CHARACTERISATION AND APPLICATION OF BAMBARA PROTEIN-POLYSACCHARIDE COMPLEX COACERVATES IN ENCAPSULATION OF BIOACTIVE COMPOUNDS

By

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DECLARATION

I hereby declare that the work reported in this thesis and submitted at the Department of Biotechnology and Food Technology at the Durban University of Technology for a Masters Degree is my original work. I confirm that it has not been previously submitted for a degree at any Higher Education Learning Institution.

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As the candidate's supervisor I agree to the submission of this thesis

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DEDICATION

This thesis is dedicated to my parents Makemore and Shamiso Busu.

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ABSTRACT

Bambara groundnut (*Vigna subterranea*) is a leguminous crop that is indigenous to Africa. In South Africa, the legume is cultivated in KZN, Limpopo and Mpumalanga where it is considered a traditional food. Bambara groundnut is a good source of protein (15 – 28 %) and contains substantial amounts of starch. The legume thrives well in areas of low agricultural input. Despite its good protein content, bambara groundnut is mostly cultivated in rural areas for by subsistence farmers. In recent years, there has been increased interest in bambara groundnut protein as an alternative protein source. The purpose of this study is to investigate the complexation behavior of bambara protein with gum Arabic and test the application of the formed complexes in encapsulation and delivery of bioactive compounds. In the first part of this study, four protein fractions extracted at different pH including the salt-solubilisation method were complexed with gum Arabic. The protein content as well as physicochemical properties (SDS-PAGE, FTIR, Zeta potential, SEM) of the protein fractions and resulting bambara protein-gum Arabic (BPI-GA) complexes were then investigated. In subsequent parts of the study, bambara protein extracted by the salt-solubilisation method was complexed with gum Arabic. The influence of ionic strength and biopolymer mixing ratio on complex formation was investigated. Subsequently, the emulsification properties, foaming properties, encapsulation efficiency and release properties of the formed complexes were also investigated under simulated gastric and intestinal pH conditions.

The salt-soluble fraction showed the highest protein content (82%) whilst the lowest protein content (76%) was recorded at pH 2. The FTIR analyses revealed an increase in β-sheet content with decrease in pH of extraction. Complexation of the protein fractions with GA resulted in the optimal pHs of interaction shifting towards acidic regions (pHopt: 4.8 to 2.9) as pH of protein extraction became more acidic. Upon complexation, protein fractions produced coacervate yields ranging between 41 - 68%, with the pH 2 fraction recording the lowest (41%) yield. Further, addition of gum arabic seemed to broaden the turbidity profiles. When assessed by SEM, the particles appeared as spherical and aggregated structures between 100-200 nm.

The addition of salt and varying biopolymer ratios impacted the formation of BPI-GA complexes. A higher protein ratio (16:1) in the BPI-GA mixture resulted in wider and normally distributed turbidity profiles. However, a higher ratio of gum arabic resulted in two pronounced peaks in the turbidity profiles, which resembled the two-step nucleation process for complex formation. High protein ratio (16:1) coupled with high ionic strength (40 mM) resulted in highly aggregated spherical particles. These spherical particles looked more uniform in size when compared to those formed from a lower protein ratio and ionic strength. This observation was confirmed by AFM, which showed highly aggregated but uniform-sized spherical particles under 200nm in size. Simultaneous increase of ionic strength and biopolymer ratio could therefore be a way of producing uniform-sized particles. BPI-GA complexes showed better emulsifying, foaming and encapsulating properties compared to BPI alone. The Gelatine: Gum Arabic control recorded the highest values for all the functional properties. Addition of 10 mM NaCl slightly improved the emulsifying capacity as well as stabilizing the foams. The release profiles indicated that BPI-GA complexes were more stable compared to BPI alone. However, the presence of salt did not have a major impact on the release properties of BPI-GA complexes.

In general, extracting proteins at various pH influenced its composition, secondary structure as well as complexation behavior with gum Arabic. The pH fractionation method could be an alternative way of producing acid-stable protein and consequently designing carrier systems with improved acid-stability. The influence of ionic strength on BPI-GA complexation was observed to be dependent on biopolymer mixing ratio. Hence, simultaneously increasing biopolymer ratio and ionic strength could be a way of producing uniformly-sized particles. The presence of salt significantly influenced the emulsifying, foaming and encapsulating properties but had minimal effect on the release profiles.

PREFACE

This thesis is organized into six chapters and presented in the format submitted for publication. Chapter one is a general introduction to the thesis. Chapter two presents a critical review of relevant literature. Chapter three focuses on the impact of protein fractionation on protein-polysaccharide complexation whilst chapter four covers the influence of salt concentration and biopolymer mixing ratios on protein-polysaccharide interactions. In chapter five, the emulsifying, encapsulating and release properties of the bambara protein-gum arabic complexes are presented. Chapter six summarises and concludes the work as well as providing recommendations for future work in the same field.

Contents

Table of figures

List of Tables

ABBREVIATIONS

SDS-PAGE – Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis

BPI-GA – Bambara protein-gum arabic complex

DSC – Differential Scanning Calorimetry

SEM – Scanning Electron Microscope

GRAS – Generally Regarded As Safe

FTIR – Fourier Transform Infra-Red

AFM – Atomic Force Microscopy

DLS- Dynamic Light Scattering

BPI – Bambara protein isolate

GDL – Glucono delta lactone

WPI – Whey Protein Isolate

LMP - Low Methoxy Pectin

β- Lg – Beta Lactoglobulin

SPI – Soy Protein Isolate

DTT – 1,4-Dithiothreitol

PPI – Pea protein isolate

pI – Isoelectric point

kDa – kilo-Dalton

GA – Gum arabic

Nm – Nano-meter

mM – milli-Molar

AA – Amino acid

pH_{opt} – pH optimum

PUBLICATIONS AND CONFERENCES ATTENDED

Publications

Nyasha M. Busu, Eric O. Amonsou (2019). Fractionation pH of bambara groundnut (Vigna subterranea) protein impacts the degree of complexation with gum arabic. *Food hydrocolloids*, *87*, pp. 653-660.

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Conferences attended

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CHAPTER ONE

1.0 INTRODUCTION

The world's population is predicted to reach 9 billion by 2050 and 11 billion by 2100 (United Nations, 2017). In order to meet the protein demand for this growing population, food production has to double (FAO, 2009). Animals are the world's major source of protein. However, due to the negative environmental impact associated with animal husbandry coupled with chronic diseases associated with meat consumption (Tullo & Guarino, 2018), there has been increased interest in plant-based proteins (FAO, 2009; Liu et al., 2009b). Soybean and peas are widely used plant proteins in the food industry. However, in light of the changing climatic conditions, these legumes may not be sufficient to meet the present and future protein demands. Consequently, alternative plant protein sources that are resilient to Sub-Saharan climatic conditions may play a role in addressing future protein needs.

Bambara groundnut (*Vigna subterranea*) is an African legume with potential as an alternative protein source. In light of the changing climate, bambara is resilient to the sub-Saharan climatic conditions and grows well in areas of low agricultural input. The legume is common in Zimbabwe, Nigeria and Mali. It has been reported to be cultivated in Thailand as well. In South Africa, the main producing areas of bambara are the Limpopo, KwaZulu Natal and Free State provinces (Hillocks et al., 2012). Bambara groundnut is a good source of protein (19 - 27%) just like peas (23 – 26%), kidney bean (18 – 24%) and soybean (36.5 – 56%), (Adebowale and Maliki, 2011; Wani et al., 2015; Kinsella, 1979). However, it remains marginalized. Research efforts to facilitate bambara utilization focused on characterization of the protein, with many studies looking into the nutritional fortification of cereal-based foods with bambara protein. The protein composition studies revealed vicillin as the major storage protein in bambara groundnuts (Kudre and Benjakul, 2013; Arise et al., 2017; Diedricks et al., 2019). The presence of phaseolin, proteinase inhibitor and heat shock protein has also been reported in some landraces of bambara (Arise et al., 2017). According to Diedericks, (2014), bambara protein showed similar emulsifying properties (79%) when compared to soybean protein (82 %). Mubaiwa et al., (2018) reported that bambara protein exhibited foaming properties of up to 80%. However, to further increase the awareness and

utilization of bambara protein, its functionality could further be explored into other areas of growing interest such as in protein- polysaccharide complexation.

Protein-polysaccharide complexation is an electrostatically driven interaction between oppositely charged biopolymers, resulting in a biopolymer rich phase and a biopolymer deficient phase (Timilsena et al., 2018). Protein-polysaccharide complexes are used widely as emulsifiers, fat mimetics and delivery matrices in the cosmetics industry (Weinbreck, 2004). The addition of polysaccharides to proteins has been reported to improve a protein's functional properties. According to Chen et al., (2018), addition of sugar beet pectin to a coconut protein stabilized emulsion resulted in better viscosity and stability compared to coconut protein stabilized emulsions. Further, a study by Sun et al., (2018) showed that micro-particulated whey protein (MWP)- pectin complexes maintained a better mouthfeel due to high dispersion stability of MWP-xanthan gum complexes. Recently studies on protein-polysaccharide complexes as encapsulating and delivery matrices for bioactive compounds are becoming popular (Souza et al., 2015). For instance, Nori et al., (2011) reported that encapsulating propolis extract in a soybean protein/pectin complex preserved its antioxidant and bacteria inhibitory activities. Similar studies have also been carried out on chia seed and lentil protein (Timilsena et al., 2016). Bambara protein-polysaccharide complexes may therefore offer potential in improving protein functionality as opposed to bambara groundnut protein alone.

Bambara groundnut protein, just like soybean protein and pea protein is rich in charged amino acids such as aspartic and glutamic acid (Adebowale and Maliki, 2011). Kudre et al., (2013) also reported the presence of charged amino acids in bambara protein which are essential in protein-polysaccharide complexation. Therefore, in this research, bambara groundnut protein-polysaccharide complexes were formed under varying conditions and characterized for potential application in encapsulation and delivery of bioactive compounds.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and Background of Bambara Groundnut

Bambara groundnut (*Vigna subterranean*), is an indigenous African leguminous crop grown primarily for subsistence (Vurayai et al., 2011). The legume is believed to have originated from parts of West Africa from a tribe called the bambara, which the legume is named after (Swanevelder, 1998). Bambara groundnut is predominantly cultivated in Africa, however, it is also rarely cultivated in India, Brazil, Syria, Greece and Thailand by subsistence farmers (Kudre and Benjakul, 2013). In terms of its consumption and socioeconomic impact in semi-arid Africa, the legume ranks third after peanut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) (Hillock et al., 2012).

Bambara groundnut is classified under the *Fabacea* family, sub-family *Papilionodease* and genus *Vigna.*(Mkandawire, 2007; Murevanhema and Jideani 2013). The legume falls under the same family as peanuts *(Arachis hypogaea*) and cowpea (*Vigna unguiculata*)(Mkandawire, 2007). Two botanical varieties exist; *Vigna subterranean* variety *spontanea*, which is the wild variety whilst *Vigna subterranean* variety *subterranean* is domesticated. Bambara groundnut is an annual herbaceous legume with a compact and well-developed tap root. The seeds of the legume develop and mature underground. When unripe, the pods are yellowish in color, but turn brown when maturing and becoming wrinkled upon drying (Nti, 2009; Swanvelder, 2008). The pods can be round or oval-shaped with one or two rounded and smooth surfaced seeds. The seeds have an approximate diameter of 2cm and can be identified with patterns and colours present on their testa (Nti, 2009). The seeds vary in colour depending on the breed.

Fig 1: Bambara groundnut (www.daff.gov.za)

2.2 Production and Utilisation

Bambara groundnut makes few demands on the soil, and is known to be drought tolerant and relatively disease free. Compared to other legumes, bambara groundnut often yields well in areas of low agricultural input(Baryeh, 2001). In recent times where drought and climate change are rampant, bambara groundnut could serve as ground cover for farmers, forage for animals as well as address food security and nutrition.

Agriculturally, bambara has a deep taproot surrounded by lateral profuse roots bearing nitrogen fixing nodules (Mubaiwa et al., 2018). This makes the legume a good rotational crop when intercropped with cereals. This is useful particularly in Africa were fertilizers are beyond the reach of many. As a food, bambara is a cheap source of protein particularly in Africa where meat protein is expensive (Yusuf et al., 2008).The premature seeds can be boiled and consumed as a salted snack. In East Africa, the groundnuts are roasted, pulverized and used to make soup with the aid of spices. In South Africa, the groundnuts are cooked with maize and then pounded into a thick and sticky dough (Wasula et al., 2014). Roasted seeds can also be crushed and mixed with other vegetables for consumption as relish. Bambara groundnut has also been used in the production of vegetable milk (Murevanhema and Jideani 2013). Mkandawire, (2007) reported that bambara groundnut milk was preferred for its color comparison with soymilk.

The pharmaceutical properties of bambara are found in the whole plant, from the seeds to the leaves. Drinking water that remains after boiling bambara groundnut helps in treating diarrhoea and internal bruising. In Nigeria, the Igbos tribe use the seeds in the treatment of venereal diseases (Diedericks and Jideani, 2015). Pregnant women also chew raw bambara seeds to alleviate nausea. Mixing the crushed seeds with water can be used to treat cataracts, anaemia as well as ulcers (Diedericks and Jideani, 2015). Recent studies have also shown that bambara protein isolates lower plasma cholesterol, prevent cancer, diabetes, obesity and protect against bowel and kidney disease (Kudre et al., 2013).

2.3 Composition of Bambara Groundnut in Comparison to Other Legumes

According to Mazahib et al., (2013), the high carbohydrate (54-65 %) and relatively high protein (18-28 %) content as well as fat (5-6.5 %) make bambara groundnut a complete food. Bambara groundnut seeds have a balanced essential amino acid profile and a relatively higher proportion of lysine (6.8 %) and methionine (1.3 %) than other legumes like peanuts (Murevanhema and Jideani 2013). Bambara is also a good source of fiber, calcium, iron and potassium.

Table 1: Chemical composition of selected legumes

Legume type	Species	Protein	Carbohydrate	Fat	Ash	Fibre
Bambara groundnut	*Voandzeia subterranea*[a]	32.4	51.79	7.35	5.78	2.68
Bambara groundnut	*Vigna subterranea*[b]	20.6	56.51	6.6	3.25	6.34
Cowpea	*Vigna unguiculata*[c]	24.13	56.6	4.37	4.73	0.97
Chick pea	*Acararietinum*[e]	22.83	57.19	5.43	3.04	3.50
Pigeon pea	*Cajanus cajun*[c]	24.46	56.83	4.78	4.58	1.10
Soybean	*Glycine max*[d]	42.80	19.80	22.80	5.20	2.30

[1]Values are reported in % dry basis. Sources [a]Chinedu and Nwinyi (2012), [b]Arise et al., (2015), [c]Mazahib et al., 2013, [e]Qayyum et al., (2012)

2.4 Bambara Protein

Recently, bambara protein has been a subject of study for many researchers. The functionality, physicochemical composition as well as protein structure have been widely studied with reason to improve its utilization. For instance, Kudre et al. (2013) investigated protein isolates from bambara

protein using SDS PAGE. The major bambara protein bands had molecular weights of 58 and 66 kDa under both reducing and non-reducing conditions. In addition, Adebowale and Maliki, (2011) reported that bambara protein isolates were not dissociated by DTT suggesting that they do not contain disulphide bonds. This suggested that 7S vicillin is the major storage protein in bambara protein. Further, a study by Okpuzor et al., (2010) indicated that bambara protein contained 7S vicilin as the major storage protein, but also contained other fractions like seed storage protein B, α 8S globulin and β isoform precursor among others. Similarly, other legumes have also been found to contain the 7S vicillin as the major protein fraction with marginal amounts of 11S globulin and other legumin fractions. Kudre et al. (2013) reported that some subunits of mung bean are stabilized by disulphide bonds, an indication of the presence of 11S fractions. Arise et al.,(2015) also found similar results on bambara protein and further validated through 2 D electrophoresis that 7S fraction is the major storage protein in bambara groundnut.

In comparison to other legume proteins such as soybean and pea, bambara protein has been reported to have good functional properties. A report by Adeleke et al., (2018) recorded an oil and water absorption capacity of over 79% and 108% respectively. Further, Goudoum et al., (2016) reported a 135% water absorption capacity for bambara protein isolate. These values indicate that bambara protein is a potential and alternative protein source for use as a functional ingredient.

2.5 Protein Extraction Methods

The selection of appropriate conditions and technology for protein extraction is essential as it can influence the physicochemical, functional and nutritional properties of protein isolates (Du et al., 2018; Piotrowicz and Salas-Mellado, 2017). Protein extraction processes such as pin-milling plus air-classification are generally applied in industries to starch-rich legume seeds such as peas and beans. These extraction methods usually yield about 60–75 % of protein content. Protein isolates extracted through wet processes (e. g. alkaline extraction/isoelectric precipitation, acid extraction) normally have protein contents of 70 % to 85 % (Boye et al., 2010).

2.5.1 Salt- soluble protein extraction

The salt extraction principle is based on the ionic strength to dissolve and fractionate the two major storage proteins (globulin and albumin) found in legumes (Boye et al., 2010). Albumin contains a

high glycoprotein (up to 45%) which increases its solubility in water by enhancing the protein-water interaction. On the other hand, the low glycoprotein (3.9%) present in globulin contributes to its insolubility in water, thereby making it extractable by salt (Mundi and Aluko 2012). The salt extraction method has been used to isolate protein from plant sources such as canola and flaxseed (Karaca et al., 2015), kidney bean (Mundi and Aluko, 2012), soybean and bambara (Adebowale and Maliki, 2011). Dialysis of the salt extract against double distilled water followed by centrifugation yields a globulin-rich precipitate and an albumin-rich supernatant (Mundi and Aluko, 2012).

2.5.2 Acid/alkali extraction

The acid extraction principle is similar to that of alkaline extraction except that initial protein extraction is conducted under acidic conditions. Legume protein solubility is high at very high acid conditions (pH< 4.0), therefore low pH is used to solubilize the protein followed by isoelectric precipitation or membrane separation (Boye et al., 2010). Past studies reported about 95.7% protein for kidney bean (Alli et al., 1993), 91.2% and 91.9% protein for faba and pea bean protein isolates respectively when citric acid solution or direct acidification method was used.

2.5.3 Isoelectric protein precipitation

The commonly used method for extracting leguminous protein is the aqueous alkali or acid solubilisation followed by isoelectric precipitation (IEP) (Boye et al., 2010). This technique takes advantage of the differential solubility of legume proteins which is high at alkaline or acidic pH thereby necessitating solubilisation of the proteins (Boye et al., 2010). Addition of salt may be necessary to increase protein solubilisation and recovery. Salt assists with salting-in the protein. The concentration and choice of salt is selected according to the salting-in characteristics of the protein to be isolated (Lam et al., 2018). The solubilized protein is then precipitated out of solution at the isoelectric point of the protein. In some cases, the isoelectric precipitated protein may have a high protein content. For instance, Adebowale and Maliki, (2011), reported protein content of 90% and 92% for bambara and soybean isoelectric precipitated protein isolates respectively. In some instances, the protein recovery of protein isolates extracted in this way is poor. This is probably due to inadequate solubilisation or loss of protein from the supernatant during centrifugation (Malomo and Aluko, 2015). A limitation of this extraction method is that isoelectric precipitated isolates contain high amounts of ash generated during the acid-base neutralization

procedure (Lam et al., 2018; Malomo and Aluko, 2015). This may result in contamination of the protein isolate. Further, the conditions used in a particular process can affect the isolate yield, purity and functionality. For instance, pH levels of 11 and above are associated with starch swelling, which may result in starch contamination of the protein isolate (Lam et al., 2018). Also, long standing times may be associated with higher isolate yield and may lead to protein denaturation (Lam et al., 2018).

2.6 Effect of Extraction Methods on Amino acid Composition and Functionality

Protein functionality is dictated to a larger extent by the protein's physicochemical and structural properties as influenced by treatment and environmental conditions (Jarpa-Parra et al., 2014). Several protein extraction protocols particularly those highlighted by (Osborne and Mendel, 1917) have been used in literature. In recent years there has been an increase in technical methods of protein extraction such as ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE) (Amponsah and Nayak, 2016) . These techniques are gaining momentum due to advantages of short extraction time, high extraction yield and low solvent amount (Amponsah and Nayak, 2016). For instance, Yagoub et al., (2017) reported that UAE for rapeseed increased the protein extraction by 43.3 % over the alkaline extraction. They further reported that the ultrasonic assisted protein extraction from rapeseed increased the disulphide bonds and decreased sulfhydryl bonds, hydrophobicity, and fluorescence intensity compared to the conventional extraction. It was concluded that sonication improved the yield and changed protein conformation, possibly providing a future functional protein.

In most research studies, the commonly used method of protein extraction is the traditional alkaline extraction followed by isoelectric precipitation. But majority of these studies vary the parameters of protein extraction based on different factors such as type of plant. For instance, lentil protein extraction carried out by Jarpa-Parra et al., (2014) made use of lentil flour slurry prepared with distilled water at pH 9, whereas Klemmer et al., (2012) on extraction of pea protein used $K_2H_2PO_4$ containing 0.1 M NaCl. This may have varying consequences on the results obtained. As such there is lack of systematic studies showing how extraction parameters impact protein yield, purity, amino acid composition and protein functionality. For instance, Mwasaru et al., (1999) reported that 36.7 % and 40.2 % of protein was extracted from cowpea and pigeon pea respectively using the micellization method compared to 42.0 % and 39.7 % extracted using the isoelectric

precipitation technique respectively. Further, Arise et al. (2015) reported lower foaming, emulsifying and water holding capacity for isoelectric precipitated bambara protein isolate in comparison to the micellised isolate.

The protein composition and charge configuration of a legume may also vary depending on the extraction pH used. For instance, Jiang et al., (2014) investigated the influence of pH shifting (pH 1.5 and 12) and ultrasound combined treatments on the physicochemical properties of pea protein isolate (PPI) fractions. The observations showed that protein isolates extracted at pH 12 had improved surface hydrophobicity and more protein sub-units when compared to samples treated under acidic conditions (Jiang et al., 2014). In a different study, Jiang et al., (2010) further concluded that extreme alkaline (pH 12) and acidic (pH 1.5) medium treatments can significantly modify structure of both β-conglycinin and glycinin components of soy protein isolate (SPI). In addition, Ursu et al., (2014) reported that proteins extracted from micro-algae at pH 7 and pH 12 showed a 5.4 % difference in aspartic acid content, 5.8 % difference in glutamic acid content and 6.3 % difference in lysine and arginine content.

Table 2: Difference in amino acid contents between selected legumes

Legume type	ASP	GLU	ARG	LYS	HIS	ALA	ILE	LEU	MET	PHE	PRO	VAL	TRP	CYS	SER	THR	TYR	GLY
Bambara	9.6	15.4	5.9	6.3	3.0	3.5	3.8	7.3	1.3	5.3	2.7	4.3	7.3	ND	3.2	2.8	3.3	3.1
Cowpea	12.2	18.9	6.8	6.9	2.5	4.4	4.6	7.7	1.2	5.7	3.9	5.4	3.8	1.0	5.5	3.8	3.2	4.1
Soybean	11.6	19.1	7.7	6.4	2.8	4.3	4.0	7.8	1.4	5.2	ND	5.0	ND	ND	5.6	4.1	3.8	4.7
Peanut	11.2	18.7	11.4	3.4	2.1	4.0	3.1	6.2	1.1	3.6	4.3	3.8	0.9	1.2	4.9	2.7	5.1	4.6

Amino acid values are expressed in g/100g protein. Recalculation has been made where necessary. ND: Not determined. 2 Sources :a Kudre et al. (2013); bAmonsou et al. (2012); cLiu et al. (2013); Pastor-Cavada et al. (2014); Adebowale et al. (2011); fLatif et al. (2013) and Elhardallou et al. (2015). 3 FAO/WHO recommended pattern for pre-school children age ; 2-5 years

The main amino acids present in legume storage protein are glutamic and aspartic acid which may include glutamine and asparagine, respectively. These amino acids account for 25-40% of the total amino acids in leguminous seeds (Kudre et al. 2013; Latif et al. 2013; Pastor-Cavada et al. 2014). However, higher amounts of arginine have also been reported in faba bean, peanut, soybean and cowpea (Latif et al. 2013; Pastor-Cavada et al. 2014; Elhardallou et al. 2015). Adebowale and Maliki, (2011) reported an arginine content of approximately 8.1 % for bambara groundnut.

The lysine content of bambara (6.3 g/100 g protein) is similar to that of mung bean (Kudre et al. 2013) and soybean (Adebowale and Maliki, 2011). Based on FAO/WHO (2007) reference, indigenous legumes such as bambara groundnut, mung bean and faba bean contained adequate sources of lysine similar to soya bean (Arise et al., 2017). Thus, indigenous legumes offer significant nutritional supplementation to cereals that are known to be deficient in lysine. Moreover, the methionine content of bambara (Kudre et al. 2013) is higher than that of marama bean (Amonsou et al. 2012) and faba bean but similar to black bean (Kudre et al. 2013) and soya bean (Adebowale and Maliki, 2011).

2.7 Protein-polysaccharide Complexes

The formation of self-assembled colloidal particles of proteins and polysaccharides is termed protein-polysaccharide complexation or coacervation. Protein-polysaccharide complexation was first studied by De Jong and Kruyt (1929) between gelatine and gum arabic (Weinbreck, 2004). Complexation or coacervation is defined by Wang and Schlenoff, (2014) as the phase separation of a colloidal system into biopolymer-rich and biopolymer-deficient phases induced by modification of the media environment. Coacervation can be categorized as simple and complex. Simple coacervation involves the use of one biopolymer whose precipitation is facilitated through varying ionic strength or addition of a more hydrophilic compound which then leads to salting out of the biopolymer (Schmitt and Turgeon, 2011). Complex coacervation on the other hand is driven by attraction between two oppositely charged polymers to yield a polymer rich phase which is the coacervate and a polymer poor region which is the equilibrium solution (Schmitt and Turgeon, 2011).

Depending on the medium conditions, biopolymer characteristics, biopolymer concentration and mixing ratio, a protein-polysaccharide mixture may result in associative (attractive) or segregative phase separation. Under very dilute biopolymer mixtures, proteins and polysaccharides remain co-

soluble with limited interactions within the aqueous solution (Gharsallaoui et al., 2010). Varying the biopolymer concentration, ionic strength and pH results in fluctuating repulsive and attractive forces. This eventually leads to phase separation of the biopolymer mixture.

Segregative phase separation occurs when two biopolymers are thermodynamically incompatible. This phase is driven by electrostatic repulsive forces between proteins and polysaccharides carrying similar net charges. Separation leads to both a protein-rich and polysaccharide-rich phase (Doublier et al., 2000). In contrast, associative phase separation (also known as complexation) occurs when the biopolymers carry opposite net charges and experience electrostatic attractive forces (De Jong, 1934). This charge interaction leads to separation into a biopolymer-rich (proteins + polysaccharides) and a solvent-rich phase.

