Investigation of Effect of Glycation and Denaturation on Functional Properties of Cowpea Proteins

Mohamed Abdullah Ahmed

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ABSTRACT

There is increasing demand in the European Union for vegetable protein used in animal feed as well as for use in human foods such as preparation of culinary sauces, mayonnaise and baking. Millions of tons of soy proteins are imported into the European Union and the price is increasing annually. A market replacement share by a similar vegetable protein by a few percent represents tens of millions of euros. Cowpea (Vigna unguiculata L. Walp) is a well-established crop around the world and the production of this grain has been increasing in Africa. Yet the exploitation of the cowpea proteins as alternative to soy protein remains to be investigated.

As with other grain legumes, cowpea has high protein quality due to its high levels of lysine which may be significant in balancing the deficiencies of this essential amino acid in cereal-based diets. The primary limitation to the improvement of cowpea proteins is the lack of information on the technology and characterisation of the cowpea protein isolate, in comparison with well-established soy protein isolate. More importantly, data on the evaluation of the functional properties of cowpea protein isolate and modification of the protein structure for enhancing the functional behaviours are lacking. Therefore, the major purpose of this study has been focused on the isolation and characterisation of cowpea proteins, as well as evaluation of the functional properties of the resultant products for appropriate food applications. The feasibility of glycation and/or denaturation in order to improve the functional properties of the proteins is also included.

The first phase of the study optimised the extraction conditions of cowpea protein which resulted in 89% yield and 90% protein content reported here for the first time. Its physicochemical and functional properties were compared to that of commercial soy protein isolate (SPI) and whey protein concentrate (WPC 60). Compared to SPI, cowpea protein isolate (CPI) had similar viscosity and solubility, but lower water holding and fat absorption capacity, however the latter were comparable to that of WPC 60. The gelation properties of CPI under different conditions are reported here for the first time.

The second phase of the study involved the thermal modification of cowpea protein isolate (CPI) in solution by **a)** denaturation and **b)** simultaneous denaturation and glycation with endogenous sugars and carbohydrates via the Maillard reaction.

Changes in physicochemical and functional properties were determined and compared to that of SPI. Generally, glycated and denatured cowpea protein isolate (GCPI) exhibited better functional properties than denatured CPI and native CPI. GCPI showed improved solubility, emulsifying activity and stability, viscosity and foam stability whereas denatured cowpea protein isolate (DCPI) exhibited better water holding capacity, oil absorption capacity and gelation properties.

The third phase of the project studied the application of cowpea flour, CPI and modified CPI in bread, mayonnaise and cakes. The fortification of bread with 5% cowpea flour produced comparable textural and sensory properties to the control. The protein isolate (CPI) could be incorporated in bread to 2% and GCPI up to 4% without adversely affecting the bread physical properties such as crumb hardness and sensory attributes. CPI could be incorporated in cakes to 20% while both DCPI and GCPI could be incorporated to 40%. In mayonnaise, replacement of egg yolk with 20%GCPI resulted in similar textural properties to the control, however sensory evaluation reported a beany flavour. The effect of cowpea protein on pasting behaviour of rice starch was investigated. The addition of GCPI had greater effect on pasting behaviour of rice starch than CPI.

Dedicated to my family

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I, Mohamed A. Ahmed, hereby declare that I am the author of this thesis. All the work described in this thesis is my own except where stated in the text. Results presented in this work have not been used in any previous application for a higher degree. All sources of information have been consulted by myself and are acknowledged by means of references.

Mohamed A. Ahmed

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ABBREVIATIONS

AACC: American Association of Cereal Chemists

BSA: Bovine serum albumin

CF: Cowpea flour

CPI: Cowpea protein isolate

DCPI: Denatured cowpea protein isolate

dH_2O: Distilled H_2O

DSC: Differential scanning calorimetry

DTNB - 5,5′-dithio-bis(2-nitrobenzoic acid)

EDTA: Ethylenediaminetetraacetic acid

FTIR: Fourier transform infrared

GCPI: Glycated cowpea protein isolate

GD: Glycation degree

GS: Gel strength

IP: Isoelectric point

M: Molar

MW: Molecular weight

nm: Nanometre

O/W: Oil in water emulsion

OPA: O-phthaldialdehyde

PAGE: Polyacrylamide Gel Electrophoresis

pH: Power of hydrogen

rpm: Rotations per minute

RT: Room temperature (22 ± 3°C)

SDS: Sodium dodecyl sulphate

SH: Sulfhydryl

SPC: Soy protein concentrate

SPI: Soy protein isolate

Td: Denaturation temperature

TPA: Texture profile analysis

WHC: Water holding capacity

WPC: Whey protein concentrate

WPC 60: Whey protein concentrate (60% protein)

WPC-GOS: whey protein isolate-galacto-oligosaccharide

ΔH: Denaturation enthalpy

Chapter One

General Introduction

1.1. Food proteins and their importance in our diet

Proteins are complex, organic compounds composed of up to several hundred amino acids (**Figure 1.1**), and cross-linked between chains by sulfhydryl bonds, hydrogen bonds and van der Waals forces. There is a greater diversity of chemical composition in proteins than in any other group of biologically active compounds. The proteins in the various animal and plant cells confer on these tissues their biological specificity. Proteins are one of the essential nutrients in our diet and are vital to living muscle tissue. They have been considered as the building blocks of nutrition for the reason that they are broken down by means of digestive enzymes into amino acids which further help in building and repairing of body tissues (Ory, 1985). Therefore the key nutritional importance of proteins refers to their role in providing the amino acids for the body. The structures of the 20 amino acids that are commonly found in proteins are shown in **Table 1.1**. Fourteen of those can be created by our bodies, on condition that adequate levels of the necessary precursors are present in our diet. Nevertheless, there are eight amino acids which are impossible to be synthesised by the human body and therefore must come from food. These include: tryptophan, lysine, methionine, valine, threonine, phenylalanine, leucine, isoleucine, and for young children histidine which is believed to be essential to growing.

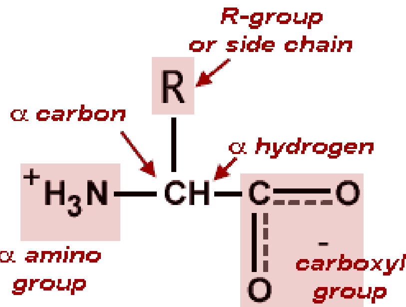


Figure 1.1 General structure of an amino acid. *Amino acids, with rare exception, contain α carbon that is connected to an amino (NH_3) group, a carboxyl group (COOH), and a variable side group (R).*

Consequently these eight are known as essential amino acids and must be provided in the diet in order for growth and health preservation. For this reason model food proteins would create foods which provide and preserve a perfect balance of amino acids in ideal forms which must be easy to digest and be absorbed into the human body.

Table 1.1 Structures and Abbreviations of the "Standard" Amino Acids of Proteins.

Name Three-letter Symbol, and One-letter Symbol	Structural Formula
Amino acids with nonpolar side chains	
Glycine Gly G	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-H$
Alanine Ala A	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-CH_3$
Valine Val V	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-CH(CH_3)_2$
Leucine Leu L	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-CH_2-CH(CH_3)_2$
Isoleucine Ile I	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-\overset{CH_3}{\underset{H}{C^*}}-CH_2-CH_3$
Methionine Met M	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-CH_2-CH_2-S-CH_3$
Proline Pro P	COO^-, H, C_2, $\overset{H_2}{C_3}$, CH_2 (4), CH_2 (5), N_1-H
Phenylalanine Phe F	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-CH_2-C_6H_5$
Tryptophan Trp W	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-CH_2-$ (indole, positions 1–7, $N-H$)
Amino acids with uncharged polar side chains	
Serine Ser S	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-CH_2-OH$
Threonine Thr T	$H-\overset{COO^-}{\underset{NH_3^+}{C}}-\overset{H}{\underset{OH}{C^*}}-CH_3$

***Table 1.1* (continued)**

Name Three-letter Symbol, and One-letter Symbol	Structural Formula
Asparagine Asn N	COO^- / $H-C-CH_2-C(=O)-NH_2$ / NH_3^+
Glutamine Gln Q	COO^- / $H-C-CH_2-CH_2-C(=O)-NH_2$ / NH_3^+
Tyrosine Tyr Y	COO^- / $H-C-CH_2-C_6H_4-OH$ / NH_3^+
Cysteine Cys C	COO^- / $H-C-CH_2-SH$ / NH_3^+
Amino acids with charged polar side chains	
Lysine Lys K	COO^- / $H-C-CH_2-CH_2-CH_2-CH_2-NH_3^+$ / NH_3^+
Arginine Arg R	COO^- / $H-C-CH_2-CH_2-CH_2-NH-C(-NH_2)=NH_2^+$ / NH_3^+
Histidine His H	COO^- / $H-C-CH_2-$(imidazole ring, NH^+, N–H) / NH_3^+
Aspartic acid Asp D	COO^- / $H-C-CH_2-C(=O)-O^-$ / NH_3^+
Glutamic acid Glu E	COO^- / $H-C-CH_2-CH_2-C(=O)-O^-$ / NH_3^+

* *Taken from Dawson, R.M.C., Elliott, D.C., Elliott, W.H., and Jones, K.M., (1986). Data for Biochemical Research (3rd ed.), pp. 1–31, Oxford Science Publications.*

The three-letter and single-letter abbreviations in common use are given

Moreover, proteins in food applications have important functions that are related to the physico-chemical characteristics necessary for lending fine product quality (Nakai, 1996). It is known that good taste, colour, aroma and texture play a very important role in determining the advantages of specific edible proteins in human food systems. In addition, there is a difference in the required characteristics for the different food applications. For example, in the case of comminuted meat, the absorption of

moisture is required and it should form a gel when heated; in the case of whipped topping, the required property is to produce a thermostable foam and in the case of beverages, protein solubility is required. Proteins from specific raw materials have traditionally been utilized in different applications. For instance, proteins from egg white are utilised to obtain a foamed texture and, egg yolk proteins are used for emulsification of oil and water.

1.2. Sources of proteins

Proteins are divided in terms of the source between animal proteins and vegetable proteins. In addition there is the so-called single-cell protein from microbial origin.

1.2.1. Animal sources

It is well known that animal proteins are an integral part of human diet and it has been demonstrated that there is a direct correlation between monetary income of people and their consumption of protein. Animal meat is considered a source of high quality protein because it provides enough essential amino acids which are necessary for the human body. However, due to the limited amount and high cost of production, animal proteins are highly priced so that they are not affordable in some parts of the world particularly in developing countries. Consequently, finding other sources of protein is needed in place of the animal proteins.

Egg, milk and meat are the main sources of animal proteins. Muscles are the main portions of the edible animal tissues, and they contain a high nutritional value protein. Meats and fish proteins provide us with sufficient amounts of lysine, methionine and tryptophan i.e. the essential amino acids.

Proteins from egg and milk symbolize a very valuable source of proteins because their essential amino acids content is high (Robinson, 1987). These are often used as reference proteins for nutritional purposes. Besides being consumed as traditional food types, these proteins have been subjected to modification and fractionation which lead to a variety of fractions of egg and dairy being commercially available (Pomeranz, 1991). For instance, currently there is a wide utilization of egg white and yolk in a variety of foods e.g. baked goods, salad dressing and ice cream etc., depending on their physico-chemical characteristics. Similarly, dairy materials have been fractionated and

processed to result in products such as casein, protein hydrolysates, lactose, fat and whey fractions currently applied in a variety of food products.

1.2.2. Single-Cell Proteins

Single cell protein (SCP) is a relatively new source of protein. Its production began in the late 1960s (Ugalde & Castrillo, 2002).The term typically pertains to the dried biomass product which is produced by microorganisms in culture systems. These potential novel protein sources (including algae, bacteria, fungi and yeasts) can be used as a food component or as a food for human consumption (Food Grade) and can be used as animal feed (Feed grade), although commercial production of SCP is presently limited. This is because of high operating and toxicological assessments costs. On the other hand, SCP production has a number of advantages such as the short generation time and high protein content obtained; as well as readily available raw materials which include wastes from other industries (Sadler, 1994).

1.2.3. Vegetable Proteins

According to the list of amino acids, there are two types of protein: complete and incomplete. Complete proteins are those that contain all the essential amino acids and are usually of animal origin; incomplete proteins are of plant origin and are those which lack one or more essential amino acids. An opportunity exists to obtain the complete set of necessary protein without use of animal products, achieved by careful combination of vegetable proteins. Consequently protein from plant origin can be considered is the most promising source because it is plentiful and has lower cost than animal protein.

Naturally, vegetable protein differs in terms of amount, type, and quality. Seed is the part of the plant that contains high protein such as soybean, cottonseed, sesame and peanut. Many kinds of seeds are presently processed on industrial scale in many parts of the world such as soybean due to its high protein content. After oil is extracted from soybean, protein is isolated from the remaining defatted products (Singh et al., 2008). There are other types of seeds in the leguminous family such as lupin (King et al, 1985), chickpea (Paredes-Lopez et al, 1991), and winged bean (Okezie and Bello, 1988), which are processed on industrial scale to isolate proteins.

Most legume proteins are relatively rich in lysine, so they are considered as high quality protein compared to that of cereals and other plant proteins. Even though these plants are poorer than animal protein in phytates and methionine, these essential amino acids could be supplemented.

Vegetable protein sources are many and varied, including leaf tissues, legumes, cereals and oilseeds. Globally, particularly in the developing world, roughly 90% of the protein intake and 88% of the human energy needs come from vegetable sources. (Salunkhe & Deshpande, 1991).

Cereals such as wheat, millet, rye, sorghum and rice are utilized in diets as an important source of protein worldwide and are characterised by a high level of starch, low lipid content, and medium level of protein (8-14%). On the other hand barley maize and triticale are used to feed animals, particularly in developed countries worldwide.

Oilseeds such as peanut, cottonseed, canola and sunflower are utilized worldwide as a source of edible oil and protein. Defatting procedures which may cause protein denaturation may be the reason for the limited use of these proteins, in addition to the existence of anti-nutritional components such as phytates, glucosinolates, trypsin inhibitor and phenolics.

Humans have used some three thousand species of plants in their diet and at least one hundred and fifty species are commercially produced for use. Most of the world's population depends on about twenty different plant crops, which are in general divided into cereals, legumes, nuts etc. In the context of human protein nutrition, the most important vegetable proteins are cereal grains and food legumes (Young and Pellett, 1994).

Table 1.2 shows the supplies of protein that are derived from plant or animal sources. On an international basis, plants provide ≈65% of the supply of edible protein worldwide. Meanwhile animal products provide ≈25%. However, there are clear differences in per capita protein consumption of animal protein sources between the developing and developed regions (Table 1.2; FAO/Agrostat, 1991). For example in the Middle East approximately 17% food protein consumed is derived from animal

products, whereas for the populations of the North America approximately 73% of food protein consumed is derived from animals (Young & Pellett, 1994).

Table 1.2 The world protein supplies per capita per day for selected regions*.

	Carbohydrate (%)	Animal protein (%)	Plant protein (%)	Energy (Kcal)
Region				
Developing	73	14	77	2846
Far East	75	11	81	2450
Middle East	67	17	78	2954
Africa	72	12	79	2363
Latin America	65	29	57	2732
Economic class				
Least developed	76	9	83	2058
Low income	74	11	81	2409
Developed	53	61	42	3417
Western Europe	51	60	42	3457
North America	48	73	34	3650
Oceania	50	66	33	3240
World	67	25	65	2710

**Based on FAO/Agrostat (1991).*

1.3. Development of legume grains as a source of proteins

The term legume is widely used this study and refers to the grains from plants belonging to the Leguminosae family. Interest in legume grains as protein sources has been steadily increasing due to the expanding population, health concerns, varying levels of income and religious beliefs (Chrtková, 1983). Legumes are considered a source of both protein and energy in many countries. Advantages are that they can be stored for a long time, even in the case of poor environmental conditions; their processing requires minimum equipment; and they can be easily transported.

Beans are a common name for large plant seeds of the genera Fabaceae (formerly Leguminosae). Consumption of beans is higher in parts of the world where animal proteins are scarce and expensive e.g. African, Asian and Latin American countries (Agbenorhevi et al., 2007; Ofuya, 2006; Rayus-Duarte et al., 1998).

Intensive research has been undertaken on soybeans during the past decade. They are a valuable commodity, from both nutritional and economic standpoint because of their high protein and oil content. As well as traditional use, soybeans have been widely used as processed products, as either protein isolates or concentrates or as oil added to

an assortment of foods. Comparatively little has been done to enhance the production, quality and yield of other grains such as beans, lupines and peas.

The Food and Agriculture Organization (FAO) reported that there are 5 minor and 10 primary pulses, which are cultivated in over 105 countries. Globally, pulses are the most important crops after cereals. Regarding production, cowpea (5.7 metric tons (mt)) along with other pulses such as dry beans (19.7 mt), chickpea (9.7 mt) and lentil (3.6 mt) are the most important (FAO, 2010). From an area of 70.6 million ha worldwide, production of pulses in 2009 was 61.5 million tons with a yield of 871 kilogram per hectare. Dry beans contributed most of this global pulses production with about 32% production followed by another pulses such as dry peas, chickpea and broad beans etc. in which cowpea took sixth place in the production (Nadarajan, 2011). Less-developed countries contribute about 74% to the pulses production and 26% is derived from developed countries (Aski et al., 2013).

Cowpea (Vigna unguiculata L. Walp) is a type of bean commonly known in Arabic countries and internationally as Lubia. It is widely grown in most tropical regions and in sub-Saharan Africa as an intercrop with other legumes and cereals (Singh & Rachie, 1985). It is an ancient grain legume crop, which is believed to have originated in Africa, Asia and even South America and became later introduced to the Indian subcontinent (Allen, 1983). Cowpea is one of the common names in English and it is also known as Southern pea, black-eye pea, niebe, crowder pea, frijole and coupe (Craufurd et al., 1996; Hall et al., 1997; Islam et al., 2008; Taiwo, 1998). All these common names are scientifically identified as Vigna unguiculata (L.) Walp, also known as Vigna sinensis (L) in older references (Gómez, 2011). **Figure 1.2** shows cowpea crop grown in Libya.

Figure 1.2 Cowpea (Vigna unguiculata L. Walp) crop grown in Libya.

Cowpea is a drought tolerant crop and is an important grain legume throughout the tropics and subtropics, covering many countries in the world. In the year 2003, about 12.4 million hectares of land was used to cultivate cowpeas globally, with Central and West Africa contributing about 8 million hectares (Mokgope, 2007). World cowpea production was 3,721,850 metric tons (mt) during the year 2003, with Africa and Asia contributing 90% and 7.6% respectively (FAO Statistical Database, 2004). In Nigeria, for example, cowpea grain production has increased substantially from 2,150,000 metric tons (mt) in 2000 to 2,815,000 mt reported in 2005 (**Table 1.3**).

Table 1 .3 Cowpea production in Nigeria (2000–2005)

Year	Production (mt)	Area (ha)	Yield (kg/ha)
2000	2,150,000	3,583	600
2001	2,172,000	3,620	600
2002	2,311,000	3,669	630
2003	2,459,000	3,726	660
2004	2,631,000	3,987	660
2005	2,815,000	4,141	678

Source: (FAO, 2010)

There are several varieties of cowpea seeds. Factors such as seed cultivar or variety and origin affect properties such as seed colour and seed composition (Taiwo, 1998). An example of cowpea seeds is shown in **Figure. 1.3**.

Figure 1.3 Cowpea beans (Black eyed kind).

Cowpea is a member of the starchy legumes, which are known as pulses and forms part of a staple diet in most African and Asian countries (Aykroyd & Doughty, 1964). It contains 18.3-35.0% protein and 31.5-48.0% of starch (Chavan et al, 1989) and has been consumed by humans since the earliest practice of agriculture (Phillips & McWaters, 1991). It is consumed as boiled foodstuff using fresh or rehydrated seeds or processed into flour to make other food products.

This kind of legume is the most economically important indigenous legume crop. (Langyntuo et al., 2003). In view of increasing cultivation of cowpea globally, there is need for increased utilization of cowpea, especially the nutritious cowpea seed or bean. In the present study cowpea beans are therefore being investigated as an additional source of protein concentrate and isolate for use in human food products.

1.4 Cowpea bean structure and composition.

The cowpea bean is made up of cotyledons, germ and a seed coat and hilum (Chavan et al., 1989). The beans may vary in colour, shape and size. The size ranges from 2 to 12 mm long, the weight ranges from 5-30 g/100 beans and the shape is globular to kidney shaped (Chavan et al., 1989). The bean coat colour ranges from white, purple to black. The seed composition, predominantly the proteins and starch varies considerably according to cultivar and seed origin (Taiwo, 1998).

The nutrient content of cowpeas varies mainly because of genetic background as well as climate, fertilisation, season and agronomic practices (Kochhar et al., 1988). Most of the nutrients are concentrated in the cotyledons as they make up most of the bean weight. The proximate composition of cowpeas is shown in **Table 1.4** (Chavan et al., 1989).

Table 1. 4 Proximate composition of cowpeas*

Constituents	Range (%)
Crude Protein (N * 6.25)	18.3-35.0
Crude Fat	0.7-3.5
Crude Fibre	2.7-7.0
Starch	31.5-48.0
Ash	2.5-4.9

**From Chavan et al. (1989)*

Because of the potential of cowpeas as an inexpensive source of significant amounts of protein, calories, and B vitamins such as folacin, niacin, and riboflavin, it should be considered as a valuable food ingredient.

To incorporate a new source of protein into food, its functional properties (solubility, water and oil binding properties, foaming and emulsifying properties, etc.) need to be evaluated because they can help predict the behaviour of protein ingredient and provide criteria to choose a suitable food product. Such information would help to restrict the amount of tests on a large scale (Hermansson, 1979). Currently, utilization of protein from cowpea is limited, unlike that of soybean, partly due to lack of knowledge as well as its inferior quality in the crude form. Up to now there have been some studies about cowpea protein, most of which are reported in the present introduction. However, information regarding potential food applications of cowpea protein is still limited.

1.5 Protein composition of cowpea seeds.

For the classification of plant storage proteins, Osborn`s method is still used. This depends on the relative solubility of the target protein in standard solvents (Osborn, 1924). Seed storage proteins were initially classified according to their solubility, into albumins (water soluble), globulins (saline soluble), prolamins (alcohol soluble) and glutelins (acid or alkali soluble).

Some studies have focused on the protein composition of cowpea seeds. A study on the conditions for the extraction of cowpea proteins was reported in 1979 (Sefa-Dedeh, and Stanley, 1979a), followed by a preliminary characterization of the water-extractable proteins (Sefa-Dedeh, and Stanley, 1979b). A major globulin was first identified in 1957 (Joubert, 1957). This 7S or vicilin-like fraction (G-1), termed vignin, was subsequently shown to be a heterogeneous globulin of about 170 kDa (Cerdeira et al., 1985; Khan et al., 1980). Vicilin from cowpea has been shown to strongly associate with chitin, chitosan, and fully acetylated chitin (Sales et al., 1996), indicating a potential antimicrobial function. Most of the bean protein is found in the globulin fraction, which constitutes 72% of the extractable protein, (Murray et al. 1983). Therefore, this fraction, as the major seed protein component in cowpea, is also responsible for the nutritional value of the seed (Bressani, 1985). This confirms the finding that globulins are the major protein component in the storage tissues of legume

cotyledons (Derbyshire et al., 1976). **Figure 1.4** shows protein composition of cowpea cotyledons (Freitas et al., 2004).

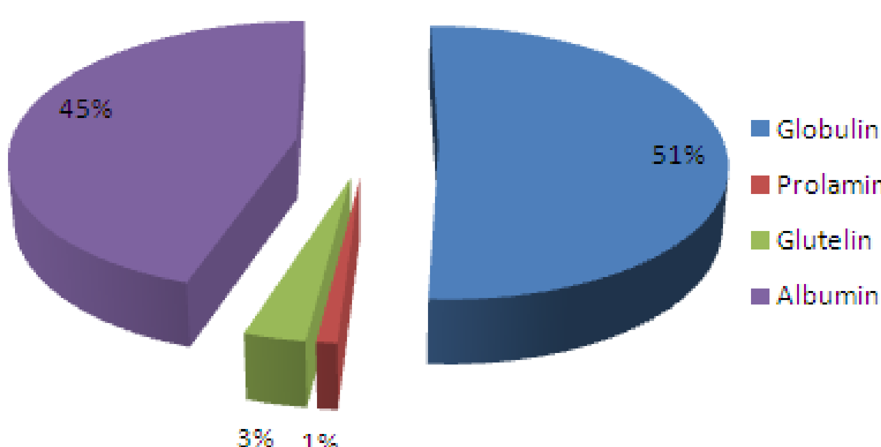


Figure 1.4 Protein composition of cowpea cotyledons (Freitas et al., 2004)

1.6 Health benefits of cowpea beans

Beans have long been known as a good source of vegetable protein, dietary fibre, minerals (P, K, Ca, Mg) and vitamins (thiamine and niacin). There is considerable evidence in the literature that beans and other food products high in water-soluble dietary fibre are able to lower blood cholesterol (Uebersax et al., 1991). Anderson (1985) reported that bean products can reduce serum cholesterol levels. Consumption of 100-135 g of dry beans per day reduced serum cholesterol by about 20% thus reducing the risk of heart disease by 40%. Nutritional therapy combining fibre foods with bean is tolerated and related with no major side effects, with the exception of a reported increase in eructation and flatulence. Moreover being a good source of soluble and insoluble dietary fibre and having the health- related benefits associated with these types of dietary fibre, beans have an added benefit of being a low-cost fibre source. Cowpea is high in complex carbohydrates and low in fat. These make it ideally suitable to helping consumers meet the dietary goals of increasing the intake of complex carbohydrates and reducing fat intake (Huges, 1991).

1.7 Nutritional qualities of cowpea proteins

Cowpea is a good source of crude protein being rich in glutamic acid, aspartic acid and lysine (Prinyawiwatkul et al., 1996). The quality of protein depends on its essential amino acid composition, bioavailability and digestibility of amino acids (FAO/WHO, 1990). Cowpea proteins digestibility could be improved by heating which inactivates antinutritional factors such as protease inhibitors, amylase inhibitors and phytic acid (Umoren et al., 1997). Conversely thermal processing of vegetable protein products can produce oxidized forms of sulphur amino acids (such as methionine sulfone, methionine sulfoxide and cysteic acid), Maillard compounds, D-amino acids, and cross-linked forms of amino acids like isopeptides, lanthionine and lysinoalanine, which reduces the protein digestibility and amino acid bioavailability with lysine being the most sensitive amino acid to nutritive damage (Papadopoulos, 1989; Gilani et al., 2005).

Many methods exist to measure protein quality. The protein efficiency ratio (PER) is the standard to measure protein quality. This method involves the feeding of rats a test protein and determining the weight gain/protein consumed (g/g). Hoffman and Falvo (2004) reported that nutritional value of protein is regarded as excellent if it has a PER higher than 2.5. Cowpea protein has PER values of 1.34-1.84, depending on the varieties (Umoren et al., 1997). Some types of processing could enhance the PER value of cowpea protein. For example, El-Niely (2006) reported that irradiation significantly enhanced PER values of cowpea protein, in a dose-dependent manner. Umoren et al. (1997) reported that autoclaving substantially increased PER values of cowpeas to 1.94-2.56.

Net protein utilization (NPU) and biological value (BV) are biological methods to measure the nitrogen retention, the difference being that NPU is calculated from nitrogen ingested whereas BV is from nitrogen absorbed (Umoren et al., 1997). It has been also reported that NPU and BV of raw cowpeas range from 34.9-40.7 and from 39.5-48.9 respectively. Umoren et al. (1997) reported that autoclaving significantly increased the NPU and BV of cowpeas to 44.9-52.9 and 48.4-58.2, respectively.

1.8 Utilisation of cowpea as a protein and energy source

Legumes serve as alternatives or supplements to animal proteins, particularly in parts of the world where there is a scarcity of animal proteins due to socioeconomic

constraints. Cowpeas are a nutritious legume crop that is of considerable importance in Nigeria and other Sub-Sahelian countries. They constitute a significant proportion of the total dietary protein and energy intake of peoples in many parts of Africa and worldwide (Ologhbo and Fetuga ,1987; Davio and William, 1976). Proteins utilized in food processing are of various origins, and can be roughly grouped into animal proteins (gelatine), vegetable proteins (e.g. soya protein), and animal-derived protein (milk proteins) (Ogunwolu et al., 2010). According to Moure et al (2006), proteins that are essential to growth and health are required in the developing countries of the world, because of the chronic problem of protein-energy malnutrition.

Shortages and high prices have recently caused restriction of animal proteins in the diets of many families in the developing countries of the world. However, vegetable proteins that are cheaper and available are of great potential as a direct food for human consumption. Many of the vegetable proteins require processing to provide a food material that has acceptable organoleptic properties for human consumption (Cherry et al., 1975). According to McWatters and Cherry (1981), protein components of plants are regarded as important source of ingredients in food systems, because of their unique functional properties, such as emulsification, fat and water absorption, texture modification, colour control and whipping properties. Ragab et al. (2004) reported that emulsifying and foaming properties of cowpea protein are higher than those of other vegetable proteins. Moreover, water-, fat-holding capacities, and other properties are good and therefore, it can be used in food formulation systems.

In Africa cowpea use is limited primarily to boiled whole seeds or traditional food preparations such as akara (**Figure 1.5**) and moi-moi prepared from cowpea flours or pastes, the quality of which is determined by the physicochemical properties of the proteins and starches. By creating variety in the foods made from cowpeas, its utilization may be enhanced. Thermal processing and irradiation can change the physicochemical properties of cowpea proteins and starches and possibly lead to modification of functional properties of cowpea flours and pastes. Such potential modification may enable a wider application of cowpea flours and pastes in food systems. In many parts of Africa, cowpea is prepared for consumption in grain, split and ground forms. The ground form has traditionally been a favourite of rural

households in northern Ghana because cowpea flour is less susceptible to post-harvest pest damage and can be used in many different dishes (Nyankori, 2002).

Figure 1.5 Akara (cowpea fried food)

1.9 Cowpea flour production

Despite their potential as a low-cost source protein, cowpeas are not optimally utilised in most advanced countries largely due to difficulty in raw material processing (McWatters et al, 1992). Although cowpea bean grains are typically consumed in combination dishes or alone as a boiled vegetable, substantial interest has developed in recent times in increasing usage of cowpea grains in other forms of food (McWatters, 1990).

Successful production of legume flours depends upon the technique of their preparation which affects the end product. Legume flour is a product prepared from ground legume meal after defatting, having protein content of 50-65% (Codex Alimentarius Commission, 1989). High quality legume flours can be prepared by using a double drum drier. Removal of the seed coat from dark-coloured or highly pigmented cowpea varieties is needed prior to flour production to avoid dark specks that are not agreeable for many uses (Beuchat et al., 1985).

Mass preparation of cowpea flour from dry grains is a simpler technology than that used for soybean and other oilseed flour preparations (McWatters, 1990). However the defatting step required for the preparation of flour from peanut seeds (Prinyawiwatkul et al., 1993) and soy bean grains is not necessary for cowpea grains because of its low fat content. Cowpea flour in Africa is usually sold whole or mixed with other flours, mainly in bulk or packaged in unlabelled packets (Nyankori, 2000), similar to that in **Figure 1.6**.

Figure 1.6 Packed unlabelled cowpea flour

Ashaye et al. (2001) prepared cowpea flour (**Figure 1.7**) by sorting cowpea grains to remove extraneous material, rinsing, soaking (20 min), dehulling by washing with water, drying the cotyledons (24 h, 55°C) and grinding of the dry cotyledons in a hammer mill.

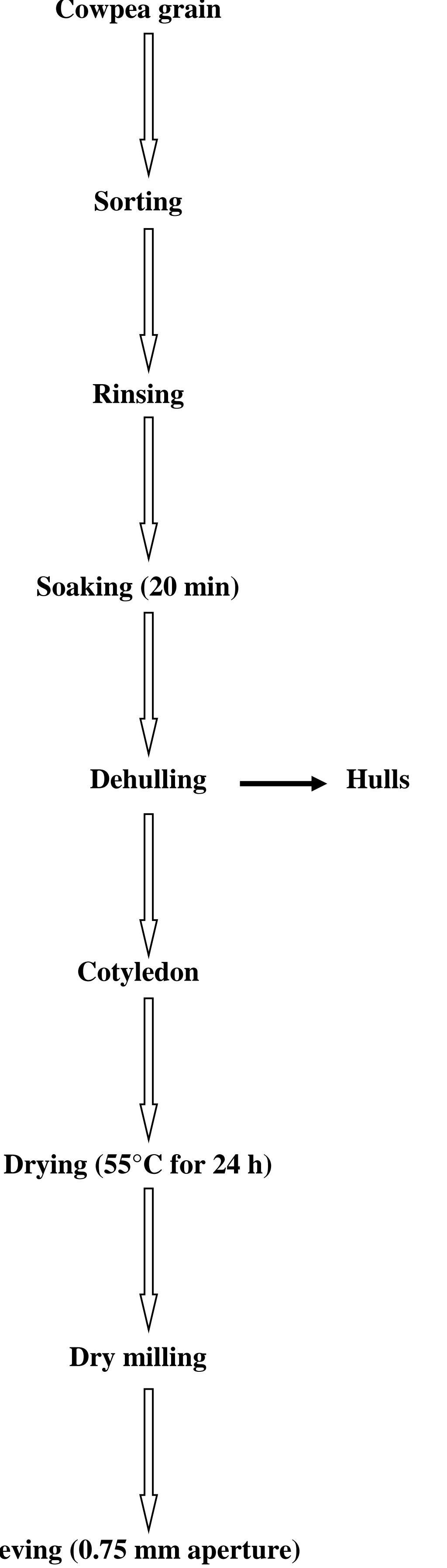


Figure 1.7 Preparation of cowpea flour (taken from Ashaye et al., 2001)

1.10 Uses of cowpea flour in foods

Like peanut and soybean flours, cowpea flour is high in protein, therefore representing a good source of nutrition and potential functional food ingredient in many products such as macaroni, cookies, bread, biscuits, chips, extruded snacks, weaning foods, etc. For less-developed countries that depend on imported wheat flour, cowpea flour would have additional economic advantages when used to partially replace wheat flour in different food products (Okaka and Potter 1977). The use of cowpea flours in bakery products provides a means for enhancing the nutritional quality of these wheat flour based foods without sacrificing the quality or palatability of the product It has been reported that the use of 10% or more drum dried cowpea flour for supplementing bread resulted in significantly decreased loaf volume (Okaka and Potter, 1977). However, addition of surfactants (calcium or sodium stearoyl-2-lactylate) reduced the effect but the addition of 30% of cowpea flour was not acceptable even with the addition of calcium or sodium stearoyl-2-lactylate. Conversely Mustafa et al. (1986) reported that the replacement of 10% wheat flour by non-decorticated cowpea flour resulted in acceptable bread and increased loaf volume; but when the addition of cowpea flour was increased to more than 10%, the loaf volume decreased and the distinct beany flavour was strongly imparted.

Supplementing bread with non-fermented and fermented cowpea flour increased the protein content of bread from 10.8% to 13.2% and 14.2% respectively without any appreciable difference in loaf volumes and with a slightly darker colour compared to that of the control (Lu and Sanni-Osomo, 1988).

Addition of 0-24% of cowpea flour to tortillas (unleavened), to replace wheat flour, resulted in acceptable tortillas without any appreciable difference in quality compared to that of a control with wheat flour only (Holt, 1990).

Biscuits (McWatters, 1980) and sugar cookies (McWatters, 1978) were supplemented with 10-30% non-decorticated cowpea grains flour without significant difference in dough handling characteristics and baking properties compared to the control.

The use of 10%, 20% and 30% cowpea meal in buttermilk doughnuts resulted in acceptable sensory qualities compared to those of 100% wheat flour products. Batters

including cowpea meal were well suited to mechanical cutting and frying, but they absorbed too much oil during the frying process, which could be reduced by using cowpea fine powder flour instead of cowpea meal. The use of soy flour in doughnut formula also enhanced the sensory qualities (McWatters, 1982).

Muffin (chemically leavened quick bread) containing 43% cowpea flour to replace soft wheat flour was successfully prepared by Holt et al (1992).

Chinese noodles were prepared by Chompreeda et al. (1988) by using 4-12% cowpea flour with 7-21% defatted peanut flour. The increase of cowpea flour level up to 21% produced a product with acceptable sensory quality and increased protein content to 21%. Cowpea meal was used to prepare yoghurt-like products and imitation milk (Schaffner and Beuchat, 1986) and McWatters (1977) prepared ground beef patties with 5% cowpea flour supplementation and the sensory quality compared favourably with controls.

Prinyawiwatkul et al (1996) evaluated extruded cowpea flour alone and/or mixed with other legume flours for nutritional and functional qualities, total and reducing sugars, and available lysine and reported that the products were more nutritious, stable, and more convenient than traditional products. However due to high protein content in cowpea flour, it was difficult to puff it in pure state by extrusion.

Snack foods including snack chips have been studied as novel and inventive products to increase the consumption of cowpea (Phillips et al., 2003). Snack chips prepared from cowpea, wheat flour and cornmeal were positively accepted by West African consumers, however not accepted by American consumers who were unfamiliar with the cowpea flavour (Ward et al., 1998). Researchers of Bean/Cowpea Collaborative Research Support Program (CRSP) have investigated cowpea flour as an ingredient in weaning food products for poor countries in need of foods for malnourished children. It has been found that the composite weaning mixtures with cowpea and maize and either peanut or soybean flours that were cooked by extrusion successfully supplied enough protein for weaning children (Mensa-Wilmot et al. (2001).

1.11 Effect of processing on nutritional quality of cowpea protein isolate

Cowpea has a low fat content, and for this reason defatting treatment has only a small effect in increasing protein content in the protein extraction process. It is known

that protein concentrate yields increase when removing non-protein fractions such as carbohydrates, soluble minerals and low molecular weight nitrogen compounds from full fat or from defatted meals or flours at neutral or acidic pH. The preparation of protein isolates consists of an aqueous solubilisation of protein and carbohydrates at neutral or in alkaline medium and the selective recovery of the solubilised protein by precipitation at isoelectric pH of 4, separation by centrifugation to concentrate precipitated protein, washing and neutralization by adjusting the pH to neutral pH before drying (L'hocine et al., 2006; Moure et al., 2006). In general protein isolate has higher protein content than that of concentrate and defatted flour. The preparation and extraction process affects the efficiency of protein extraction. The researchers explained that the higher fat content leads to decrease in the efficiency of protein extraction from defatted flour because of the formation of an emulsion during extraction.

Different amounts of protein fractions with different solubility characteristics can be produced from cowpea grains, depending on the variety and amount of extraction aqueous media used. Globulin protein fractions are pointed out to be the major cowpea grain protein, ranging from 48 to 90%. The remaining soluble fractions are albumins, glutelins and prolamins with varying concentrations (Chavan et al., 1989). Each protein fraction can also be divided into subunits with different molecular masses. It was mentioned that heating of cowpea grain proteins substantially decreases the yield a of globulin and albumin protein fractions with associated increase in glutelin content. (Nugdallah and El Tinay, 1997).

1.12 Process for preparation of cowpea protein concentrate

The generic term "concentrate" is customarily used for products having the content of protein of 65% to less than 90% (Codex Alimentarius Commission, 1989). Cowpea protein concentrate could have a protein content as high as 82% (Aremu, 1990) and can be prepared by following the same methods of production of protein concentrate as used for other legumes. For example, commercial soy protein concentrate can be produced by three basic processes as presented in **Figure 1.8.** These processes aim to remove water-soluble non-protein constituents from defatted soybean flour or flakes by aqueous ethanol (60-80%) extraction, acid leaching at isoelectric of pH 4.5 and moist heat-water leaching. These processes render the proteins insoluble, facilitating the removal of soluble carbohydrates by centrifugation. The precipitated protein with remaining

carbohydrates are dispersed in water, and adjusted to pH 7.0, followed by spray drying to produce protein concentrates.

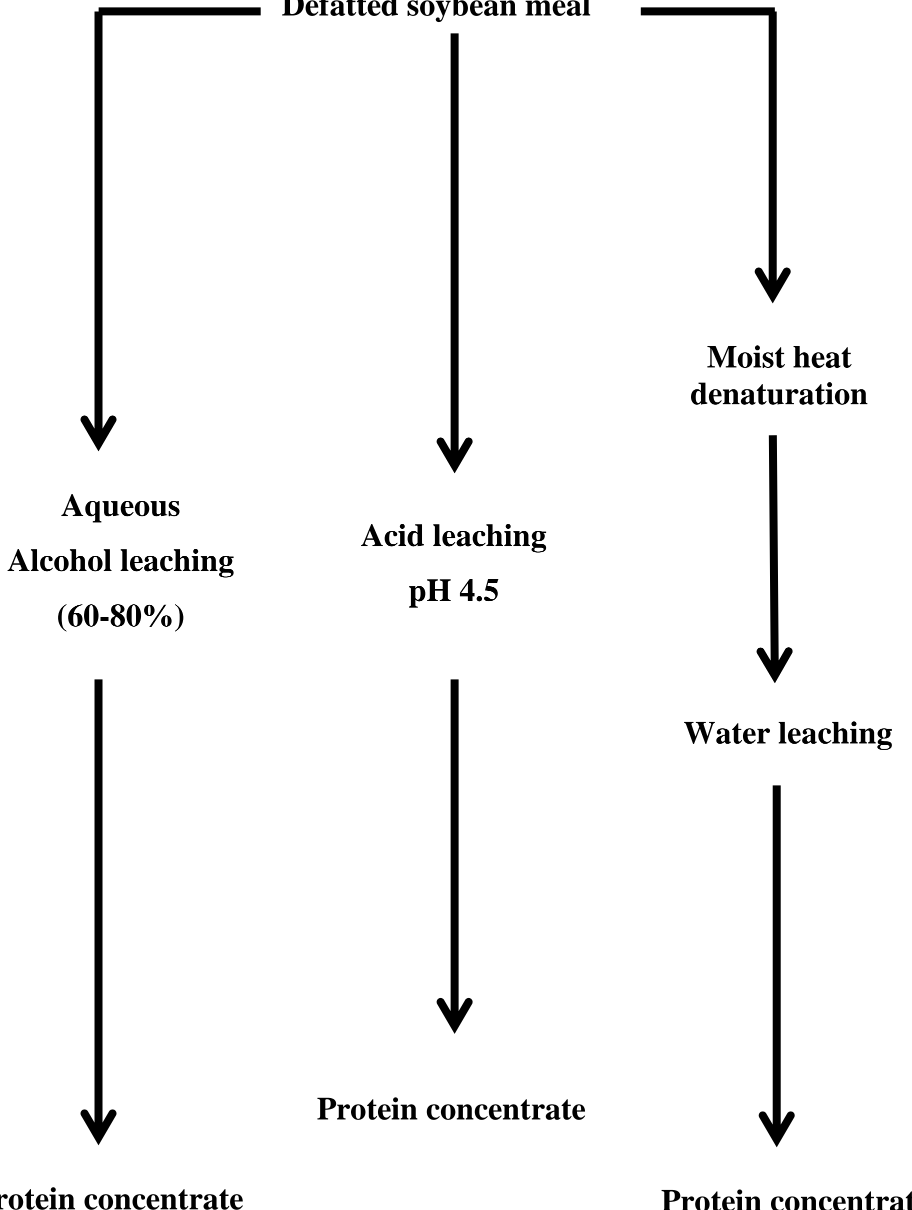


Figure 1.8 Process for the preparation of soy protein concentrate (taken from Circle and Smith, 1978)

Cowpea protein concentrate was produced from cowpea seeds as a by-product of starch production (Tomalsquim et al., 1971; Olvera-Novoa et al., 1997) as shown in **Figure 1.9**.

