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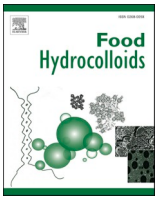

Heat-induced dissociation and association of proteins in hempseed protein bodies

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ABSTRACT

Protein bodies (PBs) are naturally occurring storage organelles in seeds. In hempseeds, the major storage proteins, including edestin (11S globulin) and albumin, are primarily located in the crystalloids and proteinaceous matrices of hemp protein bodies (HPBs), respectively. The retention of native PB structures in flours and dry-fractionated protein ingredients has important implications for protein functionality and digestibility, especially when heat treatment is applied during processing. While the thermal behaviour of hempseed proteins has been studied in protein isolate systems, to the best of our knowledge, it has not yet been explored in HPB systems. In this study, we isolated native HPBs using an enzymatic method. Aqueous suspensions of HPBs (4 % protein, w/w) were heated at selected temperatures (60–100 °C) and pH 7 for 20 min, followed by hydrolysis with trypsin at pH 7 and 37 °C for 120 min. The thermal aggregation of proteins in HPBs was characterised using confocal laser scanning microscopy (CLSM) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The hydrolysis of HPBs by trypsin was monitored over 120 min by measuring the degree of protein hydrolysis (DH) and analysing SDS-PAGE. Aggregation of edestin in HPBs, primarily driven by disulfide bond formation, occurred upon heating, most noticeably at temperatures above 80 °C. Heating increased DH and altered protein degradation patterns of both acidic and basic subunits of edestin. This may be related to conformational changes in the HPB structure resulting from heat-induced dissociation-association of multiple HPB protein fractions, including 11S edestin, 7S globulin, and 2S albumin. These findings contribute to our understanding of the structure-hydrolysis relationships of HPBs, potentially leading to their use as a new plant-based material for food applications.

1. Introduction

Protein bodies (PBs) are subcellular organelles where the majority of seed storage proteins accumulate, serving as a source of amino acids and energy during germination (Galili & Herman, 1997). Morphologically, PBs typically exhibit a spherical shape with diameters ranging from 0.1 to 25 μm. They are described as single-membrane-bound organelles, within which internal inclusions are embedded in an albumin-rich proteinaceous matrix, all enclosed by an external membrane. Two distinct types of inclusions, the globoid and the crystalloid, commonly occur within PBs in seeds (Do et al., 2024; Huang, 1985; Pernollet, 1978). The crystalloid is a globulin storage inclusion (Angelo et al., 1968), while the globoid is a non-proteinaceous inclusion that serves as the main accumulation site for phytic acid and cations (Wang & Guo, 2021).

Protein bodies can withstand a range of thermal and non-thermal food processing technologies, such as milling, dry fractionation, and hydrothermal cooking, as evidenced by the retention of their structural integrity throughout these processes (Gulati et al., 2018; Pelgrom et al., 2014; Rom et al., 1992; Tanaka et al., 1978). For this reason, PBs often remain intact in various plant protein ingredients, including flours and mildly fractionated protein fractions, which are subsequently incorporated into food products (Gulati et al., 2018; Lv et al., 2023; Pelgrom et al., 2014). Several studies have demonstrated that microstructural changes in PBs during cooking are mainly responsible for the increased resistance of some cereal proteins to enzymatic hydrolysis, resulting in lower protein digestibility (Duodu et al., 2002; Gulati et al., 2018; Oria et al., 1995; Rom et al., 1992). In light of the growing interest in milder and more sustainable extraction methods that preserve the native structures and functionality of seed PBs (Pulivarthi et al., 2023), a detailed understanding of their structural modifications during food processing and the implications for enzymatic hydrolysis is critical for developing foods with improved techno-functional properties and

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Abbreviations

CLSM	confocal laser scanning microscopy
HPB	hemp seed protein body
MW	molecular weight
PB	protein body
pI	isoelectric point
SDS-PAGE	sodium dodecyl sulphate polyacrylamide electrophoresis
SEM	scanning electron microcopy
β-ME	β-mercaptoethanol
AS	acidic subunit
BS	basic subunit
–SH	sulfhydryl
S–S	disulphide
OPA	o-Phthaldialdehyde
ANS	1-anilino-8-naphthalene-sulfonate
SP, soluble protein	
TCA	trichloroacetic acid
Ho	protein surface hydrophobicity
DH	degree of protein hydrolysis
HAA	hydrophobic amino acid.

nutritional value from plant protein sources.

Our research group recently conducted a study on the isolation of PBs from hemp seeds for their potential use as a source of high-quality proteins for food applications (Do et al., 2024). Microstructural and chemical analysis of native hemp protein bodies (HPBs) revealed that 11S legumin-type globulin (edestin) is the most abundant storage protein, located in the crystalloid. Additionally, albumin is present in smaller amounts, presumably within the proteinaceous matrix (Do et al., 2024). Hempseed proteins comprise approximately 65–80 % globulin (mainly edestin) and 20–30 % albumin. Native edestin exists as a hexameric globular protein with a molecular weight (MW) of approximately 320 kDa, composed of six identical monomers. Each monomer (~52 kDa) consists of an acidic subunit (AS, ~34 kDa) and a heterogenous basic subunit (BS, ~20 and 18 kDa) (Potin & Saurel, 2020). Each edestin monomer contains five cysteine residues: two form an intermolecular disulphide bond between the AS and the BS, another two form an intramolecular disulphide bond within the AS, while the remaining cysteine has a free sulfhydryl (–SH) group (Docimo et al., 2014).

Heat treatments, such as pasteurisation and ultra-high temperature, are widely employed in the processing of plant-based protein ingredients to ensure microbiological safety and improve protein digestibility (Meganaharshini et al., 2023; Pulivarthi et al., 2023). However, heating causes native proteins to undergo denaturation (unfolding) and subsequent aggregation of the unfolded molecules. The thermal behaviour of hempseed proteins has been studied in protein isolates obtained through alkaline extraction and isoelectric precipitation (Raikos et al., 2015). Hemp protein isolate (HPI) predominantly consists of globulin (edestin) in a partially or fully denatured state. The albumin fraction is largely removed during the precipitation of the globulin fraction at its isoelectric point (Hadnađev et al., 2018). Upon heating, the high free –SH content of globulin in HPI (Tang et al., 2006) promotes disulphide bond (S–S) formation via –SH oxidation and/or SH-induced S–S interchange reactions, leading to protein aggregation (Raikos et al., 2015). Heating HPI at 75–80 °C induces conformational changes in the edestin molecule (denaturation), exposing trypsin cleavage sites and thereby increasing the rate of proteolysis. In contrast, heating HPI at a higher temperature of 90 °C produces the opposite effect—the aggregation of edestin can attenuate its hydrolysis rate by shielding these cleavage sites (Yin et al., 2008).

Unlike the globulin-rich HPI, HPBs exhibit greater compositional and structural complexities, with coexisting albumins, globulins, and phytate. These native protein assemblies comprise multiple structural components held together by covalent and/or non-covalent bonds. Such complexities pose challenges for studying the thermal characteristics of HPBs, which, to the best of our knowledge, have yet to be thoroughly investigated. Here, we report for the first time on the thermal behaviour of isolated HPBs in aqueous suspensions and evaluate how it proceeds at the molecular level—particularly regarding the dissociation-association reactions of major HPB protein fractions during heating. We also examine heat-induced structural modifications of proteins in HPBs in relation to the trypsin hydrolysis of edestin subunits.

2. Materials and methods

2.1. Materials

Commercial dehulled hemp seeds, also known as hemp hearts, were purchased from Hemp Connect Ltd. (Levin, New Zealand). Viscozyme® L (Batch KTN02310, Novozymes, Bagsværd, Denmark) was kindly donated by Azelis NZ Ltd. (Auckland, New Zealand). This is a blend of beta-glucanases, pectinases, hemicellulases, and xylanases with an optimum pH range of 3.3–5.5 and an optimum temperature range of 40–50 °C. Trypsin from porcine pancreas (T0303, activity 15156 BAEE units/mg protein) was obtained from Sigma Aldrich Ltd. (St. Louis, MO, USA). The Pierce™ Bicinchoninic acid (BCA) Protein Assay Kit (catalogue number 23225) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals and reagents were of analytical grade and were obtained from Sigma Aldrich Ltd., unless otherwise specified. Milli-Q water from a Millipore Reference Milli-Q water purification system (Merck, Germany) was used for all experiments.

2.2. Preparation of HPB suspension

Protein bodies were isolated from hemp hearts using an aqueous enzymatic method, as previously described (Do et al., 2024). In brief, the hemp hearts were incubated with Viscozyme® L in 50 mM citrate buffer at pH 5 and 50 °C for 24 h, under continuous gentle stirring and occasional sonication. During this enzymatic process, the hemp cotyledon cell walls were hydrolysed, releasing intracellular PBs and oil bodies into the reaction buffer. Following centrifugation (4000 rpm, 30 min), the PB-rich pellet was collected and washed successively with absolute acetone and Milli-Q water to remove residual oil and soluble carbohydrates, respectively. The compositional analysis (% w/w, dry weight basis) of the washed HPBs showed a high protein purity (90.8 %) and minimal contamination with oil (0.95 %) and carbohydrate (0.28 %) (Do et al., 2024).

The washed HPBs were suspended in water, and the pH was adjusted to 7 with 3M NaOH. The HPB suspension was then diluted with water to a final protein concentration of 4 % (w/v), based on protein concentration determination using the bicinchoninic acid (BCA) assay, with bovine serum albumin as the standard. To completely solubilise the HPBs for the BCA assay, a sample treatment reagent containing 2.0 % SDS and 0.8 % sodium hydroxide was applied, followed by heat treatment at 100 °C for 10 min (Liu & Pan, 2017).

2.3. Heat treatment

Aliquots of the HPB suspension (4 % protein, w/v, pH 7) were transferred into centrifuge tubes and heated in a thermostatic shaking water bath (BS-11, Jeio Tech Co. Ltd., Korea) at temperatures of 60, 70, 80, 90, and 100 °C for exactly 20 min (excluding the time taken to reach the target heating temperature). Heating was then terminated by immediately cooling the tubes in an ice bath for 5 min. All samples were stored at room temperature until further analysis.

