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Purification and characterization of a Na^+/K^+ dependent alginate lyase from turban shell gut *Vibrio* sp. YKW-34

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Abstract

An alginate lyase with high specific enzyme activity was purified from *Vibrio* sp. YKW-34, which was newly isolated from turban shell gut. The alginate lyase was purified by in order of ion exchange, hydrophobic and gel filtration chromatographies to homogeneity with a recovery of 7% and a fold of 25. This alginate lyase was composed of a single polypeptide chain with molecular mass of 60 kDa and isoelectric point of 5.5–5.7. The optimal pH and temperature for alginate lyase activity were pH 7.0 and 40 °C, respectively. The alginate lyase was stable over pH 7.0–10.0 and at temperature below 50 °C. The alginate lyase had substrate specificity for both poly-guluronate and poly-mannuronate units. The k_{cat}/K_m value for alginate (heterotype) was $1.7 \times 10^6\ s^{-1}\ M^{-1}$. The enzyme activity was completely lost by dialysis and restored by addition of Na^+ or K^+. The optimal activity exhibited in 0.1 M of Na^+ or K^+. This enzyme was resistant to denaturing reagents (SDS and urea), reducing reagents (β-mercaptoethanol and DTT) and chelating reagents (EGTA and EDTA).
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Keywords: Alginate lyase; *Vibrio* sp.; Na^+ or K^+ dependent; Purification; Characterization

1. Introduction

Alginate is a kind of linear polysaccharide composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues linked with 1,4-*O*-glycoside bonds. The homopolymeric blocks of M and G, and their alternating sequence are coexistent in alginate molecule [1]. The most important commercial sources of alginate are species of *Laminaria*, *Macrocystis* and *Ascophyllum* [2]. Alginate lyase, characterized as mannuronate lyase (EC 4.2.2.3) and guluronate lyase (EC 4.2.2.11), can catalyze the degradation of alginate by the β-elimination mechanism yielding products with an unsaturated uronic acid at the non-reducing terminus [3].

The application of alginate lyase to the production of alginate oligosaccharides is becoming increasingly important. Because of special chemical properties and biological activities, alginate oligosaccharides are widely used in food industry, biotechnology area and medical field [4–6]. Another application of the alginate lyase is to partly hydrolyze the cell wall of brown algae, in which the digestion of the brown algae was facilitated and its protoplasmic detritus could be developed as a feed for animals [7,8]. Due to the broad application of alginate lyase, it has been isolated from a variety of sources, such as marine mollusks (*Haliotidae* [9] and *Littorinidae* [10]) and various species of bacteria [11,12].

Vibrio sp. YKW-34 that has a high cell wall hydrolytic activity on brown algae was isolated from the gut of the turban shell, *Turbinidae batillus cornutus* [13]. In the present study, the alginate lyase produced by this strain was purified and its various properties were analyzed.

2. Materials and methods

2.1. Materials

Alginate (Satialgine S-1100, M/G = 1.2) was purchased from Systems Bio-industry (WI, USA). The M and G blocks of alginate were donated by Ocean University of China (Qingdao, China). Marine broth medium was purchased from Difco Laboratories (Detroit, MI, USA). DEAE Sepharose FF, Phenol Sepharose 6 FF, and Sephacryl S-100 HR were from Amersham Pharmacia Biotech. (Uppsala, Sweden). Polyacrylamide and molecular mass marker were from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals used in this study were of guaranteed reagent grade.

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2.2. Bacterial strain and growth media

For enzyme production, the *Vibrio* sp. YKW-34 was grown in 1-l flasks, containing 200 ml of artificial seawater (2.5% NaCl, 0.5% $MgSO_4 \cdot 7H_2O$, 0.1% KCl, 0.02% $CaCl_2$, 0.01% K_2HPO_4, 0.002% $FeSO_4 \cdot 7H_2O$) supplemented with 0.2% alginate and 0.2% KNO_3 with initial pH of 8.0 adjusted by 1N of NaOH. The seed was cultured in marine broth under the condition described in previous report [13] for 12 h to reach a cell density of 1.0×10^7 CFU/ml. Twenty milliliters of the culture was subsequently inoculated into each flask. The flasks were incubated at 25 °C with shaking at 110 rpm for 48 h.

