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Thermosensitive properties of poly(proline)-based polypeptide having an amino-acid of low hydrophobicity

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Summary

Several types of poly(Gly-co-Pro) and poly(Ala-co-Pro) were synthesized. Their molecular conformations and thermosensitive properties were investigated in an attempt to find new thermosensitive materials. These polypeptides were assumed the polyproline-II structure in the temperature range of 20 to 80ºC. They also exhibited cloud points in light transmittance, indicating the phase transition. The transition temperatures increased with decrease in hydrophobicity of the polypeptides, that is, in the order poly(proline), poly(Ala-co-Pro) and poly(Gly-co-Pro).

Introduction

The phenomenon of thermosensitive water-soluble polymers has gained interest in recent years from the viewpoint of designing drug delivery systems. Heskins and Guillet showed that poly(*N*-isopropylacrylamide) (polyNIPAAm) has a lower critical solution temperature (LCST) of 32ºC in water [1]; and that the thermosensitive behavior of polyNIPAAm and its derivatives has been investigated extensively [2-4]. These polymers are utilized in peptide drug delivery systems in the form of nanoparticles composed of novel graft copolymers having a hydrophobic backbone and hydrophilic branches [5-7]. However, their use is limited to oral applications because of the non-biodegradable nature of synthetic polymers.

By contrast, polypeptides are biodegradable and biocompatible. Thus, they have been studied for biomedical applications [8-11]. It is well known that polypeptides such as poly(proline) [12], tropocollagen [13] and elastin-model polypeptides [14] exhibit heat precipitation in water. This phenomenon is very interesting for biomedical applications.

In our previous study, as a first trial for designing the thermosensitive material, we attempted to control the heat precipitate temperature of poly(proline) by introducing Hyp(Bzl) residue (a more hydrophobic amino-acid than Pro residue) into it [15]. The transition temperature of these polypeptide was found to be highly dependent on the

Hyp(Bzl) residue content. This indicates that the transition temperature of poly(proline)-based polypeptide can be controlled by varying the hydrophobicity of polypeptides. But it has not been known that the thermosensitive properties of poly(proline)-based polypeptides were made with less hydrophobic amino-acid than Pro residue.

In the present study, polypeptides, poly(Gly-co-Pro), having different hydrophobicity were used as poly(proline)-based copolypeptides for designing thermosensitive material. It is known that Gly residue is hydrophilic than Pro residue [16, 17]. Thus, Gly residue was selected as a controlling residue of hydrophobicity in poly(proline)-based polypeptide. Ala residue takes intermediate hydrophobicity between Pro and Gly reside [16, 17]. Therefore, it also seems interesting to ascertain the thermosensitive properties of poly(Ala-co-Pro). Poly(proline)-based polypeptides, poly(Gly-co-Pro) and poly(Ala-co-Pro), were synthesized by Pro and one of Gly-Pro and Ala-Pro with diphenylphosphoryl azide (DPPA). Towards this end, we investigated how slight difference in hydrophobicity of polypeptides affect their molecular behavior during heat precipitation.

Experimental

Materials

Proline and Gly-Pro were purchased from Peptide Institute Inc. (Osaka, Japan). Ala-Pro was purchased from Sigma-Aldrich Japan K. K. (Tokyo, Japan). Diphenylphosphoryl azide (DPPA) and *N,N*-dimethylformamide (DMF) were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). These materials were used as received.

Polymerization

The typical procedure was as follows [18, 19]: To a stirred suspension of Pro and Gly-Pro in DMF (0.5 ml/mg) containing 2.5, 5.0 or 10.0 mol% Gly residue in the total amino-acid residue were 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 h and further at room temperature for 3 days. To the reaction mixture was added a large volume of ethyl acetate, and the precipitate was collected by centrifugation to be dried *in vacuo*. The product was recrystallized from formic acid and diethyl ether. Each synthesized copolypeptide is abbreviated as poly(Gly-co-Pro)-x, where x is the molar percent of Gly residue in poly(Gly-co-Pro). Poly(Ala-co-Pro) and poly(proline) free of Gly residue was synthesized and purified by the same method as described above. Scheme 1 schematizes the synthesis of poly(Gly-co-Pro). Polymerization results are summarized in Table 1.

