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Physicochemical Properties of Poly-γ-glutamic Acid Produced by a Novel *Bacillus subtilis* HA Isolated from *Cheonggukjang*

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Abstract

A novel bacterium isolated from *Cheonggukjang* was identified as a glutamate-dependent *Bacillus subtilis* HA with 98.3% similarity to *Bacillus subtilis* Z99104. Optimization of poly-γ-glutamic acid (γ-PGA) production by modulating fermentation factors including carbon sources, nitrogen sources, inorganic salts and fermentation time was investigated. Optimum culture broth for γ-PGA production consisted of 3% glutamate, 3% glucose and various salts, resulting in the PGA production of 22.5 g/L by shaking culture for 72 hr at 37°C. Average molecular weight of γ-PGA was determined to be 1,220 kDa through MALLS analysis. The γ-PGA solution showed a typical pseudoplastic flow behavior, and a great decrease in consistency below pH 6.0 regardless of the same molecular weight of γ-PGA. The molecular weights of isolated γ-PGA were drastically decreased by heat treatment in various acidic conditions, resulting in different hydrolysis of γ-PGA. The consistency of γ-PGA solution was greatly decreased with increase heating time in acidic conditions.

Key words: *Bacillus subtilis*, poly-γ-glutamic acid, consistency index, molecular weight

INTRODUCTION

Poly-γ-glutamic acid (γ-PGA) is generally produced by bacteria of the *Bacillus* genus (1). It is an anionic homo-polyamide made of d- and l-glutamic acid units connected by amide linkages between α-amino and γ-carboxylic acid groups (2). Therefore, the γ-PGA polymer is structurally and fundamentally different from proteins (3). γ-PGA is water-soluble, biodegradable, edible as well as non-toxic to humans and the environment. γ-PGA and its derivatives offer a wide range of unique applications. It has been used as a drug carrier (4,5), curable biological adhesive (6,7), biodegradable fibers (8), and highly water absorbable hydrogels (9).

Many researchers have tried to clarify the metabolic pathway and the enzymes related to γ-PGA synthesis and polymerization so as to enhance the productivity. Several γ-PGA producing *Bacillus* strains have been isolated from highly salty seasoning of fermented soybean foods (10,11). Several *Bacillus* sp. produce γ-PGA as a viscous material or a capsular component. These strains are most useful in terms of industrial applications and have been studied most intensively. In order to enhance the γ-PGA productivity, several investigations of the nutrient requirements for γ-PGA production have revealed that the requirements varied according to the strain used (1).

According to the nutrient requirements, γ-PGA producing bacteria can be divided into two groups; one that requires the addition of l-glutamic acid in the medium to stimulate γ-PGA production, one that does not require l-glutamic acid. The γ-PGA as viscous material has particular viscoelastic properties. Its production is dependent upon the type of microorganisms and environmental factors such as temperature and nutrient composition (1,12).

γ-PGA is a high molecular-weight polypeptide consisting of γ-linked glutamic acid units and its α-carboxylic acid side chains can be chemically modified for a drug delivery carrier (13). For the production of γ-PGA-cisplatin conjugate with anti-tumor effects, the average molecular weight of γ-PGA was reduced by heating after HCl treatment (14). γ-PGA is unique homo-biopolymer with various applications in food and medicinal field so that the characterization of its physicochemical properties will allow us to provide useful information for future research and applications.

The present study was carried out to optimize higher production of γ-PGA by a novel *B. subtilis* HA isolated from traditional fermented soybean food, characterize its physicochemical properties and modulate the molecular weight of γ-PGA.

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MATERIALS AND METHODS

Materials and reagents

All chemicals were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise indicated. Glutamate was purchased from the Yakuri Pure Chemicals Co., Ltd. (Kyoto, Japan). BCA protein assay kit was purchased from the Bio-Rad (Hercules, CA, USA). Nutrient and MRS broth were purchased from the Difco (Detroit, MI, USA).

Isolation of γ-PGA producing *Bacillus* sp.

