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Biodegradation of Poly(α-amino acid) *in Vitro*

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ABSTRACT: The preparation of copolypeptides consisting of L-glutamic acid and *N*-hydroxyalkyl-L-glutamine, or hydrophobic L-amino acids such as L-alanine, L-leucine, or L-valine, was performed to determine the effects of copolymer composition and sequential distributions on the rate of degradation by papain in a PECF at pH = 7.4 and 37°C to simulate *in vivo* polymer degradation. Random copolymers consisting of γ-BLG and γ-MLG, L-ala, L-leu, or L-val were synthesized by the NCA method. Hydrophilic copolymers were obtained by successive reactions of side chains by anhydrous HBr or aminoalcohol treatment. All the samples were found to be extensively degraded by random-chain fracture with papain. Further, the degradation data for these samples followed the Michaelis-Menten rate law, being of the first order in papain concentration. The nature of side chains are important to the rate of degradation by papain and the uncharged side chains of copolymer samples give values much higher than the negatively charged side chain of L-glutamic acid at pH = 7.4. The order of the rate of degradation among the former group was: L-ala > L-leu > L-val > *N*-hydroxyalkyl-L-glutamine.

KEY WORDS Biodegradation / Poly(α-amino acid) / Papain / The Michaelis–Menten Rate Law / The Hydrophobic Effect / Copolymer Composition / Sequential Distribution /

Poly(α-amino acid)s and their copolymers are very useful for biodegradable medical applications such as temporary artificial skin substitutes in burn therapy, temporary barriers to prevent adhesion between natural tissue planes damaged either by accident or surgery between the pericardium and heart wall during open-heart surgery, polymer carriers for conjugates coupled to proteins for therapeutic use and drug delivery systems. Especially, water-soluble poly(α-amino acid)s should be very useful for protein conjugates and drug delivery systems. Poly(α-amino acid)s are typical biodegradable polymers and thus these controlled-release systems offer the distinct advantage such that no residual polymer remains following drug release or polymer biodegradation. Anderson, *et al.*[1] have shown that the rate of *in vivo* degradation of synthetic poly(α-amino acid)s can be controlled by varying the hydrophilicity of the side chain groups. The degradation was attributed to cleavage of the poly(α-amino acid) chain by proteolytic enzymes, such as endopeptidase cathepsin B, released during acute and chronic stages of the inflammatory response. For practical use of poly(α-amino acid)s in biomedical material fields, it should be worthwhile to investigate the partial modification of side chains to understand how to control the rate of degradation by proteolytic enzymes in detail.

In this paper, the preparation of copolypeptides consisting of L-glutamic acid and *N*-hydroxyalkyl-L-glutamine, or hydrophobic L-amino acids such as L-alanine, L-leucine, or L-valine, was performed to clarify the effects of copolymer composition and sequential distribution, the exact arrangement of two comonomers along copolymer chains, on the rate of degradation by papain in a pseudo-

extracellular fluid (PECF)[2] at a pH of 7.4 and 37°C to simulate *in vivo* polymer degradation. Papain is a well characterized plant thiol endopeptidase[3] with a broad range of specificity. It is closely related to cathepsin B, a thiol endopeptidase that has been isolated from mammalian spleen, liver, kidney, and lung, and is released by the cells in response to inflammation.[4]

The hydrolysis of poly-L-glutamic acid by papain has been extensively studied by Miller at various pH,[5,6] since it has been found to react with poly-L-glutamic acid.