2.3. Enzyme activity assay

The alginate lyase activity was quantified by spectrometric determination of reducing sugars by Nelson method [14] unless otherwise mentioned. The reaction mixture, containing 100 μl of enzyme solution, 100 μl of 0.1% alginate and 200 μl of 20 mM Tris–HCl (pH 8.0) with 0.2 M NaCl, was incubated at 30 °C for 1 h. The formed reducing sugars were then determined by Nelson reagent. Enzyme activity (U/ml) was defined as the amount of enzyme required to liberate 1 μmol reducing sugar (mannuronic acid or guluronic acid)/min.

When comparing the specific activity of the purified alginate lyase with others, the alginate lyase activity was also measured by ultraviolet absorption method at 235 nm, in which one unit was defined as an increase of 0.1 in absorbance at 235 nm/min [11].

2.4. Protein determination

Protein concentration was determined by the method of Bradford [15] using bovine serum albumin as the calibration standard. The relative protein contents of chromatography fractions were estimated by absorbance at 280 nm.

2.5. Enzyme purification

All the purification procedures were carried out at 4 °C. 400 ml of culture fluid was centrifuged at $12{,}000 \times g$ for 15 min, and the cell free supernatant was concentrated by ultrafiltration (10-kDa cutoff membrane, Millipore, Bedford, MA) to 80 ml followed by dialyzed against distilled water overnight. The carbohydrates and degradation products of alginate were removed by polyethyleneimine (PEI) precipitation method [16] with some modification. 1% PEI was added dropwise with stirring, and the precipitation was removed by centrifugation ($10{,}000 \times g$; 15 min). The lowest quantity of PEI required to remove carbohydrate was determined by measuring the alginate lyase activity in the supernatant. The supernatant was dialyzed against buffer A (20 mM Tris–HCl buffer, pH 8.0) for overnight and applied to a DEAE-Sepharose FF column (2.6 cm × 30 cm), which was equilibrated with buffer A in advance. Bound proteins were eluted with 800 ml linear gradient of 0–1 M of NaCl in buffer A at a flow rate of 1.5 ml/min (8 ml/tube). Main active fractions (fraction numbers 35–41) were pooled, concentrated to 10 ml by ultrafiltration and dialyzed against buffer A. Ammonium sulfate was added to the dialyzed solution to a final concentration of 1.7 M. The enzyme solution was then applied to a Phenol Sepharose 6 FF column (1.6 cm × 10 cm), which was equilibrated with buffer A containing 1.7 M ammonium sulfate. Bound proteins were eluted with 450 ml linear gradient of 1.7–0 M ammonium sulfate in buffer A at 1.5 ml/min (6 ml/tube). Main active fraction (fraction numbers 50–53) was pooled, concentrated to 2 ml and dialyzed against buffer A containing 0.15 M NaCl. The dialyzed enzyme solution was applied to a Sephacryl S-100 HR column (1.6 cm × 60 cm) equilibrated with buffer A containing 0.15 M NaCl. Bound proteins were eluted with the same buffer at a flow rate of 0.5 ml/min (2 ml/tube). Active fractions (fraction number 22–28) were pooled and used for further study. The molecular mass was determined using the same column with a range of molecular mass markers: β-galactosidase (116 kDa), albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

2.6. Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [17] on 10% polyacrylamide gel with 0.1% SDS. Protein bands were detected with Coomassie Brilliant Blue stain. Molecular mass marker for SDS-PAGE was consisted of albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and trypsin inhibitor (20.1 kDa).

2.7. Determination of isoelectric point

The purified enzyme solution was focused on a Rotofor cell (Bio-Rad Laboratories, CA, USA) according to the Rotofor system instruction manual using ampholytes with pH range of 3–10, and 20 fractions were collected after focusing. Alginate lyase activity assay was monitored in each fraction. p*I* value was determined by the protein samples provided by the starter kit: phycocyanin (p*I* 4.5–5.5, blue), hemoglobin (p*I* 6.0–7.5, red) and cytochrome (p*I* 8.0–9.0, orange).

2.8. Effects of temperature and pH on alginate lyase activity and stability

The effect of temperature on alginate lyase activity was determined by carrying out the enzyme activity assay at each temperature (10–90 °C) at pH 8.0. The effect of pH on alginate lyase activity was assayed by replacing Tris–HCl buffer (pH 8.0) with 10 mM of each buffer: acetic acid buffer (pH 4–6), phosphate buffer (pH 7 and 8) and glycine-NaOH buffer (pH 9–11) at 30 °C. The temperature stability of the alginate lyase was determined by preincubating the enzyme solution at each temperature (10–90 °C) at pH 8.0 for 1 h, and then the residual enzyme activity was determined. The pH stability of the alginate lyase was determined by preincubating the enzyme solution at each pH (4–11) at 30 °C for 1 h, and then the residual enzyme activity was determined by adjusting the pH with Tris–HCl (0.1 M, pH 8.0). The relative activity was defined as the percentage of activity determined with respect to the maximum alginate lyase activity.

