Extraction, gelation and microstructure of Bambara groundnut vicilins

Diedericks, C. F., de Koning, L., Jideani, V. A., Venema, P., & van der Linden, E.

This is a "Post-Print" accepted manuscript, which has been Published in "Food Hydrocolloids"

This version is distributed under a non-commercial no derivatives Creative Commons

(CC-BY-NC-ND) user license, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited and not used for commercial purposes. Further, the restriction applies that if you remix, transform, or build upon the material, you may not distribute the modified material.

Please cite this publication as follows:

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]. <https://doi.org/10.1016/j.foodhyd.2019.105226>

You can download the published version at:

<https://doi.org/10.1016/j.foodhyd.2019.105226>

Extraction, gelation and microstructure of Bambara groundnut vicilins

Claudine F. Diedericks^a, Linda de Koning^a, Victoria A. Jideani^b, Paul Venema^{a*}, Erik van der Linden^a

^a Laboratory of Physics and Physical Chemistry of Foods, Wageningen University, P.O. Box 17, 6700 AA Wageningen, The Netherlands

^b Department of Food Science and Technology, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa

* Corresponding author: paul.venema@wur.nl

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.

Highlights

- Pure vicilins extracted from Bambara groundnut
- Vicilin forms the major storage protein in Bambara groundnut black-eye seeds
- Bambara groundnut vicilin gel networks are weak-link fractal structures

Keywords

Bambara groundnut

Vicilin

Plant protein

Protein gel

Fractal dimension

Scaling

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 forms 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; Messian, Chihi, Sok, & Saurel, 2015; Messian, 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-Cartryse, & 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. Materials and Methods

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 μ m mesh sieve (flour particle size $D_{0.5} = 20.7 \pm 0.1 \mu$ m) 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. Extraction of Bambara groundnut vicilin

Vicilin was extracted from defatted BGN flour using an optimized Osborne fractionation method (Fig. 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.

2.3. 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.

2.4. Molecular weight determination of BGN vicilin

2.4.1. 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.

2.4.2. 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 NaNO₃ + 0.02% NaN₃) was filtered over a 0.2 µm filter and in line over a 0.025 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.

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.

2.5. Isoelectric point (pI) determination of BGN vicilin

The pl 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.

2.6. *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.

2.7. *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).

2.8. *Gelation functionality of BGN vicilin*

2.8.1. *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.

2.8.2. *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 ($\tan \delta$) were recorded as a function of temperature.

2.8.3. 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:

$$G' \sim (c - c_p)^t \quad (1)$$

where the $\sim$ 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:

$$G' \sim c^w \quad (2)$$

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-link¹ and the weak-link² 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) and limit of linearity/critical strain (γ_0) is given by:

$$\gamma_0 \sim c^n \quad (3)$$

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

$$w = \frac{1}{3-D_f} \text{ and } n = \frac{1}{3-D_f}$$

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

$$\beta = 1 + (2 + x)(1 - \alpha).$$

2.9. Microstructure of BGN vicilin gels

2.9.1. 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 μ l 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).

2.9.2. 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

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

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.

3. Results and Discussion

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-Cartryse, & 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.

3.2. Molecular weight characterisation

3.2.1. Gel electrophoresis

Native-PAGE profiles of BGN vicilin and BGN flour are presented in Fig. 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).

To determine the molecular weight of the subunits of BGN vicilin, SDS-PAGE was performed under reducing and non-reducing conditions (Fig. 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.

3.2.2. SEC-MALLS analysis

The elution profile of BGN vicilin revealed one major peak eluting between 50–55 min (Fig. 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.

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. 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.

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. 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.

3.5. Thermal properties of BGN vicilin

The thermal transition parameters of BGN vicilin as evaluated by differential scanning calorimetry are shown in Fig. 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; Messian et al., 2015; Tang & Sun, 2011b). No endothermic peaks were visible upon reheating of the BGN vicilin solutions, thus indicating irreversible denaturation.

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. 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. 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.

3.7. Applicability of gelation models to BGN vicilin gels

3.7.1. 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. (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. 9, different symbols indicate different extraction batches), which yielded an average $c_p = 0.45 \pm 0.25$.

