γ-Polyglutamic Acid Produced by *Bacillus subtilis* (natto): Structural Characteristics, Chemical Properties and Biological Functionalities

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γ-(D,L)-Polyglutamic acid [γ-(D,L)-PGA, or γ-PGA] produced by *Bacillus subtilis* (natto) both on laboratory and pilot scale fermenter systems has been characterized. γ-PGA in its free acid form (H^+) is insoluble in water. The salt forms of K^+, Na^+, NH_4^+, Ca^{2+}, and Mg^{2+} of γ-polyglutamates are fully soluble in water. The structural characteristics of the salts of γ-Polyglutamates (Na^+, K^+, NH_4^+, Ca^{2+}, and Mg^{2+}) were determined with ^{1}H- and ^{13}C-NMR spectroscopy and FT-IR spectroscopy. The thermal properties were determined with thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). The typical physical and chemical properties including pH-titration curve, concentration-dependent viscosity, temperature-dependent viscosity, and heavy metal binding properties with Pb^{2+}, Cd^{2+}, and Cu^{2+} were also determined. The biological functionalities were partially characterized with *in vivo* feeding studies with broilers and egg-layers, and activation of GTF (Glucose tolerance factor) activity with rat 3T3-L1 cell culture studies. γ-(D,L)-Polyglutamic acid [γ-(D,L)-PGA] appears to have five different conformations depending on the environmental conditions. They are the α-helix, β-sheet, helix-to-random coil transition, random-coil and enveloped aggregate. The conformational states, hydrogen bonding and polyanionic nature make γ-PGA a versatile multi-functional biopolymer possessing many useful biological functionalities.

Keywords: γ-PGA; γ-Polyglutamic acid; Sodium γ-polyglutamate; *Bacillus subtilis* (natto); Calcium absorption.

INTRODUCTION

γ-Polyglutamic acid was first discovered as a major constituent in the capsule of *Bacillus anthracis*.[1] It is a naturally occurring biopolymer consisting of a mono amino acid - glutamic acid, in a certain combination of D-form and L-form,[2-9] or all D-form as first reported by Ivanovics and Brucker.[10] γ-(D,L)-polyglutamic acid [γ-(D,L)-PGA, or γ-PGA] is a very unusual poly-anionic biopolymer consisting of only γ-peptide linkage between α-amino and γ-carboxyl groups as shown in Fig. 1.[11,12] γ-PGA may be bio-synthesized following the *de novo* pathway in the solid state fermentation, or the salvage[2] bioconversion pathway in the submerged fermentation, in which, γ-PGA was found to be largely accumulated during the stationary stage of cell growth.

γ-PGA has been reported to be the major component in the viscous sticky mucilage in natto produced by *Bacillus subtilis*,[2,13,14] and being isolated from filtrate of the culture media of Bacillus bacteria.[10-16] Natto, used as a health food in Japan for more than a thousand years, is produced by steaming small soybeans and fermented them with *Bacillus subtilis* (natto).[3,5,6,16-18] This group of strains is classified as *Bacillus subtilis* according to "Bergeys' Manual of Determinative Bacteriology, 3rd Edition, but because of their requirement of biotin for nutrition, growth, and production of the sticky material γ-PGA, therefore, *Bacillus subtilis* (natto) is still generally considered to be of a differ-

Dedicated to the memory of the late Professor Ho Tong-Ing.

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ent species from *Bacillus subtilis* by the Japanese natto industry. γ-(D)-polyglutamic acid, γ-(L)-polyglutamic acid, and γ-(D,L)-polyglutamic acid are collectively called γ-polyglutamic acid (γ-PGA).

In the past few years, γ-PGA has been proposed for potential applications in wide areas including food, cosmetics, agriculture, sewer treatment, biodegradable plastics and other industrial applications. An extensive review on the progress of microbial biosynthesis and the application of γ-PGA was done by Ing-Lung Shih et al.,[19] recently. But the specific structural characteristics and unique biological functionalities of γ-PGA and its derivative salts remain unclear and still very confusing; it is also known as a nontoxic and environmentally friendly natural biodegradable biopolymer. It is our purpose to elucidate more insights into the structural characteristics and their biological functionalities for further development and more practical applications.

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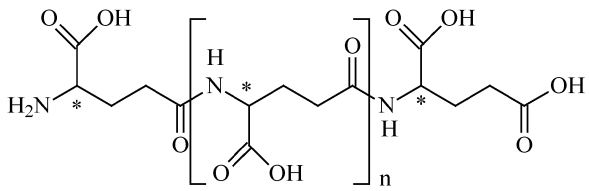
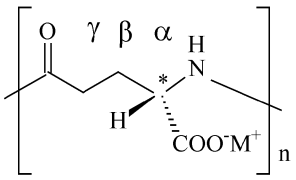


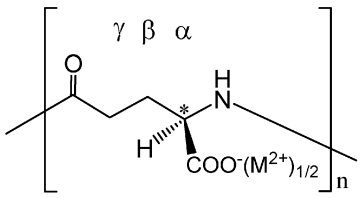
γ-(D,L)-Polyglutamic acid (or γ–PGA)

B



Repeating unit of M^+ γ-(L)-polyglutamate, [M^+ γ-(L)-PGA]

C



Repeating unit of $(M^{2+})_{1/2}$ γ-(D)-polyglutamate, [$(M^{2+})_{1/2}$ γ-(D)-PGA]

Fig. 1. The chemical structure of γ-(D,L)-polyglutamic acid [(γ-(D,L)-PGA), or γ-PGA] and the salts of γ-PGA. (A) the H form of γ-PGA; (B) the M(I) γ-PGA; (C) the $M(II)_{1/2}$ γ-PGA; where M(I) = K^+, Na^+, or NH_4^+ and M(II) = Ca^{++}, or Mg^{++}.

MATERIALS AND METHODS

Bacterial strain and fermentation

Bacillus subtilis (natto) from stock culture collection was selected, based on its superior γ-PGA productivity and growth requirement, for laboratory development and pilot scale production of γ-PGA. Batch cultures of *Bacillus subtilis* (natto) were carried out in a 5-liter laboratory fermenter system, and a 600-liter pilot plant fermenter system was used for process development, optimization and further process scale-up engineering design requirements.

Culture conditions and cultivation

Nutrient Broth (Difco Laboratories) plates containing 1.5% agar were routinely used for culturing *Bacillus subtilis* (natto). The seed culture was continued for 7 days at 40 °C to induce spore formation. After cultivation, 10 mL of sterilized standard saline (0.9% NaCl) was used to suspend the spores. 1 mL. portions of the spore suspension (~5×10^8 cells/mL) were divided into several micro tubes and stored frozen at -80 °C for later cultivation use.

For cultivation in a 5-liter laboratory fermenter (Biostat, B. Braun Biotech Intl.), 3-liters of production medium comprising 8% glucose, 10% sodium L-glutamate, 0.05% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.01% $MnSO_4.H_2O$, 1.5% Peptone, 0.02% $CaCl_2$, 50% biotin, 1.0% yeast extract, and 3.0% NaCl was used. 0.05% Silicone oil (Dow Corning Silicone) was used as antifoaming agent. After routine steam sterilization, the acidity of the medium was adjusted to pH 7.3 with a 5% NaOH sterilized solution. The stock spore solution (1 mL) was diluted with standard saline and then added to the medium to start the cultivation. The temperature and agitation were maintained at 37 °C and 350 rpm, respectively. The aeration was maintained at 0.1 vvm for 20 hours and then increased to 1 vvm after 20 hours of cultivation. 20 mL of the culture sample was removed from the fermenter at appropriate time intervals and stored at -20 °C for further analysis. The concentration of γ-PGA in culture broth obtained by batch fermentation was typically 23-35 g/L.

Extraction, purification, and quantification of γ-PGA

Since γ-PGA is an extra-cellular fermentation prod-

uct with a molecular weight (M.Wt.) in the range from 100,000 to 2,500,000 daltons, the culture medium becomes highly viscous in the final fermentation stages due to the accumulation of γ-PGA in the culture broth. It is necessary to reduce the viscosity of the culture broth for efficient extraction and recovery of γ-PGA products. The cells were first thermally deactivated at 100 °C and an equal volume of de-ionized water was added to the culture broth to reduce the viscosity; enough activated carbon and Celites as filter aid were used to decolorize, and the broth solution was subsequently separated with a filter press, and the cell free solution was then clarified with an ultrafiltration membrane system. A volume ratio of 1:3x alcohol[3] was normally used to precipitate γ-PGA from the cell-free culture broth solution. The γ-PGA precipitates were then recovered by centrifugation. Thoroughly washing the γ-PGA with alcohol or chilled de-ionized water is required to obtain a high purity product. Purification of γ-PGA is the method developed by Goto and Kunioka.[18] The standard curve between γ-PGA concentration and gel-permeation chromatography (GPC) peak area was generated using the purified γ-PGA. The concentration and the molecular weight of γ-PGA were measured at 35 °C using a Perkin-Elmer Model # Series 200 GPC system equipped with TOSOH G6000PWXL and G300SW columns (Tosho, Tokyo, Japan), UV detector (Perkin-Elmer Model 785A), Refractive Index (RI) detector (Waters Model 410), a Du Pond Instrument column heating unit and a Waters Model 730 data module. Pullulan standards of narrow polydispersity (Polyscience Corporation) were used to construct a calibration curve from which molecular weights of γ-PGA were calculated without further correction. The mobile phase contained 50 mM NaCl, 50 mM K_2HPO_4, 0.05% (w/v) sodium azide. Broth samples for various medium conditions at various culture times were collected by removal of a small aliquot from the cultures. Molecular weight of γ-PGA in the broth was routinely monitored. Broth samples were first filtered through a 0.45 μm cellulose acetate syringe filter unit (Uniflo Plus, Schleischer & Schuell Inc. Keene, NH, USA) to remove the cells; the filtrate was then injected into the GPC column to perform the analysis of γ-PGA content in the broth. The mobile phase flow rate was maintained at 0.5 mL/min; the columns and UV and RI detector cells were maintained at 30 °C. The γ-PGA concentrations in culture filtrate solutions ranged from 0.5 to 6 mg/mL, and the injection volume was 50 μL. Dilution of the culture filtrate was sometimes required to keep within this concentration range for better reproducibility. The peak area corresponding to the eluted γ-PGA was measured digitally.

Preparation of purified γ-PGA

Purified γ-PGA was produced by repeating twice the procedure of dissolution and then dialysis with a 30,000 daltons nominal molecular weight limiting membrane to pH 2.0, re-crystalization with alcohol precipitation and then removing the residual water and alcohol from γ-PGA by drying *in vacuo* under 0.1 mmHg and 50 °C for 48 hours.

Nuclear magnetic resonance (NMR) spectroscopy

Measurement of proton (^{1}H)-NMR-spectra were performed with a Varian NMR spectrometer at 400 MHz. ^{1}H-NMR chemical shifts in parts per million (ppm) are reported using HDO at 4.8 ppm as an internal reference. The concentration of the γ-PGA sample and its derivatives are 0.02% (w/v) in D_2O solutions. The pH of samples was adjusted by adding aliquots of concentrated NaOD solutions. The pH values are direct meter readings of a digital pH meter without correction for deuterium effects. The temperature was 30 °C. Peak areas for ^{1}H-NMR spectra were measured by digitally integrating and are reported as relative peak areas representing a given number of hydrogens. The chemical shift of ^{1}H spectra was measured relative to the 3-(trimethylsily) propionic acid-d_4 sodium salt (TSP) signal as 0 ppm.

Measurement of carbon (^{13}C)-NMR spectra was performed with a Brucker WP-270SY at 67.9 MHz in 10 mm tubes. The concentration of γ-PGA samples was 0.06% (w/w) in D_2O solutions, the temperature was 30 °C, and concentrated NaOD solutions were used to adjust pH values. The chemical shift of ^{13}C spectra was measured relative to the dioxane signal from 76.8 ppm.

