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1. INTRODUCTION

Marama bean (*Tylosema* species) represents a potentially valuable source of leguminous oilseed protein in Southern Africa. The protein content (30-39%) of marama bean (Amarteifio & Moholo, 1998; Holse, Husted & Hansen, 2010) is similar to that of soya beans (Mujoo, Trinh & Ng, 2003) and peanuts (Venkatachalam & Sathe, 2006). Marama is a drought-tolerant legume that grows in the wild in Botswana, Nambia and parts of South Africa (Gauteng, North-West and Limpopo provinces) (Coetzer & Ross, 1976; Castro, Silveira, Coutinho & Figueiredo, 2005). Therefore, it has great potential as an alternative to these other oilseed legumes. However, marama is not yet commercially cultivated or utilised.

Storage proteins from protein rich sources, particularly soya bean have been applied in food systems to improve functionality. Chemical characteristics of the proteins in terms of subunit compositions and hydrophobicity have been found to significantly influence the functional properties of proteins (Nakai, 1983; Utsumi & Kinsella, 1985). Solubility and conformational behaviour of proteins primarily determine the functionality of proteins in food systems (Tolstoguzov, 1993). Marama bean is an indigenous and under-researched legume. As result, there is a very limited scientific knowledge on the characteristics of its storage protein (Ripperger-Suhler, 1983; Maruatona, Duodu & Minnaar, 2010). From the amino acid profile, the tyrosine content of marama bean flour is almost five times higher than that of soya bean flour (Ripperger-Suhler, 1983; Maruatona *et al.,* 2010). Tyrosine is involved in protein crosslinking (Takasaki *et al.,* 2005). The presence of tyrosine residues in high amount in marama protein may lead to structural stability of its protein as suggested for gluten (Tilley, Benjamin, Bagorogoza Okot-Kotber, Prakash & Kwen, 2001). This may be of interest in food applications.

Marama storage protein, like soya bean protein may be applied in food systems to improve functionality. However, to determine the potential use of marama protein as a functional ingredient in food systems, the knowledge of protein composition and structure as well as its functionality is indispensable.

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2. LITERATURE REVIEW

This chapter is divided into three parts. The first part is a review on the marama bean. It mainly discusses the distribution, habitat and the morphological characteristics of the marama bean. The second part provides a review on the seed microstructure and protein composition of legumes. The third part is a review on functionality of legume proteins with respect to thermal and rheological properties and how these properties are influenced by the protein composition.

2.1. Distribution, habitat and morphological characteristics of marama seed

The pods, produced by yellow flowers borne in raceme, are firm with varying sizes and shapes. Morphological characteristics of marama seeds are also dependent on the species. Pollen can be used to identify species within the genus *Tylosema* with 95% reliability using dichotomous keys to species (Castro *et al.,* 2005). Detailed taxonomic classification of the various species within the genus *Tylosema* has been provided by Castro *et al*. (2005). The discussion here is limited to distribution and habitat of the species, pods and seed morphology from the identified species. The distribution of the two most commonly found species (*T. esculentum* and *T. fassoglense*) is shown in Figure 2.1.

Tylosema esculentum (Burch) A. Schreib is mostly found in sandy soil, but can be found in flat grass lands especially in dolomite and limestone outcrops, occurring at 900-1100 m altitude. It is naturally distributed in the northern areas of Namibia, Botswana, Gauteng and Limpopo in South Africa. The flowering period is from October/November to March. The pod is ovate to ovate-oblong, sometimes circular with length and width varying from 1.3-1.8 cm and 1.2-1.5 cm, respectively. The seeds contained in pods are brownish black and very dark. The seeds are ovate to circular, 1.3-1.8 cm long and 1.2-1.5 cm wide. Each seed can weigh between 20-30 g. Synonyms for this specie are *Bauhinia esculenta* (Bruch.), *Burkeana esculenta* (Benth) and *B. bainesii* (Coetzer and Ross, 1976; Castro *et al*., 2005).

