Available online at www.sciencedirect.com
SCIENCE DIRECT®
Thermochimica Acta 409 (2004) 201–206
thermochimica acta
www.elsevier.com/locate/tca

The effects of urea and *n*-propanol on collagen denaturation: using DSC, circular dicroism and viscosity

R. Usha*, T. Ramasami

Department of Biophysics, Central Leather Research Institute, Sardar Patel Road, Adyar, Chennai 600020, India

Received 19 June 2003; received in revised form 27 July 2003; accepted 31 July 2003

Abstract

The effect of urea and *n*-propanol on circular dichroism (CD) and viscosity of purified type1 collagen solution at various temperatures and differential scanning calorimetry (DSC) of rat-tail tendon (RTT) collagen fibre have been studied. CD reveals a spectrum with a positive peak at around 220 nm and a negative peak at 200 nm characteristics of collagen triple helix. The molar ellipticity decreases as the concentration of urea increases up to particular concentration (collagen solution treated with 265 μM of urea) and after that it increases (collagen solution treated with 500 μM of urea). There is a linear decrease in molar ellipticity as the concentration of *n*-propanol increases. Denaturation temperature of urea and *n*-propanol treated with purified collagen solution has been studied using viscosity method. Additives such as urea and *n*-propanol decrease the thermal stability of collagen triple helix in solution and in RTT collagen fibre. Thermal helix to coil transition of urea and *n*-propanol treated collagen depends on the degree of hydration and the concentration of these additives. Thermodynamic parameters such as the peak temperature, enthalpy of activation, and energy of activation for collagen-gelatin transition for native, urea and *n*-propanol treated RTT collagen fibre has been calculated using DSC. The change in the thermodynamic parameters has been observed for native, urea and *n*-propanol treated RTT collagen fibres. The experimental results show that the change in the water structure, dehydration and desolvation induced by different additives such as urea and *n*-propanol on RTT may vary with the type of denaturation.

Keywords: Differential scanning calorimetry; Rat-tail tendon; Urea; *n*-Propanol; Denaturation; Circular dichroism

1. Introduction

Leather is a well-exploited industrial commodity manufactured from skin. It derives its unique mechanical function and matched properties for application from the three-dimensional architecture of skin. It is established that leather derives its mechanical strength from the structural stability of the main constituent of skin, viz. collagen [1]. Although there are several types of collagen the predominant form of collagen of interest to leather science is the type 1 collagen.

The stability of the triple helix in collagen depends on inter- and intra-molecular hydrogen bonds [2,3]. The thermal denaturation of collagen depends on its water content [4–6], pH of the environmental medium [7,8], and the degree of cross-linking [9,10]. Certain additives denature collagen by weakening hydrophobic bonds while others either interact with peptide hydrogen bonds or by combining with charged sites on the protein or by some composite effect. Additives such as alcohols, urea, and dioxane salts interact with native proteins to alter their stability. Differences in the mechanism of denaturation for different additives are usually reflected on the characteristic thermodynamic functions.

The heat transformation of collagen to gelatin is interpreted as a disintegration of the collagen triple helical structure into random coils. This is accompanied by a change in physical properties such as viscosity, sedimentation, diffusion, light scattering, and optical activity. The overall helix to coil transition process has been monitored by circular dichroism (CD). The thermal unfolding was monitored by changes in ellipticity at 230 nm [11–13] or by differential scanning calorimetry (DSC) [14–17]. The mechanical properties as well as thermally induced isometric tension reflected the structural stability of collagenous tissue. These two seem to measure the components of the stability differently [18].

In this work, CD and viscosity have been used on purified type 1 collagen solution and DSC on rat-tail tendon (RTT)

* Corresponding author. Tel.: +91-44-24911386; fax: +91-44-24911589.
E-mail address: usharamamoorthy54@yahoo.co.in (R. Usha).

0040-6031/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0040-6031(03)00335-6

collagen fibre to understand the effect of the additives on collagen denaturation.

2. Materials and methods

2.1. Sample preparation

Collagen fibres were teased out from tails of 6-month-old male albino rats (Wistar strain) and thoroughly washed and stored at −20 °C until needed.

