Biosci. Biotechnol. Biochem., **68** (9), 1875–1881, 2004

Thermal Behavior of Fowl Feather Keratin

Koji TAKAHASHI,[1,†] Hirosaburo YAMAMOTO,[1] Yoshiko YOKOTE,[2] and Makoto HATTORI[1]

[1]*Department of Applied Biological Science, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan*
[2]*Department of Chemistry, Faculty of Science, Josai University, Saitama 350-0248, Japan*

Received March 19, 2004; Accepted June 22, 2004

Differential scanning calorimetry (DSC) was applied to elucidate the thermal behavior of fowl feather keratins (barbs, rachis, and calamus) with different morphological features. The DSC curves exhibited a clear and relatively large endothermic peak at about 110–160 °C in the wet condition. A considerable decrease in transition temperature with urea and its helical structure content estimated by Fourier transform infrared spectroscopy (FT-IR), and the disappearance of one of the diffraction peaks with heating at 160 °C for 30 min, indicated that DSC could be used to evaluate the thermal behavior of keratin. Barbs showed a lower denaturation temperature than rachis and calamus. The pulverized samples showed a slightly higher denaturation temperature than the native samples. In the dry condition, thermal transition occurred in a markedly higher temperature region close to 170–200 °C. It is hence concluded that fowl feather keratins have very high thermal stability, and that the elimination of water brings about even greater thermal stability.

Key words: keratin; thermal stability; differential scanning calorimetry (DSC); Fourier transform infrared spectroscopy (FT-IR); X-ray diffraction

Feather proteins are highly insoluble in normal protein solvents and indigestible by proteases due to the large degree of crosslinking by cystine residues and hydrogen bonds, as Akahane *et al.* reported that the amino acid composition of feather keratins showed high contents of serine, proline, glycine, and cystine.[1)] The primary structures of feather keratins have been studied by using the main component obtained from those samples by S-carboxymethylation and enzymatic digestion. A comparison of the sequences of the main component from the five species, fowl, duck, pigeon, kite, and pheasant, has shown that sequences of 80–97 amino acid residues long were common to all species with 82% similarity.[2)] Attempted prediction of the secondary structure of the sequence from fowl keratin by the methods of Lewis *et al.*[3)] and Chou and Fasman[4,5)] indicated that the β-sheet content of this region was about 30%, and that such other structures as turn and coil covered the remaining parts.[2)] Tsuboi *et al.* have reported from the results of infrared and Raman microscopy that the anti-parallel pleated sheet and unordered structural contents were respectively about 50%,[6)] although the details of the structures have not yet been elucidated. Two types of physical models for feather keratin have been proposed to accommodate most of the pertinent X-ray diffraction and electron microscopic data. One is a netlike array of ellipsoidal particles,[7–9)] and the other is an arrangement of helical structures.[10)] As an extension of the latter, Fraser *et al.* have proposed a twisted-sheet model for the fibrous structure of feather keratins from X ray diffraction data, which consists of units of a pleated sheet containing four chains and forming a helical structure.[11)] Filshi and Rogers have reported on the basis of electron microscopic examination that feather calamus contained microfibrils approximately 30 Å in diameter embedded in an amorphous matrix.[12)] But, the true chain configuration based on the sequences and its arrangement within the fibrous structure of fowl keratin still remain to be elucidated.

These peculiar and stable structures of feather keratin suggest that its thermal transition probably occurs at a considerably higher temperature than that of the other proteins, if indeed it occurs at all, but no research has been reported so far on the thermal behavior of feather keratin. In this study, therefore, differential scanning calorimetry (DSC) was applied to elucidate the thermal behavior of fowl feather barbs, rachis, and calamus with different morphological features, and to investigate the strong effect of water on this thermal behavior.

Materials and Methods

Sample. Barbs, rachis, and calamus (Fig. 1) obtained from the feathers of female White Leghorn chickens by

† To whom correspondence should be addressed. Fax: +81-42-360-8830; E-mail: k-taka@cc.tuat.ac.jp
Abbreviations: DSC, differential scanning calorimetry; FT-IR, Fourier transform infrared spectroscopy

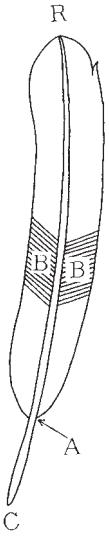

Fig. 1. Schematic Representation of Fowl Feather.
B, barbs; R-A, rachis; A-C, calamus.

cutting with scissors were thoroughly defatted with ethanol and ether. The defatted samples were immersed again in ethanol, which was progressively replaced with water by a stepwise-decrease in ethanol concentrations of 80%, 60%, 40%, and 20% to obtain wet samples. These wet samples were lyophilized to obtain dry samples. Pulverized wet samples were prepared by pulverizing the wet samples 10 times with a Polytron (Kinematica, Luzern, Switzerland) in an ice bath at 24,000 rpm for 30 sec, with a 60-sec interval between each pulverization, and the filtering (no. 101 filter paper, Advantech, Tokyo, Japan). This study used wet samples, dry samples, and pulverized wet samples.