Scheme 1. Synthesis of poly(Gly-co-Pro).

Table 1. Preparation of polypeptides.

Polypeptide	Reactants	Reactant ratio	Yield (%)	Content[a] (mol%)		Mn[c]
				Pro	Xaa[b]	
Poly(proline)	Pro		32.5	100	0	2.0×10^3
Poly(Gly-co-Pro)-2.8	Pro, Gly-Pro	95: 5	80.2	97.2	2.8	2.1×10^3
Poly(Gly-co-Pro)-5.4	Pro, Gly-Pro	90:10	87.0	94.6	5.4	2.2×10^3
Poly(Gly-co-Pro)-11.9	Pro, Gly-Pro	80:20	76.7	88.1	11.9	2.1×10^3
Poly(Ala-co-Pro)-3.1	Pro, Ala-Pro	90:10	68.7	96.9	3.1	1.6×10^3
Poly(Ala-co-Pro)-6.6	Pro, Ala-Pro	80:20	86.1	93.4	6.6	1.6×10^3

[a]The composition of polypeptides were determined by amino acid analysis.
[b]Xaa = Gly or Ala residue.
[c]The averaged molecular weight was determined by MALDI-TOF-MS.

Measurement

Conformation of the polypeptides was determined by the measurement of circular dichroism (CD). CD spectra of the synthesized polypeptides were measured in water over the temperature range of 20 to 80ºC by JASCO J-720 (Jasco. Co.). The ellipticity was expressed as mean residual molar ellipticity [θ] in degrees $\cdot$ cm^2 $\cdot$ $dmol^{-1}$. The thermosensitivity of the polypeptides was characterized based on turbidity measurements. There were prepared polypeptide solutions at various concentrations from 160 mg/ml to 2.5 mg/ml. Each solution was heated at a rate of 1ºC/min and the transmittance of the solution at 500 nm was measured by a U-3210 type spectrometer (Hitachi. Co.). The transition temperature was defined as the temperature at 50% transmittance.

Results and Discussion

Poly(Gly-co-Pro) and poly(Ala-co-Pro) were obtained by polymerization of proline and one of Gly-Pro and Ala-Pro, respectively, in the presence of DPPA as a coupling reagent [18, 19]. Poly(proline) was obtained by the same method as described above, except for not using dipeptide. It is generally presumed that the sequence of the Gly or Ala residue would break down the polyproline-II structure and form another structure. Thus, dipeptides, Gly-Pro and Ala-Pro, were used in order to avoid the Gly and Ala sequence. Several types of poly(Gly-co-Pro) (Gly content = 2.8, 5.4, and 11.9 mol%) and poly(Ala-co-Pro) (Ala content = 3.1 and 6.6 mol%) were synthesized by controlling the Gly-Pro or Ala-Pro content in the reaction mixture. The results of the synthesis of poly(proline), poly(Gly-co-Pro) and poly(Ala-co-Pro) are summarized in Table 1. The averaged molecular weight of these polypeptides was about 2000. The residual Gly content in poly(Gly-co-Pro) increased with the concentration of Gly-Pro in the reaction mixture. The Ala content in poly(Ala-co-Pro) was also increased with the concentration of Ala-Pro in the reaction mixture. Amino-acid analysis was reveal the composition of the copolypeptide, based on which the reactivity of monomers such as Pro and Gly-Pro can be compared. Figure 1 compares the molar fraction of Gly-Pro in the monomer and copolymer, calculated based on amino-acid analytical data. The slope in Figure 1 is nearly 1, which means that Gly-Pro has a similar reactivity to Pro.