2.9. Effects of metal ions on alginate lyase activity

The purified alginate lyase was dialyzed against 20 mM Tris–HCl buffer (pH 8.0). The effects of metal ions on enzyme activity were determined by adding 100 μl of each metal ion to the reaction mixture with or without 0.1 M NaCl. The relative activity was defined as the percentage of activity determined with respect to that measured under the standard condition described previously. In order to evaluate the optimal concentrations of Na^+ and K^+ for enzyme activity, alginate lyase activity was determined at different concentrations of Na^+ and K^+. The relative activity was defined as the percentage of activity determined with respect to the maximum alginate lyase activity.

2.10. Effects of possible inhibitors and activators on alginate lyase activity

The purified alginate lyase was dialyzed against 20 mM Tris–HCl buffer (pH 8.0). The effects of chelators, denaturants, and reducing reagents on enzyme activity were determined by adding 100 μl of each reagent solution to the standard reaction mixture. The relative activity was defined as the percentage of activity determined with respect to that measured under the standard condition.

2.11. Substrate specificity

The purified enzyme was incubated with two types of homopolymeric substrate (M and G blocks) to evaluate the substrate specificity. In each case, the reaction was carried out as described previously by replacing alginate with 0.1% M or G blocks.

2.12. Kinetic parameters

K_m and V_{max} values for the alginate lyase acting on alginate at different concentrations of 0.05–0.5 mg/ml were calculated by linear regression analysis of Lineweaver–Burke [18], double-reciprocal, plots of initial velocity data obtained under the condition described above.

3. Results and discussion

3.1. Purification of the alginate lyase

The alginate lyase was purified from the cell free culture supernatant of *Vibrio* sp. YKW-34 by PEI precipitation, DEAE Sepharose, Phenol Sepharose and Sephacryl chromotographies (Fig. 1). The purification procedure from culture fluid was summarized in Table 1. In the cultural fluid, the protein concentration was as low as 0.135 mg/ml, whereas the carbohydrate concentration was high (data not shown). The PEI precipitation could fractionate the enzyme better on the DEAE-Sepharose chromatography (Fig. 1A) than without PEI (Fig. 1A insert), though this caused about 30% loss in the enzyme yield. The PEI was also useful to decrease the viscosity of culture filtrate, and thus facilitating the ultrafiltration membrane filtration. The alginate lyase with the highest protein and activity peaks was further purified by Phenol Sepharose (Fig. 1B) and Sepacryl chromotographies (Fig. 1C). The alginate lyase was finally purified by 25-fold with a yield of 7%.

The target protein turned out to be a negative charged protein with p*I* value of 5.5–5.7 and molecular mass of 60 kDa (will be shown in Section 3.3). The carbohydrates in the culture fluid, including the undegraded alginate and its degradation products with different molecular sizes, were acidic [19]. The similarity in charge and size made it difficult to separate small amount protein from large amount of carbohydrates. Therefore, PEI was used to separate carbohydrate from the crude cultural fluid. PEI precipitation method was applied to remove the bulk of the nucleic acids and acidic carbohydrates in the extract from plant sources [16] as well as to purify the extracelluar alginate lyase in the cultural filtrate of a marine bacterium [19].

3.2. Specific activity of the alginate lyase

The specific activity of the purified alginate lyase was determined as 55.93 and 3731 U/mg by reducing sugar method and ultraviolet absorption method, respectively. Commonly used methods for alginate lyase activity assay include ultraviolet absorption method [11,20,21], reducing sugar method [12,22], and TBA method [19,23–25]. Iwamoto et al. [11] compared the results of TBA method and ultraviolet absorption method for alginate lyase activity assay. Based on the above results of specific activity and the data acquired by Iwamoto et al., the

