Beyond a
Subsistence Crop

Structure and functionality
of Bambara groundnut proteins

Claudine Florett Diedericks

Propositions

1.   Vicilin, the major storage protein in Bambara groundnut seeds, controls the gelation behaviour of the protein isolates.
        (this thesis)

2.   Soaking and roasting can be both beneficial and detrimental to Bambara groundnut protein functionality.
        (this thesis)

3.   Making major crops readily available hampers food security.

4.   The wording in relation to the scholarly record by the editors and publisher of *Aging, Neuropsychology and Cognition*, disputes the purpose of a retraction statement as put forward by Dougherty (M.V. Dougherty, 2017. Metaphilosophy, 48, 258–283).

5.   Collaboration is an art form negated by necessity.

6.   The tip of the iceberg in academia and stuttering is governed by either fear or self-actualization.

Propositions belonging to the thesis, entitled

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Structure and functionality of Bambara groundnut proteins'

Claudine Florett Diedericks
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Thesis

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General Introduction

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1.1.   Introduction

Protein transition is a term which is increasingly advocated as we come to realise the importance of shifting from a largely animal-based protein diet to one rich in plant proteins. Rooted in the advancement of food security and sustainability as prominent focus areas of the United Nations 2030 Agenda for Sustainable Development (United Nations, 2015), several drivers of the protein transition have been highlighted. These include amongst others the reduction of environmental impact, meeting the protein demands of a growing population to eradicate hunger, establishing protein systems which are resilient in light of climate change, and addressing public health through dietary intervention (Pyett, de Vet, Trindade, van Zanten, & Fresco, 2019). When considering these aspects of the protein transition, it has been recognised that leguminous crops and especially those considered as pulses, play an important role. Leguminous crops form part of the plant family Leguminosae and are generally divided into two groups, i.e. (i) oilseeds which refer to those crops primarily cultivated for oil extraction, and (ii) pulses which are exclusively harvested for their edible dry seeds (Tiwari, Gowen, & McKenna, 2011). In terms of commercialised legume protein ingredients, soybeans which are considered as oilseeds are the most widely used and well-characterised for their functionality in food systems (Nishinari, Fang, Guo, & Phillips, 2014; Thrane, Paulsen, Orcutt, & Krieger, 2017). In recent years however, the need to establish a wider net of alternative legume protein sources have been recognised to not only secure a wider food supply, but also to limit the large-scale deforestation associated with the cultivation of soy crops (Nadathur, Wanasundara, & Scanlin, 2017). To that end, pea can be considered as the largest non-soy protein alternative (Stone, Wang, Tulbek, & Nickerson, 2019), whilst there are many other pulse crops which are of increasing interest. One such a crop is Bambara groundnut [BGN], which was further investigated as a novel plant protein source in this thesis.

1.2.   Bambara groundnut—potential beyond a subsistence crop

1.2.1.   *Cultivation practices and seed characteristics*

Bambara groundnut (*Vigna subterranea* (L.) Verdc.), as the name implies, is a pulse crop of which the fruit (pod) development occurs below the soil surface. The pods contain one to two seeds, with varietal differences mostly based on the colour of

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the seeds (see Fig. 1.1). Although originating in West Africa, BGN is also cultivated in other parts of sub-Saharan Africa and Southeast Asia, albeit as primarily a subsistence crop in rural areas (Feldman, Ho, Massawe, & Mayes, 2019; Van Wyk & Gericke, 2017). The average yields for BGN seeds as recorded in 2017 were 715 kg/ha, whereas yields for peas and soybeans were almost three (1990 kg/ha) to four (2854 kg/ha) times higher (FAO, 2019). Considering the many advantages of BGN, research efforts are nowadays geared towards highlighting the crop as a viable agricultural commodity as a means of addressing food security and nutrition-related aspects. One major advantage of this crop, is its ability to produce reasonable yields under extreme climatic conditions such as droughts. Under various levels of drought stress (mild to severe), it has been shown that BGN exhibits all three indicators for drought-tolerance, i.e. tolerance, escape and avoidance mechanisms. Drought tolerance refers to the ability of plants to resist dehydration by means of osmotic adjustment and protein stabilisation. Drought escape refers to the rapid development and reproduction of plants before the onset of severe drought conditions, whilst drought avoidance refers to the increased water-use efficiency of plants through increased root growth, vegetative growth limitation or a reduction in transpiration (Feldman et al., 2019; Kooyers, 2015). These are important traits in the current context of climate change, where climate-resilient crops with the ability to adapt to increasing heat stresses are becoming of increased importance in our agricultural systems (Calles, Xipsiti, & del Castello, 2019). Furthermore, as a legume crop BGN is able to fixate nitrogen and as such improve soil fertility, whilst it also performs well in intercropping systems with local staple cereals (Cleasby, Massawe, & Symonds, 2016).

Fig. 1.1. Bambara groundnut seed varieties; differentiated on seed coat colour.

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In terms of the nutritional composition of its seeds, BGN is often described as a complete food comparing favourably to other seeds in the *Vigna* genus, as well as to those of soybean (*Glycine max*) and pea (*Pisum sativum* L.) as shown in Table 1.1. Although varying levels of total protein are often recorded between different studies, BGN seeds are still considered as a high-quality protein source being rich in some essential amino acids (Adebowale, Schwarzenbolz, & Henle, 2011; Yao et al., 2015). It is therefore recognised that BGN can contribute to dietary diversification, especially in the local cultivation areas where child malnutrition still prevails. Here it should be noted that strategies to overcome certain limiting factors which hampers the utilisation of BGN as a preferred food, such as the hard-to-cook properties of the seeds and the low social perception of BGN as a "women's crop" which differentiates it from the major (export) cereal crops generally produced by men, are important to strengthen the position of this crop in the local context (Feldman et al., 2019; Mubaiwa, Fogliano, Chidewe, & Linnemann, 2017).

Table 1.1. Nutritional composition of Bambara groundnut in comparison to other legume seeds.

	Bambara groundnut[a]	**Cowpea**[b]	**Soybean**[c]	**Pea**[d]
	(g/100 g)			
Protein	24.0–27.3	22.4–26.3	32.5–36.5	21.6–23.1
Carbohydrates	55.8–61.7	65.9–69.0	30.2–31.3	61.2–68.4
Fibre	3.4–3.7	3.4–3.8	8.6–9.3	2.1–4.7
Fat	5.3–6.6	1.4–2.1	14.9–19.9	0.9–2.4
Ash	3.0–3.8	2.8–3.3	4.8–4.9	2.8–3.7

Data sources: [a] (Arise, Amonsou, & Ijabadeniyi, 2015; Kaptso et al., 2015), [b] (Khattab, Arntfield, & Nyachoti, 2009), [c] (Amonsou, Taylor, & Minnaar, 2011; USDA, 2019), [d] (Khattab et al., 2009; Ma et al., 2011).

1.2.2. *Bambara groundnut protein functionality*

The inherent characteristics of BGN seeds makes the crop an ideal candidate as a novel plant protein source, when considering aspects of the protein transition. In recent years, many studies have focussed on the extraction and functionality characterisation of BGN proteins; in some instances reporting similar properties to that of commercialised (soy) plant proteins. Functional properties which are mostly investigated for BGN proteins relates to foaming and emulsification, whilst water and

oil absorption capacities and the minimum protein concentration required to form a solid-like material have also been reported. An overview of some functional properties of BGN proteins are shown in Table 1.2. When comparing the different studies, some general commonalities can be identified. Firstly, in studies where various protein extraction methods were employed, the observed functionalities within the same varieties were reportedly different. This is to be expected, considering that pulse protein constituents are characterised by molecular differences with different sensitivities to extraction conditions, i.e. changes in environmental conditions (Tiwari et al., 2011). It is therefore recognised that knowledge on a protein's molecular structure (also other physicochemical properties) and its interaction to the environment, is essential to establish the link to the observed functionalities—known as the structure-function relationship (Nadathur et al., 2017). This structure-function characterisation thus becomes important when establishing novel proteins as food ingredients.

As also identified from the BGN literature, there remains a gap between linking the observed macroscopic functionality to the molecular and mesoscopic structural properties of BGN proteins. This is especially evident in the gelation characterisation of BGN proteins, which up till now was only investigated in terms of the minimum concentration where a gel could be visually observed. For this reason, gelation characterisation linked to the structural properties of BGN proteins formed an integral part of this thesis.

1.3. **Plant proteins extraction and gelation functionality**

Plant proteins which are considered as food ingredients are primarily obtained from the storage proteins, which are stored in protein bodies within seed cells. The storage proteins can be categorised into different groups according to several classification systems, although the Osborne classification system which is based on solubility still remains the most widely used (Nadathur et al., 2017; Shewry & Casey, 1999). According to this system four groups can be identified, i.e. (i) Albumins—these are low molecular weight (M_w) proteins with a high solubility in water, (ii) Globulins—proteins soluble in dilute salt solutions which are characterised by their high M_w comprising multi-subunit molecules, (iii) Prolamins—these proteins are soluble in aqueous ethanol solutions, and (iv) Glutelins—alkali extractable proteins. The most abundant proteins in legume seeds are the globulins, which are further classified as the 7S (vicilin) or 12S (legumin) proteins based on their sedimentation coefficients.

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Table 1.2. Functional properties of Bambara groundnut proteins.

Functional property	Extraction method
Solubility	(i) Isoelectric precipitation [IEP] (pH 12, pH 4.5), 85.2% protein content
	(ii) IEP (pH 10, pH 4.5) with/without heat treatment and EDTA, 84–87% protein content
Emulsification	(i) IEP (pH 10, pH 4.5) with/without heat treatment and EDTA, 84–87% protein content
	(ii) IEP (pH 8, pH 4) 78–80% protein content, or salt solubilisation aqueous extraction (0.5 M NaCl) 55–59% protein content
	(iii) IEP (pH 9, pH 5) 90–91% protein content, or salt solubilisation extraction (0.8 M NaCl) 75–78% protein content
Foaming	(i) IEP (pH 8, pH 4) 78–80% protein content, or salt solubilisation aqueous extraction (0.5 M NaCl) 55–59% protein content
	(ii) IEP (pH 9, pH 5) 90–91% protein content, or salt solubilisation extraction (0.8 M NaCl) 75–78% protein content
Gel formation	(i) IEP (pH 9, pH 5) 90–91% protein content or salt solubilisation extraction (0.8 M NaCl) 75–78% protein content
	(ii) IEP (pH 4) extraction without solubilisation, protein content not reported

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Main findings	Reference
o Highest solubility at pH 10 (81%) and 0.45 M NaCl (65%) o Lowest solubility in pH range 4–5 and without added salt	(i) (Kudre, Benjakul, & Kishimura, 2013)
o Highest solubility (85% in deionised water, 89% in 0.45 M NaCl) in presence of EDTA and extraction temperature of 70°C	(ii) (Kudre & Benjakul, 2014)
o Highest emulsifying activity observed at lowest BGN protein concentrations, in presence of EDTA and 70°C extraction o Emulsion stability index increased at increasing protein concentrations	(i) (Kudre & Benjakul, 2014)
o Emulsifying activity and stability highest for salt solubilised proteins in comparison to IEP extracted proteins	(ii) (Arise et al., 2015)
o Salt solubilised proteins have overall higher emulsifying activity and stability compared to IEP extracted proteins	(iii) (Adebowale et al., 2011)
o Higher foam capacity and stability observed at acidic pH for salt solubilised proteins in comparison to IEP extracted proteins	(i) (Arise et al., 2015)
o Higher foam stability observed for proteins obtained via salt solubilisation	(ii) (Adebowale et al., 2011)
o Minimum gelling concentration observed at 8% w/v for IEP proteins and 6% w/v for salt solubilised proteins	(i) (Adebowale et al., 2011)
o Minimum gelling concentrations ranged from 13–20% w/v	(ii) (Adeleke, Adiamo, & Fawale, 2018)

The vicilin proteins are trimeric proteins (M_w around 150–170 kDa) which are devoid of disulphide bonds, whilst the legumin proteins are hexamers (M_w around 300–400 kDa) which are characterised by disulphide linked subunits (Derbyshire, Wright, & Boulter, 1976; Duranti & Gius, 1997). It is therefore also these proteins which form the bulk of the protein constituents when extracted from the seeds.

1.3.1. *Fractionation techniques for extraction of plant protein ingredients*

There are various techniques employed for the extraction of proteins from legume seeds, with most classified as either wet or dry fractionation techniques. Classified under the former, is the isoelectric precipitation method which is the most commonly used for the extraction of commercial protein isolates. This method is based on the solubilisation of proteins under alkaline conditions, followed by isoelectric precipitation which is generally in a range between pH 4–5 for pulse proteins. The majority of proteins extracted with this method are the vicilin and legumin proteins, which are characterised by varying protein content depending on the source and processing conditions (Arntfield & Maskus, 2011; Boye, Zare, & Pletch, 2010). Such proteins are generally characterised as either protein concentrates (protein content between 65–80%) or protein isolates (protein content higher than 90%) (Stone et al., 2019), although in many studies these terms are also interchangeably used. By modifying the isoelectric precipitation method through the inclusion of membrane separation techniques and/or salt solubilisation steps, the various fractions of the storage proteins, i.e. albumins, legumins and vicilins, can be successfully isolated (Rubio et al., 2014). Other wet fractionation techniques which are considered as mild fractionations, include the aqueous extraction method which are based on solubilisation in water. As reported for yellow pea, aqueous extraction can yield protein fractions with protein contents between 46–57% (Kornet et al., 2019; Pelgrom, Boom, & Schutyser, 2015).

Dry fractionation techniques refer mostly to the combination of milling and air classification to obtain protein- and starch-enriched fractions. Air classification techniques as a means of processing pulse crops received wide interest in the '70s and '80s, with renewed recognition in recent years as sustainable techniques being less resource- and energy-intensive in comparison to wet fractionation processes (Arntfield & Maskus, 2011; Schutyser, Pelgrom, van der Goot, & Boom, 2015). Milling is used as a size reduction technique to liberate cellular components and in the process separate the starch granules (size range for various legume species 4–85 µm) from the protein bodies (size range 0.1–25 µm) (Assatory, Vitelli, Rajabzadeh,

& Legge, 2019; Do & Singh, 2019). The resultant flour after milling is subjected to air classification, where based on size and/or density differences, the smaller protein-enriched fractions are separated from the larger starch-enriched fractions in a circulating stream of air. Such protein-enriched fractions have a typical protein content in the range of 30–60% (Schutyser et al., 2015; Stone et al., 2019). Based on the current literature, it is recognised that dry fractionation processes are increasingly investigated in the context of an alternative to wet fractionation. However, such techniques are not yet suited to the production of high-purity protein isolates, which are often highlighted as being important to establish the structure-function relationship of (novel) proteins.

1.3.2. *Gelling behaviour of plant proteins*

Gelation is a phenomenon which is important to the formation of distinct textural properties observed in many foods such as gelatine desserts, yoghurts and boiled eggs. Recognised as a complex process encompassing several aspects, gel formation can be simplistically defined as the formation of a 3D continuous gel network which is characterised by the conversion of a fluid to a solid (Foegeding, 2015; Van der Linden & Foegeding, 2009). Protein gel formation can be induced by physical (e.g. heat or pressure) or chemical (e.g. acid or enzymatic) means, with heat-induced gelation being the most commonly applied for globular plant proteins. The heat-induced gelation of proteins is a multi-step process, which involves dissociation and unfolding of the native structures, followed by association and further aggregate formation into a gel network under specific environmental conditions (Arntfield & Maskus, 2011; Clark, Kavanagh, & Ross-Murphy, 2001; Totosaus, Montejano, Salazar, & Guerrero, 2002). The gelling behaviour of soy proteins have been extensively studied, as it was recognised that an understanding of the gelation functionality as influenced by the structural properties of soy proteins, would facilitate their use in food systems (Nakamura, Utsumi, & Mori, 1986). Today, commercialised soy proteins as well as pea proteins can be applied as gelling agents in many food systems, as a large body of knowledge is available on the gelling behaviour of the protein isolates and their different globulin fractions. Here, the importance of the compositional and structural properties of proteins in relation to gelation functionality is again highlighted. As shown for example by O'Kane, Vereijken, Gruppen, & Van Boekel (2005) for pea protein isolates, gel strengths as determined by rheological measurements differed significantly for protein isolates from different cultivars. This was attributed to the legumin fractions which had different disulphide bonding ability, whilst the higher presence of the vicilin α subunits in some cultivars resulted in stronger repulsive

forces and therefore weaker gels. When investigating the gel formation of novel plant proteins, it thus remains important to understand the molecular composition of the proteins in relation to their gelling behaviour, beyond the often empirically reported least or minimum gelling concentrations.

Considering again the protein transition, the (partial) replacement of animal proteins with plant proteins without negatively affecting functionality, is a research area of continued interest. Whey protein concentrates (≥25% protein content) and whey protein isolates (>90% protein content) are dairy proteins obtained from whey or milk serum after precipitation of caseins from milk; consisting of different fractions with β-lactoglobulin (M_w ranging between 18.4–36.9 kDa) and α-lactalbumin (M_w 14.2 kDa) forming the major components (Farrell Jr. et al., 2004; Kilara & Vaghela, 2018). Whey proteins find wide application as low cost functional food ingredients and are especially recognised for their gelation functionality (Havea, Watkinson, & Kuhn-Sherlock, 2009; Morr & Ha, 1993; Steventon, Gladden, & Fryer, 1991). It has been recognised that the combination of whey proteins with plant proteins offers novel structuring and nutritional possibilities to foods compared to the single protein systems. As such, there are many studies focussing on the heat-induced gelation of whey proteins in admixture with soy proteins (Comfort & Howell, 2002; Jose, Pouvreau, & Martin, 2016; McCann, Guyon, Fischer, & Day, 2018; Roesch & Corredig, 2005), whilst the combination with other legume proteins are limited to pea (Wong, Vasanthan, & Ozimek, 2013) and rapeseed (Ainis et al., 2019; Ainis, Ersch, & Ipsen, 2018). From these studies, the parameters of importance can be narrowed down to the concentrations at which the single proteins have been added to the mixture (mixing ratios) and changes in the environmental conditions (pH, ionic strength). When mixing soy and whey proteins for example at concentration ratios of 6% to 6% and 11.2% to 4.8%, similar gelation kinetics and final gel strengths were observed which were comparable to the single 6% whey protein gels. The microstructure of these gels were however different, appearing more homogeneous for single whey proteins whilst the addition of soy had a coarsening effect (McCann et al., 2018). Overall, it appears that whey proteins are mainly responsible for the gel network formation when mixed with most plant proteins, whilst the latter are evenly distributed within this major gel network. Finally, the vast knowledge available on the gelation behaviour of (soy) plant proteins, offers a valuable basis from which to investigate the gelation of BGN proteins beyond the currently known least gelation concentrations.

1.4.   Aim and outline of this thesis

Bambara groundnut is a pulse crop which despite its undervalued standing, is continually recognised for its many benefits as a viable crop to address current and future-related food concerns. An area which has received increasing research interest, is that of value-addition to the crop through formulation of BGN products and ingredients, at laboratory scale. Commercialisation is however hampered due to several factors, of which the lack of knowledge between the structural aspects of BGN fractions and their observed macroscopic functionality can be considered as one. Hence, the aim of this thesis was to gain insight into this structure-function relationship, by investigating the gel formation of BGN proteins obtained from different processing streams. Through this approach, we could identify the interplay between the effects of processing on the structural and physicochemical properties of BGN proteins and their resultant gelation functionality.

In **Chapter 2**, extraction processes were optimised to obtain the high purity vicilin protein fractions, which are the known major storage proteins present in pulse seeds. These fractions were characterised for their physicochemical and structural properties, with valuable techniques employed such as size-exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS), which provided detailed information on the molecular weight and size characteristics. The gelling behaviour of BGN vicilin fractions were further investigated in terms of their rheological responses and gel microstructures, whilst also linking to scaling models as a means of describing the resultant gel networks. The same approach was also applied in **Chapter 3**, where the proteins of interest were now the so-called BGN protein isolates as obtained with the commonly utilised isoelectric precipitation method. In general, the protein isolates are considered as a mixture of proteins with a lower purity in comparison to the vicilin fractions. In addition, the applicability of mild fractionation techniques to obtain protein-enriched flour fractions were also explored. In **Chapter 4**, the synergistic enhancement of BGN protein isolates in combination with whey protein isolates was investigated, as a means of determining the potential of BGN proteins as a partial animal protein replacer. The rheological responses and gel microstructures of the mixed gels were determined as a function of pH and mixing ratios. **Chapter 5** addressed the effect of pre-treatments which are commonly applied in practice to overcome negating factors such as the hard-to-cook and hard-to-mill properties of the seeds. The effect of soaking and/or roasting on the seed microstructures and the composition of their resulting flour and protein isolate fractions were investigated. Here, SEC-MALLS and microscopic techniques (cryo-scanning electron microscopy and confocal laser scanning microscopy) were

valuable tools in identifying the differences between treatments. Finally in **Chapter 6**, an overview of the main findings of this thesis are presented on the basis of the interplay between processing, structure and functionality; whilst also linking to the observed challenges and considerations for further research.

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2

Extraction, gelation and microstructure of Bambara groundnut vicilins

2

Abstract

Nowadays there is a growing interest in exploiting new sources of plant proteins as functional ingredients in food products. In recent years, Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] has been explored as such a potential plant protein source, as a means of value-addition to this leguminous crop. To elucidate on the macroscopic functionality of BGN protein isolates, the focus of our study was on the extraction and characterisation of the vicilin protein fraction as the known major storage protein present in legume seeds. BGN vicilin had a high protein content (91%) and formed the largest component in relation to the other protein fractions. Together with molecular weight profiles obtained with gel electrophoresis and size-exclusion chromatography coupled with light scattering, the purity of vicilin and its presence as the predominant protein fraction in BGN black-eye seeds was confirmed. The isoelectric point (pH 5.3), solubility profile (highest solubility 86% at NaCl concentrations above 200 mM) and thermal denaturation temperature (92°C) of BGN vicilin correspond to the range reported for other legume vicilins. Furthermore, the gelation behaviour of BGN vicilin gels were investigated using dynamic oscillatory measurements. These data were further analysed with scaling models, which revealed that fractal scaling was best suited for description of the BGN vicilin gel networks. The gel microstructures were visualised with confocal laser scanning and scanning electron microscopy.

2.1. Introduction

The world's population is expected to increase to 9.8 billion by 2050 and 11.2 billion by 2100 (United Nations, 2017) and as such the demand for highly nutritious foods will also increase. Plant proteins are more sustainable and cost-effective compared to animal proteins and for that reason there is a growing interest in exploiting new sources of plant proteins as functional ingredients in food products (Adebowale, Schwarzenbolz, & Henle, 2011; Nadathur, Wanasundara, & Scanlin, 2016). Legume seeds accumulate large amounts of proteins during their development and are therefore considered as promising protein sources (Duranti & Gius, 1997).

Bambara groundnut (BGN) is a legume that is still quite unknown and underutilised as a functional ingredient in food products. It is extensively grown throughout Africa and in some parts of Asia, Northern Australia, and Central and South America. BGN is a tough crop which can grow under harsh conditions such as drought and low soil fertility (Arise, 2016; Baryeh, 2001). It has a high protein content (18–27%) which is equal or even higher compared to other legumes and it is rich in essential amino acids, hence it can enhance the nutritional value of food products (Kaptso et al., 2015; Yao et al., 2015). One of the reasons the crop remains underutilised in food applications is due to a lack of information on the functional properties of its ingredients and their underlying interactive mechanisms. Considering the continued strive towards energy-efficient production of plant enriched fractions with native functionality (Schutyser & Van der Goot, 2011), it would be attractive to obtain the minimally processed fractions of BGN as a means of sustainably exploiting the crop. For this reason, knowledge of the interactions between BGN fractions are of importance as a means of creating new possibilities for their usage. Hereto it is necessary to first characterise and understand the behaviour of individual components, which makes the investigation of the pure BGN protein fractions of interest.

Previous research focussed amongst others on the physicochemical and gelation characteristics of BGN protein isolates extracted with the isoelectric precipitation method. It was shown that BGN proteins are able to form a gel upon heating (Adebowale et al., 2011; Arise, 2016). Gelation is an important phenomenon responsible for the formation of distinct textures characteristic of many foods such as cheeses, boiled eggs and jams. Protein gelation not only provides a desirable texture, structure or stability to food products, but it also makes it possible to entrap other food ingredients such as (flavour-containing) oil droplets in its matrix, which contribute to sensory attributes (Renkema, 2001). It is for this reason that the investigation of the main gelling mechanisms of biopolymer systems form the basis

towards understanding how structure formation at the molecular scale determines functionality at the macroscopic scale. Such understanding enables the use of individual components and mixtures in novel ways (Ersch, 2015; Norton & Frith, 2001).

Vicilin proteins are considered as the major storage proteins in legume seeds and are known for their ability to form gels. As highlighted in several studies (Bora, Brekke, & Powers, 1994; Mession, Chihi, Sok, & Saurel, 2015; Mession, Sok, Assifaoui, & Saurel, 2013; Mohamad Ramlan et al., 2004; Nakamura, Utsumi, & Mori, 1986; Tang, 2008), knowledge of the thermal and gelation behaviour of vicilins from various legumes are of importance to facilitate their use as food ingredients. Depending on their source, descriptive names for vicilin are often assigned such as β-conglycinin from soybean (*Glycine max*) and phaseolin from kidney bean (*Phaseolus vulgaris*), whereas those from emerging legume crops are simply referred to as vicilins (Rangel, Domont, Pedrosa, & Ferreira, 2003; Tang & Ma, 2009). The structure of vicilins can exhibit considerable variation due to post-translational processing and differences in agronomic and environmental factors, which results in differences in their physicochemical and functional properties (Maruyama et al., 1999; Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, & Blecker, 2012; Shewry, Napier, & Tatham, 1995; Tandang-Silvas, Tecson-Mendoza, Mikami, Utsumi, & Maruyama, 2011). Vicilins are also thought to be the major proteins present in BGN seeds, however only one study according to our knowledge have focussed on the extraction of these vicilin-like fractions, without establishing the purity (i.e. vicilin free from contaminating proteins such as legumin) and without functionality characterisation (Arise, Nwachukwu, Aluko, & Amonsou, 2017). Hence, the aim of our study was to extract pure vicilin proteins whilst establishing their presence as major protein fractions in BGN black-eye seeds, followed by physicochemical and gelation functionality characterisation.

2.2. Materials and Methods

2.2.1. *Materials*

Bambara groundnut [BGN] seeds were obtained from Thusano Products (Louis Trichardt, Limpopo, South Africa). The black-eye variety was selected and screened for defects and foreign materials. After sorting the seeds were dehulled (combination of automatic and manual dehulling), coarse-milled into grits with a pin mill (Condux-Werk LV 15M, Wolfgang bei Hanau, Germany) followed by fine-milling with a rotor mill (Fritsch GmbH Pulverisette 14, Idar-Oberstein, Germany) fitted with a 0.5 mm

mesh sieve ring. The resultant flour was defatted with n-Hexane (1:3 w/v) at room temperature for 1 h under continuous stirring, after which the hexane was decanted and the process repeated for two consecutive washings. In the final washing step the hexane was removed through vacuum filtration and the flour collected and air-dried overnight. The defatted flour was sieved through a 315 μm mesh sieve (flour particle size $D_{0.5}$ = 20.7±0.1 μm) with an air jet sieve (Hosokawa Alpine E200 LS, Augsburg, Germany) and stored in airtight containers at -20°C. All chemicals used were of analytical grade.
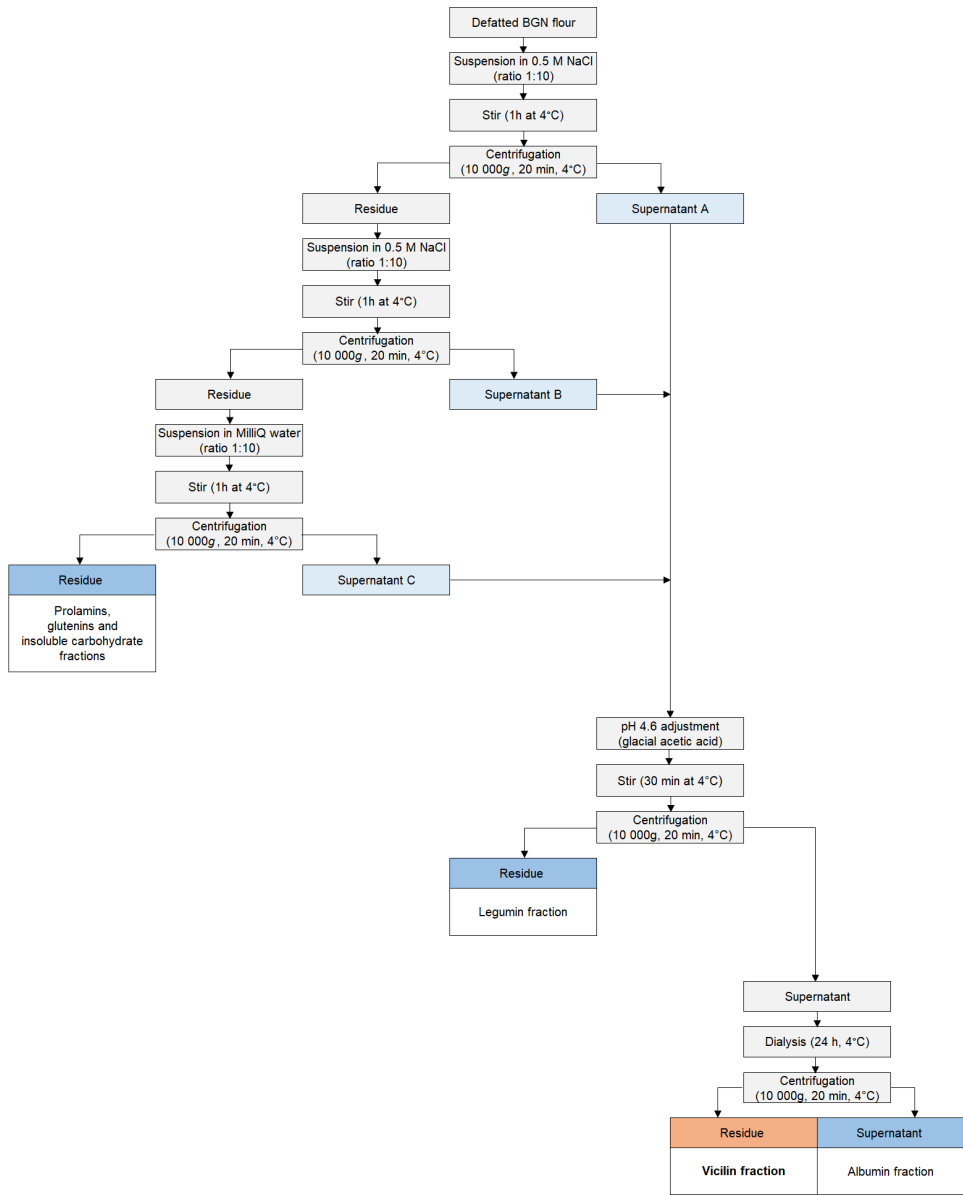
2.2.2. *Extraction of Bambara groundnut vicilin*

Vicilin was extracted from defatted BGN flour using an optimized Osborne fractionation method (Fig. 2.1) modified from Fido, Mills, Rigby, & Shewry (2004) and Arise, Nwachukwu, Aluko, & Amonsou (2017). Briefly, defatted flour (1:10 w/v) was dispersed in 0.5 M NaCl (pH 6.1, unadjusted), stirred at 4°C for 1 h and centrifuged at 4°C (10 000 *g*, 20 min) with an Avanti J-26 XP centrifuge (Beckman Coulter, USA). This procedure was repeated twice (final extraction with deionised water) to obtain three supernatants containing the globulins, albumins, sugars and soluble non-starch polysaccharides. The pooled supernatants (supernatants A ,B and C) were adjusted to pH 4.6 with glacial acetic acid (precipitation of legumin) in an ice bath, stirred at 4°C for 30 min and centrifuged. The supernatant (containing vicilin, albumin and soluble sugars/non-starch polysaccharides) obtained after centrifugation was dialysed (4°C) against several changes of distilled water until the conductivity of the water remained constant. Following dialysis the retentate was centrifuged, the residue freeze dried and designated as the vicilin fraction.

The total yield was expressed as the dry weight of vicilin per weight defatted flour, whereas the protein content of both the vicilin and flour was added to the dry weight ratio of vicilin to flour to obtain the protein yield.

2.2.3. *Compositional analysis of BGN vicilin*

The protein content was measured with the Dumas nitrogen combustion method (FlashEA 1112 series, Thermo Scientific, The Netherlands), using a nitrogen-to-protein conversion factor of 5.7. The oil content was determined with the standard Soxhlet extraction method, with petroleum ether as extraction solvent. Protein and oil determination were carried out in at least triplicate measurements. The moisture and ash contents were gravimetrically determined in duplicate, by oven drying at 105°C overnight and igniting at 525°C for 6 h respectively. Carbohydrate content was determined by difference.

Fig. 2.1. Bambara groundnut vicilin extraction scheme.

2.2.4. *Molecular weight determination of BGN vicilin*

Polyacrylamide gel electrophoresis (PAGE)

Native-PAGE analysis was performed to establish the electrophoretic profile of BGN vicilin proteins in their native state. A 45 μl 0.11% (w/v) vicilin solution containing 2 mM NaCl was mixed with 15 μl of NativePAGE™ sample buffer 4X (Invitrogen, USA). The sample mixture was loaded in aliquots of 15 μl onto the NativePAGE™ Novex® (4–16% Bis-Tris) gel which resolves proteins in the molecular weight range of 15–1000 kDa. Separation was performed with the NativePAGE™ running buffer 20X at a constant voltage of 200 V. The NativeMark™ unstained protein standard (Invitrogen, California, USA) containing eight protein bands (molecular weight range ~20–1200 kDa) was used as molecular weight markers. After separation the bands were stained with a Coomassie® G-250 stain and after destaining, scanned using a calibrated densitometer (Bio-Rad GS-900™, USA).

SDS-PAGE analysis was performed under reducing and non-reducing conditions to determine the purity of the vicilin extract. For reducing conditions, 39 μl of a 0.11% (w/v) vicilin solution containing 2 mM NaCl was mixed with 15 μl of NuPAGE® LDS sample buffer (Invitrogen, USA) and 6 μl NuPAGE® reducing agent. In the case of non-reducing conditions, 45 μl of vicilin solution was mixed with 15 μl of sample buffer. Both samples were heated at 70°C for 10 min, and aliquots of 15 μl loaded onto the NuPAGE® Novex® (4–12% Bis-Tris) gels which resolves proteins in the molecular weight range 2–200 kDa. The NuPAGE® MES SDS running buffer 20X was added and separation performed at a constant voltage of 200 V. The Mark12™ unstained standard was used as molecular weight markers. Staining and scanning of the gels were carried out as explained above for the Native-PAGE analysis.

High performance size-exclusion chromatography coupled with multi-angle light scattering (HPSEC-MALLS)

The HPSEC system consisted of four TSK gel analytical columns (PW$_{XL}$ guard, G6000 PW$_{XL}$, G4000 PW$_{XL}$ and G3000 PW$_{XL}$) (Tosoh Bioscience LLC, USA) connected in series and thermostated at 35°C with a temperature control module (Waters). The mobile phase (100 mM NaNO$_3$ + 0.02% NaN$_3$) was filtered over a 0.2 μm filter and in line over a 0.25 μm filter, in-line vacuum degassed (1200 series degasser, Agilent Technologies, USA) and pumped (1200 series binary pump, Agilent Technologies) with a flow rate of 0.5 ml/min. BGN vicilin solutions were placed in a thermally controlled sample holder at 10°C and 200 μl was injected (1260 series autosampler, Agilent Technologies) onto the columns.