2.4. Confocal laser scanning microscopy (CLSM)

Microstructural characteristics of HPBs were examined using a confocal laser scanning microscope (Model Zeiss LSM900 with Airyscan 2, Carl Zeiss, Jena, Germany) equipped with a 63 × oil immersion objective. Samples (500 μL) were selectively stained with Fast Green FCF (30 μL, 1 mg/mL in Milli-Q water) to visualise proteins. Stained samples were placed on concave microscope slides, covered with glass coverslips, and viewed under the microscope. Fast Green FCF was excited with a helium-neon laser at 633 nm, and the emitted light was collected between 638 and 750 nm. Representative confocal micrographs were captured using Zeiss ZEN 3.1 (Blue Edition) imaging software (Carl Zeiss, Germany) and processed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.5. Determination of soluble protein content

Unheated and heated HPB suspensions were centrifuged at 10,000 rpm and room temperature for 20 min. The resulting supernatants were then diluted 10-fold with Milli-Q water prior to the analysis of protein content using the Pierce™ BCA protein assay kit, following the manufacturer's instructions. The soluble protein content of the HPB suspension was calculated using the following equation:

$$\text{Soluble protein content } (\%) = \frac{SP}{TP} x100 \quad (1)$$

Where *SP* is the protein content of the supernatant and *TP* is the total protein content of the suspension.

2.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of HPB suspensions, along with their respective supernatants and pellets, was performed before and after heating, following the method described by Manderson et al. (1998). Prior to SDS-PAGE, freshly prepared HPB suspensions (4 %, w/v) and their supernatants were diluted 1:20 and 1:2 with Milli-Q water, respectively. These samples were then mixed with sample buffer containing 62.5 mM Tris-HCl buffer (pH 6.8), 10 % glycerol, 2 % SDS, and 0.0025 % bromophenol blue at a 1:1 (v/v) ratio. The freeze-dried pellets were mixed with the sample buffer to achieve a final protein concentration of 0.1 % (w/v). For reduced SDS-PAGE, β-mercaptoethanol (β-ME) was added to the sample buffer at 5 % (v/v).

All samples were heated at 95 °C for 10 min. Then, 7 μL of each sample was loaded onto freshly prepared Tris-glycine gels and run at a constant voltage of 120 V for approximately 90 min. After electrophoresis, the gels were stained in a 0.3 % (w/v) Coomassie brilliant blue solution prepared in 20 % (v/v) isopropanol and 10 % (v/v) glacial acetic acid, and subsequently destained in a 10 % (v/v) isopropanol and 10 % (v/v) glacial acetic solution.

The destained gels were scanned using a molecular imager Gel Doc XR system (Bio-Rad Laboratories, Richmond, CA, USA). Densitometric analysis of protein bands was performed using ImageJ software. Relative band intensities (or proportions) of protein fractions compared to total proteins (expressed as a percentage) in each lane were calculated from the areas under the corresponding peaks on the densitograms. This analysis allowed relative comparisons of the protein composition across samples, independent of the total protein loaded.

2.7. Determination of surface hydrophobicity

The surface hydrophobicity of HPBs was measured using 1-anilino-8-naphthalene-sulfonate (ANS) as a hydrophobic fluorescence probe, following the method described by Cao et al. (2022). HPB suspensions (4 %, w/v) were serially diluted with a 10 mM sodium phosphate buffer (pH 7) to achieve final protein concentrations ranging from 0.005 to 0.02 % (w/v). An 8 mM ANS stock solution (pH 7) was prepared in the same buffer. For fluorescence measurements, 10 μL of the ANS solution was added to 2 mL of the diluted HPB sample. A 200 μL aliquot of this mixture was then transferred into a well of a black, clear-bottom 96-well microplate. Fluorescence intensity was recorded using a Varioskan™ LUX multimode microplate reader (Thermo Scientific, USA), with excitation set at 390 nm and emission at 470 nm, employing a slit width of 5 nm and a scanning speed of 5 nm/s. The buffer with ANS and HPB samples without ANS were used for control measurements. The index of protein surface hydrophobicity (H_O) was calculated from the initial slope of the plot of fluorescence intensity versus protein concentration, derived using linear regression analysis.

2.8. Determination of total free sulfhydryl (–SH) content

The total free –SH content was determined using Ellman's reagent, following the method described by Wang et al. (2022) with minor modifications. HPB suspensions (4 %, w/v) were diluted 10-fold with Milli-Q water. In a 2 mL microcentrifuge tube, 0.4 mL of the diluted protein suspension was mixed with 1.2 mL of Tris-glycine buffer (pH 8.0, containing 86 mM Tris, 90 mM glycine, 4 mM EDTA, and 2.5 % SDS). To this mixture, 21.3 μL of Ellman's reagent (4 mg DTNB (5'-,5-dithiobis-(2-nitrobenzoic acid)) dissolved in 1 mL of Tris-glycine buffer) was added. The resulting mixture was incubated at room temperature for 1 h on an orbital shaker in the dark, then centrifuged at 10,000 rpm for 15 min. The absorbance of the supernatant was measured at 412 nm using a UV–vis spectrophotometer (Genesys 10S, Thermo Scientific, USA). A solution containing only the Tris-glycine buffer and Ellman's reagent was used as a blank control. The total free –SH content (μmol/g of protein) was calculated using the following equation:

$$-SH\ (\mu mol/g) = \frac{D \times A_{412} \times 10^6}{\varepsilon \times C} \quad (2)$$

Where A_{412} is the absorbance at 412 nm, D is the dilution factor, C is the concentration of the HPB suspension (mg/mL), and ε is the molar absorptivity constant (1.36×10^4 mol/(L.cm)).

2.9. Trypsin-catalysed hydrolysis of HPBs

Hydrolysis of HPBs was characterised using trypsin. Enzyme solution was prepared by dissolving trypsin in a phosphate buffer (50 mM, pH 7). Unheated or heated HPB suspension (4 % w/v, pH 7) was pre-incubated in a shaking water bath at 37 °C for 10 min. Following this, the pre-heated trypsin solution was added to the HPB suspension to achieve an enzyme/substrate ratio of 1:200 (w/w). The resulting reaction mixture was incubated at 37 °C, 170 rpm, and pH 7. A combination of trypsin and pH 7 was selected to minimize any potential effects of pH on the structure of HPBs, while remaining within the optimal range for trypsin activity (pH 7–9, according to the manufacturer's specifications). Controls (test protein in reaction buffer without trypsin) and blanks (trypsin in reaction buffer without test protein) were also prepared and subjected to the same hydrolysis conditions to assess the chemical stability of PBs and the auto-digestion of trypsin, respectively.

Aliquots of hydrolysates were withdrawn from the reaction mixture at various time intervals (0, 1, 5, 10, 15, 30, 45, 60, 90, and 120 min). A set of aliquots (100 μ L) was immediately transferred to Eppendorf tubes containing 150 μL of Milli-Q water and 250 μL of reducing sample buffer, resulting in a final protein concentration of 1 mg/mL for Tris-Tricine SDS-PAGE. These reducing samples were heated at 95 °C for 10 min prior to gel electrophoresis. Additionally, another set of aliquots (100 μ L) was diluted to 0.5 mL with Milli-Q water in Eppendorf tubes and heated in a boiling water bath for 5 min to stop trypsin activity. These samples were then snap frozen and stored at −20 °C until OPA analysis.

2.10. Degree of protein hydrolysis

The degree of protein hydrolysis (DH) was determined using the o-Phthaldialdehyde (OPA) spectrophotometric assay, as described by Nielsen et al. (2001) with minor modifications. This assay is based on the colorimetric reaction between free amino groups released during protein hydrolysis and the OPA reagent. A fresh OPA reagent was prepared in Milli-Q water, containing final concentrations of 3.81 % sodium tetraborate decahydrate, 0.1 % SDS, 0.08 % OPA (pre-dissolved in 2 % ethanol), and 0.088 % dithiothreitol. The reagent was protected from light by covering it with aluminium foil and was used immediately. A reference standard curve was constructed using L-serine at concentrations ranging from 12.5 to 100 mg/L in Milli-Q water.

Prior to the assay, 0.83 mL of 5 % (w/v) trichloroacetic acid (TCA) solution was added to 0.5 mL of hydrolysate samples. The mixtures were then vortex-mixed and centrifuged at 10,000 rpm and room temperature for 30 min to precipitate intact and unhydrolysed proteins (Zahir et al., 2018). The resulting supernatants, containing small peptides and amino acids, were used for the assay. Next, 200 μL of the OPA reagent was added to 20 μL of the hydrolysate supernatants, L-serine standards, and Milli-Q water (blank) in wells of a 96-well polystyrene microplate. The contents of the wells were mixed using a digital microtiter shaker (IKA-Schüttler MTS 4, Janke & Kunkel GmbH & Co KG IKA-Labortechnik Staufen, Germany) and allowed to stand for 2 min in the dark at room temperature. The absorbance was then measured at 340 nm using a microplate reader (Synergy 2, BioTek Instruments, Agilent Technologies, USA).

The absorbance values were converted to concentrations of free amino groups (meqv serine-NH_2/L) based on the standard curve. The DH (%) was calculated using the following equation:

$$DH\% = \frac{NH_{2T} - NH_{2T0}}{NH_{2TOT} - NH_{2T0}} x100 \quad (3)$$

where NH_{2T} and NH_{2T0} are the concentrations of free amino groups in samples at time t and time 0 of hydrolysis, respectively, and NH_{2TOT} is the total content of free amino groups in samples after complete hydrolysis with 6M HCl at 110 °C for 24 h.

2.11. Kinetic analysis of trypsin hydrolysis of HPBs

The kinetics of trypsin hydrolysis of HPBs were evaluated using SDS-PAGE followed by densitometric analysis. Densitometric measurements of protein bands on Tricine SDS-PAGE gels were conducted using ImageJ software. The intensity of the protein bands was monitored over the 120-min hydrolysis period, and the experimental data was fitted to a three-parameter exponential decay model using SigmaPlot 15.0 (Systat software, IL, USA). The model is represented by the following equation:

$$[I_t] + B = [I_0]e^{-kt} + B \quad (4)$$

Where $[I_t]$ denotes the relative intensity of the protein band at time t, $[I_0]$ denotes the initial intensity of the protein band at 0 min, k is the first-order hydrolysis rate constant (min^{-1}), and B indicates the background density parameter at the corresponding migration distance on the gel.

