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Enzymatic Hydrolysis of Multi-Component Random Copolypeptides *in Vitro*

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ABSTRACT: Multi-component random copolypeptides membranes consisting of L-cystein (C), L-alanine (A), and L-glutamic acid (G), were synthesized by *N*-carboxy-amino acid anhydride (NCA) method following deblocking of the protective units of side chains by hydrogen fluoride (HF) treatment. All the water soluble samples were found extensively degraded by random chain fracture with papain. Degradation data for these samples followed the Michaelis–Menten rate law, being of the first order to the enzyme concentration. Hydrogels were prepared by intermolecular crosslinking between L-cystein residues (cystine bonding). The swelling ratio and enzymatic degradation behavior of these hydrogels were investigated in a pseudo-extracellular fluid (PECF) at pH 7.4 and 37°C as a function of copolymer composition to simulate *in vivo* polymer degradation. The rate of enzymatic hydolysis of hydrogels was highly dependent on the swelling ratios of the samples.

KEY WORDS Multi-Component Random Copolypeptide / Enzymatic Hydrolysis / Papain / the Michaelis–Menten Mechanism / Cystine Bonding / Hydrophobicity /

Poly(α-amino acid)s and their copolymers have potential for biodegradable 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 after surgery between the pericardium and heart wall during open-heart surgery, polymer carriers for protein conjugates and drug delivery systems. Poly(α-amino acid)s are typical biodegradable polymers. 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. 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 materials, 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.

In this study, the preparation of multi-component random copolypeptides hydrogels consisting of L-cystein, L-alanine, and L-glutamic acid was performed to clarify the effects of the nature of side chain of the comonomer and copolymer composition on the rate of degradation by papain in a pseudo-extracellular fluid (PECF)[2] at a pH 7.4 and 37.0°C to simulate *in vivo* polymer degradation from an applicable point of view for bio-

Table I. Pseudo-extracellular fluid (PECF) ($NaHCO_3$, K_2HPO_4, NaCl, KCl)

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

medical materials. The composition of PECF is listed in Table I. One of the most typical points in this study is that copolypeptide hydrogels are obtained by crosslinking not using any chemical crosslinkers but cystine bonding between L-cystein residues of copolymers which is similar to that of natural proteins.

Papain 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 isolated from mammalian spleen, liver, kidney, and lung, and is released by cells in response to inflammation[6].

EXPERIMENTAL

Materials

Synthesis of Mother Polymers. *N*-carboxyanhydrides (NCA) of L-amino acids were prepared by the phosgenation of the corresponding L-amino acids in tetrahydrofuran (THF) and purified by multiple recrystallization.

Solutions (0.1 M, *ca.* 2 wt%) of the mixture of γ-benzyl L-glutamate NCA (G-NCA), L-alanine NCA (A-NCA) and *S*-benzyl L-cystein NCA (C-NCA) in 1 : 1 (v/v) mixture of dioxane/methylene dichloride were prepared and polymerization was initiated with triethylamine (TEA) at total of the NCA monomers : TEA equals to 25 : 1.

The copolymerization reaction was followed by CO_2 evolution according to the method of Patchornik and Shalitin.[7] Solvents used for synthesis and TEA were purified by the usual method described in the literature. All starting polymer samples were precipitated by adding about 4 times the amount of methanol in volume including 5 vol% of 0.1*N* HCl to the polymer solution at 4°C. The precipitation products were washed in methanol and dried under reduced pressure at 50°C. The preparative data of the mother polymers is listed in Table I.

Preparation of Hydrophilic Samples. Deblocking reaction of γ-BLG and L-cys(Bzl) residues in copolymers was performed by hydrogen fluoride (HF) treatment. The aqueous polymer solution was dialyzed exhaustively against distilled water, filtered through a Millipore filter and lyophilized. Completion of the deblocking reaction was ascertained by UV spectra measurement. The composition of these copolymers was determined by amino acid analysis.

Papain (3.5 m Anson μmg^{-1}, No. 7144) was purchased from Nakarai Tesque Co. (Kyoto, Japan), and used without further recrystallization. Papain was activated in the PECF solution as proposed by Homsey at pH 7.4 with 0.01 M cystein and 0.01 M EDTA.