Tylosema fassoglense (Schewinf.) Torre & Hillc occurs mainly in eastern and central tropical Africa. It grows in open woodland on red dolomite soil, in forest, and secondary shrub land. *T. fassoglense* sometimes occurs in cultivated areas, on sandy soil, rocky or clay soil, periodically flooded, at 30-2100 m altitude. The flowering

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period begins from October/November to February. The pods are obovate and oblong to obovate, with length and width varying between 5-10 cm long and 3-5 cm wide. The seeds are suborbicular or ellipsoid, 1.5-2.8 cm long and 1-2 cm wide. They are chestnut- brown to blackish. Synonyms for this specie are *Bauhinia fassoglensis*, *B. cissoids*, *B. welwitschii* and *B. kirkii* (Coetzer & Ross, 1976; Keegan & van Staden, 1981; Castro *et al*., 2005).

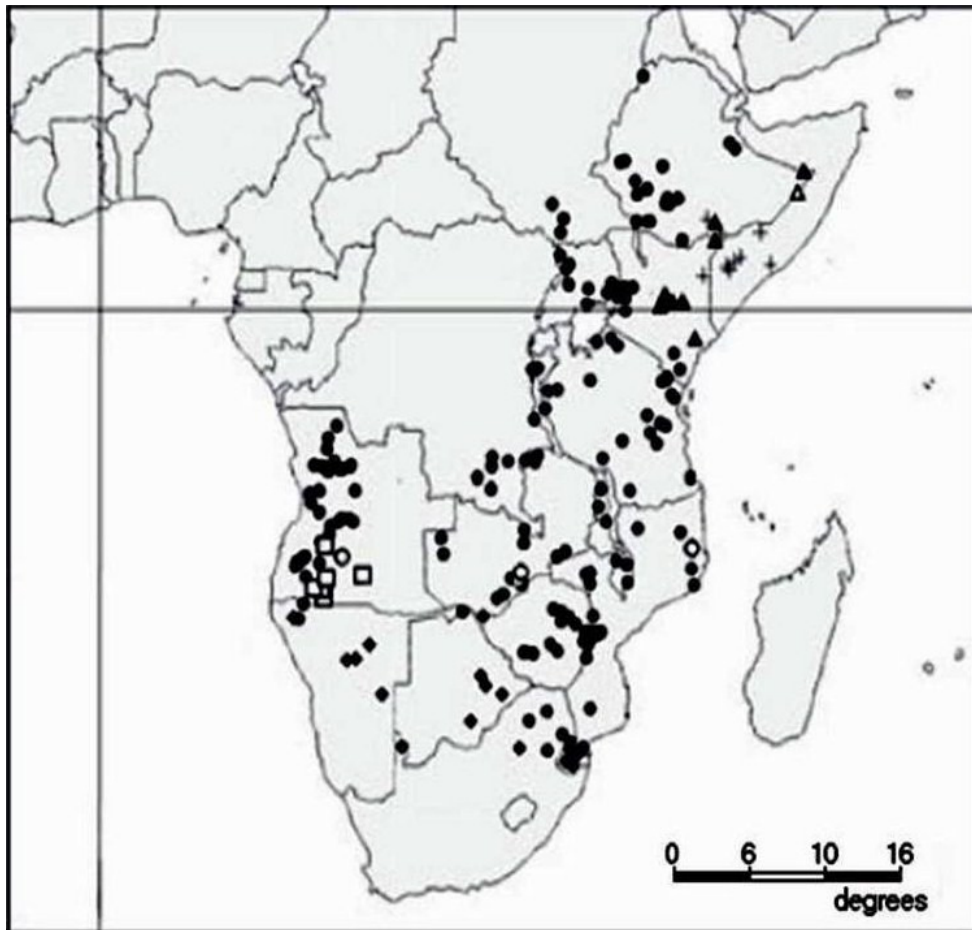

Figure 2.1 Distribution map of *T. esculentum* (◆), *T. fassoglense* (●) (Castro *et al.*, 2005)

Tylosema humifusum (Pic.Serm. & Roti Mich.) Brenan grows in limestone soil and sandy plains with open vegetation of grass and low shrubs, at 400-1000 mm in Somalia and Northern Kenya. The pods are rhombic, 4.0-4.5 cm long and 2.5-3.5 cm wide. They are brownish with small longitudinal and oriented light-brown grooves. The seeds are sub-circular, compressed with length and width of 20 mm and 18 mm respectively (Coetzer & Ross, 1976; Castro *et al*., 2005).

