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Influence of oxazolidines and zirconium oxalate crosslinkers on the hydrothermal, enzymatic, and thermo mechanical stability of type 1 collagen fiber

Mahdi A. Haroun[a,*], Palmina K. Khirstova[b], Gurashi A. Gasmelseed[c], Antony D. Covington[d]

[a] Department of Chemical and Environmental Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 UPM, Serdang, Malaysia
[b] People's Hall 11113, P.O. Box 6272, Khartoum, Sudan
[c] Juba University, Leather Dept. P.O. Box 12327 Khartoum, Sudan
[d] Leather Centre, University College Northampton, Northampton, UK

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ABSTRACT

Stabilization of type I rat tail tendon (RTT) collagen by crosslink agent oxazolidine and zirconium oxalate was studied to understand the effect on the thermal, enzymatic and mechanical stability of collagen. The results show that both oxazolidine and zirconium oxalate imparts thermal stability to collagen, and oxazolidine exhibits a marked increase in the peak temperature and enthalpy changes when compared to both native and zirconium oxalate tanned RTT. There is a decrease in the peak temperature and the enthalpy changes of oxazolidine tanned RTT fibers after treatment with urea, suggesting the possibility of alterations in the secondary structure of collagen after tanning. Oxazolidine, which forms carbocationic intermediates species in solution, have better crosslinking with collagen as seen from viscometry studies and hence provides better enzymatic stability to collagen than zirconium, which largely forms monomeric species in solution. Zirconium does not seem to change the tensile strength of RTT fibers significantly in wet condition as well as oxazolidine.

1. Introduction

Collagen is the major structural component of connective tissues. It is an important biomaterial finding several applications as prosthesis, artificial tissue, drug carrier, cosmetics and leather industry [1]. This widespread use of collagen emphasizes the need to understand the mechanism of stabilization of collagen against biodegradation and heat, as these studies will have far reaching implications in both industrial and biological applications of collagen.

Skin matrix is primarily composed of triple helical collagen units; the fibrillar arrangement of these units is stabilized by various non-covalent and covalent intermolecular crosslinks [2–5].

Collagen is resistant to all enzymes except collagenase. Collagenases are a class of metalloproteinase, which are available from different sources. The mode of action of collagenase has been found to be dependent on the source from which it is obtained.

Mammalian collagenase from human-fibroblast cleave collagen at a single site (leu-Ileu) breaking it into a 3/4 and 1/4 fragment [6], whereas, bacterial collagenases from *Clostridium histolyticum* cleave collagen at multiple sites [7]. Various studies have been carried out to understand the activity of collagenase on collagen [7]. Recently, atomic force microscopy (AFM) imaging has been used to directly observe the degradation of type I collagen fibrils by collagenase [8]. The thermal stability of collagen and the influence of various factors on the denaturation temperature of collagen have been studied widely [9,10]. Also the dimensional stability of collagenous tissues has been examined under various environmental conditions to elucidate the role of intermolecular forces in stabilizing molecular forces that lead to mechanical stability [10–12].

It is well known that collagen crosslinked with various crosslinking agents such as aldehydes and metal ions which is made resistant against the degradation by collagenase and also that the thermal and mechanical stability of collagen is increased owing to the crosslinks formation [13]. This crosslinking is the main objective of tanning, a process that converts raw animal hide/skin into leather.

The role of metals as a protein stabilizer has gained an attention in the tanning industry [14,15]. Tanning, as a process introduces intermolecular crosslinks between collagen molecules, thereby improving the thermal, enzymatic and mechanical stability of the fibrous network [16,17]. In zirconium tanning, various theories have been proposed to explain the mechanism of binding of zirconium to collagen. It has been shown that zirconium orthosulfate, which was used in tanning was predominantly anionic or nonionic in character and binds with amino groups of collagen [18]. Williams Wynn

* Corresponding author.
E-mail address: Mahdiupm@hotmail.com (M.A. Haroun).

