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Soybean germination limits the role of cell wall integrity in controlling protein physicochemical changes during cooking and improves protein digestibility

Mostafa Zahir, Vincenzo Fogliano, Edoardo Capuano *

Food Quality and Design Group, Wageningen University and Research, Wageningen, the Netherlands

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ABSTRACT

Previous studies showed that *in vitro* digestibility of proteins in cooked beans is modulated by heat treatment and that the effect may be different whether proteins are heated in intact cotyledon or in a bean flour. In this study, germinated and non-germinated soybean cotyledons and flour were boiled at 100 °C for varying times (30, 90, or 180 min). After grinding, the level of trypsin inhibitors, protein aggregation, surface hydrophobicity, the secondary structure, and *in vitro* digestibility were studied. The presence of an intact cell wall during cooking increased protein denaturation temperature by about 10% and reduced the denaturation of trypsin inhibitors, and induced distinct changes in protein surface hydrophobicity and secondary structure. These physicochemical properties translated into an increment in protein degree of hydrolysis (DH, 72%) of protein cooked for 30 min as flour compared to proteins cooked in intact soybean tissues (64%). Increase in cooking times (90 and 180 min) resulted in limited improvement in the protein digestibility and changes in protein physicochemical properties for both boiled cotyledons and flour. Soybean germination resulted in distinct changes in protein physicochemical properties and higher protein DH% of raw soybean (61%) compared to non-germinated raw soybean (36%). An increase in protein digestibility of germinated soybean was also observed after boiling for both cotyledon and flour. However, significant differences in DH% were not observed between proteins boiled in intact cotyledon and in a flour. This work provides extra knowledge of the role of cellular integrity on protein properties in plant foods and suggests that germination or grinding before cooking may increase protein digestibility.

1. Introduction

Globally, the consumption of plant-based proteins has gained popularity in recent decades due to the increasing demand for sustainable and healthy foods. Soybean is one of the major sources of plant-based protein for human consumption (Day, 2013), but plant protein utilization by humans is still too low compared to that of animal proteins (Becker & Yu, 2013; Sá, Moreno, & Carciofi, 2019; Young & Pellett, 1994). Knowledge of the correlation between soybean processing and protein digestibility can be applied to maximize utilization by humans. In previous studies (Zahir, Fogliano, & Capuano, 2018, 2020) we have shown that the cellular integrity of soybean and processing methods lead to large differences in soybean protein digestibility. In particular, we demonstrated that protein digestibility in soybean particles obtained by first milling and then boiling was higher than protein digestibility of soybean particles of the same size that were first boiled and then milled. This difference is mainly driven by a different degree of tissue integrity being the fraction of broken cells higher when the soybean was first milled and then boiled. Another recent study has indicated that the protein conformational change of bean proteins is different according to whether they are heated inside intact cells (i.e. in the crowded bean intracellular environment) or in a flour (i.e. open environment due to the loss of cellular integrity) (Rovalino-Cordova, Fogliano, & Capuano, 2019). That study showed that differences in protein secondary structure may, at least partially, explain the higher digestibility of bean proteins heated in flour.

The cellular structure and intercellular matrix may have a protective effect on proteins preventing the heat-induced conformational changes. Differential scanning calorimetry studies have shown a difference in protein denaturation temperature between soft and hard beans

* Corresponding author at: Food Quality Design, WU Agrotechnology & Food Sciences, Bornse Weilanden 9, 6700 AA Wageningen, the Netherlands.
E-mail address: edoardo.capuano@wur.nl (E. Capuano).

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(BernalLugo, Parra, Portilla, PenaValdivia, & Moreno, 1997; Garciavela & Stanley, 1989). The authors attributed the thermal stability of hard beans proteins to differences in the cell wall composition which would limit cell swelling, starch gelatinization, and protein denaturation. However, there is very limited information about the role of cell integrity in controlling the protein physicochemical changes during cooking. In particular, it is not known if protein can respond differently to the hydrolysis process during digestion, depending on whether the thermal treatment occurred in a tightly packed intracellular environment (intact cotyledon) or in a relatively open environment (flour). Comparison of the protein physicochemical properties and digestibility in both boiled germinated and non-germinated soybean can be used to determine the extent to which the cell-matrix may affect the way proteins respond to heat treatments and consequently affect its digestion. Germination is indeed known to produce a change in the cell wall architecture and the cytoplasmic matrix which may modulate the effect of the cell-matrix on heat-induced changes in proteins properties and digestibility (Gronwald, Jung, Litterer, & Somers, 2009; Martín-Cabrejas et al., 2008; Zahir, Fogliano, & Capuano, 2020). These insights might further lead to the possibility of re-design soybean processing to enhance the nutritional utilization of soybean proteins.

The purpose of the present study was to determine the effect of the encapsulation of proteins within intact soybean cotyledon cells on protein conformational changes during cooking and the impact of these changes on protein digestibility. To address this, cotyledons of non-geminated and germinated soybean were boiled either before or after milling and protein digestibility was investigated after grinding of the tissues to a fine flour (32–45 μm). The grinding process was performed to isolate the effect of the presence of cell wall on protein conformation from the well-established barrier effect towards digestive enzymes. Quantitative chemical analyses of some protein physicochemical properties such as the level of trypsin inhibitors, surface hydrophobicity, and secondary structure were performed to provide more understanding of the impact that cell integrity during cooking may have in modulating protein digestibility.

2. Materials and methods

2.1. Materials

Dried soybean seeds were purchased from De Vlijt (Wageningen, The Netherlands) and stored at room temperature.

Porcine pepsin (P6887, 3.200–4.500 U/mg protein), pancreatin (P1750, 4X USP), Porcine bile extract (B8631), pefabloc® SC, sodium dodecyl sulfate (SDS), o-phthaldialdehyde (OPA), DL-dithiothreitol (DTT), L-serine, trypsin (bovine), N-α-benzoyl-DL-arginine-pnitroanilide hydrochloride (DL-BAPA), and bromophenol blue (BPB) were purchased from Sigma-Aldrich, USA. Trichloroacetic acid (CAS 76–03-9) and disodium tetraborate decahydrate (CAS 1303–96-4) were bought from Merck & Co. (Darmstadt, Germany). NuPAGE® 4–12% Bis-Tris Gels, NuPAGE® LDS sample buffer (4× concentrated), MES running buffer, and Mark 12 Unstained Standard for SDS-PAGE analysis were provided by Thermo fisher scientific (Van Allen Way Carlsbad, CA, USA). Other chemicals used in this study were of analytical grade.

