Enzymatic Hydrolysis of Water-Soluble Random Copolypeptides

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SYNOPSIS

Two component water-soluble random copolypeptides consisting of *N*-hydroxyethyl L-glutamine and hydrophobic L-amino acids such as L-alanine, L-leucine, L-phenylalanine, or L-valine, were prepared by carrying out aminolysis reactions with 2-amino-1-ethanol (E) on starting copolymers consisting of γ-benzyl L-glutamate (B) and the corresponding hydrophobic L-amino acid. The effects of copolymer composition and sequential distributions, as well as molecular conformations, on the rate of degradation by bromelain in a PECF at pH 7.4 and 37.0°C to simulate *in vivo* polymer degradation. All the samples were found to be extensively degraded by random chain fracture with bromelain. 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 and the molecular conformations are important to the rate of degradation by bromelain.

INTRODUCTION

Poly(α-amino acid)s and their copolymers offer potential for biodegradation medical applications such as temporary artificial skin substrates 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 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) chains 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 areas, it is worthwhile to investigate the partial modification of side chains to understand how to control the rate of degradation by proteolytic enzymes in detail.

On the other hand, it is well known that copolymer properties can be profoundly influenced by the sequential distribution of the comonomers in the copolymer chain as well as the copolymer molar composition.

In this paper, the preparation of copolypeptides consisting of *N*-hydroxyethyl L-glutamine and a hydrophobic L-amino acid, such as L-alanine, L-leucine, L-phenylalanine, or L-valine, was performed to clarify the effects of copolymer molar composition and the sequential distribution on the rate of degradation by bromelain in a pseudoextracellular fluid (PECF)[2] at a pH 7.4 and 37.0°C to simulate *in vivo* polymer degradation.

Bromelain is a well-characterized plant thiol endopeptidase[3–5] with a broad range of specificity. It is closely related to cathepsin B, a thiol endopeptidase that has been isolated from mammalian

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spleen, liver, kidney, and lung, and is released by the cells in response to inflammation.[6] The hydrolysis of poly(*N*-hydroxyethyl L-glutamine) by papain has been already extensively studied by us[7-9] at various pH regions.

To prepare copolymers of sufficiently high molecular weight with higher efficiency, it is desirable to perform copolymerizations up to somewhat higher conversion. In this study, therefore, the copolymerizations were stopped at a conversion level of about 50 mol %. Thus, the change in composition of the monomer should be taken into consideration in obtaining accurate monomer reactivity ratios, r_1 and r_2, from the higher conversion data. So, a least-square method, taking into consideration the change in composition of the monomer was used in this study. The change in comonomer composition with conversion can be calculated by the Skeist equation.[10]

EXPERIMENTAL

Materials

N-carboxyanhydrides (NCA) were prepared by phosgenation of the corresponding α-amino acids in tetrahydrofuran and purified by multiple recrystallization. Solutions (0.1*M*, ca. 2 wt %) of γ-benzyl L-glutamate NCA and a hydrophobic L-amino acid, such as L-alanine, L-leucine, L-phenylalanine, or L-valine, NCA in 1 : 1 (v/v) mixture of dioxane/methylene dichloride were prepared and the polymerization initiated with triethylamine at a monomer : initiator ratio of 50. The copolymerization reaction was followed by CO_2 evolution according to the method of Patchornik and Shalitin.[11] The copolymerization was stopped at about 50 mol % conversion. All solvents used for synthesis and the initiator were purified by the usual methods described in the literature. The polymers formed were precipitated by adding four times the amount of methanol in volume including 5 vol % of 0.1*N* HCl to the polymer solution at 4°C. Then the precipitation products were washed in pure methanol and dried under reduced pressure at 50°C. The aminolysis reaction of γ-benzyl L-glutamate residues was carried out by 2-amino-1-ethanol.[7] The completion of the aminolysis reaction was ascertained by using a UV spectra measurement. The composition of these copolymers before aminolysis reaction was determined by amino acid analysis. After the aminolysis reaction, the aqueous polymer solution was dialyzed exhaustively against distilled water, filtered through a Millipore filter, and lyophilized.

