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^{13}C CP/MAS NMR Study of the Conformation of Stretched or Heated Low-Sulfur Keratin Protein Films

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ABSTRACT: ^{13}C CP/MAS NMR spectra of the low-sulfur *S*-(carboxymethyl)kerateine film extracted from wool (SCMKA) were measured under the unstretched, stretched, and heated conditions, where the film was prepared by casting from aqueous solution. From the observed ^{13}C chemical shifts, intensities, and half-widths of the main-chain carbonyl-carbon peaks, it was concluded that β-sheet and random-coil forms appear upon stretching and heating, respectively, and the α-helix form decreases. ^{13}C dipolar-dephasing times (T_{DD}) of the carbonyl carbons in SCMKA films were determined from dipolar-dephasing experiments in order to explore the molecular motions. From these results, it was found that the mobility of the main chains of SCMKA does not change upon stretching or heating.

Introduction

Since fibrous proteins generally have period amino acid sequences and higher order structure, clarification of their fine structure becomes very important, not only when discussing their physical and chemical properties but also when obtaining the information about the molecular design of synthetic polypeptides. Wool keratin consists of several kinds of proteins and can be separated into three main fractions after reducing the disulfide bonds and protecting the thiol groups with iodoacetic acid to form *S*-(carboxymethyl)kerateine (SCMK).[1-3] The low-sulfur protein of SCMK (SCMKA) is one of the intermediate filaments and is considered to originate chiefly from the microfibrils of wool.[4] The α-helix content of SCMKA in an aqueous solution was determined to be about 50% by means of ORD[5] and CD.[6] In addition, the sequence of seven amino acid residues, (a–b–c–d–e–f–g)$_n$, in which the hydrophobic and hydrophilic residues are located at a and d positions and other positions, respectively, has been determined in SCMKA as well as tropomyosin, myosin, and paramyosin, which assume a coiled-coil α-helix rope.[4,7]

Recently, the structural changes in SCMKA films under stretching and heating have been investigated by means of X-ray diffraction[8] and FT-IR spectroscopy.[9] From these studies, it is found that the β-sheet form in SCMKA films appears upon stretching and the α-helix form disappears upon heating.

Carbon-13 cross polarization/magic angle spinning (CP/MAS) NMR is a very powerful tool for conformational characterization of polypeptides and proteins in the solid state because the ^{13}C NMR chemical shifts depend on their main-chain conformations such as α-helix, ω-helix, 3_1-helix, 3_{10}-helix, and β-sheet forms, as reported in experimental and theoretical studies.[10-28] Furthermore, the side-chain conformations of the L-phenylalanine and L-tyrosine residues of oligopeptides or homopolypeptides in the solid state have been determined,[29] and quantitative determination of the conformations and the dynamic analyses of silk fibroin proteins[30,31] have been performed by means of ^{13}C CP/MAS NMR spectroscopy. In our previous study,[32] the major conformation and its content of four kinds of SCMK and native wool fiber have been successfully determined, and the existence of the coiled-coil structure in SCMKA, α-helix-rich fragment of SCMKA, and native wool fiber has been confirmed by means of ^{13}C CP/MAS NMR spectroscopy.

In this work, we aim to measure ^{13}C CP/MAS NMR spectra of stretched or heated SCMKA films in the solid state and to clarify their conformational changes by stretching and heating. Moreover, the molecular motion of stretched or heated SCMKA films is discussed by the dipolar-dephasing (DDph) ^{13}C NMR experiment.

