Short-form paper:

Cloning, expression, and characterization of a new *β*-agarase from *Vibrio* sp. CN41

Running title: A novel *β*-agarase from *Vibrio* sp. CN41

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Abstract

A new agarase, Aga41A, cloned from *Vibrio* sp. CN41, consisted of 990 amino acids with only 49% amino acid sequence identity with known *β*-agarases. Aga41A belongs to the GH50 family, but yields neoagarotetraose as the end product. Aga41A was expressed and characterized.

Main text

Agarase is a key enzyme that allows certain bacteria to degrade agar, and also an important enzyme for a variety of applications, e.g., in food and cosmetics (6, 10). According to their cleavage pattern, agarases are classified into *α*- and *β*-agarases, which hydrolyze *α*-1,3 and *β*-1,4 linkages of agarose, respectively (3). Meanwhile, agarases are grouped into GH (glycoside hydrolase) families based on their amino acid sequence identities (8), i.e., GH16, GH50, and GH86 families identified as *β*-agarases, and the GH96 family identified as *α*-agarases. Agarases from different families have various catalytic domains and cleavage modes of agarose, and hence different end products. The known GH50 agarases produce generally DP2 (neoagarobiose), or DP2 and DP4 (neoagarotetraose). Typically, the GH16 agarases yield DP4 as the main end product, while the GH86 agarases produce DP8 (neoagarooctaose) and DP6 (neoagarohexaose) (4).

Currently, approximately 40 agarases have been characterized and deposited in the CAZy database (4). The majority are GH16, while a few are GH50 and GH86 agarases. Almost all agarase genes were previously cloned by constructing genomic libraries (e.g., 5, 11, 12, 15) or sequencing purified proteins followed by genomic DNA library screening or degenerate PCR

(e.g., 2). Our goal was to find new agarases for industrial application using simpler approaches. Here we report the cloning of a novel GH50 agarase from *Vibrio* sp. CN41 using homology-based cloning followed by SiteFinding PCR (18), a chromosome walking method to amplify the flanking sequences.

A *Vibrio* strain, CN41, which formed depressions on agar plate surfaces, was isolated from Zhejiang coastal sediments at the East China Sea. The 16S rRNA gene (GenBank number: HM536934) amplified from genomic DNA of *Vibrio* strain CN41 using primers 27F and 1492R (1), had 99% identity with *Vibrio azureus* strain LC2-005^{T}.

The amino acid sequences of agarases from the CAZy database were aligned using the program ClustalW (19) to find conserved sequences for homology cloning. A phylogenetic tree (not shown) was constructed by MEGA 4.0 using the Maximum Parsimony algorithm (17). Agarases clustered into distinct families revealed by the phylogenetic tree. Block Maker (7) was used to find conserved protein blocks in the GH16, GH50, and GH86 families. Degenerate primers for conserved protein blocks were then designed for each family using the program COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) (14) to assess which families occurred in *Vibrio* strain CN41. Only one fragment was amplified with the GH50 primers (Table S1) and sequenced, confirming it is in the GH50 family. SiteFinding PCR (18) was used to amplify the flanking sequences with six nested gene-specific primers (GSP1-6, Table S1). Specific products were amplified for both upstream and downstream sequences after a single SiteFinding cycle and two rounds of nested PCR. The complete coding region of the agarase gene (henceforth referred to as *aga*41A) was assembled and analyzed by the software DNAMAN (Lynnon Corp.). The gene *aga*41A contained 2973 bp,

encoding 990 amino acids with a putative signal peptide of 42 amino acid residues at the N-terminus and a GH50 family catalytic module at the C-terminus. The agarase Aga41A has greatest amino acid sequence identity (49%) with two agarases from *Vibrio* sp. JT0107 and *Agarivorans* sp. QM38 (Fig. 1). The two most similar GH50 agarases have not been characterized, although they have been sequenced and expressed (9, 16). Therefore, information on their biochemical properties is not available for reference and comparison with Aga41A.

To characterize the Aga41A agarase, the *aga*41A gene was cloned into the plasmid pET-28b (+) and transformed into *E.coli* BL21 (DE3) for expression, which was induced by 1 mM of IPTG at 30ºC for 7 h. A soluble protein containing Aga41A and histidine tag was expressed and purified 13.2-fold by the Ni-NTA affinity chromatography (QIAGEN), with a yield of 64 mg/L of culture broth. The purified recombinant Aga41A ran as a single band on an SDS-PAGE gel (Fig. S1), corresponding to the predicted molecular weight of ~110 kDa.

