Temperature Dependence of pH for Solubilized Collagen Solutions

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SYNOPSIS

The temperature dependence of the pH for an acid-soluble collagen in solution was measured, so that the pH largely increased in the temperature region including ca. 40°C of the thermal denaturation temperature. As the changes of the optical rotation, α_D, and the electrical conductivity, σ, were observed in this temperature region, the change of the pH is due to the thermal denaturation of collagen. The differential curve of pH vs. t gave clear deflection points and a large peak at ca. 40°C. The thermal denaturation temperature, t_d, could be estimated from the peak temperature. The t_d obtained by the measurement of the α_D, $t_{d\alpha_D}$, decreased with the decrease in the heating rate and with the increase in the concentration of collagen. However, the t_d obtained through the measurement of the pH, $t_{d\mathrm{pH}}$, was independent of the variations of the heating rate and the concentration of collagen. These measurements were carried out for different kinds of collagens prepared by various methods. Some of them had one kind of peak; others had two kinds of peaks. The $t_{d\mathrm{pH}}$ correlated with the $t_{d\alpha_D}$ for different kinds of collagen preparations. Therefore, the measurement of the temperature dependence of the pH was useful for the determination of the t_d. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Collagen is a cylindrical molecule that has a triple-helical structure like the poly-L-proline II structure in its native state.[1,2] It is not stable to thermal treatment in solution and changes to gelatin with a random-coil structure at the thermal denaturation temperature, t_d. Many researchers[3,4] have studied thermal denaturation by measuring the temperature dependence of the viscosity or the optical rotation, α_D. In our previous article,[5] the electrical conductivity, σ, of the collagen solutions was found to decrease stepwise in the temperature region of the thermal denaturation, in spite of the decrease of its viscosity. Therefore, we concluded that the number of free protons, which are considered as carrier ions of electrical conduction, decreased with the thermal denaturation. We explain this phenomenon as follows: Before the thermal denaturation, both hydrophilic and hydrophobic side chains of collagen are outside the molecule. Once 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 protons are trapped with hydrophilic side chains of the denatured collagen, σ decreased with a decreasing number of them, owing to the thermal denaturation. On the basis of this result, we could expect that the pH of the collagen solutions increase with the thermal denaturation. In this article, for different kinds of collagens prepared by various methods, we investigated the effect of measuring conditions on the observed t_d by measuring the temperature dependence of the pH and discussed that the method detecting the t_d with this measurement of the pH is available for the determination of the t_d, compared with that of the t_d by measuring the temperature dependence of the α_D.

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EXPERIMENTAL

Materials

To examine the temperature dependencies of the pH and the α_D for different kinds of collagens in solution, acid-soluble collagen[4] (ASC, Nippi Co., Tokyo, Japan), pepsin-solubilized collagen[6] (PC1, Koken Co., Tokyo, Japan), proctase-solubilized collagen[7,8] (PC2, Nippi Co.), and alkali-solubilized collagen[9] (AC, Nippi Co.) were used as commercially available collagens. ASC is soluble in a dilute acid solution and is an intact collagen. PC1 and PC2 are collagens from which both terminal nonhelical regions containing most of the intra- and intermolecular cross-links are removed by hydrolysis catalyzed by pepsin and proctase. AC is a collagen from which both terminal nonhelical regions are removed by hydrolysis with sodium hydroxide and methylamine. Three kinds of pepsin-solubilized collagens (PL1, PL2, and PH) that were prepared from lime splits and a commercially available hide powder (Marshall Laboratory Co., U.S.A.) were used as prepared collagens. They are listed in Table I. These pepsin-solubilized collagens were prepared by the method described in our previous article.[5]

Measurement of pH and Thermal Denaturation Temperature

Collagens were dissolved in 1 m*M* HCl solution below 10°C. The pH of their solutions was measured automatically by using a Horiba (Kyoto, Japan) pH meter F-16 connected to a personal computer with an RS-232C interface, while raising the temperature from 20 to 50°C linearly in a water bath. The differential coefficient (dpH/dt) for pH vs. t was calculated using a personal computer. We defined $t_{d\text{pH}}$, a thermal denaturation temperature, for the temperature where dpH/dt showed a maximum value.