2.2. 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 [19].

2.3. Hydrogen bond breaking reagent

Urea used in the experiments was of analytical grade. Urea solutions of 1, 3, and 6 M were prepared on a weight basis. The collagen fibres were equilibrated in urea solution before each experiment.

2.4. Preparation of aqueous alcoholic mixtures

Alcoholic solvents, viz. methanol, ethanol, and *n*-propanol, used were of analytical grade. They were used without further purification. The collagen fibres were equilibrated in aqueous–alcoholic media where alcoholic proportions varied as 1, 7, 10, and 20 mol% [20].

2.5. Circular dichroism (CD)

Collagen was treated with different concentrations of urea and *n*-propanol to study the conformational stability of collagen. Purified collagen solution was incubated in the presence of denaturants at 25 °C for 24 h and the spectra were recorded at 25 °C using a Jasco spectropolarimeter. A scan speed of 20 nm min^{-1} was used with an average of five scans per sample. A slit width of 1 nm and a time constant of 1 s were used. A 1 mm cell was used for the experiments. A reference spectrum containing acetic acid was also recorded. The CD spectra of the samples were obtained after subtracting the reference spectrum. Spectra were expressed in terms of molar ellipticity [21]. Changes in the conformation of collagen on addition of various amounts of urea and *n*-propanol were recorded.

2.6. Viscosity measurements

Viscosities were determined using a thermostated water bath with quartz Ubbelohde's viscometer.

2.7. Differential scanning calorimetry (DSC)

Known amount of native RTT (generally 1–2 mg) was immersed in 1 and 3 M urea and 1 and 10 mol% *n*-propanol for 24 h. The soaked samples were blotted uniformly and hermetically encapsulated in aluminum. The samples were placed in a differential scanning calorimetric cell of a Perkin-Elmer DSC-7 instrument. The heating rate was maintained constant at 6 K min^{-1}. The peak temperature (T_p) for collagen-to-gelatin process was recorded and enthalpy changes (ΔH) and energy of activation (E_a) for this transition were computed using standard methods [22,23].

3. Results and discussions

3.1. Circular dichroism (CD)

UV CD spectra of protein solutions provide information about secondary structure contents of proteins [24]. CD has been used to characterize collagen-like peptides [25,26]. The effect of urea on the CD of lysozyme and alcohol on denatured proteins has been reported [27,28]. Far UV CD spectra of collagen solutions treated with various concentrations of urea and *n*-propanol at 25 °C are shown in Figs. 1 and 2. Addition of urea and *n*-propanol to collagen solution did not alter the CD spectra significantly (Figs. 1 and 2 for θ_{220}) at a given concentration. The CD spectrum reveals spectrum at peak at around 220 nm characteristic of the triple helix, which decreases during denaturation. The CD spectra of collagen treated with urea and *n*-propanol exhibit patterns characteristic of random conformation. In general, by increasing the concentration of urea and *n*-propanol, a slight decrease in molar ellipticity around 220 nm and an increase around 200 nm were observed. The molar ellipticity decreases as the concentration of urea increases up to particular concentration (collagen solution treated with 265 μM of urea) and after that it increases (collagen solution treated with 500 μM of urea) as given in Table 1. In the case of *n*-propanol, from 2 to 10 ($\times 10^{-2}$ M), there is a linear decrease in ellipticity at 220 nm and an increase at 200 nm has been observed. It has been reported that on complete denaturation, the positive peak at 220 nm disappears completely and the negative band has found red shifted [29]. In this

Table 1
Molar ellipticity (deg cm^2 dmol^{-1}) at θ_{220} and θ_{200} for purified rat-tail tendon collagen solution treated with various concentrations of urea

Specification	θ_{220}	θ_{200}
Native collagen	46655	−348980
Collagen + 65 μM urea	25771	−343807
Collagen + 130 μM urea	25412	−324039
Collagen + 200 μM urea	24754	−331777
Collagen + 265 μM urea	43340	−373317
Collagen + 500 μM urea	61052	−534955

