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cDNA cloning of an alginate lyase from abalone, *Haliotis discus hannai*

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Abstract

An alginate lyase, termed HdAly in the present paper, was isolated from the hepatopancreas of abalone, *Haliotis discus hannai*, by ammonium sulfate fractionation, followed by TOYOPEARL CM-650M column chromatography. Enzymatic properties of HdAly were similar to those of previously reported *Haliotis* and *Turbo* poly(M) lyases, e.g., it preferentially degraded a poly(β-D-mannuronate)-rich substrate with an optimal pH and temperature at pH 8.0 and 45 °C, respectively. In order to determine the primary structure of abalone lyase that is still poorly understood, cDNAs for HdAly were cloned by PCR from the abalone hepatopancreas cDNA library and sequenced. From the nucleotide sequences of the cDNAs, the sequence of 909 bp in total was determined, and the amino acid sequence of 273 residues was deduced from the translational region of 822 bp locating at nucleotide positions 27–848. The N-terminal region of 16 residues, except for the initiation Met in the deduced sequence, was regarded as the signal peptide since it was absent in the HdAly protein and showed high similarity to the consensus sequence for signal peptides of eukaryote secretary proteins. This suggests that HdAly is initially produced as a precursor possessing the signal peptide in hepatopancreatic cells and then secreted into digestive tract as the mature form. Thus, the mature HdAly was regarded to consist of 256 residues with the calculated molecular mass of 28895.5 Da. The amino acid sequence of HdAly showed 85 and 28% identity to those of *Turbo cornutus* alginate lyase SP2 and the C-terminal region of *Chlorella* virus lyase-like protein CL2, respectively, while it showed no significant identity to those of any bacterial alginate lyases. In order to provide the basis for the structure–function studies and various applications of the abalone lyase, a bacterial expression system was constructed by means of the HdAly-cDNA and pET-3a expression plasmid. Although the active recombinant HdAly was hardly produced at a cultivation temperature 37 °C in *Escherichia coli* BL21 (DE3), a small amount of soluble and active enzyme could be produced when the temperature was lowered to 19 °C.

Keywords: Alginate lyase; Abalone; Haliotis; Primary structures; cDNA cloning

1. Introduction

Alginate is a major structural heteropolysaccharide in brown algae that is comprised of (1→4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G), which are arranged as homopolymeric M- and G-blocks and alternating or random heteropolymeric M/G-blocks.[1] Alginate lyase is the enzyme that degrades the alginate by a β-elimination mechanism forming 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate at the new nonreducing terminus.[2,3] This enzyme has been shown to distribute in mollusks,[4–6] seaweeds,[7,8] and bacteria,[2,3,9–13] and utilized for the analysis of the fine structure of alginates[1,2,14–16] and production of protoplasts from seaweeds.[6,17–19] Based on the substrate specificity, the alginate lyase was primarily classified into poly(M)-lyase [EC 4.2.2.3] and poly(G)-lyase [EC

Abbreviations: SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

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4.2.2.11] acting preferentially on the poly(M)- and poly(G)-blocks, respectively. While, multifunctional enzymes that can act on both poly(M)- and poly(G)-blocks have been found in *Alteromonas* sp.[11,20] The alginate lyases from mollusks such as *Haliotis*, *Dlabella*, *Littorina*, *Aplysia*, and *Turbo* are classified into poly(M)-lyase.[4–6,21–25]

Up to now, primary structure analyses of alginate lyases have been performed mainly on bacterial enzymes, and more than 20 genes of bacterial enzymes have been cloned.[1,2,11–13] Further, the three-dimensional structure of a bacterial enzyme A1-III from *Sphingomonas* sp. A1 was recently solved,[26] and interest in this bacterium is now focused on the biochemical pathways for utilization of alginate.[27,28] Compared with those advances in bacterial alginate lyases, studies on structures of the molluscan enzymes are still limited, i.e., the amino acid sequence of a molluscan enzyme has been determined only in *Turbo cornutus* SP2 by the protein method,[29] while no cDNA or gene for the molluscan enzymes has been cloned. The amino acid sequence of *Turbo* SP2 shows practically no homology with any bacterial enzymes, but a 19.8% identity with a C-terminal region of the alginate lyase-like protein CL2 from a *Chlorella* virus is indicated.[30] The biological implication for this similarity between *Turbo* and *Chlorella* virus proteins remains obscure. The higher order structure of *Turbo* SP2 was suggested to be rich in the β-structure,[24] which is different from bacterial enzymes that contain substantial amount of α-helices.[2,10,15,26] These structural features in *Turbo* SP2 lead us to consider that molluscan alginate lyases may generally possess such structural characteristics distinct from those of bacterial enzymes. In order to verify this point, it is essential to determine primary structures of many other molluscan enzymes and study them comparatively.

Therefore, in the present study, we focus on the abalone alginate lyase that has been investigated for a long time, together with the *Turbo* enzyme. The primary structure has not been determined. First, we isolated and characterized the alginate lyase from the common Japanese abalone, *Haliotis discus hannai*, and demonstrated that this enzyme is a typical molluscan poly(M)-lyase. Then, we amplified the cDNA for the abalone lyase by PCR to deduce the amino acid sequence. Further, we attempted to construct the bacterial expression system for the abalone lyase in order to provide the basis for structure–function analyses. We have also considered the utilization of the recombinant enzyme for various applications such as the manufacture of oligoalginates that have been shown to be beneficial for therapeutic utility and food additives. To our knowledge, this is the first report on the cDNA cloning and bacterial expression of molluscan alginate lyase

2. Experimental

2.1. Materials

Abalone, *H. discus hannai*, was purchased from a local market in Hakodate, Hokkaido Prefecture, Japan. TOYOPEARL CM-650M was purchased from Toyo Soda Mfg, Co. (Tokyo, Japan). Lysylendopeptidase, Oligotex-dT(30), TaKaRa *Taq* DNA polymerase, 5′- and 3′-Full RACE kits, and restriction endonucleases were purchased from TaKaRa (Tokyo, Japan). The pCR-TOPO 2.1 TA cloning kit was purchased from Invitrogen (CA, USA). The other chemicals used were reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Preparation of crude alginate lyase

