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ORIGINAL RESEARCH PAPER

Cloning, expression and characterization of a new ι-carrageenase from marine bacterium, *Cellulophaga* sp.

Su Ma · Yu-Long Tan · Wen-Gong Yu · Feng Han

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Abstract
Purpose of work The purpose of this study is to report a ι-carrageenase which degrades ι-carrageenan yielding neo-ι-carratetraose as the main product in the absence of NaCl.
The gene for a new ι-carrageenase, CgiB_Ce, from *Cellulophaga* sp. QY3 was cloned and sequenced. It comprised an ORF of 1,386 bp encoding for a protein of 461 amino acid residues. From its sequence analysis, CgiB_Ce is a new member of GH family 82 and shared the highest identity of 32 % in amino acids with ι-carrageenase CgiA2 from *Zobellia galactanovorans* indicating that it is a hitherto uncharacterized protein. The recombinant CgiB_Ce had maximum specific activity (1,870 U/mg) at 45 °C and pH 6.5. It was stable between pH 6.0–9.6 and below 40 °C. Although its activity was enhanced by NaCl, the enzyme was active in the absence of NaCl. CgiB_Ce is an endo-type ι-carrageenase that hydrolyzes β-1,4-linkages of ι-carrageenan, yielding neo-ι-carratetraose as the main product (more than 80 % of the total product).

Keywords Carrageenase · Cellulophaga · Characterization · Cloning · Oligosaccharide

Introduction

Carrageenan is a generic name for a family of polysaccharides, extracted from species of red seaweeds (*Rhodophyta*). These polysaccharides mainly consist of alternating 3-linked β-D-galactopyranose (G-units) and 4-linked α-D-galactopyranose (D-units) or 3,6-anhydro-α-D-galactopyranose (DA-units), forming the disaccharide repeating unit of carrageenans. Further classification of carrageenans is based on the position and number of sulfate groups per different repeating disaccharide unit, e.g. κ-(3,6-anhydro-α-D-galactopyranosyl-1,4-4-sulfate-β-D-galactose), ι-(2-sulfate

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S. Ma · Y.-L. Tan · W.-G. Yu · F. Han
Key Laboratory of Marine Drugs, Chinese Ministry of Education, Ocean University of China, Qingdao 266003, China
e-mail: masu277@gmail.com

Y.-L. Tan
e-mail: yulongtan212@gmail.com

W.-G. Yu
e-mail: yuwg66@ouc.edu.cn

S. Ma · Y.-L. Tan · W.-G. Yu · F. Han
Shandong Provincial Key Laboratory of Glycoscience and Glycotechnology, Ocean University of China, Qingdao 266003, China

S. Ma · Y.-L. Tan · W.-G. Yu · F. Han (✉)
Laboratory of Glycobiology, School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao 266003, China
e-mail: fhan@ouc.edu.cn

-3,6-anhydro-α-D-galactopyranosyl-1,4-4-sulfate-β-D-galactose) and λ-carrageenan (2,6-sulfates-α-D-galactopyranosyl-1,4-2-sulfate-β-D-galactose) (Campo et al. 2009). Carrageenan oligosaccharides (COS) exhibit various biological activities, such as antioxidant (Ren et al. 2010), antiviral (Wang et al. 2011), antitumor and immunomodulation activities (Yuan et al. 2006) and therefore have captured more and more attention.

Enzymes that degrade different carrageenans are named as κ-, ι- and λ-carrageenases and belong to different glycoside hydrolase (GH) families in the carbohydrate-active enzymes (CAZy) database (Cantarel et al. 2009). Specific enzyme degradation can keep the native structure of carrageenans without the risk of modification and is more safe and controllable than other degradation methods. GH family 82 comprises nine ι-carrageenases obtained from a number of bacteria but only four have been purified and characterized (Barbeyron et al. 2000; Hatada et al. 2011). The main products of ι-carrageenan hydrolyzed by ι-carrageenases CgiA and CgiA2 from *Zobellia galactanivorans* or ι-carrageenase CgiA from *Alteromonas fortis* were neo-ι-carratetraose and neo-ι-carrahexaose which were hard to separate. The ι-carrageenase A94Cgi from *Microbulbifer thermotolerans* produced a high ratio of neo-ι-carratetraose in a mixture of products but it showed little activity in the absence of NaCl which might increase the expenditure on reactor repair and oligosaccharide desalting (Hatada et al. 2011). Therefore, the discovery of new ι-carrageenases for the further utilization in industrial production and purification of ι-COS is essential and important.