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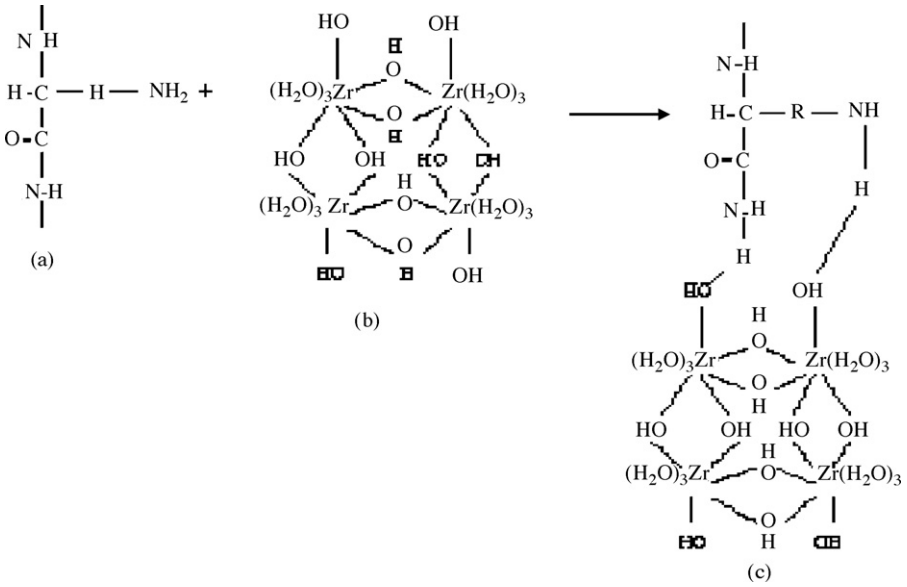

Fig. 1. Schematic representation of the interaction of collagen with zirconium: (a) polypeptide chain of collagen representing the basic amino acid residues; (b) hydrate zirconium negatively charged because of the presence of hydroxyl groups; (c) complex of hydrated zirconium and polypeptide chain of collagen, exhibiting charged interactions.

[19] studied the fixation of zirconyl chloride and suggested that it formed coordination compounds with carboxyl groups in protein.

Covington [20] said that zirconium salts are characterized by eight coordination and high affinity for oxygen, resulting in a tetrameric core structure; the basic unit of structure is four Zr ions at the corners of a square, linked by diol bridges, above and below the plane of the square (Fig. 1). By hydrolysis or basification, the tetrameric units can polymerize, by forming more diol or sulfato bridges. In this way, zirconium species may be cationic, neutral or anionic and large ions can form. So tanning may involve all the polar side chains of collagen, those bearing carboxy, amino or hydroxyl groups (Fig. 1) [20].

On the other hand oxazolidines (Fig. 2) are heterocyclic derivatives obtained by the reaction of amino-hydroxy compounds with aldehydes. It was observed that oxazolidines react with the hide protein and impart special characteristics to leather when tanned in combination with synthetic tanning agents (syntans), chrome and vegetable tanning materials. The bifunctional behaviour of these oxazolidines is responsible for their reaction with proteins.

It has been postulated that a carbocationic intermediate may be provided as a result of ring opening to form an *N*-hydroxymethyl compounds, which then is able to interact with collagen amino side chains through the formation of covalent bonds in a pH dependent manner (Fig. 2) [20].

There are number of methods available for examining the physical state of collagen in solution. But those measurements are not applicable for fibrous tissue itself. Differential scanning calorimetry (DSC) is a useful technique to characterize collagen with various crosslinking agents. Crosslinks cause an increase in the melting temperature because they decrease the entropy of melting transition [21]. DSC has also been applied to the study of the denaturation process which occurs in biological macromolecules such as collagen [12,22]. The isothermal measurement of rate of collagen denaturation, measured continuously using calorimetric method was used to determine rate constants for collagen denaturation in tendons.

In the present study, an attempt has been made to understand the stabilization of collagen by oxazolidine and zirconium oxalate, to study the role and effect of coordinate and covalent crosslink on the hydrothermal, enzymatic and thermo mechanical stability of collagen. Rat tail tendon (RTT), which contains predominantly type 1 collagen, has been chosen for the study. Oxazolidine and zirconium oxalate are widely used in the leather industry as tan-

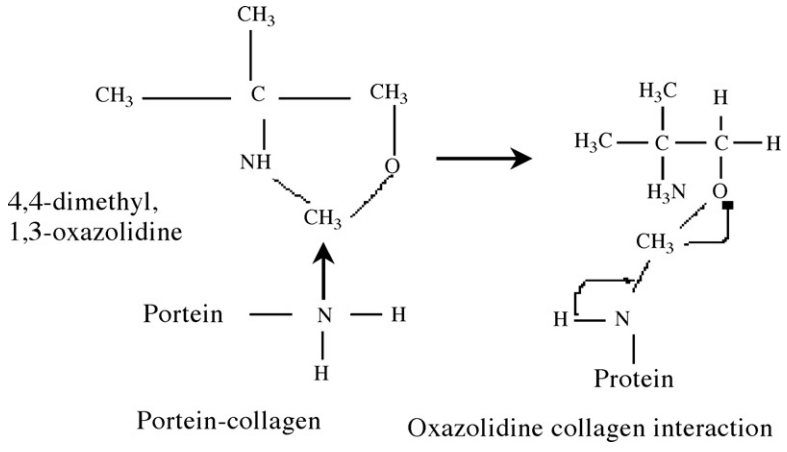

Fig. 2. Schematic representation of the interaction of 4, 4-dimethyl-1, 3-oxazolidine with protein collagen.

