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Cross-linking and film-forming properties of transglutaminase-modified collagen fibers tailored by denaturation temperature

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ABSTRACT

Transglutaminase (TGase) catalyzing crosslinking between intra- and inter-chain glutamine and lysine peptide residues has been used for modifying protein's structure. However, its enzymatic performance on albuminoids such as collagen was still not completely illustrated. Herein, we investigated the crosslinking efficiency of films preheated at different temperatures and estimated its physicochemical properties. The electrophoresis results showed that the extracted collagen had typical triple helix structure but reduced and even disappeared as temperature increased. Accordingly, X-ray diffraction (XRD) indicated that the amount of triple helices decreased, corresponding to the decreases in thermal stability and mechanical properties of films. TGase crosslinking decreased the thickness of all films, while mechanical properties and thermal stability had a significant improvement especially at 45 °C and 65 °C. With the proper equilibrium of denature temperature and TGase crosslinking, the tailored film-forming properties of collagen can offer a potential to engineer collagenic material for biodegradable and edible packaging applications.

1. Introduction

Collagen, a promising biomaterial, is also the most abundant and widely distributed functional protein in mammalian body. Up to date, 28 different types of collagen have been identified in literature (Ricard-Blum, 2011). Type I collagen consists of two homologous chains (α1) and one supplementary chain (α2) in a natural state that varies slightly in its chemical composition, which constitutes the triple helical region (Zeugolis & Raghunath, 2011). These chains are polypeptides with the repetitive Gly-X-Y amino acid sequence and CO and NH groups on the chains connected by hydrogen bonds (Chak, Kumar, & Visht, 2013). The random coil extensions are located at the amino/N-terminal and carboxy/C-terminal ends of the collagen molecule, named N- and C-telopeptides respectively, which is mainly responsible for collagen-collagen crosslinking (Oechsle, Bugbee, Gibis, Kohlus, & Weiss, 2017). Owing to its excellent biocompatibility, weak antigenicity and good biodegradability, collagen has been widely used in many fields, such as medicine, food, and biomedical applications (Sinthusamran, Benjakul, & Kishimura, 2013).

Accordingly, as a desirable edible packaging material, collagen with native triple helices and fibril networks exhibited superior features such as higher enthalpy, greater network structure and better mechanical properties than gelatin (Zhang, Li, & Shi, 2006). However, once collagen was extracted from its original source and then subject to reprocessing, it will exhibit different properties than it was in the original state, such as weak mechanical properties, thermal instability and easily degradable. To overcome these shortcomings, it is essential to improve the physiochemical properties and the ability to resist external attack by using various methods.

Cross-linking is usually a process of linking polymer chains by covalent or non-covalent bonds and forming tridimensional networks (Wihodo & Moraru, 2013). The protein films' structure can be tailored by chemical, enzymatic and physical means of crosslinking (Buchert et al., 2010). Among them, enzymatic cross-linking is one of the most accepted ways to tailor the protein properties due to its advantage of alternative, nontoxic and efficient for modifying protein materials (Stachel, Schwarzenbolz, Henle, & Meyer, 2010). Transglutaminase (TGase, EC 2.3.2.13) is an class of transferases obtained from microorganism and it has been widely applied to improve food texture, protein solubility, emulsification, foaming, viscosity and water-holding

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capacity of protein (Gaspar & Góes-Favoni, 2015). TGase catalyzes acyl transfer reaction between γ-carboxyamide groups of peptide-bound glutamine residues (acyl donor) and ε-amino groups of lysine residues (acyl acceptor). Therefore, the polymerization of proteins can be achieved as a result of the formation of intra- or intermolecular ε-(γ-glutamyl) lysine bonds (Bae, Darby, Kimmel, Park, & Whiteside, 2009), including myofibrillar proteins, gelatin, soy proteins, fish proteins and cereal proteins (Buchert et al., 2010). Hence, TGase has been widely used to improve the mechanical and barrier properties of biopolymer-based films, such as gelatin (Liu et al., 2016), egg white protein (Peng et al., 2017), whey protein and its combinations with pectin, chitosan (Azeredo & Waldron, 2016

Jiang et al., 2016). However, the efficiency of crosslinking on albuminoids such as collagen induced by TGase is rarely reported in literature. For instance, gelatin is a hydrolysis product of collagen with thermal pre-treatment in the acid or alkaline condition, consisting of the amorphous primary chains and some smaller peptide fragments (Duconseille, Astruc, Quintana, Meersman, & Sante-Lhoutellier, 2015). Water molecules are embedded in the mesh of these chains to form a solution due to the destruction of collagen triple helix in the process of gelatinization, which provide the feasibility for TGase crosslinking. Accordingly, enhanced crosslinking efficiency and mechanical strength were achieved in bovine skin gelatin films (Wang, Liu, Ye, Wang, & Li, 2015).

