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RESEARCH ARTICLE

Exploring genotypic diversity and processing effects on protein quality for nutritional and functional enhancement in pigeon pea (*Cajanus cajan* L.)

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Abstract

Pigeon pea protein isolate (PpPI) offers a sustainable and high-quality plant-based protein alternative. This study investigated the *in-vitro* protein digestibility of thirty pigeon pea genotypes, identifying two contrasting lines—Pusa Arhar 2018-4 (low digestibility) and ICP 1452 (high digestibility). The cytotoxicity, amino acid bioavailability, and gene expression modulation induced by PpPI hydrolysates, along with the impact of thermal processing, were evaluated using Caco-2 cells. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay confirmed that PpPI hydrolysates were non-cytotoxic in both control and autoclaved samples. The amino acid bioavailability assay revealed that neutral and polar amino acids (glycine, alanine, serine, proline, and leucine) had higher transport efficiency (17.43–27.90%) than charged amino acids (9.08–18.76%). Autoclaving significantly improved amino acid transport, with bioavailability ranging from 3.18–21.93% in Pusa Arhar 2018-4 and 4.38–34.84% in ICP 1452. Gene expression analysis using Caco2 cell line showed upregulation of the peptide transporter gene PepT1 (1.04–1.87-fold in Pusa Arhar 2018-4; 1.07–1.89-fold in ICP 1452), with significantly higher expression in autoclaved samples ($p < 0.05$). SREBP2, a key cholesterol metabolism regulator, was downregulated in both genotypes, though not significantly affected by thermal processing. These findings highlight the potential of autoclaved PpPI hydrolysates to improve amino acid bioavailability and intestinal gene expression, providing key insights for selecting pigeon pea genotypes with superior protein quality to guide breeding for enhanced nutritional functionality.

Keywords: Pigeon pea protein isolate, PepT1, SREBP2, amino acid bioavailability, cytotoxicity

Introduction

The increasing awareness of the importance of high-quality proteins in the diet has driven researchers to explore eco-friendly and sustainable alternatives. A promising solution is the replacement of animal-based proteins with plant-derived sources. Animal proteins are not only costly and limited in availability but are also associated with significant environmental impacts, including freshwater depletion, climate change, biodiversity loss, and various human health concerns. Among plant-based proteins, soy, wheat, peas, and potatoes currently dominate the global market (Hadnadev et al. 2017). However, expanding the range of protein-rich crops is essential to meet the growing global demand for sustainable and health-promoting dietary options. Pigeon pea (*Cajanus cajan* L.) has emerged as a promising yet underutilized plant protein source. It is widely available, cost-effective, nutritionally valuable, and has been linked to numerous health benefits. Despite its potential to improve food security and protein intake, especially in

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developing countries, pigeon pea remains underexploited when compared to more commercially recognized legumes (Haji et al. 2024). Leveraging its genetic diversity presents an important opportunity for breeding programs to improve both agronomic performance and nutritional quality traits such as protein digestibility and functional peptide bioavailability. A key challenge in the modern food system is not only to meet the rising demand for plant-based proteins but to ensure these proteins are of high quality, promote health, and are produced sustainably. Many dietary proteins and their derived peptides possess physiological functions beyond basic nutrition. Protease-derived peptides from various food sources have demonstrated bioactivities such as antioxidant, antimicrobial, anticancer, hypocholesterolemia, antihypertensive, and immunomodulatory effects (Kitts and Weiler, 2003; Görgüç et al. 2020). While extensive research has been conducted on other legumes for their bioactive potential, the characterization and functional evaluation of pigeon pea protein isolate (PpPI) remain limited, despite its rich protein content and wide availability. Processing methods such as heat treatment, soaking, dehulling, enzymatic hydrolysis, fermentation, and germination have been shown to improve plant protein quality by enhancing digestibility and reducing anti-nutritional factors (Kalpanadevi and Mohan 2013; Samtiya et al. 2020). In an earlier study, Dutta et al. (2024) autoclaving was identified as an effective thermal processing technique that significantly improved the digestibility of pigeon pea protein by inducing structural modifications and reducing compounds that impair protein absorption.