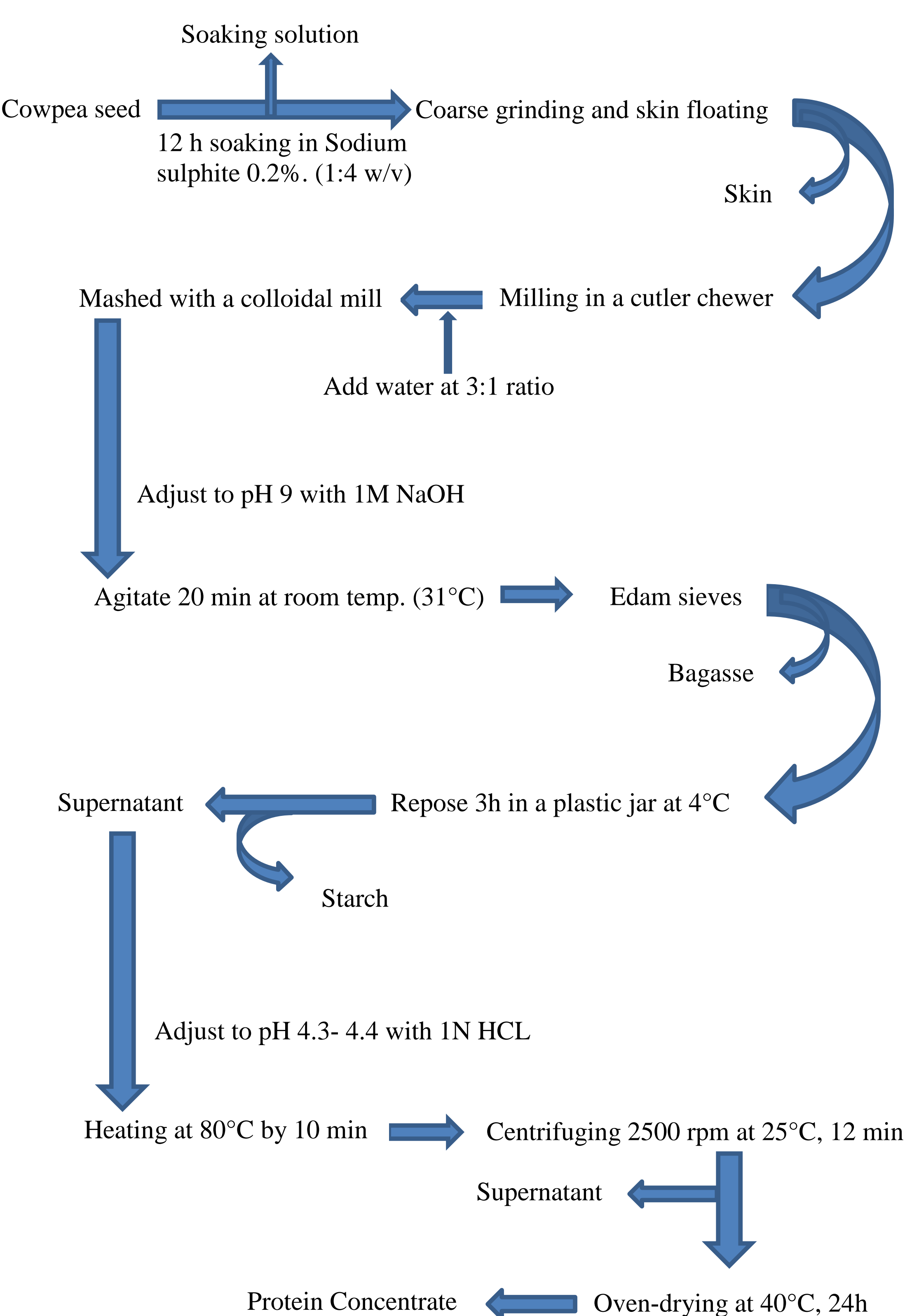


Figure 1.9 Process for preparation of cowpea protein concentrate (CPC) from seeds (Olvera-Novoa et al., 1997)

1.13 Process for preparation of cowpea protein isolates

Protein isolates (ca. 90% protein) is an enriched protein product that can be prepared by wet methods. These methods were originally developed for preparation of soybean protein isolates (L'hocine et al., 2006). In order to prepare protein isolates, after defatting of legume flakes or flour, the protein is solubilised using dilute alkaline solution. The insoluble material such as oligosaccharides and minerals is removed by subsequent centrifugation. Acidification of the supernatant by using food grade acid such as hydrochloric acid (pH 4.5) causes precipitation of the proteins iso-electrically. The curd protein is subsequently washed to remove the non-protein solubles. The recovered protein is neutralized (pH 7) and spray dried. **Figure 1.10** shows the flow chart for the production of protein isolate from soybean (L'hocine et al., 2006).

Liu (1996) and Gueguen (1983) reported that flour particle size and other factors such as the type of the solubilising agent, pH of solubilisation and pH of precipitation, affect the protein isolate yield prepared by isoelectric precipitation processes. A decrease in protein yield was demonstrated when flour with larger particle sizes was used, compared to that of smaller particle sizes. Gueguen (1983) found that when sodium and potassium hydroxide are used to solubilise protein, similar yields of protein are recovered. On the other hand, calcium hydroxide was shown to solubilise less than 10% of pea protein, apparently because of the "salting-out influence of the calcium ions. Sodium hydroxide (NaOH) is the most common reagent utilized to solubilise plant proteins in the food industry.

The protein isolate products can be dried using several techniques such as drum, spray and freeze drying. Drum-dried and freeze-dried isolates have been pointed out to have dark colour, while spray-dried isolate has light colour and taste. The darkening of products dried by freeze-drying is due to polyphenol oxidation while the darkening of products dried by drum-drying is due to the Maillard reaction (Sumner et al., 1981).

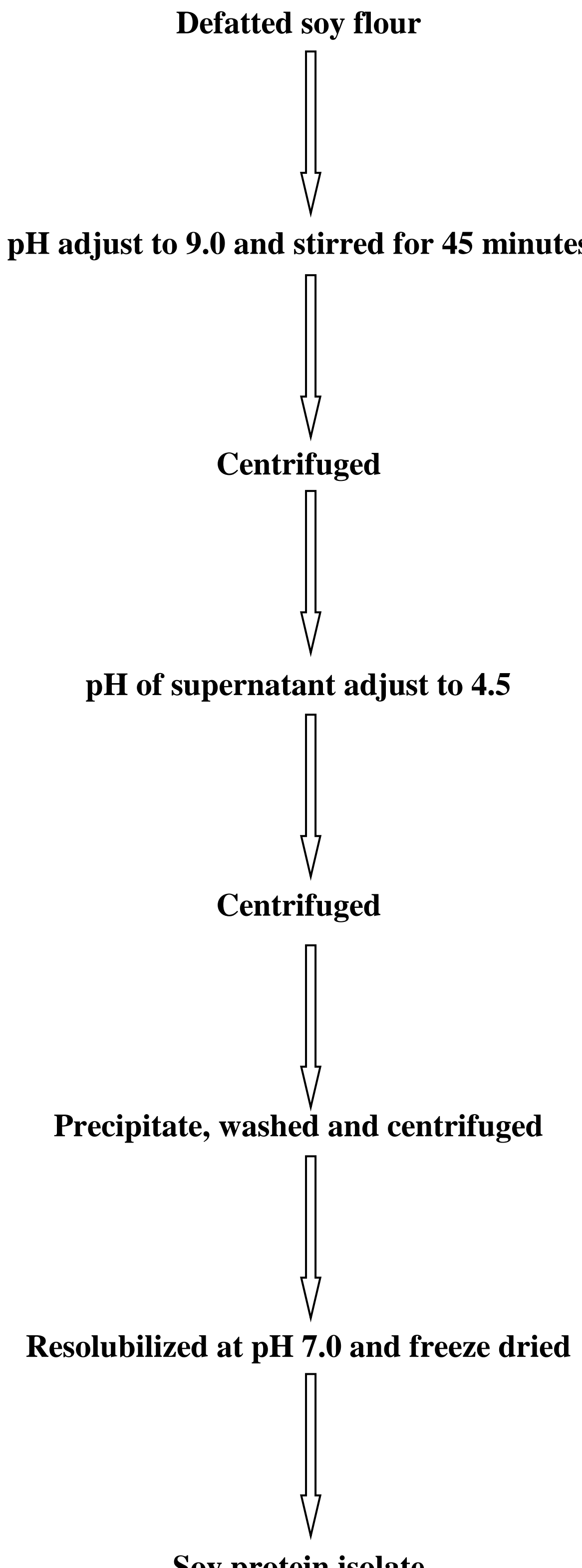


Figure 1.10 Process for soy protein isolate production (L'hocine et al., 2006).

1.14 Functional properties of pulse proteins

Functional properties of plant proteins determine their potential applications in food, which are in turn determined by their physicochemical characteristics (Hermansson, 1979). There are many definitions for functional properties of proteins but they have similar meanings. For example, Pour-EI (1981) has defined functional properties of food proteins as any characteristic of a food that affects its use. Kinsella and Melachouris (1976) defined functional properties as "those chemical and physical propertie which affect the behaviour of food proteins in food systems during preparation, storage, and consumption". Functional properties of food proteins such as solubility, texture, viscosity, water- and fat-binding, foam, emulsion, and gel characteristics are of special interest. **Table 1.5** shows a summary of functional properties which important for food proteins.

Table 1.5 Functional properties of food proteins important in food systems

Property	Functional criteria
Hydration	Gelling, solubility, swelling, syneresis, thickening, water absorption, wettability
Organoleptic	Colour, flavour, grittiness, mouthfeel, odour, smoothness, texture
Surface	Emulsification, flavour binding, foaming (aeration-whipping), lipid binding, protein-lipid film formation
Structural/ rheological	Adhesion, aggregation, chewiness, cohesiveness, dough formation, elasticity, extrudability, fibre formation, gelation, grittiness, network cross-binding, stickiness, texturisability, viscosity
Other	Compatibility with additives, enzymatic antioxidant

Taken from Kinsella (1979)

The functional properties that affect the function of food proteins in food applications are adhesion or cohesion, aeration or foamability, coagulation, colour, flavour, gelation, emulsification, solubility, texturization, viscosity, water and oil

absorption. Of these, foamability, solubility and emulsification are common to investigate since these properties form the basis of various food systems. When new or improved food proteins are used in conventional foods, it is very important to investigate the functional properties of the produced protein to determine in which kind of food they can be supplemented and be used successfully. Consequently, certain important properties of proteins are now discussed.

1.14.1 Solubility

The solubility of a protein determines its ability to associate with water and is a function of many parameters including solvent, pH, temperature and ionic strength. Protein solubility is often the first property determined at each stage of preparation of a protein ingredient. Such investigations as the solubility profile as a function of pH, of heat treatment, and of ionic strength are the most often used. Solubility properties under different conditions are frequently useful in measuring the functional application of proteins as well as in the optimization of extraction and processing procedures of proteins (Rupnow, 1992). Factors that affect solubility include protein structure and composition, conditions and methods of extraction, processing, or treatments used to enhance proteins, influence protein foamability and emulsifying properties (Cherry, 1990). Most of the alpha-helices of globular proteins are amphiphilic (Mandel-Gutfreund and Gregoret, 2002; Eisenberg et al., 1982). It is known that alpha helices in globular proteins usually consist of two types of residues, hydrophobic and hydrophilic, with the number of each type being roughly equal (Chou et al., 1997). Solubility is affected by many factors, such as ionic hydration, hydrophobic residues, pH, charges, electrostatic repulsion, and denaturation, (Moure et al., 2006).

Figure 1.11 depicts protein solubility at high pH, isoelectric pH and low pH. In the pH-solubility profiles, cowpea proteins exhibit a u-shaped curve in which the minimum solubility of proteins is observed at pH 3.5 to 5.5 and maximum solubility at alkaline pH (Horax et al., 2004; Ragab et al., 2004). Changes in pH have their greatest disruptive effect on hydrogen bonding and salt bridges. For example the polypeptide polylysine is composed entirely of the amino acid lysine which has an amino group on its side chain. At acidic pH all of the side chains are positively charged and they repel each other, causing the molecule to uncoil. At basic pH, however, the side chains are

neutral, they do not repel, and the molecule does coil into an alpha-helix (Vojisavljevic, 2007).

The degree of insolubility reflects the extent of aggregation and denaturation of protein that can influence its emulsification, hydration, foaming, and gelling properties. While partial aggregation or denaturation may enhance certain properties, initial solubility of proteins helps the homogeneous dispersion through the aqueous phase or emulsion and is a necessity for their utilisation in beverages (Rupnow, 1992; Kinsella, and Melachouris, 1976).

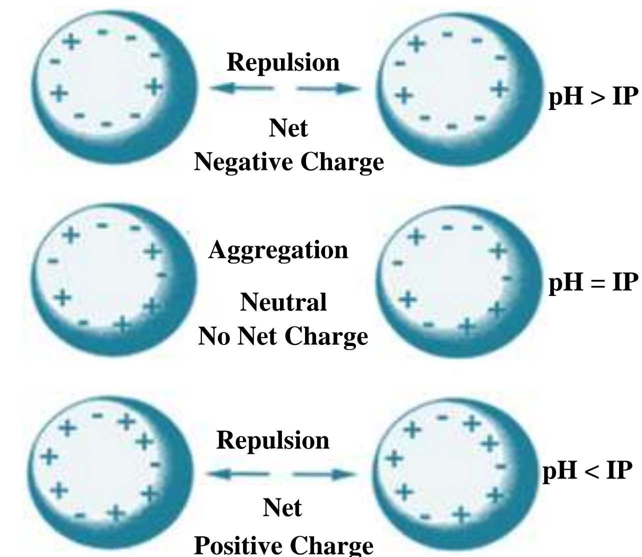


Figure 1.11 Protein at high pH, isoelectric pH and low pH

Protein solubility is seemingly the most important functional property in foods. Good solubility of protein is frequently considered to be a prerequisite for its performance in many food products (Kinsella and Melachouris, 1976). For example, for beverages, salad dressings and soups the solubility of the proteins is maybe the most important measure. Moreover, to get best functionality in foods that need emulsification, foaming and gelation properties, a comparatively soluble protein is also required (Damodaran, 1996). Because of the importance of the solubility of proteins in various food applications, it is relevant to make sense of the intrinsic and extrinsic factors that influence their solubility (Damodaran, 1996).

1.14.2 Water and oil binding capacity

In food systems protein can interact with water and oil due to its hydrophilicity and hydrophobicity. Water holding or absorption capacity of protein is its ability to absorb as well as retain water in various foods such as doughs and comminuted meat products (Kinsella and Melachouris, 1976; Rupnow, 1992). Water holding or absorption is commonly determined as the amount of water retained by a protein powder sample after blending with water and emptying the excess water by a centrifuging technique. Zayas (1997) reported that water holding capacity of protein is its ability to hold its own and added water through the application of force. Likewise, oil binding capacity is the determination of protein-oil interaction which is also necessary in various foods such as doughnuts, meat and sausages.

Chou and Morr (1979) demonstrated that water holding capacity varies as a function of several factors such as the hydrophilic–hydrophobic balance of amino acids in the protein molecule and carbohydrate fractions associated with the protein.

As stated by Hayta et al. (2002), the oil absorption capacity of food material depends on the type and content of hydrophobic fraction present in the matrix structure. The presence of hydrophobic amino acids in the structure of protein may be responsible for its tendency for oil absorption. The existence of several nonpolar side chains may bind the hydrocarbon chains of oil, thereby resulting in higher oil binding capacity (Thanatcha and Pranee, 2011).

Amino acids are classified as polar or non-polar, according to the properties of the side-chains (**Table 1.1).** A further subclassification of acidic-polar when the side chain contains a carboxylic acid (aspartic, glutamic) and basic-polar when the side chain contains an amino group (lysine, arginine, histidine) can also be introduced.

The nonpolar amino acids contain mostly hydrocarbon R groups that do not bear positive or negative charges. Nonpolar (i.e., hydrophobic) amino acids play a significant role in maintaining the three-dimensional structures of proteins, for the reason that they interact poorly with water forming only van der Waals interactions with water molecules (Creighton, 1983). Two types of hydrocarbon side chains are found in this group: aromatic and aliphatic. Polar amino acids have functional groups capable of hydrogen bonding, so they easily interact with water. Asparagine, glutamine, serine, threonine and tyrosine

belong to this category. The side chains of protein have a wide chemical variety which is vital for the unique functions of biological proteins.

1.14.3 Emulsifying properties

Emulsifying properties of proteins are the ability to stabilise an emulsion. This property is important for a lot of food applications such as soups, salad dressings, mayonnaise and cakes (Jackman et al., 1989). These properties are generally discussed in terms of emulsifying activity, emulsifying capacity and emulsifying stability (Pearce and Kinsella, 1978). Emulsions are considered to consist of at least one lipophilic liquid and at least one polar hydrophilic liquid (Al-Malah et al., 2000).

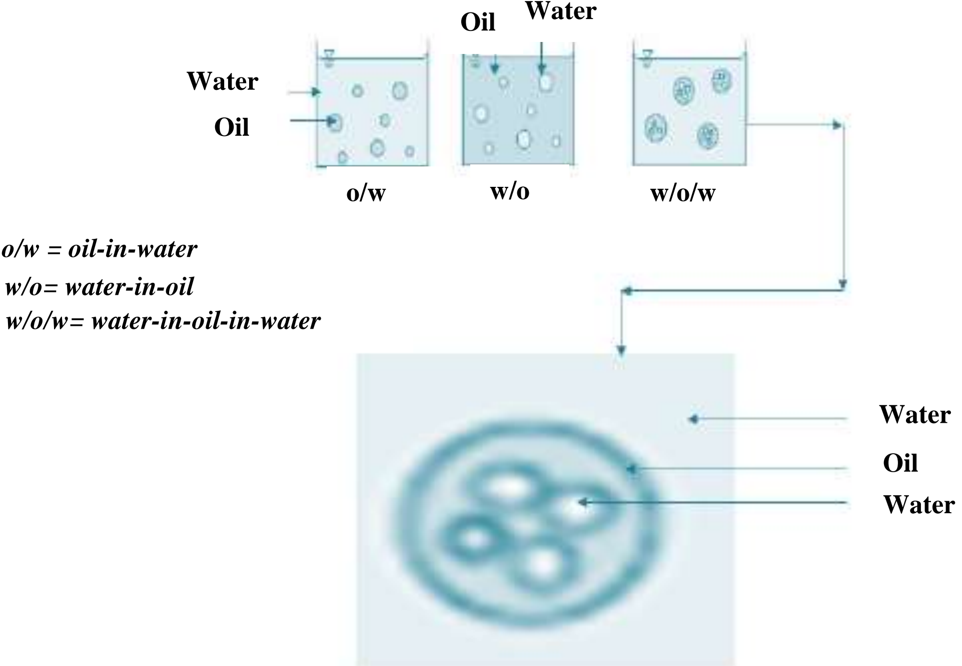
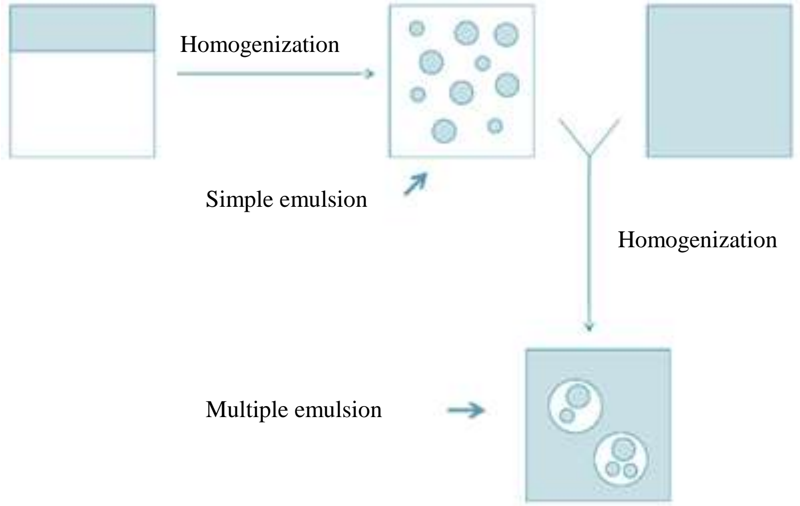


Figure 1.12 Principle types of emulsions (Taken from Schubert et al., 2006)

Dispersions of an oil or lipophilic phase in a hydrophilic or watery phase are the two basic types of emulsions. Water and oil are the two most used liquids for the production of food emulsions worldwide, these types of emulsions are named as water-in-oil (W/O) emulsions and oil-in-water (O/W) emulsions. More complex types of emulsions consist of many phases, three or more, which can be prepared by, for example, dispersing a W/O into a watery phase (W), leading to a water-in-oil-in-water emulsion type (W/O/W) (Schubert et al., 2006). **Figure 1.12** and **Figure 1.13** depict the basic types of emulsions.

Emulsifying capacity is defined as the maximum quantity of oil that can be emulsified by a standard amount of protein under specified conditions before the emulsion collapses. Emulsion activity and emulsion stability refer to the ability of a protein to form an emulsion that remains stable for a certain time under particular conditions (Pearce and Kinsella, 1978, Kinsella and Melachouris, 1976). Emulsion stability is defined as the ability of emulsion droplets to remain dispersed without creaming, flocculation or coalescing (Zayas, 1997). Wang and Kinsella (1976) reported that salt, pH of the medium, protein concentration, and solubility influence the emulsifying capacity of plant proteins. Other factors are for example, equipment design, shape of container, rate of oil addition, type of oil used as well as nature of proteins (Christian and Saffle, 1967). Therefore emulsifying characteristics are not just a characteristic of the protein under investigation but also a characteristic of the emulsion system, the method and equipment used to prepare the emulsion.

In certain foods, a natural protein ingredient is an effective stabilizer. Proteins are effective surface-active agents because they possess the capacity to lower interfacial tension between hydrophobic and hydrophilic components in foods. A stabilizing effect of proteins in the emulsion system results from the formation of a protective barrier around fat droplets, preventing emulsion coalescence (Zayas, 1997).



► *Simple emulsion: regular emulsion oil-in-water (O/W); inverse emulsion water-in-oil (W/O)*
► *Multiple emulsion: O/W/O, W/O/W*

Figure 1.13 Simple emulsion and multiple emulsion

1.14.4 Foaming properties

Foams are important for many food applications such as leavened bakery, meringues and whipped toppings products (Tovmsend and Nakai, 1983). Foaming or whipping characteristics of protein are defined as its ability to form stable foam by air incorporation and by beating. The ability of protein to form foam is commonly determined by measuring the increase in volume of foam after whipping. The term foam stability refers to the ability of the foam to retain some of its properties constant over time (for instance, bubble size and/or foam volume and/or liquid content) (Denkov and Marinova, 2006). The typical foam structure is shown in **Figure 1.14**. Many studies were performed to evaluate the foaming properties of food proteins under a variety of conditions (Kinsella and Melachouris, 1976; Cherry and McWatters, 1981), Sathe and Salunkhe (1981) and Sathe et al., (1982 demonstrated that g temperature, pH, the presence of sugars and salts, influence the foaming properties of proteins from lupin and great northern bean seed.

The foaming properties of proteins are influenced by the source of the protein, and methods and thermal parameters of processing, including protein isolation, temperature, pH, protein concentration, mixing time, method of foaming. Among many factors influencing foaming capacity of proteins the type of foaming equipment and method of agitation are important. It is known that soluble proteins are good foaming agents. Different proteins possess different foaming powers; however, surface tension does not change proportionally to foaming power indicating that good emulsifiers are not necessarily good foamers. For example, the surface tensions of soybean protein and egg albumin were low and close to haemoglobin, but the foaming power of haemoglobin was considerably higher (Zayas, 1997).

There are comparatively little data on foaming properties of cowpea proteins. Butt and Batool (2010) found that cowpea protein isolate has higher foaming capacity than that of pigeon pea protein isolates, and more stable foam than that from mungbean protein isolates. Horax et al. (2004) found that cowpea protein isolates have low foaming capacity and less stable foam compared to that from soy protein isolates.

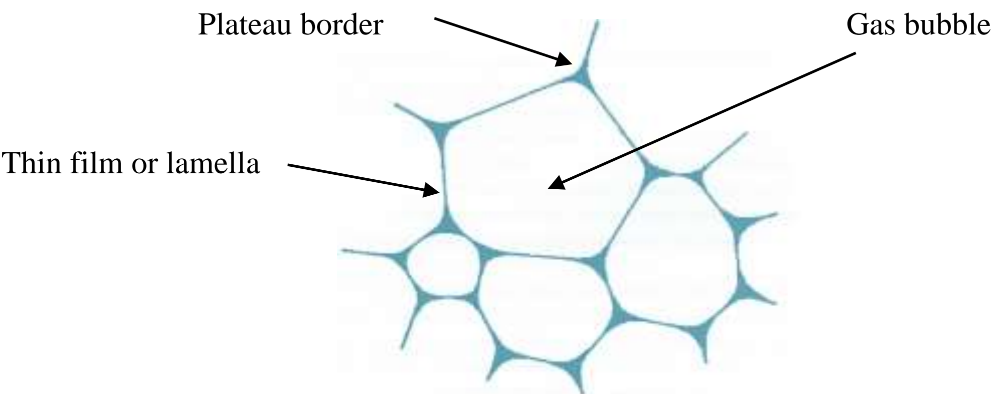


Figure 1.14 The structure of foam bubbles (taken from Wilde and Clark, 1996)

1.14.5. Gelation and viscosity

Thermally induced gelation of proteins provides textural and sensory properties in food products. A gel is an intermediate state between a solid-like rheological behaviour and a liquid-like rheological behaviour and consists of a dispersing medium (water or other solvents), and a dispersed phase (polymers or colloids) and can be very close to a solid or to a liquid (Nishinari, 2009). **Figure 1.15** depicts gel formation from a thermally induced protein solution. Gels that are formed from protein consist of a continuous network of denaturated and aggregated proteins (Alting, 2003).

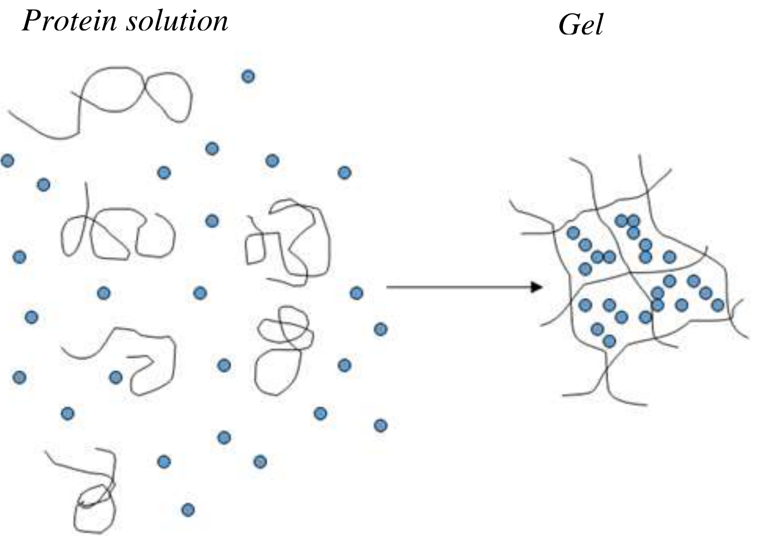


Figure 1.15 Gel formation from a protein solution

Gel formation is influenced by the extent of crosslinking of the initial molecules present, and this is dependent on environmental conditions, temperature, heating time, pH, and concentration (Mcklem, 2002). The molecular structures of coarse-aggregated and fine-stranded networks are schematically shown in **Figure 1.16**. The type of gel depends on the environmental conditions during its formation. If the ionic strength is increased, or when the pH approaches the isoelectric point of the proteins, the gel can become coarser (Renkema, 2001). For instance, a fine stranded network structure of glycinin was obtained in the pH range of 7.0 to 7.6 (Hermansson 1985, Nakamura et al. 1984). Changing of the protein concentration, ionic strength or pH, in the case of globular proteins, caused the gel network structure to change as well as contributing to the gel strength, as shown in **Figure 1.17** (Suhaimi, 2005).

Viscosity is an internal characteristic of a fluid that is usually expressed in centipoise (cP). It is the term for the resistance that a fluid has to movement and flowing. It is one of the most prominent sensory and physical properties of liquid and semisolid foods (Christensen, 1987). It describes the resistance to flow revealed by a solution and is interpreted as $\eta = \tau/\gamma$. In case of Newtonian fluid, shear rate γ (sec^{-1}) is related to shear stress τ ($dynes/cm^{-2}$) and therefore, the viscosity η is independent of the shear rate (the rate at which the solvent or solution is sheared). However, semi-solid and liquid foods usually demonstrate very complex flow characteristics and most demonstrate non-Newtonian flow properties.

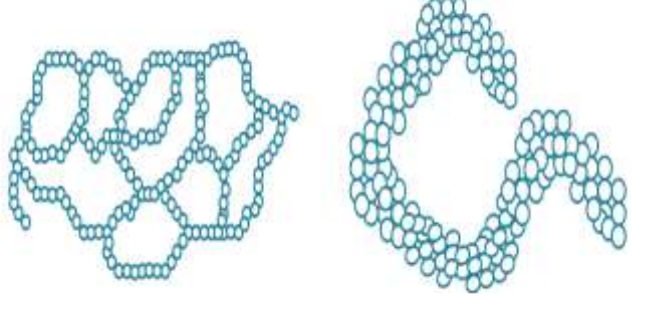


Figure 1.16 A Schematic diagram of gel network (from Renkema, 2001)

Pea protein isolates showed similar thickening characteristics to soy protein isolate (Gueguen and Lefebvre, 1983). Protein isolates from some legumes exhibited non-

Newtonian, time-dependent behaviour (Gueguen and Cerletti, 1994). Other researchers investigated the influences of some solutes on the viscosity of soy protein isolate suspensions (Hermansson, 1975; Babajimopoulos et al., 1983). It was noted that addition of glucose or sucrose, or sodium chloride led to reduction in the viscosity of soy protein slurries.

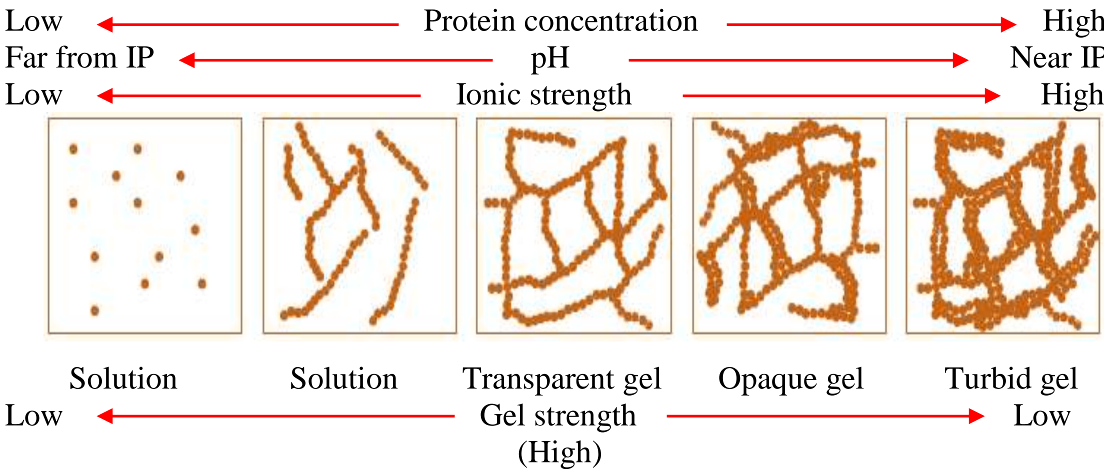


Figure 1.17 A typical model for the protein network by changing the concentration, ionic strength or pH (Taken from Yasir, 2005)

1.14.6 Protein denaturation and associated molecular interactions

Many treatments can be used to modify protein structure to improve its specific functional properties. It has been reported that the functionality of soy protein depends on the degree of denaturation, dissociation, as well as aggregation of glycinin and β-conglycinin fractions (Sorgentini et al, 1995). The conformation of a protein while it is in its activated or functional state is named the "native state" of the protein. Protein denaturation is commonly defined as any change of original native structure of a protein which does not alter the sequence of amino acids (Adler-Nissen, 1976). Denaturation takes place for the reason that the bonding interactions accountable for the secondary and tertiary structure are disrupted; these comprise hydrogen bonds and electrostatic interactions, hydrophobic interactions, ionic bonds, and covalent bonds. (Cramp, 2007).

1.14.6.1 Electrostatic Interactions and Hydrogen Bonds

The protein chemistry in gelation may include many interactions, including hydrogen bonds and electrostatic interactions, hydrophobic interactions, ionic bonds, and covalent bonds. Protein structural changes due to denaturation provide the

opportunity for gelation interactions to take place. Hydrogen bonds are stronger interactions occurring between the hydrogen atom in a polar bond and the unshared electron pair in a nearby electronegative atom or ion (Brown et al., 2000). Hydrogen bonds in proteins can exist between proteins, or between the water and amino groups of proteins (**Figure 1.18**).

Water **Amino Acid** **Water**

Figure 1.18 Hydrogen bonding between the amine group on an amino acid and water and a carboxylic group on an amino acid and water.

Electrostatic interactions are known to play a important role in protein structure and function (Roy and Taraphder, 2007). Electrostatic interactions are collectively known as van der Waals forces and include dipole-dipole interactions, London dispersion forces, and hydrogen-bonding. These forces are less than 1/6 as strong as covalent or ionic bonds (Cramp, 2007; Brown et al., 2000). The electrostatic interaction between peptides or proteins can be reduced by 0.3M NaCl; the counter- ions of NaCl interrupt electrostatic interactions causing breakdown of electrostatic bonds (Zhong et al., 2006). London dispersion forces (**Figure 1.19**) are weak interactions that can take place in proteins between two molecules when one instantaneous dipole moment induces an adjacent instantaneous dipole moment in an adjacent molecule (Brown et al., 2000).

$\delta+$ C—H $\delta-$ ▪▪▪▪▪▪▪▪ $\delta+$ C—H $\delta-$

Figure 1.19 London dispersion forces – induced dipoles (Brown et al., 2000).

1.14.6.2 Hydrophobic interactions

Hydrophobicity is the molecular driving force behind many important biological processes, e.g. protein folding (Li et., 2007). Hydrophobic interactions are stronger than hydrogen bonds, and are resulting from the repulsion of water by hydrophobic molecules (Cramp, 2007). The hydrophobic amino acid side chains interact with each other rather than with water, reducing the number of water molecules needed to form an ordered structure nearby the hydrophobic groups, and raising the entropy of the system (**Figure 1.20**) (Brown et al., 2000).

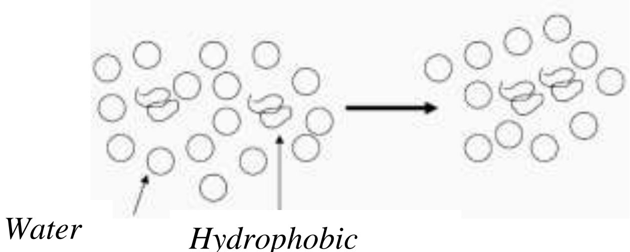


Figure 1.20 Hydrophobic interaction.

1.14.6.3 Covalent bonds

Covalent bonds are very strong and involve a chemical link between molecules (Brown et al., 2000). Identification of covalent bonds can be performed by dispersing protein samples in solvent including 0.2M 2-Mercaptoethanol, which reduces disulphide bonds to sulfhydryl groups (Zhong et al., 2006). Disulphide bonds are covalent bonds that may break and form under appropriate thermal conditions, such as the disulphide bonds between 11S acidic and basic subunits in soy protein (**Figure 1.21**) (Wolf, 1993).

COO^- COO^-

H_3N— C —H H_3N— C —H

CH_2 CH_2

S ——— S

Figure 1.21 Covalent disulphide bonds between two cysteine residues (taken from Wolf, 1993).

1.15 Changes in functional properties due to modification

The feasibility of using alternative sources of protein like trash fish, grain, microbes, and leaf etc. as food proteins is often limited due to their low biological value, undesirable organoleptic properties, toxic constituents, and poor functional properties. These problems may be overcome by physical or mechanical treatment or by microbial, enzymatic, or chemical modification (Kinsella and Melachouris, 1976).

Protein modification usually refers to the intentional alteration of protein structure by physical, enzymatic, or chemical agents to improve functional properties. Thus, modification in conformation at all levels of organization, i.e., primary, secondary, and tertiary structures may include disruption and reformation of covalent bonds and secondary forces, using physical (thermal and pressure), chemical or enzymatic treatments (Matheis and Whitaker, 1984).

Enzymes mostly modify food proteins by hydrolysis of peptide bonds. Since they are expensive, this process is not commonly applied at present. Lee et al (1984) reported that the immobilisation of enzymes may permit repeated use of enzymes and therefore reduce operational costs. Currently chymotrypsin and papain are the favoured enzymes used to enhance the nutritional value and functional properties of proteins (Phillips et al., 1994).

Changing the processing conditions such as pH and temperature are examples of the enabling physical modification of food proteins. These usually involve the use of heat to obtain partial denaturation. Legume proteins are known to have compact globular structures and are hence resistant to proteolytic enzyme attack *in vivo*, often resulting in incomplete digestion of these proteins. Denaturation enhances protein functionality by means of increasing the accessibility of buried reactive areas of the molecules (Gueguen and Cerletti, 1994). Schwenke et al. (1990) have noted that heating resulted in a higher water adsorption capacity as well as improved thickening characteristics of faba bean protein isolates.

Chemical modification of food proteins includes the hydrolysis of the peptide bonds and derivatisation of the amino acid side chains (Howell, 1996). This modification can be done by treatment with different agents such as acids and alkalis, alkylation, esterification, acetylation, oxidation and reduction. But, many of these

methods are unsuitable for food purposes. The concerns of chemical modification of food include deterioration of organoleptic properties, toxicity, loss of nutritional value, reversibility of modification and reaction with other food consumed. Furthermore, possible obstacles to the utilization of chemical modification of proteins entail ethical, legal, economic, and cultural aspects (Feeney and Whitaker, 1985). However, this kind of modification is commonly to enhance the functional properties of vegetable and other food proteins (Gueguen and Cerletti, 1994).

In general, physical, enzymatic or chemical modification of proteins has the possibility to control the functional properties, microbiological stability, acceptability and nutritional value of food products. There are many barriers in the commercial production of these modified foods such as the expensive, time-consuming procedure of safety assessment of these modified proteins (Howell, 1996).

1.15.1 Protein glycation

Non-enzymatic glycosylation of proteins, called glycation, is a spontaneous reaction between reducing carbohydrates and free amino groups (e.g. in amino acids or the ε-amino group of lysine in proteins, as well as the α-amino groups of terminal amino acids) leading to the formation of Schiff base (**Figure 1.22**). This intermediate is unstable and undergoes rearrangement to form stable Amadori products. Subsequently, the Amadori products degrade into dicarbonyl intermediates. These compounds can form cross-links and stable end products, called advanced glycation end products (AGEs) (Martins et al. 2000).

The water activity, type of sugar present, and type of amino acids available are important factors in the regulation of the Maillard reaction. Different sugars show different rates of reactivity. For instance, hexoses are less reactive than pentoses. When considering hexoses, glucose is less reactive than mannose, which is less reactive than galactose (Izydorczyk, 2005). In general, non-enzymatic browning occurs in most food with water activities in the range of 0.3 to 0.7. Increasing water activity decreases the reaction rate due to a dilution influence (Stamp and Labuza, 1983). On the other hand, a further decrease in water activity usually increases the browning rate, except in food systems where it limits the movement of the reducing sugars and amino acids (Eichner and Karel, 1972). Izydorczyk (2005) reported that the Maillard reaction may proceed more rapidly at lower water activities. Protein–polysaccharide interactions

depend on the intrinsic properties of polysaccharides such as branching, electric charge, molecular weight etc. (Nagy et al., 2009).

During non-enzymatic glycation, a wide range of reaction products are formed with a significant importance for the nutritional value of foods. This value can be reduced by decreasing the digestibility or changing the immunoreactivity of proteins (Maleki et al. 2000; Chung and Champagne 2001), or by a possible formation of toxic and mutagenic compounds. However, the nutritional value of proteins can be improved by the formation of antioxidative and antimutagenic products (Martins et al. 2001).

The interactions between proteins and carbohydrates in foods have attracted considerable attention during the past decade. This may be attributed to the ubiquitous nature of protein glycation reactions and the modification of some functional properties of proteins after their conjugation with carbohydrates (Easa, et al., 1996; Kato et al., 1990; Kato et al., 1991; Nakamura, et al., 1992). In addition, the application of glycated food proteins in the food industry presents fewer safety issues when compared with chemically modified food proteins (Kato, et al., 1996).

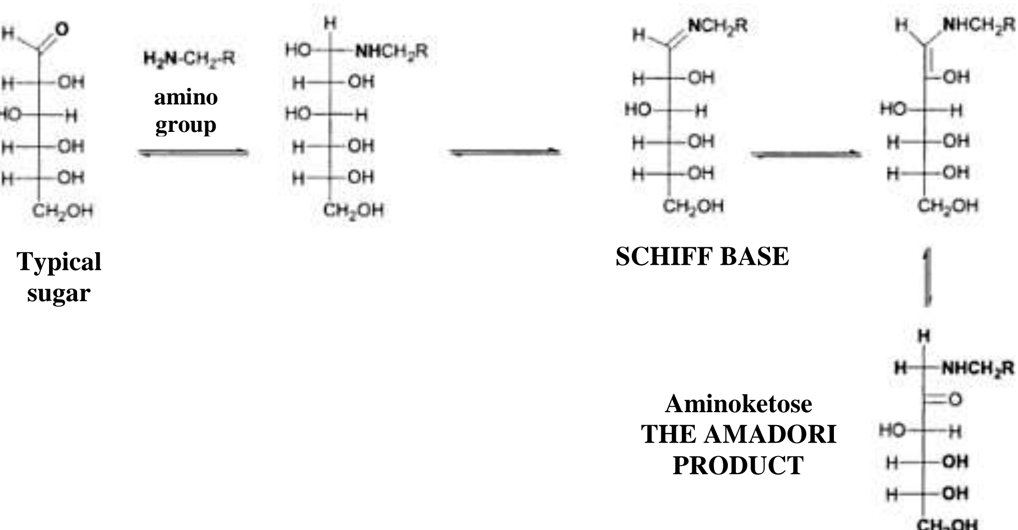


Figure 1.22 The early stages of the Maillard reaction (taken from Fayle and Gerrard, 2002).

Maillard reaction products (MRPs) are formed following heat processing of food and can potentially alter the functional and/or the biological properties. The current trend is to use MRPs as commercial food additives in applications as emulsifiers,

antioxidants and antibacterial agents (Cloos and Christgau, 2002; Kato, 2002; van Boekel, 1998; Volkin et al., 1995; Ames, 1992).