Experimental Section

Materials. S-Carboxymethylated kerateine (SCMK) was prepared from Merino 64's wool according to the procedure of O'Donnel and Thompson[2] and was fractionated into three fractions: low-sulfur proteins (SCMKA), high-sulfur proteins, and high-glycine–tyrosine proteins by isoelectric precipitation according to the procedure of Dowling and Crewther.[3] The purification of SCMKA was performed by dialysis of its aqueous sodium tetraborate solution against deionized water. The amino acid composition of SCMKA determined by a Hitachi L-8500 amino acid analyzer after hydrolysis with 6 N HCl at 110 °C for 20 h is listed in Table I. The molecular weights of SCMKA obtained here were estimated to be 66–46K by the SDS-PAGE method (the SDS-PAGE pattern showed five clear bands, but further fractionation was not performed in this work). The amino acid composition and the molecular weight of SCMKA obtained here agree with the results of previous studies.[2-4,33]

The SCMKA film was prepared by dropping a 2 wt % aqueous solution of SCMKA on a Teflon plate and air-drying at room temperature. The thickness of the film was ca. 100 μm. It was uniaxially stretched to the desirable ratio by a hand-stretching apparatus in ethanol/water (7:3, v/v) and air-dried. The heated film was prepared by heating at 200 °C for 3 h under nitrogen atmosphere. The samples for ^{13}C CP/MAS NMR measurement were cut into small pieces with scissors and then stacked in a desiccator.

The sodium salt of poly(L-aspartic acid), which was purchased from Sigma, Ltd., is used in order to obtain reference data with respect to the ^{13}C dipolar-dephasing time.

Measurements. ^{13}C CP/MAS NMR spectra were recorded on a JNM GSX-270 NMR spectrometer operating at 67.8 MHz with a CP/MAS accessory. Samples (ca. 100–200 mg) were contained in a cylindrical rotor made of zirconia and spun at a speed of up to 4–4.5 kHz. Contact time was 2 ms, and repetition time was 5 s. Spectral width and data points were 27 kHz and 8K, respectively. Spectra were usually accumulated 400–2000 times to achieve a reasonable signal-to-noise ratio. The ^{13}C NMR chemical shifts were calibrated indirectly through external adamantane (29.5 ppm relative to tetramethylsilane). Spinning sidebands appearing due to insufficient spinning rate were removed by the total suppression of the sidebands (TOSS) method[34] except for the DDph experiment.[35] ^{13}C dipolar-dephasing time (T_{DD}) was determined by measuring the ^{13}C CP/MAS NMR spectra, varying the delay time, τ, for the proton dipolar

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Table I
Amino Acid Composition of SCMKA

amino acid	residues/1000 residues	amino acid	residues/1000 residues
Asx[a]	85	Met	7
Thr	52	Ile	36
Ser	93	Leu	96
Glx[b]	148	Tyr	38
Pro	39	Phe	29
Gly	80	Lys	32
Ala	63	His	8
Val	61	Arg	73
CM-Cys[c]	60		

[a] Asx means Asp and Asn. [b] Glx means Glu and Gln. [c] CM-Cys means (carboxymethyl)cysteine.

decoupling and data acquisition in the range of 17–130 μs. T_{DD} is given by

$$I(\tau) = I_0 \exp(-\tau/T_{DD})$$

where I_0 is signal intensity at $\tau = 0$. Since T_{DD} corresponds to the ^{13}C spin–spin relaxation time, it is possible to discuss the molecular motion using T_{DD}. The advantage of the ^{13}C DDph experiment is that the accumulation time is very short to achieve a reasonable signal-to-noise ratio compared with that for obtaining ^{13}C spin–lattice relaxation time, $T_1{}^C$, because T_{DD} is 10–100 μs but $T_1{}^C$ is 0.1–1000 s in the solid state.

Results and Discussion

Assignment of ^{13}C Peaks. ^{13}C CP/MAS TOSS NMR spectra of unstretched, stretched, and heated SCMKA films in the solid state are shown in Figure 1. The peaks of the carbonyl, aromatic, C_α methine, and side-chain aliphatic carbons appear at about 172–175, 115–158, 45–60, and 10–40 ppm, respectively. The observed ^{13}C chemical shift values in SCMKA films are summarized in Table II together with those of SCMKA lyophilized product, which has been reported previously.[32] The assignments can be made in a straightforward manner by using reference data with respect to homopolypeptides in the solid state.[10–12,15,21,23,26–28] Although the observed ^{13}C chemical shift values for the carbon groups in all the samples are nearly the same, the expanded carbonyl signals are significantly different from each other as shown in Figure 2.

