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An advance for removing antinutritional protease inhibitors: Soybean whey purification of Bowman-Birk chymotrypsin inhibitor by combination of two oppositely charged polysaccharides

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ABSTRACT

Two successive and selective coacervations induced by chitosan (Ch) and carrageenan (CG) were applied to remove antinutritional protease inhibitors and purify Bowman–Birk protease inhibitor (BBI) from soybean whey. At the first coacervation induced by Ch (66.7, 200, and 510 kDa), only Kunitz trypsin inhibitor (KTI) and BBI complexed with Ch were extracted, while β-amylase and soybean agglutinin remained in supernatant. The binding constants for the interaction increased on the order Ch-66.7 < Ch-200 < Ch-510. At the second selective complexation, we observed a competitive binding behavior between KTI/BBI and CG. At a mixing weight ratio of 3:1 (pH 3.0 for ι-CG, and pH 3.11 for λ-CG), the preferential binding of KTI to CG led to the single enrichment of BBI in the supernatant. Our results indicated that the purified BBI was a good source for further study of its anti-carcinogenic properties, due to its high bioactivity (669.5 U/mg chymotrypsin-inhibitory activity and 2260 U/mg trypsin-inhibitory activity).

1. Introduction

Complex coacervation based on protein–polysaccharide interaction provides a new strategy for selective removal of unneeded proteins or purification of target proteins without affecting their natural biological activities. A previous report showed that carboxymethyl cellulose can be used to fractionate whey protein through their ability to form a complex (Hansen, Hidalgo, & Gould, 1971), and pectin was successfully used to precipitate whey protein with a recovery of 90% (Serov, Antonov, & Tolstoguzov, 1985). Additionally, about 90% of acid-soluble proteins can be recovered from rapeseed-protein isolates waste water using sodium alginate and carrageenan (Gillberg & Ternell, 1976).

Selective complexation can be achieved in a mixed multi-proteins/polysaccharide system based on preferential interactions formed through electrostatic interactions. Selectivity in this case is defined as one protein transferring into the condensed phase prior to another (Wang, Gao, & Dubin, 1996). When two proteins have a

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different isoelectric point (pI), one target protein can form an insoluble complex with polysaccharides at appropriate pH, whereas the other cannot. The relative pH values for β-lactoglobulin and bovine serum albumin (BSA) complexation show salt dependence meaning that ionic strength determines the order of coacervation, and the pH controls the yield of the target protein (Xu, Mazzawi, Chen, Sun, & Dubin, 2011). For two proteins with similar pI, such as BSA and β-lactoglobulin (4.9 and 5.2, respectively), selective complexation can also occur due to protein charge anisotropy (Du, Dubin, Hoagland, & Sun, 2014).

Soybean whey proteins (SWPs) can be nutritional and functional ingredients for many food formulations upon their proper recovery and process (Martos, Préstamo, & Rupérez, 2006). SWPs are mainly composed of Kunitz trypsin inhibitor (KTI, 20 kDa), Bowman–Birk protease inhibitor (BBI, 7.9 kDa), soybean agglutinin (SBA, 120 kDa), lipoxygenase (102 kDa) and β-amylase (61.7 kDa) (Iwabuchi & Yamauchi, 1987; Sorgentini & Wagner, 1999). Soybean oligosaccharides, mainly stachyose and raffinose, are non-digestible oligosaccharides belonging to the raffinose family, also known as prebiotics, which promote the growth of helpful microorganisms in the colon (Tenorio, Espinosa, Préstamo, & Rupérez, 2010). After heat treatment of soy flakes, there still remain ~20% BBI and KTI residual traces that need to be removed, in order to increase the viability of SWPs for use in the animal and human diet (Friedman & Brandon, 2001).

Many methods were developed to identify chymotrypsin BBI, which is a potential anticancer agent, present in soybean seed (Gillman, Kim, & Krishnan, 2015; Muzard, Fields, O'Mahony, & Lee, 2012), soymilk (Arques et al., 2014), or soybean-derived products. However, the extraction and purification of BBIs from soybean proteins is still performed by acid precipitation, salting-out and gel-filtration chromatography (Bowman, 1946), or the combination of ion-exchange, affinity, and gel-filtration chromatography (Prasad, Dutta-Gupta, & Padmasree, 2010). Generally, these approaches are limited by low productivity, the time-consuming nature of the method, and high-solvent consumption. There remains limited information regarding the BBI purification based on protein-polysaccharide coacervation.

In our previous work, we achieved selective coacervation of KTI with chitosan (Ch) under salt-free, oligosaccharides-free, and small-molecules-free conditions due to the special structure of Ch (Li et al., 2014). Moreover, the purification of SBA, KTI, and BBI was achieved by a combination of ammonium-sulfate fractionation and protein-polysaccharide coacervation under salt-free conditions (Li, Hua, Chen, Kong, & Zhang, 2016). However, these studies were performed under "ideal" or "pretreated" conditions, with only single proteins involved and via salt-free or ammonium-sulfate fractionation, and the supernatant remaining after precipitation cause serious pollution of environment. Therefore, in this study, we investigated a more advanced approach for the removal of antinutritional factors and purification of one or more proteins from untreated soybean whey. Our goal was that the method would not be affected by the presence of high concentrations of carbohydrates and mineral salts, non-protein nitrogen, or unprecipitated proteins in original samples of soybean whey.

