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Short communication

Carbodiimide stabilizes the ultrasound-pretreated camelina protein structure with improved water resistance

Xiangwei Zhu[a], Donghai Wang[b], Xiuzhi Susan Sun[a,*]

[a] Bio-Materials and Technology Laboratory, Department of Grain Science and Industry Kansas State University, Manhattan, KS 66506, United States
[b] Department of Biological and Agricultural Engineering, Kansas State University, Manhattan, KS 66506, United States

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ABSTRACT

Camelina protein showed poor water resistance, which restrained its industrial application, such as for adhesives or coatings. In this research, the effect of ultrasound pretreatment and carbodiimide coupling on water resistance of camelina protein isolate (CPI) was discussed. Camelina protein was extracted from defatted camelina meal by alkali solubilization and acid precipitation and treated by high intensity ultrasound. Both CPI and ultrasound-modified CPI (UCPI) were cross-linked by Ethyl-3-(3-dimethyl-aminopropyl-1-carbodiimide) (EDC). The cross-linked CPI exhibited increased molecular weight and particle size due to the amide bond formation between free amino groups and carboxyl groups. Accordingly, microstructures of the coupled protein became rigid and condensed with increased aqueous stability. The crosslink degree of UCPI was higher than CPI, leading to UCPI's better water resistance, more compact microstructures and larger particle size.

1. Introduction

To date, the limited fuel resources and corresponding environmental concerns caused by the petroleum manufacturing has urged us to develop alternative bio-based materials (Sun, 2011). Compared with other natural polymers, plant proteins, exhibiting a wide range of functional properties, was potentially utilized for bio-based resins or composites. Some popular proteins such as soy, canola, and zein have already been developed into applied biopolymers (Teng et al., 2012). However, large-scale production of those edible proteins obviously depletes the human food supplies. Consequently, alternative non-edible protein sources were expected.

Proteins from camelina provide such potential. Camelina sativa, a member of the mustard family, is a widely grown dryland oilseed crop in the North America and Europe. It was mostly utilized as biodiesel sources in the past few decades (Nguyen et al., 2013). In addition, it has drawn increasing attention due to the abundant polyunsaturated fatty acids in the camelina oil, offering superior food and health benefits (Kasetaite et al., 2014). Accordingly, the accumulating interests for camelina oil resulted in increased production of camelina meal (CM) that contained 40% crude protein (Li et al., 2014). As we previously reported, camelina protein (CP) contained four major fractions, albumins (water-soluble), globulins (5% NaCl-soluble), prolamins (70% ethanol-soluble), and glutelins (0.1 N NaOH-soluble) (Zhu et al., 2016), but the potential antinutritional toxic compound (glucosinolates) in the meal restricted the proteins for food uses (Matthäus and Zubr, 2000). Thus, the non-edible camelina protein became a suitable source for non-food bioproducts such as adhesives.

Similar to other plant proteins, research effort has focused on turning CP into useful biopolymers. Zhao et al. developed CP-based film and discussed the cysteine-induced unfolding to its physiochemical properties (Zhao et al., 2014). Our group also investigated the CP's adhesion properties on wood by different isolation processes (Li et al., 2015). However, these results indicated that like many other pristine proteins, CP exhibited poor water resistance, which was due to CP's limited molecular weight and non-stabilized structures (Li et al., 2015; Zhu et al., 2016). In addition, in aquatic environment, hydrophilic amino acids tended to absorb water, and protein–protein interactions were then weakened (Sun, 2011). In order to resolve this problem, scientists have utilized many coupling reagents, such as glutaraldehyde, PEG, or carbodiime to stabilize the protein structures to improve water-resistance. These investigations have been limited toproteins from soy, canola, and zein et al. (Chien et al., 2012; Kim et al., 2004; Wang et al., 2014). Thus, this triggered us to symmetrically investigate the coupling

Abbreviations: DCM, defatted camelina meal; CP, camelina protein; CPI, camelina protein isolate; UCPI, ultrasound-modified camelina protein isolate; EDC, ethyl-3-(3-dimethyl-aminopropyl-1-carbodiimide); NHS, *N*-hydroxysuccinimide.

* Corresponding author.
E-mail address: xss@ksu.edu (X.S. Sun).

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effect on CP structure with aiming to improve CP's water resistance for alternative biopolymers.

Ethyl-3-(3-dimethyl-aminopropyl-1-carbodiimide) (EDC), a zero-length cross-linker, was used to stabilize protein structure for bio-based materials(Nam et al., 2008; Usha and Sreeram, 2012). In addition to selecting the appropriate coupling agents, unfolding was important to protein's reactivity and further crosslink (Puppo et al., 2004; Renkema et al., 2002). Ultrasound technique, a widely used green approach, was effective in unfolding the protein structure with increased free functional groups for further stabilizations (Hu et al., 2013a; O'Donnell et al., 2010; Xue et al., 2013). Thus, the objective of this study was to investigate the EDC coupling effects on water resistance of camelina protein isolated (CPI) with ultrasound pretreatment. The coupling processes effects were characerized by analyzing the free amino groups, molecular weight, particle size, and the microstructures of the treated camelina proteins.