Bromelain (EC 3.4.22.4, from Pineapple Stem, No. B-2252, Sigma) was purchased from Nakarai Chem. Co., Kyoto, Japan, and used without further recrystallization. Unless stated, all measurements were made in a system containing 10 m*M* cystein and 40 m*M* ethylenediaminotetraacetate (EDTA) in the PECF solution. The composition of the PECF was listed in Table I.[2]

Measurements

The intrinsic viscosity [η] (dL/g) of starting polymers was determined in dichloroacetic acid (DCA) at 25.0°C using an Ubbelohde-type viscometer. The experimentally determined values of the yield of polymer (P), the compositions of the γ-benzyl L-glutamate component at the initial monomer mixture (F_1), and in copolymer (cf_1), together with the [η] value, are summarized in Table II.

Molecular weights of the water-soluble samples were estimated at pH 7.4 and 37.0°C in PECF by sedimentation equilibrium using a MOM type 3170-b ultracentrifuge equipped with a Reyleigh interference optical system and a 12 mm double sector cell.[12] Their intrinsic 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 samples are summarized in Table III.

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

The molecular weight distribution curves of these samples were investigated by gel permeation chromatograph (GPC) on a Toyo-Soda high-speed liquid chromatography HLC-803D equipped with TSK-Gel Type G-4000SW, C-No. SW46A0015 in PECF at 25°C.

Table I Pseudoextracellular Fluid (PECF) ($NaHCO_3$, K_2HPO_4, NaCl, KCl)

Ion	Concentration Physiological	PECF (meq/L)
Na^+	145	145
K^+	5	5
Cl^-	113	118
HCO_3^-	30	30
HPO_4^{--}	2	2

Table II Copolymerization Data of Original Copolypeptides

Sample Code	F_1 (mol %)	cf_1 (mol %)	P (%)	$[\eta]$ (dL/g), DCA, 25°C
B-1	100	100	79	1.33
BA-1	78	86	48	1.45
BA-2	70	78	44	1.20
BA-3	60	70	49	1.64
BA-4	50	66	45	1.85
BA-5	35	51	44	1.60
BL-1	70	85	42	1.35
BL-2	60	75	46	1.08
BP-1	80	86	40	1.88
BP-2	70	76	48	1.79
BV-1	65	85	41	0.98
BV-2	58	75	47	0.80

All kinetic measurements were made in PECF 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 $\frac{1}{2}$ its initial value. A viscosity–kinetic measurement was performed as follows: An aliquot 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.

To convert the reduced viscosity to intrinsic viscosity, the Huggins expression,[13] $\eta_{sp}/C = [\eta] + k'[\eta]^2C$, relating reduced viscosity to concentration was followed in the concentration range of interest and the constant in the Huggins expression was within the same error as that for molecular weight. The intrinsic viscosity as a function of the digestion time may thus be calculated in this way.

Table III Preparative Data of Water-Soluble Copolypeptides

Sample Code	HEG (mol %)	$[\eta]$ (dL/g), PECF, 37°C	M_w	b_0
B(E)-10	100	0.320	69,400	0
B(E)-20	100	0.264	44,700	0
B(E)-30	100	0.184	25,300	0
B(E)-40	100	0.125	13,600	0
BA(E)-10	86	0.294	54,200	−30
BA(E)-20	78	0.276	50,300	−70
BA(E)-30	70	0.338	69,800	−80
BA(E)-40	66	0.480	98,000	−90
BA(E)-50	51	0.410	84,500	−135
BL(E)-10	85	0.328	53,900	−120
BL(E)-20	75	0.315	49,500	−180
BP(E)-10	86	0.335	64,000	−150
BP(E)-20	76	0.418	75,200	−240
BV(E)-10	85	0.138	14,800	0
BV(E)-20	75	0.180	23,300	0

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. A straight line is drawn so as to fit the experimental points for B(E) homopolymer. From this straight line, the empirical parameters, K' and a, in the equation: $[\eta] = K'M^a$ for B(E) homopolymer were obtained. In regard to the copolymer 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 copolymers from the intrinsic viscosity experimental data.

Type of Degradation

To determine whether random degradation of the main chain of a polypeptide is dominant in the reaction with bromelain, GPC analyses of partially degraded polypeptides were carried out. Figure 2 illustrates GPC curves for B(E)-20 sample. From Figure 2, it may be concluded that B(E)-20 sample

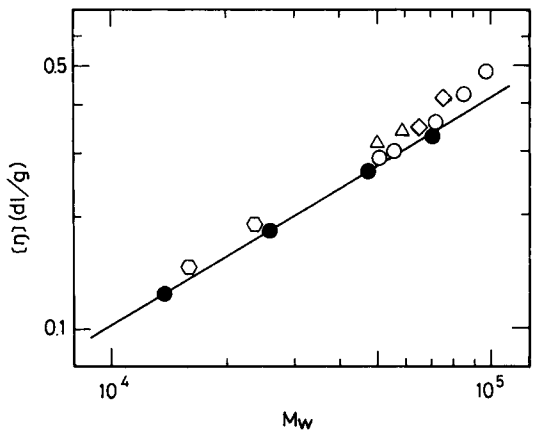

Figure 1 The intrinsic viscosity $[\eta]$ vs. molecular weight M_w at pH 7.4 and 37.0°C: (●) B(E); (○) BA(E); (△) BL(E); (◇) BP(E); (○) BV(E).

