J. Ocean Univ. China (Oceanic and Coastal Sea Research)
DOI 10.1007/s11802-013-1994-2
ISSN 1672-5182, 2013 12 (1): 155-159
http://www.ouc.edu.cn/xbywb/
E-mail:xbywb@ouc.edu.cn

Purification and Characterization of a New Thermostable κ-Carrageenase from the Marine Bacterium *Pseudoalteromonas* sp. QY203

LI Shangyong[#], JIA Panpan[#], WANG Linna, YU Wengong, and HAN Feng[*]

Key Laboratory of Marine Drugs, Chinese Ministry of Education; *Provincial Key Laboratory of Glycoscience and Glycotechnology of Shandong Province and School of Medicine and Pharmacy*, *Ocean University of China*, *Qingdao* 266003, *P. R. China*

(Received March 27, 2012; revised May 9, 2012; accepted July 6, 2012)
© Ocean University of China, Science Press and Springer-Verlag Berlin Heidelberg 2013

Abstract A new extracellular κ-carrageenase, namely CgkP, 34.0 kDa in molecular weight, was purified from *Pseudoalteromonas* sp. QY203. CgkP showed relatively high activity at acidities ranging from pH6.0 to pH9.0 and temperatures ranging from 30℃ to 50℃ with the highest activity at 45℃ and pH7.2. Sodium chloride increased its activity markedly, and KCl increased its activity slightly. The divalent and trivalent metal ions including Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Al^{3+} and Fe^{3+} significantly inhibited its activity, while Mg^{2+} did not. CgkP remained 70% of original activity after being incubated at 40℃ for 48 h, and remained 80% of the activity after being incubated at 45℃ for 1 h. It exhibited endo-κ-carrageenase activity, mainly depolymerizing the κ-carrageenan into disaccharide and tetrasaccharide. CgkP was more thermostable than most of previously reported κ-carrageenases with a potential of being used in industry.

Key words κ-carrageenase; purification; characterization; thermostability; *Pseudoalteromonas*

1 Introduction

Carrageenans are sulfated galactans consisting of D-galactose residues linked by alternating α-1, 3 and β-1, 4 glycosidic bonds, which are commercially important components of the cell walls of red seaweeds (Liu *et al*., 2010). The three most industrially exploited carrageenans, namely κ-, ι- and λ-carrageenans, are distinguishable each other; they contain one, two and three ester-sulfate groups per repeating disaccharide unit, respectively (Guibet *et al*., 2007). Carrageenans are widely used in industry owing to their unique physicochemical properties. The κ-carrageen-derived sulfated oligosaccharides have been reported to have anti-viral and anti-tumor (Mou *et al*., 2003), anti-inflammation, antioxidant and immunolore- gulation activities (Yuan *et al*., 2006).

The κ-carrageenases (EC 3.2.1.83) cleave the internal β-1, 4 linkages of κ-carrageenans, yielding oligogalactans of either neocarrabiose or neoagarobiose series. Several κ-carrageenases have been found in marine bacteria such as *Pseudoalteromonas carrageenovora* (Collén *et al*., 2009), *Pseudomonas elongata* (Khambhaty *et al*., 2007), *Zobellia galactanovorans* (Potin *et al*., 1991) and *Vibrio* sp. (Toshiyoshi *et al*., 1999). The genes of the κ-carrageenases have been cloned from *Pseudoalteromonas carrageenovora* (Barbeyrona *et al*., 1994) and *Cytophaga drobachiensis* (Barbeyron *et al*., 1998). The κ-carrageen at high concentrations is highly viscous at room temperature and an inhibitor of κ-carrageenase degradation. The most commonly used method of obtaining low viscous κ-carrageen is to increase solution temperature; unfortunately most reported κ-carrageenases are not stable at temperartures above 40℃ (Zhou *et al*., 2008).

In this study, a new κ-carrageenase,namely CgkP, was purified from a marine bacterium *Pseudoalteromonas* sp. QY203 and characterized. CgkP was relatively thermostable, promising for industrial applications.

