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# ADSORPTION COMPLEXES OF COLLAGENOUS POLYPEPTIDE-IONIC SURFACTANT IN AQUEOUS MEDIUM

## 2.[1] THE INFLUENCE OF ADSORPTION COMPLEXES OF POLYPEPTIDE-IONIC SURFACTANT ON THERMAL STABILITY OF COLLAGEN TYPE I

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In the first part of this study it has shown that the interaction between collagenous polypeptides (collagen type I with native structure of triple helix and collagen hydrolyzates with no significant secondary structure) and ionic surfactants (sodium dodecyl sulfate and hexadecyl trimethylammonium bromide) involves the formation of micelle-like clusters of ionic surfactant adsorbed onto polypeptide backbone (polypeptide-surfactant complexes) at surfactant concentrations much lower than its *cmc*. The present investigation is focused on the influence of polypeptide-ionic surfactant complexation on thermal denaturation of the collagen type I extracted from calfskin and young horse tendons. On the basis of capillary viscometry, a good correlation between denaturation temperatures and activation energies of viscous flow for the dilute aqueous collagen-ionic surfactant solutions at pH 2.5 and 8.0 has been found. Beginning from that, it has established that the presence of ionic surfactant reduces thermal stability of the two collagens, with a more pronounced effect in the case of sodium dodecyl sulfate. Accordingly, it has been proposed a thermal denaturation mechanism of the collagen triple helix in the presence of ionic surfactants.

## INTRODUCTION

The manifold biomedical applications of proteins require preliminarily knowing of their thermal, chemical and mechanical stability under given employing conditions of them. More than a third of the proteins synthesized by the living cells of mammalian and human body consist of collagens and, among them, the collagen type I is the majority structural protein.

As a consequence of an excellent biodegradability and a low immunogenic effect, the collagen type I finds a lot of applications in biomedical field, cosmetics or pharmaceutical industry.[2-6] From this point of view, there are at least two main reasons which entail assessing the thermal stability of collagen: (a) the behavior of the collagen native structure in the collagen systems under employing conditions (depending on temperature, pH, ionic strength) and (b) carrying over the collagen-based materials during the course of their manufacture-end use period. The term of thermal stability in the case of the collagen type I refers to the temperature endurance of secondary and tertiary structures embedded into the structural feature of the collagen triple helix. The thermal denaturation of this distinctive conformation implies a destruction process giving rise to a final state of more or less random coil within which the three polypeptide chains (two α1(I) an one α2(I)) are completely

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detached.[7] Considering all these facts, the denaturation temperature or the helix-coil transition temperature ($T_d$) is defined as the temperature at which a half of the collagen molecules having initially the native conformation are denatured. Some previous studies found that $T_d$ of fibrillar collagen in aqueous solutions is close to the physiological temperature of the source body, whereas $T_d$ of the same collagen, but in an aggregation state (e.g. fibers), increases by more than $20^0$C.[8-10] One of the most important parameter which alters the thermal stability of collagen is the pH of the environment.[11-15] A number of experimental findings show that the maximum of thermal stability of the collagen type I molecules in aqueous solution, at a given ionic strength, is obtained at physiologic pH.[10]

This study carries forward the previous investigations,[1] with the main aim in establishing the influence of adsorption collagen-ionic surfactant (IS) complexes on the thermal stability of collagen in solution, being well-known that both collagen and surface-active agents represent common components of soap, shampoos, creams and other cosmetics.[6]

## RESULTS AND DISCUSSION

The assessment of the influence of adsorption collagen-IS complexes on thermal stability of the collagen type I rests on $T_d$'s determination in aqueous IS solutions. The experimental conditions were established in order to avoid the formation of insoluble collagen-IS complexes. As it has been shown elsewhere,[1] an IS may be adsorbed onto a polypeptide backbone carrying a net-electric charge of the same sign as for that of IS because there is a non-zero fraction of ionic groups with the opposite charge anchored to the polypeptide chain (depending on the pH of solution) which favor the onset and the development of polypeptide-IS complexation. Beginning from this, two general kinds of aqueous systems have been studied: collagen-sodium dodecyl sulfate (SDS) at basic pH (higher than isoelectric pH of collagen, IEP) and collagen-hexadecyl trimethylammonium bromide (HTAB) at acidic pH (lower than IEP). At the same time, two concentration behaviors of surfactant were used, for both the case of the calfskin collagen (CSC) and the case of the collagen extracted from young horse tendons (HTC): bellow critical micelle concentration (*cmc*) and above *cmc*.

### 1. Collagen-HTAB systems

The increase of temperature of a collagen solution leads to the thermal degradation of protein during which the triple helical structure is unfolded resulting almost randomly coiled arrangements (gelatin). Such a transformation is accompanied by profound changes in physical properties of protein such as viscosity, solubility or optical activity. The pH of the collagen-cationic surfactant (HTAB) systems was fixed at 2.5, bellow IEP of collagen, in order to avoid the protein precipitation. The collagen concentration was kept constant at 0.05 wt% throughout the collagen-HTAB solutions.

