MOLECULAR LEVEL UNDERSTANDING OF TANNING USING AN ORGANO-ZIRCONIUM COMPLEX

by

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ABSTRACT

Organo-zirconium (Organozir) is a zirconium tanning salt complexed with organic moieties. The development of this tanning salt as a potential substitute for chromium has been reported in our earlier work. In the present investigation, studies to understand the mechanism of tanning using organozir at the molecular level have been attempted. The thermal and enzymatic stability of collagen brought about by organozir have been studied using hydrothermal shrinkage measurement, differential scanning calorimetry (DSC) and hydroxyproline estimation respectively. It has been observed that collagen tanned with organozir exhibits a hydrothermal shrinkage temperature of 94°C. The denaturation peak temperature observed from DSC, which indicates the helix → coil transition of collagen, also occurs at 94°C. Mechanistic insights into the stability of collagen against collagenase have been addressed by studying the conformational changes occurring in collagen as a result of interaction with organozir using circular dichroism (CD). The interaction with organo-zirconium complex results only in minor changes in the triple helical conformation of collagen. This indicates that organozir stabilizes collagen against collagenase degradation by blocking the active site in collagen.

INTRODUCTION

The present day research in leather sector is an eco-driven research. Many chemicals hitherto considered as harmless are soon becoming classified under the category of chemicals harmful to the environment. Chromium is one such chemical. Chromium(VI) is a known carcinogen and chromium(III) is considered as non-toxic. However, reports on chromium(III) bringing about DNA and protein damage under certain ligand environments are emerging.[1,2] Hence, alternatives to chromium for tanning are being sought actively. Our previous attempt to seek a potential alternative to chromium resulted in the development of an organo-zirconium complex, organozir.[3,4] Mechanistic insight into tanning with organozir was attempted through indirect studies.[5] Tanning studies using deaminated and acetylated hide powder and urea treatment indicated possible involvement of both electrovalent and hydrogen bonding forces in the stabilization process of collagen. But the molecular level interaction of organozir with collagen is yet to be elucidated. Hence, in this present investigation, an attempt has been made to unravel the molecular level interaction of organozir with collagen. Rat tail tendon (RTT), which predominantly contains Type 1 collagen, as in skin/hide, has been tanned with organozir to study the influence of the same on the thermal and enzymatic stability of collagen.

The molecular stability of collagen arises from the interplay of a wide range of forces and the role of these forces in the stabilization process of collagen has been elucidated.[6-9] Native collagen is susceptible to attack by collagenase at physiological pH and temperature.[10-12] Differential scanning calorimetry (DSC) is a useful technique to study the thermal denaturation of collagen because the position, height, width, area and symmetry of the thermogram peak provide valuable information about the denaturation process. Using DSC collagen denaturation can be studied in collagen fibres at different levels of hydration.[13-15] The conformational behavior of collagen in solution can be examined using circular dichroism (CD). The alterations brought about by organo-zirconium complex in the conformation of collagen and collagenase can thus be elucidated. The stability of the protein against enzymatic activity can be known by estimating the hydroxyproline content after subjecting the protein to enzyme degradation. Hence, in this study, the effect of cross-linking brought about by organozir on the thermal and enzymatic stability of collagen has been studied using differential scanning calorimetry (DSC), circular dichroism (CD) and hydroxy proline estimation.

MATERIALS AND METHODS

Sample Preparation

Tails were excised from 6-month old male albino rats (Wistar strain) and frozen at -20°C, the maximum storage time being 1 month. On removal from the freezer, tails were

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ıwed and tendons were teased out. Teased collagen fibres ere washed with 0.9 % NaCl at 4°C, to remove the adher- ; soluble proteins. The rat tail tendons (RTT) were ıshed extensively in double distilled water at 4°C. ganozir was prepared as described previously.[3] RTT res were tanned with 1 % solution of organozir for 24 hrs a pH of 4.0.

