Modeling the Thermal Denaturation of the Protein–Water System in Pulses (Lentils, Beans, and Chickpeas)

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Abstract

Thermal treatment applied during the cooking of pulses leads to denaturation and even aggregation of the proteins, which may impact protein digestibility. Thermal transitions of lentil, chickpea and bean proteins were studied using DSC. Protein-enriched samples were obtained by dry air classification of dehulled seeds and were heated up to 160 °C, with water contents ranging from 0.2 to 4 kg/kg dry basis. The DSC peaks of the resulting endotherms were successfully modeled as overlapping Gaussian functions. The denaturation temperatures were modeled as a function of temperature according to the Flory-Huggins theory. The modeling allows to calculate the degree of protein transition for any temperature and moisture condition. The denaturation diagrams reflect the different protein compositions of lentil, chickpea and bean (particularly the 11S/7S globulin ratio). Chickpea proteins were more thermally stable than those from lentil and bean. Proteins underwent an irreversible transition, suggesting that unfolding and aggregation were coupled.

Keywords

Protein denaturation, DSC, Mathematical modeling, Pulses, Globulin, Peak desummation

1. Introduction

According to the FAO[1], pulses are legume crops that are harvested for their edible dry seeds but are not used for oil extraction. The International Year of Pulses (2016) highlighted their important nutritional benefits as part of sustainable food production targeting food security and nutrition. The seeds are rich in carbohydrates including starch, as well as in proteins and dietary fibers, and are a source of minerals and vitamins.[2] In particular, their high protein content (15-30 % dry weight)[3] makes pulses an excellent source of plant-based proteins for the formulation of food and ingredients. However, the impact of processing on protein bioavailability and digestibility is important to take into account. In particular, thermal treatment during cooking leads to protein denaturation and aggregation[4], the latter being one of the main adverse effects on protein digestibility.[5,6]

Inter and intra molecular bonds that stabilize the native structure of the protein are allowed to break down during heating, depending on the temperature applied.[4] This transition from a native state to a more disordered arrangement is called denaturation. Pulse proteins, which are oligomeric proteins with quaternary structure, undergo reversible dissociation into monomers.[7] Both the tertiary and secondary structure are affected with irreversible unfolding that generally remains partial.[4] Denaturation of pulse proteins occurs between 80 °C and 100 °C in excess water.[8–10] This spatial reorganization leads to exposure of the reactive groups, especially the hydrophobic amino acid residues embedded in the core of the native protein.[4] As a consequence, the formation of new bonds and interactions between partially unfolded peptide chains is favored. The protein aggregation that follows the denaturation process results in the formation of soluble or insoluble high molecular weight complexes. Protein aggregation reduces access to certain essential amino acids that are specific for protease action, and that are trapped inside the aggregate structures.[11] Heat-induced aggregation can thus impair protein digestibility of cooked pulses[6], to an even greater extent if aggregation is high.[5] Therefore, a better

62 understanding of the denaturation-aggregation status of proteins is needed, as a function of the
63 cooking conditions to enable more accurate monitoring of the nutritional aspect. It should be
64 noted that the presence of other factors that affect digestibility such as heat-sensitive anti-
65 nutritional factors (enzymes inhibitors) also requires monitoring.
66 Previous studies on the denaturation of legume proteins mainly focused on thermal stability at
67 constant water content. However, cooking in water implies that pulses undergo hydration. The
68 water content in the seed increases from the initial value to reach equilibrium moisture.[12] Phase
69 transitions of other components of the seed, *i.e.* gelatinization of starch, also result in varying
70 hydration rates, meaning the water content in the protein pool changes during cooking.
71 Denaturation-aggregation thus needs to be described as a function of the water content. A
72 similar approach to the one developed for the modeling of gelatinization-melting of starch at
73 increasing temperature and water content[13] should be suitable. In the present study, the phase
74 transitions of proteins were monitored using differential scanning calorimetry (DSC), a reliable
75 and convenient thermo-analytical method to study the endothermic and exothermic changes
76 associated with the denaturation and aggregation of proteins.[14,15] To avoid the structural
77 modifications usually caused by chemical extraction reported in most studies, we chose to work
78 on protein-enriched samples obtained using a physical separation method only, *i.e.* air
79 classification. The study was focused on beans, lentils and chickpeas, which are widely
80 produced and the most consumed pulses in France.[16]
81

82 ## 2. Materials and methods

83 ### *2.1 Raw material*

84 Green lentils (*L. culinaris,* var. Anicia), chickpeas (*C. arietinum*, var. Elvar) and navy beans
85 (*P. vulgaris*, var. Linex) were provided by Cibèle (Saint-Georges-Sur-Arnon, France), Moulin

Marion (Saint-Jean-sur-Veyle, France) and Cavac (La Roche-sur-Yon, France), respectively. The lentils were harvested in 2017 and the chickpeas and beans in 2018. All the seeds were stored in a vacuum pack at 7 °C until use.

2.2 Sample preparation

Protein-enriched samples were prepared by dry fractionation and air classification as described in Lefèvre *et al.*[13] Briefly, lentil seeds were dehulled by dry abrasion and residues of the hull were removed by sieving through a 2-mm mesh screen. Chickpea and bean seeds were crushed using a cutting mill equipped with a 8-mm sieve and residues of the hull were removed with a vibrating sieve. For all pulses, the removed hulls represented 10-12% of the raw weight, and the process induced 6-8% of losses. The dehulled and crushed pulses were ground into flour in a high speed impact mill equipped with a pin mill. The resulting flours were separated into coarse and fine fractions using an ATP air classifier (Hosokawa Alpine, Augsburg, Germany) with the following optimized speeds: 6 500 rpm for lentils, 10 000 rpm for chickpeas and 8 000 rpm for beans. The resulting fine fractions were stored at 4 °C until use and are hereafter referred to as protein-enriched samples.

2.3 Sample characterization

Water content of the protein-enriched samples was calculated on a wet basis by drying 5 g of each sample for 2 h at 132 °C (± 2 °C) using the NF EN ISO 712 standard method (2010). Starch content was measured using the enzymatic procedure of Holm *et al.*[17] Protein content was measured using the Kjeldhal method according to NF EN ISO 20483, with 5.4 as nitrogen conversion factor.[18] Lipid content was measured using the gravimetric ether extraction method according to AOAC 2003.05.[19] Soluble and insoluble fibers were measured using a TDF Analyzer (Ankom, Macedon, USA) according to AOAC 2011.25 and AACC 32-50.01.[20] Phytates were extracted according to a published method[21] with some modifications. A 100 mg-

sample was added to a Pyrex vial with 5 mL of 0.5 M HCl. The capped vial was heated under stirring for 6 min in boiling water and then centrifuged for 20 min at 4 500 *g*. The supernatant was recovered and filtered through a 0.2 µm syringe filter (Acrodisc) and 0.5 M NaOH was added for neutralization and the solution was then diluted (1:10). Phytic acid content was measured by high performance ion chromatography (HPIC) using an ICS-2500 Dionex chromatograph (Sunnyvale, CA, USA). Fiber and phytate measurements were made in triplicate and all other measurements were made in duplicate.