Elemental analysis

Elemental analysis of purified γ-PGA in the free acid form was carried out on a sample which had been dried to constant weight (0.1 mmHg, 50 °C, 48 hrs) in the presence of phosphorus penta-oxide and then carefully stored in screw-cap glass vials in a desiccator prior to analysis. The analysis was performed at National Taiwan University Microanalysis Laboratory where the γ-PGA samples were handled as a highly moisture sensitive material. Elemental analysis was conducted with an elemental analyzer (Perkin-

Elmer Model CHN-2400).

Measurement of Infra-red (IR) spectra

Measurement of IR spectrum was performed using an IR spectrophotometer (Nicolet-Magna-IR 550, USA) with KBr pellet.

Thermal analysis

Differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA) were used to determine the crystalline melting point (T_m), decomposition temperature (T_d), and stability and which are concerned with chemical transformation of the polymer. Melting and decomposition temperatures represent morphological changes and stability of the polymer. The measurements were made with a Universal v2511TA Instrument.

Measurement of viscosity

Measurement of viscosity was performed using a Brookfield viscometer Model DV-I+ at 25 °C. The pH-titration curve, the typical pH-dependent viscosity, the temperature-dependent viscosity, and the concentration-dependent viscosity were obtained for a 4% sodium γ-polyglutamate (apparent M. Wt. 980 k Daltons) solution.

RESULTS

The glutamic acid added to the culture media was efficiently bio-converted into γ-PGA in the stationary phase via a salvage bioconversion pathway. The typical concentration of γ-(D,L)-polyglutamic acid produced by Vedan's 5-L laboratory scale fermenter system was 28 ~ 35 g/L. The apparent molecular weight of γ-PGA was estimated to be over 2×10^6 daltons.

Samples of purified γ-polyglutamic acid (PGA, H form), sodium γ-polyglutamate (γ-PGA, Na^+ form) calcium γ-polyglutamate (γ-PGA, Ca^{2+} form), magnesium γ-polyglutamate (γ-PGA, Mg^{2+} form), ammonium γ-polyglutamate (γ-PGA, NH_4^+ form) were produced and determined; the results are summarized in Table 1,[11,12] and further discussed in the following.

The infrared spectra of γ-polyglutamic acid (PGA, H form) and γ-polyglutamate salts in KBr pellet show a characteristic strong amide absorption at about 1620-1655 cm^{-1}, a weaker carbonyl absorption at about 1395-1454 cm^{-1}, and a strong hydroxyl absorption at 3400-3450 cm^{-1}. The absorption peak at 3400-3450 cm^{-1} was characteristic of OH stretching from the bound hydroxyl group and adsorbed water molecules. The peaks in the range from 2900 cm^{-1} to

Table 1. Summarized data of chemical shifts of ^1H and ^{13}C, FT-IR absorption peaks, and thermal analysis for ã-PGA and the mineral salts: Na^+, K^+, NH_4^+, Ca^{++}, and Mg^{++} of ã-polyglutamates[11,12]

Item	H	Na^+	K^+	NH_4^+	Ca^{++}	Mg^{++}
^1H-NMR(400 MHz, D_2O, 30 °C)						
Chemical shift in ppm: α CH		3.98	4.00	3.68	4.18	4.08
β CH_2		1.98, 1.80	1.99, 1.80	1.68, 1.48	2.16, 1.93	2.05, 1.88
γ CH_2		2.19	2.19	1.93	2.38	2.31
^{13}C-NMR(67.9 MHz, D_2O, 30 °C)						
Chemical shift in ppm: α CH		56.43	62.21		62.21	62.10
β CH_2		31.61	35.16		36.17	35.11
γ CH_2		34.01	39.74		39.68	39.60
CO		182.21	182.11		182.16	182.12
COO^-		182.69	185.46		185.82	185.16
FT-IR absorption (KBr), cm^{-1}						
C=O, stretch	1739					
Amide I, N-H bending		1643		1643	1622	1654
Amide II, stretch		1585				
C=O, symmetric stretch	1454	1402		1395	1412	1411
C-N, stretch	1162	1131		1139	1116	1089
N-H, oop bending	698	707		685	669	616
O-H, stretch	3449	3436		3443	3415	3402
Thermal analysis:						
Hydrated water (%)	0	10	42		20	40
Dehydration temperature, °C		109	139		110	122
Melting point, T_m, °C	206	160	193, 238	219		160
Decomposition temp., T_d, °C	209.8	340	341	223	335.7	331.8

2800 cm^{-1} were indicative of aliphatic N-H stretching. The absorption peaks around 1600-1660 cm^{-1} and 1390-1450 cm^{-1} were characteristic of amide groups and C=O groups. The strong absorption peaks observed in the range from 1085 cm^{-1} to 1165 cm^{-1} are characteristic of C-N groups. The IR spectra of the γ-polyglutamic acid and the γ-polyglutamates conform to the presence of carboxyl, hydroxyl, carbonyl and amide groups.

Dehydration temperatures are: 109 °C, 139 °C and 122 °C for Na^+, K^+ and Mg^{++} salts of γ-PGA, respectively. The melting points (T_m) are 206 °C, 160 °C, 193 °C & 238 °C, 219 °C, 101 °C and 122 °C for H^+, Na^+, K^+, NH_4^+, Ca^{2+} and Mg^{2+} salts of γ-PGA, respectively. The decomposition temperatures (T_d) are 209.8 °C, 340 °C, 341 °C, 223 °C, 335.7 °C and 331.8 °C for the H, Na^+, K^+, NH_4^+, Ca^{2+} and Mg^{2+} salts of γ-PGA, respectively. The hydrated waters are found to be10%, 42%, 20% and 40% for Na^+, K^+, Ca^{2+} and Mg^{2+} salts of γ-PGA, respectively. The large amount of the hydrated water reflects strong water binding ability and the hygroscopic nature of these products.

DISCUSSION

γ-Polyglutamic acid (γ-PGA, H form)

Zanuy[23] reported that the conformational preferences of the naturally occurring γ-poly (D-) glutamic acid in the un-ionized state showed that a left-handed helix with 3_{19}, a 19-membered ring hydrogen bonds set between the CO of the amide group *i* and the NH of amide group of (*i* + 3) is the most stable conformation for the γ-polyglutamic acid, and only weak intra-molecular interactions between the oxygen of the carbonyl side groups and the NH of the backbone amide groups were detected. γ-PGA (H form) is not soluble in water but only the organic solvent-DMSO. The typical elemental analysis of γ-(D,L)-polyglutamic acid sample is shown below:

Elemental analysis of purified γ-polyglutamic acid (γ-PGA) with an apparent molecular weight of 1.23×10^6 daltons

Found:	C: 44.86%,	H: 5.91%,	N: 10.49%,	S: 0%
Calculated:	C: 46.51%,	H: 5.43%,	N: 10.85%,	S: 0%

The experimental results showed in close conformity to the calculated formular composition. No contamination of S or $SO_4^=$ was found.

FT-IR spectrum of γ-polyglutamic acid (γ-PGA, H form) in KBr pellet show: C=O stretching band at 1739 cm^{-1}: C=O symmetric absorption band at 1454 cm^{-1}; C-N stretchinh at 1162 cm^{-1}; N-H oop bending at 698 cm^{-1}; and O-H stretching band at 3449 cm^{-1}. Amide I, N-H bending and Amide II, stretching bands are not observable presumably due to strong hydrogen bonding.

Thermal analysis results for γ-polyglutamic acid (γ-PGA, H form) show the melting point™ and the decomposition temperature (T_d) are 206 °C and 209.8 °C, respectively.

Sodium γ-Polyglutamate (γ-PGA, Na^+ form)

The ionized salts of γ-PGA are no longer in handedness helix structures, but behave like they are in the open random-coil state, which are soluble in water solutions and tasteless. The anionic α-carboxyl group in each of the glutamic acid moieties is reactive and available for binding to the cationic entity of the other molecule or biopolymer, or for further modification as a free carboxylic acid. The typical ^{1}H-NMR, ^{13}C-NMR, FT-IR, TGA, pH titration curve, pH-dependent viscosity, temperature-dependent viscosity and concentration-dependent viscosity are shown in Figs. 2, 4, 5b, 7, 9, 10, respectively. Samples of sodium γ-polyglutamate with apparent molecular weight of 1,230,000 daltons pH 6.8 were used for the analytical studies.

^{1}H-NMR for sodium γ-polyglutamate in D_2O shows the chemical shifts are: 3.98 ppm for α-CH proton; 1.98 ppm and 1.80 ppm for β-CH_2 proton; and 2.19 ppm for γ-CH_2 proton. ^{13}C-NMR for sodium γ-polyglutamate shows the chemical shifts are: 56.43 ppm for α-CH_2 group; 31.61 ppm for β-CH_2 group; 34.01 ppm for γ-CH_2 group; 182.21 ppm for CO group; and 182.69 ppm for COO^- group.

The FT-IR absorption bands for sodium γ-polyglutamate in KBr pellets are: Amide I, N-H bending band at 1643 cm^{-1}; Amide II, stretching band at 1585 cm^{-1}; C=O symmetric stretching band at 1402 cm^{-1}; C-N stretching band at 1131 cm^{-1}; N-H oop bending band at 707 cm^{-1}; and O-H stretching band at 3436 cm^{-1}.

The thermal analysis results for sodium γ-polyglutamate show: hydrated water is 10%, dehydration temperature is 109 °C, the melting point (T_m) is 160 °C and the decomposition temperature (T_d) is 340 °C.

Potassium γ-Polyglutamate (γ-PGA, K^+ form)

Potassium γ-polyglutamate is an ionic salt and very soluble in water. Its ^{1}H-NMR shows similar chemical shifts

to those of sodium γ-polyglutamate, but the ^{13}C-NMR shows smaller chemical shifts for the three groups of α-, β- and γ-carbon as compared to the corresponding groups in sodium γ-polyglutamate, and the chemical shift of α-COOH coalesces toward that of a carbonyl group, which may represent some difference in structural conformation and chemical functionality, as shown in containing 42% hydration water as compared to only 10% hydration water found in sodium γ-polyglutamate. Samples of potassium γ-polyglutamate with an apparent molecular weight of 980,000 daltons were used in the analytical studies.

^{1}H-NMR for potassium γ-polyglutamate in D_2O shows the chemical shifts are: 4.00 ppm for α-CH proton; 1.99 ppm and 1.80 ppm for β-CH_2 proton; and 2.19 ppm for γ-CH_2 proton. ^{13}C-NMR for potassium γ-polyglutamate shows the chemical shifts are: 62.21 ppm for α-CH_2 group; 35.16 ppm for β-CH_2 group; 39.74 ppm for γ-CH_2 group; 182.21 ppm for CO group; and 185.46 ppm for COO^- group.

The Thermal analysis results for potassium γ-polyglutamate show: hydrated water is 40%, dehydration temperature is 139 °C, the melting points (T_m) are 193 °C & 238 °C and the decomposition temperature (T_d) is 341 °C.

Calcium γ-Polyglutamte (γ-PGA, Ca^{++} form)

The calcium salt of γ-PGA is very soluble in water, even at neutral pH. It is tasteless. The characteristic coordinated complex ionic structure between calcium ion and γ-polyglutamate ion largely contributes to the solubility and stabilizes the calcium ion in aqueous or body fluids at neutral pH conditions, which may have important signifi-

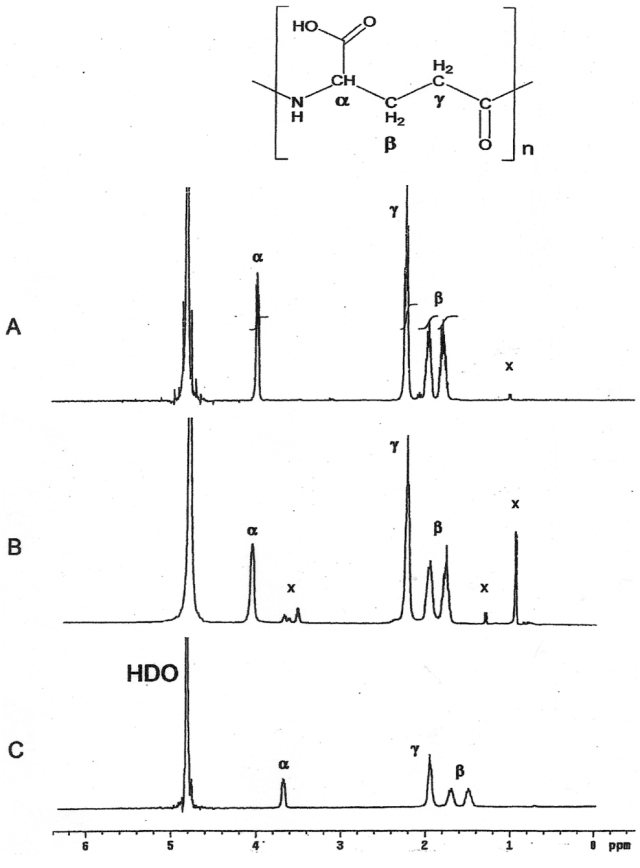


Fig. 2. 400 MHz ^{1}H-NMR spectra of M^+ γ-polyglutamate in D_2O at pH 6.8 and 30 °C. Chemical shift was measured in ppm units from the internal standard. (A) sodium γ-polyglutamate, (B) potassium γ-polyglutamate, (C) ammonium γ-polyglutamate. x indicates impurity peak.

