

Molecular Cloning and Characterization of a Novel β -Agarase, AgaB, from Marine *Pseudoalteromonas* sp. CY24*

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Agarases are generally classified into glycoside hydrolase families 16, 50, and 86 and are found to degrade agarose to frequently generate neoagarobiose, neoagarotetraose, or neoagarohexaose as the main products. In this study we have cloned a novel endo-type β -agarase gene, *agaB*, from marine *Pseudoalteromonas* sp. CY24. The novel agarase encoded by *agaB* gene has no significant sequence similarity with any known proteins including all glycoside hydrolases. It degrades agarose to generate neoagarooctaose and neoagarodecaose as the main end products. Based on the analyses of enzymatic kinetics and degradation patterns of different oligosaccharides, the agarase AgaB appears to have a large substrate binding cleft that accommodates 12 sugar units, with 8 sugar units toward the reducing end spanning subsites +1 to +8 and 4 sugar units toward the non-reducing end spanning subsites –4 to –1, and enzymatic cleavage taking place between subsites –1 and +1. In addition, ¹H NMR analysis shows that this enzyme hydrolyzes the glycosidic bond with inversion of anomeric configuration, in contrast to other known agarases that are retaining. Altogether, AgaB is structurally and functionally different from other known agarases and appears to represent a new family of glycoside hydrolase.

Agar, usually obtained from the species of marine red algae, *Gelidium* and *Gracilaria*, is composed of agarose and agar-pectin (1). Agarose is a linear polymer of alternatively arranged 3-O-linked β -D-galactopyranose and 4-O-linked 3,6-anhydro- α -L-galactopyranose. Agar-pectin has the same basic framework but contains substituent groups such as sulfate, pyruvate, and methoxyl (2). In agarose, the α (1→4)-linked units exhibit 3,6-anhydro bridges upon the disorder-order conformational transition that stabilize the molecules into double helices, leading to the formation of pseudocrystalline fibers made of aggregates of double-stranded agarose chains (3). Carrageenans, the other category of red algal gel-forming polysaccha-

rides from Gigartinales and Solieriales, differ from agars in the D-configuration of the (1→4)-linked galactose residues and in the density of ester-sulfate substituents per digalactose repeating unit (one in κ -carrageenan, two in ι -carrageenan and three in λ -carrageenan). Agarases are glycoside hydrolases (GHs)² that hydrolyze agarose. They are grouped into α and β types that hydrolyze α -1,3 linkages and β -1,4 linkages, respectively (4). Agarases have been isolated from several bacteria genera, including *Cytophaga* (5), *Pseudomonas* (6–9), *Pseudoalteromonas* (10), *Zobellia* (11), *Streptomyces* (12), *Alteromonas* (13), *Vibrio* (14, 15), *Microscilla* (16), *Microbulbifer* (17), and *Aeromonas*. Most known agarases are β -agarases, with the exception of two α -agarases found from *Alteromonas agarlyticus* GJ1B (18, 19) and *Thalassomonas* sp. JAMB-A33 (20). More frequently, β -agarases hydrolyze agarose yielding neoagarobiose, neoagarotetraose, or neoagarohexaose as the main products (17, 21–23). Agarases have potential applications in the food, cosmetic, and medical industries for the production of oligosaccharides from agar or agarose (24, 25). Moreover, agarases can be used to degrade the cell walls of marine algae for the preparation of protoplasts and for the extraction of labile substances with biological activities (26).

Glycoside hydrolases are a widespread group of enzymes displaying a great variety of protein folds and substrate specificities. They function via general acid/base catalysis mediated by two major mechanisms, either an overall retention or an inversion of anomeric configuration (27). “Retaining” enzymes utilize a double-displacement mechanism involving a covalent glycosyl-enzyme intermediate, whereas “inverting” enzymes utilize a single-displacement reaction. In 1991 a classification of glycoside hydrolase was proposed on the basis of similarities in sequence (28). A sequence-based family contains glycoside hydrolases folding in the same pattern and functioning with the identical mechanism. Furthermore, the evolutionarily, structurally, and mechanistically related families were grouped together in higher hierarchical level termed “clans” (29). Currently, more than 100 families and 14 clans have been reported with details available at CAZy website. The known β -agarases are grouped into glycoside hydrolase families 16, 50, and 86, and family GH 16 is the most abundant with the β -agarases. Members of families GH 50 and 86 in clan GH-A share the same structure of (β/α)₈ barrel in which the acid/base and the nucleophilic residues are located at the COOH-terminal end of

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² The abbreviations used are: GH, glycoside hydrolase; FACE, fluorophore-assisted carbohydrate electrophoresis; kbp, kilobase pair(s); DP, degree of polymerization.

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strands β_4 and β_7 , respectively (30, 31). Members of family GH 16 in clan GH-B display the general β -jelly roll topology with two predominantly parallel or antiparallel β -sheets forming a long open substrate binding cleft, and two catalytic residues are located in the cleft (11).

We reported here the cloning, expression, purification, and characterization of a novel β -agarase AgaB from *Pseudoalteromonas* sp. CY24. We determined also the relationship between the structure of β -agarase AgaB and its enzymatic activity by analyzing the enzymatic kinetics and the degradation products of different unlabeled and labeled oligosaccharide substrates. Altogether, AgaB is structurally and functionally different from other known agarases and appears as the first representative of a novel family of glycoside hydrolases.

EXPERIMENTAL PROCEDURES

Unless otherwise stated, all chemicals used were of analytical grade or higher. Enzymes were purchased from TaKaRa (Dalian, China). All DNA manipulations and *Escherichia coli* transformations were performed using standard procedures as described previously (32).

Bacterial Strains, Plasmids, and Culture Conditions—*E. coli* DH5 α harboring pBluescript II KS(+) was cultured at 37 °C in Luria-Bertani (LB) medium containing 50 μ g/ml ampicillin. *E. coli* BL21 (DE3) containing pET-24a(+)-*agaB* was incubated at 37 °C in LB medium supplemented with 30 μ g/ml kanamycin. The agarase-producing bacterial strain was cultured using an optimal medium consisting of 2.5% NaCl, 0.25% casein, 0.5% MgSO₄·7H₂O, 0.1% KCl, 0.02% CaCl₂, 0.06% NaH₂PO₄, 0.002% FeSO₄·7H₂O, and 1.5% agar (or 0.1% for liquid medium).

