Food Chemistry 460 (2024) 140561

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Exploring protein structural adaptations and polyphenol interactions: Influences on digestibility in pigeon pea dal and whole grains under heat and germination conditions

Minakshi Dutta[a], R. Dineshkumar[a], C.R. Nagesh[a], Y. Durga Lakshmi[a], Brijesh Lekhak[a], Navita Bansal[a], Suneha Goswami[a], Ranjeet Ranjan Kumar[a], Aditi Kundu[b], Pranab Kumar Mandal[c], Bindvi Arora[d], Ranjeet Sharad Raje[e], Somnath Mandal[f], Achchhelal Yadav[g], Aruna Tyagi[a], S.V. Ramesh[h], G. Rama Prashat[e,*], T. Vinutha[a,*]

[a] *Division of Biochemistry, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India*
[b] *Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India*
[c] *National Institute for Plant Biotechnology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India*
[d] *Division of Food Science & Post Harvest Technology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India*
[e] *Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India*
[f] *Department of Biochemistry, Uttar Banga Krishi Viswavidyalaya, Coochbehar, West Bengal, India*
[g] *Division of Agricultural Physics, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India*
[h] *ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala, 671 124, India*

ARTICLE INFO

Keywords:
Protein digestibility
Polyphenol interaction
Pigeon pea dal & grains
Thermal treatment
Autoclaving
Germination
Antinutritional factors

ABSTRACT

Pigeon pea, a protein-rich legume with low protein digestibility (PD) due to its high polyphenol content and other antinutritional factors (ANFs). Consequently, processing methods are crucial to improve PD. We investigated the effects of thermal treatments (cooking, hydrothermal, autoclaving, infrared rays) treatments and germination on modulation of PD, its properties and association with ANFs in two distinct genotypes based on polyphenol content: high (Pusa Arhar 2018–4) and low (ICP-1452). Treatments improved *in vitro* PD and essential amino acid content, with autoclaving showing significantly higher PD (ICP-1452: 90.4%, Pusa-Arhar 2018–4: 84.32%) ascribed to disruption of tight protein matrices. Significant increase in β-turn, reduction in protein: starch, protein: polyphenol interactions as well as breakdown of storage proteins revealed by the analysis of protein structural properties. This study suggests thermal treatments, particularly autoclaving, can enhance pigeon pea protein's nutritional quality for its utilization as a new ingredient in development of healthy foods.

1. Introduction

Pigeon pea (*Cajanus cajan*) is the second most consumed (15–20%) pulse in India, next only to chickpea, owing to its high protein content, simple cooking and preference by Indian consumers (Sarkar et al., 2020). Pigeon pea contains protein in the range of 18–25%, carbohydrate (57.6%), fat (0.993%–1.75%) and minerals (mg/kg DW) such as potassium (105.17 to 144.07), magnesium (8.95 to 12.67), calcium (7.74 to 12.27), iron (0.247 to 0.543), zinc (0.122 to 0.313), manganese (0.061 to 0.432) and copper (0.087 to 0.134) (Gerrano et al., 2022; Kuraz Abebe, 2022; Sharma et al., 2011). Pigeon peas not only have balanced sources of carbohydrates, protein, minerals, and essential amino acids (EAAs) especially lysine which is a limiting amino acid in cereals (Duranti, 2006), but also contains vitamins, fibre, and as well bioactive compounds (Wood & Grusak, 2007). The pulses including pigeon pea are deficient in sulfur amino acids such as cysteine and methionine (Venkidasamy et al., 2019). Nevertheless, unlike soybean, chickpea, garden peas and mung bean, the nutritional quality of dietary proteins of pigeon pea is limited by protein digestibility due to the presence of polyphenols (0.3–1.83%) and other antinutritional factors like phytic acid (0.2–0.9%), enzyme inhibitors and negligible content of lectins below toxicity level (400 unit/g) (Kachare et al., 2019, Sun et al.,

---

* Corresponding authors.
E-mail addresses: rama.g@icar.gov.in (G. Rama Prashat), vinutha.t@icar.gov.in (T. Vinutha).

https://doi.org/10.1016/j.foodchem.2024.140561
Received 2 January 2024; Received in revised form 18 July 2024; Accepted 19 July 2024

2020, Singh & Eggum, 1984, Godbole et al., 1994, Duhan et al., 1999, Singh, 1988, Grant et al., 1983, Igbedioh et al., 1994). Hence enhancement of plant protein digestibility by employing food processing techniques is a critical aspect of the development of high-quality food products. Several processing techniques, such as heat treatments, soaking, dehulling, enzymatic hydrolysis, fermentation, and germination have demonstrated their effectiveness in diminishing the presence of antinutritional components and as well as polyphenols in legumes and plant-based proteins, which can otherwise hinder digestibility (Kalpanadevi & Mohan, 2013; Samtiya et al., 2020). Reduction of these detrimental factors through application of food processing techniques offers an opportunity to elevate the nutritional quality of plant proteins thereby enhancing better absorption by the human body (Gu et al., 2022; Sá et al., 2020). Dietary protein quality is determined by the digestion of proteins and absorption of their constituent amino acids (digestibility) and utilization of absorbed amino acids to support the whole-body protein synthesis (availability) (WHO, 2007). Considering the requirements for EAAs and the fact that protein is the only dietary source of EAAs, a recommended dietary allowance (RDA) for protein thus does not only entail a quantitative aspect but also has a qualitative dimension. To the best of our knowledge, till date no studies on the effect of thermal treatments on protein digestibility of pigeon pea are available except for microwave treatment (Sun et al., 2020). Therefore, investigation of the effect of processing methods in modulating protein digestibility, properties and its association with antinutritional factors is of paramount importance for the benefit of consumers and plant protein industries.

Polyphenols in particular is reported to interfere with protein digestibility either by forming complex with protein digestive enzymes or protein substrates which can have diverse outcomes, including inhibition, activation, or no observable effect at all (Velickovic & Stanic-Vucinic, 2018). Interestingly many studies have reported a negative correlation of polyphenol content with *in vitro* protein digestibility (IVPD) (Singh, 1993; Świeca et al., 2013; Lamothe et al., 2014).

Although pigeon pea is largely produced and consumed in India, due to its low protein digestibility which not only least preferred by protein-food industries but also compromises the consumer's health benefits, hence the objective of the present study is to assess the effect of various thermal treatments, including pressure cooking, autoclaving, hydrothermal and infrared heating, as well as non-thermal treatment such as germination on improvement of the IVPD. This objective was achieved by examining how these treatments modulate digestibility of proteins that co-exist with antinutritional factors, especially polyphenols which are abundantly present in the seed matrix. To understand how polyphenols affect the digestion of proteins, two different pigeon pea genotypes with the contrasting polyphenol compositions were chosen in this study. All the structural, molecular analysis and studies related to IVPD; *in vitro* protein-polyphenol interactions were carried out in these two genotypes.

2. Materials and method

2.1. Materials

Seeds of pigeon pea genotypes (30) were obtained from the Division of Genetics, ICAR-IARI. These seeds were cleaned and analyzed for their polyphenol content. Among these, two genotypes for high and low polyphenol content were selected. The seeds of these two selected genotypes were divided into two parts. One part was cleaned and dehulled to obtain split dal, which was then subjected to four different thermal treatments. The other part, consisting of whole grains, was used for germination. Gallic acid pure (98%), SDS-PAGE sample loading buffer (6×) was purchased from G-Biosciences (USA), 3 colour pre-stained Protein Ladder (10-250 kDa) was obtained from Genetix (India). Pepsin from porcine gastric mucosa, pancreatin, 5,5′-dithiobis- [2-nitrobenzoic acid] (DTNB), 2,4,6-Trinitrobenzenesulfonic acid (TNBS), Folin & Ciocalteu's phenol reagent, N-α-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), L-Glutathione reduced were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phytic acid assay kit was obtained from Megazymes® (Ireland).

2.2. Processing treatment

2.2.1. Thermal treatments

The split dal from the two genotypes (Pusa Arhar 2018-4 and ICP 1452) were subjected to different thermal treatments. For pressure cooking (C): previously soaked (12*h*) dals cooked in a temperature-controlled container at 100 °C while maintaining a static pressure of 15 psi for 30 min. For hydrothermal treatment (HT): coarsely ground to particle size of 2.5 μm and steamed for 15 min at 100 °C and pressure cooked. For autoclaving (AC): previously soaked (12 h) dal autoclaved at 15 psi and 121 °C for one hour. For infrared heating (IRH): previously soaked (12 h) dals spread in a uniform 0.5 cm thick layer and placed 11 cm below an NIR lamp with a power of 150 watts having a wavelength (0.7–1.0 μm); density of 480 watts m^{-2} and temperature 40 °C was maintained in the chamber for 15 min followed by pressure cooking (Vinutha et al., 2022). The processed samples were subsequently subjected to drying at 55 °C in a hot air oven, followed by pulverization to create a fine powder. The resulting powder was stored and utilized for subsequent analyses.

2.2.2. Germination (G)

The whole grains of were soaked in distilled water overnight (12 h) at room temperature. Soaked seeds were allowed to germinate for 72 h and sprouts that had grown to a length of 1.5 cm were collected, dried and then pulverized to fine powder and stored for further analysis.

2.3. Profiling of total, free and bound polyphenol content

Total polyphenol (TP), free fraction (FF) and bound fraction (BF) content was determined following the method enumerated by Carbonaro et al. (2000) with minor modifications. Sample was treated in 0.1 M NaOH (0.1 g of the samples/mL) and centrifuged at 20,000*g* for 20 min to obtain the supernatant which was assessed for TP content by measuring the absorption at 328 nm and comparing it to a standard gallic acid. The protein present in the supernatant was precipitated by 5% trichloroacetic acid and subsequently centrifuged at 20,000*g* for 20 min and absorbance of the supernatant was measured at 328 nm for FF content. To account for the contribution of proteins to the overall absorption bovine serum albumin solution was used as control. The BF content was then calculated from the difference between TP and FF.

2.4. Total protein content and amino acid profiling

To estimate the total protein content in the sample, the nitrogen content of the samples was determined by Kjeldhal method (AOAC, 2006). The resulting nitrogen value was then multiplied by 6.25 to obtain the total protein content. Amino acid profiling and quantification of amino acid were done following the method described by Sarika et al. (2017) using the Shimadzu LC-30 UPLC system of the Nexera X2 series with minor modifications. The standard amino acid solution, comprising 17 high-purity amino acids at 0.1 mol/L, was used, and L-norleucine served as the internal standard. The standard amino acids were eluted through the C18 column using mobile phase consists of acetic acid (0.5%): acetonitrile (80:20, *v*/v), with flow rate of 1.75 mL/min. The samples were derivatized with fluorenyl methoxy carbonyl chloride (FMOC) and eluted using mobile phase comprised a buffer phase (tetramethylammonium chloride and sodium acetate trihydrate, pH 3.5) and an organic phase (acetonitrile and methanol in a 49:1 ratio). Samples were run isocratically with a 90:10 ratio of Solvent A (buffer phase) to Solvent B (organic phase), where Solvent A and B had a ratio of 9:1 and 1:9, respectively.

Amino acid scores of the EAAs were determined using the reference pattern of FAO (1991).

$$EAAS = \frac{Grams\ of\ EAA\ in\ 100g\ of\ test\ protein}{EAA\ in\ 100g\ FAO\ reference\ protein} \times 100$$

2.5. In vitro simulated gastrointestinal digestion

In vitro protein digestibility (IVPD) was measured following the method given by Vitali et al. (2009) with few modifications. Briefly, flour sample of 0.5 g was mixed with 12.5 mL of trypsin solution (0.5 mg/mL pepsin in dissolved in distilled water; pH 2) and incubated at 37 °C in an incubator shaker (Orbitek, Scigenics Biotech) for 2 h. Then the solution was neutralized to pH 7 with (with 6 M NaOH) and 2 mL of pancreatin (5 mg/mL pancreatin dissolved in phosphate buffer, pH 8.2) and incubated for 24 h at 37 °C with continuous shaking. To stop the enzymatic reaction 7 mL of 10% TCA was added and centrifuged at 4100*g* for 20 min. The residue was collected, washed and the protein content was determined by Kjeldahl method (AOAC, 2006). The protein content in the supernatant was calculated by subtracting the protein in the pellet from the protein content of the sample.