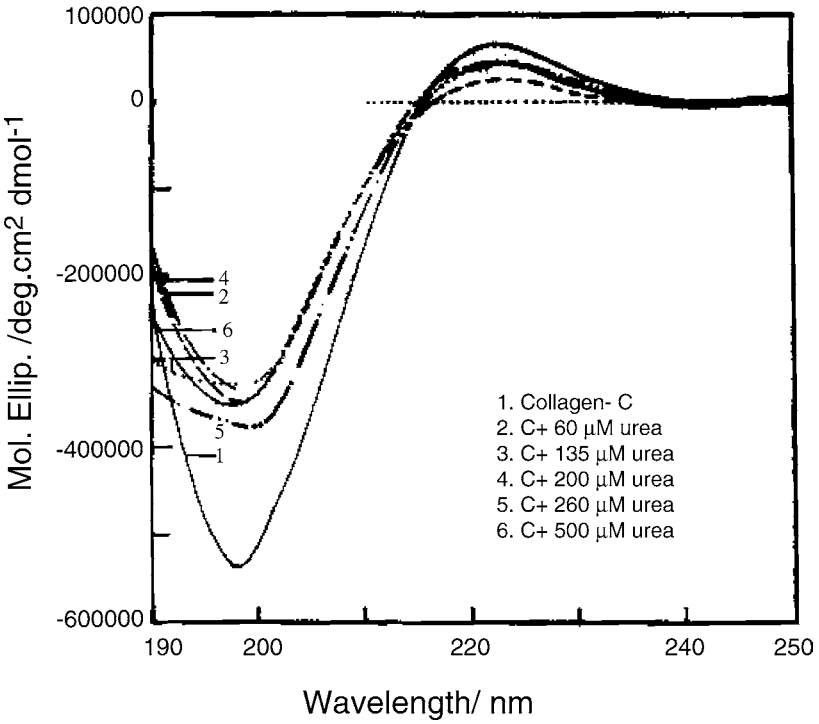

Fig. 1. CD spectra of purified type 1 collagen solution treated with various concentrations of urea.

