Effect of Transglutaminase on the Functional Properties of Gelatin Obtained from Chrome-tanned Pigskin

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Abstract: In this paper, microbial transglutaminase (mTG) was used to modify chrome-tanned pigskin gelatin, and the functional properties of gelatin such as viscosity, water solubility, melting temperature, and Bloom strength were studied under various reaction conditions. The viscosity of gelatin was changed obviously with mTG treatment, especially when the concentration of gelatin solution was higher than 15% (W/V), the viscosity of gelatin solution was too high to determine by using a Pinkevich viscometer (Φ10mm). When the gelatin concentration was 15%, enzymatic cross-linking also induced a significant decrease of water solubility, and in the reaction process, the gelatin lost its capability to undergo thermally reversible transitions and became insoluble in boiling water. Unexpectedly, the Bloom strength of gelatin was evidently decreased compared to the control sample without mTG treatment, indicating that the treatment with mTG had a significant effect on the structure of gelatin gel. We propose that the intermolecular covalent cross-links between specific glutamine and lysine should block the formation of a collagen-like triple-stranded helix among polymer chains in certain regions, and lead to the decrease of Bloom strength.

Key words: microbial transglutaminase; gelatin; Bloom strength; covalent crosslink

1 Introduction

One of the most significant problems of the leather industry is waste generation. The tanning industry is a generator of liquid wastes as well as tanned and non-tanned solid wastes. One ton of wet salted hide yields only 200kg of leather, but over 600Kg of solid waste [1]. Solid waste from tanning operations produce serious environmental impact: WHO describes Cr⁶ as toxic and carcinogenic. The solid leather waste has potential for recycling into useful and high added value products due to its collagen substrate. Nowadays, gelatin manufacture is a fashion way for recycling these solid leather wastes [2–4].

Gelatin is a protein produced by partial hydrolysis of collagen extracted from the bones, connective tissues, organs, and some intestines of animals such as domesticated cattle, porcine and horses. The natural molecular bonds between individual collagen strands are broken down into a form that rearranges more easily. And its chemical composition is, in many respects, closely similar to that of its parent collagen. Gelatin presents different and multi-functional properties, which make it suitable for use as an ingredient to improve elasticity, consistency and stability. Gelatin melts when heated and solidifies when cooled again. Together with water, it forms a semi-solid colloid gel. Gelatin forms a solution of high viscosity in water, which sets to a gel on cooling.

The quality of any gelatin depends on the species from which it is extracted and on the manufacturing method. The quality of gelatin obtained from chrome-tanned waste leather is poor because of the
excessive chemical and thermal pre-treated for tanned waste dechroming. The main difference in properties of gelatin obtained from chrome-tanned waste leather and natural gelatin are that the former have low melting temperature and low Bloom strength but relatively high viscosities. Only a few commercial uses for these gelatins could be applied where without gel formation is required, and for the majority, high rheological properties are indispensable. In order to improve the quality of gelatin, gelatin-modifying materials such as oxidized starch\(^5\), dextran dialdehydes\(^6\), MgSO\(_4\) and glycerol was used. Transglutaminases (TGases; EC 2.3.2.13) are widely distributed in various organisms, including vertebrates, invertebrates, plants, and microorganism, and are reportedly responsible for certain biological events such as epidermal keratinization, blood coagulation, and regulation of erythrocyte membranes. TGases catalyze an acyl-transfer reaction in which \(\gamma\)-carboxamide groups of peptidebound glutamine residues act as the acyl donor and, generally, the \(\varepsilon\)-amino groups of lysine residues or some naturally occurring primary amino groups are the acyl acceptor. Thus the polymerization of proteins can be achieved as a result of the formation of intermolecular or intramolecular \(\varepsilon\)-(\(\gamma\)-glutamyl)-lysine bonds. Recently, a microbial transglutaminase (mTG) isolated from the culture medium of Streptocverticillium molbairaense has become commercially available\(^7\). Unlike TGases from many other sources, the mTG possess many features, including Ca\(^{2+}\) independence, a broader substrate specificity for acyl donors, a smaller molecule size, and a higher reaction rate, which make them suitable for industrial applications. Currently, this mTG has been successfully applied in the food industry for improving the physical properties and texture of protein-related foods.\(^8\) More recently, the use of mTG to modify gelatin has also been reported\(^9,10\).

The objective of the study was to improve gelling properties of the gelatin extracted from chrome-tanned waste of porcine skin by incorporating mTG as a cross-linking reagent at varying concentrations of mTG and gelatin. The effect of mTG on the gelling properties was examined by determining the changes of viscosity, water solubility, melting temperature and Bloom strength.

2. Experimental

2.1 Materials

The source of transglutaminase was a commercial product obtained from Yiming Biological Products Co., Ltd (JiangSu, China). As determined by a colorimetric hydroxamate method\(^11\), the enzyme activity of mTG was 102 U/g of powder. The gelatin was provided by a gelatin factory in QuFu (Shandong, China), and the gelatin was produced using chrome-tanned porcine skin. All other regents used in the paper were of analytical grade.