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Static light scattering was measured at 665 nm at 15 angles between 32° and 144° (DAWN HELEOS II, Wyatt Technologies, USA). UV absorption was measured at 280 nm (1260 series, MWD, Agilent Technologies) to detect proteins and polyphenols. The sample concentration was measured by refractive index (RI) detection, held at a fixed temperature of 35°C (ERC-7510, Erma Optical Works). Data collection and processing were performed using ASTRA 6 software (Wyatt Technologies). For normalization, alignment and band broadening the reference material bovine serum albumin (BSA) with a M_w of 67 kDa was used.

2.2.5. *Isoelectric point (pI) determination of BGN vicilin*

The pI of BGN vicilin solutions (0.5% w/v, 100 mM NaCl) was determined in duplicate with the ZS Zetasizer Nano (Malvern Instruments Ltd., UK) in a pH range of 3.5–6.0. Samples were injected (1 ml) into the capillary cell and the electrophoretic mobility measured at room temperature.

2.2.6. *Vicilin solubility as a function of NaCl concentration*

The solubility of BGN vicilin solutions (11% w/v) were determined at various NaCl concentrations (0.05–0.5 M) at pH 7, by stirring the solutions at room temperature followed by centrifugation at 20°C (3350 *g*, 30 min) in a Hermle Z 306 benchtop centrifuge (HERMLE Labortechnik GmbH, Germany). The supernatant and residue were freeze dried, and the solubility expressed as a percentage of the dry residue weight per total initial dry weight. It should be noted that the apparent solubility as measured here holds true under the conditions (centrifugation speed, ionic strength) applied and could vary upon changes in these parameters.

2.2.7. *Thermal stability analysis of BGN vicilin*

A differential scanning calorimeter (Perkin Elmer STA 6000, USA) was used to determine the thermal properties of BGN vicilin solutions. Approximately 60 mg of 11% (w/v) BGN vicilin solutions prepared with 200 mM NaCl and without (no added salt) were placed in stainless steel pans which were hermetically sealed. The pans were heated at a rate of 10°C/min from 15–110°C and subsequently cooled at the same rate to 15°C. The denaturation temperature (T_d) and enthalpy of denaturation (ΔH) were determined from the thermograms as analysed using the manufacturer software (Pyris, PerkinElmer).

2.2.8. *Gelation functionality of BGN vicilin*

Minimum gelling concentration

A minimum gelling concentration was determined experimentally using the test tube inversion method, described as the lowest concentration where the sample does not flow in an inverted tube (O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004a). BGN vicilin solutions (pH 7, 200 mM NaCl) were prepared in 3 ml tubes in a concentration range of 2–11% (w/w). The samples were heated in a water bath at 95°C for 30 min, cooled to room temperature for 1 h and overnight at 4°C before the tubes were inverted.

Small deformation rheology

Oscillatory shear measurements were carried out in a MCR302 controlled stress rheometer (Anton Paar, Austria) equipped with a sandblasted concentric cylinder geometry (CC17). Vicilin solutions at various concentrations (pH 7, 200 mM NaCl) were subjected to a constant strain of 1% and frequency of 1 Hz (within the linear viscoelastic region) throughout the experiment. A thin layer of paraffin oil was added to the samples to prevent evaporation, before the samples were heated from 20 to 95°C, kept at 95°C for 30 min and cooled to 20°C. The temperature was kept constant at 20°C for 25 min; heating and cooling rates were set at 3°C/min. The storage modulus (G'), loss modulus (G") and loss tangent (tan δ) were recorded as a function of temperature.

Theoretical models describing gelation

One may distinguish different gelation models to describe the process of gelation and the resulting properties of gels. These include amongst others branching, percolation and fractal models. Branching models are based on theory developed by Flory (1941) and Stockmayer (1944). The percolation model allows for a broader applicability compared to single-branch theories as it allows for cyclic connections, whereby a critical concentration can be identified where below this concentration separate clusters are observed and above this concentration one infinite cluster. As such, the percolation model has been applied to, through critical exponents, describe gelation behaviour and rheological properties (Van der Linden & Foegeding, 2009). Another means of modelling gelation is through space-filling by fractal aggregates or flocs, known as fractal scaling (Van der Linden & Foegeding, 2009).

In the percolation model, scaling of G' is given by:

$$G' \sim (c - c_p) \qquad (2.1)$$

where the ~ symbol indicates "proportional to" and c is the concentration of monomers in the system, c_p is the critical percolation threshold concentration and t is the scaling exponent.

For the fractal model, scaling of G' is given by:

$$G' \sim c^w \qquad (2.2)$$

where the scaling exponent w is related to the fractal dimension, D_f, of the gel network and c_p assumed to be zero. The D_f can be determined through several experimental techniques such as light scattering, gel permeability, rheology and microscopy (Bi, Li, Wang, & Adhikari, 2013; Bremer, Van Vliet, & Walstra, 1989; Hagiwara, Kumagai, & Matsunaga, 1997; Hagiwara, Kumagai, & Nakamura, 1998; Vreeker, Hoekstra, den Boer, & Agterof, 1992). Light scattering is considered to be the most appropriate technique in characterising fractal structures in dilute systems, but poses limitations in concentrated systems. To overcome these limitations, rheological measurements have been identified as a better suited technique in the characterisation of gel structures in concentrated systems (Ould Eleya, Ko, & Gunasekaran, 2004).

Various models have been developed as a means of relating the scaling exponent to the fractal dimension. One such model is the one developed by Shih, Shih, Kim, Liu, & Aksay (1990) which relates to the existence of two regimes, i.e. the strong-link[1] and the weak-link[2] regime, based on the strength of the inter- and intra-floc links. In the strong-link regime the elasticity parameter (G') follows the scaling in eq. (2.2) and limit of linearity/critical strain (γ_o) is given by:

$$\gamma_0 \sim c^n \qquad (2.3)$$

where $w = \frac{3+x}{3-D_f}$ and $n = -\frac{1+x}{3-D_f}$; x represents the fractal dimension of the backbone of the aggregates with an assumed value of 1.3. Similarly in the weak-link regime one has $w = \frac{1}{3-D_f}$ and $n = \frac{1}{3-D_f}$

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[1] Strong-link regime—neighbouring (inter-)floc links have higher elasticity vs links in the (intra-)flocs
[2] Weak-link regime—higher elasticity in links in the (intra-)flocs vs links between neighbouring (inter-)flocs

A refinement of the Shih et al. (1990) model was proposed by Wu & Morbidelli (2001), in which an elastic constant (α) varying between 0–1 was introduced to account for contributions from both the inter- and intra-floc links. This constant allows for determination of a transition (intermediate) regime, where $w = \frac{\beta}{3-D_f}$ and $n = \frac{3-\beta-1}{3-D_f}$; with $\beta = 1 + (2+x)(1-\alpha)$.

2.2.9. *Microstructure of BGN vicilin gels*

Confocal laser scanning microscopy (CLSM)

Vicilin solutions were prepared at five protein concentrations (5.5%, 7.4%, 9.2%, 11% and 12.7% w/w) in the presence of 200 mM NaCl and labelled non-covalently with a 0.005% (w/w) final concentration of the fluorescent dye, Rhodamine B. Gels were prepared for microstructural analysis in sealed glass chambers (Gene Frame 125 μl adhesives, Thermo Fisher Scientific, UK) which were heated in a water bath at 95°C for 30 min, cooled to room temperature for 1 h and overnight at 4°C. Gelled samples were imaged with a Zeiss LSM 510 META confocal microscope equipped with an Axiovert 200M inverted microscope, using a 40x NA 1.3 oil immersion objective. Rhodamine B was excited at 543 nm (He-Ne laser).

Scanning electron microscopy (SEM)

Vicilin protein gels at three protein concentrations (5.5%, 9.2% and 12.7% w/w) in the presence of 200 mM NaCl were prepared for SEM analysis in 10 ml pre-lubricated syringes by heating in a water bath at 95°C for 30 min. After cooling overnight at 4°C, the gels were prepared for imaging as described by Urbonaite et al. (2016). Briefly, gel pieces were cut and incubated for 8 h in an aqueous 2.5% (v/v) glutaraldehyde solution to allow crosslinking of the proteins. The excess glutaraldehyde was removed by placing the gel pieces into deionised water overnight under gentle rotation, after which the deionised water was stepwise replaced with ethanol. The gel pieces were subjected to critical point drying in a Leica Automated Critical Point Dryer (EM CPD300, Leica, Austria), followed by fracturing and adhesion on sample holders with Carbon Adhesive (Electron Microscopy Sciences, USA). Subsequently, the solvent was evaporated and the samples sputter coated with a 15 nm thick layer of Tungsten (MED 020, Leica, Austria) before analysis in a field emission SEM (Magellan 400, FEI, The Netherlands), where a working distance of 4 mm and SE detection at 2 kV and 13 pA were applied.

2.3. Results and Discussion

2.3.1. Chemical composition and protein yield

BGN vicilin was characterised with a protein content of 91.4 ± 1.0 g/100 g, with minor amounts of fat (0.4%), moisture (0.2%), ash (2.2%) and carbohydrates (5.8%). These values are comparable to those found for pea vicilin, with a reported protein content of 90.0 g/100 g (N x 5.6) and carbohydrates (6.8%) being the largest non-protein component (Rubio et al., 2014).

The BGN vicilin dry weight yield per 100 g defatted flour was 6.6 g; whilst in comparison the yield for legumins was 5.1 g, 6.3 g for albumins and 20.4 g for the fraction containing prolamins, glutelins and insoluble carbohydrates. When taking into consideration the protein content of each fraction, a protein yield of 46.4% was calculated for BGN vicilins, 29% for legumins, 20.5% for albumins and 4.1% for the remainder fraction. The protein yield of BGN vicilin is thus indicative of its presence as major protein fraction in the seeds. The legumin to vicilin (L / V) ratio was 0.62. The L / V ratio of pulses varies according to certain agronomical factors such as varieties and species, whilst processing and environmental factors also have an effect on this ratio and consequently the physicochemical properties of the protein fractions (Singhal, Karaca, Tyler, & Nickerson, 2016). Related to the agronomical factors, Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, & Blecker (2012) have shown that the L / V ratio in smooth pea seeds are significantly impacted by cultivation year, soil type, seeding density and cultivation location; as such presenting a possible means of producing seeds with specific L / V ratios. In a study by Tzitzikas, Vincken, De Groot, Gruppen & Visser (2006), a large variety of pea lines (59 genotypes) were investigated for differences in amongst others their protein content and globulin composition. Expressed as a ratio of vicilin to legumin, a range of 1.3–8.2 have been identified which shows vicilin content of all pea lines being higher than legumin. Furthermore, Barac et al. (2010) have shown that pea genotypes with high vicilin content and/or low legumin content had a higher protein extractability (isoelectric protein precipitation) whilst also influencing functionality. The similarities in BGN and pea vicilin protein composition could prove to be beneficial in establishing the functional properties of BGN vicilin.

2.3.2. Molecular weight characterisation

Gel electrophoresis

Native-PAGE profiles of BGN vicilin and BGN flour are presented in Fig. 2.2. Two major bands of approximately 170 kDa and 385 kDa were identified in all samples.

The broad band at 170 kDa corresponds to the trimeric structure of vicilin varying between 150–190 kDa, whereas the band at 385 kDa appears to correspond to the hexamer legumin. Legumin which is also classified as a globulin storage protein, have a reported molecular weight range of approximately 320–380 kDa and consists of six subunit pairs which are linked by disulphide bonds (Barac et al., 2010; Shewry et al., 1995). However, assuming that the BGN vicilin protein is of high purity, the band at 385 kDa could be attributed to the hexameric structure of vicilin, since vicilin is known to undergo reversible aggregation when subjected to changes in environmental conditions such as ionic strength (Shewry et al., 1995). Furthermore, BGN vicilin in solution without salt addition or pH adjustment (pH 6.1), shows two smaller bands at approximately 555 kDa and 678 kDa. This could be attributed to aggregate formation as was found for phaseolin (600–653 kDa), the vicilin-like fraction present in French and common beans (Blagrove, Lilley, Van Donkelaar, Sun, & Hall, 1984; Shewry et al., 1995).
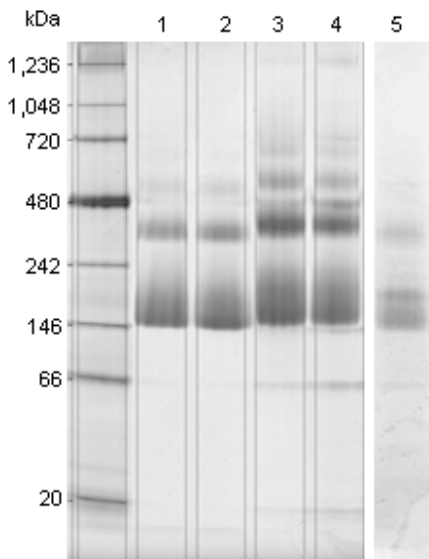
Fig. 2.2. Native-PAGE patterns of BGN vicilin in lane 1 and 2 (pH 7, 2 mM NaCl), BGN vicilin in lane 3 and 4 (pH 6.1, unadjusted) and BGN defatted flour in lane 5.

To determine the molecular weight of the subunits of BGN vicilin, SDS-PAGE was performed under reducing and non-reducing conditions (Fig. 2.3). The similar bands obtained under both conditions is indicative of the purity of the vicilin protein, since it lacks disulphide bonds amongst its subunits. As such the vicilin polypeptides would not be dissociated under reducing conditions, thus exhibiting similar electrophoretic patterns in the absence and presence of the reducing agent; contrary to the other major storage proteins legumin and albumin (Mession et

al., 2015; Tang, 2008; Tang & Sun, 2011a). In the only existing study as per our knowledge on BGN vicilin extraction, it was reported that clear differences in the electrophoretic bands were observed under reducing and non-reducing conditions, which corresponded to the bands of the BGN protein isolate containing a mixture of proteins (Arise et al., 2017). Comparatively, we have been able to show that pure vicilins free from contaminating proteins can be extracted from BGN flour. Three major polypeptide bands were observed at approximately 53 kDa, 65 kDa and 118 kDa which corresponds to the bands previously reported for BGN protein isolates and BGN vicilin-like protein (Arise et al., 2017; Busu & Amonsou, 2018; Kudre, Benjakul, & Kishimura, 2013). The band at 53 kDa is often denoted as the β subunit in legume seed vicilins and is thus indicative of the major subunit composition of BGN vicilin, this being the largest band present. Similarly, the band at 65 kDa is found in many vicilin proteins and is often denoted as the α subunit of vicilin, also known as convicilin (Chang, Alli, Molina, Konishi, & Boye, 2012; Maruyama et al., 1998; O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004b; Tzitzikas et al., 2006). The band at 118 kDa corresponds to the bands identified for BGN protein isolates under non-reducing conditions and to the high molecular weight bands identified in pea vicilin and red kidney bean vicilin-rich protein isolate (Adebowale et al., 2011; Rubio et al., 2014; Tang, 2008). The similarities of the electrophoretic profiles of BGN vicilin to its protein isolates and other legume vicilins, serves as another indication of its presence as major protein fraction in BGN seeds and its purity when extracted with the method as modified in our laboratory.
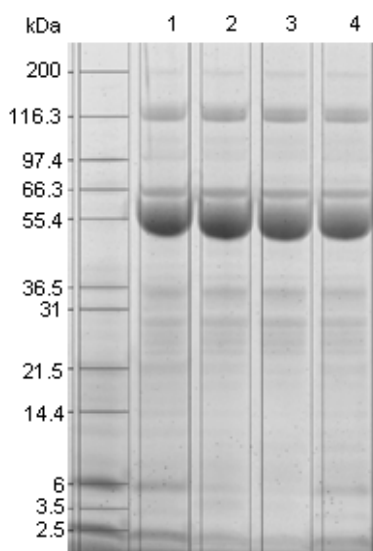
Fig. 2.3. SDS-PAGE patterns of BGN vicilin in lane 1 and 2 (pH 7, 2 mM NaCl) under reducing conditions, and BGN vicilin in lane 3 and 4 (pH 6.1, unadjusted) under non-reducing conditions.

SEC-MALLS analysis

The elution profile of BGN vicilin revealed one major peak eluting between 50–55 min (Fig. 2.4). From the elution peaks a molecular weight of 196 kDa was calculated, which is similar to the gel electrophoretic analysis, corresponding to the trimeric structure of vicilin and serves as a confirmation of its purity.
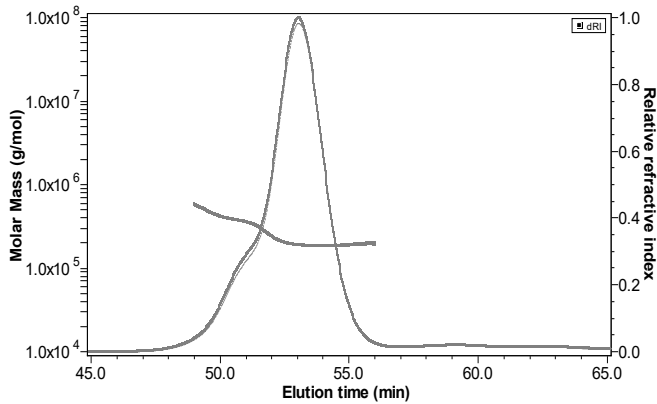
Fig. 2.4. SEC-MALLS elution profile of BGN vicilin. Replicate indicated with dashed line.

2.3.3. Isoelectric point (pI) determination of BGN vicilin

The electrophoretic mobility of BGN vicilin was measured as a function of pH, as shown in Fig. 2.5. At increasing pH from 3.5–6.0, the electrophoretic mobility changed from positive to negative which is attributed to two mechanisms, i.e. dissociation of functional groups or adsorption of ions on the surface (Salgin, Salgin, & Bahadir, 2012). The pI, which is the pH where the electrophoretic mobility is neutral, was determined to be at pH 5.3; corresponding to the pI measured for a salt-soluble BGN protein extract (pH 5.3) and a BGN vicilin-like extract (pH 5.2). Similarly, a pI range of pH 4.7–5.6 have been identified for vicilins from soybeans, kidney beans, red and mung beans (Arise, 2016; Busu & Amonsou, 2018; Kuipers et al., 2006; Tang & Sun, 2011b). The variation in pI can be ascribed to various factors such as a protein's subunit composition and differences in a protein's ionic environment (Kuipers et al., 2006; Salgin et al., 2012). The absolute measurement values were lower compared to those previously reported for BGN protein fractions and other legume vicilins, as is expected in the presence of salts.
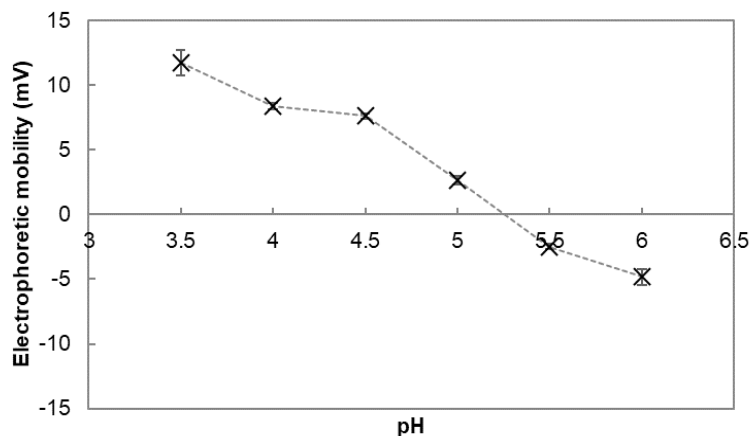
Fig. 2.5. Electrophoretic mobility of BGN vicilin as a function of pH.

2.3.4. Vicilin solubility as a function of NaCl concentration

The solubility of BGN vicilin was determined at increasing NaCl concentrations as shown in Fig. 2.6. The highest solubility (86%) was found at NaCl concentrations above 200 mM, whilst at lower concentrations (50 mM and 100 mM NaCl) the vicilin solubility was less than 10% with an increase in solubility up to 40% observed at 150 mM NaCl. Considering that vicilin is part of the globulin storage proteins which are soluble in dilute salt solutions, this finding is as expected. This general trend was also observed by Tavano & Neves (2008), where the solubility of chickpea vicilin increased upon increasing NaCl concentration with an 80% solubility in the presence of 200 mM NaCl. As found by Maruyama et al. (1999) for soy vicilin (β-conglycinin), the solubility of the β subunit was dependent on salt concentration, whereas the solubility of the α and α' subunits with their high hydrophilic extension regions remained independent of salt concentration. The insolubility of BGN vicilin at low ionic strength is therefore a further indication that the subunit composition of this fraction is largely composed of β subunits.

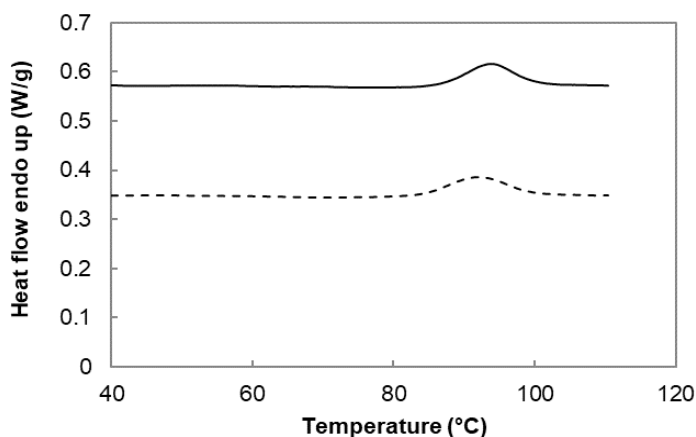### *2.3.5. Thermal properties of BGN vicilin*

The thermal transition parameters of BGN vicilin as evaluated by differential scanning calorimetry are shown in Fig. 2.7. One endothermic peak was observed, representing the denaturation temperature (T_d) of BGN vicilin at 92°C and 94°C in respectively

the absence and presence of NaCl. Various T_d values of BGN protein isolates have been reported in a range of 62–108°C, with two endothermic peaks observed in some cases which were related to the denaturation of vicilin and legumin (Adebowale et al., 2011; Arise et al., 2017; Kaptso et al., 2015; Kudre et al., 2013). The T_d of BGN vicilin is comparable to other legume vicilins, which were reported to be higher than 90°C (Tang, Sun, & Yin, 2009). In studies on the subunit composition of soybean vicilin (β-conglycinin) the thermal stability was attributed to the different subunits, with the highest T_d (90.8°C) found for the β subunit and the lowest for the α subunit. Furthermore it was shown that the thermal stability of a heterotrimer is determined by the subunit with the lowest T_d (Maruyama et al., 1998). Comparing the T_d values for BGN vicilin, it provides a further indication of the subunit composition being largely comprised of β subunits.

Upon NaCl addition the T_d of BGN vicilin increased slightly, as was also observed in previous studies for BGN protein isolates and proteins from mung bean, black bean and cowpeas at varying degrees (Kimura et al., 2008; Kudre et al., 2013). Similarly, a slight increase in enthalpy (ΔH) from 1.9 J/g to 2.4 J/g (corresponding to 372.4 kJ/mol and 470.4 kJ/mol respectively) was observed, which is indicative of the increased thermal stability of BGN vicilin in the presence of salt. Comparatively, ΔH values of 0.4–0.6 J/g were reported for salt-extracted BGN proteins, 7.6 J/g for pea vicilins and 9.8–13.6 J/g for vicilins from kidney, red and mung beans (Adebowale et al., 2011; Message et al., 2015; Tang & Sun, 2011b). No endothermic peaks were visible upon reheating of the BGN vicilin solutions, thus indicating irreversible denaturation.
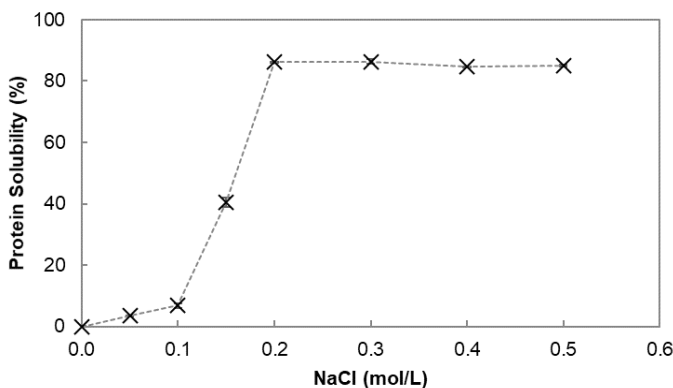
**Fig. 2.6.** Effect of NaCl concentration on BGN vicilin solubility.

Fig. 2.7. Thermogram for BGN vicilin solutions (11% w/v, pH 7) with 200 mM NaCl (solid line) and without added salt (dashed line).

2.3.6. *Gelling properties of BGN vicilin*

The minimum protein concentration where a self-supporting gel could be visually observed, was found at 5.5% (see inset Fig. 2.8). This concentration is comparable to that observed by Adebowale et al. (2011) for salt-extracted BGN proteins (4.5–4.7%), whereas the minimum gelling concentration for BGN protein isolates were reportedly higher at 7.2–7.3%. Furthermore, it also appears that BGN vicilin can form a gel at lower concentrations compared to its soybean equivalent β-conglycinin (extracted under different conditions), for which a minimum gelation concentration of 12.5% was reported (Mohamad Ramlan et al., 2004).

The viscoelasticity of BGN vicilin gels was probed by dynamic oscillatory measurements. The sol-gel transition indicating the formation of a gel network as determined at the G'/G" cross-over point was independent of the protein concentration (5.5–12.8%) as observed during isothermal heating (95°C), whereas for the lowest concentration at 3.7% no gel was formed. Soon after the cross-over point, G' is much larger than G" and the gelation behaviour is interpreted in terms of G'. Furthermore, the linear viscoelastic region appeared to be independent of protein concentration at a limiting strain of about 10% as shown in Fig. 2.8. Three distinct regions in G' magnitude levels could also be identified, i.e. for the lowest concentration below the gelation point, gels at protein concentrations close to the gelation point and those BGN vicilin gels at higher concentrations far from the gelation point.
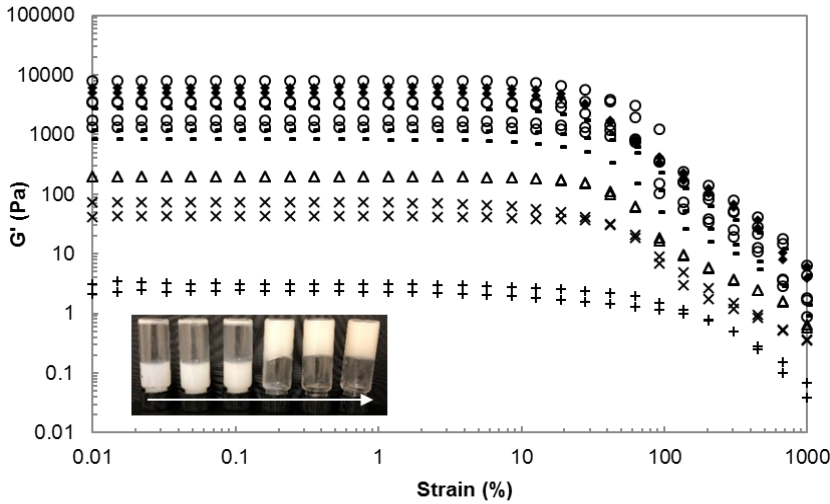
Fig. 2.8. Strain sweep of BGN vicilin at various concentrations (pH 7, 200 mM NaCl). Symbols indicate vicilin concentrations: ♦12.9%, ○11%, −9.2%, Δ7.4%, x5.5%, +3.7%. **Inset:** Minimum gelling concentration determination for BGN vicilins; increasing concentrations (1.8%, 2.8%, 3.7%, 5.5%, 7.4% and 11.1%) from left to right.

### 2.3.7. *Applicability of gelation models to BGN vicilin gels*

Percolation approach

The elastic moduli (G') dependence on protein concentration of BGN vicilin gels were determined by following the fitting procedure as described by Van der Linden & Sagis (2001) for ovalbumin gels (and other protein systems—casein, soy glycinin, soy β-conglycinin—as obtained from literature data). Through this approach c_p and t, as defined from eq. (2.1), were determined by a multi-step procedure. First, c_p was determined using a graphical deduction from plots of $G'^{1/t}$ vs c, with those values providing the best linear fits used in the second step for determination of the actual t. From our results the best fits were obtained for scaling exponents t = 5.0 and 5.5 (example Fig. 2.9, different symbols indicate different extraction batches), which yielded an average c_p = 0.45 ± 0.25. Using the c_p values obtained from the best linear fits, the scaling exponent (t = 4.91 ± 0.34) could be determined by plotting G' vs $c-c_p$ (example Fig. 2.10, different symbols indicate different extraction batches). The scaling exponent for BGN vicilin is much higher than the predictions from the

isotropic percolation model (t = 2.06 ± 0.6) (Mitescu & Musolf, 1983); a model which was shown to be applicable to various (plant) protein systems describing their apparent homogeneous network structures (Van der Linden & Sagis, 2001; Zhang et al., 2010). The higher t as observed for the BGN vicilin gels could be indicative of non-homogeneous network structures, as similar higher values were found for pre-sheared systems which are expected to be of such a nature (Kanai, Navarrete, Macosko, & Scriven, 1992; Trappe & Weitz, 2000). This presumed inhomogeneity therefore points more into the direction of a fractal scaling approach (where c_p is assumed to be zero), an assumption which is further cemented by the low critical percolation concentration (c_p = 0.45). It can thus be expected that the fractal scaling model would be better suited to describe the network structures of BGN vicilin gels as opposed to the isotropic force percolation model.
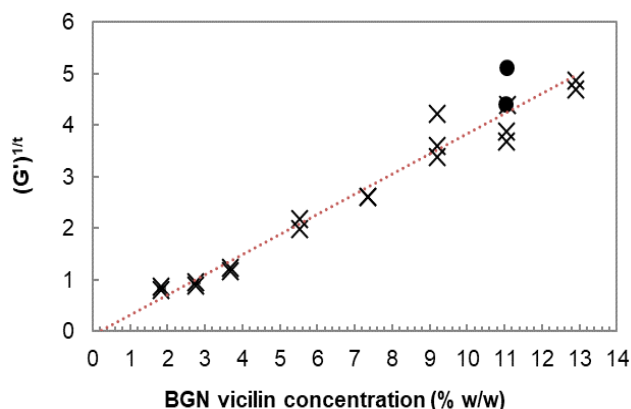
Fig. 2.9. $(G')^{1/t}$ (t=5.5) as a function of vicilin concentration (pH 7, 200 mM NaCl). Different symbols (●X) indicate data points obtained from different vicilin batches.

Fig. 2.10. G' as a function of $(c-c_p)$ to determine the scaling exponent as a power-law function. Different symbols (●X) indicate data points obtained from different vicilin batches.
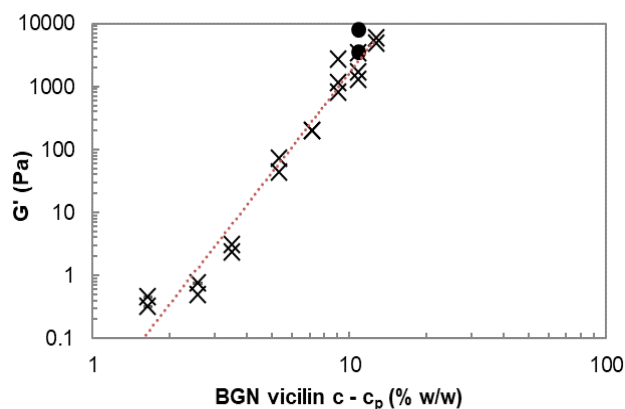
Fractal scaling
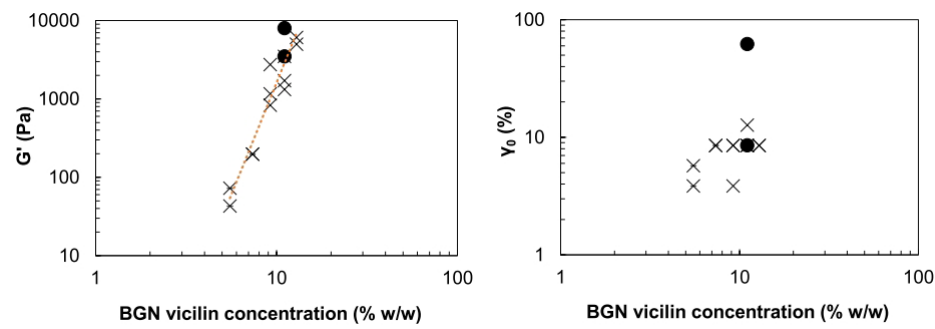
The scaling behaviour of the storage modulus (G') at the end of cooling (20°C) for BGN vicilin gels as a function of protein concentration is shown in Fig. 2.11 (different symbols indicate different extraction batches). A positive power-law behaviour was evident with increasing vicilin concentration which can be fitted to the form $G' \sim c^w$ (eq. 2). Similarly, scaling of the critical strain (γ_0) to vicilin concentration is also shown in Fig. 2.11, where a positive slope could be deduced according to eq. (3) ($\gamma_0 \sim c^n$). It should be noted that although the limiting strain values appeared to be independent of protein concentration (Fig. 2.8, section 2.3.6), differences could still be distinguished when considering the definition of a 5% deviation in G' from its maximum value. The scaling exponents were used to calculate the fractal dimensions (D_f) of BGN vicilin gels on the basis of the scaling models by Shih et al. (1990) and Wu & Morbidelli (2001), and are shown in Table 2.1.

As a first approximation from the Shi et al. model the positive scaling exponent n is indicative of a weak-link gel. Subsequently $w = \frac{1}{3 - D_f}$ from the Shi et al. model for gels in the weak-link regime was used to calculate the D_f of 2.83. This value corresponds to the D_f(2.82) reported for heat-induced bovine serum albumin (BSA) gels prepared at pH 5.1 (100 mM NaCl) and pH 7 (300 mM CaCl$_2$). In these systems however the γ_0 notably increased with increasing BSA concentrations and the scaling exponents (w and n) were similar, which is indicative of a weak-link regime (Hagiwara et al., 1998). The differences in exponents for BGN vicilin and the apparent independence of γ_0 to the protein concentration, thus raises the question if the fractal structures are truly in the weak-link regime. In a similar observation for β-lactoglobulin protein gels, the exponents were also different (w=3.24, n=0.36)[3], but due to the positive n value was characterised as weak-link gels with a D_f of 2.69 (Hagiwara et al., 1997). When recalculated by Wu & Morbidelli (2001), it was revealed that these β-lactoglobulin gels were actually in the transition regime as indicated by the α parameter (α=0.76).

To confirm if BGN vicilin gels are truly in the weak-link regime, the D_f was estimated with the Wu & Morbidelli model, followed by determination of the β constant and α parameter. The α parameter was calculated as 0.98, a value close to 1, confirming the weak-link regime for BGN vicilin gels which translates to rigid aggregate structures. Considering the general trend for various protein/colloidal gels systems which shows both strong- and weak-link behaviour based on the change

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[3] n exponent not reported in original article; value reported here was estimated by (Wu & Morbidelli, 2001).

in aggregation conditions (Hagiwara et al., 1997; Ikeda, Foegeding, & Hagiwara, 1999), it is reasonably expected that BGN vicilin gels at 200 mM NaCl concentration is indeed characterised by a weak-link fractal structure.

Fig. 2.11. (Left) Scaling behaviour of storage modulus G' and (Right) critical strain γ_0 as a function of BGN vicilin concentration (pH 7, 200 mM NaCl). Different symbols (●X) indicate data points obtained from different vicilin batches.

Table 2.1 Fractal dimension and related structural parameters of BGN vicilin gels (pH 7, 200 mM NaCl).

