Autoclaving-Induced Changes in the Functional Properties of Protein Isolates from Pigeon Pea

Abstract

Pigeon pea (*Cajanus cajan*) is a protein-rich legume crop widely cultivated in tropical and subtropical regions, serving as a vital dietary protein source, particularly where protein malnutrition is a concern. The protein fractions in pigeon pea seeds are primarily composed of albumin, globulin, glutelin, and prolamin, with globulins accounting for nearly 60% of the total protein. Despite its nutritional value, pigeon pea protein remains underutilized in plant protein market. This study investigated the impact of autoclaving treatment on the functional properties of protein isolates obtained from two pigeon pea genotypes, Pusa Arhar 2018-4 and ICP 1452. Key functional properties, including protein solubility, surface hydrophobicity, water holding capacity (WHC), oil holding capacity (OHC), emulsifying properties, and foaming properties, were analysed. Autoclaving significantly reduced protein solubility across all pH levels due to thermal denaturation and aggregation, which enhanced hydrophobic interactions. Surface hydrophobicity increased by 10.68% and 16.18% for Pusa Arhar 2018-4 and ICP 1452, respectively, as a result of protein unfolding and exposure of hydrophobic regions. WHC decreased significantly, while OHC increased due to enhanced binding affinity for oil molecules. Emulsifying activity index (EAI) and stability index (ESI) decreased after autoclaving, reflecting reduced interfacial activity and emulsion stability. Conversely, autoclaving significantly improved foaming capacity by 22.90% and 34.31% for Pusa Arhar 2018-4 and ICP 1452, respectively, with greater foaming stability observed over extended durations. These findings highlight the potential of autoclaving to modulate the functional properties of pigeon pea protein isolates, enhancing their suitability for diverse food applications.

Keywords: Pigeon pea, protein isolates, functional properties, protein solubility, emulsification properties, autoclaving

1. Introduction

Pigeon pea is an important legume crop with high protein content of 18–25%. It is widely cultivated in tropical and subtropical regions, as an important dietary protein source, particularly in regions of Asia and Africa where protein malnutrition remains a concern (Sharma *et al.*, 2011; Pranati et al., 2024). The storage proteins in pigeon pea are mainly categorized into four main types: albumin, globulin (further divided into 7s vicilin and 11s legumin fractions), glutelin and prolamin (Singh and Jambunathan, 1982). Among these, globulin is the predominant protein in the cotyledons, accounting for nearly 60% of the total protein, followed by albumin, while prolamin represents the smallest fraction. In terms of amino acid composition, similar to other legumes, pigeon pea seeds are rich in lysine, leucine, aspartic acid, glutamic acid and arginine, but deficient in sulphur-containing amino acids such as methionine and cysteine. Albumin storage proteins are particularly rich in methionine and cysteine compared to the legumin fraction of globulins, whereas vicilin proteins are abundant in methionine but lack cysteine (Gupta et al., 2001). The rising popularity of plant proteins has been remarkable and continues to grow, driven by consumers' increasing awareness of food ingredients, their preference for clean and lean protein options, and concern for natural, sustainable, and eco-friendly food sources (Akharume et al., 2020). Pigeon pea serves as a promising alternative plant-based protein source, offering diversity in a market largely dominated by soy and pea proteins. For a plant protein to be considered a good source, it must not only possess high protein quality but also exhibit an excellent functional property profile. The functional properties of proteins refer to any physicochemical characteristics that influence how proteins behave and perform during food processing, as reflected in the quality of the final product. Key functional properties such as solubility, emulsification, gelation, and foaming play a vital role in determining the protein isolate's suitability and performance across various food formulations and applications. These properties arise from intricate interactions involving the composition, structure, conformation, and physicochemical attributes of the proteins (Kinsella and Melachouris, 1976). Thermal treatment plays a pivotal role in enhancing the functional properties of protein isolates by modifying their native structure through controlled time and temperature exposure (Mir et al., 2021; Raikos, 2010). In our earlier study (Dutta et al., 2024), we reported an improvement of protein