The IVPD was determined using the formula:

$$IVPD = \frac{Protein\ content\ in\ the\ supernatant}{Protein\ content\ in\ the\ sample} \times 100$$

2.6. Degree of hydrolysis (DH)•

The DH was carried out by method given by Adler-Nissen (1979). To determine DH 50 μL of digested, undigested and the amino acid mixture was taken. Digested sample was taken from the supernatant produced in Section 2.7 and amino acid mixture was taken from the syringe filtered sample from Section 2.6. The percentage of hydrolysis was calculated using the following formula:

$$DH(\%) = \frac{At - Ao}{Amax - Ao}$$

A_t = Absorbance of digested sample
A_0 = Absorbance of undigested sample
A_{max} = Absorbance of total amino acid content

2.7. Free sulfhydryl group (SH) content

The determination of free SH content was done by the method previously reported by Pan et al. (2020) with slight modification. Briefly, 5 mg of samples was suspended in 1 mL of Tris-glycine buffer (86 mM Tris, 90 mM glycine, 4 mM EDTA) containing 8 M urea at pH 8.0. This mixture was then incubated overnight at 23 °C with continuous shaking at 400 rpm and centrifuged at 3000*g* for 10 min at 23 °C, the supernatant was collected and then diluted to a final concentration of 0.1 mg/mL using Tris-glycine buffer (pH 8.0). The resulting solution was subjected to a reaction with Ellman's reagent in 100:1, *v*/v ratio for 10 min at room temperature. Absorbance of the mixture was measured at 412 nm. The free SH group was calculated using the following formula:

$$SH(\mu mol/g) = \frac{73.53^*(Asample - Ablank)^*D}{C}$$

Here, D = dilution factor and C = protein concentration (1 mg/mL).

2.8. In vitro studies on interaction of protein isolate/polyphenol complex (PPC)

2.8.1. Preparation of protein isolate (PI)

Protein isolates (PIs) were prepared by the method given by Papalamprou et al. (2010) with few modifications. In brief, 100 g of defatted flour was mixed with distilled water at a ratio of 1:10 (*w/v*). The mixture was then adjusted to pH 8.5 using 1 M NaOH and stirred at 500 rpm for 45 min at room temperature, followed by centrifugation at 4500*g* for 20 min at 4 °C. The resulting supernatant was collected, and the pellet was used for additional round of extraction (1:5 w/v ratio with distilled water, pH 8.5). The pH of the combined supernatants was then adjusted to 4.5 using 0.1 M HCl and the solution was then centrifuged at 4500*g* for 20 min at 4 °C. The resulting precipitate was washed twice with distilled water (4 °C), re-dispersed in distilled water with pH adjusted to 7 using 1 M NaOH, and finally, subjected to freeze-drying. The protein content of the resultant protein isolate was determined by Kjeldahl method (AOAC, 2006).

2.8.2. Preparation of total polyphenol extract

The total polyphenol was extracted by the method given by Putra et al., 2022. using Soxhlet apparatus. Flour sample (5 g) was mixed with 70% methanol at a temperature of 60 °C for 2 h. After extraction the solvent was evaporated using a rotary evaporator and the residue was collected. The polyphenol content was determined by Folin-Ciocalteu's colorimetric approach as previously described by Carbonaro et al. (2000).

2.8.3. Preparation of protein isolate/polyphenol complex (PPC) under in vitro conditions

The PI and polyphenol conjugate were prepared following the method given by Hao et al., 2022. In brief, PI solutions were prepared fresh in phosphoric acid buffer (5 mM, pH 7.0) (10 mg/mL) and stirred overnight at 4 °C for complete hydration, and polyphenol stock was prepared in 50% ethanol solution. The polyphenol extract was then added to the PI solution based on the inherent polyphenol content of the sample per g of protein. The solution is mixed thoroughly and incubated at 25 °C for 2 h in the dark. Centrifuged for 20 min at 3434*g*. The supernatant was decanted and the pellet was freeze-dried to yield the PPC and the IVPD of the PPC was determined by a method given in Section 2.5.

2.9. Profiling of storage protein fractions (albumin and globulin) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Albumin and globulin fractions of the seed protein were extracted using a method described by Jakubczyk et al. (2019) with a few modifications. Briefly 1 g of defatted sample was combined with 4 mL of distilled water and subsequently centrifuged at 8000*g* at 4 °C for 20 mins. The resulting supernatant, which contained the soluble albumin, was collected and kept. The pellet was resuspended in Tris–HCl buffer (100 mM Tris–HCl, 0.5 M NaCl, pH 8.1) followed by centrifugation at 10,000 rpm at 4 °C for 20 min. The supernatant containing globulin was collected. The protein fractions (albumin and globulin) were analyzed in SDS-PAGE (Laemmli 1970). The SDS-PAGE was performed using 3% stacking gel (*w/v*, pH 6.8) and 12% separating gel (w/v, pH 8.8).

2.10. Secondary structure determination by FTIR

The protein secondary structure of flour samples obtained from native and treated flour was examined using FTIR spectroscopy (Bruker, Germany). Finely ground flour samples with a uniform particle size were mixed with KBr at a ratio of 1:90 (*w/w*). Subsequently, the resulting pellets were scanned in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} using a total of sixty-four scans to capture both background and sample spectra. Baseline calibration and deconvolution were performed in the range of 1500–1800 cm^{-1} using ORIGIN pro 8 SR0 (v.8.0724, origin lab corporation, USA) and the relative amounts of different secondary structure of the samples were determined.

Table 1
Total protein content (%) and Essential amino acid content (g/100 g protein) of native, germinated and thermal treated pigeon pea varieties.

	Pusa Arhar 2018–14							ICP 1452						
	Dal	Cooking	HT	AC	IRH	WG	G	Dal	Cooking	HT	AC	IRH	WG	G
Total protein content%	22.79 ± 0.04	21.21 ± 0.05	21.65 ± 0.07	20.56 ± 0.21	21.65 ± 0.16	22.96 ± 0.05	23.31 ± 0.04	21.31 ± 0.09	20.03 ± 0.04	20.34 ± 0.12	19.9 ± 0.08	20.08 ± 0.02	21.87 ± 0.16	22.83 ± 0.04
Lysine	6.37 ± 0.009	5.42 ± 0.007	5.9 ± 0.007	5.85 ± 0.007	5.83 ± 0.005	6.25 ± 0.007	6.29 ± 0.007	6.98 ± 0.04^{a}	5.74 ± 0.05^{b}	5.06 ± 0.05^{a}	5.70 ± 0.03^{b}	5.17 ± 0.04^{a}	6.29 ± 0.08^{c}	6.32 ± 0.06^{c}
Histidine	3.33 ± 0.004^{f}	2.96 ± 0.004^{c}	2.89 ± 0.007^{b}	2.94 ± 0.004^{c}	2.82 ± 0.004^{a}	3.1 ± 0.004^{e}	3.04 ± 0.009^{d}	3.28 ± 0.03^{c}	2.92 ± 0.02^{a}	2.92 ± 0.04^{a}	3.12 ± 0.04^{b}	2.82 ± 0.05^{a}	3.56 ± 0.02^{d}	3.74 ± 0.01^{e}
Leucine	8.66 ± 0.02^{d}	8.93 ± 0.03^{a,b,c}	9.2 ± 0.009^{b,c,d}	9.09 ± 0.01^{c,d}	9.19 ± 0.01^{a}	8.17 ± 0.01^{b,c,d}	9.57 ± 0.02^{a,b}	6.49 ± 0.11^{a}	7.51 ± 0.2^{b}	7.96 ± 0.14^{b}	8.26 ± 0.25^{c}	8.33 ± 0.16^{d}	6.31 ± 0.15^{a}	7.52 ± 0.2^{b}
Isoleucine	4.5 ± 0.04^{e}	3.98 ± 0.01^{b,c}	3.7 ± 0.01^{a}	3.97 ± 0.01^{b}	3.94 ± 0.01^{b}	4.13 ± 0.02^{d}	4.07 ± 0.01^{c,d}	4.91 ± 0.13^{d}	4.14 ± 0.26^{a}	4.31 ± 0.21^{b}	4.58 ± 0.21^{c}	4.21 ± 0.27^{a,b}	4.73 ± 0.16^{c}	4.32 ± 0.45^{b}
SAA^{A}	2.33 ± 0.05^{b}	2.21 ± 0.02^{c}	2.27 ± 0.007^{e}	2.31 ± 0.01^{d}	2.15 ± 0.008^{e}	2.28 ± 0.01^{a}	2.22 ± 0.02^{f}	2.24 ± 0.11^{b}	2.16 ± 0.08^{a,b}	2.14 ± 0.44^{a}	2.23 ± 0.27^{b}	2.12 ± 0.34^{a}	2.23 ± 0.1^{b}	2.13 ± 0.35^{a}
Phenylalanine +Tyrosine	10.28 ± 0.004	11.95 ± 0.01	11.3 ± 0.01	11.62 ± 0.01	11.29 ± 0.02	10.26 ± 0.004	10.88 ± 0.007	10.99 ± 0.19^{a}	11.89 ± 0.25^{b,c}	11.51 ± 0.33^{a,b}	11.73 ± 0.29^{b,c}	11.25 ± 0.39^{a}	11.16 ± 0.07^{a}	12.09 ± 0.34^{c}
Valine	4.33 ± 0.03^{a}	4.61 ± 0.03^{c}	4.6 ± 0.01^{c}	4.52 ± 0.01^{b}	4.69 ± 0.01^{c}	4.79 ± 0.009^{d}	5.13 ± 0.01^{e}	4.11 ± 0.33^{a}	4.38 ± 0.45^{b}	4.66 ± 0.05^{c}	3.99 ± 0.3^{a}	4.77 ± 0.09^{c}	3.81 ± 0.1^{a}	3.76 ± 0.12^{a}
Threonine	3.3 ± 0.004	3.57 ± 0.01	3.41 ± 0.01	3.72 ± 0.007	3.38 ± 0.02	3.53 ± 0.01	4.3 ± 0.007	3.25 ± 0.05^{b}	3.76 ± 0.07^{c,d}	3.71 ± 0.03^{c}	3.89 ± 0.21^{d}	3.70 ± 0.37^{c}	2.59 ± 0.08^{a}	3.22 ± 0.46^{b}
Total EAA (g/100 g protein)	43.03 ± 0.03^{c}	43.63 ± 0.03^{e}	43.36 ± 0.03^{d}	44.07 ± 0.01^{f}	43.35 ± 0.02^{b}	42.54 ± 0.02^{a}	45.5 ± 0.02^{g}	41.81 ± 0.18^{b}	42.52 ± 0.29^{b,c}	42.29 ± 0.009^{b}	43.6 ± 0.21^{d}	42.54 ± 0.17^{d,e}	40.76 ± 0.11^{a}	43.12 ± 0.28^{c,d}

Sulfur Amino Acid (SAA)- Methionine + Cysteine.
Values are means ± standard error ($n = 3$).
For both the variety, means with the same letters were not significantly different ($p < 0.05$).

