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Thermal Denaturation of Pea Globulins (*Pisum sativum* L.)—Molecular Interactions Leading to Heat-Induced Protein Aggregation

Jean-Luc Mession,*[,†] Nicolas Sok,[†] Ali Assifaoui,[†,‡] and Rémi Saurel[†]

[†]Agrosup Dijon, UMR PAM 02.102, Equipe PAPC (Procédés Alimentaires et PhysicoChimie), 1 Esplanade Erasme, 21000 Dijon, France

[‡]School of Pharmacy, Université de Bourgogne, 7 bd Jeanne d'Arc, 21079 Dijon, France

Supporting Information

ABSTRACT: The heat-induced denaturation and aggregation of mixed pea globulins (8%, w/w) were investigated using differential scanning calorimetry (DSC), SDS-PAGE, and size-exclusion chromatography (SEC-HPLC). DSC data showed that the pea proteins denaturation temperature (T_d) was heating-rate dependent. The T_d value decreased by about 4 °C by lowering the heating rate from 10 to 5 °C/min. The SDS-PAGE analysis revealed that protein denaturation upon heating at 90 °C was mainly governed by noncovalent interaction. The SEC-HPLC measurements indicated that low-denatured legumin (≈350−410 kDa) and vicilin/convicilin (≈170 kDa) globulins were heat-denatured and most of their subunits reassociated into high-molecular weight, soluble aggregates (>700 kDa). The addition of *N*-ethylmaleimide slightly modified the aggregation route of pea globulins. However, partially insoluble macroaggregates were produced in the presence of dithiothreitol, reflecting the stabilizing effect of disulfide bonds within legumin subunits.

KEYWORDS: *Pisum, pea globulins, legumin 11S, vicilin 7S, heat-induced denaturation and aggregation, interaction*

1. INTRODUCTION

Physicochemical properties of proteins depend on solvent parameters (pH, ionic strength, temperature). Solvent modifications could induce destabilization of protein native structure and irreversible molecular rearrangements. Conditions allowing globular proteins gelation to occur are of primary interest, since better control would help to modulate textural and rheological characteristics of the resulting gelled foodstuff.[1] Such a protein "functionalization" was achieved by heat treatment, entailing unfolding of the compact globulin structure and subsequently protein aggregation.

On account of worldwide pressures on water and energy demands, the growing use of plant proteins for human food rather than animal ones appears more sustainable.[2,3] Although mostly produced for livestock diet, pulse proteins would exhibit relevant physicochemical properties to valuate, owing to both their low cost and reduced environmental impact.[4] Therefore emphasis would be to substitute, at least partially, ingredients from animal production, e.g., whey proteins.[5] Alternative protein sources such as pea would counterbalance as well the leader position of soy protein isolates on the global market.[6]

Pea proteins are mainly composed of globulins (≈70%), i.e., legumin 11S and vicilin/convicilin 7S. They represent ≈23% (w/w, on a dry basis (d.b.)) of the dry seeds.[7,8] The hexameric legumin molecular weight (M_w) ranges from ≈330 to ≈410 kDa, whose subunits of ≈60 kDa are constituted of one acidic α (≈35−43 kDa) and one basic β (≈19−23 kDa) polypeptide bound together via a disulfide bridge.[9] Vicilin and convicilin are trimeric proteins (150 and 180−210 kDa, respectively). The great vicilin heterogeneity originates from the in vivo proteolysis of a precursor (≈50 kDa), producing small fragments: α (≈20 kDa), β (≈13 kDa), and γ (≈12−16 kDa).[10] Two cleavage sites are possible, which were not reported to modify basically vicilin M_w nor charge.[8] Usually a purified fraction of vicilin is contaminated by a third trimeric globulin named convicilin, whose subunits are ≈71 kDa in weight.[11] Despite their quite similar amino acid composition, convicilin differs from vicilin by its N-extension highly charged close to the C-terminus.[8] In contrast to vicilin/convicilin practically devoid of sulfur-amino acid, each legumin subunit would contain up to four methionine and seven cysteine residues.[7,11] Heterogeneity among pea globulin is as well ascribed to a legumin-to-vicilin ratio ranging from 2 to 4, related to pea cultivar and genotype.[8]

Only a few studies deal with thermal gelation of pea globulins.[12−19] In addition to processing parameters applied to allow their gelation, pea globulins heterogeneity influenced the textural properties of heat-set gels. Pea globulins were recognized for the lower gelling ability than that for their soy counterparts; gelation of pea proteins appeared to be governed mainly by nonspecific interactions, whereas higher involvement of sulfhydryl/disulfide bonds (S^-/SS) exchange reactions was reported for soy proteins.[15]

Alternatively, gelation of globular proteins could be carried out at ambient temperature.[20] In the cold-set gels, denaturation and aggregation are dissociated from the gelation step. First the protein solution is preheated above the denaturation temper-

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ature to induce globulin unfolding, which rearranged into soluble aggregates. After cooling, the second step consists of reducing gradually electrostatic repulsions between preaggregated proteins, to allow their assemblage into structured network.[21] In the presence of calcium cations with amounts up to 20 mM, Maltais and co-workers[22] produced preaggregated soy globulins cold-set gels, yielding a wide range of structural properties. Complexity could be as well incremented by adding a gelling polysaccharide to the preaggregated protein solution, before the second step of the cold-set gelation process. Otherwise, Pires Vilela et al.[23] performed the gelation of a mixed system made of preaggregated soy proteins in admixture with gellan gum by slow calcium diffusion. Enhanced firmness and lower syneresis than that of single-protein gels were highlighted. These authors ascribed such a synergistic effect to gelation entrapping phase separation,[24] the latter phenomenon occurring generally when dissimilar biopolymers in structure are mixed together. Microstructures of interest could be kinetically entrapped by gelation of the unstable mixture, before any macroscopic split in its appearance could be noticed.[1] Since literature is scarce regarding thermal denaturation of plant proteins, especially pea globulins, emphasis was to investigate first of all the influence of the heating step on protein aggregation. This would allow better control of the textural properties, enabling versatile applications of the gelled biomaterial.

In this present study, to complete our previous work regarding pea globulins extraction and physicochemical characterization,[25,26] we intended to inquire thermal denaturation and aggregation of pea globulins, at pH 7.5 and low ionic strength. First thermal properties of pea globulins were assessed using DSC. Then SDS-PAGE and SEC-HPLC analyses were applied to provide information on molecular interactions and M_w of heat-induced protein aggregates, respectively.