2.2. Samples preparation and heat treatment

Dried soybean seeds (100 g) were soaked in tap water at a 1:3 (w/v) ratio and kept at 5° C for 12 h. The excess water after soaking was discarded and the seed coat was removed manually. Dehulled soybean cotyledons were cooked as whole cotyledons or as fine flour of size range 32–45 μm obtained by intensive milling and sieving. The cooking was conducted in a water bath at 100 °C for varying times (30, 90, or 180 min) to evaluate the effects of heat treatment time. Boiled cotyledons and control samples (uncooked cotyledons) were ground for 5–10 min using Retsch Cryo Mill (Retsch Technology GmbH, Haan, Germany) sieved to obtain a fine flour of the same size of the flour that was heated (32–45 μm). Samples were then dried at 40 °C until a moisture content of 10% was achieved and total protein content was estimated by the Dumas combustion method according to Zahir et al. (2018, 2020). Dried samples were stored until further analysis.

In a separate experiment, the effect of germination was investigated. For this, dried seeds were germinated following the procedure described in detail in our previous work (Zahir et al., 2020). Briefly, dried seeds were disinfected with 0.07% sodium hypochlorite solution and soaked in tap water for 6 h prior germination process that was continued for 4 days at 27 °C under darkness. The sprouts and seeds coats of germinated seeds were removed and the germinated cotyledons were boiled and prepared using the same conditions as described for non-germinated soybean samples. An overview of the experimental plan is given in Fig. 1.

2.3. In vitro digestion and protein hydrolysis quantification

The digestion experiments were carried out based on the recommendations of the Infogest consortium (Brodkorb et al., 2019; Minekus et al., 2014). In brief, a mixture of sample flour (size 32–45 μm) and ultrapure water was prepared to normalize the protein content in all samples before *in vitro* digestion process. The sample mixture was suspended in a simulated salivary fluid (with no salivary α-amylase) for 2 min. Subsequently simulated gastric fluid (containing pepsin) was incorporated and the pH of the mixture was adjusted to 3 and incubated at 37 °C with constant mixing for 2 h. Following the gastric phase, the gastric chyme was combined with simulated intestinal fluid containing fresh bile and pancreatin solution. The pH of the mixture was then adjusted to 7 and incubated at 37 °C for 2 h. Aliquots of the digestion mixture (1 mL) were taken at different time points during simulated intestinal digestion. The activity of enzymes was stopped using 5% trichloroacetic acid (TCA) and samples were stored at −20 °C until further analysis. The concentration of free amino groups was determined by OPA method as described by Nielsen, Petersen, and Dambmann (2001) with minor modifications as described by Zahir et al. (2018, 2020). The degree of protein hydrolysis (DH%) was quantified based on the concentration of free amino groups in enzymatically hydrolyzed samples, non-hydrolyzed samples, and completely hydrolyzed samples in 6 N HCl at 110 °C for 24 h as detailed by Zahir et al. (2018, 2020).

2.4. Proteins thermal properties

Thermal properties of soybean protein were determined by the use of a differential scanning calorimeter (Perkin Elmer DSC with stainless steel large volume cups) in triplicate. For this, soybean particle size range 2000–3000 μm and fraction with size range 32–45 μm were used to simulate encapsulated protein and free protein samples respectively. Fifty mg of prepared sample (~50% moisture) was weighed into a DSC pan and the pan was then hermetically sealed and equilibrated at 25 °C for 5 h at room temperature. The thermograms were obtained at a heating rate of 10 °C min^{-1} from 25 °C to 160 °C. A sealed empty pan was used as a reference. The onset peak temperature (T onset), the end peak temperature (T end), the temperature at the peak maximum (Tp), which is generally used to indicate the temperature of denaturation (Liu et al., 2008) and the enthalpy of denaturation (ΔH, J/g dry matter) were obtained from thermogram analysis using the PyrisTM operation software (PerkinElmer, Ltd., United Kingdom).

2.5. Trypsin inhibitor activity

Trypsin inhibitor activity assay was assessed according to the modified procedure proposed by Liu (2019) with slight modifications. In short, the extraction of trypsin inhibitor was performed by mixing one gram of soybean flour (32–45 μm) with 50 mL of 10 mM NaOH (pH 8.4) and kept under constant stirring at room temperature for 3 h. The