At first, distribution of the alginate lyase in digestive fluid and tissues of abalone was examined as follows: digestive fluid (~ 2.5 mL) of an abalone (shell size 8 × 6 cm) was aspirated with a 1-mL plastic syringe from the digestive tract between the stomach and hepatopancreas and centrifuged at 100,000*g* for 1 h to remove insoluble materials. The stomach, intestine, and hepatopancreas of the abalone were carefully excised and rinsed with 10 mM sodium phosphate (pH 7.0). The tissues were then separately homogenized in 2.5 mL of 10 mM sodium phosphate (pH 7.0) and extracted at 4 °C for 30 min. The tissue extracts were obtained by centrifugation at 100,000*g* for 1 h. As shown in Table 1, total lyase activity was the highest in the digestive fluid, while the activity in the hepatopancreas extract was approximately one-half of that in the digestive fluid. The activity in the stomach and intestinal extracts were one-tenth or less than that in the digestive fluid. Thus, it was indicated that the alginate lyase was produced in the hepatopancreas and secreted into the digestive fluid. Therefore, alginate lyase was prepared from the digestive fluid and hepatopancreas according to the method of Boyen and coworkers[6] with some modifications: digestive fluid (~ 25 mL) and the hepatopancreas (~ 125 g) from 10 abalones were combined and suspended in 250 mL of 10 mM sodium phosphate (pH 7.0) containing 0.2% sodium azide, 0.1 mM phenylmethanesulfonyl fluoride and 1 mM EDTA, and the enzyme was extracted at 4 °C for 30 min with occasionally stirring. The extract was obtained by centrifugation at 100,000*g* for 1 h. Then, solid $(NH_4)_2SO_4$ was added to the supernatant to make 70% saturation, and the precipitates formed were spun down by centrifugation at 10,000*g* for 20 min. This procedure removed most of cellulases, which frequently obstruct dialysis by tearing cellulose tubes, from the alginate lyase in the supernatant. Then, the alginate lyase was precipitated by further addition of solid $(NH_4)_2SO_4$ to

Table 1
Distribution of alginate lyase in digestive fluid and tissues of abalone

Samples	Total activity (U)[a]	Specific activity (U mg^{-1})	Total protein (mg)
Digestive fluid	1100	18.0	61
Stomach [b]	80	10.0	8
Intestine [b]	20	0.4	50
Hepatopancreas [b]	510	2.0	255

[a] One unit of lyase activity is defined as the amount of enzyme that increases the absorbance at 235 nm to 0.01 for 1 min.
[b] Extract from each tissue.

make 100% saturation, and collected by centrifugation at 10,000*g* for 20 min. The precipitates were dissolved in and dialyzed against 10 mM sodium phosphate (pH 7.0), and the small amount of precipitates formed during the dialysis was removed by a brief centrifugation. The crude alginate lyase thus obtained was subjected to TOYOPEARL CM-650M column chromatography (see under Section 3).

2.3. Substrates

Sodium alginate (*Macrocystis pyrifera* origin, Sigma-Aldrich (MO, USA)) was dissolved in distilled water to make 1% (w/w) and heated at 90 °C for 1 h before use. Poly(M)-rich, Poly(G)-rich, and random(MG) substrates were prepared by the method of Gacesa and Wusteman.[31] Mannuronate and guluronate contents in the substrates were estimated by the method of Morris and coworkers.[32] The mannuronate content in the original alginate was ~60%, while those in the poly(M)-rich substrate and the random(MG) substrate were estimated as 86 and 64%, respectively. The guluronate content in the poly(G)-rich substrate was 99%.

2.4. Lyase activity assay

The alginate lyase activity was assayed in a 3-mL of reaction mixture containing 0.1% substrate, 50 mM sodium phosphate (pH 7.0) and an appropriate amount of enzyme at 30 °C. The reaction was initiated by the addition of the enzyme (usually 0.05–0.1 mL). The progress of the reaction was monitored by measuring the absorbance at 235 nm with a spectrophotometer (HITACHI Model 200-10, Tokyo, Japan) equipped by a temperature-control device (TAITEC SP-12R, Tokyo, Japan). One unit of lyase activity is defined as the amount of enzyme that increases Abs_{235nm} to 0.01 for 1 min. For the measurement of pH dependence, the pH of the reaction mixture was varied with 50 mM sodium phosphate (pH 5.0–11.5). Temperature dependence of the activity was measured at 15–55 °C in 50 mM sodium phosphate (pH 7.0).

2.5. Size estimation of oligo-alginates

The sizes of oligo-alginates resulted by the lyase digestion of poly(M)-rich block substrate were estimated by gel filtration through Bio-Gel P2 (Bio-Rad Laboratories, CA, USA) or thin-layer chromatography (TLC) on HPTLC-60 plates (GL Sciences, Tokyo, Japan). The size estimation was confirmed with a JMS-700TZ ESI mass spectrometer (JEOL, Tokyo, Japan)

2.6. SDS-PAGE

SDS-PAGE was carried out with 0.1% SDS–10% polyacrylamide slab gel by the method of Porzio and Pearson.[33] After the electrophoresis, the gel was stained with 0.2% Coomassie Brilliant Blue R250, and the background of the gel was destained with 5% MeOH–7% AcOH.

2.7. Protein determination

Protein concentration was determined by the biuret method[34] or the method of Lowry and coworkers[35] using bovine serum albumin (BSA) as a standard protein.

2.8. Circular dichroism

Circular dichroism of the abalone lyase was measured at 15 °C in a solution containing 50 mM sodium phosphate (pH 7.0) and 0.5 mg mL^{-1} of enzyme in a cell with a light pathlength of 1 mm by using a J-600 spectropolarimeter (JASCO, Tokyo, Japan).

2.9. Determination of amino acid sequence

The amino acid sequence of the N-terminus of the abalone alginate lyase was determined with an ABI 473A protein sequencer (Applied Biosystems, CA, USA), and the specimen that had been transferred on a PVDF membrane after SDS-PAGE. To determine the amino acid sequences of internal regions, peptide fragments of the lyase were prepared as follows: 1 mg of the abalone alginate lyase was reduced with 5.0 mM

2-mercaptoethanol in the presence of 8 M urea and 50 mM Tris–HCl (pH 8.0), and carboxymethylated with 5.1 mM monoiodoacetic acid according to the method of Crestfield and coworkers.[36] The thus reduced-carboxymethylated enzyme was dialyzed against 0.1 mM sodium bicarbonate and lyophilized. The lyophilized enzyme was dissolved in 1 mL of 10 mM Tris–HCl (pH 9.0) and digested with 1/100 (w/w) of lysylendopeptidase at 37 °C for 5 h. The digests were applied to an HPLC (HITACHI LP1000, Tokyo, Japan) equipped with a Mightysil RP-18 GP column (4.6 × 150 mm, KANTO Chem., Tokyo, Japan), and peptide fragments were eluted separately by a linear gradient of 0–50% of CH_3CN including 0.1% of trifluoroacetic acid. The amino acid sequences of the fragments were analyzed by the protein sequencer.