2. MATERIALS AND METHODS

Extraction of Pea Globulins. Low-denatured globular pea proteins isolate (PPI) was extracted from defatted Nutralys S85 M isolate (Roquette SA, Lestrem, France), as previously described.[25,26] PPI contained 92.5% (w/w) proteins, as assessed by the Kjeldahl method (nitrogen-to-protein conversion factor of 6.25),[27] and 7.36% (w/w) ashes (d.b.).

All the others reagents and chemicals purchased from Sigma-Aldrich were of analytical grade.

Preparation of Heat-Induced Aggregates. The PPI powder was suspended at 16% (w/w) in deionized water (DW) and stirred at room temperature until complete dissolution (≈2 h). The protein solution was desalted by extensive dialysis against water, with a protein solution-to-water 1:10 (4 changes for 24 h at 4 °C). The pH was 7.4 ± 0.15. The protein concentration was adjusted to 8% (w/w) with DW. This solution was named LPP-control, containing low-denatured pea proteins according to the described extraction procedure.[25]

The influence of structure disturbing agents NEM or DTT on the thermal denaturation of LPP was sought out. *N*-Ethylmaleimide (NEM) and dithiothreitol (DTT) agents inhibit sulfhydryl/disulfide bonds (S^-/SS) exchange reactions by blocking free sulfhydryl groups and reducing disulfide bonds, respectively.[18] Each reagent powder was poured separately to the LPP solution up to 10 mM concentration and stirred at room temperature until complete dissolution (≈2 h). The resulting solutions were named LPP+NEM and LPP+DTT.

Each LPP solution (≈25 mL) was poured in a glass vessel (50 mL) hermetically sealed and placed in an mineral oil bath previously equilibrated at 40 °C and then heated at 4.3 ± 0.2 °C/min from 40 to 90 °C, incubated at 90 °C for 1 h, cooled in ice for at least 2 h, and thereafter stored at 4 °C until further use. APP samples (APP-control, APP+NEM, and APP+DTT solutions) were centrifuged (12000g, 25 min, 25 °C), and supernatants were kept for further analysis.

Differential Scanning Calorimetry (DSC). Protein denaturation was assessed by DSC. Onset temperature (T_{onset}), temperature of denaturation (T_d). and enthalpy of denaturation (ΔH_d) were determined using a Q20 calorimeter (TA Instruments, Newcastle, DE, USA), beforehand calibrated with indium.[15] Each LPP solution at 8% (w/w) was weighed in an aluminum pan, hermetically sealed, and heated from 20 to 110 °C at either 5 or 10 °C/min rates. An additional LPP solution in 100 mM NaCl and pH 7.2 was prepared to compare data with that obtained previously.[26] An empty pan served as reference. One replicate of each sample was reheated after cooling to check that denaturation was irreversible. Thermograms were computed with the TA Universal Analysis Program (Version 4.5).

Free Sulfhydryl Contents Determination. The free sulfhydryl content of protein samples was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), namely, Ellman's reagent.[28] Protein solutions were extensively dialyzed at 4 °C against a 0.1 M Na_2HPO_4 buffer at pH 7.5. The reaction mixture was prepared by mixing the protein sample at 1% (w/w) final concentration with guanidium hydrochloride (GDNHCl, 2 M) and DTNB (1 mM), stirred vigorously by vortex, and kept in a dark area at room temperature for 15 min. Absorbance was recorded at 412 nm using a molar extinction coefficient ε for DTNB at 13250 $M^{-1}\cdot cm^{-1}$. The free sulfhydryl content was expressed as μM $S^-/g_{protein}$.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein samples were run on acrylamide−bisacrylamide continuous gels (8%T, 2.7%C) and 0.1% (w/v) SDS.[29] Each protein sample at 1% (w/w) final concentration was mixed with DW (control) or one of the following denaturing reagent solutions: SDS (5% (w/v)), SDS+urea (5% (w/v) and 3 M, respectively), or SDS+DTT (for each 5% (w/v), mixtures were heated at 100 °C for 10 min) and then kept for ≈16 h at room temperature. Each reaction mixture was centrifuged (10000*g*, 25 min, 25 °C) and then diluted to 5 $mg_{protein}$/mL in the sample buffer (62.5 mM tris-HCl, pH 6.8; 10% (w/v) glycerol, 0.005% (v/v) bromophenol blue, and 2% (w/v) SDS). Twenty microliter protein samples were applied on each well. Gels were run at 10 mA and 185 V for ≈6 h. Wide range M_w markers (S8445, Sigma-Aldrich) were deposited on a separate lane. Proteins were stained with Coomasie Blue R-250. Polypeptide composition by densitometric analysis on destained gels was carried out using SigmaScan Pro (Systat Software Inc., Version 5, San Jose, CA).[25]

High-Performance Liquid Chromatography (HPLC). The size exclusion chromatography (SEC-HPLC) system was composed of a Waters 600s controller, a 616 pump, and a 996 photodiode array detector (Waters, Milford, MA). A Superdex 200 10/300 GL column model (10 × 300 mm; GE Healthcare, Uppsala, Sweden) was chosen. The Superdex column was pre-equilibrated with a 50 mM Na_2HPO_4, pH 7.2, and 50 mM NaCl elution buffer. The flow rate was 0.4 mL/min. Otherwise a column model Discovery BIO GFC 500 (4.6 × 300 mm, Supelco, Bellefonte, PA) was used in another data set of SEC-HPLC measurements, pre-equilibrated with a 50 mM Na_2HPO_4, pH 7.2, and 100 mM NaCl elution buffer. The flow rate was 0.15 mL/min. Each column was precalibrated separately with a set of wide M_w protein standards (MWGF 1000-1KT 29−700 kDa, Sigma-Aldrich). Each protein sample prepared initially at 8% was diluted at either 0.8% or 0.5% (w/w) in DW and filtered with a 0.22 μm filter prior to analysis. A volume of 50 μL was injected to the HPLC system by means of a Waters 717 autosampler. Spectra were recorded and processed using the Waters Empower PDA software (Version 3).

