International Journal of Biological Macromolecules 112 (2018) 93–100

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: https://www.journals.elsevier.com/ijbiomac

Expression and characterization of a κ-carrageenase from marine bacterium *Wenyingzhuangia aestuarii* OF219: A biotechnological tool for the depolymerization of κ-carrageenan

Jingjing Shen, Yaoguang Chang *, Feng Chen, Shujun Dong

College of Food Science and Engineering, Ocean University of China, 5 Yushan Road, Qingdao 266003, China

ARTICLE INFO

Article history:
Received 3 August 2017
Received in revised form 2 December 2017
Accepted 12 January 2018
Available online xxxx

Keywords:
κ-Carrageenase
GH16
Hydrolysis pattern
κ-Carrageenan
Degradation

ABSTRACT

κ-Carrageenases are desirable tools for tailoring the molecular weight, physicochemical properties and functionalities of κ-carrageenan, a macromolecule widely utilized in various industries. In this study, a novel GH16 family κ-carrageenase, designated as Cgk16A, was cloned from the genome of marine bacterium *Wenyingzhuangia aestuarii* OF219 and expressed in *Escherichia coli*. Its biochemical properties, kinetic parameters and hydrolytic pattern were characterized. The enzyme demonstrated a low optimal reaction temperature (25 °C) and a cold-adapted feature. As an endo-acting glycoside hydrolase, Cgk16A degraded κ-carrageenan in a random manner, and it was competent to prepare the degradation products with varying degrees of polymerization. The mass spectrometry analysis revealed that the end products of Cgk16A were majorly composed of κ-carrageenan tetrasaccharide with a minor portion of disaccharide. The enzyme showed higher enzyme-substrate affinity over all hitherto characterized GH16 κ-carrageenases, indicated by its low K_m value (0.17 μM). Cgk16A could be employed as a potential biotechnological tool for depolymerizing κ-carrageenan, which would facilitate the future application of κ-carrageenan and its degradation products.

1. Introduction

Carrageenan is a family of sulfated polysaccharides obtained from red algae (Rhodophyta). Depending on the occurrence of 4-linked 3,6-anhydro-α-D-galactopyranose and the sulfation pattern, carrageenan can be classified to several different types, including κ-, ι- and λ-carrageenan [1]. κ-Carrageenan is composed of alternating 4-linked-α-D-3,6-anhydrogalactose (DA) and 3-linked-4-O-sulfated-β-D-galactopyranose (G4S) residues (Fig. S1). It is well-known for its ability to form gels and its wide applications in various industries as the gelling agent, thickener and stabilizer [2,3]. In addition, various bioactivities of κ-carrageenan have been established, such as antiviral effect and anticoagulation [4,5]. Although there is a controversy over its inflammation-inducing toxicity [6,7], it is generally regarded as safe (GRAS) by the US Food and Drug Administration [8].

The physicochemical properties of κ-carrageenan, such as viscosity, gel strength and stabilizing ability, were highly depended on its molecular weight [9–11]. Therefore, κ-carrageenan with different performance and application potential could be prepared by tailoring the degree of polymerization. In addition, depolymerized products of κ-carrageenan have also drawn a considerable interest in the functional food and medicine fields due to their diverse activities [12]. It has been proven that low molecular weight κ-carrageenans can exhibit antioxidant [13], immunomodulation [14], antiangiogenic [15] and antitumor [14,16] effects.

Enzymatic hydrolysis is a favorable procedure for degrading polysaccharides. κ-Carrageenases (EC 3.2.1.83) are a group of glycoside hydrolases that could specifically cleave the linkages in the backbone of κ-carrageenan without the risk of modifying the substituted moieties in native structure [17]. They hydrolyze the internal β(1 → 4) glycosidic linkages of κ-carrageenan yielding neo-disaccharide series ($[DA\text{-}G4S]_n$) [18].

