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Purification and Comparison of Properties of the C-Terminus Truncated Agarase of *Pseudomonas* sp. W7

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Abstract Three plasmids derived from the β-agarase gene (*pjaA*) of *Pseudomonas* sp. W7 were expressed in *Escherichia coli* AD494(DE3) pLysS with lactose as an inducer. These products corresponded to the complete (PjaA) and the two C-terminal truncated (PjaAI and PjaAII) forms of β-agarase. The PjaAI and the PjaAII were originated from exonuclease III treatment from PjaA by deleting 127 and 182 amino acid residues-encoded nucleic acids at 3' region, respectively. The molecular weights of the purified proteins were 71 kDa, 58 kDa, and 50 kDa on SDS-PAGE, respectively. The K_m value of PjaAI was lower than that of the PjaA, and the catalytic efficiency (k_{cat}/K_m) of PjaAI was increased to 5 times. The enzyme of PjaAI retained more than 90% activity at 50°C. In contrast to the PjaAI, the remaining activity of the PjaA was only 20% at the same temperature.

Key words: Agarase, overexpression, C-terminal deletion, *Pseudomonas* sp.

Agarases are produced by a number of different organisms, from bacteria to algae. In bacteria, agarases have been isolated from *Pseudomonas* sp. [3, 7], *Pseudoalteromonas* sp. [22], *Alteromonas* sp. [1, 16], *Cytophaga* sp. [21], *Bacillus* sp. [9], and *Vibrio* sp. [2, 20] with a diverse range of molecular weights and biochemical properties. The agarases are divided into two groups by their mode of action as α-agarase and β-agarase.

The studies conducted on agarases have been focused on the biochemical properties, including substrate degradation and cloning to reveal the nucleotide sequences of agarases from a diverse range of organisms. We have also characterized some biochemical properties and nucleotides of the agarase gene from *Pseudomonas* sp. W7 and revealed some important features. This agarase (PjaA) was classified as β-agarase by their substrate degrading activity and showed a halophilic property. The agarase activity was the highest with 0.9 M NaCl concentration, and this was an extremely high concentration, compared with agarase from *Pseudomonas* sp. In addition, the alignment of amino acid sequences revealed a high similarity with the known amylases [7, 10].

In many amylases, the C-terminal region plays an important role in enzyme function. Some truncations have been reported to affect the thermostability, substrate binding, and catalytic ability of the enzyme [5, 14, 17]. Furthermore, the proteolytic cleavage of the C-terminal region was induced after germination, resulting in the change of thermostability. Some amylases also show changes of the biochemical properties by C-terminal deletion. β-Amylase also undergoes proteolytic germination in the C-terminal region and shows increased affinity for starch and increased thermostability [11]. α-Amylase from *Altermonas haloplanctis* also has a long propeptide in its C-terminal region, which helps secretion of the enzyme to extracellular space in *E. coli* [6]. In spite of the resemblance of amino acid sequences between the PjaA protein and amylase, the role of the C-terminal region for the agarase activity has not yet been identified. In this study, we purified the C-terminal truncated agarases after overexpression in *E. coli* and investigated the relationship between the C-terminal lengths and the enzyme properties.

Materials and Methods

Overexpression of pEAG3 and its Derivatives

The recombinant plasmids were previously constructed [10]. *E. coli* strains harboring the recombinant plasmids (Table 1) were grown at 37°C in LB broth with gentle shaking. One percent (v/v) of overnight cultured cells was

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Table 1. *E. coli* strains and plasmids used in the present study.

Bacterial strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
E. coli		
AD494(DE3)pLysS	*Δara-leu7697 ΔlacX74 ΔphoAPvuII phoR ΔmalF3* F'[$lac^+(lacI^q)pro$] *trxB*::*kan*(DE3) pLysS (Cm^R)	Novagen
BL21(DE3)	F *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	Novagen
Plasmids		
pET22b(+)	His tag fusion expression vector; T7 promoter, six His tag coding sequence	Novagen
pEAG 3	pET22b(+) containing a 1,926 bp fragment of *pjaA*	[10]
pEAG 3-1	381 bp deletion from pEAG3	[10]
pEAG 3-2	546 bp deletion from pEAG3	[10]

reinoculated to 1 l of LB broth, and lactose was added to a final concentration of 1 mM when the absorbance at OD_{600} reached 0.6. After 3 h for induction, the cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl (pH 8.0), and disrupted by sonification on ice. The soluble fractions were collected by centrifugation at 10,000 ×g for 30 min, and they were applied to Ni-IDA affinity chromatography (Novagen, Madison, WI, U.S.A.). The protein was eluted with 20 mM Tris-HCl (pH 8.0) containing 0.5 M imidazole [8, 13, 15]. Finally, the collected elutants were dialyzed in 20 mM Tris-HCl (pH 8.0) at 4°C. SDS-PAGE was used to estimate the amount of expressed proteins and the molecular weight of purified agarases.