In general, the biochemical properties of Aga41A correspond to those of GH50 agarases. Agarase activity was assayed by the DNS (3,5-dinitrosalicylic acid) method (13) with heat-inactivated enzyme used as a negative control. One unit of enzymatic activity was defined as the amount of protein required to produce 1 μmol of reducing sugars from agarose per minute under standard assay conditions. Standard assay conditions for enzyme activity were as follows: 10 μl of enzyme (0.05 mg/ml) incubated in 1 ml of NTA-0 buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5) containing 0.25% agarose (w/v) for 30 min at 40ºC. Results showed that Aga41A was most stable below 40ºC and from pH 7.0 to 8.6, with the maximum activity at ~40ºC at pH 7.5 under the conditions used. The K_m and V_{max}, calculated from

Lineweaver-Burk double reciprocal plot for agarose, were 3.54 mg/ml and 3 U/mg, respectively.

However, Aga41A produced DP4 as the only end product from agarose (characterized as below), which is uncommon in the GH50 agarases. Agarose hydrolysates were investigated over 36 h by thin layer chromatography (TLC) (Fig. 2A). DP8, DP6, and DP4 were produced sequentially over the time course (Fig. 2A). DP4 was the final product, further confirmed by mass spectrometry which indicated a molecular mass of 653 Da, the molecular weight of DP4 (630 Da) plus Na (23 Da). Therefore, Aga41A is an endo-hydrolytic agarase and cleaves agarose, DP8, and DP6 to produce DP4 as the final product, but does not digest DP4 further (Fig. 2B).

In conclusion, Aga41A belongs to the GH50 family based on amino acid sequence identity, but produces an uncommon end product (DP4). This novel agarase provides a chance to investigate the cleavage mode of agarases in different families. Aga41A has the potential in industrial applications to allow production of the oligosaccharide, DP4, without the need to purify it from mixed end products.

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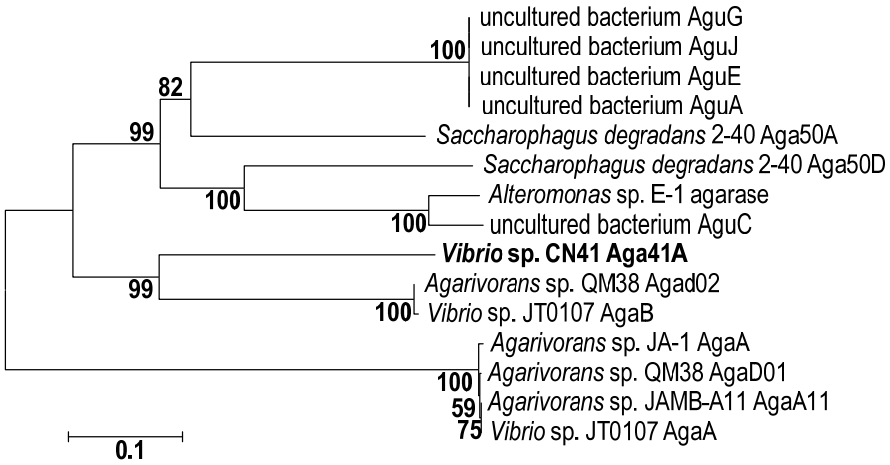
Figure legends

Fig. 1. Phylogenetic relationship of GH50 agarases based on inferred amino acid sequences. The tree was constructed using MEGA 4.0 with the Neighbor-Joining algorithm and 1,000 bootstrap replicates. Scale bar indicates 0.1 substitution per site.

Fig. 2. TLC analysis of recombinant Aga41A reaction products. (A) Reactions were performed at 40°C in NTA-0 buffer containing 1% agarose and sampled at the following time intervals: 0.5, 1, 2, 12, 24, and 36 h. (B) Reactions were performed under the standard assay conditions using different substrates: DP8 (lane 1), DP6 (lane 2) and DP4 (lane 3). Gal, galactose; Std, standard; DP4, neoagarotetraose; DP6, neoagarohexaose; DP8, neoagarooctaose.



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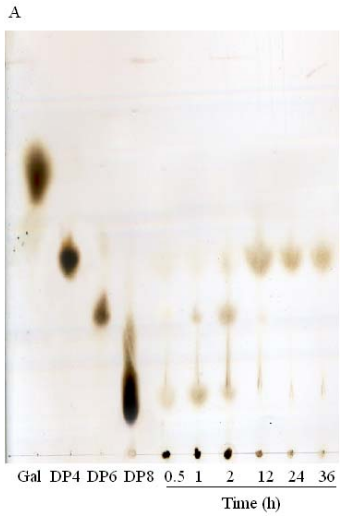

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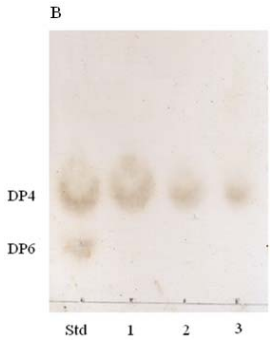

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