As in the case of the first part[1] of the study, ultraviolet-circular dichroism (UV-CD) (Fig.1), electron microscopy (Fig.2) and capillary viscometry were used to characterize the collagens (CSC and HTC) at molecular scale. The main characteristics of CSC and HTC arisen from the measurements are outlined in the table 1. The results confirm the occurrence of the unmodified secondary and tertiary structures (triple helix) of the collagens. At the same time, the molecular weight of HTC is higher than that of CSC as resulted from viscosity measurements. This fact indicates a certain degree of HTC self-assembly likely due to some intermolecular crosslinks. The arborescent, branched shape of HTC (at molecular level) revealed by electron microscopy (Fig.2b) is in a good accordance with the molecular weights obtained for HTC and CSC.

The isoelectric ranges found by visual observation of the non-uniformity of the aqueous dispersions of the collagens (collagen concentration – 0.10 wt%) at various pHs are the following: 4.6-6.0 for CSC and 4.1-6.0 for HTC.

The choice to employ the capillary viscometry in studying collagen denaturation in solution[19-22] builts upon both simplicity and very high sensitivity of the method. In such investigations either relative viscosity or reduced viscosity of a protein solution (with a constant protein concentration) as a function of temperature is usually followed. From this standpoint, the thermal denaturation of a protein in solution is attended by an abrupt decline of relative (or reduced) viscosity within a narrow range of temperature. The abscissa of the inflexion point on the relative/reduced viscosity-temperature dependence is considered as $T_d$.

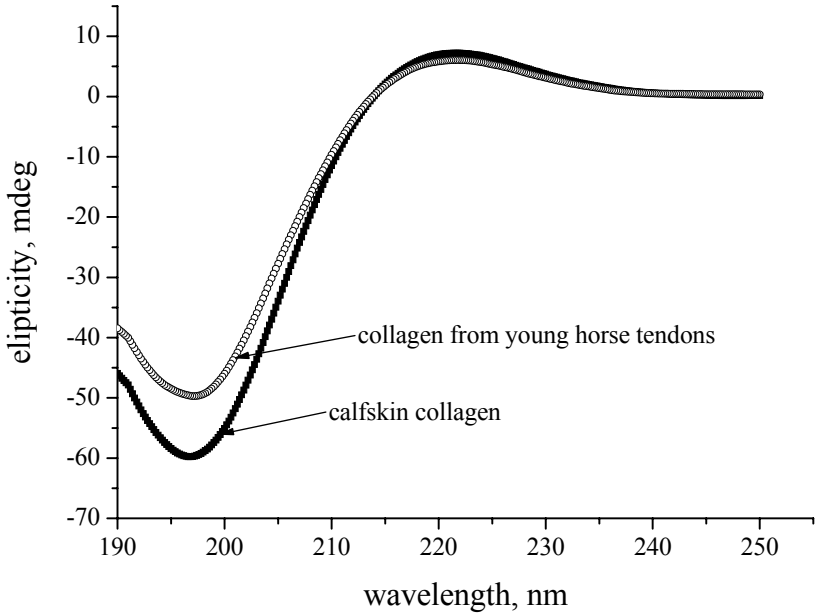
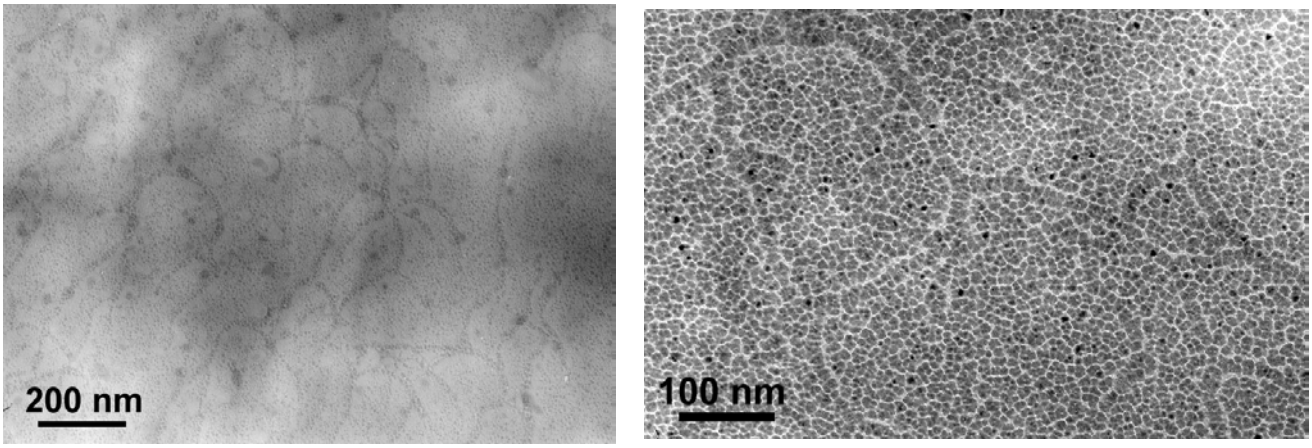



Fig. 1 – CD spectra of collagen type I in 0.1 M acetic acid solution (concentration of protein 0.05% by weight).




a b

Fig. 2 – TEM images at molecular scale for (a) CSC and (b) HTC .