digestibility by employing various processing treatments like cooking, hydrothermal treatment, autoclaving, infrared heating and germination. Among all the treatments autoclaving showed a highest improvement of protein digestibility. The beneficial effects of heat treatment on pigeon pea protein isolates, along with enhanced protein digestibility, can expand their potential applications across a wide variety of food products. Therefore, this study aims to investigate the effect of autoclaving on the functional properties of protein isolates derived from two pigeon pea genotypes, Pusa Arhar 2018-4 and ICP 1452.

2. Materials and methods

2.1. Materials

Seeds of the two genotypes (Pusa Arhar 2018-4 and ICP 1452) were collected form Division of Genetics, ICAR-IARI, New Delhi. 8-Anilino-1-naphthalenesulfonic acid ammonium salt (ANS reagent), Bovine serum albumin obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulphate was obtained from Amresco (Krackeler Scientific, Albany, New York).

2.2. Pigeon pea protein isolates (PpPIs)preparation

PpPIs were prepared following the method described by (Papalamprou et al., 2010), with slight modifications. Briefly, 100 g of defatted flour was mixed with distilled water in a 1:10 (w/v) ratio. The pH of the mixture was adjusted to 10.5 using 1 M NaOH, and it was stirred at 500 rpm for 45 minutes at room temperature (20-22 °C). The mixture was then centrifuged at 4500×g for 20 minutes at 4 °C and the supernatant was collected. The pellet was re-suspended in distilled water at a 1:5 (w/v) ratio, the pH was again adjusted to 10.5, and the mixture was stirred for another 45 minutes before being centrifuged under the same conditions. The supernatants from both extractions were combined, and their pH was adjusted to 4.5 using 0.1 M HCl to precipitate the protein, which was allowed to settle overnight. The precipitated protein solution was centrifuged at 4500×g for 20 minutes at 4 °C. The resulting precipitate was washed twice with chilled distilled water (4 °C), re-dispersed in distilled water, and the pH was adjusted to 7 using 1 M NaOH. Finally, the sample was freeze-dried to obtain the PpPI powder. The protein content of the isolate was analysed using the Kjeldahl method (AOAC, 2006).

2.3. Protein Solubility

The percent protein solubility was determined using the method described by (Lan et al., 2018) using Bradford method. For this process, 100 mg of the sample was weighed and 10 mL of distilled water was added. The pH of the solution was adjusted to 2, 4, 6, 8, 10 and 12 using 2M NaOH and 0.1N HCl. Samples were collected at each pH level and analysed using the Bradford method. For the Bradford assay, 2 mL of Bradford reagent was added to a test tube, followed by 990 µL of water and 10 µL of the pH-adjusted sample. A blank was prepared using 1 mL (1000 µL) of distilled water. Both the blank and samples were incubated in the dark for 10 minutes. Absorbance was measured at 595 nm using a spectrophotometer. A standard curve was generated using Bovine Serum Albumin (BSA), and the protein concentration of the samples was determined from the curve. The percent protein solubility was then calculated using the following formula:

Protein Solubility (%) = (Protein content in supernatant/total protein content) x 100

2.4. Surface hydrophobicity (H_o)

Surface hydrophobicity was evaluated using the method described by Hao et al., (2022) with 1-anilinonaphthalene-8-sulfonic acid (ANS) as fluorescent probe. The protein-polyphenol reaction solution was diluted with sodium phosphate buffer (5 mM, pH 7.0) to achieve final protein concentrations of 0.05, 0.1, 0.15, 0.20, and 0.25 mg/mL. A 5 mL aliquot of the diluted protein solution was taken, and 25 µL of 8 mmol/L ANS solution was added, followed by thorough mixing. The fluorescence intensity of the sample was measured using RF-540 fluorescence spectrometer (Shimadzu, Tokyo, Japan) with an excitation wavelength of 390 nm and an emission wavelength of 470 nm. The slope of the resulting curve represents the surface hydrophobicity of the protein in the sample.