Poly(proline) is thermosensitive on having type-II structure [20-22]. Thus, it is necessary to consider the conformational properties of the polypeptides as a function

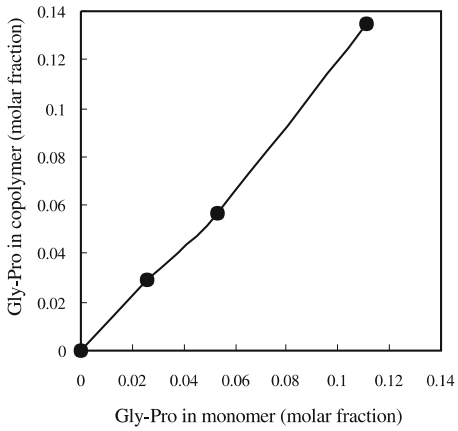

Figure 1. Content of Gly-Pro in monomer and copolymer.

of the temperature variation of the aqueous solution. The CD spectra of poly(proline) and poly(Gly-co-Pro) at 20ºC in water exhibited a strong negative band around 206 nm and a weak positive band around 229 nm as shown in Figure 2. The same manner was obtained for poly(Ala-co-Pro). These spectra corresponded almost exactly to the standard spectrum of polyproline-II structure [23-25].

Previous studies have shown that the type II structure of poly(proline) does not almost undergo an unfolding transition in the temperature range of 5 to 90ºC [15, 25-26]. The CD spectrum of poly(proline) revealed a little, insignificant dependency on temperature.

The CD spectra of poly(Gly-co-Pro) and poly(Ala-co-Pro) also showed a little dependency on temperature. However, the dependence was not so significant in the temperature range of 20 to 80ºC. That is, for poly(Gly-co-Pro) containing 5.4 mol% of Gly, the values of $[\theta]_{min}$ were -32200, -31000, -29600, and -28800 at 20, 40, 60, and 80ºC, respectively (Figure 3). CD spectra of poly(Ala-co-Pro) also takes large negative molar ellipticity at 206 nm in the temperature range of 20 to 80ºC. Thus, it appears that this polypeptide does not almost undergo an unfolding transition in this temperature range.

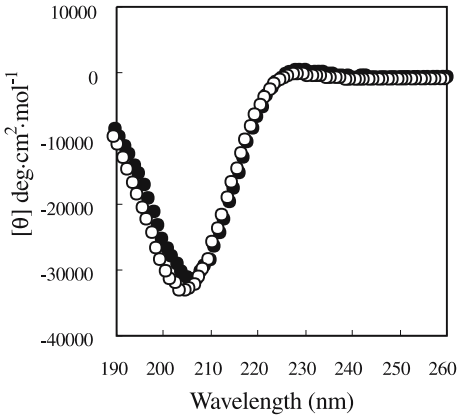

Figure 2. CD spectra of (●) poly(proline) and (○) poly(Gly-co-Pro) at 20ºC.

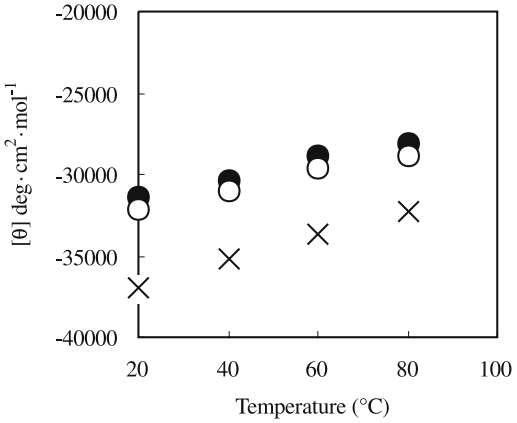

Figure 3. Temperature dependence of the molar ellipticity at 206 nm for (●) poly(proline), (○) poly(Gly-co-Pro) and (×) poly(Ala-co-Pro).

By the way, Pro residue can make a peptide bond to yield a cis-trans isomer, and Gly residue has no side chain so it is free in structure. Then, poly(proline) and poly(Gly-co-Pro), comprising these residues, are seemed to exhibit comparatively small ellipticity. Another hand, Ala residue can not make a peptide bond to yield a cis-trans isomer, and in addition to it has a side chain. Therefore, poly(Ala-co-Pro) is seemed to exhibit large ellipticity compared with poly(proline) and poly(Gly-co-Pro) (Figure 3). These results indicate that poly(proline), poly(Gly-co-Pro) and poly(Ala-co-Pro) have a polyproline-II structure in water over the temperature range 20-80ºC even though they may deviate from the standard polyproline-II structure due to local distortions.
In our previous study, we investigated with respect to the light transmittance of poly(proline) and more hydrophobic polypeptide, poly(Hyp(Bzl)-co-Pro) [15]. Then, it was clarified that the light transmittance of these polypeptide change drastically at specific temperature. The light transmittance of the aqueous solution of poly(Gly-co-