**1.1. Aqueous solutions of CSC-HTAB.** The determination of $T_d$ was performed at two different HTAB concentrations: $3x10^{-4}$M (bellow *cmc*) and $3x10^{-3}$M (above *cmc*). The surfactant concentrations were chosen as a rough indicative taking into account the results of the study on HTAB adsorption onto collagen at acidic pH (2.5)[1], which prove that the onset of adsorption (critical aggregation concentration, *cac*) occurs at HTAB concentration of about $1.5\text{-}2.1x10^{-4}$M and the saturation adsorption ($T_2$) corresponds to the cationic surfactant concentration of $7.5x10^{-4}$M. In this respect, the investigations are focused on the study of the influence of the collagen-HTAB adsorption complexes on thermal stability of collagen at the surfactant concentration within its *cac* and *cmc*, on the one hand, and higher than $T_2$, on the other hand. In aqueous solution, pH 2.5, the *cmc* of HTAB is found to be $7.4\text{-}7.5x10^{-4}$M as it resulted from steady state fluorescence of pyrene and surface tension measurements.[1]

Study of thermal denaturation of CSC in the presence of HTAB evidences a noticeable diminution of $T_d$ as surfactant concentration increases (Fig.3a). This is an indirect confirmation of the fact that the progressively increasing value of the positive net-charge of the collagen-HTAB complexes formed as HTAB concentration rises favors unfolding of the native conformation of collagen at temperatures lower than those for collagen solution with no surfactant.

*Table 1*

Some experimental features of collagen type I as resulted from circular dichroism, electron microscopy and capillary viscometry

| Sample | UV-CD, pattern location | | | | TEM | Capillary viscometry | |
|---|---|---|---|---|---|---|---|
| | positive peak, nm | negative peak, nm | crossover point, nm | Rpn* | | intrinsic viscosity,** [η], dL/g | viscosity average molecular weight,** $\overline{M}_v$, a.m.u. |
| CSC | 221.6 | 196.8 | 214 | 0.12 | hair-shaped structures of 250-280 nm in length, low degree of intermolecular association | 12.26 | $3.6 \times 10^5$ |
| HTC | 221.8 | 197.2 | 213.8 | 0.12 | arborescent structures, some branching degree | 14.25 | $3.9 \times 10^5$ |

* The absolute value of the ratio between the maximum intensities corresponding to the positive peak and negative one, respectively

** $\overline{M}_v$ was obtained on the basis of the intrinsic viscosity determination in accordance with the experimental conditions required to properly employ the Mark-Houwink-Sakurada equation[16-18]

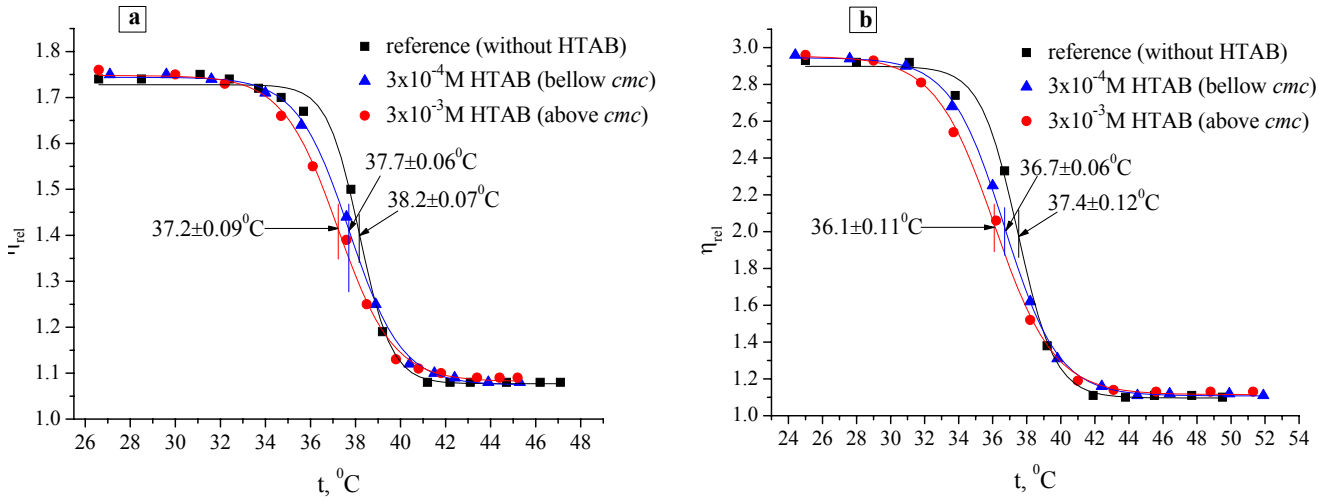



Fig. 3 – Relative viscosity-temperature dependences for acetic collagen solutions in the presence of HTAB at pH 2.5: (a) CSC and (b) HTC.

