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Review

Poly-γ-glutamate synthetase of *Bacillus subtilis*

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

Poly-γ-glutamate (PGA) is a most promising biodegradable polymer. In extracellular mucilage-producing *Bacillus subtilis*, the *pgsBCA* genes encode the membrane-associated PGA synthetase complex. It was recently speculated that PGA synthetase consists of both the intact 44 kDa and the in-phase overlapping 33 kDa-*ywsC* (corresponding to *pgsB*) gene products. This review covers current research into *B. subtilis* PGA synthetase and discusses the structural and functional features of the enzyme.

Keywords: Poly-γ-glutamate; Synthetase; Amide ligase; Membranous enzyme; Complex; *Bacillus subtilis*

1. Introduction

Poly-γ-glutamate (PGA) is an unusual anionic polypeptide in which D- and/or L-glutamate is polymerized via γ-amide linkage (Fig. 1). Therefore, it is an optically active polymer having a chiral center in every glutamate unit. So far, many PGA producers have been found. *Bacillus anthracis* produces PGA composed only of D-glutamate, i.e. D-PGA, as the main component of the capsule [1]. DL-PGA, composed of 50–80% D- and 20–50% L-glutamate [2,3], is abundantly contained in the extracellular mucilage of *Natto*, a traditional Japanese fermented food made from soybeans by *Bacillus subtilis* (formerly *Bacillus natto*). In addition, the *B. subtilis* PGA is usually obtained as a mixture of the polymers with apparently various molecular masses (10–1000 kDa), as PGA depolymerases accumulate in the culture medium during PGA production [4]. *Bacillus licheniformis* produces stereochemically various PGAs (D-glutamate contents, 10–100%) [5]. *Bacillus megaterium* also synthesizes DL-PGA (D-contents, 20–50%) with a high-molecular-mass (about 1000 kDa) [6]. Comparatively low-molecular-mass L-PGA (about 10 kDa) was isolated from alkalophilic *Bacillus halodurans* [7]. It was recently reported that *Natrialba aegyptiaca*, an extremely halophilic archaeon, produced highly elongated L-PGAs (>1000 kDa) extracellularly [3,8]. *Natronococcus occultus* is also an L-PGA-producing archaeon [9]. In eukarya, *Hydra* produces L-PGA [10]. PGA is thus quite various in stereochemical composition and molecular size.

PGA is substantially non-toxic to humans and the environment and even edible. The potential applications of PGA and its derivatives have been the focus of study by various industries. The ester derivatives of PGA have potential as biodegradable substitutes for currently used non-biodegradable materials:

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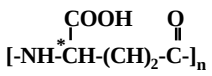

Fig. 1. Structure of poly-γ-glutamate (PGA). The chiral center of the glutamate unit in PGA is indicated by an asterisk.

thermoplastics, fibers, films, and membranes [11]. Its derivatives, however, remain to be improved, as DL-PGA that is currently available shows irregular stereochemistry. Generally, the thermoplasticity of degradable polyesters is significantly influenced by the homogeneity of the stereochemical composition [12]. Other PGA derivatives, which exhibit very high water-absorption capability and serve as both flocculants and heavy metal- and radionuclide-binding agents, have also been developed [2]. Many attempts, therefore, are being made to develop such environmental uses of PGA. Furthermore, a wide range of unique applications of PGA, including the use as cryoprotectants, bitterness-relieving agents, thickeners, animal feed additives, osteoporosis-preventing factors, humectants, drug deliverers, gene vectors, curative biological adhesives, dispersants, and enzyme-immobilizing materials, have been established [2,13].

As described earlier, PGA is indeed a most promising biopolymer, but, for the acceptance of the polymer in practical, industrial uses, two major problems remain to be solved: how to control its structural diversity and how to produce it in more abundance and at a moderate price. This review deals with recent information on *B. subtilis* PGA synthetase, which would not only deepen the understanding of the mechanism of PGA synthesis but also provide an insight into the development of PGA utility.

2. The PGA synthetase-encoding genes of *B. subtilis*

2.1. γ-Glutamyltranspeptidase, which is irrelevant to the PGA synthesis

First, we review recent information on γ-glutamyltranspeptidase (GGT), which has been assumed to function as the PGA synthetase in *B. subtilis* [14]. It is well known that many PGA-producing *B. subtilis* possess small cryptic plasmid(s) [15], unlike *B. subtilis* 168, which is assumed not to produce PGA [16]. Hara et al. [17] reported that the *B. subtilis* (*natto*) plasmids encode the GGT responsible for PGA synthesis. The GGT, however, shows no similarity to the *B. subtilis* (*natto*) GGT identified later [18]. A recent study proved that the curing and transformation of the plasmids have no effect on the PGA productivity of *B. subtilis* [2,19]. Plasmid-free PGA producers of *B. subtilis*, e.g. *B. subtilis* (*chungkookjang*), have been also isolated [20]. Thus, plasmids do not encode any gene that is important for PGA synthesis.

