Anomalous Behavior of Electrical Conductivity of Solubilized Collagen Solutions with Thermal Denaturation

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SYNOPSIS

The temperature dependence of the electrical conductivity, σ, for a collagen solution was measured in the temperature region including the thermal denaturation temperature, t_d. The σ increased with temperature, t, but decreased at ca. 40°C, and then again increased. In this temperature region, a change of optical rotation, α_D, was observed. The change is due to the thermal denaturation. The differential curve of σ vs. t gave clear deflection points and a large peak at ca. 40°C. The t_d could be estimated from the peak temperature. The t_d decreased with the increase in the concentration of collagen and with the decrease in the heating rate. These measurements were carried out for the collagen prepared by various methods. Some of them showed one peak; others had two peaks. The t_d obtained by the measurement of σ correlated with that obtained by the measurement of α_D. The activation energy of σ, ΔE_a, obtained from the linear relationship between log σ and $1/T$, increased with the concentration of collagen, but was unchanged for the heating rate. The ΔE_a obtained for various types of collagen showed a constant value. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

Collagens have been rarely utilized industrially, except for leather, glue and gelatin, as most of them are insoluble in an organism. However, some solubilization methods for those insoluble collagens[1,2] have been developed and have been utilized for medical and cosmetics materials.[3,4] We have studied the solubilization of collagens with pepsin from limed splits, which are split hides treated with lime and sulfur compound solutions, and are byproducts in the leather-manufacturing process, and the recovery of the solubilized collagen.[5] Collagen is a cylindrical molecule that has a helical structure in its native state. It is not stable to heat in solution and changes to random-coil gelatin at the thermal denaturation temperature, t_d. It is important to know the thermal denaturation behavior of solubilized collagen solution if it is to be used in industry. Thermal denaturation has been investigated by measurements of the temperature dependence of the viscosity and optical rotation, α_D, by many researchers.[6,7] It was found that some commercially available collagens, and ones that were prepared from the various kinds of hides through a solubilizing treatment, had two kinds of t_d in the temperature dependence of α_D. Thus, when the temperature dependence of the electrical conductivity, σ, for collagen solutions was examined, it was found that σ of collagen solutions decreased abruptly at t_d as determined by the temperature dependence of α_D. In this article, to determine the t_d of collagens prepared by various methods, we measured the temperature dependence of σ and discuss the influences of measuring conditions on the t_d.

EXPERIMENTAL

Materials

Commercially available acid-soluble collagen[7] (ASC, Nippi Co., Tokyo, Japan), pepsin-solubilized[1] or

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Table I Commercially Available and Prepared Collagens

Code	Source	Solubilization Method
Commercially available collagen		
ASC[a]	Insoluble collagen from bovine corium	Extracted with acid solution
PC1[b]	Insoluble collagen from bovine corium	Solubilized with pepsin
PC2[a]	Insoluble collagen from bovine corium	Solubilized with proctase
AC[a]	Insoluble collagen from bovine corium	Solubilized with NaOH and CH_3NH_2
Prepared collagen		
PL1	Limed split crushed with a homogenizer	Solubilized with pepsin
PL2	Limed split crushed with a mill	Solubilized with pepsin
PH	Commercially available hide powder[c]	Solubilized with pepsin

[a] Products of Nippi Co.
[b] Product of Koken Co.
[c] Product of Marshall Laboratory Co.

proctase (protease produced by *Aspergillus niger* var. *macrosporus*)-solubilized[8,9] collagen (PC1, Koken Co., Tokyo, Japan or PC2, Nippi Co.), alkali-solubilized collagen[2] (AC, Nippi Co.), and collagens that we prepared from hides (PL1, PL2, or PH) are listed in Table I. The preparation method of collagens is as follows: Limed splits, which were produced in the leather-manufacturing process, were crushed with a homogenizer or a mill. These, and commercially available hide powder (Marshall Laboratory Co., U.S.A.), were solubilized with pepsin (1 : 10,000, Nacalai tesque Inc., Kyoto, Japan, added 1/5 of substrate) in 0.1*M* CH_3COOH (200 times to substrate) for 24 h at 20°C, and then the solubilized mixture was centrifuged at 15,000 rpm for 10 min. The supernatant was salted out with 10% NaCl for the supernatant and centrifuged at 15,000 rpm for 10 min. The sediment was dissolved in 0.1*M* CH_3COOH and centrifuged at 15,000 rpm for 10 min. Furthermore, salting out and dissolution were repeated. The supernatant was dialyzed against distilled water and lyophilized.[5]