On the basis of the shape of the relative viscosity-temperature dependences, the data were fitted with a Boltzmann type equation for reverse sigmoids:

$$\eta_{rel} = (\eta_{rel})_{min} + \frac{(\eta_{rel})_{max} - (\eta_{rel})_{min}}{1 + e^{\frac{t - t_{inf}}{b}}} \qquad (1)$$

where $(\eta_{rel})_{min}$ and $(\eta_{rel})_{max}$ are the lowest and the highest values of relative viscosities measured for a collagen solution within employed temperature range, t – temperature (in $^0$C), $t_{inf}$ – the abscissa of the inflexion point which coincides with $T_d$ (in $^0$C) and b – a parameter which is a measure of the sigmoid's dip. The main fitting parameters are shown in table 2.

**1.2. Aqueous solutions of HTC-HTAB.** The tendency observed in the case of CSC systems keeps up for HTC ones as well (Fig.3b). Even though HTC possesses a crosslinking degree higher than that of CSC, which is ascertained by both electron microscopy and viscometry, actually HTC thermal denaturation in the presence of the cationic surfactant is not slowed down to occur but the difference between $T_d$ of HTC without surfactant and for HTC at maximum concentration

of HTAB is $1.3^0$C against $1.0^0$C obtained for CSC. At the same time, the occurrence of a difference in denaturation temperatures for HTC and CSC, at the same protein concentration and pH, is caused by the different nature in mammalian species and the kind of tissue from which the collagens were extracted.

*Table 2*

Statistical parameters of Boltzmann fittings for temperature dependence of relative viscosity for the studied systems

| Sample | Surfactant concentration, mole/L | Degrees of freedom, DF | Sum of squared residuals over degrees of freedom, $\chi^2$/DF | Coefficient of determination (R-squared), $R^2$ |
|---|---|---|---|---|
| CSC, pH 2.5 | 0 | 11 | 0.00031 | 0.99759 |
| CSC-HTAB, pH 2.5 | $3x10^{-4}$ (bellow *cmc*) | 8 | 0.00009 | 099928 |
| | $3x10^{-3}$ (above *cmc*) | 9 | 0.00021 | 0.99811 |
| HTC, pH 2.5 | 0 | 7 | 0.00196 | 0.99816 |
| HTC-HTAB, pH 2.5 | $3x10^{-4}$ (bellow *cmc*) | 8 | 0.0004 | 0.99957 |
| | $3x10^{-3}$ (above *cmc*) | 7 | 0.00092 | 0.99900 |
| CSC, pH 8.0 | 0 | 9 | 0.00078 | 0.99361 |
| CSC-SDS, pH 8.0 | $3x10^{-3}$ (bellow *cmc*) | 8 | 0.00006 | 0.99946 |
| | $3x10^{-2}$ (above *cmc*) | 8 | 0.00004 | 0.99963 |
| HTC, pH 8.0 | 0 | 7 | 0.00024 | 0.99953 |
| HTC-SDS, pH 8.0 | $3x10^{-3}$ (bellow *cmc*) | 8 | 0.00033 | 0.99932 |
| | $3x10^{-2}$ (above *cmc*) | 7 | 0.0009 | 0.99814 |

## 2. Collagen-SDS systems

**2.1. Aqueous solutions of CSC-SDS.** As mentioned above, the experimental conditions in preparing collagen-IS mixtures were chosen so that only solutions (monophasic systems) were eventually obtained. Thus, all the collagen-SDS systems are prepared at basic pH (pH 8.0, above the isoelectric range of collagen) due to the use of an anionic surfactant. Resembling to collagen-HTAB solutions, in the case of collagen-SDS mixtures the surfactant concentration obeyed the same behavior: $3x10^{-3}$M (above *cac* and bellow *cmc*) and $3x10^{-2}$M (above $T_2$). The value of *cmc* of SDS in water at pH 8.0 and $25^0$C is $7x10^{-3}$M and its *cac* and $T_2$ (in aqueous solution of CSC 0.05 wt%, pH 8.0) are $1.3\text{-}1.4x10^{-3}$M and $2.1x10^{-2}$M, respectively.[1] The effect of temperature changes on relative viscosity of CSC solutions in the presence of SDS is plotted in figure 4a. The well-known denaturing action of SDS on proteins is obvious in the case of CSC as well: in regard to the reference solution of collagen, with $T_d$ of $40.1^0$C, the presence of SDS induces a significant decline of $T_d$ to $38.8^0$C and $37.6^0$C, respectively, as surfactant concentration rises. It is important to note that $T_d$ of CSC at basic pH ($40.1^0$C) is neatly higher than that of collagen at acidic pH ($38.2^0$C). This could be a direct consequence of a lower mean number of carboxylate groups (belonging to aspartic acid and glutamic acid residues) at pH 8.0 in comparison with that of the ammonium groups (belonging to arginine, lysine and hydroxylysine residues) at pH 2.5 that each CSC molecule individually carries. Indeed, by using the Henderson-Hasselbalch equation, the percent proportion of the existing carboxylate groups (accounted with respect to the overall number of carboxyl groups) at a given pH, $\alpha_{-COO^-}$, can be written as:

$$\alpha_{-COO^-} = \frac{10^{pH-(pKa)_{-COOH/-COO^-}}}{1+10^{pH-(pKa)_{-COOH/-COO^-}}} \cdot 100 \qquad (2)$$

and the percent proportion of the ammonium groups (accounted with respect to the overall number of amine groups), $\alpha_{-NH_3^+}$, as follows:

$$\alpha_{-NH_3^+} = \frac{1}{1+10^{pH-(pKa)_{-NH_3^+/-NH_2}}} \cdot 100 \qquad (3)$$

where $(pKa)_{-COOH/-COO^-}$ and $(pKa)_{-NH_3^+/-NH_2}$ are the acidity exponents associated to the

chemical pairs written as indices, 2.04 and 9.00, respectively.[1] Thus, at pH 2.5 the two percent proportions are: $\alpha_{-COO^-}$ = 74% and $\alpha_{-NH_3^+}$ = 100%. Similarly, at pH 8.0: $\alpha_{-COO^-}$ = 100% and $\alpha_{-NH_3^+}$ = 91%. For an almost equal number of both amine and carboxyle groups occurring onto each collagen molecule, it is easy to observe that the net-positive charge of collagen at acidic pH is greater than its negative net-charge at basic pH, what could lead to the conclusion that the net-charge size of collagen (strongly associated with the strength of electrostatic intermolecular repulsions) crucially brings about its thermal stability (lower at acidic pH and greater at basic pH).

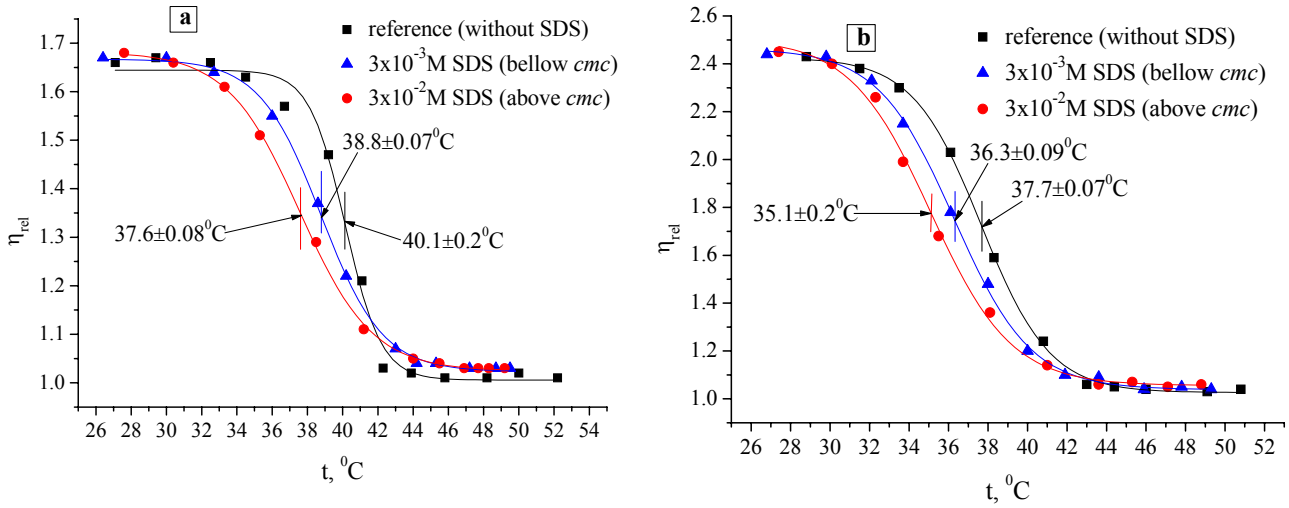



Fig. 4 – Relative viscosity-temperature dependences for acetic collagen solutions in the presence of SDS at pH 8.0: (a) CSC and (b) HTC.

