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ORIGINAL RESEARCH PAPER

Cloning, expression and characterization of a new agarase-encoding gene from marine *Pseudoalteromonas* sp.

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Abstract The β-agarase gene *agaA*, cloned from a marine bacterium, *Pseudoalteromonas* sp. CY24, consists of 1,359 nucleotides encoding 453 amino acids in a sequence corresponding to a catalytic domain of glycosyl hydrolase family 16 (GH16) and a carbohydrate-binding module type 13 (CBM13). The recombinant enzyme is an endo-type agarase that hydrolyzes β-1,4-linkages of agarose, yielding neoagarotetraose and neoagarohexaose as the predominant products. In two cleavage patterns, AgaA digested the smallest substrate, neoagarooctaose, into neoagarobiose, neoagarotetraose and neoagarohexaose. Site directed mutation was performed to investigate the differences between AgaA and AgaD of *Vibrio* sp. PO-303, identifying residues $V_{109}VTS_{112}$ as playing a key role in the enzyme reaction.

Keywords β-Agarase · Carbohydrate binding module · Glycoside hydrolase · Neoagaro-oligosaccharide · *Pseudoalteromonas* sp. CY24

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Introduction

Agar is an abundant biopolymer found in the cell walls of some red algae and is composed of agarose and agarocolloid. Agarose is a neutral polysaccharide of alternatively arranged 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactopyranose. Agarocolloid is a heterogeneous mixture of smaller molecules that occur in lesser amounts, whose structures are similar but slightly branched and sulfated. Agarases are the glycoside hydrolases (GH) that hydrolyze agarose. They are classified into two groups according to the mode of action on agarose: α-agarase and β-agarase, which, respectively, hydrolyze α-1,3-linkages and β-1,4-linkages in agarose. Agarases are useful enzymes for the structural analysis of the cell wall and for protoplast isolation from agarophyte algae (Araki et al. 1994). Neoagaro-oligosaccharides, which have D-galactose residues at their reducing ends, are produced only by enzymatic depolymerization of agarose by β-agarase and have attracted increasing interest because of their wide applicability in the food, cosmetic and medical industries.

Based on amino acid sequence homology, known β-agarases are classified into three glycoside hydrolase families (http://afmb.cnrsmrs.fr/CAZY), namely families GH16, GH50 and GH86. Family 16 comprises more than 20 agarases isolated from a number of marine bacteria including genera *Agarivorans*, *Janthinobacterium* (Shi et al. 2008), *Microbultifer* (Ohta et al. 2004), *Pseudoalteromonas*, *Pseudomonas* (Belas

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et al. 1988), *Vibro* (Dong et al. 2007a; Zhang and Sun 2007), *Zobellia galactanivorans*, etc. All β-agarases in GH16 feature a common catalytic motif, (E[ILV]D[IVAF][VILMF](0,1)E) (Juncosa et al. 1994) and act by a general acid catalysis in which two acidic residues participate in a double replacement reaction, resulting in the retention of configuration at the anomeric carbon (Johansson et al. 2004). Structures of only two agarases have been determined: β-agarases A and B from *Zobellia galactanivorans* Dsij. These both degrade agarose and also agarose oligosaccharides, that must comprise at least six sugars, to yield neoagarotetraose as a main product. The active site cleft of the two enzymes accommodates eight sugar units spanning subsites −4 to +4 of the substrate (Allouch et al. 2003).

We cloned a novel gene encoding β-agarase AgaB from *Pseudoalteromonas* sp. CY24 in previous work (Ma et al. 2007). In this paper, we describe the cloning, expression and characterizing of another β-agarase gene *agaA*.

Materials and methods

Bacterial strain, plasmid, medium and culture conditions

Pseudoalteromonas sp. CY24 was originally isolated from the seacoast area of Qingdao, China and was maintained at 25°C on agar medium at 25°C (Ma et al. 2007). *E. coli* DH5α and plasmid pBluescript II KS(+) were used for preparation of recombinant plasmids. *E. coli* BL21 (DE3) and pET-24a(+) (Novagen) were used for enzyme expression. *E. coli* cells were cultured in Luria–Bertani (LB) broth with 100 μg ampicillin/ml or 30 μg kanamycin/ml when necessary. Agarose, neoagaro-tetraose (DP4), -hexaose (DP6) were purchased from Sigma and -octaose (DP8), -decaose (DP10) were prepared in our laboratory. Oligonucleotides used for gene cloning and site-directed mutagenesis are shown in Supplementary Table 1. All DNA manipulations were carried out according to standard protocols.