Due to the many inconsistencies in reports of GGT by Hara et al. [16,17,21], the roles of GGT in PGA synthesis remained uncertain. Abe et al. [4] reported that the GGT purified from the culture media of *B. subtilis* (*natto*) digested PGA in an exopeptidase-like fashion. GGT can be considered an extracellular PGA *exo*-depolymerase. Inconsistency between GGT activities and PGA productivities in various PGA producers was also demonstrated [22]. It was recently reported that the PGA productivity of the *B. subtilis* (*natto*) mutant defective in the *ggt* gene remains unchanged [2]. Cloning and disruption experiments of the PGA synthetase-encoding genes (see later; [23,24]) as well as the recent studies on the *B. subtilis* GGT [2,4,22], have entirely disproved the speculation that GGT functions as *B. subtilis* PGA synthetase.

2.2. Identification of the PGA synthetase-encoding genes

A genuine PGA synthetic system (i.e. a PGA synthetase) from *B. subtilis*, which synthesizes in vitro high-molecular-mass PGA, has not been purified due to its extreme instability [25]. Ashiuchi et al. [23] introduced a genetic approach into the study of the PGA synthetase and for the first time identified an operon responsible for the PGA synthesis of *B. subtilis*. The *pgs* operon (*pgs*: poly-γ-*g*lutamic acid *s*ynthesis; accession no. AB016245) is composed of three genes, *pgsB*, *-C*, and *-A* (Fig. 2), which are highly homologous to *B. anthracis capBCA* genes [14]. The *Escherichia coli* clone harboring all of the *pgsBCA* genes produces extracellular PGA [23], whereas the *pgs* null mutant of *B. subtilis*, designated hereafter *B. subtilis* MA-11, cannot synthesize the polymer [24]. Accordingly, the *pgs* operon, i.e. *pgsBCA*, encodes the sole machinery for the production of extracellular PGA in *B. subtilis*.

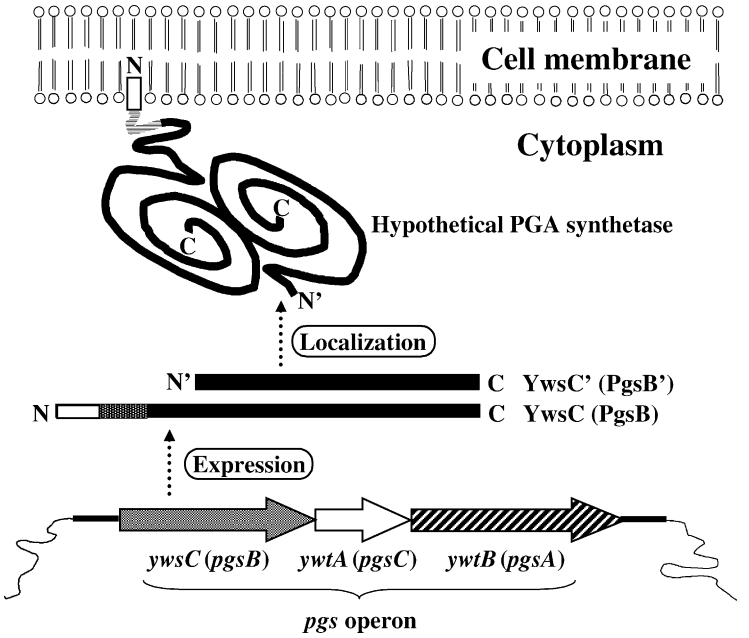

Fig. 2. Expression of the hypothetical PGA synthetase in *B. subtilis*. The YwsC protein is the intact 44 kDa-*ywsC* (*pgsB*) gene product with the membrane-anchoring (shown by a white column) and the ATP-binding (shown by a gray column) sequences at its N-terminus, but the YwsC′ protein is the in-phase overlapping 33 kDa-*ywsC* (*pgsB*) gene product lacking these important regions in catalysis and localization. The hypothetical PGA synthetase consists of the YwsC and YwsC′ proteins [26].