ning agents to convert the raw hides and skins into leather. Hence, understanding the effect of them on the dimensional stability of collagen will also aid in understanding the process of tanning. The thermal stability of collagen has been investigated by measuring the hydrothermal shrinkage of the collagen fibers, the characteristic temperature at which collagen fiber shrinks to one-third its original length and by differential scanning calorimetric (DSC) measurements. DSC allows the study of enthalpy changes, energy of activation and hydrothermal isometric tension associated with collagen transitions in RTT collagen fiber which are associated with the denaturation of collagen, together with the temperature at which the phenomenon occurs [12,22]. The enzymatic stability of collagen has been ascertained by estimating viscosity and the amount of hydroxyproline released from collagen after subjecting it to collagenase degradation.

2. Materials and methods

2.1. Materials

Zirconium oxalate (C_4O_6Zr, molecular weight 267.2592) was supplied by Solon (OH, USA). Oxazolidine (4,4-dimethyl-1,3-oxazolidine) from Angus Chemicals (Chicago, USA). *C. histolyticum* type I collagenase purchased from Sigma Chemicals Co., USA were used. All other chemicals were of analytical grade.

2.2. Preparation of rat tail tendone (RTT) fibers

Collagen fibers teased out from the tails of 6 months old albino rats (Wistar strain) were used for the study. Teased collagen fibers were washed with 0.9% NaCl at 5 °C, to remove the adhering soluble proteins. The RTT was washed extensively with deionized water at 5 °C. Four different set experiments of tanning were carried out with these fibers.

Two set of the tendons were treated with 2 and 5% zirconium oxalate at pH 2.5 for 24 h, respectively. And another set was tanned with 2 and 5% oxazolidine (Neosyn TX) at pH 6 and 8 for 24 h.

2.3. Measurement of samples

The diameter of the fibers in wet condition was measured using a filar micrometer attached to an optical microscope. Area of cross-section of the fibers was calculated from the diameter assuming a cylindrical shape for the fiber. Areas of cross-section were compiled from the average of diameters measured at least in five locations along the length of the fiber.

2.4. Differential scanning calorimetry studies for thermal properties

The thermal shrinkage temperature of native, oxazolidine, and zirconium oxalate tanned RTT fibers were studied by using a differential scanning calorimeter (DSC 7, PerkinElmer). The temperature of the instrument was calibrated using indium as the standard. The range of heating of the sample from 30 to 100 °C. The samples were first air dried at ambient temperature and the weights recorded. 1–2 mg of RTT were crosslinked in oxazilidine (2, 5, 9 and 12%) solution at pH 5 and 8 and zirconium oxalate (2, 5, 9 and 12%) solution at pH 2.5 for 24 h, then oxazolidine and zirconium tanned RTT fibers were swollen in water and 6 M urea solution for a period of 24 h. The samples were sealed in a DSC cell and heated at a constant rate of 5 °C/min. The peak temperature T_p and the onset temperature T_s (in °C), and the enthalpy changes ΔH (in J g^{-1}) associated with the phase change for the shrinkage process for native, oxazolidine, and zirconium oxalate tanned RTT fibers before and after urea treatment were determined [12]. After the DSC curve has been obtained, it was fitted to the following equation:

$$d\alpha/dT = \beta k_0 e^{-E_a/RT}(1-\alpha)^n$$

where α is the degree of chemical reaction, n the order of the reaction, k the reaction rate, and β is the proportionality constant. E_a is the activation energy for the phase transition. This equation represents the theoretical shape of the DSC curve that is determined in PC series, DSC standard program. The resultant values of k_0, E_a and n are calculated [23].

