Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Expression, crystallization and preliminary X-ray analysis of an anomeric inverting agarase from *Pseudoalteromonas* sp. CY24

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Received 17 August 2010
Accepted 13 October 2010

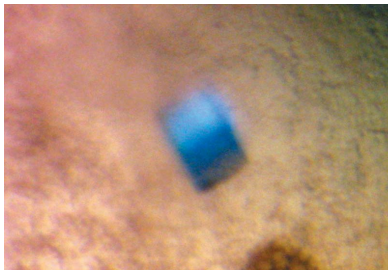
AgaB from *Pseudoalteromonas* sp. CY24 is a novel agarase that hydrolyzes agarose to generate products with inverted anomeric configuration and that has been proposed to have a larger catalytic cleft than other β-agarases. Here, the expression, purification, crystallization and data collection of AgaB in both wild-type and selenomethionine-substituted forms is described. The crystals of wild-type AgaB diffracted to 1.97 Å resolution and belonged to space group $C222_1$. The selenomethionine derivative crystallized in space group $I222$. The phasing problem was solved by the multiwavelength anomalous dispersion (MAD) method. These results will facilitate detailed structural and enzymatic analysis of AgaB.

1. Introduction

Agarose is a linear polymer that consists of alternately arranged (1,3)-linked β-D-galactopyranose and (1,4)-linked 3,6-anhydro-α-L-galactopyranose units (Araki, 1937). The neutral charge and chemical simplicity of agarose make it less likely to interact with biomolecules. Therefore, it is widely used for electrophoretic separation and in column-based chromatography. In nature, agarose is degraded by agarases, which can be classified into α and β types that hydrolyze the α-1,3-linkages and β-1,4-linkages, respectively (Duckworth & Turvey, 1969; Day & Yaphe, 1975). The β-agarases are grouped into families 16, 50 and 86 of the glycoside hydrolases (GHs) on the basis of protein-sequence similarity. GH family 16 adopts a β-jelly-roll fold and includes glycoside hydrolases with various substrate specificities. GH families 50 and 86 display $(\beta/\alpha)_8$-barrel topology and only contain β-agarases (http://www.cazy.org/Glycoside-Hydrolases.html).

AgaB from *Pseudoalteromonas* sp. CY24 is a functionally novel β-agarase (Ma *et al.*, 2007). The main products of the hydrolysis of agarose by this enzyme are neoagarooctaose and neoagarodecaose, which are larger products than those obtained using any other known agarases. Moreover, AgaB hydrolyzes the glycosylic bond with inversion of anomeric configuration, in contrast to the retaining mechanism of other agarases (Fig. 1). *BLAST* analysis revealed that AgaB (GenBank AAQ56237.1) has 100% protein-sequence identity and 97% DNA-sequence identity to rAgaC (GenBank BAF03590.1) from *Vibrio* sp. PO-303 (Dong *et al.*, 2006). Interestingly, rAgaC also lacks the ability to hydrolyze neoagaroctaose and smaller neoagaro-oligosaccharides (Dong *et al.*, 2006). AgaB and rAgaC may belong to a novel GH family, as they are only distantly related to other known glycoside hydrolases.

Agarase is of great interest for applications in the food, cosmetics and medical industries involving the production of oligosaccharides (Kobayashi *et al.*, 1997). To date, only a few agarases have been successfully expressed in *Escherichia coli*. Here, we report a facile protocol for the expression of soluble AgaB protein. The addition of glucose to the culture medium or a reduction of the amount of certain nutrients greatly improves the solubility of recombinant AgaB. Wild-type and selenomethionine-substituted (SeMet) AgaB proteins were purified and crystallized. The results will shed light on the structure and mechanism of AgaB.