Effect of Stretching. The expanded ^{13}C CP/MAS TOSS spectra of SCMKA films in their carbonyl regions are shown in Figure 2. It can be seen that the carbonyl ^{13}C signal consists of two major peaks and two minor peaks. It can be concluded that one major peak at about 176 ppm comes from the main-chain carbonyl carbon in the right-handed α-helix (α_R-helix) form, and another major peak as a shoulder at about 172 ppm comes from those in the β-sheet form, because the ^{13}C chemical shift value of the main-chain carbonyl carbon in polypeptides in the solid state is strongly influenced by the conformation of the main chain but not by the type of amino acid and/or a specific amino acid sequence, as demonstrated already.[10–28] The ^{13}C peaks of the main-chain carbonyl carbons in the α_R-helix and β-sheet forms appear at 175.8 ± 0.8 and 170.9 ± 1.2 ppm, respectively.[15,23] It is also concluded that one minor peak at about 180 ppm probably comes from the side-chain carbonyl carbons of L-aspartic acid C_γ, L-glutamic acid C_δ, and carboxymethyl-L-cysteine C_ϵ, and another minor peak at about 166 ppm comes from the rotor used (the cap of the rotor is made of polyimide[36]). The four peaks are decomposed by computer-fitting, and their ^{13}C chemical shift values are found to be at about 176, 172, 180, and 166 ppm, respectively. As an example, the observed and deconvoluted peaks of the carbonyl carbons of the unstretched SCMKA film are shown in Figure 3. The ^{13}C chemical shift values for the four deconvoluted peaks were determined to be 166.1, 172.5, 176.4, and 180.5 ppm, respectively; half-widths were 2.8, 4.5, 4.0, and 3.6 ppm, respectively; relative intensities were 6.2%, 36.1%, 50.2%, and 7.5%, respectively. Using these results, the relative intensity of the peak at 180.3 ppm was calculated to be 8% (=7.5/(36.1 + 50.2 + 7.5) × 100). Since it is assumed that the main-chain carbonyl-carbon ^{13}C signal mainly consists of two peaks, the relative intensities of the two peaks at 172.5 and 176.4 ppm in the unstretched film were calculated to be 58% (=50.2/(50.2 + 36.1) × 100) and 42% (=100 − 58), respectively. Table III summarizes the ^{13}C chemical shifts, half-widths, and relative intensities of the carbonyl carbons in SCMKA films as determined by computer-fitting. The relative intensities of the unstretched film agree with those of the lyophilized product of SCMKA, as reported previously.[32] This finding indicates that the conformation of SCMKA film cast from aqueous solution is nearly the same as that of the lyophilized product. The relative intensity of the downfield peak at about 176 ppm corresponds to the α-helix content because the peak comes from the main-chain carbonyl carbons in the α_R-helix form as mentioned above. The α-helix content decreases as the stretching ratio is increased. On the other hand, the relative intensity of the upfield peak at 172 ppm, which comes mainly from the main-chain carbonyl carbons in the β-sheet form, increases as the stretching ratio is increased.