Measurement of Optical Rotation and Thermal Denaturation Temperature

To compare this method with the traditional one, the temperature dependence of the α_D was measured using a Horiba high-sensitivity polarimeter SEPA-200 (Ref. 10) by increasing the temperature from 20 to 50°C, and then these data were fed into a personal computer and the differential coefficient ($-d\alpha_D/dt$) of $-\alpha_D$ was calculated. We defined $t_{d\alpha_D}$, a thermal denaturation temperature, for the temperature where $-d\alpha_D/dt$ showed a minimum value. The correlation between the helix fraction,[3,4] X, and the ratio of the component L denatured at the lower temperature to that of the component H denatured at the higher temperature, L/H, was determined by the method reported in the previous article.[5]

Determination of Chain Compositions by Sodium Dodecyl Sulfate–Polyacrylamide Gel Slab Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel slab electrophoresis, SDS-PAGE, was carried out by

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[c]
AC[a]	Insoluble collagen from bovine corium	Treated with NaOH and CH_3NH_2 and then extracted with CH_3COOH
Prepared collagen		
PL1	Limed split pulverized with a homogenizer	Solubilized with pepsin
PL2	Limed split pulverized with a mill	Solubilized with pepsin
PH	Commercially available hide powder[d]	Solubilized with pepsin

[a] Product of Nippi Co.
[b] Product of Koken Co.
[c] Proctase produced by *Aspergillus niger* var. *macrosporus*. Product of Meijiseika Co.
[d] Product of Marshall Laboratory Co.

Laemmli's method[11] for collagens denatured at 100°C for 5 min. Their chromatograms were obtained by the reported method.[5]

RESULTS AND DISCUSSION

Characterization of Collagens

To characterize collagens prepared by various methods shown in Table I, the temperature dependence of the α_D or the X and SDS-PAGE were investigated. In Table II, t_L, the lower t_d, t_H, the higher t_d, and the L/H ratio obtained by measuring the temperature dependence of the α_D or the X for various collagen preparations are shown. ASC and AC showed only one $t_{d\alpha_D}(t_H)$, but the $t_{d\alpha_D}$ for AC was lower than that for ASC. The others had two kinds of $t_{d\alpha_D}$. There were small differences between both t_d for PC1 and PC2, but the L/H ratio of PC2 was larger than that of PC1. For pepsin-solubilized collagens that were prepared from limed splits and a commercially available hide powder, their L/H ratios increased in the order of PL1, PL2, and PH, but there were small differences among their t_d.

On the SDS-PAGE chromatogram, the collagen were separated into subunits: α_1, α_2, β_{12}, β_{11}, and γ, due to the mobility. Each molecular weight of α_1 and α_2 is 100,000.[12] The β_{12} is bound between α_1 and α_2 by cross-linking and the β_{11} is bound between two α_1 by cross-linking. The γ is bound among two α_1 and one α_2 by cross-linking. To investigate chain compositions of collagens prepared by various methods, SDS-PAGE was carried out, and these chromatograms are shown in Figure 1. In the chromatogram of ASC, components between β_{11} and γ were observed, but there were no components having fragments of α : α_f. In the chromatogram of PC1, components of α_f were observed. In the chromatogram of PC2, components between β_{11} and γ and those of α_f were observed. The relative mobility of each subunit for AC decreased and each peak was relatively broad. In this chromatogram, α_2, α_1, and β, which consists of β_{11} and β_{12}, were observed but γ was not detected. In the chromatograms of PL1, PL2, and PH, components between β_{11} and γ, the one between α_1 and α_2, and those of α_f were observed. The ratio of α_f for PL1, PL2, or PH was larger than that for PC1. The component between α_1 and α_2 in these chromatograms became clear gradually in the order of PL1, PL2, and PH.