Statistical Analysis. Analysis of variance was conducted using Bonferroni's test (5% p), using a standard statistical software. This procedure evidenced significance of results ($p \leq 0.05$) according to treatments applied on protein samples.

3. RESULTS AND DISCUSSION

Thermal Stability. T_{onset}, T_d, and ΔH_d values of LPP-control at 8% (w/w) and at pH 7.5 were examined by DSC at two heating rates (Table 1). The T_{onset} and T_d values give

Table 1. Thermal Parameters of Globular Pea Proteins (LPP, 8% (w/w)) at pH 7.5 and Effect of Some Protein Structure Disturbing Agents[a]

(A) Heating Rate = 10 °C/min			
sample	T_{onset} (°C)[b]	T_d (°C)[c]	ΔH_d (J/g $_{protein}$)[d]
LPP-control	70.5 ± 0.1 a	82.4 ± 0 a	9.5 ± 0.25 a
LPP−NaCl[e]	77.2 ± 0.2 b	87.5 ± 0.7 b	13.9 ± 0.4 b
LPP+NEM[f]	68.3 ± 0.5 c	80.3 ± 0.2 c	10.6 ± 1.1 a
LPP+DTT[g]	68.7 ± 0.2 c	82.1 ± 0.1 a	7.7 ± 1.4 a
(B) Heating Rate = 5 °C/min			
sample	T_{onset} (°C)	T_d (°C)	ΔH_d (J/g $_{protein}$)
LPP-control	67.1 ± 0.2 a	78.5 ± 0.2 a	9.2 ± 1.2 a
LPP−NaCl		not determined	
LPP+NEM	66.2 ± 0.4 a	78.0 ± 0 a	10.1 ± 0.7 a
LPP+DTT	66.6 ± 0 a	79 ± 0 a	9.4 ± 0.1 a

[a]All data were given as mean ± SD of triplicate measurements. Means in a column bearing the same letter are not significantly different ($p \geq 0.05$). [b]Onset temperature. [c]Denaturation temperature. [d]Enthalpy of denaturation. [e]In 100 mM NaCl, pH 7.2. [f]LPP prepared with NEM (10 mM). [g]LPP prepared with DTT (10 mM).

information on temperature-induced protein unfolding and protein thermal stability, respectively. The enthalpy changes (ΔH_d) reflected the extent of ordered structure of the globulin as the transition from native to denatured state took place.[30] Thermograms displayed only one broad endothermic peak, corresponding to the overlapping denaturation of 7S and 11S pea globulin fractions.[16,17] At 10 °C/min, thermal parameters of LPP-control were found within the same range as those reported at pH 7 and no salt added (Table 1A); Shand and co-workers[15] and Sun and Arntfield[16] measured T_d and ΔH_d values of around 84 °C, 7.3 J/g and 86.2 °C, 15.8 J/g $_{protein}$, respectively, for laboratory-prepared pea globulin isolates. In the present study, protein extraction using diluted (0.25−0.5 M) acid/alkali solutions was milder than that performed by Shand et al.[15] However, acidic precipitation would be more detrimental for pea globulins native structure than the salt extraction applied by Sun and Arntfield.[17] Partial protein denaturation due to extraction procedure would enhance their aggregation during heat treatment. The loss of protein structure integrity consists of redistribution of noncovalent and covalent bonds.[18] Rupture of hydrogen bonds is endothermic, while weakening then disruption of hydrophobic bonds and protein aggregation are exothermic reactions. The concomitance of endothermic and exothermic reactions may result in no variation or a decrease in the ΔH_d value.[19,31]

Effect of NaCl. Thermal stability of LPP increased significantly by addition of 100 mM NaCl (Table 1A). Thus a higher level of energy was required to induce globulin unfolding. The stabilizing effect of NaCl is assigned to the nonspecific charge-shielding effect between charged groups of proteins, reducing inter- and intrachain repulsions.[16,18] NaCl may also reinforce intramolecular hydrophobic interaction, thereby promoting salting-out effect and aggregation.[31]

The thermal parameters of the LPP−NaCl sample were close to values measured for globulins extracted previously from dry pea seeds.[26] As the isolate S85 M batch originated from these latter ones, this result corroborated the low-denaturing extraction of LPP from the soluble fraction of the present commercial isolate.[25]

Effect of Heating Rate. Both T_{onset} and T_d values for LPP-control sample decreased by about 4 °C by reducing the heating rate from 10 °C/min to 5 °C/min, whereas ΔH_d values were not affected (Table 1). Sun and Arntfield[17] found that temperature at which protein started to aggregate was heating rate-dependent; by reducing the heating rate from 4 to 1 °C/min, a 2 °C decrease of T_d was recorded, while T_{onset} and ΔH_d values of pea globulins were not affected. Likewise DSC data reflected that the energy required to induce LPP denaturation decreased with heating rate.[1,16] The lower thermal parameter values of LPP-control than those reported by Sun and Arntfield[17] would indicate the greater loss of ordered secondary structures of the studied pea globulins prior to heat treatment. By contrast with the authors cited above,[17] the LPP thermal denaturation was triggered at temperature slightly lower than 70 °C. Regardless of the heating rate applied, the temperature difference between T_{onset} and T_d values of the pea proteins used by Sun and Arntfield[17] and LPP-control in the present study was ≈20 and ≈12 °C, respectively. The narrower temperature range observed for the LPP-control sample denotes a higher cooperative transition from native to a denatured protein state than that encountered by the authors cited above.[17] The width of the endothermic transition was related to the interdependent forces binding together the conformational domains constitutive of seed globulins. The wide structural diversity of these globulins, also their extraction procedure, could explain the differences noted by several authors.[15,16,30]