Measurement of the Electrical Conductivity and the Thermal Denaturation Temperature

Collagens were dissolved in 1 m*M* HCl at 5°C. The σ of collagen solutions was measured automatically with a Horiba (Kyoto, Japan) DS-15 connected to a personal computer while raising the temperature from 20 to 50°C linearly in a water bath as shown in Figure 1, and the differential curve ($d\sigma/dt$) for the σ vs. t was calculated using a personal computer. We defined the minimum in $d\sigma/dt$ vs. t as the thermal denaturation temperature, $t_{d\sigma}$.

Measurement of the Optical Rotation and the Thermal Denaturation Temperature

To compare this method with a traditional method, α_D was measured with a Horiba SEPA-200 high-sensitivity polarimeter.[5] The temperature dependence of $-\alpha_D$ was measured, and $t_{d\alpha}$ was determined by using the calculated $-d\alpha_D/dt$ as well as measurement of the thermal denaturation temperature, $t_{d\sigma}$.

Measurement of Sodium Dodecyl Sulfate–Polyacrylamido Gel Slab Electrophoresis

The measurement of sodium dodecyl sulfate–polyacrylamido gel slab electrophoresis, SDS-PAGE, was carried out by Laemmli's method[10] for the collagen denatured at 100°C for 5 min, as follows: The concentration of the separating gel was 5% and was adjusted to pH 8.8 with 1.5*M* tris-hydrochloric acid buffer solution. The concentration of the stacking gel was 4.5% and was adjusted to pH 6.8 with 0.5*M*

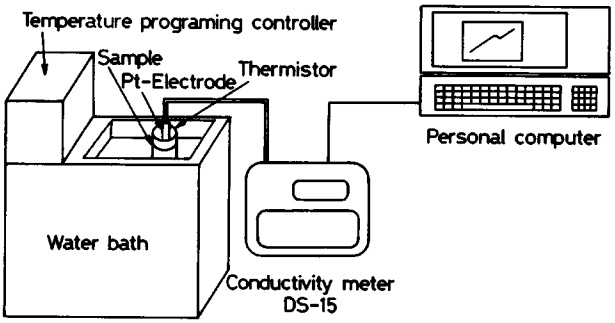

Figure 1 Schematic drawing for the measurement of electrical conductivity.

tris-hydrochloric acid buffer solution. The electrophoresis was carried out at 150 V for 2 h using a tris-glycine buffer solution. After the gel was dried, its chromatogram was recorded using a densitometer (He—Ne laser, 633 nm). The relative intensity was calculated by standardization of the area given by integration of the chromatogram for each collagen to be one, after the background was corrected.

RESULTS AND DISCUSSION

Characterization of Collagen

To characterize collagens prepared by the various methods shown in Table I, measurement of the temperature dependence of α_D or SDS-PAGE was carried out. The thermal denaturation of the ASC solution occurred in one step over a narrow temperature range (ca. 3°C), but for solubilized collagens, it occurred in two steps. We proposed the measuring method of t_d for those collagens, as follows: The helix fraction (X)[6,7] at t°C was calculated by the following equation;

