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Purification and Characterization of a New κ-Carrageenase from the Marine Bacterium *Vibrio* sp. NJ-2

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The carrageenan-degrading marine bacterium *Vibrio* sp. strain NJ-2 was isolated from rotten red algae, and κ-carrageenase with high activity was purified from the culture supernatant. The purified enzyme with molecular mass of 33 kDa showed the maximal activity of 937 U/mg at 40°C and pH 8.0. It maintained 80% of total activity below 40°C and between pH 6.0 and 10.0. The kinetics experiment showed the K_m and V_{max} values were 2.54 mg/ml and 138.89 mmol/min/mg, respectively. The thin layer chromatography and ESI-MS analysis of hydrolysates indicated that the enzyme can endolytically depolymerize the κ-carrageenan into oligosaccharides with degrees of depolymerization of 2–8. Owing to its high activity, it could be a valuable tool to produce κ-carrageenan oligosaccharides with various biological activities.

Keywords: κ-Carrageenase, purification, characterization, oligosaccharides

Introduction

Carrageenans are linear sulfated galactans extracted from several kinds of red seaweeds and share a common backbone of D-galactose with altering α-1,3 and β-1,4 linkages [2, 6]. They are classified into three main groups (λ-, ι-, and κ-carrageenan) according to the number and position of the sulfated ester groups. Carrageenans have been extensively used in the food, cosmetic, and pharmaceutical industries owing to their various biological activities [4], among which κ-carrageenan is the main industrially exploited one. κ-Carrageenan shows various kinds of physiological activities, including antitumor [8], anti-coagulation [21, 23], anti-inflammation [24], anti-thrombosis, and viral inactivation effects [19], which depend on the different degrees of sulfate esterification of the molecules. However, the shortcomings of polysaccharides, such as the high molecular weight and poor tissue-penetrating ability, have greatly limited their potential applications in humans [9]. To improve the activity of carrageenan in humans, it is a good way to degrade carrageenan into oligosaccharides, which possess much better biological activities and tissue-penetrating ability.

κ-Carrageenase, a member of family 16 of the glycoside hydrolases (GH 16), hydrolyzes β-1,4 linkages in κ-carrageenan. It can specifically cleave the internal β-1,4 linkages of κ-carrageenan and yields a series of homologous even-numbered oligosaccharides (Fig. 1). Thus, κ-carrageenase is a valuable tool to produce oligosaccharides with various physiological activities. The enzyme has been purified from several marine bacteria, such as *Alteromonas* [1], *Cytophaga* [18], *Pseudoalteromonas* [5], *Pseudomonas* [16], and *Vibrio* [17] strains. However, the enzymes with high activity for depolymerization of carrageenan polysaccharides are rather rare. Moreover, many carrageenases have not been purified to homogeneity and are not very well characterized. Therefore, none of these carrageenases is commercially available. In the present study, a novel κ-carrageenase with high activity, being purified from the marine bacterium *Vibrio* sp. NJ-2, has been characterized. The enzyme could effectively degrade κ-carrageenan into even-numbered oligosaccharides, and thus it may hold great potential to produce oligosaccharides in industry.

Materials and Methods

Isolation and Identification of the Bacterium

The carrageenan-degrading marine bacterial strains were isolated

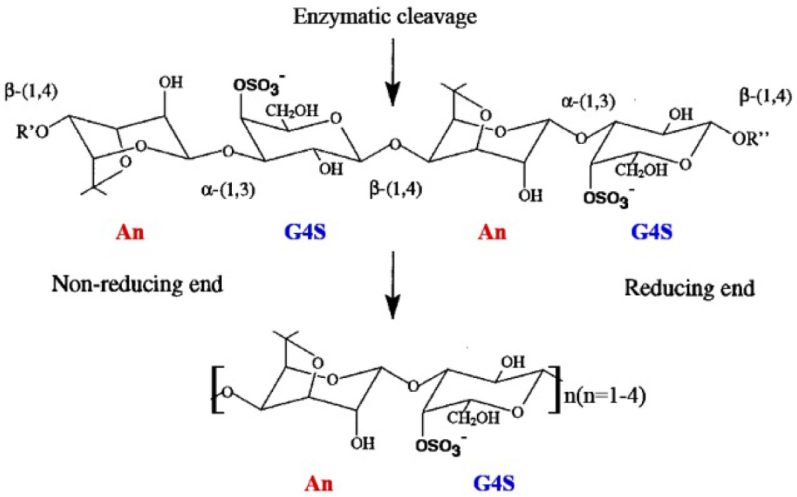

Fig. 1. Schematic diagram showing two disaccharide-repeating units of κ-carrageenan, where the site of enzymatic hydrolysis is specific at the internal κ-1,4 linkage of the κ-carrageenans.

from rotten red algae from the Yellow Sea as follows. The samples were collected from the coast of the Yellow Sea, washed with sterilized seawater, and then spread on agar plates containing 0.1% κ-carrageenans, 0.05% $(NH_4)_2SO_4$, and 0.001% $FeCl_3$. The plates were incubated at 30°C for 36 h and the positive colonies showing clear zones were picked out from the selection plates. The 16S rDNA sequence of the stain NJ-2 was amplified by PCR from the genomic DNA and then sequenced. The sequence was blasted and aligned with closely related sequences retrieved from NCBI using the BLASTn and Clustal X programs, respectively.

