined with the tryptic peptide fragments by a matrix-assisted laser desorption ionizationtime of flight mass spectrometry (MALDI-TOF MS) using an ABI Proteomics Analyzer 4700 (Applied Biosystems) in a MS/MS mode with DeNovo Explorer software.

Cloning of cDNA for LbAly35

The hepatopancreas cDNA from LbAly35 was synthesized with a cDNA synthesis kit (TaKaRa). cDNAs encoding LbAly35 were amplified by PCR from the hepatopancreas cDNA with AmpliTaq Gold DNA polymerase (Applied Biosystems) and degenerated primers synthesized on the basis of the N-terminal and internal amino-acid sequences of LbAly35. PCR was performed in 20 μL of reaction mixture containing 50 mM KCl, 15 mM Tris–HCl (pH 8.05), 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 2.5 mM $MgCl_2$, and 5 pmol μL^{-1} primers, 1 ng μL^{-1} template DNA, and 0.5 units μL^{-1} AmpliTaq Gold DNA polymerase (Applied Biosystems). A successive reaction at 96°C for 20 s, 55°C for 20 s and 72°C for 45 s was repeated for 30 cycles with Thermal Cycler Dice mini (TaKaRa). cDNAs encoding 5'- and 3'-terminal regions of LbAly35 mRNA were amplified with 5'- and 3'-Full RACE kits (TaKaRa, Tokyo, Japan), respectively. The PCR products were cloned with a pCR-TOPO2.1 TA cloning kit (Invitrogen) and subjected to nucleotide sequence analysis using a BigDye-terminator Cycle sequencing kit (Applied Biosystems) and an ABI 3130*xl* Genetic Analyzer (Applied Biosystems).

Results

Comparison of thermal stability between LbAly35 and HdAly

LbAly35 and HdAly were incubated at 40°C and 45°C for various time intervals, and the activity remaining after the incubation was determined (Fig. 1). The first order inactivation rate constants for LbAly35 and HdAly were calculated as 0.04/min and 1.80/min at 45^{o}C and 0.003/min and 0.06/min at 40^{o}C, respectively. These results indicated that thermal stability of LbAly35 was 20-45 times higher than that of HdAly under these conditions. These results confirmed that *Littorina* alginate lyase was significantly heat-stable compared with other molluscan alginate lyases such as abalone HdAly.

cDNA cloning and primary structure analysis for LbAly35

The N-terminal amino-acid sequence of 20 residues of LbAly35 was determined as ASGTELFRHTTFTDGSISEA by the protein sequencer. This sequence matched with that previously reported on *Littorina* enzymes [28] and showed less than 14% amino-acid identities with those of abalone HdAly [26], abalone HdAlex [27], turban shell SP2 [25], and sea hare AkAly30 [30]. This suggests that the primary structure of LbAly35 is much deviated from those of other molluscan alginate lyases. On the other hand, internal amino-acid sequences of two tryptic fragments, i.e., TL(I)SSGIFR and L(I)PGL(I)WGGAMK, were determined by the MALDI-TOF/MS analysis. These sequences were also matched with those determined in the previous study [28]. Thus, we synthesized the degenerated forward and reverse primers, Fw1and Rv1, on the basis of the N-terminal and the internal amino-acid sequences, respectively (Table 1). The PCR using Fw1 and Rv1 primers successfully amplified a cDNA encoding the internal region of LbAly35, i.e., LbAly35-cDNA-1 (317 bp) (Fig. 2). Then, the cDNA encoding 3′- and 5′-terminal regions of LbAly35-cDNA-1 were amplified by 3′- and 5′-RACE PCRs using appropriate specific primers (Fig. 2 and Table 1). Finally, a cDNA covering entire translational region of LbAly35, cDNA-Full (1,068 bp), was amplified with FullFw and FullRv primers (Figs. 1 and 2). In the 3'-terminal region of the cDNA, a putative polyadenylation signal sequence, CATAAA, and a poly (A)+ tail were found. The nucleotide and deduced amino-acid sequences are available from the DNA Data Bank of Japan (DDBJ) with the accession number AB704758.