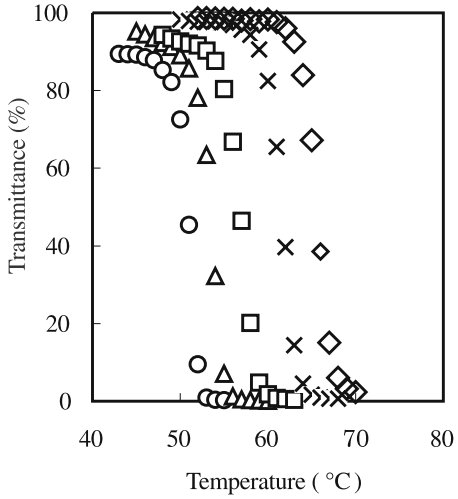

Figure 4. Temperature dependence of the light transmittance of poly(Gly-co-Pro) solutions: (○) 120 mg/ml, (△) 80 mg/ml, (□) 40 mg/ml, (×) 20 mg/ml and (◇) 10 mg/ml.

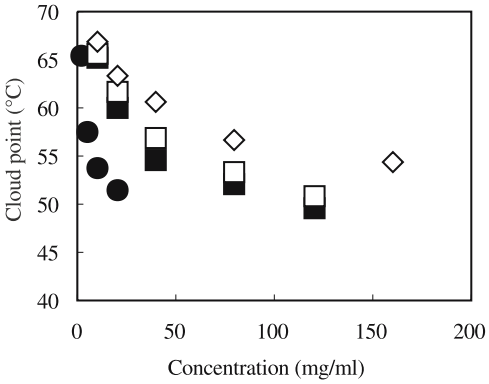

Figure 5. Concentration dependence of the cloud point temperature of aqueous solution of: (●) poly(proline), (■) poly(Gly-co-Pro)-2.8, (□) poly(Gly-co-Pro)-5.4 and (◇) poly(Gly-co-Pro)-11.9.

Pro) also changed drastically at a specific temperature (Figure 4), indicating that poly(Gly-co-Pro) chains also undergo molecular aggregation at higher temperature.

For poly(Gly-co-Pro) containing 2.8 mol% of Gly, the transition temperature decreased with poly(Gly-co-Pro) concentration: 65.3, 60.0, 54.5, 52.0 and 49.5ºC at 10, 20, 40, 80 and 120 mg/ml, respectively (Figure 5). For poly(Gly-co-Pro) with 5.4 mol% of Gly, the transition temperature also decreased with concentration: 65.6, 61.6, 56.8, 53.4 and 50.9ºC at 10, 20, 40, 80 and 120 mg/ml, respectively. For poly(Gly-co-Pro) with 11.9 mol% of Gly, the transition temperature also decreased with concentration: 80.4, 66.9, 63.3, 60.6 and 56.6ºC at 10, 20, 40, 80 and 160 mg/ml, respectively.

The light transmittance of the aqueous solution of poly(Ala-co-Pro) also changed drastically at a specific temperature. For poly(Ala-co-Pro) containing 3.1 mol% of Ala, the transition temperature decreased with poly(Ala-co-Pro) concentration: 67.4, 62.1 and 56.7ºC at 5, 10 and 20 mg/ml, respectively (Figure 6). The solubility of this polypeptide was limited at 20mg/ml. This was probably due to the strong hydrophobic

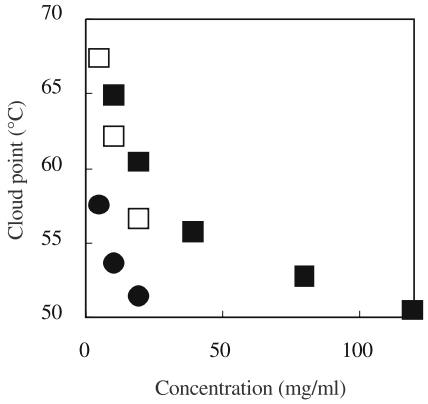

Figure 6. Concentration dependence of the cloud point temperature of aqueous solution of: (●) poly(proline), (■) poly(Ala-co-Pro)-3.1 and (□) poly(Ala-co-Pro)-6.6.

