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Influence of pH fractionation and salt on Bambara protein–gum arabic interaction and insoluble complex formation

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Abstract

Proteins exhibit remarkable interaction with polysaccharides to form complexes with novel functionality. Nevertheless, the formation of insoluble complexes close to the protein isoelectric point (pI) results in protein–protein interference, low coacervates yield, and limited application in acidified food systems. Hence, pH fractionation with salt was used to extract Bambara groundnut protein to induce a shift in optimum pH (pH_{opt}) of insoluble complexes formed with gum arabic, ultimately improving protein and coacervates yields. Turbidimetric analyses were employed to monitor the interaction between Bambara groundnut protein and gum arabic. Protein isolates from optimised fractionation conditions and insoluble complexes were characterised. The most effective fractionation condition was achieved at pH 2.95, 0.28 M NaCl, producing a substantial pHopt shift to 3.4. This shift was associated with the formation of spherical microparticle complexes and an impressive 70% coacervate yield. Changes in patterns of protein–gum arabic interaction were associated with an increased content of β-sheet, enrichment of legumin subunits, and basic amino acids of the isolate. This approach successfully shifted the formation of insoluble complexes towards a high-acid pH environment, notably away from the pI. Findings from this study create a prospect for future utilisation of pulse grain protein-gum arabic complexes in acidic beverage development.

Keywords: Bambara groundnuts; gum arabic; interaction; insoluble complex.

Practical Application: The study creates a prospect for future utilisation of pulse grain protein-gum arabic complexes in acidic beverage development.

1 INTRODUCTION

Proteins can interact with polysaccharides to form coacervates with novel surfaces and excellent functional properties. Protein-polysaccharides complexation has attracted much attention, particularly in the encapsulation and controlled release of bioactive ingredients, emulsion stabilisation, meat mimetics, and improvement of the viability of probiotics during storage (Li et al., 2022). Complexing proteins with polysaccharides is a pH-induced associative phase separation driven by electrostatic attraction between oppositely charged biomolecules, leading to biopolymer and solvent-rich phases (Zhang et al., 2021). During acid titration of a protein-polysaccharide mixture, maximum coacervation takes place when both biopolymers are at electrical equivalence (pH_{opt}), followed by a dissolution of the complex at $pH_{\Phi 2}$. The complex dissolution occurs due to protonation of the carboxyl group of the polysaccharide backbone (Carpentier et al., 2021). The electrostatic interaction also produces soluble and insoluble complexes at pH_c and $pH_{\Phi 1}$, respectively.

The optimum complexation (pH_{opt}) usually occurs within a narrow pH range (4.0–4.5) close to the isoelectric point of protein (Carpentier et al., 2021). Within the narrow pH range, protein–protein interaction has been found to interfere with the desired protein–polysaccharide complexation (Archut et al., 2023). Large aggregates are reportedly formed at the expense of smaller sizes (Jaramillo et al., 2012), which does not improve their bioavailability as a delivery vehicle for bioactive compounds in the acidified food system (Chen et al., 2020). Hence, the application of these complexes might be affected by the optimum complexation pH (pH_{opt}) obtained, particularly for those acidified beverages with pH ≥ 4.6 (Singh et al., 2022), which are prepared close to the isoelectric point of proteins. Although there are few exceptions where the pH_{opt} of the complexes has been formed below pH 4.0 (Archut et al., 2023), protein modification may be required to alter their complexation behaviour with anionic polysaccharides. To achieve the formation of coacervates below the isoelectric point of protein, Busu and Amonsou (2019) fractionated Bambara proteins prior to complexation. Protein fractions extracted at pH 2 had an optimum complexation pH value of 2.9, which was below the isoelectric point of proteins. However, further research may be required to improve the coacervate and protein yield, which appeared low (41 and 16%, respectively) in the acidified range.

The extraction condition can be manipulated to improve the protein yield and achieve the desired composition or structural modification to shift the optimum complexation pH of the protein with polysaccharide away from the isoelectric region. Although Busu and Amonsou (2019) successfully shifted the optimum complexation pH as described above, these authors did not include any

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salt in their protein extraction medium, which could be one of the reasons for the recorded low yield. Furthermore, these authors did not investigate the effects of mildly acidic pH extraction conditions. A previous report by Gulzar et al. (2017) demonstrated that mild acidic pH with salt could produce additional protein subunits in comparison with the conventional alkaline extraction. These studies suggest there is an opportunity to improve both the protein, coacervate yield, and properties by improving the efficiency of the process. The extraction conditions employing based on ionic strength and pH fractionation were anticipated to modify the protein composition and structure to generate new protein subunits that can shift the optimum complexation pH and properties of the complexes formed with gum arabic. Hence, in this study, a response surface methodology (RSM) was used to optimise the fractionation conditions of Bambara protein with a view to improving the protein yield and modifying the protein structure and composition by shifting the critical structure formation pHs away from the protein isoelectric region during complexation with gum arabic. In total, 13 experimental runs with extraction conditions of pH (2.0–9.0) and NaCl concentration (0.0–0.6 M) were generated as independent variables using a central composite design (CCD). Complexation behaviours were assessed by turbidimetric analyses focusing on pHs of critical structure formation. Changes in structure and conformation of fractionated proteins were analysed to understand protein interaction with gum-arabic (a model polysaccharide). This study demonstrated the use of pH fractionation and ionic strength as an extraction protocol to structurally modify Bambara protein and cause a shift in pH_{opt} during complexation with gum arabic and improved coacervates yield.