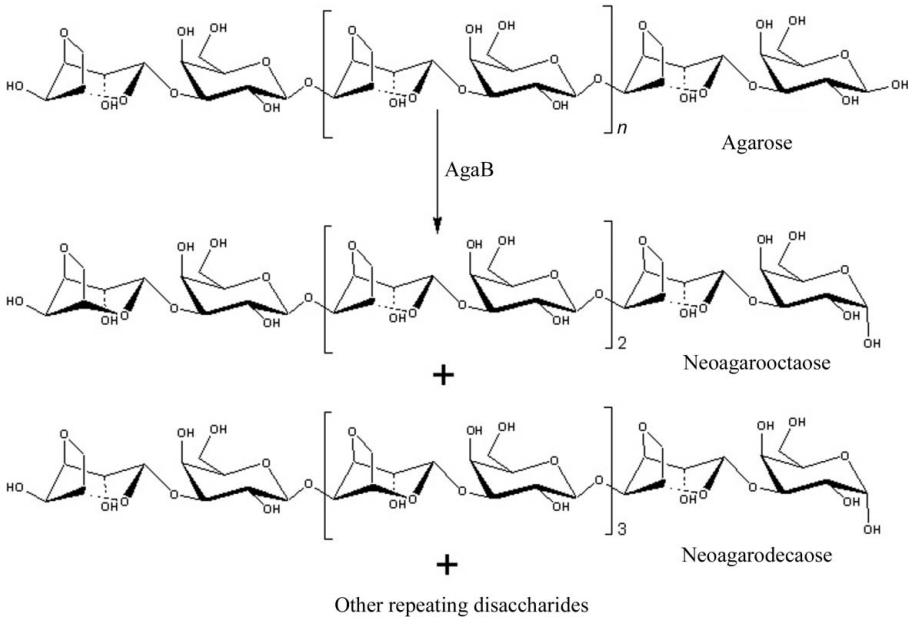


Figure 1
Scheme of the enzymatic reaction of AgaB. The hydrolysis products of agarose mainly consisted of neoagarooctaose and neoagarodecaose.

2. Materials and methods

2.1. Cloning and protein expression

The DNA sequence encoding *Pseudoalteromonas* sp. CY24 AgaB (amino acids 39–478) was amplified by PCR using the oligonucleotide primers 5′-GACT**GGATCC**GCTAACTATACGGCCAGCAATGC (forward; *Bam*HI restriction site indicated in bold) and 3′-GGGT-**CTCGAG**CTATTGGCAAGTATAACCTGACACAACG (reverse; *Xho*I restriction site indicated in bold). The inserts were cloned into expression vector pSJ2 (a derivative of pET21a and a gift from Professor Zhaohui Xu of the University of Michigan, USA) using standard molecular-cloning methods. The wild-type protein was expressed as an N-terminally His_8-tagged fusion protein in *E. coli* BL21 (DE3) at 289 K in LB medium supplemented with 1% glucose and 100 µg ml^{-1} ampicillin by the addition of 1.0 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG). After culturing for 24 h, the cells were harvested by centrifugation for 10 min at 2500*g*.

2.2. Expression of selenomethionine-labelled protein

Because there were no methionine residues in the AgaB protein sequence, a triple mutant, L122M/L242M/L366M, was constructed to assist in phase determination. The mutations were introduced by PCR into the pSJ2-agaB template DNA using the QuikChange kit (Stratagene) according to the manufacturer's instruction. The SeMet protein was expressed in *E. coli* B834 (DE3) plysS strain (Novagen) at 289 K in modified M9 medium by the addition of 0.05 m*M* IPTG. 150 ml of the modified M9 medium consisted of 0.3 ml 1 *M* $MgSO_4$, 15 ml 10× M9 (20 g l^{-1} ammonium chloride, 60 g l^{-1} KH_2PO_4 and 120 g l^{-1} Na_2HPO_4), 0.3 ml 12.5 mg ml^{-1} $FeSO_4{\cdot}7H_2O$, 3 ml 20%(*w*/*v*) glucose, 1.5 ml 4 mg ml^{-1} amino-acid mix I (all amino acids except Met, Tyr, Trp and Phe), 1.5 ml of 2 mg ml^{-1} amino-acid mix II (Met, Tyr, Trp and Phe), 0.15 ml 1 mg ml^{-1} vitamins (riboflavin, niacinamide, pyridoxine monohydrochloride and thiamine) and 0.6 ml 10 mg ml^{-1} selenomethionine (Acros). The cells were harvested by centrifugation after growth for 24 h.