Purification of κ-Carrageenase

Strain NJ-2 was propagated in medium containing 0.1% κ-carrageenans, 0.05% $(NH_4)_2SO_4$, and 0.001% $FeCl_3$ in sterile seawater with shaking for 48 h at 30°C. The culture medium was centrifuged (10,000 ×*g*, 60 min) and the cell-free supernatant was fractionated at 40% and 80% ammonium sulfate saturation. The precipitated protein with 40% ammonium sulfate saturation was discarded, and the precipitated protein with 80% ammonium sulfate saturation was suspended in distilled water and dialyzed in a dialysis bag (MWCO: 8,000–14,000 Da) against distilled water, and the freeze-dried successively. Protein contents were determined by the Bradford method [3]. The obtained enzyme powder was dissolved in 5 ml of Tris-HCl buffer (pH 8.0) and the end concentration was 4%. Then the enzyme solution was applied to Sephadex G-200 (Pharmacia Company, Stockholm, Sweden) column chromatography (100 × 1.6 cm) equilibrated with the same buffer, and eluted at a flow rate of 0.1 ml/min. The elutes were monitored continuously at 280 nm for protein and fractions were assay for activity against κ-carrageenan. All the fractions of the first peak from Sephadex G-200 column chromatography containing κ-carrageenase activity were gathered, concentrated, and applied to another column of Sephadex G-75, and further equilibrated with the same eluent. Fractions were collected and monitored for the presence of κ-carrageenase. The purity of the fractions was assessed by SDS-PAGE. Pure fractions with activity were stored at –20°C.

Assay of Enzymatic Activity and Substrate Specificity

The κ-carrageenase activity was determined by measuring the increase in the concentration of reducing sugar by the DNS method as described previously [11]. The assay system consisted of 1.0 ml of substrate (0.5% κ-carrageenan in Tris-HCl buffer (pH 8.0)) and 1.0 ml of enzyme, and boiled (for 10 min). Inactivated enzyme with the same treatment was used as a control. One unit of κ-carrageenase activity was defined as the amount of enzyme needed to release 1 μmol of reducing sugars (D-galactose equivalent) per minute.

Biochemical Characterization of the Purified Enzyme

The optimum temperature for κ-carrageenase activity was determined under the standard assay condition by varying the incubation temperature from 20°C to 80°C. The thermal stability of the κ-carrageenase was determined by incubating the enzyme solution at each temperature (20–80°C) for 0.5 h and then measuring the residual enzyme activity. The activity of untreated enzyme was regarded as 100% and the relative activity was determined.

The effect of pH on κ-carrageenase activity was assayed by replacing Tris-HCl buffer (pH 8.0) with Na_2HPO_4-citric acid buffer (pH 4.0–7.0), Tris-HCl buffer (pH 8.0 and 9.0), and glycine-NaOH buffer (pH 10.0–11.0) at 40°C. The pH stability of the κ-carrageenase was determined by pre-incubating the enzyme solution at each pH (4.0–11.0) at 35°C for 6 h and then the enzyme activity was determined in the same pH buffer. The activity of untreated

Table 1. Purification of the κ-carrageenase from *Vibrio* sp. NJ-2.

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Folds	Recovery (%)
Crude medium	1000	51.88	1349	26	1	100
$(NH_4)_2SO_4$ precipitation	70	2.202	947	130	5	70.2
Sephadex G-200 filtration	6	0.688	519	754	29	38.5
Sephadex G-75 filtration	3	0.272	255	937	40	18.9

enzyme was regarded as 100% and the relative activity was determined.

Kinetics Study of the Enzyme

The initial reaction rate of the enzyme was assayed at various concentrations of κ-carrageenan (0.5–10 mg/ml) by the DNS method and the reaction system was incubated at 40°C and pH 8.0. The Michaelis constant (K_m) and the reaction rate at infinite substrate concentration (V_{max}) were determined according to the Lineweaver-Burk plotting method [7].

