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Thermal Stability of Collagen in Intervertebral Disc Tissues

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Abstract—The thermal stability of collagen in intervertebral disc tissues was studied using differential scanning calorimetry. It was found that the melting of collagen in a native tissue was complete at 62–75°C (ΔH = 62.4 J/g) under heating excised annulus fibrosus and nucleus pulposus samples. On heating an intact structure up to 80°C, the denaturation of collagen did not occur. It was shown that the degradation of a proteoglycan component in the test tissues had no effect on the thermal stability of collagen.

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New technologies, such as intradiscal thermal annuloplasty, are in active current use in medicine for the treatment of spine diseases [1]. For the successful use of this technology, it is of importance to study processes that occur in the connective tissues of an intervertebral disc under laser heating. The intervertebral disc is composed of an annulus fibrosis and a nucleus pulposus. Their main structural component is the fibrillar protein collagen immersed in a gel of proteoglycans [2].

The thermal stability of collagen has long been studied. The collagen–water systems with various component concentrations and intact collagen have been considered [3, 4]. It was noted that, in the tropocollagen–water (10–98%) system and in intact collagen with various moisture contents, the temperature of denaturation increased with decreasing percentage of water, whereas the heat of denaturation of intact collagen was independent of the rate of heating. Miles and Ghelashvili [5] reported the quantitative characteristics of the transition and data that demonstrated a relationship between the heat of collagen denaturation ΔH_m, the denaturation temperature T_m, and the degree of hydration. The heat effect of denaturation increased with water content. Thus, at water contents from 0.07 to 1 mol, the heat of collagen denaturation was 11.6 J/g dry sample, whereas this value increased to 58.55 J/g dry sample at water contents from 30 to 460 mol.

The denaturation of collagen is a first-order phase transition [6]. The occurrence of two phases (crystalline and amorphous) is characteristic of collagen, as supported by X-ray diffraction analysis. In the course of denaturation under equilibrium conditions, the volume and temperature of the system remained constant; this is the characteristic property of first-order phase transitions. This condition for collagen is met in the case of very slow crystallization or very slow melting of the protein, as well as in the combination of these conditions. The last traces of crystallinity always disappeared at a strictly specified temperature because the melting of polymers occurs over a narrow temperature range. Mandelkern [6] noted that the fibrillar tension affect the structure stabilization of collagen fibers and increases the denaturation temperature of collagen, that is, enhances its thermal stability.

Bass et al. [7] studied the thermal behaviors of both an entire system that included vertebral parts and an intervertebral disc and excised annulus fibrosus fragments. They found that, on heating both of the materials to 85°C in an aqueous medium, collagen denaturation did not occur in the vertebrae–intervertebral disc system, whereas collagen underwent complete denaturation in the excised sample of annulus fibrosus tissue. Thus, the integrity of the vertebral system affects the thermal stability of collagen. The fibrillar tension, which takes place in the entire vertebrae–intervertebral disc system and is fully absent from the excised fragment of annulus fibrosus tissue, causes an increase in the temperature of denaturation. An alternative explanation may consist in the effect of proteoglycans, which stabilize the matrix. However, the thermal behavior of the proteoglycan component of tissues is not clearly understood.

This work was devoted to a study of the effect of glycosaminoglycans and proteoglycans on the thermal stability of collagen in the annulus fibrosus and nucleus pulposus tissues of an intervertebral disc.

MATERIALS AND METHODS

Sample Preparation

The intervertebral discs and the tissue samples of the annulus fibrosus and nucleus pulposus of an intervertebral disc were excised (post mortem after no longer than 5 h) from the tails of 12-month-old or younger calves. Vertebral segments (1 cm in height and

1–2 cm in diameter) containing an intervertebral disc were mechanically separated from and chords and muscle tissues. The samples of annulus fibrosus and nucleus pulposus tissues were also mechanically extracted. Visually, the tissue structure was homogeneous with no damages. The entire intervertebral discs were studied immediately after the removal. A portion of the samples of annulus fibrosus and nucleus pulposus tissues was dried at room temperature after the removal and kept at –20°C. These storage conditions did not cause structural changes and had no effect on the thermal properties of the tissues [8]. Tissue fragments of size $3 \times 3 \times 1$ mm were used for studying the isolated annulus fibrosus and nucleus pulposus tissues of the intervertebral disc. Before studying by differential scanning calorimetry (DSC), all of the samples were kept in a 0.15 M NaCl solution for swelling.