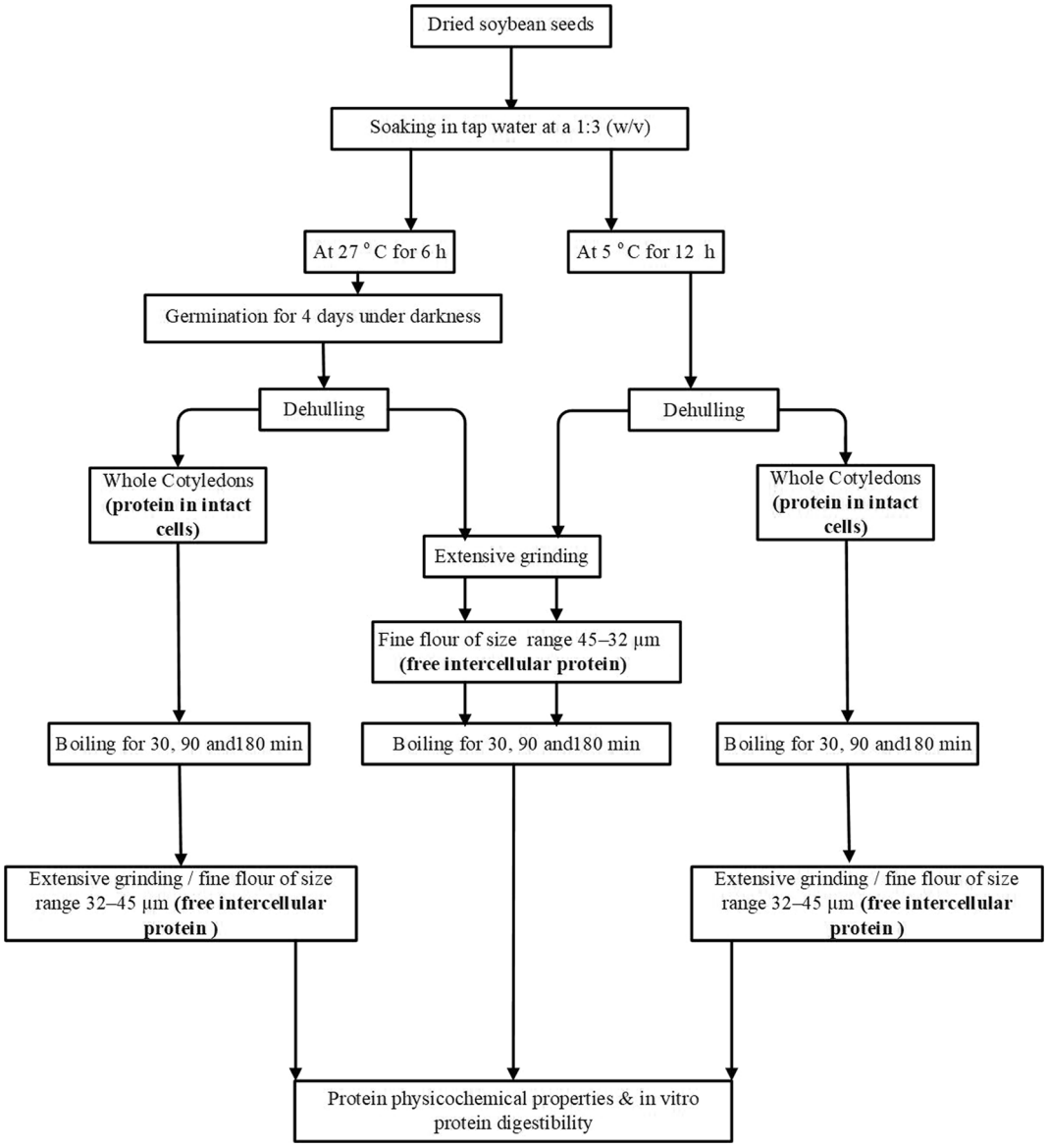

Fig. 1. Scheme for preparing samples for *in vitro* protein digestion and protein physicochemical properties analysis.

resulting dispersion was centrifuged at 2000*g*, for 5 min. One mL of samples supernatant was diluted so that 2.0 mL of a dilute sample caused 30–70% inhibition of trypsin activity. In determining trypsin inhibitor, a reaction between trypsin (type 1 × from bovine pancreas) and N-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride (DL-BAPA) in presence of trypsin inhibitor extract (soybean flour extracts) was performed for 10 min at 37 °C and stopped by acetic acid. The absorbance at 410 nm for the sample reading was measured after clarification by centrifugation (at 2500*g* for 10 min). The absorbance of this sample is indicated as A_{410S}. The reaction was also run in the absence of inhibitors by replacing the sample extract with an equal amount of water. This corresponding absorbance is known as the standard reading or reference reading in some other literature and is indicated here as A_{410R}. In addition, reagent blanks for the sample readings (indicated as A_{410SB}) and a reagent blank for the reference/water readings (A_{410RB}) were also run by adding the acetic acid solution before the trypsin solution. The trypsin inhibitor units (TIU) per mg sample was calculated based on the following equation:

$$\text{TIU/mg sample} = \frac{\{[(A_{410R} - A_{410RB}) - (A_{410S} - A_{410SB}) \times 100] \times \text{mL diluted soy extract}\}}{(\text{mg sample per mL diluted soy extract used for the assay})}$$

where $(A_{410\ R} - A_{410RB}) = A_{410CR}$, corrected reference reading of A_{410}, $(A_{410S} - A_{410SB}) = A_{410CS}$, corrected sample reading of A_{410}, $(A_{410CR} - A_{410CS})/ A_{410CR}$ should be in the range of 0.30–0.70 that is 30–70% of trypsin inhibition by given dilute soy extract.

2.6. Protein surface hydrophobicity

Protein surface hydrophobicity of samples was determined based on the interaction between hydrophobic chromophore bromophenol blue

(BPB) and protein according to Chelh, Gatellier, and Sante-Lhoutellier (2006). Sample (fine flour with size 32–45 μm prepared as described in 2.2 section) containing ~5 mg protein was dispersed in 1 mL of 20 mM phosphate buffer (pH 6.0). The mixture was combined with 200 μL of 1 mg/mL BPB (in ultrapure water) and mixed well. The tube contained 1 mL of phosphate buffer and 200 μL of 1 mg/mL BPB was used as a reference sample. Both sample and reference were kept under constant stirring at room temperature for 10 min. The dispersion was then centrifuged at 2000*g* for 15 min and diluted 10 fold with phosphate buffer. The absorbance of the diluted supernatant was measured at 595 nm against a blank phosphate buffer. The amount of BPB bound was estimated by the following equation:

$$Bound\ \ BPB(\mu g) = 200\ \ \mu g \times \frac{(A595\ \ Control - A595\ \ Sample)}{A595\ \ Control}$$

where A595 was the absorbance at 595 nm.

2.7. Protein aggregation

Soybean protein aggregation formed upon cooking was monitored using gel electrophoresis under non-reducing conditions. The method used was adapted from previous studies (Yasir, Sutton, Newberry, Andrews, & Gerrard, 2007; Zenker et al., 2019) with some medications. Sample of fine flour size ranges (32–45 μm) was combined with NuPAGE® LDS buffer (4× concentrated), and ultrapure water in a ratio 5/5/10 (w/v/v). The mixture was kept under stirring for 10 min and then (10 μL) was taken and loaded onto the gel wells. Mark 12 Unstained Standard (Invitrogen) was used as MW marker. Gels were run at 120 V for ~1.5 h using MES buffer and stained with Coomassie Brilliant Blue R-250 for ~2 h. Afterward, gels were then fixed in washing buffer (10% absolute ethanol and 7.5% glacial acetic acid) and the gels images were acquired using Lab software TM (Bio-Radd Laboratories, California, USA).