Molecular cloning and DNA sequencing of the β-agarase gene

Clones exhibiting agarolytic activities were screened from a genomic DNA library of CY24. One of the positive clones, which harbored a 3.5 kb *Hin*dIII insert, was sequenced. Subcloning identified a single gene responsible for the agarolytic activity. This gene was 1.4 kb in length and was designated *agaA*. The reading frames, theoretical isoelectric point (pI) and molecular weight (MW) were predicted using the DNATools program. Putative prokaryotic signal sequence and the domain architecture were inspected using SignalP Server http://www.einet.net/review/33240-849313/SignalP_server.htm. Database searching was performed with BLAST or PSI-BLAST. Multiple sequence alignment of various *agaA* homologues was performed with ESPript2.2 (Easy Sequencing in PostScript) (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) (Gouet et al. 2003).

Construction of the recombinant plasmid containing β-agarase gene *agaA*

The primers agaAF and agaAR, incorporating an *Nde*I site or an *Xho*I site into the 5′-end, were used to amplify the ORF of *agaA*. After gel purification, the PCR products were digested with *Nde*I and *Xho*I, and ligated into *Nde*I/*Xho*I digested pET-24a(+), yielding a recombinant plasmid pET-agaA, which was then transferred into *E. coli* BL21(DE3).

Point mutations

Site-directed mutagenesis was performed with the Quikchange Site Directed Mutation Kit (Stratagene) according to the manufacturer's instructions. After a sequence check, mutated genes were expressed in *E. coli* BL21(DE3).

Expression and purification of recombinant β-agarase

Transformed *E. coli* BL21(DE3) was grown at 37°C to mid-growth phase in LB medium containing 30 μg kanamycin/ml. The culture temperature was shifted to 25°C and expression of recombinant agarase AgaA was induced with 0.1 mM IPTG. After 16 h, the culture supernatant was harvested and brought to 60% saturation with solid ammonium sulfate. The suspension was stirred gently at 4°C for 1 h and centrifuged at 15,000*g* for 20 min. The precipitate was dissolved in 20 mM phosphate buffer (pH 8.0) containing 2 M NaCl and loaded onto a phenyl

Sepharose high performance column. Proteins were gradient eluted with elution buffer (20 mM phosphate buffer, pH 7.5) stepwise. The active fractions eluted by 30% elution buffer were concentrated by centrifugal ultrafiltration (Centricon-10) (Millipore). Pooled enzyme solution was purified further with Superdex 75 column. The amount of protein was measured using BCA protein assay reagent (Pierce Biotechnology, USA), utilizing bovine serum albumin as the standard protein.

Enzyme activity assay

Agarase activity was determined by the enzymatic production of reducing sugars from agarose (Miller 1959; Ma et al. 2007). Simply, the enzyme was incubated in 20 mM phosphate buffer (pH 6.5) containing 0.25% (w/v) agarose at 40°C for 10 min. The reaction was ended by adding of 3,5-dinitrosalicylic acid and the mixture was then incubated for 10 min in boiling water for color development. After cooling, the absorbance was measured at 520 nm. The amount of reducing sugar liberated was measured using D-galactose as a standard. One unit of enzyme was defined as the amount of protein that liberates 1 μmol reducing sugar per min under these assay conditions.

Analysis of effects of temperature and pH on enzyme activity

The optimal temperature of AgaA was determined in the buffer used in the standard assay condition at temperatures ranging from 10 to 60°C. The pH dependence was assayed at 40°C in the pH range from 4.92 to 8.34 using the sodium phosphate buffer.

Hydrolysis product analysis

The dynamics of hydrolysis of agarose (0.25%) were measured with 0.01 μM AgaA at 40°C. Aliquots were withdrawn and boiled to inactivate the enzyme. Then the solution was freeze-dried and resuspended in distilled water. The final products were subjected to fluorophore-assisted carbohydrate electrophoresis (FACE) (Yu et al. 2002). Simply, oligosaccharides were reductively aminated with monopotassium 7-amino-1,3-napthalenedisulfonic acid (ANDS) and sodium cyanoborohydride. The labeled mixture was electrophoresed through gradient polyacrylamide gel.

The digests of different degree polymerization (DP) of oligosaccharides were spotted on a silica gel 60 aluminium sheet (Merck), and developed with a solvent of *n*-butanol/acetic acid/water (2:1:1, by vol.). The oligosaccharides were identified by spraying the sheet with a modified diphenylamine-aniline reagent followed by heating at 100°C for 5 min (Aoki et al. 1990).

To characterize the cleavage pattern, DP8 and DP10 were fluorescently labeled as described above and purified with a Sephadex G10 column before degradation. Degradation products were analyzed with PAGE.

Accession number of cloned gene

The nucleotide sequence reported in this paper was deposited into the GenBank with an accession number of AY150179.