Using the c_p values obtained from the best linear fits, the scaling exponent ($t = 4.91 \pm 0.34$) could be determined by plotting G' vs $c - c_p$ (example Fig. 10, different symbols indicate different extraction batches). The scaling exponent for BGN vicilin is much higher than the predictions from the isotropic percolation model ($t = 2.06 \pm 0.6$) (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.

3.7.2. 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. 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. 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. 8, section 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 1.

Table 1 Fractal dimension and related structural parameters of BGN vicilin gels (pH 7, 200 mM NaCl).

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

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 CaCl₂). 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 ($\alpha=0.76$).

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 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.

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 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. 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. 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.

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)

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

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.

Conflicts of interest

The authors declare that this study does not have any conflict of interest.

Acknowledgements

We would like to thank Vanessa Stolten for assistance with the BGN vicilin extraction. Financial support from the National Research Foundation (NRF, grant number 94568) of South Africa provided to Claudine Diedericks is acknowledged.

References

- Adebowale, Y. A., Schwarzenbolz, U., & Henle, T. (2011). Protein isolates from Bambara groundnut (*Voandzeia Subterranean* L.): Chemical characterization and functional properties. *International Journal of Food Properties*, 14(4), 758–775.
<http://doi.org/10.1080/10942910903420743>
- Arise, A. K. (2016). *Composition and functional bioactive properties of Bambara groundnut protein and hydrolysates*. Durban University of Technology.
- Arise, A. K., Nwachukwu, I. D., Aluko, R. E., & Amonsou, E. O. (2017). Structure, composition and functional properties of storage proteins extracted from bambara groundnut (*Vigna subterranea*) landraces. *International Journal of Food Science and Technology*, 52(5), 1211–1220. <http://doi.org/10.1111/ijfs.13386>
- Barac, M., Cabrilo, S., Pesic, M., Stanojevic, S., Zilic, S., Macej, O., & Ristic, N. (2010). Profile and functional properties of seed proteins from six pea (*Pisum sativum*) genotypes. *International Journal of Molecular Sciences*, 11(12), 4973–4990.
<http://doi.org/10.3390/ijms11124973>
- Baryeh, E. A. (2001). Physical properties of bambara groundnuts from Botswana. *Journal of Food Engineering*, 47, 321–326. <http://doi.org/10.1016/j.jfoodeng.2008.04.006>
- Bi, C. H., Li, D., Wang, L. J., & Adhikari, B. (2013). Viscoelastic properties and fractal analysis of acid-induced SPI gels at different ionic strength. *Carbohydrate Polymers*,

92(1), 98–105. <http://doi.org/10.1016/j.carbpol.2012.08.081>

Blagrove, R. J., Lilley, G. G., Van Donkelaar, A., Sun, S. M., & Hall, T. C. (1984). Structural studies of a french bean storage protein: phaseolin. *International Journal of Biological Macromolecules*, 6(3), 137–141. [http://doi.org/10.1016/0141-8130\(84\)90054-0](http://doi.org/10.1016/0141-8130(84)90054-0)

Bora, P. S., Brekke, C. J., & Powers, J. R. (1994). Heat induced gelation of pea (*Pisum sativum*) mixed globulins, vicilin and legumin. *Journal of Food Science*, 59(3), 594–596. <http://doi.org/10.1111/j.1365-2621.1994.tb05570.x>

Bremer, L. G. B., Van Vliet, T., & Walstra, P. (1989). Theoretical and experimental study of the fractal nature of the structure of casein gels. *Journal of the Chemical Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases*, 85(10), 3359–3372. <http://doi.org/10.1039/F19898503359>

Busu, N. M., & Amonsou, E. O. (2018). Fractionation pH of bambara groundnut (*Vigna subterranea*) protein impacts degree of complexation with gum arabic. *Food Hydrocolloids*, 87, 653–660. <http://doi.org/10.1016/j.foodhyd.2018.08.044>

Chang, Y., Alli, I., Molina, A. T., Konishi, Y., & Boye, J. I. (2012). Isolation and characterization of chickpea (*Cicer arietinum* L.) seed protein fractions. *Food and Bioprocess Technology*, 5(2), 618–625. <http://doi.org/10.1007/s11947-009-0303-y>

Duranti, M., & Gius, C. (1997). Legume seeds: Protein content and nutritional value. *Field Crops Research*, 53(1–3), 31–45. [http://doi.org/10.1016/S0378-4290\(97\)00021-X](http://doi.org/10.1016/S0378-4290(97)00021-X)

Ersch, C. (2015). *Protein mixtures: Interactions and Gelation*. Wageningen University.

