Synthesis and Properties of High Molecular Weight Polypeptides Containing of Tryptophan[1)]

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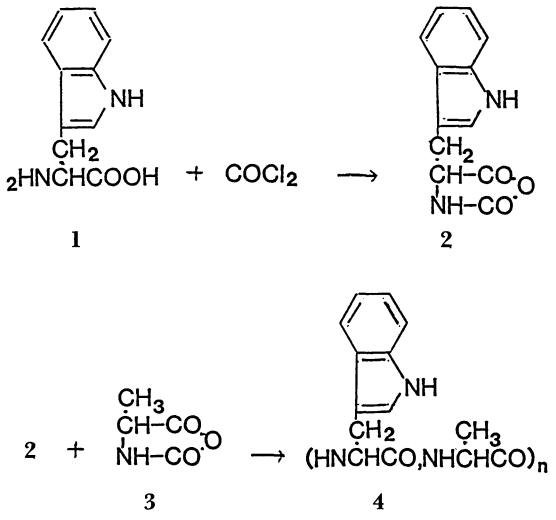
High purity *N*-carboxy-DL-tryptophan anhydride (DL-4-indoylmethyl-2,5-oxazolidinedione, DL-Trp NCA) was synthesized in good yield. Copolypeptides with a random sequence of DL-Trp with L-alanine (Ala) and DL-alanine (DL-Ala) were synthesized by copolymerization of the corresponding *N*-carboxy-α-amino acid anhydrides (NCA) in solution. Copolypeptides of DL-Trp with L-Ala and DL-Ala were partially soluble in water. The water-soluble copolypeptides of DL-Trp and L-Ala mostly gave disordered conformations, while the water-insoluble copolypeptides were found in a mixture of α- and disordered conformations. The solubility of the copolypeptides was not influence by the tryptophan content in the polypeptides but, rather, by the conformation of the polypeptides.

Tryptophan (Trp, **1**) is widely found in native proteins or several peptide hormones such as snake venom neutrotoxin, ACTH, glucagon, and tyrocidin as a minor component of one to two percent. A very small amount of tryptophan residue influences the emergence of their toxicity or higher-order conformations.[2,3)] Tryptophan has a side chain comprising the indole nucleus, which is characteristic of both a hydrophilic aromatic group and a hydrophobic indol nitrogen capable of hydrogen bonding as a donor. Tryptophan has an interesting structual arrangement in water-soluble proteins, such as cobrotoxin.[2,3)]

High molecular weight synthetic copolypeptides containing the tryptophan residue are therefore both interesting and important in the conformational and biological approach to proteins.

E. Kachalski, et al. first reported the synthesis of poly(DL- or L-tryptophan), which are obtained by the polymerization of *N*-carboxy-DL or L-tryptophan anhydride (Trp NCA) at 150 °C in a high vacuum.[4)] Cosani, et al. have reported on a block copolymer (ethyl DL-glutamate)$_m$(L-Trp)$_n$. High-molecular-weight and synthetic random copolypeptides of tryptophan, however, are very little known.[5)]



In this paper we report on the copolymerization of DL-Trp NCA (**2**) with L-Ala NCA (**3**) to produce high molecular-weight copolypeptides (**4**). In addition, the solubility and conformation of the copolypeptide in solvents and in the solid state were studied.

Experimental

Synthesis of NCA (2,3). All NCA were prepared in high purity and good yield by a previously reported method.[6)] DL-Trp NCA was reprecipitated volumetrically by adding hexane to the ethyl acetate solution of NCA, producing high-purity DL-Trp NCA in 46% yield; mp 135—138 °C. Found: C, 61.16; H, 4.56; N, 11.46%. Calcd for $C_{12}H_{10}O_3N_2$: C, 62.60; H, 4.34; N, 12.17%.

Polymerization of NCAs. NCA were copolymerized in 1,2-dichloroethane or ethyl acetate at the concentrations given in Table 1.