To minimize variations among three replicate gels, the band intensity at time t was normalised to the initial intensity at 0 min. This normalisation approach allowed the calculation of % residual protein band ($=[I_t]/[I_0] \times 100$) for effective plotting of hydrolysis data (He et al., 2015).

3. Results and discussion

3.1. Effect of heating on HPB morphology

CLSM examination of HPB suspensions, subjected to various heat treatments (60–100 °C, 20 min) at pH 7, revealed differences in their aggregation behaviour (Fig. 1). Our previous study showed that, all pH levels (2–13), except neutral pH, induced varying degrees of HPB aggregation (Do et al., 2024). Therefore, pH 7 was chosen for all heating experiments to eliminate the influence of this extraneous variable and better understand the true effect of heating on HPB aggregation. However, this pH inevitably caused some structural damage to the HPBs. Specifically, Fig. 1A shows an unheated suspension containing spherical HPBs (~5 μm in diameter) alongside some smaller protein particles. These particles were likely formed as a consequence of the rupture of intact HPBs. Some HPBs appeared to have lost their internal contents, possibly due to the solubilisation of albumin-rich proteinaceous matrices and edestin crystalloids (indicated by white circles). Similar observations of isolated HPBs dispersed in water at neutral pH have been previously reported (Do et al., 2024).

Heat treatments altered the aggregation behaviour of HPBs in suspension systems. Specifically, a moderate temperature of 60 °C did not induce significant HPB aggregation (Fig. 1B). HPBs began to form loose clumps at 70 °C (Fig. 1C), although many remained as separate individual particles. Discrete clusters were observed at 80–90 °C (Fig. 1D and E), which further agglomerated into larger aggregates at 100 °C (Fig. 1F), indicating that higher temperatures increased the extent of HPB aggregation. Additionally, HPBs largely retained their original spherical/globular shape after heating up to 90 °C. However, at the elevated temperature of 100 °C, some HPBs appeared to have lost their initial structure, with their borders becoming indistinguishable from those of neighboring particles (Fig. 1F).

These microscopic observations support previous studies (Batterman-Azcona & Hamaker, 1998; Gulati et al., 2018; Rom et al., 1992), which indicated that heat treatment caused PB aggregation but had little influence on PB structure in some cereals. Gulati et al. (2018) observed, using CLSM and scanning electron microscopy (SEM), the formation of large aggregates of millet PBs through hydrophobic associations upon cooking in water (100 °C, 20 min). Interestingly, the heat-treated millet PBs exhibited no apparent morphological changes, possibly because the storage proteins remained intact and were mostly confined to rigid crystalloids. Similarly, cooking in water (100 °C, 20 min) did not alter the shape of PBs present in both corn and sorghum when viewed under SEM (Batterman-Azcona & Hamaker, 1998; Rom et al., 1992)

3.2. Heat-induced aggregation of proteins in HPBs

The effect of heating on protein aggregation was investigated using Glycine SDS-PAGE (Fig. 2). Under non-reducing conditions, Fig. 2A clearly shows a progressive reduction in the intensity of the edestin band (AS–BS, ~52 kDa) as the temperature increased, suggesting that heating caused extensive aggregation of edestin. A dramatic reduction in the band intensity was particularly noticeable at 80 °C. Continued heating to 100 °C resulted in complete disappearance of this band, indicating that virtually all edestin was present in the form of high-MW protein aggregates and could not enter the stacking gel. Under reducing conditions, these aggregates completely dissociated into three clearly visible bands: the AS (~34 kDa) and the BS (~20 and 18 kDa) (Fig. 2B). Additionally, a small portion of albumin was also detected, evidenced by the appearance of polypeptide bands with a MW of <20 kDa on the gels. Taken together, these findings suggest that the heat-induced aggregation of proteins in HPBs was primarily driven by disulphide bond formation in edestin.

Our SDS-PAGE results are consistent with those previously reported in HPI (Raikos et al., 2015) and corroborate earlier CLSM findings (Fig. 1), showing that protein aggregation in HPBs occurred to a significant extent at temperatures above 80 °C. Notably, although HPBs appeared to remain largely intact after heating at 90–100 °C (Fig. 1E and F), the edestin contained in compact crystalloids had undergone extensive aggregation (Fig. 2A). Similarly, Batterman-Azcona and

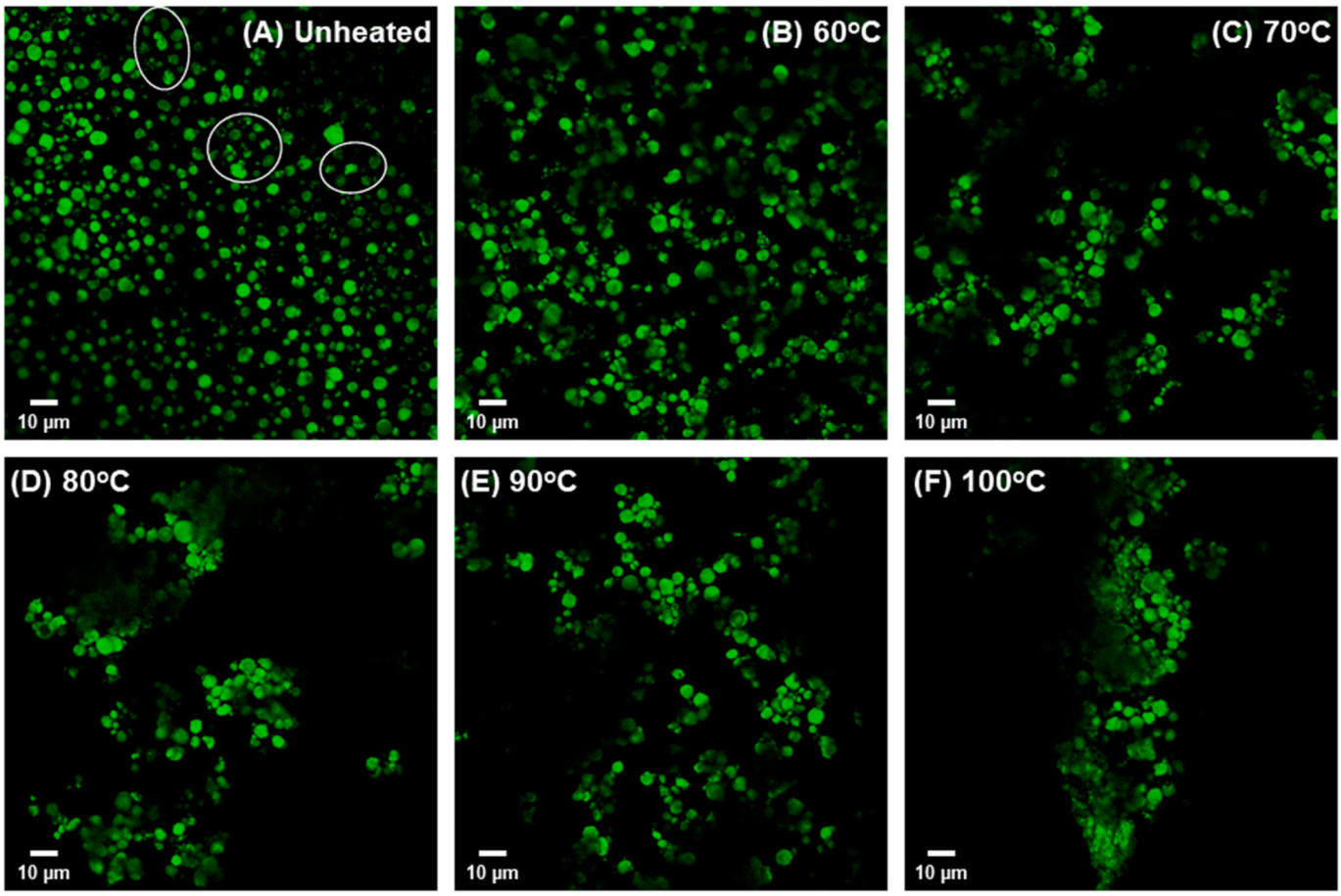

Fig. 1. Representative CLSM images of HPB suspensions before (A) and after heating at (B) 60 °C, (C) 70 °C, (D) 80 °C, (E) 90 °C, and (F) 100 °C. Proteins were stained green with Fast Green FCF. Scale bar = 10 μm. White circles indicate areas in the unheated suspension where HPBs have ruptured or lost their internal contents.

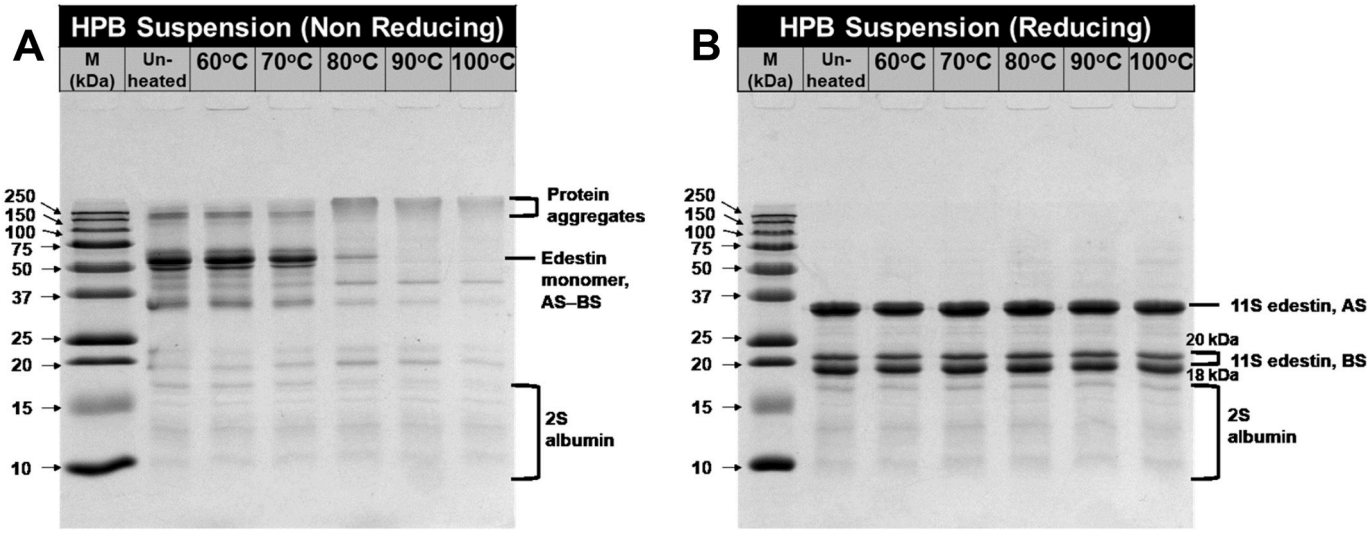

Fig. 2. Non-reduced SDS-PAGE (A) and reduced SDS-PAGE (B) of unheated and heated HPB suspensions. Abbreviations: M, molecular weight marker in kDa; AS–BS, edestin; AS, acidic subunit; BS, basic subunit.