The Maillard reaction involving proteins and reducing saccharides has been the subject of much investigation recent years. Until quite recently, studies involving the Maillard reaction had been the domain of food flavour chemists, studying the chemistry of flavour development in amino acid-reducing sugar model systems. In the last two decades, however, the Maillard reaction has attracted a great deal of interest among researchers in other disciplines, including nutrition, toxicology, physiology, and pathology. Most of this interest has been spurred on by the effects of glycation on the structural and functional modification of proteins in food systems, as well as the physiological and pathological consequences of protein glycation in biological systems. The positive attributes of the Maillard reaction are realized mainly in food systems. These attributes may be divided into two categories, sensorial and textural. The sensory attributes of the Maillard reaction include the development of desirable colour, volatile and non-volatile flavour and aroma compounds during food preparation. The textural attributes include the improvement of protein solubility, water-holding capacity, thermal stability (Darewicz et al., 1998; Shu et al., 1996; Kato et al., 1996), gelling (Cabodevila et al., 1994), and emulsifying properties (Saeki, 1997; Shu et al., 1996; Matsumodi et al., 1995; Kato et al., 1993). The functionality of the conjugated proteins increase with increasing polysaccharide chain length and content (Shu et al., 1996). Recently, with the development of more sensitive analytical techniques, several studies have reported other favourable consequences of the Maillard reaction in food systems, namely, the formation of compounds with antioxidant (Chuyen, 1998; Wijewickreme and Kitts, 1998), anti-bacterial (Einarsson and Eriksson,1990), anti-mutagenic, and anti-carcinogenic properties (Aeschbacher, 1990). It is known that the glycation of protein by sugars negatively affects the nutritional value of proteins (Friedman 1996). Mori and Nakatsuji (1977) reported that the reduction of the nutritive value of protein depends on the reduction in intestinal absorption of the Maillard-induced lysine derivatives. Along with the change in physiological properties of Maillard reaction products, there are many factors that can adversely affect the nutritional quality, such as the formation of toxic compounds during the heat treatment of food (Friedman 1999). In fact, the reduction of nutritional quality is mainly pertinent in infant nutrition where milk is the only source of proteins. An additional important nutritional consequence of the Maillard

reaction is the formation of antioxidative materials in food (Chiu et al., 1991). There are also some data on the formation of antibacterial materials (Einarsson et al., 1988).

Table 1.6 shows some investigational studies of the functionality of protein–carbohydrate conjugates via the Maillard reaction published recently.

Table 1.6 Some experimental studies of the functionality of Maillard-type protein–carbohydrate conjugates published recently (2004–2011). All studies were in the dry state.

Protein	Carbohydrate	Reference
β-Casein	Dextran	Mu et al., 2006
β-Lactoglobulin	Chitosan	Miralles et al., 2007
β-Lactoglobulin	Dextran	Dunlap and Côté, 2005; Jiménez-Castaño et al., 2005; Jiménez-Castaño et al., 2007
β-Lactoglobulin	Acacia gum	Schmitt et al., 2005
Ovalbumin	Dextran	Choi et al., 2005
Sodium caseinate	Dextran	Fechner et al., 2007
Sodium caseinate	Maltodextrin	Morris et al., 2004
Sodium caseinate	Pectin	Einhorn-Stoll et al., 2005
Soybean protein	Dextran	Diftis et al., 2005
Soybean protein	Porphyran	Takano et al., 2007
Soy protein	Acacia gum	Mu et al., 2011
Soy protein	Glucose	Tian et al., 2011
Soy protein	Fructose	van de Lagemaat et al., 2007
Soy protein	Glucomannan	Zhang and Chi, 2011
Soy protein hydrolysate	Curdlan	Junfeng et al., 2006
Whey protein	Carboxymethyl-cellulose	Kika et al., 2007
Whey protein	Dextran	Wooster and Augustin, 2007
Whey protein	Dextran	Zhu et al., 2010
Whey protein	Maltodextrin	Akhtar and Dickinson, 2007
Whey protein	Maltopentaose	Li et al., 2005
Whey protein	Pectin	Einhorn-Stoll et al., 2005; Neirynck et al., 2004

Utilisation of cowpea proteins holds much promise in the improvement of new formulated foods. However, the cowpea proteins have not been fully studied, particularly the modification in order to improve their functional properties. The present study aims to investigate the functional properties of cowpea protein, and to study its possible utilization in food application such as bread in order to provide guideline information for better use of this agricultural raw material. The investigations of functional properties of cowpea protein offer the chance to understand the mechanisms of its function in food systems to expand and modify on present capabilities.

1.16 Objectives of the Current Project

1.16.1 General Aims of the Project

The general objective was to determine key functional properties of cowpea protein in comparison with those of a commercial soy protein ingredient, as well as the changes in its functional properties brought about by modifications. Our hypothesis was that denaturation of proteins, combined with glycation with endogenous sugars would lead to improve functional properties such as foaming and emulsifying properties. An additional objective was to study the feasibility of addition of native and modified protein to different kinds of food products, in order to provide guideline information for better use of these proteins.

1.16.2. Experimental Aims of the Project

The specific objectives are to:

1. Optimise the production process of cowpea protein isolates.
2. Modify cowpea proteins by denaturation and glycation in solution.
3. Study the physicochemical properties of native and modified proteins.
4. Determine key functional properties of native and modified cowpea protein isolate in comparison with those of soy protein isolate.
5. Evaluate the effect of addition of these proteins on physical and sensory qualities in model food application systems.

Chapter Two

Materials and Methods

2.1 Materials

Cowpea seeds were obtained from a local store (Bismallah, Edinburgh). Whey protein concentrate (WPC 60, 60% protein) was obtained from FrieslandCampina, Netherlands. Whey protein concentrate-galactooligosacharides (WPC-GOS, 50% protein) and soy protein isolates (SPI) (Solae, 90% protein), were kindly provided by Nandi Proteins Limited Company, Edinburgh, Scotland, UK. Wheat grains (Robigus), a soft type of wheat that contains 9% protein and 0.44% ash with 12% moisture, were supplied by W. N. Lindsay Ltd, Tranent, UK. Glycine, DNTB, EDTA, Coomassie Brilliant Blue G-250, 2-mercaptoethanol, 1-anilinonaphthalene-8-sulphonate (ANS), SDS, Tris, acetic acid, 95% ethanol, urea, enzymes and all other and reagents and chemicals were obtained from Sigma-Aldrich Company, UK. Electrophoresis and all electrophoresis materials were purchased from Invitrogen, UK. The yeast (Allinson's baking yeast), sunflower oil and the rest of the ingredients were from ASDA supermarket in Edinburgh, UK.

2.2 Methods

2.2.1 Preparation of cowpea flours

Flour samples were obtained by milling the beans in a DLFU-mill from Buhler-Miag (Braunschweig, Germany). The flour samples were sieved with a 600 micrometer (µm) screen and defatted by extraction with cold acetone for 1 h at 4°C (flour/solvent ratio of 1:3 w/v) according to the procedure described by Wang et al. (1999). The resulting slurry was centrifuged at 5000 x g for 10 min. at room temperature, the pellet was air-dried overnight, ground, and stored in an airtight container at 5ºC for further use.

2.2.2 Chemical analyses of cowpea flour

2.2.2.1 Moisture content

The moisture content was determined by drying 3g sample in an air oven to a temperature maintained at 105 ± 5 °C as per procedure given in AACC (2000) method No. 44-15A.

2.2.2.2 Total ash

The ash content was determined by putting 4g dried sample in a muffle furnace and after ignition maintaining a temperature of 550 °C as described in AACC (2000) method No. 08-01.

2.2.2.3 Crude protein

The nitrogen was determined by a Kjeldahl procedure according to AACC (2000) method No. 46-10. The factor 6.25 was used to convert nitrogen to crude protein.

2.2.2.4 Crude fat

The method employed was that of solvent extraction using a Soxhlet extraction as described in method No. 30-10 (AACC 2000). 2 g of sample were taken in a thimble and placed in the extraction tube of the Soxhlet apparatus. About 250 ml of hexane were added in the 500 ml bottom flask of the apparatus and connected to the Soxhlet apparatus. The fat was extracted by running hexane over the sample at the rate of 3-4 drops per sec for about 5 h. The content of the flask was transferred to a pre-weighed petri dish and dried on a hot plate for 10 min at a temperature of 40-50ºC. The petri dish was cooled in a desiccator and weighed. Fat percentage was calculated according to the following formula.

$$\text{Crude fat} = \frac{\text{Weight of fat in sample}}{\text{Weight of sample}} \times 100 \qquad \textbf{Equation 2.1}$$

2.2.2.5 Crude fibre

Crude fibre content was determined by following the method No. 32-10 as described in AACC (2000). 2 g fat and moisture free sample was taken and placed in a 1000 ml beaker. 200 ml solution of 1.25 % H_2SO_4 was added in the beaker. The sample was then digested by boiling for 30 min. Then it was filtered by using suction apparatus. The residue was washed with hot water until becoming acid free. The residue was then again transferred to a 1000 ml beaker and boiled with 200 ml solution of 1.25 % NaOH for 30 min. It was again filtered and the residue was transferred to a pre-weighed crucible and dried in an oven at 100 ºC for 24 h till constant weight was obtained. Then the dried residue was charred on a burner and ignited in a muffle furnace at 550-600ºC for 5-6 hours, cooled in desiccators and weighed. The loss in weight during incineration

represents the weight of crude fibre in the sample. The crude fibre % was calculated by using the following formula.

$$\text{Crude fibre} = \frac{\text{Weight of residue} - \text{Weight of ash}}{\text{Weight of sample}} \times 100 \qquad \textbf{Equation 2.2}$$

2.2.2.6 Nitrogen free extract

The nitrogen free extract was calculated by using the following expression:

$$\text{NFE} = 100 - (\text{Moisture\%} + \text{ash\%} + \text{crude protein\%} + \text{crude fat\%} + \text{crude fibre \%}) \qquad \textbf{Equation 2.3}$$

2.2.2.7 Starch

The starch content in cowpea flour was determined according to AACC method No. 76-11 (AACC, 2000). Triplicate samples of cowpea flour dispersions (100 mg) were prepared in 0.2 ml ethanol (80%). Thermo-stable ά-amylase (300 units) was then added to dispersions and vigorously vortexed. The mixtures were then incubated for 6 minutes at 50°C with occasional shaking. Amyloglucosidase (20 units) and sodium acetate buffer (4 ml, 200 mM, pH 4.5) were added and the mixtures were stirred and incubated at a temperature of 50°C for 30 minutes. The mixtures were then transferred from the tubes to 100 ml volumetric flasks and the volumes were adjusted by using dH_2O. The contents were centrifuged at 5000 x g for 10 minutes. Triplicate aliquots (0.1 ml) of each sample were transferred to test tubes. Glucose oxidase peroxidase reagent (GOPOD) was then added to blank and samples and incubated at 50°C for 20 minutes. Genesys 6 Spectrophotometer (Thermospectronic, USA) was used to measure the absorbance of blank, glucose control and test samples at 510 nm.

2.2.2.8 Total dietary fibre (TDF)

The TDF content in cowpea flour was determined according to AACC method No. 32-05 (AACC, 2000). Triplicate samples of cowpea flour dispersions (300 mg) were prepared in sodium phosphate buffer (10 ml, 0.1 M, pH 7.5) and incubated in a shaking water bath at 95-100°C for 35 minutes with 1 ml heat-stable α-amylase (Sigma-Aldrich, A-3306). The mixtures were then cooled to 60°C and incubated at this temperature for 30 minutes with pepsin solution (60 units) with continuous agitation. The pH was adjusted to 4.0- 4.7 by adding 1N HCl and the contents were then incubated at 60°C for 30 minutes with 100 µl amyloglucosidase (Sigma-Aldrich,

A-9913) with constant agitation. The fibre contents were then precipitated by the addition of ethanol (95%) in the ratio 1:4. The contents were filtered and washed twice with ethanol. Residues were finally dried, cooled, weighed and corrected for protein and ash contents. A blank was run at the same time through the entire procedure along with experimental samples in order to determine any contribution from reagents to the residue.

2.2.2.9 Insoluble dietary fibre (IDF)

The IDF content in cowpea flour was determined according to AACC method No. 32-20 (AACC, 2000). Triplicate samples of cowpea flour dispersions (300 mg) were prepared in sodium phosphate buffer (10 ml, 0.1 M, pH 7.5) and incubated in a shaking water bath at 95-100°C for 35 minutes with 1 ml heat-stable α-amylase (Sigma-Aldrich, A-3306). The mixtures were then cooled to 60°C and incubated at this temperature for 30 minutes with pepsin solution (60 units) with continuous agitation. The pH was adjusted to 4.0- 4.7 by adding 1N HCl and the contents were then incubated at 60°C for 30 minutes with 100 µl amyloglucosidase (Sigma-Aldrich, A-9913) with constant agitation. The residue was filtered, washed and rinsed with dH_2O and filtered. The resultant residue was then washed twice with ethanol (95%), dried, weighed and corrected for protein and ash contents. A blank was run concurrently through entire procedure along with experimental samples in order to determine any contribution from reagents to resultant residue.

2.2.2.10 Soluble dietary fibre (SDF)

The SDF content in cowpea flour was calculated by using the following expression:

$$SDF = TDF - IDF \qquad \textbf{Equation 2.4}$$

2.2.2.11 Sugars (mono-, di- and oligosaccharides)

Sugars were extracted from cowpea flour using ten volumes of hot ethanol (80% w/v) as in Ofuya (2006), with modifications. The mixture was then put in shaking water bath (60°C) for 1 hour. After extraction, the sample was centrifuged for 20 min at 2000 rpm and the extract was filtered and concentrated to 2ml under vacuum by using a rotary vacuum evaporator at 60°C, made to 4 ml with deionized water (dH_2O) and redistilled again. This was repeated three times to get ethanol-free extract. Next, the

extract was deproteinized with 10% lead acetate (24 drops/6 ml) and filtered. Next, the sample was treated with saturated monopotassium phosphate (2 drops) to eliminate excess lead and filtered and stored at a 0°C. Next, the sample was analysed by high performance anion exchange chromatography with pulsed amperometic detection (HPAE-PAD). The column was a Carbopac PA-100, 4 x 250mm/ PA-1 Guard, 4 x 50mm, with a flow rate of 1 mL/min. Samples were pumped with a post-column pump with water as solvent at a temperature of 30°C.

2.2.2.12 Total sugar content

The total sugar content of CPI, DCPI and GCPI was determined by a spectrophotometric method using anthrone reagent as described by Sharma and Sangha (2009). Triplicate samples of cowpea flour (100 mg) were placed into a boiling tube. The samples were hydrolysed with 5 mL of 2.5 N HCl in a boiling water bath for 3 h and then cooled to room temperature. The mixtures were neutralised with Na_2CO_3 and the volume was made up to 100 mL and centrifuged. The supernatant was collected and 1.0 ml was transferred to the 10 ml volumetric flask, the volume was made up to 10.0 ml with dH_2O. The anthrone reagent was freshly prepared before use by mixing 0.2 g of anthrone (0.2%) with 100 mL of ice-cold 95% H_2SO_4, protected from light in a dark bottle and used within 10 h. Anthrone reagent (4.0 ml) was then carefully added to each tube of standard solutions (glucose) and test solution protein samples. Tubes were then heated for 8 min in a constant boiling water bath, cooled rapidly and the absorbance of the green to dark green coloured solution was measured at 630 nm against reagent blank. Triplicate absorbance readings were taken from each sample. The total sugar content was calculated with the help of a standard calibration curve of glucose.

2.2.3 Preparation of cowpea of protein samples

2.2.3.1 Size reduction of flour

Cowpea flour from 2.2.1 was size-separated on the test sieve shaker (Endecotts) by using 100-mesh (0.15 mm, width) size screen. The sample that remained upon the screen was reground, sieved, and the portion that passed through the mesh sieve was mixed with the first portion. This was named in the present study as cowpea flour (FP).

2.2.3.2 Preparation of CPI and DCPI

Preparation was carried out by a slight modification of the method described by El-Adawy (1996), as outlined in **Figure 2.1**. Cowpea flours were mixed with five and

ten fold quantity of distilled water (dH_2O) (w/v) at room temperature (RT). The pH of the mixtures were then adjusted to two different values of pH (9 and 10) with 2N NaOH and stirred gently for 1hour at different temperatures ranging from 20 to 55ºC. The mixture was centrifuged (5000 x g) for 30 min at RT to remove the starch and fibre fractions. The pH of the supernatant was adjusted to two different values of pH (4.0 and 4.5) by drop wise addition of 2N HCI whilst stirring. The precipitated proteins were centrifuged (5000 x g) for 30 min at RT, washed twice with dH_2O, resuspended in water and neutralized to pH7 with 0.1N NaOH, and dialysed (cut off 10 KDa) overnight at 4°C against dH_2O. To prepare DCPI a 500 ml sample of CPI suspension (during preparation), after removing the starch and fibre fractions , was heat treated for 2 hours in a shaking water bath at 85°C ± 3°C followed by cooling to RT by immersion of the flask in cold water. For measurements of effect of heating temperature on solubility and turbidity, aliquots of 100 ml were removed from the heat-treated solution at 30 minute intervals of increasing temperature and cooled to RT. The non-heated and heat treated samples were freeze-dried and referred to as cowpea protein isolate (CPI) and denatured cow pea protein isolate (DCPI) respectively.

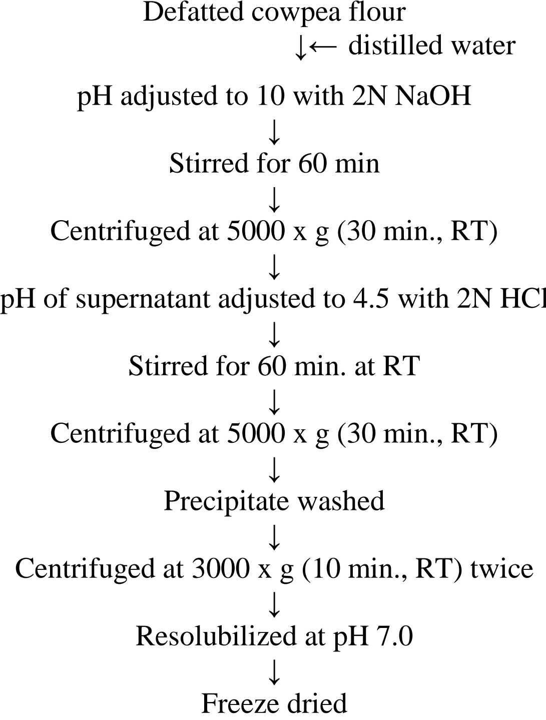


Figure 2.1 Procedure for preparation of cowpea protein isolate (CPI)

2.2.3.3 Preparation of GCPI

As outlined in **Figure 2.2**, a 2 litre dispersion of defatted cowpea flour sample in distilled water (5% w/v) was adjusted to pH 10 with 2N NaOH, and stirred for 1 hour at RT. One 500 ml sample was removed to serve as non-modified control. The suspension was heated for 2 hours in a shaking water bath at 85°C ± 3°C followed by cooling to RT by immersion of the flask in cold water. For measurements of effect of heating temperature on solubility and turbidity, aliquots of 100 ml were removed from the heat-treated solution at 30 minute intervals of increasing temperature and cooled to RT. The samples were centrifuged (5000 x g) for 30 min at RT to remove the starch and fibre fractions. The pH of the different supernatants was adjusted with 2N HCl to 4.5 to precipitate the proteins; the precipitated proteins were centrifuged at 5000 x g for 30 min at RT, washed twice with dH_2O, resuspended in water, neutralized to pH7 with 0.1N NaOH, dialysed (cut off 10 KDa) overnight at 4°C against dH_2O and freeze-dried. The sample is referred to as glycated cowpea protein isolate (GCPI).

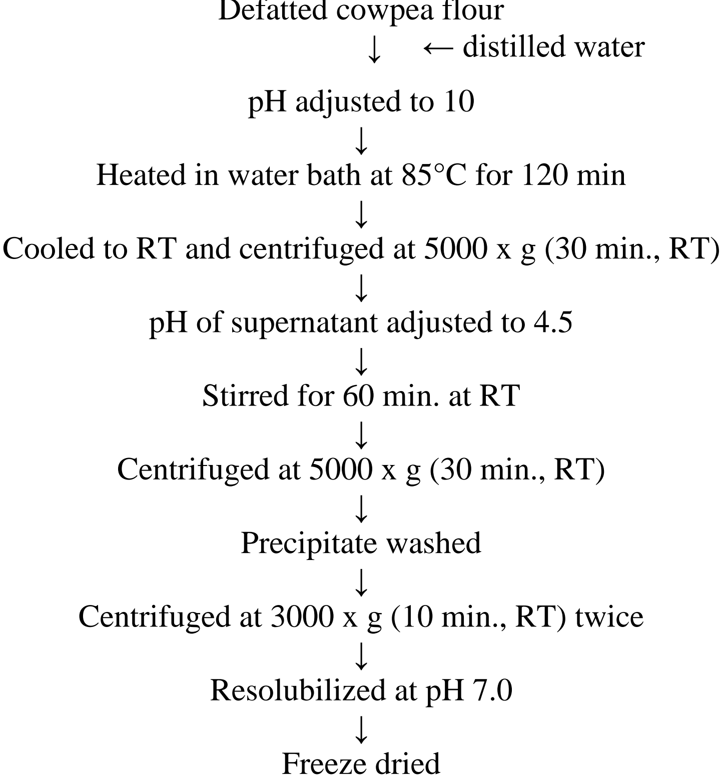


Figure 2.2 Procedure for modification of cowpea protein (GCPI)

2.2.3.4 Determination of protein contents and yields

The nitrogen was determined by a Kjeldahl procedure according to AACC (2000) method 46-10. The factor 6.25 was used to convert nitrogen to crude protein. Triplicate samples of cowpea protein isolates were analysed for their protein contents and yields. The protein contents of the isolates were determined as in section **2.2.1.3** by the Kjeldahl procedure according to AACC (2000) method 46-10.; the factor 6.25 was used to convert nitrogen content to protein content. Protein yield was calculated as:

$$\text{Yield (\%)} = \frac{\text{weight (g) of protein isolate x protein content (\%) of protein isolate}}{\text{10 g (weight of cowpea flour) x protein content (\%) of cowpea flour}} \text{ X 100}$$

Equation 2.5

2.2.3.5 Protein amino acid analysis

Amino acid analysis of cowpea protein samples was performed by using an HPLC technique. 10 mg of protein sample was hydrolysed with 200 μl of cold performic acid. The contents were then mixed by placing the tube in an ultrasonic bath for 10 min, capped and left to stand overnight at 5°C. 50 mg of sodium metabisulphite was carefully added. The contents were immediately vortexed and 0.8 ml of 7.5N HCl was added. The contents were mixed by placing in a sonic bath for 15 min. The tube was unsealed and placed into a heating block previously heated to 110°C for 1 hour then the tube was sealed and the contents were hydrolysed for a further 23 hours. After hydrolysis, samples were evaporated and taken in the sodium carbonate buffer, pH 9.7.

2.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the procedure of procedure of Wu and Hojilla-Eva (2005), by using Pre-cast native PAGE 10-20% Tris-glycine gradient gels in an electrophoresis unit (XCell Surelock™ Mini Cell, Invitrogen Life Technologies, Paisley, UK), at constant voltage 180V for approximately 45 min. Samples (2μg protein/μL) were prepared in non-reducing sample buffer (120M Tris-HCl, pH6.8, 20% glycerol, 4% SDS, and 0.008% bromophenol blue; while reduced sample buffer added 10% β-Mercaptoethanol). Running buffer was 10x SDS-PAGE buffer (1% SDS, 0.25M Tris-HCl and 1.92M glycine). The molecular weight markers (Plus2 pre-stained (1x)

MW 4-250 KDa) and samples were run under reducing conditions (2-mercaptoethanol), and non-reducing conditions.

2.2.5 Glycoprotein staining

GelCode® Glycoprotein Staining Kit was used to conduct the glycoprotein staining. The separated protein was fixed by immersing the gel in 50% methanol for 30 min. The gel was then washed twice with 3% acetic acid for 10 min. The gel was transferred to the oxidizing solution and gently agitated for 15 minutes. The gel was then washed three times with 3% acetic acid for 5 min before transferring to the GelCode® Glycoprotein Staining Reagent. The gel was then incubated for 5 min with the reducing solution before being washed with 3% acetic acid and then with dH_2O. Glycoproteins appear as magenta bands.

2.2.6 Determination of protein solubility

For effects of pH on protein isolate solubility, protein samples (20 mL, 0.4% w/v) were suspended in a 25 ml beaker at pH 2-14 at room temperature and stirred for 30 min. The respective slurries were then centrifuged at 5000 rpm for 20 min. The soluble protein contents were then determined by the Bradford procedure as described by Kruger (1994). Percent protein solubility was expressed as below:

$$\text{Solubility (\%)} = \frac{\text{protein in the supernatant (mg/ml)}}{\text{initial protein (mg/ml)}} \times 100 \qquad \textbf{Equation 2.6}$$

2.2.7 Measurement of viscosity

2.2.7.1 Viscosity with a Brookfield viscometer

A Brookfield viscometer (Model DV-II + Brookfield Engineering Laboratories, Inc., Stonghton, MA.) equipped with a No. 1 spindle was used to measure apparent viscosity of the CPI compared with the SPI, at different pH levels according to Philip et al. (2007). Protein solution samples were heated at 40 and 60°C. Measurement was performed in duplicate with values reported in centipoises (cPs) units.

2.2.7.2 Viscosity with a Bohlin Gemini rheometer

A Bohlin Gemini rheometer (Malvern Instruments Limited, Worcestershire, U.K.), was used to measure the viscosity of protein sample solutions and emulsions using controlled shear rate (0.10 – 100 1/s). A cone and plane geometry with a cone angle of 4° (C4/40) and a cone diameter of 40 mm was used and data were recorded for samples in duplicate. Protein and samples, during testing, were held at 25°C and 22°C (room temperature) respectively.

2.2.8 Differential scanning calorimetry (DSC)

The thermal characteristics of protein samples were assessed by differential scanning calorimetry (DSC) using a DSC 2010 instrument (TA Instruments, New Castle, USA) according to Sorgentini et al. (1995) and Renkema, (2001). Approximately 6 mg of samples were placed in DSC hermetic aluminium pans. Analysis was performed at a temperature gradient of 20-140°C at a rate of 10°C/min. An empty aluminium pan was used as reference. The DSC data were analysed with universal analysis software (TA universal analysis, TA instruments).

2.2.9 Determination of gelation properties of cowpea protein

2.2.9.1 Preparation of gels

Protein gels at preferred protein concentrations (**Section 2.2.3.2**) were made in beakers (10 ml). Aqueous dispersions (5 ml) of protein samples were prepared in dH_2O and mixed to get a uniform suspension. The pH of suspensions was adjusted to 7.5 by addition of 1N NaOH. The aluminium foil was used to cover the beakers to prevent evaporation during heating. The gelation was done by heating the suspensions in a water bath at 90ºC for 60 min. Gels were kept overnight in the fridge (4ºC) for analysis of rheological properties.

2.2.9.2 Determination of effect of protein concentration on gelation properties

Cowpea protein dispersions of different concentrations ranging from 8 to 16 % (w/v) were made and the pH values were adjusted to pH 7.5 with 1N NaOH. Next, the dispersions were heated (90ºC, 60 min) for gelation properties studies.

2.2.9.3 Determination of effect of heating temperature on gelation properties

Protein dispersions (14 % w/v, pH 7.5) were heated at 80°, 85°C, and 90ºC for 60 min for gelation properties studies.

2.2.9.4 Determination of effect of heating time on gelation properties

Protein dispersions (14 % w/v, pH 7.5) were heated at 90ºC for different times ranging from 10 to 60 min for gelation properties studies.

2.2.9.5 Determination of effect of pH on gelation properties

The pH of protein dispersions (14 % w/v) was adjusted to different pH values ranging from 3 to 11 with 1N NaOH or 1N HCl as necessary. Next, 5ml was taken from each dispersion and heated at 90 ºC for 60 min for gelation properties studies.

2.2.9.6 Determination of gel strength

Triplicate samples of cowpea protein gels were compressed to 50% deformation using a Zwick/Roell type Z010 machine. Gel strength (N) was then calculated according to Boye (1995) as the force necessary to fracture the gels (breaking force).

2.2.9.7 Determination of water holding capacity (WHC).

Water holding capacity (WHC) of cowpea protein gels was determined by using the centrifugation technique according to Mao et al. (2001) with modifications. 1.4g of each protein sample was weighed and placed in centrifuge tubes, 10 ml of dH_2O added, the pH was adjusted to pH 7.5 using 1N NaOH and heated at 90ºC for 60 min to form gels. The gels were kept overnight at 4°C and centrifuged at 6000 x g for 30 min and the separated water (supernatant layer) was measured. The WHC of gel was expressed as:

$$\text{WHC}(\%) = \frac{W_1 - W_2}{W_1} \times 100$$

Equation 2.7

Where W_1 = water content of the sample; W_2= separated water.
The measurements were done in triplicate.

2.2.10 Determination of foaming capacity and foam stability

Foaming capacity (FC) and foam stability (FS) were determined by the method described by Lin et al. (1974), with some modifications. 100 ml of 1% (w/v) aqueous protein dispersions were homogenized for 3 min. Dispersion volume was recorded before and after foaming. FC was expressed as mL foam/mL liquid after stirring. The FS was recorded after 30 min storage at room temperature.

2.2.11 Determination of water holding capacity

Water holding capacity was evaluated according to method described by Makri et al. (2005) with slight modifications. A five gram of each sample was vortexed with 30 ml of dH_2O in a centrifuge tube for 2 min and allowed to stand at RT for 45 min and then centrifuged at 10,000 rpm for 20 min. The supernatant was carefully decanted (discarded) and the weight of each sample was noted. The water holding capacity was expressed as the number of g water held by 1.0 g of protein sample. The estimations were done in triplicate.

2.2.12 Determination of oil absorption

Fat absorption capacity was evaluated according to the method described by Bencini (1986) with slight modifications. The procedure was similar to that for water absorption capacity except using sunflower oil instead of water. The result was expressed as g of sunflower oil absorbed per g of protein sample.

2.2.13 Determination of emulsifying properties

The emulsions were prepared to measure the emulsifying activity index (*EAI*) and the emulsifying stability index (*ESI*) of CPI and GCPI. The pure sunflower oil/water emulsions, stabilized with cowpea protein samples were prepared from 50 g of 1% aqueous protein suspension and 30 ml of sunflower oil by vigorous stirring for 5 min at RT. The mixture was then homogenised at 13,000 rpm for 2 min. 50 µL portions of the emulsions were transferred by pipette at 0 and 10 min after homogenization from the bottom of each container. The portions were diluted with 10 mL of 0.1% SDS solution. Absorbance of each diluted sample was measured at 500 nm using a Genesys 6 Spectrophotometer (Thermospectronic, USA). The absorbances measured immediately and at 10 min after emulsion formation were then used to calculate the *EAI* and the *ESI* according to Klompong et al. (2007).

The *EAI* was expressed as:

$$\text{EAI (m}^2\text{/g)} = 2T\ A_0 \times \text{dilution factor}/C \times \varphi \times 10\,000 \qquad \textbf{Equation 2.8}$$

Where T = 2.303; dilution factor = 200; A_0 = absorbance measured immediately (at 0 min) after emulsion formation; C = weight of protein/unit volume (g mL^{-1}) of aqueous phase before emulsion formation; Φ = oil volume fraction of the emulsion.

And the ESI was expressed as:

$ESI\ (min) = A_0 \times \Delta t / \Delta A$ **Equation 2.9**

Where $\Delta t = 10$ min and $\Delta A = A_0 - A_{10}$.

Each sample was prepared in triplicate for EAI and ESI measurement.

2.2.14 Turbidity measurements

1 ml samples of the supernatants of centrifuged, heat -treated cow pea flour dispersions (100 ml samples taken at different intervals of heat treatment of the 2 litre dispersion) were transferred to glass cuvettes. Turbidity measurements were carried out at wavelength of 600 nm as described by Tay et al. (2005) using a Genesys 6 spectrophotometer (Thermospectronic, USA).

2.2.15 Determination of hydrophobicity

Surface hydrophobicity indices (S_o) of solutions of CPI and DCPI (2 hours of heat treatment at 85°C) were determined by using 1-anilino-naphthalene-8-sulphonic acid (ANS) as the fluorescence probe as described by Kato and Nakai (1980), with slight modifications. 1mL of protein samples ranging from 0.2 to 1 mg/ml in 8mM sodium phosphate buffer pH 7.0, containing 0.002% SDS were mixed with 15 ml of ANS solution (8mM in 0.01 M sodium phosphate buffer, pH 7.4) and left to stand for 1 h at RT. The fluorescence intensity of protein was determined with a fluorescence spectrophotometer (Model 203, Stable Micro System Ltd.), at λ_{ex} = 390 nm and λ_{em} = 470, excitation and emission wavelengths respectively. The initial slope of the plot of fluorescence intensity versus protein concentration was used as an index of hydrophobicity.

2.2.16 Determination of glycation degree

A spectrophotometric assay was used to measure the free amino groups of 5% (w/v) solutions of protein samples (2 hours of heat treatment at 85°C) at pH7 by the orthophthaldialdehyde (OPA) method described by Achouri et al., 2005. The OPA reagent was freshly prepared before use by mixing 40 mg of OPA (dissolved in 1ml of ethanol), 1.905 g disodium tetraborate decahydrate and 0.05g of SDS (dissolved in 40ml of dH_2O). The volume of solution was brought to 50 ml with dH_2O and 2.35ml of 2-mercaptoethanol was added. 100µl of the sample was added to 1.8ml of OPA reagent and allowed to stand for 5 min at room temperature. The absorbance was measured at

340 nm using a Genesys 6 spectrophotometer (Thermospectronic, USA). A calibration curve of leucine was obtained by preparing standards with concentrations of 0.25-2 mM. Three replicates were performed for each measurement.

The glycation degree (GD) was calculated using the following equation:

$$GD\% = (A_0 - A_t / A_0) \times 100 \qquad \textbf{Equation 2.10}$$

Where A_t, = absorbance of the sample; A_0= absorbance of the control.

2.2.17 Monitoring of browning

Browning of cowpea extract during modification treatment (2 hours of heat treatment at 85°C), as in section 2.2.3.3, was measured by using a spectrophotometric assay as absorbance at 420 nm as described by Brands et al., (2002). Moreover the samples (1ml) were diluted in 20% (w/v) SDS (2ml) to reduce light scattering. The unheated sample was used as blank and the sample results were calculated by subtracting blank value from the sample readings.

2.2.18 FTIR measurements

The infrared measurements were performed by using a Fourier transform infrared spectrometer (Satellite FTIR Spectrometer, Mattson). The protein samples were prepared using a potassium bromide (KBr) pellet technique at 1:100 ratio and scanned in the range of 400-4000 cm^{-1} to present the spectra in absorbance units. All samples were considered in triplicate.

2.2.19 Determination of free and total sulfhydryl (SH) groups

The sulfhydryl (SH) content of 5% w/v solutions of CPI and GCPI (2 hours of heat treatment at 85°C) was determined by a colorimetric assay using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) as described by Campbell et al. (2009). Free SH groups (SH_F) were determined by addition of 300 µl of the sample to 5 ml Tris-glycine buffer (0.086M Tris, 0.09M glycine, 0.004M Na_2EDTA, pH 8), followed by addition of 200 µl 0.02M DTNB buffer. The solution was vortexed and left at to react at RT for 15 min before recording the absorbance was at 412 nm using a spectrophotometer. The blank for each measurement was the sample prepared using the described procedure but omitting the DTNB. For determination of total SH groups (SH_T), 300µl of each sample

was added to 5ml Tris-glycine buffer pH 8 containing 6M urea, 0.5% SDS and 0.06ml of β-Mercaptoethanol (β-ME). 200μl of 0.02M DTNB buffer was then added and absorbance at 412 nm was measured as described for free SH-groups. The % denaturation was calculated as (Free SH/ Total SH) x 100.

2.2.20 Determination of increase in dough volume

The increase in dough volume during fermentation was determined using a graduated beaker according to the procedure described by Shah et al (2006). After mixing, the dough was placed into a graduated beaker and allowed to rise for 60 min at 28°C. The height of the dough was measured on the graduated surface of the beaker before and after fermentation and the net increase in volume was calculated. Dough rising was calculated as the ratio of (increase in dough height/initial height of the dough) multiplied by 100.

2.2.21 Determination of dough textural properties

The doughs for rheological measurement were prepared as for baking (bread) experiments but without yeast. The textural characteristics of dough were preformed in a Zwick/Roell type Z010 machine according to and Autio et al. (2001). A round plastic box with an inner diameter of 68 mm and a height of 20 mm was filled with the dough and the expelled dough was carefully trimmed off with a knife to achieve an even surface. Doughs were compressed with a plunger (sample area, 314 mm^2). Compression range was 50% and the compression rate 2 mm/sec. When the dough was compressed from 0 to 50%, the force as a function of time was registered. The maximum force was taken as a hardness value and the average value of ten replicates is reported. The above experiments were conducted at ambient temperature.

2.2.22 Determination of water absorption of dough flour

Water absorption during mixing was evaluated according to AACC standard method 88-04 (Anonymous, 1995) with some modifications. Five grams of each dough flour sample was vortexed with 30 ml of dH_2O in a centrifuge tube for two min and allowed to stand at RT for 45 min and then centrifuged at 10,000 rpm for 20 min. The supernatant was carefully decanted and the weight of each pellet sample was noted. The water absorption was expressed as the ratio of water absorbed by flour in grams per 100 g of the sample (dry matter).

2.2.23 Preparation of bread loaves

As outlined in **Figure 2.3**, the bread was prepared by the straight dough method 10-10B of AACC (2000), using the recipe given in **Table 2.1.** Dough was prepared from wheat flour or wheat-cowpea composite flours. Wheat-cowpea composite flours were prepared by mixing CPI or GCPI with wheat flour at 2, 4 and 6% (w/w). The ingredients were mixed together in a food mixer (Breville, SHM2) and kneaded at setting 2 for 5 minutes, followed by incubation at 30°C for 50 min to allow for the yeast to begin the fermentation process. Each dough preparation was rolled into a ball, put into a bread pan, covered and allowed to rise at 35°C for 30 min. The risen dough preparations were baked at 200°C for 30 min., in a Russell Hobbs 14552 Mini Oven and allowed to cool for 1 hr before loaf volume and texture determinations were carried out.

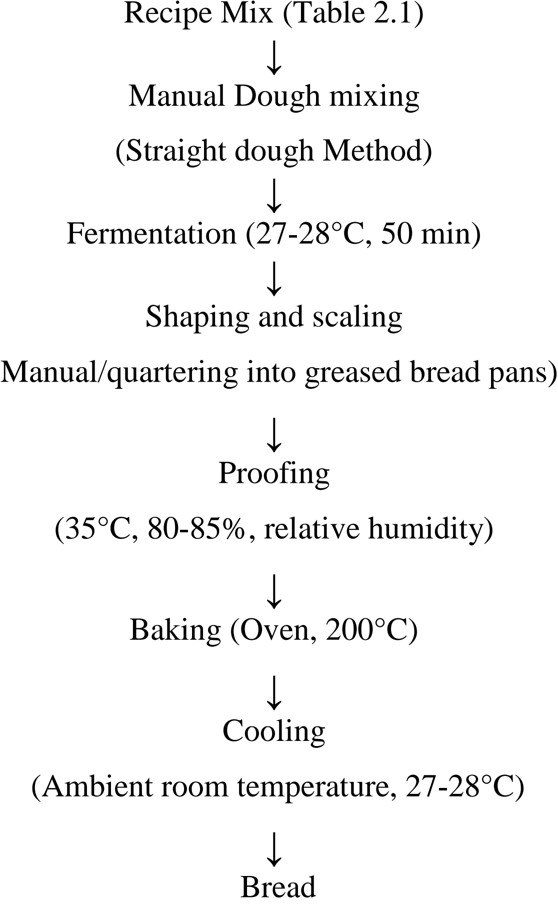


Figure 2.3 Flow chart for the process of preparation of bread

Table 2.1 Recipe used for preparation of breads

Component	Bread Composition (%)
Flour*	60.70
Yeast	0.60
Sugar	1.82
Salt	0.60
Fat	3.18
Ascorbic acid	75ppm
Water	33.10

*Wheat or wheat-cowpea protein composite flour

2.2.24 Preparation of cakes

The recipe used was soft wheat flour 100 g, fresh eggs 140 g, sunflower oil 100 mL, sugar 100 g, baking powder 4 g, and mono-and di-glyceride emulsifier 8 g, The eggs, sugar and emulsifier were mixed in a food mixer (Breville, SHM2) for 3 min at high speed until the mixture became creamy. Flour and baking powder were added to the mixture and mixed for 3 min at low speed. Sunflower oil was then slowly poured in and the batter was mixed using a plastic spoon. The mixture was then transferred into a baking pan and baking was immediately performed in a Russell Hobbs 14552 Mini Oven at 180°C for 20 min.

For the purpose of studying the application of cowpea proteins in cakes, CPI, DCPI and GCPI were used to replace egg by 20 and 40%. In order to compensate the water content in eggs, cowpea protein sample was dissolved in an appropriate amount of water in the food mixer before adding the remainder of the eggs, sugar and emulsifier. The procedure followed was the same as above.

2.2.25 Preparation of mayonnaise

The recipe used was two egg yolks (33 g), pepper 0.3 g, sugar 2.5 g, white vinegar 17 mL, salt 2.5 g and sunflower oil 70 mL. First egg yolks, pepper, salt and 5 mL of white vinegar were placed into a food mixer (Breville, SHM2). Beating continued at the high speed and oil was added drop by drop. When batter became creamy the remaining sunflower oil was slowly added while beating continuously at a middle speed. After all the sunflower oil was added the remaining white vinegar was whisked in using a plastic spoon. The product was allowed to cool for 2 h in a cool place before sensory evaluation and texture determinations were carried. In order to study the application of cowpea proteins in mayonnaise, CPI, DCPI and GCPI were used to replaced egg yolk at levels of 20 and 40%.

2.2.26 Proximate analysis

Proximate analysis of the flour and bread loaves was carried out using official AOAC methods (AOAC, 1990), for moisture (14.004), crude fat (14.081), crude fibre (7.0006), ash (14.006) and crude protein (47.021). Nitrogen to protein conversion factor of 6.25 was used. Carbohydrate was calculated by subtraction.

2.2.27 Determination of bread loaf and cake volume

The volume of bread loaves and cakes was determined by the rapeseed displacement method as described by Giami et al. (2004) using sesame in place of rapeseed. The sample was weighed and placed in a 2 litre container. The sesame seeds in a measuring cylinder were poured over the loaf in the box and levelled with a spatula. The volume of the spilled sesame seeds was noted as the volume of the loaf. For bread, the specific loaf volume (SLV) was calculated as cubic centimetres per gram (cm^3/g), and the formula was "specific volume of loaf = v/wt (cm^3/g)". All measurements were done in triplicate.

2.2.28 Determination of bread crumb and cake hardness

The crumb hardness was determined after cooling for 1 hour using a Zwick/Roell type Z010 texture analyser based on Method 74-09 of AACC (2000). A cylindrical die compressed the bread or cake slices up to 40% in two cycles. At a compression of 25% the compression force value (CFV) was measured for each sample.