2.4 Differential scanning calorimetry

Thermal denaturation of proteins was studied by DSC as described in Lefèvre *et al.*[13] Briefly, a Perkin DSC 8500 (Perkin Elmer, Norwalk, USA) was calibrated using indium as standard. Protein-enriched samples and deionized water were weighed in stainless steel pans to obtain a water content X ranging from 0.2 to 4 kg kg^{-1} dry basis (db). This range covers the progressive hydration of proteins while the pulses are cooking. The pans were heated from 20 °C to 160 °C at a rate of 10 °C/min, using an empty sealed pan as reference. All measurements were duplicated. A blank thermogram (empty pans in both reference and sample ovens) was recorded daily. The heat flow (mW) of the sample pans minus the variation in the heat flow of the blank during heating was recorded using Pyris Thermal Analysis Software (Perkin Elmer, Norwalk, USA). Table 1 lists the experimental conditions applied for the different water contents. In the study conditions (*i.e.* at $T \leq 160$ °C), the lowest water content to be able to detect a complete DSC signal was 0.2, 0.3 and 0.4 kg kg^{-1} db, for beans, lentils and chickpeas, respectively. When the heat flows were similar for close water contents, fewer intermediate water contents were used. In addition, double scans were performed with an intermediate water content of 0.8 kg kg^{-1} db. Samples were heated from 20 °C to 80 °C, 110 °C or 160 °C at a rate of 10 °C/min, then cooled to 20 °C and heated a second time from 20 °C to 160 °C at a rate of 10 °C/min.

[Insert Table 1 here]

2.5 Modeling the protein denaturation diagram

DSC thermograms record heat flow (φ) as a function of temperature (T) at each water content. Phase transitions of the sample components result in peaks in the endotherm. The DSC thermograms obtained from the protein-enriched samples are discussed as if the peaks were only due to the phase transition of proteins. This hypothesis will be confirmed in the results part.

2.5.1 Modeling the DSC peaks

Thermal denaturation of the protein-enriched samples resulted in single or double peaks in the DSC signal depending on the variety of the pulse concerned. The shape of all the endotherms depended on water content. Therefore, heat flows were analyzed according to the desummation procedure described in Lefèvre *et al.*[13] on pulse starches. First, baseline subtraction and non-dimensionalization were performed on heat flow values in all pulses. In the lentil and chickpea samples, the heat flow was composed of two roughly overlapping endotherms depending on the water content, termed P1 and P2. The dimensionless heat flows ($\overline{\varphi}$) were fitted to a sum of two Gaussian functions (i = P1 or P2) as follows:

$$\overline{\varphi} = \sum_{i=\mathrm{P1,P2}} \frac{\beta_i}{\Delta T_i \sqrt{2\pi}} \exp\left(-\frac{1}{2}\left(\frac{T - T_i}{\Delta T_i} \right)^2 \right) \quad (1)$$

where T_i (°C) is the temperature at maximum peak i; ΔT_i (°C) controls the width of the peak i and is related to the full width at half maximum (FWHM) of the peak i according to $2\sqrt{2\ln(2)} \times \Delta T_i = \mathrm{FWHM}$; β_i is the dimensionless area of peak i. Therefore:

$$\int_{-\infty}^{\infty} \overline{\varphi} = \beta_{\mathrm{P1}} + \beta_{\mathrm{P2}} = 1 \quad (2)$$

In bean, the heat flow had a single endotherm (i = P1) irrespective of water content, so $\beta_{P1} = 1$. Eq. (1) was similar to the equation used previously for modeling starch conversion heat flow[13] which was composed of overlapping gelatinization and melting peaks. The parameters from Eq. (1) depend on the water content X in the samples.

2.5.2 Peak temperature

The temperature at maximum peak T_i (°C) is the denaturation temperature of the proteins responsible for transition i (i = P1 or P2). The Flory–Huggins equation[22] was used to describe the relation between T_i and the volume fraction of the water (ϕ) in the protein-water mixture:

$$\frac{1}{T_i} - \frac{1}{T_{i,0}} = \frac{R}{\Delta h_{i,0}} \frac{v_a}{v_w} \left(\phi - \chi_i \, \phi^2 \right) \tag{3}$$

where $\Delta h_{i,0}$ (J mol^{-1}) is the change in the molar enthalpy of denaturation per repeating unit (amino acid); v_a / v_w is the ratio of the molar volume of the repeating unit ($v_a = 74.5 \times 10^{-6}\,\mathrm{m^3 mol^{-1}}$)[23] to the molar volume of the water ($v_w = 18.1 \times 10^{-6}\,\mathrm{m^3 mol^{-1}}$) and, therefore, $v_a / v_w = 4.1$; R is the gas constant (8.31 J mol^{-1} K^{-1}); $T_{i,0}$ (K) is the denaturation temperature of the pure polymer and χ_i is the Flory interaction parameter. To calculate ϕ, the density of water was taken to be 1 000 kg m^{-3} and the density of protein was attributed an average value of 1 350 kg m^{-3}.[24] Therefore, ϕ (m^3 m^{-3}) was expressed as a function of water content X:

$$\frac{1}{\phi} = 1 + \frac{x_p^{db}}{1.35 X} \tag{4}$$

where x_p^{db} (kg kg^{-1} db) is the mass fraction of protein in the protein-enriched samples.

2.5.3 Peak width

176 The width-related parameter ΔT_i represents the cooperativity of protein unfolding process and
177 the presence of multiple protein domains with heterogeneous thermal stabilities.[4,25] ΔT_i was
178 likely to decrease as a function of water content. The following empirical relation was used to
179 describe ΔT_i as a function of X:

180 $$\Delta T_i = \Delta T_{i,\infty} + \left(\Delta T_{i,0} - \Delta T_{i,\infty}\right)\exp\left(-\frac{X}{\gamma_i}\right) \quad (5)$$

181 where $\Delta T_{i,0}$ (°C) and $\Delta T_{i,\infty}$ (°C) are the values for ΔT_i, with zero and excess water contents,
182 respectively; γ_i is the rate parameter of decrease for the two correlations.