2.5. Water Holding Capacity (WHC)

PpPI (0.5 g) was transferred to a dry centrifuge tube and 10 mL of distilled water was added. The mixture was stirred magnetically at 25 °C for 30 minutes and subsequently centrifuged at 4000 rpm for 30 minutes. After carefully decanting the supernatant, the centrifuge tube was tilted at a 45° angle for 30 minutes to drain any excess water. The total weight of the centrifuge tube and the sediment was then recorded. The water-holding capacity (WHC) was determined using the following formula-

WHC % = (m2- m1)/0.5 × 100

Where, m1 is the mass of PpPI and centrifuge tube (g), m2 is the mass of sediment and centrifuge tube (g)

2.6. Oil Holding Capacity (OHC)

The sample was placed in a dry centrifuge tube and combined with 3 mL of soybean oil. The mixture was vortexed at 25 °C (room temperature) for 30 minutes and subsequently centrifuged at 4000 rpm for 30 minutes. After decanting the supernatant, the centrifuge tube was tilted at a 45° angle for 30 minutes to remove any remaining oil. The sediment's mass was recorded and the oil holding capacity (OHC) was calculated using the following formula-

$$OHC\% = (m2 - m1)/0.5 \times 100$$

Where, m1 is the mass of PpPI and centrifuge tube (g), m2 is the mass of sediment and centrifuge tube (g)

2.7. Emulsification properties

The Emulsification ability index (EAI) and Emulsification stability index (ESI) was performed following the methodology of (Quintero-Quiroz et al., 2022). In brief, 1.5 mL of oil was added to 4.5 mL of a protein solution (0.5% w/v) prepared in 0.01 M phosphate buffer at pH 7. The mixture was homogenized at room temperature for 2 minutes. Next, 250 µL of the emulsion was combined with 50 mL of a 0.1% sodium dodecyl sulphate solution, and the absorbance was measured at 500 nm using a UV/VIS spectrophotometer (UV-1700, Shimadzu). The emulsifying activity index (EAI) and emulsifying stability index (ESI) were calculated using the following formula:

$$EAI\ (m^2/g) = (2*2.303*A0*N)/(c*\varphi*10000)$$

$$ESI\ (min) = (A0*t)/\Delta A$$

2.8. Foaming Capacity and Foaming Stability

Foaming capacity and stability were assessed using a modified method from Wani et al. (2015). Briefly, 0.5 g of PpPI was dissolved in 50 mL of distilled water in a beaker. The mixture was homogenized for 2 minutes at 20,000 rpm, then transferred immediately to a graduated cylinder and the initial volume (V1) was recorded. Foam capacity (FC) was calculated using the formula:

$$FC\ (\%) = (V1-V2)/V0 \times 100$$

Where, 0 represents the total volume of the protein suspension, while V2 refers to the total volume of the protein suspension immediately after homogenization (at 0 minutes).

The total volume was recorded at 15, 30, 45 and 60 min, respectively. Foam stability (FS) was calculated by the equation:

FS (%) =Vt/V0 × 100

3. Result and Discussion

3.1. Protein solubility

Protein solubility is determined by the balance between protein-protein and protein-solvent interactions. Hydrophobic interactions stimulate protein-protein interactions, leading to precipitation, while protein-solvent interactions enhance hydration and solubility. These interactions are influenced by environmental conditions such as pH, ionic strength and temperature, as well as the type of solvent used. Additionally, processing methods, including extraction and post-extraction treatments, also affect protein-solvent interactions (Karaca et al., 2011). Solubility of the PpPIs from control and autoclaved pigeon pea were analyzed at different pH range of 2-12 for both the genotypes (Pusa Arhar 2018-4 and ICP 1452), with results presented in Figure 1. The analysis revealed that autoclaving significantly ($p<0.05$) reduced protein solubility at all tested pH levels, with reduction ranging from 35.85-75.28%. The results also observed that both the control and autoclaved treated protein isolates showed the lowest solubility at pH 4 (5.19-12.5%), whereas the highest solubility was observed at pH 12 (43.47-70.46%). Protein solubility serves as the foundation for other protein functional properties, is among the most crucial characteristics of proteins. It is closely linked to emulsification, foaming and various other protein properties. The results of this study indicated that both control and autoclaved protein isolates exhibited the lowest solubility at pH 4, within the isoelectric point range of most proteins, where electrostatic repulsion is minimal and protein-protein interactions are maximized. Conversely, the highest solubility was observed at pH 12, where increased electrostatic repulsion and protein unfolding promote better protein-solvent interactions. The reduction in solubility by autoclaving treatment could be attributed to the thermal denaturation and aggregation of proteins caused by autoclaving, which likely enhances hydrophobic interactions and decreases protein-solvent interactions. Several previous studies have similarly reported a decrease in protein solubility following thermal treatment in

protein isolates from quinoa, rice bran and cowpea, among others (Peyrano et al., 2016; Lv et al., 2017; He et al., 2022).

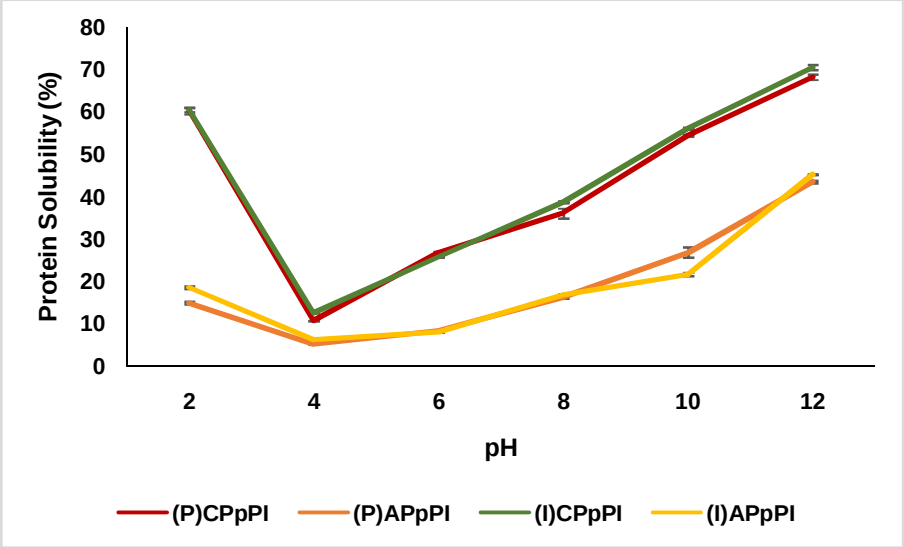

Figure 1. Protein solubility (%) of control and autoclaved PpPI. (P) CPpPI- Control PpPI of Pusa Arhar 2018-4. (P) APpPI- Autoclaved PpPI of PusaArhar 2018-4, (I)CPpPI - Control PpPI of ICP 1452, (I)APpPI- Autoclaved PpPI of ICP 1452.

3.2. Surface hydrophobicity(H_0)

Surface hydrophobicity (H_0) measures the number of hydrophobic groups on the surface of the protein contributed by non-polar amino acids. In this study the H_0 of the control and autoclaved PpPIs of both the genotypes was carried out (Figure 2). After autoclaving treatment, the H_0 of the PpPI was found to be increased significantly ($p<0.05$) by 10.68% and 16.178% in Pusa Arhar 2018-4 and ICP 1452respectively. Autoclaving led to a significant increase in H_0 for PpPI in both genotypes. The increase in surface hydrophobicity can also be linked to heating, as it may alter the protein's conformation, leading to the exposure of hydrophobic regions that are typically hidden within the protein structure. Yeom et al. (2009) also reported similar increase in surface hydrophobicity after autoclaving treatment in rice bran protein isolates.