2.2.29 Texture analysis of mayonnaise

Texture parameters (hardness, and adhesiveness) of mayonnaise were determined by using a Zwick/Roell type Z010 machine with a 20 mm diameter cylindrical probe. The mayonnaise samples were penetrated by using force load of 0.1 N at a length of 40 millimetres and crosshead speed of ten millimetres forming.

2.2.30 Sensory evaluation

Sensory evaluation of bread, cake and mayonnaise samples was performed after 24 hours after production to evaluate overall acceptability of the products. The bread and cake samples were sliced into pieces of uniform thickness and served with water. Twenty panel members were randomly selected from students of the School of Life Sciences, Heriot Watt University, UK. Panellists evaluated product samples on a 9 point hedonic scale (Larmond, 1977) with 9 = liked extremely, 8 = liked very much, 7 = liked, 6 = liked mildly, 5 = neither liked nor disliked, 4 = disliked mildly, 3 = disliked, 2 = disliked very much and 1 = disliked extremely. Samples were presented to a panel of judges with 3-coded digit numbers. Examples of the forms for sensory evaluation for bread, cake and mayonnaise are given in Appendices I, II and III, respectively.

2.2.31 Determination of effect of cowpea proteins on pasting behaviour of starch

To evaluate of the effect of cowpea protein on pasting properties of the starch, CPI/rice starch mixture solution (10 g starch, 2 g CPI and 100 mL H_2O), GCPI/ rice starch (10 g starch, 2 g GCPI and 100 mL H_2O) and rice starch solution alone (10 g starch and 100 mL H_2O) samples were measured on a Bohlin Gemini rheometer (Malvern Instruments Limited, Worcestershire, U.K.). A cone and plane geometry with a cone angle of 2° (C 2/40) and a cone diameter of 40 mm was used and data were recorded for samples in duplicate. Samples, during testing, were equilibrated at 25°C and then heated from 25°C to 95C° at rate of 2°C/min and under constant shear rate at 100 rev/min, keeping the temperature at 95°C for 10 min and 40 sec, and cooling down to 25°C at the same rate as the heating (2°C/min) and under the same constant shear rate at 100 rev/min. The oscillation frequency was 1 Hz. The strain applied was 0.01. Thus, from the pasting behaviour of GCPI/ rice starch systems, i.e., curves of viscosity as function of temperature, the key pasting parameters can be measured (**Figure 2.4**). These include:

- The peak viscosity (Peak vis), the maximum viscosity of the sample (as shown in **Figure 2.4**).
- The final viscosity (Final vis), which in the present study, corresponds to the viscosity at 25°C. (as shown in **Figure 2.4**)
- The onset temperature (T_{onset}), when the viscosity tends to increase. From the curve (**Figure 2.4**), a starting point was drawn through the first temperatures at which point the viscosity is constant. Then a line was drawn straight down the leading edge of the region at which point the viscosity tends to increase. T_{onset} is considered the intersection point of these 2 lines as shown in **Figure 2.4**.
- The peak temperature (T_{peak}) is considered the temperature at the maximum viscosity. T_{peak} is the intersection between straight lines drawn from either side of the peak vis (point T_{peak} **on Figure 2.4**).

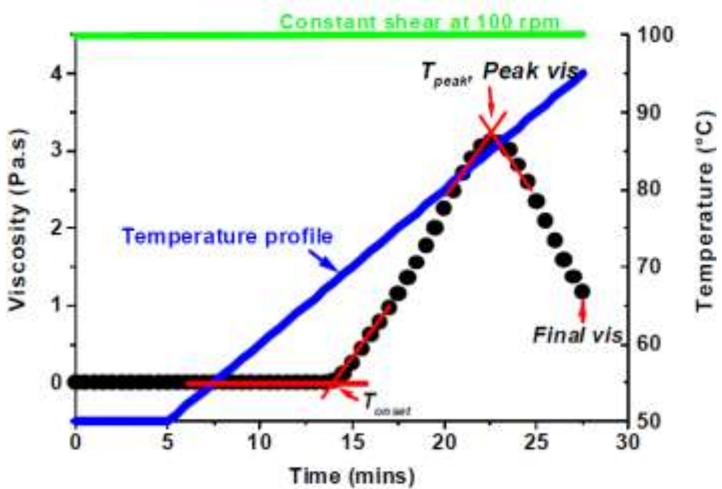


Figure 2.4 Pasting curve of starch/ milk protein (black solid symbols). Temperature profile used (blue colour), Tonset, Tpeak, Peak vis and Final vis (indicated by arrows) obtained from the curve (from Noisuwan, 2009)

2.2.32 Statistical analysis

The determinations were performed in triplicate (n=3) and mean ±standard deviation (SD) values were calculated. Data obtained in each chapter were analysed by one-way ANOVA. The comparison between means (3 replications) was performed at the 95% significance level ($p \leq 0.05$) by the least significant difference test (LSD). The analyses were carried out using SPSS version 10 for Windows (SPSS Inc., NY, USA).

Chapter Three

The Extraction and Evaluation of Cowpea protein isolate

3.1 Introduction

Cowpea is a leguminous crop commonly grown in Africa (AATF, 2005). It is a small dicotyledonous seed, which is either oval (Giami, 2005), globular or kidney (Taiwo, 1998) shaped. The chemical compositional properties of cowpeas vary considerably according to varietal differences (Longe, 1980). Cowpea (Vigna unguiculats L) is a good source of proteins. USDA (2008) reported that cowpea seed contains 24% crude protein. However, the successful application of proteins will depend on their protein content and their other chemical composition, which in turn, will be dependent on isolation conditions of the proteins such as extraction temperature, extraction and precipitation pH. On the other hand, the physico-chemical properties of these proteins will affect their optimal utilisation, which will be also dependent on isolation conditions and the nature of these proteins. Meanwhile, isolation techniques are very important for effective yield of cowpea protein.

In this study extraction of cowpea protein was compared to soy protein because both are seed proteins and the process for soy protein has been commercialised and is currently the most widely implemented protein extraction from pulses. Alkaline extraction-isoelectric precipitation is one of the most widely applied methods in protein extraction due to its efficiency and ease of operation (Chew et al., 2003). It has been used in the soy and cottonseed protein isolating industries for many years (Dennison 1999; Lewis 1996; Sanchez-Vioque, 1999; Steytler 1996). Change in pH of the protein isolation process can be used to increase yield and it can be easily accepted by the food industry without heavy investment in new machinery. Moreover, this method has no significant impact on the amino acid profile of protein (Chew et al., 2003).

Globulins are the major protein in cowpea seed ranging from 48 to 90% (Chavan et al., 1989; Chan and Phillips, 1994; Freitas et al., 2004). Chan and Phillips (1994) also reported that cowpea proteins contain 67% and 25% globulins and albumins, respectively. Freitas et al (2004) found that cowpea proteins contain 51% globulins and 45% albumins. Chavan et al (1989) found 48% - 90% globulins, 3% - 15% albumin, 7% - 23% glutelins and 5% - 13% prolamins. Although, in contradiction to these findings, Ragab et al (2004) found that 71% and 11% of cowpea proteins were albumins and globulins, respectively. These differences may be due to effects of one or more environmental factors such as temperature, light, soil type, and nutrition (Ayerza, 2009).

The cowpea globulin, vignin, vicilin type glycoprotein is a 7S (Svedberg unit) composed of 3 main units (α-, β-, and γ-vignin) (Aliuko and Yada, 1995; Freitas et al., 2004). The 7S component was equated by Daniellson (1949) with the globulin, vicilin. The 11S and 7S globulin fractions have been extensively investigated as the two fractions make up more than 50% of the total soya bean protein. Bekhit (2007) reported that cowpea contains large amounts of vicilin 7S globulin and a lesser of amount legumin-like 11S globulins. The 7S fraction of soy protein accounts for 37% of the water extractable soy protein, the majority of which is β-conglycinin (7S) (Thanh and Shibasaki, 1977; Koshiyama, 1968). Glycinin (11S) makes up 31% of the protein, and consists of the major soybean reserve protein, 11S globulin (glycinin)

As for soybean, pea and other legume seeds, cowpea proteins extraction can be done from the seeds by a great many methods such as wet methods and dry processing using pin milling and air classification techniques. Seeds are pin milled to produce flours with a specific density and particle size. Such flours can be separated into fine fraction (protein) and coarse fractions (starch) using an air classifier (Sosulski, 1982). It was reported that protein yields produced by wet extraction are as pure as those by other methods. Also, it was reported that functional properties of proteins obtained by aqueous processing are as good as those of the proteins obtained by dry processes (Gueguen, 1991). Therefore an alkaline extraction-isoelectric precipitation method was chosen for this study as a wet process in order to isolate cowpea protein. This process involved solubilisation of cowpea proteins at pH 10 (with 1N NaOH) and exclusion of insoluble material by a centrifugation technique. The proteins were precipitated by adding HCl, 1N until they reached their isoelectric point.

The purpose of the current study was reported in this chapter to examine the effect of different conditions such as extraction temperature, and extraction and precipitation pH on protein yield in order to provide information for optimising isolation conditions of cowpea proteins; and to determine their physico-chemical and functional properties. Cowpea protein isolates (CPIs) were prepared from cowpea seeds flour on a laboratory scale. The proximate composition of cowpea flour and protein isolate was determined and the effects of isolation conditions on protein yield were studied. Among the conditions investigated were extraction temperature, extraction pH and precipitation pH, as well as extracting ratio. Commercial soy protein isolate (90% protein) was used for

comparison. SDS-PAGE electrophoresis was used to analyse protein patterns and amino acid composition, thermal properties and functional properties were also determined for further characterisation. Results from this study gave detailed information on cowpea protein isolates, their extraction and their functional properties that will facilitate an estimation of their potential as food ingredients.

3.2 Materials and Methods

The materials and methods used within this study were described in chapter 2.

3.3 Results and Discussion

The present study was designed to investigate the best procedures to isolate CPI and to determine the functional quality. The CPI was prepared by isoelectric precipitation (IP) and investigated for functional properties. The studied parameters and their results are discussed in this section. The section is divided into 11 subsections. The first subsection discusses the cowpea seed flour proximate composition. The second subsection discusses the cowpea protein yield. The third subsection discusses the protein composition of CPI. The fourth subsection discusses the amino acid composition of cowpea protein isolate. Further subsections were focused on physicochemical properties of cowpea protein such as solubility, viscosity, and gelation etc.

3.3.1 Proximate composition of cowpea flour

The determination of quality of raw materials is actually dependent on proximate composition which often is considered the basis for establishing the overall acceptance and nutritional value to the consumers. Cowpea flour was analysed for its proximate composition as in **Table 3.1**. From the table, results of the proximate analysis show that cowpea was composed of 25.56% protein, 1.20% fat, 4.57% fibre, 3.29% ash and 58.09% carbohydrate.

Table 3.1 Proximate composition of cowpea flour

Parameters [a]	%
Moisture content	7.46 ±0.26
Crude fat	1.20 ±0.06
Crude protein [b]	25.56 ±0.09
Crude fibre	4.57 ±0.03
Total CHO [c]	58.09 ±0.31
Ash	3.29 ±0.02

[a] *Mean ± standard deviation of three replicates*

[b] *N x 6.25*

[c] *Total Carbohydrate*

It has been described that cowpea has substantial amounts of good quality proteins and fibres (Prinyawiwatkul et al., 1996). As shown in **Table 3.5**, cowpea fine powder flour (FP) contained 26.14% protein, 56.33% carbohydrate, 2.19% fibre, 2.61% ash and 1.03% fat, respectively. The cowpea flour was obtained by milling, sieving and defatting the beans as described in section 2.2.1, while the cowpea fine powder flour was obtained by size-separating of cowpea flours on the test sieve shaker by using a 100-mesh (0.15 mm, width) size screen. The sample that remained upon the screen was reground, sieved, and the portion that passed through the mesh sieve was mixed with the first portion. This was named in present study as cowpea flour (FP). The results are similar to those previously obtained (Sosulski et al., 1987; Abdalla et al., 2001; Ragab et al., 2004; Khalid et al., 2012). The results indicated high protein (26.14%) and low fat (1.03%) in cowpea flour.

Similarly the results of high protein and low fat content of cowpea seeds were also reported for other legumes (Adsule, and Akapopunam, 1996).

Table 3.2 Sugars and starch content of cowpea flour

Parameters	Value (%)
Glucose	0.30 ±0.01
Fructose	0.45 ±0.03
Sucrose	1.70 ±0.11
Raffinose	0.74 ±0.09
Stachyose	2.67 ±0.12
Starch	40.55 ±2.04

**Mean ± standard deviation of three replicates*

Table 3.2 presents the sugars and starch content. The cowpea flour used in this study contained 40.55% starch, a significantly higher content when compared with that reported for other kinds of legumes, soybean, 10.9 -11.7% (Stevenson et al., 2006), wrinkled pea, 26.57% - 32.55% (Dostálová et al., 2009) and pigeonpea, 33% (Rangaswamy et al., 2005). The contents of cowpea sugars were 0.30, 0.45, 1.70, 0.74, and 2.67% for glucose, fructose, sucrose, raffinose and stachyose respectively. The data

are similar to those previously obtained (Mwangwela, 2006; Longe, 1980). The verbascose could not be quantitatively estimated due to the unavailability of standard verbascose. Researchers reported that the verbascose content in cowpeas ranges from 0.6% to 3.5%, on a dry weight basis (Mwangwela, 2006; Longe, 1980; Reddy et al., 1980; Prinyawiwatkul et al., 1996).

According to Morrow (1991), Ruiz et al. (1996), Tharanathan and Mahadevamma (2003) and Larrauri (1999) cowpea and other legumes were considered common dietary fibre sources; all of them contained varying proportions of soluble and insoluble fractions. In this study, insoluble dietary fibre (IDF), soluble dietary fibre (SDF) and total dietary fibre (TDF) of cowpea flour were 9.90, 1.11, 11.01g/100g CF (cowpea flour) respectively as shown in **Table 3.3**.

Table 3.3 Dietary fibre content of cowpea flour

Parameters	Value (%)
IDF[1]	9.90 ±0.41
SDF[2]	1.11 ±0.35
TDF[3]	11.01 ±0.7

**Mean ± standard deviation of three replicates*

[1] IDF stands for "Insoluble dietary fibre";

[2]SDF stands for "Soluble dietary fibre";

[3]TDF stands for "Total dietary fibre".

3.3.2 Yield of cowpea protein

Protein samples were prepared from cowpea flour (CF) by alkaline extraction-isoelectric precipitation (IP). The cowpea flour was extracted by using fine powder (FP) to distilled water ratios of 1:5, and 1:10. The protein was extracted by adjusting the water to pH 9 and 10 (alkaline extraction) and then precipitated by adjusting the water to pH 4 and 4.5 (isoelectric precipitation). **Table 3.4** shows the effects of extraction ratio (Ext. Ratio), extraction pH (Ext. pH), and precipitation pH (Ppt. pH) on the protein yield and protein contents of cowpea protein isolate. The highest yield of protein (89.05%) was obtained from an extraction ratio 1:10 with an extraction and precipitation pH 10 and 4.5 respectively. This was followed by 1:5 extraction ratio when the protein was extracted by using extraction pH 10, and precipitated at pH 4.5

which gave 85.16% protein yield. The lowest yield (61.63%) was obtained from an extraction ratio 1:5 with an extraction and precipitation pH 9 and 4.0 respectively. The highest content of protein (90.76%) was obtained using the $CPIP_8$ method, at the same time as the lowest content (50.22%) was obtained using the $CPIP_1$ method as shown in **Table 3.4**. It is known that the solubility of a protein depends on the net charge on the surface of a protein. The net charge depends on the pH of the solvent and the number and identities of the amino acids that make up the protein. The hydrophilic amino acids include arginine, aspartic acid glutamic acid and lysine. Depending on the pH of the solvent the charges of the amino acid's side chains change. Lysine and arginine begin to lose their positive charge as the pH increases. If the pH decreases on the other hand, aspartic acid and glutamic acid begin to lose their negative charges. A protein becomes more soluble if there is a net charge at the protein surface, since it prefers to interact with water, rather than with other protein molecules (Ahmed et al, 2010).

The picture of fine powder cowpea flour (FP), cowpea protein isolate (CPI), and soy protein isolate (SPI) is shown in **Figure 3.1**. Commercial SPI (90% protein) was added to the picture for comparative purpose.

Table 3.4 Protein yield of CPI produced by using different combinations of Extraction and Precipitation pH

Samples	Ext. Ratio	Ext. pH	Ppt. pH	Protein Content (%)	Protein Yield (%)
$CPIP_1$	1:5	9.0	4.0	50.22 ±0.15	61.63 ±0.34
$CPIP_2$	1:5	9.0	4.5	77.04 ±0.27	71.11 ±0.22
$CPIP_3$	1:5	10.0	4.0	80.03 ±0.17	81.64 ±0.19
$CPIP_4$	1:5	10.0	4.5	84.56 ±0.24	85.16 ±0.31
$CPIP_5$	1:10	9.0	4.0	57.44 ±0.21	63.42 ±0.14
$CPIP_6$	1:10	9.0	4.5	75.22 ±0.23	69.06 ±0.25
$CPIP_7$	1:10	10.0	4.0	77.26 ±0.31	83.57 ±0.22
$CPIP_8$	1:10	10.0	4.5	90.76 ±0.11	89.05 ±0.10

**Data were the mean value of three replicates ± deviation standard.*

CPIP stands for "Cowpea protein-isoelectric point precipitated";

Ext stands for "Extraction";

Ppt stands for "Precipitation".

It has been reported that about 95.7% of the total cowpea protein could be extracted by solvents (distilled water, 1.0 M NaCl, 70% ethanol and 0.2% NaOH) on

the basis of solubility and the remaining percentage is believed to be insoluble proteins and non-protein nitrogen (Ragab et al., 2004). Although many studies have indicated that globulins are the major protein in cowpea seed ranging from 48 to 90% (Chavan et al., 1989; Chan and Phillips, 1994; Freitas et al., 2004), Ragab et al (2004) reported values for albumin (71.4%), globulin (11.1%), glutelin (11.0%) and prolamin (2.20%). The cowpea protein composition differences reported may be due to effects of one or more environmental factors. The effects of temperature, light, soil type, and nutrition can affect cowpea seed protein (Ayerza, 2009).

Figure 3.1 Protein Samples: fine powder cowpea flour (FP), cowpea protein isolates (CPI,) and soy protein isolate (SPI). SPI was a commercial sample (90% protein) added to the picture for comparative purpose.

Extraction pH of 9 and 10, extraction ratios of 1:5 and 1:10, and isoelectric precipitation at pH of 4.0 and 4.5 were done to obtain eight cowpea protein fractions of protein contents ranging from 50.22 to 90.76% and protein yields ranging from 61.63 to 89.05%. The CPI yield was increased with increasing extraction temperature from 25 to

55°C (**Figure 3.2**). When temperature is increased, molecules move faster due to increase in kinetic energy. The increase in water temperature causes more hydration of fibres associated with proteins, increasing contact of proteins with water. Meanwhile, as the protein warms, the non-polar side-chains become more accessible for interactions with the solvent because of the increased molecular motion from higher temperatures, which facilitates the increase in solubility (Wang, 1999). Alli (1977) reported that elevating the extraction temperature (ET) from 20 to 45°C was associated with increased yield of white kidney bean protein; Davidson et al. (1979) found that increasing extraction temperature (ET) increased the yield of protein isolate from soybean. These previous studies confirm what is indicated by the **Figure 3.2**.

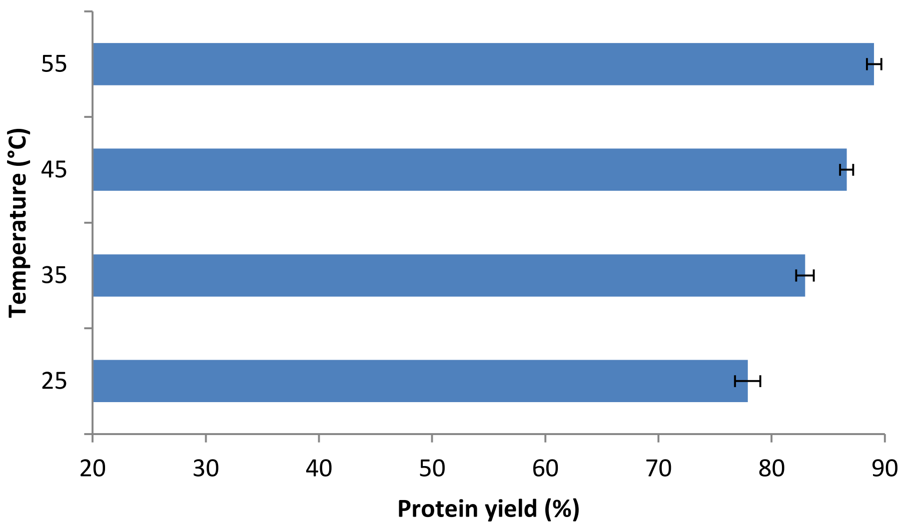


Figure 3.2 Effect of extraction temperature on protein yield

In the present study cowpea protein isolate (CPI) prepared from cowpea seeds flour amounted to 89.05% yield compared to that reported by El-Jasser (2010) who obtained 51% yield by using pH extraction and precipitation 9 and 4 respectively, at room temperature. This higher yield of extraction might be because of fine grinding of CF and removing of impurities by sieving before extraction along with some extraction conditions i.e. temperature, pH and extraction ratio.

This study reports for the first time a method that results in a yield of 89%, which is feasible for industrial scale implementation without heavy investment in new machinery. **Appendixes IV-VI** show some of the equipment used in a factory for soy protein extraction, including the decanter centrifuge and clarifier centrifuge (for

separating the solids and solutions), batch tank for stirring and pH adjustment, pasteuriser (heat treatment), and spray drier. Furthermore, this method has no significant impact on the amino acid profile of protein (Chew et al., 2003).

3.3.3 Proximate composition of cowpea protein samples

Protein composition is important in evaluating the quality of raw materials and often the basis for affecting the functional properties of the protein in food systems. Codex Alimentarius Commission (1989) reported that a product is defined as protein isolate, if it contains protein content equal to or higher than 90%, protein concentrate if it has protein content ranging from 65%-90%, and as protein flour when it has protein content of 50%-65%. All protein samples in the present study were estimated for their proximate compositions as shown in **Table 3.5**. From the table, protein content of cowpea fine powder was 26.14%; cowpea protein isolate, 90.76; and soy protein isolate was 90.0%. Liquid drainage after precipitation was analysed for protein content. It was found that 3.1% protein of cowpea protein was lost with this liquid. The content of protein (90.76%) of CPI obtained in this study was in agreement with Codex Alimentarius Commission (1989) for labelling a protein extract as Protein Isolate. From the results and according to the CODEX Standard (Codex Alimentarius Commission, 1989), it was demonstrated that, cowpea protein isolate (90.67% protein content) was obtained only from the extraction pH of 10, extraction ratios of 1:10, and isoelectric precipitation at pH of 4.5 (method CPIP8). However, cowpea protein samples of protein content 77.04, 80.03, 84.56, 75.22, and 77.26% which were produced by the CPIP methods CPIP2, CPIP3, CPIP4, CPIP6 and CPIP7 respectively could be considered as Cowpea Protein Concentrates, meanwhile those of protein content 50.22 and 57.44% which were produced by the CPIP methods CPIP1 and CPIP5 could be considered as Cowpea Protein Flour.

Similar results were found in the literature (Horax et al., 2004; Mwasaru et al., 2000). The protein content of CPI was not significantly different from that of soy protein isolate (SPI), which is commercially widely used in the food industry. Some scientists like Khalid et al. (2012) reported lower protein content in CPI i.e. 76.0%. The two protein isolates (CPI & SPI) were investigated for their properties.

The proteins are actually polymers made of amino acids and as reported by Butt and Batool (2010), the relative proportion of proteins represents their quality and this, in

turn, is dependent on the genetic makeup of legumes. Meanwhile some environmental factors, may affect protein contents of these protein isolates. The plants are affected by many factors such as day length, high temperature and relative humidity, and their potential yields may not be realized in all environments (Uncu and Arioglu, 2005). Plant breeders make use of genotype-environment interactions for the identification of high yield cultivars of the required quality. Previous studies have reported the effect of factors such as climatic conditions, soil characteristics, agronomic practice, genotypic characteristics (Piper and Boote, 1999), and water stress (Noureldin et al., 2002) on the protein content of soybean. Generally, protein content decreases in areas with high temperatures (Kane et al., 1997; Gunasekera et al., 2006). According to Miladinovic et al. (2006), the protein content of soybean seeds grown in environments at similar latitudes had significant differences. Sogut (2006) found significant effects of interaction between year and environment on protein content in soybean seed. Arslanoglu et al (2011) reported that genotype-environment interaction was significant for protein content, and Sudaric et al. (2006) also mentioned that protein content was affected by environmental changes.

Table 3.5 Proximate composition of cowpea protein isolate (CPI) compared to soy protein isolate (SPI) and cow pea fine powder FP

Protein samples[1]	Components (g/100g flour)					
	Moisture	Crude protein[5]	Crude fat	Crude fibre	Total ash	Total CHO[6]
FP[2]	7.06 ±0.22	26.14 ±0.13	1.03 ±0.04	2.19 ±0.02	2.61 ±0.02	56.33 ±2.12
CPI[3]	5.40 ±0.16	90.76 ±0.56	0.50 ±0.01	1.07 ±0.05	1.95 ±0.03	4.38 ±0.04
SPI[4]	5.14 ±0.11	90.00 ±1.03	0.52 ±0.02	0.49 ±0.01	3.18 ±0.01	0.36 ±0.01

[1] *Mean ± standard deviation of three replicates*

[2] *FP stands for cowpea fine powder;*

[3] *CPI stands for "Cowpea protein Isolate";*

[4] *SPI stands for "Soy protein Isolate";*

[5] *N x 6.25*

[6] *Total Carbohydrate*

3.3.4 Amino acid composition of cowpea protein isolate

The amino acid compositions of cowpea protein isolate (CPI) and soy protein isolate (SPI) are presented in **Table 3.6**. The amino acid composition of CPI was done in this study and the values of SPI were obtained from Tian et al. (2011) and these show similar patterns of amino acid for both CPI and SPI. Tryptophan is not shown in the table because it was decomposed by the hydrolysis with HCl. As can be seen from the table (**Table 3.6)**, the levels of amino acids such as glycine, leucine, lysine, phenylalanine and valine in CPI are higher than those in SPI but the levels of some other amino acids such as aspartic and histidine were low in CPI in comparison with those in SPI. As shown also, the sulphur amino acid (methionine) was low in these two types of protein (CPI & SPI). The low levels of methionine in both CPI and SPI have been found in other legume seed proteins as reported by other studies (Sahasrabudhe et al., 1981; Sosulski and McCurdy 1987; Paredes et al., 1991).

Table 3.6 Amino acid profile of cowpea protein isolate (CPI)

Name (amino acid)	CPI[1] (g/100g)	SPI[2] (g/100g)
Aspartic acid	11.84	12.1 ±0.3
Threonine	05.05	03.1 ±0.0
Serine	01.27	03.0 ±0.1
Glycine	04.22	04.3 ±0.1
Alanine	09.74	04.5 ±0.0
Valine	01.18	05.3 ±0.1
Methionine	01.26	01.1 ±0.0
Isoleucine	06.91	05.2 ±0.2
Leucine	15.13	08.5 ±0.2
Tyrosine	03.66	03.2 ±0.0
Phenylalanine	08.57	05.9 ±0.2
Lysine	09.72	06.4 ±0.2
Histidine	01.98	02.7 ±0.0
Arginine	09.30	08.0 ±0.2
Proline	01.15	04.9 ±0.1

[1]Data are means ± standard deviation of duplicate determinations.

[2]Data from Tian et al. (2011). CPI[1] stands for "Cowpea protein isolate"; SPI[2] stands for "Soy protein isolate".

3.3.5 SDS-PAGE electrophoresis

Electrophoresis was performed with samples using SDS-PAGE in order to identify the proteins in cowpea protein isolate (CPI) and to compare them with those in soy protein isolates (SPI) on the basis of molecular size. The procedure followed was that described by Wu and Hojilla-Evangelista (2005) and Coomassie brilliant blue was used to stain the gels. Details of the method are presented in Chapter 2 (2.2.4). Protein pattern staining of CPI and SPI are shown in **Figure 3.3**. Cowpea fine powder flour (FP), cowpea protein isolate (CPI), and soy protein isolate (SPI) contained approximately similar bands ranging from 36 to above 110 KDa with major polypeptides (36 and 50 KDa) observed in the cowpea fine powder flour (FP) and cowpea protein isolate (CPI). On the other hand, the major polypeptides of soy protein isolate (SPI) were 38 and 58 KDa. The major constituents in legume seeds proteins are globular proteins (El-Adawy, 1996; Abrol and Ahmad, 2003). According to Paredes et al (1991) chickpea proteins have molecular sizes similar to cowpea proteins. In previous studies conducted via SDS-PAGE also minor and major protein bands of kidney and navy bean proteins appeared to have similar molecular sizes to cowpea proteins (Horax et al., 2004; Kohnhorst et al., 1990).

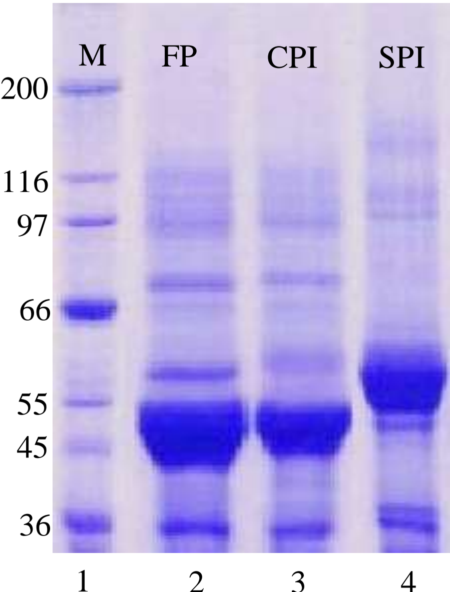


Figure 3.3 Protein staining of cowpea fine powder flour (FP), cowpea protein isolate (CPI) and soy protein isolate (SPI). Lane1 = Marker (KDa); Lane2 = cowpea fine powder flour (FP); Lane 3 = cowpea protein isolate (CPI); Lane 4 = soy protein isolate (SPI).

3.3.6 Solubility of cowpea protein isolate

Solubility characteristics of proteins under different conditions are often important in determining their functional applicability and in the improvement of protein extraction. Solubility is an important functional property, as it has effects on other functional properties of proteins (Sikorski 2001).

In the present study, the solubility profiles of the samples are shown in **Figure 3.4**. This includes cowpea protein isolate (CPI) and soy protein isolate (SPI). Both two samples gave the solubility curves indicating a typical behavior at isoelectric point, which is around pH values in the range of pH 4 to pH 5, similar to those reported for proteins from other legume seeds, lablab protein concentrate (Melaku, 1998), winged bean flour (Narayana and Narasinga Rao, 1982), chick pea flour (Bencini, 1986) and cowpea flour (Elkhalifa, 1997). However, CPI had much higher solubilities than SPI, particularly in the neutral and alkaline pH range. This is similar to what was obtained for CPI which had high solubility values at alkaline pH (Horax et al., 2004). The extraction of proteins at pH 10 could explain higher solubility of CPI obtained in the present study.

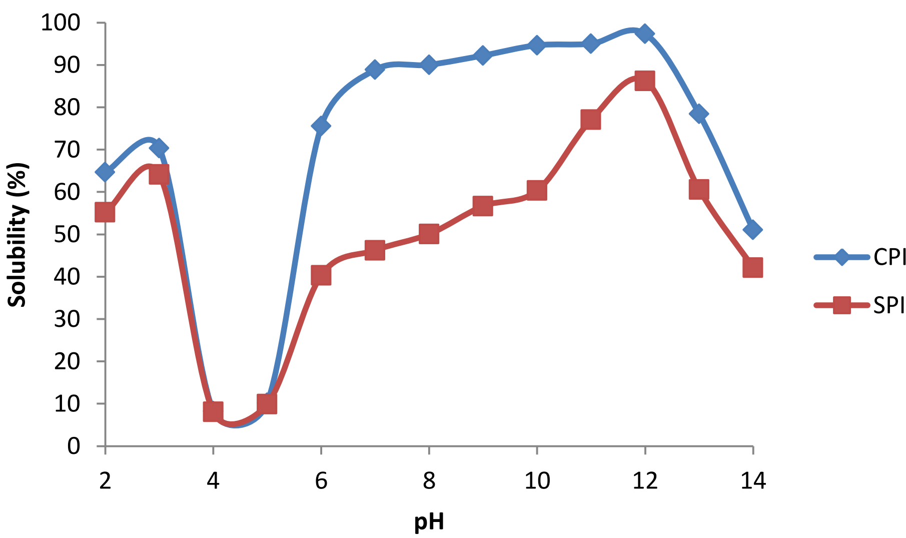


Figure 3.4 Effects of pH on protein isolate solubility of CPI (0.4% w/v) and SPI (0.4% w/v). CPI = (Cowpea Protein Isolate; SPI = Soybean protein Isolate

Solubility of protein of CPI was minimum (4-6% soluble protein) at pH 4.0-5.0. This is due primarily to both the net charge of peptides, which increases as pH values move away from the isoelectric region and surface hydrophobicity that reinforces the

aggregation and precipitation through hydrophobic interactions (Sorgentini and Wagner, 2002). Khalid et al. (2012) observed an isoelectric pH of 5.0 for cowpea protein implemented on commercial scale for the purpose of raising the global utilization of cowpea seed and raising the obtainability of cheap, good quality protein for human consumption. The pH-dependency of protein recovery from plant has been observed previously for Cashew nut (Ogunwolu et al., 2010).

Damodaran (1996) reported that pH and ionic strength influence electrostatic forces which affects the solubility. The insolubility of most proteins at their isoelectric point (IP) is due to neutralisation of charge repulsion that occurs between protein molecules (Damodaran, 1996). Diversity of isolation steps of proteins may have different effects on surface charge and the difference in solubility between CPI and SPI in the neutral pH range may be due to this reason.

It is known that the solubility of proteins depends on the ionic strength along with function of the nature of ions (Thijs and Jörg, 2008). Protein solubility at various pH values could be determined by the prevalent charge on the protein constituent amino acids (Adebowale et al., 2005) as shown in **Figure 3.5**.

$R\text{-}CH(NH_2)\text{-}COO^-$ (II) $\underset{OH^-}{\overset{H^+}{\rightleftharpoons}}$ $R\text{-}CH(^+NH_3)\text{-}COO^-$ (I) $\underset{OH^-}{\overset{H^+}{\rightleftharpoons}}$ $R\text{-}CH(^+NH_3)\text{-}COOH$ (III)

Figure 3.5 Protein solubility at various pH values (from Adebowale et al., 2005)

It is a zwitterion which prevails at the region of isoelectric pH in protein. Lowest solubility occurs at this pH due to lowest repulsion between the constituent amino acids. The balance in negative and positive charges reduces the electrostatic repulsion, and this, in turn, reduces protein solubility at the region of isoelectric point.

Once pH of the protein solution is reduced below the region of isoelectric point, cation III takes preponderance while in alkaline medium, anion II predominates

(Adebowale et al., 2005). In these cases, electrostatic repulsion is enhanced which improves solubility as is shown in **Figure 3.4** at pH 3 and pH 7-12.

Similar results for cowpea proteins have been previously reported by some researchers (Khalid et al., 2012; Naczk et al., 1986). However, variety of the materials and the conditions of processing significantly affect proteins solubility (Tian, 1998).

The commercial preparation of soy proteins may lead to partial denaturation. Smith and Circle (1978) reported that moist heat treatment rapidly insolubilises soy proteins. Moreover, the initial fat content of soybean is relatively high which requires extraction by solvent and subsequent removal of this solvent. These treatments in the addition to heat in processing lead to reduction of solubility (Tian, 1998). In the case of cowpea, these problems do not happen because cowpea has low fat. The high solubility of CPI in the low pH range (4.5 to 6) indicates that this protein may be ideal in the formulation of acidic food (Kinsella, 1979) compared to SPI.

3.3.7 Viscosity of CPI as influenced by pH and temperature

As shown in **Figure 3.6** the viscosity of CPI was higher than that of SPI, at the same temperatures. It was still comparable to SPI even when heated at 60°C, which indicates an acceptable range according to Schenz and Morr, (1996). Kulkarni et al. (1991) reported that most of the food products especially infant foods produced at the moderate (40°C) and high (60°C) temperatures will provide a good viscosity for infant food formulation.

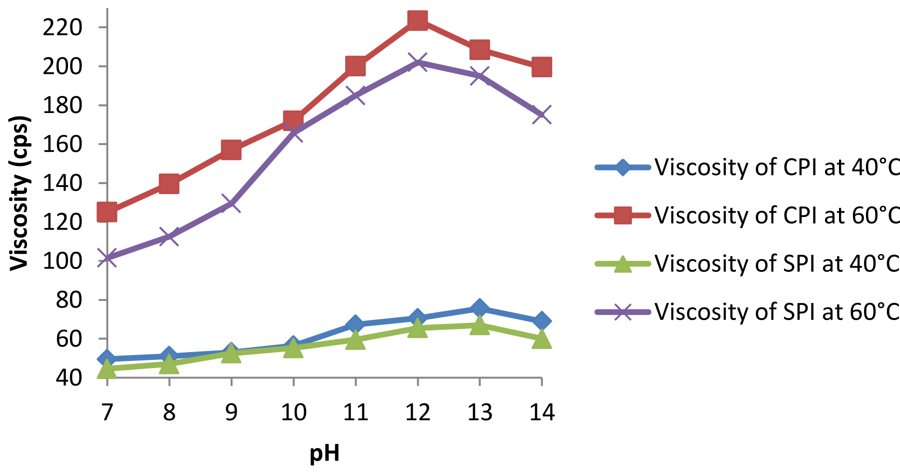


Figure 3.6 Viscosity of CPI (10% w/v) and SPI (10% w/v) as influenced by pH and temperature CPI = Cowpea Protein Isolate; SPI = Soy protein Isolate

Both CPI and SPI exhibited a gradual increase in the viscosity at 60°C as the pH was increased; this could be related to solution conditions, some of which are: temperature, ionic strength and pH which affect viscosity values of protein solutions. It was observed that the viscosity of CPI at high temperature (60°C) was significantly ($p<0.05$) higher than that at moderate temperature (40°C). Modler and Emmons (1977) reported a decrease in viscosity of globular proteins when the temperature and pH are decreased and an increase as the temperature and pH are increased. Krase et al (2002) reported that solubility of proteins plays an important role in their rheological properties. Knowledge of the viscosity of protein is of practical importance in product formulation, mouthfeel properties and processing texture control (Kinsella, 1979). The present results are in agreement with results from a study of Bian et al (2003) who reported that the viscosity of soy protein increased as the temperature increased. It has been reported that low viscosity of SPI may be useful in the development of juice-based beverages, high protein drinks and its utilization in infant food formulation in order to avoid the adverse consequences that occur in the case of high viscosity (Sze-Tao and Sathe, 2000).

The present results demonstrate that CPI would have similar applications potential to SPI.

3.3.8 Thermal properties of cowpea protein isolate

The different structural and conformational properties of proteins shown by heating can be revealed by differential scanning calorimetry (DSC). Although the thermal properties of CPI were determined using a DSC by Horax et al. (2004), further research is necessary to confirm and to identify the exact denaturation temperature of this protein.

The thermal properties of the samples are shown in **Table 3.7** as indicated by using Differential scanning calorimetry (DSC) in order to investigate the thermal stability of cowpea protein isolate (CPI) in comparison to soy protein isolate (SPI). The thermal stability indicates proteins resistance to aggregation in response to heating. As can be also seen from **Figure 3.7** which demonstrates DSC thermograms of CPI and SPI, cowpea proteins had one exothermic transition peak at 84.21°C with denaturation enthalpy (ΔH) 8.56 J/g which corresponds to the 7S component, while soy proteins had two exothermic transitions conforming to 7S and 11S globulins (Hua et al., 2005).

Table 3.7 shows the denaturation temperature (Td) and denaturation enthalpy (ΔH) of the protein isolates. There is no significant difference (P>0.05) between Td of 7S subunits of CPI (84.21°C) and of SPI (82.49°C) (Table 3.7). ΔH of CPI (8.56 J/g protein) was much higher than ΔH of 7S subunits of SPI that was 1.25 J/g protein. Td and ΔH of 11S subunits of SPI were 96.11°C and 3.88 J/g protein, respectively. This means that more heat is required to denature SPI than CPI.

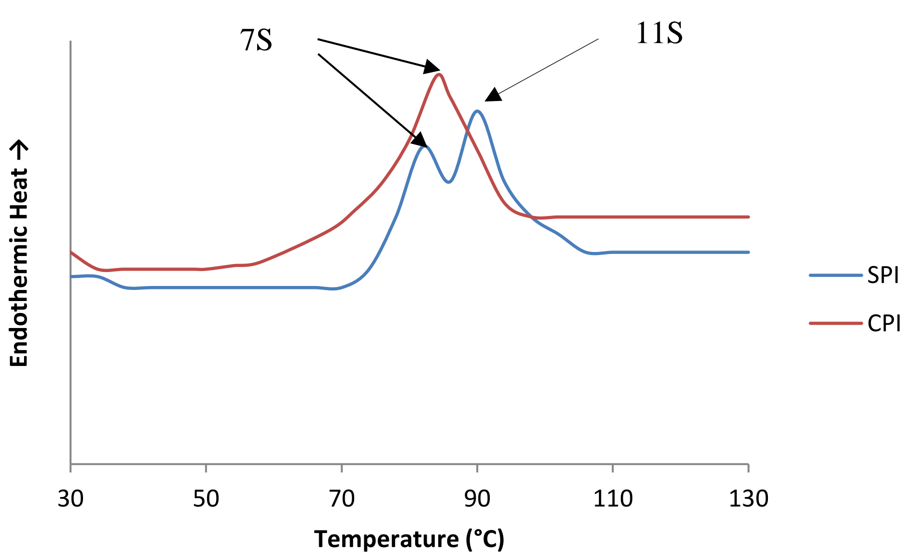


Figure 3.7 DSC thermogram of CPI and SPI; 7S globulin; 11S globulin.

Horax et al. (2004) who investigated the characterization of cowpea protein isolate prepared from three cowpea varieties, reported that CPI has 85.2 - 88.4°C and 8.42 - 10.33 J/g denaturation temperature and enthalpy respectively. The high ΔH of CPI indicates a high level of 7S globulin compared to that in SPI, which contains less 7S globulin than 11S globulin. This ΔH value also demonstrated a high heat stability of CPI in comparison with SPI. However, because there is no significant difference in the denaturation temperature (Td) between CPI and SPI, the stability to heat of these two proteins might be similar to each other. This information may be helpful in the design of proper heat treatment for the application and addition of CPI in food products, especially those that need heat-induced gelation for emulsion stability and structure.

