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Thermal denaturation of fish collagen in solution: A calorimetric and kinetic analysis

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ABSTRACT

To further understand the thermal stability of fish collagen in solution, the thermal denaturation of collagen from the skin of snakehead (*Channa argus*) was studied by differential scanning calorimetry. Isoconversional and multivariate non-linear regression (Multivar-NLR) methods showed that the thermal denaturation behavior of fish skin collagen solution (FSCS) could be best described by a three-state model (a reversible step followed by an irreversible one), which approximated to single-step process at low heating rate (β) or low temperature. FSCS had relatively lower denaturation temperature (T_{max}), enthalpy and apparent activation energy than bovine hide collagen under the same conditions. Based on Multivar-NLR, $T_{max\,(\beta=0.001\,K/min)}$ of FSCS was simulated to be 21 °C, comparing with $T_{max\,(\beta=2–8\,K/min)}$ (30.4–32.2 °C); however, the optimal operating temperature of FSCS would be below 10 °C for anti-denaturation, indicating that FSCS should be handled at much lower temperature than conventionally determined.

1. Introduction

Collagen is a structural protein in the extracellular matrix (ECM) and comprises nearly one-third of the total protein in animals [1]. Among various collagens, type I collagen is the most abundant and has been widely applied in cosmetics, pharmaceutical and biomedical industries due to its advantages of biocompatibility, biodegradability and weak antigenicity [2]. The structure of type I collagen molecule is the right-handed triple helix that is composed of two α_1 chains and one α_2 chain, each containing $(Gly\text{-}X\text{-}Y)_n$ repeats where Gly is glycine, and X and Y are usually imino acids such as proline and hydroxyproline [3]. The molecular structure of collagen is mainly stabilized by the steric hindrances originated from the pyrrolidine rings of imino acids and the hydrogen bonding effects due to the hydroxy group of hydroxyproline [3]. All the practical applications of type I collagen are based on its native triple helix structure that determines its specific functions. However, as a protein, collagen is susceptible to heat which can induce the triple helix to collapse, resulting in the thermal denaturation of collagen into gelatin. Consequently, the knowledge of the thermal denaturation of collagen is important to understand its thermal stability, which is fundamental to the practical handlings of this biomaterial [4,5]. Although much has been reported on the thermal denaturation of collagen in different forms including crude tissues [6], gels [7] and fibrils [5,8], there is little information about its thermal denaturation in solution that might be involved during its extraction, its processing from solution to solid states (e.g., fibers and films), as well as its direct use as injectable biomaterials [9].

Differential scanning calorimetry (DSC) has been proved to be one of the best techniques to study the thermal denaturation of collagen, due to the appreciable heat absorption when the denaturation occurs [4,6–8,10,11]. However, isothermal calorimetry is difficult to perform since the experimentally accessible temperature region is narrower than the actual temperature interval where the thermal denaturation of collagen occurs [6]. In contrast, non-isothermal calorimetry is convenient and its use for the kinetic analysis of thermal degradation of polymers was suggested by Budrugeac [12]. Furthermore, according to the proposal of the International Confederation for Thermal Analysis and Calorimetry (ICTAC) Kinetics Project, multi-heating-rate and isoconversional methods can be used to explore the kinetics and mechanisms of complex processes [13]. Such methods, nevertheless, have rarely been applied to the kinetic analysis of the thermal denaturation of collagen in solution.

At present, the main sources of type I collagen are the connective tissues (e.g., skins and tendons) of mammals, which may carry potential risks of disease transmission such as foot and mouth disease (FMD), transmissible spongiform encephalopathy (TSE) and

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bovine spongiform encephalopathy (BSE). Accordingly, fish offals including skins, scales and bones have been considered as the desired safe collagen sources [2,14–16]. While a number of fish collagens have been reported as alternatives for mammalian collagen, the knowledge of the thermal denaturation of fish collagen is far from enough to support its industrial applications. Up to now, the literatures concerning its thermal denaturation have been only focusing on its denaturation temperature, whereas little information is available about how the denaturation occurs, how the denaturation process can be controlled through the regulation of temperature and how the denaturation behavior differs from that of mammalian collagen. Moreover, it should be noted that the thermal stability of collagen has direct correlation with the amounts of imino acids; the higher the imino acid content, the better the thermal stability [1,16]. Since obvious differences always exist in the imino acid composition of fish collagen compared with that of mammalian collagen [14–16], the thermal denaturation behaviors of these two collagens might be also quite different.

Snakehead (*Channa argus*) is a commercially important freshwater fish native to Asia and Russia [2], and large quantities of skins are generated as byproducts during its filet processing. These abundantly available skins have attracted recent attention as potential collagen sources and thus snakehead skin collagen was chosen for this work. Here, the thermal denaturation of snakehead skin collagen in solution was studied using non-isothermal calorimetry and three types of kinetic methods, i.e., the Kissinger method, the isoconversional methods, as well as the multivariate non-linear regression method. These technologies were applied in combination to determine the kinetic parameters and mechanisms of the denaturation process, and both of the non-isothermal and isothermal denaturation behaviors of snakehead skin collagen were simulated. The fundamental data are expected to further understand the thermal stability of fish collagen in solution and to present guidelines for its anti-denaturation during industrial applications.

