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Investigation on enzymatic degradation of γ-polyglutamic acid from *Bacillus subtilis* NX-2

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Abstract

The preparation of γ-polyglutamic acid (γ-PGA) from *Bacillus subtilis* NX-2 has been previously investigated, and its depolymerization during the batch culture was studied in this paper. The results suggested that the γ-PGA depolymerase was present and active extracellularly in the culture. The *ywtD* gene from *B. subtilis* NX-2, encoding the γ-PGA depolymerase was cloned and expressed in *Escherichia coli*. The YwtD protein was purified by metal-chelating affinity chromatography. YwtD was proved to be an endo-hydrolase enzyme and exhibited a remarkable activity in γ-PGA degradation at a wide range of temperature (30–40 °C) and pH (5.0–8.0). On an optimal condition of 30 °C and pH 5.0, an efficient γ-PGA enzymatic degradation was achieved. The molecular weight of γ-PGA could be reduced within the range of 1000–20 kDa and the polydispersity also decreased as a function of depolymerization time. Therefore, a controllable degradation of γ-PGA could be available by enzymatic depolymerization.

Keywords: γ-Polyglutamic acid; Depolymerase; *Bacillus subtilis*; Molecular weight; Polydispersity

1. Introduction

γ-Polyglutamic acid (γ-PGA) is a naturally occurring polyamide made of D- and L-glutamic acid monomers via γ-amide linkages. It is synthesized as the major component of capsules and slimes by several Gram positive bacteria (e.g. *Bacillus subtilis*, *B. licheniformis*, *B. anthracis*, *B. megaterium*) [1–3]. γ-PGA is water-soluble, biodegradable, edible and non-toxic toward human and environment. Recent research has reported that γ-PGA is potential as a good candidate for various applications [1–3]. A key property of γ-PGA required for practical applications is molecular weight. γ-PGA derived from microbial can have molecular weights ranging from 100 to over 1000 kDa [1,2]. To obtain γ-PGA with expected molecular weights, a substantial amount of effort has been devoted within the last few years to γ-PGA degradation by chemical and physical methods [4,5]. However, the poor control of the molecular cleavage as well as the increasing polydispersity during the hydrolysis prevents these methods from being satisfactory. The enzymatic degradation under mild conditions offered a controllable degradation of biopolymers and γ-PGA depolymerase could serve as a potent tool for such purposes.

Although γ-PGA is highly resistant to proteolytic attack because of its structural features, γ-PGA with various molecular masses (10–1000 kDa) are found in γ-PGA producing cultures [6]. It has been believed that, an enzymatic γ-PGA-degrading are operative at some point during growth and γ-PGA production. Birrer et al. found that cells of *B. licheniformis* ATCC9945 grown on poly(γ-D-glutamic acid) express a depolymerase activity, which seems to be associated with the cell surface [7]. King et al. successfully separated γ-PGA hydrolase that were bound to γ-PGA from *B. licheniformis* ATCC 9945a, and suggested that the polyglutamyl γ-hydrolase cleaves γ-PGA to γ-D-oligo-(glutamic acid) [8]. Kunioka and Goto observed that γ-PGA incubated with the cell-free broth of *B. subtilis* IFO3335 was depolymerized over time, in contrast to the former, the depolymerase seems to be excreted into the medium [9]. Tanaka et al. isolated and characterized an extracellular γ-PGA hydrolase from a filamentous fungus [10]. Suzuki and Tahara cloned the *ywtD* gene of *B. subtilis* IFO16449 in *Escherichia coli* which is located downstream of the γ-PGA biosynthesis genes (*ywtABC*) of *B. subtilis* 168 and characterized the novel γ-PGA-hydrolyzing

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enzyme operative as an endo-hydrolase [11]. However, the depolymerization of γ-PGA in batch culture as well as the enzymatic depolymerization of γ-PGA concerning the molecular weight and polydispersity has not been extensively studied.

In this paper, a systematic study of γ-PGA depolymerase activity as a function of the culture time and nutrient conditions, including the location and characteristics of γ-PGA depolymerase from *B. subtilis* NX-2, was carried out. The *ywtD* gene of *B. subtilis* NX-2, encoding a γ-PGA depolymerase was cloned and expressed in *E. coli*. The YwtD protein was purified by metal-chelating affinity chromatography. A controllable enzymatic degradation of γ-PGA through optimizing pH and temperature conditions was achieved. The changes of the molecular weight and the molecular weight distribution were measured as a function of depolymerization time.