Table 3. 7 Thermal properties of cowpea and soy protein isolate samples[1] as determined by DSC

	7S				11S			
Protein isolate[2]	**Onset (°C)**	**End (°C)**	**Td (°C)[3]**	**ΔH (J/g)[4]**	**Onset (°C)**	**End (°C)**	**Td (°C)**	**DH (J/g)**
CPI	76.98 ± 1.08	93.91 ± 1.25	84.21 ±1.84	8.56 ±0.97	-	-	-	-
SPI	78.44 ± 1.53	88.02 ± 1.41	82.49 ±1.75	1.25 ± 0.23	89.98 ± 0.46	101.99 ± 0.95	96.11 ± 0.58	3.88 ± 0.61

[1]*Mean ± standard deviation of three replicates*

[2]*CPI = (Cowpea protein isolate; SPI = Soybean protein isolate*

[3]*Td = denaturation temperature;*

[4]*ΔH = Denaturation enthalpy*

3.3.9 Comparisons of selected functional properties of CPI against SPI, WPC 60 and WPC-GOS

Functional properties of protein refer to its physical and chemical properties which affect its utilisation in food applications (Zayas, 1997). The functional properties of CPI (90% protein) prepared on lab scale were compared to those of commercially prepared, commercially available whey protein concentrate (60% protein) (WPC 60), commercially available soy protein isolate SPI (90% protein) and a Maillard reaction formed conjugate of whey protein isolate and galacto-oligosaccharide (WPC-GOS, 50% protein) which is not commercially available. Whey proteins are now viewed as valuable ingredients due to recent discoveries of functionality roles. The food/beverage industry has recently recognized the application and marketing benefits of soy protein. Therefore, in this study we compared cowpea protein functionality with WPC on the one hand and with WPC-GOS on the other hand, since comparison of the functional properties of cowpea protein and whey protein are needed to identify specific ingredient applications and marketing strategies. It is important to mention here that the modification of WPC-GOS, which was conducted by conjugation of WPC with galacto-oligosaccharides, has improved its functional properties, which indicates that a similar modification could lead to improvement of the functional properties of cowpea proteins.

Foam capacity (FC), foam stability (FS), water holding capacity (WHC), fat absorption capacity (FAC), emulsifying activity index (EAI) and emulsifying stability index (ESI) are shown in figures 3.8, 3.9, 3.10, 3.11, 3.12 and 3.13 respectively.

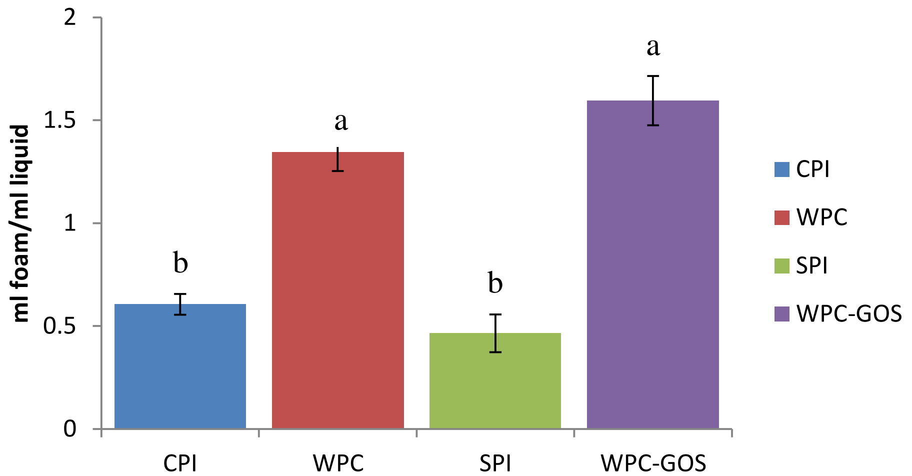


Figure 3.8 Foam capacity of CPI compared with WPC, SPI and WPC-GOS (1% w/v). Means not indicated by the same superscript are significantly (P<0.05) different.

The whipping characteristics of proteins were determined according to Mwasaru et al. (1999). Foams consist of air cells trapped inside a liquid and separated by a thin liquid lamellar phase (Britten and Lavoie, 1992). Foams can be formed wherever energy is expanded due to their large liquid-gas interfacial area and, once formed, are basically unstable (Hailing and Walstra, 1981). Natural proteins can be used as protein foaming agents. Proteins form a stabilising film around air bubbles by means of rapid adsorption, which promotes foam expansion (Britten and Lavoie, 1992). Protein foams are used to enhance texture, appearance and consistency of foods and they are important in various food industries (Vani and Zayas, 1995). The FC (**Figure 3.8**) of CPI was lower than those of WPC and WPC-GOS but similar to that of SPI. The FS (**Figure 3.9**) was lowest for CPI. Although the FS (**Figure 3.9**) of CPI is lower than other proteins samples, its FC (**Figure 3.8**) was similar to that of SPI. Although not as good as WPC 60, CPI could be potentially applied to dairy-free food applications such as: meringues, soft ice creams, whipped toppings, milk shake mixes, etc. Foaming aids in air incorporation, leavening, and texturization in baked goods, meringues and whipped toppings etc. (Malekian et al., 2000). Kinsella and Melachouris (1976) reported that protein solubility affects foaming properties of protein, thus the foaming properties of CPI indicate promising food applications. Kinsella (1985) reported that low foaming capacity was due to lesser solubility, inadequate electrostatic repulsions and excessive protein-protein interactions. The results of the current investigation are in agreement with the finding of Butt and Batool (2010).

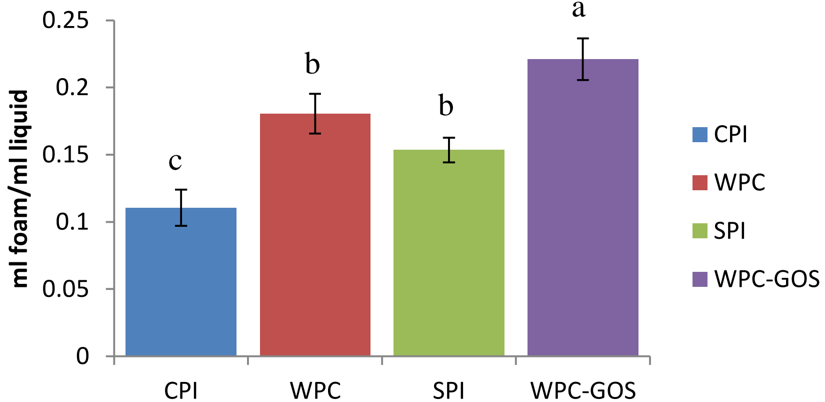


Figure 3.9 Foam stability of CPI compared with WPC, SPI and WPC-GOS (1% w/v). Means not indicated by the same superscript are significantly (P<0.05) different.

In foods protein can interact with water and oil because of its hydrophilicity and hydrophobicity. Water holding capacity of protein is its ability to hold its own and added water through the application of force (Zayas, 1997). The terms water holding ability, water hydration capacity, water absorption, and, water binding are all used to denote the amount of water that a protein can absorb and retain in food applications (Quinn and Paton, 1979). The WHC (**Figure 3.10**) of CPI is significantly lower than that of SPI and WPC-GOS but greater than that of WPC. As shown in **Figure 3.10**, CPI had a WHC of 1.98 g water/g protein that is in agreement with that obtained by Ragab et al (2004) and that by Prakash and Narasinga (1986) and similar to that of protein concentrates (Lin and Zayas, 1987). The high value of WHC might be due to the fact that this protein had good ability to swell for the reason that it contained high content of protein, which could be accountable for the increased WHC (Kinsella, 1979). The WHC of CPI is significantly greater than that of WPC. Therefore, incorporation of CPI in food products could enhance the mouth feel by increasing the moisture therefore it might be useful in viscous foods to increase viscosity (Abulude et al., 2006). On the other hand, WHC of CPI is significantly lower than that of SPI.

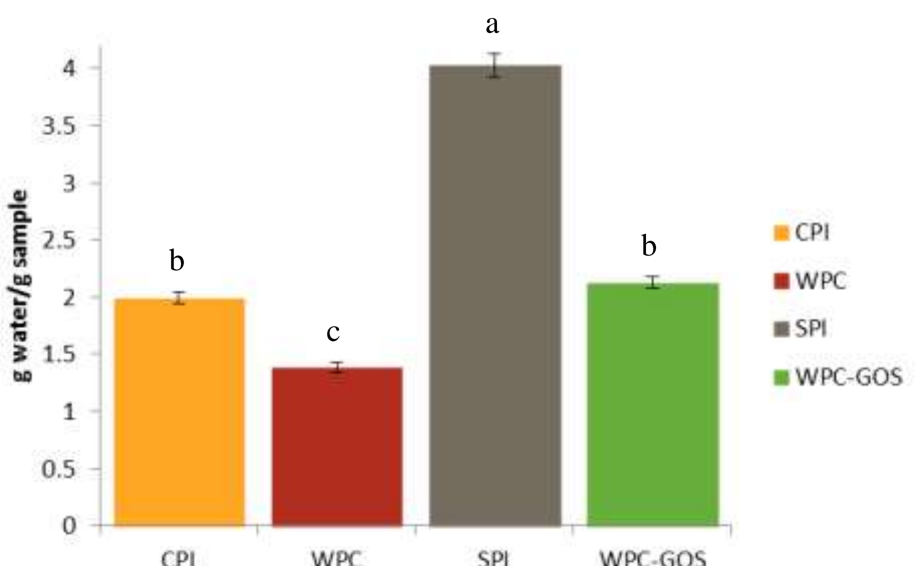


Figure 3.10 Water holding capacity of CPI compared with WPC, SPI and WPC-GOS (16% w/v). Means not indicated by the same superscript are significantly (P<0.05) different.

Fat absorption capacity (FAC) is important in food applications, since fats and oils play important roles to improve the mouth-feel and flavour retention (Kinsella and Melachouris, 1976; Prinyawiwatkul et al., 1997; Serdaroğlu et al., 2005). It is

commonly determined by adding liquid oil to a powdered protein, mixing together and holding, centrifuging, and measuring the amount of absorbed oil (Lin et al, 1974). In the current study, the FAC (**Figure 3.11**) of CPI was found to be lower than SPI but higher than WPC.

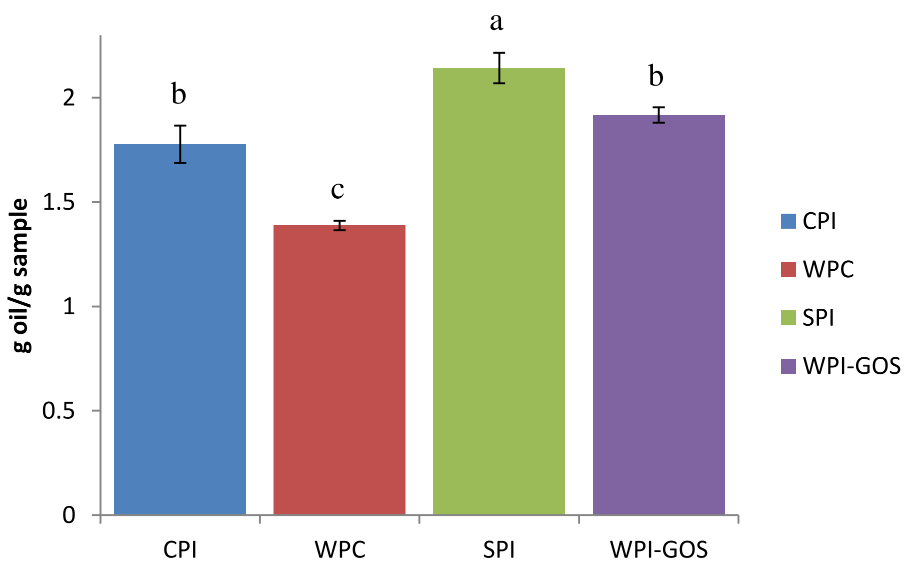


Figure 3.11 Fat absorption capacity of CPI compared with WPC, SPI and WPC-GOS (16%w/v). Means not indicated by the same superscript are significantly (P<0.05) different.

The result obtained for CPI (1.76 g oil/g protein) is lower than that reported by Sefa-Dedeh, and Yiadom-Farkye (1988) but higher than that obtained by Ragab et al (2004). They explained the mechanism of physical entrapment of oil and many authors have related it to the different conformational features and the nonpolar side chains of the proteins (Ragab et al., 2004). The FAC of CPI is lower than that of SPI but greater than that of WPC. In general CPI had good FAC and this makes the CPI suitable for many kinds of meat analogs such as breading and batter mixes, where it helps to improve mouthfeel and retain juiciness.

Makri et al. (2005) pointed out that proteins, being surface active agents, help in the formation and stabilization of emulsion due to electrostatic repulsion that they create by absorbing to the oil droplet surface. An emulsion is defined as a two-phase liquid system, one dispersed in the other as droplets (Damodaran, 1996). Emulsifying capacity is usually defined as the volume of oil emulsified by protein before the emulsion breaks down. Kinsella and Melachouris (1976) mentioned that emulsion activity and emulsion stability refer to the capacity of a protein to form an emulsion that continues unaffected

for a certain time under specified conditions. The common emulsions are oil-in-water systems, one of which is mayonnaise; and water-in-oil types, one of which is margarine (McWatters and Cherry, 1981). In the current study, as shown in **Figure 3.12,** the EAI of CPI is lower than those of other proteins samples but the differences were not significant. ESI (**Figure 3.13**) of CPI is significantly lower than SPI and WPC-GOS but there was no significant difference between CPI and WPC.

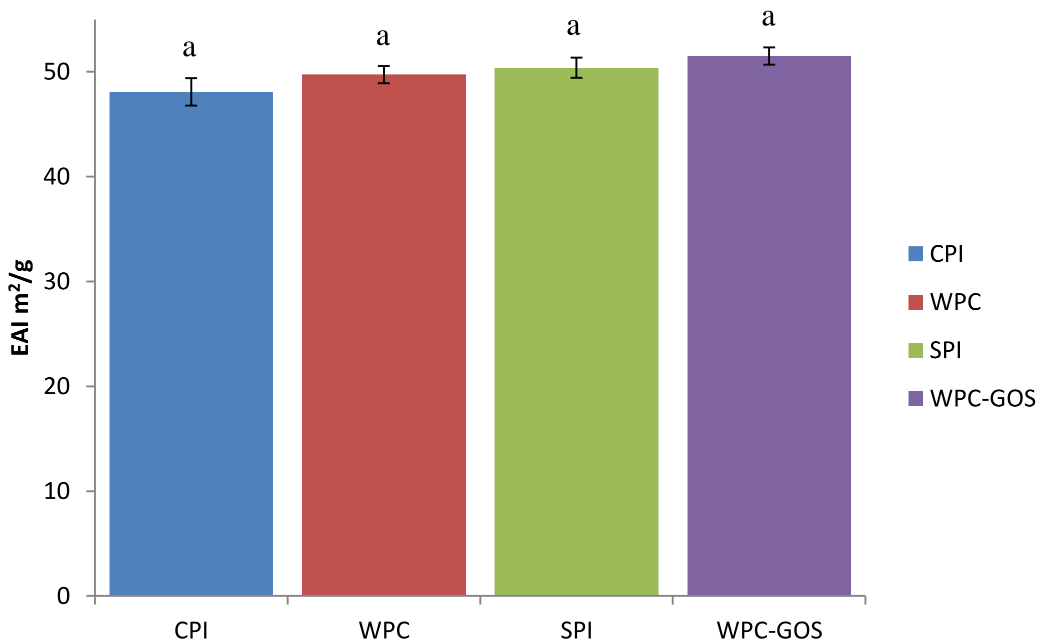


Figure 3.12 Emulsifying activity index of CPI compared with WPC, SPI and WPC-GOS (1% w/v). Means not indicated by the same superscript are significantly (P<0.05) different.

The emulsion properties of CPI are in concordance with those reported earlier by Mwasaru et al. (2000), Horax et al. (2004) and Ragab et al (2004).

Protein solubility largely affects foaming, emulsification and gelation functionalities (Kinsella and Melachouris, 1976), consequently the high solubility of CPI indicates promising food applications. CPI is a novel and exciting product and its functional properties are very interesting. It should have several applications and use in the food industries.

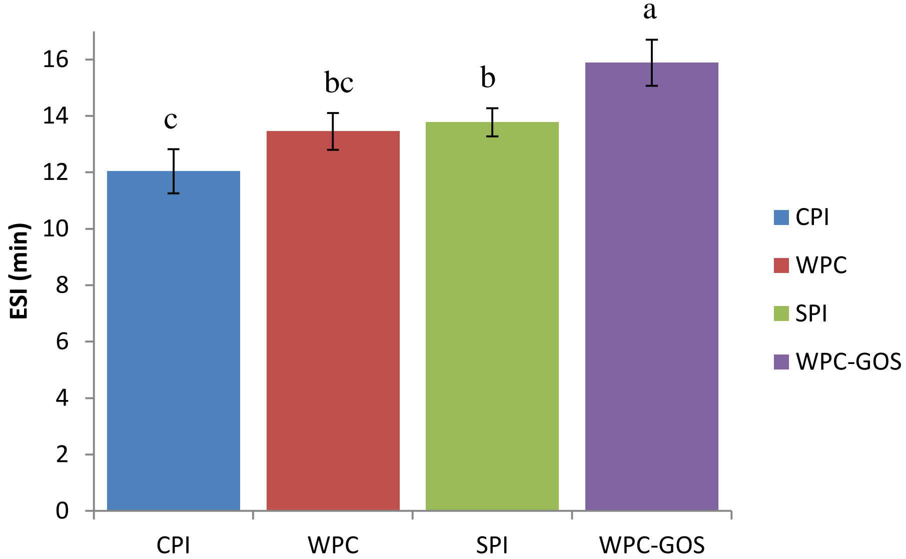


Figure 3.13 Emulsifying stability index of CPI compared with WPC, SPI and WPC-GOS (1%). Means not indicated by the same superscript are significantly (P<0.05) different.

3.3.10 Gelation properties

The formation of gels is an orderly aggregation of denatured molecules, dependent on protein concentration, pH, temperature and time of heating (Circle et al., 1964). Effect of protein concentration, heating temperature, heating time and pH on gel strength (GS) and water holding capacity (WHC) of CPI are presented in Figures: 3.15, 3.16, 3.17 and 3.18 respectively. The GS gradually increased from 0.60 at 12% protein concentration to 1.29 N at 16% protein concentration (**Figure 3.14**). Hillier et al. (1980) and Hegg (1982) mentioned that the GS of heat-induced gels, with respect to globular food proteins, is affected by the protein concentration. As reported by Shimada and Matsushita (1980), the lowest concentrations of the proteins p-conglycinin and glycinin isolated from soy bean by isoelectric precipitation method (IP) by using the procedure described by Thanh and Shibasaki (1976) needed to form gels were 10 and 12 % respectively. Whereas Damodaran (1988) following the same procedure, reported that the minimum concentration of glycinin protein required to create a gel was 9 %. It was reported that the heat treated soy glycinin undergoes gel formation or disaggregation which relies on protein concentration; high concentrations of glycinin protein favored gel formation while low concentrations favoured disaggregation (Mori et al., 1982). Damodaran (1989) found a linear relationship between gel strength and protein

concentration for both p-conglycinin and soy isolate. As shown also in **Figure 3.14**, the gel formed by 12% protein concentration had 93% WHC and this gradually increased when protein concentration of CPI increased to up to 16%. Little published information on gelation of CPI is available. Although Ragab et al (2004) who investigated heated CPI (90 °C for 10 min) reported that CPI is unable to form a gel, even after cooling, Khalid et al (2012) reported that the critical protein concentration to form gel for both defatted cowpea flour and cowpea protein isolates dispersion was 12.0% (w/v). The results of this study also indicate that the gels obtained from 12% protein concentration for cowpea protein isolates belong to the colloidal dispersion or weak-gel category. Most previous studies focused on soy isolate gels. It was found that soy isolate gels have a high WHC in comparison with those from milk protein (Smith et al., 1960). On the other hand, severa1 researchers reported that glycinin tofu has higher WHC than that made from p-conglycinin.

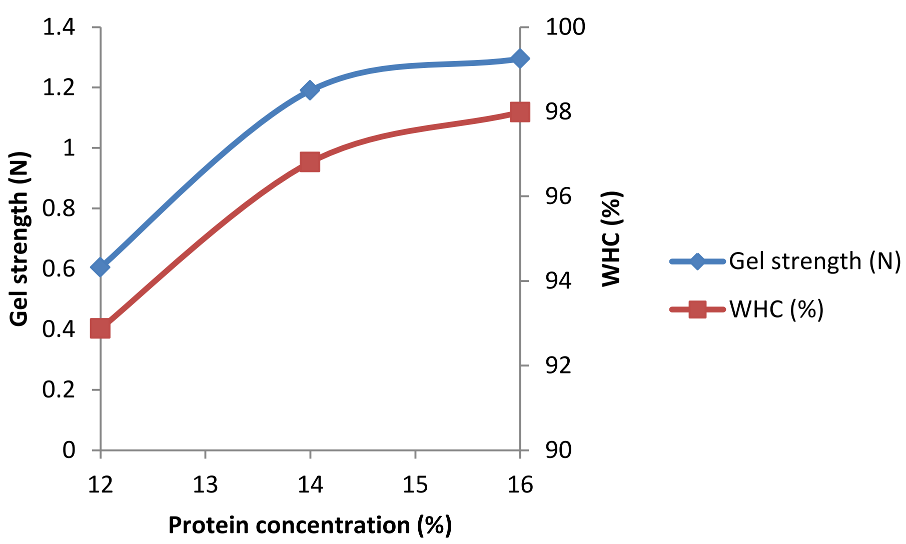


Figure 3.14 Effect of protein concentration on gel strength and WHC of CPI gel. Samples were measured in duplicate.

As shown in **Figure 3.15,** no gels were formed at the temperatures below 80°C. This result is in agreement with the theory that heat denaturation of protein is a prerequisite for gel formation by globular proteins (Utsumi et al., 1997). In the case of SPI, heating temperature must be greater than 60°C to induce dissociation of globulins (in quaternary

structure) as well as to create unfolding of the subunits of protein (Kangll et al., 1991). Gel Strength (GS) tended to increase when temperature increased from 80 °C to 90 °C. Similar results for SPI gels at different temperatures were reported by Furukawa et al. (1980) who observed that GS increased when the temperature increased. In the case of whey protein concentrate (WPC), an increase in GS was observed when temperature increased from 75 °C to 100 °C (Boye et al., 1995). The conformational changes of protein molecules that are followed by aggregation might be the reason for the increase in GS which is affected by the increase in heating temperature (Utsumi et al. 1997). The marked increase in GS of CPI at 90 °C may be attributed to the formation of the 3-dimensional (3D) network obtained specifically at this degree of temperature (Boye et al., 1995).

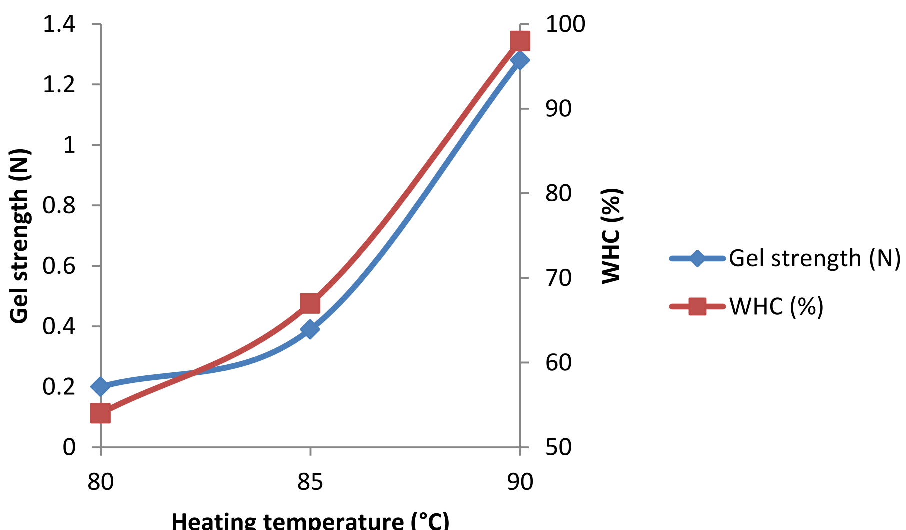


Figure 3.15 Effect of heating temperature on gel strength and WHC of CPI (16% protein). Samples were measured in duplicate.

As shown in **Figure 3.15**, the gels prepared at 90°C had significantly higher value of WHC than those prepared at 80°C. In the case of soy protein isolate, Ramadan (2000) reported that the complex quaternary structure of glycinin may be affected by increasing the temperature; which might lead to a transition from a disordered aggregated gel to ordered gel structure which consists of strands. Stanley and Yada (1992) reported that heating of whey protein at temperatures higher than the gelation temperature led to phase separation and caused lower WHC.

Generally temperature affects extraction of protein and as shown in **Figure 3.2**, CPI yield tended to increase with increasing extraction temperature from 25 to 55°C.

As shown in **Figure 3.16,** the gel strength (GS) of CPI tended to increase from 0.71 N to 1.3 N when the heating time was increased from 10 to 40 min (at 90 °C); however there is no significant trend towards an increase in the gel strength between 40 min and 60 min. It was reported by Catsimpoolas and Meyer (1970) that the use of low temperatures in heating resulted in a long time for gel formation and led to formation of weak gels, while high temperatures resulted in a short time for gel formation. Zayas (1997) recommend that glycinin should be well unfolded to develop the appropriate 3-dimensional (3D) network in order to form a gel.

As demonstrated in **Figure 3.16**, the WHC of CPI gels (at 90 °C) was increased from 75% at 10 min to 97% at 40 min of heating and remained constant at 97 % between 40 min and 60 min (**Figure 3.16**). It can be mentioned that the increase in WHC of CPI was confined to the first phase of the formation of gel. Similar results on WPC gels were reported by Boye (1995).

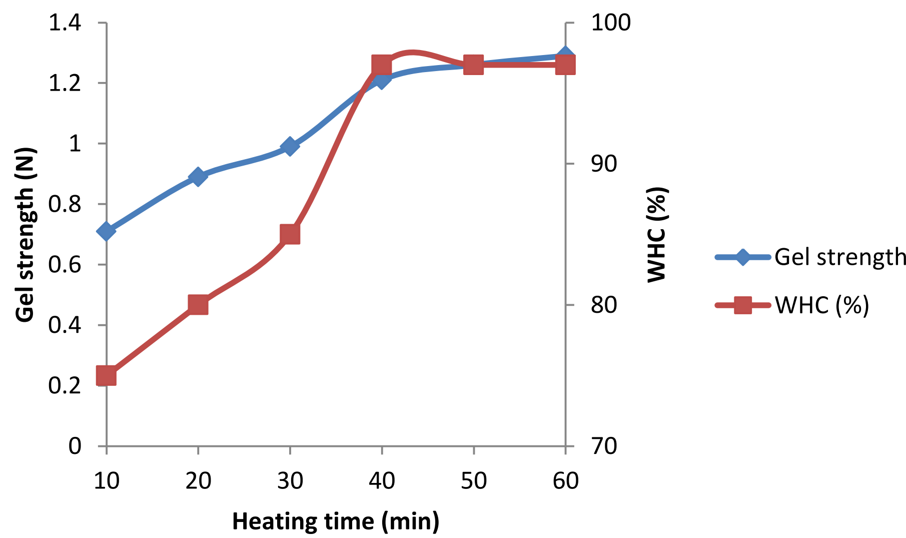


Figure 3.16 Effect of heating time at 90•C on gel strength and WHC of CPI (16% protein). Samples were measured in duplicate.

As shown in **Figure 3.17**, at pH value 7 a harder gel, with gel strength 1.3 N was observed in comparison with gels at pH values above. On the other hand, at pH 11, a softer gel was observed with GS 0.4 N. This may be attributed to intermolecular

repulsion forces (IRF) which act at the high level of net charge in order that interactions between protein and solvent are preferred rather than those between proteins (Hermansson 1978). Meanwhile, there is no gel observed at pH 4 and 5, but white, opaque coagulums were established. This may be due to the absence of repulsive forces (RF) which give rise to lower hydration, lower expansion and subsequently leading to formation of weaker gels (Cheftel and Cuq,1983). The relationship between pH and gelation of β-Iactoglobulin (β-lg), at protein concentration of 12%, was studied by Standing and Hermansson (1991) and it was observed that aggregated, untransparent gels were formed in the pH range of 4 to 6 while transparent firm gels were formed below pH 4 or above pH 6. In the case of whey protein (15 %), it was found that coarse, opaque, coagulum gels were observed in the pH range of 4 to 6 while in the pH range of 8 to 10 (alkaline region), firm gels were formed in comparison to soft gels observed at pH values 2 to 3 (Boye et al., 1995).

As also shown in **Figure 3.17**, WHC tended to decrease from 70 % to 64 % when increasing the pH from 3 to 4. Lower WHC was observed in the pH region 4 to 5. From pH 6 to pH 7, WHC tended to increase from 75% to 97%, remained constant in the pH region from 7 to 10, and then decreased to 80% at pH 11. Similar results on soy protein concentrate (SPC) were reported by Hutton and Campbell (1977), lower WHC (241g of water/100g of protein) of SPC was obtained at pH 5 (isoelectric pH) and then increased when the pH increased, while higher WHC (349 g of water/ 100 g of protein) was obtained at pH 7.

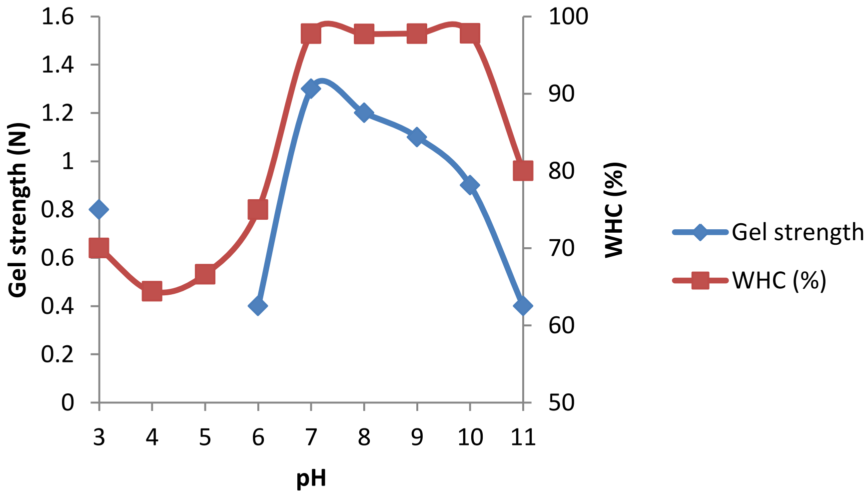


Figure 3.17 Effect of pH on gel strength and WHC of CPI (16% protein). Samples were measured in duplicate.

Generally it can be mentioned that with regard to the CPI in the present study, no gels were formed below a concentration of 12%. In the case of soy protein isolate, the minimum protein concentration needed to form gels was 8% (Catsimpoolas and Meyer, 1970). Hence CPI did not show better gelation properties than those of soy protein isolate, however they were similar to those of SPI. So, from the obtained results, it can be noted that the maximum peak force value of CPI gels was 1.3 Newton even at the highest concentration (16%). Hsu et al. (1982) reported similar results for yellow pea protein isolates at the protein concentration of 10%.

3.4 Conclusions

In this chapter, protein isolation from cowpea on the basis of liquid extraction ratio, extraction pH, precipitation pH and extraction temperature has been extensively studied. The results from the present study suggest that for the future production of CPI, the use of alkaline solution at pH10 with extraction ratio of 1:10 and extraction temperature of 55°C is recommended in terms of the highest yield. The extraction of CPI by this method which resulted in 89% yield and 90% protein content is a feasible way to obtain the cowpea protein isolates and this would be also feasible for upscaling to industrial scale. This is the first description of the identification of optimum protein extraction conditions that resulted in high 89% yield and 90% content.

The findings of this study also show that:

- CPI had significantly (at least 20%) better solubility than SPI at pH 6-9 (**Figure 3.4**).
- CPI had similar viscosity at 60°C to SPI at the same protein concentration (**Figure 3.6**).
- CPI and SPI had similar denaturation temperature; no significant difference in Td (**Figure 3.7**).
- CPI had similar foam capacity to SPI, but lower foam stability (**Figure 3.8** and **Figure 3.9**).
- CPI had significantly lower WHC than SPI, but similar to WPC 60 and WPC-GOS (**Figure 3.10**).
- CPI had significantly lower FAC than SPI, but similar to WPC 60 and WPC-GOS (**Figure 3.11**).
- CPI had similar EAI to SPI (**Figure 3.12**), but significantly lower ESI than SPI (**Figure 3.13**).

Recently, supplemented products have been a substantial growth category for food applications. According to the findings the CPI demonstrated good functional properties that confirmed that the method used in its isolation was efficient and it could be utilised in protein supplementation in bakeries etc.

This is the first time that the gelation properties of CPI have been described under different conditions. The CPI was investigated for its gelling properties and the conclusions drawn from these investigations are that increasing protein concentration, heating temperature, heating time and pH (7-10) enhanced the gel formation.

- The minimum protein concentration of CPI to form heat induced gels was 12 %.
- Increasing protein concentration from 12% to 16% enhanced the gel formation.
- Better gel with highest values of gel peak force (gel strength) and WHC were formed when CPI was heated at 90°C for 60 min.
- The hardest gel, with highest WHC was observed at pH 7 while a much softer gel was obtained at pH 11.
- No gel was formed at pH 4 and 5; instead white, opaque coagulums were formed.

Chapter Four

Modification and applications of cowpea protein isolate

4.1 Introduction

The interaction between proteins and reducing sugars in foods has attracted considerable attention during the past decade. This may be attributed to the ubiquitous nature of protein glycation reactions due to the Maillard reaction and the modification of some functional properties of proteins after their conjugation with carbohydrates (Easa, et al., 1996; Kato et al., 1990, 1992; Nakamura, et al., 1992). Also, the application of glycated food proteins in the food industry presents fewer safety issues when compared with chemically modified food proteins (Kato, et al., 1996). The positive characteristics that occur due to the Maillard reaction are mainly comprehended in food applications. These characteristics are divided into two groups, sensorial and textural. The first group, sensory characteristics, includes the development of food colour, flavour and aroma compounds during food production. On the other hand, the textural characteristics include the improvement in the solubility, water absorption capacity, gelling, thermal stability and emulsifying properties of protein and until the present, most glycation reactions of proteins and reducing sugars to improve functional properties have occurred in the dry state (Cabodevila et al., 1994; Saeki, 1997; Darewicz et al., 1998; Kato et al., 1993; Kato et al., 1996; Matsumodi et al., 1995; Shu et al., 1996). The functionality of the conjugated proteins improved with increasing chain length and content of polysaccharide (Shu et al., 1996). In recent times, with the development of more analytical techniques, a number of studies have reported other favourable consequences of this known reaction in food applications, such as the formation of compounds with antioxidant, anti-bacterial, anti-mutagenic, and anti-carcinogenic properties (Wijewickreme and Kitts, 1998; Einarsson and Eriksson,1990; Chuyen et al., 1998; Aeschbacher, 1990).

It is now known that the importance of cowpea proteins can be improved by greater utilization in various human food applications. Due to the functional properties of cowpea proteins, they could be utilized as ingredients in a range of preparations, and they also have potential to be used in many food applications such as high protein snack foods, meat analogues etc., and therefore, they can promise huge possibilities in the improvement of new formulated foods. However, the cowpea proteins have not been fully studied, particularly the modification in order to improve their functional properties. In this chapter the effect of Maillard reaction of CPI and reducing sugars in the liquid state on functional properties is investigated. Most studies on improvement

of functional properties of food proteins by the Maillard reaction have been done on dry powders heat treated in ovens at controlled humidity (Tian et al., 2011; Mulsow et al., 2009; Ledesma-Osuna et al., 2008; Aminlari et al., 2005; Achouri et al., 2005; Li et al., 2005). Very little information is available on the effect of glycation in solution on protein functionality.

The effects of the Maillard reaction may be positive and negative. The positive attributes of the reaction are realized mainly in food systems. These attributes may be divided into two categories, textural and sensory. The textural attributes, include the water-holding capacity, improvement of protein solubility, and thermal stability. The sensory attributes of the Maillard reaction include the development of desirable colour, volatile and non-volatile flavour and aroma compounds during food preparation (Darewicz et al., 1998; Shu et al., 1996), gelling (Cabodevila et al., 1994), and emulsifying properties (Saeki, 1997; Shu et al., 1996; Kato et al., 1993).

The negative aspects of the Maillard reaction may be considered from the nutritional, toxicological and physiological points of view. Nutritionally, the reaction reduces the quality of food proteins by reducing the bioavailability of lysine (an essential amino acid), and by destroying other essential amino acids such as tryptophan and cysteine (Piuo ferrato et al., 1998; Birlouez-Aragon, 1997; Hurrell, 1990).

The study reported in this chapter investigated the effect of following modifications of CPI in solution:

(a) Denaturation.
(b) Combined denaturation and glycation via the Maillard reaction.

The effects of these modifications on physico-chemical and functional properties of CPI were studied and the results are reported here.

4.2 Materials and Methods

The materials and methods used within this study were described in chapter 2.

4.3 Results and Discussion

For this work, cowpea protein solution (a part of protein solution was taken before precipitation in section 2.2.2.2) was heated at 85°C for 0 to 120 min. These investigations were intended to study the effect of the heating at 85°C for 2 hours on glycation and resulting protein functionality.

The present study was designed to investigate the modification of cowpea protein isolate during the extraction process (chapter 3) to investigate the effect of glycation with its endogenous sugars on functional properties of cowpea protein isolate. When the protein was extracted by adjusting to pH 10.0 (alkaline extraction) as described in section 2.2.3, a part of solution was taken before precipitation at pH 4.5 (isoelectric precipitation). This part of the solution was heated at 85°C for times from 0 to 120 min; with the aim to glycate proteins with endogenous reducing sugars and oligosaccharides present in the solution. This was named as glycated cowpea protein isolate (GCPI). The effect of heat treatment of cowpea protein solution and resulting product was determined. The studied parameters and their results are discussed in this section.

4.3.1 The physicochemical properties of modified cowpea protein

A number of causes can lead to protein denaturation (unfolding), i.e. the loss of native structure, which involves breaking and creating of bonds, depending on environmental factors such as pH and temperature. The increase of temperature leads to denaturation, i.e. to increase in the degree of structural disorder of proteins (Bellavia, 2010). It is commonly known that proteins can be denatured through exposure to heat.

Glycation of proteins with reducing sugars or oligosaccharides via Maillard-type reaction can be produced by the reaction of the ε-amino groups of proteins with the carbonyl group of saccharides under controlled pH, temperature and relative humidity (Izydorczyk, 2005; Nakamura et al., 1992). **Figure 4.1** shows an example of the classical pathway of protein glycation by glucose leading to advanced glycation end-products (AGEs) via Amadori products. The initial reaction between glucose and protein amino group forms a reversible Schiff base which rearranges to a fructosamine

group or Amadori product. With time Amadori products may form AGEs via dicarbonyl intermediates such as protein-bound 1,4 deoxyglucosone.

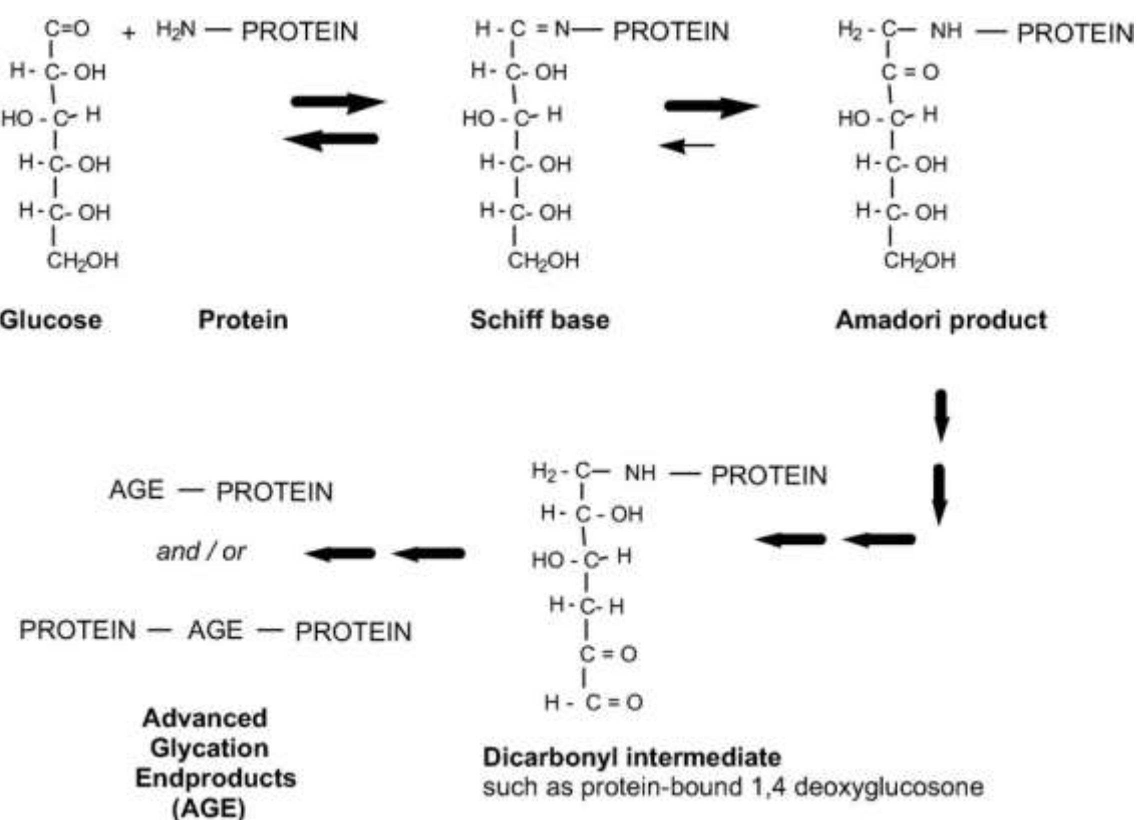


Figure 4.1 Example of the classical pathway of protein glycation by glucose leading to AGEs via Amadori products (Peyroux and Sternberg, 2006).