2.5. Hydrothermal tension experiments

Hydrothermal tension experiments were carried out in an Instron testing machine model 1112. Using appropriate load cells in the range of 0.1–500 kg could be measured. The sensitivity of the load cells was 2% at the maximum range. A liquid cell container was kept on the heater whose input supply voltage was adjusted to get a required heating rate of 3 °C/min. The fibers were immersed in the respective solutions in the liquid cell with one end attached to the frame and the other end attached to the load cell. The shrinkage temperature and tension were continuously recorded. As the fiber is held at constant length during the experiment, the temperature at which the tension begins to increase is recorded as the shrinkage temperature (T_s). The temperature at which the tension (which increases progressively with shrinkage) reaches the maximum is the temperature at maximum tension (T_t). The corresponding tension is defined as the isometric tension (I_t) [24,25].

2.6. Viscosity measurements

Viscosity measurements were performed using an Ostwald type viscometer (10-mm diameter) of 2 ml capacity. The viscometer was thermo stated at 25 ± 1 °C. The viscometer was calibrated with 10 and 20% sucrose solution. The intrinsic viscosity of the sucrose solution was found to be in close agreement with the reported values. The Collagen solutions (CCOL, w/w) were prepared by dissolving collagen in 0.5 M acetic acid. Solution with pH value of 2.4 was centrifuged at 9000 × g for 10 min to remove entrapped air-bubbles, the supernatant was then dialysed against several changes of 0.02 M Na_2HPO_4, and the precipitate that formed was collected, dissolved in 0.5 M acetic acid then dialysed extensively against 0.1 M acetic acid. The flow times of collagen samples were measured after a thermal equilibrium time of 30 min. The collagen concentration (0.6×10^{-6} M) was fixed and the measurements were carried out with oxazolidine and zirconium oxalate concentrations of 0.6×10^{-6} to 30×10^{-6} M for both crosslinkers. The viscosity measurement was based on the flow rate of collagen solution through the capillary of an Ostwald viscometer. In these experiments the viscosity contribution (η) due to collagen was measured as a function of the measured concentration of the added complex. The flow time was measured with a digital stopwatch at least three times and the average was taken. The viscosity was calculated from the relation, $(\eta = t - t_o/t_o)$, where t_o is the flow time of buffer (pH 4.2, $I = 2 \times 10^{-2}$ M) and t is the flow time for each sample. Plots of relative viscosity, η/η_o (η and η_o are viscosity of collagen in the presence and absence crosslinkers) vs. [crosslinkers]/[collagen], were calculated [26].

2.7. Studying of enzymatic stability

The treatment of native, oxazolidine and zirconium oxalate treated collagen fibers with collagenase was carried out in 0.04 M $CaCl_2$ solution buffered at pH 7.0 with 0.05 M Tris–HCl. The

collagen–enzyme ratio was maintained at 50:1. The samples were incubated at 37 °C. The designated samples were collected at various time intervals ranging from 20 to 150 h. The cleavage of native, oxazolidine and zirconium oxalate treated with collagen was monitored by the production of the soluble form of hydroxyproline from insoluble collagen [27]. By using the method of Woessner [28] the amount of hydroxyproline (μg) in the collagenase hydrolysate released at different time intervals after acid hydrolysis of the sample was determined.

2.8. Tensile strength and elongation percentage

Tensile strength measurements were made using a liquid cell attachment designed for an Instron testing machine model 1112 and 0–500 g load cell. The sensitivity of the load cell was 2% at the maximum range. The specimen length was 1 cm and the elongation rate used was 0.5 cm min^{-1}. The tensile strength and percent elongation of native, oxazolidine and zirconium oxalate treated RTT in water medium at room temperature were calculated.

3. Results

3.1. Hydrothermal stability analysis

Hydrothermal stability or shrinkage temperature of the collagen fibers is a measure of the stability of the matrix as a whole, which arises due to the long range ordering of the matrix. The shrinkage temperatures and the temperature at maximum tension of the native, oxazolidine, and zirconium oxalate treated with RTT fibers were measured using a differential scanning calorimeter (DSC 7, PerkinElmer). The shrinkage temperature for native collagen fiber was found to be 65 °C (Table 1).

The RTT fibers treated with different oxazolidine (2, 5, 9, and 12%) solution at pH 5 and 8 exhibited stability against wet heat even at low concentration (2%), whereas the RTT fibers treated with 2, 5, 9, and 12% zirconium oxalate produced shrinkage temperature of 73, 75, 75, and 76 °C, respectively (Table 1).

It can be seen from Table 1 that oxazolidine exhibit an increase in the shrinkage temperature when compared to both native RTT and zirconium oxalate.

This enhanced hydrothermal stability of oxazolidine tanned RTT is due to new crosslinks formed and consequent changes

Table 1
Values of shrinkage temperature and temperature at maximum tension on native, oxazolidine, and zirconium oxalate crosslinked RTT fibers.