From the differences of the L/H ratios calculated by the temperature dependence of the X and the

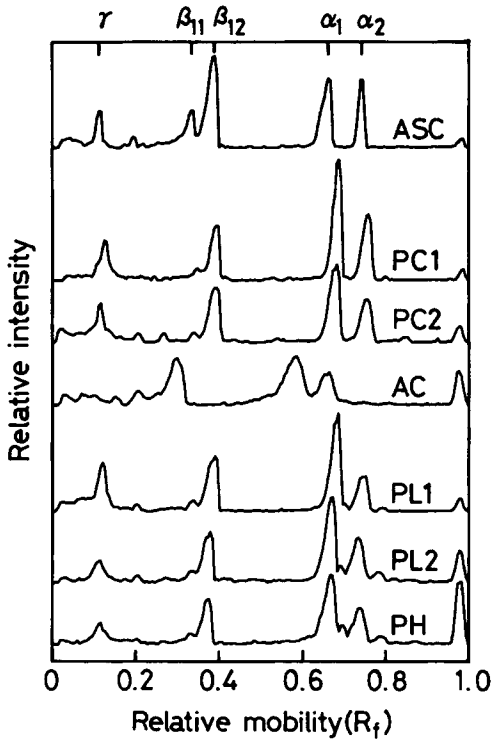

Figure 1 Variations of the SDS-PAGE chromatograms for different kinds of collagens prepared by various methods and from several sources. The indicated subunit positions are those of ASC.

Table II Thermal Denaturation Temperature ($t_{d\alpha_D}$) Determined from the Temperature-dependent Curves of the Optical Rotation[a] and the Ratio of Component *L* to That of Component *H* (*L*/*H*) for Various Collagen Preparations in 1 m*M* HCl

Collagen Sample Code	Concentration of Collagen (Wt %)	$t_{d\alpha_D}$ (°C)[b]		
		t_L	t_H	L/H
ASC	0.19	[c]	40.7	—
PC1	0.19	34.9	40.1	0.09
PC2	0.20	35.1	39.8	0.37
AC	0.20	[c]	33.3	—
PL1	0.20	34.8	40.1	0.13
PL2	0.20	35.0	40.0	0.33
PH	0.20	35.0	39.3	0.44

[a] Heating rate: 0.3°C/min.
[b] t_L: The lower thermal denaturation temperature. t_H: The higher thermal denaturation temperature.
[c] There was only one peak in the $-d\alpha_D/dt$.

chain compositions analyzed by SDS-PAGE for collagens prepared by various methods, it was concluded that the ratio of component L increased as the content of chain components besides the collagen subunits increased. Therefore, pepsin-solubilized and proctase-solubilized collagens have the triple-helical structure at 20°C, but might contain the component whose subunits were degraded partially. The amount can be estimated from the L/H ratio.[13]

Temperature Dependencies of pH and Optical Rotation

The temperature dependencies of the pH and the α_D for ASC are shown in Figure 2. The pH increased slightly with temperature and then increased stepwise at ca. 40°C. It is well known that changes in some physical properties of collagen solutions are caused by the helix–coil transitions. In the temperature region where the pH increased, the α_D changed markedly. Thus, this change corresponded to the thermal denaturation of collagens in solution. The differential curve had a peak at ca. 40°C. However, for some of solubilized collagens, the pH changed in two steps and there were two kinds of peaks in the dpH/dt curves as shown in Figure 3. One was that at ca. 35°C and the other at ca. 40°C as well as the measurement of the α_D. We found that the measurement of the temperature dependence of the pH could be used to determine the t_d of collagens in solution.

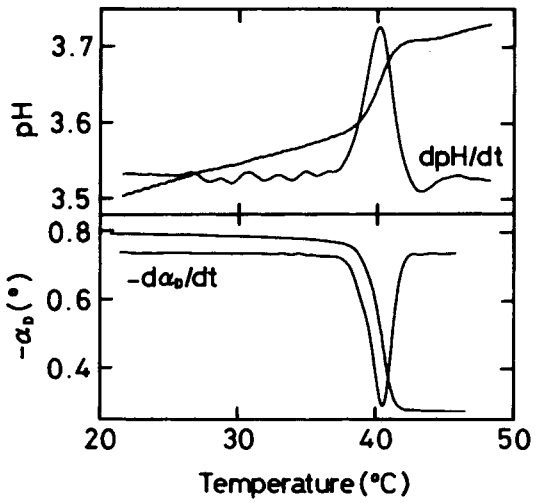

Figure 2 Temperature dependencies of the pH and the $-\alpha_D$ for ASC. Concentration of collagen: 0.19%; heating rate: 0.3°C/min; solvent: 1 mM HCl.