Results

Cloning and sequence analysis of *agaA*

The *β*-agarase gene from *Pseudoalteromonas* sp. CY24 genomic library was cloned and sequenced as described in materials and methods. The open reading frame of *agaA* consisted of 1,359 nucleotides, encoding a putative protein of 453 amino acid residues, with a predicted molecular weight of 48.4 kDa and isoelectric point of 5.35. The *N*-terminal of the protein functions as a signal peptide for the export of the enzyme. Splicing of the signal peptide may occur between residues 23 and 24. The mature AgaA protein has a catalytic domain of glycosyl hydrolase family 16 (GH16) and a carbohydrate-binding module type 13 (CBM13).

Expression and purification of the recombinant AgaA

Purified recombinant enzyme formed a single protein band of 48 kDa on SDS-PAGE (Fig. 1), which is in

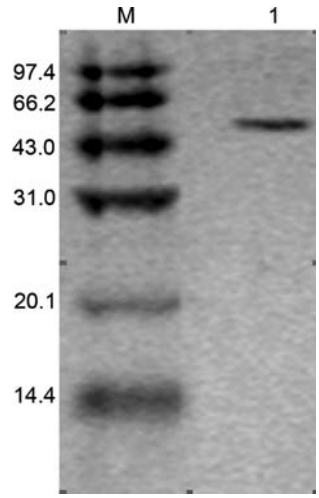

Fig. 1 SDS-PAGE analyzes of the purified recombinant protein AgaA. *Lane M*, low molecular weight standards (in kDa). *Lane 1*, purified AgaA

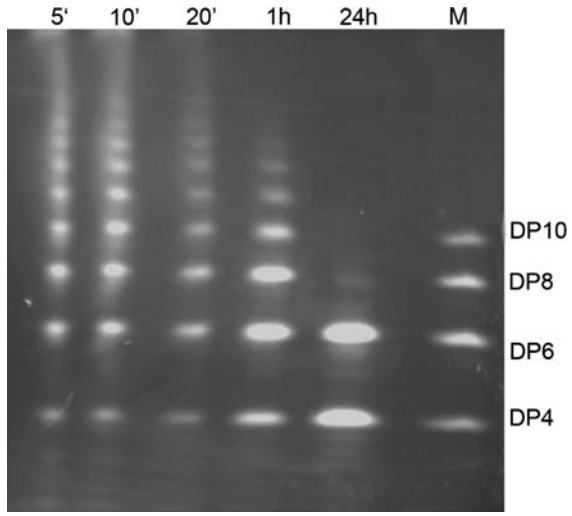

Fig. 2 FACE analysis of the products of agarose hydrolysis with AgaA. Agarose (50 ml, 0.25%) was hydrolyzed by AgaA (0.01 μM) at 40°C. At intervals aliquots from the reaction mixture were taken out and analyzed by the FACE method. The lane marked M was loaded with neoagarotetraose (DP4) and neoagarohexaose (DP6) (Sigma), neoagarooctaose (DP8) and neoagarodecaose (DP10) (prepared from the hydrolysis product of AgaB from *Pseudoalteromonas* sp. CY24)

agreement with that estimated from the DNA sequence. The specific activity of the recombinant β-agarase was 482 U/mg.

Effects of temperature and pH on enzyme activity and stability

The optimum temperature for AgaA was around 40°C and it retained its activity when the temperature was between 4 and 30°C for 1 h. The optimal pH was 6.5 and it was stable in the pH range from 5 to 9 (data not shown).

Hydrolysis product analysis

The products of the enzyme reactions over the course of time were analyzed by FACE (Fig. 2). AgaA displayed an endo-type activity, cleaving the glycosidic linkages in a random manner and giving various DPs at the initial stage. After 1 h of incubation, the polymer size decreased, with a concomitant increase in tetramers and hexamers (DP4 and DP6). After 24 h of incubation, the main products were DP4 and DP6, accounting for (41%) and (43.5%), respectively. The ^{13}C-NMR spectra of DP6 was shown in a previous paper (Li et al. 2007).

The enzyme degraded agarose oligosaccharides comprised of at least eight sugars (Fig. 3) and it cut the fourth and the sixth glycosidic linkage of DP8