Specification	Shrinkage temperature (T_s), °C	Temperature at max. tension (T_t), °C
Native RTT in water	65	68
Oxazolidine crosslinked RTT at pH 6		
2% solution	87	>94
5% solution	85	>94
9% solution	86	>94
12% solution	88	>94
Oxazolidine crosslinked RTT at pH 8		
2% solution	87	>94
5% solution	88	>94
9% solution	88	>94
12% solution	89	>94
Zirconium crosslinked RTT at pH 2.5		
2% solution	73	>98
5% solution	75	>98
9% solution	75	>98
12% solution	76	>98

Note: The values are mean ± S.D. of three values.

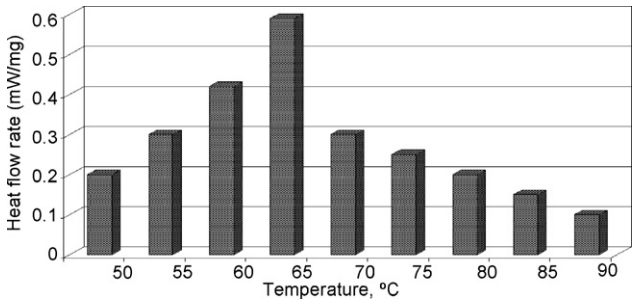

Fig. 3. DSC thermogram of native collagen fiber.

in the tertiary structure of collagen. In the case of oxazolidine tanning there is a general belief that the dimensional changes occurring beyond shrinkage temperature may be partially reversible [29]. This is unique in the hydrothermal stability of tanned RTT fibers.

In case of zirconium an increase in the shrinkage temperature were observed when compared to native RTT. This may be due to a net increase in the number of intermolecular crosslinks arising from electrostatic, co-ordinate, and covalent interactions between the Zr(IV) complexes and collagen.

In order to examine the role of secondary structure in the hydrothermal stability of tanned RTT fibers, oxazolidine tanned RTT fibers are tested in urea solution. It is known that urea is capable of breaking down hydrogen bonds in collagen molecules [30]. The shrinkage temperature of oxazolidine tanned RTT fibers in varying amounts of urea solutions are given in Table 2. The decrease in hydrothermal stability of oxazolidine tanned RTT in urea solution is well known according to the previous studies [30].

In case of zirconium oxalate there is decrease in shrinkage temperature of tanned RTT fibers when tested in varying amounts of urea solutions as compared with that of zirconium oxalate tanned RTT in water. This decrease in the shrinkage temperature in urea solutions reveals alterations in the secondary structure of collagen are feasible even after tanning. The results clearly show that zirconium impart thermal stability to collagen.

There are three possible mechanisms through which zirconium can bind to collagen.

These are (i) polar binding of anionic sites of zirconium species with polar amino groups; (ii) polar binding of cationic sites of zirconium species with polar carboxyl groups; and (iii) co-ordinate and covalent bonding between Zr(IV) ion and oxygen atoms of carboxyl groups present in collagen {the nitrogen atoms of amino or imino groups playing part in such coordination, the evidence of that is zirconium salts are characterized by eight coordination and high affinity for oxygen, resulting in a tetrameric core structure; the basic unit of structure is four Zr ions at the corners of a square, linked by diol bridges, above and below the plane of the square (Fig. 1)} (i.e. zirconium can simultaneously bind to two or more protein strands) [31].

The endothermic phenomenon including heat flow rate of native, oxazolidine and zirconium oxalate tanned RTT fibers was shown in Figs. 3–5.

In these figures a single peak is observed through all the studied figures, which explain an endothermic phenomenon in which a phase transition involving changes in the lattice and long range order is realized.

The peak temperature for the shrinkage phenomenon, the changes in enthalpy and energy of activation associated with the phase change for the native, oxazolidine and zirconium oxalate tanned RTT are given in Table 3.

The peak temperatures for the shrinkage process for native and zirconium oxalate tanned RTT collagen fibers in water are 65 and

Table 2
Hydrothermal shrinkage temperature of oxazolidine and zirconium oxalate (2 and 5%) solution crosslinked RTT fibers in water and different urea solutions.