This section describes the physicochemical properties of denatured and glycated cowpea protein isolates. Samples, cowpea flour (CF), cowpea protein isolate (CPI), denatured cowpea protein isolate (DCPI) and denatured plus glycated cowpea protein isolate (GCPI) were prepared as in section **2.2.3.1**. CF was prepared as size-separated cowpea flour on the test sieve shaker (Endecotts) by using 100-mesh (0.15 mm, width) size screen to get fine powder and to remove the remaining husk from flour produced in the first stage of production as in section **2.2.1.** Beans were milled in a DLFU-mill from Buhler-Miag (Braunschweig, Germany), sieved with a 600 micrometre (μm) screen and defatted by extraction with cold acetone for 1 h at 4°C (flour/solvent ratio of 1:3 w/v). The resulting freeze-dried cowpea flour in this treatment was named CF. CPI and DCPI were prepared as described in section **2.2.3.2** and as shown in **Figure 2.1**. For CPI, cowpea flour was mixed with 10 fold quantity of distilled water (w/v) at room

temperature. The pH of the mixture was then adjusted to 10 with 2N NaOH and stirred gently for 1hour at 55℃. The mixture was centrifuged to remove the starch and fibre fractions. The pH of the supernatant was adjusted to value 4.5 by drop wise addition of 2N HCI. The precipitated proteins were centrifuged, washed with dH_2O, resuspended in water and neutralized to pH 7 using 0.1N NaOH, and dialysed (cut off point of dialysis membrane 10 KDa) overnight at 4°C against dH_2O. This was freeze-dried and referred to as CPI. For DCPI, a part from protein suspension taken during the preparation of CPI (above), after removing the starch and fibre fractions, was heat treated for 2 hours in a shaking water bath at 85°C ± 3°C followed by cooling to RT by immersion of the flask in cold water. The pH of the supernatant was adjusted to value 4.5 by drop wise addition of 2N HCI. The precipitated proteins were centrifuged, washed with dH_2O, resuspended in water and neutralized to pH 7, and dialysed against dH_2O as for CPI. The freeze-dried product was referred to as DCPI. GCPI was prepared as described in section **2.2.3.3** and as shown in **Figure 2.2**. A 2 litre dispersion of defatted cowpea flour sample in distilled water (5% w/v) was adjusted to pH 10 with 2N NaOH, and stirred for 1 hour at RT. The suspension was heated for 2 hours in a shaking water bath at 85°C ± 3°C followed by cooling to RT by immersion of the flask in cold water. The samples were centrifuged to remove the starch and fibre fractions. The pH of the supernatant was adjusted with 2N HCl to 4.5 to precipitate the proteins; the precipitated proteins were centrifuged, washed with dH_2O, resuspended in water, neutralized to pH7 with 0.1N NaOH, dialysed against dH_2O and freeze-dried. The samples are referred to as glycated cowpea protein isolate (GCPI). A summary of preparation of the samples used in this study is shown in **Table 4.1.**

One of the oldest techniques used to follow the course of denaturation is to determine changes in solubility. Proteins vary importantly in their resistance to solubilisation and some proteins that are important in food applications are insoluble in their native state. The loss of solubility of protein is one of the last stages of changes in its structure that must have occurred. Thus, this is a rather basic measure of protein denaturation. The heating at 85°C for 120 min led to a decrease in solubility of both CPI and CF suspension corresponding to increased heating time (**Figure 4.2**), which corresponded in turn to increase in turbidity of supernatant of centrifuged heat treated cowpea flour dispersion (**Figure 4.2**). Turbidity measurement at 600 nm could be used as an indicator of protein denaturation (Kitabatake and Kinekawa, 1995 and Molina and

Wagner, 1999). The solubility of CF suspension tends to decrease from 88.86 to 69.56% which was still higher than that of control (CPI under the same conditions). Thus, it seems likely that protein denaturation induced by heat treatment may be reduced by the presence of carbohydrates and sugars present in CF.

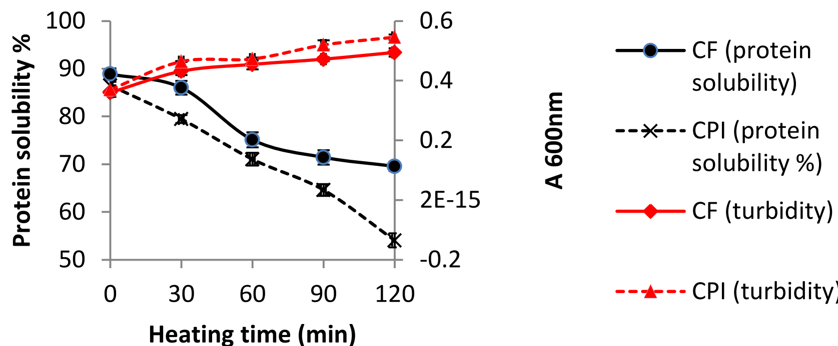


Figure 4.2 The effect of heat treatment at 85 ºC (pH 10) on solubility and turbidity of CPI (0.4% w/v) and CF (0.4% w/v). Error bars indicate the standard deviation of triplicate measurements. CPI= cowpea protein isolate, extracted from cowpea flour as presented in Table 4.1 (A); CF= cowpea flour, extracted from cowpea grains as presented in Table 4.1 (D).

4.3.1.1 Solubility

When proteins are exposed to high temperature, a loss of solubility occurs, depending on the type of protein as well as the severity of the heating. Investigation of the thermal denaturation of proteins is very important in food and biological industries. It is well-known that thermal properties of protein are affected by many factors such as protein concentration, ionic strength and pH. Ashie et al (1999) reported that addition of sugars, as in the manufacture of surimi (fish-based food product), increases protein stability against pressure treatment and that these solutes act by altering the structure of water. Molecules such as sucrose and trehalose are referred to as kosmotropes (water structure makers or order makers) (Plumridge and Waigh, 2002). Braga (1986) who used the DSC technique to investigate the effect of sugar additives on the thermal denaturation of ovalbumin, noted that when the sugar was added, the denaturation temperature peaks tended to shift higher.

Denaturation (unfolding) of protein could cause improvement of the water absorption by the protein. Denaturation by heat treatment can expose hydrophobic and SH groups located in the interior of the protein molecule, which can lead to irreversible protein aggregation, thus resulting in a reduction in solubility (Renkema et al., 2000).

Table 4.1 Composition and treatments of samples

	Product	Composition	Treatment
A	CPI (5% total solids)	90.76% ±0.11 protein, 04.38% ±0.09 CHO	Native extraction from cowpea flour (section **2.2.3.2)**
B	DCPI (5% total solids)	90.81% ±0.23 protein, 04.53% ±0.04 CHO	Extraction after heat treatment of a part of protein suspension taken during the preparation of CPI, after removing the starch and fibre fractions, at 85°C/2 hours (section **2.2.3.2)**
C	$GCPI_1$ (5% total solids)	90.02% ±0.11 protein, 05.93%±0.07 CHO	Extraction after heat treatment of cowpea flour (in aqueous state) at 85°C/2 hours (section **2.2.3.3)**
D	CF suspension (5% total solids)	26.14% ±0.13 protein, 56.33% ±2.12 CHO	Extraction by milling the cowpea grains, and by size-separating the flour on the test sieve shaker by using 100-mesh (0.15 mm, width) size screen to get fine powder and to remove the husk (section **2.2.1** & section **2.2.3.1)**

CPI= cowpea protein isolate; DCPI= denatured cowpea protein isolate GCPI= glycated cowpea protein isolate; CF= cowpea flour.

In the present study, such a process applied to cowpea proteins may indicate notable accompanying denaturation, but the GCPI, which was produced from heat treatment of CP suspension at 85°C for 2 hours, was more soluble than DCPI that was produced from heat treatment of CPI under the same conditions (**Figure 4.3**).

4.3.1.1.1 Protein solubility as function of pH

Solubility of proteins is one of their most essential functional properties, because of its impact on other functional properties (Sikorski 2001). Solubility of proteins is important in a lot of protein-based formulations, such as food emulsions (Hayakawa and Nakai 1985). Solubility of protein is an indication for protein isolates behaviour in the food products and reflects the extent of denaturation of protein either by chemical or by heat treatment under different pH conditions (Horax et al., 2004). High solubility of food proteins is number one for best functionality in food applications. Solubilities of CPI, DCPI and GCPI were determined in aqueous solutions as a function of pH and compared to that of SPI. The change of solubilities in the range of 2.0 to 14.0 of pH values are shown in **Figure 4.3**.

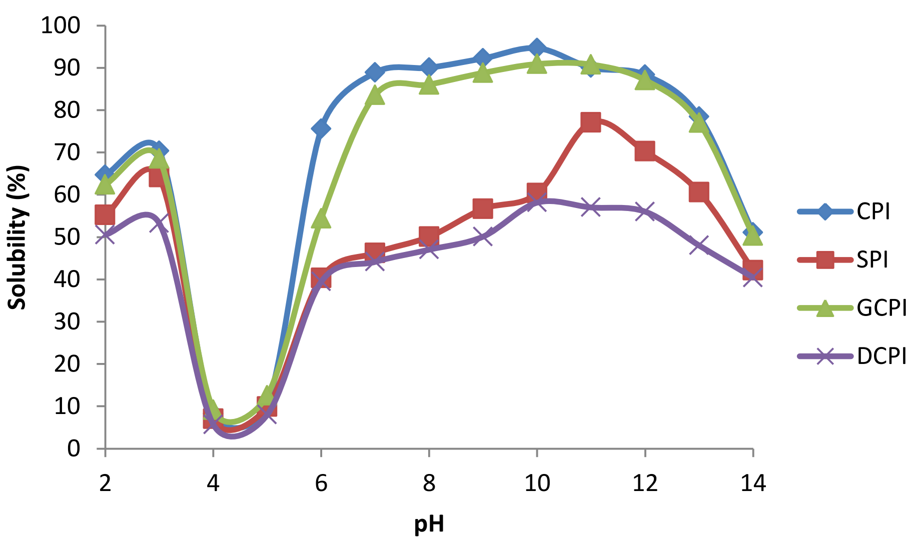


Figure 4.3 Effects of pH on protein isolate solubility (0.4% w/v). CPI = cowpea protein isolate (Table 4.1, A); DCPI = Denatured cowpea protein isolate (Table 4.1, B); GCPI = Glycated cowpea protein isolate (Table 4.1, C); SPI = Soy protein isolate (90% commercial product, 90% protein, 0% carbohydrate). Samples were measured in duplicate.

The solubility of both CPI and GCPI was remarkably higher than that of SPI between pH 6 and 10. As can be seen the solubility of GCPI is slightly lower than CPI for the reason that GCPI is, in part, denatured due to the heating treatment in glycation. However, as can be seen in **Figure 4.3**, the solubility of GCPI was higher than that that of DCPI and SPI, especially between pH 6 and 10. In general, the decrease in the solubility of a protein takes place strikingly at its IP region where the protein has about zero net charge and it tends to aggregate due to the electrostatic interactions of protein induced by the charge asymmetry (Mu et al., 2011). The result of the present study suggested that GCPI produced by glycation via the heat treatment of cowpea flour before protein extraction, has significantly ($P < 0.05$), 35.42% and 33.32% more solubility than DCPI and SPI, respectively above pH 6. Campbell et al. (2003) reported that the heat treatment at 70°C for10 min led to increase in the solubility of SPI for the reason that a hydrophilic layer was formed around the protein. The glycation via the Maillard reaction can improve the solubility of heat treated globular proteins, based on the fact that hydrophobicity of protein molecules will tend to decrease due to the Maillard reaction. This means the presence of more polar groups on the surface area of the unfolded protein, resulting in improved solubility in aqueous media (Campbell et al., 2003).

4.3.1.2 Glycation

The second column in **Table 4.2** shows significant reduction in free amino groups of DCPI and GCPI (85 ºC for 2 hours) compared to CPI, indicating a glycation degree (GD) of 17.12% for DCPI and of 49.31% for GCPI which is attributed to Maillard reaction with reducing sugars and polysaccharides present in the cowpea flour. The presence of SDS and mercapto-ethanol in the reaction buffer ensures complete unfolding of protein to enable the OPA reagent to react with all free amino groups, which could otherwise be masked. As shown in the second column in **Table 4.2** also, the DCPI lost approximately 17% of its free amino groups during heat treatment. This could be due to reaction with residual reducing sugars present in CPI and also due to structural modifications of the CPI, such as protein polymerization and crosslinking (Chevalier et al., 2001; Jimenez-Castano et al., 2005; van de Lagemaat et al., 2007). Polymerization occurs by the reaction between an ε-amino group (a primary amine) in a protein- bound lysine side chain and an amide group in a protein-bound glutamine, resulting in crosslinks between protein molecules (Babiker et al., 1998).

Figure 4.4 shows the changes in free amino groups during the extended heating duration of CF and CPI (which became DCPI). It can be seen from **Figure 4.4** that free amino groups decreased from 2.16 to 1.09µg/µl after 120 min of heating time. Meanwhile, as shown in the same figure (**Figure 4.4**) also, when CF suspension was heated, a brown colour increase occurred at 420 nm after a certain duration of heating time which was higher than that for CPI (or DCPI). This confirms that the Maillard reaction was more active in CF than CPI.

As shown in **Table 4.1**, the mean values for total carbohydrates of CF, CPI, DCPI and GCPI were 56.33% ±2.12, 04.38% ±0.09, 04.53% ±0.04 and 05.93%±0.07, respectively. CF contained the highest amount of carbohydrates, so, this resulted in higher reduction of free amino groups and development of absorbance at 420 nm than that of DCPI and CPI (**Figure 4.4**).

In the present study, the GCPI is prepared in the same conditions as the CF samples shown in **Figure 4.2**, i.e. GCPI prepared by heating the CF suspension at 85°C for 2 hours**.** Accordingly, as shown **in Figure 4.5**, there is a significant difference in total carbohydrates between CPI and GCPI. For that reason, the attachment of carbohydrates made CF suspension more soluble than CPI at all heating duration times studied **(Figure 4.2).** It can be mentioned that glycation was confirmed by determination of the total carbohydrate content of GCPI in comparison to CPI (control) (**Figure 4.5**). It was calculated that 1.55% more total carbohydrates were bound to GCPI produced from CF suspension after heat treatment at 85°C for 2 hours. This difference might account for the increase of Maillard reaction in the case of GCPI. Characterisation of the sugars involved in glycation was not attempted in this study. There is no mass spectrometry of glycated proteins in this study because the equipment was not in working order and the time was limited.

Figure 4.6 shows the glycation degree (GD) of cowpea flour suspension at different reaction times. GD of protein extracted increased gradually from 15 to 90 min and then steadied, indicating that more cowpea protein and carbohydrate was conjugated when heated at 85ºC.

Table 4.2 Free amino groups, free SH groups and surface hydrophobicity index of CPI, DCPI and GCPI (85 ºC for 2 hours)

Protein sample	Free amino groups µg/µl	Free SH-groups content µmol/g	Total SH-groups content µmol/g	Surface hydrophobicity S_o
CPI	2.160 ± 0.16a	0.972 ± 0.09a	1.537 ± 0.08a	385.9 ± 10.6c
DCPI	1.797 ± 0.17b	0.657 ± 0.05c	1.056 ± 0.07c	601.1 ± 7.2a
GCPI	1.095 ± 0.11c	0.804 ± 0.06b	1.362 ± 0.09b	540.3 ± 5.9b

**Means in the same column not followed by the same superscript letter are significantly different ($p \leq 0.05$).*

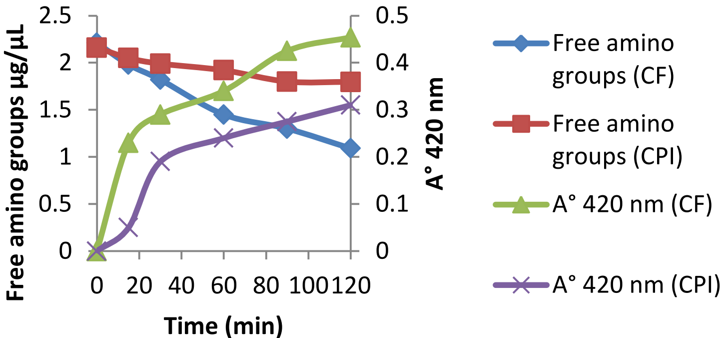


Figure 4.4 Monitoring of free amino groups and browning of cowpea protein extraction (5% w/v) heated in a water bath at 85°C. CPI= cowpea protein isolate, extracted from cowpea flour as in presented Table 4.1 (A); CF= cowpea flour, extracted from cowpea grains as presented in Table 4.1 (D). Samples were measured in duplicate.

From the FTIR spectroscopy of CPI and GCPI (**Figure 4.7**), the absorbance of C–O stretching in the C–OH group (1023 cm^{-1}) and O-H (H-bonded) stretching vibration (3430.2 cm^{-1}) in GCPI increased compared to CPI. **Table 4.3** demonstrates the functional groups and the corresponding modes of vibration taken from the FTIR spectra of possible Maillard reaction products. In this study, the peak at 1023.6 cm^{-1} may be due to C-O stretching in the C-OH group and C-C stretching in the carbohydrate structure. Also, it may be due to C-N stretching. The peak at 3430.2 cm^{-1} may be mostly due to O-H (H-bonded) stretching vibration indicating increase of water binding. Although, FTIR analysis shows the functional groups of Maillard reaction products, it is not suitable for measuring the compound compositions of Maillard reaction products.

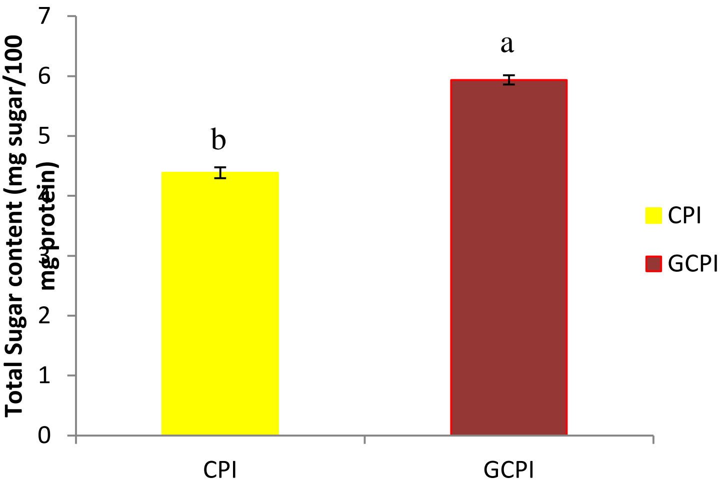


Figure 4.5 The content (mg sugar/100 mg protein) of total sugars bound to proteins before (CPI) and after (GCPI) glycation as determined by spectrophotometric method using anthrone reagent. CPI= cowpea protein isolate, Table 4.1 (A); GCPI = Glycated cowpea protein isolate, Table 4.1 (C). Means not indicated by the same superscript are significantly (P<0.05) different.

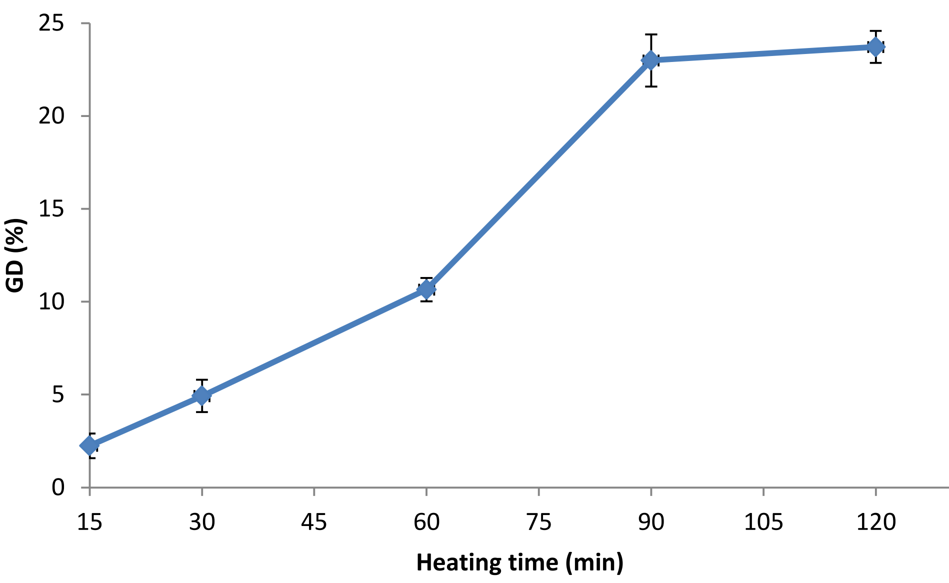


Figure 4.6 Glycation degrees (GD) of cowpea flour suspension (Table 4.1, D) at different reaction time. Error bars indicate the standard deviation of triplicate measurements.

Table 4.3 Functional groups and vibrational modes taken from the FTIR spectra of CPI and GCPI.

Wavenumbers (cm^{-1})		Functional Group	Mode of Vibration
CPI[1]	GCPI[2]		
3429	3430	O-H (H-bonded)	Stretching
1648	1653	-C=O	Stretching
		-C=N	Stretching
1465	1457	O-H (C-OH groups)	Bending

CPI[1]= cowpea protein isolate; GCPI[2]= glycated cowpea protein isolate.

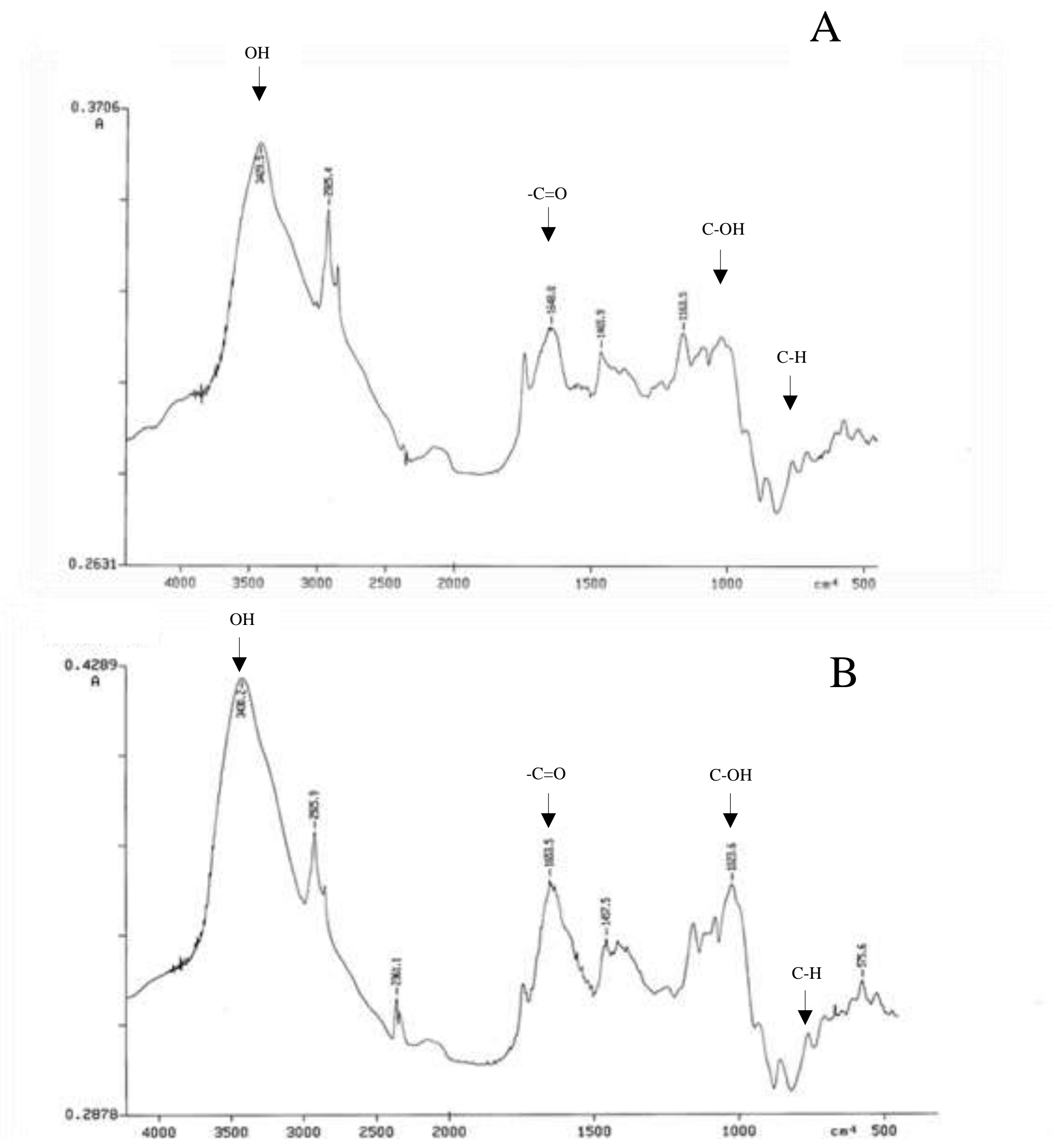


Figure 4.7 FTIR spectroscopy of cowpea protein isolate (A), and glycated cowpea protein isolate (B)

4.3.1.3 Denaturation

The figures in the third and fourth columns of **Table 4.2** depict a significant decrease of both free (SH_F) and total thiol groups (SH_T) for DCPI and GCPI compared to CPI ($p \leq 0.05$). However the values for DCPI are lower than for GCPI.

Measurement of the former gives an indication of the degree of unfolding of protein: an increase in thiol groups indicates moderate unfolding associated with the molten globule state, whereas a decrease in thiol groups indicates the formation of disulphide bonds and a more advanced degree of denaturation. The reduction in SH_T compared to control confirms the formation of disulphide bonds (Cabodevila et al., 1994) as urea and SDS dissociate protein-protein interaction (aggregation) by hydrophobic and electrostatic interactions, which could otherwise mask the free thiol groups. The significant decrease in free and total SH for DCPI compared to the control indicates the formation of disulphide bonds. Comparatively the decrease for GCPI was lower indicating that fewer disulphide bonds were formed in GCPI. These results support the evidence that the higher sugar content of GCPI protected CPI from denaturation. The decrease in free and total SH groups of GCPI correlates with increase in surface hydrophobicity as shown in the fourth column of Table 4, which is also an indication of denaturation degree of protein (Zhang and Chi, 2011 and Funtenberger et *al*, 1997).

4.3.1.4 Viscosity

The viscosities of CPI and GCPI suspensions (8% w/v) as function of shear rate are shown in **Figure 4.8.** It was observed that the viscosity of the GCPI revealed the highest value when increasing shear rates, which indicates shear thinning behaviour (Bourne, 2000). The difference in viscosity between CPI and GCPI positively correlates with carbohydrates attached to GCPI (**Figure 4.5**), which is consistent with the result of viscosity as shown in **Figure 4.8**. The high viscosity of GCPI might be related to the molecular movements, formation of physical barriers and interfacial film formation by protein product ingredients (Maskan and Göğüş, 2000). This increase in viscosity would be of relevance in mayonnaise manufacture, as partial or full replacement of egg.

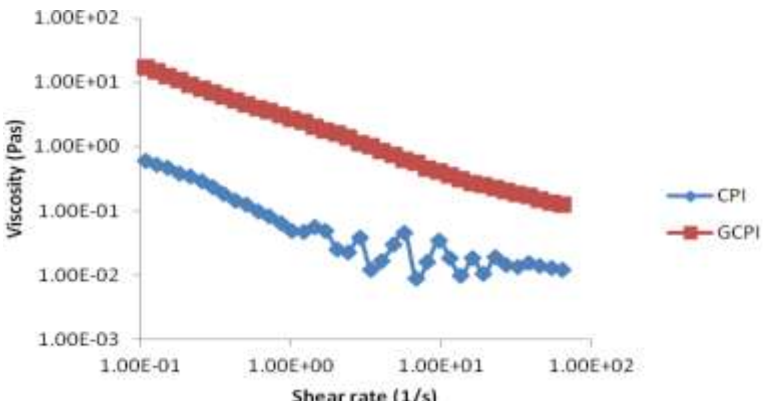


Figure 4.8 Viscosity of 8% cowpea protein (w/v) native or glycated. . CPI = cowpea protein isolate (Table 4.1, A); GCPI = Glycated cowpea protein isolate (Table 4.1, C).

4.3.1.5 Electrophoretic mobility and glycoproteins staining

Figure 4.9 shows protein pattern and glycoprotein staining of native and modified cowpea protein isolates. It was shown that cowpea proteins contained bands in the range of 36 to around 116 KDa with major bands at 36 and 50 KDa (**Figure 4.9, A**). It was noted that molecular weight proteins below 36 KDa were observed in both native and modified protein samples as shown in **Figure 4.9 (A)**.

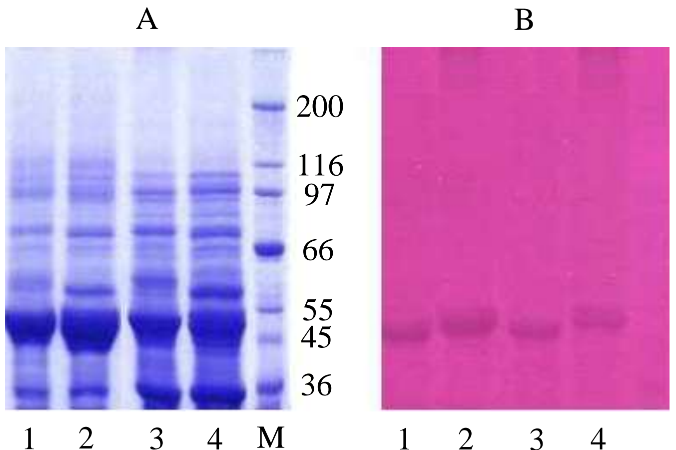


Figure 4.9 SDS PAGE of cowpea protein isolates under reducing and non-reducing conditions (A), and staining of the glycoproteins in the gels (B). Lane1 = CPI (non-reduced); Lane 2 = GCPI (non-reduced); Lane 3 = CPI (reduced); Lane 4 = GCPI (reduced). CPI = (Cowpea Protein Isolate (Table 4.1, A); GCPI = Glycated Cowpea Protein Isolate (Table 4.1, C).

El-Adawy (1996) reported that globular proteins were the major constituent in all legume seeds. In comparison, it was reported that extracted kidney and navy bean proteins show minor bands with molecular sizes of 26 to 28 kDa and major bands ranging from 43 to 47 kDa (Kohnhorst et al., 1990).

As shown in **Figure 4.9 (B)**, the stained bands reveal a broad smearing pattern at the entrance to the separating gel, supporting the formation of high molecular weight conjugates. The one band (around 50 kDa) in CPI (lanes 1 and 3) migrated further into the gel than the band seen in GCPI (lanes 2 and 4), which was to be expected based on the difference in molecular size between native and glycated cowpea protein. The increase in molecular weight might be caused by protein aggregation as well as glycation. Only one band was visible for both CPI and GCPI, but the band of GCPI was distinct and heavier (**Figure 4.10** and **Figure 4.11**) than that of CPI and tended to shift higher (around 55kDa) which suggested that cowpea protein already contained glycoprotein at 50kDa and more reacted with cowpea oligosaccharides due to glycation. Due to the fact that SDS does not bind to carbohydrate, the more glycated protein migrated with decreased mobility in SDS-PAGE (**Figure 4.9 B**). This reduces the net charge to mass ratio of protein-SDS complex, resulting in an increased apparent molecular weight (Westemeier, 1997). As observed by SDS-PAGE (**Figure 4.9 A**), cowpea seeds globulin had relative molecular masses between 45 and 66 kDa. These correspond to the typical molecular mass of 7S storage proteins according to data previously reported (Khan et al., 1980; Macedo et al., 1995; Pedalino et al., 1992). Cowpea seeds contain a large quantity of vicilin 7S globulin and a lesser quantity of legumin-like 11S globulins (Bekhit, 2007). Freitas et al. (2004) reported that globulins constitute 51 % of the seed cowpea protein and that albumins constitute 45 %.

It is known that glycoproteins are proteins that contain sugars and/or oligosaccharide chains (glycans) covalently attached to their polypeptide side-chain. In the case where carbohydrate is grafted to the protein in a co-translational or post-translational modification, the procedure is known as glycosylation or enzymatic glycosylation. Glycoproteins are generally important integral membrane proteins, as they play a role in cell-cell interactions (Funakoshi and Suzuki, 2009. Glycation by the Maillard reaction is a non-enzymatic process in which carbohydrate is attached to protein. The results of **Figure 4.9** indicate that the 50 kDa band of native CPI stained by glyco-protein staining is a glycosylated protein, whereas the one at 55 kDa of GCPI contains additional sugars or oligosaccharides added by the process of glycation due to the Maillard reaction.

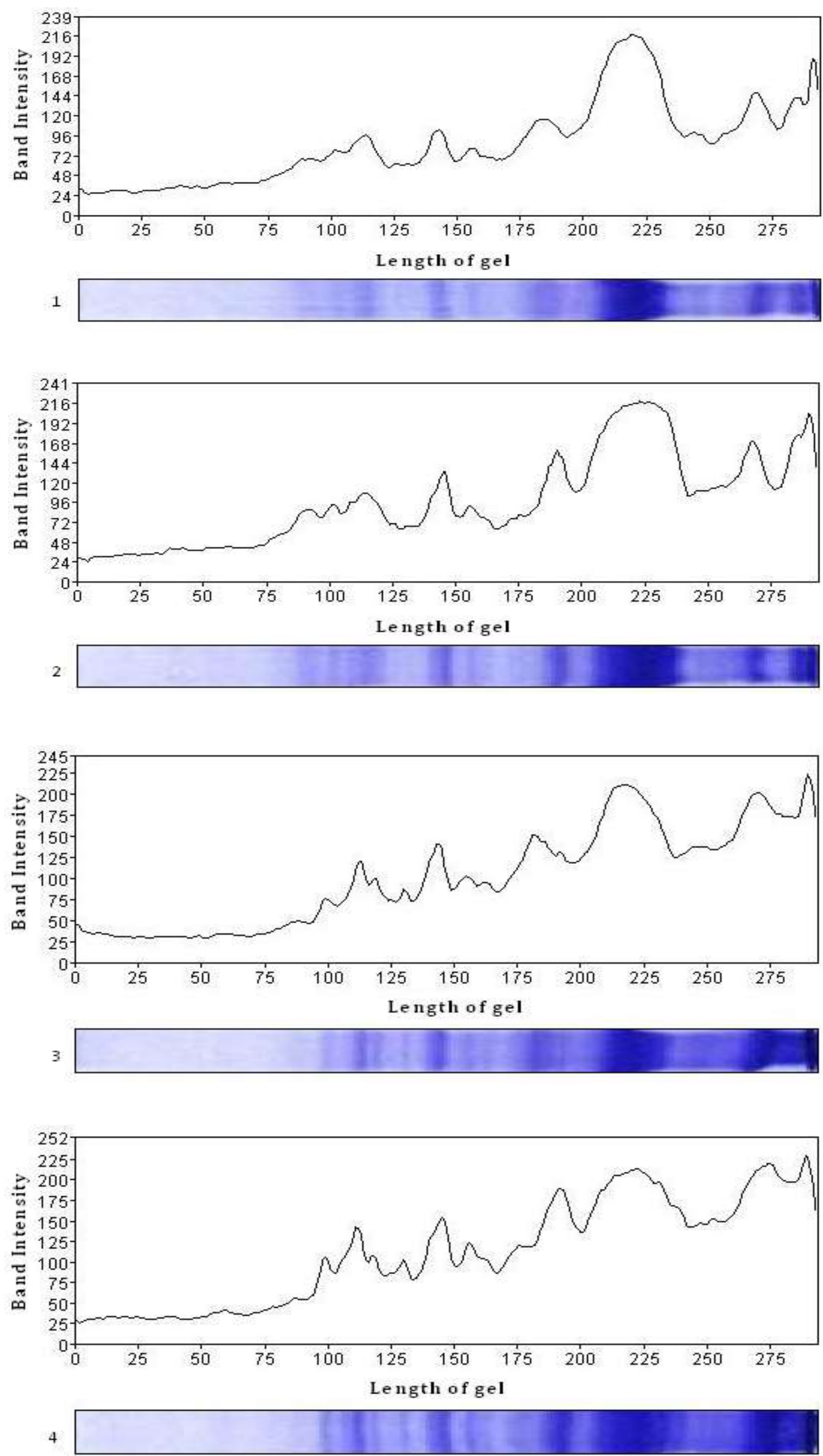


Figure 4.10 The comparative densitometric analysis of the protein staining of cowpea protein samples. Lane1 = CPI (non-reduced); Lane 2 = GCPI (non-reduced); Lane 3 = CPI (reduced); Lane 4 = GCPI (reduced).

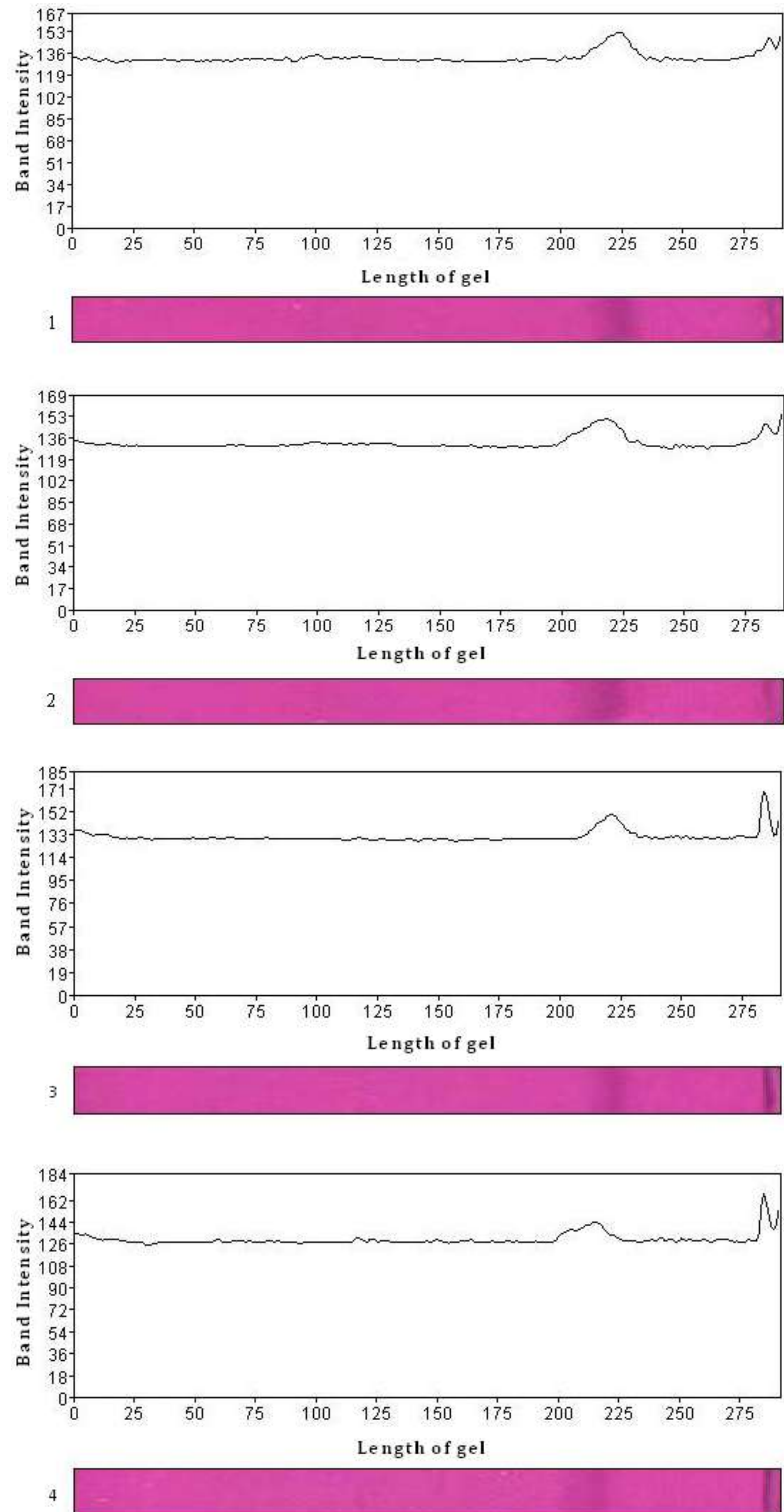


Figure 4.11 The comparative densitometric analysis of the glycoprotein staining of cowpea protein samples. Lane 1 = CPI (non-reduced); Lane 2 = GCPI (non-reduced); Lane 3 = CPI (reduced); Lane 4 = GCPI (reduced).

4.3.1.6 Thermal properties

The thermal characteristics of native and modified cowpea protein isolate were determined by Differential scanning calorimetry (DSC). Proteins were extracted as described in section 2.2.3 and the values of protein content are shown in **Table 4.1** (CPI, 90.76% protein, 04.38% carbohydrate; GCPI, 90.02% protein, 05.93% carbohydrate). The DSC thermogram (**Figure 4.12**) shows one exothermic transition peak of CPI at 84.21°C with denaturation enthalpy (ΔH) 8.56 J/g. The results show that the denaturation temperature peak of GCPI tended to shift higher (88.11°C). The denaturation temperature and ΔH are shown in **Table 4.4**.

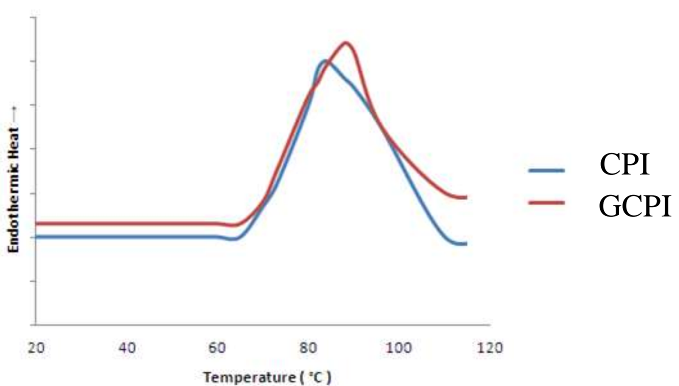


Figure 4.12 DSC thermogram of native (CPI) and glycated (GCPI) cowpea protein isolates. (CPI = Cowpea Protein Isolate; GCPI = Glycated Cowpea Protein Isolate).

Table 4.4 Thermal properties of cowpea protein sample[1]

Protein samples	Td (°C)[2]	ΔH (J/g)[3]
CPI	84.21 ±0.24	8.56 ±0.97
GCPI	88.11 ±0.32	9.95 ±1.23

[1] *Mean ± standard deviation of three replicates, CPI*

CPI= cowpea protein isolate;

GCPI= glycated cowpea protein isolate.

[2] *Td = denaturation temperature;*

[3] *ΔH = denaturation enthalpy*

These results indicate that glycation by the Maillard reaction increases the heat stability of the protein, which confirms the findings of Zhuo et al. (2013), and Liu et al. (2012).

4.3.2 Functional properties of modified CPI

4.3.2.1 Emulsifying properties

Figure 4.13 shows the emulsifying activity index (EAI) and emulsifying stability index (ESI) of cowpea protein extracted from CF suspension during different extended heating times. CF consisted of 26.14% protein (**Table 4.1, D**). The GCPI was extracted by heating the cowpea flour suspension at 55°C and pH 10 for 1 hour as described in section **2.2.2.3.** A part from this was taken as control and the other parts were heated at 85°C for 30, 60, 90, 120 and 150 min. The protein was extracted by cooling the heated CF suspension to RT by immersion of the flask in cold water; then centrifuged to remove the starch and fibre fractions; the pH of the supernatants was adjusted to 4.5 to precipitate the proteins; the precipitated proteins were centrifuged, washed with dH_2O, resuspended in water, neutralized to pH 7, dialysed against dH_2O and freeze-dried. The sample is referred to as glycated cowpea protein isolate (GCPI). The emulsions were prepared at pH 7 from GCPI which was extracted at different heating times from CF suspension. The preparation of emulsions and methods of determinations of EAI and ESI are described in section 2.2.13.