In the cDNA-Full, an open reading frame of 891 bp was found in nucleotide positions from 75 to 965 (Fig. 2). Accordingly, an amino-acid sequence of 296 residues was deduced from the translational region of 891 bp. The N-terminal region of 22 residues of the deduced sequence except for the initiation Met, KAETQLCLCLVVLVTVLSGVNP, was predicted as the signal peptide for secretion according to the method of von Heijne [37] and the following region of 10 residues, STSHQSNTKR, was regarded as a propeptide-like region of this enzyme since this region was absent in the purified LbAly35 protein (Fig. 3). Accordingly, the mature LbAly35 was considered to consist of 263 residues with the calculated molecular mass of 29,409.9 Da. The molecular mass was much smaller than that estimated by the SDS-PAGE, i.e., ~35,000 Da. The reason for this inconsistency in molecular masses has remained unclear; however, we now consider that the post-translational glycosylation may take place in this enzyme increasing the molecular mass since turban-shell and abalone alginate lyases have been suggested to be glycosylated [25, 26].

The basic local alignment search (BLAST) on sequence databases revealed that the deduced amino-acid sequence of LbAly35 showed considerable similarity to PL-14 alginate lyases from mollusks and *Chlorella* virus. Namely, LbAly35 shared 48%, 48%, 47%, 40% and 23% amino-acid identities with those of abalone HdAly [26], turban-shell SP2 [25], abalone HdAlex [27], sea hare AkAly30 [30] and *Chlorella* virus vAL-1 [34], respectively (Fig. 4). These high sequence identities indicated that LbAly35 is also a member of PL-14 despite of the significantly deviated N-terminal sequence. It has been shown that highly conserved regions occur among PL-14 enzymes (boxed in Fig. 4). These regions correspond to the strands A3-A6 and loop L1 which constitute the active cleft of vAL-1 and AkAly30 [30, 34]. In the vAL-1 sequence, amino-acid residues, K197, H213, R221, Y233 and Y235 were located on the surface of the active cleft contributing to the catalytic action and/or substrate binding. These residues were completely conserved in LbAly35 as K100, H117, R125, Y137 and Y139 (Fig. 4). However, another residue, S219, which was also found to play key roles in the catalytic reaction and/or substrate binding in vAL-1, was replaced by T123 in LbAly35. Two cysteine pairs, C106-C115 and C145-C150, which were suggested to form disulfide bonds in turban-shell SP2 [25], were also conserved in LbALy35 as C112-C121 and C150-C157 (Fig. 4). On the other hand, N105 which has been suggested as a carbohydrate-chain anchoring residue in SP2 [25] and also conserved in HdAly and HdAlex, was replaced by K110 in LbAly35. Thus, except for some replacements, most of the conserved and catalytically important amino acid residues of PL-14 enzymes were conserved in LbAly35. From these characteristics in the primary structure, we conclude that LbAly35 is a new member of PL-14.

Discussion

The small marine snail *L. brevicula* was shown to be a good source for alginate lyase [38]. Several alginate lyase isozymes were detected in the hepatopancreas extract of *L. brevicula* after the Biogel-alginate affinity chromatography and one of these isozyme designated as “alginate lyase VI” was isolated [23, 39]. The enzymatic properties of alginate lyase VI were investigated but no primary structure data of this enzyme was provided. In our previous study we also purified three alginate lyase isozymes, LbAly35, LbAly32 and LbAly28, with the molecular masses of 35, 32, and 28 kDa, respectively, from *L. brevicula* [28]. The N-terminal amino acid sequences of *L. brevicula* alginate lyases showed a similarity less than 15% to those of other molluscan PL-14 enzymes and practically no similarity to any protein sequences currently deposited in the databases. In addition, these *Littorina* alginate lyase isozymes showed no cross-reactivity with rabbit anti-abalone alginate lyase antiserum [28]. These results led us to consider that primary/higher-order structures of *Littorina* alginate lyases are somewhat different from those of other molluscan alginate lyases. Therefore, in the present study, we determined the complete amino acid sequence of the major alginate lyase isozyme LbAly35 and compared it with those of other molluscan alginate lyases.