	(Shih et al., 1990) Model	(Wu & Morbidelli, 2001) Model		
w	D_f	D_f	β	α
5.72	2.83	2.83	1.06	0.98

2.3.8. *Microstructure of BGN vicilin gels*

CLSM and SEM were used to probe the microstructure of BGN vicilin gels at different length scales. As discussed in section 2.3.6, the minimum gelling concentration was determined at 5.5% (w/w) vicilin concentration, thus imaging was performed in the concentration range 5.5–12.7%. From the CLSM images (length scale 50 μm, Fig. 2.12), no structural information could be distinguished for the protein concentration range investigated, indicating a homogeneous system at this length scale. From these images a D_f close to 3 can be assumed, which is consistent with the D_f of 2.83 as determined from rheological data. In the corresponding SEM images (Fig. 2.12) however, structures were observed in a range of length scales from 3 μm down to sub-micron lengths. On these length scales inhomogeneous aggregate structures

were visible, which corresponds to the inhomogeneity as implied from the high scaling exponent obtained from the percolation model for BGN vicilin gels and from the D_f value of 2.83.
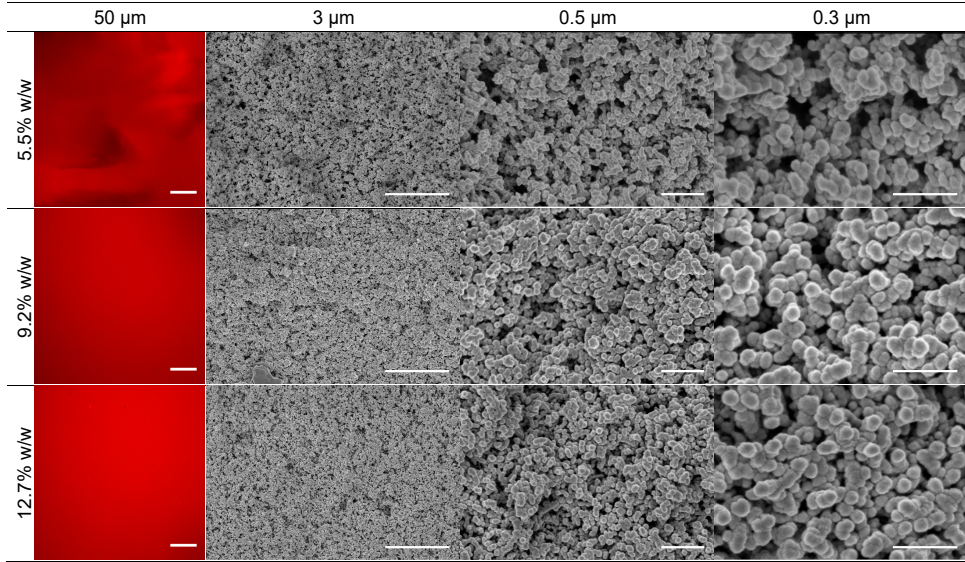
Fig. 2.12. Microstructure of BGN vicilin gels at various protein concentrations (pH 7, 200 mM NaCl) on different length scales as imaged with CLSM (first column) and SEM (last three columns). For the CLSM images the inserted scale bar corresponds to 50 μm, whilst for the SEM images the scale bars correspond to 3 μm, 0.5 μm and 0.3 μm, respectively.

2.4. Conclusions

Vicilin proteins were successfully extracted from BGN black-eye flour using a fractionation method modified in our laboratory. Through several physicochemical analyses, we were able to establish the purity of BGN vicilin and its presence as the major protein fraction in the seeds. The gelation of BGN vicilin at various protein concentrations (pH 7, 200 mM NaCl) was well characterised by fitting rheological parameters to two gelation models; i.e. the isotropic percolation model and the fractal scaling model. The latter was best in describing the gel network structures, with a scaling behaviour evident from the power-law relationship of the elasticity and

critical strain as a function of protein concentration. A fractal dimension (D_f) of 2.83 was determined by both the Shih et al. (1990) and Wu & Morbidelli (2001) scaling models, characterising vicilin gels as weak-link fractal structures. The microstructural images were consistent with the rheological data. The work we have presented here is the first of this nature providing insight into the physicochemical and gelation properties of Bambara groundnut vicilin, in terms of colloidal aggregation models.

Acknowledgements

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3

Physicochemical properties and gelling behaviour of Bambara groundnut protein isolates and protein-enriched fractions

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Abstract

Plant proteins, and specifically those from legume crops, are increasingly recognised as sustainable and functional food ingredients. In this study, we expand on the knowledge of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] proteins, by characterising the composition, microstructure and rheological properties of BGN protein isolates obtained via wet extraction and protein-enriched fractions obtained via dry fractionation. The BGN protein isolates were compared in the context of the major storage protein, vicilin, as previously identified. Molecular weight analysis performed with gel electrophoresis and size-exclusion chromatography coupled to light-scattering, revealed some major bands (190 kDa) and elution patterns with molecular weights (205.6–274.1 kDa) corresponding to that of BGN vicilin, whilst the thermal denaturation temperature (T_p 91.1°C, pH 7) of BGN protein isolates also coincided to that of the vicilin fraction. Furthermore, the concentration dependence of the elastic modulus G' of the BGN protein isolates, closely resembled that of BGN vicilin (both upon NaCl addition); suggesting that vicilin is the main component responsible for gelation. Confocal laser scanning and scanning electron micrographs revealed inhomogeneous aggregate structures, which implies that fractal scaling were better suited for description of the BGN protein isolate gel networks. Concerning the BGN protein-enriched fractions, both rotor and impact milling with air jet sieving and air classification, respectively, were successfully applied to separate these fractions from those high in starch; as evident from compositional analysis, particle size distributions and microscopic imaging. When considering sustainability aspects, dry fractionation could thus be a viable alternative for producing BGN protein-enriched fractions.

3.1. Introduction

Pulses are recognised as an important class of legumes due to their nutrient-rich composition and associated health benefits. In recent years, there have been an increased effort in highlighting the importance of pulses, as seen by the declaration from the United Nations for 2016 as the International Year of the Pulses (Calles, Xipsiti, & del Castello, 2019). Similarly, research efforts are increasingly geared towards establishing ingredient functionality of pulse crops, with a large focus on protein functionality. This focus stems from the necessity of producing protein-rich foods from plant proteins as opposed to animal proteins, to contribute positively to not only a nutritious diet but also to food security and sustainability (Boye, Zare, & Pletch, 2010; Day, 2013; Stone, Wang, Tulbek, & Nickerson, 2019). Nowadays in the current context of climate change, the utilisation of pulses as "climate-smart crops" becomes of more importance as food sources which are able to mitigate and adapt to climate change (Calles et al., 2019).

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] is one such pulse crop which fulfils the aforementioned criteria. Largely cultivated in West Africa, BGN is a drought-tolerant and high-quality protein crop which compares favourably in terms of protein content (up to 27% reported) to other legumes such as soybean (36.5%) and yellow pea (25%) (Arise, Amonsou, & Ijabadeniyi, 2015; Boye et al., 2010; Feldman, Ho, Massawe, & Mayes, 2019). BGN is increasingly recognised as a crop to be valorised; this is seen through the concerted efforts amongst international organisations who are focussed on optimising the crop's performance under various climatic conditions (Feldman et al., 2019). Noteworthy in terms of protein functionality, is the foaming capacity and emulsifying ability of BGN protein isolates and concentrates which were found to be similar (if not higher) compared to soybean protein isolates (Adebowale, Schwarzenbolz, & Henle, 2011). Here it should be noted that soybean proteins are the most widely used and commercially available plant proteins; known for their ability to impart various functionalities such as gelation, emulsification and water-binding in food systems (Day, 2013; Nishinari, Fang, Guo, & Phillips, 2014; Thrane, Paulsen, Orcutt, & Krieger, 2017). This oilseed can therefore be considered as a benchmark for comparison to new plant protein sources. Another relevant functionality of BGN proteins is their gelling behaviour, which was mostly investigated in terms of establishing the protein concentration above which a solid-like material is formed, without any further characterisation of the gels (Adebowale et al., 2011).

There are various techniques used for the fractionation of protein-enriched pulse flours or protein concentrates and isolates (Boye et al., 2010). The extraction

of protein concentrates and isolates is usually associated with wet fractionation techniques which are the most widely used industrially, whereas fractionation of pulse flours is carried out with a combination of milling and air classification processes. Protein concentrates and isolates are characterised by higher concentrations (50–90%) compared to protein-enriched flours (30–50%) (Schutyser, Pelgrom, van der Goot, & Boom, 2015; Stone et al., 2019). Furthermore, differences are also observed in functionality—proteins obtained from wet fractionation have been subjected to harsh conditions which leads to a partial loss of native functionality. In comparison, the functionality of dry fractionated protein-enriched flours is largely determined by the extent of detachment of protein bodies from other non-protein cell components, which is determined by the milling process (Pelgrom, Vissers, Boom, & Schutyser, 2013). Various milling techniques are used for processing of seeds into flour; in our study both rotor and impact milling were investigated. In rotor milling, particle size reduction is achieved through both the impact of the material against the rotor teeth and shearing between the sieve and spinning rotor teeth; whereas in impact milling this is achieved through collisions between the material particles and the mill wall (Pelgrom et al., 2013; Pollard et al., 2008). When taking into consideration the sustainability aspect of plant protein production, dry fractionation techniques are favoured as they are less resource- and energy-intensive (Schutyser et al., 2015).

Research to date on BGN proteins is largely focussed on the characterisation of the wet extracted concentrates and isolates, whereas research on the protein-enriched dry fractions is still lacking. In Chapter 2 we have explored the gelation functionality of vicilin as the major storage protein present in BGN seeds. The purity of this protein fraction was considered to be high (91% protein content) and gel formation was evident at low protein concentrations, with the resulting gels characterised as weak link fractal type structures. Similarly in this study, the aim was to determine the physicochemical properties of the less purified wet-extracted BGN protein isolates and to compare their gelation functionality to that of the high purity vicilin fraction. Through this approach, we were able to establish the role of vicilin in the structure-function behaviour of BGN protein isolates. In addition, we have also investigated the suitability of dry fractionation techniques to obtain BGN protein-enriched fractions. This is the first attempt, as per our knowledge, to obtain protein-enriched BGN fractions via dry fractionation, including an evaluation on the structural and rheological properties of these fractions.

3.2. Materials and Methods

3.2.1. Materials

Bambara groundnut black-eye variety seeds were purchased from Thusano Products (Louis Trichardt, Limpopo, South Africa). The seeds were screened for defects, dehulled and milled into flour (particle size $D_{0.5}$ = 20.7 ± 0.1 µm), followed by defatting with n-Hexane as previously described (Diedericks, De Koning, Jideani, Venema, & Van der Linden, 2019). All chemicals used were of analytical grade.

3.2.2. Extraction of Bambara groundnut protein isolates and solutions preparation

Protein isolates were extracted from the defatted flour through the commonly utilised isoelectric precipitation method (Boye et al., 2010). Briefly, defatted flour (approximately 150 g per extraction batch) was dispersed in deionised water (1:10 w/v), adjusted to pH 9.5 with 1 M NaOH and stirred for 2 h at 4°C. After stirring the dispersion was centrifuged at 4°C (4000 g, 30 min) with an Avanti J-26 XP centrifuge (Beckman Coulter, USA). The supernatant containing the solubilised proteins, sugars and soluble non-starch polysaccharides were adjusted to pH 4 with 1 M HCl for isoelectric precipitation, stirred for 2 h at 4°C followed by centrifugation. The residue obtained after centrifugation was dispersed in deionised water (1:5 w/v), adjusted to pH 7 with 1 M NaOH and stirred overnight at 4°C; after which it was freeze-dried and designated as the BGN protein isolate (BGN-PI). The total yield is expressed as the dry weight of BGN-PI per weight defatted flour, whereas the protein yield is expressed as the total protein weight fraction in the final freeze-dried product.

All BGN-PI solutions were prepared in deionised water at varying concentrations at pH 7 (adjusted with 1 M NaOH or HCl), with and without 200 mM NaCl. Dispersions were stirred overnight and stored at 4°C in the presence of 0.02% (w/w) NaN_3 to prevent microbial spoilage. For protein solutions at varying pH, BGN-PI was prepared in McIlvaine buffer at the desired pH to a final protein concentration of 9.2% (w/w).

3.2.3. Preparation of Bambara groundnut dry fractionated material

BGN protein- and starch-enriched fractions were obtained through dry fractionation by either impact milling or rotor milling. Dehulled seeds were pre-milled into grits with a pin mill (Condux-Werk LV 15M, Wolfgang bei Hanau, Germany) before milling a batch quantity of approximately 150 g with the impact mill (ZPS50

Hosokawa-Alpine, Ausburg, Germany) at 8000 rpm. The impact mill is fitted with a rotating classifier wheel through which finer particles are passed and coarser particles retained for further milling. The classifier wheel speed was set at 3000 rpm, the screw feeder rate at 5 rpm and the airflow at 52 m³/h. The impact milled (IM) flour was further air classified (ATP50 Hosokawa-Alpine, Ausburg, Germany) at a classifier wheel speed of 6000 rpm whilst the screw feeder rate and airflow were kept at the same settings. Through air classification, a protein-rich fine fraction and a starch-rich coarse fraction were obtained (Pelgrom, Boom, & Schutyser, 2015a).

BGN black-eye seeds were also dehulled and coarse-milled before rotor milling (Fritsch GmbH Pulverisette 14, Idar-Oberstein, Germany) with a 0.5 mm mesh sieve ring at 18000 rpm. The resultant rotor milled (RM) flour was defatted and subjected to air jet sieving (E200 LS Hosokawa-Alpine, Ausburg, Germany) with a sieve mesh size of 20 μm at 3000 Pa for 3 min, to obtain a protein-rich fine fraction and a starch-rich coarse fraction as comparison to the IM flour fractions. The milling yield was expressed as the dry weight of the milled flour per weight of BGN grits, whereas the yield of the fine and coarse fractions were expressed per weight of the starting flour material.

3.2.4. *Compositional analysis of Bambara groundnut protein isolates and dry fractionated flours*

BGN dry-fractionated flour fractions were evaluated for protein, starch and moisture content whilst fat, ash and carbohydrate content were additionally determined for BGN-PIs. The Dumas nitrogen combustion method (FlashEA 1112 series, Thermo Scientific, The Netherlands) was used for determination of the protein content (nitrogen-to-protein conversion factor 5.7; N x 6.25 was calculated to allow comparison to literature reported values). The Soxhlet extraction method with petroleum ether as extraction solvent was used to determine the fat content. Protein and fat determination were carried out in at least triplicate measurements. The moisture and ash contents were determined in duplicate through gravimetric analysis, by oven drying at 105°C overnight and igniting at 525°C for 6 h respectively. Carbohydrate content was calculated by difference and total starch content was analysed with the Megazyme Total Starch/α-Amylase Assay Kit (Megazyme International Ltd., Bray, Ireland).

3.2.5. Particle size distribution and microstructure of Bambara groundnut dry fractionated flours

The particle size distributions of BGN flour fractions were analysed with a laser diffraction particle size analyser fitted with an Aero S dry dispersion unit (Mastersizer 3000, Malvern Instruments Ltd., UK). A pressure of 200 kPa was applied and all measurements performed in triplicate. Furthermore, all fractions were imaged with a desktop SEM (Phenom G2 Pure, Phenom-World BV, The Netherlands) for evaluation of their microstructure. The samples were attached on aluminium pin stubs with carbon adhesives (SPI Supplies/Structure Probe Inc., USA) and the stubs subsequently placed on sample holders for analysis. An acceleration voltage of 5 kV was applied.

3.2.6. Molecular weight determination of Bambara groundnut protein isolates and dry fractionated flours

Polyacrylamide gel electrophoresis (PAGE)

Native-PAGE and (reducing) SDS-PAGE were performed as previously described (Diedericks et al., 2019) to determine the electrophoretic profile of BGN proteins in their native state and to obtain information on the protein subunits, respectively. Briefly, BGN-PI solution (0.04% w/v, pH 7, no added salt) and BGN flour fractions (0.1% w/v) were mixed with the NativePAGE™ sample buffer 4X (Invitrogen, USA). The proteins were resolved on a 4–16% Bis-Tris gel in a molecular weight range of 15–1000 kDa, at a constant voltage of 200 V. The NativeMark™ unstained protein standard (Invitrogen, California, USA) with a molecular weight range of ~20–1200 kDa was used as molecular weight markers.

Similarly, SDS-PAGE analysis was performed under reducing conditions by mixing BGN-PI solution (0.04% w/v, pH 7, no added salt) and BGN flour fractions (0.1% w/v) with the NuPAGE® LDS sample buffer (Invitrogen, USA) and NuPAGE® reducing agent. Heat was applied to the sample mixture (70°C, 10 min) before the proteins were resolved on a 4–12% Bis-Tris gel in the molecular weight range 2–200 kDa. Separation was performed at a constant voltage of 200 V and the Mark12™ unstained standard used as molecular weight markers. Both Native-PAGE and SDS-PAGE gels were stained with a Coomassie® G-250 stain, followed by destaining after which they were scanned with a calibrated densitometer (Bio-Rad GS-900™, USA).

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High performance size-exclusion chromatography coupled with multi-angle light scattering (HPSEC-MALLS)

HPSEC-MALLS analysis was performed as previously described (Diedericks et al., 2019). Briefly, the HPSEC system consisted of an in-line vacuum degasser (1200 series degasser, Agilent Technologies, USA), pump (1200 series binary pump, Agilent Technologies) and four TSK gel analytical columns—PW$_{XL}$ guard, G6000 PW$_{XL}$, G4000 PW$_{XL}$ and G3000 PW$_{XL}$ (Tosoh Bioscience LLC, USA)—connected in series at a fixed temperature of 35°C. The filtered (0.2 μm filter and in-line over a 0.25 μm filter) mobile phase consisting of 100 mM NaNO$_3$ and 0.02% NaN$_3$ were eluted over the columns at a flow rate of 0.5 ml/min. BGN-PI solutions (0.5% w/w) were centrifuged and the supernatants filtered (0.2 μm filter), followed by injection of 200 μl onto the columns. Three detectors were used, i.e. static light scattering (DAWN HELEOS II, Wyatt Technologies, USA) measured at 665 nm and 15 angles (32–144°) for characterisation of molecular weight distribution, UV absorption (1260 series, MWD, Agilent Technologies) for detection of proteins and polyphenols at 280 nm, and refractive index (RI) detection (ERC-7510, Erma Optical Works) at a fixed temperature of 35°C for characterisation of sample concentration. Bovine serum albumin (M$_w$ 67 kDa) was used as reference material for normalization, alignment and band broadening. ASTRA 6 software (Wyatt Technologies) was used for all data collection and processing.

3.2.7. Isoelectric point (pI) determination of Bambara groundnut protein isolates

The electrophoretic mobility of BGN-PI solutions (0.1% w/v, no added salt) was measured in duplicate with the ZS Zetasizer Nano (Malvern Instruments Ltd., UK) to determine the pI. Samples (1 ml) were injected into the capillary cell and measured at room temperature in a pH range of 3.5–7.5.

3.2.8. Thermal stability analysis of BGN protein isolates and dry fractionated flours

The thermal properties of BGN-PI (12% w/v dry matter, varying pH 3.5–7.5) and BGN fractionated flour (12% w/v dry matter) were measured with a differential scanning calorimeter (PerkinElmer STA 6000, USA), by weighing 60 mg of each sample suspension in stainless steel pans which were hermetically sealed before analysis. Samples were heated from 10–120°C and subsequently cooled to 10°C at a rate of 10°C/min. From the thermograms, the onset temperature (T$_o$), peak temperature (T$_p$—indicating starch gelatinisation or protein denaturation) and

enthalpy of transitions (ΔH) were analysed using the manufacturer software (Pyris, PerkinElmer, USA).

3.2.9. *Gelation functionality of BGN protein isolates and dry fractionated flours*

Minimum gelling concentration

The test tube inversion method was used as previously described to experimentally determine the minimum gelling concentration of BGN-PI (pH 7, with and without 200 mM NaCl addition) and BGN protein-enriched fractions. Samples were prepared in 3 ml tubes at various concentrations by heating in a water bath at 95°C for 30 min, before cooling to room temperature for 1 h and overnight at 4°C. After cooling, the tubes were inverted to determine the lowest concentration where the sample does not flow (O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004).

Small deformation rheology

BGN-PI solutions at various concentrations (pH 7, with 200 mM NaCl and without added salt) and varying pH conditions (pH 3.5–7.5, 9.2% w/w protein concentration), and BGN protein-enriched fractions prepared at 12% (w/v) dry matter suspensions were subjected to oscillatory shear measurements using either a MCR502 or MCR302 controlled stress rheometer (Anton Paar, Austria) fitted with a sandblasted concentric cylinder (CC17) geometry, at a strain of 1% and a frequency of 1 Hz. The storage (G') and loss (G") moduli were recorded within the linear viscoelastic regime as a function of temperature—samples were initially heated from 20–95°C, kept at 95°C for 30 min before they were cooled to 20°C (3°C/min) and monitored at this temperature for 25 min.

Theoretical models describing gelation of BGN protein isolates

Two models (percolation and fractal) were applied as previously described for BGN vicilin gels (Diedericks et al., 2019), to characterise the gelation and rheological behaviour of BGN protein isolates (pH 7, with 200 mM NaCl and without added salt). In the percolation model G' scales with protein concentration according to:

$$G' \sim (c - c_p) \tag{3.1}$$

where the $\sim$ symbol indicates "proportional to", c is the protein concentration in the system, c_p is the critical percolation threshold concentration and t the scaling exponent.

In the fractal model, G' is given by:

$$G' \sim c^w \tag{3.2}$$

where the scaling exponent w is related to the fractal dimension (D_f) of the gel network (implicitly c_p is assumed to be zero). In our study D_f was determined through rheological parameters in relation to the scaling exponents by using the models of Shih, Shih, Kim, Liu, & Aksay (1990) and Wu & Morbidelli (2001).

3.2.10. Microstructure of BGN protein isolate gels

Confocal laser scanning microscopy (CLSM)

BGN-PI gels were prepared for microstructural analysis by non-covalent labelling of various BGN-PI solutions (pH 7, 200 mM NaCl) with the fluorescent dye Rhodamine B (0.005% w/w final concentration). The solutions were heated for 30 min at 95°C in sealed glass chambers (Gene Frame 125 µl adhesives, Thermo Fisher Scientific, UK) before cooling for 1 h at room temperature and overnight at 4°C. Imaging was done with a Zeiss LSM 510 META confocal microscope equipped with an Axiovert 200M inverted microscope (40x 1.3 oil immersion objectives). Rhodamine B was excited at 543 nm (He-Ne laser).

Scanning electron microscopy (SEM)

BGN-PI gels were prepared for SEM analysis in 10 ml pre-lubricated syringes through heat-induced gelation at 95°C for 30 min. Gels were evaluated at three concentrations (6.0%, 8.7% and 11.9% w/w) at pH 7 and in the presence of 200 mM NaCl. Imaging was performed as described by Urbonaite et al. (2016). Briefly, proteins were crosslinked by submerging the cut gel pieces in an aqueous glutaraldehyde solution (2.5% v/v) for 8 h. After removal of the glutaraldehyde with deionised water and subsequently with ethanol, the gel pieces were subjected to critical point drying (Leica Automated Critical Point Dryer EM CPD300, Leica, Austria), fractured and attached to sample holders with Carbon Adhesive (Electron Microscopy Sciences, USA). Before analysis in a field emission SEM (Magellan 400, FEI, The Netherlands), the solvent was evaporated and a 15 nm thick layer of Tungsten (MED 020, Leica, Austria) sputter coated onto the samples. The SEM was operated at a working distance of 4 mm with 2 kV and 13 pA SE detection.

3.3. Results and Discussion

3.3.1. *Compositional analysis and yield*

The isoelectric precipitated protein isolates had a protein content of 76.4 ± 1.3 g/100 g (N x 5.7) or 83.8 ± 1.4 g/100 g (N x 6.25). Similar to what was found for the major BGN storage protein vicilin, BGN-PI carbohydrates (9.3–16.6%, calculated by difference) constituted the largest non-protein component with other minor components of fat (0.3%), moisture (2.2%) and ash (4.5%). These values are comparable to those previously reported for isoelectric precipitated BGN proteins (Kaptso et al., 2015; Kudre, Benjakul, & Kishimura, 2013), although in our samples a lower amount of fat was detected as a result of the defatted flour. The dry weight yield and the protein yield of BGN-PI were 15.5 g and 11.6 g, respectively, per 100 g defatted flour.

BGN pre-milled seeds were subjected to two types of milling before preparation of protein-enriched fractions through dry fractionation. The composition of the resultant flour fractions are shown in Table 3.1. Although having a higher protein content, the milling yield of impact milled flour (28.9%) was much lower compared to that of the rotor milled flour (77.4%). The impact mill is a pilot scale mill which in itself is not ideal for processing of smaller batches, as particles are known to accumulate on the mill's inner walls (Pelgrom, Berghout, van der Goot, Boom, & Schutyser, 2014). In comparison, the rotor mill is considered suitable for small scale grinding under cryogenic conditions (Meghwal & Goswami, 2014). Furthermore, protein-enriched fractions with protein contents of 28.6% for the air classified impact milled flour and 32.6% for the rotor milled air jet sieved flour were obtained. The protein content of the impact milled protein-enriched fine fraction are lower compared to that reported for yellow pea (42.9 g/100 g dry matter) and lupine (46.2–53.7 g/100 g dry matter), which could be attributed to the different classifier wheel speeds and the larger batch sizes used for processing (Pelgrom et al., 2014, 2015a). The protein content of the rotor milled protein-enriched fractions were however comparable to that reported for quinoa varieties (32–32.7 g/100 g dry matter), obtained at higher sieve mesh sizes during rotor milling and air jet sieving (Opazo-Navarrete, Tagle Freire, Boom, Janssen, & Schutyser, 2018). Both impact milled and rotor milled protein-enriched (fine) fractions were characterised with a low starch content as opposed to the starch-enriched (coarse) fractions, which indicates that separation of these fractions can be achieved through air classification and air jet sieving. BGN flour is thus suitable for dry fractionation through the techniques employed.

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**Table 3.1.** Composition and milling yield of Bambara groundnut fractions obtained through dry fractionation.

	Protein (N x 5.7)[a]	Moisture	Starch	Yield (dw)
	(g/100 g)			
IM flour	21.7 ± 0.2 (23.8)	2.5 ± 0.5	29.2 ± 0.8	28.9
RM flour	17.7 ± 0.0 (19.4)	2.5 ± 0.5	37.5 ± 3.3	77.4
IM coarse	17.7 ± 0.5 (19.4)	2.5 ± 0.5	31.0 ± 0.1	3.6
RM coarse	15.4 ± 0.1 (16.9)	2.0 ± 0.0	39.0 ± 0.2	80.5
IM fine	28.6 ± 0.2 (31.4)	2.0 ± 0.0	5.0 ± 0.7	9.4
RM fine	32.6 ± 0.2 (35.7)	1.5 ± 0.5	9.1 ± 0.6	16.2

[a] N x 6.25 indicated between brackets
IM: impact milled, RM: rotor milled, dw: dry weight.

3.3.2. *Particle size distribution and microstructure of BGN dry fractionated flours*

The particle size distribution of BGN dry fractionated flours are shown in Fig. 3.1. The rotor milled flour had a wider size distribution and higher volume-averaged particle diameter ($D_{0.5}$ 41.0 µm) compared to the impact milled flour ($D_{0.5}$ 27.4 µm). The literature reported volume-averaged particle diameter of various impact milled legume flours, obtained at a similar classifier wheel speed (2900 rpm) as used in our study, ranged from 12.4–17.1 µm (Pelgrom, Boom, & Schutyser, 2015b). The differences in particle size were attributed to seed hardness, with harder seeds characterised with a higher $D_{0.5}$. The higher $D_{0.5}$ obtained for the BGN impact milled flour could thus be attributed to the hardness (hard-to-cook and hard-to-mill phenomena) associated with BGN seeds (Mubaiwa, Fogliano, Chidewe, & Linnemann, 2017). After air classification of the impact milled flour, the shift in particle size distribution curves of the protein-enriched fine fraction ($D_{0.5}$ 24.4 µm) and the starch-enriched coarse fraction ($D_{0.5}$ 222.3 µm) could be clearly distinguished. Similarly, after air jet sieving of the rotor milled flour, the protein-enriched fine fraction had a smaller volume-averaged particle diameter ($D_{0.5}$ 17.9 µm) compared to the starch-enriched coarse fraction ($D_{0.5}$ 45.5 µm). Both dry fractionation techniques are thus suitable for obtaining BGN flour rich in protein, with the rotor milling and air jet

sieving combination giving a higher separation under the applied conditions. In addition, the SEM micrographs as shown in Fig. 3.2, confirms the partial separation of the protein-rich cell fragments from the intact smooth and oval-shaped starch granules after air classification and air jet sieving. This is in agreement to the compositional and particle size distribution data, which have shown that separation of the fine protein-enriched and the coarse starch-enriched fractions was achieved.
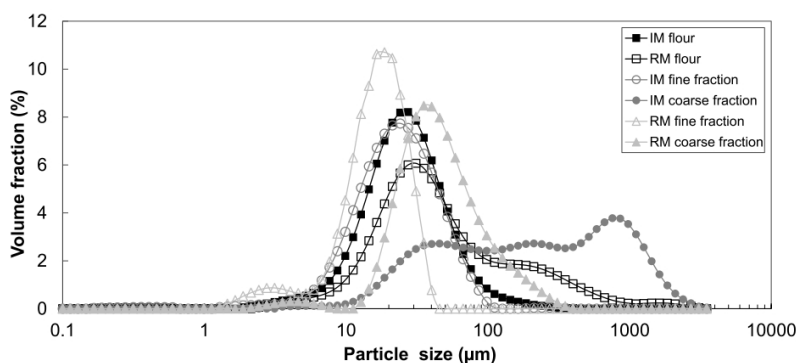
Fig. 3.1. Particle size distribution of Bambara groundnut impact milled (IM) and rotor milled (RM) flour, and their corresponding dry fractionated fine (protein-rich) and coarse (starch-rich) fractions.
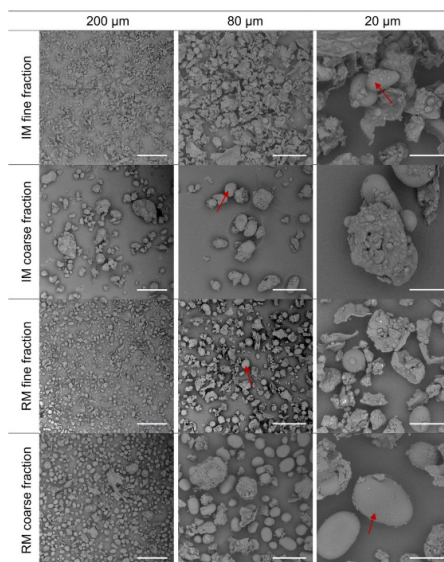

Fig. 3.2. Scanning electron micrographs of Bambara groundnut impact milled (IM) and rotor milled (RM) fine and coarse fractions. Starch granules are indicated with arrows; scale bars correspond to 200 μm, 80 μm and 20 μm, respectively.

3.3.3. *Molecular weight characterisation of BGN protein isolates and fractionated flours*

Gel electrophoresis

Native-PAGE and SDS-PAGE profiles of BGN-PI and dry fractionated BGN flour are shown in Fig. 3.3. The patterns in the Native-PAGE revealed similar bands for all samples, although lower protein concentrations in the flour samples resulted in lighter bands. The major bands of approximately 190 kDa and 360 kDa were identified in all samples. As previously reported, the band of 190 kDa relates to the trimeric structure of BGN vicilin, with a known molecular weight of 196 kDa (Diedericks et al., 2019). The band at 360 kDa is indicative of legumin, an hexameric globulin storage protein with a reported molecular weight range between 320–380 kDa which consists of six subunit pairs linked by disulphide bonds (Barac et al., 2010; Shewry, Napier, & Tatham, 1995).

Under reducing SDS-PAGE, the low molecular weight proteins present in BGN-PI (lane 7) are not recovered in the flour fractions which could be as a result of the lower protein concentration of these samples. The major bands in the approximate range of 50–63 kDa corresponds to the β and α subunits of BGN vicilin as previously identified (Diedericks et al., 2019). The lower molecular weight band at 25 kDa could be attributed to the basic subunit of legumin, whereas the band observed around 97 kDa is indicative of lipoxygenase as identified by Barac et al. (2010) for pea protein isolates. The electrophoretic profile of BGN-PI is similar to that previously reported for isoelectric precipitated proteins from different BGN varieties (Adebowale et al., 2011).
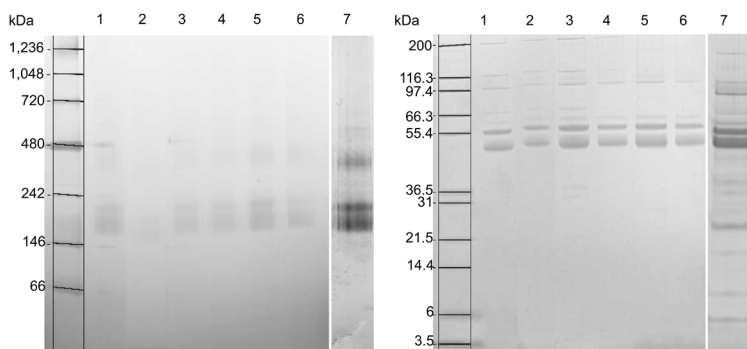
Fig. 3.3. (Left) Native-PAGE and (Right) reducing SDS-PAGE patterns in lane 1—Impact milled [IM] flour, lane 2—rotor milled [RM] flour, lane 3—RM fine fraction, lane 4—RM coarse fraction, lane 5—IM fine fraction, lane 6—IM coarse fraction and lane 7—BGN protein isolate (pH 7).

Size-exclusion chromatography

HPSEC analysis was used to characterise the molecular weight of BGN protein isolates. The protein isolates were characterised with a wide molecular weight distribution indicative of a heterogeneous protein population, as determined from combined MALLS and RI signals (Fig. 3.4). BGN-PIs obtained from different extraction batches had mostly similar peaks which reveals the reproducibility of the extraction process. The major peak, previously identified as the BGN vicilin fraction (M_w 196 kDa) (Diedericks et al., 2019), was present as the largest peak in both BGN-PIs. The molecular weights were calculated as 205 kDa and 274 kDa respectively, with the higher M_w of the latter batch caused by the presence of some larger oligomers as seen from the concave shape of the M_w slope (Pothecary, Ball, & Clarke, 2012) and confirmed by the higher radius of gyration (Rg = 35 nm). In this BGN-PI batch, a higher degree of aggregate formation was evident as also seen from the peak eluting earlier at the void volume, which gave a large light scattering signal with almost no RI and UV responses.

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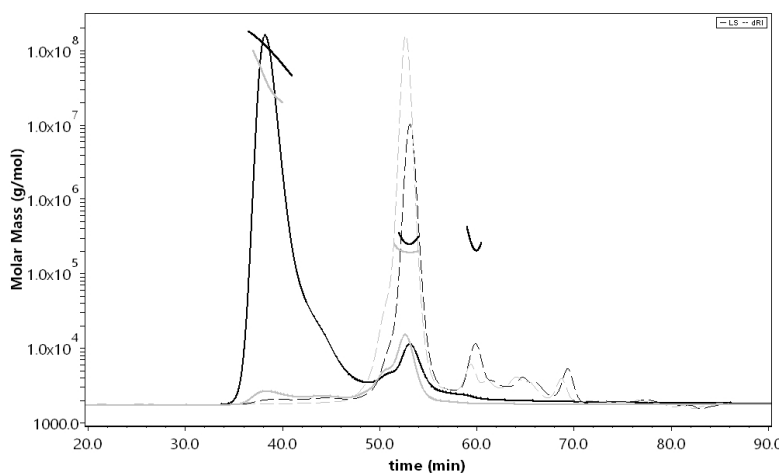
Fig. 3.4. Molar mass profiles as obtained from MALLS (straight line) and RI (dashed lines) responses of Bambara groundnut protein isolates from two different extraction batches as indicated by the black and grey lines.