2.10. cDNA cloning and nucleotide sequence analysis

Total RNA was prepared from 1 g of abalone hepatopancreas by the guanidinium thiocyanate–phenol method,[37] and mRNA was selected with Oligotex-dT(30) from the total RNA according to the manufacturer's protocol. A cDNA library was constructed from the mRNA with a TaKaRa cDNA synthesis kit using random primer. cDNAs encoding the abalone alginate lyase were amplified by PCR from the cDNA library with TaKaRa *Taq* DNA polymerase, and degenerated primers was synthesized on the basis of partial amino acid sequences of the alginate lyase. PCR was performed in 50 μL of reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM each of dATP, dTTP, dGTP, and dCTP, 1.2 mM $MgCl_2$, and 2 pmol μL^{-1} primers, 1 ng μL^{-1} template DNA, and 0.05 units μL^{-1} TaKaRa *Taq* DNA polymerase. A successive reaction at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 120 s was repeated for 30 cycles with a PC 700 Program Incubator (ASTEC, Fukuoka, Japan). cDNAs for 5′- and 3′-terminal regions of mRNA were amplified with 5′- and 3′-Full RACE kits (TaKaRa, Tokyo, Japan), respectively. The size of cDNA was estimated with 1% agarose-gel electrophoresis. The PCR products were cloned with a pCR-TOPO2.1 TA cloning kit (Invitrogen, CA, USA), and nucleotide sequences of the cDNAs were analyzed with a BigDye-terminator Cycle sequencing kit (Applied Biosystems, CA, USA) and an ABI 310 Genetic Analyzer (Applied Biosystems, CA, USA). Restriction enzymes and T4 DNA ligase were purchased from TaKaRa (Tokyo, Japan).

2.11. Bacterial expression of recombinant enzyme

Nde I and *Bam* HI restriction sites were introduced to the 5′- and 3′-termini of the abalone lyase cDNA by PCR, and the cDNA was inserted between *Nde* I and *Bam* HI restriction sites of pET-3a expression plasmid (Novagen, WI, USA). The plasmid was introduced to *Escherichia coli* BL21(DE3) (Novagen, WI, USA), and the recombinant enzyme was expressed by the induction with 1 mM isopropyl 1-thio-β-D-galactoside. The bacteria containing the recombinant lyase were collected by centrifugation at 5000*g* for 10 min and suspended with 10 vol of the lysis buffer containing 8% (w/v) sucrose, 0.5% (w/v) TritonX-100, 50 mM Tris–HCl (pH 8.0), 50 mM EDTA, and 0.1 mg mL^{-1} lysozyme, and the cells were lysed by repeating freeze and thaw cycles. The cell lysate was centrifuged at 100,000*g* for 1 h, and the lyase activity in the supernatant was determined.

3. Results

3.1. Purification and characterization of abalone alginate lyase

The crude alginate lyase from abalone was subjected to a TOYOPEARL CM-650M column (1.3 × 18 cm) pre-equilibrated with 10 mM sodium phosphate (pH 7.0), and the adsorbed proteins were eluted by a linear gradient of 0–0.3 M NaCl in 10 mM sodium phosphate (pH 7.0). As shown in Fig. 1, a single fraction exhibiting lyase activity against sodium alginate was eluted at around 0.13 M NaCl. According to SDS-PAGE followed by densitometry, the molecular mass and purity of this enzyme were estimated as 28,000 Da and more than 95%, respectively. The thus purified enzyme is

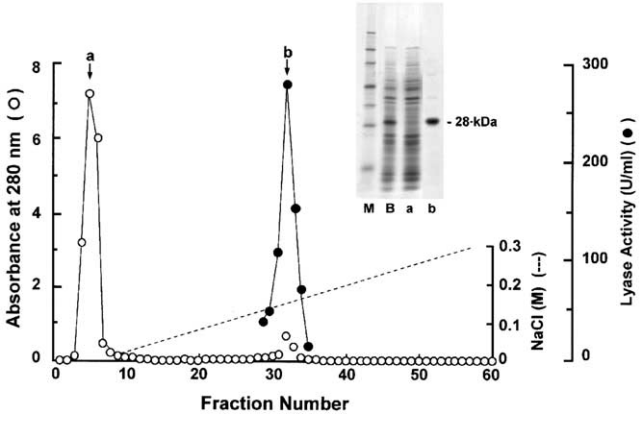

Fig. 1. Purification of HdAly by TOYOPEARL CM-650M column chromatography. The alginate lyase partially purified by ammonium sulfate fractionation between 70–100% saturation was dialyzed against 10 mM sodium phosphate (pH 7.0), then subjected to a column of TOYOPEARL CM-650M (1.3 × 18 cm). The adsorbed proteins were eluted with a 0–0.3 M NaCl linear gradient in 10 mM sodium phosphate (pH 7.0). Each fraction contained 7.0 mL. A 0.05-mL portion of the fraction was used for lyase assay. The SDS-PAGE patterns of the sample before chromatography (B) and fractions indicated by the arrows (a and b) are shown in the inset. M, molecular weight markers.

termed HdAly in the present study. HdAly was regarded as a monomeric enzyme since the molecular mass estimated by gel-filtration on Sephacryl S-200 was 30,000 Da, which is fairly consistent with that estimated by SDS-PAGE (data not shown). By this procedure, 3.2 mg of HdAly with a specific activity being 489 times higher than that of crude enzyme was purified (Table 2). The optimal pH and temperature of the HdAly were at pH 8.0 and 45 °C, respectively (Fig. 2). HdAly showed the highest preference to poly(M)-rich substrate, while practically no activity was shown for poly(G)-rich substrate (Fig. 3). Therefore, HdAly was regarded as a poly(M)-lyase like other molluscan alginate lyases.[4–6] According to the results of Bio-Gel P2 gel filtration (Fig. 4), HdAly appeared to cleave the glycosidic bond between the third and forth residues from the reducing terminus in a pentamer unit yielding tri- and disaccharides. Then, the action of HdAly to the tetra- and trisaccharides obtained in the Bio-Gel P2 gel filtration was further investigated by TLC. As shown in Fig. 5, HdAly could slowly degrade the tetrasaccharide into the trisaccharide and probably unsaturated monosaccharide (invisible in the TLC), but it hardly degraded the trisaccharide. This implies that the spacial expanse of the substrate-binding site in the HdAly is close to the size of tetra- to pentasaccharide.

Fig. 6 shows that circular dichroism of HdAly is of a typical β-structure being similar to that of *Turbo* enzyme.[24] This indicated that HdAly, as well as the *Turbo* enzyme, possess a β-structure as a major structural constituent.

