Biodegradation of the Cross-Linked Cationic Poly(amino acid) Hydrogels by Proteolytic Enzymes

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Received March 6, 1995; Revised Manuscript Received June 27, 1995[⊗]

ABSTRACT: The degradation of the cross-linked cationic poly(amino acid)−glutaraldehyde (GA) hydrogels by some proteolytic enzymes has been investigated using lysine (Lys)- and ornithine (Orn)-containing homo- and copolypeptides. From the experimental results, the following findings were obtained. The polylysine (PLL)−glutaraldehyde (GA) gels are degraded by trypsin, but not by chymotrypsin and papain. Optimal conditions for degradation of the PLL−GA gels are trypsin units over 500, pH 7−11, 0.5−2 M salts, and higher reaction temperatures. A pH region below 5 and a salt concentration over 4 M inhibit trypsin activity toward the PLL−GA gels. Copoly(Lys^1−Tyr^1)-GA gels are degradable by trypsin, chymotrypsin, and even papain. Polyornithine (PLO) gels behave like PLL gels but exhibit no degradation by enzymes. Copoly(Lys^1−Orn^1) gels are degradable by trypsin, suggesting potential for a controlled biodegradation. The results might offer some clues to the understanding of the degradation of natural protein adhesives.

Introduction

It has been widely observed in aqueous systems that insolubilized water-soluble proteins are surface adhesive in biological systems.[1,2] For example, mussels secrete a soft semitransparent gelly thread emerging sideways from a flattened ovoid disk in 10 min when it is dark.[3] The transparent thready gel turns, successively, white turbid, pale yellow after a few hours, brownish red overnight, and finally into a strong green thread after several days, exhibiting a high tensile strength of 200−1000 kgf/cm^2. This hardening process is due to auto-cross-linking by an oxidase tyrosinase.[4] Thus, mussels attach not only to rocks but also to plastics, glass, slate, and metals by approximately 100 disks and threads, which are called byssus. These attachment byssus are biodegradable by microorganisms, keeping marine ecology clean.[5]

Apart from marine adhesion processes, cross-linked biological polymers in watery systems have long been an important class of materials and are used in a diverse assortment of applications as hydrogels including medical wound dressing; progress continues in developing approaches to describe the molecular structure of cross-linked polymers.[6,7]

We have investigated the polymer chemistry of adhesive proteins in watery systems. During the investigation we conducted insolubilizing experiments of some marine adhesive protein models such as barnacle arthropodin using an oxidase[8] and also reported insolubilizing experiments including gel formation of simplified water-soluble cationic lysine and ornithine polypeptides using organic cross-linking agents such as dialdehydes.[9−14] In our ongoing study, this paper describes the biodegradation characteristics of cationic cross-linked polypeptide hydrogels using hydrolytic enzymes.

Experimental Section

Materials. Poly(L-lysine) (PLL; DP 240), poly(L-ornithine) (PLO; DP 290), copoly(Lys^1−Orn^1) (DP 620), copoly(Lys^1−Tyr^1) (DP 250), and copoly(Orn^1−Tyr^1) (DP 140) were synthesized according to the amino acid *N*-carboxyanhydride procedure followed by polycondensation. The DPs (average degrees of polymerization) of the polypeptides were estimated from viscometry as described in our previous paper.[10]

Enzymes, trypsin (from porcine pancreas; EC 3.4.21.4; activity, 5200 USP units/mg), α-chymotrypsin (from bovine pancreas; EC 3.4.21.1; activity, min 1000 USP chymotrypsin units/mg), and papain (from Carica papaya; EC 3.4.22.2; activity, over 3000 USP units/mg), were purchased from Wako Pure Chemical Industries, Ltd.

