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Citation	Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 157(4), 317-325 https://doi.org/10.1016/j.cbpb.2010.07.006
Issue Date	2010-12
Doc URL	http://hdl.handle.net/2115/44107
Type	article (author version)
File Information	Isolationand.pdf



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**Isolation and characterization of two alginate lyase isozymes, AkAly28 and AkAly33, from the
common sea hare *Aplysia kurodai***

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Keywords: *Aplysia kurodai, gastropod, alginate lyase, PL-14, alginate, brown seaweed*

Abstract

Two alginate lyase isozymes, AkAly28 and AkAly33, with approximate molecular masses of 28 kDa and 33 kDa, respectively, were isolated from the digestive fluid of the common sea hare, *Aplysia kurodai*. Both of AkAly28 and AkAly33 were regarded as the endolytic polymannuronate (poly(M)) lyase (EC 4.2.2.3) since they preferably degraded poly(M)-rich substrate producing unsaturated tri- and disaccharides and rapidly decreased the viscosity of sodium alginate solution in the initial phase of degradation. Optimal pH and temperature of the two enzymes were similarly observed at pH 6.7 and 40 °C, respectively. Temperature that caused a half inactivation of the two enzymes during 20-min incubation was also similar to each other, i.e., 38 °C. However, NaCl requirement and activity toward oligosaccharide substrates of the two enzymes were significantly different from each other. Namely, AkAly28 showed practically no activity in the absence of NaCl and the maximal activity at NaCl concentrations higher than 0.2 M. While AkAly33 showed ~20% of maximal activity despite the absence of NaCl and the maximal activity at around 0.1 M NaCl. AkAly28 hardly degraded oligosaccharides smaller than tetrasaccharide, while AkAly33 could degrade oligosaccharides larger than disaccharide producing disaccharide and 2-keto-3-deoxy-gluconaldehyde (an open chain form of unsaturated monosaccharide). Analysis of the N-terminal and internal amino-acid sequences of AkAly28 and AkAly33 indicated that both of the two enzymes belong to polysaccharide lyase family 14.

1. Introduction

Herbivorous marine invertebrates such as sea urchin, abalone, and sea hare feed on seaweeds (Mai et al., 1995; Takami et al., 1998; Johnston et al., 2005). To obtain carbohydrate nutrients, these invertebrates digest seaweeds' structural and storage polysaccharides, e.g., cellulose, alginate, mannan, starch and laminarin, with appropriate polysaccharide-degrading enzymes in their digestive fluid (Suzuki et al., 2003; Shimizu et al., 2003; Suzuki et al., 2006; Ootsuka et al., 2006; Nishida et al., 2007; Hata et al., 2009; Nikapitiya et al., 2009; Kumagai and Ojima, 2009 and 2010; Zahura et al., 2010). The thus produced oligosaccharides and monosaccharides are assimilated directly by animals themselves or through the fermentation by intestinal bacteria (Erasmus et al., 1997; Sawabe et al., 2003). Among the seaweeds' polysaccharides, alginate in brown seaweeds appears to be the most abundant carbohydrate. For example, the alginate content in the frond of *Laminaria* sp. is usually more than 20% (w/w) in dry weight while other polysaccharides are less than 5%. Correspondingly, an alginate-degrading enzyme, i.e., alginate lyase (EC 4.2.2.3), is also abundant in the digestive fluid of abalone and turban shell which are fond of brown seaweeds (Shimizu et al., 2003 and Muramatsu et al., 1977). Alginate is a heteropolyuronide comprising 1,4-linked β -D-mannuronate (M) and its C5 epimer α -L-guluronate (G). These uronide units are arranged as homopolymeric G and M blocks, and heteropolymeric MG blocks (Haug et al., 1967; Gacesa P., 1988; Gacesa P., 1992; Wong et al., 2000). Alginate lyase splits the glycosyl linkages of alginate by the β -elimination mechanism producing oligosaccharides possessing an unsaturated uronic acid (4-deoxy-L-*erythro*-hex-4-eno-pyranosyl-uronic acid) at the newly formed non-reducing terminus. To date general properties of alginate lyases from several gastropods have been investigated; however, the physiological significance of this enzyme in the assimilation of alginate in gastropods still remains obscure.

To enrich the general information about the physiological roles of alginate lyases in gastropods, it seems necessary to study comparatively the enzymatic properties of various gastropod alginate lyases and

the reaction products produced by the gastropod enzymes. To date gastropod alginate lyases have been isolated from abalone *Haliotis rufescens* and *H. corrugate* (Nakada et al., 1967), *H. tuberculata* (Boyen et al., 1990; Heyraud et al., 1996), *H. discus hannai* (Shimizu et al., 2003; Suzuki et al., 2006), *H. iris* (Hata et al., 2009); turban shell *Turbo cornutus* (Muramatsu et al., 1977); small marine snail *Littorina* sp. (Elyakova et al., 1974), *Omphalius rusticus* and *L. brevicula* (Hata et al., 2009); and sea hare *Dolabella auricular* (Nisizawa et al., 1968). In addition, alginate lyase activities were detected in sea hare *Aplysia depilans*, *A. californica* and *A. juliana* ((Boyen et al., 1990; Wakabayashi et al., 1999). Most of the above gastropod enzymes have been identified as an endolytic polymannuronate lyase (poly(M) lyase (EC 4.2.2.3)) which produces unsaturated tri- and disaccharide as major products. Exceptionally, one enzyme that exolytically acts on polymer substrate, i.e., HdAlex, has been isolated from abalone *H. discus hannai* (Suzuki et al., 2006). This enzyme degraded not only polymer alginate but also unsaturated trisaccharide, which had been produced by the abalone endolytic enzyme HdAly, to unsaturated disaccharide and 2-keto-3-deoxygluonaldehyde (an open chain form of unsaturated monosaccharide; term α -keto acid in the present paper). The primary structures of HdAly and HdAlex were analyzed by the cDNA method and the amino-acid identity between the two deduced sequences was 67% (Shimizu et al., 2003; Suzuki et al., 2006). On the other hand, the amino-acid sequence of an endolytic enzyme SP2 from turban shell was determined by the protein method (Muramatsu et al., 1996). Amino-acid identity among the above three gastropod enzymes was approximately 60%. According to the hydrophobic cluster analysis of the primary structure (Henrissat and Davies, 1997), these gastropod enzymes were classified to polysaccharide-lyase family 14 (PL-14) (<http://www.cazy.org/>). Except for the above abalone and turban-shell enzymes, other gastropod alginate lyases have not so extensively investigated.

The common sea hare, *A. kurodai*, is a typical herbivorous marine gastropod possessing various polysaccharide-degrading enzymes in its digestive fluid. Recently we have succeeded to isolate two kinds

of polysaccharide-degrading enzyme, i.e., β -1,3-glucanase and β -1,4-mannanase, from the digestive fluid of this animal (Kumagai and Ojima, 2009; Zahura et al., 2010). During the purification of these enzymes, we detected considerably high alginate lyase activity in the crude enzyme preparation. Although alginate lyase activities were detected in the digestive fluid of *A. depilans* and *A. californica* (Boyen et al., 1990) and buccal juice of *A. juliana* (Wakabayashi et al., 1999), no alginate lyase from *Aplysia* sp. has been purified. Therefore, in the present study, we isolated alginate lyases from *A. kurodai* and characterized their basic properties.