2.2 Moisture Determination of Gelatin

In order to know the accurate concentration of the gelatin solution prepared subsequently, it is necessary to know the moisture content of the gelatin. The moisture of gelatin was determined according to Chinese standards GB/T 5009.3-2003\(^12\).

2.3 Preparation of Samples

Gelatin gels of different concentration (6.67%, 9%, 12% and 15%) were formed by mixed the dry gelatin powder with distilled water, and the mixtures were left at room temperature for 2h to allow the gelatin to absorb water and swell. The mixtures were then incubated at 45°C for 30 min in a temperature-controlled water bath with occasional stirring. The pH was adjusted to 6.5±0.1 with 0.2mol/L NaOH. The mTG (4U/g gelatin) was added after total dissolution of the dry gelatin in water, and the gelatin-mTG solutions were incubated at 45°C for 4h, followed by a heating step of 90°C for 5min to inactivate the mTG.
To study the effect of mTG concentration on gel properties of the gelatin, mTG at various concentrations (1, 4, 10 and 15 U/g of gelatin) were added in 6.67% gelatin solution which the pH was adjusted to 6.5. The mixture was then incubated at 45℃ for 4h and the mTG was denatured as described above.

2.4 Viscometry of mTG cross-linked gelatin solution

The viscosity of gelatin in solution was determined using a Pinkevich viscosimeter (Φ10mm). Soon after the mTG was added, 10mL gelatin-mTG solution was drawn into the Pinkevich viscosimeter and the viscosities were determined at 45℃ every 20min in the same temperature-controlled water bath as the gelatin-mTG reacted in. And the results were converted to mPa.S with the equation: η=Kt.

2.5 Solubility in water

The denatured gelatin-mTG solutions were cooled in a refrigerator at 7℃ for 16h, and froze at -20℃ for 24h. The frozen samples were lyophilized with alpha 1-2 freeze dryer (CHRIST, German). About 50 mg of the dry samples were immersed in 20mL distilled water and incubated for 24 h at 25℃ differently. The insoluble material was separated by centrifugation at 10,000rpm for 10 min. Nitrogen contents of the supernatant were determined by the way of Kjeldahl method. The control experiment was made with the same method except for the gelatin without modification by mTG. The solubility of the gelatin was evaluated on the base of nitrogen that was dissolved in the water and was expressed as the percentage of nitrogen contained in the control experiment. All the results were averages of three determinations.

2.6 Melting temperature

To study the melting temperature, each sample was poured in a 50mL colorimetric tube. All gels were stored in a refrigerator, at a temperature of 7℃, for 16h before use. One glass bead, 5mm in diameter, was place on the surface of gel in each colorimetric tube, and the gel samples were heated with the temperature climbing 0.5℃ every minute. Their temperatures were monitored with a digital thermometer until the glass bead dropped to the graduation mark of 25mL of colorimetric tubes. We considered the melting temperature as the temperature in which the gel lost its gel appearance and became a viscous solution.

2.7 Bloom strength

The gel strength of gelatin is a measure of the rigidity of a gel formed from a 6.67% solution and prepared according to certain arbitrary prescribed conditions. Bloom is a measure of force (weight) required to depress a prescribed area of the surface of the sample a distance of 4 mm. In this paper, the gel strength was determined on 6.67% gels modified with mTG at various concentrations. Cooled the solutions in a refrigerator at 7 ℃ for 16h, and the gel strength at 8-9 ℃ was determined on a JS-1 Bloom Tester ( Tianjin Medical Equipment Research Institute, Tianjin, China ) with a local cell of 5kN, cross-head speed 1mm/s, equipped with a 1.27cm diameter flat-face cylindrical plunger. The dimensions of the sample were 3.3cm diameter and 6cm height. The maximum force, taken when the plunger had penetrated 4mm into the gelatin gels, was the average of three determinations.

3 Results and discussion

3.1 Moisture of Gelatin

The result of moisture in gelatin used in the study is 11.51%. As the gelatin solutions were prepared, the moisture content of the gelatin was taken into account.

3.2 Viscosity
Being a polymer, gelatin's macromolecular nature produces a viscosity in solution which at most temperatures and concentrations displays rheological properties Newtonian in nature. The viscosity characteristics displayed by a given gelatin grade are primarily related to the molecular weight distribution of the gelatin molecules.

As shown in Fig. 1 the viscosity of gelatin solutions increase evidently with the treatment by mTG. Transglutaminase is an enzyme capable of catalysing acyl-transfer reactions, introducing covalent cross-links between gelatin molecules. Therefore, the molecular weight of the gelatin is enlarging, and the viscosity is increasing. Gelatin concentration also impacts the viscosity greatly, the viscosity-enhancing effect was more pronounced at higher gelatin concentration. The viscosity increased from 5.2mPa.s to 7.6mPa.s after 4h reaction when the gelatin concentration was 6.67%, but as far as 12% gelatin the viscosity hit 41.4mPa.s from 20.35mPa.s. Especially, when the concentration of gelatin solution was higher than 15%, the viscosity was too great to determine using the Pinkevich viscosimeter. Many pervious studies [13, 14] have approved that there is a network structure formed due to intermolecular covalent cross-linking among gelatin chains as shown in Fig. 2. It is indicated that with the concentration of gelatin solution increasing, the network structure was tighter and more complex. The formation of the tight network structure generates considerable changes in the viscoelastic properties of gelatin solution.

