Effects of processing on the characteristics of flour and protein isolates produced from *Lablab purpureus*

Submitted in complete fulfillment for the Degree of Master of Applied Sciences (Food Science and Technology) in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa

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Reference Declaration

I, Mr. Tremayne Sheldon Naiker - 21301361 and Prof. John Jason Mellem do hereby declare that in respect of the following dissertation – Title: **Effects of processing on the characteristics of flour and protein isolates produced from *Lablab purpureus***

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Authors Declaration

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof. John Jason Mellem**, **Prof. Eric Oscar Amonsou**, and **Prof. Himansu Baijnath**.

To the Lord almighty, my saviour

"Through it all I have learnt to trust in him"

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Publications and Conference Outputs

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Preface

The following dissertation is organized into six chapters and is presented as follows:

Chapter 1:

Introduction (describes problem statement, aims and contribution to knowledge relating to research).

Chapter 2:

Literature review (review of previous related studies and potential knowledge gaps, research aims and objectives).

Chapter 3 (Research objective 1):

The effect of soaking, steaming and dehydration on the microstructure, physicochemical properties and *in vitro* starch digestibility of flour produced from *Lablab purpureus* (L.) Sweet (hyacinth bean).

Chapter 4 (Research objective 2):

The effect of steam and dehydration on the nutritional quality and functional properties of protein isolates produced from *Lablab purpureus* (L.) Sweet (hyacinth bean).

Chapter 5 (Research objective 3):

The emulsifying properties of Ca^{2+}-induced *Lablab purpureus* (L.) Sweet (hyacinth bean) protein nanoparticles.

Chapter 6:

Summary and conclusions (general discussion of key research findings, limitations, recommendations and future work).

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Abstract

The utilization of legumes by food industries has grown considerably in intermediate forms other than whole grains. Thus, continuous work is focused on modifying legume-based raw materials for improving its techno-functional properties whilst preserving its nutritive value. The study conducted was aimed at analyzing the effects of processing treatments on the characteristics of flour and protein isolates produced from *Lablab purpureus* (L.) Sweet (hyacinth bean). Flour was produced from legume grains subjected to steaming (S+A) and dehydration (S+A+D) treatments, following soaking (S). Protein isolates were produced from respective flour fractions using isoelectric precipitation. Samples produced from steaming and dehydration was found useful for potential application as functional food ingredients for nutritional intervention. The respective flour samples contained improved resistant starch (23.44 g/100 g dry starch) content. Swelling and solubility indices were found to be greater at lower temperatures mainly attributed to the pre-gelatinization of starch granules. Thus, they may be potentially suitable for ingredient application in texture modified foods.

Protein isolates produced displayed traits typical of high-quality proteins and demonstrated exceptional functionality. The rapid increase in predicted biological values observed suggested improved protein digestibility potential. Samples contained significant concentrations of branched chain and aromatic amino acids highlighting potential health benefits. Protein nanoparticles were produced using Ca^{2+}-induced aggregation (0.00-6.50 mM) from hyacinth bean protein isolate (2% m/v, pH 7). This was to examine its potential for development as food-grade Pickering emulsion stabilizers. Protein solutions containing high Ca^{2+} concentrations resulted in higher dynamic viscosities (mPa.s). Protein nanoparticles (~172.38 nm) were formed at 3.50 mM Ca^{2+}. The z-average diameter of aggregates was dependent on Ca^{2+} concentration. Results satisfied certain criteria for nanoparticles to potentially function as Pickering stabilizers. However, nanoparticles were susceptible to 4 M Urea and emulsion creaming became more apparent as storage progressed. The study conducted provides valuable information on how processing could be useful for obtaining value-added legume grain ingredients for potential food applications. Such approach could diversify the use of hyacinth bean and help improve the competitiveness of the legume grain sector.

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Chapter 1: Introduction

The global population is expected to grow by over a third or 2.30 billion people by 2050. Thus, the Food and Agricultural Organization (FAO) has forecast worldwide protein shortages. It has been forecasted that almost all growth is to take place in developing countries (FAO, 2017). This presents the developing world with foreseeable challenges in providing poor and under-nourished populations with safe, nutritious and wholesome food resources. Food legumes have been regarded as valuable sources for human nutrition (Bhat and Karim, 2009). *Lablab purpureus* (*L.*) Sweet (hyacinth bean) is an indigenous drought tolerant leguminous crop with multiple uses from food, to soil improvement and protection. The legume grains are sources of protein, carbohydrates, dietary fiber, mineral elements, and contain relatively low levels of anti-nutritional factors (Maass et al., 2010, CABI, 2014, NAS, 1979).

In Southern Africa, hyacinth bean has received minimal attention with respect to broadening the food base and is currently used by large scale farmers as a forage plant (Makgoga, 2013). The utilization of legume grains by food industries has grown considerably in intermediate forms (e.g. flour, protein concentrates and isolates) other than whole grains. The techno-functional properties of legume flour are said to be limited, thus continuous efforts are aimed at modifying legume-based materials for providing end products with desirable traits and functional properties. The design of food processing equipment and operations were largely adapted to transform and preserve food resources. However, more recently it is viewed as an option to produce raw materials with desirable properties whilst preserving their nutritional value, rather than the use of high-grade raw materials in food applications (Bußler et al., 2015).

The common methods used for preparing legume grains are soaking, steaming, microwaving, boiling, and roasting. Soaking of grains prior to cooking has been associated with a decrease in cooking time and it is said to initiate processes, such as gelatinization and protein denaturation (Fabbri and Crosby, 2016). Damodaran et al. (2008) reported that the functionality of proteins is largely governed by its complex chemical make-up, response to external environmental factors (e.g. temperature, pH) and interaction with other food constituents. Aguilera (2005) confirmed that improvement to the quality of food related materials will be largely determined on interventions made at the microscopic level, as many microstructural elements (e.g. fibers, starch, protein) that critically contribute to their techno-functionality, identity and quality are below the ~100 µm range.

Therefore, this research work was focused on examining the effects of processing on the characteristics of flour and protein isolates produced from hyacinth bean legume grains. This is aimed at producing materials with improved nutritional and functional properties for promoting their utilization as functional ingredients in food products. The development of food-grade Pickering emulsion stabilizers is fast attracting interest as they're recognized as safe compared to inorganic materials. Pickering emulsions offer some promising applications in functional foods development including the encapsulation and delivery of food bioactives (Xiao et al., 2016). Therefore, protein nanoparticles will be produced from hyacinth bean protein isolate. This is to examine its particle and emulsifying properties for its potential to function as food-grade Pickering emulsion stabilizers.

Chapter 2: Literature Review

2.1. *Lablab purpureus* (hyacinth bean)

Lablab purpureus (L.) Sweet (*Dolichos bengalensis*, *Lablab niger*, *Dolichos lablab*, *Lablab vulgaris*), commonly referred to as hyacinth bean is an ancient leguminous crop species that falls under the family Fabaceae (Figure 1).

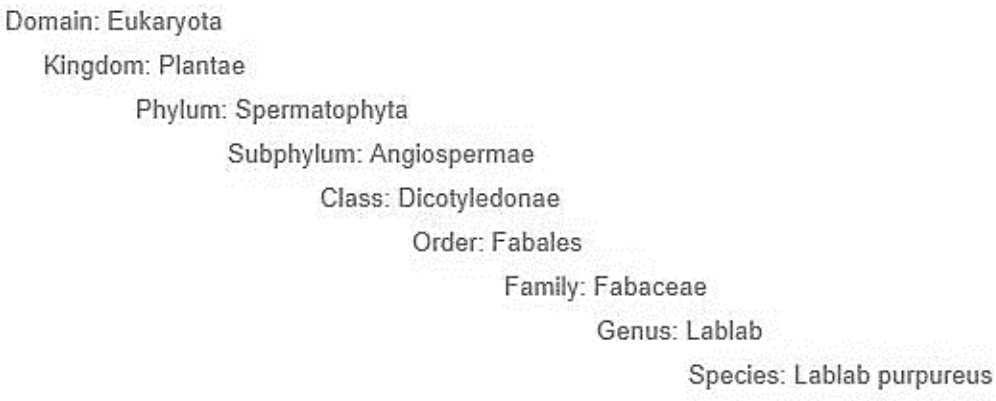

Figure 1: Taxonomic tree, Genus: Lablab, Species: *Lablab purpureus* (CABI, 2014).

Hyacinth bean has been used for as long as 3500 years and is widely distributed in Africa, the Indian sub-continent more specifically South East Asia. An embryological study conducted revealed a close relationship between *Phaseolus vulgaris*, *Vigna catjang*, and *Dolichos lablab*. However, there was a distant relationship of this group with *Glycine max*. Despite the agro-morphological diversity of hyacinth bean in South-Asia, its origin is Africa. This is due to Africa being recognized as the only continent where wild plants have occurred naturally (Pengelly and Maass, 2001).

In parts of Bangladesh hyacinth bean has been identified as the third most important vegetable after eggplant (*Solanum melongena*) and taro (*Colocasia spp.*). For decades, it has been recognized as one of the agro-morphologically versatile tropical leguminous crop species. In eastern southern Africa, it is used mainly as a garden plant other than a field crop. This was confirmed by market surveys suggesting a high demand for the crop in Kenya. Since the 1970s, hyacinth bean has been recognized as a neglected crop in large areas despite its adaptation to harsh climatic conditions, tradition and great diversity (Smartt, 1985, Grotelüschen, 2014). The crop usually grows well in warm climate (18-30°C), however cool seasons (temperature ranging from 14-28°C) are preferred. It requires rainfall (750-2500 mm/year) or irrigation during the first months with sowing done between July-August.

It is adaptable in regions that are arid, semi-arid (200-2500 mm of annual precipitation), and humid rainforest regions (22-35°C), warm-temperate, subtropical, both lowlands and highlands (grown widely up to 2100 m altitude in the New Guinea highlands). After 2-3 months of sowing, hyacinth bean is said to be drought-tolerant. The well-developed deep root system that penetrates to water sources more than 2 m below the soil surface permits growth to persist long into dry seasons. It is highly adaptable for growth in different types of soil (deep sands to heavy black clays) and is said to tolerate different pH conditions ranging from 5 to 7.5. Germination is epigeal and normally takes 5 days, whereby the legume grains remain viable for 2-3 years. Early-maturing varieties can be grown annually producing pods 60 days after sowing and continues up to 120 days. The green bean, pod and leaf are known for its use as a vegetable. Other uses include forage/green manure, pulse (also used as 'dhal'), ornamental, and herbal medicine. In recent times, it has been recognized for its bio-functional properties for use as a nutraceutical (Maass et al., 2010, Yuan et al., 2009).

Despite vastly available germplasm, there are only a few varieties that are recognized in countries that cultivate hyacinth bean commercially. Varieties of the species show significant differences in fruit and growth phenological traits that influence pod length, diameter, thickness, and yield which are the main criteria for marketing quality. Other variable characteristics of the species may exist amongst the flowers (color, abundance, fragrance, corolla size, peduncle length), leaves (size, shape, hairiness, color), physiology (flowering time, drought tolerance, seedling vigor, day length sensitivity, disease and pest resistance, maturation time, and legume grain viability) and legume grains (size, shape, and color), (University of Agricultural Sciences GKVK, 2013).

The leaves are broad and ovate-rhomboid in shape measuring 7-15 cm in length. They are often crinkly and appear rounded below. The flowers are large in a long, erect raceme, purple or white, two to four at nodes. The legume grains vary in size and color with an average weight of 100 grains ranging between 25-40 g. There are four recognized grain colors, Black, Khaki, Chocolate, and Buff. The legume grains are characterized by their flattened form, and large hilum. The hilum is typically white, prominent and oblong, usually covering one third of the seed (Figure 2). The stems are upright and cylindrical, twining up to 6-10 meters in length (Makgoga, 2013, NAS, 1979).

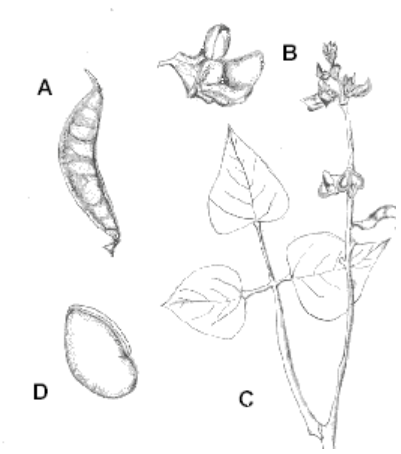

Figure 2: Plant fractions of *Lablab purpureus*. (A. Pod; B. Flower; C. Stem with leaves and flowers; D. Grain) (Murphy and Colucci, 1999).

The two recognized botanical types of hyacinth bean are the garden and field types (erect and bushy). The garden type is twining, late maturing, grown on supports, and is used mainly as a green vegetable. The crop has numerous excellent qualities, from its ability to produce large volumes of green materials with high protein concentration, to a source of food, fodder, and soil protection in harsh climatic conditions. However, in Africa both the legume grains and immature pods are known to be a lesser food source despite the grains containing up to 25 g of protein (Table 1).

Table 1: Comparative nutritional values for distinct parts of *Lablab purpureus*, compiled by James A. Duke, US. Department of Agriculture (NAS, 1979)

Part	Calories	Protein (g)	Fat (g)	Total Carbohydrates (g)	Fiber (g)	Ash (g)
GF	312.00	25.00	2.70	65.20	16.10	7.10
GF	312.00	24.80	2.40	65.60	15.20	7.20
S	382.00	25.10	1.70	68.90	7.80	4.00
S	381.00	25.50	1.10	69.60	9.60	3.60
S	380.00	24.50	1.40	69.90	7.80	4.30
L	284.00	22.00	3.70	55.90	61.40	12.80

GF (Green Fruit), S (Seed), L (Leaf).

Compared to other leguminous crop species, the leaves are rich in protein (28%), and iron (155 mg per 100 g of leaves, dry weight). The grains are a source of fiber that is important for controlling health related illnesses such as heart disease, cancer, diabetes, and obesity. It is also recognized for use as a laxative, febrifuge, diuretic, carminative, anthelmintic, aphrodisiac, anaphrodisiac, antispasmodic, digestive, and stomachic agent (Yuan et al., 2009, Smartt, 1985, Naeem et al., 2009).

The grains are reported to be sources of vitamins A, B and C, and have a lower proteinase activity (2.40-3.20 units/mg seed) in comparison to most other legumes. It also contains tyrosinase which is used for the treatment of hypertension. Protein isolated from the seed has great potential for usage as an additive to improve cake quality (Brad 2003, Naeem et al. 2009, Maass et al. 2010). As a food, boiled grains can be eaten as shelly bean and can be prepared in numerous ways. In Indonesia, the grains are served raw for tempeh (traditional fermented food) that is typically made from soybeans (Reimar V, 1963, University of Agricultural Sciences GKVK, 2013).

2.2. Grain Processing and Dehydration

2.2.1. Water in food materials

Water is omnipresent and influences the quality attributes and physical properties of food materials through various roles. It is well documented for providing an environment during which various sugars, salts, acids and alternative comparatively tiny hydrophilic molecules are dissolved (Figure 3). Water plays a vital role within the structure and properties of food macromolecules. It interacts with suspended mixture particles, gels and will function as a plasticizer for increasing the elasticity of molecules.

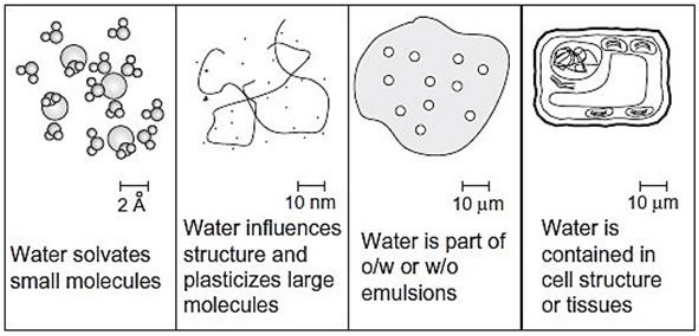

Figure 3: Roles of water in food product materials (Kerr, 2013).

Water and environmental conditions (i.e. ionic content and pH) is thought to influence the conformation of enzymes and their functionality, whereby at low water activity most enzymatic reactions are slow. At a molecular level, water contributes to the flow behavior of dispersions by increasing the space of empty areas during which different molecules can move. In dense systems, molecular chains are largely constrained as a result of entanglements. Water separates the side groups and chains of larger molecules. This allows for easier reptation and on a macroscopic level influences the textural and rheological properties of food materials. Generally, high moisture levels result in higher water activity, higher freezing point, greater flexibility, higher specific heat, greater thermal conductivity, lower viscosity, osmotic pressure, and boiling point. At low moisture levels several macromolecules may form non-crystalline solid states. In biological materials, more than one dynamic water structure exists and has a considerable influence on water removal as well as biological activity (Damodaran, 2017).

2.2.2. Pre-treatment procedures

The objective of soaking grains is to increase the moisture level within the kernel to the required level for ensuring uniform starch gelatinization during cooking. Soaking is known to reduce grain mutilation from osmotic pressure and reduces time for pre-cooking by creating fissures within the kernel.

However, extended soaking may result in the loss of water-soluble vitamins, flavor, and mineral elements. Grain quality and costs are largely determined by soaking parameters used and its profound effect on uniformity throughout grain mass during the process of soaking (Carlson et al., 1979, Velupillai, 1993). The purpose of boiling grains is for gelatinizing starch present in the endosperm. This is an irreversible process carried out by increasing the temperature, whereby starch granules that have absorbed water undergo structural changes from crystalline to an amorphous state. Following starch gelatinization, water enters the interchain space through the disruption of hydrogen bonds between starch chains. For starch gelatinization to occur sufficient moisture, and heat transfer around gelatinization temperature are required for grains under treatment (Velupillai, 1993, Ahmad and Noomhorm, 2013).

A variety of processes have been developed for producing value-added grain merchandise. These processes include dry heat, freeze drying, chemical, gamma irradiation, freeze thaw, gun puffing treatments, step wise hydration-cook-dry and soak-cook-dry methods. Freeze thaw method, freeze drying, and gun puffing are uneconomical as a result of the prices of machine investment. Soak-cook-dry methods combined with dry heat have been recognized for its economy and simplicity (Sabularse et al., 1991, Alfy et al., 2016). Baz et al. (1992) proposed an advantageous preparation process for parboiled rice. The final grain product was suitable for consumption after simmering for 8-10 minutes. The rice was subjected to cooking (90-100°C, 1-10 minutes) in order to partially hydrate the grains for providing uniform moisture distribution. Following cooking, steaming was conducted at pressures between 250- and 2.000-mm Hg above atmospheric pressure for 1.50-30 minutes. Drying was conducted under stationary conditions using a conventional belt dryer and thereafter, with a vibrating dryer (i.e. high velocity belt dryer, vibrating fluid bed dryer).

2.2.3. Dehydration

Dehydration or drying refers to the removal of large amounts of water from food materials, thus contributing to preservation and reducing costs related to transportation and storage. This is accomplished by creating a large difference in water activity between the material and environment. Food preservation against microbiological and chemical forms of deterioration is said to be achieved by reducing the water activity of materials below 0.70-0.80. Previous studies have revealed that there are more than two types of water with different relaxation time constants and rates of rotational motions. A traditional model further explains that water exists in bulk and bound phases (Figure 4A). During the later phases of the drying process, there is water present that requires more energy to be removed. This water is said to not be removed except by freeze-drying (Kerr, 2013).

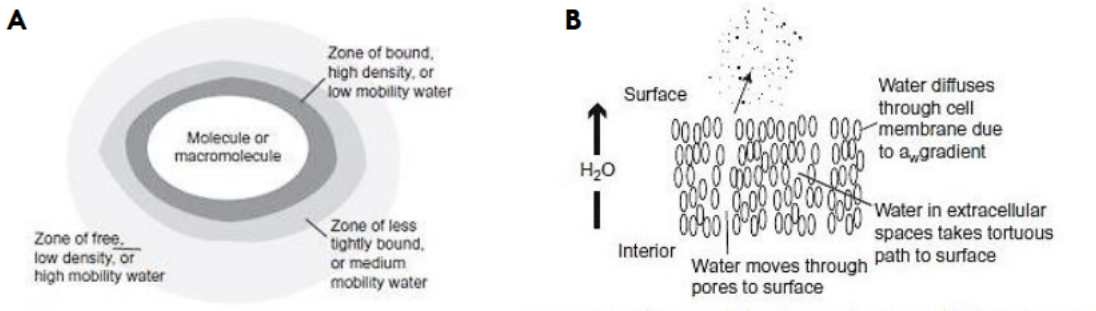

Figure 4: The proposed state of water (A) near food molecules, (B) movement from interior regions of food materials to the surface during drying (Kerr, 2013).

Proteins and polysaccharides containing polar or ionic groups has a greater binding affinity to bound water. Thus, extra energy is required to dissociate from these groups than from other water molecules. In the cell organelles water is often found compartmentalized, whereby it must move through the cell membrane by diffusion. In the case of damaged cells, this water is required to move around the cell wall and membrane fragments (Leung, 2017). For spaces present between cells, extracellular water that may be trapped must follow a tortuous path to the material surface. In food materials containing biological tissues, a variety of factors affect the ability of water to diffuse and reach the material surface where it undergoes transformation from a liquid to gaseous state (Figure 4B). Hot air dryers, osmotic dryers and freeze dryers are among the different unit operations available for drying grains. Hot air dryers are operated between 40-80°C, whereby drawn air generated by a fan is passed across a bank of heaters (Figure 5A).

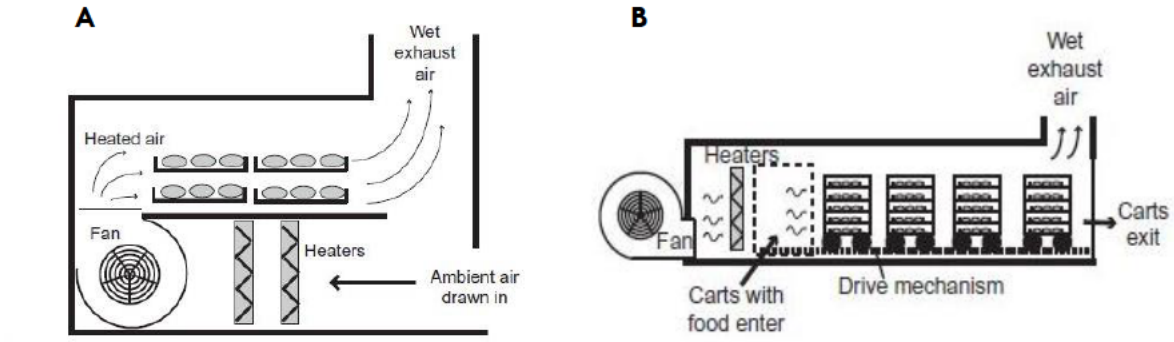

Figure 5: (A) Batch type hot air dryer (B) Continuous tunnel dryer, (Kerr, 2013).

Continuous dryers have evolved to improve the throughput of products. In tunnel drying, insulated chambers (10-15 m) containing series of trolleys are driven. This provides a semi-continuous movement (Figure 5B). In co-current model tunnel dryers, the air moves in the same direction as the product. This allows for the moist and coolest product to be exposed to the least humid air. In the initial phases, rapid drying is promoted when the product remains near wet bulb temperature.

The product that is most dried at the end of the tunnel is exposed to lower air temperature. Therefore, the product may have underwent fewer quality changes with respect to browning or case-hardening. In counter-current tunnel drying, air enters in the opposite direction to the product movement. Some of the associated advantages of counter-current drying is that the lowest moisture product contacts the driest and highest temperature air. This allows for the removal of excess moisture from the respective materials that was hardest to remove. The major physical changes that occur during drying include bed porosity, particle density, shrinkage, bulk density and may vary due to moisture removal, shrinkage of structure and internal collapse. The extent of these changes also depends on the type of food material, dryer and conditions employed (Lewicki, 1998).

During drying, cellular structures may shrink back into the spaces left by water that was removed. This may lead to a shrunken appearance and is typical of air-dried products. During progressive drying more moisture is removed and the structure becomes porous, rigid, less elastic and may enter a glassy state. This results in an increase or decrease to the apparent density which may include the solid structure and air voids incorporated. Structural changes to food materials during drying affects their rehydration properties. Osmotic drying creates less porous and denser materials. Freeze drying creates the least shrinkage in materials but with higher porosity and minimal changes to specific volume. In terms of color and appearance, browning is a problem particularly in materials subjected to hot-air drying. However, minimal changes are observed in freeze dried and osmotically dried materials. Color changes occur most dramatically at elevated drying air temperatures, whereby materials become dark with varying degrees of red and yellow. Texture of materials is also affected by drying parameters employed, whereby dried foods become increasingly firmer at low moisture levels. In some foods, a glassy state is reached which may contribute to the desired crispness in dehydrated snack foods. However, this characteristic is undesirable in dried fruits (Mayor and Sereno, 2004).

In terms of food powders, desirable textural properties are related to bulk density, particle size and ability to disperse and rehydrate in water. Dried food materials often result in large changes to taste profiles but is not always associated with poor quality. These materials are known to retain fat, protein, fiber and carbohydrates in a denser form compared to their moist precursors. In terms of fruits and vegetables, leaching out of vitamins and minerals may occur during drying preparations and pre-treatment procedures. However, minerals are not expected to be destroyed during drying. Vitamins are heat sensitive and are most susceptible to degradation during drying process. Vitamin loss may be dependent on the type of material, drying preparation, process and parameters employed (Kerr, 2013).

2.3. Microstructure characteristics of legume grains

The fractionation of legume grains by food industries is continuously generating interest for extending the use of its main components (i.e. starch, protein, and dietary fiber). The microstructure of dry legumes (peas, beans, lentils) is said to be different compared to oilseed grains. This is mainly due to their respective starch and oil contents. In oil-based legumes, the protein bodies are surrounded by a network of lipid bodies whereas in dry legume grains the cotyledon cells are formed by starch granules covered by a protein matrix (Błaszczak et al., 2007). The microstructure of legume cotyledons is known to influence the physical properties of grains, and purity of protein isolates.

However, the utilization of legume starch and proteins in food products are limited due to the relatively high costs of their isolation. In terms of starch, the common difficulties known in its isolation are due to the presence of cotyledon cell wall materials (i.e. hydratable fine fiber) and the strong adherence of large amounts of insoluble proteins to starch. Various processing treatments are applied on legumes (soaking, cooking, autoclaving, microwaving, etc.) mainly to significantly enhance their nutritional value through the inactivation of anti-nutritional factors. However, these treatments have been found to affect legume starch and protein structures, thereby altering their functionality (Otto et al., 1997).

Acevedo et al. (2017) has evaluated the effects of germination, soaking-cooking and microwaving treatment on the microstructure of pigeon pea (*Cajanus cajan* L.) legume grains. Soaked grains were germinated using a damp cloth containing sodium hypochlorite for 5 days. Soaking-cooking (SC) was conducted in boiling water for 20, 40 and 60 minutes using a grain to distilled water ratio of 1:10 (m/v). Grains were subjected to microwaving treatment for 10 minutes at different potencies (50, 70 and 100%) in a microwaving oven. All pre-treated grains were dried in a hot air oven (55°C for 24 h) to constant weight. The cotyledon cells formed by starch granules were identified as the main storage reservoir of legume grains. Most starch granules were found to have retained their shape, size, and smooth surface. The average size of starch granules reported was 24.10 x 17 µm. In raw grains starch granules were found to have a smooth and oval surface (Figure 6a).

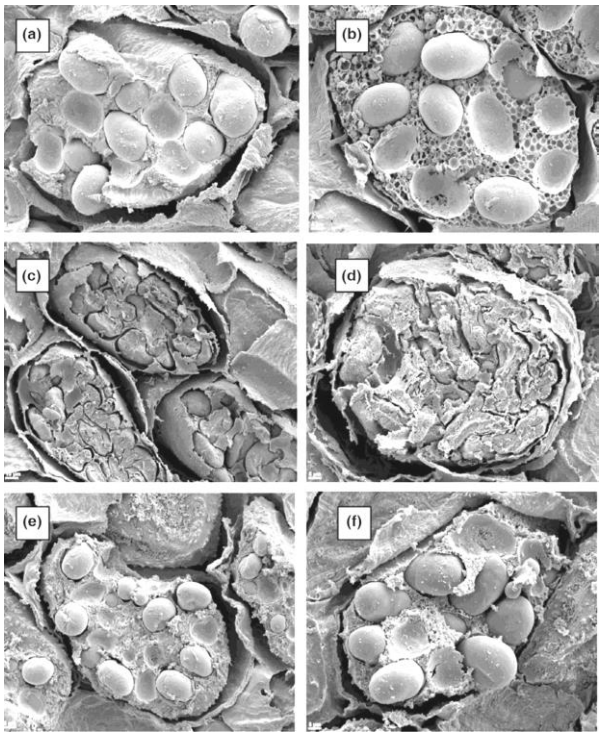

Figure 6: Scanning electron microscopy of pigeon pea grains: (a) Raw, Bar=5 µm; (b) Germinated, Bar = 5 µm; (c) Soaking-Cooking (6 h–20 minutes), Bar = 10 µm; (d) Soaking-Cooking (6 h-60 minutes), Bar = 5 µm; (e) Microwaving 50%, Bar = 5 µm; (f) Microwaving 100%, Bar = 5 µm (Acevedo et al., 2017).

In germinated grains, minimal changes to the cell wall were observed in comparison with its native state (Figure 6b). Soaking-cooking (6 h–20 minutes) was found to have preserve the structure of starch granules. However, their surface appeared to be flattened and the protein matrix exhibited contractions because of the heat applied. It was observed that samples produced by soaking-cooking (6 h–60 min) resulted in dissemination of compartmentalization between starch granules and the protein matrix (Figure 6c). The microwaving treatments didn't show significant size variations in starch granules. However, the structure of the protein matrix was affected (Figures 6e and f). This was attributed to the denaturation of protein that was caused by microwaving heating.

Aguilera et al. (2009) has examined the influence of soaking, cooking, and dehydration treatments on the microstructural characteristics of flour produced from lentil for their effective utilization in various food applications. In this sense, dehydration of food materials is associated with numerous advantages and the adaptation of processing strategies may result in the improved utilization of legume grains. This could further facilitate the development of economical viable products (Vega-Mercado et al., 2001, Aguilera et al., 2003).

Soaked legume material was cooked by boiling for 30 minutes and dehydrated (75±3°C for 6 h) using an air forced tunnel. The scanning electron micrographs (SEM) of raw and processed lentil reveal that starch granules were the major storage components. In raw samples they appeared spherical-oval (22 µm) in shape. The granules were surrounded by well-defined protein bodies and was characterized by a smooth surface (Figure 7).