Effects of Protein Structure Disturbing Agents. The addition of NEM or DTT up to 10 and 20 mM was assessed with regards to thermal stability of the LPP samples. Since the values obtained at either 10 or 20 mM concentration of each agent were not significantly different, only data in the presence of 10 mM are discussed. By comparison with LPP-control, it was noted for LPP+NEM and LPP+DTT samples a slight but significant decrease in T_{onset} values at 10 °C/min. Regarding T_d, it decreased for LPP+NEM, whereas no change was evidenced for LPP+DTT (Table 1A). Thus at 10 °C/min heating rate, NEM and DTT would facilitate slightly unfolding of LPP. In contrast, at 5 °C/min, the presence of NEM or DTT did not affect thermal parameters of protein samples (Table 1B). In addition, no difference in ΔH_d values was noted regardless of the protein sample investigated. Therefore, the extent of the loss of protein-ordered secondary structure was similar regardless of LPP treatment and heating rate.[16] Since vicilin 7S proteins are usually depleted of sulfur-amino acid, their DSC characteristics were generally not altered by the presence of NEM or DTT.[32,33] Regarding native legumin 11S, interchain and intrachain disulfide linkages were rather located inside the subunits, while noncovalent interaction stabilized protein structure at a quaternary level.[34,35] Heat-induced disruption of legumin oligomeric structure would be a prerequisite to allow exposure of reactive sulfhydryl groups initially buried in the hydrophobic core of subunits.[35] On the assumption that interchains S^-/SS exchange reactions occurred by heat treatment, the L_α and L_β polypeptides constitutive of each legumin 11S subunit would dissociate to a large extent. At 5 °C/min, denaturation was initiated at lower temperature than that noticed at higher heating rate. It would foremost be ascribed to the disruption of noncovalent interactions binding together subunits of LPP. In contrast at 10 °C/min, simultaneous unfolding at several levels of protein structure was suggested. By increasing the heating rate, denaturation and aggregation would be more likely concomitant events. This is supposed to affect accessibility of both free sulfhydryl groups and disulfide bonds at temperatures rapidly above ≈70 °C.

The occurrence of S^-/SS exchange reactions during heat denaturation of LPP-control would require a sufficient level of globulin unfolding, enabling the exposure of a part of free sulfhydryl groups and disulfide bonds of legumin subunits. Regarding the minor effects of NEM and DTT agents on LPP thermal parameters, legumin was possibly "diluted" with vicilin proteins. In addition, the limited effects of NEM and DTT may originate from the compact structure of legumin, hard to unfold extensively upon heat treatment.[34] This was consistent with the thermal parameters of a purified pea legumin fraction which were not altered in the presence of 20 mM NEM, as reported by O'Kane et al.[14] Thus it is generally stated that S^-/SS exchange reactions play a facultative role upon pea globulins heat denaturation.[18,19]

Complete and irreversible denaturation was verified on the thermogram by reheating after cooling one replicate of each LPP sample: no endothermic peak was evident.

Determination of Free Sulfhydryl Content. Free sulfhydryl contents of unheated and heated protein samples were evaluated in the presence of an excess of GDNHCl (Table 2). GDNHCl is a strong denaturing agent, disrupting noncovalent bonds which stabilized protein conformation.[18] Extensive dialysis of samples was required to remove excess or nonreacted NEM or DTT agents prior to Ellman's titration.

Table 2. Free Sulfhydryl Contents in Samples Containing Low-Denatured (LPP) and Aggregated Globular Pea Proteins, Heated at 90 °C (APP, Prepared at 8% (w/w) LPP and at pH 7.5)

sample	free sulfhydryl groups (μg $S^-/g_{protein}$)[a]
LPP-control	2.1 ± 0.1 a
LPP+NEM[b]	2.0 ± 0.0 a
LPP+DTT[c]	13.3 ± 2.4 b
APP-control	2.9 ± 0.1 c
APP+NEM	3.5 ± 1.1 c
APP+DTT	13.3 ± 1.3 b

[a]Determined at pH 7.5 according to Ellman's titration,[28] using ε(412 nm) = 13 250 $M^{-1}\cdot cm^{-1}$ for DTNB. All data were given as mean ±SD of triplicate measurements. Means bearing the same letter (a–c) were not significantly different ($p > 0.05$). [b]LPP and corresponding APP samples prepared with NEM (10 mM). [c]LPP and corresponding APP samples prepared with DTT (10 mM).

The free sulfhydryl contents of LPP-control and LPP+NEM samples were not significantly different. NEM appeared to be ineffective to block free sulfhydryl groups as they would not be accessible in a low-denatured state.[15] Sulfur-containing residues would be inaccessible, since they were reported to be buried inside pea globulin molecules, especially legumin 11S, in a low-denatured state.[35]

As mixed pea globulins have a very low free sulfhydryl content, a relatively small number and possibilities of S^-/SS exchange reactions during heat denaturation and aggregation are expected.[19] Surprisingly, heating LPP increased accessibility and thus exposure of free sulfhydryl groups, even higher for APP+NEM than that for APP-control. This uncommon tendency contradicted data emphasized by Tang et al.[36] on phasolein vicilin 7S, which differs from pea vicilin by its disulfide bonds content. Conversely in the present study, higher number of free sulfhydryl groups for APP-control and APP +NEM samples could reflect disruption of more disulfide bonds within LPP than newly established during APP formation. From LPP to APP-control, thermally induced conformational changes were supposed to open stiff local bends of polypeptide chains, where a few sulfhydryl groups could be located initially in the case of the unheated pea globulins. Upon heating, a newly established disulfide bond could release another sulfhydryl group previously involved in the broken disulfide bond, suggested to be more accessible than the first (free) sulfhydryl group. Besides, Ellman's reagent exhibited higher reactivity for the APP-control than that for the LPP-control sample; the addition of GDNHCL up to 3 M was found to break more easily thermal aggregates than the unheated pea globulins, leading apparently to higher free sulfhydryl group contents in the case of the aggregates. Concerning LPP+NEM and the corresponding APP+NEM sample, the situation would be more complex. During heat-induced denaturation, a competitive kinetics is hypothesized, occurring between the blocking effect of NEM toward the sulfhydryl groups carried by the unfolded polypeptide chains and protein aggregation. When the buried sulfhydryl groups are exposed upon heating, NEM would inhibit partially S^-/SS exchange reactions, entailing enhanced polypeptide segments flexibility.[19] Such a phenomenon would favor nonspecific interactions between unfolded LPP, which were suggested to increase protein aggregation rate. NEM reactivity would be further reduced to block just exposed free sulfhydryl groups, since unfolded polypeptide chain aggregated quickly via hydrophobic interactions. This would indicate that the thermal aggregation of unfolded pea proteins was predominantly governed by noncovalent interactions.[18]

The free sulfhydryl group contents for LPP+DTT and the corresponding APP+DTT sample were identical, though about four fold higher than in other samples. The reduction of disulfide bonds of legumin subunits apparently reached 100% level with a concentration up to 10 mM DTT, thereby allowing their titration by Ellman's reagent in both unheated and heated samples. Likewise, this indicated that no new disulfide bond was established by thermal aggregation. The assumption that most of the sulfur-containing residues are involved into disulfide bonds, located deeply in the hydrophobic core of 11S globulin subunits, may corroborate such a result.[14,34] Hence in the present study, the total sulfhydryl content of LPP is accounting for about 13 μM equivalent $S^-/g_{protein}$. Half of the value after subtracting the free S^- value from the total S^- value was defined as the SS content of LPP, estimated at around 5 $\mu M/g_{protein}$.