Differential scanning calorimetry (DSC). DSC of the wet samples, urea-treated samples, pulverized wet samples, and dry samples was performed with a SSC-5020 DSC 100 apparatus (Seiko, Chiba, Japan) as described by Takahashi *et al.*,[13] except that a heating rate of 5 K/min was used. A silver sample capsule (15 μl) was pretreated at 250 °C for 20 min to prevent any exothermal effect from the capsule at around 170 °C. A sample (about 3 mg dry basis) was loaded into the capsule, and pressed with a silver disc (0.2 mm thick) to bring the sample into full contact with the bottom of the capsule before sealing it. Water and an empty capsule were used for the wet samples and dry samples respectively as references. After the DSC run, the lid of the capsule was removed with a clean nipper. The opened capsule and contents were dried at 110 °C for 8 h, and then incinerated at 550 °C for 8 h. The sample weight in the capsule was determined from the reduced weight after incineration,.

X-ray diffractometry. X-ray diffractometry of the rachis sample was carried out by an X-ray diffractometer (Rint Rapid, Rigaku Co., Tokyo, Japan) with a copper target at 40 kV and 36 mA producing CuKa of 1.54 Å, collimator of 10 μm dia., and a goniometer oscillating 0°–30° about the w axis and −20°–20° about the ø axis. Data were recorded with an imaging plate system and collected for values of 2θ between 7° and 60°. The excess water on the surface of the wet rachis and heated rachis (at 160 °C for 30 min) was wiped off with filter paper, and X-ray diffractometry was completed within 10 min after quickly mounting one piece of the rachis sample on the sample stage.

Fourier transform infrared spectroscopy (FT-IR). Rachis was treated by immersion in 2, 4, 6, or 8 M urea at 40 °C for 4 h, and then carefully sliced with a surgical knife. A slice (about 0.5 μg) was quickly mounted on a diamond window (3 mm in dia.) for permeation measurement. The spectrum was quickly recorded by a Jeol JIR-5500 FT-IR spectrometer equipped with a micro-facility and MCT detector. Background measurements were taken using a small amount of each urea solution at the same concentration as that used for treating the sample. For each spectrum, a 100-scan interferogram was collected at 4 cm^{-1} resolution, and apodised with a triangular function to give the spectrum in its transmission mode. The second-derivative spectrum was obtained using GRAMS/32 ver. 5.0. Smoothing was accomplished with a nine-point Savisky-Golay function, and the secondary structural contents were estimated from the peak area of the second-derivative spectrum using a base line drawn through the peak by the Savisky-Golay method. The peaks in the wavenumber region of 1,600–1,700 cm^{-1} were generally assigned according to the result of Dong *et al.*,[14] as follows: 1,656 cm^{-1}, helix; 1,632 cm^{-1}, 1,641 cm^{-1}, and 1,695 cm^{-1}, β-sheet; 1,668 cm^{-1} and 1,686 cm^{-1}, turn; 1,616 cm^{-1}, others. But, in this experiment the peak at 1,656 cm^{-1} was assigned to a helical structure shown in the twisted-sheet model indicated by Fraser *et al.*,[11] because there is no report of feather keratin having an α-helix, while the peak at 1,660 cm^{-1} appearing for urea-treated rachis was assigned to be derived from the peak at 1,656 cm^{-1}. FT-IR spectra for the dry calamus were also obtained by heating at 5 °C interval for 10 min from 80 °C to 200 °C according to the method described above.

Analytical methods. The amino acid compositions of the barbs, rachis, and calamus were determined as described previously.[15] The amount of solubilized keratin was determined by the micro-biuret method.[16]

Results and Discussion

Features of the fowl feather preparations

The moisture level of the barbs, rachis, and calamus obtained from the wet samples by lyophilization was determined to be 6.3%, 8.2%, and 9.2% respectively, by drying at 110 °C for 8 h. The amino acid compositions of the barbs, rachis, and calamus demonstrated the absence

of hydroxyproline and hydroxylysine (Table 1), indicating no contamination by other dermal scleroproteins like collagen. All the samples showed a high content of cystine (41–44 residues/1,000 residues), whereas lanthionine artificially converted from cystine was not detected. These samples had very high contents of serine, proline, and glycine (143–152, 104–124, and 113–136 residues/1,000 residues respectively), whereas there was a very small content of methionine in barbs and rachis, and it was absent in calamus. The amino acid composition is similar to that of the whole fowl feather reported by Akahane *et al.*[1] The solubility of barbs in 50 mM glycine NaOH buffer containing 5 mM $CaCl_2$ and NaCl (pH 11) at 40 °C and 50 °C for 12 h was determined by the micro-biuret method,[16] and showed only 0.85% and 1.65% at each temperature, indicating a high resistance to solubilization. Such high insolubility or stability of fowl feather keratin was thought to be due mainly to many intra-and inter-molecular disulfide bonds.