183 *2.5.4 Amplitude of the peak area*

184 The effect of water content on the relative peak areas β_i was also investigated. With increasing
185 water content, β_{P1} was likely to decrease. The following empirical equation was used to
186 describe this relation for lentil and chickpea:

187 $$\beta_{P1} = \beta_{P1,\infty} + \left(\beta_{P1,0} - \beta_{P1,\infty}\right)\exp\left(-X\right) \quad (6a)$$

188 where $\beta_{P1,0}$ and $\beta_{P1,\infty}$ are the dimensionless area of the first peak, with zero and excess water
189 contents, respectively. β_{P2} was calculated by combining Eq. (2) and Eq. (6a) as follows:

190 $$\beta_{P2} = 1 - \left[\beta_{P1,\infty} + \left(\beta_{P1,0} - \beta_{P1,\infty}\right)\exp\left(-X\right)\right] \quad (6b)$$

191 *2.5.5 Degree of protein denaturation*

192 The degree of protein denaturation τ was defined for any temperature T and water content X
193 as the ratio between the change in enthalpy calculated from the beginning of the first peak to T,
194 and the change in the whole enthalpy from the beginning of the first peak to the end of the
195 second peak (Eq. (7a)). Denaturation refers to the global transition of proteins, which includes
196 unfolding and potential aggregation.

197 $$\tau = \int_0^T \overline{\varphi}(T,X)\,dT \Big/ \int_0^\infty \overline{\varphi}(T,X)\,dT \qquad (7a)$$

198 Using the error function to calculate integral Gaussian functions, Eq. (7a) becomes Eq. (7b) for
199 lentil and chickpea (with i = P1 and P2) and for bean (with only i = P1).

200 $$\tau = \sum_{i=\mathrm{P1,P2}} \beta_i \times \frac{1}{2}\left(1 + \mathrm{erf}\left(\frac{T - T_i}{\Delta T_i \sqrt{2}}\right)\right) \qquad (7b)$$

201 Finally, isovalue lines of the degree of protein denaturation τ were represented as a
202 temperature T versus water content X diagram.

203 *2.5.6 Identification of parameters*

204 The modeling of protein denaturation diagram involves 9 parameters for lentil ($T_{i,0}$, $\Delta h_{i,0}$, χ_i,
205 ΔT_i, β_{P1}), 13 parameters for chickpea ($T_{i,0}$, $\Delta h_{i,0}$, χ_i, $\Delta T_{i,0}$, $\Delta T_{i,\infty}$, γ_{P2}, $\beta_{\mathrm{P1},0}$ and $\beta_{\mathrm{P1},\infty}$) and
206 4 parameters for bean ($T_{\mathrm{P1},0}$, $\Delta h_{\mathrm{P1},0}$, χ_{P1}, ΔT_{P1}), with i = P1 or P2. The overall procedure[13] was
207 used to identify all the parameters. At the same fitting session, all dimensionless heat flow
208 thermograms were fitted to the model combining Eq. (3), (5) and (6a) in Eq. (1). The curve
209 fitting toolbox (Matlab software, version R2019b, The MathWorks Inc., Natick, USA) with the
210 Levenberg-Marquardt algorithm was used as a solver.

211 *2.5.7 Statistical methods*

212 All parameter values presented in this study are given with a 95% confidence interval. The root
213 mean square error (RMSE) was calculated between experimental and predicted dimensionless
214 heat flows for all the experimental conditions.

215

216 ## 3. Results and Discussion

217 *3.1 Characterization of the samples*

[Insert Table 2 here]

The chemical composition of the protein-enriched fractions obtained after dry fractionation and air classification are listed in Table 2. Proteins were the main component in the fine fractions, with x_p^{db} ranging from 38.2% of db weight for chickpea to 55.8% db for lentil. These values are in good agreement with protein concentration range of 40-60% db reported in literature for air-classification on pulse flours.[26–28] Protein contents of the dehulled seeds were 24.1% db, 19.3% db and 23.2% db in lentil, chickpea and bean, respectively. The dry air-classification process can thus be considered as an efficient physical method to produce protein-enriched samples while avoiding the structural modifications that may occur with chemical extraction. Tyler *et al.*[27] already reported good protein separation efficiency using air-classification, particularly for green lentil and navy bean. Insoluble dietary fibers (IDF) were the second component in the protein-enriched samples, ranging from 11.1% db for lentil to 18.5% db for chickpea. Other components listed in Table 2 represented all together 25-37% db of the samples and between 2 and 11% db each. The interactions between proteins and those non-proteins components may affect the thermal stability of proteins[29] and the water repartition due to the competition for available water[30]. These interactions may impact the denaturation temperature of proteins and were intrinsic to the protein-enriched samples studied here. There have not been investigated further.

3.2 DSC characteristics of the protein-enriched fractions

[Insert Fig. 1 here]

[Insert Fig. 2 here]

[Insert Table 3 here]

For each pulse, Fig. 1 presents a selection of three DSC thermograms at $X = 0.4$, 1.0 and 2.0 kg kg^{-1} db. All transition peaks were observed above 60 °C, so the temperature range of interest was between 60 and 160 °C. According to the protein enrichment of the fine fractions

after air-classification in comparison with other components (Table 2), we confirmed the hypothesis that the DSC thermograms were mainly due to protein denaturation. Indeed, the second main component of the samples, IDF, underwent no thermal transition below 200 °C.[31] Denaturation of phytates and alpha-galactosides also occurred at temperatures exceeding the conditions of the study, whereas the melting point of lipids was lower than the temperature range of interest. Thus, only the gelatinization of residual starch could lead to additional DSC peaks in the temperature range of interest. However, the starch content was less than 6% db in all samples, resulting in a low contribution to the total enthalpy of the observed transition. The DSC peak were thus mainly attributed to the phase transitions of proteins.

The experiments revealed that the thermal behavior of proteins depended on both the water content and the type of pulse (Fig. 1). The DSC thermograms for lentils showed two roughly overlapping peaks P1 and P2, depending on the moisture content of the sample. With lower water content (i.e. $X \leq 2$ kg kg^{-1} db), a biphasic endotherm was observed. With excess water, a single broad peak was observed, suggesting that both peaks are merged as modeled by the Gaussian functions (Fig. 1c). The endotherm for chickpea had two distinct peaks, P1 and P2, regardless of the water content. The shape and size of the peaks changed with the moisture content. The endotherm for bean had a single peak, P1, of constant size and shape, regardless of the water content. All DSC thermograms were successfully modeled as shown by the low RMSE between experimental and predicted heat flows (Fig. 1). The parameters presented in Table 3 had low confidence intervals, which allows accurate modeling of experimental data. Modeling is particularly useful to understand the superposition of P1 and P2 endotherms.