2. Materials and methods

2.1. Materials

The animal, *A. kurodai* (body length and weight, ~12 cm and ~150 g, respectively), was collected from the coast of Hakodate, Hokkaido Prefecture of Japan, in July 2008. Approximately 150 mL of digestive fluid was obtained from the gastric lumen of 20 animals by squeezing the stomach after dissection. The digestive fluid was dialyzed against 2 mM sodium phosphate buffer (pH 7.0) for 2 h and centrifuged at 10,000 \times g for 10 min to remove insoluble materials. The supernatant was used as a crude enzyme for the purification of alginate lyase. TOYOPEARL DEAE-650M was purchased from Toyo Soda Mfg, Co. (Tokyo, Japan), and Mono-S 5/50GL and Mono-Q 5/50GL were from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England). Sodium alginate (*Macrocystis pyrifera* origin) was purchased from Sigma-Aldrich (St. Louis, USA). The other chemicals used were reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Substrates

Sodium alginate was dissolved in 10 mM sodium phosphate buffer (pH 7.0) to make 1% (w/v) and heated at 90 °C for 1 h before use. Poly(M)-rich, Poly(G)-rich, and poly(MG)-rich substrates were prepared by the method of Gacesa and Wusteman (1990). Mannuronate and guluronate contents in the substrates were estimated by the method of Morris and coworkers (1980). The mannuronate content in the original alginate was estimated to be 60%, while those in the poly(M)-rich substrate and the poly(MG)-rich substrate were 86% and 64%, respectively. The guluronate content in the poly(G)-rich substrate was 99%. Unsaturated oligomannuronates (unsaturated disaccharide–hexasaccharide, Δ M– Δ M6) were prepared by the digestion of poly(M)-rich substrate with the abalone endolytic enzyme HdAly as described previously (Shimizu et al., 2003; Suzuki et al., 2006).

2.3. Alginate lyase activity

Alginate lyase activity was assayed in a 1-mL reaction mixture containing 0.15% (w/v) substrate, 0.15 M NaCl, 10 mM sodium phosphate (pH 7.0) and an appropriate amount of enzyme (usually 5–10 units) at 30 °C. The progress of the reaction was monitored by measuring the absorbance at 235 nm with a Model 3010 spectrophotometer (HITACHI, Tokyo, Japan) equipped by a temperature-control device SP-12R (TAITEC, Tokyo, Japan). One unit (U) of alginate lyase was defined as the amount of enzyme that increases Abs_{235nm} to 0.01 for 1 min. pH dependence of the enzyme was determined at 30 °C in reaction mixtures adjusted to pH 4–11 with 50 mM sodium phosphate buffer. Temperature dependence was measured at 10–60 °C in 10 mM sodium phosphate (pH 7.0). Thermal stability was assessed by measuring the activity remaining after the heat treatment of the enzyme at 15–45 °C for 20 min. NaCl dependence of the enzyme was measured in reaction mixtures containing 0–0.5 M NaCl.

2.4. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 10% (w/v) polyacrylamide slab gel containing 0.1% (w/v) SDS according to the method of Porzio and Pearson (1977). After the electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol–10% (v/v) acetic acid, and the background of the gel was destained with 5% (v/v) methanol–7% (v/v) acetic acid. Protein Marker, Broad Range (New England BioLabs, Ipswich, MA, USA) was used as a molecular mass marker.

2.5. Thin-layer chromatography

The reaction mixture (70 μ L) containing 1.0% (w/v) poly(M)-rich substrate or alginate oligosaccharides, 0.15 M NaCl, 10 mM sodium phosphate buffer (pH 7.0) and 0.5 U enzyme was incubated at 30 °C. At appropriate time intervals aliquots (each 10 μ L) of the reaction mixture were withdrawn and heated in boiling water for 2 min to terminate the reaction. The reaction products were then subjected to thin-layer chromatography (TLC) using TLC-60 plate. The degradation products were developed with 1-butanol–acetic acid–water (2:1:1, v:v:v) and the sugars fractionated on the plate were stained by heating the TLC plate at 110 °C for 5 min after spraying with 10% (v/v) sulfuric acid in ethanol. To detect the unsaturated sugars and α -keto acid on the plate, thiobarbituric acid (TBA) staining was carried out according to the method of Lanning and Cohen (1951). Unsaturated oligosaccharide markers were prepared by the digestion of poly(M)-rich substrate with abalone crude enzyme (Suzuki et al., 2006).

2.6. Analysis of oligosaccharides by anion-exchange chromatography

Production of oligosaccharides by alginate lyase was analyzed by anion-exchange chromatography with a Shimadzu LC-20AT HPLC (Shimadzu, Kyoto, Japan) equipped with a TSK-GEL DEAE-2SW (4.6 mm \times 25 cm) column (Tosoh Corporation, Japan). The degradation products of poly(M)-rich substrate

produced by alginate lyases was subjected to the HPLC and the oligosaccharides adsorbed were eluted with a linear gradient of 0-0.15 M NaCl. Elution of the oligosaccharides was detected by monitoring absorbance at 235 nm with a Shimadzu SPD-20A UV detector.

2.7. Determination of partial amino-acid sequences

The N-terminal amino-acid sequence of alginate lyase was determined with an ABI Procise 492 protein sequencer (Applied Biosystems, Foster City, CA, USA). Internal amino-acid sequences were determined with peptide fragments prepared either by tryptic digestion or by digestion with lysylendopeptidase at 37 °C for 12 hours. The tryptic fragments were subjected to a matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using an ABI Proteomics Analyzer 4700 (Applied Biosystems, Foster city, CA, USA) and the amino-acid sequences of the fragments were determined by MS/MS mode with DeNovo Explorer software. While the lysylendopeptidyl fragments were separated by reverse phase HPLC quipped with a Mightysil Rp-18 (4.6 mm × 150 mm) column and fragments were subjected to both MALDI TOF-MS and ABI Procise 492 protein sequencer. Homology searches for the amino-acid sequences on 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.

2.8. Protein determination

Protein concentration was determined by the biuret method (Gornall et al., 1949) or the method of Lowry et al. (1951) using bovine serum albumin fraction V as a standard protein.