Fido, R. J., Mills, E. N. C., Rigby, N. M., & Shewry, P. R. (2004). Protein extraction from plant tissues. In P. Cutler (Ed.), *Methods in Molecular Biology* vol. 244, (pp. 21–27). New Jersey: Humana Press Inc.

Flory, P. J. (1941). Molecular size distribution in three dimensional polymers. I. Gelation. *Journal of the American Chemical Society*, 63(11), 3083–3090. <http://doi.org/10.1021/ja01856a061>

Hagiwara, T., Kumagai, H., & Matsunaga, T. (1997). Fractal analysis of the elasticity of BSA and β -lactoglobulin gels. *Journal of Agricultural and Food Chemistry*, 45(10), 3807–3812. <http://doi.org/10.1021/jf970348m>

Hagiwara, T., Kumagai, H., & Nakamura, K. (1998). Fractal analysis of aggregates in heat-induced BSA gels. *Food Hydrocolloids*, 12(1), 29–36. [http://doi.org/10.1016/S0268-005X\(98\)00043-5](http://doi.org/10.1016/S0268-005X(98)00043-5)

Ikeda, S., Foegeding, E. A., & Hagiwara, T. (1999). Rheological study on the fractal nature of the protein gel structure. *Langmuir*, 15(25), 8584–8589. <http://doi.org/10.1021/la9817415>

Kanai, H., Navarrete, R. C., Macosko, C. W., & Scriven, L. E. (1992). Fragile networks and rheology of concentrated suspensions. *Rheologica Acta*, 31(4), 333–344.

- Kaptso, K. G., Njintang, Y. N., Nguemtchouin, M. M. G., Scher, J., Hounhouigan, J., & Mbofung, C. M. (2015). Physicochemical and micro-structural properties of flours, starch and proteins from two varieties of legumes: bambara groundnut (*Vigna subterranea*). *Journal of Food Science and Technology*, 52(8), 4915–4924. <http://doi.org/10.1007/s13197-014-1580-7>
- Kimura, A., Takako, F., Meili, Z., Shiori, M., Maruyama, N., & Utsumi, S. (2008). Comparison of physicochemical properties of 7S and 11S globulins from pea, fava bean, cowpea, and French bean with those of soybean-French bean 7S globulin exhibits excellent properties. *Journal of Agricultural and Food Chemistry*, 56(21), 10273–10279. <http://doi.org/10.1021/jf801721b>
- Kudre, T. G., Benjakul, S., & Kishimura, H. (2013). Comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut. *Journal of the Science of Food and Agriculture*, 93(10), 2429–2436. <http://doi.org/10.1002/jsfa.6052>
- Kuipers, B. J. H., Van Koningsveld, G. A., Alting, A. C., Driehuis, F., Voragen, A. G. J., & Gruppen, H. (2006). Opposite contributions of glycinin- and β -conglycinin-derived peptides to the aggregation behavior of soy protein isolate hydrolysates. *Food Biophysics*, 1(4), 178–188. <http://doi.org/10.1007/s11483-006-9019-y>
- Maruyama, N., Katsube, T., Wada, Y., Oh, M. H., Barba De La Rosa, A. P., Okuda, E., Nakagawa, S., & Utsumi, S. (1998). The roles of the N-linked glycans and extension regions of soybean β -conglycinin in folding, assembly and structural features. *European Journal of Biochemistry*, 258(2), 854–862. <http://doi.org/10.1046/j.1432-1327.1998.2580854.x>
- Maruyama, N., Sato, R., Wada, Y., Matsumura, Y., Goto, H., Okuda, E., Nakagawa, S., & Utsumi, S. (1999). Structure-physicochemical function relationships of soybean β -conglycinin constituent subunits. *Journal of Agricultural and Food Chemistry*, 47(12), 5278–5284. <http://doi.org/10.1021/Jf0117053>
- Mertens, C., Dehon, L., Bourgeois, A., Verhaeghe-Cartryse, C., & Blecker, C. (2012). Agronomical factors influencing the legumin/vicilin ratio in pea (*Pisum sativum* L.) seeds. *Journal of the Science of Food and Agriculture*, 92(8), 1591–1596. <http://doi.org/10.1002/jsfa.4738>
- Mession, J. L., Chihi, M. L., Sok, N., & Saurel, R. (2015). Effect of globular pea proteins fractionation on their heat-induced aggregation and acid cold-set gelation. *Food Hydrocolloids*, 46, 233–243. <http://doi.org/10.1016/j.foodhyd.2014.11.025>
- Mession, J. L., Sok, N., Assifaoui, A., & Saurel, R. (2013). Thermal denaturation of pea globulins (*Pisum sativum* L.) - Molecular interactions leading to heat-induced protein aggregation. *Journal of Agricultural and Food Chemistry*, 61(6), 1196–1204.