3. A hypothetical PGA synthetase

Recently, the *ywsC* gene, which corresponds to the *pgsB* gene in the *pgs* operon, was found to encode both the intact 44 kDa protein, YwsC, and the in-phase overlapping 33 kDa protein, YwsC′ [26]. As shown in Fig. 2, the YwsC protein possesses the membrane-anchoring and the ATP-binding sequences at its N-terminus, but the YwsC′ protein lacks these important regions in catalysis and localization. Urushibata et al. [26] proposed that PGA synthetase (EC 6.3.2.-) consists of both YwsC and YwsC′ proteins, namely YwsCC′, and described that this hypothetical PGA synthetase is quite stable and resistant to a high concentration of detergent, e.g. 0.2% Triton X-100, contrary to findings in previous reports [24,25]. They also reported that the YwsCC′ enzyme produced PGA directly and effectively from only the L-glutamate monomer in the presence of Mn^{2+}. As the essential nature of the *ywsC* gene in the production of DL-PGA of *B. subtilis* (*natto*) has been shown [26], the enzymatically-synthesized PGA would be

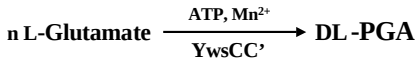

Fig. 3. Proposed reaction mechanism of the hypothetical PGA synthetase, YwsCC′.

the DL-copolymer (Fig. 3). It has been proved that the *pgsB* (corresponding to *ywsC*) gene product does not catalyze the glutamate isomerization [23,24]. The structural analysis of the synthesized PGA must give a clue to the interesting reaction mechanism of the hypothetical PGA synthetase, YwsCC′. Nevertheless, the sound data, including the stereochemistry, molecular size and yield of the PGA, have yet to be shown.

4. Re-characterization of the hypothetical PGA synthetase

Ashiuchi and his co-workers have found that the hypothetical PGA synthetase, YwsCC′, is produced in cells of the *E. coli* clone harboring the *ywsC* (*pgsB*)

Table 1
Enzymatic PGA synthesis with the hypothetical PGA synthetase, YwsCC′, and the membrane-associated proteins from *B. subtilis* (*chungkookjang*) and *B. subtilis* MA-11

Biocatalyst	PGA synthesis[a]
The purified YwsCC′	–[b]
The membrane-associated protein from *B. subtilis* (*chungkookjang*)	+[c]
The membrane-associated protein from *B. subtilis* MA-11	–

[a] These enzymatic PGA syntheses were conducted according to the method of Urushibata et al. [26].
[b] High-molecular-mass PGA was not synthesized.
[c] High-molecular-mass PGA was synthesized.

gene and have purified it to apparent homogeneity by the method of Urishibata et al. [26]. The fact that high-molecular-mass PGA is produced by the *E. coli* clone where the *pgs* operon including the *ywsC* (*pgsB*) gene was expressed [23] implicates that all of the *pgs* gene products including YwsCC′ can fold into active forms in the *E. coli* clone cells. In addition, *E. coli* cells substantially exhibit no PGA productivity; thus, their use as host cells is more advantageous to avoid contaminating genuine PGA than the use of *B. subtilis* (*natto*) cells showing a high PGA productivity [26]. The PGA synthesis by the purified YwsCC′ was carried out according to the procedures reported previously [26]. The observation, contrary to what would be expected, indicated that the purified YwsCC′ had little activity for PGA synthesis under any conditions tested (Table 1). Their attractive hypothesis, therefore, has not been re-conformed yet.

5. Enzymatic PGA synthesis by the cell membrane-associated protein from *B. subtilis*

More recently, the first success in the enzymatic synthesis of highly elongated PGA (>1000 kDa) with the cell membrane-associated protein of *B. subtilis* (*chungkookjang*), but not with its cytoplasmic protein, in the presence of ATP and glutamate was reported (Table 1; [2,20]). When D-glutamate was used as the substrate, the elongated PGA chain consisted only of D-glutamate; when L-glutamate was used, the elongated chain was composed only of L-glutamate. These findings provided deep insights into the synthesis of the structurally controlled PGAs. On the other hand, the fact that the PGA synthetic activity is completely lost in the presence of a high concentration of detergent, e.g. 0.2% Tween X-100, suggests that either the membranous PGA synthetase is probably maintained at an active form through the interaction with cell membranes or a certain essential factor in catalysis is readily librated from the membranes by detergent, or both. PGA was not synthesized by the membrane-associated protein of *B. subtilis* MA-11 (Table 1), indicating that the *pgsBCA* gene products are responsible for the membranous PGA synthetic activity.