The γ-PGA producing *Bacillus* strain was isolated from the traditional fermented soybean food, *Cheonggukjang*, through serial dilution and inoculated on MRS agar plate and incubated at 37°C for 24 hr. The *Bacillus* strain producing mucilage can be easily distinguished on MRS agar plates, since it grows with a sticky and rough morphology (15). The mucilage producing strains were isolated bacteria. To isolate a bacterial strain producing γ-PGA efficiently, the *Bacillus* strain isolated was cultured and then transferred to the defined media for γ -PGA production. After incubation at 37°C for 24 hr, the strains producing viscous mucilage were selected and analyzed further. The defined medium for γ-PGA production was 2% glucose, 2% glutamate, 0.05% K_2HPO_4, 0.05% $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$ and 50 μg biotin according to the modified method of Goto and Kunioka (16). For solid medium 1.5% agar was added.

Identification of γ-PGA producing bacterium

For 16S rDNA sequencing the DNA from bacteria producing high mucilage isolated pure culture was prepared with Wizard® genomic DNA purification kit (Promega, USA) according to the procedure recommended by the manufacturer. The universal primer used to amplify bacterial 16s rDNA were NS1 (5'-GTAG TCATATGCTTGTCTC-3') and NS8 (5'-TCCGCAGG TTCACCTACGGA-3'). The 16s rDNA products were purified using the Wizard® SV Gel and PCR clean-up system (Promega, USA). The purified PCR products were analyzed using an ABI PRISM 3700 DNA Analyzer. DNA sequencing was performed by the Korean Culture Center of Microorganism (KCCM), Korea. DNA sequences sharing 99% identity with known sequences were assigned to the phylotype at *Bacillus subtilis*. It was assigned and deposited in the Korean Culture Center of Microorganism as KCCM 10775P. Sequences of insets were compared to the ribosomal DNA sequence in GenBank using BLAST program. Multiple alignments were performed with the Clustal X and Mega 2 program (17).

Microorganisms and culture conditions

Bacillus subtilis HA was used in this experiment. To prepare the starter culture *B. subtilis* HA was grown on MRS agar plate at 37°C for 24 hr, and then transferred to a 500 mL flask containing 150 mL of nutrient broth. The medium was cultured in a shaking incubator (SI-900R, Jeio Tech Co., Ltd., Korea) at 180 rpm and 37°C for 24 hr.

Optimization of γ-PGA production by *B. subtilis* HA

To optimize the γ-PGA production, carbon and nitrogen sources were investigated. The optimum medium for γ-PGA production consists of 1 g Na_2HPO_4, 1 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.02 g $MnSO_4 \cdot 5H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 30 g glutamate, and 30 g glucose in 1 liter distilled water according to the modified method of Goto and Kunioka (16). 3% (v/v) of starter culture was inoculated into 500 mL Erlenmeyer flask containing 100 mL medium incubated at 37°C on a rotary shaker at 170 rpm for 72 hr.

Isolation and purification of γ-PGA

The γ-PGA was purified by the modified purification method of Choi and Lee (18). For the purification of γ-PGA from *B. subtilis* HA, cells were harvested from the culture broth by centrifugation at 24,900×*g* for 10 min. Three volume of ethanol were added to culture supernatant mixed well and then centrifuged (24,900×*g*, 10 min, 4°C). Precipitated materials (crude γ-PGA) were harvested and dried in a vacuum drying oven (VO-200, Sunil Eyela Co., Ltd., Korea) at 50°C (16). The precipitate was dissolved in distilled water. It was adjusted to pH 2.0 by adding 6 N HCl and left overnight at 4°C. Then the supernatant was obtained after centrifugation and adjusted to pH 6.5 with 6 N NaOH. Three volumes of ethanol were added to the supernatant, and the mixtures were mixed well and then centrifuged (24,900×*g*, 10 min, 4°C). The resultant precipitate was collected by centrifugation at 24,900×*g* for 10 min and then dissolved in distilled water. The γ-PGA solution was dialyzed in de-ionized water and then lyophilized.