2.11. *Confocal laser scanning microscopy (CLSM)*

The protein and polyphenol in the treated and native flour sample were visualized by using CLSM (Leica TCS-SP2, Germany). Sample was prepared using method described by (Vinutha et al., 2022) with a few modifications, briefly flour sample of 100 mg was soaked in 1 mL deionized water, vortexed for 1 min followed by heating for 10 min (100 °C) and samples were mixed properly. Protein molecules are stained using Rhodamine B (0.0025%) and polyphenols were stained using 0.01% toluidine blue O. All samples were preserved in the dark condition immediately to avoid fluorescent quenching. Then the sample was visualized under CLSM with the fluorescence excitation and emission wavelength at 543 nm and 545–660 nm respectively for Rhodamine B; 488 nm and 530 nm respectively for Toluidine blue O.

2.12. *Microstructure imaging by scanning electron microscope (SEM)*

Microstructure of the flour matrix of treated and native samples was visualized using SEM (Jeol JSM 6610LV, Japan). The flour samples were uniformly spread over the SEM stub having double sided adhesive tape using a rubber suction bulb and coated with gold-palladium (JEOL, JEC-3000FC). The images for all the samples were taken at x400 magnification.

2.13. *Trypsin inhibitor (TI) assay*

TI activity was determined indirectly following the method described by Kakade (1969) with few modifications. 0.5 g of sample was taken for extraction in 25 mL of water by stirring for 3 h followed by centrifugation at 12,000 rpm for 20 min at 4–6 °C. The resultant supernatant was diluted 10 times to obtain TI source. Two sets of extract (0-1 mL) were pipetted, endogenous (E) and test (T). Trypsin enzyme was added to the test set followed by adding substrate BAPNA (α-Benzoyl-D, L-arginine 4-nitroanilide hydrochloride) to all the tubes. One tube with only enzyme and substrate without adding sample served as standard (S). Then the absorbance of the resultant solution was recorded at 410 nm. The protein content was determined using Lowry method.

A graph of ΔA (A_{test} -$A_{endogenous}$) was plotted against volume of abstract and the extract required to inhibit 50% (S/2) of trypsin activity. The aliquot size is considered to be one unit of TI activity which corresponds to that amount of TI in μg of protein present in the aliquot. The TI activity is expressed in trypsin inhibitor unit (TIU) per gram of sample.

2.14. *Phytic acid (PA)*

For PA determination Megazyme® kit was used. Sample (1 g) was extracted with 20 mL of hydrochloric acid (0.66 M) overnight at room temperature. 1 mL of the extract taken and centrifuged at 13,000 rpm for 10 min. Immediately 0.5 mL of the resulting supernatant was taken and neutralized by addition of 0.5 mL of sodium hydroxide solution (0.75 M). Extract was pipetted into two different tubes for determining free and total phosphorus in the sample respectively. Total inorganic phosphorus (Pi) was released by using enzymes phytase and alkaline phosphatase. Then the phosphorus present in the solution is determined by the absorbance at wavelength 655 nm. Phosphorus calibration curve was prepared using standard phosphorus solutions and PA content was calculated using Mega-Calc.

2.15. *Statistical analysis*

All assays and experiments were performed in three technical replicates and the results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out by one-way ANOVA (Analysis of variance) using IBM SPSS Statistics 19 (SPSS Inc., USA). Significant

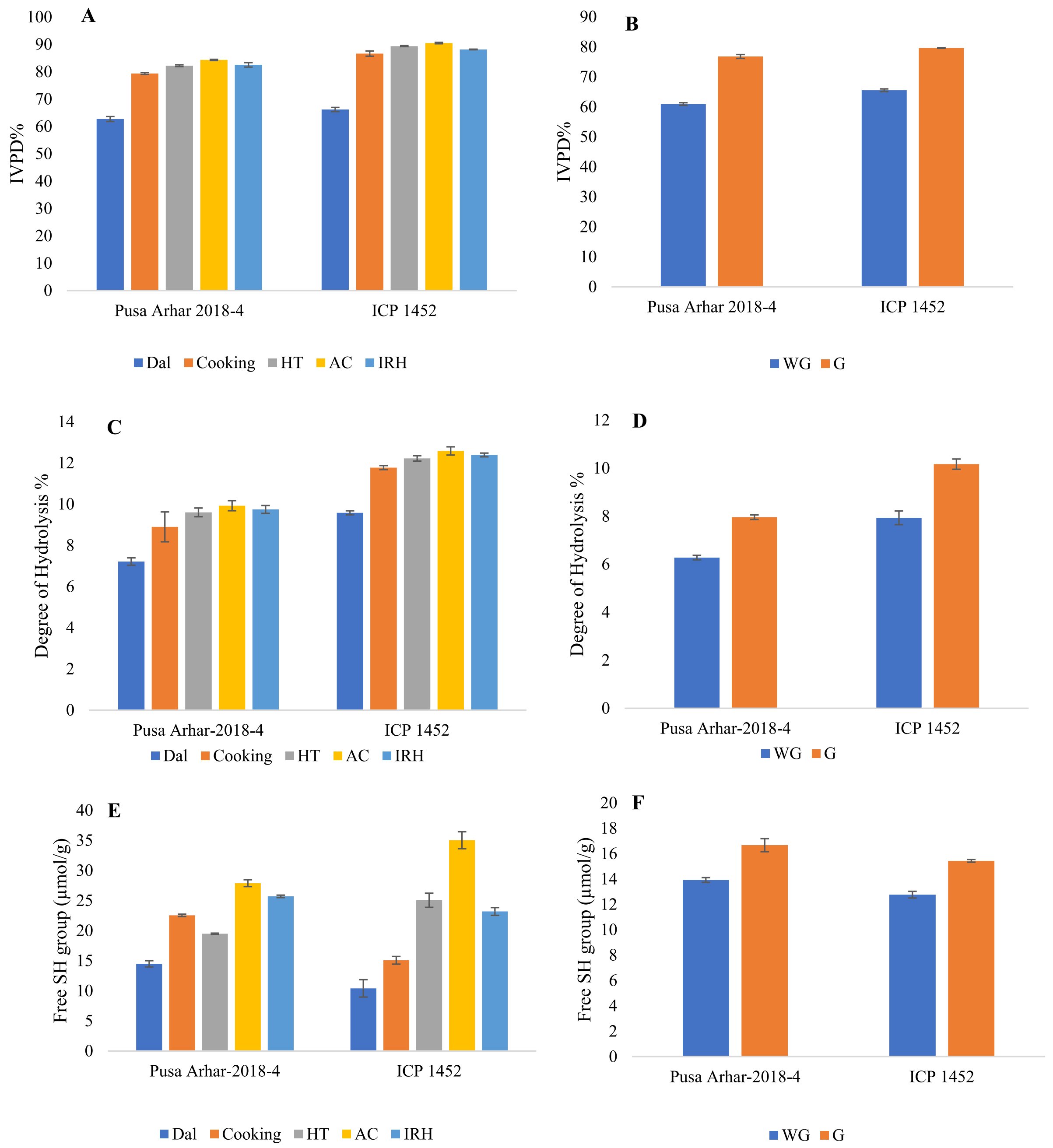

Fig. 1. Graphical representation of changes in protein properties after thermal treatment and germination. Values are means of three replicates with standard error. IVPD (%), degree of hydrolysis (%) and free sulfhydryl group content (µmol/L) after thermal treatment in left panel (A, C, E) and after germination in right panel (B, D, F) respectively.

differences were assessed using *t*-test and defined at a 5% level ($p < 0.05$).

3. Results

3.1. Profiling of polyphenol composition in pigeon pea genotypes

The TP, FF and BF in the whole grains and dals of 30 different pigeon pea genotypes are presented in Supplementary Table 1 and Supplementary Fig. 1. Whole grains exhibited TP content in the range of 215.33 ± 0.98–1250.22 ± 1.69 mg GAE/100 g, FF of 51.90 ± 1.01–571.95 ± 0.63 mg GAE/100 g and BF of 110.03 ± 0.51–678.27 ± 3.2 mg GAE/100 g, whereas dals showed TP content in the range of 173.03 ± 1.09–652.21 ± 1.65 mg GAE/100 g, FF of 39.75 ± 0.39–275.37 ± 0.98 mg GAE/100 g and BF of 98.06 ± 0.81–376.84 ± 1.2 mg GAE/100 g. Among all the genotypes whole grain and dal of genotype Pusa Arhar 2018–4 characterized with darker seed coat colour showed high content of TP, FF and BF. Whereas whole grain and dal of genotype ICP 1452 showed lowest content of TP and BF and Bahar genotype showed lowest FF, and these two genotypes are characterized with light seed coat colour. Thus, whole grains of pigeon pea genotypes showed large variation in polyphenol content. Additionally, whole grains showed higher polyphenol content (5.9–82.6%) compared to dehulled cotyledons, suggesting polyphenol content is majorly associated with the seed coat colour and pertinently present in the seed husks, and also a significant amount of polyphenol is present in bound form in whole grain and dal of

Table 2

Percent content of β-sheet, β-turn, α-helix, and Random coils in control and treated Pusa Arhar 2018–4 (high polyphenol containing) and ICP1452 (low polyphenol containing) pigeon pea variety.

	Pusa Arhar 2018–4						ICP-1452					
	β-sheet		α-helix		β-turn		β-sheet		α-helix		β-turn	
	Dal	Whole grain	Dal	Whole grain	Dal	Whole grain	Dal	Whole grain	Dal	Whole grain	Dal	Whole grain
Control	26.8 ± 0.19^{d}	$26.42 \pm 0.31^{c,d}$	16.98 ± 0.023^{d}	18.92 ± 0.11^{e}	12.41 ± 0.16^{a}	13.85 ± 0.11^{b}	27.16 ± 0.11^{d}	28 ± 0.15^{d}	18.98 ± 0.06^{a}	17.83 ± 0.03^{b}	25.51 ± 0.64^{a}	25.96 ± 0.07^{a}
Cooking	$25.63 \pm 0.25^{b,c}$	–	10.96 ± 0.39^{b}	–	$15.6 \pm 0.18^{c,d}$	–	$25.98 \pm 0.16^{b,c}$	–	11 ± 0.08^{a}	–	26.78 ± 0.092^{d}	–
Hydrothermal	25.35 ± 0.37^{b}	–	$10.59 \pm 0.12^{a,b}$	–	15.21 ± 0.07^{c}	–	25.17 ± 0.28^{a}	–	11.23 ± 0.08^{a}	–	26.61 ± 0.092^{c}	–
Germination	–	24.02 ± 0.31^{a}		12.8 ± 0.09^{c}	–	15.22 ± 0.09^{c}	–	$27.85 \pm 0.32^{d,e}$		12.27 ± 0.1^{b}	–	26.71 ± 0.026^{b}
Autoclaving	$25.96 \pm 0.08^{b,c,d}$	–	9.97 ± 0.23^{a}	–	15.9 ± 0.09^{d}	–	$25.41 \pm 0.11^{a,b}$	–	10.71 ± 0.05^{b}	–	22.76 ± 0.059^{e}	–
Infrared	25.35 ± 0.11^{b}	–	11.23 ± 0.29^{b}	–	15.83 ± 0.0^{d}	–	26.41 ± 0.13^{c}	–	10.22 ± 0.2^{b}	–	26.78 ± 0.028^{e}	–

- Values are means ± standard error ($n = 3$).
- For both the variety, means with the same letters were not significantly different ($p < 0.05$).

pigeon pea seeds (Supplementary Fig. 1). These results presented herein confirm the earlier findings in various legume species such as faba bean, kidney bean, cow pea *etc.* (Carbonaro et al., 2000; Gao, Ma, Wang, & Feng, 2017; Sashikala et al., 2015; Segev et al., 2010; Singh, 1993). Based on these results, Pusa Arhar 2018–4 (P^{+}), having high polyphenol content, and ICP 1452 (P^{-}), having low polyphenol content, were selected for further biochemical and protein quality analysis.