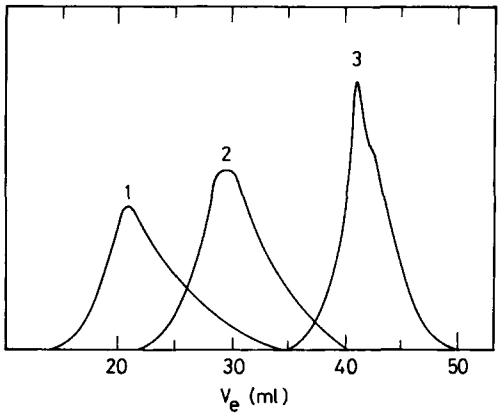

Figure 2 GPC elution curves for reaction products of B(E)-20 at pH 7.4 in PECF by bromelain. [E] = $2.0 \times 10^{-5} M$. (1) Original; (2) 60 min of digestion; (3) 300 min of digestion.

is dominantly degraded by a random main-chain cleavage as in the case of the degradation of B(E) homopolymer by endopeptidases such as papain, chymotrypsin, ficin, or pepsin, previously reported.[7-9] The same was observed previously in the case of the degradation of PLGA reported by Miller.[14]

Rate of Degradation

Typical plots of 1/M against the bromelain digestion time are shown in Figure 3. The procedure in this study 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 have rather narrow molecular weight distributions, the aminolysis reaction of side chains of γ-benzyl L-glutamate residues will break a few peptide bonds which both theoretically[15] and experimentally[16] widens the molecular weight distribution considerably. Thus, original samples 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 GPC profiles in Figure 2 and plots of $1/M$ against bromelain digestion time in Figure 3 indicate random degradation, and since the original samples have random distributions, the 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

It is known that all peptidases yield data which can be analyzed in the framework of the Michaelis–Menten mechanism. The rate of 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 B(E)-10, BA(E)-20, and BL(E)-20; they indicate the first-order behavior and are typical of endopeptidase degradation. The order of the rate of degradation, V, among the samples was BA(E)-20 > BL(E)-20 > B(E)-10 under the same experimental condition.

Next, the rate of degradation, V, was evaluated as a function of substrate concentration. Figure 5 illustrates the experimental results for B(E)-10 and BA(E)-20. The rate of bond breaking was calculated from the plots similar to Figure 3, by measuring the

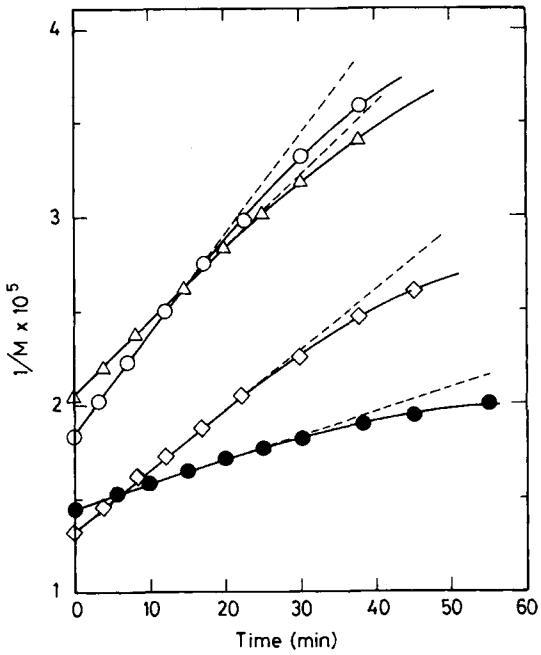

Figure 3 Typical plots of $1/M$ for samples as a function of bromelain digestion time at pH 7.4 and 37.0°C. [E] = $2.0 \times 10^{-5} M$. (●) B(E)-10; (○) BA(E)-20; (△) BL(E)-20; (◇) BP(E)-20.