EXPERIMENTAL

Materials

First, random copolypeptides: poly(γ-benzyl L-glutamate-*co*-γ-methyl L-glutamate) (GM), poly(γ-benzyl L-glutamate-*co*-L-alanine) (GA), poly(γ-benzyl L-glutamate-*co*-L-leucine) (GL), and poly(γ-benzyl L-glutamate-*co*-L-valine) (GV), covering the whole range of copolymer composition were synthesized by the NCA method so as to obtain precise information on sequential distributions in copolymer chains. The G-NCA and M, A, L or V-NCA, in desired molar ratios, were dissolved in a 1 : 1 (v/v) mixture of dioxane and dichloromethane. The total concentration of both NCA's was kept at 3 wt%. The polymerization was initiated with triethylamine at an NCA-to-initiator molar ratio of 50. All starting copolymer samples were purified and fractionated as described in the previous paper.[8] The debenzylation of BLG residues in copolymers was performed by anhydrous HBr treatment according to the method of Idelson and Blout.[9] Copolymer samples: PLAE consisting of *N*-hydroxyethyl-L-glutamine (E) and L-glutamic acid (A), and PLAP consisting of *N*-hydroxypropyl-L-glutamine (P) and L-glutamic acid (A), were obtained by successive reactions of the GM copolymers with HBr and aminoalcohols, respectively, that is, after debenzylation of BLG residues in GM copolymers by the above method, aminoalcoholysis of MLG residues was carried out by 2-amino-1-ethanol and 3-amino-1-propanol, respectively. The aqueous polymer solution was dialyzed exhaustively against distilled water, filtered through a Millipore filter and lyophilized. The yield was 88 to 92% for the series of the side chain reaction. The composition of these copolypeptides was determined by elemental and amino acid analysis. Papain (3.5 m Anson u mg^{-1}, No. 7144) was purchased from Nakarai Chem. Co. and used without further recrystallization. The papain was activated with the PECF solution proposed by Homsey at pH = 7.4 with 0.01 M cystein and 0.04 M EDTA.[10] The other chemicals were of analytical grade.

Measurements

Sample molecular weight was estimated

Table I. Preparative data of samples

Sample code	COOH mol%	$[\eta]$ dl g^{-1} (PECF, 37°C)	$M_{w.o}$	b_0
G(A)-1	100	0.802	48,800	0
G(A)-2	100	0.494	31,700	0
G(A)-3	100	0.315	21,200	0
G(A)-4	100	0.160	11,700	0
PHEG-1	0	0.264	44,700	0
PHEG-2	0	0.184	25,300	0
PHEG-3	0	0.125	13,600	0
PHPG-1	0	0.371	87,100	−75
PHPG-2	0	0.320	69,400	−75
PLAE-1	73	0.954	83,400	0
PLAE-2	51	0.728	85,100	0
PLAE-3	19	0.567	98,100	0
PLAP-1	73	1.098	84,500	0
PLAP-2	51	0.823	97,800	0
PLAP-3	19	0.544	91,000	0
GA(A)-1	94	0.841	92,400	0
GA(A)-2	88	0.880	102,000	−30
GA(A)-3	76	0.500	54,200	−75
GA(A)-4	66	0.865	98,900	−120
GL(A)-1	85	1.211	118,000	−45
GL(A)-2	75	1.062	72,000	−110
GL(A)-3	68	1.220	125,000	−180
GV(A)-1	89	0.780	97,500	
GV(A)-2	76	0.960	108,100	

from equilibrium runs on a 0.01 M phosphate buffer of the polymers using a MOM type 3170-b ultracentrifuge at pH = 7.4 and 37.0°C. Viscosities were measured with an Ubbelohde type viscometer on the same buffer at pH = 7.4 and 37.0°C. The preparative data of these water-soluble copolypeptide samples are summarized in Table I.

The molecular weight distribution curves of these copolypeptide samples were investigated by gel permeation chromatograph (GPC) on a Toyo-Soda High-speed Liquid Chromatography HLC-803D equipped with TSK-Gel Type G4000SW, C-No. SW46A0015 in PECF at 25°C.

The chain conformation of these copolypeptides in PECT was examined by optical rotatory dispersion (ORD) measurements. The Moffitt–Yang parameter b_0 was evaluated from the ORD data obtained with a Yanagimoto OR-100 Type spectropolarimeter, using a tungsten lamp at 37.0°C. Table I also includes the experimental data of b_0 for these copolypeptides in PECF at pH = 7.4 and 37.0°C.

All kinetic measurements were made in PECF solution using a modified Ubbelohde type viscometer. The initial rate of degradation V was calculated by measuring the time necessary for the molecular weight to drop to 1/2 its initial value. A viscosity-kinetic measurement was performed as follows: An aliquate of the polymer solution (10 ml unless stated) was pipetted into the viscometer. After determining the viscosity of a solution, 0.2 ml of the stocked enzyme solution was added and the viscosity was taken periodically with stirring. Unless otherwise stated, the enzyme concentration was controlled to $[E] = 5.10 \times 10^{-6}$ M after being mixed with the polymer solution in the viscometer.

