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Poly-γ-glutamic Acid Biosynthesis by a Novel γ-glutamyltranspeptidase from *Bacillus cereus*

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Abstract: A novel γ-glutamyltranspeptidases (GGT) for the synthesis of poly-γ-glutamic acid (γ-PGA) is presented. Soil samples from the mangrove sediment were collected. One strain designated L-12 was identified whose cell extract could convert glutamine into γ-PGA. The 16S rDNA gene analysis of L-12 revealed its phylogenetic relatedness to *Bacillus cereus*. The GGT produced by L-12 was purified 20.3-fold. The optimum pH and temperature for γ-PGA synthesis by this GGT were 7.5 and 20°C, respectively. The results indicated that L-12 could be used as a novel bacterial GGT source for synthesizing γ-PGA production. To our knowledge, this is the first report for GGT to γ-PGA biosynthesis.

Key words: Poly-γ-glutamic acid, biosynthesis, γ-Glutamyltranspeptidases, *Bacillus cereus*, glutamine

INTRODUCTION

Poly-γ-glutamic acid (γ-PGA) is a natural biopolymer consisting of D- or L-glutamic acid units which are connected by amide linkages between α-amino and γ-carboxyl groups. With its biological properties such as water solubility, biocompatibility, nontoxicity, edibility and degradability, γ-PGA possesses various applications in food, cosmetics, medicine, agriculture and the environment (Zhang *et al*., 2012a).

The γ-PGA can be produced by chemical synthesis, microbial transformation and enzymatic synthesis. Presently, most studies about the production of γ-PGA were focused on microbial transformation by the several *Bacilli* species (Zhang *et al*., 2012b). However, this method has several disadvantages, such as increased viscosity of medium upon γ-PGA production which could limit cells growth, the instability of γ-PGA Mw according to a range of factors and amounts of by-products in the culture mixture which lead to difficulty in waste management and product purification (Yong *et al*., 2011). Therefore, the enzymatic synthesis process is regarded as another environmentally friendly strategy for γ-PGA production.

γ-Glutamyltranspeptidases (GGTs, EC 2.3.2.2) which are widely distributed in the organism, catalyze not only the cleavage of the γ-glutamyl bond of γ-glutamyl compounds, but also the transfer of their γ-glutamyl group to other amino acids or peptides (Imaoka *et al*., 2010). By employing various γ-glutamyl acceptors, some researchers have developed GGT as the biocatalyst to produce various functional γ-glutamyl compounds, such as γ-L-glutamyl-L-DOPA, γ-glutamyl taurine, γ-D-glutamyl-L-tryptophan, β-N-(γ-L(+)-glutamyl) phenylhydrazine, γ-glutamyl ethylamide (L-theanine) and γ-glutamyl glutamine (Shuai *et al*., 2011). However, these γ-glutamyl compounds are all dipeptides compounds and it has not been reported to obtain highly homogeneous polymer from GGT, due to its transpeptidation is a dynamic reversible reaction which has an optimum depending on reaction conditions and the intrinsic kinetic characteristics of the enzymes (Suzuki *et al*., 2007).

In the present research, we have found microbial strains which can produce GGT with novel properties for γ-PGA biosynthesis. As a result, a newly isolated candidate was identified as *Bacillus cereus*. The Mw of γ-PGA production by this enzyme is about 6.5 kDa.

MATERIALS AND METHODS

Chemicals: All chemicals were analytical grade and purchased from Sigma-Aldrich (China).

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Sreening for GGT producers: Screenings were carried out by the spread-plate methods. Samples collected from the mangrove sediment, Guangdong, China, were suspended in sterilized water and spread on selective medium agar plates (5% glutamine, 0.5% $NaHPO_4$, 0.3% KH_2PO_4, 5% NaCl, 0.01% $MgSO_4$, $7H_2O$, 0.02% $CaCl_2$, 0.01% γ-glutamyl-p-nitroanilide (γ-GpNA) and 2% agar, pH 6.0~7.0). After incubation at 30°C for 2~3 days, colonies which exhibited yellow were selected and streaked on additional new plates until homogenous colonies were observed. The purified strains were stored at 4°C.

Preparation of cell extracts: The strains were grown in an Erlenmeyer flask containing the liquid medium (1% glutamine, 1% glucose, 0.5% yeast extract and 5% NaCl) for 48 h. Cells were harvested by centrifugation (10,000 g for 10 min at 4°C) and washed twice with cold 50 mM Tris-HCl buffer (pH 7.2) and the cell pellet were resuspended in one volume of the above buffer followed by sonication on ice. Lysate was centrifuged at 14,000 g for 10 min to remove debris. The supernatant was obtained for GGT analysis or purification.