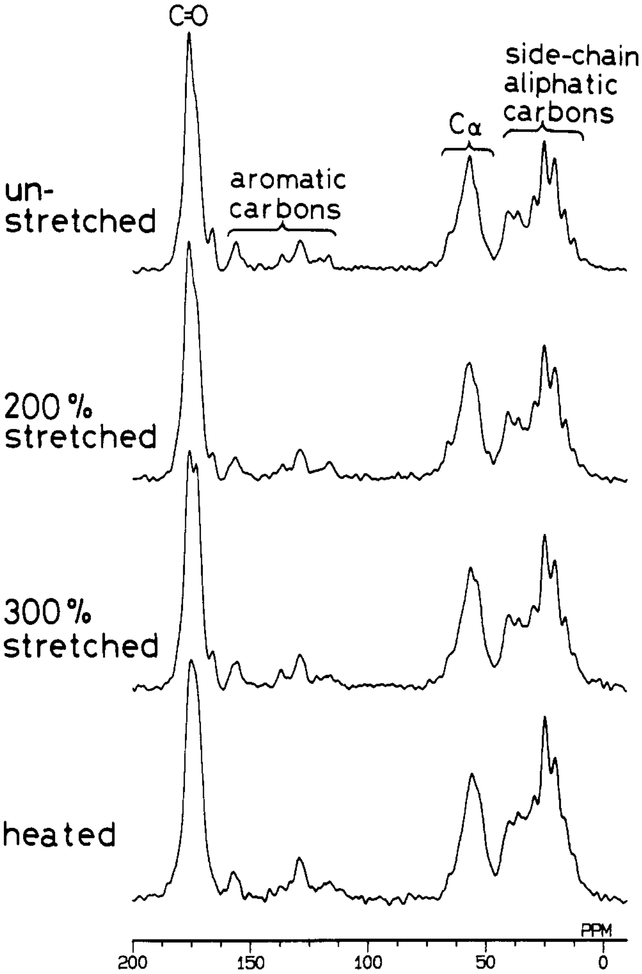

Figure 1. ^{13}C CP/MAS TOSS NMR spectra of SCMKA films cast from aqueous solution.

The ^{13}C CP/MAS DDph NMR spectra of the SCMKA films were measured, and the aliphatic region is shown in

Table II
Observed ^{13}C NMR Chemical Shifts for SCMKA Samples in the Solid State

chemical shifts,[a] ppm					
lyophilized product[b]	unstretched film	200% stretched film	300% stretched film	heated film	assignment[c]
176.0	176.2	176.1	175.9	175.1	carbonyl carbon (αh)
d	*d*	*d*	173.1	*d*	carbonyl carbon (βs)
155.1	155.9	156.4	155.5	154.3	Tyr C_ζ and Arg C_z
e	136.4	136.2	137.0	*e*	Phe C_γ
129.1	128.8	128.9	129.1	129.3	Phe $C_{\delta,\epsilon,\zeta}$ and Tyr C_γ
e	116.3	116.2	*e*	*e*	Tyr C_e
d	*d*	65.8	*d*	65.2	Val C_α and Ile C_α (αh)
56.9	56.7	56.9	56.3	55.7	C_α methine carbons
40.3	40.3	40.4	40.2	39.9	mainly Gly C_α
35.6	36.2	36.0	36.0	36.1	*f*
		33.3	33.2	33.4	*f*
28.8	29.3	29.1	29.6	29.2	*f*
25.0	25.0	24.9	24.9	24.7	*f*
(*d*)	(*d*)	(23.5)	(23.8)	(23.8)	Leu $C_{\delta1}$
20.7 (20.9)	20.6 (21.0)	20.6 (21.1)	20.6 (20.9)	20.5 (20.4)	Thr C_γ, Val C_γ and Leu $C_{\delta2}$
16.2 (16.5)	16.3 (16.5)	16.0 (16.2)	16.2 (16.4)	16.7 (*d*)	Ala C_β (αh) and Ile $C_{\gamma'}$
12.1 (12.2)	12.3 (12.1)	12.6 (12.7)	12.4 (12.5)	12.6 (12.2)	Ile C_δ

[a] The numbers in parentheses are chemical shifts of the peaks observed in ^{13}C CP/MAS DDph spectra (τ is about 60 μs). [b] From ref 32. [c] The assignment was made by reference data of homopolypeptides in the solid state (refs 15, 23, and 32). αh and βs in parentheses mean the α_R-helix and β-sheet forms, respectively. [d] Observed as the shoulder peak. [e] Observed as the weak and broad peak. [f] The methine and/or methylene carbon peaks of the side chains but unassigned to individual amino acid residue at this stage.