Thermal behavior in the wet condition

The endothermic transition of fowl feather rachis in water occurred in a considerably higher temperature region of 110–160 °C with two endothermic peaks than those of globular proteins and scleroprotein such as collagen (Fig. 2 and Table 2). The lower temperature peak (peak 1) was relatively weak and broad in shape,

Table 1. Amino Acid Compositions[a] of Barbs, Rachis, and Calamus from Fowl Feather

	Barbs	Rachis	Calamus	Whole[b]
Hyp	0	0	0	0
Asp	53	51	59	63
Thr	48	45	44	53
Ser	152	151	143	157
Glu	87	83	83	86
Pro	121	124	104	117
Lan	0	0	0	—
Gly	115	113	136	115
Ala	57	56	74	56
Cys	44	43	41	42
Val	77	75	68	77
Met	3	5	0	3
Ile	48	45	35	43
Leu	72	70	87	74
Tyr	17	16	18	16
Phe	28	33	33	36
Hyl	0	0	0	—
Lys	9	8	10	12
His	5	4	5	3
Arg	47	46	47	47

[a]Residues/1,000 residues.
[b]Akahane *et al.*[1]

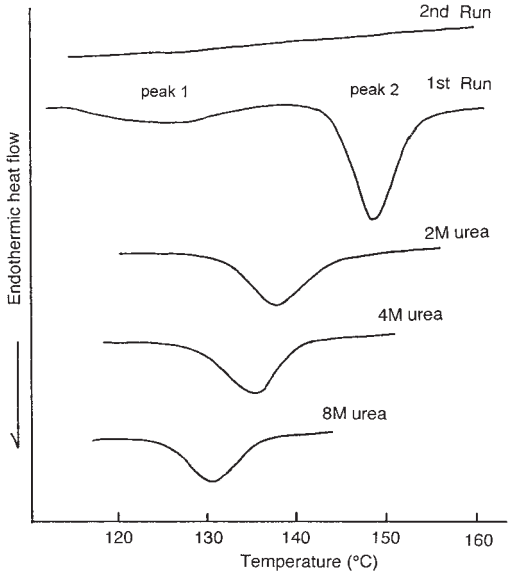

Fig. 2. DSC Curves for Wet Fowl Feather Rachis with and without the Presence of Urea.

After immersing rachis in 2–8 M urea at 40 °C for 4 h, DSC was carried out using an airtight silver sample capsule at a heating rate of 5 K/min.

Table 2. Thermal Characteristics of Barbs, Rachis, and Calamus from Fowl Feather with or without Urea-treatment, or with Pulverization Evaluated by DSC under the Wet Condition

	Peak 1				Peak 2			
	Transition temp. (°C)			Enthalpy (mJ/mg)	Transition temp. (°C)			Enthalpy (mJ/mg)
	*T*o	*T*p	*T*c		*T*o	*T*p	*T*c	
Barbs								
native	116.2 ± 1.1	127.1 ± 1.4	—	7.2 ± 0.8	—	140.3 ± 1.3	147.6 ± 2.4	17.5 ± 0.7
pulverized	130.2 ± 1.1	140.7 ± 0.4	—	2.0 ± 0.3	—	151.4 ± 0.8	157.0 ± 0.6	17.0 ± 0.9
Rachis								
native	113.3 ± 1.7	124.0 ± 0.8	133.6 ± 0.5	4.1 ± 1.8	143.1 ± 0.9	148.3 ± 0.4	153.3 ± 0.5	18.4 ± 1.3
2 M urea	—	—	—	—	132.2 ± 0.5	137.3 ± 0.1	142.8 ± 0.7	10.2 ± 1.3
4 M urea	—	—	—	—	131.8 ± 0.6	135.4 ± 0.3	141.1 ± 0.3	10.9 ± 1.6
8 M urea	—	—	—	—	125.0 ± 0.3	129.8 ± 0.5	134.8 ± 0.8	9.4 ± 2.1
pulvrized	—	—	—	—	146.4 ± 1.6	152.8 ± 0.7	158.5 ± 0.7	14.5 ± 1.4
Calamus								
native	112.6 ± 1.1	122.2 ± 0.7	131.1 ± 0.4	2.6 ± 1.1	143.5 ± 1.2	148.1 ± 0.7	152.4 ± 0.9	9.8 ± 0.5
pulverized	110.1 ± 2.3	126.8 ± 1.1	—	8.3 ± 0.9	—	146.5 ± 1.2	153.8 ± 1.9	20.5 ± 0.9

*T*o, oneset temp.; *T*p, peak temp.; *T*c, conclusion temp. Mean ± S.D. (n = 7).