2. Materials and methods

2.1. Micro-organism

B. subtilis NX-2 was isolated from soil sample [12]. Rosetta (DE3) were used in this study. *E. coli* was routinely grown at overnight in Luria–Bertani medium. Plasmids of pET15b and pGEM were from Novagen Co. US.

2.2. Flask culture conditions

B. subtilis NX-2 was first inoculated into 50 mL of seed medium containing glucose 20 g L^{-1}, yeast extract 5 g L^{-1}, $K_2HPO_4{\cdot}3H_2O$ 2 g L^{-1}, L-glutamic acid 10 g L^{-1}, $MgSO_4$ 0.2 g L^{-1} in 500 mL flask and aerobically incubated at 32.5 °C for 16 h with shaking at 220 rpm. Seed culture (0.8 mL) was then transferred to 500 mL flask containing 80 mL of basal medium comprising glucose 40 g L^{-1}, L-glutamate 40 g L^{-1}, NH_4Cl 8 g L^{-1}, $K_2HPO_4{\cdot}3H_2O$ 2 g L^{-1}, $MgSO_4$ 0.2 g L^{-1}, the final pH was adjusted to 7.5. The flask culture was incubated at 32.5 °C in a rotary shaker at 220 rpm.

To investigate the nutrient condition corresponding to the presence of active depolymerase in batch culture, the medium formulation was also prepared with 1% (w/v) γ-PGA in stead of glucose.

2.3. Batch culture conditions in bioreactor

The reactor runs were performed in a Bioflo 110 bioreactor (New Brunswick Scientific, Edison, NJ, USA). The medium was autoclaved in the reactor (121 °C for 15 min). *B. subtilis* NX-2 seed culture (80 mL) was used to inoculate 4 L of the bioreactor culture medium. The aeration rate into the bioreactor was kept at 1.2 vvm. Temperature was controlled at 32.5 °C and the initial medium pH adjusted at 7.5 while agitation was kept at 400 rpm. At predetermined intervals, sample aliquots of the culture were withdrawn for the determination of biomass, residual substrate and γ-PGA production.

2.4. Preparation of γ-PGA

γ-PGA was isolated and purified by a previously reported method [13].

2.5. γ-PGA depolymerase investigations in batch culture

A batch production of γ-PGA was carried out in a 7.5 L bioreactor. In the late stationary phase, when the carbon source (glucose) was depleted and the reduction of γ-PGA molecular weight occurred, a 100 mL sample of the batch culture was withdrawn and divided into two aliquots. The cells were removed from one aliquot by filtration through a 0.45 μm cellulose acetate syringe filter unit (Shanghai Anpel Co., China). To minimize microbial contamination of the filtrate, the filter unit was pretreated by rinsing with a 1.5% aqueous formaldehyde solution, glass-distilled deionized water, 70% ethanol, and sterile glass-distilled deionized water. The filtrate was collected in a sterile flask and 4 mL aliquots of the filtered solution were aseptically transferred to two sterile 20 mL test tubes. One test tube was placed in boiling water for 5 min in order to deactivate the possible extracellular depolymerase and the other remained no treatment. A third test tube contained 4 mL of the non-filtered culture with no treatment. The test tubes were placed in a shaker incubator at 37 °C (250 r/min). Samples were removed at 24 h intervals and analyzed by GPC.

2.6. Production and simple purification of the mature γ-PGA depolymerase

2.6.1. Cloning of ywtD gene

A sense primer 5′-CCGATGTTAAAACTGCAAAAAGAG-3′, and an antisense primer 5′-GCAATGACGAAAATGGTGTGG-3′, were designed on the basis of the sequence of the *ywtD* gene of *B. subtilis* 168 [14]. A DNA fragment was amplified by PCR with the two primers and *B. subtilis* NX-2 genome DNA as a template, and the amplified fragment was inserted into pGEM-T, generating pGEM-T-*ywtD* to analysis the nucleotide sequence. Then the fragment was amplified again by PCR with primers 5′-CCTCGAGAACACACTGGCAAACTG-3′ and 5′-GCTGAGCTTATTGCACCCGTATACTTC-3′, and was inserted into pET15b digested by Xho I and Bpu1102 I. The resulting plasmid pET15b-*ywtD*, named pYWTD, was transformed into *E. coli* Rosetta (DE3) by the method previously reported [11,15].