From these enzymatic and structural properties, HdAly was found to be a typical molluscan alginate lyase like the previously reported *Haliotis* and *Turbo* poly(M) lyases.[4–6]

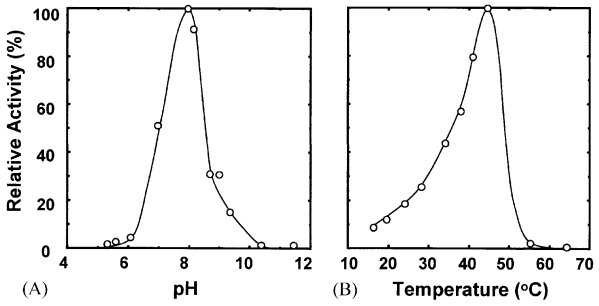

Fig. 2. pH and temperature dependence of HdAly. (A) Activity of HdAly was measured at 30 °C in a reaction mixture containing 50 mM sodium phosphate with pHs indicated in the abscissa; (B) activity of HdAly was measured at various temperatures in a medium containing 50 mM sodium phosphate (pH 7.0).

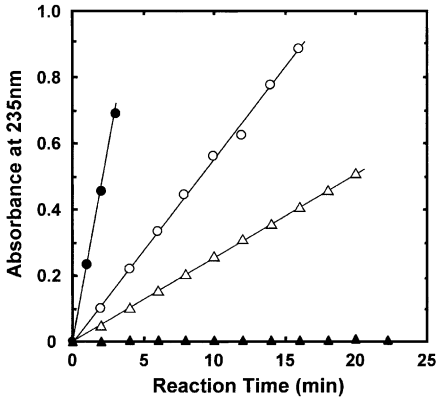

Fig. 3. Substrate specificity of HdAly. Activity of HdAly was measured with either the sodium alginate (○), poly(M)-rich substrate (●), random(MG) substrate (△), or poly(G)-rich substrate (▲) in a concentration of 0.1%.

3.2. Partial amino acid sequence of HdAly

In order to obtain information on the primary structure of HdAly, partial amino acid sequences were analyzed. As shown in Table 3, the N-terminal amino acid sequence of 21 residues for HdAly was determined as AVLWTHKEFDPANYRNGMHAL with a specimen blotted to a PVDF membrane. This sequence showed ~81% amino acid identity with the corresponding sequence of *Turbo* SP2,[29] i.e., 17 residues out of 21 residues were identical between two sequences (see Fig. 11). In addition, the amino acid sequences of four major fragments, L4, L5, L7, and L8, that had been prepared by lysylendopeptidase digestion followed by HPLC, showed 67–90% identity with corresponding sequences of *Turbo* SP2. Among the peptides, L5 was regarded as the fragment locating in the C-terminus of HdAly according to the comparison with the sequence of *Turbo* SP2. These results indicate that HdAly possesses high structural similarity to *Turbo* SP2.

Table 2
Purification of abalone alginate lyase

Preparation	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Extracts	6171	16,739	2.7	100	1
Crude enzyme [a]	80	7349	92	43.9	34
HdAly	3.2	3467	1324	20.7	489

[a] The preparation after the ammonium sulfate fractionation.

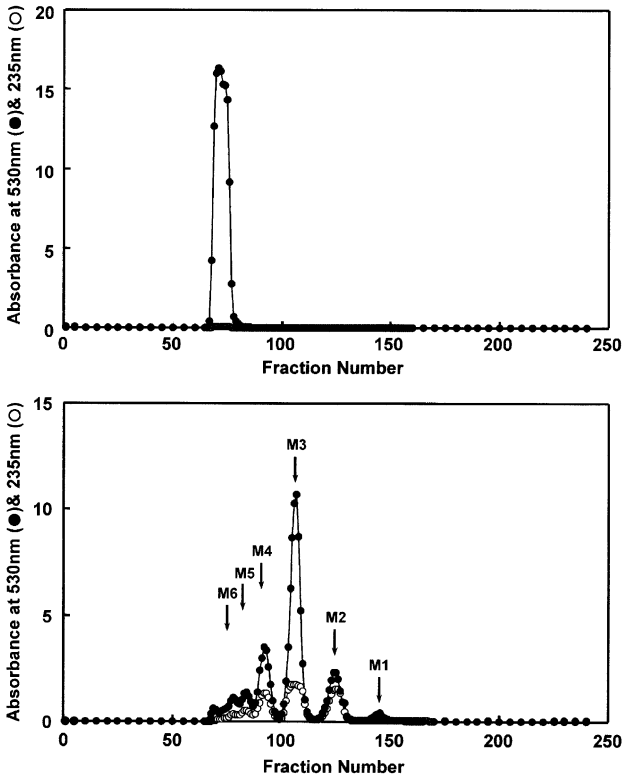

Fig. 4. Gel-filtration of poly(M)-rich substrate and its digests by HdAly through Bio-Gel P2. (A) The poly(M)-rich substrate (50 mg) was applied on the column (2.6 × 94.5 cm) and eluted with 50 mM sodium phosphate (pH 7.0) at a flow rate of 48 mL h^{-1}; (B) the poly(M)-rich substrate was digested with 1000 U of HdAly at 30 °C for 6 h and subjected to gel-filtration. Each fraction contained 2.0 mL. Elution of uronates was monitored by the carbazole–sulfuric acid reaction (Abs 530 nm) (●), and uronates with unsaturated non-reducing end were detected by Abs at 235 nm (○). The elution pattern of the digests (B) was not changed by either the extension of digestion time or re-addition of 500 U of HdAly after the 6-h digestion.

3.3. Cloning of cDNA for HdAly

The complete amino acid sequence of HdAly was determined by a cDNA method. First, the cDNA, named cDNA-NC, covering almost entire amino acid sequence of HdAly, was amplified by PCR with the AlgFw–AlgRv primer pair designed on the basis of amino acid sequences of the N-terminal region and L5 of the HdAly (Table 4, Fig. 7). By nucleotide sequence analysis, the cDNA-NC was found to consist of 758 bp encoding the amino acid sequence of 252 residues that shows ~85% identity with the sequence of *Turbo* SP2. All the amino acid sequences of L4, L5, L7, and L8 peptides can be assigned to the deduced sequence (Fig. 8), indicating that the cDNA thus cloned is of the HdAly protein. Next, 5′- and 3′-RACE PCRs were performed with primers shown in Table 3, and cDNA-5RACE (178 bp) and cDNA-3RACE (328 bp) were

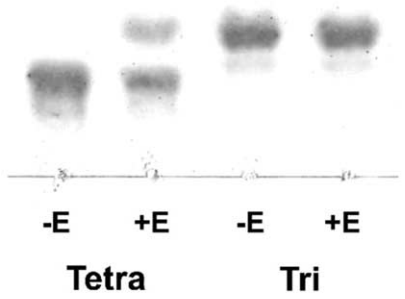

Fig. 5. TLC of tetra- and trisaccharides digested with HdAly. Tetra- and trisaccharides obtained in Bio-Gel P2 gel-filtration (see Fig. 4) were separately pooled, desalted by the gel-filtration through the same column in water, and lyophilized. These oligosaccharides were dissolved in 20 μL of 10 mM sodium phosphate (pH 7.0) to make a final concentration 1 μg μL^{-1} and digested with 0.06 U μL^{-1} of HdAly at 30 °C for 20 h. The digests (~2 μg each) were applied to a TLC plate (GL Science, HPTLC-60) and developed with a solvent of 2:1:1 butanol–acetic acid–water (vol/vol). Visualization of the oligosaccharides was performed by heating the TLC plate at 130 °C for 10 min after spraying 10% sulfuric acid in ethanol (vol/vol). Tetra and Tri, tetra- and trisaccharides, respectively. $-E$ and $+E$, without and with HdAly, respectively.