Up to date, seven enzymes with validated κ-carrageenase activity and clear protein sequence have been studied. Their biochemical properties and catalysis behavior have been characterized at different degrees, which were summarized in the Supplementary data 1. According to the classification based on amino acid sequence similarities, all those κ-carrageenases belong to the GH16 family in the carbohydrate-active enzymes (CAZy) database [19]. Recently, a bacterium *Wenyingzhuangia aestuarii* OF219 was isolated from marine sediment by our lab, and the genome of this bacterium has been clarified due to its versatility in utilizing polysaccharides (manuscript in preparation). An open reading frame (ORF) (located from 403,434 to 405,182 in the complementary strand, GenBank accession number MF289779) was annotated coding a putative GH16 protein, which would be a candidate

* Corresponding author.
E-mail address: changyg@ouc.edu.cn (Y. Chang).

https://doi.org/10.1016/j.ijbiomac.2018.01.075
0141-8130/

gene for novel κ-carrageenases. In this report, the ORF, hereinto designated as gene *cgk16A*, was cloned and heterologously expressed in *Escherichia coli*. The produced protein correspondingly coded as Cgk16A were subsequently characterized, and its feasibility for preparing depolymerized products of κ-carrageenan was evaluated.

2. Materials and methods

2.1. Bioinformatics analysis

Domain architectures were predicted by using a combination of bioinformatics analysis tools including dbCAN [20], CD-search [21] and SignalP 4.1 [22]. Similarity between sequences was evaluated by using BLASTP [23]. Multiple sequence alignments were implemented utilizing ClustalX [24], and the phylogenetic tree was constructed using the neighbor-joining method with MEGA6 [25]. The theoretical molecular weight of proteins was calculated by the program Compute pI/Mw [26].

2.2. Gene cloning and protein expression

Genomic DNA of *W. aestuarii* OF219 was extracted from the culture using the TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China). The gene *cgk16A* without the predicted signal sequence was amplified by PCR using the forward and reverse primers 5′-GACACGGATCCACTTCTCAAAATTTAAGACCGCTTAATGC-3′ and 5′-GACACCTCGAGTTAATCTATAATTATTTTTTGGGTTTTAATTCC-3′. The PCR product was subsequently digested with *Bam*HI and *Xho*I and ligated into the pET 28a(+) vector (Novagen, San Diego, CA, USA) which introduced a $(His)_6$ tag at the N-terminus. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) competent cells (Biomed, Beijing, China). The transformant was cultured in the Luria-Bertani (LB) media (Hope Bio-Technology, Qingdao, China) containing 30 μg/mL kanamycin at 37 °C until OD_{600} reached 0.4. Isopropyl β-D-1-thiogalactopyranoside was then added to a final concentration of 0.5 mM for inducing protein expression. After 12 h cultivation at 16 °C, cells were harvested by centrifugation and disrupted by sonication in 20 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 8.0).

Unless otherwise stated, the purification steps were performed using an ÄKTA™ Prime Plus system (GE healthcare, Uppsala, Sweden) at 4 °C. To purify the $(His)_6$-tagged recombinant protein, supernatant of the cell lysate was loaded onto the HisTrap™ HP columns (GE healthcare, Uppsala, Sweden) and eluted by a linear gradient of 0–0.5 M imidazole in 20 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 8.0) with 0.3 M NaCl. The active fractions were desalted by HiTrap™ Desalting columns (GE healthcare, Uppsala, Sweden) with 20 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 8.0). The purity and molecular weight (M_w) of purified enzyme were subsequently assessed by SDS-PAGE on 5% stacking gel and 10% running gel. Protein bands were stained with Coomassie brilliant blue. The M_w of the purified protein was calculated with molecular weight markers (Page Ruler™ prestained protein ladder, Fermentas, Waltham, MA, USA). The resulted purified recombinant enzyme was employed in the following characterizations.

2.3. κ-Carrageenase activity assay

The κ-carrageenan employed as substrate was purchased from Sigma (St. Louis, MO, USA). Its weight-average molecular weight was determined as 396.1 kDa by using high performance size exclusion chromatography combined with multi-angle laser light scattering [27].

