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ROLE OF SOLVENTS IN STABILITY OF COLLAGEN

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The influence of hydroxymethyl chain length of the solvents on collagen was established with conformational stability and thermal stability. Thermal stability of monomeric collagen and RTT fibres (rat tail tendon) treated with methanol, ethylene glycol (EG) and glycerol were reported using the melting temperature for helix-coil transition and the peak temperature for collagen-gelatin transition. Both melting temperature and peak temperature increases as the hydroxymethyl chain length increases. Conformational stability of collagen solution treated with lower and higher concentrations of methanol, ethylene glycol and glycerol indicates that aggregation of collagen molecule is more at higher concentrations of these solvents. The concentration dependence is greater for the increased number of OH groups. Since protein aggregation is associated with neuro degenerative diseases, aggregation of collagen molecule in the presence of solvents is of great importance for biomedical application.

Keywords: *collagen, conformational and thermal stability, ethylene glycol, glycerol, methanol*

Introduction

Collagen is an exracellular protein responsible for most of the mass and strength of structural tissues such as bone, tendons, skin and cartilage. Collagen molecule is characterized by a triple helical structure and the structural element of fibril forming collagen family [1, 2]. The nature of the triple helical conformation had been elucidated initially through X-ray diffraction [3] and later confirmed by X-ray crystallographic [4] and computational studies of collagen like peptides [5, 6]. Collagen fibres in vivo must be stable enough to withstand the disruptive influence of thermal agitation, but capable of assembling into component molecule. In solution, the unfolding of fibrous collagens is within only a few degrees of the animal body temperature. There is an increase in the transition temperature when the molecules are aggregated to form fibres [7]. Investigation on the influence of various organic solvents on the thermal denaturation temperature (T_m) of soluble collagen provides information about the possible mechanisms involved in pertrabational activity. Sugars and polyols have been widely used to protect the native structure of proteins and the enzyme activity from thermal denaturation. The extent of stabilization by different sugars and polyols is discussed in terms of their different influences on the structure of water. The hydroxymethyl chain length of polyols were found to be decisive factors for their stabilizing effect on collagen structure. The effect of aliphatic alcohols on the dimensional stability of collagen has been reported earlier [8–10]. The influence of a number of glycols and alkyl-substituted glycols on the thermal stability of acid soluble calfskin collagen has been reported [11]. Thermodynamics and kinetic examinations of protein stabilization by glycerol [12] and the solubility of amino acids and related compounds in ethylene glycol solution have been reported [13]. The melting behaviour of collagen fibres in ethylene glycol has been discussed in terms of polymer-in-a box mechanism and polymer-melting mechanisms [14, 15]. In this present investigation thermal and conformational stability of collagen in ethylene glycol and a comparison of the melting temperature of collagen with increasing hydroxymethyl chain length of solvents-methanol, ethylene glycol and glycerol have been studied.

Experimental

Sample preparation

Collagen fibres were teased out from tails of six-month-old male albino rats (Wistar strain) and thoroughly washed and stored at –20°C until needed.

Preparation of collagen solution

Collagen extracted from RTT by known procedures was used for CD and viscosity studies. The collagen content of the solution was estimated by the standard procedure [16, 17]. SDS PAGE checked purity of collagen.

Preparation of solvent mixtures

Methanol, ethylene glycol and glycerol are of HPLC grade (Merck India) analytical grade. They were used without further purification.

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Circular dichroism

The CD spectra expressed in terms of molar ellipticity for the pure aqueous solution of collagen and samples equilibrated with methanol, ethylene glycol and glycerol were analyzed at 25°C using a Jasco spectropolarimeter. A scan speed of 20 nm min^{-1} with slit width of 1 nm and a time constant of 1 s was used with an average of five scans in a 1 mm cell for the samples. Conformation of native collagen in 5 mM acetic acid was recorded in the far UV region [18]. Changes in the conformation of collagen on addition of various amounts of methanol, ethylene glycol and glycerol were also recorded. The concentration of collagen was $2 \cdot 10^{-6}$ M and the concentrations of solvents were of micro (600 micro molar) and mill molar (10^{-3} M).