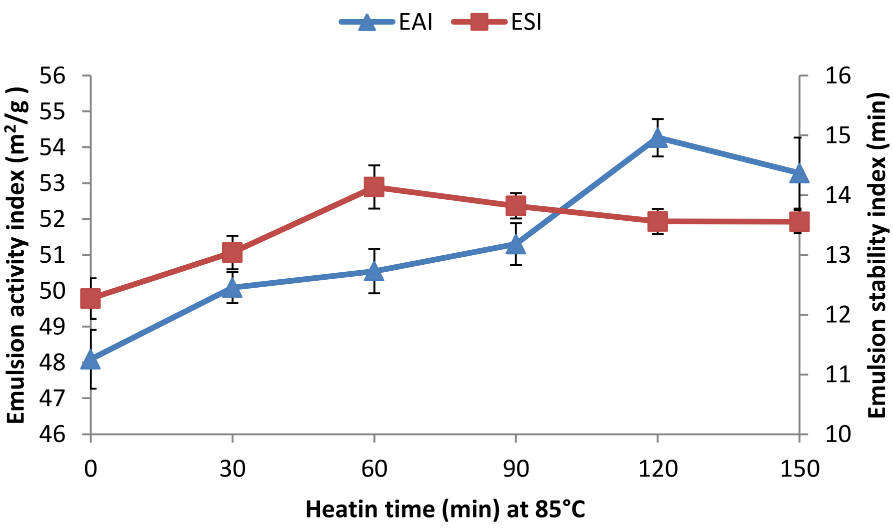


Figure 4.13 Emulsifying activity index (EAI) and Emulsifying stability index (ESI) values of cowpea protein extracted from cowpea flour suspension at different extended heating time (85•C for varying times). Error bars indicate the standard deviation of triplicate measurements..

As shown in **Figure 4.13**, the EAI and ESI of the control which was extracted from CF suspension before heating treatment were 48.09 m^2/g EAI and 12 min ESI, respectively. Heating of CF suspension at 85°C for 2 hours, significantly (P< 0.05) increased the EAI and ESI of GCPI. The heating led to gradual and substantial (P<0.05) increase in EAI of this protein from 50.08 to 54.27m^2/g after 30 and 120 min respectively. Moreover, the heating resulted in a significant (P < 0.05) increase in ESI during extended heating time. These results indicate that the denaturation and/or glycation could improve emulsifying properties of cowpea protein.

Figure 4.14 compares the EAI of DCPI and GCPI at 7.0 (neutral) and 4.0 (acidic) pH. CPI, DCPI and GCPI were isolated from cowpea flour as described in section 2.2.3 and the values of protein content are shown in **Table 4.1** (A, B & C). The preparation of emulsions and methods of determinations of EAI and ESI are described in section 2.2.13. The pH of protein solutions was adjusted to 4.0 and 7.0 using either hydrochloric acid (HCl) or sodium hydroxide (NaOH).

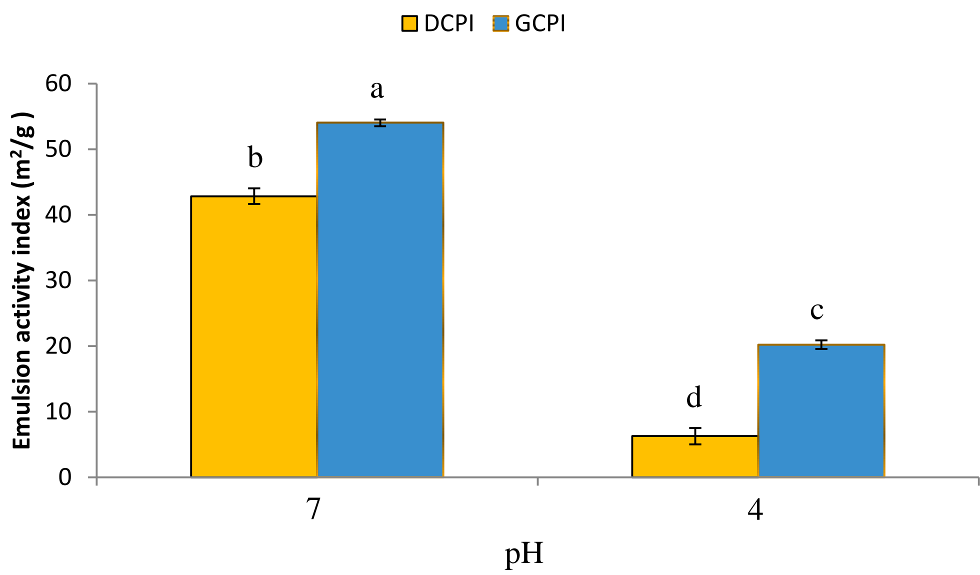


Figure 4.14 EAI of DCPI and GCPI (120 min) at pH 7.0 (1) and pH 4.0 (2).
Means not indicated by the same superscript are significantly (P<0.05) different.

The EAI of GCPI at these two pH values was significantly (P < 0.05) higher than that of DCPI. The effect of the heating (85°C, for 2 hours) on the EAI at acidic pH was remarkable in comparison to that at neutral pH value. EAI of GCPI increased to 54.01 and 20.19 m^2/g at pH 7.0 and pH 4.0 respectively. This might be due to the remarkable

difference in solubility between DCPI and GCPI at neutral and acidic pH values as can be seen in **Figure 4.2**. The EAI of the two protein samples (DCPI & GCPI) showed lower values at the acidic pH than those at the neutral pH value. Similar results were found for soy protein-polysaccharide conjugates glycated in the dry state (Diftis and Kiosseoglou 2003; Mu et al., 2011). The emulsifying properties of GCPI indicated that this modified protein would be of relevance in food manufactures such as sauces.

Schwenhe (1997) reported that glycation of proteins renders them more hydrophilic. Consequently, controlled heating improves protein solubility or protects protein from loss of solubility and therefore, the EAI and ESI of GCPI was higher than DCPI. The improved hydrophilic nature could have improved the emulsifying properties of the protein. Improvement in emulsion properties may be due to polarity and increasing solubility of the protein (Schwenhe 1997). Glycation might be the key to protect the functional properties of cowpea protein in food applications that require heat treatments.

Until the present, the use of cowpea protein as a food functional ingredient, such as foaming or emulsifying agent has been limited compared to soy protein. The current results show that glycation of cowpea protein with its own endogenous sugars via Maillard reaction could improve its functionality and create a promising protein source for the food applications.

The solubility of protein is a key requirement for the film formation for the reason that on the oil-water interface, rapid migration and adsorption is essential. Felix et al. (1990) have found a positive relationship between the emulsifying capacity of proteins and their solubility. A number of different studies have shown that the pH influences indirectly the emulsifying capacity of proteins by affecting their solubility. However, McWatters and Holmes (1979) working with peanut proteins demonstrated that the emulsifying properties cannot be predicted solely on the basis of solubility level, as some proteins like egg white and gelatin perform well at their iso-electric points. Nakai et al (1980) reported that the solubility, the surface hydrophobicity and molecular flexibility influence the emulsification behaviour of globular proteins. Solubility of proteins plays a role in emulsifying properties, but 100% solubility is not an absolute requirement. Although less soluble proteins do not perform well as emulsifiers, no reliable relationship exists between solubility and emulsifying properties in the 25-80%

solubility range (Silva-Ríos, 2013; Fennema, 2000). The stability of protein film at the oil-water interface is dependent on favorable interaction with both the oil and aqueous phases, so some degree of solubility is likely to be necessary (Fennema, 1976).

The viscosity of the protein samples emulsions was directly proportional to the emulsion behaviour. As shown in **Figure 4.15,** the viscosity of the GCPI emulsion was higher than that of the CPI, probably because of the glycation of cowpea proteins by carbohydrates. Diftis et al. (2005) reported that conjugation of soy protein isolate with dextran via the Maillard reaction produced a solid-like emulsion due to bridging flocculation, which supported the findings presented in this study, in which the higher viscosity of GCPI emulsions were related to greater ESI. The GCPI demonstrate higher viscosity than CPI **Figure 4.15**; this might be due to the degree of branching by the formation of high molecular weight complexes between cowpea proteins and carbohydrates.

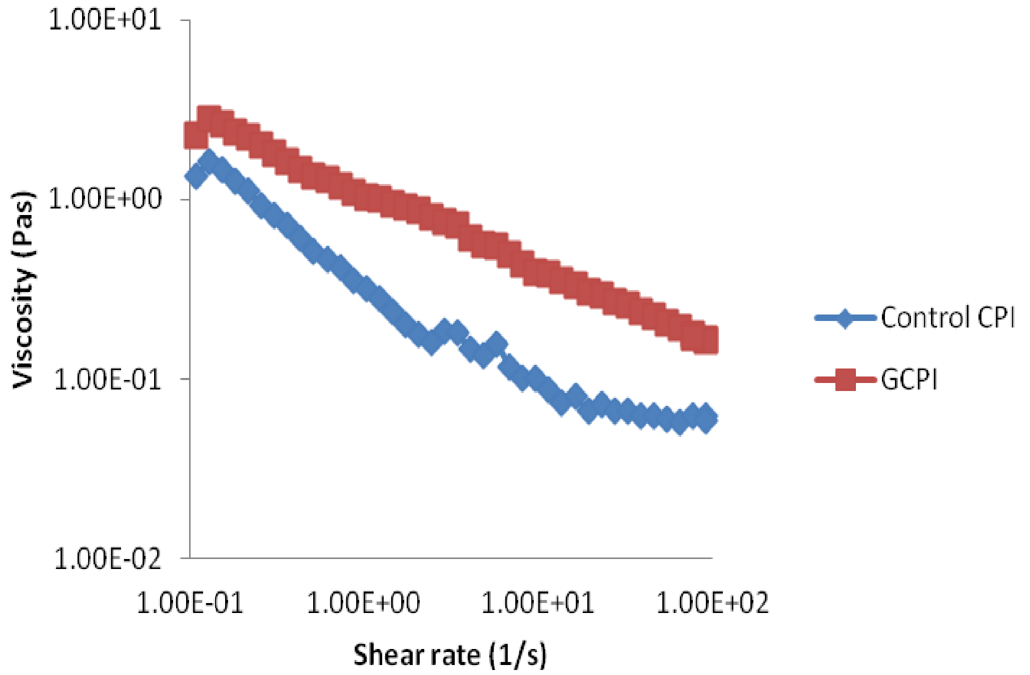


Figure 4.15 Viscosity of emulsions containing 0.25% CPI or GCPI. CPI = cowpea protein isolate (Table 4.1, A); GCPI = Glycated cowpea protein isolate (Table 4.1, C).

According to Stokes Law (**Equation 4.1**), higher viscosity of an emulsion, in the continuous phase, may increase stability.

$$v = 2r^2 (\Delta\rho) g/9\eta \qquad \textbf{Equation 4.1}$$

Where η = the viscosity of the continuous phase, g = gravity, $\Delta\rho$ = the change in density among the oil and continuous phase, r = the droplet radius, and v = the droplets velocity (in the continuous phase),

In a sense, a higher continuous phase viscosity leads to a decrease in the movement or velocity of the dispersed oil droplets, based on Stoke's Law (**Equation 4.1**). Consequently, over a standard time, droplet-droplet encounters will decrease and the emulsions will be stable for longer.

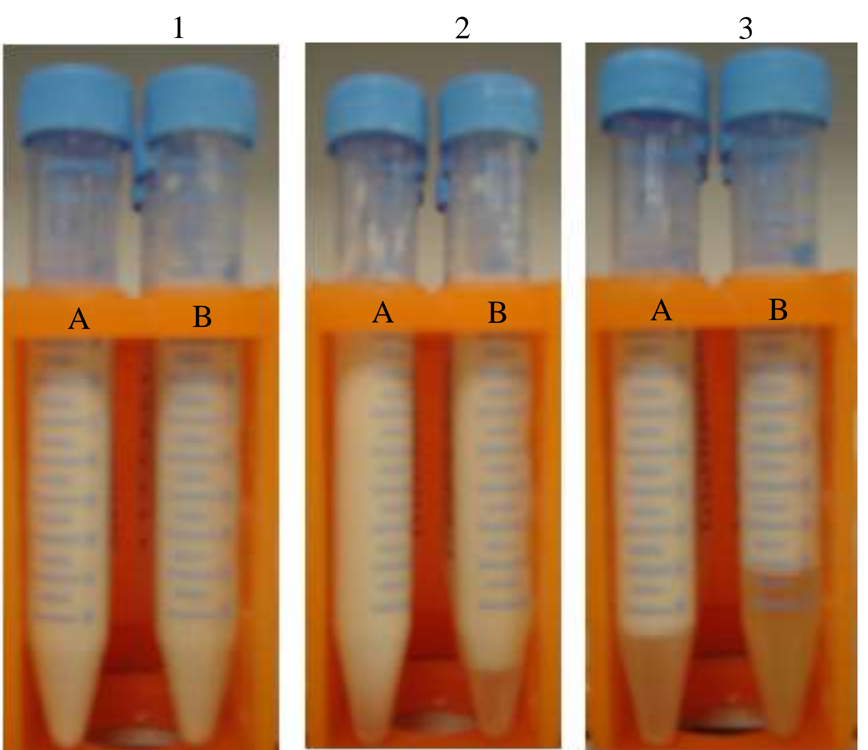


Figure 4.16 Creaming behaviour of GCPI (A) and CPI (B) emulsions after different storage duration time: 0 day (1), 1 day (2) and 10 days (3), at pH 7.0

As shown in **Figure 4.16**, no creaming occurred after one day but after ten days there was some creaming of GCPI which was significantly less than that of CPI. This indicated that cowpea protein-saccharide glycation could produce stable emulsions. The presence of GCPI in the continuous phase at the time of the formation of emulsions (oil-in-water) enhanced the stability of emulsions. This result was established by the increased creaming stability and viscosity (Dickinson and Galazka, 1991).

4.3.2.2 Water holding capacity

The ability of the cowpea protein isolates to adsorb water was assessed using an excess water-centrifugation method (section 2.2.9.7). The water holding capacity difference of the native and modified cowpea protein isolates is shown in **Figure 4.17**. CPI, DCPI and GCPI were isolated from cowpea flour as described in section 2.2.3 and the values of protein content are shown in **Table 4.1** (A, B & C).

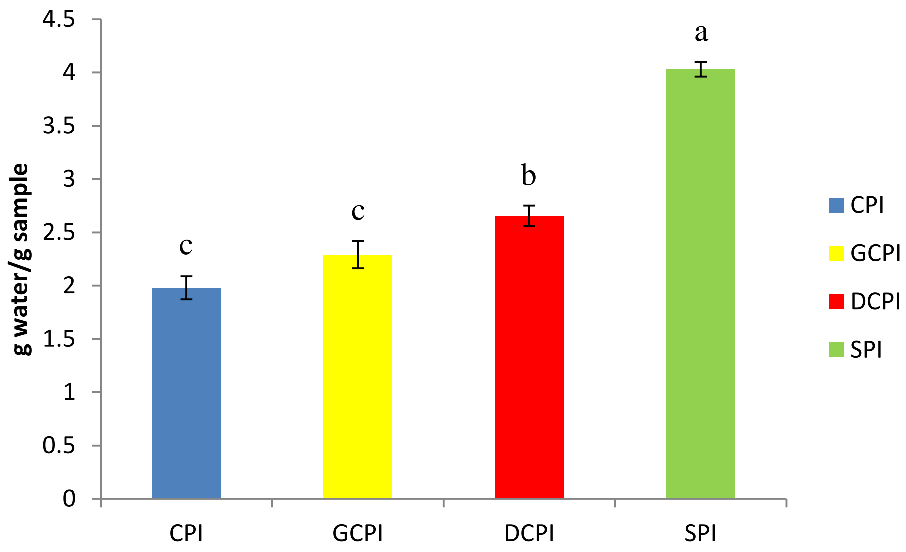


Figure 4.17 Water holding capacity of CPI, GCPI and DCPI at pH 7.5. CPI contained 90.76% protein and 4.38% carbohydrate; GCPI contained 90.02% protein and 5.93% carbohydrate; DCPI contained 90.81% protein and 4.53% carbohydrate. Means not indicated by the same superscript are significantly (P<0.05) different.

The water holding capacity of cowpea proteins was increased from 1.98 g water/g protein for CPI to 2.29 and 2.65 g water/g protein for GCPI and DCPI respectively. This indicates that water holding capacity is significantly improved more by denaturation than glycation. The increased net negative charge of denatured and glycated protein would increase the number of potential water-binding sites, which particularly improve protein-water interaction. Furthermore, this improvement in water holding capacity is partly due to the general expansion and unfolding of protein molecules (Beuchat, 1977). This increase in water holding capacity by denaturation would be of relevance in many foods such as comminuted meat and doughs.

4.3.2.3 Oil absorption capacity

The oil absorption capacity of CPI, DCPI and GCPI is demonstrated in **Figure 4.18**. The oil absorption capacity was measured as described in section 2.2.12. Five grams of each sample was mixed with 30 ml of sunflower oil in a centrifuge tube, allowed to stand at RT for 45 min and then centrifuged. The supernatant was carefully decanted and the weight of each sample was noted. The oil absorption capacity was expressed as the number of g oil held by 1.0 g of protein sample. Glycation has been shown to have effects on the oil absorption capacity of the cowpea protein. The oil holding capacity of cowpea proteins was increased ($P<0.05$) from 1.76 g oil/g protein for CPI to 1.89 (6.8% increase) and 2.02 (12.8% increase) g oil/g protein for GCPI and DCPI respectively. Kinsella and Melachouris (1976) reported that oil absorption capacity of protein is partially related to the physical confinement of oil by means of the protein matrix, as a result the source of the protein might be important.

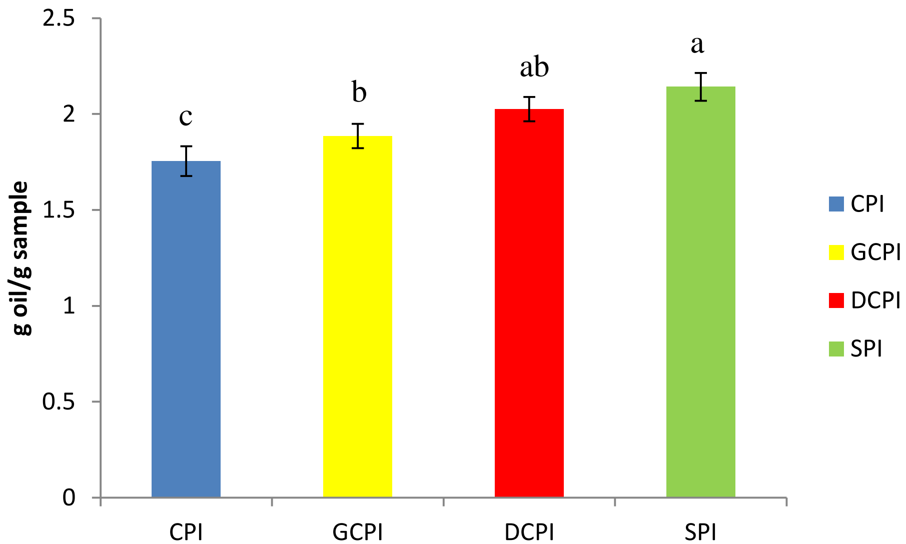


Figure 4.18 Oil absorption capacity of CPI, GCPI and GCPI. CPI contained of 90.76% protein and 4.38% carbohydrate; GCPI contained 90.02% protein and 5.93% carbohydrate; DCPI contained 90.81% protein and 4.53% carbohydrate. Means not indicated by the same superscript are significantly (P<0.05) different.

The relatively high absorption capacity of oil may be attributed to the denaturation degree of protein (Liu and Hung, 1998). This increase in oil absorption capacity would be of relevance in many food system such as meat, sausages, and doughnuts.

4.3.2.4 Foaming properties

Foaming refers to the ability of a protein to form foam by means of air. Foam capacity is as a result of solubilised protein. The ability of proteins to form a film rapidly during whipping is a key point in the manufacture of cakes, ice-creams, confectionaries etc. Foam stability is of importance since the utility of whipping agents correlates to their ability to keep the whip constant with time. The formation of foams by proteins involves the dispersal of soluble proteins at the air–water interface in addition to rapid conformational change as well as rearrangement at the interface (Damodaran et al., 1994; Xie and Hettiarachchy, 1998; Park et al., 2000).

The effect of glycation and denaturation on foam capacity and stability of cowpea proteins is shown in **Figures 4.19** and **4.20** respectively. There were no statistically significant differences as regards foam capacity between CPI and GCPI but the DCPI had significantly (P<0.05) lower foam capacity. The foam stability values of DCPI and GCPI were higher (11.4% and 10.75% increase respectively) than CPI (see **Figure 4.20**). This increase could be due to denaturation and/or glycation. Although increase in foam stability is relatively small, this could still translate to significant importance in bakery applications for example.

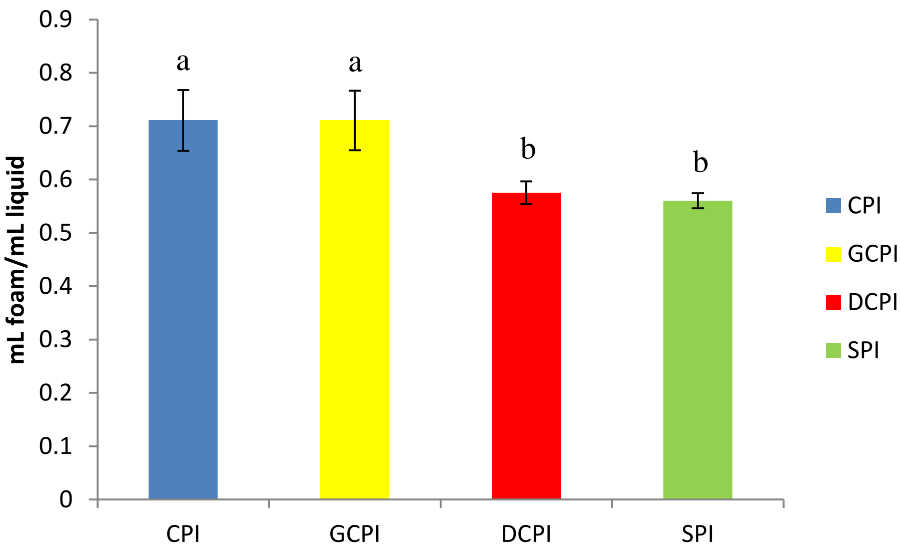


Figure 4.19 Foam capacity of CPI, GCPI and DCPI.
Means not indicated by the same superscript are significantly (P<0.05) different.

There are many conditions affecting foaming properties of proteins such as the type of protein, pH, temperature, degree of denaturation and whipping methods (Saetae and Suntornsuk, 2011). The DCPI showed a low foam capacity value. The foam capacity value of GCPI was significantly (P<0.05) higher than DCPI. This indicates that glycation might lead to a delay in the denaturation of protein when heated in the same conditions in the presence of cowpea's own carbohydrates. The higher capacity value of CPI is due to its higher solubility where soluble proteins can decrease surface tension at the interface between the surrounding liquid and air bubbles. The high protein solubility is a precondition for good foaming properties (Cheftel et al., 1985; Halling and Walstra, 1981).

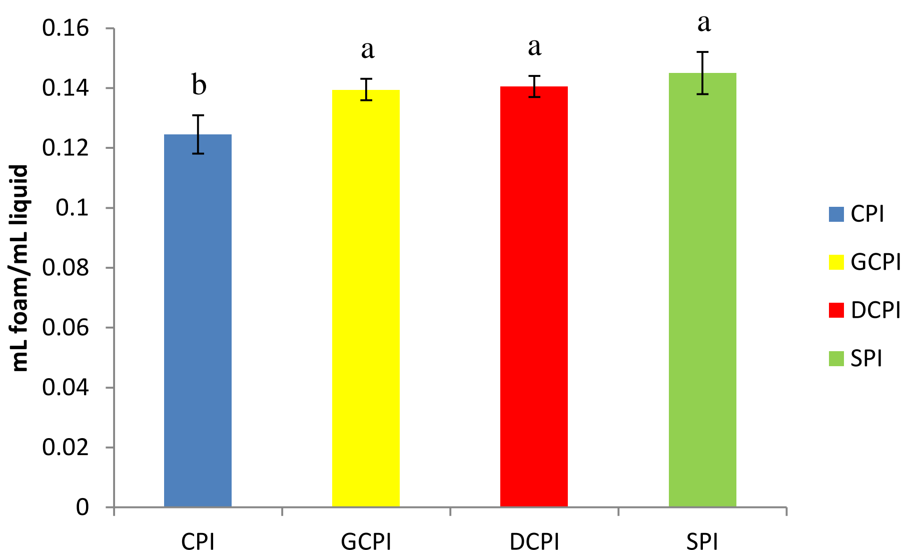


Figure 4.20 Foam stability of CPI, GCPI and DCPI.
Means not indicated by the same superscript are significantly (P<0.05) different.

Glycation has been reported to improve the foam stability of some plant proteins such as those from soybean (Achouri et al., 2005; Bobálová et al., 2010).

4.3.2.5 Gelation properties

The majority of food gels based on protein are induced due to heating. The formation of gels is influenced by pressure, heat, pH, or shearing, as well as the presence of various solvents. In the present research it has been found that cowpea proteins form gels by heat treatment (90°C) with maximum peak force value of 1.3

Newton (section 3.3.10) at pH 7.0 and 16% protein concentration as a critical protein concentration. The aim of this section is to determine and discuss the effect of denaturation and glycation on thermal gelation properties of cowpea protein isolate. When the glycated protein was tested it was noted that glycation produced some improvement of the gelation properties (**Figure 4.21**). The gel strength tended to increase from 1.3 N for CPI to 1.39 and 1.44 N for GCPI and DCPI respectively but still lower than that of SPI. The increase of gel strength of DCPI was significantly (P≤0.05) different from CPI but that for GCPI was not substantial. Therefore denaturation and glycation appeared to enhance the functional properties of cowpea proteins but the gel induced from the DCPI suspension was higher than that from GCPI. It is well known that gelation is dependent on the denaturation of protein, followed by its intermolecular association to induce matrices, which trap water and other food ingredients (Hui, 2006). It has been reported that the addition of locust bean gum to soy protein isolate led to an increase in the strength of gels independent of the protein state (native or denatured), albeit gels of native soy protein isolate were less deformable, weaker and showed lower water holding capacity than the gels of heat-denatured soy protein isolate (Perrechil, 2013).

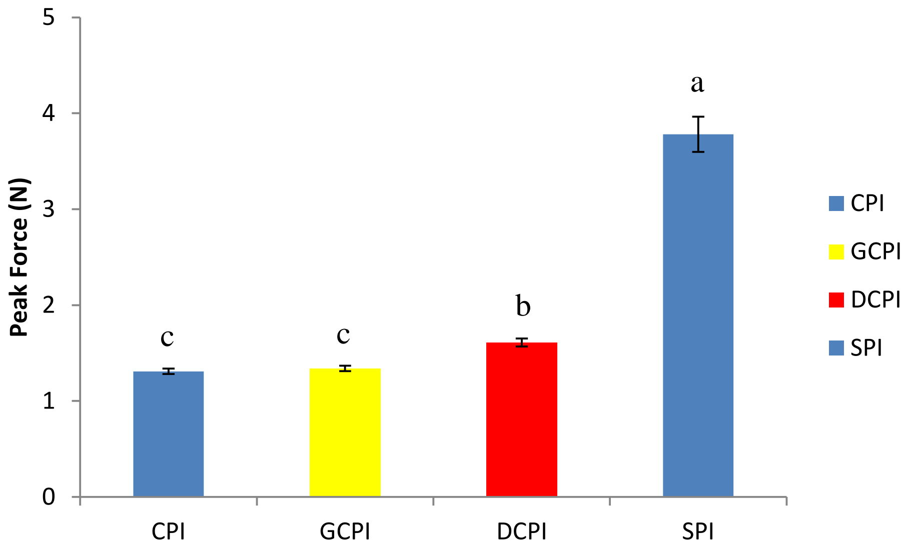


Figure 4.21 Gel peak forces of CPI, GCPI and DCPI.
Means not indicated by the same superscript are significantly (P<0.05) different.

Soy protein isolate has been conjugated to xylose via the Maillard reaction using a wet heat procedure at 121°C for 60 min, resulting in enhancements in gelation

functionality and production of a higher elasticity gel with a higher breaking force (Cabodevila et al. 1994). It has been reported that soy protein can form irreversible gel under heating conditions of 100°C for half an hour and the disulphide bonds must be a part of the gelation mechanism (Circle et al., 1964).

The present study confirms that the properties of texture of the cowpea protein isolate gels could be modulated by the denaturation. However the increase in gelation for GCPI was not significant compared to the control CPI.

4.4 Conclusions

In summary, the functional characteristics of cowpea protein were greatly improved by thermal denaturation and by glycation via the Maillard reaction. In the present study, the functional properties of cowpea proteins have been extensively investigated and compared to that of soy protein isolate (SPI). Glycated cowpea protein isolate (GCPI) and denatured cowpea protein isolate (DCPI) were prepared from cowpea flour dispersion as described before. Compared to denaturation alone, combined denaturation and glycation appears to exhibit better functional properties of cowpea protein including solubility, emulsifying activity and stability, viscosity, and foam stability. On the other hand, denaturation appears to exhibit better water holding capacity, oil absorption capacity and gelation properties, indicating that these modifications are suitable to modify this type of protein for many food applications. The functional properties of CPI, GCPI and DCPI compared to SPI are shown in **Table 4.5.** SPI is extensively used in the food industry in various applications. These results show that CPI could deliver similar functional properties to SPI. Therefore protein preparations with selected degrees of denaturation and glycation could present unique functional characteristics, which in turn, may serve as specific applications in particular food systems.

Table 4.5 The functional properties of CPI, GCPI and DCPI compared to SPI

Functional properties	CPI	DCPI	GCPI	SPI
WHC	+	+++	++	++++
OAC	++	++++	+++	++++
FC	+++	++	+++	+
FS	+	++	++	+++
EAI	++	+	++++	+++
ESI	+	++	+++	++++

WHC = water holding capacity; OAC = oil absorption capacity; FC = foam capacity; FS = foam stability; EAI = emulsifying activity index; ESI = emulsifying stability index; CPI= cowpea protein isolate; DCPI = denatured cowpea protein isolate; GCPI = glycated cowpea protein isolate

In this study, it was apparent that every functional property of cowpea protein isolate was improved by denaturation and/or by combined denaturation and glycation. Hence,

selection of products for which the modified protein will complete its functions is based on the way that cowpea protein isolate has been modified.

It is important to mention that, the solubility of the cowpea flour suspension showed resistance to heat treatment more than that of native CPI. Since the Maillard reaction is considered to be relatively safe, denatured and/or glycated cowpea protein could be produced under relatively simple processing conditions and provide a new and promising ingredient for food applications.

Chapter Five

Applications of cowpea protein isolate in foods

5.1 Introduction

Plant protein sources provide 65% of the world's supply of edible protein, with the main contributors being cereal (47%) and pulses, nuts and oil seeds (8%) (Young and Pellett, 1994). In nations such as those in Western Europe, approximately 35% of dietary proteins are obtained directly from plants, while in developing nations close to 80% of the proteins are from plants (Millward, 1998). Although plants can provide all of human protein needs, a misconception persists that they are nutritionally inferior to animal proteins. This derives from complex social and cultural attitudes towards meat and because of the scientific tradition of protein quality evaluation in animals (Millward, 1998). In fact, the important nutritional question is whether plant proteins are easily acceptable.

The utilisation of legume proteins in food applications is expected to increase significantly in the future in order to meet the worldwide quest for cheap sources of protein. Among the justifications for interest in vegetable proteins is the ever increasing number of vegetarians and the increasing costs of animal protein sources (Sethi and Kulkarni, 1994). On the other hand, soybean grains have been the major vegetable protein resource for foods for a long time, including bakery and dairy products, confectionery and meat or fish products. Surely, soy protein has made a substantial influence in the food applications. Cowpea protein isolate, which has now been shown in the present study to be isolated using processing conditions similar to that of soy protein isolate, and also to exhibit good functional properties in comparison to soy protein isolate, offers substantial potential in a lot of food applications. Cowpea flour has been previously used as protein supplement in bread, biscuits, buttermilk doughnuts, muffin (chemically leavened quick bread), Chinese noodles, yoghurt-like products, imitation milk and snack chips (Mustafa et al., 1986; McWatters, 1980; McWatters, 1982; Holt et al., 1992; Chompreeda et al., 1988; Schaffner and Beuchat, 1986; Phillips et al., 2003). The results indicated that the baking and sensory properties of the products were not unfavourably affected by the cowpea flour. But if unheated cowpea flour was used at a higher level, unfavourable influences of the protein on dough or baking characteristics including crumb and crust texture and colour of the products were noted (Sales, 1980; Onayemi and Potter, 1976). Cowpea flavours may be a main limitation in the utilisation of cowpea flour and cowpea protein concentrate. In

contrast to cowpea flour, no research data are available on the investigation of potential food utilisation of cowpea protein isolates in food applications.

The previous chapter in this study has demonstrated that cowpea protein isolate has good functional properties. Since the good applications of vegetable-derived proteins will mostly depend on the functional and physical properties they impart to foods and to their acceptance by consumers (McWatters, 1980), the choice of appropriate food systems for evaluating the possibility of the new vegetable protein ingredients is very important. For the present study, bread, cake and mayonnaise have been selected as classic foods to study the potential of cowpea protein isolates (CPI; DCPI; GCPI) as additional additives for bread and as replacement for egg in cake and mayonnaise. Whereas foaming ability, water holding capacity and oil absorption capacity of the proteins are important in bread and cake, the emulsifying properties are also desirable in cake, and the emulsifying capacity and stability are important in mayonnaise.

Proteins as food ingredients have other important functions besides nutritional value, i.e., those relating to the physico-chemical characteristics necessary for producing good quality product (Nakai, 1996). Good aroma, taste, colour and texture are very essential in evaluating the application potential of specific proteins in food applications. In addition, different food products have need of different properties, for example, in comminuted meat, the protein should absorb water and form a gel, in a beverage it should be soluble, and in a whipped topping the protein should produce stable foam. Proteins from specific raw materials have been utilised to give the various functional properties necessary in various applications. In view of that, where foaming behaviours are essential to the texture properties, egg white protein has been utilised; while if emulsification is essential, egg yolk has been used.

Although the uses of cowpea are still limited in food industries, there is great interest in this legume as a source for creating of new protein-rich food products. Like soybean products, cowpea flour or proteins can be added into textured protein, cereal and bakery products, milk products, meat products, and other applications.

5.2 Materials and Methods

The materials and methods used within this study were described in chapter 2.

5.3 Results and Discussion

The study in each product would be focused on the suitable amount of the cowpea protein isolate sample for addition into food product and comparison of GCPI and DCPI with CPI. Sensory and physical properties of products were evaluated in this study.

5.3.1 Incorporation of cowpea flour and protein in bread

Bread, cakes and biscuits etc. constitute a large part of the modern human diet. Commercial bread and biscuits contain around 7–8% protein, which is relatively low. Most of these products can easily be enriched and fortified with low cost proteins (Sharma et al., 1998; Indrani et al., 2007). Bread was probably the first ever produced processed food, and still remains the most universally accepted. Though it is not a highly nutritional source of protein, it is nonetheless a principal source of both calories and protein in most countries. Some 70 % of the world's protein supply comes from vegetable sources and 30% from animal sources (Forsum, 1973). Bread making in Libya depends on imported sources of wheat, grains and its flour. Consequently Libya imports annually about 95% of its requirements of wheat grains mainly for bread making and other bakery products from European countries (FAO, 2013; Gadan et al, 2006;). The quality of most of these products is between medium to poor and hence affects the quality of the bread. Bread improvers such as ascorbic acid and calcium iodate are used in the local market to improve the quality of the flour (Shalgam and Shoelya, 2001).

In the present study, in order to investigate the effect of cowpea flour and proteins on wheat bread, flour samples containing wheat and cowpea flours or proteins were formulated at different levels (w/w) of cowpea flour or protein substitutions for bread making. Proximate analyses of the samples i.e. moisture, crude protein, ash and crude fat, were carried out according to American Association of Cereal Chemists (AACC)

methods. Carbohydrate was calculated by difference. Straight dough method was used to bake bread, according to the recipe indicated in **Table 2.1** (chapter 2). Doughs were prepared from wheat flour with and without the addition of different quantities of cowpea flour. The ingredients were mixed for five minutes. The dough was baked at 200°C for 30 min., in a loaf pan in a Russell Hobbs Mini Oven as shown in **Figure 2.3** (chapter 2). Water absorption of the composite flour was determined and loaf volume and specific loaf volume were measured. Sensory evaluation was performed 24 hours after baking to evaluate flavour and overall acceptability of the bread sample. The bread samples were sliced into pieces of uniform thickness and served with water. Panellists evaluated products samples on a 9 point hedonic scale (Larmond, 1977) with 9 = liked extremely, 8 = liked very much, 7 = liked, 6 = liked mildly, 5 = neither liked nor disliked, 4 = disliked mildly, 3 = disliked, 2 = disliked very much and 1 = disliked extremely. Samples were presented to a panel of judges with a 3-coded digit number. Crumb hardness as texture was measured 1 hour after cooling using the Zwick/Roell type Z010 equipment.

In order to investigate the effect of cowpea flour on wheat bread, flour samples containing wheat and cowpea flours were formulated at 0, 5, 15 and 20% (w/w) level of cowpea flour substitutions of wheat flour.

As shown in **Table 5.1**, the protein contents of the composite breads ranged from 6.1–9.9%. Protein contents increased significantly with increasing levels of cowpea flour in the composite flours. Water absorption increased with increasing contents of cowpea flour in the blends (**Figure 5.1**).

Table 5.1 Proximate Analysis of Bread

	Chemical composition of bread (g/100g sample)					
CF[1] (%)	Moisture	Protein	Lipid	CF[2]	TA[3]	CHO[4]
0	28.5^{c}	6.1^{d}	1.7^{a}	0.08^{c}	3.0^{c}	58.92^{a}
5	29.6^{b}	7.4^{c}	1.6^{a}	1.4^{b}	3.4^{bc}	54.80^{b}
15	31.1^{a}	8.6^{b}	1.3^{b}	2.3^{ab}	3.9^{ab}	49.70^{c}
20	29.9^{b}	9.9^{a}	0.9^{c}	3.4^{a}	4.9^{a}	47.50^{d}

[1]CF= Cowpea flour; [2]CF= Crude fibre; [3]TA= Total Ash; [4]CHO= Carbohydrate.

Means in the same column not indicated by the same superscript are significantly (P<0.05) different.

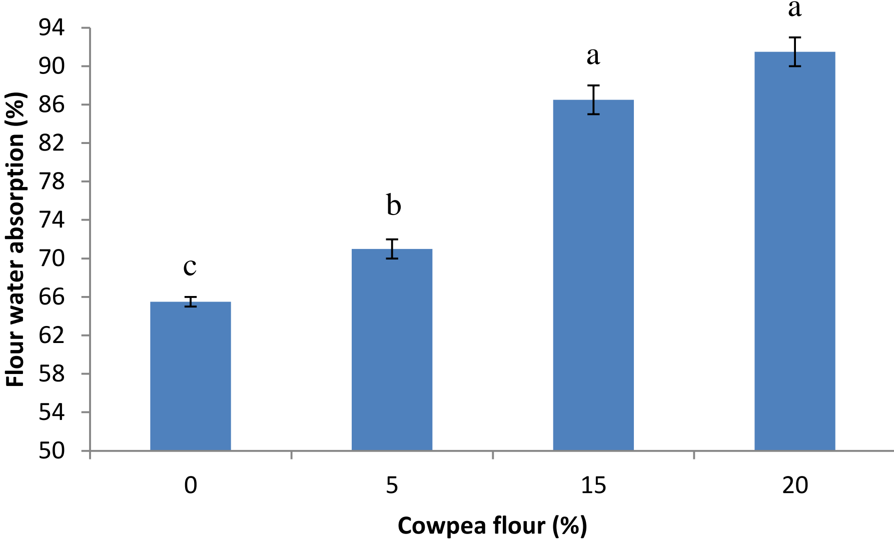


Figure 5.1 Water absorption of wheat-cowpea flour composite
Means not indicated by the same superscript are significantly (P<0.05) different.

Figure 5.2 presents the average results of sensory attributes of cowpea flour addition. As can be seen, difference in the overall acceptability of 0% (control), and 5% level of cowpea flour was insignificant (P>0.05) On the other hand, results showed that cowpea flour incorporation at the level of 20% led to a poorer overall acceptability.

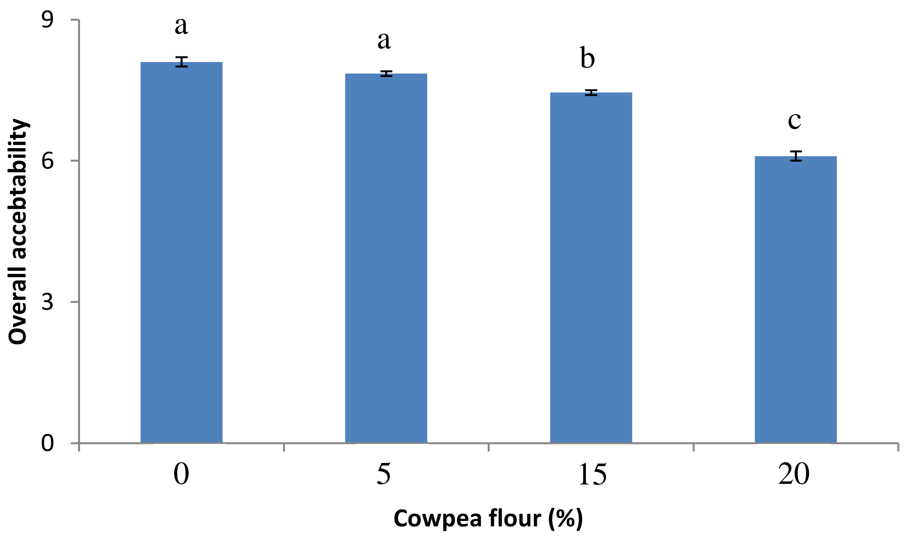


Figure 5.2 Sensory evaluation of breads made with different levels of cowpea flour

Means not indicated by the same superscript are significantly (P<0.05) different.

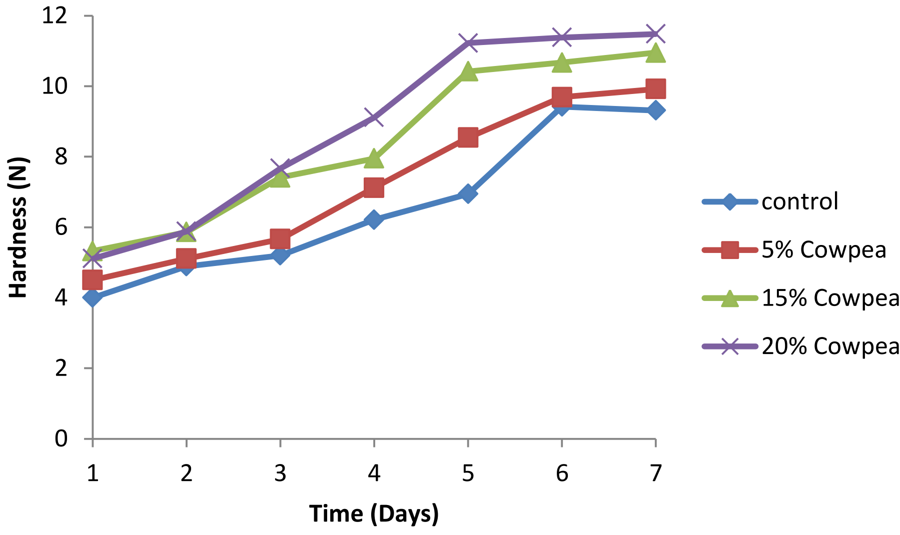


Figure 5.3 Hardness of bread crumbs prepared with different levels of cowpea flour. Samples were measured in duplicate.