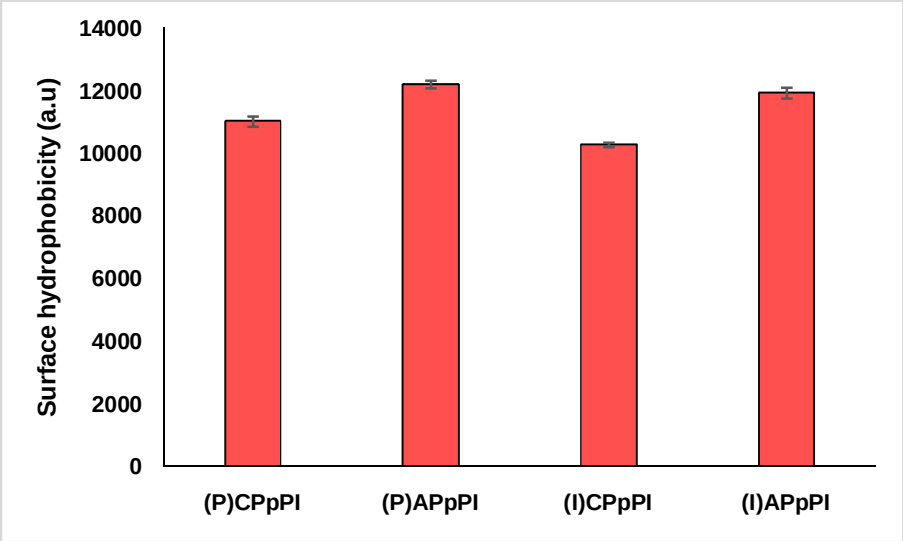

Figure 2. Surface hydrophobicity of control and autoclaved PpPI. (P) CPpPI- Control PpPI of Pusa Arhar 2018-4. (P) APpPI- Autoclaved PpPI of PusaArhar 2018-4, (I)CPpPI - Control PpPI of ICP 1452, (I)APpPI- Autoclaved PpPI of ICP 1452.

3.3. Water holding capacity (WHC) and oil holding capacity (OHC)

WHC and OHC of the PpPIs from control and autoclaved pigeon pea were analysed and the results were represented in Figure 3. The WHC of control PpPI was found to be 274.50% for Pusa Arhar 2018-4 and 275.49% for ICP 1452. The WHC was found to be decreased significantly ($p<0.05$) after autoclaving, 210.28% for Pusa Arhar 2018-4 and 209.8% for ICP 1452. Whereas the OHC of the control PpPIs were found to be 125.82% for Pusa Arhar 2018-4 and 124.13% for ICP 1452. The OHC of the PpPI was found to be increased significantly ($0<0.05$) after autoclaving treatment. The OHC of autoclaved PpPI was found to be 186.44% for Pusa Arhar 2018-4 and 189.25% for ICP 1452.WHC and OHC are essential functional attributes of protein isolates, directly influenced by their structural properties. The significant reduction in WHC for both genotypes, likely due to decreased solubility and increased surface hydrophobicity, which may have impaired the water-binding capacity PpPIs. Conversely, the OHC of PpPIs showed a marked increase following autoclaving, potentially due to protein denaturation and the exposure of hydrophobic groups, enhancing the binding affinity for oil molecules. A similar enhancement in OHC after thermal treatment was reported for quinoa protein isolates by Mir et al. (2021).