2.3. Protein purification

The cell pellets were homogenized and sonicated in lysis buffer consisting of 25 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 5 m*M* *β*-mercaptoethanol. Cell debris was removed by centrifugation for 60 min at 15 000*g*. The supernatant was loaded onto a nickel-affinity column (GE Healthcare) and washed with 10 m*M* imidazole in lysis buffer. The recombinant protein was eluted from the affinity resin with 300 m*M* imidazole in lysis buffer and digested with His-tagged TEV protease. The released His tag and His-tagged protease were removed after loading the protein samples onto a second nickel-affinity column. A further step consisting of anion-exchange chromatography (HiTrap DEAE, GE Healthcare) and gel-filtration chromatography (Superdex 200 16/60, GE Healthcare) yielded highly

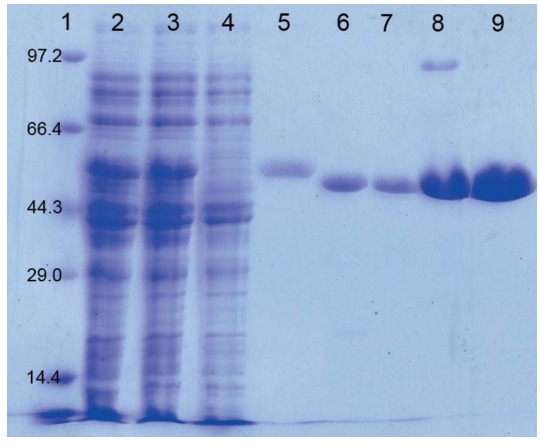
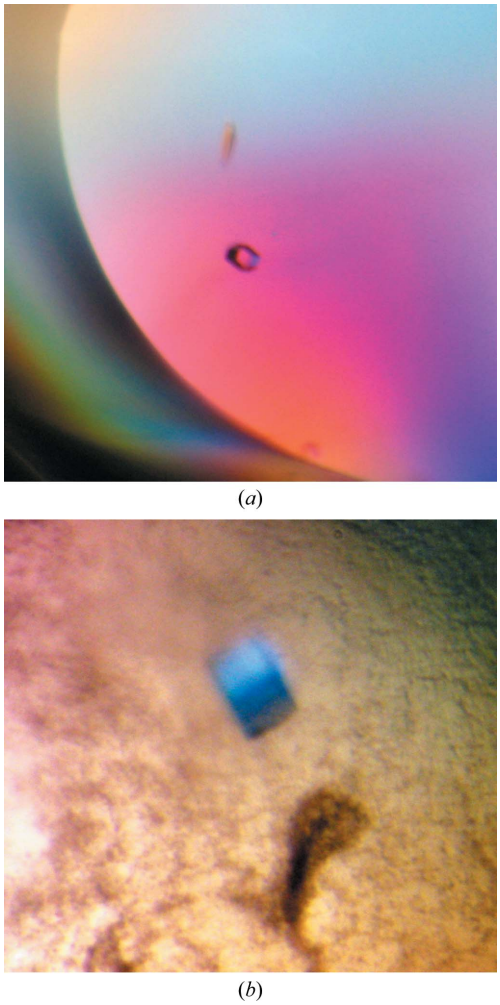


