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Purification and Refolding of a Novel β-Agarase from Inclusion Body of *E. coli*

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Abstract *β*-agarase AgaB appears to represent a new family of glycoside hydrolase; it is structurally and functionally different from other known agarases. In the present study, AgaB was expressed with a temperature-inducible expression system in *E. coli* BL21 (DE3) as a fusion protein bearing a C-terminal hexahistidine tag. The protein existed mainly in the form of inclusion body. After being washed and solubilized, AgaB in inclusion body was denatured and purified to electrophoretic purity by immobilized metal affinity chromatography. The purified AgaB was then refolded using a simple pulse dilution method, and the refolded AgaB showed a high specific hydrolysis activity of about 1600 units /mg protein. Forty milligrams of refolded pure protein were obtained from 1L of culture.

Key words *β*-agarase; inclusion body; refolding; immobilized metal affinity chromatography

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1 Introduction

Agarases produced by agar-degrading bacteria are classified into two groups based on their modes of action, namely, *α*-agarase and *β*-agarase. They hydrolyze *α*-1, 3 linkage and *β*-1, 4 linkage of agarose respectively (Duckworth and Turvey, 1969). Agarases are widely used in food, cosmetic and medical industries for the production of oligosaccharides from agar (Kobayashi *et al*., 1997; Yoshizawa *et al*., 1995). Neoagaro-oligosaccharides produced by *β*-agarase inhibit the growth of bacteria, decrease the degradation rate of starch, and are used as low-calorie additives to improve food quality (Ohta *et al*., 2004). The polysaccharide fractions prepared from marine algae using *β*-agarase have macrophage-stimulating activity and are usable in physiologically functional foods with protective and immunopotentiating activities (Yoshizawa *et al*., 1995). Moreover, agarases can be used to degrade the cell wall of marine algae for extraction of labile substances with biological activities and for preparation of protoplasts (Araki *et al*., 1998). In biotechnology, they can also be used to recover DNA fragments from agarose gel after electrophoresis.

The *agaB* gene encoding a novel *β*-agarase AgaB was cloned from marine *Pseudoalteromonas* sp. CY24①. It has been suggested that AgaB may represent a new family of glycoside hydrolases. Structurally, AgaB has no significant similarity to any known glycoside hydrolases. Functionally, AgaB hydrolyzes agarose, producing neoagarooctaose and neoagarodecaose as the major end product, whereas most of *β*-agarases hydrolyze agarose, producing mainly low degree of neoagaro-oligosaccharides. Although AgaB has been produced in pET24a (+)/*E. coli* BL21 (DE3) system, only approximately 3 mg of AgaB could be obtained extracellularly per liter of culture supernatant①. Thus, there is a strong demand on a rapid, large-scale production of recombinant AgaB to explore its potential valuable applications. In this paper, we report that a significant amount of pure AgaB proteins could be successfully obtained with one-step purification followed by refolding of AgaB from *E. coli*.

2 Materials and Methods

Expression vector pBV220 was kindly provided by Prof. Fang (Academy of Military Medical Sciences, Beijing, China).

2.1 Construction of Expression Plasmid pBV-AgaB

DNA manipulation and *E. coli* transformation were performed following standard procedures (Sambrook *et al*., 1989). Gene encoding AgaB was amplified by PCR from genomic DNA of *Pseudoalteromonas* sp. CY24

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① Ma, C.P., 2005. Molecular cloning and characterization of a β-agarase belonging to a novel family of glycoside hydrolase from marine *Pseudoalteromonas* sp. CY24. (In contribution).

as a template, using forward primer (5'-CCG **GAA TTC** CAT ATG TTA AAG CGC CAC CAA G-3') containing an *EcoRI* site (bold) and reverse primer (5'-TGC A**CT GCA G**CT AGT GGT GGT GGT GGT GGT GTT GGC AAG TAT AAC CTG-3') containing a *PstI* site (bold) and a hexa-histidine tag (underlined). The PCR product was digested with *EcoRI* and *PstI* and cloned into the *EcoRI* and *PstI* sites of pBV220 vector, yielding the recombinant plasmid pBV-AgaB. The insert was confirmed by DNA sequencing.

