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Alteration of the Thermodynamic Characteristics of Corneal Collagen Denaturation as a Result of Nonenzymatic Glycation

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Abstract—The thermal stability of the scleral and corneal tissues after in vitro treatment with ribose, threose, and glyceraldehyde was investigated. The thermal transition temperature and the enthalpy of collagen fiber crosslinking were determined by differential scanning calorimetry (DSC). The resistance of the tissues toward trypsin was also determined after heating tissue samples in the DSC furnace. It was shown that the denaturation temperature of scleral and corneal samples treated by crosslinking agents increased, but the enthalpy of denaturation decreased. It is suggested that crosslinking in the collagen matrix of the cornea and sclera prevents complete collagen denaturation if the temperature does not rise up to 110°C.

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Pathologies in the structure of collagen fibers brought about by high myopia are due to decreasing crosslink density in the sclera. As a result, the biomechanical properties of the sclera are disturbed and the pathology progresses irreversibly [1, 2].

The techniques being developed for the prevention and treatment of progressive myopia are based on scleroplastic operations or treatment of the scleral or corneal tissue with crosslinking agents [1]. However, the introduction of advanced techniques into medical practice is impossible without careful in vitro and in vivo investigations.

Collagen is the basis of the intracellular matrix of the cornea and sclera. The overall collagen content of the sclera and cornea is, respectively, some 80 and 90% based on dry weight. Collagen fibrils in the cornea form a unique structure: they are stacked in lamellas lying strictly parallel, which is responsible for the exclusive transparency of the corneal tissue. Fibrils are not orderly arranged in the sclera; therefore, the sclera is untransparent, and alteration of its structure does not change the properties of the eyeball [3].

In the sclera, as in other collagen-containing tissues, collagen macromolecules can be crosslinked by monosaccharides, their metabolism products, and other compounds containing aldehyde and alcoholic functionalities. Crosslinking can occur naturally in the aging body, as a result of diabetes mellitus, and under the action of intentionally introduced reagents. This complex multistage process starts with aminocarbonylation, which produces Schiff bases (aldimines), the latter undergoing intramolecular rearrangement to 1-amino-1-deoxyketoses (Amadori compounds). Subsequent dehydration, condensation, spontaneous degradation of Amadori compounds to yield more reactive saccharoses (tetroses, pentoses, and deoxyglucosones), fragmentation, oxidation, and cyclization produce various nitrogen- and oxygen-containing heterocyclic compounds [4, 5].

Yellow-brown final glycation products are formed as a result of these processes [6]. With increasing crosslink density in collagen fibers, the Young modulus increases [7] and the thermostability and stability toward proteolytic enzymes rise [7–9]. An improved thermal stability is expressed as an increased denaturation temperature T_d.[1] Thus, T_d is an important criterion, a measure of the crosslink density of collagen fibers.

This work is intended to investigate the thermal stability of the sclera and cornea, the alteration of the T_d of collagen, and the stability toward trypsin of the scleral and corneal tissues treated with crosslinking agents.

MATERIALS AND TECHNIQUES

Tissue Sample Preparation

Sclera and cornea tissue from rabbit were obtained no later than 6 h post mortem. Sclera and cornea samples were washed with water, dried in air for 24 h, and cut into samples 3–4 mg in weight based on dry weight.

Treatment with Crosslinking Agents

Tissue samples 5–10 mg in weight were treated with 1 M solutions of monosaccharides (from Sigma) in an incubation buffer (0.15 mol/L NaCl, 25 mmol/L EDTA

[1] The denaturation temperature is the temperature at which the ordered structure of the collagen triple strand is destroyed to form a random coil.

Table 1. Water and collagen contents in intact samples ($P = 0.95$, $n = 3$)

Tissue	TGA water content, wt %	Collagen water content in intact samples, wt %
Sclera	19.3 ± 2.5	64.56 ± 2.0
Cornea	15.2 ± 3.0	76.32 ± 2.7

(from Quality Biological, Inc.), 5000 penicillin units, 5 mg streptomycin (from PanEco) per 1000 mL of the buffer with pH 7.4).

Three sclera and three cornea samples were used in each experiment. Crosslinking was performed as follows: (1) with ribose for 1 and 5 days at 37°C, (2) with threose for 5 days at 37°C, and (3) with glyceraldehyde for 12 h at 25°C.

Differential Scanning Calorimetry (DSC)

Tissue samples 1–5 mg in weight based on dry weight were sealed in an aluminum cap. An empty cap was used as the reference. Thermal analysis was carried out by differential scanning calorimetry (DSC) on a Mettler Toledo DSC 822 instrument at temperatures from 25 to 110°C. The temperature variation rate was 10 K/min.