**2.2. Aqueous solutions of HTC-SDS.** The denaturing action of SDS remains strong enough in the case of HTC as well (Fig.4b). Hereby, a decrease by 2.6$^0$C of $T_d$ of HTC in the presence of anionic surfactant of 3x10$^{-2}$M is observed in comparison with surfactant-free collagen solution. At the same time, $T_d$ of HTC keeps almost constant as pH changes. Considering the findings just discussed above, this means that the net-charge size of collagen at employing pHs is approximately changeless. Such a status can be achieved if the ratio between the overall number of carboxyl groups and that of amine ones is more than unity so that the positive net-charge at pH 2.5 be almost equal to the negative net-charge at pH 8.0. This most likely appears when the total number of amino groups anchored onto the collagen molecule decreases compared to the number of carboxyl groups. The depicted situation occurs especially in the case of intermolecular crosslinking of collagen. The collagen into extracellular matrix, depending on the nature and type of tissues, may form crosslinks between triple helixes by means of lysine and hydroxylysine residues. Consequently, the side chains of lysine and hydroxylysine residues loose their amine groups (under lysyl oxidase action) carrying out aldehyde groups. The latters can react with ε-amine groups of lysine/hydroxylysine residues belonging to neighbor collagen molecules giving rise to intermolecular crosslinks (Schiff bases).[23] On this basis, the fact that $T_d$ of HTC at pH 2.5 and 8.0 is roughly the same is thus accountable.

To further confirm the formation of adsorption complexes of collagen-IS during thermal denaturation of collagen, the activation energies of viscous flow for the investigated systems were determined.

## 3. The assessment of the activation energies of viscous flow for the studied collagen-IS solutions

For a liquid of which flowing particles (ions, molecules, aggregates) do not undergo structural transformations as temperature changes (such as helix-coil transition), its dynamic viscosity obey a temperature dependence given by an Arrhenius type equation:[24]

$$\eta = A \cdot e^{\frac{E_a}{RT}} \qquad (4)$$

where $E_a$ is the molar activation energy of viscous flow, A – a preexponential factor, R – ideal gas constant and T – absolute temperature. Such a dependence is considered to be valid if the majority of protein molecules do not possess a significant secondary structure. Under these conditions, the values of activation energy of viscous flow are shown in table 3 for both collagen and collagen-IS solutions.

*Table 3*

Activation energy values and fitting parameters associated to the viscosity-temperature dependences for the collagen/collagen-surfactant solutions at temperatures higher than the denaturation temperatures of collagen

| Sample | [IS], mole/L | Exponential fitting, $\eta = f(T)$ | | | | Linear fitting (semi-log plot), $\ln\eta = f(1/T)$ | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $E_a$,* kcal/mol | DF | $\chi^2$/DF | $R^2$ | $E_a$,* kcal/mol | DF | $\chi^2$/DF | $R^2$ |
| CSC, pH 2.5 | 0 | 3.61±0.10 | 4 | 0.000003 | 0.99703 | 3.60±0.10 | 4 | 0.000006 | 0.99702 |
| CSC-HTAB, pH 2.5 | $3x10^{-4}$ | 4.86±0.26 | 3 | 0.00001 | 0.99101 | 4.83±0.26 | 3 | 0.00003 | 0.99110 |
| | $3x10^{-3}$ | 5.11±0.17 | 4 | 0.000008 | 099518 | 5.09±0.17 | 4 | 0.00002 | 0.99545 |
| HTC, pH 2.5 | 0 | 3.60±0.07 | 3 | 0.000002 | 0.99880 | 3.60±0.07 | 3 | 0.000004 | 0.99885 |
| HTC-HTAB, pH 2.5 | $3x10^{-4}$ | 4.42±0.23 | 3 | 0.00003 | 0.99149 | 4.39±0.23 | 3 | 0.00008 | 0.99189 |
| | $3x10^{-3}$ | 5.21±0.21 | 3 | 0.00003 | 0.99492 | 5.19±0.22 | 3 | 0.00008 | 0.99476 |
| CSC, pH 8.0 | 0 | 3.79±0.14 | 4 | 0.00001 | 0.99432 | 3.77±0.14 | 4 | 0.00003 | 0.99456 |
| CSC-SDS, pH 8.0 | $3x10^{-3}$ | 4.79±0.21 | 4 | 0.00001 | 0.99180 | 4.76±0.21 | 4 | 0.00004 | 0.99218 |
| | $3x10^{-2}$ | 6.28±0.20 | 5 | 0.00002 | 0.99458 | 6.23±0.20 | 5 | 0.00005 | 0.99494 |
| HTC, pH 8.0 | 0 | 4.41±0.12 | 4 | 0.000006 | 0.99667 | 4.40±0.12 | 4 | 0.00002 | 0.99691 |
| HTC-SDS, pH 8.0 | $3x10^{-3}$ | 6.67±0.42 | 4 | 0.00013 | 0.98270 | 6.55±0.43 | 4 | 0.00028 | 0.98347 |
| | $3x10^{-2}$ | 6.02±0.19 | 3 | 0.00001 | 0.99682 | 5.98±0.20 | 3 | 0.00003 | 0.99682 |

* The activation energy values of viscous flow of water are 3.58±0.06 kcal/mol (from exponential fitting) and 3.56±0.07 kcal/mol (from linear fitting)

Increasing the activation energies of flow for the collagen solutions as IS concentration increases typifies an indirect marked evidence on formation and size development of collagen-IS complexes. In proportion as the size of adsorption complexes is rising, the denaturing effect of IS is growing. The experimental data show however that the denaturing action of SDS is neatly higher than that of HTAB, a highlight derived from the comparison of $T_d$ values of collagen in SDS and HTAB solutions, on the one hand, and also comparing the activation energies of viscous flow for the mentioned systems. In addition, analyzing these data the following important outcomes are revealed:

(a) The activation energies of flow for the surfactant-free solution of CSC and HTC at pH 2.5, accounting the experimental error, are practically equal even though the molecular weights of the two collagens are different. This ascertainment was still expected, because the collagen type I, in the denatured state, is mainly occurred as unfolded polypeptide chains (with no secondary structure) denoted α1(I) and α2(I). Depending on the inter- and intramolecular crosslinking degree, the system in the same denatured state may also contain dimmers (two polypeptide chains are hooked together yielding $\beta_{11}$ or/and $\beta_{12}$) or/and trimers (γ, three polypeptide chains without secondary structure are held together by crosslinks). Due to the fact that the positive net-charge of the collagenous polypeptides is large enough at pH 2.5, the molecular conformations are three-dimensionally more extended (as a result of electrostatic repulsions between the same-sign charges which are dominant), what induces a high anisotropy of the

spatial distribution of molecular structure. This favors directional flows in order to minimize the friction against the surrounding molecules. So, even in the case of HTC, with a molecular weight higher than of CSC, the majority number fraction of denatured protein occurs as α1(I) and α2(I) chains. Therefore, the multimer structures (β, γ), due to their anisotropy, do not bring about a further significant contribution to the activation energy of viscous flow compared with that issued for CSC under the same conditions.

(b) The comparison of the activation energies for CSC-HTAB and HTC-HTAB asserts that the adsorption of cationic surfactant increases the net-charge of denatured collagen structure with keeping and even enhancing the anisotropy of conformations of denatured polypeptide chains. Thus, the activation energies of viscous flow for the CSC and HTC solutions are the same at a given concentration of HTAB. Obviously, the increase of surfactant concentration magnifies the size of collagenous polypeptide-IS complexes which, in its turn, induces a certain increase of the activation energy of viscous flow.

(c) At basic pH (8.0) the conformations of polypeptide chains have a lower spatial expansion and, consequently, a lower degree of anisotropy, all of this being due to the negative net-charge at pH 8.0 rather less than the positive net-charge of a polypeptide chain at pH 2.5. In this way, as a matter of course the activation energy of viscous flow for HTC solution is higher than that for the case of CSC solution (the denatured multimer species, β and γ, are more numerous in the case of HTC than in that of CSC). On the same reason, the activation energies corresponding to CSC and HTC solutions at pH 8.0 are greater than those for the same solutions at pH 2.5.

(d) As in the case of the collagen-HTAB solutions, increasing SDS concentration results in formation of adsorption polypeptide-surfactant complexes which, by their spatial size, enhances activation energy of viscous flow excepting the HTC-SDS system at SDS concentration of $3x10^{-2}$M for which further investigations are required.

Hence, the thermal denaturation mechanisms of the collagen type I in the presence of SDS and HTAB are similar and consist preliminarily in yielding of some protein-IS complexes within which, depending on surfactant concentration, the micelle-like structures adsorbed onto collagen triple helix (original sites of bonding are side groups carrying the charge of the opposite sign against that of IS)[1] enhances both the net-charge of collagen and the strength of intra- and interchain electrostatic repulsions within the collagen triple helix. At low temperatures, the potential effect of such repulsive interactions does not alter the conformational integrity of the native structure of collagen but, as temperature increases, it is undoubtedly that the denaturation of protein is more easily carried out (at lower $T_d$). In this respect, Fathima and Dhathathreyan,[25] studying thermal stability of the collagen type I (derived from rat tail) in the presence of SDS and HTAB, suggest a rather strange denaturation mechanism. They claim that at working pH (4.2), probably bellow IEP of the protein, the denaturation process consists of collagen micellization induced by SDS occurrence whereas this behavior does not take place in the presence of HTAB. Although the data presented in the paper[25] are rather inconclusive, they no way support the proposed mechanisms. Moreover, this statement of the authors[25] is objectionable from at lest two points of view:

(a) Collagen micellization in the presence of SDS at pH 4.2 is a precipitation in fact as a result of the formation of insoluble collagen-SDS complexes due to attractive interactions between dodecyl sulfate ions and ammonium groups predominantly occurred onto collagen triple-helix. In such a way, the triple-helical conformation of collagen can by no means be altered but even reinforced. Furthermore, as SDS concentration rises enough, the resolubilization of the collagen precipitate would normally take place. It is important to mention that a "micellization" is also brought about near by IEP of collagen, the process known as collagen fibrillogenesis, but even in the salting-out stages during the course of extraction and purification steps of collagen. None of those processes lead to the triple-helix unfolding.

(b) "Collagen micellization" in the presence of HTAB at the same pH had to occur simply but no assignation regarding the possible denaturation mechanism in this case was claimed.

