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Low-temperature crosslinking of proteins using non-toxic citric acid in neutral aqueous medium: Mechanism and kinetic study

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ABSTRACT

For the first time, mechanism and kinetics of citric acid crosslinking of protein in neutral aqueous condition under low temperature are reported. Crosslinking was a conventional method to improve properties of protein products to replace petroleum-derived polymers. However, current crosslinking methods were toxic, expensive or required harsh conditions. Non-toxic carboxylic acid could effectively enhance wet performance properties of multiple protein products under mild conditions, which were critical for preserving the activities during immobilization of enzymes. In this study, more than one carboxyl groups in one citric acid molecule were found capable of reacting with protein in aqueous medium at pH 6.8 under 50 and 75 °C, verifying occurrence of crosslinking. The pseudo second-order reaction had activation energy as low as 24.956 kJ mol^{-1}, indicating its easy occurrence. Mild crosslinking using citric acid might provide alternative to accelerate low-cost and efficient enzyme immobilization as well as industrial utilization of protein-based products.

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1. Introduction

In order to replace synthetic polymers derived from depleting petroleum, biobased materials, such as proteins, is attracting more attention as substitutes (Raquez et al., 2010). Proteins from agricultural byproducts and wastes could be prospective candidates, due to their annual renewability, low cost and large availability (Hernandez-Izquierdo and Krochta, 2008; Poole et al., 2008). Plant proteins, such as wheat gluten, zein from corn, sorghum protein and soy protein, are byproducts or coproducts of traditional food industry, rapidly expanding bioethanol and biodiesel industries (Taheripour et al., 2010), while animal proteins, especially chicken feathers and coarse wool fibers, are wastes in poultry industry and textile industry (Xu et al., 2014), respectively. All these proteins were developed into films (HernandezIzquierdo and Krochta, 2008), fibers (Reddy and Yang, 2005), adhesives (Chen et al., 2013) and other forms of products, demonstrating their processability and high potential for industrial applications. However, brittleness, water/moisture sensitivity and poor dry/wet mechanical properties remain major drawbacks that restrict wide applications of protein-based materials. On the other hand, immobilization of enzymes is very important for industrialization of these high-cost biocatalysts (Rodrigues et al., 2013). Immobilized enzymes usually have better stability to heat and operation, could be readily removed and recycled from the reaction medium. However, as proteins, most enzymes are susceptible to high temperature, extreme pH and other harsh treatment conditions, and thus require mild reaction conditions (Jia et al., 2014). Chemical crosslinking is a facile approach to improve water stability and mechanical properties of protein-based materials and to immobilize enzymes. However, most of the current crosslinking methods were either inexpensive-efficient-unsafe or expensive-inefficient-safe. Formaldehyde and glutaraldehyde are two most potent crosslinkers for proteins, with low cost. However, they were proved carcinogens (Feron et al., 1991) and could cause environmental pollution (Di Stefano et al., 1999). Most other crosslinkers, such as carbodiimides and genipin, which were proved safe, had relatively low crosslinking efficiency, and were expensive (Van Vlierberghe et al., 2011). Thus, there is still need for a safe crosslinker with high efficiency and low cost.

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Citric acid, one of the most widely used polycarboxylic acids, could be the crosslinker with abundance, safety, low cost and adequate efficiency. Due to lack of molecular-level elaboration of interaction, it remained controversial to define the roles of citric acid as a plasticizer or as a crosslinker in processing of biobased macromolecules. Initially, with three carboxylic groups and one hydroxyl group in one molecule, citric acid was expected to reduce intermolecular force by disrupting hydrogen bonds among macromolecules, and thus was regarded as a plasticizer. In processing of starch (Ma et al., 2008), chitosan (Yokoyama et al., 2002) and other polysaccharides, citric acid functioned as a typical plasticizer by increasing the flexibility and decreasing the tensile strength. However, according to the limitedly available literature, the plasticizing effects of citric acid in proteins were not as good as that for polysaccharides. The tensile strength, breaking elongation and modulus all decreased when zein and gelatin films were plasticized by citric acid (Cao et al., 2009; Parris and Coffin, 1997). In addition, the puncture strength of soy protein films decreased and then increased from about 0.15 N to 0.25 N as the weight ratio of citric acid over protein increased from 1.75 to 2.5% (w/w) (Eswaranandam et al., 2004). It was speculated that the drastically reduced pH after addition of citric acid induced precipitation of proteins, and thus adversely affected film forming capability of proteins. Therefore, it could also be assumed that maintaining the neutral or alkaline pH of protein solution during addition of citric acid might retain the film forming properties and facilitate function of citric acid as a plasticizer.