2.8. Protein secondary structure

Protein secondary structure was studied using the Fourier transform infrared (FTIR) spectroscopy technique. Fine flour samples with size range of 32–45 μm prepared as described in section (2.2) were used for FT-IR analysis. The spectra of the samples were analyzed in the wave number range from 400 to 4000 cm^1 with 32 scans in a Bruker Tensor 27 (Bruker Optics, Ettlingen, Germany). The secondary structure of soybean protein that is commonly based on the amide I band (1600–1700 cm^{-1}) was analyzed using Origin lab® software (Northampton, MA, USA) and following the procedure of Rovalino-Córdova, Fogliano, and Capuano (2019) with some modifications. Briefly, before the curve fitting a straight baseline correction was performed in the region (1600–1700 cm^{-1}). The identified peaks with the amide I (1600–1700 cm^{-1}) were assigned to their particular substructure of protein secondary structure according to the assignments of Long et al. (2015). The relative composition of each secondary structure component was estimated by dividing the individual component peak area on the total area, obtained as a result of the calculations of the area of the entire peaks.

2.9. Statistical analysis

The data are presented as mean ± standard deviation (SD) of at least three replicates. The significant differences in means were determined at the $p < 0.05$ level using IBM SPSS statistics 25 link (NY: IBM Corp). The difference between DH% at each digestion time point, trypsin inhibitor units and the amount of bound BPB were assessed by two-way ANOVA. The factors tested were boiling time, and soybean structure (cotyledons and flour), as well as their interaction (boiling time × structure set), independently for boiled samples of non-germinated and germinated samples. The statistical difference between boiled non-germinated and germinated samples was determined by Student's paired *t*-test. The difference in the values of each thermal protein property among samples was assessed by ANOVA followed by Tukey's test. Pearson correlation was used to measure the association between the means of protein physicochemical and means of *in vitro* digestibility of final products (120 min of intestinal digestion) at a significance level of 0.05.

3. Results

3.1. In vitro protein digestibility

In vitro protein digestibility, expressed as a degree of hydrolysis (DH %), at different duodenal digestion time points, is presented in Fig. 2. Fig. 2-A clearly shows that protein digestibility increased after cooking. The increase in DH% due to the differences in boiling times and /or in the level of integrity of boiled soybean (cotyledons and flour) was modest. However, we found statistical differences in DH% values among

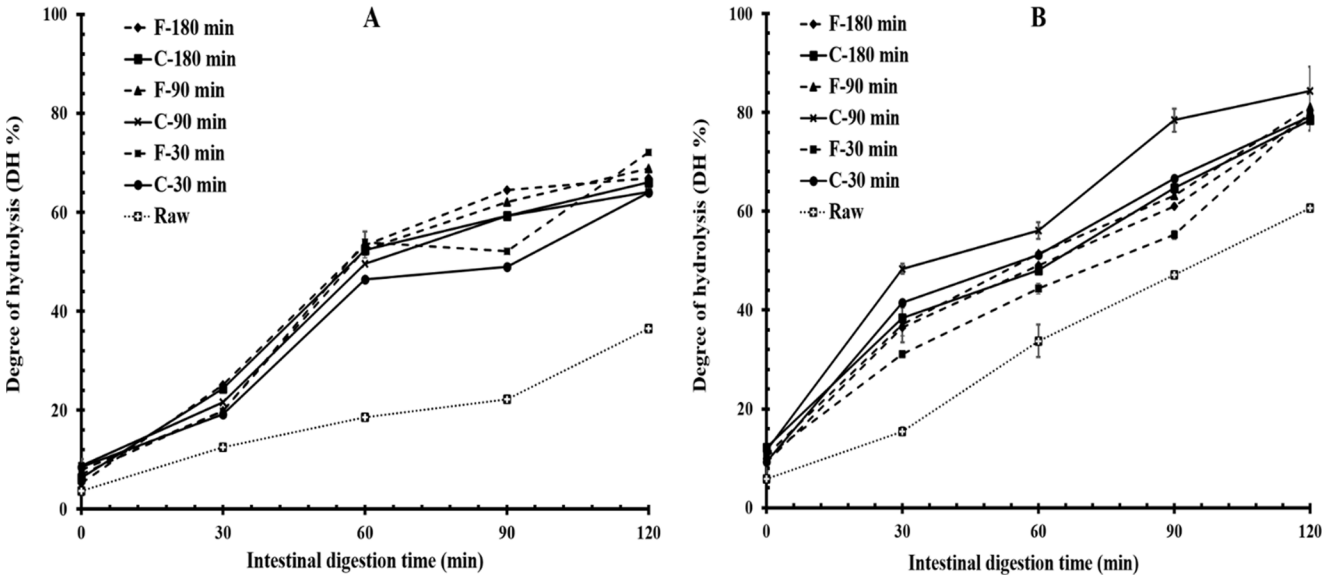

Fig. 2. Degree of protein hydrolysis (DH%) in soybean particles (32–45 μm) prepared from boiled soybean for varying times (30, 90, or 180 min) as intact cotyledons (C) or flour (F). A = non-germinated soybean, B = germinated soybean. Raw samples of non-germinated and germinated soybean were used as control samples.

non-germinated samples ($p < 0.01$) for all intestinal digestion time points. At the end of the intestinal digestion, the DH% in the flour samples was significantly higher than the DH% in the cotyledon samples ($p \leq 0.01$). The effect of the boiling time was also significant ($p \leq 0.01$), but due to the interaction, the trend was different in flour compared to the intact cotyledon. Protein digestibility of raw soybean was markedly increased after germination. For the germinated samples, significant differences in DH% were found for all intestinal digestion time points, except for 120 min of duodenal digestion. Student's paired *t*-test showed that there was a significantly higher DH% in germinated compared to non-germinated samples ($p \leq 0.01$) at each intestinal time point. The data in Fig. 2, A shows that in non-germinated samples the thermal treatment applied to the flour induces a small but significant increase in protein digestibility compared to when the same treatment is applied to the intact cotyledon. The data in Fig. 2 B demonstrated that the combination of a mild treatment such as germination and a short thermal treatment significantly improved protein digestion in soybean.

3.2. Thermal properties of encapsulated and free proteins of soybean

Monitoring of thermal denaturation of soybean protein using DSC technique usually requires soybean flour or isolated soybean protein as substrate materials. In this study, it was observed that the denaturation temperatures (Td) of soybean protein fractions 7S and 11S were higher for particles of 2000–3000 μm compared to the fine particle of 32–45 μm where the proteins were free of any physical boundary (see Table 1). The same trend was observed for germinated soybean, although the difference between big particles and fine particles was negligible, (see Table 1). Statistically significant differences were observed between big particles and fine particles for both non-germinated and germinated soybean for each thermodynamic property investigated, indicating that the cell wall barriers may delay the heat-induced denaturation of protein when packed inside the intact cells.