2.6.2. Production and purification of YwtD in E. coli

E. coli Rosetta (DE3) harboring pYWTD was grown at 37 °C in Luria–Bertani medium containing 100 μg mL^{-1} of ampicillin and 34 μg mL^{-1} chloramphenicol. When cell growth had reached an optical density at 600 nm of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mmol L^{-1}, followed by cultivation for 8 h. Cells were harvested from 1000 mL of culture by centrifugation, resuspended in 50 mL of 10 mM phosphate buffer (pH 7.0), and then disrupted by sonic oscillation for 15 min. The YwtD protein was purified by metal-chelating affinity chromatography [11]:

the supernatant was applied to a column equilibrated with N_i-NTA SepharoseFF (4 °C). The column was washed with 10 mM imidazole buffer (10 mM imidazole, 20 mM Tris–HCl, 0.3 M NaCl, 20% glycerol, pH 8.0), and then histidinetagged protein was eluted with imidazole buffer (20 mM Tris–HCl, 0.3 M NaCl, 20% glycerol, pH 8.0) with a gradient of imidazole from 100 to 300 mM. The purified YwtD was collected and concentrated.

2.7. Enzymatic depolymerization of γ-PGA

The depolymerase activity of YwtD towards γ-PGA was investigated in a reaction mixture (7 mL) containing 10 mg mL^{-1} γ-PGA, 0.05 mol L^{-1} sodium citrate buffer (pH 5, 6 and 7) and 10 μg mL^{-1} YwtD. A reaction mixture in the absence of YwtD served as a blank for comparison to the former. Portions (60 mL) of the reaction mixtures were withdrawn at 6, 10, 24, 48 and 72 h, and then boiled for 5 min to terminate the reaction. After termination of the reaction, the mixture was centrifuged at 8000 × *g* for 10 min. A 2.5 mL sample of the supernatant was withdrawn and subjected to GPC.

The effect of γ-glutamyltranspeptidase (GGT) on depolymerization of γ-PGA was also studied. A reaction mixture containing GGT (1 μg mL^{-1}) was experimented as mentioned above. GGT was prepared as described previously [16]. Considering that high hydrolase activity of GGT was detected at neutral, the experiment was carried out at pH 7.0.

2.8. Analysis

2.8.1. Biomass determination

Cell dry weight (CDW) was determined by centrifuging 1.5 mL aliquots in microfuge tubes, washing the cell with diluted water, and drying under vacuum at 80 °C.

2.8.2. Quantification of carbon source in culture

The concentration of glucose and L-glutamate remaining in the broth were analyzed by a biosensor equipped with both glucose and L-glutamic acid oxidase electrode (Institute of Biology, Shandong Academy of Sciences SBA-40C) after separation of the cells by centrifugation [12].

2.8.3. γ-PGA quantification and molecular weight measurement

The number- and weight-average molecular weight (M_n and M_w) along with polydispersity (M_w/M_n) of γ-PGA were measured by gel permeation chromatography (GPC) using an *Alltech* system controller equipped with Shodex OH pak SB800 Series columns (SB 806 M HQ), a refractive-index (RI) detector (Schambeck SDF RI2000), and a UV detector (Thermo LINEAR UV201). Pullulan standards of narrow polydispersity (Shodex Co.) were used to construct a calibration curve from which molecular weights of γ-PGA were calculated with no further correction. A calibration curve was generated using purified γ-PGA as a standard to relate the γ-PGA GPC peak area, based on which the amount of γ-PGA was calculated [13].

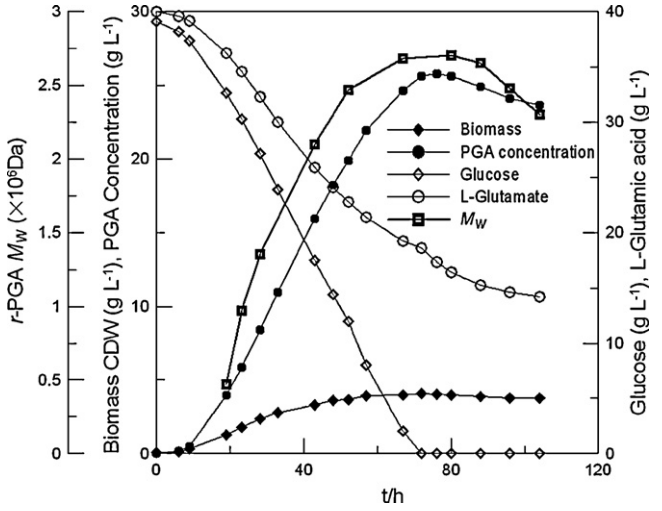

Fig. 1. Profile of batch production of γ-PGA from *B. subtilis* NX-2 in fermentor (culture medium: glucose 40 g L^{-1}, L-glutamic acid 40 g L^{-1}, $(NH_4)_2SO_4$ 5 g L^{-1}, $K_2HPO_4{\cdot}3H_2O$ 20 g L^{-1}, $MgSO_4$ 0.1 g L^{-1}, $MnSO_4$ 0.03 g L^{-1}, initial pH 7.5).