Determination of Collagen Concentrations

To perform analysis for collagen, the samples were hydrolyzed with a mixture of concentrated (12 M) hydrochloric acid and fluoroacetic acid (99 wt %) in a volume ratio of 2 : 1 for 1 h, and the solution was evaporated in a LABCONCO concentrator. A Hitachi 835 amino acid analyzer (Japan) was used for determining the amino acid composition of the hydrolyzate. In this case, cation-exchange separation and a spectrophotometric reaction with ninhydrin were preliminarily performed in accordance with a standard procedure [9]. The collagen content was determined from the amount of the collagen-specific amino acid hydroxyproline (Hyp), which accounts for 13.3% of the molecular weight of collagen [10].

To determine the hydroxyproline content of nucleus pulposus tissues after enzymatic treatment, we used a spectrophotometric reaction with *p*-dimethylaminobenzaldehyde after the preliminary selective oxidation of hydroxyproline with chloramine in an evaporated hydrolyzate [11].

Determination of Glycosaminoglycan Concentrations in the Samples

The concentrations of glycosaminoglycans were determined using a spectrophotometric reaction with dimethylmethylene blue in accordance with a published procedure [12]. Dry samples of weight 3 to 10 mg were transferred into solution with the use of papain (2.9 mU/mg, 2.4 mg/ml) in an incubation buffer solution (EDTA, 25 mmol/l; streptomycin, 200 μg/ml; penicillin, 200 U/ml; and NaCl, 0.15 mol/l) for 4 h at 60°C. An aliquot portion (50–100 μl) of the test solution and 2.5 ml of a dimethylmethylene blue solution with a concentration of 16 mg/l (Basic Blue 24; Sigma, Germany) were placed in a spectrophotometric measurement cell. The absorbance of solution was measured on a Varian Cary 3E spectrophotometer at a wavelength of 540 nm against a reference solution of dimethylmethylene blue. Calibration was performed with solutions of chondroitin sulfate A (Sigma) with concentrations of 125 to 500 μg/ml.

Enzymatic Treatment of the Tissues

The samples of annulus fibrosus and nucleus pulposus tissues were treated with 1.5 ml of an enzyme solution at 37°C for a day. The solutions of the enzymes trypsin (Sigma) with a concentration of 1 mg/ml and chondroitinase ABC (Sigma) with a concentration of 0.2 mU/ml in the incubation buffer.

Thermal Treatment

Vertebral segments containing the intervertebral disc were placed in a 0.15 M NaCl solution preheated to 80°C and kept in this solution for 15 min. Upon completion of the thermal exposure, the samples of annulus fibrosus and nucleus pulposus tissues were mechanically removed from the intervertebral discs.

Differential Scanning Calorimetry (DSC)

The thermal behavior of the samples was studied on a Mettler Toledo DSC 822e differential scanning calorimeter. The samples of weight 5–10 mg were tightly closed in standard aluminum dishes (40 ml). An analogous empty dish was used as a reference sample. The heating from 25 to 100°C was performed at a rate of 10 K/min. The heat effect was referred to the collagen content.

RESULTS AND DISCUSSION

Biochemical Analysis

Table 1 summarizes data on the concentrations of collagen and glycosaminoglycans obtained in both intact and enzymatically treated samples.

Data on the concentrations of main components in intervertebral disc tissues obtained in intact samples are consistent with published data [2]. The Hyp/Hyl ratio allowed us to determine the type of tissue collagen. This value varied from 14 to 22 or from 4.2 to 6 for type I collagen or type II collagen, respectively. The experimental results suggest that nucleus pulposus tissues mainly contained type II collagen, whereas annulus fibrosus tissues contained both type I and type II collagen [13]. This is consistent with the results of immunochemical analysis [14].