3.3. Trypsin inhibitor activity

Thermal inactivation of trypsin inhibitor in non-germinated and germinated soybean is shown in Fig. 3. Cooking either as a whole cotyledon or as flour resulted in substantial inactivation of the trypsin inhibitors. It is quite clear from Fig. 3a and b, that ~90% inactivation has been achieved during the first 30 min of cooking. The effect of cooking time, cellular integrity, and their interaction were all significant for both germinated and not germinated samples ($p \leq 0.01$). A significant reduction in trypsin inhibitor activity (44%) was observed in raw samples after germination process ($p < 0.001$) (see Fig. 3.b). Statistically significant differences were observed between boiled samples of non-germinated and germinated soybean at each cooking time ($p \leq 0.03$).

3.4. Protein aggregation

Fig. 4 shows non-reducing SDS-PAGE images of non-germinated and germinated soybeans boiled for 30, 90, and 180 min as whole cotyledons or fine flour. The smearing on the top of SDS PAGE gel is likely due to high-MW protein aggregates formed by S-S bridging and other covalent cross-linking. This is because it is possible that the SDS-PAGE condition, although under non-reducing condition, could damage the non-covalent interaction between proteins. Using native PAGE which maintains both the proteins' secondary structure and native charge density, could be the best technique to know the aggregation state of a protein (Shi et al., 2013; Stegemann, Ventzki, Schrödel, & de Marco, 2005). In the non-reducing SDS-PAGE condition used in our study, all samples formed protein aggregates. The highest intensities of the smearing on the top of SDS PAGE gel were detected for non-germinated samples, whereas the lowest intensities were detected for germinated soybeans that were boiled either as cotyledons and flour (image B). However, we observed the formation of aggregates also in raw samples (Lanes 2 in both images) likely as a result of extensive grinding. It has been shown that ball milling treatment may form bigger aggregates of soybean protein through intermolecular S-S (Liu et al., 2017). The SDS-PAGE profile based on MW distribution of protein shows clear differences between uncooked (raw) non-germinated soybean samples (Fig. 4, A) and raw germinated samples (Fig. 4, B). The raw sample (lane 1- A) shows a typical profile of soybean proteins is made up of several polypeptides in the molecular weight range of 10 to 140 kDa. The band of molecular weights around 21 kDa corresponding to the trypsin inhibitor subunit was detected in both raw and germinated soybean (Lane 2 in Fig. 4, A and B). The new bands appearing at approximately 26 kDa (Lane 2 in Fig. 4.B) might originate from the proteolysis of soybean storage proteins during germination. (Gonzalez-Montoya, Hernandez-Ledesma, Silvan, Mora-Escobedo, & Martinez-Villaluenga, 2018; Zahir et al., 2020). The intensity of major bands was gradually reduced after boiling (Fig. 4. A-Lanes 3–8) while those subunits disappeared after combining germination and boiling treatment (Fig. 4.B -Lanes 3–8).

3.5. Protein surface hydrophobicity

Fig. 5 shows protein surface hydrophobicity that is expressed as the amount of hydrophobic chromophore bromophenol blue (BPB) that is bound to the hydrophobic domains of the proteins. Fig. 5 shows a significant increase in the amount of bound BPB after boiling and germination. The amount of bound BPB detected for raw soybean (control sample) was approximately 50% lower compared to the value of its germinated counterpart and the non-germinated boiled for 30 min. the effect of cooking time, cellular integrity, and their interaction were all significant in germinated samples ($p < 0.01$), whereas only the effect of cooking time was significant in non-germinated samples ($p < 0.01$).

Table 1
DSC thermal characteristics for soybean particles (2000–3000 μm) and fine flour (32–45 μm) prepared from non-germinated and germinated soybeans.

Samples		β-Conglycinin (7S)				Glycinin (11S)			
		T onset (°C)	T end (°C)	T d (°C)	ΔH (J/g)	T onset (°C)	T end (°C)	T d (°C)	ΔH (J/g)
Non- germinated Soybean	Particles (2000–3000 μm)	79.96 ± 1.3 ^{a}	87.02 ± 0.7^{a}	82.75 ± 0.9^{a}	0.16 ± 0.1^{c}	100.84 ± 0.6a	109.17 ± 0.4^{a}	104.65 ± 0.45^{a}	1.24 ± 0.22^{a}
	Particles (45–32 μm)	72.36 ± 1.5 ^{bc}	79.95 ± 1.9^{b}	76.76 ± 1.8^{b}	0.24 ± 0.1^{b}	91.4 ± 2.8b	99.52 ± 1.42^{d}	95.62 ± 0.31^{d}	0.26 ± 0.12^{b}
Germinated Soybean	Particles (2000–3000 μm)	76.95 ± 0.8 ^{a}	83.18 ± 1.7^{a}	80.19 ± 1.1^{ab}	0.102 ± 0.06^{d}	95.4 ± 0.1a	104.4 ± 0.5^{ce}	99.90 ± 0.5^{be}	0.65 ± 0.05^{b}
	Particles (45–32 μm)	72.04 ± 0.5 ^{bc}	80.73 ± 3.2^{c}	76.96 ± 2.1^{bc}	0.36 ± 0.2^{a}	96.1 ± 2.2a	104.7 ± 0.68^{be}	98.95 ± 0.70^{ce}	0.66 ± 0.50^{b}

Data are expressed as mean ± standard deviation of three replicates and analyzed with ANOVA followed by Tukey's test). Column data with different letters are significantly different ($p < 0.05$).