olation and Characterization of Type I Collagen

id soluble rat tail tendon (RTT) collagen type I was iso- ed according to the method described by Chandrakasan al.[16] The procedure included acetic acid extraction and ting out with NaCl. The purity of collagen preparation ıs confirmed by SDS- polyacrlyamide gel electrophoresis. ıe collagen concentration in the solutions was determined ɔm the hydroxyproline content according to the method of ɔessner.[17]

vestigation on the Enzymatic Stability

ostridium histolyticum Type I collagenase purchased ɔm Sigma Chemicals Co., USA was used. The treatment native and treated collagen fibres with collagenase was rried out in 0.04 M $CaCl_2$ solution buffered at pH 7.0 with)5 M Tris-HCl. The collagen:enzyme ratio was main- ned at 50:1. The specimens were incubated at a tempera- e of 37°C. Samples of collagenase treated fibers have en collected at various time intervals ranging from 15 to 0 hrs. The cleavage of native and tanned collagen was ɔnitored by the production of soluble form of hydrox- roline from insoluble collagen.[18] The percentage (%) of droxyproline in the collagenase hydrolysate withdrawn at fferent time intervals was determined using the method of oessner[17] after acid hydrolysis of the sample.

ıermal Analysis

ydrothermal Shrinkage Measurement: The hydrothermal ıbility of the tanned fibres was determined by the standard ethod using micro-shrinkage tester.[19] A small strip of fibre ıs cut and placed on a water grooved microscopic slide. ıe slide in turn was placed on a heating stage along with a icroscope mounted above the heating stage. The rate of ating was maintained at 2°C/min. The temperature at ıich fibre shrinks to one third of its length was taken as e shrinkage temperature.

ılorimetric Measurement: The native and organozir nned collagen RTT fibres were blotted uniformly to move excess adhering water and hermetically encapsulat- in aluminium pans. The samples were fused in a differ- ıtial scanning calorimetric cell of a Netzsch DSC 200 PC fferential scanning calorimeter. The temperature was cali- ated effectively using indium as standard. The heating te was maintained constant at 5°C/min. The denaturation temperature T_D (in °C) and the enthalpy changes ΔH (in J/g) associated with the phase change for the shrinkage process for native and organozir tanned fibres were measured.

Estimation of Zirconium Content in Treated Collagen Fibers

The amount of zirconium present in the collagen fibers treated with organozir was estimated using a Jobin-Yvon (JY 24 series) atomic emission spectrophotometer. A small amount of sample was accurately weighed and was digested using nitric and sulfuric acid mixture (in the ratio of 6:4). The contents were then made up to a known volume. The instrument was first stabilized over a period of one hour and calibrated using carbon as reference. The maximum absorption wavelength chosen for zirconium was 267.863 nm, where the interference of one metal over the other was not observed. Standard solutions of zirconium were prepared and the linearity for the intensity vs concentration curve was confirmed. Then the made up sample was analyzed for zirconium after suitable dilutions and the % ZrO_2 was calculated. The free zirconium in the organozir treated collagen fibres of known mass was estimated after washing the same for 2 hrs in a known volume of double distilled water. The wash liquor was estimated for zirconium following the above procedure.

Circular Dichroic (CD) Measurements

CD spectral studies of collagen with organozir: Circular dichroic spectra were measured using Jasco 715 Circular Dichroism spectropolarimeter using a quartz cell with a light path of 1 mm at 0.2 nm intervals, at 25°C, with 3 scans averaged for each sample. CD spectra were recorded in the far UV region (190 - 250 nm), under nitrogen, to estimate the conformational changes of organozir treated collagen sample. Concentration of collagen was kept constant at 0.6×10^{-6} M throughout and the concentration of organozir was varied from 0.6×10^{-6} M to 30×10^{-6} M.

Results And Discussion

The % ZrO_2 present in the collagen fibres treated with organozir was found to be 6% on the dry weight of collagen. There is no significant change in the % ZrO_2 content of the organozir treated collagen fibres (5.9%) after washing with water for 2 hrs. Hence, it is clear that the organic matrix complexed with the zirconium helps in the irreversible fixation of the zirconium on to the collagen matrix.