Isolation and Identification of Strain CY24—Strain CY24 was isolated from seawater collected from the coastal sea areas of Qingdao, China. Diluted seawater was plated on solid medium containing agar as the sole carbon source as described above and incubated at 25 °C for 48 h. Pit or clear zone-forming colonies were picked out and purified under the same conditions. Morphological, physiological, and biochemical characteristics and 16 S rRNA phylogenetic analysis were used to identify the isolated strain to genus (33, 34).

Enzyme Activity Assaying—The activity of agarase was assayed using 3,5-dinitrosalicylic acid method (35). An appropriately diluted enzyme solution was incubated at 40 °C in 20 mM sodium phosphate buffer (pH 6.0) containing 1 ml of 0.25% (w/v) agarose. Ten minutes later 750 μ l of 3,5-dinitrosalicylic acid was added, and reaction mixture was boiled for 10 min before adding 5 ml of H₂O. The absorbance of reducing sugar was measured at 520 nm and compared with the standard curve of D-galactose. One unit of enzyme was defined as a 1- μ mol increment/min in the reducing end.

Cloning of the *agaB* Gene—Genomic DNA of strain CY24 was isolated using WizardTM Genomic DNA extraction kit (Promega) following the manufacturer's instructions. The genomic DNA was digested with restriction endonuclease PstI and fractionated through 1.0% agarose gel electrophoresis. The fragments ranging from 5.0 to 12.0 kbp in length were retrieved using Qiagen gel extraction kit, ligated into pBluescript II KS(+), and transferred into *E. coli* DH5 α , forming a genomic DNA library. Clones expressing agarase activity were screened

out when they functioned to generate clear zones after incubation at 25 °C for 48–72 h. Six agarase-producing clones were identified in about 8000 transformants that were named pBA1–pBA6. The inserts of positive clones were subjected to single and double digestion with various restriction endonucleases with their physical maps constructed after agarose gel electrophoresis. The inserts of positive clones ranged from 6.7 to 10.3 kbp in length and shared a common PstI fragment of 6.7 kbp. To localize the boundary of agarase-encoding gene, the 6.7-kbp PstI insert of pBA1 was sequenced and submitted to double digestion with appropriate restriction enzymes resulting in four independent subclones, namely pBAS1–pBAS4. Subclone pBAS4 with a 3.2-kbp BamHI-KpnI DNA fragment was found to possess agarase activity and contain a complete open reading frame.

Sequence Analysis and Classification of *agaB* Gene—Sequence analysis of *agaB* gene was performed using PredictProtein software, ExPASy Server, and SignalP 3.0 Server. Representative sequences of each glycoside hydrolase family were extracted from the Swiss-Prot or GenBankTM sequence data banks or entered manually from the literature. Some of these sequences were edited to separate their multiple constitutive catalytic domains. Sequence comparisons were conducted with BLAST program and the hydrophobic cluster analysis.

Expression of Recombinant AgaB—The *agaB* gene, including its signal sequence, was PCR-amplified from subclone pBAS4 using the sense primer 5'-GGAATTCATATGTTAAAGCGCCACCAAGC-3' and the antisense primer 5'-CCGCTCGAGCTATTGGCAAGTATAACCT-3' containing NdeI and XhoI sites (underlined), respectively. The PCR product was purified and digested by NdeI and XhoI and cloned into the pET-24a(+) expression vector linearized with the same restriction enzymes. The resulting pET-24a-*agaB* was transformed into *E. coli* BL21 (DE3) competent cells. The *E. coli* BL21 (DE3) cells carrying the agarase gene (pET-24a-*agaB*) were grown at 37 °C in LB medium containing 30 μ g/ml kanamycin sulfate until the cell density reached 0.5 A₆₀₀. At this point a final concentration of 0.1 mM isopropylthio- β -galactopyranoside was added. The cultivation was continued further at 25 °C and 250 rpm for 18–24 h. *E. coli* BL21 (DE3) bearing pET-24a(+) was used as the negative control.

Purification of Recombinant AgaB—The purification of recombinant AgaB was carried out at temperatures below 4 °C. Cell and medium were separated by centrifugation at 10,000 \times g for 10 min. The supernatant was brought to 60% (w/v) saturation with solid ammonium sulfate and centrifuged again. The protein in pellet was dissolved at 4 °C overnight, ultra-filtrated, and desalted. The protein was purified further by loading on a DEAE-Sepharose Fast Flow column (Amersham Biosciences) and washing with 20 mM phosphate buffer (pH 6.5) and eluting with 2 M NaCl in 20 mM phosphate buffer (pH 7.5). The elution fractions with agarase activity were then subjected to purification through a phenyl-Sepharose high performance column (Amersham Biosciences) equilibrated with 20 mM sodium phosphate buffer (pH 7.5) containing 2 M NaCl. The column was washed with equilibration buffer, and the protein was eluted using 20 mM phosphate buffer (pH 6.5) directly. Finally, the fractions with agarase activity were loaded on a Superdex75

column (30 \times 1 cm, Amersham Biosciences) equilibrated ahead of use, and the enzyme was eluted with 0.2 M NaCl in 20 mM phosphate buffer (pH 6.5). SDS-PAGE was performed to detect the purified enzyme as described by Laemmli (36).

Biochemical Characterization of AgaB—The optimum temperature of AgaB was determined by monitoring enzymatic activity at temperatures ranging from 10 to 60 °C and pH 6.0 for 10 min. The thermostability of AgaB was evaluated by measuring the residual activity of the enzyme after incubation at different temperature for 1 h. The pH dependence of AgaB was assayed at 40 °C in a pH range from 5.7 to 7.0. The effect of pH on AgaB stability was determined by measuring its residual activity after incubation at 4 °C and various pH values (4.0–10.6) for 1 h. The effects of various metal ions and chelators on AgaB activity were examined by determining the activity in the presence of 1 mM of various ions or chelators. In addition, the substrate speciality of AgaB was detected at 40 °C using 0.25% agar, agarose, κ -carrageenan, ι -carrageenan, λ -carrageenan, alginate, and chitosan as the substrates.

Hydrolysis Products Analysis of AgaB—The dynamics of hydrolysis of agarose (0.25%) with AgaB (0.01 μ M) at 40 °C was traced for up to 24 h. An aliquot of hydrolysis product was taken out at different times and analyzed using fluorophore-assisted carbohydrate electrophoresis (FACE) (37).