Analysis of purified γ-PGA

Protein content remaining in the purified PGA was determined by the dye binding method of Bradford (19). Total sugar content was determined by the modified phenol/sulfuric acid method (20). A 1 mL of purified γ-PGA solution was mixed with 25 μL phenol reagent (80%, v/v), and then added with 2.5 mL of concentrated sulfuric acid rapidly to the solution surface without allowing it to touch the sides of the tube. The solution was undisturbed for 10 min before shaking vigorously, and then determined the absorbance at 485 nm. The intensity of

the orange color is proportional to the amount of total carbohydrates present. The standard curve for calculating total carbohydrate was constructed with glucose as a standard sugar.

Molecular weight analysis of γ-PGA

To determine the average molecular weight of γ-PGA the purified γ-PGA and its hydrolysate were dissolved in distilled water and filtered through a 0.45 μm micro-filter membrane prior to injection into the size exclusion chromatograph coupled with a multi angle laser light scattering system (21). The molecular weight range for light scattering is determined by the size of the dissolved polymer molecules without standard curve and using the refractive indices of solvent and polymer. A HPSEC system equipped with a multi-angle laser-light scattering detector (DAWN EOS; Wyatt Technology, SantaBabara, CA, USA) and refractive index detector (Optilab rEX; Wyatt Technology, SantaBabara, CA, USA) was used to determine the weight-average molecular weights of the γ-PGA and its hydrolysate. Shodex OHpak SB-805 column was used with a flow rate 1 mL/min, and the mobile phase was distilled water containing 0.1 M $NaNO_3$.

The concentration and molecular weight of γ-PGA were also measured using an Autochro-GPC system (Young In Scientific Co., Seoul, Korea) equipped with a Waters 410 Refractometer (Milford, MA) and a Shodex OHpak SB 800 HQ series column (SB 802.5, SB 805). The freeze-dried sample was suspended in dH_2O. The eluant containing 0.1 M Na_2SO_4, 0.05% (w/v) NaN_3 was brought to pH 4.0 using glacial acetic acid, and the flow rate was set at 1.0 mL/min (22). The molecular weight and amount of γ-PGA was calculated using the peak area of the GPC measurements with purified γ-PGA as a standard.

Physical properties of γ-PGA

Rheological properties of the viscous culture broth and γ-PGA solution (1～3%, w/v) were determined using a Rheometer System (HAAKE RheoStress 1, Germany) equipped with a cone plate device (Platte PP35 Ti, 3.5 cm diameter, 2°). The flow behavior was determined by the shear rate (1～100/sec) at 20^oC, and consistency index ($Pa \cdot sec^n$) and flow behavior index were evaluated by the power-law model (23).

Modulation of the molecular weight according to the pH, acids and heat-treatment

Changes in consistency index and molecular weight of γ-PGA (1%, w/v) according to heat and acid treatment with HCl, citric acid and acetic acid were determined. The pH of γ-PGA was adjusted to pH 2, 3, 4, 5, 6, and 7 with 6 N HCl and 6 N NaOH then followed by heat-treatment at 100^oC for 60 min for HCl. pH 2 was maintained for acetic acid and citric acid with heat-treatment at 100^oC for 20 min. The consistency index and molecular weight of the γ-PGA solution were measured periodically (5～60 min).

Statistical analysis

All the data were statistically analyzed by Student's *t*-test, and $p<0.05$ was considered to be statistically significant. Data were analyzed using SPSS™ version 12.0 for Windows (SPSS Inc., Chicago, IL), and the results were expressed as mean±standard deviation (SD).

RESULTS AND DISCUSSION

Characterization of the γ-PGA producing strain

The morphology of the newly isolated bacterium was rod-shape (0.7～0.8 μm length and 1.38～1.41 μm breadth) as determined by scanning electron microscopic analysis. The strain grew well on MRS agar plate at 37～42^oC, while no growth was observed at 25^oC and 60^oC. The strain was able to perform starch and casein hydrolysis, gelatin liquefaction, nitrate reduction and catalase test, but negatively reacted to citrate utilization and oxidase test. The strain could grow in nutrient broth with 7% NaCl but the growth was inhibited at 9% NaCl. From 16s rDNA partial sequencing, the strain belonged to the genus *B. subtilis* and was designated as *Bacillus subtilis* HA. *B. subtilis* HA was found to be closely related to *B. subtilis* Z99104 with 98.3% similarity.