Thermogravimetric Analysis (TGA)

In order to determine the residual water content, air-dried 1–5 mg tissue samples were heated in the cell of a Mettler Toledo TGA 851 thermogravimetric analyzer at 10 K/min in the range 40–160°C; then, the samples were exposed for 15 min at 160°C. The residual was used to normalize DSC peak areas.

Enzymatic Treatment of Denatured Issues

After recording DSC traces, sclera and cornea samples heated in the DSC furnace were treated with 1% aqueous solution of trypsin at room temperature for 24 h in order to evaluate the collagen denaturation depth during heating. Trypsin is known to split (solubilize) only fully denatured collagen fibrils with the triple strand, and it does not affect collagen that has retained its tertiary structure [10, 11].

RESULTS AND DISCUSSION

Table 1 displays the residual water content in dried samples of intact issues as determined by TGA and the collagen percentage in dry tissue samples calculated using data from [3].

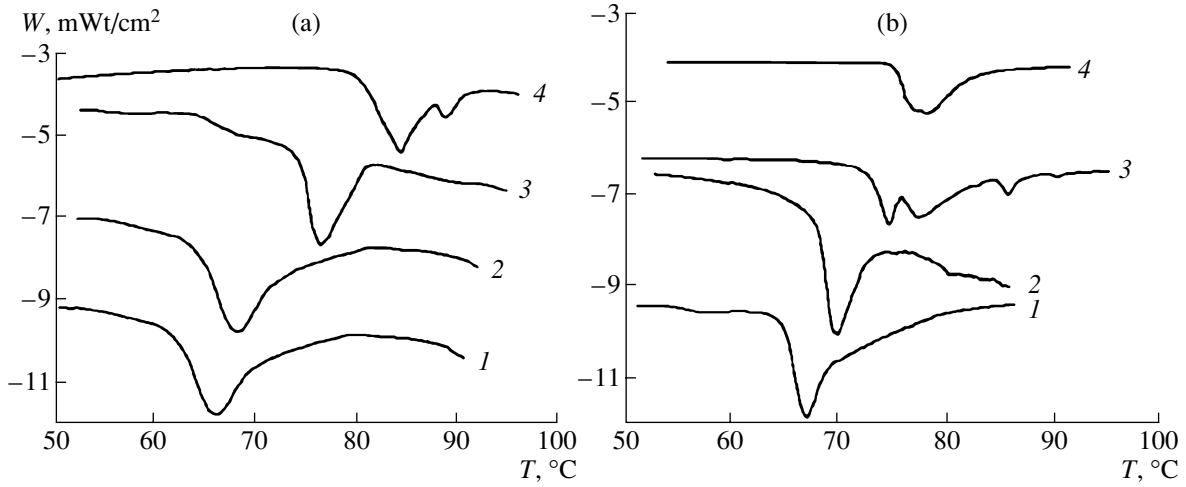
Table 2 compiles the denaturation temperature and the heat of denaturation for sclera and cornea samples, as well as the solubilities of samples in trypsin after heating inside the calorimeter furnace. For the crosslinked samples, the heats of denaturation were calculated on the assumption that the collagen content of dry crosslinked samples does not differ substantially from the collagen content in well dried intact tissues.

Intact Sclera and Cornea Samples

Example DSC traces for intact sclera and cornea samples are displayed in the figure (curve *1*). The endothermic peaks are clearly seen in the DSC trace; their peak temperatures are 66–67°C. The transition parameters (temperature and heat) are indicated in Table 2. The heat referred to pure collagen is 54.2 ± 2.4 J/g for the sclera and 49.8 ± 3.1 J/g for the cornea, which corresponds to collagen denaturation (ΔH_m for collagen containing some water ranges from 45 to 65 J/g [12]). Heated samples dissolve in trypsin, which indicates the full denaturation of their collagen.

Table 2. Temperatures and heats of denaturation for sclera and cornea samples before and after crosslinking ($P = 0.95$, $n = 3$)