To identify the structure of final hydrolysis products, oligosaccharides with different degree of polymerization (DP) were purified from a hydrolytic mixture of agarose by AgaB. Agarose was fully hydrolyzed, and the reaction mixture was centrifuged and concentrated by rotary evaporation. The concentrated mixture was loaded on a Bio-Gel P6 column (Bio-Rad), equilibrated ahead of use, and eluted with 0.5 M NH_4HCO_3 . The collected fractions were rotary-evaporated repeatedly to eliminate NH_4HCO_3 and dried under a vacuum. For ^{13}C NMR analysis the purified neoagarooctaose (DP8), neoagarodecaose (DP10), neoagarododecaose (DP12), and neoagarotetradecaose (DP14) (10 mg each) were dissolved in 1 ml of 99.9% D_2O and lyophilized. Such operation was repeated three times to replace all exchangeable protons. The spectra were recoded by a JNM-ECP600 (JEOL) spectrometer, with 2,2-dimethyl-2-silapentane-5-sulfonic acid as the internal standard. A matrix-assisted laser desorption ionization time-of-flight mass spectrum was performed on BiflexIII (Bruker Daltonics, Inc., Billerica, MA) operating in positive-ion mode with 2,5-dihydroxybenzoic acid as a matrix. All detected oligosaccharide ions were observed as pseudomolecular ions $[\text{M}+\text{Na}]^+$ and $[\text{M}+\text{K}]^+$.

Substrate Binding Subsites of AgaB—The kinetic parameters for oligosaccharides were determined at 25 °C in 20 mM sodium phosphate buffer (pH 6.0). Substrate concentration was varied in the range 5–40 mg/ml, and the reaction products were quantified by high performance anion exchange chromatography with pulsed amperometric detection (Dionex) as described previously (11, 38). All values were determined in triplicate. The Michaelis constant (K_m) and the turnover number (K_{cat}) were determined by a nonlinear regression program.

To determine the smallest substrate of AgaB and the number of substrate binding subsites in its catalytic cleft, a hydrolysis reaction was carried out using 10 mg/ml different oligosaccharides in 10 μ l of reaction mixture (pH 6.0). According to the

different DP of oligosaccharides, the reaction mixture was adjusted with different concentrations of AgaB and incubated at 25 °C for different times. Hydrolysis product was then analyzed using FACE.

To determine the direction and location of the substrate in the active site of AgaB, the reducing end of DPs 14, 12, 10, and 8 were fluorescently labeled using monopotassium 7-amino-1,3-naphthalenedisulfonic acid and sodium cyanoborohydride reagents. The labeled products were purified by Sephadex G10 column and then degraded by AgaB. The degradation product was subjected to gradient (18–25%) discontinuous PAGE using a vertical slab gel electrophoresis system (Amersham Biosciences).

^1H NMR Spectroscopy Analysis—For ^1H NMR spectroscopy analyses, agarose powder (0.5 g) was melted in 50 ml of D_2O (containing 2,2-dimethyl-2-silapentane-5-sulfonic acid) and allowed to cool down to 40 °C. After recording the spectrum of the substrate, AgaB was added at a final concentration of 2.4 nM. Aliquots were taken out every 10 min for 2 h, then at 18 h after the addition of the enzyme (*i.e.* when enzymic hydrolysis was complete and the mutarotation equilibrium had been reached) and directly recorded at 60 °C with a JNM-ECP600 spectrometer. NMR signals were assigned according to Jam *et al.* (39) and Lahaye (40) and based on a COSY (correlation spectroscopy) spectrum of neoagarooligosaccharides.

RESULTS

Isolation and Identification of Strain CY24—Based on sole carbon source culture and clearing zone screening on agar plates, 50 agarase-producing bacterial strains were obtained from seawater. Of them, the one (CY24) showing the highest activity was selected for the isolation of agarase-encoding gene. This strain was assigned to the genus *Pseudoalteromonas* based on its morphological, physiological, and biochemical characteristics and its 16 S rRNA gene sequence (data not shown) and named as *Pseudoalteromonas* sp. CY24.

Cloning and Sequence Analysis of agaB Gene—As described under “Experimental Procedures,” the agarase-positive subclone pBAS4 was obtained which contained a single open reading frame, 1437 bp in length. This open reading frame was named *agaB* gene and deposited in the GenBankTM under accession number AY293310. A putative promoter sequence, 5'-TTGGCA-3' for the -35 region and 5'-TATAGT-3' for the -10 region, is present 150 bp upstream from the initiation codon with 15-bp spacing. The putative ribosome-binding site, 5'-AAGGAG-3', is located 9 bp upstream from the initiation codon ATG. A typical stem loop sequence downstream the stop codon TAG was found as a ρ -independent transcriptional termination site. The predicted product of *agaB* gene is a protein of 478 amino acids with a theoretical molecular mass of 50.9 kDa. The amino acid residue composition of AgaB is not average. The four amino acids (Ala, Asn, Ser, and Val) account for 41.4%, and the other three (Cys, Met, and Trp) account for only 0.8% of the total. As PredictProtein software predicted, AgaB is a globular protein and has strong solvent accessibility composition. Hydrophobic cluster analysis suggested that a strong helical transmembrane motif located at the region of amino acid 20–38. Using SignalP 3.0 Server predicted that the most

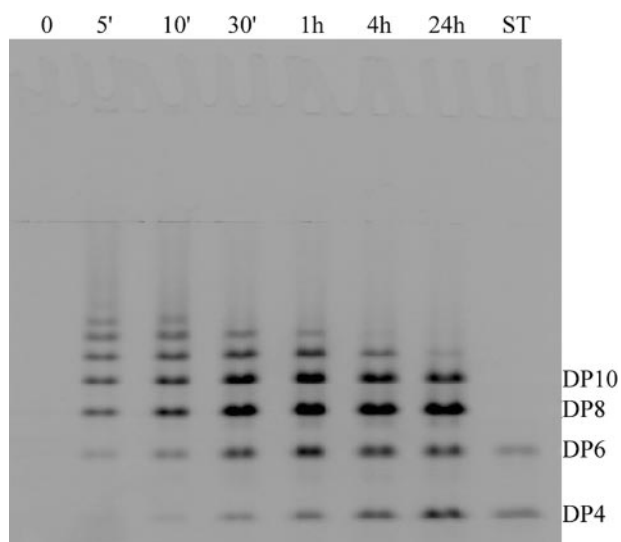


FIGURE 1. FACE analysis of the products of agarose hydrolysis by AgaB. Agarose (50 ml, 0.25%) was hydrolyzed by AgaB (0.01 μ M) at 40 °C. At intervals aliquots from the reaction mixture were taken out and analyzed by FACE method. The lane marked ST was loaded with neoagarotetraose (DP4) and neoagarohexaose (DP6) (Sigma) as the standard. After hydrolysis for 24 h, the main products were neoagarooctaose (DP8) and neoagarodecaose (DP10) with lesser amounts of DPs 4 and 6.

probable cleavage site of signal peptide was between Ala³⁸ and Ala³⁹. ExPASy server calculated the molecular mass and isoelectric point of the mature enzyme deduced from its amino acid sequence were 47.2 kDa and 4.83 respectively.