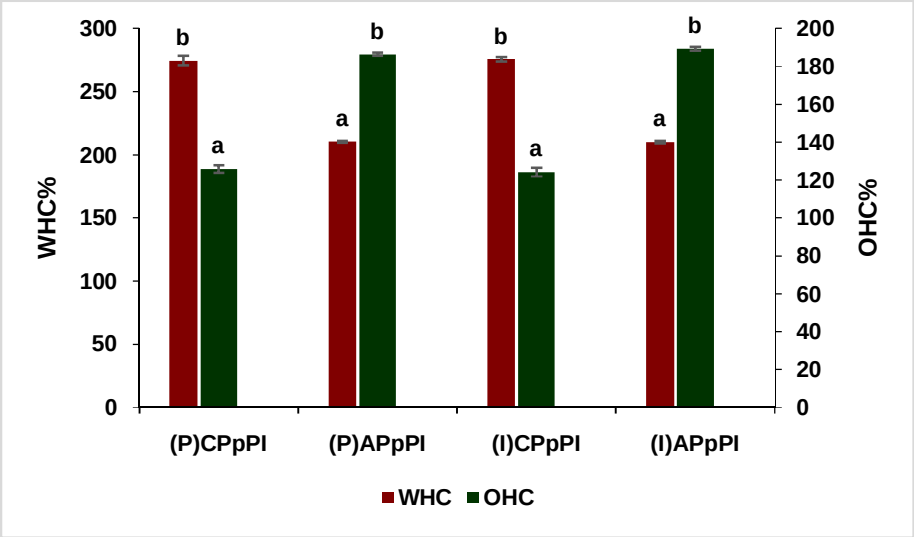

Figure 3. Water holding capacity (%) and oil holding capacity (%) of control and autoclaved PpPI. (P) CPpPI- Control PpPI of PusaArhar 2018-4, (P) APpPI- Autoclaved PpPI of PusaArhar 2018-4, (I)CPpPI - Control PpPI of ICP 1452, (I)APpPI- Autoclaved PpPI of ICP 1452

3.4. Emulsification properties

Emulsifying properties, such as emulsifying activity index (EAI) and emulsifying stability index (ES), are essential functional attributes with significant applications in food systems. EAI refers to the protein's ability to bind oil and water at the interface to form an emulsion, which is influenced by factors such as the size, shape, charge, composition, and hydrophobicity of protein molecules (Ragab et al., 2004). On the other hand, ESI indicates the stability of the emulsion over time under specific conditions and is determined by the strength of these interactions (Karaca et al., 2011; Ma et al., 2011). The EAI and ESI of PpPIs from untreated and autoclaved pigeon pea seeds were evaluated, and the results are depicted in the Figure 4. The EAI was recorded as 21.83m^2/g for Pusa Arhar 2018-4 and 20.19m^2/g for ICP 1452 in untreated samples. Following autoclaving, a significant decrease in emulsification ability was observed, with the EAI decreasing to 16.39m^2/g for Pusa Arhar 2018-4 and 16.34m^2/g for ICP 1452. Similarly, the ESI, which measures the stability of emulsions over time, was significantly improved in autoclaved samples. Untreated PpPIs exhibited an ES of 72.66 min for Pusa Arhar 2018-4 and 75.33 min for ICP 1452. However, after autoclaving, the ESI was found to be 29 min for Pusa Arhar 2018-4 and 30.33 min for ICP 1452. The emulsifying property of proteins can be assessed through emulsifying activity and emulsion stability, which reflect the protein's capacity to create emulsions at the interface between water and

oil (Singh and Sit, 2023). These findings suggest that autoclaving negatively impacts the proteins' ability to form emulsions, likely due to structural alterations that reduce their interfacial activity and destabilize interactions within the emulsion system. Previously, Singh and Sit (2023)reported that autoclaving negatively impacts the emulsification ability and stability of proteins by causing protein aggregation, structural disruption, and a reduction in droplet size, leading to smaller and less stable emulsions in manila tamarind protein isolate. Similarly, He et al. (2022) also reported a negative impact of high temperature (121˚C) on emulsification properties of quinoa protein isolates.