Investigation on the Thermal Stability of Organozir Tanned Collagen

The shrinkage temperatures of the native and organozir tanned RTT fibres measured using micro-shrinkage tester are shown in Table I. The shrinkage temperature for native

TABLE I
Hydrothermal Shrinkage Temperatures of Native and Organozir Tanned Rat Tail Tendon Fibres

Nature of fibre	Shrinkage temperature T_s (°C)
Native	62 ± 1
Organozir tanned	94 ± 1

Note: The values are a mean ± SD of three experiments.

collagen fibre is 62°C. The RTT fibres tanned with organozir exhibit a wet-heat stability of 94°C. Shrinkage temperature of the collagen fibres is a measure of the stability of the matrix as a whole, which arises due to the long range ordering of the matrix, and increase in shrinkage temperature represents an increase in the stability of the matrix through the interaction processes between collagen and organozir. There are three possible mechanisms through which zirconium can bind to collagen. These could be (i) polar binding of anionic sites of zirconium species with polar amino groups, (ii) polar binding of cationic sites of zirconium species with carboxyl groups and (iii) covalent bonding between neutral sites and oxygen atoms of carboxyl groups present in collagen, the nitrogen atoms of amino or imino groups playing no part in such coordination.[21] Organozir is a complex of zirconium that is stabilized with an organic matrix based on a polymeric network carrying suitable ligating sites such as phthalate, citrate and salicylate.[3] Thus, the stable complexes present in the organozir display high crosslinking ability (both electrostatic and coordinate covalent) with the collagen network, hence increasing the long-range order of the collagen to withstand heat up to 94°C. The shrinkage process has been considered as a phase transition involving the conversion of crystalline triple helical collagen structure to an amorphous random coil form.[22] The physical change in the structure of collagen observed at the shrinkage temperature is the reduction in the length of the fibre to about 1/3 its original length. Earlier, differential scanning calorimetry was used to analyze the endothermic helix → coil transition of collagen fibres in various aqueous environments and to determine the corresponding enthalpy changes (ΔH) and temperatures of collagen → gelatin transition.[23] The typical DSC thermograms of native and organozir crosslinked RTT fibres are shown in Fig. 1A and 1B respectively. During collagen denaturation the phase transition in the form of changes in the lattice occurs with the absorption of heat. The onset temperature, denaturation peak temperature and enthalpy changes are given in Table II. The endotherm peak at 35-37°C is the melting temperature peak of collagen. It could be seen that organozir crosslinked RTT exhibits a marked increase in the denaturation peak temperature when compared to native RTT. This may be due to a net increase in the number of intermolecular crosslinks arising from both electrostatic and co-ordinate covalent interactions owing to the presence of organic ligands in the complex. Therefore, the rise in denaturation temperature with organozir reflects the increase in average number of crosslinks per molecule.

TABLE II
Values of Onset Temperature (T_o), Denaturation Temperature (T_D) and Enthalpy Change (ΔH) of Native and Organozir Tanned Rat Tail Tendon Fibres

Sample	T_o (°C)	T_D (°C)	ΔH (J/g)
Native	61 ± 1	65 ± 1	14 ± 0.5
Organozir tanned	90 ± 1	94 ± 1	11 ± 0.5

Note: The values are a mean ± SD of three experiments.

Investigation on the Enzymatic Stability of Organozir Tanned Collagen

The stability of the organozir tanned collagen fibres against enzyme degradation was studied by estimating the hydroxy proline content release after treatment of organozir tanned collagen with collagenase. The extent of hydrolysis of native and organozir tanned collagen by collagenase examined over a range of time period is given in Table III. The table clearly displays the stabilization effect of organozir on the collagen fibres, where a marked decrease in the release of hydroxyproline as against the untreated collagen fibres is