3. Results and discussion

3.1. γ-PGA depolymerase investigations in batch culture

The production of γ-PGA by *B. subtilis* NX-2 has been previously investigated [12]. It was observed that *B. subtilis* NX-2 largely produced capsular γ-PGA during early stationary phase (Fig. 1). The molecular weight of γ-PGA attained the maximum of 2700 kDa after 64 h of cultivation. Glucose was completely utilized and a maximum γ-PGA concentration of 25 g L^{-1} was observed at 72 h. The yield and molecular weight of γ-PGA remained unvaried at 72–80 h. The residual L-glutamic acid in the culture could be consumed as the alternative carbon source. A remarkable degradation of γ-PGA occurred after 88 h of cultivation, when the free L-glutamic acid decreased to a critical concentration of 15 g L^{-1}. These results suggested that the γ-PGA depolymerase was present and active during the late stationary phase after 88 h of cultivation, meanwhile, glucose was exhausted and free L-glutamic acid in the culture was decreased to the critical concentration of 15 g L^{-1}.

In order to localize the depolymerase, aliquots of culture broth after 88 h of cultivation were withdrawn and filtered to remove the cells while the γ-PGA product and possibly extracellular γ-PGA depolymerase remained in the filtrate. One filtered aliquot was incubated at 30 °C for 67 h to study whether the filtrate showed the presence of an active γ-PGA depolymerase, the other was treated with boiling water prior to incubation so as to inactivate the suspected depolymerase enzyme. A non-filtered aliquot was also inoculated as a blank. By comparison of incubations carried out using the filtered or non-filtered aliquots, any molecular weight decrease could be determined that was attributed to the presence of active depolymerase.

A dramatic loss of molecular weight was observed in both of non-filtered and filtered aliquots during 48 h incubation, while only small changes in molecular weight were detected for the filtered aliquot treated in boiling water (Fig. 2). These results

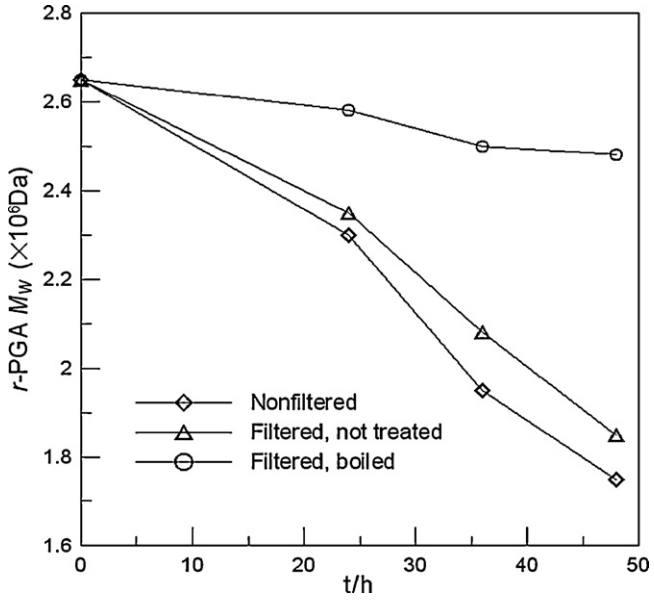

Fig. 2. Results of a study to determine the presence of γ-PGA depolymerase enzyme in culture.

indicated that the γ-PGA depolymerase was located and active extracellularly in the culture.

To further investigate the nutrient condition and culture time corresponding to the presence of active depolymerase in batch culture, 10 g L^{-1} γ-PGA instead of glucose and different level of glutamic acid (0, 10 and 20 g L^{-1}) were contained in the mineral medium. Cell viability and molecular weight of γ-PGA were monitored over time. From Fig. 3, it was found that during the first 24 h of incubation, the cell growth was significantly detected when L-glutamic acid (15, 20 g L^{-1}) was used as the additional carbon source, but occurred slowly with non-glutamic acid added. Interestingly, during the following 24 h, a significant degradation of γ-PGA was observed when non-glutamic acid added and 15 g L^{-1} L-glutamate supplied. In the case of 20 g L^{-1} L-glutamic acid supplied, the molecular weight of γ-PGA remained almost unvaried. These results indicated that the presence of γ-PGA depolymerase was concerned with the nutrient conditions and active as a function of culture time. In the absence of glucose, L-glutamate in the culture was consumed as the alternative carbon source, when L-glutamate was supplied lower than 15 g L^{-1}, γ-PGA was observed degraded during the incubation, which was consistent with the results observed during the late stationary phase of batch culture (Fig. 1).

