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Biosynthesis and chemical modification of poly(γ-glutamic acid)

Mária Borbély[1,*], Yukio Nagasaki[2], János Borbély[3], Kesuo Fan[1], Amrut Bhogle[1], and Martin Sevoian[1,]**

[1]Department of Veterinary and Animal Science, [3]Department of Polymer Science and Engineering, University of Massachusetts, Amherst, MA 01003, USA
[2]Department of Materials Science and Technology, Science University of Tokyo, Tokyo, Japan

SUMMARY

The water soluble poly(γ-glutamic acid) (γ-PGA) was synthetized by bacteria. A series of new, less water soluble α-esters were prepared by repeated esterification.
The characterization of the resulted polymers was performed by 200 MHz ^{1}H and 75.4 MHz ^{13}C NMR spectroscopy.

INTRODUCTION

The bacterially synthetized water solule γ-PGA has been described in the 1950s (1). It was demonstrated that in this extracellular polyamide formed by *Bacillus licheniformis* (previously named as *Bacillus subtilis*), the repeating glutamic acid units were linked between γ-carboxy functional groups (2). Thorne and Leonard determined the effect of some metallic ions on the growth, the peptide production and the stereochemistry of the γ-PGA (3). In other papers the process of the chemical modification of the γ-PGA has been reported (4-6).
Poly(γ-glutamic acid) has a good solubility in water and in DMSO, but it is less soluble in other solvents. It has been found that the esterification of the carcoxyl groups at the α-position in the glutamic acid units decreased the solubility of the polymer in water while the same increased in other solvents (7).
The secondary structure of synthetically polymerized γ-PGA has been described as α-helix in a water solution (8). A remarkable influence of metal ions on the NMR signals has also been reported (9).
In this paper, we describe a procedure to synthetize various less water soluble α-esters as well as a route to get a higher degree of esterification using the natural origin γ-PGA. Finally, the characterization of the polymer by NMR spectroscopy is reported.

EXPERIMENTAL

Organism:

Bacillus licheniformis (ATCC 9945a) obtained from Dr. Thorne's laboratory (University of Massachusetts, Amherst) is acknowledged.

*Permanent address: Veterinary Institute, Debrecen, Hungary
**Corresponding author

Medium:
Medium E (1) was prepared for the growth of γ-PGA: L-glutamic acid, 20 g; citric acid, 12 g; glycerol, 80 g; NH_4Cl, 7.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_2 \cdot 6H_2O$, 0.04 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; $MnSO_4 \cdot H_2O$, 0.08 g in 1 liter distilled water ; pH adjusted to 7.4 with NaOH. The medium was autoclaved at 121 ° C at 15 lbs/sqinch pressure for 20 minutes.

Culture and Production of Polymer:
Culture plates (nutrient agar with 0.5 % yeast extract) were inoculated with a spore suspension of *Bacillus licheniformis* and incubated overnight at 37 ° C. A highly mucoid colony was used to inoculate Medium E (50 ml) and incubated at 37 ° C on an orbit shaker (150 rpm) for 48 hours. This suspension was used as a seed to inoculate large scale cultures (2 liter volume), which were incubated on the shaker for five days.

Polymer Isolation and Purification:
After incubation the contents of the culture flasks were centrifuged (Sorvall RC-5B, 8000 g, 4 ° C, 40 minutes) to separate the cells from the polymer solution. Two and one-half volumes of cold 95 % ethanol were slowly added to the supernatant while stirring and kept in refrigerator overnight. The liquid was decanted and the precipitated polymer was dissolved in distilled water. The polymer solution was dialyzed against 4 volumes of distilled water, concentrated and freeze dried.