The multiple or overlapping peaks observed by DSC showed that the thermal stability of the proteins varied. This is linked to the different protein fractions in the seed. The main proteins in pulses are globulins and albumins in varying proportions depending on the species and variety of the pulse concerned.[3,32] Almost all pulses contain two main types of globulins with

sedimentation coefficients around 7S and 11S called vicilin and legumin, respectively.[33,34] The temperature, shape and size of the denaturation endotherms presented in Fig. 1 were thus determined mainly by the thermal denaturation of 7S and 11S globulins. Vicilin has a trimeric structure stabilized by hydrophobic interactions, electrostatic and hydrogen bonds. Legumin has a quaternary structure made of three dimers held together by non-covalent bonds. In each dimer, acidic and basic subunits are associated with disulfide bridges.[7] The covalent bonds give legumin higher thermal stability than vicilin, because more energy is needed to break the bonds.[35] In excess water, 7S and 11S globulins unfold at 80-85 °C and 90-100 °C, respectively.[10,36] In lentil, endotherms P1 and P2 were attributed to the denaturation of 7S and 11S globulin, respectively, with peak temperatures at 83 °C and 91 °C (with $X = 3$ kg kg^{-1} db). $\beta_{P1,0}$ and $\beta_{P1,\infty}$ did not differ significantly in lentils, β_{P1} thus remained constant and equal to 0.65 irrespective of the water content (Table 3). This value suggests that the P1 transition was slightly predominant than to P2 transition. In chickpea, endotherms P1 and P2 were again attributed to the successive denaturation of 7S and 11S globulin, respectively, with peak temperatures at 83 °C and 103 °C (with $X = 3$ kg kg^{-1} db). β_{P1} was lower than 0.5, except at the lowest water contents ($X \leq 0.5$ kg kg^{-1} db). This trend suggests that the legumin transition was predominant in chickpea. This result is in good agreement with the high 11S/7S ratio commonly reported for several chickpea species.[37–39] The decreasing function for β_{P1} (Fig. 2e) suggests that part of the 7S globulin was solubilized without denaturation with increasing water content. This hypothesis is consistent with the decrease in total enthalpy of phase transition observed before non-dimensionalization (from 21 to 10 J g^{-1} protein when X increased from 0.4 to 1 kg kg^{-1} db). In some cases, denaturation peaks of vicilin and legumin in chickpea have been reported to overlap.[10,37] In bean, the single peak P1 at 92 °C (with $X = 3$ kg kg^{-1} db) was attributed to the predominant vicilin fraction, as already suggested in several bean varieties.[9,14,40] The diversity of DSC signals between lentil, chickpea, and bean clearly reflects

the variations in the 11S/7S ratio between pulses, as already reported.[26,41] In this sense, DSC analysis can be used to rapidly determine globulin ratios when determination using chromatography and electrophoresis on protein isolates is not possible.

3.3 Modeling of peak width and temperature

No significant difference was observed between $\Delta T_{i,0}$ (°C) and $\Delta T_{i,\infty}$ in lentil and bean, the peak widths ΔT_i were thus constant and equal to $\Delta T_{i,\infty}$ (Table 3). This low value (3-4 °C) reflected highly cooperative unfolding for vicilin and legumin, irrespective of the water content.[25] In chickpea, ΔT_i decreased with a reduction in water content, as shown by the decreasing exponential law (Fig. 2 and Table 3). The progressive sharpening of both P1 and P2 peaks showed that increasing moisture led to a more cooperative denaturation of chickpea vicilin and legumin.[4] The heterogeneous composition of the protein-enriched samples may also play a role in the DSC characteristics. The lower protein content in chickpea than in lentil and bean (Table 2) may lead to a broader and more water-sensitive endotherm in the DSC thermogram due to the lower protein purity and hence, possible interaction with residual components, in particular with the higher IDF content.[29]

The denaturation temperatures T_i of lentil, bean and chickpea proteins decreased with an increase in water volume fraction (Fig. 2). Temperatures were modeled using the Flory-Huggins theory for a polymer in solution (Eq. 3). The coefficient of determination (R^2) was 0.98-0.99 for T_{P1} and 0.96-0.99 for T_{P2}. The fitting strength shows that this model fits both 7S and 11S protein denaturation in all pulses studied. Concerning the P1 transition, the denaturation temperatures of pure polymer were similar for lentil and bean, with a $T_{P1,0}$ value of 165.2 °C and 166.2 °C, respectively. $T_{P1,0}$ was 30 °C higher for chickpea. Again for the P2 transition, $T_{P2,0}$ was 20 °C higher for chickpea legumin compared to lentil legumin (Table 3).

316 The denaturation temperatures of pure protein varied more between pulses than the denaturation
317 temperatures of gelatinization and melting of pure starch, obtained with the same Flory-
318 Huggins model.[13] The change in molar enthalpy of denaturation $\Delta h_{i,0}$ ranged from 41.2 kJ mol^{-1}
319 to 65.8 kJ mol^{-1} for vicilin and from 41.9 to 45.1 kJ mol^{-1} for legumin (Table 3). These
320 enthalpies were higher than the change in molar enthalpy of gelatinization and melting of
321 starch.[13] The Flory interaction parameter χ_i was low (0-0.14) compared to the range 0.48-0.51
322 previously reported for cereal, potato or pulse starches.[13,42–44] These values suggests that the
323 interactions between water and proteins were low.[44] To conclude, all the values determined for
324 Flory-Huggins parameters ($T_{i,0}$, $\Delta h_{i,0}$, χ_i) differ from those previously obtained for starch
325 gelatinization and melting modeling. The Flory-Huggins theory is thus an accurate and useful
326 tool for predicting the impact of moisture on the denaturation temperatures of pulse proteins.

327 *3.4 Relevance of the predicted denaturation temperatures*

328 Denaturation temperatures T_i modeled using the Flory-Huggins parameters described above
329 were consistent with denaturation temperatures already reported for lentil, chickpea and bean
330 proteins. For lentil in excess water (X = 10 kg kg^{-1} db), two overlapping peaks were found at
331 77.9 °C and 83.7 °C, respectively. At the same water content and also in green lentil proteins,
332 Barbana and Boye[36] observed a similar result with a broad peak at 79.6 °C. The denaturation
333 temperatures of green lentil proteins were lower than those of red lentil proteins, which have
334 been reported to range from 87 °C in excess water to 123 °C with limited water.[36,45] For
335 chickpea, T_{P1} and T_{P2} were predicted to be 83.3-84.9 °C and 101.9-103.6 °C, respectively, when
336 X ranged between 3 and 4 kg kg^{-1} db. These values are in good agreement with the denaturation
337 temperatures of chickpea proteins reported in previous studies for the same range of water
338 content.[46–48] These authors reported broad peaks ranging from 85 °C to 100 °C, depending on
339 the varieties and on the extraction methods used. Our results differ slightly from the two peaks

observed by Chang *et al.*[37] at 79.3 °C and 96.3 °C. At higher water content (X = 9 kg kg^{-1} db), Withana-Gamage *et al.*[10] reported a vicilin transition at 80-85 °C depending on the variety of chickpea, which is similar to our T_{P1} at 80.5 °C. The same authors reported that the legumin transition occurred at 89.2-93.2 °C, which is lower than our T_{P2} predicted at 99.0 °C. For bean in excess water (X = 10 kg kg^{-1} db), we found a single peak at 87.1 °C. This temperature was slightly lower than the peak temperature of 90.6 °C reported by Rui *et al.*[9] in white bean, the same variety as the navy bean we used in the present study. Yin *et al.*[40] and Law *et al.*[49] also reported higher peak temperatures for red bean and rice bean, respectively (92.5 °C and 91.9 °C, respectively). Meng and Ma[14] observed a major peak at 86.4 °C close to our result, and a shoulder at 92.2 °C for red bean globulin, at the same water content. Their measurements on fractionated fractions showed that the DSC signal could be split into a biphasic endotherm for 7S vicilin (peaks at 88 °C and 94 °C) due to non-glycosylated and glycosylated subunits, and a minor endotherm for 11S legumin (peak at 89.5 °C), overlapping the first one. In the present study, it is possible to hypothesize that a peak at a higher temperature than T_{P1} but with a lower amplitude may be hidden under P1 endotherm, corresponding with the denaturation of residual 11S legumin.