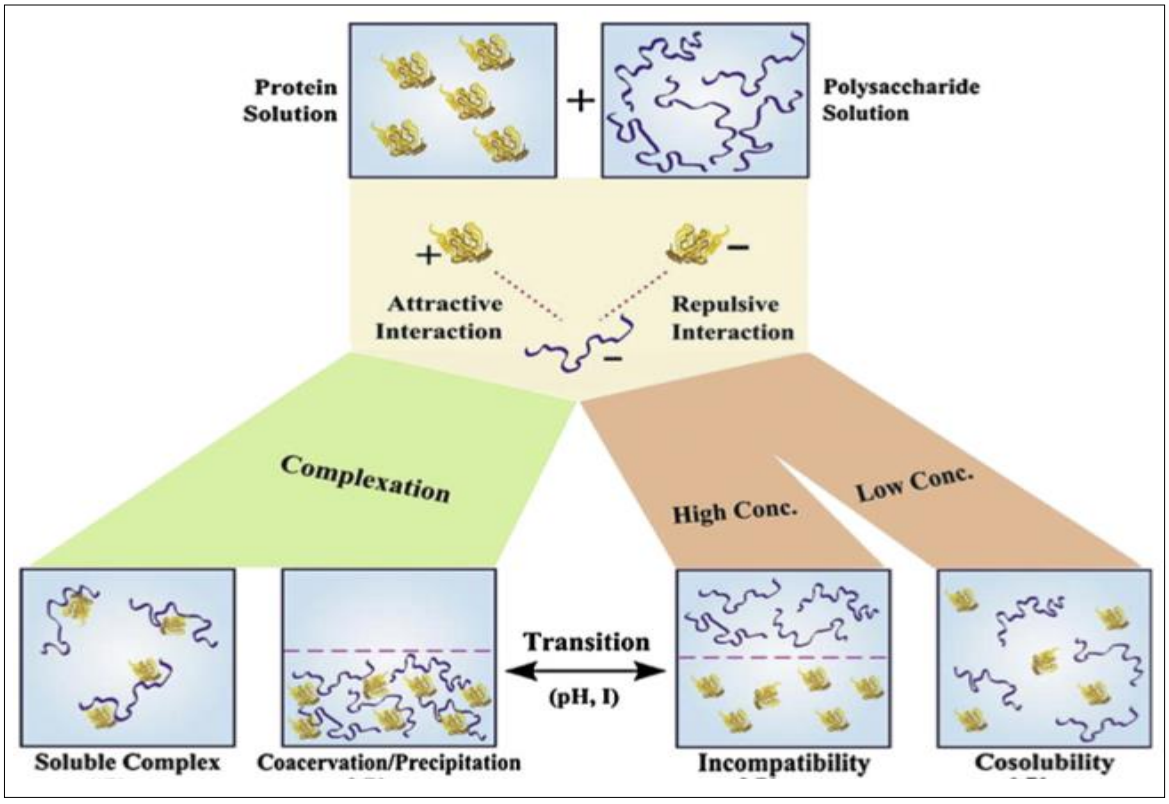

Fig 2: Complex coacervation schematic diagram (Schimmt and Turgeon 2011)

Coacervation is believed to follow two pH-induced structure forming events associated with the formation of soluble and insoluble complexes during acidification. As proteins take on a net positive charge below the isoelectric point (pI), initial attraction leads to a slight change in turbidity

and the formation of soluble complexes which corresponds to a critical pH_c (Liu, 2009a). As the solution is acidified further, complexes continuously grow in size and number until reaching a second critical $pH_{\phi 1}$ which is the formation of insoluble complexes. At this pH, solutions change from transparent to cloudy, and macroscopic changes in turbidity ensue until reaching a maximum pH_{opt}, where opposing charged biopolymers reach an electrical equivalence giving a neutral complex (Weinbreck, 2004). As pH is lowered further towards pKa of the polysaccharides, reactive sites on the anionic polysaccharide become protonated leading to the progressive loss of the complex structure and turbidity until reaching complete dissolution at $pH_{\phi 2}$ (Aryee and Nickerson, 2012).

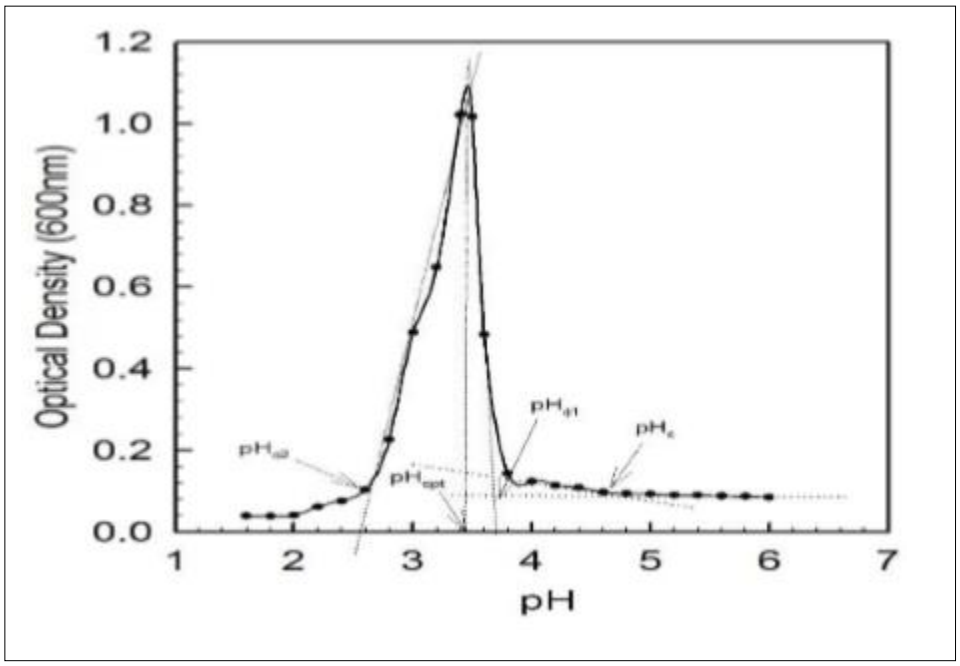

Fig 3: Schematic diagram showing critical transitions pH on a turbidity profile (Aryee and Nickerson, 2012)

2.7.1 Complexation by covalent interactions

Protein-polysaccharide complexes produced through conjugation are useful in the food industry as emulsifiers, stabilisers and fat mimetics. The Maillard reaction is a modification reaction recognized as suitable for producing specialty ingredients for food applications (Spotti et al., 2014). The Maillard reaction involves non-enzymatic condensation reaction between a reducing

sugar and an available Ɛ-amino group of the amino acid resulting in an Amadori rearrangement (Kasran et al., 2013).

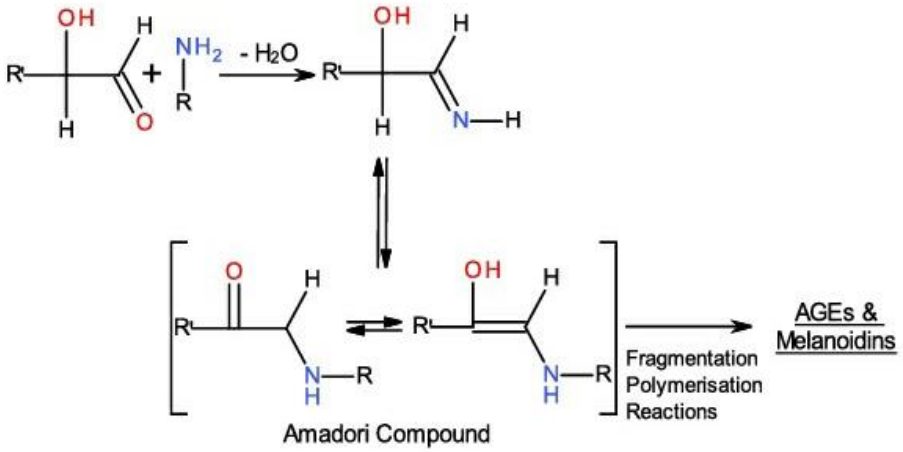

Figure 4: Schematic representation of Maillard reaction showing a reducing end of polysaccharide reaction with a free amino group of protein (Evans et al., 2013)

Condensation of a carboxyl group of the reducing sugar with the Ɛ-amino group of the protein is sufficient for conjugation via the formation of a covalent bond. These conjugates have been shown to be stable to changes in pH, ionic strength and temperature (Schmitt and Turgeon, 2011). Moreover, Akhtar and Dickinson, (2007) reported that significant improvements in interfacial functionality can be achieved through protein-polysaccharide conjugation. For instance, Xue et al., (2013) reported that emulsifying properties of SPI were significantly improved after conjugation with gum arabic. Furthermore, Yin et al., (2012) reported that although pea protein was denatured during the Maillard reaction, the emulsion droplet size did not increase. Instead, denaturation of the protein caused a significant decrease in emulsion droplet size which resulted in shelf-stable emulsions. The finding was due to the irreversible oil-water interfacial films, whereby the polysaccharide was fixed onto the droplet surface (Yin et al. 2012).

2.7.2 Complexation by electrostatic interactions:

Electrostatic interactions between proteins and polysaccharides result in attraction or repulsion between biopolymers in aqueous solution. If the two biopolymers are oppositely charged, complexation or attractive phase separation occurs. When the biopolymers are likely charged, repulsion or segregative phase separation occurs (Kasran et al., 2013). Complexation between proteins and anionic polysaccharides occurs below the protein isoelectric point (pl), whilst

complexation with cationic polysaccharides occurs above the protein isoelectric point (Takahashi et al., 2013). Most plant proteins (pI ~ 4-5) can form complexes with anionic polysaccharides (pKa ~ 2-3) in the intermediate region of pH where the two macromolecules carry opposite net charges (pI>pH<pKa) (Timilsena et al., 2016).

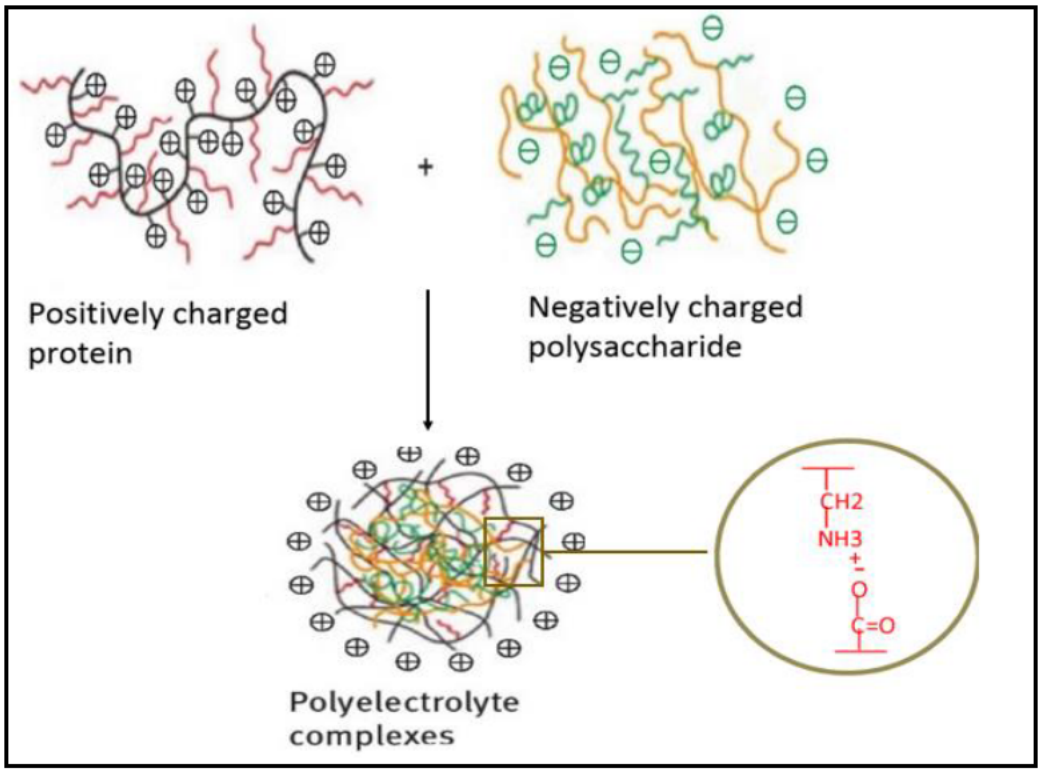

Fig 5: Electrostatic interactions between a positively charged protein and negatively charged polysaccharide (Jones and McClements, 2011)

2.8 Factors Affecting Coacervation

Interactions between biopolymers play a significant role in controlling structure, texture and stability of the coacervates. The degree of complexation depends on a number of intrinsic and extrinsic factors. Intrinsic factors include the distribution of ionizable groups on the surface of the protein, ease of protein unfolding, backbone flexibility and charge density of the polysaccharide (Dickinson, 1998; Kasran et al., 2013). Extrinsic factors include pH, ionic strength, biopolymer mixing ratio, temperature as well as techno-functional aspects such as homogenizing speed.

2.8.1 Effect of pH on coacervation

The degree of ionization of the amino group of proteins is dependent on pH of the medium in which they exist. When the pH of a solution is close to the isoelectric point (pI) of the protein, the protein has minimal charge and consequently limited interaction with the polysaccharide. At pH = pI, the protein is neutral and precipitates out of solution. Optimal coacervation occurs when charges between proteins and polysaccharides reach electrical equivalence, a point where they both carry equal and opposite charges. It has been observed that optimal coacervation occurs within a narrow pH range between the pI of the protein and pKa of the polysaccharide. (De Kruif et al., 2004). Weinbreck, (2004) investigated the interaction of gum arabic (GA) and whey protein isolate (WPI) and observed that at a pH below 2.3, the carboxyl group of the gum arabic (pKa = 2.2) became protonated whilst the WPI (pI~5) carries a net positive charge. When the pH was above pH 2.2, both GA and WPI carried a net negative charge and in both instances, the biopolymers do not react with each other. This is in agreement with ר(Jun-xia et al. 2011) who reported that optimum complexation between SPI and GA occurred at pH 4. Beyond pH 3.5, coacervate yield decreased significantly and this was attributed to the protonation of carboxylic groups on the gum arabic. In a separate study, Wee et al., (2014) studied complexation between puka gum (extracted from *Meryta sinclaii*) and WPI. They observed that soluble complexes formed at pH 5.7 which is above the pI of WPI (5.1) whilst the pHopt was observed at pH 4.7. The formation of soluble complexes at a pH above WPI was attributed to the interactions between positive patches of WPI and negatively charged carboxyl groups on puka gum. According to Wee et al., (2014), increasing ratio of protein to polysaccharide resulted in an increase of the pHopt as a result of the presence of more positively charged protein molecules.

2.8.2 Effect of ionic strength

The number of ions present in a biopolymer solution is an important factor affecting complex coacervation. The presence of NaCl at sufficiently high concentrations has the effect of screening surface charges on both the protein and the polysaccharide. This results in reduced electrostatic interactions and prevents complex formation (Liu et al., 2009b). Previous studies have shown that at low concentrations, NaCl ions associate with the protein structure, thus altering its conformation to expose additional charged groups. On the other hand, low ionic strength has an effect of overcoming short-range repulsions between polymers, leading to exposure of more sites on the

protein and eventually increasing electrostatic interactions and enhancing complex coacervation (Eghbal and Choudray, 2018). For instance, Jun-xia et al., (2011) observed that an increase in NaCl concentration significantly decreased coacervate yield and increased the protein content in the supernatant, an indication of the dissociation of complexes. Weinbreck, (2004) observed similar results on a WPI/GA complex system. The addition of NaCl causes a significant decrease of the $pH_{\phi 1}$ and results in the polymers interacting at lower pH values were they both carry negative charges (Weinbreck, 2004).

At low ionic strengths, sulfated polysaccharides of relatively high charge density can form fairly strong reversible complexes with proteins, even at neutral or alkaline pH (i.e. well above pI of protein) (Kasran et al., 2013; Dickinson, 1998). Further, Liu et al., (2009b) reported that NaCl of less than 45 mM enhanced complexation between WPI and carrageenan whilst complexation was inhibited at ionic strengths higher than 1 M. Different types of salts also gives different effects on complexation. For instance, when CaCl2 was added to whey protein-carrageenan mixture, the complexes were formed at pH 8, a phenomenon which was attributed to calcium bridging (Weinbreck, 2004). Further, the addition of $MgCl_2$ and $BaCl_2$ downshifted pHopt and reduced coacervation yield more than incorporation of NaCl (Niu et al., 2015). This was attributed to the incorporation of divalent cations in the complexes.

2.8.3 Effect of biopolymer mixing ratio

Optimal coacervation is observed when equal opposite charges on the protein and polysaccharides are neutralized. The total charges available for coacervation are determined by the biopolymer mixing ratio. Adjusting biopolymer mixing ratio to favour maximum charge neutralisation is imperative to keep the binding affinity constant (Antonov et al., 2018). (Liu et al., 2009b) and (Klassen, et al. 2011) investigated the effect of biopolymer mixing ratio on pea protein-gum arabic and canola protein –alginate complexes respectively. Both authors hypothesized that mixing ratio dependence is a result of initial complexes forming from protein-protein aggregates available per polysaccharide chain. It was therefore concluded that biopolymer mixing ratio follows a progressive growth of protein- protein aggregates with increasing biopolymer concentration until

a critical size is reached (Liuet al., 2009b). Numerous studies have reported results that agree with this hypothesis. For instance, De Kruif et al., (2004) and Liu et al., (2009b) studied the complex coacervation of whey protein-carrageenan and pea protein isolate-GA respectively. They observed that protein-polysaccharide saturation occurred at mixing ratios of 30:1 and 4:1, respectively. A study by Aryee and Nickerson (2012) on lentil protein isolates (LPI) and gum arabic indicated that gum arabic interacted with all positive charges of LPI at a mixing ratio of 1:1 at which the zeta potential was 0 mV. Beyond this mixing ratio, excess amount of protein in the solution contributed to the formation of LPI-LPI aggregates rather than complexation. In a study by Niu et al., (2015), increasing protein-polysaccharide ratio of ovalbumin-gum arabic from 1:1 to 24:1 shifted the turbidity curve to higher pH values. However, the highest turbidity value which indicated maximum coacervation was observed at a ratio of 2:1.

2.8.4 Effect of polysaccharide charge density

Biopolymer charge density determines the strength of attraction and plays an important role in the outcome of complexation. Depending on the strength of attraction, protein-polysaccharide electrostatic interactions may result in complex coacervates, soluble complexes or amorphous co-precipitates. The main classes of positively charged residues in most proteins are the $–NH_{3+}$ groups (pKa ~11). An increase in net negative charges on the protein results in enhancement of electrostatic protein–protein repulsion (Curtis et al., 2002). Further, it reduces protein–polysaccharide attraction by screening the interactions of the positively charged groups. In polysaccharides, carboxylated polysaccharides (e.g. alginate, pectin) have a weaker charge density when compared to sulfated polysaccharides (e.g. carrageenan, chitosan) (Aryee and Nickerson, 2014). The molecular attraction of protein bound $-NH_{3+}$ groups to $–OSO_{3-}$ groups is much stronger than for $–CO_{2-}$ groups. According to De Kruif et al., (2004) weak polysaccharides have a low linear charge density and form complexes with oppositely charged protein. However, strong polyelectrolytes have a higher charge density and form precipitates with proteins over a narrower pH range. For instance, a study by Souza and Garcia-Rojas, (2017) on the effect of charge density on thermodynamics of coacervation revealed that polymers with sulphated polysaccharides such as chitosan release more heat compared to those with carboxyl groups. Moreover, Stone et al., (2013) reported a decrease in optical density (OD) of canola protein isolate (CPI) aggregates upon addition of carrageenan gum. This was attributed to the presence of strong electrostatic forces induced by sulphate groups of the free carrageen molecules.

2.9 Application of Plant Protein-polysaccharide Complexes

Protein-polysaccharide complexes have gained momentum in the food industry due to their functionality. Polysaccharides have the ability to increase bulk viscosity in emulsions, thus improving stability against coalescence. When mixed with proteins, polysaccharides interact with the latter by hydrophobic interactions, hydrogen bonding and electrostatic interactions (Stone et al., 2013). Protein-polysaccharide complexes are believed to be more surface active than individual proteins or polysaccharides, therefore they are regarded as better emulsifiers and stabilisers in emulsion systems. They have also been used as fat replacers in foods due to their ability to mimic sensory, optical and rheological properties of lipid droplets (Oduse et al., 2017). According to Oduse et al., (2017), whey protein-pectin particles showed higher foaming and emulsifying properties when compared to whey protein alone. In a separate study by Wang et al., (2016), heated soy protein isolate complexed with a carrageenan soluble complex was found to have the highest emulsifying and foaming properties.

Tran and Rousseau (2013) reported greater emulsion stability for soy soluble polysaccharide-SPI complexes. They attributed this observation to the ability of polysaccharides to coat the protein's surface thus preventing protein–protein interactions. According to Hayati et al., (2009), when polysaccharides bind to proteins at the interface, they change the rheology of the emulsion. However, this laso depends on the type of polysaccharide used. For instance, the addition of gum arabic to an egg-protein-stabilised emulsion turned the rheology of the emulsion from a Newtonian fluid to a Bingham plastic while the use of other polysaccharides such as locust bean, guar gum (GG) and carboxymethycellulose resulted in shear thinning behavior (Hayati et al., 2009).

Another important application of protein-polysaccharide complexes is the encapsulation of bioactive compounds and nutraceuticals. Encapsulation is the process during which bioactive compounds (core material) are entrapped inside a thin layer of coating material (shell) that release their contents at controlled rates, specific sites and over a prolonged period of time (Nedovic et al., 2011). Proteins and polysaccharides including gums, either individually or in complexed form commonly used as shell materials for encapsulation of bioactive compounds (Timilsena et al., 2017). The shell matrix creates a physical barrier between the core and the external environment so that the core is protected from direct sunlight, moisture, heat or oxygen (Timilsena et al., 2018).

In recent years, microencapsulation has been employed in the cosmetic, pharmaceutical as well as the food industry for controlled release and targeted delivery of the core compound. Several studies have shown that bioactive compounds can be encapsulated and protected within a protein-polysaccharide wall. For instance, Jun-xia et al., (2011) successfully encapsulated sweet orange oil within a soybean protein isolate-gum arabic complex and observed that spray-dried capsules had smooth surfaces compared with the freeze-dried capsules. Similarly, (Mendanha et al., 2009) investigated soy protein-pectin complexes to encapsulate casein hydrolysate and reported that the two biopolymers form a stable wall material around a compound. (Timilsena et al., 2016) investigated the use of chia seed protein-chia seed polysaccharide in encapsulation of chia seed oil. Protein-polysaccharide complexes may therefore have potential as future carrier matrices for bioactive compounds.

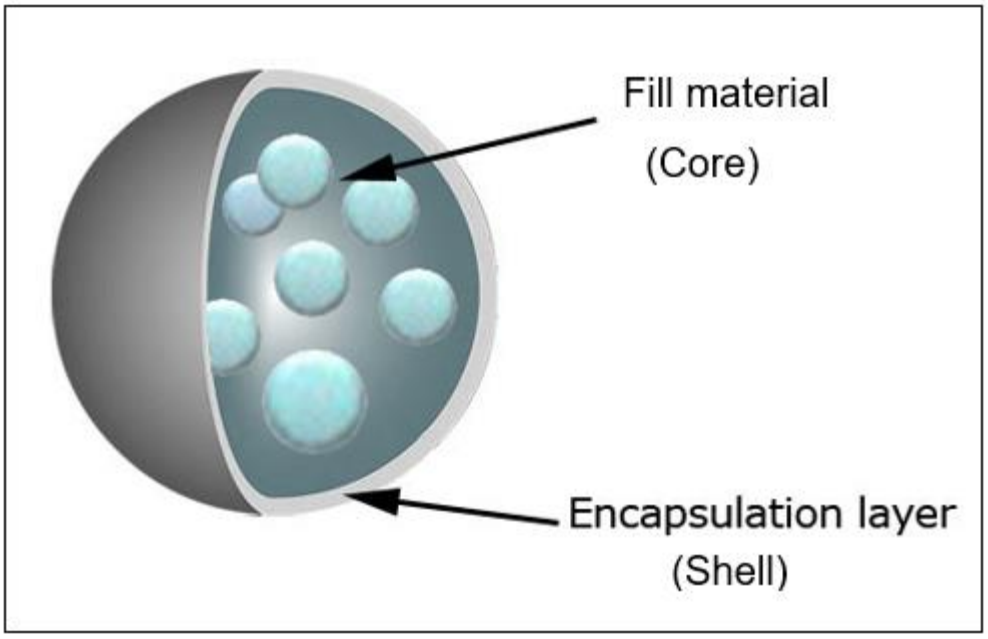

Fig 6: Schematic representation of encapsulation (Schimmt & Turgeon, 2011)

2.10 Encapsulation Techniques

The selection of encapsulation techniques is governed by physical and chemical properties of the core material, the coating material as well as the intended application of the food ingredients (Desai and Jin-Park, 2005). Encapsulation techniques should be able to incorporate bioactive compounds into food matrices whilst ensuring minimal impact on the organoleptic properties of the product as well as maintaining the physicochemical stability of the complexes. The technique should

maximize the assimilation of encapsulated compounds upon consumption and ensure controlled release in response to a specific environmental stimulus (Đorđević et al., 2015)

Table 3: Overview of encapsulation techniques (Đorđević et al., 2015)

Method	**Commonly encapsulated actives**	**Properties of methods**	**Properties of encapsulation system**
Spray drying	Hydrophobic compounds Lyophilized proteins	Easy handling. Low operating costs, high production rates. Reproducibility. Wide choice of encapsulating materials. Encapsulation efficiency 10–90%. Difficult to control particle size. Not suitable for high temperature-sensitive actives.	Spherical particles (powders). Good stability of encapsulates. Non-uniform particles. Tendency of particles to aggregate
Spray cooling/ chilling	Hydrophobic compounds	Lower operating costs compared to spray-drying. Suitable for heat-sensitive actives. Encapsulationefficiency10–100%. Particle size is not easily controllable. Moderate yields for small batches.	Water insoluble micro-capsules. Rapid release of the actives. Special storage conditions required.
Extrusion processes	Cells Plant compounds Enzymes Proteins	Cost-effective. Gentle/no organic solvents, extreme temperatures or pH conditions are required. Can be performed under both aerobic and anaerobic conditions. Higher production capacity due to multiple nozzle systems. Encapsulation efficiency 20–50%. Difficult to scale-up	Spherical particles. Burst release of actives from matrix type.

		Difficult to process high viscous polymer solutions.	
Emulsification process	Hydrophilic and hydrophobic/lipophilic compounds	Easier to scale up than extrusion. Microgel particles must be separated from the liquid bath. More expensive compared to extrusion. Process difficult to control.	Spherical particles. Burst release of actives from matrix type.
Complex coacervation	Hydrophobic compounds Flavours Essential oils	Expensive. Complex mechanisms. Sensitive to environmental conditions. Suitable for heat-sensitive actives. Encapsulation efficiency 40–90%. Use of organic solvents.	Spherical, multi-nucleate particles. Heat-resistant. Controlled release of actives. Good storage stability.

2.10.1 Encapsulation by coacervation

Encapsulation by coacervation involves four main steps of emulsification, coacervation, gelation and hardening of the capsules (Eghbal and Choudhary, 2018). According to Timilsena et al., (2016), applying mild heating maybe necessary for the purposes of increasing mechanical and thermal stability of the formed capsules. Encapsulation by coacervation is beneficial for its high encapsulation efficiency as well as the controlled release properties which may be triggered by mechanical stress, temperature or presence of enzymes and acids (Wang and Schlenoff, 2014; Eghbal and Choudhray, 2018).

Complex coacervation has been used for the encapsulation of a wide range of hydrophobic oils. Different oils such as sweet orange oil, flax seed oil and canola oil among others have been encapsulated. Encapsulation by coacervation has also been used to encapsulate probiotic for delivery in the gastro-intestines. For instance, Eratte et al., (2015) successfully co-encapsulated tuna oil and *Lactobacillus casei* in WPI-GA complexes for delivery in the gastro-intestines. Many studies have shown the encapsulation effectiveness of these protein-polysaccharide complexes. For instance, Jain et al., (2015) reported 70 % encapsulation efficiency for β-carotene encapsulated in WPI-GA complexes.

Encapsulation studies using plant protein-polysaccharides as carrier particles have been on the rise. However, some studies have reported the unsuccessful encapsulation of oils. A study by Liu et al., (2010) on encapsulation using PPI-GA complexes revealed that the complex system failed to

encapsulate flax oil when compared to a Gelatin-GA complex system. This was attributed to the different chemical structure of plant protein versus animal protein. It was suggested that the smaller and flexible molecular structure of gelatin allows it to quickly align at the oil interface as opposed to the bigger and rigid structure of pea protein. Fortunately, one way of improving encapsulation efficiency of plant protein-polysaccharide complex systems could be through cross-linking. A study by Yuan et al., (2017) investigated the encapsulation of algal oil in SPI-chitosan complexes and observed an encapsulation efficiency of over 97% and 75% for transglutaminase cross-linked and non-cross linked complexes respectively. Yuan et al., (2017) further reported that hardening the capsules with transglutaminase improved the oxidative stability of SPI-chitosan capsules.

Many factors such as the core to wall ratio and drying techniques may affect the encapsulation efficiency, release properties and integrity of carrier particles. For instance, Timilsena et al., (2016) studied complex coacervation of chia seed protein-chia seed gum to encapsulate chia seed oil. They reported that capsules obtained by complex coacervation followed by spray drying showed higher encapsulation efficiency and increased oxidative stability than microcapsules obtained by simple emulsification followed by spray drying. Qv et al., (2011) observed that spray drying lutein encapsulating Gelatin-GA particles gave a smoother surface compared to freeze-drying. The core to wall ratio may also affect encapsulation efficiency. Jun-xia et al., (2011) reported that increasing the core load significantly decreased the encapsulation efficiency but increased the size of the particles. A general trend as observed by Hogan (2001) was that encapsulation efficiency decreases as core-wall ratio increases.