Hamaker (1998) demonstrated the formation of large disulphide-linked protein aggregates on SDS-PAGE after cooking corn flour in boiling water for 20 min, despite no apparent physical deformations of corn PBs as observed by transmission electron microscopy (TEM). It was proposed that zeins (the storage proteins of corn) are neither static nor always confined to PB crystalloids; instead, they can undergo significant structural modifications during heating. Additionally, Hamaker, Kirleis, Butler, Axtell, and Mertz (1987) proposed that the structural strengthening at the surface and interior of PBs in sorghum during cooking is due to the formation of disulphide bonds in kafirins (the storage proteins of

sorghum). This hypothesis supports our observations that most HPBs did not dissociate and retained their structural integrity after heating.

To further explore the molecular interactions driving the heat-induced aggregation of proteins in HPBs, we examined the effect of heating on their surface hydrophobicity (H_O) and total free –SH content, as shown in Fig. 3.

Changes in H_O are often employed to predict protein conformational changes, and monitoring changes in fluorescence intensity due to ANS binding to exposed hydrophobic sites on protein surfaces provides valuable insights into the thermal denaturation and aggregation of proteins. As expected, the H_O of HPBs increased steadily with increasing temperatures (60–80 °C), reaching a maximum at 80 °C (Fig. 3A). This increase is likely attributed to the heat-induced unfolding of proteins on HPB surfaces. However, when the temperature was further raised from 80 to 100 °C, a pronounced decline in H_O was observed, probably related to the formation of HPB protein aggregates. These findings extend those of previous studies (Shen & Tang, 2012; Wang et al., 2014), which demonstrated that heating unfolds compact, native globular proteins, exposing hydrophobic residues initially buried in the protein's interior and causing a corresponding increase in H_O. Denatured (unfolded) proteins, being thermodynamically unstable, tend to associate with one another to form soluble or insoluble aggregates stabilised by hydrophobic forces, leading to a decrease in H_O.

As previously demonstrated by SDS-PAGE (Fig. 2), we also identified disulphide bonds as the main covalent interaction involved in the formation of HPB protein aggregates. To substantiate this finding, we investigated changes in the total free –SH content of HPBs in response to increased heating temperature. As illustrated in Fig. 3B, the total –SH content of unheated HPBs was 32.02 ± 0.18 μmol/g, which falls within the range previously reported for hemp proteins (32.82–34.85 μmol/g) and is considerably higher than that of soy or pea proteins (Kahraman et al., 2022; Xu et al., 2022). The relatively higher –SH content in hemp proteins is associated with a greater level of sulphur-containing amino acids (cysteine) and a stronger tendency for covalent aggregation through disulphide bonds (Tang et al., 2006; Xu et al., 2022).

It was evident from Fig. 3B that heat treatments up to 70 °C progressively decreased the total –SH content of unheated HPBs. Heating HPBs at 80 °C led to a sharp reduction (~70 %) in the total –SH content, which continued to drop only slightly with further heating from 80 to 100 °C. These results may indicate heat-induced formation of disulphide (S–S) bonds in HPBs through SH oxidation and/or SH/S–S interchange reactions, thereby reducing the amount of detectable free –SH groups (B. Wang et al., 2022). We postulate that disulphide bond formation occurs primarily within the edestin crystalloids (intra-HPB protein aggregation), and to a lesser extent, at their outer surfaces (inter-HPB protein aggregation).

Our quantitative data presented here highlight the involvement of both hydrophobic forces and disulphide linkages in the thermal denaturation and aggregation of HPB proteins. This was particularly evident when HPBs were heated at temperatures ≥80 °C, as reflected in distinct changes in their H_O and total free –SH content, consistent with earlier SDS-PAGE and CLSM results. Our results are strongly supported by the differential scanning calorimetry (DSC) data for edestin, as reported by Tang et al. (2006), with onset and peak denaturation temperatures of 86 and 95 °C, respectively.

3.3. Heat-induced dissociation-association behaviour of HPB protein fractions

Glycine SDS-PAGE was used to visualise and quantify the distribution of proteins between the pellet and supernatant following the centrifugation of unheated or heated HPB suspensions (Fig. 4). To account for differences in protein concentration, the uncentrifuged suspension was diluted 40-fold, while the supernatant was diluted only 4-fold prior to loading. The same dilution factor was applied to all supernatant samples, ensuring that the amount of protein loaded onto the gel was proportional to the protein concentration in the supernatant.

Under non-reducing conditions (Fig. 4A), two major protein bands present in the supernatant of unheated HPBs were identified as edestin (~52 kDa) and albumin (<20 kDa), both of which had been released from HPBs and remained non-sedimentable after centrifugation. Visual inspection of their electrophoretic patterns revealed that the edestin band progressively disappeared from the supernatant, while the albumin band appeared to increase in intensity with heating. Additionally, stained materials remaining in the wells and appearing on top of the resolving gel were clearly observed at temperatures ≥80 °C, indicating the formation of high-MW protein aggregates that did not sediment under the centrifugation conditions used. It is important to note that this observation strongly depends on the applied centrifugation conditions, as higher centrifugal force/longer times may result in the sedimentation of these protein aggregates as well.

Reduced SDS-PAGE (Fig. 4B) showed the presence of five major HPB protein fractions in the supernatant: (1) 7S vicilin-type globulin (~48 kDa); subunits of 11S legumin-type globulin (edestin), namely (2) AS ~34 kDa, (3) BS ~20 kDa, and (4) BS ~18 kDa; and (5) 2S albumin polypeptides (<20 kDa). Densitometric analysis of the relative proportions (percentage distribution) of these HPB protein fractions as a function of heating temperature revealed two opposite trends, as illustrated in Fig. 5. Specifically, the proportions of both the 7S globulin and the BS of edestin decreased significantly, whereas the proportions of

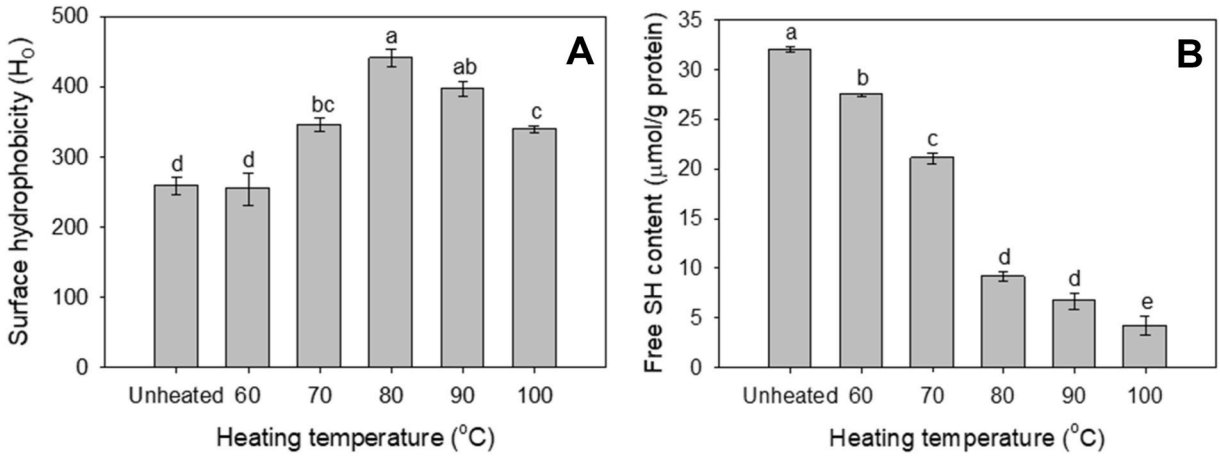

Fig. 3. Surface hydrophobicity (A) and total free sulfhydryl (–SH) content (B) of unheated and heated HPBs. Error bars represent the standard deviation. Different letters within each plot indicate significant differences at $p < 0.05$, as determined by Tukey's significant difference test.