Trypsin is an endogenous proteolytic enzyme, which catalyzes the hydrolysis of peptide bonds containing lysine and arginine residues. Enzymatic treatment with trypsin causes the defragmentation of proteoglycans by degrading their core protein and facilitates the transfer of individual oligopeptides with attached glycosaminoglycans into solution [15].

Table 1. Biochemical composition of intact and enzymatically treated annulus fibrosus and nucleus pulposus tissues from the intervertebral disc

Tissue	Treatment	Amount of Hyp per dry residue (mg/100 mg)	Hyp/Hyl ratio	Concentration of chondroitin sulfate based on dry sample weight (%)
Annulus fibrosus (AF)	Intact sample	9.6 ± 0.5	8.7 ± 1	10.28 ± 1.46
	Chondroitin ABC lyase	8.5	9.6 ± 1	5.19 ± 0.66
	Trypsin	9.0 ± 0.5	6.6 ± 1	5.81 ± 4.70
Nucleus pulposus (NP)	Intact sample	4.5 ± 0.5	5.8 ± 0.5	39.97 ± 1.17
	Chondroitin ABC lyase	9.0 ± 0.5	–	8.24 ± 0.34
	Trypsin	7.1 ± 0.5	–	8.16 ± 0.59

Chondroitinase ABC is a specific enzyme that cleaves 4- and 6-chondroitin sulfate and dermatan sulfate. This effect of the enzymes explains a dramatic decrease in the concentration of chondroitin sulfate in the annulus fibrosus and nucleus pulposus tissues of the intervertebral disc after the treatment of the tissues with trypsin and chondroitinase ABC.

Thermal Behavior of Annulus Fibrosus and Nucleus Pulposus Tissues

Figures 1 and 2 show the thermograms of the intact and enzymatically treated annulus fibrosus and nucleus pulposus samples of the intervertebral disc. Table 2 summarizes the heat effects and characteristic temperatures.

The heat effect (ΔH) is close to the enthalpy ΔH_m of collagen melting. Any effects were absent from thermograms upon repeatedly heating. For example, the thermogram of the repeated heating of a sample of native nucleus pulposus tissue is given (Fig. 1, curve *4*). After heating in a calorimeter cell, all of the samples fully dissolved in trypsin, which does not affect intact collagen fibrilles but acts on only degraded polypeptide collagen chains [8]. All of the above facts demonstrate that the complete denaturation of collagen occurred upon thermal action on the fragments of intervertebral disc tissues.

It is likely that the difference in the heat effects of collagen denaturation for the annulus fibrosus and the nucleus pulposus is related to different stabilities of collagen in these tissues. In turn, the stability of collagen depends on different degrees of matrix organization [16]. In the nucleus pulposus, the degree of aggregation of proteoglycans is low; therefore, the collagen and proteoglycan components are weakly interrelated. In the annulus fibrosus, proteoglycans form aggregates with hyaluronic acid, and the degree of interaction between two subsystems is much higher [2]. This fact was supported by a lower sensitivity of the characteristics of collagen melting (T_m and ΔH_m) in the annulus fibrosus to treatment with chondroitinase ABC, which degrades the glycosaminoglycans of a proteoglycan component.

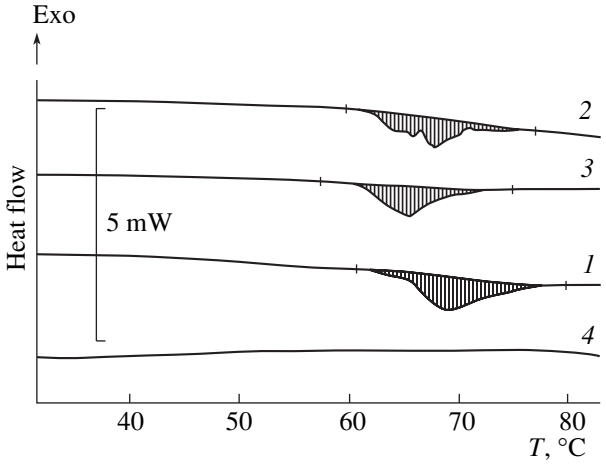

Fig. 1. Typical DSC traces of nucleus pulposus tissues: (*1*) intact sample, (*2*) treatment with chondroitinase ABC, (*3*) treatment with trypsin, and (*4*) repeated heating of an intact sample.