Figure 2
Purification steps of SeMet AgaB monitored using 12% SDS–PAGE. Lane 1, molecular-weight markers (labelled in kDa); lane 2, total sample of bacterial lysates after sonication; lane 3, supernatant after centrifugation; lane 4, nonbound material after the first nickel column; lane 5, eluate after the first nickel column; lane 6, protein after cleavage with His-tagged TEV protease; lane 7, nonbound AgaB after the second nickel column; lane 8, eluate after the HiTrap DEAE column; lane 9, eluate after gel-filtration chromatography.

purified and homogeneous protein (Fig. 2). The yield was 15 mg per litre of culture medium and the protein was concentrated to 10 mg ml^{-1} for crystallization using an Amicon Ultra-4 centrifugation tube (Millipore, 10 000 molecular-weight cutoff). All purification steps were carried out at 277 K. The masses of native and SeMet AgaB were measured by AutoFlex MALDI-TOF/TOF mass spectrometry (Bruker, Germany) with α-cyano-4-hydroxycinnamic acid (CCA) as the matrix.

2.4. Activity assay

The activities of wild-type and SeMet AgaB were determined using the 3,5-dinitrosalicylic acid method (Ma *et al.*, 2007). In brief, 50 µl diluted enzyme solution (in 20 m*M* sodium phosphate pH 6.0, 200 m*M* NaCl) was mixed with 450 µl 20 m*M* sodium phosphate buffer pH 6.0 containing 0.25%(*w*/*v*) agarose and incubated at 313 K for 10 min. The reaction solution was then mixed with 375 µl 3,5-dinitrosalicylic acid and boiled for 10 min before adding 2.5 ml H_2O. The amount of reducing sugar was monitored by measuring the absorbance at 520 nm and was calibrated with the standard curve of D-galactose. One unit of enzyme was defined as a 1 µmol increment per minute in the reducing end.



(*a*)

(*b*)

Figure 3
Crystals of (*a*) native and (*b*) SeMet AgaB. The maximum dimensions of the native crystal are about 0.1 × 0.05 × 0.05 mm and the maximum dimensions of the SeMet crystal are about 0.15 × 0.1 × 0.1 mm.

2.5. Crystallization

All crystallization experiments were performed at 293 K using the sitting-drop vapour-diffusion method. Initial screening trials were set up using Crystal Screen, Grid and Natrix reagents from Hampton Research and Wizard kits from Emerald BioSystems. 1 µl protein solution at a concentration of 10 mg ml^{-1} in storage buffer (25 m*M* Tris–HCl pH 8.5, 100 m*M* NaCl and 1 m*M* DTT) was mixed with 1 µl well solution and equilibrated against 100 µl well solution in 96-well sitting-drop plates (catalogue No. 3773, Corning). The crystals were further optimized by modifying the pH, the precipitant concentration and the types of additive. Native AgaB protein crystals were grown in the condition 0.1 *M* HEPES pH 7.0, 30% PEG 4000, 0.10 *M* ammonium sulfate and 15% glycerol and appeared after six months (Fig. 3*a*). SeMet AgaB crystals were grown in the condition 0.1 *M* Bicine pH 9.0, 0.5 *M* NaCl, 0.01 *M* $MgCl_2$ and 0.01 *M* cetyltrimethylammonium bromide (CTAB) and appeared after one week (Fig. 3*b*).

2.6. Data collection

Prior to data collection, the crystals were flash-frozen in liquid nitrogen and tested on an in-house X-ray generator at Shanghai Institute of Biochemistry and Cell Biology. The native crystals were directly frozen in their mother liquor. The cryoprotectant used for the SeMet crystals consisted of 0.1 *M* Bicine pH 9.0, 0.5 *M* NaCl, 0.01 *M* $MgCl_2$, 0.01 *M* CTAB and 40% glycerol. Promising crystals were saved and shipped in a travel Dewar to beamlines BL-6A and BL-17A at the Photon Factory (KEK, Tsukuba, Japan). Diffraction data were collected on an ADSC Q270 detector at 100 K using one single crystal (Table 1). Intensity data were integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The data set for the native crystal was collected at a wavelength of 0.9780 Å and processed to 1.97 Å resolution. After crystal annealing, a multiple-wavelength anomalous diffraction (MAD) data set for the SeMet crystal was collected at wavelengths of 0.9789, 0.9641 and 0.9793 Å.