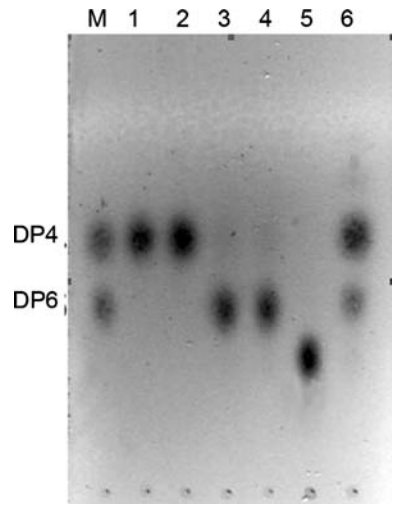

Fig. 3 TLC analysis of the hydrolysis products of neoagaro-oligosaccharides with AgaA. Neoagaro-oligosaccharides at a concentration of 10 mg/ml in 20 mM phosphate buffer (pH 6.5) were hydrolyzed by AgaA at room temperature for 24 h. The enzyme concentration used for DP4 and DP6 was 10 μM and for DP8 was 1 μM. *Lane M*, DP 4 and DP6 as the standards; *Lane 1*, DP4; *Lane 2*, hydrolytic products of DP4; *Lane 3*, DP6; *Lane 4*, hydrolytic products of DP6; *Lane 5*, DP8; *Lane 6*, hydrolytic products of DP8

from the reducing end mainly in a symmetrical mode (Fig. 4).

Point mutations

Sequence comparison of AgaA and its homologues was carried out with ESPript software, using *Zobellia galactanivorans* agarase A whose structure is known (PDB1O4Y) as the template. The result indicated that the amino acids of the catalytic domain have 98% similarity with β-agarase AgaD of *Vibrio* sp. PO-303 (Dong et al. 2007b) (only six residues, 103, 109-112 and 182 are different, Supplementary Fig. 1). In contrast to AgaA, AgaD digested agarose into DP2 and DP4 as the predominant products, and its activity was only 15% of AgaA. Therefore, AgaA was distinct from AgaD.

A series of mutations was introduced by site-directed mutagenesis to evaluate amino acids that confer the great difference between AgaA and AgaD (103, from N to T; 109-112, from VVTS to GCHL; 182, from I to S). The results indicated that none of the replacements changed the substrate cleavage pattern (data not shown), while that at 109–112 significantly decreased the enzyme activity (Table 1).

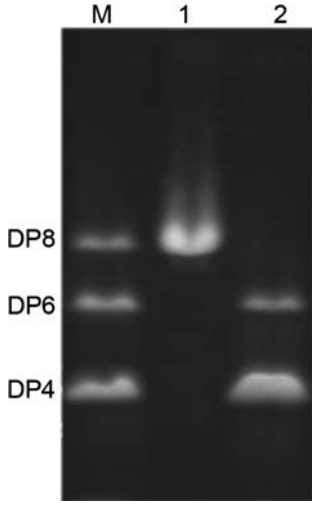

Fig. 4 PAGE analysis of hydrolysis products of fluorescently labeled DP8 with AgaA. *Lane M*, DP 4, 6, and 8 as the standards; *Lane 1*, fluorescently labeled DP8; *Lane 2*, hydrolytic products of fluorescently labeled DP8

Table 1 Effects of mutation sites on the activity of AgaA

Strain	Specific activity (U/mg)
Wild-type	482 (±9)
103 (N → T)	466 (±12)
109–112 (VVTS → GCHL)	36 (±5)
182 (S → I)	475 (±19)

Activity was determined with 1 μM wild type enzyme or mutated enzyme in 20 mM phosphate buffer (pH 6.5) containing 0.25% (w/v) agarose at 40°C for 10 min. Values in parentheses represent the standard deviations of triplicate experiments

Discussion

We have cloned a new β-agarase gene, *agaA*, from the marine strain *Pseudoalteromonas* sp. CY24. The recombinant β-agarase hydrolyzed agarose into DP4 and DP46 (nearly equal amounts), but did not cleave DP4 or DP6. In contrast, most agarases of GH16 hydrolyzing agar and agarose frequently generate DP2, or DP4 as the main products (Ohta et al. 2004; Jam et al. 2005; Dong et al. 2007a, b).

Oligomeric saccharides were used to investigate the nature of the binding site. Unlike agarases A and B from *Zobellia galactanivorans*, which had eight substrate binding sites but fragmented DP8 only into tetrasaccharides, AgaA cleaved the fourth and the sixth glycosidic bonds of the smallest substrate DP8 from the reducing end and produced DP2, DP4, DP6 as the final products. According to the final AgaA-hydrolysis products of agarose and the AgaA-cleavage sites of DP8, we presumed that there were six key substrate binding sites and four additional sites in the catalytic cleft. The six key sites were positioned from -2 to $+4$ and the four additional sites from -4 to -3 and from $+5$ to $+6$, respectively, following the subsite naming convention (Davies et al. 1997). To these sites, substrate DP10 may have at least three binding patterns with the most probable one being the -4 to $+6$ subsites. This has been confirmed in this study (Supplementary Fig. 2). These properties of AgaA distinguish it from other members of GH16.

Amino acid replacement identified residues 109–112 on the β8 strand are crucial for enzyme activity. We presume that these sites might contribute to the substrate binding and we will use AgaD to verify these presumptions. Altogether, our results indicated that AgaA is a new agarase.

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