TLC and ESI-MS Analysis of Degradation Products

Enzymatic hydrolysis of κ-carrageenan was conducted under standard condition with 0.2% κ-carrageenan as the substrate, by incubating for 20 min, 40 min, 1 h, 2 h, 3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 20 h, 22 h, 24 h, 30 h, 36 h, 48 h, 60 h, and 72 h, respectively. After incubation, the mixture solutions were boiled for 10 min and then centrifuged at 12,000 rpm for 10 min to remove the unsolved materials. The hydrolysates were loaded onto a carbograph column (Alltech, Grace Davison Discovery Sciences, UK) to remove salts after removing proteins, and then concentrated, dried and re-dissolved in 1 ml of acetonitrile/1 mM NH_4HCO_3 (1:1 (v/v)).

The products of different reaction times were analyzed by TLC with the development system containing butanol-acetic acid-water (2:1:1). In order to further determine the composition and degree of polymerization (DP) of the products, ESI-MS was employed. In brief, 2 ml of supernatant was loop-injected to Micromass Q-TOF and Q-TOF Ultima instruments (Waters, Manchester, UK) after centrifugation. The oligosaccharides were detected in a negative-ion mode using the following settings. The spray voltage was set at 4 kV, with a sheath gas (nitrogen gas) flow rate of 30 arbitrary units, an auxiliary gas (nitrogen gas) flow rate of 5 arbitrary units, a tube lens voltage of −250 V, a capillary temperature of 350°C, and a capillary voltage of −48 V. The scan rate was normal, and the type was full, with a microscan number of 3, searching for a mass range of *m/z* 200–2,000.

Results and Discussion

Isolation and Identification of Strain NJ-2

The strain NJ-2 was isolated and purified. The 16S rDNA sequence of the strain was sequenced and submitted to GeneBank (Accession No. KT765103). According to phylogenetic analysis of the 16S rDNA sequence, the strain was assigned to the genus *Vibrio* and named *Vibrio* sp. NJ-2.

Purification and Substrate Specificity of κ-Carrageenase

As shown in Table 1, the κ-carrageenase was purified by a series of purification procedures and finally yielded a significantly high activity of 937 U/mg and a fold of 40, while only 18.9% recovery was achieved. The result of SDS-PAGE showed that the purified enzyme was a single protein band with molecular mass of 33 kDa (Fig. 2). The molecular mass of carrageenase from different sources differed from one other. For instance, the κ-carrageenase from marine strain WZUC10 has a molecular mass of 45 kDa [25]. Three isozymes with molecular masses of 39, 58, and 100 kDa from *Cytophaga* have also been reported [18]. Moreover, the enzyme from *Pseudoalteromonas elongata*

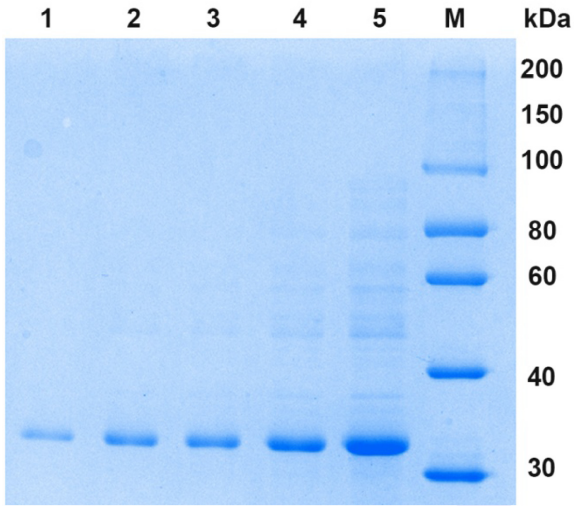

Fig. 2. SDS-PAGE of the enzyme for each purification step.
Lane M, PAGE ruler; Lane 1, sample of medium supernatant cultivated for 48 h; Lane 2, sample after $(NH_4)_2SO_4$ precipitation; Lane 3, condensed sample after $(NH_4)_2SO_4$ precipitation; Lane 4, sample after Sephadex G-200 filtration; Lane 5, condensed sample after SephadexG-75 filtration.