Optimization of medium for γ-PGA production

Modified medium of Goto and Kunioka (16) was used initially for the production of γ-PGA. It was found that *B. subtilis* HA produce optimum γ-PGA through submerged fermentation at 72 hr with optimum temperature 37^oC. On the other hand, it was reported that the optimum production of γ-PGA from *B. subtilis* TAM4 and *B. licheniformis* was observed at 30^oC (24,25).

To optimize the culture medium for γ-PGA production, tests of the effects of various carbon and nitrogen sources, magnesium and manganese were conducted. To determine the best carbon source for the γ-PGA production by *B. subtilis* HA, six different carbon sources were separately provided with 30 g/L concentration. The *B. subtilis* HA grew well in media supplemented with maltose, glycerol, or mannose as a carbon source, but those carbon sources had little effect on the γ-PGA production. Among the carbon sources tested, glucose yielded the highest γ-PGA production (11.1 g/L)(Fig. 1). The maximum γ-PGA production was obtained with 30 g/L of glucose (Table 1).

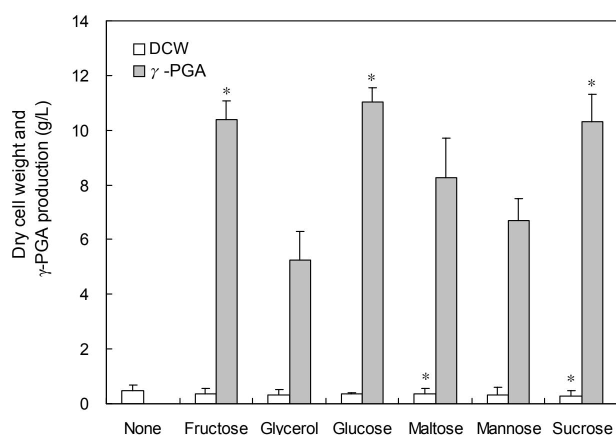

Fig. 1. Effect of various carbon sources on dry cell weight and γ-PGA production in shake-flask cultures of *B. subtilis* HA. All carbon sources were added to 30 g/L. The culture was incubated at 37°C on a rotary shaker at 170 rpm for 48 hr using the modified medium of Goto and Kunioka (16). DCW: dry cell weight. Data shown are mean±SD values. A value with an asterisk is significantly different from the control group by *t*-test ($^{*}p<0.05$).

Table 1. Effects of glucose, ammonium sulfate, magnesium sulfate, manganese sulfate and calcium chloride concentrations on the γ-PGA production in shake-flask culture of *B. subtilis* HA

Sources	Concentration (g/L)	γ-PGA production (g/L)	Cell growth (g/L)
Glucose	0	−	0.39±0.05
	10	7.92±0.63	0.48±0.05
	20	9.61±0.48	0.54±0.03
	30	11.05±0.51	0.46±0.03
	40	9.34±0.60	0.43±0.05
	50	8.69±1.50	0.43±0.05
Ammonium sulfate	0	0.97±0.15	0.56±0.66
	5	11.05±0.51	0.32±0.03
	10	10.55±0.05	0.32±0.01
	15	10.54±0.25	0.45±0.01
Magnesium sulfate	0	−	0.12±0.00
	0.5	11.05±0.51	0.26±0.03
	1.0	10.02±0.26	0.31±0.01
	1.5	9.62±0.50	0.27±0.03
	2.0	9.42±0.40	0.19±0.01
	2.5	9.05±0.14	0.18±0.00
	3.0	8.01±0.38	0.18±0.01
Manganese sulfate	0	11.04±1.00	0.20±0.07
	0.02	11.05±0.51	0.32±0.03
	0.04	9.00±1.00	0.35±0.08
	0.06	8.17±0.72	0.30±0.01
	0.08	6.00±0.41	0.29±0.02
	0.10	5.49±0.57	0.28±0.07
Calcium chloride	0	8.67±1.15	0.23±0.08
	0.2	12.17±1.26	0.26±0.06
	0.4	17.33±0.76	0.27±0.07
	0.6	14.43±0.81	0.23±0.06
	0.8	14.1±1.01	0.23±0.06

Data shown are mean±SD values.