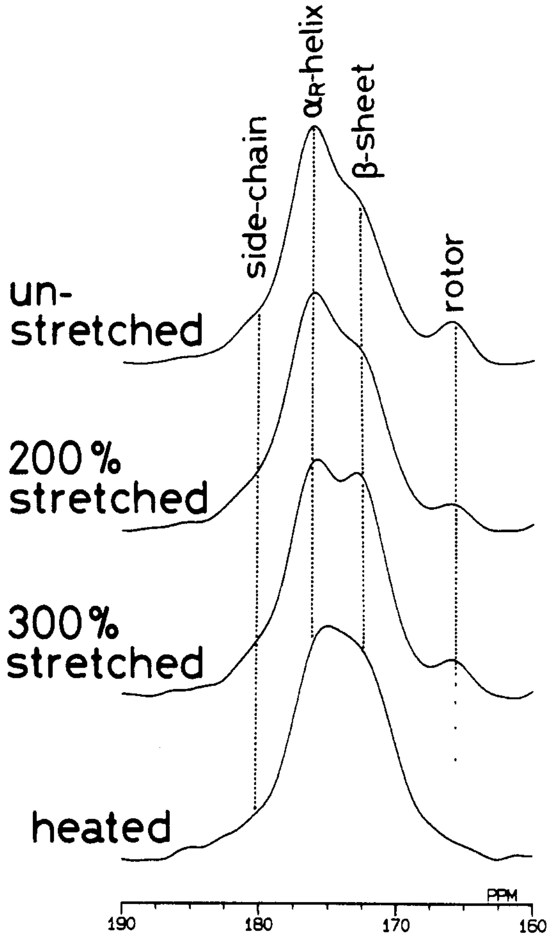

Figure 2. Expanded ^{13}C CP/MAS TOSS NMR spectra for the carbonyl-carbon region in SCMKA films cast from aqueous solution.

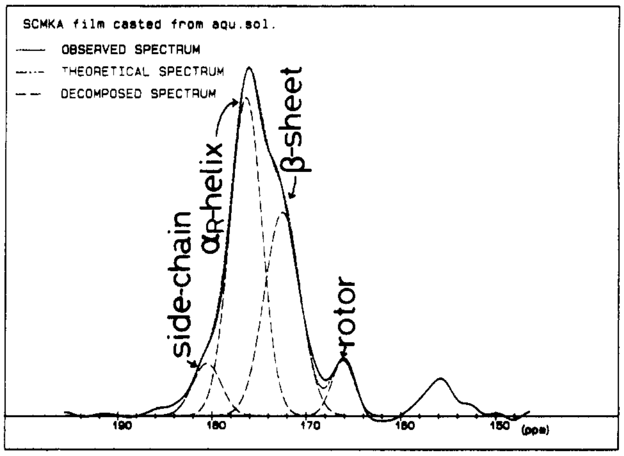

Figure 3. ^{13}C NMR spectra for the carbonyl-carbon region in the unstretched SCMKA film, deconvoluted by the computer fitting with Gaussian functions.

Figure 4, where τ is about 60 μs. Only the peaks of the methyl carbons clearly appear, at about 11–23 ppm, because the T_{DD} values of the methyl carbons, which undergo rapid rotation about the C_{3v} axis, are longer than those of the methine and methylene carbons. Especially, the peaks of the L-leucine $C_{\delta1}$ methyl carbons clearly appear at about 23 ppm in the ^{13}C CP/MAS DDph NMR spectra only (as compared with Figure 1). The observed ^{13}C chemical shifts and their assignments are summarized in Table II. In our previous study,[39] two peaks at 15.8 and 16.7 ppm in ^{13}C CP/MAS spectrum of tropomyosin in the solid state can be assigned to the L-alanine C_β carbons in the external and internal sites of the coiled-coil structure, respectively, from the difference in the mobility of these carbons and the reference data of poly(L-alanine). The peaks at 16.2–16.4 ppm in SCMKA films are contributed by the L-alanine C_β (in the α_R-helix form) and L-isoleucine $C_{\gamma'}$ carbons whose relative ratio is ca. 3:2. These peaks appear downfield from the C_β carbon peak of poly(L-alanine) in the α_R-helix form (15.7 ppm[40]) as well as the lyophilized product of SCMKA. This indicates that the coiled-coil structure exists in SCMKA films and is maintained in spite of the appearance of the β-sheet form by stretching.