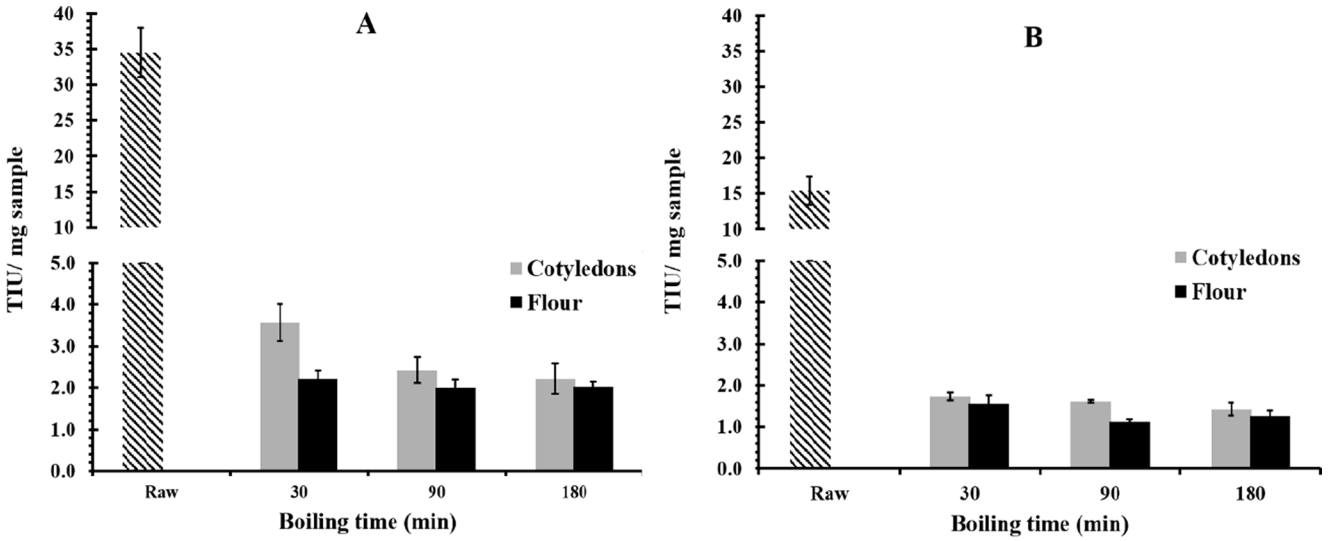

Fig. 3. Trypsin inhibitor units in in soybean particles (32–45 μm) prepared from boiled soybean for varying times (30, 90, or 180 min) as intact cotyledons (C) or flour (F). A = non-germinated soybean, B = germinated soybean. Raw samples of non-germinated and germinated soybean were used as control samples.

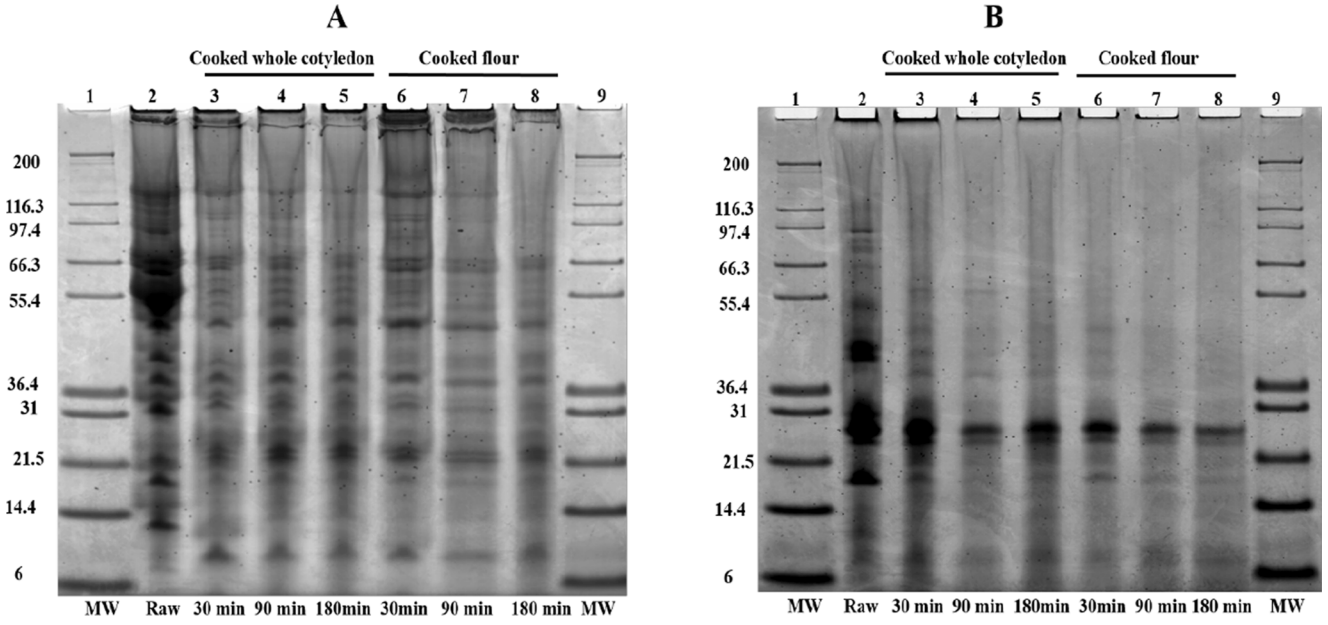

Fig. 4. Non-reducing SDS-PAGE images of protein profiles of in soybean particles (32–45 μm) prepared from boiled soybean for varying times (30, 90, or 180 min) as intact cotyledons (C) or flour (F). A = non-germinated soybean, B = germinated soybean. Raw samples of non-germinated and germinated soybean were used as control samples.

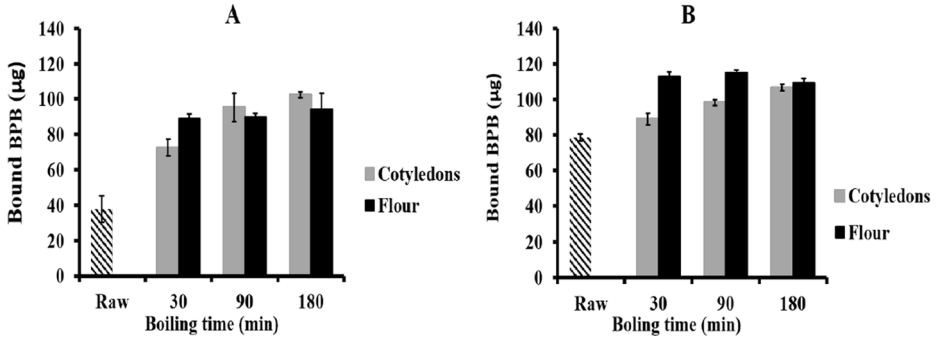

Fig. 5. Protein surface hydrophobicity of in soybean particles (32–45 μm) prepared from boiled soybean for varying times (30, 90, or 180 min) as intact cotyledons (C) or flour (F). A = non-germinated soybean, B = germinated soybean. Raw samples of non-germinated and germinated soybean were used as control samples.