2.10.2 Encapsulation of bioactive compounds

Bioactive compounds are defined as extra-nutritional constituents that typically occur in small quantities in foods (Kris-Etherton et al., 2002). Bioactive compounds can be divided into bioactive molecules (vitamins, bioactive peptides, lipids etc.) as well as bioactive living cells (probiotics) (Van-Immerseel et al., 2010). Despite their health benefits, bioactive compounds are highly unstable during manufacturing, storage and transportation. They become highly unstable when they are exposed to elevated temperatures, light, oxygen and extreme pH. In the human gastrointestinal tract, bioactive compounds still remain vulnerable to extreme pH (Kris-Etherton et al., 2002).

The instability of bioactive compounds in foods results in impartation of bad flavour and stringency to food matrices. Further, their bioavailability in the body may also be compromised. Encapsulation therefore plays a role in ensuring stability of the bioactive compound in food matrices as well as the gastrointestinal tract. Moreover, encapsulation promotes controlled release at the desired target, thus promoting bioavailability (Đorđević et al., 2015).

2.11 Choice of Biomaterials

2.11.1 Gelatine

Gelatine is a protein of animal origin obtained from acid/basic hydrolysis of collagen. Acid hydrolysis yields gelatine A whilst basic hydrolysis yields gelatine B. Gelatine consists of a large number of glycine, proline and 4-hydroxy proline residues (González-Paz et al., 2013). The isoelectric point of gelatine depends on the method of extraction. Acidic gelatine has an isoelectric point of 5 whilst basic gelatine has an isoelectric point of 9. The quality of gelatine is correlated with its physical, chemical and structural characteristics (Brychcy et al., 2015). Under specific conditions (temperature, solvent and pH) gelatine macromolecules display flexibility sufficient to realize a wide variety of conformations. This makes it possible to vary the gelatine characteristics depending on its molecular structure (Brychcy et al., 2015).

A gelatine-gum arabic complex system is one of the classic complex systems to be studied by Bungenberg de Jong and Kruyt in 1929 (De Kruif et al., 2004). Gelatine is primarily used as a gelling agent in food pharmaceuticals and cosmetic industries. The polysaccharide also has emulsifying properties, hence has also been used as a carrier compound for encapsulation and sustained release of bioactive compounds. For instance, Shaddel et al., (2018) encapsulated black raspberry water extracts in double emulsions formulated from gelatine-gum arabic. Further, Qv et al., (2011) reported 85 % lutein encapsulation efficiency on a gelatine-gum arabic complex system.

2.11.2 Beta (β) carotene as the core compound

Carotenoids are lipophilic compounds that contribute the yellow to red color in many foods. β -carotene serves as a vitamin A precursor and is mainly found in red palm oil, palm fruits, leafy green vegetables, carrots, sweet potatoes, pumpkins, mangoes and papayas (Pattani, 2010). A characteristic feature of carotenoids is the chemical structure of their backbone molecule.

Carotenoids such as β-carotene have a 40-C backbone which consists of conjugated double bonds. This feature allows carotenoids to take up excess energy from other molecules through a non-radiative energy transfer mechanism (Ranjan et al., 2014). This characteristic is responsible for the antioxidant activity where the major role is to quench singlet oxygen. β –carotene is

known to have exceptional antioxidant and free radical scavenging potential (Qian et al., 2012). It has also been shown to enhance immune function, decrease risk of degenerative diseases such as cancers, cardiovascular diseases as well as cataracts (Ranjan et al., 2014).

The conjugated structure of β -carotene is highly susceptible to conditions encountered during processing and storage. Extensive exposure to light, extreme heat, oxygen and acids may cause isomerisation of t*rans*- carotenoids which is the stable form, to *Cis*- carotenoids, the unstable form (Ranjan et al., 2014). This can result in color loss as well as reduction in pro-vitamin A and free radical quenching activity (Gomes et al., 2013), hence the need to encapsulate β –carotene. Many studies have successfully encapsulated β –carotene. Jain et al., (2015) successfully encapsulated β -carotene in WPI-GA. The study indicated that the encapsulation efficiency was greater than 70 % with sustained release behaviour. Further, Jain et al., (2016) encapsulated β –carotene in casein-gum tragacanth complexes and reported an encapsulation efficiency of 79% as well as improved stability of the bioactive compound.

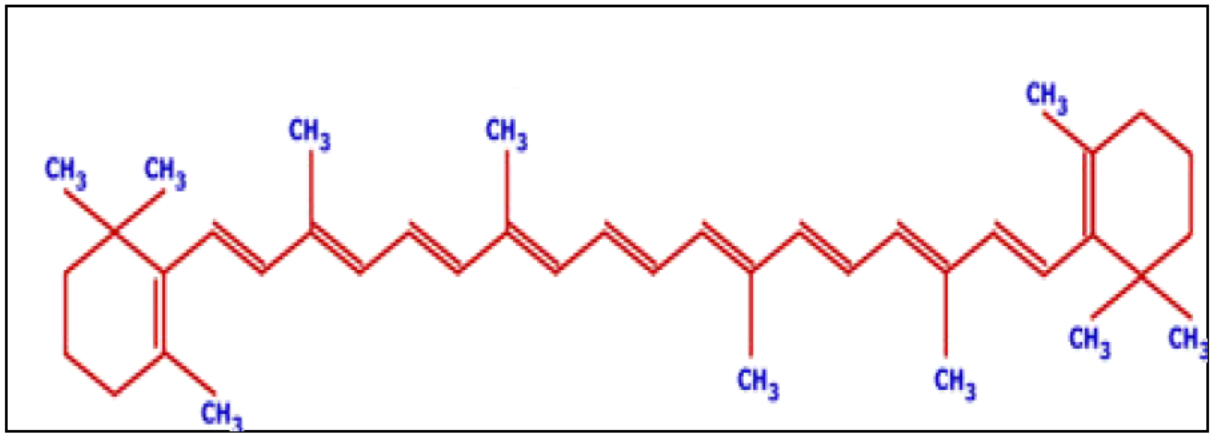

Fig 7: Schematic presentation of chemical structure of beta carotene (Pattani, 2010)

2.12 Polysaccharides

Polysaccharides are a class of carbohydrates with polymeric oligosaccharides formed through glycosidic linkages among the multiple monosaccharide repeat units (Holdt and Kraan, 2011). In nature, the main sources of polysaccharide origins are plants (pectin, cellulose), animals (chitosan, chitin, glycosaminoglycan), microbial (dextran, xanthan gum, gellan gum) and algal (agar, alginate, carrageenan). Depending on composition of the monosaccharide units, polysaccharides can be classified as homopolymers (formed from the same monosaccharide repeat units; e.g starch, cellulose), or heteropolymers (formed from different monosaccharide units like chitosan) (Holdt and Kraan, 2011). Polysaccharides are promising biomaterials due to their abundance in nature, low cost, biodegradability, non-toxicity and water solubility. In addition, polysaccharides have a larger number of reactive functional groups on their backbone which can be easily derivable and contributes to their structural and functional diversity (hydroxyl, carboxyl and amino) (Debele et al., 2016).

2.12.1 Gum Arabic (GA)

Gum arabic is a complex exudate of the *Acacia senegal* and *Acacia seyal* tree belonging to the Leguminose family. It is non-digestible and its low viscosity in solutions renders it popular for its emulsifying and stabilizing properties (Featherstone, 2015). Moreover, it is characterized as a non-starch polysaccharide which is resistant to intestinal enzymes and is fermentable in the gut to liberate short chain fatty acids (Nie et al., 2013).

Gum Arabic is an anionic polysaccharide which is highly branched and contains protein fractions in its structure. The structure of gum arabic is composed of a main chain made up of (1,3) and (1,6) linked β-D-galactopyranosyl units with (1,6)-linked β-D-glucopyranosyl uronic acid units. (Williams and Phillips, 2009). Gum Arabic has also been reported to contain approximately 2 % glycoprotein of approximately 250 kDa (Aryee and Nickerson 2012). The second fraction is the arabinogalactan-protein fraction which constitutes approximately 10% of the gum. The arabinogalactan chains are covalently linked to a polypeptide backbone. The third fraction is a glycoprotein which represents approximately 1% of the total gum and it contains 20-50% protein composed of different amino acids (Aryee and Nickerson 2012).

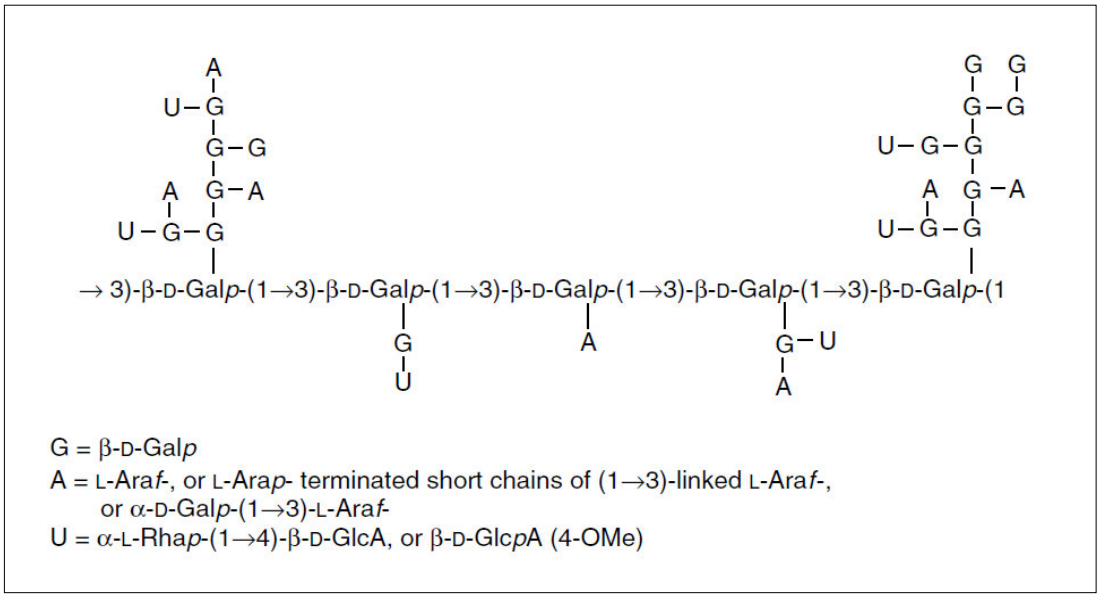

Fig 8: A schematic structure of gum arabic (Izydorczyk et al., 2005)

Gum Arabic is usually used as an emulsifier in the production of flavour oil-water emulsions for application in beverages due to its hydrophobic peptides and hydrophilic polysaccharides which align at the oil/water interphase (Weinbreck, 2004; Featherstone, 2015). Due to its excellent emulsification properties, gum Arabic has been one of the extensively used polysaccharide in microencapsulation. De Jong and Kruyt (1929) were the first to investigate the coacervation phenomenon on a gum Arabic-gelatin system. The complex system was further investigated by other scholars such as (De Kruif et al., 2004). The complexation potential of other animal proteins with gum arabic has also been investigated. For instance, (Weinbreck, 2004) successfully encapsulated sunflower oil, lemon and orange oil in a whey protein isolate (WPI)/GA complex system and observed that a pH of 4 produced over 80 % encapsulation efficiency. Further, Prata et al., (2008) investigated the integrity of oil-containing gelatin-gum arabic complexes after chemically and enzymatically cross-linking them with glutaraldehyde and transglutaminase respectively.

The interaction of gum arabic with plant proteins has also been investigated. For instance, (Liu , 2009a) investigated complex coacervation between pea protein isolate (PPI) and gum Arabic and observed that at pHs lower than the pKa of gum Arabic (1.88), the complexes dissociated as the gum Arabic chains became less protonated. However, not much information is available on the encapsulating properties of gum Arabic with plant proteins. A study by Liu et al., (2010) showed

that PPI-GA complex failed to encapsulate flax oil whilst a Gelatine-GA complex system had an encapsulation efficiency of 40 %. Therefore, it may be interesting to investigate the encapsulation efficiency of plant protein-gum arabic complex systems.

2.12.2 Xanthan gum (XG)

Xanthan gum is a microbial polysaccharide produced by *Xanthomonas campestris* through a fermentation process (Featherstone, 2015). It is a high molecular weight exo-polysaccharide that has branched polymeric chains. Xanthan gum has a cellulosic backbone with tricassharide side chains (Izydorczyk et al., 2005). The trisaccharide side chains are comprised of D-glucosyl, D-mannosyl and D-glucuronyl acid residues in a 3:3:2 molar ratio with varying proportions of o-acetyl and pyruvl residues (Nie et al., 2013; Jindal et al., 2013). Approximately half of the terminal D-mannosyl units are linked to a pyruvate group at C4 and C6 positions, whereas the non-terminal residue is acetylated at C6. The structure of xanthan gum is a linear 1-4 –D-glucose similar to the cellulose backbone (Faria et al., 2011). The molecular weight of xanthan gum ranges from 2×10^6 to 20×10^6 Da. The thermal stability of xanthan gum against hydrolysis is far better than many other water soluble polysaccharides possibly because of the ordered helical structure that protects its molecules from de-polymerization. The carboxyl groups on the side chains of XG make it an anionic polysaccharide (Feng et al., 2014). Moreover, the hydroxyl groups also make XG a suitable candidate for electrostatic or covalent interactions with proteins (Kumar et al., 2018).

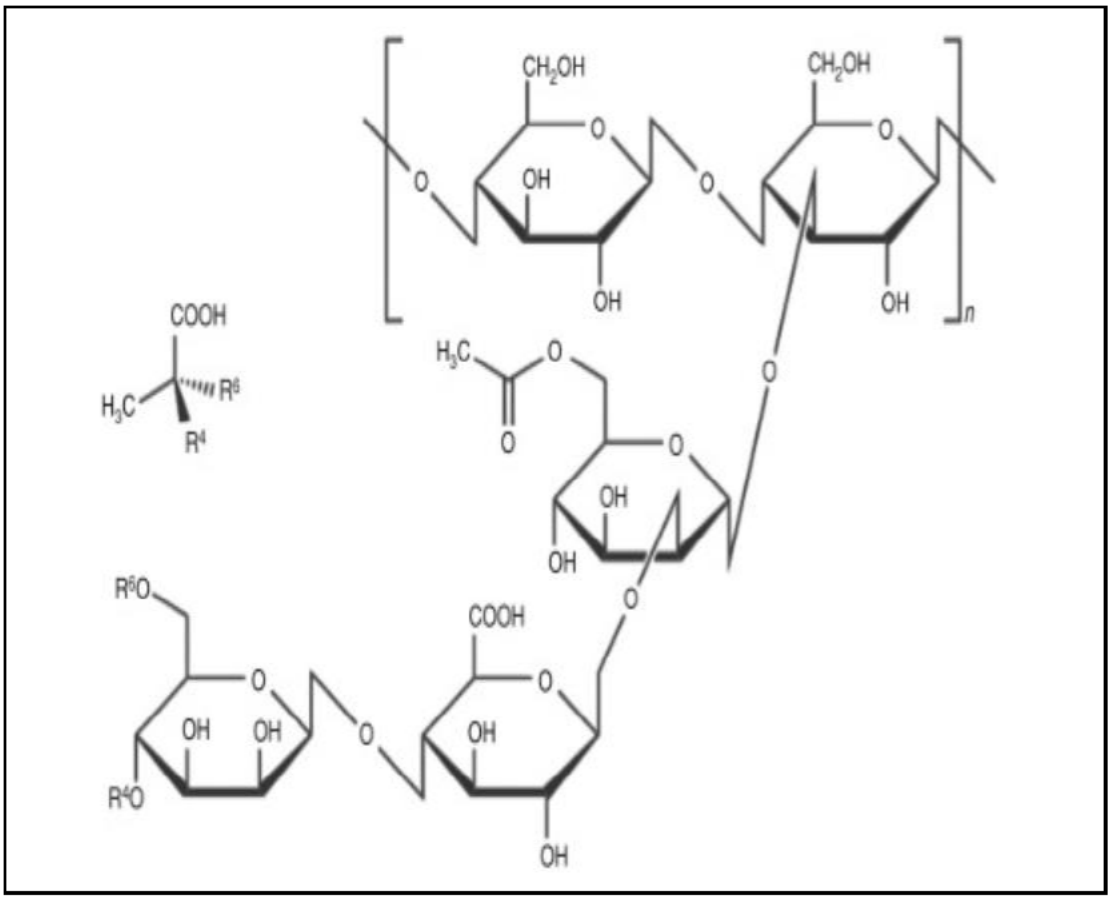

Fig 9: Chemical structure of xanthan gum (Jindal et al., 2013)

Xanthan gum has been extensively used in a wide range of applications in the food industry as well as in cosmetics and water treatments (Chang et al., 2015). But in complexation with proteins, XG is rapidly gaining attention. This could be due to the stability of xanthan gum across a broad range of temperatures (up to 90° C), salt concentrations and pH (2-11) (Jindal et al., 2013). Xantham is rapidly gaining momentum as an encapsulating matrix. For instance, Bertrand et al., (2007) reported that the addition of (0.01 – 0.06% w/v) XG to WPI resulted in improved gel strength at pH 6.0-6.5. Benichou et al., (2007) also reported that 0.1 wt% XG added to heat-denatured WPI resulted in an increase in its opacity, gelation rate and final rigidity. Numerous studies have also shown that XG complexes well with plant proteins such as SPI and PPI. For instance, Laneuville et al., (2000) investigated the preparation methods of whey protein-xanthan gum complexes and reported that Pr: Ps ratio of 5:1 and 10:1 produced principally smaller sized complexes than higher ratios.

2.12.3 Pectin

Pectins constitute a family of hetero-polysaccharides that are primarily found in the cell walls of terrestrial plants (Maxwell et al., 2012). Industrial sources of pectin include apple peels, citrus peels and sugar beet pulp. Pectin naturally acts as a soluble dietary fiber but finds its functionality in food systems as a gelling agent in jams, jellies and stabilization of fruit juices. Pectins are a family of covalently linked polymers rich in D-galacturonic acid and containing L-Rhamnose, D-galactose, L-arabinose and 13 other different monosaccharides though 20 different linkages (Kaya et al., 2014). The carboxyl group of galacturonic acid can be methyl-esterified while the hydroxyl group at the O-3 position and O-2 position of GalA can be O-acetyl-esterified. Most plant derived pectins have been identified to 3 main structural types including homogalacturonan (HG), rhamnogalacturonan-1(RG-I) and substituted galacturonans (GS). (Wang et al., 2018; Naqash et al., 2017) Over the years, many pectin structural elements have been described and all pectins are believed to essentially contain the same repeating elements, though they may differ in amounts and fine chemical structure Voragen et al., (2009). Pectins are identified by two parameters; the degree of methyl-esterification (DM) of the carboxyl group and the distribution of these methyl-esters along the pectin backbone. Based on the degree of esterification, pectins are divided into low-methyl-esterified pectin (LMP), DM <50%, and high-methyl-esterified pectin (HMP), DM>50% (Ru et al., 2012).

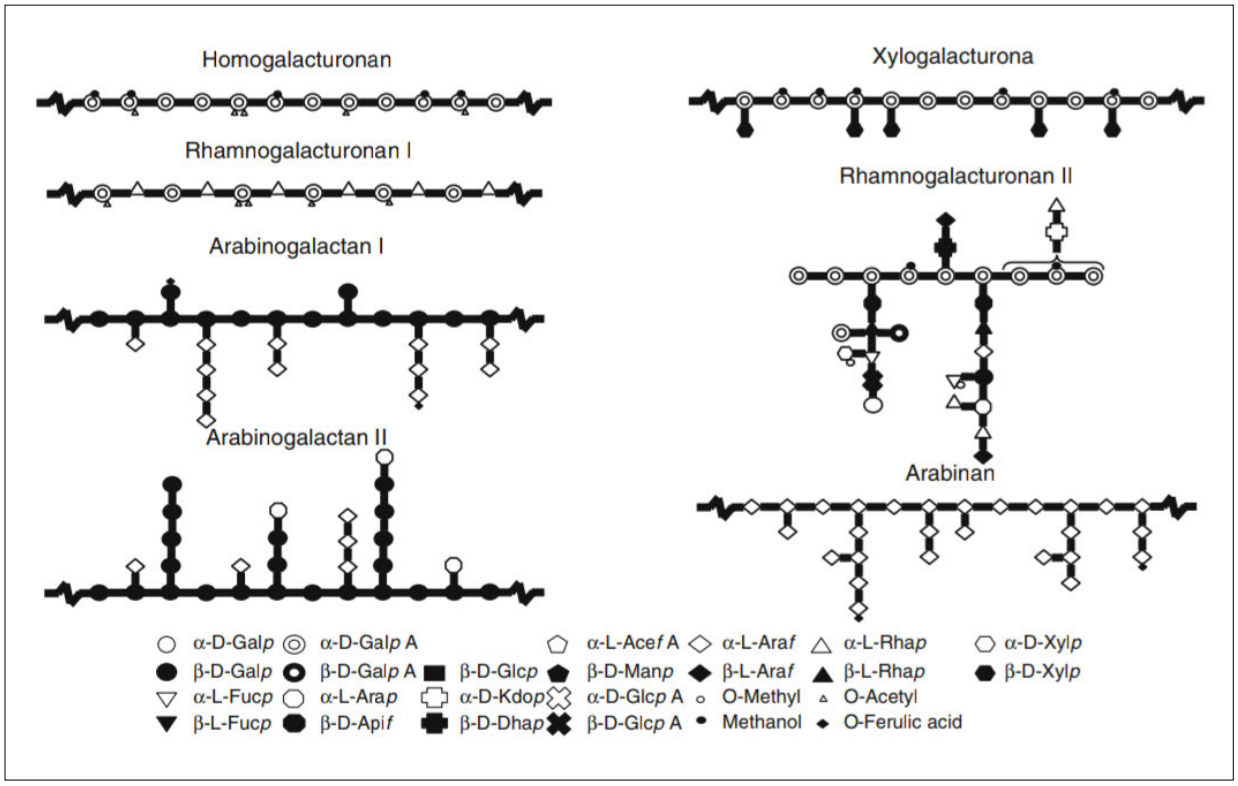

Fig 10: Schematic structure of pectin (Voragen et al., 2009)

Studies have shown that pectin has good emulsifying properties. Furthermore, the interaction of pectins with polysaccharides has been widely investigated. For instance, Ru et al., (2012) investigated the influence of ionic strength and biopolymer ratio on serum albumin-pectin complex. The study showed that increasing NaCl concentration from 0.01 to 0.04 M shifted the critical structure formation pHs to lower values. Similarly, other scholars who worked on different complex systems such as PPI-alginate reported similar results (Klemmer et al., 2012). Furthermore, Oduse et al., (2017) investigated the foaming and emulsifying properties of WPI-Pectin complexes and reported that all WPI-Pectin complexes showed higher foaming capacity and stability when compared to the native WPI alone

2.13 Hypotheses:

1. Bambara total storage protein will produce a better protein-polysaccharide complex yield compared to other protein fractions. In comparison to other protein fractions, it is expected that the total storage protein will have more charged amino acids such as glutamic acid, lysine, histidine etc., which will increase the sites for electrostatic interaction between the positively charged NH_3 group of bambara protein and the negative COOH group of the polysaccharide (Ducel et al., 2009).

2. Optimum complexation between bambara protein and gum Arabic is expected to occur at a higher protein ratio as opposed to gum arabic, a pH close to the isoelectric point of the protein and ionic strength of 10 mM. Bambara protein (pI ~~ 4.5) consists of charged amino acids e.g. arginine, lysine, glutamine and is positively charged at pH lower than its pI. gum Arabic [pKa(COOH) ~~ 1.88] on the other hand is deprotonated (negatively charged) at a pH above its pKa (Liu, et al., 2009a). The oppositely charged biopolymers will therefore electrostatically interact to form a complex. A higher protein ratio will ensure that sufficient protein molecules are available per polysaccharide thus allowing for charge balancing and enhanced electrostatic interactions among the biopolymers (Aryee and Nickerson, 2012).

3. BPI: GA complexes are expected to show better emulsifying, foaming and encapsulating properties. Gum arabic is an emulsifying agent due to the hydrophobic peptides and hydrophilic polysaccharides in its structure, and hence will aid alignment around the oil/water interphase (Featherstone, 2015). In addition, polysaccharides coat the protein and prevent protein-protein interactions that lead to flocculation (Lam and Nickerson, 2013). They also increase viscosity of the aqueous phase and may influence the viscoelastic behaviour and thickness of the adsorbed macromolecular layer, thus increasing foam stability (Oduse et al., 2017). Further, BPI: GA complexes will show improved stability to release the core compound compared to BPI alone. Stabilising bonds such as hydrogen bonds and Van der Waals interactions are generated during complexation and will contribute towards stabilising the formed BPI-GA wall.

2.14 Aim:

To form and characterize bambara groundnut protein-polysaccharide complex cocervates and test their application in encapsulation and delivery of bioactive compounds with the view of adding value and improving utilisation.

2.15 Objectives:

1. To determine the physicochemical properties (SDS-PAGE, FTIR, amino acid analysis and particle size) of bambara protein fractions and their influence on degree of complexation.
2. To evaluate bambara groundnut protein-polysaccharide complex formation as a function of pH, biopolymer mixing ratio and NaCl and further investigate the microstructure and surface properties of the formed complexes.
3. To determine some functional properties (e.g. Emulsifying, Foaming, Encapsulating and Release properties) of BPI-GA complexes using β-carotene as the model core compound.

CHAPTER THREE

Influence of pH fractionation of bambara protein on complexation with gum arabic

Abstract

Bambara groundnut is a protein-rich legume of African origin. This study investigated the influence of fractionation pH on degree of complexation of bambara protein isolate (BPI) with gum arabic (GA). BPI extracted at pH 2, pH 7, pH 9 and by the traditional salt solubilisation method were complexed with GA. BPI-GA complexes appeared as spherical particles (average size: 100-200 nm) that were aggregated. Optimal pHs of biopolymer interactions decreased (pH_{opt}: 4.8 to 2.9) as protein extraction pH became more acidic. pH 2 fraction had the lowest coacervates yield (41%). ζ-potential profiles of protein fractions displayed similar pH dependent patterns, but with different pHs of net neutrality. The pH 2 fraction showed a high molecular weight protein (100 kDa), which was absent in other fractions. It also contained the highest content of basic amino acids. Protein extraction pH could be manipulated to produce better acid- stable structures on complexation with polysaccharides.

Keywords:

Bambara, gum Arabic, complexation, protein

3.0 INTRODUCTION

Proteins and polysaccharides are widely distributed in nature and these polymers have been employed in various food applications for the development of novel ingredients. Protein-polysaccharide interactions are important in structure formation and controlling the physical and functional properties of foods. Other areas of protein-polysaccharide applications include emulsion stabilization, fat mimetic and meat replacers (Yin et al., 2012). Recently, there is a growing interest in understanding protein-polysaccharide complex coacervation as well as the application of the formed protein-polysaccharide complexes in encapsulation and controlled delivery of bioactive compounds.

Schmitt and Turgeon (2011) define coacervation as phase separation of a mixture system into biopolymer-rich and biopolymer-deficient phases. Coacervation can be simple or complex. Simple coacervation involves one biopolymer whose phase separation is facilitated through salting out of the biopolymer. Complex coacervation, on the other hand, involves the interactions between two oppositely charged biopolymers to yield a biopolymer rich phase which is the complexes or coacervates and a polymer poor region which is the equilibrium solution (Schmitt and Turgeon 2011). A mixture of protein and polysaccharide in solution may result in either complexation, precipitation or segregative phase separation depending on several factors including environmental pH, ionic strength and charge density of the biopolymers (Elmer et al., 2011; Esfanjani and Jafari, 2016). Among these factors, the solution pH is important in protein-polysaccharide complex formation as this impacts on the charge configuration of the biopolymers. Biopolymers carrying opposite charges will tend to attract each other, resulting in protein-polysaccharide complexes; whilst those of equal charge will repel, resulting in segregation or phase separation (Liu et al., 2009a).

Most research on protein-polysaccharide complexation mainly focused on understanding the mechanism of interaction between animal proteins, particularly those derived from milk (casein, whey, β-lactoglobulin) and polysaccharides (gum arabic, chitosan, pectin) (Weinbreck, 2004; Jones et al., 2009; Oduse et al., 2017). The growing consumer demands for natural products has prompted studies on complexation of plant protein with polysaccharides, hence legume proteins from soya and peas have been explored (Yin et al., 2012; Elmer et al., 2011). So far, the traditional alkaline solubilisation, followed by isoelectric precipitation and dialysis recovery is the most

common method used to extract plant protein intended for complextion with polysaccharides (Klemmer et al., 2012; Zhang et al., 2015). Optimum complexation between these proteins and polysaccharides has been generally observed within a narrow pH range: 3.5 - 4.0 (Aryee and Nickerson, 2014; Timilsena et al., 2016). This could limit the use of formed complexes in certain applications, especially in the formulation of acidic beverages ($2.5 < pH < 6.0$, according to the South African Food Regulation, Government Gazette, 1985) and the delivery of bioactive compounds due to greater instability under gastric conditions (at pH 2).