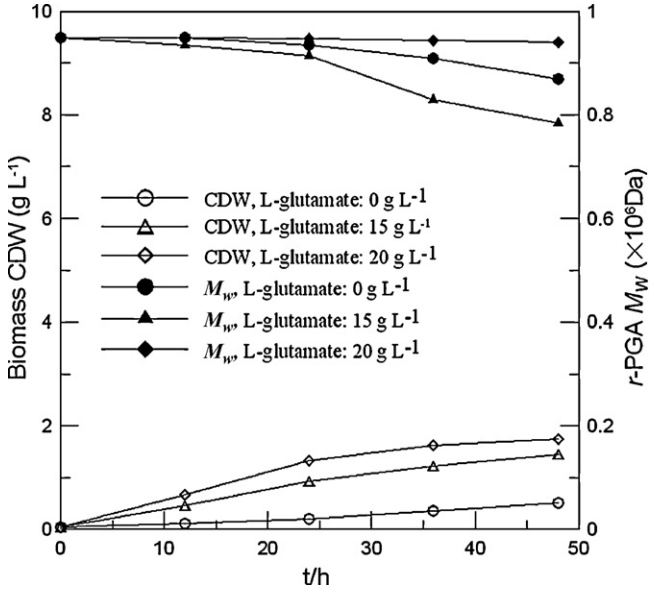

Fig. 3. Results of a study to determine the nutrition conditions concerned with the presence of γ-PGA depolymerase enzyme in culture.

3.2. Production and purification of the mature γ-PGA depolymerase

In order to obtain effectively the mature γ-PGA depolymerase, the enzyme was produced in cells of the *E. coli* Rosetta (DE3) clone harboring the plasmid pET15b-*ywtD* (pYWTD). As the N-terminus of the enzyme was modified with the histidine-tag, the YwtD protein could be purified by metal-chelating affinity chromatography (see Section 2). The purified YwtD protein exhibited a single band (45 kDa) on sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4).

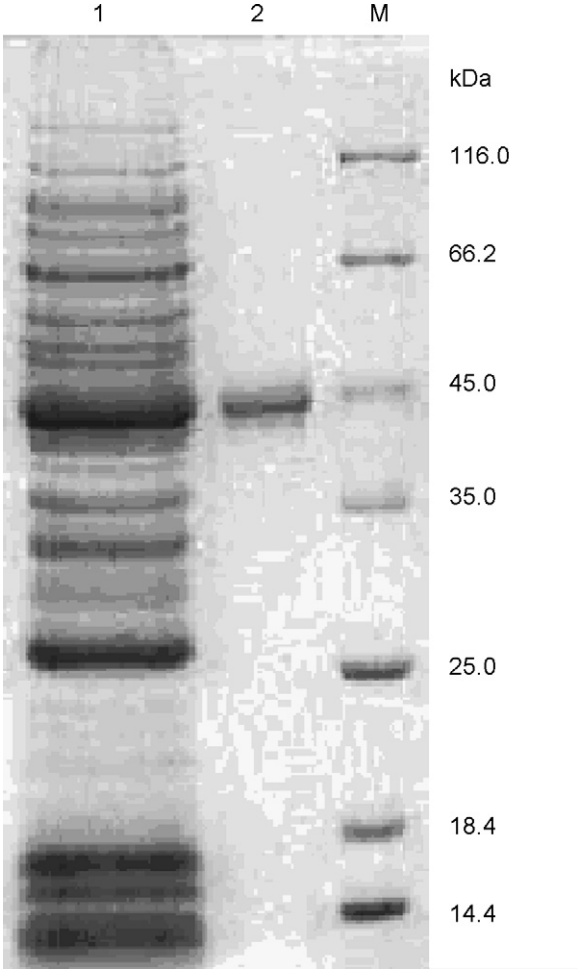

Fig. 4. SDS-PAGE analysis of the purification of the protein YwtD (lane M, the LMW marker kit; lane 1, the crude extract of the *E. coli* Rosetta (DE3)/pYTWD clone; lane 2, the purified YwtD).