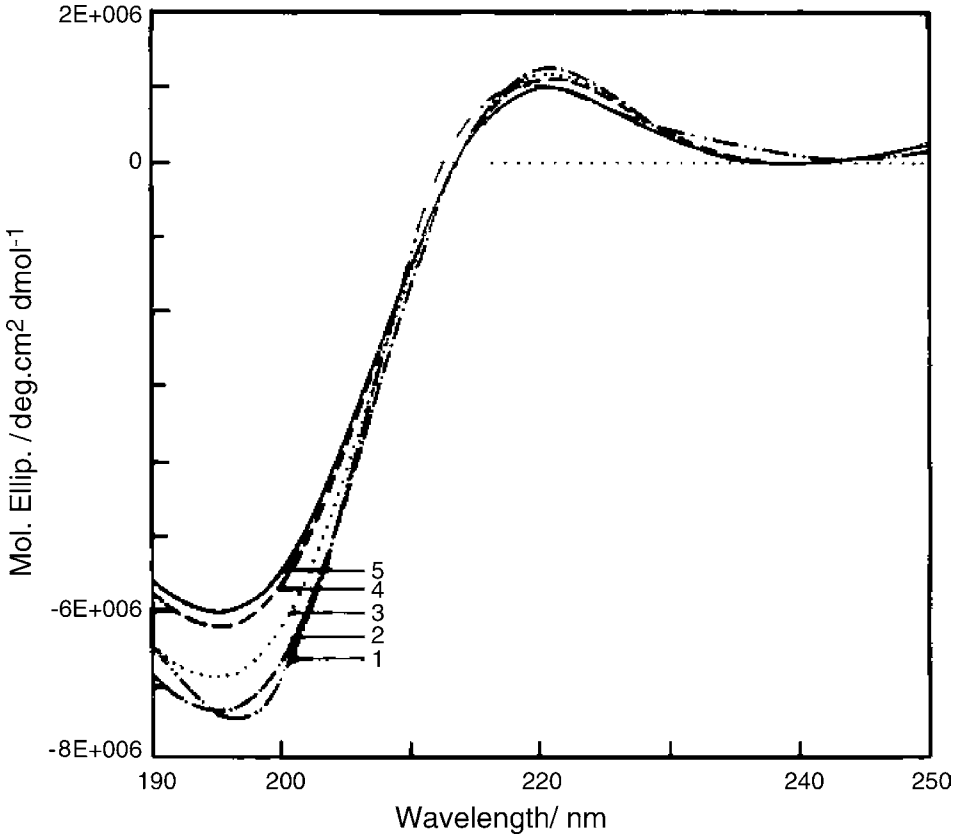

Fig. 2. CD spectra of purified type 1 collagen solution treated with various concentrations of *n*-propanol: (1) collagen + 10×10^{-2} M *n*-propanol, (2) collagen + 7.5×10^{-2} M *n*-propanol, (3) collagen + 5×10^{-2} M *n*-propanol, (4) collagen + 2.5×10^{-2} M *n*-propanol, and (5) native collagen.

Table 2
Rpn (ratio of positive to negative band) values of native collagen and collagen treated with various concentrations of urea

Serial no.	Specification	Rpn
1	Native collagen	7.5
2	Collagen + 65 μM urea	13.34
3	Collagen + 130 μM urea	12.75
4	Collagen + 200 μM urea	13.4
5	Collagen + 265 μM urea	8.6
6	Collagen + 500 μM urea	8.76

investigation, there is neither significant change in the red shift of the negative band nor any disappearance of the positive band at 220 nm. Partially denatured collagen was found to give CD spectra with lower intensity, red shifted crossover points and a higher ratio of the intensity of short wavelength band to the intensity of the long wavelength band [30]. The ratio of positive to negative band (Rpn) of the native and urea treated collagen solution is given in Table 2. This ratio increases as the concentration of urea increases up to a particular concentration after that it decreases. The decrease of the Rpn indicates that collagen is folded or precipitated at an extreme urea concentration. Low concentration of urea stabilizes protein. The stability increases as the denaturants concentration is raised to the limit of its sub-denaturing effect. With further increase in the concentration, the protein unfolding effect of the denaturant beats its own stabilizing effect.

3.2. Viscosity

Thermal stabilities of a purified collagen solution and of solutions treated with urea and *n*-propanol are discernible from the corresponding viscosity curves in Fig. 3. The temperature at which the change in viscosity was half the maximum value was taken as the denaturation temperature. The denaturation temperature of purified collagen solution and treated with urea (0.1 M) and *n*-propanol (1 μM) (at a given concentration) are 37, 35, and 36 °C, respectively. Additives such as urea and *n*-propanol decrease the thermal stability of collagen triple helix in solution and in RTT collagen fibre. The helix to coil transition of urea and *n*-propanol treated collagen depends on the degree of hydration and the concentration of these additives. The viscosity of purified type 1 collagen solution decreases by the addition of the denaturing agents and it depends on the concentration of the specific additives.

3.3. Differential scanning calorimetry (DSC)

The endothermic helix to coil transition by DSC in soluble collagen or in the fibrous form has been analyzed by DSC and the corresponding enthalpy changes and temperature of collagen to gelatin transition determined [31–35]. The peak temperature, enthalpy changes, and energy of activation associated with the phase change (collagen–gelatin transition) for native RTT in water, 3 M urea, and 10 mol% of *n*-propanol are presented in Table 3.