3. Results

3.1. Purification of alginate lyase from *A. kurodai*

The sea hare alginate lyase was isolated as follows. The crude enzyme from *A. kurodai* was subjected to ammonium sulfate fractionation and the precipitates formed between 60-90% saturation of ammonium sulfate were collected by centrifugation at 10,000×g for 15 min. The precipitates were dissolved in and dialyzed against 10 mM sodium phosphate buffer (pH 8.0) and applied to a TOYOPEARL-DEAE 650M column (2.5 cm × 42 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0-0.2 M NaCl (total 600 mL) in 10 mM sodium phosphate buffer (pH 8.0) at a flow rate of 15 mL/h and the eluent was collected as 5-mL fractions. Alginate lyase activity was detected in two peaks separately eluted at 0.05 M and 0.10 M NaCl. The first peak fractions consisted of 3–4 major proteins with ~30 kDa according to SDS-PAGE while the second peak fractions 7-8 protein bands with 25–40 kDa. Thus in the present study we used the first peak fractions for further purification because of their higher purity. The fractions were pooled, lyophilized and dialyzed against 2 mM sodium phosphate buffer (pH 8.0) and subjected to an AKTA-FPLC (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England) equipped with a Mono-Q 5/50GL column pre-equilibrated with the same buffer. The proteins were eluted with a linear gradient of 0–0.2 M NaCl (total 40 mL) at a flow rate of 1.0 mL/min and the eluent was collected as 1-mL fractions. In this chromatography, alginate lyase was eluted in both passed through fractions and fractions eluted by 0.08 M NaCl (Fig. 1A). The latter fractions showed a single band with ~33 kDa on SDS-PAGE and thus we used these fractions as a purified *Aplysia* alginate lyase, AkAly33 (Fig. 2). While the passed through fractions were dialyzed against 2 mM sodium phosphate buffer (pH 6.0) and subjected to a Mono-S 5/50GL column pre-equilibrated with same buffer. The adsorbed proteins were eluted with a linear gradient of 0–0.5 M NaCl (total 30 mL) at a flow rate of 1.0 mL/min, and the alginate lyase showing a single band with ~28 kDa on SDS-PAGE was eluted at 0.5 M NaCl after the linear gradient (Figs. 1B and 2). Thus, we named this enzyme AkAly28 as another *Aplysia*

alginate lyase. By the above purification procedure, AkAly33 was purified 26-fold at a yield of 3.8% and the specific activity 2057 U/mg, while AkAly28 was purified 73-fold at a yield of 4.9% and the specific activity 5741 U/mg (Table 1).

3.2. Basic properties of AkAly28 and AkAly33

Optimal pH and temperature of both AkAly28 and AkAly33 were similarly observed at 6.7 and 40 °C, respectively (Fig. 3A & B). The temperature that caused a half inactivation of AkAly28 and AkAly33 during 20-min incubation was also similar to each other, i.e., 38 °C (Fig. 3C). On the other hand, the effect of NaCl on the activity was significantly different between two enzymes. Namely, AkAly28 showed no activity in the absence of NaCl and required NaCl at concentration higher than 0.2 M for the maximal activity (Fig. 3D). On the other hand, AkAly33 showed ~20% of maximal activity even in the absence of NaCl and the maximal activity at around 0.25 M NaCl. These results indicated that NaCl acted as a strong activator for both AkAly28 and AkAly33; however, the NaCl dependency was much heavier in AkAly28 than AkAly33.

3.3. Degradation of polymer substrates by AkAly28 and AkAly33

To examine substrate specificity of AkAly28 and AkAly33, sodium alginate, poly(M)-rich, poly(G)-rich and poly(MG)-rich substrates were subjected to lyase reaction. As shown in Fig. 4A and B, both AkAly28 and AkAly33 exhibited the highest activity toward poly(M)-rich substrate, moderate activity toward sodium alginate and weak activity toward poly(MG)-rich substrate, but no activity toward poly(G)-rich substrate. These results indicate that both enzymes are classified to poly(M) lyase (EC 4.2.2.3). When degraded sodium alginate, AkAly28 and AkAly33 rapidly decreased its viscosity in the early phase of the reaction (Fig. 5). Accordingly, actions of these enzymes were regarded as endolytic.

The degradation products of poly(M)-rich substrate produced by AkAly28 and AkAly33 were analyzed by TLC. As shown in Fig. 6A and B, both AkAly28 and AkAly33 produced tri- and disaccharide as major degradation products along with various sizes of intermediary oligosaccharides. However, relative amounts of tri- and disaccharide produced by the two enzymes were significantly different in the prolonged reaction time. Namely, the amount of disaccharide produced by AkAly33 in reaction time 2–6 h was obviously larger than that by AkAly28 (Fig. 6A and B). This difference may be ascribable to the difference in the oligosaccharide-degrading activity between AkAly28 and AkAly33. Namely, AkAly33 appeared to degrade poly(M)-rich substrate into various sizes of oligosaccharides in the reaction time up to 1h and further degraded the thus formed oligosaccharides to disaccharide and monosaccharide (α -keto acid) in the reaction time 2–6 h (Fig. 6B). On the other hand, AkAly28 readily degraded poly(M)-rich substrate to oligosaccharides; however, this enzyme was considered to be incapable of degrading trisaccharide even by the prolongation of reaction time. To confirm the difference in the trisaccharide-degrading activity between AkAly28 and AkAly33, we further investigated the degradation products by anion-exchange chromatography using a TSK-GEL DEAE-2SW column. As shown in Fig. 7A, trisaccharide was produced by AkAly28 as a major product and disaccharide was not so much produced even by the prolongation of reaction time to 6 h. On the other hand, both di- and trisaccharide were readily produced by AkAly33 in 1-h reaction and much higher amount of disaccharide was produced in 6-h reaction (Fig. 7B). These results strongly suggested that AkAly33 degraded trisaccharide in the prolonged stage of the reaction. It should be noted that α -keto acid was also efficiently produced along with the production of disaccharide according to the TBA-stained TLC (see Fig. 6). However, the α -keto acid was hardly detected in HPLC (Fig. 7) since the open chain form of α -keto acid, i.e., 2-keto-3-deoxy-gluconaldehyde, does not exhibit absorbance at 235 nm.

Then, we further examined the activities of AkAly28 and AkAly33 toward various sizes of oligosaccharides, i.e., unsaturated disaccharide–heptasaccharide (ΔM – $\Delta M6$). As shown in Fig. 8B, AkAly28 could degrade oligosaccharides larger than pentasaccharides producing trisaccharide as a major product; however, it hardly degraded oligosaccharides smaller than pentasaccharides. On the other hand, AkAly33 could degrade oligosaccharides larger than disaccharide producing disaccharide and α -keto acid (Fig. 8C). From these results, we may conclude that AkAly28 is the enzyme that preferably degrades substrates larger than trisaccharide while AkAly33 is the enzyme that can degrade not only polymer substrate but also oligosaccharides producing disaccharide and α -keto acid. By the actions of these two enzymes, *Aplysia* may efficiently degrade alginate substrate to disaccharide and α -keto acid in the digestive fluid.

3.4. N-terminal and internal amino-acid sequences of AkAly28 and AkAly33

The N-terminal amino-acid sequences of 40 residues for AkAly28 and AkAly33 were determined by the protein sequencer (Table 2). These sequences showed 57.5% amino-acid identity to each other; however, they showed no appreciable similarity with the sequences of known alginate lyases. This suggests that AkAly28 and AkAly33 are structurally related isozymes but distinct from other gastropod alginate lyases. On the other hand, proteolytic fragments of AkAly28 and AkAly33 showed considerable sequence identity with those previously determined in abalone and turban shell alginate lyases. Namely, the sequences of two tryptic fragments of AkAly28, KGSFSPLHDKR (T-1) and GRFKFK (T-2), showed 27% and 50-67% identities to the residues 51-61 and 159-164, respectively, of both abalone HdAly (Shimizu et al., 2003) and turban shell SP2 (Muramatsu et al., 1996). In addition, the amino-acid sequences of two lysylendopeptidyl fragments, MPGLFGGEDGDGAYK (L-1) and WNSVSEEVHINTVGK (L-2), showed 47% identity to the residues 96-111 and 170-184, respectively, of both HdAly and SP2. In these sequences,

residues 97-102 and 178-181 are known as the highly conserved regions among PL-14 enzymes (Suzuki et al., 2006; Yamamoto et al., 2008).