<http://doi.org/10.1021/jf303739n>

- Mitescu, C. D., & Musolf, M. J. (1983). Critical exponent for 3-D percolation conductivity, revisited. *Journal de Physique Lettres*, 44(16), 679–683.
- Mohamad Ramlan, B. M. S., Maruyama, N., Takahashi, K., Yagasaki, K., Higasa, T., Matsumura, Y., & Utsumi, S. (2004). Gelling properties of soybean β -conglycinin having different subunit compositions. *Bioscience, Biotechnology, and Biochemistry*, 68(5), 1091–1096. <http://doi.org/10.1271/bbb.68.1091>
- Nadathur, S. R., Wanasundara, J. P. D., & Scanlin, L. (2016). Proteins in the Diet: Challenges in Feeding the Global Population. In *Sustainable Protein Sources* (pp. 1–19). Elsevier Inc. <http://doi.org/10.1016/B978-0-12-802778-3.00001-9>
- Nakamura, T., Utsumi, S., & Mori, T. (1986). Mechanism of heat-induced gelation and gel properties of soybean 7S globulin. *Agricultural and Biological Chemistry*, 50(5), 1287–1293. <http://doi.org/10.1080/00021369.1986.10867555>
- Norton, I. T., & Frith, W. J. (2001). Microstructure design in mixed biopolymer composites. *Food Hydrocolloids*, 15(4–6), 543–553. [http://doi.org/10.1016/S0268-005X\(01\)00062-5](http://doi.org/10.1016/S0268-005X(01)00062-5)
- O’Kane, F. E., Happe, R. P., Vereijken, J. M., Gruppen, H., & van Boekel, M. A. J. S. (2004a). Characterization of pea vicilin. 2. Consequences of compositional heterogeneity on heat-induced gelation behavior. *Journal of Agricultural and Food Chemistry*, 52(10), 3149–54. <http://doi.org/10.1021/jf035105a>
- O’Kane, F. E., Happe, R. P., Vereijken, J. M., Gruppen, H., & van Boekel, M. A. J. S. (2004b). Characterization of pea vicilin. 1. Denoting convicilin as the α -subunit of the *Pisum* vicilin family. *Journal of Agricultural and Food Chemistry*, 52(10), 3141–8. <http://doi.org/10.1021/jf035104i>
- Ould Eleya, M. M., Ko, S., & Gunasekaran, S. (2004). Scaling and fractal analysis of viscoelastic properties of heat-induced protein gels. *Food Hydrocolloids*, 18(2), 315–323. [http://doi.org/10.1016/S0268-005X\(03\)00087-0](http://doi.org/10.1016/S0268-005X(03)00087-0)
- Rangel, A., Domont, G. B., Pedrosa, C., & Ferreira, S. T. (2003). Functional properties of purified vicilins from cowpea (*Vigna unguiculata*) and pea (*Pisum sativum*) and cowpea protein isolate. *Journal of Agricultural and Food Chemistry*, 51(19), 5792–5797. <http://doi.org/10.1021/jf0340052>
- Renkema, J. M. S. (2001). *Formation, structure and rheological properties of soy protein gels*. Wageningen University.
- Rubio, L. A., Perez, A., Ruiz, R., Guzman, M. A., Aranda-Olmedo, I., & Clemente, A. (2014). Characterization of pea (*Pisum sativum*) seed protein fractions. *Journal of the Science of Food and Agriculture*, 94(2), 280–287. <http://doi.org/10.1002/jsfa.6250>
- Salgin, S., Salgin, U., & Bahadir, S. (2012). Zeta potentials and isoelectric points of biomolecules: The effects of ion types and ionic strengths. *International Journal of*