Here we report the cloning and expression of a new ι-carrageenase from *Cellulophaga* sp. QY3, CgiB_Ce, that belongs to the GH family 82. It exhibited high activity towards ι-carrageenan without NaCl and yielded neo-ι-carratetraose as the main product (more than 80 % of the total product).

Materials and methods

Bacterial strains, plasmids, culture conditions and chemicals

Cellulophaga sp. QY3 was isolated from the red alga *Grateloupia livida* collected from Qingdao coastal waters of China Yellow Sea and preserved in China Center for Type Culture Collection (CCTCC) under the accession number M209082. Strain QY3 was identified by analysis of the partial sequence of its 16S rRNA gene (GenBank accession number DQ311648). *Escherichia coli* strains DH5α (Gibco BRL) and BL21(DE3) (Novagen, USA) were grown at 37 °C in LB broth or on LB agar supplemented with ampicillin (50 μg/ml) or kanamycin (30 μg/ml) when relevant. The pMD18-T (TaKaRa, Dalian, China) and pET-28a (+) (Novagen, USA) plasmids were employed as gene cloning and expression vectors, respectively. Oligonucleotides used for the cloning and expression of CgiB_Ce are shown in Supplementary Table 1.

Cloning of the ι-carrageenase gene

Degenerate primers (PcgiB-F1, PcgiB-R1) were designed according to the conserved sequences of GH family 82 ι-carrageenases to amplify part of CgiB_Ce-encoding gene using the genomic DNA of *Cellulophaga* sp. QY3 as the template. A 273-bp DNA fragment was obtained and sequenced. SiteFinding-PCR (Tan et al. 2005) was used to amplify the flanking sequences with six nested gene-specific primers (SFP1&2, Up-cgiB-R1&2, Down-cgiB-F1&2). The PCR products were purified, sequenced, and assembled with the initial *cgiB_Ce* fragment to obtain the full-length gene. The DNA sequence for *cgiB_Ce* was deposited in GenBank under the accession number JX871895.

Sequence analysis of the ι-carrageenase gene and its product

The putative ORFs were identified by DNATools program. Theoretical molecular weight and isoelectric point were calculated using Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html). The signal peptide was predicted using SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP). Multiple sequence alignments between CgiB_Ce and other known ι-carrageenases were obtained using the ClustalX program. The phylogenetic tree was constructed by MEGA 4.0 (Tamura et al. 2007) and the neighbor-joining method.

Expression and purification of recombinant CgiB_Ce

The primers PcgiB-F2 and PcgiB-R2 were designed to introduce *Nde*I and *Xho*I sites encompassing *cgiB_Ce*.

After gel purification, the PCR product was digested with *Nde*I and *Xho*I, and ligated into similarly digested pET-28a (+). The resulting plasmid, pET28-cgiB, was then transformed to *E. coli* BL21 (DE3).

Escherichia coli BL21 (DE3) cells harboring pET28-cgiB were grown at 37 °C in LB medium containing 30 μg kanamycin/ml until the OD_{600} reached 0.4, and IPTG was then added to give 0.1 mM. Cultivation was continued further for 36 h at 16 °C and 100 rpm. Cells were harvested by centrifugation (6,000×*g*, 10 min), resuspended in phosphate buffer (20 mM, pH 6.5; 500 mM NaCl), and disrupted by ultrasonication. The supernatant obtained by centrifugation (10,000×*g*, 30 min) was loaded onto a HiTrap HP column (1 ml, GE Healthcare). The recombinant proteins were eluted by a linear gradient of imidazole (5–500 mM) in phosphate buffer (20 mM, pH 6.5; 500 mM NaCl). Active fractions were stored at −20 °C.

The purity and molecular weight of CgiB_Ce were determined using SDS-PAGE. Protein was measured using the Bradford method with BSA as standard.

ι-Carrageenase activity assay

ι-Carrageenase, 100 μl, was incubated with 900 μl substrate solution consisting of 0.2 % (w/v) ι-carrageenan (type V, from *Eucheuma spinosa*, Sigma-Aldrich) in 20 mM phosphate buffer (pH 6.5) at 45 °C for 20 min. The reducing oligosaccharide products in the reaction mixture were assayed using the 3,5-dinitrosalicylic acid method. 1 U of ι-carrageenase activity was defined as the amount of enzyme required to liberate 1 μmol reducing sugar as neo-ι-carratetraose per min under the above conditions.