Protein Quantification and Enzyme Assays

The concentrations of total proteins in the cell extracts and the purified enzymes were determined by the Bradford method [4], using bovine serum albumin (Sigma, MO, U.S.A.) as a standard.

Agar plate containing 1% (w/v) agar in 20 mM Tris-HCl (pH 8.0) and 0.9 M NaCl was used to detect the agarase activity. Twelve μg of the purified enzymes were applied on the agar plate and incubated at 37°C for 12 h. The enzyme activity was detected by the transparent halo on the plate, which was visualized by adding the Lugol (0.05 M I_2 in 0.12 M KI) solution.

Kinetics Determination

The agarase activity was also determined by the Somogy-Nelson method [18]. As a substrate solution, 0.3% (w/v) agar was completely melted in 20 mM Tris-HCl (pH 8.0) and 0.9 M NaCl. The agarase activity was assayed by measuring the absorbance at OD_{510}, where (+) galactose was used as a standard. One unit of the enzyme was defined as the amount that liberated 1 μmole of reducing sugar per min. The kinetic parameters were determined by measuring the initial velocities of the enzyme reactions under different concentrations of agar solutions by using the standard Michaelis-Menten approach. The Lineweaver-Burk plot was used to calculate the Michaelis constant (K_m) and the velocity of reaction (V_{max}).

Thermal Stability

Thermal stability was determined by measuring the residual activities after preincubating the enzymes at different temperatures. Twelve μg of purified enzymes were preincubated for 30 min at temperatures ranging from 40°C to 80°C. Then, the reaction mixtures were incubated for 1 h at 37°C with the agar solution. The enzyme activity was determined by using the standard enzyme assay described above.

RESULTS

Production of Agarases

In our previous reports, we described the deletion of the agarase gene by exonuclease III to generate the 3' deleted mutants pEAG3-1 and pEAG3-2 from pEAG3 and the detection of the overexpressed proteins (PjaA, PjaAI, and PjaAII) by using *E. coli* BL21(DE3) [10]. In this study, these agarase genes were transferred into *E. coli* AD494 (DE3)pLysS, which was a mutant deficient in the *trxB* gene encoding the thioredoxin reductase, to permit the potential formation of disulfide bond in *E. coli* [23]. In addition, lactose was used as an inducer for overexpression

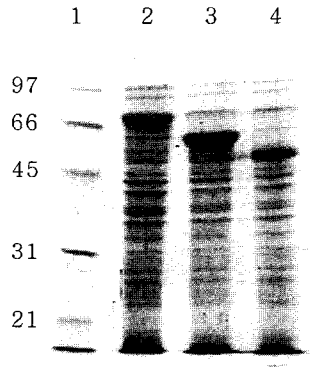

Fig. 1. SDS-PAGE of overexpressed agarases from *E. coli* AD494(DE3)pLysS harboring pEAG3 and the truncated plasmids. Lane 1: molecular weight marker; lane 2: PjaA (pEAG3); lane 3: PjaAI (pEAG3-1); lane 4: PjaAII (pEAG3-2).

