



**IMPACT OF BAMBARA GROUNDNUT PROTEIN
MODIFICATION ON COMPLEXATION BEHAVIOUR WITH
GUM ARABIC FOR IMPROVED STABILITY AND
FUNCTIONALITY IN FOOD APPLICATIONS**

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DECLARATION

I, **Abiola A. Ojesanmi**, hereby declare that the work reported in this thesis and submitted to the Department of Biotechnology and Food Science at Durban University of Technology for the degree of Doctor of Food Science and Technology is my original work. I confirm that it has never been previously submitted for a degree at a Higher Education Learning Institution.

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DEDICATION

This doctoral thesis is dedicated to God Almighty, the treasure of my heart and my soul.

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ABSTRACT

Protein-polysaccharides complexation has attracted considerable research interest over the past several decades, particularly regarding its role in the encapsulation of bioactive compounds, emulsion stabilization, meat mimetics, and fat replacers. However, complexes usually form within a narrow pH range characterized by weak interactions owing to a tightly packed structure and limited molecular flexibility of the native proteins. Consequently, this restricts the number of oppositely charged groups on globular proteins that can interact with polysaccharides and limits the applications of complexes in certain foods, particularly in acidic beverages. Enhancing the interactions between globular proteins and polysaccharides to achieve stable complex formation requires modifications to the protein structure, composition, and functional properties. Hence, the purpose of this study was to evaluate the influence of various protein modification methods on their physicochemical and structural properties, as well as how these structural changes affect their ability to form complexes with polysaccharides. To achieve this aim, pH-induced fractionation and ionic concentration were utilized as a protocol for extracting and structurally modifying Bambara protein prior to complexation with gum arabic. Additionally, limited trypsin hydrolysis and atmospheric cold plasma were done to modify the protein, and their impact on the stability and properties of complexes was investigated. Finally, encapsulation of riboflavin in a model beverage using Bambara protein hydrolysates (DH 5)- gum arabic soluble complex as a delivery system was determined.

Response surface methodology was employed to optimise the pH-induced fractionation of Bambara protein. Thirteen experimental trials were conducted under varying extraction conditions of pH (2.0 to 9.0) and NaCl concentrations (0.0 to 0.6 M), which were considered as independent variables within a central composite design. Complexation behaviour was evaluated through turbidimetric analyses, while structural and conformational changes in fractionated proteins were examined to elucidate their interaction with gum-arabic. Bambara protein fraction obtained at pH 2.95, 0.28 M NaCl produced the most pronounced shift in pH_{opt} (3.4) with spherical microparticles complex and 70% coacervate yield. The modification of protein-gum arabic complexation profile was corroborated by increased content of β -sheet, enriched legumin subunits, and basic amino acids of the protein fraction.

Trypsin hydrolysis was done at a controlled degree of 2, 5, and 7.5 prior to complexation with gum arabic focusing on the critical pH structures formations. The hydrolysates were characterized using SEC-HPLC, zeta potential, surface hydrophobicity, and intrinsic fluorescence. Electrostatic interactions between the biopolymers were monitored through turbidimetry analysis. The stability property of the complexes was investigated using differential scanning calorimetry and rheometry. Among the hydrolysates, DH 5 produced the most stable soluble complexes over a broader pH range (7.0 - 4.3), compared to DH 2, DH 7.5, and the unhydrolyzed isolate showed enhanced binding strength and structural stability. This was evidenced by its rheological fingerprints, with DH 5 showing a more pronounced plateau value of G' , indicating a stronger molecular interaction and binding strength between the hydrolysate and gum arabic. This suggests a higher degree of internal structure and makes the complex more stable. Additionally, DH 5 showed a shift in thermal transition temperature, increased negative charge, and surface hydrophobicity.

Bambara protein was subjected to atmospheric cold plasma treatment (2, 5 and 10 min) prior to complexation with gum arabic. The impact of the exposure time on composition, structural changes of protein and complex behaviour was analysed. The optimum complexation, pH_{opt} of cold plasma treated Bambara protein-gum arabic complexes significantly shifted to acidic pH following the treatment in an exposure time-dependent manner. The structural changes coincide with the observed shift from α -helix to β -sheet structure, indicating opening of the structure of protein. Crosslinking effects of cold plasma corroborated the breaking of the cross-linking disulfide bonds and oxidative modification of proteins, respectively by the chemically reactive species. The variation in the protein composition was associated with a significant rise in positively charged amino acids throughout the cold plasma treatment compared to the untreated protein. The slight red shift in fluorescence intensity data of the plasma protein suggests partial unfolding of the protein structure, correlating with increased hydrophobicity and negative surface charge. The difference between the G' and G'' values of the insoluble complex for the untreated proteins increased from < 1 log to ~ 1 log after 10 min exposure time indicating the formation of a stable network structure. The complexes formed at optimal pH (pH_{opt}) of 3.2 and 3.0 could be useful in acidic beverages and may serve as fat replacers in low-fat food products. Hence, cold plasma treatment for 5 min could be recommended for a more stable structure. The complexes formed at optimal pH (pH_{opt}) of 3.2 and 3.0 could be useful in acidic beverages and may serve as fat replacers in low-fat food products. Hence, cold plasma treatment for 5 min could be recommended for a more stable structure.

Three methods of protein modifications were employed in this study, pH fractionation in the presence of salt, limited enzymatic hydrolysis and emerging atmospheric cold plasma technology. Due to the observed structural changes following each method of modification, a choice of modification strategy depends on the intended objective. The pH fractionation with salt may be adequate for protein sources where high yield is required to produce mild acidic food products. Limited enzymatic hydrolysis and polysaccharides could be appropriate for food products with more stable and better network structure in a wider pH range while atmospheric cold plasma being a non-thermal treatment may be appropriate for heat sensitive food products and where crosslinking of the protein is required.

The results of this study are uniquely differentiated by the key variables associated with each modification method, namely: exposure time in cold plasma treatment, enzyme selection in limited hydrolysis, and the range of pH and salt concentrations applied during the extraction–fractionation process. This variability highlights the complexity of the research topic and suggests that further investigation is needed to fully understand the relationships between these variables. Additionally, there is a need to determine different results that could be produced when the variables are extended and their applications in other food products.

PREFACE

This thesis comprises eight chapters, with the experimental work presented in manuscript format. Chapter One provides a general introduction to the study. Chapter two critically reviews the existing research on legume proteins, nutritional profile, physicochemical, structural composition, and functional properties of leguminous proteins, such as solubility and emulsification properties. The complexation of protein and polysaccharides was critically reviewed. A concise overview was provided focusing on the structural and functional changes in Bambara proteins employing limited enzymatic hydrolysis along with atmospheric cold plasma technology to modify structures of the proteins preceding complexation to impact their functionality and widen the food applications of the complexes. The next chapters were divided into three parts, the first section discussed how optimization of Bambara protein extraction conditions (pH and ionic strength) caused a structural modification of the proteins and their impact on complexation behaviour with gum arabic (chapter 3). The second part reported how the degree of hydrolysis broadened the region of formation of soluble complexes and stabilized the complex solutions prepared from Bambara protein hydrolysates and gum arabic (chapter 4). The third section discussed the utilization of atmospheric cold plasma to effect structural modification of Bambara proteins and the effect of exposure time on their complexation behaviour (chapter 5). Chapter six showed the application of the Bambara protein hydrolysate (DH 5) - gum arabic soluble complex in encapsulation efficiency and release property of riboflavin (Vit B2) using a model beverage compared to Bambara protein isolate-gum arabic soluble complex. Chapter seven offers a general discussion of all the results, while Chapter eight presents the thesis conclusions along with key recommendations.

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ABBREVIATIONS

ANOVA: Analysis of variance

BPH: Bambara protein hydrolysate

CD: Circular Dichroism

DSC: Differential scanning calorimetry

FAO: Food and Agriculture Organization of the United Nation

FI: Fluorescence intensity

FTIR : Fourier Transition Infra-Red

G : Loss modulus

G : Storage modulus

G : Complex modulus

HCl : Hydrochloric acid

H : Protein denaturation enthalpy

HPLC : High-Performance Liquid Chromatography

kDa: Kilo-Dalton

ME: Mercaptoethanol

MW: Molecular weight

ACP: Atmospheric cold plasma

PAGE: Polyacrylamide gel electrophoresis

SD: Standard deviation

SDS: Sodium dodecyl sulfate

SEM: Scanning electron microscopy

TCA: Trichloroacetic acid

Td: Peak Temperature of Transition

TEMED: Tetramethyl Ethylene-Diamine

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1 CHAPTER ONE

1.1 Introduction

Bambara groundnut (*Vigna subterranea*) is considered an underutilized legume with the potential to contribute significantly to food and nutrition security on a global scale. It is widely cultivated in Africa due to its excellent nitrogen-fixing capacity and high protein content (19-25%) (Arise *et al.* 2014). Bambara groundnut is drought-resistant compared to pea and soybean, which have failed to thrive under extreme drought conditions (Kudre *et al.* 2013; Majola *et al.* 2021). The amino acid characterization of Bambara protein indicates that it is a rich source of lysine, much like other grain legumes. This positions it as a prime choice for use in food products, especially as a supplement to cereals that lack sufficient (Arise *et al.* 2017). Bambara protein also provides a notable amount of methionine (1.6 g per 100 g of protein) and cysteine (1.2 g per 100 g of protein), which are amino acids typically limited in most legumes. The main storage protein in Bambara groundnut is vicilin, which exhibits excellent solubility and strong emulsifying properties (Adebowale *et al.* 2011; Alabi *et al.* 2020). However, most plant proteins including Bambara is seldom optimal for functional food application in their native forms, hence the need for modification

Complexation of protein with polysaccharides is a good modification strategy that can optimize plant protein functionality, facilitating their use in food applications. Complex coacervation refers to an associative phase separation phenomenon driven primarily through electrostatic attractions between oppositely charged biomacromolecules, producing two distinct layers: a dense, biopolymer-dense coacervate phase and a dilute, solvent-rich supernatant layer (Stone, Teymurova, and Nickerson 2014). In recent decades, protein-polysaccharide complex coacervation has drawn considerable attention, especially for its applications in encapsulating active ingredients, stabilizing emulsions, developing meat alternatives, and creating fat replacers (Patel 2020; Li *et al.* 2022). Electrostatic interactions take place within two stages: first, the development of a soluble complex at the critical pH (pH_C), followed by an increase in turbidity as acid titration progresses. This is followed by the formation of an insoluble complex at $pH_{\phi 1}$, marked by a sharp increase in turbidity and a visible transition from a clear to a cloudy solution. Maximum coacervation occurs when both biopolymers are at electrical equivalence

(pH_{opt}) and finally dissolution of the complex (pH_{q2}), resulting from carboxyl group protonation in the polysaccharide backbone. Modification through complexation of protein-polysaccharide has been reported to play a significant role in controlling the functional and structural properties of food systems (Warnakulasuriya and Nickerson 2018). Previous studies by Xie and Hettiarachchy (1997) showed improved solubility and emulsification properties of the complex formed from soy protein isolate-xanthan gum over a pH range of 3.0-9.0, an ionic strength of 0.1 to 1.0 M NaCl, and at a temperature of 85 °C for 1 hr. However, Stone et al. (2013) reported contrasting findings, noting that complexation of canola protein isolate with carrageenan had a significant negative impact on solubility, emulsion, and foaming capacities, including stability. This effect may be due to the assembly of insoluble complex granules, propelled by both electrostatic and hydrophobic interactions. These interactions are largely influenced through alterations in structure within the biopolymer (Sanchez et al., 1997). Furthermore, the characteristics of the resulting complexes appear to strongly depend on the protein source and the specific polysaccharide used (Stone et al., 2013). Typically, optimum complex coacervation is confined to a narrow pH range of 4.0 -4.2, near the isoelectric point of the protein, where some properties like solubility and emulsifying ability of the protein are minimal (Mession *et al.* 2012; Hasanvand and Rafe 2018; Carpentier *et al.* 2022). Furthermore, the process of producing soluble complexes is associated with relatively weak interactions, which result from the rigid and densely packed conformation of native globular proteins (Razi, Fahim, Amirabadi, & Rashidinejad, 2023). As a result, only a limited number of oppositely charged groups on globular proteins can interact with polysaccharides (Tolstoguzov, 2017). This limitation is a barrier to the applications of complexes in certain foods particularly in acidic beverages. Hence, further modification to enhance the functionality of protein could be explored.

Atmospheric cold plasma is an innovative, non-thermal, eco-friendly technique increasingly utilized to enhance safety of food, modify starch, and induce structural changes in proteins (Min et al., 2017; Thirumdas, Kadam, & Annapure, 2017). It is a technology that modifies the functionalities of biomolecules through interactions with ions, free electrons, reactive oxygen, or nitrogen species (Yan *et al.* 2017; Dharini, Jaspin, and Mahendran 2022). These interactions cause structural changes in atmospheric cold plasma-treated proteins. Atmospheric plasma-induced oxidation altered the amino acid functional group residues in grass pea protein isolate (GPPI), promoting cross-linking between tyrosine residues and resulting in the formation of di-tyrosine (Mehr, Koocheki, and Milani, 2020). Oxidation causes proteins to unfold, which

increases their surface hydrophobicity and promotes aggregation (Zhang et al., 2020b). The formation of free carbonyl groups and hydroxylation of tryptophan amino acid responsible for the shortfall of fluorescence emission intensity and conformational changes of the treated grass pea protein, were reported (Mehr and Koocheki 2020). In another study, Ji et al. (2018) observed an enhancement in β -sheet and random coil structures, accompanied by a depletion in α -helix and β -turn levels in pea protein isolate. This represents a shift from a well-ordered, compact protein secondary structure becoming a less ordered, flexible configuration, caused by the impact of high-energy ions from atmospheric plasma. Additionally, conformational changes induced by plasma treatment altered the surface charges of amino acid residues, resulting in an increased negative charge on grass pea protein (Mehr & Koocheki, 2020). This structural modification of protein after plasma treatment could impact the degree of complexation. The influence of exposure time of cold plasma modification on the complexation behaviour of protein with polysaccharide is yet to be investigated.

Furthermore, in addition to non-thermal physical modification with atmospheric cold plasma, enzymatic modification is another promising approach that can cause structural rearrangements in proteins, improving the functional properties (Watanabe and Arai, 1992). For example, enzymatic hydrolysis lowers the molecular weight of proteins, expands the quantity of ionizable groups, alters their secondary and tertiary structures, and brings hydrophobic groups that were originally buried within the core of proteins to the surface (Jin et al., 2020). Hydrolysis beyond 10% can generate small protein fragments devoid of the desired surface properties (Kong *et al.* 2023). A mild enzymatic hydrolysis has been reported to improve the functional properties of proteins (Zang et al., 2019). Based on this, it was hypothesized that limited trypsin hydrolysis would partially unfold the protein, generating flexible, soluble peptides with improved charge characteristics, which should strengthen interactions with gum arabic.

Currently, only a few studies on the modification of protein from Bambara groundnut in relation to complexation have been reported. Busu and Amonsou (2019) were able to manipulate the extraction pH of Bambara protein in combination with acacia gum (gum arabic) and shifted the optimum complexation pH from 4.8 to 2.9. However, despite this success, a low coacervation yield of 41% and protein yield of 16% was recorded. In this study, modification strategies such as optimization of extraction conditions, use of atmospheric cold plasma, and limited trypsin hydrolysis of Bambara proteins would be explored as promising

alternatives to produce protein-polysaccharides complexes, which can be applied in low-acid beverage formulation.

The present work explores the contribution of optimized extraction conditions, limited trypsin hydrolysis, and atmospheric cold plasma on the structure, physicochemical changes, and complexation behaviour (critical structure-forming pH_s, binding strength, and stability) of Bambara groundnut protein with gum arabic.

2 CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Legume Grain Proteins

2.1.1 Nutritional properties

Legumes are gaining more interest as food ingredients, and this is because of their nutrient-rich, cost-effectiveness, and high functionality (Johnston et al. 2015; Feyzi et al. 2018; Kebede 2020; Goldstein and Reifen 2022). Legumes are rich in carbohydrates and protein, and also contain useful amounts of dietary fiber (Dhull, Punia, Kidwai, et al., 2020; Siddiq & Uebersax, 2012). For example, an underutilized drought-tolerant legume, Bambara groundnut comprises roughly 64.4% carbohydrates, 23.6% protein, 6.5% fat, and 5.5% dietary fiber, and is particularly rich in essential minerals, underscoring its nutritional significance. Bambara grains come in a variety of colours, including red, black, cream, purple, yellow, brown, and white. Figure 2.1 illustrates the grain colors of various accessions, including Maikai, Bidillali, Duna, Roko, Katawa, Karu, Ex-Sokoto, Maibergo, Cancaraki, Jatau, and Giwathe (Khan *et al.* 2021). Legumes are mainly made up of starch (65%–72%) and dietary fiber (10%–20%) (Keskin et al., 2022). The starch is mostly slowly digestible resistant starch, which gives legumes a low glycemic index. In addition, the high protein content of legumes has enabled their processing into flours, protein concentrates, or protein isolates, thereby making them suitable for the formulation of nutritious and functional foods (Lu *et al.* 2020).

Protein composition of grain legumes is dependent on legume species (Table 2.1). These indigenous legumes are comparable to commonly used proteins from soybean (Chen *et al.* 2020b) and pea proteins (Lu *et al.* 2020). Seed proteins are classified as structural, storage, or biologically active, with most legume proteins being storage proteins. During seed maturation, storage proteins are produced and stored in the endosperm of monocotyledons and embryos of dicotyledons. Their presence in mature seeds is called “protein bodies” (membrane-bound organelles). Storage proteins are important because they determine not only the overall protein composition of leguminous seeds but also their quality (amino acid composition) for various nutritional and functional uses (Carbonaro and Nucara 2022). The amino acid composition impacts the functional properties of proteins because it determines the ratio of polar to non-polar groups, as well as the balance of positive, negative, and neutral groups, thereby modifying

surface hydrophobicity and electrical charge interactions. Thus, the structural characteristics of plant proteins are important considerations when optimizing their functional properties.



Figure 2.1 Seed colour of some accessions of Bambara groundnut (a) Duna (b) Roko (c) Katawa (d) Ex-sokoto (e) Maikai (f) Maibergo (g) Karu (h) Cancaraki (i) Bdillali (j) Jatau (k) Giwa (Khan *et al.* 2021).

Table 2.1. Protein nutritional characterization of legumes worldwide (Zhang *et al.* 2024a).

Common names	Latin name	Protein content (%)	amino acid composition (AAC)	Reference
Soybean, Soy	<i>Glycine max</i>	30–45	It provides all nine essential amino acids (EAA), but is deficient in sulfur-containing amino acids.	Zhang et al. (2021)
Pea	<i>Pisum sativum</i>	20–25	It provides all nine essential amino acids, and lower concentrations of sulfur amino acids and tryptophan, and higher concentrations of basic and acidic amino acids.	Shanthakumar et al. (2022)
Fava bean	<i>Vicia faba</i>	27–34	It provides all nine essential amino acids, but lack of sulfur-containing amino acids and tryptophan.	Nivala et al. (2021)
Mung bean, Green bean	<i>Vigna radiata</i>	25–28	It contains sufficient quantities of all amino acids including lysine, except methionine, cystine, and tryptophan.	Du et al. (2018)
Azuki bean, Red bean	<i>Vigna angularis</i>	21–23	It provides all nine essential amino acids.	Wang et al. (2022)
Kidney bean	<i>Phaseolus vulgaris</i>	23–25	Sulfur-containing amino acids (cysteine and methionine) were limiting but the proteins were rich in acidic amino acids.	Mundi and Aluko (2012)
Lentil , Hyacinth bean	<i>Lablab purpureus</i>	21–31	It contains all essential amino acids, but is usually deficient in sulfur-containing amino acids (methionine and cysteine) and tryptophan.	Hang et al. (2022)
Chickpea	<i>Cicer arietinum</i>	15–25	It contains all essential amino acids, but sulfur-containing amino acids as limiting amino acids.	Zia-Ul-Haq et al. (2007)

2.1.2 Amino acid composition

The amino acid composition of legume proteins varies substantially according to the species (Table 2.2), environmental factors, growing conditions, genetic architecture, and isolation method of the storage proteins. Legume proteins are high in lysine, compensating for the lysine shortage in grains (Gupta, Gupta & Kumar 2021). Although the major amino acids in legume storage proteins are acidic amino acids, which contain asparagine and glutamine, correspondingly making up 25-40% of the amino acid composition (Kudre, Benjakul, and Kishimura 2013; Arise *et al.* 2017; Gunes and Can Karaca 2022). For example, leucine and arginine were reported to be high in fava beans (Multari, Stewart & Russell 2015) while cowpea has lysine as the most abundant amino acid (Shevkani *et al.* 2015; Awika and Duodu 2017).

Sulphur-containing amino acids, methionine, and cysteine are the deficient amino acids in legume grain proteins, while the other essential amino acids are adequately present (threonine, leucine, isoleucine, valine, lysine, histidine, tryptophan) (Aremu *et al.* 2015). Interestingly, a significant content of sulphur-containing amino acids was present in Bambara storage proteins (cysteine-1.2g/100g protein, methionine-1.6g/100g protein) (Alabi *et al.* 2020). Although legumes are low in sulphur-containing amino acids, this can enhance calcium retention, in contrast to animal proteins (Maphosa, Jideani, and Adeyi 2017). Proline has been reported to impart rigidity to the structure, contributing to the protein conformational changes of proteins (Damodaran, Parkin & Fennema 2008). The ring-shaped side chain of proline causes a twist in the configuration of proteins, thereby preventing the formation of beta-pleated conformation. Beta-pleated is the conformation responsible for increasing the protein solubility by preventing the occurrence of aggregation in the protein. The properties of the chemical group of amino acid strongly determine its functional qualities, and the overall of a protein molecular surface charge is equally dictated by the relative acidic to basic amino acid ratio, which has an impact on the protein solubility, which is a critical functional property in legume proteins (Esteghlal *et al.* 2019; Day, Cakebread, and Loveday 2022).

Table 2.2. The composition of amino acids in 10 legumes (g/100 g protein)

Amino acid	BGN	CP	SB	AB	LP	LB	LT	CK	BB	KB
Arginine	4.0	1.6	7.2	1.3	3.9	2.2	2.2	1.8	0.7	1.5
Aspartic acid	5.0	2.8	11.7	2.4	3.9	2.9	3.1	2.3	0.8	2.9
Histidine	2.2	0.7	2.5	0.5	1.0	0.6	0.8	0.5	0.2	0.7
Serine	3.2	1.2	5.1	1.0	1.9	1.1	1.3	1.0	0.3	1.3
Glutamic acid	16.5	4.5	18.7	3.1	8.7	4.2	4.4	3.4	1.3	3.6
Proline	3.2	1.1	5.5	0.9	1.5	1.0	1.2	0.8	0.3	1.0
Glycine	3.3	1.0	4.2	0.8	1.5	1.1	1.1	0.8	0.3	0.9
Alanine	3.5	1.1	4.3	1.2	1.3	1.1	1.2	0.8	0.3	1.0
Lysine*	3.0	1.6	6.4	1.5	1.9	1.8	2.0	1.3	0.5	1.6
Threonine*	2.5	0.9	3.9	0.7	1.3	0.9	1.0	0.7	0.3	1.0
Valine*	3.8	1.1	4.8	1.0	1.5	1.2	1.4	0.8	0.3	1.2
Isoleucine*	3.8	1.0	4.5	0.8	1.6	1.0	1.2	0.8	0.3	1.0
Leucine*	6.8	1.8	7.8	1.7	2.7	1.8	2.0	1.4	0.6	1.9
Tyrosine*	3.2	0.8	3.1	0.6	1.4	0.7	0.8	0.5	0.2	0.7
Phenylalanine*	4.3	1.4	4.9	1.1	1.4	1.1	1.4	1.0	0.3	1.3
Tryptophan*	0.7	0.3	1.3	0.9	0.3	0.3	0.3	0.2	0.1	0.3
Cystine**	0.5	0.3	1.3	0.2	0.4	0.4	0.4	0.3	0.1	0.3
Methionine**	2.0	0.3	1.3	0.2	0.3	0.3	0.2	0.3	0.1	0.4

BGN: Bambara groundnut; CP: Cowpea; SB: Soybean; AB: Adzuki beans; LP: Lupin beans; LB: lima beans; LT: Lentils; CK: Chickpea; BB: Broad beans; KB: Kidney beans. (Olaleke, Olorunfemi and Akintayo 2006; Leonard 2012; N'Dri Yao *et al.* 2015; Faostat and Production 2016).

2.1.3 Functional properties

Legume proteins exhibit functional properties such as emulsification, gelling, solubility, oil and water absorption, and rheological behavior (Barac *et al.* 2015; Lam *et al.* 2018). The solubility profile of the protein determines its functional property as a food ingredient for industrial use (Kumar *et al.* 2022); hence, high solubility is a requirement for food applications. The protein extraction method (pH of the system, salt type, and level), source of legume protein, and hydrophobic regions that promote aggregation may impact the solubility of the protein (Barac *et al.* 2015; Shevkani *et al.* 2019).

Generally, at the isoelectric point, legume proteins are typically less soluble (pH 4.0 to 5.0) due to the lack of electrostatic repulsion between like-charged molecules (Zeece, 2020). Proteins

that are low in charge aggregate due to hydrogen bonds, hydrophobic interactions, and Van der Waals forces; once the size and quantity of aggregates are adequate, the protein precipitates. Proteins that are low in charge due to non-covalent forces; once the size and quantity of aggregates are adequate, the protein precipitates (Goldstein & Reifen, 2022). For example, solubility profile of native legume-derived proteins, including fava bean (Liu *et al.* 2019), faba bean (Eckert *et al.* 2019), chickpea (Huang *et al.* 2022), Bambara groundnut (Ruzengwe, Amonsou & Kudanga, 2023), soybean (Han *et al.* 2024), and lentil proteins (Vogelsang *et al.* 2023), was considerably diminished in comparison with the modified proteins. The functionality of legume protein can often be improved either by using physical, chemical, or enzymatic methods.

In a research carried out by Huang *et al.* (2022), improvement in the rheological properties, protein solubility, and thermal properties of high-pressure homogenized chickpea protein were improved relative to the native protein, which enhanced the food application of the legume protein. Similarly, the gel property of laccase-modified Bambara groundnut protein was enhanced in an enzyme dose-dependent manner (Ruzengwe, Amonsou & Kudanga, 2023), which can contribute to better texture of foods. The limitation in the functionality of unmodified legume proteins impedes their use in food applications and trade development in the food industry.

Emulsification is another critical property for adding proteins into food formulations. The stabilizing potential of proteins for emulsions is a key functional ability, determining their effectiveness in food products. Emulsion is a mixture of two separate liquid phases, where one liquid exists as tiny droplets distributed throughout the carrier phase of the other (Shevkani, Singh, Chen, Kaur & Yu, 2019). The ability of proteins to emulsify depends on the dissolution and hydrophobicity behaviour (Boye, Zare, and Pletch 2010; Lam *et al.* 2018). The stabilization mechanism of proteins involves adsorption at the phase boundary to reduce interfacial tension (Shevkani *et al.*, 2019; Lam *et al.*, 2018). Furthermore, variations in the emulsifying capacities of pulse proteins are often linked to differences in their composition and flexibility of the molecular (Yan *et al.* 2021). For example, lentil protein has been demonstrated to be a suitable emulsifier in salad dressing, baked foods, and as a stabilizer in encapsulating agents (Khazaei *et al.* 2019). Similarly, better emulsion stability was reported for legume flours such as chickpea, pea, soybean, lentil, and fava bean compared with cereal flours (wheat, barley, and oat), owing to their greater protein content (Stone *et al.* 2019).

2.1.4 Significance of legume protein in food applications

Legume proteins are increasingly used as food ingredients to meet the growing consumer demand in food systems. Vegetable proteins are used as alternatives in a range of food uses, such as replacing meat in meat analogs, using mung bean protein instead of eggs, and substituting soy or almond milk for cow's milk in products like vegan cheese (Mefleh *et al.* 2022; Wang *et al.* 2022; Guyony, Fayolle, and Jury 2023; Vashisht *et al.* 2024). The functional property of the legume grain proteins are key factors when designing protein-based food products. For example, to produce good quality beverages, the solubility and viscosity of protein are paramount (da Silva Matos *et al.* 2024); water-retention and lipid-binding characteristics are needed in dough formation and use of meat gel (Ren *et al.* 2022; Guo, Cui and Meng 2023). Emulsifying properties are important for products like coffee whiteners (e.g., soy creamer), while foaming properties are essential for items such as whipped toppings (Deotale *et al.* 2020).

Additionally, in bakery products like bread and pastries, legume proteins contribute to greater elasticity and higher protein levels. Protein powders offer a convenient means to increase protein consumption, while vegan and vegetarian foods like tempeh, plant-based sausages, and tofu serve people who survive on diets that are plant-based. Moreover, the incorporation of plant proteins into cereals and breakfast items is increasing to enhance their nutritional benefits (Santos, Pintado & da Silva, 2022; Shrestha *et al.*, 2024). The rising demand for sustainable, plant-based foods has driven widespread adoption of plant proteins, providing consumers with healthier and more ethical options. Moreover, the use of legume proteins in plant-based foods is generating new prospects for food manufacturing formulations. However, legume proteins have several drawbacks, such as low solubility, beany flavour, and potential allergenicity. These limitations have significantly restricted their use at an industrial level.

The protein quality of Bambara groundnut (*Vigna subterranea*) is significantly limited by its anti-nutritional factors, particularly phytic acid, tannins, oxalates, and protease inhibitors, which impair digestibility and amino acid availability (Mashau *et al.*, 2025). These compounds interact with proteins and digestive enzymes by forming insoluble complexes and inhibiting proteolytic activity, thereby reducing enzymatic hydrolysis and nutrient absorption. For instance, phytic acid chelates minerals and binds proteins, while tannins form indigestible

protein–polyphenol complexes that limit accessibility to digestive enzymes. Additionally, these anti-nutrients suppress the activity of digestive enzymes such as trypsin, further lowering protein utilization efficiency (Adebo et al., 2024). Consequently, raw Bambara groundnut typically exhibits moderate protein digestibility (~70%), which improves substantially (to >80%) when anti-nutritional factors are reduced through processing methods like dehulling, germination, or fermentation.

Protein quality in Bambara groundnut (*Vigna subterranea*) can be improved through several strategies that mitigate anti-nutritional factors. During pH fractionation, proteins are selectively precipitated at their isoelectric points, leaving polyphenols, tannins, and phytic acid in the supernatant, which reduces co-precipitation and enhances protein digestibility. Similarly, adjusting salt concentration alters protein solubility and disrupts protein–polyphenol interactions, weakening the binding of inhibitory compounds (Mashau et al., 2025). Controlled enzymatic hydrolysis further enhances protein availability by partially cleaving proteins into smaller peptides, decreasing binding sites for tannins and other polyphenols, degrading protease inhibitors, and improving enzyme accessibility and amino acid release without compromising overall nutritional value (Mashau et al., 2025). Additionally, atmospheric cold plasma (ACP) generates reactive oxygen and nitrogen species that oxidatively modify tannins, phytic acid, and protease inhibitors, reducing their ability to form insoluble complexes with proteins. This non-thermal treatment increases in vitro protein digestibility and mineral bioavailability in legumes, including Bambara groundnut, by 10–20% without significant protein denaturation (Li et al., 2024; Mashau et al., 2025). Overall, the presence of these anti-nutrients is a key limiting factor in the biological value of Bambara groundnut protein, although their effects can be mitigated by appropriate processing.

2.2 Protein Modification Techniques

Utilization of plant proteins in the food system is on the increase due to greater consumer or market acceptability and low cost compared to animal protein (Andreani *et al.* 2023). However, most plant proteins are seldom optimal for functional food application in their native forms, so there is a need for modification to obtain optimum functionality. There are various methods for modifying structural characteristics and enhancing the operational properties of legume proteins. Each approach exhibits advantages and limitations, determined by factors like the

protein source and profile, cost, operational parameters, feasibility, and intended application. Chemical, enzymatic, and physical techniques can be employed to modify proteins.

Chemical modifications involve interactions among proteins and chemical agents, causing the breaking of bonds that alter the native protein structure (Akharume, Aluko & Adedeji, 2021). Such proteins altered chemically are reported to offer better functional attributes than their native counterparts. Despite this, the production of harmful chemical residues, costs, consumer concerns, and compliance issues limit the commercialization of chemical modification techniques (Nasrabadi, Doost, and Mezzenga 2021). Consequently, chemical modification is a less commonly used technique, especially as consumers increasingly prefer foods free from artificial additives. Physical methods, including pulsed electric fields, ozone treatment, oscillatory magnetic fields, high pressure, ultraviolet radiation, and, in new developments, cold plasma technology is increasingly being adopted as alternatives to heat processing, driven by consumer demand for safe, wholesome, and additive-free foods (Nema *et al.* 2022). Many of the physical approaches can be applied at a commercial scale; however, a reassessment of the energy and cost-effectiveness is necessary to ensure that they achieve the target objectives of sustainability (Nasrabadi, Doost & Mezzenga 2021).

2.2.1 Protein extraction methods

Extraction methods significantly influence the structural composition of leguminous proteins (Yang *et al.* 2021a). Protein extraction can be difficult due to their sensitivity to the environment, which can cause changes in their structural properties in relation to the surrounding solvent (Gadalkar and Rathod 2020). Technologies, including dry and wet methods, are being developed to maximize extraction yield, ensure protein purity, retain functionality, and speed up the alkaline extraction process (Eze *et al.* 2022). Acid, salt, and alkaline solutions are commonly used in wet protein extraction, with each method presenting distinct benefits and limitations for protein solubility and quality (Hadidi, Aghababaei & McClements 2023). The ability of these methods to solubilize protein is determined by factors including flour characteristics, particle size, solvent ratio, extraction conditions (pH and temperature), and the ionic strength or salt present in the extractant (Hewage *et al.* 2024).

2.2.1.1 Impact of extraction conditions on the structure of proteins

Choosing the right variables for protein isolation is crucial in the manufacturing, as they can affect the final product's composition, technological properties, and amino acid profile. Protein recovery yield represents a crucial metric for evaluating the efficiency and practicality of an isolation method, which underscores the use of salt-assisted extraction to enhance protein solubilization and recovery. Salts such as NaCl have been reported to enhance protein yield when used in combination with pH fractionation (Langton et al., 2020). The addition of salt during extraction exploits the 'salting-in' and 'salting-out', which is typically accompanied by a desalting process, such as dialysis. The selection of salt type and concentration, or combinations thereof, depends on the target protein's salting-in behavior and the salting-out response of non-target proteins, as precipitation varies among proteins.

For example, Hu *et al.* (2017) compared the influence of pH and salt during walnut protein extraction using sodium sulfosuccinate (AOT) micelle-mediated methods, micelle-assisted enzymatic approach, and water-based isolation techniques. The study demonstrated that the extraction yield, and functional behaviour, like foaming, solubility, water retention or oil binding, and emulsifying capacities of the protein, were significantly influenced by the extraction methods. In an additional investigation by (Cui *et al.* 2020), four separate varieties, Agassiz, (A), Spider (S), Trapeze (T), and ND Trial(N) of pea protein isolate were obtained via alkaline-based extraction with isoelectric point precipitation, using the extraction pH 8.5, 9.0, 9.5 or 10.0. A higher legumin amount in cultivars T and N and lower percentages of free sulfhydryl but higher disulphide groups in cultivars A and S were observed. Varieties T and N exhibited a slightly higher proportion of α -helix structures, while cultivars A and S showed a greater β -turn content, indicating a more rigid and tightly folded globulin structure, as β -turns limit the conformational flexibility of the peptide chain (Marcelino & Gierasch, 2008). Additionally, the proteins displayed minimized surface tension, which promotes improved foaming capacity and stability.