$$X = \frac{[\alpha]_D^t - [\alpha]_D^{45}}{[\alpha]_D^{20} - [\alpha]_D^{45}}$$

where $[\alpha]_D^t$, $[\alpha]_D^{20}$, and $[\alpha]_D^{45}$ were the specific optical rotations at t°C, 20°C, and 45°C, respectively. The differential coefficient, dX/dt, was calculated. For solubilized collagens whose thermal denaturation occurred in two steps, e.g., PL2, we defined lower, middle, and higher temperatures for peaks in the dX/dt curve as t_L, t_M, and t_H, and X values for respective temperatures as X_L, X_M, and X_H as shown in Figure 2. If the component denatured at the lower temperature, component L, and the component denatured at the higher temperature, component H, exist and the thermal denaturation of component L terminates and that of component H starts nearly at t_M, the change of X_M and the ratio of amounts between component L and H, L/H, correlates to each other by definition of X. The amount of component L is proportional to "$1 - X_M$" and the amount of component H is proportional to "X_M." Thus, L/H is calculated on the relationship of X_M by the following equation: $L/H = (1 - X_M)/X_M$. In Table II, t_L, t_H, L/H, and X_M for collagen prepared with various methods are indicated. ASC and AC had one $t_{d\alpha}$, but $t_{d\alpha}$ of AC was lower than that of ASC. However, the others had two $t_{d\alpha}$. For PC1 and PC2, there were no differences between t_L and t_H, but L/H of PC2 was larger than that of PC1. For solubilized collagens that we prepared from the various kinds of hides, in the order of PL1, PL2, and PH, their L/H increased as their t_H were slightly lowered, but their t_L hardly varied. We confirmed their reproducibilities by measuring three or four times.

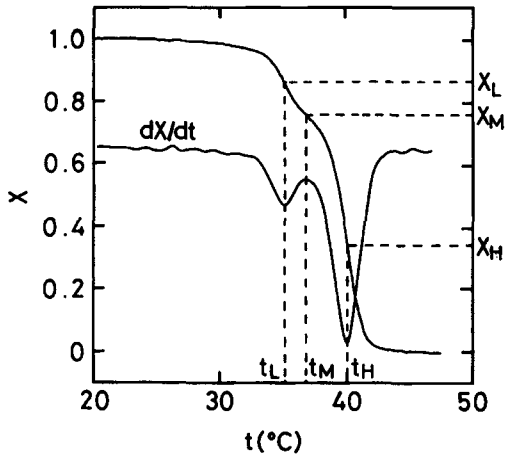

Figure 2 Temperature dependencies of helix fraction, X, and dX/dt for pepsin-solubilized collagen, PL2. Concentration of collagen: 0.20%; solvent: 1 mM HCl; heating rate: 0.3°C/min.

Table II The Thermal Denaturation of Temperature ($t_{d\alpha}$) Determined from the Temperature Dependence Curves of the Optical Rotation,[a] the Ratio of Rate of the Component L to That of Component H (L/H), and X_M for Various Collagen Preparations in 1 mM HCl

Sample Code	Concentration of Collagen (wt %)	$t_{d\alpha}$ (°C)		L/H	X_M
		t_L	t_H		
ASC	0.19	[b]	41.1	—	
PC1	0.19	36.4	40.1	0.08	0.992
PC2	0.20	35.7	40.3	0.37	0.731
AC	0.20	[b]	33.2	—	
	0.20	[b]	33.4	—	
PL1	0.19	35.4	41.2	0.14	0.874
	0.19	34.3	40.1	0.14	0.880
	0.22	35.1	40.4	0.13	0.885
PL2	0.20	35.3	40.0	0.33	0.753
	0.19	34.3	39.6	0.32	0.758
	0.22	34.9	40.0	0.31	0.765
PH	0.20	36.0	39.5	0.48	0.678
	0.19	34.7	39.5	0.45	0.691
	0.22	34.6	39.5	0.47	0.678
	0.20	34.6	39.6	0.42	0.703

[a] Heating rate: 0.3°C/min.
[b] There was a peak in the $d_{\alpha D}/dt$ curve.

Collagen is a molecule having a helical structure with a molecular weight of 300,000 and consists of two similar amino acidic residual sequential polypeptide chains, α_1, and one polypeptide chain, α_2, having a different amino acid residue sequence from α_1, whose molecular weights are 100,000, respectively.[11] In the SDS-PAGE chromatogram, collagen subunits are resolved into α_2, α_1, and β_{12}, which is bound between α_1 and α_2 by cross-linking; β_{11}, which is bound between two α_1 by cross-linking; and γ, which is bound among two α_1 and one α_2 by cross-linking, due to the facility for migration. To investigate chain compositions of collagens prepared by various methods, SDS-PAGE was carried out, and these chromatograms are shown in Figures 3 and 4. In the chromatogram of ASC, a band between β_{11} and γ was found, but there was no band for a component having smaller molecular weight than that of α: α_f. In the chromatogram of PC1, bands between α_1 and β_{12} and those of α_f were found. In the chromatogram of PC2, bands between β_{11} and γ, bands between α_1 and β_{12}, and those of α_f were found. The relative mobility of each subunit for AC was lowered and each peak was relatively broad. In this chromatogram, α_2, α_1, and β, which consists of β_{11} and β_{12}, were found, but γ was not observed. In chromatograms of PL1, PL2, and PH, bands between β_{11} and γ, bands between α_1 and α_2, and those of α_f were found. The rate of α_f for PL2 and PH was larger than that for PC1, and the bands between α_1 and α_2 in these chromatograms were found more and more clearly in the order of PL1, PL2, and PH, as shown in Figure 4.