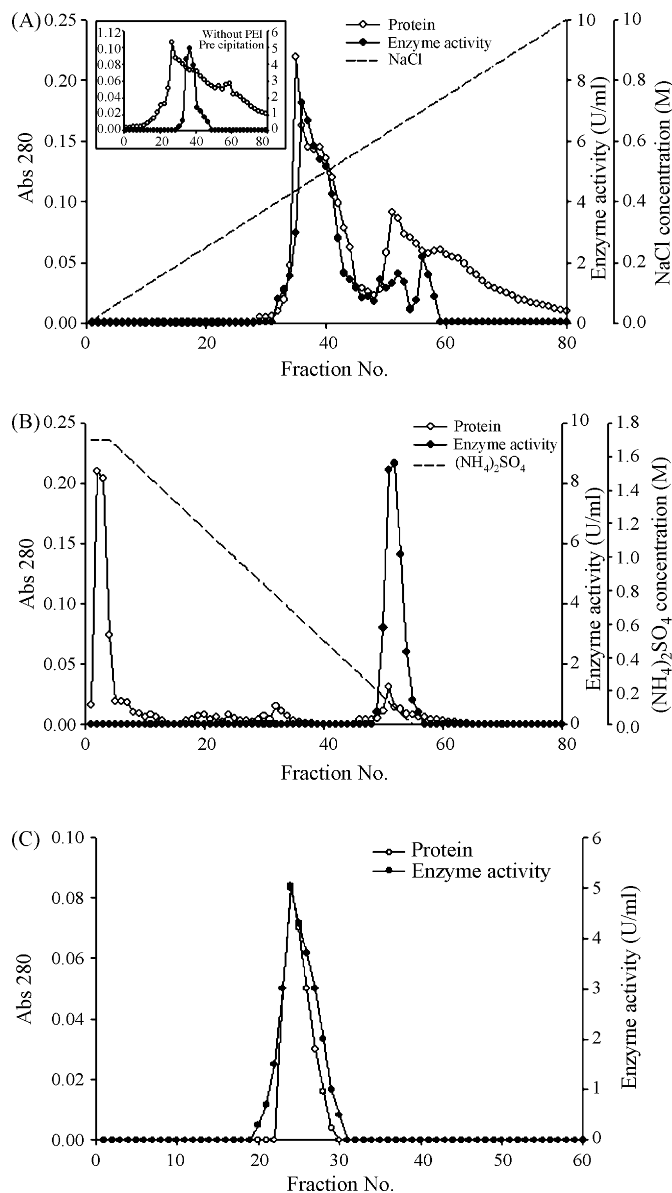

Fig. 1. Purification of the alginate lyase produced by *Vibrio* sp. YWK-34. (A) DEAE Sepharose FF column chromatography of culture fluid with and without (the inset) previous PEI precipitation. (B) Phenol Sepharose 6 FF column chromatography. (C) Sephacryl S-100 HR column chromatography. Protein concentration was determined by its absorbance at 280 nm and alginate lyase activity was determined by Nelson method using alginate as the substrate.

Table 1
Purification of alginate lyase from *Vibrio* sp. YKW-34

Step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture fluid	400	54.00	119.64	2.22	100	1
PEI precipitation	80	37.52	86.01	2.29	72	1
DEAE Sepharose	56	8.57	29.99	3.50	25	2
Phenol Sepharose	24	0.43	15.58	36.23	13	16
Sephacryl	14	0.14	7.83	55.93	7	25

Alginate lyase activity was measured by Nelson method described in the text. One unit of the enzyme activity was defined as the amount of enzyme required to liberate 1 μmol reducing sugar/min.

Table 2
Comparison of specific activity, molecular mass, p*I* and optimal temperature and pH of alginate lyases from different microorganisms

Microorganism	Specific activity[a]		Mr (kDa)	p*I*	Optimal *T* (°C)	Optimal pH	Reference
	Reported value	Converted to A_R					
Vibrio sp. YKW-34	$A_R = 55.93$ $A_U = 3731$	55.93	60	5.5–5.7	40	7.0	
Vibrio sp. 510-64	$A_R = 25.00$	25.00	34.6	–	35	7.5	[12]
Pseudoalteromonas sp.	$A_R = 0.40$	0.40	25, 79	–	35–45	7.0–8.0	[22]
Alteromonas sp.	$A_U = 1122.8$	16.84	33.9	3.8	–	7.5–8.0	[11]
Pseudomonas sp.	$A_U = 222.8$	3.34	36	–	–	7.5	[20]
Corynebacterium sp.	$A_U = 57$	0.86	27	7.3	55	7.0	[21]
A marine bacterium	$A_T = 48$	45.00	29	4.2–5.0	–	7.8	[19]
Vibrio sp.	$A_T = 1.93$	1.81	57, 47	4.6	–	8.2	[23]
Enterobacter sp.	$A_T = 42.9$	40.22	31	8.9	35	7.5	[24]
Pseudomonad sp.	$A_T = 73.0$	68.44	50	–	30	7.5	[25]