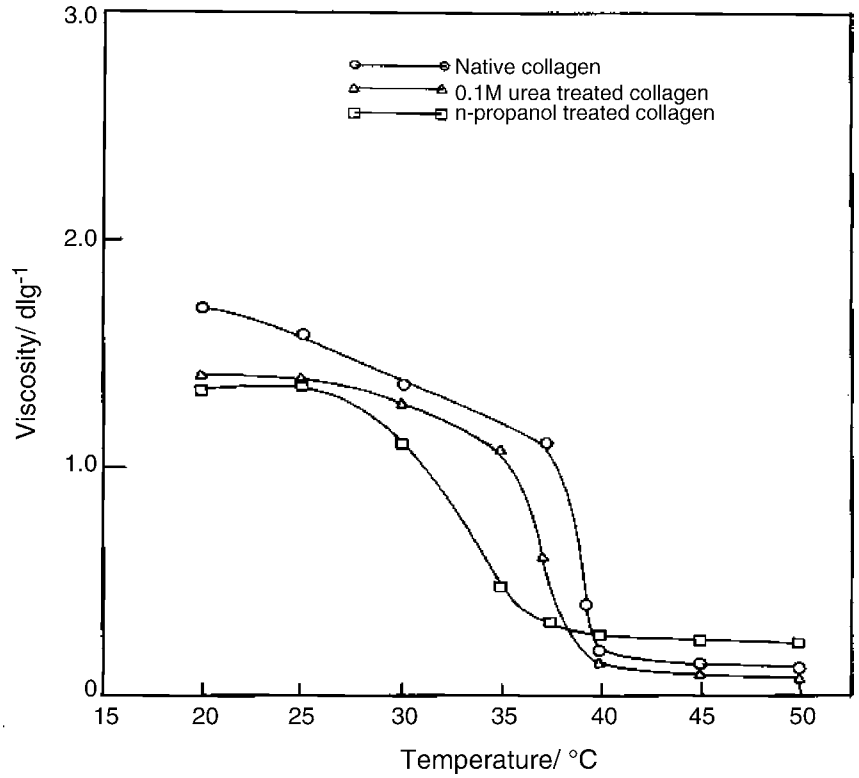

Fig. 3. Viscosity curves of native collagen; urea (0.1 M) and *n*-propanol (1 μM) treated purified type 1 collagen solution at different temperatures.

Table 3
Comparison of thermodynamic parameters (DSC) of native RTT swollen in water, 3 M urea, and 10 mol% of *n*-propanol

Specification	$T_p \pm 1$ (°C)	ΔH (J g^{-1})	E_a (kJ mol^{-1})	ln(k_0)
Native RTT	67	47 ± 1	1180 ± 25	415 ± 9
Native RTT swollen in 10 mol% *n*-propanol	59.5	43 ± 1	595 ± 5	220 ± 5
Native RTT swollen in 3 M urea solution	53	39 ± 0.5	1096 ± 23	401 ± 7

Mean ± standard deviation of six determinations. T_p: peak temperature, ΔH: change in enthalpy, E_a: energy of activation, k_0: rate of shrinkage.