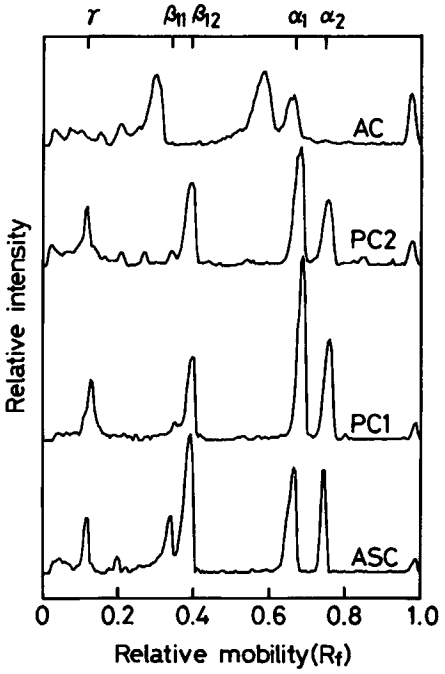

Figure 3 Variations of SDS-PAGE patterns for commercially available collagens. Indicated subunit positions are those of ASC.

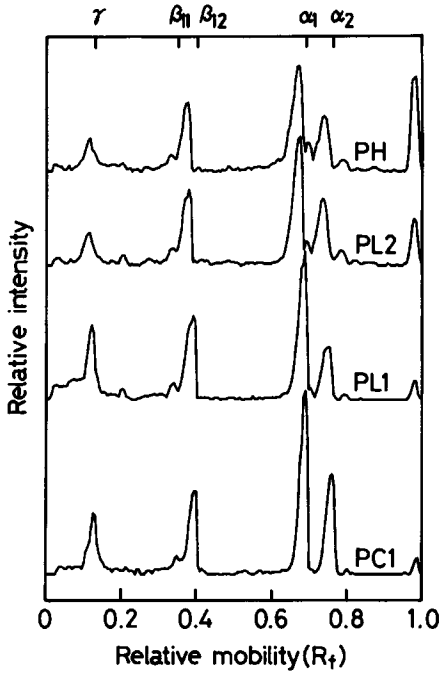

Figure 4 Variations of SDS-PAGE patterns for pepsin-solubilized collagens from hides. Indicated subunit positions are those of pepsin-solubilized collagen, PC1.

From the L/H values calculated by measuring the temperature dependence of X and the chain composition analyzed by SDS-PAGE for collagens prepared by various methods, it was found that the collagen having a larger ratio of components L contained more chain components besides the collagen subunit.

Temperature Dependence of σ and α_D

The temperature dependencies of σ and $d\sigma/dt$ for ASC are shown in Figure 5. The σ increased with temperature, then decreased stepwise at ca. 40°C, and then increased again. It is well known that changes in some physical properties of collagen solution are caused by helix–coil transitions. In the temperature region where σ decreased, the $-\alpha_D$ changed markedly, as shown in Figure 6. Thus, these changes were thought to correspond to the thermal denaturation. The differential curve had a minimum at ca. 40°C, and t_d was estimated from the peak temperature. However, as shown in Figure 7, for some

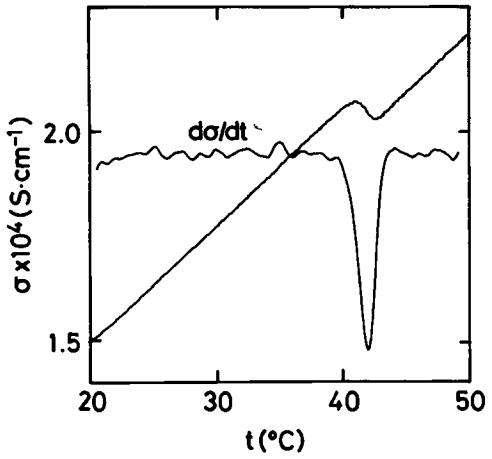

Figure 5 Temperature dependencies of σ and $d\sigma/dt$ for ASC. Concentration of collagen: 0.19%; solvent: 1 mM HCl; heating rate: 0.3°C/min.