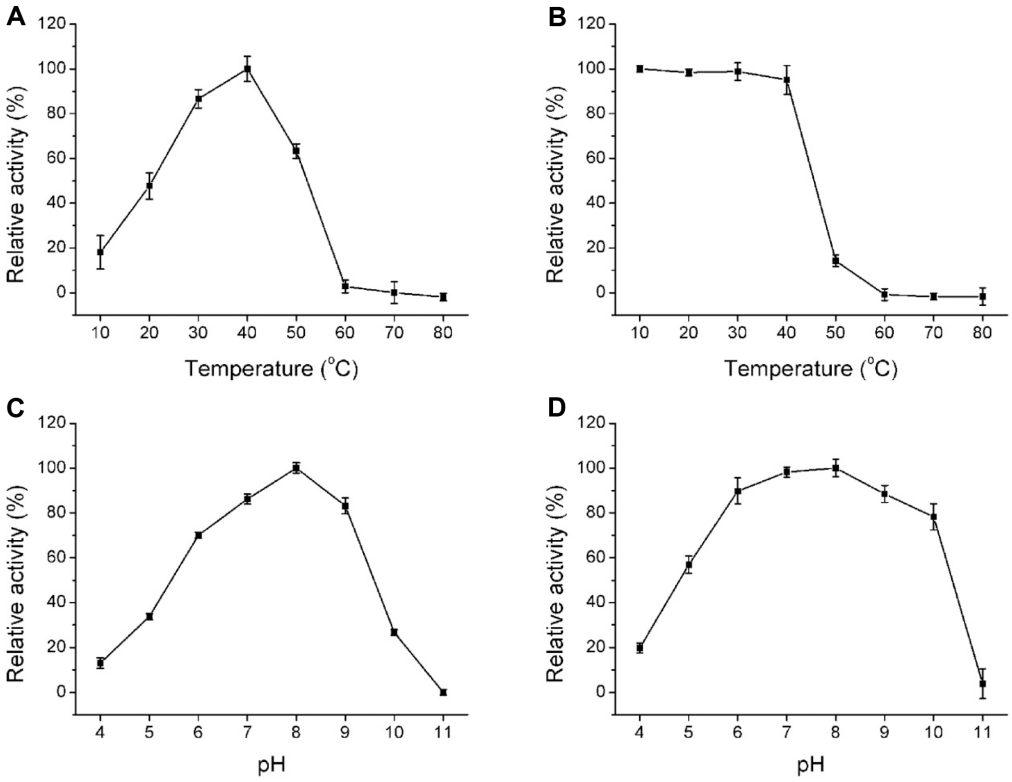

Fig. 3. Effects of pH and temperature on the activity and stability of the carrageenase.
(**A**) The optimal temperature of the enzyme was determined by measuring the activities at various temperatures (10–80°C). (**B**) The thermostability of the carrageenase was determined by measuring the residual activities after the enzymes were incubated at different temperatures (10–80°C) for 0.5 h. (**C**) The optimal pH of the enzyme was determined by measuring the activities at 40°C in Na_2HPO_4-citric acid buffer (pH 4.0–7.0), Tris-HCl buffer (pH 8.0 and 9.0), and glycine-NaOH buffer (pH 10.0–11.0). (**D**) The pH stability of the enzyme. The residual activities were measured at 40°C in Tris-HCl buffer (pH 8.0) after incubation in the above buffers at 4°C for 24 h. The highest activity was set to be 100%. Each value represents the mean of three replicates ± standard deviation.

has a molecular mass of 128 kDa [10].

The enzyme effectively degraded κ-carrageenan, but showed no activity towards λ- and ι-carrageenans (data not shown).

Biochemical Characterization of the Enzyme

The enzyme showed maximum activity at 40°C (Fig. 3A) and was stable below 40°C (Fig. 3B). This enzyme possessed approximately 90% activity after incubation at 40°C for 30 min and was gradually inactivated as temperature increased. The enzyme showed optimal activity at pH 8.0 (Fig. 3C) and retained more than 80% activity after being incubated at a broad pH range of pH 6.0–10.0 for 24 h (Fig. 3D). However, this enzyme was mostly stable at pH 8.0. Thus, it was an alkaline-stable carrageenase and it could retain stability in a broader pH range. Most of characterized enzymes showed the maximum activity around 40°C, whereas CgkLL1 from *Pseudoalteromonas. porphyrae* [13] and CgkAJ5 from *Pseudoalteromonas* sp. AJ5-913 [15] both had the optimal reaction temperature of 55°C. Therefore, owing to its high activity and thermal stability, the enzyme would be a potential tool for preparation of carrageenan oligosaccharides and detailed structural analysis of carrageenan.

The effects of metal ions (1 mM) on the enzyme are shown in Table 2. The monovalent ions, such as Na^+ and K^+, showed activation effects on enzyme activity. Specifically, the enzyme activity was boosted by various concentrations of NaCl, where the activity reached the maximum with 300 mM NaCl, being boosted around four times compared with that without NaCl. It is reasonable that because the enzyme was purified from a marine-derived bacterium, a certain degree of NaCl concentration was indispensable for the survival of the strain and activation of the enzyme. Moreover, the carrageenase was activated by NaCl in a broad concentration range. Therefore, it was a salt-activated

Table 2. Effects of metal ions and chemical agents on the activity of the enzyme.