[a] Specific activities of alginate lyases were determined by three methods in different literatures: A_R (μmol/mg/min) is by reducing sugar method, A_U ($0.1\Delta_{235}$nm/mg/min) is by ultraviolet absorption method and A_T (μmol/mg/min) is by TBA method.

following Eqs. (1) and (2) were established, respectively,

$$A_R = 0.015\ A_U \quad (1)$$

$$A_T = 0.016 A_U \quad (2)$$

where A_R (μmol/mg/min) is enzyme activity determined by reducing sugar, A_U ($0.1\Delta_{235}$nm/mg/min) by ultraviolet absorption method and A_T (μmol/mg/min) is by TBA method.

The specific enzyme activity, a measure of enzyme efficiency, is an important parameter to describe the property of an enzyme. However, it is hard to compare the specific activity of different alginate lyase directly because of different assay method and activity unit. The above Eqs. (1) and (2) facilitated the comparison of the specific activity among different alginate lyases. After converting the specific activity reported as A_U or A_T to the equal value of A_R, the specific activities of various alginate lyases could be compared. As shown in Table 2, the specific activity of the purified alginate lyase from *Vibrio* sp. YKW-34 was higher than those of other alginate lyases [11,12,19–24], but only lower than that of an alginate lyase from *Pseudomonad* sp. described by Davidson et al. [25].

3.3. Determination of molecular mass and isoelectric point

The protein pattern after each purification procedure is shown in the SDS-PAGE (Fig. 2). The single band after the final procedure indicated that the alginate lyase was composed of one polypeptide chain with the molecular mass of around 60 kDa, which was coincident with the result estimated by Sephacryl S-100 gel filtration chromatography. Molecular masses of other alginate lyases were in a range of 25–79 kDa (Table 2). The molecular mass of alginate lyase from *Vibrio* sp. YKW-34 belonged to this range.

The purified alginate lyase focused mainly in the 4th fraction near the anode, and the isoelectric point was estimated to be 5.5–5.7. Hence, this alginate lyase was characterized as a negative charged acidic protein. As shown in Table 2, p*I* values of most alginate lyases derived from marine microorganisms were lower than 7.0 [11,19,23]. Only a few alginate lyases showed p*I* values above 7.0 [21,24].

3.4. Effects of temperature and pH on alginate lyase activity and stability

The temperature profile of the alginate lyase is shown in Fig. 3A. The optimal temperature of this alginate lyase was 40 °C. This enzyme was active at temperature down to 30 °C, but the activity decreased at temperature below 30 °C or above 40 °C. The alginate lyase possessed more than 70% activity after

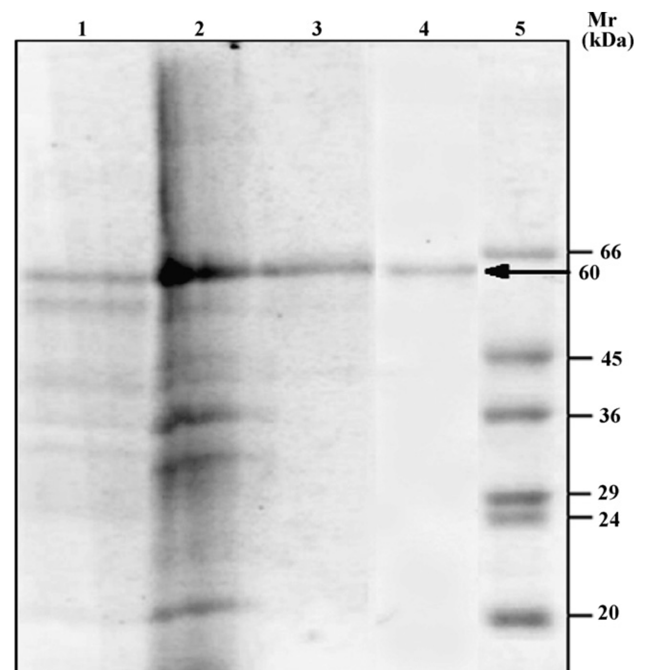

Fig. 2. SDS-PAGE analysis of samples from various purification steps containing the alginate lyase produced by *Vibrio* sp. YWK-34. Lanes: 1, culture fluid; 2, after DEAE Sepharose; 3, after Phenol Sepharose; 4, after Sephacryl and 5, molecular mass marker.