3.2. Effect of thermal and germination treatments on total protein content and amino acid composition

Total protein content from native and treated seed flours of both pigeon pea genotypes showed a decline after thermal treatments but exhibited significant increase following germination (Table 1 and Supplementary Fig. 2 A and B). The protein content in the native dal was found to be 22.79 g/100 g and 21.32 g/100 g for P^{+} and P^{-} respectively, which declined significantly during treatment by 4.79–10.2% and

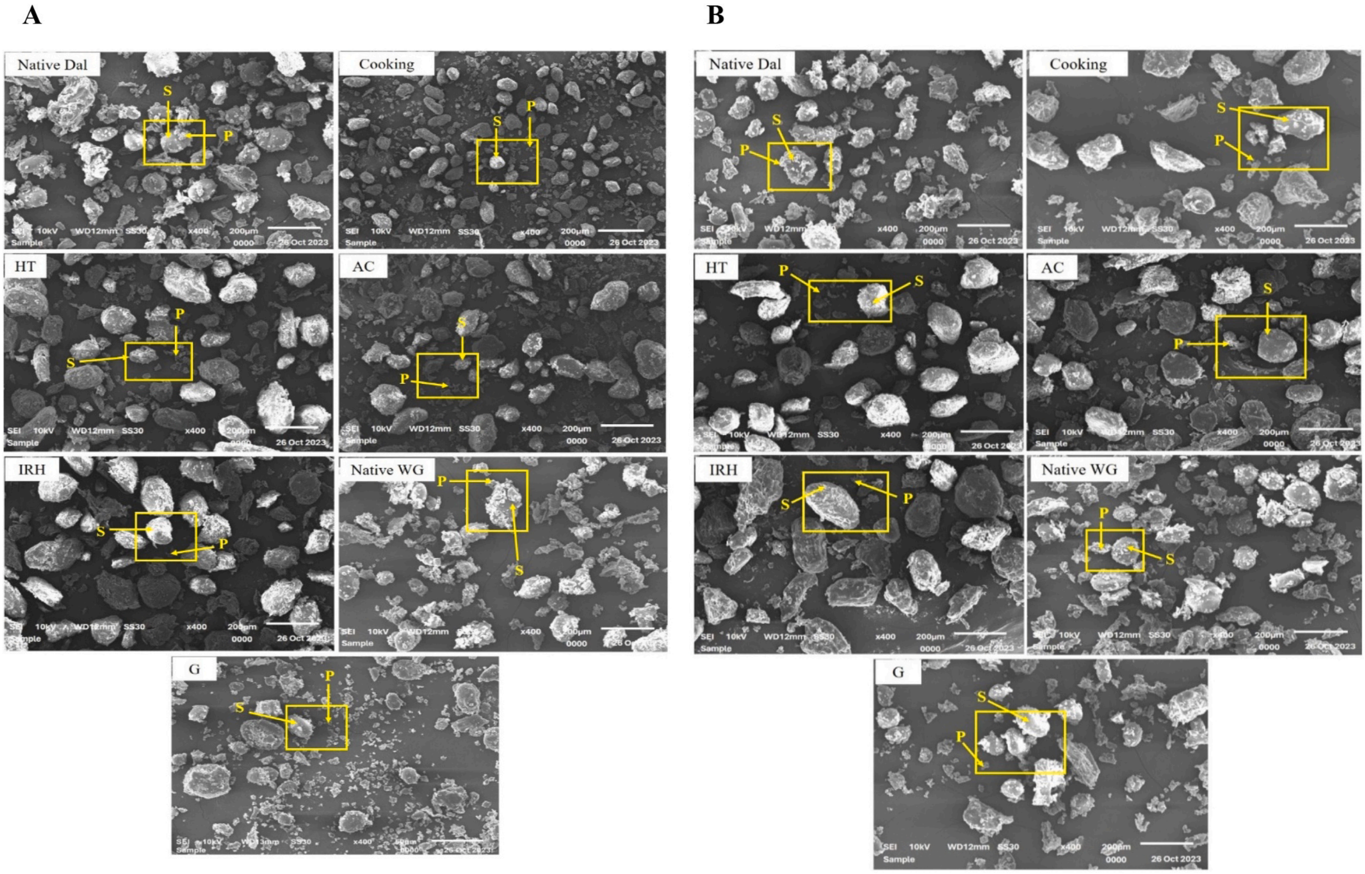

Fig. 2. SEM images of native, thermal treated and germinated pigeon pea flour of Pusa Arhar 2018–4 (A), ICP-145 (B). Marked area showing the protein and starch interaction (S- Starch, P-Protein). (A) Pusa Arhar 2018–4, (B) ICP-145.

4.51–6.61% respectively. AC treatment showed the highest reduction in protein content in both the genotypes. However, germination treatment significantly increased the protein content. The protein content in whole grain was found to be 22.96 g/100 g and 21.87 g/100 g for P^+ and P^- respectively, which showed an increment of 1.29% (P^+) and 4.39% (P^-), respectively during germination. The increase in the protein content during germination may be attributed to the biosynthesis of new protein during the process of germination. On the other hand, the decline in protein content during thermal treatment can be explained due to the loss of water-soluble proteins and also due to the process of protein denaturation. Alterations of protein content following the thermal treatment and germination have been reported in pearl millet and various legumes such as mung beans, chickpeas, cowpeas, peas, African yam beans, field beans, and lupins (Vinutha et al., 2022; Uppal & Bains, 2012; Habiba, 2002; Onyeike & Omubo-Dede, 2002; Vaga et al., 2017).

Table 1 presents the EAA composition in native and treated flour. The EAAs content was found to be increased (0.68–6.95%) in both the genotypes due to thermal treatments and germination. Thermal treatments and germination showed a decline in sulfur amino acid (0.14–7.72%) and lysine (7.27–24.2%) contents, whereas increased leucine (3.15–28.35%) and aromatic amino acid content (3.3–16.27%) was observed. The decreased sulfur amino acid during thermal treatment could be due to the decomposition of these amino acids following an exposure to high temperature. Whereas decreased sulfur amino acid in germinated seed could be attributed to utilization of sulfur amino acid from storage protein to the growing seedling by metabolizing into sulfate ions (Anderson & Fitzgerald, 2003). Likewise, the decrease in lysine concentration that has been noticed may be the result of the Maillard reaction, which is the condensation of amino acid groups in proteins (or in peptide linkage or even free amino acids) with glycosidic sugars (Candela et al., 1997). Similar alterations the contents of amino acids were reported in chickpeas, kidney bean, chickpea, lentil and faba beans after thermal processing and germination (Mubarak, 2005; Ziena, 1989; Candela et al., 1997; El-Adawy, 2002).

3.3. *Influence of thermal treatments and germination on protein properties*

3.3.1. *In vitro protein digestibility and degree of hydrolysis*

The native whole grain of P^+ and P^- showed IVPD values of 60.95% & 65.54%, and native dal of P^+ and P^- showed 62.73% & 66.2% respectively. Both the genotypes showed a substantial increase in IVPD, ranging from 76.8 ± 0.65% to 84.32 ± 0.23% in P^+ and from 79.64 ± 0.1% to 90.4 ± 0.24% in P^-. Notably, AC showed the highest enhancement of IVPD in both the genotypes (Fig. 1. A and B) was due to the prevalence of higher temperature for longer duration of time (121 °C for 60 min) than cooking (100 °C for 30 min), hydrothermal treatment (steaming for 15 min followed by additional cooking for 30 min at 100 °C) and infrared heating (40 °C for 15 min followed by additional cooking for 30 min at 100 °C) which enabled higher rate of penetration of steam into seed matrices might possibly allowed higher denaturation and stimulated folding process of proteins was evident by higher β-turn content (Table 2) and disruption of tight protein matrices visualized by SEM image analysis (Fig. 2). Loosening of protein matrices and protein structural changes into high β-turn content was sufficient enough for higher accessibility to protease enzymes that contributed to significantly increased IVPD after AC treatment (Fig. 5). Similar to the present study, previous findings by Arise et al. (2022), Zhang et al. (2019), Bressani and Elias (1977) and Jood et al. (1989) on jack bean, sweet potato protein, chick pea, blackgram, common bean, have reported that autoclaving resulted in the highest increase in IVPD compared to other thermal processing methods. Increased IVPD in peanut, sesame seeds, pea, black grams, chickpeas, lentils, red and white kidney beans after thermal treatments (boiling, autoclaving, microwave cooking, roasting *etc.*) was also reported by Embaby, 2010; Park et al., 2010; Rehman & Shah, 2005. Thermal treatment and germination showed a trend of increasing DH in dal and wholegrains of both the genotypes which indicated the increase of proteolysis after thermal treatment and germination. The DH was found to be significantly increased ($p < 0.05$) by 22.92–31.37% in P^+ and 23.43–37.67% in P^- after thermal treatments and germination. The highest increase in DH was observed after AC in both the genotypes (Fig. 1. C and D). The increase in DH was positively correlated with the increase in IVPD after thermal treatments and germination in both the genotypes (Supplementary Fig. 4). Improvement of DH after thermal treatment was reported in soybean, lentil, black turtle bean and increase in protein digestibility along with DH was found in germinated pigeon pea seeds (Zahir, Fogliano, & Capuano, 2018; Zhang et al., 2019; Ohanenye, Sun, Sarteshnizi, & Udenigwe, 2021). However, thermal treatment and germination enabled better IVPD and DH in the genotype having low polyphenol (P^-) compared to the genotype having high polyphenol (P^+). Thus, these results clearly demonstrate that inherent polyphenol content in pigeon pea can hinder proteolysis by complexing with protein thus form tight protein matrices that consequently lead to low accessibility to digestive enzymes and hence reduced IVPD was observed in genotype having high polyphenol (P^+) compared to the genotype having low polyphenol (P^-).

3.3.2. *Free sulfhydryl (FSH) group*

The FSH group from native and treated flour of pigeon pea are shown in Fig. 1. E and F and it was observed that thermal treatments and germination significantly ($p < 0.05$) improved the FSH by 14.09–29.93% in P^+ and 19.51–33.7% in P^-. AC showed the highest increase in FSH group in both the genotypes. Previously the increase in FSH group was reported in quinoa albumin, sesame protein after heat treatment and germination (Yang et al., 2022; Di et al., 2022). The increase in the FSH group in thermal treated and germinated seeds suggest the loosening of the compact structure of protein matrices due to protein denaturation and breakdown of storage proteins, which was further vindicated by SEM images, FTIR analysis (observed change in the absorbance in the amide I range) and a decrease in the band intensity in SDS-PAGE (Fig. 4). Higher FSH content was due to a conversion of in native protein structure into a denatured protein form which showed a positive correlation with the increase in IVPD of treated seeds, indicating a better access to the digestive enzymes (Supplementary Fig. 4).

3.4. *Secondary structure analysis by FTIR*

The FTIR spectra displayed distinct peaks in the amide I region (Supplementary Fig. 3), specifically at 1700 cm^{-1} and 1600 cm^{-1}, changes in this amide I region underlie the changes in the secondary structure of the protein molecule. These peaks are characteristic of various secondary structures in proteins: 1623–1641 cm^{-1} and 1674–1694 cm^{-1} for β-sheets, 1648–1657 cm^{-1} for α-helices, and 1660–1684 cm^{-1} for β-turns. In this study, the β-turn content in native flour of whole grain and dal was 12.41% and 13.85% respectively in P^+ and 15.97% and 16.4% respectively in P^-. This was found to increase significantly ($P < 0.05$) after thermal treatments and germination by 9.89–28.1% in P^+ and 6.59–24.26% in P^- (Table 2). However, after thermal treatment and germination there was a significant decrease in α-helix content (32.36–41.25% in P^+ and 31.14–46.14% in P^-) and a small and non-significant decrease in β-sheets content (3.15–9.08% in P^+ and 0.51–7.3% in P^-). The significant increase in β-turn was observed after thermal treatment and germination which might enable increased IVPD. Thus, this result clearly indicated that improvement of IVPD after thermal treatment and germination was primarily due to higher β-turn content which in turn enabled higher accessibility to protein digestive enzymes. The higher content of β-turn in low polyphenol containing variety suggests that thermal treatment might exposed β-turn on the protein surface because of low hindrance by polyphenols for accessibility of proteases, hence increased IVPD in low polyphenol containing variety (P^-).