In case of AkAly33, the sequence GMFFSTFFGGSKK of a tryptic fragment T-3 showed 69% and 77% identities to the residues 216-228 and 233-245 of HdAly and HdAlex, respectively. This region is also highly conserved among PL-14 enzymes. The sequence LPGLFGGEK of a lysylendopeptidyl fragments L-3 showed 67% and 78% identity to the residues 96-104 in the catalytic domains of HdAly and HdAlex, respectively. The sequence YDVYFENFGFGIGGK of a lysylendopeptidyl fragment L-4 showed 73% identity to the residues 80-95 in the catalytic domains of HdAly and HdAlex. The K95 was predicted as a key residue for the catalytic action of HdAly (Yamamoto et al. 2008).

These amino-acid identities between *Aplysia* enzymes and abalone and turban shell PL-14 enzymes indicate that both AkAly28 and AkAly33 also belong to PL-14.

4. Discussion

Alginate from brown seaweeds has been widely used in various industrial fields such as food and pharmaceutical industries because of its ability to form highly viscous solution as sodium salt and elastic gel upon chelating divalent metal ions (Onsøyen E., 1996). Recently, degradation products of alginate produced by alginate lyase were shown to exhibit various biological activities, e.g., promotion of root growth in higher plants (Tomoda et al., 1994; Sutherland IW., 1995; Xu et al., 2003), acceleration of growth rate of *Bifidobacterium* sp. (Akiyama et al., 1992), induction of production of cytotoxic cytokines in human mononuclear cells (Natsume et al., 1994; Iwamoto et al., 2003), suppression of IgE (Yoshida et al., 2004), antitumour and antibacterial effects (Hu et al., 2004 and 2005). These facts led us to consider that alginate lyase is applicable to extend practical uses of alginate and its oligosaccharides. Besides the practical uses, alginate lyase is known as an important enzyme for the seaweed-feeding gastropods like

abalone, turban shell and sea hare. This enzyme is considered to provide carbon and energy sources for the gastropods through the degradation of seaweeds' alginate. Compared with abalone and turban shell alginate lyases, other gastropod enzymes have not been so well characterized. Thus, in the present study, we isolated alginate lyases from the common sea hare *A. kurodai* and determined their general properties.

Two alginate lyase isozymes, AkAly28 and AkAly33, were successfully isolated from the digestive fluid of *A. kurodai*. The specific activity of AkAly28 was 2-times higher than that of AkAly33 and the yield of AkAly28 was also higher than that of AkAly33 (Fig. 2 and Table 1). In the TOYOPEARL CM-650M chromatography, another alginate lyase(s) showing endolytic poly(M) lyase activity was also detected. Although we have not purified this enzyme yet, this suggests that at least three kinds of poly(M) lyases are present in the digestive fluid of *A. kurodai*. The presence of multiple alginate lyases may relate to the specific food habit of sea hare, i.e., this animal usually feeds various kinds of brown seaweeds belonging to Laminariales and Fucales. Simultaneous actions of multiple alginate lyases may be advantageous on the degradation of alginate in the cell-wall matrices with different structures from various seaweeds.

The optimum pH of both AkAly28 and AkAly33 was at around 6.7 whereas those of other gastropod alginate lyases were usually observed at weak alkaline pH, e.g., optimal pHs of alginate lyases from *H. discus hannai*, *H. iris*, and *O. rusticus* were at 8.0-8.5, and that of a *Littorina* enzyme was pH 7.5 (Hata et al., 2009). It is noteworthy that AkAly33 retained relatively high activity in a wide pH range compared with AkAly28, e.g., it showed more than 15% of maximal activity at pH 5.0 - 9.0 and about 40% even at pH 5.5 where AkAly28 showed no activity. Similar acid-tolerance property was also reported in a *Littorina* enzyme, which retained 90% or higher activity even after the incubation at 30 °C for 15 min at pH 3-11 (Hata et al., 2009). The optimum temperature of both AkAly28 and AkAly33 were 40 °C and the temperature that caused a half inactivation of these enzymes during 20-min incubation was 38 °C. These

values are fairly consistent with those of other gastropod alginate lyases. Both AkAly28 and AkAly33 showed high NaCl dependency. In the absence of NaCl, AkAly33 showed ~20% maximal activity but AkAly28 no activity, and both enzymes required 0.2-0.25 M NaCl to exhibit maximal activities. Similar NaCl requirement was also shown in a *Littorina* enzyme, e.g., it showed 20% maximal activity in the absence of NaCl and maximal activity at 0.05 M NaCl (data not shown). Whereas less NaCl requirement was observed in abalone enzyme HdAly which exhibited 70% maximal activity in the absence of NaCl and maximal activity above 0.05 M NaCl (data not shown). Thus, NaCl requirement seemed to be a common property among the molluscan alginate lyases although its extent differs depending on the animal species.

Activation of alginate lyase by monovalent and divalent metal ions was previously reported in the enzymes from marine bacteria (Hu et. al., 2006). Whereas, alginate lyases from soil and terrestrial bacteria did not require metal ions to express their optimum activities (Preston et. al., 2000; Cao et. al., 2007). These facts may reflect an aspect of physiological adaptation of the alginate lyases to sea water environment which contains various divalent metal ions and ~0.6 M NaCl. Thus, it is reasonable to consider that the NaCl requirement of molluscan alginate lyases is also ascribable to the adaptation to sea water environment. In case of an enzyme from halophilic bacteria, mechanisms for the activation by NaCl were explained by the changes in the enzyme structure into a suitable form for approaching to, or binding with, the substrate (Lanyi, 1974). Activation mechanisms for molluscan alginate lyase by NaCl are still remained obscure.

AkAly28 and AkAly33 preferably degraded poly(M)-rich substrates and rapidly decreased the viscosity of alginate solution, thus both of the two enzymes were regarded as endolytic poly(M) lyase (EC 4.2.2.3) like other gastropod alginate lyases (Muramatsu et. al., 1977; Heyraud et al., 1996; Shimizu et al., 2003; Hata et al., 2009). However, the activities toward oligosaccharides were considerably different

between two enzymes. Namely, AkAly28 degraded tetra- and pentasaccharide producing tri- and disaccharide but not degraded trisaccharide, while AkAly33 could degrade trisaccharide to disaccharide and α -keto acid (Fig. 8B and C). These differences between AkAly28 and AkAly33 may imply that the roles of two enzymes are somewhat different in the digestive fluid. For example, AkAly28 acts on larger alginate substrates producing mainly trisaccharide and AkAly33 degrades the trisaccharide producing disaccharide and α -keto acid. We previously isolated two alginate lyase isozymes from the digestive fluid of *H. discus hannai*, i.e., endolytic HdAly and exolytic HdAlex (Shimizu et al., 2003; Suzuki et al., 2006). HdAly produced unsaturated trisaccharide and HdAlex degraded the trisaccharide to disaccharide and α -keto acid. Thus, AkAly28 and AkAly33 of *A. kurodai* may correspond to HdAly and HdAlex of *H. discus hannai*, respectively.

