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Cloning, Expression, and Characterization of a New β-Agarase from *Vibrio* sp. Strain CN41▽†

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A new agarase, $AgaA_{CN41}$, cloned from *Vibrio* sp. strain CN41, consists of 990 amino acids, with only 49% amino acid sequence identity with known β-agarases. $AgaA_{CN41}$ belongs to the GH50 (glycoside hydrolase 50) family but yields neoagarotetraose as the end product. $AgaA_{CN41}$ was expressed and characterized.

Agarase is a key enzyme that allows certain bacteria to degrade agar and is also an important enzyme for a variety of applications, e.g., in food and cosmetics (6, 9). 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). Agarases are also grouped into glycoside hydrolase (GH) families based on their amino acid sequence identities (8), e.g., GH16, GH50, and GH86 families are identified as β-agarases and the GH96 family is identified as α-agarases. Agarases from different families have various catalytic domains and cleavage modes for 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 agarases, while a few are GH50 and GH86 agarases. Almost all agarase genes were previously cloned by constructing genomic libraries (see, e.g., references 5, 10, 11, and 14) or sequencing purified proteins followed by genomic DNA library screening or degenerate PCR (see, e.g., reference 2). Our goal was to find new agarases for industrial application by using simpler approaches. Here, we report the cloning of a novel GH50 agarase from *Vibrio* sp. strain CN41 by using homology-based cloning followed by SiteFinding PCR (17), 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 amplified from genomic DNA of *Vibrio* sp. strain CN41 by 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 (18) to find conserved sequences for homology cloning. A phylogenetic tree (not shown) was constructed by MEGA 4.0, using the maximum-parsimony algorithm (16). 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 by using the program COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) (13) to assess which families occurred in *Vibrio* sp. strain CN41. Only one fragment was amplified with the GH50 primers (see Table S1 in the supplemental material) and sequenced, confirming it is in the GH50 family. SiteFinding PCR (17) was used to amplify the flanking sequences with six nested gene-specific primers (GSP1 to GSP6; 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 $agaA_{CN41}$) was assembled and analyzed by the software DNAMAN (Lynnon Corp.). The gene $agaA_{CN41}$ contained 2,973 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 $AgaA_{CN41}$ has greatest amino acid sequence identity (49%) with two agarases from *Vibrio* sp. strain JT0107 and *Agarivorans* sp. strain QM38 (Fig. 1). The two most similar GH50 agarases have not been characterized, although they have been sequenced and expressed (15, 19). Therefore, information on their biochemical properties is not available for reference and comparison with $AgaA_{CN41}$.

To characterize the $AgaA_{CN41}$ agarase, the $agaA_{CN41}$ gene was cloned into the plasmid pET-28b(+) and transformed into *Escherichia coli* BL21(DE3) for expression, which was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 30°C for 7 h. A soluble protein containing $AgaA_{CN41}$ and a histidine

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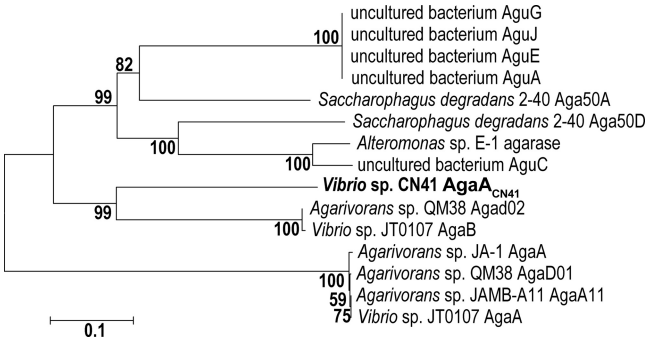

FIG. 1. Phylogenetic relationship of GH50 agarases based on inferred amino acid sequences. The tree was constructed using MEGA 4.0 with the neighbor-joining algorithm and 1,000 bootstrap replicates. The scale bar indicates 0.1 substitutions per site.

tag was expressed and purified 13.2-fold by Ni-nitrilotriacetic acid (NTA) affinity chromatography (Qiagen), with a yield of 64 mg/liter of culture broth. The purified recombinant AgaA$_{CN41}$ ran as a single band on an SDS-PAGE gel (see Fig. S1 in the supplemental material), corresponding to the predicted molecular mass of ~110 kDa.

In general, the biochemical properties of AgaA$_{CN41}$ correspond to those of GH50 agarases. Agarase activity was assayed by the DNS (3,5-dinitrosalicylic acid) method (12), 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 (wt/vol) for 30 min at 40°C. Results showed that AgaA$_{CN41}$ was most stable below 40°C and from pH 7.0 to 8.6, with the maximum activity at ~40°C 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, AgaA$_{CN41}$ produced DP4 as the only end product from agarose (characterized as described 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 mass of DP4 (630 Da) plus Na (23 Da). Therefore, AgaA$_{CN41}$ is an endohydrolytic agarase and cleaves agarose, DP8, and DP6 to produce DP4 as the final product but does not digest DP4 further (Fig. 2B).

In conclusion, AgaA$_{CN41}$ 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. AgaA$_{CN41}$ has the potential in industrial applications to allow production of the oligosaccharide DP4 without the need to purify it from mixed end products.

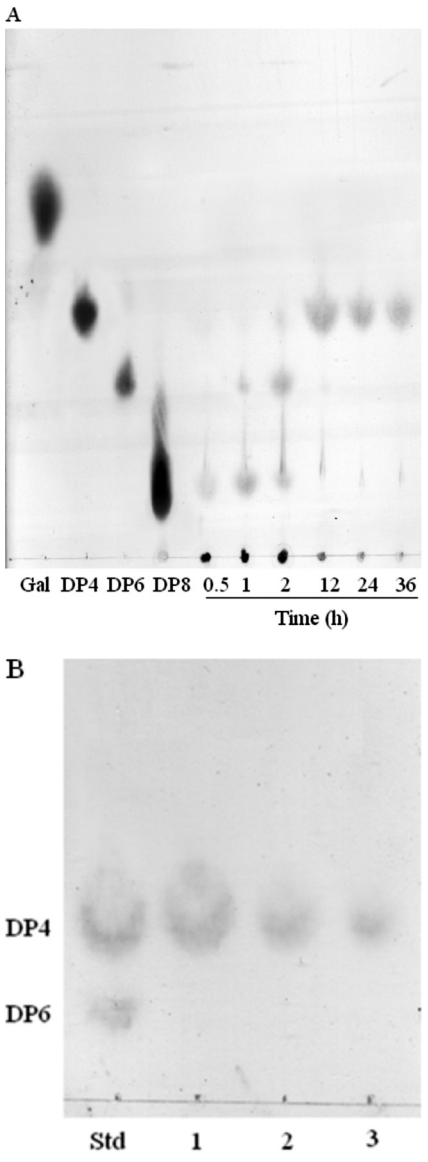

FIG. 2. TLC analysis of recombinant AgaA$_{CN41}$ reaction products. (A) Reactions were performed at 40°C in NTA-0 buffer containing 1% agarose and sampled at the following time intervals: 0.5, 1, 2, 12, 24, and 36 h. (B) Reactions were performed under the standard assay conditions using different substrates, namely, DP8 (lane 1), DP6 (lane 2), and DP4 (lane 3). Gal, galactose; Std, standard; DP4, neoagarotetraose; DP6, neoagarohexaose; DP8, neoagarooctaose.

Nucleotide sequence accession number. The 16S rRNA gene and *agaA*$_{CN41}$ amplified from genomic DNA of *Vibrio* sp. strain CN41 were deposited in GenBank under accession numbers HM536934 and HM563685, respectively.

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