Copolypeptides Soluble in Water. The copolypeptide (1g) was placed into water (100 ml) at 20—30 °C, and kept in a refrigerator for 24 h at about 8 °C. An insoluble polymer was separated on a sintered-glass filter and then washed with water and a small amount of ethanol. The residue was dried in vacuo at room temperature. The filtrate was lyophilized under reduced pressure. A water-soluble copolypeptide was obtained.

Viscosity Measurement. The viscosities of the copolypeptides were measured in a solution of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) using ubbelohde viscometer at 25 °C.

Gel Filtration. High-performance liquid chromatography was performed on a Shimadzu LC-6A. A column (1.3×30 cm, void volume 1×10^5) TSK Gel G3000SW was calibrated in Tris HCl (Buffer pH 7.5) using carbonic anhydrase (MW 29000), albumine, bovine serum (MW 66000), and Cytochrome C (MW 12400) as standards of the molecular weight.

Spectroscopic Measurements. Tryptophan components in the copolymers were estimated from the UV absorbance at

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268 nm for solutions of the copolymers in HFIP. Circular dichroism (CD) spectra were recorded on a JASCO J-20A at wavelengths of 300 to 190 nm at 21—23 °C, using a 0.1 mm path length cell, at a concentration of about 0.03 mol/1000 cm³ (residue basis) in HFIP.

The reduced mean residue ellipticity, $[\theta]'$, was defined as

$$[\theta]' = 3 \cdot \theta / C \cdot L \cdot 10 \cdot (n^2+2),$$

where θ is the observed ellipticity in deg, L the optical path length in cm, C the polymer concentration in mol/1000 cm³ (residue basis) and n the refractive index (n_D^{20} 1.275) of the solvent.[7] The infrared spectra were recorded on a Nippon Densi JIR FX 6160 by KBr disks or nujol mull.

Results

DL-Trp NCA was copolymerized with L-Ala NCA and DL-Ala NCA in solution to produce copolypeptides. The polypeptides were obtained by filtration of the polymerization mixture and then dried in vacuo. The polymerization conditions and properties of copolypeptides are shown in Table 1.

Yields of copolypeptides were remarkably decreased by increasing the fed Trp NCA. The composition of the Trp residue in the copolymer formed was normally less than that of the initial monomer ratio. Katchalski reported the polymerization of Trp NCA in the fusion state because of difficulty in the polymerization of Trp NCA in solution.[4] High-purity DL-Trp NCA polymerized at a slower rate than that of Ala NCA in a solution of 1,2-dichloroethane with butylamine as an initiator. In a previous paper we reported that when the polymerization of DL- or L-Ala NCA is initiated with butylamine, the initiation reaction is completed within 60 min, for a monomer conversion of about 10%. The polymerization reaction after this time takes place for 23 h at a conversion of about 70%; it must be proceeded only through the propagation reaction. This suggests that the initiation rate is faster than that of propagation reaction.[8] Most polymerizations of NCAs in acetonitrile were considered regarding this mechanism. The polymerizaton of NCA in 1,2-dichloroethane may proceed by almost the same mechanism as in acetonitrile. The copolymerization of L-Ala NCA with DL-Trp NCA suggests that the propagation reaction of the Trp residue with DL-Trp NCA is slower than the other propagation i.e., a reaction of the Ala residue with Ala NCA and Trp NCA, and a reaction of the Trp residue with Ala NCA.

The water-soluble parts of the obtained copolypeptides could be increased by increasing the amount of Trp NCA fed. In the copolypeptides of DL-Ala and DL-Trp, the water-soluble parts were not proportional to the contained DL-Trp residue. Although all of the copolypeptides formed were soluble in dichloroacetic acid and trifluoroacetic acid, very soon a blue precipitate separated out. Cosani determined the degree of polymerization of the copolymer by the ratio of the initiator to the amount of NCAs fed.[5] The degree of polymerization, however, did not always depend on the ratio of the monomer with the initiator when the polymerization was propagated by activated NCA.[9] The viscosity of the copolypeptides was thus measured in a solution of HFIP. The results are given in Table 1. No relationship has been studied between the viscosity of the HFIP solution and the molecular weight of the polypeptides. The molecular weights of poly(γ-benzyl L-glutamate) (poly(OBzlGlu)) and poly(DL-Ala) are given by the equations (1)[10] and (2),[8] respectively, and $[\eta]$ is given by the equation (3):