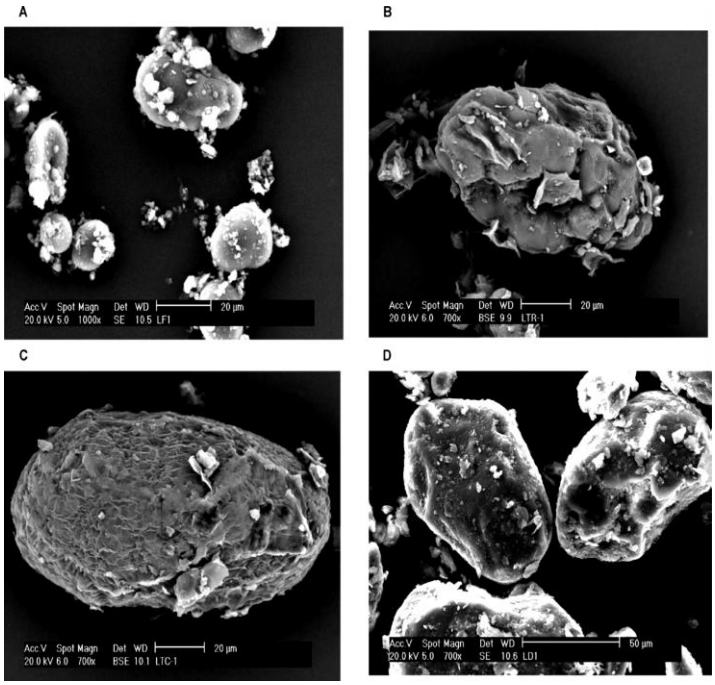

Figure 7: SEM of lentil flours: (A) raw; (B) soaked; (C) soaked + cooked; (D) soaked + cooked + dehydrated. Bar size = 20 µm (Aguilera et al., 2009).

In samples produced by soaking, pronounced changes to starch granules were observed. They appeared larger, slightly eroded and was attributed to the water absorption properties of starch (Figure 7B). The size of starch granules appeared to increase (121-125 µm) extensively for samples produced by cooking after soaking. Overall, starch granules kept their internal integrity, but their surface was flattened as an effect of heat. Samples produced by dehydration following soaking and cooking resulted in smaller starch granules compared to cooked grains. This was mainly attributed to losses of holding water.

2.4. The physicochemical properties of legume flour

The physicochemical properties of food and its components are functional and chemical which food manufacturers take into consideration when making decisions concerning the quality of ingredients/products. The suitability for using food ingredients in various food systems is largely dependent on their physicochemical properties. The nutritive value of food legumes is their ability to provide nutrients (protein, carbohydrates, vitamins, and minerals) that is digestible in presence/absence of anti-nutritional compounds. The composition for pulses (pea, lentil, and bean) from various locations is shown (Table 2). The protein content of most legumes ranges between 17-30% dry weight basis.

Table 2: The nutritional composition of various pulses adapted from (Boye et al., 2010a)

Pulse	Variety	Composition (g/100 g of sample)				
		Protein	Fat	Fibre	Ash	Carbohydrates
Pea	Pigeon	19.39	3.24	5.56	4.05	–
	Cowpea	22.53	1.60	5.33	3.81	–
	Lencolen	34.70	2.40	4.25	3.93	54.72
Lentil	Giza	27.50	1.16	–	4.03	63.40
	Family 91	26.70	1.24	–	3.41	64.60
	Pakistani	26.40	1.25	–	1.46	64.50
	Giza 9	31.40	1.15	6.75	4.16	56.53
Bean	Kidney	23.58	0.83	24.90	3.83	60.01
	Red kidney	16.89	1.64	30.34	1.14	–
	V.C 2010	26.40	1.75	6.15	4.50	61.20

Traditional food processing (soaking, decortications, germination, fermentation and cooking) methods have been found to significantly influence the nutritive values of food legumes. Anti-nutritional factors (ANF) present in legumes are essential for its further application in various food systems. They have been found to reduce nutrient utilization and/or intake in various legume-based foods. Some of the common ANFs found in food legumes include phytates, tannins, trypsin inhibitors (protease), cyanogenic 12 glycosides, glycosides, flavonoids, oxalates, alkanoids, gossypol, cardiac haemagluttinins (lectins), and coumarins (Shivachi et al., 2012). Soaking, boiling, and germination have been found to increase the utilization of legumes. Shaahu et al. (2014) examined the influence of processing (decortication, roasting and boiling in tap water) on the ANFs, proximate, mineral, and amino acid composition of *Lablab purpureus*. All ANFs examined were significantly reduced by the applied processing techniques. Boiling was found to be the best method for the reduction of tannins (37%), alkaloids (33%), oxalates (38%), trypsin inhibitors (100%) and HCN (89%).

Soaking legumes in a fresh water solution containing sodium chloride prior to cooking have been found to improve their nutritional quality. Complex sugars, belonging to the raffinose family have the potential to cause gastric issues (flatulence) if they're not broken-down during digestion. The method of cooking legumes in fresh water containing sodium chloride can result in a tender skin. This was caused by calcium and magnesium ions found in pectin of cell walls replaced by sodium ions (Fabbri and Crosby, 2016). In addition, soaking of legumes has been associated with a reduction in cooking time having been found to initiate processes such as gelatinization and protein denaturation which softens the texture.

Shivachi et al. (2012) has associated prolonged cooking time to be responsible for the underutilization of legumes in many diets. This is due to prolonged cooking reducing the nutritive values of legumes, with respect to vitamins and certain amino acids. Furthermore, legumes collected from gene bank accessions have been found to take a longer time to cook in comparison with farmers' collections. Thus, variation in cooking time maybe influenced by several factors including type of water, energy source, genetic characteristics, size and age of legumes.

Osman (2007) has studied the effects of processing on the chemical composition of *D. lablab* flour produced from legume grains obtained in Saudi Arabia. Grains were prepared by roasting, soaking overnight in water (1:10 m/v), cooking in water for 30 minutes in a pressure cooker, germination at room temperature for 5 days, and autoclaved for 121°C for 20 minutes under 15 lb/in. The moisture content of samples was significantly increased by soaking, germination, and decreased by roasting treatment. Germination was found to further increase the protein content of the prepared flour, whilst other processes used reduced this parameter. The decrease in protein content was attributed to the possibility of leaching out of soluble proteins. Cooking was found to significantly reduce fat content and was attributed to the possibility of the presence of lipolytic enzyme activity which breaks down triacylglycerides to simple fatty acids, sterol esters and polar lipids.

Mubarak (2005) has associated the dehulling of legume grains prior to soaking to greatly retain mineral elements of mung bean (*Phaseolus aureus*) grains. Germination showed increases to Ca, K, P, Mg, Fe, and Mn. Loss in divalent metal concentrations was attributed to the formation of phytate-cation protein complexes actioned by the binding of proteins. Na, Mg, Fe concentrations were not significantly reduced by pressure-cooking and could be due to cooking by steam only minimizing leaching out of solubles (Table 3).

Table 3: Effect of traditional food processing on the mineral content of mung bean grains (mg/100 g dry weight basis) (Mubarak, 2005)

Element	Raw	Dehulling	Soaking	Germination	Boiling	Autoclaving	Microwave cooking
Na	12.00	10.20	9.60	11.60	8.20	8.95	8.10
K	3.62	2.90	2.35	3.95	2.90	2.88	2.80
Ca	84.00	80.00	81.00	88.50	75.00	80.00	78.00
P	391.00	385.00	381.00	406.00	368.00	370.00	365.00
Mg	55.60	54.30	49.90	56.60	44.00	48.00	47.80
Fe	9.70	8.60	8.40	9.65	7.90	8.10	8.00
Mn	1.70	1.50	1.40	1.70	1.30	1.55	1.40

Ghavidel and Prakash (2007) reported germination was found to improve protein, thiamin, and *in vitro* digestibility of iron, calcium for legumes studied. The concentrations of ANFs phytic acid and tannin were significantly reduced by 47–52% and 43-52% respectively, compared to the control. Flour produced from dehulled germinated grains improved the overall quality of legumes. This was due to the improvement of nutrient bioavailability and reduction of ANFs. In heterogenous food systems, functional properties (i.e. gelation, oil absorption, water holding capacity, swelling, and bulk density) are affected by interactions of major and minor chemical food constituents.

Starch concentrations significantly differ amongst various legumes, whereby it has been reported that raw lentil contains 51%-59% (dry matter) of starch. The gelatinization of starch serves as an important property, whereby starch absorbs water resulting in a loss to granular organization (Sotomayor et al., 1999, Copeland et al., 2009, Kaur et al., 2010). In legume flour, starch molecules may interact with protein, fiber and mineral elements. This was found to extensively affect their thermal properties which may include parameters such as heat capacity, and gelatinization temperature (Divekar et al., 2017, Ma et al., 2016). Li and Zhu (2017) studied the influence of starch interactions on the physicochemical properties of whole grain quinoa flour. Pearson correlation analysis revealed significant correlations present amongst starch and properties of quinoa flour. Food lipids and protein were also found to have greatly contributed to the functionality of whole grain quinoa flour.

During thermal treatment the structure and physicochemical properties of starch are altered extensively. Thermal treatment of starches has been found to either result in gelatinization or retrogradation of starch granules. This has been proven to influence functionality and is largely dependent on the type of starch present and degree of modification (Ma et al., 2011). Flours produced from broad bean, black bean, lentil and chickpea were subjected to heat moisture (HMT) and annealing (ANN) treatments. HMT treated flours resulted in a significant decrease on all thermal parameters investigated compared to ANN treatment.

There were distinct differences observed for the gelatinization temperature range (T_c–T_o) between ANN (1.26-2.15°C broad bean and black bean), HMT (2.41-5.33°C for black bean and lentil) and native (1.85-5.32°C for chickpea and lentil) flours. The differences observed were attributed to the molecular rearrangement of starch containing flour that may have occurred during HMT and ANN treatments. Thus, heat transfer into starch granules could have been delayed due to increases in starch crystallinity (Chávez-Murillo et al., 2018, de la Rosa-Millán et al., 2017).

Chau and Cheung (1998) compared the functional characteristics of flour produced from *Phaseolus calcaratus*, *Phaseolus angularis*, and *D. lablab* with soybean. The pH values for the respective flour dispersions (10% m/v) ranged between 6.53-6.65. Flour produced from soybean legume grains was found significantly denser compared to *P. calcaratus*, *D. lablab* and *P. angularis*. Benítez et al. (2013) reported similar bulk densities (0.80-0.98 g/mL) for mucuna, dolichos, cowpea, and jack bean grains, whereby flour produced from dolichos and cowpea were found significantly denser compared jack bean and mucuna flours.

Water and other food components (proteins and polysaccharides) are essential constituents that influence the rheological and textural properties of foods. Within a protein matrix, proteins can retain water against a gravitational force. This is referred to as water holding capacity which is the sum of physically entrapped, hydrodynamic and bound water. It is regarded to be more important than water binding capacity in many food applications. Water holding, and absorption capacities are important in food applications such as custards, sausage manufacture, and baking of dough. These applications require water to be absorbed without protein dissolution, thus achieving viscous body thickening (Seena and Sridhar, 2005).

Other factors that may influence water absorption of food materials include cell wall material and starch content (Damodaran et al., 2008). Chau and Cheung (1998) found significant differences amongst the water and oil holding capacities (WHCs and OHCs) for flours produced from *P. angularis*, *P. calcaratus*, *D. lablab* and soybean. Soybean flour had a greater WHC compared to the other three flours and this was attributed to its higher protein content. The protein content for flours produced from *P. angularis*, *P. calcaratus*, *D. lablab* ranged between 30.40-32.10%, whereas soybean flour had a protein content of 52.8%. The WHC of *D. lablab* was found comparable to flour produced from faba bean, lentil and lima bean ranging between 1.04-1.08 g/g. WHC for flours produced from four non-conventional legumes (*Stizolobium niveum*, *Canavalia ensiformis*, *Vigna unguiculata*, *Lablab purpureus*) were studied by Benítez et al. (2013). Differences to capacities observed were attributed to constituents that have high affinity for water molecules such as polar amino acid residues, and polysaccharides.

Oil holding capacity of flour is an important property required for flavor retention in many food applications. The basic mechanism for OHC is due to the physical entrapment of oil by capillary action. Factors that may influence this property include the hydrophobicity, and the different proportions of non-polar amino acid residues of proteins. OHCs for soybean flour was found significantly greater compared to *P. angularis*, *P. calcaratus* and *D. lablab* flours. Lablab had similar OHCs compared to chickpea, cowpea, green gram, and lentil. Emulsion properties of different legume flours are largely influenced by protein specificity, in addition to heating and aging of emulsions having a significant influence on emulsion flocculation stability and coalescence. Protein specificity is thus controlled by interactions involving adsorbed protein molecules. These may include formation of hydrogen, hydrophobic, and disulfide bonds. Emulsion formation are significant in product applications such as formation of frozen desserts, baking and whiteners (Damodaran. et al., 2008).

The influence of pH on the emulsifying activity (EA) of flours produced from *P. calcaratus*, *D. lablab*, and *P. angularis* was compared to soybean flour. At pH 4 minimum emulsion activity (45.80-54.20%) was recorded for flours produced, however at pH 2 (54.00-57.70%) and pH 10 (56.60-62.00%) there was a sizeable increase in activity. Minimum EA observed at pH 4 was attributed to the protein of the flours produced reaching their isoelectric points, which is around pH 4. Similarly, the emulsion stabilities (ESs) of flours produced were found to be pH dependent. Heating of different emulsions at 80°C did not affect the ES as greater than 80.20% of ES remained at pH values investigated. The ES profiles of flours produced from *P. calcaratus*, *D. lablab* and *P. angularis* showed similarities. At pH 4 the ESs (11.40-22.10%) of the mentioned flours were lower compared to soybean flour. Either side of pH 4, an increase in ES was noted mainly attributed to an increase in protein solubility (Chau and Cheung, 1998).

Food foams are formed mechanically or by supersaturation. In addition, the following is required for their formation: conformational change, rearrangement and rapid adsorption at the air-water interface during shear, viscoelastic film formation through intermolecular interactions. Rapid adsorption at the air-water interface is essential for an increase in the capacity of foams and viscoelastic film formation respectively. Viscoelastic film formed is said to assist in the stabilization of foam structure as time progresses (Damodaran. et al., 2008). Foam capacities (FCs) for flours were found pH dependent and higher around pH 4 ranging between pH 2 (106-111%) and pH 10 (114-122%). This was attributed to increases in protein flexibility allowing for diffusion to occur more rapidly at the air-water interface resulting in the encapsulation of air particles. Soybean flour had greater FCs and produced more stable foams over time when compared to the other flours studied. This was due to its higher protein content (Chau and Cheung, 1998).

2.5. *In vitro* starch digestibility characteristics

The concept of functional foods in promoting health has been developed during the past two decades. Food legumes and their bioactive properties (sources of resistant starch, saponins, phenolic compounds, dietary fiber, oligosaccharides, antioxidants, and phytochemicals including phytates) have made them unique functional foods. Legumes have a high content of indigestible fibers and low glycemic index (GI) which aid diabetic individuals' glycemic control. In addition, they assist in the prevention of insulin-resistance representing the prodrome to type 2 diabetes. The inhibition of α-amylase and β-glucosidase activity also known as the hypoglycemic effect has been reported similar for legumes and antidiabetic drugs. In addition, planning diets with the consumption of legumes has been associated with weight management, improvement of dyslipidemia, and attenuates postprandial glycemic response (Duranti, 2006, Bahadoran and Mirmiran, 2015).

In the early 1980s, the glycemic index (GI) was defined to serve as a numeric expression for the available carbohydrates in foods (usually containing 50 g available carbohydrate) on blood glucose concentration over a set period. There are three classifications that make up the GI index of foods, low (< 55), moderate (56–69), and high (> 70). Glycemic index is often expressed relative to a standard usually glucose or white bread (Sadler, 2011). Previous GI research revealed that foods containing similar concentrations of carbohydrates have different glycemic responses (Figure 8).

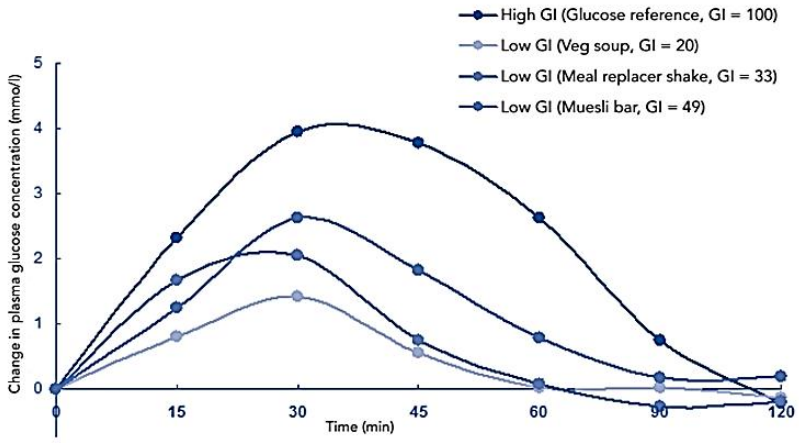

Figure 8: Blood glucose response curves for high and low GI foods (Sadler, 2011).

Food-related factors that have been reported to influence the glycemic impact of a food include the nature of carbohydrates available in food, in terms of the nature of starch and its hydrolysis products, physical properties (i.e. hydration properties, particle size), the contents of monosaccharides (galactose, fructose, mannose, glucose), disaccharides (sucrose, lactose), and oligosaccharides (maltodextrins).

In addition to food form and the degree of cooking, other food components such as dietary fiber, fat, protein, phytochemicals, and organic acids may have a significant influence on blood glucose response (Sadler, 2011). The rate of starch hydrolysis in the digestive tract is an important factor on GI values, for example, amylopectin has been reported to undergo hydrolysis more rapidly in comparison to amylose by pancreatic α-amylase. In terms of starch, there are certain fractions that digest rapidly known as rapidly digestible starch (RDS), slowly digestible starch (SDS) and a fraction that resist digestion known as resistant starch. Other factors that may have a significant influence on starch hydrolysis include the accessibility of digestive enzymes to starch. This may be further influenced by the physical properties of food (i.e. structure, particle size) (Sajilata et al., 2006).

Resistant starch (RS) refers to the starch fraction that is not hydrolyzed by digestive enzymes in the small intestine. It passes to the large intestine where it's a substrate for bacterial fermentation. It is calculated as the difference between total starch and the sum of both SDS and RDS fractions. RDS and SDS are calculated as the amount of glucose (converted to starch) released between 0, 20 and 120 minutes of *in vitro* digestion (Singh et al., 2010). Starch retrogradation prior to gelatinization serves as a mechanism for resistant starch formation and reduction to digestibility, whereby processing techniques may affect both these processes. In terms of legumes, the crystalline structure (type C) is more stable to that found in cereal grains (type A). Thus, processing of cereal grains results in lower resistant starch concentrations compared to legumes. It has been found that starches produced from several legumes subjected to steam treatment had resulted in higher yields of indigestible RS (19-31%, DM basis) compared to raw legumes and were 3 to 5 times higher than raw legumes. Furthermore, it was found that dry treatment resulted in higher RS contents compared to wet treatment in several legumes, cereals, and tubers (Bhat and Karim, 2009, Sajilata et al., 2006).

Flour produced from *P. angularis*, *P. calcaratus*, and *D. lablab* legume grains subjected to different cooking times were examined by Cheung and Chau (1998), for resistant starch (RS) and non-starch polysaccharides (NSP). The whole legume grains were boiled in tap water for time intervals ranging between 30-120 minutes prior to drying and milling. Findings revealed that the total dietary fiber contents of flour produced from grains that were processed was significantly higher to raw grains. The maximum percent increase in total dietary fiber were observed between 60-120 minutes of cooking time and ranged between 18.60-47.80%. In addition, cooking time was found directly proportional to concentrations of NSP and RS.

Increases to resistant starch concentrations were attributed to the presence of cell-enclosed and retrograded starch formed during cooking. Proteins (exogenous or endogenous) have been found to significantly influence starch digestibility and functional properties in cereals and various food products, whereby exogenous proteins are said to possibly aid starch digestion (Tinus et al., 2012). Protein barrier present around starch granules was confirmed by pronase hydrolysis resulting in a significant increase to *in vitro* starch digestibility. This has made starch readily available for hydrolysis by digestive enzymes (amylase and amyloglucosidase). Endogenous proteins are said to hinder starch digestion as reported in sorghum and pasta. Starch granules in sorghum are reported to be encapsulated by protein bodies making sorghum less digestible (Singh et al., 2010). Thus, starch digestion could be enhanced or reduced by proteins present in the food matrix. Therefore, understanding starch and protein digestion and nutrient synergy are essential for process and product designs. Piecyk et al. (2019) investigated the effects of processing on *in vitro* starch digestion of grass pea flour (Table 4).

Table 4: Starch fraction and pGI analysis of flour produced from processed legume grains (g/100 g dry matter) (Piecyk et al., 2019)

Variety	Sample	RDS	SDS	RS	pGI
	RF	14.40 ± 0.40^{b}	21.70 ± 1.60^{f}	13.50 ± 1.40^{c}	64.90 ± 2.70^{b}
	CF	46.60 ± 0.40^{g}	0.60 ± 0.40^{a}	2.50 ± 0.00^{a}	95.70 ± 0.30^{f}
Derek	C & FF	42.60 ± 0.80^{e}	2.60 ± 0.40^{b}	4.50 ± 0.40^{b}	91.30 ± 0.60^{e}
	CSF	41.20 ± 0.40^{d}	4.80 ± 0.40^{c}	12.50 ± 0.00^{c}	78.10 ± 0.10^{cd}
	C & FSF	39.10 ± 0.90^{c}	6.50 ± 0.80^{d}	13.20 ± 0.10^{c}	77.20 ± 1.00^{cd}

Data with the same superscript letters in the columns are not significantly different ($p < 0.05$). [RDS (Rapidly digestible starch), SDS (slowly digestible starch), RS (resistant starch), pGI (predicted glycemic index), RF (raw flour), CSF (cooked seed flour), C & FSF (cooked & frozen seed flour), CF (cooked flour), C & FF (cooked & frozen flour)].

The analysis of *in vitro* starch digestibility was carried out on raw flours. The raw flour slurries were heated for 30 minutes (cooked flour - CF). Cooked and frozen flour (C & FF) was prepared in the first stage as cooked flour, then the samples were cooled for 1 h at room temperature and frozen (-18°C, 21 days). Prior to analyses samples were defrosted at room temperature for 2 h. Overall, the flour produced from cooked grass pea grains was found suitable for application in food products for decreasing GI values and to enrich them in resistant starch. Flour samples produced by cooking resulted in a rapid increase to RDS, and a decrease in SDS contents. Following heat treatment in an aqueous environment, increases to starch digestibility may be dependent on the denaturation degree of proteins. It has been reported that proteins form layers around starch granules. Flour samples produced by cooking caused a significant decrease in RS content (2.50%) and was attributed to the complete gelatinization of starch.

The cooling period after cooking (30 minutes) was said to have minimal effects on starch retrogradation. In CSF, higher RS values (12.50%) were found compared to CF. This value is low compared to rapidly digesting (56.00-65.50%), slowly digesting (5.10-9.20%) and resistant starch (29.40-34.80%) contents found in Indian lentil (*Lens culinaris*) cultivars of flour (Kaur et al., 2010).

This was due to the retrogradation of starch during the drying of grains and was confirmed by the content of retrograded starch. The storage of flour after cooking and freezing was found to decrease RDS and cause a small increase in SDS contents. Thus, it was found that the RS content increased only in C&FF compared to CF. Sajilata et al. (2006) related dehydration of amylose rich legumes to high contents of resistant starch. On drying, dispersed polymers of gelatinized starch undergo retrogradation to a semi-crystalline form that's highly resistant to digestion by pancreatic α-amylase. Furthermore, when gelatinized starches are cooled/dried, a certain portion can retrograde to a less soluble form that is resistant to acid and amylase action leading to a fall in catalytic efficiency, resulting in low glycemic indices. Starch hydrolysis was monitored between 0 and 180 minutes (Figure 9).

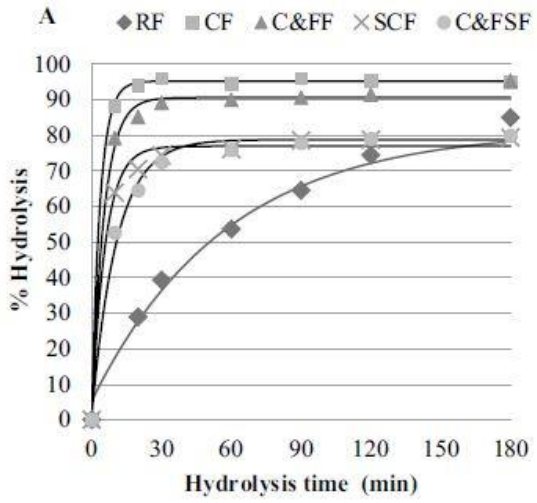

Figure 9: *In vitro* starch hydrolysis curve of grass pea flour: raw (RF), cooked (CF), cooked & frozen (C&FF), from cooked seed (CSF) and from cooked & frozen seed (C&FSF) (Piecyk et al., 2019).

There was a sharp increase in the content of digested starch in CF followed by an overall decrease in the rate of starch digestion. These differences were reflected in pGI value and were found significantly higher in cooked flours (Table 4). Results conclude that high pGI values for cooked flours were largely affected by a low concentration of flour used, relatively long cooking period (30 minutes) and therefore a high degree of starch disintegration.

2.6. Protein characteristics of legume grains

Globulins and albumins are the major proteins found in legume protein, whereby they are salt and water soluble respectively. Albumins (MM of 5000-80000 Da) are made up of enzymatic proteins that include lectins, amylase and protease inhibitors. Globulins are the major storage protein fraction found in legumes and are further sub-divided into legumin (11S) and vicilin (7S). These sub-unit ratios vary significantly amongst legume varieties. Legumin (11S) contain higher amounts of sulfur-containing amino acids (methionine and cysteine) compared to vicilin (7S). These fractions are built on polymorphic sub-units encoded by multigene families, whereby 11S legumins (hexameric quaternary structure) are made up of acidic (MM of 40000 Da) and basic (MM of 20000 Da) subunits. The 7S vicilin (trimeric structure) have molecular masses ranging between 175000 –180000 Da (Boye et al., 2010a).

The protein sub-unit composition for varieties of *P. vulgaris* was determined by Rui et al. (2011) using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). This was performed under both non-reducing and reducing conditions in the presence of β-mercaptoethanol (β-ME) respectively (Figures 10A, B).

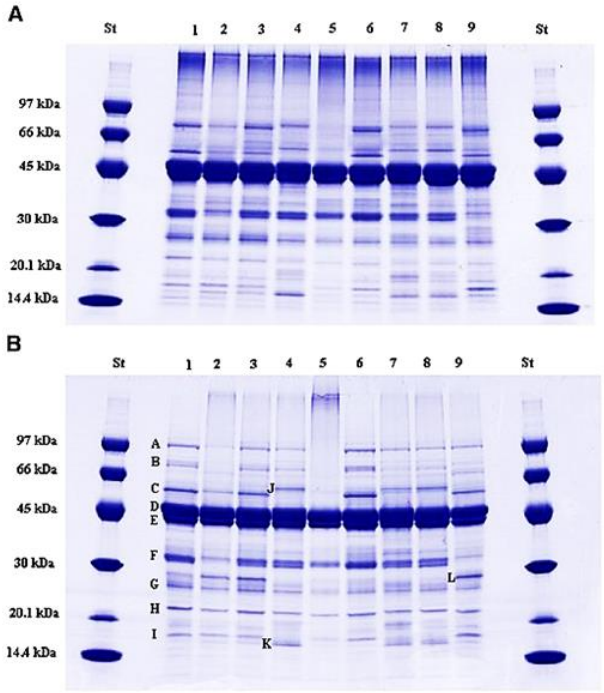

Figure 10: SDS-PAGE (A) under non-reducing conditions and (B) under reducing conditions of *Phaseolus vulgaris* bean protein isolates. Lanes 1-9, White bean, pink bean, pinto bean, cranberry bean, black bean, great northern bean, light red kidney bean, dark red kidney bean, and small red bean respectively (Rui et al. 2011).

Large similarities were reported under both sets of conditions for protein isolates used. Major bands were observed under reducing (barely affected by) and non-reducing conditions around 47 kDa and were considered as 7S vicilin. These bands are largely present in legume protein. Less prominent bands dissociated after the addition of β-ME were reported as 11S legumin. Minor differences were reported in the profiles of all nine varieties of legume protein, whereby black bean significantly varied compared to the other varieties. Intense bands were observed at a high molecular mass range under reducing conditions (lane 5, Figure 10B).

These intense bands were considered protein aggregates stabilized by molecular forces other than disulfide bonds and may possibly be hydrogen bonds. Thus, β-ME was unable to break down the structure completely. Minor proteins found in legumes may include prolamins (soluble in alcohol), and glutelins (soluble in dilute acid or alkali detergents). Prolamins and glutelins are characterized by the proportion of proline/glutamine and methionine/cysteine respectively. Glutelins have a higher concentration of sulfur containing amino acids, thus are of nutritional interest (Rui et al. 2011).

Subagio (2006) characterized the protein isolate of hyacinth bean from Indonesia for the development of protein as a food ingredient. The protein isolates were produced using an isoelectric method and was fractionated using Osborne sequencing based on solubility in some solvents. The dominant fractions found were globulin (55%), NaOH soluble (27%) and albumin (18%). The amount of 7S globulin found were high (20.50%) and 11S globulin low only accounting for 9.44% total protein. Overall, the total amounts of globulins found in this study were very low, only accounting for 30% of total protein. This may be attributed to other globulins (2S and 15S) not being analyzed, or the current sequencing method used not being able to analyze 7S and 11S globulins optimally.

2.6.1. Protein denaturation and functionality

The difference in free energy (energy required to unfold a native protein structure) between the native and denatured states contributes to the stability of the native structure for protein molecules. Protein denaturation is monitored by examining changes to the physical and chemical properties of protein molecules. Therefore, it can be defined as the transformation of a folded protein molecule structure formed under physiological conditions, to an unfolded state under non-physiological conditions. Many monomeric globular proteins denature when changes to their physical and chemical property(s), (y), are monitored as a function of temperature/denaturant concentration (Figure 11).

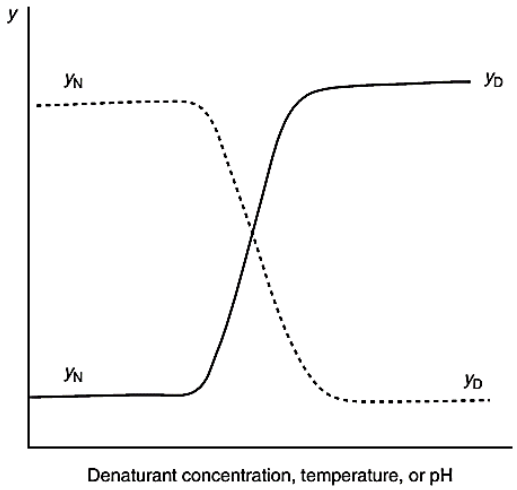

Figure 11: Protein denaturation curves, yN (native) and yD (denatured) states respectively (Damodaran. et al., 2008).