The extraction method of proteins may affect their composition and structure, which in turn could impact their complexation with polysaccharides. According to a previous study by Ursu et al., (2014), proteins extracted from micro-algae at pH 7 and pH 12 showed a 5.4 % difference in aspartic acid content, 5.8 % difference in glutamic acid content and 6.3 % in lysine and arginine content. A separate study on marama bean showed that some basic 11S subunits were lacking in protein extracted at slightly acidic conditions (pH 6) compared to that obtained at pH 8 (Gulzar et al., 2017). These variations in protein subunits and amino acid content could impact on protein complexation with polysaccharides. We therefore anticipate that the pH of extraction could be manipulated to alter the charge configuration on protein and possibly the optimum pH of complexation with polysaccharide.

Bambara groundnut (*Vigna subterranea*) is the third most important legume in Africa after peanuts and cowpea. Bambara, just like cowpea and soybean, is a good source of protein (19-25 %) (Eltayeb et al., 2011; Kudre and Benjakul, 2013). Furthermore, it is drought tolerant and therefore, well adapted well to the changing climate. Recent studies on bambara focused on structure-composition and functionality (e.g. emulsification and foaming) of bambara protein extracted by alkaline solubilisation followed by isoelectric precipitation (Adebowale and Maliki, 2011; Arise et al., 2015). In this study, the influence of extraction pH of bambara groundnut protein on composition, structure of the protein fractions and their degree of complexation with gum arabic were investigated. Gum arabic has been widely used in complexation studies both with animal and plant protein isolates. It is mainly an anionic arabinogalactan polysaccharide with a low charge density and weak reactive sites, thus making it a suitable choice for complexation with proteins (Aryee and Nickerson, 2014; Butstraen and Salaün, 2014). Hence gum Arabic was used in this study.

3.1 Materials and Methods

3.1.1 Materials

Bambara groundnuts were purchased from farmers in Umbumbulu areas of KwaZulu Natal Province, South Africa. Commercial gum arabic (*Acacia senegal)*, glucono-delta-lactone and other laboratory grade chemicals were purchased from Sigma-Aldrich.

3.1.2 Flour preparations

Bambara groundnuts were cleaned and soaked in water for 6 hours and then de-hulled using a pestle and mortar. De-hulled and dried grains were milled into flour using a Kenwood food blender (BL487, South Africa) and sieved through a 355 μm screen sieve. Prepared flour was then defatted twice using n-hexane at a ratio of 1:10 w/v for 2 hours at room temperature. The defatted flour was air-dried at room temperature for 24 hours overnight. The fat content of the defatted flour was less than 0.01% as determined by AOAC (2000). Defatted flour was subsequently kept separately in air tight containers at 4^{o} C.

3.1.3 Extraction of bambara protein fractions.

Protein fractions were extracted from defatted flour using a method described by Amonsou et al., (2012) with 30 mM Tris-HCl buffers at pH 2.0, 7.0 and 9.0 respectively. Flour slurries were prepared at a ratio of 1:10 w/v and protein extraction was carried out in a shaking water bath at 40^{o}C for 1 hour. The major storage protein fraction was extracted using the traditional alkaline method with 30 mM Tris-HCl buffer, pH 8 containing 0.5 M NaCl, at flour to solvent ratio of 1:20. This material was referred to as "the salt-soluble fraction." Crude protein content (N x 5.71) of the flour was determined by combustion analysis (AOAC, 2000).

3.1.4 Protein content and yield

The protein concentrations of isolates and flour were determined using the Bradford method (Bradford, 1976). The protein yield was determined as the dry mass of protein isolate per mass of the defatted flour (Arise et al., 2015)

3.1.5 SDS-PAGE

This was performed as described by Amonsou et al., (2012) under reducing and non-reducing conditions. Under reducing conditions, a loading buffer containing 1, 4-Dithiothreitol (DTT) was

mixed with protein samples, whereas DTT was absent under non-reducing condition. The protein samples were then heated in a water bath (100 ^{o}C) for 5 min. A 12% separating gel (pH 8.8) and 5% stacking gel (pH 6.8) was prepared according to the method of (Laemmli, 1970). Protein samples (10 µl) were loaded in each lane. A mixture of standard proteins (10– 250 kDa) was used as molecular weight markers. After electrophoresis, the gel was stained in 0.25% Coomassie Brilliant Blue G-250.

3.1.6 FTIR

The secondary structure of protein fractions was determined by FTIR spectroscopy (Carbonaro and Nucara, 2010). Protein samples were finely crushed with ground potassium bromide (KBr) salt and pressed under vacuum. The sample was scanned at 4 cm resolution from 4 000 cm^{-1} to 400 cm^{-1} and read from 32 cycles.

3.1.7 Amino acid determination

The amino acid content of the protein fractions was analysed by the AccQ-Tag derivatization method according to (Yusuf et al., 2008) with minor modifications. Briefly the sample was placed in a 0.5 mL hydrolysis tube and dried down. The tube was placed in a vessel containing an acid hydrolysis solution of 6 M HCl. The vessel was flushed with nitrogen to prevent oxidation during hydrolysis and was afterwards placed at 110°C for 24 hours. The sample was loaded into a UPLC and thereafter calculations were done to determine the total amino acids present in the sample.

3.1.8 Zeta potential

The zeta potential of homogenous protein and gum arabic solutions were measured over a pH range of 3 to 8 at 1-unit increment using Malvern Mastersizer 2000 ***(***Malvern Instruments, Westborough, MA, USA***).***

3.1.9 Turbidimetric analysis

This was carried out according to the methods outlined by (Liu et al., 2009a), (Klemmer et al., 2012) with minor modifications. Stock solutions (1.0%, w/v) of BPI (pH 2, pH 7, pH 9 and salt-soluble fraction) and GA (1.0 % w/v) were prepared separately by dissolving each powder in Milli-QTM water under constant stirring (500 rpm) for 2 h at room temperature (21-22^{o}C), and then overnight at 4°C to help facilitate protein dissolution. The BPI solutions were heated in a water

bath at 80^{o}C for 15 mins before mixing with the GA. Total biopolymer concentration of 0.1 % (w/v) for 4:1 ratio BPI: GA mixtures were prepared at a total volume of 30 ml.

Acidification of the mixed as well as homogenous solutions from pH 8 to 1.50 was performed using a combination of 0.05 % (w/v) glucono-delta-lactone (GDL) and a gradient concentration of HCl. Homogenous BPI and GA solutions were prepared as controls. Changes in the optical density of the solution were recorded over a pH range of 8.00 to 1.50 using a UV/Vis spectrophotometer at 600 nm using plastic cuvettes (1 cm path length). Charged neutral complex formation (pHopt) was determined graphically as the point which corresponds to the maximum optical density at 600 nm.

3.1.10 Coacervate yield

The coacervate yield was determined according to the method outlined by (Jun-xia et al., 2011). BPI and GA solutions were mixed together at a ratio of 4:1 to a total biopolymer concentration of 0.1% (w/v). The pH was adjusted (1 M HCl) to correspond with the pH_{opt} values obtained and the mixture was stirred with a magnetic stirrer for 30 minutes at 4 ˚C to promote coacervation. The coacervates were recovered by centrifugation at 3500 rpm for 15 minutes. The supernatant was carefully discarded and the coacervates collected and dried in an oven at 105 ˚C to a constant mass. The coacervates yield was calculated using the following equation.

$$\mathrm{CY}\ (\%) = \frac{\mathrm{m}i}{\mathrm{m}o}\ \mathrm{X}\ 100$$

Where CY is the percentage yield of coacervates, mi and mo are the dried mass (mg) of coacervates and total mass of both BPI and GA in the formulation respectively.

3.1.11 SEM

The method according to (Jun-xia et al., 2011) was used. Dry particles were mounted on circular aluminium stubs with double sided adhesive tape and subsequently coated for 250 s with 15nm gold. Examination and photography was done using a FEGSEM (Zeiss ultra plus, Germany) on one side of two-way adhesive tape and observed at voltage of 5.0 kV.

3.1.12 Statistical analysis

Protein samples and coarcervates were prepared at least twice and physicochemical analyses were carried out in triplicates where necessary. Data were analyzed using one-way analysis of variance

(ANOVA) and the means were compared using the Fisher Least Significant Difference (LSD) test ($p<0.05$).

3.2 Results and Discussion

3.2.1 Proximate composition

As expected, the major component of bambara grain was carbohydrate (60%) followed by protein (24%). The chemical composition data compare favourably with the 26-27% protein content reported by Arise et al., (2017). Other pulses such as cowpea, kidney beans (Kudre and Benjakul, 2013) and black bean (Carrasco-Castilla et al., 2012) also compare favourably with the results that were observed. Although, the protein content of bambara was lower than that of soybean, it can still be considered a good source of protein. Bambara flour was generally low in fat and ash, which is consistent with previous reports (Arise et al., 2017; Adebowale and Maliki, 2011).

3.2.2 Protein yield and content

The extraction yield was significantly affected by pH of the extraction medium. The protein yield was minimal at pH 2 (16 %) whilst pH 7, pH 9 and the salt-soluble fractions had fairly similar yields (52-56 %). The variation in protein yields could be attributed to differences in solubility behaviour under varying pH conditions and the presence or absence of salt. The low yield at pH 2 could be explained by the low solubility of protein in highly acid medium. Protein solubility is principally governed by a balance in the proportion of interactions between oppositely charged residues (lysine/arginine and aspartic/glutamic acid). Therefore, charged amino acids would be involved in the association-dissociation phenomena of protein sub-units (Carbonaro et al., 1993). It is therefore reasonable to propose that as solution pH was lowered towards the pKa of acidic amino acids, (pKa of aspartic and glutamic acids 2.77 and 3.22 respectively), they become protonated. This results in reduced electrostatic repulsive forces which consequently lead to increased protein-protein interactions and partial precipitation of protein subunits (Schmitt and Turgeon, 2011).

The protein contents of fractions varied significantly with the pH of extraction. The salt-soluble fraction showed the highest protein content (82 %) whilst the lowest protein content (76 %) was recorded at pH 2. The pH 7 and 9 fractions recorded protein contents of 80 % and 81 %

respectively. Salt-soluble globulin is often the major storage protein of most leguminous grains. Thus, the protein content of the salt-soluble bambara fraction is in agreement with previous reports (Arise et al., 2017; Adebowale and Maliki, 2011).

3.2.3 SDS-PAGE

Bambara protein fractions showed three major bands of molecular weights 81, 55 and 50 kDa (Fig 11). However, the pH 2 fraction showed an additional band at approx. 100 kDa, similar to defatted flour. Although its protein yield was low, pH 2 fraction seemed to produce a more representative SDS-PAGE profile of the storage protein. Comparable SDS-PAGE patterns have been reported for bambara protein isolates extracted at pH 12, (Kudre et al., 2013) pH 9, (Arise et al., 2015) and pH 7 (Adebowale and Maliki, 2011).

In the presence of dithiothreitol (DTT), the 81 kDa protein band was reduced and two lower molecular weight bands (64 kDa and 18 kDa) appeared, suggesting that this protein subunit was stabilised by disulphide bonds and could possibly belong to the 11S legumins. Furthermore, a band at approximately 25 kDa observed for defatted flour and pH 2 fraction could correspond to the basic legumin (MW – 20-25 kDa). This is because legumins exist as hexamers (MW approx. 360 kDa) composed of six acidic and six basic polypeptides linked by single disulphide bonds (Kudre et al., 2013). The protein bands reported at 100, 50 and 55 kDa may correspond to 7S vicillin subunits which are glycoprotein devoid of disulphide bonds and are frequently non-covalently associated in trimers.

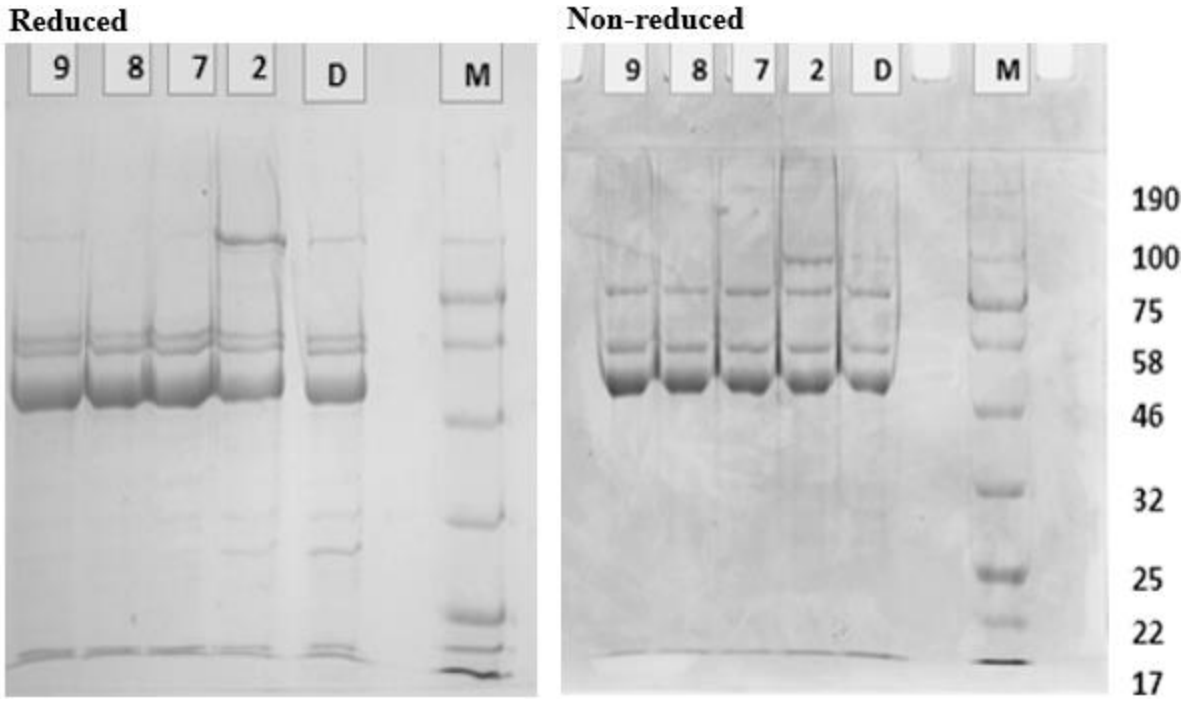

Figure 11: SDS-PAGE for bambara protein fractions (BPI) extracted at different pH.
M = marker, D = defatted flour, 2 = pH 2 fraction, 7 = pH 7 fraction, 8 = salt-soluble fraction, 9 = pH 9 fraction

3.2.4 Amino acid analysis

The amino acid profiles of fractions varied significantly with the pH of extraction, particularly for the charged amino acids including glutamic acid, aspartic acid, arginine and lysine (Table 4). Charged amino acids are important in complexation with polysaccharides as they provide sites of interaction for complexation (Schmitt and Turgeon, 2011). Glutamic and aspartic acid were the major amino acids of bambara protein fractions. Similarly, these were the major amino acids found in other legumes such as soybean, mung bean, fenugreek, (Alain et al., 2007; Feyzi et al., 2015). Vasconcelos et al., (2010) recorded approximately 15 % and 12 % of glutamic acid and aspartic acid respectively for cowpea protein extracted with salt. These amino acids values agree with the values obtained in this study. The pH 2 fraction recorded the highest content of basic amino acids.

Table 4: Amino acid composition (g/100 g protein) of protein fractions

	pH 2	pH 7	Salt-soluble	pH 9
Essential AA				
Threonine	4.5	2.2	2.5	2.5
Lysine	5.5	3.8	5.2	4.6
Histidine	3.3	4.0	2.2	4.0
Methionine	1.8	1.4	1.5	1.3
Valine	5.4	4.8	5.1	4.9
Isoleucine	6	5.5	6.2	5.3
Leucine	10	8.7	9.6	9.0
Phenylalanine	7.10	8.3	8.2	7.7
Sub-total	**43.7**	**38.7**	**40.5**	**39.2**
Non-essential AA				
Serine	5.6	5.7	5.7	5.3
Arginine	6.8	6.7	7.1	6.7
Glycine	4.8	4.1	3.9	3.7
Tyrosine	6.1	4.5	4.3	4.2
Aspartic acid	9.9	10.6	11.1	10.7
Glutamic acid	11.2	16.6	17.1	17.1
Alanine	4.4	36	3.86	3.6
Proline	5.5	5.4	5.21	4.9
Sub-total	**54.2**	**57.4**	**58.2**	**56.1**
Recovery	**97.8**	**96.0**	**98.6**	**95.4**
Total polar AA	**56.1**	**54.1**	**55.0**	**55.0**
Total polar charged AA	**36.7**	**41.7**	**42.6**	**43.1**

3.2.5 FTIR

The amide I region (1600-1700 cm $^{-1}$) of the bambara protein fractions was analysed as it is considered a better predictor of the protein secondary structure due to the predominant C=O stretching band with little contributions from C-N stretching (Grdadolnik, 2002). As shown in Table 5, β-sheet structures appeared as the dominant conformation in all the fractions and this is consistent with reports in literature (Shevkani et al., 2015). The pH of extraction slightly influenced the structural conformations of protein fractions. An increase in β-sheet content was generally observed at acidic pH. Peaks corresponding to alpha helix, beta sheets and beta turns were identified at varying proportions in the different protein fractions. Although, all the protein fractions showed peaks corresponding to α- helix, β-sheets and β –turns, the pH 2 fraction

contained more β-sheet structures. This could be attributed to protein denaturation and unfolding at extreme pH (Mauri & Anon, 2006).

Table 5: Secondary structure band assignment of bambara protein fractions extracted at different pH

References	*Peak range (cm^{-1})	Secondary structures	pH 2 (%)	pH 7 (%)	Salt soluble (%)	pH 9 (%)
Carbonaro et al., (2010)	1612 – 1694	β sheets	51.7±0.07^{a}	49.1±0.28^{b}	42.8±0.70^{d}	41.4±0.56^{c}
Murayama and Tomida (2004)	1630 – 1660	α helix	19.2±0.57^{a}	19.5±0.34^{a}	6.1±0.28^{b}	23.3±0.23^{c}
Pelton and McLean (2000),	1662 – 1684	β turns	22.4±0.4^{a}	23.3±0.4^{a}	49.0±0.56^{b}	27.2±0.84^{c}
Carbonaro et al., (2010)	1640 – 1650	Random coil	7.7±0.56^{a}	9.6±0.56^{b}	4.32±0.56^{c}	8.14±0.70^{ab}

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

3.2.6 Zeta potential

Zeta potential of protein fractions and gum arabic as a function of pH is shown in Fig 12. The ζ-potential profiles of protein fractions showed similar pH dependent patterns. As the pH decreased from 8 to 3, the zeta potential of protein fractions decreased from maximum negative to maximum positive, with some variation in absolute zeta values. The zeta potential of gum arabic remained negative over the complete pH range that was studied. This trend is consistent with previous reports by (Ladjal-Ettoumi et al., 2016*)*. The negative zeta potential from aspartate and glutamate side chain (pka 3.65 and 4.25, respectively may be contributing to overall negative zeta potential of studied fraction at pH above 5.5. Aspartic acid /Asparagine and Glutamic acid /glutamine were the predominant amino acids in protein fractions (Table 4). The isoelectric point (that is pH at ζ=0 mV*)* were not substantially different (approx. 4.5) among protein fractions, except for the salt

soluble fraction, which had the highest pH (approx. 5.3) at ζ=0 mV. This could be explained by differences in protein composition.

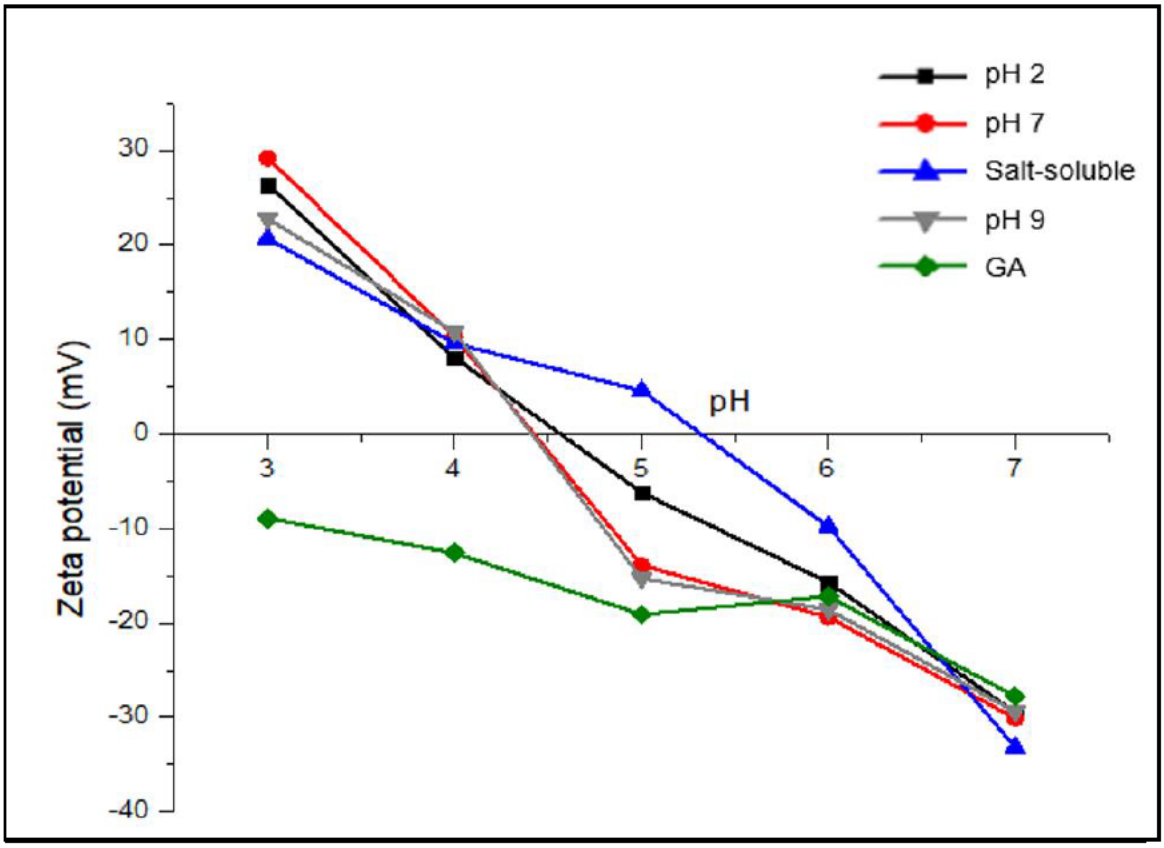

Figure 12: Zeta potential of gum arabic and protein fractions as a function of pH

3.2.7 Effect of fractionation pH on complexation

The influence of protein fractions obtained at pH 2, pH 7 and pH 9 and by salt solubilisation on complex formation with gum arabic was investigated at a constant ratio of 4:1, BPI: GA. The choice of the 4:1 ratio was based on preliminary experiments. Figure 13 shows the turbidity profiles for bambara protein fractions-gum arabic (BPI-GA) complexes and homogenous biopolymers respectively as a function of pH.

The addition of gum arabic seemed to broaden the turbidity curves of the protein-polysaccharide mixtures when compared to those of homogenous samples. The presence of gum arabic in the mixture may have induced electrostatic repulsive forces to hinder protein-protein aggregation,

which resulted in delayed formation of critical structures and a broadened curve (Klemmer et al., 2012; Elmer et al., 2011).

Generally, homogenous protein fractions showed symmetric normal distribution curves during titration whereas the BPI-GA mixed profiles appeared skewed towards the acidic regions. This shift towards acidic pH in the presence of gum Arabic could suggest the formation of more stable structures in acidic regions relative to protein-protein aggregates in the absence of a polysaccharide. Similar results have been observed with optimum pH of interaction ranging between 3 and 3.5 (Liu et al., 2009a; Aryee and Nickerson, 2014). However, the extent of the acidic shift in this study varied with the protein fractions, suggesting that extraction pH of protein has an impact on the degree of complexation with polysaccharides and the stability of complexes formed thereof. The differences in amino acid composition across the pH fractions could be responsible for the shift. For instance, the pH 2 fraction recorded the highest basic amino acid content whilst salt-soluble fraction recorded the highest acidic amino acid content. The point of optimum coacervation for the pH 2 fraction-GA complex was pH 2.9, which suggests that complexes formed at this pH would be stable in acidic conditions and can be used as carrier and protective matrices of bioactive compounds.

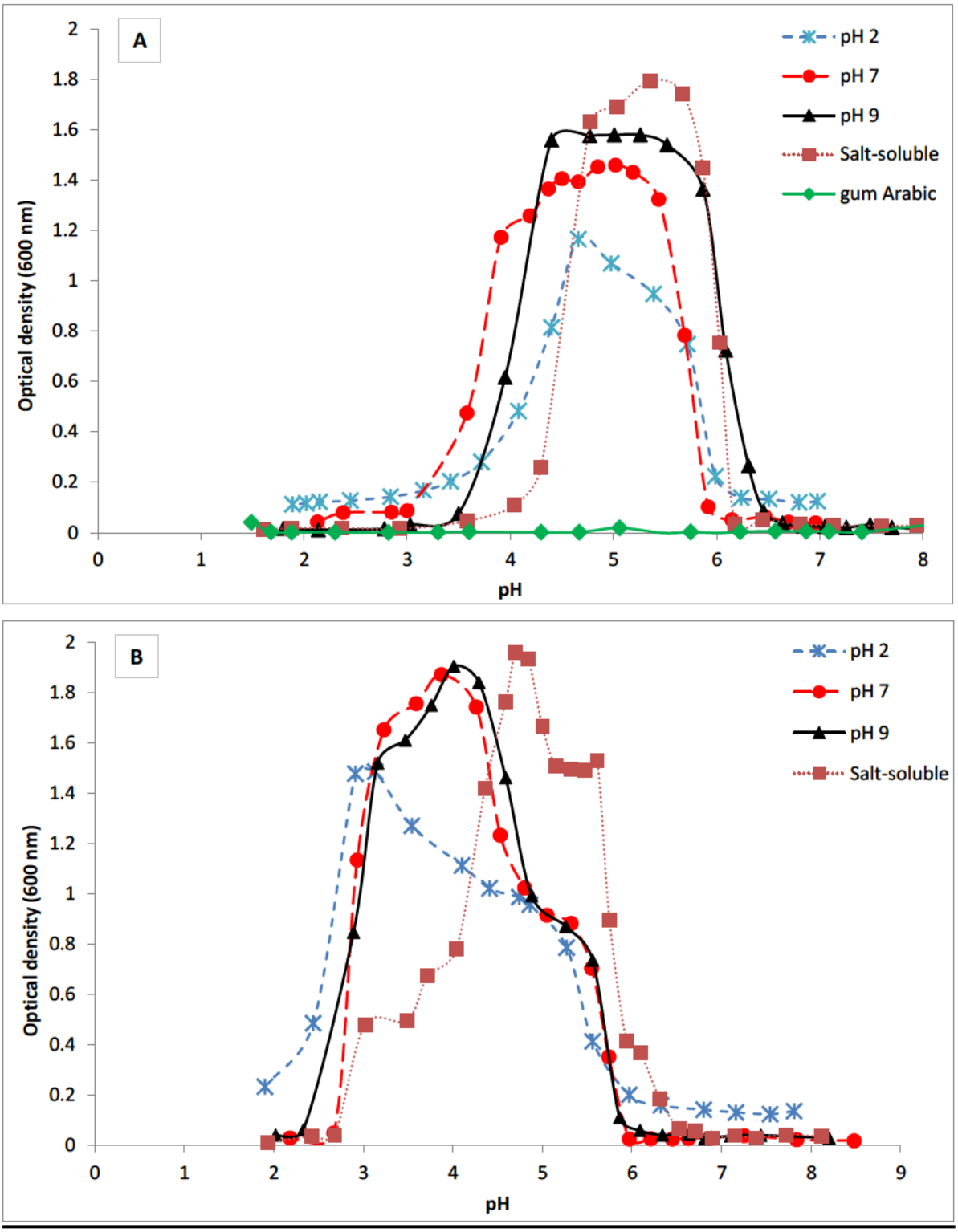

Figure 13: Mean optical density of (**A**) homogenous bambara protein fractions (BPI) and gum arabic (GA), (**B**) the respective protein-polysaccharide complexes as a function of complexation pH

3.2.8 Coacervate yield

Generally, the coacervate yield increased with increasing pH of extraction medium (Fig 14). The pH 9 fraction showed the highest coacervate yield (68 %) whilst the pH 2 fraction recorded the lowest coacervate yield (41 %). The low coacervate yield for the pH 2 fraction could be attributed to protonation of carboxyl groups on gum Arabic molecule at the point of optimal coacervation (pH = 2.91), which consequently hindered protein-polysaccharide interactions (Liu et al., 2009a). Carboxyl groups of gum Arabic become protonated at pH values close to their pKa (approx. 2.2), thus shielding the charges and interrupting the electrostatic interactions between the biopolymers. The zeta potential of gum arabic becomes less negative as pH is reduced, an indication of the protonation of the carboxyl groups on the gum arabic. The high coacervate yield for the pH 9 fraction can be attributed to the charge configuration of the biopolymers at the pH of optimum interaction (4.05). As indicated (Fig 12), the zeta potential of GA at pH 4.05 is -12.6 mV whilst that of the pH 9 fraction is 10.8 mV. The interaction of these biopolymers at a point close to the electrical neutrality therefore resulted in a high coacervate yield (Liu et al., 2009a).