$$[\eta] = 2.78 \times 10^{-5} M^{0.87} \quad (1)$$

$$[\eta] = 5.1 \times 10^{-4} M^{0.74} \quad (2)$$

and

$$[\eta] = (\ln \eta_{rel})/c, \quad \eta_{sp} = \eta_{rel} - 1. \quad (3)$$

Here, $[\eta]$ is the intrinsic viscosity, M the molecular

Table 1. Synthesis and Properties of Copolypeptides of L-[a] and DL-Alanine[b] with DL-Tryptophan

Entry No.	NCA (Ala/Trp) Ratio	NCA Concentration mol dm⁻³	Conversion %	Copolypeptides Water soluble part(A) %	Ala/Trp	η_{sp}/c[c]	Water insoluble part(B) %	Ala/Trp	η_{sp}/c[c]
1[a]	95/5	0.12	94	2	97.6/2.4	0.35	98	97.3/2.7	1.12
2[a]	91/9	0.19	92	14	94.2/5.8	0.26	86	94.4/5.6	0.48
3[a]	84/16	0.17	86	20[f]	89.8/10.2	0.16	75	88.1/11.9	0.28
4[a]	81/19	0.17	86	24	90.4/9.6	0.17	76	88.6/11.4	0.19
5[a]	71/29	0.15	67	31	85.5/14.5	0.13	69	71.6/28.4	0.19
6[a]	61/39	0.14	48	37	84.2/15.8	0.12	63	70.6/29.4	0.19
7	0/100	0.21	36	18	0/100	0.58[d]	82	0/100	
8[b]	97/3	0.47	98	33	97.8/2.2	0.78	67	95.4/4.6	
9[b]	84/16	0.38	98	41	96.2/3.8	0.98	59	88.6/11.4	
10[b]	75/25	0.34	36	17	87.5/12.5	0.43[e]	83	75.8/24.2	

a) Polymerization was initiated with butylamine (BA). at an NCA-to-BA molar ratio of 200 at 30 °C for 15 d in 1,2-dichloroethane. b) Polymerization was initiated with BA at an NCA-to-BA molar ratio 100, at 30 °C for 14 d in ethyl acetate. c) c=0.25 g/100 cm³ in HFIP. d) c=0.1 g/100 cm³. e) c=0.25 g/100 cm³ in mixed solvent HFIP/DCE: 4/1. Insoluble part of 7—10b have low solubility to measure the viscosity of solutions. f) Yield after cutting off a low molecular weight by dialysis tube of 3000 dalton.

weight of the polymer, c the concentration of the polymer (g/100 cm^3), η_{rel} the relative viscosity, and η_{sp}/c the specific viscosity of the dichloroacetic acid solution. The specific viscosity of the poly(OBzl-Glu), with a molecular weight of 20000, is 0.04 in a solution of HFIP at a concentration of 0.5 g/100 cm^3 at 30 °C. The specific viscosity of poly(DL-Ala) with a molecular weight of 6000 (determined by the specific viscosity 0.45 in dichloroacetic acid) was 0.4 in HFIP at 25 °C at a concentration of 0.25 g/100 cm^3. T. Ozaki, et al.[11] reported that in the oligomeric peptides derived from OBzlGlu the formation of α-helix begins in the range from the hexapeptides to the octapeptides derived from γ-benzyl L-glutamate. Most oligomeric peptides are capable of forming a secondary structure, such as an α-helix or β-sheet conformation, in a range which is almost the same as the chain length of poly(OBzlGlu). Gel permitation chromatography was carried out in order to evaluate the relation between the specific viscosity and molecular weight of copoly(Trp, Ala). The result (Fig. 1) shows that the specific viscosity of a copolymer (No. 3,(A)) with a molecular weight of 15000—18000, (plotted as a calibration curve for the elution parameter) is 0.16 in HFIP. This suggests that these copoly(Trp, Ala) have a chain length which is capable of constructing a secondary conformation, such as an α-helix or β-structure. Poly(L-Ala) is insoluble in water, and poly(DL-Trp) partly soluble. Entry 1 in Table 1 is for a copolypeptide formed containing 2.2% of a water-soluble copolypeptide which contains 2.4 mol% of the Trp residue. The indol nucleus of the Trp residue exerts a hydrophilic group in the copolypeptides. The insoluble part of the copolypeptides mentioned in the same entry, however, contained 2.7 mol% of the Trp residues. The tryptophan content in the water-soluble copolypeptide was less than that in the water-insoluble copolypeptide in each entry. The viscosity of the water-soluble parts was lower than that of the water-insoluble parts in each entry. This suggests that the solubility of the copolypeptides in water is essentially independent of the rate of the tryptophan residue in the copolypeptides.