To convert the reduced viscosity to intrinsic viscosity, the Huggins expression,[11] $\eta_{sp}/C = [\eta] + k'[\eta]^2 C$, relating reduced viscosity to concentration was followed in the concentration range of interest and the constant in the Huggins expression was within the error same as that for molecular weight. The intrinsic viscosity as a function of the time may thus be calculated in this way.

Table II. Pseudo-extracellular fluid (PECF)[2] ($NaHCO_3$, K_2HPO_4, NaCl, KCl)

Ion	Concentration physiological	PECF
		meq l^{-1}
Na^+	145	145
K^+	5	5
Cl^-	113	118
HCO_3^-	30	30
HPO_4^{--}	2	2

RESULTS AND DISCUSSION

Relation between Intrinsic Viscosity and Molecular Weight

The dependence of the intrinsic viscosity of these samples on molecular weight is shown in

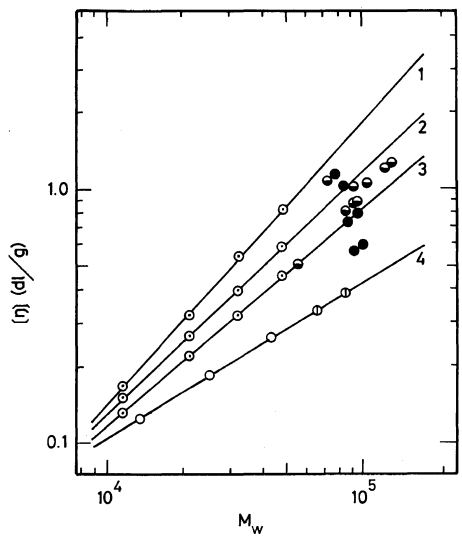

Figure 1. The intrinsic viscosity $[\eta]$ *vs.* molecular weight M_w for (1) G(A) in 0.10 M phosphate buffer (pH = 7.4), (2) G(A) in 0.50 M phosphate buffer (pH = 7.0), (3) G(A) in 1.00 M phosphate buffer (pH = 6.9), and (4) PHEG and PHPG in 0.10 M phosphate buffer (pH = 7.4) at 37.0°C. Plots were experimental data for the copolymers, PLAE and PLAP (●), and GA(A), GL(A), and G(V) (◒).

Figure 1. This figure also shows log-log plots of the intrinsic viscosity as a function of molecular weight for poly-L-glutamic acid, G (A), with several ionic strengths; number 1 to 3 denote the molecular weight dependence of the intrinsic viscosity for G(A) in 0.10 M, 0.50 M, and 1.00 M of phosphate buffer at pH = 6.9 to 7.4, respectively. Number 4 in the figure denotes the relation obtained for poly(*N*-hydroxyalkyl-L-glutamine) (PHEG and PHPG) in 0.10 M phosphate buffer at pH = 7.4 and 37.0°C. A straight line is drawn so as to fit the experimental points for each case. From this straight line, the empirical parameter K' and a in the equation; $[\eta] = K' M^a$, for each case were obtained. In regard to the copolypeptide samples, it would be impossible to obtain different molecular weight fractions with exactly the same copolymer composition and their molecular weight distributions. Thus, an imaginary straight relation was used to distinguish the molecular weights of the copolypeptide samples from the intrinsic viscosity experimental data. This relation was obtained by synchronizing with those of lines 1 to 4 obtained with G(A) samples in the figure.

Type of Degradation

To check whether random degradation of the main-chain of a polypeptide is dominated by the reaction with papain, GPC analyses of partially degraded polypeptides were carried out. Figure 2 illustrates GPC curves for polypeptide samples. From Figure 2, it may concluded that each sample is dominantly degraded by a random main-chain fraction as in the case of the degradation of G(A) by endopeptidases such as chymotrypsins, elastase, ficin, papain, pepsin, or subtilisin, previously reported by Miller.[6] Dickinson and Hiltner[12] reported that the main products of PHEG by papain digestion are oligomers of polymeri-