Specification	Shrinkage temperature ($T_s \pm 1$) for crosslinked RTT fibers (°C) at			
	pH 5		pH 8	
	2%	5%	2%	5%
Oxazolidine crosslinked RTT in				
Water	85	87	87	88
1 M urea	85	84	86	83
3 M urea	82	84	84	86
6 M urea	82	83	84	85
8 M urea	82	83	84	85

Specification	Shrinkage temperature ($T_s \pm 1$) for crosslinked RTT fibers (°C) at pH 2.5	
	2%	5%
Zirconium crosslinked RTT in		
Water	73	75
1 M urea	70	72
3 M urea	69	70
6 M urea	68	69
8 M urea	66	65

Note: The values are mean ± S.D. of three values.

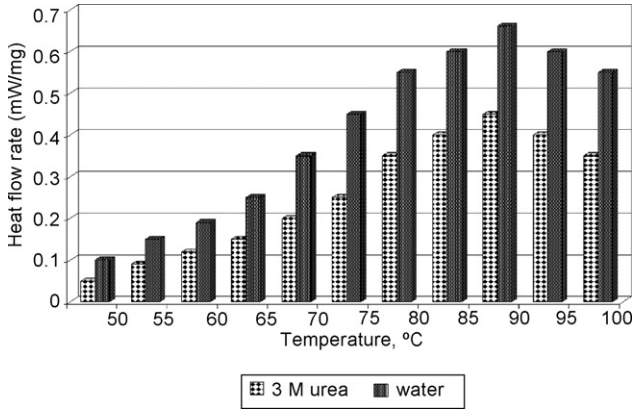

Fig. 4. DSC thermogram of oxazolidine crosslinked collagen fibers in water medium and 6 M urea solution.

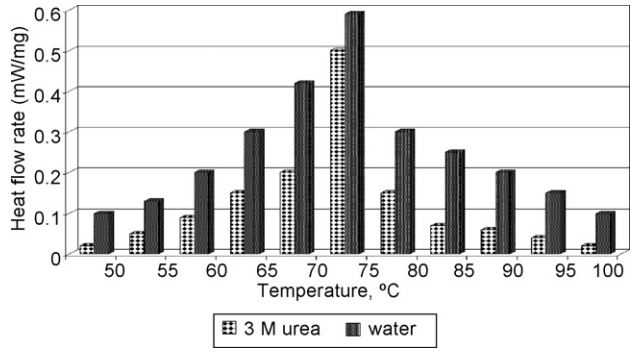

Fig. 5. DSC thermogram zirconium oxalate crosslinked collagen fibers in water medium and 6 M urea solution.

75 °C, respectively. But there is no significant change in the enthalpy values for the native and zirconium oxalate tanned RTT. Measured curves of oxazolidine tanned RTT fibers has been recorded before and after the treatment with 6 M urea and are given in Fig. 4. It is seen that the peak temperature for oxazolidine tanned RTT before treatment with urea is 88 °C with an enthalpy value of $58 \pm 0.4\,J\,g^{-1}$ (Table 3). Oxazolidine tanned RTT exhibits a marked increase in the peak temperature as well as in the enthalpy changes when compared to both native and zirconium oxalate tanned RTT fibers. This may be due to a net increase in the number of Carbocations intermediate which may be provided as a result of ring opening, which then is able to interact with collagen amino side chains through the formation of covalent bonds in a pH dependent manner (Fig. 2). Therefore, the rise in melting temperature with crosslinking agent reflects an increase in the average number of crosslinks per molecule [32].

The decrease in the peak temperature and the enthalpy changes of oxazolidine tanned RTT fibers after the treatment with 6 M urea solutions reveal that the alterations in the secondary structure of collagen are possible even after tanning.

The energy of activation associated with the phase change increases for crosslinked RTT compared to the native ones. The energy that is needed for the conversion of collagen to gelatin transition is more due to the additional crosslink introduced by oxazolidine and zirconium. The activation energy decreases from 1990 to 1560 kJ mol^{-1} in the case of oxazolidine tanned RTT fibers in 6 M urea suggesting that some of the hydrogen bonds were broken in the presence of urea even before heating. Comparing the shape of the calorimetric curve of zirconium crosslinked RTT in water medium and 6 M urea solution (Fig. 5) and the corresponding activation energy values suggest that a process with large activation energy has a sharp peak with high normalized peak height, whereas one that has a low energy of activation displays a broad peak [33]. The rate of shrinkage for native, oxazolidine,

Table 3
Comparison of thermodynamic parameters (DSC) of native, oxazolidine, and zirconium crosslinked RTT fibers in water and urea solutions.