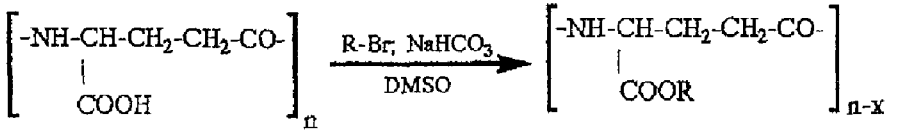
Esterification of γ-PGA:
First esterification: to a 2 % solution of the free acid form of polymer in DMSO solution, a two molar excess of $NaHCO_3$ and a five molar excess of different alkyl bromide were added. Samples were taken at various times and were precipitated in ether containing 1 % methanol. The precipitate was washed with acetone and dried in vacuo.
Second esterification: the dried and partly esterified polymer from the first esterification was reacted a second time under the same conditions. The reaction was allowed to continue for various times and the polymer was precipitated in 10 volumes of cold, aqueous hydrochloric acid (pH=2). The precipitation was filtered, washed in water, acetone or hexane and further purified by Soxhlet extraction and dried in vacuo.

Characterization of Polymers:
The molecular weight (M_w) was determined by Gel Permeation Chromatography, using a Waters E LC equipment (column: ASAHIPAK GSM-700, 7.5 mm i.d. and 500 mm length; eluent: 50 mM phosphate buffer solution (pH=7) with 100 mM NaCl; flow rate: 1 ml/min). The degree of esterification (conversion) was determined by NMR, using 200 MHz for ^1H and 75.4 MHz operation frequency for ^{13}C experiments.

RESULTS AND DISCUSSION

Esterification of Polymer:
Using the conditions reported above for fermentation, 3-4 g polypeptide were obtained from one liter Medium E, which is less than previously reported (1) but proved to be very pure. The estimated molecular weight was $2.9 \cdot 10^5$ - $1.4 \cdot 10^6$, calculated from the GPC chromatograms.

Experiments were carried out to find the relation between reaction time and conversion, using the following esterification reaction at room temperature (Scheme 1).
It was found that under the indicated condition the highest possible conversion was about 45 mol % at 40 hours esterification time (Figure 1).



R= C_2H_5, C_4H_9, C_8H_{17}

Scheme 1. The esterification of poly(γ-glutamic acid)

After a maximum value the conversion decreased, presumably because of some hydrolytic reaction.

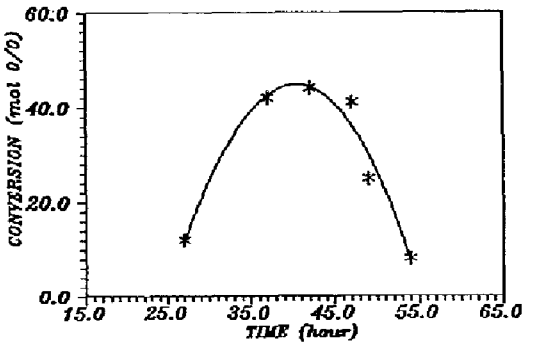


Figure 1. Degree of esterification (conversion) as a function of time during the first esterification of the natural origin γ-PGA (R=C_8H_{17})

Table 1. Degree of esterification obtained at various reaction times with different R-groups in the first and second reactions

Sample	R-group	Reaction	Time (hour)	Conversion (mol %)
1	C_2H_5	1st	40	45
2	"	2nd	16	75
3	"	2nd	23	90
4	"	2nd	40	89
5	C_4H_9	1st	42	30
6	"	2nd	16	43
7	"	2nd	23	52
8	"	2nd	48	98
9	C_8H_{17}	1st	48	25
10	"	2nd	16	47
11	"	2nd	23	59

NMR Characterization:

^{1}H and ^{13}C NMR measurements were applied to study the polymer structure and to determine the degree of esterification. Figure 2 indicates the ^{1}H NMR spectra of the original γ-PGA (in D_2O and DMSO-d_6), sample 5, 7, and 4 (in DMSO-d_6).