2 MATERIALS AND METHODS

2.1 Materials

Bambara groundnuts, *Vigna subterranea*, were collected from Jozini, Kwazulu-Natal province in South Africa. Glucono-d-lactone and gum arabic from *Senegal acacia* trees were purchased from Sigma Aldrich, South Africa, including other laboratory-grade chemicals.

2.1.1 Preparation of defatted Bambara flour

Bambara groundnuts were sorted, soaked in water for 8 h, dehulled, oven-dried at 40°C, milled, and defatted using n-hexane at a ratio of 1:5 (g/mL) for 4 h. The defatting was repeated, and the fat content was 0.75% according to Crespo and Yusty (2005). The residual hexane evaporated by exposing the defatted flour in a fume hood overnight.

2.1.2 Extraction of Bambara protein

Protein isolate was extracted from defatted Bambara groundnut flour using the method of Amonsou et al. (2012) by suspending the flour in Tris–HCl buffer solution (1 g of flour:10 ml of buffer) under constant agitation for 2 h. Statistical Software Design Expert version 11 (Stat Ease Inc., United States) was used to generate 13 experimental runs with CCD. The extraction pH (2.0–9.0) and NaCl concentration (0–0.6 M) were varied as independent variables. Centrifugation of the suspension was achieved using an Eppendorf centrifuge at 10,000 × g for 30 min at 4°C, and the pellet was discarded. The supernatant was dialysed against distilled water with a 12-kDa cellulose membrane for 5 days at 4°C, and the water changed two times daily. Following the completion of dialysis, the precipitate was freeze-dried and stored at -20°C for further analysis.

2.1.3 Protein content and yield

The protein content of the isolates was determined according to the method described by Lowry et al. (1951) and the total protein of the defatted flour (N X 6.25) by the Kjeldahl method. The yield of the isolate was taken as % protein recovered to that of the defatted flour, according to Arise et al. (2015).

2.1.4 Turbidimetric analysis of Bambara protein and Gum arabic

Stock solutions of Bambara protein isolate (BPI) and gum arabic (GA) were prepared according to the modified method of Busu and Amonsou (2019). 1% w/v of BPI and that of GA were dissolved separately in Milli-QTM water and allowed to continuously stir at 22°C for 2 h. Solubility of BPI was improved by stirring overnight (12 h) at 4°C. Thereafter, the BPI solution was subjected to heating in a water bath at 80°C for 15 min prior to mixing with GA to have a mixture volume of 30 ml. BPI:GA mixing ratio of 4:1 at a concentration of 0.1% (w/v) was adopted based on the preliminary studies. The solution pH was adjusted to pH 8 using 0.1 M NaOH prior to the commencement of the turbidimetric analysis. The turbidimetric titration through acidification was carried out with the use of an internal acidifier (0.05 w/w glucono-d-lactone, GDL), thereby reducing the dilution effect on the sample solution from pH 8 to 3.9. The pH was further reduced using gradients of HCl concentrations to mitigate the impact of dilution (0.05, 0.5, 1, and 2 M HCl was used to reduce pH from 3.9 to 3.4, 3.4 to 2.8, 2.8 to 2.3, and 2.3 to 1.5, respectively). Changes in optical density were recorded with a UV-VIS spectrophotometer using plastic cuvettes at 600 nm. The maximum optical density corresponds to the maximum complexation pH (pH_{opt}). The data obtained from the turbidimetric titration was fitted into a quadratic polynomial model to describe the complexation (BPI:GA) behaviour.

2.1.5 Determination of coacervate yield

The BPI:GA complex was prepared by mixing the individual biopolymer at a ratio of 4:1 to a total concentration of 0.1% w/v. The pH of the mixture was adjusted to the optimum complexation pH (pH_{opt}) using 1 M HCl. Stirring was carried out for 30 min at 4°C to facilitate complexation between the biopolymers. The suspension mixture was centrifuged at 8,500 x g for 15 min to recover the coacervates (Plati et al., 2021). This was followed by freeze-drying while the supernatant was discarded (Equation 1).

$$\text{Coacervate yield (\%)} = \frac{Freeze\ dried\ mass\ of\ coacervate}{Total\ weight\ of\ biopolymers} \quad (1)$$

2.1.6 Experimental design

RSM was done to determine the influence of extraction pH and NaCl concentration using CCD on optimum complexation pH (pH_{opt}) and coacervate yield of BPI:GA complexes. Statistical Software Design Expert version 11 (Stat Ease Inc., United States) was

used to generate 13 experimental runs. The behaviour of the complexes (BPI:GA) was designed using a quadratic polynomial model regression equation, which was used to describe the complexation behaviour. All experiments were carried out in randomised order to reduce external effects on the response (Equation 2).

$$Y = \beta_0 + \sum_{K=1}^{2} \beta_i X_i + \sum_{K=1}^{2} \beta_{ii} X_i X_i + \varepsilon \quad (2)$$

Y: the response variable;

β_o: a constant;

β: the linear;

β_{ii}: the interactive coefficient;

X_i: the level of the independent variable;

ε: the random error.