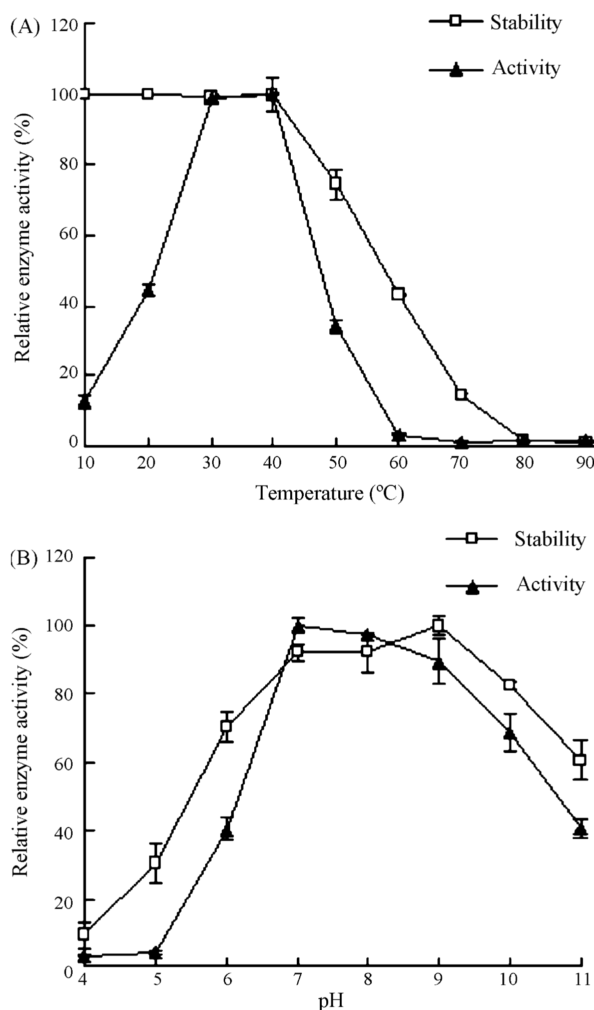

Fig. 3. Effects of temperature and pH on stability and activity of the alginate lyase produced by *Vibrio* sp. YWK-34. (A) Temperature profiles were checked at different temperatures (10–90 °C) in 0.1 M Tris–HCl (pH 8.0). (B) pH profiles were checked at 30 °C in different buffers (pH 4.0–11.0). Alginate lyase activity was determined by Nelson method using alginate as the substrate. The relative activity was defined as the percentage of activity determined with respect to the maximum alginate lyase activity.

incubation at 50 °C for 1 h, and gradually inactivated as temperature increased. These results indicated that the alginate lyase was thermostable up to 50 °C. Comparing with other alginate lyases (Table 2), the optimal temperature of this alginate lyase was higher than those of other alginate lyases [12,24,25], and similar to that of the *Pseudoalteromonas* sp. alginate lyase [22]. Only the alginate lyase from *Corynebacterium* sp. showed a higher optimal temperature of 55 °C but it was not stable at this temperature [21].

The pH profile of the alginate lyase is shown in Fig. 3B. The maximum alginate lyase activity was observed at pH 7.0, and more than 70% of the alginate lyase activity still remained after incubation at a pH range of 7.0–10.0 for 1 h. The optimal pH of this alginate lyase was similar to that of the alginate lyase from *Corynebacterium* sp. [21], and lower than those of other alginate lyases (Table 2). The pH stability over a wide pH facilitated the industrial application of this alginate lyase at neutral and alkaline pH conditions.

Table 3
Effects of metal ions on the alginate lyase activity purified from *Vibrio* sp. YKW-34

Metal ions	Relative activity (% ± S.D.)[a]			
	Without NaCl		With NaCl	
	0.05 M[b]	0.1 M	0.05 M	0.1 M
None	0 ± 0.25	0 ± 0.25	79.4 ± 2.14	100 ± 0.63
Na^+	79.4 ± 0.32	100 ± 0.63	98.6 ± 1.07	89.5 ± 2.27
K^+	88.4 ± 0.25	100 ± 0.20	99.2 ± 2.15	88.7 ± 2.52
Mg^{2+}	0 ± 0.13	0 ± 0.13	100 ± 1.07	100 ± 1.78
Ca^{2+}	0 ± 0.25	0 ± 0.51	100 ± 1.23	100 ± 0.95
Al^{3+}	0 ± 0.38	0 ± 0.38	100 ± 0.97	100 ± 1.73
Ba^{2+}	0 ± 0.13	0 ± 0.25	100 ± 1.66	100 ± 2.02
Fe^{2+}	0 ± 0.63	0 ± 0.13	73.5 ± 1.82	70.3 ± 2.42
Mn^{2+}	0 ± 0.25	0 ± 0.13	68.0 ± 0.83	65.6 ± 7.66
Cu^{2+}	0 ± 0.25	0 ± 0.51	59.4 ± 1.57	58.2 ± 2.44
Hg^{2+}	0 ± 0.38	0 ± 0.51	37.3 ± 1.21	35.4 ± 4.65
Fe^{3+}	0 ± 0.63	0 ± 0.63	32.2 ± 3.58	30.2 ± 0.75
Ag^+	0 ± 0.13	0 ± 0.25	29.5 ± 0.31	26.0 ± 6.37

