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Solution Property and Irradiation Effect of Random Copolypeptides Composed of Ala and Pro Residues

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Summary

Poly(proline) and random copolypeptide composed of Pro and Ala residues were synthesized, and their solution properties and molecular conformation were investigated. Aqueous solutions of the polypeptide were irradiated with γ-rays above the transition temperature. It was shown that the transition temperature of the aqueous solution of the copolypeptide is influenced by Ala-residue content and γ-ray irradiation.

Introduction

Stimuli-responsive polymers have recently attracted attention [1-3]. A typical example, poly(N-isopropyl acrylamide) (PNIPAAm), which have thermo-responsive properties, has been investigated as an interesting polymer for biomaterials and drug delivery systems [4, 5]. Aqueous solution of PNIPAAm has a lower critical solution temperature (LCST) at approximately 31 ºC, and, the LCST of the PNIPAAm-related copolymers is controlled by the monomer composition. Aqueous solution of poly(N-vinylisobutyramide) (PNVIBA) whose monomer unit is very analogous to that of PNIPAAm also exhibited LCST at 35ºC . The thermo-responsive behavior of PNVIBA and its related polymers was also investigated [6-9]. These polymers were applied to the peptide-drug delivery system using nanoparticles composed of novel graft copolymers having hydrophobic backbone and hydrophilic branches [10, 11]. However, they are limited to oral use because of the non-biodegradable property of the synthetic polymers. Polypeptides, on the contrary, are biodegradable and biocompatible. Tropocollagen, elastin-related poly(pentapeptide) and poly(proline) exhibit thermo-responsive properties in water [12-14]. In our previous works, we

showed that proline-related random copolypeptides poly(Pro-co-Xaa) exhibit thermo-responsive properties in water, and also that such property can be controlled by hydrophobicity of the amount of non-Pro residue [15-17]. Biodegradable hydrogels have been prepared from proteins and synthetic polypetides such as collagen [18, 19], gelatin, elastin-like polypeptide, poly(Gly-Val-Gly-Val-Pro) [20-22] and poly(glutamic acid) [23, 24] by cross-linking formation using γ- and UV-irradiation technique or cross-linking regents, and their properties have been also investigated for biomedical use.

We synthesized the previous poly(Pro-co-Xaa)s by condensation polymerization of dipeptide Xaa-Pro (or Pro-Xaa) and proline, that is, they were designed as the molecules having no Xaa-Xaa portions in the amino-acid sequence for stabilizing polyproline-II conformation that is the structural base forming inter-molecular packing of polypeptide chains. In the present work, for forming the Ala-Ala sequence which is designed as a suitable component creating cross-linking by γ-irradiation, we tried condensation polymerization using alanine and proline as monomer amino-acid, and their solution properties as a function of irradiation were investigated.

Experimental

Materials

Proline was purchased from Peptide Institute Inc. (Osaka, Japan). Alanine, diphenylphosphoryl azide (DPPA) [25] as condensing reagent, N, N-dimethylformamide (DMF) as solvent and trifluoroacetate(TFA) were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Synthesis of poly(proline) and poly(Pro-co-Ala)

Copolypeptides were synthesized by standard DPPA procedure. To a stirred suspension of Pro and Ala mixed in DMF (0.5ml/mg) containing 10 and 20 mol% Ala residue, respectively, in the reaction mixture, was added 1.3-fold molar excess of DPPA and 2.3-fold molar excess of triethylamine. The mixture was stirred at 5-10ºC for 1 hour and further at room temperature for 3 days. To the reaction mixture was added a large volume of methanol or ethylacetate, and the precipitate was collected by centrifugation to be dried in vacuum. The product was recrystallized from TFA and diethyl ether. Each synthesized copolypeptide is abbreviated as PA-x, where x is the molar percent of Ala residue in poly(Pro-co-Ala). Copolypeptides were characterized by H^1-NMR and Gel permeation chromatography (GPC). Poly(proline) (PP) free of Ala residue was synthesized and purified by the same method as described above.