2.2 Expression of AgaB

Ten microlitres of LB medium containing 50 μg mL^{-1} ampicillin was inoculated with a single colony of *E. coli* BL21 (DE3) transformed with pBV-AgaB and was then incubated overnight at 37℃. Fresh LB medium was inoculated 1:100 with the overnight culture and incubated at 30℃ until OD_{600} of about 0.6 was reached. Expression of AgaB-His6 was induced by incubation at 42℃ for 4 h, and cells were harvested by centrifugation (12000 g, 10 min, 4℃) and stored at –20℃ until use.

2.3 Isolation and Solubilization of Inclusion Body

The frozen cells were resuspended in 100 mL of lysis buffer (50 mmol L^{-1} Tris-HCl, 1mmol L^{-1} EDTA, and 0.1mol L^{-1} NaCl, pH 8.0; 100 μg mL^{-1} lysozyme and 0.1% Triton X-100), and incubated at room temperature for 15 min. The cells were sonicated in an ice/water bath for 20 cycles of pulsing for 10 s and cooling for 10 s. The power was set at 30% of the full (750W). The lysate was centrifugated at 4℃, 12000 g for 15 min. The pellet, containing AgaB inclusion body, was washed twice with 50 mL of wash buffer (0.1 mol L^{-1} NaCl, 1% TritonX-100, and 50 mmol L^{-1} Tris-HCl, pH 8.0). The pellet (about 70 mg) was dissolved and denatured in Buffer A (8 mol L^{-1} urea, 0.5 mol L^{-1} NaCl, and 20 mmol L^{-1} sodium phosphate (PB), pH 8.0), and incubated at room temperature for 30 min. The insoluble material was removed by centrifugation at 12 000g for 30 min.

2.4 Purification of AgaB

A HiTrap Chelating HP column (1mL, Amersham Biosciences) was equilibrated on a FPLC apparatus (Pharmacia) with 10 bed volumes of Buffer A. After loading the supernatant of solubilized inclusion body, the column was washed with the same buffer until the A_{280} absorbance reached the baseline. Then the bound protein was eluted with 50% Buffer B (8 mol L^{-1} urea, 0.5 mol L^{-1} NaCl, 20 mmol L^{-1} PB, pH 4.0). All flow rates were 0.5 mL min^{-1}. The eluted fractions were monitored with a UV detector and further analyzed by SDS-PAGE.

2.5 Refolding of AgaB

The purified AgaB was diluted to 0.3 mg mL^{-1} with Buffer A. Three milliliters of solution was added to 300 mL of renaturing buffer in three times, one milliliter each time, and in an interval of 30 min. The solution was incubated at 4℃ for 24 h with stirring. Once folding was judged to be complete, the solution was desalted and concentrated with Amicon Ultra-15 centrifugal filter (Millipore) by centrifugation at 400 g and 4℃ for 30 min. The supernatant of the refolded AgaB was collected and stored at –20℃.

2.6 Measurement of AgaB Activity

Ten microlitres of diluted solution of the refolded AgaB was incubated in 990 μL 20mmol L^{-1} PB (pH 6.0) containing 0.25% agarose at 40℃ for 10min. The enzymatic activity of AgaB was expressed as the initial rate of agarose hydrolysis by measuring the release of reducing ends, using the 3, 5-dinitrosalicylic acid procedure (Miller, 1959). One unit of enzymatic activity was defined as the amount of proteins that produced 1μmol of reducing sugar per minute under assaying condition. D-galactose was used as the standard.

2.7 Characterization of the Enzyme

The optimum temperature was determined by monitoring enzyme activity at temperature ranging from 10 to 60℃. The enzyme was incubated with substrate solution (pH6.0) for 1 h at different temperatures. Then the thermostability of the enzyme was evaluated by measuring the residual activity. The pH dependence of AgaB was detected at 40℃ between pH 5.7 and 7.0, and the effect of acidity on the enzyme stability was determined by measuring the residual activity after incubating AgaB at 4℃ for 1 h at various pHs (pH 4.0-10.6). The effects of various metal ions and chelators on enzyme activity were also examined by determining the residual activity in the presence of 1 mmol L^{-1} of the various compounds under the same condition.