Viscosity measurements

Viscosity measurements were determined at 20–50°C using quartz Ubbelohde'a viscometer. The concentration of collagen is 0.25 mg mL^{-1} and the concentrations of the solvents are $3 \cdot 10^{-2}$ M for methanol, $1.5 \cdot 10^{-2}$ M for ethylene glycol and $1 \cdot 10^{-2}$ M for glycerol.

Differential scanning calorimetry (DSC)

Known amount of native RTT (generally 1–2 mg) was immersed in 3:1 ratio of water, methanol, ethylene glycol and glycerol overnight. The soaked samples were blotted uniformly and hermetically encapsulated in aluminum pans. The samples were placed in a differential scanning calorimetric cell of a Seiko 52 WH DSC 220C module instrument. The heating rate was maintained constant at 6°C min^{-1}. The peak temperature (T_p) for collagen-to-gelatin process was recorded.

Results and discussion

Circular dichroism

Collagen exhibits a unique CD spectrum with a small positive peak between 220 and 225 nm and a large negative peak at 197 nm [19–22]. The CD spectrum of native collagen and those treated with micro molar concentrations of ethylene glycol and glycerol are given in Fig. 1 and the molar ellipticity with Rpn values are given in Table 1. There is no significant change in the spectrum and Rpn values as well as in the molar ellipticity of native, ethylene glycol and glycerol treated collagen. The corresponding CD spectrum of the different samples is given in Fig. 2. There is a decrease in molar ellipticity at 220 and an increase at 197 nm as the chain length of the solvent increased. The decrease in the dichroic intensity at 220 nm has been observed for collagen treated with chromium complexes and formaldehyde and glutaraldehyde and is possibly due to aggregation of collagen molecule in the presence of these cross-linking agents through coordinate covalent and covalent linkages respectively [23, 24]. Partially denatured collagen gives CD spectra with lower intensity and red shifted crossover points and higher ratio of positive to negative band (Rpn). This is used in

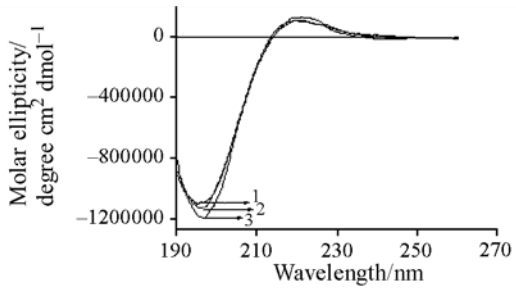

Fig. 1 CD spectra of 1 – native collagen, 2 – collagen+ 600 μM ethylene glycol, 3 – collagen+ 600 micro molar glycerol

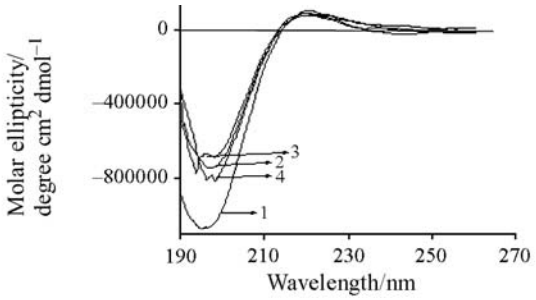

Fig. 2 CD spectra of 1 – native collagen, 2 – collagen+(10^{-3} M) of methanol, 3 – collagen+(10^{-3} M) of ethylene glycol, 4 – collagen+(10^{-3} M) of glycerol

Table 1 Molar ellipticity and Rpn ratio of native monomeric collagen treated with 600 μM ethylene glycol and glycerol

Specification	Molar ellipticity (degree cm^2 $dmol^{-1}$)		Rpn ratio
	220 nm	197 nm	
Native collagen	105179	–1.10518E+06	0.095
Collagen+600 μM ethylene glycol	101217	–1.13528E+06	0.089
Collagen+600 μM glycerol	118248	–1.19319E+06	0.099