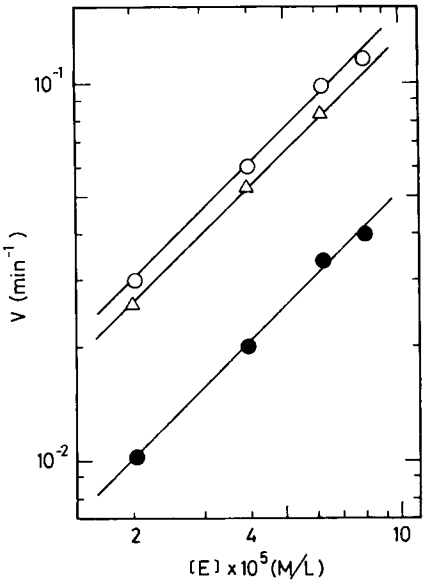

Figure 4 Dependence of reaction rate V (min^{-1}) on bromelain concentration at pH 7.4 and 37.0°C in PECF: (●) B(E)-10; (○) BA(E)-20; (△) BL(E)-20.

time necessary for the molecular weight to drop to $\frac{1}{2}$ 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. Figure 6 illustrates Lineweaver–Burk plots for B(E)-10 and BA(E)-20 with data obtained in Figure 5. As expected, the degradation follows the Michaelis–Menten rate law within experimental error since the rate of degradation, V, is dependent on the number of bonds and not macromolecules, which for 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 samples which were calculated from the experimental findings.

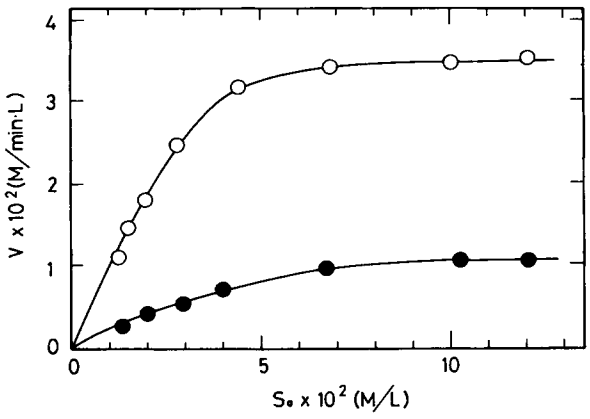

Figure 5 Plots of reaction rate V (min^{-1}) as a function of polymer substrate concentration S_o (M/L) at pH 7.4 and 37.0°C in PECF: (●) B(E)-10; (○) BA(E)-20.

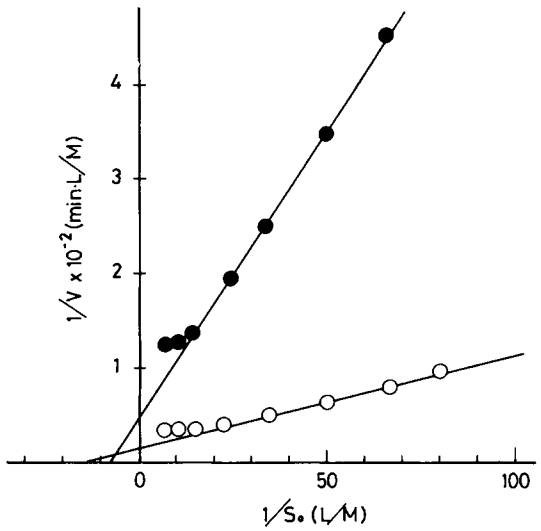

Figure 6 Lineweaver–Burk plots with the data obtained in Figure 5: (●) B(E)-10; (○) BA(E)-20.

Effects of Hydrophobic Side Chains on Degradation Rate

The relation between the composition of BA(E), BL(E), BP(E), and BV(E) copolymers and the rate of degradation, V, by bromelain was investigated under the same experimental conditions. Table V 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 *N*-hydroxyethyl L-glutamine (HEG) residue in the copolymers. The order of the rate of degradation among the copolymers was BA(E) > BL(E) > BP(E) > BV(E), on the same order of HEG molar percent. Figure 7 illustrates the experimental results of BA(E) copolymers in Table V relating the rate of degradation, V_{av}, with the molar percent of

Table IV Michaelis Parameters for Samples in PECF at pH 7.4 and 37.0°C

Sample Code	E_o (M/L)	K_m (M/L)	V_m (M/min L)
B(E)-10	2.0×10^{-5}	12.5×10^{-2}	2.1×10^{-2}
BA(E)-20	2.0×10^{-5}	6.7×10^{-2}	7.2×10^{-2}
BL(E)-20	2.1×10^{-5}	8.5×10^{-2}	5.8×10^{-2}

