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Characterization of acylated pepsin-solubilized collagen with better surface activity

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ABSTRACT

A new kind of acylated collagen with water solubility and better surface activity was prepared via reaction of pepsin-solubilized calf skin collagen with lauroyl chloride and succinic anhydride in this paper. The equilibrium surface tension and the isoelectric point were 55.92 mN/m and 4.93 respectively, suggesting that acylated collagen had surface activity as well as water solubility. Meanwhile, the results of Fourier transform infrared spectroscopy analyses and electrophoresis patterns demonstrated that the triple helix conformation of collagen was not destroyed, but the subunits of acylated collagen shifted to higher molecular weight than those of native collagen. Scanning electron microscope and differential scanning calorimeter measurements revealed that lyophilized acylated collagen exhibited relatively well-distributed pore structure and its denaturation temperature was about 9.0 °C higher than that of native collagen. Additionally, the increase of the diameter of the fibrils was observed by atomic force microscopy. Acylated collagen with water solubility and better surface activity might broaden the application of collagen-based materials to cosmetics, drug delivery and pharmacotherapy.

1. Introduction

Type I collagen, with an approximate molecular weight of 300 kDa [1], is characterized by the presence of a triple helix structure that is composed of two α1 chains and one α2 chain [2]. Each polypeptide chain contains the repeating typical tripeptide units Gly-X-Y, where Gly is glycine, X and Y are often proline and hydroxyproline [3]. Proline and hydroxyproline belong to hydrophobic and hydrophilic amino acids, respectively. In the side chains, collagen is also composed of other hydrophobic amino acid residues including phenylalanine and leucine residues, and hydrophilic ones, such as lysine and arginine residues. Both hydrophobic and hydrophilic parts might endow native collagen with surface activity theoretically.

Gómez-Guillén [4] reviewed that collagen surface activity was based on the presence of charged groups in the side chains as well as the collagen sequence containing hydrophilic and hydrophobic amino acid residues. As early as 1973, Satterlee et al. [5] pointed out that, when adequately hydrolyzed by pancreatin, papain and pepsin, the emulsifying activity of collagen hydrolyzate obtained from beef and pork skin was similar to that of nonfat dry milk. Additionally, the emulsifying activity of collagen derived from the skin of hake (*Merluccius merluccius* L.) and trout (*Salmo irideus* Gibb) was found to be the highest at the pH ranging from 1.0 to 3.0, as investigated by Montero and Borderías [6]. In a later study, Kim and Park [7] extracted acid-soluble collagen from the skin of Pacific whiting, which presented slightly lower emulsifying activity than a commercial emulsifier, Tween-80. Usually, being a fibrous protein, collagen with integrated triple helix structure obtained by the treatment of acetic acid (pH 2.5–3.0), could not dissolve in water [8]. However, surface active agent, generally speaking, is able to dissolve in water.

To endow the water solubility of collagen, Zhang et al. [9] and Sripriya et al. [10] prepared water-soluble and polyanionic collagens via introducing a large amount of carboxyl groups based on the method of acylation of collagen with succinic anhydride, however, the obtained water-soluble and polyanionic collagens exhibited weak surface activity as a result of the strong hydrophilicity brought by the high acylation ratios of 90% and 96%, respectively. Usually, the surface activity of proteins is related to the number of hydrophobic and hydrophilic groups and a suitable ratio of hydrophobic to hydrophilic groups would lead to a better surface activity [11]. Toledano et al. [12,13] reported the effect of the ratios of saturated fatty acid residues (C_4–C_{16}) to gelatin on the surface activity of gelatin by reacting with N-hydroxysuccinimide esters of saturated fatty acids at the degrees of 10–25% and 60–85%,

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respectively, and then found that the better surface activity was obtained at the higher ratio of fatty acid residues to gelatin, as determined by surface tension, emulsifying activity and foam property. To investigate the emulsifying activity of chickpea protein, the ratios of acetyl residues to chickpea protein were regulated via acetylating ε-amino groups of lysine with acetic anhydride at the degrees of 6% and 49%, respectively, and the results showed that the moderately acetylated protein (6%) presented higher emulsifying activity than the highly acetylated one (49%) at low salt concentrations (<0.5 mol/L) [14]. In order to endow native collagen with water solubility and surface activity, appropriate hydrophobic and hydrophilic groups could be introduced into the side chains of native collagen. Therefore, the modified collagen might be suitable for promoting the dispersion and the sustained release for hydrophobic drugs [15,16] under physiological pH conditions.

According to the illustration of Scheme 1, the authors had prepared water-soluble collagen with surface activity by the acylation of pepsin-solubilized calf skin collagen with lauroyl chloride and succinic anhydride at various weight ratios. After optimization, acylated collagen with water solubility exhibited better surface activity at the lauroyl chloride/succinic anhydride ratio of 1:0.2 (w/w). Therefore, in the present work, the authors mainly focused on the characterizations of this acylated collagen that displayed the specific properties of water solubility and better surface activity. The surface activity and water solubility were evaluated by the value of surface tension and isoelectric point (pI), respectively. The secondary structure and the surface morphology were analyzed by Fourier transform infrared spectroscopy (FTIR), sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and scanning electron microscope (SEM). Furthermore, the fibril morphology was performed by atomic force microscopy (AFM) and the thermostability was detected by differential scanning calorimetry (DSC).