The optimisation objective was to minimise pH_{opt} and maximise coacervate yield, and this was found at a point that maximises the desirability function.

2.1.7 Verification and selection for optimum extraction conditions

The optimum values for BPI:GA complexes were compared with the predicted values for validation at a 95% confidence interval. Four extraction conditions were chosen from the RSM data for comparison with the optimum. This was based on maximising the coacervate yield and minimising the pH_{opt}.

2.2 Characterisation of optimised Bambara protein isolates

2.2.1 Amino acid composition

The amino acid profile of Bambara proteins was determined based on the principle of reverse-phase chromatography with pre-column derivatisation following acid digestion using the HPLC PICO-TAG system by Bidlingmeyer et al. (1984). The proteins were hydrolysed using 6 mol/L of HCl at 116°C for 24 h before subjected to chromatographic analysis. The methionine content was determined after performing acid oxidation. The digests were separated on a cationic column (4.6 × 150 mm) using a gradient of sodium citrate buffers (pH 3.45 and pH 10.85) at a flow rate of 0.45 mL/min.

2.2.2 Zeta potential

Zeta potential measurement of Bambara proteins was determined as a function of pH using zetasizer Nano zs (Malvem Instruments, Westborough MA, United States). Freshly prepared BPI dispersions were diluted to 2 mg/ml with deionised water and hydrated for 1 hour, which was vortexed intermittently. The sample solution was filtered using a 0.22 µm HA Millipore membrane prior to measurement. The measurement was carried out over a pH range of 3–8 for all the protein fractions and gum arabic.

2.2.3 Gel electrophoresis

Reducing and non-reducing sodium dodecyl sulphate (SDS)-PAGE electrophoresis were both carried out according to Laemmli (1970). 6 mg Bambara protein/ml was mixed with Tris–HCl buffer, and pH was adjusted to 8.0 in combination with 10% (w/v) SDS, which forms the reducing buffer, while that of the non-reducing buffer contains 10% v/v ß-mercaptoethanol. Heat was applied to the sample solutions at 95°C for 10 min, after which cooling was achieved prior to centrifugation at 10,000 × g for 15 min. A volume of 15 µL of aliquot was loaded into a 4–12% gradient gel, and the polypeptides were separated using the mini-PROTEIN system (BIO-RAD, Hercule, United States). Protein markers with a broad range of 10–200 kDa and Coomassie brilliant blue were used as a standard and to stain the gel, respectively.

2.2.4 Secondary structure of optimised Bambara proteins

FTIR analysis was carried out on the protein isolates by an infrared spectrophotometer (Agilent Cary 630 FTIR, CA, United States) at 4,000–400 cm^{-1}, scanning the background of each sample with air and reading from 32 cycles.

2.2.5 Scanning electron microscopy of BPI-GA complexes

BPI:GA complexes were directly mounted on a circular aluminium stub with double-sided sticky tape, which was coated for 250 s with 15 nm gold. This was followed by examination and scanning using a FEGSEM (ZEISS Ultra Plus, Germany) scanning electron microscope at an accelerated voltage of 5.0 kV. A calibrated scale bar on the monograph was used for the measurement of the sample sizes.

2.3 Statistical analysis

All experiments were carried out in triplicates, unless otherwise stated. Data were further subjected to RSM using the CCD for two factors, namely, pH and ionic strength. The model fitting followed a polynomial regression model. The mean coacervate yield and pH_{opt} were compared using a paired two-sample t-test in Microsoft Excel version 2016. In all tests, $p < 0.05$ was considered a statistically significant difference.

3 RESULTS AND DISCUSSION

3.1 Protein yield and content of Bambara protein isolates

The percentage yield and purity of protein fractions obtained through RSM design were significantly different (Table 1). Generally, increasing the ionic strength of the extraction solvent seemed to favour protein yields in a pH-dependent manner. A mild acidic pH (5.75) resulted in significant low protein yields independent of the ionic strength. Although the yield (63%) of protein fractions obtained at pH 3.5 with 0.5 M was lower than that of the traditional alkaline control method, this fraction showed comparable protein content (94%) to the control. Previous extraction at a lower pH (pH 2) without salt resulted in a yield of 16% (Busu & Amonsou, 2019), which is significantly low compared to the yield obtained (63%) in this study; this is a significant improvement for Bambara protein.