Table 2
Relative content of different secondary structures in in soybean particles (32–45 μm) prepared from boiled soybean for varying times (30, 90, or 180 min) as intact cotyledons (C) or flour (F); A = non-germinated soybean, B = germinated soybean.

A- Non-germinated -boiled samples								
Amide I band components	Wavenumber (cm^{-1})	Raw	Boiling time					
			30 min		90 min		180 min	
			C	F	C	F	C	F
A1	1618	8.0	10.0	5.1	6.8	6.8	5.0	3.5
β-sheet	1610–1640 1670–1680	37.4	25.6	20.6	20.5	24.4	21.2	25.8
Random coil	1640–1650	7.1	13.5	18.4	15.1	18.5	20.2	17.1
α-helix	1650–1660	15.0	17.1	27.5	24.8	21.3	23.5	24.3
Turn	1660–1670 1680–1700	24.2	27.3	24.3	27.1	24.0	25.8	25.9
A2	1682	8.3	6.5	4.1	5.6	5.0	4.4	3.3
B- Germinated -boiled samples								
Amide I band components	Wavenumber (cm^{-1})	Raw	Boiling time					
			30 min		90 min		180 min	
			C F		C F		C F	
			C	F	C	F	C	F
A1	1618	4.8	6.3	2.2	6.8	5.0	4.7	4.1
β-sheet	1610–1640 1670–1680	25.0	25.7	24.4	20.5	26.6	20.5	27.6
Random coil	1640–1650	19.7	16.7	23.4	19.8	20.6	26.9	20.6
α-helix	1650–1660	20.1	21.2	25.0	26.1	20.7	25.0	17.6
Turn	1660–1670 1680–1700	24.8	25.0	20.2	22.4	23.9	19.1	23.3
A2	1682	5.6	5.0	4.9	4.3	3.2	3.8	6.7

3.6. Protein secondary structure changes

The curve-fitting of amide I bands in FTIR spectra (see Fig. S1) of cooked non-germinated and germinated soybean allowed to identify peaks within amide I (1700–1600 cm^{-1}). The relative contents in secondary structures are presented in Table 2. Compared with raw samples, random coil, α-helix and β-turn contents of boiling-treated samples increased while the content of β-sheets decreased. Increasing cooking time of both germinated and non-germinated cotyledon and the corresponding flours resulted in modest changes, suggesting very limited changes in protein secondary structure between intact cotyledons and flour cooked for different times (30, 90 or 180 min). No clear trend was observed when boiled samples of non-germinated and germinated soybean are compared. Comparing uncooked samples, germinated one showed the lowest content of β-sheet and the highest contents of the random coil, α-helix, and β-turn.

4. Discussion

In a recent study, we provided evidence that protein digestibility is higher in soybean particles that have been produced by milling soybean before boiling compared to particles produced after boiling (Zahir, Fogliano, & Capuano, 2018) This was explained by the higher fraction of broken cells produced when milling is applied before boiling compared to the fraction of broken cells produced when milling is applied after boiling. Another study suggests that conformational changes in protein secondary structure induced by thermal treatment may be different depending on whether proteins are heated within intact cells or outside the cellular environment and that proteins heated outside intact cells are better digested *in vitro* (Rovalino-Córdova et al., 2019). To get additional insight on the effect of cellular integrity on changes in protein properties and digestibility we have investigated protein physicochemical properties and *in vitro* digestibility of protein from non-germinated and germinated soybean boiled for different times as a whole cotyledon (i.e. intact cells) or flour (i.e. broken cells). Quantitative chemical analyses of protein of heated soybean (cotyledon and flour) showed that the physicochemical properties of soybean proteins were influenced by the presence of intact cotyledon cells during cooking (see Table 1, 2 Figs. 3 and 5). Furthermore, proteins boiled in intact cotyledons showed relatively lower digestibility compared to proteins that boiled in soybean flour, but these differences were less substantial in non-germinated samples (see Fig. 2). These results, therefore, prove that the structural integrity of the soybean tissue and the chemical environment surrounding proteins would produce differential conformational changes influencing soybean protein digestibility.

The other interesting observation was that cooking time had a limited effect on protein digestibility. The effect of thermal treatment on protein digestibility is far from trivial and strongly depends on the complex interplay between protein denaturation (which increases digestibility) and aggregation/cross-linking (which decreases digestibility). In legumes, thermal treatments are often reported to increase protein digestibility (Dallas et al., 2017; Torres, Rutherfurd, Munoz, Peters, & Montoya, 2016; Zahir et al., 2018) but the modulating effect of varying cooking time is not clear or neglected. Here we show that thermal treatment increases protein digestibility but only a limited improvement is achieved by prolonging the boiling from 30 min to 180 min in non-germinated samples. This is in line with Torres et al. (2016) who reported that extending autoclaving from 5 to 20 min does not improve the protein digestibility of legumes. Another study found no improvement in the *in vitro* protein digestibility when selected legumes were autoclaved at 121 °C for 10, 20, 40, 60, 90 min, and autoclaving for 10 min showed the highest protein digestibility (Rehman & Shah, 2005) Similarly, no linear relationship between heat intensity and protein digestibility was observed when beans were boiled for 25, 30, 35, 40 and 45 min (Abd El-Moniem, 1999).

To better understand the links between protein properties and protein digestibility, several protein properties have been investigated. The denaturation temperature of soybean proteins was lower when soybean cotyledon cells were previously disrupted (flour) before DSC analysis (Table 1) compared to soybean particles that have little or no physical damage to the cells. The lower protein denaturation temperature of soybean flour may depend on the ability of soybean flour to better absorb water. An early study reported that the denaturation temperatures of isolated soybean protein shifted to lower temperatures when the water/protein ratio was higher and indeed the water uptake can play a pivotal role in the denaturation rate (Kitabatake, Tahara, & Doi, 1990;