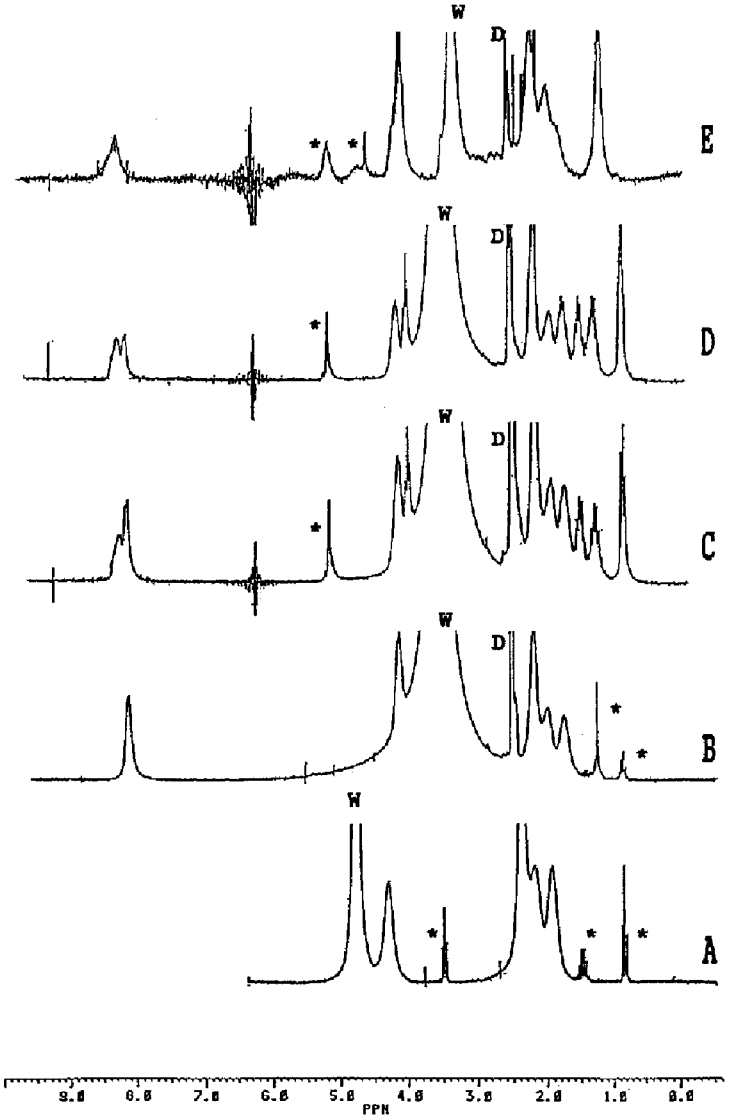


Figure 2. ^{1}H NMR spectra of γ-PGA (A: in D_2O and B: in DMSO-d_6); C: sample 5, D: 7 and E: 4 (in DMSO-d_6); * indicates impurities; ^{W}water; ^{D}peak of solvent.

Table 2 summarizes the chemical shifts of γ-PGA in D_2O, DMSO-d_6 and formic acid formyl-d_1 (Fd_1) solvents.

Table 2. ^1H NMR chemical shifts of γ-PGA

Peak	Chemical Shift (δ=ppm)		
	solvent		
	D_2O	DMSO-d_6	Fd_1
Amide	-	8.10	7.78
α-CH	4.27	4.12	4.50
β-CH_2	2.15; 1.90	1.97; 1.73	2.20; 2.01
γ-CH_2	2.35	2.18	2.46

The chemical shifts of amide and α-CH protons usually indicate the secondary structure of poly α-amino acids (10, 11). According to literature (12) we assume random coil in D_2O, helix in DMSO-d_6 and sheet-like structure in Fd_1 solvent. These results will be published (13).

The chemical shifts of amide protons of ester derivatives move towards downfield comparing to γ-PGA. The ratio of the two amide peaks corresponds to the acid and ester forms.

^{13}C NMR assignments of γ-PGA and sample 7 in DMSO-d_6 are summarized in Table 3.

Table 3. ^{13}C NMR chemical shifts of γ-PGA and sample 7*

Peak	Chemical Shift (δ=ppm)	
	γ-PGA	sample 7
β-CH_2	27.54	27.57; 26.98[2*]
γ-CH_2	31.97	31.94; 31.78[2*]
α-CH	51.98	52.12; 52.02[2*]
CO	171.94	171.90; 172.25[3*]
COOH	173.96	173.95
COOR	-	171.63

*Chemical shifts of Butyl side chains are: 13.59; 18.59; 30.18 and 64.14 ppm

[2*] In amino acid residues with COOR pendant group

[3*]CO peak of E residues in EE' diads (E: glutamic acid with COOH; E': glutamic acid with COOR pendant groups

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