3.2 Turbidity profile of Bambara proteins: Gum arabic mixture

Changes in turbidity as a function of pH were monitored to investigate the complexation behaviours of protein fractions with gum arabic. The turbidity profiles of Bambara protein and gum arabic revealed some differences in complexation behaviours across the pH range of 2–8 (Figure 1A–1D). The turbidity curves show some variations in the critical pH region at pH_{opt} (optimum complexation pH). Although the optimum pH of complexation values was generally observed in the pH range of 4.0–4.5, which may correspond to protein isoelectric point (pI) (Busu & Amonsou, 2019), a notable shift in pH_{opt}, below the pI range, was observed for the protein isolates produced at pH 3.5 and 2.57 containing 0.5 M and 0.25 M NaCl, respectively (Table 1). These two fractions obtained at relatively high acidic conditions recorded an average pH_{opt} of about 3.5, and coacervates yielded 73% (Table 1). A similar shift was observed by Busu and Amonsou (2019) for the protein extracted at pH 2, but these authors reported a low yield of coacervates. The highest coacervate yield is achieved at the maximum optical density (OD_{max}), which is equivalent to the optimum complexation pH. It is worth noting that the OD_{max} for each protein fraction varies significantly with the lowest (1.1–1.2 nm) observed at alkaline extraction pH (8.0 and 8.9) dependent on the ionic strength. The OD_{max} for the fractions extracted at strong acidic pH 2.57 and 3.5 were 1.4 and 1.6 nm, respectively. Mild acidic pH 5.75 (at the central points of the design) was on the average of 1.3 nm, resulting in 58 and 38% increases in coacervate yield for both strong and mild acidic pHs compared to the coacervate yield of fractions obtained at alkaline pH 8.0 and 8.9. The low value of OD_{max} for fractions obtained at alkaline pH is responsible for the low yield (41%) obtained, which might be due to the degree of ionisation of the side chains of the proteins. High coacervate yield has been reported to portray strong electrostatic interaction between the biopolymers leading to a more turbid solution (Hachfi et al., 2023).

3.3 Fitting of the models

The $p < 0.05$ of the models indicates that the model terms are meaningful and have a substantial effect on the responses (Baylan & Çehreli, 2018). According to the p-values (Table 2), extraction pH, NaCl concentration, and the square of interaction had a significant effect on the pH_{opt} and coacervate yield. The predicted R^2 and adjusted R^2 for coacervate yield and pH_{opt}, respectively, are in reasonable agreement with each other that shows difference of less than 0.2. This indicates a good correlation between the actual and predicted values and good representation of the whole sample. The lack of fit values was 0.2317 and 0.1729 for pH_{opt} and coacervate yield respectively and was found to be insignificant ($p > 0.05$), showing that the model was well fitted into the experimental data. These results indicate that the selected models effectively correspond to the data for all the response variables.

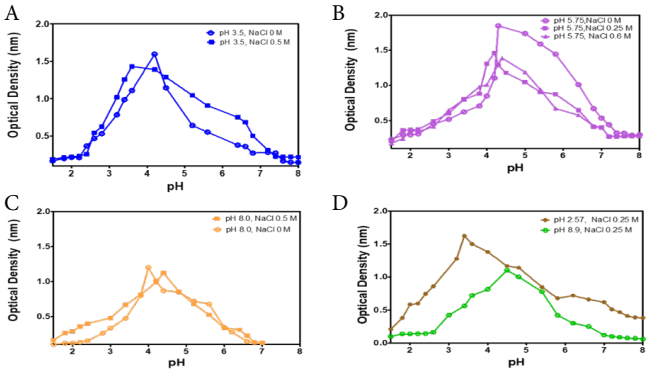


Figure 1. (A–D) Turbidity profile of Bambara protein isolates:Gum arabic complexes. A single graph was chosen as a representative out of the five central points (pH 5.75 and 0.25 M NaCl) on CCD (B).

Table 1. Protein content and yield of Bambara protein fractions extracted at varying pH and NaCl concentrations.

pH	NaCl (M)	Protein yield (%)	Protein content (%)	pH_{opt}	Coacervate yield (%)
3.50	0.00	$39.18^{a} \pm 0.30$	$89.56^{e} \pm 0.58$	$4.10^{cd} \pm 0.07$	$64.18^{c} \pm 3.28$
5.75	0.00	$38.24^{a} \pm 2.47$	$80.95^{a} \pm 0.56$	$4.30^{cd} \pm 0.35$	$68.44^{cd} \pm 5.49$
8.00	0.00	$58.44^{cd} \pm 0.23$	$85.23^{c} \pm 0.59$	$4.00^{bc} \pm 0.14$	$44.65^{b} \pm 3.73$
2.57	0.25	$59.66^{cd} \pm 6.19$	$88.26^{d} \pm 0.03$	$3.40^{a} \pm 0.28$	$74.80^{d} \pm 5.49$
5.75	0.25	$46.27^{b} \pm 0.69$	$87.46^{d} \pm 0.15$	$4.20^{cd} \pm 0.14$	$71.67^{cd} \pm 1.48$
5.75	0.25	$43.17^{ab} \pm 1.18$	$83.10^{b} \pm 0.58$	$4.20^{cd} \pm 0.14$	$71.79^{cd} \pm 2.62$
5.75	0.25	$46.71^{b} \pm 3.82$	$88.26^{d} \pm 0.07$	$4.00^{bc} \pm 0.14$	$66.56^{cd} \pm 1.88$
5.75	0.25	$47.4^{b} \pm 3.56$	$85.31^{c} \pm 0.15$	$4.20^{cd} \pm 0.14$	$67.01^{cd} \pm 1.73$
5.75	0.25	$45.35^{b} \pm 3.62$	$87.92^{d} \pm 0.01$	$4.20^{cd} \pm 0.28$	$71.47^{cd} \pm 2.26$
8.93	0.25	$67.09^{e} \pm 1.21$	$93.55^{g} \pm 0.52$	$4.50^{d} \pm 0.14$	$34.93^{a} \pm 4.31$
8.00	0.50	$75.86^{f} \pm 2.40$	$91.62^{f} \pm 0.20$	$4.40^{cd} \pm 0.04$	$46.37^{b} \pm 3.03$
3.50	0.50	$63.15^{de} \pm 2.76$	$94.33^{g} \pm 0.01$	$3.60^{ab} \pm 0.28$	$71.38^{cd} \pm 2.43$
5.75	0.60	$55.61^{c} \pm 3.34$	$89.34^{e} \pm 0.14$	$4.40^{cd} \pm 0.07$	$66.21^{cd} \pm 8.02$