In food processing heat is the commonly used denaturing agent whereby protein denaturation is said to further influence the functional properties of food during processing. Thermal processing is a recognized process for improving the palatability and nutritional quality of proteins. This is achieved by the inactivation of protease inhibitors. Denaturation of proteins has also been reported to extensively influence protein functionality. This is dependent on the level of protein denaturation, whereby it is said that limited protein denaturation could be beneficial to protein functionality. However, extensive denaturation could have the opposite effect. This is typically observed in relation to surface properties. Denaturation is a co-operative process in globular proteins, once a protein molecule begins to unfold the entire molecule completely unfolds with a slight increase in temperature/denaturant. This ultimately means that globular proteins can't exist in intermediate states (Damodaran et al., 2008).

The increase in water content of foods facilitates the thermal denaturation of protein by decreasing peak denaturation temperature (T_d). The thermal stability of protein powders has been found to decrease by increasing the water content (0-0.35 g water/g protein). In a dry state, proteins have a static structure consisting of polypeptide segments restricting mobility. At high water contents swelling of the protein occurs resulting in increased chain mobility and flexibility. This occurs as a result of hydration and penetration of water into the surface. Thus, the protein molecule begins to take on a dynamic molten structure. The application of heat provides greater access of water to salt bridges and peptide hydrogen bonds compared to the dry state. This results in a lower peak denaturation temperature (T_d). In aqueous solutions, various salts and sugars are said to affect the thermostability of proteins. Sucrose, glucose, lactose, and glycerol have been found to stabilize proteins against thermal denaturation.

Furthermore, the peak denaturation temperatures of soy protein, β-lactoglobulin, serum albumin, and oat globulin were found to significantly increase upon addition of 0.50 M NaCl (Damodaran. et al., 2008).

The thermal properties (onset and peak denaturation temperature, enthalpy) for protein isolates produced from nine *Phaseolus vulgaris* legume varieties were examined by Rui et al. (2011). The single endothermic peak observed amongst samples was determined to likely represent the denaturation of 7S vicilin. The thermal stability of most globular proteins is represented by their peak denaturation temperature (T_d). Cranberry and light red kidney bean protein isolates had lower thermal stabilities compared to the other samples. This was due to their lower peak denaturation temperatures (Table 5).

Table 5: Thermal properties of protein isolates produced from *Phaseolus vulgaris* bean (Rui et al., 2011)

Variety	T_o (°C)	T_d (°C)	ΔH (J/g)	$\Delta T_{½}$ (°C)
White	85.33 ± 0.13^{b}	90.58 ± 0.50^{a}	10.98 ± 0.04^{bc}	4.98 ± 0.04^{d}
Pink	84.70 ± 0.06^{c}	89.55 ± 0.06^{b}	12.14 ± 0.53^{a}	5.15 ± 0.13^{cd}
Pinto	85.73 ± 0.08^{ab}	$91.04\pm\pm0.38^{a}$	10.42 ± 0.43^{c}	5.07 ± 0.10^{cd}
Cranberry	78.42 ± 0.41^{e}	84.99 ± 0.19^{d}	10.41 ± 0.16^{c}	7.43 ± 0.06^{a}
Black	84.18 ± 0.10^{d}	88.97 ± 0.21^{c}	10.43 ± 0.59^{c}	5.17 ± 0.15^{c}
Great Northern	84.41 ± 0.22^{cd}	89.26 ± 0.54^{bc}	11.09 ± 0.27^{bc}	$5.19\pm\pm0.11^{c}$
Light Red kidney	77.13 ± 0.44^{f}	$82.14\pm\pm0.35^{e}$	6.72 ± 0.52^{d}	5.98 ± 0.14^{b}
Dark Red kidney	85.77 ± 0.10^{a}	91.14 ± 0.06^{a}	11.06 ± 0.69^{bc}	4.97 ± 0.03^{d}
Small Red	85.46 ± 0.10^{ab}	90.81 ± 0.13^{a}	11.78 ± 0.08^{ab}	5.03 ± 0.09^{cd}

Values followed by different superscript letters in the same column are significantly different ($p<0.05$), T_o - onset temperature, T_d - peak denaturation temperature, ΔH - enthalpy of denaturation, $\Delta T_{½}$ - peak width at half-height.

In the formulation of various food products, legume proteins as isolates or concentrates are used as functional ingredients for increasing nutritional quality and providing desirable sensory characteristics such as flavor, structure, texture, and color. It has been said that the desirable effects of heating legumes can be attributed to denaturation of the globulin. It is normally resistant to denaturation and digestion in its native state (Sashikala et al., 2015).

2.6.2. Nutritional quality evaluation and functional properties

The parameters such as protein efficiency ratio or net protein utilization, biological values and protein digestibility are often used to evaluate the nutritional quality of food proteins. These parameters are largely dependent on the ratios of essential to non-essential amino acids and their digestibility (Table 6).

Table 6: Amino acid content of various leguminous grains (Boye et al., 2010a)

	Amino acid	Pea	Chickpea	Lentil	Bean	Soy
		Pisum sativum	*Cicer arietinum*	*Lens culinaris*	*Phaseolus lunatus*	*Glycine max*
		g/16 g N	g/16 g N	mg/g (dwb.)	(dwb. %)	-
Essential AA	Isoleucine	3.33	4.10	5.06	5.30	1.94
	Leucine	6.58	7.00	8.09	9.00	3.26
	Lysine	6.84	7.70	5.69	7.70	2.69
	Methionine	**1.03**	**1.60**	**1.18**	**1.30**	**0.61**
	Phenylalanine	4.19	5.90	5.55	6.00	2.16
	Threonine	3.59	3.60	5.62	4.90	1.62
	Tryptophan	0.94	1.10	ND	ND	0.50
	Valine	3.89	3.60	7.24	5.90	2.06
	Arginine	6.84	10.30	9.10	6.90	3.17
	Histidine	2.52	3.40	6.84	3.20	1.15
Non-essential AA	Alanine	4.27	4.40	21.32	4.70	1.79
	Aspartic acid	10.68	11.40	11.17	12.00	4.79
	Cysteine	**1.55**	**1.30**	**0.44**	**1.10**	**0.70**
	Glutamic acid	16.92	17.30	24.22	15.10	7.66
	Glycine	4.32	4.10	10.22	4.20	1.77
	Proline	3.76	4.60	8.88	4.70	2.04
	Serine	4.79	4.90	11.20	7.20	1.92
	Tyrosine	3.16	3.70	5.05	3.40	1.53

dwb. (dry weight basis), ND-not determined.

Generally, legume proteins are reliable sources of arginine, glutamic acid, aspartic acid, lysine, and leucine. However, they lack sulfur containing amino acids (methionine and cysteine) and tryptophan. Proteins that have a high Fischer ratio containing high concentrations of branched-chain amino acids and low level of aromatic amino acids are reportedly beneficial to health (Maheri-Sis et al., 2008, Oomah, 2001). The impact of traditional food processing techniques (dehulling, soaking, germination, boiling, autoclaving and microwave cooking) on the protein quality of mung bean legume grains (*Phaseolus aureus*) were examined by Mubarak (2005). It was reported that all processes, apart from dehulled and germinated, significantly reduced concentrations of sulfur containing amino acids, threonine, tryptophan, and lysine (Table 7).

Table 7: Effect of traditional processing on the amino acid content of mung bean grains (g/16 g N) (Mubarak, 2005)

Amino acid	Raw	Dehulling	Soaking	Germination	Boiling	Autoclaving	Microwave cooking	FAO/WHO (1973)
Tyrosine	3.27	3.27	3.11	3.28	3.23	3.14	3.20	-
Phenylalanine	5.66	5.68	5.60	5.70	5.67	5.69	5.65	-
Total aromatic amino acid	8.93	8.95	8.71	8.98	8.90	8.83	8.85	6.00
Theonine	3.15	3.19	3.10	3.20	3.20	3.17	3.18	4.00
Cystine	0.75	0.76	0.64	0.77	0.76	0.65	0.75	-
Methionine	1.92	1.92	1.70	1.95	1.83	1.85	1.80	-
Total sulfur amino acids	2.67	2.68	2.34	2.72	2.59	2.50	2.55	3.50
Leucine	8.36	8.40	8.25	8.53	8.44	8.50	8.43	7.00
Isoleucine	4.74	4.70	4.64	4.70	4.40	4.30	4.37	4.00
Lysine	4.19	4.21	4.15	4.26	4.05	4.00	4.02	5.50
Valine	5.20	5.21	5.23	5.20	5.20	5.30	5.18	5.00
Tryptophan	0.97	0.97	0.95	1.00	0.89	0.94	0.88	1.00
Total essential amino acid	38.20	38.30	37.30	38.60	37.70	37.40	37.50	36.00
Aspartic acid	13.50	13.50	13.80	13.50	13.80	13.80	13.90	-
Glutamic acid	21.70	21.60	21.60	21.50	21.80	21.70	21.80	-
Proline	4.23	4.22	4.35	4.20	4.36	4.26	4.37	-
Serine	4.95	4.95	4.96	4.80	4.90	4.86	4.96	-
Glycine	4.26	4.25	4.35	4.20	4.40	4.38	4.47	-
Alanine	4.35	4.33	4.53	4.41	4.58	4.50	4.66	-
Arginine	6.33	6.33	6.50	6.35	6.00	6.52	5.92	-
Histidine	2.49	2.50	2.58	2.42	2.54	2.54	2.55	-
Total non-essential amino acids	61.80	61.70	62.70	61.40	62.30	62.60	62.50	-
Leucine/ isoleucine ratio	1.76: 1	1.78: 1	1.77:1	1.81:1	1.91:1	1.97:1	1.92:1	1.8:1

Compared to FAO/WHO references, it was found that grains subjected to thermal processing resulted in higher concentrations of total aromatic amino acids, leucine, isoleucine and valine. *In vitro* protein digestibility (IVPD) of mung bean grains was found significantly higher after germination and autoclaving compared to the other processing techniques used (Table 8). The reasons for this noticeable improvement could be due to protein denaturation, destruction of trypsin inhibitors or a reduction in phytic acid, and tannins. There was a distinct improvement in protein efficiency ratio (PER) values found after after germination, boiling, and autoclaving processes. However, cooking and soaking processes had an adverse effect on the content of essential amino acids and the resulting index.

Table 8: Effect of processing on the protein quality of mung bean grains (Mubarak, 2005)

Property	Raw	Dehulling	Soaking	Germination	Boiling	Autoclaving	Microwave cooking
In-vitro protein digestibility (%)	80.20^{e}	84.30^{d}	87.40^{c}	89.10^{a}	87.80^{c}	88.70^{a}	88.20^{b}
Protein efficiency ratio (PER)	4.29	4.30	4.24	4.37	4.32	4.35	4.32
Essential amino acid index (%)	67.80	67.90	65.60	68.90	65.90	65.70	65.60
Chemical score (CS) (%)	76.20	76.50	66.90	77.50	73.60	71.40	73.10
First limiting amino acid	Lysine 76.20	Lysine 76.50	Cys+Meth 66.90	Lysine 77.50	Lysine 73.60	Cys+Meth 71.40	Cys+Meth 72.90
Second limiting amino acid	Cys+Meth 76.30	Cys+Meth 76.60	Lysine 75.50	Cys+Meth 77.70	Cys+Meth 74.00	Lysine 72.70	Lysine 73.10

$^{a-e}$ Means in the same row with different superscript letters are significantly ($p<0.05$) different.

It was found that the chemical score and concentration of limiting amino acids are dependent on processing treatments having significantly varied in the study conducted. Mung bean grains lysine and sulfur containing amino acids varied as first and second limiting amino acids for processes used (Mubarak, 2005). Protein ingredients derived from soybean, whey and wheat are typically used by food industries. However, food manufacturers are looking for alternative protein sources due to concerns over dietary preferences (allergenicity, Halal, vegetarianism etc.). Previous studies have reported that the functional properties of legume protein have been found comparable to frequently used soy and whey protein. These functional properties have been exploited in the development of various food formulations including soups, ready to eat snacks, bakery and extruded products (Adebiyi and Aluko, 2011, Boye et al., 2010a).

The functional properties of proteins have been classified based on hydration (solubility, water and oil absorption), surface characteristics (emulsification, foaming, and formation of protein-lipid films), structure and rheological characteristics (aggregation, elasticity, viscosity, adhesiveness). The important functional properties of proteins in food applications include water and fat holding capacities, solubility, emulsifying, foaming, gel forming, and rheological behaviors. These properties are associated to environmental factors, processing conditions, structure, molecular size, and charge distribution of protein molecules (Day, 2013, Tang and Sun, 2011).

Shevkani et al. (2015a) studied the functional properties of protein isolates produced from different kidney bean and field pea lines. The isolates for different lines showed exceptional functional properties and were found significantly different (Table 9). The charge on proteins were found positively correlated to foaming, emulsion properties and solubility behavior. Other recognized contributing factors were amount, composition, structure of proteins and processing conditions.

The results collected provided information on the use of kidney bean and field pea proteins for utilization as functional ingredients. The use of proteins derived from kidney bean was found suitable for application in food products that require strong gel formation (analogues, meat curds, gels etc.). Water and fat absorption capacities were found higher for field pea proteins and this makes it suitable for ingredient application in products where proteins are the main linkages between fat and water. This may include products such as breads, muffins, cakes, and cold meat.

Table 9: Functional properties of protein isolates from different kidney bean and field pea lines Shevkani et al. (2015a)

	PS (%)	EAI (m^2/g)	ESI (min)	FC (%)	FS (%)
Kidney bean					
EC 572723	71.80±0.40^{c}	26.60±0.80^{d}	21.80±3.20^{a}	97±5.00^{b}	91±0.20^{a}
Pi 244719	65.80±0.70^{ab}	22.30±2.70^{c}	23.60±2.70^{a}	110±4.70^{c}	95±0.80^{b}
Pi 312296	68.70±1.40^{bc}	15.80±1.50^{b}	78.90±5.10^{c}	83±8.40^{a}	92±0.90^{a}
Pi 249554	67.00±1.30^{b}	21.30±0.50^{c}	27.30±3.10^{a}	105±7.10^{bc}	95±1.30^{b}
PLB 10 1	78.50±1.80^{e}	20.30±1.50^{c}	78.20±3.70^{c}	121±7.10^{d}	90±1.50^{a}
Mean	*70.40*	*21.30*	*46.00*	*103*	*92*
Field pea					
IC 394027	64.20±1.10^{a}	14.10±1.30^{b}	95.40±1.80^{d}	87±0.10^{a}	95±1.40^{b}
IC 342028	73.40±0.30^{d}	13.30±1.70^{ab}	52.60±0.90^{b}	108±2.40^{c}	95±2.10^{b}
IC 291541	79.90±3.40^{e}	11.80±2.20^{a}	66.70±3.50^{bc}	132±2.40^{e}	96±2.80^{b}
IC 381453	74.90±0.40^{d}	14.00±0.90^{b}	88.40±5.00^{cd}	118±7.10^{c}	94±0.30^{b}
IC 299013	70.90±0.30^{c}	12.30±1.50^{a}	87.20±10.20^{cd}	105±2.80^{bc}	94±0.40^{b}
Mean	*72.70*	*13.10*	*78.10*	*110*	*95*

Means with similar superscript letters in a column did not differ significantly ($p > 0.05$). [PS -protein solubility; EAI -emulsifying activity index; ESI -emulsion stability index; FC -foaming capacity; FS -foam stability]

The physicochemical and functional properties of commercial isolated yellow field pea protein isolates were determined. Samples were subjected to high hydrostatic pressure (HHP) treatments (200, 400 and 600 MPa) (Chao et al., 2018). The functional attributes of protein isolate samples were found to be significantly different and were attributed to substantial changes in protein structure caused by HHP treatments. HHP treatment was recognized as an effective processing method for enhancing the utilization of pea proteins as functional ingredients in food product formulations. The mechanism responsible for emulsion and foam formation were found to be different. The effects of HHP treatment on emulsion formation were found to be dependent on pH and processing procedure. In terms of foam formation and stabilization, structural changes were observed to have a negative impact.

Protein concentration and HHP treatment were found to positively influence foam formation. The encapsulation of a greater number of air bubbles may be caused by the abundance of protein molecules. Samples treated with HHP had higher foam capacities compared to the control with exception for samples with a protein concentration of 25 mg/mL treated at 600 MPa. The results obtained suggest that pressure-induced protein unfolding improves foam formation by enhancing air bubble encapsulation. The protein solubility of samples showed the typical pattern of plant storage proteins. This was accompanied with a minimal solubility at pH 4–6 and increases above and below this range (Figure 12).

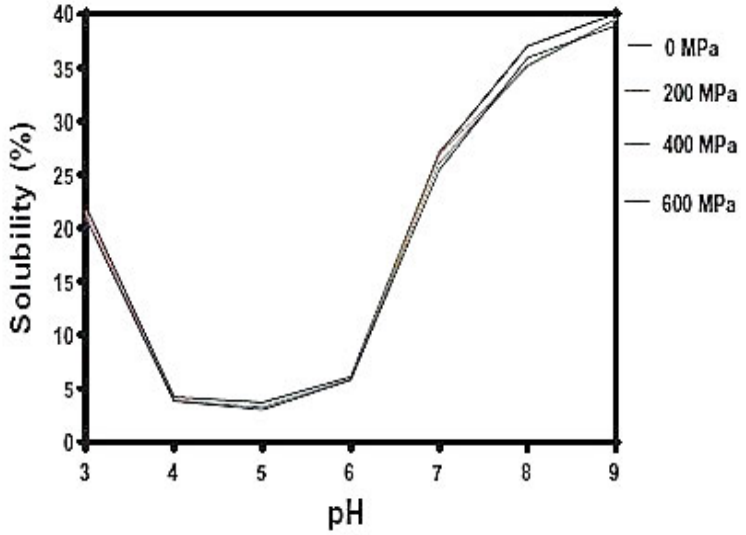

Figure 12: Protein solubility of control and high pressure-treated pea proteins (Chao et al., 2018).

There was no significant difference between the protein solubility of HHP treated samples. This indicates that similarly soluble protein aggregates were formed at treatments of 200, 400 and 600 MPa.

2.6.3. Utilization of native and modified legume proteins as emulsion stabilizers

Plant proteins have received considerable attention from food industries in the early years of the 21st century as a potential substitute to animal and dairy proteins. This is mainly attributed to their availability, feasibility and as comparable nutritional and functional properties. Proteins from food legumes have been regarded a poor man's meat in various parts of the world due to their low price in comparison to animal proteins. In addition, it has been reported that they are 2-3 times richer in protein compared to cereal grains. Briefly, in chickpeas, lentils, dry peas, beans protein contents have been found in the range of 17-30% and 35.00-49.60% in soybean (Boye et al., 2010a).

The interfacial properties, hence amphiphilic nature of legume derived proteins provides them with excellent emulsion characteristics. They are among the key driving forces behind their utilization as stabilizers of emulsion-based delivery systems. In addition, they have been widely reported for their effective use as emulsifiers of oil-in-water (O/W) emulsions contributing to their stability against coalescence and flocculation (Boye et al., 2010b, Dickinson, 2010, Joshi et al., 2012). Carbonaro et al. (2012) reported that high β-sheet (~30-44%) and low α-helix (~12-20%) contents make legume proteins unique in structure. Thus, compared to animal (β-sheet ~4-11% and α-helix ~4-26%) and cereal proteins (β-sheet ~20-30% and α-helix ~28-29%) this permits them to produce better conformational changes at the oil-water interface.

In the process of emulsification, a homogenous dispersion is formed with the help of an emulsifier prior to the mixing of two immiscible liquids (oil and water). The emulsifier aids in reducing the surface tension between the two liquids, thus forming a protective layer around droplets formed by adsorbing on the surface. During adsorption at the interface proteins undergo tertiary and secondary structural modification. This facilitates maximum interaction between hydrophobic segments and phases to take place. The behavior of proteins at the interface plays a major role in the determination of the physicochemical stability of emulsions. Further aggregation of adsorbed protein molecules through hydrophobic or disulfide bonding contributes to the formation of a viscoelastic layer (Figures 13A-C).

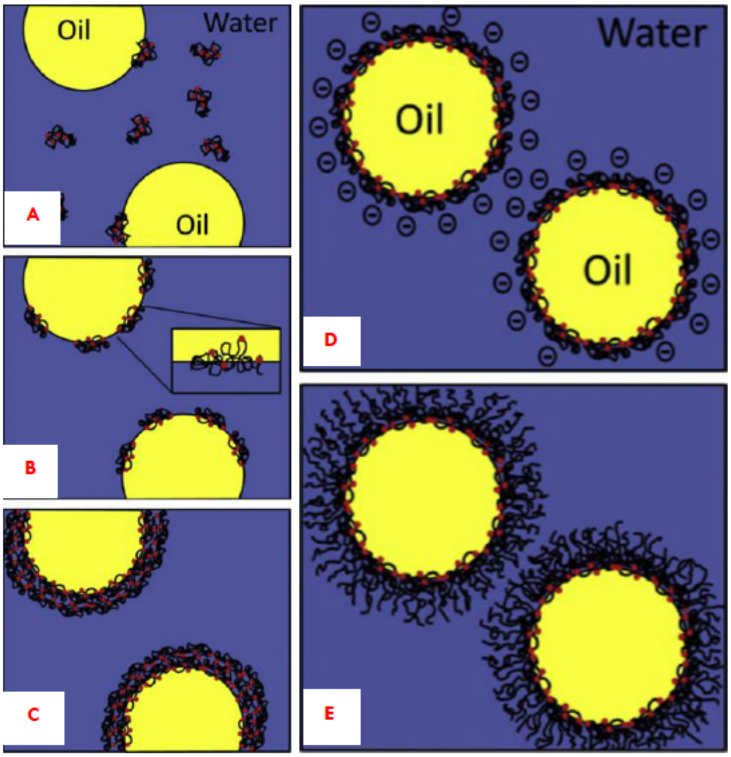

Figure 13: The role of globular proteins in the formation and stabilization of oil-in-water emulsions. (A) Movement of proteins towards oil-water interface (B) Adsorption and reorientation (C) Formation of viscoelastic film (D) Electrostatic repulsion and (E) steric stabilization. The red dots represent hydrophobic moieties of proteins (Sharif et al., 2018).

The inter-droplet repulsive interactions (both electrostatic and steric) that take place determine the stability of emulsions (Damodaran, 2005, Evans et al., 2013, Lam and Nickerson, 2013) as shown on Figures 13D-E. During the storage of emulsions stabilized by native proteins, physical (creaming, coalescence, and flocculation) and chemical (oxidation and hydrolysis) instability may occur. This is due to the formation of non-covalent bonds at the interface that is mainly influenced by environmental related factors (pH, temperature, and ionic strength), molecular size and concentration of emulsifiers, surface hydrophobicity, and homogenization intensity (Lam and Nickerson, 2013, McClements, 2004).

It has been widely reported that exceptional emulsion properties are warranted by lower molecular size, higher surface hydrophobicity, solubility, surface charge, and flexibility. Some of the limitations that affect the physicochemical and emulsifying properties of legume protein may include low water solubility and surface activity at neutral pH. In the case of high solid contents, increased emulsion viscosity may also limit emulsion properties. In terms of application these limitations may cause draw backs for use as wall material in the encapsulation process. To overcome these issues the structure of native legume protein is modified.

Some of the approaches used to modify protein structure include physical, chemical and enzymatic modifications or in combinations (Can Karaca et al., 2015, Liu et al., 2014, Nishinari et al., 2014). Some of the typical physical modification procedures used to modify protein structure includes heat, high pressure, sonication, and extrusion treatments whereby heat is the most commonly used method. The effect of heat on the emulsification and conformational properties of kidney beans protein was examined (Tang and Ma, 2009). A fixed heating temperature of 95°C at varying time intervals of 15, 30, 60, and 120 minutes was used. Significant improvements to functional properties were observed which were attributed to conformational changes to protein secondary structure because of short time heat exposure (15 and 30 minutes). Similarly, emulsions stabilized using soy protein showed better stability due to an increase in surface hydrophobicity and in the formation of disulfide bonds (Wang et al., 2012). This was due to modifications on structural and interfacial properties of soy protein achieved through heat treatment (90°C for 20 minutes).

There have been several drawbacks for the use of chemical modification for achieving desirable structural conformation of proteins. This is mainly due to the use of harsh temperatures, pH, solvents, and other conditions (González-Pérez and Arellano, 2009). This is achieved by attaching low molecular weight molecules from protein through deamidation, acylation, esterification, methylation, alkylation or succinylation. These desirable changes can also be induced by attaching high molecular weight molecules, thus forming protein-polysaccharides based conjugates through a Maillard reaction (Arogundade et al., 2012). Cationization is used for the modification of polysaccharides and animal proteins achieved through the addition of quaternary ammonium groups. Nesterenko et al. (2014) microencapsulated water-soluble ascorbic acid and fat soluble α-tocopherol in emulsions stabilized using soy proteins modified by cationization and acylation.

It was found that emulsions formed by soy proteins modified by cationization showed reduction in droplet size and viscosity. The range of retention efficiency (RE) was 38.30-94.80% for all prepared emulsions, whereby the RE of α-tocopherol was found to be the greatest for emulsions stabilized by acylated soy proteins. The use of divalent Ca^{2+} ions has been documented to function as a salt-bridge to favor protein aggregation by shielding negatively charged groups on proteins. The structure of Ca^{2+}-induced protein aggregates is said to be maintained by hydrophobic interactions (Cao et al., 2015, Zhang et al., 2012). Nap et al. (2018) studied the effect that Ca^{2+} ions have on the structure of weak polyelectrolyte chains end-tethered to nanoparticles (Figure 14).

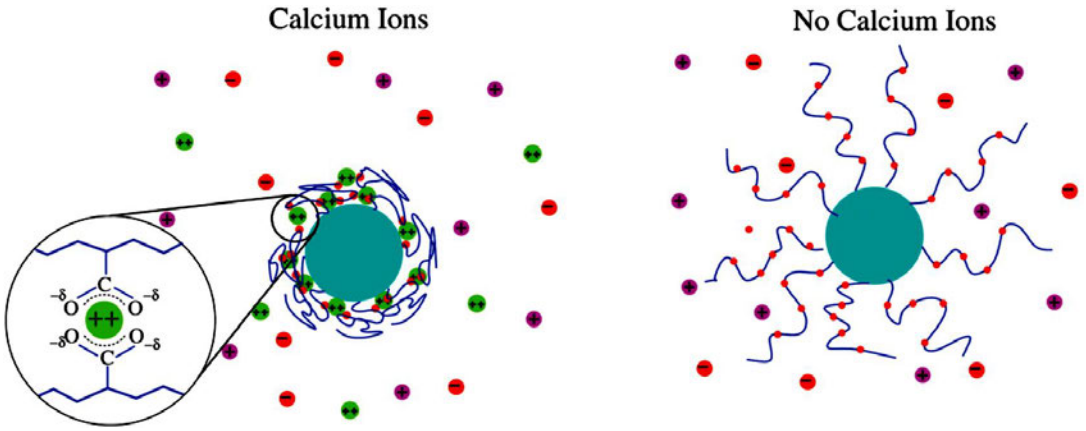

Figure 14: Calcium bridged state of two carboxylic acid monomers associated with one calcium ion illustrating the effect of calcium ions on the structure of a polyelectrolyte layer (Nap et al., 2018).

It was observed that calcium ions form complexes with acidic monomers that are less hydrophilic, thus increased hydrophobicity caused the polymer layer to collapse. Liu et al. (2017) fabricated soy protein nanoparticles using Ca^{2+}-induced aggregation with subsequent GAD (glutaraldehyde) crosslinking to perform as Pickering oil-in-water emulsion stabilizers. The concept of Pickering emulsions was recognized since the publication of Pickering in 1907. The use of solid particles in oil-in-water (o/w), water-in-oil (w/o), or even multiple emulsions was observed to function as emulsifiers in place of surfactants in a fundamentally different way (Figure 15). The stabilization of emulsion droplets by solid particles takes place by adsorption at the surface of droplets. The anchoring of solid nanoparticles to the oil-water interface occurs due to partial wetting on the surface of the nanoparticles by water and oil. The high resistance to coalescence, thus the availability of stable millimeter-sized droplets are major benefits for Pickering emulsions.

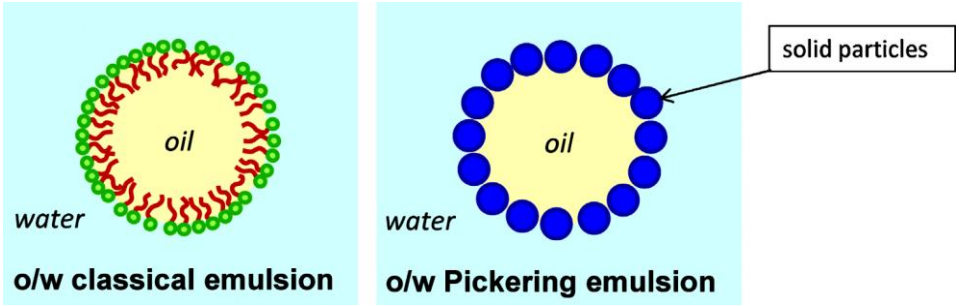

Figure 15: Comparison of classical (surfactant-based) and Pickering oil-water emulsions (Chevalier and Bolzinger, 2013).

Some of the promising applications for Pickering emulsions are for encapsulation and delivery of bioactive compounds, food texture modification and the potential for producing emulsions with flexible interfacial properties.

It is confirmed that the formation and stabilization of Pickering emulsions are largely dependent on proper fabrication procedures or modification of colloidal particles (Pickering, 1907, Xiao et al., 2016). Colloidal particles from food origin are advantageous over inorganic particles. This is attributed to their GRAS (generally recognized as safe) status, amenability for surface modification, biocompatibility, feasibility and degradability. To date, only a limited number of food proteins have been confirmed to potentially function as Pickering stabilizers. This includes the use of zein (de Folter et al., 2012), however an anti-solvent process is required to transform water-insoluble protein into water-soluble colloidal particles in order to function as effective Pickering stabilizers.

Others include whey (Destribats et al., 2014, Shimoni et al., 2013), pea (Liang and Tang, 2014), and soy (Liu and Tang, 2016b, Liu and Tang, 2016a, Liu and Tang, 2016c, Liu and Tang, 2013) proteins. Solid stabilizing particles (or sub-micron, ~100 nm) are often smaller compared to emulsion droplets. This allows for the stabilization of droplets as small as a few micrometers in diameter and for the stabilization of larger droplets (Chevalier and Bolzinger, 2013). It was found for soy protein that increasing Ca^{2+} concentration led to a progressive increase in turbidity and particle size (60-130 nm). This confirmed protein aggregation had taken place and formation of protein nanoparticles. It was found that increasing GAD concentrations had resulted in larger particle sizes, with higher surface charge and lower surface hydrophobicity. The emulsions produced followed the rule of limited coalescence and had a very low surface coverage (3.80-12.60%). The emulsion droplet sizes were found to significantly decrease with increasing soy protein nanoparticle concentrations (0.04-0.40% m/v).

Kargar et al. (2012) studied the potential ability of food-grade particles [Microcrystalline cellulose (MCC) and modified starch (MS)] to enhance the oxidative stability at the droplet interface of 20% sunflower oil-in-water emulsions. Lipid oxidation was measured over 7-days by placing emulsions in an oven immediately after formation at 40°C to accelerate oxidation rate. Mechanical, thermal and physicochemical methods have been developed to truly accelerate stability measurements of dispersions. These methods cause the true state of dispersions to change or at least be stressed.