The partial amino-acid sequences of AkAly28 and AkAly33 indicated that these enzymes are the members of PL-14 which includes abalone and turban-shell enzymes (Muramatsu et al., 1996; Shimizu et al., 2003; Suzuki et al., 2006). The N-terminal amino-acid sequences of AkAly28 and AkAly33 showed practically no identities with those of abalone and turban shell enzymes; however, the internal sequences of AkAly28 and AkAly33 showed considerably high similarity with the corresponding sequences of abalone and turban shell enzymes (Table 2). The K95, which was predicted as a key residue for the catalytic action of HdAly (Yamamoto et al. 2008), was conserved in a lysylendopeptidyl fragment (L-4) of AkAly33. The importance of this lysine residue was also reported in the *Chlorella* virus PL-14 enzyme (Ogura et al, 2009). These amino-acid sequence analyses for AkAly28 and AkAly33 indicate that these enzymes are the members of PL-14 like abalone and turban shell enzymes although the sequences AkAly28 and AkAly33 may be somewhat diverged from other gastropod enzymes. To determine the structural characteristics of *Aplysia* alginate lyases and investigate the regions relating to their catalytic actions, we recently cloned

cDNAs encoding the *Aplysia* alginate lyases. Complete primary structure of *Aplysia* alginate lyase will be published elsewhere.

Acknowledgements

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

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Legends to figures

Fig. 1. Purification of alginate lyases from *A. kurodai* by AKTA-FPLC. (A) Mono-Q column chromatography of the active fractions obtained by TOYOPEARL-DEAE 650M chromatography. (B) Mono-S column chromatography of the passed through fractions in Mono-Q column chromatography. Conditions for the chromatographies are described under “Materials and Methods”. Activity levels for fractions are indicated with shaded bars.

Fig. 2. SDS-PAGE for the *Aplysia* alginate lyase in various purification steps. Mk, molecular mass markers; Lane 1, Proteins precipitated between 60 and 90% saturation of ammonium sulfate; Lane 2, The first peak fractions obtained by DEAE TOYOPEARL-650 M chromatography; Lane 3, AkAly33 purified by Mono-Q column chromatography; Lane 4, AkAly28 purified by Mono-S column chromatography.

Fig. 3. Effects of pH, temperature, and NaCl on AkAly28 and AkAly33. (A) pH dependence was measured at 30 °C in reaction mixtures adjusted to pH 4-11 with 50 mM sodium phosphate buffer. (B) Temperature dependence was measured at 10-60 °C in a reaction mixture containing 0.15% sodium alginate, 0.15 M NaCl and 10 mM sodium phosphate (pH 7.0). (C) Thermal stability was assessed by measuring the activity remaining after the heat-treatment at 15-45 °C for 20 min. (D) NaCl dependence was measured in a reaction mixture containing various concentrations of NaCl, 0.15% sodium alginate, 10 mM sodium phosphate (pH 7.0) and 1 U/mL enzyme at 30 °C. ○, AkAly28; ●, AkAly33.

Fig. 4. Substrate preference of AkAly28 and AkAly33. Activity was measured in a reaction mixture containing either the sodium alginate (○), poly(M)-rich substrate (●), poly(MG)-rich substrate (Δ) or

poly(G)-rich substrate (▲) in a concentration of 0.15% (w/v). Degradation of substrates was monitored by measuring the increase in absorbance at 235 nm. A, AkAly28; B, AkAly33.

Fig. 5. Decrease in the viscosity of sodium alginate solution by the digestion with AkAly28 and AkAly33.

The reaction was carried out at 30 °C in an Ostwald-type viscometer, in a mixture containing 0.12% sodium alginate, 0.15 M NaCl, 10 mM sodium phosphate (pH 7.0), 10 U/mL AkAly28 (○) or 7 U/mL AkAly33 (●).

Fig. 6. TLC for the degradation products of poly(M)-rich substrates produced by AkAly28 and AkAly33.

Poly(M)-rich substrate (1.0% (w/v)) in 10 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl was degraded by AkAly28 (A) and AkAly33 (B) at 30 °C for 6 h. The aliquots of reaction products (each 2 μL) were applied to TLC-60 plate and developed with 1-butanol–acetic acid–water (2:1:1, v:v:v). Total sugars separated on the plate were visualized by spraying with sulfuric acid in ethanol (A and B, left), while unsaturated sugars and α-keto acid were detected by TBA staining (A and B, right). M, marker oligosaccharides; α-keto, α-keto acid (an open chain form of 2-keto-3-deoxy-gluconaldehyde); ΔM, unsaturated disaccharide; ΔM2, unsaturated trisaccharide; ΔM3, unsaturated tetrasaccharide; ΔM4, unsaturated pentasaccharide; ΔM5, unsaturated hexasaccharide.

Fig. 7. Anion-exchange chromatography of the degradation products of Poly(M)-rich substrate. (A)

Poly(M)-rich substrate (1.0% (w/v)) was degraded by 100 U/mL of AkAly28 at 30 °C and the degradation products obtained at reaction time 0 h, 1 h and 6 h were subjected to TSK-GEL DEAE-2SW anion-exchange chromatography. Elution of oligosaccharides was detected with a Shimadzu SPD-20A UV detector at 235 nm. (B) Poly(M)-rich substrate was degraded by 100 U/mL of AkAly33 as in (A). ΔM, unsaturated disaccharide; ΔM2, unsaturated trisaccharide.

Fig. 8. TLC for the degradation products of unsaturated oligosaccharides produced by AkAly28 and AkAly33. (A) Unsaturated oligosaccharide substrates, Δ M- Δ M6. (B) The oligosaccharides degraded by AkAly28 at 30 °C for 2 h. (C) The oligosaccharides degraded by AkAly33 as in (B). The reaction products developed on TLC plate were visualized by TBA staining. M, marker; Δ M, unsaturated disaccharide; Δ M2, unsaturated trisaccharide; Δ M3, unsaturated tetrasaccharide; Δ M4, unsaturated pentasaccharide; Δ M5, unsaturated hexasaccharide; Δ M6, unsaturated heptasaccharide.

Table 1. Summary of the purification of AkAly28 and AkAly33.

Samples	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude ^a	2163	79.06	171007	1	100
AS ^b	324.26	390.06	126481	4.93	73.96
DEAE ^c	8.64	2631.03	22732	33.28	13.29
AkAly33 ^d	3.15	2057.14	6480	26.02	3.79
AkAly28 ^e	1.46	5740.74	8382	72.61	4.90

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

^b Fraction precipitated between 60 and 90% saturation of ammonium sulfate.

^c Active fractions obtained by DEAE TOYOPEARL-650M chromatography.

^d AkAly33 purified by Mono-Q column chromatography.

^e AkAly28 purified by Mono-S column chromatography.