Mean ± SD (n =2). Mean values with different subscript letters in a column are significantly different ($p < 0.05$).

3.4 Effect of process parameters on pH_{opt} and coacervate yield

The 3D surface plots showed that at acidic extraction pH with NaCl concentration between 0.2 M and 0.3 M, a pronounced effect on the pH_{opt} was observed (Figure 2A). The pH_{opt} reduced with a decrease in extraction pH. Regression analysis for studying the effect of the independent variables on pH_{opt} suggested that the model was significant at $p \leq 0.05$. A significant negative effect of interaction between extraction pH and NaCl concentration on coacervate yield was observed (Figure 2B). This suggests that the level of one of the predictors can be increased while the other decreases for constant coacervate yield. The negative coefficient of the independent variables at the quadratic level suggests a maximum coacervate yield at acidic pH, and it decreases with an increase in extraction pH. The low coacervate yield occurred with BPI extracted at alkaline pH, where there was a low degree of ionisation of BPI and GA.

3.5 Verification and selection of optimal range for extraction conditions

Determination of criteria for the optimum conditions was the final stage of the design, which was based on maximising the coacervate yield and pH_{opt} below the isoelectric point of the protein while all the independent variables were kept within the range. These optimum conditions were performed at Design-Expert software, and the optimal range for pH_{opt} and coacervate yield was determined from RSM data. Based on the statistical analysis, optimal conditions with maximum desirability were pH 3.50 with 0.50 M NaCl, pH 2.57 with 0.25 M NaCl, pH 2.62 with 0.04 M NaCl, and pH 2.95 with 0.28 M NaCl. The predicted responses were pH_{opt} 3.51, coacervate yield 75.50%; pH_{opt} 3.20, coacervate yield 66.98%; pH_{opt} 3.47, coacervate yield 62.99%; and pH_{opt} 3.33, coacervate yield 70.21%. The optimised extraction conditions were further validated by complexing the resultant Bambara protein isolates with gum arabic; this was done in triplicates. The validation of the optimised conditions through turbidity titration indicated pH_{opt} 3.60 coacervate yield 71.1% (pH 3.5, 0.50 M NaCl), pH_{opt} 3.20 coacervate yield 64.69% (pH 2.57, 0.25 M NaCl), pH_{opt} 3.60 coacervate yield 62.14% (pH 2.62, 0.04 M NaCl), and pH_{opt} 3.40 coacervate yield 69.74% (pH 2.95, 0.28 M NaCl). The experimental values for the responses were similar ($p > 0.05$) to the respective predicted ones, confirming the validation of the experimental design and demonstrating the validity and good adequacy of the RSM. The proteins that were fractionated under optimised conditions were further characterised to understand their complexation behaviour, and these include pH 3.50 with 0.50 M NaCl, pH 2.57 with 0.25 M NaCl, pH 2.62 with 0.04 M NaCl, and pH 2.95 with 0.28 M NaCl.

Table 2. Quadratic model for pH_{opt}, coacervate yield, and linear model regression coefficient for Bambara protein isolates and gum arabic complexes.

	pH_{opt}	p-value (pH_{opt})	Coacervate yield	*p*-value (coacervate yield)
Intercept	4.15		70.95	< 0.0001
A-pH	+0.3945	< 0.0001	-12.56	0.1986
B-NaCl	+0.129	0.0199	+1.68	0.3600
AB	+0.0000	1.0000	-1.48	0.0001
A^2	-0.1038	0.0405	-8.72	0.0202
B^2	+0.1704	0.0112	-4.10	
Adjusted R^2	0.91		0.94	
Predicted R^2	0.77		0.81	
R^2	0.95		0.97	

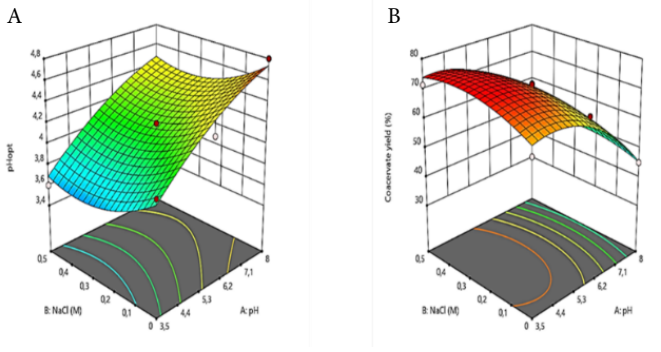


Figure 2. Response surface plots showing the interaction effects of pH and NaCl concentration on (A) optimum complexation pH (pH_{opt}) and (B) coacervate yield of Bambara protein isolate and gum arabic complexes.