Hydrogels were prepared by cystine bonding between L-cystein residues in copolymers with potassium ferricyanide oxidation. In the case of CAG(A) random copolymers, we reported previously that C-NCA, A-NCA, and G-NCA copolymerized in an ideally random fashion and that the L-cystein residue was well dispersed in CAG copolymer chains.[8] Thus, rather homogeneous cystine bonding between copolymer chains may be expected.

Measurements

Molecular Characterizations. Intrinsic viscosity $[\eta]$ ($dl\,g^{-1}$) of starting polymers was determined in dichloroacetic acid (DCA) at 25°C using an Ubbelohde-type viscometer and is listed in Table II. $[\eta]$ of the water soluble samples after deblocking reaction were

Table II. Copolymerization data of original samples

Sample code	Composition/mol%			$[\eta]$/dl g^{-1} (DCA, 25°C)
	L-Cys	L-Ala	L-Glu	
CAG-1	4	24	72	1.22
CAG-2	6	25	69	1.40
CAG-3	10	28	62	1.45
CAG-4	35	32	33	1.28

Table III. Preparative data of water soluble samples

Sample code	L-Cys	$[\eta]$/dl g^{-1}
	mol%	(PECF, 37°C)
CAG(A)-1	4	0.488
CAG(A)-2	6	0.510
CAG(A)-3	10	0.552
CAG(A)-4	35	0.470

measured with an Ubbelohde-type viscometer at pH 7.4 and 37°C in PECF. The preparative data of these water soluble samples are summarized in Table III.

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

All kinetic measurements of the enzymatic hydrolysis of the water soluble samples were achieved using an Ubbelohde type viscometer in PECF. 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. Viscosity-kinetic measurement was performed as follows: An aliquote of the polymer solution (10 ml unless otherwise stated) was pipetted into the viscometer. After determining the viscosity of a solution, 0.2 ml of the stocked papain solution was added and viscosity was taken periodically with stirring. Unless otherwise stated, the papain 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,[9] $\eta_{sp}/C = [\eta] + k'[\eta]^2 C$, 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. $[\eta]$ values as a function of digestion time may thus be calculated in this way. The molecular weights of these samples were calculated from $[\eta]$ values by the equation;

$$[\eta] = 6.58 \times 10^{-6} M_w^{1.08} \quad (1)$$

obtained for Poly(L-glutamic acid) (PLGA) under the same experimental conditions.[10]

Swelling Ratios of Hydrogels. The swelling ratio q of a hydrogel in PECF was determined by equilibrating the hydrogel in PECF at 37°C. The hydrogel was removed, blotted to remove surface PECF and weighed until a constant weight was achieved. The hydrogel was then dried in a vacuum oven. q was defined as the ratio of the weight of PECF swelling of the hydrogel to that of the dried one.

Enzymatic Hydrolysis of Hydrogels in Vitro. Enzymatic hydrolysis studies *in vitro* were carried out using papain. The hydrogel was exposed to PECF at pH 7.4 and 37°C. The hydrogel was removed from the enzyme solution at appropriate time intervals, weighed, and then vacuum dried at 60°C to constant weights.

RESULTS AND DISCUSSION

Type of Degradation

To determine whether random degradation of the main chain of a polypeptide is dominant in the reaction with papain, GPC analyses of partially degraded polypeptides were carried out. Figure 1 illustrates GPC curves for CAG(A)-2. From Figure 1, it may concluded

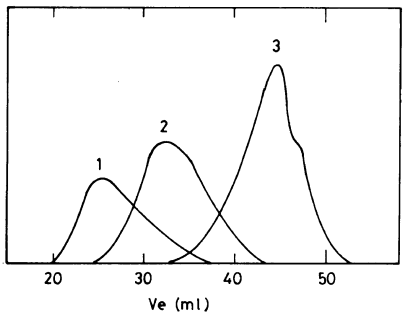

Figure 1. GPC elution curves for reaction products of CAG(A)-2 at pH 7.4 and 25°C in PECF by papain. $[E]=9.0\times10^{-6}$ M. (1) original, (2) 1 h of digestion, and (3) 10 h of digestion.