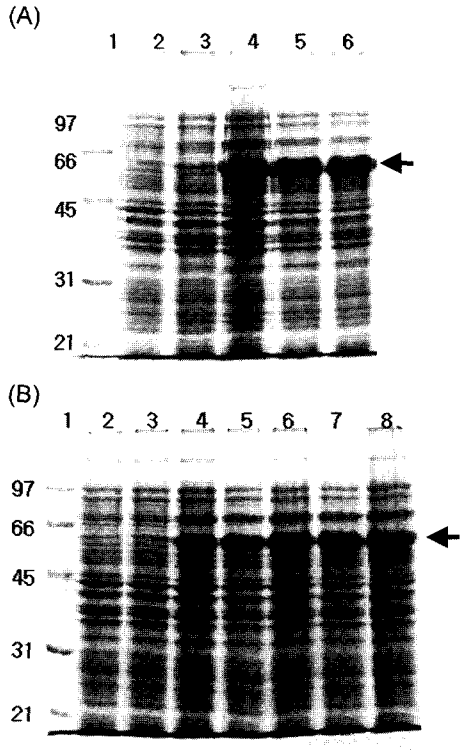

Fig. 2. Effect of lactose concentrations on the expression of agarase (A) and time-course yield (B) of the PjaAI produced from *E. coli* AD494(DE3)pLysS.
(A) Lane 1: molecular weight marker; lane 2: no lactose; lane 3: 0.1 mM lactose; lane 4: 0.5 mM lactose; lane 5: 1 mM lactose; lane 6: 10 mM lactose. (B) Lane 1: molecular weight marker; lane 2: not induced; lane 3: 1 h; lane 4: 2 h; lane 5: 3 h; lane 6: 4 h; lane 7: 5 h; lane 8: 6 h.

of the corresponding genes. The overexpressed agarases in *E. coli* AD494(DE3)pLysS were identified by SDS-PAGE of cell lysates (Fig. 1). The amount of proteins expressed in *E. coli* BL21(DE3) and AD494(DE3)pLysS was similar, but specific activities of the total cell lysates were 0.9 U/mg from *E. coli* BL21(DE3) and 1.5 U/mg from *E. coli* AD494(DE3)pLysS.

This result suggested that the internal cysteine bonds in agarase were probably influenced by the deficiency of thioredoxin reductase. Furthermore, the agarase activity by lactose induction was about 2-folds higher than that by IPTG induction in *E. coli* AD494(DE3)pLysS. It has been known that the overexpression by lactose has a potential risk on the repression of expression by glucose, because glucose can be generated from lactose in *E. coli* [19]. To examine the influence of lactose concentrations and cultivation times on the expression of agarase, we cultivated *E. coli* AD494(DE3)pLysS harboring pEAG3-1 plasmid with the elevated concentrations of lactose up to 10 mM. When 0.5 mM to 10 mM lactose was added to the medium, the expressions of agarase protein were not affected by the lactose (Fig. 2A). The expression of PjaAI protein induced with 0.5 mM lactose for various induction times was investigated. The agarase protein accumulated in the cytoplasm after 2 h. Based on the induction, any repression by lactose could not be found (Fig. 2B), thus indicating that lactose is actually a good inducer for the overexpression of agarase in this system.

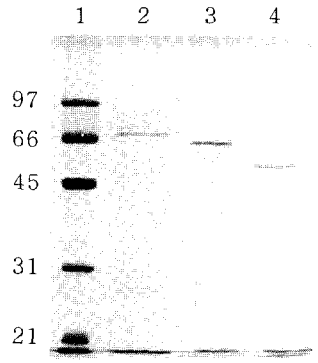

Fig. 3. SDS-PAGE of the purified agarases from *E. coli* AD494(DE3)pLysS harboring pEAG3 and the truncated plasmids.
Lane 1: molecular weight marker; lane 2: PjaA (pEAG3); lane 3: PjaAI (pEAG3-1); lane 4: PjaAII (pEAG3-2).

Purification of PjaA and Truncated Agarases

To investigate the effect of C-terminal deletion on the enzyme activity, we purified the truncated agarases (PjaAI, PjaAII) and the intact form of recombinant agarase (PjaA). The soluble fractions of cell lysates were loaded onto the Ni-IDA column and eluted with 0.5 M imidazole. The eluted agarases were dialyzed against 20 mM Tris-HCl (pH 8.0) and analyzed by SDS-PAGE (Fig. 3). The purified recombinant proteins exhibited a molecular size of 71 kDa,

Table 2. Comparison of agarase activities through the purification steps with various recombinant plasmids.