2 Materials and Methods

2.1 Bacterial Strains, Media and Culture Conditions

The strain QY203 was isolated from the decayed red algae collected from the coast of Qingdao, China. It was cultured in a fermentation medium (30 g L^{-1} NaCl, 3 g L^{-1} $MgSO_4·7H_2O$, 0.2 g L^{-1} $CaCl_2$, 0.1 g L^{-1} KCl, 0.02 g L^{-1} $FeSO_4$, 3 g L^{-1} Casein, 1.5 g L^{-1} Na_2HPO_4, 1 g L^{-1} NaH_2PO_4 and 2 g L^{-1} κ-Carrageenan) at 25℃ for 48 h in a shaker (150 r min^{-1}). *Escherichia coli* DH5α was used as the host of recombinant plasmids. *E. coli* cells were grown at 37℃ in Luria-Bertani (LB) broth or on LB agar supplemented with (100 μg ampicillin) mL^{-1} if necessary.

* Corresponding author. E-mail: fhan@ouc.edu.cn
# These authors contributed equally to this work.

2.2 Molecular Identification of Strain QY203

Identification of strain QY203 was accomplished using the 16S rRNA gene (rDNA) sequencing. Briefly, the 16S rDNA was amplified from genomic DNA by PCR using the bacterial specific primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') with a GeneAmp 9700 PCR system. The PCR product was inserted into pMD18-T vector (Sangon Biotech, China) and sequenced with a BigDye terminator sequencing method. Sequence analysis was performed by comparing the DNA sequences with those retrieved from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple sequence alignments were performed using ClustalX 1.83 with the phylogenetic trees constructed with MEGA 4.0 (Tamura *et al*., 2007).

2.3 Purification of CgkP

Enzyme purification was always carried out at 4℃. The bacterial culture was centrifuged at 6000×g for 15 min. Into the supernatant, ammonium sulfate was added to a final saturation of 40%. After standing for 2 h, 200 mL of the mixture was loaded onto a Phenyl-Sepharose column (1.6 cm×20 cm) equilibrated with 20 mmol L^{-1} phosphate buffer (pH 7.2), then eluted with a linear gradient of $(NH_4)_2SO_4$ (1.5–0 mol L^{-1}, 100 mL) at a flow rate of 1 mLmin^{-1}. The fractions (25 mL) showing κ-carrageenase activity was loaded onto a column of HiTrap Q (1.6 cm×10 cm) equilibrated with 20 mmol L^{-1} phosphate buffer (pH 7.2). After washing, a linear NaCl gradient (0–1 mol L^{-1}, 100 mL) was superimposed on the starting buffer at 1 mL min^{-1}. The active fractions were stored at −20℃. Protein concentration was determined by referring to bovine serum albumin (BSA) standard following Bradford method. Sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) was performed as the described (Laemmli *et al*., 1973).

2.4 Assay of CgkP Activity

Unless stated otherwise, 0.9 mL of κ-carrageenan (2 g L^{-1} in 20 mmol L^{-1} sodium phosphate buffer, pH 7.2) was incubated with 0.1 mL of the enzyme at 45℃ for 15 min. The reaction was stopped by heating at 100℃ for 10 min. The reducing sugar released was quantified with dinitrosalicylic acid (DNS) reagent (Miller, 1959). One unit of κ-carrageenase activity was defined as the amount of enzyme releasing 1 μmol reducing sugar (measured as galactose) from κ-carrageenan per minute.

2.5 Characterization of the Purified CgkP

The optimal acidity of the enzyme was determined by using 50 mmol L^{-1} Na_2HPO_4-citric acid (pH 4.0–7.0), 50 mmol L^{-1} Na_2HPO_4-NaH_2PO_4 (pH 6.6–8.6), 100 mmol L^{-1} Tris-HCl (pH 7.6–9.0) and 50 mmol L^{-1} Gly-NaOH (pH 9.0–11.0) buffers in the assay system. To determine acidity stability, the residual activity was measured after enzyme was incubated in above buffers (pH 4.0–11.0) at 4℃ for 6 h. The optimal temperature of the enzyme was determined by measuring the activity at various temperatures (10–60℃). The thermostability of the enzyme was determined by measuring the residual activity after the enzymes in 20 mmol L^{-1} phosphate buffer (pH 7.2) were incubated at 35℃, 40℃, 45℃ and 50℃, respectively, for various times.