6. Biochemical analysis of the membranous PGA synthetase

6.1. Probable reaction mechanism

Previously, Troy [25] and Gardner and Troy [27] proposed a reaction mechanism of a membranous D-PGA synthetase from *B. licheniformis* that is closely similar to those of multienzyme systems [28] such as Gramicidin S synthetase, which was accompanied by the cleavage of ATP into AMP. According to the mechanism, ATP first activates L-glutamate and L-glutamyl-γ-adenylate is then formed. This activated form of L-glutamate is bound to a catalytically essential sulfhydryl group in the enzyme (or in an acceptor) and isomerized into the D-isomer. The γ-D-glutamyl moiety was transferred to a growing poly-γ-D-glutamyl chain bound to another sulfhydryl group of the synthetase (or an acceptor). The elongated D-PGA chain was eventually transferred to the NH_2 terminus of an endogenous D-PGA acceptor. Until recently, this hypothesis has been believed without any confirmation.

However, the enzymologic analyses of the *pgsBCA* gene products revealed that ATP is hydrolyzed into ADP but not into AMP in PGA synthesis [24]. It is assumed that the membranous PGA synthetase from *B. subtilis* belongs functionally to the amide ligase family [29]. Fig. 4 illustrates the probable reaction mechanism of the *B. subtilis* PGA synthetase. According to the general principal of amide ligation, the phosphoryl group of ATP is first transferred to a terminal carboxyl group of an acceptor, e.g. low-molecular-mass PGA, through substrate-dependent ATP hydrolysis; then, an

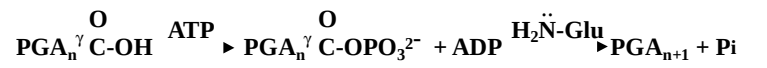

Fig. 4. Proposed reaction mechanism of the membranous PGA synthetase from *B. subtilis*.

amide linkage is formed by nucleophilic attack of an amino group of either a D- or L-glutamate monomer (as a donor) into the phosphorylated carboxyl group. The main chain of PGA is highly elongated when a series of the reaction occurs iteratively and successively at an active site of the enzyme. A bacterial polyamino acid other than PGA, i.e. cyanophycin, is abundantly accumulated into cells of cyanobacteria and algae [30]. Detailed analysis of the purified cyanophycin synthetase, an enzyme belonging to the ligase family [29], demonstrated that low-molecular-mass cyanophycin is essential as the primer molecule for the synthesis of the highly elongated cyanophycin [31]. For the enzymatic PGA synthesis, the acceptor described earlier would correspond to such primer molecule. It remains to be investigated whether low-molecular-mass PGA functioning as the primer molecule in catalysis exists in *B. subtilis* cells, especially in the membranes.

6.2. Function of each Pgs component

Based on the kinetic data of the glutamate-dependent ATPase activities of the PgsB, -C, and -A components and their structural features, the functions of each Pgs component have been proposed [24]. The PgsA component functions as the PGA transporter that effectively removes the reaction products charged highly negatively (eventually PGA) from an active site of the enzyme and is important for the enlargement (elongation) of PGA. A structural feature seen in amide ligases [29] has been found in the PgsB component. Orthologues of the PgsC component, the most hydrophobic component [24], have yet to be identified from organisms other than PGA producers. It is suggested that an active site of the PGA synthetase is constituted mainly of PgsB and -C. Generally, amide ligases show strict stereospecificity for amino acid substrates [29], but the PGA synthetase, PgsBCA, can recognize both enantiomers of glutamate as the substrate. Accordingly, the membranous PGA synthetase from *B. subtilis* is comformationally unique and atypical among amide ligases studied so far.

7. Future prospects

PGA possesses enormous potentialities as a new macromolecular material. However, for the practical use of PGA, there are some problems to be solved. First, the cost for PGA production is estimated to be much higher than that of conventional thermoplastic materials. Next, it is quite difficult to synthesize PGA by means of modern industrial (organic) chemistry. Nevertheless, a mass-production system of PGA may be established by the application of current molecular enzymology. The demands of public opinion will also push for the development of such earth-friendly technology. Besides, the detailed analyses of PGA synthetases would deepen the understanding of the general principles in the syntheses of highly elongated, structurally and functionally modified peptides and might allow the design of an unprecedented protein-synthesis system that is independent on living bodies, on cells, and even on DNA and presumably even the creation of atypical but functional proteins, e.g. an enzyme consisting only of D-amino acids. We hope that this review contributes to the further developments of the enzymatic syntheses of naturally occurring polyamino acids including PGA and to the establishment of a new field in biochemistry, namely green-technology or green-engineering.

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