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INFLUENCE OF CYCLODEXTRINS ON THE PHYSICAL PROPERTIES OF COLLAGEN

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ABSTRACT

Collagen and cyclodextrins are well known as triple helical protein and oligosaccharides. Cyclodextrins are the simplest organic compounds, which exhibit complex formation with other organic molecules and hence are excellent models of enzymes. This property of cyclodextrins to form inclusion complexes is being exploited in the present investigation. In fibrillogenesis, collagens with intact telopeptide form fibrils more rapidly, than those with degraded telopeptides. Since hydrophobic aminoacid residues are found in the telopeptide region and that the telopeptide is involved in fibrillogenesis, there is a possibility that the cavity of cyclodextrin encapsulate the hydrophobic amino acid residues, thereby interrupting the crosslinking of collagen. Hence cyclodextrins were added to collagen in different percentages (concentrations) and fibril formation experiments were performed. Similarly cyclodextrins were added to different concentration of collagen and the viscometric properties of collagen were studied. Results show that the cyclodextrins interact with collagen though the pathway of interaction is yet be studied in detail.

KEY WORDS: Collagen, Cyclodextrin, Telopeptide, Fibrillogenesis, Interaction, Hydrophobic amino acid residues, Crosslinking.

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INTRODUCTION

The prevalence of type I collagen and the facility with aggregates of type I collagen may be dissociated into monomers has contributed immensely to the information on collagen in general[1]. The collagen triple helix is flanked at both the ends by non-helical aminoacids which extends from 25 to 26 amino acids in length. Important stabilizing features for the collagen triplex helix include the presence of Gly as every third residue, a high content of amino acids, and inter chain hydrogen bonds[2]. The telopeptide segments during fibrillogenesis, interacts with the highly conserved triplet sequences and intermolecular cross links are formed through condensation reactions[3]. The extent of conformational changes taking place in the telopeptides before or during the formation of fibrils is yet to be described fully. In fibrillogenesis collagen ith intact telopeptides form fibrils more rapidly than those with degraded telopeptides[4]. Much evidence has shown that proteolytic treatment of the native collagen results in the cleavage of some telopeptides and this loss decreases the rate of fibrillogenesis[5]. Telopeptide regions are rich in charged residues. Cyclodextrins comprise a family of three well known industrially produced major and several rare minor oligosaccharides. The most characteristic property of cyclodextrins is its ability to from inclusion complexes; with a variety of guest molecules[6]. Cyclodextrins are the most important examples of simple organic compounds, which exhibit complex formation with other organic molecules and hence, are excellent models of enzymes. This property of cyclodextrins to form inclusion complexes is being exploited in the present investigation. Cyclodextrins are also shown to function as aggregation suppressors for wide range of proteins[7]. Although we have investigated the micelle formation on various collagens and peptides in solutions and their interactions with different surfactant micelles and additives in the recent past[8-12] in order to understand the location, solubilisation, molecular dynamics, thermodynamics, conformations and geometries in these complex systems; the interaction of various cyclodextrins with collagen has not been examined so far to the best of our knowledge. Therefore in this communication, it is worthwhile to report the interaction of different cyclodextrins with collagen with a view to understand the fibril formation and various physicochemical properties of collagen which will stimulate further studies.

MATERIALS AND METHODS

(i) Isolation and purification of collagen

Collagen was extracted form rat tail tendons and purified as suggested by Miller and Rhodes in 1982[13]. The tail tendons were teased out from four month old male albino rats of wistar strain. These tendons were placed in 0.9% NaCl after washing in cold distilled water overnight at 0°C. The tendons were then taken out and washed in cold distilled water and stirred overnight in 0.5M acetic acid at pH 3.0. The protein inhibitors: N-ethyl maleimide (2M) and phenyl methyl sulfonylfluoride (1mM) were added. The resulting solution was centrifuged at a speed of 50,000 g and the supernatant was collected. The collagen solubilized in the supernatant was reprecipitated by the drop wise addition of 25% NaCl (w/v) to a final concentration of 5% (w/v) NaCl. The precipitated collagen was then removed by centrifuging the solution at 35,000g for 30min. Again the residue was dissolved in 0.5M acetic acid for overnight stirring. The suspension was again centrifuged at 50,000g for 45min. and the supernatant was dialyzed against 0.02M disodium hydrogen phosphate. After dialysis the precipitated solution was centrifuged at 35,000g and the precipitate was redissolved in 0.5M acetic acid. The collagen dissolved in 0.5M acetic acid was centrifuged at 50,000g for 45min. The supernatant was dialyzed against 0.05M acetic acid and the solution was centrifuged at 50,000g for 45min and the supernatant was collected at 4°C. The purity of the collagen was checked using SDS-PAGE.