Li, Wei, Fang, Zhang, & Zhang, 2014). Limited water availability inside cotyledon cells has been reported to have protective effects on beans protein denaturation during thermal treatment (Garciavela & Stanley, 1989). On top of that, studies on macromolecular crowding in fundamental biology show that the thermodynamic properties of intracellular proteins are different from free proteins. This also results in protection towards denaturation (Eggers & Valentine, 2001). In the case of flour germinated soybean, there was no considerable difference in protein denaturation temperatures between big particles and fine particles compared to what was observed in non-germinated soybean. The increase in soybean cell wall porosity, the reduction in the tightly packed intracellular environment, and the change in protein macrostructure caused by the germination process (Zahir et al., 2020) may facilitate the ability of proteins to absorb water from the matrix and unfold. However, how much the lower sensitivity of proteins to denaturation when boiled in intact cells contributes to the observed lower digestibility under typical boiling conditions, is difficult to say. On one hand, the small differences in protein sensitivity to thermal denaturation may explain the differences in residual trypsin inhibitor activity which is one of the primary factors adversely affecting the soybean protein digestibility (Burova, Varfolomeeva, Grinberg, Haertle, & Tolstoguzov, 2002). The low residual trypsin inhibitory activity found in soybean either boiled as whole cotyledons or as flour (Fig. 3) is consistent with previous literature (Dipietro & Liener, 1989; Liu, 2019; Mostafa, Rahma, & Rady, 1987). In Fig. 3 however, besides the obvious reduction in TIU with cooking times, it is clear that the thermal inactivation of trypsin inhibitor activity was influenced by the structural integrity of boiled soybean with more trypsin inhibitors denatured when proteins are heated outside intact cells. Germination contributes to reducing trypsin inhibitor activity, which becomes especially relevant at shorter cooking times. Fig. 3 shows a substantial difference in trypsin inhibitor between non-germinated and germinated soybean in the raw state and at the short boiling time (30 min).

On the other hand, the lack of the typical DSC endothermic peak of protein denaturation for previously boiled soybean samples suggests that proteins were already completely denatured after 30 min of boiling (data not shown). These observations are in good agreement with the complete denaturation detected for commercial soybean isolate that underwent thermal processing before isolation (Arntfield & Murray, 1981; Tang, Choi, & Ma, 2007). A different level of denaturation is also unable to explain the surface hydrophobicity data. Protein denaturation and dissociation of the quaternary structure lead to protein unfolding which increases surface hydrophobicity (Wang, Li, Jiang, Qi, & Zhou, 2014). In the present study, protein surface hydrophobicity was drastically increased after germination/cooking but the effect of the structure was only significant in germinated samples (Fig. 5). Wang et al. (2014) reported that surface hydrophobicity of isolated soybean protein first increased with the heat treatment but the formation of protein aggregates from dissociated and denatured polypeptides may limit further increase in surface hydrophobicity or even produce a decrease in it. We observed a limited further increase in protein surface hydrophobicity upon long thermal processing times for both non-germinated and germinated samples, but no clear decrease. This suggests that the level of aggregation of soybean proteins has been limited which is also supported by the SDS-PAGE profiles in Fig. 4 where no formation of new aggregates is visible compared to the raw material. The intensity of the aggregates formed upon heating is less visible in germinated samples, a good indication that protein of germinated soybean was partially hydrolysed. It must be stressed however that denaturing conditions used in the electrophoretic analysis may have removed aggregates formed by hydrophobic interactions. The germination process instead was associated with an increase in protein surface hydrophobicity. This is not surprising as the amount of protein surface hydrophobicity was already reported to be proportional to the growth of hypocotyls length (Liu, Yin, & Li, 2014). The authors suggested that changes in protein conformation during germination due to the action of endogenous proteases may increases surface hydrophobicity.

Previous studies have shown the secondary structure of protein may affect its digestibility, for instance, a high amount of the β-sheet structure might partially limit the access of proteolytic enzymes (Carbonaro, Maselli, & Nucara, 2012; Rovalino-Córdova et al., 2019; Yu, 2005). Similarly, less organized secondary structures like random coils may increase the susceptibility of proteins to proteases (Carbonaro et al., 2012). In our work, we see changes in the relative contributions of each secondary structure induced by the heat treatment and germination. In our study, boiled non-germinated cotyledon and both cooked germinated cotyledons and flour were accompanied by an increase in contents of the random coil, α-helix, and β-turn and a decrease in β-sheet conformation as shown in Table 2. Other studies showed the effect of heat treatment was more intense in non-germinated samples (reduction of β-sheets, an increase of random coils) and less in germinated samples. Germination per se induced an increase in the random coils. Some studies reported that heat treatment induced an increase in the content of α- helix and β-turn structure, and a decrease in the content of β-sheet structures (Hu et al., 2013; Wang et al., 2014). However, Zhang and coworkers found that the secondary structure contents (α-helix, β-sheet, and random coils) of soybean protein isolated did not change significantly after heat treatment (Zhang, Chen, Qi, Sui, & Jiang, 2018).

To find insight into the protein physicochemical factors that most affect *in vitro* digestibility at the end of intestinal digestion, a correlation analysis was performed and it is shown in Table 1-S. As shown in Table 1-S, protein digestibility was inversely related to trypsin inhibitor ($r = -0.851 p = 0.001$), β-sheet ($r = -0.626$, $p = 0.017$) and β-turn (-0.442, $P = 0.113$). On the other hand, protein digestibility was linearly related to the surface hydrophobicity ($r = 0.876, p = 0.001$) random coil ($r = 0.782$ $p = 0.001$) and α-helix ($r = 0.530$, $p = 0.051$). This correlation analysis confirms that the denaturation of proteins, including trypsin inhibitors and the modification of the secondary structures, may all affect protein digestibility. However, none of these factors has a very strong correlation with DH%, and can fully explain DH %. The example of TIU is indicative when e.g. germinated raw and cooked non-germinated samples are compared.

5. Conclusions

Investigation of the role played by cellular integrity on proteins physicochemical changes during cooking is of particular interest since it might provide useful information on the improvement of soybean protein digestibility. The current *in vitro* study showed that protein conformational changes induced by heat and *in vitro* digestibility of isolated proteins depend on the physical state of the soybean materials (cotyledon or flour) during cooking, i.e. whether the proteins are heated in intact cells or not. Digestibility of isolated proteins is higher when proteins were cooked as flour than when they are cooked in a whole cotyledon but these differences are bigger in non-germinated compared to germinated samples. Furthermore, this study gave insights into the role of the germination process in inducing distinct changes in soybean protein physicochemical properties already in the raw material that translated into an increment in protein digestibility regardless of the cooking time. This study provides extra knowledge about the role of cell wall intactness in protein physiochemical changes and digestion suggesting germination or grinding soybean seeds before heating treatment could be employed to improve the digestibility of soybean proteins.

CRediT authorship contribution statement

Mostafa Zahir: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Writing - original draft. **Vincenzo Fogliano:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **Edoardo Capuano:** Conceptualization, Data

curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110254.

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