Electrophoretic Patterns of LPP-Control, LPP+NEM, LPP+DTT, and Corresponding APP Samples under Denaturing (SDS) and Denaturing/Reducing (with SDS +DTT) Conditions. More efficient than classical denaturing agents (urea, GDNHCL), the SDS detergent at a few tens of millimolar concentration binds strongly proteins and unfolds their conformation by increasing net negative charge between polypeptide segments.[31]

Pea Globulins Heterogeneity. Numerous bands were shown for LPP-control (Figure 1, lane 1). The band P_1 at less than 14 kDa could evidence traces of pea albumin PA_1 or dissociated vicilin fragments β (13 kDa), γ (12–16 kDa) or β:γ.[8,10] Polypeptides V_1–V_4 in the range of 20–36 kDa could correspond to fragments α (≈20 kDa), α:β (≈30–36 kDa), and β:γ (≈25–30 kDa), whereas V_5 was the main, uncleaved subunit α:β:γ (≈52 kDa). The band P_2 at ≈26 kDa appearing slightly on lanes 2*, 3, 5*, 7*, 8, and 9* may be remaining pea albumin PA_2 (indicated by dotted circles). The polypeptide at ≈89 kDa was probably lipoxygenase Lip (or Lox_{1-2}).[16] The

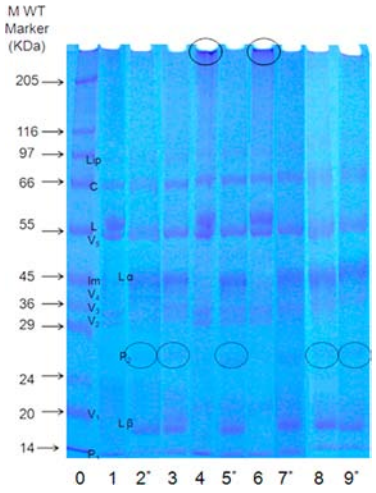

Figure 1. Electrophoretic patterns (continuous SDS-PAGE, 8%T) of low-denatured (LPP) and aggregated pea proteins, heated at 90 °C (APP, prepared at 8% (w/w) LPP and at pH 7.5). Some LPP and their corresponding APP samples were prepared with either dithiothreitol (DTT) or *N*-ethylmaleimide (NEM), for each 10 mM concentration. The samples on lanes 2, 5, 7, and 9 bearing the asterisk * were treated under reducing conditions with SDS+DTT reagents (for both 5% (w/v)), prior to SDS-PAGE analysis. Lane 0, molecular weight (M_w) markers; lanes 1−2*, LPP-control; lane 3, LPP+DTT; lanes 4−5*, APP-control; lanes 6−7*, APP+NEM; lanes 8−9*, APP+DTT. On the lanes, P_{1-2}, bands of pea albumins PA_{1-2} or vicilin fragments; V_{1-5}, bands attributed to vicilin 7S subunits; Im, impurity; L, legumin 11S subunit; L_α and L_β, acidic and basic polypeptides of legumin, respectively; C, convicilin; Lip, lipoxygenase. The solid and dotted circles highlight bands corresponding to high-M_w aggregates (>200 kDa) produced by heat treatment and presumably pea albumin PA_2, respectively. Note that LPP-control and LPP+NEM (not presented here) samples exhibited identical electrophoretic patterns.

strong band at ≈56 kDa was attributed to the legumin L main subunits, which were disrupted under reducing conditions into acidic L_α (≈38−40 kDa) and basic L_β (≈20−22 kDa) polypeptides, initially linked via interchain disulfide bond (lane 2*).[9,15] An impurity Im at ≈40 kDa overlapped with the band L_α under reducing conditions. This impurity was possibly unprocessed legumin subunits, which were not associated in vivo with L_β.[8,34] For the LPP+DTT sample, the disulfide bonds between the polypeptides L_α and L_β were reduced (lane 3). Moreover, a densitometric analysis carried out on three lanes gave the following composition, as percentages of the total polypeptide bands: P_1, 9.4 ± 1.6%; V_1, 1.9 ± 0.4%; V_2−V_4, 15.2 ± 2.3%; Im, 5.4 ± 0.6%; V_5, 16.0 ± 1.4%; L, 39.6 ± 3.3%; C, 9.9 ± 1.2%; Lip, 2.7 ± 0.9%. This composition was close to that found previously for globulins extracted from pea seeds.[26] With regards to a higher legumin amount than that determined elsewhere in several protein isolates from different pea cultivars,[19] legumin here would have a very small content of free sulfhydryl groups. By comparison, the LPP-control and one of the protein isolates used by O'Kane et al.,[19] containing 28% legumin amount, exhibited free sulfhydryl group contents of ≈5.3 and ≈11.2 μmol S^-/g_{legumin}, respectively. The cysteine content of a pea protein sample is cultivar-specific.[16,18]

Effect of Heat Denaturation on Electrophoretic Pattern. High-M_w polymerized proteins (>200 kDa) were caught at the top of lanes 4 and 6 (Figure 1). Protein denaturation was reflected by the diffusive and unresolved bands of very low electrophoretic mobility.[15,37] Meanwhile, minor changes regarding subunits M_w and relative polypeptide composition of heat-denatured protein samples suggested that aggregation was mainly ascribed to noncovalent interactions.[37] The latter ones were disrupted in the presence of SDS. A strong band at the bottom of the well was observed for both APP-control and APP+NEM samples. A fraction of large heat-induced protein aggregates was presumably formed via covalent interaction, which did not enter the electrophoresis gel (indicated by solid circles in Figure 1). This could reflect also the reduced reactivity of NEM toward restricted S^-/SS exchange reactions. Covalent bonding occurring locally in the deep of the protein structure during heat treatment cannot be excluded, though interchains bonding would remain limited given the poor accessibility of free sulfhydryl groups. The low rate and possibilities of S^-/SS exchange reactions among pea globulins could be related to several factors, i.e., (i) pea legumin and cysteine contents variability from one cultivar to one another, (ii) position/exposure of sulfhydryl groups on the polypeptide chains, and (iii) overall low reactivity of disulfide bonds, deeply buried within the hydrophobic core of 11S globulins.[15−19,36,38]