Sequence Comparison and Classification of *agaB* Gene—BLAST results indicated that the similarity of the deduced amino acid sequence of *agaB* gene with the existed proteins was very low ($E > 0.1$). The sequence was further compared by hydrophobic cluster analysis plots because of its established effectiveness in the comparison of widely divergent proteins. However, the hydrophobic cluster analysis result showed that AgaB has no similarity at all with glycoside hydrolases or with carbohydrate binding modules. To test the reliability of our analysis and to possibly detect other agarase sequences sharing similarity with the protein, the signature motifs of families GH 16, 50, and 86 were undertaken, but no consensus signature was found in AgaB. All these findings confirmed that AgaB is not obviously similar to any previously defined glycoside hydrolases.

Expression and Purification of AgaB—Subclone pBAS4 carrying *agaB* gene showed very low extracellular agarase activity. To obtain a sufficient amount of the enzyme, the *agaB* gene was expressed in the pET24a(+)/*E. coli* BL21 (DE3) system. The recombinant AgaB was secreted into medium under the control of its own signal peptide. The maximal extracellular production of AgaB was induced by 0.1 mM isopropylthio- β -galactopyranoside at 25 °C for 18–24 h. The recombinant AgaB was purified 476-fold by ammonium sulfate precipitation, weak anion-exchange chromatography, hydrophobic interaction chromatography, and gel filtration chromatography. The specific activity of 5000 units mg^{-1} and a final yield of 21.2% were achieved. Approximately 3.4 mg of recombinant enzyme was obtained per liter of culture supernatant. The purified enzyme gave a single band with an apparent molecular mass of 50.4 kDa

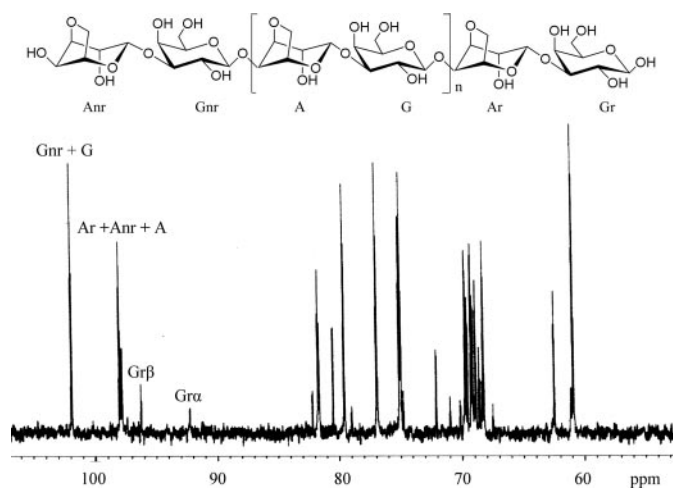


FIGURE 2. The ^{13}C NMR spectrum of the neoagarooctaose (DP8) purified from the hydrolyzed products of agarose by AgaB. The upper trace is the structure of agarose. Peak assignments are labeled according to the nomenclature defined in upper panel. A and G refer to the anhydrogalactose and galactose residues, *nr* and *r* refer to the non-reducing and reducing ends of agarose oligomers, and *n* refers to the number of disaccharide repeating units.

on SDS-PAGE. This value is in good agreement with the value estimated from the deduced amino acid sequence of mature enzyme.

General Properties of AgaB—AgaB was a true agarase. The pure AgaB hydrolyzed agarose but not κ -carrageenan, ι -carrageenan, λ -carrageenan, alginate, and chitosan. The optimal temperature was found to be 40 °C. The activity of AgaB was stable at low temperature and retained more than 90% activity until 35 °C. Its optimum pH was 6.0. AgaB was very stable at pH 5.7–10.6, retaining more than 95% activity. AgaB activity was inhibited completely by Hg^{2+} and slightly by Cu^{2+} , Mn^{2+} , and EDTA. In contrast, Ca^{2+} and Ba^{2+} slightly increased the activity.

Hydrolysis Product Analysis of Agarose by AgaB—The hydrolysis of agarose by AgaB was accompanied by a rapid fall in specific viscosity and a marked increase in reducing power. As shown in Fig. 1, in the initial stage the enzyme hydrolyzed agarose to generate oligosaccharides with various DPs. This hydrolysis pattern indicates that this enzyme is an endo-type agarase. After hydrolysis for 24 h, the main products were DPs 8 and 10 with lesser amounts of neoagarotetraose (DP4) and neoagarohexaose (DP6) as identified by FACE. Because of the lack of suitable standards, the main products were purified and identified by mass spectrometry (data not shown) and ^{13}C NMR spectroscopy (Fig. 2). The ^{13}C NMR spectra of these hydrolysis products showed the typical pattern of neoagarooligosaccharide. There were evident resonances at about 97 and 93 ppm that are characteristic for the β and α anomeric forms, respectively, of galactose residues at the reducing end of the neoagarooligosaccharides. There was no evidence of a signal at 90.72 ppm, a finding that could be attributed to hydrolyzed α -(1,3) linkages (22, 41). All these evidenced that AgaB was a β -agarase.

The Substrate Binding Subsites of AgaB—To determine the number of subsites in the binding cleft of AgaB, we have carried out the analyses of enzymatic kinetics and reaction products of AgaB by using oligosaccharides of defined DP as substrates.

TABLE 1
Kinetic parameters for the hydrolysis of oligosaccharides by AgaB

Substrates	K_m	K_{cat}	K_{cat}/K_m
	mM	s^{-1}	
DP8	ND ^a	ND	ND
DP10	12.81	0.19	0.015
DP12	12.39	4.77	0.385
DP14	8.36	5.16	0.617

^a ND, not detectable.