Table 2. Partial amino-acid sequences of AkAly28 and AkAly33

Peptides ^a	Sequences ^b	Similar regions of other enzymes ^c
AkAly28		
N-terminus	ASTLWSVGSVPHSTDVSSILGHFAPYYHEWGDDSI S TSTK	
T-1	<u>KGSF</u> <u>SPL</u> HDKR	HdAly, SP2 (51-61)
T-2	<u>GRF</u> <u>KFK</u>	HdAly, SP2 (159-164)
L-1	MP <u>GLFGG</u> EDGDGAYK	HdAly (96-111)
L-2	WNSVSEE <u>VHINTV</u> GK	SP2 (170-184)
AkAly33		
N-terminus	DTVIWSLSSVPLSSDTDVILQNF ^b GPMYHDFGDDSI S TSTK	
T-3	<u>GMFF</u> STFFGG S KK	HdAly (216-228); HdAlex (216-228)
L-3	<u>LPGLFGG</u> EK	HdAly (96-104); HdAlex (96-104)
L-4	<u>YDVYFENFGFG</u> I G GK	HdAly (80-95); HdAlex (80-95)

^aT-1 – T-3, tryptic fragments; L-1 – L4, Lysylendopeptidyl fragments. ^bResidues conserved among PL-14 enzymes are underlined and a catalytically important lysine in PL-14 enzymes is shown as the bold letter. ^cResidue numbers for similar sequence regions in abalone HdAly and HdAlex, and turban shell SP2 are shown in the parentheses.

Fig.1

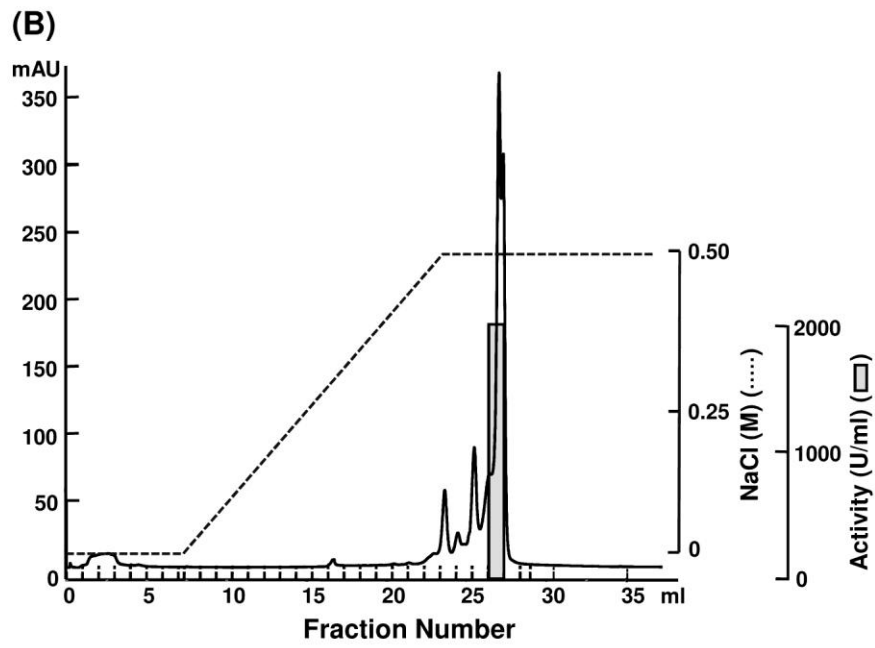
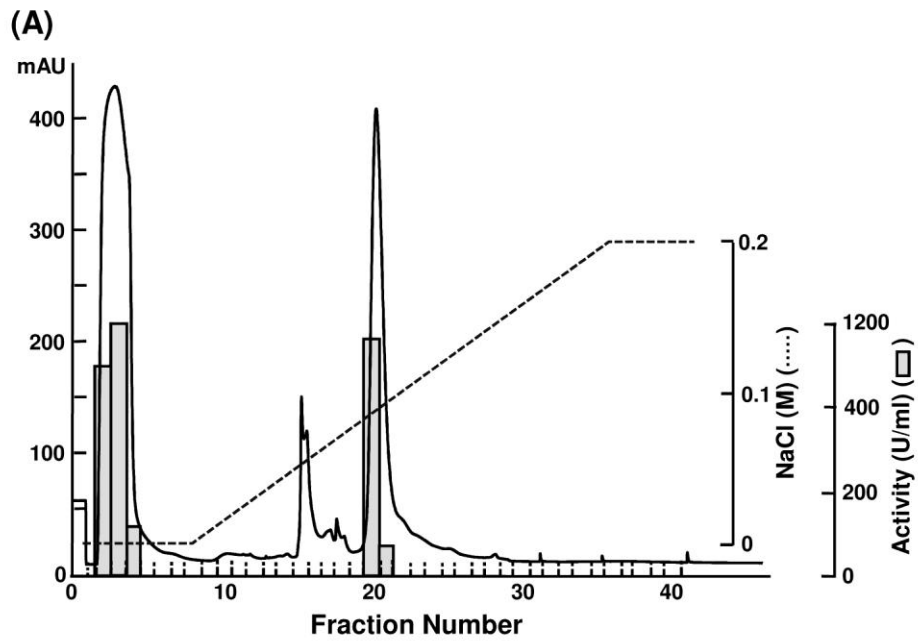