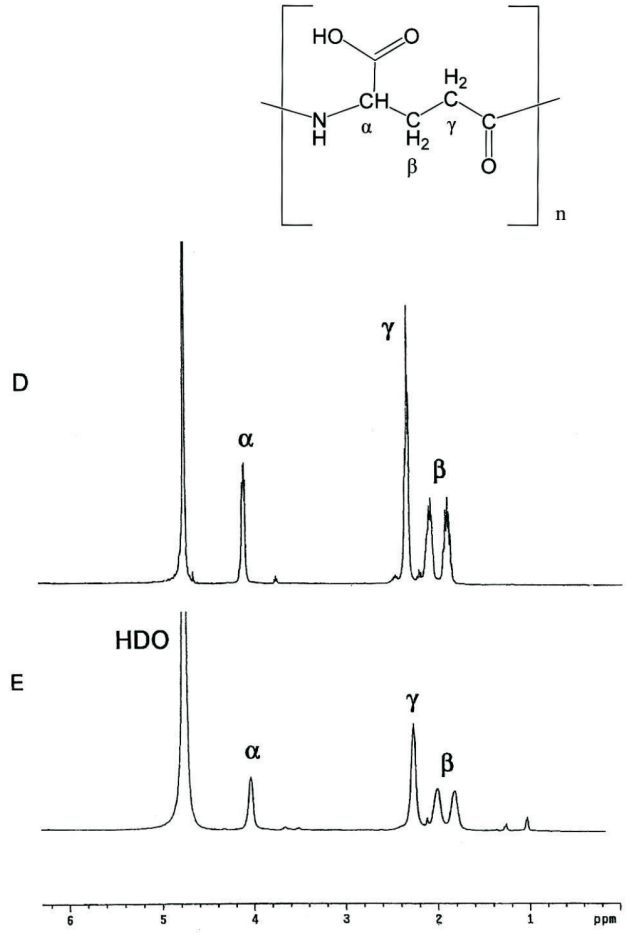


Fig. 3. 400 MHz ^{1}H-NMR spectrum of M^{2+} γ-polyglutamate in D_2O at pH 6.8 and 30 °C. (D) calcium γ-polyglutamate, (E) magnesium γ-polyglutamate. Chemical shift was measured in ppm units from the internal standard.

cance in health-care functionalities such as providing calcium bio-availability and facilitating the calcium absorption and bone formation in osteoblast cells or even reducing the osteoporosis conditions and the over-all growth and health conditions. The typical ^{1}H-NMR, ^{13}C-NMR, FT-IR, TGA, and pH-titration curves are shown in Figs. 3, 4, 6, 7, and 9, respectively. Samples of calcium γ-polyglutamate with an apparent molecular weight of 490,000 daltons and pH 6.8 were used for the analytical studies.

^{1}H-NMR for calcium γ-polyglutamate in D_2O shows the chemical shifts are: 4.18 ppm for α-CH proton; 2.16 ppm and 1.93 ppm for β-CH_2 proton; and 2.38 ppm for γ-CH_2 proton. ^{13}C-NMR for calcium γ-polyglutamate show the chemical shifts are: 62.21 ppm for α-CH_2; 36.17 ppm for β-CH_2; 39.68 ppm for γ-CH_2; 182.16 ppm for CO; and 185.82 ppm for COO^-.

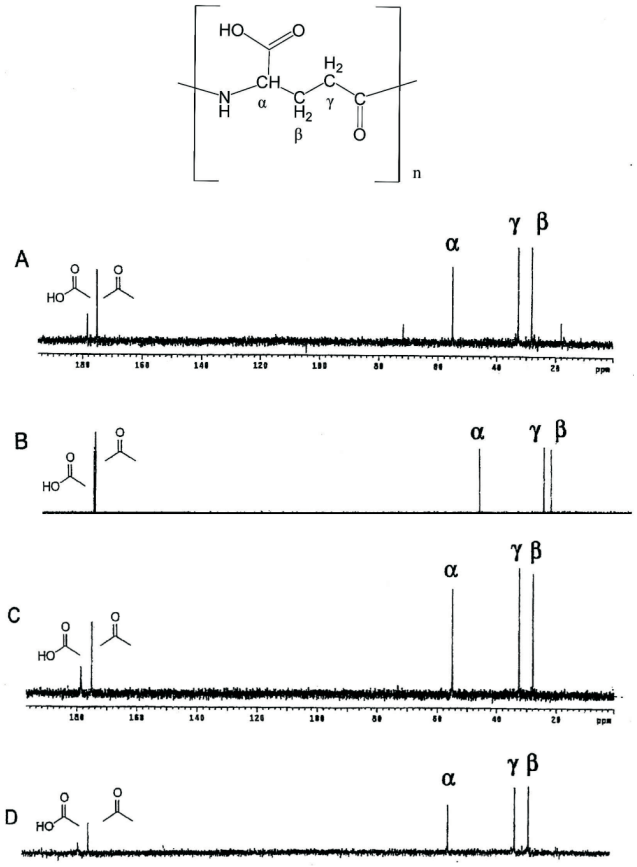


Fig. 4. ^{13}C-NMR spectra (67.9 MHz) of γ-polyglutamate in D_2O at pH 6.8 and 30 °C. Chemical shift was measurement in ppm units relative to the internal reference. (A) potassium γ-polyglutamate, (B) sodium γ-polyglutamate, (C) calcium γ-polyglutamate, (D) magnesium γ-polyglutamate.

The FT-IR absorption for calcium γ-polyglutamate in KBr pellets are: Amide I, N-H bending band at 1622 cm^{1}; Amide II, stretching band not observed presumably due to strong ionic complexation; C=O symmetric stretching band at 1412 cm^{-1}; C-N stretching band at 1116 cm^{-1}; N-H oop bending band at 669 cm^{-1}; and O-H stretching band at 3415 cm^{-1}.

The Thermal analysis results for calcium γ-polyglutamate show: hydrated water is 20%, dehydration temperature is 110 °C, the melting point (T_m) is not observed and the decomposition temperature (T_d) is 335.7 °C.

Magnesium γ-Polyglutamate (γ-PGA, Mg^{2+} form)

The magnesium salt of γ-PGA is also very soluble in water or aqueous solution at neutral pH conditions. It is tasteless as well. The coordinated ionic complex structure between magnesium ion and γ-polyglutamate ion is apparently similar to that of calcium γ-polyglutamate, also the magnesium γ-polyglutamate has a much higher hydrated water content of 40% as compared to 20% hydrated water in calcium γ-polyglutamate. The higher water hydration may reflect its difference in structural conformation and coordination in ionic structure. The typical ^{1}H-NMR, ^{13}C-NMR, FT-IR, and DSC, are shown in Figs. 3, 4, 6, 8, respectively. Samples of magnesium γ-polyglutamate with an apparent molecular weight of 890,000 daltons and pH 6.8 were used in the analytical studies.

^{1}H-NMR for magnesium γ-polyglutamate in D_2O shows the chemical shifts are: 4.08 ppm for α-CH proton; 2.05 ppm and 1.88 ppm for β-CH_2 proton; and 2.31 ppm for γ-CH_2 proton. ^{13}C-NMR for magnesium γ-polyglutamate show the chemical shifts are: 62.10 ppm for α-CH_2 group; 35.11 ppm for β-CH_2 group; 39.60 ppm for γ-CH_2 group; 182.12 ppm for CO group; and 185.16 ppm for COO^- group.

The FT-IR absorption for magnesium γ-polyglutamate in KBr pellets are: Amide I, N-H bending band at 1654 cm^{1}; Amide II, stretching band not observed presumably due to strong ionic complexation; C=O symmetric stretching band at 1411 cm^{-1}; C-N stretching band at 1089 cm^{-1}; N-H oop bending band at 616 cm^{-1}; and O-H stretching band at 3402 cm^{-1}.

The Thermal analysis results for magnesium γ-polyglutamate show: hydrated water is 40%, dehydration temperature is 122 °C, the melting point (T_m) is 160 °C and the

decomposition temperature (T_d) is 331.8 °C.

Ammonium γ-Polyglutamate (γ-PGA, NH_4^+ form)

The ammonium salt of γ-PGA is soluble in water at neutral pH. It is also tasteless. The ammonium γ-polyglutamate is in linear coil conformation similar to that of the sodium γ-polyglutamate under neutral pH conditions. The ^{1}H-NMR, FT-IR, DSC, and pH-titration curve are shown in Figs. 2, 5b, and 9, respectively. Samples of the ammonium γ-polyglutamate with an apparent molecular weight of 890,000 daltons, pH 6.8 were used in the analytical studies.

^{1}H-NMR for ammonium γ-polyglutamate in D_2O shows the chemical shifts are: 3.68 ppm for α-CH proton; 1.68 ppm and 1.48 ppm for β-CH_2 proton; and 1.93 ppm for γ-CH_2 proton.

The FT-IR absorption in KBr pellets are: Amide I, N-H bending band at 1643 cm^{-1}, Amide II, stretching band not observed presumably due to strong ionic complexation or strong hydrogen bonding, C=O symmetric stretching band at 1395 cm^{-1}, C-N stretching band at 1139 cm^{-1}, N-H oop bending band at 685 cm^{-1}, O-H stretching band at 3443 cm^{-1}.

The Thermal analysis results show the melting point (T_m °C) and the decomposition temperature (T_d °C) are 219 °C and 223 °C, respectively.