The denaturation temperature of proteins can differ considerably among pulse species, and also between varieties of the same species. In this study, the denaturation temperature of green lentil and navy bean proteins was lower than the denaturation temperatures reported for red lentil and for red and rice beans, respectively. A difference of up to 10 °C has been reported among the denaturation temperatures of proteins in different bean varieties.[9,49] This variability is linked to the different proportions of storage proteins.[32] It is important to underline that the DSC characteristics are also affected by environmental and processing conditions, and protein extraction methods.[15,36,45] Studying pure vicilin and legumin fractions or a globulin mixture may also lead to slight variations in the denaturation temperatures.[14,15,37] The purity degree of

the protein sample is important because the thermal stability of proteins may be affected by interactions between protein and non-protein components.[29] All these factors can explain the variations in denaturation temperatures, number and shape of DSC peak between studies on a similar pulse cultivar.

3.5 Thermal aggregation of proteins

[Insert Fig. 3 here]

Double scans were performed in order to determine if the observed transitions were reversible, *i.e.* still observable on the DSC signal after a preheating treatment. The first heating treatments are hereafter referred to as preheating. The DSC signals of the second heating treatments are presented in Fig. 3 and showed that a peak disappears when the temperature of the preheating exceeds the temperature of the transition considered. For all pulses, a preheat treatment to 80 °C did not damage the protein, since the DSC thermograms were similar to the control. This is consistent with the higher denaturation temperatures described above. When lentil and chickpea samples were preheated to 110 °C (Fig. 3a and b), only one transition was observed at a temperature corresponding to the P2 transition of the untreated sample (115-120 °C). This means that the vicilin transition P1 was no longer detected, while the legumin peak P2 was still observed. In addition, the legumin peak was no longer detected when the sample was preheated to 160 °C. These results confirm that 11S globulin is more thermally stable than 7S globulin. Also in bean, vicilin was irreversibly damaged at 110 °C (Fig. 3c). In conclusion, both types of protein were irreversibly damaged when the thermal treatment exceeded their denaturation temperature. Ricci *et al.*[29] also observed irreversible denaturation process using DSC. The authors reported additional reversible glass transition for lentil, bean and chickpea ($X = 0.15$ kg kg^{-1}) ranging from 150 to 170 °C. The differences in water content, heating temperatures, protein purity and pulse variety may explain why a similar glass transition was not observed on the DSC thermograms in Fig. 3.

The irreversibility of the transition suggests that thermal denaturation involves both unfolding of the structure and aggregation between protein chains.[10,11] Partially denatured proteins may form a soluble and insoluble complex with new interactions. German *et al.*[8] showed that the dissociation of 11S purified soy proteins during heating was followed by aggregation of the released basic subunits into insoluble complexes. In the presence of 7S proteins, basic 11S subunits and 7S subunits can form soluble complexes. Denaturation and aggregation seemed to be coupled in the present study, or at least not separable using DSC analysis. Sirtori *et al.*[35] also reported irreversible denaturation of pea globulins. These authors reported that the vicilin peak disappeared from the DSC thermogram when samples were pre-heated to 200 °C, or heated at 100 °C for a long time (120-300 min). The 11S peak was visible in all conditions, even after harsh treatment at 200 °C, but its detergent solubility decreased.

3.6 Modeling the denaturation diagram of proteins

[Insert here] Fig. 4.

The results obtained with desummation modeling allowed us to calculate the degree of protein denaturation (including unfolding and aggregation) for any temperature T and water content X. Some isovalue lines of degree of protein denaturation are presented as a T versus X diagram in Fig. 4. The differences we observed in the denaturation temperatures between the three pulses, related to their respective protein composition, are still visible in the diagram. The higher 11S/7S ratio for chickpea led to isovalue lines of denaturation degree at higher temperatures than for bean and lentil proteins. For example, to achieve 99% protein denaturation at $X = 4$ kg kg^{-1} db, lentil and bean should be heated to 96-98 °C while chickpea should be heated to 111 °C. The fact that the thermal behavior depends on the species and variety of pulse means that extrapolating this diagram to other legumes will be tricky, in contrast to extrapolating the starch gelatinization diagram.[13] The specific denaturation characteristics of each pulse need to be known to accurately predict the denaturation state of the proteins.

In addition, some studies[35,50] demonstrated that the length of the thermal treatment and the moisture conditions have an effect on the degree of denaturation of pulse proteins. This result suggests that protein denaturation-aggregation should be considered as a kinetic process. From this point of view, our diagram represents a short heating time (8 minutes heating at between 80 °C and 160 °C). With longer heating times, the final diagram would probably be different.

To conclude, the DSC experiments on protein-enriched fractions of pulses and the desummation method of the thermograms were suitable for modeling the thermal denaturation of pulse proteins. The temperature, shape and size of the DSC peaks were consistent with the protein composition of each pulse, in particular the 11S/7S globulin ratio. The single transition observed for bean was characteristic of 7S vicilin denaturation, which is the main globulin in this pulse. However, an endotherm with two transitions was observed for chickpea and lentil. Its thermal characteristics are in good agreement with the denaturation of 7S vicilin and then 11S legumin at higher temperatures. Irreversible denaturation of both protein types was observed, suggesting that unfolding and aggregation were coupled under the heating conditions used in the study. The three obtained diagrams of protein denaturation enable monitoring of the phase status of lentil, chickpea and bean proteins, in different T and X conditions. However, the diagrams cannot be used to generalize denaturation temperature ranges to all cultivars and has to be studied with a case-by-case approach as composition may vary.

Since the unfolding and aggregation caused by processing, such as heating, modifies the functionality and nutritional quality of pulse proteins, denaturation diagrams are key parameters to improve the cooking process of pulses. The next step in this work will be to combine the phase transition models for starch and for proteins to provide a model of physicochemical changes at the seed scale. However, the present results should be interpreted with caution, as the proteins may behave differently when purified or mixed with other components of the seeds.