Enzymatic Reaction. The substrate gels were prepared as follows. As an example, to a solution containing 20 mg of PLL (96 μmol of residue) in 0.1 mL of distilled water (pH 5) was added $^1/_{10}$ mol equiv glutaraldehyde (GA; 9.6 μmol). The massive gels formed were washed throughly with distilled water and used intact for the degradation experiments by the addition of 3000 units each of the enzymes. As a quantitative evaluation of degradation, the gels prepared were washed thoroughly with distilled water and methanol, dried, and ground into finely divided particles, designated here as microgels. To the microgel particles swollen in distilled water and in salt solutions in the quartz cells was added directly 100−1500 units of each enzyme as powders.[15]

The enzymatic degradations of the cross-linked gels were measured, with stirring, using a Jasco UVIDEC-1 spectrophotometer and were judged from the decrease of transmittance at 650 nm. The pH of the degradation systems was controlled in the range pH 3−12 by the addition of NaOH or HCl (0.01−0.1 M) and was measured with a Beckman digital pH meter.

Results and Discussion

Since the late 1940s the digestion of non-cross-linked PLL by a variety of proteases such as trypsin has been reported in many earlier papers, in which they reported that the main degradation process to oligopeptides occurred within 30 min.[16−19] This paper first describes the degradation of cross-linked PLL and related hydrogels.

Orn is an unnatural cationic amino acid and has one less methylene group in the side chains than Lys. Since the gel preparation by GA was reported in detail in our previous papers,[12,14] we avoid here repeating the description except for additional information. When allowed to stand without mixing or stirring, the Lys- and/or Orn-containing polypeptides and GA systems formed soft and solid gels after 20−40 h. The swelling and color characteristics between PLL and added organic cross-linkers including GA were also described in the previous

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1995.

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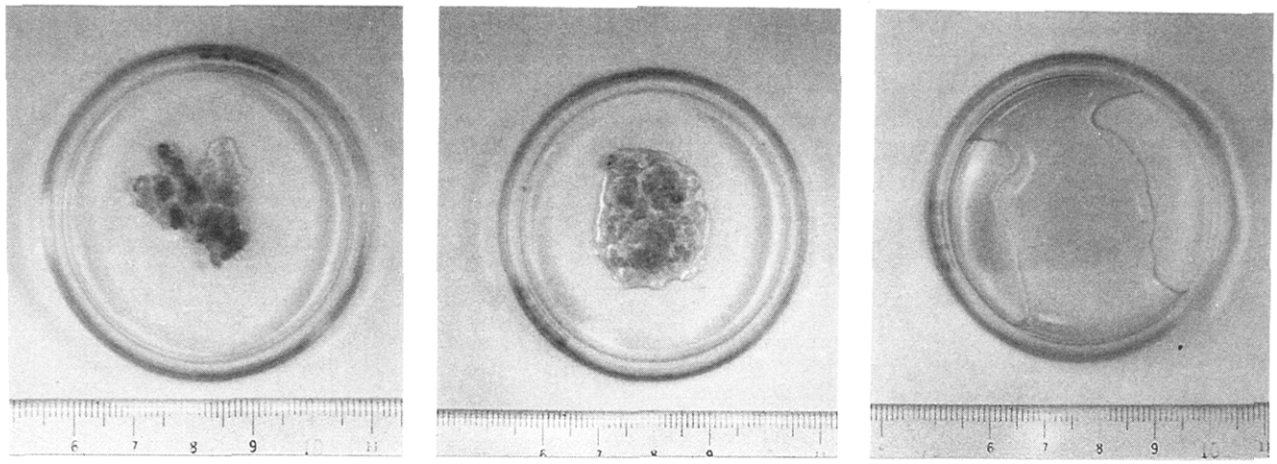

a b c

Figure 1. Photographs of the biodegradation process of the copoly(Lys[1]Tyr[1])−1/5 GA gel by 3000 units of chymotrypsin: (a) 0, (b) 6, (c) 10 h.