Therefore, it is essential to further investigate how autoclaving affects not only protein digestibility but also amino acid bioavailability, peptide transport, and associated health-promoting properties, such as lipid-lowering effects. Additionally, evaluating genotypic variation in these traits across diverse pigeon pea lines can provide valuable phenotypic markers for breeding programs. Identifying genotypes with naturally higher digestibility, improved peptide transport capacity, and favorable bioactivities can help breeders develop nutritionally enhanced pigeon pea cultivars tailored for both food functionality and human health. Hence, the present study aimed at evaluating the *in-vitro* protein digestibility (IVPD) of 30 pigeon pea genotypes to identify those with high and low digestibility, which could aid in selecting superior genotypes for breeding programs.

Materials and methods

Materials

Pigeon pea seeds were procured from the Division of Genetics, ICAR-IARI, New Delhi. Pepsin from porcine gastric mucosa, pancreatin, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent, KAPA SYBR Fast qPCR master mix (Sigma-Aldrich, USA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin were obtained from Gibco. SuperScript cDNA synthesis kit (170-8891) was obtained from BioRad. The experimental work was conducted in the year 2024.

In-vitro protein digestibility (IVPD) assay

Pigeon pea seeds of 30 genotypes were sorted to remove broken, damaged ones and any foreign materials. After cleaning, they were finely ground and *in-vitro* protein digestibility was determined following the method proposed earlier by Dutta et al. (2024). Briefly, 0.5 g flour sample was mixed with 12.5 mL of trypsin solution (0.5 mg/mL pepsin dissolved in distilled water, pH 2) and incubated at 37°C in an incubator shaker (Orbitek, Scigenics Biotech) for 2 hours. The solution was then neutralized to pH 7 using 6 M NaOH, followed by the addition of 2 mL of pancreatin (5 mg/mL dissolved in phosphate buffer, pH 8.2) and incubated at 37°C with continuous shaking for 24 hours. To terminate the enzymatic reaction, 7 mL of 10% TCA was added, and the mixture was centrifuged at 4100g for 20 minutes. The residue was collected, washed, and analyzed for protein content using the Kjeldahl method (AOAC, 2006).

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

Preparation of pigeon pea protein isolate (PpPI)

PpPIs were prepared by the method given by Papalamprou et al. (2010) with a 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 minutes at room temperature (20–22°C), followed by centrifugation at 4500g for 20 minutes at 4°C. The resulting supernatant was collected, and the pellet was re-suspended in distilled water at a ratio of 1:5 (w/v) with pH adjusted to 8.5. After an additional 45 minutes of stirring, followed by centrifugation (4500×g, 20 minutes, 4°C), both supernatants were combined. The pH of the combined supernatants was then adjusted to 4.5 using 0.1 M HCl and left overnight to precipitate the protein. The protein precipitate 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 the Kjeldahl method (AOAC, 2006). For autoclaving treatment, PpPI was autoclaved at 15 psi and 121°C for one hour, dried and used for further investigation.

Preparation of PpPI hydrolysate by simulated gastric digestion

PpPI hydrolysate was prepared by simulated gastric

digestion using the method given by Liu et al. (2021) with slight modification. Briefly, 2.5 g of PpPI was suspended in 50 mL of Milli-Q H_2O in sterilized 125 mL Erlenmeyer flasks. The pH was adjusted to 2.3 using 6N HCl, and 625 U/mL of porcine pepsin was added. The samples were incubated for 1 hour at 37°C with gentle agitation. After pepsin digestion, NaOH was used to adjust the pH to 8.0. Subsequently, 0.5% W/W porcine pancreatin was added to each sample, and the samples were incubated for 4 hours at 37°C with gentle agitation. After incubation, the samples were heat-inactivated at 95°C for 5 minutes and centrifuged at 16,000 x g for 30 minutes at 4°C to isolate the soluble protein fraction. After centrifugation, the soluble fraction was sterile filtered (0.2 µm) and stored at -80°C. The soluble protein content was determined by the Bradford method (Bradford, 1976).

Cell culture and *viability assay*

Human intestinal Caco-2 cells were obtained from NCCS Pune and grown in DMEM supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 humidified air at 37°C (Heraeus HERAcell 150 Incubator, Thermo Electron Corporation). Cells were sub-cultured upon reaching approximately 80–90% confluency and the medium was changed every second day.