Abbreviations used

db	Dry basis
DSC	Differential scanning calorimetry
FWHM	Full width at half maximum (°C)
P1	First peak of the DSC thermogram
P2	Second peak of the DSC thermogram
R	Gas constant (J mol^{-1} K^{-1})
RMSE	Root mean square error
T	Temperature (°C)
T_i	Temperature at maximum peak i (°C)
X	Water content (kg kg^{-1} dry basis)
x_p^{db}	Mass fraction of protein (kg kg^{-1} dry basis)

Greek symbols

β_i	Dimensionless area of peak i
$\beta_{\mathrm{P1},0}$	Dimensionless area of first peak (P1) when water content is zero
$\beta_{\mathrm{P1},\infty}$	Dimensionless area of first peak (P1) in excess water
$\Delta h_{i,0}$	Change in molar enthalpy of transition i per repeating unit (J mol^{-1})
ΔT_i	Parameter related to the width of peak i (°C)
$\Delta T_{i,0}$	Parameter related to the width of peak i when water content is zero (°C)
$\Delta T_{i,\infty}$	Parameter related to the width of peak i in excess water (°C)
v_a	Molar volume of repeating unit amino acid (m^3 mol^{-1})
v_w	Molar volume of water (m^3 mol^{-1})
τ	Degree of protein denaturation
φ	Heat flow (W)
$\overline{\varphi}$	Normalized dimensionless heat flow
ϕ	Volume fraction of water in protein-water mixture (m^3 m^{-3})
χ_i	Flory interaction parameter for i transition

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Figure captions

Fig. 1. DSC thermograms of lentil, chickpea and bean protein-enriched fractions for water contents $X = 0.4$ kg kg^{-1} db (a), $X = 1.0$ kg kg^{-1} db (b) and $X = 2.0$ kg kg^{-1} db (c). The experimental dimensionless heat flows (dots) were modeled using the overall procedure described in Lefèvre *et al.*[13] The predicted dimensionless heat flows and P1 and P2 endotherms are represented by the solid, dashed, and dotted lines, respectively. RMSE were calculated between experimental and predicted heat flows for the different water contents.

Fig. 2. Parameters of the Gaussian functions T_i (a,b), ΔT_i (c,d) and β_{P1} (e) represented as a function of water volume fraction (ϕ) or water content (X) in the water-protein mixture for the lentil, chickpea and bean protein-enriched fractions. Dots represent the primary parameters of the Gaussian function obtained using the desummation method by fitting the experimental dimensionless heat flows to Eq. (1) for each water content. The solid lines represent the modeling of the Gaussian parameters using the overall identification procedure for every water contents in the same fitting session.

Fig. 3. DSC thermograms of protein-enriched fractions of lentils (a), chickpeas (b) and beans (c) using the double scan procedure, for $X = 0.8$ kg kg^{-1} db. Samples were pre-heated from 20 °C to 80 °C, 110 °C or 160 °C followed by standard heating from 20 °C to 160 °C. A control sample (only standard heating from 20 °C to 160 °C) is presented for the sake of comparison.

Fig. 4. Modeled denaturation diagram of lentil, chickpea and bean proteins obtained with the desummation procedure. Isovalue lines showing the degree of protein denaturation (unfolding and aggregation coupled) are presented in a temperature T versus water content X diagram.

Tables

Table 1. Experimental conditions of the database according to the type of pulse: water content (X) of protein samples analyzed with DSC from 20 °C to 160 °C.

X (kg kg^{-1} db)	Lentil	Chickpea	Bean
0.2	nd	nd	×
0.3	×	nd	–
0.4	×	×	×
0.6	×	×	×
0.8	×	×	×
1.0	×	×	×
1.2	–	×	×
1.4	–	×	×
1.6	–	×	×
1.8	–	×	×
2.0	×	×	×
2.2	–	–	–
2.4	–	–	–
2.5	–	×	×
2.6	–	–	–
2.8	–	–	–
3.0	×	×	×
3.5	×	–	×
4.0	×	–	–
Total measurements	18	22	26

×: measured in duplicate.

nd: incomplete signal detected by DSC at $T \leq 160$ °C;

–: not tested.

Table 2. Chemical composition of protein-enriched fractions as a fine fraction obtained after dry fractionation and air classification (g/100g dry weight basis)

Pulse	Moisture	Starch	Proteins	IDF	SDF	Lipids	Phytates	Alpha-galactosides
Lentil	7.9 ± 0.1	3.0 ± 0.6	55.8 ± 0.1	11.1 ± 1.4	3.1 ± 0.6	2.2 ± 0.1	3.5 ± 0.1	6.3 ± 0.5
Chickpea	5.3 ± 0.1	5.6 ± 0.7	38.2 ± 1.5	18.5 ± 1.1	2.7 ± 0.8	11.5 ± 0.1	2.2 ± 0.1	5.2 ± 0.8
Bean	5.8 ± 0.1	4.2 ± 0.3	49.4 ± 0.1	13.9 ± 3.1	3.7 ± 1.4	2.7 ± 0.2	2.8 ± 0.1	6.3 ± 0.1

IDF: insoluble dietary fibers; SDF: soluble dietary fibers

Table 3. Parameters of Eqs. (3), (5) and (6) used to describe the heat flows (Eq. (1)) for lentil, chickpea and bean protein-enriched samples (mean values ± 95 % confidence interval). The values were used to calculate the degree of protein denaturation $\tau = \sum_{i=\text{P1, P2}} \beta_i \times \frac{1}{2}\left(1+\text{erf}\left(\frac{T-T_i}{\Delta T_i\sqrt{2}}\right)\right)$ with two peaks (P1 and P2 for lentil and chickpea) or one peak (P1, bean).

Protein-enriched sample	$\frac{1}{T_i}-\frac{1}{T_{i,0}}=\frac{R}{\Delta h_{i,0}}\frac{v_g}{v_w}\left(\phi-\chi_i\,\phi^2\right)$						$\Delta T_i=\Delta T_{i,\infty}+\left(\Delta T_{i,0}-\Delta T_{i,\infty}\right)\exp\left(-\frac{X}{\gamma_i}\right)$					$\beta_{\text{P1}}=\beta_{\text{P1},\infty}+\left(\beta_{\text{P1},0}-\beta_{\text{P1},\infty}\right)\exp\left(-X\right)$	
	$T_{\text{P1},0}$ (°C)	$T_{\text{P2},0}$ (°C)	$\Delta h_{\text{P1},0}$ (kJ mol^{-1})	$\Delta h_{\text{P2},0}$ (kJ mol^{-1})	χ_{P1}	χ_{P2}	$\Delta T_{\text{P1},0}$ (°C)	$\Delta T_{\text{P2},0}$ (°C)	$\Delta T_{\text{P1},\infty}$ (°C)	$\Delta T_{\text{P2},\infty}$ (°C)	γ_{P2}	$\beta_{\text{P1},0}$	$\beta_{\text{P1},\infty}$
Lentil	165.2 ± 0.4	201.1 ± 3.3	57.6 ± 0.4	45.1 ± 2.9	0.00 ± 0.05	0.05 ± 0.05	4.2 ± 0.1	4.0 ± 0.2	4.2 ± 0.1	4.0 ± 0.2	1	0.65 ± 0.01	0.65 ± 0.01
Chickpea	198.1 ± 8.5	224.7 ± 4.6	41.2 ± 5.2	41.9 ± 2.5	0.12 ± 0.05	0.14 ± 0.03	6.5 ± 0.3	7.0 ± 1.6	2.2 ± 0.2	3.9 ± 0.1	0.3 ± 0.1	0.59 ± 0.02	0.12 ± 0.01
Bean	166.2 ± 0.3		65.8 ± 0.3		0.00 ± 0.05		3.5 ± 0.1		3.5 ± 0.1			1	1