The circular dichroism spectra observed for water-insoluble copolypeptides (Table 1) are shown in Figs. 2-1 and -2. The CD spectra of the polypeptides in the helical, β-sheet, and random forms have been studied extensively.[12-17] The ellipticity bands between 185 and 225 nm are associated with the α-helix, β-sheet, and random-coil peptide. The negative dichroisms near 206 and 220 nm have been used for assigning the helix, and the strong negative band near 190 nm for the random-coil of the polypeptides. The insoluble polymer (No. 1—4 in Fig. 2) exhibited a maximum peak at about 190 nm, a minimum at 204 nm, and a shoulder peak at about 220 nm. For all four poly-

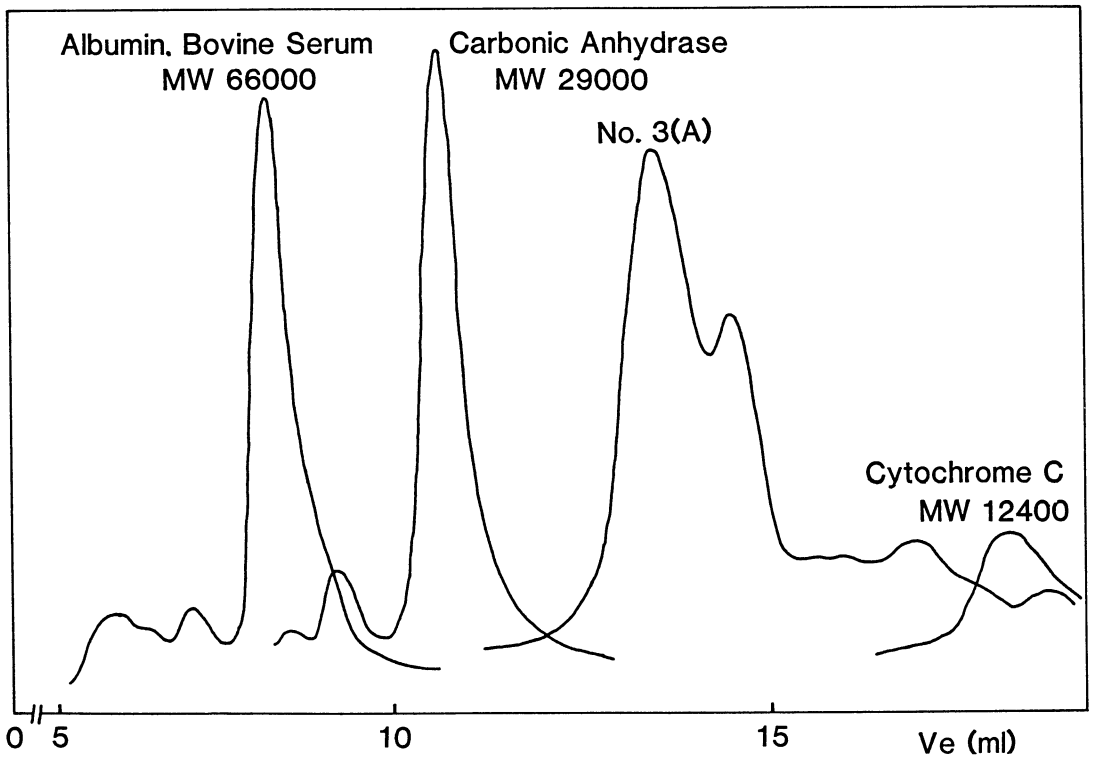

Fig. 1. GPC elution curve of water-soluble copolymer No. 3(A) after dialysis and cutting off MW below 3000) and standard proteins. Column; TSK Gel G3000SW, Developer: 100 mM Tris (pH 7.0), Flow: 0.3 ml min^{-1}, Wave: 280 nm.