2. Experimental

2.1. Materials

Type I collagen was extracted from calf skin as previously described by Zhang et al. [17]. The obtained collagen solution was lyophilized by a freeze dryer (Labconco Freeze Dryer FreeZone 6 Liter, USA) at −50 °C for 2 days and stored at 4 °C until used. The results of SDS-PAGE showed that the molecular weight of collagen, the electrophoresis patterns of which displayed two α bands (α1 and α2) and one β band, was about 300 kDa. Lauroyl chloride and succinic anhydride were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of acylated collagen

The preparation of acylated collagen is shown in Scheme 1. For step 1, native collagen was acylated with lauroyl chloride by the method of Genin et al. [18] with some modification. Lyophilized collagen (1.0 g) was cut into small pieces and added into 100 mL dimethyl sulfoxide (DMSO) containing two equivalent of triethyl amine (Et_3N), and then lauroyl chloride (1.0 g) dissolved in DMSO (10 mL) was added dropwise into the reaction system. The reaction system was stirred at 20 °C for 24 h, then the sample was centrifuged and washed five times with DMSO. Subsequently, the intermediate product was dissolved and dialyzed against 0.1 mol/L acetic acid for 3 days.

For step 2, succinic anhydride was used to acylate the intermediate product obtained in the step 1 according to the method of Potorac et al. [19] with slight modification. The intermediate product solution was adjusted to pH ∼ 9.0 by addition of 0.1 mol/L NaOH. Then, a solution of succinic anhydride (0.2 g) in DMSO (10 mL) was gradually added and the pH value was readjusted and maintained constantly at about 9.0 during reaction, by adding 1.0 mol/L NaOH. After stirring for 4 h at 20 °C, the product was purified by dialyzed against deionized water. Finally, the obtained acylated collagen was lyophilized by a freeze dryer at −50 °C for 2 days and stored at 4 °C until used. The preparation of acylated collagen was done by three times and native collagen obtained in Section 2.1 was used as a control.

2.3. Surface tension measurements

Acylated collagen solution with the concentration of 4 mg/mL was prepared by dissolving in water and then diluted to the desired concentration for each measurement. Native collagen was dissolved in 0.1 mol/L acetic acid to reach a final concentration of 4 mg/mL and then used as the reference. Prior to running tests with acylated collagen solutions, the surface tensiometer was calibrated with ultra pure water. After equilibration for about 36 h, the surface tension of all the samples was determined at 20 °C with a surface tensiometer (dataphysics OCA-H200, Germany) and was measured three times for each concentration.

2.4. pI of acylated collagen

0.5 mg/mL native and acylated collagen solutions were prepared by dissolving in 0.1 mol/L acetic acid and water, respectively. The solutions were titrated with 0.25 mol/L NaOH or 0.25 mol/L HCl and the pH value interval was 0.5 pH. Then, the Zeta potential at a given pH value was recorded by a Zeta potential titration apparatus (Malven Zetaweight Nano ZS, UK) with triplicate at 20 °C and the pI was determined at the pH value where the Zeta potential was zero.

2.5. FTIR spectra

Lyophilized native and acylated collagens were equilibrated in a desiccator containing silica gel for 3 days at 20 °C before testing. Subsequently, 2 mg lyophilized sample was triturated with 200 mg potassium bromide (KBr) and prepared as pellets. The FTIR spectra were obtained using a Nicolet iS10 spectrometer (Thermo Fisher Scientific, USA) at the resolution of 4 cm^{-1} in the wave number region from 400 cm^{-1} to 4000 cm^{-1} and the spectra plots represented the average of 32 scans.

2.6. SDS-PAGE patterns

SDS-PAGE was performed by the method of Lamemmli [20] with some modification, using 7.5% resolving gel and 4% stacking gel. Native and acylated collagen solutions (4 mg/mL) were mixed with a sample buffer containing 0.5 mol/L Tris–HCl (pH 6.8), sodium dodecyl sulfate (SDS), glycerol and bromophenol blue, to reach a final concentration of 1.0 mg/mL, and then these mixed solutions were boiled for about 5 min. After electrophoresis for approximate 120 min, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 for 45 min and destained using 7.5% acetic acid/5% methanol solution.

2.7. SEM observation of acylated collagen

Lyophilized acylated collagen was coated with gold and the morphology conformation was observed by a scanning electron microscopy (Inspect F, Fei Company, USA). Native collagen was used as the reference and the analyses were made at three different points to confirm the consistency of the observed morphologies. The average pore size of each sample was analyzed by the manual