The LPP and APP samples were mixed with various reagents which disrupted physical and/or chemical interactions stabilizing protein structure (lanes 5* and 7*, APP-control and APP+NEM samples treated with SDS+DTT prior to SDS-PAGE analysis are illustrated here). Electrophoretic patterns of samples diluted in DW and treated with SDS or SDS+urea were not found to differ; hydrogen bonding would contribute to a small extent to the stabilization of pea proteins structure in both low-denatured and thermally aggregated states.[35] The SDS amount up to ≈35 mM brought by the electrophoresis sample buffer was sufficient to dissociate subunits constitutive of both unheated and heated pea proteins, which are held together via noncovalent weak interactions.[10,11,34] Thus thermal aggregation was conducted presumably via preponderant hydrophobic bonding.[14,18,19] After an additional SDS+DTT treatment of APP-control and APP+NEM samples, the strong band attributed to high-M_w aggregates disappeared (lanes 4, 5* and 6, 7*). This underpinned that a few heat-induced disulfide bonds were established, presumably in-between a minor part of legumin subunits. As evaluated by densitometry, the band L intensity was however not significantly different from LPP-control and LPP+NEM to the corresponding APP samples. This traduced the little involvement of S^-/SS exchange reactions. Moreover, heat treatment did not disrupt legumin subunits, as no band corresponding to dissociated L_α and/or L_β polypeptides was noticed on the lanes 4 and 6.

In the presence of DTT, the L_α and L_β polypeptides were dissociated for both unheated and heated samples (lanes 3 and 8−9*). Thereby, it remained unclear whether unheated legumin underwent modifications of its overall oligomeric structure upon DTT addition. However, DSC data did not indicate any strong decrease of T_d for LPP+DTT. Hence, legumin structure integrity would be preserved despite the addition of DTT. Moreover, the development of turbidity of the LPP+DTT solution while heating was noted. The resulting APP+DTT solution was whitish. Small precipitates were collected by centrifugation, ascribed to insoluble macro-aggregates. The combination of heat treatment with DTT up to 10 mM would induce strong unfolding of legumin oligomeric structure, where disruption of disulfide bonds between acidic L_α and basic L_β legumin polypeptides was expected. From structural data, the L_β polypeptide was

hydrophobic and buried in the core of legumin structure, while L_α was rather hydrophilic and located at the periphery.[35] Thereby the lower steric hindrance may account for the higher exposure of L_β polypeptides, promoting hydrophobic interaction during heat treatment and macroaggregates formation, which further precipitated. Hence, preservation of disulfide bonds would prevent overaggregation of pea globulins upon heating.

Effect of Heat Treatment on Chromatographic Profiles at 215 nm of Pea Globulins. Spectra of LPP and APP samples were divided into five elution volume ranges (i–v), to differentiate protein fractions according to their M_w range (Figures 2 and 3). The relative content of each fraction was calculated by considering the total spectrum integrated area as 100% (Table 3). The APP+DTT sample was not injected for safety; the large particles in suspension did not allow proper filtration and consequently their complete removal prior to the SEC analysis. A wavelength of 215 nm was chosen since the volume of the Superdex column (≈24 mL) diluted protein samples and thus its signal intensity at 280 nm was low.

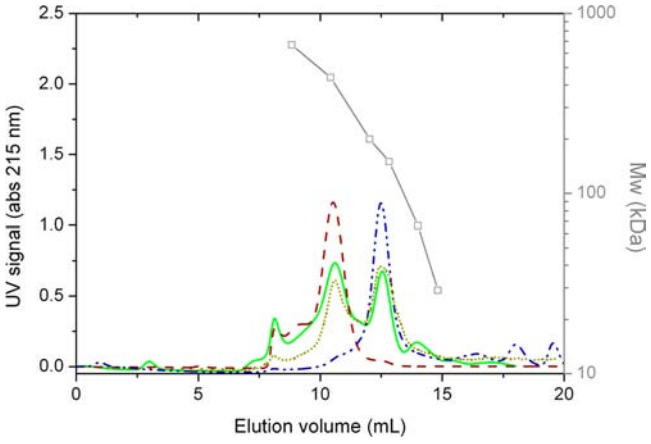

Figure 2. SEC elution profiles of unheated, low-denatured pea proteins (LPP), obtained with a Superdex 200 10/300 GL column. The flow rate was 0.4 mL/min. Samples are LPP-control (light green —); LPP+DTT (yellowish green ···); legumin 11S (dark red ––), and vicilin 7S (blue –··) enriched fractions. The column was previously calibrated with M_w standards (-□-, right axis).

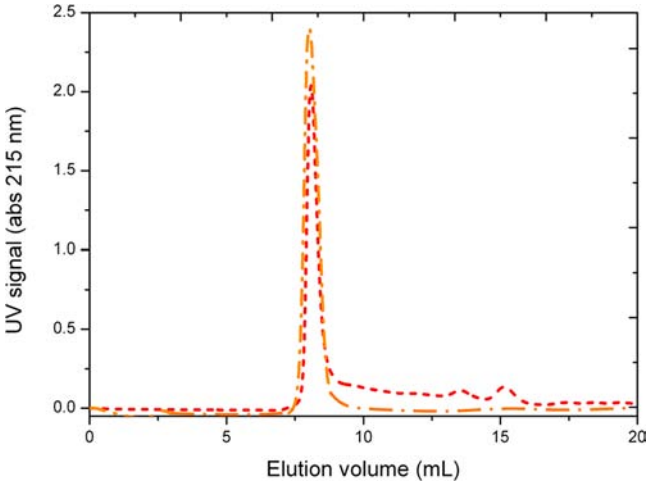

Figure 3. SEC elution profiles of aggregated pea proteins, heated at 90 °C (APP, prepared at 8% (w/w) protein concentration and at pH 7.5). SEC measurement parameters are given in Figure 2. Samples are APP-control (red ––) and APP+NEM (orange —·).