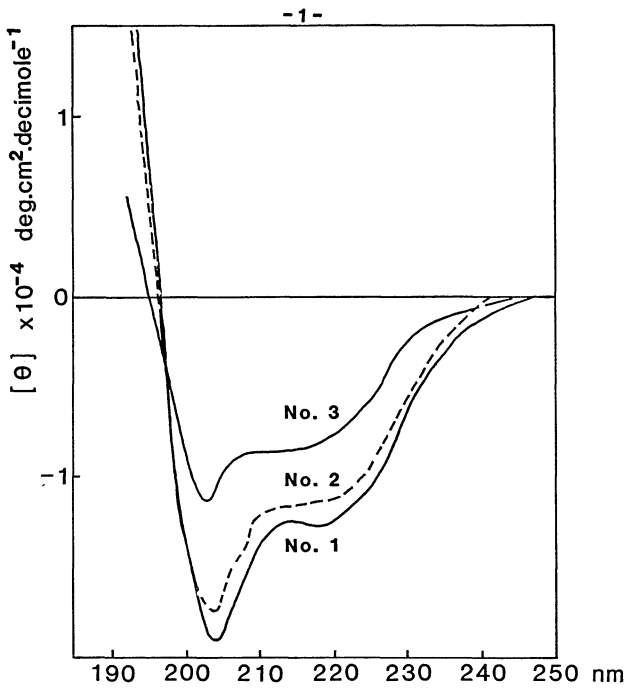

Fig. 2-1. Circular dichroism spectra of water-insoluble copolypeptides(B) shown in Table 1.

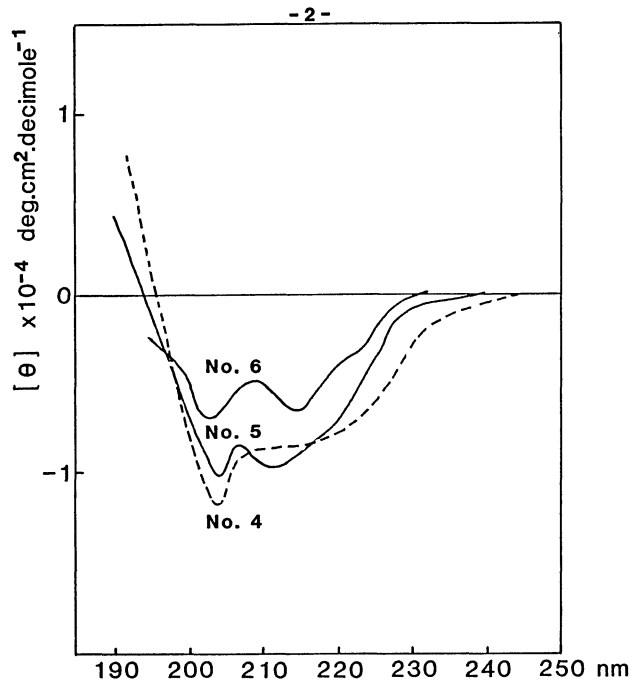

Fig. 2-2. Circular dichroism spectra of water insoluble copolyptides(B) shown in Table 1.

mers given in Fig. 2, the spectra indicate that a right-handed α-helix conformation can be assumed. The intensity of negative peak, which is proportional to the helix content, was decreased by an increase in the tryptophan residue. Polymers No. 5 and 6 given in Fig. 2 have two negative peaks at 212 and 216 nm, respectively, in addition to those of Entries 1—4. These differences in the spectrum patterns could result in an overlapping pattern of the α-helix with β I. The CD spectra of many water-soluble copolypeptides shown in Table 1 (Fig. 3) gave a minimum peak at 197 nm. Furthermore, a minimum peak was observed at