The N-terminal regions of molluscan alginate lyases appeared to be deviated depending on the order of enzyme-source animal. Namely, alginate lyases from *H. discus hannai* and *T. cornutus,* which belong to Archeogastropoda, shared 80% amino-acid identity with each other in the N-terminal 20 amino acid residues (Fig. 4). On the other hand, the alginate lyase LbAly35 from *L. brevicula*, which belongs to Mesogastropoda, showed only 15% and 20% identities in the N-terminal sequences with *Haliotis* and *Turbo* enzymes, respectively. Whereas, AkAly30 from *A. kurodai*, which belongs to Opisthobranchia, showed only 5%, 10% and 15% identities with *Haliotis*, *Turbo* and *Littorina* enzymes, respectively. The high divergence of N-terminal sequence at order level may cause some differences in enzyme properties. For example, the above molluscan alginate lyases were shown to be different from each other in the degree of temperature and pH stabilities [24, 26, 28, 30]. The temperatures that caused a half inactivation during 20-min incubation were 43^{o}C, 48^{o}C and 50^{o}C for *Haliotis*, *Aplysia* and *Littorina* alginate lyases, respectively [28, 30]. The higher thermal stability of *Littorina* enzyme than abalone enzymes was also confirmed in the present study (Fig. 1). The degree of stability for the molluscan enzymes appeared to be related to the habitat temperatures for the enzyme-producing animals. Namely, *Haliotis* inhabits under the tidal zone where habitat temperature is modestly changed around 10-15^{o}C. Whereas *A. kurodai* inhabits in the region where habitat temperature frequently increases above 30^{o}C in summer [29, 30]. On the other hand, *Littorina* inhabits a tidal zone where the habitat temperature changes from 15^{o}C to 40^{o}C in a day due to the exposure to direct sunshine as well as the ebb and flow of the tide [28]. Thus, the molluscan alginate lyases may have been evolved concomitantly with the adaptation of the animals to the temperature environment. Thus, the highly deviated N-terminal region may relate to the molecular adaptation to temperature conditions. It may be possible to investigate the significance of N-terminal region in the heat stability of alginate lyase by using chimeric alginate lyases with replaced N-terminal regions among heat-stable and heat-unstable enzymes. We are now constructing a bacterial expression system for this enzyme to produce above chimeric enzymes.

The deduced amino-acid sequence of LbAly35 was comprised of a putative signal peptide region of 22 residues, a propeptide-like region of 10 residues, and a mature enzyme domain of 263 residues (Fig. 3). The occurrence of propeptide-like region is not in common among gastropod molluscan alginate lyases and absent in the deduced amino-acid sequence of abalone HdAly [26] and HdAlex [27]. Whereas the corresponding region of 9 residues was found in the deduced sequence of sea hare AkAly30 [30]. The physiological roles of such propeptide-like regions are still obscure in alginate lyases; however, propeptides 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 [40].

The amino-acid sequence of mature LbAly35 domain showed 40% to 48% identities with those of abalone HdAly, turban-shell SP2, abalone HdAlex, sea hare AkAly30 and 23% identity with *Chlorella* virus vAL-1 (Fig. 4). These high sequence similarities indicate that LbAly35 is also a member of PL-14. Three-dimensional structure of the catalytic domain of *Chlorella* virus vAL-1 was solved and the amino-acid residues responsible for the catalytic action were shown to be K197, S219, R221, Y233 and Y235. These amino-acid residues were present in β-strands A3-A6 and loops L-1–L-2 which were surrounding the active cleft of this enzyme [34]. These residues were also found in sea hare AkAly30 as K99, S126, R128, Y140 and Y142 in sea hare AkAly30 [30]. In case of LbAly35, among these 5 residues, K100, R125, Y137 and Y139 were conserved. The occurrence of such residues in LbAly35 suggested that

LbAly35 possessed the catalytic sites similar to that of other PL-14 molluscan alginate lyases. Thus, LbAly35 was also classified as a member to PL-14 alginate lyase.