Various nitrogen sources like yeast extract, tryptone, beef extract, peptone, $(NH_4)_2SO_4$, NH_4Cl were tested for γ-PGA production. Among the nitrogen sources tested, $(NH_4)_2SO_4$ yielded the highest γ-PGA production (Fig. 2). The maximum γ-PGA yield (11.1 g/L) was obtained in defined medium supplemented with 5 g/L of $(NH_4)_2SO_4$ as the nitrogen source (Table 1). Mg^{2+} and Mn^{2+} have been reported to significantly stimulate γ-PGA production by their addition to the medium (25,26). γ-PGA was not produced without the addition of Mg^{2+} but addition of a small amount of Mg^{2+} resulted in the stimulation of γ-PGA production. The maximum production of γ-PGA was achieved with the addition of 0.5 g/L of $MgSO_4 \cdot 7H_2O$ to the medium. However, the amount of produced γ-PGA decreased proportionally to the amount of Mn^{2+} added to the medium. The results indicate that *B. subtilis* HA require Mg^{2+} but not Mn^{2+} for the production of γ-PGA. Production of γ-PGA also increased by adding 0.4 g/L of $CaCl_2 \cdot 2H_2O$ to the medium (Table 1). Thus, the addition of $CaCl_2 \cdot 2H_2O$ increases γ-PGA production. From above results, the optimal composition of defined medium for production of γ-PGA was determined as below: 1 g Na_2HPO_4, 1 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.4 g $CaCl_2 \cdot 2H_2O$, 30 g glutamate, 30 g glucose and 500 μg biotin per 1 liter culture broth.

γ-PGA production was evaluated in the defined medium with optimal nutrient composition as shown in Fig. 3, the γ-PGA production and consistency index increased rapidly during the fermentation for 72 hr, showing a γ-

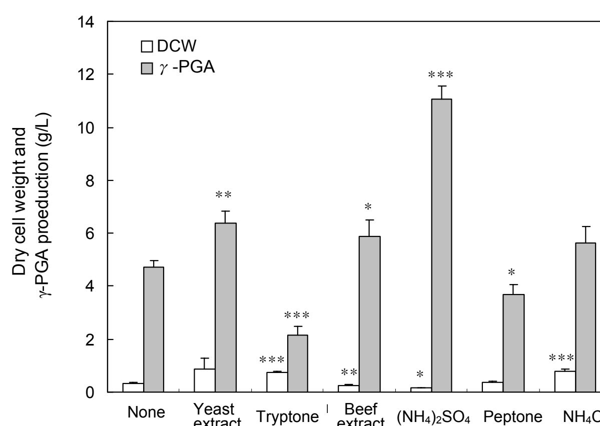

Fig. 2. Effect of various nitrogen sources on dry cell weight and γ-PGA production in shake-flask cultures of *B. subtilis* HA. All nitrogen sources were added to 5 g/L. The culture was incubated at 37°C on a rotary shaker at 170 rpm for 48 hr using the modified medium of Goto and Kunioka (16). DCW: dry cell weight. Data shown are mean±SD values. A value with an asterisk is significantly different from the control group by *t*-test ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

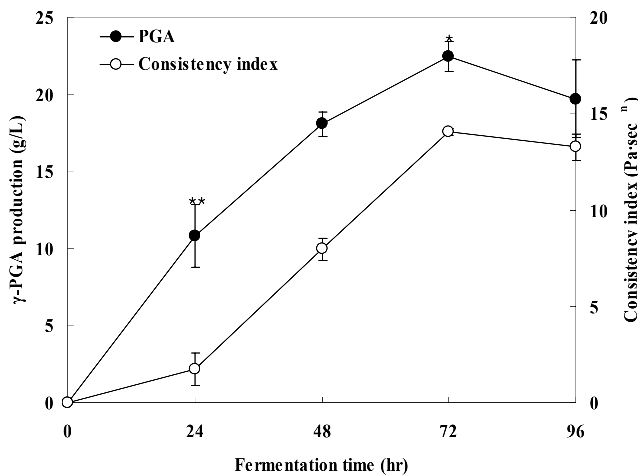

Fig. 3. Comparison of γ-PGA production and consistency index according to fermentation time in shake-flask cultures of *B. subtilis* HA. The culture was incubated at 37°C on a rotary shaker at 170 rpm. Data shown are mean±SD values. A value with an asterisk is significantly different from the control group by *t*-test ($^{*}p<0.01$, $^{**}p<0.001$).