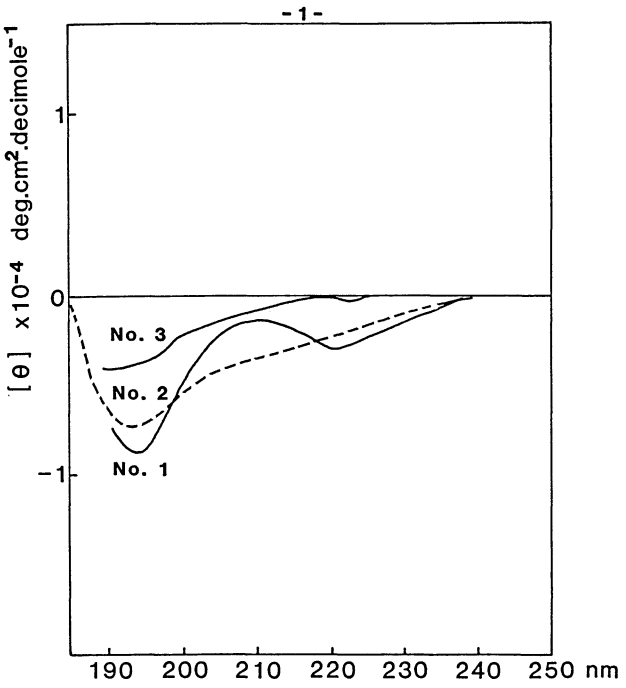

Fig. 3-1. Circular dichroism spectra of water-soluble copolypeptides(A) shown in Table 1.

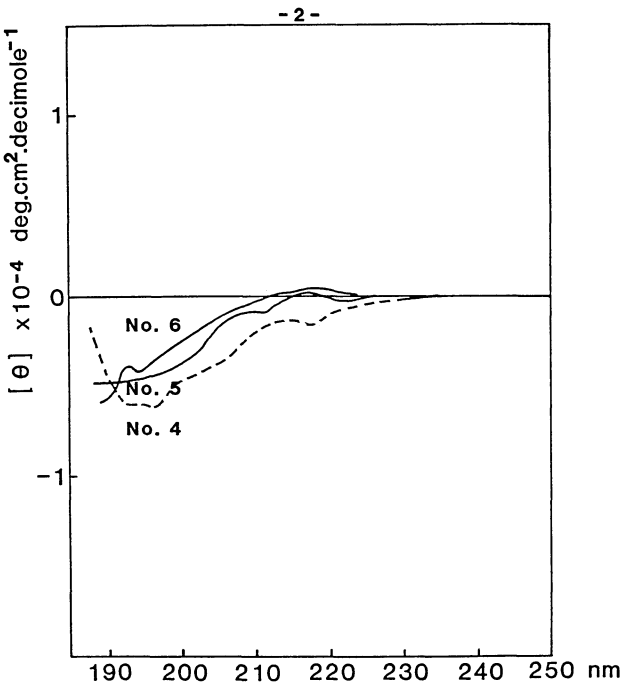

Fig. 3-2. Circular dichroism spectra of water-soluble copolypeptides(A) shown in Table 1.