Salvage Bioconversion Pathway for Production of γ-Polyglutamic Acid

The submerged fermentation process provides an economical advantage for industrial scale production of γ-polyglutamic acid. γ-Polyglutamic acid is first biosynthesized inside the cell body and subsequently released into the fermentation broth. The salvage bioconversion pathway as shown in Fig. 11, was first proposed by Hara T. et al.[24] for the racemization of L-glutamic acid into D-glutamic acid and co-polymerization of D- and L-glutamic acids into γ-polyglutamic acid. L-glutamic acid serves as an inducer and feed stock for the production of γ-polyglutamic acid. In this bioconversion pathway, most of the L-glutamic acid is first racemized into D-glutamic acid, then both L- and D-glutamic acids are co-polymerized into the end-product γ-polyglutamic acid. Both D- and L-glutamic acid optical enantiomers exist in γ-PGA produced by Bacillus subtilis,[2-9] and were reporte[21] to be mostly in a ratio of 50-70% of D-glutamic acid to 50-30% of L-glutamic acid due to the specific glutamate racemase activity in the *Bacillus subtilis* (natto) strains. Manganese ion was reported as play an important role in regulating the ratio of D:L in the γ-PGA biosynthesis.[4,22] The sequence of both L- and D-glutamic acids in γ-polyglutamic acid remains unknown. Table 2 shows the bacteria producing γ-polyglutamic acid con-

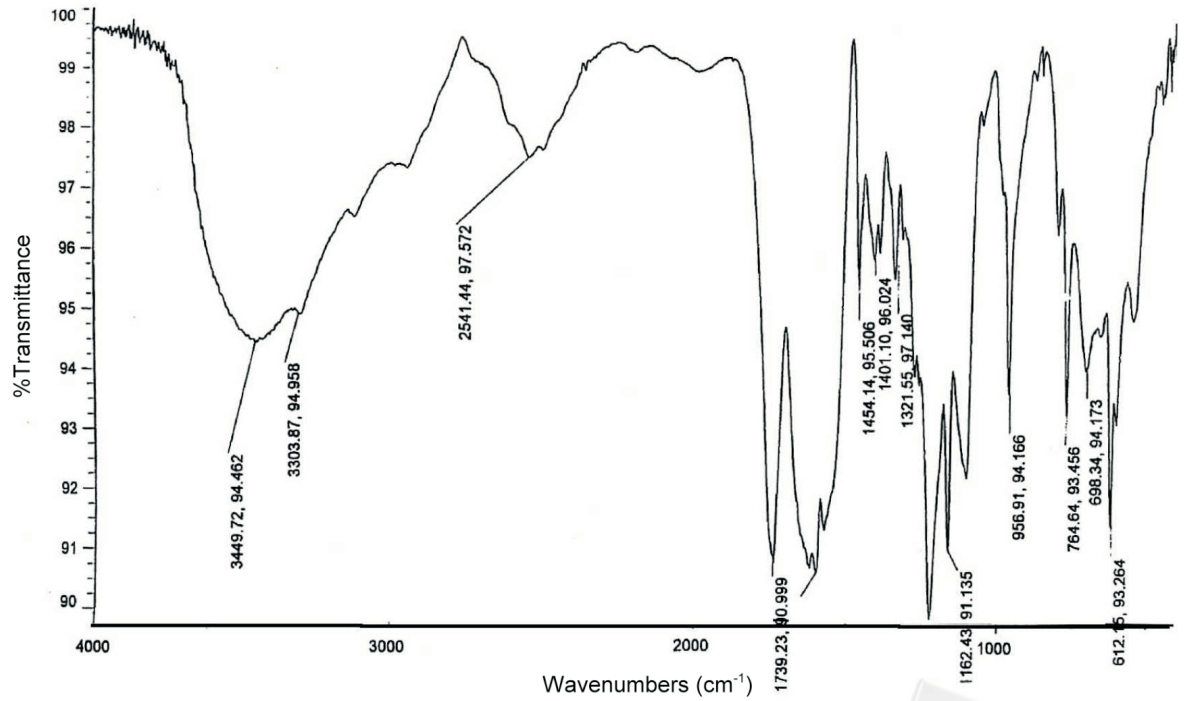


Fig. 5a. Infrared (FT-IR) absorption spectrum of γ-polyglutamic acid (H form) in KBr pellet.

taining different ratios of D- to L-glutamic acid. The ratios of D:L in the γ-(D,L)-polyglutamic acid produced by Vedan's 5-L laboratory scale fermenter system was determined and found to be 52% of D-form: 48% of L-form. The

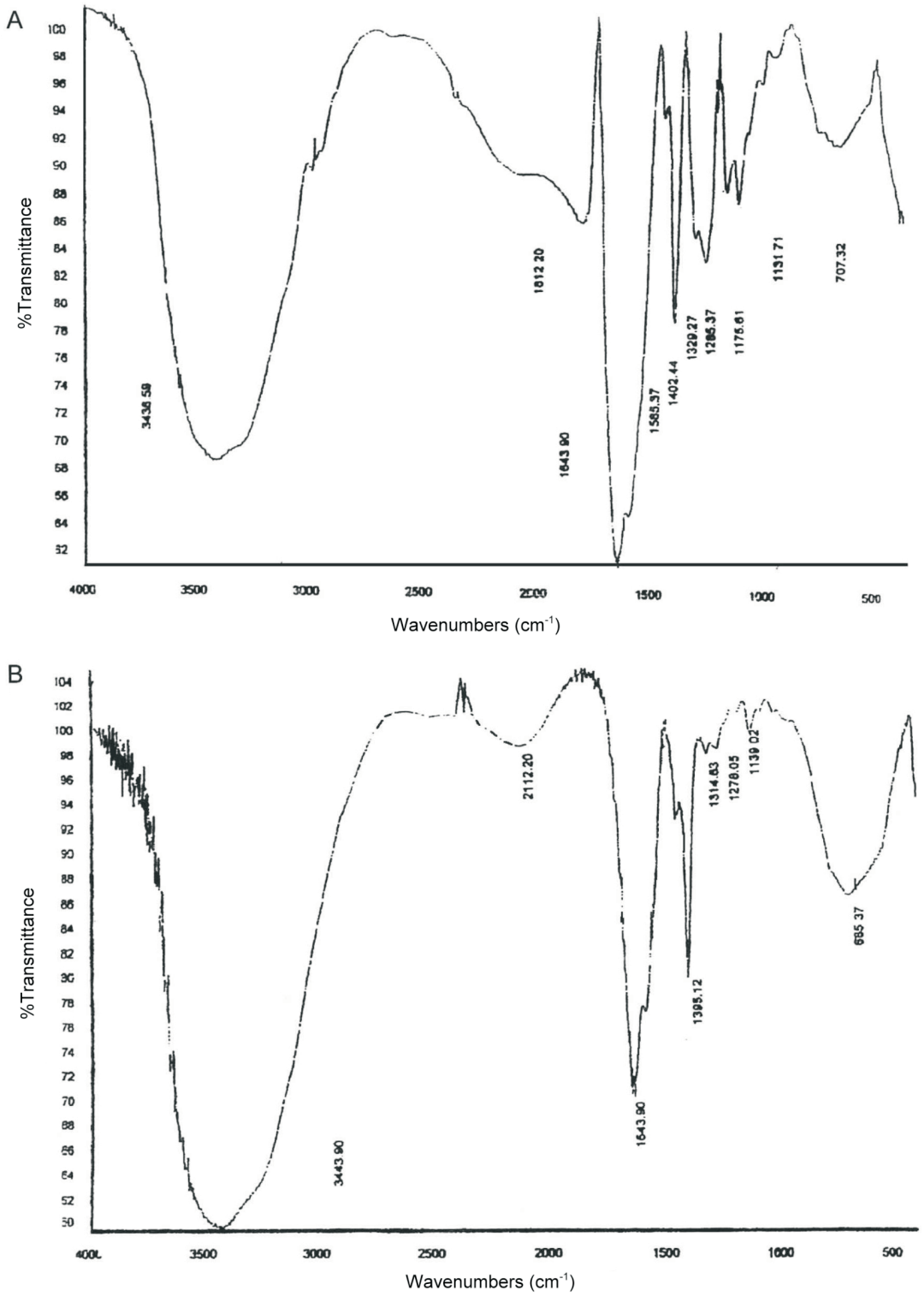


Fig. 5b. Infrared (FT-IR) absorption spectra of γ-polyglutamates in KBr pellet. (A) sodium γ-polyglutamate, (B) ammonium γ-polyglutamate.

analytical results on the γ-polyglutamic acid produced from a 600 L pilot fermenter system conform to those produced from the 5-L fermenter system. Fig. 12 shows the time course of a typical growth of sodium γ-polyglutamate

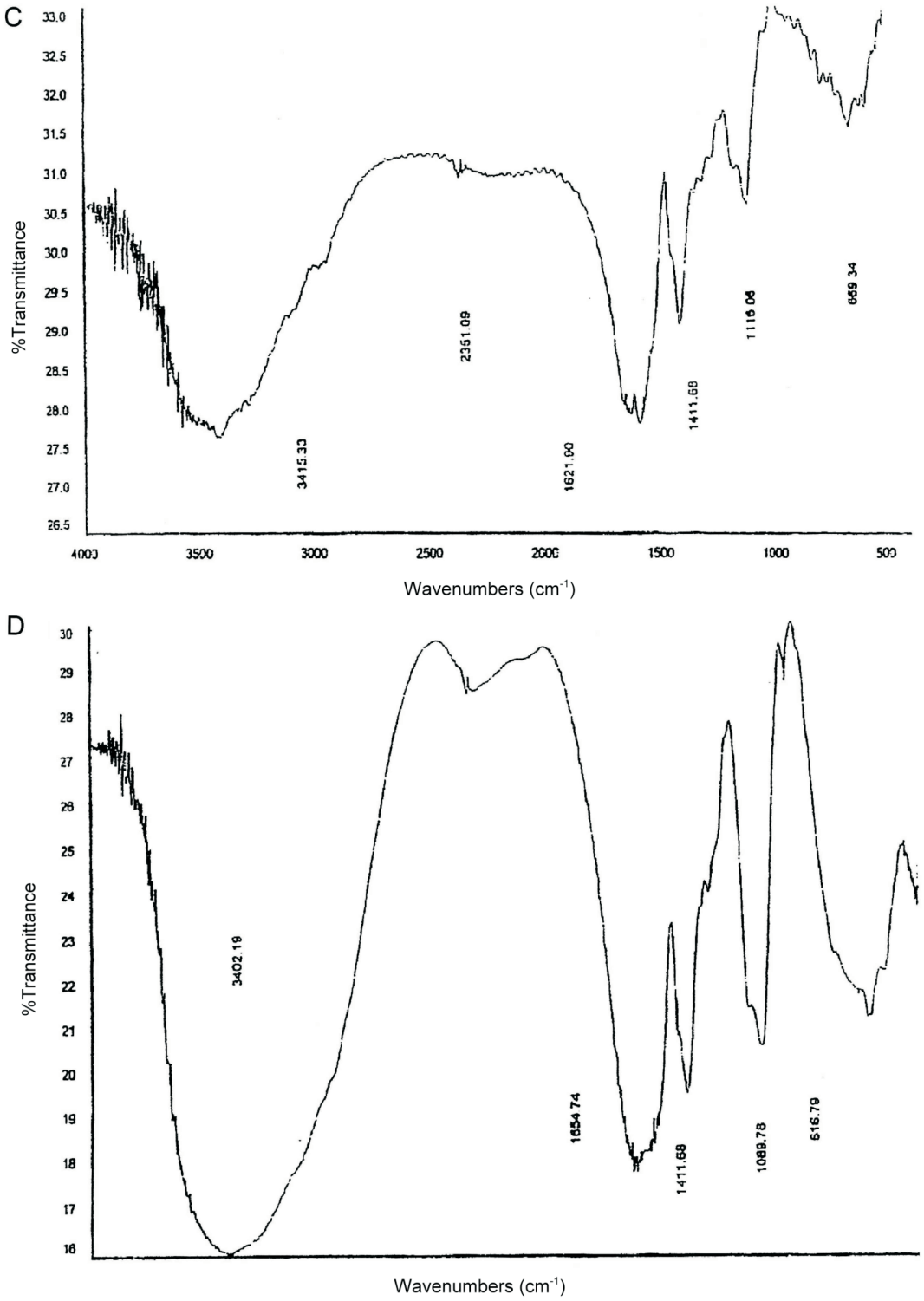


Fig. 6. Infrared (FT-IR) absorption spectra of γ-polyglutamates in KBr pellet. (C) calcium γ-polyglutamate, (D) magnesium γ-polyglutamate.

in a 600 liter fermenter.