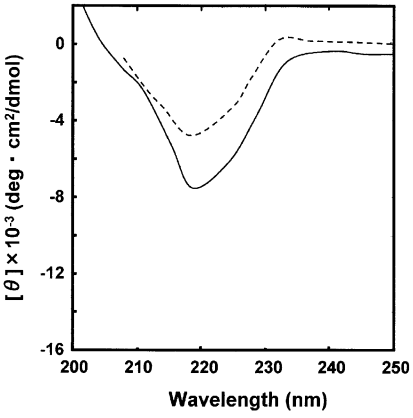

Fig. 6. Circular dichoroism of HdAly. The circular dichroism of HdAly (solid line) was measured in a cell with 1 mm light pathlength at 15 °C. The dotted line indicates the circular dichroism of *Turbo* SP2 measured by Muramatsu and coworkers.[29]

Table 3
Partial amino acid sequences of the HdAly

Peptide	Sequences
N-terminus [a]	AVLWTHKEFDPANYRNGMHAL (1–21)[b]
L4	DHQIDGFCD (138–146)
L5	NFVLSTDSGHPT (242–255)
L7	GRYSSHGP (52–59)
L8	ASVDLDGIFFSTFFGGHDST (210–229)

[a] Determined with the intact HdAly.
[b] Numbers in the parentheses indicate the residual numbers corresponding to the sequence of *Turbo* SP2.[29]

Table 4
Nucleotide sequences for PCR primers

Primer name	Sequence [a]
PCR	
AlgFw	5′-GCNGTNYTNTGGACNCAYAA-3′
AlgRv	5′-TGNCTRWSNCCNGTRGGNYG-3′
5′-RACE	
5F2	5′-TGTCCAGTTCTTTGCAACGC-3′
5R2	5′-TTGCGGTAGTTTGCTGGGTC-3′
3′-RACE	
3F	5′-GATGGGTCTATCAAGGTGTGG-3′
3Adapt	5′-CTGATCTAGAGGTACCGGATCC-3′
Confirmation	
Full5F	5′-GCTGCGGCAGAAAGTCGACCAG-3′
Full3R	5′-GATGTTGGCGTGTACATG-3′

[a] R, adenine or guanine; M, adenine or cytosine; Y, cytosine or thymine; N, adenine or guanine or cytosine or thymine.

amplified, respectively. By overlapping the nucleotide sequences of cDNA-5RACE, cDNA-NC, and cDNA-3RACE, in this order, the nucleotide sequence of total 909 bp was determined (Figs. 7 and 8). The reliability of this sequence was confirmed with cDNA-Full (872 bp) that was newly amplified with a specific primer-pair, Full5F–Full3R (Table 4). The translational initiation codon ATG was found in nucleotide positions from 27 to 30 and termination codon TAA from 846 to 848 (Fig. 8). In the 3′-terminal region, a putative polyadenylation signal sequence, TAAAAA and a poly (A+) tail were found. Accordingly, the sequence of 278 amino acids was deduced from the translational region of 822 bp. The N-terminal region of 16 residues except for the initiation Met, i.e., ELAFLLFLAIGASVEG, is regarded as the signal peptide of HdAly since it is deficient in the HdAly protein and shows high similarity to the consensus sequence for signal peptides of eukaryote secretory proteins.[38] This suggests that HdAly is produced in hepatopancreatic cells as a precursor possessing the signal peptide and secreted into digestive tract as the mature form. Thus, the mature HdAly was concluded to consist of 256 residues with a calculated molecular mass of 28895.5 Da.

3.4. Bacterial expression of HdAly

By using the cloned cDNA for HdAly, we attempted to construct bacterial expression system that would provide the basis for structure–function studies and various applications. In order to express HdAly in mature form, cDNA-EX encoding the 256 residues of the mature HdAly was amplified by PCR from the cDNA-Full with the forward primer ExFw (5′-CGAACATATGG-TATTGTGGACACATAAG-3′) including an *Nde* I site whose inner ATG is applicable as a translational initiation codon in pET-3a, and the reverse primer (5′-GATGTTGGATCCTACATGTGTACA-3′) including a *Bam* HI site. The amplified cDNA-EX was digested with *Nde* I and *Bam* HI and inserted between *Nde* I and *Bam* HI sites of pET-3a. The expression plasmid was then introduced to *E. coli* BL21 (DE3), and the recombinant HdAly was expressed as follows: a single colony of transformed bacteria was inoculated to a 2 mL of Luria-Bertani's broth containing 50 μg mL^{-1} ampicillin and incubated at 37 °C overnight. The culture was added to a 50 mL of the broth and incubated at 37 °C for 4 h, and then isopropyl 1-thio-β-D-galactoside was added to make 1 mM. At various time intervals, 0.5 mL of the culture was pipetted off and spun down at 5000*g* for 5 min. The pellet was dissolved in 0.1 mL of 10 M urea, 2% SDS, 50 mM Tris–HCl (pH 7.6), and 0.6 M 2-mercaptoethanol, and then subjected to SDS-PAGE. As shown in Fig. 9(A), a protein with ~28,000 Da was increasingly produced during the incubation, with a leaky expression in the absence of IPTG. The N-terminal amino acid sequence of the 28,000 Da protein was determined as MVLWTHKEFDPANYR- by the protein sequencer with a specimen blotted to a PVDF membrane. This sequence is completely consistent with the sequence expected from the cDNA-EX accompanied by the substitution of N-terminal Ala to Met due to the

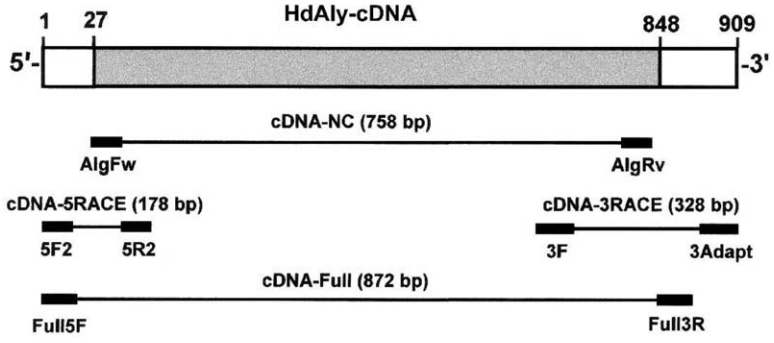

Fig. 7. Schematic diagram of cDNA for HdAly. Closed and opened boxes indicate coding and non-coding regions of HdAly-cDNA, respectively. The numbers in the top of the boxes indicate the nucleotide positions. Relative positions for cDNA-NC, cDNA-5RACE, cDNA-3RACE, and cDNA-Full are shown by lines, while the primers used for PCRs are shown as bold lines.