Additives	Concentration (mmol/l)	Relative activity (%)
Control	0	100 ± 2.1
	100	273 ± 5.2
Na^+	300	369 ± 3.8
	500	221 ± 2.7
K^+	1	153 ± 1.9
Mg^{2+}	1	112 ± 2.8
Ca^{2+}	1	108 ± 1.5
Mn^{2+}	1	39 ± 2.5
Cu^{2+}	1	32 ± 1.7
Zn^{2+}	1	21 ± 2.4
Ni^{2+}	1	49 ± 5.7
Ba^{2+}	1	98 ± 2.3
Fe^{2+}	1	51 ± 3.6
Fe^{3+}	1	38 ± 7.2
EDTA	1	71 ± 2.5
SDS	1	56 ± 2.7

carrageenase. As shown in Table 2, divalent ions such as Mg^{2+} and Ca^{2+} showed activation effects on enzyme activity, whereas other ions like Mn^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}, and Fe^{3+} showed inactivation effects on the activity of the carrageenase. Additionally, chemical agents such as EDTA and SDS showed inactivation effects on the activity of the enzyme. The effects of metal ions on the activity of the enzymes were similar, as shown in Table 3. CgkP from *Pseudoalteromonas* sp. QY203 [12] and CgkZM2 from *Zobellia galactanivorans* [14] were both activated by Na^+, K^+, and Mg^{2+}. The inhibitory ions of these enzymes were Cu^{2+}, Pb^{2+}, and Zn^{2+}.

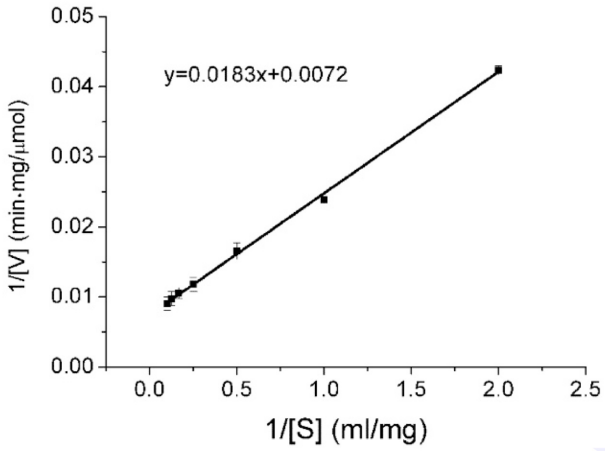

Fig. 4. Lineweaver-Burk plot for determining the kinetic parameters of the enzyme with various concentrations of κ-carrageenan (0.5–10 mg/ml) at 40°C and pH 8.0.

Kinetics Analysis of the Enzyme

As calculated from the Lineweaver-Burk curve (Fig. 4), the apparent K_m and V_{max} values were 2.54 mg/ml and 138.89 μmol/min/mg, respectively. Compared with the K_m values of other κ-carrageenases, such as those from *Pseudoalteromonas porphyrae* (4.4 mg/ml) [13], *Vibrio* sp. CA-1004 (3.3 mg/ml) [17], *Tamlana* sp. HC4 (7.63 mg/ml) [20], *Pseudoalteromonas* sp. AJ5-913 (9.8 mg/ml) [15], and *Pseudoalteromonas elongata* (6.6 mg/ml) [10], our enzyme showed higher affinity to the κ-carrageenan substrate.

Action Mode and Analysis of Degradation Products

The action mode of the enzyme was monitored by thin layer chromatography. As shown in Fig. 5, at the initial stage of the hydrolysis reaction, the polysaccharide substrate was hydrolyzed into the oligomers with high

Table 3. Comparison of the enzyme with carrageenases from other strains.

Enzyme	Source	pH	Temperature (°C)	Cation activators	Cation inhibitors	K_m (mg/ml)	Products	Ref.
CgkNJ	*Vibrio* sp.	8.0	40	Na^+, K^+, Mg^{2+}, Ca^{2+}	Cu^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}	2.54	2, 4, 6, 8	This study
Cgkp	*Pseudoalteromonas* sp. QY203	7.2	45	Na^+, K^+, Mg^{2+}	Cu^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Al^{3+}	-	2,4	[22]
CgkN5-2	*Cellulophaga lytica* N5-2	7.0	35	-	-	1.647	2, 4, 6, 8	[25]
CgkLL1	*Pseudoalteromonas porphyrae*	8.0	55	Mg^{2+}, Ba^{2+}	Cu^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}		-	[20]
CgkZM2	*Zobellia galactanivorans*	6.0	39	Na^+, Ca^{2+}	Cu^{2+}, Pb^{2+}, Mn^{2+}	0.842	4, 6, 8, 10	[23]
CgkCA	*Vibrio* sp. CA-1004	8.0	40	-	Cu^{2+}, Pb^{2+}, Zn^{2+}	3.3	2, 4	[14]
CgkHC4	*Tamlana* sp. HC4	8.0	30		Cu^{2+}, Pb^{2+}, Zn^{2+}	7.63	2	[24]
CgkAJ5	*Pseudoalteromonas* sp.	8.0	55	-	-	9.8	2, 4, 6, 8, 10	[21]