PGA production of 22.5 g/L. After then, γ-PGA production and consistency index decreased slightly. This may be due to the partial enzymatic hydrolysis of the γ-PGA in late stage of fermentation. Such a hypothesis was supported for *natto* mucilage, which was hydrolyzed by depolymerase produced by *Bacillus* sp., resulting in decreased viscosity (27). Considering that the utilization of glutamate in defined medium, *B. subtilis* HA could produce 22.5 g/L of γ-PGA from 30 g/L glutamate. Compared to previous reports about the γ-PGA yield (3,28,29), the conversion of glutamate in optimized culture medium by *B. subtilis* HA is considered to be efficient.

Physico-chemical properties of γ-PGA

The molecular weight and the ratio of carbohydrate to protein for purified γ-PGA were investigated. The molecular weight of purified γ-PGA was determined by GPC analysis. Purified γ-PGA as standards were used to construct a calibration curve, from which unknown molecular weights of γ-PGA were calculated (Fig. 4A). The molecular weight of purified γ-PGA was determined to be 1220 kDa. As shown in Fig. 4B, purified γ-PGA showed the retention time of 8.14 min in HPLC chromatogram. The purified γ-PGA consisted of approximately 96.9% γ-PGA, 0.1% protein content and 3.0% total sugar (carbohydrate) content. It has been reported that the molecular weight of γ-PGA varies according to the culture conditions and strain types, ranging from 100 to 2,000 kDa (30). The consistency index was sharply increased with increasing γ-PGA concentration as shown in Table 2. When the consistency index of γ-PGA (30

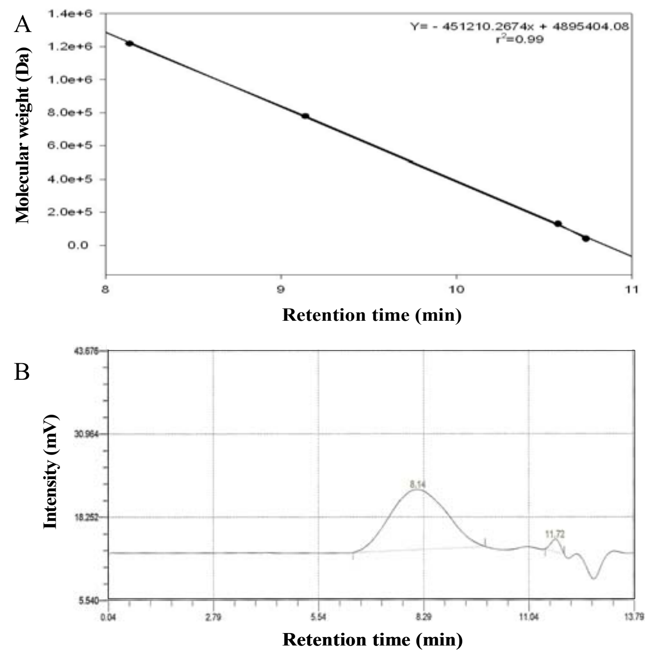

Fig. 4. Standard curve of molecular weight of γ-PGA and HPLC chromatogram of molecular weight of purified γ-PGA. A: Standard curve of molecular weight of γ-PGA by using GPC column. The molecular weight was determined by SEC-MALS. The molecular weight of γ-PGA was 1220 kDa, 243 kDa, 63 kDa and 24 kDa, respectively. B: 8.14 min (γ-PGA), 11.72 min (by-product).

g/L) solution was compared with that of γ-PGA (10 g/L) under 100/sec of shear rate, its value increased 4 fold. The rheological characteristics of γ-PGA solution revealed the typical pseudoplastic properties, showing the decrease in the flow behavior index with increasing γ-PGA concentration.