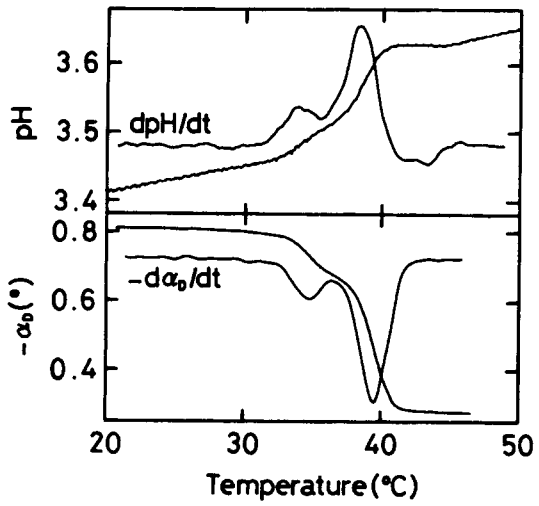

Figure 3 Temperature dependencies of the pH and the $-\alpha_D$ for PL2. Concentration of collagen: 0.2%; heating rate: 0.3°C/min; solvent: 1 mM HCl.

Thermal Denaturation Temperature

Variations of the t_d were observed with measuring conditions such as the heating rate and the concentration of collagen. We tried to clarify these effects on the observed $t_d(t_H)$ for PC1, which has the least amount of component L (below 10%) in solubilized collagens except for AC. As shown in Figure 4, the $t_{d\alpha_D}$ was constant in the rate less than ca. 0.3°C/min and increased with increase of the heating rate more than that. It was considered that the $t_{d\alpha_D}$ was independent of the heating rate in the region below ca. 0.3°C/min. On the other hand, the t_{dpH} seemed to be almost constant in spite of variation of the heating rate and was lower than the $t_{d\alpha_D}$ within this measuring condition. The $t_{d\alpha_D}$ decreased with the concentration of collagen. However, the $t_{d\text{pH}}$ was hardly influenced by the concentration of collagen and was relatively lower than the $t_{d\alpha_D}$ as shown in Figure 5. In the measurement of the temperature dependence of the pH, it increased more as the concentration of collagen increased up to ca. 0.2%. It was suggested that the number of protons that caused the electrical conduction decreased due to the interaction between protons and hydrophilic groups of the denatured collagen : gelatin as described in our previous article.[5]

We carried out these measurements under the same conditions on various collagens prepared with different methods and from different sources, as shown in Table I. In the measurement of the pH, ASC and AC had only one peak, whereas PC2, PL1, PL2, and PH had two kinds of peaks on their dif-

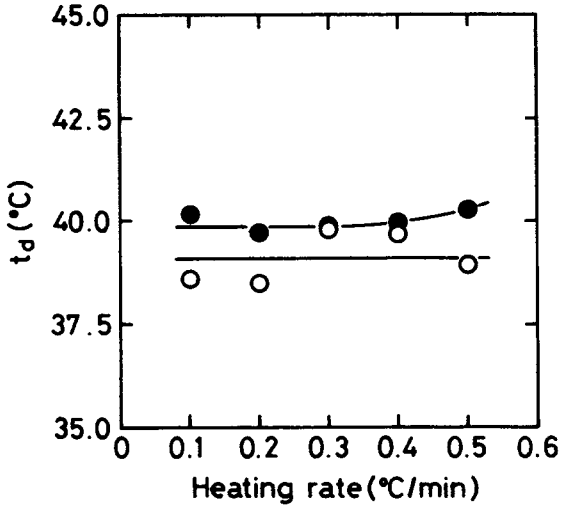

Figure 4 Variation of the thermal denaturation temperature, t_d, with the heating rate for pepsin-solubilized collagen, PC1: (○) measured by the pH, t_{dpH}; (●) measured by the optical rotation, $t_{d\alpha_D}$. Concentration of collagen: 0.18–0.20%; solvent: 1 m*M* HCl.

