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Thermosensitive property of poly(Hyp(Bzl)-co-Pro)

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Summary

Several types of poly(Hyp(Bzl)-co-Pro)s were synthesized. Their molecular conformations and thermosensitive properties were investigated in an attempt to find new thermosensitive materials. These polypeptides assumed the polyproline-II structure in the temperature range of 20 to 80°C. They also exhibited cloud points in light transmittance, indicating the phase transition. The transition temperatures decreased with increasing hydrophobicity of the polypeptide.

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 32°C in water [1]; the thermosensitive behavior of polyNIPAAm and its derivatives has been investigated extensively [2-4]. Akashi et al. synthesized poly(*N*-vinylisobutylamide) (polyNVIBA) and showed that polyNVIBA, whose monomer unit resembles that of polyNIPAAm, undergoes phase transition at 35°C in water upon heating [5]. The thermosensitive behavior of polyNVIBA and related polymers has been investigated [6], and it was shown that their LCSTs in water vary depending on the chemical structure of their side-chain groups. 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 [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. However, only elastin-model polypeptides have been studied so far. It is also well known that polypeptides have specific ordered structures strongly associated with their amino-acid sequence. For example, it is generally recognized that poly(proline) forms the polyproline-II structure in water [15-18] because of the conformational restrictions imposed by the Pro residue. Thus, it is conceivable that

designing of polypeptides will lead to a new class of functional materials.
The present study attempted to control the precipitation temperature of poly(proline) during heat precipitation by designing poly(proline)-based copolypeptides of varying hydrophobicity, containing proline residue as a major component and *O*-benzylhydroxyproline (Hyp(Bzl)) residue as a minor component. Towards this end, we investigated how slight differences in hydrophobicity of polypeptides affect their molecular behavior during heat precipitation.

Experimental

Materials

Proline was purchased from Peptide Institute Inc. (Osaka, Japan). Boc-Hyp(Bzl)-OH, HCl · H-Pro-OMe, 1-ethyl-3(-3-dimethylaminopropyl)-carbodiimide hydrochloride (WSC · HCl), 1-hydroxybenzotriazol, diphenylphosphoryl azide (DPPA), 4N-hydrogen chloride in dioxane (4N-HCl/dioxane), 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.

Monomer Peptide

HCl · H-Hyp(Bzl)-Pro-OH.
Boc-Hyp(Bzl)-OH and HCl · H-Pro-OMe were reacted using 1-hydroxybenzotriazol and WSC · HCl for 24 h in DMF. The product was washed with 5% aqueous citric acid solution and 5% aqueous $NaHCO_3$ solution and further purified by chromatography on silica column with chloroform as eluent. The purified oily product was deprotected by 1N-NaOH and 4N-HCl/dioxane to produce a white powder. TLC was carried out on Merck silica gel 60 plates with the following solvents: R_f^1, $CHCl_3$-MeOH-H_2O (8:3:1, v/v); R_f^2, MeOH; R_f^3, $CHCl_3$-MeOH (1:1, v/v). R_f^1 0.32, R_f^2 0.48, R_f^3 0.26. Found: C 57.40; H 6.37; N 7.83. Calcd. for $C_{17}H_{23}O_4N_2Cl$: C 57.54; H 6.35; N 7.90. m/z = 319.94.

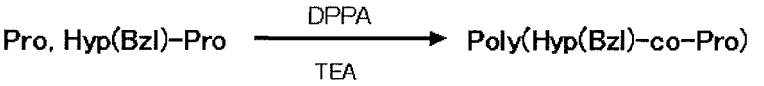
Polymerization

The typical procedure was as follows [19, 20]: To a stirred suspension of Pro and Hyp(Bzl)-Pro in DMF (0.5 ml/mg) containing 2.5, 5.0 or 10.0 mol% Hyp(Bzl) residue in the reaction mixture were added 1.3-fold molar excess of DPPA and 2.3-fold molar excess of triethylamine. The mixture was stirred at 5-10°C for 1 h and further at room temperature for 3 days. To the reaction mixture was added a large volume of methanol, 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(Hyp(Bzl)-co-Pro)-x, where x is the molar percent of Hyp(Bzl) residue in poly(Hyp(Bzl)-co-Pro). Poly(proline) free of Hyp(Bzl) residue was synthesized and purified by the same method as described above. Scheme 1 schematizes the synthesis of poly(Hyp(Bzl)-co-Pro). Polymerization results are summarized in Table 1.

Table 1. Analytical data of synthesized polypeptides.