Analysis of reaction product and hydrolytic pattern

Analysis and fractionation of digested and undigested ι-carrageenan samples were monitored by gel filtration chromatography on a Superdex peptide 10/300 GL column (GE Healthcare) with 0.1 M $NaNO_3$ as an eluent using an AKTA FPLC system (GE Healthcare) equipped with a refractive index detector. To identify the structure of hydrolysis products, the eluted fractions were analyzed using negative-ion ESI-MS and ^{1}H- and ^{13}C-NMR spectrometry as described previously (Jouanneau et al. 2010; Yang et al. 2009).

Results and discussion

Sequence analysis of the ι-carrageenase gene

The ORF of the gene *cgiB_Ce* consisted of 1,386 bp with deduced amino acid size of 461 (49.9 kDa) including a putative signal peptide of 23 amino acid residues. The mature protein had a calculated molecular weight of 48,071 Da and a pI of 4.79. Except for an uncharacterized ι-carrageenase, Celly_2571, from *Cellulophaga lytica*, CgiB_Ce had the highest identity of 32 % with ι-carrageenase CgiA2 from *Z. galactanovorans*. The enzyme was categorized as a GH family 82 member based on the sequence analysis of

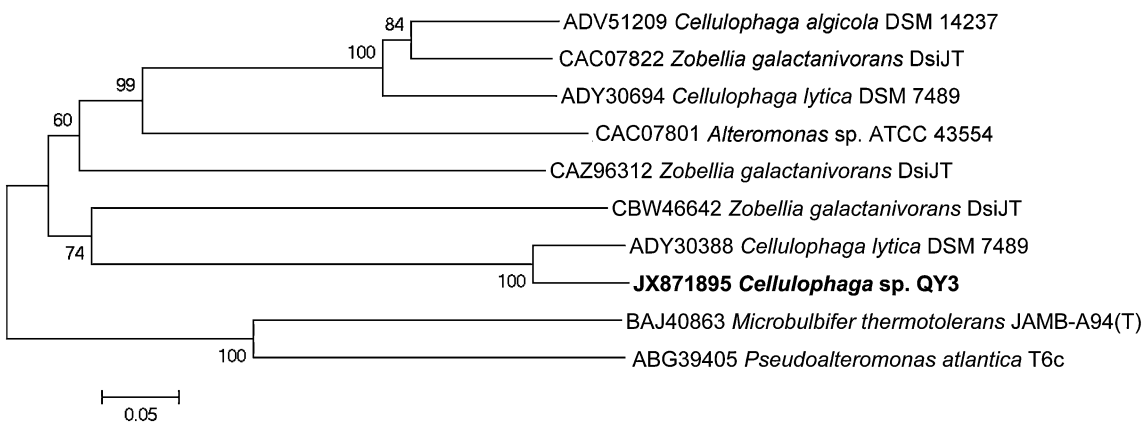

Fig. 1 Phylogenetic analysis of ι-carrageenase CgiB_Ce. The GH family 82 ι-carrageenase protein sequences were aligned using ClustalX, and phylogenetic tree was constructed using MEGA 4.0 via the neighbor-joining method

the catalytic domain. A phylogenetic tree was constructed for all ι-carrageenases from GH family 82 (Fig. 1). CgiB_Ce, along with Celly_2571 (GenBank ADY30388) and CgiA3 (GenBank CBW46642), formed a deeply branched cluster in the phylogenetic tree and was thus clearly distinct from other carrageenases in GH family 82.

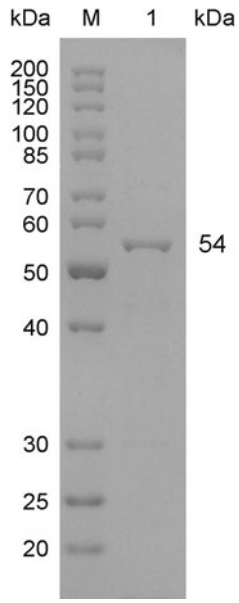

Fig. 2 SDS-PAGE of recombinant CgiB_Ce. *Lane M* molecular weight markers, *Lane 1* purified ι-carrageenase CgiB_Ce

Purification and biochemical characterization of CgiB_Ce

Recombinant CgiB_Ce was purified to homogeneity with a specific activity of 1,870 U/mg and a final yield of 77 %. It showed a single band with an apparent molecular weight of 54 kDa on SDS-PAGE (Fig. 2).