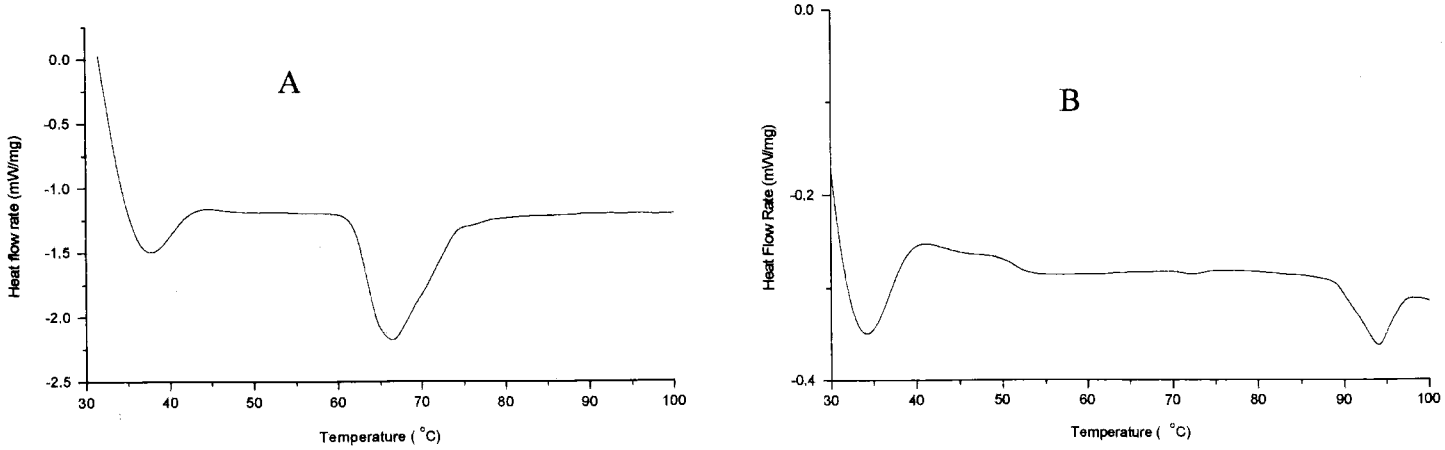

Figure 1. - Differential scanning calorimetry (DSC) thermograms of native and organozir tanned collagen fibres. (A) native and (B) organozir.

TABLE III
The Release of Hydroxyproline per mg of Native and Organozir Tanned Collagen Fibres on Treatment with Collagenase at Various Intervals of Time

Time of incubation (hrs)	Hydroxyproline (μg)	
	Native	Organozir tanned collagen fibres
15	25.1	0
30	52.2	0.2
45	76.8	0.35
60	97.4	0.5
75	119	0.67
90	128.6	0.76
105	132.1	1.2

observed. Native RTT collagen fibres have undergone extensive hydrolysis with the treatment of collagenase, about 132 μg of hydroxyproline has been released per mg of collagen. Mammalian collagenase is highly specific in its activity against collagen where it specifically cleaves collagen at 772-773 amino acid residue of Gly-Ile or Gly-Leu bond in the polypeptide chain of collagen.[24] In the present investigation, bacterial collagenase has been used for understanding the enzymatic stability of collagen and it cleaves the peptide bond between Y and Gly (Y is most frequently a neutral amino acid) at several regions in the collagen triple helix.[25] The organo-zirconium complex has provided the stability to triple helical collagen against the degradation by bacterial collagenase. The nature of inhibition of organo-zirconium tanned collagen fibres against collagenase could be due to (i) blocking of the specific sites in the collagen, (ii) conformational changes in collagen which would have rendered collagen as an unknown substrate for collagenase to recognize, (iii) interaction of organo-zirconium with collagenase so that the activity of collagenase is lost. Since, estimation of zirconium in collagen fiber shows that washing of fiber with water does not lead to loss of zirconium, it is evident that organozir binds to collagen irreversibly. Hence, on treating organozir treated collagen with collagenase, one does not expect any organozir to bind to collagenase. Hence, the possibility of any interaction between organozir and collagenase is ruled out. Study on the conformational changes in collagen with the interaction of organo-zirconium complexes can provide insights on the issues that have been tried to address.