2.6 Mode of κ-Carrageenan Degradation by CgkP

Mixtures of 5 mL CgkP (2 U mL^{-1}) and 50 mL κ-carrageenan (2 g L^{-1} in 20 mmol L^{-1} phosphate buffer, pH 7.2) were incubated at 45℃ for up to 30 min. An aliquot of hydrolysis product (0.5 mL) was taken out at different times (1, 5, 10, 15 and 30 min) in order to determine the viscosity and reducing sugar. Viscosity was measured as described previously by Kobayashi *et al*. (2009).

2.7 Analysis of CgkP Degrading Products

Five milliliters of purified enzyme (5 U mL) was incubated with 20 mL of κ-carrageenan (2 g L^{-1} in 20 mmol L^{-1} phosphate buffer, pH 7.2) at 37℃ overnight. Thin-layer chromatography (TLC) analysis was performed on a HPTLC plates (Merck, Germany) with a solvent of n-butanol/acetic acid/water (2:1:1) (Toshiyoshi *et al*., 1999). The end products of κ-carrageenase degrading reaction were fractionated on a Biogel-P6 column (1.6 cm×100 cm). Elution was performed with 0.2 mol L^{-1} NH_4HCO_3 at a flow rate of 0.05 mL min^{-1}.

3 Results and Discussion

3.1 Identification of Strain QY203

Sixty-two colonies (strains) were isolated, of them, 39 produced κ-carrageenase and 15 showed high κ-carrageenase activity (>6 U mL^{-1}) after being cultured at 25℃ for 48 h. Of the 15 strains with high enzyme activity, strain QY203 showed the highest κ-carrageenase activity (12 U mL^{-1}). The 16S rDNA of strain QY203 (GenBank accession number JQ669941) was 1430 bp in length. BLASTn search revealed that the highest similarity of 99.4% existed between the strain QY203 and *Pseudoalteromonas* sp. P102. The phylogenetic tree (Fig.1) shows that the strain QY203 belongs to genus *Pseudoalteromonas*. Strain QY203 is a species in genus *Pseudoalteromonas*, which was designated as *Pseudoalteromonas* sp. QY203.

3.2 Purification and Properties of CgkP

The enzyme was purified 88.3 folds through $(NH_4)_2$-SO_4 precipitation, hydrophobic chromatography and anionic-exchange chromatography (Table 1). The specific activity and yield were 1121.7 U mg^{-1} and 26.9%, respectively.

The purity of the purified CgkP was confirmed by SDS-PAGE (Fig.2). A single protein band was stained, which was estimated to be 34.0 kDa in molecular weight. and the Mws of the κ-carrageenases from the genus *Pseudoalteromonas* was at least 40.0 kDa. For example,

Mw of κ-carrageenase from *Pseudoalteromonas porphyrae* LL1 was 40.0 kDa (Liu *et al*., 2010), Mw of κ-carrageenase CgkA from *Pseudoalteromonas carrageenovora* (ATCC 43555) was 44.4 kDa (Barbeyron *et al*., 1994) and *Pseudoalteromonas elongata* yielded κ-carrageenase with a Mw of 128.0 kDa (Khambhaty *et al*., 2007).

