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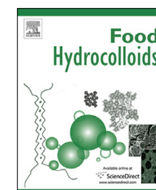

Effect of globular pea proteins fractionation on their heat-induced aggregation and acid cold-set gelation

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ABSTRACT

This report focuses on cold-set gelation of pea (*Pisum sativum* L.) proteins and related globulin fractions, namely Vicilin 7S and Legumin 11S. Protein thermal denaturation and aggregation were investigated using differential scanning calorimetry (DSC) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and sulfhydryl (S^-)/disulfide (S–S) bond assessment. While the denaturation temperature (T_d) of pea proteins increased from about 69 to 77 °C with increasing legumin content, the disulfide-linked acidic (α) and basic (β) legumin subunits (56–58 kDa) denatured and aggregated in a temperature range of 75–85 °C. Dissociation of legumin oligomers and their rearrangements via hydrophobic interactions and sulfhydryl/disulfide bonds exchange reactions would occur concomitantly during the heat-treatment, giving rise mainly to high-molecular weight aggregates of random structure. However thermal denaturation and aggregation of vicilin molecules would involve solely non-covalent interactions. Then glucono-δ-lactone (GDL) acid-induced gelation of protein thermal aggregates was evaluated by means of G' and G'' moduli. The preheated mixed pea globulins and vicilin-enriched samples gave rise to increased final moduli values of the acid gels, while legumin-enriched samples displayed low gelling properties. Improving functional properties of pea proteins would help to promote their application in plant-based gelled products such as tofu, alternatively to their soy counterparts.

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1. Introduction

Demographic change in combination with rapid urbanization and economical growth tend to increase dramatically worldwide pressure on land, water and natural resources. (Lundqvist et al., 2007). In spite of the wide inequalities in access to water and food, increases in income in most of developing countries are associated with modifications in food consumption, with a marked preference for animal-based food to the detriment of vegetarian products. However, given the heavy ecological debt of meat production systems, the need to develop alternative protein sources of reduced bioenergy and water demands for human diet is of primary interest. Thus one crucial challenge for food researchers would be to increase the awareness on the utilization of sustainable plant proteins, displaying both satisfying nutritional value and functionality (Boye, Zare, & Pletch, 2010). The development of novel and attractive plant protein-based foodstuffs would ultimately encourage the partial replacement of ingredients provided by animal production (meat, eggs, milk).

Soybean represent two-thirds of the world plant protein consumption (USDA, 2014). Despite its widespread utilization in many "vegetarian" meat-like products, the dependence on soy importations for many countries is in discordance with improving efficiencies of food production (O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004a, 2004b, 2004c). Mainly used as protein source in animal feed, pea (*Pisum sativum* L.) proteins could represent an alternative to soybean as functional ingredient in human food (Marcone, Kakuda, & Yada, 1998a).

Dry pea seeds contain about 20–25% crude protein, among them 70% are storage globulins (Boye et al., 2010). Salt-soluble globulins are composed of two fractions, namely legumin 11S and vicilin/convicilin 7S (Tzitzikas, Vincken, de Groot, Gruppen, & Visser, 2006). These are constituted of heterogeneous subunits assembled into high molecular weight (Mw) oligomers. Pea legumin 11S is hexameric (330–410 kDa), and its subunits (mainly 60–65 kDa) dissociate into acidic α (40 kDa) and basic β (20 kDa) polypeptides by disulfide bond reduction (O'Kane et al., 2004c). Vicilin and convicilin 7S are trimeric (150 kDa and 180–210 kDa, respectively) (Tzitzikas et al., 2006). Vicilin is a combination of heterogeneous polypeptides, since the major subunits (48–52 kDa) can be cleaved *in vivo* into low-Mw fragments (12–16, 20, 25–30

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and 30–36 kDa) (O'Kane et al., 2004a, 2004b). The convicilin subunits (70 kDa) display about 80% amino-acids sequence homology with the uncleaved vicilin subunits, though are distinguishable by their highly charged *N*-terminal extension region close to the *C*-terminus, and also the absence of *in vivo* cleavage (Tzitzikas et al., 2006).

Focusing on one essential functional property, gelation of globular proteins is based on their ability to form three-dimensional network (Bryant & McClements, 1998). To induce protein–protein interaction, a denaturing thermal process is usually applied. This leads to protein unfolding, exposure of initially buried reactive residues and subsequent aggregation caused by different molecular interactions, possibly involving non-covalent and/or covalent bonds. Many recent reports pointed out the lower textural features of pea protein heat-set gels than those obtained with their soy counterparts, though processed under the same conditions (O'Kane et al., 2004c; O'Kane, Vereijken, Gruppen, & van Boekel, 2005; Shand, Ya, Pietrasik, & Wanasundara, 2007; Sun & Arntfield, 2010, 2011, 2012). The type and composition of the pea proteins used and the different procedures of each study affected gelation properties. As reported by many authors, the commercial pea protein isolates utilized exhibited poor gelling properties, since proteins were extensively denatured during their large-scale production (Shand et al., 2007). Besides, pea proteins heat-induced gelation would require concentrated pea protein suspensions in the presence of high salt concentrations (Sun & Arntfield, 2010, 2011, 2012). Moreover, slow heating/cooling rates applied would be required for pea protein molecules to form gel network of enhanced elasticity (Sun & Arntfield, 2011). Nevertheless, Shand et al. (2007) indicated that the number of cross-links within the gelled network increased with the availability of soluble pea protein aggregates provided by heat-treatment.