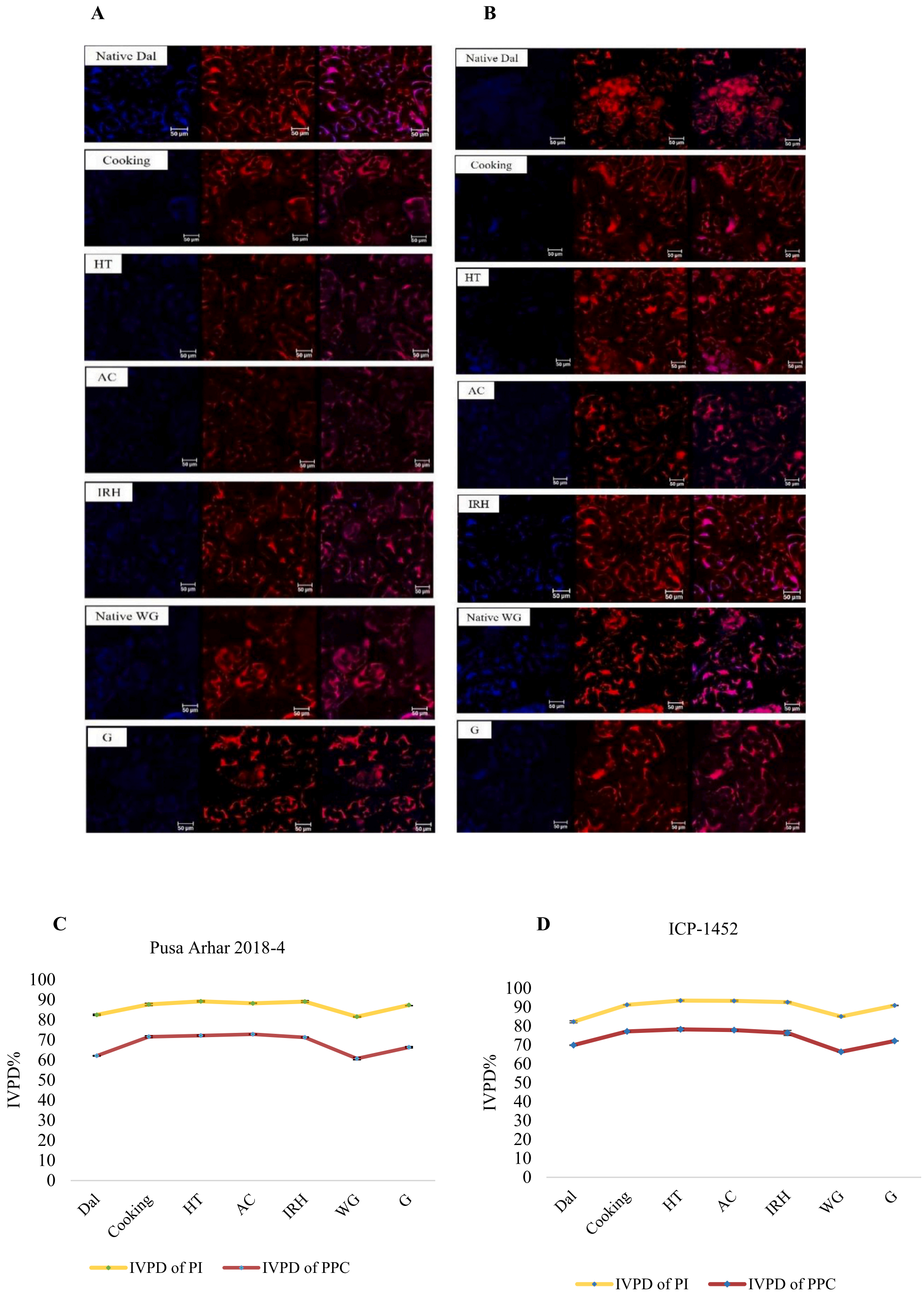

Fig. 3. Confocal laser scanning micrographs of polyphenol and protein pigeon pea flour of Pusa Arhar 2018–4 (A) and ICP1452 (B) depicting protein (red), polyphenol (blue) and protein-polyphenol overlay images. IVPD % of protein isolates (PI) and protein-polyphenol complex (PPC) of Pusa Arhar 2018–4 (C) and ICP1452 (D). Values are means of three replicates with standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. *Microstructure images of pigeon pea flours by SEM*

The microstructure of flour samples from native and treated flour of both the genotypes (P^+ and P^-) was analyzed through SEM (Fig. 2. A and B). The distinct structures of starch granules and protein molecules are observed. In native flours, starch molecules appear as oval-shaped, smooth-surfaced granular structure, while the protein molecules present as irregularly clustered entities with rough surfaces and were observed to be tightly adhering to the surface of the starch granules (Möller et al., 2021). Following a thermal treatment, the starch molecules underwent gelatinization, losing its granular structure to present a larger and rougher appearance causing the release of adhered protein molecules into the flour matrix. Additionally, the disrupted interaction between the proteins and starch molecules offered an enhanced protease accessibility. Additionally in the treated flours, smaller fractions of denatured proteins were also observed, making them more accessible to digestive enzymes and thus contributing to the improved digestibility of the protein (Sun et al., 2020). However, the changes in starch granule were not prominent in the germinated seed flour derived from both the genotypes. A small fraction of protein was

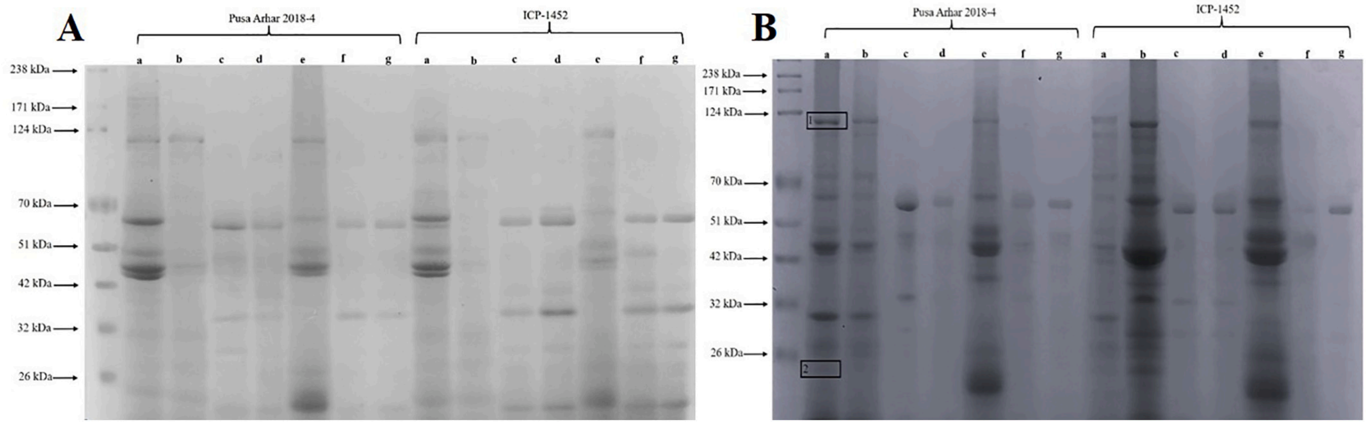

Fig. 4. SDS-PAGE image of globulin (A) and albumin (B) fractions in untreated and treated seeds of Pusa Arhar 2018–4 and ICP-1452. 1. Lipoxygenase 2. Trypsin inhibitor WG-Whole grain, HT-Hydrothermal, AC- Autoclaved, IRH- Infrared heating G- Germination.

observed implying the degradation of protein molecules during the germination process that in turn could have contributed to the increased IVPD.

3.6. *Confocal laser scanning microscopy*

The native and treated flours of both the genotypes (P^+ and P^-) were examined using CLSM (Fig. 3. A and B). The microstructure of flour highlighted the presence of protein (red) and polyphenol (blue). In native flour the polyphenol molecules were more prominent than in treated and germinated seeds, and showed a close interaction with protein which can be seen as purple colour due to fusion of protein (red) and polyphenol (blue) molecules. This interaction appeared less prominent in thermal treatment and germination, as indicated by a faint purple colour. Hence germination and thermal processing reduce the interactions between protein and polyphenol which in turn facilitates the high accessibility for protein degrading enzymes thereby improving IVPD. Furthermore, compared to the protein molecules in native flour, molecules in thermally treated and germinated flour show a disorganized and non-compact arrangement due to protein denaturation and degradation.

3.7. *Effect of polyphenol on the in vitro protein digestibility*

To determine the effect of polyphenol on the IVPD, protein polyphenol complex (PPC) was allowed to form under *in vitro* conditions and IVPD was evaluated. This approach serves as an indirect measure to understand how polyphenols influence digestibility. The result of IVPD of PPC showed a positive effect of thermal treatment and germination on IVPD (Fig. 3. C and D). In P^+, native whole grain PI showed IVPD of 81.48%, and dal PI exhibited 82.42%. After subjecting the samples to thermal treatments and germination, the IVPD of PI ranged from 87.45% to 89.03% indicating an improvement in IVPD of PI after thermal treatment and germination. However, when these isolates formed complexes with polyphenol extract, a significant decrease in IVPD was observed. The reduction was 25.57% in whole grain and 24.73% in dal. Interestingly, PPC derived from thermally treated and germinated seeds showed a significant ($p < 0.05$) increase in IVPD compared to PPC from native flour (9.29% to 17.27%) in P^+. A similar trend was observed in P^-, where native whole grain PI had an IVPD of 83.16%, and for dal, it was 84.94%. After thermal treatment and germination, the IVPD of PI increased to a range of 90.76% to 93.37%. However, upon complex formation of native PI with polyphenol extract, there was a significant decrease in IVPD. The reduction was 21.97% in whole grain PPC and 15.05% in dal PPC. PPC derived from thermally treated and germinated samples displayed a significant increase (8.7%–11.97%) in IVPD compared to ones derived from native flours. It was also observed that the inhibitory effect of the polyphenol extract is more pronounced in the genotype with higher polyphenol content (P^+) compared to the genotype with lower polyphenol content (P^-). Following thermal treatment and germination, denaturation of PI and degradation respectively, could have caused a decreased interaction with polyphenols, [as evident from CLSM images (Fig. 3)] and hence facilitated increased IVPD. Decreased IVPD of protein complexed with polyphenol has been reported in the instances of soy protein isolate (SPI)/black soybean seed coat extract (BE) complexes, whey protein/fruit phenolics complex (chlorogenic acid and catechin) and whey protein isolates (WPI)/caffeic acid complex (He et al., 2015; de Morais et al., 2020; Ren et al., 2018).