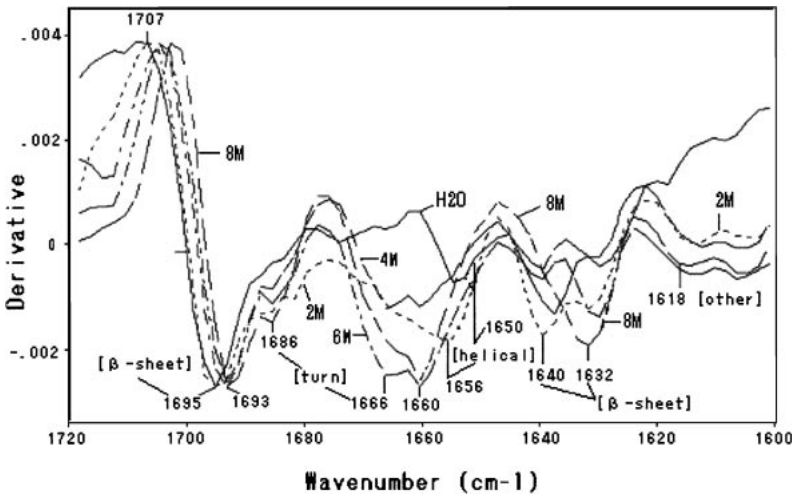

Fig. 3. Second-derivative FT-IR Spectra for Fowl Feather Rachis Treated with Urea.

After immersing rachis in 2–8 M urea at 40 °C for 4 h and slicing it, a slice was quickly mounted on a diamond window. A 100-scan interferogram was quickly collected at 4 cm^{-1} resolution and apodised with a triangular function to give the spectrum in the transmission mode. The second-derivative spectrum was obtained by using GRAMS/32 ver. 5.0, and smoothing was accomplished with a nine-point Savisky-Gloay function.

whereas the higher one (peak 2) was strong and sharp. Since the enthalpy for peak 2 was 4 times as much as that for peak 1, peak 2 is considered to be the main thermal transition for fowl feather rachis. After the first DSC run, the sample was quickly cooled to room temperature, and the second run was done. The DSC curve of the second run indicated no endothermic peak (Fig. 2). Hence the endothermic transition detected is considered to be irreversible.

Immersion of rachis in 2–8 M urea at 40 °C for 4 h resulted in a marked decrease in the transition temperature and enthalpy with increasing concentrations of urea (Table 2). The relationship between the transition temperature (peak temperature, Y) and the urea concentration (X) can be expressed by the following regression equation with a high correlation coefficient: $Y = 0.311X^2 - 4.67X + 147.5$ ($R = 0.978$). For the transition enthalpy (Y), the following regression equation was obtained: $Y = 0.272X^2 - 3.16X + 17.4$ ($R = 0.920$). These concentration-dependent decreases suggest that urea destabilized the higher-order structure of rachis, probably due to destruction of hydrogen bonding. FT-IR was applied to evaluate changes in the secondary structure of rachis resulting from the urea treatment. The second-derivative FT-IR spectra of the native and urea-treated rachis showed obvious absorption peaks in the wavenumber region of 1,550–1,750 cm^{-1} of amide I (Fig. 3), which was assigned to helical (1,650 cm^{-1} and 1,656 cm^{-1}), β-sheet (1,632 cm^{-1}, 1,641 cm^{-1}, and 1,695 cm^{-1}), turn (1,668 cm^{-1} and 1,686 cm^{-1}), and other (1,616 cm^{-1}) structures corresponding to the model by Fraser *et al.*[11] The secondary structural contents of native rachis indicated a β-sheet content of about 78%, which is considerably higher than the value predicted previously,[2,6] probably due to a difference between the result based on the whole sequence of keratin by FT-IR and the result based on the partial sequence from the enzymatic digest that was predicted from its primary structure and from the results of Raman microscopy. Figure 4 indicates that the helical structural content of native rachis was markedly decreased by the urea treatment, and that a slight decrease in the β-sheet structure and a large increase in the turn structure occurred. Since the helical structure has been proposed to be an internal structure constituting units of a fibrous structure,[11] distortion of the helix is considered to have

