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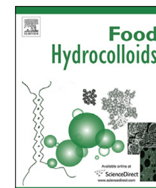

Two-step complex behavior between Bowman–Birk protease inhibitor and ι-carrageenan: Effect of protein concentration, ionic strength and temperature

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Complex behavior of Bowman–Birk protease inhibitor (BBI) with ι-carrageenan (LC) as a function of pH, protein to polysaccharides ratio and salt concentration was studied by turbidimetric titration, dynamic light scattering (DLS) and isothermal titration calorimetry (ITC). At fixed BBI/LC weight ratio of 5:1, turbidity and DLS results showed that pH_c and $pH_{\varphi 1}$ shift to the lower pH values with the increase in ionic strength (I), whereas the former occurred at the pH lower than isoelectric point (pI = 4.2) of BBI at $I \geq 100$ mM NaCl. ITC results showed that BBI binding to LC involves a two-step process with an increasing exothermic enthalpy at the first binding step. The further insight of BBI-LC complexation was studied as a function of BBI concentration, ionic strength and temperatures using ITC. The critical molar ratio ($r_{critical}$) between two binding steps was independent of protein concentration, ionic strength and temperature, although the heat flow obviously decreased with the increasing I (0–200 mM) and slightly increased with the elevated temperature (25–45 °C). The negative heat capacity (ΔC_p) and the gain in nonionic contribution (ΔG_{no}) indicated the involvement of nonelectrostatic interactions (e.g., hydrophobic effect) for the first binding step.

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1. Introduction

Proteins and polysaccharides are present together in many kinds of food systems, and the interactions between these both types of food macromolecules play significant roles in controlling the structure, texture, and stability of food systems through their thickening or gelling behavior and surface properties (Schmitt & Turgeon, 2011). It has been shown that polysaccharide-protein electrostatic interaction is influenced by a number of factors, particularly the polysaccharide to protein ratio, the pH, and the ionic strength (Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004). Complex formation occurs between oppositely charged biopolymers through electrostatic attractions and is widely applied to enzyme immobilization (Brena & Batista-Viera, 2006), antigen delivery, protein fractionation (Xu, Mazzawi, Chen, Sun, & Dubin, 2011), and food ingredient encapsulation and stabilization of food products (Giroux & Britten, 2011).

In China, millions of tons of soybean whey are produced every year. Bowman-Birk protease inhibitor (BBI), one of the most important nutritional and functional ingredients of soybean whey, contains 71 amino acids with 7 disulfide bonds, which makes the molecule very stable within the pH range encountered in most foods (Qi, Song, & Chi, 2005). This protein contributes to the nutritional quality of soybeans due to its high cystine content. The potential therapeutic value of BBI has been explored for many years. Bioactive proteins isolated from soybeans whey, such as the protease inhibitor BBI concentrate and purified BBI are now being intensively studied as cancer-preventive agents (Armstrong et al., 2000; Losso, 2008).

Carrageenans, sulphated linear polysaccharides of D-galactose and 3,6-anhydro-D-galactose, are widely used as a thickening, gelling and stabilizing agent as well as fat substitutes in the food industry (Gu, Decker, & McClements, 2005). κ- and ι-carrageenan in aqueous solution undergo a thermoreversible conformational transition from coil at elevated temperatures to helix (ordered) at low temperatures followed by aggregation and network formation at high polysaccharide concentration through sulfate groups and the 3,6-anhydro-D-galactopyransyl ring (Campo, Kawano, da Silva,

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& Carvalho, 2009). For λ-Carrageenan, it has a random coil conformation at all temperatures and unable to form gels, due to the presence of three sulfate groups (Gu et al., 2005).

As soybean-derived serine protease inhibitor, BBI has been identified in soymilk, infant formula, tofu, bean curd, and fermented soybean products (Hernández-Ledesma, Hsieh, & Ben, 2009). Carrageenan and soybean proteins are both widely used in food industry as gelling or viscous agents (Karaca, Low, & Nickerson, 2015; Prajapati, Maheriya, Jani, & Solanki, 2014), and the presence of interaction between BBI and carrageenan was unavoidable, special in the course of carrageenan–assisted tofu preparation (Saha & Bhattacharya, 2010). It has been reported that negatively charged carrageenan, could be used to recover soybean whey protein efficiently, and potential removal of antinutritional factors (Kunitz trypsin inhibitor and BBI) by carrageenan will be another interests (Smith, Nash, Eldridge, & Wolf, 1962). Changes in structural and thermal properties in protein–carrageenan systems were also observed. Carrageenan and milk proteins form a complex that could aggregate and stabilize matrix during gelation (Spagnuolo, Dalgleish, Goff, & Morris, 2005). The addition of carrageenan to β-lactoglobulin produced a slight increase in the thermal denaturation temperature of the globulin (Jones, Decker, & McClements, 2010). Uruakpa and Arntfield (2006) found that the addition of κ-carrageenan to commercial canola proteins increased surface hydrophobicity of the protein. However, there is still very little information available on the details of thermodynamic features of carrageenan –BBI complex formation/coacervation and on the nature of noncovalent interactions at the origin of these phenomena.