Cell viability was assessed by using the MTT assay following the method given by Kouadio et al. (2005). Cells were seeded into 96-well plates at a density of 6×10^3 cells per well and cultured at 37°C in an atmosphere of 5% CO_2. The cells were allowed to adhere for 24 hours. Test compounds, consisting of PpPI hydrolysate at concentrations ranging from 0 to 250 µg/mL, were added to DMEM supplemented with 10% fetal bovine serum (FBS). After treatment, the solvent was removed, and 100 µL of filtered MTT solution was added to each well and incubated for 2 hours. Following incubation, the MTT solution (0.5 mg/mL) was aspirated, and 100 µL of MTT lysis buffer (8 mM HCl and 10.5% NP-40 in DMSO) was added to each well. After 5 minutes of gentle shaking, absorbance at 575 nm was measured using a Synergy H1 microplate reader (BioTek, Bad Friedrichshall, Germany). Cell viability percentage was calculated by the following formula:

$$\text{Cell viability rate} = \frac{\text{Experimental absorbance}}{\text{Control absorbance}} \times 100$$

Amino acid transport studies

Amino acid bioavailability was analyzed following the method given by Goulart et al. (2014) with some modifications. For the amino acid transport study, Caco-2 cells were seeded onto polyethylene terephthalate (PET)-transwell inserts (1×10^5 cells/cm^2 per insert; pore size 0.4 µm, area 1.12 cm^2) in 6-well plates (Corning Life Sciences). After seeding, the cells were cultured for 14–15 days before conducting the experiments. Prior to introducing the digests, the medium in both compartments was aspirated and replaced with preheated (37°C) transport buffer containing 130 mmol L^{-1} NaCl, 10 mmol L^{-1}KCl, 1 mmol L^{-1} $MgSO_4$, 5 mmol L^{-1} glucose and 50 mmol L^{-1} HEPES. The digest was added to a concentration 100 µg/mL in the transport buffer. The cells were incubated for 2 hours, after which the apical and basal solutions were collected and the amino acid content was profiled by UPLC. The transport experiments were performed in triplicate in independent experiments. A cell control, consisting of a blank without the sample or digestive enzymes, was also included in the study design.

Expression analysis of PepT1 and SREBP2

To investigate the effects of PpPI hydrolysate on the expression pattern of PepT1 and SREBP2, Caco2 cell monolayers cultured on 6-culture plate were treated with PpPI hydrolysates at a concentration of 100µg/mL in DMEM medium and incubated for 24h. Caco2 cells were harvested after washing with 2 mL of a chilled 50 mM PBS of pH 7.4 with a cell scrapper. Total RNA was extracted from all selected varieties at both stages using the Trizol reagent. cDNA was synthesized with the iScript cDNA Synthesis Kit (BIO-RAD). Quantitative real-time PCR was performed using the SYBR Green One-Step qRT-PCR kit. The reaction volume for qRT-PCR was set at 20 µL, and the cycling conditions were as follows: an initial pre-denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. β-actin was used as the internal reference gene. Gene expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method (Sen Roy and Seshagiri, 2013). The primer sequences utilized in the experiment are provided in Table 1.

Table 1. Primer sequences of the gene SREBP2, PepT1 and β-actin with Tm (°C) values

Gene name	Oligo Id	Sequence	Tm (°C)
SREBP2	HsSREBF2 FP	TTCCTGTGCCTCTCCTTTAAC	62.1
	Hs SREBF2 RP	TCATCCAGTCAAACCAGCC	63.8
PepT1	HsSLC15A1 FP	CCCTGATTGTGTTTGTCCTTG	64.2
	HsSLC15A1 RP	AATGCCTTACTCCGATGCC	63.9
β-actin	HsACTB FP	GTCTTCCCCTCCATCGTG	63.3
	HsACTB RP	GTACTTCAGGGTGAGGATGC	61.2

Results

In-vitro protein digestibility assay

In-vitro protein digestibility of thirty pigeon pea genotypes was analyzed initially. From the results, it was observed that the IVPD of the pigeon pea seeds was in the range of 60.95±0.011 % to 65.54±0.21 % with a significant variation. The significantly ($p<0.05$) highest protein digestibility was observed in ICP 1452, whereas the lowest was observed in Pusa Arhar 2018-4. The results of IVPD of 30 genotypes are represented in Fig. 1. These two genotypes were further selected for the extraction of PpPIs and further assays.

Cytotoxicity assay of PpPI hydrolysate

The percentage of cell viability after treatment with protein hydrolysates was assessed using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). This colorimetric assay evaluates cellular metabolic activity as a measure of viability, proliferation or cytotoxicity. It is based on the reduction of the yellow tetrazolium salt (MTT) into insoluble purple formazan crystals by mitochondrial dehydrogenases in metabolically active cells. The cell viability results for PpPI hydrolysates at concentrations ranging from 50 to 250 µg/mL as depicted in Fig. 2. The findings revealed that PpPI hydrolysates exhibited >100% cell viability across all tested concentrations, indicating no cytotoxic effects in both the control and autoclaved PpPI hydrolysates.