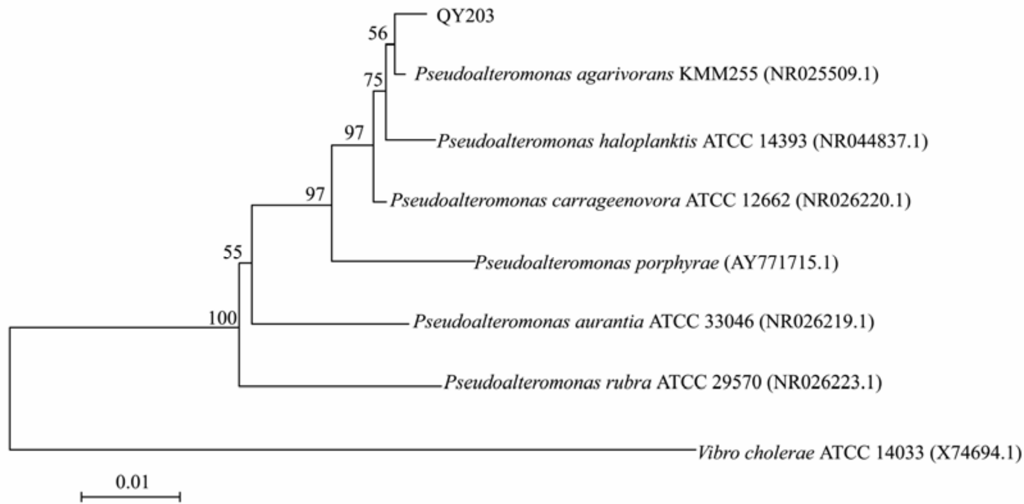

Fig.1 Phylogenetic tree of strain QY203 and related bacteria based on a maximum parsimony analysis of 16S rDNA sequences. Number after the strain names are culture collection numbers followed by GeneBank accession numbers of 16S rDNA sequences.

Table 1 Summary of CgkP purification

Purificaion step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Folds	Recovery (%)
Crude extract	2504	197.6	12.7	1	100
$(NH_4)_2SO_4$ precipitation	2264	142.6	15.9	1.3	90.4
Phenyl HP	1595	1.8	886.1	69.8	63.7
DEAE HP	673	0.6	1121.7	88.3	26.9

Note: The observables were measured at least three times.

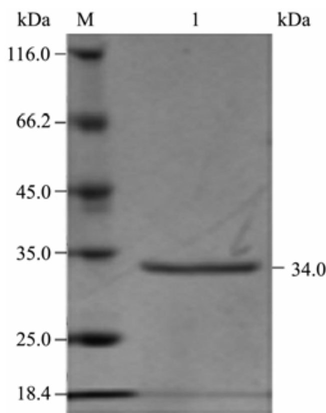

Fig.2 SDS-PAGE of CgkP. Lane M, molecular weight markers, lane 1, purified CgkP. The gel was stained with Coomassie Brilliant Blue.

The activity of CgkP in phosphate buffer was the highest at pH 7.2 (Fig.3A) and stable within a range of pH 6.0–9.0 (Fig.3B). The optimal pH value of CgkP was different from that of κ-carrageenases purified from *Pseudoalteromonas porphyrae* LL1 and *Pseudoalteromonas carrageenovora* ATCC 43555 (pH 8.0) (Liu *et al*., 2010). The optimal temperature of CgkP was 45℃ (Fig.3C). CgkP remained 95% and 80% of activity after incubation at 40℃ and 45℃ for 1 h, respectively (Fig.3D). CgkP still retained 70% activity after being incubated at 40℃ for 48 h. It retained 40% and 20% activity even being incubated at 50℃ for 1 h and 3 h, respectively. The κ-carrageenases from *P. porphyrae*, *P. carrageenovora* and *Cytophaga drobachiensis* were stable only at temperatures below 30℃ (Liu *et al*., 2011). The κ-carrageenases from *Cytophaga* sp. MCA-2 and *Pseudoalteromonas* sp. WZUC10 were stable at 40℃ for less than 2 h (Zhou *et al*., 2008). Although the κ-carrageenase from *Tamlana* sp. HC4 was stable at temperatures below 45℃ for 2 h, only 10% activity was retained after being incubated at 50℃ for 30 min (Sun *et al*., 2010). In comparison, CgkP was thermostable, promising for industrial application.

CgkP was active without NaCl; however, its activity was enhanced markedly by NaCl. The activity of CgkP was increased by 240% by 300 mmol L^{-1} NaCl (Table 2). Its activity was also enhanced by K^+. In comtrast, divalent and trivalent metal ions including Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Al^{3+} and Fe^{3+} significantly inhibited the activity of CgkP; while Mg^{2+} was not effective on its activity. The chelating agent EDTA inhibited the activity of CgkP, suggesting that it was a metalloenzyme (Bernardo *et al*., 2004). Detergent SDS reduced the activity of CgkP, which was similar to most κ-carrageenases reported previously.