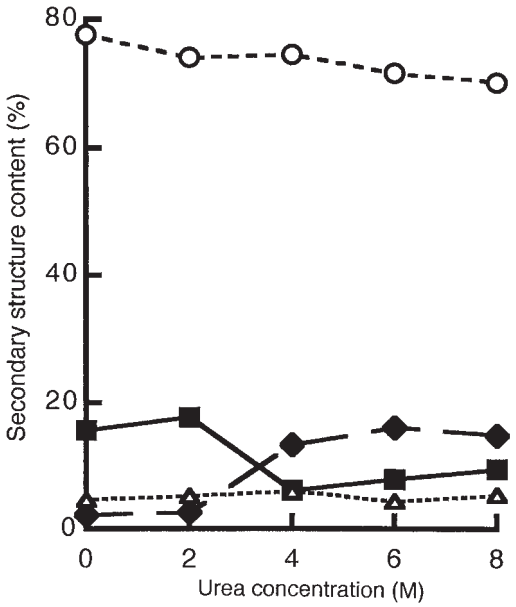

Fig. 4. Effect of Urea on the Secondary Structural Contents of Rachis Estimated by FT-IR.

○, β-sheet; ■, helical; ◆, turn; △, others. The secondary structural contents were estimated from the peak area of the second-derivative spectrum.

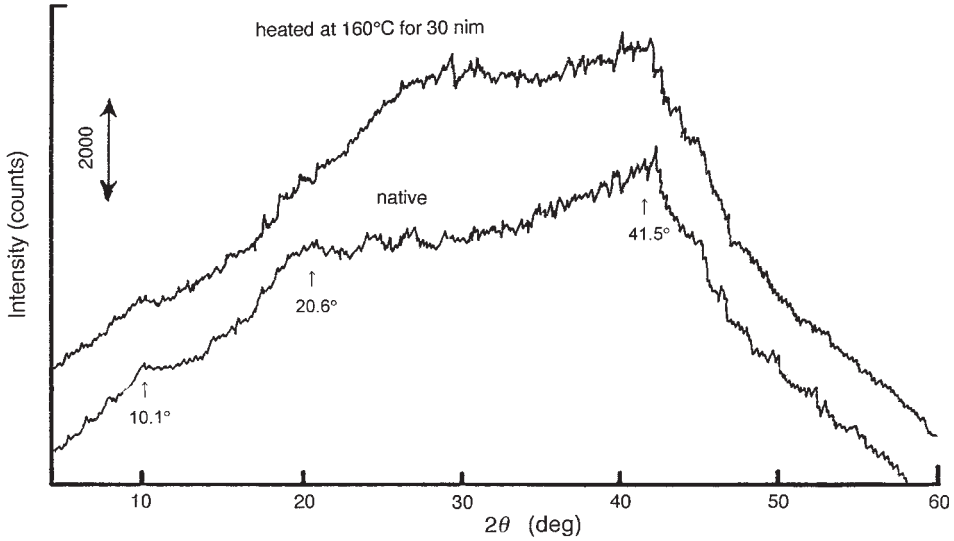

Fig. 5. X-ray Diffractograms for Fowl Feather Rachis Heated at 160 °C for 30 min as Compared with the Native Condition. See Materials and Methods for the X-ray diffractometry conditions.

brought about disarray of the helical and fibrous structural arrangement, probably resulting in the reduced thermal stability evaluated by DSC. The X-ray diffractogram of native rachis showed three small diffraction peaks at 2θ 10.1°, 20.6°, and 41.5° (Fig. 5). This indicates that rachis had fibrous structures with an ordered arrangement and packing, this being related to the arrangement of microfibrils in feather which was found to be less ordered than that in wool.[12] But, the rachis sample heated at 160 °C for 30 min, at which temperature the endothermic transition observed by DSC had completely ceased, showed disappearance of the diffraction peak at 20.6° (spacing, $d = 4.31$ Å). The diffraction peaks at 10.1° and 41.5° remained, presumably due to the high thermal stability of the structure, rich in intra- and inter-disulfide bonds. It is considered from these results that DSC can be used to evaluate the thermal behavior on denaturation of higher-order structures of rachis keratin stabilized with hydrogen bonds that constitute an ordered molecular arrangement.