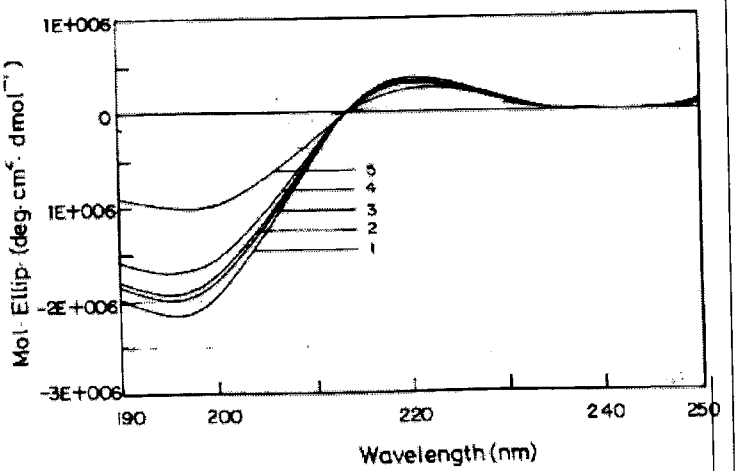

Figure 2. - Far-UV CD spectra of native (0.6 μM) and organozir treated collagen at concentrations (1) 0 μM, (2) 0.6 μM, (3) 6 μM, (4) 15 μM and (5) 30 μM.

TABLE IV
Rpn Ratio of Organozir Treated Collagen

Concentration of organozir (μM)	Rpn (Characteristic Ratio)
0	0.117
1	0.12
10	0.125
25	0.137
50	0.17

Investigation on the Conformational Behavior of Organozir Tanned Collagen

In order to investigate whether organozir stabilizes collagen against enzyme by changing the conformation of collagen, CD spectral studies were carried out. The CD spectra of collagen in the presence of increasing concentrations of organozir are shown in Fig. 2. In the far UV region, collagen exhibits its minimum at 197 nm and a maximum at 220 nm with a cross over point at about 210 nm. The maximum at 220 nm in CD spectrum of native collagen solution is characteristic of triple folded helix.[26] It could be seen from the Fig. 2 that there are no major alterations in the conformation of collagen. Increase in metal ion concentration has resulted in only minor changes in the amplitude of the spectra, a slight unwinding of triple helices and no further modifications are observed. This decrease in dichroic intensity at 197 and 220 nm in a concentration dependent manner could be due to the crosslinking of native collagen molecules with the zirconium complex. This also indicates that the change in CD of collagen in the presence of organozir is not due to loss of triple helicity because it is known that collagen on complete denaturation undergoes drastic changes like the disappearance of the positive peak and red shifting of the negative peak.[27,28] The parameter Rpn, denotes the ratio of positive peak intensity to negative peak intensity. It is a characteristic ratio for the triple helical conformation of collagen and collagen-like peptides.[28] The Rpn values for collagen solution and collagen solution treated with organozir are given in Table IV. The Rpn ratio for collagen solution of concentration, 0.6 μM is found to be 0.117. The

Rpn values of the collagen solutions treated with organozir are close to the Rpn values exhibited by the collagen and hence it is evident that no major secondary structural changes take place in collagen on treatment with the organo-zirconium complex. Hence, the stability imparted to collagen by organozir against collagenase, should arise from blocking of the active site in collagen by organozir.

Conclusions

An attempt has been made to gain an insight into the mechanism of zirconium tanning by studying the thermal and enzymatic stability of organozir, an organo-zirconium complex tanned collagen. The hydrothermal stability of the organozir tanned collagen fibres has been found to be about 94°C. The conformational changes of collagen brought about by organozir have been investigated. Estimation of zirconium content in the treated collagen fibres indicated that there was no free zirconium available in the collagen matrix. Hence, the enzymatic stability of the matrix is attributed to the reactivity of organozir with collagen, which blocks the active sites in collagen.

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