2. Materials and methods

2.1. Materials

Defatted camelina meal (DCM) with 32.4% crude protein (db), and 11.0% moisture content (db) was provided by Field Brothers Inc. (Pendroy, MT, US). *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), ninhydrin reagent, SDS Gel Preparation Kit, hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. High-intensity ultrasound treatment of camelina protein

CPI was separated from DCM as previously described (Zhu et al., 2016) and characterized (Supporting information). CPI dispersion (2.5%, w/v) was prepared to pH 8 with gently stirring at ambient temperature for 4 h. An ultrasound processor model VCF 1500 (SONICS & MATERIALS INC, Newtown, CT,USA) was used to sonicate 100 mL of CPI dispersions in an ice-water bath at 20 kHz at 375W for 20 min (pulse duration of on-time 1 min and off-time 1 min). After that, samples were lyophilized and stored at 4 °C refrigerator in air tight containers until used.

2.3. Preparation of CPI and UCPI dispersions

CPI and UCPI were dissolved in deionized water at a solid/liquid ratio of 1:20 (w/v) with pH adjusting to 8, and gently stirred for 1 h. EDC was added with NHS as the catalyst in the ratio of EDC (mmol)/NHS (mmol)/protein (g) = 0:0:1; 0.1:0.1:1; 0.25:0.25:1; 0.4:0.4:1, namely CPI-0, 0.1, 0.25, 0.4 or UCPI-0, 0.1 0.25, 0.4. The protein dispersions were adjusted to pH 8 again and conditioned 30 min, followed by the oil bath at 75 °C for 20 min to stimulate the crosslink reaction. After that, CPI and UCPI dispersions were gently stirred and cooled at room temperature for 6 h before further characterizations.

2.4. Characterization

Based on the reaction between the amino group and ninhydrin, the concentration of the amino group can be tested by the changes in absorbance at 570 nm (Moore et al., 1958; Sarin et al., 1981). Crosslink degree (CD) was calculated using the equation:

$$CD = \frac{A_0 - A_t}{A_0} \times 100\% \quad (1)$$

A_0: free amino groups of heated CPI or UCPI solution (no crosslink reagent)

A_t: free amino groups of EDC modified CPI or UCPI solution

SDS-PAGE was performed on a 4% stacking gel and 12% separating gel with a discontinuous buffer system, as described by Laemmli (LAEMMLI, 1970). EDC coupled CPI and UCPI samples (5 mg/mL in buffer containing 2% SDS, 25% glycerol, and 0.01% bromphenol blue) were incubated for 1 h at room temperature, then heated at 95 °C for 5 min and cooled in the ice bath for 3 min. Aliquots (15 mL) of the prepared samples were loaded onto the gels.

Particle sizes of samples were measured by light scattering using Horiba Laser Scattering Particle Size Distribution Analyzer LA-910 and reported as volume-mean diameter.

2.5. Water resistance

Water resistance was determined by measuring the protein-loss in soaking test. CPI and UCPI dispersions were prepared for the water soaking test. In a typical experiment, dispersion was uniformly brushed onto an aluminum lids and cured at 110 °C for 2 h. After recovering to room temperature, the protein-covered container was soaked in distilled water for 20 h, followed by 100 °C drying for 30 min. Protein lost could be calculated as

$$Protein\ \ loss = \frac{W_i - W_f}{W_i - W_g} \times 100\% \quad (2)$$

where W_g is the weight of the aluminum lids, W_i is the weight of the aluminum lids and protein after 110 °C curing, and W_f is the weight of the aluminum lids and protein after 100 °C drying.

The soaked proteins were collected and lyophilized. The morphology of soaked and non-soaked camelina protein was observed with a Hitachi S-3500N scanning electron microscope (Ltd., Tokyo, Japan)

3. Results and discussion

3.1. Protein crosslink degree

The crosslink degree was evaluated by the levels of free amino groups of CPIs and UCPIs. Expectedly, with increasing EDC content, the amount of free amino groups kept decreasing for both CPI (1.94 to 1.18 mmol/ml) and UCPI (1.89 to 1.02 mmol/mL) (Table 1). After the ultrasound treatment, the amount of CPI's free amino groups increased from 1.27 to 1.54 mmol/ml, and it confirms the protein's unfolding effect. The crosslink degree of UCPI-0.25, 0.4 were higher than CPI-0.25, 0.4. It demonstrates the stimulating effect of ultrasound treatment on UCPI coupling. Increased free amino groups were created for UCPI during the ultrasound treatment, and thus the reactivity of UCPI with EDC increased. Similar observation was also reported for other proteins. During the high-intensity ultrasound processing, strong cavitation force could unfold protein's secondary and tertiary structures, and thus the protein would be physically denatured and exhibit increased free sulfhydryl groups, increased surface hydrophobicity, and reduced particle size (Hu et al., 2013a,b; Xue et al., 2013). In addition, the characteristics of the denatured proteins varied under different ultrasound intensities. For example, Hu et al. (2013b) found that soy protein particle size was reduced after ultrasound treatment at 400W for 40 mins; however, they found the particle size increased when ultrasound intensity further increased, due to the excessive free radical present in the solution, which caused aggregation. For camelina protein, we found that it exhibits smallest particle size (7.68 um) when it was treated at 375 W for 20 min. The reduced time and intensity also suggest that CPI's secondary and tertiary structure were more liable to dissociate than soy protein.

In the presence of EDC, the cross-linked CPI and UCPI also exhibited increased molecular weight (Fig. 1a). There are four major subunits for pristine CPI and UCPI (Lane1 and Lane5), distributing