Finally, we made a phylogenetic tree on the basis of amino acid sequences of PL-14 alginate lyases from mollusks and *Chlorella* virus (Fig. 5). LbAly35 was found to form a cluster with other molluscan PL-14 alginate lyases but deviated from *Chlorella* virus, vAL-1. The relationship among molluscan enzymes in the tree appeared to be well consistent with the orders of enzyme-source animals. This indicates that molecular deviation of alginate lyase well reflects the phylogenetic relationship of molluscan species.

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Legends to figures

Fig. 1 Heat-inactivation profiles for LbAly35 and HdAly.

LbAly35 (●, ▲) and HdAly (○, △) were incubated at 40°C (●, ○) and 45°C (▲, △) for various time intervals, and the activity remaining after the incubation for LbAly35 was determined in the standard assay conditions. Logarithm values for relative activities are plotted against heating time.

Fig. 2 Schematic diagram for LbAly35-cDNA.

Closed and open boxes indicate coding and non-coding regions of LbAly35-cDNA, respectively. The numbers in the top of the figure indicate the nucleotide positions. Relative positions for LbAly35-cDNA-1, cDNA-3RACE, cDNA-5RACE, and cDNA-Full are indicated with thin lines along with the positions of PCR primers indicated with bold lines.

Fig. 3 The nucleotide and deduced amino-acid sequences of LbAly35-cDNA.

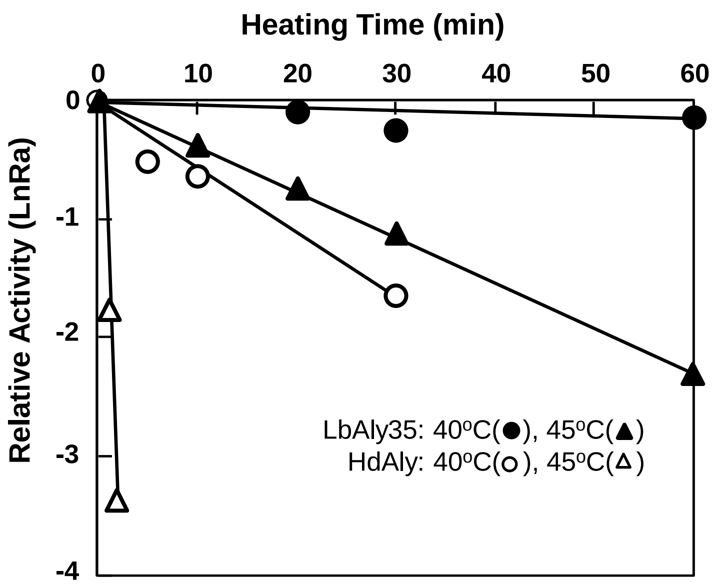
Residue numbers for both nucleotide and amino-acid sequences are indicated in the right of each row. The translational initiation codon ATG, termination codon TAG, and a putative polyadenylation signal, CATAAA, are boxed. A putative signal peptide is indicated by a dotted underline. The positions of FullFw and FullRv 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, AB704758.

Fig. 4 Alignment of amino acid sequences of LbAly35 with those of other PL-14 enzymes.

The amino-acid sequence of LbAly35 was aligned with those of abalone HdAly [26] and HdAlex [27], turban-shell SP2 [25], sea hare AkAly30 [30] and *Chlorella* virus vAL-1[34]. Identical, highly conservative, and conservative residues among sequences are indicated by asterick (*), colon (:), dot (.), respectively. The amino acid residues conserved among PL-14 enzymes are boxed and the catalytically important amino-acid residues for PL-14 enzymes are shaded. Highly deviated N-terminal region among molluscan enzymes at their order level are indicated by a dotted box.