(ii) Determination of the concentration of collagen

To 1ml of collagen solution 1ml of 6N HCL was added and maintained at a temperature of 110°C for 20hrs in sealed tubes. After hydrolysis the sample was evaporated to dryness, the residue was dissolved in water and made up to a known volume. The hydroxyproline was estimated by the method of Woesner[14]. The collagen content of the sample was then calculated by multiplying the hydroxyproline content by a factor 7.46 and was expressed as µg/100ml.

(iii) Fibril formation studies

The collagen fibrils were reconstituted from purified collagen solution by mixing with phosphate buffer (0.2M) and sodium chloride (2M) in an ice bath [15]. The pH of the solution was adjusted with 1.25N sodium hydroxide to 7.4. The final composition of each constituent in the mixture was: collagen: 3.3mg/ml, phosphate buffer: 0.02M and sodium chloride: 0.13M. The temperature of the solution was raised slowly to 35°C. The collagen gel formed in a time of 20-30mins was followed by measuring the turbidity at 313nm using a UV-VIS spectrometer. The rate of the fibril formation of collagen without cyclodextrins and with α / β or γ cyclodextrins in all the experiments have been calculated as the time taken to reach half the maximum absorption.

(iv)Viscosity Measurement of collagen

The viscosity of different concentrations of collagen was measured by an Ostwald viscometer[47]. The viscometer was calibrated with 10%, 20% sucrose solutions in water and the results are in good agreement with the values available in the literature. The viscometer gives a flow time of 240 seconds for water at 25°C. The temperature of measurement was accurate within ±0.1°C. Different sizes of viscometers, with differing flow times were also used in order to estimate the correct viscosity values. Regarding details of viscosity measurements, we refer to our earlier publications[16-20].

RESULTS AND DISCUSSION

Since the cavity of the cyclodextrin molecule is hydrophobic in nature, it is said only hydrophobic molecules get encapsulated in the cavity of these molecules to form inclusion complexes. Hence, 1mM stock solution of the α, β and γ cyclodextrins were prepared in phosphate buffer. 300µl of collagen and 100µl of 0.1M acetic acid were neutralized with 1.25M sodium hydroxide solution. Fig.1 shows the absorbance at 313nm as a function of time for normal collagen in the absence of the cyclodextrins.

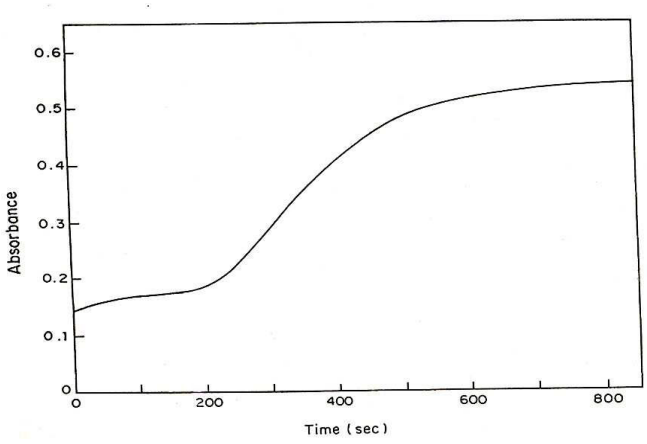

Figure 1
Plot of UV absorbance Vs Time measured at 313nm for normal collagen in the absence of the cyclodextrin