Table 2 Molar ellipticity and Rpn ratio of native monomeric collagen and treated with 10^{-3} M methanol, ethylene glycol and glycerol

Specification	Molar ellipticity (degree cm^2 dmol^{-1})		Rpn ratio
	220 nm	197 nm	
Native collagen	105179	–1.10518E+06	0.095
Collagen+1.5·10^{-3} M methanol	114842	–1.17956E+06	0.097
Collagen+5.0·10^{-3} M ethylene glycol	84671.5	–79343.1	0.107
Collagen+5.0·10^{-3} M glycerol	80535.3	–84403.9	0.095

establishing helical conformation in solution. The molar ellipticity and the Rpn ratio of native, methanol, ethylene glycol and glycerol treated collagen at higher concentrations are given in Table 2. The deviations of the spectral properties increases as the hydroxymethyl chain length increases. Possibly, the chain length of hydrocarbon chain of the organic solvent molecule destroys its ability to destabilize partially the triple helix. CD spectral changes with small deviations of Rpn ratio in the presence of methanol, ethylene glycol and glycerol is indicative of conformational changes in the collagen molecule without denaturation. CD spectral studies indicate that as the hydroxymethyl chain length and concentration increases the aggregation of the collagen molecule increases. In other words, the concentration dependence is greater for increased number of OH groups. It has been reported that the aggregation process involves conformational changes of the whole proteins or of a specific domain and it can be due to the association of partially unfolded molecules [25].

It has been reported that methanol and ethanol promote the fibrillogenesis of collagen. Ethylene glycol has a much weaker inhibitory effect, whereas glycerol strongly inhibits fibril formation process in collagen [26, 27]. The effect of various concentrations of ethylene glycol on the conformational stability of collagen was studied and CD spectrum of various concentrations of ethylene glycol treated collagen is given in Fig. 3. There is a linear decrease in molar ellipticity at 220 nm and an increase at 197 nm has been observed as the concentration of EG is increased. This may be due to the aggregation of collagen molecule in the presence of EG. The Rpn ratio of native collagen and treated with various concentrations of ethylene glycol is given in Table 3. There is no significant change in the Rpn ratio for native, and EG treated collagen. CD spectral changes with identical Rpn values (~0.1) are indicative of conformational changes in the collagen molecule in the presence of EG. There is also the possibility that collagen molecules in solution may exist in forms other than the simple monomeric triple helical state.

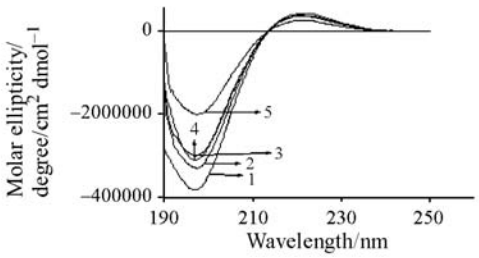

Fig. 3 CD spectra of 1 – native collagen, 2–5 – increasing concentrations of ethylene glycol

Table 3 Rpn ratio of monomeric collagen solution treated with various concentrations of ethylene glycol

Specification	Rpn ratio
Native collagen	0.109
Collagen+3·10^{-3} M ethylene glycol	0.109
Collagen+5·10^{-3} M ethylene glycol	0.118
Collagen+6.5·10^{-3} M ethylene glycol	0.115
Collagen+8·10^{-3} M ethylene glycol	0.106

Lowering the dielectric constant of the solution by adding organic solvents might be expected to enhance intermolecular electrostatic interaction [28, 29].

Melting temperature

Polar functional groups eliminate any destabilizing influence on the protein. Therefore methanol, ethylene glycol and glycerol were chosen as the stabilizing reagents for collagen. Collagen molecules are more stable when precipitated as fibres than the same molecules in solution. The melting temperature of collagen solution treated with increasing number hydroxymethyl group that is methanol, ethylene glycol and glycerol using viscosity method was given in Table 4. The temperature at which the change in viscosity was half maximum value was taken as the melting temperature. The melting temperature increases as the number of hydroxymethyl group increases. Specific viscosity for these stabilizing reagents treated collagen solution at various temper-