Physical forces (i.e. centrifugal, magnetic, and electric) have been used to accelerate concentration changes or phase separation of dispersions (upper part Figure 16). However, phenomena such as Ostwald ripening and phase transitions (lower part Figure 16) can't be accelerated using these physical forces. Thus, the combination of traditional aging tests alongside new accelerated tests is used to significantly speed up stability evaluation.

Coalescence and flocculation/agglutination (middle part Figure 16) may be accelerated by physical and/or thermal approaches (Lerche and Sobisch, 2011). Temperature is the common method used for accelerated stability testing of suspensions and emulsions. In addition to the enhanced sedimentation and creaming, Ostwald ripening, phase inversion, coalescence and flocculation may be accelerated by higher temperatures. Samples are typically sealed and stored at higher temperatures (40-60°C) in an oven for weeks or months. At given time the state of the dispersion is analyzed to detect destabilization phenomena (Figure 16).

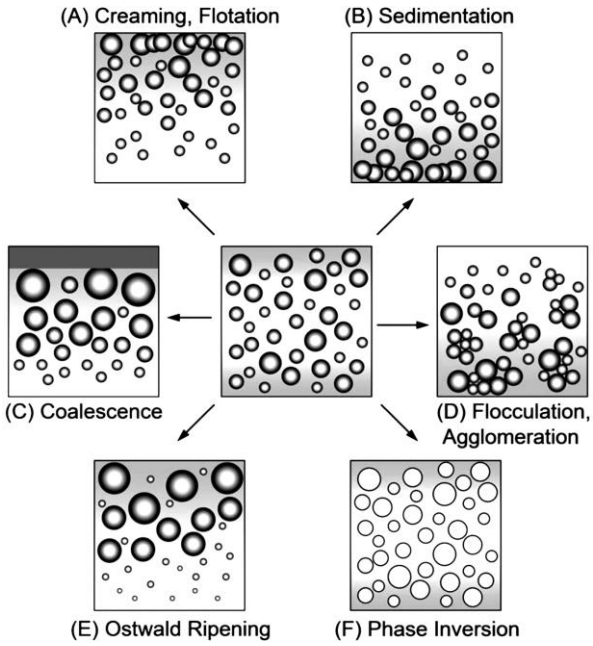

Figure 16: Pathways the state of a dispersion may change after formulation by physical and/or physicochemical phenomena (Lerche and Sobisch 2011).

The rationale for this approach is based on a moderate energy barrier that impairs stability of dispersions. This prevents colliding particles to be attracted by van der Waals forces and thus stick to each other. Increasing temperature is said to impart higher kinetic energy to particles increasing the probability that the energy barrier is overcome, and the particles will aggregate/coalesce. In case of coalescence, the higher collision energy thins the fluid layer (film rupture) between two particles and they coalesce. The increase of flocculation with increasing temperature may be interpreted in a similar way. In case of Ostwald ripening, the solubility of dispersed phase and diffusion increases at higher temperatures and accelerates ripening (J. Mc Clements, 2005).

It was found that an increase to MCC and MS particle concentration from 0.10-2.50% was found to decrease droplet size and enhance the physical stability of emulsions.

The emulsions are physically stabilized by particles due to their packed arrangement at the droplet interface and enhanced viscosity of the continuous phase. The enhanced viscosity occurs because of the particles forming a network in the aqueous phase. Furthermore, this prevents droplets from colliding in the system. The stability of droplets against coalescence was monitored over 40-days. It was observed that higher particle concentration resulted in a reduction in droplet size and an increase in physical stability of emulsions (Table 10).

Table 10: The effect of particle concentrations on the mean droplet size ($D_{3,2}$) of 20% oil-in-water emulsions stabilized with MCC or MS (Kargar et al., 2012)

Particle concentration (%)	Particle type	$D_{3,2}$ (μm)		
		Day 0	Day 21	Day 40
0.10	MCC	14.40±0.40	-	-
0.50		11.50±0.20	-	-
1.00		10.40±0.01	10.40±0.10	10.30±0.06
1.50		10.10±0.03	10.10±0.02	10.10±0.20
2.00		8.90±0.01	8.80±0.20	8.70±0.30
2.50		10.10±1.00	10.30±0.07	10.30±0.03
0.10	MS	14.50±0.90	22.10±0.90	–
0.50		8.10±0.10	7.80±0.09	9.40±0.40
1.00		5.20±0.60	6.30±1.80	6.40±0.40
1.50		5.10±0.02	5.50±0.07	5.90±0.10
2.00		6.40±0.06	6.60±0.10	7.70±0.30
2.50		5.10±0.01	5.00±0.02	6.20±0.30

Data represents mean ± standard deviation.

This was attributed to the ability of the particles to form and maintain a thick interfacial layer around droplets. Furthermore, by increasing particle concentration the rate at which surface coverage occurs around the droplet increases and this enhances the resistance of the emulsion droplets to aggregate or coalesce. The effect of pH on surface coverage (zeta-potential) and mean droplet size ($D_{3,2}$) was studied on MCC and MS particles (Figures 17A-B).

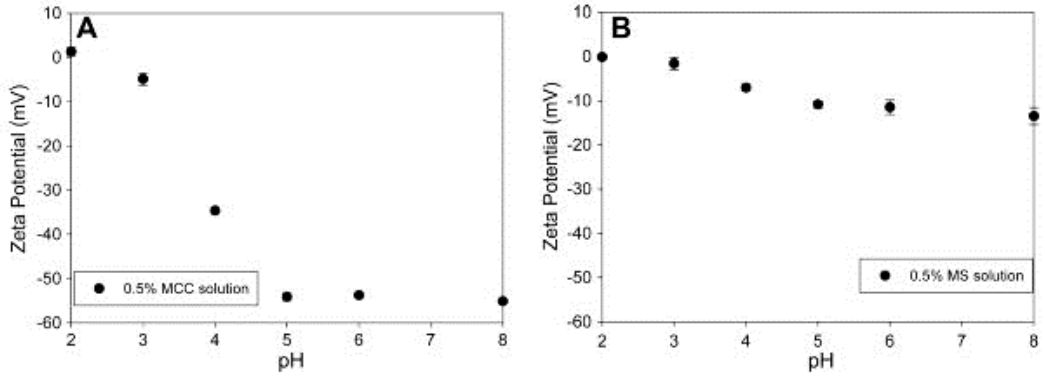

Figure 17: The effect of pH on zeta potential for (A) 0.50% MCC particles and (B) 0.50% MS particles. Data at points represent mean values±standard deviation (n=3) (Kargar et al. 2012).

Particles became more negatively charged as the pH increases and eventually plateauing at pH above 5. Emulsions stabilized by MCC particles at pH 4 were found to be very stable over 40-days. This was attributed to a reduction in droplet collision caused by high electrostatic repulsive force on the droplet surface. After 20 days, the droplet sizes for the emulsions prepared with MS particles (pH 2) significantly increased from 4.80±0.20 μm to 11.60±1.10 μm (Table 11).

Table 11: The effect of pH on the mean droplet size ($D_{3,2}$) of 20% oil-in-water emulsions stabilized by 1.50% MCC or MS (Kargar et al., 2012)

pH	Particle type	$D_{3,2}$ (μm)		
		Day 0	Day 21	Day 40
2	MCC	–	–	–
4		9.20±0.02	9.20±0.05	9.40±0.10
6		9.60±0.01	9.40±0.40	9.60±0.10
8		8.90±0.10	9.10±0.20	9.10±0.20
2	MS	4.80±0.20	11.60±1.10	–
4		5.10±0.02	5.40±0.05	5.90±0.17
6		6.50±0.07	7.40±0.80	7.40±1.70
8		6.50±0.80	8.70±0.03	8.40±0.20

Data represents mean ± standard deviation.

This was caused by minimal electrostatic force occurring between droplets stabilized by uncharged MS particles. This causes droplets to coalesce in comparison to emulsions stabilized by negatively charged particles at higher pH.

2.7. Aims and Objectives

2.7.1. Aim

To implement processing strategies for improving the nutritional and functional properties of intermediates produced from hyacinth bean legume grains for potential application as functional food ingredients.

2.7.2. Objectives

1. To determine the effect of processing on the microstructure, physicochemical properties and *in vitro* starch digestibility of hyacinth bean flour produced from legume grains subjected to steaming, and dehydration treatments prior to soaking respectively.
2. To determine the effect of soaking, steaming and dehydration processing treatments on the nutritional quality and functional properties of protein isolates produced from respective hyacinth bean flour fractions.
3. To determine the emulsifying properties of Ca^{2+}-induced hyacinth bean protein nanoparticles.

Chapter 3: The effect of soaking, steaming and dehydration on the microstructure, physicochemical properties and *in vitro* starch digestibility of flour produced from *Lablab purpureus* (L.) Sweet (hyacinth bean)

Abstract

Hyacinth bean flour was produced from legume grains subjected to steaming and dehydration treatments, prior to soaking. This was to improve its utilization as potential functional food ingredients. The morphological characteristics of starch granules extensively changed in response to processing. There was distinct variation in the chemical composition of samples studied. Potassium (215.33-326.33 mg/100 g), sodium (19.30-24.57 mg/100 g) and phosphorous (148.67-178.33 mg/100 g) were among the main contributors to total variation whereby individual minerals exhibited different leaching responses to processing treatments. There were significant correlations ($p<0.05$) present amongst total starch (48.66-51.02 g/100 g), chemical constituents and physicochemical properties studied. High water penetration potential was identified for samples produced by steaming and dehydration. This was attributed to high levels of swelling and water solubility at lower temperatures (60-70°C). In samples produced by steam-dehydration a significant improvement in resistant starch (23.44 g/100 g dry starch) content was observed.

3.1. Introduction

Food legumes are recognized as the second most valuable source for human nutrition. Hyacinth bean is an indigenous drought tolerant leguminous crop to Africa. It is known in various parts of the world by different names, lablab bean, dolico lablab, dolic lablab, dolique lablab, labelabe, faselbohne, helmbohne. The legume grains are sources of protein, carbohydrates, dietary fiber, minerals, and anti-nutritional factors (tannins, trypsin inhibitors, phytic acid) (Maass et al., 2010, NAS, 1979). In Southern Africa, it has received minimal attention with respect to broadening the food base and is used by large scale farmers as a forage plant for soil improvement. Various processing methods (e.g. steaming, microwaving, boiling, roasting and pressure cooking) have been used to increase the utilization of legume grains through the reduction of anti-nutritional factors (Fabbri and Crosby, 2016, Makgoga, 2013). However, few studies have reported the effect of dehydration on the nutritional quality of legumes. Dehydrated food ingredients may present advantages associated with prolonged preservation time, convenient storage conditions (reduction of volume and weight), soft and homogenous texture (Martín-Cabrejas et al., 2009).

Therefore, flour was produced from hyacinth bean legume grains subjected to subsequent soaking, steaming and dehydration treatments.

This was carried out to improve nutritional quality and functionality for potential application as functional ingredients in the production of legume-based food products. The cotyledon cells are the main storage reservoir of legume grains which are mainly formed by starch deposited in partially crystalline granules covered by a protein matrix. Soaking of legume grains has been associated with a decrease in cooking time and is said to initiate processes, such as the gelatinization of starch and protein denaturation (Copeland et al., 2009, Kaur et al., 2010). The gelatinization of starch serves as an important property, whereby starch absorbs water resulting in a loss to granular organization. The peak gelatinization temperature for black bean, broad bean, chickpea and lentil flours were found in the range of 67.26-71.18°C (Tinus et al., 2012, Chávez-Murillo et al., 2018).

In response to thermal treatments, the crystalline structure of legume starch (type C) is known to be more stable than that of cereal grains (type A). Starch retrogradation has been reported by several authors as the mechanism responsible for the reduction in digestibility of flour produced from various legumes and peas (Sajilata et al., 2006, Eyaru et al., 2009, Piecyk et al., 2019). This may be attributed to cooling of gelatinized starch polymers that may retrograde to a less soluble form resistant to digestion by pancreatic α-amylase. This resistance to digestion may result in reduced glycemic responses which is significant for avoiding health illnesses (e.g. hyperglycemia, hypoglycemia and ketoacidosis). Thus, flour produced from hyacinth bean legume grains subjected to dehydration prior to soaking and steaming would result in greater starch fractions resistant to enzymatic hydrolysis.

Numerous studies have reported the effects of processing on the physicochemical properties of legume flour. However, minimal work has been reported on the interactions between chemical food constituents and the physicochemical properties of legume flour as affected by processing. Li and Zhu (2017) analyzed the functional properties of whole grain quinoa flour to composition using principal component and Pearson correlation analyses. Legume flour is predominantly composed of starch (35.00-52.00%) and is recognized for its unique properties (i.e. low gelatinization temperature, freeze thaw stability) (Tinus et al., 2012). Therefore, starch present in flours produced would significantly influence its physicochemical properties. Findings are expected to provide food processing industries with valuable knowledge on nutrient loss and preservation from a statistical perspective, and to assist with the development of legume-based ingredients for food applications.

3.2. Materials and methods

3.2.1. Materials

Hyacinth bean legume grains were obtained from Reservoir Hills, Umgeni River Valley (Kwa-Zulu Natal Province), South Africa. The herbarium voucher containing specimens was deposited in the Ward Herbarium (UDW), University of KwaZulu-Natal, Westville Campus, Durban. All chemicals and reagents used in the study were of analytical grade.

3.2.2. Processing

The processing of legume grains was carried out based on methods by Shimelis and Rakshit (2007) and Martín-Cabrejas et al. (2009) with minor modifications. Briefly, hyacinth bean legume grains (300 g) were soaked (1:3 m/v) at room temperature for 12 h in distilled water (pH 7) and dehulled. Samples of soaked grains were steamed using an autoclave (10.547 kg/m^2 pressure, 121°C for 30 minutes). Pre-treated grains were dried in an oven set at 55°C for 12 h. A portion of soaked-autoclaved grains was subjected to dehydration (60-80°C for 6 h) using a counter current tunnel dryer. Thereafter, the respective grain portions were milled into flour and screened through a sieve (180 µm). Defatting of flour was carried out using *n*-hexane (1:3 m/v) for 90 minutes at room temperature. After defatting flour sample were left under a laminar fume hood for removal of excess *n*-hexane and named as follows: (S) Soak, (S+A) Soak and Autoclave, (S+A+D) Soak, Autoclave and Dehydrated.

3.2.3. Chemical analysis

The chemical composition of flour samples was determined using methods by AOAC (1990), protein (960.10), ash (923.03), fat (920.85), moisture (925.10), and mineral analysis (984.27). The contents for protein was determined using the Kjeldahl method (N × 5.71), ash in a muffle furnace (600°C for 6 h), fat by Soxhlet method, moisture in a drying oven (105°C for 3 h), and respective minerals were analyzed using ICP-MS (Inductively coupled plasma-mass spectrometry). Total starch content was determined using methods described by Goñi et al. (1996). Briefly, samples were dispersed in 6 mL of 2 M KOH and shaken at room temperature for 30 minutes. Thereafter, suspensions were incubated for 45 minutes at 60°C with 0.40 M sodium acetate buffer pH 4.75, amyloglucosidase (Sigma A-7420, 28 U/mL), and α-amylase (Sigma A-3176). A glucose oxidase and peroxidase (GOD-POD) kit was used to quantify the glucose concentration of samples against standard D-Glucose at 510 nm. Factor conversion from glucose to starch content was 0.90. The total dietary fiber content was estimated as the difference between total flour weight and moisture, protein, fat, ash, starch contents (Li and Zhu, 2017).

Apparent amylose content (%) was determined at 625 nm (UV Spectrophotometer, U-2900) using standard amylose blends from potato starch (Williams et al., 1970).

3.2.4. Scanning electron microscopy

Scanning electron micrographs (SEM) of hyacinth bean flour samples were viewed using a Quanta 250 field emission gun microscope at an accelerated voltage of 10 kV, working distance of 8 mm and coupled to an Everhart-Thornley-Detector operated. Briefly, particles of flour were placed on double-sided adhesive tape and mounted on aluminum stubs (Ø = 12 mm). Samples were then covered with platinum in a sputtercoater (Q150T ES, Quorum Technologies Ltd, UK) [t = 60 s, I = 30 mA].

3.2.5. Physicochemical properties

3.2.5.1. Foaming properties

The foaming properties of flour suspensions (2% m/v) were evaluated at room temperature (Seena and Sridhar, 2005). The foam capacity (%) was determined by recording the initial and final volumes (mL) for suspensions following homogenization (2 minutes) using a Silverson homogenizer. After 60 minutes of incubation at room temperature, the final volume for the suspensions was recorded to calculate foam stability (%).

3.2.5.2. Emulsifying properties

The emulsion capacity (%) of samples were calculated using the heights of the emulsified layer and the total contents of the tube. Emulsions formed were subjected to heating (80°C for 30 minutes) followed by centrifugation (Eppendorf 5810 R) at 1100 × *g* for 5 minutes. The final height of the emulsified layer was recorded to determine the stability (%) of emulsions (Sridaran et al., 2012).

3.2.5.3. Water and oil absorption capacities

Water and oil absorption capacities (g/g) were determined following centrifugation at 3000 × *g* for 10 minutes. The gain in mass of flour suspensions prepared with water/canola oil was determined to calculate water and oil absorption capacities (Sofi et al., 2013).

3.2.5.4. Swelling power and water solubility index

Swelling power (SP_f) and Water solubility index (WSI_f) was determined using a method by Li and Zhu (2017) with minor modifications. Flour samples (W_0, 0.25 g, dwb.) were weighed into pre-weighed 15 mL centrifuge tubes containing 10 mL deionized water. The flour suspensions were incubated for 1 h at 60, 70, 80 and 90°C. Upon cooling, suspensions were centrifuged at 3000 × *g* for 30 minutes.

The supernatants were poured into pre-weighed aluminum pans before drying to a constant weight (W_1) at 105°C. The mass of the sediment remaining in the respective centrifuge tubes was recorded (Ws). The WSI_f and SP_f were calculated as (g/g) for flour samples at the respective incubation temperatures using equations 1 and 2:

$$WSI_f = \frac{W_1}{W_0} \quad \text{..... (1)}$$

$$SP_f = \frac{W_s}{W_0 \times (1 - WSI_f)} \quad \text{..... (2)}$$

3.2.5.5. Bulk density

The ratio of mass to volume were determined for flour samples. The bulk densities were expressed as the weight of sample per unit volume (g/mL).

3.2.6. *In vitro* starch digestibility

In vitro starch digestibility was determined according to methods by Englyst et al. (1992) and Sopade and Gidley (2009) with minor modifications. Briefly, 500 mg of sample was stirred in 1 mL of porcine α-amylase (Sigma A-3176; 250 U/mL of carbonate buffer pH 7). Thereafter, 5 mL of pepsin (Sigma P-6887) solution prepared in 0.02 M HCl was added and incubated at 37°C for 30 minutes. The digesta was neutralized with 5 mL of 0.02 M NaOH, and further adjusted with 25 mL of 0.20 M sodium acetate buffer pH 6, followed by the addition of pancreatin (Sigma P-1750; 2 mg/mL of acetate buffer) mixture and amyloglucosidase (Sigma A-7420; 28 U/mL of acetate buffer). Starch hydrolysis (%) was determined by quantification of glucose in the digesta between 0 and 180 minutes of digestion using a GOD-POD kit. The factor conversion from glucose to starch content used was 0.90.

3.2.6.1. Determination of Rapidly digestible starch (RDS), Slowly digestible starch (SDS), and Resistant starch (RS) fractions

RDS and SDS were measured as the percentage of total starch hydrolyzed (g/100 g of dry starch) at 20 and 120 minutes of enzymatic digestion respectively. Resistant starch was measured as the difference between the total starch and the sum of RDS and SDS contents for respective flour samples.

3.2.6.2. Expected glycemic index

The expected glycemic index (eGI) was estimated using methods described by Siddhuraju and Becker (2005). The HI (Hydrolysis Index) was calculated as the ratio of areas under the hydrolysis curves (AUC) for flour and glucose:

$$AUC = C_{\infty}(t_f - t_0) - (\frac{C_{\infty}}{k})[1 - \exp[-k(t_f - t_0)]]$$

where, C_{∞} corresponds to the glucose concentration at equilibrium (t_{120}), t_f is the final time (180 minutes), t_0 is the initial time (0 minutes), and k is the kinetic constant. Thereafter, the expected glycemic indices (eGI) for respective samples were determined using equation:

$$eGI = 39.71 + (0.549 \times HI).$$

3.2.7. Statistical analysis

The means of replicate data (n=3) were subjected to analysis of variance (ANOVA) for determination of significant differences ($p<0.05$) using IBM SPSS software (IBM Corporation, New York, USA). Principal component analysis was used to visualize similarities and differences amongst samples. The relationship between the chemical food constituents and the physicochemical properties of flour were analyzed using Pearson correlation analysis.

3.3. Results and discussion

3.3.1. Chemical composition

Hyacinth bean flour samples produced are predominantly composed of starch (48.66-51.02%) and protein (27.35-29.47%) respectively (Table 12). Samples produced from legume grains subjected to steaming (S+A) and dehydration (S+A+D) significantly reduced ash (2.64-3.92%), fat (0.43-0.76%) and amylose (20.18-27.91%) contents. However, these processes were found to considerably improve total dietary fiber contents (6.21-12.48%). Findings were in accordance to work conducted on legume varieties lentil, lablab bean, kariya grains and field peas, whereby processing treatments were found to significantly influence chemical composition (Aryee and Boye, 2017, Fawale et al., 2017, Shaahu et al., 2014).

Table 12: Chemical composition for hyacinth bean flour produced from differently processed grains

Sample	Protein	Fat	Total Starch	Ash	Moisture	Total Dietary Fiber	Amylose
S	29.47±1.21^{a}	0.76±0.23^{b}	51.02±0.08^{a}	3.92±0.33^{a}	8.62±0.09^{a}	6.21±1.22^{a}	27.91±0.28^{a}
S+A	27.35±0.10^{b}	0.44±0.40^{b}	48.90±0.02^{b}	2.64±0.11^{b}	8.70±0.47^{a}	11.97±0.29^{b}	26.81±0.20^{b}
S+A+D	27.84±1.12^{b}	0.43±0.03^{b}	48.66±0.02^{b}	2.67±0.18^{b}	7.92±0.54^{b}	12.48±1.43^{b}	20.18±0.31^{c}

Data denotes mean±standard deviation (n=3). Values with different superscript letters are significantly different ($p<0.05$). [S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated)].

Principal component analysis was used to explain the variation in chemical composition of samples (Figure 18a-b). The first principal component accounted for 83.954% of total variation, whereby protein, total dietary fiber (6.21-12.48%), moisture (7.92-8.70%), and amylose (20.18-27.91%) were recognized as the main contributors to variation. In terms of mineral composition, potassium (215.33-326.33 mg/100 g), sodium (19.30-24.57 mg/100 g) and phosphorous (148.67-178.33 mg/100 g) were main contributors to variation present in samples (Figure 18c-d). Magnesium (2752.00-3437.67 mg/100 g) was the most abundant mineral element found in flour samples produced, followed by potassium (215.33-326.33 mg/100 g), phosphorous (148.67-178.33 mg/100 g), calcium (40.07-71.43 mg/100 g), sodium (19.30-24.57 mg/100 g), iron (4.57-10.17 mg/100 g), manganese (3.97-5.43 mg/100 g), zinc (3.57-4.90 mg/100 g) and copper (1.08-1.33 mg/100 g) respectively.

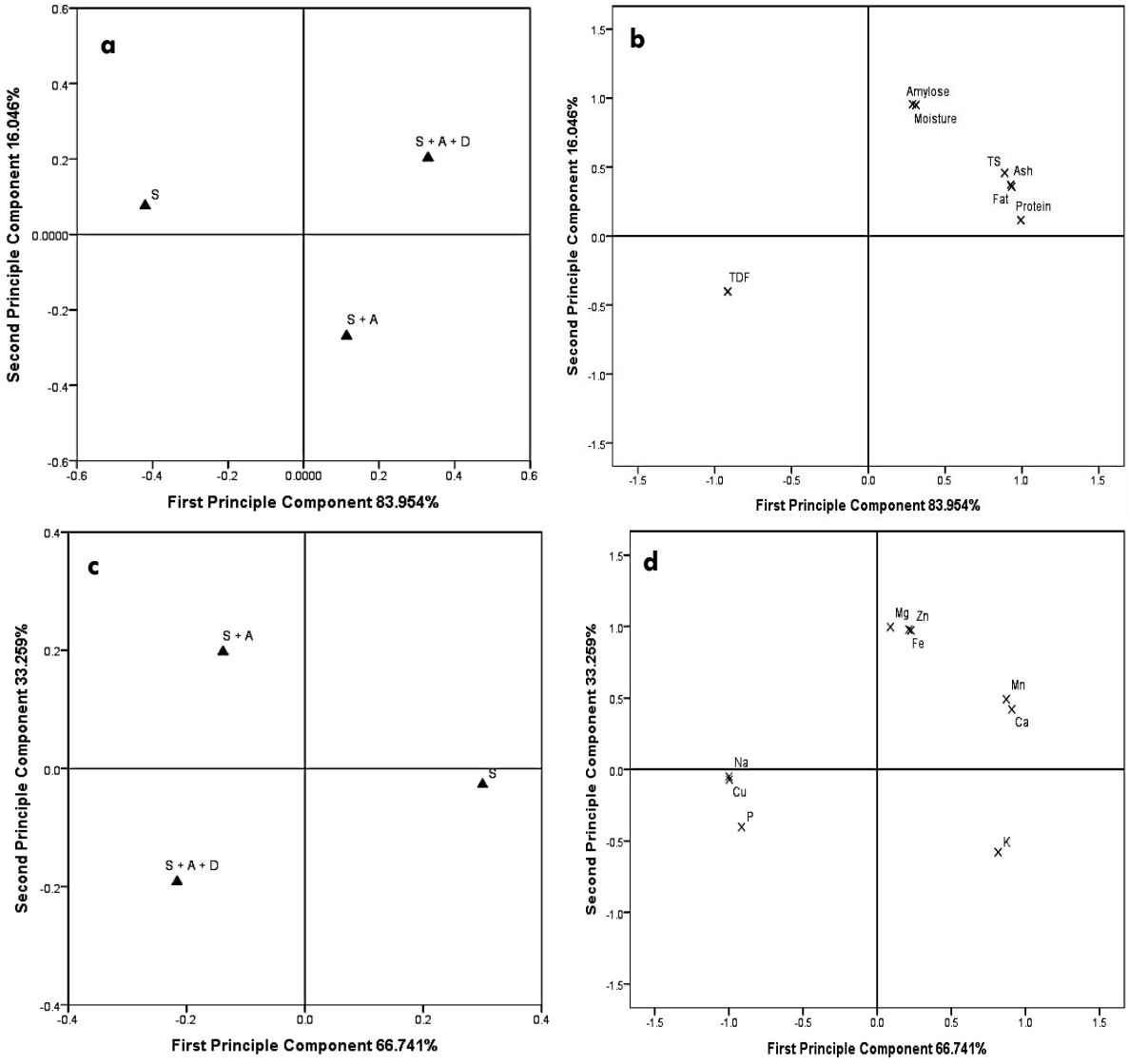

Figure 18: Principal component analysis of hyacinth bean flour samples showing score (left) and loading (right) plots of (a-b) chemical and (c-d) mineral composition: S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated).

The differences in mineral concentrations (Table 13) observed could be attributed to the leaching out of minerals into water following processing of legume grains (Xu et al., 2016). Flour produced from legume grains subjected to soaking (S) was found to have the greatest retention of minerals. Steam (S+A) and dehydration (S+A+D) treatments were found to significantly reduce the concentrations of magnesium and copper. However, improvement in concentrations for calcium, iron, zinc, and manganese were observed compared to soaking (S). Sánchez-Arteaga et al. (2015) suggested that high concentrations of minerals (Ca, K and Na) could impact the stability of legume proteins by shifting the denaturation process to slightly higher temperatures.

Several significant correlations ($p<0.05$) existed between the chemical constituents studied (Appendices 1A-C). Starch present in flour was found positively correlated to sodium ($r=0.993$) in steam-dehydrated (S+A+D) samples. Naiker et al. (2019) has reported similar findings for cultivars of cowpea flour, whereby sodium and calcium were found positively correlated to starch. However, negative correlations existed between starch, calcium ($r=-0.995$) and copper ($r=-0.999$) for steamed samples (S+A). The correlations observed amongst chemical constituents suggest an inherent association, implying that starch present in hyacinth bean flour produced by steam-dehydration is associated with high concentrations of sodium, however starch present in flours produced by steaming may be associated with low concentrations of calcium and copper. Therefore, it can be confirmed that the individual minerals present in flour produced from legume grains subjected to different process treatments exhibited different leaching responses.

Table 13: Mineral element concentration (mg/100 g) for flour produced from differently processed hyacinth bean grains

Sample	Ca	Mg	K	Na	P	Cu	Fe	Mn	Zn
S	40.07±0.69^{b}	3437.67±3.32^{a}	326.33±1.81^{a}	24.57±5.09^{a}	178.33±1.62^{a}	1.33±0.23^{a}	4.57±0.25^{a}	3.97±0.06^{a}	3.57±0.15^{a}
S+A	71.43±4.32^{a}	3194.33±0.85^{a}	228.00±1.83^{b}	19.30±1.14^{a}	148.67±0.95^{a}	1.08±0.37^{a}	5.33±0.93^{b}	5.30±0.35^{b}	3.77±0.21^{a}
S+A+D	71.43±0.82^{a}	2752.00±3.90^{b}	215.33±3.84^{b}	21.30±4.36^{a}	149.33±2.11^{a}	1.17±0.35^{a}	10.17±2.84^{c}	5.43±0.12^{b}	4.90±0.75^{b}

Data denotes mean±standard deviation (n=3). Values with different superscript letters are significantly different ($p<0.05$). [S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated)].