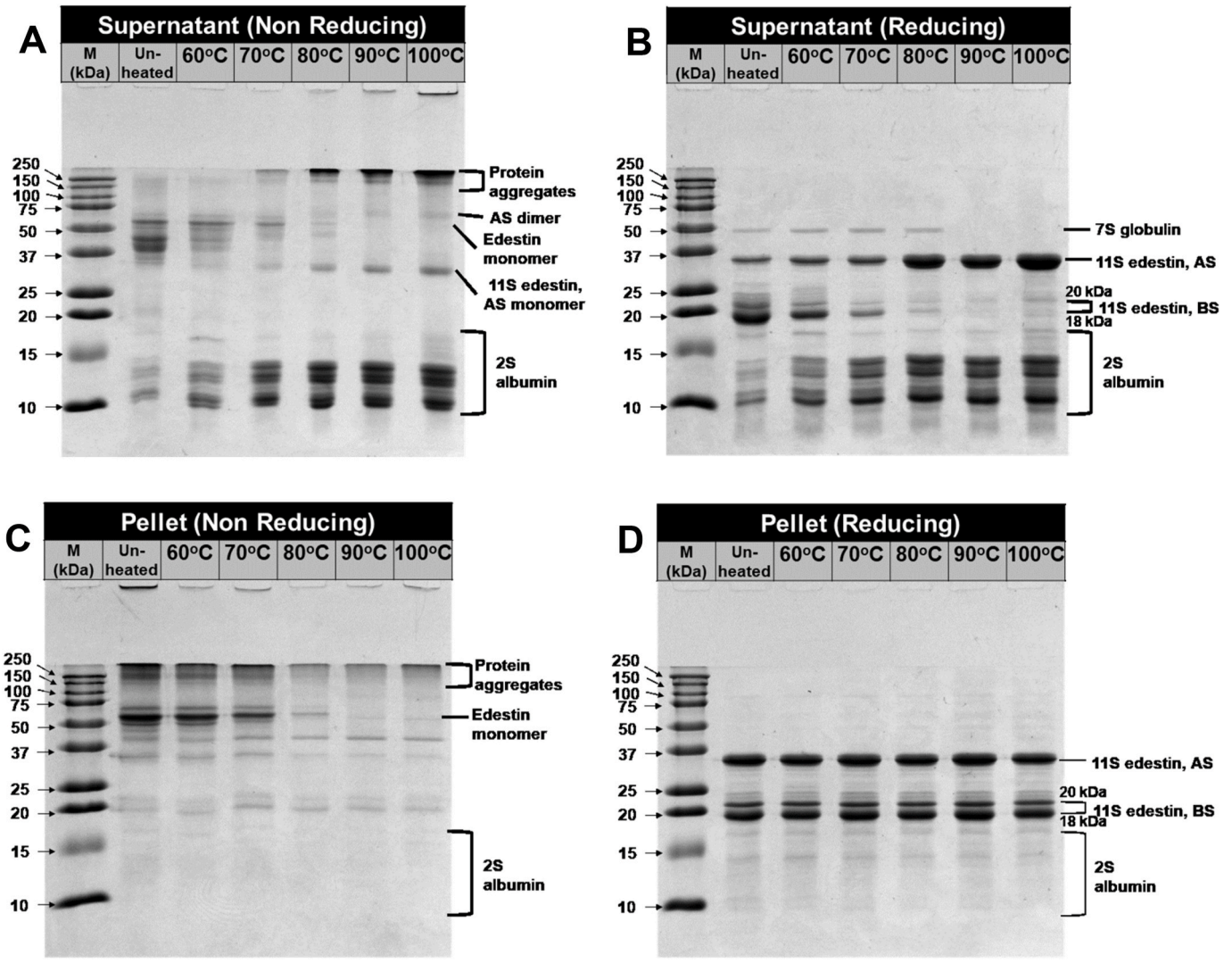

Fig. 4. Non-reduced and reduced SDS-PAGE of (A and B) supernatant fractions and (C and D) pellet fractions, separated by centrifugation of HPB suspensions. Abbreviations: M, molecular weight marker in kDa; AS–BS, edestin; AS, acidic subunit; BS, basic subunit.

both the 2S albumin and the AS of edestin increased significantly upon heating HPBs above 80 °C. This suggests the formation of heat-induced aggregates of the former fractions, which predominantly migrated to the pellet, and the concomitant release of the latter fractions into the supernatant.

Furthermore, the electrophoretic patterns of the pellet (Fig. 4C and D) followed trends similar to those observed in the uncentrifuged suspension (Fig. 2A and B) under both non-reducing and reducing conditions. This suggests that the pellet, composed of insoluble HPB proteins, constituted the bulk of the uncentrifuged suspension. The distribution of proteins between the supernatant and the pellet cannot be directly calculated from the SDS-PAGE results but can be quantitatively determined using the BCA protein assay. The soluble protein (SP) content was calculated as the protein content of the supernatant relative to the total protein content of the HPB suspension and plotted as a function of heating temperature (60–100 °C) in Fig. 6.

As shown in this figure, heating at temperatures ≤90 °C did not significantly alter the SP content. Only the heat treatment at 100 °C increased the SP content ($n = 3$, $p < 0.05$), from 9.2 ± 0.7 % (unheated) to 12.0 ± 0.9 % (heated). These results suggest that the release of the AS of edestin and albumin from HPBs into the supernatant during heating may compensate for the loss in protein solubility caused by the thermal aggregation of the BS of edestin and 7S globulin already present in the aqueous phase, resulting in no significant change in SP content. At 100 °C, the former effect would dominate, leading to an overall increase in SP content. However, this increase (~2.8 %) was rather small in magnitude and should be interpreted with caution. Interestingly, Sorgentini, Wagner, and Anon (1995) found that centrifuging heat-treated aqueous dispersions of native soy protein isolate (5–15 % w/w, 100 °C for 30 min) produced soluble fractions that were depleted of the BS of glycinin and subunits of 7S globulin but highly enriched in the AS of glycinin and albumin.

The observed opposite trends in the solubilisation/aggregation behaviour of the different HPB protein fractions may reflect differences in their intrinsic hydrophilicity/hydrophobicity characteristics, which are influenced by their amino acid composition. Based on published data on edestin subunit composition, both the 20 kDa BS and 18 kDa BS of edestin contain a higher proportion of hydrophobic amino acids (HAAs)—such as alanine, proline, valine, methionine, and phenylalanine—compared to the AS of edestin (Kim & Lee, 2011). This supports our SDS-PAGE results (Fig. 4B), which show that the AS remained soluble in the supernatant even at high temperatures, despite its dissociation from the oligomeric structure of edestin, whereas the more hydrophobic BS aggregated readily during heating.

The thermal behaviour of the edestin subunits parallels that of the 11S globulin subunits in soybeans and legumes (Carbonaro et al., 1997;

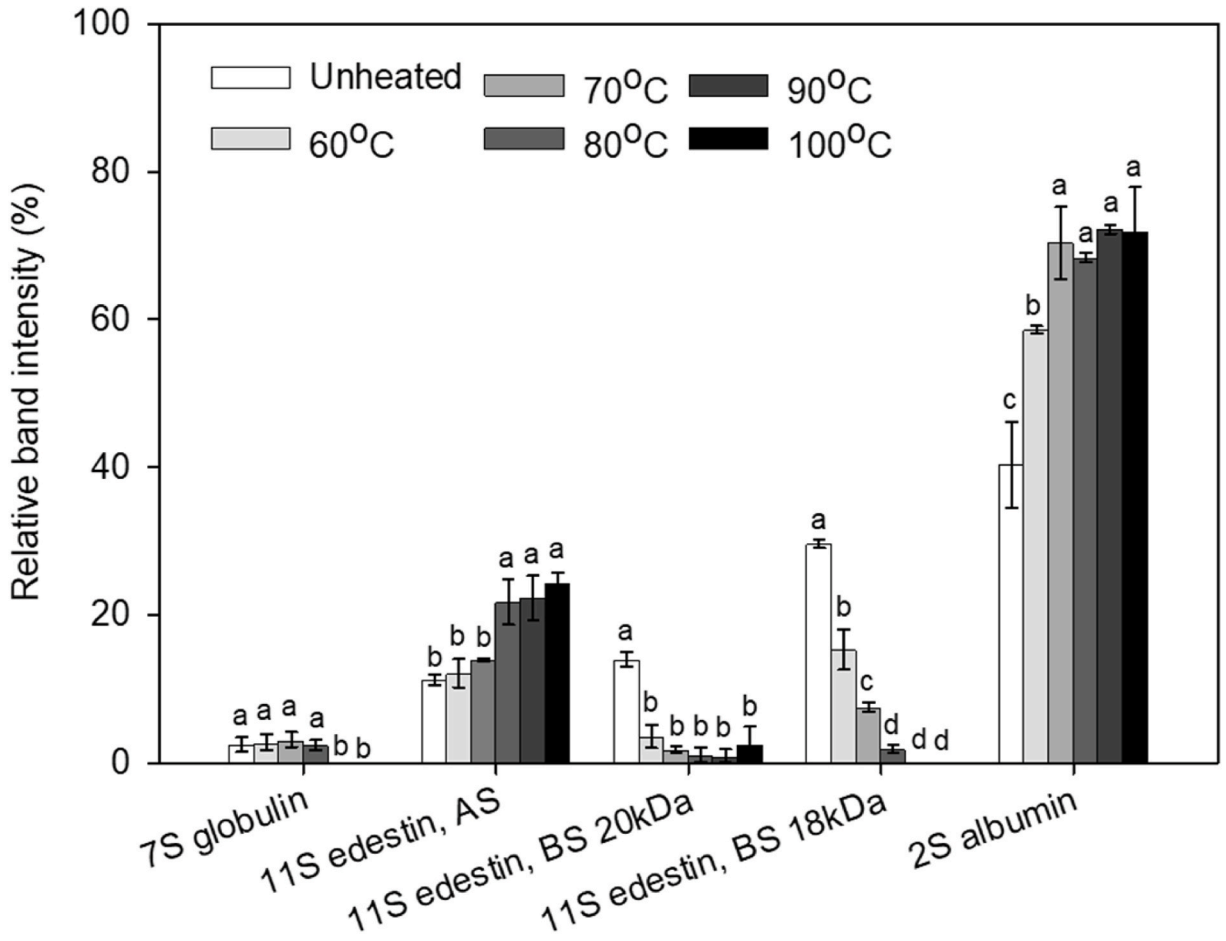

Fig. 5. Changes in the relative proportions (%) of individual protein fractions in the supernatant, obtained by centrifuging HPB suspensions, as a function of heating temperature. Error bars represent the standard deviation. Different letters within the same HPB protein fraction indicate significant differences at $p < 0.05$, as determined by Tukey's significant difference test.

Yamagishi et al., 1980). According to Carbonaro et al. (1997), the protein fraction that retained solubility in water after cooking legumes (120 °C, 20 min) exhibited a very high charge density, characterised by high levels of arginine and glutamic acid, and low levels of HAAs. This fraction likely corresponded to the AS of 11S globulin due to its hydrophilic character, as indicated by its amino acid composition. Yamagishi et al. (1980) further suggested that the BS of soybean 11S globulin (glycinin), which has a higher proportion of HAAs than the AS of glycinin, is stabilised by the hydrophilicity of the AS in an aqueous system. The release of the AS from the oligomeric glycinin structure upon heating may expose the BS to the aqueous environment, making them more susceptible to aggregation through hydrophobic interactions and disulphide bonds.