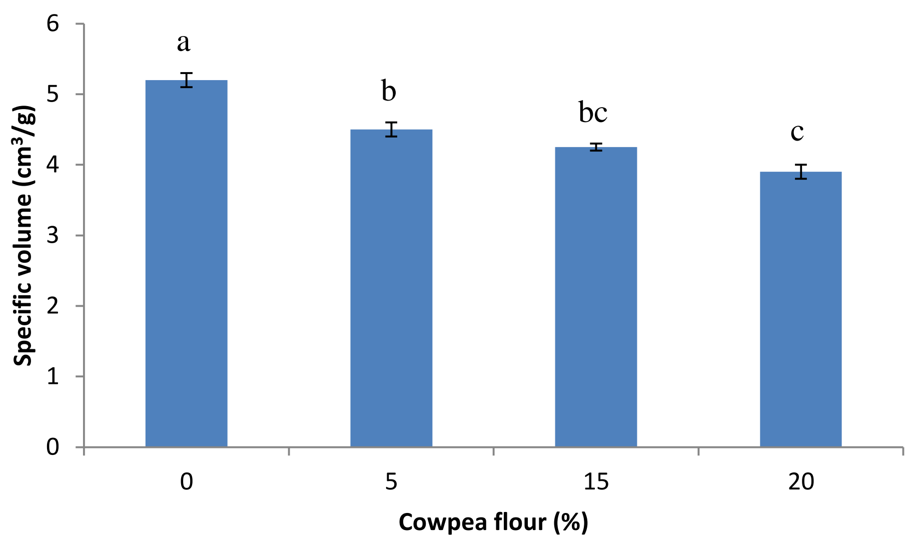


Figure 5.4 Influence of cowpea flour on loaf volume

Means not indicated by the same superscript are significantly (P<0.05) different.

As shown in **Figure 5.3** and **Figure 5.4**, for bread hardness and bread specific volume respectively, cowpea flour increased the hardness of the bread crumb and decreased the specific volume of breads, whilst 5% level of cowpea flour decreased the specific volume of bread but crumb hardness remained similar to the control up to three days. It must be mentioned that cowpea flour resulted in an increase in crumb moisture. This could indicate a positive trend as increased crumb softness might relate to the moisture retention capacity. **Figure 5.3** shows increased crumb hardness.

In order to investigate the effect of cowpea protein isolates, CPI, DCPI and GCPI on wheat bread, protein samples containing wheat and cowpea flours were formulated at 0, 2, 4 and 6% (w/w) level of cowpea flour substitutions for bread making. The dough and bread properties of wheat cowpea protein composite flours are presented in **Figure 5.5** and **Figure 5.6**. Both water absorption of dough and weight of bread loaf increased with increasing contents of cowpea protein isolate in the blends. Differences in the values for water absorption at the levels of 4% and 6% for both CPI and GCPI were significant ($P \leq 0.05$). Vose (1980) reported that the increase in the water absorption produced by the addition of pea and soy protein isolate might be related to their water holding capacity. The moisture content of bread in the present study increased as the amount of protein added increased (**Table 5.2**). The moisture content of the composite breads ranged from 37.61 to 40.05 for control (wheat flour only) and GCPI (6%), respectively.

Table 5.2 shows the proximate analysis of control bread and protein-added breads. The fat and ash content of all samples were not statistically different. On the other hand, each sample contained statistically different amount of protein. The protein content of bread increased as the amount of protein added increased. The protein contents of the composite breads ranged from 7.5 to 10.76%. Protein contents increased significantly with increasing levels of cowpea protein isolate in the composite flours.

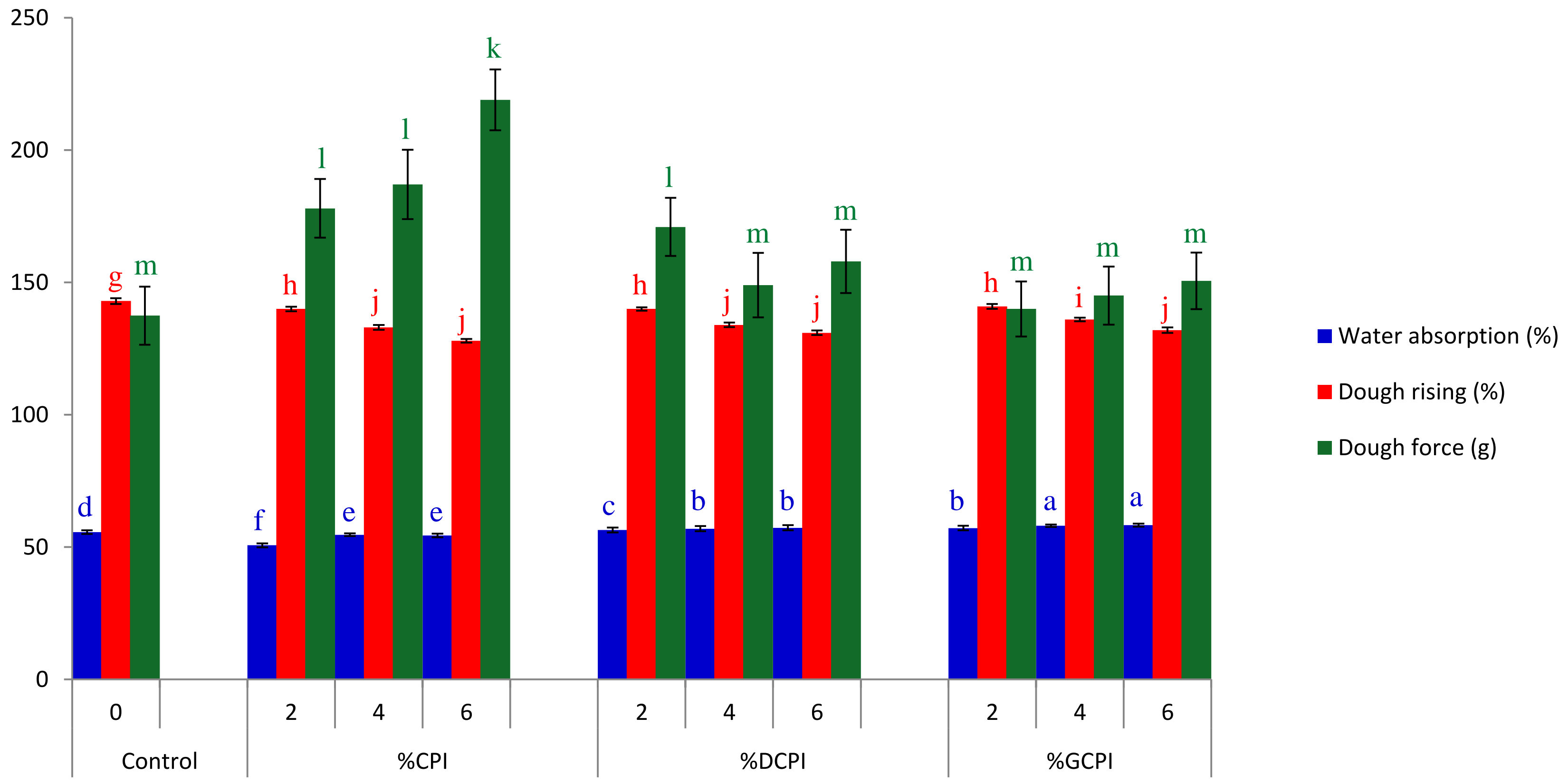


Figure 5.5 Properties of dough containing cowpea protein. CPI= cowpea protein isolate; DCPI= denatured cowpea protein isolate; GCPI= glycated cowpea protein isolate. *Means in the same colour bar not indicated by the same superscript letter are significantly different (p≤0.05).

The force required to compress the dough (peak value, hardness) containing CPI, DCPI and GCPI respectively is shown in **Figure 5.5**. Results show that the bread dough containing the CPI was the hardest requiring more strength to compress it to the required extent. Dough containing GCPI was the softest requiring the least force to compress it. Control dough samples (wheat flour only) and dough samples containing GCPI respectively had almost similar textural properties. It is possible that dough made with GCPI is generally more cohesive and viscous and hence softer. When 4% GCPI was incorporated into wheat dough, water absorption was increased by 2.4% ($P< 0.05$). Addition of 4% CPI decreased water absorption of dough by 1.0% ($P< 0.05$). Kenny et al. (2001) found that the addition of heat treated whey protein concentrate (4%) increased water absorption of frozen dough compared with that of native whey protein concentrate which significantly reduced water absorption. He and Hoseney (1990) reported that bread with higher moisture content was significantly firmer and fresher and aged at a slower rate than that of bread with lower moisture content. These results indicate that denatured protein (DCPI) improved the water absorption property of cowpea protein whereas it is even more improved by denatured and glycated protein (GCPI).

The specific volume of cowpea composite flour breads decreased significantly with increasing cowpea protein as shown in **Figure 5.6**. Increasing GCPI concentrations resulted in bread samples with increased moisture content, albeit decreased specific volume in bread. The latter might be due to the significant decreasing trend of the volume of risen dough. Differences in the values for moisture content at the levels 2% and 4% were not substantial but the moisture content increased significantly with increasing CPI or GCPI at the level of 6%, whilst the weight of bread loaf values were significantly increased. CPI increased the hardness of the breadcrumb (**Figure 5.6**) and decreased the specific volume of breads. GCPI decreased the specific volume of bread and reduced crumb hardness compared to CPI and DCPI to levels similar to that of the control. Although GCPI showed increase in crumb moisture, the change was not significant compare to the control. It nevertheless indicates a positive trend as increased crumb softness might relate to the moisture retention capacity as reported by Shittu et al., (2007).

Table 5.2 Results of proximate analysis of flours, protein powders and the respective bread loaves. Values are the average of triplicate determinations. Means labelled with different upper case letters are statistically different ($p \leq 0.05$).

	Components (g/100g flour)					
Flour	Moisture	Protein	Lipid	Crude fibre	Total Ash	Carbohydrate (by difference)
Wheat	13.30	9.70	1.47	0.84	0.47	74.22
Cowpea	8.10	25.20	1.63	4.40	3.70	57.17
CPI	4.40	89.70	0.44	1.07	1.35	3.04
DCPI	4.63	89.51	0.45	1.04	1.30	3.07
GCPI	4.69	88.43	0.47	1.02	1.29	4.10
Bread						
Control	37.61^{b}	7.50^{f}	2.56^{a}	0.08^{c}	3.00^{a}	49.25^{a}
*CPI						
2%	37.71^{b}	8.58^{de}	2.51^{a}	0.10^{c}	3.29^{a}	47.81^{b}
4%	37.98^{b}	9.66^{bc}	2.48^{a}	0.46^{b}	3.41^{a}	46.01^{c}
6%	40.01^{a}	10.76^{a}	2.40^{a}	1.23^{a}	3.48^{a}	42.03^{d}
*DCPI						
2%	37.91^{b}	8.35^{ef}	2.47^{a}	0.09^{c}	3.22^{a}	47.96^{b}
4%	38.33^{b}	9.42^{cd}	2.46^{a}	0.41^{b}	3.39^{a}	45.99^{c}
6%	40.03^{a}	10.60^{a}	2.44^{a}	1.21^{a}	3.42^{a}	42.30^{d}
*GCPI						
2%	38.03^{b}	8.33^{ef}	2.40^{a}	0.13^{c}	3.11^{a}	48.00^{b}
4%	38.75^{b}	9.40^{cd}	2.21^{a}	0.40^{b}	3.20^{a}	46.04^{c}
6%	40.05^{a}	10.52^{ab}	2.11^{a}	1.30^{a}	3.39^{a}	42.63^{d}

**% cowpea proteins in composite bread*

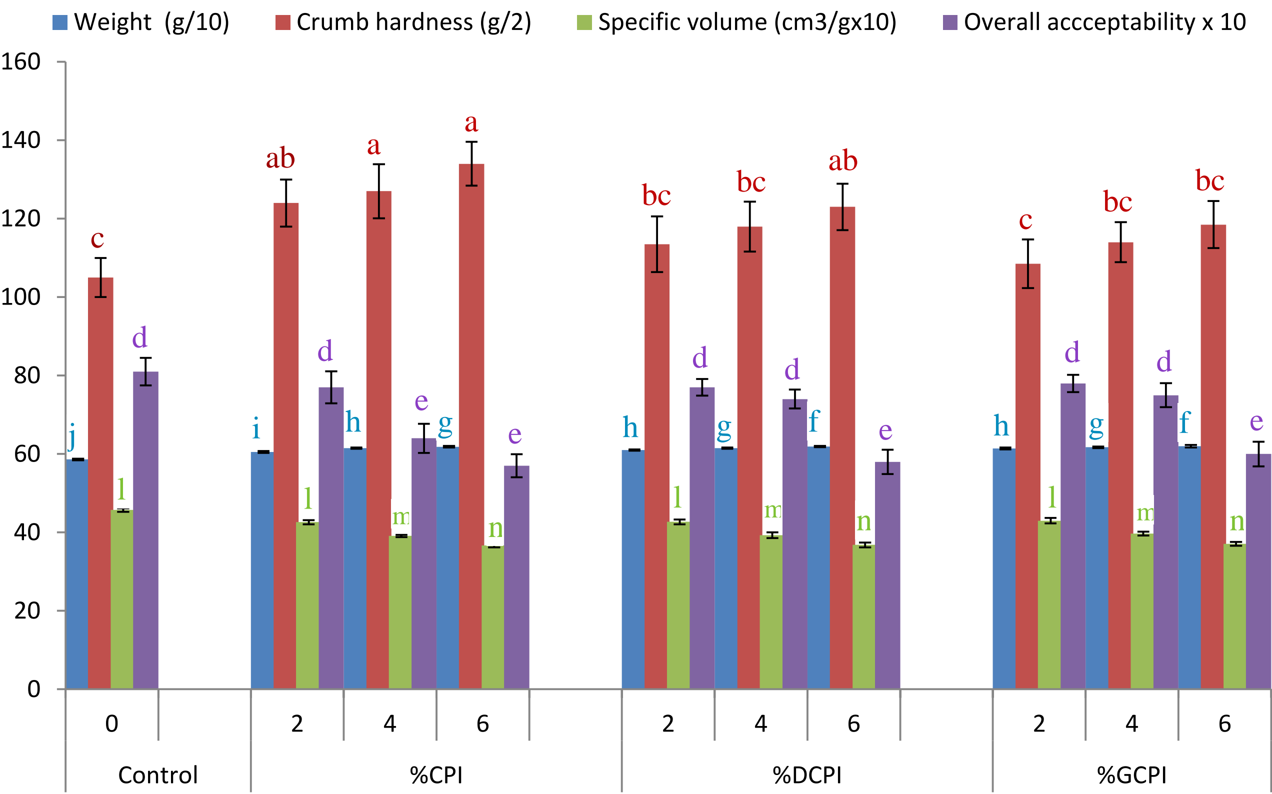


Figure 5.6 Weight, crumb hardness, specific volume and overall acceptability of bread containing cowpea protein. CPI= cowpea protein isolate; DCPI= denatured cowpea protein isolate; GCPI= glycated cowpea protein isolate. *Means in the same colour bar not indicated by the same superscript letter are significantly different (p≤0.05.

This study presents unique enhancement of softness attributes of bread for the first time by the incorporation of denatured- glycated cowpea protein (GCPI). Although the wheat protein in bread is subject to denaturation and Maillard reaction during baking of bread, these findings indicate a prerequisite for modification of added protein prior to baking. Apart from decreased volume, the GCPI at level up to 4% did not adversely affect the bread physical properties. The overall acceptability (**Figure 5.6**) was unaffected, as well as the crumb texture.

5.3.2 Incorporation of cowpea protein isolate in cake

For the purpose of studying the application of cowpea proteins in cakes, CPI, DCPI and GCPI were used to replace egg by 20 and 40%. In order to compensate the water content in eggs, the cowpea protein sample was dissolved in an appropriate amount of water in the food mixer before adding the remainder of the eggs, sugar and emulsifier. The procedure was described in section 2.2.24 (chapter 2).

Table 5.3 Characteristics of laboratory prepared sponge cakes

Level of Addition of Protein	Cake Volume (mL)	Firmness (gf)
Control[1]	960.01 ±11	554.72 ±09
CPI[2]		
20%	955.22 ±10	510.87 ±08
40%	885.13 ±12	434.39 ±11
DCPI[3]		
20%	943.00 ±11	457.85 ±08
40%	870.16 ±09	512.91 ±12
GCP[4]		
20%	951.03 ±12	442.55 ±09
40%	880.00 ±08	505.77 ±10

**Means ± standard deviation of three replicates. Control[1] = 100% egg proteins; CPI[2]= cowpea protein isolate; DCPI[3]= denatured cowpea protein isolate GCPI[4]= glycated cowpea protein isolate.*

Table 5.3 presents the characteristics of laboratory prepared sponge cakes supplemented with GCPI., In terms of volume and firmness of the cakes with 20% replacement of cowpea proteins for egg proteins, the volume of cakes was similar to that of the controls. Meanwhile, the volume of the cakes produced from GCPI was not substantially higher than that prepared with 20% substitution by CPI. The cake volume

was reduced somewhat at the level of 40% replacement of GCPI for egg proteins. The reduction in cake volume when the level of cowpea protein increased may be due to the difference in foaming properties of these proteins, particularly for DCPI which have lower foam capacity than that of CPI and GCPI. Liu (1996) and Sathe et al. (1981) reported that the fortification of breads with non- wheat flours such as legume proteins, reduced the loaf volume. This reduction in volume is generally due to the influence on gluten, which plays an important role in dough formation, and the texture of the bread. In the present study, the purpose was to use cowpea protein to replace egg protein in cake. If cowpea proteins were used to replace egg protein at levels of 40%, the volume of the cakes decreased more compared with that at a level of 20%.

In further evaluation of the texture of cakes, firmness values were determined using a texture analyser Zwick/Roell (model BDOFBO5. TS, Herefordshire, Germany) (**Table 5.3**). The apparent decrease in firmness of the crumb, at levels of 40% replacement with cowpea proteins, did not represent a softening which may have been wished for. The lower value in firmness of CPI (at level of 40%) reflects the more crumbly texture which led to loss of coherence during the compressive force that was applied to the slice during measuring.

In comparison, GCPI gave improved coherence compared to CPI and it was softer than the control. No oily-surface was noted in the crumb and the cake coherence was good.

The sensory evaluation of cakes produced with replacement of egg by cowpea proteins is presented as shown in **Table 5.4**. Twenty members of varying backgrounds were asked to evaluate the cakes for overall acceptability along with the presence of a cowpea flavour. No difference was found at a level of 20% substitution of egg by GCPI. However the overall acceptability of both CPI and DCPI was slightly decreased at the same level, whereas that of GCPI was the closest to the control. Moreover, the panellists commented that after substitution at a level of 20% and 40% with CPI, DCPI and GCPI, all of the cakes were acceptable. In relation to cowpea flavour, the panel members did not detect any beany flavour at 20% substitution levels. At 40% substitution level, the cowpea flavour was detectable, but some panel members definitely noted that they favoured cowpea flavour in the cakes, reflecting the variety of cultures and dietary backgrounds of the panel members. Some panel members also noted that an adverse

influence of the CPI and DCPI was the crumbly mouthfeel, coarse of the cakes supplemented at 40%, whereas that of GCPI was acceptable and similar to the control.

Table 5.4 Sensory evaluation of cakes

	Overall acceptability	Cowpea protein taste
Control[1]	9.0 ±0.14	-
CPI[2]		
20%	7.4 ±0.13	-
40%	5.3 ±0.10	+
DCPI[3]		
20%	8.2 ±0.09	-
40%	7.1 ±0.08	+
GCPI[4]		
20%	8.6 ±0.15	-
40%	7.5 ±0.12	+

**Means ± standard deviation of three replicates. Control[1] = 100% egg proteins; CPI[2]= cowpea protein isolate; DCPI[3]= denatured cowpea protein isolate GCPI[4]= glycated cowpea protein isolate.*

*** Sensory evaluation scores for overall acceptability, 1 = disliked extremely, 2 = disliked very much, 3 = disliked, 4 = disliked mildly, 5 = neither liked nor disliked, 6 = liked mildly, 7 = liked, 8 = liked very much and 9 = liked extremely.*

5.3.3 Incorporation of cowpea protein isolate in mayonnaise

In order to investigate the application of cowpea proteins in mayonnaise, CPI, DCPI and GCPI were used to replace egg yolk at levels of 20 and 40%. The mayonnaise was prepared described in section 2.2.25 (chapter 2).

Table 5.5 depicts the texture parameters determined for laboratory prepared mayonnaise. The GCPI (20%) samples and controls were similar to each other for firmness and adhesiveness values which were higher ($P>0.05$) than those of the CPI and DCPI. In general, it can be seen from **Table 5.5**, in terms of firmness and adhesiveness of the mayonnaise, with 20% replacement of GCPI proteins for egg proteins, the quality of the produced mayonnaise was similar to that of the control and higher in quality products that prepared with 20% substitution by CPI.

Table 5.5 Characteristics of laboratory prepared mayonnaise

Level of Addition of Protein	Firmness (gf)	Adhesiveness (x 10^{-3} Nm)
Control[1]	212.10 ±8.28[a]	19.71 ±0.62[a]
CPI[2]		
20%	147.11 ±3.23[b]	14.01 ±0.40[b]
40%	65.26 ±1.15[d]	7.18 ±0.33[d]
DCPI[3]		
20%	155.03 ±4.26[b]	14.49 ±0.42[b]
40%	106.05 ±1.18[c]	9.81 ±0.24[c]
GCPI[4]		
20%	198.21 ±5.29[a]	18.23 ±0.58[a]
40%	150.91 ±3.23[b]	14.48 ±0.46[b]

**Means in the same column not followed by the same superscript letter are significantly different ($p \leq 0.05$).*
Control[1] = 100% egg proteins; CPI[2]= cowpea protein isolate; DCPI[3]= denatured cowpea protein isolate GCPI[4]= glycated cowpea protein isolate.

The highest force values for both control and GCPI mayonnaise were found to be 212 and 198 g respectively. The firmness of mayonnaise produced with 40% replacement of GCPI proteins for egg proteins was reduced but still better than that of both CPI and DCPI which had declined more significantly.

The sensory evaluation of mayonnaise produced with replacement of cowpea proteins is presented in **Table 5.6**. Sensory evaluation was performed after 24 hours of production to evaluate overall acceptability of the products. Overall acceptance was evaluated using a 1-9 hedonic scale in terms of colour, flavour and texture. Moreover, the panellists were asked to give comments on the flavour of the products. The overall acceptability of the mayonnaise produced from CPI, DCPI and GCPI was similar at the level of 20% replacement of egg yolk with cowpea proteins. The cowpea flavour was not detectable at this level. However, with substitution at a level of 40%, the cowpea flavour of the mayonnaise was generally detected.

The major negative observation related to the oily, coarse mouth-coating and the watery texture. When the emulsifying characteristics of cowpea proteins were investigated, the proteins played a role as an emulsifier in typical oil/water systems, where the

hydrophilicity of the proteins is more required. Moreover, in the preparation of mayonnaise, the content of oil is very high as well and the lipophilicity of the emulsifier is mostly necessary. The results of the functional investigations demonstrated that cowpea proteins showed high solubility but low fat-binding abilities. This shows that they might lack sufficient hydrophobic groups so that they could not well produce amphiphilic functions in very high-fat foods such as mayonnaise.

Table 5.6 Sensory evaluation of mayonnaise

	Overall acceptability	Cowpea protein taste
Control[1]	7.0 ±0.11	-
CPI[2]		
20%	6.0 ±0.10	-
40%	5.1 ±0.08	++
DCPI[3]		
20%	6.2 ±0.07	-
40%	5.4 ±0.12	+
GCPI[4]		
20%	6.6 ±0.09	-
40%	5.8 ±0.07	+

** Mean ± standard deviation of three replicates. Control[1] = 100% egg proteins; CPI[2]= cowpea protein isolate; DCPI[3]= denatured cowpea protein isolate GCPI[4]= glycated cowpea protein isolate.*

*** Sensory evaluation scores for overall acceptability, 1 = disliked extremely, 2 = disliked very much, 3 = disliked, 4 = disliked mildly, 5 = neither liked nor disliked, 6 = liked mildly, 7 = liked, 8 = liked very much and 9 = liked extremely.*

5.3.4 Effect of cowpea protein isolate on starch pasting behaviour

The overall aim was to investigate if interactions between rice starch and cowpea proteins (CPI and GCPI) do occur during heat treatment and to identify the mechanisms underlying these interactions. Rice starch contains very little protein and would be a suitable ingredient for this study.

The effect of cowpea protein isolate on starch pasting behaviour was determined as described in chapter 2 (section 2.2.31). CPI/rice starch mixture solution (10 g starch,

2 g CPI and 100 mL H_2O), GCPI/ rice starch (10 g starch, 2 g GCPI and 100 mL H_2O) and rice starch solution alone (10 g starch and 100 mL H_2O) samples were measured on a Bohlin Gemini rheometer (Malvern Instruments Limited, Worcestershire, U.K.). Samples during testing, were equilibrated at 25°C and then heated from 25°C to 95° at a rate of 2°C/min and under constant shear rate at 100 rev/min, keeping the temperature at 95°C for 10 min and 40 sec, and cooling down to 25°C at the same rate as the heating (2°C/min) and under the same constant shear rate at 100 rev/min. The oscillation frequency was 1 Hz. The strain applied was 0.01. Thus, from the pasting behaviour of protein/ rice starch systems, i.e., curves of viscosities as function of temperature, key pasting parameters were measured as shown in **Figure 2.4** (chapter 2).

The results of the viscosity changes for the cowpea proteins on their own during the pasting experiment are plotted in **Figure 5.7**, where it can be obviously seen that the viscosities of the CPI and GCPI solutions increased with increasing temperature due to denaturation and aggregation of cowpea proteins.

The different pasting results for the addition of CPI and GCPI to rice starch are plotted in **Figure 5.7**. The key parameters extracted from curves in this Figure are reported in **Table 5.7**. These results clearly demonstrate that both CPI and GCPI affected differently and markedly the pasting behaviour of rice starch.

The addition of CPI decreased the T_{onset} and the T_{peak} of rice starch/CPI mixture. The peak viscosity was clearly decreased. On the other hand, GCPI demonstrated a higher T_{onset} and T_{peak} with higher peak and final viscosity than that of CPI.
Glycation delayed the pasting of rice starch, shifting the curves to higher temperature. This could be of important application in UHT (ultra high temperature) treatment of sauces in the food industry where delayed gelation of starch is beneficial.

Ribotta and Rosell (2010) who studied the effect of soy protein isolate on corn cassava gelatinization reported that addition of soy protein isolate to cassava starch led to increase of pasting temperature of the mixture of soy protein isolate/cassava starch.

Lim and Narsimhan (2006) reported that soy protein isolates increased overall viscosity and pasting temperature of soy protein/starch/ fructose corn syrup mixtures.

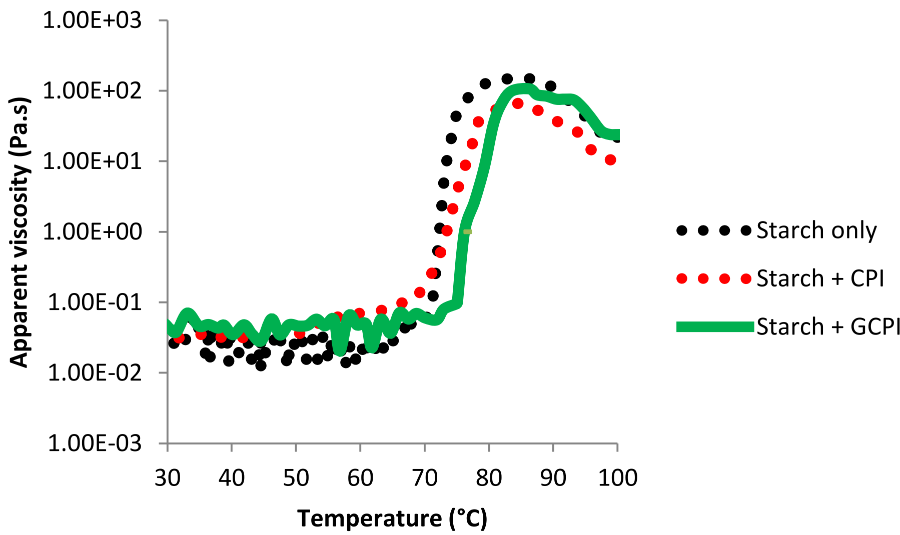


Figure 5.7 Apparent viscosities as a function of temperature for rice starch with cowpea protein isolate. Starch only = rice starch solution alone (10 g starch and 100 mL H_2O). Starch + CPI = CPI/rice starch mixture solution (10 g starch, 2 g CPI and 100 mL H_2O); Starch + GCPI = GCPI/ rice starch (10 g starch, 2 g GCPI and 100 mL H_2O).

GCPI increased the T_{onset} and T_{peak} when added to rice starch due to the presence of cowpea's own saccharides in GCPI as saccharides are known for their influence on the pasting and gelation properties of starch (Kelly et al., 1995). Perry and Donald (2002) pointed out that sugars reduce the level of solvent plasticization leading to an increase in the starch gelatinization temperature. The effect of saccharides on starch gelatinization showed different behaviours depending on preparation methods and type of saccharides and starch concentration.

Saccharide effects on starch swelling and gelatinization have been widely reported by researchers such as decreasing the swelling of starch granules or increasing gelatinization temperatures of starches (Hyang Aee et al., 1998; Chinachoti et al., 1990; Maaurf et al., 2001; Baek et al., 2004). Functionality is important for starches in a wide range of food applications. Starch provides texture to many foods such as pasteurised custard that requires UHT (Ultra high temperature) and provides a consistent shelf-stable product that consumers rely upon (Satin, 2000).

Table 5.7 The pasting parameters* for rice starch/water mixtures and rice starch/cowpea protein mixtures.

Protein	T_{onset} (°C)	T_{peak} (°C)	Peak viscosity (Pa.s)	Final viscosity (Pa.s)
None[1]	67.5 ±0.01	84.4 ±0.10	151 ±2.10	25.7 ±0.33
CPI[2]	66.1 ±0.11	85.3 ±0.20	67.8 ±1.80	12.1 ±0.21
GCPI[3]	73.0 ±0.14	86.3 ±0.13	33.60 ±0.24	2.13 ±0.01

* *Mean ± standard deviation of three replicates.* None[1] *= rice starch solution alone (10 g starch and 100 mL H_2O).* CPI[2] *= CPI/rice starch mixture solution (10 g starch, 2 g CPI and 100 mL H_2O);* GCPI[3] *= GCPI/ rice starch (10 g starch, 2 g GCPI and 100 mL H_2O).*

Kohyama and Nishinari (1991) reported that most saccharides exhibit an anti-plasticizing effect and reduce the amount of amylose leaching. The saccharides generally increase the gelatinization temperature of starch because of the retardation of the swelling of granules (Kohyama and Nishinari, 1991; Eliasson, 1992; Hyang Aee et al., 1998; Maaurf et al., 2001; Gonera and Cornillon, 2002; Krüger et al., 2003; Baek et al., 2004) as well as due to structural phase changes of water. It was reported that sucrose decreased the effect of water as a plasticizer (Freidman, 1995).

Rheological measurements of starch paste have been used to characterise the effect of sugars on starch behaviour. Prokopowich and Biliaderis (1995) reported that the addition of sugars such as ribose and sucrose to starch-sugar-water mixtures at a ratio of 1:0.5:1.5 (w/w), inhibited chain reorganization in gels, as followed by dynamic rheometry and differential scanning calorimetry in the order, ribose > sucrose > water alone.

Katsuta et al. (1992) reported that disaccharides more effectively compete for available water than monosaccharides and inhibit starch swelling. Sugars stabilize the firm gel structure through the formation of sugar bridges between amorphous regions and melted crystalline regions.

5.4 Conclusions

In the present study, the functional properties of thermally modified cowpea proteins have been extensively evaluated. Generally, glycated cowpea proteins isolate (GCPI) exhibits good functional properties, better than DCPI and CPI.

The control bread samples (wheat flour only) and bread samples containing 5% cowpea flour had almost similar properties, while bread made with 20% cowpea flour gave lower overall acceptability. To conclude, I can suggest that the fortification of bread with 20% cowpea flour produced a high protein product. This could be used as protein-rich food for the relief of malnutrition in poor countries. The protein content of wheat/cowpea composite breads ranged from 6.1 – 9.9%. Water and oil absorption capacities of the flour blends increased with increasing cowpea flour contents.

The addition of CPI in wheat flour at the levels of 2, 4 and 6% led to a decrease in water absorption of dough and decrease in the loaf volume of bread. This study has shown that CPI could be incorporated in bread to 2% in order to enhance nutritional quality without affecting the sensory attributes of the product.
GCPI led to increase in water absorption and decrease in the loaf volume of bread. Apart from decreased volume, the glycated cowpea protein isolate at the level up to 4% did not adversely affect the bread physical properties as the crumb hardness value did not significantly differ from that of the control. The sensory attributes were also unaffected.

This concept can be further developed in the bakery industry. The target consumers would be health conscious people who seek the benefits of high protein bread.

In case of cakes and mayonnaise, flavour problems or bean flavour resulting from cowpea protein ingredients were more evident in mayonnaise than in cakes. Baking temperature of cake probably minimised the influence of volatile odour components in the cowpea proteins. CPI could be incorporated in cakes to 20% while both DCPI and GCPI could be incorporated in cakes to 40%. As a result, cowpea proteins offer good potential as a new ingredient in cakes. On the other hand, although cowpea proteins resulted in beany off-flavour in mayonnaise, they still resulted in stable emulsions.

GCPI replacement of egg yolk (20%) resulted in firmer texture than CPI and DCPI which was not significantly different compared to the control (**Table 5.5).**

Table 5.8 shows the improvement of functionality of cowpea protein in food applications. The softness of bread crumb was improved by GCPI more than by DCPI. The softness of cake was improved by GCPI more than by DCPI. In the case of firmness of the mayonnaise, with 20% replacement of glycated cowpea proteins for egg proteins, GCPI gave a value comparable to the control, whereas values were lower for both DCPI and CPI (**Table 5.5**).

Table 5.8 Improvement of functionality of cowpea protein in food applications

food applications	**CPI**	**DCPI**	**GCPI**
Bread			
Crumb softness	+	++	+++
Cake			
Softness	+	++	+++
Mayonnaise			
Firmness	+	++	+++

CPI= cowpea protein isolate; DCPI = denatured cowpea protein isolate; GCPI = glycated cowpea protein isolate

The effect of cowpea protein on pasting behaviour of rice starch was investigated. The addition of GCPI inhibited gelation of rice starch significantly more than CPI.

Chapter Six

General conclusions and recommendations for future study

6.1 General Conclusions

6.1.1 Summary of the results

Cowpea (*Vigna unguiculats L*) protein isolates, which offer potential as a protein source for food applications, have been studied in the present research. Three major parts were included and the results are reported. The first phase of the program involved the extraction conditions of cowpea proteins, which provided the positive information for the production of the protein isolates. The protein isolate was further extensively characterised in terms of proximate composition, amino acid analysis, gel electrophoresis, and functional properties in comparison to commercial soy protein isolate. The second phase involved the modification of cowpea proteins via two procedures, glycation via the Maillard reaction and thermal denaturation in the wet state. Based on the two protein isolates obtained, the third part of the research covered the evaluation of the applications of the proteins in model food systems.

6.1.2 Extraction and characterisation of cowpea proteins

Cowpea proteins have been extracted from cowpea seed flour with dH_2O (distilled water) and recovered by isoelectric precipitation. The use of alkaline solution at pH 10 with extraction ratio of 1:10 and extraction temperature of 55°C is recommended in terms of the highest yield. The extraction of cowpea protein isolate by this method which resulted in 89% yield and 90% protein content is a feasible way to obtain the cowpea protein isolates and this would be also feasible for upscaling to industrial scale. This is the first description of the identification of optimum protein extraction conditions where yield and content are high. The use of alkaline solution (pH 10) is an economic method for extraction of the proteins because it gave the highest recovery. Cowpea protein isolate and soy protein isolate presented approximately similar amino acid profiles. Similar to other legumes, the amino acids containing sulphur in cowpea protein are limited. When cowpea protein isolates were further characterised using gel electrophoresis, cowpea protein isolate, and soy protein isolate contained similar bands. Generally cowpea protein isolate was found to possess some favourable functional properties such as relatively high solubility and foam capacity when compared to soy protein isolate. Therefore, it can be concluded that:

- CPI had significantly (at least 20%) better solubility than SPI at pH 6-9.

- CPI had similar viscosity at 60°C to SPI at the same protein concentration.
- CPI and SPI had similar denaturation temperature; there was no significant difference in the denaturation temperature.
- CPI had similar foam capacity to SPI, but lower foam stability.
- CPI had significantly lower WHC than SPI, but similar to WPC 60 and WPC-GOS.
- CPI had significantly lower FAC than SPI, but similar to WPC 60 and WPC-GOS.
- CPI had similar emulsion activity index (EAI) to SPI, but significantly lower emulsion stability index (ESI) than SPI.

The findings of the present study also show that cowpea protein isolate has a good viscosity comparable to soy protein isolate at moderate and high temperatures, which indicates an acceptable range. So, cowpea protein isolate would have similar applications potential to soy protein isolate. It could provide a good viscosity for food products especially infant foods produced at moderate and high temperatures such as infant food formulations.

6.1.3 Modification cowpea proteins

In the present study, the functional characteristics of cowpea protein were greatly improved by thermal denaturation and by glycation via the Maillard reaction. Compared to denaturation, combined denaturation and glycation appears to result in better functional properties of cowpea protein including solubility, emulsifying activity and stability, viscosity, and foam stability. The denaturation alone appears to result in better water holding capacity, oil absorption capacity and gelation properties, indicating that these modifications are suitable to modify this type of protein for many food applications. To conclude, although the water holding capacity of cowpea protein isolate was less than that of soy protein isolate, this property was enhanced by glycation and even more by denaturation. The oil absorption capacity of cowpea protein isolate was enhanced by denaturation more than by glycation which had a comparable effect to that on soy protein isolate. The foaming capacity of cowpea protein isolate was greater than that of soy protein isolate. Both denaturation and glycation enhanced the foaming stability of cowpea protein isolate more than that of the control. The emulsifying activity index which was less than that of soy protein isolate was enhanced by glycation, and the emulsifying index was enhanced by denaturation and even more by glycation.

It was apparent that every functional property of cowpea protein isolate was improved by denaturation and/or by glycation. Hence, selection of products for which the modified protein will optimise its functions is based on the way that cowpea protein isolate has been modified.

6.1.4 Applications of cowpea proteins in model food systems

The functional properties of cowpea proteins have been extensively evaluated. Generally, glycated cowpea proteins isolate exhibited good functional properties. The glycated cowpea protein isolate at the level up to 4% did not adversely affect the bread physical properties of bread as the crumb hardness values did not significantly differ from those of the control. The sensory attributes were also unaffected. This concept can be further developed in the bakery industry. The target consumers would be health conscious people who seek the benefits of high protein bread.

Cowpea proteins offer good potential as a new ingredient in cakes when fortified to 20% for CPI and to 40% for DCPI and GCPI. On the other hand, cowpea proteins still seem to have potential importance in mayonnaise if applied at a lower amount (< 20%) due to their good emulsifying properties. This offers potential to be utilised as a new protein to replace egg proteins. The addition of GCPI inhibited gelation of rice starch more than CPI. This indicates potential application in enhancing starch stability during thermal food processing.

6.2 Recommendations for future study

There are presently no standard methods available for the investigation of the functional properties of proteins including emulsifying properties, protein-water interactions, foaming properties, gelation and viscosity, as well as flow properties. The variations in equipment, preparation and sample size used from laboratory to laboratory make it difficult to compare the previous studies. In future it is necessary to develop methods. These need to be standardised and accepted internationally in order that comparable results can be obtained by different laboratories.

Modified cowpea protein isolates showed a potential to be a fine functional ingredient food protein. Some further studies should be done for the good understanding of its properties. The studies include:

- Functional properties of denatured and glycated cowpea protein as affected by environmental factors such as ionic strength, pH, and thermal treatment.
- Changes in cowpea protein molecules and extent of modification.
- Effect of denaturation and/or glycation on protein nutritional quality.
- Structural properties of food product containing CPI determined by scanning electron microscopy.
- Elaboration of the study of effect of glycation with different oligosaccharides and polysaccharides on enhancing functionality of cowpea protein isolate in different food applications.
- Characterisation of glycated sugars by mass spectrometry.

It is expected that the obtained data can be used for process adjustment and production of modified cowpea protein isolate on a large scale basis.

The results of this thesis should initiate wider studies on the effect of glycation of plant proteins with endogenous sugars in solution. Almost all previous studies on glycation were conducted in the dry state (**Table 1.7**), and not with endogenous sugars or fibres.

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Appendix I Sensory Evaluation of Breads

Product: Pan Bread Date: Name:

I. Colour: Please rate each sample for Overall acceptability (√)

	Very poor						very good		
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

II. **Texture: a. Overall acceptability:** Please (√) (1-9 hedonic scale)

	Very poor						very good		
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

b. Comment: How do you describe the texture of each sample? For example hardness or softness, good or bad coherence, adhesiveness, crumbly, oily mouthcoating, coarseness or finesse (You may not need all of them)

Sample 1	
Sample 2	
Sample 3	
Sample 4	

III. **Flavour: a. Overall acceptability:** Please (√) (1-9 hedonic scale)

	Very poor						very good		
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

b. Comment: How do you describe the flavour of each sample? For example any bean flavour which are not acceptable, or other.

Sample 1	
Sample 2	
Sample 3	
Sample 4	

Appendix II Sensory Evaluation of Cakes

Product: Sponge Cake Date: Name:

I. Colour: Please rate each sample for Overall acceptability (√)

	Very poor						very good		
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

II. **Texture: a. Overall acceptability:** Please (√) (1-9 hedonic scale)

	Very poor						very good		
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

b. Comment: How do you describe the texture of each sample? For example hardness or softness, good or bad coherence, adhesiveness, crumbly, oily mouthcoating, coarseness or finesse (You may not need all of them)

Sample 1	
Sample 2	
Sample 3	
Sample 4	

III. **Flavour: a. Overall acceptability:** Please (√) (1-9 hedonic scale)

	Very poor						very good		
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

b. Comment: How do you describe the flavour of each sample? For example any bean flavour which is not acceptable, fair or strong, poor flavour from egg, or other.

Sample 1	
Sample 2	
Sample 3	
Sample 4	

Appendix III Sensory Evaluation of Mayonnaise

Product: Mayonnaise Date: Name:

I. Colour: Please rate each sample for Overall acceptability (√)

	Very poor						very good		
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

II. **Texture: a. Overall acceptability:** Please (√) (1-9 hedonic scale)

	Very poor						very good		
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

b. Comment: How do you describe the texture of each sample? For example good or bad coherence, adhesiveness, crumbly, oily mouthcoating, coarseness or finesse (You may not need all of them)

Sample 1	
Sample 2	
Sample 3	
Sample 4	

III. **Flavour: a. Overall acceptability:** Please (√) (1-9 hedonic scale)

	Very poor						very good		
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

b. Comment: How do you describe the flavour of each sample? For example any bean flavour which is not acceptable, fair or strong, poor flavour from egg, or other.

Sample 1	
Sample 2	
Sample 3	
Sample 4	

Appendix IV *Decanter (left) and clarifier (right) centrifuge.*

Appendix V *Batch tank.*

***Appendix VI** Spray drier.*