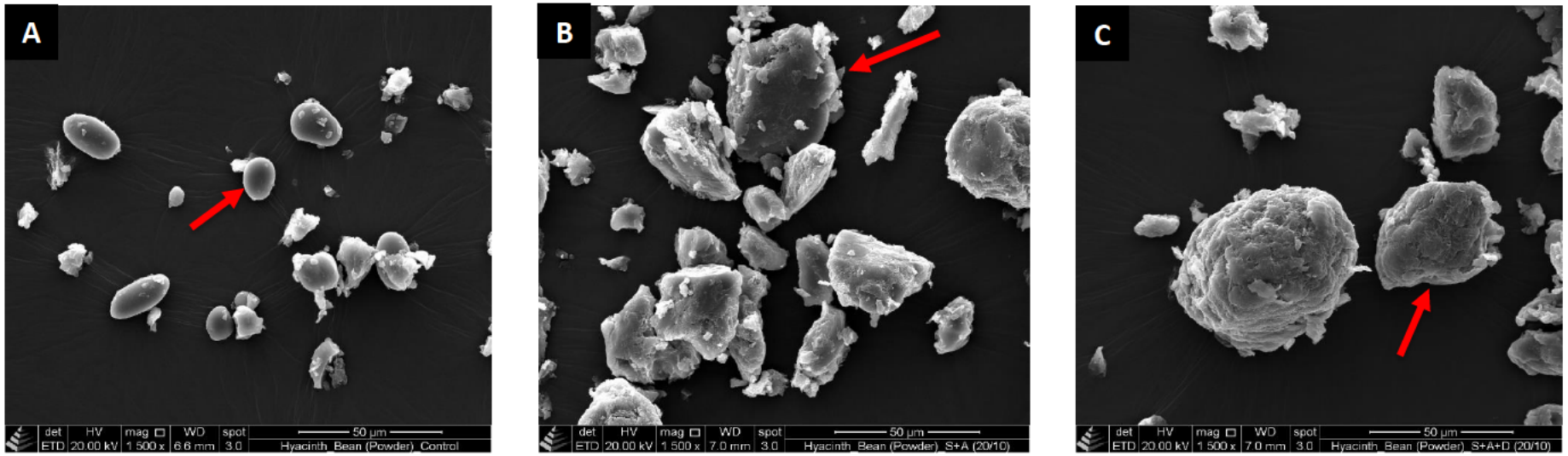

Figure 19: SEM of hyacinth bean flours: (A) Soak, (B) Soak + Autoclave, (C) Soak + Autoclave + Dehydrated. Bar size = 50 µm.

3.3.2. Scanning electron microscopy

Scanning electron micrographs of flour samples are shown on (Figures 19A-C). The representative storage component pointed out amongst flour samples were considered as starch granules. The extent of their morphological changes was found dependent on processing treatments. In flour samples produced from legume grains subjected to soaking (S), starch granules were characterized by a smooth surface and were surrounded by irregular shaped protein bodies (Figure 19A). The structure of these granules was at large uniform and appeared to be spherical-oval in shape. There were large morphological changes to starch granules observed for samples produced by steaming (S+A) and dehydration (S+A+D). Both processes resulted in the extensive enlargement on the surface of starch granules (Figures 19B-C). This may be attributed to the water absorption of starch that could have occurred at higher temperatures due to the rupture of granules in the presence of heat.

Similarly, Aguilera et al. (2009) reported cooking after soaking extensively enlarged starch granules present in chickpea and lentil. Both legumes reached similar sizes (121-125 μm) representing enlargements of 7.30 and 5.50 times greater than raw starch granules in chickpea and lentil, respectively. Morphological characteristics of flour samples produced by steaming (S+A) and dehydration (S+A+D) were found similar. However, smaller starch granules were present in dehydrated samples which may be attributed to the loss of holding water (Figure 19C). Minor breakage to starch granules were evident in flour samples produced by steam and dehydration. However, the integral granule structure was maintained, and protein matrix attached to starch granules were still visible.

3.3.3. Physicochemical properties

The bulk densities for hyacinth bean flour samples produced were found in the range (0.66-0.87 g/100 mL). The variation in physicochemical properties studied is explained by principal component analysis (Figure 20). The first principal component explains 84.733% of the total variation whereby, water absorption capacity, swelling and solubility indices were among the main contributors.

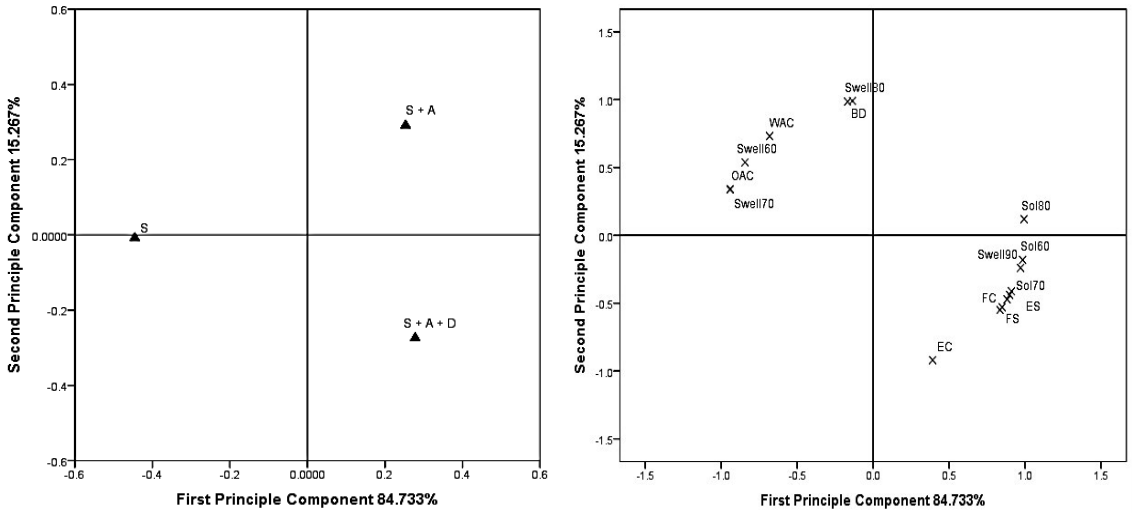

Figure 20: Principal component analysis for the functional properties of hyacinth bean flour samples showing score (left) and loading (right) plots: S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated).

3.3.3.1. Foaming properties

Foaming properties is largely determined by the ability of proteins to form cohesive viscoelastic film through intermolecular interactions. This is achieved by protein conformational change and rearrangement at the air-water interface. Therefore, stable foams are characterized by the formation of a resistant amorphous film (solid surface) by decreasing the surface tension with increasing viscosity on the solution surface (Rodríguez-Miranda et al., 2012). Flour samples produced from legume grains subjected to different process treatments extensively influenced foam capacities (61.35-79.08%) and stabilities (69.09-72.25%) (Table 14). Steam (S+A) and dehydration (S+A+D) processes were found to impair foaming ability and stability. Juárez-Barrientos et al. (2017) had confirmed that native proteins present in Mexican jackfruit seed flour contributes to more stable foams in comparison to samples produced from seeds subjected to boiling.

Table 14: Physicochemical properties for hyacinth bean flour

Sample	Foam capacity (%)	Foam stability (%)	Emulsion capacity (%)	Emulsion stability (%)	WAC (g/g)	OAC (g/g)	Bulk density (g/100 mL)
S	79.08±3.07^{b}	72.25±0.83^{a}	33.17±2.45^{a}	93.70±4.94^{a}	0.72±0.02^{a}	0.85±0.07^{b}	0.66±0.00^{a}
S+A	61.35±3.07^{a}	69.09±5.78^{a}	27.69±1.00^{c}	59.49±5.07^{b}	2.28±0.07^{b}	1.02±0.03^{a}	0.72±0.01^{b}
S+A+D	62.41±2.68^{a}	69.36±1.06^{a}	22.03±2.08^{c}	66.04±4.56^{a}	2.66±0.09^{c}	0.97±0.02^{a}	0.87±0.01^{c}

Data denotes mean±standard deviation (n=3). Values with different superscript letters are significantly different ($p<0.05$). [S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated)].

Based on Pearson correlation analysis, chemical food constituents (i.e. protein, starch, fiber) extensively influenced foaming properties in soaked (S) samples (Appendix 2A). Protein (r= -0.991, $p<0.05$) and moisture (r= -0.991, $p<0.05$) impaired foam stability, however ash (r= 0.996, $p<0.05$) and total dietary fiber (r= 0.991, $p<0.05$) were found to significantly promote stability. Protein dispersions containing sugars (i.e. lactose, sucrose) have been found to impair foam formation but improve the stability of foams (Damodaran et al., 2008). Mineral elements contributed to the formation and stabilization of foams in samples produced by steaming (S+A) and dehydration (S+A+D). In the present study, manganese (r= -0.999, $p<0.05$) and zinc (r= -0.999, $p<0.05$) impaired foam capacity and stability in flour samples produced by steaming (S+A) respectively (Appendix 3B). Zhu and Damodaran (1994) has provided evidence of the interactions between divalent cation salts to foamability and stability of whey protein isolate.

3.3.3.2. Emulsifying properties

The emulsion capacities (22.03-33.17%) and stabilities (59.49-93.70%) were found to be significantly different amongst all processed flour samples (Table 14). Steaming (S+A) and dehydration (S+A+D) treatments extensively impaired emulsion properties. Chemical food constituents extensively influenced emulsion formation and stabilization. In soaked (S) and steamed (S+A) samples, protein (r=0.995, $p<0.05$) and total dietary fiber (r=-0.995, $p<0.05$) promoted and impaired emulsion stability respectively (Appendix 2A). The behavior of proteins at the interface plays a major role in the determination of the physicochemical stability of emulsions. Further aggregation of adsorbed protein molecules through hydrophobic or disulfide bonding contributes to the formation of a viscoelastic layer. The inter-droplet repulsive interactions (both electrostatic and steric) that takes place determines the stability of emulsions (Evans et al., 2013).

3.3.3.3. Water and Oil absorption capacities

All samples produced showed similar oil absorption capacities (OAC) (0.85-1.02 g/g). The oil absorption capacity of flour samples helps to improve mouth feel and the retention of flavor. It refers to the physical entrapment of oil within the protein. The forces involved in lipid–protein interaction are non-covalent bonds such as electrostatic, hydrophobic, and hydrogen bonds (Du et al., 2014). The water absorption capacities (WAC) (0.72-2.66 g/g) was found to be greater for samples produced from grains subjected to steaming (S+A) and dehydration (S+A+D) (Table 14). Acevedo et al. (2017) reported similar findings for pigeon pea (1.72-1.74 g/g), dolichos bean (2.36-2.63 g/g) and jack bean (2.87-2.99 g/g) flours subjected to soaking-cooking. This change may be attributed to the exposure of hydrophilic groups from chemical food constituents (i.e. protein, starch) that may have high affinity for water.

Several significant correlations ($p<0.05$) were observed between chemical food constituents and water absorption capacity. For samples produced from legume grains subjected to steaming and dehydration, mineral elements extensively influenced the respective parameter (Appendix 3B and 3C). Sodium ($r= 0.998$), magnesium ($r= 0.999$), potassium ($r= 0.989$), and phosphorous ($r= 0.998$) facilitated the absorption of water in both steam (S+A) and steam-dehydrated (S+A+D) samples respectively. Moisture ($r= 0.990$) was found significantly positively correlated to water absorption in samples produced by steam-dehydration (Appendix 2C).

3.3.3.4. Swelling power and Water solubility index

Swelling and water solubility indices were found significantly different amongst flour samples across incubation temperatures (60-90°C). Soaking temperature and time have been reported to greatly influence the hydration capacity of legume grains, whereby elevated temperatures increase the diffusion rates of various constituents (i.e. starch) resulting in high water absorption capacities (Campos-Vega et al., 2018). For samples produced by soaking (S), swelling and water solubility (g/g) was directly proportional to temperature (Table 15).

Table 15: Water solubility index and swelling power at different incubation temperatures for flour produced from hyacinth bean grains

		Temperature (°C)			
	Sample	**60**	**70**	**80**	**90**
Water Solubility Index (g/g)	S	0.35±0.01^{a}	0.44±0.00^{a}	0.46±0.01^{a}	0.48±0.03^{b}
	S+A	0.14±0.07^{b}	0.18±0.03^{b}	0.20±0.03^{b}	0.16±0.08^{a}
	S+A+D	0.21±0.01^{c}	0.23±0.01^{c}	0.38±0.22^{a}	0.23±0.00^{a}
Swelling Power (g/g)	S	3.80±0.04^{b}	5.06±0.24^{a}	7.13±0.79^{b}	10.28±0.92^{c}
	S+A	7.24±0.19^{a}	8.88±0.34^{b}	8.53±0.43^{a}	8.26±0.94^{a}
	S+A+D	6.98±0.02^{a}	7.76±0.11^{c}	12.61±1.50^{a}	9.04±0.10^{a}

Data denotes mean±standard deviation (n=3). Values with different subscript letters are significantly different ($p<0.05$). [S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated)].

The improved solubility indices observed at higher temperatures could be attributed to the leaching out of soluble sugars and amino acids during the disruption of starch granules. However, this pattern was not observed for samples produced by steam (S+A) and dehydration (S+A+D) treatments. A reduction in swelling power and water solubility occurred between 80-90°C respectively. This was due to the pre-gelatinization of starch granules after legume grains were subjected to steaming and dehydrated prior to milling. The significance of this result means that high water penetration can be achieved for flours subjected to low incubation temperatures.

For samples produced by soaking, starch and sodium were found significantly positively ($p<0.05$) correlated to swelling power and water solubility (Appendix 4A and 5A). Therefore, greater swelling and solubility indices for flours produced from grains subjected to soaking are associated with high starch and sodium contents. Furthermore, the reduction of amylose present in starch granules at higher temperatures is associated with favorable swelling and solubility properties. Other contributing factors that may have contributed to variations in swelling and solubility behavior of flours produced may include differences in starch granule crystallinity, viscosity patterns, and internal organization because of negatively charged phosphate groups. In addition, complexes that may form between amylose, protein and lipids during starch gelatinization may also prove to significantly influence the properties of starch present in legume flour (Ali et al., 2016, Maaran et al., 2014).

3.3.4. *In vitro* starch digestibility

Starch hydrolysis (%) for the respective flour samples was found to increase over time (0-180 minutes) (Figure 21).

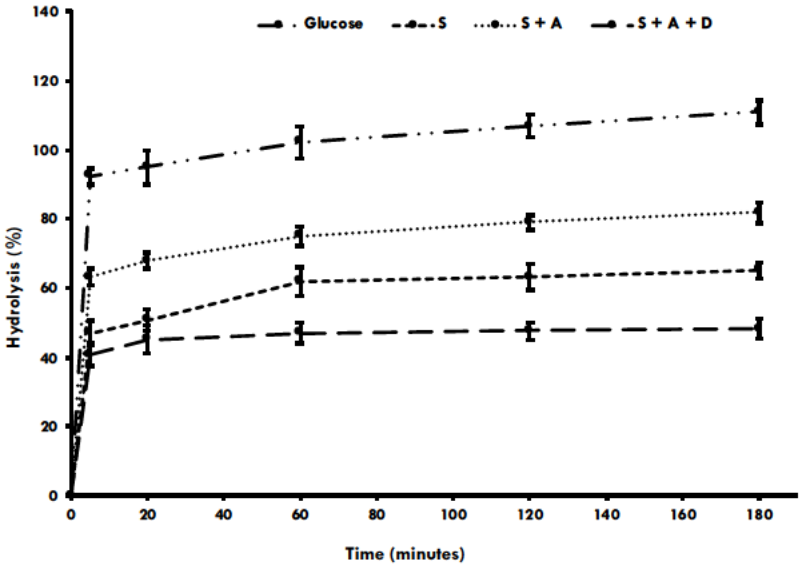

Figure 21: Starch hydrolysis for processed hyacinth bean flour at different time intervals under controlled incubation temperature (°C). S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated).

The rapidly digestible starch (RDS) fraction is the less desirable form of dietary starch and was found in the range of (20.39-28.56 g/100 g). It is associated with a rapid increase of postprandial plasma glucose and insulin (Table 16). Cooking of legume grains in excess water has been found to increase the concentration of rapidly digestible starch (RDS) despite various studies reporting that legume starch presents slow digestion properties (Jukanti et al., 2012).

Table 16: Starch fraction (g/100 g of dry starch) analysis and expected glycemic indices (eGI) for hyacinth bean flour

Sample	TS	RDS	SDS	RS	K	HI	eGI
S	51.02±0.56^{a}	25.91±0.85^{a}	6.33±0.80^{a}	18.78±0.79^{a}	0.12±0.04^{a}	0.99±0.01^{a}	40.26±0.01^{a}
S+A	48.90±0.89^{b}	28.56±1.01^{b}	8.37±0.95^{b}	11.97±0.88^{b}	0.16±0.01^{a}	1.26±0.04^{b}	40.40±0.01^{a}
S+A+D	48.66±1.01^{b}	20.39±0.95^{c}	4.83±0.89^{c}	23.44±0.87^{c}	0.09±0.02^{b}	0.78±0.03^{a}	40.14±0.03^{a}

Data denotes mean±standard deviation (n=3). Values with different superscript letters are significantly different ($p<0.05$). [S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated), TS (Total starch), RDS (Rapidly digestible starch), SDS (Slowly digestible starch), RS (Resistant starch), K (Kinetic constant), HI (Hydrolysis index), eGI (Expected glycemic index)].

The use of hydrothermal treatments has been found to decrease the digestion rate of various legume starches by amylolytic enzymes. This resulted in relatively high amounts of SDS and RS fractions (de la Rosa-Millán et al., 2017). The resistant starch content of flour samples ranged between (18.78-23.44 g/100 g). Some of the positive effects of resistant starch on human health may include increase absorption of minerals, reducing plasma triglyceride and cholesterol levels, improving colonic health, and interventions of insulin resistance (Fuentes-Zaragoza et al., 2010).

The resistant starch content was found to be greater in samples produced by dehydration prior to steaming and soaking (S+A+D). This may be attributed to the dehydration of gelatinized starch following steaming (S+A) resulting in the retrogradation of amylose present in starch to a less soluble form that is resistant to enzymatic digestion. Retrogradation of amylose is the main mechanism behind the formation of resistant starch. Thus, when legume grains were subjected to steaming prior to dehydration, the amylose in starch granules swelled resulting in the disintegration of the crystalline structure of amylopectin. Thereafter, swelling and thickening of starch takes place and once the legume grains are dehydrated retrogradation of starch occurs (Sajilata et al., 2006).

SEM images confirmed minor breakage to starch granules had taken place in flour samples produced from legume grains subjected to dehydration. Arıcı et al. (2016) had reported a similar pattern of results found, whereby resistant starch content of taro flours generally increased with air velocity (0.50-2.00 m/s) at a drying temperature of 50°C. The expected glycemic indices (eGI) for samples were not significantly different ($p<0.05$). However, they were considered low GI ranging between (40.14-40.40). The results obtained offer opportunities for the development of legume-based food ingredients characterized by the slow release of glucose (low glycemic index).

3.4. Conclusion

The study conducted confirmed the effects of soaking, steaming and dehydration of flour produced from hyacinth bean legume grains. SEM images revealed morphological characteristics of starch were largely dependent on processing treatments. Principal component analysis confirmed significant variation present in samples chemical composition and physicochemical properties. It was confirmed that individual mineral elements exhibited different leaching responses in flour produced from legume grains subjected to the different process treatments. There was great interaction present amongst starch, mineral elements and physicochemical properties. Steam-dehydration (S+A+D) of legume grains prior to soaking resulted in flour with a high resistant starch content and improved water swelling and solubility properties at lower temperatures. Thus, this makes it suitable for ingredient application in texture-modified food products that require rehydration for preserving food structure or creating a new one to serve a functional purpose. Furthermore, these products may potentially be recognized for low glycemic responses which are vital for avoiding health illnesses.

Chapter 4: The effect of steam and dehydration on the nutritional quality and functional properties of protein isolates produced from *Lablab purpureus* (L.) Sweet (hyacinth bean)

Abstract

The study conducted was focused on investigating the effect of steaming and dehydration on the nutritional quality and functional properties of protein isolates produced from hyacinth bean. This was for obtaining value-added legume derived protein ingredients for potential food applications. Protein isolates were prepared by isoelectric precipitation from legume grains subjected to subsequent soaking, steaming and dehydration respectively. Samples produced displayed traits of high-quality proteins. Leucine (10.10-10.34 g/100 g) and glutamic acid (16.35-16.70 g/100 g) were the major essential and non-essential amino acids found amongst samples. The major protein polypeptides present amongst samples were vicilin (7S) and legumin (11S). Steaming was identified as useful for producing protein ingredients for nutritional intervention with potential health benefits. Samples recorded improved Fischer's ratio (1.57-1.78), BCAA to tyrosine ratio (3.51-4.28), predicted biological values (44.84-54.70), foaming and emulsion properties. Dehydration extensively preserved the nutritional qualities and functionality of steamed samples. Findings have the potential to diversify the use of hyacinth bean and help improve the competitiveness of the legume grain sector.

4.1. Introduction

Food proteins are among the vital macro nutrients and are recognized for their importance in the human diet for energy, normal body growth, and function. By 2050 the global population is expected to grow by over a third or 2.30 billion people. It is forecasted that almost all growth is expected to take place in developing countries. Thus, the Food and Agricultural Organization (FAO) has forecasted worldwide protein shortages by 2050 (FAO., 2017). In Africa and the Indian sub-continent, the prevalence of protein malnutrition is severely affecting people due to socio-economic reasons. Since the beginning of the 21st century, plant proteins have been identified as potential substitutes to animal and dairy proteins. This is mainly due to their availability, comparable nutritional and functional properties. Legume grains are 2-3 times higher in protein compared to cereal grains, ranging from 17-30% in chickpeas, lentils, dry peas, beans and 35-49.6% in soybeans (Sharif et al., 2018, Boye et al., 2010a).

Hyacinth bean is an indigenous leguminous crop to Africa that grows well in semiarid and arid soils. In Southern Africa it is used by farmers as a forage plant and has received minimal attention with respect to broadening the food base. The legume grains are sources of functional protein due to the good balance of essential to non-essential amino acids, and their bioavailability (Maass et al., 2010). The use of traditional processing treatments (i.e. steaming, microwaving, boiling, roasting, and pressure cooking) have been found to increase the consumption of legumes (Fabbri and Crosby, 2016, Mondor et al., 2009, Pelgrom et al., 2013).

Dehydration is an effective method for preserving food by imposing a difference in water activity between food and environment by removing relatively large amounts of water. Dehydrated foods are known for presenting taste and similar characteristics to traditional foods allowing for accurate amounts of energy and nutrients to be known in prepared dishes. Legumes have been regarded suitable for dehydration as they are sources of dietary protein that complement cereals, roots and tubers. Based on technology, dehydration of beans is segmented into spray dried, drum dried, vacuum dried, and freeze dried. Based on reports, spray dried currently has the maximum market share followed by freeze-dried method (Kerr, 2013, Market Reports, 2019).

Soaking of grains prior to thermal processing has been said to reduce cooking time and initiate protein denaturation. The desirable qualities of legumes can be achieved through the denaturation of major storage protein fractions (i.e. globulin). In globular proteins, once a protein molecule begins to unfold the entire molecule completely unfolds with a slight increase in temperature. Heat is the commonly used denaturing agent in food processing and is recognized for improving the palatability and nutritional quality of proteins as well as influencing functionality (Damodaran et al., 2008). The parameters that determine the nutritional quality of food proteins are largely dependent on the content of limiting and essential amino acids relative to nutritional requirements. The nutritional adequacy of limiting amino acids has been questioned due to its limited availability for synthesis that may result in impaired growth and diseases. Therefore, this work presents simple application on how diverse processing strategies maybe useful for obtaining legume-derived protein ingredients with improved nutritional quality and functional properties for food application.

4.2. Materials and methods

4.2.1. Materials

Hyacinth bean legume grains were obtained from Reservoir Hills, Umgeni River Valley (Kwa-Zulu Natal Province), South Africa. The herbarium voucher containing specimens will be deposited in the Ward Herbarium (UDW), University of KwaZulu-Natal, Westville Campus, Durban. All chemicals and reagents used in the study were of analytical grade.

4.2.2. Processing conditions

Processing was conducted using methods described by Shimelis and Rakshit (2007) and Martín-Cabrejas et al. (2009) with minor modifications. Legume grains (300 g) were soaked (1:3 m/v for 12 h) at room temperature in distilled water (pH 7) and dehulled. A fraction of soaked grains was subjected to steaming using an autoclave (10.547 kg/m2 pressure, 121°C for 30 minutes). Pre-treated grains were dried in an oven set at 55°C for 12 h. Thereafter, a fraction of soaked-autoclaved grains was loaded onto shelves and dehydrated using a counter-current tunnel dryer (60 – 80°C for 6 h). The respective grain portions were milled into flour and screened through a sieve (180 µm). Flour fractions were named as follows: S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated).

4.2.3. Protein isolates preparation

The respective protein isolates were produced by isoelectric precipitation using methods described by Rui et al. (2011) with simple modifications. The respective flours produced were defatted using *n*-hexane (1:3 m/v) for 90 minutes at room temperature. Defatted flours were left under the laminar flow overnight for the removal of excess *n*-hexane. The flours were dispersed in distilled water (pH 7, 1:20 m/v) and shaken for 1 h at 55°C. The resultant slurry was centrifuged at 11 000 × *g* at 20°C for 30 minutes (Eppendorf 5810R). The precipitates were discarded, and the supernatants collected. The supernantants were stored at 4°C overnight to allow for the precipitation of starch. Thereafter, samples were centrifuged using the mentioned conditions and the supernatants obtained were subjected to acid precipitation (pH 4.5) using 1 M HCl solution. The recovered precipitates were then washed, resolubilized in distilled water (pH 7) and freeze-dried.

4.2.4. Determination of protein content and yield

The protein content (N × 5.71) of samples was determined following methods by (AOAC, 1990). Protein yield (%) was estimated as the protein content of isolates relative to defatted flour:

$$\text{Protein Yield (\%)} = \frac{\text{Protein content isolate (\%)} - \text{Protein content flour (\%)}}{\text{Protein content isolate (\%)}} \times 100$$

4.2.5. Amino acid analysis

Amino acids were analyzed using methods described by Alaiz et al. (1992). Briefly, protein isolate samples were incubated at 110°C for 24 h before hydrolysis using 6 M HCl. Thereafter, amino acids were analyzed using high-performance liquid chromatography (HPLC) that was equipped with a 300 × 3.90 mm i.d. reversed-phase (RP) column (Novapack C_{18}) maintained at 18°C following derivatization with diethyl ethoxymethylenemanolate. Tryptophan content was determined by HPLC-RP chromatography after hydrolysis under alkaline conditions (Yust et al., 2004). The contents of cysteine, and methionine were determined after oxidizing with performic acid (Gehrke et al., 1985).

4.2.6. Nutritional quality evaluation

The nutritional quality of hyacinth protein isolates were evaluated by FAO/WHO/UNU (1985) guidelines as performed by Vioque et al. (2012):

- *Amino acids score (chemical score)*:

$$= \frac{\text{amino acid in test sample}}{\text{amino acid recommended by FAO (1985)}} \times 100$$

- *Protein efficiency ratios (PER)*:

$$\text{PER}_1 = -0.684 + 0.456 \times \text{Leu} - 0.047 \times \text{Pro}$$
$$\text{PER}_2 = -0.468 + 0.454 \times \text{Leu} - 0.105 \times \text{Tyr}$$
$$\text{PER}_3 = -1.816 + 0.435 \times \text{Met} - 0.780 \times \text{Leu} + 0.211 \times \text{His} - 0.944 \times \text{Tyr}$$

- *Predicted biological value (BV)*:

$$\text{BV} = 10^{2.15} \times \text{Lys}^{0.51} \times (\text{Phe} + \text{Tyr})^{0.60} \times (\text{Met} + \text{Cys})^{0.77} \times \text{Thr}^{0.24} \times \text{Trp}^{0.21}$$

whereby each amino acid symbol represents:

% amino acid/% amino acid FAO/WHO/UNU (1985), if % amino acid ≤ % amino acid FAO pattern, or:
% amino acid FAO/WHO/UNU (1985)/% amino acid, if % amino acid% ≥ amino acid FAO pattern.

4.2.7. Protein electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under non-reducing (absence of β-mercaptoethanol [β-ME]) and reducing conditions (presence of β-ME). Samples were denatured in sample buffer (0.60 M Tris-HCl pH 6.8, SDS, Sucrose, Bromophenol blue 0.50%) by heating to 95°C for 5 minutes. Samples (15 µg/µL) were loaded onto 12% gradient gel polyacrylamide under a constant voltage (200 V). After staining with Coomassie brilliant blue R250 for 45 minutes, gradient gels were destained with 10% methanol containing 7% acetic acid until prominent and weaker bands became apparent (Laemmli, 1970).

4.2.8. Functional properties

4.2.8.1. Foaming properties

Foaming properties were determined using methods by Adebiyi and Aluko (2011) with simple adjustments. Protein suspensions (500 mg in 5 mL of 0.10 M phosphate buffer pH 7) were homogenized for one minute. Volumes of suspensions were noted before and after homogenization to calculate foam capacity (FC):

$$\text{FC}\ (\%) = \frac{\text{Volume after homogenization (mL)} - \text{Volume before homogenization (mL)}}{\text{Volume after homogenization}}$$

After 1 h of incubation time at room temperature the final volume of the foam was recorded to calculate foaming stability (FS):

$$\text{FS}\ (\%) = \frac{\text{Final volume after incubation (mL)}}{\text{Initial volume after homogenization (mL)}}$$

4.2.8.2. Emulsion properties

Protein isolate (500 mg) was dispersed into 5 mL of 0.10 M phosphate buffer pH 7 and homogenized with 5 mL of sunflower oil (Arise et al., 2017). Emulsions formed were centrifuged at 1100 × *g* for five minutes. The heights of the emulsified layer and contents were recorded to calculate emulsion capacity (EC):

$$\text{EC}\ (\%) = \frac{\text{Height of the emulsified layer (mL)}}{\text{Total height of contents (mL)}}$$

Following incubation (80°C for 30 minutes) and centrifugation using the mentioned conditions, the emulsion stability (ES) was calculated:

$$\text{ES}\ (\%) = \frac{\text{Height of emulsified layer after heating (mL)}}{\text{Height of emulsified layer before heating (mL)}}$$

4.2.8.3. Water and oil holding capacities

Protein isolates (1 g) were dissolved in 5 mL of distilled water/sunflower oil in a 15 mL pre-weighed centrifuge tube. The respective suspensions were vortexed and incubated at room temperature. Following centrifugation at 4000 × *g* for 30 minutes, the supernatants were drained for 15 minutes and the final mass of the tube was recorded to determine water/oil holding capacity (%) of the remaining residue (Mundi and Aluko, 2012).

4.2.8.4. Protein solubility

Protein isolates (100 mg) were dispersed into distilled water and adjusted (pH 3-9) with 1 M HCl/NaOH to final volume of 10 mL respectively (Kudre et al., 2013). Following incubation for 1 h at room temperature, the suspensions were centrifuged at 8000 × *g* for 15 minutes. The protein concentration (%) for the supernatants at the respective pH values was determined to calculate protein solubility (%):

$$\text{Protein Solubility (\%)} = \frac{\text{Protein concentration (\%) of supernatant}}{\text{Protein concentration (\%) of sample}}$$

4.2.9. Statistical analysis

Data reported were subjected to analysis of variance (ANOVA) for determination of significant differences ($p<0.05$) using IBM SPSS software (IBM Corporation, New York, USA). Principal component analysis was used to visualize differences in amino acid profiles for the respective samples.

4.3. Results and discussion

4.3.1. Protein content and yield

The protein content for isolates ranged between 80.60-86.25% (Table 17). The purity of protein isolates has been found largely dependent on the nature of plant material and extraction procedure (Malomo and Aluko, 2015).