Plasmids		Inducing agent	Total protein (mg)	Total activity (units)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
pEAG3	Cell lysate	Lactose	51.7	77.9	1.5	100	1
	Purified enzyme		0.7	3.5	5.0	4.5	3.3
pEAG3-1	Cell lysate	Lactose	50.8	101.6	2.0	100	1
	Purified enzyme		1.1	34.1	31.0	33.6	15.5
pEAG3-2	Cell lysate	Lactose	52.7	5.5	0.1	100	1
	Purified enzyme		0.6	0.7	1.2	12.7	12.0

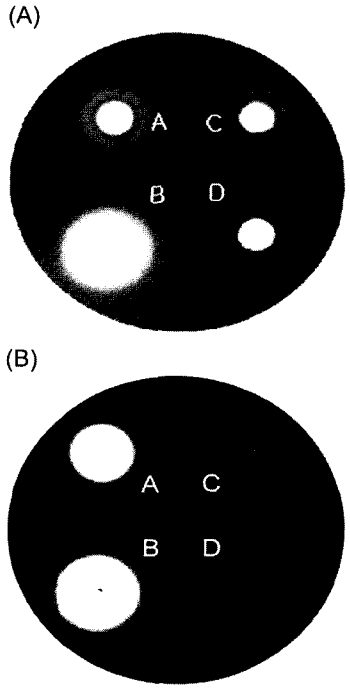

Fig. 4. Agarase activities of the purified proteins (20 μg) from *E. coli* AD494(DE3)pLysS (A) and colonies harboring recombinant plasmid (B).
(A) A: PjaA (pEAG3); B: PjaAI (pEAG3-1); C: PjaAII (pEAG3-2); D: 20 mM Tris-HCl (pH 8.0). (B) A: PjaA (pEAG3); B: PjaAI (pEAG3-1); C: PjaAII (pEAG3-2); D: pET22b (+).

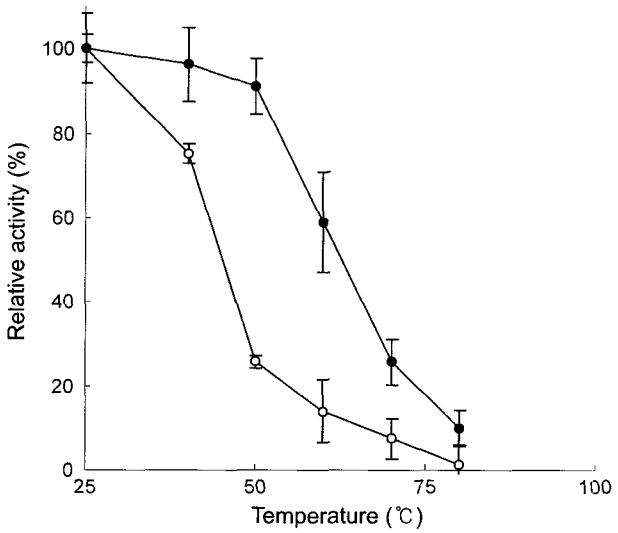

Fig. 5. Stabilities of the PjaA and the PjaAI proteins at the various temperatures.
Each enzyme was preincubated at different temperatures for 30 min and the residual activity was measured. The open circle means the agarase activity of PjaA, and the solid circle means activity of PjaAI.

58 kDa, and 50 kDa, respectively, which agreed with the sizes calculated from the nucleotide sequences. Table 2 shows the purification steps with Ni-IDA affinity chromatography of the truncated agarases. The purification yield and the specific activity were highly related to the lengths of the C-terminus. The specific activities of the three purified agarases (PjaA, PjaAI, and PjaAII) were 5.0 U/mg, 31.0 U/mg, and 1.2 U/mg, respectively. The agarase activities of the purified proteins were measured by using an agar plate. As shown in Fig. 4A, the PjaAI showed the highest enzyme activity. When the colonies were grown on the LB agar plate, the colony containing pEAG3-1 plasmid showed the largest halo (Fig. 4B). These results implied that the C-terminal region did not affect the agarase secretion into the extracellular space, but it was important for the enzyme activity to take place.

Thermostability of Agarase

The thermostabilities of PjaA and PjaAI were measured from the irreversible thermal inactivation curves (Fig. 5). The elevated temperatures led the intact form (PjaA) to decrease the agarase activity significantly. Only 20% of the activity were retained at the temperature of 50°C, whereas the PjaAI was still stable at this temperature and 60% of the agarase activity were retained at 60°C. The agarase from *Pseudoalteromonas* isolated from marine environment showed a similar thermostability pattern to the PjaA [22]. However, most agarases from marine microorganisms, except for thermophiles, are not stable at temperatures over 50°C [9].