This result was in agreement with that calculated from the deduced amino acid sequence of the *ywtD* gene.

3.3. γ-PGA enzymatic degradation

3.3.1. Effect of pH and temperature on γ-PGA enzymatic degradation

Effects of pH and temperature on γ-PGA enzymatic degradation were investigated by measuring the resulting molecular weights as a function of depolymerization time (Figs. 5A and 6A). The purified γ-PGA with molecular weight of about 1000 kDa was used in the experiments. The optimal temperature and pH conditions were determined by measuring the percentage molecular weight reduction after 24 h (Figs. 5B and 6B). This method is similar to that reported by Thompson et al. [17] for the enzymatic hydrolysis of xyloglucan by xyloglucan endo-hydrolase.

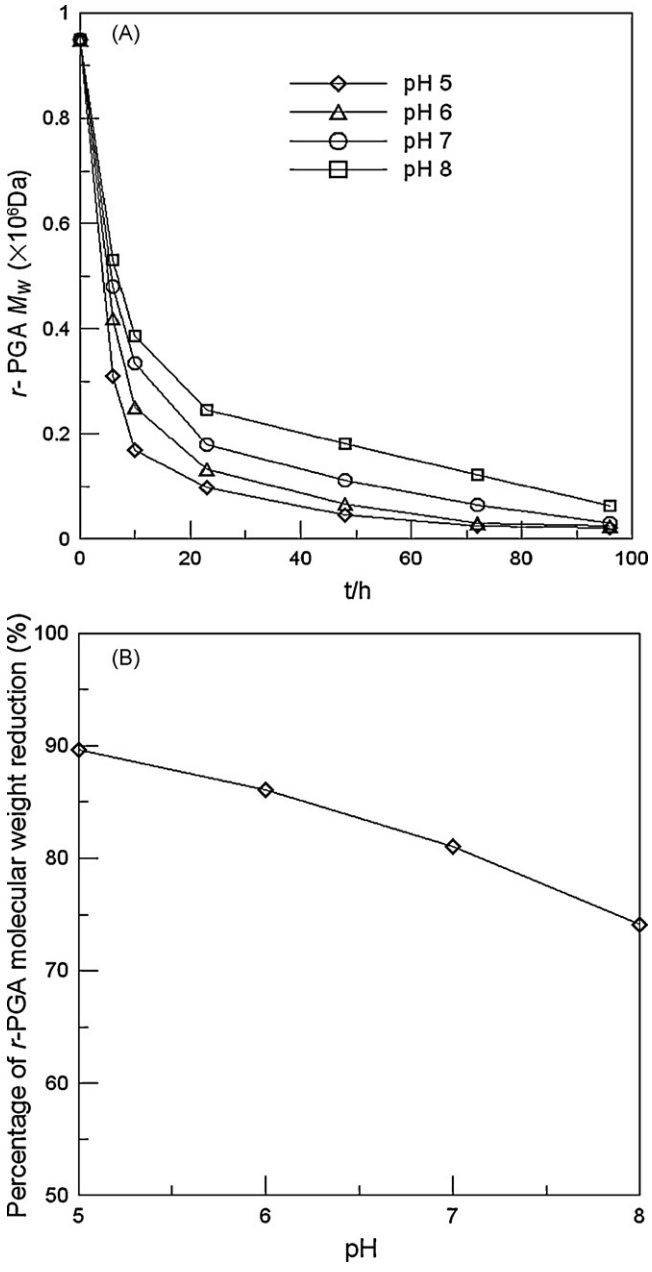

Fig. 5. Effect of pH on γ-PGA enzymatic degradation (temperature condition: 30 °C). (A) Effect of pH on γ-PGA depolymerization as a function of degradation time. (B) Effect of pH on molecular weight reduction after 24 h.