Table 17: Protein content and yield for hyacinth bean protein isolate samples

Sample	Flour (%)	Isolate (g/100 g)	Protein yield (%)
S	29.47±0.76^{a}	80.60±0.30^{a}	63.44±0.25^{a}
S+A	27.35±0.24^{b}	82.37±0.22^{b}	66.80±0.43^{b}
S+A+D	27.84±0.10^{b}	86.25±0.29^{c}	67.72±0.71^{b}

Data denotes mean±standard deviation (n=3). Values with different superscript letters are significantly different ($p<0.05$). [S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated)].

This means that the preparation method used in the study was suitable for removing other components (i.e. lipids, starch) during the extraction process. The protein yield (63.44-67.72%) of samples was found comparable to those reported for beach pea (67.90-77.30%), and pigeon pea (49.70-63.60%). Factors that may have contributed to variations in protein yield could be due to differences in protein fraction distribution and solubility behavior in extraction solvents (Chavan et al., 2001).

4.3.2. Nutritional quality evaluation

Table 18 shows the amino acid analysis of hyacinth bean protein isolates produced from legume grains subjected to different processing treatments. Total variation is further explained by principal component analysis (Figure 22). The various amino acids studied were found distant from the origin of the biplot suggesting variation amongst samples. The first and second principal components extracted accounted for 62.990% and 37.010% of total variation respectively.

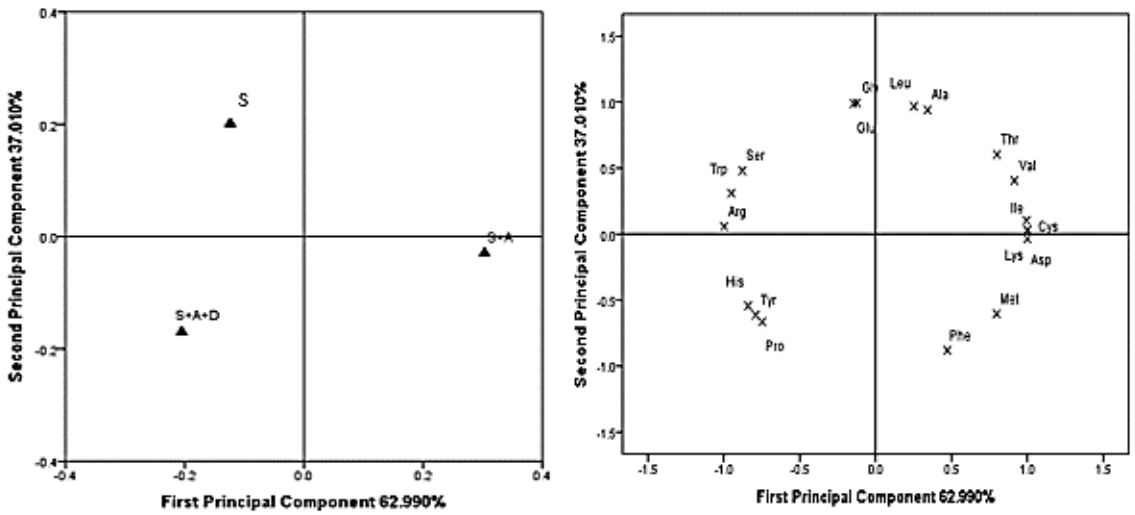

Figure 22: Principal component analysis of hyacinth bean protein isolates showing score (left) and loading (right) plots of amino acids composition: Soak (S), Soak+Autoclave (S+A), and Soak+Autoclave+Dehydrated (S+A+D).

Leucine (10.10-10.34 g/100 g) and glutamic acid (16.35-16.70 g/100 g), which may include glutamine were the major essential and non-essential amino acids found amongst samples respectively. All protein isolates contained significant amounts of aromatic (10.87-12.01 g/100 g) and branched chain amino acids (18.53-19.34 g/100 g). However, samples produced by steaming (S+A) resulted in a higher Fischer's ratio (1.57-1.78) and BCAA to tyrosine ratio (BTR) (3.51-4.28). It has been reported that the severity of hepatic damage increases with decreasing Fischer's ratio and BTR. Thus, administering BCAA booster to these patients may be useful (Ishikawa, 2012a, Ishikawa, 2012b).

Table 18: Amino acid composition (g/100 g) of hyacinth bean protein isolates produced from legume grains subjected to different processing treatments.

Amino Acid (AA)	S	S+A	S+A+D	FAO recommendation[b]
Essential AA				
Histidine	2.60±0.00	2.09±0.00	2.44±0.00	1.9
Tryptophan	2.01±0.00	1.86±0.01	1.83±0.00	1.1
Threonine	3.35±0.00	3.95±0.00	3.50±0.01	1.4
Lysine	3.07±0.00	3.89±0.00	3.73±0.00	-
Methionine	0.78±0.00	0.87±0.00	0.95±0.00	-
Valine	4.69±0.01	4.80±0.01	4.74±0.01	3.5
Isoleucine	3.64±0.00	4.20±0.00	4.05±0.00	2.8
Leucine	10.20±0.00	10.34±0.01	10.10±0.00	6.6
Phenylalanine	6.26±0.00	6.35±0.00	6.91±0.00	-
Subtotal Essential AA:	36.60	38.35	38.25	-
Nonessential AA				
Serine	5.80±0.00	5.24±0.00	4.95±0.00	-
Cysteine	1.18±0.00	1.33±0.00	1.31±0.00	-
Arginine	9.20±0.00	8.75±0.00	8.80±0.00	-
Glycine	5.77±0.00	5.82±0.00	5.62±0.00	-
Aspartic acid	9.95±0.01	10.20±0.02	10.15±0.00	-
Glutamic acid	16.62±0.00	16.70±0.00	16.35±0.00	-
Alanine	3.68±0.00	3.74±0.00	3.65±0.00	-
Proline	5.90±0.00	5.32±0.00	5.80±0.00	-
Tyrosine	5.28±0.00	4.52±0.00	5.10±0.00	-
Subtotal Nonessential AA:	63.38	61.62	61.73	-
Phenylalanine+Tyrosine (AAA)	11.54	10.87	12.01	6.3
Methionine+Cysteine	1.96	2.20	2.26	2.5
Isoleucine+Leucine+Valine (BCAA)	18.53	19.34	18.89	-
Fischer's ratio (BCAA/AAA)	1.61	1.78	1.57	-
BCAA to Tyrosine ratio (BCAA/Tyrosine)	3.51	4.28	3.70	-
Arginine+Lysine	12.27	12.64	12.53	-
Basic	14.87	14.73	14.97	-
Acidic	26.57	26.90	26.50	-
Hydrophobic	27.98	28.90	28.16	-
Hydrophilic	9.15	9.19	8.45	-

Data denotes mean±standard deviation (n=2). [S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated)], AAA (Aromatic amino acid), BCAA (Branched chain amino acid), [b]FAO (1985)

Furthermore, proteins with a high Fischer ratio containing high concentrations of branched-chain amino acids and low level of aromatic amino acids are health beneficial (Oomah, 2001, Maheri-Sis et al., 2008).

Samples produced by steaming (S+A) contained significant amounts of Arginine+Lysine (12.27-12.64 g/100 g). The respective amino acids have been associated with beneficial hypocholesterolemic effects in improving cardiovascular health, and help in hypertension regulation (Vallabha et al., 2016, Malomo et al., 2014). Several nutritional parameters were determined using amino acid composition data for samples and FAO/WHO/UNU guidelines (Table 19). Amino acid scores (AAS) are used to predict dietary protein quality of food samples. The scores determined lysine and sulfur containing amino acids (Methionine+Cysteine) as the first (52.93-67.07) and second (78.40-90.40) limiting amino acids amongst samples respectively.

Table 19: Evaluation of nutritional parameters for hyacinth bean protein isolate

Parameters	S	S+A	S+A+D	FAO recommendation[b]
Amino acid scores (AAS)				
Histidine	136.67	110.16	128.42	1.9
Tryptophan	182.73	169.09	166.36	1.1
Threonine	239.29	282.14	250.00	1.4
Lysine	52.93	67.07	64.31	5.8
Methionine+Cysteine	78.40	88.00	90.40	2.5
Valine	134.00	137.14	135.43	3.5
Isoleucine	130.00	150.00	144.64	2.8
Leucine	154.55	156.67	153.03	6.6
Phenylalanine+Tyrosine	183.17	172.54	190.63	6.3
First limiting amino acid	Lysine	Lysine	Lysine	-
Second limiting amino acid	Methionine+Cysteine	Methionine+Cysteine	Methionine+Cysteine	-
Predicted biological value (BV)	44.84	54.70	53.41	-
Protein efficiency ratio $(PER)_1$	3.69	3.78	3.65	-
Protein efficiency ratio $(PER)_2$	3.61	3.75	3.58	-
Protein efficiency ratio $(PER)_3$	2.04	2.80	2.18	-

[S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated), [b]FAO (1985)

Samples produced by steaming (S+A) and dehydration (S+A+D) contained higher contents for lysine and sulfur containing amino acids compared to soaking (S) but were still inadequate to fulfill FAO requirements. Siwela and Amonsou (2016) reported similar lysine contents (3.50 g/100 g) for *Bauhinia* grain proteins. Therefore, consumption of legume derived protein ingredients produced by steaming and/or dehydration with cereal or soybean grains may potentially contribute to a well-balanced essential amino acid profile rich in lysine and sulfur-containing amino acids. These results are in accordance to previous work conducted on mung bean grains (Mubarak, 2005). Protein efficiency ratios (1, 2, 3) greater than 2 were recorded amongst samples. This was considered typical of high-quality proteins, whereby low-quality proteins have PER values below 1.50 (Friedman, 1996).

The PER values recorded were predominantly higher than those of conophor nut (2.39-2.60), lupin (2.22–2.34) and *Vicia faba* (2.60-2.90) protein isolates (Iyenagbe et al., 2017, El-Adawy et al., 2001, Vioque et al., 2012). Predicted biological values (BV) are a measure of the amount for absorbed protein from a food source. Samples produced by steaming (S+A) and dehydration (S+A+D) resulted in a significant increase to predicted biological values (44.84-54.70).

This indicates improved protein digestibility potential which could be attributed to reduction in anti-nutrients. Leguminous grains contain significant concentrations of trypsin inhibitors, phytic acid, and oligosaccharides which are known to diminish the bioavailability of trace elements and proteins. Shaahu (2014) confirmed that boiling grains in water significantly reduced the concentrations of tannins, alkaloids, oxalates, and trypsin inhibitors in Highworth variety of Lablab bean. The predicted biological values were comparable with those reported for soybean curd (Pedroche et al., 2004).

4.3.3. Electrophoresis

The predominant polypeptides identified amongst samples (lanes 1-3) under non-reducing and reducing conditions had an estimated molecular mass of 55 kDa (Figure 23).

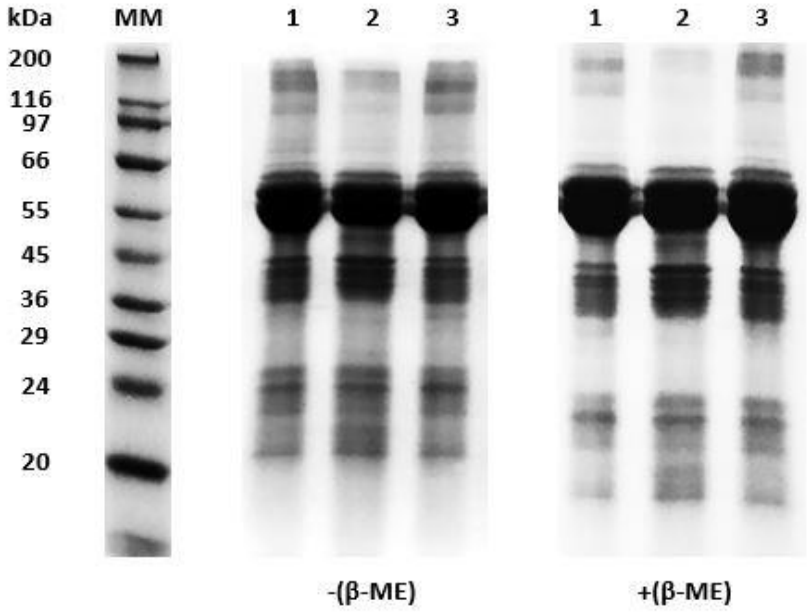

Figure 23: SDS-PAGE profiles under non-reducing (-β-ME) and reducing (+β-ME) conditions: 1) Soak (S), 2) Soak+Autoclave (S+A), 3) Soak+Autoclave+Dehydrated (S+A+D), Molecular Marker (MM).

This may correspond to vicilin (7S) sub-units that are largely present in legume proteins. Romero et al. (1975) reported vicilin (7S) has an oligomeric protein consisting of three polypeptide sub-units α-, β-, and γ- with molecular masses ranging between 43-53 kDa. The smaller polypeptides observed between 20-45 kDa were considered acidic and basic sub units of legumin (11S). The electrophoresis patterns observed is in accordance with work conducted on *Phaseolus* and field pea protein isolates (Tang, 2008, Shevkani et al., 2015b). Under reducing conditions (presence of β-mercaptoethanol) a few faint protein bands were observed amongst samples (lanes 1-3), thus suggesting the presence of disulfide bonds. Aryee and Boye (2017) has reported similar protein patterns under the mentioned conditions for lentil protein isolates.

4.3.4. Functional properties

4.3.4.1. Foaming properties

The foaming properties of proteins have been found useful in food products (e.g. baked foods, ice-cream mixes, and whipped toppings) that require aeration and overrun. Protein sources with good foaming properties are characterized by the ability of surfactant molecules to rapidly adsorb at the air-water interface and rearrange to form a cohesive viscoelastic film through intermolecular interactions (Wani et al., 2015). Foaming capacity (101.56-115.80%) and stability (55.45-70.43%) amongst protein isolate samples was found significantly different ($p<0.05$) (Table 20).

Table 20: Functional properties of hyacinth bean protein isolates produced from processed legume grains

Sample	Foam capacity (%)	Foam stability (%)	Emulsion capacity (%)	Emulsion stability (%)	Water holding capacity (g/g)	Oil holding capacity (g/g)
S	101.56±5.03^{a}	55.45±2.19^{a}	43.71±4.03^{a}	64.36±4.92^{a}	2.77±0.54^{a}	3.40±0.38^{a}
S+A	115.80±5.29^{d}	68.97±2.52^{d}	66.90±2.76^{d}	80.15±3.03^{d}	2.26±0.32^{a}	4.33±0.47^{b}
S+A+D	105.43±2.65^{e}	70.43±0.34^{b}	64.67±1.16^{e}	76.25±4.59^{e}	2.40±0.30^{a}	3.76±0.65^{a}

Data denotes mean±standard deviation (n=3). Values with different superscript letters are significantly different ($p<0.05$). [S (Soak), S+A (Soak+Autoclave), S+A+D (Soak+Autoclave+Dehydrated)].

Samples produced from legume grains subjected to steaming (S+A) were found to extensively improve foaming properties. The net charge of amino acid residues and degree of solubility have been found to significantly affect foaming properties of proteins (Lawal et al., 2005).

Amino acid composition data confirmed samples produced by steaming (26.90, 14.73 g/100 g respectively) contained a high ratio of acidic (including Aspartic acid, Glutamic acid) to basic (including Lysine, Arginine, Histidine) amino acids followed by soaking (26.57, 14.87 g/100 g respectively) and dehydration (26.50, 14.97 g/100 g respectively). The charged amino acid residues are mostly located on the surface of the protein molecule. Tang et al. (2009) associated the relative ratio of acidic and basic amino acids to the net charge on the surface of proteins.

This may indicate that the net charge of isolates produced by steaming (S+A) was highest, followed by soaking (S) and dehydration (S+A+D). Therefore, during homogenization the high charge on proteins may have weakened hydrophobic interactions increasing protein solubility and flexibility. This facilitates protein molecules to spread more quickly on the air-water interface, encapsulating air particles and increasing foamability. This observation is in accordance to a study conducted on the functional characterization of kidney bean and field pea protein isolates (Shevkani et al., 2015b).

4.3.4.2. Emulsion properties

The nature of proteins (i.e. composition of polar, non-polar, charged and non-charged amino acid residues) that possess hydrophilic and hydrophobic properties, thus interacting with both water and oil components could be used as emulsifiers in food systems. Oil-in-water emulsion formation is based on the adsorption of proteins at the surface of oil droplets in the form of a densely packed layer and are largely dependent on protein solubility and hydrophobicity (Ulloa et al., 2011). The emulsion capacity (43.71-66.90%) and stability (64.36-80.15%) was found significantly different ($p<0.05$). Samples produced by steaming (S+A) and dehydration (S+A+D) treatments resulted in higher emulsion capacities (Table 20). The high emulsion capacities observed for samples maybe attributed to high contents of hydrophobic amino acid residues, thus facilitating rapid initial adsorption at the oil-water interface. The stabilization of the resultant emulsions formed could be due to the presence of strong protein–protein interactions at the oil–water interface preventing oil droplet coalescence (Dickinson, 2010).

4.3.4.3. Water and oil holding capacities

Water and oil holding capacities of protein isolates may contribute to the texture, mouthfeel and flavor retention of products. This makes it suitable for ingredient application in products where hydration and shortening are desired such as in baked products, soups, and meat extenders (Adebowale et al., 2011). Processing treatments (soaking, steaming and dehydration) had no significant influence on water holding capacities (2.26-2.77 g/g).

However, isolates produced by steaming (S+A) resulted in a higher oil holding capacity (3.40-4.33 g/g) and could be attributed to the exposure of more hydrophobic amino acid residues (Table 20). The water and oil holding capacities were found comparable to field pea (4.20 and 6.40 g/g, respectively) and kidney bean (3.00 and 5.90 g/g, respectively) protein isolates (Shevkani et al., 2015b, Tang et al., 2006).

4.3.4.4. Protein solubility

The solubility profile for protein isolates can be considered as a guide to their functionality as it relates to many functional properties. The protein solubility for samples was investigated between pH 3-9 and resembled a V-shaped curve. Protein solubility patterns observed were typical of plant storage proteins, whereby minimum solubility occurred near the isoelectric point (pH 4-5) and extensively increased at both acidic and alkaline pH values (Figure 24). Subagio (2006) confirmed the low solubility of hyacinth bean protein isolate in the pH range 3–5.5.

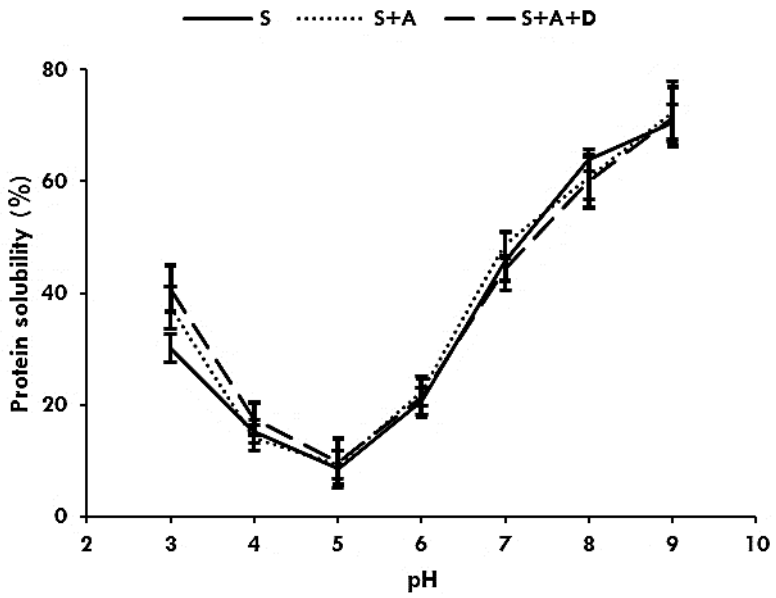

Figure 24: Protein solubility profiles for protein isolates produced from processed hyacinth bean legume grains: Soak (S), Soak+Autoclave (S+A), and Soak+Autoclave+Dehydrated (S+A+D). Data reported represents mean and standard deviations (n=2).

This is primarily due to to the lack of electrostatic repulsion that promotes aggregation and precipitation through hydrophobic interactions. The large ratio of acidic (Aspartic acid and Glutamic acid) to basic (Lysine, Arginine, and Histidine) amino acid residues amongst samples may have contributed to minimum solubility at pH 4–5 (isoelectric pH) and maximum solubility at alkaline pH. It is said that proteins are electrically neutral at their isoelectric points.

Thus, differences in solubility around isoelectric pH could be due to the presence of positively and negatively charged amino acid residues on the surface. This could contribute to the hydrophilicity of proteins. Therefore, if the hydration repulsion forces were greater than hydrophobic attractions then proteins may have varied in solubility around isoelectric pH (Damodaran et al., 2008).

The differences in solubility patterns at high acidic and basic pH values could be attributed to differences in net charges of amino acid residues. In a study conducted on kidney bean and field pea protein isolates it was observed that the solubility of proteins was closely dependent upon the charge on proteins and might be related to protein structure such as α-helix, β-sheet and β-A (Shevkani et al., 2015b). Karaca et al. (2011) reported a positive correlation between protein solubility and surface charge for isolates prepared from faba bean, lentil and pea proteins.

4.4. Conclusion

Hyacinth bean protein isolates produced displayed traits of high-quality proteins. Samples produced from legume grains subjected to steaming have been recognized as protein ingredients with potential health benefits and useful for nutritional intervention. This was due to an extensive improvement in nutritional and functional parameters investigated. The protein ingredients obtained could offer positive hypocholesterolemic effects for improving cardiovascular health. Dehydration caused minimal change to nutritional qualities and functionality demonstrated by steaming, thus extensively contributed to preservation. The underutilization of many indigenous crops is mainly attributed to both limited research and marketing. Thus, findings could markedly influence the value of hyacinth bean by diversifying its use through possible applications in foods that require protein enrichment such as in cereal based foods and in the production of gluten-free products. Furthermore, results may aid broadening segmentation of dehydration technology in existing markets for obtaining ingredients and products from legume grains with maximum quality.

Chapter 5: The emulsifying properties of Ca^{2+}-induced *Lablab purpureus* (L.) Sweet (hyacinth bean) protein nanoparticles

Abstract

Nanoparticles can be used as emulsifiers for the stabilization of Pickering emulsions. The development of food-grade Pickering stabilizers is fast attracting interest as these are recognized as safe compared to inorganic materials. Hyacinth bean is an indigenous under-utilized leguminous crop in Africa. The amphiphilic nature of legume proteins warrants its utilization as emulsion stabilizers. Hyacinth bean protein nanoparticles were produced using Ca^{2+}-induced aggregation (0.00-6.50 mM) and were characterized based on size (dynamic light scattering) and surface property (zeta-potential). The viscosity profiles of protein solutions displayed shear thinning behavior which was considered characteristic of globular proteins. Protein aggregation was found dependent on Ca^{2+} concentration, therefore the z-average diameter of protein nanoparticles increased at higher Ca^{2+} concentrations. Nanoparticles were relatively stable against 120 mM DTT at different pH and protein concentrations. The surface charge for nanoparticles became increasingly negative at higher pH. Oil-in-water emulsions (10%) stabilized by protein nanoparticles were subjected to accelerated storage (1 week/ 55 °C) and characterized based on microstructure, droplet size (static light scattering) and stability. Emulsions were stable during storage with minimal changes to mean droplet size observed. Findings would be significant for the development of hyacinth bean protein nanoparticles as potential food-grade Pickering stabilizers.

5.1. Introduction

Plant proteins have attracted interest as potential substitutes to animal and dairy proteins in the formulation of different products. However, apart from soy protein and wheat gluten they remain largely underutilized by the food industry. This is mainly due to insufficient structure–function information relating to their performance. Legumes are important agricultural crops grown all over the world and are 2 to 3 times higher in protein than cereal grains (Karaca et al., 2011, Boye et al., 2010a). *Lablab purpureus* (L.) Sweet (hyacinth bean) is an indigenous leguminous crop to Africa, whereby its legume grains are a source of functional protein. The protein isolate has received considerable attention for its use as an additive for improving cake quality, in addition to its numerous techno-functional properties (i.e. emulsion, foaming) relating to food applications (Maass et al., 2010, Subagio 2006). The two major storage protein fractions present in legumes are albumins (~10-30%) and globulins (~70%).

Globulins have limited water solubility mainly due to its high molecular weight and extra hydrophobic surface (Day, 2013, Sharif et al., 2018). It has been widely reported that good emulsification properties of proteins are warranted by lower molecular size, higher surface hydrophobicity, surface charge, and flexibility (Can Karaca et al., 2015, Liu et al., 2012, Nishinari et al., 2014). Kimura et al. (2008) confirmed the emulsifying properties of protein fractions in globulins isolated from various legumes (fava bean, pea, black and red cowpea).

In recent times, the development of food-protein based particles to perform as Pickering emulsion stabilizers has fast attracted interest. Pickering emulsions refer to dispersions of any type (oil-in-water, water-in-oil, multiple) stabilized by solid particles in place of surfactants. They retain the basic properties of classical emulsions stabilized by surfactants, so that they can be substituted for a classical emulsion in most applications. Pickering emulsions stabilized by nanoparticles offer promising applications for encapsulation and delivery of bioactive compounds, food texture modification and the potential for producing emulsions with flexible interfacial properties. The preferred use of biopolymer-based particles over inorganic sources to function as stabilizers is due to their generally recognized as safe status, amenability for surface modification, biocompatibility, feasibility and degradability (Berton-Carabin and Schoen, 2015, McClements and Gumus, 2016).

The amphiphilic nature of legume proteins is among the driving forces behind its utilization as emulsion stabilizers. This provides them with the potential to simultaneously remain in the aqueous phase and adsorb at the surface of oil droplets (Yang et al., 2013, Chevalier and Bolzinger, 2013). The fabrication methods of protein nanoparticles used to stabilize Pickering emulsions have been found to largely influence their properties. Some of these methods include complexation with biopolymers (e.g. anionic polysaccharides) and the use of enzymatic crosslinking agents (e.g. transglutaminase) (Totosaus et al., 2002). Liu and Tang (2013) reported that heat-induced soy protein nanoparticles could effectively stabilize oil-in-water Pickering emulsions. Protein nanoparticles may also be induced by the addition of divalent ions (Ca^{2+}). This takes place by Ca^{2+} functioning as a salt-bridge to favor aggregation by shielding negatively charged groups on proteins (Zhang et al., 2012).

In principle, for nanoparticles to function as Pickering emulsion stabilizers its particle size should be substantially smaller than the targeted emulsion droplet size. This is for stabilizing emulsion droplets as small as a few micrometers in diameter. Nanoparticles should be partially wetted in the continuous and dispersed phases and should not be soluble in either phase.

This allows for particles to preserve proper partial wettability to gain enough interfacial adsorption efficiency. Furthermore, strong internal integrity of particles in the presence of intra-particle disulfide bonds are required for maintaining structural integrity once adsorbed at the emulsion interface (Xiao et al., 2016, Chevalier and Bolzinger, 2013). Therefore, the purpose of the study was to characterize Ca^{2+}-induced hyacinth bean protein nanoparticles for its potential development as food-grade Pickering emulsion stabilizers. Firstly, the effect of protein aggregation as affected by Ca^{2+} concentration was characterized based on turbidity by sample absorption, viscosity and particle size. Consequently, Ca^{2+}-induced protein nanoparticles at different pH conditions and concentrations were characterized in terms of surface charge and particle stability. The integrity of particles was assessed in the presence of protein reducing agents to dissociate intra-particle interactions. Lastly, the emulsification performance of Ca^{2+}-induced protein nanoparticles were evaluated as stabilizers for the production of oil-in-water emulsions.

5.2. Materials and methods

5.2.1. Materials

Hyacinth bean legume grains were obtained from Reservoir Hills, Umgeni River Valley (Kwa-Zulu Natal Province), South Africa. The herbarium voucher containing specimens will be deposited in the Ward Herbarium (UDW), University of KwaZulu-Natal, Westville Campus, Durban. All chemicals and reagents used in the study were of analytical grade. Water was obtained from a Milli-Q water purification system (Advantage A10 Water Purification System).

5.2.2. Preparation of hyacinth bean protein isolate

Protein isolate was produced from defatted hyacinth bean flour using isoelectric precipitation as described by Rui et al. (2011). Briefly, legume grains were soaked (1:3 m/v) in Milli-Q water, dehulled and dried to constant weight in an oven (55°C). Legume grains were milled into flour using an Alpine Fine impact mill 100 UPZ II (Hosokawa Alpine AG, Augsburg, Germany) at maximum speed: 15000/min. Thereafter, flour produced was screened through a sieve (180 µm) and subjected to defatting at room temperature using *n*-hexane (1:3 m/v) for 90 minutes. Following defatting, the flour was dispersed in Milli-Q water (pH 7, 1:20 m/v) and incubated for 1 h at 55°C. The resultant slurries were centrifuged (Thermo Scientific™ Sorvall™ LYNX 6000 superspeed) at 11 000 × *g* at 20°C for 30 minutes and the precipitates discarded. The collected supernatants were stored at 4°C overnight to allow for precipitation of starch and other minor constituents.

Thereafter, the supernatants were subjected to acid precipitation (pH 4.50) using 1 M HCl solution. The recovered precipitates were then washed, resolubilized in Milli-Q water (pH 7) and freeze-dried.

5.2.3. Preparation of protein nanoparticles

Protein nanoparticles were produced by Ca^{2+}-induced aggregation (Liu et al., 2017). Firstly, hyacinth bean protein stock solution (2% m/v) was prepared and adjusted to pH 7 using 1 M NaOH/ HCl. Sodium azide (0.02% m/v) was added to prevent microbial growth. The solution was left under stirring conditions overnight at 4°C for the complete hydration of proteins. Thereafter, under stirring conditions calcium chloride was gradually added to hyacinth bean protein dispersions at different concentrations (0.00-6.50 mM). The protein dispersions pH 7 were maintained using 1 M NaOH and stored overnight (25°C/ 185 rpm). Following incubation, the absorption of suspensions was measured using a UV-Vis spectrophotometer (Analytik-Jena, Specord 200) at 600 nm. Hyacinth bean protein nanoparticles were successfully produced when absorption values increased by at least 50% with no distinct precipitation.

5.2.4. Characterization of hyacinth bean protein nanoparticles

5.2.4.1. Dynamic light scattering

The particle size distributions of Ca^{2+}-induced hyacinth bean protein nanoparticles were determined by dynamic light scattering (DLS) at 25°C. This was performed using a Zetasizer Nano ZS instrument equipped with a He-Ne laser (λ =633 nm; Malven instruments, U.K.). The intensity weighted hydrodynamic diameter (z-average) for respective samples were calculated based on diffusion coefficients by the Stokes-Einstein equation as described by Liu and Tang (2014) using the viscosity (0.8872 mPa.s) and refractive index (1.33) of water for the dispersant and the refractive index of protein (1.46).

5.2.4.2. Viscosity measurement

The viscosity of hyacinth bean protein nanoparticle dispersions (0.00-6.50 mM $CaCl_2$) were determined using a rheometer (Thermo Scientific™ HAAKE™ MARS™ 60, Modular advanced rheometer system) with a double gap cylinder rotor (CC27 DG/ Ti). Samples were analyzed at 25°C in controlled shear stress mode by increasing the shear stress from 0.00 Pa – 0.40 Pa (linear). The viscosity of samples was then calculated from the resulting shear rate.