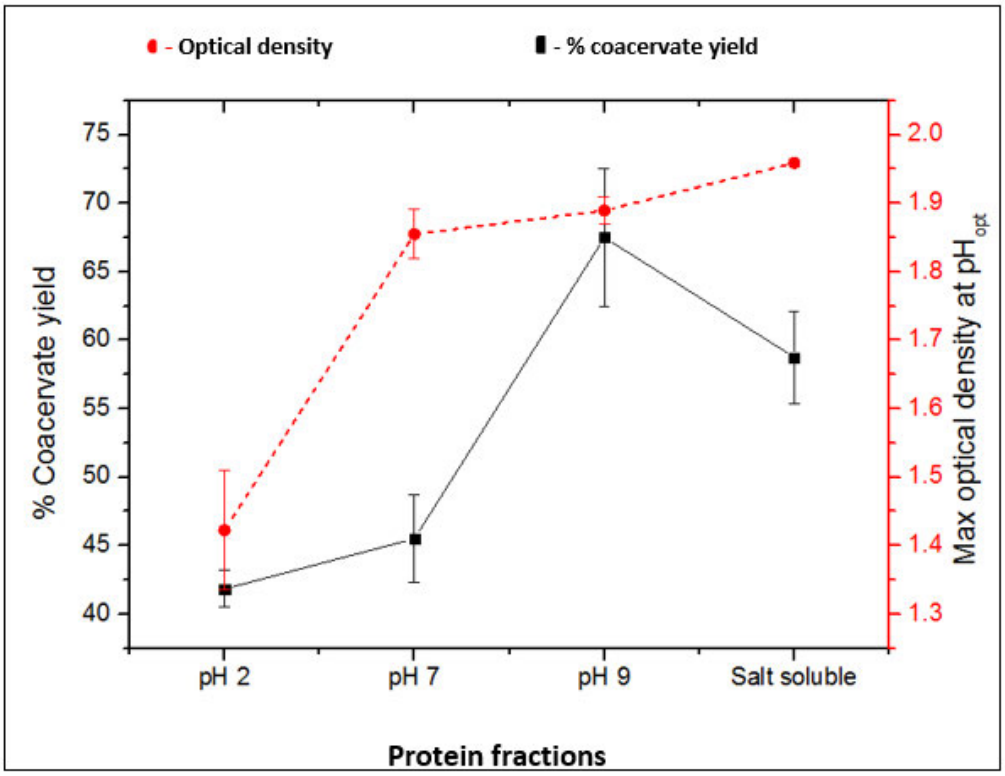

Figure 14: Percentage coacervate yield and optical density (OD) obtained at 600 nm of bambara protein fractions-gum arabic complexes (BPI-GA).

3.2.9 SEM

The formation of coacervates was confirmed by FEGSEM (Fig 15). The formed particles appeared as spherical and aggregated structures. These particles had average size range of 100-200 nm. Larger particle sizes have been reported in literature (Jones et al., 2009). The pH 2-GA and pH 9-GA complexes showed less aggregation and seemed to have more a homogenous particle size.

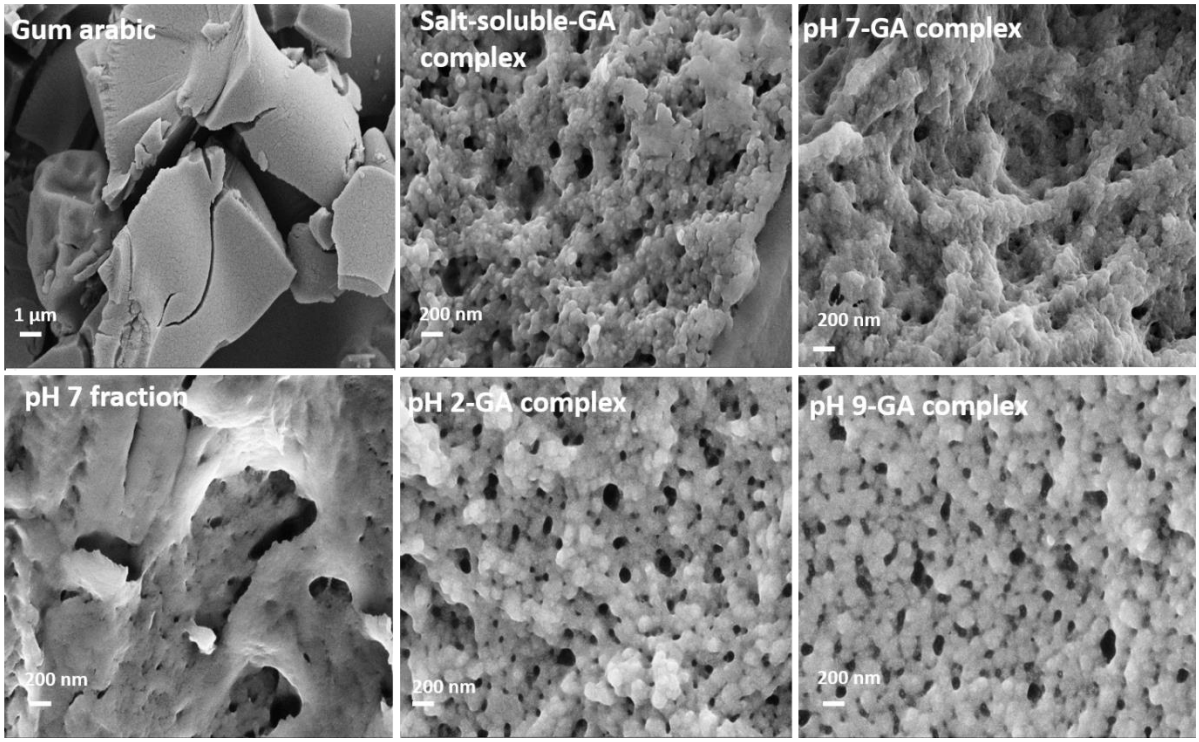

Figure 15: SEM micrographs of BPI-GA complexes in comparison to homogenous gum arabic and pH 7 protein fraction

3.3 Conclusion

The fractionation pH of bambara protein influenced the degree of complexation with gum arabic. The vicillin fraction seemed to be the most abundant sub-unit in all protein fractions. Protein fractions, particularly pH 2 fraction showed a strong secondary structure dominated by β-sheet conformation. The addition of gum arabic to protein fractions seemed to delay critical structure formation. The shift of pH-opt to acidic regions seemed to increase with decrease in fractionation pH. In comparison to homogenous protein fractions and gum arabic, spherical particles within the nano-range (200 nm) were formed for all protein fractions-GA complexes. Complexing

polysaccharides with protein fractionated under different pH conditions could therefore be an alternative way of designing carrier systems with improved acid-stability.

CHAPTER FOUR

Influence of ionic strength and biopolymer ratio on formation of bambara protein-gum Arabic complexes

Abstract

Bambara protein extracted using the traditional salt solubilisation method was complexed with gum Arabic. The influence of ionic concentration (0, 10, 40 mM) and biopolymer ratio (4:1 and 16:1) on complex formation was investigated. High concentration of protein produced wider and normally distributed turbidity profiles. However, increasing the gum Arabic produced turbidity profiles with two pronounced peaks that resembled the nucleation process. A 40 mM ionic strength in the 4:1 complex resulted in reduced turbidity whereas that of the 16:1 ratio increased. The zeta potential of 16:1 ratio appeared spread out over the voltage range whereas that of the 4:1 ratio seemed to follow a pattern. By AFM, the 4:1,10 mM complex showed minimal aggregation compared to 16:1, 40 mM complex which produced more uniformly sized particles under 200 nm. Generally, high protein ratio and salt concentration could be important in producing uniform sized particles for industrial applications.

Keywords:

Bambara; Protein; Polysaccharide; Complexation; Ionic strength; Biopolymer ratio

4.0 INTRODUCTION

Protein-polysaccharide complexes are important structural biomaterials that can be used as encapsulation matrices for the delivery of nutraceuticals (Benbettaïeb et al., 2019; Klemmer et al., 2012). The growing demand for plant protein utilisation in various applications including complex formation creates an opportunity to explore alternative sources of proteins. Recent investigations on plant-based protein complexation with polysaccharides however have been limited to soybean and pea proteins (Aryee and Nickerson, 2014; Jun-xia et al., 2011).

Bambara groundnut, a legume of African origin, is a potential alternative for protein-polysaccharide complex formation due to its high protein content (19 – 27 %) that is similar to pulse grains such as peas (Boye et al., 2010; Ladjal-Ettoumi et al., 2016). The amino acid profile of bambara protein is also rich in charged amino acids such as glutamic acid, making it a good candidate for electrostatic complexation with polysaccharides such as gum arabic (Eltayeb et al., 2011; Kudre and Benjakul, 2013). In our previous study, 40 - 65% coacervate yield was obtained for bambara protein-gum arabic complexes. Furthermore, we established that protein fractionation pH gives different amino acid composition and charge configuration, which subsequently impacts the degree of protein-polysaccharide interactions. However, the study did not investigate how other parameters such as ionic strength and biopolymer ratio could impact on the degree of bambara protein complexation with gum arabic.

Previous research has investigated the effect of salt concentration and biopolymer ratio in complexation. Findings from these research showed that high concentrations of NaCl could inhibit coacervation due to charge screening whilst low concentrations could enhance complexation by inducing slight conformational changes to the protein structure thereby exposing reactive sites (Liu et al., 2010; Santos et al., 2018). For instance, Aryee and Nickerson, (2014) studied the influence of NaCl (0 – 50 mM) on pea protein-gum arabic complexation at a 1:1 biopolymer mixing ratio. According to these authors, salt concentrations of less than 7.5 mM enhanced complexation but higher concentrations (> 50 mM) inhibited complexation by blocking charges on the biopolymers. In a separate study, Klemmer et al., (2012) showed that increasing protein ratio on pea protein-gum Arabic complexes (1:1 – 20:1, PPI: GA) resulted in normally-distributed turbidity profiles, indicating the formation of uniform-sized particles. Other studies conducted on whey protein-gum arabic complexes showed that the extent of ionic strength required to inhibit or enhance

coacervation is dependent on the biopolymer mixing ratio (Weinbreck, 2004; De Kruif et al., 2004). Based on available data, it would be important to know how salt concentration and biopolymer ratio impact complexation behaviour, in order to facilitate the application of Bambara protein in complexation with polysaccharide. We therefore aim to build on our previous study by investigating the influence of biopolymer mixing ratio and ionic strength on bambara protein-gum arabic interactions.

4.1 Materials and Methods:

4.1.1 Materials

Mature and sun-dried bambara groundnut seeds were purchased from farmers in Umbumbulu, KwaZulu Natal Province, South Africa. Commercial gum arabic, glucono-delta-lactone and other laboratory grade chemicals were purchased from Sigma-Aldrich.

4.1.2 Flour preparations

Bambara flour was prepared as described in chapter 3.

4.1.3 Extraction of bambara storage protein

Total storage protein was extracted from defatted flour using the traditional alkaline method according to Amonsou et al., (2012). Protein concentration of isolate was determined using the Bradford method (1976).

4.1.4 Turbidimetric analysis

This was carried out as described in chapter 3 with the exception that the effect of NaCl (0,10, 20 and 40 mM) and biopolymer weight mixing ratio BPI:GA (4:1 and 16:1) on complex coacervation were also investigated.

4.1.5 Surface charge

The zeta potential was measured according to the method of (Souza and Garcia-Rojas, 2017). The zeta potential of homogenous 0.1 % (w/v) protein and 0.1 % (w/v) gum arabic solutions were measured over a pH range of 3 to 7 at 1-unit increment using Malvern Master-sizer 2000**,** Malvern Instruments, Westborough, MA, USA**.**

4.1.6 Atomic Force Microscopy (AFM)

AFM was carried out using a method by Jones et al., (2009). Protein-polysaccharide complex solutions were diluted using Milli-Q water to approximately 0.0033% protein concentration (w/v). Samples were then mounted on a newly cleaved mica slide (PELCO Mica, 9.9 mm disks; Ted Pella, Redding, CA) by injecting approximately 2 µl of the dilute sample onto the surface. The slides were then fixed to magnetic steel wafers using adhesive strips (PELCO tabs; 12 mm OD; Ted Pella, Redding, CA). These samples-mica assemblies were loosely covered in petri dish and placed in a desiccator overnight to dry the samples thoroughly. When dry, the sample-mica assemblies were scanned using a CP-II atomic force microscope (Force constant 3N/m, Multi 75Al; Ted Pella, Redding, CA). Various scanning windows were analysed under air (10 x 10µm, 5 x 5µm, 3 x 3 µm) in contact mode with a scan speed of 0.8 Hz and a force of 60 nN.

4.1.7 Statistical analysis

All experiments were conducted in duplicate. The data were analyzed using one-way analysis of variance (ANOVA) and the means were compared using the Fisher Least Significant Difference (LSD) test ($p<0.05$).

4.2 Results and Discussion:

4.2.1 Influence of biopolymer ratio on protein-polysaccharide complexation

A preliminary experiment was carried out to evaluate the effect of biopolymer mixing ratio on protein-polysaccharide interactions and establish the appropriate mixing ratio for subsequent experiments. Generally, increasing the protein ratio in the protein-polysaccharide mixture (Fig 16b) resulted in wider and normally distributed turbidity profiles. However, a higher ratio of gum arabic in the mixture (Fig 16a) produced turbidity profiles with two pronounced peaks (peak i and ii).

The first peak (i) fell within the pH optimum of homogenous BPI which translated to the isoelectric point of bambara protein. These peaks resemble a two-step nucleation process believed to be fundamental in the process for protein-polysaccharide formation (Weinbreck, 2004; Liu et al., 2009b). Peak (i) could be associated with the formation of protein-protein aggregates and peak (ii), the adsorption of gum arabic onto the protein aggregates (Jarpa-Parra et al., 2014; Timilsena et al.,

2018). Further, the normal turbidity profiles formed with increasing protein ratio have a close resemblance with that of homogenous bambara protein. This indicates that gum arabic had minimal effect on complex formation at higher protein ratios. On the other hand, the wider turbidity profiles could be a result of GA, which caused sufficient electrostatic repulsion to delay the formation of protein-protein aggregation. Similar observations were reported by (Klemmer et al., 2012; Elmer et al., 2011). Data from our preliminary experiment suggests that it may not be necessary to increase GA ratio, therefore in subsequent experiments, ratios 4:1 and 16:1 were used to investigate the influence of salt.

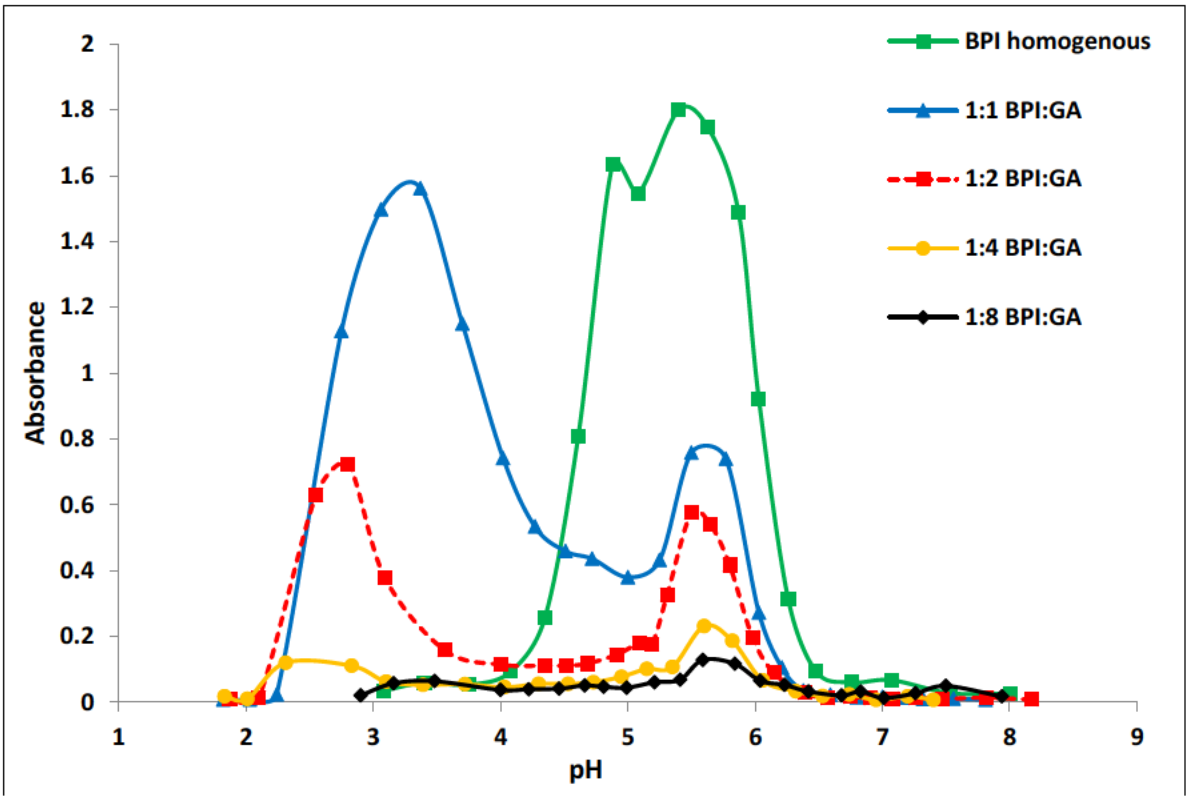

Fig 16A: Influence of higher gum arabic ratio (GA>BPI) on BPI: GA complexation.

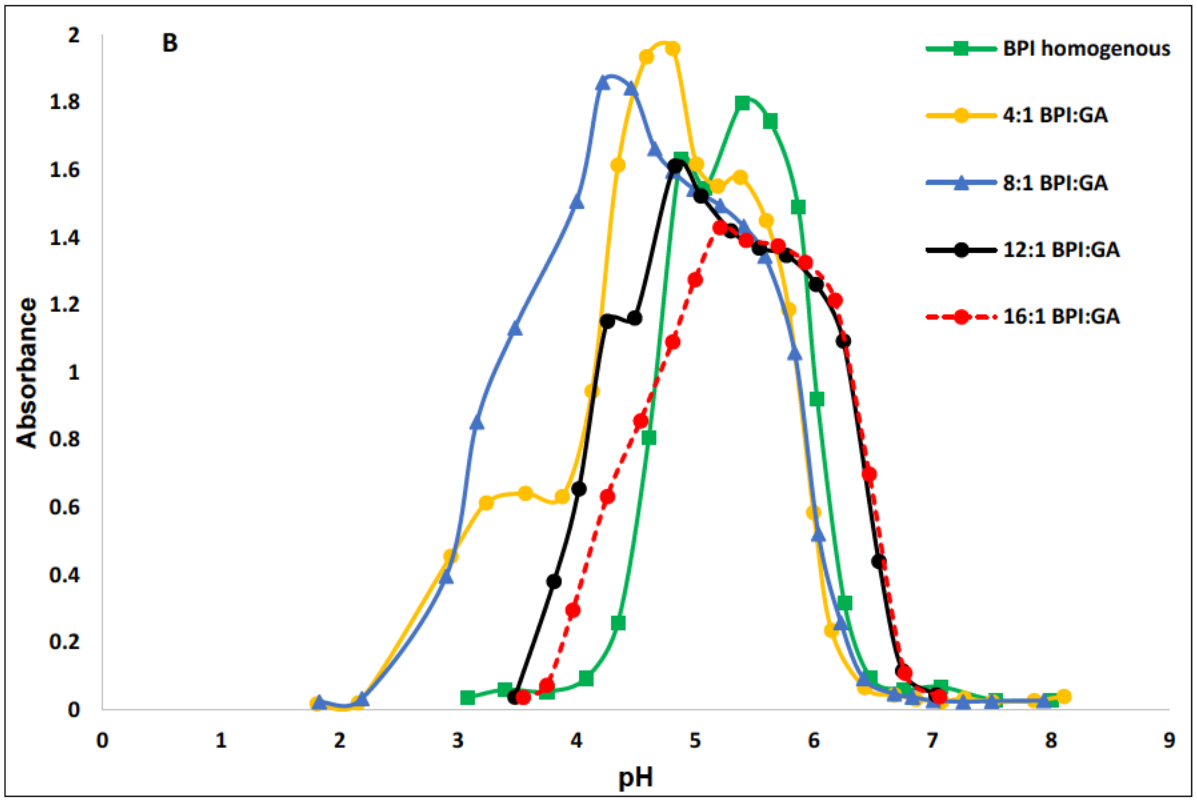

Fig 16B: Influence of higher protein ratio (BPI>GA) on BPI: GA complexation.

4.2.2 Influence of biopolymer mixing ratio and ionic strength on degree of complexation

Bambara protein: gum arabic complexes at 16:1 ratio produced normally-distributed turbidity profiles compared to the 4:1 ratio (Fig 17). Further, increasing the ionic strength in the 4:1 ratio seemed to shift critical structure formation towards acidic pH. Moreover, increasing ionic strength in the 4:1 mixing ratio reduced the absorbance of the profiles whereas that of 16:1 ratio increased. According to Jones et al., (2009), the addition of ions could shift critical pHs to more acidic values to compensate for the partial blockage of charges induced by the ions. This may explain the shift to acidic pH in the 4:1 ratio. Charge neutralisation due to high protein may also explain why critical pHs were not affected by increasing ionic strength on the 16:1 ratio (Weinbreck, 2004; Yuan et al., 2017). Compared to the irregularly shaped 4:1 ratio turbidity profiles, the 16:1 ratio profiles suggest formation of uniformly-sized particles over the studied pH range. This may possibly be a way of producing uniformly-sized particles for use in the food industry.

The influence of salt concentration on protein-polysaccharide interactions has been explained by scholars such as (Yuan et al., 2017; Liu et al., 2009b). Low ionic concentrations have been shown to enhance complexation by unfolding the protein to expose more reactive sites (Klemmer et al., 2012; Liu et al., 2009b). On the other hand, high ionic strength inhibit complexation by screening available charges from interacting with polysaccharides (Aryee and Nickerson, 2014). Although these phenomena are true for the 4:1 ratio, they did not apply to the 16:1 ratio possibly because of the high protein ratio. Observations from this study suggest that the influence of ionic strength depends on the biopolymer mixing ratio as well (Weinbreck, 2004). Therefore, higher salt concentrations may necessitate the use of higher protein to produce uniformly-sized particles.

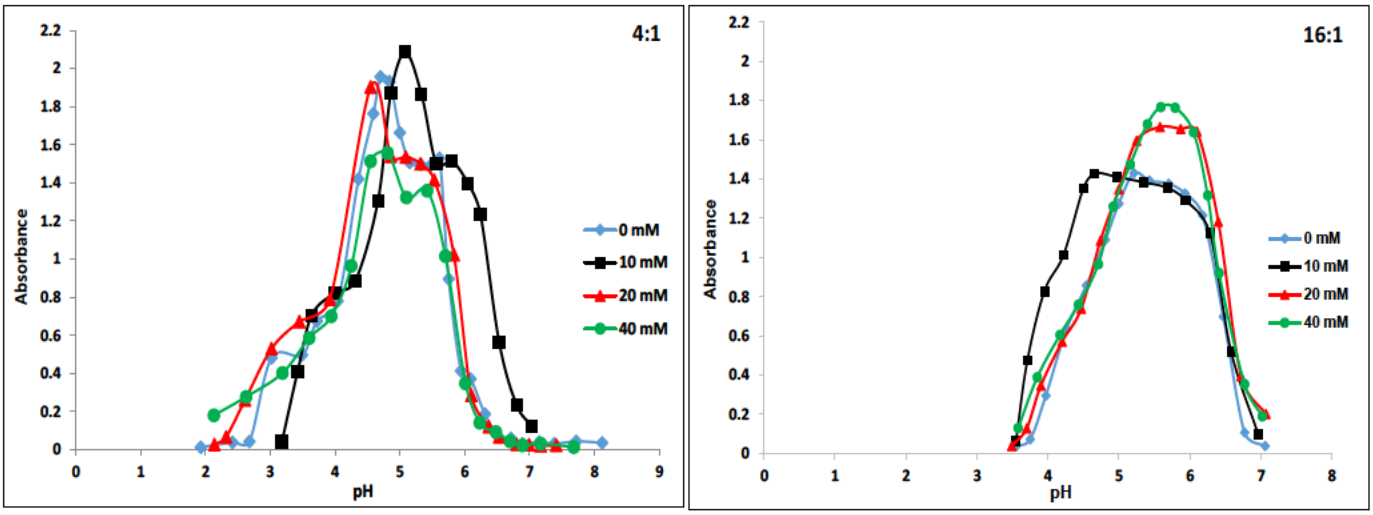

Fig 17: Comparison of turbidity measurements of BPI-GA complexes as a function of synergistic influence of biopolymer ratio and ionic strength.

4.2.3 Zeta potential

The zeta potential of homogenous BPI decreased from maximum negative to maximum positive as pH decreased from 8 to 3. The isoelectric point for the BPI was at approximately pH 4, which is consistent with other studies on bambara protein (Arise et al., 2017; Kudre et al., 2013). On the other hand, the zeta potential of gum arabic remained negative over the complete pH range that was tested. This is because gum arabic is an anionic biopolymer containing carboxyl functional groups with a pKa of 2.2. Similar results have been reported in literature (Karaca et al., 2015).

Further, the zeta potential measurements for BPI-GA complexes went from maximum negative at pH>4 to maximum positive at pH<4. The zeta potential of the 16:1 mixing ratio at various ionic strengths appeared dispersed compared to those of 4:1 mixing ratio which seemed to follow a pattern. Changes in surface charge from negative to positive may be due to gradual protonation of the carboxyl groups and amino groups on the proteins and gum arabic (Santos et al., 2018). The negative zeta potential from pHs above 4.5 can be attributed to the glutamic and aspartic acid which are deprotonated at that pH range (pKa 3.65 and 4.25 respectively) (Ladjal-Ettoumi et al., 2016).