Chemical Structural Characteristics

Glutamic acid, the building monomer of γ-polyglutamic acid, possesses three chemically active functional groups: α-NH_2, α-COOH, and γ-COOH. The chemical reactivity of the three functional groups follow the order: α-NH_2 > α-COOH, > γ-COOH. The hydrogen dissociation constants of the three groups are: $pK_\alpha(= pK_1) = 2.13 \sim 2.2$, $pK_\gamma(= pK_2) = 4.25 \sim 4.32$, and $pK_3 = 9.7 \sim 9.95$. In a chemically catalyzed polymerization of glutamic acid, the condensation would take place between the more reactive α-COOH group and α-NH_2 group, resulting in the formation of an α-peptide bond, and the end product will be

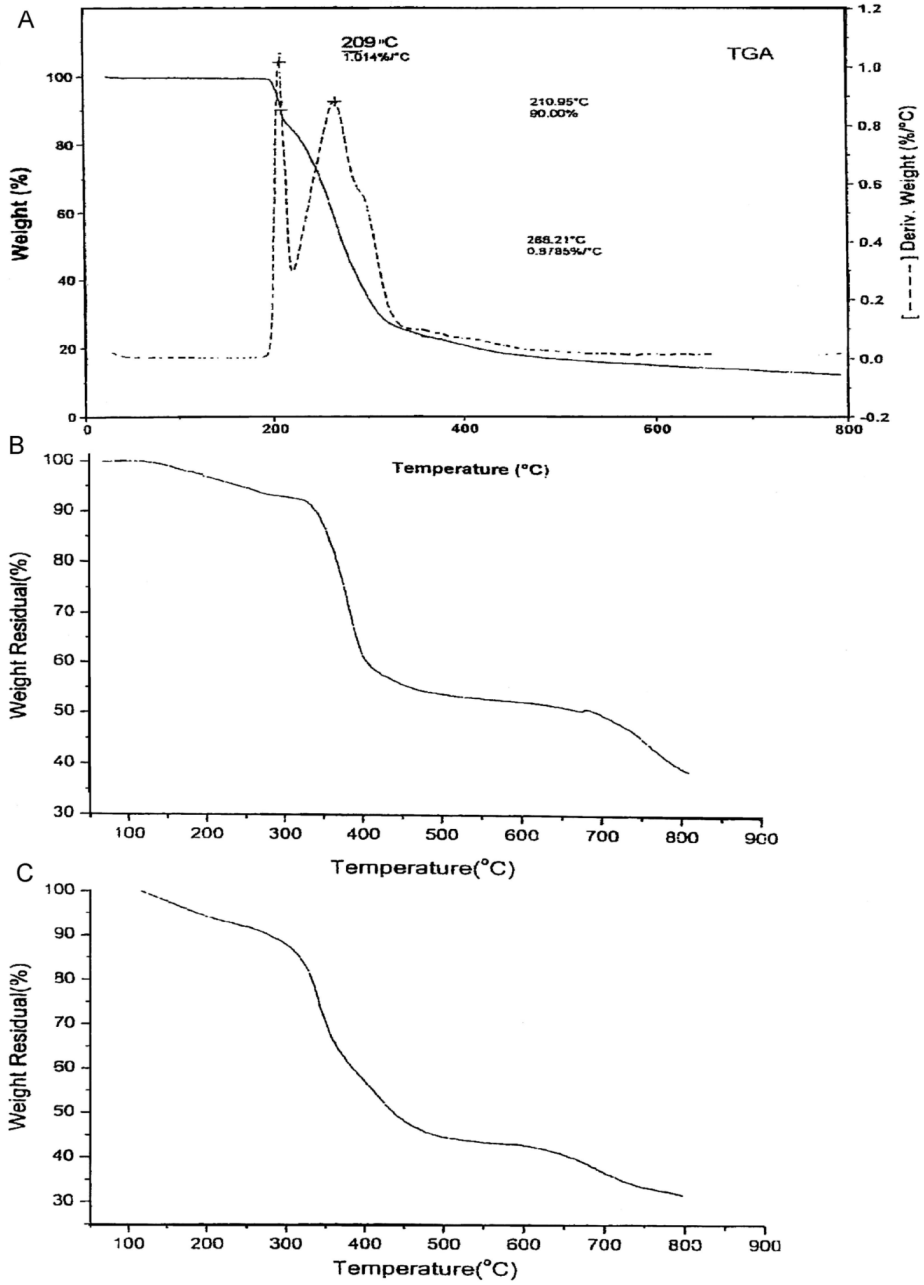


Fig. 7. Thermal properties of γ-polyglutamic acid and γ-polyglutamates. T_d (decomposition temperature) was measured with thermal gravimetric analysis (TGA) by heating at a rate of 10 °C/min. (A) γ-polyglutamic acid, (B) sodium γ-polyglutamate, (C) calcium γ-polyglutamate.

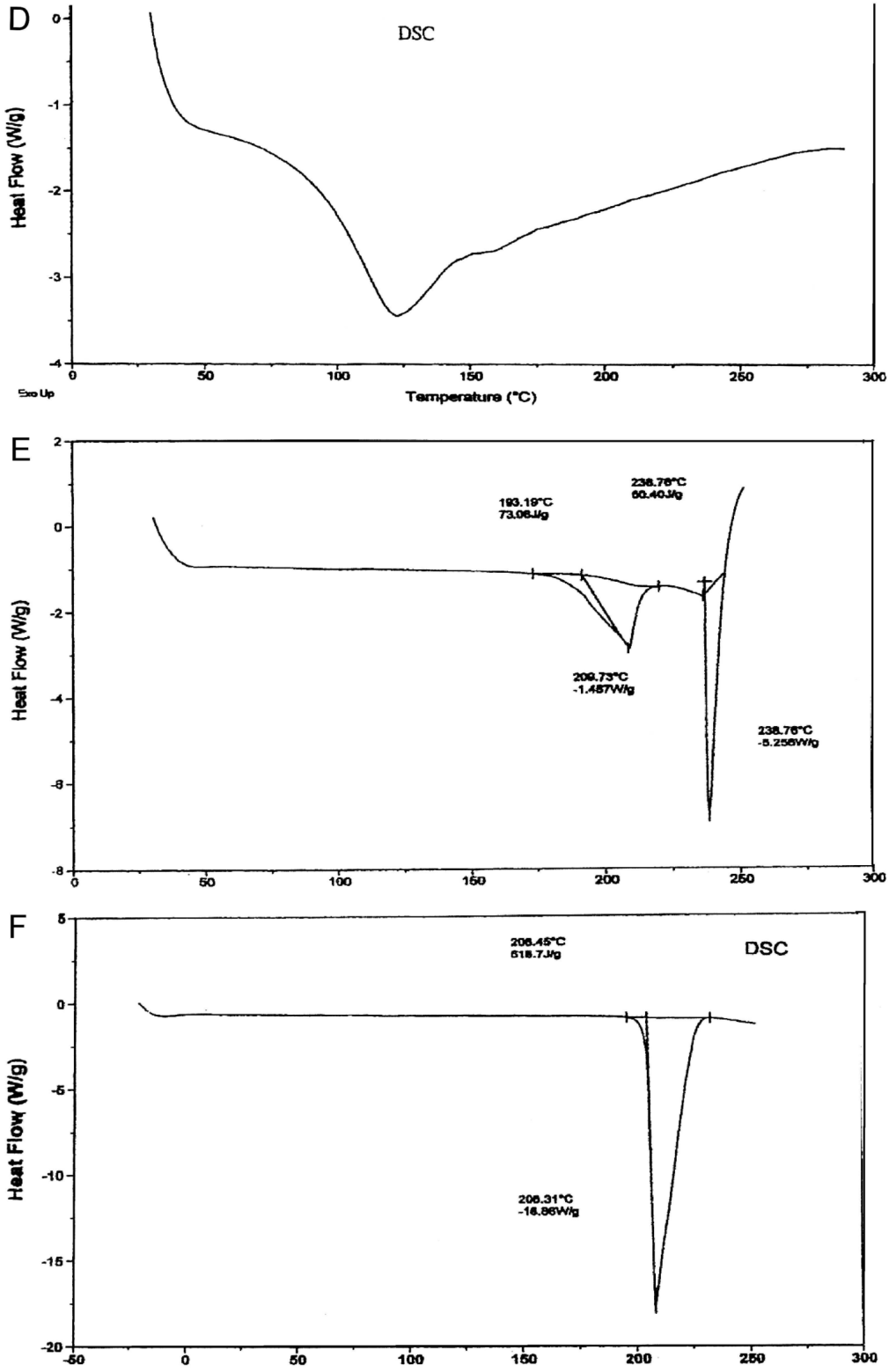


Fig. 8. Thermal properties of M^{2+} γ-polyglutamates. T_m (endothermic melting point) was measured with differential scanning calorimeter (DSC) by heating at a rate of 10 °C/min. (D) magnesium γ-polyglutamate, (E) potassium γ-polyglutamate, (F) γ-polyglutamic acid.

Table 2. The bacteria producing γ-(D,L)-PGA[11,12]

γ-PGA producing bacteria	Mol. wt., Mw	Ratio of D: L		Yield (g/L)	References
		D- (%)	L- (%)		
B. subtilis (natto) MR141	2.1×10^6	80	20	2~4	Ogawa et al. (1997)[2]
B. subtilis TAM-4	$6.0 \times 10^5 \sim 1.6 \times 10^6$	78	22	22.1	Ito et al. (1996)[3]
B. licheniformis ATCC9945A	$1.4 \times 10^4 \sim 9.8 \times 10^5$	45~85	55~15	16~20	Cromwick et al. (1995)[4]
B. subtilis IFO3335	$10^5 \sim 2 \times 10^6$	17	83	9.6	Kunioka et al. (1994)[5]
B. subtilis F-2-01	5.0×10^6	55	45	25~50	Kubota et al. (1993)[6]
B. subtilis var. polyglutamicum	1.1×10^6	40	60	16~20	Unpublished
B. subtilis NRRL B-2612	2.0×10^4	52	48	10~14	Ward et al.[7]
B. natto		58	42		Saito et al. (1974)[8]
B. anthracis	$2.1 \sim 5.3 \times 10^4$	100	0	1~2	Ivanovics et al. (1937)[10]
B. licheniformis A35		59	41	8.1	Cheng et al. (1989)[9]
B. subtilis (natto) Vedan	$1.5 \sim 3.0 \times 10^6$	52	48	30~45	Unpublished (Vedan)

α-polyglutamic acid. But in the submerged fermentation process, most L-glutamic acids are enzymatically racemized to D-glutamic acids, and both D- and L-glutamic acids are co-polymerized through the formation of γ-peptide bonds between less reactive γ-COOH groups and α-NH_2 groups, resulting in the formation of end product, γ-(D,L)-polyglutamic acid. α-Peptide bond is the normal bonding found in the protein structures, which can be decomposed by the most proteases. γ-Peptide bond in γ-polyglutamic acid can

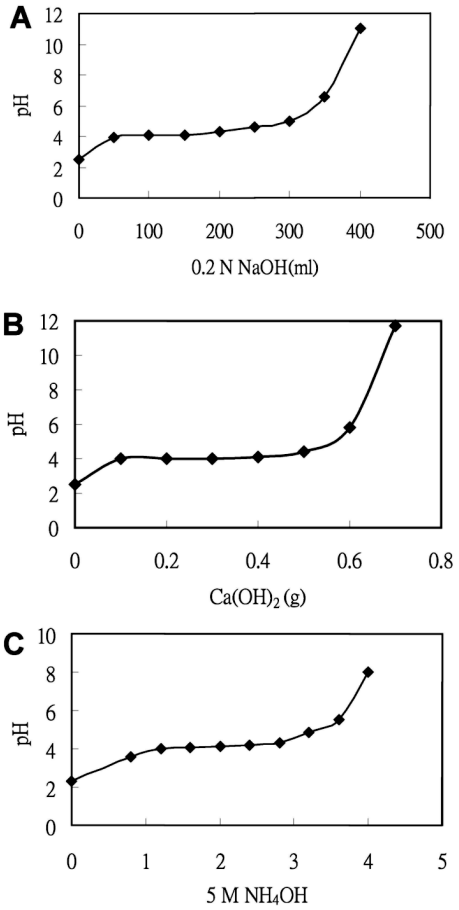


Fig. 9. pH-titration curve of γ-polyglutamic acid (γ-PGA) at 25 °C. (A) 10% γ-PGA with 0.2 N NaOH, (B) 2% -PGA with $Ca(OH)_2$, (C) 4% -PGA with 5 NH_4OH.