Chromatographic Profile of Unheated LPP Samples. For the LPP-control sample, two major peaks were detected within the fractions ii + iii and iii + iv (Figure 2). In their order of detection, the two peaks were attributed to legumin 11S and vicilin/convicilin 7S, with a mean M_w of about 410 and 170 kDa, respectively (Table 3). The fraction iii was an overlapping zone within spectra, where legumin and vicilin proteins coeluted. Peak identification was consistent with elution profiles of legumin 11S- and vicilin 7S-enriched fractions, which were analyzed by SDS-PAGE in our previous study.[26] Purity of each enriched globulin fraction was also estimated around 80%. The minor peaks detected in both LPP-control and vicilin samples at ≈54, 30, and >30 kDa would contain vicilin fragments in small amounts.[32] Several authors evaluated the mean M_w of 7S and 11S pea globulins in the range of ≈150–200 and ≈330–410 kDa, respectively.[9−11,32,34] The discrepancies in M_w determination for most of seed globulins could originate from the great heterogeneity in their constitutive subunits and molecular shape.[38] Moreover, in the present study, it is supposed that nonspecific interactions between the Superdex column matrix, made of cross-linked agarose–dextrane beads, and glycoproteins altered protein retention time, even if the column was chosen according to a previous report on pea vicilin.[32] To compare with SDS-PAGE data of LPP-control, hexameric legumin, trimeric vicilin, and convicilin M_w would be 6 × 56 = 336 kDa, 3 × 52 = 156, and 3 × 65 = 205 kDa, respectively. Those calculated values would be more consistent for low-denatured pea globulins. Besides, it was checked that the addition of NEM up to 10 or 20 mM did not modify elution profile of LPP. The unheated pea globulin structures were unaffected by NEM (spectrum not shown).

In Figure 2, a shouldered peak eluted early (≈8.2 mL). Its relative protein content (fractions i + i′) was about 12% (Table 3). First it could correspond to high-M_w aggregates yielded by the industrial process (>700 kDa). Those remained in the soluble protein extract at a low level (≈2%) of the total protein composition, as discussed in a previous work.[25] Such an early peak was as well noticed for the laboratory-prepared isolate from pea seeds, though of lower intensity than that observed for LPP-control (spectrum not shown).[26] Thus several compounds were detected here. According to Marcone et al.,[34] such a peak close to the void volume of the column was typical of plant globulins. Unidentified large-sized material responsible for protein solution turbidity could elute here. In addition, this early peak was observed on the legumin-enriched sample spectrum, accounting for about 20% of the relative protein content (fractions i + i′). This was higher than that found for LPP-control and was attributed by Gatehouse et al.[9] to polymerized species of legumin 12–15S. For LPP+DTT, it was noteworthy that the relative protein content for this early peak decreased by about threefold by comparison with LPP-control. However, the fraction ii, consisting of legumin 11S, was unaffected. Noncovalent interactions stabilizing the oligomeric structure of unheated legumin 11S would prevent the release of dissociated L_α and L_β polypeptides, while a fraction of legumin would have undergone polymerization via disulfide bonds. Apparently DTT disrupted the 12–15S polymers into trimeric 7S protein material, as suggested by the higher relative protein fraction iv for the LPP+DTT sample than that for LPP-control.

Chromatographic Profiles of APP Samples. In Figure 3, the total integrated areas of APP-control and APP+NEM

Table 3. Percentage of Integrated Area from SEC-HPLC Spectra at 215 nm, of Low-Denatured (LPP) and Aggregated Pea Proteins, Heated at 90 °C (APP, Prepared at 8% (w/w) LPP and at pH 7.5), As Presented in Figures 2 and 3, Respectively[a]

elution volume range (mL)	<8.7 ± 0.1	(8.7 ± 0.1)−(9.1 ± 0.1)	(9.1 ± 0.1)−(11.2 ± 0.3)	(11.2 ± 0.3)−(12 ± 0.1)	(12 ± 0.1)−(13.6 ± 0.4)	>13.6 ± 0.4
M_w (kDa)	>650	650−620	620−360	356−260	260−60	<60
protein fraction	i	i′	ii	iii	iv	v
sample	**relative percentage of protein fraction (%)**					
Leg[b]	5.1 ± 1.9 a	13.8 ± 3.2 a	71.0 ± 4.1 a	8.0 ± 2.3 a	2.1 ± 1.2 a	0
Vic[c]	0	0	0	17.8 ± 5.1 b	60.4 ± 7.1 b	21.7 ± 5.5 b
LPP-control (=LPP+NEM[d])	4.1 ± 0.96 a	7.6 ± 2.3 a	33.2 ± 4.2 b	11.0 ± 2.7 b	28.1 ± 5.9 c	15.3 ± 1.5 b
LPP+DTT[e]	1.4 ± 0.4 b	1.8 ± 0.3 b	32.4 ± 0.8 b	10.7 ± 4.0 b	40 ± 4.8 d	14.2 ± 0.6 b
APP-control	61.3 ± 6.3 c	4.2 ± 1.3 a	9.8 ± 4.1 c	2.4 ± 1.3 c	6.4 ± 3.1 e	14.7 ± 3.3 b
APP+NEM	83.9 ± 6.8 d	11.4 ± 6.4 a	2.8 ± 0.4 d	0	0	0
APP+DTT	not determined					

[a]All data were given as mean ±SD, calculated on at least four spectra. Spectra were divided into five protein fractions (i−v), their relative fraction (%) was calculated by the ratio with the total integrated area (summed fractions i + i′ + ii + iii + iv + v, taken as 100%). Means bearing the same letter (a−e) were not significantly different ($p > 0.05$) [b]Legumin-enriched fraction. [c]Vicilin-enriched fraction. [d]LPP and corresponding APP samples prepared with NEM (10 mM). [e]LPP and corresponding APP samples prepared with DTT (10 mM).