Here, we developed an approach enabling removal of antinutritional protease inhibitors and purification of BBI from original soybean whey based on two successive and selective coacervations induced by two oppositely charged polysaccharides. To remove these protease inhibitors, the initial complexation between SWPs and Ch was investigated as a function of pH, protein to polysaccharides ($R_{SWPs\text{-}Ch}$) ratio, and molecular weight (MW). We also used fluorescence spectroscopy to determine the binding affinity between SWPs and Chs of different MWs. At the second complexation, the competitive binding interaction between BBI/KTI and carrageenan (CG) was studied as a function of pH, mixing-weight ratios and polysaccharide type. The combination of two oppositely charged polysaccharides, Ch and CG, offered a novel approach for removing antinutritional protease inhibitors and purifying target proteins without affecting their natural biological activities.

2. Materials and methods

2.1. Materials

Cooled defatted soybean meal (containing 52.4% crude protein), was purchased from Shandong Wonderful Industrial & Commercial Co.Ltd (Shandong, China). Chitosans powders of different MW (510, 200, or 66.7 kDa) with deacetylation degrees of 85 ± 2% were provided by Golden-Shell Pharmaceutical Co., Ltd. (Zhejiang, China). GENUVISCO ι-carrageenan [LC, MW ~607 kDa, measured by size-exclusion chromatography-high-performance liquid chromatography (SEC-HPLC)], was purchased from CPKelco (Lille Skensved, Denmark). λ-CG (λG; MW ~520 kDa), was purchased from Sigma-Aldrich (St. Louis, MO, USA). HCl, H_2SO_4, NaOH, Na_2CO_3, $Na_2C_4H_4O_6$, NaOH, $NaHCO_3$, $CuSO_4$, Glycerol, Ammonium persulfate (AP), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), Sodium dodecyl sulfate-sodium salt (SDS), Phenol, Tris (hydroxymethyl)aminomethane (Tris), Glycine, Dimethyl sulfoxide, Acetic acid, 2-Mercaptoethanol, Coomassie Brilliant Blue G-250 were of analytical grade. Trypsin, Chymotrypsin, Tricine, N_α-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPA), Benzoyltyrosine 4-nitroanilide Benzoyl-L-tyrosine *p*-nitroanilide (BTpNA), N-Tris (Hydroxymethyl)Methylglycine (Tricine), Acrylamide, Bicinchoninic acid, Trifluoroacetic acid, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of soybean whey

Soybean whey was prepared according to our previous work (Li et al., 2016). Firstly, every 300 g defatted soybean (Glycine max) meal was suspended in 3000 mL deionized water and stirred for 30 min, followed by removal of the insoluble precipitate (1000–1200 g, 30.3%–36.4%) by centrifugation (10,000*g* for 30 min) and collection of about 2100–2300 g supernatant (63.6%–69.7%). After adjusting to pH 4.5 by 2.0 M HCl, the supernatant was centrifuged again to remove the soybean curd (146.68–183.32 g, accounting for ~4.4%–5.56%). After standing at 4 °C for 48 h, remaining SPI residue was removed by centrifugation (10,000*g* for 30 min), followed by another centrifugation (10,000*g* for 30 min) at pH 8.0 by adding 2.0 M NaOH to remove undesired precipitates (64.4–97.34 g, 1.95%–2.95%). By this method, about 1946.68–2153.22 g (58.9%–65.2%) soybean whey was prepared, and stored at 4 °C until use. The composition of soybean whey was as follow: ~protein 0.41% (w/v), carbohydrates 0.84% (w/v), moisture 98.4% (w/v) and ash 0.28% (w/v).

2.3. Turbidimetric titration

Stock solutions of 0.4% Ch (containing 0.4% (v/v) acetic acid) and 0.2% (w/w) CG (LC and λG) were prepared by dispersing 0.4 g chitosan or 0.2 g ι-carrageenan and 0.2 g λ-carrageenan sample powers, respectively, in deionized water and stirring for 3 h to 5 h at room temperature (25 ± 1 °C), then diluted with deionized water to 100 mL.

2.3.1. SWP-Ch systems

Turbidity titration of SWP/Ch (66.7, 200, and 510 kDa) mixtures were performed by mixing different volumes of soybean whey and Ch stock solutions at an initial pH of 3.0. The final mixing weight ratios were 20:1, 10:1, 5:1, 2:1 and 1:1. Different concentrations of NaOH (1, 0.5, or 0.25 M) were added to gradually alkalify the