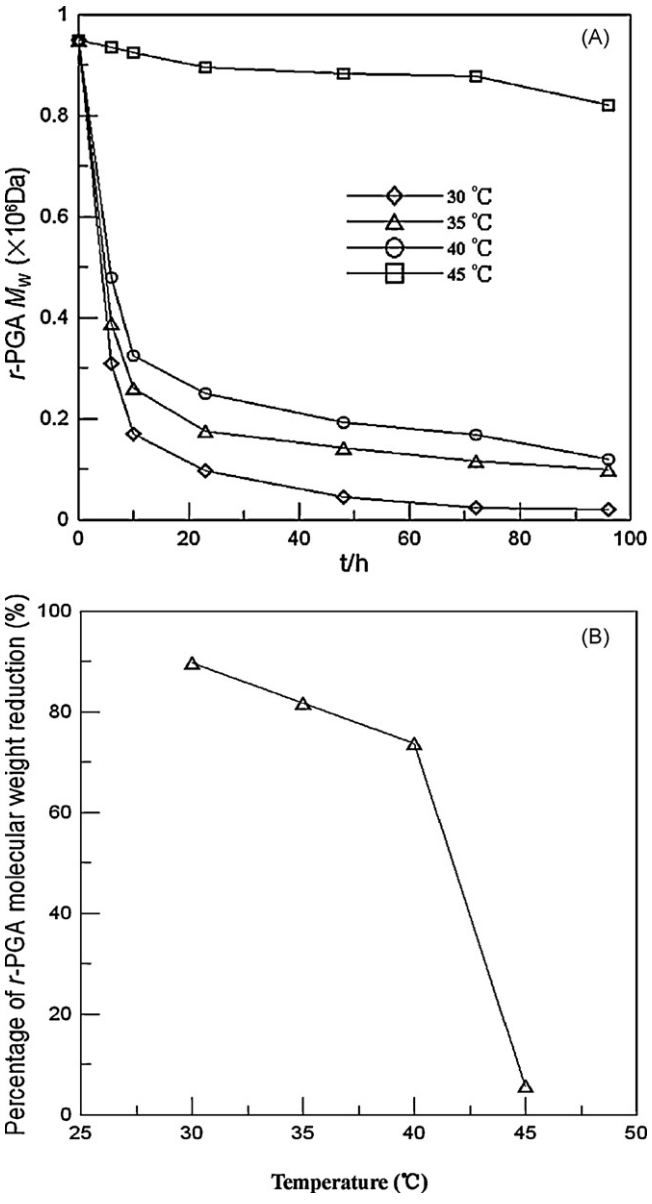

Fig. 6. Effect of temperature on γ-PGA enzymatic degradation (pH condition: 5.0). (A) Effect of temperature on γ-PGA depolymerization as a function of degradation time. (B) Effect of temperature on molecular weight reduction after 24 h.

The active range of YwtD was wide for both temperature (30–40 °C) and pH (5.0–8.0). At the upper temperature limit of 45 °C, no remarkable changes of molecular weights were observed possible due to the loss of depolymerase activity. The optimal conditions for γ-PGA enzymatic degradation were determined at 30 °C and pH 5.0, for that an efficient degradation of γ-PGA could be achieved within the range of 1000–20 kDa.

Molecular weight reduction as a function of depolymerization time at pH 5.0 was measured by GPC (Fig. 7). The

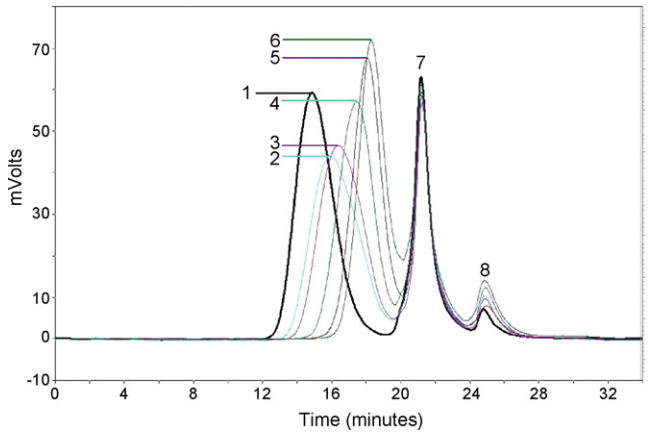

Fig. 7. The enzymatic degradation of γ-PGA at pH 5.0 determined by GPC (GPC trace of (1) 0 h, (2) 6 h, (3) 10 h, (4) 24 h, (5) 48 h, (6) 72 h, (7) salt, (8) impurity).

corresponding signal bands of degraded γ-PGA were observed shifted as a function of depolymerase time while no shifts was observed in the absence of YwtD, indicating the depolymerase activity of YwtD. None free glutamic acid was observed during hydrolysis, suggesting that YwtD functioned as an endo-hydrolase enzyme. This was consistent with the findings of other researchers [11,18]. According to the profile of enzymatic degradation, the molecular weight of γ-PGA was reduced within the range of 1000–20 kDa. γ-PGA of an expected molecular weight could be available at the predetermined interval. Polydispersity also decreased as a function of hydrolysis time (Fig. 8), suggesting that a resulting product with a relatively narrow polydispersity could be attained. The results indicated that a controllable degradation of γ-PGA could be achieved by enzymatic degradation, and promisingly supply the expected molecular weights adequate to the potential applications of γ-PGA.