Effect of Heating. The ^{13}C CP/MAS NMR spectra of a heated SCMKA film are shown in Figure 1, and the expanded carbonyl and aliphatic regions are shown in Figures 2 and 4, respectively. The ^{13}C CP/MAS NMR spectra of the heated SCMKA film show that each peak is broader than that of the other films without heat treatment. In particular, the carbonyl and methyl peaks are especially broad as shown in Figures 2 and 4. From the deconvoluted results of the ^{13}C carbonyl signal of the

Table III
Observed ^{13}C NMR Chemical Shifts, Half-Widths, Relative Intensities, and ^{13}C Dipolar-Dephasing Times, T_{DD}, of the Carbonyl Carbons in SCMKA Films[a]

	^{13}C chemical shift, ppm	half-width, ppm	relative intensity, %	T_{DD}, μs
Main-Chain Carbonyl-Carbon Peaks at Upfield				
unstretched	172.5	4.5	42[b]	77
200% stretched	172.5	4.5	46[b]	70
300% stretched	172.4	4.2	53[b]	72
heated	172.1	5.1	53[b]	65
Main-Chain Carbonyl-Carbon Peaks at Downfield				
unstretched	176.4	4.0	58[b]	73
200% stretched	176.4	4.0	54[b]	73
300% stretched	176.2	3.7	47[b]	86
heated	175.8	4.4	47[b]	80
Side-Chain Carbonyl-Carbon Peaks				
unstretched	180.5	3.6	8[c]	76
200% stretched	180.5	3.6	9[c]	166
300% stretched	180.1	3.5	9[c]	161
heated	180.1	5.0	10[c]	143

[a] Determined by computer fitting. [b] For only main-chain carbonyl carbons (see text). [c] For all carbonyl carbons in the sample (see text).

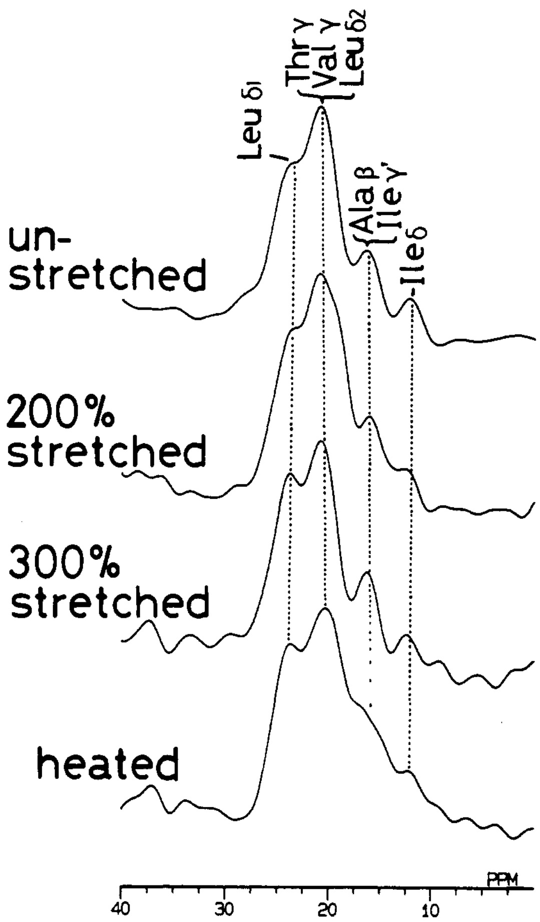

Figure 4. Expanded ^{13}C CP/MAS DDph NMR spectra for the aliphatic carbons region in SCMKA films where τ is about 60 μs.