3.3.4. Isoelectric point (pI) determination of BGN protein isolates

The isoelectric point of BGN-PI was determined around pH 4.3 by neutral electrophoretic mobility, as shown in Fig. 3.5. This is in the typical range for legume proteins where pI's between pH 4–5 have been identified (Boye et al., 2010). At increasing pH from 3.5–7.5, the electrophoretic mobility changed from positive to negative, with absolute measurement values comparable to those previously reported for BGN isoelectric precipitated proteins (Busu & Amonsou, 2018).
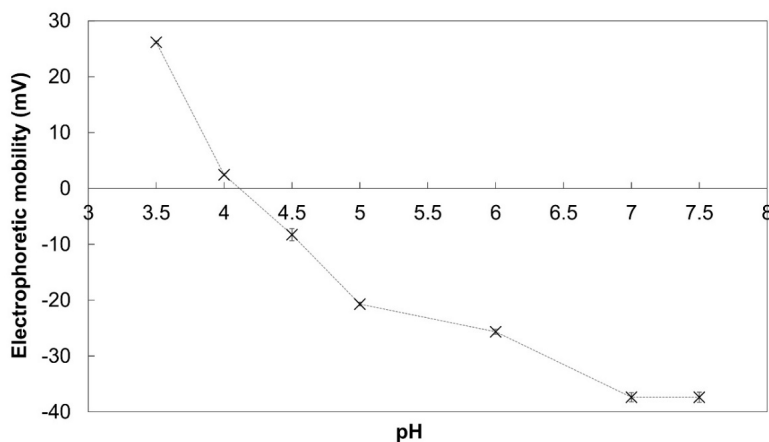

Fig. 3.5. Electrophoretic mobility of BGN protein isolates as a function of pH.

3.3.5. Thermal properties of BGN protein isolates and dry fractionated flour

The thermal transition parameters of BGN-PI obtained from differential scanning calorimetry are shown in Fig. 3.6. The onset and peak denaturation temperatures of BGN-PI increased sharply up to pH 4.5 (close to the pI) before reaching a maximum at pH 5 (T_p 93.3°C), followed by a gradual decrease to a plateau at neutral pH (T_p 91.1°C). The enthalpy of denaturation showed an opposite trend, with a plateau at acidic pH up to pH 5 (ΔH 0.48 J/g) before sharply increasing to pH 6 and reaching a maximum at pH 7 (ΔH 0.91 J/g). The thermal stability of (plant) proteins is known to be affected by changes in pH. Arntfield & Murray (1981) observed the same effect of pH on fababean proteins and attributed the lower T_p and ΔH at acidic pH to the increased positive charges which weakens the protein structure (due to

unfolding); whilst maximum values were recorded around the pI at net neutral charge. The highest thermal stability for canola protein isolates was determined at pH 7, which was attributed to an increase in covalent and non-covalent interactions (Kim, Varankovich, & Nickerson, 2016). The T_p of BGN-PI at pH 7 is similar to that previously reported for BGN vicilin (T_p 92°C), which highlights the presence of vicilin as the major protein fraction in BGN-PI (Diedericks et al., 2019).

   The BGN protein-enriched fine fractions were characterised with higher T_p and lower ΔH values compared to the flour and starch-enriched coarse fractions (Table 3.2). Compared to the peak denaturation temperature and enthalpy of BGN-PI at neutral pH, the protein-enriched fractions were characterised with lower values. This is as expected for heterogeneous systems, where thermal transitions are influenced by exothermic and endothermic contributions from both protein and starch components (Henshaw, McWatters, Akingbala, & Chinnan, 2003). The impact and rotor milled BGN flour and coarse fractions were characterised with a T_p in a similar range as previously reported for starches (T_p 73.1–81.7°C) from different BGN varieties (Kaptso et al., 2015; Oyeyinka, Singh, & Amonsou, 2017). Compared to impact milled pea flour and their fine fraction, where two distinct peaks attributed to starch gelatinisation (T_p 66.3–70.3°C) and protein denaturation (T_p 88.2–89.2°C) were observed (Pelgrom et al., 2015a), only one distinct peak could be measured for all BGN flour and fractions.
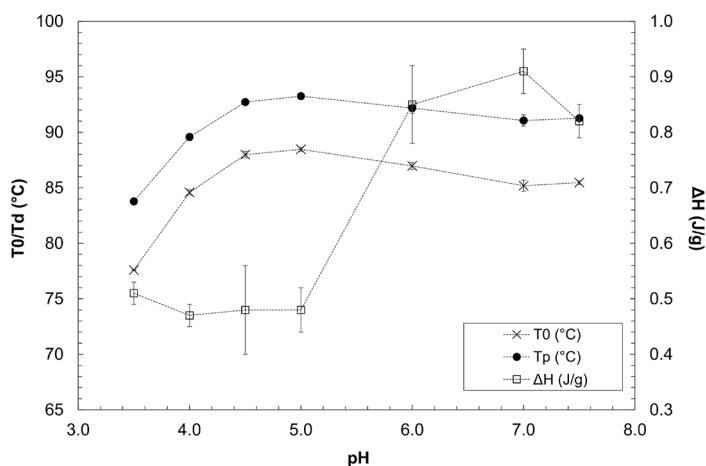
Fig. 3.6. Thermal properties of BGN-PI solutions (9.2% w/w) as a function of pH. T_o: onset temperature; T_p: denaturation temperature; ΔH: enthalpy.

**Table 3.2.** Thermal properties of dry fractionated Bambara groundnut flours.

	T_o (°C)	T_p (°C)	ΔH (J/g)
IM flour	73.8 ± 0.2	79.2 ± 0.0	0.63 ± 0.19
RM flour	69.9 ± 0.5	76.5 ± 0.1	0.63 ± 0.06
IM coarse	74.6 ± 0.1	79.5 ± 0.1	0.57 ± 0.02
RM coarse	71.8 ± 0.3	77.5 ± 0.0	0.71 ± 0.15
IM fine	82.4 ± 0.2	86.2 ± 0.3	0.17 ± 0.02
RM fine	72.0 ± 0.1	80.8 ± 4.4	0.34 ± 0.26

T_o: onset temperature, T_p: peak temperature, ΔH: enthalpy, IM: impact milled, RM: rotor milled.

3.3.6. *Gelling properties of BGN protein isolates and dry fractionated flour*

The viscoelasticity of BGN-PI gels was probed by dynamic oscillatory measurements at various protein concentrations (with and without the addition of 200 mM NaCl). Gel formation as indicated by the G'/G" cross-over point was observed during isothermal heating at 95°C for all BGN-PI concentrations in the absence of salt, except for the 6.9% (w/w) solution where G" remained higher than G' indicating predominant viscous (liquid-state) behaviour. The cross-over point was independent of ionic strength. However, upon salt addition gel formation occurred at lower concentrations (5.2% w/w), presumably as a result of decreased electrostatic repulsion which allows attractive interactions to dominate (Ikeda, Foegeding, & Hagiwara, 1999), and overall higher gel strengths were observed. This observation is also seen in the minimum gelling concentrations (see insets Figs. 3.8 and 3.9), which were found at 8.6% (w/w) and 5.5% (w/w) for BGN-PI without salt and BGN-PI in the presence of 200 mM NaCl, respectively. Furthermore, the shift to higher G' values for the BGN-PI gels with 200 mM NaCl and at similar protein concentrations as the samples to which no salt was added (Fig. 3.7), indicates a higher gelation efficiency of BGN-PIs in the presence of salt. The gelation behaviour of BGN-PI (pH 7, 200 mM NaCl) is also similar to that of BGN vicilin under the same conditions (Diedericks et al., 2019), which implies that the vicilin fraction controls the gelation of the isoelectric precipitated protein isolates. The linear viscoelastic region was mostly independent of protein concentration with a limiting strain (γ_o) around 50% (Fig. 3.8) for BGN-PI gels without salt, whereas in the presence of

200 mM NaCl the γ_o decreased to around 20% (Fig. 3.9). The strain sweeps are comparable to that found for BGN vicilin gels in three distinct concentration regimes (Diedericks et al., 2019).
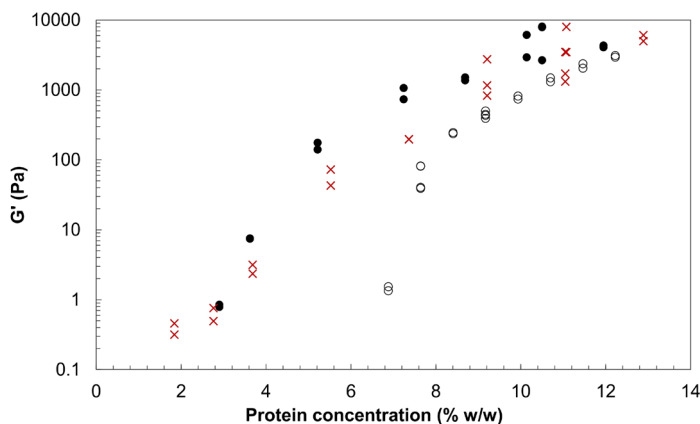
Fig. 3.7. Elastic moduli (G') dependence on Bambara groundnut protein concentration as influenced by salt addition. o—BGN-PI (without salt), ●—BGN-PI (200 mM NaCl), x—BGN vicilin (200 mM NaCl).
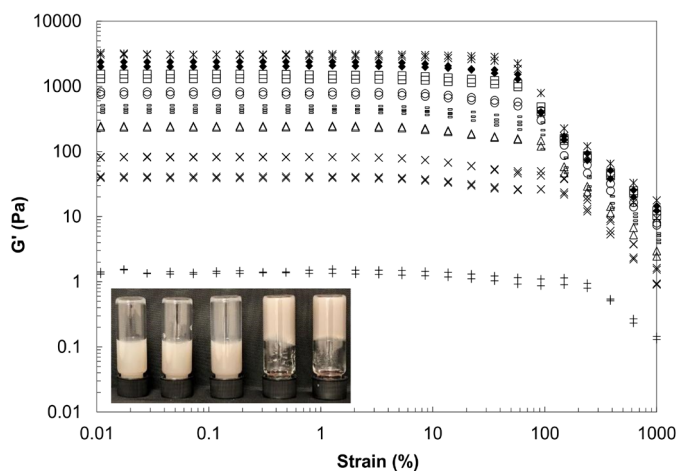
Fig. 3.8. Strain sweep of Bambara groundnut protein isolate solutions (pH 7, no added salt) at various concentrations (ж12.2%, ♦11.5%, □10.7%, o9.9%, −9.2%, Δ8.4%, x7.6%, +6.9%). **Inset:** Minimum gelling concentration determination; increasing protein concentrations (3.7%, 5.5%, 7.4%, 8.6%, 10%) from left to right.
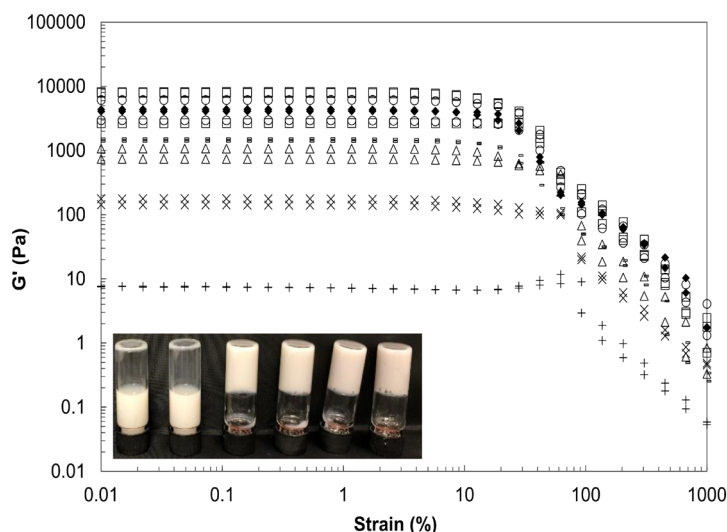
Fig. 3.9. Strain sweep of Bambara groundnut protein isolate solutions (pH 7, 200 mM NaCl) at various concentrations (♦11.9%, □10.5%, o10.1%, −8.7%, Δ7.2%, x5.2%, +3.6%). **Inset:** Minimum gelling concentration determination; increasing protein concentrations (2.8%, 3.7%, 5.5%, 7.4%, 8.6%, 10%) from left to right.

In addition, the viscoelasticity of BGN-PI gels (9.2% w/w) was determined as a function of pH (Fig. 3.10). Gel formation was evident at all pH values, with the G'/G" cross-over point observed during the first 15 min of the initial heating step (20–95°C) for BGN-PIs in the pH range 3.5–6, whereas at higher pH (7 and 7.5) the sol-gel transition was observed after 32 min during isothermal heating (95°C). The delayed sol-gel transition at pH 7 and 7.5 is as a result of the higher thermal stability of BGN-PI at this pH, as discussed in section 3.3.5. Furthermore, gels in the acidic pH range were characterised with higher elasticity compared to those at neutral/ alkaline pH. The more acidic gels (pH 3.5–5) were also characterised with a lower limiting strain ($\gamma_o \sim 5\%$) compared to gels at pH 6 ($\gamma_o \sim 30\%$) and pH 7 and 7.5 ($\gamma_o \sim 60\%$).

The minimum gelling concentration of BGN protein-enriched fine fractions as obtained from impact milled and rotor milled flour, were determined at 12% (w/v) dry weight and 11% (w/v) dry weight respectively. Taking into consideration the protein content of these fractions, self-supporting gels were visually observed at minimum protein concentrations (N x 5.7) of 3.4% for the impact milled protein-enriched fraction and 3.6% for the rotor milled fraction as shown in Fig. 3.11. These

fractions were further probed with dynamic oscillatory measurements at their observed minimum gelling concentrations. In both protein-enriched fractions, no clear sol-gel transition was observed as G' remained higher than G" throughout the measurements. The average final G' values for the impact milled and rotor milled protein-enriched fractions were 292.5 ± 103.5 Pa and 80.5 ± 32.6 Pa respectively. The impact milled flour are thus characterised with a higher gel strength compared to the rotor milled flour, under the specific conditions used.
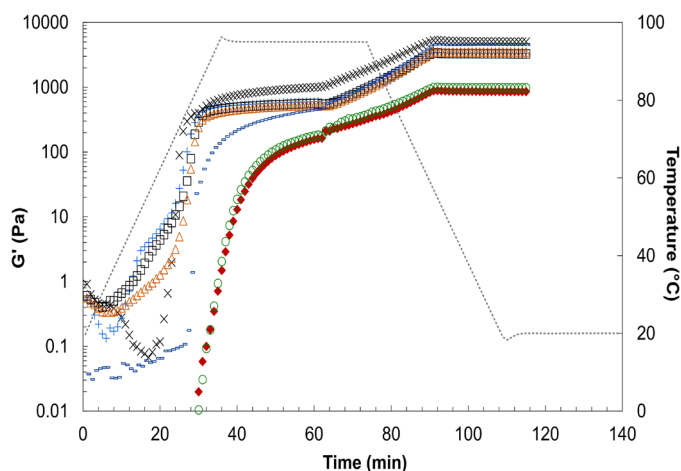
Fig. 3.10. Effect of pH on the elasticity (G') of heat-induced Bambara groundnut protein isolate gels (9.2% w/w protein concentration). Different symbols indicate BGN-PIs at different pH: x3.5, +4.0, □4.5, Δ5.0, −6.0, ♦7.0, ○7.5.
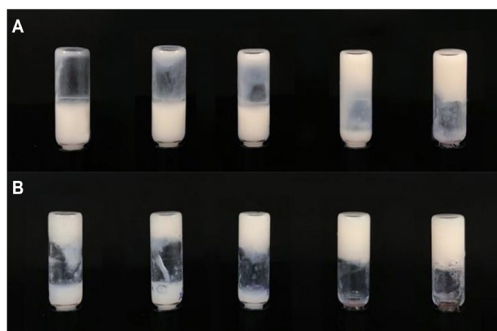
Fig. 3.11. Minimum gelling concentration determination of (A) impact milled and (B) rotor milled Bambara groundnut protein-enriched flour fractions. Increasing dry weight concentrations 8–12% from left to right.

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3.3.7. *Applicability of gelation models to BGN protein isolate gels*

Percolation approach

The percolation model was applied to determine the dependence of the elastic moduli (G') on BGN protein concentrations. Through a multi-step fitting procedure as described by Van der Linden & Sagis (2001) for various protein systems, the critical percolation concentration (c_p) and the scaling exponent (t) as defined from eq. (3.1) were determined. As shown in Table 3.3, BGN-PI gels with 200 mM NaCl addition were characterised with lower c_p and t values compared to the gels without added salt. This t value (t = 2.0) is also lower compared to that found for the major BGN protein fraction vicilin (t = 4.91), whilst it is comparable to predictions from the isotropic percolation model (Mitescu & Musolf, 1983). This implies that the elasticity of BGN-PI gels in the presence of salt can be predicted with the percolation model, which is indicative of a system with an homogeneous structure. The decrease in c_p from 6.5% to 2.9% is comparable to that reported for protein isolates from kidney bean at pH 2, where a decrease in critical percolation concentrations were observed at increasing ionic strengths (Zhang et al., 2010). Similarly, a decrease in c_p was observed for ovalbumin gels in the presence of salt, which is attributed to the change in electrostatic interactions favouring protein-protein attraction (Van der Linden & Sagis, 2001).

Table 3.3. Scaling parameters of BGN protein isolate gels at pH 7 as obtained with the percolation and fractal scaling models.

NaCl (mM)	Percolation parameters		Fractal scaling parameters			
	c_p	t	w	$D_f{}^a$	β	α
0	6.50	2.72	8.43	2.49	0.94	1.02
200	3.70	2.00	4.43	2.77	1.05	0.98

[a] Fractal dimensions as determined with the Shih et al. (1990) and Wu & Morbidelli (2001) models

Fractal scaling

The elastic moduli (G') plotted as a function of protein concentration revealed a power-law relation as shown in Fig. 3.12, from which positive scaling exponents (w) could be extracted as defined from eq. (3.2). The scaling exponents were used to

determine the fractal dimensions (D_f) from the Shih et al. (1990) and Wu & Morbidelli (2001) models, whilst additional parameters (β—constant which ranges between 1 and 3+x depending on value of α; α—elastic constant used a measure to validate the actual gel regime) were also calculated from the latter model as shown in Table 3.3. In addition, a negative critical strain (γ_o) exponent (-0.37) was found for BGN-PI gels without salt addition, whilst a positive slope (0.72) was found for gels in the presence of 200 mM NaCl. According to the Shih et al. (1990) model, this is indicative of a strong-link and a weak-link regime, respectively. Taking into consideration the α parameter, which is close to 1, a weak-link regime is confirmed (Wu & Morbidelli, 2001) for both BGN-PI gel systems. These gels are thus characterised as rigid aggregate structures, which are in agreement to the findings for BGN vicilin gel systems (Diedericks et al., 2019).

Considering the apparent applicability of both the percolation and fractal scaling models to BGN-PI gels, it can be concluded that predictions from these two models do not provide a clear picture in terms of the critical gelling concentration (c_p) and fractal dimensions of these systems.

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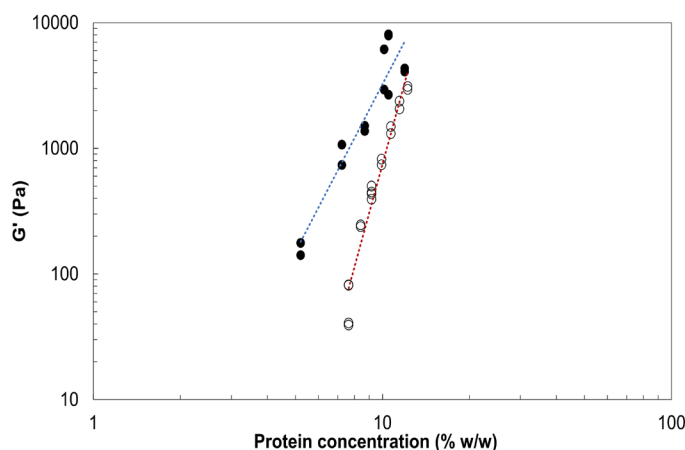
Fig. 3.12. Scaling behaviour of storage modulus G' as a function of BGN-PI concentration. o—BGN-PI (without salt), ●—BGN-PI (200 mM NaCl).

3.3.8. *Microstructure of BGN protein isolate gels*

CLSM and SEM (Fig. 3.13) were used to probe the microstructure of BGN protein isolate gels at 200 mM NaCl concentration, as a means of comparison to the microstructures of BGN vicilin gels as previously reported (Diedericks et al., 2019). From the CLSM images (length scale 50 µm), some inhomogeneities are visible at higher protein concentrations, which was not observed in the case of BGN vicilin gels. In the corresponding SEM images, comparable structures as were found for the BGN vicilin gels were observed in a range of length scales from 3 µm down to 0.3 µm, which was attributed to inhomogeneous aggregate structures. This apparent inhomogeneity thus highlights the inconsistencies of the percolation approach, where homogeneous structures were implied from the scaling exponent.
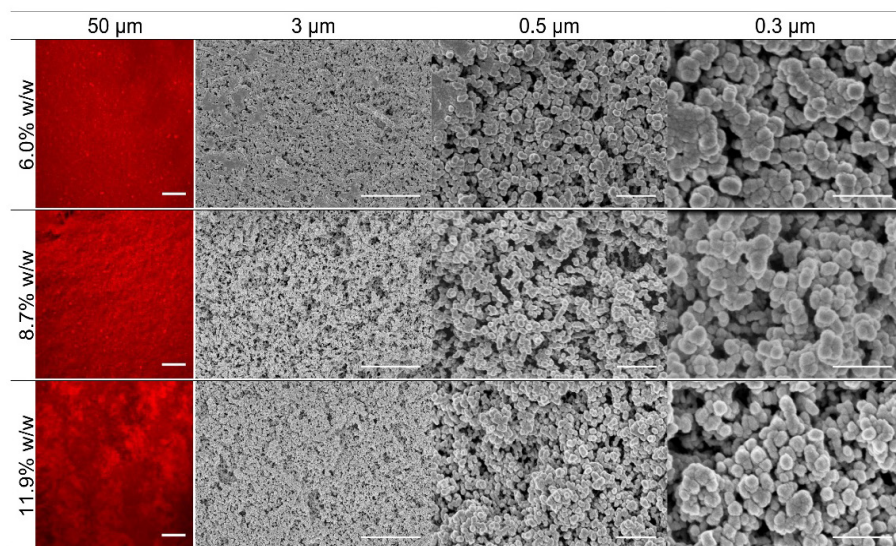
Fig. 3.13. Microstructure of BGN protein isolate gels at various protein concentrations (pH 7, 200 mM NaCl) on different length scales as imaged with CLSM (first column) and SEM (last three columns). For the CLSM images the inserted scale bar corresponds to 50 µm, whilst for the SEM images the scale bars correspond to 3 µm, 0.5 µm and 0.3 µm, respectively.

3.4. Conclusions

Protein isolates were extracted from Bambara groundnut black-eye seeds through isoelectric precipitation. Molecular weight analysis revealed that vicilin was the major fraction present in BGN-PI, which was also supported by the denaturation temperature of the protein isolates at pH 7 coinciding with that of BGN vicilin. Furthermore, the role of BGN vicilin as the major protein fraction in the protein isolate mixture was also identified in their gelling behaviour. Upon 200 mM NaCl addition, a clear shift in G' patterns was observed for BGN-PI gels which was consistent with that of BGN vicilin gels at the same salt concentration. In terms of characterisation of the gel networks, two scaling models (isotropic percolation and fractal scaling) were applied which appeared to both be applicable in describing the gel network structures. However, the microstructural images revealed inhomogeneous structures which highlighted the inconsistencies of the percolation model. As such, caution should be applied when using these scaling models in drawing conclusions on the processes governing the gelation of BGN-PIs. Furthermore, we were also able to successfully apply dry fractionation techniques to obtain protein-enriched BGN fractions. Two milling techniques, i.e. impact milling with air classification and rotor milling with air jet sieving were evaluated, with the latter providing a fraction with higher yield and protein content under the conditions applied. In terms of functionality, gel formation was evident at low protein concentrations for both impact milled and rotor milled fractions (3.4% and 3.6%, respectively), with the impact milled fraction characterised with a higher gel strength (G') as measured at the minimum gel concentration.

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Acknowledgements

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4

Effect of pH and mixing ratios on the synergistic enhancement of Bambara groundnut-whey protein gels

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Abstract

Plant and animal-based protein mixtures are increasingly recognised as a new group of functional ingredients offering novel structuring capabilities. When combining new sources of plant and animal proteins, it is important to gain a mechanistic understanding of such mixtures to enable their use as food ingredients. In this study we have investigated the synergistic enhancement of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) protein isolates [BGN-PI] when combined with whey proteins [WPI] in heat-induced gelled mixtures. Mixed proteins were characterised for their rheological and structural properties at 12% (w/w) total protein concentrations at three mixing ratios (70:30, 50:50 and 30:70) and three pH values (pH 3, 5 and 7), with comparison to the single proteins systems. At acidic pH (pH 3) WPI dominated the gel formation of the mixed gels with BGN-PI having no effect, whilst close to the isoelectric point of both proteins at pH 5, BGN-PI lowered the gel strength of the mixtures. Synergistic enhancement was observed at pH 7, where independent of the BGN-PI concentration in the mixtures, the mixed gels were characterised with similar high gel strengths compared to that of the single BGN-PI. Hence, BGN-PI dominated the elasticity of the mixed gel networks at neutral pH, which to the best of our knowledge appears novel for a plant protein in admixture with WPI.

4

4.1.   Introduction

In recent years, plant proteins have become of increasing interest from not only a nutritional perspective, but also as a means of creating a more sustainable and secure food supply (Day, 2013; Nadathur, Wanasundara, & Scanlin, 2017). Pulses which are an important group of plant proteins, were shown to reduce on average 40% of carbon emissions and 17% of land use, when producing 1 kg of proteins consisting of equal amounts milk and pulses (Calles, Xipsiti, & del Castello, 2019; Nijdam, Rood, & Westhoek, 2012). As such there is also an increasing trend to establish the protein functionality of pulse (and other legume) crops in admixture with animal-derived proteins (Ben-Harb et al., 2018; Comfort & Howell, 2002; Wong, Vasanthan, & Ozimek, 2013).

However, when blending plant and animal-based proteins it is important to understand the mechanical and structural properties of these blends, considering that challenges in textural and other sensorial properties can arise (Ainis, Ersch, & Ipsen, 2018; Jose, Pouvreau, & Martin, 2016). Soy proteins as commercially available plant proteins have been extensively studied for their rheological behaviour in combination with whey proteins, which are an important group of proteins well-known for their gelation functionality in foods. The addition of soy to whey protein systems at different ratios resulted in gels with similar gel strengths, but with visibly different microstructures (McCann, Guyon, Fischer, & Day, 2018). As also shown by Roesch & Corredig (2005), the concentration of soy to whey proteins is an important factor in the gelation kinetics, with soy present in lower amounts in the mixtures behaving in a similar manner as the single whey protein gels whereas at higher concentrations there is a difference in gel network development. Similarly, Jose et al. (2016) have shown that both soy and whey proteins contributed to the formation of a gel network when mixed at different ratios, however noting the decrease in gel strength upon increasing concentrations of soy proteins in the mixed protein gels. In comparison to soy proteins, research on other plant proteins and specifically pulse proteins in admixture with whey proteins, and their effect on gelation functionality, is limited (Ainis et al., 2019, 2018; Wong et al., 2013). Hence, considering again the shift towards sustainable and "climate-smart crops" (Calles et al., 2019; Nadathur et al., 2017), it becomes increasingly important to not only diversify our diets with untapped protein sources such as indigenous pulses and ancient grains, but to also establish the functionality of such proteins as food ingredients.

As a high-quality protein pulse crop widely grown in West Africa (Feldman, Ho, Massawe, & Mayes, 2019), Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] proteins has shown promising gelling behaviour upon heat-induced gelation

(**Chapter 2** and **3**). As such to further explore its potential as a sustainable plant protein source, the aim of this study was to determine the effect of BGN proteins in admixture with whey proteins on the rheological properties and network structures of the resulting gels. The single BGN and whey proteins were investigated and compared to the mixed protein systems, at varying pH and at different mixing ratios. As such, we could gain insights into the structuring ability of BGN proteins in mixed systems and establish the synergistic enhancement under the conditions investigated.

4.2. Materials and Methods

4.2.1. Materials

Whey protein isolate (WPI) (BiPro JE-099-2-420) with a protein content of 97.7% (specified by manufacturer) was obtained from Davisco Foods International Inc. (Le Sueur, USA). Black-eye Bambara groundnut seeds were obtained from Thusano Products (Louis Trichardt, Limpopo, South Africa) and processed into defatted flour as described in the preceding chapters. All chemicals used were of analytical grade.

4.2.2. Extraction of Bambara groundnut protein isolates and solutions preparation

Bambara groundnut protein isolate (BGN-PI) was extracted from defatted black-eye flour with the isoelectric precipitation method as described in **Chapter 3**. Briefly, the flour was dispersed in deionised water at a ratio of 1:10 (w/v) and the pH adjusted to 9.5 with 1 M NaOH before being stirred for 2 h at 4°C. The solubilised proteins were obtained by centrifugation (Avanti J-26 XP, Beckman Coulter, USA) at 4000 g (30 min, 4°C) in the supernatant, which were then adjusted to pH 4 with 1 M HCl for isoelectric precipitation. The precipitated proteins were recovered in the residue after a second centrifugation step and readjusted to pH 7, before being freeze-dried as the protein isolates. The protein content of the BGN-PIs was 72.6% as determined with the Dumas nitrogen combustion method (N x 5.7).

Stock solutions of WPI and BGN-PI were prepared in deionised water to reach at least 12% w/w final protein concentrations at pH 3, 5 or 7 (adjusted with 1 M NaOH or 1 M HCl), under continuous overnight stirring at 4°C. Following dissolution, the stock solutions were mixed at three ratios of WPI to BGN-PI (70:30, 50:50 and 30:70) to a total protein concentration of 12% w/w. All samples were stored at 4°C in the presence of 0.02% (w/w) NaN_3 to prevent microbial spoilage.

4.2.3. Determination of free thiol groups

The accessible thiol groups in WPI and BGN-PI were determined with the Ellman's assay (Ellman, 1959) as described in the protocol by Aitken & Learmonth (2009). Briefly, 10 mM of 5,5′-dithiobis(2-nitrobenzoicacid) (DTNB) were dissolved in 0.1 M phosphate buffer at pH 8 and a volume of 100 μl added to a cuvette containing 3 ml denaturing buffer (6 M guanidinium chloride in 0.1 M Na$_2$HPO, pH 8) for absorbance reading at 412 nm with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies Inc., USA). The protein solution (100 μl) was then added to the cuvette and after thorough mixing, the absorbance read at 412 nm. A reference cuvette was prepared in the same manner containing 100 μl reaction buffer (0.1 M phosphate, pH 8) without DTNB. The absorbance values determined at 412 nm were used to calculate the thiol concentrations, using an extinction coefficient of 13600 M/cm (Cornacchia, Forquenot De La Fortelle, & Venema, 2014).

4.2.4. Visual observation and microstructure of protein gels

A volume of 12 ml of the stock solutions and their mixtures at the different ratios were prepared in pre-lubricated 30 ml cups. These samples were subjected to heat-induced gelation by heating in a water bath at 95°C for 30 min, after which they were cooled at room temperature for 1 h and overnight at 4°C. Following cooling, the cups were inverted to visually evaluate the gels which were formed.

Scanning electron microscopy (SEM) was used to evaluate the microstructure of the single and mixed WPI and BGN-PI gels. Gels were prepared in pre-lubricated 10 ml syringes after which it was prepared for SEM imaging as previously described for BGN protein gels. In brief, gels were cut into pieces and cross-linked in aqueous glutaraldehyde solution (2.5% v/v) for 8 h, after which the glutaraldehyde was stepwise removed with deionised water and ethanol under gentle rotation. The gel pieces were then dried through critical point drying (Leica Automated Critical Point Dryer EM CPD300, Leica, Austria), the dried pieces fractured and attached to the sample holders with Carbon Adhesive (Electron Microscopy Sciences, USA). The solvent was subsequently evaporated and the samples sputter-coated with a 15 nm thick layer of Tungsten (MED 020, Leica, Austria), before analysis in a field emission SEM (Magellan 400, FEI, The Netherlands). The SEM was operated at a working distance of 4 mm with 2 kV and 13 pA SE detection.

4.2.5. *Small deformation rheology*

Dynamic oscillatory measurements were performed in a controlled stress rheometer (MRC302, Anton Paar, Austria) fitted with a sandblasted concentric cylinder (CC17) geometry. The single and mixed 12% (w/w) total protein solutions at varying pH, and single BGN-PI at corresponding concentrations in the mixtures (i.e. 3.6%, 6% and 8.4% w/w) at pH 7, were heated from 20°C to 95°C at 3°C/min, held at 95°C for 30 min, followed by cooling to 20°C (3°C/min) and a final holding step for 25 min at 20°C. A thin layer of paraffin oil was placed on top of the samples during measurement to avoid evaporation. Rheological parameters were recorded at constant strain (1%) within the linear viscoelastic regime and a frequency of 1 Hz.

In addition, the same measurements were performed for single WPI, single BGN-PI and the 6% WPI/6% BGN-PI mixture at pH 7 to which *n*-ethylmaleimide (NEM) was added. NEM is a thiol blocking agent which is used to evaluate the role of disulphide bonds during gelation (Alting, Hamer, De Kruif, & Visschers, 2000), and was added to the protein solutions in excess of 10x or 20x the free thiol groups present in the protein isolates.

4.3. Results and Discussion

4.3.1. *Visual observation and microstructural characterisation of single and mixed whey and Bambara groundnut protein gels*

Gel formation occurred in all protein systems (12% w/w total protein concentrations) at all pH values, with distinct physical differences as shown in Fig. 4.1. The single WPI gels at pH 7 were transparent with a smooth surface, whilst at pH 3 the gels were transparent but with an irregular and sticky surface. This is in agreement to previous observations for whey protein gels, which at low pH below and at neutral pH above the isoelectric point (pI ≈ 4.9) are known to form fine-stranded networks with respectively rigid strands or flexible curved strands (Cornacchia et al., 2014; Foegeding, 2006; Langton & Hermansson, 1992). At pH 5 the single WPI gels were opaque and white, which is attributed to the low electrostatic repulsion near the pI, resulting in the formation of particulate gel networks (Ako, Nicolai, Durand, & Brotons, 2009; Foegeding, 2006). In contrast to the WPI gels, single BGN-PI gels were opaque and white to slight yellowish at all pH values. The turbidity and crumbly nature of these gels could be attributed to the presence of larger aggregates/irregularly-shaped particles in the BGN-PI gel networks (Jose et al., 2016; Renkema, Lakemond, De Jongh, Gruppen, & Van Vliet, 2000), which as shown for lupine

protein isolates are typically induced by freeze drying (Berghout, Venema, Boom, & van der Goot, 2015). The mixed protein gels at pH 3 and pH 7 were visually characteristic of both single protein systems, whereas at pH 5 at all mixing ratios the gels were visually comparable to the single BGN-PI gel.
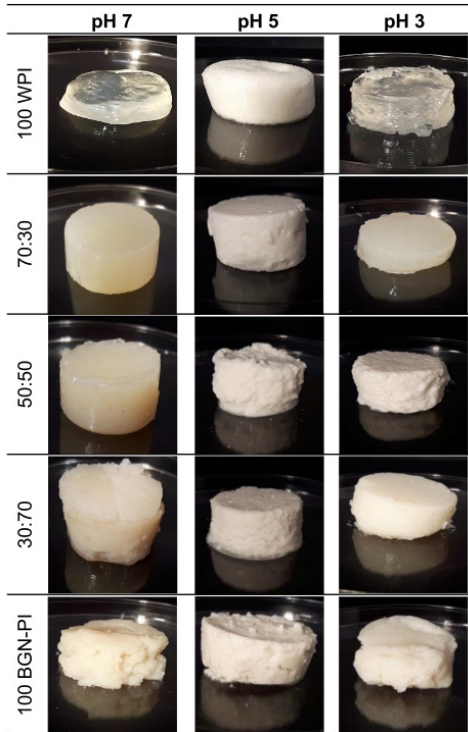
Fig. 4.1. Physical appearance of single and mixed whey and Bambara groundnut protein gels (12% w/w total protein concentrations) at varying pH. The ratios correspond to the following concentrations: 70:30—8.4% WPI/3.6% BGN-PI, 50:50—6% WPI/6% BGN-PI, 30:70—3.6% WPI/8.4% BGN-PI.