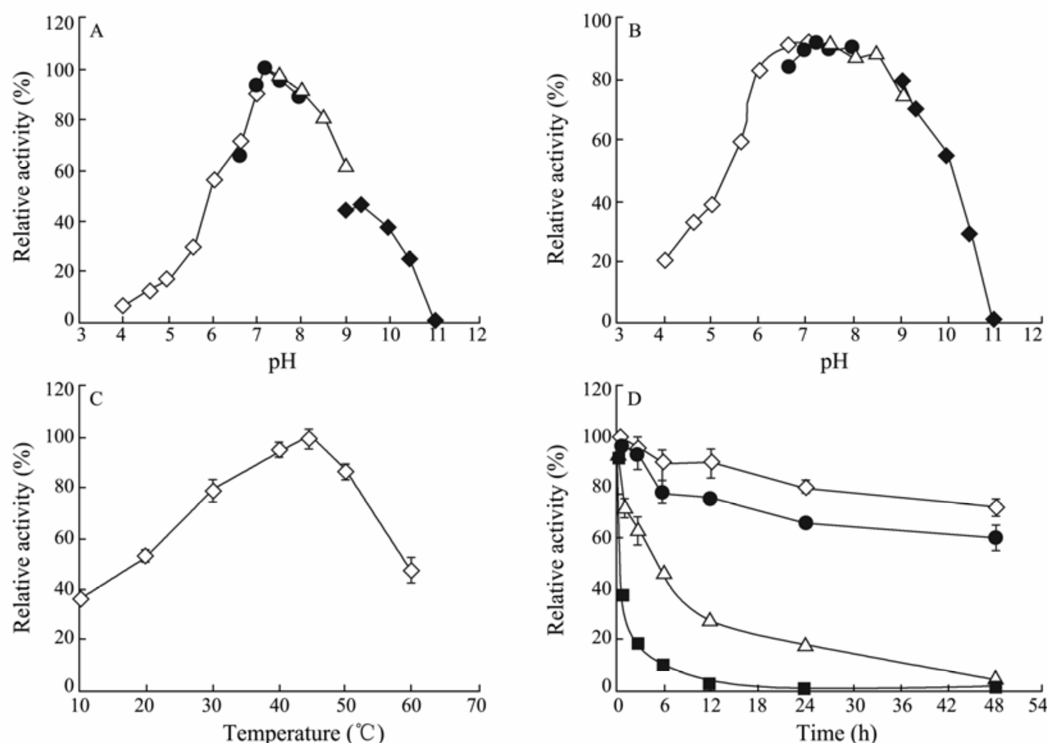

Fig.3 Effects of acidity and temperature on the activity and stability of CgkP. (A) The optimal acidity of CgkP. Buffers were 50 mmol L^{-1} Na_2HPO_4-citric acid (◇), 50 mmol L^{-1} Na_2HPO_4-NaH_2PO_4 (●), 100 mmol L^{-1} Tris-HCl (△) and 50 mmol L^{-1} Gly-NaOH (◆). (B) The acidity stability of CgkP. The same buffers were used as above. (C) The optimal temperature of CgkP. (D) The thermostability of CgkP. CgkP was incubated at 35℃ (◇), 40℃ (●), 45℃ (△) and 50℃ (■) for up to 48 h.

Table 2 Effect of metal ions, chelators and detergents on κ-carrageenase activity of CgkP

Additives	Concentration (mmol L^{-1})	Relative activity (%)
None	–	100±2.2
NaCl	100	245±3.6
	300	340±5.5
	500	175±3.9
KCl	1	128±1.4
$CuCl_2$	1	9±2.7
$MnCl_2$	1	45±4.3
$NiCl_2$	1	14±1.6
$BaCl_2$	1	94±0.3
$CaCl_2$	1	86±5.8
$ZnCl_2$	1	32±2.9
$MgCl_2$	1	107±3.5
$AlCl_3$	1	51±3.3
$FeCl_3$	1	57±0.7
SDS	1	76±1.8
EDTA	1	82±2.5

Notes: Activity without addition of metal ions or chemical agents was defined as 100%. Data were shown as means ± SD (n=5).