Changes in the molecular weight and consistency index of γ-PGA according to pH, heat-treatment and acids

Changes in the molecular weight of γ-PGA in aqueous solution were investigated by heat treatment at 100°C. Generally, γ-PGA is water soluble anionic polypeptides which undergo structural changes related to the degree of ionization of γ-carboxyl group of γ-PGA (31). In the heat treatment, the molecular weight of γ-PGA was drastically reduced by lowed the pH in acid conditions. In

Table 2. Consistency index and flow behavior index at various γ-PGA concentrations

γ-PGA concentration (g/L)	Power law model[1)]	
	K ($Pa·sec^n$)	n
10	5.01±1.00	0.42±0.01
20	10.93±0.75	0.38±0.01
30	19.90±0.12	0.34±0.03

[1)]Power law model, $\sigma=K\cdot(\dot{\gamma})^n$; σ, shear stress (Pa); $\dot{\gamma}$, shear-rate (1/sec); K, consistency index ($Pa·sec^n$); n, flow behavior index (dimensionless).

the case of heating at 100°C and pH 2.0, the molecular weight of γ-PGA was rapidly reduced to 243 kDa, 63 kDa and 24 kDa by increasing heating times to 20 min, 40 min and 60 min, respectively (Fig. 5A). On the other hand, the molecular weight of γ-PGA was gradually reduced by the heat treatment at pH 6.0. At pH 7.0 the γ-PGA molecular weight remained constant even with heat-treatment (Fig. 5A). Thus, it is concluded that the molecular weight of γ-PGA can be modulated by adjusting pH in acidic conditions as well as heat treatment. The consistency of γ-PGA solution was greatly decreased by increasing heating time at 100°C. Furthermore, the consistency decrease was greatly dependent upon the pH value in acidic conditions (Fig. 5B). It has been reported that the γ-PGA polymer with un-ionized acid posses a helical conformation, and the polymer with ionized salt behaves like in the random coil state (32). Thus, it implied that a helical conformation of γ-PGA polymer due to un-ionized carboxylic groups in acidic conditions resulted in the decrease in consistency.

To evaluate the effects of acid types on the reduction of molecular weight of γ-PGA, citric acid and acetic acid as food ingredient were compared with hydrochloric acid. As shown in Fig. 6A, γ-PGA solution without heat treatment showed slightly different molecular weights in the case of the treatment with different acids. Among them acetic acid treatment showed a weak hydrolysis of γ-PGA compared with treatment of HCl or citric acid. This implies that γ-PGA in acid solution may be partially hydrolyzed. Furthermore, the heat treatment resulted in a drastic hydrolysis of γ-PGA. During heat treatment the hydrolytic degradation of γ-PGA in acetic acid solution was much weaker than that in HCl or citric acid. The molecular weight of γ-PGA solutions treated with HCl, citric acid or acetic acid tended to decrease with increasing heating time (Fig. 6A). The molecular weight of γ-PGA with 1220 kDa was gradually decreased to below 500 kDa by heating for 20 min. γ-PGA without heat treatment showed a different consistency index according to acid types used. The γ-PGA solution including acetic acid showed a higher initial consistency index compared to that of HCl or citric acid (Fig. 6B). In addi-