ϕ : volume fraction of water (m^3 m^{-3})

X : water content (kg kg^{-1} db)

γ_{P1} was fixed at 1.

$\beta_{\text{P2}} = 1-\beta_{\text{P1}}$

Figures

Fig. 1.

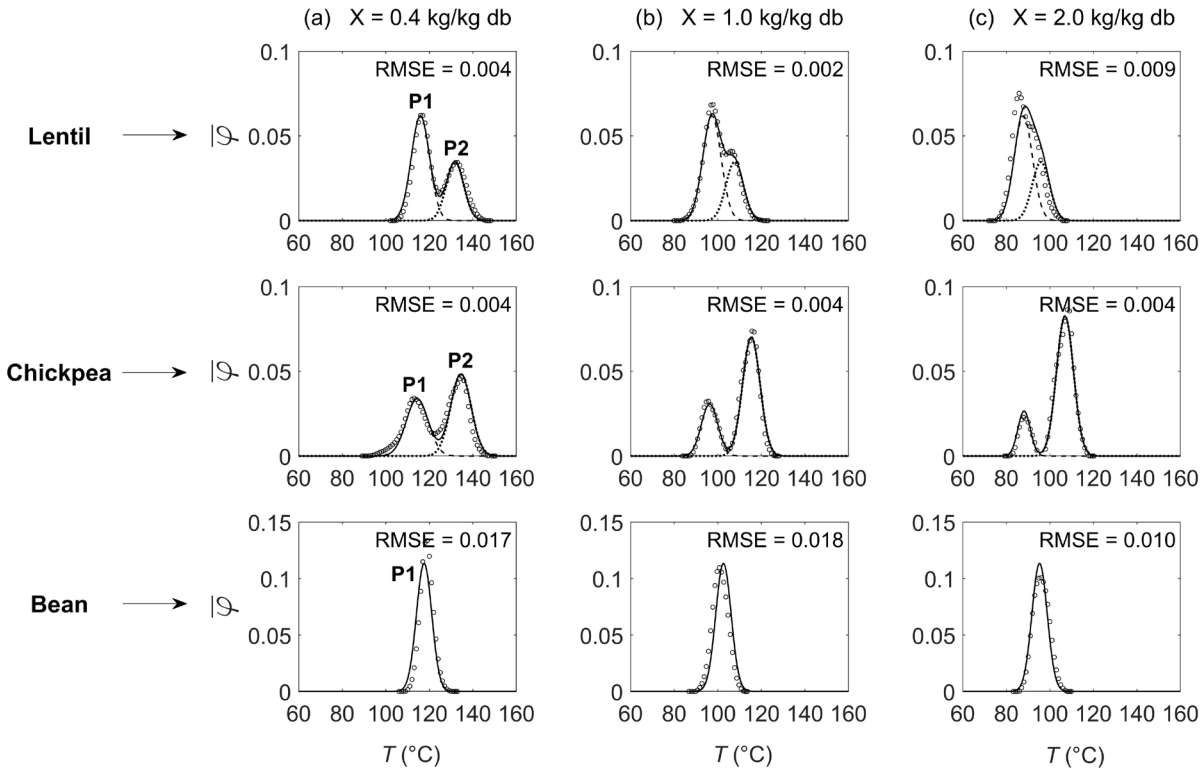


Fig. 2.

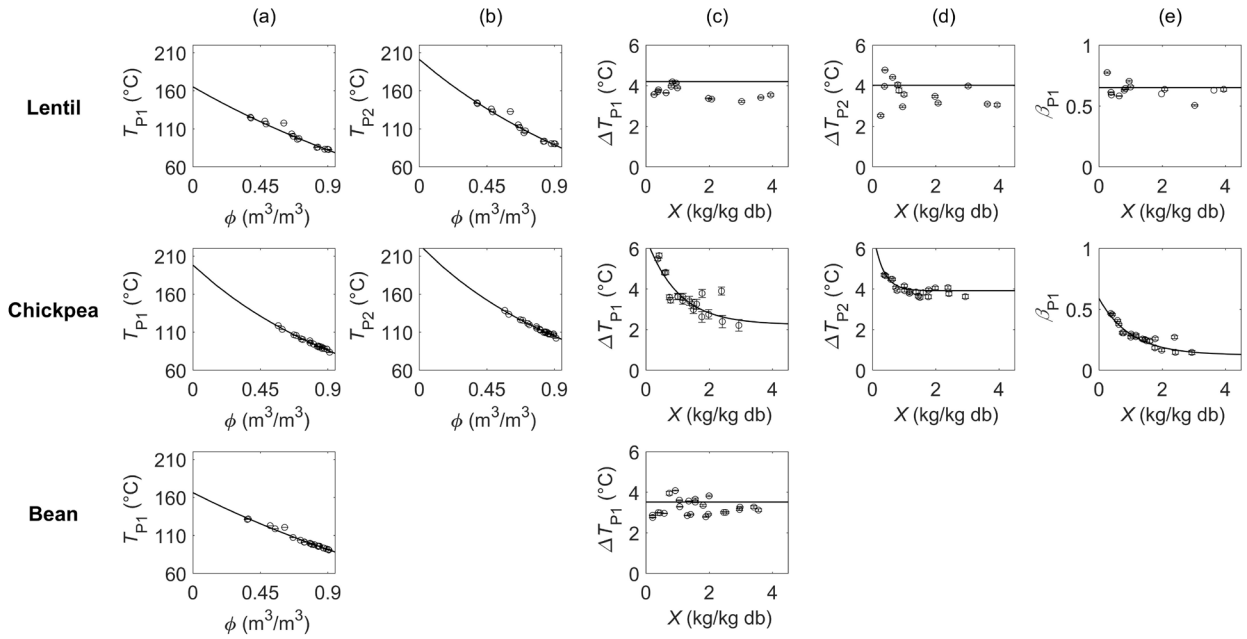

(a)
(b)
(c)
(d)
(e)
Lentil
Chickpea
Bean
T_{P1} (°C)
T_{P2} (°C)
ΔT_{P1} (°C)
ΔT_{P2} (°C)
β_{P1}
ϕ (m^3/m^3)
X (kg/kg db)

Fig. 3.