Polypeptide	Reactants	Reactant ratio	Yield (%)	Content (%)[a]		Mn[b]
				Pro	Hyp(Bzl)	
Poly(proline)	Pro	—	32.5	100	0	2.0×10^3
Poly(Hyp(Bzl)-co-Pro)-0.9	Pro, Hyp(Bzl)-Pro	95:2.5	29.7	99.1	0.9	1.8×10^3
Poly(Hyp(Bzl)-co-Pro)-2.5	Pro, Hyp(Bzl)-Pro	90: 5	29.9	97.5	2.5	1.9×10^3
Poly(Hyp(Bzl)-co-Pro)-6.0	Pro, Hyp(Bzl)-Pro	80:10	30.2	94.0	6.0	1.8×10^3

[a]The composition of polypeptides was determined by amino-acid analysis.
[b]The averaged molecular weight was determined by MALDI-TOF-MS.



Scheme 1. Synthesis of poly(Hyp(Bzl)-co-Pro).

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 80°C by JASCO J-720 (Jasco. Co.). The ellipticity was expressed as mean residual molar ellipticity [θ] in degrees · cm^2 · $dmol^{-1}$. The thermosensitivity of the polypeptides was characterized based on turbidity measurements. Polypeptide solutions at various concentrations from 20 mg/ml to 2.5 mg/ml were prepared. Each solution was heated at a rate of 1°C/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.

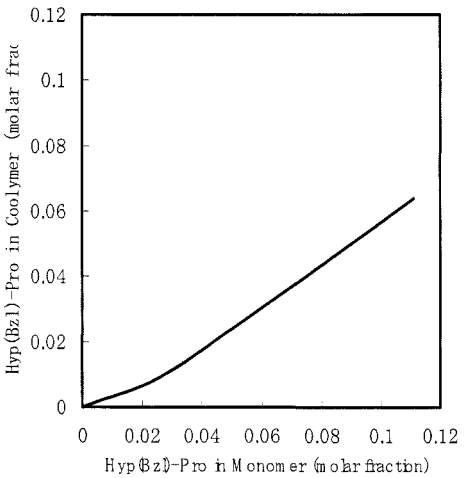


Figure 1. Content of Hyp(Bzl)-Pro in monomer and copolymer.

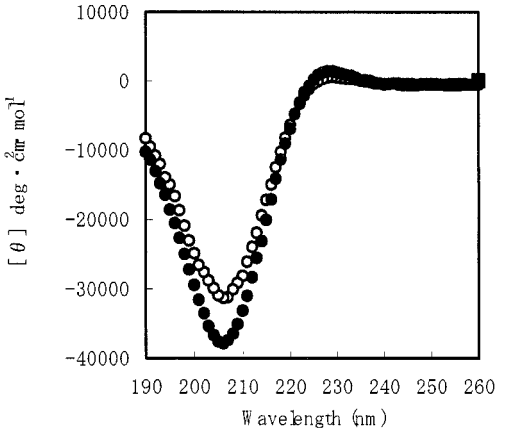


Figure 2. CD spectra of (○) poly(proline) and (●) poly(Hyp(Bzl)-co-Pro) at 20°C.

Results and Discussion

Poly(proline) and poly(Hyp(Bzl)-co-Pro) were obtained by polymerization of proline or proline and Hyp(Bzl)-Pro, respectively, in the presence of DPPA as a coupling reagent [19, 20]. It was presumed that the sequence of the Hyp(Bzl) residue would break down the polyproline-II structure and form another structure. Thus, the dipeptide Hyp(Bzl)-Pro was used in order to avoid the Hyp(Bzl) sequence. Several types of poly(Hyp(Bzl)-co-Pro) (Hyp(Bzl) content = 0.9, 2.5, and 6 mol%) were synthesized by controlling the Hyp(Bzl)-Pro content in the reaction mixture. The averaged molecular weight of these polypeptides was about 2000. The residual Hyp(Bzl) content in poly(Hyp(Bzl)-co-Pro) gradually increased with the concentration of Hyp(Bzl)-Pro in the reaction mixture. This is probably due to the difference in reactivity between proline and Hyp(Bzl)-Pro. Amino-acid analysis would reveal the composition of the copolypeptide, based on which the reactivity of monomers such as Pro and Hyp(Bzl)-Pro can be determined. Figure 1 compares the molar fraction of Hyp(Bzl)-Pro in the monomer and copolymer, calculated based on amino-acid analysis data. The slope in Figure 1 is less than 1, which means that Hyp(Bzl)-Pro has a lower reactivity than Pro. The results of the synthesis of poly(proline) and poly(Hyp(Bzl)-co-Pro) are summarized in Table 1.