Table 1. Degradation of Cationic Polypeptide Gels by Enzymes

gel	enzymes[a]		
	trypsin	chymotrypsin	papain
PLL−1/10 GA[b]	○	×	×
PLO−1/10 GA	×	×	×
copoly(Lys[1]Orn[1])−1/10 GA	○	×	×
copoly(Lys[1]Tyr[1])−1/5 GA	○	○	△
copoly(Orn[1]Tyr[1])−1/5 GA	×	×	×

[a] Enzymes used were 3000 units each. Symbols: ○ denotes the rapid transformation to liquids within 10 h; △ denotes the slow or partial degradation within 60 h; × denotes almost no degradation over 60 h. [b] Polypeptide gels used were prepared from polypeptides of 20 mg each.

paper.[12] For the preparation of copoly(Lys[1]−Tyr[1]) gels, concentrations as high as 1/5 mol equiv GA are necessary for gel formation, which is a higher amount of GA than required for the other polypeptides. Copoly(Lys[1]−Tyr[1])−1/5 GA gel was somewhat fragile due to its few cross-linking bridgeheads since the phenolic hydroxyl groups of Tyr exhibit low cross-linking units by GA.

As an example, Figure 1 shows the photographs of the biodegradation process of the copoly(Lys[1]−Tyr[1])−1/5 GA gel by 3000 units of chymotrypsin. The gel was degraded to liquid after 10 h by chymotrypsin and after 6 h by trypsin. The biodegradation of polypeptide gels wtih different amino acid compositions is summarized in Table 1. The circle in Table 1 denotes the rapid complete transformation of gels to liquids within 10 h, the triangle slow or partial degradation within 60 h, and the cross almost no degradation over 60 h. The results in Table 1 illustrate that each enzyme recognized specifically the gels as substrates and digested the peptide bonds. Papain, which has low substrate specificity, exhibited slower biodegradation ability toward the copoly(Lys[1]−Tyr[1])−1/5 GA gel than trypsin and chymotrypsin, and very low degradation may occur but not result in PLL−GA (1/10) gels. Of the three enzymes used in the present paper the degradation ability of trypsin is the highest toward cationic polypeptide gels containing Lys.

PLO−1/10 GA gel and copoly(Orn[1]−Tyr[1])−1/5 GA gel exhibited no or a very low degradation by the three enzymes under the present experimental conditions.

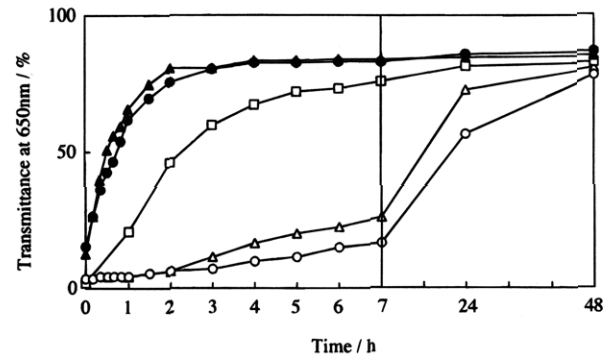

Figure 2. Trypsin amount dependence of the degradation of the PLL−1/10 GA microgels at 25 °C and pH 7: (▲) 1500, (●) 1000, (□) 800, (△) 500, (○) 100 units.

The reason is due to the Orn residues which is an unnatural amino acid and cannot be digested by these three proteolytic enzymes.

When the polypeptide microgels are suspended in aqueous media as finely powdered particles, the transmittance of light at 650 nm is less than 10% and increases as the microgels degrade. In this paper we judged that the change of the transmittance is covariant with the degree of degradation and that when the change reached the equilibrium time, the degradation ended. Furthermore, the time from the addition of enzymes to the equilibrium was defined here as the net degradation time.