2.8 Hydrolysis Product Analysis of AgaB

Fluorophore assisted carbohydrate electrophoresis (FACE) was used to identify the hydrolysis products of AgaB. Enzymatic hydrolysis of agarose was carried out by adding refolded AgaB (60 μg) into 50 mL 20 mmol L^{-1} PB (pH 6.0) containing 0.25 % agarose at 25℃. After incubation for 24 h, the reaction supernatant was fluorescently labeled using monopotassium 7-amino-1, 3- napthalenedisulfonic acid (ANDS) and sodium cyanoborohydride reagents (Yu *et al*., 2002). The degradation products were subjected to density gradient (18% to 25%) discontinuous polyacrylamide gel electrophoresis (DGGE) performed on a vertical slab gel system (Amersham). Six microlitres of the sample were loaded and subjected to electrophoresis at constant 300V for 2 h. Finally, the gel was visualized under UV light and photographed.

2.9 Determination of Protein Concentration

Protein concentration was determined using Bradford Kit according to the manufacture's instruction (Beyotime Institute of Biotechnology, Haimen, China).

3 Results and Discussion

In this study, the *agaB* gene was amplified from genomic DNA of *Pseudoalteromonas* sp. CY24. As shown in Fig.1, the *agaB* gene was inserted into the *E. coli* expression vector pBV220. A hexa-histidine (H_6) affinity tag was added to the C-terminus of AgaB to facilitate its purification.

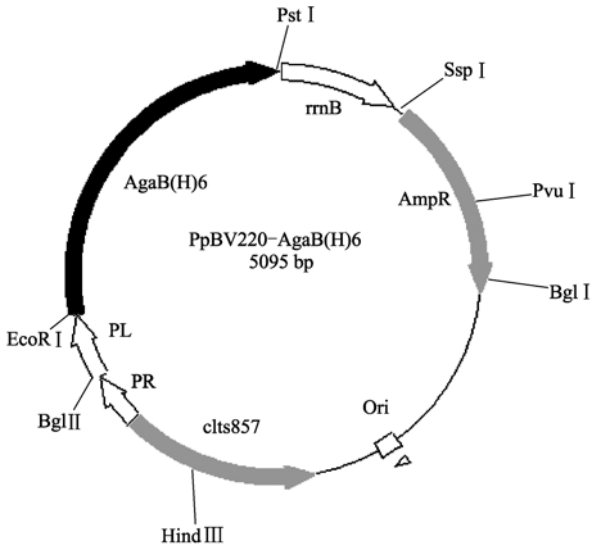

Fig.1 Map of the plasmid pBV-AgaB for expression of AgaB-His.

3.1 Expression of Recombinant AgaB

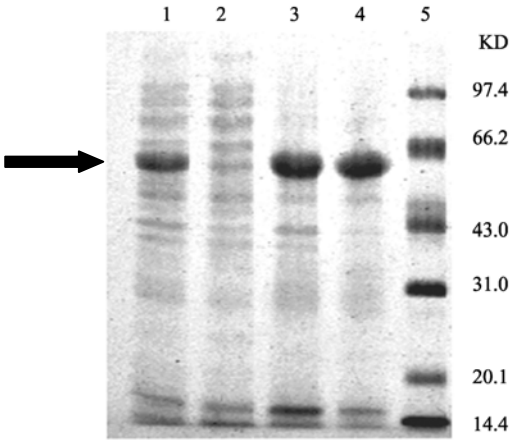

Fig.2 SDS-PAGE analysis of recombinant AgaB. All samples were analyzed on a 15% gel and stained with Coomassie Blue. Lane 1, whole cell lysate of the induced bacteria; Lane 2, supernatant of whole cell lysate; Lane 3, inclusion bodies; Lane 4, washed inclusion body; Lane 5, protein molecular weight standards. The bold arrow points to the band corresponding to AgaB.