5.2.4.3. Relative stability

The relative stability of protein nanoparticle dispersions was determined at different pH conditions (5-9) using dissociation tests performed by Liu et al. (2017) with simple modifications. Briefly, protein nanoparticle dispersions (2-10% m/v) were treated with reducing agents, 4 M Urea and 120 mM DTT. The mixtures were adjusted to their respective pH using 1 M NaOH/ HCl and left to stand at room temperature for 30 minutes. The initial absorbance for sample suspensions was set as 100%. Thereafter, the absorbance of respective mixtures was determined at 600 nm using a UV-Vis spectrophotometer.

Thus, the relative stability of the respective mixtures was expressed as:

$$\text{Relative stability (\%)} = \frac{\mathrm{A_t}}{\mathrm{A_0}} \times 100$$

where A_t and A_0 are absorbance values at 600 nm before (initial) and after treatment with various reagents respectively.

5.2.4.4. Surface charge

The zeta-potential of suspensions containing protein nanoparticles was determined at different pH values (5-9) based on electrophoretic mobility in an electrophoresis cell (Model DTS 1060C, Malvern Instruments Ltd., Malvern, Worcestershire, UK), Zetasizer Nano ZS instrument.

5.2.5. Preparation and characterization of emulsions

5.2.5.1. Emulsion preparation

Hyacinth bean protein nanoparticles (pH 7; containing 0.02%, m/v, sodium azide) were diluted with Milli-Q water to different protein concentrations (0.80-2.00%, m/v). Then, 1 mL of canola oil was mixed with diluted suspensions containing protein nanoparticles to obtain 10% oil-in-water emulsions. Homogenization was performed at ~10000 rpm for 5 minutes using an Ultra Turrax homogenizer (model IKA-ULTRA-TURRAX T25 basic, IKA Works, Inc. Wilmington, NC). The resultant emulsions were subjected to accelerated storage for 7 days at 55°C (Liu and Tang, 2016a, Lerche and Sobisch, 2011).

5.2.5.2. Emulsion microstructure

The emulsion microstructure was visualized using Cryo-SEM (Cryo-scanning electron microscopy). Briefly, samples were frozen by plunging in nitrogen slush (ca. -210°C) and immediately transferred to the cryo-chamber (PP2000 T, Quorum Technologies Ltd., Laughton, United Kingdom) that was pre-cooled to -135°C. Inside the cryo-chamber, samples were fractured with a probe and sublimated at -90°C for 15 minutes.

Thereafter, samples were sputter coated with platinum in an Argon atmosphere (60 s coating at ca. 5-10 mA current) and transferred to the cryo stage in the SEM chamber (T = -135°C). Imaging was carried out with a Quanta 250 FEG field emission scanning electron microscope (FEI, Brno, Czech Republic) under high vacuum (~$3 \cdot 10^{-7}$ mbar) with an Everhart-Thornley detector under a working distance of 5 mm and an accelerating voltage of 10 kV.

5.2.5.3. Creaming Index (CI)

The creaming stability for prepared emulsions were evaluated over a 7-day accelerated storage (55°C) period by methods described by Keowmaneechai and McClements (2002). The creaming index (CI) was calculated based on the emulsions that separated into a top cream layer and a bottom serum layer (H_S) during storage:

$$\text{Creaming Index (\%)} = \frac{H_S}{H_e} \times 100$$

were H_S is the serum layer and H_e the total height of the respective emulsions.

5.2.5.4. Droplet size and distribution

The droplet size distribution and Sauter mean diameter $D_{3.2}$ [µm] of emulsion samples was determined periodically over the 7-day storage period by static light scattering (Mastersizer 2000 with Hydro 2000SM sample dispersion unit, particle refractive index: 1.47).

5.2.6. Statistical analysis

All measurements were performed at least in duplicate. The significant differences ($p<0.05$) of data reported were determined by analysis of variance (ANOVA) using IBM SPSS software (IBM Corporation, New York, USA).

5.3. Results and discussion

5.3.1. Characterization of Ca^{2+}-induced hyacinth bean protein nanoparticles

The absorption of suspensions progressively increased at high Ca^{2+} concentrations. Figures 25A-B presents a visual observation and the photometric measurements of the absorption for hyacinth bean protein isolate dispersions subjected to Ca^{2+}-induced aggregation (0.00-6.50 mM; pH 7).

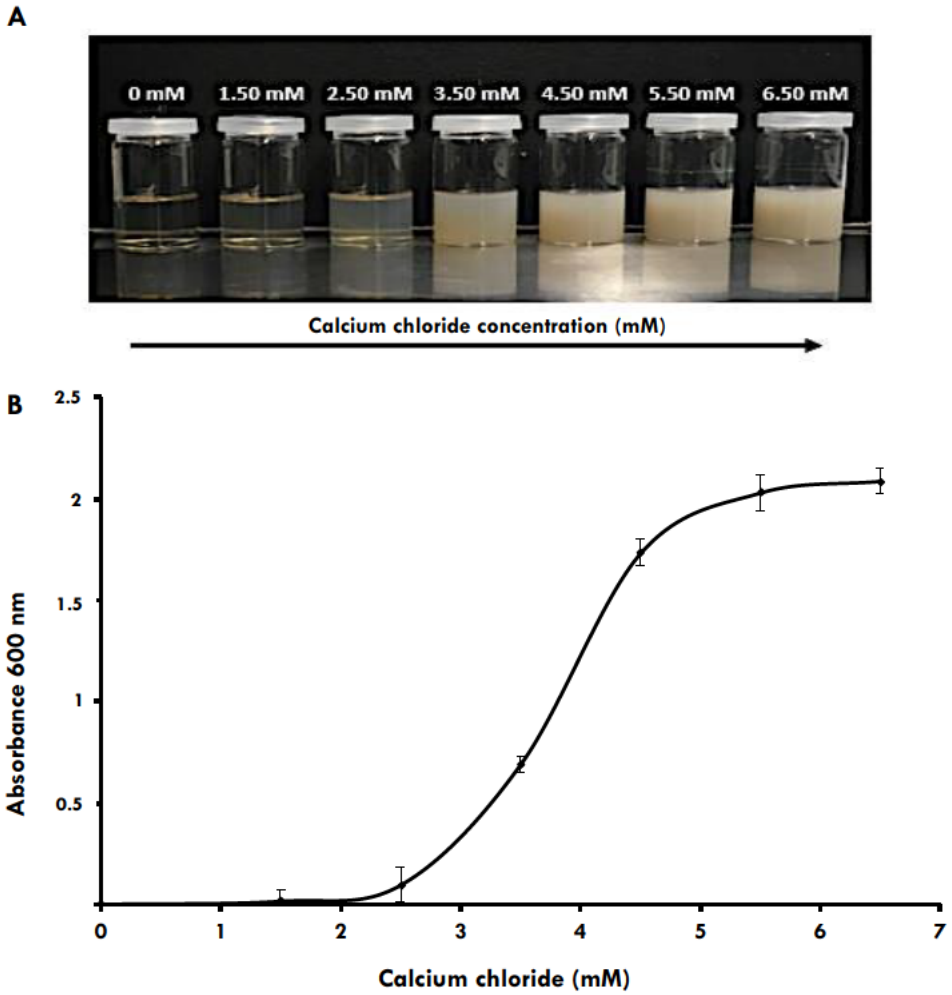

Figure 25: (A) Visual observation of hyacinth bean protein isolate (2% m/v, pH 7) at different Ca^{2+} concentrations (0.00-6.50 mM), (B) changes in absorption for respective samples at 600 nm. Data for absorption represents means and standard deviations (n=3).

There was a strong increase in absorbance values from 2.50 mM Ca^{2+} indicating progressive sample absorption. The absorbance values following 4.50 mM Ca^{2+} were nearly constant indicating that absorption amongst the respective samples are similar. These results indicate that aggregation of hyacinth bean proteins is highly dependent on Ca^{2+} concentration. These results suggest that aggregation of hyacinth bean proteins is dependent on Ca^{2+} concentration. It has been reported that soy protein aggregation is highly dependent on Ca^{2+} concentration, whereby the capacity of soy proteins to bind Ca^{2+} at pH 6.20 can reach 51.70 mg of Ca per g of protein (Cao et al., 2015, Canabady-Rochelle et al., 2009). Zhang et al. (2012) reported a 50% increase in the turbidity of sample suspensions to represent the formation of protein nanoparticles.

The particle size distribution and z-average diameter (Figures 26A-B) of protein dispersions were determined by dynamic light scattering (DLS). The size distributions of dispersions presented a PdI in the range of 0.31-0.52 which indicates the existence of a narrow to moderate polydisperse population.

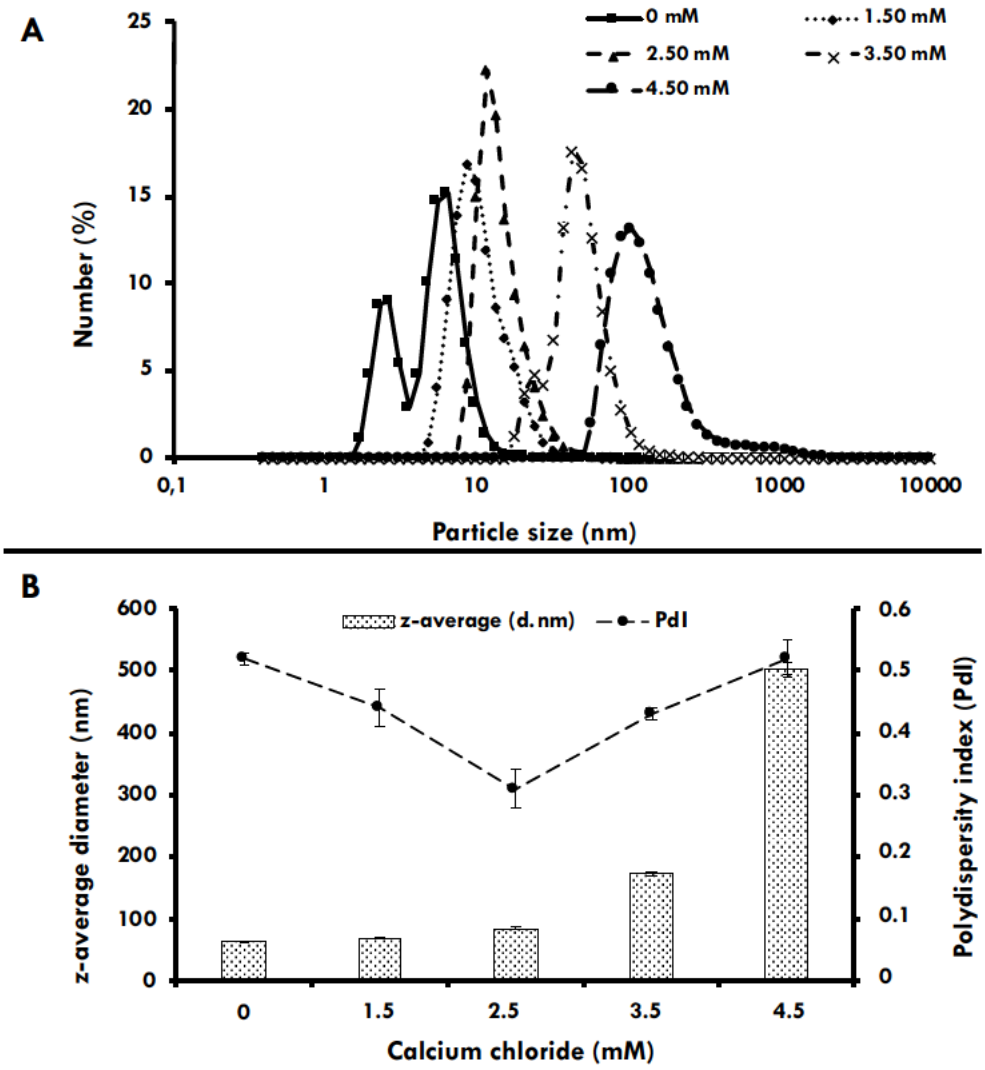

Figure 26: (A) Number based particle size distribution, (B) z-average diameter and polydispersity index (PdI) of hyacinth bean protein isolate (2% m/v, pH 7) at different calcium chloride concentrations (0.00-4.50 mM). Data for z-average and polydispersity indices represents mean and standard deviations (n=2).

Number based particle size distributions were used to detect for the possible presence of several particle populations amongst samples. The z-average diameter for untreated hyacinth bean protein dispersions (2% m/v; 0 mM Ca^{2+}) was determined at 63.44 nm. Furthermore, two distinct peaks were present within the range of 1-10 nm (Figure 26A). This may indicate that most proteins present were in its native state in the respective sample.

Subagio (2006) studied the protein fractions present in hyacinth bean legume grains. It was confirmed that globulins (~55%) are the major storage proteins present in hyacinth bean protein isolate. This is further divided into two fractions 7S Globulin (~20%) and 11S Globulin (~9%). Thus, this could be attributed to naturally occurring protein fractions found in hyacinth bean.

There was a distinct shift in the major size distribution peak observed between 2.50 mM-3.50 mM Ca^{2+}. Consequently, this resulted in a sharp increase in z-average diameter (85.94-172.38 nm). Liu and Tang (2014) observed similar particle sizes for aggregates prepared from soy (50-200 nm) and pea (134-165 nm) protein isolates using heat followed by the addition of NaCl and using acidic pH treatments respectively. When the Ca^{2+} concentration was further increased to 3.50 mM and above this resulted in a dramatic increase to z-average diameter (172.38-502.88 nm) and polydispersity indices (0.43-0.52). This reveals that particle sizes have increased and the width of their distributions. These observations are in accordance to a study conducted by Liu et al. (2017) on Ca^{2+}-induced soy protein aggregates. Furthermore, the aggregation of proteins observed in the present study well support observations for sample absorption. Thus, protein aggregation is dependent on the applied Ca^{2+} concentration.

The viscosity of a solution relates to its resistance to flow under an applied force and its behavior maybe dependent on the solute type (size, shape, flexibility, hydration). Figure 27 presents the viscosity profile for Ca^{2+}-induced protein aggregates at the respective concentrations (0.00-6.50 mM). Protein solutions containing high Ca^{2+} concentration showed higher viscosities. Overall, the samples did not display Newtonian behavior (shear stress not directly proportional to shear rate), thus a mean viscosity could not be reported.

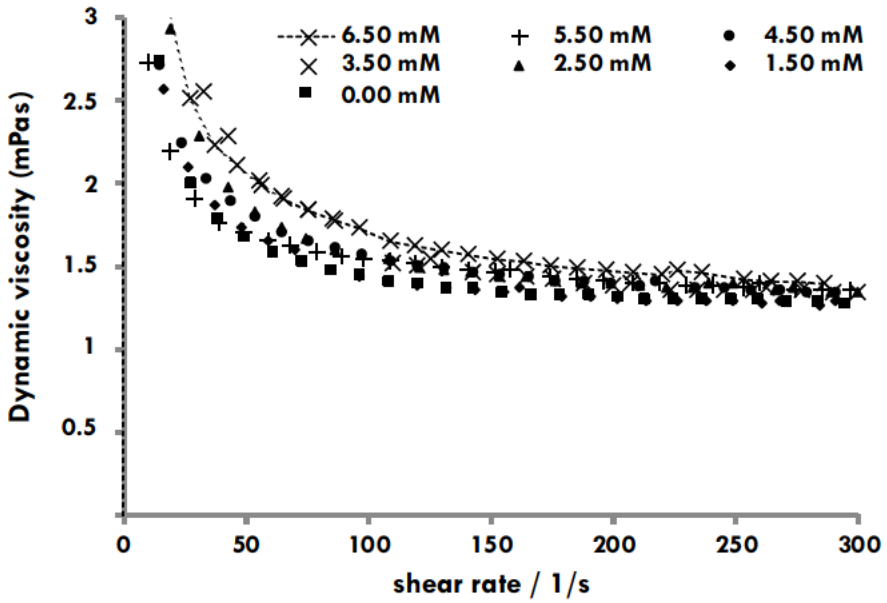

Figure 27: Viscosity profile of Ca^{2+}-induced hyacinth bean protein aggregates (pH 7) at different concentrations (0.00-6.50 mM). Data represents mean values for duplicate measurements.

This observation for protein solutions was in good accordance with literature. Primozic et al. (2017) confirmed shear thinning behavior for lentil protein isolate solutions whereby dynamic viscosity decreased with an increase in shear rate. This may be attributed to the tendency of protein molecules to orient its main axes in the direction of flow. Furthermore, the dissociation of weakly held dimers and oligomers into monomers may have also contributed to shear-thinning. For solutions containing globular proteins (soy, whey), it is known when shearing flow is stopped viscosity is regained (Damodaran et al., 2008). Since the Ca^{2+}-induced aggregation of hyacinth bean protein was found relatively independent of Ca^{2+} concentrations $\geq$ 4.50 mM, the following experiments involving particle characterization were performed on nanoparticles induced by 3.50 mM Ca^{2+}.

5.3.2. Particle characteristics: Effect of pH and concentration

The effect of concentration and pH on the internal integrity of hyacinth bean protein nanoparticles were evaluated using relative stability tests (Figure 28).

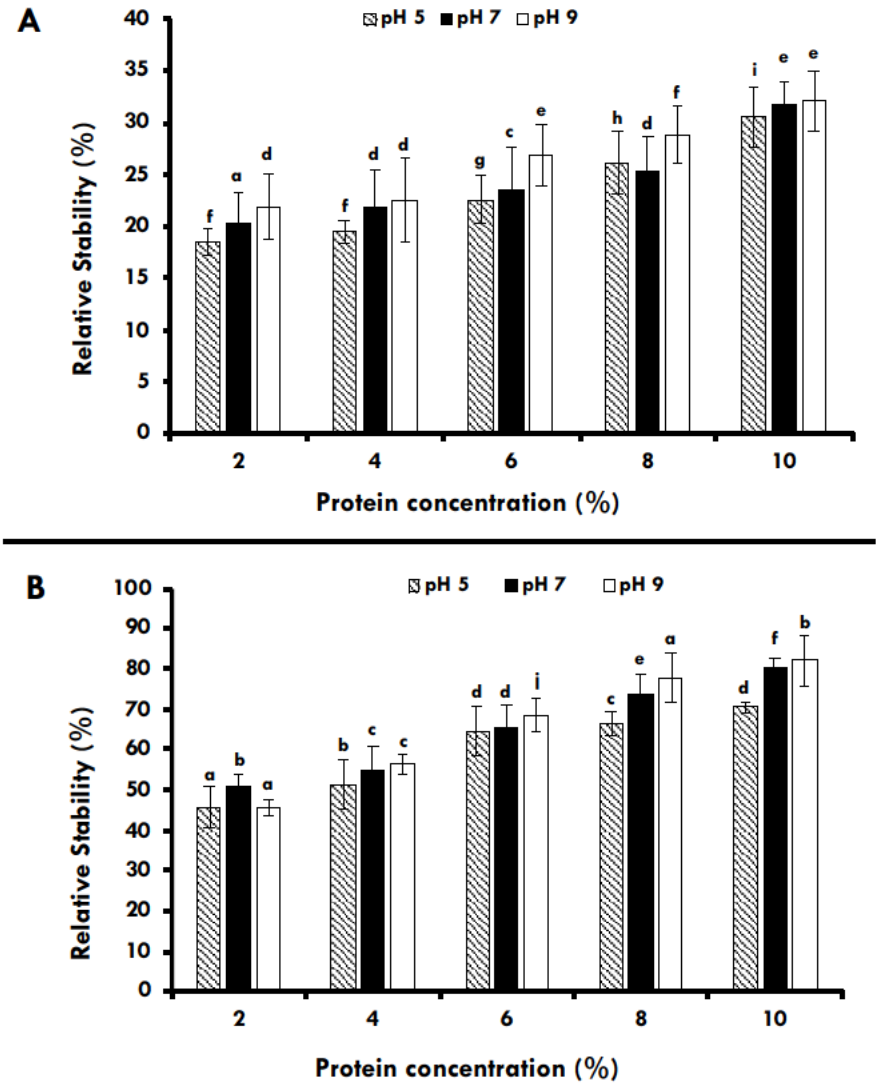

Figure 28: The influence of pH on the relative stability of hyacinth bean protein nanoparticle dispersions (2-10% m/v, 3.50 mM Ca^{2+}) subjected to (A) Urea 4 M and (B) DTT 120 mM treatments. Data at respective protein concentrations represented with different alphabets (a, b) are significantly different ($p < 0.05$). Data represents means and standard deviation (n=3).

In principle, nanoparticles should be able to preserve proper partial wettability to gain enough interface absorption efficiency to function as Pickering emulsion stabilizers. Therefore, strong internal integrity of nanoparticles, hence the presence of intra-particle disulfide bonds are required to maintain their structural integrity once adsorbed at the emulsion interface (Xiao et al., 2016, Schmitt et al., 2010). Protein studies are often conducted in complex solutions, thereby chemical denaturation of proteins is one of the primary methods used for assessing their stability, structure and function. Bennion and Daggett (2003) reported that chemical denaturation of proteins using Urea occurs through direct and indirect mechanisms. The initial step involves the expansion and solvation of the hydrophobic core by water and then by Urea.

Thus, the weakening of the water structure by Urea allowed free water to compete with intraprotein interactions. Different reducing agents, Urea (4 M) and DTT (120 mM) were used to dissociate intra-particle interactions (i.e. hydrogen bonds, hydrophobic interactions and disulfide bonds) at different particle concentrations under varying pH conditions.

In the presence of 4 M Urea and 120 mM DTT, the relative stability of protein nanoparticles was found to be significantly different ($p<0.05$) at different concentrations for the pH conditions studied. Therefore, environmental conditions need to be carefully considered for particles to perform as Pickering stabilizers as its stability may vary extensively. Under Urea and DTT, particle stability improved as concentration increased across the pH range investigated. However, it was observed that protein nanoparticles at different concentrations were susceptible to the presence of Urea for the respective pH range. These results may indicate that the structure of the Ca^{2+}-induced protein nanoparticles was largely maintained by hydrogen bonds and hydrophobic interactions. Cao et al. (2015) has confirmed that the structure of Ca^{2+}-induced protein aggregates are maintained by hydrophobic interactions.

Protein nanoparticles at different concentrations treated with DTT was found more stable compared to Urea at different pH conditions. This may be attributed to the presence of disulfide bonds maintaining the internal integrity of these nanoparticles (Destribats et al., 2014). It is known that the net surface charge on proteins must be large enough to overcome various attractive forces (e.g. hydrophobic, van der Waals and depletion). This is to stabilize electrostatic repulsive forces between oil droplets of emulsions (McClements, 2004). Figure 29 shows zeta-potential comparison for solutions containing hyacinth bean protein nanoparticles (3.50 mM Ca^{2+}) and untreated hyacinth bean protein (native) as influenced by pH. At pH 7, native (-41.88 mV) and solutions containing Ca^{2+}-induced protein nanoparticles (-11.96 mV) both carried a net negative charge above their pI values (pI ~4.50 for globulins; ~6.00 for albumins) (Swanson, 1990). This indicates that protein dispersions are stable due to electrostatic repulsive forces.

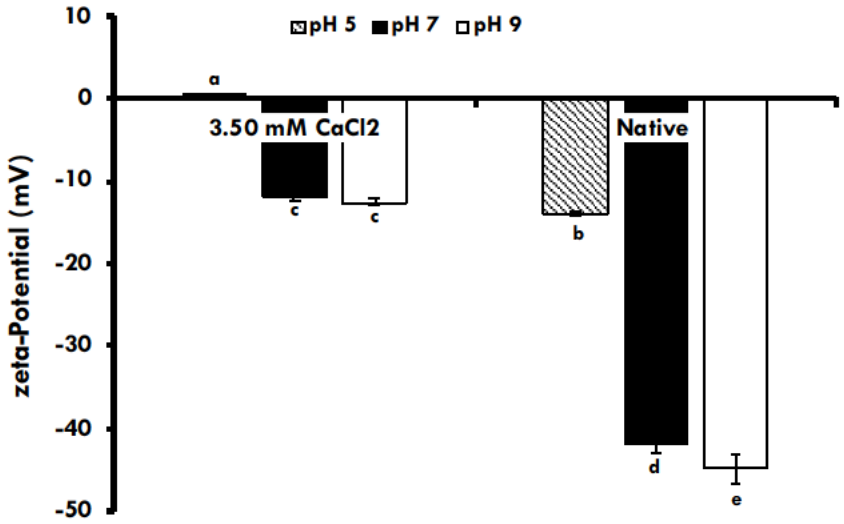

Figure 29: The influence of pH on zeta-potential of protein dispersions (2% m/v, native and formed with 3.50 mM Ca^{2+}). Data at respective protein dispersions represented with different alphabets are significantly different ($p<0.05$). Data represents means and standard deviation (n=3).

A rapid decline in the magnitude of zeta-potential was observed for solutions containing Ca^{2+}-induced protein nanoparticles (-12.60 to 0.50 mV) compared to the native (-44.88 to -13.97 mV) across the pH range studied confirming the electrostatic screening by Ca^{2+} binding. The magnitude of zeta-potential for both samples was found to sharply increase as pH conditions became more alkaline (pH 5 to 9). This may be caused by the electrostatic repulsion amongst proteins being weakened at higher pH, thus preventing its dissociation. The higher zeta-potential values at pH 5 (-13.97 to 0.50 mV) may be attributed to the deprivation of water by aggregated proteins that may have impaired the ionization of charged groups in proteins (Teng et al., 2012).

5.3.3. Characterization of emulsions

The emulsification performance of hyacinth bean protein nanoparticles (3.50 mM Ca^{2+}, pH 7) was evaluated in 10% oil-in-water emulsions at different concentrations (0.80-2.00% m/v). Emulsions stabilized by protein nanoparticles were subjected to 7-day accelerated storage period (~55°C) for accelerating physicochemical related phenomena (i.e. creaming, Ostwald ripening, phase inversion, coalescence and flocculation) (Figure 30).

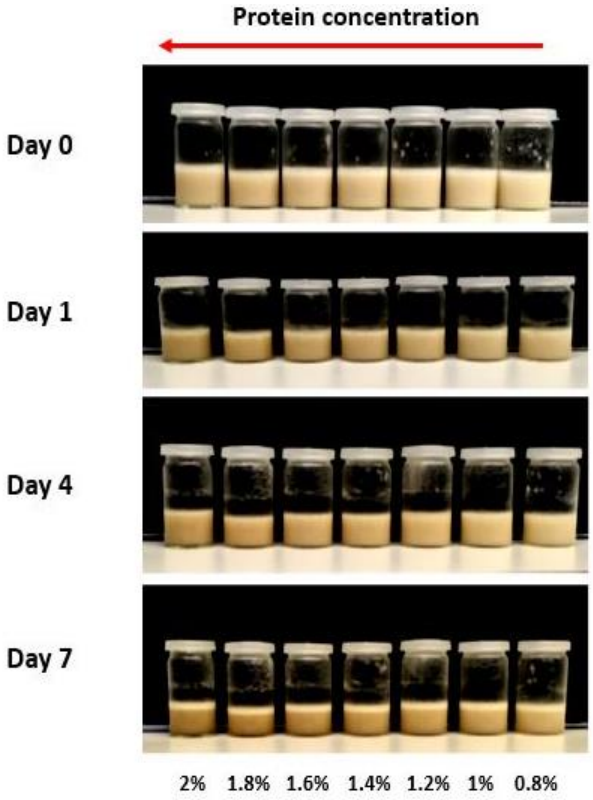

Figure 30: 10% oil-water emulsions stabilized by different concentrations of 3.50 mM Ca^{2+} hyacinth bean protein nanoparticle dispersion (0.80-2.00%, pH 7).

The rationale for temperature accelerated storage is based on the presence of a moderate energy barrier which imparts stability to emulsion dispersions by preventing the colliding particles to come close enough to be attracted by van der Waals forces and thus stick to each other. Therefore, increasing temperature imparts higher kinetic energy to the particles increasing the probability that this barrier is overcome, and the particles will aggregate/coalesce (Lerche and Sobisch, 2011). Oil-in-water emulsions produced were found relatively stable over the accelerated storage period. Droplet size distribution profiles for emulsions stabilized with different concentrations of protein nanoparticles were found similar on day(s) 0 and 7. There was no distinct shift of major peaks observed in droplet size distribution during the 7-day period, whereby similar peaks were detected between 10-100 µm (Figure 31).

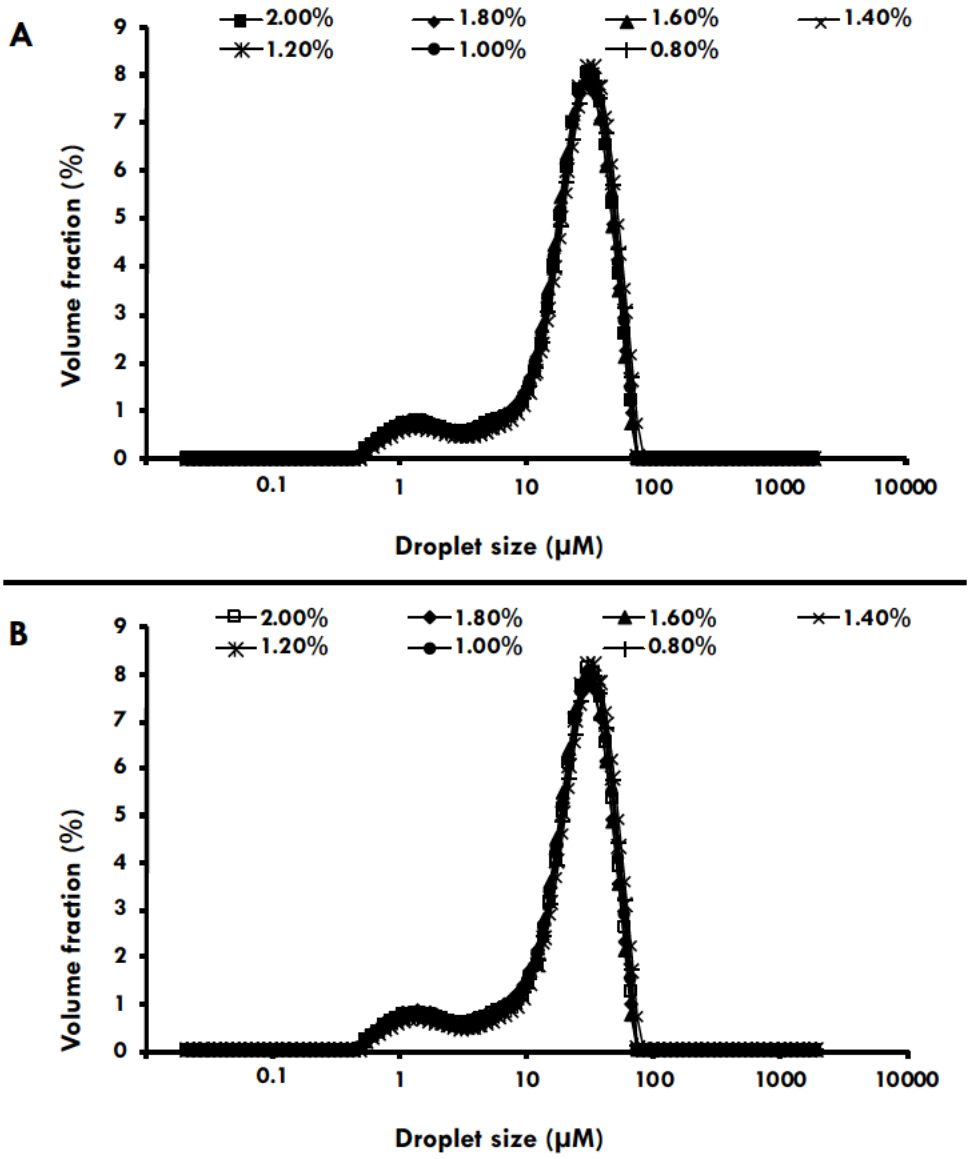

Figure 31: Droplet size distribution for 10% oil-water emulsions [Day 0 (A) and Day 7(B)] stabilized by different concentrations of 3.50 mM Ca^{2+} hyacinth bean protein nanoparticles (0.80-2.00%, pH 7).