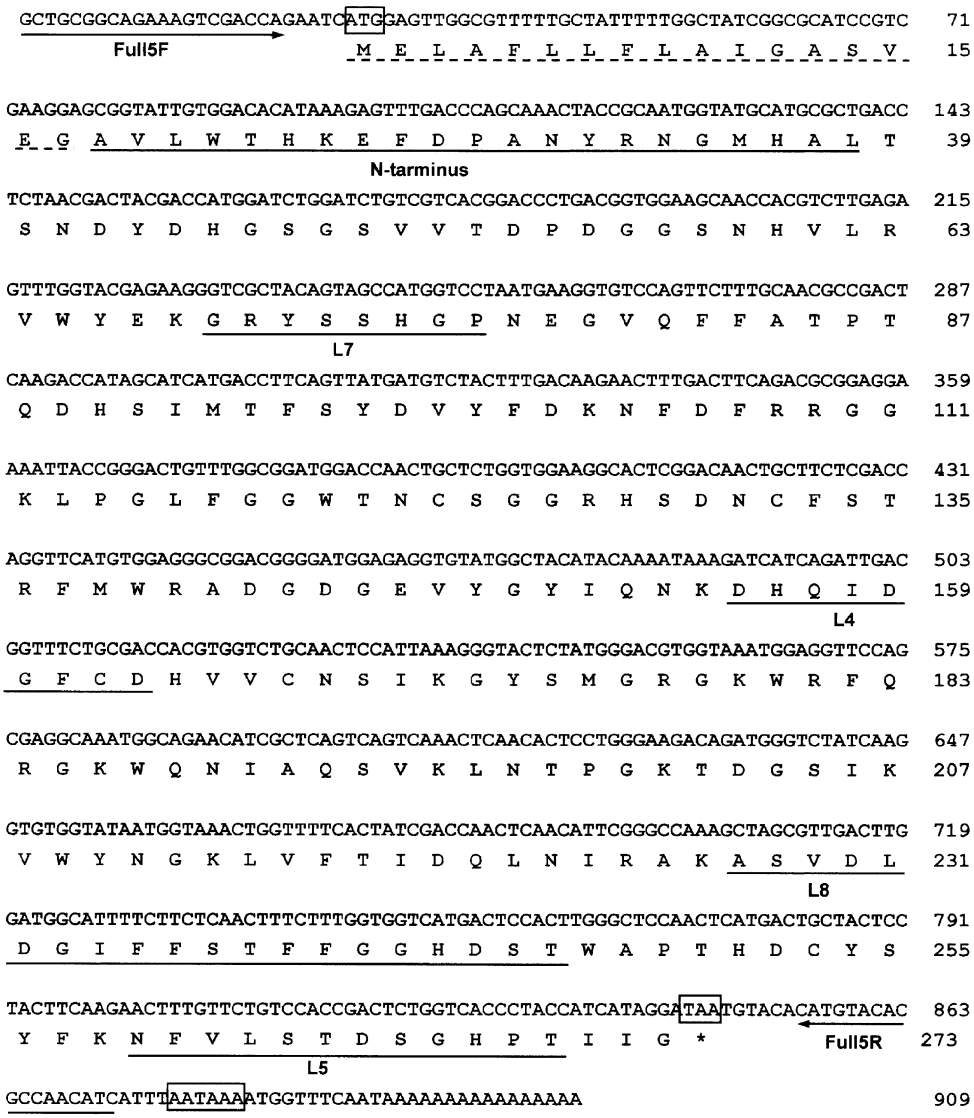

Fig. 8. The nucleotide and deduced amino acid sequences of HdAly-cDNA. Residue numbers for both nucleotide and amino acid are indicated to the right of each row. The translational initiation codon ATG, termination codon TAA, and a putative polyadenylation signal AATAAA are boxed. A putative signal peptide is indicated by a dotted underline. The amino acid sequences determined with intact HdAly (N-terminus) and peptide fragments, L4, L5, L7, and L8 are indicated by lines under the amino acid sequence. The positions of Full5F and Full5R primers are indicated by arrows under the nucleotide sequence. The sequence data are available from the DNA Data Bank of Japan with an accession number, AB110094.

manipulation of the 5′-terminus of the cDNA. Thus, the 28,000 Da protein was identified as the recombinant HdAly produced by *E. coli*. The molecular mass of the recombinant HdAly appeared to be slightly lower than that of native enzyme. This is probably ascribable to the lack of a carbohydrate chain that has been reported to anchor on Asn-105 in *Turbo* enzyme[29] (see Fig. 11). Densitometric analysis revealed that the amount of the recombinant HdAly reached a maximum in 1.5–2 h of the induction. However, the recombinant HdAly could not be extracted as an active enzyme from the bacteria with the lysis buffer described in the Section 2, indicating that the enzyme formed inclusion bodies in the cell. Accordingly, the insoluble recombinant HdAly was dissolved in 8 M urea–5 mM 2-mercaptoethanol, and dialyzed against 10 mM sodium phosphate (pH 7.0) to promote renaturation; however, no active enzyme was obtained. Therefore, we further investigated the expression conditions suitable for producing the active and soluble HdAly, and found that the temperature for cultivation of bacteria should be lowered below 20 °C to decrease the expression rate and avoid thermal denaturation of the enzyme. Thus, the active HdAly came to be produced in the soluble fraction by the cultivation at 19 °C. However, the amount of soluble HdAly was still very small, i.e., at most 0.2% of the total expressed