In our work, two tandem strong promoters, P_R and P_L in pBV220 were used to drive the expression of recombinant AgaB. The promoters were completely repressed at 30℃. The expression of recombinant AgaB-His was induced after the temperature was shifted to 42℃. This expression system controlled by temperature shift is much more simple and cheaper than that controlled by a chemical inducer. SDS-PAGE analysis revealed that the recombinant AgaB accumulated up to 50% of the total protein of *E. coli* cells (Fig.2). Protein in inclusion body is of great advantage for protein purification, as IB is readily prepared and contains only a few impurities (Mayer and Buchner, 2004). After cells being harvested, they were disrupted by sonication. Inclusion bodies were collected and washed with 1% TritonX-100 to remove cell debris and other contaminants (Fig.2).

3.2 Solubilization and Purification of Inclusion Bodies

Inclusion body can be solubilized completely by 8 mol L^{-1} urea. Solubilized IB was not pure enough, which may associate with other proteins that may interfere with its refolding. Purification ahead of refolding may be required to minimize such interference.

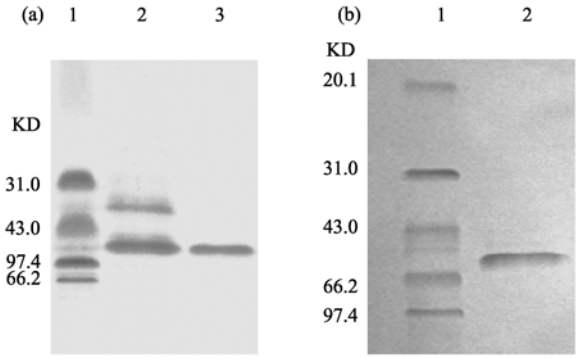

Fig.3 (a) SDS-PAGE analysis illustrating the purification of AgaB after purification using Ni^{2+} chelating column. Lane 1, protein molecular weight standards; Lane 2, inclusion body dissolved in 8 mol L^{-1} urea; Lane 3, purified denatured protein of AgaB by IMAC. (b) Lane 1, protein molecular weight standards; Lane 2, the supernatant of refolded protein (concentrated 100-fold from dilution buffer).

Table 1 Purification of His-tagged AgaB from 1 L of *E. coli* BL21 (DE3) cells

Purification step	AgaB-His† (mg)	Purity†† (%)
Total cell lysate	85	52.7
Denatured inclusion bodies	74	85
Nickel chelating chromatography	61	98
Refolded protein	44	100

Notes: † Yield was determined using a Bradford assay;
†† Purity was estimated from SDS gel.

HisTrap HP is a ready-to-use column, prepacked with precharged Ni Sepharose. This prepacked column is ideal for preparative purification of His-tagged recombinant proteins by immobilized metal ion affinity chromatogra-

phy (IMAC). Herein, the inclusion body was solubilized with Buffer A (8.0 molL^{-1} urea) and loaded onto the column and, subsequently, the majority of them bound with Ni^{2+}-Sepharose. Then the denatured AgaB was eluted from the column in one-step and analyzed by SDS-PAGE. As showed in Fig.3a, the purity of approximately 100% was reached. The results of this purification are summarized in Table 1.

3.3 Refolding of AgaB

Refolding is a process that leads to a change in protein conformation from unfolded to folded (native) state. It is initiated by reducing concentration of denaturant used to solubilize IB (Tsumoto *et al*., 2003). Optimal procedure to reduce denaturant concentration and assistance of refolding by solvent additives play key roles in protein refolding. In general, in particular for refolding by dilution, low concentration of urea is included in refolding solvent (De Bernardez Clark *et al*., 1998). In our study, one molar per liter of urea is low for efficient refolding, but enough to maintain solubility and flexibility of folding intermediates. However, urea alone is insufficient for efficient refolding. The addition of co-solutes is often essential to facilitate refolding. Without co-solutes, refolding generates a varying degree of aggregates. As shown in Table 2, the optimal composition of refolding buffer for AgaB was 20 mmol L^{-1} PB, 1 mol L^{-1} urea, 500 mmol L^{-1} L-arginine, 10% glycerol, pH 7.0. In addition, the optimal temperature of refolding and the initial concentration of denatured protein were also investigated (Table 2). It was found that 4℃ and 300 μg mL^{-1} were the most appropriate.