The kinetic parameters of AgaB on different oligosaccharides were shown in Table 1. DP10 was a very poor substrate with by far the lowest K_{cat} and considerable K_m of all the substrates tested. The K_{cat} increased with increasing chain length of the substrate, while K_m values appeared to be relatively independent of DP. Catalytic efficiency factor K_{cat}/K_m increased sharply (about 26 times) as the DP increased from 10 to 12, whereas it was similar between

DPs 12 and 14. No degradation was detected for oligosaccharides with $DP \leq 8$. Therefore, AgaB is more active on higher oligosaccharides, and DP10 is the shortest substrate oligomer that can be recognized and cleaved by AgaB.

Product distributions were shown in Fig. 3, and these data provide excellent insights into the relative cleavage point and products assignment. Agreeing reasonably well with kinetic parameters under stringent condition (high enzyme concentration and prolonged incubation time), DP10 could be thoroughly degraded into DPs 4 and 6, indicating a single productive binding mode in which the -4 to $+6$ subsites (following the subsite naming convention of Davies *et al.* (42), $-n$ represents the non-reducing end, and $+n$ represents the reducing end) were filled. One cleavage site was also observed for DP12, the cleavage mode being that in which the -4 to $+8$ subsites were occupied and not the symmetrical (-6 to $+6$) mode, and four units were needed from the non-reducing end, similar to cleavage of DP10. Results with DP14, as might be expected, are more complex, with two productive binding modes seen and the preferred cleavage mode being that in which -6 to $+8$ subsites are filled. The preference for four sugars in the glycon ($-n$) sites and eight sugars in the aglycon ($+n$) sites, thus, suggests that a minimum of 12 sites make up the active site.

The Hydrolysis Mechanism of AgaB—Glycoside hydrolase hydrolyzes the glycosidic bond via two major mechanisms, overall retention or inversion of anomeric configuration. To determine the hydrolysis mechanism of AgaB, the hydrolysis products of agarose by AgaB at different time were analyzed by recording the 1H NMR spectra at 600 MHz

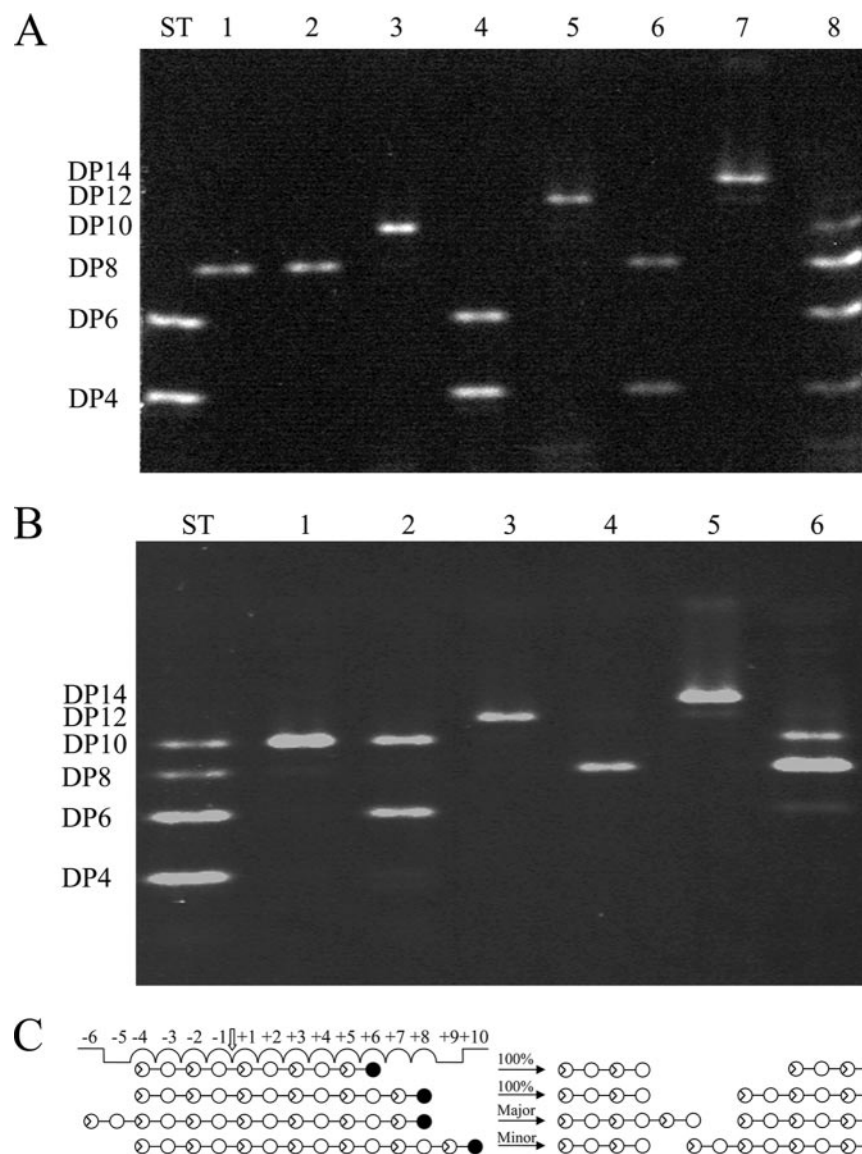


FIGURE 3. A, FACE analysis of hydrolysis products of neoagarooligosaccharides (10 mg/ml) by AgaB. The lane marked ST was loaded with DPs 4 and 6 as the standard. Lanes 1, 3, 5, and 7, DPs 8, 10, 12, and 14 without AgaB, respectively. Lanes 2, 4, 6, and 8, DPs 8, 10, 12, and 14 were degraded by AgaB with different enzyme concentrations and incubation times, respectively. DP14 was completely hydrolyzed by 0.10 μM AgaB within 12 h, giving principally DPs 8 and 6 ($76 \pm 3\%$) with a small amount of DPs 10 and 4 ($24 \pm 3\%$) but not DPs 12 and 2. DP12 was fragmented in DPs 8 and 4 by 0.28 μM AgaB within 12 h, and the hydrolytic product of DP10 was DPs 4 and 6 by 3.20 μM AgaB within 48 h, whereas DP8 couldn't yet be hydrolyzed with higher enzyme concentration (8 μM) after prolonged incubation (up to 96 h). DP2, neoagarobiose; DP4, neoagarotetrose; DP6, neoagarohexaose; DP8, neoagarooctaose; DP10, neoagarodecaose; DP12, neoagarododecaose; DP14, neoagarotetradecaose. B, PAGE analysis of hydrolysis products of fluorescently labeled oligosaccharides by AgaB. The lane marked ST was loaded with DPs 4, 6, 8, and 10 as the standard. Lane 1, 3, and 5, fluorescently labeled DPs 10, 12, and 14 without AgaB, respectively. Lanes 2, 4, and 6, fluorescently labeled DPs 10, 12, and 14 degraded by AgaB, respectively. The hydrolysis conditions were described as above. C, schematic representation of the hydrolysis pattern determined for AgaB. White circles represent β -linked D-galactose units; white circles with triangular inserts represent α -linked L-3,6 anhydro-galactose units; black circles represent the reducing ends. The β -1,4 bond cleaved during catalysis is labeled by arrow.