DSC for barbs and calamus of fowl feathers in water showed similar thermal denaturation to rachis with two endothermic peaks, whereas the higher-temperature peak (peak 2) of barbs was about 8 °C (Tp) lower than that of rachis and calamus (Fig. 6 and Table 2). As already described, these keratin molecules had similar amino acid composition (Table 1), and similar N-terminal region, central region, and C-terminal region.[2] Therefore, the different denaturation temperature for barbs is considered to possibly have been caused by differences in morphological structure such as molecular arrangement and packing, and not by a difference in the protein molecule itself. Since both rachis and calamus constitute the shaft part of the feather, these are considered to have substantially similar structural stability. The enthalpy of barbs and rachis was almost the same, whereas that of calamus was considerably less

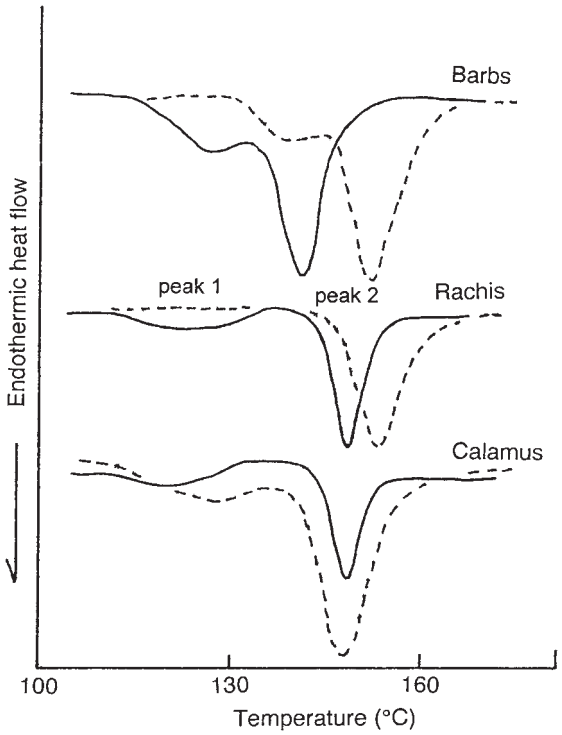

Fig. 6. DSC Curves for Wet Fowl Feather Barbs, Rachis, and Calamus as Compared with Those for the Pulverized Samples.
——, native sample; - - - - -, pulverized sample. See Materials and Methods for the DSC conditions.

(Table 2). Since the pulverized calamus contains powdery parts that were mostly lost during filtration, resulting in a substantial decrease in the enthalpy as described in the thermal behavior of pulverized calamus, the powdery parts are considered to be amorphous. The very low enthalpy of calamus is thus believed likely to have been caused by its higher content of amorphous components than those in rachis.

Table 3. Thermal Characteristics of Barbs, Rachis, and Calamus from Fowl Feather Evaluated by DSC under the Dry Condition

	Peak 1				Peak 2			
	Transition temp. (°C)			Enthalpy (mJ/mg)	Transition temp. (°C)			Enthalpy (mJ/mg)
	*T*o	*T*p	*T*c		*T*p1	*T*p2	*T*c	
Bards	188.4 ± 0.8	191.8 ± 0.8	195.8 ± 0.9	28.0 ± 2.3				
Rachis	175.4 ± 0.8	180.2 ± 0.6	185.2 ± 0.8	6.2 ± 0.8	191.5 ± 0.4	201.3 ± 1.1	207.1 ± 1.2	24.7 ± 1.9
Calamus	172.3 ± 2.1	176.6 ± 2.0	182.1 ± 1.8	10.0 ± 0.7				

*T*o, oneset temp.; *T*p, peak temp.; *T*c, conclusion temp. Mean ± S.D. (n = 7).

The DSC curves for the pulverized samples in water are shown in Fig. 6. The lower-temperature peak (peak 1) apart from that for calamus, was reduced or disappeared, and the higher-temperature peak (peak 2) shifted to a higher temperature region when compared with the results for the native samples (Table 2). This suggests that shear loading resulting from pulverization might have induced an ordered molecular rearrangement of the fowl feather keratin molecules to provide slightly higher stability. In case of calamus, the powdery parts and needle-like parts were formed by pulverization, but the powdery parts could not be recovered, because they stuck to the filter paper. Consequently, the pulverized calamus sample was composed mostly of the needle-like parts. Since the denaturation temperature of the pulverized calamus was similar to the native value, these needle-like parts are considered to play an important role in the thermal transition of an ordered structure. That is, the powdery parts are substantially amorphous. The enthalpy values for the pulverized samples were similar except for calamus, which had a much higher enthalpy (about twice as high) due to its lack of powdery parts.