The κ-carrageenase activity was assayed by incubating enzyme with 1 mg/mL κ-carrageenan in 20 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 8.0, containing 0.1 M NaCl) at 25 °C for 5 min. The released reducing sugar in the incubation was quantified by the para-hydroxybenzoic acid hydrazide (pHBAH) method (pHBAH was purchased from Sigma-Aldrich, St. Louis, MO, USA) [28]. One unit of κ-carrageenase activity was defined as the amount of enzyme required to produce 1.0 μmol reducing sugar (equivalent to D-galactose) per minute. The protein concentration was measured by a kit based on bicinchoninic acid assay (BCA Protein Assay Kit, Beyotime Biotechnology, Shanghai, China), with bovine serum albumin as the standard.

2.4. Biochemical characterization

The optimal temperature for the activity of purified recombinant protein was investigated by incubating enzyme and substrate at temperatures ranging from 15 to 60 °C. The thermal stability was evaluated by pretreating enzyme at 4 °C, 20 °C, 25 °C, 30 °C and 40 °C before enzymatic activity assay.

The optimal pH was determined by assessing the activity in various buffers containing 0.1 M NaCl. The followed buffers were used: 20 mM citrate–phosphate buffer for pH 4.0–7.0; 20 mM NaH_2PO_4-Na_2HPO_4 buffer for pH 7.0–9.0; and 20 mM Na_2CO_3-$NaHCO_3$ buffer for pH 9.0–11.0. The pH stability was measured by incubating the enzyme in these buffers at 4 °C for 1 h, and the residual activity was measured after adjusting pH back to 8.0.

To examine the impacts of metal ions and chemicals, enzymatic activities were monitored by adding them to the mixture with a final concentration of 1 mM. In addition, the impact of NaCl concentration on the enzyme was examined by assaying protein activities in the presence of salt at different concentrations (0–1.0 M).

2.5. Determination of kinetic parameters

Kinetic parameters of the enzyme were determined by measuring initial reaction rates in the presence of 0.01–2.50 μM κ-carrageenan. Conditions except the substrate concentration were identical with those of the standard enzymatic activity assay (described in 2.3). The K_m and V_{max} were calculated by fitting activity results and substrate concentrations against the Michaelis–Menten equation. And the K_{cat} and K_{cat}/K_m values were subsequently deduced from V_{max} and K_m.

2.6. Analysis of reaction products and hydrolytic pattern

For investigating the hydrolytic pattern of the enzyme, 5 U enzyme was mixed with 100 mg κ-carrageenan in 20 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 8.0, containing 0.1 M NaCl) at 25 °C. Aliquots of reaction mixture were withdrawn at intervals and heated at 100 °C for 10 min to deactivate the enzyme. The products were analyzed using high performance size exclusion chromatograph coupled with a refractive index detector (HPSEC-RID) (Aglient 1260, Aglient Technologies, Santa Cruz, CA, USA). A TSKgel SuperAW4000 column (Tosoh Corporation, Kanagawa, Japan) (0.2 M NaCl as the eluent, flow rate 0.6 mL/min) and a Superdex peptide 10/300 GL column (GE healthcare, Uppsala, Sweden) (50 mM ammonium formate as the eluent, flow rate 0.5 mL/min) were respectively employed to examine the global profile and oligosaccharide pattern of the reaction products.

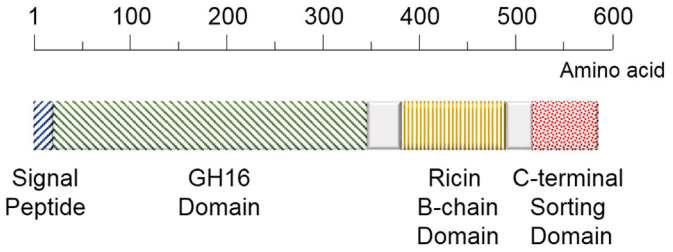

Fig. 1. A schematic of the modular arrangement of Cgk16A. The signal peptide (1–20 amino acids) was found by using the SignalP 4.1 server. The GH16 family glycoside hydrolase catalytic domain (21–347 amino acids) was identified by the dbCAN. The ricin B-chain domain (378–490 amino acids) and the Por secretion system C-terminal sorting domain (516–581 amino acids) were predicted by the CD-search.