The microstructures of the gels at pH 7 were further investigated with SEM. As shown in Fig. 4.2, the gel microstructures appeared homogeneous at larger length scales and became increasingly coarser at smaller length scales. Comparing the single WPI and BGN-PI gels, the BGN-PI gel network appeared to form coarser aggregates with large pores, whilst a more uniform network structure with some strands were visible in the WPI gels (see **Appendix** for additional SEM images at varying magnifications). These are however small differences which do not directly explain the vast differences between the single protein gels as observed on a macroscopic scale. As already alluded to, freeze-dried BGN-PI contains large insoluble materials which, independent of pH, are subsequently also present in the resultant gel network. These large materials would scatter more light as such causing

the observed turbidity. It is thus expected that at higher magnifications (at smaller length scales) that structural differences between the single protein systems would become more prominent. Similar microstructures to the single BGN-PI systems were observed for the mixed protein gels, although appearing more dense with smaller pores at increasing BGN-PI concentrations. BGN-PI can thus replace WPI at varying concentrations without largely affecting the gel network structures. In comparison, Roesch & Corredig (2005) observed distinct differences in gel microstructures of soy and whey protein mixtures, where soy proteins caused phase separation at higher ratios (70:30) or large particulate aggregates at low ratios (30:70) in mixed gels with 10% (w/w) total protein concentrations.
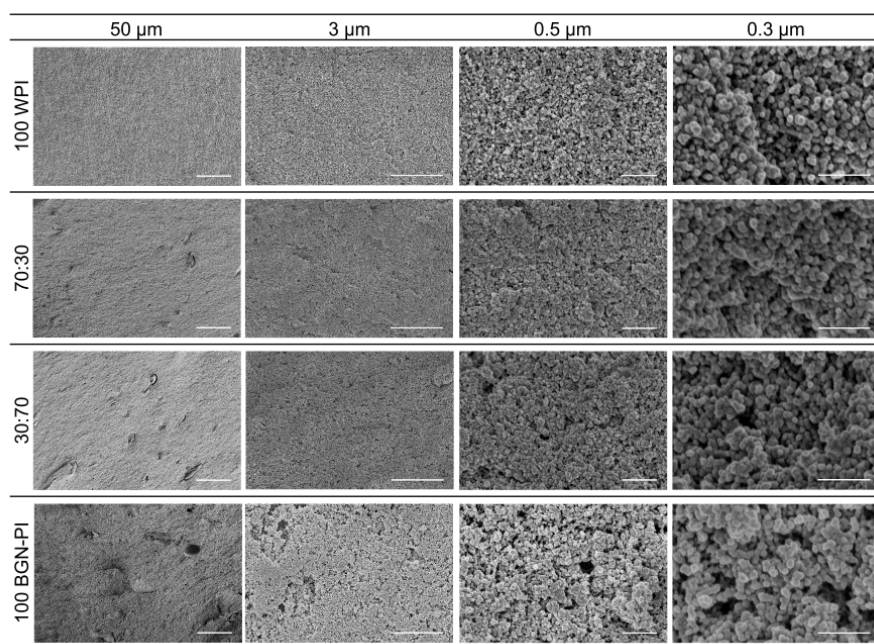

Fig. 4.2. Microstructures of single and mixed whey and Bambara groundnut protein gels at pH 7 and 12% (w/w) total protein concentrations as imaged with scanning electron microscopy. Scale bars correspond to 50 μm, 3 μm, 0.5 μm and 0.3 μm respectively.

4.3.2. *Rheological behaviour of single and mixed whey and Bambara groundnut protein gels*

The heat-induced gelation kinetics in terms of the storage modulus G' of WPI and BGN-PI gels and their mixtures, at varying pH, are shown in Fig. 4.3. At neutral pH, single BGN-PI gels were characterised with higher elasticity compared to single WPI gels. These differences in gel strength can be attributed to the type (non-covalent vs covalent disulphide bonds) and extent of interactions occurring in the gel networks, as reported for other whey and plant protein mixtures (Ainis et al., 2018; McCann et al., 2018). In these studies however, the gel strength at pH 7 of the plant proteins (soy or rapeseed) was lower compared to that of WPI at the same concentrations, which was attributed to predominantly non-covalent hydrophobic or hydrogen interactions occurring in the plant proteins and disulphide interactions in WPI. The higher gel strength of single BGN-PI in our study could thus be indicative of the extent of disulphide interactions contributing to the gel network. As reported by Kudre & Benjakul (2014), the reduction in sulfhydryl groups and increasing disulphide content of BGN-PI upon heating were evident of aggregate formation through disulphide interactions.

Considering that vicilins as the major storage proteins present in BGN seeds are devoid of disulphide bonds amongst the subunits (Diedericks, de Koning, Jideani, Venema, & van der Linden, 2019), such interactions would be driven by the sulphur-rich albumin and legumin fractions. In addition, considering that electrostatic repulsion is also known to contribute to gel formation of denatured proteins (Ako et al., 2009), these interactions could also contribute to the gel network of BGN-PI which at pH 7 is furthest from its isoelectric point (pI = 4.3). The G' development of the mixed gels at pH 7, at all mixing ratios, were initially similar to the elasticity curve of the WPI gels, before sharply increasing to reach a similar gel strength as the BGN-PI gels. These results are in agreement to the observed microstructures, which for the mixed protein systems closely resembled the network structure of the single BGN-PI gel with coarser aggregates. The effect of the single BGN-PI at the corresponding concentrations to which they were added to the mixture, was also determined. As shown in Fig. 4.4, these gels were characterised with lower elasticity compared to the mixtures. It would thus appear that independent of the concentration, BGN-PI is able to exert a strengthening effect on the gel networks. The hypothesis by Ainis et al. (2018), i.e. that the rheological responses of mixed protein systems are governed by the protein which forms a stronger network, could thus to some extent be applied to our systems (at pH 7).
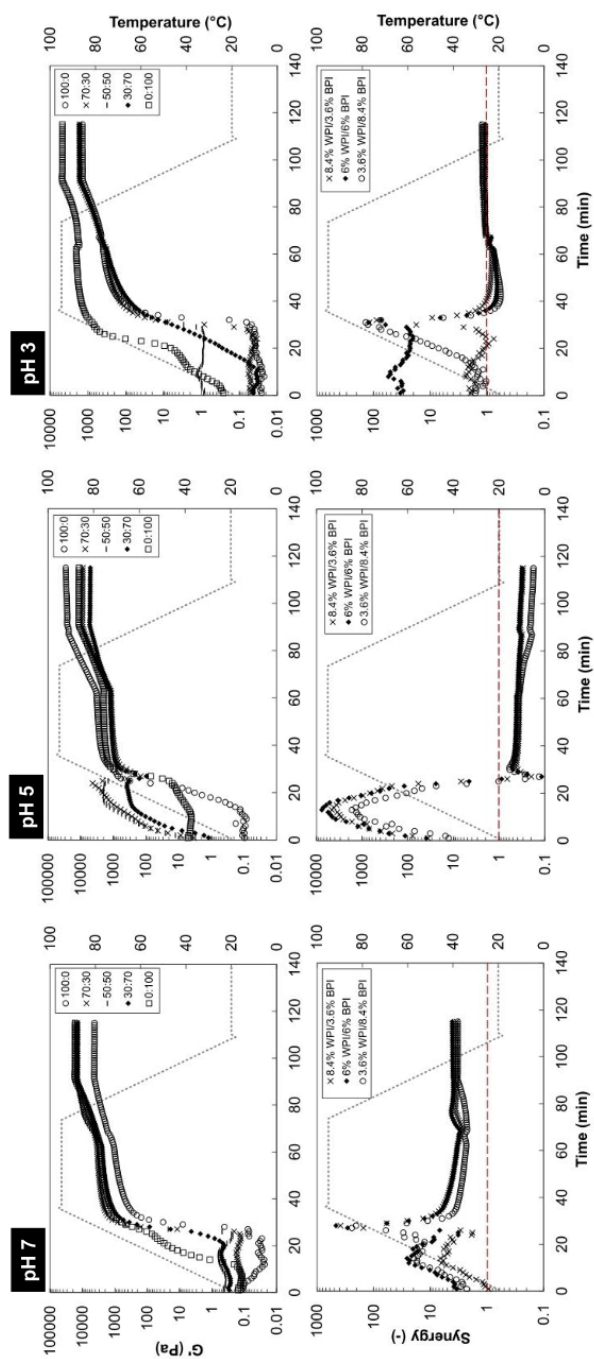
Fig. 4.3. Elastic moduli (G′) development and degree of synergy of heat-induced whey and Bambara groundnut protein gels at different mixing ratios at pH 7 (left), pH 5 (middle) and pH 3 (right). The ratios correspond to the following protein concentrations: 100:0—12% WPI, 70:30—8.4% WPI/3.6% BGN-PI, 50:50—6% WPI/6% BGN-PI, 30:70—3.6% WPI/8.4% BGN-PI, 0:100—12% BGN-PI. The dashed line indicates an S ratio of 1.
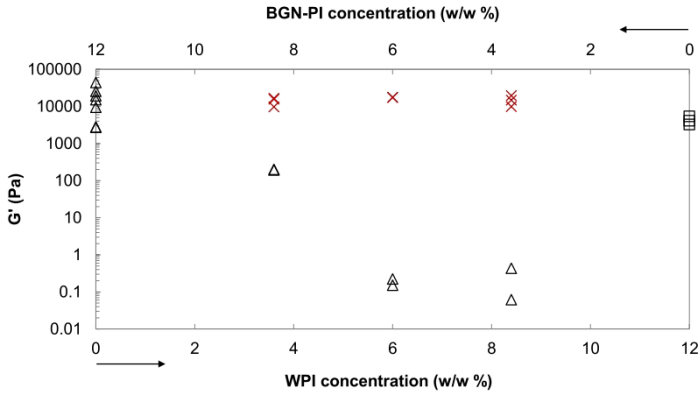
Fig. 4.4. Elastic moduli (G') at pH 7 after cooling at 20°C of single Bambara groundnut proteins (Δ), single whey proteins (□) and mixed WPI/BGN-PI (x) at the corresponding concentrations of WPI (bottom x-axis, increasing concentrations left to right) and BGN-PI (top x-axis, increasing concentrations right to left).

In addition, the sharp increase in G' of the mixed gels occurs close to the gelation point (i.e. the G'/G" cross-over point) of WPI. In mixtures of soy and whey proteins, the coinciding gelation points of the single WPI and the mixed protein systems were attributed to the denaturation of the major whey protein fraction, β-lactoglobulin (Jose et al., 2016; McCann et al., 2018). As such it is evident that whey proteins also participate in the network formation of the mixed protein systems at neutral pH. This observation is also confirmed by the deformation of the network structures under applied strain (Jose et al., 2016), as shown in Fig. 4.5. The linear viscoelastic region (LVR) of the mixed gels at the 8.4% WPI/3.6% BGN-PI and 6% WPI/6% BGN-PI mixing ratios closely resembled that of the single WPI gels (strain up to 100%), whereas the 6% WPI/6% BGN-PI gels were characterised with an LVR between that of single BGN-PI (strain around 10%) and WPI.

Similarly at pH 3, single BGN-PI gels are characterised with higher gel strengths in comparison to single WPI gels (Fig. 4.3). At pH 3, both WPI and BGN-PI are below their isoelectric points where non-covalent hydrogen bonds and hydrophobic interactions, as opposed to stronger disulphide bonds, are expected to dominate (Otte, Zakora, & Qvist, 2000; Shimada & Cheftel, 1989). This is highlighted in the final gel strength of the single protein systems, which at pH 3 is

much lower compared to the higher pH values. The onset of gelation of the mixed protein systems (at all mixing ratios) coincided with that of single WPI, whilst their gelation profiles and final gel strengths also closely corresponded to that of WPI. This is evident that WPI dominates the elasticity in the mixed protein systems at pH 3, with BGN-PI having no effect. In comparison at pH 5, WPI gels were characterised with the highest elasticity compared to single BGN-PI and mixed protein systems (Fig. 4.3). At pH 5, WPI is close to its isoelectric point and the high gel strengths can be attributed to a combination of covalent disulphide bonds and hydrophobic interactions (Cornacchia et al., 2014; Otte et al., 2000). The elasticity curves of the mixed protein gels at the 8.4% WPI/3.6% BGN-PI and 6% WPI/6% BGN-PI mixing ratios, resembled that of the single WPI gels, whilst their final gel strengths were similar to that of the single BGN-PI gels. The lowest elasticity was determined in the mixture where BGN-PI was in excess (3.6% WPI/8.4% BGN-PI), which implies a negating effect on gel strength upon increasing BGN-PI concentrations. The onset of gelation of the mixed protein systems occurred almost immediately at the start of the temperature sweep, with a sharp increase in G' reaching an initial maximum around 80°C, followed by a sudden decrease and gradual increase to similar gelation profiles of the single systems. This initial elasticity behaviour could be attributed to aggregate formation resulting from electrostatic interactions between WPI and BGN-PI before heating, where at pH 5 both proteins are close to their isoelectric points (Nicolai, Britten, & Schmitt, 2011). The aggregation rate is further enhanced upon increasing temperatures, as such resulting in the formation of the initial gel network which are disrupted close to the denaturation temperature (~83°C) of β-lactoglobulin (Ainis et al., 2018), as seen in the sudden drop in G'. In addition, when comparing the LVR of the gels at pH 5 and pH 3 to that at neutral pH, it is evident that the deformation under an applied strain of the gel networks are pH dependent. The limiting strain was around 10% for the gels at lower pH (Fig. 4.5), indicating the higher strain sensitivity and brittle nature of these gels (Langton & Hermansson, 1992; Stading & Hermansson, 1991).

Furthermore, as previously reported for other plant and whey protein systems, synergistic effects can be quantified by relating the final G' of the mixtures to either the G' of the protein being replaced, or to the sum of the separate effects produced by the individual proteins at corresponding concentrations (Ainis et al., 2018; Jose et al., 2016; Wong et al., 2013). To evaluate if synergies occurred upon mixing of WPI and BGN-PI at 12% (w/w) total protein concentrations, the following equation as proposed by (Wong et al., 2013) was used:
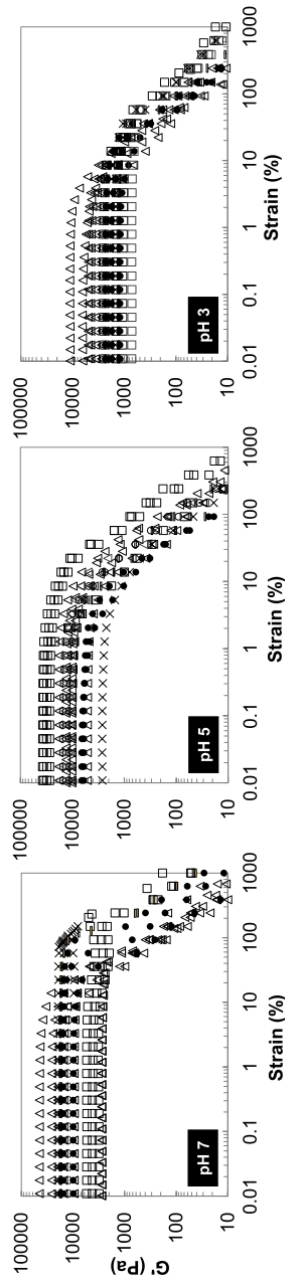
Fig. 4.5. Strain sweep of heat-induced whey and Bambara groundnut protein gels at different mixing ratios at pH 7 (left), pH 5 (middle) and pH 3 (right). Symbols correspond to: □—12% WPI, ∆—12% BGN-PI, x—8.4% WPI/3.6% BGN-PI, - —6% WPI/6% BGN-PI, •—3.6% WPI/8.4% BGN-PI.

$$S = \frac{G'mixture}{G'WPI} \qquad\qquad (4.1)$$

where the ratio S is indicative of the degree of synergism between the proteins, with S ≠ 1 defined as the presence of one protein exerting an effect on the other protein in the mixture, and S > 1 defined as synergy in the mixed protein system (Ainis et al., 2018). Wong et al. (2013) defines S < 1 as negative enhancement, which can be interpreted as either non-synergistic effects or antagonistic effects. The degree of synergy at the different mixing ratios for all pH values are also shown in Fig. 4.3. Synergistic effects were observed at pH 7 for all mixed protein systems with the highest synergy observed in the 6% WPI/6% BGN-PI gels, although upon cooling the S ratio was similar for all mixtures. Compared to whey and pea protein mixtures (10% w/v total protein) at pH 6 and pH 8 where the highest synergies were observed at the lowest pea concentration (ratio 8:2 WPI to pea) (Wong et al., 2013), BGN-PI can replace WPI at higher concentrations whilst positively influencing the gel strength. At pH 5, the S ratio of all mixtures were below 1 with the lowest synergy observed for the gel containing BGN-PI in excess; hence confirming the negating effect of BGN-PI on the gel strength at this pH. The S ratio at pH 3 for all mixed protein systems was close to 1, which indicates that BGN-PI had no effect on the gelation behaviour of the mixtures. Ainis et al. (2018) observed a similar trend for rapeseed and whey protein mixtures, where at pH 3 the S ratio was close to 1 and the elasticity of the mixtures compared closely to that of the single WPI gel.

4.3.3. *Effect of disulphide interactions on gelation kinetics of single and mixed whey and Bambara groundnut protein systems at pH 7*

Disulphide interactions are known to stabilise whey protein gel networks at neutral or alkaline pH, as such contributing to higher gel strengths under these conditions (Shimada & Cheftel, 1989). The high elasticity of the single and mixed protein systems at pH 7 was therefore indicative of the role of disulphide interactions in the formation of the gel networks. To determine if disulphide bridges were indeed present and their effect on gel formation, the single proteins and the 6% WPI/6% BGN-PI mixed system were subjected to heat-induced gelation in the presence of the thiol blocking agent, NEM. As shown in Fig. 4.6, overall lower gel strengths were observed at a concentration of NEM in 10x excess of the free thiol concentrations quantified per protein system, i.e. 9.2 μmol/g protein and 28.2 μmol/g protein for BGN-PI and WPI, respectively. The gelation profiles of the single BGN-PI and

the mixed protein system with NEM remained similar to the control gels, whereas the initial gelation kinetics of the single WPI appeared to deviate from the control. This observation was more prominent for single WPI gels where NEM was present at higher concentrations, resulting in a decrease in elasticity during initial gel formation. Heat-induced gel formation for whey proteins is generally reported as a two-stage process, i.e. disulphide interactions which forms the initial junction zones through sulfhydryl/disulphide interchange reactions followed by strengthening of the gel network through non-covalent interactions (Alting et al., 2000; Shimada & Cheftel, 1989). These results confirm the important role of disulphide interactions in gel formation of WPI gels, which to a lesser extent also influenced the gel networks of single BGN-PI and the 6% WPI/6% BGN-PI mixed protein system.

The influence of NEM on BGN-PI could be attributed to various factors, as also found for cultivar-specific pea protein isolates where the addition of NEM resulted in either a decrease or increase of the gel elasticity. The differences in gel behaviour in these pea protein systems were ascribed to the reactive residues' spatial proximity upon unfolding of the legumins, or to increased repulsive forces amongst the vicilin α-subunits at high concentrations (O'Kane, Vereijken, Gruppen, & Van Boekel, 2005). Roesch & Corredig (2005) also studied the effect of NEM on soy and whey protein mixtures in a ratio of 70:30 (1.4% w/v total protein concentration) and observed that both disulphide bridging and non-covalent interactions were important for complex formation. As such it can be concluded that both disulphide interactions and non-covalent interactions contribute to the gel formation of the single and mixed protein gels.
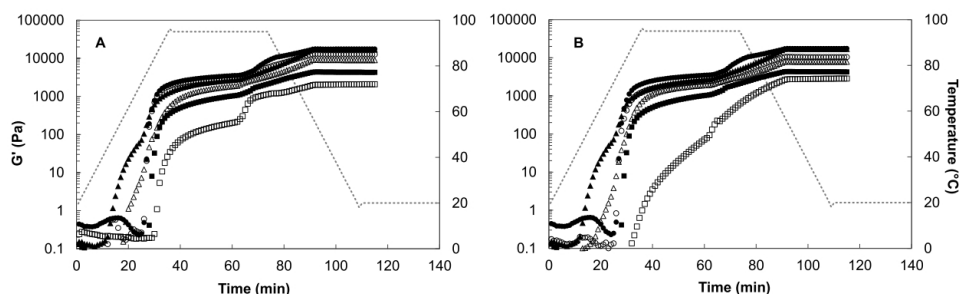
Fig. 4.6. The effect of NEM in (A) 10x excess and (B) 20x excess of thiol concentrations on the elastic moduli (G') development of heat-induced single and mixed whey and Bambara groundnut protein gels at pH 7. Symbols correspond to: ■—12% WPI, ▲—12% BGN-PI, ●—6% WPI/6% BGN-PI without NEM; open symbols correspond to the same protein concentrations with NEM.

4.4. Conclusions

The addition of BGN-PI to WPI at different mixing ratios was shown to synergistically enhance gel formation at neutral pH, independent of the concentration of BGN-PI in the mixtures, as also evident from the similar gel microstructures of the mixed gels to that of single BGN-PI. This apparent beneficial influence of BGN-PI on whey proteins at neutral pH is noteworthy, considering that plant proteins are known to have a negating effect on gel strengths when replacing whey proteins at higher concentrations. The role of disulphide interactions was shown to be important in the network formation of single WPI gels; to a much larger extent compared to single BGN-PI and mixed protein gels. Under acidic conditions, BGN-PI had either no effect on the viscoelastic properties of mixed protein gels (pH 3) or at increasing concentrations in the mixed protein systems resulted in lower gel strengths (pH 5). Hence, it can be concluded that BGN-PI as a novel plant protein has the ability to enhance or change the viscoelastic behaviour of gel networks when combined with whey proteins.

Acknowledgements

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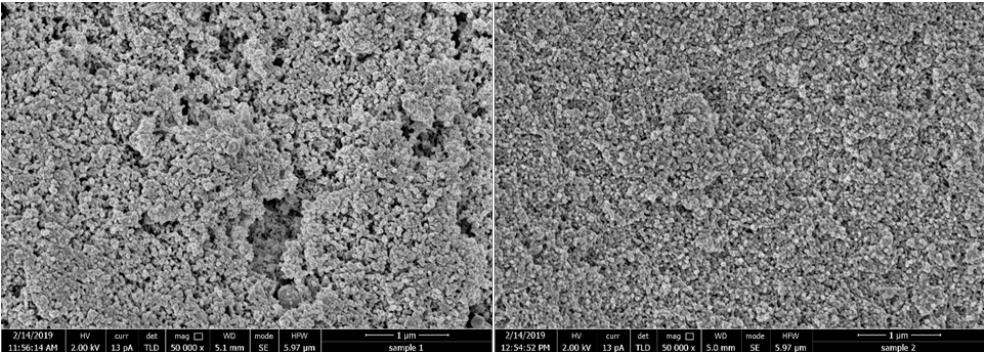
Appendix: Supplementary Material
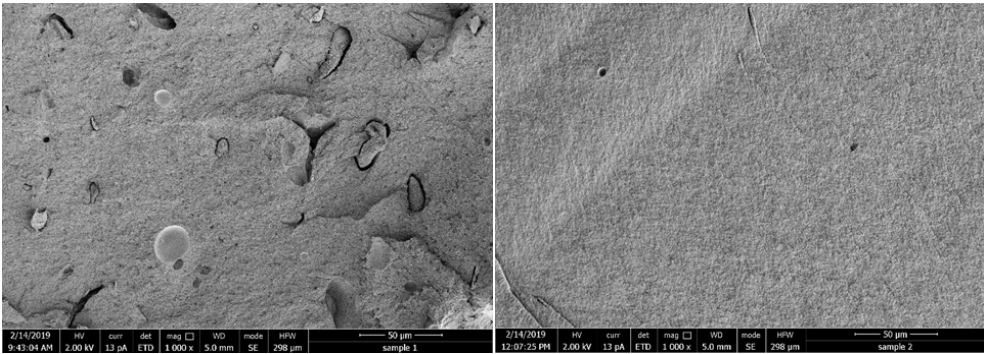
Fig. S4.1. Microstructures of (left) Bambara groundnut and (right) whey protein isolate gels at pH 7 and 12% (w/w) concentrations; 50 µm length scale.
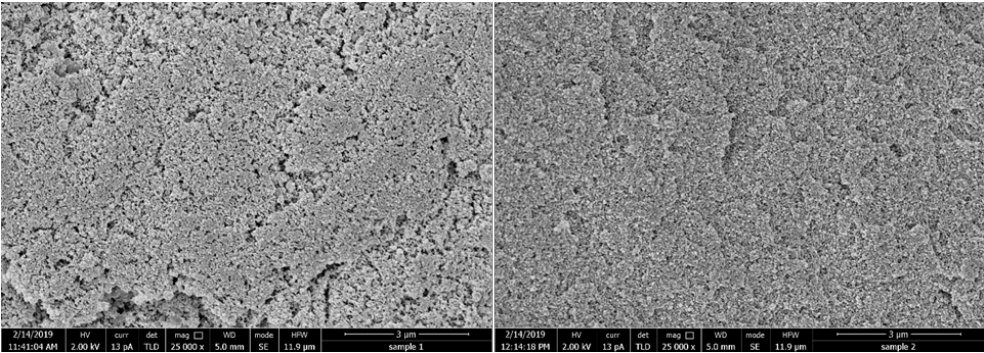
Fig. S4.2. Microstructures of (left) Bambara groundnut and (right) whey protein isolate gels at pH 7 and 12% (w/w) concentrations; 3 µm length scale.

Fig. S4.3. Microstructures of (left) Bambara groundnut and (right) whey protein isolate gels at pH 7 and 12% (w/w) concentrations; 1 µm length scale.

Fig. S4.4. Microstructures of (left) Bambara groundnut and (right) whey protein isolate gels at pH 7 and 12% (w/w) concentrations; 500 nm length scale.
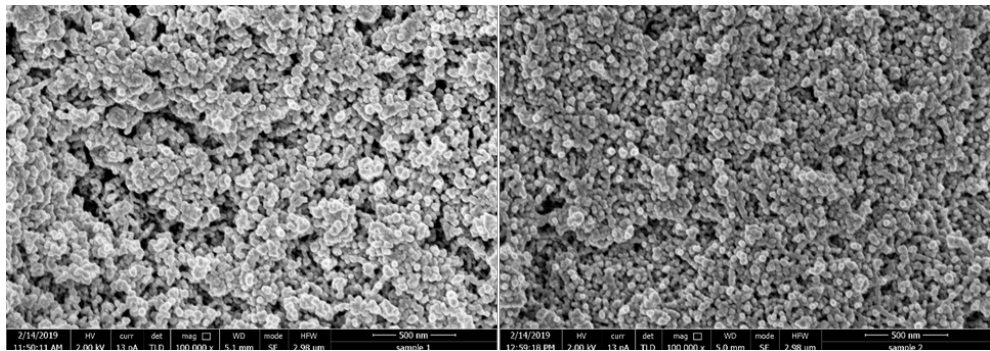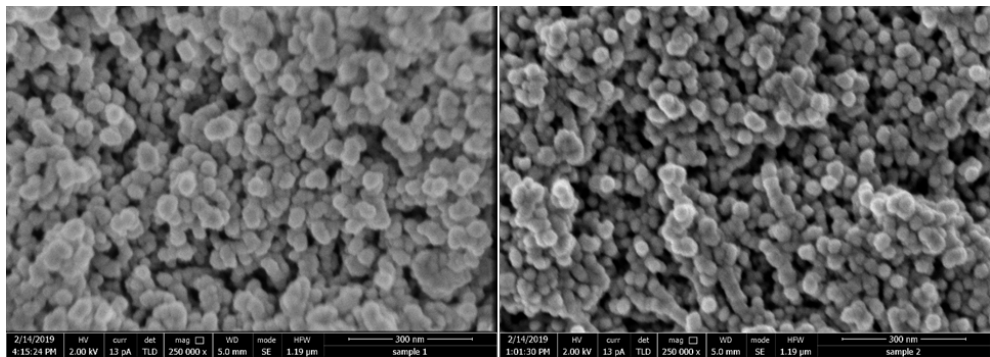

Fig. S4.5. Microstructures of (left) Bambara groundnut and (right) whey protein isolate gels at pH 7 and 12% (w/w) concentrations; 300 nm length scale.

5

Effect of processing on the microstructure and composition of Bambara groudnut (*Vigna subterranea* (L.) Verdc.) seeds, flour and protein isolates

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Abstract

Pre-processing treatments are generally employed to circumvent negating effects, such as the hard-to-cook and hard-to-mill properties, associated with legume seeds. Several studies have investigated the effects of soaking and roasting on the macroscopic qualities of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] seeds, yet knowledge is lacking on the effects of these treatments on the microstructural and molecular properties of the seeds and resulting fractions (i.e. flour and protein isolates). Size-exclusion chromatography coupled with light scattering results have shown that roasting induces thermal aggregation in BGN proteins, resulting in the formation of insoluble aggregates. As such the molecular composition of samples which were roasted and soaked-roasted were comparable, yet different to the control (untreated seeds), whereas the samples which were subjected to soaking compared favourably to the control. The morphology of the seeds were also well characterized by microscopic techniques, including a confocal imaging technique which appears novel for legume seeds, revealing cotyledon cells with several starch granules embedded in a matrix of protein bodies. The findings of this study provide valuable insights on the microstructural and molecular compositional changes occurring in BGN seeds and fractions when subjected to soaking and/or roasting, which could be linked to some macroscopic properties as previously reported.

5

5.1. Introduction

Nowadays, there is a renewed and growing interest in the consumption of plant proteins. This stems from the necessity to (partially) transition from animal-based protein diets, as a means of creating a more sustainable, nutritious and secure food supply (Aiking, 2011; Day, 2013). Next to cereal grains, legumes are one of the most important plant groups for human protein nutrition, with specifically pulses (dry seeds) forming an important part of the traditional diets in many developing countries (Calles, Xipsiti, & del Castello, 2019; Day, 2013; Tiwari, Gowen, & Mckenna, 2011). Yet, despite their nutritional importance, many (indigenous) pulses remain to be cultivated by small scale farmers and are yet to be exploited commercially as protein sources (Calles et al., 2019).

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] is such a pulse crop, which despite its high nutritional value are still characterized as an underutilized subsistence crop. Widely grown in sub-Saharan Africa and Southeast Asia, BGN is considered as a high-quality protein food, with a reported protein content up to 27% and higher methionine levels compared to other grain legumes (Arise, Amonsou, & Ijabadeniyi, 2015; Feldman, Ho, Massawe, & Mayes, 2019; National Research Council, 2006). Also known to withstand harsh environmental conditions, BGN can be considered as a valuable crop in the current context of sustainability and climate change (Calles et al., 2019; Feldman et al., 2019), thus making it an important crop to be valorised beyond its local boundaries. As with other pulses however, there are several factors associated with the limiting use of BGN; amongst others the hard-to-cook (HTC) and hard-to-mill (HTM) properties of the seeds which also have an influence on the nutritional quality (Gwala et al., 2019; Martín-Cabrejas, Esteban, Perez, Maina, & Waldron, 1997; Mubaiwa, Fogliano, Chidewe, & Linnemann, 2017; National Research Council, 2006). These factors are especially limiting in the local areas of consumption, considering that extensive cooking times in comparison to other legumes are required to soften BGN seeds, with firewood being the predominant source of energy (Mubaiwa et al., 2017).

To circumvent these negating factors, the seeds are subjected to various pre-treatments before cooking or processing into flour and resulting products. These include amongst others soaking, roasting, germination and fermentation, or a combination of these treatments. The effect of these treatments on the functionality, nutritional composition and physical properties of BGN seeds have been the subject of several studies (Adegunwa, Adebowale, Bakare, & Kalejaiye, 2014; Barimalaa & Anoghalu, 1997; Ijarotimi & Esho, 2009; Mazahib, Nuha, Salawa, & Babiker, 2013; Mubaiwa, Fogliano, Chidewe, & Linnemann, 2018; A. T. Oyeyinka, Pillay, Tesfay,

& Siwela, 2017; Yusuf, Ayedun, & Sanni, 2008); of which the outcomes provided valuable knowledge in terms of pre-processing geared towards specific applications and the influence of varietal differences. However, to gain further insight on the effect of these pre-processing treatments on the seed qualities, it is important to also investigate the resulting microstructural changes.

The aim of this study was therefore to gain insight into the microstructural changes of BGN seeds, upon various pre-processing treatments. In order to link the microstructural characteristics to the reported macroscopic properties of the BGN seeds and resulting flour, we have obtained seeds of the same varieties (red and black-eye) and subjected to the same treatments (soaking and/or roasting) as used by Mubaiwa et al. (2018). The microstructure of the seeds were well characterized with microscopic techniques, which included a novel means of imaging legume seeds based on the inherent autofluorescence of the protein bodies present in BGN seeds. In addition to the flour, we have also investigated the effect of these treatments on the protein isolates extracted from the flour, by means of size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). This is a well-known technique used amongst others for monitoring and characterizing the (thermal) aggregation behaviour of proteins, as it allows for the accurate determination of absolute molecular weight (M_w) (Choi & Ma, 2006; Ye, 2006; Zhao, Mine, & Ma, 2004). Through this approach we have demonstrated for the first time, as per our knowledge, the molecular changes occurring upon roasting and/or soaking of BGN seeds, as such providing further insights into their previously reported macroscopic properties.

5.2. Materials and Methods

5.2.1. Materials

Bambara groundnut [BGN] seeds were obtained from the Director of Research and Specialist Services (red variety) and Dee Spice Private Company (black-eye variety) in Zimbabwe. The seeds were subjected to three different treatments as described by Mubaiwa et al. (2018)—soaking, roasting and combined soaking and roasting—with raw, untreated seeds used as the control. Briefly, seeds were soaked by immersion in deionized water for 24 h at room temperature followed by oven drying at 50°C for 48 h. Roasted seeds were prepared by dry-roasting in a coffee roaster (KN-8828-2K, Pullman Espresso Accessories, Australia) at various temperature intervals ranging from 70–179°C. A third batch of seeds were subjected to soaking and drying, followed by dry-roasting.