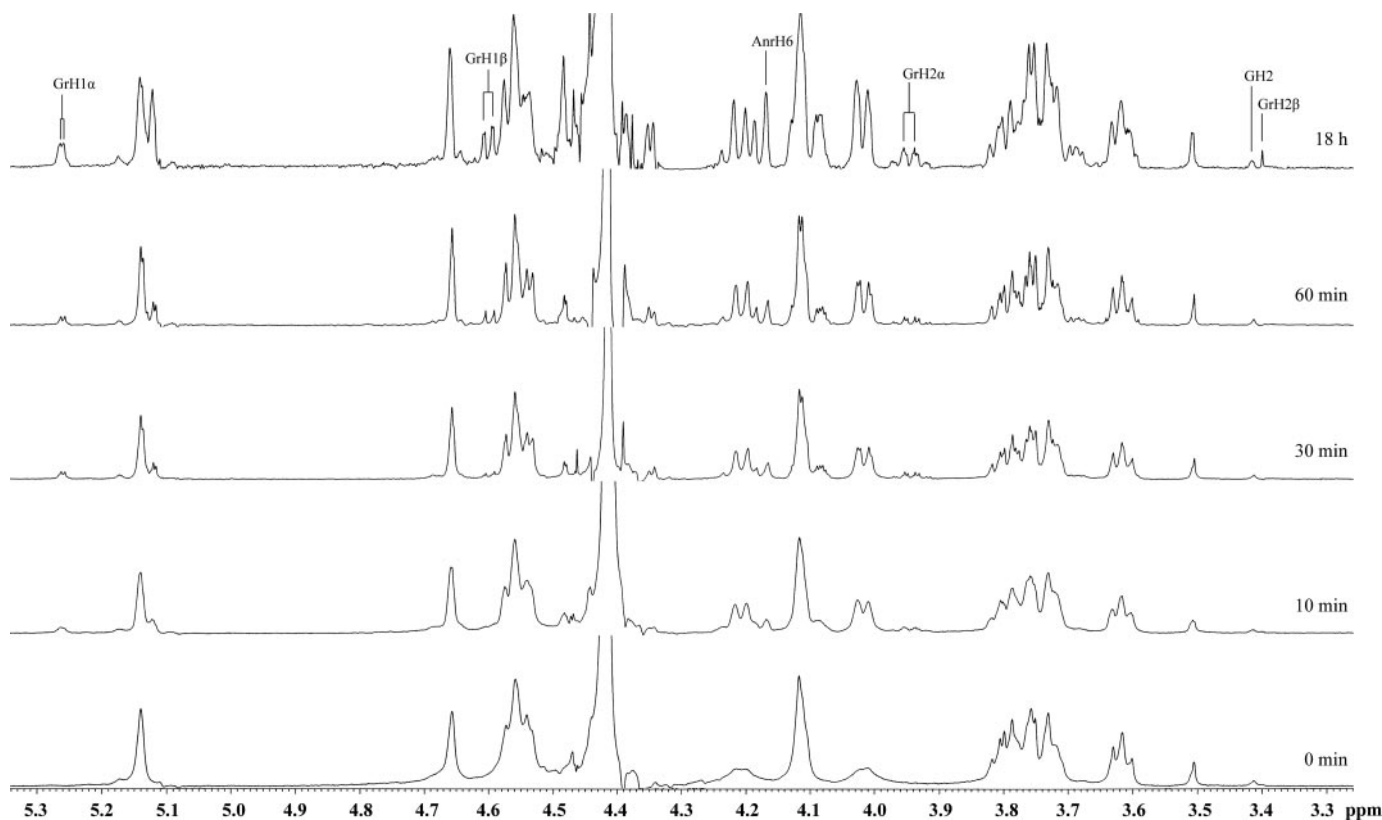


FIGURE 4. ^1H NMR monitoring of the hydrolysis of agarose by AgaB. From bottom to top, initial substrate and substrate were incubated after the addition of AgaB (2.4 nM) for 10 min, 30 min, 1 h, and 18 h. Peak assignments are labeled according to the nomenclature of Fig. 2. A and G refer to the anhydrogalactose and galactose residues, *nr* and *r* refer to the non-reducing and reducing ends of agarose oligomers.

using 2,2-dimethyl-2-silapentane-5-sulfonic acid as the internal standard. The ^1H NMR signals were assigned on the basis of ^1H , ^1H COSY, and heteronuclear multiple quantum coherence spectra of neoagarohexaose and neoagarooctaose. Compared with the ^1H NMR spectrum of agarose (Fig. 4), the spectra recorded from the reaction mixture, including that sampled at as early as 10 min after the addition of the enzyme, showed several new resonances. These corresponded on the one hand to protons on the non-reducing ends released by hydrolysis (e.g. Anr H6) and on the other hand to the release of α -anomers (GrH1 α , GrH2 α). Only after 30 min of hydrolysis, a signal corresponding to the presence of β -anomers (GrH1 β) was clearly visible. Therefore, AgaB inverts the anomeric bond configuration, producing α -anomers that progressively give rise to β -anomers when mutarotation takes place.

DISCUSSION

We have cloned a novel β -agarase-encoding gene, *agaB*, from marine *Pseudoalteromonas* sp. CY24. The deduced protein of *agaB* gene was 478 amino acids in length with a theoretical molecular mass of 50.9 kDa. To identify proteins with amino acid sequences related to AgaB, the DNA and protein sequences of AgaB were used as a query against the FASTA and BLAST data bases. The results of bioinformatics analysis revealed that the amino acid sequence of AgaB does not bear any detectable similarity to any of the established glycoside hydrolase families. As suggested by Prof. Bernard Henrissat (Universités d'Aix-Marseille I and II, Marseille,

France), who established the classification system of glycosidases, AgaB should be the representative of a novel CAZY family.