Amino acid bioavailability assay

The amino acid bioavailability of the control and treated PpPIs was assessed using the Caco-2 cell line. Figure 3 illustrates the levels of individual amino acids detected on the basal side after 2 hours of cell incubation. Neutral and polar amino acids like glycine, alanine, serine, proline, valine, threonine, isoleucine, leucine, methionine, phenylalanine and tyrosine showed a higher bioavailability, ranging from 17.43-27.90% compared to positively and negatively charged amino acids like aspartic acid, glutamic acid, histidine and arginine, which showed bioavailability in the range of 9.08-18.76%. The autoclaved PpPI hydrolysates

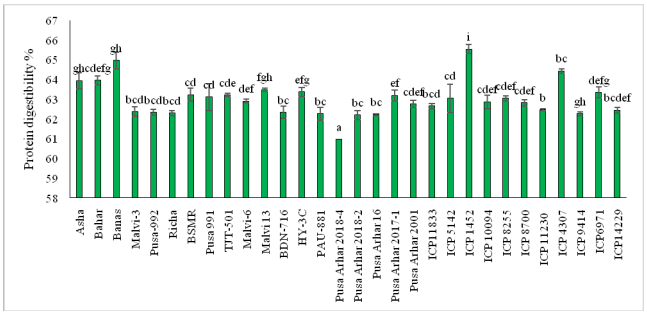

Fig. 1. *In-vitro* protein digestibility (IVPD) assay of thirty pigeon pea genotypes. Values are mean of three replicates ± SE

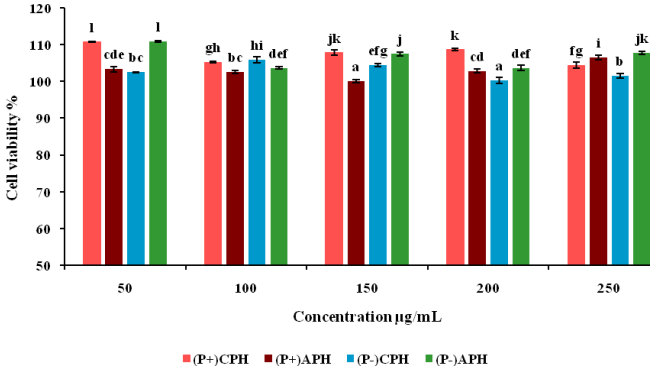

Fig. 2. Cell viability (%) of control and autoclaved PpPI. (P^+) CPH- Control PpPI hydrolysates of Pusa Arhar 2018-4, (P^+) APH- Autoclaved PpPI hydrolysates of Pusa Arhar 2018-4, (P^-)CPH - Control PpPI hydrolysates of ICP 1452, (P^-)APpPI- Autoclaved PpPI hydrolysates of ICP 1452.Values are mean of three replicates ± SE

showed significantly ($p<0.05$) higher bioavailability than the control PpPIs, *i.e.*, 3.18-21.93% for Pusa Arhar 2018-4 and 4.38–34.84% ICP 1452.

Expression analysis of protein and lipid metabolism genes

The expression of the genes PepT1 and SREBP2 was evaluated in Caco-2 cells treated with control and autoclaved protein hydrolysates. PepT1 (Peptide Transporter 1), a proton-coupled oligopeptide transporter predominantly expressed in the intestinal brush border membrane, plays a crucial role in protein absorption in humans. On the other hand, SREBP2 (Sterol Regulatory Element-Binding Protein 2) is essential for cholesterol regulation. Under cholesterol-depleted conditions, SREBP2 undergoes proteolytic cleavage, releasing its NH_2-terminal domain, which activates the promoters of SREBP2-regulated genes, including those coding for the LDL receptor, HMG-CoA synthase and HMG-CoA reductase. The gene expression