Likewise, Yang *et al.* (2021a) explored 3 different extraction methods using ultrafiltration combined with dialysis, isoelectric precipitation after alkaline extraction, and, micelle-assisted extraction and precipitation for pea protein isolate. It was reported that only alkaline extraction with isoelectric point precipitation and micelle-assisted extraction and precipitation retained globulin, while ultrafiltration contained albumin. Pea proteins extracted using an alkaline solution exhibited a higher 11S/7S ratio with more impact on the protein conformation

(exposure of hydrophobic region) as opposed to those recovered through salt solution. The salt-dialysis method impeded protein unfolding and formed a coarse particulate structure, which produced weak gels. In contrast, the alkaline extraction–isoelectric precipitation approach partially denatured the proteins, exposing reactive groups that promoted aggregation during gelation, thereby hindering progressive unfolding and yielding a weak, loosely connected gel network. Previous studies refer to the use of pH fractionation and salt as micellization and it has been designed to reduce denaturation and preserve the functional attributes of the proteins. This is possible due to the ability of the extraction method to preserve the protein structure and improve the functional properties such as solubility, foaming capacity, emulsifying properties, and gelation properties, compared to other methods like isoelectric precipitation (Adebowale, Schwarzenbolz & Henle 2011).

Extraction above a certain pH has been highlighted to negatively alter the protein's structural integrity. For instance, Quinoa protein denatured more when the extraction pH exceeded 10 (Ruiz *et al.*, 2016). Moreover, using an alkaline pH above 10 or extending the extraction time increased protein denaturation, reducing its solubility and functionality (Bengoechea *et al.* 2017; Hou *et al.* 2017). Likewise, Miranda *et al.* (2022) gave a report on no changes in the SDS-PAGE and surface charge result of lentil protein using different extraction methods: alkaline extraction enhanced by enzymes and ultrasound. This result contradicts some previous studies where there was a significant effect of ultrasonic-assisted salt extraction affecting surface charge of plant sources through changes in protein secondary structure or amino acid content (Rahman and Lamsal 2021; Zhao *et al.* 2021b; Eze *et al.* 2022). The structural modification can cause pH-induced protonation of carboxyl and deprotonation of amino groups in proteins (Kore *et al.* 2022).

2.2.2 Limited enzymatic hydrolysis as protein modification tool

Degree of hydrolysis, DH, above 10% is termed intensive or extensive hydrolysis while DH below 10% is called limited or controlled hydrolysis (Rezvankehah *et al.* 2021). Extensive hydrolysis can enhance the role of proteins in nutrition and therapy, while controlled cleavage is effective in improving their techno-functional attributes (Eckert *et al.* 2019). When focusing on the superior functional properties of protein hydrolysates, extensive hydrolysis is not ideal, as it produces numerous small peptides that lack the necessary surface characteristics (Ding *et*

al. 2021). Hydrolysates from protein having the same degree of hydrolysis (DH) can exhibit different structures and functional characteristics, based on the protein substrate and the catalyst employed. Protein hydrolysates with the same DH can vary in structure and function depending on the substrate and enzyme (Shahbal *et al.* 2023).

2.2.2.1 Mechanism of limited hydrolysis

Mechanism of enzymatic hydrolysis starts with the breaking of the protein backbone bonds in the protein substrate, followed by the formation of new amino moieties. A new amino group is formed from each of the peptide cleaved, and there is a linear increase in the amino nitrogen using the newly formed amino groups (Ghosh, Prasad & Saha 2017). The hydrolysis process involves a peptidase-catalyzed nucleophile-driven bond formation by a water molecule involved in amide bond cleavage connecting the amine and carboxylic acid groups of two neighbouring amino acids. The liberated amino and carboxyl groups are in their protonated or dissociated forms, depending on the pH of the medium. The amino units are protonated (NH_3^+), and the carboxyl groups are not dissociated at low pH levels (below the carboxyl group pKa, which is 3.1 to 3.6).

Protons are used during hydrolysis at such pH levels. The medium's pH rises gradually as a result. The carboxyl groups are ionized as COO^- , and the amino units are uncharged when hydrolysis takes place at high pH (above the amino units pKa, which is 7.5 to 7.8). Because the amino groups (NH_2) are uncharged and the carboxyl groups (COO^-) are dissociated, protons are released, lowering the medium pH. The release and absorption of protons are almost equal at mid-level pH (pH 4 to 7), which keeps the medium pH relatively constant (Adler-Nissen 1979; Belitz, Grosch & Schieberle 2009).

2.2.2.2 Impact of limited hydrolysis on protein structure

Enzyme action can degrade or restructure proteins to produce the required functionality, depending on the functionality of relevance. The extent of enzymatic hydrolysis correlates with the degree of hydrolysis (DH), which indicates the proportion of peptide bonds split. It serves as a common measure of enzymatic hydrolysis. A universal way for calculating DH does not exist; instead, several techniques are widely applied, such as O-phthalaldialdehyde (OPA), amino

acid nitrogen, pH-stat, TNBS, measurement of soluble nitrogen after trichloroacetic acid precipitation (SN-TCA) and formol titration methods (Rutherford 2010).

Previous studies on limited enzymatic hydrolysis have confirmed the significance of DH in the structural modification of proteins. For instance, Liu *et al.* (2019) hydrolysed Fava bean protein isolate using alcalase to achieve DH 0, 4, 9, and 15. DH 4% produced suitable lower molecular weights which were not excessively shortened with a more flexible peptide structure. Higher degrees of hydrolysis (9% and 15%) excessively reduced the molecular weight of the protein (all below 15 kDa) and caused a pronounced drop in surface hydrophobicity coupled with an increased surface charge, which could negatively influence the emulsifying activity. A degree of hydrolysis of 4% proved most effective, providing an optimal molar mass for stable boundary layers, an elevated surface net charge to enhance electrostatic repulsion, and higher hydrophobicity for improved interface attachment. Limited hydrolysis was recommended by the authors to improve emulsifying property and achieve stability against oxidation in protein emulsion.

Similarly, the degree of hydrolysis of 6.5% with pea protein produced lower molecular weight peptides (< 15 kDa), which is similar to that of Liu *et al.* (2019) at DH 9 and 15. Although the fluorescence emission and surface hydrophobicity were both reduced for the two studies, at different DHs. Higher onset and denaturation temperatures were observed for the pea proteins (Chen and Campanella 2022). The two findings are consistent with the report of Xu *et al.* (2016) on rice protein hydrolysates; there was a reduction in the molecular weight, increased flexibility, and surface hydrophobicity for DH 0.5 and 2%, which later decreased at DH 6% and consequently increased the protein solubility.

Additionally, in a study conducted by Jin *et al.* (2020), a shift in tertiary conformation in molecular weight to < 30 kDa of dehulled walnut protein using trypsin was reported. DH 7.1 showed a higher surface hydrophobicity and intrinsic fluorescence intensity compared to DH 1 and 3. Shuai *et al.* (2022) studied how varying degrees of hydrolysis of 2, 4, 6, and 8% affected the structural and functional characteristics of pea protein, employing four different proteases, including flavourzyme, neutrase, alcalase, and trypsin. At the same degree of hydrolysis, the enzymes improved pea protein characteristics in the following order: trypsin > alcalase > neutrase > flavourzyme.

Trypsin-digested pea protein possessed finer particles, a molar mass, smaller sulfhydryl content, higher solubility, and intensified fluorescence, while the upward change in DH

improved the foaming and emulsification of the protein. This first caused an increase and then decreased, which later attained the optimum at 6% DH. Overall, the role of DH should not be overstated, which means that it is the structure of the resulting peptides, rather than the degree of hydrolysis, that dictates the functional properties of a protein hydrolysate.

2.2.2.3 Choice of enzyme

Choosing the right enzyme allows controlled substrate modification because of its specificity for certain cleavage sites. Enzymes with proteolytic activity cleave peptide bonds, yielding peptides of different lengths and free amino acids (Ningrum *et al.* 2023). Proteases are categorized as endopeptidases or exopeptidases according to where they act on the peptide chain and their mode of catalysis. The degree of hydrolysis largely relies on the choice of enzyme and the parameters of the process, such as time, temperature, and pH. The resulting fragments play a key role in peptide hydrophobicity, which in turn affects their techno-functional properties (Singhal *et al.*, 2016). Enzymes act by cleaving proteins at target areas.

For instance, trypsin cleaves basic residues (lysine, arginine), chymotrypsin targets aromatic residues (phenylalanine, tyrosine, tryptophan), and microbial transglutaminase (TG) catalyzes glutamine–lysine cross-linking (Ma *et al.* 2010; Gaspar and de Góes-Favoni 2015; Akharume, Aluko and Adedeji 2021). Trypsin catalyzes the hydrolysis of peptide bonds at the carboxyl side of lysine or arginine residues, breaking the bond between the COOH group of these amino acids and the adjacent NH₂ group (Fig. 2.2). Cleavage takes place more slowly when the basic residue is adjacent in sequence to proton-donating residue or cystine the reaction is completely blocked when the residue is followed by proline, as demonstrated in a study by Perutka & Šebela (2018) and does not occur at all when the residue is followed by proline. As demonstrated by Ding *et al.* (2021), trypsin is more peptide-specific than alcalase in that trypsin produced soy protein fragments that exhibited markedly better emulsifying properties compared to those generated by alcalase. The quality of emulsion produced from trypsin hydrolysates was caused by an efficient reduction in the size of soy protein aggregates, which fabricated soy protein fragments at DH 8.0% and assisted in forming fine emulsion droplets. Xu *et al.* (2020) investigated the influence of individual activity of alcalase and flavourzyme hydrolysis on chickpea protein isolate. Alcalase was reported to be more effective in terms of the DH (25.8%), reflecting cleavage of a higher total peptide linkages compared to its counterpart, Flavourzyme (11.9%), which is a function of the enzyme specificity for available catalytic sites. Alcalase, due to its broad catalytic specificity, effectively breaks down larger

peptides for extensive hydrolysis, whereas flavourzyme predominantly cleaves terminal peptide bonds (Fuentes, Richard & Chen 2021). Peptide bonds within the globulins were broken by alcalase, but flavourzyme cleavage effect was minimal, indicating that more flavourzyme-sensitive sites are embedded rather than exposed on the surface of the globulin structure.

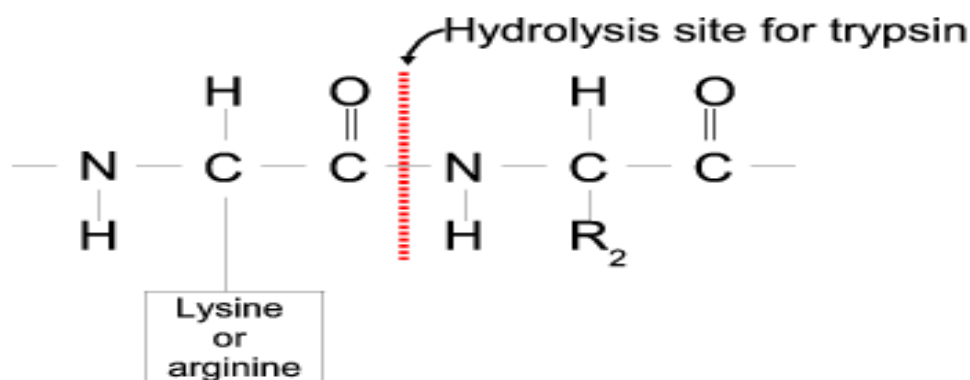


Figure 2.2. Enzymatic cleavage site of trypsin on peptide bond during hydrolysis. (Smyth 1967)

2.2.2.4 Impact of limited protein hydrolysis on its interaction with polysaccharides

Enzymatic modification has been proven to alter the structures of proteins, exposing more charged amino acid side chains and increasing the protein flexibility. The structural alterations following the hydrolysis of protein are expected to influence the protein-polysaccharides complexation. For example, Zhao *et al.* (2022) attributed a rise in solubility of the complex formed between soy cleaved peptides and gum arabic in the extent of hydrolysis-dependent manner to the partial loosening of the protein conformation. The flexibility of the structure increased the binding of soy protein hydrolysates to gum arabic and caused more GA-binding sites to become exposed. Compared with the emulsion of nonhydrolysed protein, the emulsifying activity and stability indices of emulsion produced from the hydrolyzed soy protein-gum arabic complexes were significantly higher which was due to the small particle size and emulsion droplet size and homogeneity. The zeta potential of the complexes formed with the hydrolysates was well above ± 30 mV which is considered a highly stable system. The surface of the soy protein hydrolysate–gum arabic complex was rich in negatively charged groups, and the resulting electrostatic repulsion enhanced emulsion stability. In contrast,

emulsions of unhydrolyzed soy protein with gum arabic contained numerous aggregates, the binding forces within the complex were relatively weak, and the overall emulsion stability was poor.

In another study, scallop *Patinopecten yessoensis* male gonad protein hydrolysates from trypsin were complexed with κ -carrageenan/xanthan gum, soluble complex started forming at pH 8.5, and the insoluble complex shifted from 4.2 to 2.8, and optimum complexation pH was between 2.4-1.4 in mixing ratio-dependent manner. The gel formed from the protein-polysaccharide hydrolysates complex exhibited higher gel strength in an acidic pH with a tightly packed structure and highly organized microstructures featuring smaller pore sizes and more uniform denser networks in acidic regions (Yan *et al.* 2022). Xu *et al.* (2022) used fungal protease 400 (F400) to partially hydrolyze soy protein isolate (HSPI) with a degree of hydrolysis from 1.5 to 5% and complexed with sodium alginate (SA). SDS-PAGE result of the hydrolysates showed that F400 selectively hydrolyzed 7S protein while preserving the basic subunit of the 11S protein. A degree of hydrolysis of 2% exhibited improved emulsifying capacity and stability, attributed to increased polypeptide flexibility and greater exposure of the proteins' hydrophobic regions. However, increasing the degree of hydrolysis from 2% to 5% resulted in a reduction of emulsifying activity. The turbidity profile showed an optimum pH being formed at a biopolymer mixing ratio (HSPI: SA) of 7:1, at pH 3.5, insoluble complex at pH 4.5, FTIR result confirmed interaction electrostatic $-\text{NH}^{3+}$ in hydrolyzed soy-derived protein isolate and $-\text{COO}^-$ in sodium alginate. Isothermal calorimetric analysis is used to elucidate the interactions, binding strength, and thermodynamic properties of the complex coacervate reactions (Kayitmazer 2017). The Isothermal calorimetric analysis result showed that the peak heights initially declined and subsequently stabilized suggesting progressive occupancy of HSPI binding sites. Additionally, certain points deviated between the integrated enthalpy changes and the baseline in the 45 °C titration curve, likely due to the higher reaction temperature promoting assembly of larger cohesive units during the complex reaction (Kaspchak, Mafra and Mafra 2018). The complexes produced were applied to the microencapsulation of sweet orange oil which presented a better heat resistance relative to the complex formed with unhydrolyzed soy protein-sodium alginate. However, the complexation of protein hydrolysates with polysaccharides offers a practical approach for forming a robust framework. Nevertheless, only a limited number of studies have addressed the complexation of hydrolyzed protein in combination with polysaccharides.

2.2.3 Physical modification

Physical approaches to food processing—high pressure, magnetic fields, UV radiation, ozone, pulsed electric fields, heat, and atmospheric cold plasma (ACP)—are increasingly used as alternatives to chemical modification to meet consumer demand for additive-free, safe, and whole foods (Nema *et al.* 2022). Heat-treating kidney, red bean, and mung bean protein isolates at 90 °C for 30 minutes markedly enhanced emulsion capacity, solubility, and surface hydrophobicity relative to untreated isolates (Karaca 2021). This is consistent with the results of Peng *et al.* (2016), who observed improved emulsifying, interfacial properties, and surface hydrophobicity after the thermal treatment of pea protein isolate compared to native pea protein. Chao and Aluko (2018) also reported conformational changes and increased surface hydrophobicity following treating pea protein isolate across a broader temperature range of 50–100 °C for 30 minutes. Emulsion capacity was improved at pH 7 resulting in smaller emulsified oil droplets than untreated pea protein. At pH 5.0, however, the average oil droplet size of heat-treated pea protein-stabilized emulsions was higher than that of native protein-stabilized emulsions. At the same time, the foaming capacity was not improved regardless of the pH. These outcomes were linked to the partial unfolding of protein structure due to protein denaturation which increased the charged residues on the protein surface and exposed the capacity, emulsifying activity, and gel strength. This result was associated to the observed alterations in hydrophobicity and particle size due to the ultrasonication treatment (Wang *et al.* 2020). Saricaoglu (2020) used high-pressure homogenization (25–150 MPa) to optimize the functional behavior of lentil protein isolate in a recent study. According to the author, increasing the pressure of 50–150 MPa disentangled the protein and increased the solubility, emulsification, and foaming behavior of lentil protein. However, untreated lentil protein formed weak gel-like structures. Therefore, in this study, the focus is on atmospheric cold plasma, a non-thermal modification method.

2.2.3.1 Mechanism of atmospheric cold plasma

Non-thermal plasma technology is an emerging technique applied directly or indirectly to food biomolecule surfaces for protein modification and sterilization (Akharume, Aluko & Adedeji, 2021). Plasma is a reactive gas containing ROS, including atomic oxygen (O), molecular oxygen (O₂), ozone (O₃), hydroxyl radicals (OH); reactive nitrogen species (RNS) like nitric oxide (NO) and nitrogen dioxide (NO₂); as well as ultraviolet (UV) radiation and free radicals,

and positive and negative charged ions (Tijani 2021; Wende and Brandenburg 2022). Non-thermal plasma is produced by applying energy to a neutral gas for ionization. The energy is stored as kinetic energy and internal excitation, imparting varying degrees of freedom to species (Jiang, Cheng & Sun 2020). Atmospheric cold plasma can be applied using three main approaches: remote exposure, direct exposure, and electrode contact. In the remote exposure method, food materials are positioned away from the plasma-generating source, allowing secondary reactive species produced from air and other gases to interact with the food. Direct exposure involves placing food materials directly within the plasma generation source, also known as active plasma, comprising both short- and long-lived reactive species. In the electrode contact approach, food is positioned on a plasma-generating electrode, where it is exposed to emitted chemical species and ion bombardment (Ganesan *et al.* 2021). For the effectiveness of the chosen method, the area of the target material in atmospheric cold plasma must be monitored, usually between ≤ 1 cm to 20 cm. Treatment time is another important factor, influenced by physical, chemical, and processing conditions such as surface topography, pH, texture, and the presence of active components in the food. Additionally, the temperature of the medium is maintained at a constant 35 °C, a feature particularly suitable for heat-sensitive foods. Other factors affecting the properties of the generated plasma include voltage, frequency, exposure mode, type of carrier gas, energy discharge source (electrical, thermal, optical, electromagnetic, etc.), and the type of electrode used (Akharume and Adediji, 2023).

2.2.3.2 Effect of atmospheric cold plasma application on protein structure and its interaction with polysaccharides

Atmospheric cold plasma has been regarded as one of the ways to physically modify proteins. Reports have shown that the reactive species can introduce polar functional groups on the surface of the proteins, thereby causing structural changes (Bu *et al.* 2023). Modification of Bambara protein using cold plasma is scanty, unlike other legume proteins such as mung bean protein, grass pea protein, kidney bean, and adzuki bean flour, which are extensively plasma modified (Rahman & Lamsal, 2023; Mehr & Koocheki, 2020; Wu *et al.* 2023).

For example, Alabi *et al.* (2023) explored the use of plasma-modified water to modify the molecular structure, physicochemical traits, and functional behavior of Bambara groundnut globulin. A significant shift from helical structure to β -turns was observed following cold plasma-activated water treatment, with a decline in the glutamic acid content. A slight

bathochromic shift in fluorescence intensity, which correlated with increased surface hydrophobicity suggesting protein structure unfolding, was reported. However, the authors observed there was a similar trend for the electrophoretic patterns, surface charge, and solubility profiles of Bambara groundnut globulin, with and without plasma treatment. The emulsifying ability of the protein was reduced while foaming stability was stable as high as 15 mg protein per mL. The indirect mode of plasma exposure in this study might be a contributing factor to the structural modification recorded in the globulin (Sruthi *et al.* 2022).

The main amino acid targets for reactive species are sulfur-containing and aromatic residues, followed by five-membered ring amino acids, and finally basic aliphatic amino acids, in decreasing order of reactivity (Zhou *et al.* 2016). According to (Dong *et al.* 2017), the covalent bonds in the protein molecules are broken down due to the intense plasma energy. This enables the reactive molecules to oxidize the side chains of sulphur-containing amino acids leading to cleavage of disulfide (S–S) bonds. The cleavage of S–S bonds may result in the formation of SH or SO groups, which can further disrupt protein conformation and cleave polypeptide chains. Additionally, oxidation can cause protein fragmentation, cross-link formation, unfolding, and other conformational changes (Segat *et al.* 2015). Free sulphydryl groups undergo reversible oxidation to sulphenic acid and irreversible oxidation to sulphinic acid and sulphonic acid.

Thiol group oxidation in cysteine and methionine induced by reactive oxygen species leads to the formation of disulfide bonds and changes them into cystine and methionine sulfoxide, respectively. The formation of a covalent intra or interprotein crosslink from the oxidation of cysteine through the formation of disulphide bridges is fundamental to the protein conformation. Cleavage of peptide bonds occurs within amino acid groups due to their exposure to reactive oxygen species from atmospheric cold plasma (Stadtman and Levine 2003). For example, alkoxyl radicals and alkyl peroxide derivatives of proteins undergo oxidation by the α -amidation or diamide pathways (Fig. 2.3), likewise, glutamyl and aspartyl amino acid residues when interacting with reactive species of atmospheric cold plasma (Fig. 2.4). Carbonylation is another oxidation-induced reaction that occurs when the side chains of proline, arginine, and lysine, threonine, tryptophan and histidine, are oxidized by reactive species (Surowsky, Bußler & Schlüter 2016).

Carbonylation leads to the introduction of reactive aldehyde or ketone groups in protein structure which are reliable markers of protein oxidation. In aromatic amino acids, oxidation

primarily occurs through interactions with reactive species such as hydroxyl ($\cdot\text{OH}$) or atomic oxygen radicals. Atomic oxygen in an excited state is effective at removing a hydrogen radical from the protein's backbone, creating radical sites that encourage further interaction that fragments the protein. (Misra *et al.* 2016). Tyrosine residue also undergoes crosslinking leading to the formation of 3,4, dihydroxyl (DOPA) derivatives and di-tyrosine crosslinked derivatives (fig. 2.5). A significant increase in α -helix was reported after atmospheric pressure plasma jet treatment for 8 mins for both air and N_2 at an input voltage of 75 V, the β -sheet content decreased after plasma treatment compared with untreated (Choi *et al.* 2017).

In contrast with the report of (Dong *et al.* 2017), following the DBD plasma exposure (60 V input voltage) of zein at 1.0 ± 0.2 A current for 4 min. The study found a lower content of β -sheet, while the α -helix and β -turn increased. The variations observed in the α -helix and β -sheet in the two studies might be due to variations in equipment and processing conditions. The plasma flow of atmospheric pressure plasma jet exerts a direct influence on a particular region, while dielectric barrier discharge cold atmospheric plasma affects a wider area. In addition, the distinction of reactive oxygen/nitrogen species induced by different feeding gases also generated different results. In addition, the reduction in the content of β -sheet may be attributed to conversion to β -turn, which is where the disulfide bonds are formed (Jiang, Cheng & Sun 2020).

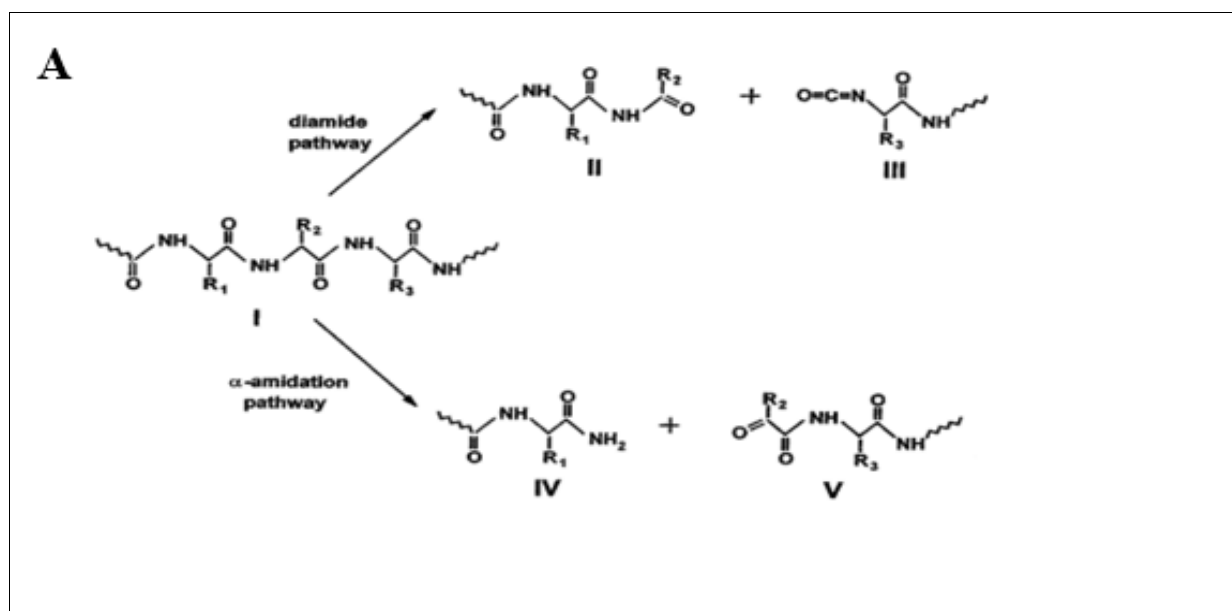


Figure 2.3. Free radical-mediated cleavage of by the diamide and α -amidation pathways. (Stadtman and Levine 2003).

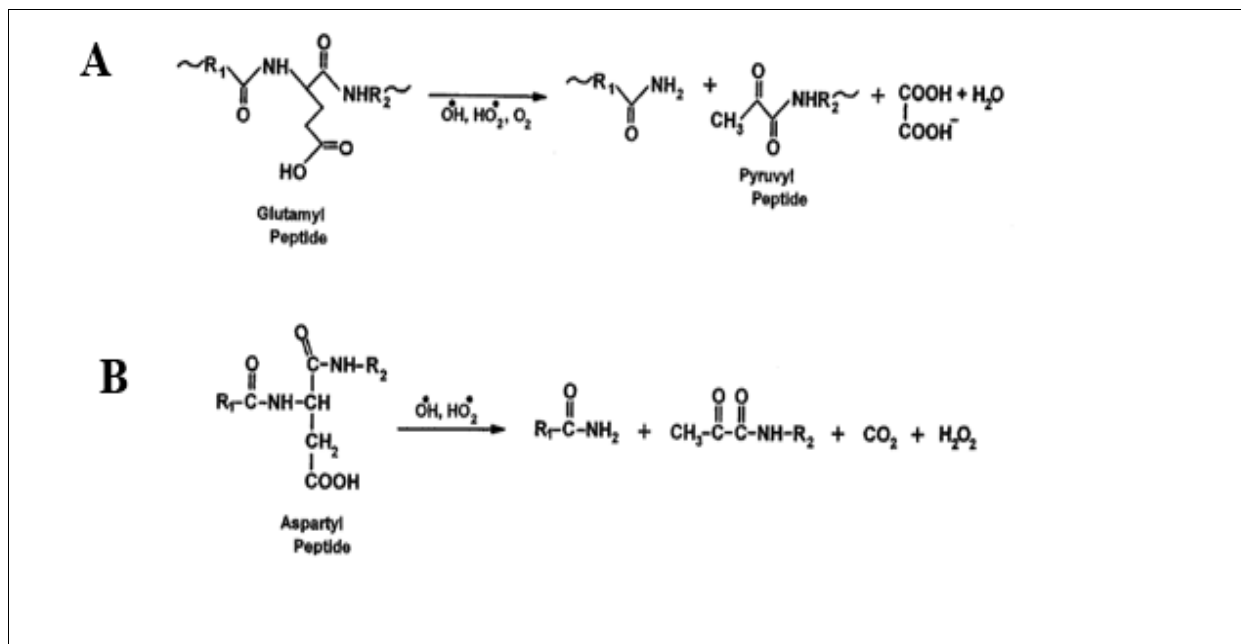


Figure 2.4 Free radical-mediated (A) oxidative cleavage of glutamyl residues and (B) cleavage through oxidation of aspartyl residue. (Stadtman and Levine 2003).

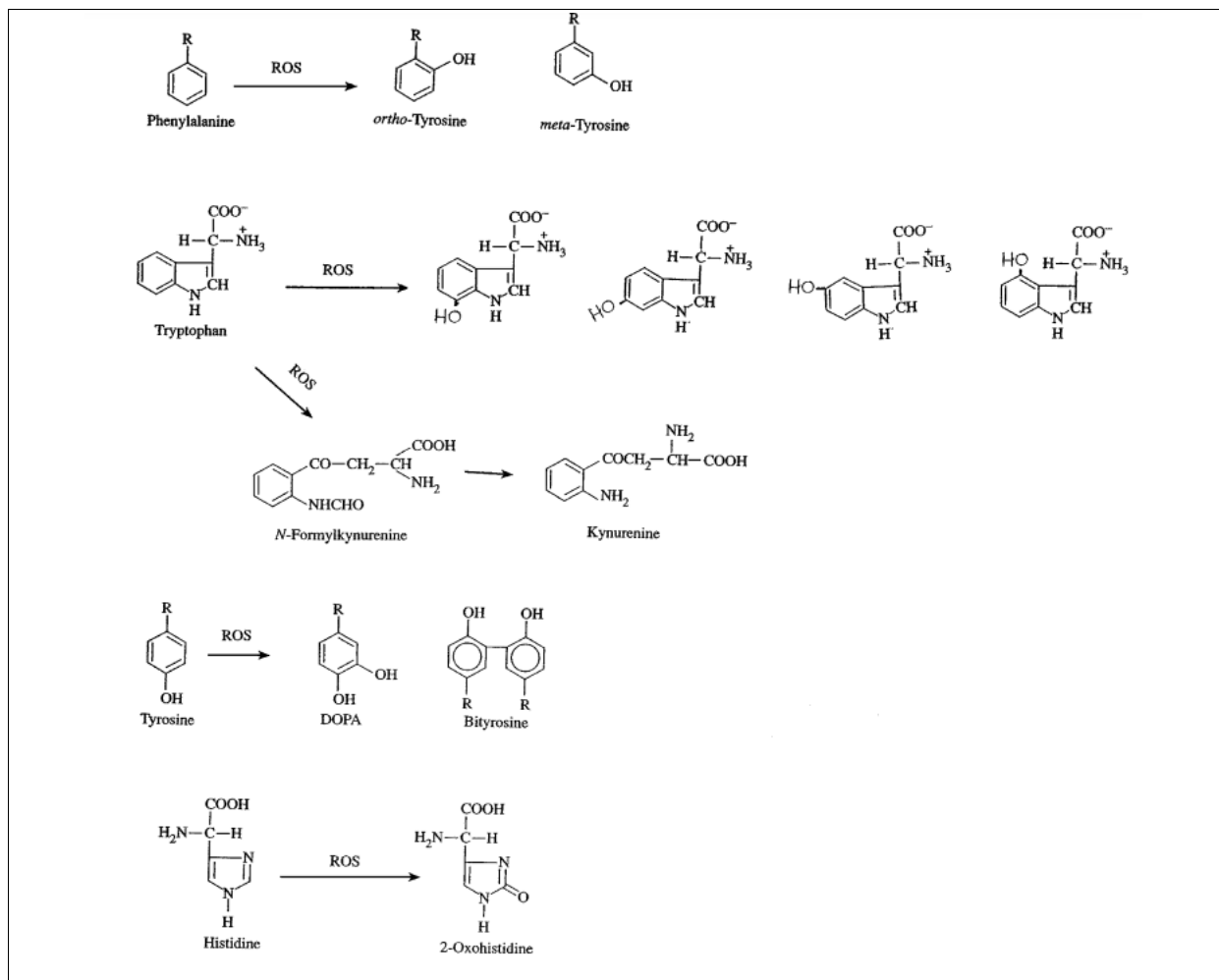


Figure 2.5 Oxidative modification of aromatic amino acids (Stadtman and Levine 2003).

2.2.3.3 Impact of atmospheric cold plasma on protein-polysaccharides complexation

Atmospheric cold plasma has been extensively applied to structurally alter proteins, which has immensely improved their functionality. However, there is scanty research work done to explore the impact of cold plasma on the complexation of protein with polysaccharides. Although some other physical modifications have been used to improve the interaction of protein with polysaccharides, such as ultrasonication. Similar to atmospheric cold plasma, power ultrasound is a non-thermal emerging technique used to effect structural modification of biopolymers. Multiple physical phenomena result from the bubble created during sonication imploding. These include strong shear stresses and high-speed collisions between particles, which cause disruption in both mechanism and chemistry resulting in the disruption of hydrogen bonds, disulphide bonds, and the spatial organization of polysaccharides, which modifies the secondary and tertiary conformations of proteins (Thirunavookarasu, Kumar, and Rawson 2022).

For instance, (Chen *et al.* 2019) stated that the treatment of whey protein–gum arabic complexes with ultrasonic strengthens the interaction and shortens the formation time likewise Zhao *et al.* (2021) observed an increase in binding affinity of the protein and polysaccharides during complex formation, which decreases the time of formation. This result was attributed to the cavitation created during ultrasound treatment, which exposed more amino acids in proteins and carbonyl groups in polysaccharides (Zhao *et al.* 2021b). The equilibrium among electrostatic, hydrogen bonding, and hydrophobic forces generates a dense interfacial layer that inhibits coalescence, and this is the mechanism responsible for protein–polysaccharide interaction. Protein–polysaccharide complex stability and formation are influenced by several factors, including pH, ionic strength, polymer type, molecular weight, medium temperature, and related charge (Ghosh and Bandyopadhyay 2012).

Ultrasonication treatment of protein-polysaccharide complex has led to the promotion of a structural network through various bonds between proteins and polysaccharides, which contributed to the superior functional behaviour of the complexes. Following the use of ultrasonic treatment (> 300 W), the complex of whey protein-gum acacia showed higher hydrophilic groups, resulting in enhanced solubility relative to native proteins and

polysaccharides. There was a decline in surface tension of the protein-polysaccharide emulsion and particle size and an increase in thermal stability, antimicrobial, antioxidant, and storage stability of the complexes (Chen *et al.* 2019). The result was due to the cavitation caused by high-intensity ultrasound treatment, which induced breaking of bonds and depolymerization of the molecules, increasing their oil and water solubility of the complexes (Wang *et al.* 2016).