- Electrochemical Science*, 7(12), 12404–12414.
- Schutyser, M. A. I., & Van der Goot, A. J. (2011). The potential of dry fractionation processes for sustainable plant protein production. *Trends in Food Science and Technology*, 22(4), 154–164. <http://doi.org/10.1016/j.tifs.2010.11.006>
- Shewry, P. R., Napier, J. A., & Tatham, A. S. (1995). Seed Storage Proteins: Structures and Biosynthesis. *The Plant Cell Online*, 7(7), 945–956. <http://doi.org/10.1105/tpc.7.7.945>
- Shih, W.-H., Shih, W. Y., Kim, S.-I., Liu, J., & Aksay, I. A. (1990). Scaling behavior of the elastic properties of colloidal gels. *Physical Review A*, 42(8), 4772–4779.
- Singhal, A., Karaca, A. C., Tyler, R., & Nickerson, M. (2016). Pulse proteins: From Processing to Structure-Function Relationships. In *Grain Legumes* (pp. 55–78). <http://doi.org/10.5772/64020>
- Stockmayer, W. H. (1944). Theory of molecular size distribution and gel formation in branched polymers: II. General cross linking. *The Journal of Chemical Physics*, 12(4), 125–131. <http://doi.org/10.1063/1.1723922>
- Tandang-Silvas, M. R. G., Tecson-Mendoza, E. M., Mikami, B., Utsumi, S., & Maruyama, N. (2011). Molecular design of seed storage proteins for enhanced food physicochemical properties. *Annual Review of Food Science and Technology*, 2(1), 59–73. <http://doi.org/10.1146/annurev-food-022510-133718>
- Tang, C. H. (2008). Thermal denaturation and gelation of vicilin-rich protein isolates from three *Phaseolus* legumes: A comparative study. *LWT - Food Science and Technology*, 41(8), 1380–1388. <http://doi.org/10.1016/j.lwt.2007.08.025>
- Tang, C. H., & Ma, C. Y. (2009). Heat-induced modifications in the functional and structural properties of vicilin-rich protein isolate from kidney (*Phaseolus vulgaris* L.) bean. *Food Chemistry*, 115(3), 859–866. <http://doi.org/10.1016/j.foodchem.2008.12.104>
- Tang, C. H., & Sun, X. (2011a). Structure-physicochemical function relationships of 7S globulins (vicilins) from red bean (*Phaseolus angularis*) with different polypeptide constituents. *Food Hydrocolloids*, 25(3), 536–544. <http://doi.org/10.1016/j.foodhyd.2010.08.009>
- Tang, C. H., & Sun, X. (2011b). A comparative study of physicochemical and conformational properties in three vicilins from *Phaseolus* legumes: Implications for the structure-function relationship. *Food Hydrocolloids*, 25(3), 315–324. <http://doi.org/10.1016/j.foodhyd.2010.06.009>
- Tang, C. H., Sun, X., & Yin, S. W. (2009). Physicochemical, functional and structural properties of vicilin-rich protein isolates from three *Phaseolus* legumes: Effect of heat treatment. *Food Hydrocolloids*, 23(7), 1771–1778. <http://doi.org/10.1016/j.foodhyd.2009.03.008>
- Tavano, O. L., & Neves, V. A. (2008). Isolation, solubility and in vitro hydrolysis of chickpea