Characterization	Peak temperature ($T_p \pm 1$ (°C))	Activation energy (kJ mol^{-1})	Rate of shrinkage ($\ln k_o$ (S^{-1}))	Enthalpy change (ΔH)
Native RTT	65	1140 ± 20	409 ± 12	45 ± 1
Native RTT in 3 M urea	55	1105 ± 19	398 ± 9	35 ± 0.3
Oxazolidine crosslinked RTT in water	88	1980 ± 15	495 ± 8	58 ± 0.4
Oxazolidine crosslinked RTT in 6 M urea	82	1560 ± 20	470 ± 11	40 ± 1
Zirconium crosslinked RTT in water	75	1998 ± 17	555 ± 7	48 ± 2
Zirconium crosslinked RTT in 6 M urea	68	1470 ± 24	345 ± 9	37 ± 1

and zirconium oxalate crosslinked RTT are given in Table 3. The rate of shrinkage $\ln k_0$ (S^{-1}) increases with degree of crosslinking. The rate of shrinking is dependent on the energy of breaking of intermolecular hydrogen bonds and the resulting increase in structural disorder. In general zirconium tanned collagen fiber are known to resist thermal shrinkage to relatively higher temperature compared to native fibers, and oxazolidine is believed to impart crosslink of varying thermal stability. The enthalpy changes associated with the phase transition need to implicate the thermo mechanical events associated with secondary, tertiary and quaternary structures while the composite enthalpy changes need to derive contributions from the extent of ordering in all the levels of collagen structure.

Therefore the overall thermal concluded that oxazolidines and zirconium oxalate tanning, as a result of its general chemical characteristics, impart hydrothermal stability to collagen fibrils predominantly by interaction with basic groups [34], which are located at the 'band' region in the collagen structure [35]. The band structure of collagen contains sterically bulky acidic and basic amino acid side chains, which are open and accessible for interactions with tanning materials. Thus, the reaction of tanning materials with the collagen brings about orderliness, which necessitates higher heat energy for the denaturation to take place.

3.2. Viscosity analysis of the collagen oxazolidines–zirconium system

In order to understand the interaction of oxazolidine and zirconium oxalate with collagen, the influence of the two crosslinkers (oxazolidine and zirconium) on the viscosity of collagen was studied. Relative viscosity was measured for collagen in the presence of varying concentrations of the oxazolidine and zirconium oxalate from the flow rate of the solution. A plot of relative viscosity (η/η_o) against $1/R$ (R = [collagen]/[crosslinkers]) is shown in Fig. 6. In the case of collagen treated with oxazolidine, the relative viscosity was found to increase with increasing oxazolidine concentration, while that for zirconium oxalate treated with collagen it was found to decrease with increasing metal ion concentration when compared to the viscosity of native collagen (Fig. 6).

Such increase in viscosity has been attributed to aggregation of protein [36]. The increase in viscosity of collagen on treatment with oxazolidines observed here is again a proof for the aggregation of collagen in the presence of oxazolidine, which has resulted in the better crosslinking with collagen when compared to zirconium oxalate [36]. Zirconium (IV) oxalate on the other hand, is a monomeric species and has no replaceable aqua ligand with the soluble collagen, and hence incapable to increase the relative viscosity [37].

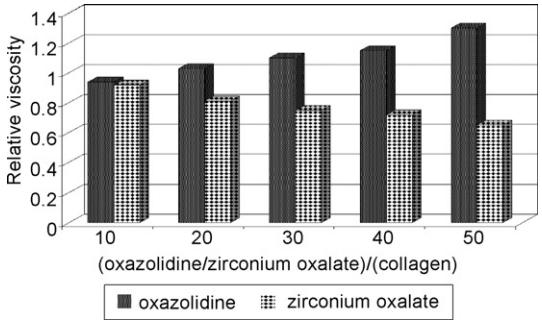

Fig. 6. Relative viscosity of collagen in the presence of different concentrations of oxazolidine and zirconium oxalate.

Table 4
Hydroxyproline released per mg of native, oxazolidine, and zirconium oxalate treated collagen fibers on treatment with collagenase at different intervals.

Incubation time (h)	Hydroxyproline amount (μg)		
	Native	Oxazolidine	Zirconium oxalate
20	28.6	0.10	0.21
40	58.1	0.21	0.30
60	85.4	0.35	0.45
80	105.2	0.55	0.60
100	129	0.69	0.75
120	143	0.88	0.88
140	165.2	0.99	1.0

3.3. Analysis of enzymatic stability of the collagen–oxazolidine, zirconium collagenase ternary system

The stability of the oxazolidine and zirconium oxalate treated collagen fibers against enzyme degradation was studied by analyzing the rate of hydrolysis of collagen on treatment with collagenase. The extent of solubilization of collagen by collagenase examined over a period of time is given in Table 4. The table clearly showed that the stabilization effect on the collagen fibers treated with zirconium oxalate where a marked decrease in the release of hydroxyproline is observed as against the untreated collagen fibers. Native RTT collagen fibers have undergone extensive hydrolysis with the treatment of collagenase (132 μg of hydroxyproline were released per mg of collagen).