Furthermore, Xiong *et al.* (2019) reported increased shelf-life stability and decreased surface tension of the emulsion produced from myofibrillar protein xanthan gum complexes following the ultrasonic treatment. The droplet size and apparent viscosity of the emulsions were reduced, which altered the flow behaviour of the emulsion. Circular dichroism demonstrated a decline in α -helix and an increase in β -sheets content of the ovalbumin xylose complex, which was directly proportional to the ultrasound treatment time (Fu *et al.* 2019), while FIIR spectra of soy protein-lentinan complex inferred a reduction and generation of some new functional groups during the complexation process (Wen *et al.* 2020). The interaction between the NH_2 groups of amino acids and polysaccharides leads to a reduction in free NH_2 groups and the formation of new functional compounds, such as Amadori products and Schiff bases, was reported (Matsumura and Rajan 2021). However, Albano and Nicoletti (2018) reported no impact of ultrasound treatment on the electrostatic interactions and resulting functional groups in whey protein concentrate and pectin complex. Similar to the report of Xiong *et al.* (2019) on ultrasound-modified myofibrillar protein–xanthan gum complex, no significant difference in the functional groups present in the complexes in comparison to untreated samples.

Additionally, ultrasound-processed whey protein–gum arabic complex triggered molecular unfolding and exposure of tryptophan of the protein molecule, leading to a transition in λ_{max} to higher values, contrary to the report of (Ma *et al.* 2019) on soy protein-citrus pectin complex which had no significant difference in the λ_{max} . Therefore, the implication is that there are several factors responsible for the structural modification of protein-polysaccharides complexes using ultrasonication which are but not limited to the energy delivered by the applied technique and the protein classification (Thirunavookarasu, Kumar & Rawson 2022).

2.3 Protein-polysaccharide complexation

Electrostatic interactions between acidic and basic residues, macroions along the macromolecular chains, and other side groups result in complexation between two

biopolymers. The parameters that govern the strength of the interaction are the net opposite charge, the form, size, and flexibility of the macroions, and the reactivity of the side chain groups (peptide-bound amino acids and acidic sugar units in polysaccharides). Molecular interactions between protein and polysaccharides bring about a unique structure, forming soluble and insoluble complexes with novel functionalities that are useful in various food applications. The interactions involve non-covalent linkages, including hydrophobic, electrostatic, van der Waals, and hydrogen linkages.

2.3.1 Mechanism of protein-polysaccharide complexation

The electrostatic interaction follows two structure-forming events of initial formation of soluble complex at pH_C with an increase in turbidity during acid titration (Paliya *et al.* 2023). The mechanism of soluble complex formation involves initial contact and adsorption of the protein to the polysaccharide through random collisions facilitated by Brownian motion. To optimize interactions, the protein, and polysaccharide may undergo further conformational changes. Proteins can partially unfold to reveal more binding sites, while polysaccharides can stretch to enhance contact points (Weiss *et al.* 2019; Clement and Jim 2021). The soluble complex forms when the binding interactions linking the protein and polysaccharide are balanced with the solubility of the complex in the aqueous environment which is reflected by a slight rise in turbidity (fig. 2.6). Then, followed by the subsequent formation of an insoluble complex at $\text{pH}_{\phi 1}$ corresponding to a swift rise in turbidity and the transition from transparency to turbidity. Optimum complex coacervation is achieved when the biopolymers attain electrostatic equivalence, maximizing charge neutralization and phase separation (pH_{opt}) and then finally dissolution of the complex ($\text{pH}_{\phi 2}$) as a result of protonation of carboxyl group of polysaccharide backbone (Warnakulasuriya and Nickerson 2018; Carpentier *et al.* 2022). The net charge of anionic polysaccharides diminishes during complexation due to the increasing attachment of positively charged protein groups and the resultant complex solubility and hydrophilicity decrease as a direct result. It also reduces the quantity of positively charged groups accessible, lowering the protein isoelectric point (pI) (Bushra *et al.* 2023).

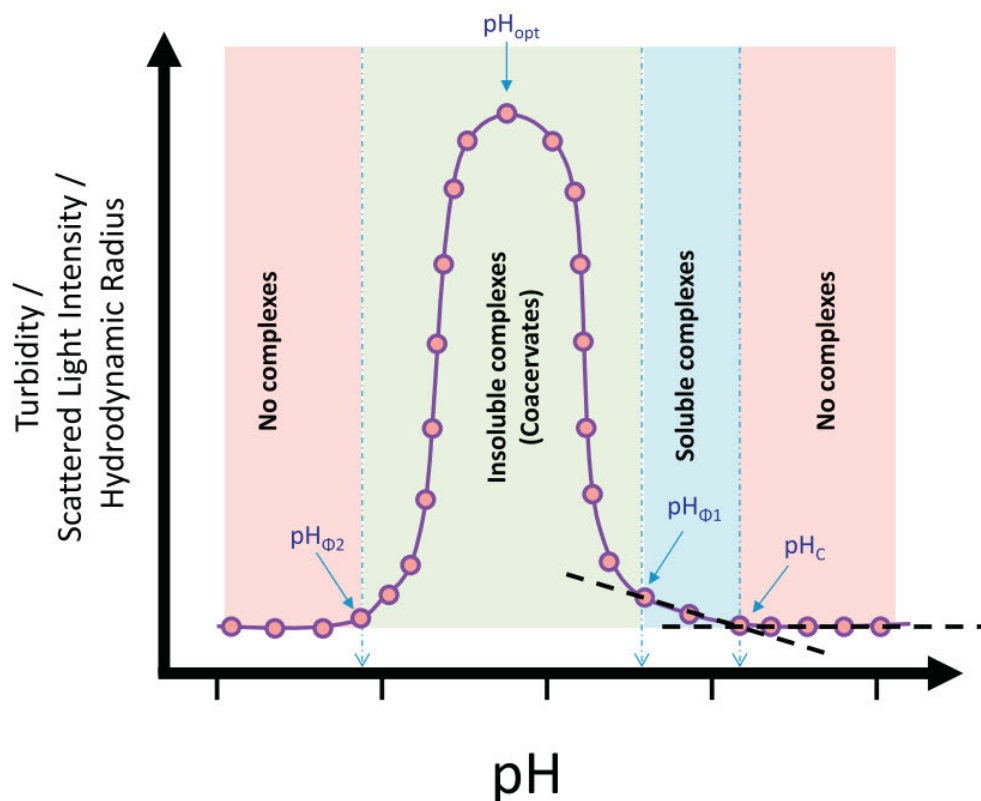


Figure 2.6. Illustration of the turbidity profile highlighting important pH transitions (Moschakis and Biliaderis 2017).

2.2.3.2. Protein-polysaccharides interactions

The importance of protein-polysaccharide interactions in food systems cannot be overemphasized owing to their capacity to control the structure, texture, shelf life, sensory and digestive properties of many food products (Quiroga Ledezma, 2018). Recent research has revealed that protein-polysaccharide complexes have more promising applications than single components in food systems, owing to protein-polysaccharide interactions (Xue and Luo 2023). Foods with emerging textures or optimized sensory properties can be created by utilizing non-covalent interactions between proteins and polysaccharides (Zhang *et al.* 2024).

Non-covalent binding is the dominant binding between proteins and polysaccharides, which includes ionic interactions, electrostatic forces, van der Waals force, hydrogen bonding, and hydrophobic interactions (Lin *et al.* 2024). The interaction can be influenced by a lot of factors, such as pH, ionic strength, temperature, nature of the biopolymers, and mass ratio (Li *et al.* 2022).

Electrostatic interactions are predominantly mediated between anionic moieties on polysaccharides and cationic residues on proteins, driving complex formation and stabilization (Cortez-Trejo *et al.* 2022). The surface charge of a protein depends on its isoelectric point and the solution pH, while the presence of salts can further influence the net charge of the protein (Li, Wang, Hu, Wu & Meeren, 2022). Sun *et al.* (2016) demonstrated electrostatics was the dominant interaction between modified starches and casein, although both hydrogen bonding and electrostatic interaction were affected by steric hindrances.

Hydrogen bonding occurs due to the attraction between hydrogen atoms and electron-rich electronegative atoms (Karas, Wu, Das & Wu, 2020). In a protein-polysaccharide complex, the prevalence of —OH groups in polysaccharides makes hydrogen bonds the dominant hydrophilic interactions (Lopez-Bar *et al.* 2017). These hydroxyl groups form interactions with polar amino acid side chains as well as with the amide and carbonyl groups along the protein backbone (Fernandez-Alonso *et al.* 2012; Lopez-Bar *et al.* 2017). According to Lopez-Bar *et al.* (2018), structural analysis of extruded samples using Fourier-transform infrared spectroscopy showed an improved hydrogen bonding formation between hydrolyzed pea protein and wheat starch. This was attributed to the hydrolyzed pea protein containing more exposed carboxyl groups, which increased the propensity to establish hydrogen bonds with wheat starch. Hydrogen bonds may cause the complex conformation to become densely packed and permit surface-bound water to be replaced by intra- and intermolecular hydrogen bonds. This results in stalled domains that restrict structural rearrangement and cause protein-polysaccharide complex particles to act like a solid (Sayed *et al.* 2023).

In addition, there can also be hydrophobic interactions, whereby hydrophobic residues inside native proteins could bind to polysaccharide granules (Aryee *et al.* 2018).

Negatively charged polysaccharides may engage in ionic interactions with amino acid residues; however, research on these interactions remains scarce (Majumdar, Sen, & Ray, 2019).

According to (Liu *et al.* 2023), the complexation process is a result of the interplay of entropic and enthalpic forces and is dependent on environmental and structural factors such as pH, charge density, temperature, ionic strength, polymer type, and mixing ratio.

2.2.3.3. Factors influencing protein-polysaccharide interactions

2.2.3.3.1. pH

Proper pH control is essential to maintain opposite charges on proteins and polysaccharides, facilitating the formation of stable complexes via electrostatic interactions (Cortés-Morales, Mendez-Montealvo and Velazquez 2021). pH influenced protein–polysaccharide interactions by modulating the ionization of functional side chains, including amino and carboxylic groups. (Behrouzain, Razavi & Joyner, 2020). Proteins, as naturally occurring biopolymers, exhibit amphoteric charge behavior, typically with an isoelectric point around 5.0 (Samrot *et al.* 2020). Electrostatic attraction is almost impossible when an anionic polysaccharide and a protein are combined at a pH above the protein isoelectric point, pI, as both acquire a negative net. As the pH falls below the protein's pI, electrostatic forces intensify, promoting the formation of polysaccharide–protein complexes. The type of polysaccharide and protein, along with their functional groups, determines the characteristics of the resulting complex, making it highly system-specific.

Each protein–polysaccharide system has a pH at which the opposing charges of the two polymers are balanced and at their maximum. This specific pH is typically identified as the electrical equivalence pH (EEP) (Raoufi *et al.* 2020). At EEP, the electrostatic interactions are strongest, leading to maximal complex formation and an increased volume of the coacervate phase (Muhoza *et al.* 2022). EEP can be determined by measuring the ζ -potentials of each biopolymer over a broad pH range and finding the pH where their ζ -potentials are equal and oppositely charged (Schmitt *et al.* 2009).

2.6.3.2. Ionic strength

The salt type and concentration (e.g., Na^+ , K^+ , Ca^{2+} , Mg^{2+}) play a significant role in shaping the structures of protein–polysaccharide complexes driven by electrostatic attraction, as salts can shield charged sites of both protein and polysaccharide molecules and disrupt electrostatic attractions between them (Lin and Miao 2021). Interaction with microions reduces the net charge carried by proteins and polysaccharides, resulting in a decrease in electrostatic attraction between macromolecules at high salt concentrations. In addition, high salt concentrations lead to charge screening of proteins and polysaccharides, thereby weakening the electrostatic interactions between the biopolymers and reducing the formation and stability of soluble complexes (Cortez-Trejo *et al.* 2022). The effect of microion concentration on protein–polysaccharide complexes is limited when the ionic strength is low.

Proteins and polysaccharides carry enough charges to enable electrostatic interactions, but added salt changes the dielectric properties of the solution, negatively affecting complex formation. Compared to monovalent cations, divalent and multivalent cations more efficiently reduce electrostatic interactions, enabling inhibition of polysaccharide–protein complexation at lower ionic strengths. However, salt-induced suppression can be mitigated by diluting the solution, since the resulting lower ionic strength promotes the reformation of protein–polysaccharide complexes (Schmitt *et al.* 2009).

2.6.3.3. Charge density

Biopolymer charge density, representing the number of charges or reactive sites per unit surface length, plays a crucial role in governing electrostatic interactions during coacervation (Jin *et al.* 2020). There have been some interesting findings about the influence of the charge density of biopolymers. The local electrostatic density of proteins is extremely reliant on pH; proteins exhibit a net negative charge at pH levels above their isoelectric point (pI) and a net positive charge below the pI. Positively charged regions on the protein surface are known as charge patches (Li *et al.* 2022).

In a study conducted by Zheng, Van der Meeren, and Sun (2024), a larger positively surface-charged patch on the protein surface enhances its binding affinity to flexible polyanions compared to proteins possessing several smaller patches. Similarly, Pak *et al.* (2016) demonstrated that the charge per unit length/area of the polymers involved in complexation determines the magnitude of electrostatic interactions and the composition of the resulting complexes. Strong polyelectrolytes typically form precipitates with proteins, whereas weakly charged polyelectrolytes such as gum arabic produce coacervates (Roy 2024). Further, flexible proteins (e.g., gelatin, casein) can strongly bind to polysaccharides compared to globular proteins, since they have increased conformational flexibility, which enables them to associate with reactive polysaccharide sites during coacervation. Consequently, the interactions between polyanions are strongly influenced by their charge density and molecular rigidity (Mella and Tagliabue 2022).

Moreover, the conformation of proteins can change with pH, as demonstrated by Rani, Sogi, and Gill (2021), who found that α -gliadin exhibited a mono-dispersed random coil structure under varying pH conditions. Solvent pH affects the ionization of polysaccharide chains

according to the pKa of their reactive sites. At extremely low pH, dissociation is suppressed, and further complexation does not occur (Li *et al.* 2024). Conversely, low-charge-density polysaccharides tend to interact with proteins only when the proteins are in a more charged, ordered helical conformation rather than in a less charged, disordered random coil state (Roy 2024).

2.3.2 Choice of polysaccharides

Polysaccharides are composed of monosaccharide units connected by diverse glycosidic bonds, which give rise to a variety of structures and functional properties (Chaudhary, Jain, and Jaiswar 2022). The behavior of polysaccharides in solution is influenced by their structure and charge density, allowing them to act as rigid, highly charged rods or as flexible, less ordered coils (Wang *et al.* 2021). The extent of branching in polysaccharides can generate steric hindrance, preventing proteins from accessing reactive sites and forming strong intermolecular interactions or stable complexes (Luo *et al.* 2021).

For example, the structural features of pectins, such as blockiness and methyl esterification, influence their charge distribution; highly blocky pectins carry greater local charge densities than those with random methyl ester patterns (Archut, Drusch, and Kastner 2022). Thus, pectins exhibiting higher local charge densities tend to form complexes more effectively with proteins, owing to their greater binding affinity relative to less charged pectins. However, polysaccharides with very high charge densities, such as chitosan and carrageenan, tend to promote precipitate formation. Additionally, factors like the degree of branching and molecular weight of polysaccharides can influence their distribution and continuity within these mixed systems (Ćirić *et al.* 2020). Polysaccharides with higher branching and lower molecular weight generally disperse more than those with fewer branched chains and higher molecular weight, which are easy to agglutinate and form a continuous and heterogeneous structure (Su *et al.* 2021). In addition, polysaccharides at low concentrations can increase the density of protein-polysaccharide clusters, while high concentrations of polysaccharides can disrupt the protein network, as intertwining two separate networks into a rigid structure becomes difficult (Yang *et al.* 2021b). Among several polysaccharides extensively employed in complex formation are gum arabic, chitosan, cellulose, pectin, carrageenan, guar gum, tragacanth gum, and xanthan gum (Carpentier *et al.* 2021; Cortés-Morales, Mendez-Montealvo, and Velazquez 2021; Sarraf,

Naji-Tabasi and Beig-babaei 2021). Gum arabic is extensively utilized in the food industry for its distinctive properties, including low viscosity.

2.3.2.1 Gum arabic structure and application

Gum arabic is a natural polymer and weak electrolyte with a low amount of surface charge. Gum arabic is a complex anionic water-soluble polysaccharide, an exudate of *Senegal acacia* trees, made up of 1,3-linked β -D-galactopyranosyl units, with side chains attached to the main chain through 1,6 linkages (fig 2.7). Acacia gum may be divided into three primary components using hydrophobic interaction chromatography: arabinogalactan-peptide (AGp), arabinogalactan-protein (AGP), and glycoproteins (GP) (Rhazi *et al.* 2020). AGP features a coiled structure capable of penetrating the lipid phase at many places, allowing for optimal interface anchoring (Han, Gu *et al.* 2019; Han, Hu *et al.* 2019). Higher AGP content demonstrated greater emulsifying capacity to some extent, and higher AGP content was closely linked to the interfacial characteristics of GA (Sánchez-Reséndiz *et al.* 2018). Gum arabic has some desirable properties such as low viscosity, edibility, low cost, generally regarded as safe status (GRAS), good binding, thickening properties, and most importantly, an excellent emulsifying property. Based on these properties, gum arabic has found applications in food formulations such as ice creams, jellies, candies, beverages, syrups, and chewing gums. Previous studies have reported gum arabic as a flavour encapsulator and additive for stabilizing citrus oil emulsions in soft drink formulations (Schultz 2021). In addition, the combination of polysaccharides with legume grain protein in complexation has been previously reported to enhance the functional properties of protein-polysaccharide coacervates.

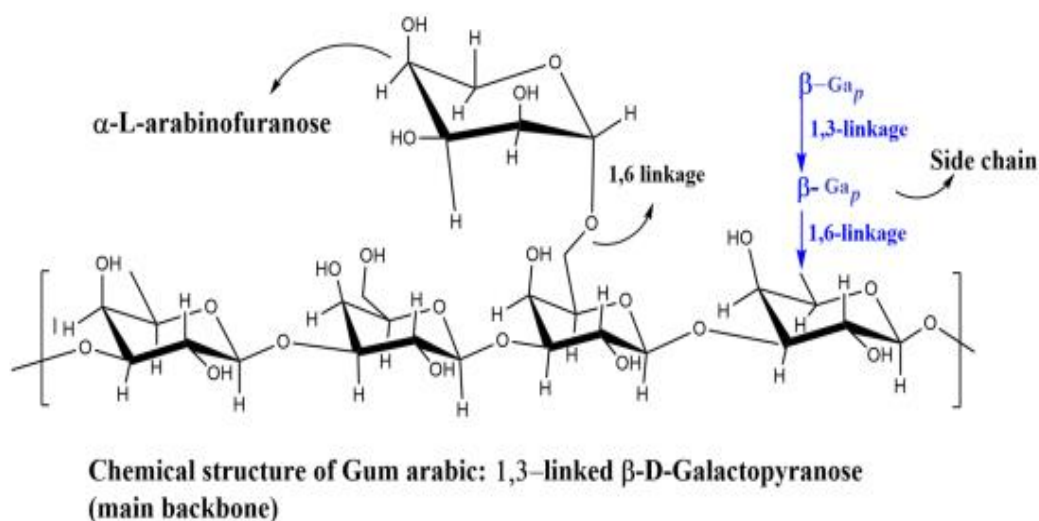


Figure 2.7. Chemical structure of gum arabic (Mate and Mishra 2020).

2.3.2.2 Encapsulation of bioactive compounds

Complexation has been proven to efficiently encapsulate and protect sensitive flavour and bioactive molecules against degradation from processing conditions such as shear, heat, light, moisture, oxygen, and temperature. For instance, Fan *et al.* (2021) explored soy protein isolate-fucoidan complex to successfully encapsulate curcumin which improved long-term storage stability and controlled the release rate of curcumin in simulated gastrointestinal fluids. The dense core-shell structure that was formed under pH modulation from 7 to 4 contributed to stability. The synergistic effect of the biopolymers on the curcumin provided a stable delivery system for the bioactive compound. Creating efficient bioactive delivery mechanisms, or microencapsulation vehicles, without compromising the sensory quality or shelf life of the product is the major problem facing food manufacturers. In another study conducted by Ha (2022), α -tocopherol was microencapsulated using complex from isolates from pea protein and gum tragacanth. The complex formed provided gastrointestinal protection for the active ingredient at a biopolymer ratio of 1:1 by producing microcapsules with stability against pH. This property was due to the granule size within liquid suspensions and microcapsules, encapsulation capacity, and profile of active release. The increase in polysaccharide ratio is

reflected in the increase in the droplet size of the formation of aggregates through electrostatic connections. Unlike the complex with tara gum, no change in droplet size was detected, indicating that no complex was formed due to the absence of electrostatic interactions.

In another study (Jin *et al.* 2016), heat-treated/ alcalase hydrolyzed soy protein-dextran glycosides nanogel was explored to encapsulate riboflavin following sonication. The structural stability of the carrier complex was supported by the weak intermolecular forces, causing high encapsulation efficiency of the riboflavin.

2.3.2.3 Fat replacement

Recently, research has focused on low-fat foods rich in mono- and polyunsaturated fatty acids without trans fats by employing protein-polysaccharide complexes as fat replacers (Hong *et al.* 2023). Fat replacement in foods has been demonstrated by Huang *et al.* (2022) who were able to produce gel from the complex of soy protein isolate and konjac glucomannan which was identical to the original pork following 20 min boiling. The lubrication and swallowing feeling of natural fat was replicated when the protein-to-polysaccharide ratio was 1:4 likewise the frictional behaviour and texture. In another study conducted by de Souza Paglarini, Martini, and Pollonio (2019), inulin, κ - and ι -carrageenan- soy protein isolate was used to successfully decrease overall pork fat content and enhance the fibre content while maintaining the original texture, rheology, and colour with a better fatty acid profile having reduced saturated fat content and increased unsaturated fat content. Another food product generally loved by both adults and children is ice cream rich in in saturated fatty acids (Clarke and Cox 2024). (Sun *et al.* 2015) were able to form a gel using soy protein isolate-cellulose nanofibers by cold induction which was similar to cream by substituting 10% of the cream to achieve low-fat and calorie food products.

Composite gel made from pea protein-agar gum complex has effectively replaced animal fat by lowering fat levels while increasing the water content, protein, and dietary fiber content of the minced meat blend and improving the temperature at which oxidation begins (Guo *et al.* 2018). In addition to fat substitutes, various types of oily meals emulsions using protein and cellulose as stabilisers have also been formulated. These include oil-based powders (Zhang *et al.* 2022), food-grade foam and oleogel (Zhang *et al.* 2020a; Urbánková *et al.* 2021).

2.3.2.4 Emulsion stabilizers

Moreover, particle-stabilised emulsions (pickering emulsions) employing proteins and polysaccharides as natural biopolymers have also become a focus of growing attention in recent years. This has enabled the application of edible colloidal particles to maintain emulsion stability, making it possible to use biodegradable, sustainable, and label-friendly biopolymer particles that are acceptable as food-grade materials (Rayner *et al.* 2014). For instance, three types of starch granules, including dissolved and non-solvent-precipitated were complexed with octenyl succinic anhydride to explore the stability of pickering emulsions. The size and stability of the emulsion droplets were affected by the different forms and sizes of emulsifiers made from waxy maize. The emulsions were able to maintain their stability even after a year of storage because the non-solvent precipitates provided the best emulsification characteristics determined by droplet size and microstructural distribution, and stability (Saari *et al.* 2019). Proteins in their native state have also been employed as colloidal particle-stabilised particles in oil-dispersed in water high internal phase colloidal systems. According to recent research by Xu *et al.* (2018b), native ovalbumin, with a small particle size (nanometres), demonstrated a superior particle-based stabiliser for high internal phase emulsions stabilised in a water matrix due to its great stability within the molecular structure, which guarantees that the material will remain particulate during interfacial adsorption. These pickering emulsions have reduced volatile oil vaporization, exceptional stability against heating and long-term storage, and a distinct responsiveness to freeze-thaw cycles. However, to enhance the emulsifying capacity of a protein-polysaccharides complex, certain native protein modification is required. According to Xu, Tang, and Binks (2020), glycated bovine serum albumin-galactose mixture had a significantly better capacity for refolding and a far stronger structural integrity. Comparing native bovine serum albumin to high internal phase emulsions in terms of emulsification and stabilization, the authors demonstrated improved results. The underlying mechanism for enhancing emulsification efficiency in glycated bovine serum albumin results in the formation of a nanostructure with a core enclosed by a hydrophilic galactose layer, with a hydrophilic galactose shell encircling the interior part of protein. The self-supporting gel network exhibited by these high internal phase emulsions helps to improve stability during prolonged storage, resilience to extreme heat, and freeze-thaw reversibility.

2.3.3 Challenges in the use of native protein in complexation with polysaccharides

The growing interest in the utilization of legume proteins as a multifunctional substitute for proteins from animal sources for food applications is well justified. However, there are challenges arising from the use of native proteins in complexation with polysaccharides. For instance, generally, optimum complexation usually forms within a narrow pH range (4.0–4.3) near the protein's isoelectric point, solubility and emulsifying properties are poor within a narrow pH range (4.0–4.3) close to the iso-electric point of protein where some functional properties such as solubility and emulsifying ability of the protein are poor (Mession *et al.* 2012; Kaur *et al.* 2023). Consequently, this narrow pH range has limited the application of complexation in certain foods and the release of bioactive compounds owing to increased instability in gastric conditions.

In addition, the formation of complexes within a narrow pH range is characterized by weak interactions due to the compact globular structure and molecular rigidity of the native proteins. Consequently, this restricts the number of oppositely charged groups on the globular proteins that can interact with polysaccharides and limits the applications of complexes in certain foods, particularly in acidic beverages. Hence, further modifications to enhance the flexibility of the protein and the functionality of protein are highly required.

2.4 Conclusion

The addition of polysaccharides to protein has been shown to improve food systems with a unique and improved property than individual biopolymers. The protein-polysaccharide complexes can be controlled by adjusting environmental factors to tailor the textural properties and techno-functional characteristics. However, despite numerous studies on protein–polysaccharide complexes for food applications, several challenges and knowledge gaps persist regarding the critical structure formations of complexes during complexation as a result of the dense structure of native globular proteins, limiting the molecular interactions between the biopolymers and consequently the food applications. Therefore, a research study focusing on the globular protein modifications and how the modification protocols impact the interactions between modified proteins and polysaccharides, forming soluble and insoluble complexes, is

imperative. The findings from this study will create opportunities to expand the food applications of the biopolymer complexes in the food industry.

2.5 Hypothesis

- a. pH fractionation and NaCl concentration as an extraction protocol of protein could alter the structure and modify the protein which can consequently influence the interaction with gum arabic. In a study conducted by Cui *et al.* (2020), significant changes were reported in the protein compositions of four cultivars of yellow pea protein, low content of legumin and higher vicilin in two cultivars, while less β -Turn and more aggregates were observed in the other two cultivars, which consequently improved the protein solubility. This can influence the protein interaction with polysaccharides.
- b. Limited hydrolysis (DH 2, 5, and 7.5) with trypsin will improve the flexibility and solubility of the native compact globular Bambara proteins thereby increasing the binding affinity and molecular interaction which may influence their complexation behavior with gum arabic. Klost and Drusch, (2019) showed notable changes in zeta potential result of pea protein hydrolysate in a degree of hydrolysis-dependent manner and a shift in the isoelectric point. A significant increase in solubility was reported as a result of increased amount of terminal carboxyl- and amino groups.
- c. There will be introduction of functional groups due to the amino acids oxidation in the plasma-treated Bambara protein. This may induce conformational changes and alteration of surface charges of the protein as a function of exposure time. As reported by Courtinlo *et al.* (2019) longer processing times can increase the number of collisions in the treated plasma samples which in turn increase the effectiveness of the plasma modification.

2.6 Aim

This research aims to investigate the impact of extraction conditions (pH fractionation and NaCl concentration), limited enzymatic hydrolysis, and atmospheric cold plasma on the structure, physicochemical changes, and complexation behaviour of protein from Bambara groundnut with gum arabic to enhance complex utilisation in the encapsulation of riboflavin as a model bioactive compound.

2.7 Objectives

The specific objectives are

- a. To optimize protein extraction conditions (pH and salt concentration) for improved yield while keeping the optimum pH of complexes of Bambara protein and gum arabic complexes below the isoelectric point (pI) of the protein using response surface methodology.
- b. To determine the impact of the degree of limited trypsin-mediated hydrolysis on the formation and stability of Bambara protein-gum arabic soluble complexes.
- c. To determine the effect of atmospheric cold plasma on the structural, conformational changes and functional properties of Bambara protein and gum arabic complexation behaviour.
- d. To determine the encapsulation efficiency and release profile, microstructure of Bambara protein hydrolysate-gum -gum arabic soluble complexes in a model beverage using riboflavin as the bioactive compound.

3 CHAPTER THREE

OPTIMISATION OF PH FRACTIONATION PROCESS FOR IMPROVED BAMBARA GROUNDNUTS PROTEIN-GUM ARABIC COMPLEXATION BELOW THE PROTEIN ISOELECTRIC POINT

Abstract

Protein yield from Bambara groundnut was improved in moderately acidic pH and the impact on the complexation behaviour with Gum arabic was investigated. Central composite design of response surface methodology (RSM) was employed and two independent extraction variables, pH (2.0-9.0) and NaCl concentration (0.0-0.6 M) were analysed to optimize the response variables. Changes in optical densities of Bambara protein isolates (BPI) -Gum arabic complexes were studied during turbidimetry analysis. Optimized BPI were further characterized for morphology and physiochemical properties as well as FTIR to determine the factors responsible for their complexation behaviour. Protein yield improved significantly by ~2 folds (63%) at moderately acidic pH with dependency on ionic strength compared with protein at the same pH without salt addition which suggests support from ionic strength. Protein fraction extracted at pH 2.95, 0.28 M NaCl formed better complex more outside the isoelectric point region (3.4) with high coacervate yield (~70%) and this was evidenced by the amino acids profile and surface charges of the protein. The fraction consists of more β -sheet structure, enriched legumin subunits and more positively charged amino acids which favour their complexation with gum arabic. This study was able to provide a mechanism that can produce proteins to complex below the isoelectric point of protein, and it enhanced the utilization of the complexes in acidified medium, especially those beverages prepared around pH 3.

3.1 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 (Eratte *et al.* 2018), emulsion stabilization (Keum *et al.* 2024), meat mimetics (Kim *et al.* 2024)

and improvement of the viability of probiotics during storage (Bora *et al.* 2023). Complexing protein with polysaccharides is a pH-induced associative phase separation driven by electrostatic attraction between oppositely charged biomolecules leading to biopolymer and solvent-rich phases (Stone, Teymurova and Nickerson 2014). The electrostatic interaction produces soluble complex at pH_c and thereafter, insoluble complex, denoted by $pH_{\phi 1}$. During pH titration of 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 carboxyl group of polysaccharide backbone (Carpentier *et al.* 2021; Li *et al.* 2021).

The optimum complexation (pH_{opt}) has been found to occur within a narrow pH range (4.0 - 4.5) close to the isoelectric point of protein (Hasanvand and Rafe 2018; Carpentier *et al.* 2021). Within the narrow pH range, protein-protein interaction has been found to interfere with the desired protein-polysaccharide complexation (Archut, Drusch and Kastner 2022). In addition to that, large aggregates are reportedly formed at the expense of smaller sizes (Jaramillo, Roberts and Coupland 2011) which does not improve their bioavailability as a delivery vehicle for bioactive compounds in the acidified food system (Chen *et al.* 2022). Furthermore, the poor dispersibility of complexes in acidified beverages is equally a challenge which has limited the food application (Yildiz *et al.* 2018). Although there are few exceptions where the complexes have been formed below pH 4.0 (Archut, Drusch and Kastner 2022; Tian *et al.* 2024), protein modification may be required to alter their complexation behaviour with anionic polysaccharides. To achieve the formation of coacervates below the isoelectric point of the protein, Busu and Amonsou (2019) fractionated Bambara proteins prior to complexation. Proteins fraction extracted at pH 2 had an optimum complexation pH value: 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.

A promising way to enhance protein yields in the acidified regions will be through modification using ionic strength and pH variation. The extraction conditions were anticipated to modify the protein composition and structure to generate new protein subunits that can impact the optimum complexation pH shifting and properties of complexes formed with gum arabic. The synergistic effect of extraction pH and ionic strength of protein has been reported to enhance protein extractability and recovery, thereby increasing the yield (Devnani, Moran and Grossmann 2023). A previous study by Busu and Amonsou (2019) on fractionation pH of Bambara protein as described above did not include mild acidic extraction conditions and salt. Extraction of

marama protein at mild acidic pH with the addition of 0.5 mol L⁻¹ NaCl has been reported to modify protein composition and structure (Gulzar, Taylor and Minnaar 2017) which may change the complexation properties of protein with polysaccharide.

In this study, a response surface methodology was used to optimise the pH fractionation process of Bambara protein with a view to improve the protein yield and modify the protein structure and composition to shift the critical structure formation pHs away from protein isoelectric region during complexation with gum arabic. Thirteen experimental runs with extraction conditions of pH (2.0 to 9.0) and NaCl concentration (0.0 to 0.6 M) were generated as independent variables using a central composite design. Complexation behaviours were assessed by turbidimetric analyses. Changes in structure and conformation of fractionated proteins were analysed to understand protein interaction with gum-arabic, used as model polysaccharide. The current study reports an improved protein and coacervate yield in the acidic region and a shift in pH_{opt} away from isoelectric point which can enhance the food application of the complexes in acidic beverages.

3.2 Materials and methods

3.2.1 Materials

Bambara groundnut, *vigna subterrenea* were collected from Jozini, province of Kwazulu-Natal in South Africa. Glucono-δ-lactone and gum arabic from *Senegal acacia* trees were purchased from Sigma Aldrich, South Africa including other laboratory-grade chemicals.

3.2.2 Preparation of defatted Bambara flour

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

3.2.3 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 (1g of flour:10 ml of buffer) under constant agitation for 2 hours. Statistical Software Design Expert version 11 (Stat Ease Inc., USA) was used to generate 13 experimental runs with a central composite design. The extraction pH (2.6 to 8.9) and NaCl concentration (0 to 0.6 M) were varied as independent variables. Centrifugation of the suspension was achieved using Eppendorf centrifuge at 10,000 x g for 30 minutes 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 was changed 2 times daily. Following the completion of dialysis, the protein isolate was freeze-dried and stored at -20°C for further analysis.

3.2.4 Protein content and yield

The protein content of the isolates was determined using Lowry method according to Rosebrough, Farr, and Randal (1951) and the total protein of the defatted flour (N X 6.25) by Kjeldahl method. The yield of the isolate was taken as % protein recovered to that of the defatted flour (Arise, Amonsou and Ijabadeniyi 2015).

3.2.5 Determination of coacervate yield

BPI: GA complex was prepared by mixing the individual biopolymer at a ratio 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 minutes at 4°C to facilitate complexation between the biopolymers. The suspension mixture was centrifuged at 8500 x g for 15 minutes to recover the coacervates (Plati *et al.* 2021). This was followed by freeze drying while the supernatant was discarded.