heated film (Table III), it is seen that the half-widths are greater than those of other films, although the ^{13}C chemical shifts are hardly changed. This shows the existence of various conformations and/or different microenvironments in the heated film. From this, it can be said that the random-coil form appears by heating at 200 °C for 3 h. Sakabe et al. have reported that the X-ray diffraction of the α-helix form completely vanishes in the SCMKA film heated at 200 °C for 3 h in vacuo.[8] The results obtained here, however, indicate that the α_R-helix form remains to an appreciable extent in the heated film, although the random-coil form appears on heating. In our previous study,[32] the relative intensities in the main-chain downfield carbonyl-carbon peaks in the other two SCMK samples, i.e. the high-sulfur and high-glycine–tyrosine proteins, were determined to be 25% and 8%, respectively (their X-ray diffraction patterns show only the existence of β-sheet form[41,42]). The same result was obtained on the nonhelical fragment of SCMKA after partial hydrolysis with α-chymotrypsin[43] (it has been reported that the X-ray diffraction pattern of this fragment shows no reflections[44]). These results indicate that the relative intensity of the main-chain downfield carbonyl-carbon peak is small in those proteins in which the existence of the α-helix form is not identified by means of X-ray diffraction. Since the ^{13}C chemical shift value of the main-chain carbonyl carbons is strongly influenced by the local conformation mentioned above, it may be suggested that the α_R-helix form remains locally in the heated film as shown from the ^{13}C NMR data. The difference between the X-ray diffraction and NMR spectroscopy suggests that only the packing of the ordered structure (the α_R-helix form) in the SCMKA film is disrupted by heating, secondary structure being retained.

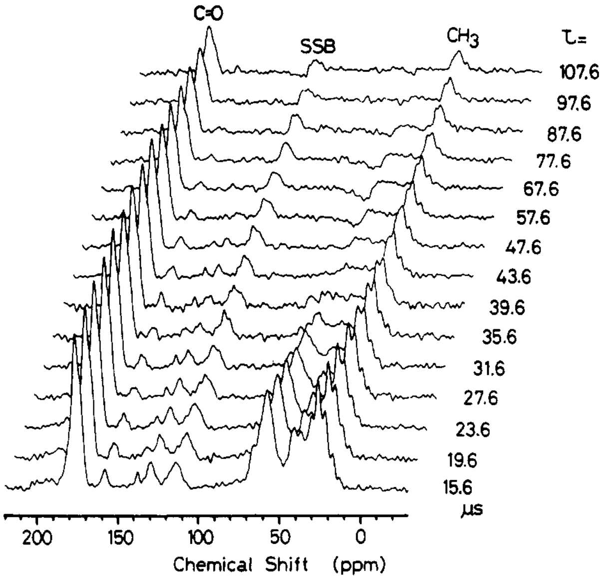

Figure 5. ^{13}C CP/MAS DDph NMR spectra of the unstretched film at various τ.

Molecular Motion. Figure 5 shows the ^{13}C CP/MAS DDph NMR spectra of the unstretched film at various values of τ. The T_{DD} values of the carbonyl carbons were determined from the deconvoluted relative intensities of each peak in the range of τ = 17–130 μs. The plots of the peak intensity against τ show no multiple components, and thus all the T_{DD} values were obtained as one component. The T_{DD} values of SCMKA films are summarized in Table III. The T_{DD} values of the main-chain carbonyl carbons in SCMKA films were nearly equal compared with that of the sodium salt of poly(L-aspartic acid) (56 μs). Recently, Pivcová et al. have reported from spin-locking CP/MAS experiments that the mobility of poly(L-aspartic acid) is much less than that of poly(alkyl methacrylate)s.[45] Therefore, it can be said that the mobility of the main-chain carbons in all samples is limited at room temperature. This supports the quantitative analysis for the α-helix form done from the ^{13}C carbonyl signals. Because a comparison of the intensities of ^{13}C CP/MAS NMR signal is not appropriate, the mobilities of the carbons were substantially different from each other.

On the other hand, the T_{DD} value of the side-chain carbonyl carbons in the stretched and heated SCMKA films is longer than that of the main-chain carbonyl carbon. This indicates that the mobility of the side chains becomes higher by stretching or heating. Therefore, it was suggested that the packing of the ordered structure in SCMKA film is released by stretching or heating.

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