Similar to the BS of edestin, hemp 7S globulin is characterised by a high percentage of HAAs (45–47 %) (Eckhardt et al., 2024; Sun et al., 2021). This high HAA content may enhance its propensity for complexation with the BS of edestin through strong hydrophobic interactions and disulphide bonds (Wang et al., 2008). This interaction may explain the total disappearance of the 7S globulin band when heated above 80 °C (Fig. 4B). These findings complement earlier reports on soybean proteins (Petruccelli & Anon, 1995; Wang et al., 2014), which indicated that heating at neutral pH drives hydrophobic association and subsequent disulphide bonding between the β subunit of 7S globulin (β-conglycinin) and the BS of 11S globulin (glycinin), triggering the formation of insoluble protein aggregates.

In contrast, albumin in hemp seeds generally has a lower content of aromatic amino acids and HAAs and exhibits higher solubility compared to hemp globulins (Malomo & Aluko, 2015). This may explain why 2S albumin remained unaffected in the presence of the reducing agent and did not aggregate like 7S and 11S globulins (Fig. 4A and B). Instead, albumin appeared to partially leach from HPBs into water during heating, as evidenced by the observed increase in its relative proportion in the supernatant with increasing temperature (Fig. 5). Similarly, Van de Vondel, Lambrecht, and Delcour (2022) found that 2S albumin in quinoa seeds did not aggregate but partially leached into water during boiling.

Moreover, as mentioned above, non-sedimentable protein aggregates, along with several types of soluble protein species, were detected electrophoretically in the supernatant by non-reduced SDS-PAGE after heating HPBs at temperatures between 80 and 100 °C (Fig. 4A). Yamagishi et al. (1980) reported that heating causes the release of AS from its association with BS in the native oligomeric structure of soybean glycinin. This process is accompanied by the self-assembly of AS into dimers or higher oligomeric aggregates through disulphide bonds. Based on these findings, it can be inferred that the soluble proteins in the supernatant, after separation from the HPB pellet, possibly consisted of monomer (~34 kDa), dimer (~68 kDa), and oligomers of the AS of edestin, which could be identified in Fig. 4A by their electrophoretic mobility.

Overall, our analysis of the solubilisation/aggregation behaviour of the different HPB protein fractions suggests heat-induced dissociation-association of proteins in HPBs—a phenomenon that will be discussed in detail in Section 3.5.

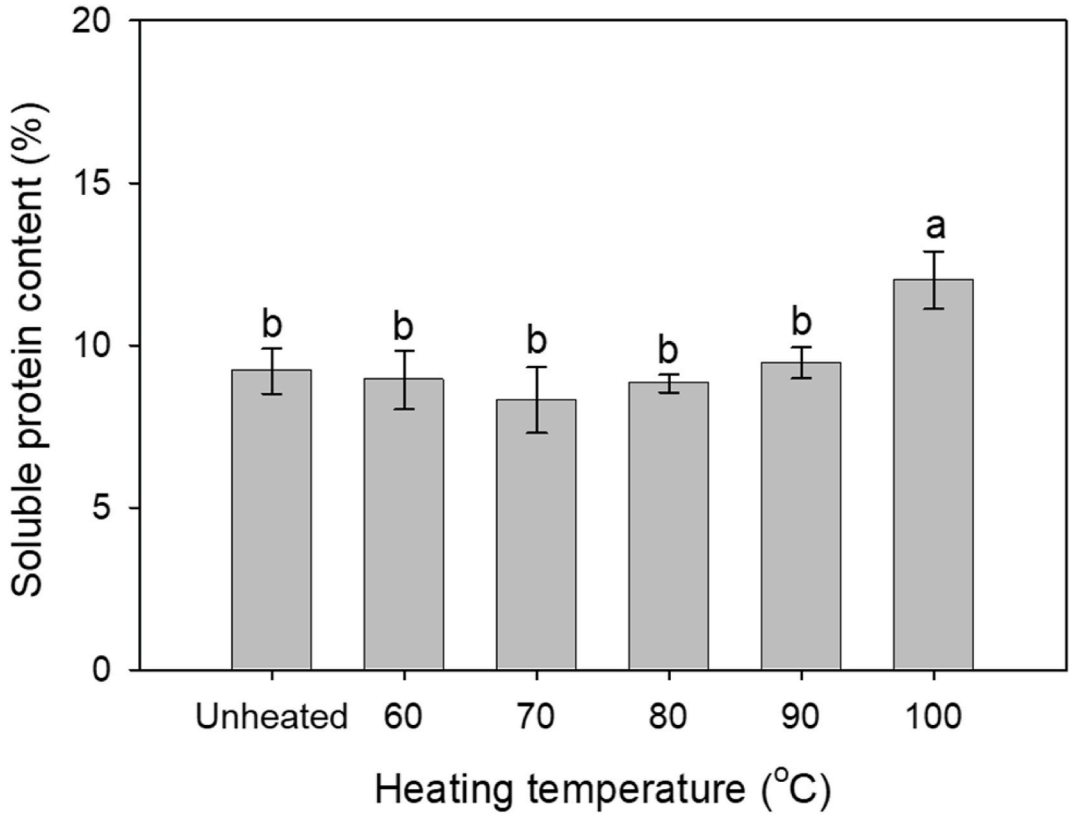

Fig. 6. Changes in soluble protein content of HPB suspension (%) as a function of heating temperature. Error bars represent the standard deviation. Different letters indicate significant differences at $p < 0.05$, as determined by Tukey's significant difference test.

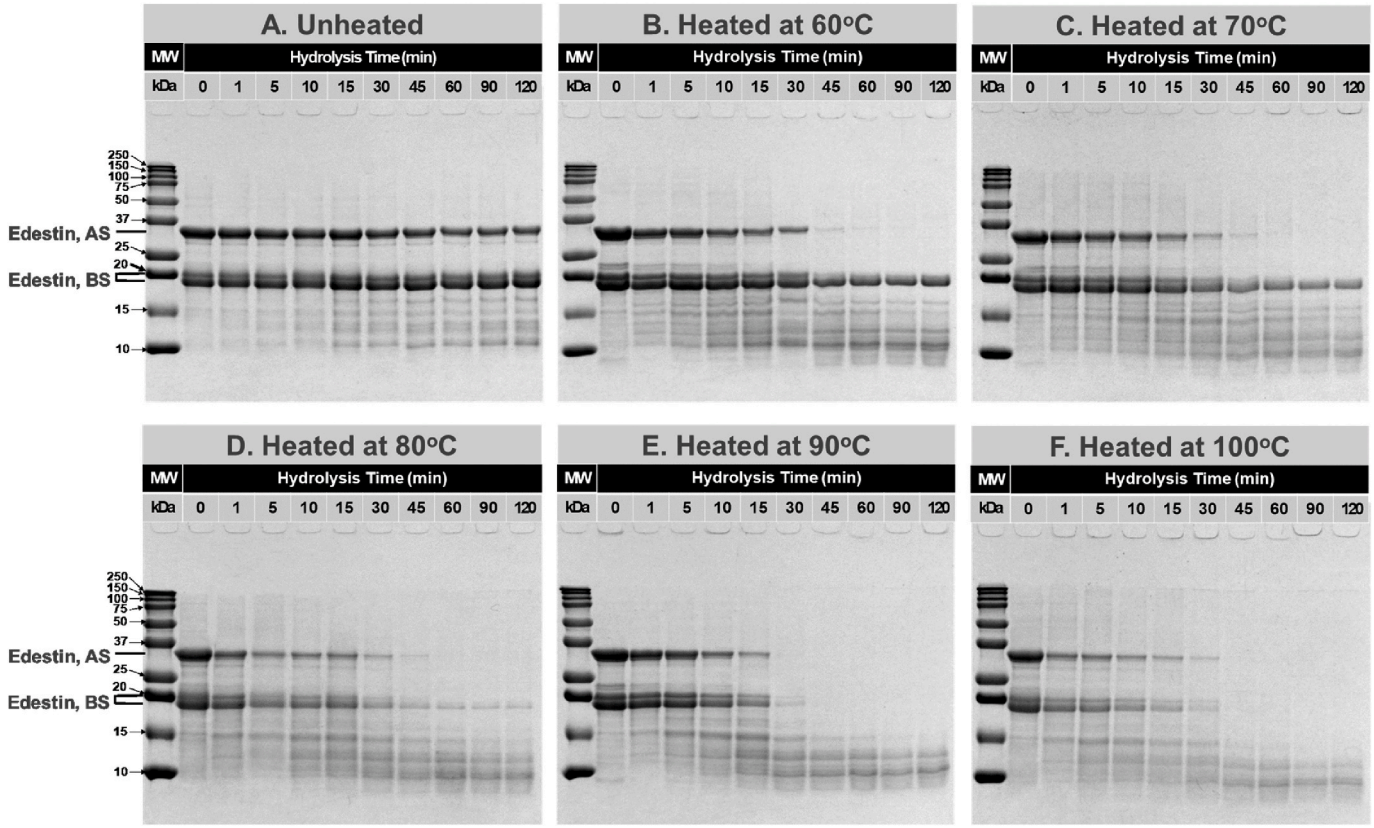

Fig. 7. Reduced SDS-PAGE analysis of trypsin hydrolysis of unheated and heated HPB suspensions: (A) unheated, (B) 60 °C, (C) 70 °C, (D) 80 °C, (E) 90 °C, and (F) 100 °C. Lane MW shows molecular weight markers in kDa. The number above each lane indicate the duration of hydrolysis (min).

3.4. Effect of heating on trypsin hydrolysis of HPBs

The trypsin-catalysed hydrolysis of HPBs was monitored over a period of 120 min using reduced Tricine SDS-PAGE (Fig. 7). Significant differences in the degradation patterns of two prominent protein bands of HPBs—the AS (~34 kDa) and the BS (~18–20 kDa) of edestin—were visually observed between samples subjected to different heat treatments.