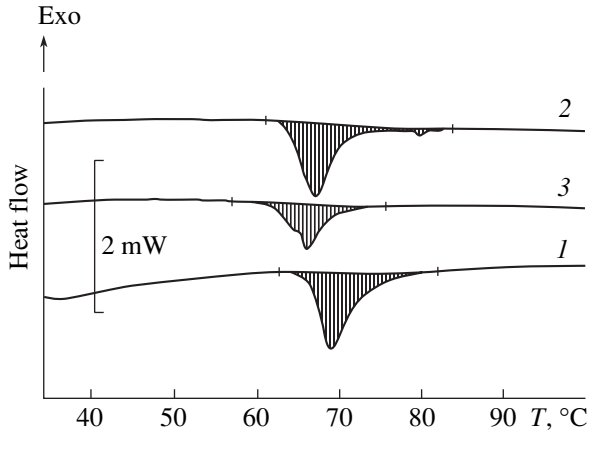

Fig. 2. Typical thermograms of annulus fibrosus tissues: (*1*) intact sample, (*2*) intact, and (*3*) enzymatically treated samples.

Table 2. Characteristics of transitions in intact and enzymatically treated annulus fibrosus and nucleus pulposus tissues

Sample	Peak temperature T_p, °C	Heat effect of collagen denaturation ΔH_m, J/g
1(NP) intact	68.44	62.4
2(NP) chondroitinase ABC	67.27	61.5
3(NP) trypsin	65.09	53.6
4(AF) intact	68.7	57.5
5(AF) chondroitinase ABC	65.5	55.2
6(AF) trypsin	65.7	32.1

Trypsin also facilitates the removal of the stabilizing proteoglycan component from tissues. However, DSC demonstrated a considerable decrease in the heat effect of collagen denaturation in this case. It is likely that trypsin modifies a collagen network to cause this considerable change in the heat effect, as compared with that in the native samples of the annulus fibrosus and nucleus pulposus tissues of the intervertebral disc. The heat effect decreased most strongly in the annulus fibrosus tissues of the intervertebral disc. It is likely that, in this case, the fibrillar network of collagen exhibits a very high organization [2], and enzymatic treatment with trypsin results in a considerable change in this organization.

The complete denaturation of constituent collagen is common to all of the samples of the annulus fibrosus and nucleus pulposus tissues of the intervertebral disc (both intact and subjected to enzymatic treatment).

Effect of Thermal Treatment on Intact Intervertebral Discs

In the treatment of annulus fibrosus and nucleus pulposus tissues with trypsin after the thermal treatment of an intact intervertebral disc, the dispersion of these tissues in an enzyme solution was observed. However, thermal analysis data indicate that collagen macromolecules retained a triple-strand structure. Figure 3 shows the thermograms of annulus fibrosus samples after thermal treatment and the thermograms of the dispersions of these tissues obtained after enzymatic treatment with trypsin. The heat effects and peak temperatures for these samples are almost coincident with the corresponding characteristics of the samples of native annulus fibrosus tissues. Thus, the heat effect of denaturation of annulus fibrosus collagen after thermal treatment was 20.92 J/g. For a dispersion, the heat effect of collagen denaturation was equal to 16.21 J/g; however, these data were obtained with a moist sample. Note the same tendency for a decrease in the heat effect of collagen denaturation after treatment with trypsin as that found previously for the fragments of annulus fibrosus tissues treated with the specified enzyme.

However, heat effects in nucleus pulposus collagen were much lower than in intact samples. The heat effect of denaturation for nucleus pulposus collagen subjected to thermal treatment was 19.62 J/g, whereas the heat effect of collagen denaturation for a dispersion was equal to 2.8 J/g. Data on the dispersion of nucleus pulposus tissue are also given for a moist sample. Figure 4 shows the thermograms of nucleus pulposus tissues.