Measurement and γ-irradiation

10ml samples of each aqueous solution of polypeptides (20 or 50mg/ml) were prepared in 20ml glass tubes with glass stopper and irradiated above the LCST for 2 hours with γ-rays from a ^{60}Co source (1.44kGy/h) in the irradiation pool at Research Institute for Advanced Science and Technology, Osaka Prefecture University. Circular dichroism (CD) spectra of the synthesized polypeptides were measured in water at 5ºC by JASCO J-820 (Jasco. Co., Japan). The ellipticity was expressed as

mean residual molar ellipticity [θ] in degrees • cm^2 • $dmol^{-1}$. The thermo-responsive property of the polypeptides was characterized by turbidity measurements. Each solution was heated at a rate of 0.5ºC/min. The transition temperature was defined as 50% transmittance of polypeptide solution during the heating process. The transmittance of the γ-irradiated solution at 500nm was also measured by a circular dichroism spectrometer.

Results and Discussion

Characterization of poly(proline) and poly(Pro-co-Ala)

The characterization results of polypeptides are summarized in Table 1.

Table 1. Preparation of polypeptides using DPPA.

Polypeptide	Reactant ratio (mol%)	Yield (w%)	Content[a] (mol%)		Mn.[b] ($\times 10^5$)	Mw/Mn
			Pro	Ala		
PP	Pro/Ala=100/0	39.1	100	0	1.3	-
PA-8.8	Pro/Ala=90/10	58.9	91.2	8.8	1.9	1.2
PA-16.8	Pro/Ala=80/20	54.6	83.2	16.8	2.0	1.2

a) The composition of polypeptides was estimated by ^{1}H-NMR measurement.
b) The averaged molecular weight was estimated by GPC measurement with calibration using the polyethylene glycol standard.

CD spectra of non- and γ-irradiated PP, PA-8.8 and PA-16.8

As shown in Figure 1, the CD spectrum of PP in water (20 mg/ml) at 5ºC exhibited a strong negative band at 206nm and a weak positive band at 229nm which correspond to the standard spectrum of polyproline-II conformation [26, 27]. The CD spectra of PA-8.8 and PA-16.8 in water at 5ºC also exhibited a strong negative band at 205nm and a weak positive band at 228nm, and the negative-band strength is slightly smaller than that of PP. This spectrum suggests that PA-8.8 and PA-16.8 maintain polyproline-II conformation in water at 5ºC as a whole in spite of the local deformation of polyproline-II conformation induced by the Ala residue.
As shown in Figure 2, the CD spectrum of γ-irradiated PA-16.8 in water (20 mg/ml) at 5ºC presented a negative band at 204nm and a positive band at 230nm, and the strength of these bands was slightly smaller than that of non-irradiated ones. However, the spectrum-change by γ-irradiation for PP and PA-8.8 was less significant than that for PA-16.8. The result of the CD spectra of PA-16.8 indicates that drastic conformation change has not occurred by γ-irradiation, because the CD bands are not shifted, i.e. the CD bands showed the typical CD pattern of polyproline-II conformation. However, slight decrease of band strength suggests that conformation of PA-16.8 was locally changed. Furthermore, it is an important factor that oxygen is present in aqueous solution of polypeptide, because this triggers the occurrence of free radical. It is surmised that the free radical causes the radical reaction of the methyl group of Ala residues. However, radiolysis of backbone (peptide bond) has not occurred at 2.8 kGy [28].

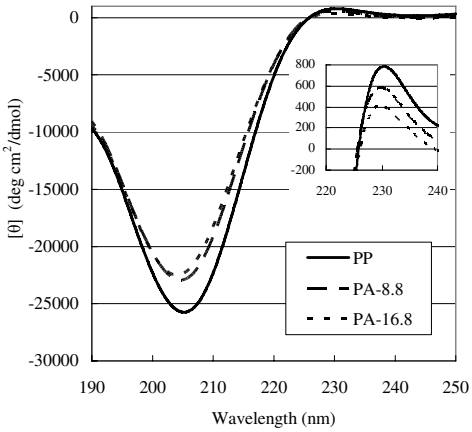

Figure 1. CD spectra of non-irradiated PP, PA-8.8 and PA-16.8 at 20 mg/ml.