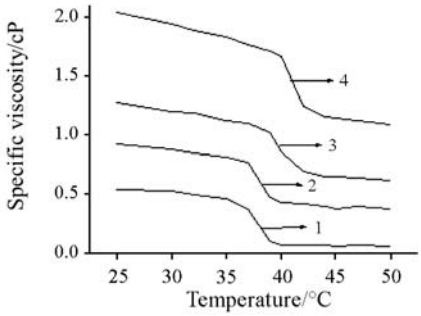

Fig. 4 Temperature *vs.* specific viscosity of 1 – collagen solution and treated with 2 – $3 \cdot 10^{-2}$ M methanol, 3 – $1.5 \cdot 10^{-2}$ M ethylene glycol and 4 – $1 \cdot 10^{-2}$ M glycerol

Table 4 Melting temperature of collagen (0.25 mg/mL) treated with methanol, ethylene glycol and glycerol

Specification	Melting temp. T_m/°C
Native collagen	37.8
Collagen+ $3 \cdot 10^{-2}$ M methanol	39.8
Collagen+ $1.5 \cdot 10^{-2}$ M ethylene glycol	48.8
Collagen+ $1.0 \cdot 10^{-2}$ M glycerol	–

atures is given in Fig. 4. Since the specific viscosity was high for glycerol treated collagen it is not possible to measure the melting temperature. It has been reported that the protein stabilization by glycerol may be enhancement of the structure of the medium or the solvation layer of the protein [12]. The melting temperature increases, as the hydroxymethyl chain length increases and can be explained in terms of lowering of the dielectric constant would have on intramolecular interaction. The stabilizing effect of polyhydric alcohols on collagen may be the result of a decrease in the hydrogen bond rupturing capacity of the media.

Differential scanning calorimetry

Collagen molecules embedded within the lattice of a fibre are substantially more thermally stable than the same molecules in dilute solution. Differential scanning calorimetry studies of rat-tail tendons treated with 3:1 ratio of water and methanol, ethylene glycol and glycerol are given in Fig. 5. The peak temperature for the shrinkage processes is given in Table 5. The peak temperature increases as the number of hydroxymethyl group increases. The decrease of the length of unbroken hydrocarbon chain of the organic solvent molecule destroys its ability to

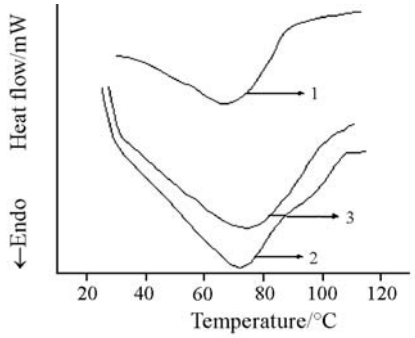

Fig. 5 DSC curve of native RTT fibres treated with 3:1 ratio of water and 1 – methanol, 2 – ethylene glycol and 3 – glycerol

Table 5 Peak temperature for collaen to gelatin transition of native RTT fibres treated with 3:1 ratio of water and methanol, ethylene glycol and glycerol

Specification	Peak temp. T_p/°C
Native RTT treated with methanol	68.5
Native RTT treated with ethylene glycol	72.5
Native RTT treated with glycerol	75.5

destabilize partially the triple helix. The increase in the melting temperature of collagen as the chain length of hydroxymethyl group increases may be due to sufficient decrease in the environmental dielectric constant. This overcomes the destabilizing tendencies by enhancing the strength of the hydrogen-bonded network within the protein molecule.

Conclusions

The stabilizing effect of methanol, ethylene glycol and glycerol on collagen was established with melting temperature for helix to coil transition, peak temperature for collagen to gelatin transition and the conformational stability. The stability increases as $CH_3OH \geq C_2H_4(OH)_2 \geq C_3H_5(OH)_3$. The aggregation of the collagen molecule is more as the concentrations as well as the hydroxymethyl chain length of the solvents increases. Protein aggregation is associated with neurodegenerative diseases [25]. Hence, a study of this kind is useful for biomedical application.

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