1. Fibril Formation Studies

Collagen fibril formation is an endothermic process involving the formation of hydrophobic and electrostatic interactions between adjacent molecules, with the accompanying release of water molecules[21]. Several studies[22-24] suggest that the role of inhibitors of fibril formation is predominantly one of weakening the hydrophobic interactions between collagen molecules. With α cyclodextrin, the lag phase increases showing that there may be some hindrance in the nucleation of the fibers to form fibrils (Fig.2). Table 1 shows the rate of fibril formation of collagen in the presence of various concentration of α cyclodextrins. Also the rates of fibril formation are the same for collagen with 5% and 25% α cyclodextrin (table 1). But they are lesser than the control when the percentage of α cyclodextrin in collagen is 12.5% and 50%. Also a hump in the lag phase is observed for 50% α cyclodextrin (Fig.2). On the addition of β cyclodextrin (Fig 3) there appears a slight shoulder in the lag phase. When the rates of fibril formation are compared, the rates are almost the same for 5%, 12.5% and 25.5% of β cyclodextrin (Table 2) with collagen. For fibril formation of collagen with 50% β cyclodextrin, the rate is a little higher than the control. A hump is observed in the lag phase for the 50% β cyclodextrin also (Fig 3). For fibril formation experiments with γ cyclodextrin (Fig.4), the rate of fibril formation of collagen with 25% γ cyclodextrin is the same as that of the control collagen (Table 3). The curve for fibril formation with 25% γ cyclodextrin shows a hump in the lag phase. With 5% γ cyclodextrin the ratio is slightly lesser and for 12.5% and 50% γ cyclodextrin, the rate of fibril formation is higher than that of the control collagen (Fig 4). The presence of "knitted" structure for collagen has already been observed[25]. It has also been demonstrated that a 3- dimensional array of fibrous elements in intimate association with a non- fibrous swelling component must incorporate a system of repeated inter fibril connections in order to sustain compressive loading[26]. α -helical coiled coils are simple structures that are stabilized by hydrophobic and electrostatic interactions between α-helices[27]. In coiled coil helices, the apolar residues form the hydrophobic interface between the helices. The structure is stabilized by the packing of "knobs" formed by the hydrophobic side chains of one helix into "holes" formed by the spaces between the side chains of neighboring helices[28]. Although the hydrophobic interactions seem to represent the major driving force for the stabilization of coiled coil structures[29], ionic interactions between the side chains of different helices are considered relevant to stability, orientation and stoichiometry of coiled coils[30-32].

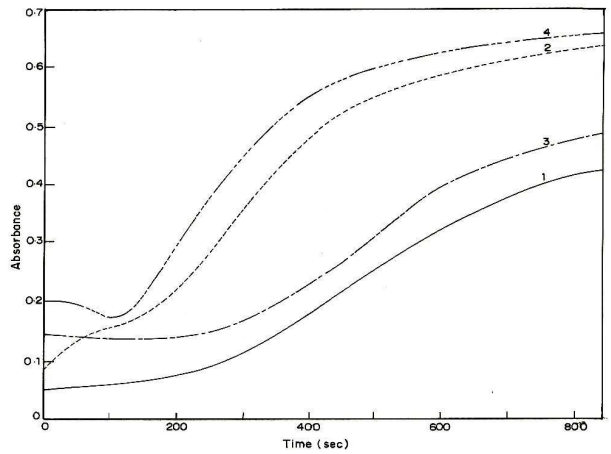

Figure 2

Plot of UV absorbance Vs Time measured at 313nm for collagen with different concentration of α cyclodextrin. In Fig.2 curve (1) represents concentration of α cyclodextrin of 0.0165mM, (2) 0.125mM, (3) 0.25mM and (4) 0.5mM.