Assay of GGT activity: GGT activity was assayed by measuring the amount of p-nitroaniline released from γ-GpNA. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of p-nitroanilide per minute with γ-GpNA as the substrate.

Identification of isolate strain: One microorganism, the GGT from which could convert glutamine into γ-PGA, was identified based on sequence analysis of 16S rDNA. Using consensus primers, the approximately 1.5-kb 16S rDNA sequence was obtained and aligned to submitted sequences in NCBI database using the basic local alignment search tool (BLASTN) system. The analyzed sequences were compiled in an alignment with MEGA 4.0 and the phylogenetic tree was then constructed with the neighbor-joining algorithm. The bootstrap values for constructed tree were based on 100 replicates.

Purification of GGT: The purification of GGT was performed according to the method of Shuai *et al.* (2011). The purity and molecular mass of the purified GGT was estimated with SDS-PAGE using a 5% stacking gel and a 12.5% resolving gel. This was followed by staining with 0.1% Coomassie brilliant blue R-250.

Γ-PGA biosynthesis and identification: The mixture of 20% (w/v) L-glutamine or D-glutamine, 100 U GGT and 100 mL 50 mM Tris-HCl buffer (pH 7.0) was incubated and shaken at 150 rpm for 6 h at 25°C. The reaction was stopped by boiling the mixture for 10 min. γ-PGA produced was monitored by SDS-PAGE analysis (Pereira *et al*., 2012) or ultraviolet (UV) spectrophotometric method (Zeng *et al*., 2012). To search for the optimum reaction pH and temperature for γ-PGA production by the partially purified GGT, the pH was varied from 6.0 to 8.0 and temperature was varied in the range of 10~30°C.

RESULTS AND DISCUSSION

Screening for strains producing GGT: In this study, we first screened the yellow color colonies, GGT producers, on the selective medium and analyzed the hydrolysis activity of each isolate using the γ-GpNA as substrate. Isolates with higher hydrolysis activities were chosen and their γ-PGA production abilities were then assessed by using glutamine as the substrates.

The SDS-PAGE analysis showed that one isolate, named L-12, exhibited γ-PGA productivity and the product were identified by comparison of that without the addition of GGT (Fig. 1). Although several microbial sources of GGT were reportedly able to convert into various γ-glutamyl compounds, these compounds are mainly dipeptides compounds, such as γ-L-glutamyl-L-DOPA, γ-glutamyl-L-taurine, γ-D-glutamyl-L-tryptophan and so on, limited to the enzymes' transpeptidation ability (Suzuki *et al*., 2007). In the present work, all the isolated GGT producers tested, L-12 was found to produce the GGT with synthesizing γ-PGA ability. To our knowledge, this is the first report for GGT to γ-PGA biosynthesis so far. This observation suggested that the mechanism of transpeptidation action of GGT might be different between various microorganisms. Furthermore, the conditions of γ-PGA biosynthesis by GGT from L-12 used in this study yielded a polymer with a median value of Mw about 6.5 kDa. The Mw of γ-PGA here is within the low end of the Mw range previously reported (10~1000 kDa), but the low Mw γ-PGA could be preferable for drug delivery applications, allowing diffusion of the molecules (Pereira *et al*., 2012).

Identification of the strain: Because of the synthesizing γ-PGA ability of the GGT from L-12, identification of the strain was carried out. The strain was identified as *Bacillus cereus* based on its 16S rDNA gene sequence (1,434 bp) which was aligned with sequences available in the GenBank databases at NCBI using MEGA4.0 software. The phylogenetic tree clearly demonstrated that the isolated strain has maximum homology (more than 96% match) with the strain *B. cereus* and further named as *B. cereus* L-12.

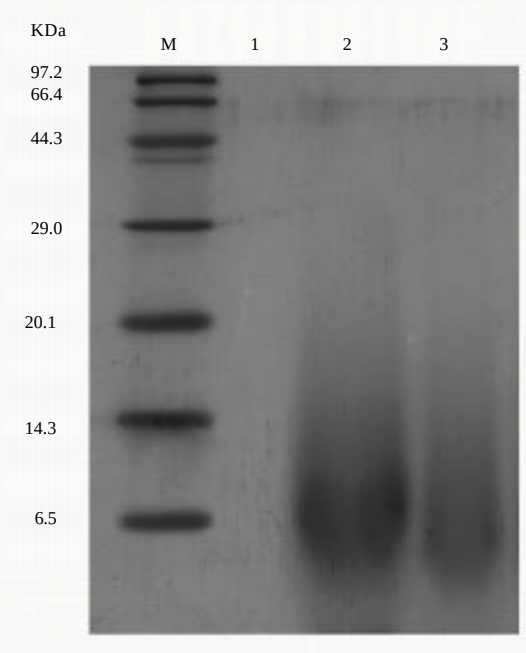

Fig. 1: SDS-PAGE for Mw estimation of γ-PGA produced by GGT from *B. cereus* L-12. Lane M, standard protein molecular mass marker (TaKaRa, sizes in kilodalton are indicated on the right); Lane 1: Without GGT; Lane 2: γ-PGA produced by cell extracts; Lane 3: γ-PGA produced by purified GGT