3.6 Characterisation of optimised Bambara protein isolates

3.6.1 Amino acid profile

The amino acid composition of the protein was carried out to determine how the variation in the composition influenced the complexation behaviour with gum arabic. Extraction conditions of Bambara protein significantly impacted the amino acid composition (Table 3). Acidic amino acid groups were observed to be the major residues in the Bambara proteins. Although the medium pH is one of the factors controlling the electrostatic interactions between amino acids and gum arabic, the side chains of serine and threonine have been reported to show strong interactions with saccharides leading to complex formation (Ozdemir et al., 2016). The highest content of serine and threonine was found in the protein fraction extracted at pH 2.95, 0.28 M, relative to the control (pH 8.0, 0.50 M NaCl). Similarly, the basic amino acids in the protein fraction were 36% higher than the traditional alkaline control protein. Arginine, lysine, and histidine are positively charged groups, which suggest sufficient positively charged regions providing more electrostatic interaction sites for the negatively charged carboxylic group of gum arabic (Derkach et al., 2022). From this study, it appears that the fractions extracted at moderately acidic pH 2.95, 0.28 M contain more basic amino acids compared to the control and other fractions, which possibly influenced the complexation behaviour in terms of pH_{opt} shift and coacervate yield (70%).

3.6.2 Zeta potential

Zeta potential of the proteins was carried out to understand surface charge configuration and how they may have impacted complexation behaviour with gum arabic. Bambara proteins

had net positive and negative charge at pHs below and above the isoelectric point (pI) of the protein, respectively, while gum arabic (GA) solution exhibited negative charge at all the pHs tested (Figure 3). Isoelectric points (pI) of the protein fractions ranged between 4.5 and 5.0 except for the fraction extracted at pH 2.95 with 0.28 M NaCl that has the neutrality at 4.0. It is assumed that differences in pI values indicate disparity in amino acid composition (Klassen & Nickerson, 2012). Moreover, it is worth noting that the fraction with the highest content of basic amino acids (pH 2.95, 0.28 M NaCl) had the lowest value for pI. Since complexation is electrostatically driven at pH values below pI (< 5), strong interactions between positively charged BPI and negatively charged GA molecules were favoured, leading to the formation of coacervates. At pH 5, the negative value for the fraction at pH 2.95, 0.28 M NaCl, increased (-19.7 Mv) relative to other fractions, which might have increased its affinity to bind with the carboxyl group of gum arabic.

3.6.3 Gel electrophoresis profile of optimised Bambara Protein isolates

An SDS-PAGE profile was carried out to investigate the effect of the extraction conditions on the molecular weight distribution of Bambara protein fractions. Under non-reducing conditions, the general profile shows major bands that correspond to vicilin (7S) ~52 kDa (lanes 1–5); however, significant variation in the protein distribution was observed in the various fractions (Figure 4). Additional bands appeared at ~71, ~86, and ~32 kDa (α- and β-legumin, 11S) in the fraction obtained at pH 2.95, 0.28 M NaCl (lane 1) compared to the traditional alkaline control method (lane 2). Under non-reducing conditions, a new fairly intense band of low molecular weight, ~22 kDa, corresponding to β-legumin (Lei et al., 2022) appeared in fractions obtained at pH 2.95 with 0.28 M NaCl (lane 10) and control pH 8.0, 0.5 M NaCl (lane 9). Feng et al. (2023) reported relatively high stern-volmer quenching constant values of both 11S

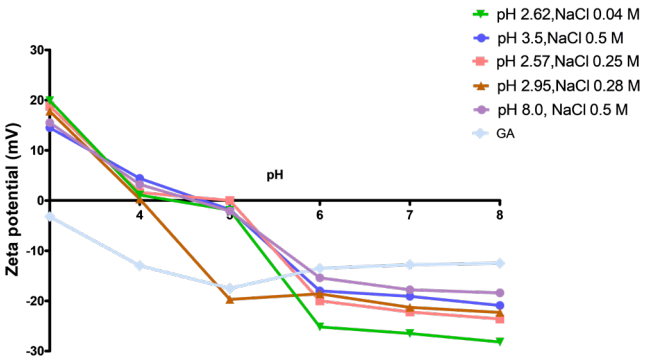
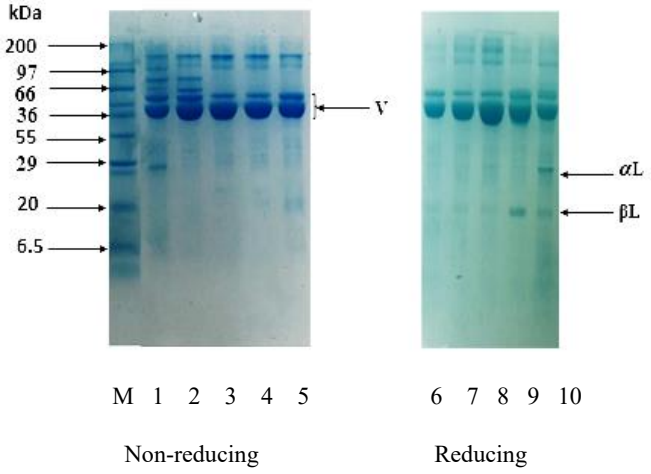


Figure 3. Zeta potential of gum arabic (GA) and BPI solutions at optimised extraction conditions.