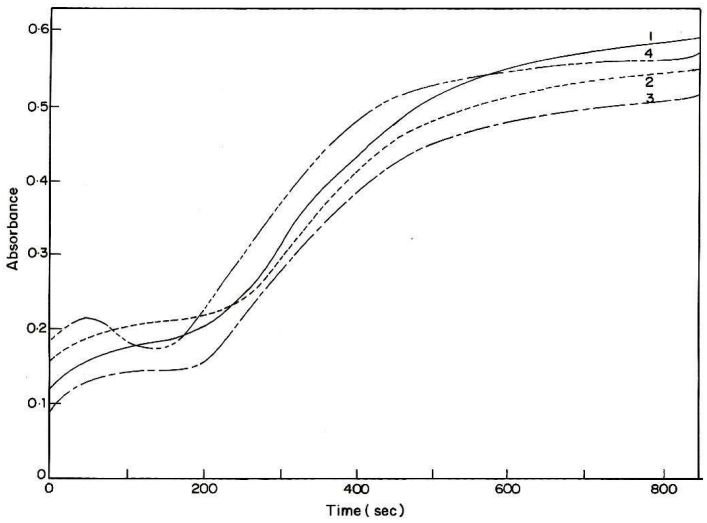

Figure 3

Plot of UV absorbance Vs Time measured at 313nm for collagen with different concentration of β cyclodextrin.In Fig.3 curve (1) represents concentration of β cyclodextrin of 0.125mM, (2) 0.125mM, (3) 0.25mM and (4) 0.5mM.

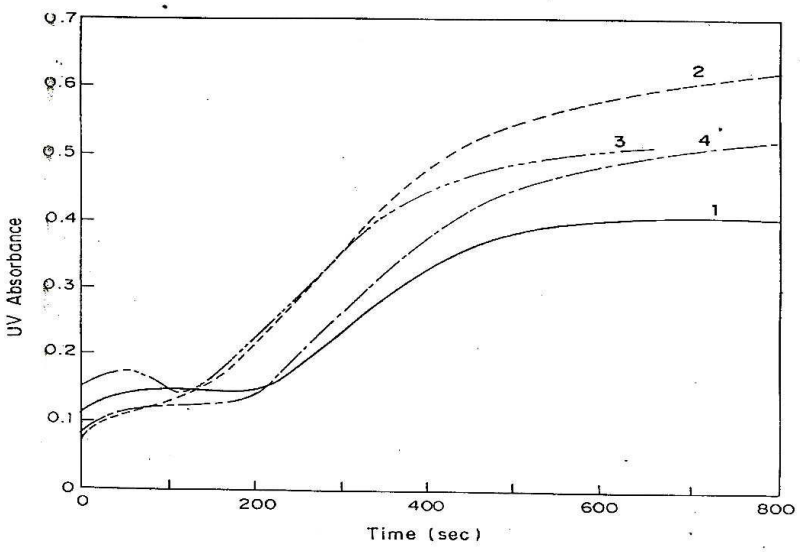

Figure 4

Plot of UV absorbance Vs Time measured at313nm for collagen with different concentration of γ cyclodextrin.In fig.4 curve (1) represents concentration of γ cyclodextrin of 0.0165mM, (2) 0.125mM, (3) 0.25mM and (4)0.5mM.

Table 1

Rate of fibril formation on the addition of α cyclodextrin

Sample	Initial absorption	Rate ×10-3	Final absorption
Control	0.10	1.2±0.4	0.5
5% α CD	0.05	0.7±0.3	0.4
12.5% α CD	0/09	1.4±0.5	0.6
25.0% α CD	0.15	0.8±0.6	0.5
50.0% α CD	0.2	1.6±0.4	0.7

Table 2
Rate of fibril formation on the addition of β cyclodextrin

Sample	Initial absorption	Rate ×10-3	Final absorption
Control	0.14	1.2±0.5	0.50
5% α CD	0.13	1.1±0.3	0.58
12.5% α CD	0.15	1.18±0.6	0.55
25.0% α CD	0.10	1.24±0.6	0.50
50.0% α CD	0.18	1.49±0.4	0.57

Table 3
Rate of fibril formation on the addition of γ cyclodextrin

Sample	Initial absorption	Rate ×10-3	Final absorption
Control	0.14	1.2±0.3	0.50
5% α CD	0.12	1.11±0.4	0.42
12.5% α CD	0.08	1.44 ±0.6	0.62
25.0% α CD	0.08	1.26±0.7	0.52
50.0% α CD	0.15	1.32 ±0.5	0.52