A notable feature not only of agarases but of very many glycoside hydrolases is their modularity (43). Proteins are rarely found as single catalytic domain entities but instead display a modular structure with one (occasionally more) catalytic domain linked to one or several non-catalytic modules. However, no obvious modular structure was found in AgaB. Based on phylogenetic evidence, enzymes in a family share a common ancestor. Even though these proteins have now diverged significantly in their primary sequences, they usually feature a common catalytic motif. The active sites of glycoside hydrolases require the two amino acids, glutamic or aspartic acid, both of which are highly conserved in each glycoside hydrolase family. AgaB lacks the motif E(I/L/V)D(I/V/A/F)(V/I/L/M/F)_(0,1)E, i.e. the catalytic site typical of clan B of glycoside hydrolases (44, 45, 46) that encompasses families GH 7 and 16 (30). Furthermore, no conserved motifs from other glycoside hydrolases were found in AgaB. This indicates that AgaB may have evolved along a separate evolutionary line. Therefore, this unique β -agarase AgaB will become a useful material for studying the structure, function, and evolution of agarases.

AgaB hydrolyzes agarose to give DPs 8 and 10 as the main products. Of note is the inability of this enzyme to cleave neoagarooligosaccharides with $\text{DP} \leq 8$, and its activity on DP10 is extremely low, whereas this enzyme has a high activity on DPs

12 and 14. This is in good agreement with its product specificity. Therefore, AgaB was functionally different from currently known β -agarases grouped into families GH 16, 50, and 86. Some agarases of family GH 16 degrade agarose and agarose oligosaccharides comprising at least six sugars to yield DP4 as a main product (10, 11). AgaA of family GH 50 from *Vibrio* sp. strain JT0107 degrades not only agarose but also agarose oligosaccharides comprising at least four sugars to yield neoagarobiose (14). AgrA from *Pseudoalteromonas atlantica* T6c in family GH 86 degrades agarose and agarose oligosaccharides more polymerized than hexamers to yield DP6 as the main product (47). These results suggest that the extraordinary structure of β -agarase AgaB was more adapted to degrade longer oligosaccharides (DP \geq 12). It is possible that high subsite occupancy is required to provide sufficient energy for the substrate to adapt the distorted conformation.

AgaB did not bear any detectable similarity to any of the established glycoside hydrolase families. This hindered AgaB from successful modeling based on the structure of an enzyme with different substrate specificity. As evidenced in α -amylases and arabinanase (48–50), the application of different oligomeric substrates is an effective way to explore the nature of the binding site and the process of catalysis for glycoside hydrolases. Recently, Allouch *et al.* (11) determined the subsite numbers of β -agarases A and B from *Zobellia galactanivorans* Dsij in family GH 16 using this strategy and found that the result was in good agreement with crystal structural analysis. These two enzymes adopt a jellyroll fold with a long and open active site cleft that accommodates a maximum of eight sugar units with subsites -4 through -1 from the catalytic site toward the non-reducing end and subsites $+1$ through $+4$ toward the reducing end of the substrate. In this study the number of substrate binding subsites of AgaB was determined using the same strategy. Based on the analyses of enzymatic kinetics and degradation patterns of different oligosaccharides, we can infer that the active cleft of AgaB consists of 12 glycosyl binding subsites with the 8 sugar units toward the reducing end in the active site channel (spanning subsites $+1$ to $+8$) and the four sugar units toward the non-reducing end (spanning subsites -4 to -1). These results clearly indicate that the catalytic cleft of AgaB is larger than that of β -agarases A and B from *Z. galactanivorans* Dsij in family GH 16 (11) and differs from other glycoside hydrolases with known structures, such as κ -carrageenase and ι -carrageenase (51, 52). Consistent with this subsite organization, AgaB releases mainly DPs 8 and 10 from agarose, whereas most other β -agarases give shorter products.

^1H NMR monitoring of the anomeric configuration of the hydrolysis products of AgaB indicates that this enzyme inverts the anomeric bond configuration, producing α -anomers that progressively give rise to β -anomers when mutarotation takes place (Fig. 4). This result demonstrates that AgaB hydrolyzes the $\beta(1\rightarrow4)$ linkages of agarose by a one-step nucleophilic substitution, a mechanism that results in the inversion of the anomeric configuration and precludes transglycosylation (27). This finding contrasts with the case of other agarases, which retain glycoside hydrolases with transglycosylating properties (39). The hydrolytic mechanism is known to be absolutely conserved within glycoside hydrolase families (30, 53). Therefore, this

result further confirms that AgaB is very different from known agarases.

Altogether, we show here that AgaB is a novel endo-type β -agarase that is structurally and functionally different from other agarases. AgaB degrades agarose with an inverting mechanism to yield DPs 8 and 10 as the main end products. Moreover, AgaB has a large active site cleft that accommodates 12 substrate binding subsites. Therefore, our results clearly indicate that β -agarase AgaB appears to define a new family of glycoside hydrolase. Further studies on three-dimensional structure of β -agarase AgaB should give more precise information for the structure-function relationship of this novel enzyme.