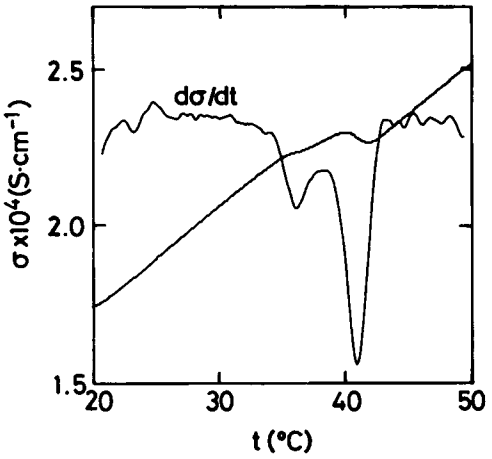

Figure 7 Temperature dependencies of σ and $d\sigma/dt$ for pepsin-solubilized collagen, PL2. Concentration of collagen: 0.20%; solvent: 1 mM HCl; heating rate: 0.3°C/min.

of solubilized collagens, σ changed in two steps and there were two minima in the temperature dependence of $d\sigma/dt$. One was at ca. 35°C and the other at ca. 40°C. We found that the measurement of the temperature dependence of σ could be used to determine t_d.

Thermal Denaturation Temperature

Variations of t_d were observed with measuring conditions such as the heating rate and the concentration of collagen. We tried to clarify influences of them on observed t_d using PC1. As shown in Figure 8, t_d slightly increased up to a rate of ca. 0.3°C/min and then increased more so with further increase of the heating rate. It was considered that t_d was independent of the heating rate to ca. 0.3°C/min. The $t_{d\sigma}$ was a little higher and was more influenced by the heating rate than was the $t_{d\alpha}$ over ca. 0.3°C/min.

However, the t_d slightly decreased to ca. 0.2% and then more so with the increase of the concentration of collagen. The $t_{d\alpha}$ was a little higher and was less influenced by the concentration of collagen than was $t_{d\alpha}$, as shown in Figure 9.

We carried out these measurements under the same conditions (heating rate: 0.3°C/min; concen-

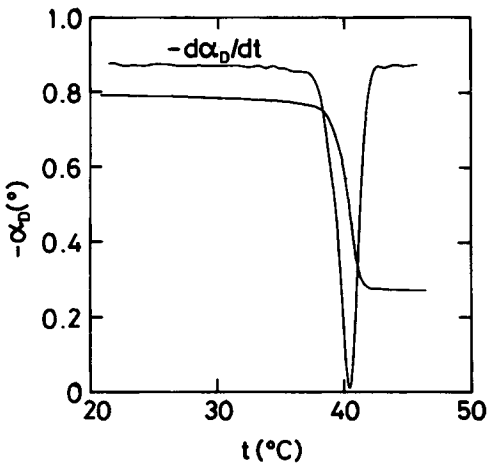

Figure 6 Temperature dependencies of $-\alpha_D$ and $-d\alpha_D/dt$ for ASC. Concentration of collagen: 0.19%; solvent: 1 mM HCl; heating rate: 0.3°C/min.

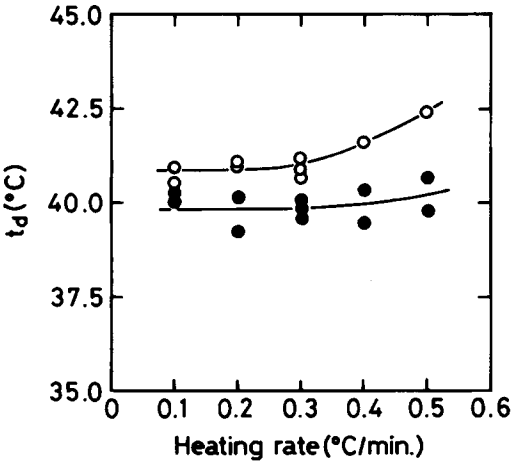

Figure 8 Variations of the thermal denaturation temperature, t_d, with heating rate for pepsin-solubilized collagen, PC1: (○) $t_{d\sigma}$ measured by electrical conductivity; (●) $t_{d\alpha}$ measured by optical rotation. Concentration of collagen: 0.18–0.19%; solvent: 1 mM HCl.