The unheated sample exhibited considerable resistance to complete proteolysis, as evidenced by only a slight decrease in the intensity of the AS band and minimal change in the intensity of the BS band after 120 min of hydrolysis (Fig. 7A). In contrast, all heated samples exhibited much greater susceptibility to proteolysis, as indicated by the marked decreases in the intensities of both subunit bands during hydrolysis (Fig. 7B–F). Specifically, HPBs heated at 60–80 °C showed almost complete disappearance of the AS band and a sharp decrease in the intensity of the BS band after 60 min of hydrolysis (Fig. 7B–D). Further heating of HPBs to 90 or 100 °C resulted in rapid and complete disappearance of both edestin subunit bands after only 30 min of hydrolysis (Fig. 7E and F). In all samples, as the hydrolysis progressed, decreases in the intensity of the edestin subunit bands were accompanied by substantial increases in the intensity of protein bands with MWs lower than that of the BS (<18 kDa). These SDS-PAGE results indicate that heating significantly improved the hydrolysis of HPBs by trypsin, and this effect was further enhanced with increasing temperature.

Using densitometry, the intensities of residual protein bands at each time point of interest during hydrolysis were quantitatively determined. This allowed us to construct plots of the percentage of the AS or BS remaining intact over time (Fig. 8A and B) to trace the kinetics of proteolysis. The kinetic data were fitted to a first-order exponential decay model. The R^2 values for the AS and BS hydrolysis curve fits were above 0.85 for all samples (results not shown), except for the BS of the unheated sample ($R^2 = 0.022$), which was due to its complete resistance to trypsin cleavage throughout the entire 120 min of hydrolysis.

As shown in Fig. 8A and B, the fitted decay curves closely followed the experimental data, confirming the high R^2 values. The first-order hydrolysis rate constants (k) derived from the exponential curve fitting are presented in Fig. 8C. A comparison of the rate constants, both within and across subunits, revealed two key trends: (1) k generally

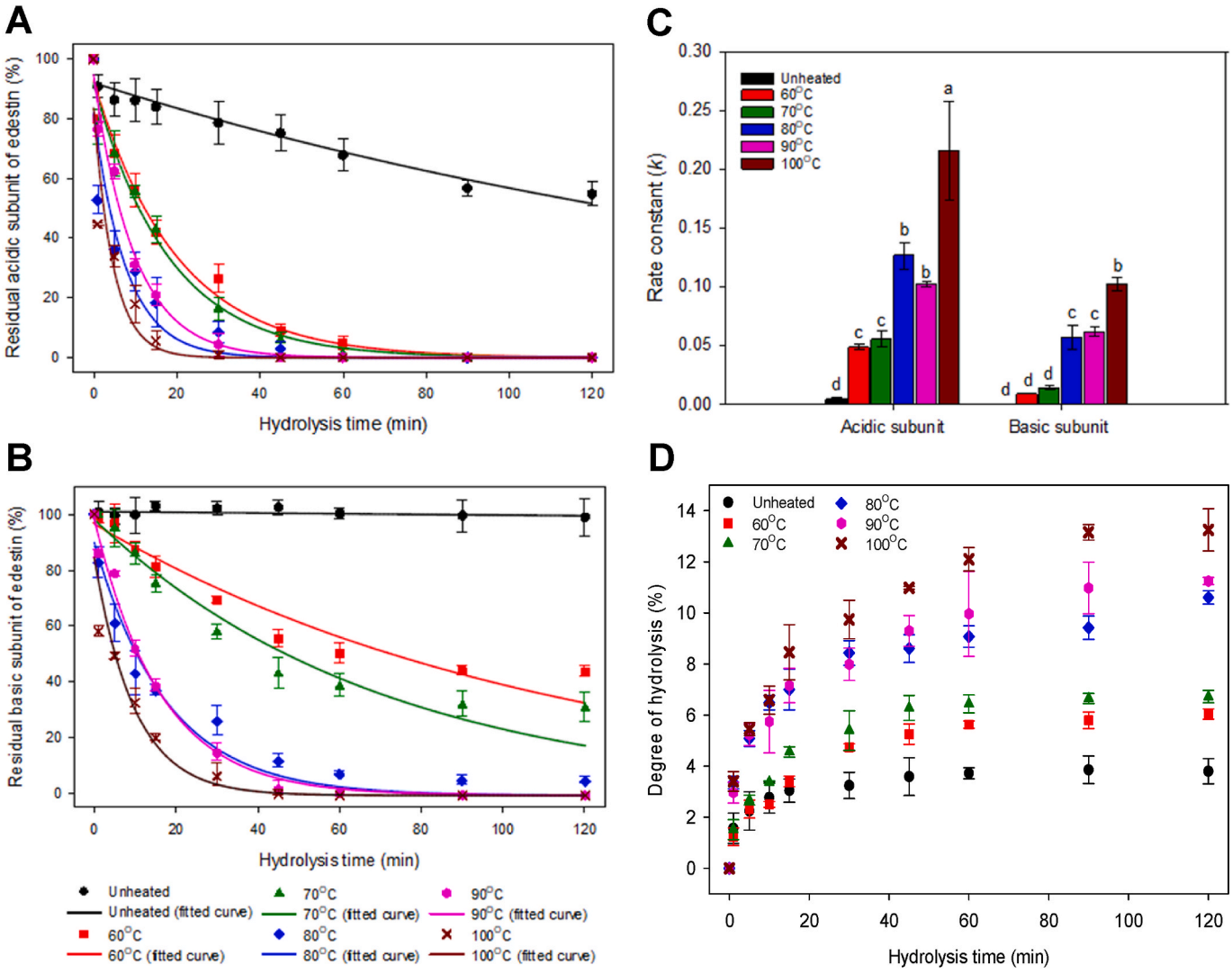

Fig. 8. Densitometric quantification of the acidic subunit (A) and the basic subunit (B) of edestin remaining (%) after trypsin hydrolysis of unheated HPBs (●) and HPBs heated at 60 (■), 70 (▲), 80 (◆), 90 (●), and 100 °C (×). Fitted exponential decay curves for the time-dependent hydrolysis of HPBs are represented by solid lines. (C) Comparison of hydrolysis rate constants (k) for the acidic subunit and the basic subunit of edestin in unheated HPBs (■) and in HPBs heated at 60 (■), 70 (■), 80 (■), 90 (■), and 100 °C (■). Different letters indicate significant differences at $p < 0.05$, as determined by Tukey's significant difference test. (D) Changes in the degree of hydrolysis (%), estimated using the o-Phthaldialdehyde assay, as a function of time during trypsin hydrolysis of unheated and heated HPBs. Error bars represent the standard deviation.

increased with higher heating temperatures for the same subunit, and (2) the *k* value for the AS was significantly higher than that for the BS at the same heating temperature. The degree of hydrolysis (DH) curves (Fig. 8D) exhibited trends similar to those observed in the proteolysis kinetics of HPBs, as derived from SDS-PAGE and quantitative densitometry (Fig. 8A and B).

The kinetic data presented here provide new insights into the susceptibility of HPBs to proteolysis. The resistance of unheated HPBs to trypsin appears to be largely ascribed to the stability of their native conformations at pH 7, at which the proteolysis was carried out. At this pH, the presence of HPB structural barriers, including intact membranes and albumin-rich proteinaceous matrices, could impede access of trypsin to edestin contained within crystalloids during hydrolysis. Additionally, the constrained packing of edestin molecules within crystalloids and the highly ordered edestin hexamer structure (Patel et al., 1994) may also restrict the availability of cleavage sites for trypsin. Moreover, phytic acid deposited in globoids may play a role in limiting the rate of protein hydrolysis by forming trypsin-resistant complexes with edestin (Wang & Guo, 2021). Although this is outside the scope of the current study, it could have a significant influence on protein digestion and absorption, which would warrant further investigation.

The HPB structural barriers could be partially or completely disrupted upon heating, which consequently improved the rate and extent of hydrolysis of the edestin subunits. In fact, heating HPBs in water may have caused disruption of their intact structures and the release of albumin from the proteinaceous matrix into the aqueous phase, as suggested by our earlier SDS-PAGE results (Fig. 4A and B). We also propose that heating may have disrupted the edestin hexamer structure and denatured the monomeric subunits. This process may have unfolded polypeptide chains originally packed into compact, native protein conformations, thereby exposing susceptible bonds to cleavage by trypsin.

Yin et al. (2008) reported similar observations for the trypsin hydrolysis of HPI, providing evidence for the opposing effects of thermal denaturation and subsequent aggregation of edestin on proteolysis. Heating HPI at 75–80 °C increased the hydrolysis rate by denaturing the proteins. In contrast, heating at 90 °C inhibited the hydrolysis rate due to protein aggregation, which limited access of trypsin to cleavage sites. In the present study, we noted that both edestin subunits of HPBs heated at 90 °C were degraded more slowly during the first 30 min of hydrolysis compared to those of HPBs heated at 80 °C, while further hydrolysis caused a reversal of proteolysis rates (Fig. 8A and B). This indicated that the initial resistance of edestin to trypsin attack, conferred by thermal aggregation of HPBs at 90 °C, was ultimately overcome as the hydrolysis reaction progressed. After heating HPBs at 100 °C, as noted above, both edestin subunits were readily and completely hydrolysed within 30 min. This led us to hypothesize that all potential cleavage sites (especially Lysine and Arginine bonds) in the denatured proteins were made available to trypsin, and that trypsin's access to these sites was not hindered by extensive HPB protein aggregation (Deshpande & Damodaran, 1989a; Park & Russell, 2000).

Moreover, the overall proteolysis rates of the AS differed considerably from those of the BS of edestin. At all temperatures tested, the BS was hydrolysed much more slowly than the AS (Fig. 8C). These differences may, in part, be related to the structural organisation of the subunits in the edestin molecule and the packing of these molecules in the crystalloid, which is not fully understood and warrants further investigation. According to a widely accepted model for the structure of 11S globulins, as is the case with edestin, each molecule consists of six pairs of monomers held together by non-covalent bonds. Each monomer pair consists of an AS and a BS, linked by one or more disulphide bonds (Jensen & Grumpe, 1983). It is generally understood that the BS forms the hydrophobic core of the globular protein, while the AS is located on the protein surface, with hydrophilic unordered regions (loops) exposed to the solvent and enzymes (Subirade et al., 1994). Therefore, it is likely that the surface location of the AS within the edestin crystalloids of HPBs provides shielding from trypsin-mediated cleavage, thereby slowing down the hydrolysis of the BS core (Carbonaro, Grant, & Cappelloni, 2005).