Figure 2 shows the enzyme concentration dependence of the degradation of the PLL−1/10 GA microgels by trypsin at 25 °C and pH 7. When 100 units of trypsin was added, it took over 48 h to degrade the microgels. Increased trypsin amounts of 500, 800, 1000, and finally 1500 units shorten the degradation time to 48, 24, 4, and 2 h, respectively. Thus, 1000−1500 units of trypsin was found to be a suitable amount for a fast degradation of the PLL microgels, and since lower levels of the enzyme such as 100 units digested and degraded the microgels completely after several days even at the latest cases.

Trypsin as a protease has been studied for a very long time. Among many studies, the reported digestion conditions of trypsin were optimal pH 8−9 and wide-

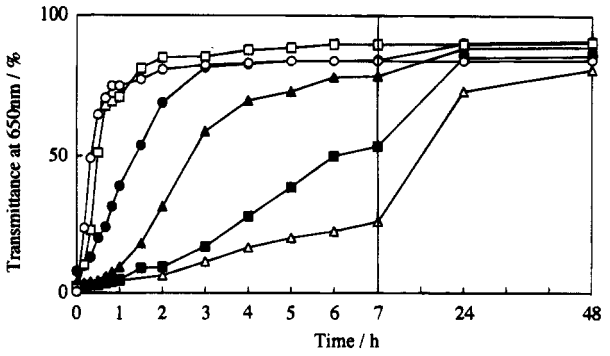

Figure 3. NaCl concentration dependence of the degradation of the PLL−$^1/_{10}$ GA microgels by 500 units of trypsin at 25 °C and pH 7: (△) 0, (○) 0.5, (□) 1, (●) 2, (▲) 3, (■) 4 M.

Table 2. Relationship between the Swelling Degree and the Tryptic Degradation Time of the PLL−$^1/_{10}$ GA Gels

amount of enzyme units	pH	NaCl (M)	swelling ratio	degradn time (h)
1000	2	0	<4.7	>48
1000	5	0	5.9	>48
1000	7	0	6.1	4
1000	11	0	5.7	4
500	7	0	6.1	48
500	7	0.5	2.1	2
500	7	1	1.7	3
500	7	2	1.4	4
500	7	3	1.2	6
500	7	4	1.1	24

temperature dependence 0−80 °C,[20-23] and Ca^{2+} increased its stability.[24]

The pH dependence profile of the degradation of the PLL−$^1/_{10}$ GA microgels by 1000 units of trypsin at 25 °C was examined. At pH 7 and 11, the degradation proceeded quickly (4 h), and no significant difference was found between the two pH's. Trypsin degraded the gels in the pH region between neutral and weak alkaline conditions, which coincided well with the optimal pH 8 of this enzyme. Figure 3 shows the NaCl concentration dependence profile of the degradation of the PLL−$^1/_{10}$ GA microgels by 500 units of trypsin at pH 7 and 25 °C. The degradation time of the microgels by trypsin was 2 h in 0.5 M NaCl. When the NaCl concentrations were increased to 1, 2, 3, and 4 M, the degradation times became longer: 3, 4, 6, and 24 h, respectively. However, the degradation time of the microgels by trypsin in 4 M NaCl was shorter than that in salt-free water. It was clear that the presence of NaCl in the systems enhanced the degradation of the PLL−GA gels, but a salt concentration over 2 M needed longer degradation times than did diluted salt systems. This optimal salt concentration of the degradation of the PLL−GA microgels at 2 M NaCl was contrary to our expectations;[21] we anticipated the degradation time would become longer in higher salt concentrations. The activation of trypsin by NaCl (0−4 M) was also confirmed by the hydrolysis of an authentic substrate N-α-benzoyl-L-arginine ethyl ester at pH 7 and 25 °C, as well.[25]

In order to clarify the reasons for the results in Figure 3 and to discuss the mechanism of the enzymic digestion in more detail, we summarize the relationship between the degradation time and the swelling degree of the PLL−$^1{}_{10}$ GA microgels by changing the pH of the systems, the enzyme units, and the NaCl concentration at pH 7 and 500 units of enzyme in Table 2. As described above, when the pH was raised from 5 to 7, the degradation reaction toward the PLL−GA microgels was accelerated, while the swelling degree of the gels