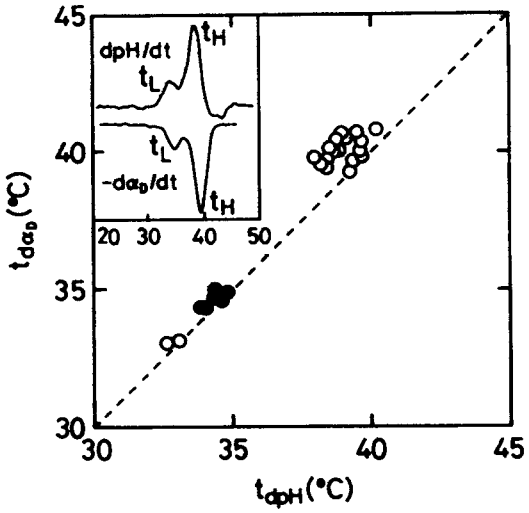

Figure 6 Relationship between the thermal denaturation temperature determined by the pH, t_{dpH}, and the optical rotation, $t_{d\alpha_D}$, for various collagen preparations: (○) the higher thermal denaturation temperature, t_H; (●) the lower thermal denaturation temperature, t_L. Solvent: 1 m*M* HCl.

ferential curves. However, for PC1, two kinds of peaks were observed on the plots of $-dt\alpha_D/dt$ vs. t, but the peak at the t_L was not detected in the dpH/dt curve. To examine the correlation between the t_{dpH} and the $t_{d\alpha_D}$, the $t_{d\alpha_D}$ was plotted against the t_{dpH}. It was observed that the correlation existed between them, whose t_L were measured for other collagens except for ASC, AC, and PC1 and whose t_H were measured for all collagens listed in Table I, as shown in Figure 6. The correlative equation was determined by a least-squares method and is shown as follows:

$$t_{d\alpha_D} = 1.108\ t_{dpH} - 3.201\ (\gamma = 0.9811)$$

As they agreed with each other within about 1–2°C and the correlative coefficient, γ, was 0.9811, the temperature dependence of the pH was useful for the determination of the t_d. In this measuring method of the t_d, the data were fed automatically into a personal computer and the temperature for the maximum on the differential curve calculated from these data was determined as the t_d; so, it was clarified that the t_d of solubilized collagens that denatured in two steps was determined more easily and more rapidly by using of this method than that by measuring the temperature dependence of the α_D. Therefore, this is a new method for detecting the t_d of collagens in solution.

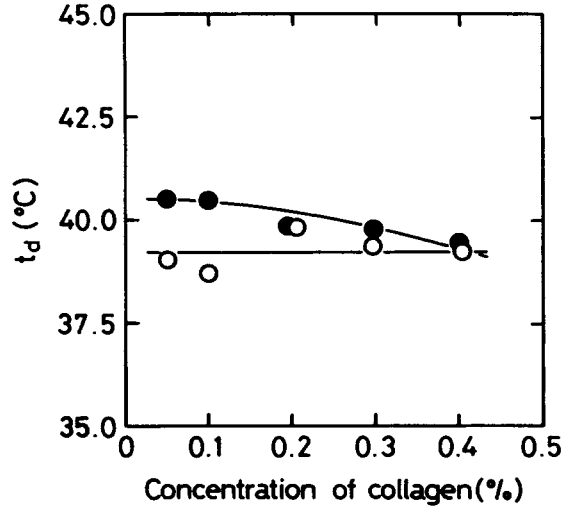

Figure 5 Variation of the thermal denaturation temperature, t_d, with the concentration of pepsin-solubilized collagen, PC1: (○) measured by the pH, t_{dpH}; (●) measured by the optical rotation, $t_{d\alpha_D}$. Heating rate: 0.3°C/min; solvent: 1 m*M* HCl.

CONCLUSIONS

Collagen is not stable to thermal treatment in solution and is easy to be denatured. The determination of the t_d is important to prepare solubilized collagens and to utilize them in industry. The mea-

surement of the temperature dependence of the pH was useful for the determination of the t_d for various collagen preparations in solution, because the $t_{d\text{pH}}$ and the $t_{d\alpha_D}$ agreed with each other to within about 1–2°C and the correlative coefficient, γ, between both was 0.9811. As the determination of the t_d was carried out automatically by using a personal computer, the t_d was determined more easily and more rapidly by using this method than by measuring the temperature dependence of the α_D. Therefore, we developed the new measuring method of the t_d for various collagen preparations in solution.

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