2. Materials and methods

2.1. Materials

Fish collagen was prepared from the skin of snakehead (*C. argus*) according to the method of Zhang et al. [15]. Briefly, the descaled skin was first pretreated with 0.1 mol/L NaOH, 3% H_2O_2 and 0.5% non-ionic detergent in series to remove non-collagenous components, and then pepsin-solubilised collagen was extracted from the pretreated skin by 0.5 mol/L acetic acid containing 1% pepsin (EC 3.4.23.1, 1:10,000, Sigma Chemical Co.). Type I collagen was purified via a combination of refrigerated centrifugation, salting-out with 0.7 mol/L NaCl and dialysing in 0.01 mol/L acetic acid. Then the purified collagen was lyophilized in a freeze-dryer (Labconco FreeZone 2.5L, USA). Bovine hide type I collagen was prepared by the method previously described [9], and was also purified and lyophilized as above. Both of the fish skin collagen and the bovine hide collagen were stored with silica gel at 4 °C within one month before use. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli [17], using the discontinuous Tris–HCl/glycine buffer system with 7.5% separation gel and 4% stacking gel. Both of the two collagens displayed two α bands (α_1 and α_2) and one β band with regard to the SDS-PAGE patterns, indicating the similar molecular weight of about 300 kDa. All the chemicals used were of analytical grade.

2.2. Amino acid determination

Amino acid analysis was performed according to Zhang et al. [15]. The lyophilized snakehead skin collagen (10 mg) was hydrolyzed in 6 mol/L HCl solution at 110 °C for 24 h. The hydrolysate was further processed and subjected to an amino acid analyzer (835-50, HITACHI Co., Tokyo, Japan).

2.3. Preparation of collagen solutions

Fish skin collagen solution (FSCS) and bovine hide collagen solution (BHCS) with concentrations of 8, 12, 16 and 20 mg/mL were prepared by dissolving the lyophilized collagens in 0.1 mol/L acetic acid, respectively. All the collagen solution samples were degassed by refrigerated centrifugation and then stored at 4 °C before calorimetry.

2.4. Calorimetry

DSC was carried out by using DSC 200PC (Netzsch-Gerätebau GmbH, Wittelsbacherstrasse, Germany) as described [4]. FSCS and BHCS samples (6.0 ± 0.1 mg) were sealed in 100 μL aluminum pans and the pan containing 6.0 mg of 0.1 mol/L acetic acid was used as the reference. Firstly, DSC thermograms were recorded by heating FSCS (16 mg/mL) and BHCS (16 mg/mL) from 4 to 60 °C at heating rates of 2, 3, 5 and 8 K/min, respectively, and the dependences of the temperature of the peak maximum (T_{max}) and the enthalpy of the denaturation (ΔH) on heating rate (β) were investigated. Secondly, the FSCS and BHCS samples with different concentrations (8, 12, 16 and 20 mg/mL) were heated at a heating rate of 5 K/min to study the dependences of T_{max} and ΔH on concentration. All measurements were performed thrice and the DSC data were evaluated using Proteus® from Netzsch.

2.5. Kinetic analysis

Kinetic analyses of the DSC data were performed according to the general algorithm suggested previously [12], which was in accordance with both of the ICTAC-2000 project results [13] and the recent ICTAC Kinetics Committee recommendations for kinetic computations [18]. In brief, isoconversional methods were used to study the changes of activation energy during the thermal denaturation of FSCS, followed by the use of a non-linear regression method to evaluate probable mechanisms. Additionally, the Kissinger method was applied in the first place for determining the apparent activation energy corresponding to the maximum of the collagen denaturation rate [5].

2.5.1. Kissinger method [19]

The Kissinger method is based on the following equation assuming that the reaction rate reaches the maximum at T_{max}:

$$\ln \frac{\beta}{T_{max}^2} = \ln \frac{AR}{E} - \frac{E}{RT_{max}} \quad (1)$$

where A is the Arrhenius pre-exponential factor, R is the gas constant, E is the activation energy, and T_{max} is in the form of absolute temperature. A value of E could be obtained from the slope of the plot $\ln(\beta/T_{max}^2)$ vs. $(1/T_{max})$.

2.5.2. Isoconversional methods

The dependence of E on the degree of conversion (α) (a ratio of the heat released between the start of a reaction and the actual time to the total heat), can be estimated by using isoconversional methods without any knowledge of the reaction model [8]. It had been reported that a variation of E with α is unambiguously the sign of a multi-step process and the shape of E_α (the value of E corresponding to a given α) dependence could provide important insights into the mechanism of the process [20]. The differential