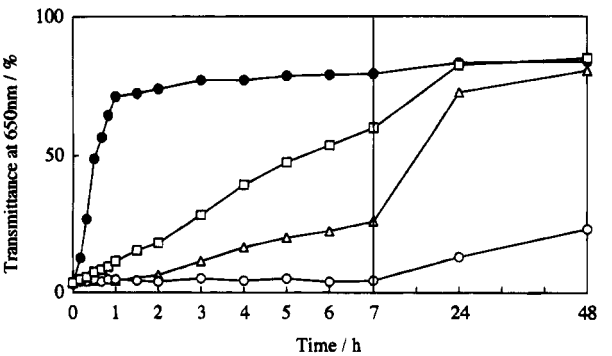

Figure 4. Temperature dependence of the degradation of the PLL−$^1/_{10}$ GA microgels by 500 units of trypsin at pH 7: (○) 5, (△) 25, (□) 40, (●) 60 °C.

without salts exhibited the least change and even at pH 2 and 11 the gels themselves were in the swollen states (swelling ratios 4.7−6.1). In these swollen states active trypsin easily penetrates into the gels, as do solvents, and degrades them (pH 7−11). On the other hand, when the NaCl concentration in the systems at pH 7 was increased from 0.5 to 4 M, the gels were in their contracted states and enzymes could not penetrate inside. The degradation time of the PLL−GA microgels then became much longer, from 2 to 24 h, as shown in Table 2. Thus, since tryptic digestion toward the PLL−GA gels is activated by the increased NaCl concentrations (less than 3 M), as an additional experiment, the effect of the added salts was examined using NaBr, KCl, and NH_4Cl. The difference between activation by the salts was in the 10% range, suggesting no significantly salt species dependence. In this connection, the activating effect of divalent metal ions, such as Ca^{2+} and Co^{2+}, on a variety of aminopeptidases has long been known. The reason has been explained that Ca^{2+} could reduce autolysis by combining with trypsin.[25] Likewise, monovalent cations are considered to reduce autolysis of trypsin.

Figure 4 shows the temperature dependence profile of the degradation of the PLL−$^1/_{10}$ GA microgels by 500 units of trypsin at pH 7. The degradation time was shortened when the temperature was raised. The PLL microgels were degraded after several days at 5 °C, after 48 h at 25 °C, after 24 h at 40 °C, and after 1 h at 60 °C. From these temperature dependence features, it was confirmed that the degradation of the PLL microgels by trypsin proceeded faster at higher temperatures. In contrast to other common enzymes, it is known that trypsin can display activity even at temperatures higher than 80 °C.[23] Due to these temperature characteristics, it is concluded that trypsin degrades the PLL microgels according to rate kinetics rather than to enzyme kinetics.

As described above, enzymes degrade the PLL−GA gels by changing the external conditions such as added amounts, pH, and salt concentrations, and so on. However, if the degradation by enzymes could be controlled by internal molecular design, a material science advance would be possible. This viewpoint was examined using some copolypeptide−GA gels, to which the unnatural amino acid Orn was incorporated. We could choose many kinds of unnatural amino acids. However, if in cases where an unnatural amino acid is chosen to be incorporated the favorable PLL−GA gel characteristics such as the swelling properties and ion adsorption ability are changed greatly or lost, the introduction of the unnatural amino acid is undoubtedly