spectra were slightly lower (≈90%) than that calculated from their corresponding LPP spectra. This confirmed that practically all the protein thermal aggregates were water-soluble. Both APP-control and APP+NEM spectra showed a straight peak detected in the high-M_w range (>700 kDa). These APP samples contained mainly protein aggregates, eluting in both cases at around 8.2 mL. Their main peak overlapped with the early peak evidenced on the LPP spectra. As described by Li et al.[37] for heat-denatured soy proteins, a high initial protein concentration (>5% (w/w)) yielded large thermal aggregates, eluting close to the void volume of the gel filtration column. In the present case, the exclusion limit of the Superdex column was 1250 kDa, according to the supplier. The relative protein contents of fractions (i + i′) were about 67% and 95% for APP-control and APP+NEM, respectively (Table 3). The relative protein fraction ii decreased by about 3- and 11-fold from LPP-control to APP-control and from LPP+NEM to APP+NEM samples, respectively. The relative protein fraction iv decreased by about 4-fold from LPP-control to APP-control, while this was absent on the APP+NEM spectrum. Most of globulins were heat-denatured, and the dissociated subunits rearranged into high-M_w aggregates. Upon heating, the presence of NEM appeared to particularly enhance pea globulin structure unfolding, as a higher globulin fraction was involved into large aggregates formation. In contrast, it was noted on the APP-control spectrum an extended shouldering within fractions (ii + iii + iv), where the initial legumin 11S and vicilin 7S peaks could not be resolved accurately. A fraction of unfolded globulins is supposed to coexist with large aggregates, since DSC data indicated that the APP samples did not contain remaining low-denatured proteins, by reheating procedure. Besides, the protein material detected in fraction v would not contribute markedly to the aggregation phenomenon, as its integrated area was not affected by heat treatment. The minor peaks at ≈65 and ≈30 kDa could consist of thermally dissociated subunits which did not aggregate, such as convicilin subunits for the former one. O'Kane et al.[13,14] reported that the highly hydrophilic N-extension of convicilin could disturb gelation of an enriched vicilin isolate; a critical convicilin polypeptide content of about 13% among pea globulins of an isolate was found to decrease its gelling ability, i.e., pronounced weakening of the protein network induced by heat treatment.[19] Similarly in the present study, the convicilin content close to 10%, as determined by densitometry, is hypothesized to hinder protein thermal aggregation.

Regarding the APP+NEM sample (Figure 3), NEM would intensify thermal aggregation of dissociated subunits. The intermediate fractions (ii−iv) within APP+NEM spectrum decreased by about fourfold as compared to that calculated for APP-control (Table 3). Upon heating, it was expected that NEM promoted larger protein aggregates formation than those generated in the APP-control sample.[39] As described for several plant globulins, the extent of thermal aggregation depends upon content and exposure rate of their hydrophobic residues. Choi and Ma[39] emphasized that NEM in combination with a high ionic strength enhanced macroaggregates formation and then partial precipitation of heat-denatured buckwheat legumin 11S. NEM would favor as well in the present study hydrophobic residue exposure of unfolded LPP and possibly increased aggregation rate of legumin via nonspecific interaction. In contrast with the authors cited above, the desalting procedure of the LPP solutions prevented strong imbalance in hydrophobicity of thermally aggregated protein material and their precipitation in solution.

It was elucidated with the Discovery BIO GFC 500 column of wide exclusion limits that the thermal aggregates were large and polydisperse in size, accounting for a mean M_w of about 1.4×10^3 kDa for both APP-control and APP+NEM samples (Supporting Information). This aggregates mean M_w matches quite well with the exclusion peak of the Superdex column.

It was checked that neither the M_w nor the aggregates peak intensity of APP samples changed within a five days storage period at 4 °C. The aggregated proteins in both APP-control and APP+NEM samples were also stable to dilution (SEC data from samples injected at 0.5% (w/w) not shown).

Proposal of an Aggregation Route of Mixed Pea Globulins. Aggregation of unfolded proteins is related to the balance between electrostatic repulsions due to their net surface charge and heat-induced hydrophobic attractive interaction.[20] As suggested by the heating rate dependence of T_d values of LPP-control (Table 1), an increase of the heating rate is suggested to entail a lower extent of rupture of hydrophobic

bonds during protein unfolding. Thereby aggregates that are more compact and endowed with a higher thermal stability are suggested to be formed. Conversely, a lower heating rate of ≈4 °C/min would decrease protein denaturation rate. This is expected to promote molecular rearrangements in a linear fashion; i.e., protein aggregation occurs when unfolded chains have a rather specific orientation.[14,17] Such a tendency is enhanced by a high level of electrostatic repulsion between protein molecules, since the LPP solutions studied were at pH 7.5 and in the absence of salts; this prevented protein aggregation prior to their thermal denaturation.[1,16] Nevertheless, the aggregation route of globulins is related to other factors.[1] Due to high LPP concentration and incubation temperature of ≈11 °C higher than T_d, protein thermal aggregation in a dense (concentrated) media would lead to large and amorphous aggregates. First the aggregation rate of globulins increases with heating temperature.[37] Moreover, the predominance of physical nonspecific interactions between unfolded proteins would generate aggregates exhibiting disordered structures. The random association of their dissociated subunits is assumed; the slight gain of free sulfhydryl groups content from low-denatured to aggregated proteins is consistent with the extensive exposure of buried hydrophobic groups (Table 2).[40] Likewise, the formation upon heating of high-M_w aggregates would reduce rapidly the accessibility of intermolecular disulfide bonds. Given the heterogeneity of pea globulins subunits, composition, and charge, further studies on the thermal aggregation route of vicilin and legumin separated fractions should be undertaken.[18,19]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

A complementary analysis of chromatographic profiles of unheated/heated pea globulins, using a column of wide exclusion limits. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: +33 380 774 030. Fax: +33 380 396 647. E-mail: jean-luc.mession@laposte.net.

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Notes

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■ ABBREVIATIONS USED

SEC-HPLC, size-exclusion chromatography; DSC, dynamic scanning calorimetry; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; M_w, molecular weight; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; S^-/SS, sulfhydryl/disulfide bond exchange reactions; GDNHCl, guanidium hydrochloroide; PPI, globular pea proteins isolate; L_α, acidic polypeptide of legumin 11S subunit; L_β, basic polypeptide of legumin subunit; LPP, low-denatured pea proteins; APP, aggregated globular pea proteins; T_{onset}, onset temperature; T_d, denaturation temperature; ΔH_d, denaturation enthalpy; DTNB, dithiobis(2-nitrobenzoic acid); DW, deionized water; UV, ultraviolet

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