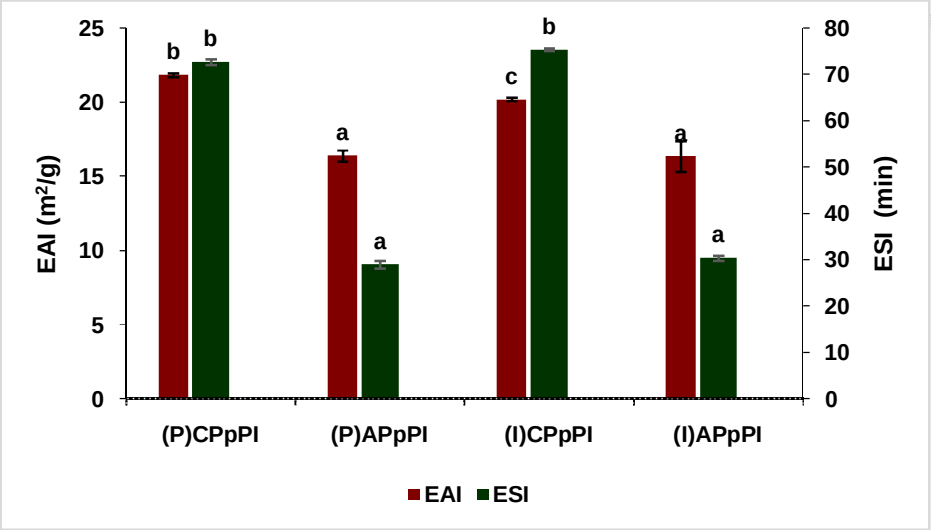

Figure 4. Emulsification ability index (m^2/g) and emulsification stability index (min) of control and autoclaved PpPI. (P) CPpPI- Control PpPI of PusaArhar 2018-4, (P) APpPI- Autoclaved PpPI of PusaArhar 2018-4, (I)CPpPI - Control PpPI of ICP 1452, (I)APpPI- Autoclaved PpPI of ICP 1452.

3.5. Foaming capacity and foaming stability

Foam formation and stability primarily influenced by the interfacial film created by proteins, which regulates air bubbles in suspension and reduces the rate of coalescence. Enhanced foaming capacity indicates a higher ability to incorporate air bubbles, while foam stability refers to the protein's ability, along with other protein components, to form a robust and cohesive film around the air bubbles, preventing air diffusion from them (Ma et al., 2011). The foaming capacity of the control PpPIs was observed to be 89.16% for Pusa Arhar 2018-4 and 84.05% for ICP 1452. Autoclaving treatment significantly ($p<0.05$) increased the foaming capacity of the protein isolates

by 22.90% in Pusa Arhar 2018-4 and 34.31% for ICP 1452. The foaming stability was determined at different time periods of 15, 30, 45 and 60 mins and results are represented in Figure 5. While foaming stability decreased over time for all samples, the autoclaved PpPIs exhibited greater stability than the control PpPIs at extended durations. At 45 minutes, the foaming stability was 14.26% for Pusa Arhar 2018-4 and 11.83% for ICP 1452, while at 60 minutes, it increased to 15.92% for Pusa Arhar 2018-4 and 16.91% for ICP 1452. These results demonstrate that autoclaving improves both the foaming capacity and long-term stability of the protein isolates. Although foaming stability decreased over time for all samples, the autoclaved PpPIs exhibited greater stability at longer durations (45 and 60 minutes), suggesting that autoclaving improved the protein's ability to form a cohesive and robust interfacial film. Thermal treatments caused unfolding of proteins exposes buried hydrophobic groups, enhancing the protein's interfacial properties and facilitating its quick adsorption at the air-water interface, thereby improving foaming capacity and stability(Zhao et al., 2020; Mir et al., 2021).