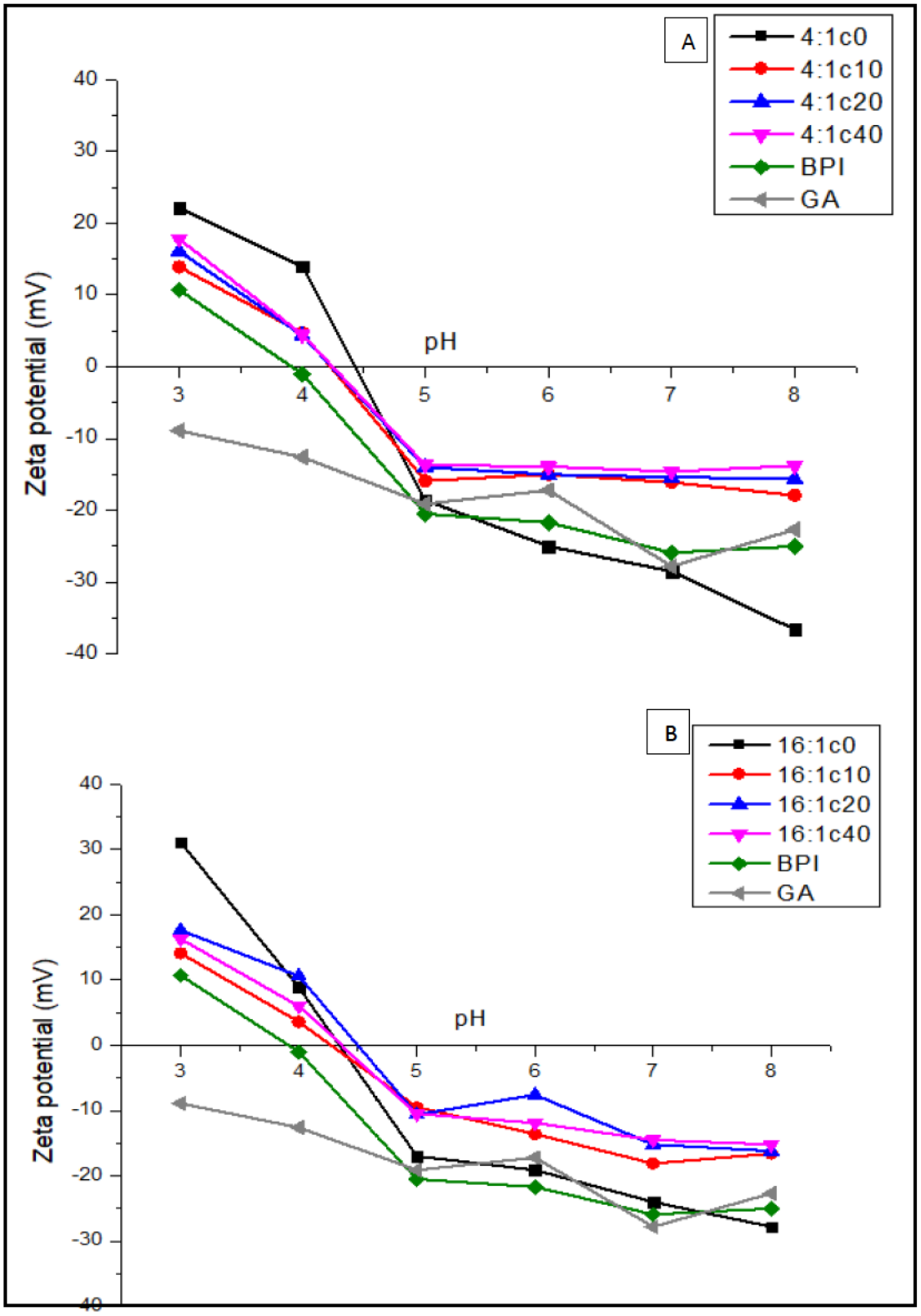

Fig 18: Zeta potential of BPI: GA complexes as influenced by biopolymer ratios and ionic strength

4.2.4 AFM

The morphology of BPI-GA complexes at various ionic strengths and biopolymer ratios were observed by AFM (Fig 4). Generally, the 4:1, 10 mM complex showed particle sizes ranging between 100 – 250 nm with minimal aggregation whilst the 16:1, 40 mM showed highly aggregated particles. However, a closer look at the 16:1, 40 mM revealed spherical structures under 200 nm in size. Furthermore, the particles in the 16:1, 40 mM complex appeared more uniform in size compared to the 4:1, 10 mM complex. The increased aggregation in the 16:1, 40 mM complex can be attributed to the high protein content, which resulted in protein-protein aggregation. Moreover, the higher ionic strength could also be responsible for protein unfolding to expose surface charges on the protein backbone thus further propagating protein-protein aggregation (Ladjal-Ettoumi et al., 2016). The uniformity of particle size in the 16:1, 40 mM was confirmed by turbidity profiles observed in this study. Therefore, increasing protein ratio as well as salt concentration could be an important way of producing uniform sized particles for application in food industries. The homogenous bambara protein isolate showed protein bodies ranging between 1 – 1.5 µm in size.

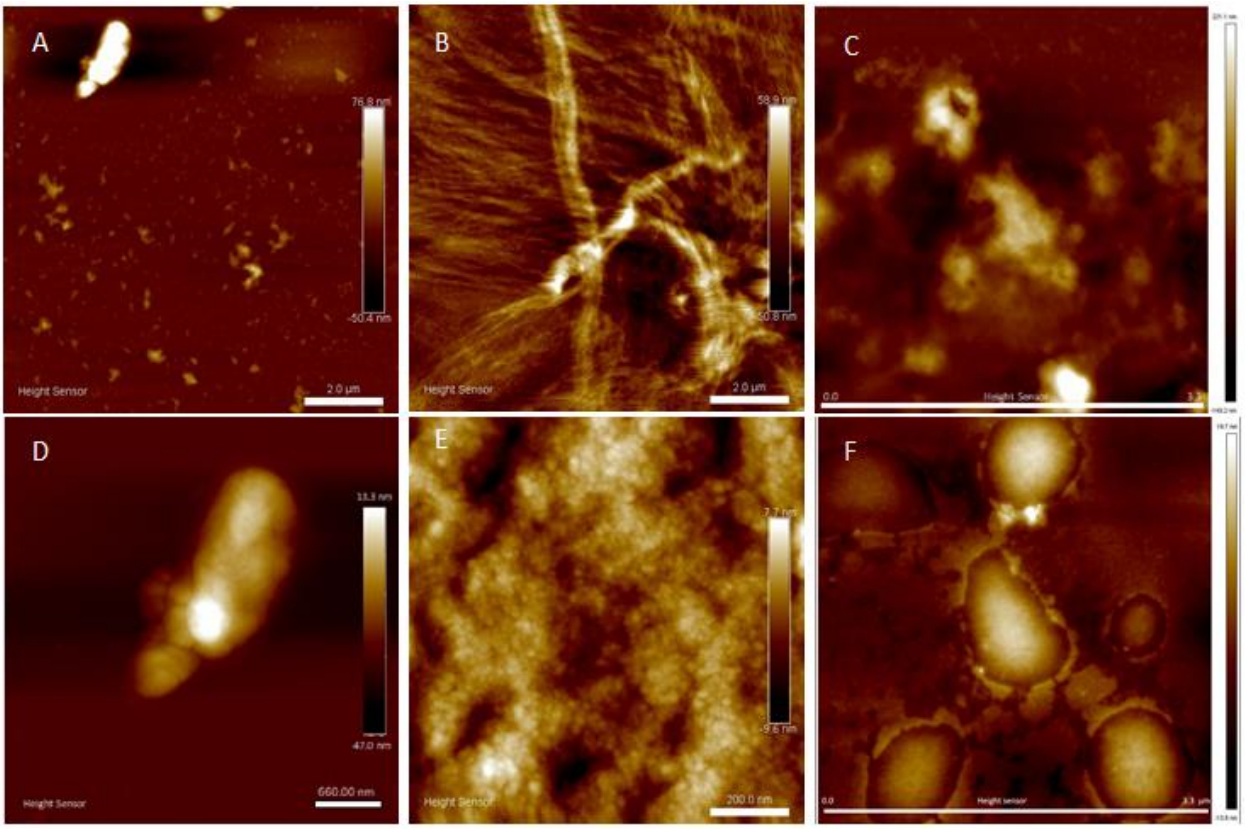

Fig 19: AFM images of selected BPI-GA complex combinations as influenced by ionic strength and biopolymer ratio - ***Key** – **(A)** 4:1-10 mM, **(B)** GA**, (C)** 16:1-40 mM, (**D)** Enlargement of A, **(E)** Enlargement of C, **(F)** BPI

4.3 Conclusion

Solution ionic strength and biopolymer ratio impacted bambara protein-gum arabic complexation. An initial increase of gum arabic produced two peaks in the profile believed to resemble the nucleation process. Further increase however resulted in inhibition of complexation. The influence of ionic strength on complexation was observed to be dependent on the biopolymer mixing ratio. Zeta potential of the 16:1mixing ratio appeared dispersed compared to that of the 4:1 ratio which seemed to follow a distinct pattern. In comparison to homogenous bambara protein and gum arabic, spherical particles were formed for both 16:1, 40 mM and 4:1, 10 mM complexes. Further, particles formed from the 16:1, 40 mM complex seemed to be uniform in size. Therefore, simultaneously increasing biopolymer ratio and ionic strength could be a way of producing uniformly-sized particles.

CHAPTER FIVE

Encapsulation, release and interfacial properties of bambara protein-gum arabic complexes.

ABSTRACT

Foaming, emulsifying, encapsulating and release properties of bambara protein-gum arabic (BPI-GA) complexes were investigated. The effect of NaCl (10 mM) on these functional properties was also investigated. A Gelatine-GA complex system was used as a control in this study. Gelatine-GA complexes recorded the highest emulsifying, foaming and encapsulating properties compared to B PI-GA complexes. Similarly, BPI-GA complexes in the presence of NaCl showed better functional properties compared to those without salt. In comparison to the BPI-GA complexes, the emulsion capacity for BPI was 14.2% lower, and 26.2% lower for the encapsulating efficiency. Although there was an insignificant difference in the first hour between foams stabilized by BPI-GA complexes, the presence of salt seemed to improve the stability of foams after 3 hours. BPI alone recorded the highest release capacity in both the acidic and alkaline simulations. However, BPI-GA complexes in both the alkaline and acidic simulations showed a more stable release pattern. BPI-GA + 10 mM NaCl released up to 72% in the 2 hour simulation whilst BPI + 0 mM NaCl released up to 76%. The Gelatine-GA complex recorded the least release capacity in both the acidic and alkali simulations. Generally, complexing proteins with polysaccharides improved the functionality of proteins. Furthermore, the addition of low salt concentrations into protein-polysaccharide complexes seems to enhance their functional properties.

5.0 INTRODUCTION

The application of protein-polysaccharide complexes as functionality improvers has resulted in increased research attention towards proteins and polysaccharides. In emulsions, polysaccharides have been shown to increase the thickness of interfacial layers, enhance hydrophilicity and steric repulsion. However, they display lower surface activity if used alone (Liu et al., 2010). Proteins on the other hand are good emulsifiers but lack the stabilising effect of polysaccharides (Yin et al., 2012). Therefore, controlled protein-polysaccharide interactions may improve the emulsifying and other functional properties.

In a study by (Liu et al., 2010), pea protein isolate (PPI) stabilised emulsions had lower emulsifying capacity compared to those stabilised with PPI-GA complexes. In addition, Yin et al., (2012) reported that soy protein-soybean polysaccharide complexes produced more stable emulsions compared to those stabilised by soy protein isolate alone. Previous studies have also shown that protein-polysaccharide complexes can be applied as encapsulating matrices for protection and delivery of bioactive compounds (Li et al., 2015; Shishir et al., 2018; Bustos-Garza et al., 2013). For instance, the complexation of soy protein-gum arabic produced better encapsulation efficiency of tomato oleoresin compared to soy protein alone (Li et al., 2015). But apart from soybean and pea protein, other marginalised protein sources such as bambara groundnut could serve as potential encapsulating and release matrices.

Bambara protein has been shown to exhibit good emulsifying and foaming properties. A study by Arise et al., (2017) showed that bambara protein stabilised emulsions by over 70%. Further, Kudre et al., (2013) reported that the emulsifying properties of bambara protein (82 %) were comparable to those of soybean protein (86 %). Therefore bambara protein may offer potential as encapsulating matrices for bioactive compounds when complexed with polysaccharides. In our previous study, bambara protein-gum arabic complexes were prepared. Further, 10 mM NaCl was shown to enhance complexation at 4:1 biopolymer ratio. However, the encapsulation efficiency and release properties of the formed complexes were not investigated. This study therefore seeks to investigate the functionality of bambara protein-gum arabic complexes in comparison to BPI and Gelatine-GA complexes. Gelatine has been widely used in complexation studies with other proteins due to its linear structure and good solubility (Anvari and Joyner, 2017). Gelatine was the first protein to

be investigated in a Gelatine-GA complex system by De Jong and Kruyt (1929), therefore it was used as a reference sample.

5.1 Materials and Methods

5.1.1 Materials

Bambara groundnuts were purchased from farmers in Umbumbulu areas of KwaZulu Natal Province, South Africa. Gelatine was purchased from Sigma-Aldrich and was used in all experiments in this study as a standard for comparison with bambara protein. Gelatine has been widely used in complexation studies with polysaccharides such as gum arabic. Other laboratory grade chemicals were purchased from Sigma-Aldrich.

5.1.2 Emulsifying properties

Emulsifying properties were measured according to methods adapted from (Guo and Mu, 2011; Marco and Rosell, 2008). Emulsions were prepared at 4:1 protein-polysaccharide ratio both in the absence and presence of NaCl (10 mM). Protein and polysaccharide solutions were prepared as previously described in previous research chapter 1. The oil-in-water emulsions were prepared by adding 30 % canola oil to the continuous phase, followed by homogenisation using the ultrasonicator at power setting of 20 %. Fifty microliter aliquots of each emulsion were pipetted at 0 and 120 min and added to 5 ml of 0.1% (w/v) SDS. The absorbance of the emulsions was measured using a spectrometer at 500 nm. The emulsifying activity was expressed as the absorbance at 0 min, and the emulsion stability was expressed as

$$ES\ (\%) = \frac{\text{Abs at } 120 \text{ min}}{\text{Abs at } 0 \text{ min}} \text{ x } 100$$

5.1.3 Foaming properties

Foaming properties were determined using a method by Arise et al., (2015). Suspensions were prepared by dispersing 300 mg protein in 5 mL of 0.1 M phosphate buffer at pH 7.0. Sample suspensions were homogenised using the ultrasonicator at power setting of 20 % for 5 min and volumes were recorded before and after homogenisation.

Foam stability at 25 ^{o}C was determined as the volume of foam that remains at 1 hour, 3 hour and 5 hour intervals and expressed as percentage of the initial volume.

5.1.4 Protein-polysaccharide complex carrier preparation

Protein-polysaccharide complexes were prepared as previously described in research chapter 1 with the exception that 30% β-carotene oil was added before the addition of (0.1 w/v %) gum arabic. Complexes were prepared at ionic strength (0 and 10 mM NaCl) and biopolymer ratio (4:1) based on our previous study. The protein-polysaccharide: β-carotene emulsion was then vigorously homogenised using an ultrasonicator at power setting 20%. The pH of the emulsion was adjusted to 4.5 to facilitate complexation and encapsulation. The mixture was left to stir for 30 min at 4^{o}C and thereafter left to sediment for 1 hour. The sediment was carefully collected and dried in an oven at 30^{o}C for 2 hours. The complex carriers were stored in an air tight container at 4^{o}C.

5.1.5 Encapsulation efficiency

Surface oil was determined using the method of Liu et al., (2010). Briefly, 1 g of oven-dried complex carriers was dispersed in 30 ml of hexane and vigorously shaken for 30 s. The solvent was filtered with Whatman No. 41 filter paper into a beaker and allowed to evaporate overnight in a fume hood. To remove the remaining hexane, the beaker was heated at 105 °C, for 15 min followed by cooling in a desiccator. The surface oil content (defined as oil that was readily extracted by a single hexane wash) was calculated and expressed as grams of oil per 100 g of the complex carriers.

Encapsulation efficiency was calculated according to a method by (Alexe and Dima, 2014),

$$\mathrm{EE} = \frac{Total\ oil\ added - surface\ oil\ not\ encapsulated}{Total\ oil\ added\ to\ the\ mix} \text{ x } 100$$

5.1.6 In-vitro release studies

Release studies were carried out according to methods adapted from (Maltais et al., 2009). Complex carriers, (2 g) were dispersed in 10 ml of gastric acid medium pH 1.2, (2 g NaCl, 7 ml of 37% HCl, and 0.1% (w/v) pepsin). The mixture was incubated at 37°C for 2 hours with constant stirring. To simulate intestinal digestion, 2g of complex carriers were dispersed in an intestinal alkaline medium containing 1.0% (w/v) pancreatin (pH 7.4). Digestion was followed for 2 hours at 37°C with stirring. Samples were taken hourly and quantification was obtained by measuring absorbance at 280 nm and calculated as follows:

(1) % β carotene released + matrix degraded at 1 hour interval $= \frac{carotene\ released}{Total\ carotene\ initially\ added}\ x100$

Matrix degradation was measured on complexes without β-carotene to ensure accuracy of β carotene absorbance. Protein [released] is the protein concentration (g/L) and protein [total] is the total protein concentration in the gels.

Matrix degradation was calculated as:

(2) % matrix degraded at 1 hour interval $= \frac{Protein\ [released]}{Protein\ [total]}\ x100$

The amount of β-carotene released at 1 hour interval was then calculated as follows:

(3) % β-carotene oil released at 1 hour interval = Equation (1) – Equation (2)

5.2 Results and Discussion

5.2.1 Emulsification properties

Emulsifying capacities of the BPI-GA complexes were relatively similar (52% for 4:1 + 0 Mm NaCl and 65% for 4:1 + 10 Mm NaCl), but differed significantly from those of BPI alone (43%). As expected, Gelatin: GA recorded the highest emulsifying capacity (74%) and stability (90%) followed by BPI: GA in the presence of salt (4:1, 10 mM) whose emulsifying capacity and stability were 65% and 86% respectively (Fig 20). BPI recorded the least emulsifying properties (44% and 62%) possibly due to poor solubility at the pH of emulsion preparation. The high emulsifying properties of the BPI-GA complex in the presence of salt may be due to the partial unfolding of bambara protein during complexation with gum arabic (Liu et al., 2010). Moreover, the presence of salt may have also promoted unfolding of the protein resulting in enhanced alignment onto oil droplets and consequently improved emulsion capacity (Evans et al., 2013). The high emulsifying capacity of Gelatin: GA complex may be attributed to the smaller and linear size of gelatin. Commercial gelatin has an average MW ranging from 40 to 80 kDa (Liu et al., 2010), which is much smaller in size compared to legumin (~338 kDa) and vicillin (~117 kDa) observed by (Diedricks et al., 2019) in BPI. In addition, compared to BPI's globular, rigid conformation, gelatin has linear chains which are more flexible. Therefore, gelatine molecules can easily migrate to and

align themselves at the oil-water interface in a short time compared to BPI molecules (Liu et al., 2010).

The addition of polysaccharides to emulsions has been reported to enhance emulsion stability by increasing thickness of the interfacial layer and enhancing hydrophilicity of the oil droplets (Evans et al., 2013). Similarly, emulsions stabilised by bambara protein-gum arabic complexes were more stable compared to those stabilised by bambara protein alone. Polysaccharides coat the protein and prevent protein-protein interactions that lead to flocculation (Lam and Nickerson, 2013). The data obtained on the emulsifying properties of BPI-GA complexes could indicate that BPI-GA complexes have potential as encapsulating matrices for bioactive compounds.

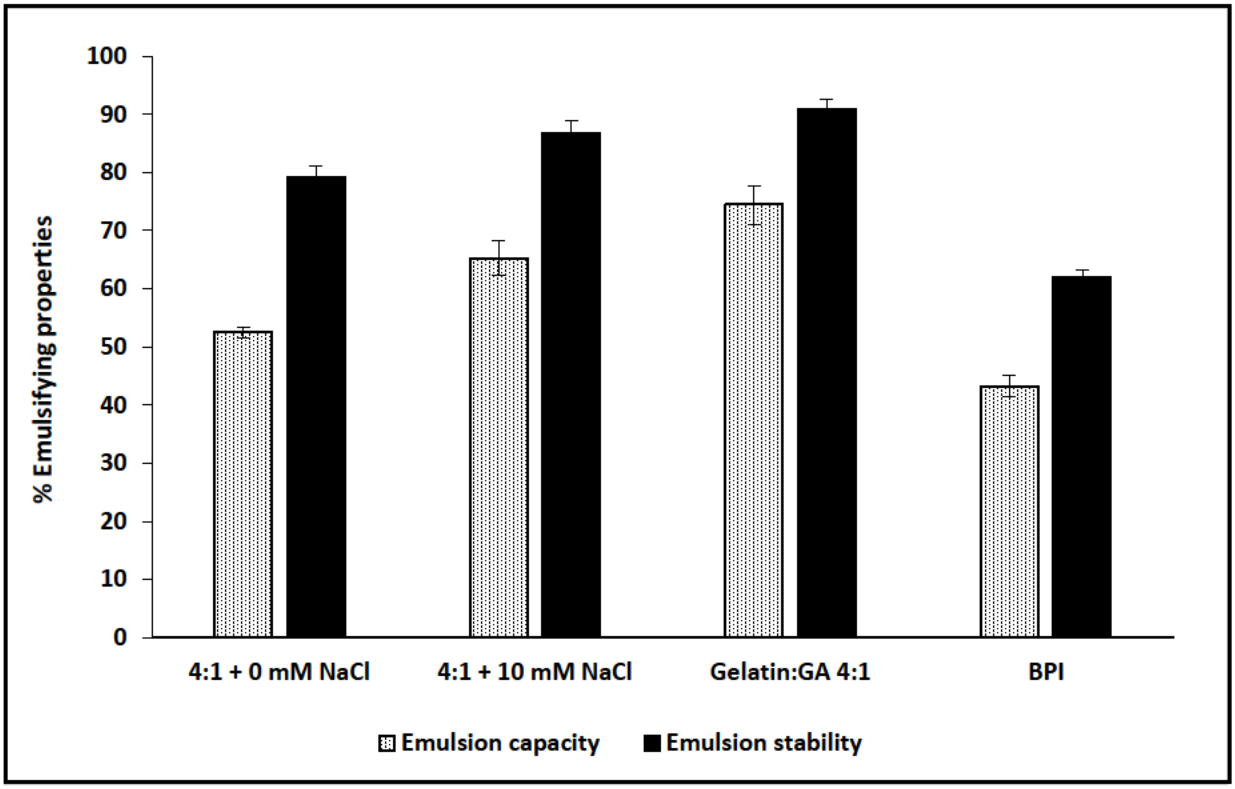

Fig 20: Emulsifying properties of BPI: GA complexes compared to Gelatin: GA and BPI alone

5.2.2 Foaming properties

Generally, complexing bambara protein with gum arabic improved the foaming properties (Fig 21). The addition of salt seemed to further improve foaming capacity and stability. Gelatine: GA showed higher foam capacity (85%). BPI: GA complexes in the presence of salt also exhibited a

slightly higher foam capacity (64%) compared to complexes in the absence of salt (56%). Low salt concentrations bind to charged groups on the protein surface and promote electrostatic interactions with water thus improving protein solubility, protein flexibility and limits intermolecular cohesion (Miquelim et al., 2010; Oduse et al., 2017). A study by (ErCelebi and Ibanoglu, 2009) showed that the foam capacity of Whey protein isolate-Guar gum (WPI-GG) complexes improved under low NaCl concentration (0.05 M) due to increased protein solubility and flexibility.

Foams stabilised with the Gelatine: GA complex recorded the highest foam stability. There was insignificant change in the foaming stability of the BPI-GA complexes in the first hour. However, a substantial decrease in foam stability was noted after 3 hours in all samples. This can be attributed to coalescence of air bubbles due to disruption of hydrophobic bonds (Eltayeb et al., 2011). After 5 hours, foams stabilised with Gelatine: GA complex were most stable, followed by BPI: GA complexes in the presence of salt. Low salt concentrations (<0.05 M) have been reported to enhance foam stability (Ahmed et al., 2012). Moreover, the presence of polysaccharides in foams has been reported to have a stabilising effect. Polysaccharides increase the viscosity of the aqueous phase and may influence the viscoelastic behavior and thickness of the adsorbed macromolecular layer and hence increasing foam stability (Oduse et al., 2017).

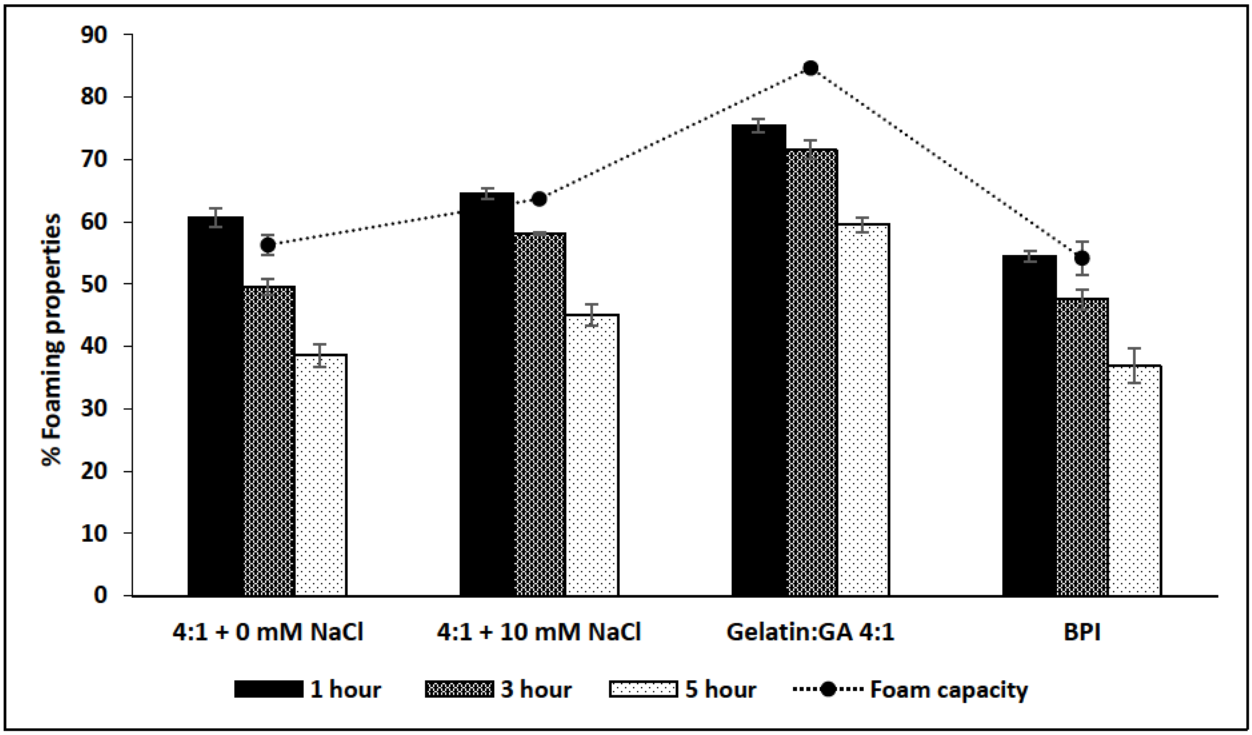

Fig 21: Foaming properties of the BPI: GA complexes compared to Gelatin: GA and BPI carried out over a period of 5 hours

5.2.3 Encapsulation properties

The encapsulation efficiency (EE) of BPI-GA complex in the presence of salt was 14 % higher than in the absence of salt (Fig 22). As expected, BPI recorded the least encapsulation efficiency compared to the complexes. The higher EE of BPI-GA complexes in the presence of salt may have been due to improved protein solubility due to presence of salt (Liu et al., 2010).

The Gelatine-GA complex system recorded the highest encapsulating capacity (67%). Numerous studies have investigated Gelatine-GA complex systems in the area of complexation and have been shown to have high encapsulating properties. For instance, a study by Lescano et al., (2015) on encapsulation of *Acrocomia aculeata* oil reported 59 – 97% encapsulation efficiency using a Gelatin-GA complex system. Moreover, a study by (Liu et al., 2010) showed that Gelatine-GA complexes encapsulated 40% of flax oil. However, in the same study, PPI-GA complexes failed to encapsulate the flax oil. This was attributed to the linear, flexible and smaller gelatin size (40 to 80 kDa) which allows gelatin to rapidly align at the oil interface thus enhancing encapsulation as opposed to the globular and rigid structure of pea protein (legumin ~360 kDa and vicillin ~150 kDa) (Liu et al., 2010). This could also justify why BPI-GA complexes had lower EE as compared to Gelatin-GA complexes.