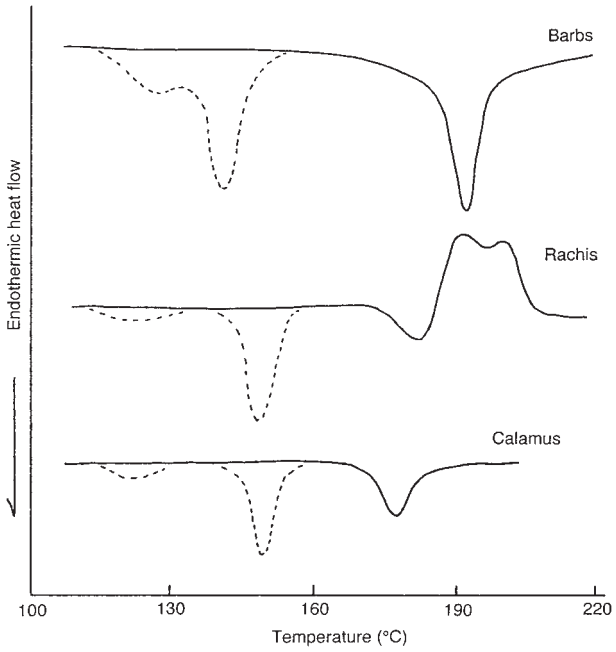

Fig. 7. DSC Curves for Dry Fowl Feather Barbs, Rachis, and Calamus as Compared with Those for the Wet Samples.
——, dry sample; - - - - -, wet sample. See Materials and Methods for the DSC conditions.

Thermal behavior in the dry condition

In the dry condition, the endothermic peaks on the DSC curves for the fowl feather keratins (6.3–9.2%, moisture) were in a considerably higher-temperature region, close to 170–200 °C, than those for the wet samples (about 110–160 °C) (Fig. 7 and Table 3). In this higher-temperature region, the following inseparable reactions could generally be considered to take place while heating a biopolymer under normal conditions: (1) changes in the higher-order structure, (2) cleavage of the main chain and/or its limited recombination, and (3) thermal decomposition of the biopolymer containing its compositional unit residue and/or oxidative degradation with an infinite exotherm. All the fowl feather keratins (2–3 mg) heated to the conclusion temperature of the endothermic peak by the DSC apparatus were dissolved in water (50 ml) at room temperature for 2 min with vigorous stirring, but the dissolved protein could not be determined for each sample. It is thus considered that the peptide bond of the keratins was not sufficiently cleaved during the endothermic transition, suggesting that the endothermic peak corresponds with the detection of reaction 1. The second-derivative FT-IR spectra of calamus were obtained with heating from 80 °C to 200 °C (Fig. 8). The absorption peak at 1,626–1,628 cm^{-1}, which was assigned to the β-sheet, decreased markedly from 180 °C to 200 °C, corresponding to the temperature region of the endothermic transition detected by DSC. It is thus considered that DSC can be used to evaluate the thermal phase transition of the higher-order structure of feather keratin, such as disordering of its fibrous structure, while minimizing the occurrence of the second and third reactions. The remarkable increase in thermal transition indicates a very strong effect of water on the thermal stability of the higher-order structure. Since such marked effects of water on thermal transition have also been reported for several proteins,[17,18] and starch,[19] this phenomenon is considered to be common to general biopolymers, and to be caused by an increase in thermal stability with a decrease in mobility due to the elimination of moisture. In the case of rachis, the endothermic process was inseparably linked to complicated exothermic peaks. But

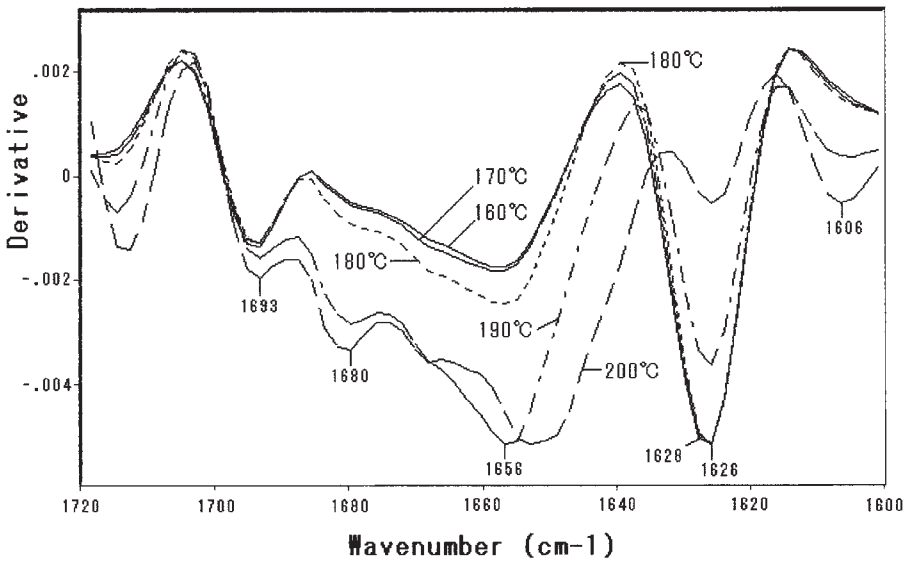

Fig. 8. Second-derivative FT-IR Spectra for Dry Fowl Feather Calamus with Heating from 160 °C to 200 °C. See Materials and Methods for the FT-IR conditions.