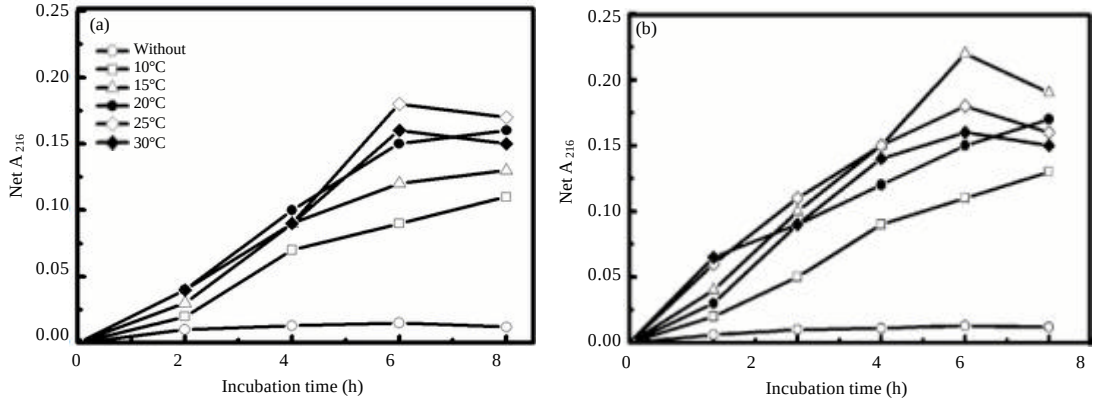

Fig. 2(a-b): Effect of (a) pH and (b) Temperature of the reaction mixture on γ-PGA synthesis. The reaction mixture contained glutamine (20%) and GGT (1 U mL^{-1})

Purification of GGT: The GGT was purified to 20.3-fold and the recovery of total activity was 31.1% by ammonium sulfate precipitation, DEAE-Sepharose FF column and Superdex-75 chromatography. The purified enzyme showed a single band by SDS-PAGE analysis which suggests that the GGT was purified to homogeneity.

Effect of pH and temperature on GGT activity from L-12 for γ-PGA production: To determine the optimal pH of the reaction mixture for the production of the γ-PGA, we measured the yield of γ-PGA at various pH values (pH 6.0-8.0). The reaction mixtures contained 20% glutamine and 1.0 U mL^{-1} GGT (final concentration), 50 mM Tris-HCl buffer and the pH were adjusted with NaOH. After incubation for several hours at 25°C, the reactions were terminated by boiling the mixture for 10 min. The concentration of γ-PGA was determined by UV spectrophotometry. The maximum yield of γ-PGA was

obtained at pH 7.5 after 6 h (Fig. 2a). The effect of temperature on the biosynthesis of γ-PGA was investigated. The pH of the reaction mixture was adjusted to 7.5 and 1.0 U mL^{-1} GGT (final concentration) was used. The highest yield of γ-PGA was obtained at 20°C after 8 h (Fig. 2b). There are many studies on γ-PGA biosynthesis, but the studies only focused on the main producers of γ-PGA, various *Bacilli* strains and improving conditions for γ-PGA productivity (Zhang *et al*., 2012a, b). No attention was paid to γ-PGA biosynthesis by enzymatic method. In our research, the enzymatic synthesis of γ-PGA catalyzed by GGT was demonstrated and the results display vast potential in industrialization.

CONCLUSION

In this study, the novel enzyme γ-glutamyltranspeptidases (GGT) of strain *Bacillus cereus* L-12 isolated from the mangrove sediment could be utilized to synthesize poly-γ-glutamic acid (γ-PGA) using glutamine as substrate. This enzyme therefore is a potential candidate for industrial production and applications. L-12 GGT is a novel enzyme that can be used in the enzymatic synthesis of γ-PGA. The maximum yield of γ-PGA was obtained at 20°C, pH 7.5, after 8 h. The Mw of γ-PGA production by this enzyme is about 6.5 kDa. The purified GGT exhibits a high molecular mass (85 kDa), possibly indicating it is a complex of γ-PGA synthetase. Further study is in progress to determine whether this high-molecular-mass GGT is a multidomain enzyme.

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