In the present case, with β cyclodextrin and γ cyclodextrin the bump in the lag phase may be due to the formation of knobs being facilitated by the presence of cyclodextrins, which are non-fibrous components during the nucleation process, leading to an increase in the local concentration of the fibrils. Again, the process may be destabilized by the dissolution of the local concentration with time, thereby, returning to normal lag phase. Also the lateral growth of fibers is not hindered, as evidenced by the overall growth phase *i.e*., the sigmoidal shape of the curve. However, we do not know the correct reason at the moment. The lag phase of collagen fibril formation with cyclodextrin probably produces fewer nucleation centers. There is a possibility that the hydrophobic amino acid residues are encapsulated by the cavity of the cyclodextrin moiety thereby reducing the nucleation centers but facilitating the lateral aggregation leading to formation of fibrils.

2. Viscometric measurements

The structure of water is important for interactions between proteins and in membrane transport phenomena. These interactions are important to study due to the fact that the exact shapes, sizes and the structures of the proteins are not clearly known. A matter of particular interest is the observation that proteins have sufficient affinity for certain solutes dissolved in aqueous solutions to allow those solutes to displace the hydration of water or atleast adsorb on the surface of the proteins. Data have been reported for instance, where the binding of certain electrolytes by proteins[33,34] indicate that interactions occur between cations and the peptide carbonyl group as well as between the anions and the protons bonded to the peptide nitrogen atoms. It is generally accepted that the stability of the α helical conformation present in globular proteins is not entirely due to hydrogen bonds, but a significant proportion of this stability also arises from the mutual interactions of nonpolar side chains of the aminoacid residues constituting the polypeptide chain[35]. However little attention has been paid so far in determining the role of these interactions called hydrophobic interactions, in the structural stability of collagen[36]. It has been established that collagen forms micelles in solutions at acidic pH in acetate buffer. The anionic (SDS) and non-ionic surfactants facilitate the micelle formation of this collagen by enhancing the hydrophobic interactions[8].

The relative viscosity and the specific viscosity of collagen alone were first determined by measuring the flow time of collagen at different temperatures. The relative and specific viscosity was again measured for collagen with α, β and γ cyclodextrin. The solutions after the addition of the three cyclodextrins were stirred well for homogeneity. Figure 5 shows the plot

of specific viscosity Vs concentration of normal collagen. From the figure it is seen that the value of the intrinsic viscosity drops at 25°C and at 37°C it drops drastically. Figure 6 shows the plot of specific viscosity Vs concentration of collagen in the presence of 0.03mM of α cyclodextrin at various temperatures. The figure shows a curve instead of a straight line. Figure 7 shows the plot of specific viscosity Vs concentration of collagen in the presence of 0.17mM of β cyclodextrin at various temperatures. It is seen that the intrinsic viscosity is higher for the collagen with β cyclodextrin than for collagen alone. Figure 8 shows the plot of specific viscosity Vs concentration of collagen in the presence of 0.23mM of γ cyclodextrin at different temperatures. Here also a curve is obtained instead of straight line. Table 5 shows a comparison of the intrinsic viscosities of different concentrations of collagen at different temperatures in the presence of β cyclodextrin. From figure 6 and 8, it is seen that the intrinsic viscosity lies between 60 & 70 dL/g for collagen with α-cyclodextrin and between 70 and 80dL/g for collagen with γ cyclodextrin respectively. For collagen with β cyclodextrin, the intrinsic viscosity is between 10 & 20dL/g. Hence it is seen that the stability of collagen is enhanced on the addition of the cyclodextrins. With the addition of α and γ cyclodextrins, the plot of specific viscosity Vs concentration of collagen deviates from that of a straight line. Such results have already been reported. Mandal et al. have reported that at higher concentrations of collagen there is a deviation from straight line[8,37]. In the present case, for the same concentration of collagen with β cyclodextrin, a straight line is observed. Hence the deviation from straight line cannot be due to the concentration of collagen. This may be due to the presence of α and ***γ*** cyclodextrins. Table 4 shows the different concentrations of α, β and ***γ*** cyclodextrin added to the collagen solution of different concentrations for study.