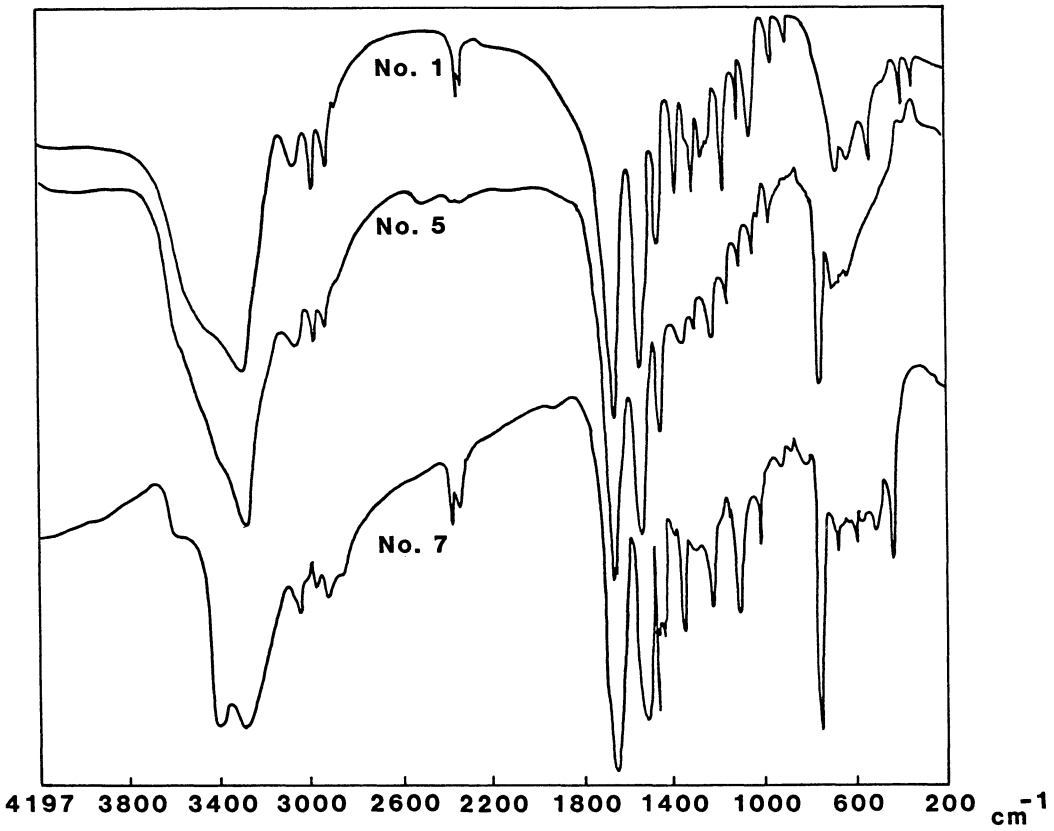

Fig. 4. Infrared spectra of water-insoluble copolypetides(B) shown in Table 1 in KBr disk.