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

3.2.6 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 stir at 22°C for 2 hours continuously. The solubility of BPI was improved by leaving it overnight at 4°C. Thereafter, the BPI solution was subjected to heating in a water bath at 80°C for 15 minutes 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 using 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 a gradient of HCl concentrations to mitigate the impact of dilution (0.05, 0.5, 1, and 2 M HCl was used to reduce pH from pH 3.9 -3.4, 3.4 - 2.8, 2.8-2.3, 2.3-1.5 respectively). Changes in optical density were recorded with UV-VIS spectrophotometer using plastic cuvettes at 600 nm. The maximum optical density corresponds to the maximum complexation pH (pH_{opt}). The behaviour of the complexes (BPI: GA) was designed using a quadratic polynomial model regression equation to describe the complexation behaviour. All experiments were carried out in randomized order to reduce external effects on the response.

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

Y is the response variable, β_0 is a constant; β is the linear and β_{ii} the interactive coefficient; X_i is the level of the independent variable and ε is the random error. The optimisation objective was to minimise pH_{opt} and maximise coacervate yield. Design Expert 11 (statEase Inc, USA) was used for numerical optimisation, and this was found at a point that maximises the desirability function.

3.2.7 Verification and selection for optimum extraction conditions

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

3.2.8 Characterization of optimized Bambara protein isolates

3.2.8.1 Amino acid composition

Bambara protein isolate was hydrolysed using 6 mol/L of HCl at 116°C for 24 hours before being subjected to reverse-phase chromatographic analysis (Bidlemeier, Cohen and Tarvin 1984). Sulphur-containing amino acid, methionine was determined after performing acid oxidation and alkaline hydrolysis respectively (Gehrke *et al.* 1985).

3.2.8.2 Zeta potential

Zeta potential measurement of Bambara protein isolates was determined as a function of pH using Litesizer Nano ZS (Anton Paar, New Castle, Delaware). Freshly prepared BPI dispersions were diluted to 2 mg/ml with deionized water and hydrated for 1 hour which was vortexed intermittently. The sample solution was filtered using 0.22 µm HA Millipore membrane prior to measurement. Measurement was carried out over a pH range of 3 to 8 for all the isolates.

3.2.8.3 Gel electrophoresis

Reducing and non-reducing SDS-PAGE electrophoresis were both carried out according to (Laemmli 1970). 6 mg Bambara protein/ ml was mixed with Tris-HCl buffer, and the pH was adjusted to 8.0 in combination with 10% (w/v) sodium dodecyl sulphate (SDS) only which forms the reducing buffer, while that of the non-reducing buffer contains 10% v/v β-mercaptoethanol. Heat was applied to the sample solutions at 95°C for 10 minutes after which cooling was achieved prior to centrifugation at 10000 x g for 15 minutes. 15 µl of aliquot was loaded into a 4 to 12% gradient gel and the polypeptides were separated using the mini-PROTEIN system (BIO-RAD, Hercules, USA). Protein marker with a broad range of 10 to 200 kDa and Coomassie Brilliant Blue was used as a standard and to stain the gel respectively.

3.2.8.4 FTIR

FTIR analysis was carried out on the isolates extracted at different pH and ionic strength at 400- 4000 cm^{-1} scanning the background of each sample with air. To achieve the deconvolution spectrum, OMNIC 6.0 was used to process the infrared spectrum. Peak strength and peak area were achieved using second-derivative processing and gaussian fitting and for the baseline correction, peak Fit V4.12 was applied. Then, the equivalent percentage secondary structure of Bambara protein fractions was calculated.

3.2.9 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 seconds with 15 nm gold. This was followed by examination and photographs were taken using FEGSEM (Zeiss Ultra Plus, Germany) at an accelerated voltage of 5.0 kV. The calibrated scale bar on the monograph was used for the measurement of the sample sizes.

3.2.10 Statistical analysis

All experiments were conducted in triplicate unless otherwise stated. The experimental design and data analysis were performed using response surface methodology (RSM) based on a central composite design (CCD) with pH and NaCl concentration as independent variables. The experimental data were fitted to a second-order polynomial regression model to evaluate the effects of the independent variables and their interaction on the response variables. Analysis of variance (ANOVA) was used to assess the significance of the regression model and individual model terms. The adequacy of the model was evaluated using the coefficient of determination (R^2), and the statistical significance of the model was determined at $p < 0.05$.

3.3 Results and discussion

3.3.1 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 resulted in significantly 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. A similar extraction condition (pH 2) without salt in the extraction of Bambara protein resulted in a yield of 16% which is quite low compared to the yield obtained (63%) in the current study (Busu and Amonsou 2019). The yield obtained in the current study is a significant improvement for Bambara protein.

3.3.2 Turbidity profile of Bambara proteins – gum arabic complexes

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-gum arabic revealed some differences in complexation behaviours across the pH range 2-8 (Figure 1A-D). The turbidity curves show some variations in critical pH region at pH_{opt} (optimum complexation pH). Although, the optimum pH of complexation values was generally observed in the pH range 4.0-4.5, which may correspond to protein isoelectric point (pI), a notable shift in pH_{opt} , below pI range was observed for the protein fractions 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 yield 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 nm -1.2 nm) observed at alkaline extraction pH (8.0 and 8.9) dependent on the ionic strength. The OD_{max} at the acidic pH (2.57 and 3.5) ranged between 1.4 nm-1.6 nm while that of mild acidic pH (5.75) was between 1.3 nm to 1.8 nm resulting in 58% and 38% increase respectively compared to the yield of fractions obtained

at alkaline pH. 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 ionization 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 (Soussi Hachfi *et al.* 2023).

Table 3.1 Protein content and yield of Bambara protein fractions extracted at varying pH and NaCl concentration.

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

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

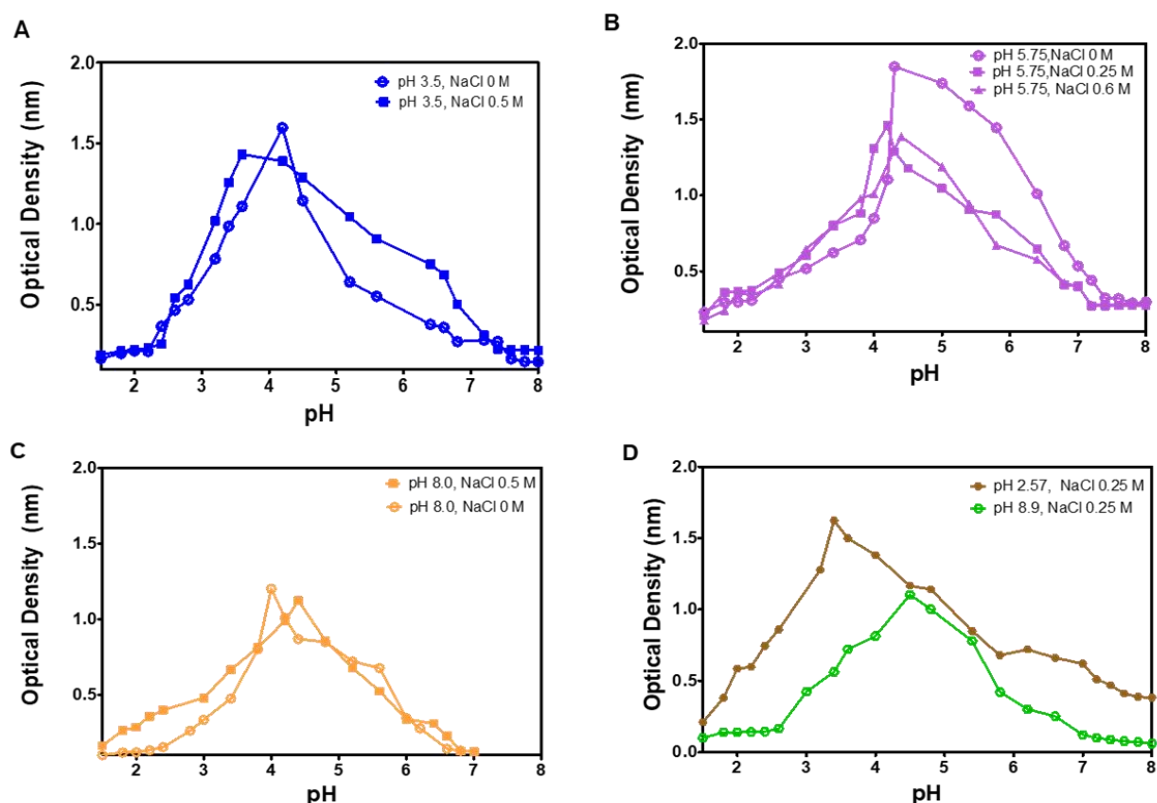


Figure 3.1 Turbidity profile of Bambara protein isolates: gum arabic complexes. A single graph was chosen as a representative out of the five central points (pH 5.75 and 0.25 M NaCl) on central composite design (Fig. 1B).

3.3.3 Fitting of the models

The p-values (< 0.0001) for the quadratic model demonstrated how well the model predicts the experimental response values, and because it was significant ($p < 0.05$), the model was validated (Table 2). The significance of the model terms is analysed using the p-value. The p-value < 0.05 of the model indicates that the model terms are meaningful and have a substantial effect on the responses. According to the p-values (Table 3), extraction pH, NaCl concentration, and the square terms of the variables had a significant effect on pH_{opt} and coacervate yield. The lack of fit values were 0.2317 and 0.1729 for pH_{opt} and coacervate yield, respectively, and were 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.

Table 3.2 Quadratic model for pH_{opt}, coacervate yield and linear model regression coefficient for Bambara protein isolates and Gum Arabic complexes.

Coefficient	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	

*P ≤ 0.05

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

The 3-D 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 (Fig. 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 (Fig. 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 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 low degree of ionization of BPI and

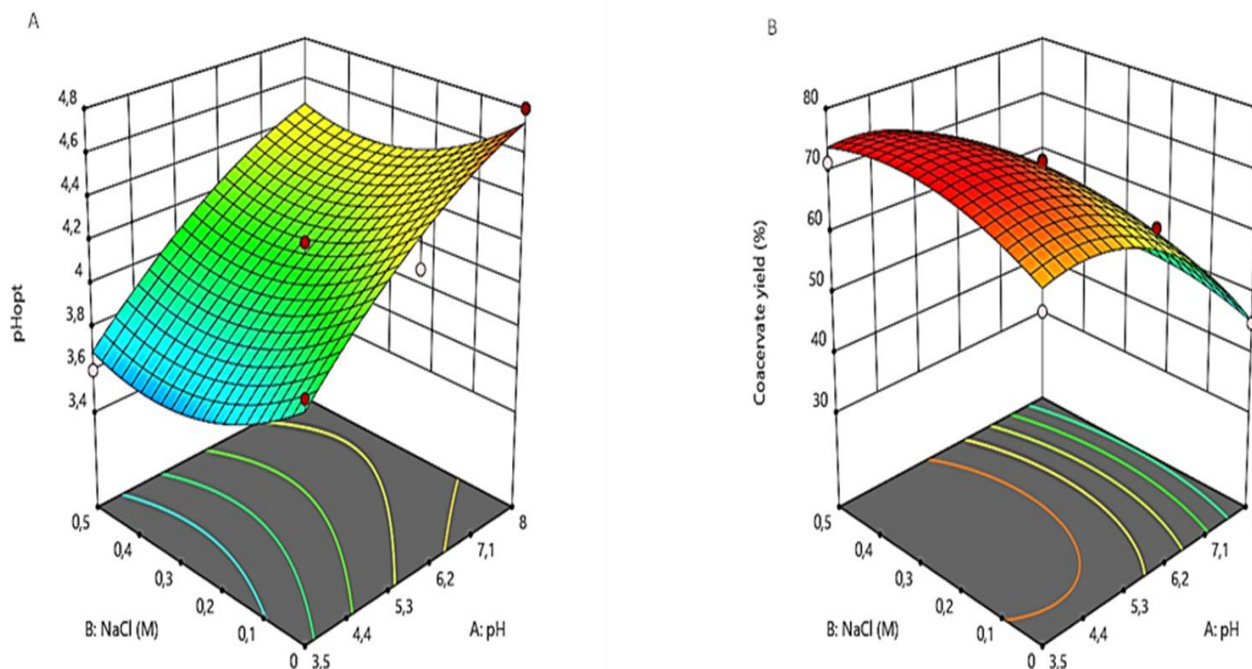


Figure 3.2 Response surface plots showing the interaction effects of pH and NaCl concentration on (A) optimum complexation pH (pH_{opt}) and (B) coacervate yield of Bambara protein isolate- gum arabic complexes.

3.3.5 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 maximizing the coacervate yield and minimizing the pH_{opt} 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 3.5 with 0.5 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} 3.5, coacervate yield 62.99%, pH_{opt} 3.2, coacervate yield 66.97%, pH_{opt} 3.47, coacervate yield 65.56% and pH_{opt} 3.33, coacervate yield 70.21% respectively. The optimized extraction conditions were further validated by complexing the resultant Bambara protein isolates with gum arabic, this was done

in triplicates. The validation of the optimized conditions through turbidity titration indicated pH_{opt} 3.6 coacervate yield 62.14%, pH_{opt} 3.4 coacervate yield 66.69%, pH_{opt} 3.5 coacervate yield 65.03% and pH_{opt} 3.4 coacervate yield 69.74% (table not shown). 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 adequacy of the response surface methodology. The proteins that were fractionated under optimized conditions were further characterized to understand their complexation behaviour and these include pH 3.5 with 0.5 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.

3.3.6 Characterization of optimized Bambara protein isolates

3.3.6.1 Amino acid profile

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 Bambara proteins. Although the medium pH is one of the factors controlling the electrostatic interactions between amino acids and gum arabic, the side chain of serine and threonine has been reported to show strong interaction with saccharides leading to complex formation (Ghosh and Bandyopadhyay 2012; 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, 0.5 M NaCl). Similarly, the basic amino acids were 36% higher than the traditional alkaline control protein. Arginine, lysine and histidine are positively charged groups which suggests sufficient positively charged regions providing more electrostatic interaction sites for the negatively charged carboxylic group of gum Arabic (Schmitt and Turgeon 2011; Derkach, Voron'ko and Kuchina 2022).

Table 3.3 Amino acid composition (g/100 g protein) of Bambara protein isolates extracted at optimized 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	0.80	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	7.13	12.61	17.00
PUAA	5.74	6.74	5.20	7.27	9.94

3.3.6.2 Zeta potential

Zeta potential of the proteins was carried out to understand the surface charge configuration of the proteins and how they may have impacted complexation behaviour with gum arabic. Bambara proteins had net positive and negative charge at pH below and above the isoelectric point (pI) of the protein respectively while gum arabic (GA) solution exhibited negative charge at all the pH tested (Fig. 4). Isoelectric points of the protein fractions ranged between 4.5 to 5.0 except for the fraction extracted at pH 2.95 with 0.28 M salt that has the neutrality at 4.0. It is

assumed that differences in pI values indicate disparity in amino acid composition (Klassen and Nickerson 2012). 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 (Doublier *et al.* 2000). Close to pI, BPI binds GA leading to an increase in negative charge (Jaramillo, Roberts and Coupland 2011), and consequently extending the formation of complex which possibly shifted the point of maximum complexation towards the moderately acidic pH. This suggests that the closer the pI to the acidic region the lower the pH_{opt}. For example, at pH 5, the negative value for the fraction at pH 2.95, 0.28 M salt increased (-19.7 Mv) relative to other fractions which might have increased its affinity to bind with the carboxyl group of gum arabic. Although both BPI and GA are negatively charged at pH 6.0-8.0, the positive patches on the surface of BPI weakly interacted with gum arabic (Schmitt and Turgeon 2011).

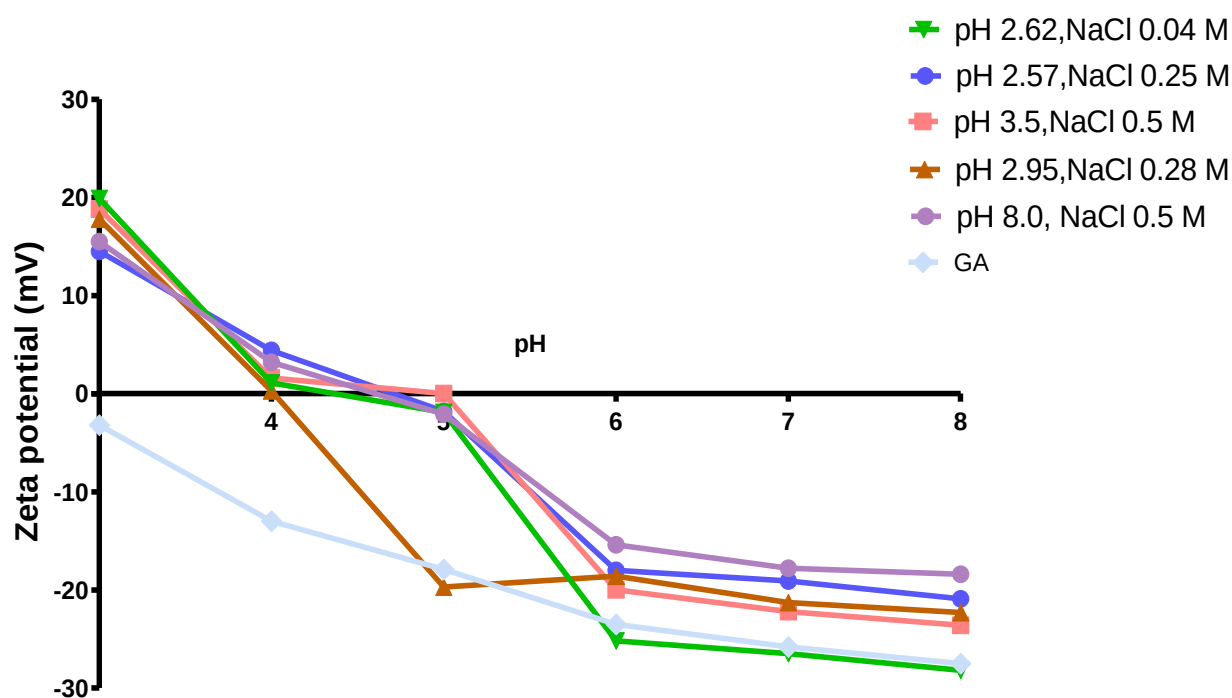


Figure 3.3 Zeta potential of gum arabic (GA) and BPI solutions at optimized extraction conditions.

3.3.6.3 Gel electrophoresis profile of optimized Bambara protein isolates

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 which correspond to vicilin, ~52 kDa (lanes 1-5), however significant variation in the protein distribution was observed in the various protein fractions (Fig. 3.). Similar bands appear in lanes 3-5 while there are additional bands at ~71 kDa, ~86 kDa and ~32 kDa (α and β -legumin) in lane 1 relative to the traditional alkaline control method (lane 2). Protein fraction obtained at pH 2.95, with 0.28 M salt (lane 9) and control; pH 8.0, 0.5 M salt (lane 8) show a new fairly intense band of low molecular weight, ~22 kDa under the non-reducing condition corresponding to β -legumin (Lei et al., 2022). The subunits (legumin and vicilin) observed in Bambara proteins have a high proclivity to form complexes with gum arabic, however, legumin was reported to complex slightly better than vicilin. (Feng *et al.* 2023). The contents of α and β isolated at pH 2.95, and 0.28 M salt were significantly higher than those fractionated at other pHs and traditional alkaline control methods. This suggests more reactive sites for electrostatic interaction with gum arabic during complexation which might explain the reason behind the pH_{opt} shift.

3.3.6.4 FTIR

FTIR analysis provided information about the modifications that occur in the secondary structure of the protein fractions. The spectra analysis of Bambara proteins in the amide-I region revealed three major Gaussian bands centred at 1612-1694, 1646-1664, and 1662-1684 corresponding to β -sheet, α -helix, and β -turn respectively (Pelton and McLean 2000; Schmidt, Giacomelli and Soldi 2005; Carbonaro and Nucara 2010). Generally, β -sheet is the dominant structure for all the protein fractions (Table 5). The high content of α -helix suggests a folded and rigid structure which 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. The transition from α -helix to β -sheets takes place during protein unfolding due to low pH treatment (He *et al.* 2021). This might suggest significant structural changes leading to dissociation and rearrangement of protein molecules at low pH and the presence of NaCl (Srivastava *et al.* 2020; Guckeisen, Hosseinpour and Peukert 2021). Partial protein unfolding unlocks the previously hidden hydrophobic residues of protein to be available

for interaction. In addition to the above-mentioned modification, proline was found to be higher in fraction obtained at pH 2.95, 0.28 M salt than other fractions including the control. High proline content has been linked to less folded structure and high solubility in acidic pH (Alabi *et al.* 2020).

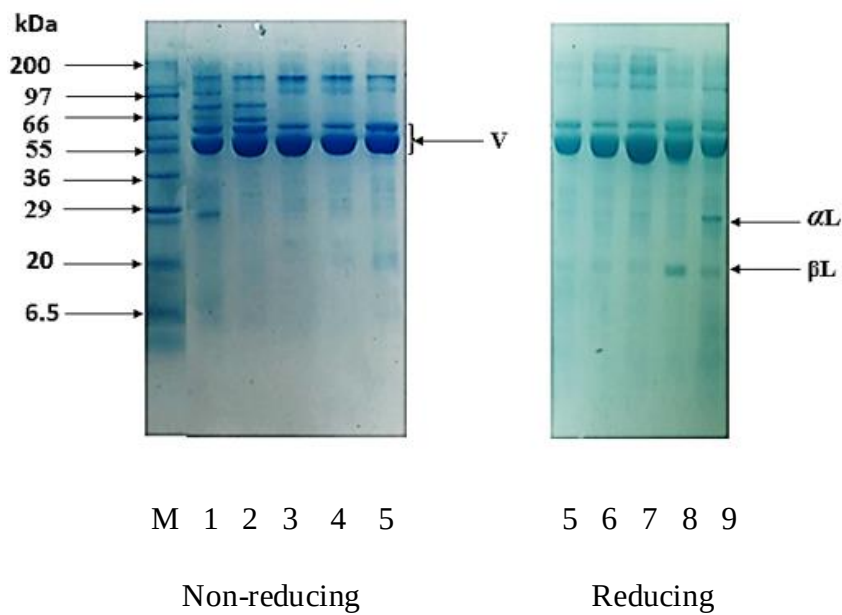


Figure 3.4 SDS-PAGE of Bambara protein isolates extracted at optimized conditions and

L: Legumin, V: vicilin, β L: Basic legumin, α L: Acidic legumin, M: Marker, lanes 1 and 9: pH 2.95, 0.28 M NaCl, lanes 2 and 8: pH 8.0, 0.5 M NaCl, lanes 3 and 7: pH 3.5, 0.5 M NaCl, lanes 4 and 6: pH 2.57, 0.25 M NaCl, lanes 5 and 5: pH 2.62, 0.04 M NaCl

Table 3.4 Secondary structure of Bambara protein isolates extracted at optimized conditions.

Optimized extraction conditions	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

3.3.7 Scanning electron microscopy of BPI-GA complexes

The microstructure of particles of (BPI) and their BPI-GA complexes were observed by FEGSEM (Fig. 5). For lyophilized droplets of BPI solution, the structural surface was relatively intact, exhibited mainly spherical structures with a lack of cracks, and a compact surface morphology typical of dehydrated products obtained by freeze-drying technique. The surface of the lyophilized complex showed a sponge-like porous microstructure. The electrostatic interactions between BPI and GA led to a spongy three-dimensional microstructure, with a porous surface consisting of cavities and intertwined vacuoles of different sizes. These vacuoles constitute potential points inside which the entrapment and protection of sensitive bioactive compounds may take place (Hasanvand and Rafe 2018). Among the complexes, the pH 2.95, 0.28 M NaCl BPI-GA complex provided a more homogenous, highly organized, dense structure with well-connected agglomerated particles and well-constructed pores. Similar porous structures have been reported for various complex coacervation systems, such as soy protein isolate – chitosan, pea protein isolate – beet pectin, hemp protein – gum arabic complexes (Huang *et al.* 2012; Lan *et al.* 2020; Plati *et al.* 2021).

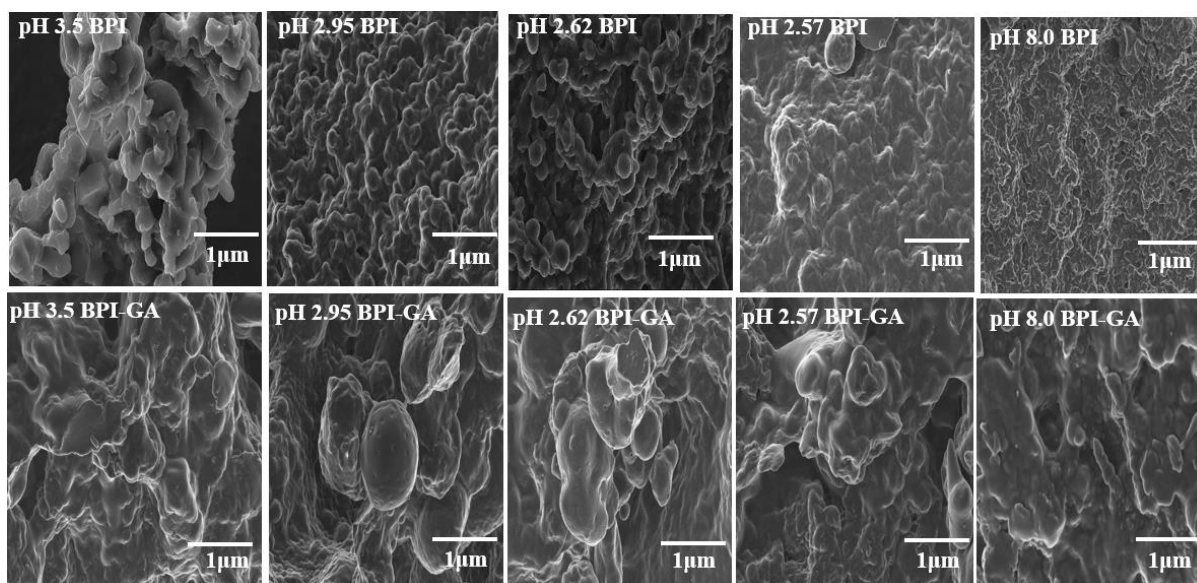


Figure 3.5 SEM micrograph of Bambara protein isolates (BPI) at optimised extraction conditions and their complexes with Gum arabic (GA)

3.4 Conclusion

Large aggregates are reportedly formed at the expense of smaller sizes at a narrow pH range (4.0 - 4.5) where optimum complexation usually occurs which are mostly appropriate as a delivery vehicle for bioactive compounds in the acidified food system. The composition and structure of Bambara protein as a globular protein were significantly altered during the extraction, varying the extraction pH and NaCl concentration. This influenced their electrostatic interaction with Gum arabic which brought about a shift in optimum complexation pH (pH_{opt}). pH_{opt} shifted from pH 4.0 to 3.4 as the extraction pH of BPI moved from alkaline to acidic region with a coacervate yield of ~70% at optimum conditions at pH 2.95, 0.28 M. The positive zeta potential values obtained at pH 4 below the protein pI resulted in strong interaction at the optimum complexation pH. Significant variation in the amino acid composition of the proteins was observed with the highest content of basic amino acids in the protein extract pH 2.95, 0.28 M NaCl. An increase in protein yield was observed at acidic and alkaline pH with an increase in ionic strength. SEM analysis revealed a compact and a regular polymeric network characterized by heterogenous sized vacuoles suggesting a high integrity of encapsulating materials. The composition and structure of Bambara protein as a globular

protein was significantly altered during the extraction. This influenced their electrostatic interaction with Gum arabic which brought about a shift in optimum complexation pH (pH_{opt})

3.5 Research output

a) Journal article

1. Abiola Adenike Ojesanmi, Eric O Amonsou. Influence of pH fractionation and salt on Bambara protein -gum arabic interaction and insoluble complex formation. Journal of Food Science and Technology 44: e00341

b) Conference abstracts

4. Abiola Adenike Ojesanmi and Amonsou EO (2022). Optimization of protein extraction conditions and its impact on the optimum pH of Bambara protein-gum arabic complexes. Presented at the 2022 AOCS Annual Meeting and Expo: Sustainable Protein Forum, 4th -6th Oct, Chicago, USA (Oral presentation).
5. Abiola Adenike Ojesanmi, Amonsou, EO.(2023). Optimization of protein extraction conditions and its impact on the optimum pH of Bambara protein-gum arabic complexes. Presented at the SAAFoST 25th Biennial Congress: Future Foods Smarter and More Sustainable Solutions, 28th-30th August, Cape Town, South Africa (Poster presentation)

4 CHAPTER FOUR

IMPACT OF LIMITED TRYPSIN HYDROLYSIS ON BAMBARA PROTEIN - GUM ARABIC INTERACTION AND FORMATION OF SOLUBLE COMPLEXES

Abstract

Soluble protein-polysaccharide complexes are of growing interest for food and beverage applications. However, due to the compact structure of globular proteins, forming stable complexes across a wide pH range is challenging. In this study, the effects of limited trypsin hydrolysis on Bambara protein and its interaction with gum arabic to form these complexes were investigated. Proteins were hydrolyzed to degrees of hydrolysis (DH) 2, 5, and 7.5, and analyzed for structural characteristics. Among studied hydrolysates, DH 5 produced the most stable soluble complexes over a broader pH range (7.0-4.3), showing enhanced binding strength and structural stability. This was evidenced by its rheological fingerprints, a shift in thermal transition temperature, increased negative charge, and surface hydrophobicity. Limited hydrolysis at DH 5 is the most effective in enhancing the formation and stability of Bambara protein-gum arabic soluble complexes. The stability of Bambara protein-gum arabic soluble complexes for bioactive compound encapsulation may be further investigated to support industrial applications.

Keywords: Bambara groundnut, gum arabic, soluble complex, binding strength, molecular interaction

4.1 Introduction

Complexes of protein and polysaccharides are essential in the food industry for encapsulation of bioactive compounds, emulsion, and forming stabilization, as well as texture modification. The polyelectrolyte nature of proteins and polysaccharides can induce a non-covalent electrostatic interaction between the biopolymers, leading to the formation of soluble and insoluble complexes within the acid dissociation constant (pKa) of polysaccharides and the

isoelectric point of proteins, pI (Pilevaran et al., 2021). Both the soluble and insoluble complexes play important roles in food systems. However, the application of insoluble complexes is restricted in clear liquid food due to high sedimentation, which can disrupt the desired uniform structure (Qiu et al., 2025). Hence, soluble complexes are preferred over insoluble ones for selected food applications. These complexes have been employed in a range of food applications which include the encapsulation and delivery of nutraceuticals (Wang et al., 2024), emulsion stability (Shen et al., 2022; Wang et al., 2022a), and solubility enhancement of beverage model system (Qayum et al., 2023). Shen et al. (2022) have observed a substantial improvement in the emulsifying ability of pea protein under acidic conditions through the formation of soluble complexes between pea protein-dextran sulphate. Pea protein-tragacanth gum complex showed the ability to encapsulate α -tocopherol (Carpentier et al., 2021).

The mechanism of soluble complex formation involves initial contact and adsorption of the protein to the polysaccharide through random collisions facilitated by Brownian motion. To optimize interactions, the protein and polysaccharide may undergo further conformational changes. Proteins can partially unfold to reveal more binding sites, while polysaccharides can stretch to enhance contact points (Clement, & Jim, 2022; Weiss et al., 2019). The soluble complex forms when the attractive interactions between the protein and polysaccharide are balanced with the solubility of the complex in the aqueous environment. Hence, during complexation, critical pHs of structure formation are monitored to prevent dissolution and balance solubility in an aqueous environment. Usually, the formation begins at the critical pH (pH_c), characterized by submicron-sized particles that appear as transparent or opaque solutions (Li et al., 2022).

Despite these potentials, soluble complexes usually form within a narrow pH range characterized by weak interactions due to folded structure and limited molecular flexibility of the native proteins (Razi et al., 2023). Consequently, this restricts the number of oppositely charged groups on globular proteins that can interact with polysaccharides (Tang et al., 2024). Previous studies on complexation demonstrated the possibility of pH broadening in the formation of soluble complexes. Although the studies were not specifically targeted towards enhancing the soluble complex profile, they demonstrate that the pH of formation and stability of soluble complexes can be improved. For instance, the turbidity profile of the heated and unheated dispersions of pea protein-gum arabic showed a slight shift in pH of soluble complex formation from 6.7 (unheated) to 6.2 (heated) (Guldiken et al., 2023). Liu et al. (2010) also

observed a shift in pH_c to lower values in the turbidity profiles of pea protein isolate-gum arabic complexes following heating of the biopolymers at higher temperatures. The results from these studies suggest that modification strategies may be used to improve the formation of soluble complexes, although the narrow pH range remains a challenge.

A green and sustainable modification with high sensitivity for industrial scale-up is required, such as enzymatic hydrolysis. Limited enzymatic hydrolysis of proteins is an innovative approach that can be employed to modify protein structure and improve their interaction with polysaccharides, thereby enhancing complex formation. It is a technology that is highly reproducible, does not require harsh conditions, is easier to control, and retains the nutritional quality of proteins (Chen et al., 2023). A high degree of hydrolysis ($> 10\%$) is undesirable for this study as it could lead to excessive small protein fragments lacking the desired surface properties (Kong et al., 2023). A limited degree of enzymatic hydrolysis has been reported to improve the solubility, surface hydrophobicity, charge characteristics, and molecular flexibility of proteins and thereby increase their functional property (Zang et al., 2019). This study, therefore hypothesized that limited enzymatic hydrolysis using trypsin will partially unfold the protein structure and produce more flexible soluble peptides with improved charge characteristics, which are expected to increase the intermolecular interaction with gum arabic. So far, to the best of our knowledge, no study has investigated the impact of the degree of limited trypsin hydrolysis on the stability and properties of soluble proteins-polysaccharides complexes. Hence, this study aimed to investigate the impact of limited trypsin hydrolysis (Degree of Hydrolysis, DH 2, 5, and 7.5) on the formation of Bambara protein and its interaction with gum arabic to form these complexes. Gum arabic, GA consists of a complex mixture of arabinogalactan-proteins, glucuronic acid, rhamnose, and other sugars, which offers multiple sites for interaction with proteins through electrostatic interaction (Li et al., 2018). The formation of hydrogen bonding between the hydroxyl group on gum arabic and polar amino acids of proteins further stabilized the complex. Limited hydrolysis can enhance the formation of soluble complexes with gum arabic, increasing the binding sites for interaction and flexibility of the protein. However, there is a need to establish optimal hydrolysis conditions that promote the formation and stability of soluble complexes, alongside a comprehensive understanding of their structural and functional characteristics.

4.2 Material and Methods

4.2.1 Materials

Bambara groundnuts (*Vigna subterranea*) grains were obtained from Jozini, KwaZulu Natal province of South Africa. Trypsin from bovine pancreas (10,000 BAEE units/mg protein), commercial gum arabic (*Acacia senegal*), glucono-delta-lactone, and other laboratory-grade chemicals were purchased from Sigma-Aldrich. Tris, glycine, sodium hydroxide, hydrochloric acid, sodium phosphate monobasic, sodium phosphate dibasic, ANS (1,8-anilinonaphthalenesulfonate), β -mercaptoethanol (2-ME), ammonium sulfate were purchased from Sigma-Aldrich, South Africa. BSA standard was purchased from Bio-Rad Hercules, USA.