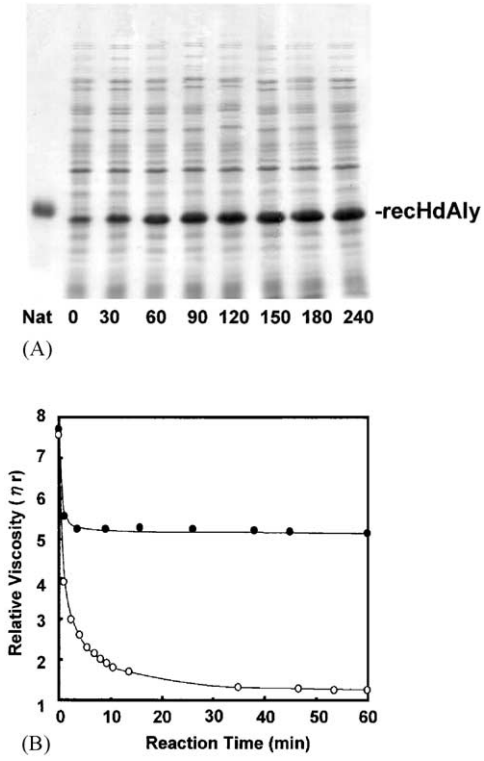

Fig. 9. Bacterial expression of HdAly. (A) Bacterial expression of HdAly. *E. coli* BL21 (DE3) containing recombinant pET-3a plasmid was cultivated at 37 °C and induced by 1 mM IPTG. An aliquot of the culture (0.5 mL) was pipetted off at appropriate time (0–240 min after the induction), and the protein composition of the bacterial pellet was analyzed by SDS-PAGE. recHdAly, recombinant HdAly; Nat, the HdAly purified from abalone hepatopancreas. (B) Degradation of sodium alginate by the recombinant HdAly expressed at 19 °C. The reaction was performed in an Ostwald-type viscometer at 30 °C, in a mixture containing of 0.15% sodium alginate, 50 mM sodium phosphate (pH 7.0), and 0.98 U of the recombinant HdAly. The flow time of the buffer was 45 s. ○, soluble fraction in the lysis buffer containing 8% (w/v) sucrose, 0.5% (w/v) TritonX-100, 50 mM Tris–HCl (pH 8.0), 50 mM EDTA, and 0.1 mg mL^{-1} lysozyme; ●, insoluble fraction suspended in the same buffer (amount of the active HdAly was not determined).

HdAly. In order to improve the yield of recombinant HdAly, we are now investigating optimal conditions for the expression. The degrading activity of the recombinant HdAly on sodium alginate is shown in Fig. 9(B).

4. Discussion

In the present study, an abalone alginate lyase, HdAly, was isolated by ammonium sulfate fractionation followed by TOYOPEARL CM-650M column chromatography (Fig. 1, and Table 2). By PCR using the primers synthesized on the basis of partial amino acid sequences of HdAly, cDNAs encoding HdAly were successfully cloned, and the complete amino acid sequence of HdAly was determined (Figs. 7 and 8).

In order to clarify structural characteristics of HdAly, we analyzed the primary structure by means of a hydropathy plot[39] and secondary structure prediction.[40] As shown in Fig. 10, the hydropathy plot of HdAly exhibited a characteristic pattern, i.e., the hydrophobic and the hydrophilic regions repeated alternately along the sequence. While, the secondary structure prediction indicated that HdAly consists of mainly β-sheet, turns, and random coils, but contains substantially no α-helix. This tendency is prominently seen in the N-terminal ~1/3 region, i.e., around residues 16–83, for example. From these analyses, we now assume that the major structural motif of HdAly is 'β-sheet-turn-β-sheet'. This assumption is in accordance with the result obtained by the circular dichroism measurement for HdAly indicating a protein rich in β-structure (Fig. 6). Since bacterial alginate lyases are known to be rich in α-helix[2,10,15,21] e.g., A1-III from *Sphingomonas* sp. A1 is constructed from the (α6/α5)-barrel motif,[21] the three-dimensional structure of HdAly is expected to significantly differ from those of bacterial alginate lyases. Therefore, we further searched for uronate lyase-relating enzymes that possess β-sheet as a major structural motif. As a result, pectate lyases PelC and PelE from *Erwinia chrysanthemi*[41,42] that exhibit a β-sheet rich topology termed 'parallel β-helix structure' were found. The hydropathy plots and predicted secondary structure of the pectate lyases were also similar to those of HdAly, although the amino acid sequence identity between the pectate lyases and HdAly were less than 18% (data not shown). In order to clarify whether or not HdAly possesses a structural motif similar to the pectate lyases, the three-dimensional structure analysis is essential.

The amino acid sequence of HdAly was compared with that of *Turbo* SP2[29] and the C-terminal region of *Chlorella* virus CL2 (219 residues)[30] (Fig. 11). The sequence of HdAly showed 85 and 28% identity to those of *Turbo* SP2 and *Chlorella* virus CL2, respectively. In HdAly sequence, an Asp corresponding to the carbohydrate chain-anchoring residue in *Turbo* SP2 (Asp-105) and 5 Cys (Cys-106, 115, 145, 150, and 236) that have been implied as important residues for catalytic activity and/or disulfide bonding[29] are all conserved. However, in the amino acid sequence in *Chlorella* virus CL2, those conserved residues are all substituted by different amino acids or deleted. This may indicate that the conserved Asp and Cys in the molluscan enzymes is not directly participating in the alginate degrading activity because the *Chlorella* virus CL2 can depolymerize alginate. On the other hand, the carbohydrate chain-anchoring to Asp in the molluscan enzymes may correlate to the stability of the enzyme. Namely, the bacterially expressed HdAly that is considered to possess no carbohydrate chain appeared more