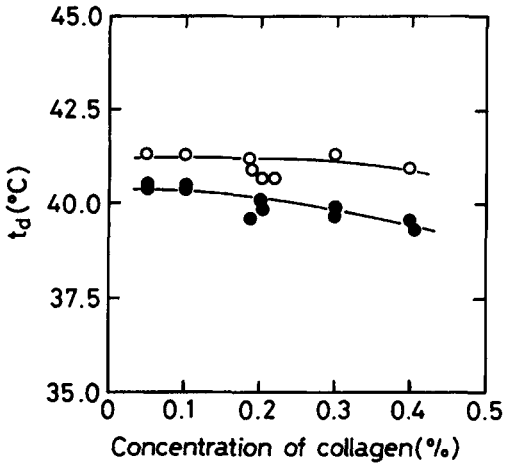

Figure 9 Variations of the thermal denaturation temperature, t_d, with concentration of pepsin-solubilized collagen, PC1: (○) ($t_{d\sigma}$) measured by electrical conductivity; (●) ($t_{d\alpha}$) measured by optical rotation. Solvent: 1 mM HCl; heating rate: 0.3°C/min.

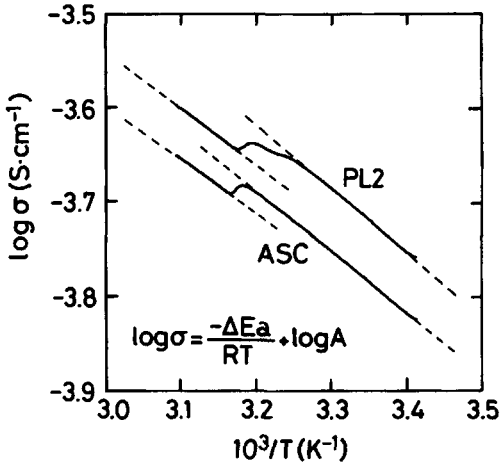

Figure 11 Plots of log σ vs. $1/T$ for collagens. Solvent: 1 mM HCl; heating rate: 0.3°C/min.

tration of collagen; 0.19–0.2%) on collagens prepared by different methods and from different sources as shown in Table I. On the measurement of σ, ASC and AC had one denaturation peak in a differential curve, whereas PC2, PL1, PL2, and PH had two denaturation peaks. However, on PC1, two $t_{d\alpha}$ were found but a lower $t_{d\sigma}$ was not observed. It was found that correlations existed between $t_{d\sigma}$ and $t_{d\alpha}$, whose t_L were measured for other collagens except for ASC and AC and whose t_H were measured for all collagens listed in Table I. They agreed with each other within about 1–2°C, as shown in Figure 10. Therefore, the new measurement method of t_d from the temperature dependence of σ was carried out automatically and more easily than by measuring the temperature dependence of α_D, the traditional method.

Activation Energy

The activation energy for electrical conductivity, ΔE_a, was obtained by the normal method from plots of log σ vs. $1/T$ before and after thermal denaturation, as shown in Figure 11. Influences of some fac-

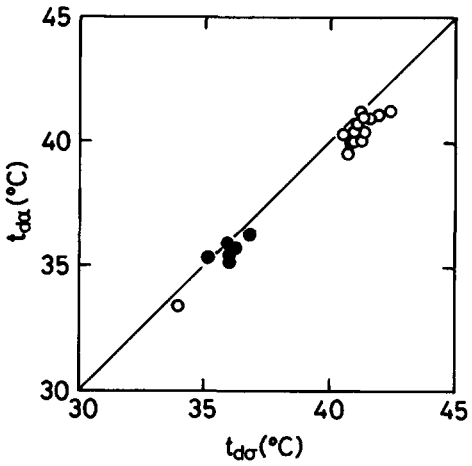

Figure 10 Relationship between the thermal denaturation temperature determined by electrical conductivity, $t_{d\sigma}$, and optical rotation, $t_{d\alpha}$, for various collagens: (○) higher thermal denaturation temperature, t_H; (●) lower thermal denaturation temperature, t_L. Solvent: 1 mM HCl; heating rate: 0.3°C/min.