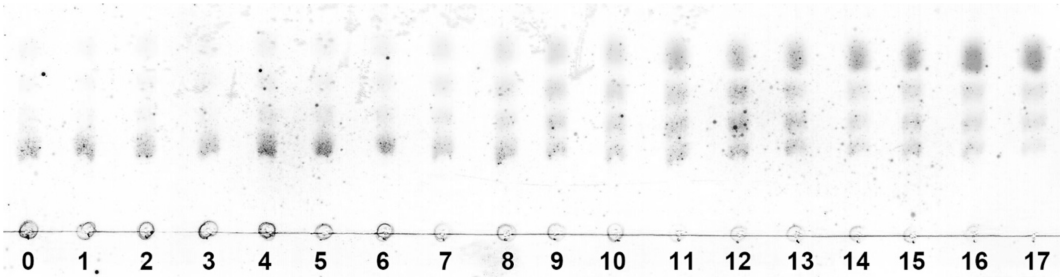

Fig. 5. TLC analyses of the degradation products of the enzymes after different incubation times (20 min, 40 min, 1 h, 2 h, 3h, 6 h, 9 h, 12 h, 15 h, 18 h, 20 h, 22 h, 24 h, 30 h, 36 h, 48 h, 60 h, and 72 h).
Lanes 0–17 present the hydrolysates of different incubation times.

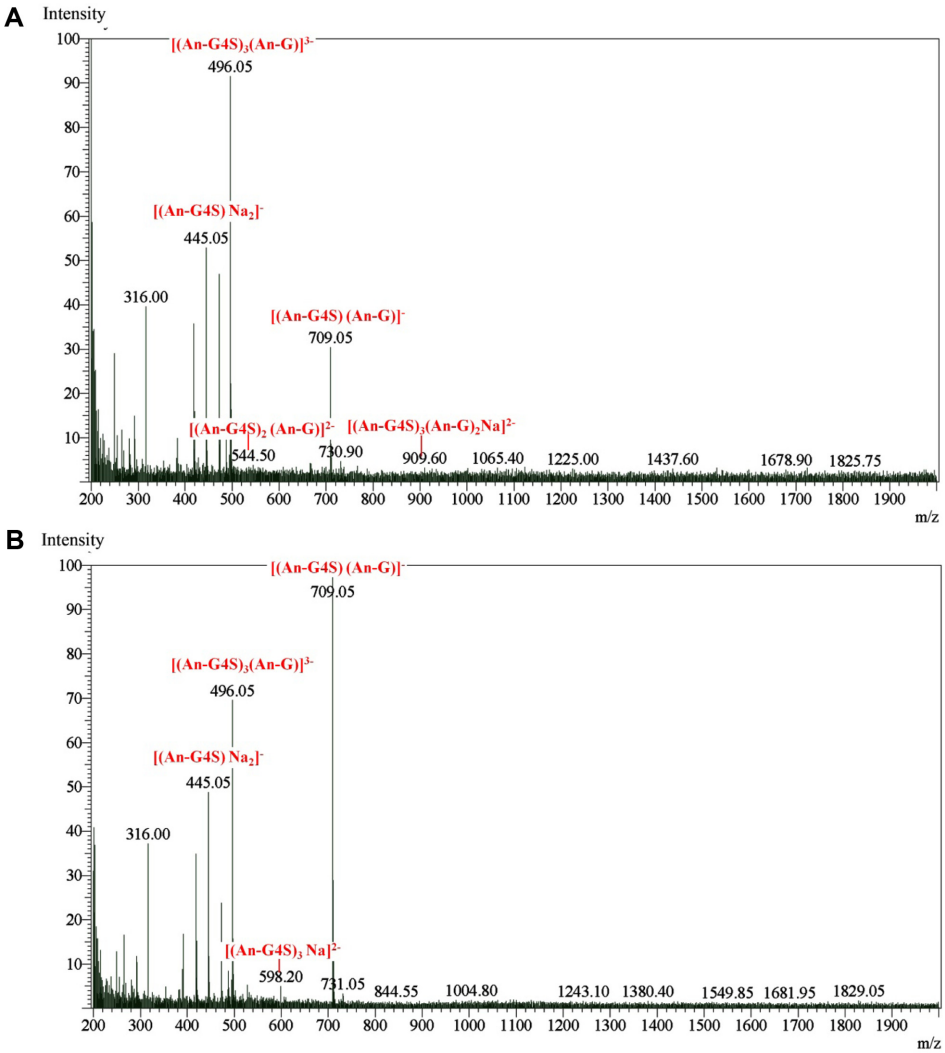

Fig. 6. ESI-MS analysis of the degradation products of the enzyme incubated with 0.2% κ-carrageenan for 12 h and 72 h, respectively.