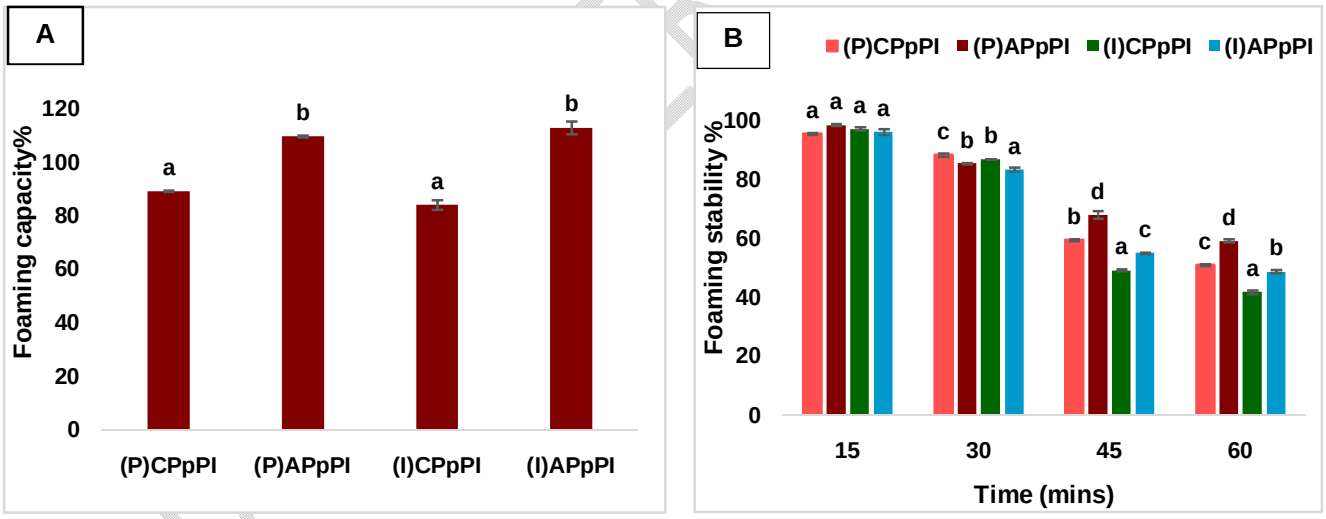

Figure 5. A. Foaming capacity (%) and B. foaming stability (%) of control and autoclaved PpPI. (P) CPpPI- Control PpPI of PusaArhar 2018-4, (P)APpPI- Autoclaved PpPI of PusaArhar 2018-4, (I)CPpPI - Control PpPI of ICP 1452, (I)APpPI- Autoclaved PpPI of ICP 1452.

4. Conclusion

The findings of the study revealed how autoclaving impacts the functional properties of protein isolates from pigeon pea genotypes Pusa Arhar 2018-4 and ICP 1452. Autoclaving significantly influenced key properties, including solubility, surface hydrophobicity, WHC, OHC, emulsification, and foaming characteristics. The reduction in protein solubility at all pH levels was attributed to thermal denaturation and protein aggregation, which increased hydrophobic interactions while decreasing protein-solvent interactions. Enhanced surface hydrophobicity, reflected protein structural alterations, leading to the exposure of previously buried hydrophobic regions. WHC declined significantly due to reduced water-binding capacity, while OHC increased, likely due to protein denaturation and improved affinity for oil molecules. Although emulsifying activity and stability decreased post-autoclaving, the treatment enhanced foaming capacity and long-term stability, highlighting its potential to improve specific functional properties. These changes have practical implications for food applications, where functional properties such as foaming, emulsification, and oil binding play critical roles in determining product quality. Overall, autoclaving emerges as a promising processing method to optimize the functional attributes of pigeon pea protein isolates, broadening their applicability in a variety of food formulations. Further research is recommended to explore the underlying molecular mechanisms and evaluate the potential of these protein isolates in real-world food systems.

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