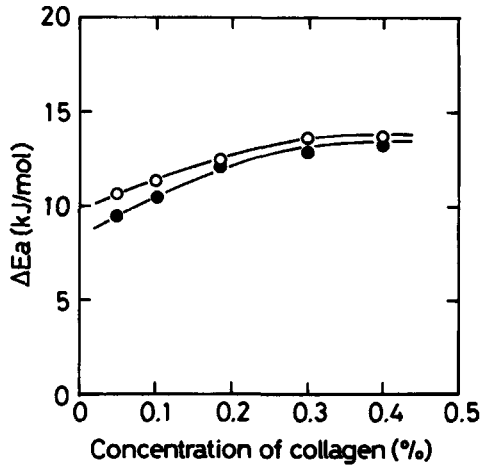

Figure 12 Variations of the activation energy of electrical conductivity, ΔE_a, with concentration of pepsin-solubilized collagen, PC1: (○) before thermal denaturation; (●) after thermal denaturation. Solvent: 1 mM HCl; heating rate: 0.3°C/min.

tors on ΔE_a were examined. The ΔE_a for PC1 was not influenced by the heating rate and was unchanged by thermal denaturation. The ΔE_a increased slightly with the concentration of collagen, as shown in Figure 12. This phenomenon indicates the decrease of the mobility of the carrier ion owing to the increase of the viscosity of solution with the concentration of collagen. The ΔE_a for collagen prepared by different methods and from different sources, listed in Table III, were unchanged. Their ΔE_a before or after the thermal denaturation for various collagen preparations (concentration of collagens: 0.19–0.2%) were 11.9–12.5 or 11.0–12.1 kJ/mol, respectively.

In this study, the carrier ions for conduction were considered to be protons, based on the ΔE_a value (ca. 10 kJ/mol). Generally, σ increases with increasing number of carrier ions and decreasing viscosity. As the viscosity of collagen solutions decrease with thermal denaturation, the protons move more rapidly in the solution after the thermal denaturation of collagen than before. Therefore, σ is expected to increase after the thermal denaturation. However, σ decreases with the thermal denaturation. Further, the pH of collagen solution increased with the thermal denaturation. Therefore, we conclude that this result means that the number of free carrier ions (protons) decreases with the thermal denaturation and we explain this phenomenon as follows: Before the thermal denaturation, both hydrophilic and hydrophobic side chains of collagen are outside the molecule. Once the thermal denaturation occurs, hydrophobic side chains are included into the molecule and hydrophilic side chains remain outside the denatured collagen. As some of the free carrier ions are trapped with hydrophilic side chains of a denatured collagen, σ decreases with a decreasing number of them owing to the thermal denaturation.

Table III The Activation Energy of Electrical Conductivity (ΔE_a) for Various Collagen Preparations in 1 m*M* HCl

Sample Code	Concn of Collagen (wt %)	Before Denaturation (kJ/mol)	After Denaturation (kJ/mol)
ASC	0.19	12.2	11.2
PC1	0.19	12.5	12.1
PC2	0.20	12.3	11.4
	0.20	12.2	11.2
AC	0.20	12.4	11.2
	0.20	12.4	11.0
PL1	0.19	12.5	11.4
PL2	0.20	12.4	11.1
PH	0.20	11.9	11.0

Heating rate: 0.3°C/min.

CONCLUSIONS

The measurement of the temperature dependence of σ was useful for the determination of t_d. It was found that $t_{d\sigma}$ and $t_{d\alpha}$ agreed each other within about 1–2°C. Therefore, the measurement of t_d was carried out automatically and more easily by using this method than by measuring the temperature dependence of α_D. The variation of ΔE_a for native or denatured collagens was not influenced by the heating rate, but increased slightly with the concentration of collagen. The changes of ΔE_a with the thermal denaturation were not observed. The carrier ions for conduction were to be protons based on the ΔE_a value, and the decreasing number of free carrier ions (protons) with thermal denaturation was concluded from the result that σ decreased in spite of a decrease of the viscosity of the collagen solutions.

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