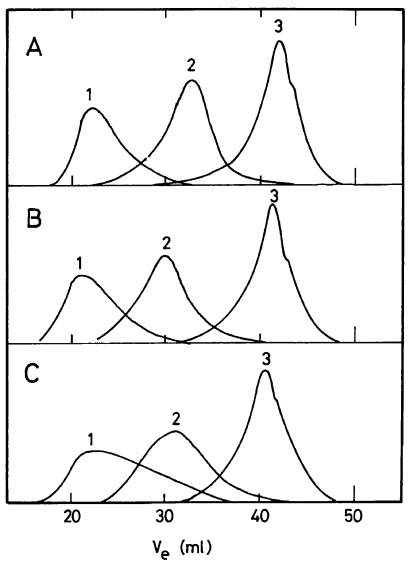

Figure 2. GPC elution curves for reaction products of polypeptides in PECF solution by papain. (A) G(A)-1 for; (1) original, (2) 180 min, and (3) 720 min of digestion, (B)PLAP-3 for; (1) original, (2) 60 min and (3) 600 min of digestion, and (C) GL(A)-2 for; (1) original, (2) 30 min and (3) 300 min of digestion, respectively. Papain concentration $[E] = 8.5 \times 10^{-6}$ M.

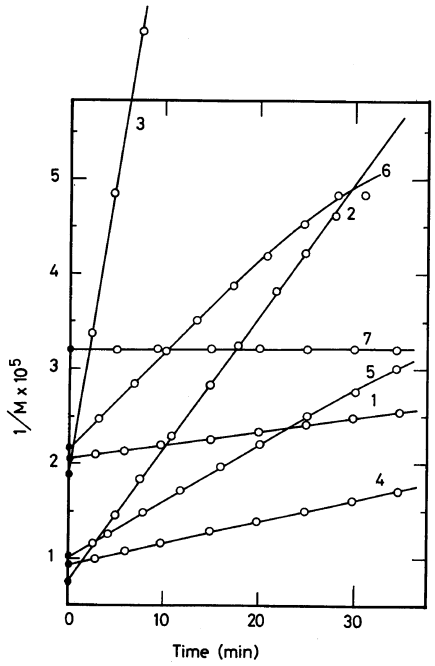

Figure 3. Typical plots of 1/M for samples as a function of papain digestion time. Papain concentration $[E] = 5.1 \times 10^{-6}$ M at pH = 7.4 and 37.0°C. Numerals in the figure denote (1) G(A)-1, (2) GL(A)-2, (3) GA(A)-3, (4) GV(A)-2, (5) PLAP-2, (6) PHEG-1, and (7) G(A)-D-1.

zation degree 4 to 9, rather than monomers. The same was observed previously in the trypsin-catalyzed hydrolysis of poly-L-lysine.[13]

Rate of Degradation

Typical plots of 1/M against the papain digestion time are shown in Figure 3. The procedure in this paper for converting reduced viscosity to the molecular weight is strictly valid only if the molecular weight distribution during degradation differs but little from that in the original sample. Even if starting polymer samples such as PBLG, GM, GA, GL, and GV, have rather narrow molecular weight distributions, debenzylation with HBr to give L-glutamic acid residues will break a few peptide bonds[14] which both theoretically[15] and experimentally[16] widens the molecular weight distribution considerably. Thus, initial samples such as G(A), GA(A), GL(A), and GV(A), should have very nearly random or Gaussian molecular weight distribution. Theoretically, a plot of 1/M against degree of polymerization should be linear for the random degradation of an initially random distribution.[15] For random degradation of a random distribution, the molecular weight will drop to one half its initial value when one bond has been broken per initial molecular weight. Since the chromatograms in Figure 2 and plots of 1/M against papain digestion time in Figure 3 indicate random degradation, and since the original samples have random distributions, our procedure used to convert reduced viscosity to molecular weight is completely justified. As shown in Figure 3, linear relations were obtained for all samples, but when the reaction proceeded over a rather long period, its rate dropped off slightly, indicating loss of enzyme activity. This and the order of rate of degradation will be discussed in the following.