degree of depolymerization (DP). As the reaction proceeds, the products with low DP (2–4) appeared, while the oligomers with DP of 8 decreased. The results indicated that the enzyme hydrolyzed the carrageenan in an

endolytic manner and produced a series of even-numbered oligosaccharides with low DPs. After incubation for 72 h, the dimers accounted for a large fraction of the hydrolysates. Owing to the presence of sulfate groups on the oligosaccharides, all mass spectral experiments were performed in negative-ion mode. The mass spectra of the degradation products obtained at 12 and 72 h are shown in Fig. 6. The hydrolysates prepared for 12 h contained oligosaccharides with high DP, including hexasaccharide, octasaccharide, and decasaccharide. The peak at *m/z* 544.50 is assigned to hexasaccharide ($[(A\text{-}G4S)_2(An\text{-}G)]^{2-}$). The peak corresponding to *m/z* 496.05 is assigned to octasaccharide ($[(An\text{-}G4S)_3(An\text{-}G)]^{3-}$). The peak at *m/z* 909.60 is likely to be decasaccharide ($[(An\text{-}G4S)_3(An\text{-}G)_2Na]^{2-}$). Moreover, the peaks of dimers and tetramers also appeared as 445.05 ($[(An\text{-}G4S)\ Na_2]^{-}$) and 709.05 ($[(An\text{-}G4S)\ (An\text{-}G)]^{-}$). With the progressing hydrolysis reaction, the oligomers with high DP decreased, while disaccharides and tetrasaccharides increased, as shown in Fig. 6B. Almost all of the characterized enzymes were endolytic, as shown in Table 3. Generally, carrageenases from different organisms degrade κ-carrageenan substrate to yield distinct oligosaccharides. The Cgkp from *Pseudoalteromonas* sp. QY203 [12] and CgkCA from *Vibrio* sp. CA-1004 [17] yielded dimers and tetramers as the main products. The distribution of degradation products of CgkN5-2 from *Cellulophaga lytica* N5-2 [22], CgkZM-2 from *Zobellia galactanivorans* [14], and CgkAJ5 from *Pseudoalteromonas* sp. AJ5-913 [15] were similar, as they all produce even-numbered oligosaccharides.

In conclusion, in this study, a k-carrageenase with high activity was purified from the culture supernatant of *Vibrio* sp. NJ-2. The purified enzyme with a molecular mass of 33 kDa showed the maximal activity of 937 U/mg at 40°C and pH 8.0. It maintained 80% of total activity below 40°C and between pH 6.0 and 10.0. The metal ions Na^+, K^+, Mg^{2+}, and Ca^{2+} enhanced the enzyme activity. The TLC and ESI-MS analyses of the hydrolysates indicated that the enzyme endolytically depolymerized the κ-carrageenan into oligosaccharides with degrees of depolymerization of 2–8. Because of its high activity, it could be a valuable tool for the production of κ-carrageenan oligosaccharides with various biological activities.