4.2.2 Preparation of defatted flour

Bambara groundnut grains were sorted, soaked in water for 8 h, dehulled, oven-dried at 40 °C, milled into flour using a Kenwood food blender (BL487, South Africa), and sieved through a 355 μ m screen sieve. The flour was defatted using n-hexane at a ratio of 1:5 (g/mL) for 4 h following the method described by Arise et al. (2014). The defatting was repeated according to Crespo and Yusty (2005). The residual hexane evaporated by exposing the defatted flour in a fume hood overnight.

4.2.3 Extraction of Bambara protein isolate

The major storage protein fraction was extracted using the traditional alkaline method (Amonsou et al., 2012) with 30 mol/m³ Tris-HCl buffer, pH 8, containing 500 mol/m³ NaCl, at flour to solvent ratio of 1:20 under constant agitation for 2 hours. Centrifugation of the suspension was achieved using an Eppendorf centrifuge at 10,000 x g for 30 minutes 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 was changed 2 times daily. Following the completion of dialysis, the protein isolate was freeze-dried and stored at -20°C for further analysis. This material was referred to as the salt-soluble fraction. The protein content of Bambara protein was determined using the modified Lowry method (Markwell et al., 1978), and the yield was calculated as % protein in the extract compared to that of the defatted flour.

4.2.4 Enzymatic hydrolysis of Bambara protein isolates

Limited enzymatic hydrolysis of Bambara protein isolate was carried out using the optimized method by Chen and Campanella (2022). Bambara protein isolates and the hydrolysates (5%, w/v, protein basis) were dispensed in deionized water in a reaction vessel equipped with a stirrer. The dispersion was heated to 37 °C before the addition of trypsin (0.00032 µL/100 mg sample). The dispersion was incubated for 20, 40, and 90 min, and pH was kept constant by adding 1000 mol/m³ NaOH or 1000 mol/m³ HCl. Inactivation of trypsin was done by heating the mixture in a water bath at 85 °C for 10 min and then cooled in ice water. This was followed by centrifugation at 8000 xg for 20 min at 4 °C to precipitate the undigested proteins, and the supernatant, Bambara protein hydrolysate (BPH), was collected, lyophilized for 72 h at a condenser temperature of -50 °C, and kept at -20 °C for three days until further analysis. Bambara protein solutions (5%, w/w) without adding trypsin at pH 7.8 were used as a control.

4.2.5 Degree of hydrolysis (DH)

O-phthaldialdehyde (OPA) method was used to determine the free amino groups in Bambara proteins after hydrolysis according to Auwal et al. (2019). OPA reagent (6 mol/m³) dissolved in methanol was freshly prepared, containing 100 mol/m³ sodium tetraborate decahydrate containing 0.2% (w/v) of sodium-dodecyl-sulfate (SDS), and β-mercaptoethanol. 10 µL of Bambara proteins were mixed with 200 µL of OPA reagent in a 96 well-clear plate and incubated for 100 s at 35 °C, and then the absorbance at 340 nm on a microplate reader was taken. Glycine was used as the standard, and the number of free amino groups in the hydrolysate was calculated as serine-NH₂ moieties. The total number of primary amino groups in the protein samples was determined by acid hydrolysis using 6000 mol/m³ of HCl at 110 °C for 24 h. Among all the degrees of hydrolysis recorded, only the samples with DH < 10% and significant differences in their turbidity profile were chosen for this study.

The DH (%) was calculated as follows = $100 \times \frac{(OPA)_{hydrolysate}}{(OPA)_{acid\ digest}}$

4.2.6 Turbidity profile of Bambara protein hydrolysates- gum arabic complexes

Stock solutions of BPH, DH 2, 5, and 7.5, and gum arabic were prepared according to Busu and Amonsou (2019) at a mixing ratio of 4:1 (protein basis) and concentration of 0.1% (w/v). The solubility of unhydrolyzed Bambara protein isolate (BPI) and BPHs was improved by leaving it overnight (12 h) at 4 °C, while that of gum arabic was dissolved separately in ultrapure water produced by MilliPore Milli-Q system available from Millipore Corporation (Milli-Q water) and allowed to stir at 22 °C for 2 h continuously. The mixed solution pH was adjusted to pH 8 with 1000 mol/m³ NaOH, and then dropwise addition of 1000 mol/m³ HCl was used to achieve turbidimetric titration upon acidification at room temperature (25 °C). Diluting effects were mitigated to a minimum by using an internal acidifier (0.05 w/w) glucono-d-lactone, GDL, and a gradient of HCl concentrations based on pH (500 mol/m³ > pH 4.0; 1000 mol/m³ > pH 3; 2000 mol/m³ > pH 1.5). Optical density changes were monitored by a UV-VIS spectrophotometer using plastic cuvettes at 600 nm (1 cm path length). The maximum optical density corresponds to the maximum complexation pH (pH_{opt}). According to the previous study, as Yan et al. (2023) have stated, the critical pH points including pH_c, pH_{cmax}, pH_{φ1} and pH_{opt} and pH_{φ2} were graphically determined from the pattern as indicated by the slight rise in turbidity, the intersection between two curve tangents, a rapid rise in turbidity and the highest turbidity, respectively.

4.2.7 Size exclusion chromatography

Bambara protein hydrolysates and unhydrolyzed isolate were subjected to high-performance size exclusion chromatography (Shimadzu Prominence-I LC-2030C, Japan). Briefly, the analytical column (300 mm × 7.8 mm Yarra 3 μm SEC 2000 column) (Phenomenex, Torrance, CA) was connected at a fixed temperature of 30 °C. The filtered mobile phase consisted of 0.1 mL/100 mL TFA (solvent B). 10 μL aliquot of Bambara proteins (10 mg/ mL) dissolved in sodium phosphate buffer (pH 7) was injected into the columns for 60 min at a flow rate of 0.2 mL/min using a linear gradient (0–100%). Peak detection was at a wavelength of 214 nm. A protein molecular weight standard with different protein sizes ranging from 15–600 kDa was used.

4.2.8 Zeta potential

Bambara protein hydrolysates and gum arabic solutions were subjected to zeta potential measurement over a pH range of 3–8 at 1-unit increment using Litesizer Nano ZS (Anton Paar, New Castle, Delaware).

4.2.9 Surface hydrophobicity

Surface hydrophobicity of the unhydrolyzed protein isolates and hydrolysates was determined by the fluorescence method using a hydrophobic probe, 8-anilino-1-naphthalenesulfonic acid (ANS) as the hydrophobic probe (Sun et al., 2023). Protein stock solution (10 mg/mL) was prepared in 0.1 mol/L sodium phosphate buffer, pH 7.0, followed by dilution to 0.0009–0.01%. A 20 μ L aliquot of ANS solution (8 mol/m³) was added to 4 mL of each dilution and mixed thoroughly. Fluorescence was then measured at excitation and emission wavelengths of 390 and 470 nm, respectively. The slope of the plot of fluorescence versus protein concentration was taken to be the hydrophobicity.

4.2.10 Intrinsic fluorescence spectroscopy

The method described by Cui et al. (2023) was used to record intrinsic fluorescence spectra on the JASCO FP-6300 spectrofluorometer (JASCO, Tokyo, Japan) at 25 °C with a 1 cm path length cuvette. Protein stock solutions (unhydrolyzed and hydrolysates) (10 mg/mL) were prepared in 0.1 mol/L sodium phosphate buffer, pH 7. The buffer was then used to dilute the respective stock solution to 0.002%, and fluorescence spectra were recorded at excitation wavelengths of 275 nm (tyrosine and tryptophan) with emission recorded from 280 to 500 nm.

4.2.11 Frequency sweep of Bambara protein-gum arabic soluble complexes

The rheological properties of Bambara protein hydrolysates/isolate-gum arabic soluble complex solutions were performed using a rheometer (MCR 102, Anton Par, New Castle, Delaware) equipped with parallel plate geometry (diameter of 50 mm and 1 mm gap) as previously described by Deng et al. (2018). The oscillatory frequency sweep (0.01–100 Hz)

was measured after cooling to 25 °C using a 0.5% strain to ensure the measurements were being performed within the LVR. All samples were covered by a thin layer of paraffin oil to minimize evaporation during measurements. The measurements were performed within the linear viscoelastic region of the complexes, so the structures were not destroyed. The dynamic rheological parameters used to evaluate the soluble complexes network were the storage modulus (G'), loss modulus (G''), $\tan \delta$, and complex modulus (G^*). The slope of the G^* was analyzed using the power-law model, which states that:

$$G^* = A_n W^{n^*}$$

G^* is the complex modulus, A_n is a measure of the strength of the electrostatic interactions, and the angular frequency range 0.1–100 rad/s is represented by W and n^* , the power law exponent which is a measure of the elastic characteristic of the complexes.

4.2.12 Apparent viscosity of Bambara protein-gum arabic soluble complexes

The viscosity of the Bambara protein hydrolysates-gum arabic soluble complex solutions was determined at 25 °C in an MCR102 rheometer (Anton Paar, New Castle, Delaware) (Xing et al., 2024). The shear stress was measured with the application of a given shear rate from 1 s⁻¹ to 300 s⁻¹.

4.2.13 Differential scanning calorimetry (DSC)

Thermal stability of Bambara proteins was measured using a differential scanning calorimeter (model DSC-7, PerkinElmer, Norwalk, CT, USA) according to Wan et al. (2023). Each sample, 3 mg was weighed into a hermetically sealed standard aluminium pan. DSC analysis was carried out in the scanning range of 20 – 200 °C, heating rate 5 °C/min, nitrogen flow rate 10 mL/min. A sealed, empty pan was used as a reference. The onset temperature (T_o), denaturation temperature (T_p), and enthalpy change (ΔH) of the thermal transitions were analyzed using the Universal Analysis 2000 software (TA instruments).

4.2.14 Statistical analysis

Each analysis was carried out in duplicate unless otherwise stated. Experimental data were analyzed using analysis of variance (ANOVA) and means were compared using Fisher Least Significant Difference (LSD) test ($p < 0.05$).

4.3 Results and discussion

4.3.1 Fat content of Bambara groundnut flour

The fat content of the flour was 0.75%, suggesting a successful removal of fat, which can interfere with the protein extraction (Mamoudou, & Mune, 2024). Low fat content facilitates protein extraction by improving the purity of the protein isolate.

4.3.2 Protein content of the isolate

The protein content of Bambara protein isolate was 91%. The high protein content suggested it is of relatively high purity. The protein content obtained in this study is higher than that of yellow peas, green lentils, and red lentils, but it is comparable to the value found for mung bean (Shrestha et al., 2023; Gao et al., 2020).

4.3.3 Degree of hydrolysis, DH

The effect of enzyme reaction time on DH was assessed during hydrolysis. As expected, DH increased progressively with the duration of exposure. DH values of 2 and 5 were obtained at 20 and 40 minutes, respectively, while reaching a DH of 7.5 required more than twice the time needed to reach DH 5. Trypsin specifically cleaves peptide bonds in proteins at the carboxyl side of lysine and arginine residues. The relatively inner localization of these polar residues within the parent protein necessitates extended exposure to achieve higher DH. The DH data from this study are comparable to those reported by Jin et al. (2020) on walnut protein using the same enzyme. According to Ding et al. (2021), an extended incubation period allows sufficient interaction of the enzyme with the protein active sites. Globular proteins are good

sources of lysine and arginine, which further supports the choice of trypsin for hydrolysis (Gupta, & Uversky, 2023).

4.3.4 Turbidity profile

The formation of protein and gum arabic complex was monitored through acid titration of the mixture from pH 8 to 1.8 to detect the critical structures forming stages (Fig. 4.1). At high pH, a minimal interaction between protein with polysaccharide was observed until the critical pH_c of structure forming pH was reached. Marginal differences were observed in sample mixtures at this stage. However, substantial variations in the interaction of protein in a range of pH_c to $pH_{\phi 1}$ correspond to pH range of the soluble complex forming stage (Table 4.1). Notable DH 5 showed a significantly wider pH range compared to DH 2 and DH 7.5 as indicated in the shaded portion of the turbidity curves. The optimum pH of soluble complex formation estimated by the tangent was fairly similar, revolving around pH 5. The soluble complex is formed when the attractive interactions between the protein and polysaccharide are balanced with the solubility of the complex in the aqueous environment. This balance is crucial to maintain the complex in a dissolved state rather than precipitating out. Consequently, the variation in the behaviour of the hydrolysates in complex formation was due to a number of factors including the degree of exposure and difference in charge configuration as shown in zeta potential analysis at specific pH conditions and degree of protein flexibility. Limited hydrolysis at DH 5 enhances the flexibility while maintaining the overall structure of the protein. As can be seen from the figure, DH 5 showed more negative absolute values compared to DH 2 and DH 7.5.

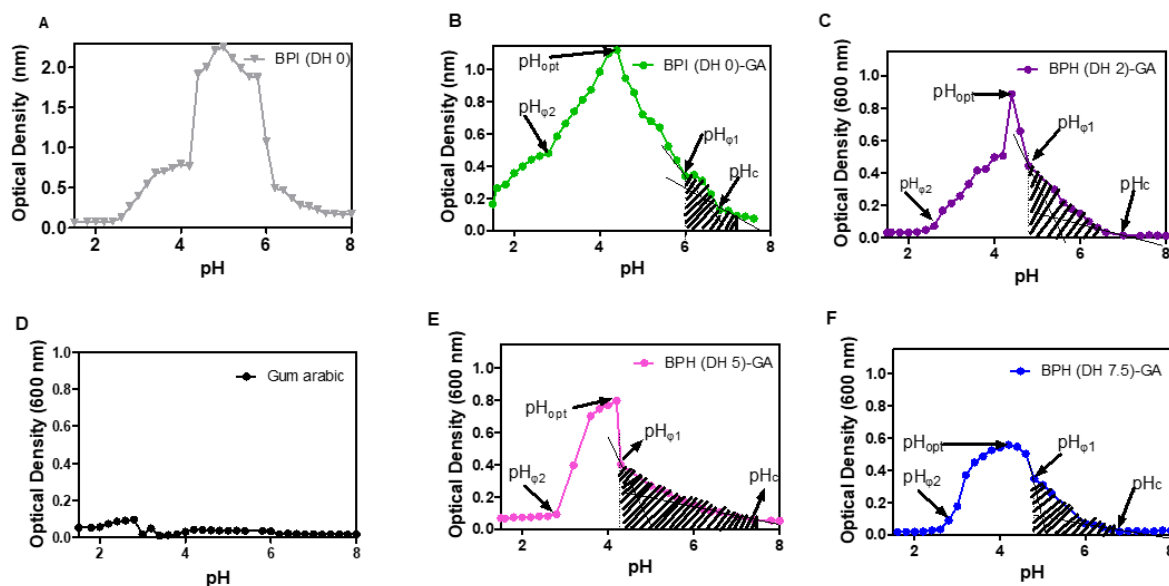


Figure 4.1. Turbidity profile of Bambara protein hydrolysates – gum arabic showing optimum complexation, pH_{opt} and region of soluble complexes, pH_c - $pH_{\phi 1}$. BPI-Bambara protein isolate, BPH-Bambara protein hydrolysates, DH-degree of hydrolysis, GA-Gum arabic.

Table 4.1. Critical structure forming pH_s for Bambara proteins-gum arabic complexes

Samples	pH range for soluble complexes (pH_c - $pH_{\phi 1}$)	pH_{cmax}	pH_{opt}	$pH_{\phi 2}$	Yield of soluble complex (%)
BPI (DH 0)-GA	$6.80^a \pm 0.10$ - $6.00^c \pm 0.10$	$6.20^a \pm 0.01$	$4.40^a \pm 0.10$	$2.80^a \pm 0.00$	$38.87^d \pm 0.02$
BPH (DH 2) - GA	$7.00^a \pm 0.10$ - $4.80^b \pm 0.20$	$5.40^b \pm 0.03$	$4.40^a \pm 0.10$	$2.60^a \pm 0.20$	$56.08^c \pm 0.04$
BPH (DH 5) - GA	$7.00^a \pm 0.10$ - $4.30^a \pm 0.10$	$4.60^d \pm 0.02$	$4.20^a \pm 0.10$	$2.80^a \pm 0.10$	$71.59^a \pm 0.01$
BPH (DH 7.5) - GA	$6.80^a \pm 0.10$ - $4.80^b \pm 0.00$	$5.10^c \pm 0.01$	$4.20^a \pm 0.20$	$2.80^a \pm 0.10$	$57.82^b \pm 0.02$

Mean $\pm$ SD (n=2). Different letters superscripted along the column were significantly different at $p < 0.05$. Protein content of Bambara isolate is 91%. BPI- Bambara protein isolate, BPH- Bambara protein hydrolysate, DH- Degree of hydrolysates, GA- Gum arabic, pH_{opt} - optimum pH of insoluble complex formation, pH_c - pH of soluble complex formation, pH_{cmax} - maximum pH of soluble complex formation, $pH_{\phi 1}$ - onset pH of soluble complex formation, $pH_{\phi 2}$ - pH of insoluble complex dissolution.

4.3.5 Size exclusion chromatography

Bambara protein hydrolysates were subjected to size exclusion chromatography kinetics to assess the extent of hydrolysis on protein size (Fig. 4.2). The unhydrolyzed protein had two predominant peaks with molecular weights of 533 and 318 kDa identified as globulin storage protein (Husband et al., 2024). As seen from the graph, the higher the extent of hydrolysis, the more the number of peaks corresponding to the peptides generated. As expected, extended hydrolysis (DH 7.5) led to the production of a greater number of peptides with sizes ranging from 34 to 11 kDa while DH 5 had 144, 49, and 35 KDa molecular weights. The reduction in molecular weight was anticipated to increase the molecular flexibility and the negative surface charge of the proteins as the hydrolysis progresses (Klost & Drusch 2019). This promotes the intermolecular interactions among the bio-polymer molecules and creates inequality in their net charge which is a favourable condition for the formation of soluble complexes (Warnakulasuriya & Nickerson 2018; Huang et al., 2023).

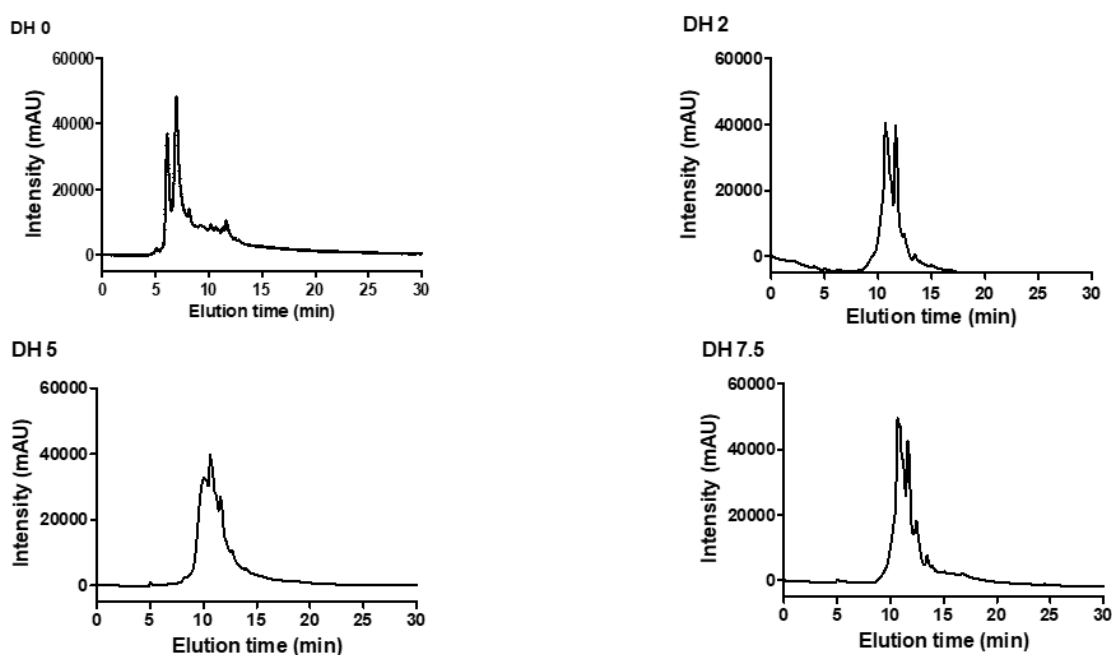


Figure 4.2. Size exclusion chromatography elution profile of Bambara proteins and hydrolysates at varying degree of hydrolysis. DH-Degree of hydrolysis

4.3.6 Zeta Potential

Zeta potential provides is a measure of the net surface charge and potential charge distribution at the interface. Although the surface profile displays a similar pattern consisting of positive and negative charges above and below protein pI respectively, hydrolysis significantly shifted the pH of electrical neutrality, which may correspond to variation in pI of hydrolysate in comparison with unhydrolyzed protein (Fig. 4.3). This confirming an enhancement in the solubility around the isoelectric point of native protein. The conformational change in protein structure after hydrolysis was reflected in the variation in the net surface. The exposure caused by the hydrolysis must have added additional charged amino acid residues, which can alter the absolute overall surface charge and potentially increase the net positive charge below the pI (Damodaran & Parkin, 2017). Notable, DH 5 hydrolysate showed an intermediate absolute negative value compared to DH 2 and DH 7.5 around pH 5. The negative absolute value of DH 5 was lower than that of gum arabic suggesting inequality in the charge density of the biopolymers. This possibly favour positive interaction and binding of the hydrolysates to gum arabic (Klost & Drusch 2019; Nasrabadi et al., 2021). The highest absolute negative ζ -potential value was recorded at DH 7.5 (-30.1 mV) as compared to the highest ζ -potential value in unhydrolyzed protein (-19 mV). An increase in the number of exposed ionizable amino and carboxyl groups following the hydrolysis could also be a contributing factor to the high negative net charge of the hydrolysates (Eckert et al., 2019). Additionally, the increase in electronegativity of the hydrolysates with increasing DH could be related to the increase in the number of charged amino acids and peptides produced by hydrolysis. This suggests a greater binding affinity between the biopolymers since more carboxylic and amino groups would be released as the peptide bonds are cleaved by trypsin. A rise in net negative charge is due to carboxylic groups dissociating into carboxylate ions (COO^-), since the hydrolysis was kept at pH 7.8, which is higher than the isoelectric point of most amino acids and peptides (Mahmoud et al., 1992; Nawaz et al., 2022). A report of lower zeta potential of -37 mV for lentil protein hydrolysates compared to the native proteins (-35 mV) was observed by Avramenko et al. (2013). Stability and strong electrostatic repulsive forces among the protein particles have been linked to a net negative surface charge above the pI of proteins (Wijaya, 2019).

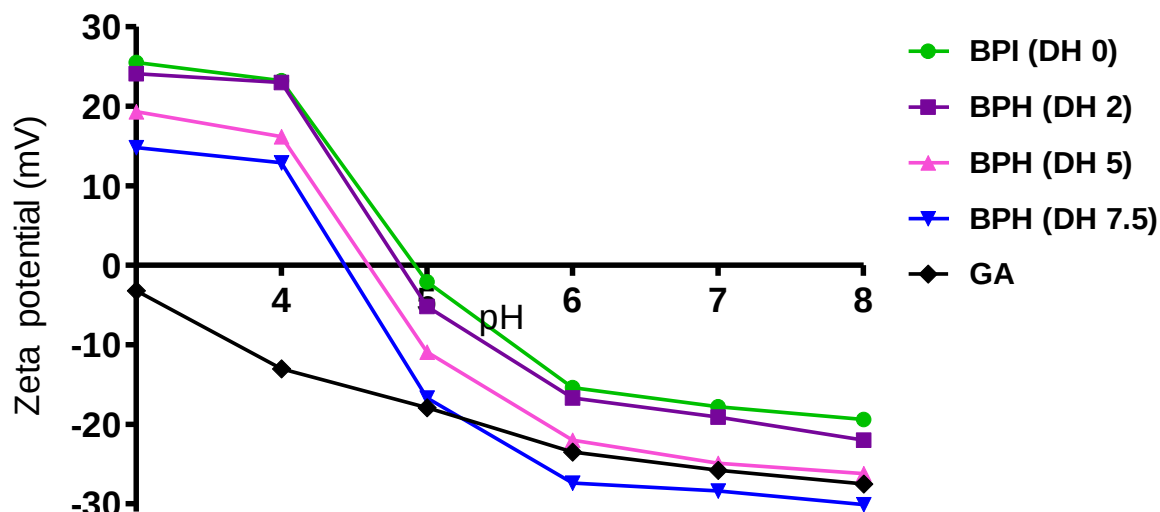


Figure 4.3. Zeta potential profile of Bambara protein isolates, corresponding hydrolysates, and gum arabic as a function of pH. BPI-Bambara protein isolates BPH-Bambara protein hydrolysate.

4.3.7 Surface hydrophobicity

Surface hydrophobicity of protein hydrolysates increased throughout the trypsin-induced hydrolysis with DH 7.5 having four-fold higher values than that of unhydrolyzed protein (Fig. 4.4A). The increase in surface hydrophobicity was associated with partial unfolding of the protein structure and it is related to the cleaving of small peptides into the solution (Kamani et al., 2022; Wang et al., 2022b). Consequently, the unfolding resulted in the exposure of hydrophobic amino acids on the surface of the protein which were previously buried in the interior part of the protein (Lv et al., 2023). An increase in surface hydrophobicity is an indication of high solubility of protein which is an index for high intermolecular interaction (Ma et al., 2023).

4.3.8 Intrinsic fluorescence

Fluorescence spectroscopy is an effective method for detecting changes in protein tertiary structure. The fluorescence intensities of the hydrolysates were found to be higher than that of the unhydrolyzed protein, increasing with an increase in the degree of hydrolysis (Fig. 4.4B). The maximum emission fluorescence wavelength ($\lambda_{\max}$) of unhydrolyzed Bambara protein was 336 nm, while the $\lambda_{\max}$ with increasing DH were 338, 342, and 348 nm for DH 2, 5, and 7.5,

respectively. The intensity of the fluorescence is related to the exposure of the aromatic amino acids to the reactive solution, which is reflective of changes in the protein structure (Shi et al., 2023). A red shift in λ_{max} observed with increasing DH suggests a loose, flexible structure and exposure of tryptophan to the aqueous environment resulting in water-dependent chromophore fluorescence quenching and increasing the reactive sites of the protein (Markwell et al., 1978).

4.3.9 Frequency sweep of Bambara protein-gum arabic soluble complexes solutions

The formation of soluble complexes between proteins and polysaccharides, such as gum arabic, involves various physicochemical interactions that can stabilize the mixture solution. Dynamic rheological properties were performed on the solution of Bambara protein-gum arabic soluble complexes to provide insight into their macroscopic structures and binding strength (Fig. 4.5A-B). As can be seen in Figure 4.5A, both moduli showed frequency dependency. G' was significantly higher than G'' along the entire frequency range for all complexes, suggesting a dominance of elastic behaviour and presence of structure. A gel-like network structure for complexes such as soy b-conglycinin -soluble soy polysaccharides/gum arabic (Zang et al., 2019) was previously reported. As the DH increased from 2 to 5, both G' and G'' values increased. However, a further increase to DH 7.5 led to a decrease in G' and G'' , with DH 5 exhibiting the most pronounced plateau in G' . These trends may be attributed to the differences in the molecular structure of the hydrolysates, which likely affected their interaction strength, and the structure formed with gum arabic (Badjona et al., 2025). A higher G' stands for higher elasticity and indicates a stronger molecular interaction and binding strength between the hydrolysate and gum arabic resulting in a higher degree of internal structure, and this makes the complex more stable (Wang & Selomulya, 2022a).

Tan δ for BPI and complexes produced with the isolates and hydrolysates were within 0 and 1 (Fig. 4. 5B), tan δ less than 1 suggests that the particles are highly associated due to the colloidal forces such as electrostatic forces, repulsive and attraction forces, and van der Waals forces (Liang et al., 2007).

Power law equation was used to fit the frequency sweep data (Table 4.2), and all the samples fitted well ($R^2 > 0.9503$) in the model. Protein hydrolysates with DH 5 showed the highest A_n

value, suggesting that it has produced the soluble complex with a higher magnitude of interaction and binding strength to the polysaccharide. The n^* value decreased gradually; low n^* values are characteristic of an elastic network, and n^* value close to 1 indicates viscous gel behaviour (Behrouzian et al., 2016).

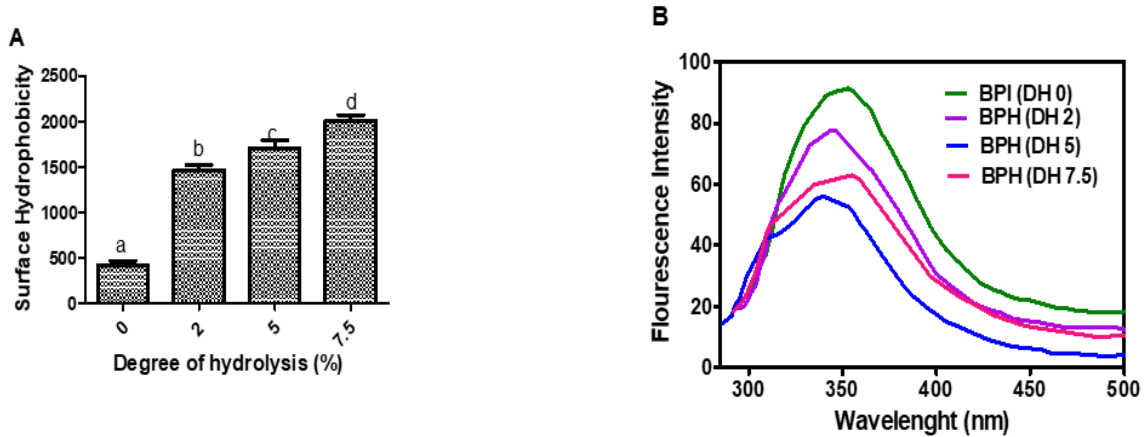


Figure 4.4 Surface hydrophobicity and intrinsic fluorescence spectra of Bambara protein isolates and hydrolysates as a function of degree of hydrolysis. BPI-Bambara protein isolate, BPH-Bambara protein hydrolysates.

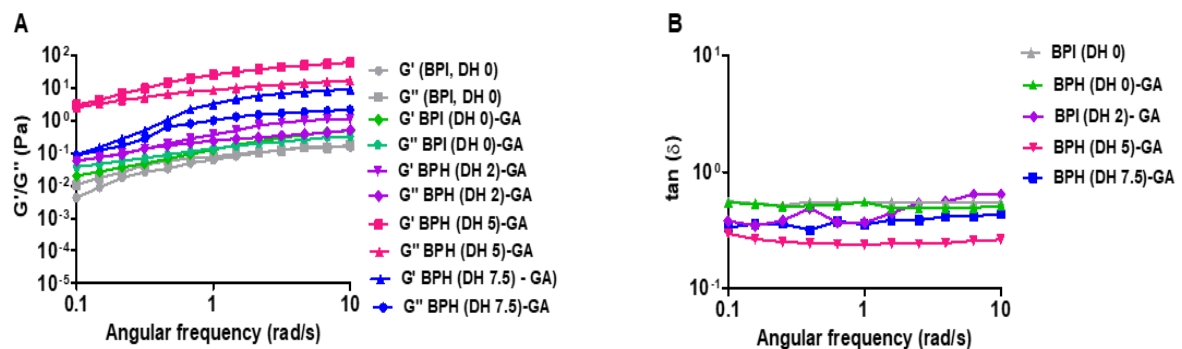


Figure 4.5. Frequency sweep curve for Bambara protein hydrolysates-gum arabic soluble complexes showing dynamic storage modulus, G' and loss modulus, G'' and Loss factor, Tan δ . Bambara protein isolate-Gum arabic (BPH-GA), Bambara protein hydrolysates-Gum arabic (BPH-GA)

4.3.10 Apparent viscosity of Bambara protein-gum arabic soluble complex solutions

The viscoelastic behaviour highlights the impact of protein hydrolysis on the magnitude of the interaction with polysaccharides. The shear-dependent viscosities for BPI and the complexes at different degrees of hydrolysis depict the non-newtonian shear-thinning behaviour (Fig. 4.6). The flow curve can provide insights into the flow behaviour and structural integrity of the complex. A pronounced shear-thinning behaviour often indicates strong interactions and a well-structured network. Agarwal et al. (2023) have related the shear thinning behaviour of pea/quinoa protein and locust bean gum mixtures to the interactions between the biopolymers. A better structural integrity indicating a stronger interaction between the pea protein and locust bean gum was reported for a pronounced shear thinning behaviour. The graph in this study revealed the effect of variation in the degree of hydrolysis on the viscosity of the complex system. It is interesting to note that viscosity decreased with increasing DH. A maximum viscosity was found with a complex formed with Bambara protein isolate which reflects the lowest solubility.

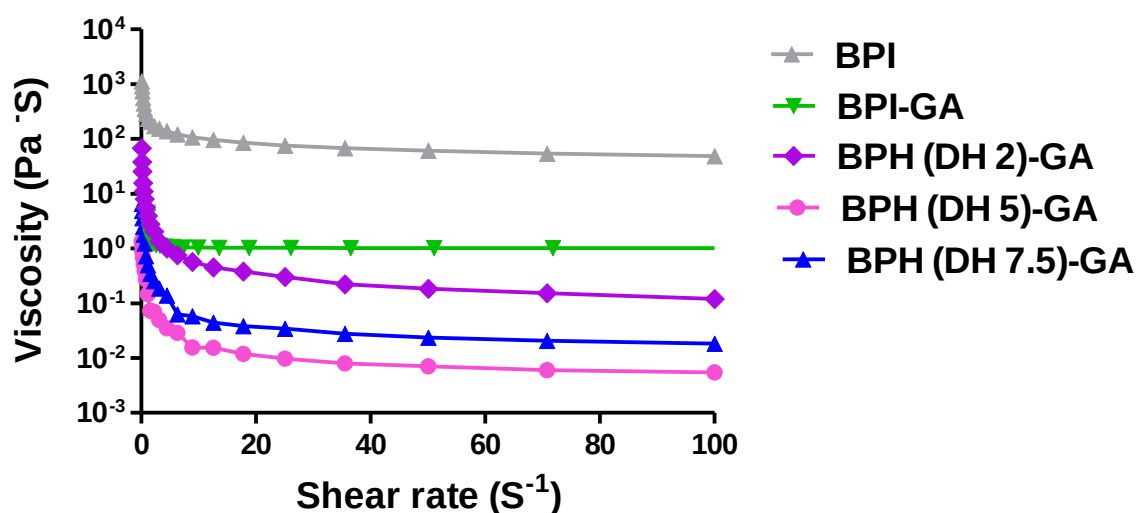


Figure 4.6. The apparent viscosity of Bambara protein hydrolysates-gum arabic soluble complexes.