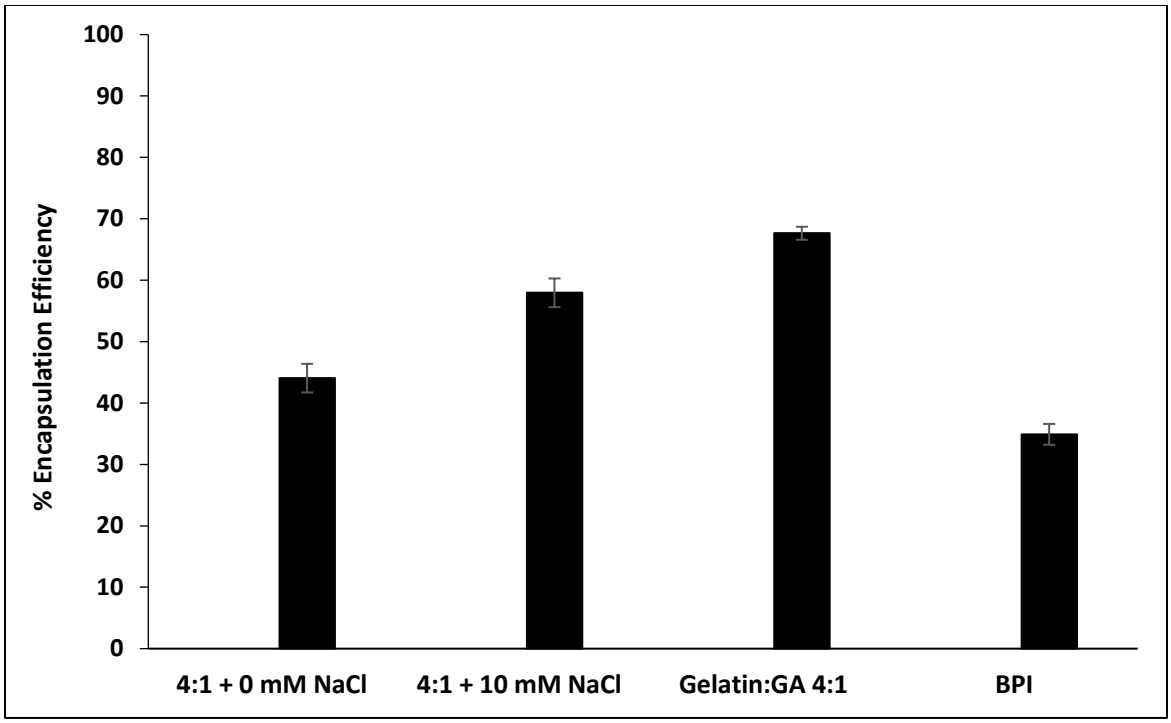

Fig 22: Encapsulation efficiency of bambara protein: gum Arabic compared to Gelatin: GA and BPI alone.

5.2.4 Release properties

Release properties were investigated under gastric (pH 1.2, pepsin enzyme) and intestinal (pH 7.4, pancreatin enzyme) simulation conditions. Generally, the acidic profile (pH 1.2, pepsin) showed a slow progression of less than 16% in the first hour for the BPI-GA complexes with and without salt whilst BPI released up to 70% β-carotene in the first hour (Fig 23a). The alkaline profile (pH 7.4, pancreatin) showed a faster progression with up to 60% of oil released for both the BPI-GA complexes and BPI releasing up to 80 % in the first hour (Fig 23b).

The initial slow release for the acidic profile for both BPI-GA complexes may be attributed to stabilising bonds such as hydrogen bonds and Van der Waals interactions which come into play during complexation and are not easily dissociated by pH (Dickinson, 2008). A sharp increase in release (up to 60%) was observed in the second hour at the simulation pH 2. At this pH, both the bambara protein and gum arabic carry positive charges. This results in disintegration of the complex leading to increased release of the core compound (Teng et al., 2013). Moreover, pepsin is known to attack peptide bonds preferentially at the C-terminal side of hydrophobic aromatic

amino acids such as tyrosine, tryptophan and phenylalanine (Maltais et al., 2009). Bambara protein has been reported to be rich in these hydrophobic aromatic amino acids (Kudre et al., 2013; Arise et al., 2015).

In the alkaline profile, a slow release of both the BPI-GA complex carriers was observed in the first 30 minutes. The subsequent fast progression may be attributed to the peptide-bond hydrolysing activity of pancreatin which may have weakened bonds in the carier wall (Maltais et al., 2009). Moreover, the pancreatic amylase enzyme present in enzyme pancreatin may have hydrolysed 1, 4-glycoside linkages present in gum arabic, thus further weakening the protein-polysaccharide wall (Maltais et al., 2009). BPI as expected showed a fast release progression from the beginning till the end of the simulation. This could be due to the absence of stabilising bonds that were generated during protein-polysaccharide complexation. These findings suggested that although mild salt levels improved degree of interaction between proteins and polysaccharides, the role of salt in stabilising complex carriers is minimal if not insignificant.

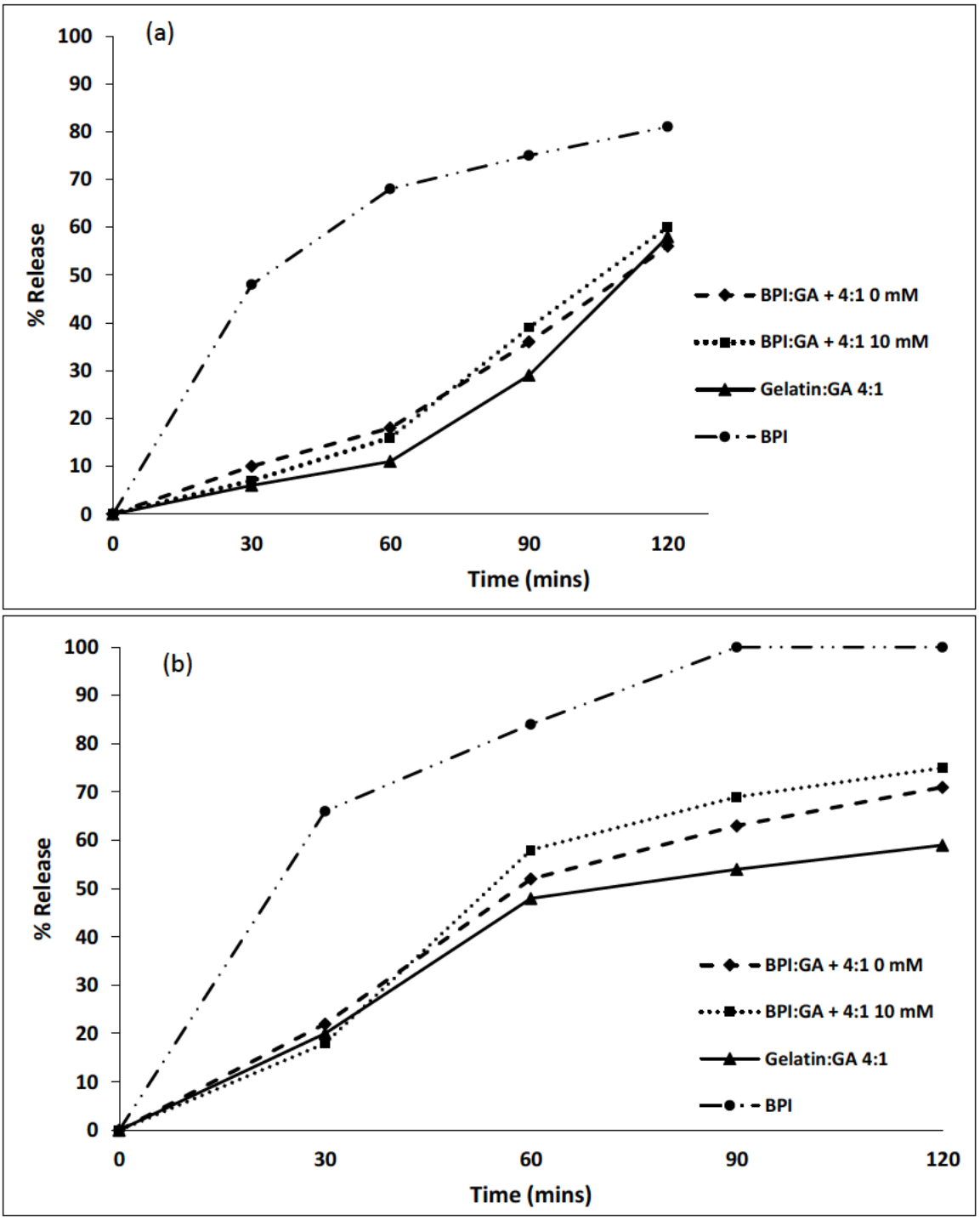

Figure 23: Time course for release of encapsulated β-carotene oil prepared by complex coacervation (a) Gastric simulation (pH 1.2 + pepsin), (b) Intestinal simulation (pH 7.4 + pancreatin)

5.3 Conclusion

Gelatin-GA complexes produced higher emulsifying and encapsulating capacities compared to BPI-GA complexes. Emulsions stabilised by BPI-GA complexes were more stable compared to those stabilised by bambara protein alone. Further, complexing bambara protein with gum arabic improved the foaming properties. The presence of salt in the complexes seemed to slightly improve the emulsifying, foaming and encapsulating properties. Based on the release profiles, BPI showed a fast release progression whilst BPI-GA and Gelatine-GA complexes showed slower release in both acidic and alkali simulations, thus demonstrating the positive effect of complexation on release properties. Furthermore, the presence of salt improves the degree of interaction between proteins and polysaccharides as well as protein functionality. However, the role of salt in enhancing or minimising the release capacity was minimal.

CHAPTER SIX

6.1 Summary and Conclusions

The first part of this study demonstrated the influence of bambara protein extraction pH on degree of complexation with gum arabic. The physicochemical properties of the protein fractions as a consequence of extraction pH were investigated. As expected, the extraction pH significantly influenced the amino acid composition, structural conformation and degree of interaction of the protein fractions with gum arabic. Protein fractions extracted through salt solubilisation method followed by isoelectric precipitation (storage protein) produced a higher protein yield compared to other methods of extraction. Further, protein content of the storage protein fraction was highest (82 %) whilst the pH 2 fraction recorded the least protein content of 76 %. Although the pH 2 fraction recorded the least protein content and yield, its SDS-PAGE profile was more representative of the storage protein in the defatted flour. Observations made on the defatted flour and pH 2 fraction suggested the presence of a 25 kDa band which corresponded to a basic legumin that has been reported in other legumes such as soybean and peas. The amino acid profile varied significantly with the pH of extraction. Glutamic and aspartic acid were the major amino acids in all the bambara protein fractions. These charged amino acids are important for complexation as they provide sites of interaction for complexation. It was noted that the secondary structure of all the protein fractions was slightly influenced with the pH of extraction. The β-sheet conformation appeared as the most dominant conformation in all the fractions, particularly in the pH 2 fraction. This could have been due to the acidic medium of extraction which may have caused protein denaturation and unfolding.

Upon complexing bambara protein with gum arabic, a shift of the optimum pH of complexation towards acidic regions was observed. This suggested the formation of complexes that are stable in acidic conditions. However, the extent of the shift varied with pH of extraction. This observation suggested that stable complexes under different acidic conditions can be fabricated through manipulating the protein extraction pH. For instance, the optimum pH of complexation for pH 2 fraction was 2.9 whilst that of the salt-soluble fraction was 4.01. Therefore, extracting proteins fractions at different pH conditions and complexing them with gum arabic could be an alternative way of designing carriers with improved acid stability.

The second phase of this study investigated the influence of ionic strength and biopolymer ratio on formation of bambara protein-GA complexes. Generally increasing protein ratio in the protein-polysaccharide mixture resulted in wider and normally distributed turbidity profiles. A higher GA ratio on the other hand caused the formation of two distinct peaks in the profiles. It was observed that the 16:1 BPI-GA sample had normally distributed profiles, which suggested the formation of uniformly sized particles across the studied pH range. The regularity of the turbidity profile was further enhanced by gradually adding NaCl up to 40 mM, hence it was concluded that the 16:1, 40 mM BPI-GA sample had uniformed sized particles. The presence of these uniform-sized particles in the 16:1, 40 mM BPI-GA sample was confirmed by AFM. It was therefore concluded that simultaneously increasing ionic strength and biopolymer ratio could be an important way of producing uniform size particles for application in the food industry.

In the third study, some of the functional, encapsulation and release properties of bambara protein isolate, gelatine-GA complexes and BPI-GA complexes in the presence and absence of NaCl were investigated. In comparison to BPI alone, BPI-GA complexes generally showed improved emulsifying and foaming properties. These functional properties were further improved by the addition of 10 mM NaCl. However, in comparison to gelatine-GA complexes, the functional properties of BPI-GA complexes regardless of the presence of salt were much less than those of a Gelatine-GA complex system. This was expected due to the smaller, linear and more flexible structure of gelatin which allows gelatine it to easily migrate and align itself on the air/water interface. BPI-GA complex in the presence of 10 mM NaCl showed better encapsulation when compared to either BPI alone or BPI-GA in the absence of salt. However, the role of ionic strength in stabilizing the complexes against fast release of beta carotene was minimal.

In conclusion, fractionation pH impacted the degree of interaction of protein with polysaccharides as well as the stability of the formed complexes. As observed, the total storage protein showed a higher BPI-GA complex yield whilst the pH 2-GA complex showed better acid-stability. Biopolymer ratio and presence of salt also influenced BPI-GA interaction. A biopolymer ratio of 4:1 BPI: GA and ionic strength of 10 mM produced higher turbidity values on the turbidity profiles, which translates to higher complex yield. However, a 16:1 biopolymer ratio and 40 mM ionic strength produced uniform-sized particles. Hence, simultaneously increasing biopolymer ratio and ionic strength could be useful in producing uniformly-sized particles. Complexing bambara protein

with gum arabic improved the emulsifying, foaming and encapsulating properties of bambara protein. Further, BPI: GA complexes showed a slower release progression compared to BPI alone.

6.2 Recommendations

Bambara protein has huge potential as both a functional and nutritional ingredient in the food industry. However, the lack of knowledge on the physicochemical and other functional properties impedes its utilisation. Further research can be carried out to investigate other extraction and pre-treatment methods such as cold-plasma and how they may improve bambara protein functionality. Methods of improving protein yield for protein fractions extracted at acidic pH may also be investigated. For instance, in the first objective of this study, the pH 2 fraction produced a small yield although it showed potential when complexed with gum arabic. Therefore different fractionation procedures to improve complex yield between proteins and polysaccharides maybe investigated.

Although the influence of ionic strength and biopolymer ratio on BPI-GA complexes was investigated, the synergistic influence of these and other factors was not fully elucidated in this study. It may be beneficial for future studies to investigate the combined influence of factors that affect protein-polysaccharide complexation on a Response Surface Methodology model. Moreover, the influence of different types of salts may bring about different observations, and hence may be worth investigating. Also, studies on the interaction of bambara protein with different polysaccharides such as guar gum or xanthan gum can be initiated.

Observations from this study showed that BPI-GA complexes have potential as carrier matrices for bioactive compounds. However, more studies are needed to investigate the effect of cross-linking, heating and the addition of solute (e.g. sucrose) to improve the stability of complexes and consequently the release profile of encapsulated compounds therein.

REFERENCES

Adebowale, Y. A., Maliki, K., 2011. Protein isolates from Bambara groundnut (Voandzeia Subterranean L.): Chemical characterization and functional properties. *International journal of food properties* 14, 758-775.

Adeleke, O.R., Adiamo, O.Q. and Fawale, O.S., 2018. Nutritional, physicochemical, and functional properties of protein concentrate and isolate of newly-developed Bambara groundnut (Vigna subterrenea L.) cultivars. *Food science & nutrition*, *6*(1), pp.229-242.

Ahmed, S.H., Babiker, E.E., Mohamed, I.A., Eltayeb, M.M. and Ahmed, S.O., 2012. Effect of sodium chloride concentration on the functional properties of selectec Legume flours. *African Journal of Food, Agriculture, Nutrition and Development*, *12*(6).

Akhtar, M. and Dickinson, E., 2007. Whey protein–maltodextrin conjugates as emulsifying agents: An alternative to gum arabic. *Food Hydrocolloids*, *21*(4), pp.607-616.

Alain, M. M. M., Israel, M. L., Rene, M, S., 2007. Improving the nutritional quality of cowpea and bambara bean flours for use in infant feeding. *Pakistan Journal of Nutrition* 6, 660-664.

Alexe, P. and Dima, C., 2014. Microencapsulation in food products. *AgroLife Scientific Journal*, *3*(1), pp.9-11.

Alli, I., Gibbs, B.F., Okoniewska, M.K., Konishi, Y. and Dumas, F., 1993. Identification and characterization of phaseolin polypeptides in a crystalline protein isolated from white kidney beans (Phaseolus vulgaris). *Journal of agricultural and food chemistry*, *41*(11), pp.1830-1834.

Amonsou, E. O., Taylor, J. R. N., Beukes, M., Minnaar, A., 2012. Composition of marama bean protein. Food Chemistry 130, 638-643.

Amponsah, A. and Nayak, B., 2016. Effects of microwave and ultrasound assisted extraction on the recovery of soy proteins for soy allergen detection. *Journal of food science*, *81*(11), pp.T2876-T2885.

Antonov, Y.A., Zhuravleva, I.L., Cardinaels, R. and Moldenaers, P., 2018. Macromolecular complexes of lysozyme with kappa carrageenan. *Food hydrocolloids*, *74*, pp.227-238.

Anvari, M. and Joyner, H.S., 2017. Effect of formulation on structure-function relationships of concentrated emulsions: Rheological, tribological, and microstructural characterization. *Food hydrocolloids*, *72*, pp.11-26.

Arise, A. K., Amonsou, E. O., Ijabadeniyi, O. A., 2015. Influence of extraction methods on functional properties of protein concentrates prepared from South African bambara groundnut landraces. *International Journal of Food Science & Technology* 50, 1095-1101.

Arise, A. K., Nwachukwu, I. D., Aluko, R. E., Amonsou, E. O., 2017. Structure, composition and functional properties of storage proteins extracted from bambara groundnut (Vigna subterranea) landraces. *International Journal of Food Science & Technology* 52, 1211-1220.

Aryee, F. N. and Nickerson M. T., 2014. Effect of pH, biopolymer mixing ratio and salts on the formation and stability of electrostatic complexes formed within mixtures of lentil protein isolate and anionic polysaccharides (κ-carrageenan and gellan gum). *International Journal of Food Science & Technology* 49, 65-71.

Aryee, F.N. and Nickerson, M.T., 2012. Formation of electrostatic complexes involving mixtures of lentil protein isolates and gum Arabic polysaccharides. *Food Research International*, *48*(2), pp.520-527.

Baryeh, E.A., 2001. Physical properties of bambara groundnuts. *Journal of food engineering*, *47*(4), pp.321-326.

Benichou, A., Aserin, A., Lutz, R. and Garti, N., 2007. Formation and characterization of amphiphilic conjugates of whey protein isolate (WPI)/xanthan to improve surface activity. *Food Hydrocolloids*, *21*(3), pp.379-391.

Benbettaïeb, N., Karbowiak, T. and Debeaufort, F., 2019. Bioactive edible films for food applications: Influence of the bioactive compounds on film structure and properties. *Critical reviews in food science and nutrition*, *59*(7), pp.1137-1153.

Bertrand, M.E. and Turgeon, S.L., 2007. Improved gelling properties of whey protein isolate by addition of xanthan gum. *Food Hydrocolloids*, *21*(2), pp.159-166.

Boye, J.I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E. and Rajamohamed, S.H., 2010. Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, *43*(2), pp.537-546.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.

Brychcy, E., Malik, M., Drożdżewski, P., Król, Ż. and Jarmoluk, A., 2015. Physicochemical and antibacterial properties of carrageenan and gelatine hydrosols and hydrogels incorporated with acidic electrolyzed water. *Polymers*, *7*(12), pp.2638-2649.

Bustos-Garza, C., Yanez-Fernandez, J., Barragan-Huerta, B. E., 2013. Thermal and pH stability of spray-dried encapsulated astaxanthin oleoresin from Haematococcus pluvialis using several encapsulation wall materials. *Food research international* 54, 641-649.

Butstraen, C. and Salaün, F., 2014. Preparation of microcapsules by complex coacervation of gum Arabic and chitosan. *Carbohydrate polymers* 99, 608-616.

Carbonaro, M. and Nucara, A., 2010. Secondary structure of food proteins by Fourier transform spectroscopy in the mid-infrared region. *Amino acids* 38, 679-690.

Carbonaro, M., Vecchini, P., Carnovale, E., 1993. Protein solubility of raw and cooked beans (Phaseolus vulgaris): role of the basic residues. *Journal of Agricultural and Food Chemistry* 41, 1169-1175.

Carrasco-Castilla, J., Hernandez-Alvarez, A. J., Jimenez-Martinez, C., Jacinto-Hernandez, C., Alaiz, M., Giron-Calle, J., Vioque, J., Davila-Ortiz, G., 2012. Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. *Food Chemistry* 135, 1789-1795

Chen, H., Ji, A., Qiu, S., Liu, Y., Zhu, Q. and Yin, L., 2018. Covalent conjugation of bovine serum album and sugar beet pectin through Maillard reaction/laccase catalysis to improve the emulsifying properties. *Food hydrocolloids*, *76*, pp.173-183.

Chinedu, S.N. and Nwinyi, O., 2012. Proximate analysis of Sphenostylis stenocarpa and Voadzeia subterranean consumed in South–Eastern Nigeria. *Journal of Agricultural Extension and Rural Development*, *4*(3), pp.57-62.

Chang, I., Im, J., Prasidhi, A.K. and Cho, G.C., 2015. Effects of Xanthan gum biopolymer on soil strengthening. *Construction and Building Materials*, *74*, pp.65-72.

Curtis, R.A., Ulrich, J., Montaser, A., Prausnitz, J.M. and Blanch, H.W., 2002. Protein–protein interactions in concentrated electrolyte solutions. *Biotechnology and bioengineering*, *79*(4), pp.367-380.

Department of Agriculture, Forestry and Fisheries, Production guideline of Bambara groundnut (2016), http://www.daff.gov.za/docs/Brochures/Bambara.pdf (accessed 31 July 2016)

De Jong, H.B., 1934. Complex relationships in lyophilic colloidal systems. I. General introduction. *Recueil des Travaux Chimiques des Pays-Bas*, *53*(2), pp.163-170.

De Jong, B., 1929. HG; Kruyt, H. R. Koazervation. In *Proc. K. Ned. Akad. Wet* (Vol. 32, pp. 849-856).

Debele, T.A., Mekuria, S.L. and Tsai, H.C., 2016. Polysaccharide based nanogels in the drug delivery system: Application as the carrier of pharmaceutical agents. *Materials Science and Engineering: C*, *68*, pp.964-981.

De Kruif, C.G., Weinbreck, F. and de Vries, R., 2004. Complex coacervation of proteins and anionic polysaccharides. *Current opinion in colloid & interface science*, *9*(5), pp.340-349.

Desai, K.G.H. and Jin Park, H., 2005. Recent developments in microencapsulation of food ingredients. *Drying technology*, *23*(7), pp.1361-1394.

Diedericks, C.F., 2014. *Functional properties of bambara groundnut (Vigna subterranea (L.) Verdc.) non-starch polysaccharides in model and food systems* (Doctoral dissertation, Cape Peninsula University of Technology).

Diedericks, C.F. and Jideani, V.A., 2015. Physicochemical and functional properties of insoluble dietary fiber isolated from bambara groundnut (Vigna subterranea [L.] Verdc.). *Journal of food science*, *80*(9), pp.C1933-C1944.

Diedericks, C.F., de Koning, L., Jideani, V.A., Venema, P., van der Linden, E., 2019. Extraction, gelation and microstructure of Bambara groundnut vicilins, *Food Hydrocolloids* doi: https://doi.org/10.1016/j.foodhyd.2019.105226

Dickinson, E., 1998. Stability and rheological implications of electrostatic milk protein–polysaccharide interactions. *Trends in Food Science & Technology*, *9*(10), pp.347-354.

Dickinson, E., 2008. Interfacial structure and stability of food emulsions as affected by protein–polysaccharide interactions. *Soft Matter*, *4*(5), pp.932-942.

Doublier, J.L., Garnier, C., Renard, D. and Sanchez, C., 2000. Protein–polysaccharide interactions. *Current opinion in Colloid & interface Science*, *5*(3-4), pp.202-214.

Đorđević, V., Balanč, B., Belščak-Cvitanović, A., Lević, S., Trifković, K., Kalušević, A., Kostić, I., Komes, D., Bugarski, B. and Nedović, V., 2015. Trends in encapsulation technologies for delivery of food bioactive compounds. *Food Engineering Reviews*, *7*(4), pp.452-490.

Du, M., Xie, J., Gong, B., Xu, X., Tang, W., Li, X., Li, C. and Xie, M., 2018. Extraction, physicochemical characteristics and functional properties of Mung bean protein. *Food Hydrocolloids*, *76*, pp.131-140.

Eghbal, N. and Choudhary, R., 2018. Complex coacervation: Encapsulation and controlled release of active agents in food systems. *Lwt*, *90*, pp.254-264.

Elhardallou, S.B., Khalid, I.I., Gobouri, A.A. and Abdel-Hafez, S.H., 2015. Amino acid composition of cowpea (Vigna ungiculata L. Walp) flour and its protein isolates. *Food and Nutrition Sciences*, *6*(9), p.790.

Elmer, C., Karaca, A. C., Low, N. H., Nickerson, M. T., 2011. Complex coacervation in pea protein isolate–chitosan mixtures. *Food research international* 44, 1441-1446.

Eltayeb, A. R. S., Ali, A. O., Abou-Arab, A. A., Abu-Salem, F. M., 2011. Chemical composition and functional properties of flour and protein isolate extracted from Bambara groundnut (Vigna subterranean). *African Journal of Food Science* 5, 82-90.

Eratte, D., McKnight, S., Gengenbach, T.R., Dowling, K., Barrow, C.J. and Adhikari, B.P., 2015. Co-encapsulation and characterisation of omega-3 fatty acids and probiotic bacteria in whey protein isolate–gum Arabic complex coacervates. *Journal of functional foods*, *19*, pp.882-

892.

Erçelebi, E.A. and Ibanoğlu, E., 2009. Rheological properties of whey protein isolate stabilized emulsions with pectin and guar gum. *European Food Research and Technology*, *229*(2), pp.281-286.

Esfanjani, A. F. and Jafari, S. M. 2016. Biopolymer nano-particles and natural nano-carriers for nano-encapsulation of phenolic compounds. *Colloids and Surfaces B: Biointerfaces* 146, 532-543.

Evans, M., Ratcliffe, I. and Williams, P.A., 2013. Emulsion stabilisation using polysaccharide–protein complexes. *Current Opinion in Colloid & Interface Science*, *18*(4), pp.272-282.

Food and Agriculture Organization of the United Nations (FAO), 2009, October. Global agriculture towards 2050. In *High level expert forum* [Accessed 6 November]

Faria, S., de Oliveira Petkowicz, C.L., De Morais, S.A.L., Terrones, M.G.H., De Resende, M.M., de França, F.P. and Cardoso, V.L., 2011. Characterization of xanthan gum produced from sugar cane broth. *Carbohydrate Polymers*, *86*(2), pp.469-476.

Featherstone, S. ed., 2015. *A Complete Course in Canning and Related Processes: Volume 3 Processing Procedures for Canned Food Products*. Woodhead Publishing.

Feng, E., Ma, G., Wu, Y., Wang, H. and Lei, Z., 2014. Preparation and properties of organic–inorganic composite superabsorbent based on xanthan gum and loess. *Carbohydrate polymers*, *111*, pp.463-468.

Feyzi, S. Varidi, M. Zare, F. Varidi, M. J., 2015. Fenugreek (Trigonella foenum graecum) seed protein isolate: extraction optimization, amino acid composition, thermo and functional properties. *Journal of the Science of Food and Agriculture* 95, 3165-3176.

Gharsallaoui, A., Yamauchi, K., Chambin, O., Cases, E. and Saurel, R., 2010. Effect of high methoxyl pectin on pea protein in aqueous solution and at oil/water interface. *Carbohydrate Polymers*, *80*(3), pp.817-827.

Goudoum, A., NGAMO TINKEU, L.S., Madou, C., Djakissam, W. and Mbofung, C.M., 2016. Variation of some chemical and functional properties of Bambara groundnut (Voandzeia Subterranean L. Thouars) during sort time storage. *Food Science and Technology*, *36*(2), pp.290-295.

González-Paz, R.J., Lligadas, G., Ronda, J.C., Galià, M., Ferreira, A.M., Boccafoschi, F., Ciardelli, G. and Cádiz, V., 2013. Study on the interaction between gelatin and polyurethanes derived from fatty acids. *Journal of Biomedical Materials Research Part A*, *101*(4), pp.1036-1046.

Gomes, G.V.D.L., Borrin, T.R., Cardoso, L.P., Souto, E. and Pinho, S.C.D., 2013. Characterization and shelf life of β-carotene loaded solid lipid microparticles produced with stearic acid and sunflower oil. *Brazilian Archives of Biology and Technology*, *56*(4), pp.663-671.

Grdadolnik, J. 2002. FTIR investigation of protein conformation. Bulletin of the Chemists and Technologists of Macedonia 21, 23-34.

Gulzar, M., Taylor, K., Minnaar, A., 2017. Influence of extraction pH on the foaming, emulsification, oil-binding and visco-elastic properties of marama protein. *Journal of the Science of Food and Agriculture* 97, 4815-4821.

