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Can green solvents be alternatives for thermal stabilization of collagen?

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ABSTRACT

"Go Green" campaign is gaining light for various industrial applications where water consumption needs to be reduced. To resolve this, industries have adopted usage of green, organic solvents, as an alternative to water. For leather making, tanning industry consumes gallons of water. Therefore, for adopting green solvents in leather making, it is necessary to evaluate its influence on type I collagen, the major protein present in the skin matrix. The thermal stability of collagen from rat tail tendon fiber (RTT) treated with seven green solvents namely, ethanol, ethyl lactate, ethyl acetate, propylene carbonate, propylene glycol, polyethylene glycol-200 and heptane was determined using differential scanning calorimetry (DSC). Crosslinking efficiency of basic chromium sulfate and wattle on RTT in green solvents was determined. DSC thermograms show increase in thermal stability of RTT collagen against heat with green solvents (>78 °C) compared to water (63 °C). In the presence of crosslinkers, RTT demonstrated thermal stability >100 °C in some green solvents, resulting in increased intermolecular forces between collagen, solvent and crosslinkers. The significant improvement in thermal stability of collagen potentiates the capability of green solvents as an alternative for water.

1. Introduction

The triple helix structure of collagen is stabilized by intra-chain hydrogen bonds and intermolecular water bridges between the polypeptides [1]. Additionally, restriction of backbone rotation of imino acids namely, proline and hydroxyproline and hydrophobic interactions between nonpolar residues on the surface also stabilizes collagen [2]. For industrial applications of gelatin and leather, collagen is subjected to various physico-chemical operations, resulting in generation of heat that leads to degradation and destabilization. Hence, intermolecular crosslinkers such as aldehydes [3], and genipin [4], tanning agents such as basic chromium sulfate (BCS) [5,6], vegetable tannins like wattle (*Acacia*) [7], metal ions [8,9] are being used for imparting thermal, conformational and enzymatic stability in aqueous medium.

Although BCS and wattle in aqueous medium show appreciable tanning efficiency, compelling issue of indefinite water usage necessitates exploration of greener and cleaner processing [10]. The green solvents selected for this study were based on GlaxoSmithKline solvent selection guide [11]. The selection criteria were based on the values of LD_{50} (lethal dose >5000 mg/kg), low vapor pressure and dielectrics constant being close to that of water. In support of this, seven green solvents viz., ethanol (EtOH), ethyl lactate (EL), propylene carbonate (PC) from polar group of green solvents, propylene glycol (PG), polyethylene glycol-200 (PEG) from glycol family of solvents, ethyl acetate (EA), heptane (HP) from non-polar group of green solvents, have been chosen. The physico-chemical properties of these green solvents have successfully replaced hazardous solvents like toluene and xylene [12]. Due to its biodegradability and recyclability, these green solvents are being used for various industrial applications [13–18]. In order to use green solvents for industrial applications of collagen, the major criterion to be assessed is its thermal stability.

Denaturation of collagen is a complex process involving interplay of kinetics and thermodynamics. The most accepted model of protein denaturation follows a single step first order process as described by Lumry–Eyring [19] in the following equation:

$$N \xrightarrow{k} D \quad (1)$$

where N and D, are the native and denatured state, k is the rate constant dependent on temperature and follows Arrhenius equation. Apart from Lumry–Eyring model, several reports have adapted isoconversional and multivariate non-linear regression methods that describe denaturation of collagen in aqueous medium as a set of

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kinetic triplets [20–22]. Since this study focuses on denaturation of collagen in green solvents at a constant heating rate, Lumry–Eyring model has been followed. The thermal stability of collagen fiber increases as the hydration level decreases, as a result of reduction in hydrogen bonds or reducible covalent crosslinks. The denaturation temperature collagen fiber ranges from 57 to 65 °C, depending on the number of water molecules surrounding collagen [23]. Interaction of RTT with different aliphatic alcohols, viz., methanol, ethanol and *n*-propanol, ethylene glycol [24] and certain substituted glycols [25,26] have shown to exhibit marginal thermal stability in the range of 63–70 °C.

Therefore, this study aims to assess dimensional and thermal stability of collagen in green solvents at the inter-fibrillar level using optical microscopy and differential scanning calorimetry, respectively. Furthermore, the tanning efficiency of principle crosslinking agents, BCS and wattle in green solvents were also determined.

2. Materials and methods

2.1. Materials

RTT fibers were teased from the tails of 6 month old male albino rats (Wistar strain). Ethanol was purchased from Hayman speciality products, ethyl acetate from Hi-Media, heptane from Ranbaxy fine chemicals Ltd., ethyl lactate from Sigma-Aldrich, propylene glycol purified, propylene carbonate for synthesis and polyethylene glycol 200 from Loba Chemie and used without further purification for this study. All solvents purchased were of analytical grade. Basic chromium sulfate (BCS) and wattle were of commercial grade.