Table V The Average Rate V_{av} of the Copolymers

Sample Code	HEG (mol %)	$S_0 \times 10^2$ (M/L)	V_{av} (M/min L)
B(E)-10	100	4.05	1.05×10^{-2}
B(E)-40	100	4.20	0.94×10^{-2}
BA(E)-10	86	3.90	2.65×10^{-2}
BA(E)-20	78	3.88	3.58×10^{-2}
BA(E)-30	70	4.12	4.56×10^{-2}
BA(E)-40	66	4.05	4.85×10^{-2}
BA(E)-50	51	4.20	5.67×10^{-2}
BL(E)-10	85	3.79	2.08×10^{-2}
BL(E)-20	75	4.05	3.00×10^{-2}
BP(E)-10	86	3.90	1.65×10^{-2}
BP(E)-20	76	3.83	2.45×10^{-2}
BV(E)-10	85	4.10	1.30×10^{-2}
BV(E)-20	75	4.15	1.55×10^{-2}

HEG residue. 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.

Sequential Distributions of Comonomers in Copolymers

It was suggested in the previous section that the sequential distribution of comonomers in copolymer chains is another major factor determining the rate of degradation of copolypeptides. Thus, it is important to understand the sequential distributions of comonomers. To do so, it is necessary to obtain precise values for the monomer reactivity ratios, r_1 and r_2, from copolymerization experimental data. Using the experimental finding obtained in Table II, we integrated the copolymerization equation derived by Skeist,[10] taking into consideration the change in monomer composition with conversion. The instantaneous mole fraction of 1-monomer, F_1, in the monomer mixture as a function of conversion, P, is calculated by the differential equation of Skeist indicated as

$$dF_1/dP = (F_1 - f_1)/(1 - P) \quad (1)$$

where P is the apparent molar conversion and f_1 is the mole fraction of 1-component in the polymer formed during the differential time interval (the instantaneous copolymer composition of 1-monomer residue) and is given by the following equation[17]:

$$f_1 = \frac{F_1(r_1F_1 + 1 - F_1)}{F_1(r_1 + 1 - F_1) + (1 - F_1)[F_1 + r_2(1 - F_1)]} \quad (2)$$

Using F_1 calculated from eqs. (1) and (2), the cumulative copolymer composition of 1-monomer, cf_1, in copolymer is calculated from

$$cf_1 = [F_1 - (1 - P)F_1]/P \quad (3)$$

where F_1 is the initial mole fraction of 1-component in copolymerization.

The best possible values of r_1 and r_2 were calculated by the computor procedure, which was used to obtain the best fit to the experimental data (Table II) by trial-and-error selections of r_1 and r_2. The mode of calculation is reported elsewhere in detail.[18] The numerical values obtained with each cases are summarized in Table VI, together with the data for BL, BP, and BV in the same experimental conditions, which reported in the previous paper by us.[19,20]

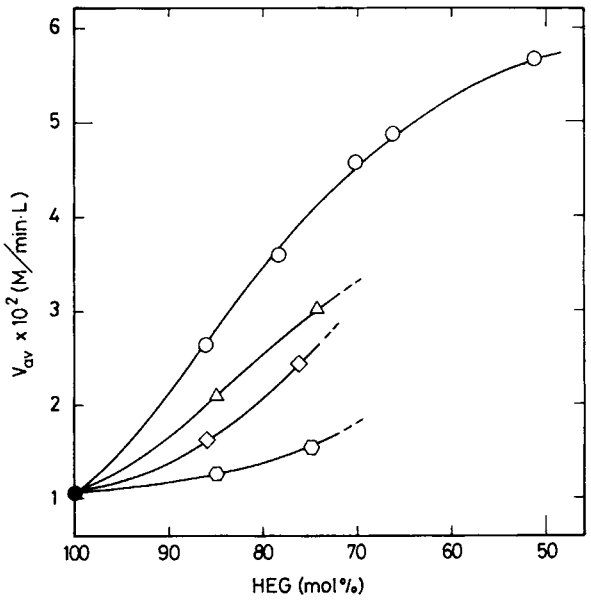

Figure 7 Plots of the average rate of bromelain digestion V_{av} (min^{-1}) as a function of the cumulative copolymer composition of HEG monomer residue for copolymers at pH 7.4 and 37.0°C in PECF.