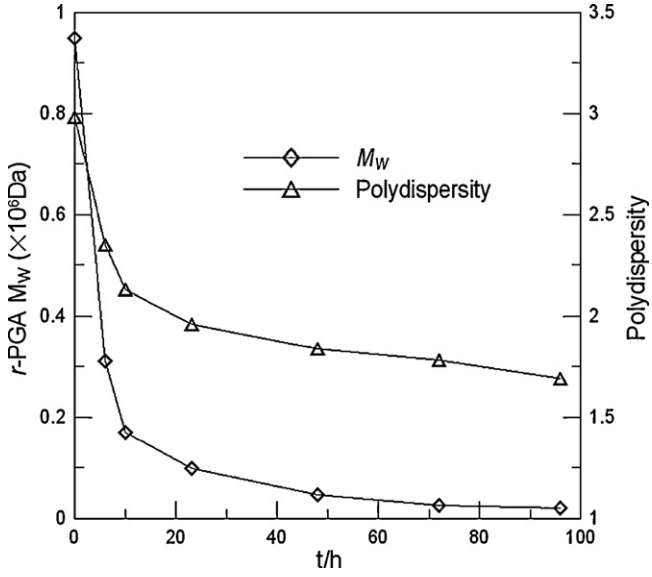

Fig. 8. Results as a function of enzymatic degradation time for molecular weight and polydispersity of γ-PGA determined by GPC at pH 5.0.

Table 1
The effect of GGT on enzymatic degradation of γ-PGA

	Time (h)	Glutamic acid (g L^{-1})	γ-PGA M_w (×10^6 Da)
GGT	0	–	0.950
	24	–	0.947
	48	–	0.945
	72	–	0.945
YwtD	0	–	0.950
	24	–	0.180
	48	–	0.112
	72	–	0.065
YwtD + GGT	0	–	0.950
	24	–	0.178
	48	0.125	0.095
	72	0.278	0.040

–, representing not detected.

3.3.2. *Effect of GGT on γ-PGA degradation*

The activity of GGT was detected during the cultivation of *B. subtilis* NX-2 [16]. It has been reported that *B. subtilis* GGT showed exo-γ-glutamyl hydrolase activity towards γ-PGA and was involved in γ-PGA degradation in vivo to yield the constituent amino acids [19]. However, as shown in Table 1, in the absence of YwtD, GGT showed little hydrolase activity towards γ-PGA. In the presence of YwtD, it was found that no free glutamic acid was detected until the γ-PGA molecular weight was reduced to about 100 kDa at 48 h, 0.125 g L^{-1} of free glutamic acid was detected in the reaction mixture. These results indicated that GGT showed little hydrolase activity to γ-PGA with molecular weight above 100 kDa. The enzymatic degradation of γ-PGA by YwtD appeared to yield the fragmentation appropriate for hydrolysis by GGT. GGT could play a unique role required by stationary phase cells to supply the free gluamic acid as alternative carbon source.

4. Conclusion

In this paper, γ-PGA depolymerase activity during the batch culture was investigated. The results showed that the presence of γ-PGA depolymerase was concerned with the nutrient conditions and active as a function of culture time. γ-PGA depolymerase was located and active extracellularly in the culture during the late stationary phase, at that time glucose was exhausted and free L-glutamic acid in the culture was decreased to a critical concentration of 15 g L^{-1}.

The *ywtD* gene from *B. subtilis* NX-2, encoding the γ-PGA depolymerase was cloned and expressed in *E. coli*. YwtD was proved to be an endo-hydrolase enzyme and exhibited a remarkable activity in γ-PGA degradation at a wide range of temperature (30–40 °C) and pH (5.0–8.0). The optimal conditions for γ-PGA enzymatic degradation were determined at 30 °C and pH 5.0. The molecular weight of γ-PGA could be degraded within the range of 1000–20 kDa and the polydispersity decreased as a function of depolymerization time. Compared with the physical and chemical method of γ-PGA degradation, enzymatic degradation offered a mild and controllable method to obtain the expected molecular weight and narrow polydisper-

sity without disturbing the chemical constitution of the polymer. Based on this research, the preparation of γ-PGA via enzymatic degradation adaptable to practical applications will be anticipated available in the future work.

Acknowledgements

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