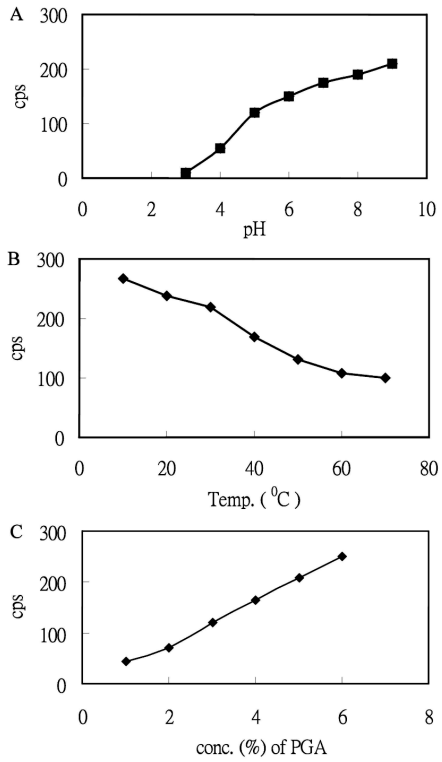


Fig. 10. (A) pH dependence of the viscosity of 4% sodium γ-polyglutamate at 25 °C; (B) Temperature dependence of the viscosity of 4% sodium γ-PGA, pH 6.8; (C) Concentration dependence of the viscosity of 4% sodium γ-PGA, pH 6.8.

only be hydrolyzed by γ-GTP (γ-glutamyltranspeptidase), which is rare in nature. None of the general proteases can hydrolyze γ-polyglutamic acid; therefore, γ-polyglutamic acid possesses a certain resistance against microbial attack.

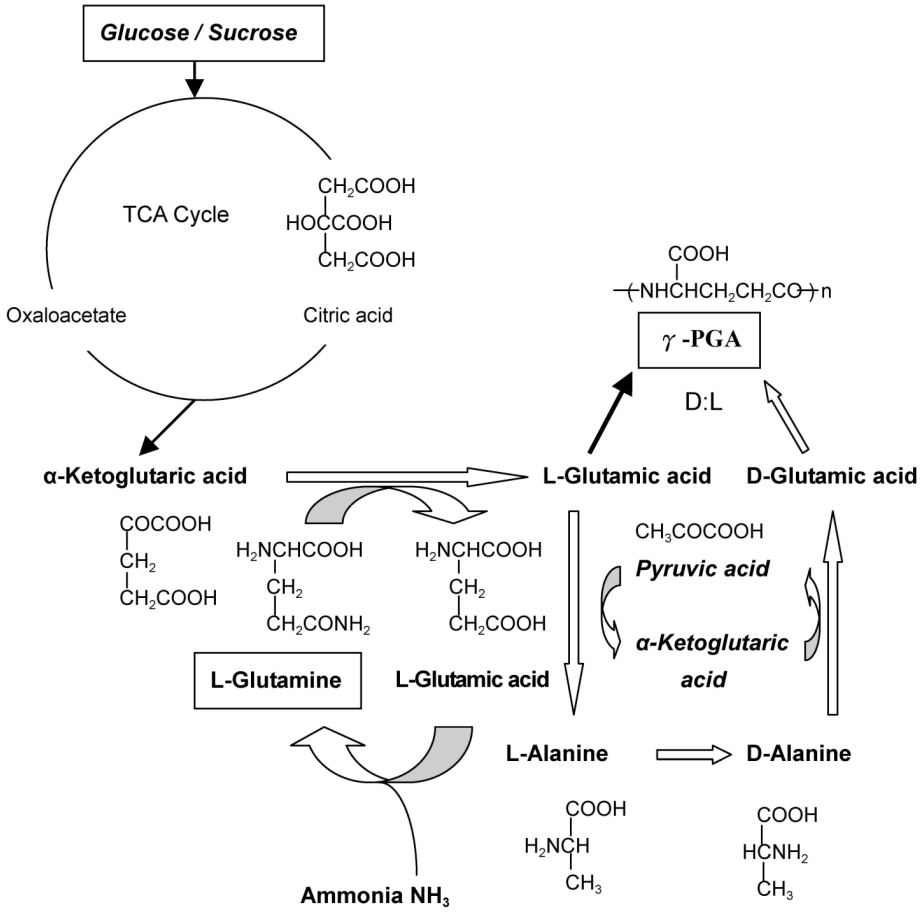


Fig. 11. γ-PGA biosynthesis via salvage pathway. The microbes are stimulated to produce γ-PGA by the addition of L-glutamic acid.

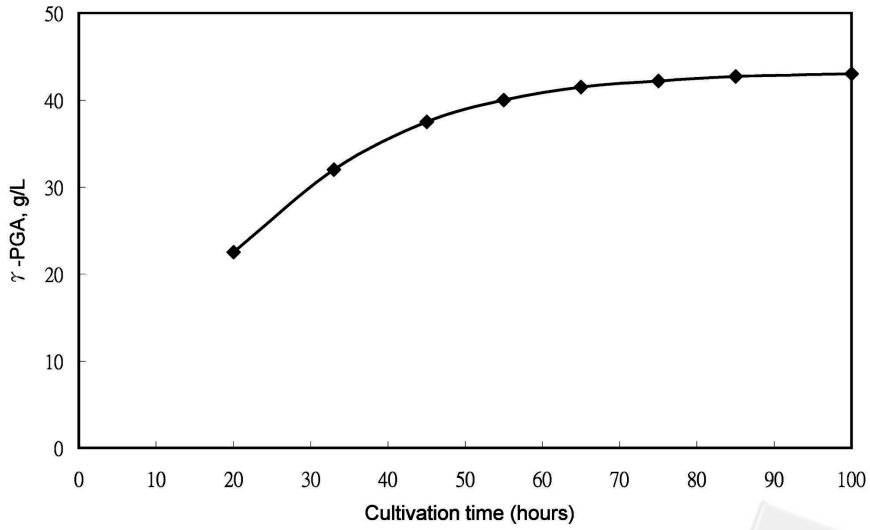


Fig. 12. Typical growth of sodium γ-polyglutamate in a 600 liter fermenter system.

In γ-polyglutamic acid (γ-PGA, H form), four strong intra molecular hydrogen bondings existed between the carbonyl group of the γ-peptide bond and the amino group of the other γ-peptide bond,[23] as compared to only one hydrogen binding occurring in an average of 3.6 amino acid residues found in proteins in nature. They are designated as 3_{19}, 3_{17}, 3_{14} and 3_{12} and formed within every 3 glutamic moieties. ORD (Optical Rotatory Dispersion)[24] study showed that such strong hydrogen bondings make the γ-polyglutamic acid (γ-PGA, H form) form a tightly compacted α-helix conformation, resulting in a strong hydrophobic character and becoming insoluble in water at pH 2.0. As pH rises, hydrogen bonding breaks up, α-helix conformation changes into linear random-coil conformation, and α-COOH group ionizes to form α-COO^- anion. At pH 4.09, there are approximately 50% of the α-helix conformation changed into random-coil transition, or there exists about 50% γ-polyglutamic acid in poly anionic random-coil conformation, as shown in the pH-titration curve in Fig. 9. At pH 6.5 or higher, γ-polyglutamic acid essentially exists in linear random-coil conformation only and shows a poly-anionic characteristic, which allows γ-polyglutamic acid more functionality for binding to cationic molecules or surfaces, and makes it a novel multi-functional biopolymer for applications in a wide range of fields.

Chelation with Heavy Metals (Pb^{2+}, Cd^{2+}, Cu^{2+})

Chelation of Pb^{2+} with sodium γ-polyglutamate (γ-PGA, Na^+ form)

A 100 mL working solution of 2% sodium γ-polyglutamate (in Na^+ form) was prepared by dissolving sodium γ-polyglutamate with a molecular weight of 890×10^3 in de-ionized water, and pH was adjusted to 6.0 with 10% NaOH solution. A 25 mL working solution of 10^{-2} M $Pb(NO_3)_2$ was prepared from Lead Nitrate (C.P. grade, from Merck). 15 mL of a series of different concentrations of $Pb(NO_3)_2$ in the final concentrations of 0.09, 0.12, 0.17, 0.22, 0.36, and 0.45 mM/L were prepared and 25 mL of 0.016% sodium γ-polyglutamate (in Na^+ form) was added to each of the $Pb(NO_3)_2$ solutions, mixed well and pH was then adjusted with 10% NaOH solution to pH 6.0; de-ionized water was added to a total final volume of 50 mL. The mixtures were mixed well and left standing for 30 minutes for complete chelation. The precipitates were filtered using an ultrafiltration membrane filtration cartridge with a molecular weight cut-off of 20,000 daltons to separate the precipitates. The residual Pb^{2+} ion concentrations in clear filtrate were then determined using an ICP/MS analyzer. The results are shown in Table 3.

The above results showed that Pb^{2+} ions were chelated with γ-polyglutamate and formed stable white precipitates in the form of complex aggregates of Pb^{2+} and γ-PGA. The saturated absorption capacity is about 4 mM of Pb^{2+} per gram of sodium γ-polyglutamate.

Chelation of Cd^{2+} with sodium γ-polyglutamate (γ-PGA, Na^+ form)

Cadium Nitrate, $Cd(NO_3)_2$ (C.P. grade, from Merck) was used to replace $Pb(NO_3)_2$, and the same experiment procedure with absorption of Pb^{2+} in the previous section was repeated with a series of different final concentrations of 0.09, 0.12, 0.17, 0.21, 0.34, and 0.42 mM/L $Cd(NO_3)_2$. The results are shown in Table 4.

The above results showed that Cd^{2+} ions were chelated with γ-polyglutamate and formed stable precipitates in the form of complex aggregates of Cd^{2+} and γ-PGA. The saturated absorption capacity is about 3.7 mM of Cd^{2+} per gram of sodium γ-polyglutamate.

Chelation of Cu^{2+} with sodium γ-polyglutamate (γ-PGA, Na^+ form)

$Cu(NO_3)_2$ (C.P. grade, from Merck) was used to replace $Pb(NO_3)_2$, and the same experiment procedure with the absorption of Pb^{2+} in the previous section was repeated

Table 3. Chelation of Pb^{2+} ion with sodium γ-polyglutamate (γ-PGA, Na^+ form) at 25 °C, pH 6.0, and 0.008% sodium γ-polyglutamate

Original conc. of Pb^{2+}, $[Pb^{2+}]_o$, mM/L	Conc. of Pb^{2+} in filtrate, mM/L	Pb^{2+} adsorbed by γ-PGA, mM/L	Pb^{2+} adsorbed by γ-PGA, mM/g
0.09	0.00	0.09	1.09
0.12	0.00	0.12	1.48
0.17	0.00	0.17	2.15
0.22	0.00	0.22	2.66
0.36	0.03	0.33	4.07
0.45	0.14	0.31	4.12

Table 4. Chelation of Cd^{2+} ion with sodium γ-polyglutamate (γ-PGA, Na^+ form) at 25 °C, pH 6.0, and 0.008% sodium γ-polyglutamate

Original conc. of Cd^{2+}, $[Cd^{2+}]_o$, mM/L	Conc. of Cd^{2+} in filtrate, mM/L	Cd^{++} adsorbed by γ- PGA, mM/L	Cd^{2+} adsorbed by γ- PGA, mM/g
0.09	0.00	0.09	1.15
0.12	0.00	0.12	1.52
0.17	0.00	0.17	2.09
0.21	0.00	0.21	2.65
0.34	0.04	0.30	3.71
0.42	0.12	0.30	3.73

Table 5. Chelation of Cu^{2+} ion with sodium γ-polyglutamic acid (γ-PGA, Na^+ form) at 25 °C, pH 6.0, and 0.008% sodium γ-polyglutamic acid

Original conc. of Cu^{2+}, $[Cu^{2+}]_o$, mM/L	Conc. of Cu^{2+} in filtrate, mM/L	Cu^{2+} adsorbed by γ-PGA, mM/L	Cu^{2+} adsorbed by γ-PGA, mM/g
0.08	0.00	0.08	1.03
0.11	0.00	0.11	1.42
0.16	0.00	0.16	1.99
0.20	0.00	0.20	2.56
0.33	0.00	0.33	4.16
0.42	0.03	0.39	4.85

with a series of different final concentrations of 0.08, 0.11, 0.16, 0.20, 0.33, and 0.42 mM/L $Cu(NO_3)_2$. The results are shown in Table 5.