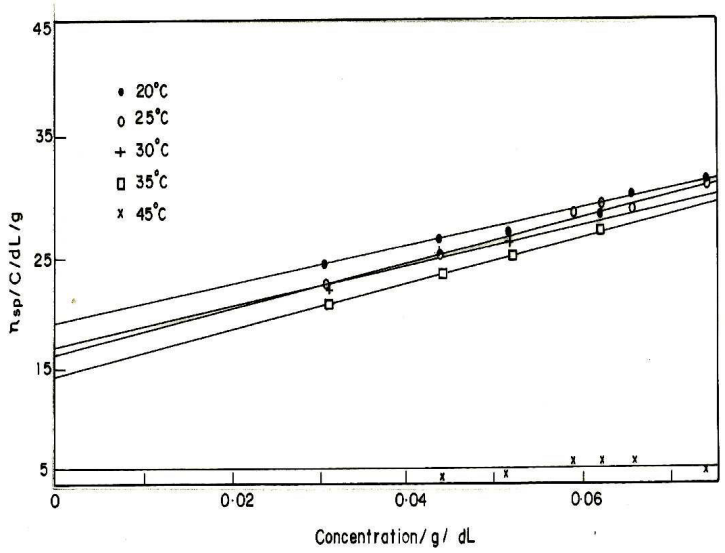

Figure 5
Plot of η_{sp}/c Vs Concentration for normal collagen at different temperatures.

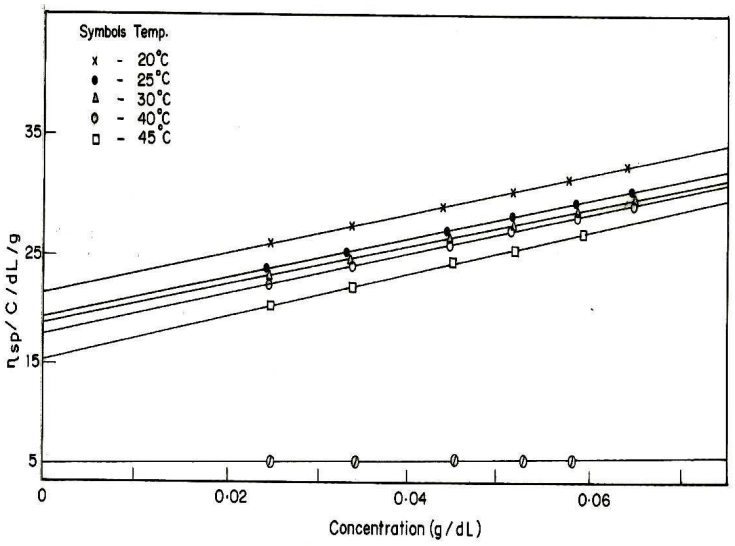

Figure 6
Plot of η_{sp}/C Vs Concentration for collagen in presence of 0.03mM α cyclodextrin at different temperatures.

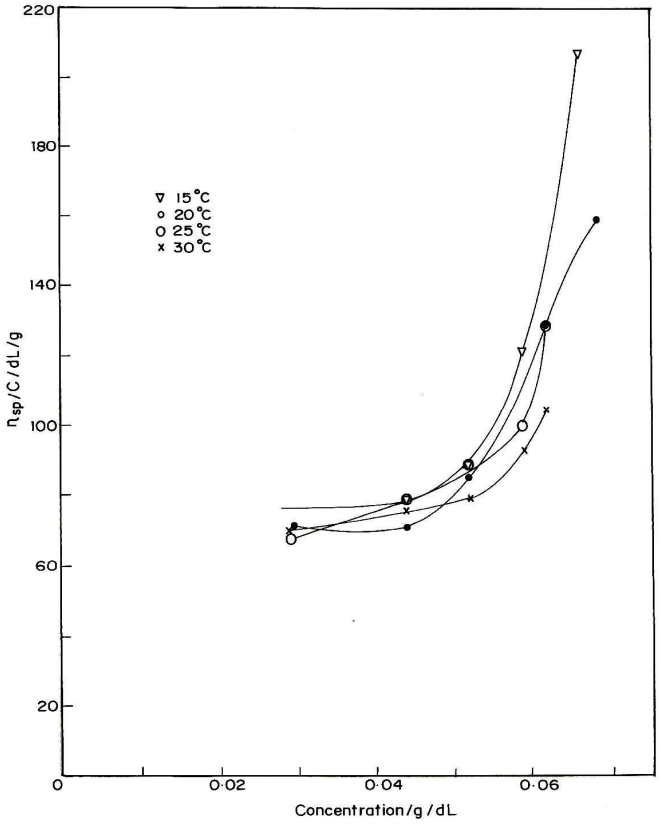

Figure 7
Plot of η_{sp} /C Vs Concentration for collagen in 0.17mM of B cyclodextrin at different temperature.

