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Binding of phytate to soybean protein during the heat treatment of soymilk and its effect on protein aggregation

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ABSTRACT

To investigate the interactions between phytates and soybean proteins during heat treatment, as well as their effects on the heat-induced protein aggregation and the formation of soymilk protein particles, the amount of free phytate, calcium, and magnesium in both unheated and heated soymilk were measured by equilibrium dialysis. It was found that about one-third of the free phytate became attached to proteins and was transferred to the particulate protein fraction after heat treatment. By heating purified soy protein solutions, it was shown that the soy glycinin had considerably higher phytate-binding capacity than β-conglycinin and that the binding reaction only occurred when the glycinin was denatured by being either heated to a temperature higher than 75 °C or treated with urea and SDS. It was assumed that when soymilk was heated to high temperatures, there was a critical "opening period" when the compact structure of glycinin unfolded and dissociated into the basic and acidic polypeptides, with plenty of the inner basic amino acids exposed to the surface, which attracted phytate and other denatured negatively charged protein fractions. The competitive binding of phytate with the basic polypeptides inhibited the binding of α/α' subunits and acidic polypeptides to some extent, resulting in a smaller ratio of particulate proteins. Phytate enhanced the solubility and inhibited the thermal aggregation of glycinin by increasing the negative charges on the protein surface.

1. Introduction

As the primary phosphorus reserves in mature soybean, phytate normally accounts for 1–2% of the seeds on a dry basis, which equals to 70–80% of their total phosphorus (Hou & Chang, 2003). Microstructural and chemical evidence provided by Prattley and Stanley (1982) demonstrated that phytate located in the protein bodies of soybean, likely in the form of a soluble protein-cation-phytate complex. Calcium and magnesium, being the main divalent cations in soybean, were analyzed to be in the concentrations of 140–280 mg/100 g seeds and 240–340 mg/100 g seeds, respectively. They are able to bind to proteins through either carboxyl (Bao, Lv, Yang, Ren, & Guo, 2008) or imidazole (Kroll, 1984) groups or form complexes with phytate. Factors affecting the binding nature and the types of resultant complexes include pH, the existence of metal ions, and also the structural and charging properties of a certain protein (Grynspan & Cheryan, 1989; Kies, De Jonge, Kemme, & Jongbloed, 2006; Okubo, Myers, & Iacobucci, 1976; Prattley, Stanley, & van de Voort, 1982). As a result, phytate and metal ions exist in various forms in soybean and soymilk, and their binding interactions are likely to be altered along with the changes in the protein structure and surface charges during processing (heat treatment, pH adjustment, chemical modification, etc.), which has not been well understood so far.

There has been evidence showing that the unique charging and structural properties of phytate, as well as its extensive chelating capability exert some significant impact on the stability, solubility and aggregation behaviors of plant proteins (Saito, Kohno, Tsumura, Kugimiya, & Kito, 2001; Wang, Xie, & Guo, 2015), but they are as yet not fully understood or investigated. It has been well accepted that phytate exerts an important buffering effect in soymilk coagulation, either by acid or by Ca^{2+}/Mg^{2+}, resulting in a slower protein cross-linking so that the gels exhibit finer gel network, softer texture, and higher water holding capacity (Ishiguro, Ono, Wada, Tsukamoto, & Kono, 2006; Toda, Takahashi, Ono, Kitamura, & Nakamura, 2006; Wang & Guo, 2016). Bye, Cowieson, Cowieson, Selle, and Falconer (2013) observed the dual effects of sodium phytate on the structural stability and solubility of proteins. It was suggested that the phytate-protein interaction was so complicated that it can serve as either a kosmotrope or a chaotrope, dependent on the net charges on the protein surface and the phytate/protein ratios. Soybean proteins are much

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more complex than the generally used model proteins, e.g. bovine serum albumin and lysozyme. Thus, the roles played by the coexisting phytate in the solubility, structural stability, as well as the aggregation propensity of proteins in a natural soymilk system require further confirmation.

Glycinin (also known as 11S protein) and β-conglycinin (or 7S proteins, β-CG) are the main storage proteins in soybean, which account for more than 80% of total protein content (Nishinari, Fang, Guo, & Phillips, 2014). By heating soymilk, the native proteins therein undergo unfolding, dissociation, and further aggregation, resulting in the formation of soymilk protein particles (d > 40 nm) and soluble proteins (d < 40 nm) (Ono, Choi, Ikeda,& Odagiri, 1991; Guo, Ono, & Mikami, 1997; Ren, Tang, Zhang, & Guo, 2009; Chen & Ono, 2014). Various factors, such as 11S/7S ratio (Guo & Ono, 2005), heating methods (Peng et al., 2017; Zuo, Chen, Shi, Wang, & Guo, 2016), and coexisting solutes (e.g. surfactant and NaCl, Ren et al., 2009), were reported to affect the formation of protein particles. Nonetheless, it remains unclear whether phytate involves in or affects the structure of protein particles.

In the present study, the existing forms of phytate, calcium, and magnesium in raw and heated soymilk were compared and discussed at length. The mechanism of the interactions between phytate and soybean proteins during heat treatment was investigated. Also, the effects of phytate-binding on the aggregation of proteins were studied in order to supplement the existing theories concerning soymilk protein particle structure and the roles played by phytate in soymilk and tofu processing.