The control (raw) and treated seeds were dehulled (automatic and manual dehulling where necessary) and coarse-milled with a pin mill (Condux-Werk LV 15M, Wolfgang bei Hanau, Germany) into grits, followed by fine-milling with a 0.5 mm mesh sieve ring fitted on a rotor mill (Fritsch GmbH Pulverisette 14, Idar-Oberstein, Germany) to obtain flour. The flour was defatted with n-Hexane (1:3 w/v) under continuous stirring for 1 h at room temperature, the hexane decanted and the procedure repeated twice. In the final step, vacuum filtration was used to remove the hexane and the flour collected and air-dried overnight. The defatted flour was sieved (315 µm mesh sieve) with an air jet sieve (Hosokawa Alpine E200 LS, Augsburg, Germany) and stored at -20°C in airtight containers. All chemicals used were of analytical grade.

5.2.2. *Extraction of Bambara groundnut protein isolates*

The commonly utilised isoelectric precipitation method as described in **Chapter 3** was used to extract protein isolates from the defatted flour prepared from the control and treated BGN seeds. Briefly, defatted flour dispersed in deionized water (1:10 w/v) was adjusted to pH 9.5 with 1 M NaOH to solubilize the proteins, before isoelectric precipitation at pH 4 (adjusted with 1 M HCl), followed by centrifugation (4000 g, 30 min) with an Avanti J-26 XP centrifuge (Beckman Coulter, USA) to obtain the protein-rich residue. This residue was adjusted to pH 7 before being freeze-dried as the protein isolate (BGN-PI).

The total yield was expressed as the dry weight of BGN-PI per weight defatted flour, whereas the total protein content as determined with the Dumas nitrogen combustion method (FlashEA 1112 series, Thermo Scientific, The Netherlands) was used to express the protein yield of the final freeze-dried material (nitrogen-to-protein conversion factor N x 5.7).

5.2.3. *Microstructure of Bambara groundnut seeds*

Cryo-planing, cryo-scanning electron microscopy (cryo-SEM) and energy-dispersive x-ray spectroscopy (EDS)

The control (untreated) red and black-eye seeds, as well as the roasted black-eye seeds, were prepared for cryo-SEM imaging by imbibing the seeds between sheets of wet tissue paper in a closed container for 48 h at room temperature (see Fig. 5.1). After the imbibition process the seeds were sampled at a specific location from a longitudinal section, the samples mounted to the sample holder and frozen in melting propane or ethane. The frozen samples were transferred to a cryo-ultramicrotome (Leica MZ6 or Leica EM UC7; Leica Microsystems, Amsterdam, The Netherlands) where the initial planing were carried out with a glass knife, followed by final planing

with a diamond knife. Known as cryo-planing, this technique is advantageous as it creates flat internal surfaces which are largely devoid of artefacts due to the low operational temperatures (Nijsse & Van Aelst, 1999). Once a smooth area was obtained, the samples were transferred to a SEM preparation chamber (Gatan Alto 2500; Gatan, Abingdon, UK) for brief sublimation (freeze-etching) followed by sputter coating with a thin layer of platinum. The samples were then transferred into the SEM analysis chamber for observation at -125°C at an acceleration voltage of 5 kV (JSM-6490LA SEM; Jeol, Tokyo, Japan) or 3 kV (Auriga field emission SEM; Zeiss, Jena, Germany). In addition, energy dispersive X-ray spectroscopy (Aztec X-Max 80mm²; Oxford Instruments, Abingdon, UK) was performed to identify the elemental composition of the samples.
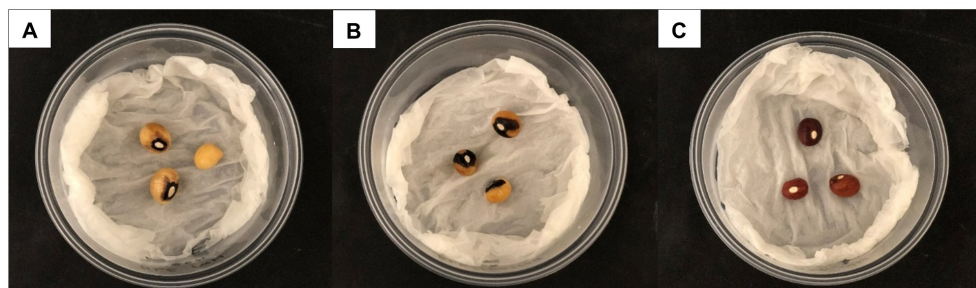
Fig. 5.1. Imbibition of Bambara groundnut seeds for 48 h at room temperature (20°C). (A) Black-eye control; (B) Black-eye roasted; (C) Red control.

Confocal laser scanning microscopy (CLSM)

CLSM was performed to visualize the morphology of a dry BGN (black-eye) seed. The seed was sampled with a razor blade on the outer edge of the cotyledon, the cut surface placed on a cover slip with immersion oil and observed with a Leica TCS SP5 confocal microscope equipped with a Leica DMI6000B-CS inverted microscope. Three objectives were used, i.e. 10x dry objective (0.4 NA), 40x (1.25 NA) and 63x (1.4 NA) oil immersion objectives. After a signal hunt, the protein bodies could be detected in fluorescence mode and the oil bodies in reflection mode, both at an excitation wavelength of 476 nm (Argon laser) and emission wavelengths of 481–795 nm and 472–481 nm, respectively. In addition, the starch granules and cell walls were visualized in the "dark" areas where no fluorescence or reflection were detected.

5.2.4. *High performance size-exclusion chromatography coupled with multi-angle laser light scattering (HPSEC-MALLS) analysis of BGN flours and protein isolates*

HPSEC-MALLS was performed as previously described (Diedericks, de Koning, Jideani, Venema, & van der Linden, 2019). The HPSEC system consisted of four TSK gel analytical columns (Tosoh Bioscience LLC, USA) which were thermostated at 35°C and connected in series, a vacuum degasser (1200 series degasser, Agilent Technologies, USA) and a pump (1200 series binary pump, Agilent Technologies). The filtered mobile phase (100 mM $NaNO_3$ + 0.02% NaN_3) were pumped through the system at a flow rate of 0.5 ml/min, and 200 µl of the BGN flour dispersions and protein solutions (0.5% w/w) were injected onto the columns with an autosampler (Agilent Technologies). Three detectors measuring static light scattering, UV absorption and refractive index (RI) were used for characterization of the samples. ASTRA 6 software (Wyatt Technologies) was used for collection and processing of data. The reference material bovine serum albumin (BSA, M_w 67 kDa) was used for normalization, alignment and band broadening.

5.2.5. *Statistical Analysis*

Analysis of variance was performed on the total protein data, obtained from at least triplicate measurements, and Duncan's multiple range test was used to determine significant differences ($p \leq 0.05$) among the means (IBM SPSS Statistics 25, Chicago, USA).

5.3. Results and Discussion

5.3.1. *Protein composition and yield*

The yield and protein content of BGN flours and protein isolates are shown in Table 5.1. Compared to the control flours, the protein content of the soaked and roasted black-eye flour was significantly higher, whereas in the red variety the protein content of the roasted and soaked-roasted flour was significantly lower. Overall these values are lower in comparison to that reported by Mubaiwa et al. (2018), which could be attributed to the extra fine milling step and lower sieve mesh size employed in their study. The protein content of the protein isolates from both varieties was in a similar range, with that of the soaked BGN-PIs being comparable to the control samples. In contrast, the protein content of the roasted and soaked-roasted BGN-PIs decreased significantly compared to that of the control. During roasting, samples are exposed

to high temperatures for short times, which can cause denaturation of proteins to varying extents depending on their thermal stability (Meng & Ma, 2001; Tiwari et al., 2011). Roasting also had the largest effect (in reference to the control samples) on protein yield in both varieties, with the roasted and soaked-roasted BGN-PIs characterized with the lowest yields in a range of 3.2–5.5 g protein per 100 g defatted flour. In applications where the protein content and/or yield are of importance, soaking would thus be the best suited as a pre-processing treatment.

Table 5.1. Protein content of Bambara groundnut flour and protein isolates, and dry weight yield of protein isolates.[a]

| Variety	Sample	Protein content flour	Protein content BGN-PI	Yield (dw) BGN-PI
		(g/100 g)		
Black-eye	Control	14.8 ± 0.1^a	68.3 ± 0.8^{ab}	11.5
	Soaked	15.8 ± 0.3^b	66.2 ± 0.7^{ac}	12.1
	Roasted	15.6 ± 0.3^b	61.6 ± 1.0^d	4.4
	Soaked-roasted	10.8 ± 0.1^c	61.8 ± 3.6^d	3.2
Red	Control	14.7 ± 0.3^a	72.0 ± 0.9^e	14.7
	Soaked	14.8 ± 0.1^a	70.5 ± 1.0^{be}	12.5
	Roasted	13.0 ± 0.2^d	63.3 ± 2.1^{bd}	5.5
	Soaked-roasted	12.7 ± 0.2^d	61.4 ± 0.3^d	3.4

[a] Protein content values of flour and BGN-PI (Bambara groundnut protein isolate) presented as means ± standard deviations; means within a column followed by the same superscript letter are not significantly different (p > 0.05). Dw refers to the dry weight yield of BGN-PI per 100 g defatted flour.

### 5.3.2. *Microstructural characteristics of control and processed BGN seeds*

The microstructures of BGN control and roasted seeds were well characterized with the microscopic techniques employed. An overview of the cotyledon cells in the control red BGN seed, as obtained with cryo-SEM and shown in Fig. 5.2A–B, reveals the presence of several oval-shaped starch granules surrounded by numerous protein

bodies. This morphological structure of cotyledon cells has also been reported for other pulse seeds such as cowpea and faba bean, whilst the starch granules have the same shape as previously reported for BGN starches (Oyeyinka, Singh, & Amonsou, 2017; Swanson, Hughes, & Rasmussen, 1985). In general, starch granules appear darker than protein bodies due to a lower amount of electrons reflected from their surface. At higher magnifications (Fig. 5.2C–D), the protein bodies appeared more distinct whilst ranging in colour/intensity from grey to black. This colour/intensity difference was also observed for protein bodies of yellow pea, which was attributed to differences in electron density caused by the orientation of the protein bodies, the planing depth or the formation of ice-crystals during cryofixation (Kornet et al., 2019). The size of starch granules and protein bodies ranged from 7–45 μm and 1–5 μm, respectively, which corresponds to that previously reported for these cell components from BGN and other legume seeds (Amonsou, Taylor, & Minnaar, 2011; Do & Singh, 2019; Kaptso et al., 2015; Kornet et al., 2019; Swanson et al., 1985; Wolf, 1970). Oil bodies (<0.5 μm) were also present in the cells, with most of them accumulating at the cell walls as shown in Fig. 5.2E. Furthermore, the corresponding EDS elemental map (Fig. 5.2F) provided further evidence of the composition of the structures—the carbon signal corresponded to the oil bodies, whilst the oxygen and nitrogen signals corresponded to the starch granules and the protein bodies, respectively.

Similar to the red BGN seeds, the cryo-SEM images of the control black-eye seeds (Fig. 5.3A–D) revealed cotyledon cells rich in starch granules and protein bodies, both in a similar size range as found for the red variety. However, the protein bodies appeared more uniform in colour, whilst the number of starch granules per cell were lower in the black-eye seeds with no granules visible in the cells close to the hull (see area indicated in Fig. 5.3A). In addition, CLSM imaging of the dry seeds (Fig. 5.3E–F) provided a structural overview which was in good agreement to the cryo-SEM images of the imbibed seeds. The principle of this microscopic technique is based on the detection of fluorescing (or reflected) light following illumination of a sample with a scanning laser light. The structures of interest are generally stained with fluorescent dyes, although intrinsic fluorescence (autofluorescence) found in biological samples such as aromatic amino acids, phenolics and structural proteins are often advantageously used, as minimal sample preparation is required (Holopainen-Mantila & Raulio, 2016; Monici, 2005). In our samples, autofluorescence was found for the protein bodies, whilst the oil bodies were visualized through reflectance and the non-illuminated areas corresponded to the starch granules. Here it should be noted that this can be considered as a novel means of CLSM imaging for legume seeds, which holds true for these specific samples imaged under the specified conditions.

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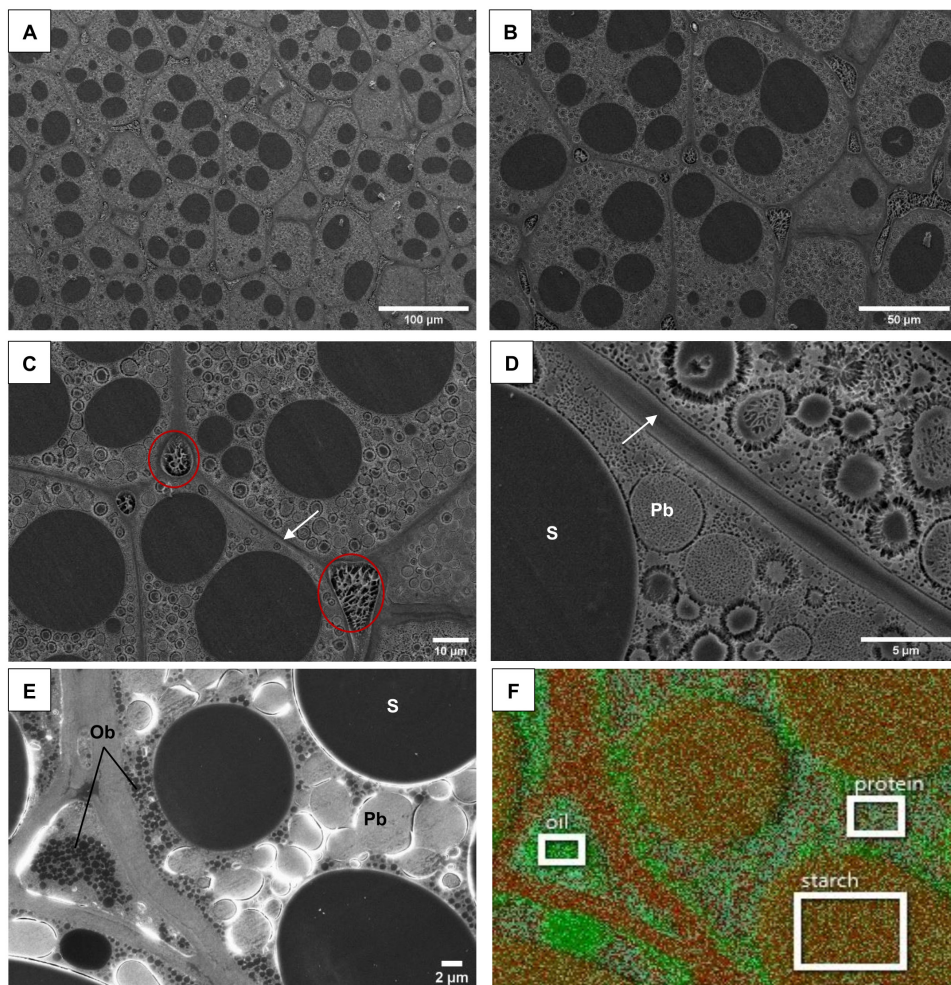
Fig. 5.2. Morphology of BGN red control seeds after imbibition for 48 h, as imaged with cryo-SEM at different magnifications. (A)–(B) overview of the cotyledon cells; (C)–(D) at higher magnifications the protein bodies appear more distinct, arrows indicate cell walls and the circled areas indicate intercellular spaces; (E) oil bodies visible as dark spots (<0.5 μm) mostly concentrated at the cell walls; and the corresponding EDS elemental map (F) with carbon indicated in green, oxygen in red and nitrogen in blue. S: starch granules, Pb: protein bodies, Ob: oil bodies.
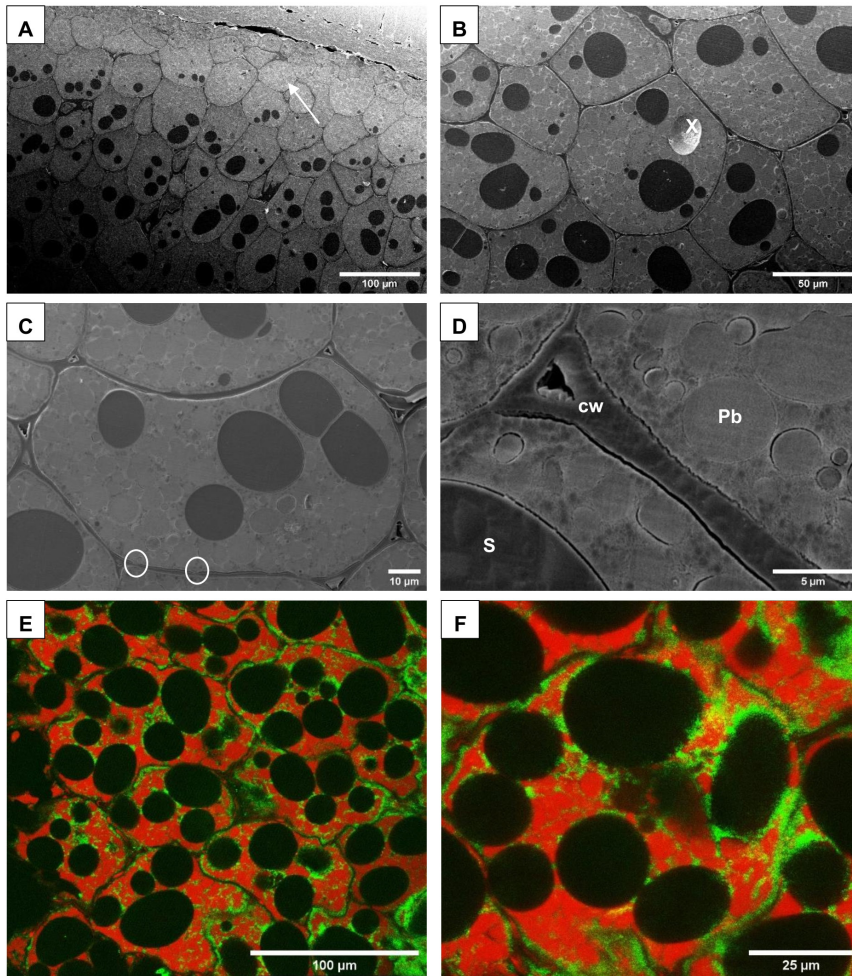
Fig. 5.3. Morphology of BGN black-eye control seeds imbibed for 48 h as imaged with cryo-SEM and dry seeds as imaged with CLSM, at different magnifications. (A)–(B) overview of the cotyledon cells, arrow indicates cells close to the hull devoid of starch granules and X indicates an empty starch cell; (C)–(D) at higher magnifications the plasmodesmata (circled areas) and oil bodies along the cell wall are visible; (E–F) CLSM micrographs showing protein bodies in red, oil bodies in green and starch in black. S: starch granules, Pb: protein bodies, cw: cell wall.

The microstructure of the roasted black-eye BGN seeds is distinctly different compared to the control seeds, as shown in Figure 5.4A–D. The protein bodies could not be distinguished, as they became aggregated with other intracellular components and the intact starch granules. Whilst the cell walls remained swollen, no water was absorbed by the aggregated cell components as seen by the large ice-crystal layer between the cell components and the cell walls. The aggregated cell material is indicative of the dry state, leaving an imprint which can be linked to that in the neighbouring cells; whilst the large ice-crystal layer would likely facilitate a higher fluid entrapment as seen by the higher water absorption capacities for roasted (and soaked-roasted) BGN seeds reported by Mubaiwa et al. (2018). Similar morphological characteristics were reported for navy beans—SEM images revealed the destruction of outer structures of the cotyledon upon roasting, whilst starch granules and other intracellular materials became partly aggregated (Aguilera, Lusas, Uebersax, & Zabik, 1982). The morphology of the soaked and soaked-roasted seeds were not microscopically examined. It is however expected that the soaked seeds will resemble that of the imbibed control seeds with swollen intercellular spaces due to the longer imbibition time and the imbibition method (immersion in water), whilst the morphology of the soaked-roasted seeds is expected to resemble that of the roasted seeds (Gowen et al., 2006; Mubaiwa et al., 2018; Swanson et al., 1985). These microstructural differences can also be linked to the higher dehulling and milling efficiencies of the roasted seeds as reported by Mubaiwa et al. (2018), which can be attributed to an increase in fracture points upon roasting, caused by increased mechanical stresses and vapor pressures (Aguilera et al., 1982; Köksel, Sivri, Scanlon, & Bushuk, 1998). Additional images of the cotyledon cells of the raw and roasted BGN seeds at various magnifications are provided as supplementary material (**Appendix**).

5.3.3. *Chromatographic profiles and molecular weight characterization of BGN flours*

The chromatograms of BGN black-eye and red flours as detected by UV and MALLS detectors are shown in Figs. 5.5 and 5.6, respectively. The UV elution patterns were comparable for both BGN varieties, with several peaks observed for all treatments. In general, the elution patterns of the control and soaked flours were comparable to that reported for yellow pea flour, where the larger peaks eluting first were ascribed to the globulin proteins and the smaller peaks eluting later to the smaller proteins such as the albumins and the vicilin subunits (Kornet et al., 2019). The large peak eluting around 53 min for the control and soaked BGN flours corresponds to that of
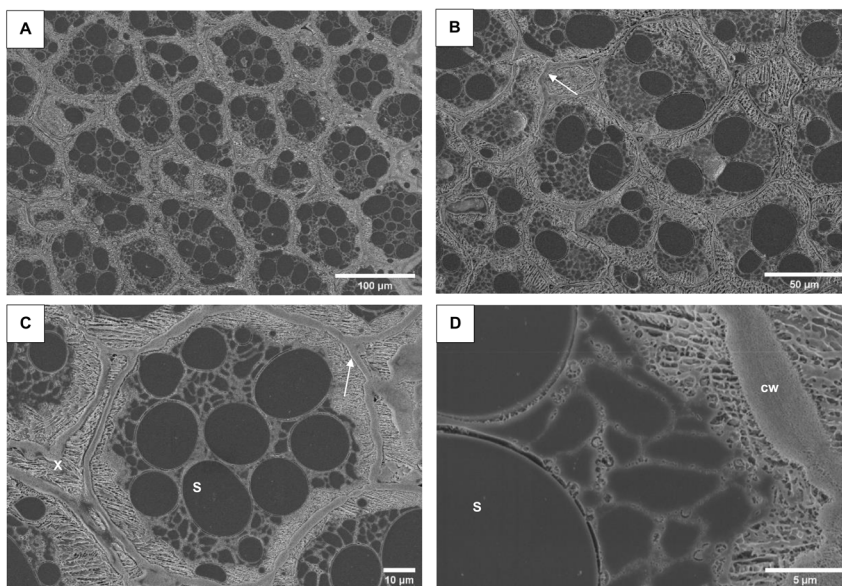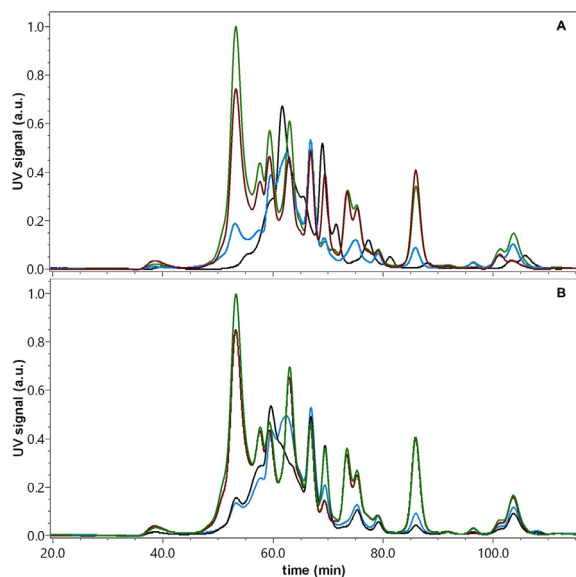
Fig. 5.4. (A)–(D) Morphology of BGN black-eye roasted seeds imbibed for 48 h, as imaged with cryo-SEM at different magnifications; arrows indicate cell walls and the area marked with an X indicates an intercellular space. S: starch granules, cw: cell wall.

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Fig. 5.5. SEC-UV elution profiles of (A) black-eye and (B) red Bambara groundnut flours subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted.
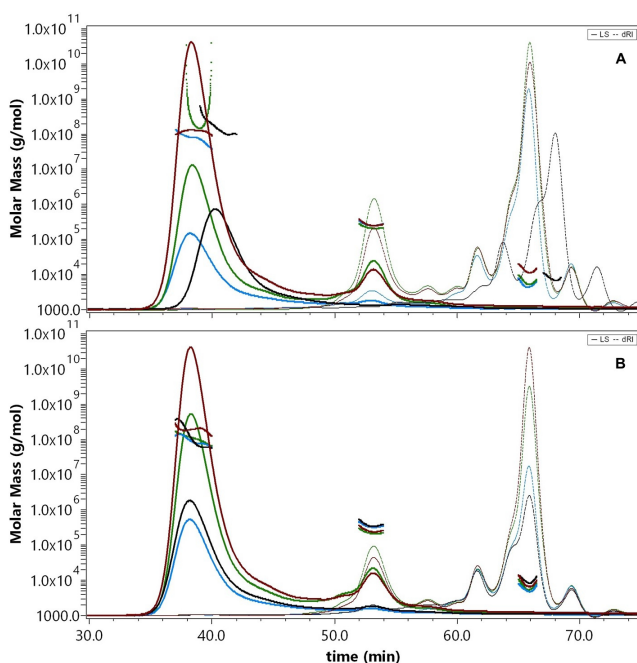
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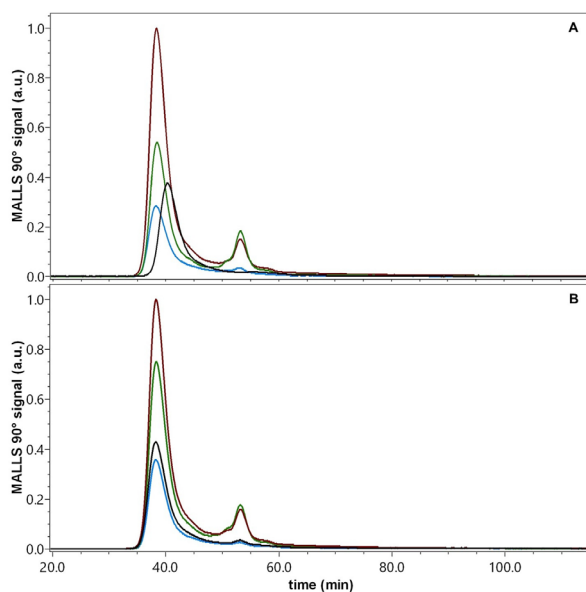
Fig. 5.6. SEC-MALLS elution profiles of (A) black-eye and (B) red Bambara groundnut flours subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted.

the major storage protein fraction, vicilin, as reported in our previous study (Diedericks et al., 2019). In comparison, this peak almost disappeared in the roasted and soaked-roasted flours. This can be attributed to the denaturation of the proteins upon roasting and subsequent formation of larger, insoluble aggregates, which were mostly removed through centrifugation before size-exclusion analysis (Choi & Ma, 2006). This observation is also consistent in the MALLS elution patterns, where (small) peaks corresponding to the vicilin fraction were visible for the control and soaked flours, with almost no response detected for the samples subjected to roasting. In addition, a large peak appeared around 38 min for the control flours, and to a lesser extent for the processed flours, which are indicative of soluble aggregated protein fractions or high-molecular-weight Maillard reaction products (MRPs). The latter can be formed through the interaction of reducing sugars such as glucose and fructose, with proteins and amino acids. This is generally observed by a significant decrease in the free sugars upon roasting, indicating their thermal degradation and subsequent reactivity (Oracz & Nebesny, 2019). However, in the study by Mubaiwa et al. (2018), roasting had no significant influence on the fructose content of the seeds. In addition, considering that the large peak was also present in the control flours, it is more likely evident of soluble protein aggregates. These fractions are present in small amounts as deduced from the corresponding UV and RI responses, which were much smaller compared to the MALLS signal (Hoffmann, Sala, Olieman, & De Kruif, 1997; Zhao et al., 2004).

The molar mass distribution profiles were determined from combined MALLS and RI signals. As shown in Fig. 5.7, the peaks eluting between 52–54 min which were attributed to the major storage protein fraction vicilin, were mostly characterized with constant molecular weights as indicated by the straight lines above the peaks—the slight upwards curving of the M_w slopes is however indicative of the presence of some larger oligomers (Pothecary, Ball, & Clarke, 2012). This is also reflected in the M_w values of the control black-eye (266 kDa) and red (257 kDa) BGN flours, which were slightly higher compared to that previously reported for the trimeric BGN vicilin fraction (196 kDa) (Diedericks et al., 2019). These differences are as expected, considering that vicilin is a purified protein fraction obtained from the flour which is inherently more heterogeneous. In comparison, the largest effect on the M_w for this fraction was seen in the red soaked-roasted (347 kDa) and roasted (378 kDa) flour, as well as the roasted black-eye flour where this elution peak disappeared completely. The effect of roasting was also evident on the size, i.e. radius of gyration (R_g), which increased from 34 nm for the red control flour to 51 nm and 55 nm for the soaked-roasted and roasted flours, respectively. An increased R_g is evident of aggregate formation, as was also observed for β-lactoglobulin solutions

upon heating (Hoffmann et al., 1997). The sharp declining M_w slopes for the peaks eluting earlier is indicative of aggregates with heterogeneous sizes (Choi & Ma, 2006) and with M_w values >1 MDa, these fractions are confirmed as large soluble aggregates eluting at the void volume. The formation of these large aggregates can be linked to the low foaming capacity and stability of roasted and soaked-roasted BGN flours as reported by Mubaiwa et al. (2018), as the molecular unfolding and subsequent aggregation of proteins are known to negatively affect their foaming properties (Pozani, Doxastakis, & Kiosseoglou, 2002). In addition, prominent RI peaks characterized with concave M_w slopes were identified at later elution times, which are indicative of lower molecular weight fractions also with heterogeneous sizes. Similar to the fractions identified in yellow pea flour (Kornet et al., 2019), these later eluting fractions are comparable in M_w to the smaller albumins and vicilin subunits, with M_w values ranging from 5–13 kDa. Overall, the molecular composition of the roasted and soaked-roasted flours were comparable, whereas the soaked flour closely resembled that of the control.


Fig. 5.7. Molar mass profiles as obtained from MALLS (straight line) and RI (dashed lines) responses of (A) black-eye and (B) red Bambara groundnut flours subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted.

5.3.4. *Chromatographic profiles and molecular weight characterization of BGN protein isolates*

To further elucidate on the effect of soaking and roasting on the composition of the BGN seeds, the extracted protein isolates were also subjected to HPSEC analysis. Fig. 5.8 shows the corresponding UV elution profiles of the black-eye and red BGN-PIs. Similar to what was found for the flour, the major peak which was attributed to the trimeric vicilin fraction eluting between 52–54 min, was observed in both varieties and for all treatments. In the red variety this peak appeared smaller for the soaked-roasted sample, whereas in the black-eye variety this peak was slightly smaller for all processed samples compared to the control. In addition, two large peaks which were partially resolved appeared directly after the major eluting fraction for the roasted and soaked-roasted samples in both varieties. A similar observation was made for buckwheat globulin upon heating at 100°C and various time intervals, which was attributed to the dissociation of the major (hexameric) protein into monomers, as suggested by a decrease in the major peak and a simultaneous increase in a subsequently eluting peak (Choi & Ma, 2006). Similarities were also found in the elution patterns of dry beans and lentils, where after cooking the major peaks appeared at higher retention times (Carbonaro, Cappelloni, Nicoli, Lucarini, & Carnovale, 1997).

As seen in the MALLS chromatograms (Fig. 5.9), the large peak eluting around 38 min which was identified as large, soluble aggregates in the flour was also present in the BGN-PIs. In addition, a second (partially resolved) peak appeared as a shoulder to the large peak in all samples, but more prominently for the roasted and soaked-roasted BGN-PIs. Similar structural changes were observed for kidney bean, red bean and mung bean to varying extents, where after heating at 95°C the formation of soluble aggregates or high molecular weight oligomers were evident at lower elution volumes (Tang, Chen, & Ma, 2009). In kidney bean, these aggregates were mostly characterized as vicilin dimers which were formed upon association of the unfolded monomers (Tang & Ma, 2009). Considering that this shoulder peak is also slightly present in the control samples, it is indicative that aggregate formation also occurs during the extraction of the protein isolates; possibly during freeze drying as reported for lupin protein isolates (Berghout, Venema, Boom, & van der Goot, 2015). Furthermore, the peaks eluting after the vicilin peak in the UV chromatograms are not present in the MALLS elution profiles; which are thus indicative of smaller-sized particles/aggregates.

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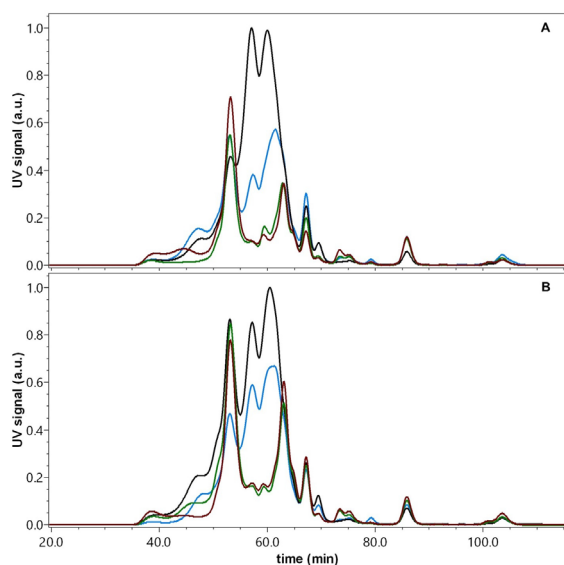
Fig. 5.8. SEC-UV elution profiles of (A) black-eye and (B) red Bambara groundnut protein isolates subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted.
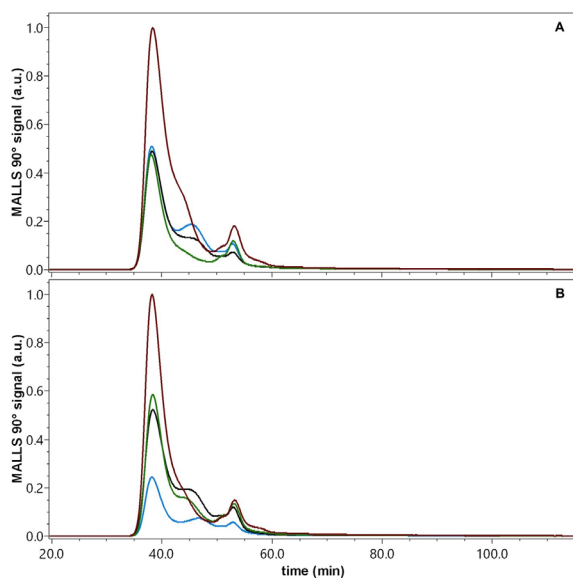
Fig. 5.9. SEC-MALLS elution profiles of (A) black-eye and (B) red Bambara groundnut protein isolates subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted.

The RI and MALLS signals were combined to obtain the molar mass distribution profiles of the BGN-PIs, as shown in Fig. 5.10. The M_w values for the vicilin fraction (eluting between 52–54 min) in the control samples were 279 kDa for the black-eye and 302 kDa for the red variety. As seen from the slight upwards curving of the M_w slopes, these fractions also contain some larger oligomers as were found in the corresponding flour samples. These M_w values are comparable to that found for BGN-PIs from different batches of black-eye seeds as found in **Chapter 3**, which highlights the reproducibility of the protein extraction process. In contrast to the M_w values calculated for this peak in the roasted and soaked-roasted flours, lower values were reported for both BGN-PIs ranging from 207–210 kDa and 206–220 kDa, respectively. Similarly, the R_g for these samples were also lower compared to the control, ranging from 25–35 nm for the roasted BGN-PIs and an average of 31 nm for the soaked-roasted BGN-PIs. This can be attributed to the association-dissociation behaviour of the oligomers upon heating, similar to what was found for globulin proteins from soybean, oat and buckwheat (Choi & Ma, 2006; Mori, Nakamura, & Utsumi, 1982; Zhao et al., 2004). In these proteins it was found that heating caused the formation of soluble aggregates through association of the oligomers, followed by dissociation of these aggregates into monomers (upon further heating) and re-association into high molecular weight aggregates and finally insoluble aggregates. This thermal aggregation effect is seen in the M_w values of the large peaks at earlier retention times (around 38 min) and the subsequent shoulder peaks which appeared before the vicilin fractions. In these peaks, the M_w values were smaller (<2600 kDa) for the roasted and soaked-roasted samples compared to the control and soaked samples (>6100 kDa). This suggests that the aggregates of the roasted and soaked-roasted BGN-PIs became less soluble, to an extent where larger insoluble aggregates were formed, which would have been removed through centrifugation prior to HPSEC analysis (Choi & Ma, 2006).