2.2. Sample treatment

The RTT were washed twice in cold distilled water at 4 °C. Each tendon was weighed using balance with 10 μg accuracy. RTT was treated with two different compositions of solvents, i.e., 100% solvent, 0% water (v/v) and 50% solvent and 50% water (v/v) for 24 h at 25 °C. To investigate the efficiency of crosslinkers, 1% (w/v) of BCS or wattle was added to the vials containing solvent/solvent:water mix. RTT was then placed in the vial. The vial was gently agitated manually for couple of minutes for the RTT to interact with water, crosslinking agents and solvent.

2.3. Investigation of dimensional stability by microscopy

Optical micrograph using Aven Inc., Digital Mighty scope, 1.3 M (Product code: 48708-25, Made in Taiwan) of 10× resolution were obtained to monitor the changes occurring in the dimensions of RTT. The brightness intensity and contrast was fixed independently according to the dimensions of RTT. Optical micrograph of RTT in water was taken at the zeroth hour and further incubated in different solvents (*of different compositions and in the presence/absence of crosslinking agents*) for 24 h. After incubation, the changes in the diameter of RTT were observed using optical microscope. The resultant swelling of RTT was measured by the ruler in pixels and calculated in mm, by the Visual eye, Version 6.0, software as per manufacturer's instructions.

2.4. Investigation of thermal stability by calorimetric method

The peak temperature T_p and enthalpy, ΔH, of RTT crosslinked with BCS and wattle in the presence of solvents was determined using differential scanning calorimeter (Q series 200, TA instruments, USA). Pre-weighed RTT was incubated in green solvents/water for 24 h. After incubation period, RTT was removed and weighed and air-dried for different time intervals. Based on weight loss, moisture content of samples (treated RTT fibers) was maintained as 43% (±2%). Predetermined weights of samples were sealed in the hermetic aluminum Tzero pans (TA instruments, Waters LLC, USA). The temperature was increased from 30 to 150 °C at the rate of 5 °C/min to determine the denaturation temperature (also termed as peak temperature, T_p) of RTT. The onset temperature, peak temperature and total enthalpy of transition for each sample were generated using Universal TA Analysis software, version 3.9A (TA Instruments, Waters LLC). All experiments were carried out in triplicate. The results presented here are average of three independent experiments, and the standard error was calculated as the standard deviation divided by the square root of the number of replicates. Students' *t*-test was performed to analyze whether the data is statistically significant.

The endothermic process of denaturing RTT can be described using Arrhenius equation. The relationship between enthalpy evolved and temperature can be used to calculate activation energy (E_a) using the following equation [27]:

$$\ln\left(\ln\left(\frac{H_t}{H_t - H}\right)\right) = -\frac{E_a}{R}\left(\frac{1}{T_p} - \frac{1}{T}\right) \quad (2)$$

where H_t is the total enthalpy process, H is the enthalpy evolved at a given temperature, T, T_p is the temperature at the peak of endotherm and R is universal gas constant. H_t and H were obtained from area under the endotherm curve. A plot of $\ln(\ln(H_t/(H_t - H)))$ vs $1/T$ should give a straight line whose slope is $-E_a/R$.

3. Results

The objective of this study is to determine the thermal stability of collagen fiber in green solvents, which may potentially replace water for collagen-based industrial applications. The dimensional and thermal stability of RTT collagen fiber were assessed using optical microscopy and differential scanning calorimeter, respectively. The thermal behavior of collagen in green solvents would provide better insights on the stability of collagen in different non-aqueous medium. Furthermore, the effect of crosslinkers, BCS and wattle on collagen in green solvents was also observed. Although, BCS and wattle are immiscible in all green solvents, the high penetration characteristics of BCS and wattle enables it to interact with the hydration shell of collagen at slightest agitation thus resulting in crosslinking [6]. Crosslinking was validated by observing lack of swelling effect of RTT in the presence of solvents and loss in diameter of RTT due to dehydration caused by BCS [28]. Table 1 shows comparative analysis of thermal stability of RTT collagen fibers in different green solvents in the presence and absence of crosslinkers, BCS and wattle.

3.1. Influence of ubiquitous solvent—Water on RTT

3.1.1. Dimensional stability of RTT in water

The dimensional stability of RTT collagen fiber in its native state shows macroscopic banded or wave pattern that is typical of helical microstructure of collagen [29]. Under stretching or hydration conditions, the wave pattern disappears. The dimensional integrity of RTT fiber in water was in accord with reported results showing banded pattern (Fig. 1a).

3.1.2. Thermal stability of RTT in water

The peak temperature (T_p) of RTT in water is about 63 °C [30]. Our results are consistent with that reported in literature [7], showing 64 °C for RTT in water (Fig. 2a). DSC thermogram of native RTT showed an additional peak at 112 °C, which corresponds to bound water present in collagen fiber (Supplementary Fig. S2). The enthalpy of denaturation of RTT collagen fiber has been found to