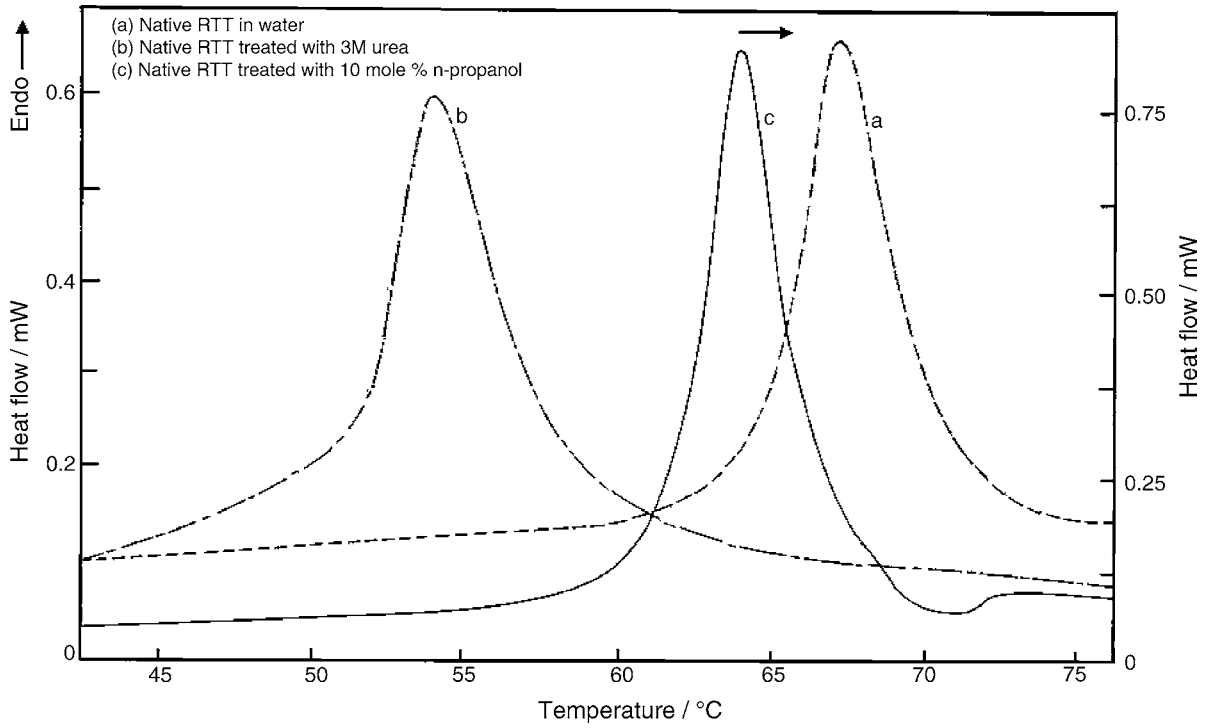

Fig. 4. Comparison of thermogram of (a) native RTT, (b) 6 M urea treated RTT (*y*-axis left side), and (c) 10 mol% *n*-propanol treated RTT (*y*-axis right side).

The comparison of the thermogram of native, 3 M urea and 10 mol% of *n*-propanol treated RTT presented in Fig. 4 indicate that the thermogram is broader in 3 M urea treated RTT compared to that of native RTT. The broadening of DSC scan in macromolecular system refers to the smaller cooperatives among the participating subunits. This could be a disorganization of the fibre matrix. In alcoholic media the shape of the thermogram is sharper compared to that in urea. A sharper transition means a strong interdomain connection when the system behaves as a single big unit. The loosening of the structural water cannot be the explanation of this sharp transition because it should appear as a significant change in the heat capacity, which cannot be seen, from the DSC scans. However, all these thermodynamic parameters determine the native state stability.

4. Conclusion

The non-random distribution of ionizable and hydrophobic side chains along the repeating unit results in the occurrence of charged and hydrophobic patches on the surface that contribute to stabilization of higher order structures through electrostatic and hydrophobic interactions on the stability of collagen. Hence, it is important to study the effect of hydrophobic interactions of collagen. The role of these bonds in stabilizing collagen can be investigated if specific reagents can be found to independently influence the stability.

There are reports that urea and guanidine hydrochloride promoting the solubility of the hydrophobic residues in aqueous solution may also disrupt the hydrophobic interactions [36]. Repulsive forces of water molecules influence hydrophobic interactions. Urea can participate in hydrogen bonding with water and therefore compete with these hydrophobic forces. The hydrophobic bond is due to the entropy gained from the release of highly organized water from the hydrophobic molecules; the displacement of water requires breaking some of its hydrogen bonds. Therefore, hydrophobic binding is endothermic opposing the ionic component of binding.

It is believed that hydrophobic forces influence intra- and inter-molecular interactions in collagen, which are weakened in the presence of nonpolar groups from the solvent environment. In this study of collagen with *n*-propanol or in

the presence of varying amounts of urea, it is necessary to include the effects of solvation of the protein in solvent media, which reflects the ways in which these additives would interact with the protein and with the water of solvation. The change in the water structure, dehydration, and desolvation induced by different additives such as urea and *n*-propanol on RTT may vary with the type of denaturation.

To summarize, the phase changes corresponding to helix–coil transition and collagen to gelatin transition are associated with major changes in protein–water interactions. Therefore, urea and *n*-propanol seem to influence the CD and viscosity data of monomeric collagen solution and DSC data of RTT collagen fibre significantly.