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References

1. Barbeyron T, Henrissat B, Kloareg B. 1994. The gene encoding the kappa-carrageenase of *Alteromonas carrageenovora* is related to beta-1,3-1,4-glucanases. *Gene* **139:** 105-109.
2. Barbeyron T, Michel G, Potin P, Henrissat B, Kloareg B. 2000. Iota-carrageenases constitute a novel family of glycoside hydrolases, unrelated to that of kappa-carrageenases. *J. Biol. Chem.* **275:** 35499-35505.
3. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
4. Campo VL, Kawano DF, Silva Jr DBd, Carvalho I. 2009. Carrageenans: biological properties, chemical modifications and structural analysis – a review. *Carbohydr. Polym.* **77:** 167-180.
5. Collen PN, Lemoine M, Daniellou R, Guegan JP, Paoletti S, Helbert W. 2009. Enzymatic degradation of kappa-carrageenan in aqueous solution. *Biomacromolecules* **10:** 1757-1767.
6. De Ruiter GA, Rudolph B. 1997. Carrageenan biotechnology. *Trends Food Sci. Technol.* **8:** 389-395.
7. Hoylaerts MF, Bollen A, De Broe ME. 1990. The application of enzyme kinetics to the determination of dissociation constants for antigen-antibody interactions in solution. *J. Immunol. Methods* **126:** 253-261.
8. Hu XK, Jiang XL, Aubree E, Boulenguer P, Critchley AT. 2006. Preparation and in vivo antitumor activity of kappa-carrageenan oligosaccharides. *Pharm. Biol.* **44:** 646-650.
9. Kalitnik AA, Barabanova AOB, Nagorskaya VP, Reunov AV, Glazunov VP, Solov'eva TF, Yermak IM. 2013. Low molecular weight derivatives of different carrageenan types and their antiviral activity. *J. Appl. Phycol.* **25:** 65-72.
10. Khambhaty Y, Mody K, Jha B. 2007. Purification and characterization of kappa-carrageenase from a novel gamma-proteobacterium, *Pseudomonas elongata* (MTCC 5261) syn. *Microbulbifer elon* comb. nov. *Biotechnol. Bioprocess Eng.* **12:** 668-675.
11. Kidby DK, Davidson DJ. 1973. A convenient ferricyanide estimation of reducing sugars in the nanomole range. *Anal. Biochem.* **55:** 321-325.
12. Li SY, Jia PP, Wang LN, Yu WG, Han F. 2013. Purification and characterization of a new thermostable kappa-carrageenase from the marine bacterium *Pseudoalteromonas* sp. QY203. *J. Ocean Univ. China* **12:** 155-159.
13. Liu GL, Li Y, Chi Z, Chi ZM. 2011. Purification and characterization of kappa-carrageenase from the marine bacterium *Pseudoalteromonas porphyrae* for hydrolysis of kappa-carrageenan. *Process Biochem.* **46:** 265-271.
14. Liu ZM, Li GY, Mo ZL, Mou HJ. 2013. Molecular cloning, characterization, and heterologous expression of a new kappa-carrageenase gene from marine bacterium *Zobellia* sp. ZM-2. *Appl. Microbiol. Biotechnol.* **97:** 10057-10067.

15. Ma YX, Dong SL, Jiang XL, Li J, Mou HJ. 2010. Purification and characterization of kappa-carrageenase from marine bacterium mutant strain *Pseudoalteromonas* sp. AJ5-13 and its degraded products. *J. Food Biochem.* **34:** 661-678.
16. Ostgaard K, Wangen B, Knutsen S, Aasen I. 1993. Large-scale production and purification of κ-carrageenase from *Pseudomonas carrageenovora* for applications in seaweed biotechnology. *Enzyme Microb. Technol.* **15:** 326-333.
17. Potin P, Sanseau A, Le Gall Y, Rochas C, Kloareg B. 1991. Purification and characterization of a new kappa-carrageenase from a marine *Cytophaga*-like bacterium. *Eur. J. Biochem.* **201:** 241-247.
18. Sarwar G, Matayoshi S, Oda H. 1987. Purification of a kappa-carrageenase from marine *Cytophaga* species. *Microbiol. Immunol.* **31:** 869-877.
19. Serkedjieva J, Hay AJ. 1998. In vitro anti-influenza virus activity of a plant preparation from *Geranium sanguineum* L. *Antiviral Res.* **37:** 121-130.
20. Sun FX, Ma YX, Wang Y, Liu QA. 2010. Purification and characterization of novel kappa-carrageenase from marine *Tamlana* sp. HC4. *Ch. J. Oceanol. Limnol.* **28:** 1139-1145.
21. Yamada T, Ogamo A, Saito T, Uchiyama H, Nakagawa Y. 2000. Preparation of *O*-acylated low-molecular-weight carrageenans with potent anti-HIV activity and low anticoagulant effect. *Carbohydr. Polym.* **41:** 115-120.
22. Yao Z, Wang FF, Gao Z, Jin LM, Wu HG. 2013. Characterization of a kappa-carrageenase from marine *Cellulophaga lytica* strain N5-2 and analysis of its degradation products. *Int. J. Mol. Sci.* **14:** 24592-24602.
23. Yao Z, Wu H, Zhang S, Du Y. 2014. Enzymatic preparation of kappa-carrageenan oligosaccharides and their anti-angiogenic activity. *Carbohydr. Polym.* **101:** 359-367.
24. Yuan H, Zhang W, Li X, Lü X, Li N, Gao X, Song J. 2005. Preparation and in vitro antioxidant activity of κ-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives. *Carbohydr. Res.* **340:** 685-692.
25. Zhou MH, Ma JS, Li J, Ye HR, Huang KX, Zhao XW. 2008. A kappa-carrageenase from a newly isolated *Pseudoalteromonas*-like bacterium, WZUC10. *Biotechnol. Bioprocess Eng.* **13:** 545-551.