Fig. 2

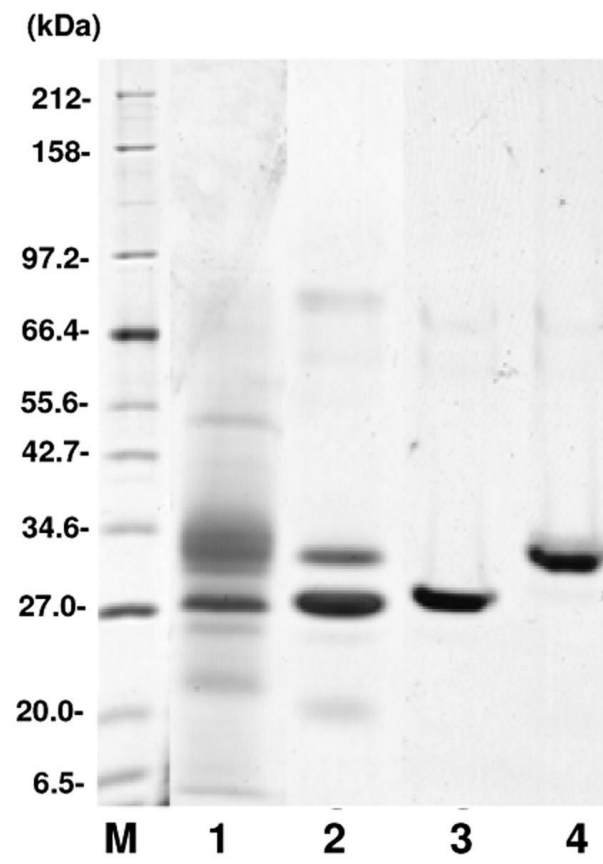


Fig. 3

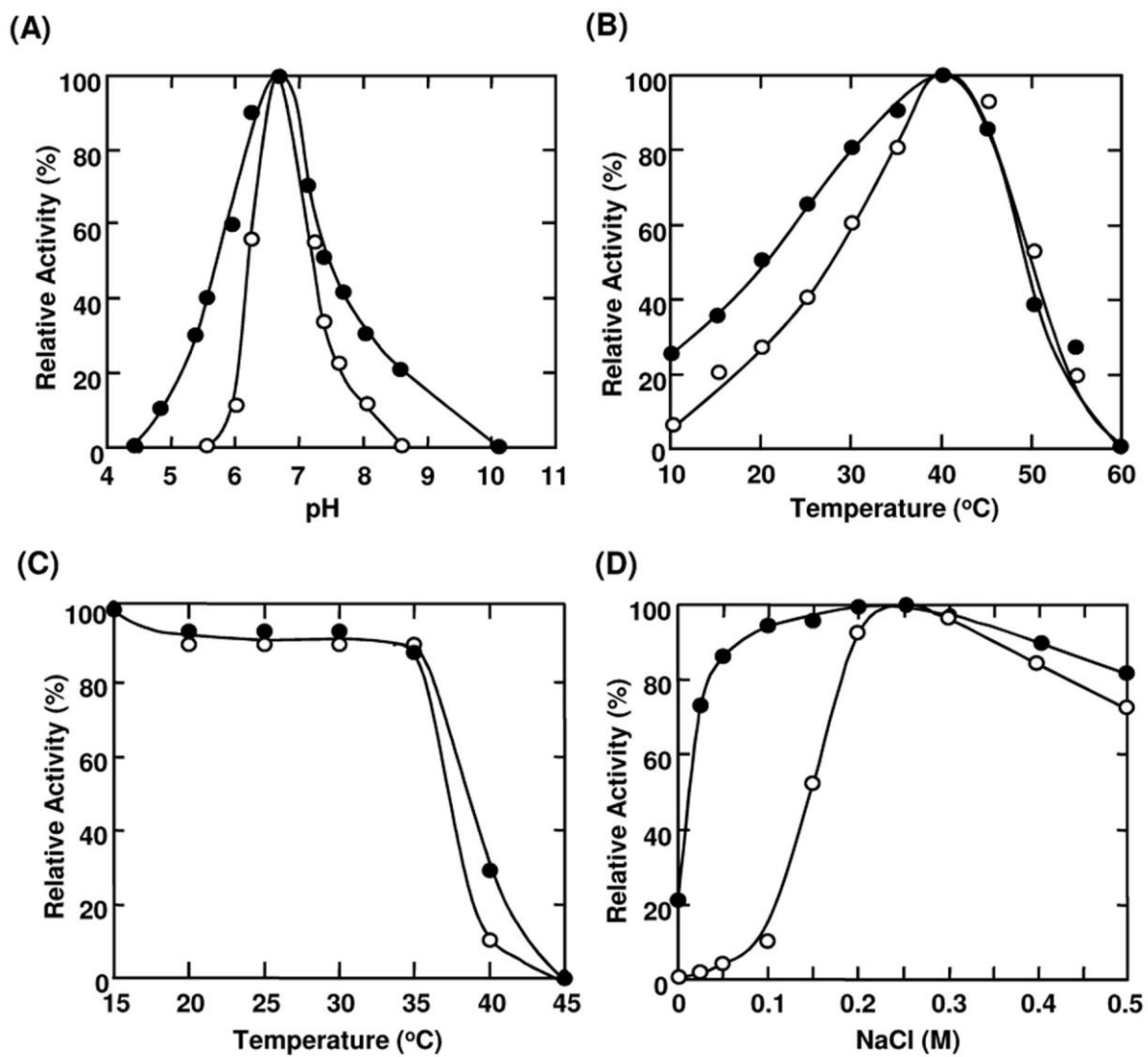


Fig. 4

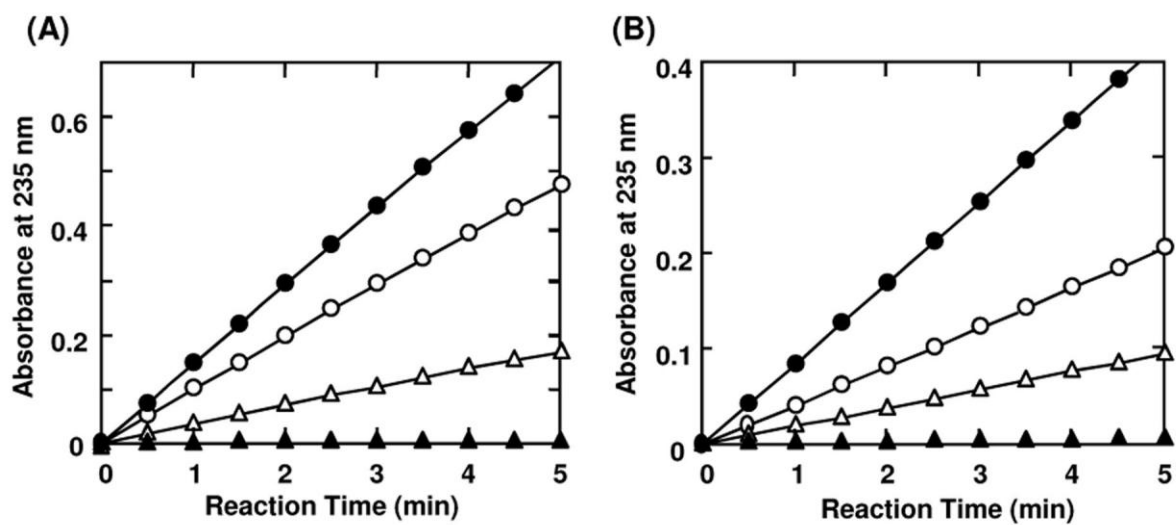