Rate Law

All peptidases yield data which can be analyzed in the framework of the Michaelis–Menten mechanism. The rate of the biodegradation of samples was expected to be first order in enzyme concentration. It was necessary to confirm this since the enzyme concentration varied over a wide range so that the rate could always be measured. Figure 4 shows the expected experimental results for G(A)-1, PHEG-1, and GA(A)-3; they indicate first order behavior and are typical of endopepti-

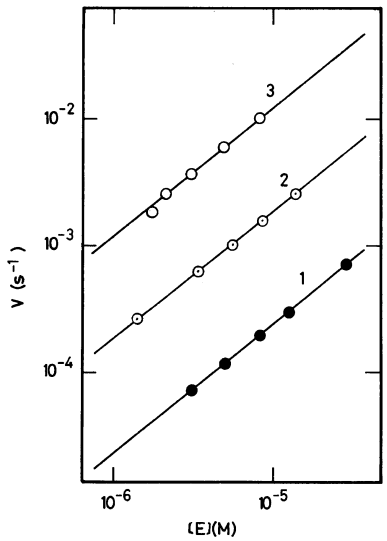

Figure 4. Dependence of reaction rate V (s^{-1}) on papain concentration for (a) G(A)-1, (2) PHEG-1, and (3) GA(A)-3.

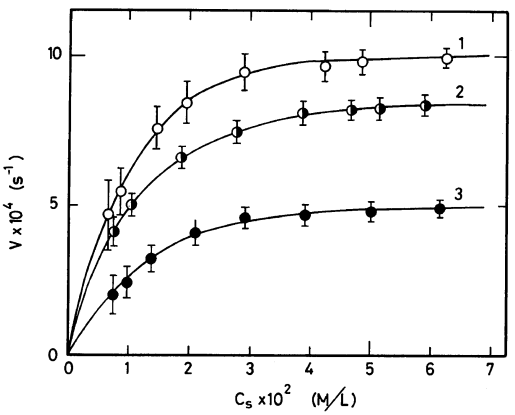

Figure 5. Plots of rate V as a function of substrate concentration C_s (M/L) in PECF solution at pH = 7.4 and 37.0°C for (1) PHEG-1, (2) PLAP-2, and (3) GL(A)-1. Papain concentration [E] = 5.10×10^{-6} M.

dase degradation. The rate of degradation V for GA(A)-3 by papain was about 40 times that for G(A)-1 under the same experimental conditions, pH = 7.4 and 37.0°C.

Next, the rate of degradation V was evaluated as a function of substrate concentration. Figure 5 illustrates the experimental results for PHEG-1, PLAP-2, and GL(A)-1. The rate of bond breaking was calculated from plots similar to Figure 3, by measuring the time necessary for the molecular weight to drop to 1/2 its initial value, which corresponds to less than 0.5% breaking of the total bonds. Under given experimental conditions, a plot of reciprocal rate against reciprocal substrate concentration permits evaluation of the two constants (V_m and K_m) in the Michaelis–Menten rate law. As expected, the degradation follows the Michaelis–Menten rate law within experimental error. Since the rate V is dependent on the number of bonds and not macromolecules, the Michaelis constants were calculated in terms of residue concentrations, which far high sample molecular weights are nearly equal to the peptide concentration. Table IV summarizes the values of the Michaelis–Menten parameters, V_m and K_m, for PHEG-1, PLAP-2, and GL(A)-1, respectively, calculated from the experimental findings in Figure 5.

Effects of Hydrophobic Side Chains on Degradation Rate

The relation between the composition of GA(A), GL(A), and GV(A) copolymers and the rate of degradation V by papain was investigated under the same experimental conditions. Table III summarizes the experimental data for the average rate of degradation V_{av} for several repeated runs for each case as a function of molar percent of L-glutamic acid residue (COOH) in the copolymers. In Figure 6, the order of the degradation rate among the copolymers was GA(A) > GL(A) > GV(A). These experimental values do not fall on straight lines with copolymer composition, thus, other factors such as sequential distributions of comonomers influence the rate of degradation.