CgiB_Ce was most active at 45 °C and pH 6.5 in 50 mM phosphate buffer (Fig. 3a, c). The enzyme was stable between pH 6.0–9.0 (Fig. 3b). It retained 70 % of the original activity after incubation at the temperatures below 40 °C for 1 h (Fig. 3d). The effects of metal ions, detergents and chelating agents on the activity of CgiB_Ce were shown in Table 1. Although its activity was enhanced by NaCl, the enzyme was active in the absence of NaCl.

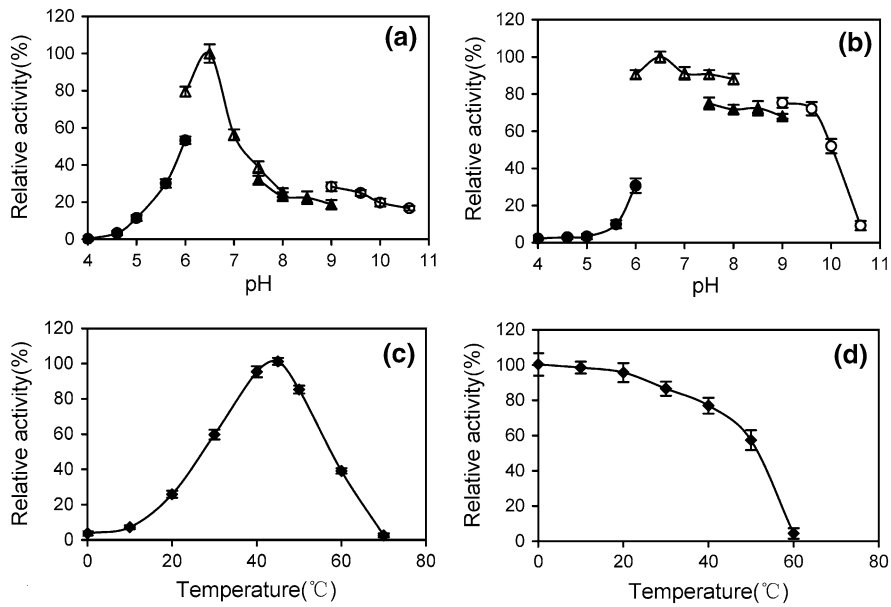

Fig. 3 Effects of pH and temperature on the activity and stability of CgiB_Ce. **a** The optimal pH of CgiB_Ce was determined by measuring the activity at 45 °C in 50 mM citrate acid buffer (pH 4.0–6.0; *closed circles*), phosphate buffer (pH 6.0–8.0; *open triangles*), Tris/HCl buffer (pH 7.5–9.0; *closed triangles*) and glycine/NaOH buffer (pH 8.6–10.6; *open circles*). **b** pH stability of CgiB_Ce. The residual activity was measured at 45 °C in 50 mM phosphate buffer (pH 6.5) after incubated in the pH range of 4.0–10.6 with the above buffers for 24 h at 4 °C. **c** The optimal temperature of CgiB_Ce was determined by measuring the activity in 50 mM sodium phosphate buffer (pH 6.5) at various temperatures (0–70 °C). **d** Thermostability of the purified CgiB_Ce. CgiB_Ce was incubated in 50 mM buffers (pH 6.5) without substrate for 1 h at 0–60 °C, respectively. Residual activities were measured at 45 °C

Table 1 Effect of metal ions, chelators and detergents on the activity of CgiB_Ce

Reagent added	Concentration (mM)	Relative activity (%)	Reagent added	Concentration (mM)	Relative activity (%)
None	–	100 ± 2.4			
NaCl	50	129 ± 3.1	NiCl	1	128 ± 2.1
	100	144 ± 2.7	LiCl	1	114 ± 1.9
	300	156 ± 3.2	$CuCl_2$	1	13 ± 1.7
	500	166 ± 2.6	$ZnCl_2$	1	51 ± 1.3
	700	148 ± 2.1	$BaCl_2$	1	118 ± 2.2
KCl	1	116 ± 1.9	$CaCl_2$	1	122 ± 1.6
	10	134 ± 3.6	$MgCl_2$	1	149 ± 2.9
	50	125 ± 2.8	$FeCl_2$	1	174 ± 3.5
	100	123 ± 2.3	$MnCl_2$	1	173 ± 3.1
SDS	1	2.9 ± 0.7	$FeCl_3$	1	89 ± 2.3
EDTA	1	10.4 ± 1.1	$AlCl_3$	1	44 ± 1.8