Table 4.2. Effect of degree of hydrolysis on power law model of Bambara proteins-gum arabic soluble complexes

Samples	A_n (Pa)	n^*	R^2
BPI (DH 0)	$1.2600^e \pm 0.1052$	$0.4361^a \pm 0.0281$	0.9709
BPI-GA (DH 0)	$19.1200^d \pm 0.9752$	$0.2613^b \pm 0.0192$	0.9503
BPH-GA (DH 2)	$54.3900^c \pm 1.2440$	$0.2065^c \pm 0.0091$	0.9809
BPH-GA (DH 5)	$67.3200^a \pm 0.5933$	$0.1368^e \pm 0.0038$	0.9915
BPH-GA (DH 7.5)	$57.3900^b \pm 0.9559$	$0.1547^d \pm 0.0070$	0.9780

a Results were represented as mean values $\pm$ standard deviation of duplicate tests.

b Different letters superscripted along the column were significantly different at $p < 0.05$.

BPI- Bambara protein isolate, BPH- Bambara protein hydrolysate, GA- Gum arabic,

DH- Degree of hydrolysis, A_n – measure of the strength of the interaction, n^* - power law exponent.

4.3.1 Thermal stability

Differential scanning calorimeter thermograms of Bambara protein isolate, hydrolysates, and their corresponding soluble complexes provided the thermal stability, such as T_d (denaturation temperature) and ΔH (enthalpy change) of the samples. All samples showed a similar endothermic peak over a wide temperature range (Fig. 4.7). The T_d of Bambara protein was less depth and occurred at 52.61 °C. The T_d of gum arabic appears at 88.11 °C, indicating higher heat resistance of gum arabic than Bambara protein isolate. This may be because gum arabic has a larger molecular weight and more stable polysaccharide chains than the proteins. The T_d of the BPI-GA complex appears at 66.22 °C, and the peak was larger than that of BPI, which suggested that the expansion of the spatial structure of the BPI-GA complex required a higher temperature and heat than that of Bambara protein. This also implied that the thermal stability of the BPI-GA soluble complex is superior to that of BPI (Lan et al., 2018). The denaturation temperature of the soluble complex formed with the hydrolysates shifted to higher values in a degree of hydrolysis-dependent manner, having DH 5 as the highest (T_d for DH 2- 76.29 °C, DH 5- 94.24 °C, and DH 7.5- 92.90 °C). This indicates the formation of a new network structure and enhanced thermal stability of Bambara protein hydrolysates-gum arabic soluble complexes (Lan et al., 2018). Soluble complex formation increased protein stability against thermal denaturation (Chen et al., 2020). The high denaturation temperature of the soluble complexes formed with the hydrolysates might be associated with the lower molecular

weight polypeptides, as indicated in the size exclusion chromatography result (Fig. 4.2) (Li et al., 2020; Peng et al., 2020). In addition, this might be due to a stable hydrogen bond and greater hydrophobic content in the hydrolysates. The highest enthalpy of denaturation (ΔH) was recorded for gum arabic (Table 4.3), indicating a more folded structure since ΔH represents the proportion of undenatured biopolymer and is correlated with the extent of the folded structure (Latorre, & Velazquez, 2021; Ma et al., 2019). The impact of the unfolded structure of GA was significant in the ΔH values for the complexes. The lowest ΔH value recorded for BPI might be due to an increase in the stability of hydrophobic interaction, as corroborated by the hydrophobicity and fluorescence data (Fig. 4.4A & B). The complexation of Bambara protein hydrolysates with gum arabic had a significant influence on the thermal stabilities of the BPH-GA soluble complexes.

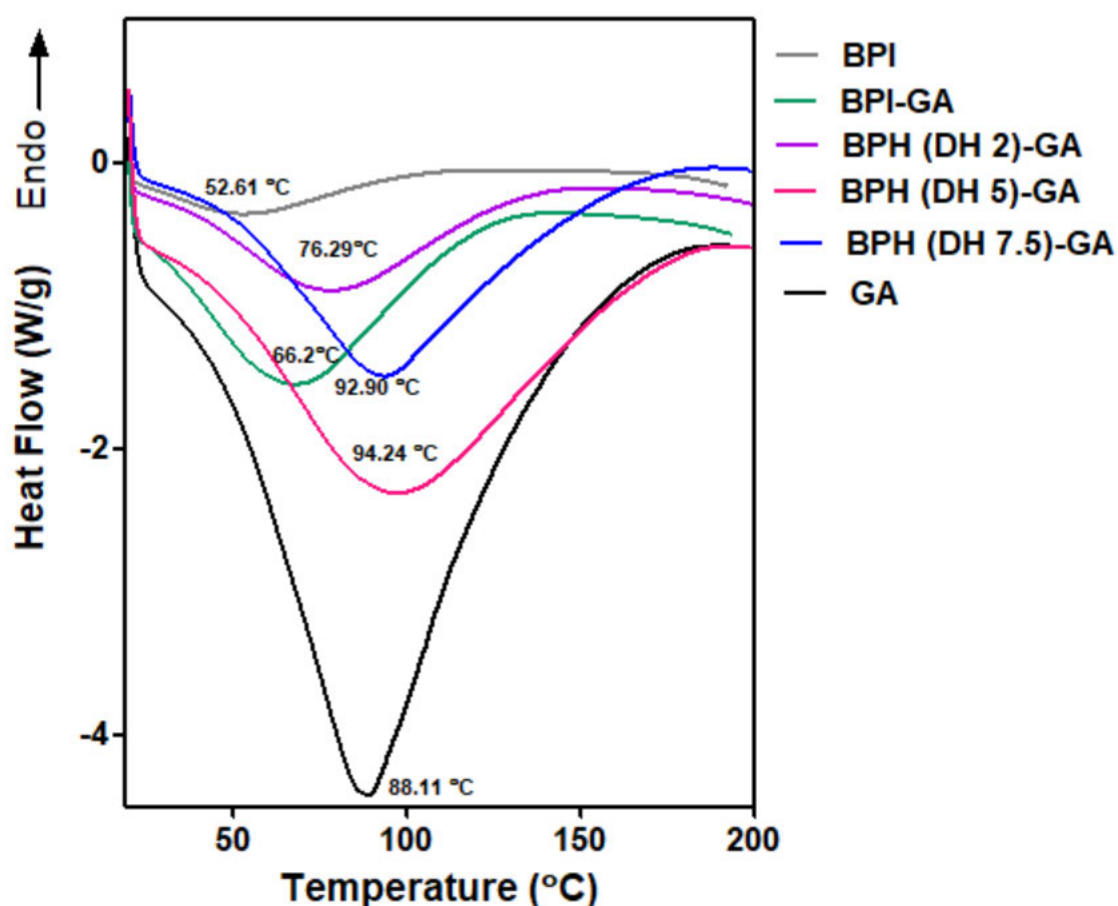


Figure 4.7 Thermal properties of Bambara protein hydrolysates-gum arabic soluble complexes

Table 4.3. Thermal properties of Bambara protein hydrolysates-gum arabic soluble complexes

Samples	Onset temperature, T_o (°C)	Peak temperature, T_d (°C)	Enthalpy, ΔH (J/g)
BPI	20.05	52.61	227.567
BPI-GA	20.16	66.22	1021.40
BPH (DH 2)-GA	20.37	76.29	722.77
BPH (DH 5)-GA	20.15	94.24	2751.10
BPH (DH 7.5)-GA	20.18	92.90	1597.50
GA	20.93	88.11	3478.90

BPI-Bambara protein isolate, GA-gum arabic, BPH- Bambara protein hydrolysates, DH-degree of hydrolysis.

4.4 Conclusion

Limited enzymatic hydrolysis using trypsin was explored to partially unfold the Bambara protein structure and produce more flexible soluble peptides with improved charge characteristics. The pH range of soluble complex formation for all the hydrolysates was substantially broadened with the widest range of formation at DH 5. The observed broad range at DH 5 might be supported by the synergistic effect of limited peptides generated, the increased negative charges and a significant shift in the pH of net neutrality compared to DH 2 and DH 7.5. The increased surface hydrophobicity and a red shift in intrinsic fluorescence might be attributed to the conformational changes of the protein. Overall, limited trypsin hydrolysis at DH 5 may be a better approach due to the wider range of stability with a better network structure in complexation with polysaccharides.

4.5 Research output

a) Journal article

1. Ojesanmi, A. A., & Amonsou, E. O. (2025). Impact of limited trypsin hydrolysis on Bambara protein-gum arabic interaction and the formation of soluble complexes. *Food Bioscience*, 106854.

b) Conference

1. Abiola Adenike Ojesanmi and Eric O. Amonsou. Improvement of Bambara protein-gum arabic interaction and soluble complex formation using tryptic limited hydrolysis. 38th EFFoST International conference, *2024 Future Food System: Innovation through Progress at Scientific Interfaces*, Bruges, Belgium, 12th – 14th November 2024 (Oral presentation).

5 CHAPTER FIVE

IMPACT OF ATMOSPHERIC COLD PLASMA APPLICATION ON BAMBARA PROTEIN AND ITS INTERACTION WITH GUM ARABIC

Abstract

Atmospheric cold plasma, ACP, an innovative nonthermal process, may be a useful tool for biopolymer modification. In this study, atmospheric cold plasma modification of Bambara protein and its interaction with gum arabic were investigated. Specifically, the impact of exposure time on composition, structural changes of protein, and complex behaviour was analysed. Hence, Bambara protein was subjected to atmospheric cold plasma treatment (2, 5 and 10 min) prior to complexation with gum arabic. Positively charged amino acids increased notably throughout the treatment, while a decrease in thiol content weakened the intermolecular forces within the protein structure. Carbonyl content significantly increased in an exposure time-dependent manner, suggesting protein oxidation mediated by the reactive species. A slight transition from α -helix to β -sheet, accompanied by a redshift in fluorescence emission observed, suggesting the denaturation of the protein conformation, correlating with an upward trend in hydrophobicity following the cold plasma treatment. The structural modification led to increased surface charge of the proteins and a remarkable shift of the optimum complexation pH, pH_{opt} of the complexes in the order of 10 min > 5 min > 2 min. The disparity between G' and G'' values rose from < 1 log to ~ 1 log for 10 min treated Bambara protein-gum arabic complex, indicating the development of a robust stable network structure. This research shows that ACP can notably alter protein structure, thus offering significant potential for enhancing pulse protein-polysaccharides complexation behaviour in protein-based foods.

Keywords: Atmospheric cold plasma, exposure time, Bambara groundnut protein, modification, gum arabic, complexation, pH_{opt} .

5.1 Introduction

Atmospheric cold plasma, ACP is a developing non-thermal, environmental-friendly approach useful in addressing safety of food, starch modification and effecting structural changes in protein molecules (Feizollahi et al., 2020; Min et al., 2017; Thirumdas et al., 2017). ACP has some outstanding advantages over other traditional thermal methods in that it has shorter treatment time coupled with lower energy requirements and no thermal damage to physical and nutritional properties of food due to the use of lower temperature (Feizollahi et al., 2018; Misra & Roopesh, 2021; Mandal, Singh, & Singh, 2018). It is a technology that modifies both the structures and functionalities of biomolecules through interactions with reactive oxygen and nitrogen reactive entities, including anions, free radicals, ultraviolet photons, electrons, ions, and other energetic particles (Alabi et al., 2023). These particles can cleave covalent bonds and trigger various chemical reactions, such as crosslinking or cleavage of the protein backbone. Furthermore, amino acid side chains are modified by oxidation, leading to changes in the secondary and tertiary structure of proteins through the cleavage of disulfide and hydrogen bonds (Kopuk et al, 2022; Zhu et al., 2018).

In the past few years, several authors have modified protein using cold plasma to effect structural changes in proteins. For instance, Mehr and Koocheki (2020) reported the introduction of new reactive chemical groups into grass pea protein isolate structure following the treatment using atmospheric cold plasma, leading to the formation of free carbonyl group and di-tyrosine. Surface hydrophobicity of mushroom (*Agaricus bisporus*) was enhanced due to partial protein unfolding during oxidation, which in turn caused aggregation (Zhu et al., 2022). Additionally, Zhou et al. (2023) reported molecular rearrangement of Zein-alginate nanoparticles following the cold plasma treatment resulting to smaller particle size with better dispersibility, larger specific surface area and higher surface charge. However, the limitation of narrow pH range of formation of protein-polysaccharides complexes remains a challenge, as well as complex being formed near the isoelectric point of protein, where some functional properties like emulsification and solubility are poor (Liu et al., 2010). This limits the use of the complexes in specific food systems, such as acidic beverages. Therefore, it is a more feasible approach to investigate the effect of cold plasma on proteins in an exposure time-dependent manner prior to complexation with polysaccharides, with emphasis on the critical pH values for structure formation.

A shift in the critical structure-forming pHs of protein-polysaccharide complexes indicates a change in the charge-charge interactions between the protein and polysaccharide molecules. This directly affects the stability of the complexes and determines their food applications.

The mechanism driving the complex coacervation primarily involves noncovalent intermolecular interaction, where oppositely charged groups on the protein and polysaccharide molecules attract each other, leading to aggregation and precipitation. When the charge neutralization reaches a critical point, optimum complexation pH (pH_{opt}) is attained. The formation of an insoluble complex is sustained as long as oppositely charged groups are present on the biopolymers' backbone. The —COOH groups of polysaccharides interact not only with protein side chains containing polar groups but also with the amine and carbonyl groups of the protein backbone (Fernandez-Alonso et al., 2012; Lopez-Bar et al., 2017). Furthermore, hydrophobic interactions between protein and polysaccharide may be involved, where hydrophobic residues in the proteins could bind to carboxyl groups of polysaccharide granules (Aryee et al., 2018). Ionic interactions can occur between anionic polysaccharides and protein amino acid residues.

Although protein modifications using cold plasma can be found in literature (Santhosh et al., 2024; Wang et al., 2024; Xu et al., 2024), however, to our understanding, no work has demonstrated the potential of ACP treatment in regulating protein functional properties and the influence of cold plasma exposure time on complexation behaviour with gum arabic with focus on the critical structure-forming pHs. Therefore, this study aims to elucidate the mechanism and formation of Bambara protein with gum arabic complexes following cold plasma treatment, identify the impact of exposure time on the critical structures pHs and their rheological properties. Hence, Bambara protein was subjected to direct atmospheric cold plasma treatment as a function of exposure time. The oxidation markers, free-sulfhydryl and carbonyl group were determined to establish the effect of reactive species on the Bambara plasma-treated proteins. Amino acid composition was done to determine the changes in composition and structural changes of the plasma treated proteins were analysed using circular dichroism, surface hydrophobicity and intrinsic florescence.

5.2 Materials and Methods

5.2.1 Preparation of defatted flour

Bambara groundnut defatted flour was prepared following the procedure outlined in section 3.2.2.

5.2.2 Protein isolate extraction from Bambara groundnut

The salt-soluble protein fraction from defatted Bambara flour was extracted according to section 4.2.3.

5.2.3 Cold plasma treatment of Bambara groundnut proteins

The protein samples were exposed to an atmospheric plasma jet operating at an electrical conductivity of 120 V (APJ) (Enercon Industries, Menomonee Falls, Wisconsin, USA). The plasma generated was passed through a heat exchanger connected to a chilling unit. Treatment was done at room temperature (25 °C) for 2, 5, and 10 min in a 150 mL beaker using 32 g of the lyophilized protein isolate for individual treatment time, and the samples were continuously stirred at low speeds. The system operated at an input power of 750 W using nitrogen as the carrier gas. A control sample was not subjected to cold plasma treatment. 2, 5, and 10 min exposure time to plasma were selected in this study, as prolonged treatments have been found to negatively impact protein functionality while also leading to high energy consumption (Mollakhalili-Meybodi, Yousefi, Nematollahi, & Khorshidian, 2021).

5.3 Analysis of cold plasma-treated Bambara proteins

5.3.1 Amino acid composition

The amino acid composition of plasma-treated Bambara groundnut proteins was determined following the procedure outlined in section 3.2.8.1.

5.3.2 Zeta potential

Zeta potential of the cold plasma-treated protein and gum arabic was measured according to section 3.2.8.2. The diluted dispersion of 0.2% w/v was hydrated for 1 h and subsequently filtered through a 0.45 μm HA Millipore membrane prior to analysis.

5.3.3 Intrinsic fluorescence intensity

Intrinsic fluorescence spectra of plasma treated protein was carried out according to section 4.3.4.

5.3.4 Surface Hydrophobicity

Surface hydrophobicity of the plasma-treated protein was done as described in section 4.3.3.

5.3.5 Thiol content quantification

Variations in thiol groups were measured using a modified methodology of Ruzengwe, Amonsou, & Kudanga (2023). Plasma-treated Bambara protein solutions (100 mg/mL) were prepared in 5 mol/L GuHCl/Tris-Glycine, vortexed at 1500 rpm for 10 min using an SI-100 vortex mixer (MRC, United Kingdom), and then centrifuged at $16,000 \times g$ for 5 min. To 100 μL of the supernatant, 150 μL of GuHCl/Tris-Glycine and 50 μL of Ellman's reagent were added. The mixture was incubated at room temperature for 5 min, and absorbance was measured at 412 nm using a Cary 100 CONC UV–Vis spectrophotometer (Varian Inc., USA). A cysteine standard curve was employed to calculate the values, which are expressed as nanomoles of total sulfhydryl per milligram of protein (Li et al., 2013).

5.3.6 Carbonyl content quantification

The carbonyl content of Bambara protein was measured using a modified method based on Xu et al. (2024). Briefly, 0.4 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was mixed with 0.3 mL of protein solution (20 mg/mL in distilled water) and incubated at room temperature for 60 min. Then, 0.7 mL of trichloroacetic acid (TCA) was added, and the mixture was placed in an ice bath for 10 min. Following centrifugation at $11,000 \times g$ for 3 min, the

resulting pellet was washed three times with 1 mL of ethyl acetate:ethanol (1:1, v/v) to remove excess DNPH. The washed pellet was dissolved in 0.5 mL of 6 M guanidine hydrochloride, and the absorbance at 370 nm was measured using a spectrophotometer (Varian Inc, USA). Carbonyl content was expressed as a percentage relative to the control.

5.3.7 Circular Dichroism

Circular dichroism spectra of plasma-treated Bambara proteins were recorded using a J-810 spectrofluorometer (JASCO, Tokyo, Japan). The near-UV CD spectrum measurements were performed at concentration of 1 mg/ml of protein in 10 mM potassium phosphate buffer (pH 7.0) at 25 °C. The sample solution was filtered through a 0.02- μ m membrane before the analysis. The far-UV range (190–240 nm) was used to assess secondary structure, while the near-UV range (250–340 nm) was used for tertiary structure analysis, following the method of Omoni and Aluko (2006). The potassium phosphate buffer solution was used as blank solution for all samples.

5.3.8 Turbidity analysis of complex formation of plasma-treated Bambara proteins with gum arabic

Plasma-treated Bambara proteins and gum arabic (GA) stock solutions were formulated following the method of Busu and Amonsou (2019) with slight modifications. The biopolymers were dispersed separately in Milli-QTM water at 1% w/v and constant stirring for 2 h at 25 °C, left at 4 °C overnight to improve the solubility. Stock solutions were formulated to achieve a total biopolymer concentration of 0.1% (w/w) across a range of BPH: GA blending ratios of 4:1. Glucono- δ -lactone was used to minimize the effect of dilution in the solution (Lin et al., 2022). Changes in optical density OD, were measured as a function of pH (2.0–8.0) at 600 nm using a UV-Vis spectrophotometer with 1 cm path length plastic cuvettes. Critical pH values for the formation of soluble (pH_c) and insoluble ($\text{pH}_{\phi 1}$) protein–polysaccharide complexes, as well as the pH at which complexes dissolve ($\text{pH}_{\phi 2}$), were determined graphically by identifying the intersection of two tangent lines on the turbidity curves (Weinbreck et al., 2003; Liu, Low, and Nickerson, 2009). The pH corresponding to the maximum optical density was designated as pH_{opt} .

5.3.9 Scanning electron microscopy

Plasma-treated and untreated Bambara protein isolate–gum arabic complexes were placed on circular aluminum stubs using double-sided adhesive tape and then coated with a 15 nm layer of gold for 250 seconds. The samples were then examined and imaged using a FEGSEM (Zeiss Ultra Plus, Germany) at an accelerating voltage of 5.0 kV, with the calibrated scale bar on the micrographs used to measure sample sizes.

5.4 Rheological measurement of cold plasma-treated Bambara protein–gum arabic complexes

5.4.1 Frequency sweep

Rheological properties of plasma-treated Bambara protein–gum arabic complexes were measured using a MCR102 rheometer (Anton Paar, Austria) with a 50 mm parallel plate and 1 mm gap, following Deng et al. (2018). Oscillatory frequency sweeps (0.01–100 Hz) were conducted at 25 °C using a 0.5% strain to remain within the linear viscoelastic region (LVR). A thin layer of paraffin oil was applied to all samples to reduce evaporation during measurement. The dynamic rheological behavior of the soluble complex network was assessed through key parameters, namely the storage modulus (G'), loss modulus (G''), $\tan \delta$, and complex modulus (G^*).

5.4.2 Apparent viscosity

The flow behavior of Bambara protein–gum arabic complex solutions was investigated at 25 °C using an MCR102 rheometer (Anton Paar, New Castle, Delaware) (Xing et al., 2024). Shear stress was recorded across a range of shear rates from 1 s⁻¹ to 300 s⁻¹.

5.5 Statistical analysis

Each analysis was carried out in duplicate unless otherwise stated. Experimental data were analyzed by ANOVA, and mean differences were assessed using Fisher's Least Significant Difference (LSD) test at $p < 0.05$.

5.6 Results and discussion

5.6.1 Turbidity measurements of plasma-treated Bambara protein-gum arabic

The complex coacervation behaviour of cold plasma-treated Bambara protein with gum arabic showed some significant differences relative to the untreated protein (Fig. 5.1 A and B). In the absence of protein, gum arabic solutions remained transparent across all pH values, indicating no formation of light-scattering particles (Fig. 5.1A). The turbidity of the untreated Bambara protein and gum arabic was rapidly increased at pH 6 (pH_c) and reached a maximum at about pH_{opt} 4.4, which was due to a significant decrease in the protein surface charge and aggregation as it approached the isoelectric point (4.9).

The turbidity graph for the plasma-treated proteins-gum arabic complexes is significantly different with variations in the optical density, OD, and the degree of aggregation of the proteins. The complexes formed with the plasma protein treated for 10 min depicted lower OD compared to the ones treated for 2 and 5 min which indicates less aggregation (Rashwan et al. 2023). At $pH > pI$ (pH 5 to 8), relatively low turbidity (< 0.034 nm) was observed because only a few protein molecules bind to each anionic polysaccharide. Although a rise in overall negative surface proteins net charge (ζ -potentials profile) was recorded (-6.1 to -30.4 Mv) within the pH activity range, the onset of soluble complexes formation (pH_c) was possible due to the positive surface domain patches of the protein. At this stage, the particles are suspended throughout the medium, and there is random motion that facilitates collision and initiates contact between the particles, leading to adsorption and conformational changes of both the proteins (partial unfolding) and polysaccharides (stretching). More binding sites were created to enhance the non-covalent forces, including hydrogen bonding, electrostatic and hydrophobic interactions with gum arabic particles. Partial denaturation of the protein conformations uncovers more reactive domains (amino acids) to the solvent phase, thus more chances of interactions (or binding) with the polysaccharide (Ghosh and Bandyopadhyay 2012). It is important to note that the rise in the proteins net negative charge at pH above the isoelectric point influenced the delay in the onset of insoluble complex formation resulting in a shift in pH_{opt} for all the plasma-treated proteins at pH 3.5.

At $\text{pH} < \text{pI}$ (pH 3 to 5), there was an increase in protein binding, protein binding increased due to its net positive charge. This indicates a higher protein-binding capacity per mole of gum arabic, which is consistent with the greater charge density at these pH levels, as the protein is net-positively charged below pI . This suggests that the protein binding capacity (number of moles of protein-bound per mole of gum arabic) is higher, which is expected because of the higher charge density. At low pH, complexes can reach electrical neutrality or even undergo charge reversal. Electrical neutrality often results in significant aggregation and increased turbidity, as shown in Fig. 5.1B. Additionally, a single protein molecule may bind multiple polysaccharide molecules, allowing proteins to function as electrostatic bridges (Jones et al., 2010).

At pH 3, where the plasma protein possesses a strong net positive charge and the charge of gum arabic is negative, thus giving rise to the possibility of strong electrostatic complexation between the biopolymers. This explains the high binding affinity between the plasma-treated proteins and gum arabic under pH 3 conditions and the observed shift in optimum complexation pH (pH_{opt} of 3.2, 3.0, and 2.8 for 2, 5, and 10 min treatments respectively) compared to pH_{opt} of 4.4 in untreated protein. This range of pH on the zeta potential profile showed an increase in charge configuration of the biopolymers and crossing points for plasma-treated proteins and gum arabic. This indicates the opening of more binding sites by the reactive nitrogen species. The final stage of structure formation was where the net charge reduced owing to increasing addition of a proton to the carboxyl group of gum arabic limiting the complex formation with the proteins leading to full solubilization of the complexes at pH_{ϕ_2} below pH 2, the acid dissociation constant (pK_a) of gum arabic.

The observed shift in optimum complexation pH was anticipated to result from the role of nitrogen-based reactive species produced during ACP treatment of the protein, including nitric oxide (NO) and nitrogen dioxide (NO_2) (Pirie, 2023). For instance, hydroxyl species can cleave the peptide bonds that connect amino acids in proteins. Additionally, activated atomic oxygen can effectively remove hydrogen atoms from the protein backbone, creating radical sites that promote further interactions and lead to protein fragmentation (Misra et al., 2016). This fragmentation exposes buried hydrophobic amino groups, increasing the protein's flexibility and reactive sites, thereby enhancing interactions with polysaccharides to form a stronger network structure. The reactive species enhance the interfacial properties of both proteins and polysaccharides by altering their composition as well as extending the formation period, ultimately causing a shift in the critical structure-forming pH levels (Sun et al., 2022).

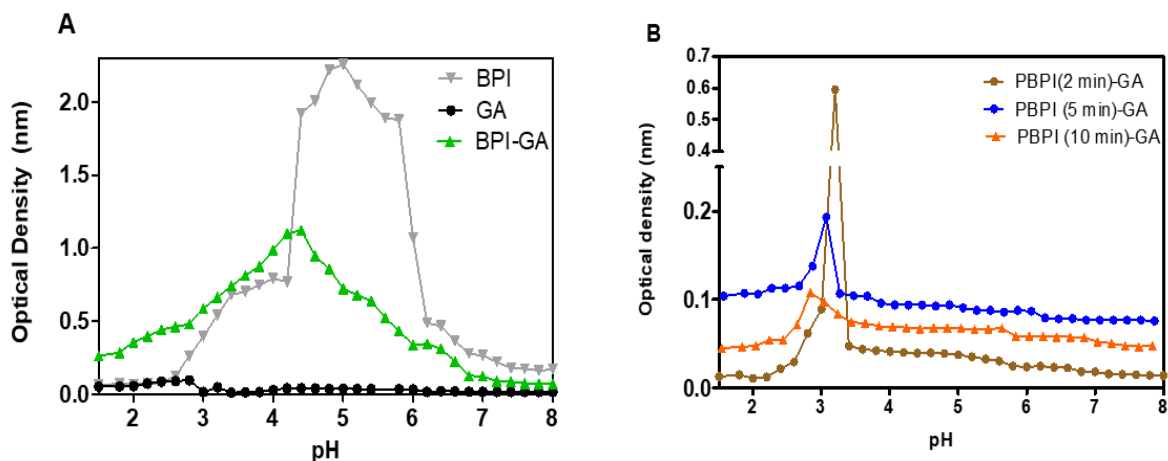


Figure 5.1 Effect of cold plasma treatment on Bambara protein–gum arabic insoluble complexes turbidity as a function of exposure time (A) Untreated Bambara proteins (B) Cold plasma-treated Bambara protein-gum arabic insoluble complexes. BPI- Bambara protein isolate, PBPI-Plasma treated Bambara protein isolates, GA- Gum arabic.

5.6.2 Amino acid composition

Treatment of Bambara proteins with cold plasma for 2, 5, and 10 min resulted in alterations of the profile of the amino acid (Table 5.1). The charged amino acids (proton-donating (acidic) and proton-accepting (basic) side chains and other accessible functional groups of poly-ions may interact molecularly during complex formation (Tolstoguzov, 2017). There was an evident increase in glutamic acid content after 2 min of exposure followed by a decrease up to 4% after 5 min plasma treatment which later increased as the exposure time was extended to 10 min. Aspartic acid increased throughout the duration of the plasma treatment. The oxidation of glutamyl residues can lead to peptide fragmentation and cleavage, as well as α -amidation, affecting the protein's structural integrity and functional properties (Olatunde, Singh, Shiekh, Nuthong, & Benjakul, 2021) which enables better dispersibility of the fragments and gives more uniform interaction with gum arabic. The formation of carbonyl derivatives is usually from lysine, threonine, arginine, and proline side chains (Akagawa, 2021). The impact of the oxidation-induced carbonyl derivatives was reflected in the lysine, arginine, and threonine

content which increased notably throughout the treatment compared to the untreated protein. Aromatic amino acids, phenylalanine, and tyrosine notably went in an upward direction after 2 and 5 min exposure followed by a decrease at 10 min. Amino acids with aromatic residues were reported to be susceptible to oxidation during plasma treatment which subsequently impacts their functional properties (Sruthi et al., 2022). Abarghoei, Goli, & Shahi (2023) gave similar results for atmospheric cold plasma-treated wheat germ protein isolate. Methionine, a sulphur-containing amino acid remained unchanged after 2 min treatment until after 5 and 10 min plasma treatment suggesting the amino acid was minimally affected by hydroxylation. The oxidation of methionine has been linked to conformational changes in proteins modulating their functionalities (Zhang, Xiao, & Ahn, 2013). The observed increase in hydrophobic amino acid content may arise from interactions between reactive species and both the polypeptide backbone and the aliphatic side chains of the proteins hydrophobic amino acid residues which exposed more binding sites to the aqueous environment to enhance molecular interaction with gum arabic. Uncharged proteins such as hydrophobic groups are weakly attracted to anionic polysaccharides indicating their role in complexation (Damodaran, Anand & Razumovsky, 1998). The interaction of the side chains of the amino acids with the reactive species of cold plasma resulted in the opening of the protein structure, resulting in to increase in flexibility and accessibility as well as producing more binding sites for stronger interaction with higher binding affinity with gum arabic (Wenske et al., 2021). The structural modifications following plasma treatments of Bambara proteins explain the molecular mechanisms that caused a delay in the onset of soluble complexes formation, and the resultant significant shift observed in pH_{opt} .

Table 5.1 Amino acid composition of untreated and plasma-treated Bambara proteins in g/100 g protein in relation to exposure time.

Amino acid	BPI (0 min)	PBPI (2 min)	PBPI (5 min)	PBPI (10 min)
His	2.34 ± 0.00 ^a	3.34 ± 0.00 ^b	3.58 ± 0.01 ^b	3.06 ± 0.01 ^a
Arg	5.18 ± 0.02 ^a	8.38 ± 0.00 ^b	8.68 ± 0.00 ^b	8.15 ± 0.01 ^b
Ser	5.08 ± 0.00 ^a	6.32 ± 0.02 ^b	6.40 ± 0.00 ^b	6.13 ± 0.00 ^b
Gly	3.75 ± 0.05 ^a	3.75 ± 0.00 ^a	3.83 ± 0.00 ^a	3.74 ± 0.00 ^a
Asp	8.47 ± 0.01 ^a	10.47 ± 0.01 ^b	10.79 ± 0.01 ^b	11.82 ± 0.03 ^b
Glu	14.03 ± 0.00 ^a	17.33 ± 0.00 ^b	16.65 ± 0.00 ^b	18.50 ± 0.01 ^b
Thr	2.91 ± 0.01 ^a	3.62 ± 0.00 ^a	3.70 ± 0.02 ^a	3.53 ± 0.00 ^a
Ala	3.91 ± 0.00 ^a	3.91 ± 0.00 ^a	3.68 ± 0.00 ^a	3.83 ± 0.00 ^a
Pro	3.01 ± 0.02 ^a	3.61 ± 0.00 ^a	3.58 ± 0.01 ^a	3.69 ± 0.00 ^a
Lys	5.09 ± 0.00 ^a	6.49 ± 0.00 ^b	4.45 ± 0.00 ^a	6.10 ± 0.01 ^b
Tyr	6.70 ± 0.00 ^a	6.90 ± 0.01 ^a	7.53 ± 0.02 ^a	6.85 ± 0.01 ^a
Met	2.46 ± 0.02 ^a	2.46 ± 0.02 ^a	2.70 ± 0.01 ^a	2.63 ± 0.03 ^a
Val	4.34 ± 0.01 ^a	4.58 ± 0.00 ^a	4.35 ± 0.00 ^a	4.36 ± 0.03 ^a
Ile	4.15 ± 0.00 ^a	4.05 ± 0.00 ^a	4.17 ± 0.01 ^a	4.20 ± 0.00 ^a
Leu	7.60 ± 0.00 ^a	7.60 ± 0.01 ^a	7.65 ± 0.02 ^a	7.69 ± 0.02 ^a
Phe	7.52 ± 0.02 ^a	7.88 ± 0.01 ^a	8.58 ± 0.03 ^a	7.72 ± 0.00 ^a
HAA	36.74 ± 0.00 ^a	37.84 ± 0.00 ^a	38.54 ± 0.00 ^b	37.66 ± 0.00 ^a
NCAA	22.50 ± 0.01 ^a	27.80 ± 0.03 ^b	27.44 ± 0.03 ^b	30.32 ± 0.01 ^c
PCAA	12.61 ± 0.01 ^a	18.21 ± 0.02 ^b	16.71 ± 0.00 ^b	17.31 ± 0.00 ^b
AAA	14.22 ± 0.03 ^a	14.78 ± 0.00 ^a	16.11 ± 0.03 ^b	14.57 ± 0.00 ^a

HAA = hydrophobic amino acid, PCAA = positively charged amino acid, NCAA = negatively charged amino acid, AAA = aromatic amino acid, BPI-Bambara protein isolate, PBPI-Plasma treated Bambara protein isolate. Mean ± SD (n = 2) Values in the same row with the same superscripts are not significantly different.