Table VI Values of r_1 and r_2 Computed from the Skeist Equation

Copolymer	Solvent	r_1	r_2
GA	DO–MC	2.36	0.50
GL	DO–MC	2.65	0.38
GP	DO–MC	1.64	0.59
GV	DO–MC	2.96	0.17

Figure 8 illustrates, for example, variation in the instantaneous mole fraction of 1-monomer, F_1, along with the instantaneous copolymer composition of 1-monomer residue, f_1, and the cumulative copolymer composition of 1-monomer, cf_1, in copolymer with the molar conversion, P, for BA-2 copolymer at an initial 1-monomer feed is $F_1 = 0.78$. It is clear from Figure 8 that when monomers start to copolymerize, the copolymer analyzed at low conversion contains significantly more 1-monomer (B-component) than 2-monomer (A-component). Since B-NCA reacts with the growing peptide chain faster than A-NCA, the relative amount of free B-NCA monomer decreases faster during the course of copolymerization. This variation in copolymer composition results in a higher concentration of the less reactive monomer, A-NCA. Consequently, there is a drift in f_1 as well as F_1 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. The reactivity of NCA is influenced by a number of factors, such as the stereochemical structure and polarity of the monomer, the kinds of initiator, and the solvent effects.

Next, defining F_{12} as the fraction of bonds of 1 and 2 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.[20] These dyad distributions as well as copolymer composition are influenced by the degree of conversion P.[17]

Figure 9 illustrates the normalized fractions of monomer dyads for BA-2 copolymer against the initial monomer composition for 30% of conversion

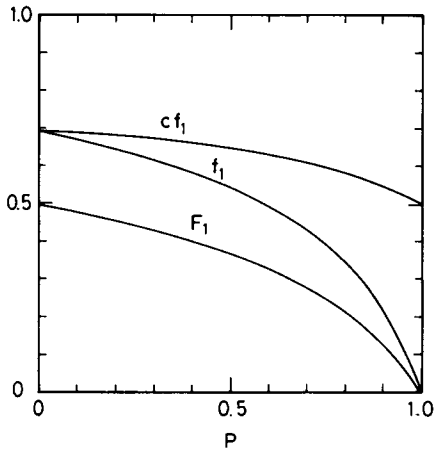

Figure 8 Variation in the mole fraction of the B-component in the monomer feed, F_1, the instantaneous copolymer, f_1, and the cumulative copolymer, cf_1, with the molar conversion, P for BA-4 ($F_1 = 0.50$).

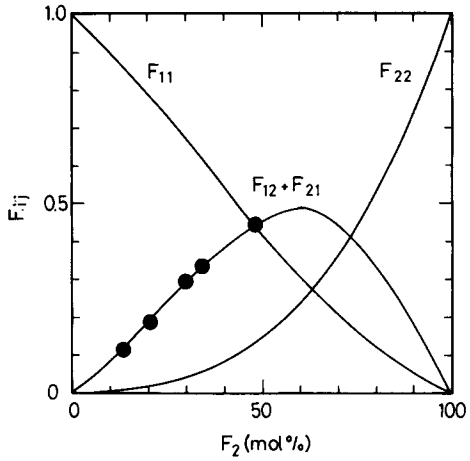

Figure 9 Normalized fractions of monomer dyads: F_{11}, $F_{12} + F_{21}$, and F_{22} as a function of the initial monomer composition of B-component for BA copolymers ($P = 0.45$).

calculated by the modified method of Harwood's system[21] on the basis of the terminal model.[20] The experimental data of the degradation rate for BA(E) copolymers were plotted by an arbitrary measure in Figure 9.

As shown in Figure 9, the agreement between the calculated and experimental values is rather good for each case. This fact indicates that, in addition to the copolymer composition, the sequential distribution of comonomers in copolymer chains becomes one of the major factors to determine the rate of biodegradation of these BA copolymers.

In conclusion, it was pointed out that random copolymers consisting of *N*-hydroxyethyl L-glutamine and a hydrophobic L-amino acid, such as L-alanine, L-leucine, L-phenylalanine, or L-valine, were found to be degraded by random chain sission with bromelain, and that the degradation data for these copolymers followed the Michelis–Menten rate law. Furthermore, the nature of side chains strongly affected the rate of degradation by bromelain and it was controlled by the comonomer composition as well as by the sequential distribution of comonomers in the copolymer chains. These findings would become very important information to design the biodegradable polymers whose rate of degradation is the most desirable for the biomedical applications.

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