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Modulation of collagen by addition of Hofmeister salts

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ABSTRACT

Collagen can be modified by addition of chaotropic or kosmotropic salts of the reversed Hofmeister series. Hence, telopeptide-poor collagen type I was suspended in H_2SO_4 (pH 2) and 0.05–0.5 M KCl and KNO_3 (chaotropes), as well as KI and KSCN (kosmotropes). Rheological parameters, including storage and loss modulus, intrinsic viscosity, and critical overlap concentration, were assessed and the microstructure was characterized by applying confocal laser scanning microscopy and scanning electron microscopy. The addition of up to 0.1 M KCl and 0.05 M KNO_3 increased the intrinsic viscosity from 1.22 to 1.51 L/g without salt to a maximal value of 1.74 L/g and decreased the critical overlap concentration from 0.66 to 0.82 g/L to a minimal value of 0.57 g/L. Higher salt concentrations increased the collagen–collagen interactions due to ions withdrawing the water from the collagen molecules. Hence, 0.1 M KSCN delivered the largest structures with the highest structure factor, area value and the highest critical overlap concentration with 17.6 L/g. Overall, 0.5 M salt led to salting out, with chaotropes forming fine precipitates and kosmotropes leading to elastic three-dimensional networks. The study demonstrated that collagen entanglement and microstructure depend strongly on the ionic strength and type of salt.

1. Introduction

Collagen denotes a family of proteins that serve as major structural components of connective tissues in vertebrates [1]. Collagen has unique physical properties, including uniformity, tensile strength, flexibility, biocompatibility, and biodegradability, and is therefore widely used in many areas. It has been used as a scaffold in tissue engineering, for implantations or wound dressing in surgical operations, as a capsule matrix material or binder in pharmaceutical applications, and for the production of gels and films in foods [2]. In the latter case, a particularly promising use of collagen has been the manufacture of coextruded sausage casings, due to the rapidly rising cost of natural, intestinal-derived ones [3]. Even though collagen casings are used extensively in the meat industry, there is very little published research focusing on the physicochemical properties of collagen formulations and its impact on the mechanical properties of the co-extruded casings [4].

Collagen type I contains uninterrupted helical regions with alternating polar and nonpolar domains leading to a lateral alignment of molecules in a quarter staggered array [5]. Hydrogen bonds between polar residues of 4-hydroxyprolin and 5-hydroxylysin, the formation of hydration networks and electrostatic interactions affect collagen stability and structure [6]. Electrostatic interactions arise from ionizable side groups present in 15–20% of all amino acid residues either in the X or in the Y position of the Gly-X-Y triplets [7]. Freudenberg et al. [8] postulated the stabilization of collagen type I with increasing ionic strength based on superior screening of charged residues and the formation of salt bridges. In a recent study, the authors demonstrated that bovine telopeptide-poor collagen type I is able to form clusters at pH 2, signifying that non-electrostatic interactions also play an essential role in the collagen arrangement [9]. Moreover, the elastic behavior of telopeptide-poor collagen gel was found to increase with increasing pH below the isoelectric point p*I* based on less electrostatic repulsion and less acid hydrolysis.

Aside from pH, the presence of certain solutes capable of altering hydration of collagen has also shown to affect the structure and assembly of collagen matrices [10,11]. For example, chaotropic ions within the Hofmeister series, so-called "water structure breakers," typically promote protein–solvent interactions thereby allowing them to be dispersed, a phenomenon known as salting-in [12]. Conversely, kosmotropic ions are known to increase water structuring

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thereby promoting protein–protein interactions leading in turn to protein precipitation, an effect also known as "salting out" [13,14]. Nevertheless, relatively little is known as to the effect of such salts on the properties of collagen Type I, especially at low pH. At pH values below the isoelectric point (p*I*) reverse effects of Hofmeister salts have been reported [15].

We hypothesized that kosmotropic anions (KI, KSCN) might increase the aggregation of collagen molecules into larger structures and decrease the critical overlap concentration, while chaotropic anions (KCl, KNO_3) might lead to a state where collagen–solvent interactions are favored. Consequently, electrostatic screening, salt-specific hydrophobic and hydration forces, and any other intra- and intermolecular interactions may lead to new collagen functionalities. Therefore, rheometry, confocal scanning laser microscopy (CLSM), and scanning electron microscopy (SEM) were conducted to characterize the counter-ion specific effect of potassium salts on the reversed Hofmeister series (chaotrope > Cl^- > NO_3^- > I^- > SCN^- > kosmotrope) at pH 2 on telopeptide-poor collagen. Telopeptide-poor collagen was applied as it is more homogeneous than native collagen, and the pH value was selected by the reason of the pH conditions of the industrial co-extrusion process and based on former studies, where this acid delivered the least effect on entanglement without hydrolysis occurring [9].