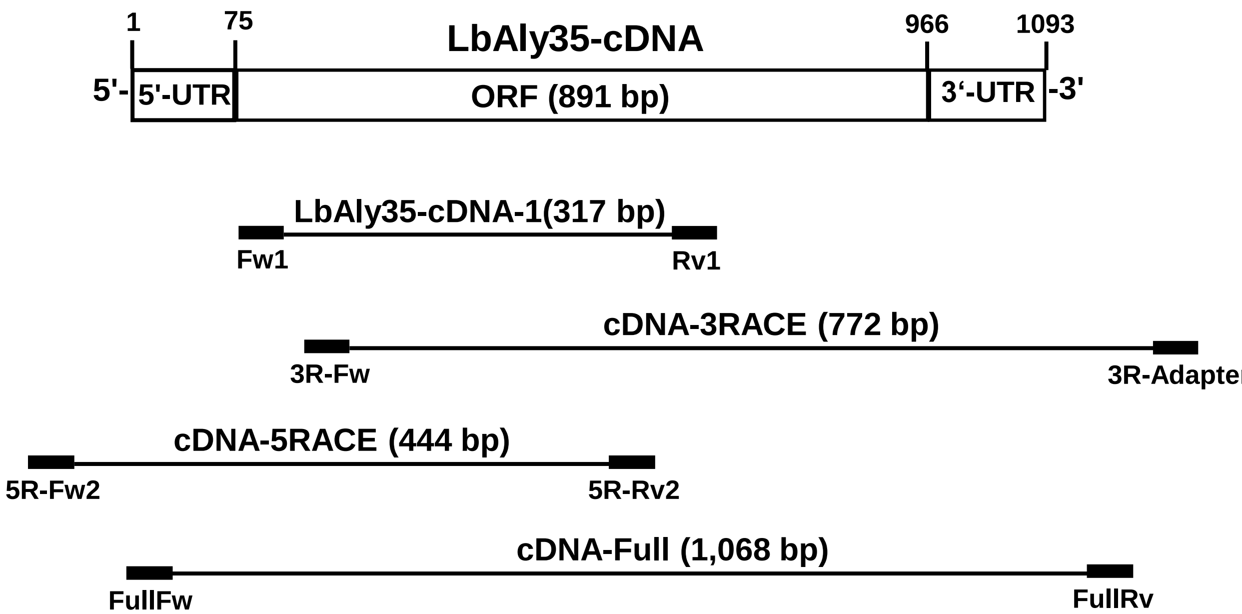
Fig. 5 Phylogenetic relationship for the PL-14 alginate lyases. The rooted scaled phylogenetic tree with branch length was drawn by the unweighted pair group method with arithmetic mean (UPGMA) using the sequences of alginate lyases from *Littorina brevicula* LbAly35 (DDBJ accession number, AB704758, boxed in the tree), *Haliotis discus discus* AlgHDD (DDBJ accession number, AB199614), *Haliotis discus hannai* HdAly (DDBJ accession number, AB110094) and HdAlex (DDBJ accession number, AB234872), *Turbo cornutus* SP2 (no accession number but see reference 25), *Aplysia kurodai* AkAly30 (DDBJ accession number, AB610185), and *Chlorella* virus vAL-1 (DDBJ accession number, AB044791). One bacterial PL-7 alginate lyase *alyVOB* from *Vibrio* sp. O2 (DDBJ accession number, DQ235161) [41] is used as an outer group.

Fig. 1.



Heating Time (min)
0
10
20
30
40
50
60
0
-1
-2
-3
-4
Relative Activity (LnRa)
LbAly35: 40ºC(●), 45ºC(▲)
HdAly: 40ºC(○), 45ºC(△)

Fig. 2.



1
75
LbAly35-cDNA
966
1093
5'-
5'-UTR
ORF (891 bp)
3'-UTR
-3'
LbAly35-cDNA-1(317 bp)
Fw1
Rv1
cDNA-3RACE (772 bp)
3R-Fw
3R-Adapter
cDNA-5RACE (444 bp)
5R-Fw2
5R-Rv2
cDNA-Full (1,068 bp)
FullFw
FullRv