The strength of attractive electrostatic interactions between proteins and polysaccharides depends to a great extent on the macromolecular charge densities (van de Velde, de Hoog, Oosterveld, & Tromp, 2015). This is clearly demonstrated using milk proteins and low methoxyl pectin vs. high methoxyl pectin, and λ-carrageenan vs. ι- or κ-carrageenans (Burova et al., 2007; Salminen & Weiss, 2014). Sulphated polysaccharides such as carrageenan also interact more strongly with proteins than carboxylated polysaccharides, such as alginates and pectins (Klassen, Elmer, & Nickerson, 2011). In our previous work, among soybean whey proteins, Kunitz trypsin inhibitors, soybean agglutinin and β-amylase, could form one-step binding behavior with ι-carrageenan (LC), whereas BBI exhibited a two-step binding behavior to LC, suggesting a very different binding mechanism for BBI interacting with carrageenan (Li, Hua, Chen, Kong, & Zhang, 2015).

The objective of this work was to study molecular binding between Bowman–Birk protease inhibitor (BBI) and ι-carrageenan (LC) as impacted by pH, protein/polysaccharide weight ratio, ionic strength and temperature. Different from the typical electrostatic force driven interaction, a very different two step binding model was observed during the BBI binding to LC when using ITC. At different ionic strength, the contributions of entropic ($-T\Delta S$) and enthalpic (ΔH) determine the value of ΔG, thereof whether the complex formation is mainly entropically driven or through favorable enthalpy change (Turgeon, Schmitt, & Sanchez, 2007). An increase in temperature enhances hydrophobic interactions and covalent bonding, whereas low temperature is favorable to hydrogen bond formation. Regarding the temperature effect, there is an indicator called molar heat capacity or heat capacity change (ΔC_p) which is highly sensitive to the interactions between macromolecule residues and solvent molecules. A large positive value is a typical signature of ionization/charge neutralization reactions, while hydrophobic interactions produce a negative ΔC_p (Gonçalves, Kitas, & Seelig, 2005). Adding one biopolymer to the other one in excess will help us to record the equilibrium point of exothermic–endothermic profile change and the change in heat effect, to analyze the possibility whether condensation of aggregated complexes and changes in biopolymer conformation could also play a role (Sperber, Cohen Stuart, Schols, Voragen, & Norde, 2010). Therefore, the influence of protein concentration, salt and temperature on the enthalpy (ΔH) and entropy (ΔS) when BBI binding to LC was studied, so as to provide insights into the origin and nature of the interactions.

2. Materials and methods

2.1. Materials

Hexane-defatted and flush-desolventized soy flake, provided by Shandong Wonderful Industrial & Commercial Co.Ltd., had a protein content of 52.4% (N × 6.25, dry base) and a nitrogen solubility index (NSI) of 85%. GENUVISCO® ι-carrageenan (LC, Mw ~1.2×10^6 Da by SEC-HPLC), was purchased from the CPKelco. (Lille Skensved, Denmark), and is just for laboratory study use. All other reagents were of analytical grade.

2.2. Preparation of soybean whey and 2S proteins

2.2.1. Preparation of soybean whey

Soybean whey was obtained according to the following procedure: defatted soybean (*Glycine max*) flake was extracted with distilled water at pH 7.0 with a water-to-meal ratio of 10: l by blending at room temperature (25 ± 2 °C) for 30 min. After centrifugation (10,000*g*, 30 min) of the suspension to remove insoluble precipitate, the supernatant was adjusted to pH 4.5 with hydrochloric acid and centrifuged at 10,000*g* for 30 min to remove the acid insoluble soy proteins. After 48 h standing at 4 °C, insoluble residues were then removed by centrifugation at 10,000*g* for 30 min. After adjusting to pH 8.0 with 1.0 M NaOH, the supernatant was centrifuged again to remove insoluble non-protein substances. The protein concentration of the laboratory-prepared soybean whey was approximately 0.4% (w/v) determined by bicinchoninic acid method (Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano, et al., 1985).

2.2.2. Preparation of 2S proteins

2S proteins from soybean whey (P-2S) were precipitated by salting out with 40% saturation of ammonium sulfate at 25 °C and centrifuged at 10,000*g* for 30 min. The precipitates containing P-2S (Kunitz trypsin inhibitor and Bowman-Birk trypsin inhibitor mixtures) were collected and dispersed in water before dialyzing using regenerated cellulose (Spectra/Por, 3.5 kDa cut off, Spectrum Medical Industries, Inc.) for 48 h at 4 °C against water containing 0.02% sodium azide (to prevent microbial growth), and freeze-dried (Labconco Corp., Kansas City, MO). The prepared P-2S was stored at −25 °C before it was used.

2.3. Preparation of Bowman–Birk protease inhibitor (BBI)

BBI was prepared by our laboratory described as follows: 0.1% (w/w) P-2S and 0.1% (w/w) ι-carrageenan solutions were adjusted to pH 7.0 separately; then protein and polysaccharides solutions were mixed together with a desired ratio of 5:1, and adjusted to pH 3.8 by addition of 0.5 M HCl with gentle stirring. After centrifugation (5000*g*, 30 min) to remove precipitates, the supernatant was collected. Supernatant made as above was adjusted to pH ≥ 7.0 to reverse polysaccharide-protein complexation; then ultrafiltration (100 kDa cut off) was performed to remove residual ι-carrageenan, the BBI solutions were dialyzed (3.5 kDa cut off) for 48 h at 4 °C against water containing 0.02% sodium azide and freeze-dried

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