The above results showed that Cu^{2+} ions were chelated with γ-polyglutamate and formed stable cyan precipitates in the form of complex aggregates of Cu^{2+} and γ-PGA. The saturated absorption capacity is about 4.5 mM of Cu^{2+} per gram of sodium γ-polyglutamate.

Conformational states in γ-polyglutamic acid and γ-polyglutamates

Apparently, γ-polyglutamic acid binds with heavy metal ions, such as Pb^{2+}, Cd^{2+} and Cu^{2+}, through chelation and followed by conformational changes from linear random-coil into enveloped aggregates and subsequently precipitates. The experimental results suggest that other heavy metal ions such as Al^{3+}, Fe^{2+}, Fe^{3+}, Cr^{3+}, Zn^{2+}, Ni^{2+}, and As^{4+} etc ions will bind with γ-polyglutamic acid in a similar chelation mechanism and followed by conformational change of γ-polyglutamic acid into enveloped aggregate precipitates. The chelations of heavy metal ions with γ-polyglutamic acid at neutral pH conditions follow the saturation binding pattern. Competitive chelation with γ-polyglutamic acid may exist for heavy metal ions existing in the solution.

It is interesting to note that both Ca^{2+} and Mg^{2+} ions bind with γ-polyglutamic acid in a total different mechanism from the chelation-aggregation pattern. The pH-titration curve of γ-polyglutamic acid with $Ca(OH)_2$ (in Fig. 9) appears to follow a coordinated ionic complex mechanism. Both ionic complexes of calcium γ-polyglutamate and magnesium γ-polyglutamate salts are totally soluble at neutral pH conditions. The capability of γ-polyglutamic acid to dissolve calcium and magnesium compounds and form stable coordinated ionic complexes may also find nutritional value in delivering more bio-available calcium and magnesium for calcium absorption by life cell systems and healthy function in bone formation in reducing old-age osteoporosis conditions. The same ionic complexing function of γ-polyglutamic acid with calcium may also find value in dissolving physiological stones or calcium scales in water systems or sewer treatment.

Our results from the saturation binding experiments on chelation of γ-PGA with the heavy metal ions of Pb^{2+}, Cd^{2+}, Cu^{2+} and Al^{3+} in the aqueous solutions at pH 6.0 showed that completely chelated γ-PGA precipitates proceed through a new conformational change from the soluble linear random-coil into insoluble enveloped and coiled aggregates, which is a new conformation much different from either of the three conformations: α-helix form,[24] par-

allel β-form,[25] and linear random-coil form,[26] already reported.

There appear to be five different conformational states that may exist for γ-polyglutamic acid and γ-polyglutamates: α-helix in un-bonded H form, β-sheet in the microbial cell envelope, helix-random-coil transition, linear random-coil at neutral pH conditions, and enveloped aggregate in chelated complexes with heavy metals. α-(L)-PGA has pK_γ (= pK_2) = 4.375 as compared to pK_2 = 3.9 in the glutamic acid. While γ-(D)-PGA has pK_α = 4.06, α-(D)-PGA has pK_γ = 5.5 ~ 6.0, and γ-(D, L)-PGA has pK_α = 4.09, as compared to pK_α = 2.2, and pK_γ (= pK_2) = 3.9 in glutamic acid. The linear random-coil conformation of γ-polyglutamic acid changes into chelated enveloped aggregate state upon binding with heavy metal ions, but changes into soluble coordinated ionic complex conformation upon binding with either calcium or magnesium ions. The strong hydrogen bindings and conformational changes of γ-polyglutamic acid make it a novel multiple-functional biopolymer with chemical characteristics suitable for application in diversified fields.

From the pH-titration curve of sodium γ-PGA (in Fig. 8), one can observe that γ-polyglutamic acid is in its un-ionized free acid form (H form) at pH 2, due to strong intra-molecular hydrogen-bonding to form stable α-helix conformations, which are very hydrophobic and insoluble in water. As pH increases, the intra-molecular hydrogen bonding in the γ-PGA is disrupted and the carboxyl groups turn into an anionic group. At pH 4.09, there are about 50% of the α-COOH groups being ionized into α-COO^- groups. Once hydrogen-bonding is disrupted and the α-COOH ionized, the γ-PGA exerted a conformational change from insoluble α-helix into the soluble linear random-coil conformation in water solution until the pH reaches a value of about 6.0. When pH reaches 6 or higher, all hydrogen-bonding disappears, and insoluble α-helix conformation of γ-polyglutamic acid transforms into soluble linear random-coil conformation, and all carboxyl groups change into free pendant anionic groups. The solubility increases as the pH of the solution increases, resulting from the increase in the dissociation of α-COOH groups and extended molecular length of γ-polyglutamate. The intrinsic viscosity is also increased with increase in pH until the pH reaches about 8.0 (in Fig. 9).

Hikichi et al.[27] concluded that at neutral pH and at room temperature there exists weak bivalent Cu^{2+} and Mn^{2+}, and γ-PGA complex in which oxygen atoms of the side-chain carboxyl group bound to both Cu^{2+} and Mn^{2+} ions, and probably the nitrogen atom of amide group of γ-PGA are bound to Cu^{2+} and that the oxygen atom of the carbonyl and carboxyl groups are bound to Mn^{2+} ions. These groups bound to Cu^{2+} and Mn^{2+} ions undergo rapid exchange between the bound and free states with water molecules. The carboxyl and amide groups undergo rapid exchange between the bound and free states at room temperature and at neutral pH. The oxygen atoms of the side-chain carboxyl and the main-chain carbonyl groups are coordinated to Mn^{2+} and also undergo rapid chemical exchange between the bound and free states. Hikichi found that all ^{1}H-NMR undergo up-field chemical shifts with increasing pH from 3 to 5, where the carboxyl is ionized. The change of the α-proton signal is most significant, reflecting proximity to the ionization-able carboxyl groups; such changes in chemical shift are indicative of conformational differences at various pH.

pH stability and temperature stability of γ-polyglutamate (Na^+ form)

From the pH-titration curve of sodium γ-PGA (in Fig. 8), one can observe that γ-polyglutamate is in its un-ionized free acid form (H form) at pH 2, due to strong intra-molecular hydrogen bonding to form stable compact α-helix conformations,[23] which are insoluble in water. As pH rises higher than 2.0, the hydrogen bonding in the γ-PGA starts to be disrupted. At pH 4.09, there are about 50% of the insoluble α-helix conformations transformed into soluble linear random-coil conformations, the α-COOH groups being ionized into anionic α-COO^- groups. The carboxyl groups turned into anionic groups and as pH continues to reach 6.0 or higher, all the intra-molecular hydrogen bonding is disrupted and all the carboxyl groups change into free pendant anionic groups, resulting in the increase in solubility and the intrinsic viscosity due to the complete conformational change from insoluble α-helixes into soluble linear random-coils.

The intrinsic viscosity is also increased with increase in pH until the pH reaches a value of about 8.0, as shown in Fig. 9. Crescenzi[26] and Rydon[24] reported that the viscosity of γ-PGA in water is strongly dependent on both pH and ionic strength due to abrupt changes that these parameters induce in the conformation of the biopolymer.

Goto[18] reported that γ-PGA in an aqueous solution was hardly hydrolyzed within 60 min. of heating at 80 °C, but was rapidly hydrolyzed by heating at 120 °C, and the

hydrolysis of γ-PGA in an aqueous solution by heating was due to a random chain scission; the activation energy of the polymer chain scission due to the hydrolysis of γ-PGA in the aqueous solution by heating was approximately 120 kJ/M. The relative bond strengths of the C-C, C-N and C-O bonds are 347 kJ/M, 305 kJ/M and 359 kJ/M. The breaking of side-chain C-O groups is highly unexpected. Kubota[28] reported that hydrolysis of γ-PGA in alkaline solution {10% γ-PGA (Na^+ form) in 0.15 equivalent of NaOH} at 90 °C resulted in an increase in the dispersity of the fragmented γ-PGA and considered that hydrolysis of the amide groups in the terminal was easier than in the main part, especially in high polymers.

BIOLOGICAL STUDIES

METHOD AND RESULTS

In Vivo field broiler feeding study

The nutritional and health aspects of γ-polyglutamic acid (Na^+ form) were studied in a field feeding to 2 sets of 4 groups of baby broilers, over a period of 25 days, at a level of 1000 ppm of γ-polyglutamic acid (Na^+ form) LM (low molecular weight), with apparent molecular weight of 250 × 10^3 daltons, as a feed supplement to the regular feed formulation. Ho[29] reported that γ-polyglutamic acid (Na^+ form) facilitated the absorption of calcium and the overall weight of both male and female broilers. The average end weight per kg of initial weight over a feeding period of 25 days are 4.712 kg for those with regular feed containing γ-polyglutamic acid (Na^+ form), and 4.629 kg for those with regular feed (control). The shank bone lengths are 9.38 cm for male and 9.18 cm for female broilers in the test set of feed containing γ-polyglutamic acid (Na^+ form), and 9.25 cm for male and 9.10 cm for female broilers in the control set with regular feed. Calcium contents in the shank bone are 17.66% for male broilers and 16.69% for female broilers in the control set with regular feed.

In Vivo field egg-layer feeding study

Egg-layers of 73 weeks old were used in this study. A total of 8 lots with 8,000 chickens per lot were used. Egg samples were collected during a 4-week period of regular feed, followed by a 4-week period of regular feed containing 1000 ppm of γ-polyglutamic acid (Na^+ form) LM (low molecular weight) with an apparent molecular weight ranging from 200 × 10^3 to 400 × 10^3 daltons and then a 2-week period of regular feed. The egg samples were tested for the strength of egg shell the thickness of egg shell pH of egg-white and egg yolk and the heights of egg white. The HU (Haugh Unit) values, which are a general fresh index or health index of the egg quality, were then calculated. Ho[29] reported that there were significant improvements in firming up of the egg yolk and egg white, and increases in egg shell strength, as shown in the reduced difference in HU and reduced difference in KGF (Eggshell strength unit) values during the period of feeding containing γ-polyglutamate (Na^+ form). The egg shell thickness, egg white height and egg yolk color are all improved during the period of feeding containing γ-polyglutamate (Na^+ form). Table 6 shows the effect of γ-polyglutamate (Na^+ form) on the quality of eggs.[29]

Table 6. The effect of γ-polyglutamic acid (Na^+ form) on the egg quality, HU value

Feeding	Sampling week	Test group HU value	Control group HU value	Difference in HU* value
Regular feed	1-4	73.7	77.0	3.4
Regular feed with 0.1% γ-polyglutamic acid (Na^+ form), LM	5-8	70.9	71.9	1.0
Regular feed	9-10	67.9	71.0	3.2

Note: 1. 73 week old egg-layers were used.
2. 4 lots with 8,000 chickens/lot were used.
3. " * " for an average value of 10 eggs.
4. HU value is defined as following:
HU = Haugh Unit--adjusted egg albumin height value
= 100 * log (H-G*0.5(30*W*0.37 – 100)/100 + 1.9
Where: H – Albumin height in mm, W – Weight of egg in gram, G - 32.2

***In Vitro* rat 3T3-L1 cell culture study**

In Vitro cell culture study – facilitating GTF (Glucose Tolerance Factor) activity by 3T3-L1 cell model.

Samples of γ-polyglutamic acid (Na^+ form) HM (high molecular weight), with an apparent molecular weight of 880×10^3 daltons and γ-polyglutamic acid (Na^+ form) LM (low molecular weight), with an apparent molecular weight of 250×10^3 daltons were used in the cell culture study.

Samples of rat 3T3-L1 pre-adipose cells were thawed and cultured in DMEM (Dulbeco's modified eagle media) containing 10% FBS (fetal bovine serum) in an incubator at 37 °C under 5% CO_2 for a week before transferring to differentiation culture. After culturing for 2 days in the differentiation culture medium DMEM containing 10% FBS, 0.5 mM IBMX (Isobutylxanthine), 1 μM DX (Dexamethasone) and 1 μg/mL insulin, the cells were transferred to the general DMEM containing 10% FBS, and continued to be cultured for another 10 days until more than 90% of cells transformed into mature adipose cells. At this time, the cells change their shape from their original star shapes into oily droplet shapes, which can be visually observed as white oily matter on the bottom of the culture plate. When the cell differentiation is completed, the following experiment can take place.