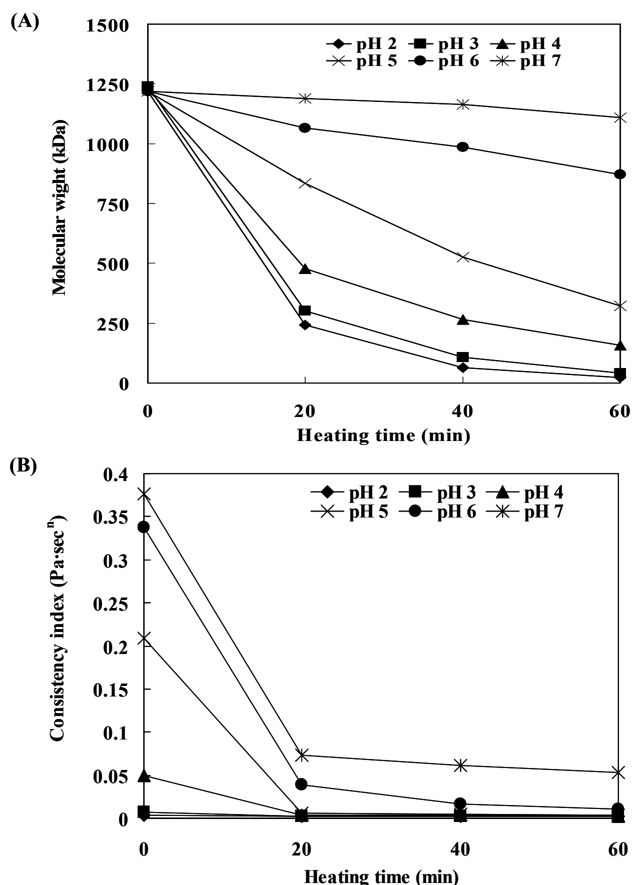

Fig. 5. Changes in molecular weight (A) and consistency index (B) of γ-PGA according to the heat-treatment at different pH. γ-PGA solution, 1% (w/v); pH control, HCl or NaOH; heating temperature, 100°C.

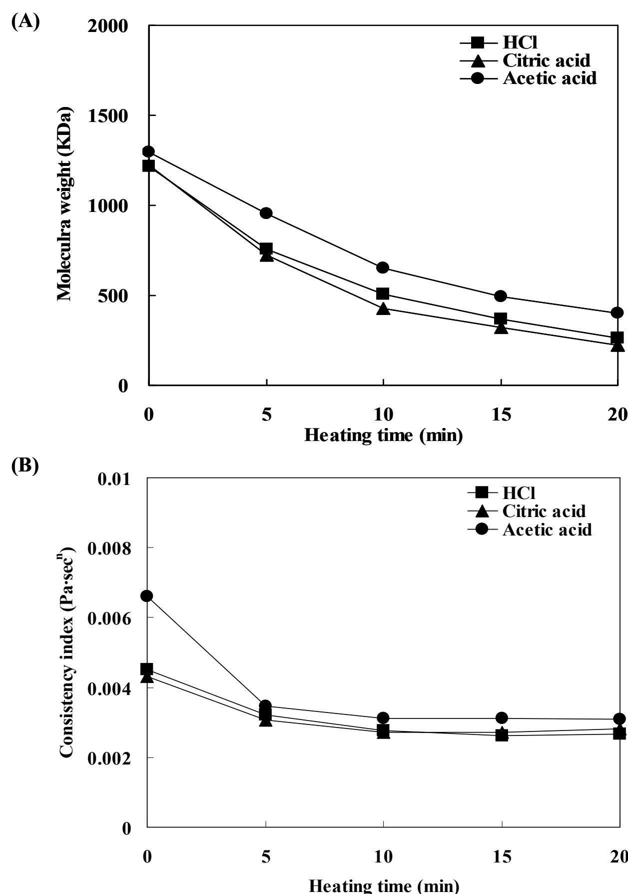

Fig. 6. Changes in molecular weight (A) and consistency index (B) of γ-PGA treated with different acids according to the heat-treatment. γ-PGA solution, 1% (w/v); heating condition, 100°C (pH 2.0).

tion, the heat treatment for 5 min resulted in the rapid decrease in consistency index regardless of acid types. Thus, it is possible to make different molecular weight of γ-PGA in edible acetic acid through heat treatment or sterilization.

The molecular weight and consistency of γ-PGA are very important physicochemical properties, for manipulating the viscosity enhancement of fruit juice, beverages and sports drinks. It also promotes the absorption of minerals and food ingredients which enhance biologically active properties such as drug carrier or sustained release materials. From the above results, the consistency and molecular weight of γ-PGA produced by *B. subtilis* HA were able to be controlled by changing pH in acidic conditions, and applying different organic acids and heat treatment.

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