Sample	Crosslinking agent	T_d, °C (peak)	ΔT_d, °C (range)	ΔH_{denat}, J/g, for issue	ΔH_{denat}, J/g, for collagen	Solubility in trypsin
Sclera	No (blank)	66.0 ± 0.5	62–74	35 ± 0.5	54.2 ± 2.4	Complete
	Rib (1 days)	66.7 ± 0.5	63–73	34 ± 0.5	52.7 ± 2.5	Complete
	Rib (5 days)	68.0 ± 0.4	67–75	30 ± 0.6	46.5 ± 2.3	With residue
	Thr (5 days)	76.0 ± 2.1	74–84	27 ± 0.4	41.8 ± 1.8	Poor
	Gly (12 h)	83.0 ± 3.1	80–92	25 ± 0.7	38.7 ± 2.2	Insoluble
Cornea	No (blank)	67.0 ± 0.5	56–75	38 ± 0.9	49.8 ± 3.1	Complete
	Rib (1 days)	68.0 ± 0.6	59–75	37 ± 0.6	48.5 ± 2.6	Complete
	Rib (5 days)	69.8 ± 0.7	68–76	33 ± 0.4	43.2 ± 2.2	With residue
	Thr (5 days)	72.0 ± 2.3	70–90	28 ± 0.5	36.7 ± 1.9	Poor
	Gly (12 h)	78.0 ± 2.9	74–88	19 ± 2.1	24.9 ± 3.5	Insoluble



Panel (a): DSC traces for sclera samples: (*1*) blanks (intact uncrosslinked samples), (*2*) samples crosslinked by ribose or 5 days, and (*3*) samples crosslinked by threose for 5 days, and (*4*) samples crosslinked by glyceraldehyde for 12 h. Panel (b): the same for cornea samples.

Sclera and Cornea Samples after Ribose Treatment

One-day exposure of samples to a ribose solution followed by DSC and trypsin treatment showed that the temperature and heat of denaturation did not differ significantly and that the samples dissolved in trypsin. After 5 days of exposure in a ribose solution, the peak temperature of the endotherm slightly increased and the heat of collagen denaturation decreased (figure, curve *2*; Table 2). The heated samples did not completely dissolve in trypsin.

Sclera and Cornea Samples after Threose Treatment

After 5 days of exposure to a threose solution, samples became deep yellow, which indicated the formation of final glycation products [6]. Representative DSC traces are displayed in the figure (curve *3*). The temperature range of collagen denaturation noticeably widens and shifts toward higher temperatures, while the heat of denaturation decreases (Table 2). The samples were treated with trypsin in order to control the completeness of denaturation of collagen fibrils; as a result, the tissues were dispersed; however, their full dissolution was not observed.

Sclera and Cornea Samples after Glyceraldehyde Treatment

Samples exposed for 12 h to a glyceraldehyde solution acquired an intense brown color. Representative DSC traces for such sclera and cornea samples are displayed in the figure (curve *4*). The curves make it clear that collagen denaturation in these samples occurs over a wide temperature range.

The peak temperatures rise even more greatly; as expected, the overall heat of the processes is smaller here than in the other samples (Table 2). When exposed to a trypsin solution, glyceraldehyde-treated eye tissue samples heated inside the calorimeter do not dissolve and do not lose their integrity.

Based on the above results, we may infer that glyceraldehyde produces the greatest crosslink density in collagen fibrils in the sclera and cornea and at the highest rate compared to the other reagents used.

The reason for which several peaks were observed in the DSC traces for glyceraldehyde-treated sclera and threose-treated cornea samples is not quite clear. However, we may suggest that these peaks result from the nonuniform distribution of crosslinks in the tissue. Both chemical and diffusion factors can affect the appearance of this nonuniformity.

The character of the interaction between the crosslinking agents used and the intact collagen structure can be inferred from the results we obtained. The ratio between the enthalpy change and entropy change during heating dictates the collagen melting temperature T_d [13]. In an endothermic process, a decrease in the heat of the process enhances the decrease in the phase-transition temperature. In the case at hand, however, the decrease in the heat of the phase transition is accompanied by the increase in the transition temperature. This means that the entropy change upon denaturation has the decisive influence on the collagen denaturation temperature. A decrease in the ΔS of this transition for the treated samples compared to the intact samples is due to the fact that nascent chemical bonds limit the number of possible configurations of collagen chains in the amorphous phase above the denaturation temperature [13]. Thus, the term "crosslinking" seems quite adequate as applied to these bonds. The systematic decrease in the heat of denaturation after crosslinking, whose trend is opposite to that of the denaturation temperature, is another interesting feature. In all probability, this means that not only do newly formed

crosslinks change the state of collagen in the amorphous phase, but they also inhibit full denaturation. This is supported by the very low solubility of the glyceraldehyde-crosslinked samples in trypsin after heating in the calorimeter furnace. A similar situation is described in works [10, 11]. The authors of the cited works, using electron microscopy, noticed the following: cow and sheep skin samples with very high crosslinking densities (these samples were either obtained from very old animals or first reduced with sodium tetrahydroborate) conserved some elements of their ordered fibrillar structure after being heated to 120°C.

In this work, we have proven the suggestion about the possibility of incomplete denaturation of collagen in crosslinked tissues; to this end, we used two autonomous and easy-to-use methods, namely, differential scanning calorimetry and enzymatic treatment.

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