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REFERENCES

1. Rochas, C., Lahaye, M., Yaphe, W., and Phan Viet, M. T. (1986) *Carbohydr. Res.* **148**, 199–207
2. Percival, E., and McDowell, R. H. (1967) *Academic press*, London, **88–98**, 127–137
3. Arnott, S., Fulmer, A., Scott, W. E., Dea, I. C., Moorhouse, R., and Rees, D. A. (1974) *J. Mol. Biol.* **90**, 269–284
4. Duckworth, M., and Turvey, J. R. (1969) *Biochem. J.* **113**, 693–697
5. Meulen, H. J., and Harder, W. (1975) *Antonie van Leeuwenhoek* **41**, 431–447
6. Belas, R. (1989) *J. Bacteriol.* **171**, 602–605
7. Ha, J. C., Kim, G. T., Kim, S. K., Oh, T. K., Yu, J. H., and Kong, I. S. (1997) *Biotechnol. Appl. Biochem.* **26**, 1–6
8. Morrice, L. M., McLean, M. W., Long, W. F., and Williamson, F. B. (1983) *Eur. J. Biochem.* **137**, 149–154
9. Morrice, L. M., McLean, M. W., Williamson, F. B., and Long, W. F. (1983) *Eur. J. Biochem.* **135**, 553–558
10. Schroeder, D. C., Jaffer, M. A., and Coyne, V. E. (2003) *Microbiology* **149**, 2919–2929
11. Allouch, J., Jam, M., Helbert, W., Barbeyron, T., Kloareg, B., Henrissat, B., and Czjzek, M. (2003) *J. Biol. Chem.* **278**, 47171–47180
12. Buttner, M. J., Fearnley, I. M., and Bibb, M. J. (1987) *Mol. Gen. Genet.* **209**, 101–109
13. Oscar, L., Luis, Q., Gina, P., and Juan, C. S. (1992) *Appl. Environ. Microbiol.* **58**, 4060–4063
14. Sugano, Y., Matsumoto, T., Kodama, H., and Noma, M. (1993) *Appl. Environ. Microbiol.* **59**, 3750–3756
15. Sugano, Y., Matsumoto, T., and Noma, M. (1994) *Biochim. Biophys. Acta* **1218**, 105–108
16. Zhong, Z., Toudkarian, A., Helinski, D., Knauf, V., Sykes, S., Wilkinson, J. E., O'Bryne, C., Shea, T., DeLoughery, C., and Caspi, R. (2001) *Appl. Environ. Microbiol.* **67**, 5771–5779
17. Ohta, Y., Hatada, Y., Nogi, Y., Li, Z., Ito, S., and Horikoshi, K. (2004) *Appl. Microbiol. Biotechnol.* **64**, 505–514
18. Hassairi, I., Ben Amar, R., Nonus, M., and Gupta, B. B. (2001) *Bioresour. Technol.* **79**, 47–51
19. Potin, P., Richard, C., Rochas, C., and Kloareg, B. (1993) *Eur. J. Biochem.* **214**, 599–607
20. Ohta, Y., Hatada, Y., Miyazaki, M., Nogi, Y., Ito, S., and Horikoshi, K. (2005) *Curr. Microbiol.* **50**, 212–216
21. Malmqvist, M. (1978) *Biochim. Biophys. Acta* **537**, 31–43
22. Vera, J., Alvarez, R., Murano, E., Slebe, J. C., and Leon, O. (1998) *Appl. Environ. Microbiol.* **64**, 4378–4383

23. Grolean, D., and Yaphe, W. (1977) *Can. J. Microbiol.* **23**, 672–679
24. Kobayashi, R., Takisada, M., Suzuki, T., Kirimura, K., and Usami, S. (1997) *Biosci. Biotechnol. Biochem.* **61**, 162–163
25. Yoshizawa, Y., Ametani, A., Tsunehiro, J., Nomura, K., Itoh, M., Fukui, F., and Kaminogawa, S. (1995) *Biosci. Biotechnol. Biochem.* **59**, 1933–1937
26. Araki, T., Lu, Z., and Morishita, T. (1998) *J. Mar. Biotechnol.* **6**, 193–197
27. Sinnott, M. L. (1990) *Chem. Rev.* **90**, 1171–1202
28. Henrissat, B. (1991) *Biochem. J.* **280**, 309–316
29. Henrissat, B., and Bairoch, A. (1996) *Biochem. J.* **316**, 695–696
30. Henrissat, B., and Davies, G. (1997) *Curr. Opin. Struct. Biol.* **7**, 637–644
31. Zverlov, V. V., Liebl, W., Bachleitner, M., and Schwarz, W. H. (1998) *FEMS Microbiol. Lett.* **164**, 337–343
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed, pp. 1.53–1.86, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Oliver, J. D. (1982) *Deep-Sea Res.* **29**, 794–795
34. Polz, M. F., and Cavanaugh, C. M. (1998) *Appl. Environ. Microbiol.* **64**, 3724–3730
35. Miller, G. L. (1959) *Anal. Chem.* **31**, 426–428
36. Laemmli, U. K. (1970) *Nature* **227**, 680–685
37. Yu, G., Guan, H., Ioanoviciu, A. S., Sikkander, S. A., Thanawiroon, C., Tobacman, J. K., Toida, T., and Linhardt, R. J. (2002) *Carbohydr. Res.* **337**, 433–440
38. Weinberger, F., Richard, C., Kloareg, B., Kashman, Y., Hoppe, H.-G., and Friedlander, M. (2001) *J. Phycol.* **37**, 418–426
39. Jam, M., Flament, D., Allouch, J., Potin, P., Thion, L., Kloareg, B., Czjzek, M., Helbert, W., Michel, G., and Barbeyron, T. (2005) *Biochem. J.* **385**, 703–713
40. Lahaye, M. (1986) *Agar from Gracilaria sp.* Ph.D. thesis, McGill University
41. Lahaye, M., Yaphe, W., Phan Viet, M. T., and Rochas, C. (1989) *Carbohydr. Res.* **190**, 249–265
42. Davies, G. J., Wilson, K. S., and Henrissat, B. (1997) *Biochem. J.* **321**, 557–559
43. Henrissat, B., and Davies, G. J. (2000) *Plant Physiol.* **124**, 1515–1519
44. Planas, A., Juncosa, M., Lloberas, J., and Querol, E. (1992) *FEBS Lett.* **308**, 141–145
45. Juncosa, M., Pons, J., Dot, T., Querol, E., and Planas, A. (1994) *J. Biol. Chem.* **269**, 14530–14535
46. Krah, M., Misselwitz, R., Politz, O., Thomsen, K. K., Welfle, H., and Borriss, R. (1998) *Eur. J. Biochem.* **257**, 101–111
47. Belas, R., Bartlett, D., and Michael, S. (1988) *Appl. Environ. Microbiol.* **54**, 30–37
48. Kandra, L., Gyemant, G., Remenyik, J., Ragunath, C., and Ramasubbu, N. (2003) *FEBS Lett.* **544**, 194–198
49. Kandra, L., Gyemant, G., Remenyik, J., Hovanszki, G., and Liptak, A. (2002) *FEBS Lett.* **518**, 79–82
50. Pitson, S. M., Voragen, A. G., Vincken, J. P., and Beldman, G. (1997) *Carbohydr. Res.* **303**, 207–218
51. Michel, G., Chantalat, L., Duee, E., Barbeyron, T., Henrissat, B., Kloareg, B., and Dideberg, O. (2001) *Structure* **9**, 513–525
52. Michel, G., Chantalat, L., Fanchon, E., Henrissat, B., Kloareg, B., and Dideberg, O. (2001) *J. Biol. Chem.* **276**, 40202–40209
53. Gebler, J., Gilkes, N. R., Claeysens, M., Wilson, D. B., Beguin, P., Wakarchuk, W. W., Kilburn, D. G., Miller, R. C., Jr., Warren, R. A., and Withers, S. G. (1992) *J. Biol. Chem.* **267**, 12559–12561