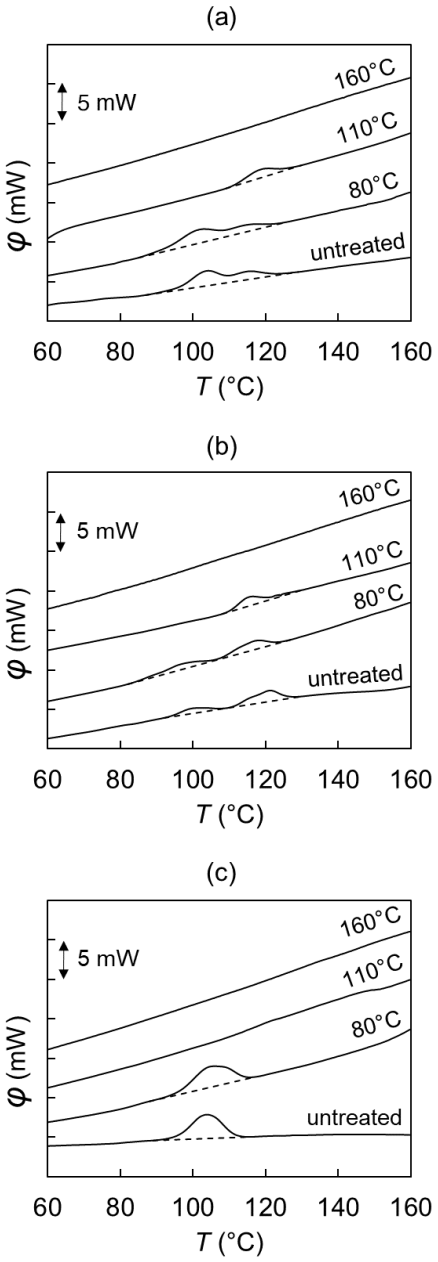
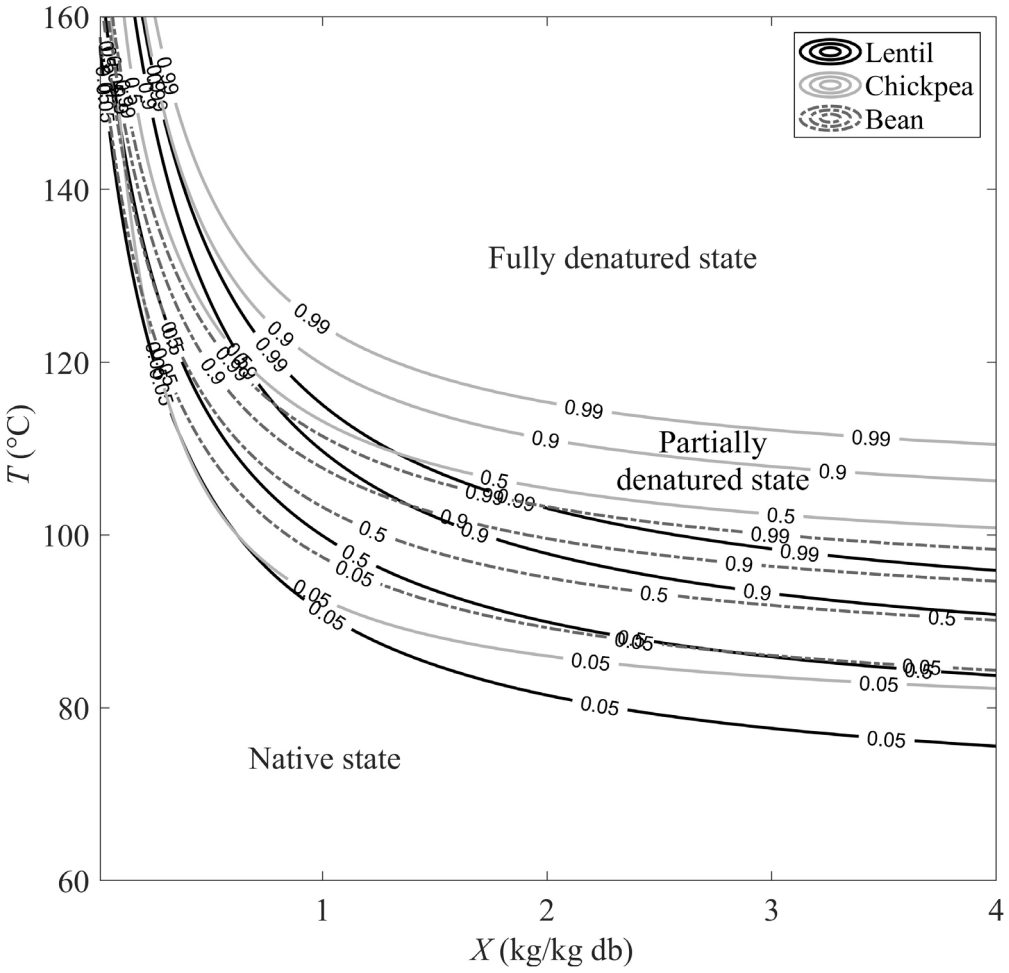
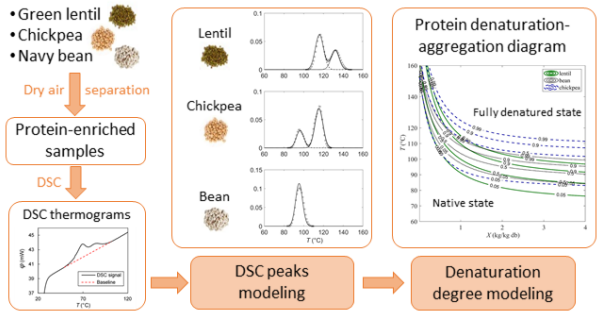

(a)
5 mW
160°C
110°C
80°C
untreated
φ (mW)
60 80 100 120 140 160
T (°C)
(b)
5 mW
160°C
110°C
80°C
untreated
φ (mW)
60 80 100 120 140 160
T (°C)
(c)
5 mW
160°C
110°C
80°C
untreated
φ (mW)
60 80 100 120 140 160
T (°C)

Fig. 4.


Lentil
Chickpea
Bean
Fully denatured state
Partially denatured state
Native state
T (°C)
X (kg/kg db)
160
140
120
100
80
60
1
2
3
4
0.99
0.9
0.5
0.05

Graphic for Table of Contents



Label : For Table of Contents Only