Kinetics

The K_m and the K_{cat} values were calculated with a nonlinear regression analysis by using the hyper program (ver. 1.1s). The K_m values of PjaA and PjaAI were 10 mg/ml and 1.7 mg/ml, respectively, and the catalytic efficiency (K_{cat}/K_m) was 3.12 min^{-1} $(mg/ml)^{-1}$ and 15.4 min^{-1} $(mg/ml)^{-1}$, respectively. The difference in K_{cat}/K_m values reflected that the PjaAI protein was more efficient than the PjaA protein in the substrate binding and sequential reaction.

DISCUSSION

We have earlier characterized some biochemical properties and the nucleotide sequence of the agarase gene (*pjaA*) from *Pseudomonas* sp. W7. This agarase was classified as a β-agarase by their substrate degrading activity and showed the halophilic property [7, 10].

In this study, we investigated the agarase activities with the C-terminal truncated by using the *pjaA* gene of *Pseudomonas* sp. W7. The truncated plasmids were overexpressed in *E. coli*, and the products were purified through metal affinity chromatography. It is known that lactose is a cheap and nontoxic material for the overexpression of protein in the *lac* based promoter. When the overexpression

of agarase genes was performed by using lactose instead of IPTG, the result showed that lactose was very effective in expression of the active agarases in this system. However, we did not find any repression by lactose, and a large amount of agarase was overexpressed at various concentrations of lactose. 0.5 mM lactose was enough to express the agarase. From the purification results, we found that the lengths of C-terminus affected the binding capacity to Ni-IDA resin. The truncated agarases are likely to have a higher binding capacity than the intact one. The purification folds were 3.3 (PjaA), 15.5 (PjaAI), and 12.0 (PjaAII). It implied that the deletion of the C-terminal region might induce the modification of the structure and consequently the histidine tail at the C-terminal region bound more easily to Ni-ligand.

In a previous report, we showed that the amino acid sequence of PjaA (from 32 to 482 residue) is 45% identical to a mature form of α-amylase from *A. haloplanctis* [10]. To survey the possible active site, the amino acid sequences of α-amylases were aligned. In the α-amylase family, the functions have been assigned to three domain types: the catalytic $(\beta/\alpha)_8$ barrel (domain A), the protruding domain B, and the domain C [12]. Domain C is made up of β-strands and is thought to stabilize the catalytic domain by shielding hydrophobic residues of the domain A from solvent. The domain search program, CD (Conserved Domain Database and Search Service, Ver. 1.53 at NCBI), with All-3551-PSSMs database showed that the PjaA had two conserved domains in the α-amylase family. The catalytic domain A, which corresponded to the amino acid sequence F^{45}-R^{385}, and the conserved C-terminal β-domain (domain C), which corresponded to D^{402} and R^{477}, could be found in the PjaA protein. The PjaAII has the additional 55 amino acids deleted from the PjaAI. Compared to the position of these conserved domains, the PjaAI has A and C domains, whereas the PjaAII has domain A and a part of domain C. The enzyme activity and the thermostability of PjaAI were remarkably higher than those of the PjaA. The PjaAI had a 5.9-folds lower K_m value than that of the PjaA, and the catalytic efficiency (k_{cat}/K_m) of PjaAI was increased 5 folds.

In many amylases, the C-terminal region plays an important role in the enzyme function. Some truncations have been reported to affect the thermostability, substrate binding, and catalytic ability of the enzyme. Deletion of the C-terminal repeated sequence in *Lactobacillus amylovorus* α-amylase increased the K_m value for soluble starch and the intact form was more thermostable than the truncated amylase [17]. The C-terminal truncated form of α-amylase from *B. subtilis* X23 was thermostable [14]. The specific activity of CGTase of *B. macerans* was increased by point mutation in the C-terminal starch binding region, but the thermostability was lower than the native protein [5]. In some amylases, the proteolytic cleavage in the C-terminal region was induced after germination, resulting in a change of thermostability. Barley β-amylase also undergoes the proteolytic process of the C-terminal region during germination, with increased affinity for starch and enhanced thermostability [11]. In this study, the C-terminal region did not affect the secretion of enzyme. The increased enzyme activity and catalytic efficiency might be due to the change of the structure, resulting in easy access to the substrate. The PjaAII protein showed low enzyme activity. These observations suggested that the conserved domain C may be responsible for the alterations in the enzymatic properties and the thermostability of PjaA to some extent.

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