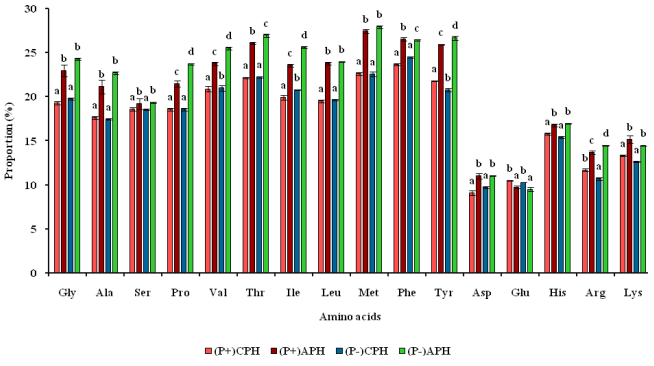

Fig. 3. Amino acid bioavailability (%) of control and autoclaved PpPI. (P^+) CPH- Control PpPI hydrolysates of Pusa Arhar 2018-4, (P^+) APH- Autoclaved PpPI hydrolysates of Pusa Arhar 2018-4, (P^-)CPH - Control PpPI hydrolysates of ICP 1452, (P^-)APpPI- Autoclaved PpPI hydrolysates of ICP 1452. Values are mean of three replicates ± SE

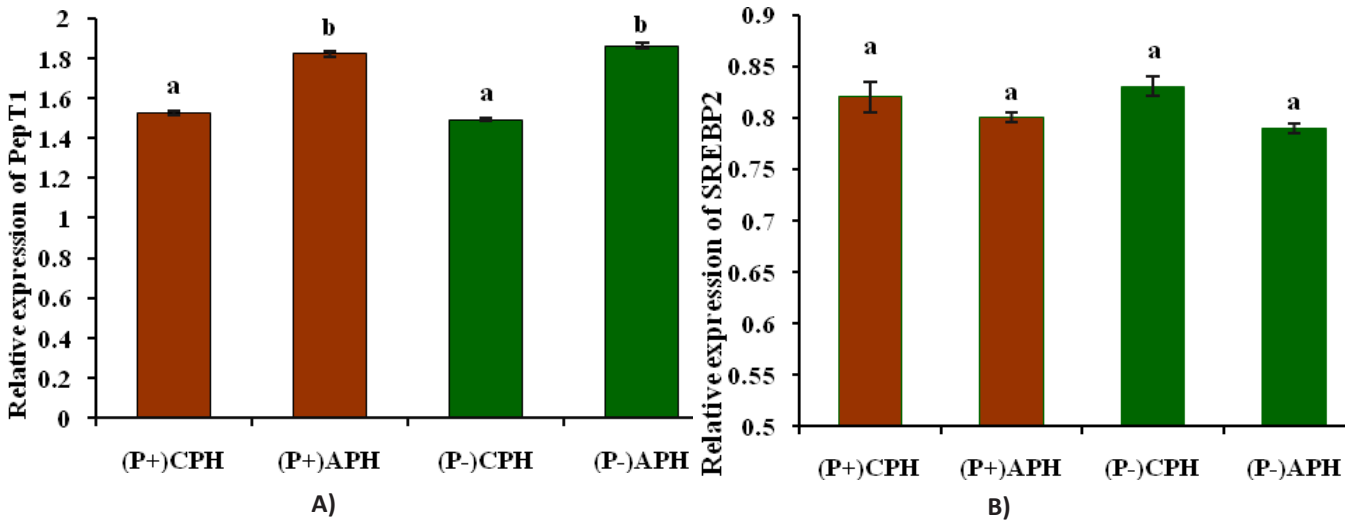

Fig. 4. Relative expression analysis of PePT1 (A) and SREBP2 (B) after treatment with PpPI hydrolysate compared to untreated control Caco2 cells. (P^+) CPH- Control PpPI hydrolysates of Pusa Arhar 2018-4, (P^+) APH- Autoclaved PpPI hydrolysates of Pusa Arhar 2018-4, (P^-)CPH - Control PpPI hydrolysates of ICP 1452, (P^-)APpPI- Autoclaved PpPI hydrolysates of ICP 1452. Values are mean of three replicates ± SE

analysis of PepT1 and SREBP2 was analysed after treatment of Caco2 cells with PpPI hydrolysates and the results are depicted in Figure 4. Treatment with protein hydrolysates led to an upregulation of PepT1 in the range of 1.04-1.87-fold in Pusa Arhar 2018-4 and 1.07-1.89-fold in ICP 1452, with autoclaved PpPI hydrolysates showing a significantly higher upregulation ($p<0.05$). In contrast, SREBP2 expression was downregulated following treatment; however, no significant difference ($p< 0.05$) was observed between the control and autoclaved PpPI hydrolysates.