[a] The activity measured by Nelson method under the standard condition described in the text was defined as 100%.
[b] The concentration is the final concentration of metal ions in the enzyme activity assay system.

3.5. Effects of metal ions on alginate lyase activity

Effects of metal ions on the purified alginate lyase activity are shown in Table 3. After dialysis to remove metal ions, no enzyme activity was observed in the presence of other metal ions, but the enzyme activity was restored in the present of Na^+/K^+. On the other hand, in the reaction mixture with NaCl, the metal ions of Fe^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{3+} and Ag^+ inhibited its activity. Since less or extra amount of Na^+/K^+ had negative effect on alginate lyase activity, the optimal concentrations of Na^+ and K^+ were investigated. The results in Fig. 4 indicated that 0.1 M of Na^+ or K^+ was the optimal concentration for alginate lyase activity.

The novel metal ion dependent property of this alginate lyase indicated that the monovalent cation such as Na^+ and K^+ acted as a cofactor in its active site. Dialysis resulted in completely

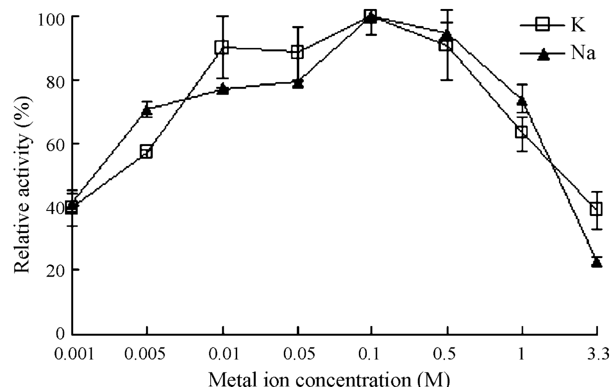

Fig. 4. Effects of the concentration of Na^+ and K^+ on activity of the alginate lyase produced by *Vibrio* sp. YKW-34. The purified alginate lyase was dialyzed against 20 mM Tris–HCl buffer (pH 8.0). Enzyme activity was assayed with K^+ and Na^+ at different final concentrations indicated according to Nelson method described in the text.

loss of the enzyme activity, whereas the application of either of the two metal ions restored its activity completely. The above characteristics showed the reversible potency in conformation of this enzyme and the exclusive dependence of Na^+/K^+ for its activity. Therefore, Na^+ was used in the enzyme assay in this study. For alginate lyase, monovalent and divalent metal ions such as Na^+, K^+, Ca^{2+}, and Mg^{2+} were commonly needed for its optimal activity but not essential [12,19,22,23]. Only the guluronate acid specific alginate lyase from *Bacillus* sp. [26] was a Ca^{2+} dependent alginate lyase. The promoting effect of metal ions on the enzyme activity may be due partially to the removal of bound water from the alginate molecule or the formation of enzyme-substrate complex [27]. The inhibition effect of heavy metal ions on enzyme activity was also demonstrated in other alginate lyases form different origins [21–23].

3.6. Effects of possible inhibitors and activators on alginate lyase activity

As shown in Table 4, β-Me, DTT and SDS increased the activity of the alginate lyase, whereas urea, EGTA and EDTA did not affect the enzyme activity. The reducing reagents, β-mercaptoethanol and DTT, were found to stimulate the alginate lyase activity by more than 30%, which indicated the possible existence of thiol in the activity catalytic site since the reducing reagent could protect thiol from being oxidized to disulfide bound [28]. The lack of quaternary structure of this alginate lyase was confirmed by the SDS-PAGE; consequently SDS could not denature it by disordering its tertiary structure, which was suggested for its common effect as well as other ionic surfactants [29]. On the other hand, SDS enhanced the enzyme activity by 30%, indicating the importance of β-strands structure in this protein because low concentration of SDS was found to stabilize β-strands [30]. The activation characteristics of *Vibrio* sp. YKW-34 by DTT and SDS were found to be novel as compared with others, in which the enzyme activity decreased [20] or were not affected [21,26]. The divalent metal ion chelators, EDTA and EGTA, were poor inhibitors for it because the divalent metal ions were not essential for the activity of this enzyme, which was similar to the case of alginate lyase from *Pseudoalteromonas* sp. [22].