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**Fig. 1** The viscosity changes of different gelatin concentrations relative to reaction time at 45°C
The viscosity of gelatin solutions treated with various mTG quantities was measured along the reaction time, respectively. As presented in Fig. 3, the addition of mTG to gelatin solutions resulted in a net increase in viscosity. Furthermore, the increase in the ratio of viscosity was promoted by increasing the amount of mTG.

### 3.3 Solubility

Gelatin is only partially soluble in cold water. However, if gelatin solutions are spray dried or lyophilized from the sol state, the resulting gelatin is "cold water soluble" and such gelatins gel quickly when immersed into cold water. The effect of mTG treatment on the solubility of gelatin in cold water (25°C) was shown in Fig. 4 and Fig. 5. As presented in Fig. 4, the solubility of gelatin was declined sharply when the concentration got 15%, and the gelatin sample lost its’ ability to undergo thermally reversible transitions and become insolubility so much as in boiling water. In boiling water, the sample could swell and absorb water, and became a hydro-gel looking like nasal mucus. But when the concentration of gelatin was low, there was not significantly change of the solubility. We supposed that in high gelatin concentration the cross-link of mTG produce a continuous three dimensional network in which the gelatin molecules were immobilized and could not disperse into water.
3.4 Melting temperature

Gel melting point is the major physical properties of gelatin gels. It is governed by molecular weight, as well as by complex interactions determined by the amino acid composition and the ratio of α/β-chains present in the gelatin \[15\]. Fig. 6 shows the effect of mTG on the gelatin melting temperature. The melting temperature of gelatin increased with treatment by mTG, when 15U/g mTG was added, the increase was about 3°C. The addition of mTG raised the melting temperature, a fact attributed to the covalent cross-linking action of the enzyme, which would increase the molecular weight of the gelatin components. Increased average molecular weight has been reported by some investigators to raise the melting temperature \[16\]. In addition, we suggested that the formation of the network structure also contribute to the raise of the melting temperature, because the covalent cross-linking bond is more stable than intermolecular hydrogen bond which stabilizes the gel network.

3.5 Bloom strength

Bloom strength is widely accepted as a measure of quality, using a gelatin with a higher Bloom strength results in an increase in gel strength at the same gelatin concentration.

Fig. 7 represents the gel strength of gelatin gels prepared with and without mTG. Unlike the viscosity and the melting temperature of gelatin, the Bloom strength was decreasing by increasing the amount of mTG, when 15U/g mTG was added, the Bloom strength decreased from 172g to 165g. With respect to the addition of mTG, it is well known that this enzyme catalyses an acyl-transfer reaction, introducing a covalent cross-link between glutamine and lysine residues. In the gelatin this type of covalent bond is likely to be formed as can be observed in the results of viscosity, solubility and melting temperature. The Bloom strength seems to be increased due to the introduction of covalent cross-link. For sure, there were many previous works \[17, 9\] reported that the transglutaminase can increase the bloom strength of gelatin. And other reports \[18, 19\] suggested that transglutaminase do not help to increase the bloom strength, and it is different from gelatins coming from and the mTG reaction condition.
The primary structure of gelatin closely resembles that of collagen, which arrange in a triple-stranded helix structure. Therefore, the gelatin has a tendency to form the structure of collagen-like triple-stranded helix at certain regions by inter-chain hydrogen bonding as shown in Fig.8. It has been reported that Bloom strength is linearly correlated with the triple-helical content of solid gelatin \[^{20}\]. We propose that the intermolecular covalent cross-links between specific glutamine and lysine should block the formation of a collagen-like triple-stranded helix among polymer chains in gel. Accordingly, the Bloom strength was decreasing with the increasing of mTG quantity.

4 Conclusions

In this paper, microbial transglutaminase (mTG) was used to modify chrome-tanned porcine skin gelatin. The functional properties of gelatin were influenced greatly with the treatment of mTG. The viscosity of gelatin was changed obviously with mTG, especially when the concentration of gelatin solution was higher than 15% (W/V) where the viscosity was too great to determine using a Pinkevich viscosimeter (Φ10mm). There was not significantly change of the solubility, when the concentration of gelatin was relatively low. But as the gelatin concentration get 15%, enzymatic cross-linking induced a significant decrease in the water solubility, and with the reaction progressing, the gelatin lost its ability to undergo thermally reversible transitions and become insoluble in boiling water. The melting temperature was improved continuously with the increasing of mTG quantities. In addition, the Bloom strength of gelatin was evidently decreased comparing with the sample without mTG treatment, indicating that the structure of gelatin gel is significantly affected by the treatment with mTG. We propose that the intermolecular covalent cross-links between specific glutamine and lysine should block the formation of a collagen-like triple-stranded helix among polymer chains, and lead to the degradation of Bloom strength.

References