Fig. 3.

```
TACGAGGGGACTGGAATTGCCTTCGTTTCATGCTGAACAATTATCATTTCTACCTGCCTTTGAGCGTTGAAAAAATGAAG    80
                                                                         M  K        2
GCCGAAACACAACTCTGTCTGTGCCTTGTGGTACTGGTCACTGTGTTGAGCGGGGTGAACCCCTCGACCTCACATCAGAG   160
 A  E  T  Q  L  C  L  C  L  V  V  L  V  T  V  L  S  G  V  N  P  S  T  S  H  Q  S    29
CAATACGAAAAGAGCGAGCGGCACAGAACTGTTTCGTCACACCACCTTCACGGATGGCAGCATCAGCGAGGCGTTGTCCG   240
  N  T  K  R  A  S  G  T  E  L  F  R  H  T  T  F  T  D  G  S  I  S  E  A  L  S     55
ACTTCCACGTGCAGAACATGTGGGGAGCCAACGCTCTCTCCGTGGTGCCAGACCCTGCTGGGGGCACAGACAAGGTGCTG   320
D  F  H  V  Q  N  M  W  G  A  N  A  L  S  V  V  P  D  P  A  G  G  T  D  K  V  L     82
AGGGTTCACTACGCGAAAGGCAGCTTCTCTCACACTCACGACAGAGACTACGGGGCGGGGTTCTACGCCACGCCTATACC   400
 R  V  H  Y  A  K  G  S  F  S  H  T  H  D  R  D  Y  G  A  G  F  Y  A  T  P  I  P   109
CCCTCGCACCGCCATGATGCTCAGCTATGACGTCTTCTTCCAGGACAACTTTCACTTCGTGTTGGGGGGGAAACTGCCGG   480
  P  R  T  A  M  M  L  S  Y  D  V  F  F  Q  D  N  F  H  F  V  L  G  G  K  L  P    135
GTCTGTGGGGAGGTGCTATGAAGAGCTGTTCCGGAGGCCGCCACTCGGATGACTGCTTCACCACACGCTTCATGTGGAGG   560
G  L  W  G  G  A  M  K  S  C  S  G  G  R  H  S  D  D  C  F  T  T  R  F  M  W  R    162
GACGGGGCGAGGGGGGAGGTGTACGTCTACCTCCCTCCAGCTGAGCAGACCGGCAGCTTCTGCAACCGTACTGACGTGGA   640
 D  G  A  R  G  E  V  Y  V  Y  L  P  P  A  E  Q  T  G  S  F  C  N  R  T  D  V  E   189
GTGCTTCCCCCTGAAGGGCAACTCGCTGGGGCGGGGCAAGTGGCACTTCAAGCTCAACCAGTGGCAGAACATGGCGCAGT   720
  C  F  P  L  K  G  N  S  L  G  R  G  K  W  H  F  K  L  N  Q  W  Q  N  M  A  Q    215
ACGTCCACCTCAACGACATCGGGCAGAGGAACGGATATGTCAAGGTGTTTGTGGACGGCCAGAAGGTGTACGAGGGGAGG   800
Y  V  H  L  N  D  I  G  Q  R  N  G  Y  V  K  V  F  V  D  G  Q  K  V  Y  E  G  R    242
GACCTGGTGCTGAGGACCAAATCCTCCATCAACATCGACGGCATGTACTTCAGCACGTTCTTCGGCGGTGCCAACAGTAG   880
 D  L  V  L  R  T  K  S  S  I  N  I  D  G  M  Y  F  S  T  F  F  G  G  A  N  S  S   269
CTGGGCCACGCCTGTCGACACCCACACCTACTTCAAGAACTTTGTCTTCTCCACTGACCCTGATCACCCAACCATGATTG   960
  W  A  T  P  V  D  T  H  T  Y  F  K  N  F  V  F  S  T  D  P  D  H  P  T  M  I    295
GCTAGAATAATATGATTGACGGACGTAAACCACGCCTGTCGATACCTTATCATCTCACTCTGATTGGCTAGAATTGTATG  1040
G  *                                                                               296
ATTGACGGACATAAACCACGCCTGTCGATACCTTAAGCAAAAAAAAAAAAAAA                             1093
```