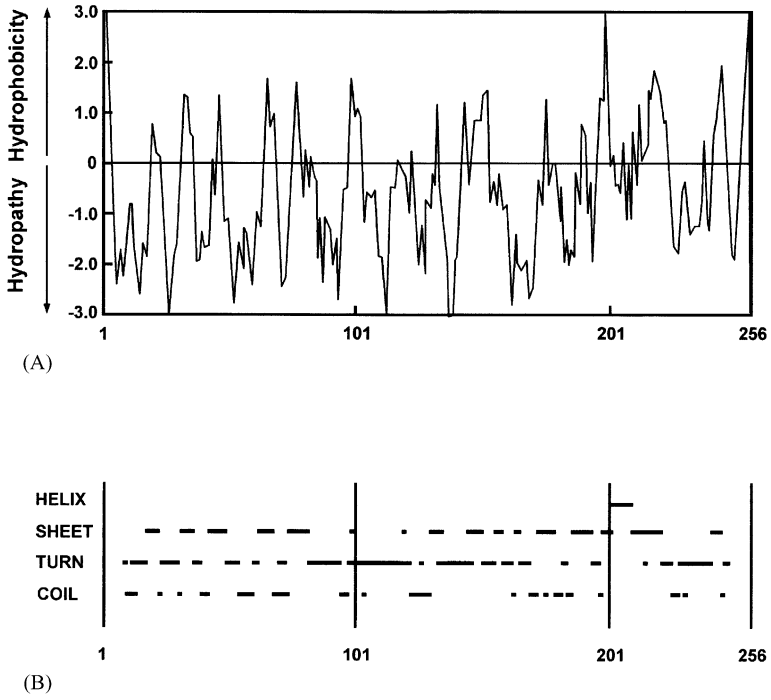

Fig. 10. Hydropathy plot and predicted secondary structure of HdAly. (A) Hydropathy plots made by the method of Kyte and Doolittle;[39] (B) predicted secondary structure made by the method of Garnier and coworkers.[40] HELIX, α-helix structure; SHEET, β-sheet structure; TURN, β-turn structure; COIL, random coil structure.

```
Abalone     1   AVLWTHKEFD PANYRNGMHA LTSNDYDHGS GSVVTDPDGG SNHVLRVWYE    50
Turbo       1   TLLWTHKEFD PNNYRDGMHA LTSNDYDHGS GKVVTDPDGG SNHVLRVWYE    50
Chlorella  87   TKNTNVISTL DLNLLTKGGG SWNVDGVNMK KSAVTTFDG- -KRVVKAVYD   135
                           *           *          **  **     *::  *:

Abalone         KGRYSSHGPN EG-VQFFATP TQ-DHSIMTF SYDVYFDKNF DFRRGGKLPG    98
Turbo           KGRWSSHGPN EG-VQFFATP TQ-DHSVMTF SYDLYLSHDF DFRRGGKLPG    98
Chlorella       KNSGTSANPG VGGFSFSAVP DGLNKNAITF AWEVFYPKGF DFARGGKHGG   185
                *   :*  *   *   * * *    : : :** ::: :::  * ** ****  *

                   ▽▽        ▽
Abalone         LFGGWTNCSG GRHSDNCFST RFMWRADGDG EVYGY----I QNKDHQIDGF   144
Turbo           LYGGWTNCSG GRHSDNCFST RFMWRKDGDG EVYAY----I PDYHHQVSGF   144
Chlorella       TFIGHGAASG YRHSKYGASN RIMWQEKGGV IDYIYPPSDL KQKIPGLDPE   235
                 : *    **  ***     *  * **:  *     * *    :       :

                ▽   ▽
Abalone         CDHVVCNSIK GYSMGRGKWR FQRGKWQNIA QSVKLNT--- -PGKTDGSIK   190
Turbo           CDHNVCNSVK GYSLGRGKWK FERGKWQNIA QHVHLNT--- -PGKTDGSIK   190
Chlorella       GQGI------ GFFQDDFKNA LKYDVWNRIE IGTKMNTFKN GIPQLDGESY   279
                           *:     *    :   * *      :**       : **

                                                              ▽
Abalone         VWYNGKLVFT IDQLNIRAKA SVDLDGIFFS TFFGGHDSTW APTHDCYSYF   240
Turbo           VWHNGKLVYT IDQLNIVSKA SVDIDGIFFS TFFGGSDSSW APTHDCYSYF   240
Chlorella       VIVNGKKE-V LKGINWSRSP ELLISRFCWN TFFGG---PL PSPKNQVAYF   325
                *  ***      :  :*       :      :: *****          :  :**

Abalone         KNFVLSTDSG HPTIIG                                       256
Turbo           KNFALSTDSS HPTIL                                        255
Chlorella       TNFQMKKYE                                               334
                **
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Fig. 11. Comparison of amino acid sequences of alginate lyases. The amino acid sequence of HdAly (Abalone) was aligned with those of *Turbo* SP2 (Turbo) and the C-terminal region of *Chlorella* virus CL2 (Chlorella). Identical and conservative residues among the sequences are indicated by '*' and ':'. Relatively high-conservative regions among the sequences are boxed. An Asp and five Cys that have been reported to carbohydrate-anchoring and catalytically/structurally important residues, respectively, in *Turbo* SP2 are indicated by reverse triangles.

liable to heat-denaturation than the native HdAly according to our preliminary examination (data not shown, but see Fig. 9). In the *Turbo* enzyme, two isoforms, SP1 (253 residues) and SP2 (255 residues) derived from the difference in C-terminal 2 residues (Ile-Lue), have been reported.[29] The physiological significance of the difference in the C-terminal length has been explained as the improvement of structural stability of the enzyme, i.e., SP2 with elongated C-terminus is more heat-stable than SP1. In HdAly, only one kind of enzyme possessing the C-terminal sequence of three residues, Ile-Ile-Gly, was found. The C-terminal sequence of HdAly seemed to be structurally equivalent to that of *Turbo* SP2 although an additional Gly is attached.

Among the sequences of HdAly, *Turbo* SP2, and *Chlorella* virus CL2, highly conserved seven regions were found (Fig. 11). Namely, the regions showing 57% or higher identity among the three proteins were ^{34}VTDPDG^{39}, ^{88}FDFRRGGK^{95}, ^{107}SGGRHS^{112}, ^{117}STRFMWR^{123}, ^{194}NGK^{196}, ^{219}FSTFFGG^{225}, and ^{239}YFKNF^{243} (residue numbers are of HdAly). These regions frequently contain basic amino acids and aromatic amino acids that have been suggested to engage in catalytic action and/or substrate binding.[1–3,29] Thus, we consider that these regions are candidates for catalytically and/or structurally important regions in these enzymes. The sequences of these enzymes showed practically no homology to those of bacterial ones as described above; however, one of the above conserved regions, i.e., ^{248}YFKNF^{253}, appeared to be similar to the YFKAGVYNQ sequence that has been suggested to participate in the catalytic action and/or structural roles in the lyases from *Vibrio halioticoli*.[12] In order to identify the residues located in the catalytic sites of the molluscan alginate lyases and also reveal the phylogenetic relationships with bacterial enzymes, further detailed studies on the primary and gene structures of molluscan alginate lyases are necessary.

In the present study, we produced active recombinant HdAly by lowering the cultivation temperature from 37 to 19 °C, although the yield was still very low. The yield is, however, expected to be improved by modifying the cultivation conditions, and also by using the expression plasmids that are liable to produce the recombinant protein into the soluble fraction or the periplasm region of the bacteria. The recombinant abalone enzyme would be useful for various applications such as the production of brown-algal protoplasts and the manufacture of oligo-alginates that are known to be useful as therapeutic agents and food additives.

HdAly acts on a pentamer unit in poly(M) substrate producing tri- and disaccharides as a major final products (Fig. 4). If the trimer and dimer are utilized by the abalone's glycolytic pathway, these oligosaccharides must be converted to monosaccharides. Since HdAly itself cannot degrade oligosaccharides smaller than a trimer, there must be an unidentified enzyme that acts on the trimer and/or dimer. We are now searching the oligoalginate-degrading enzyme from the abalone. On the other hand, acetic acid production by *V. halioticoli*, an abalone intestinal bacterium that possess alginate lyases, was recently reported, and the possible role for establishment of abalone-*V. halioticoli* association was proposed.[43] The oligoalginates produced by HdAly might be utilized through a symbiotic pathway by this bacterium. The physiological significance of HdAly in the utilization of alginate as energy and carbon sources in abalone is an important task for future research.

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