2. Materials and methods

2.1. Materials

MENG-0138 and MENG-1001 Soybean [*Glycine max* (L.) Merr.] seeds were harvested in Anhui Province, China, offered by Anhui academy of agricultural sciences. ZHONGHUANG-50 soybean was harvested in Beijing, offered by Chinese academy of agricultural sciences. All the seeds were stored in a 4 °C refrigerator before use. Phytic acid sodium salt hydrate (from rice) and orange G sodium salts were purchased from Sigma-Aldrich (Steinheim, Germany). All reagents were of analytical purity and no further purification was needed before use.

2.2. Preparation of soymilk and soymilk whey fraction

Briefly, 100 g of soybean seeds were washed and soaked in distilled water for 12 h at 4 °C. The swelled beans were drained and ground with 700 mL of distilled water in a household blender (Joyoung C-020, Shandong, China) for 2 min. The homogenate was filtered through a piece of defatted cotton sheet to remove insoluble residues. The filtrate (raw soymilk) was heated in a water bath to 95 °C, held for 5 min and rapidly cooled to 25 °C in an ice water bath. Sodium azide (NaN_3, 0.02%) was added to soymilk as a preservative.

To measure the phytate-binding capacity of soymilk whey proteins, soymilk whey was fractionated by acidification to pH 4.5. The slurry was centrifuged at 8000 × g for 10 min, after which the supernatant was neutralized to pH 6.6. The turbid solution was centrifuged (8000 × g, 10 min) again and the supernatant was collected for subsequent determination of soluble protein as well as total and free phytate.

2.3. Preparation of raw soymilk samples with different levels of free phytate

To obtain a soymilk sample almost devoid of free phytate, a hollow cylinder-like ultrafiltration membrane with a molecular weight cut-off of 10 kDa (Mosu, Shanghai, China) was used to remove small molecular compounds from freshly prepared raw soymilk. Briefly, 2000 mL of soymilk in a beaker was continuously condensed by letting out the clear yellowish ultrafiltrate. Once the soymilk was halved in volume, 1000 mL of deionized water containing 0.02% NaN_3 was added to the beaker. It took about 7 rounds of these condensation and dilution steps till the conductivity of the soymilk was about 0.7 mS/cm. During this process, the pH of the soymilk remained stable at around 6.6. The final condensed soymilk was diluted twice with Na_2HPO_4-KH_2PO_4 buffer (pH 6.6, 0.046 M, conductivity 6.0 mS/cm) so that the protein level and conductivity of the obtained desalted soymilk was similar to that of the original soymilk, but with very low free phytate concentration (ca. 0.02 mg/mL).

To further adjust the level of free phytate in the soymilk samples, different amount of sodium phytate was respectively added to one 50-mL aliquot of the desalted soymilk, to reach a free phytate content of 0, 0.2, 0.4, 0.6, 1.0 mg/mL. The samples were subsequently adjusted to the same pH (around 6.60) and conductivity (about 3.35 mS/cm), after which they were individually heated to 95 °C, held for 5 min and cooled to room temperature immediately.

2.4. Preparation of soymilk samples with different ionic strengths and denaturants

To investigate the effect of ionic strength on the binding of phytate to soy protein after heating, both original and desalted raw soymilk samples were added with different levels of NaCl (0–1 M). All the samples were heated at 95 °C for 5 min and immediately cooled down.

Further, to probe into the relationship between the protein denaturation and the extent of phytate-binding, raw soymilk was respectively treated with 1) 0.2 M 2-mercaptoethanol (2-ME); 2) 6M urea and 0.5% sodium dodecyl sulfate (SDS); 3) 0.2 M 2-ME, 6M urea and 0.5% SDS, without further heat treatment.

The total amount of protein-bound phytate in each soymilk sample was measured and calculated by the difference between total phytate content in soymilk and the amount of dialyzable phytate after various treatment.

2.5. Fractionation of soymilk by ultracentrifugation

According to the method of Guo, Ono, and Mikami (1999), raw and heated soymilk samples were centrifuged at 156,000 × g for 30 min using a Hitachi CP-80MX ultracentrifuge (Tokyo, Japan), to separate the particulate protein (d > 40 nm, precipitates) and soluble protein (d < 40 nm, supernatant) fractions. Protein concentration was measured using the method of Bradford (1976).

$$\text{The ratio of particulate proteins} = 1 - \frac{\textit{protein content in the supernatant after centrifugation}}{\textit{protein content in soymilk before centrifugation}} \times 100\%$$

2.6. Determination of phytate, calcium and magnesium contents in different soymilk fractions

The concentrations of phytate (PA), calcium (Ca) and magnesium (Mg) of both the whole soymilk and the above-mentioned supernatant in 2.4 were measured separately. The percentage of PA, Ca and Mg bound to particulate proteins were calculated by subtraction of those in the supernatant from their total amount in the soymilk, whereas those bound to soluble proteins were quantified by subtracting the amounts of free PA, Ca, and Mg from their counterparts in the supernatant.

To determine the amount of free PA, Ca and Mg in soymilk samples under various treatment, equilibrium dialysis was performed according to Ishiguro, Ono, and Nakasato (2008): a 10-cm long cellulose dialysis tube (flat sheet diameter: 25 mm, cutoff molecular weight: 12–14 kDa, Viskase, USA), filled with 5 mL of ionized water, was immersed in 100 mL of soymilk placed in a food can (98 mm × 69 mm × 50 mm) and kept at 4 °C for 24 h, after which the liquid in the dialysis tubes was