3.8. *Changes in the pattern of storage protein fractions profiling*

Major fraction of pigeon pea storage protein is globulin fraction (54–60%) (Olagunju & Omoba, 2021). SDS-PAGE was performed to profile the changes in the globulin and albumin fragments of pigeon pea seeds and their effect on IVPD after thermal treatments and germination. The globulin fraction profile of native pigeon pea flour showed two prominent bands of molecular weight near 65 and 47 kDa (Fig. 4. A) which represent the two subunits of 7 s vicilin which is the most abundant globulin in pigeon pea (Olagunju & Omoba, 2021). These subunits displayed significant reductions in band intensities, ranging from 18.11% to 72.55% in P^+ and from 49.36% to 98.12% in P^-, following both thermal treatments and germination (Supplementary Table 2). The albumin fraction profile showed four major fractions of size 94, 63, 43 and 21 kDa which were found to be prominent in native flour as compared to treated and germinated seeds. The albumin fraction in legumes contains other undesirable proteins, including lipoxygenases, TIs, lectins, as well as antigenic and allergenic compounds. The band near 94 kDa and 21 kDa represents lipoxygenases and TI respectively which were conspicuously missing after thermal treatments and germination (Fig. 4 B). The rise in IVPD observed in seeds subjected to thermal treatment and germination could be attributed to the breakdown of both globulin and albumin fractions, as observed in SDS-PAGE, which was further supported by the SEM imaging as denatured fractions of protein was evident in the thermal treated and germinated sample. Similar studies of significant reduction of storage proteins in various legumes like green gram, quinoa and other legumes after thermal treatments and its positive correlation with protein digestibility has already been reported (Sánchez-Velázquez et al., 2021; Sashikala et al., 2015; Yang et al., 2022).

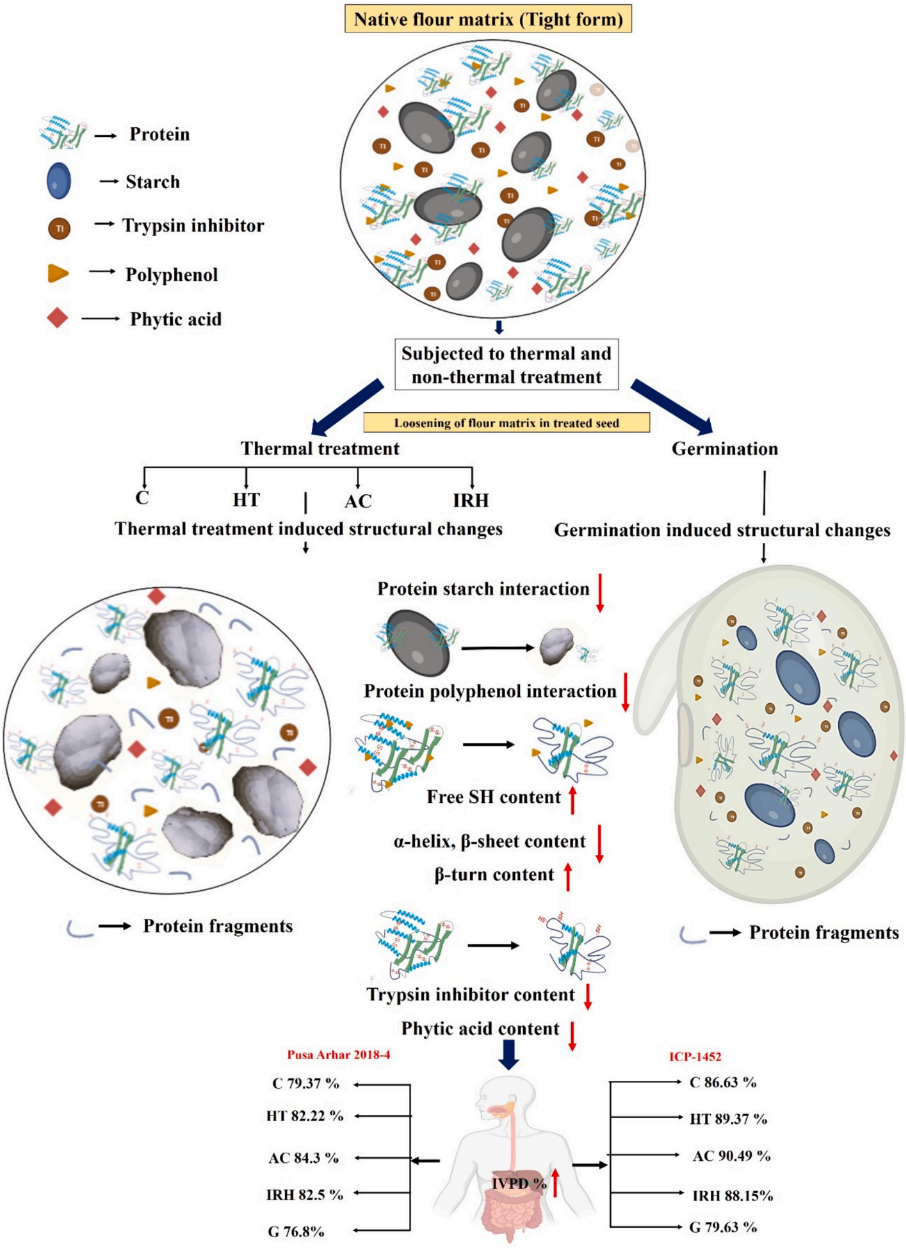

Fig. 5. Thermal treatments like cooking (C), hydrothermal (HT), autoclaving (AC), infrared heating (IRH) and germination (G) increased β-turn and random coil content, thereby increasing protein digestibility (90.49% in AC). Increased free sulfhydryl (SH) group enhanced the hydrophilicity causing an exposure of hydrophilic amino acid on the surface of protein. In addition, breakdown of storage protein, disintegration protein-polyphenol and protein-starch interactions in flour matrix attributed to loosening of the protein matrices that facilitated greater accessibility of protein hydrolysing enzymes leading to an improved protein digestibility. Inherently, higher content of polyphenols in pigeon pea has negatively impacted the protein digestibility. Protein isolated after heat treatment, when subjected to complexation with polyphenol lowered interaction due to heat induced changes in protein structure and hence increased protein digestibility.

3.9. Effect of thermal treatments and germination on antinutritional factors (ANFs)

Table 3. summarizes ANFs (polyphenol, TI, PA) content in native and treated flour of the two genotypes (P^+ and P^-). Reduction in these ANFs were observed in thermal treated and germinated flours. Among the treatments AC showed significantly higher reduction of TP content up to 36.49% followed by HT (34.54%), C (32.43%), IRH (30.03%) and G (21.59%) in P^+. In P^-, AC showed 31.7% reduction, followed by HT (28.52%), IRH (27.61%), C (24.69%) and G (9.2%). Major fraction of polyphenol in pigeon pea was present in BF form which was also found to be significantly lower after AC (64.53% in P^+ and 66.63% in P^-) as compared to C, HT, IRH and G. A small yet significant increase in FF content was observed in both P^+ (1.8–9.8%) and P^- (6.5–18.37%) following thermal treatments and germination. The TI content in native whole grain and dal was found to be 1805.65 ± 26.01 and 1617.91 ± 26.70 TIU/g respectively in P^+**,** 1497.71 ± 8.62 and 1258.21 ± 20.96 TIU/g respectively in P^-. The TI content in treated flour was found to be 508.24 ± 43.55–731.71 ± 3.09 TIU/g in P^+ and 440.68 ± 16.81–468.70 ± 26.84 TIU/g in P^-. AC showed highest reduction of TI by 68.58% in P^+ and 64.97% in P^-. PA in the native whole grain and dal showed highest amount of PA of 1.67 g/100 g and 1.75 g/100 g respectively in P^+ and 0.78 g/100 g and 0.86 g/100 g respectively in P^-. Following thermal treatment and germination, a significant reduction in PA content was observed, where IRH showed highest reduction in both the genotypes of P^+ and P^- (77.2 and 53.71% respectively).

Table 3
ANFs content in native, germinated and thermal treated pigeon pea varieties.

		Polyphenol (mg GAE/100 g)			TI (TIU/g)	Phytic acid (g/100 g)
		Total polyphenol	Free Fraction (FF)	Bound fraction (BF)		
Pusa Arhar 2018-4						
	Dal	652.21 ± 1.65^{c}	275.33 ± 0.98^{c}(21%)	376.84 ± 1.20^{c}	1617.91 ± 26.70^{f}	1.67 ± 0.15^{d}
Control	WG	1250.02 ± 1.69^{d}	571.95 ± 0.63^{d}	678.27 ± 3.20^{e}	1805.65 ± 26.01^{g}	1.76 ± 0.03^{d}
Cooking		456.29 ± 4.68^{b}(30.03%)	294.18 ± 1.85^{b}(6.83%)	$167.71 \pm 3.62^{a,b}$(55.49%)	540.61 ± 11.98(66.5%)	0.64 ± 0.05^{b}(61.2%)
HT		426.88 ± 0.82^{a}(34.54%)	288.57 ± 0.61^{a}(4.79%)	136.99 ± 0.64^{a}(63.64%)	528.47 ± 19.75^{c}(67.3%)	$0.47 \pm 0.005^{a,b}$(71.6%)
AC		414.19 ± 0.76^{b}(36.49%)	289.89 ± 1.37^{a}(5.27%)	120.0 ± 0.74^{b}(68.15%)	508.24 ± 43.55^{a}(68.58%)	0.32 ± 0.06^{a}(32.3%)
IRH		440.68 ± 7.44^{b}(32.43%)	301.11 ± 2.34^{b}(9.34%)	139.56 ± 4.79^{b}(62.96%)	583.26 ± 7.86^{b}(63.94%)	0.38 ± 0.02^{a}(77.2%)
G		980.06 ± 5.09^{d}(21.6%)	593.31 ± 1.20^{e}(3.73%)	386.75 ± 6.15^{d}(42.97%)	731.71 ± 3.09^{e}(59.4%)	0.99 ± 0.01^{c}(43.3%)
ICP1452						
Control	Dal	173.03 ± 1.09^{b}	74.96 ± 0.55^{a}	98.06 ± 0.81^{b}	$1258.21 \pm 20.96^{c,d}$	0.810 ± 0.02^{b}
	WG	215.33 ± 0.98^{d}	105.3 ± 0.65^{e}	110.03 ± 0.51^{d}	1497.71 ± 8.62^{d}	1.004 ± 0.05^{c}
Cooking		130.15 ± 3.84^{a}(24.78%)	88.74 ± 1.45^{b}(18.37%)	$41.41 \pm 2.4^{a,\ b}$(57.76%)	$468.25 \pm 19.01^{b,c}$(62.78%)	0.4 ± 0.009^{a}(50.41%)
HT		123.53 ± 0.67^{a}(28.6%)	84.35 ± 1.18^{b}(12.51%)	39.17 ± 1.05^{a}(60.04%)	440.68 ± 16.81^{a}(64.97%)	0.46 ± 0.01^{a}(42.9%)
AC		117.94 ± 1.13^{a}(31.83%)	85.29 ± 1.97^{c}(13.77%)	32.64 ± 3.05^{a}(66.7%)	468.70 ± 26.84^{a}(62.74%)	0.37 ± 0.005^{a}(53.7%)
IRH		125.10 ± 2.25^{a}(27.7%)	86.03 ± 1.42^{b}(14.76%)	39.06 ± 1.06^{a}(60.16%)	441.94 ± 34.91^{a}(64.87%)	0.53 ± 0.005^{a}(33.88%)
G		194.75 ± 2.95^{c}(9.55%)	112.21 ± 0.72^{d}(6.56%)	88.75 ± 3.66^{c}(15.82%)	680.66 ± 5.68^{b}(54.55%)	0.81 ± 0.10^{b}(18.6%)

- Values are means ± standard error ($n = 3$).
- For both the variety, means with the same letters were not significantly different ($p < 0.05$).