3. Results and discussion

AgaB from *Pseudoalteromonas* sp. CY24 has the same protein sequence as rAgaC from *Vibrio* sp. PO-303. Their expression in *E. coli* by secretion and by a direct method, respectively, have been reported previously (Dong *et al.*, 2006; Ma *et al.*, 2007). However, the AgaB purified from secreted samples was not homogeneous or sufficiently stable and only yielded microcrystals. Although the expression plasmid in this report is similar to that reported by the Araki laboratory (Dong *et al.*, 2006), mature AgaB could not be expressed as a His-tagged fusion in a soluble form in *E. coli* BL21 (DE3) cells. This problem was solved by the addition of 1% glucose to the cell-culture medium, which dramatically improved the solubility of the native AgaB protein. Soluble expression of SeMet AgaB protein was achieved by reducing the content of amino-acid mix II by 50%.

The recombinant AgaB proteins were purified to homogeneity and used for crystallization after being assayed as catalytically active forms. Crystals of the native protein appeared after six months. They diffracted to 1.97 Å resolution and belonged to the orthorhombic space group $C222_1$, with unit-cell parameters a = 49.1, b = 111.7, c = 163.8 Å. The SeMet protein crystallized within one week in a different condition which consisted of 0.1 *M* Bicine pH 9.0, 0.5 *M* NaCl, 0.01 *M* $MgCl_2$ and 0.01 *M* CTAB. The crystals diffracted to 2.64 Å resolution and belonged to the orthorhombic space group $I222$, with unit-cell parameters a = 126.8, b = 138.8, c = 147.5 Å.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

		SeMet AgaB		
	Native AgaB	Peak	High-energy remote	Edge
Beamline	BL-6A	BL-17A		
Wavelength (Å)	0.9780	0.9789	0.9641	0.9793
Crystal-to-detector distance (mm)	347.3	174.6	174.6	174.6
Oscillation range per frame (°)	1	1	1	1
Images taken	270	190	190	190
Exposure time (s)	20	2	2	2
Resolution (Å)	50.0–1.97 (2.04–1.97)	50.0–2.64 (2.73–2.64)	50.0–2.63 (2.72–2.63)	50.0–2.64 (2.73–2.64)
Space group	$C222_1$	$I222$		
Mosacity (°)	0.638	1.900		
Unit-cell parameters				
a (Å)	49.1	126.8		
b (Å)	111.7	138.8		
c (Å)	163.8	147.5		
No. of molecules in asymmetric unit	1	2		
Matthews coefficient (Å^3 Da^{-1})	2.38	3.42		
No. of observed reflections	255902	238898	229409	235138
No. of unique reflections	32447	37642	37133	37527
Completeness (%)	99.6 (98.2)	97.5 (83.1)	98.0 (93.6)	97.2 (83.7)
Average multiplicity	7.9 (7.2)	6.3 (4.9)	6.2 (4.3)	6.3 (4.6)
Average $I/\sigma(I)$	15.0 (3.8)	10.3 (2.8)	9.2 (2.5)	9.6 (2.8)
$R_{\rm merge}$† (%)	12.1 (42.9)	14.0 (51.0)	15.7 (58.0)	14.5 (51.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean of the observations $I_i(hkl)$ of reflection *hkl*.