From these observations, pea proteins cold-set gelation could represent an alternative route (Bryant & McClements, 1998). This procedure would enable better control of soluble protein aggregation; indeed heat-denaturation and gelation are divided into two steps. First, a low-concentrated protein solution (<10 wt%) at a pH far from its isoelectric point and in the absence of salt is heated to produce soluble aggregates. After cooling, gelation of the thermal aggregates is carried out by lowering electrostatic repulsions, allowing them to assemble into structured network. The second step could be achieved by adding an acidifying agent, glucono-δ-lactone (GDL). Acid-induced cold gelation is well documented for whey proteins (Alting, Hamer, de Kruif, & Visscher, 2003). In contrast, no data was available concerning pea proteins.

In a previous work, heat-treatment of a pea globulins solution resulted in high-Mw and soluble thermal aggregates (Mession, Sok, Assifaoui, & Saurel, 2013). In this regard, pea proteins extraction by an ultrafiltration/diafiltration procedure in addition with careful control of heat-treatment would promote the usefulness of aggregates as "building blocks" for cold-set gels. Hence, the present study aimed at investigating the thermal denaturation and aggregation of the different pea globulin fractions (mixed globular pea proteins: PP, legumin: Leg fraction and vicilin: Vic fraction), in terms of protein thermal stability and molecular interactions involved. Thereafter cold-gelation properties upon acidification of the thermal aggregates were compared according to the globulin fraction.

2. Material and methods

2.1. Materials

Pea proteins were extracted from smooth yellow peas (*P. sativum* L.), supplied by Roquette SA (Lestrem, France). Dehulled and ground peas contained 4.1 wt% total nitrogen (TN) on a dry basis (d.b.). The GDL powder was obtained from Prolabo (99.5% Fontenay-sous-Bois, France).

All other reagents and chemicals purchased from Sigma-Aldrich (St-Quentin Fallavier, France) were of analytical grade.

2.2. Methods

2.2.1. PP extraction and purification

Mixed globular pea proteins (PP) isolate was prepared from the defatted pea flour using a salt-extraction at pH 8, followed by tangential ultrafiltration/discontinuous diafiltration with deionized water (UF/DF), using a polyethylenesulfone membrane of 100 kDa, as previously described (Mession, Assifaoui, Lafarge, Saurel, & Cayot, 2012). During the UF step, a volume concentration ratio of 3 (VCR, mL/mL) was applied. The further discontinuous DF step consisted of a re-VCR of 3 (Taherian et al., 2011). Separation of the globulin fractions Vic and Leg was achieved by a chromatography column, pre-packed with DEAE Sepharose® Fast Flow anion-exchanger (150 mL, 15 cm bed height, GE Healthcare Amersham Biosciences Corp., Uppsala, Sweden). The pea protein concentrate obtained by UF as described above underwent the discontinuous DF step with 0.1 M Na_2HPO_4 – citric acid buffer, pH 7. Elution was performed at 20 °C using a stepwise gradient of NaCl (0, 0.06, 0.1, 0.25 and 0.5 M) in the phosphate-citrate buffer. Each eluate (150 mL) was monitored at 280 nm and analyzed by SDS-PAGE for purity. Polypeptide bands at 70, 50, 36–30 and lower than 14.2 kDa were attributed to vicilin/convicilin subunits, while the large band at 56–58 kDa belonged to legumin $\alpha\beta$ subunits (Mession et al., 2012; O'Kane et al., 2004a, 2004b, 2004c). Eluates of identical electrophoretic pattern were pooled together, and underwent for each an UF (VCR of 4) and discontinuous DF (re-VCR of 3). At the end, dried protein samples were obtained by freeze-drying, sealed and stored at 4 °C until further use. These dry samples contained mixed globular pea proteins, enriched-fractions vicilin/convicilin and legumin and were named PP, Vic and Leg, respectively.

2.2.2. Chemical analyses

Total moisture and ash contents were evaluated according to AOAC (1990) procedures. Total nitrogen (TN) and non-protein nitrogen (NPN) were determined according to EN ISO 20483:2006 and Chavan, McKenzie, and Shahidi (2001) methods, respectively. This allowed calculation of protein content, using a (protein) nitrogen-to-protein conversion factor of 6.25 (Marcone et al., 1998a; Shand et al., 2007).

2.2.3. Preparation of heat-induced aggregates

The freeze-dried PP, Vic and Leg samples were suspended in deionized water, stirred at room temperature for at least 2 h, and then centrifuged (12,000 g, 25 min, 4 °C) to obtain protein supernatants. To ensure that protein samples were at low ionic strength prior to heat-treatment, the supernatants were extensively dialyzed against a 10 mM Na_2HPO_4 buffer at pH 7.2, (ratio protein solution-to-buffer of 1:10, 4 °C, 24 h), and again centrifuged (12,000 g, 25 min, 4 °C). The PP, Vic and Leg stock solutions at 4.2, 6.2 and 3.7 wt% protein concentration, respectively, were diluted with the phosphate buffer used for dialysis to obtain various protein concentrations: 1, 2, 4 wt% for PP and Vic samples and 1, 2, 3.5 wt% for Leg samples. Protein samples (5 mL) were poured in PX/1636/04 MP tubes, hermetically sealed (SciLabware Ltd, Staffordshire, U.K.). They were placed in a water bath that was preheated at 40 °C, then heated at 1 °C/min from 40 to 85 °C, and incubated at 85 °C for 60 min. Heated tubes were subsequently cooled in ice for 30 min and stored at 4 °C overnight until further use. Some protein samples were also heated at various temperatures (70–85 °C) and times (0–60 min) for SDS-PAGE analysis (Section 2.2.5).

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