since the exotherm returned to the base line at about 210 °C, this might have been caused by some rearrangement of the higher-order structure with higher thermal stability and not by oxidative degradation of the keratin molecule. This peculiar thermal behavior should be investigated further.

References

1) Akahane, K., Murozono, S., and Maruyama, K., Soluble protein from fowl feather keratin. *J. Biochem.*, **81**, 11–18 (1977).
2) Murayama Arai, K., Takahashi, R., Yokote, Y., and Akahane, K., Amino-acid sequence of feather keratin from fowl. *Eur. J. Biochem.*, **132**, 501–507 (1983).
3) Lews, P. N., Momany, F. A., and Scherag, H. A., Folding of polypeptide chains in proteins: A proposed mechanism for folding. *Proc. Natl. Acad. Sci. USA*, **68**, 2293–2297 (1971).
4) Chou, P. Y., and Fasman, G. D., Conformational parameters for amino acids in helical, β-sheet, and random coil regions calculated from proteins. *Biochem.*, **13**, 211–221 (1974).
5) Chou, P. Y., and Fasman, G. D., Prediction of protein conformation. *Biochem.*, **13**, 222–245 (1974).
6) Tsuboi, M., Kaneuchi, F., Ikeda, T., and Akahane, K., Infrared and Raman microscopy of fowl feather barbs. *Canadian J. Chem.*, **69**, 1752–1757 (1991).
7) Bear, S. R., and Rugo, H. J., The results of X-ray diffraction studies on keratin fibers. *Ann. N.Y. Acad. Sci.*, **53**, 627–647 (1951).
8) Fraser, R. D. B., and MacRace, T. P., Molecular organization in feather keratin. *J. Mol. Biol.*, **1**, 387–397 (1959).
9) Astbury, W. T., and Beighton, E., Structure of feather keratin. *Nature*, **4784**, 171–173 (1961).
10) Ramachandran, G. N., and Dweltz, N. E., Structure of feather keratin, Collagen Proc. Symp., Univ. Madaras, India, pp. 147–177 (1960) (pub. 1962).
11) Fraser, R. D. B., MacRace, T. P., and Roger, G. E., The twisted-sheet model. In "Keratins", ed. Thomas, C. C., Springfield, Illinois, pp. 127–129 (1972).
12) Filshiie, B. K., and Rogers, G. E., Aspects of the ultrastructure of Å α-keratin, bacterial flagella, and feather keratin, Ultrastruct. Protein Fibers, Symp. Papers 19th Ann. Meeting Electron Microscope Soc. Am., Pittsburgh, Pennsylvania, pp. 123–138 (1961) (pub. 1962).
13) Takahashi, K., Shirai, K., and Wada, K., Melting behavior of gels prepared from isolated subunit of collagen. *J. Food Sci.*, **49**, 1920–1921 (1988).
14) Dong, A., Hung, P., and Caughey, W. S., Protein secondary structures in water from second-derivative amide I infrared spectra. *Biochem.*, **29**, 350–356 (1956).
15) Hattori, M., and Takahashi, K., Pepsin-solubilized elastin as a water/oil emulsifier. *Food Hydrocolloids*, **7**, 327–335 (1993).
16) Itzhaki, R. F., and Gill, D. M., A micro-biuret method for estimating proteins. *Anal. Biochem.*, **9**, 401–410 (1964).
17) Takahashi, K., Shirai, K., Wada, K., and Kawamura, A., Thermal behavior of proteins under the condition of relatively low moisture content. *Nippon Nōgeikagaku Kaishi* (in Japanese), **54**, 357–359 (1980).
18) Fujita, Y., and Noda, Y., Effect of hydration on the thermal denaturation of lysozyme as measured by differential scanning calorimetry. *Bull. Chem. Soc. Jpn.*, **51**, 1567–1568 (1978).
19) Takahashi, K., Shirai, K., and Wada, K., Structural changes in starch granules of low moisture content during heating. *Agric. Biol. Chem.*, **46**, 2502–2511 (1982).