In addition, no dramatic changes to the Sauter mean diameter ($D_{3,2}$) of prepared emulsions occurred during the storage period. Furthermore, the droplet size diameter was found similar for emulsions stabilized with protein nanoparticles at different concentrations (Table 21).

Table 21: Sauter mean diameter $D_{3,2}$ [µm] of 10% oil-in-water emulsions subjected to accelerated storage (1 week/ 55°C) stabilized by different concentrations of 3.50 mM Ca^{2+} hyacinth bean protein nanoparticles (0.80-2.00%, pH 7)

Protein concentration (%)	$D_{3,2}$ [µm]			
	Day 0	Day 1	Day 4	Day 7
2.00	7.98±0.87^{a}	8.09±0.18^{a}	8.36±0.14^{b}	7.88±0.48^{a}
1.80	7.46±0.42^{a}	8.58±0.03^{b}	8.50±0.04^{b}	7.97±0.88^{c}
1.60	7.98±0.63^{a}	8.51±0.10^{b}	7.65±0.44^{a}	8.03±0.64^{a}
1.40	8.90±0.09^{a}	9.00±0.25^{a}	8.81±0.22^{a}	8.23±0.23^{a}
1.20	8.93±0.01^{a}	8.72±0.17^{a}	8.59±0.04^{a}	8.16±0.40^{b}
1.00	8.40±0.44^{a}	8.22±0.59^{a}	8.42±0.11^{a}	8.50±0.01^{a}
0.80	8.31±0.62^{c}	8.42±0.26^{c}	8.59±0.03^{c}	8.82±0.03^{c}

Data represents mean±standard deviation (n=3). Values with different superscript letters are significantly different ($p<0.05$).

Cryo-SEM was used for potentially viewing oil-in-water droplets stabilized by 3.50 mM Ca^{2+} hyacinth bean protein nanoparticles (Figures 32A-B). The morphology of the stabilized oil droplets was spherical in shape. It appeared that Ca^{2+}-induced protein nanoparticles covered the droplet surface which may have contributed to the stability of emulsions against coalescence.

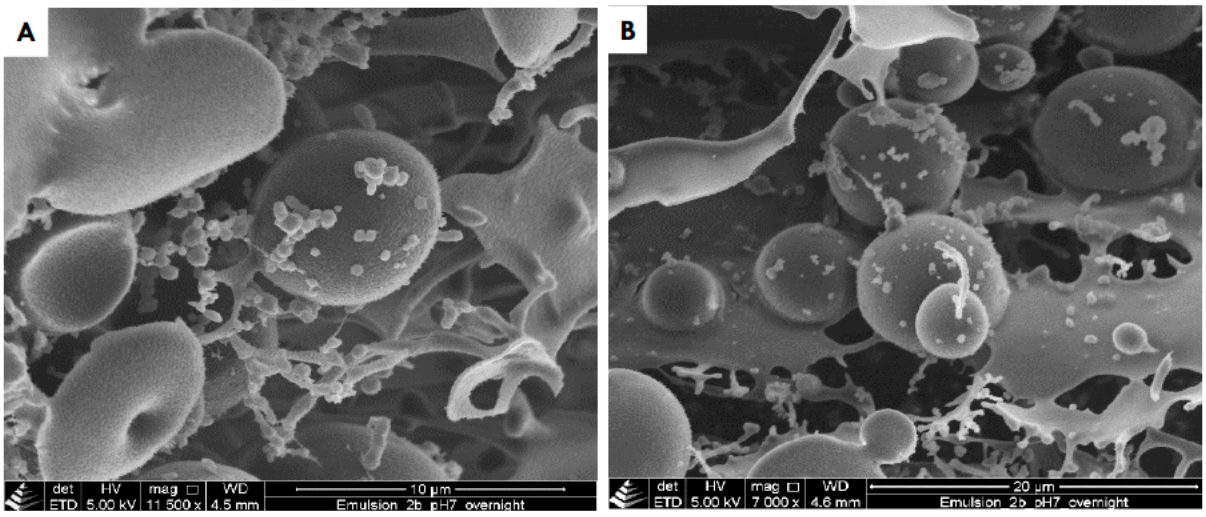

Figure 32: SEM micrographs of 10% oil-in-water emulsion droplets stabilized by 1.80% of 3.50 mM Ca^{2+} hyacinth bean protein nanoparticles (pH 7). Bar size (A) 10 µm and (B) 20 µm.

O'Sullivan et al. (2016) described smooth and spherical shaped microstructural morphology for oil-in-water droplets stabilized using 1 wt.% ultrasound treated soy protein isolate. The needle-like structures that appear in the continuous phase could be attributed to the formation of calcium crystals during sample preparation. These observations are consistent with those reported by Kargar et al. (2012), who studied the microstructure of oil-in-water Pickering emulsion droplets stabilized by microcrystalline cellulose and modified starch.

Emulsion creaming is an upward sedimentation caused by a difference in density between the oil and water phases. In comparison to water, oil droplets have a lower density allowing them to accumulate towards the emulsion surface (McClements, 2009). Emulsion stability to creaming is contributed by a lower density contrast between phases, smaller droplets and high viscosity of the continuous phase (McClements, 2007). In the present study there was no creaming present for freshly prepared oil-in-water emulsions (Day 0) stabilized with Ca^{2+}-induced protein nanoparticles (0.80%-2.00%, pH 7). However, emulsion creaming was noticeable between day(s) 1-7 during accelerated storage. The emulsion creaming first identified became more apparent as storage progressed. The creaming indices between day(s) 1 and 7 generally increased from approximately 18 to 27% (Figure 33).

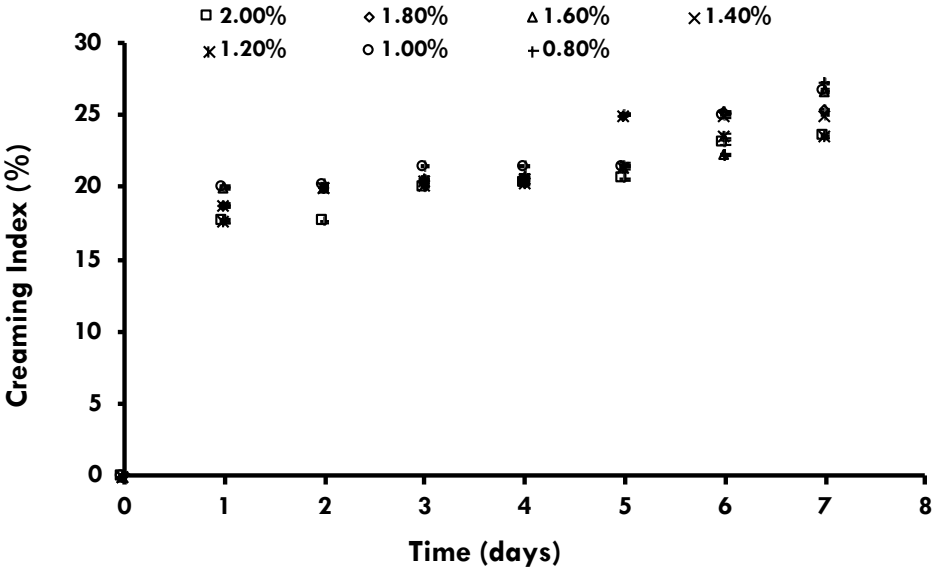

Figure 33: Creaming indices (%) for 10% oil-in-water emulsions subjected to accelerated storage (1 week/ 55°C) stabilized by different concentrations of 3.50 mM Ca^{2+} hyacinth bean protein nanoparticles (0.80-2.00%, pH 7). Data represents mean values for duplicate observations.

Pieter Walstra and Vliet (2008) reported that convection currents in dispersions caused by temperature fluctuations may strongly disturb the sedimentation of small particles (<−1 μm) resulting in increased creaming instability.

5.4. Conclusion

The particle size of hyacinth bean protein nanoparticles was largely dependent on applied Ca^{2+} concentration. The results satisfied certain criteria for hyacinth bean protein nanoparticles to potentially function as Pickering stabilizers. The size of protein nanoparticles was substantially smaller than the targeted emulsion droplet size. The relative stability of nanoparticles was found different under various pH conditions.

Thus, it is important to consider environmental conditions when developing Pickering stabilizers. Nanoparticles at different concentrations and pH were least susceptible to the presence of DTT indicating the presence of intra-particle disulfide bonds that is required to maintain particles structural integrity once adsorbed at the emulsion oil-water-interface. However, protein nanoparticles were found susceptible to the presence of Urea. Emulsion creaming became more apparent as storage progressed and maybe related to the demonstrated shear-thinning behavior of particles. Therefore, the use of appropriate cross-linking agents in fabrication procedures are recommended to develop protein-based nanoparticles with improved stability for effectively functioning as food-grade Pickering emulsion stabilizers against various stresses.

Chapter 6: General Discussion

Several different legumes (i.e. peas, beans, lentils) have been traditionally utilized as whole grains. However, there is continuous interest shown by food industries in utilizing legumes in forms such as isolates, flour, or concentrates other than whole grains. It is widely reported that changes to the quality of legume derived intermediates will be largely determined on interferences made at microscopic level. This is due to the presence of majority of microstructural elements (i.e. fibers, starch granules, protein) at this level that extensively contribute to techno-functionality in food systems (Cuq et al., 2011, Bußler et al., 2015). Thus, there is growing interest in effectively utilizing various food processing operations that is aimed at modifying intermediates for providing legume-based food products with desirable traits and functional properties

The first research study focused on examining the effect of soaking, steaming and dehydration on the microstructure, physicochemical properties and *in vitro* starch digestibility of hyacinth bean flour (see chapter 3). Briefly, hyacinth bean flour was produced from legume grains that were subjected to steaming (S+A) and dehydration (S+A+D) treatments, prior to soaking (S) respectively. The morphology of starch granules extensively changed in response to the various process treatments. Through Pearson correlation analysis, great interaction was observed amongst starch, mineral elements and physicochemical properties of flour produced from legume grains under the different process conditions.

It was found that flour produced from legume grains subjected to steam-dehydration (S+A+D) resulted in higher resistant starch content. Furthermore, it was recognized as a useful process for developing legume flour with high water swelling and solubility properties at lower temperatures for ingredient application. Dehydrated related foods are considered texture modified and specially designed for patients with chewing problems and swallowing dysfunction. These problems are common among elderly populations and are regarded to affect their perception of food, hence food choice and ultimately their ability to eat. It was concluded that steam-dehydration could be adopted as a viable processing method for the manufacture and application of legume flour in food products which require rehydration for preserving or creating a new structure to serve a functional purpose (Vega-Mercado et al., 2001, Aguilera et al., 2003, Martín-Cabrejas et al., 2009).

These ingredients could present additional advantages in the respective food formulations, for contributing to some sensory attributes (taste, and feel) that are similar to traditional foods, producing food products with a softer and homogenous texture, prolonged preservation time, easy use and storage (reduction of volume and weight), and low glycemic responses that are vital for avoiding health illnesses (i.e. hyperglycemia, hypoglycemia and ketoacidosis).

The functionality of legume derived proteins in food systems during processing, storage, and consumption are defined by their physicochemical properties. In addition, functionality arises from the composition of protein and response to external environmental factors (i.e. temperature, pH) and interaction with other food constituents (O'Connell, 2007). Thus, the second research study was based on examining the effects of steaming and dehydration on the nutritional quality and functional properties of hyacinth bean flour (see chapter 4). Briefly, protein isolates were produced by isoelectric precipitation from the respective legume flour fractions (S, S+A, S+A+D) that were used in the previous study (see chapter 3).

Through principal component analysis, it was observed that the different processes extensively influenced the amino acid profiles for the different samples. Thus, was found to significantly influence the respective samples functionality to various extents. In terms of nutritional quality, most protein efficiency ratio values were found greater than 3. Thus, protein isolate samples were considered typical of high-quality proteins. Samples produced by steaming (S+A) and dehydration (S+A+D) were identified as potentially health beneficial and useful for nutritional intervention. This was attributed to a dramatic increase to predicted biological values that further indicate their improved protein digestibility potential. There was significant improvement to the ratio of hydrophobic and hydrophilic amino acid residues found in the respective samples. In food application, this may hold potential significance in modulating taste perception (Humiski and Aluko, 2007).

Samples produced by steaming (S+A) and dehydration (S+A+D) resulted in a higher Fischer's ratio and BCAA to tyrosine ratio (BTR). It has been reported that the extent of hepatic damage increases with decreasing Fischer's ratio and BTR. Thus, administering BCAA booster to the respective patients may be useful for controlling the mentioned disorder (Ishikawa, 2012a, Ishikawa, 2012b). Furthermore, it was found that the respective samples contained significant concentrations of amino acids, Arginine and Lysine. These amino acids have been associated with beneficial hypocholesterolemic effects in improving the cardiovascular health, and helps in hypertension regulation (Vallabha et al., 2016, Malomo et al., 2014).

An extensive improvement in first (Lysine) and second (Methionine + Cysteine) limiting amino acids concentration was observed. However, based on FAO recommendations these concentrations were still inadequate to fulfil requirements. Therefore, it was concluded that consumption of legume derived protein ingredients produced by steaming (S+A) and dehydration (S+A+D) with cereal grains and soybean may potentially contribute to a well-balanced essential amino acid profile with added health benefits.

Food proteins are regarded as both nutritional and functional ingredients, whereby its emulsifying properties are recognized for contributing to the delivery of nutritional agents, functional lipids, and other materials (Foegeding and Davis, 2011, Jiang et al., 2014). Therefore, the third research study reports findings for the emulsifying properties of Ca^{2+}-induced hyacinth bean protein nanoparticles (see chapter 5). This was to examine its potential for functioning as food protein-based Pickering emulsion stabilizers. Hyacinth bean protein nanoparticles (~172.38 nm, pH 7) were produced using 3.50 mM Ca^{2+}, whereby protein aggregation was found largely dependent on Ca^{2+} concentration. Thus, z-average diameter for particles was found to sharply increase at higher Ca^{2+} concentration. Hyacinth bean protein aggregates formed at different Ca^{2+} concentrations (0.00-6.50 mM, pH 7) were characterized by a viscous flow behavior. It was observed that protein solutions containing higher Ca^{2+} concentrations have the highest viscosities.

Thus, this may present opportunities for producing protein aggregates using a non-thermal approach that can be further exploited for potential applications as microspheres in the production of biodegradable films for packaging (Falguera et al., 2011). The electrostatic screening by Ca^{2+} binding was evident, and this was confirmed through findings reported for surface charge. The results from this study satisfied certain criteria reported by Xiao et al. (2016), for the development of hyacinth bean protein nanoparticles to potentially function as Pickering stabilizers. The size of protein nanoparticles was found substantially smaller than the targeted emulsion droplet size allowing for the stabilization of droplets as small as a few micrometres in diameter. Nanoparticles at different concentrations and pH were least susceptible to the presence of DTT indicating the presence of intra-particle disulfide bonds. This is required for structural integrity once adsorbed at the emulsion interface. However, protein nanoparticles were found susceptible to the presence of Urea.

In terms of emulsification performance, creaming became more apparent as storage progressed for oil-in-water emulsions stabilized by protein nanoparticles (0.80-2.00% m/v, pH 7).

The use of appropriate cross-linking agents is recommended to develop existing nanoparticles with improved stability and emulsification performance. Glutaraldehyde (GAD) cross-linking of proteins has been widely used in literature for strengthening particle integrity (Gerrard et al., 2003, Jin et al., 2013). It is said that cross-linking of proteins is achieved through a Maillard-type reaction, however GAD is not permitted for use in the food field. Thus, alternative drug delivery systems may be considered. There is opportunity present for the development of bio-adhesives using Ca^{2+}-induced hyacinth bean protein nanoparticles considering the viscous flow behavior of these particles. Proteins are also known to contain functional groups which can be adsorbed or attached onto molecules such as antibodies by covalent bonding (Lohcharoenkal et al., 2014, Molino and Wang, 2014, Sripriyalakshmi et al., 2014).

Conclusion and Recommendations

The research work presents practical application for producing value-added legume-based ingredients for potential food applications from a processing perspective. This could influence the value of hyacinth bean by diversifying its use. Furthermore, findings may help improve the competitiveness of the legume grain sector. However, further work is required on a development platform for exploiting improved traits reported with regards to composition and functionality:

1. Development and physicochemical property assessment of dehydrated food products formulated using legume flour produced from hyacinth bean legume grains subjected to steam-dehydration.
2. Protein isolates produced from steam and dehydration resulted in significant improvement to the ratio of hydrophobic to hydrophilic amino acid residues. These residues are observed to affect and modulate taste perception. Therefore, further work is recommended on predicting the sensory properties of hyacinth bean protein isolate with respect to taste for effective ingredient application.
3. The development of bio-adhesives using Ca^{2+}-induced hyacinth bean protein nanoparticles to potentially function as drug carriers in pharmaceutics.

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Appendices

1. Pearson correlation co-efficient: Chemical composition vs mineral composition

a. Soak (S)

	Ca	Mg	K	Na	P	Cu	Fe	Mn	Zn
Protein	-,910	-,983	-,795	,130	-1.000**	,500	-,803	-,500	-,756
Fat	-,646	-,810	-,460	-,306	-,904	,082	-,471	-,822	-,963
TS (Total Starch)	-,488	-,264	-,669	.999*	-,082	,904	-,660	,822	,590
Ash	,797	,918	,642	,091	,975	-,297	,652	,679	,882
Moisture	-,910	-,983	-,795	,130	-1.000**	,500	-,803	-,500	-,756
TDF (Total Dietary Fiber)	,910	,983	,795	-,130	1.000**	-,500	,803	,500	,756
Amylose	.992*	.994*	,938	-,414	,956	-,731	,942	,225	,532

*. Correlation is significant at the 0.05 level (1-tailed).

**. Correlation is significant at the 0.01 level (1-tailed).

b. Soak + Autoclave (S+A)

	Ca	Mg	K	Na	P	Cu	Fe	Mn	Zn
Protein	-0,407	-0,024	0,189	-.991*	-0,881	-0,535	-0,404	-1.000**	-0,693
Fat	0,104	-0,521	-0,327	-0,924	-1.000**	-0,041	-0,807	-0,866	-0,961
Total Starch (TS)	-.995*	0,854	0,945	-0,381	-0,03	-.999*	0,59	-0,5	0,277
Ash	0,008	-0,436	-0,235	-0,957	-.998*	-0,136	-0,747	-0,91	-0,93
Moisture	0,011	0,419	0,217	0,962	.996*	0,155	0,734	0,918	0,923
Total Dietary Fiber (TDF)	0,407	0,024	-0,189	.991*	0,881	0,535	0,404	1.000**	0,693
Amylose	0,984	-0,965	-.999*	0,11	-0,248	0,949	-0,791	0,24	-0,533

*. Correlation is significant at the 0.05 level (1-tailed).

**. Correlation is significant at the 0.01 level (1-tailed).

c. Soak + Autoclave + Dehydrated (S+A+D)

	Ca	Mg	K	Na	P	Cu	Fe	Mn	Zn
Protein	,820	,658	,566	,970	,629	-,057	,797	,610	,857
Fat	-,129	-,363	-,469	,232	-,399	-,912	,904	,985	-,060
Total Starch (TS)	,888	,751	,670	.993*	,726	,075	,711	,500	,918
Ash	-1.000**	-,968	-,932	-,939	-,957	-,515	-,319	-,058	-.998*
Moisture	.997*	,985	,959	,907	,978	,584	,240	-,025	.990*
Total Dietary Fiber (TDF)	-,842	-,947	-,978	-,596	-,959	-,901	,254	,500	-,803
Amylose	,251	,010	-,106	,578	-,028	-,692	.998*	,979	,317

*. Correlation is significant at the 0.05 level (1-tailed).

**. Correlation is significant at the 0.01 level (1-tailed).

2. Pearson correlation co-efficient: Functional properties vs chemical composition

a. Soak (S)

	FC	FS	EC	ES	WAC	OAC
Protein	-,967	-.991*	,983	.995*	,971	,189
Fat	-,983	-,952	,967	,943	,980	,590
TS (Total Starch)	,176	,048	-,101	-,019	-,160	-,963
Ash	.999*	.996*	-.999*	-.993*	-1.000**	-,401
Moisture	-,967	-.991*	,983	.995*	,971	,189
TDF (Total Dietary Fiber)	,967	.991*	-,983	-.995*	-,971	-,189
Amylose	,850	,910	-,887	-,922	-,858	,106

**. Correlation is significant at the 0.01 level (1-tailed).

*. Correlation is significant at the 0.05 level (1-tailed).

b. Soak + Autoclave (S+A)

	FC	FS	EC	ES	WAC	OAC
Protein	.999*	,723	.991*	,914	-,982	,756
Fat	,842	,971	,926	,589	-,945	,982
TS (Total Starch)	,539	-,237	,378	,808	-,327	-,189
Ash	,890	,944	,958	,663	-,972	,959
Moisture	-,898	-,938	-,963	-,678	,976	-,954
TDF (Total Dietary Fiber)	-.999*	-,723	-.991*	-,914	,982	-,756
Amylose	-,285	,497	-,106	-,613	,052	,454

**. Correlation is significant at the 0.01 level (1-tailed).

*. Correlation is significant at the 0.05 level (1-tailed).

c. Soak + Autoclave + Dehydrated (S+A+D)

	FC	FS	EC	ES	WAC	OAC
Protein	-0,766	0,929	0,847	-0,144	0,682	-0,946
Fat	0,215	0,102	0,863	0,81	-0,333	-0,149
TS	-0,844	0,97	0,77	-0,274	0,773	-0,98
Ash	.995*	-0,976	-0,401	0,677	-0,975	0,965
Moisture	-1.000**	0,955	0,323	-0,735	.990*	-0,939
TDF	0,886	-0,696	0,168	0,97	-0,936	0,661
Amylose	-0,165	0,466	.989*	0,533	0,043	-0,507

*. Correlation is significant at the 0.05 level (1-tailed).

**. Correlation is significant at the 0.01 level (1-tailed).

3. Pearson correlation co-efficient: Functional properties vs mineral composition

a. Soak (S)

	FC	FS	EC	ES	WAC	OAC
Ca	,774	,849	-,819	-,864	-,784	,235
Mg	,903	,951	-,933	-,959	-,910	-,006
K	,614	,710	-,671	-,730	-,626	,445
Na	,128	,000	-,053	,029	-,112	-,949
P	,967	.991*	-,983	-.995*	-,971	-,189
Cu	-,262	-,383	,334	,410	,277	-,756
Fe	,624	,719	-,681	-,738	-,636	,434
Mn	,705	,608	-,650	-,585	-,693	-,945
Zn	,898	,835	-,863	-,818	-,891	-,786

**. Correlation is significant at the 0.01 level (1-tailed).

*. Correlation is significant at the 0.05 level (1-tailed).

b. Soak + Autoclave (S+A)

	FC	FS	EC	ES	WAC	OAC
Ca	-,449	,337	-,279	-,743	,227	,290
Mg	,022	-,708	-,160	,384	,212	-,673
K	,234	-,542	,054	,571	,000	-,500
Na	-,984	-,808	-1.000**	-,852	.998*	-,836
P	-,858	-,964	-,937	-,613	,954	-,976
Cu	-,573	,197	-,415	-,832	,366	,149
Fe	-,361	-,924	-,524	,002	,569	-,904
Mn	-.999*	-,723	-,991	-,914	,982	-,756
Zn	-,660	-.999*	-,785	-,341	,817	-,996

*. Correlation is significant at the 0.05 level (1-tailed).

**. Correlation is significant at the 0.01 level (1-tailed).

c. Soak + Autoclave + Dehydrated (S+A+D)

	FC	FS	EC	ES	WAC	OAC
Ca	-.996*	0,973	0,39	-0,685	0,978	-0,961
Mg	-.988*	0,89	0,157	-0,84	.999*	-0,867
K	-0,963	0,831	0,041	-0,898	.989*	-0,803
Na	-0,9	.991*	0,691	-0,382	0,84	-.996*
P	-0,981	0,872	0,119	-0,86	.998*	-0,847
Cu	-0,598	0,316	-0,579	-0,98	0,691	-0,27
Fe	-0,223	0,518	.996*	0,482	0,102	-0,558
Mn	0,042	0,274	0,938	0,696	-0,164	-0,319
Zn	-.988*	0,987	0,452	-0,633	0,961	-0,978

*. Correlation is significant at the 0.05 level (1-tailed).

**. Correlation is significant at the 0.01 level (1-tailed).

4. Pearson correlation co-efficient: Swelling and solubility vs chemical composition

a. Soak (S)

	Swell60	Swell70	Swell80	Swell90	Sol60	Sol70	Sol80	Sol90
Protein	,000	-,017	,005	0,000	,000	,500	,000	,000
Fat	-,427	-,443	-,422	-,427	-,427	,082	-,427	-,427
TS (Total Starch)	.997*	.995*	.997*	.997*	.997*	,904	.997*	.997*
Ash	,220	,237	,215	,220	,220	-,297	,220	,220
Moisture	,000	-,017	,005	0,000	,000	,500	,000	,000
TDF (Total Dietary Fiber)	,000	,017	-,005	0,000	,000	-,500	,000	,000
Amylose	-,292	-,276	-,297	-,292	-,292	-,731	-,292	-,292

**. Correlation is significant at the 0.01 level (1-tailed).

*. Correlation is significant at the 0.05 level (1-tailed).

b. Soak + Autoclave (S+A)

	Swell60	Swell70	Swell80	Swell90	Sol60	Sol70	Sol80	Sol90
Protein	,000	,012	,000	0,000	-,189	,189	-,115	,052
Fat	,500	,510	-,500	-,500	,327	,655	-,596	-,454
TS (Total Starch)	-,866	-,860	,866	,866	-,945	-,756	,803	,891
Ash	,415	,425	-,415	-,415	,235	,579	-,516	-,366
Moisture	-,397	-,408	,397	,397	-,217	-,564	,500	,349
TDF (Total Dietary Fiber)	,000	-,012	,000	0,000	,189	-,189	,115	-,052
Amylose	,971	,968	-,971	-,971	.999*	,908	-,937	-,982

**. Correlation is significant at the 0.01 level (1-tailed).

*. Correlation is significant at the 0.05 level (1-tailed).

c. Soak + Autoclave + Dehydrated (S+A+D)

	Swell60	Swell70	Swell80	Swell90	Sol60	Sol70	Sol80	Sol90
Protein	-,317	-,132	-,133	,050	-,381	-,381	-,132	-,610
Fat	-,987	-,940	-,940	,909	,643	,643	-,940	-,985
TS (Total Starch)	-,189	,000	-,001	-,082	-,500	-,500	,000	-,500
Ash	-,272	-,449	-,448	,521	,836	,836	-,449	,058
Moisture	,351	,522	,521	-,590	-,878	-,878	,522	,025
TDF (Total Dietary Fiber)	-,756	-,866	-,866	,904	1.000**	1.000**	-,866	-,500
Amylose	-,857	-,745	-,745	,687	,311	,311	-,745	-,979

*. Correlation is significant at the 0.05 level (1-tailed).

**. Correlation is significant at the 0.01 level (1-tailed).

5. Pearson correlation co-efficient: Swelling and solubility vs mineral composition

a. Soak (S)

	Swell60	Swell70	Swell80	Swell90	Sol60	Sol70	Sol80	Sol90
Ca	-,414	-,398	-,419	-,414	-,414	-,814	-,414	-,414
Mg	-,184	-,166	-,189	-,184	-,184	-,650	-,184	-,184
K	-,606	-,592	-,610	-,606	-,606	-,923	-,606	-,606
Na	.991*	.989*	.992*	.991*	.991*	,924	.991*	.991*
P	,000	,017	-,005	0,000	,000	-,500	,000	,000
Cu	,866	,857	,869	,866	,866	1.000**	,866	,866
Fe	-,596	-,582	-,600	-,596	-,596	-,918	-,596	-,596
Mn	,866	,875	,863	,866	,866	,500	,866	,866
Zn	,655	,668	,651	,655	,655	,189	,655	,655

**. Correlation is significant at the 0.01 level (1-tailed).

*. Correlation is significant at the 0.05 level (1-tailed).

b. Soak + Autoclave (S+A)

	Swell60	Swell70	Swell80	Swell90	Sol60	Sol70	Sol80
Ca	,913	,908	-,913	-,913	,974	,820	-,861
Mg	-1.000**	-1.000**	1.000**	1.000**	-,977	-,986	.996*
K	-,982	-,980	,982	,982	-1.000**	-,929	,954
Na	-,132	-,144	,132	,132	,058	-,317	,245
P	-,473	-,484	,473	,473	-,298	-,631	,571
Cu	,845	,838	-,845	-,845	,931	,729	-,778
Fe	-,915	-,920	,915	,915	-,822	-,975	,955
Mn	,000	-,012	,000	0,000	,189	-,189	,115
Zn	-,721	-,729	,721	,721	-,577	-,839	,795

*. Correlation is significant at the 0.05 level (1-tailed).

**. Correlation is significant at the 0.01 level (1-tailed).

c. Soak + Autoclave + Dehydrated (S+A+D)

	Swell60	Swell70	Swell80	Swell90	Sol60	Sol70	Sol80	Sol90
Ca	,284	,460	,459	-,531	-,842	-,842	,460	-,046
Mg	,506	,660	,659	-,719	-,947	-,947	,660	,196
K	,603	,743	,742	-,795	-,978	-,978	,743	,308
Na	-,075	,115	,114	-,196	-,596	-,596	,115	-,397
P	,538	,688	,687	-,745	-,959	-,959	,688	,233
Cu	,965	.997*	.997*	-1.000**	-,901	-,901	.997*	,826
Fe	-,825	-,703	-,704	,643	,254	,254	-,703	-,965
Mn	-,945	-,866	-,866	,822	,500	,500	-,866	-1.000**
Zn	,217	,397	,397	-,471	-,803	-,803	,397	-,115

*. Correlation is significant at the 0.05 level (1-tailed).

**. Correlation is significant at the 0.01 level (1-tailed).