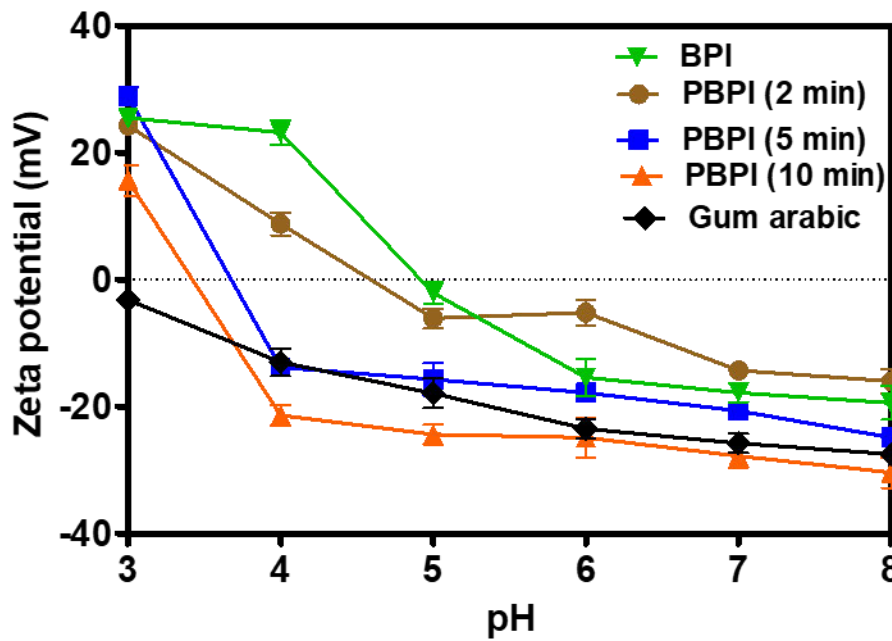


Figure 5.2 Effect of cold plasma treatment on zeta potential profile of Bambara proteins as a function of exposure time. BPI- Bambara protein isolate, PBPI-Plasma treated Bambara protein isolates

5.6.3 Zeta potential

The ζ -potentials of each biopolymer dispersion was measured to investigate the underlying mechanism behind the shift in pH_{opt} and electrostatic force between Bambara proteins and gum arabic. The negative surface charge of the plasma-treated Bambara protein increased with an increase in exposure time compared to the untreated protein (Fig. 5.2). The rise in negative charges may result from the conversion of positively charged peptide units by the reactive oxygen species to neutral ones, which were oxidised to negatively charged groups (Stadtman 2006; Liu et al. 2015). This is comparable to the report of Yu *et al.* (2021) and Alabi, Annor, and Amonsou (2023) in the plasma-treated peanut protein-sesbania gum conjugates and plasma-activated water-treated Bambara globulin, respectively, where a higher number of negatively charged carboxyl groups were introduced on the functional groups of amino acids, thereby increasing the negative surface charge of the proteins. Higher negative charges were detected at 10-minute plasma exposure, which suggests a higher density of chemical reactive species. This might have increased oxidation of the amino acid residues thereby converting them to have more negatively charged residues. Moreover, a significant shift in isoelectric point

was observed from 4.9 to 4.8, 3.6 and 3.4 for 2, 5 and 10 min plasma-treated proteins, respectively. This was reflected in the variation observed within the amino acid profile of the proteins (Lautenbach, Hosseinpour and Peukert 2021). The decrease in pH caused by plasma exposure could be attributed production certain acidic substances. Acidic H_3O^+ ions can be generated through the reaction of water molecules with hydrogen peroxide (H_2O_2) produced during plasma treatment, while nitric acid (HNO_3) and nitrous acid (HNO_2) can be produced from nitric oxide (NO) via its oxidation to nitrogen dioxide (NO_2) (Ji et al., 2018). The increased negative charges of the plasma-treated proteins must have contributed to the extension of the period of formation of soluble complex leading to the shift in pH_{opt} observed in the turbidity profile.

5.6.4 Surface hydrophobicity

Surface hydrophobicity was carried out to determine the structural changes regarding the number of surface-exposed hydrophobic regions of the protein exposed to polar solvents. The hydrophobicity of the untreated protein was substantially lower relative to the plasma-exposed proteins with the highest obtained value for the protein treated for 10 min (Fig. 5.3A). The increase in S_o observed is due to the molecular protein configuration being partially unfolded as a result of their interactions with free radicals, exposing the non-polar groups which were previously positioned within the inner region structure of the protein (Ji et al., 2018). The plasma-induced oxidation exposed the hydrophobic groups making more reactive sites available to enhance stronger binding with gum arabic (Segat et al., 2015). Li et al. (2020a) described hydrogen bonds in the protein secondary structure being broken after the cold plasma treatment, leading to a relaxed structure with greater exposure of reaction sites. The more hydrophobic groups available to interact with gum arabic, the stronger the interaction and the more the formation of insoluble complex, hence the shift in pH_{opt} towards the acidic pH. Although electrostatic attraction is the primary factor responsible for the complexation between protein-polysaccharide, it is also reported that hydrophobic interactions and hydrogen bonds serve a secondary function in maintaining the stability of the “protein-polysaccharide” insoluble complexes (Schmitt et al., 1998). Moreover, hydrophobic forces have been reported as an important force for the thermal stabilization of complexes and increased the adsorption affinity of amphiphilic proteins (Keith-Roach 2008; Matsumiya et al., 2017).

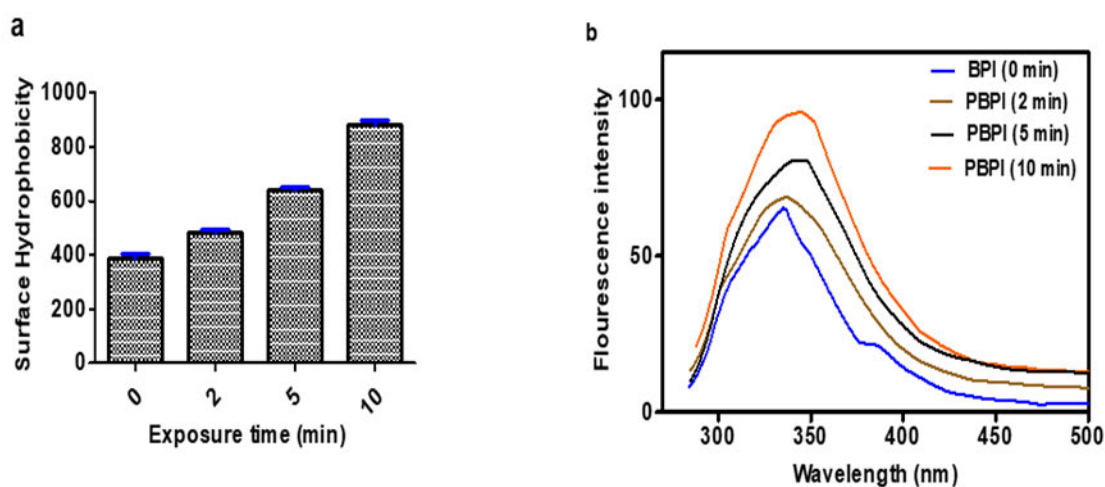


Figure 5.3. Surface hydrophobicity and intrinsic fluorescence spectra of cold plasma treated Bambara protein. PBPI- Plasma treated Bambara protein isolates, BPI-Bambara protein isolates.

5.6.5 Fluorescence emission spectra

The modifications in the three-dimensional structure of the Bambara protein were analyzed using intrinsic fluorescence spectroscopy (Fig.5.3B). A shift in the maximum intensity fluorescence from 335 nm (untreated sample) to 340 nm, 342 nm and 345 nm for proteins treated at 2, 5 and 10 min respectively was observed suggesting the tryptophan residues dominance. The treatment with plasma increased the FI_{max} of Bambara proteins throughout the exposure period. The red shift detected in fluorescence intensity after the plasma treatment suggests the conversion of tryptophan from non-polar to more polar in the aqueous solution which assists in having more reactive sites and strengthens the molecular interactions (Bubler, Ehlbeck and Schluter 2016; Mehr and Koocheki 2020). An upward trend in the fluorescence intensity has been linked to oxidative change of proteins (Xu et al., 2018a). The conformational modification indicates an increase in the intermolecular hydrophobic interactions during the treatment which agrees with the surface hydrophobicity data obtained in this study. Tertiary structural changes in Bambara protein following the use of plasma were reported to originate from the breakage of disulfide bridges and disruption of the conformation of protein (Alabi,

Annor and Amonsou 2023). This has consequently influenced the complexation behaviour of the plasma-treated proteins with gum arabic.

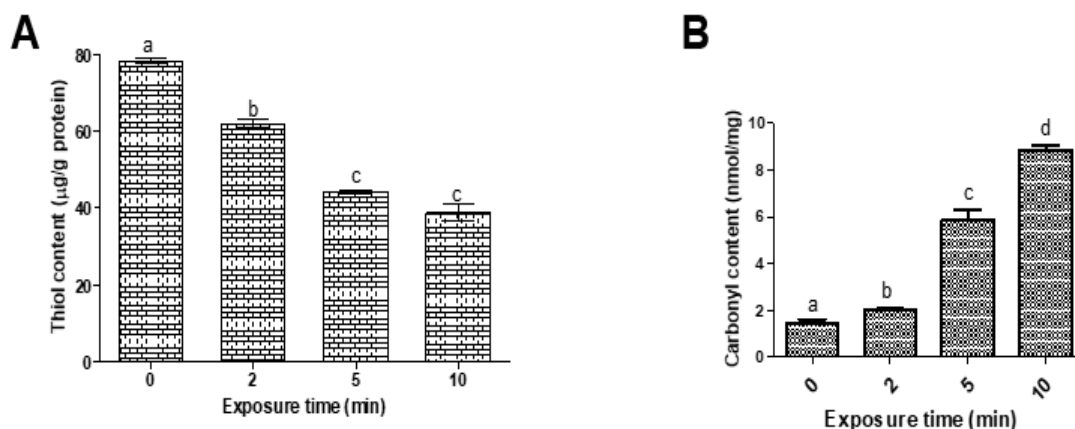


Figure 5.4. Influence of cold plasma treatment on thiol and carbonyl content of Bambara proteins with respect to exposure time.

5.6.6 Thiol content

Thiol concentration in the proteins was notably reduced following plasma treatment in an exposure time-dependent manner (Fig.5.4A). The highly reactive thiol group ($-\text{SH}$) of cysteine undergoes an oxidation reaction in the presence of reactive nitrogen species to form disulphide linkages, which can exert pronounced effects on the stability and folding of proteins (Feige, Braakman, and Hendershot 2018). The rate at which the amino acid functional group is oxidized is influenced by peptide bond flexibility, the three-dimensional conformation of the protein, the surface accessibility of amino acid residues, and the concentration of the oxidizing agent (Bommana et al., 2017). The thiol-modified state weakened the intermolecular forces within the protein structure by breaking the cross-linking disulphide bonds, thus enhancing the protein flexibility and solubility of the protein conformations (Olatunde et al., 2023). Hence, protein flexibility is anticipated to open up the protein structure to solvents and increase the accessibility for intramolecular interactions (Ravi et al., 2014). A decrease in covalent interactions within the protein molecules might allow a raise in non-covalent protein interactions with polysaccharides, leading to higher binding energy between the biopolymers,

thus elongating the formation of insoluble complex formation. This explains the shift in optimum complexation pH (pH_{opt}) of the insoluble complex as observed in the turbidity profile.

5.6.7 Carbonyl content

The content of carbonyl is a valuable index to evaluate the degree of oxidation following exposure to atmospheric cold plasma. The untreated Bambara proteins exhibited low content of carbonyl, which significantly increased in the order of 2 min > 5 min > to 10 min after plasma treatment, suggesting protein oxidation caused by the reactive species (Fig.5.4B). This may arise from the presence of charged particles becoming more energetic and denser with longer exposure time, thereby increasing the ability of reactive species to change the side chain of amino acid residues (Qin, Ting and Wendt 2010). Yu et al. (2020) reported a drastic increase in the carbonyl composition of flaxseed proteins after plasma treatment. According to Zhang et al. (2021a), the elevated levels of carbonyl residues following plasma treatment are attributed to peptide chain cleavage, resulting in protein fragmentation. Having smaller protein fragments increases the flexibility, accessibility and dispersibility of the proteins which assist in the binding with the polysaccharides during complexation. In addition, an increased number of carbonyl groups may be formed through the release of free radicals, caused by hydrogen abstraction initiated through the attack of reactive species on protein methylene carbons (Choe and Min 2006). Estévez (2011) reported the increased formation of carbonyl groups mediated by the oxidation of side chains of proline, arginine, threonine, and lysine. The conformational changes resulted in protein unfolding and a more flexible structure (Segat et al., 2015), thus enhancing the binding of plasma-treated Bambara proteins with gum arabic.

5.6.8 Circular dichroism spectroscopy

Circular dichroism was adopted to examine the influence of atmospheric cold plasma with respect to the secondary structure of the Bambara protein (Fig. 5.5). The untreated protein showed a far-UV CD spectrum exhibited distinct dichroic features, including a negative peak at approximately 195 nm, a positive peak near 207 nm, and a zero-crossing point around 200 nm. These wavelength ranges are characteristics of structural order that is predominant of alpha-helix structure (Mehr & Koocheki, 2020) and its similar to (Feyzi, Milani & Golimovahhed 2018) report on grass pea plasma treated protein. The plasma-treated protein indicated new positive peaks centered at approximately 202 nm, 210 nm, and 203 nm after 2,

5 and 10 min direct exposure to plasma treatment, respectively, with a rising pattern in the negative peak intensity observed near 210, 219 nm and 215 nm, respectively. The new peaks reflected the presence of β -sheet structure. A gradual reduction in α -helix structure with a corresponding elevation in random coil was reported for cold plasma-treated soybean protein (Zhang et al., 2020). The plasma treatment changed the molecular hydrogen linkages which maintains the secondary conformation of the plasma-treated protein and it showed to be an effective method to restructure the protein and optimize stability. A structural change from α -helical to β -sheet conformation indicates that the structure of the plasma protein was opened and became more stretched during the treatment. Protein glycosylation is mainly the condensation of the amino group in the protein and the carbonyl functional group in the sugaramino group of the protein and the carbonyl of the sugar, the α -helix is consumed in this process to stretch the protein structure (Li et al., 2013).

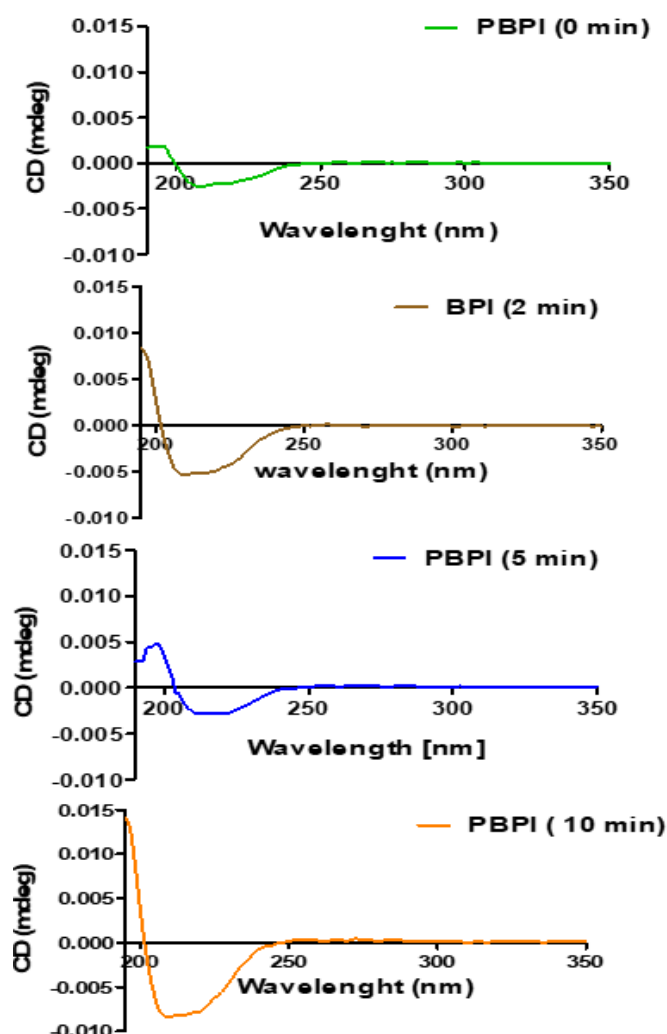


Figure 5.5. Circular Dichroism spectra for cold plasma treated Bambara proteins as a function of exposure time. BPI-Bambara protein isolates, PBPI-Plasma treated Bambara protein isolates.

5.7 Frequency sweep and apparent viscosity

Frequency sweep of the plasma-treated protein-gum arabic complexes was done to determine the degree of molecular linkages that occurred among the biopolymers. The accessibility of Bambara protein subjected to atmospheric cold plasma influenced the viscoelastic parameters across an angular frequency of 0.1 – 10 rad/s (Fig. 5.6A). In the linear viscoelastic range, LVR, the storage modulus (G') and loss modulus (G'') plasma treated Bambara proteins-gum arabic insoluble complexes increased with an increase in frequency. The insoluble complexes formed for every proteins had G' values exceeding those of G'' over the angular frequency range tested indicating a predominance of an elastic soft solid nature for the complexes (Behrouzain, Razavi, & Joyner, 2020). Atmospheric cold plasma treatment increased the difference between G' and G'' values of the insoluble complex ranging from below 1 log (untreated Bambara proteins-gum arabic insoluble complex) to ~ 1 log after 10 min exposure time. This indicates the development of a stable structured network following an exposure time of 10 min. The greater G' readings noted in the plasma-treated proteins (5 min) -gum arabic insoluble complexes compared to complexes formed with protein treated for 2 min and 10 min can results from protein polymerization and enhanced disulfide bonding as evidenced by the reduced levels of free thiol groups. Moreover, the higher G' signifies a higher degree of internal structure, making the complex more stable (Chen et al., 2024). In addition, since the structure of the complex was not destroyed (within LVE), higher G' displayed by the complex formed with protein treated for 5 min suggests higher elastic character and stronger molecular interactions within the system. Attractive interactions such as electrostatic (the predominant force), hydrophobic interactions, van der Waals, and hydrogen bonding between functional groups on the biopolymers are possible collective forces that may be responsible for the higher elastic character (Goh, Teo, Sarkar & Singh, 2020). Partial denaturation resulted in surface exposure of hydrophobic groups following cold plasma treatment. This suggests that hydrophobic interactions take place between the methyl ester groups of gum arabic and the newly exposed hydrophobic regions on the treated Bambara proteins. These interactions are facilitated by the structure of gum arabic, whose branches commonly end in rhamnose and/or glucuronic acid (Williams, 2011). $\tan \delta$ for all the complexes was within 0 and 1 (Fig. 5.6B),

$\tan \delta$ less than 1 suggests that the particles are highly associated due to the colloidal forces such as electrostatic forces, repulsive and attraction forces and van der Waals forces (Liang, Hilal, Langston, and Starov, 2007).

Apparent viscosity is a useful parameter and has different beneficial applications in the characterizing of food fluids, especially for shear-thinning fluids (Rao, 2000). Following the plasma treatments, the apparent viscosity of the complexes reduced with the increment of shear rate from 0 up to 100 s^{-1} leading to a rapid breakdown on initial shearing (Fig. 5.7). This flow behaviour indicated a non-newtonian shear-thinning flow behaviour. The reduced viscosity originates from the “adhesion” between interacting charged groups, which increases the fluidity of the complex. Following the reduction, a slower modification of the structure occurs at high shear rates. This could be ascribed to the synergistic action of the breakdown and reformation of weak linkage between plasma proteins and polysaccharides as a result of molecular collisions and Brownian motion which occurred during complexation (Xiang, Simpson, Ngadi, & Simpson, 2011).

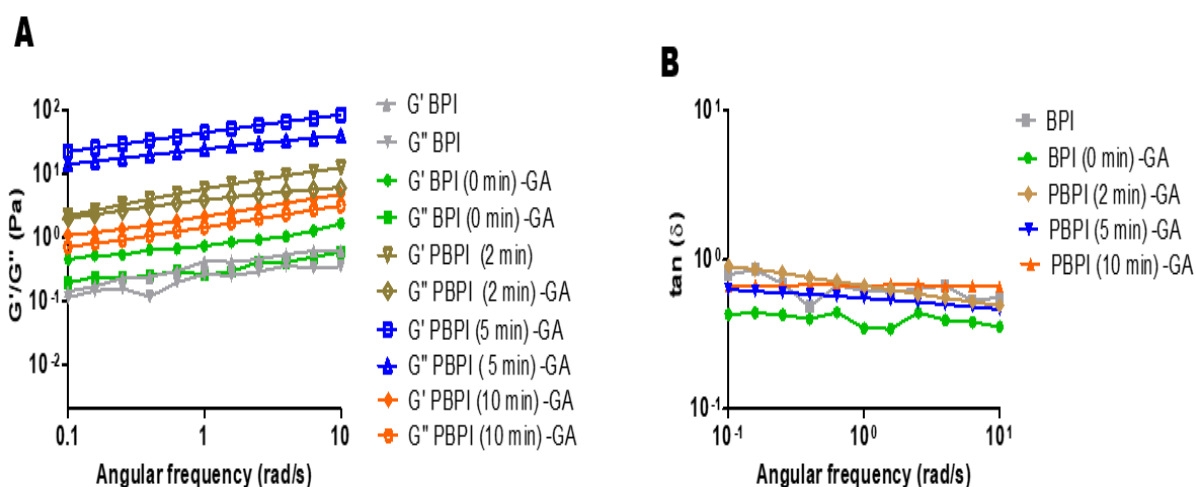


Figure 5.6. Frequency sweep curve of plasma treated Bambara protein -gum arabic soluble complexes showing dynamic storage modulus, G' and Loss modulus, G'' (B) $\tan \delta$. BPI- Bambara protein isolate, PBPI- Plasma treated Bambara protein isolates, GA- Gum arabic.

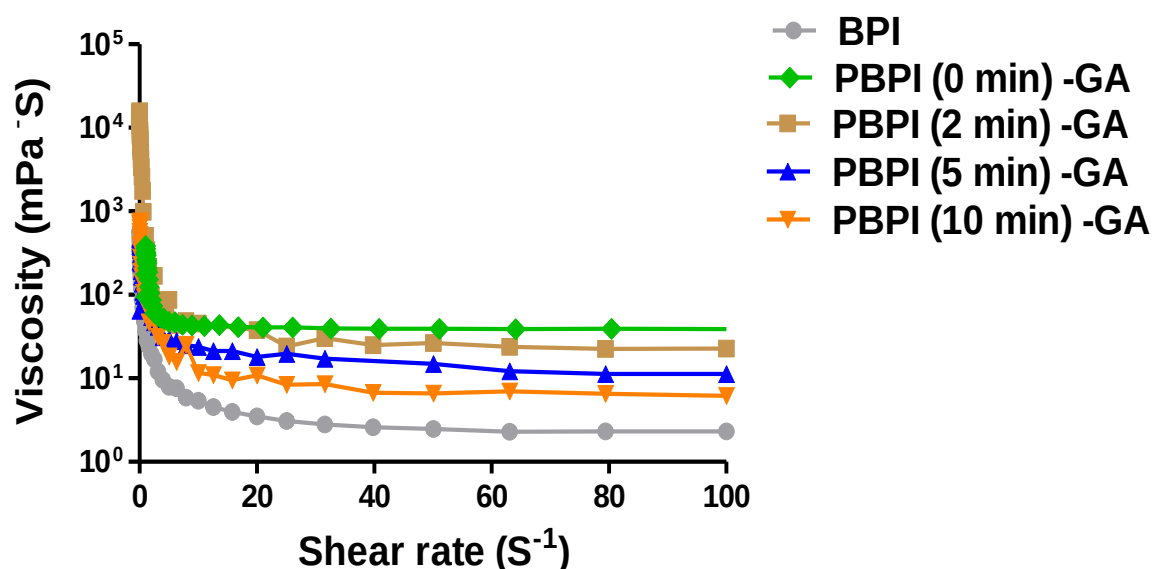


Figure 5.7. Apparent viscosity of cold plasma treated Bambara protein -gum arabic complexes. BPI- Bambara protein isolate, PBPI- Plasma treated Bambara protein isolates, GA- Gum arabic.

5.7.1 Scanning electron microscopy, SEM

Bambara proteins treated with plasma indicated substantial morphological disparities within protein particles transitioning from tightly packed to a looser configuration relative to the control samples (Fig.5.8). The complexes showed strands of a network-like structure, with aggregated particles attached. SEM results demonstrated that plasma proteins were trapped in the network formed by gum arabic. This was likely arising from the enlargement of the treated polypeptides after exposure to plasma reactive species (Ji et al., 2022; Zhou et al., 2023a), which caused the hydrophobic regions to become more exposed as reported in the surface hydrophobic data of this study (Dong et al., 2017). These findings indicate that plasma-induced collisions, friction, and shear by reactive species modified the surface morphology of Bambara proteins.

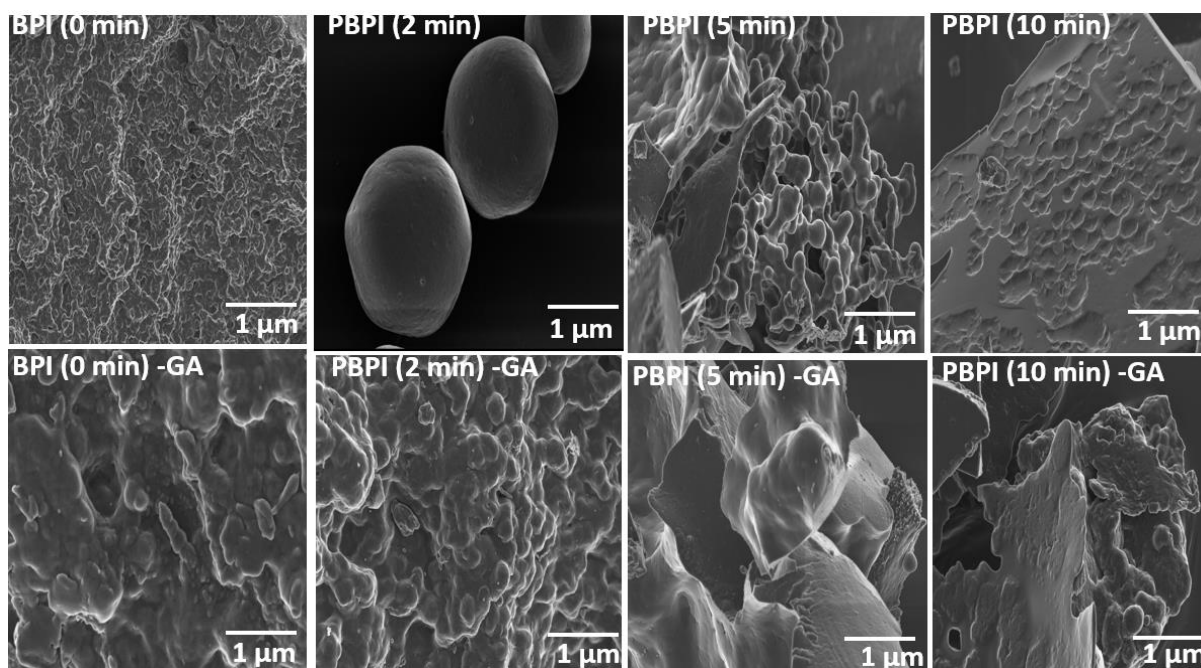


Figure 5.8. SEM micrographs of untreated Bambara protein isolate, plasma treated Bambara protein isolates- Gum arabic soluble complexes. BPI- Bambara protein isolate, PBPI- Plasma treated Bambara protein isolates, GA- Gum arabic.

5.8 Conclusion

Atmospheric cold plasma treatment of Bambara proteins prior to complexation with gum arabic introduced functional groups in the protein structure that induced conformational changes and alteration of surface charges in exposure time dependent manner. Remarkable shift was observed in the pH_{opt} towards the acidic pH following plasma treatment in exposure dependent manner. A concomitant elongation of the period of insoluble complexes formed was observed and a delay in the onset of insoluble complex formation. The pH at which the complexes are formed is crucial, as it determines their potential food applications. Complexes formed at an optimal pH (pH_{opt}) of 3.2 and 3.0 could be useful in acidic beverages such as energy drinks, soda, diet colas, and certain fruit drinks. Additionally, they may serve as fat alternatives in reduced-fat formulations like yogurt. Plasma treatment for 5 min could be recommended for a more stable structure as longer treatment notably reduced the pH_{opt} to strong acidic pH (2.8)

which might not be desirable for certain acidified foods. Plasma treatment induced crosslinking of Bambara proteins, as displayed by the decline in thiol levels, increased maximum fluorescence intensity, greater surface hydrophobicity, and oxidation of some of the amino acids. A new positive peak at around 202 nm, 210 nm and 203 nm after 2, 5, 10 min direct exposure to plasma treatment, reflecting a transformation from α -helical arrangements to β -sheet structures following plasma treatment. Atmospheric cold plasma treatment resulted in a substantial stable and structural network build up.

5.9 Research output

1. Atmospheric cold plasma modification of Bambara groundnut protein and its interaction with gum arabic: impact of exposure time. Submitted on 17th of March 2025 to Food structure: under review (see the appendices).

6 CHAPTER SIX

ENCAPSULATION, RELEASE PROPERTIES AND MICROSTRUCTURE OF BAMBARA PROTEIN-VIT B2-GUM ARABIC COMPLEXES

Abstract

This investigation explored the prospect application of Bambara hydrolysed proteins - gum arabic (BPH (DH 5)-GA) soluble complex as a delivery system for riboflavin in a model beverage. Encapsulating and release properties, microstructure and flow behaviour of Bambara protein hydrolysate (DH 5)-GA soluble complexes in model beverage were determined. Riboflavin was used as the bioactive compound.

Bambara protein hydrolysate (DH 5)-GA soluble complex was loaded with riboflavin and characterized by confocal laser microscopy scanning, CLMS, encapsulation efficiency, release property and viscosity profile. Storage stability of the carrier complexes was done by measuring the particle size distribution and polydispersity index, PDI, of the model beverage over a period of 7 days. A higher number of smaller complex particles were visible in the BPH-Vit B2-GA complex relative to the complexes from BPI-Vit B2-GA and BPI-Vit B2 possibly due to variations in the electrostatic behavior of the carrier wall polymers. Encapsulation efficiency, EE, of BPH (DH 5)-Vit B2-GA complex was notably higher (78%) than EE for BPI-Vit B2-GA and BPI-Vit B2 complexes (50 % and 41% respectively). A rapid release progression was observed in BPI-Vit B2 and BPI-Vit B2-GA complexes, while BPH (DH 5)-Vit B2-GA complex had delayed release due to tighter core-shell structure from the stabilising bonds. The apparent viscosity profile for all the complexes seems similar, ranging from 10^1 to 10^3 Pa^{-s} and depicting non-newtonian shear-thinning behaviour. A viscous biopolymer matrix can act as a reservoir, slowing down bioactive compound release. The confocal laser scanning microstructure of the BPH-Vit B2-GA complex showed a higher number of smaller particles compared to BPI-Vit B2-GA complex which showed fewer larger particles, indicating weak complex structure formation. This might be caused by the enhanced molecular flexibility of the partially hydrolysed protein having more building sites available for interaction. The particle size distribution showed that BPH (DH 5) -gum arabic soluble complexes had greater stability against aggregation compared to the complexes formed with unhydrolyzed Bambara protein isolates.

6.1 Introduction

Encapsulation technology is an important method for incorporating unstable bioactive components such as probiotics, proteins, vitamins, and other bioactive compounds into food systems (Rezvankhah et al., 2024). It improves the stability and edibility of food formulations (Zhang et al., 2020). Compared to conventional processing techniques, encapsulation technology provides an effective shield of the core material from the external environment, thereby largely preserving the functionality of active components in food (Bamidele & Emmambux, 2021). Moreover, encapsulation may aid in coating part of the bitter taste/unpleasant additives and control the wall material digestion, by regulating the release rate of the core material (Kumar et al., 2022).

There are three major issues facing the food industry, problems of instability in foods such as protein and lipids oxidation, degradation of active chemicals, and discolouration of pigments. In addition, the problem of controlling the release of probiotics, additives, and other active compounds and their effective delivery. Furthermore, how to control the organoleptic characteristics of food through adsorption or concealing flavour and odour elements. Encapsulation has been proven to address these problems in the food production. However, encapsulation faces challenges such as high energy use, environmental impact, costly preparation, and low encapsulation efficiency, instability and inability to precisely control the release (Xu et al., 2024).

In recent times, protein-polysaccharides coacervates have received growing interest to effectively encapsulate, safeguard, and regulate the release of bioactive compounds. For instance, in the encapsulation of hydrophobic (DHA) Docosahexaenoic acid molecules (an omega-3 fatty acid) using β -lactoglobulin (β -LG) in combination with low-methoxy pectin nano complexes, a prevalent role was played by β -lg because of its ability to bind ligands. Moreover, the electrostatic interaction prevented precipitation of the protein approaching its isoelectric point and DHA was protected against oxidation compared to the homogenous β -lg, however, the thermal stability of the complex was not investigated (Zimet & Livney, 2009). Another study used core-shell nanoparticles composed of zein and κ -carrageenan designed to encapsulate coenzyme Q10 and piperine, following the crossing of κ -carrageenan layer. Although the encapsulated compound exhibited improved thermal and light stability, including delayed release during in vitro digestion, the encapsulation efficiency of the complex was not evaluated (Chen et al., 2020).

Additionally, microcapsules made from pea protein-sugar beet pectin Coacervates prepared at pH 3.5 exhibited lower encapsulation efficiency compared to those formed at pH 2.5. Nonetheless, the microcapsules made at pH 2.5 had cavities and partially fragmented particles, which reduced the protection of the encapsulated oil from oxidation (Lan, Ohm, Chen & Rao, 2020). These results indicated that the pH at which coacervation occurred had a significant impact on the microstructure of the resulting coacervates and consequently reduced the encapsulation efficiency and oxidative stability.

To address the challenge of encapsulation efficiency and stability, Bambara protein (DH 5)-gum arabic soluble complex was explored to encapsulate riboflavin in a model beverage. The soluble complex (DH 5) was shown to enhance a wider pH range of soluble complex formation with better network structure and improved stability (chapter 4). However, the encapsulation efficiency and release characteristics of the resulting complex have not been explored. Hence, the encapsulation and release properties of riboflavin were evaluated using Bambara protein hydrolysate–gum arabic soluble complexes, with BPI–GA complexes serving as the reference.

6.2 Materials and methods

6.2.1 Materials

Bambara groundnuts, *vigna subterrenea* were sourced from Jozini, province of Kwazulu-Natal in South Africa. Vitamin B2 was purchased from Sigma-Aldrich and was used as a model bioactive compound. Additional laboratory-grade chemicals were supplied by Sigma-Aldrich.

6.2.2 Bambara flour preparation

The preparation of Bambara flour was carried out as detailed in Section 3.2.2.

6.2.3 Extraction of Bambara groundnut protein isolate.

Seed storage protein extracted from Bambara fat-removed flour was extracted according to the method used in section 4.2.3.

6.2.4 Bambara protein -Vit B2- gum arabic complexes carrier preparations

Bambara protein (DH 5)- gum arabic soluble complex was prepared by acid titration with the exception that 0.1% w/v of Vit B2 (Liao et al., 2021) was mixed before the inclusion of 0.1% (w/v) gum arabic. The BPH (DH 5)-GA-VB2 was homogenised using an MFC ultrasonic Homogeniser (SONIC-150W). The sample was wrapped in aluminium foil to protect the riboflavin stock solution from light exposure. The pH of the resulting mixture was adjusted to 4.6 (pH_{cmax} for the soluble complex). The complex was left to stir for 30 min, freeze-dried in a freeze-dryer. This was repeated for protein isolate-gum arabic complex and homogenous Bambara protein isolate. The complex carriers were stored in an airtight vessel at 4 °C for further analysis.