3.3 Analysis of Degradation Products

After adding CgkP, the viscosity of κ-carrageenan solution decreased rapidly in first 5 min, but changed little in following 25 min. The amount of reducing sugar (A520) increased steadily in 30 min, the whole observation period (Fig.4). These findings suggested that the enzyme was an endo-κ-carrageenase characterized by Kobayashi *et al.* (2009).

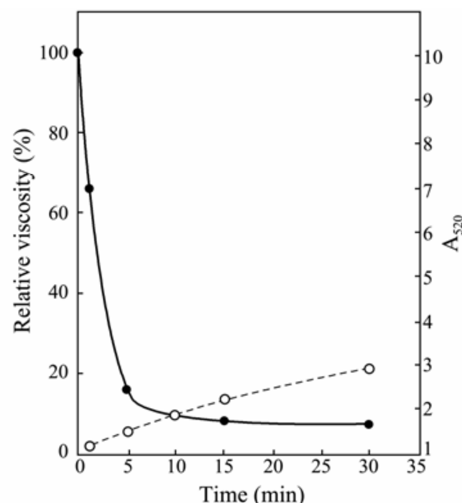

Fig.4 Decrease of κ-carrageenan viscosity during enzymatic degradation. Filled circles with a solid line, the rate of viscosity; open circles with a dotted line, the absorbance at 520 nm.

The end degrading products of κ-carrageenan by CgkP were disaccharide and tetrasaccharide as were characterized with TLC analysis (Fig.5). Out the Biogel-P6 column, most eluted hydrolysates formed two distinct peaks which were identified as κ-carrageenan-derived neocarradiaose and neocarratetraose with electrospray ionization

mass spectrometry (Data not shown). It has been documented that κ-carrageenases from *Pseudomonas* and *Vibrio* degrade κ-carrageenan into disaccharide and tetrasaccharide (Liu *et al*., 2010); while κ-carrageenase from *Pseudoalteromonas carrageenovora* ATCC43555 degrades κ-carrageenan into neocarradiaose, neocarratetraose and neocarrahexraose (Barbeyron *et al*., 1994).

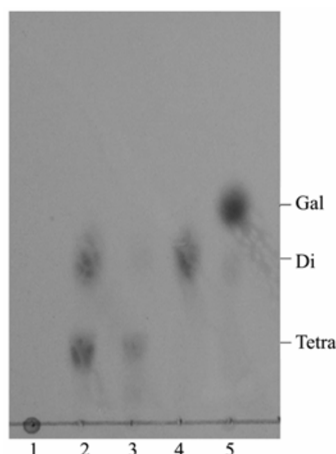

Fig.5 TLC analysis of the oligosaccharides derived from κ-carrageenan after incubation with CgkP. Lane 1, control, κ-carrageenan with inactivated CgkP; lane 2, end products of κ-carrageenan hydrolyzed by CgkP; lane 3, κ-neocarratetraose sulfate; lane 4, κ-neocarrabiose sulfate; lane 5, galactose.

4 Conclusion

An endo-κ-carrageenase, CgkP, was purified from *Pseudoalteromonas* sp. QY203 with a simple and reliable method. It is a new thermostable κ-carrageenase promising for industrial use.

Acknowledgements

This work was supported by National Science Foundation of China (31000361 and 31070712), Program for Changjiang Scholars and Innovative Research Team in University (IRT0944), Special Fund for Marine Scientific Research in the Public Interest (201005024) and the Fundamental Research Funds for the Central Universities (201013008).

References

Barbeyron, T., Gerard, A., Potin, P., Henrissat, B., and Kloareg, B., 1998. The kappa-carrageenase of the marine bacterium *Cytophaga drobachiensis*. Structural and phylogenetic relationships within family-16 glycoside hydrolases. *Molecular Biology and Evolution*, **15**: 528-537.

Barbeyrona, T., Henrissatb, B., and Kloareg, B., 1994. The gene encoding the kappa-carrageenase of *Alteromonas carrageenovora* is related to β-1,3-1,4-glucanases. *Gene*, **139**: 105- 109.