L: legumin; V: vicilin; βL: basic legumin; αl: acidic legumin; M: marker; lanes 1 and 10: pH 2.95, 0.28 M NaCl; lanes 2 and 9: pH 8.0, 0.5 M NaCl; lanes 3 and 8: pH 3.5, 0.5 M NaCl; lanes 4 and 7: pH 2.57, 0.25 M NaCl; lanes 5 and 6: pH 2.62, 0.04 M NaCl.

Figure 4. SDS-PAGE of Bambara protein isolates extracted at optimised conditions and traditional alkaline control method.

Table 3. Amino acid composition (g/100 g protein) of Bambara protein isolates extracted at optimised conditions.

Amino acid	pH 2.57, 0.25 M NaCl	pH 3.50, 0.50 M NaCl	pH 2.62, 0.04 M NaCl	pH 8.00, 0.50 M NaCl	pH 2.95, 0.28 M NaCl
Histidine	2.18	2.47	2.00	2.34	3.64
Arginine	5.07	6.20	5.05	5.18	6.50
Serine	4.00	4.73	3.69	5.08	6.32
Glycine	2.28	2.67	2.36	3.75	2.73
Aspartic acid	6.14	8.67	5.90	8.47	9.37
Glutamic acid	10.27	14.01	9.82	14.03	15.14
Threonine	1.74	2.01	1.51	2.19	3.62
Alanine	2.43	2.79	2.12	3.91	3.09
Proline	3.15	3.30	2.85	3.01	3.56
Lysine	4.75	4.84	4.01	5.09	6.86
Tyrosine	3.26	3.84	4.31	6.70	3.88
Methionine	1.63	1.98	1.99	2.46	1.95
Valine	3.62	3.99	3.23	4.34	4.24
Isoleucine	3.38	3.74	3.04	4.15	3.90
Leucine	7.03	7.64	6.16	7.60	7.84
Phenylalanine	6.49	8.50	8.77	7.52	8.08
AAA	16.41	22.68	15.72	22.50	24.51
BAA	12.00	13.51	11.06	12.61	17.00

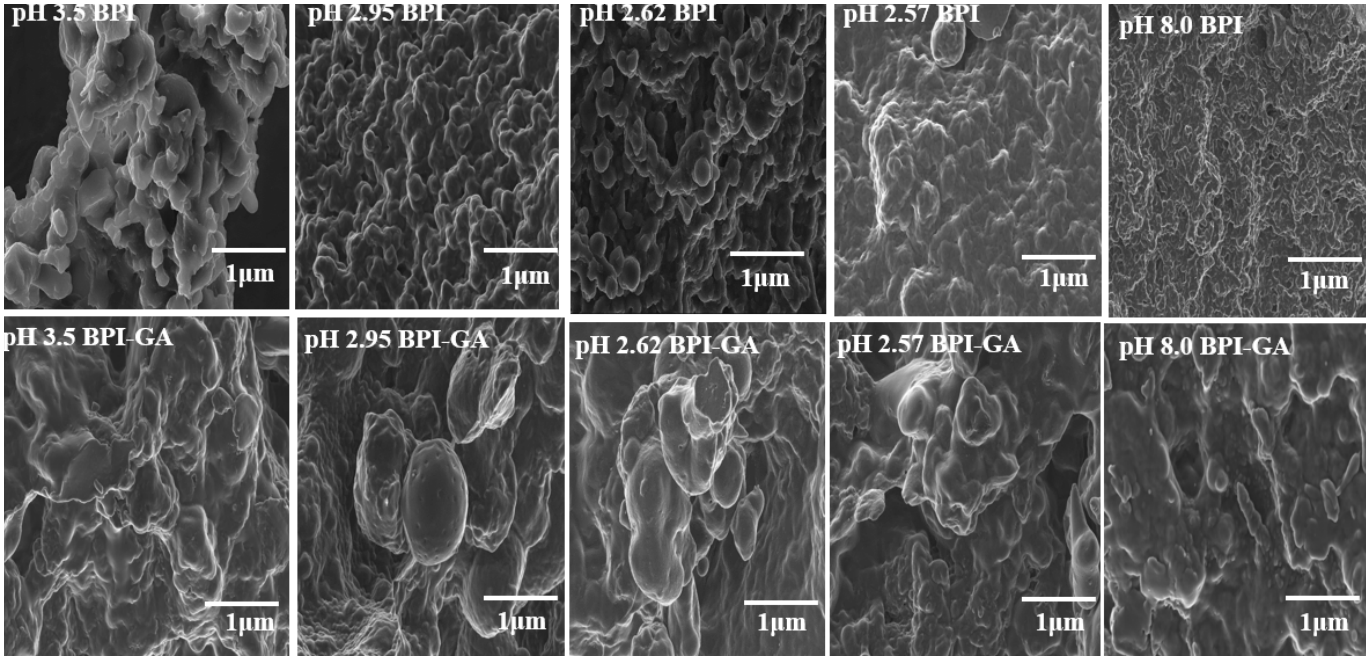


Figure 5. FEGSEM micrograph of Bambara protein isolates (BPI) at optimised extraction conditions and their complexes with gum arabic (GA).