6.2.5 Encapsulation Efficiency

Encapsulation efficiency (EE) was analysed using the approach reported by Muhoza et al. (2019) with slight modifications. In brief, 0.5 g of freeze-dried BPH-VB2-GA soluble complex was combined to 20 mL of Milli Q water then subjected to centrifugation at 14,000 × g for 10 min. After centrifugation, a filtrate with free vitamin and a concentrate with nanoparticles with encapsulated vitamin was obtained. These were used for EE determination. To calculate EE, the filtrate was assayed spectrophotometrically at 437 nm, which corresponds to the absorbance peak of riboflavin (Azevedo et al., 2014), and the amount of free vitamin was calculated from the standard calibration curve of riboflavin. Encapsulation Efficiency was calculated as follows:

$$EE \% = \frac{Vit_{total} - Vit_{free}}{Vit_{total}} \times 100$$

where Vit_{total} represents the total amount of vitamins; Vit_{free} is the amount of free vitamins in filtrate.

6.2.6 BPH-VIT B2-GA model beverage preparation

The beverage model was prepared using the method described by Molaveisi et al. (2021) with slight alterations to beverage pH. The food model included 20% w/v sucrose, 1% w/v citric acid, and 0.3% w/v ascorbic acid. The pH of the beverage was regulated to 4.6 (optimum pH for soluble complex) with 1 mol/L NaOH solution or HCl solution.

6.2.7 Controlled release property of BPH-Vit B2-GA complex

The release of vitamin B2 from the prepared complex was investigated according to Heydari-Majd et al. (2023) with slight modifications in a model beverage solution with a pH of 4.6. Briefly, 200 mg of the complex loaded with vitamin B2 was suspended in a beaker containing 20 mL of the model beverage. At regular time intervals, 3 mL of the collected solution was analyzed for absorbance at 437 nm using a UV–vis spectrophotometer. The fresh solvent with the same volume was immediately injected into the beaker. The release of the vitamins from the prepared complex was calculated using the equation:

$$\text{Release (\%)} = M_t / M_0 \times 100$$

where M_t is the concentration released at time t , and M_0 is the total loaded concentration.

6.2.8 Particle size distribution of carrier complexes

Particle size measurements of the samples were conducted using a Litesizer Nano ZS (Anton Paar, New Castle, Delaware) with the hydro EV Flexible volume wet dispersion. In brief, the 2 mg/mL sample dispersions were subjected to hydration for 1 hour in the model beverage at pH 4.6. Samples were passed through a 0.45 μm HA Millipore membrane, before analysis with particle sizes expressed as 10% (D_{10}), 50% (D_{50}), and 90% (D_{90}).

6.2.9 Viscosity profile of the carrier complexes

The samples (2 % w/w total biopolymer concentration) were placed on a dynamic rheometer (Anton Paar, Physica MCR 301, Austria) operating in oscillatory mode with a 25 mm parallel

plate setup. The plate separation was kept constant at 1 mm, and all flow and viscoelastic behavior parameters, including apparent viscosity, storage modulus, and loss modulus, were averaged over three replicates. The flow behavior was assessed using the method of Xing et al. (2024). They were first equilibrated between parallel plates for 30 seconds to achieve 25 °C. Viscosity measurements were taken while the shear rate increased linearly from 0.1 s⁻¹ to 500 s⁻¹ over 330 s, and the data were plotted on a logarithmic scale showing apparent viscosity (η) versus shear rate ($\dot{\gamma}$).

6.2.10 Confocal laser scanning microscopy, CLSM

The complexes microstructure was investigated using CLSM (LSM 710, Carl Zeiss AG, Oberkochen, Germany) according to Zhang et al. (2024). Samples were dyed with FITC and Rhodamine B at 25 °C. Dyed samples were excited by 494 and 555 nm for FITC and Rhodamine B dyeing, respectively.

6.3 Result and discussion

6.3.1 Encapsulation Efficiency

The encapsulation efficiency (EE) of the BPH Vit B2-GA complex was 78 % while that of BPI-Vit B2-GA and BPI-Vit B2 complexes were 50% and 41% respectively (Fig. 6.1). Homogeneous BPI exhibited the lowest encapsulation efficiency, whereas the higher EE of BPH-GA complexes likely reflects stronger electrostatic interactions, as shown by the turbidity profile. As a result of these interactions, a more stable structure was formed giving high binding ability of the BPH-GA complex with riboflavin. In a study by Djoullah and Saurel (2021), riboflavin was successfully encapsulated in pea protein microparticles crosslinked with transglutaminase, which improved the stability and controlled release of the vitamin. Djoullah and Saurel (2021) reported an encapsulation efficiency of 84% on 0.1% loaded riboflavin which is comparable to what was achieved in this study. Moreover, in a study conducted by Hua, Chiang and Henry (2022), Kappa-carrageenan (k-carr) served as the polysaccharide in the complex coacervation process with PPI and mung bean protein isolates along with chickpea protein to enable riboflavin encapsulation. PPI/k-carr microcapsules achieved a riboflavin

encapsulation efficiency of 65.46%, surpassing Na-alginate–chitosan microcapsules (55.70%) and soy protein–dextran nanogels (41.7–54.9%) (Jin *et al.* 2016; Danarto 2020). The EE value obtained in this study is lower than that obtained by Djoullah and Saurel (2021) and higher than that of Chiang and Henry (2022), Jin *et al.* (2016), and Danarto (2020). This might be due to the choice of enzyme and the protein source used, which influenced the electrostatic interaction and binding affinity of the complexes to riboflavin. The improved encapsulation efficiency of BPH-vitamin B2-GA likely results from a more stable complex network that preserves structure and prevents riboflavin leakage, possibly due to increased hydrogen bonding and electrostatic interactions between particles (Huang and Kuo 2016).

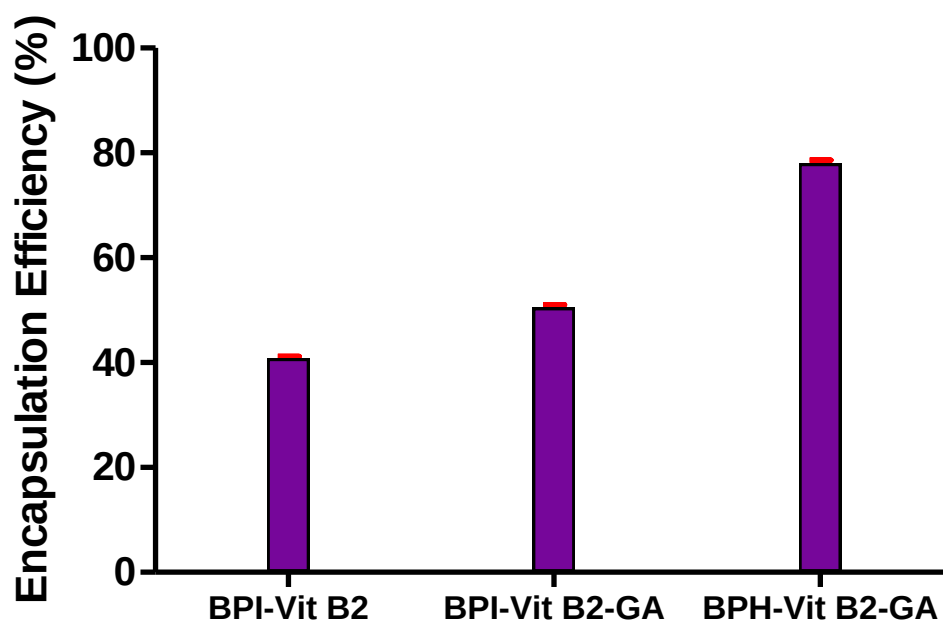


Figure 6.1. Encapsulation efficiency of the complexes

6.3.2 Release property

Release profiles were assessed in the model beverage, significant differences among the tested complexes behaviour were observed (Fig. 6.2). The model beverage showed a slow progression of riboflavin release approximately 20% in the first 30 min for the BPH-Vit B2-GA complexes whilst BPI-Vit B2-GA and BPI released up to 50% and 60% riboflavin respectively (Fig 6.2). The slow release observed for BPH-Vit B2-GA in the model beverage may be due to the establishment of a tighter encapsulated core structure from the stabilising bonds (hydrogen

bonds and electrostatic interactions) between the complexes and riboflavin which are resistant to degradation in the model beverage (Dickinson 2008; Jin et al., 2016). A sharp increase in release properties observed in the first two hours of BPI-Vit B2-GA could be due to the breakdown of the complex resulting in higher release of the core compound of the complex (Teng, Luo and Wang 2013). The BPI-Vit B2 complex had a rapid release progression from the start until 90 min. A linear trend was observed in the release rate of riboflavin starting at 90 min and 120 min up to 3 hours and later reaching a maximum for BPI-Vit B2-GA and BPI-Vit B2 complexes respectively. The lack of stabilising bonds usually formed during protein-polysaccharide complexation may be responsible for the homogenous protein isolate, BPI. These findings indicated that the complex formed with the Bambara protein hydrolysates was more stable than the complex formed with the protein isolates and exhibited notably sustained release behaviour in the model beverage.

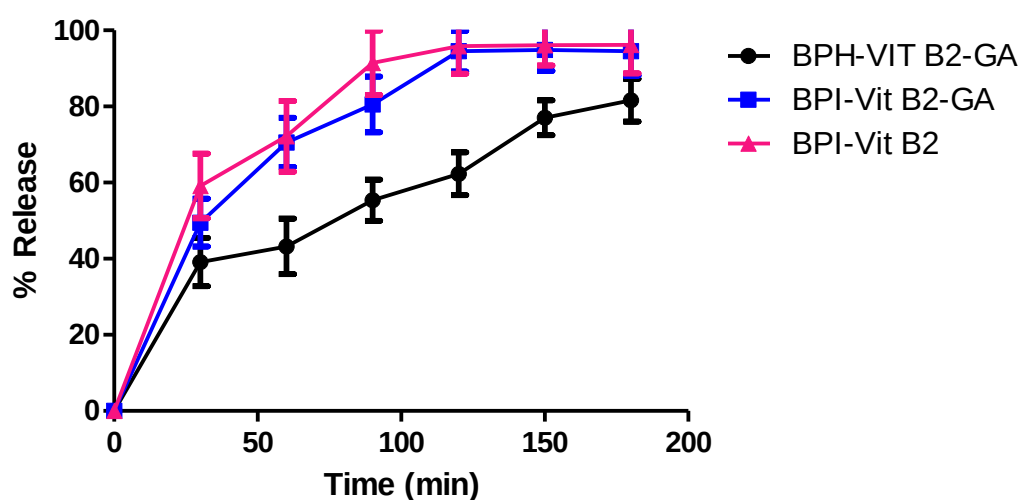


Figure 6.2. Release property of Vit B2 carrier complexes.

6.3.3 Particle size distribution, PSD

The size distribution of the particles of the complexes indicates the colloidal stability. Following the measurement done during the storage of the model beverage containing the carrier complexes on days 0, 3 and 7 postproduction, polydispersity index, PDI and PSD for BPH-Vit B2-GA complexes increased on the 3rd day and decreased on the 7th day of production, for BPI-Vit B2- GA and BPI-Vit B2 complexes, there was significant increase in PDI on days 3 and 7 compared to day 0 (Table 6.1). Generally, all the samples exhibited $PDI < 0.3$ and multimodal particle size distribution as the solutions were a mixture of biopolymers. The largest particle distribution was observed in BPI- Vit B2-GA and BPI- Vit B2 over the 7-day storage period which might be due to swelling and not necessarily flocculation since the PDI (< 0.3) is much closer to achieving a monodisperse system which indicates greater stability for a colloidal system (Igartúa, Cabezas and Palazolo 2022). The partially hydrolysed Bambara protein (DH 5)-gum arabic complex had significantly narrow particle size distribution throughout the 7 days, indicating more concentrated compared to BPI-Vit B2-GA and BPI-Vit B2. A narrow particle size distribution is preferred for commercial beverage production, this typically ensures optimal long-term stability (Piorkowski and McClements 2014). Smaller particles can be used for targeted delivery, as they can cross biological barriers more easily and have a higher surface area for bioactive binding (Martínez-Ballesta et al., 2018). In addition, the small particle size observed in the BPH-Vit B2-GA complex could be related to a more dispersible structure with a relatively high solubility as supported by the lowest value of PDI. Moreover, smaller particle size suggested a low degree of aggregation (Wu *et al.* 2020) which was supported by the lower optical density observed in the turbidity profile (chapter 4, section 4.3.3.) compared to the Bambara protein isolate-gum arabic complex and homogenous protein.

Table 6.1. Polydispersity index and D values of acidic beverages carrying riboflavin encapsulated complexes over 7 days

Carrier complex samples	PDI (%)			D10 (μm)			D50 (μm)			D90 (μm)		
Day	0	3	7	0	3	7	0	3	7	0	3	7
BPH-Vit B2-GA	22.100 ± 0.01	27.750 ± 0.10	27.050 ± 0.14	0.142 ± 0.28	0.007 ± 0.18	0.014 ± 0.05	0.099 ± 0.10	0.095 ± 0.01	0.069 ± 0.11	0.995 ± 0.02	0.232 ± 0.01	0.125 ± 0.03
BPI-Vit B2-GA	25.300 ± 0.14	26.750 ± 0.02	28.550 ± 0.13	0.016 ± 0.12	0.013 ± 0.15	0.025 ± 0.03	0.052 ± 0.03	0.094 ± 0.05	0.090 ± 0.04	0.111 ± 0.20	0.360 ± 0.02	0.200 ± 0.05
BPI-Vit B2	29.500 ± 0.08	34.250 ± 0.02	28.000 ± 0.15	0.008 ± 0.04	0.344 ± 0.02	0.020 ± 0.11	0.090 ± 0.21	0.603 ± 0.06	0.706 ± 0.15	0.252 ± 0.04	0.943 ± 0.17	0.289 ± 0.11

6.3.4 Confocal laser scanning microscopy, CLMS

CLMS was done to study the internal structure and the electrostatic interactions of the complexes. The images of the complexes showed that each sample displayed structural heterogeneity, containing regions enriched in polysaccharides and proteins (Fig. 6.3). The dark regions in the images could have been the water phase with unstained riboflavin. There were some differences in the structures formed in the carrier complexes, which is as a result of differences in the electrostatic interactions carrier wall polymers (proteins and polysaccharides). Regarding the microstructure of the BPH-Vit B2-GA complexes, a higher number of smaller complex particles were visible relative to the complexes from BPI-Vit B2-GA, which exhibited few large particles, indicating that not many protein-polysaccharide complexes were formed. This might be due to the increased molecular flexibility of the partially hydrolysed protein with more binding sites available for interaction. Additionally, lesser complex formation suggests the presence of weak attraction between the BPI-Vit B2-GA complex. Overall, all the protein-polysaccharide complexes are irregularly shaped (monodisperse) particles which support the particle size distribution result. This result is similar to the report of Arltoft, Madsen and Ipsen (2008) in the study of milk protein-carrageenan complexes.

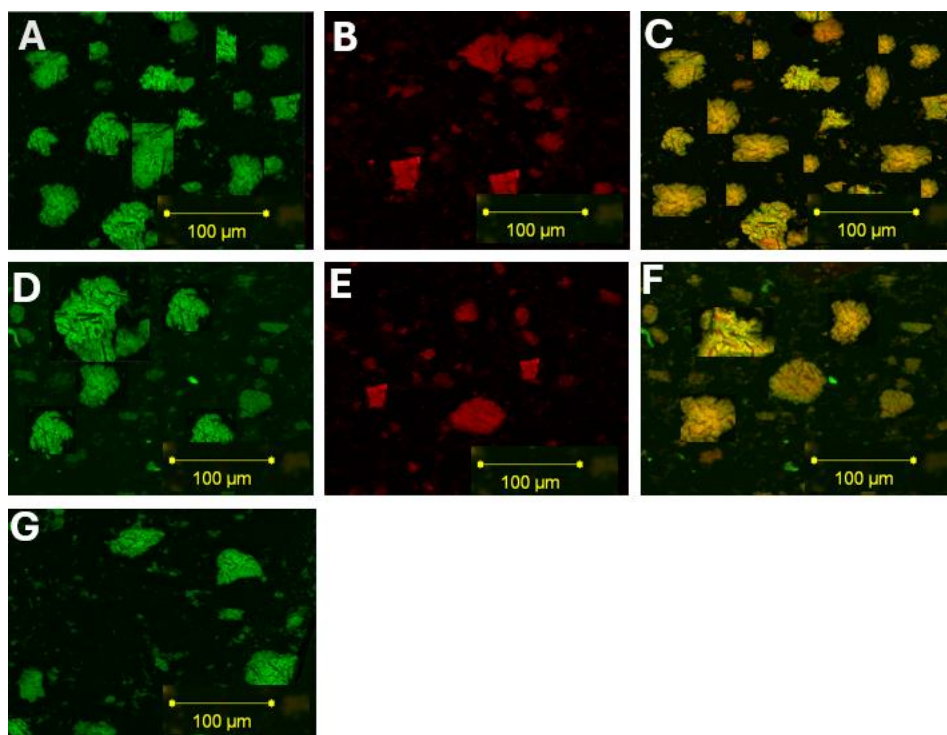


Figure 6.3. Confocal laser scanning microscopy of carrier complexes (A) Bambara protein hydrolysate (DH 5) (B) Gum arabic (C) BPH (DH 5)-Vit B2-GA complexes (D) Bambara protein isolate (E) Gum arabic (F) BPI-Vit B2-GA complexes (G) BPI-Vit B2 complexes. Green represents the protein; red represents the polysaccharide; orange or yellow represents the protein-polysaccharide complexes.

6.3.5 Apparent viscosity profile of the carrier complexes

Apparent viscosity is critical in determining a food product's rheological behavior, including ease of mixing, flow, and packaging. It provides insight into processing performance as well as the mouthfeel perceived during consumption (Shanmugam and Ashokkumar 2014). The shear-dependent viscosities for the carrier complexes depict non-newtonian shear-thinning behaviour (Fig. 6.4). The apparent viscosity was not significantly different among the complexes. However, the complex flow behaviour of the complex may originate from the strength and degree of the electrostatic interactions between two biopolymers (Schmitt, Aberkane and Sanchez 2009). However, viscosity changes may reflect electrostatic interactions or other types of intermolecular forces and hydration properties of the molecules involved. This can be explained by the formation of soluble complexes which could trap more water; meanwhile, it also can be attributed to the appearance of hydrogen bonding in the vicinity of biopolymer molecules upon the formation of soluble complexes (Yang et al., 2020). Increased

viscosity due to flocculation, potentially causing gelation, may impair the transport, handling, and dispersibility of beverages (Piorkowski and McClements 2014). In this study, there was no significant increase in size over 7 days of storage (section 6.3.3.) which is a sign of flocculation in the particle size distribution of BPH-Vit B2-GA complexes.

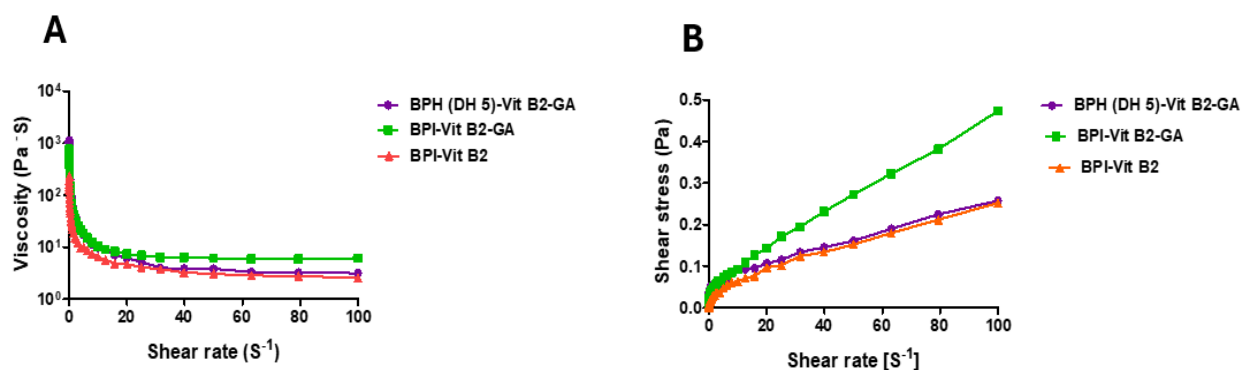


Figure 6.4. Apparent viscosity profile of the carrier complexes

6.4 Conclusion

BPH (DH 5)-GA showed effective encapsulation efficiency and slow release of Vit B2. In comparison to the homogenous BPI-Vit B2 and BPI-Vit B2-GA complexes, the beverage stabilised by the BPH-Vit B2-GA complex was more stable during 7 days of storage, as shown in the higher number of smaller complex particles. The increased molecular flexibility of the partially hydrolysed protein, providing more binding sites for interaction, must have been a contributing factor. The release pattern exhibited a rapid release trend in Bambara protein isolate and BPI-GA complexes, while BPH (DH 5)-GA complex had delayed release, indicating the beneficial impact of the limited trypsin hydrolysis on the protein structure prior to complexation.

CHAPTER SEVEN

DISCUSSION

6.5 General discussion

This research studied the role of extraction variables, limited enzymatic hydrolysis, and atmospheric cold plasma on the structure, physicochemical changes, and complexation behaviour of protein from Bambara groundnut combined with gum arabic and the controlled release of encapsulated riboflavin in a model beverage. In this thesis, the impact of optimized conditions of extraction, partial hydrolysis by trypsin and atmospheric cold plasma technology of Bambara proteins on complexation behaviour with gum arabic were investigated. Furthermore, encapsulation and release properties of riboflavin in model beverage, using soluble complexes prepared with modified Bambara protein were assessed.

The first part of the thesis reported on how protein extraction conditions including pH and ionic strength caused structural modification, which improved protein and coacervate yield and shifted the optimum complexation pH with gum arabic. The extraction parameters (pH fractionation and NaCl concentration) were optimised using response surface methodology and the protein fractions that complexed at optimum conditions were characterized (chapter 3). The second part reported on how the extent of hydrolysis impacted the development of Bambara protein hydrolysates-gum arabic soluble complexes focusing on the critical structure forming pHs (chapter 4). The third chapter discussed the impact of atmospheric cold plasma exposure time on the complexation behaviour of Bambara protein-gum arabic. The plasma-treated Bambara proteins were characterised, and the turbidity profile was investigated in addition to the dynamic shear rheology properties (chapter 5). The last part investigated the efficiency of encapsulation and release of the core compound of riboflavin using the soluble complex produced with DH 5 as well as the stability, viscosity profile and microstructure of the carrier complexes.

The formation of coacervate from legume grain proteins with polysaccharides is dependent on the structural composition of the proteins. The structures and amino acids composition of proteins influences the complexation behaviour of protein-polysaccharides coacervates (Wang et al., 2020). Optimization of the extraction pH and NaCl concentration was employed for modification of Bambara protein to produce protein fractions that are complex with gum arabic below the isoelectric point of the protein. Protein yield improved significantly by

approximately 2-fold (63%) at moderately acidic pH compared to protein extracted at the same acidic pH without salt, which suggests support from ionic strength. Optimisation of process parameters showed that acidic extraction pH with 0.2–0.3 M NaCl strongly influenced pH_{opt} , which decreased with lower extraction pH. Regression analysis indicated the model was statistically significant ($p \leq 0.05$), showing a negative interaction between extraction pH and NaCl concentration, indicating that increasing one variable while decreasing the other can maintain coacervate yield. Quadratic effects suggest maximum yield at acidic pH, with lower yields observed at alkaline extraction for Bambara protein isolates (BPI). This was because the degree of ionisation of BPI and gum arabic was low at this pH. Bambara protein fractions extracted at pH 2.95, 0.28 M NaCl formed a complex below the isoelectric point region (3.4) with a high coacervate yield (~70%). The pH_{opt} shift observed in the complex with protein fraction pH 2.95, 0.28 M NaCl is likely associated with the prevalence of β -sheet structure, both acidic and basic legumin subunits, and a high amount of positively charged amino acids in the protein.

The degree of hydrolysis of protein substantially affected the soluble complexes formed with gum arabic. Soluble complexes increased in proportion to the degree of hydrolysis from DH 2 to DH 7.5. This might be due to increased molecular flexibility and a change in surface charge distribution, causing accessibility of the ionizable amino acids and carboxyl groups after the partial trypsin hydrolysis. There is a significant broadening of the pH range of soluble complex formation for the hydrolysates, with DH 5 having ~ 3-fold increment and a broader range of pH formation compared to the complex formed with unhydrolyzed protein. The reduction in molecular weight was anticipated to increase the molecular flexibility and the negative surface charge of the proteins as the hydrolysis progresses (Klost & Drusch, 2019). This increased more binding sites and promoted strong intermolecular interactions among the bio-polymer molecules by creating inequality in their net charge, which is a favourable condition for the formation of soluble complexes (Warnakulasuriya & Nickerson, 2018; Huang, Lee, Wang, & Qiu, 2023). This was evidenced by, a shift in thermal transition temperature, increased negative charge and surface hydrophobicity. Furthermore, the Bambara protein hydrolysate-gum arabic soluble complex solution displayed shear thinning flow behaviour, similar to the unhydrolyzed protein. As the DH increased from 2 to 7.5, the values of G' and G'' became higher, further increase of DH from 5 to 7.5 caused G' and G'' values to decrease leaving DH 5 with a more pronounced plateau value of G' . A higher G' stands for higher elasticity and indicates a stronger

molecular interaction and binding strength between the hydrolysate and gum arabic resulting to a higher degree of internal structure, and this makes the complex more stable.

The complexation behaviour of plasma-treated Bambara protein with gum arabic showed some significant differences relative to the untreated protein. A significant shift in pH_{opt} was observed for the turbidity measurements. This implies a structural modification of the protein caused by the activity of reactive species of atmospheric cold plasma and the protein molecules, consequently influencing the critical structures formed (Chen et al., 2023). The critical structures pHs were delayed and shifted towards the strong acidic region in an exposure time-dependent manner, with the formation of insoluble complexes, highly dependent on the plasma exposure time. The point of formation of optimum complexation (pH_{opt}) for protein was pH 3.2, 3.0 and 2.8 for 2, 5, and 10-min treatments respectively.

The plasma treatment influenced the amino acid profile of the proteins. Aromatic amino acid residues have been reported to be susceptible to oxidation during plasma treatment, which subsequently impacts their functional properties (Surowski et. al., 2013). Phenylalanine and tyrosine went in an upward direction after 2 and 5 min treatment, followed by a downward trend after 10 min. Aspartic acid increased throughout the duration of the plasma treatment, while lysine, arginine and threonine content were significantly increased throughout the treatment. Methionine, a sulphur-containing amino acid and an excellent target for plasma oxidation was drastically reduced after 5 and 10 min plasma treatments. The oxidation of methionine has been linked to conformational changes in proteins modulating their functionalities (Adrian and Jeannette, 2014).

This consequently results in modification of protein conformation and increased their interaction with binding molecules (Wenske et al., 2021). The structural modifications following plasma treatments of Bambara proteins influenced the molecular interaction and complexation behaviour with gum arabic. Plasma treatment for 2, 5, and 10 minutes induced new positive peaks at approximately 202, 210, and 203 nm, accompanied by an increase in the intensity of negative peaks of 210, 219, and 215 nm, indicating structural changes in the protein. The new peaks indicate the presence of β -sheet. The difference between the G' and G'' values of the insoluble complex for the untreated proteins increased from $< 1 \log$ to $\sim 1 \log$ after 10 min exposure time, indicating the formation of a stable network structure.

The encapsulation efficiency, release properties, microstructure, viscosity profile and stability of Bambara protein hydrolysate (DH 5)-Vit B2-GA complex showed some notable differences. In comparison to homogenous BPI and BPI-GA, the BPH (DH 5)-Vit B2-GA complex generally showed higher encapsulation efficiency and improved release property. The stability of the Bambara protein hydrolysate carrier complex is better than other complexes. This outcome was anticipated because of the smaller particle sizes and better network structure of the BPH-Vit B2-GA complex, which allows easy migration and alignment on the air/water interface. Nonetheless, the contribution of limited hydrolysis in maintaining the stability of the complexes to avoid rapid release of riboflavin was significant.

Generally, manipulating the extraction pH and NaCl concentration to favour the proteins and reduce the interference with protein-protein interaction can widen the scope of protein-polysaccharide complexation. Hence, an effective mechanism that can produce protein fractions to complex below the isoelectric point of the protein was provided. In addition, it enhanced the utilization of the complexes in acidified medium, especially those beverages prepared around pH 3. The mechanism of soluble complex formation from pulse protein and polysaccharides starts with initial contact and adsorption of the pulse protein to the polysaccharide through random collisions facilitated by Brownian motion. The soluble complex formation is enhanced when there are conformational changes such as partial unfolding of the protein structure leading to increased solubility, as depicted in increased surface hydrophobicity value. Redshift in intrinsic fluorescence indicated a loose and flexible structure, and changes in charge configuration as reflected in zeta potential analysis following the limited trypsin hydrolysis of the protein. The partial unfolding in the proteins exposes more binding sites, while polysaccharides can stretch to enhance contact points (Clement & Jim, 2022; Weiss, Salminen, Moll & Schmit, 2019). The soluble complex is formed when the attractive interactions between the protein and polysaccharide are balanced with the solubility of the complex in the aqueous environment. This balance is crucial to maintain the complex in a dissolved state rather than precipitating out.

This study demonstrates that limited trypsin hydrolysis induced changes in the conformation of the protein. The results from this study, following the limited trypsin hydrolysis, suggested conformational changes in the protein structure. The structural modification favoured and widened the pH range of the formation of soluble complexes with a better network structure and improved stability. This indicates that the soluble complex from Bambara protein-gum

arabic interaction provided enhanced colloidal stability and a better encapsulating agent in beverage applications.

CHAPTER EIGHT

CONCLUSION AND RECOMMENDATIONS

6.6 General conclusion

Complexation behaviour of proteins and polysaccharides is largely determined by the structure and composition of the homogenous biopolymers. The composition and structure of Bambara protein were substantially altered through the extraction pH and NaCl concentration of the extraction medium. The modified structure and composition influenced their molecular interaction with gum arabic, which brought about a shift in the optimum complexation pH (pH_{opt}). A shift of pH_{opt} to a lower acidic pH suggests the formation of a stable structure in the acidic domains. An increase in protein yield was observed at both acidic and alkaline pH as the ionic strength increased. At optimum conditions, the basic amino acids content was the highest with more low molecular weight protein bands stabilized by di-sulphide bonds. A large proportion of basic amino acid in BPI extracted at pH 2.95, 0.28 M provided good interaction sites for complexation with gum arabic. Higher content of β -sheet conformation was also observed in the protein which unlocks the previously hidden hydrophobic residues of the protein structures to be available for interaction. The coacervates formed have great potential for acidified beverages.

Limited trypsin hydrolysis significantly widened the pH range of formation of soluble complexes (pH_c - $pH_{\phi 1}$), a 2-fold increment in the region of soluble complex formation for complex formed with DH 5 (pH 7.0 to 4.3) as against DH 2, 7.5 and unhydrolyzed protein. Reduction in peptide molecular weight was observed with increasing relative abundance of 144–35 kDa peptides. High net negative charge (- 30 Mv) for DH 7.5 and a slight transition in the isoelectric point (pH 4.9 to 4.3) of the hydrolysates, corroborated by exposure of the hydrophobic amino acids up to 4-fold and a red shift in maximum fluorescence wavelength in DH DH-dependent manner. The denaturation temperature of the soluble complex of the hydrolysates shifted to higher values compared to that of the isolate, having DH 5 with the maximum temperature (94.24 °C). A highly interconnected soluble complex network was formed with DH 5, showing a better stable structure compared to other complexes.

The optimum complexation, and pH_{opt} of cold plasma-treated Bambara protein-gum arabic complexes significantly shifted to acidic pH following the treatment in an exposure time-dependent manner. The structural changes coincide with the observed shift from α -helix to β -

sheet structure, indicating the opening of the protein structure. Crosslinking-induced effects on cold plasma were supported by the low thiol content and increase in carbonyl level, suggesting breaking of the cross-linking disulphide bonds and oxidation of protein, respectively by the reactive species. The variation in the protein composition was connected to the prominent increase in positively charged amino acids throughout the cold plasma treatment compared to the untreated protein. The slight red shift in fluorescence intensity data of the plasma protein suggests partial unfolding of the protein structure, correlating with the observed increased hydrophobicity and negative surface charge. The difference between the G' and G'' values of the insoluble complex for the untreated proteins increased from < 1 log to ~ 1 log after 10 min exposure time, indicating the formation of a stable network structure.

The promising protein modification approaches based on the obtained result are the adoption of pH fractionation combined with salt and trypsin-limited hydrolysis. This is based on the ability of both techniques to cause changes in Bambara protein-gum arabic interaction by shifting insoluble optimum complex formation to acidic pH and increasing the molecular flexibility of the protein with gum arabic. This enhanced the stability and broadened the potential application of Bambara protein-gum arabic complexes.

Protein modification through pH fractionation, and salt altered Bambara protein–gum arabic interactions, shifting optimal insoluble complex formation toward acidic pH. Moreover, limited hydrolysis and atmospheric cold plasma structurally modified the protein, increased the molecular flexibility and influenced the molecular interaction of the protein with gum arabic in a degree of hydrolysis and exposure time manner, respectively. This enhanced the stability and characteristics of complexes produced from Bambara protein and gum arabic. Therefore, findings from the study showed BPH (DH 5)-GA complex as a potential carrier matrix for bioactive compounds such as riboflavin and created an opportunity for future application of pulse grain protein-gum arabic complexes in acidic beverages.

6.7 Recommendations

- The enzyme-catalysed protein modification can be extended to other enzymes or a combination of enzymes prior to complexation with polysaccharides, and their impacts on complexation behaviour will be investigated.
- To examine the effect of other types of salt and pH shifting of Bambara protein isolate extraction on the formation of soluble and insoluble complexes with polysaccharides.

- To investigate the impact of indirect cold plasma treatment, such as cold plasma-activated water of Bambara protein isolate, on their molecular interaction with polysaccharides
- To develop a low-fat food product such as yoghurt which can serve as a fat replacer using the cold plasma Bambara protein-gum arabic complex with optimal pH 3.2 and 3.0
- To investigate the encapsulation and controlled release mechanism of riboflavin using the Bambara protein hydrolysate-gum arabic soluble complex under simulated gastric conditions.

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Appendices

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 pH_{opt} 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.



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Impact of limited trypsin hydrolysis on Bambara protein-gum arabic interaction and the formation of soluble complexes

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ABSTRACT

Soluble protein-polysaccharide complexes are of growing interest for food and beverage applications. However, due to the compact structure of globular proteins, forming stable complexes across a wide pH range is challenging. In this study, the effects of limited trypsin hydrolysis on Bambara protein and its interaction with gum arabic to form these complexes were investigated. Proteins were hydrolysed to degrees of hydrolysis (DH) 2, 5, and 7.5, and analyzed for structural characteristics. Among the studied hydrolysates, DH 5 produced the most stable soluble complexes over a broader pH range (7.0–4.3), showing enhanced binding strength and structural stability. This was evidenced by its rheological fingerprints, a shift in thermal transition temperature, increased negative charge, and surface hydrophobicity. Limited hydrolysis at DH 5 is the most effective in enhancing the formation and stability of Bambara protein-gum arabic soluble complexes. The stability of Bambara protein-gum arabic soluble complexes for bioactive compound encapsulation may be further investigated to support industrial applications.

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Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
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