Bernardo, R. Z., Yuridia, M. F., César, H. R., and Lourdes, V. T., 2004. Purification and characterization of lysine amino peptidase from *Kluyveromyces marxiamus*. *FEMS Microbiology Letters*, **235**: 369-375.

Collén, P. N., Lemoine, M., Daniellou, R., Guégan, J. P., Paoletti, S., and Helbert, W., 2009. Enzymatic degradation of κ-carrageenan in aqueous solution. *Biomacromolecules*, **10**: 1757-1767.

Guibet, M., Colin, S., Barbeyron, T., Genicot, S., Kloareg, B., Michel, G., and Helbert, W., 2007. Degradation of lambda-carrageenan by *Pseudoalteromonas carrageenovora* lambda-carrageenase: a new family of glycoside hydrolases unrelated to kappa- and iota-carrageenases. *Biochemical Journal*, **404**: 105-114.

Khambhaty, Y., Mody, K., and Jha, B., 2007. Purification and characterization of κ-carrageenase from a novel γ-proteobacterium, *Pseudomonas elongata* (MTCC 5261) syn. *Microbulbifer elongatus* comb. Nov. *Biotechnology and Bioprocess Engineering*, **12**: 668-675.

Kobayashi, T., Uchimura, K., Miyazaki, M., Nogi, Y., and Horikoshi, K., 2009. A new high-alkaline alginate lyase from a deep-sea bacterium *Agarivorans* sp. *Extremophiles*, **13**: 121-129.

Laemmli, U. K., and Favre, M., 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *Journal of Molecular Biology*, **80**: 575-599.

Liu, G. L., Li, Y., Chi, Z., and Chi, Z. M., 2011. Purification and characterization of κ-carrageenase from the marine bacterium *Pseudoalteromonas porphyrae* for hydrolysis of κ-carrageenan. *Process Biochemistry*, **46**: 265-271.

Miller, G. L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry*, **31**: 426-428.

Michel, G., Barbeyron, T., Flament, D., Vernet, T., Kloareg, B., and Dideberg, O., 1999. Expression, purification, crystallization and preliminary x-ray analysis of the kappa-carrageenase from *Pseudoalteromonas carrageenovora. Acta Crystallographica Section D-biological Crystallography*, **55**: 918-920.

Michel, G., Chantalat, L., Fanchon, E., Henrissat, B., Kloareg, B., and Dideberg, O., 2001. The iota-carrageenase of *Alteromonas fortis*. A beta-helix fold-containing enzyme for the degradation of a highly polyanionic polysaccharide. *Journal of Biological Chemistry*, **276**: 40202-40209.

Mou, H., Jiang, X. L., and Guan, H. S., 2003. A κ-carrageenan derived oligosaccharide prepared by enzymatic degradation containing anti-tumor activity. *Journal of Applied Phycology*, **15**: 297-303.

Potin, P., Sanseau, A., Le, G. Y., Rochas, C., and Kloareg, B., 1991. Purification and characterization of a new kappa-carrageenase from a marine *Cytophaga*-like bacterium. *European Journal of Biochemistry*, **201**: 241-247.

Toshiyoshi, A., Yoshifumi, H., and Tatsuo, M., 1999. Purification and characterization of kappa-carrageenase from a marine bacterium *Vibrio* sp. CA-1004. *Fisheries Science*, **65**: 937-942.

Sun, F. X., Ma, Y. X., Wang, Y., and Liu, Q., 2010. Purification and characterization of novel κ-carrageenase from marine *Tamlana* sp. HC4. *Chinese Journal of Oceanology and Limnology*, **28**: 1139-1145.

Yuan, H., Song, J., Li, X., Li, N., and Dai, J., 2006. Immunomodulation and antitumor activity of κ-carrageenan oligosaccharides. *Cancer Letters*, **243**: 228-234.

Zhou, M. H., Ma, J. S., Li, J., Ye, H., Huang, K., and Zhao, X., 2008. A kappa-carrageenase from a newly isolated *Pseudoalteromonas*-like bacterium, WZUC10. *Biotechnology and Bioprocess Engineering*, **13**: 545-551.

(Edited by Qiu Yantao)