Fig. 5

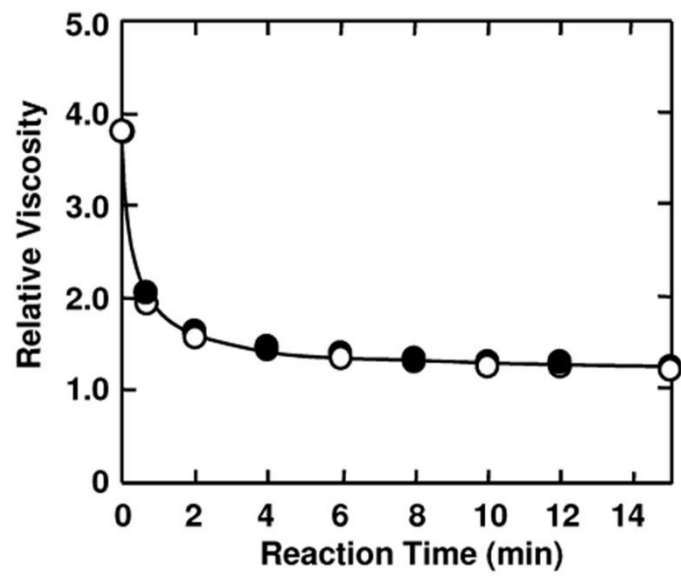


Fig. 6

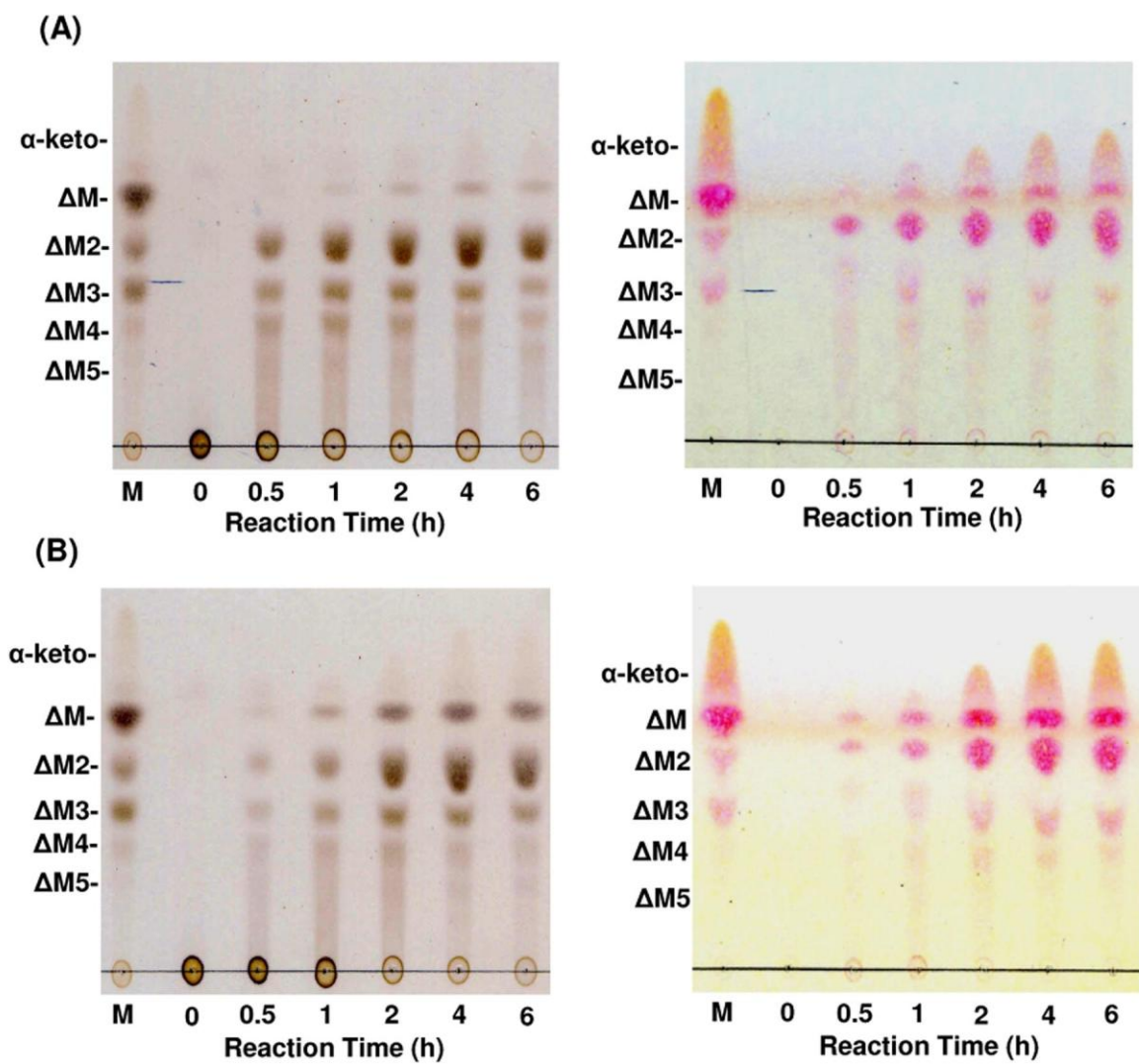


Fig. 7

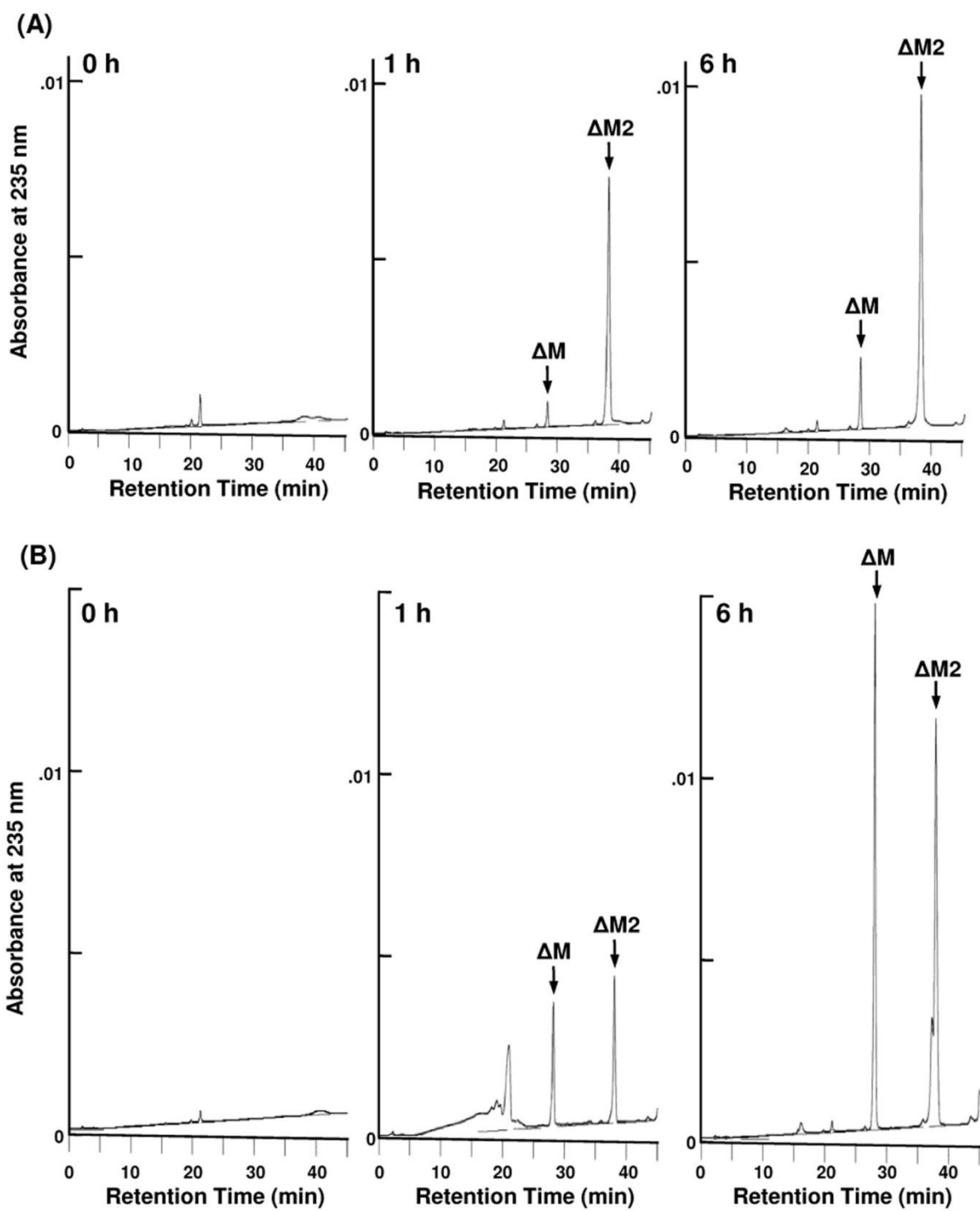


Fig. 8