interaction of the Pro residue. For poly(Ala-co-Pro) with 6.6 mol% of Ala, the transition temperature also decreased with concentration: 64.9, 60.4, 55.7, 52.7 and 50.5ºC at 10, 20, 40, 80 and 120 mg/ml, respectively.

The results shown in Figure 3 revealed that these polypeptides have the polyproline-II structure from 20 to 80ºC, which indicates that they all attained a similar conformation in an aqueous environment. Thus, they are different only in hydrophobicity. It was shown that the transition temperature of these polypeptides presents similar dependency on the concentration and Gly or Ala content(Figure 5 and 6). However, the transition temperature of poly(Ala-co-Pro) was a little than that of poly(Gly-co-Pro), and it is higher than that that of poly(proline) in the corresponding concentration. That is, the transition temperatures of poly(proline), poly(Ala-co-Pro)-6.6 and poly(Gly-co-Pro)-5.4 were 51.4, 60.4 and 61.6ºC at 20 mg/ml, respectively (Figure 5 and 6). The order of transition temperatures of three polypeptides corresponded to the order of the hydrophobic parameters (Table 2). That is, polypeptide having higher hydrophobicity presented the lower transition temperature.

The transition temperature increased with Gly content (Figure 7). Gly residue is more hydrophilic than Pro residue because of no side-chain. This means that the entropy gained with the aggregation of poly(Gly-co-Pro) is lower than that resulting from the aggregation of poly(proline). Then, the transition temperature, i.e., the temperature satisfying $\Delta G=0$, increased with mol% of Gly. The same manner was obtained for poly(Ala-co-Pro). These results correspond to the previous results in their study on the LCST of aqueous solution of poly(Hyp(Bzl)-co-Pro) [15]. In that study, they showed, for example, that the transition temperature of aqueous solutions of copolymers of *O*-benzylhydroxyproline (Hyp(Bzl)) and proline(Pro) decrease with mol% of Hyp(Bzl), which is a more hydrophobic monomer-unit than Pro.

Thermosensitive aqueous polymer solutions undergo either coil-globule transition or coacervation at the transition temperature. In the case of poly(*N*-isopropylacrylamide), which attains a random-coil conformation at lower temperature, aggregation involves coil-globule transition. By contrast, the present aggregation process appears to occur through coacervation, as evidenced by the fact that poly(Gly-co-Pro) and poly(Ala-co-Pro) attain the polyproline-II structure and that LCST was clearly affected by the polymer concentration. However, the main factor causing molecular aggregation is iceberg-like water surrounding hydrophobic groups of polymer chain [27, 28].

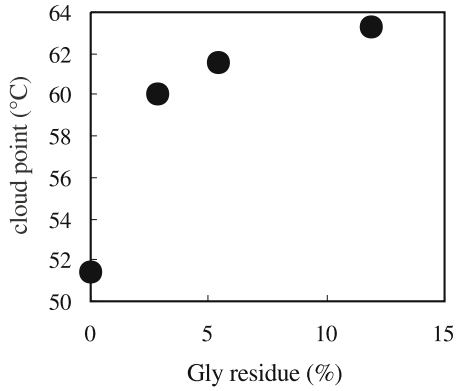

Figure 7. Cloud point temperature as a function of molar percent of Gly residue of poly(Gly-co-Pro) at 20 mg/ml.

Conclusions

Poly(Gly-co-Pro)s and poly(Ala-co-Pro)s were synthesized by using the DPPA method. These polypeptides were shown to attain the polyproline-II structure in the temperature range of 20 to 80ºC. The polypeptides possessed thermosensitive properties, depending on the hydrophobicity of the polypeptides. These results suggested that poly(proline)-based polypeptides are very suitable for use in designing thermosensitive materials.

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