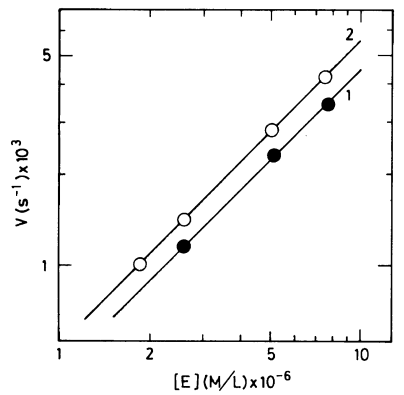

Figure 2. Dependence of the rate of the enzymatic hydrolysis V (s^{-1}) on papain concentration at pH 7.4 and 37.0°C in PECF for: (1) CAG(A)-1 and (2) CAG(A)-2.

that each sample is dominantly degraded by random mainchain cleavage as in the case of the degradation of PLGA by endopeptidases such as papain, chymotrypsin, ficin, or pepsin.[10-12]

Rate Law

All proteases yield data which can be analyzed by the Michaelis–Menten mechanism. The rate of enzymatic hydrolysis of the samples was expected to be first order to papain concentration. It was necessary to confirm this since the papain concentration varied over a wide range so that the rate could always be measured. Figure 2 shows the expected experimental results for the water

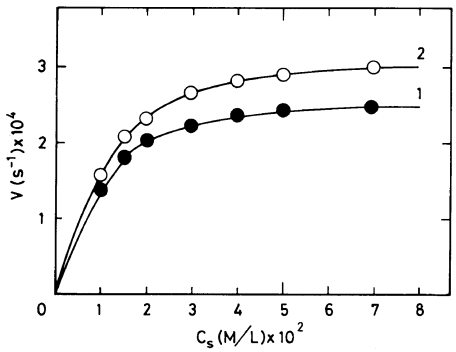

Figure 3. Plots of the rate of the enzymatic hydrolysis V (s^{-1}) as a function of polymer substrate concentration C_s ($M\,l^{-1}$) at pH 7.4 and 37.0°C in PECF ($[E]=5.1\times10^{-6}$ M) for: (1) CAG(A)-1 and (2) CAG(A)-2.

Table IV. Michaelis parameters of samples in PECF at pH 7.4 and 37.0°C

Sample code	E_o	K_m	V_m
	$M\,l^{-1}$	$M\,l^{-1}$	$M\,s^{-1}\,l^{-1}$
CAG(A)-1	5.1×10^{-6}	1.3×10^{-2}	3.3×10^{-3}
CAG(A)-2	5.1×10^{-6}	1.2×10^{-2}	3.7×10^{-3}
CAG(A)-3	5.1×10^{-6}	1.0×10^{-2}	4.5×10^{-3}

soluble samples of CAG(A), indicating first order behavior and typical of endopeptidase degradation. V was evaluated as a function of substrate concentration. Figure 3 illustrates the experimental results for CAG(A) samples. The rate of bond breakage was calculated from the plots similar to Figure 2, 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. The two parameters, V_m and K_m in the Michaelis–Menten rate law were evaluated by Lineweaver–Burk plots[13] from these experimental data obtained in Figure 3. Table IV summarizes Michaelis–Menten parameters, V_m and K_m , for samples, as calculated from the experimental findings.

Swelling Ratios of Hydrogels in PECF

q in a solvent is determined by the interac-

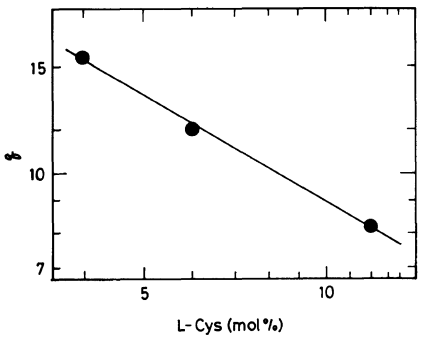

Figure 4. Swelling ratio (q) of hydrogels at 25°C as a function of L-cystein mol% in CAG(A) copolymers.

Table V. Rate of the papain hydrolysis V of samples in PECF at pH 7.4 and 37.0°C

Sample code	L-Cys	C_s	V
	mol%	$M\,l^{-1}$	$M\,s^{-1}\,l^{-1}$
CAG(A)-1	4	3.88×10^{-2}	2.42×10^{-3}
CAG(A)-2	6	4.01×10^{-2}	2.86×10^{-3}
CAG(A)-3	10	4.10×10^{-2}	3.60×10^{-3}

tion energy between the solvent molecules and polymer segments as well as elastic energy (crosslink density) for a solvent-swollen polymer. The effect of L-cystein mol% in copolymer chains on q of the cross-linked hydrogels by cystine bonding in PECF is shown in Figure 4. q in PECF decreases with increasing L-cystein mol% in copolymer chains.