Values in parenthesis are percent change in comparison to control. (WG as control for G, and dal as control for Cooking, HT, AC, IRH)

Decreased polyphenol content during thermal treatment and germination could be due to breakdown of polyphenols as observed in CLSM studies (Fig. 3) and the loss of water-soluble polyphenol during soaking prior to thermal treatments or the activation of polyphenol oxidase might have contributed to the breakdown of polyphenolic compounds in germinated seeds. Additionally, the application of heat during processing disrupts the bound polyphenol complexes, releasing previously bound phenolics into a free form. This accounts for the substantial decrease in BF content and a slight increase in FF content in the thermal treated seeds. The TIs are small proteins of low molecular weight and the significant reduction in their content could be attributed to the heat induced denaturation during thermal, whereas germination caused a mobilization of breakdown of these proteins which was visualized in SDS-PAGE image (Fig. 4). The susceptibility of PA to high temperature caused degradation and the likely cause for its reduction (Sharma et al., 2018). This reduction in ANFs after processing treatments was previously reported in cowpea, kidney beans, peas, and chickpea following different treatments like autoclaving, microwave cooking, and roasting. Reduction of TI after pressure cooking, soaking and germination in pigeon pea and other legumes and reduction of polyphenol content after thermal treatment and germination in common bean, faba bean, pigeon pea and rice were also reported (Sharma et al., 2018; Khattab et al., 2009; Singh, 1993; Carbonaro et al., 2000, Alonso et al., 2000; Duhan et al., 2001; Trugo et al., 2000).

The study's findings reveal a remarkable connection between the reduction of ANFs and the observed improvements in IVPD and DH value in the treated pigeon pea seeds. It appears that the thermal treatment and germination processes applied in this study have effectively reduced these antinutritional factors, leading to an enhanced IVPD and a more extensive DH. This suggests that the decrease in polyphenols, TIs, and PA has a positive impact on the nutritional quality of pigeon pea seeds, making them more accessible and beneficial for human consumption.

4. Conclusion

This study explores the enhancement of pigeon pea protein quality after thermal treatments and germination. The investigation includes an analysis of *in vitro* protein digestibility (IVPD), essential amino acids (EAAs) content, and factors influencing IVPD. The findings revealed that among the four thermal treatments—cooking, hydrothermal treatment, autoclaving, infrared heating—and the non-thermal treatment of germination, autoclaving exerts the most significant impact on improving IVPD. Autoclaving demonstrates the highest influence on altering protein secondary structure and reducing anti-nutritional factors (ANFs). The study further indicates that polyphenols play an inhibitory role in IVPD, and their reduction following thermal treatment and germination contributes to the overall improvement in protein quality and digestibility. The impact of polyphenols on IVPD is also evident from the two contrasting varieties used in this study, as thermal treatment had a better effect on the low polyphenol-containing variety compared to the high polyphenol-containing variety. In conclusion, autoclaving can be an effective method to improve protein digestibility of pigeon peas.

CRediT authorship contribution statement

Minakshi Dutta: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **R. Dineshkumar:** Investigation, Formal analysis, Data curation. **C.R. Nagesh:** Investigation, Formal analysis. **Y. Durga Lakshmi:** Investigation. **Brijesh Lekhak:** Investigation. **Navita Bansal:** Methodology, Investigation. **Suneha Goswami:** Validation, Methodology. **Aditi Kundu:** Methodology, Formal analysis. **Pranab Kumar Mandal:** Validation, Methodology. **Bindvi Arora:** Methodology, Formal analysis. **Ranjeet Sharad Raje:** Methodology, Formal analysis. **Somnath Mandal:** Methodology, Formal analysis. **Achchhelal Yadav:** Methodology, Formal analysis. **Aruna Tyagi:** Conceptualization. **S.V. Ramesh:** Writing – review & editing. **G. Rama Prashat:** Resources, Methodology, Formal analysis. **T. Vinutha:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

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.

Data availability

Data will be made available on request.

Acknowledgements

The authors thank University Science Instrumentation Centre (USIC), Delhi University for use of the Jeol JSM 6610LV SEM instrument, and National Institute of Plant Genome Research (NIPGR) for providing the facility of CLSM and Indian Council of Agricultural Research Indian Agricultural Research Institute (IARI) for financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140561.

References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*(6), 1256–1262.

Alonso, R., Orue, E., Zabalza, M. J., Grant, G., & Marzo, F. (2000). Effect of extrusion cooking on structure and functional properties of pea and kidney bean proteins. *Journal of the Science of Food and Agriculture, 80*(3), 397–403. https://doi.org/10.1002/1097-0010(200002)80:3%3C397::AID-JSFA542%3E3.0.CO;2-3

Anderson, J. W., & Fitzgerald, M. A. (2003). Sulphur distribution and redistribution in vegetative and generative plants. *Sulphur in Plants*, 113–134.

AOAC. (2006). *Official methods of analysis (18thed.)*. Washington, DC: The Association of Official Analytical Chemists.

Arise, A. K., Malomo, S. A., Cynthia, C. I., Aliyu, N. A., & Arise, R. O. (2022). Influence of processing methods on the antinutrients, morphology and in-vitro protein digestibility of jack bean. *Food Chemistry Advances, 1*, Article 100078.

Bressani, R., & Elias, L. G. (1977). Problem of legume protein digestibility. In *Nutritional standards and methods of evaluation for food legume breeders*. Ottawa, ON, CA: IDRC.

Candela, M., Astiasaran, I., & Bello, J. (1997). Cooking and warm-holding: Effect on general composition and amino acids of kidney beans (*Phaseolus vulgaris*), chickpeas (*Cicer arietinum*), and lentils (*Lens culinaris*). *Journal of Agricultural and Food Chemistry, 45*(12), 4763–4767. https://pubs.acs.org/action/doSearch?field1=Contrib&text1=Montserrat++Candela.

Carbonaro, M., Grant, G., Cappelloni, M., & Pusztai, A. (2000). Perspectives into factors limiting in vivo digestion of legume proteins: Antinutritional compounds or storage proteins? *Journal of Agricultural and Food Chemistry, 48*(3), 742–749. https://doi.org/10.1021/jf991005m

Di, Y., Li, X., Chang, X., Gu, R., Duan, X., Liu, F., … Wang, Y. (2022). Impact of germination on structural, functional properties and in vitro protein digestibility of sesame (Sesamum indicum L.) protein. *LWT-Food Science and Technology, 154*, 112651.

Duhan, A., Khetarpaul, N., & Bishnoi, S. (1999). Effect of various domestic processing and cooking methods on phytic acid and HCl-extractability of calcium, phosphorus and iron of pigeonpea. *Nutrition and Health, 13*(3), 161–169.

Duhan, A., Khetarpaul, N., & Bishnoi, S. (2001). Saponin content and trypsin inhibitor activity in processed and cooked pigeon pea cultivars. *International Journal of Food Sciences and Nutrition, 52*(1), 53–59. https://doi.org/10.1080/09637480020027200

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia, 77*(2), 67–82. https://doi.org/10.1016/j.fitote.2005.11.008

El-Adawy, T. A. (2002). Nutritional composition and antinutritional factors of chickpeas (*Cicer arietinum* L.) undergoing different cooking methods and germination. *Plant Foods for Human Nutrition, 57*, 83–97. https://doi.org/10.1023/A:1013189620528

Embaby, H. E. S. (2010). Effect of heat treatments on certain antinutrients and *in vitro* protein digestibility of peanut and sesame seeds. *Food Science and Technology Research, 17*(1), 31–38. https://doi.org/10.3136/fstr.17.31

FAO. (1991). *Protein quality evaluation, 51*, 1.

Gao, Y., Ma, S., Wang, M., & Feng, X. Y. (2017). Characterization of free, conjugated, and bound phenolic acids in seven commonly consumed vegetables. *Molecules, 22*(11), 1878.

Gerrano, A. S., Moalafi, A., Seepe, H. A., Amoo, S., & Shimelis, H. (2022). Nutritional and phytochemical compositions and their interrelationship in succulent pods of pigeonpea (*Cajanus cajan* [L.] Millsp.). *Heliyon, 8*(3). https://doi.org/10.1016/j.heliyon.2022.e09078

Godbole, S. A., Krishna, T. G., & Bhatia, C. R. (1994). Changes in protease inhibitory activity from pigeon pea (*Cajanus cajan* (L) Millsp.) during seed development and germination. *Journal of the Science of Food and Agriculture, 66*(4), 497–501. https://doi.org/10.1002/jsfa.2740660412

Grant, G., More, L. J., McKenzie, N. H., Stewart, J. C., & Pusztai, A. (1983). A survey of the nutritional and haemagglutination properties of legume seeds generally available in the UK. *British Journal of Nutrition, 50*(2), 207–214.

Gu, J., Bk, A., Wu, H., Lu, P., Nawaz, M. A., Barrow, C. J., … Suleria, H. A. R. (2022). Impact of processing and storage on protein digestibility and bioavailability of legumes. *Food Reviews International, 39*(7), 4697–4724. https://doi.org/10.1080/87559129.2022.2039690

Habiba, R. A. (2002). Changes in anti-nutrients, protein solubility, digestibility, and HCl-extractability of ash and phosphorus in vegetable peas as affected by cooking methods. *Food Chemistry, 77*(2), 187–192. https://doi.org/10.1016/S0308-8146(01)00335-1

Hao, L., Sun, J., Pei, M., Zhang, G., Li, C., Li, C., … Liu, L. (2022). Impact of non-covalent bound polyphenols on conformational, functional properties and in vitro digestibility of pea protein. *Food Chemistry, 383*, Article 132623. https://doi.org/10.1016/j.foodchem.2022.132623

He, Z., Yuan, B., Zeng, M., Tao, G., & Chen, J. (2015). Effect of simulated processing on the antioxidant capacity and *in vitro* protein digestion of fruit juice-milk beverage model systems. *Food Chemistry, 175*, 457–464. https://doi.org/10.1016/j.foodchem.2014.12.007

Igbedioh, S. O., Olugbemi, K. T., & Akpapunam, M. A. (1994). Effects of processing methods on phytic acid level and some constituents in bambara groundnut (*Vigna subterranea*) and pigeon pea (*Cajanus cajan*). *Food Chemistry, 50*(2), 147–151.

Jakubczyk, A., Szymanowska, U., Karaś, M., Złotek, U., & Kowalczyk, D. (2019). Potential anti-inflammatory and lipase inhibitory peptides generated by in vitro gastrointestinal hydrolysis of heat-treated millet grains. *CyTA Journal of Food, 17*(1), 324–333.

Jood, S., Chauhan, B. M., & Kapoor, A. C. (1989). Protein digestibility (in vitro) of chickpea and blackgram seeds as affected by domestic processing and cooking. *Plant Foods for Human Nutrition, 39*, 149–154.

Kachare, D. P., Satbhai, R. D., Rathod, D. B., & Naik, R. M. (2019). Evaluation of pigeon pea (*Cajanus cajan* L.) genotypes for nutritional quality. *Legume Research-An International Journal, 42*(4), 485–489. https://doi.org/10.18805/LR-3899

Kakade, M. L. (1969). An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soybean samples. *Cereal Chemistry, 46*, 518–526.

Kalpanadevi, V., & Mohan, V. R. (2013). Effect of processing on antinutrients and *in vitro* protein digestibility of the underutilized legume, *Vigna unguiculata* (L.) Walp subsp. *unguiculata*. *LWT- Food Science and Technology, 51*(2), 455–461.