- vicilin-like protein. *LWT - Food Science and Technology*, 41(7), 1244–1251.
<http://doi.org/10.1016/j.lwt.2007.08.003>
- Trappe, V., & Weitz, D. A. (2000). Scaling of the viscoelasticity of weakly attractive particles. *Physical Review Letters*, 85(2), 449–452. <http://doi.org/10.1103/PhysRevLett.85.449>
- Tzitzikas, E. N., Vincken, J. P., De Groot, J., Gruppen, H., & Visser, R. G. F. (2006). Genetic variation in pea seed globulin composition. *Journal of Agricultural and Food Chemistry*, 54(2), 425–433. <http://doi.org/10.1021/jf0519008>
- United Nations. (2017). *World Population Prospects: The 2017 Revision, Key Findings and Advance Tables. Working Paper No. ESA/P/WP/248. Department of Economic and Social Affairs, Population Division*. New York. Retrieved from https://esa.un.org/unpd/wpp/publications/files/wpp2017_keyfindings.pdf
- Urbonaite, V., Van der Kaaij, S., De Jongh, H. H. J., Scholten, E., Ako, K., Van der Linden, E., & Pouvreau, L. (2016). Relation between gel stiffness and water holding for coarse and fine-stranded protein gels. *Food Hydrocolloids*, 56, 334–343.
<http://doi.org/10.1016/j.foodhyd.2015.12.011>
- Van der Linden, E., & Foegeding, E. A. (2009). Gelation. Principles, Models and Applications to Proteins. In S. Kasapis, I. T. Norton, & J. B. Ubbink (Eds.), *Modern Biopolymer Science* (pp. 29–91). Oxford, UK: Elsevier Inc. <http://doi.org/10.1016/B978-0-12-374195-0.00002-1>
- Van der Linden, E., & Sagis, L. M. C. (2001). Isotropic force percolation in protein gels. *Langmuir*, 17(19), 5821–5824. <http://doi.org/10.1021/la010705u>
- Vreeker, R., Hoekstra, L. L., Den Boer, D. C., & Agterof, W. G. M. (1992). Fractal aggregation of whey proteins. *Food Hydrocolloids*, 6(5), 423–435.
[http://doi.org/10.1016/S0268-005X\(09\)80028-3](http://doi.org/10.1016/S0268-005X(09)80028-3)
- Wu, H., & Morbidelli, M. (2001). A model relating structure of colloidal gels to their elastic properties. *Langmuir*, 17(4), 1030–1036. <http://doi.org/10.1021/la001121f>
- Yao, D., Kouassi, K., Erba, D., Scazzina, F., Pellegrini, N., & Casiraghi, M. (2015). Nutritive evaluation of the bambara groundnut Ci12 landrace [*Vigna subterranea* (L.) Verdc. (*Fabaceae*)] produced in Côte d'Ivoire. *International Journal of Molecular Sciences*, 16(9), 21428–21441. <http://doi.org/10.3390/ijms160921428>
- Zhang, Y. H., Tang, C. H., Wen, Q. B., Yang, X. Q., Li, L., & Deng, W. L. (2010). Thermal aggregation and gelation of kidney bean (*Phaseolus vulgaris* L.) protein isolate at pH 2.0: Influence of ionic strength. *Food Hydrocolloids*, 24(4), 266–274.
<http://doi.org/10.1016/j.foodhyd.2009.10.002>

Figure Captions

Fig. 1. Bambara groundnut (BGN) vicilin extraction scheme.

Fig. 2. Native-PAGE patterns of BGN vicilins (pH 7, 2 mM NaCl) in lanes 1 and 2, BGN vicilins (unadjusted pH 6.1 and no salt addition) lanes 3 and 4, and BGN defatted flour in lane 5.

Fig. 3. SDS-PAGE patterns of BGN vicilins at pH 7 and 2 mM NaCl under reducing (lanes 1 and 2) and non-reducing (lanes 3 and 4) conditions.

Fig. 4. SEC-MALLS elution profile of BGN vicilins. Replicate indicated with dashed line.

Fig. 5. Electrophoretic mobility of BGN vicilins as a function of pH.

Fig. 6. Effect of NaCl concentration on BGN vicilin solubility.

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

Fig. 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%, ×5.5%, +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.

Fig.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.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.

Fig. 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.

Fig. 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.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

Figure 12