FullFw

Signal peptide

N-terminus

FullRv

Fig. 4.

```
LbAly35   1 ASGTELFRHTTFTDGSISEALSDFHVQNMWGANALSVVPDPAGGTDKVLRVHYAKGSFSH   60
HdAly     1    AVLWTHKEFDPANYRNGMHAL-TSNDYDHGSGSVVTDPDGGSNHVLRVWYEKGRYSS   56
SP2       1    TLLWTHKEFDPNNYRDGMHAL-TSNDYDHGSGKVVTDPDGGSNHVLRVWYEKGRWSS   56
HdAlex    1    SIVWTHNEFDPAYFRNGMHSP-VTDEDVNGSATVVPDPNGGSNLVLKVFYEKGSYSH   56
AkAly30   1   ATTVWSLSSVPHSSHVSTILGHFKPIYHDWGDDSISTSTKHSSSRALRIFYEKGSYSK   58
vAL     106      TNVISTLDLNLLTKGGGSWNVDGVNMKKSAVTTFD----GKRVVKAVYDKNSGTS  156
                     . .      .                    .     . .::  * *.  :

LbAly35     THDRDYGAGFYATP---IPPRTAMMLSYDVFFQDNFHFVLGGKLPGLWGGAMKS----CS  113
HdAly       -HGPNEGVQFFATP---TQDHSIMTFSYDVYFDKNFDFRRGGKLPGLFGGWTN-----CS  107
SP2         -HGPNEGVQFFATP---TQDHSVMTFSYDLYLSHDFDFRRGGKLPGLYGGWTN-----CS  107
HdAlex      -HGPNSKVQFFATP---TKPRVAMTLSYDVRFDPNFDFRIGGKLPGLYGGLVN-----CS  107
AkAly30     VHDHR-GAGFYSRPSAISSSVDAMILKYDVYFEN-FGFGIGGKLPGLFGGENGEGAYKCS  116
vAL         ANPGVGGFSFSAVP--DGLNKNAITFAWEVFYPKGFDFARGGKHGGTFIGHGA-----AS  209
             :       * : *         : : :::     * *  ***  * : *        .*

LbAly35     GGRHSDDCFTTRFMWRDGARGEVYVYLP-PAEQTGSFCNRTDVECFPLKGNSLGRGKWHF  172
HdAly       GGRHSDNCFSTRFMWRADGDGEVYGYIQNKDHQIDGFCDHV--VCNSIKGYSMGRGKWRF  165
SP2         GGRHSDNCFSTRFMWRKDGDGEVYAYIPDYHHQVSGFCDHN--VCNSVKGYSLGRGKWKF  165
HdAlex      GGRHSDDCFSTRFMWRDNGDGEVYGYVPDQSHQLPGFCTKN--ICDPVKGFSFGRGSWRF  165
AkAly30     GGSNPSSCFSLRLMWRKDGDGELYAYIP--TNQESGFKDRDDVIAHSTYGQSLGRGKFRF  174
vAL         GYQHSKTGASNRIMWQEKGGVIDYIYPPSDLKQKIPGLDPE----GHGIGFFQDDFKNAL  265
            *  :..   : *:**:  .    * *     .*                *   .  .  :

LbAly35     KLNQWQNMAQYVHLNDIG----QRNGYVKVFVDGQ----KVYEGRDLVLRTKSSINIDGM  224
HdAly       QRGKWQNIAQSVKLNTPG----KTDGSIKVWYNGK----LVFTIDQLNIRAKASVDLDGI  217
SP2         ERGKWQNIAQHVHLNTPG----KTDGSIKVWHNGK----LVYTIDQLNIVSKASVDIDGI  217
HdAlex      QRGVWQTIAQSIKLNTPG----STDGAIKVVYNGK----VVYASNNLALRSQSDVNIDGI  217
AkAly30     MNNKWHSISEEVHINTVG----KTDGWVKICVQAEGHSQQCYTANHLRMRNTNSHHLRGM  230
vAL         KYDVWNRIEIGTKMNTFKNGIPQLDGESYVIVNGK-----KEVLKRINWSRSPDLLISRF  320
              . *: :    ::*       . :*   :  :.:           :      .  :  :

LbAly35     YFSTFFGGANSSWATPVDTHTYFKNFVFSTDPDHPTMIG                       263
HdAly       FFSTFFGGHDSTWAPTHDCYSYFKNFVLSTDSGHPTIIG                       256
SP2         FFSTFFGGSDSSWAPTHDCYSYFKNFALSTDSSHPTIL                        255
HdAlex      FFSTFFGGSYANWAPTRDCYTWFKNFAISFDTGPEVAVG                       256
AkAly30     FFSTFFGGSEKSYAAPNDCYSYFKNFQILTPS--HAVVG                       267
vAL         DWNTFFGG---PLPSPKNQVAYFTNFQMKKYELE                            351
             :.*****     ... :  ::*.** :
```

Fig. 5.