Khattab, R. Y., Arntfield, S. D., & Nyachoti, C. M. (2009). Nutritional quality of legume seeds as affected by some physical treatments, part 1: Protein quality evaluation. *LWT- Food Science and Technology, 42*(6), 1107–1112. https://doi.org/10.1016/j.lwt.2009.02.008

Kuraz Abebe, B. (2022). The dietary use of pigeon pea for human and animal diets. *The Scientific World Journal, 2022*. https://doi.org/10.1155/2022/4873008

Lamothe, S., Azimy, N., Bazinet, L., Couillard, C., & Britten, M. (2014). Interaction of green tea polyphenols with dairy matrices in a simulated gastrointestinal environment. *Food & Function, 5*(10), 2621–2631. https://doi.org/10.1039/C4FO00203B

Möller, A. C., van der Padt, A., & van der Goot, A. J. (2021). From raw material to mildly refined ingredient–linking structure to composition to understand fractionation processes. *Journal of Food Engineering, 291*, Article 110321. https://doi.org/10.1016/j.jfoodeng.2020.110321

de Morais, F. P., Pessato, T. B., Rodrigues, E., Mallmann, L. P., Mariutti, L. R., & Netto, F. M. (2020). Whey protein and phenolic compound complexation: Effects on antioxidant capacity before and after *in vitro* digestion. *Food Research International, 133*, Article 109104. https://doi.org/10.1016/j.foodres.2020.109104

Mubarak, A. E. (2005). Nutritional composition and antinutritional factors of mung bean seeds (*Phaseolus aureus*) as affected by some home traditional processes. *Food Chemistry, 89*(4), 489–495. https://doi.org/10.1016/j.foodchem.2004.01.007

Ohanenye, I. C., Sun, X., Sarteshnizi, R. A., & Udenigwe, C. C. (2021). Germination alters the microstructure, in vitro protein digestibility, α-glucosidase and dipeptidyl peptidase-IV inhibitory activities of bioaccessible fraction of pigeon pea (*Cajanus cajan*) seeds. *Legume Science, 3*(1), e79.

Olagunju, A. I., & Omoba, O. S. (2021). Pigeon peas possess significant protein and starch fractions with potential as a functional ingredient in food applications. In *Handbook of cereals, pulses, roots, and tubers* (pp. 343–356). CRC Press.

Onyeike, E. N., & Omubo-Dede, T. T. (2002). Effect of heat treatment on the proximate composition, energy values, and levels of some toxicants in African yam bean (*Sphenostylis stenocarpa*) seed varieties. *Plant Foods for Human Nutrition, 57*, 223–231. https://doi.org/10.1023/A:1021833516234

Pan, M., Xu, F., Wu, Y., Yao, M., Xiao, X., Zhang, N., et al. (2020). Application of ultrasound-assisted physical mixing treatment improves *in vitro* protein digestibility of rapeseed napin. *Ultrasonics Sonochemistry, 67*, Article 105136. https://doi.org/10.1016/j.ultsonch.2020.105136

Papalamprou, E. M., Doxastakis, G. I., & Kiosseoglou, V. (2010). Chickpea protein isolates obtained by wet extraction as emulsifying agents. *Journal of the Science of Food and Agriculture, 90*(2), 304–313. https://doi.org/10.1002/jsfa.3816

Park, S. J., Kim, T. W., & Baik, B.-K. (2010). Relationship between proportion and composition of albumins, and *in vitro* protein digestibility of raw and cooked pea seeds (*Pisum sativum* L.). *Journal of the Science of Food and Agriculture, 90*(10), 1719–1725. https://doi.org/10.1002/jsfa.4007

Putra, L. S., Sukweenadhi, J., Nathania, C., Wibowo, E. S., Buschle-Diller, G., & Purwanto, M. G. M. (2022). Comparative study of polyphenolic compound extraction from empty palm fruit bunches and sugarcane pulp. *Heliyon, 8*(2). https://doi.org/10.1016/j.heliyon.2022.e08951

Rehman, Z.-u., & Shah, W. H. (2005). Thermal heat processing effects on antinutrients, protein and starch digestibility of food legumes. *Food Chemistry, 91*(2), 327–331. https://doi.org/10.1016/j.foodchem.2004.05.054

Ren, C., Xiong, W., Peng, D., He, Y., Zhou, P., Li, J., & Li, B. (2018). Effects of thermal sterilization on soy protein isolate/polyphenol complexes: Aspects of structure, *in vitro* digestibility and antioxidant activity. *Food Research International, 112*, 284–290. https://doi.org/10.1016/j.foodres.2018.06.034

Sá, A. G. A., Moreno, Y. M. F., & Carciofi, B. A. M. (2020). Food processing for the improvement of plant proteins digestibility. *Critical Reviews in Food Science and Nutrition, 60*(20), 3367–3386. https://doi.org/10.1080/10408398.2019.1688249

Samtiya, M., Aluko, R. E., & Dhewa, T. (2020). Plant food anti-nutritional factors and their reduction strategies: An overview. *Food Production, Processing and Nutrition, 2*, 1–14.

Sánchez-Velázquez, O. A., Ribéreau, S., Mondor, M., Cuevas-Rodríguez, E. O., Arcand, Y., & Hernández-Álvarez, A. J. (2021). Impact of processing on the *in vitro* protein quality, bioactive compounds, and antioxidant potential of 10 selected pulses. *Legume Science, 3*(2), Article e88. https://doi.org/10.1002/leg3.88

Sarika, K., Hossain, F., Muthusamy, V., Baveja, A., Zunjare, R. S., Goswami, R., … Gupta, H. S. (2017). Exploration of novel opaque16 mutation as a source for high lysine and tryptophan in maize endosperm. *Indian Journal Of Genetics And Plant Breeding, 77*(01), 59–64. https://doi.org/10.5958/0975-6906.2017.00008.6

Sarkar, S., Panda, S., Yadav, K. K., & Kandasamy, P. (2020). Pigeon pea (*Cajanus cajan*) an important food legume in Indian scenario-a review. *Legume Research-An International Journal, 43*(5), 601–610. https://doi.org/10.18805/LR-4021

Sashikala, V. B., Sreerama, Y. N., Pratape, V. M., & Narasimha, H. V. (2015). Effect of thermal processing on protein solubility of green gram (*Phaseolus aureus*) legume cultivars. *Journal of Food Science and Technology, 52*, 1552–1560. https://doi.org/10.1007/s13197-013-1149-x

Segev, A., Badani, H., Kapulnik, Y., Shomer, I., Oren-Shamir, M., & Galili, S. (2010). Determination of polyphenols, flavonoids, and antioxidant capacity in colored chickpea (*Cicer arietinum* L.). *Journal of Food Science, 75*(2), S115–S119.

Sharma, S., Agarwal, N., & Verma, P. (2011). Pigeon pea (*Cajanus cajan* L.): A hidden treasure of regime nutrition. *Journal of Functional and Environmental Botany, 1*(2), 91–101. https://doi.org/10.5958/j.2231-1742.1.2.010

Sharma, S., Singh, A., Sharma, U., Kumar, R., & Yadav, N. (2018). Effect of thermal processing on anti-nutritional factors and *in vitro* bioavailability of minerals in desi and kabuli cultivars of chick pea grown in North India. *Legume Research-An International Journal, 41*(2), 267–274. https://doi.org/10.18805/LR-3708

Singh, U. (1988). Antinutritional factors of chickpea and pigeonpea and their removal by processing. *Plant Foods for Human Nutrition, 38*, 251–261.

Singh, U. (1993). Protein quality of pigeon pea (*Cajanus cajan* (L.) Millsp.) as influenced by seed polyphenols and cooking process. *Plant Foods for Human Nutrition, 43*, 171–179. https://doi.org/10.1007/BF01087921

Singh, U., & Eggum, B. O. (1984). Factors affecting the protein quality of pigeon pea (*Cajanus cajan* L.). *Plant Foods for Human Nutrition, 34*, 273–283. https://doi.org/10.1007/BF01126556

Sun, X., Ohanenye, I. C., Ahmed, T., & Udenigwe, C. C. (2020). Microwave treatment increased protein digestibility of pigeon pea (*Cajanus cajan*) flour: Elucidation of underlying mechanisms. *Food Chemistry, 329*, Article 127196. https://doi.org/10.1016/j.foodchem.2020.127196

Świeca, M., Gawlik-Dziki, U., Dziki, D., Baraniak, B., & Czyż, J. (2013). The influence of protein–flavonoid interactions on protein digestibility *in vitro* and the antioxidant quality of breads enriched with onion skin. *Food Chemistry, 141*(1), 451–458. https://doi.org/10.1016/j.foodchem.2013.03.048

Trugo, L. C., Donangelo, C. M., Trugo, N. M. F., & Bach Knudsen, K. E. (2000). Effect of heat treatment on nutritional quality of germinated legume seeds. *Journal of Agricultural and Food Chemistry, 48*(6), 2082–2086. https://doi.org/10.1021/jf9913920

Uppal, V., & Bains, K. (2012). Effect of germination periods and hydrothermal treatments on *in vitro* protein and starch digestibility of germinated legumes. *Journal of Food Science and Technology, 49*, 184–191.

Vaga, M., Hetta, M., & Huhtanen, P. (2017). Effects of heat treatment on protein feeds evaluated *in vitro* by the method of estimating utilisable crude protein at the duodenum. *Journal of Animal Physiology and Animal Nutrition, 101*(6), 1259–1272. https://doi.org/10.1111/jpn.12646

Velickovic, T. D. C., & Stanic-Vucinic, D. J. (2018). The role of dietary phenolic compounds in protein digestion and processing technologies to improve their antinutritive properties. *Comprehensive Reviews in Food Science and Food Safety, 17*(1), 82–103.

Venkidasamy, B., Selvaraj, D., Nile, A. S., Ramalingam, S., Kai, G., & Nile, S. H. (2019). Indian pulses: A review on nutritional, functional and biochemical properties with future perspectives. *Trends in Food Science & Technology, 88*, 228–242. https://doi.org/10.1016/j.tifs.2019.03.012

Vinutha, T., Kumar, D., Bansal, N., Krishnan, V., Goswami, S., Kumar, R. R., & Praveen, S. (2022). Thermal treatments reduce rancidity and modulate structural and digestive properties of starch in pearl millet flour. *International Journal of Biological Macromolecules, 195*, 207–216. https://doi.org/10.1016/j.ijbiomac.2021.12.011

Vitali, D., Dragojević, I. V., & Šebečić, B. (2009). Effects of incorporation of integral raw materials and dietary fibre on the selected nutritional and functional properties of biscuits. *Food Chemistry, 114*(4), 1462–1469. https://doi.org/10.1016/j.foodchem.2008.11.032

Wood, J. A., & Grusak, M. A. (2007). Nutritional value of chickpea. In S. S. Yadav, B. Redden, W. Chen, & B. Sharma (Eds.), *Chickpea breeding and management* (pp. 101–142). Wallingford: CAB International. https://doi.org/10.1079/9781845932138.005.

World Health Organization. (2007). *Protein and amino acid requirements in human nutrition*. Geneva, Switzerland: WHO.

Yang, C., Zhu, X., Zhang, Z., Yang, F., Wei, Y., Zhang, Z., & Yang, F. (2022). Heat treatment of quinoa (*Chenopodium quinoa Willd.*) albumin: Effect on structural, functional, and *in vitro* digestion properties. *Frontiers in Nutrition, 9*, Article 1010617. https://doi.org/10.3389/fnut.2022.1010617

Zahir, M., Fogliano, V., & Capuano, E. (2018). Food matrix and processing modulate in vitro protein digestibility in soybeans. *Food & Function, 9*(12), 6326–6336.

Zhang, M., Huang, T. S., & Mu, T. H. (2019). Improvement of thermal, microwave and ultrasonication pretreatment on the production of antioxidant peptides from sweet potato protein via in vitro gastrointestinal digestion. *International Journal of Food Science & Technology, 54*(7), 2338–2345.

Ziena, H. M. S. (1989). *Hard-to-cook phenomenon in relation to physical, chemical and biological properties of Faba beans (Vicia faba L.)*. PhD Thesis,. Alexandria, Egypt: Faculty of Agriculture, Alexandria University.