Even though the mechanism proposed in this work in order to explain the thermal stability of the collagen type I in dilute aqueous solutions and together with ionic surfactants seems to be supported by the study of SDS and HTAB adsorption onto collagenous polypeptide[1] and the capillary viscometry data, the requirement of proper correlation between these results and the other literature data imposes developing these investigations, on the one hand, and critically and

cautiously analyzing the relationship between the experimental findings and the proposed mechanisms.

## EXPERIMENTAL

**Materials.** The collagen type I used throughout the experiments was derived from calfskin and young horse tendons, respectively. The two collagens were acquired from ICPI-Bucharest (calfskin collagen) and Poneti SA-Bucharest (horse tendon collagen) as acetic solutions (0.1 M acetic acid). The storage temperature for both stock solutions of collagen and employing freshly prepared collagen and collagen-ionic surfactant solutions was cca $4^0$C. The ionic surfactants, sodium dodecyl sulfate (99%+, Sigma Aldrich) and hexadecyl trimethylammonium bromide (>99%, Fluka) were utilized as received. The other reagents (analytical grade) were purchased from Chimopar SA-Bucharest.

**Capillary viscosity measurements.** Capillary viscometry method allows to determine the molecular weights of the two collagens and also to find the relative viscosities of the collagen and collagen-ionic surfactant solutions at various temperatures. To obtain reliable data on thermal denaturation of collagen in solution, each sample was thermostated for one hour at every working temperature before measuring the relative viscosity (flow time, in fact). In the case of determination of activation energy of viscous flow, the dynamic viscosities were obtained supposing that the solution densities are equal to those of water at the same temperatures. All the viscosity measurements are performed on a suspended-level viscometer (Ubbelohde type, SCHOTT, Mainz) equipped with the Viscosity Measuring Unit Viscoclock connected to PC to measure flow times. The procedures of employing this method was shown in detail elsewhere.[1]

**UV-Circular dichroism measurements.** These investigations were achieved by use of spectropolarimeter Jasco J-810 with a square quartz cuvette (Suprasil, path length 0.02 cm) and the following experimental parameters of spectra acquisitions: wavelength range 250-190 nm; scanning speed of spectra - 50 nm/min with 0.2 nm pitch; number of spectrum acquisitions - 4; working temperature – room temperature ($23.5^0$C); purging nitrogen gas (purity 5.5) to avoid oxygen absorption within wavelength range 210-190 nm. To derive an actual circular dichroism spectrum of collagen in dilute acetic solutions, the spectrum recorded for the acetic acid solution (0.1M) was substracted from that of the collagen in 0.1M acetic acid solution.

**Electron microscopy measurements.** The technique of mica sandwich-rotary shadowing was used to properly prepare the collagen samples to be examined under electron beam. Further features regarding the samples preparations for electron microscopy are reported in other place.[1] Samples examination was performed on a JEOL TEMSCAN 200CX equipment at a working acceleration voltage of 200 kV.

## CONCLUSIONS

In this study on the influence of collagen-IS interaction on thermal stability of collagen, a mechanism of thermal denaturation of the native structure of the collagen type I in the presence of two ionic surfactants (SDS and HTAB) was proposed. Based on the capillary viscometry data (in view of determination of $T_d$ and the activation energies of viscous flow for the investigated systems) which follow the previous study on the adsorption of SDS and HTAB, the proposed mechanism primarily consists in formation of some adsorption collagen-IS complexes within which micelle-like structures of IS adsorbed onto collagen triple helix are developed. The initiation of this process is induced by the sites (located onto collagen triple helix) carrying the opposite sign charge to that of IS. The increase of IS concentration may result in growing the spatial expansion and the number of these individual micelles and, at a limit, it is most likely to form a bilayer or, after a subsequent geometrical rearrangement, a cylindrical monolayer of IS surrounding the collagen triple helix (at surfactant concentration equal to or higher than $T_2$). The electrostatic repulsive interactions between structural elements of these adsorption complexes are not strong enough to unfold the triple helix, so that, at low temperatures, the secondary and tertiary structures of collagen remain unaffected. However, the effect of these repulsive interactions becomes visible with the increase of temperature. Thus, SDS (in a greater extent) and HTAB (in a lower degree) depress $T_d$ of collagen in dilute solution with respect to $T_d$ of collagen in surfactant-free solution.

The investigations carried on CSC and HTC (having native secondary and tertiary structures revealed by UV-CD, electron microscopy and molecular weight determinations) indicate a pretty good agreement between influence of IS concentration on $T_d$ of collagen and corresponding activation energies of viscous flow derived for the dilute collagen solutions.

Development of this study employing UV-CD at various temperatures for collagen type I-IS solutions could bring further outstanding findings on the basis of which the denaturation model proposed above could be refined in order to better describe the thermal denaturation of the collagen type I in dilute aqueous solutions.

The results regarding the decrease of $T_d$ of collagen on the presence of IS would find applications both in view of storage conditions and in view of use the systems in which the collagen and the active-surface agent are their common components (as in the case of some cosmetics and personal care products).

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