Compared to the flour, the molecular composition of the BGN-PIs from all processed seeds differed to some extent from the control samples. Overall it can be noted that BGN-PIs have a high thermal stability, despite aggregate formation, as most peaks which were found in the control BGN-PIs were also present in those subjected to heat treatment.

5.4. Conclusions

We have shown that roasting as a pre-treatment method of BGN seeds changes the molecular composition of the resulting flour and protein isolates, whereas soaking

had a minimal effect with samples comparing closely to the control. Using SEC-MALLS as a tool to accurately determine the size and weight-averaged molar masses, it was found that the major storage protein fraction vicilin—as previously identified in BGN seeds—was present as the main fraction in the control and processed flours and resulting protein isolates of both varieties. Furthermore, the thermal aggregation behaviour of BGN proteins was also evident in the roasted and soaked-roasted samples, as observed by the formation of soluble and insoluble aggregates. Yet, BGN-PIs are recognized for their high thermal stability, considering that the same elution patterns found for the control samples were also present in the samples subjected to roasting. In addition, no marked differences were observed between the red and black-eye varieties, as was also evident from microstructural imaging. These findings can be linked to some macroscopic properties of BGN seeds as reported in literature, which is valuable in terms of establishing the best pre-treatment method for a given application from both a structural and functional perspective.
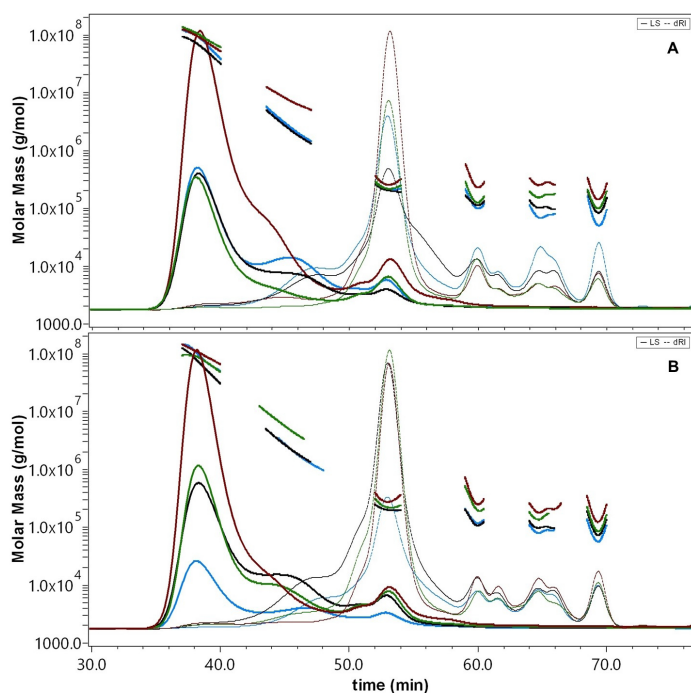
Fig. 5.10. Molar mass profiles as obtained from MALLS (straight line) and RI (dashed lines) responses of (A) black-eye and (B) red Bambara groundnut protein isolates subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted.

Acknowledgements

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Appendix: Supplementary Material
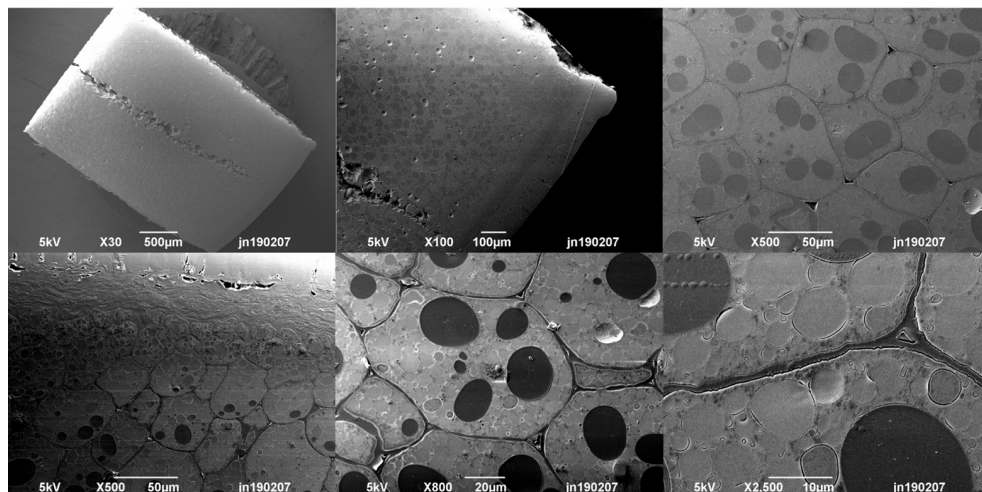
Fig. S5.1. Morphology of Bambara groundnut black-eye control (untreated) seeds imbibed for 48 h, as imaged with cryo-SEM at different magnifications.
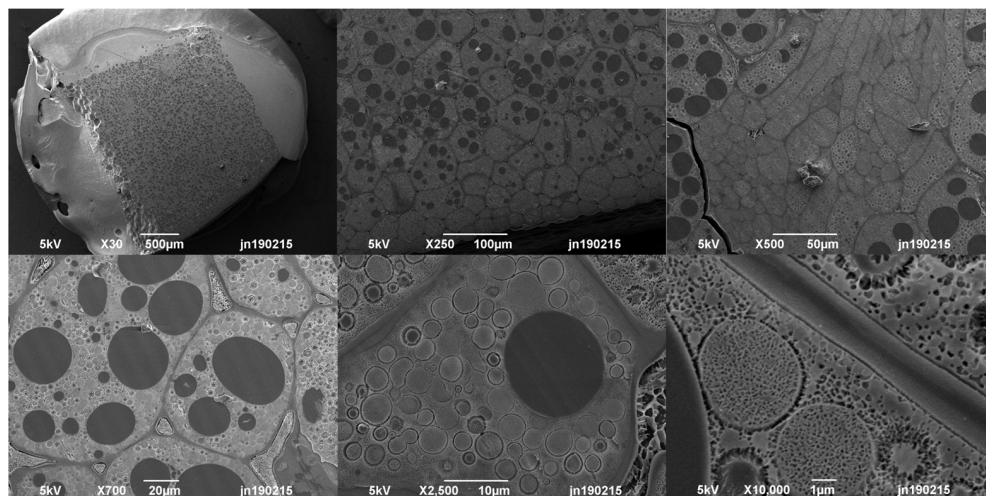
Fig. S5.2. Morphology of Bambara groundnut red control (untreated) seeds imbibed for 48 h, as imaged with cryo-SEM at different magnifications.
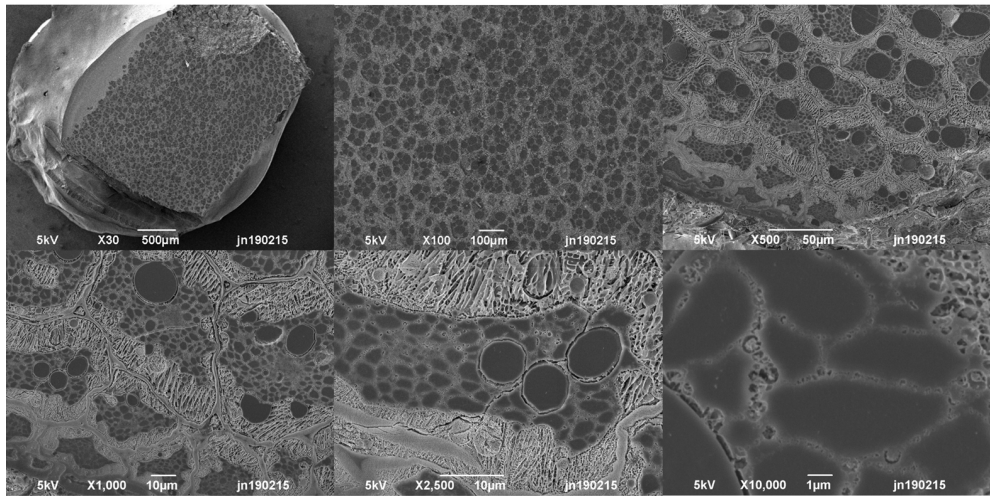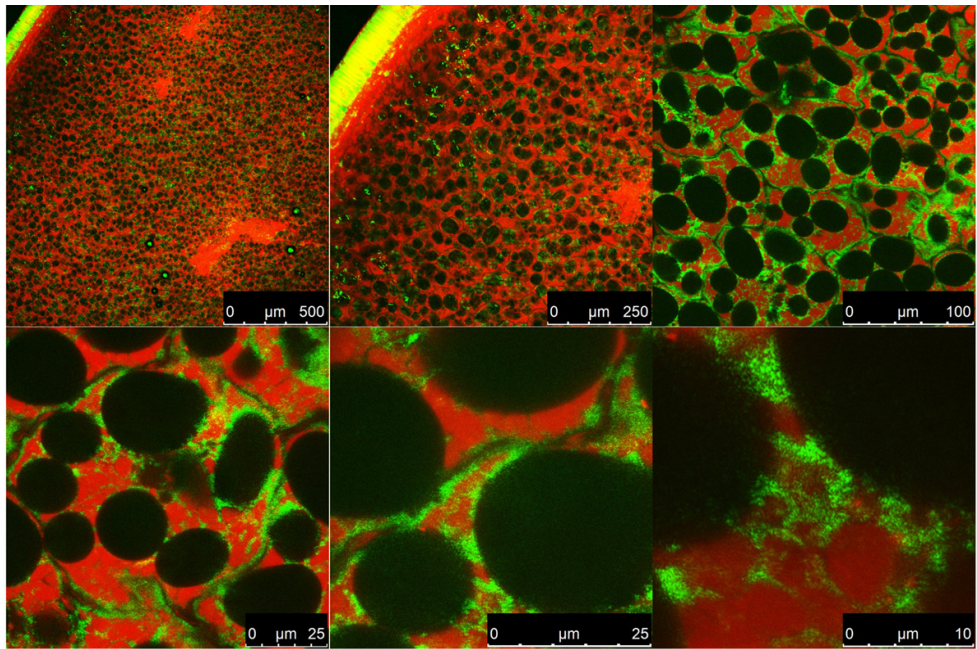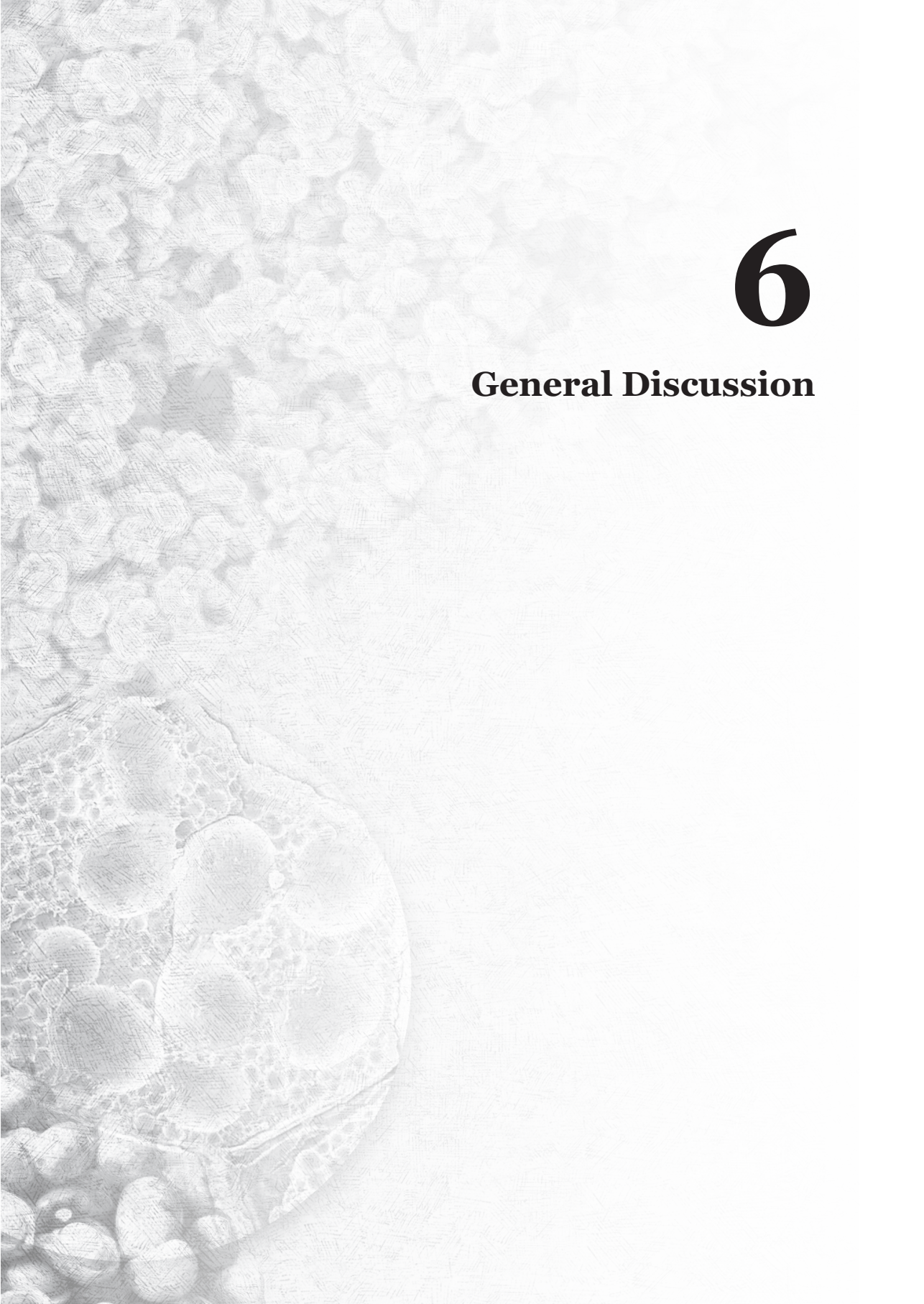
Fig. S5.3. Morphology of Bambara groundnut black-eye roasted seeds imbibed for 48 h, as imaged with cryo-SEM at different magnifications.

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Fig. S5.4. Morphology of Bambara groundnut black-eye control (untreated) dry seeds, as imaged with CLSM at different magnifications.

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General Discussion

6.1. Introduction

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] is a pulse crop praised for its nutritional composition—the seeds are characterised as a complete food—and its ability to adapt to harsh environmental conditions, which is of increased importance in the current context of climate change. Yet, despite its apparent promise, BGN remains to be classified as a "neglected and underutilised species" (NUS) or so-called "orphan" crop (Padulosi, Thompson, & Rudebjer, 2013). In recent years however, important strides have been made towards recognising the potential of BGN ingredients and products, with an increased focus on the functionality of the processed fractions obtained from the seeds. The minimum concentration at which a solid-like material is formed, portrays the extent to which gelation functionality has been investigated for BGN flour and protein systems, up till now. Considering the importance of gelation in providing specific textural (and other sensorial) characteristics to foods, it is important to understand how structure formation at the molecular scale links to the observed macroscopic functionality. By noting this gap in BGN literature in linking the structural aspects (as influenced by processing) and functionality of BGN ingredients, and considering the drive in protein transition which calls for more diverse proteins from "resource-efficient sources" (Pyett, de Vet, Trindade, van Zanten, & Fresco, 2019), we were able to formulate the aim of this thesis. Hence, the objective was to gain insights into the link between the macroscopic gel formation of BGN proteins and their structural properties. This was achieved by investigating the physicochemical properties of BGN proteins with varying purity as obtained via different extraction routes, followed by the characterisation of the resultant gel microstructures and their rheological properties. Through this approach we were able to identify how differences in processing (pre- and post-extraction) of BGN seeds affected the protein compositions, and how this in turn influenced gel formation. An overview of the main findings of this thesis is shown in Fig. 6.1, which also forms the basis for further discussions presented in this chapter.

6.2. Effect of processing on mesoscale structure

In this thesis, various means of processing were explored to obtain the fractions of interest. Here, processing is defined as any modification to the raw (control) seeds and relates to both the extraction methods employed to obtain BGN proteins of varying purity (**Chapters 2** and **3**), and to the pre-treatments applied to BGN seeds which are typically used in practice to overcome certain negating factors associated with the seeds (**Chapter 5**).
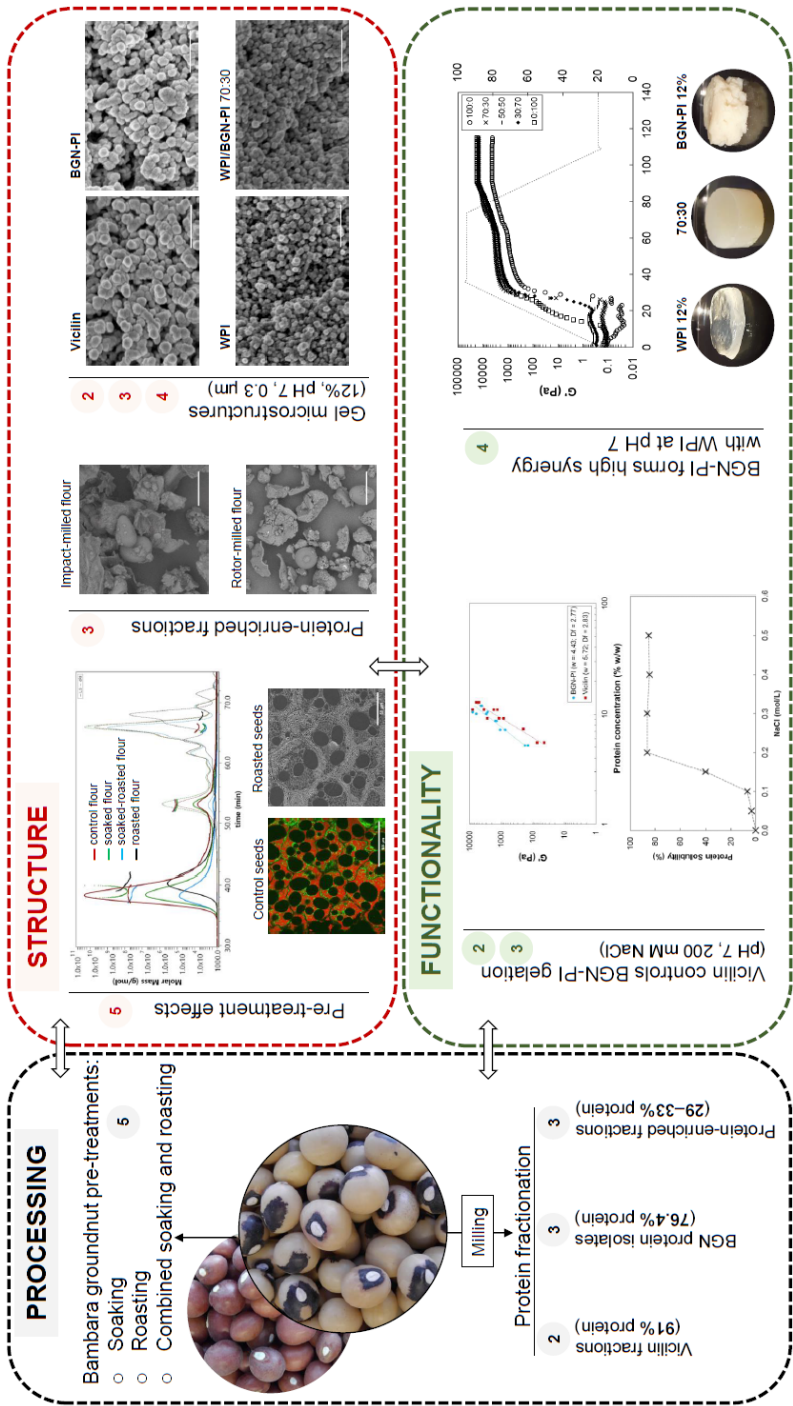
Fig. 6.1. Graphical overview of the main findings; numbers indicate the corresponding chapters in this thesis.

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6.2.1. *High purity Bambara groundnut protein fractions*

There are various techniques used for the production of protein fractions. Nowadays it is recognised that highly purified protein fractions, as obtained via conventional wet fractionation techniques, are not as sustainable (in terms of energy and water consumption) when compared to mildly processed protein-enriched fractions (Pelgrom, Boom, & Schutyser, 2015a). However, it is also recognised that the characterisation of such highly purified fractions is necessary to gain a molecular understanding, which will aid in controlled functionality when considering application of such fractions as food ingredients (O'Kane, 2004). Considering the limited knowledge on BGN proteins, it was therefore necessary to first extract and characterise the highly purified protein fractions before investigating more mildly processed fractions.

To that end, we have extracted the BGN vicilin fraction (**Chapter 2**) using an optimised Osborne fractionation method. Vicilin proteins, also denoted as the 7S globulin fractions based on their sedimentation coefficient and solubility in salt solutions (Shewry & Casey, 1999), are considered as the major storage proteins in many legume seeds. Similarly we were able to show for the first time[1], as per our knowledge, that vicilin proteins forms the major storage proteins in BGN seeds, comprising almost 50% of the total proteins present. The fractions were characterised with a high purity (91 g/100 g protein content, N x 5.7) and were free from other contaminating proteins such as legumins, as revealed by the similar bands under both reducing and non-reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Hereto the optimisation of the extraction conditions proved to be important, as the use of an alternative extraction buffer (0.2 M borate buffer at pH 9 + 0.5 M NaCl) resulted in contaminated vicilin fractions. This was observed by the differences in gel electrophoresis under non-reduced and reduced SDS-PAGE as shown in Fig. 6.2, where in the latter the band around 80 kDa disappeared whilst two bands around 22 kDa and 34 kDa became visible. These bands could be attributed to the legumin fractions, which under reducing conditions would be cleaved into the basic (21–23 kDa) and acidic (35–43 kDa) subunits (Tzitzikas, Vincken, De Groot, Gruppen, & Visser, 2006). As a further means of confirming the purity and size characteristics of the vicilin fractions, size-exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) was used as a valuable experimental tool. Comparing the vicilin proteins to the defatted flour (extraction raw material)

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[1] Amongst the BGN literature, there is one study by Arise, Nwachukwu, Aluko, & Amonsou (2017) in which vicilin fractions were extracted. In this study however, clear differences were reported for the electrophoretic patterns in the presence of reducing agents, which indicates the presence of contaminating protein fractions containing disulphide bonded polypeptides such as legumins.

and the BGN protein isolates (extraction conditions discussed in **Chapter 3** and in subsequent text to this section), a narrower molecular weight (M_w) distribution was found with the vicilin peak coinciding with the major peaks of both the BGN flour and protein isolates eluting around 52 min, as shown in Fig. 6.3—here the UV elution profiles are shown as detection response for proteins at 280 nm (Fekete, Beck, Veuthey, & Guillarme, 2014). The M_w and radius of gyration (R_g) as calculated from combined MALLS and refractive index (RI) responses of the peaks at 52 min were all comparable, ranging from 185–205 kDa and 8–12 nm, as such confirming the presence of vicilin as the major protein fraction in BGN seeds whilst also reflecting the high purity of the vicilin extract. These size characteristics are comparable to that reported for soy (i.e. β-conglycinin) and pea vicilin fractions (Guo et al., 2012; Miles, Morris, Carroll, Wright, & Newby, 1985), which considering the large body of knowledge available on these legume proteins, provides a good basis on which to model further investigations on BGN vicilin proteins.

Another method which is commonly utilised for the extraction of proteins with in general a higher purity, is isoelectric precipitation. This method has been widely described in literature for the extraction of pulse and other plant proteins, and is based on the high solubility of these proteins at alkaline conditions and their subsequent precipitation at pH values in the range of their isoelectric points (generally between pH 4–5) (J. Boye, Zare, & Pletch, 2010). This extraction technique was also applied in this thesis (**Chapter 3**) by solubilising the BGN protein isolates (BGN-PI) at pH 9.5, followed by isoelectric precipitation at pH 4; these conditions were optimised based on the highest yield and protein content when varying the solubility pH (9–10) and the extraction pH (4–5). The total protein content of BGN-PI was 76 g/100 g (N x 5.7) which is comparable to that reported for isoelectric precipitated proteins from other pulses such as lentils and chickpeas (J. I. Boye et al., 2010), but as expected constituted a lower protein purity compared to the BGN vicilin proteins. The SDS-PAGE analysis however revealed that the vicilin fractions represented the major protein group in the BGN-PIs, as also clearly confirmed with the SEC-MALLS analysis (see for example UV elution profiles in Fig. 6.3). Similar SEC-HPLC elution profiles were reported by Rangel, Domont, Pedrosa, & Ferreira (2003) for protein isolates from cowpea, which is a pulse crop also classified under the *Vigna* genus. Here the authors also identified vicilin as the major protein fraction in the cowpea protein isolates and alluded to the economic benefits of extracting the protein isolates as opposed to the purified vicilin fractions. In addition, the BGN-PIs were also characterised by the presence of larger aggregates as revealed with the SEC-MALLS analysis. Aggregate formation is typically observed in protein isolates subjected to freeze-drying, as reported by Berghout, Venema, Boom, & van der Goot

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(2015) for lupin protein isolates. To avoid freeze-dried induced aggregate formation, the authors suggested concentration of the wet protein isolates as an alternative. Thus, functionality-dependent, concentrated BGN-PIs could also be explored as a means of not only reducing aggregate formation, but also reducing the cost factor associated with freeze-drying.
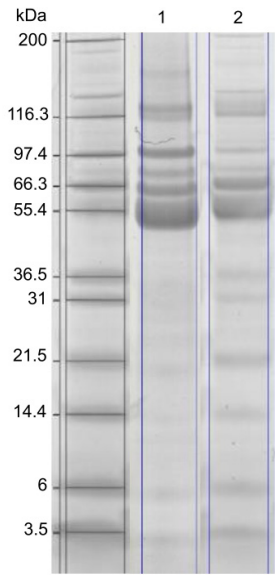
Fig. 6.2. Electrophoretic patterns of Bambara groundnut vicilin fractions (0.12% w/w, pH 7) under non-reducing (lane 1) and reducing (lane 2) conditions; as extracted with 0.2 M borate buffer (pH 9, 0.5 M NaCl).
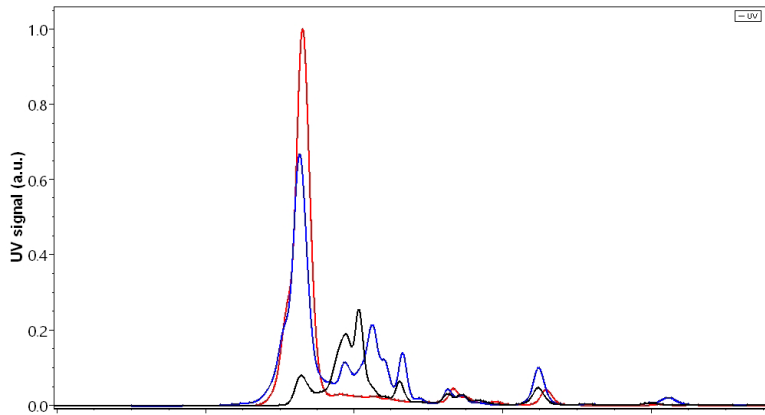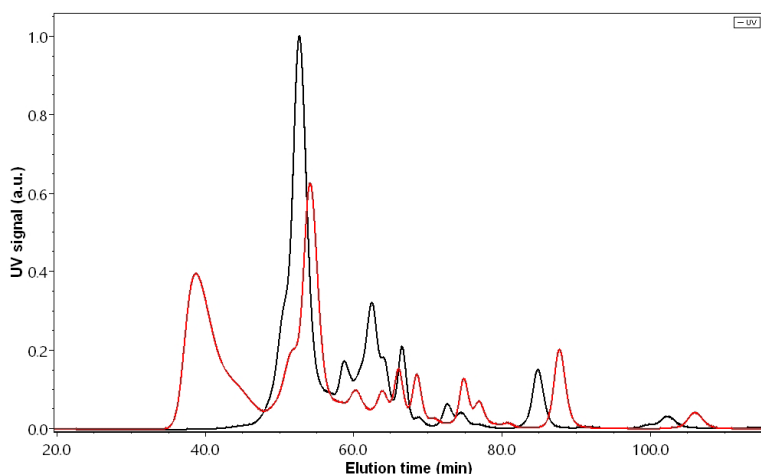

Fig. 6.3. SEC-UV elution profiles of Bambara groundnut vicilin (red), protein isolates (blue) and flour (red) fractions.

Considering the effect of polyphenol interactions on the structural characteristics of Bambara groundnut protein isolates

Another important, yet often overlooked aspect of plant protein characterisation, is the influence of protein-polyphenol interactions. Polyphenols are known to interact with proteins either covalently or non-covalently, which can lead to changes in amongst others the protein structure, thermal stability and solubility (Ozdal, Capanoglu, & Altay, 2013). These protein modifications are initiated when proteins are extracted, which typically occurs after cell rupturing and release of cell contents. This process leads to the interaction of normally separated proteins and secondary metabolites (i.e. phenolic compounds), resulting in the modification of proteins and subsequent altered nutritional and techno-functional properties (Vissers, 2017). Whilst most studies focusses on the protein-polyphenol interactions occurring upon mixing of the separate compounds, only a few studies have investigated the effect of inherent polyphenols on protein structural and physicochemical properties by "dephenolising" the starting material from which proteins are extracted (Malik, Sharma, & Saini, 2016).

In preliminary experiments, we have observed a brown discolouration of BGN-PI solutions after sonication. Browning reactions in plant foods are attributed to the formation of dark melanin pigments, initiated by the enzymatic oxidation of polyphenols. Considering that ultrasound-assisted extraction is also commonly used to extract polyphenols from plant material (Galili & Hovav, 2014), the discolouration of BGN-PI solutions was therefore indicative of polyphenols interacting with the proteins. Consequently, polyphenols were extracted from BGN flour using aqueous ethanol (70% v/v) and methanol (50%, 70% and 80% v/v) as extraction solvents. The total polyphenol content, as determined with the Folin-Ciocalteu assay, was reduced in all the solvent-extracted flour from 105 mg gallic acid equivalents (GAE)/100 g in the untreated flour to a minimum of 17 mg GAE/100 g for the 80% methanol extracted flour. BGN-PIs obtained from the 80% methanol extracted flour were further analysed with SDS-PAGE and SEC-MALLS, to determine if any structural changes occurred in the proteins. The electrophoretic patterns of the BGN-PIs obtained from the polyphenol-extracted flour were similar to that of the BGN-PIs from the untreated flour, thus indicating that no significant structural changes occurred after polyphenol removal. This is in contrast to the observation by Malik et al. (2016) for sunflower proteins where no bands were visible for the untreated protein isolates, which the authors ascribed to a higher molecular weight of proteins caused by the interactions with polyphenols. This could possibly be attributed to the high reactivity of chlorogenic acid—the main phenolic compound in sunflower seeds which is

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absent in dehulled BGN black-eye seeds—with free amino groups, as observed for soy glycinin protein and polyphenol mixtures (Harris, Jideani, & Le Roes-Hill, 2018; Malik et al., 2016; Rawel, Czajka, Rohn, & Kroll, 2002). Differences were however observed in the SEC-MALLS analysis, where the large peak eluting before the major vicilin proteins as observed for the untreated BGN-PIs (**Chapter 3**), was now characterised in the polyphenol-free BGN-PIs with a larger light scattering signal and prominent UV and RI responses (see Fig. 6.4 for SEC-UV chromatograms). The change in M_w (205 kDa to 1059 kDa) and R_g (12 nm to 80 nm) of BGN-PIs obtained from the 80% methanol extracted flour were clearly evident of large aggregate formation. Considering that the presence of protein-polyphenol complexes are known to affect retention times and the shape of peaks due to molecular interactions and possibly protein denaturation (Kroll, Rawel, & Rohn, 2003), it was expected that BGN-PIs obtained from polyphenol-free flour would be characterised with a lower amount of aggregates and a well-defined peak for the vicilin fractions. Our results indicate towards polyphenol interactions having a minor effect on BGN proteins, whilst the solvent extraction process causes protein denaturation and subsequent aggregate formation. These are however considered as preliminary observations, as further research is necessary to quantify the polyphenols present in BGN-PIs (before and after polyphenol extractions) and to evaluate the effect of ethanol and methanol (at lower concentrations) on the structural properties of BGN-PIs.

Fig. 6.4. SEC-UV elution profiles of Bambara groundnut protein isolates obtained from untreated flour (black) and 80% v/v methanol polyphenol-extracted flour (red).

6.2.2. *Applicability of mild fractionation for Bambara groundnut protein-enriched fractions*

In addition to the higher purity protein fractions, the suitability of Bambara groundnut seeds for dry fractionation was also explored in **Chapter 3**. Milling, as one of the first steps in processing of seeds for the production of flour or further extraction of individual components, are commonly reported in the BGN literature. Yet, the transition has not yet been made to explore the suitability of dry fractionation techniques in producing protein- and starch-enriched BGN fractions. Considering the increased shift towards dry fractionation and other mild fractionation techniques for the sustainable production of functional ingredients (Pelgrom et al., 2015a), the investigation into the suitability of such techniques for BGN (protein-enriched) fractions also formed a small, yet important branch to this thesis. Comparing two routes of dry fractionation—i.e. impact milling with air classification and rotor milling with air jet sieving—we were able to successfully separate the particles into fine, protein-enriched and coarse, starch-enriched fractions. Under the conditions applied, rotor milling/air jet sieving resulted in protein-enriched fractions with a smaller volume-averaged particle diameter ($D_{0.5}$ 17.9 μm) and an overall higher separation. SDS-PAGE analysis revealed two major bands around 50–63 kDa in all fractions, which corresponds to the β and α subunits of BGN vicilin. In dry fractionation, it is known that optimal separation conditions are dependent on various factors, i.e. maximum overlap between starch and flour particle size distribution curves, seed morphology, composition, etc. (Pelgrom, Boom, & Schutyser, 2015b). Similarly, we were able to identify some parameters which are important to consider when following the dry separation routes for BGN fractions as described in this thesis. Here, a smaller batch size (of starting material) was better suited for bench-top rotor milling, whereas in impact milling higher losses were recorded, as a result of the particles accumulating on the inner walls of this pilot scale mill and as such leading to a larger fouling layer. The classifier wheel speed in the impact mill was also of importance, as higher losses were recorded at higher wheel speeds, i.e. the flour yield decreased from 28.9 g/100 g at a speed of 3000 rpm, to 7.4 g/100 g and 6.3 g/100 g at a speed of 4500 rpm and 6000 rpm, respectively. The advantage of the higher classifier speeds however, is that smaller particles in the size range of the protein bodies are produced which are able to pass through the slits of the classifier wheel (Pelgrom, Vissers, Boom, & Schutyser, 2013), and as such a pre-classification took place with an increased protein content in the BGN impact milled flour as shown in Table 6.1.

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Table 6.1. Composition and milling yield of Bambara groundnut flour as obtained with impact milling at varying classifier wheel speeds.