Several methods, including dilution, dialysis, diafiltration, gel filtration and immobilization onto a solid support, may be employed to remove or reduce excess denaturing agents, allowing proteins to renature (Clark, 1998). The simplest and most widely used method for refolding is dilution. But the method of direct dilution leads to large volume of solutions and needs large volume of container, which is impractical for preparation. A 'pulsed renaturation' method, whereby the denatured protein is added to the refolding buffer in pulses (Rudolph and Fischer, 1990), is useful for preparative work (Middelberg, 2002). In pulsed dilution, after an aliquot of denatured protein solution is diluted into a refolding solvent, refolding is allowed to occur for 30 min before addition of the next aliquot (Tsumoto *et al*., 2003). In our experiment, after addition of 3 aliquots of denatured AgaB to the refolding buffer, the solution was kept at 4℃ with stirring to avoid high local concentration of protein, which may result in aggregation. The final protein concentration was up to 0.03 mg mL^{-1}. In order to check if the protein folded correctly, the diluted solution was concentrated by ultra-filtration to remove additives and the unfolded protein (Fig.3b). The measurement of activity and protein concentration of the refolded protein showed that about 40 mg pure enzymes with a high activity of 1600 units (mg protein)$^{-1}$ were obtained from 1 L culture.

Table 2 Establishment of optimal refolding conditions of denatured AgaB

Parameter	Effect
Detergents	
Tween 20	−
Temperature	
4 ℃	+
25 ℃	Mild Aggregation
30 ℃	Aggregation
37 ℃	Aggregation
Protein concentration	
300 μg mL^{-1}	+
>300 μg mL^{-1}	Aggregation
Denaturant	
1 mol L^{-1} urea	+
L-Arginine	
500 mmol L^{-1}	+++
Glycine	
10%	++

Notes: RT, room temperature; '−', no effect on refolding; '+', an increased yield of soluble protein.

3.4 Enzymatic Properties of Refolded AgaB

The properties of the refolded protein are similar to those of the native. The optimal temperature and pH of the refolded protein were 40℃ and pH 6.0, respectively. Refolded AgaB was very stable when pH changed from 5.7 to 10.6. Cu^{2+}, Mn^{2+} and EDTA were found to inhibit its activity, whereas Ca^{2+} and Ba^{2+} slightly increased its activity (data not shown). The hydrolyzing product of refolded AgaB was consistent with that of the native (Fig.4). It hydrolyzes agarose, producing neoagarooctaose and neoagarodecaose as the major end products.

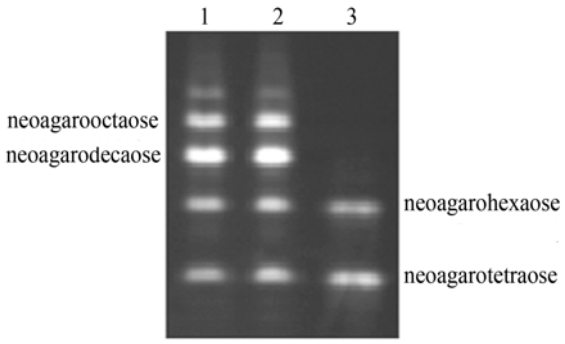

Fig.4 FACE analysis of hydrolysis product of agarose by refolded AgaB. Lane 1, hydrolysis products of agarose by native AgaB; Lane 2, hydrolysis products from agarose by pure refolded AgaB; Lane 3, neoagarotetrose (DP2) and -hexaose (DP3) (Sigma) as the standard.

Conclusion

We have developed an efficient approach to produce large amount of AgaB proteins. Even though we did not scale-up the production, a significant amount of pure

proteins could be produced cheaply and simply under moderate fermentation condition. About 40 mg pure enzymes with a high activity of 1600 units (mg protein)$^{-1}$ were obtained from 1 L culture. It is expected that the production of AgaB on an industrial scale may be realized.

Acknowledgements

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