Surprisingly, via adjusting pH to slightly alkaline, citric acid might initiate crosslinking of proteins, in addition to or other than plasticizing them. Basically, besides flexibility that could be improved via plasticization, tensile strength, wet stability, dry and wet mechanical properties of protein products could all be remarkably enhanced via incorporation of citric acid under slightly alkaline pHs. Gliadin fibers crosslinked with citric acid under various pHs showed up to 40% increase in tensile strength and 100% increase in tensile elongation (Reddy et al., 2009b). Citric acid treatments also improved both tensile strength and % elongation of normal zein fibers (Yang et al., 1996), zein nanoparticles (Xu et al., 2015), three-dimensional zein fibrous scaffolds (Cai et al., 2013), silk (Yang and Li, 1993), casein fibers (Yang and Reddy, 2012) and camelina films (Zhao et al., 2014). These evidence inferred occurrence of crosslinking of proteins.

Clarification of the reaction mechanism could be critical to optimize crosslinking process and lay foundation for the wide industrial applications of citric acid. For example, wet crosslinking using citric acid under mild conditions could be a highly potential technology for immobilization of enzymes without seriously reducing their enzymatic activities.

In this research, gliadin from wheat gluten was selected as a model protein for the mechanism study of citric acid crosslinking. Conductometric and potentiometric titrations were conducted, and indicated that the numbers of amine groups and carboxylic groups in gliadin decreased and increased, respectively. Kinetic parameters were calculated based on the titration results. It could be proved that, reaction occurred between carboxylic groups of citric acid and amine groups in gliadin.

2. Materials and methods

2.1. Materials

Wheat gluten (Whetpro 80) with about 80% protein content, was a gift from Archer Daniels Midland Co., Decatur, IL. Ethanol (94.6%), hydrochloric acid (37%), sodium hydroxide (99.0%), acetic acid (99%), isopropyl alcohol (98%), sodium carbonate (98%) and methanol (99%) were purchased from BDH Chemicals Inc. West Chester, PA. Citric acid (99%), Coomassie Brilliant Blue R-250 and sodium phosphate (98%) were obtained from EMD Chemicals Inc. Gibbstown, NJ. Tetrabutylammonium chloride (98%) was purchased from Amresco LLC, Solon, OH.

2.2. Extraction of wheat gliadin

Wheat gluten was dispersed in 70% w/w ethanol at a weight ratio of 1:4, stirred under room temperature overnight. The mixture was centrifuged at 15,000 rcf for 30 min. The supernatant was collected and dried under reduced pressure at 50 °C to remove the solvent. The obtained gliadin was then pulverized. Gliadin was a candidate protein with large availability and was suitable for industrial applications. Gliadin contains approximate 6.2 mg amino acid residues with amines in the side group per gram of protein (Reddy et al., 2009a).

2.3. Wet crosslinking of wheat gliadin with citric acid

Certain amount of wheat gliadin was dispersed in ultrapure water at a weight ratio of 1:40. The dispersion was heated under 50 °C or 75 °C for 4 h. The protein was filtered and dried under 50 °C to constant weight. About 5 g of the obtained protein was dispersed in 200 g of 0.67 mol L^{-1} citric acid solution. Sodium carbonate solution was added to adjust the pH to 6.8, under which carboxyl groups in citric acid were deprotonated. The reaction was carried out at 50 °C with stir for up to 4 h.

To wash away citric acid sorbed onto gliadin, the crosslinked gliadin samples were washed using saturated sodium chloride solution and 0.025 mol L^{-1} sulfuric acid solution for 5 times with mechanical stirring under room temperature, and then ultrapure water until citric acid could not be detected using high performance liquid chromatography (HPLC). The obtained gliadin was dried under 50 °C to constant weight.

2.4. Determination of concentration of citric acid using HPLC

The obtained gliadin was washed using ultrapure water. The rinsing water was diluted to 1 L and filtered using 4.5 μm filters for HPLC analysis. A Dionex Ultimate 3000HPLC equipped with a Dionex Acclaim Polar Advantage II C18 column (3 μm, 4.6 × 150 mm) was used for analysis. The mobile phase was 20 mM sodium phosphate (pH 4.5) with 10 mM tetrabutylammonium chloride: methanol (80:20) at a flow rate of 0.5 mL min^{-1} under 25 °C. The injection volume was 20 μL, and the wavelength was set at 205 nm on the UV/Vis detector.

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

About 1 mg of gliadin from individual treatment was mixed with 100 μL of NuPAGE LDS sample buffer (1×), heated at 70 °C for 10 min, and left standing at room temperature for 2 h. The gliadin solution was vortexed prior to loading. Each sample of 10 μL was loaded into an individual slot of the NuPAGE 4–12% Bis–Tris Gel (Life Technologies, Grand Island, NY) in the XCell SureLock™ Mini-Cell Electrophoresis System (Life Technologies, Grand Island, NY). After electrophoresis, the gel was fixed with fixing solution with 25% w/w isopropyl alcohol and 10% w/w acetic acid for 1 h, and then stained in 0.6% w/w Coomassie Brilliant Blue R-250 in 10% w/w acetic acid for 2 h at room temperature. The gel was then destained using 10% w/w acetic acid until a clear background was observed. The prestained proteins (PageRuler™ Plus Prestained Protein Ladder, 10–250 kDa, Life Technologies, Grand Island, NY) were used as standards.

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