Collagen samples treated with oxazolidine exhibit a slightly higher degree of stability compared to collagen treated with zirconium oxalate.

However, inhibition of enzymatic activity of collagenase on collagen could be most likely due to the blocking of the reactive sites in collagen by oxazolidines and zirconium oxalate, thereby making collagenase unable to degrade the substrate collagen. Table 4 shows that oxazolidines and zirconium oxalate are effective in stabilizing collagen against collagenase. This is not surprising, because oxazolidine has the capacity to form many carbocationic intermediates species, while zirconium oxalate form monomeric species which are efficient in blocking the enzyme recognition sites.

3.4. Tensile strength and extension

Tensile strength and percentage elongation of native, oxazolidine, and zirconium oxalate tanned RTT fibers (with 3, 5, 9, and 12% solutions at pH 5 and 8) in water at 25 °C are given in Table 5.

On tanning with oxazolidine, tensile strength increases significantly, until a critical concentration of oxazolidine is reached. Oxazolidine is capable of diffusing into the molecular pore dimensions. Increase in tensile strength can be interpreted in terms of the number of covalent crosslinks formed during the tanning processes. On the other hand a decrease in tensile strength at higher concentration may be due to the increase stiffness (shown by the decreasing elongation) results in a brittle fiber consequently it breaks more easily at reduced load. The bound volume measurements of oxazolidine tanned collagen provide an explanation of increased tensile strength of oxazolidine tanned RTT fiber. On the other hand the strength properties of native RTT fiber and zirconium oxalate under the same conditions of temperature vary in the range of 59 ± 4 and 45–65 MPa, respectively (Table 5). This value of native RTT fiber is nearly the same as those observed for zirconium tanned RTT fiber within experimental errors. In generals, zirconium tanning does not seem to change the tensile strength of RTT fibers significantly in wet condition as well as oxazolidine tanning, this due to the fact that deposition of material with a hardness higher

Table 5
Tensile strength and elongation, % of native, oxazolidine, and zirconium oxalate crosslinked RTT fibers at different pH levels and solution concentrations in water at 25 °C.

Characterization	Tensile strength (Mpa)		Elongation (%)	
Native RTT	59 ± 4		48 ± 4	
Characterization	Tensile strength (Mpa)		Elongation (%)	
	pH 5	pH 8	pH 5	pH 8
Oxazolidine crosslinked RTT with				
2%	145 ± 6	148 ± 6	60 ± 3	63 ± 3
5%	163 ± 9	165 ± 9	45 ± 5	48 ± 5
9%	180 ± 9	175 ± 9	30 ± 5	25 ± 5
12%	120 ± 9	118 ± 9	20 ± 5	15 ± 5
Characterization	Tensile strength (Mpa)		Elongation (%)	
	pH 2.5			
Zirconium crosslinked RTT with				
2%	61 ± 5		65 ± 8	
5%	62 ± 4		50 ± 6	
9%	65 ± 5		35 ± 6	
12%	45 ± 4		19 ± 6	

Note: The values are mean ± S.D. of 10 samples.

than that of collagen fibers may well reduce thermo mechanical properties [38].

4. Conclusions

The influence of oxazolidine and zirconium oxalate on the thermal, enzymatic and mechanical stability of type 1 collagen was investigated. DSC has been used for assessing the thermal stability of collagen. Oxazolidine and zirconium oxalate has been found to increase the hydrothermal stability of the RTT collagen fibers to about 20–23 °C more than that of the native collagen. On the basis of increase in thermal stabilization of tanning, a higher level of long range order is involved in the case of oxazolidine tanned fibers in relation to zirconium oxalate treated analogue. This could be due to the differences in the type of interaction with collagen, which is also reflected in the differences in the enzymatic and mechanical changes of collagen brought about by the two crosslinkers. Oxazolidine, which forms carbocationic species in solution, has been shown to have better crosslinking with collagen as seen from viscometry studies and hence provides better enzymatic stability to collagen than zirconium oxalate, which largely forms monomeric species in solution. In generals, zirconium does not seem to change the tensile strength of RTT fibers significantly as well as oxazolidine.

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