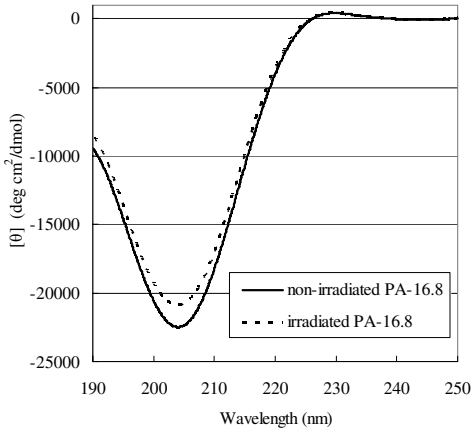

Figure 2. CD spectra of non-irradiated and irradiated PA-16.8 at 20 mg/ml.

Thermo-responsiveness properties of polypeptides

Figures 3 and 4 represent the plots of light transmittance vs. temperature for the aqueous solution of non-irradiated polypeptides at concentrations 50mg/ml and 20mg/ml, respectively. The transition temperatures of non-irradiated PP, PA-8.8 and PA-16.8 at 50 mg/ml are 59.4, 64.8 and 67.8ºC, respectively, and those at 20 mg/ml are also 65.0, 70.8 and 73.5ºC, respectively, indicating that the transition temperature of each polypeptide increases with a decrease in concentration. These results correspond to our previous works for proline-related polypeptides [15-17]. The transition temperature of the polypeptides also increases with an increase in Ala-residue content. It is known that the hydrophobicity of Pro residue is higher than that of Ala residue [29-32]. We assume that molecular aggregation in aqueous solution of poly(proline) and poly(Pro-co-Ala) is caused by molecular packing among polyproline-II conformation, that is, that molecular aggregation of the molecules having hydrophobic groups in water is caused by the decrease in number of iceberg-like water molecules [33] which forms the hydrophobic hydration onto the surface of

the hydrophobic group of amino-acids. However, the transition behavior of PA-16.8 is different from that of PP and PP-8.8, that is, the aqueous solution of PA-16.8 does not exhibit full light transmittance even at 50ºC, and its value moderately decreases with an increase in temperature in the early stage of aggregation phenomena, suggesting that the aggregation of PA-16.8 would be caused by two mechanisms. One caused by intermolecular packing among the portions forming local polyproline-II conformation as supposed for PP and PA-8.8, and the other caused by intra- and intermolecular aggregations among the hydrophobic side-chain of Ala residue locally forming non-polyproline-II conformation in addition to intra- and intermolecular aggregation caused by hydrophobic interaction between the side-chain of Ala residue and that of Pro residue.

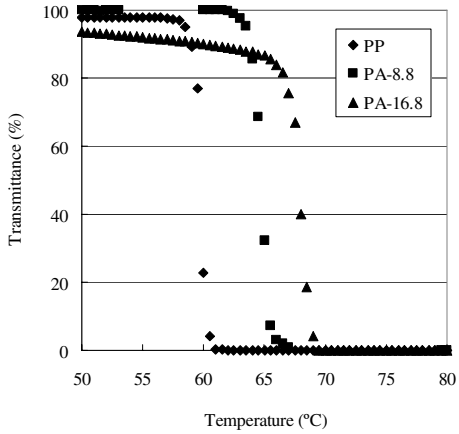

Figure 3. Temperature dependence of light transmittance at 500 nm for the aqueous solution of non-irradiated polypeptides at 50 mg/ml.

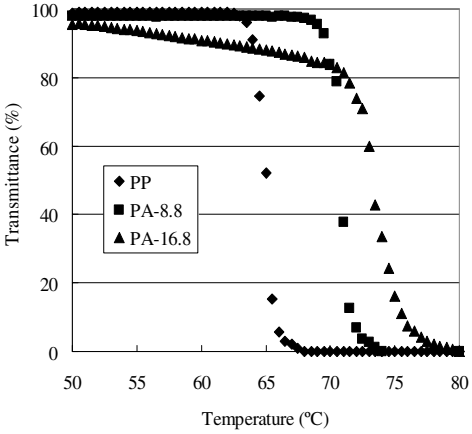

Figure 4. Temperature dependence of light transmittance at 500 nm for the aqueous solution of non-irradiated polypeptides at 20 mg/ml.