Owing to their low sequence homology to other glycoside hydrolases, attempts to solve the structure by molecular replacement using β-agarases from *Zobellia galactanivorans* Dsij (Allouch *et al.*, 2003, 2004) and other species (Henshaw *et al.*, 2006) as search models were not successful. In order to solve the phase problem, a triple mutant, L122M/L280M/L366M, was constructed by the site-directed mutagenesis method as there are no methionine residues in the 440 amino-acid chain of AgaB. The initial diffraction quality of the SeMet crystals was unsatisfactory. The spots were streaky and the mosacity was greater than 3°. After shielding the crystal from the cold gas stream with a piece of paper for about 12 s, it was exposed to the cryostream again. After this crystal annealing the diffraction spots became round and the mosacity decreased to 1.9°. This crystal was used for MAD data collection (Table 1).

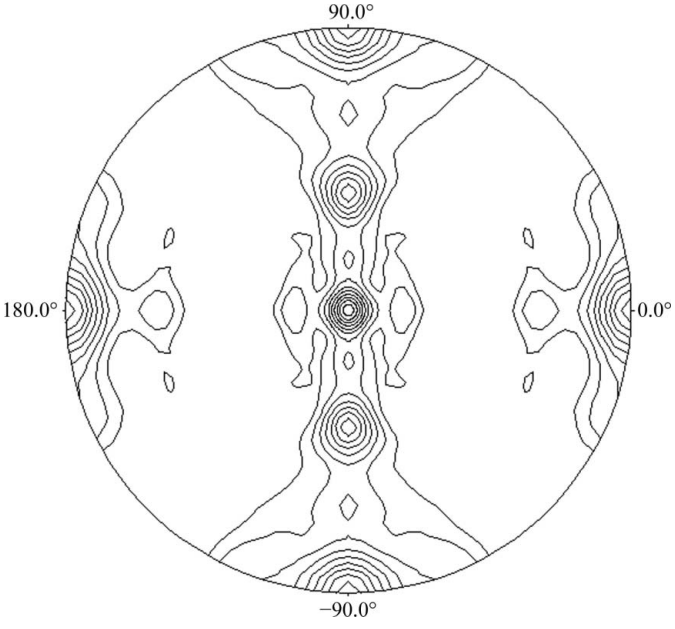


Figure 4
Self-rotation function for the SeMet peak data ($\kappa = 180°$ section). ψ is proportional to the radius, from 0° (at the centre) to 90°, and φ is labelled on the circle.

The $C222_1$ crystal of native AgaB contains one molecule per asymmetric unit with a Matthews coefficient of 2.38 Å^3 Da^{-1}, corresponding to a solvent content of 48%. The $I222$ crystal of the selenomethionine derivative is likely to contain two or three molecules per asymmetric unit, corresponding to a solvent content of 64 or 46%, respectively. A self-rotation function was computed using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) to search for noncrystallographic symmetry. SeMet peak data in the resolution range 15–3 Å were used and the integration radius was set to 29 Å, giving a predominant peak at $\varphi = 90$, $\psi = 38$, $\kappa = 180°$ as shown in Fig. 4, indicating the presence of a noncrystallographic twofold axis. This suggested that there might be two molecules and six Se atoms in one asymmetric unit.

The structure of SeMet AgaB was determined using MAD phasing with the *SOLVE* software (Terwilliger, 2003). Six Se atoms were found, confirming that there are two molecules in one asymmetric unit. Automatic model building with *RESOLVE* provided about 70% of the complete model. Structural analysis of AgaB will provide new insights into the catalytic mechanism of this novel β-agarase.

We thank the staff of the Research Centre for Proteome Analysis, Shanghai Institutes for Biological Sciences for performing the mass-spectrometric experiment, Professor Jianping Ding at Shanghai Institute of Biochemistry and Cell Biology for access to the in-house X-ray facility and the staff of beamlines BL-6A and BL-17A at the Photon Factory (Japan) for help with data collection. This work was supported by the Hundred Talents Program of the Chinese Academy of Sciences, National Natural Science Foundation of China (20872170), the National Program on Key Basic Research of China (2009CB918600) and the Key Progam of Shanghai Municipal Natural Science Foundation (08JC1412700).

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