This considerable difference between the thermal behaviors of the tissues of an entire intervertebral disc system and isolated annulus fibrosus and nucleus pulposus parts can be explained by the fact that the tension of a fibrillar collagen network was retained in the entire intervertebral disc. Therefore, collagen denaturation did not occur even on heating the tissues to 80°C and exposing them at this temperature for a long time. Indeed, the application of a tension force along the main axis of a collagen fiber caused an increase in the melting temperature of collagen as the load was increased. For example, at a tension force (p) corre-

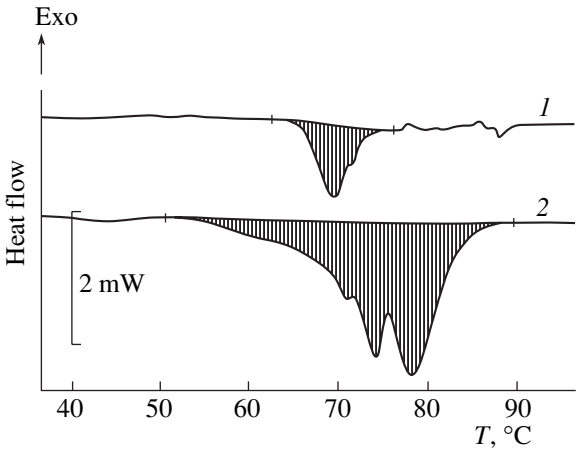

Fig. 3. DSC traces of the annulus fibrosus: (*1*) subjected to thermal treatment and (*2*) as a dispersion.

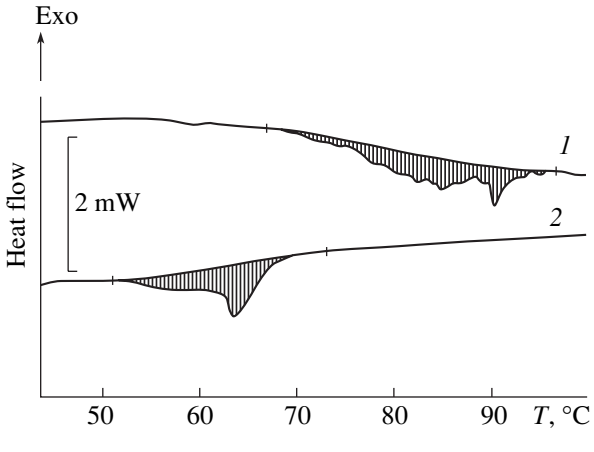

Fig. 4. DSC traces of the nucleus pulposus: (*1*) subjected to thermal treatment and (*2*) as a dispersion.

sponding to 2 atm, melting occurred at 65°C, whereas the melting point (T_m) increased to 80°C at $p = 8.8$ atm [6]. An analogous result was presented by Aksan and McGrath [17], who found that the possibility of denaturation at a constant temperature depends on the load applied to the test sample. Thus, at 67°C, denaturation in ligament tissue samples did not occur at $p \geq 6$ atm, whereas collagen underwent complete denaturation at $p \leq 1.92$ atm. In the nucleus pulposus, the fibrillar collagen network is much less pronounced, a rigid tissue structure is absent, and a portion of collagen undergoes denaturation on heating. This explains the decrease in the heat effect of denaturation on the subsequent heating in the cell of the DSC calorimeter.

In general, the above experiments demonstrated that glycosaminoglycans weakly affect the thermal stability of collagen because the heat effect of denaturation in the samples treated with chondroitinase ABC differed only slightly from the heat effect in intact samples. In this case, it is likely that enzymatic treatment with trypsin caused a modification of the collagen network because the heat effect in these samples was much lower than that in the native tissues.

The above analysis suggests that the integrity of the fibrillar structure of collagen networks strongly influences the heat effect of collagen denaturation in the annulus fibrosus and nucleus pulposus of an intervertebral disc. Collagen in the tissues that retained the fibrillar network did not undergo denaturation under thermal exposures, whereas collagen in the fragments of annulus fibrosus and nucleus pulposus tissues readily underwent denaturation on heating. Collagen fibrilles are concentrically arranged over the entire disc; this fact is responsible for the high stability of the entire system to external thermal actions. However, the above experiments demonstrated that the thermal treatment of an entire intervertebral disc caused the degradation of components that bound the collagen network. This was supported by the dispersion of thermally treated tissues under the action of trypsin.

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