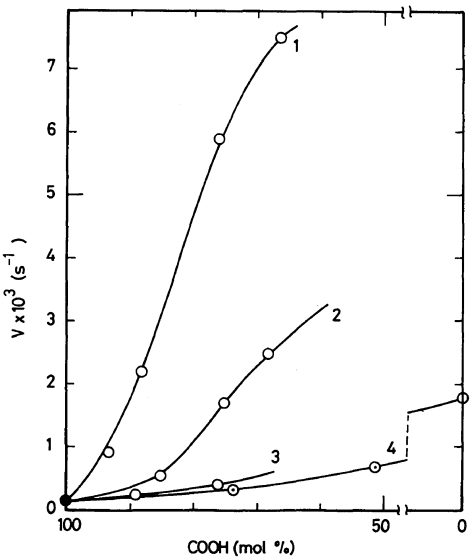

Figure 6. Plots of rate V as a function of COOH mol% of copolymers for (1) GA(A), (2) GL(A), (3) GV(A), and (4) PLAP copolymers in PECF at pH = 7.4 and 37.0°C. Papain concentration [E] = 5.10×10^{-6} M.

The Rate of PLAE and PLAP Copolymer Degradation by Papain

In Table III, the rates of degradation for PHEG and PHPG by papain is about 6 and 13 times, respectively, that of G(A) at pH = 7.4 and 37.0°C. Table III lists the experimental results for the rates of degradation of PLAE and PLAP copolymers as a function of COOH mol%. Figure 6 shows the experimental values for PLAP not to fall on the straight line connecting the V's for both homopolymers, G(A) and PHPG.

Sequential Distributions of Comonomers in Copolymer Chains

It was suggested in the previous section that the sequential distribution of comonomers in copolymer chains is a major factor determining the rate of degradation V of copolypeptides. Thus, it is important to understand the sequential distributions of comonomers.

Table III. The average rate V_{av} of the copolymers

Sample code	COOH / mol%	C_s (M/L) $\times 10^2$	V_{av} / $s^{-1} \times 10^3$
G(A)-1	100	3.94	0.14
G(A)-4	100	4.75	0.10
G(A)-D-1	100	3.69	0
PHEG-1	0	3.75	0.98
PHPG-2	0	3.81	1.75
PLAE-1	73	4.05	0.22
PLAE-2	51	4.11	0.45
PLAE-3	19	4.20	0.86
PLAP-1	73	3.45	0.30
PLAP-2	51	3.29	0.69
PLAP-3	19	4.02	1.27
GA(A)-1	94	3.92	0.98
GA(A)-2	88	3.86	2.20
GA(A)-3	76	4.12	5.88
GA(A)-4	66	4.01	7.50
GL(A)-1	85	3.88	0.47
GL(A)-2	75	3.96	1.70
GL(A)-3	68	3.90	2.45
GV(A)-1	89	3.45	0.28
GV(A)-2	76	3.90	0.35

Table IV. Michaelis parameters for samples in PECF at 37°C, pH = 7.4

Copolymer	K_m (M/L)	V_m / s^{-1}
PHEG-1	8.7×10^{-3}	1.1×10^{-3}
PLAP-2	9.4×10^{-3}	0.8×10^{-3}
GL(A)-1	10.9×10^{-3}	0.5×10^{-3}

To do so, it is necessary to obtain precise values for the monomer reactivity ratios, r_1 and r_2, from copolymerization experimental data. Figure 7 illustrates the copolymer composition curves for the copolymerization of GM, GA, GL, and GV at a conversion level from about 23 to 30 mol%. Using these experimental findings, we integrated the copolymerization equation derived by Skeist,[17] taking into consideration the change in monomer composition with conversion. In Figure 8, for example, the instantaneous mole fraction of G-monomer (F), along with the

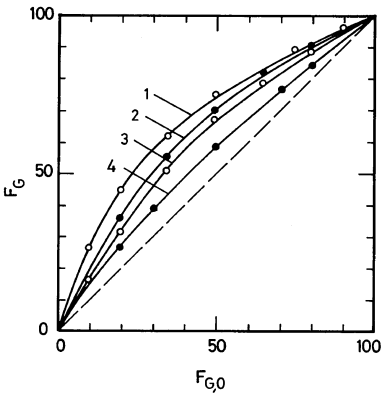

Figure 7. Copolymer composition curves for (1) GV, (2) GL, (3) GA, and (4) GM copolymers at about 23 to 30 mol% conversion.