Theoretically, for collagen, the random telopeptides provides the potential cross-linking sites for TGase though it belongs to albuminoid (water-insoluble). In vivo, collagen has been reported to be an important substrate for TGase in the extracellular matrix where it is cross-linked to other non-collagenous proteins such as osteopontin (Kaartinen, Pirhonen, Linnala-Kankkunen, & Mäenpää, 1999). Additionally, in vitro, treating type I collagen by using tissue or microbial TGase can accelerate self-assembly of collagen (Spurlin, Bhadriraju, Chung, Tona, & Plant, 2009). Jelenska et al. reported that at least partial denaturation of the native type I collagen structure was important for cross-linking with TGase. Stachel et al. confirmed that half of the crosslinking sites were located within the triple helical region of the collagen molecule (Stachel et al., 2010). Up to date, little attention has been paid to fully clarify the mechanism of collagen cross-linking with TGase, especially for the relation between crosslinking efficiency and denaturation temperature. Besides, the combined effects of denaturation temperature and TGase crosslinking on the collagen material properties still need to be estimated.

As reported previously, partial degeneration of collagen structure is beneficial to the cross-linking reaction and the high molecular will aggregate when the temperature was higher than 37 °C. It was also consistent with the conclusion of Michael L's report that five kinds of enzymes were added into collagen at optimal temperature to separate the crosslinked bonds (Miller & Johnson, 1999). Meanwhile, the denaturation temperature of pure collagen was ~49 °C and the temperature of maintaining structural integrity was ~25 °C (Bank et al., 1997). In this study, we selected 35 °C as the critical temperature of bovine collagen, and a total of five temperatures below (4 °C, 25 °C) and above (45 °C, 65 °C) the critical temperature were used for making collagen fiber suspensions. Simultaneously, the suspensions prepared in accordance with the above conditions were used to prepare films, the changes of films properties treated with and without TGase were also evaluated accordingly.

2. Materials and methods

2.1. Materials

Bovine skin was donated by Longbao Collagen Casing Co., Ltd. (Zibo, China), which was pretreated with 10 wt% lime (based on the skin) for 30 days at room temperature. Microbial transglutaminase (TGase, 100 U/g) was obtained from Yuanye company (Activa TG-S, Tianjin, China) in a powder form. Glycerol (minimum purity 99%) was purchased from Tianjin Jiangtian Chemical Technology Co. Ltd. (Tianjin, China). Other commercial chemicals were analytical grade.

2.2. Preparation of collagen fiber suspension

Collagen fibers were prepared as described previously (Wang et al., 2017). The limed bovine skins were washed thoroughly with distilled water until the pH value was close to 7. Then, the pH-neutral bovine skins were swelled by immersing it into 0.05 mol/L hydrochloric acid solution at the skin to acid ratio of 1/3 (W/V) for 24 h at 4 °C. The intact collagen fibers were torn down carefully from the swelled skin by rake tweezers and dispersed in distilled water to form 10 g·kg^{-1} collagen fiber suspensions. The suspensions were divided into two parallel groups of ten samples in total and the control group was not treated with TGase. All the suspensions included the control group were incubated at 4 °C, 25 °C, 35 °C, 45 °C and 65 °C by stirring for 4 h respectively. Then, the pH of all the suspensions were adjusted to 6 (the optimum reaction conditions of TGase) when the samples temperature reached room temperature after incubation. TGase (20 U/g on the basis of the dry collagen) was added into the experimental group and the crosslinking reaction was lasted for 4 h at room temperature. The treatment of the control group was the same as that of the experimental group, except that there was no treatment of TGase. After the reaction was completed, all samples included the control group were terminated by adding sodium chloride into the samples to a final concentration of 5% and centrifugated at 4000 g for 20 min at 4 °C to obtain the precipitation. The precipitation was then re-dissolved in 0.01 M acetic acid and dialyzed with the dialysis membranes (intercept molecular weight of 8000–14000 Dalton) at room temperature for 3 days against distilled water, the dialysis fluid was lyophilized and stored at −20 °C for use in the subsequent experiments.

2.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The molecular weight distribution of collagen was determined by SDS-PAGE. Firstly, all the lyophilized collagen were dissolved in sodium phosphate buffer (pH = 6) to obtain a solution with the concentration of 10 mg protein/ml. Then, the solution of was mixed with buffer solution at a ratio of 1:1 (v/v) respectively and denatured in boiling water for 5 min. Collagen samples and protein marker (25 μl) were loaded into the gel which consisting of 5% stacking gels and 8% acrylamide separating gels. After electrophoresis, the gel was dyed with Coomassie Brilliant Blue R-250 for 1 h and dealt with destaining solution until the clear bands appears.

2.4. Film preparation

The lyophilized collagen fiber was dissolved in sodium phosphate buffer (pH = 6) to form 10 g·kg^{-1} suspensions and mixed with 6 wt% of glycerol (on the basis of the dry collagen fiber), the air bubbles were removed by ultrasonic treatment for 30 min in an ultrasonic clear device (KQ-100E, Shufeng, China). Subsequently, 60 ml of the dispersion was immediately cast onto a polyacrylic plate (12 × 12 cm) and then air-dried in a ventilation hood at room temperature for 24 h. The newly formed films were peeled off from the plate and balanced in a desiccator (temperature was 25 °C, relative humidity (RH) was 51%) for 48 h.

2.5. Determination of film properties

2.5.1. Scanning electron microscope (SEM)

The micromorphology of films were characterized by SEM (SU1510, Hitachi, Tokyo, Japan) according to a previously reported method (Wang et al., 2017). The morphologies were observed at an acceleration voltage of 20 kV.