and 7S of soybean isolates, indicating that both proteins were prone to forming complexes with gum arabic. However, 11S demonstrated a slightly higher complexation ability than 7S. Although, in this study, legumin appeared on both fractions obtained at pH 2.95, 0.28 M NaCl and the traditional alkaline control, the fraction extracted at pH 2.95, 0.28 M NaCl had both α- and β-legumin, while the control had only β-legumin. The substantial variation observed in the protein subunits might be a contributing factor to the pH_{opt} shift.

Table 4. Secondary structure of Bambara protein isolates extracted at optimised conditions.

Secondary structure	β-Sheets	α-Helix	β-Turns
pH 3.5, 0.5 M	60.14	36.16	8.57
pH 2.95, 0.28 M	63.77	39.69	0.54
pH 2.57, 0.25 M	44.73	35.69	28.26
pH 2.62, 0.04 M	38.92	28.96	11.10
pH 8.0, 0.5 M	58.14	34.08	7.28

3.6.4 Secondary structure of optimised Bambara fractions

FTIR analysis provided information about the modification that occurs in the secondary structure of the proteins. The spectra analysis of Bambara proteins in the amide-I region revealed three major Gaussian bands centred at 1,612–1,694, 1,646–1,664, and 1,662–1,684 corresponding to β-sheet, α-helix, and β-turn, respectively (Wang et al., 2023). Generally, β-sheet is the dominant structure for all the protein fractions (Table 4). The high content of α-helix suggests a folded and rigid structure that is still hidden from the reaction sites. Interestingly, protein fractions at pH 2.62 with 0.04 M and pH 2.95 with 0.28 M salt had the lowest and highest content of β-sheets, respectively. Transition from α-helix structure to β-sheets takes place during protein unfolding (He et al., 2021). The observed changes in the secondary structure might be due to dissociation and rearrangement of protein molecules at low pH and in the presence of NaCl (Guckeisen et al., 2021).

3.6.5 Scanning electron microscopy of BPI-GA complexes

The microstructure of particles of BPI and their BPI-GA complexes were observed by FEGSEM (Figure 5). For lyophilised droplets of BPI solution, the structural surface was relatively intact, exhibited mainly spherical structures with a lack of cracks, and had a compact surface morphology typical of dehydrated products obtained by the freeze-drying technique. The electrostatic interactions between BPI and GA led to microparticles, which are predominantly spherical in shape with a rough surface and concavities. There are some observed distortions on the surface of the complexes, which are attributed to the uneven shrinkage of the particles during the drying process with rapid evaporation of water (Eratte et al., 2015). These distortions could improve the release properties of microparticles due to a greater surface area. The nanostructures are similar to those observed with pea protein — tragacanth gum complexes (Carpentier et al., 2021). The nanostructures constitute potential points inside which the entrapment and protection of sensitive bioactive compounds may take place (Taheri & Jafar, 2019). Among the complexes, the pH 2.95, 0.28 M NaCl BPI-GA complex provided a more homogenous, highly organised, dense structure with well-connected agglomerated particles. Similar structures have been reported for various complex coacervation systems, such as soy protein isolate — chitosan (Plati et al., 2021).

4 CONCLUSION

The formation of insoluble protein-polysaccharide complexes close to the protein isoelectric point constitutes a major hurdle in the application of complexes in certain foods, such as

acidified beverages. Challenges of protein–protein interference and low coacervate yield are experienced during the process of complexation. To address these challenges, a systematic manipulation of fractionation pH and NaCl concentrations using an RSM design was employed as a strategy to extract protein from Bambara groundnuts. This approach resulted in composition and structural modification of the protein fractions, consequently shifting the formation of insoluble complexes towards higher-acid pH environments and away from the protein isoelectric point. The most effective fractionation condition was achieved with the Bambara protein isolate obtained at pH 2.95, 0.28 M NaCl, producing a substantial pH_{opt} shift to 3.4 and a remarkable 70% coacervates yield. Changes in the interaction dynamics between Bambara protein and gum arabic could be attributed to increased β-sheet content, enriched legumin subunits, and higher basic amino acid contents. Furthermore, a substantial improvement in protein yield (63%) was achieved at moderately acidic pH conditions with salt compared to similar conditions with no salt, thus underscoring the supportive effect of ionic strength. Findings from this study create a prospect for future utilisation of pulse grain protein–gum arabic complexes in acidic beverage development.

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