Classifier wheel speed (rpm)	Protein (N x 5.7)[a]	Starch	Yield (dry weight)	$D_{0.5}$ particle size (μm)
		(g/100 g)		
3000	21.7 ± 0.2 (23.8)	29.2 ± 0.8	28.9	27.4 ± 0.1
4500	30.2 ± 1.1 (33.1)	37.5 ± 3.3	7.4	21.1 ± 0.8
6000	29.7 ± 0.1 (32.5)	32.5 ± 0.1	6.3	25.6 ± 0.3

[a] N x 6.25 indicated between brackets

This decrease in particle size at higher classifier wheel speeds was also seen in the SEM micrographs as shown in Fig. 6.5. It was however noted that the volume-averaged particle diameter ($D_{0.5}$) was slightly higher at 6500 rpm compared to the particles obtained at 4500 rpm. This could be attributed to increased particle-particle adhesion as the particles are further reduced below the cut point, i.e. the point at which particles have an equal chance to end up in either the fine or coarse fraction (Dijkink, Speranza, Paltsidis, & Vereijken, 2007). To reduce these particle agglomerations, the BGN flour obtained at 6500 rpm was further subjected to pressure titrations, which at higher pressures allowed increased dispersibility of the particles and as such resulted in smaller particle diameters ($D_{0.5}$ 20 μm at 250–400 kPa). Overall, we have shown that BGN seeds are suitable for dry fractionation and by varying some process parameters, the composition of protein- and starch-enriched fractions could be changed which creates the opportunity to sustainably produce application-specific BGN fractions.

6.2.3. *Effect of pre-processing on BGN seeds*

In **Chapter 5**, we have investigated the effect of three pre-treatments on the composition and microstructure of BGN seeds and resulting fractions. Our approach was to investigate processing methods which are commonly used in the local areas of consumption to overcome the hard-to-cook and hard-to-mill properties associated with the seeds. The decision for the type of pre-treatments, i.e. soaking, roasting and a combination of these two treatments, was based on the study performed by Mubaiwa, Fogliano, Chidewe, & Linnemann (2018), which allowed us to link in some
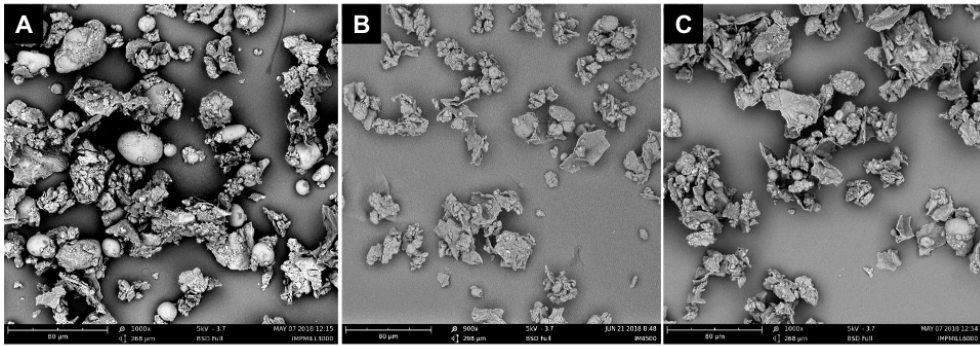
Fig. 6.5. Scanning electron micrographs of Bambara groundnut impact milled (IM) flour obtained at varying classifier wheel speeds: (A) 3000 rpm, (B) 4500 rpm and (C) 6000 rpm. Scale bars correspond to 80 μm.

instances our observations to the macroscopic functionality of the flour obtained from the pre-treated seeds, as reported by these authors. Comparing the results to the untreated, control seeds, it was evident that roasting had the largest effect on the seed microstructures and on the composition of the resulting flour and protein isolates. In terms of the seed microstructures, the seeds subjected to roasting were characterised with aggregated cell material to an extent where the protein bodies were no longer distinguishable, as observed with cryo-scanning electron microscopy (cryo-SEM). This change in microstructure could be linked to the yield (and protein content) of the protein isolates, which were threefold lower for the roasted and soaked/roasted seeds compared to the untreated seeds. Furthermore, using again SEC-MALLS as a valuable tool to monitor the changes in the molecular weight (M_w) and size (in terms of the radius of gyration R_g) characteristics of the BGN flour and protein isolates, we were able to show that roasting indeed caused the formation of large soluble and insoluble aggregates through the association-dissociation behaviour of oligomers. It has been shown that aggregate formation can be linked to decreased protein digestibility in some legume proteins subjected to heat processing (Carbonaro, Cappelloni, Nicoli, Lucarini, & Carnovale, 1997). Considering that thermal processes amongst others are commonly used to not only overcome the hard-to-cook and hard-to-mill properties of BGN and other legume seeds, but also as a means of inactivating anti-nutritional factors to improve protein digestibility (J. Boye et al., 2010), it would be worthwhile to also investigate the effect of roasting (as applied in our study) on the digestibility of BGN flour and protein isolates. This is especially relevant in the local areas of consumption which is largely in sub-Saharan Africa, where a reported 58 million (33%) children suffer from various forms of

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malnutrition (UNICEF/WHO/The World Bank, 2019). Comparatively, the seeds subjected to soaking were similar in composition to the control seeds. As such, soaking as a pre-treatment method to circumvent negating factors (i.e. hard-to-cook properties) associated with BGN seeds, can be recommended as a pre-treatment method of choice when considering compositional and functionality aspects.

In terms of the microstructural characterisation, we were able to utilise the inherent autofluorescence of BGN protein bodies, as a means of imaging the seed structures using confocal laser scanning microscopy (CLSM). In fluorescence microscopy such as CLSM, fluorescent dyes are generally used to stain the structures of interest, through sometimes elaborate sample preparation and staining techniques (Preece et al., 2015; Wood, Knights, & Chocty, 2011). By making use of the intrinsic autofluorescence of the BGN protein bodies, we were able to eliminate extensive sample preparation, and as such present an alternative means of imaging (BGN) seed structures. Although this technique of autofluorescence imaging is not novel in itself, as seen for example by the use of autofluorescence for imaging an acellular human dermis transplant (Roessner, Vitacolonna, & Hohenberger, 2012) or copepods (Michels, 2007), it does appear to be novel for legume seeds. We do expect this microscopic technique to be applicable to other pulses, however considering the novelty aspect, it would be recommended to also use additional microscopic techniques to validate the observed microstructures, especially if these are not yet reported in literature.

6.3.   Linking mesoscale structure and functionality

Considering again our aim of gaining insights between the macroscopic gel formation of BGN proteins and their structural and compositional properties, we have primarily made use of the rheological responses of the single BGN protein gel systems (**Chapters 2** and **3**) and in admixture with whey proteins (**Chapter 4**) as a basis for investigating this link. The main findings in this regard are discussed in the subsequent sections.

6.3.1.   *Bulk rheology of Bambara groundnut protein gels*

Vicilin proteins are characterised by their higher solubility in the presence of salt, as also observed for BGN vicilin fractions in this thesis (**Chapter 2**) with the highest solubility found at ≥ 200 mM NaCl concentrations. This could be linked to the presence of the β subunit as the apparent major subunit in BGN vicilin fractions, which as reported by Maruyama et al. (1999) for β-conglycinin, was insoluble at

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lower ionic strengths. This solubility dependence of the vicilin proteins as a function of ionic strength, was further used as a means to determine the role of the vicilin fractions in the gelation behaviour of the BGN protein isolates. Considering that the vicilin fractions are present as the major proteins in the BGN-PIs, as revealed by SEC-MALLS analysis, our hypothesis was that the vicilin fractions would control the gelling behaviour of the BGN-PIs. This was indeed confirmed in **Chapter 3**, where the elastic modulus (G') of the BGN-PI gels shifted to higher values in the presence of 200 mM NaCl, to closely resemble the gelation profile of the vicilin proteins at the same salt concentration. Furthermore, by applying a scaling analysis as a means of determining the relation between the structure of BGN proteins and the rheology of the resultant gels (Dhayal, 2015), we were able to provide further evidence of the governing role of BGN vicilin proteins in the gel formation of the BGN-PIs. Hereto the fractal scaling approach was applicable in describing the gel networks of both BGN vicilin proteins and BGN-PIs. By using the Shih, Shih, Kim, Liu, & Aksay (1990) and Wu & Morbidelli (2001) models as described in **Chapters 2** and **3**, the fractal dimensions (D_f) and other scaling parameters were determined as shown in Table 6.2. The scaling parameters of both vicilin and BGN-PI gels were similar, which allowed characterisation of the gels as rigid aggregate structures in the weak-link regime, i.e. the elasticity of the system is determined by the links between the clusters. Finally, the self-similarity as implied for fractal aggregates (Walstra, 2003) of the vicilin and BGN-PI gel structures, were closely comparable as observed with SEM. Through these findings we can conclude that the vicilin proteins control the gel formation of the BGN protein isolates, and as such these highly purified vicilin fractions are not necessary to achieve gelation functionality under the specified conditions.

   In terms of the dry fractionated BGN fractions, gel formation was evident at minimum gelling concentrations of 3.4% for the impact milled and 3.6% for the rotor milled protein-enriched fractions (**Chapter 2**). The corresponding rheological responses revealed higher gel strengths for the impact milled protein-enriched fractions, which is also promising when compared to the purified BGN proteins for which gel formation does not occur at these low concentrations. An important consideration for the BGN protein-enriched fractions, however, is the sedimentation of larger particles (debris) which was evident in the minimum gelling concentration experiment. Further studies are therefore necessary to characterise gel formation of the protein-enriched fractions at higher concentrations, and to determine the effect of these larger particles on the type of gel network structures formed.

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Table 6.2. Scaling parameters of Bambara groundnut vicilin and protein isolate gels as obtained with fractal scaling models (pH 7, 200 mM NaCl).

	Fractal scaling parameters[a]			
	w	D_f	β	α
BGN vicilin	5.72	2.83	1.06	0.98
BGN-PI	4.43	2.77	1.05	0.98

[a] w is the power law scaling exponent, fractal dimension (D_f) determined with the Shih et al. (1990) and Wu & Morbidelli (2001) models, β parameter and α constant determined with the latter model.

6.3.2. *Gel formation of Bambara groundnut proteins in admixture with whey proteins*

In addition to the gel functionality of the single BGN protein systems, the suitability of BGN-PIs in replacing whey protein isolates (WPI) in heat-induced gelled systems at specific mixing ratios and pH conditions were also investigated (**Chapter 4**). The functionality aspects and especially the mechanistic understanding of mixing plant and animal proteins are gaining increased interest, due to the transition towards more sustainable protein sources and to create novel structures for various food applications. The partial replacement of WPI with BGN-PI at pH 7 resulted in the synergistic enhancement of the gel networks at all mixing ratios (i.e. 30:70, 50:50 and 70:30 for 12% w/w total protein systems). Here, all mixed gels were characterised with similar rheological responses to that of the single BGN-PI gel, which had a higher gel strength compared to the single WPI gel. This observation of a higher gel strength for BGN-PI in comparison to WPI at neutral pH, is contrary to what is typically observed for other plant proteins (McCann, Guyon, Fischer, & Day, 2018). It was therefore thought that a higher extent of disulphide interactions in combination with electrostatic interactions were responsible for this apparent characteristic behaviour of BGN-PIs. In addition, microstructural characterisation of the gels at pH 7 revealed the presence of coarse aggregated structures for both BGN-PI, WPI and their mixtures at smaller length scales. These microstructures, however, did not reflect the macroscopic gel properties which for both proteins, were largely different. To some extent we could explain this observation by the large pores present in the

BGN-PI gels, but it is also recognised that these are small differences which do not directly link to the macroscopic gel characteristics. In comparison at pH 5 and pH 3, the partial replacement of WPI with BGN-PI resulted in either a decrease or no difference on the rheological responses of the mixed gels, respectively. In the latter instance, BGN-PI could be beneficially applied in food systems where the rheological response of WPI is desired, with the additional benefit of a complementary amino acid profile.

6.4. Concluding remarks

Plant proteins and more specifically pulse proteins, are increasingly recognised for not only their sustainability and nutritional benefits, but also for their novel structuring abilities as food ingredients. The subject of this thesis, Bambara groundnut, is such a pulse protein source which although being recognised for its valuable nutritional composition and remarkable cultivation character, remains to be undervalued as an indigenous crop. Hence, the aim of this thesis was to not only shed light on the potential of BGN as a novel protein source, but to bridge the gap between the observed macroscopic functionalities and the structural properties of the proteins. The findings reported in this thesis revealed that high purity BGN proteins (vicilin fractions) are not necessary to achieve the same gelation functionality as the protein isolates with a lower purity. In addition, when combined with whey protein isolates, BGN-PIs were able to change the rheological responses of the gels which provides novel reformulation opportunities. To that end, further studies delving into the phase behaviour of BGN and whey (and other) protein mixtures is necessary, to further cement our understanding of BGN proteins in mixtures. Furthermore, we have also touched upon the topic of mild processing and have shown for the first time that dry fractionated protein-enriched fractions can be obtained from BGN flour. Branching out further on the processing aspect, the effect of pre-treatments revealed that soaking is a beneficial method to address amongst others the hard-to-cook properties associated with BGN seeds, whilst retaining a similar composition as the raw seeds. Overall, the findings presented in this thesis provide new knowledge in terms of the structure-function behaviour of BGN proteins, thereby strengthening the position of BGN as an alternative pulse protein source.

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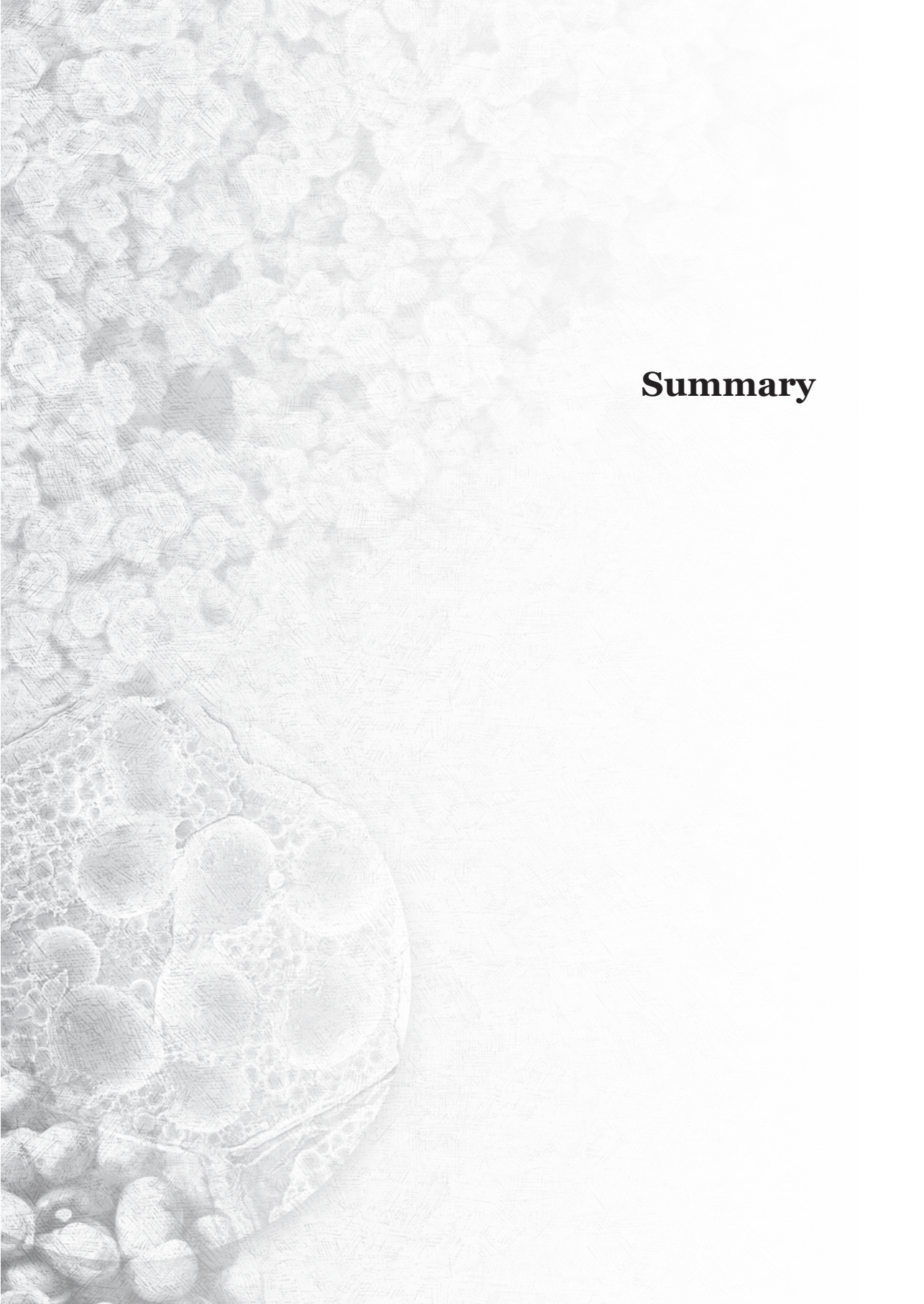
Summary

In recent years, the importance of shifting from a largely animal-based protein diet to one rich in plant proteins have become increasingly apparent. This is attributed to many factors of which most can be linked to aspects of food security, sustainability and climate change. To that end, legume crops and specifically the so-called "neglected and underutilised species" are increasingly recognised as sources with the potential to address several of these factors important to the protein transition. Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] is one such a crop which is praised as a complete food based on its optimal nutritional composition, and with a reported protein content of up to 27%, BGN is increasingly recognised for its potential as a novel protein source. Research efforts have therefore been geared towards highlighting the functionality of BGN proteins, which although being of great value, are still lacking in terms of the link between the observed macroscopic functionality and molecular and mesoscopic structural properties of the proteins. This is especially true for the gelation functionality of BGN proteins, which up till now have only been investigated for the minimum concentration above which a solid-like material can be observed after heating. The aim of this thesis was therefore to gain insights into the structural properties of BGN proteins obtained from different processing streams and their resulting gelation properties. As a basis to this aim, an overview addressing the characteristics of BGN and the reported functionality of its proteins, as well as the typical extraction processes and gelation functionality of plant proteins are presented in **Chapter 1**.

Vicilin protein fractions which are trimeric proteins with a molecular weight (M_w) around 150–170 kDa, are the known major storage proteins in most legume seeds. In **Chapter 2** we have therefore optimised extraction processes to obtain a high purity (91% protein content) vicilin fraction from BGN seeds, which were free from contaminating proteins. This was confirmed by size-exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS), where the (only) major eluting peak coincided for vicilin fractions from different extraction batches with an average M_w of 196 kDa. Furthermore, the BGN vicilin fractions were most soluble in ≥200 mM NaCl solutions, which proved to be an essential characteristic to interpret the gelation behaviour of the less pure BGN protein isolates (BGN-PIs) as found in the subsequent chapter. In terms of the gelation functionality, BGN vicilin fractions formed self-supporting gels at minimum protein concentrations of 5.5% w/w. The rheological parameters of the heat-induced BGN vicilin gels were also well-fitted to the fractal scaling model, with which the gels were characterised as weak-link fractal structures.

In **Chapter 3**, the commonly utilised isoelectric precipitation method was used to extract the BGN-PIs, which were characterised with a lower protein

content (76%) compared to the BGN vicilin fraction. The BGN-PIs were further characterised with the same experimental techniques as applied to the BGN vicilin fractions, revealing similarities which were evident of the vicilin proteins forming the major component in the BGN-PI extracts; as shown for example by the SEC-MALLS elution profiles where the largest eluting peak corresponded to that of the vicilin proteins. The rheological responses in terms of the elastic modulus G' of the BGN-PI gels, revealed a concentration dependence which closely resembled that of BGN vicilin upon 200 mM NaCl addition. Furthermore, the BGN-PI gels were also characterised as weak-link fractal structures, whilst microstructural images revealed the same inhomogeneous aggregates as observed for the BGN vicilin gel networks. In addition, we have also investigated the applicability of dry fractionation techniques as a means of obtaining protein-enriched BGN fractions. Both rotor milling combined with air jet sieving and impact milling combined with air classification, were successfully applied to separate the starch-rich coarse fractions from the protein-rich fine fractions. Gel formation was evident for protein-enriched fractions obtained from both methods, with the impact milled fractions showing higher gel strengths compared to the rotor milled fractions.

In **Chapter 4** the heat-induced gelled mixtures of BGN-PIs with whey protein isolates (WPI) were investigated for potential synergistic enhancement, at varying pH and mixing ratios. At neutral pH, the addition of BGN-PI to WPI resulted in a high synergy, with BGN-PI controlling the gelation behaviour of the mixed gels which led to higher gel strengths, independent of the mixing ratios. The microstructures of the mixed gels also appeared similar to that of the single BGN-PI gels, although when compared to the microstructure of the single WPI gels, only minor differences were evident which could not explain the large variations as observed on a macroscopic scale. Furthermore, at acidic pH, BGN-PI had either no effect (pH 3) or resulted in lower gel strengths (pH 5) of the mixed gels.

In **Chapter 5** the processing effects were investigated from an additional angle. Here three pre-treatments, i.e. soaking, roasting and a combination of the two, used in practice to circumvent negating factors (such as hard-to-cook and hard-to-mill properties) associated with BGN seeds, were investigated for their effect on the seed morphology and compositional properties of the resulting fractions. The seeds subjected to roasting were distinctly different in their morphology compared to the untreated, control seeds—cryo-scanning electron microscopic images revealed aggregated cell material indicative of the dry state, with indistinguishable protein bodies. Furthermore, SEC-MALLS analysis revealed the thermal aggregation effect on the flour and BGN-PIs, with the formation of soluble and insoluble aggregates evident in both roasted and soaked-roasted samples. In comparison, soaking had

a minimal effect, with the molecular composition of the soaked flour and BGN-PIs being comparable to that of the control samples.

This thesis concludes in **Chapter 6**, in the context of a general discussion tying together the main findings presented in each chapter. The effect of processing was discussed in terms of the pre-treatments applied to BGN seeds, the protein extraction processes employed and the molecular composition and structural properties of the resulting fractions. Furthermore, we also gave consideration to the effect of protein-polyphenol interactions in BGN-PIs, with preliminary results indicating a minimal effect of the polyphenols themselves. However, aggregate formation and possibly protein denaturation were evident from the solvent extraction process employed in polyphenol removal from BGN-PIs. Finally, the gelation functionality of the BGN protein fractions, as single systems and in admixture with whey proteins, were discussed in terms of their compositional properties. Hereto we could conclude that the vicilin fractions control the gelation behaviour of the BGN-PIs. In conclusion, the results presented in this thesis provided new knowledge and thereby a better understanding of the molecular and physicochemical properties of BGN proteins, and their resulting gelation functionality; which can be used to steer the application of BGN as a novel plant protein source.
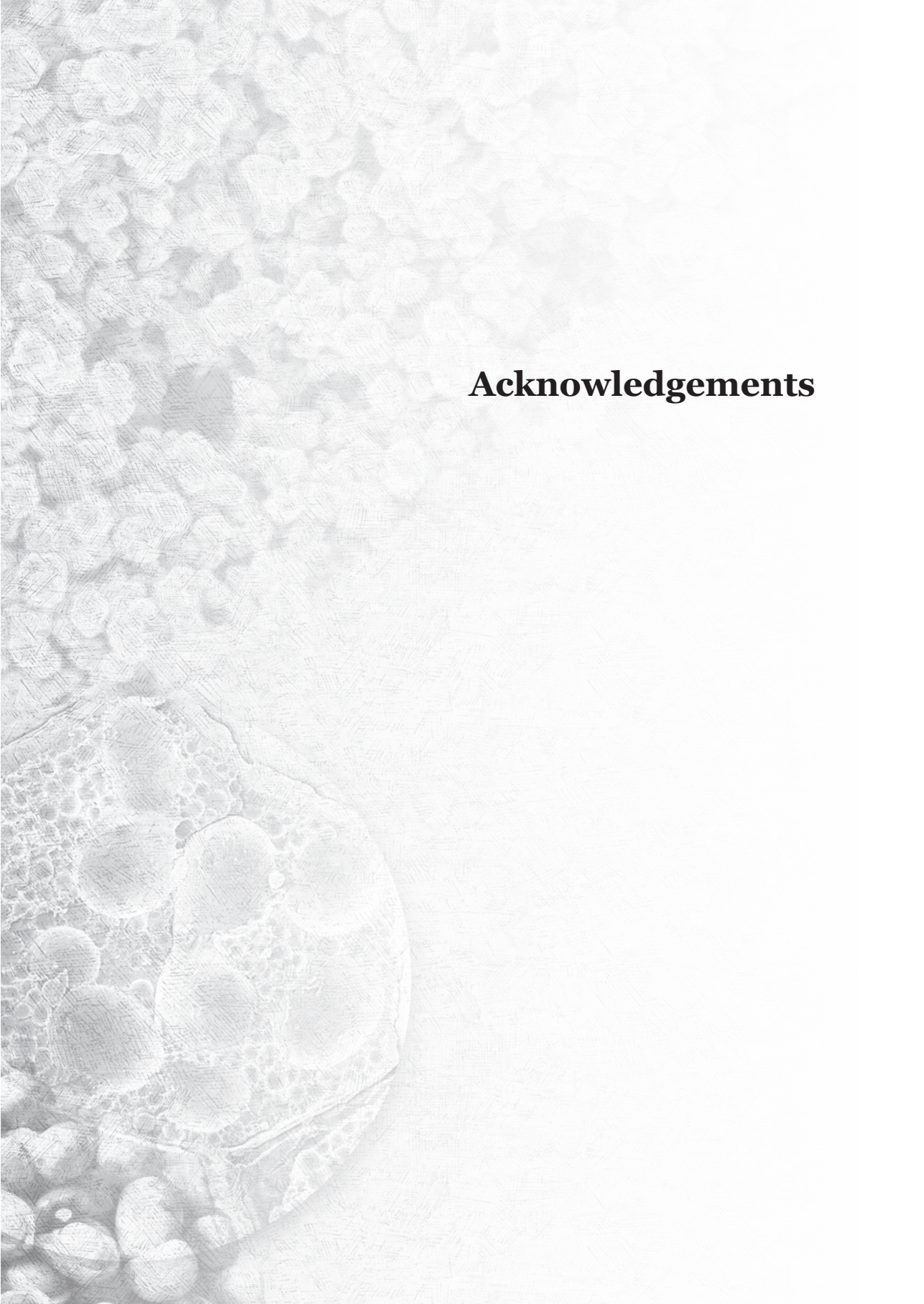
Acknowledgements

"It's not so much about the qualification and the potential doors it opens, but rather the transforming nature of the experiences gained that lead to the qualification and its subsequent impacts."
-Willie Chinyamurindi

These were the words I stumbled upon whilst browsing the pages of a South African newspaper on a mild (probably warm in Dutch terms) autumn afternoon in 2015; months before I would embark on the path towards obtaining a PhD qualification. Five years later and these words still resonate with me, eloquently capturing my own thoughts as I reflect on my PhD journey—a journey which culminated in the work presented in this thesis. I can now attest that beyond the words reflected on these pages, are the many transformative experiences to which many individuals played a significant role. It is therefore a great honour to acknowledge some of these individuals in this dedicated section.

Any doctoral student will agree that the supervisory team is crucial to the PhD experience, and for me it has been no different. **Erik**, as my promotor you have been actively involved in my project from day one. I am grateful that you gave me the opportunity to conduct my research in your group, especially on a topic about an "indigenous crop from sub-Saharan Africa" which you have never heard about before. Finding my feet has not been easy, but it was certainly made easier by your unique ability to provide guidance in such a manner which translates to (positive) new insights; on both a scientific and personal development level. **Paul**, having you as my co-promotor and daily supervisor has been an enriching experience. You are one of the most knowledgeable people I know and I have certainly learned a lot from you, stretching beyond scientific concepts to life in general. Thank you for always having an "open door" and for allowing me the freedom to grow. Above all, I am grateful for our shared sense of humour, which made for an overall pleasant collaboration amongst the three of us (i.e. Erik included). **Victoria**, I am grateful for your support as external co-promotor and mentor. You have inspired and encouraged me to start this journey, whilst reminding me to persevere when the going got tough. Thank you for always believing in me.

Fré, of course I would not have been able to extend my gratitude to my supervisors if it was not for your facilitating role as the first point of contact, as then managing director of VLAG. Thank you for your role which got the ball rolling.

Another enriching experience was that of supervising students. **Sera**, **Mabeth**, **Hongbo**, **Anne**, **Kristel**, **Bente**, **Maria**, **Linda**, **Carol**, **Liza**, **Hongyu** and **Vanessa**, thank you for showing interest in the topic of Bambara groundnut. I appreciate your valuable contributions towards this thesis and for sharing in a mutual development.

Juliet, as one of the few researchers in Wageningen working on Bambara groundnut, I was happy that we could establish a collaboration. Thank you for sharing your insights and for providing the processed seed samples, which resulted in Chapter 5 of this thesis.

Being part of the Food Physics group meant an array of different experiences, which I attribute to the melting pot of cultures, personalities and academic backgrounds represented in the group. As a South African, coming from a country known as the Rainbow Nation, I of course greatly appreciated this high level of diversity. To all my colleagues, thank you for creating and maintaining a welcoming atmosphere, both in and out of the office. Thank you for the knowledge sharing, laughter and the many eyebrow-raising moments. I am indebted to **Harry** and **Miranda** for their technical support and assistance in the lab; thank you both for always extending a helping hand when needed. **Els**, je bent waarschijnlijk de persoon wie de beste mijn Afrikaans-Nederlands verstond. Bedankt voor je altijd luisterende oor en je hulp, met alles.

As my defence day draws near, I am becoming more appreciative of the tradition of having a physical support system on that day, in the form of my paranymphs. **Marco**, I am grateful for your support throughout this PhD period, up until the end. Thank you for the many shared experiences, not only as a colleague but also as a friend. **Joy** (Joyshree), what an honour to know that I will have your support on the day of concluding this journey. I will always be grateful for attending that specific VLAG PhD week right at the beginning of this journey, where without knowing it yet then, I would be blessed with the start of a lifelong friendship. Thank you for becoming central to my support network here in Wageningen; I will always cherish our shared experiences.

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you. I am grateful for the many experiences shared amongst us, and included in "us" are of course **Elvira**, **Konstantina**, **Raisa**, **Ruth** and **Francesca**. Ladies, you have each touched my life in a special way, for that I thank you always. As for my South African counterpart (technically Lesotho as she would probably correct me), **Lerato**, thank you for your friendship. Having you as a friend in Wageningen meant I could talk about "home" in a way which made it seem less far away, know that I appreciate you. These sentiments I also extend to the other South Africans with whom I had the pleasure to connect. **Elisa**, I am grateful that my friendship with Marco also afforded us the opportunity to become friends. The last few months of writing this thesis could probably be described as the most intense part of this journey. I am therefore grateful that our paths crossed when it did **Johannes**. Thank you for reminding me that light(-heartedness) and beauty can be found in the most unlikeliest of circumstances.

Natuurlik kan ek nie hierdie gedeelte afsluit sonder om erkenning aan my familie te gee. My ouers, **Joseph** en **Josephinus**, dankie vir jul onvoorwaardelike gebede, liefde en ondersteuning. Deur vele opofferinge het jul my in staat gestel om my akademiese loopbaan na te jaag, 'n reg wat nie aan jul beskore was. Dus is ek dankbaar om hierdie boek aan julle op te dra. Uiteraard behoort hierdie mylpaal ook aan jou my broer, **Xavier**, en so ook aan die res van die familie. My hoop is dat dit ook as inspirasie sal dien vir elke kind en jongmens in ons familie en gemeenskappe; weet dat geen droom groter is as dit wat jy glo jy kan bereik. My niggie **Shakeelah** en my vriendin **Nevina**, dankie vir jul konstante ondersteuning oor die waters heen, en ook dat jul die tyd geneem het om lang vlugte aan te pak na Nederland om te kom kuier. Aan my antie **Hulda** en die res van die Nederlandse familie, dankie dat jul my met ope arms verwelkom het en altyd 'n oop deur vir my gelaat het, dit het my aanpassing hier soveel makliker gemaak.

There are yet many names which stretches beyond the pages of this book. Know that I am also immensely grateful towards you.

About the author

Biography

Claudine F. Diedericks was born on 24 July 1986 in Worcester, South Africa. After completing her undergraduate studies in Food Technology at Cape Peninsula University of Technology (Cape Town) in 2010, she enrolled for the research-based master's programme where her research focus was on the functionality of the non-starch polysaccharides (dietary fibres) of Bambara groundnut; which resulted in a South African patent published in 2014. During her master's she also received a SKILL[1] programme award, which afforded her the opportunity to follow courses for two periods at Wageningen University (The Netherlands, 2012).

After obtaining her master's degree in 2015, she received an NRF[2] scholarship to pursue a doctoral programme abroad. This enabled her to return to Wageningen University in September of the same year, where she enrolled as a PhD candidate in the Laboratory of Physics and Physical Chemistry of Foods. Her research further highlighted Bambara groundnut as a novel and sustainable (protein) food ingredient source, of which the results are presented in this thesis.

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[1] *Stimulating Knowledge Innovation through Life-long Learning*; (Dutch embassy, South Africa)
[2] *National Research Foundation* (South Africa)

List of publications

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*, 97, 105226.

Diedericks, C.F., Shek, C., Jideani, V.A., Venema, P. & Van der Linden, E. Physicochemical properties and gelling behaviour of Bambara groundnut protein isolates and protein-enriched fractions. (*Submitted, 2019*)

Diedericks, C.F., Venema, P., Mubaiwa, J., Jideani, V.A., & Van der Linden, E. Effect of processing on the microstructure and composition of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) seeds, flour and protein isolates. (*Submitted, 2019*)

Diedericks, C.F., Stolten, V., Jideani, V.A., Venema, P. & Van der Linden, E. Effect of pH and mixing ratios on the synergistic enhancement of Bambara groundnut-whey protein gels. (*To be submitted*)

Diedericks, C.F. & 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), C1933 – C1944.

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Overview of completed training activities

Discipline specific courses

2016	Multivariate analysis for food data/sciences	VLAG	Wageningen, NL
2017	Microscopy and spectroscopy in food and plant sciences	VLAG/EPS	Wageningen, NL
2017	16th European school on rheology	KU Leuven	Leuven, Belgium

Conferences

2015	Water capacity holding symposium	VLAG	Wageningen, NL
2016	16th Food Colloids conference	WUR/TNO	Wageningen, NL
2017	22nd Biennial SAAFoST conference	SAAFoST	Cape Town, South Africa
2018	Phase transitions and thermodynamics in food symposium	VLAG	Wageningen, NL
2018	17th Food Colloids conference	University of Leeds	Leeds, UK
2019	8th International Symposium on Food Rheology and structure	ETH Zürich	Zürich, Switzerland
2019	33rd EFFoST conference & Young Effost Day	WUR	Rotterdam, NL

General courses

2016	Ethics and philosophy of food science and technology	WGS	Wageningen, NL
2016	VLAG PhD week	VLAG	Soest, NL
2016	PhD workshop carousal	WGS	Wageningen, NL
2016	Applied statistics course	VLAG	Wageningen, NL
2016	Project and time management course	WGS	Wageningen, NL
2016	Presenting with impact	WGS	Wageningen, NL
2017	Reviewing a scientific paper	WGS	Wageningen, NL
2017	Scientific writing 11	WGS	Wageningen, NL
2018	R statistics course	VLAG	Wageningen,NL
2018	Chemometrics	VLAG	Wageningen, NL
2019	Career perspectives	WGS	Wageningen, NL

Optional courses and activities

2016	Preparation of research proposal		
2018	Participation and organisation of PhD study tour to Singapore and Indonesia		Singapore/ Indonesia
2015–2019	Weekly group meetings	FPH	Wageningen, NL

Colophon