When q is quite large, it is given by the following equation according to the rubber elasticity theory.[14]

$$q^{5/3}=(vM_c)(1-2M_c/M)^{-1}(1/2-\chi_1)/V_1 \quad (2)$$

where M_c is the molecular weight per cross-linked unit, M the primary molecular/weight, v the specific volume of polymer, V_1 the molar volume of solvent and the interaction parameter. The factor $(1-2M_c/M)$ expresses the correction for network inperfection resulting from chain ends. For a quite high molecular weight polymer chain, $(1-2M_c/M)$ becomes to unity. As the effective crosslink

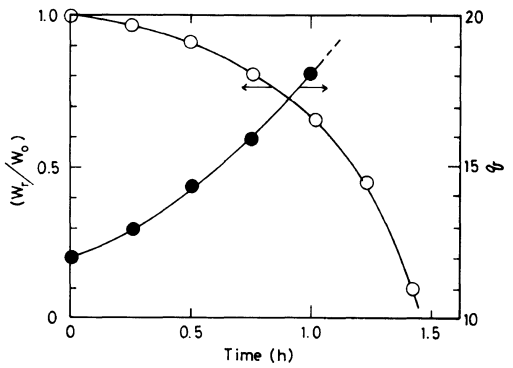

Figure 5. Dry weight ratio (W_r/W_o) and swelling ratio (q) of PECF for CAG(A)-2 hydrogel as a function of papain digestion time (h) at pH 7.4 and 37.0°C.

density, f_c is proportional to the value of M_o/M_c where M_o is the molecular weight of the repeat unit (monomeric unit), equation (2) may be simplified as

$$q^{5/3}=(vM_o)(1/2-\chi_1)/V_1 f_c \quad (3)$$

Thus, $\log q$ is in linear relation with $\log f_c$ with a slope of $-3/5$. The experimental data in Figure 4 almost fit the slope of the straight line of $-3/5$ as predicted in eq 3.

Enzymatic Hydrolysis of Hydrogels in vitro

Williams[15] has shown that numerous proteases may be present at a wound site. Enzymes of inflammatory response that likely degrade poly(α-amino acid)s include the endopeptidase cathepsin B and exopeptidase, carboxypeptidase and leucine aminopeptidase.[16] In the present investigation, thiol endopeptidase papain was selected as a commercially available analog of cathepsin B. Preweighed hydrogels were dipped in PECF including papain at 37.0°C, and the results obtained with CAG(A)-2 hydrogel are shown in Figure 5.

Degradation of the hydrogel was measured by change in bulk as well as q. As shown in Figure 5, with papain digestion, an immediate increase in q of the hydrogel was observed, while weight loss occurred slightly more slowly. An endopeptidase must make two inci-

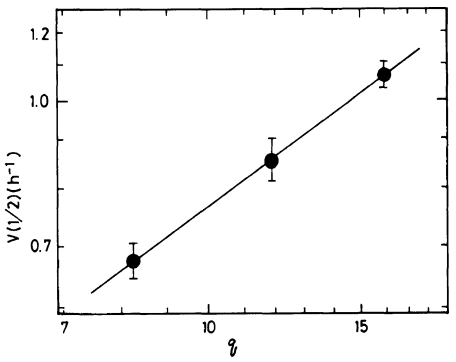

Figure 6. Rate of papain digestion V (1/2) of CAG(A) hydrogels at pH 7.4 and 37.0°C in PECF as a function of swelling ratio (q).

sions in a chain segment to produce a soluble fragment, but a single cleavage will decrease the effective crosslink density.

Figure 6 summarizes the rate of papain digestion $V(1/2)$ (h) as a function of q of PECF for CAG(A) hydrogels. $V(1/2)$ is defined as the reciprocal of the time required the sample weight to be reduced to one-half its initial value.

In conclusion, it was pointed out that random copolymers consisting of L-cystein, L-alanine, and L-glutamic acid, were found to be degraded by random chain sission with papain, and that the degradation data for these copolymers followed the Michelis–Menten rate law. The swelling ratio q of hydrogels affect the rate of enzymatic hydrolysis V of hydrogels. The latter increased with increasing q value. q may be controled by changing L-cystein mol% in copolymer chains to achieve cystine bonding. These findings are very important in the design of the biodegradable polymers whose rates of degradation are most desirable for biomedical applications.

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