Guo, Q. and Mu, T.H., 2011. Emulsifying properties of sweet potato protein: effect of protein concentration and oil volume fraction. *Food Hydrocolloids*, *25*(1), pp.98-106.

Hayati, I.N., Man, Y.B.C., Tan, C.P. and Aini, I.N., 2009. Thermal behavior of concentrated oil-in-water emulsions based on soybean oil and palm kernel olein blends. *Food Research International*, *42*(8), pp.1223-1232.

Hillocks, R.J., Bennett, C. and Mponda, O.M., 2012. Bambara nut: A review of utilisation, market potential and crop improvement. *African Crop Science Journal*, *20*(1).

Hogan, S.A., McNamee, B.F., O'Riordan, E.D. and O'Sullivan, M., 2001. Emulsification and microencapsulation properties of sodium caseinate/carbohydrate blends. *International Dairy Journal*, *11*(3), pp.137-144.

Holdt, S.L. and Kraan, S., 2011. Bioactive compounds in seaweed: functional food applications and legislation. *Journal of applied phycology*, *23*(3), pp.543-597.

Izydorczyk, M., Cui, S. W., & Wang, Q. (2005). Polysaccharide gums: structures, functional properties, and applications. *Food carbohydrates: Chemistry, physical properties, and applications*, 293-299.

Jain, A., Thakur, D., Ghoshal, G., Katare, O.P. and Shivhare, U.S., 2015. Microencapsulation by complex coacervation using whey protein isolates and gum acacia: an approach to preserve the functionality and controlled release of β-carotene. *Food and bioprocess technology*, *8*(8), pp.1635-1644.

Jain, A., Thakur, D., Ghoshal, G., Katare, O.P. and Shivhare, U.S., 2016. Characterization of microcapsulated β-carotene formed by complex coacervation using casein and gum tragacanth. *International journal of biological macromolecules*, *87*, pp.101-113.

Jarpa-Parra, M., Bamdad, F., Wang, Y., Tian, Z., Temelli, F., Han, J. and Chen, L., 2014. Optimization of lentil protein extraction and the influence of process pH on protein structure and functionality. *LWT-Food Science and Technology*, *57*(2), pp.461-469.

Jiang, J., Zhu, B., Liu, Y. and Xiong, Y.L., 2014. Interfacial structural role of pH-shifting processed pea protein in the oxidative stability of oil/water emulsions. *Journal of agricultural and food chemistry*, *62*(7), pp.1683-1691.

Jiang, J., Xiong, Y.L. and Chen, J., 2010. pH shifting alters solubility characteristics and thermal stability of soy protein isolate and its globulin fractions in different pH, salt concentration, and temperature conditions. *Journal of agricultural and food chemistry*, *58*(13), pp.8035-8042.

Jindal, M., Kumar, V., Rana, V. and Tiwary, A.K., 2013. Exploring potential new gum source Aegle marmelos for food and pharmaceuticals: Physical, chemical and functional performance. *Industrial crops and products*, *45*, pp.312-318.

Jones, O. G., Decker, E. A., McClements, D. J., 2009. Formation of biopolymer particles by thermal treatment of β-lactoglobulin–pectin complexes. *Food Hydrocolloids* 23, 1312-1321.

Jones, O.G. and McClements, D.J., 2011. Recent progress in biopolymer nanoparticle and microparticle formation by heat-treating electrostatic protein–polysaccharide complexes. *Advances in colloid and interface science*, *167*(1-2), pp.49-62.

Jun-xia, X., Hai-yan, Y., Jian, Y., 2011. Microencapsulation of sweet orange oil by complex coacervation with soybean protein isolate/gum Arabic. *Food Chemistry* 125, 1267-1272.

Karaca, A.C., Low, N.H. and Nickerson, M.T., 2015. Potential use of plant proteins in the microencapsulation of lipophilic materials in foods. *Trends in Food Science & Technology*, *42*(1), pp.5-12.

Kasran, M., Cui, S.W. and Goff, H.D., 2013. Covalent attachment of fenugreek gum to soy whey protein isolate through natural Maillard reaction for improved emulsion stability. *Food Hydrocolloids*, *30*(2), pp.552-558.

Kaya, M., Sousa, A.G., Crépeau, M.J., Sørensen, S.O. and Ralet, M.C., 2014. Characterization of citrus pectin samples extracted under different conditions: influence of acid type and pH of extraction. *Annals of botany*, *114*(6), pp.1319-1326.

Kinsella, J.E., 1979. Functional properties of soy proteins. *Journal of the American Oil Chemists' Society*, *56*(3Part1), pp.242-258.

Klassen, D.R., Elmer, C.M. and Nickerson, M.T., 2011. Associative phase separation involving canola protein isolate with both sulphated and carboxylated polysaccharides. *Food Chemistry*, *126*(3), pp.1094-1101.

Klemmer, K., Waldner, L., Stone, A., Low, N. H., Nickerson, M. T., 2012. Complex coacervation of pea protein isolate and alginate polysaccharides. Food Chemistry 130, 710-715.

Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E. and Etherton, T.D., 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *The American journal of medicine*, *113*(9), pp.71-88.

Kudre, T. and Benjakul, S., 2013. Effects of bambara groundnut protein isolate on protein degradation and gel properties of surimi from sardine (Sardinella albella). *Journal of Food Processing and Preservation* 37, 977-986.

Kudre, T. G., Benjakul, S., Kishimura, H., 2013. Comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut. *Journal of the Science of Food and Agriculture* 93, 2429-2436.

Kumar, A., Rao, K.M. and Han, S.S., 2018. Application of xanthan gum as polysaccharide in tissue engineering: A review. *Carbohydrate polymers*, *180*, pp.128-144.

Ladjal-Ettoumi, Y., Boudries, H., Chibane, M., Romero, A., 2016. Pea, Chickpea and Lentil Protein Isolates: Physicochemical Characterization and Emulsifying Properties. *Journal of Food Biophysics* 11, 43-51

Laemmli, UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Lam, A.C.Y., Can Karaca, A., Tyler, R.T. and Nickerson, M.T., 2018. Pea protein isolates: Structure, extraction, and functionality. *Food reviews international*, *34*(2), pp.126-147.

Lam, R.S. and Nickerson, M.T., 2013. Food proteins: a review on their emulsifying properties using a structure–function approach. *Food chemistry*, *141*(2), pp.975-984.

Laneuville, S.I., Paquin, P. and Turgeon, S.L., 2000. Effect of preparation conditions on the characteristics of whey protein—xanthan gum complexes. *Food Hydrocolloids*, *14*(4), pp.305-314.

Latif, S., Pfannstiel, J., Makkar, H.P.S. and Becker, K., 2013. Amino acid composition, antinutrients and allergens in the peanut protein fraction obtained by an aqueous enzymatic process. *Food chemistry*, *136*(1), pp.213-217.

Lescano, C.H., Iwamoto, R.D., Sanjinez-Argandona, E.J. and Kassuya, C.A.L., 2015. Diuretic and anti-inflammatory activities of the microencapsulated Acrocomia aculeata (Arecaceae) oil on Wistar rats. *Journal of medicinal food*, *18*(6), pp.656-662.

Li, C., Wang, J., Shi, J., Huang, X., Peng, Q. and Xue, F., 2015. Encapsulation of tomato oleoresin using soy protein isolate-gum aracia conjugates as emulsifier and coating materials. *Food Hydrocolloids*, *45*, pp.301-308.

Li, X., Fang, Y., Phillips, G.O. and Al-Assaf, S., 2013. Improved sugar beet pectin-stabilized emulsions through complexation with sodium caseinate. *Journal of agricultural and food chemistry*, *61*(6), pp.1388-1396.

Liu, S., Cao, Y., Ghosh, S., Rousseau, D., Low, N. H., Nickerson, M. T., 2009. Intermolecular interactions during complex coacervation of pea protein isolate and gum arabic. *Journal of Agricultural and Food Chemistry* 58, 552-556.

Liu, S., Low, N. H., Nickerson, M. T., 2009. Effect of pH, salt, and biopolymer ratio on the formation of pea protein isolate− gum arabic complexes. *Journal of Agricultural and Food Chemistry* 57, 1521-1526.

Liu, S., Low, N.H. and Nickerson, M.T., 2010. Entrapment of flaxseed oil within gelatin-gum arabic capsules. *Journal of the American Oil Chemists' Society*, *87*(7), pp.809-815.

Malomo, S.A. and Aluko, R.E., 2015. Conversion of a low protein hemp seed meal into a functional protein concentrate through enzymatic digestion of fibre coupled with membrane ultrafiltration. *Innovative food science & emerging technologies*, *31*, pp.151-159.

Maltais, A., Remondetto, G.E. and Subirade, M., 2009. Soy protein cold-set hydrogels as controlled delivery devices for nutraceutical compounds. *Food Hydrocolloids*, *23*(7), pp.1647-1653.

Marco, C. and Rosell, C.M., 2008. Functional and rheological properties of protein enriched gluten free composite flours. *Journal of Food Engineering*, *88*(1), pp.94-103.

Mauri, A. N. and Añón M. C., 2006. Effect of solution pH on solubility and some structural properties of soybean protein isolate films. *Journal of the Science of Food and Agriculture* 86, 1064-1072.

Maxwell, E.G., Belshaw, N.J., Waldron, K.W. and Morris, V.J., 2012. Pectin–an emerging new bioactive food polysaccharide. *Trends in Food Science & Technology*, *24*(2), pp.64-73.

Mazahib, A.M., Nuha, M.O., Salawa, I.S. and Babiker, E.E., 2013. Some nutritional attributes of bambara groundnut as influenced by domestic processing. *International Food Research Journal*, *20*(3), p.1165.

Mendanha, D.V., Ortiz, S.E.M., Favaro-Trindade, C.S., Mauri, A., Monterrey-Quintero, E.S. and Thomazini, M., 2009. Microencapsulation of casein hydrolysate by complex coacervation with SPI/pectin. *Food Research International*, *42*(8), pp.1099-1104.

Miquelim, J.N., Lannes, S.C. and Mezzenga, R., 2010. pH Influence on the stability of foams with protein–polysaccharide complexes at their interfaces. *Food hydrocolloids*, *24*(4), pp.398-405.

Mubaiwa, J., Fogliano, V., Chidewe, C., Bakker, E.J. and Linnemann, A.R., 2018. Utilization of bambara groundnut (Vigna subterranea (L.) Verdc.) for sustainable food and nutrition security in semi-arid regions of Zimbabwe. *PloS one*, *13*(10), p.e0204817.

Murayama, K. and Tomida M., 2004. Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy. *Biochemistry* 43, 11526-11532.

Mkandawire, C.H., 2007. Review of bambara groundnut (Vigna subterranea (L.) Verdc.) production in Sub-Sahara Africa. *Agricultural Journal*, *2*(4), pp.464-470.

Murevanhema, Y.Y. and Jideani, V.A., 2013. Potential of Bambara groundnut (Vigna subterranea (L.) Verdc) milk as a probiotic beverage—A review. *Critical reviews in food science and nutrition*, *53*(9), pp.954-967.

Mundi, S. and Aluko, R.E., 2012. Physicochemical and functional properties of kidney bean albumin and globulin protein fractions. *Food Research International*, *48*(1), pp.299-306.

Mwasaru, M.A., Muhammad, K., Bakar, J. and Man, Y.B.C., 1999. Effects of isolation technique and conditions on the extractability, physicochemical and functional properties of pigeonpea (Cajanus cajan) and cowpea (Vigna unguiculata) protein isolates. I. Physicochemical properties. *Food chemistry*, *67*(4), pp.435-443.

Naqash, F., Masoodi, F.A., Rather, S.A., Wani, S.M. and Gani, A., 2017. Emerging concepts in the nutraceutical and functional properties of pectin—A Review. *Carbohydrate polymers*, *168*, pp.227-239.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S. and Bugarski, B., 2011. An overview of encapsulation technologies for food applications. *Procedia Food Science*, *1*, pp.1806-1815.

Nie, S.P., Wang, C., Cui, S.W., Wang, Q., Xie, M.Y. and Phillips, G.O., 2013. The core carbohydrate structure of Acacia seyal var. seyal (Gum arabic). *Food Hydrocolloids*, *32*(2), pp.221-227.

Niu, F., Dong, Y., Shen, F., Wang, J., Liu, Y., Su, Y., Xu, R., Wang, J. and Yang, Y., 2015. Phase separation behavior and structural analysis of ovalbumin–gum arabic complex coacervation. *Food hydrocolloids*, *43*, pp.1-7.

Nori, M.P., Favaro-Trindade, C.S., de Alencar, S.M., Thomazini, M., de Camargo Balieiro, J.C. and Castillo, C.J.C., 2011. Microencapsulation of propolis extract by complex coacervation. *LWT-Food Science and Technology*, *44*(2), pp.429-435.

Nti, C.A., 2009. Effects of bambara groundnut (Vigna subterranea) variety and processing on the quality and consumer appeal for its products. *International journal of food science & technology*, *44*(11), pp.2234-2242.

Oduse, K., Campbell, L., Lonchamp, J. and Euston, S.R., 2017. Electrostatic complexes of whey protein and pectin as foaming and emulsifying agents. *International journal of food properties*, *20*(sup3), pp.S3027-S3041.

Okpuzor, J., Ogbunugafor, H.A., Okafor, U. and Sofidiya, M.O., 2010. Identification of protein types in Bambara nut seeds: Perspectives for dietary protein supply in developing countries. *EXCLI journal*, *9*, p.17.

Osborne, T.B. and Mendel, L.B., 1917. The use of soy bean as food. *Journal of Biological Chemistry*, *32*(3), pp.369-387.

Pastor-Cavada, E., Juan, R., Pastor, J.E., Alaiz, M. and Vioque, J., 2014. Protein and amino acid composition of select wild legume species of tribe Fabeae. *Food chemistry*, *163*, pp.97-102.

Pattani, K.M., 2010. Chemoprevention of Head and Neck Cancer. In *Early Diagnosis and Treatment of Cancer Series: Head and Neck Cancers* (pp. 81-93).

Pelton, J. T. and McLean, L. R. 2000. Spectroscopic methods for analysis of protein secondary structure. Analytical biochemistry 277, 167-176.

Perrechil, F.A. and Cunha, R.L., 2012. Development of multiple emulsions based on the repulsive interaction between sodium caseinate and LBG. *Food hydrocolloids*, *26*(1), pp.126-134.

Piotrowicz, I.B.B. and Salas-Mellado, M.M., 2017. Protein concentrates from defatted rice bran: preparation and characterization. *Food Science and Technology*, *37*, pp.165-172.

Prata, A.S., Zanin, M. H. A., and Grosso, I. R. M (2008). Release properties of chemical and enzymatic crosslinked gelatin-gum arabic microparticles containing a fluorescent probe plus vetiver essential oil. *Colloids and Surfaces B; Biointerfaces*, 67(2), 171-178

Qayyum, M.M.N., Butt, M.S., Anjum, F.M. and Nawaz, H., 2012. Composition analysis of some selected legumes for protein isolates recovery. *The journal of Animal and plant sciences*, *22*(4), pp.1156-1162.

Qian, C., Decker, E.A., Xiao, H. and McClements, D.J., 2012. Nanoemulsion delivery systems: Influence of carrier oil on β-carotene bioaccessibility. *Food Chemistry*, *135*(3), pp.1440-1447.

Qv, X.Y., Zeng, Z.P. and Jiang, J.G., 2011. Preparation of lutein microencapsulation by complex coacervation method and its physicochemical properties and stability. *Food Hydrocolloids*, *25*(6), pp.1596-1603.

Ranjan, S., Dasgupta, N., Chakraborty, A.R., Samuel, S.M., Ramalingam, C., Shanker, R. and Kumar, A., 2014. Nanoscience and nanotechnologies in food industries: opportunities and research trends. *Journal of nanoparticle research*, *16*(6), p.2464.

Ru, Q., Wang, Y., Lee, J., Ding, Y. and Huang, Q., 2012. Turbidity and rheological properties of bovine serum albumin/pectin coacervates: Effect of salt concentration and initial protein/polysaccharide ratio. *Carbohydrate Polymers*, *88*(3), pp.838-846.

Santos, M.B., de Carvalho, C.W.P. and Garcia-Rojas, E.E., 2018. Heteroprotein complex formation of bovine serum albumin and lysozyme: Structure and thermal stability. *Food hydrocolloids*, *74*, pp.267-274.

Schmitt, C. and Turgeon, S. L., 2011. Protein/polysaccharide complexes and coacervates in food systems. Advances in colloid and interface science 167, 63-70.

Shaddel, R., Hesari, J., Azadmard-Damirchi, S., Hamishehkar, H., Fathi-Achachlouei, B. and Huang, Q., 2018. Use of gelatin and gum Arabic for encapsulation of black raspberry anthocyanins

by complex coacervation. *International journal of biological macromolecules*, *107*, pp.1800-1810.

Shevkani, K., Singh, N., Kaur, A., Rana, J., 2015. Structural and functional haracterization of kidney bean and field pea protein isolates: a comparative study. *Food Hydrocolloids* 43, 679 -689.

Shishir, M.R.I., Xie, L., Sun, C., Zheng, X. and Chen, W., 2018. Advances in micro and nano-encapsulation of bioactive compounds using biopolymer and lipid-based transporters. *Trends in Food Science & Technology*, *78*, pp.34-60.

Souza, C.R., Oliveira, H.R., Pinheiro, W.M., Biswaro, L.S., Azevedo, R.B., Gomes, A.J. and Lunardi, C.N., 2015. Gold nanoparticle and berberine entrapped into hydrogel matrix as drug delivery system. *Journal of Biomaterials and Nanobiotechnology*, *6*(01), p.53.

Souza, C.J. and Garcia-Rojas, E.E., 2017. Interpolymeric complexing between egg white proteins and xanthan gum: Effect of salt and protein/polysaccharide ratio. *Food Hydrocolloids*, *66*, pp.268-275.

Spotti, M.J., Martinez, M.J., Pilosof, A.M., Candioti, M., Rubiolo, A.C. and Carrara, C.R., 2014. Influence of Maillard conjugation on structural characteristics and rheological properties of whey protein/dextran systems. *Food Hydrocolloids*, *39*, pp.223-230.

Stone, A.K., Cheung, L., Chang, C. and Nickerson, M.T., 2013. Formation and functionality of soluble and insoluble electrostatic complexes within mixtures of canola protein isolate and (κ-, ι- and λ-type) carrageenan. *Food research international*, *54*(1), pp.195-202.

Sun, C., Liu, R., Liang, B., Wu, T., Sui, W. and Zhang, M., 2018. Microparticulated whey protein-pectin complex: A texture-controllable gel for low-fat mayonnaise. *Food Research International*, *108*, pp.151-160.

Swanevelder, C.J., 1998. Bambara–food for Africa. *National Department of Agriculture, Government Printer, Republic of South Africa*.

Takahashi, H., Tahara, Y., Sawada, S.I. and Akiyoshi, K., 2013. Cationic amphiphilic polysaccharide nanoballs: protein stabilization and intracellular delivery by nano-encapsulation. *Biomaterials Science*, *1*(8), pp.842-849.

Teng, Z., Luo, Y. and Wang, Q., 2013. Carboxymethyl chitosan–soy protein complex nanoparticles for the encapsulation and controlled release of vitamin D3. *Food chemistry*, *141*(1), pp.524-532.

Timilsena, Y. P., Adhikari, R., Barrow, C. J., Adhikari, B., 2016. Microencapsulation of chia seed oil using chia seed protein isolate chia seed gum complex coacervates. *International journal of biological macromolecules* 91, 347-357.

Timilsena, Y.P., Akanbi, T.O., Khalid, N., Adhikari, B. and Barrow, C.J., 2018. Complex coacervation: Principles, mechanisms and applications in microencapsulation. *International journal of biological macromolecules*.

Timilsena, Y.P., Wang, B., Adhikari, R. and Adhikari, B., 2017. Advances in microencapsulation of polyunsaturated fatty acids (PUFAs)-rich plant oils using complex coacervation: A review. *Food Hydrocolloids*, *69*, pp.369-381.

Tran, T. and Rousseau, D., 2013. Stabilization of acidic soy protein-based dispersions and emulsions by soy soluble polysaccharides. *Food Hydrocolloids*, *30*(1), pp.382-392.

United Nations. (2017). World Population Prospects: The 2017 Revision, Key Findings and 743 Advance Tables. Working Paper No. ESA/P/WP/248. Department of Economic and 744 Social Affairs, Population Division. New York. Retrieved from 745 https://esa.un.org/unpd/wpp/publications/files/wpp2017_keyfindings.pdf [Accessed 5 November 2019]

Ursu, A.-V., Marcati, A., Sayd, T., Sante-Lhoutellier, V., Djelveh, G., Michaud, P., 2014. Extraction, fractionation and functional properties of proteins from the microalgae Chlorella vulgaris. *Bioresource technology* 157, 134-139.

Vasconcelos, I. M., Maia, F. M., Farias, D. F., Campello, C. C., Carvallo, C.A. F., de Azevedo Moreira, R., de Oliveira, J. T., 2010. Protein fractions, amino acid composition and antinutritional constituents of high-yielding cowpea cultivars. *Journal of Food Composition and Analysis* 23, 54-60.

Voragen, A.G., Coenen, G.J., Verhoef, R.P. and Schols, H.A., 2009. Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry*, *20*(2), p.263.

Vurayai, R., Emongor, V. and Moseki, B., 2011. Physiological responses of bambara groundnut (Vigna subterranea L. Verdc) to short periods of water stress during different developmental stages. *Asian Journal of Agricultural Sciences*, *3*(1), pp.37-43.

Wang, Q. and Schlenoff, J.B., 2014. The polyelectrolyte complex/coacervate continuum. *Macromolecules*, *47*(9), pp.3108-3116.

Wang, W., Chen, W., Zou, M., Lv, R., Wang, D., Hou, F., Feng, H., Ma, X., Zhong, J., Ding, T. and Ye, X., 2018. Applications of power ultrasound in oriented modification and degradation of pectin: A review. *Journal of Food Engineering*, *234*, pp.98-107.

Wang, W., Liu, F. and Gao, Y., 2016. Quercetagetin loaded in soy protein isolate–κ-carrageenan complex: Fabrication mechanism and protective effect. *Food research international*, *83*, pp.31-40.

Wani, I.A., Sogi, D.S. and Gill, B.S., 2015. Physico-chemical and functional properties of native and hydrolysed protein isolates from Indian black gram (Phaseolus mungo L.) cultivars. *LWT-Food Science and Technology*, *60*(2), pp.848-854.

Wasula, S.L., Wakhungu, J. and Palapala, V.A., 2014. Farmers' perceptions on adoption of Bambara nut production as a food security crop in Kakamega County, Kenya.

Wee, M.S., Nurhazwani, S., Tan, K.W., Goh, K.K., Sims, I.M. and Matia-Merino, L., 2014. Complex coacervation of an arabinogalactan-protein extracted from the Meryta sinclarii tree (puka gum) and whey protein isolate. *Food hydrocolloids*, *42*, pp.130-138.

Weinbreck, F.C.J., 2004. *Whey-protein/polysaccharide coacervates: structure and dynamics* (Doctoral dissertation).

Williams, P.A. and Phillips, G.O., 2009. Gum arabic. In *Handbook of hydrocolloids* (pp. 252-273). Woodhead Publishing.

Xue, F., Li, C., Zhu, X., Wang, L. and Pan, S., 2013. Comparative studies on the physicochemical properties of soy protein isolate-maltodextrin and soy protein isolate-gum acacia conjugate prepared through Maillard reaction. *Food research international*, *51*(2), pp.490-495.

Yagoub, A.A., Ma, H. and Zhou, C., 2017. Ultrasonic-assisted extraction of protein from rapeseed (Brassica napus L.) meal: Optimization of extraction conditions and structural characteristics of the protein. *International Food Research Journal*, *24*(2).

Yin, B., Deng, W, Xu, K, Huang, L., Yao, P., 2012. Stable nano-sized emulsions produced from soy protein and soy polysaccharide complexes. *Journal of colloid and interface science* 380, 51-59.

Yuan, Y., Kong, Z.Y., Sun, Y.E., Zeng, Q.Z. and Yang, X.Q., 2017. Complex coacervation of soy protein with chitosan: Constructing antioxidant microcapsule for algal oil delivery. *LWT-Food Science and Technology 75*, pp.171-179.

Yusuf, A., Ayedun, H., Sanni, L. O., 2008. Chemical composition and functional properties of raw and roasted Nigerian benniseed (Sesamum indicum) and bambara groundnut (Vigna subterranean). *Food Chemistry* 111, 277-282.

Zhang, Y., Tan, C., Abbas, S., Karangwa, E., Xia, S., Zhang, X., 2015. Modified SPI improves the emulsion properties and oxidative stability of fish oil microcapsules. *Food Hydrocolloids* 51, 108-117.

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Fractionation pH of bambara groundnut (*Vigna subterranea*) protein impacts the degree of complexation with gum arabic

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ABSTRACT

Bambara groundnut is a protein-rich legume of African origin. This study investigated the influence of fractionation pH on degree of complexation of bambara protein isolate (BPI) with gum arabic (GA). BPI extracted at pH 2, pH 7, pH 9 and by the traditional salt solubilisation method were complexed with GA. BPI-GA complexes appeared as spherical particles (average size: 100–200 nm) that were aggregated. Optimal pHs of biopolymer interactions decreased (pH_{opt}: 4.8 to 2.9) as protein extraction pH became more acidic. pH 2 fraction had the lowest coacervates yield (41%). ζ-potential profiles of protein fractions displayed similar pH dependent patterns, but with different pHs of net neutrality. The pH 2 fraction showed a high molecular weight protein (100 kDa), which was absent in other fractions. It also contained the highest content of basic amino acids. Protein extraction pH could be manipulated to produce better acid-stable structures on complexation with polysaccharides.

1. Introduction

Proteins and polysaccharides are widely distributed in nature and these polymers have been employed in various food applications for the development of novel ingredients. Protein-polysaccharide interactions are important in structure formation and controlling the physical and functional properties of foods. Other areas of protein-polysaccharide applications include emulsion stabilization, fat mimetics and meat replacers (Yin, Deng, Xu, Huang, & Yao, 2012). Recently, there is a growing interest in understanding protein-polysaccharide complex coacervation as well as the application of the formed protein-polysaccharide complexes in encapsulation and controlled delivery of bioactive compounds.

Schmitt and Turgeon (2011) defined coacervation as phase separation of a mixture system into biopolymer-rich and biopolymer-deficient phases. Coacervation can be simple or complex. Simple coacervation involves one biopolymer and phase separation is facilitated through salting out mechanism. Complex coacervation, on the other hand, involves the interactions between two oppositely charged biopolymers to yield a biopolymer rich phase, which is the complexes or coacervates and a polymer poor region, which is the equilibrium solution (Schmitt & Turgeon, 2011). A mixture of protein and polysaccharide in solution may result in either complexation, precipitation or segregative phase separation depending on several factors including environmental pH, ionic strength and charge density of the biopolymers (Elmer, Karaca, Low, & Nickerson, 2011; Esfanjani & Jafari, 2016). Among these factors, the solution pH is important in protein-polysaccharide complex formation as this impacts on the charge configuration of the biopolymers. Biopolymers carrying opposite charges will tend to attract each other, resulting in protein-polysaccharide complexes; whilst those of equal charge will repel, resulting in segregation or phase separation (Liu, Cao, Ghosh, Rousseau, Low & Nickerson, 2009).

Most research on protein-polysaccharide complexation mainly focused on understanding the mechanism of interaction between animal proteins, particularly those derived from milk (casein, whey, β-lactoglobulin) and polysaccharides (gum arabic, chitosan, pectin) (Bustos-Garza, Yanez-Fernandez, & Barragan-Huerta, 2013; Jones, Decker, & McClements, 2009; Shah, Davidson, & Zhong, 2012). The growing consumer demands for natural products has prompted studies on complexation of plant proteins with polysaccharides, hence legume proteins from soya and peas have been explored (Elmer et al., 2011; Yin et al., 2012; Zhang et al., 2015). So far, the traditional alkaline solubilisation, followed by isoelectric precipitation and dialysis recovery is the most common method used to extract plant proteins intended for complextion with polysaccharides (Klemmer, Waldner, Stone, Low, & Nickerson, 2012; Zhang et al., 2015). Optimum complexation between these proteins and polysaccharides have been generally observed within a narrow pH range: 3.5–4.0 (Aryee & Nickerson, 2014; Timilsena, Adhikari, Barrow, & Adhikari, 2016). This could limit the

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