Poly(proline) is thermosensitive on having type-II structure [12, 21]. Thus, it is necessary to consider the conformational properties of the polypeptides as a function of the temperature variation of the aqueous solution. The CD spectra of poly(proline) and poly(Hyp(Bzl)-co-Pro) at 20°C in water exhibited a strong negative band around 206 nm and a weak positive band around 229 nm as shown in Figure 2. These spectra corresponded almost exactly to the standard spectrum of polyproline-II structure [15-18].

Previous studies have shown that the type II structure of poly(proline) does not undergo a clear unfolding transition in the temperature range of 5 to 90°C [17, 18]. The CD spectrum of poly(proline) revealed only a slight, insignificant dependence on temperature. For example, the mean residual molar ellipticities of the negative band

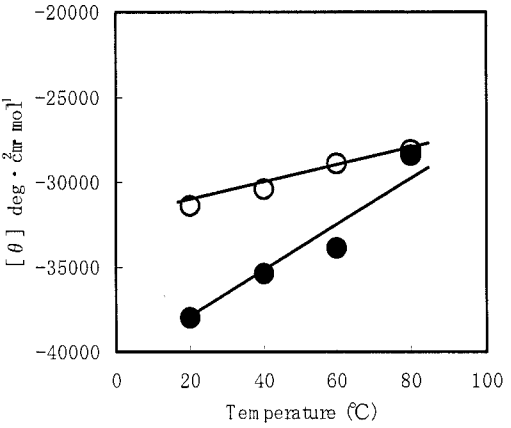


Figure 3. Temperature dependence of the molar ellipticity at 206 nm for (○) poly(proline) and (●) poly(Hyp(Bzl)-co-Pro).

around 206 nm ($[\theta]_{min}$) were, respectively, -31400, -30400, -28900, and -28100 at 20, 40, 60 and 80°C for poly(proline) (Figure 3). Thus, it appears that this polypeptide does not undergo a clear unfolding transition in this temperature range.

The CD spectra of poly(Hyp(Bzl)-co-Pro) also showed a dependence on temperature. However, the dependence was not so significant in the temperature range of 20 to 80°C. That is, for poly(Hyp(Bzl)-co-Pro) containing 6.0 mol% Hyp(Bzl), the values of $[\theta]_{min}$ were -38000, -35400, -33900, and -28400 at 20, 40, 60, and 80°C, respectively (Figure 3). These results indicate that poly(proline) and poly(Hyp(Bzl)-co-Pro) assume a polyproline-II structure in water over the temperature range 20-80°C even though they may deviate from the standard polyproline-II structure due to local distortions.

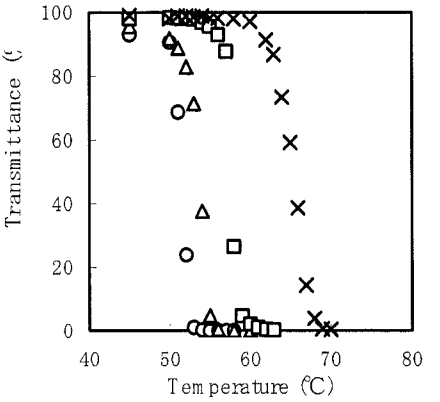


Figure 4. Temperature dependence of the light transmittance of aqueous poly(proline) solutions: (○) 20 mg/ml, (△) 10 mg/ml, (□) 5 mg/ml, and (×) 2.5 mg/ml.

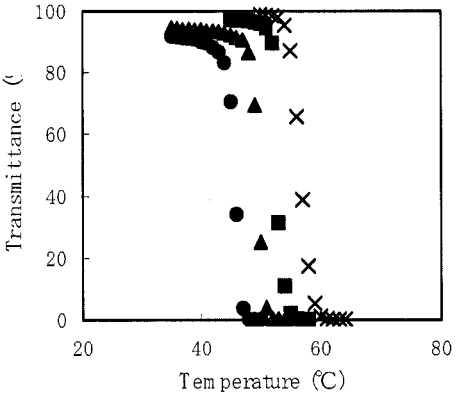


Figure 5. Temperature dependence of the light transmittance of aqueous poly(Hyp(Bzl)-co-Pro) solutions: (●) 20 mg/ml, (▲) 10 mg/ml, (■) 5 mg/ml, and (×) 2.5 mg/ml.

As shown in Figure 4, the light transmittance of the aqueous solution of poly(proline) changed drastically at a specific temperature. The transition temperature decreased with increasing poly(proline) concentration: 65.5, 57.6, 53.7 and 51.4°C at 2.5, 5, 10 and 20 mg/ml, respectively. This agreed with existing data [21-23].