The transition temperatures of irradiated PP, PA-8.8 and PA-16.8 at 50 mg/ml are 60.4, 65.3 and 67.0ºC, respectively, and those at 20 mg/ml are also 65.3, 71.0 and 72.9ºC, respectively. In the case of PP and PA-8.8, the transition temperature of each irradiated polypeptide is slightly higher than that of non-irradiated one (Figure 5, Table2). In general, the lower the molecular weight is, the higher the LCST of polymer solution is. Consequently, these results suggest that the molecular weight of PP and PA-8.8 is decreased by bond-breaking in main-chain through γ-irradiation [34-36]. It means that effect of the covalent bond breakage is more dominant than the effect of the cross-linking between adjacent polypeptide molecules for PP and PA-8.8.

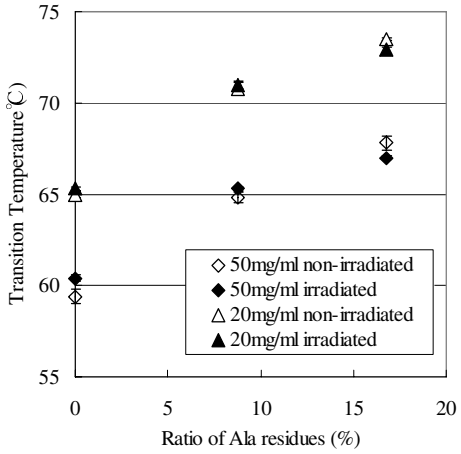

Figure 5. Plots of the transition temperature vs. Ala content (n=3).

Table 2. The transition temperature of aqueous solution of polypeptides.

	50 mg/ml			20 mg/ml		
	$T_{non\text{-}irr}$ [a] (ºC)	T_{irr} [a] (ºC)	ΔT [b]	$T_{non\text{-}irr}$ (ºC)	T_{irr} (ºC)	ΔT [a]
PP	59.4	60.4	1.0	65.0	65.3	0.3
PA-8.8	64.8	65.3	0.5	70.8	71.0	0.2
PA-16.8	67.8	67.0	-0.8	73.5	72.9	-0.6

a) The transition temperature of non-irradiated ($T_{non\text{-}irr}$) and irradiated (T_{irr}) polymers
b) $\Delta T = T_{irr} - T_{non\text{-}irr}$

However, the transition temperature of irradiated PA-16.8 is lower than that of the non-irradiated one, and the light transmittance of the former aqueous solution is also lower than that of the latter one at 50ºC as shown in Figure 6, indicating that molecular aggregation has already begun to occur below the transition temperature. These results indicate that intermolecular cross-linking might be formed by γ-irradiation at the contacted side-chains in the molecular aggregated state as it was already shown in experimental works on the hydrogel-formation of the elastin-related poly(pentapeptide)s [19]. This speculation is also supported by the CD experiments that show that the decrease of the negative band-strength of PA-16.8 by γ-irradiation is more remarkable than those of PP and PA-8.8, which indicates that formation of

polyproline-II conformation, is locally disturbed by the conformational restrictions caused by the formation of crosslinks.

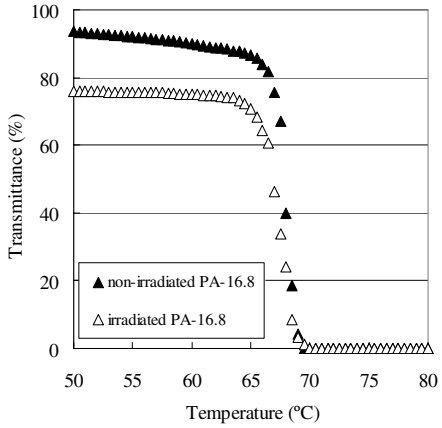

Figure 6. Temperature dependence of light transmittance at 500 nm for the aqueous solution of non-irradiated and irradiated PA-16.8 at 50 mg/ml.

Conclusions

Synthesized proline-rich copolypeptides composed of Ala and Pro residues exhibits a CD spectrum which resembles that of polyproline-II conformation, and also exhibited thermo-responsive properties in aqueous solution. The transition temperature of polypeptide increased with the Ala content, and γ-irradiation effects were specific for each polypeptide. The physical properties of proline-rich copolypeptides makes these peptides candidates for preparation of the peptide nano-particles.

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