Another possible factor contributing to the differences in the proteolysis rates between the AS and the BS of edestin is their hydrophobicity/hydrophilicity characteristics, as highlighted above in section 3.3. In fact, the BS of 11S globulins is generally characterised by a more ordered and compact structure with higher hydrophobicity, making it less accessible to proteolytic enzymes compared to its AS counterpart (Subirade et al., 1994). The lower susceptibility of the BS to proteolysis relative to the AS has been reported *in vitro* in 11S seed storage globulins from hemp (Tang et al., 2009), sesame (Orruño & Morgan, 2011), soybean (Yang et al., 2016), and coconut (Angelia et al., 2010).

It is noteworthy that the complete resistance of the edestin BS to trypsin hydrolysis in its native state was not fully overcome by heating HPBs at 60–80 °C, as evidenced by the formation of resistant polypeptides (~5–44 % of the BS remaining) after 120 min of hydrolysis (Fig. 8B). This phenomenon could be explained by the inner location of the BS within the edestin crystalloids of HPBs and its compact structure, which may lead to incomplete protein denaturation. Likewise, the compact structure of 11S globulins, such as phaseolin and vicilin, has been reported not to be completely unfolded after heating (Deshpande & Damodaran, 1989b).

Due to the stability of the BS structure against heat treatment, a higher energy input is often required to disrupt intramolecular bonds and achieve complete denaturation (Nieto-Nieto et al., 2014). In this study, we propose that complete denaturation of all edestin subunits of HPBs was achieved after heating at temperatures above 80 °C, resulting in their complete hydrolysis by trypsin into small peptides (Fig. 7E and F). Despite this, the hydrolysates retained the original spherical shape of the HPBs, as demonstrated by CLSM images in the Supplementary data (Fig. S1). These observations strongly suggest that the edestin molecules were cleaved by trypsin at multiple sites within the HPB crystalloids. However, the resulting protein fragments did not dissociate from the crystalloid structure, preventing complete disintegration of the HPBs. Similar non-dissociating cleavage fragments have also been reported in the products of trypsin hydrolysis of some 11S globulin species (Plumb & Lambert, 1990).

3.5. Proposed mechanisms for the formation of heat-induced HPB protein aggregates

Based on our results, we propose possible molecular mechanisms involved in the formation of heat-induced HPB protein aggregates. In Fig. 9, we schematically illustrate how heat treatments of HPBs induce varying degrees of denaturation and aggregation of their protein fractions, resulting in the formation of soluble and insoluble protein complexes. Trypsin-catalysed hydrolysis assays and SDS-PAGE analyses provided valuable insights, which enabled the categorisation of HPBs into three groups based on their distinct proteolytic patterns of edestin subunits, as detailed below.

3.5.1. Category 1: native HPBs

At neutral pH, isolated HPBs in their native state predominantly exist as spherical particles, consisting of edestin crystalloids enveloped by inner albumin-rich proteinaceous matrices and outer membranes. The matrix albumins in HPBs tend to partially dissolve in water, leaving behind insoluble crystalloids with attached membrane remnants. These crystalloids are often fractured, generating smaller soluble or insoluble protein particles (Do et al., 2024; Tully & Beevers, 1976). Due to their high structural integrity, native HPBs possess intact BS and only slightly cleaved AS of edestin following trypsin-catalysed hydrolysis.

3.5.2. Category 2: heated HPBs (60–80 °C)

Heating HPBs at moderate temperatures (60–80 °C) and neutral pH causes further dissolution of the matrix albumins, allowing the

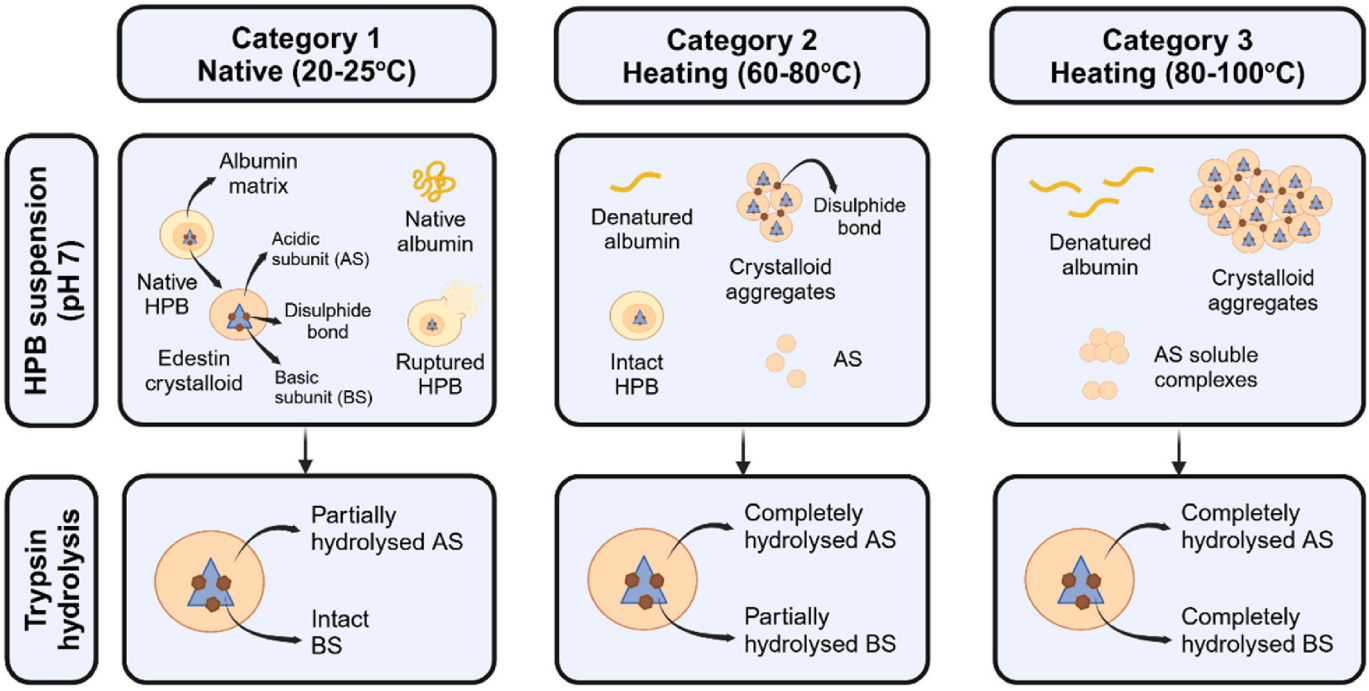

Fig. 9. Schematic illustration of the hypothetical mechanisms underlying the formation of heat-induced HPB protein aggregates and their subsequent hydrolysis by trypsin. Abbreviations: HPB, hemp protein body; AS, acidic subunit; BS, basic subunit.

remaining insoluble edestin crystalloids to interact with each other. Heating modifies the surface structure of the crystalloids by unfolding the native conformation of globular edestin, exposing buried hydrophobic groups and reactive free –SH groups (Ma et al., 2024). Subsequent SH/S–S interchange among BS and the concomitant release of AS from the oligomeric structure of edestin present on the surfaces of the crystalloid particles lead to inter-particle associations, forming crystalloid aggregates.

The heat-induced aggregation of proteins in HPBs is primarily governed by disulphide bond formation and, to a lesser extent, by hydrophobic interactions. Nevertheless, a proportion of intact HPBs and crystalloid particles do not form aggregate units and instead remain as individually separated particles in suspension, particularly those heated at 60 °C. Under these moderate heating conditions, the AS of edestin is fully denatured, while the BS is only partially denatured. Accordingly, the heated HPBs possess partially cleaved BS and completely cleaved AS after hydrolysis with trypsin.

3.5.3. Category 3: heated HPBs (80–100 °C)

Heating HPBs at higher temperatures (80–100 °C) and neutral pH leads to considerable dissolution of the matrix albumins and complete disruption of the HPB structure. Further rearrangement and cross-linking of edestin present on the surfaces of the crystalloid particles results in extensive crystalloid aggregation. The AS, released from its association with the BS in native edestin upon elevated heating, readily self-assemble into multiple disulphide-linked oligomeric species. Moreover, both edestin subunits become fully denatured when subjected to heating above their denaturation temperatures. Consequently, the heated HPBs possess completely cleaved AS and BS following hydrolysis with trypsin. This suggests that the crystalloid aggregation does not hinder access of trypsin to the cleavage sites.

4. Conclusions

The present investigation has broadened our understanding of the heat-induced inter- and intra-HPB protein modifications at the molecular level. For the first time, we have described the possible sequence of events in the thermal dissociation-association of HPB protein fractions in aqueous suspensions. Initially, the dissolution of water-soluble albumins strips away the proteinaceous matrices, facilitating heat-induced interactions among the remaining insoluble crystalloids. The oligomeric structure of edestin contained within the crystalloids dissociates into its constituent subunits. This is followed by the release of AS into the aqueous phase and the concomitant aggregation of BS at the surfaces of the crystalloid particles, resulting in the formation of crystalloid aggregates. Subsequently, the denaturation of AS may initiate further self-aggregation.

Our study also revealed significant differences in susceptibility to trypsin cleavage between the AS and BS of edestin in both native and heated HPBs. These differences are hypothesized to relate to both the subunit location within the edestin crystalloid structure and the hydrophobicity/hydrophilicity of the subunits. Future research efforts could expand to employ advanced material characterisation techniques, such as X-ray diffraction and small-angle neutron scattering, as promising tools to probe the subunit structure and assembly of edestin crystalloids in HPBs.

Finally, heat treatment can be applied to HPBs to improve their protein hydrolysis. Varying heating conditions, such as temperature and duration, can generate a range of protein hydrolysate mixtures containing different proportions of AS- to BS-derived peptides with potentially interesting functional and bioactive properties. Structural modification of HPBs through innovative processing techniques (e.g. enzymatic hydrolysis) to enhance their functionality in food systems represents an exciting avenue of research, which will be explored in subsequent studies.

CRediT authorship contribution statement

Duc Toan Do: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Aiqian Ye:** Writing – review & editing, Supervision, Methodology. **Harjinder Singh:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Alejandra Acevedo-Fani:** Writing – review & editing, Visualization, Supervision,

Project administration, Methodology, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT in order to improve readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2025.111372.

Data availability

Data will be made available on request.

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