Table V. Values of r_1 and r_2 computed from the Skeist equation

Copolymer	Solvent	r_1	r_2
GM	DO-MC	1.50	0.60
GA	DO-MC	2.36	0.50
GL	DO-MC	2.65	0.38
GV	DO-MC	2.96	0.17

instantaneous copolymer composition of G-monomer residue (f) and the cumulative copolymer composition of G-monomer (F_G) in copolymer, are indicated as functions of the conversion (P) for the GL copolymer at an initial G-monomer feed of 50 mol%. The mode of calculation is reported elsewhere in detail.[18] Finally, the computor procedure was used to obtain the best fit to the experimental data by trial-and-error selections of r_1 and r_2. The numerical values obtained are summarized in Table V.

It is clear from Figure 8 that when monomers start to copolymerize in equimolar amounts, the polymer analyzed at low conversion contains significantly more G-comonomer than L-comonomer. Since G-NCA reacts with the growing peptide chain considerably faster than L-NCA, the relative amount of free G-NCA monomer decreases during the course of copolymerization. This variation in copoly-

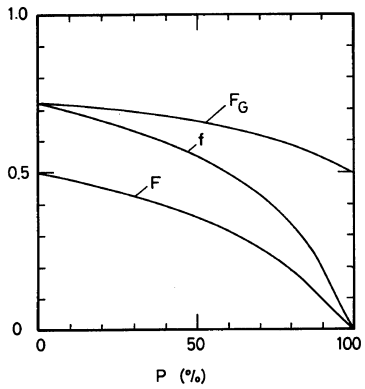

Figure 8. Variation in monomer composition (F), in the instantaneous copolymer (f), and the cumulative copolymer (F_G) with conversion (P) for GL copolymer.

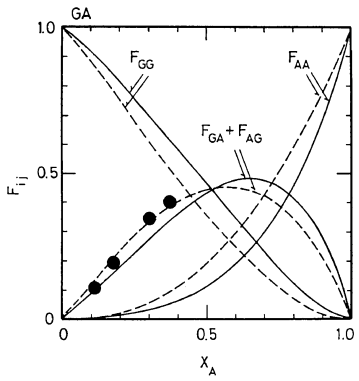

Figure 9. Normalized fractions of monomer dyads, F_{GG}, $F_{GA}+F_{AG}$, and F_{AA} as a function of the initial monomer composition of L-alanine (A) by molar content X_A for GA copolymer with (—), 25% and (----), 60% of the conversion, respectively.

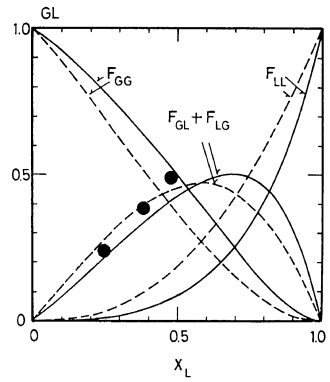

Figure 10. Normalized fractions of monomer dyads, F_{GG}, $F_{GL}+F_{LG}$, and F_{LL} as a function of the initial monomer composition of L-leucine (L) by molar content X_L for GL copolymer with (—), 25% and (----), 60% of the conversion, respectively.

mer composition results in a higher concentration of the less reactive monomer. Consequently, there is a drift in f as well as F toward the less reactive monomer with increasing P. This should be more significant with an increase in the ratio of r_1 to r_2.

Next, defining F_{ij} as the fraction of bonds of i and j in the copolymer chains (dyad), the stoichiometric and steady-state relationship for various dyad distributions were introduced using the experimentally obtained data of r_1 and r_2.[19] These dyad distributions as well as copolymer composition are influenced by the degree of conversion P.[20]

Figures 9 and 10 show the dyad distributions for GA and GL copolymers, respectively, against the initial monomer composition for 25% and 60% conversion calculated by the modified method of Harwood's system[21] on the basis of the terminal model.[19] The experimental data of the degradation rate for GA(A) and GL(A) copolymers (Figure 6), respectively, were plotted by an arbitrary measure in Figures 9 and 10.

In these Figures, agreement between calculated and experimental values is reasonably good, indicating the sequential distributions of comonomer chains to be a major factor determining the rate of biodegradation of these copolymers, and the hydrophobic side chains to contribute possibly to accelerating the rate of degradation of polypeptide chains by papain.

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