Discussion

The *in-vitro* protein digestibility (IVPD) results revealed significant variation among the thirty pigeon pea genotypes. Notably, genotype ICP 1452 demonstrated high IVPD levels alongside low polyphenol content, as previously reported (Dutta et al. 2024). Given that polyphenols are known anti-nutritional factors that impede protein digestibility (Singh, 1993), the combination of low polyphenol levels and high IVPD in ICP 1452 makes it a promising candidate for breeding programs focused on improving protein quality in pigeon pea.

The cytotoxicity assay showed no significant toxic effects of PpPI hydrolysates at the tested concentrations, confirming their safety for further functional analysis. Based on these findings, appropriate concentrations were selected for subsequent amino acid bioavailability and gene expression studies. The amino acid bioavailability analysis revealed that neutral and polar amino acids, such as glycine, alanine, serine, proline, and leucine, exhibited significantly higher bioavailability than positively or negatively charged amino acids. This is likely due to the more efficient transport mechanisms of neutral amino acids across the Caco-2 cell membrane. Autoclaved PpPI hydrolysates demonstrated enhanced bioavailability compared to untreated controls, suggesting that autoclaving facilitated protein hydrolysis and the formation of smaller molecular weight peptides, which improved amino acid release and absorption. This correlates with the higher degree of hydrolysis observed in our earlier work (Dutta et al. 2024). Previous studies also support these findings—Wang and Li (2018) and Trigo et al. (2021) reported higher transport rates for neutral peptides and greater bioavailability of neutral/polar amino acids. Short-chain peptides (<500 Da) are known to have superior cellular uptake compared to larger peptides (<2000 Da), and PepT1 is a key transporter of such low molecular weight peptides (Feng & Betti, 2017; Wang & Li, 2017). The observed upregulation of PepT1 expression in Caco-2 cells treated with both control and autoclaved PpPI hydrolysates indicates enhanced peptide uptake, pointing to improved protein absorption. This is consistent with PepT1's role in oligopeptide transport across intestinal epithelial cells (Adibi, 1997). The significantly higher PepT1 expression in autoclaved samples from both Pusa Arhar 2018-4 and ICP 1452 suggests that autoclaving may induce protein structural changes that improve digestibility and absorption. Similar upregulation of PepT1 in response to dietary peptides has been observed in other species (Bakke et al. 2010; Eickson et al. 1995). In contrast, downregulation of SREBP2, a key regulator of cholesterol metabolism, following treatment with PpPI hydrolysates may suggest a lipid-lowering effect. SREBP2 is typically activated under cholesterol-deficient conditions (Madison 2016), and its suppression by PpPIs implies the presence of bioactive peptides capable of modulating lipid metabolism regardless of thermal processing. The present findings are similar of earlier Kumar

et al. (2021) have reported hypocholesterolemic effects of pigeon pea by-product protein hydrolysates and a derived peptide (PFVKSEPIPETNNE) via SREBP2 downregulation in Wistar rats. Similarly, Zhou et al. (2022) reported decreased SREBP2 expression following treatment with buckwheat globulin hydrolysate pretreated with ultra-high pressure.

The observed variation in IVPD among pigeon pea genotypes, along with the correlation between low polyphenol content and higher digestibility in ICP 1452, underscores the potential for selecting and breeding lines with superior protein quality traits. Incorporating such genotypes into breeding programs can accelerate the development of nutritionally enhanced pigeon pea cultivars tailored for improved digestibility and bioavailability. Furthermore, identifying genotypes that naturally express favorable traits like enhanced PepT1-mediated absorption or reduced anti-nutritional factors provides a valuable foundation for marker-assisted selection and functional breeding strategies.

The findings of this study demonstrate that thermal processing, specifically autoclaving, significantly enhances the bioavailability of pigeon pea protein isolates (PpPI), as evidenced by increased transport across Caco-2 cell monolayers and the upregulation of the peptide transporter gene PepT1. Given the considerable genotypic variation observed in protein digestibility and bioactivity traits, the present study provides a valuable basis for breeding programs aimed at developing pigeon pea cultivars with superior nutritional quality. Overall, pigeon pea protein isolates, particularly from thermally processed sources, show strong potential as ingredients in functional foods and nutraceutical applications, contributing to both human health and sustainable agriculture.

Authors' contribution

Conceptualization of research (VT, SG, NKS); Designing of the experiments (MD, VT, RRK); Contribution of experimental materials (RPG, AT, AD, NKS); Execution of lab experiments (MD, BL, PV, NB); Analysis of data and interpretation (MD, NCR); Preparation of the manuscript (MD, RL, VT)

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