Values given are the average of three replications, the data were expressed as mean ± standard deviation. The activity of control (100 % relative activity) is 1,870 U/mg

The enzyme could degrade ι-carrageenan but showed no activity towards κ-carrageenan, λ-carrageenan or agarose which have the similar backbones with ι-carrageenan (data not shown).

Analysis of reaction product and hydrolytic pattern

After completion of ι-carrageenan hydrolysis by CgiB_Ce, the main product of the reaction was purified and analyzed by negative-ion ESI-MS and ^{1}H- and ^{13}C-NMR spectrometry. The spectra showed good agreement with those of neo-ι-carratetraose (data not shown), which was reported previously (Jouanneau et al. 2010). The hydrolytic pattern of CgiB_Ce was monitored by size-exclusion chromatography (Fig. 4). The rapid depolymerization, the increase of polydispersity and the production of all possible oligosaccharides showed that CgiB_Ce acted in an endolytic mode. The main product was neo-ι-carratetraose (more than 80 % of the total product).

Carrageenan oligosaccharides have gained extensive attention with various biological activities. However, oligosaccharides with similar molecular weight are hard to separate and this limits research on their structure/function relationship. A high proportion of a single homogeneous product in a mixture of products is beneficial to the oligosaccharide production (Hatada et al. 2011). Although A94Cgi produced neo-ι-carratetraose in a high ratio (more than 75 % of the total product), its activity depended strictly on the NaCl concentration. High salinity may corrode the reactor and make the purification of COS more difficult.

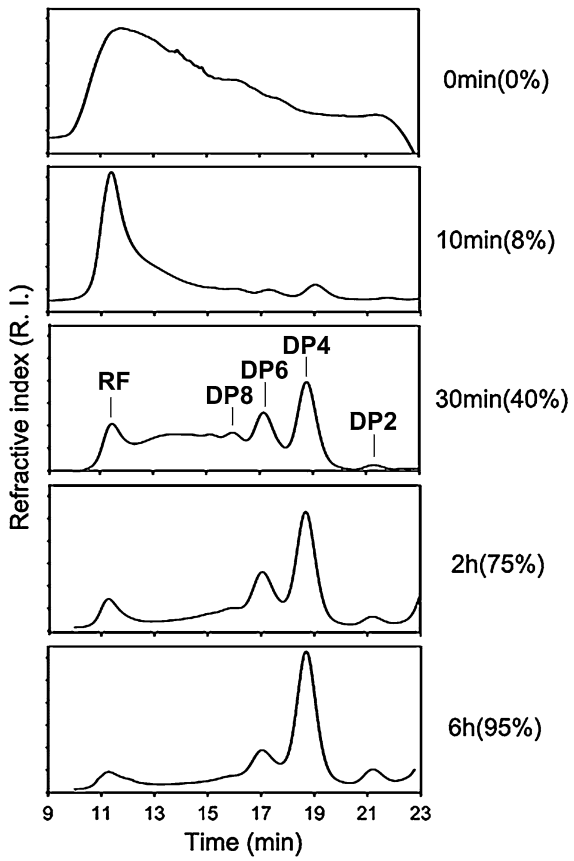

Fig. 4 Size-exclusion chromatography of the digestion products of the ι-carrageenan by CgiB_Ce. The profiles were recorded at several intervals of incubation which were correlated with percentages of degradation (in *parentheses*) determined by the reducing-sugar assay. DP2 to DP8 correspond to the peaks of the neo-ι-carrabiose to the neo-ι-carraoctaose respectively. *RF* resistant fraction

CgiB_Ce was active in the absence of NaCl, yielding neo-ι-carratetraose as the main product (more than 80 % of the total product). The discovery and study of the new ι-carrageenase CgiB_Ce is benefit not only for the production of neo-ι-carratetraose but also for its purification. Therefore, CgiB_Ce would play a significant role in further industrial application of COS.

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