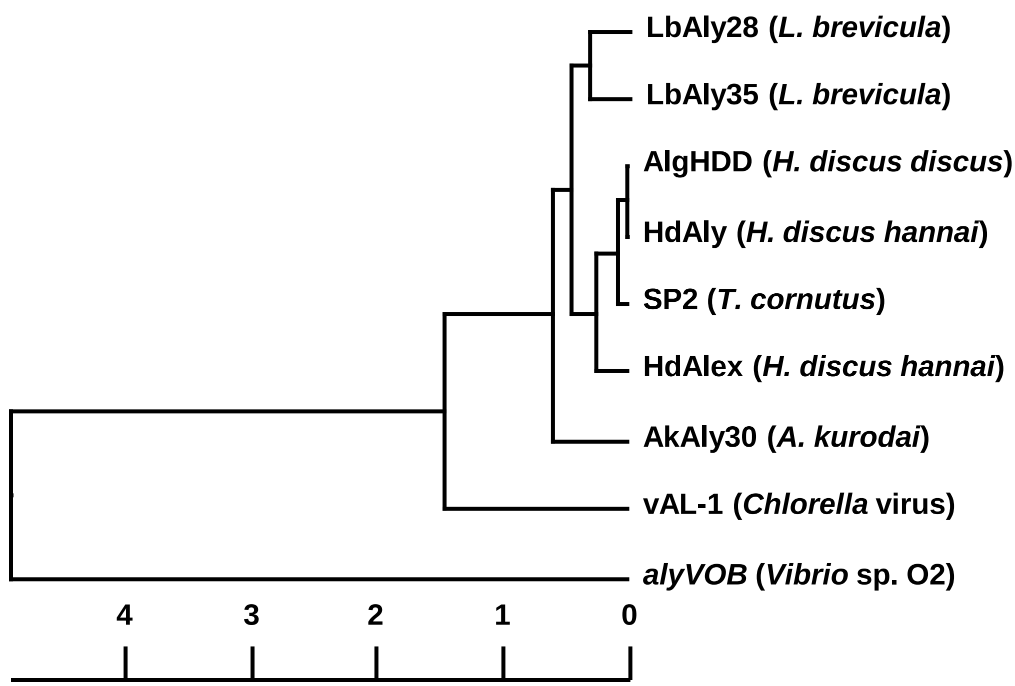

LbAly28 (L. brevicula)
LbAly35 (L. brevicula)
AlgHDD (H. discus discus)
HdAly (H. discus hannai)
SP2 (T. cornutus)
HdAlex (H. discus hannai)
AkAly30 (A. kurodai)
vAL-1 (Chlorella virus)
alyVOB (Vibrio sp. O2)
4
3
2
1
0

Table1. PCR primers used for amplification LbAly35 cDNA.

	Primer names	Sequences[a, b]
1st PCR	Fw1	5′-GARYTNTTYMGNCAYACNACNTTYACNGA-3′
		(amino-acid sequence: ELFRHTTFTD)
	Rv1	5′-TTCATNGCNCCNCCCCADATNCCNGG-3′
		(amino-acid sequence: PGLWGGAMK)
3'-RACE	3R-Fw	5′-AGGGTTCACTACGCGAATGG-3′
	3R-Adapter	5′-CTGATCTAGAGGTACCGGATCC-3′
5'-RACE	5R-Fw1	5′-GCAGCTTCTGCAACCGTACG-3′
	5R-Rv1	5′-GGAGGGAGGTAGACGTACAC-3′
	5R-Fw2	5′-GCAAGTGGCACTTCAAGCTC-3′
	5R-Rv2	5′-CCTGGAAGAAGACGTCATAG-3′
Full RACE	FullFw	5′-TACGAGGGGACTGGAATTGC-3′
	FullRv	5′-TCGACAGGCGTGGTTTACGTC-3′

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