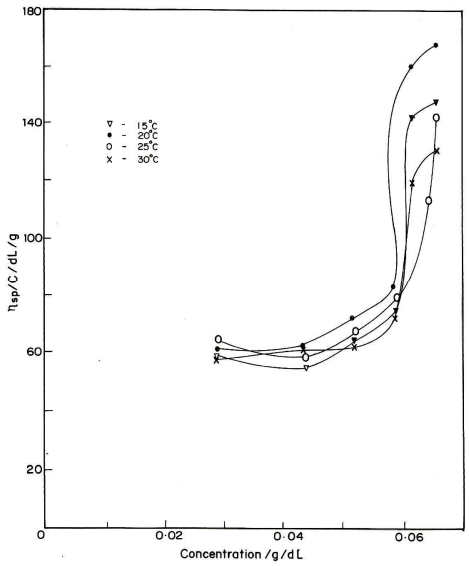

Figure 8
Plot of η_{sp} /C Vs Concentration for collagen in presence of 0.23mM of γ cyclodextrin at different temperatures.

Table 4
Concentration of α, β and γ cyclodextrin added to concentration of collagen

Concentration (g/dl)	Concentration of α, β and □ cyclodextrin (mM)		
	α CD	β CD	□ CD
0.0296	0.03	0.17	0.23
0.044			
0.052			
0.059			
0.062			
0.066			
Collagen concentration (g/dL)	Concentration of α cyclodextrin (mM)		
0.0296	0.03		
0.044			
0.052			
0.059			
0.062			
0.066			

Table 5
Intrinsic viscosity of collagen in the absence and presence of β cyclodextrin at various temperatures

Temperature (°C)	Intrinsic viscosity of collagen [η] / (dL/g)		
	[β CD]		
	0	0.17mM	0.34mM
20	17.5	21.0	22.3
25	16.5	17.5	21.0
30	14.5	17.0	21.0
35	14.5	17.0	21.5
40	-	20.0	20.0
45	-	20.0	20.3

Strand separation means final detachment of the α chains from each other, as occurs in non-cross linked molecules. However, there may be some local internal chain separation occurring early in denaturation as a multistep process, suggested by Sasaki et al., 1991 using elongational flow studies[38.] It has also been shown by comparative light scattering, viscometric and optical rotation studies that strand separation on collagen melting takes place only at the final stage of the melting process. The decrease in intrinsic viscosity with increase in temperature for normal collagen is in accordance with the suggestion of Sasaki et al., 1991. There is a possibility that the hydrophobic cavity of the cyclodextrins encapsulates the hydrophobic amino acid residues, preventing chain separation till 40°C. It is seen that without the cyclodextrins the intrinsic viscosity of collagen starts decreasing from 30°C. But with β cyclodextrins the value is almost a constant till the denaturation temperature (Table-5). When the concentration of the β cyclodextrin is increased to two fold, the intrinsic viscosity increases even more, this implies that the tolerance has increased. From (Table 5) it is seen that β cyclodextrin has an appreciable effect on the intrinsic viscosity of collagen. The table also shows that the value of intrinsic viscosity is maintained at the same value till the denaturation temperature of collagen whereas for plain collagen, the value begins to fall at a temperature near the denaturation temperature. It is also observed that when the volume of β cyclodextrin is doubled there is an increase in the value of the intrinsic viscosity. The behavior of α and γ cyclodextrin are different from that of β cyclodextrin. Further work is being carried out in these lines to find out the interactions between the telopeptides and the cyclodextrin molecules.

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