The mature adipose cells were first cultured in a sugar free DMEM medium containing 2% FBS in an incubator at 37 °C under 5% CO_2 for 1 hour, washed with PBS (0.01 M phosphate buffer solution, pH 7.4), then 200 μL of test samples which containing different concentrations of γ-polyglutamate (Na^+ form), and 200 μL of KRB (Krebs-Ringer-Bicarbonate) buffer solution containing 10 nM insulin and 2.5 g/L glucose were added, then incubated at 37 °C under 5% CO_2 for 2 hours. After centrifugation to separate the cells, the clear supernatant was taken to analyze the concentration of glucose with a glucose analyzer. The difference in amount of glucose before and after the reaction is defined as the total glucose uptake by the adipose cells. The percentage of increase in glucose up-take is defined as the GTF activity as shown in the following.

Increase in GTF activity (%) =

$$\frac{\text{Glucose uptake by the sample-Glucose uptake by the control}}{\text{Glucose uptake by the control}} \times 100\%$$

The effect of different molecular weights of γ-polyglutamate (Na^+ form) on the GTF activity is shown in Fig. 13.[31]

The results show both high and low molecular weights of γ-polyglutamate (Na^+ form) effectively increased the GTF activity, with the highest increase of 21% observed at about 300 ppm for the higher molecular weight of γ-polyglutamate (Na^+ form), and the highest GTF activity increased by approximately 33.5% as observed at the concentration range from 150-600 ppm for the low molecular weight γ-polyglutamate (Na^+ form).

The results clearly suggest that γ-polyglutamate (Na^+ form) facilitates the consumption of glucose by the GTF from adipose cells, which means more effective utilization of glucose in maintaining growth and adding health benefits to the body, and may exert good biological functionality in controlling diabetic conditions in humans.

Application of γ-polyglutamic acid (Na^+ form) in skin care products

The standard skin vital moisture creams with the following formulation, shown in Table 7, were prepared to demonstrate the effectiveness of moisturization by γ-polyglutamic acid (Na^+ form) and γ-polyglutamate hydrogels (Na^+ form). Propylene glycol was used as control for comparison, hyaluronic acid (HA) was used as relative reference, the small apparent molecular weight γ-polyglutamic acid (Na^+ form) with 200,000 to 400,000 daltons (designated as LM), high apparent molecular weight γ-polyglutamic acid (Na^+ form) with 1.15×10^6 to 1.35×10^6 daltons (designated as HM), and γ-polyglutamate hydrogels (Na^+ form) (desinated as 4% 1CL) with an apparent molecular weight from 15×10^6 to 100×10^6 daltons (cross-linked with polyglycerol polyglicidyl ether) were used as super moisturizers.

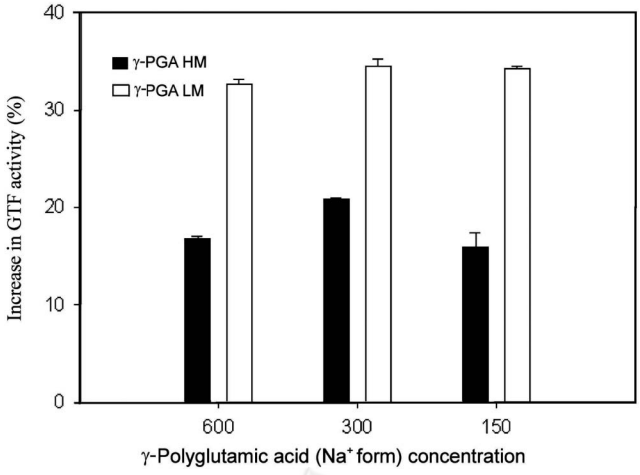


Fig. 13. The effect of γ-polyglutamic acid (Na^+ form) on GTF activity.

Table 7. The effect of γ-polyglutamic acid (Na^+ form) on the eggshell strength

Feeding	Sampling week	Test group KGF value	Control group KGF value	Difference in KGF value
Regular feed	1-4	3.3	3.8	0.5
Regular feed with 0.1% γ-polyglutamic acid (Na^+ form), LM	5-8	3.3	3.4	0.1
Regular feed	9-10	3.5	3.7	0.2

Note: 1. 73-week old egg-layers were used.
2. 4 lots with 8,000 chickens/lot were used.
3. KGF – Eggshell strength unit.

Table 8. The effect of γ-polyglutamic acid (Na^+ form) on the height of egg white

Feeding	Sampling week	Egg shell thickness mm*	Egg white height mm*	Egg yolk Color	pH of Egg-white
Regular feed	1-4	0.320	5.93	10.48	7.95
Regular feed with 0.1% γ-polyglutamic acid (Na^+ form), LM	5-8	0.327	6.00	10.90	7.91
Regular feed	9-10	0.330	5.45	10.50	7.93

The skin cream formulations were evaluated for their effectiveness of improving the elasticity of skin. A sample of 0.25 g from each of the 5 products was evenly spread over an area of 25 cm^2 on the outside skin surface of a test panelist's arm, once a day and continuously for a period of 1 month. There were 10 subjects who participated in the test. The apparent skin elasticity was measured at 23 °C and under relative humidity (RH) of 60%, with a probe of a Cutometer SEM 575 (Courage$^+$ Khazaka Electronic Gmbh, Germany), once per week and expressed in terms of the apparent elasticity index R2 value. The higher the apparent elasticity index R2 value, the better the skin elasticity. The results are shown in Table 8. The results show that the cosmetic formulations containing γ-polyglutamate (Mg^{2+} form) HM (high molecular weight), γ-polyglutamate (Mg^{2+} form) LM (low molecular weight), and γ-polyglutamate hydrogels (Na^+ form) are much better than those containing HA (hyaluronic acid) or propylene glycol in improving skin elasticity. The γ-polyglutamate hydrogels (Na^+ form) show the best result in improving skin elasticity.

CONCLUSION

γ-Polyglutamic acid and the soluble salts of γ-polyglutamates appear to be novel biopolymers with special structural characteristics and multiple functionalities good for use in many areas of industrial and economical aspects. They are natural, non-toxic, biodegradable, edible and environmentally friendly biopolymers with multiple functionalities.

The results from ^{1}H-NMR, ^{13}C-NMR, FT-IR and thermal analysis of the H form, K^+, Na^+, NH_4^+, Ca^{2+} and Mg^{2+} salts of γ-polyglutamic acid conform to their conformational changes from the insoluble α-helix state to the soluble linear random-coil conformation. The structural conformational states and special binding characteristics of the α-carboxylate anionic groups play important roles in determining the chemical and biological functionality of the biopolymers. With high hydrophilicity and reactivity important for many biological functions, the unusual polyanionic properties and structural characteristics are also significant for bioflocculant functions and detoxification of heavy metallic ions, and may also represent certain activities against pathogenic microbial activity and the growth of tumor cells as well.

The capability of γ-polyglutamic acid to dissolve calcium and magnesium compounds and form stable coordinated ionic complexes may also find nutritional value in delivering more bio-available calcium and magnesium for

Table 9. Skin vital moisture cream formulation[30]

Ingredient	Percentage, %				
	Control	A	B	C	D
Stearic Acid	5.0	5.0	5.0	5.0	5.0
Stearyl Alcohol	4.0	4.0	4.0	4.0	4.0
Wichenol 158	6.0	6.0	6.0	6.0	6.0
KOH	0.2	0.2	0.2	0.2	0.2
Propylene Glycol	5.0	–	–	–	–
γ-Polyglutamic acid (Na^+) HM	–	0.1	–	–	–
γ-Polyglutamic acid (Na^+) LM	–	–	0.1	–	–
γ-Polyglutamate Hydrogel (Na^+) 4%1CL	–	–	–	0.1	–
Hyaluronic acid (HA)	–	–	–	---	0.1
GMS 1330	2.0	2.0	2.0	2.0	2.0
Methylparaben	0.4	0.4	0.4	0.4	0.4
De-ionized Water	77.2	82.1	82.1	82.1	82.1
Vitamin E	0.2	0.2	0.2	0.2	0.2

Note: The hydrogel 4% 1 CL is the sample code of hydrogel used in the experiment. The hydrogel was made with 4% γ-polyglutamate (Na^+ form) and 1% polyglycerol polyglycidyl ether.

calcium absorption, improving healthy function in bone formation and in reducing old-age osteoporosis conditions. The same ionic complexation function of γ-polyglutamic acid with calcium and magnesium may also find value in dissolving physiological stones or calcium scales in water systems or sewer treatment.

Despite the existence of optically heterogeneous amino-acid residues in γ-polyglutamic acid, their effect on biological functionalities has not yet been reported so far; we are interested in further exploring the possible relationships between the biological functionality of γ-polyglutamic acid and its specific chemical and structural characteristics, in order to facilitate further development in the application of γ-polyglutamic acid in more specific areas including health-care, nutrition supplementation, crop and plant growth, water activity in soils, biochips and biosensors, and other environmental field applications.

Above all, γ-PGA and its derivatives provide a new line of both economical and environmental valuable biopolymers, which are of protein in nature, and are edible, water soluble, biodegradable, poly-anionic and environmentally friendly. They are stable at neutral pH, non-toxic to the human body, and versatile multifunctional biopolymers of the future. Further research and development on the applications of γ-PGA and its derivatives for many more practical applications are currently underway. Based on some of our own preliminary applications in field studies and laboratory development, we conclude that the potential applications of γ-PGA and its derivatives can be more than we expected before, and they are summarized in Table 9.

Table 10. The changes in skin elasticity over time after applying vital moisture cream. The apparent elasticity index was expressed in R2 value, and the relative apparent elasticity were expressed in $R2/(R2)_o$, %[30]

Moisturizer used	Relative Apparent Elasticity, $R2/(R2)_o$,%					
		Time, week				
	$(R2)_o$	0	1	2	3	4
5% Propylene glycol (control)	0.760	100	103.3	104.5	104.7	104.6
0.1% Hyaluronic acid (HA)	0.800	100	103.5	105.6	105.5	105.5
0.1% γ-Polyglutamate (Na^+) HM	0.825	100	105.5	105.9	106.2	106.4
0.1% γ-Polyglutamate (Na^+) LM	0.850	100	103.1	105.3	105.4	105.5
0.1% γ-Polyglutamate hydrogel (Na^+) 4% 1 CL	0.780	100	103.5	107.7	110.5	110.3

Table 11. Potential applications of γ-(D,L)-polyglutamic acid [γ-(D,L)-PGA, or γ-PGA] and its derivatives

Industrial field	Product type	Chemical/biochemical functionality
Food industry	Thickener/Stabilizer Products	Viscosity enhancement for liquid
	Texture enhancer	Texture improvement for bakery products and noodles
	Animal feed supplement	Facilitating mineral absorption, improving bone growth, egg-shell strength, and albumin firmness, decreasing body fat
	Cryoprotectant	Cryoprotection for frozen food products, improving mouth feel quality
Health-care	Nutrition supplement	Facilitating calcium absorption, improving osteoporosis conditions
Cosmetics	Moisturizer	Improving skin-care quality, reducing skin wrinkle conditions
Water treatment	Heavy metal absorbent	Removal of heavy metals and radionuclides
Wastewater treatment	Biopolymer flocculant	Substitute for polyacrylamide or PAC
Medical treatment	Controlled release drug carrier, medical bondage, suture thread	Biocompatible carrier for cancer drug, gene therapy
Hydrogels	Seed coating, soil renovation	High water absorbent
Hygiene products	Baby diapers, women's tampons	High water absorbent
Specialties	Biochip, membrane, LCD	Biocompatibility
Agriculture	Natural biocide	Inhibitor, anti-pathogen
Others	Biodegradable plastics	Biodegradability
	Biodegradable fibers	Biodegradability

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