Table 4
Effects of possible inhibitors and activators on the alginate lyase activity purified from *Vibrio* sp. YKW-34

Reagents	Relative activity (% ± S.D.)[a]	
	2 mM[b]	10 mM
β-Me	133.6 ± 3.79	130.2 ± 2.90
DTT	132.4 ± 9.25	134.4 ± 8.44
SDS	128.0 ± 3.16	132.4 ± 2.78
Urea	99.8 ± 5.43	100.1 ± 2.43
EGTA	97.6 ± 0.88	96.8 ± 1.63
EDTA	97.3 ± 9.10	98.0 ± 2.13

[a] The activity measured by Nelson method under the standard condition described in the text was defined as 100%.
[b] The concentration is the final concentration of the reagents in the enzyme activity assay system.

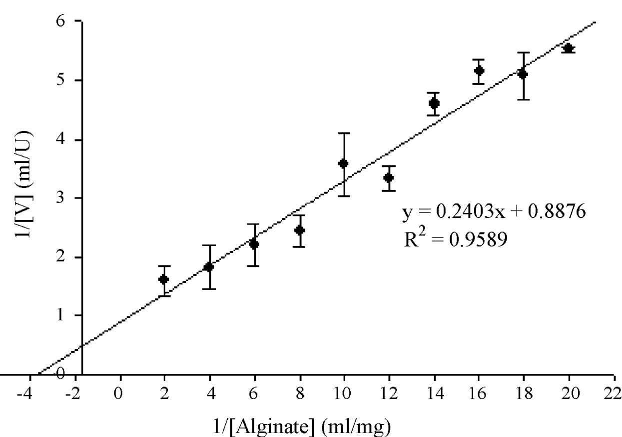

Fig. 5. Lineweaver–Burke plots for the degradation of alginate by the alginate lyase produced by *Vibrio* sp. YKW-34. Enzyme activity was determined at different alginate concentrations of 0.05–0.5 mg/ml according to Nelson method described in the text.

3.7. Substrate specificity of the alginate lyase

When alginate was substituted with poly-mannuronate and poly-guluronate as a substrate, the purified alginate lyase possessed substrate specificity for both units, and the relative activity of the alginate lyase toward M block to G block was determined to be 100–86. Alginate lyases are divided into three substrate specificities; mannuronate lyase [19,31], guluronate lyase [20,21,25] and both M and G specificity [11,22,26]. The result of the substrate specificity suggested that the alginate lyase purified from *Vibrio* sp. YKW-34 belonged to the third category.

3.8. Kinetic parameters

The K_m and V_{max} values of the alginate lyase calculated from Lineweaver–Burke plot are shown in Fig. 5. The K_m and V_{max} values were 0.27 mg/ml (6.8×10^{-6} M) and 1.13 U/ml, respectively. Based on these values, the k_{cat} (turnover number) and k_{cat}/K_m (catalytic efficiency) values were 11.3 s^{-1} and $1.7 \times 10^6\ s^{-1}\ M^{-1}$, respectively. k_{cat}/K_m values of the alginate lyase from *Alteromonas* sp. were 6.32×10^4 and $3.09 \times 10^5\ s^{-1}\ M^{-1}$ against M and G blocks, respectively [11]. Two types of alginate lyases from *Vibrio* sp. had K_m values of 5.1×10^{-5} and 5.4×10^{-5} M, respectively [23]. The K_m values of the alginate lyase from *Pseudomonad* sp. were 5.51–7.27 mM depending on alginate with different origin [25]. As compared to other alginate lyases, this alginate lyase possessed a lower K_m value and a higher k_{cat}/K_m value, which indicated its superior property.

4. Conclusions

A new alginate lyase was purified from marine *Vibrio* sp. YKW-34. This novel Na^+ and K^+ dependent alginate lyase discriminated it from other alginate lyases reported till now. Furthermore, the excellent characteristics, such as the temperature and pH stability, high specific activity, and both M and G specificity, enlightened its potential wide application. To facil-

itate the large-scale production of this enzyme, further studies on the cloning and expression are underway in this laboratory.

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