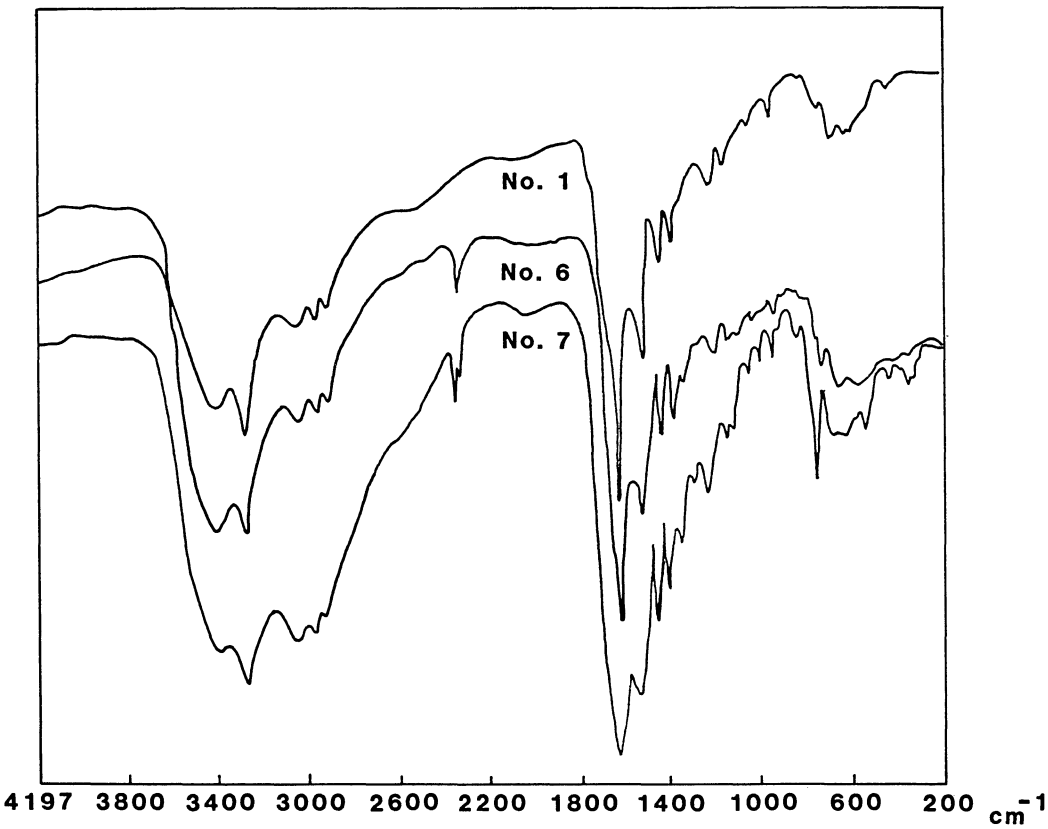

Fig. 5. Infrared spectra of water-soluble copolypeptides(A) shown in Table 1 in KBr disk.

225 nm for Entry 1 and a maximum peak at 222 nm for Entries 5 and 6. Fasman reported a strong negative peak of CD at 197 nm and a weak maximum peak at 217 nm for a poly(L-Lys) aqueous solution to be characteristic of a disordered conformation. Although the shapes were similar, the positive peak of polymer Entries 5 and 6 were higher than that of poly(L-Lys).

The infrared spectra of the copolypeptides listed in Table 1 are shown in Figs. 4 and 5. The insoluble fraction of poly(DL-Trp) gave amide I and II bands at 1654 and 1520 cm^{-1}, respectively. The insoluble fraction of polymer No. 1 gave amide I and II bands at 1656 and 1542 cm^{-1}, respectively, in addition to a band at 1306 cm^{-1}. The frequency value of these bands correspond to those for α-helical poly(L-alanine),[18-20] and the band at 1306 cm^{-1} is considered to be the characteristic of crystalline α-poly(L-Ala).[21] As is evident from the spectra in Entries 2—6 in Table 1, an insoluble polymer with a Trp residue content of 5.6—29.4 mol% gives the amide I band at 1654 and 1634 cm^{-1}, the II band at 1538 and 1305 cm^{-1}, as shown in Entry 5 of Fig. 4. The relative intensity of the 1654 cm^{-1} band was stronger than that of 1634, but almost identity upon increasing the Trp residue. Although the band at 1634 cm^{-1} corresponds to β-polypeptide,[20] the other bands for the β-conformation disappear. This may indicate that only the strongest peak of amide I for the β-conformation was observed, due to the small content of the β-conformational polypeptide. Thus, the infrared spectra coincided with the result of the CD spectra. This would also mean that the conformation of the insoluble polymers in the solid state produces little change in the solution of HFIP. The water-soluble fraction of poly(DL-Trp) No. 7 shows peaks at 1623 and 1540 cm^{-1} due to the β-conformation. The soluble fraction of Entries 1B—6B gave peaks at 1695 and 1628 cm^{-1} (amide I), due to an antiparallel and extended β-conformation, and no peak at about 610 cm^{-1} due to the α-helix conformation. Polymer 1B gave an additional peak at 445 cm^{-1}, which is characteristic of β-polyalanine. As described above, the CD spectra of the soluble polymer indicate a coiled conformation. This may indicate that the conformation of the soluble polymers in the solid state changed from β to a disordered form upon dissolution in HFIP. The infrared spectra of water-soluble copolypeptides No. 8—10 gave an amide I band at 1715—25, 1655 and amide II at 1540 cm^{-1}, characteristic of an α-helix-like conformation of (Ala-Ala-Ala-D-Ala)$_n$.[22] These bands were different from that of the water-soluble parts of Entries 1—6. For water-insoluble parts No. 8—10, bands at 1652 and 1530 cm^{-1} were observed, which are characteristic of a parallel β-conformation. These conformations were different from that of the water-insoluble parts of Entries 1—6.

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