The light transmittance of the aqueous solution of poly(Hyp(Bzl)-co-Pro) also changed drastically at a specific temperature (Figure 5), indicating that poly(Hyp(Bzl)-co-Pro) chains also undergo molecular aggregation at higher temperatures. For poly(Hyp(Bzl)-co-Pro) containing 0.9 mol% Hyp(Bzl), the transition temperature decreased with poly(Hyp(Bzl)-co-Pro) concentration: 59.8, 55.5, 50.2 and 46.7°C at 2.5, 5, 10 and 20 mg/ml, respectively (Figure 6). For poly(Hyp(Bzl)-co-Pro) with 2.5 mol% Hyp(Bzl), the transition temperature also decreased with concentration: 56.6, 52.7, 49.4 and 45.6°C at 2.5, 5, 10 and 20 mg/ml, respectively. For poly(Hyp(Bzl)-co-Pro) with 6.0 mol% Hyp(Bzl), the transition temperature was 55.4°C at 2.5 mg/ml, which was the solubility limit of this polypeptide in water. This was probably due to the strong hydrophobic interaction of the Hyp(Bzl) residue.

The results shown in Figure 3 revealed that these polypeptides assumed the polyproline-II structure from 20 to 80°C, which indicates that they all attained a similar conformation in an aqueous environment. Thus, they only differed with respect to their hydrophobicity. The transition temperature decreased with Hyp(Bzl) content (Figure 7). Hyp(Bzl) residue is more hydrophobic than Pro residue because of the hydrophobic benzyl group. This means that the entropy gain upon the aggregation of poly(Hyp(Bzl)-co-Pro) is higher than that resulting from the aggregation of poly(proline). Then, the transition temperature, i.e., the temperature satisfying $\triangle G=0$, decreased with mol% of Hyp(Bzl). These results correspond to the results reported by Akashi et al. in their study on the LCST of aqueous solution of poly(*N*-vinylalkylamide) [6]. In that study, they showed, for example, that the transition temperature of aqueous solutions of copolymers of *N*-vinylisobutylamide (NVIBA) and *N*-vinylacetoamide (NVA) decrease with mol% of NIVBA, which is a more hydrophobic monomer-unit than NVA [6]. Thermosensitive aqueous polymer solutions undergo either coil-globule transition or coacervation at the transition

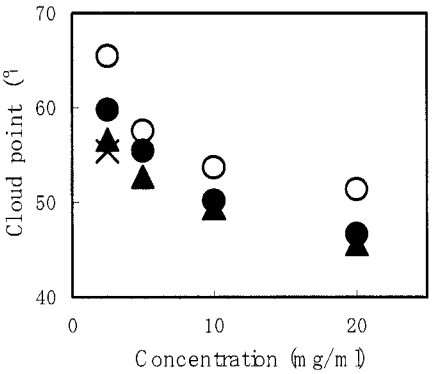


Figure 6. Concentration dependence of the cloud point temperature of aqueous solutions of: (○) poly(proline), (●) poly(Hyp(Bzl)-co-Pro)-0.9, (▲) poly(Hyp(Bzl)-co-Pro)-2.5, and (×) poly(Hyp(Bzl)-co-Pro)-6.0.

temperature. In the case of poly(vinylalkylamide), which attains a random-coil conformation, aggregation involves coil-globule transition. By contrast, the present aggregation process appears to occur through coacervation, as evidenced by the fact that poly(proline) and poly(Hyp(Bzl)-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 [24].

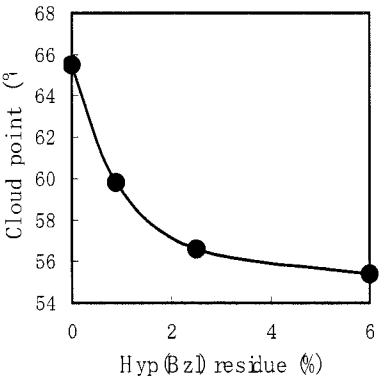


Figure 7. Cloud point temperature as a function of molar percentage of residual Hyp(Bzl) in an aqueous solution of 2.5 mg/ml poly(Hyp(Bzl)-co-Pro).

Conclusions

Poly(Hyp(Bzl)-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 80°C. The polypeptides possessed thermosensitive properties, which showed a dependence on the hydrophobicity of the polypeptides. These results suggested that poly(Hyp(Bzl)-co-Pro)s are very suitable for use in designing thermosensitive materials.

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