2. Materials and methods

2.1. Preparation of collagen suspensions

Telopeptide-poor collagen was provided by Protein Consulting (Singhofen, Germany). The sample material was produced by cleaving off telopeptides and intermolecular crosslinks from native collagen to obtain single collagen triple helices [16]. Connective content had been determined *via* chemical characterization in a previous study [9]. For preparation of suspensions and mixtures, a Stomacher Circulator 400 (Seward, West Sussex, UK) for 5 min (300 rpm) at 25 °C was used. A collagen stock suspension was prepared by dispersing collagen in 10 g/L with H_2SO_4 (Carl Roth, Germany) at pH 2. The stock suspension was stored over night to ensure proper hydration of collagen molecules. The collagen stock suspension was then diluted with H_2SO_4 to double the target polymer concentration and mixed with a double concentrated KCl, KNO3, KI, or KSCN solution in order to obtain solutions containing various salts (0.5, 0.1, and 0.05 M) and polymer (x–x%) concentrations. Finally, pH was re-adjusted to 2 and the suspension was again stored over night to allow the system to equilibrate. pK_a calculations of the collagen type I amino acids residues revealed that almost no carboxylic groups were undissociated at pH 2, maximizing the effects of the Hofmeister counter ions.

2.2. Physicochemical calculations

The ionic strength I was calculated with Eq. (1), based on the ionic strength derived from the salt I_S and derived from H_2SO_4 I_A at pH 2.

$$I = I_S + I_A. \tag{1}$$

I_S was calculated with Eq. (2), where c_i is the molar concentration of the ion i and z_i is the charge number of that ion.

$$I_S = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2. \tag{2}$$

I_A was evaluated with Eq. (3), considering the degree of ionization α_m and dissociation b_i of H_2SO_4 at pH 2.

$$I_A = \frac{1}{2}\sum_{i=1}^{n} \alpha_m\, b_i\, z_i^2. \tag{3}$$

α_m of the divalent acid with $n = 2$ and $H_{n-m}A^{m-}(m \in \{0, 1, 2\})$ was obtained from Eq. (4) and (5) with acid dissociation constants K_i and $\sum\alpha_i = 1$ [17].

$$\alpha_m = \frac{[H_3O^+]^{n-m}}{D_n}\prod_{i=0}^{m} K_i, \tag{4}$$

$$D_n = [H_3O^+]^n + K_1[H_3O^+]^{n-1} + K_1K_2[H_3O^+]^{n-2} + \cdots + K_1\,K_2\ldots K_n. \tag{5}$$

The Debye screening length κ^{-1}, based on the double-layer thickness, was calculated from Eq. (6) [18]:

$$\kappa^{-1} = \sqrt{\frac{\varepsilon_0\varepsilon_R k_B T}{2\,I\,e^2\,N_{AV}}}, \tag{6}$$

where ε_0 is the permittivity of free space, ε_R the dielectric constant of water, k_B the Boltzmann constant, T the absolute temperature, e the Coulomb electronic charge, and N_{AV} the Avogadro number.

2.3. Rheological measurements

Experiments were performed in a modular compact rheometer Physica MCR 502 (Anton Paar, Karlsruhe, Germany) at 5 °C using a single gap cylinder geometry CC27 with 28.92 mm cap diameter and 26.66 mm bob diameter (Anton Paar, Karlsruhe, Germany).

Rotational rheometry was carried out in order to determine the dynamic viscosity of the solvent η_s and of 0.1–0.5 g/L collagen suspensions η. To this purpose, shear rates were varied from 0.001 to 100 s^{-1}. The relative viscosity η_r and specific viscosity η_{sp} were calculated from Eq. (7) and (8), respectively [19]:

$$\eta_r = \frac{\eta}{\eta_S}, \tag{7}$$

$$\eta_{sp} = \frac{\eta}{\eta_s} - 1. \tag{8}$$

Moreover, the power law exponent α of the collagen suspensions was determined according to Eq. (9) [20]:

$$\eta_{sp} \sim C^{\alpha} \tag{9}$$

with the specific concentration η_{sp} being directly related to the concentration c by the power law. The intrinsic viscosity $[\eta]$ was investigated according to Huggins and Kraemer [21,22] with the reduced viscosity η_{red} and Eq. (10) and (11), respectively, while the critical overlap concentration c^* was calculated from Eq. (12) according to Nyström and Kjøniksen [23].

$$[\eta] = \lim_{c\to 0}(\eta_{red}) = \lim_{c\to 0}\left(\frac{\eta - \eta_s}{c\,\eta_s}\right), \tag{10}$$

$$[\eta] = \lim_{c\to 0}\left(\frac{\ln(\eta_r)}{c}\right) = \lim_{c\to 0}\left(\frac{\ln\left(\frac{\eta}{\eta_s}\right)}{c}\right), \tag{11}$$

$$c^* = \frac{1}{[\eta]}. \tag{12}$$

Amplitude sweeps were performed for 4 g/L telopeptide-poor collagen suspensions in order to determine the linear viscoelastic region. Frequency sweeps were conducted applying 1% strain from 0.1 to 100 Hz. The amount of 4 g/L collagen was selected based on the entanglement concentration which was found to be located at this concentration in a previous study [9]. Storage G' and loss