References

[1] A.J. Bailey, J. Soc. Leath. Technol. Chem. 79 (1992) 111.
[2] B. Brodsky, J.A.M. Ramshaw, Matrix Biol. 5 (1997) 545.
[3] D.J. Prockop, K.I. Kivirikko, Ann. Rev. Biochem. 64 (1995) 403.
[4] A. Bigi, G. Cojjazi, N. Roveri, M. Koch, Int. J. Biol. Macromol. 9 (1987) 363.
[5] G.N. Ramachandran, C. Ramakrishnan, in: G.N. Ramachandran, A.H. Reddi (Eds.), Biochemistry of Collagen, Plenum Press, New York, 1979, pp. 45–81.
[6] G.N. Ramachandran, G. Kartha, Nature 174 (1954) 269.
[7] A. Ripmonti, N. Roveri, D. Braga, Biopolymers 16 (1980) 2133.
[8] R. Usha, T. Ramasami, J. Appl. Polym. Sci. 75 (2000) 1577.
[9] R. Usha, T. Ramasami, Thermochim. Acta 356 (2000) 59.
[10] D.G. Wallace, R.A. Condell, J.W. Donovan, A. Paivinen, W.M. Rhee, S.B. Wade, Biopolymers 25 (1986) 1875.
[11] R. Gayatri, S.A. Kumar, R. Rajaram, T. Ramasami, Biochem. Biophys. Res. Commun. 283 (2001) 229.
[12] M.L. Tiffany, S. Krimm, Biopolymers 8 (1969) 347.
[13] C.M. Johnson, A.R. Fershi, Biochim. Biophys. Acta 1338 (1997) 161.
[14] C.A. Miles, A.J. Bailey, Micron 32 (2001) 325.
[15] P.E. McClain, E.R. Wiley, J. Biol. Chem. 247 (1972) 692.
[16] A. Finch, P.J. Gardner, D.A. Ledward, S. Menashi, Biochim. Biophys. Acta 365 (1974) 400.
[17] P.L. Kronick, P. Cooke, J. Polym. Sci. B: Polym. Phys. 36 (1998) 805.
[18] K.H. Gustavson, The Chemistry and Reactivity of Collagen, Academic Press, New York, 1956, pp. 140–153.
[19] J.F. Wossner Jr., Arch. Biochem. Biophys. 93 (1961) 440.
[20] R. Usha, T. Ramasami, J. Polym. Sci. B: Polym. Phys. 37 (1999) 1397.
[21] E.M. Brown, R.L. Dudley, A.R. Elsetinow, J. Am. Leath. Chem. Assoc. 92 (1997) 225.
[22] R. Usha, T. Ramasami, Thermochim. Acta 338 (1999) 17.
[23] R. Usha, T. Ramasami, J. Appl. Polym. Sci. 84 (2002) 975.
[24] S.M. Kelly, N.C. Price, Biochim. Biophys. Acta 1338 (1997) 161.
[25] K. Holmgren, L.E. Bretscher, K.M. Taylor, R.T. Raines, Chem. Biol. 6 (1999) 63.
[26] C.G. Long, E. Braswell, D. Zhu, J. Apigo, J. Baum, B. Brodsky, Biochemistry 32 (1993) 11685.
[27] K.P. Barens, J.R. Warren, J.A. Gorden, J. Biol. Chem. 247 (1972) 1708.
[28] T. Kanno, N. Tanaka, M. Kataoka, E. Takano, M. Maki, Biochim. Biophys. Acta 1338 (1997) 161.
[29] T.V. Burjanadze, M.O. Bezhitadze, Biopolymers 32 (1992) 951.
[30] C.R. Mathews, M.M. Crisanti, Biochemistry 20 (1981) 784.
[31] F. Flandin, C. Buffevant, D. Herbage, Biochim. Biophys. Acta 791 (1984) 205.
[32] J.J. Lim, Biopolymers 15 (1976) 2371.
[33] H. Hellaûer, R. Winkler, Connect. Tissue Res. 3 (1975) 227.
[34] C.A. Miles, T.V. Burjanadze, A.J. Bailey, J. Mol. Biol. 245 (1995) 437.
[35] K. Takenouchi, K. Kondo, F. Nakamura, J. Soc. Leath. Technol. Chem. 79 (1995) 188.
[36] A. Finch, D.A. Ledward, Biochim. Biophys. Acta 278 (1972) 433.