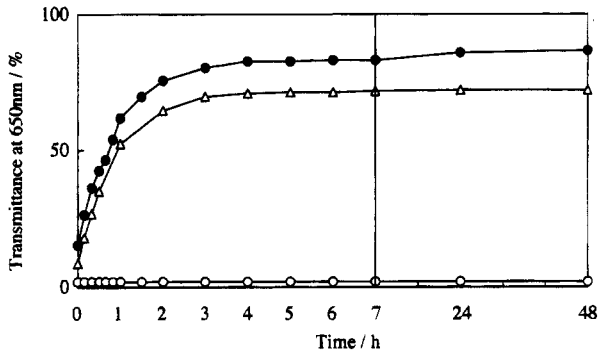

Figure 5. Degradation profile of the PLL−, PLO−, and copoly(Lys−Orn)−$^1/_{10}$ GA microgels by 1000 units of trypsin at pH 7: (○) PLO−$^1/_{10}$ GA gel; (△) copoly(Lys^1−Orn^1)−$^1/_{10}$ GA gels; (●) PLL−$^1/_{10}$ GA gel.

pointless for our purposes. From this point of view, we chose cationic Orn as the unnatural amino acid which is a lower homologue of Lys (has one less methylene group in the side chain than Lys). Figure 5 shows the degradation profile of the PLO−GA and copoly(Lys−Orn)−GA microgels by 1000 units of trypsin at pH 7. PLO−$^1/_{10}$ GA microgels were not degraded. On the other hand, trypsin degraded copoly(Lys^1−Orn^1)−$^1/_{10}$ GA slightly more slowly than the PLL−$^1/_{10}$ GA. When the molar ratios of Orn residues in copoly(Lys−Orn) and copoly(Tyr−Orn) are enriched, the degradation rate is slowed, suggesting a possibility of controlled biodegradation. In fact, after copoly(Lys^1−Orn^1)−$^1/_{10}$ GA gels were completely degraded, very small amounts of the insoluble materials were observed by the naked eye. The insoluble materials may originate from the Orn-rich domains in copoly(Lys^1−Orn^1), which trypsin could not digest.

In conclusion, we have reported here some data on the degradation of cross-linked biohydrogels using cationic Lys- and Orn-containing homo- and copolypeptides. On the basis of the experimental results, we make the following comments on the degradation of cationic PLL−GA related hydrogels: (1) PLL gels are degraded by trypsin but not by chymotrypsin and papain; (2) optimal conditions for degradation of the PLL gels are trypsin units greater than 500, pH 7−11, 0.5−2 M salts, and higher reaction temperatures; (3) a pH region below 5 and a salt concentration over 4 M inhibit trypsin activity toward the PLL gels; (4) copoly(Lys^1−Tyr^1) gels are degradable by trypsin, chymotrypsin, and even papain; (5) PLO gels behave like PLL gels but exhibit no degradation by enzymes; (6) copoly(Lys^1−Orn^1) gels are degradable by trypsin; (7) copoly(Lys−Orn) gels are controlled degradable materials depending on the Orn contents.

The degradation of the cross-linked cationic poly(amino acid)−GA gels by some proteolytic enzymes described here might not be directly related to the natural observation of the biodegradation of biological adhesives. However, the results themselves are interesting and might offer some clues to understanding degradation in biological adhesion, which includes the protein biodegradation process by microorganisms and enzymes.

These findings may also indicate promise for developing new biodegradable biohydrogel materials including medical wound dressing with very high water content. In this connection, as we have reported the selective adsorption ability of anionic molecules such as benzoic acid and acidic amino acids in the PLL−GA and PLO−GA gel matrices,[12,14] it may be possible to adsorb anionic medicines into the cationic cross-linked polypeptide gels. When the gels are biodegraded, the digested oligolysine fragments are expected to exhibit anti-infection actions as reported earlier,[26] and simultaneously the medical materials released from the inside of the gels are also expected to be effective in the cure-injured tissues of the animal organism. Thus, polylysine-related hydrogels look very promising in diverse medical applications.

Finally, biodegradation of cationic polylysine−GA-related hydrogels by microorganisms will be reported elsewhere in the very near future.

Acknowledgment. This work was supported in part by a grant from the Research Foundation for the Electrotechnology of Chuubu.

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MA950289I