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Tylosema argenteum (Chiov.) Brenan is distributed in Somalia, Southern Ethiopia and Northern Kenya. It grows on riversides or rocky soils of old alluvial slopes, up to 550 m altitude. The flowering period is from September to October. The pods are 6 cm long and 4 cm wide. The seeds from this specie are ovate-circular with length and width of 13 mm and 10 mm, respectively (Coetzer & Ross, 1977; Castro *et al*., 2005).

Tylosema angolense (P. Silveiro & S. Castro sp. nov) is a recently identified specified specie of the genus *Tylosema*. This specie was distinguished from *T. fassoglense* specie within the genus. *T. angolense* occurs in open scrub woodland, on sandy soils and sometimes on ferric soil, at 1150-1670 m altitude in Southern Angola. The flowering period falls between September and February. The pods of about 11 cm long and 6 cm wide are asymmetric, dorsally elliptic, ventrally–rhomboid, reddish during maturation and dark-brown with small longitudinal light-brown grooves when matured. According to Castro *et al.* (2005), the seeds have not been characterised because these were not seen during the period of the study.

Marama seeds (Fig. 2.2) have a very hard outer shell and decortication becomes necessary to access the inner flesh. Information on dimensional characteristics of the seed may be useful for engineering design and process control. More detailed studies on physical properties (e.g. dimensional characteristics, weight of 1000 grains, bulk density, and hardness) should also be conducted.

Figure 2.2 Marama bean seeds (*Tylosema esculentum*)

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2.2. Legume seed microstructure

The microstructure of protein bodies from oilseed legumes including soya bean and peanut has been studied extensively (Lott & Buttrose, 1978). Structurally, the protein bodies of these oilseeds such as soya bean and peanut consist of proteinaceous matrix material surrounded by a network of lipid bodies (Fig. 2.3). Protein bodies may appear oval or circular in cross-section with diameter varying from 2-15 µm (Martinez, 1979; Young *et al.*, 2004).

Although the protein bodies of different oilseed legume species may be similar in terms of their spherical shape, differences have been observed with respect to the presence or absence of crystalline inclusions within the protein bodies. The most common inclusion found within the protein bodies are the globoids, which constitute the storage sites of seed phosphorus deposited as insoluble phytate (Martinez, 1979; Lott, 1981). These globoid inclusions have bean reported in peanuts (Young *et al.*, 2004), castor bean (Tully & Beevers, 1976) and hazelnut (Lott & Buttrose, 1978). A globoid inclusion in peanut is indicated by an arrow in Figure 2.3 (B). Elemental composition analysis of spherical globoids from many legumes showed that they are rich sources of P, K, Ca and Mg (Lott & Buttrose, 1978; Lott, 1981). According to Martinez (1979) soya bean protein bodies do not contain globoids. Prattley and Stanley (1982) investigated the localisation of phytate in soya bean protein bodies, which seems to provide an explanation for the absence of globoids in soya bean. According to these authors, phytic acid in soya bean is mainly in the form of soluble protein-phytate salt. .

The microstructure of dry bean legumes was found to be different from those of oilseed legumes. This difference may be attributed to difference in seed chemical composition in terms of starch and oil contents. Unlike in oilseeds where the protein bodies are surrounded by network of lipid bodies (Lott & Buttrose, 1978; Young *et al.*, 2004), in dry beans such as cowpea, starch granules are embedded within the protein matrix (Fig. 2.4) (Biaszczak *et al.*, 2007; Sefa-Dedeh & Stanley, 1979). Similar observations were made on microstructures of pinto bean (Gujska, Reinhard & Khan, 1994) and native bean (Gujska *et al.*, 1994). The protein body size in cowpeas were reported to range from 2-6 µm (Saio & Monma, 1993), which is smaller than those of oilseed legumes such as soya bean (Martinez, 1979) and peanuts (Young *et al.*, 2004).

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The microstructure of many underutilised indigenous legume seeds including marama bean, *Bauhinia*, mucuna bean, jack bean are not known. Seed microstructures have been found to influence physical properties such as seed hardness (Aguilera & Stanley, 1999) and the purity of extracted protein isolates in terms of residual lipid retention in the isolate (Shand *et al.,* 2007). The physical location of storage protein in the seed may also affect its protein digestibility (Aguilera, 2005). Therefore, information on the protein body structure of marama bean would be required for the development of commercial processing and utilisation of marama bean protein.

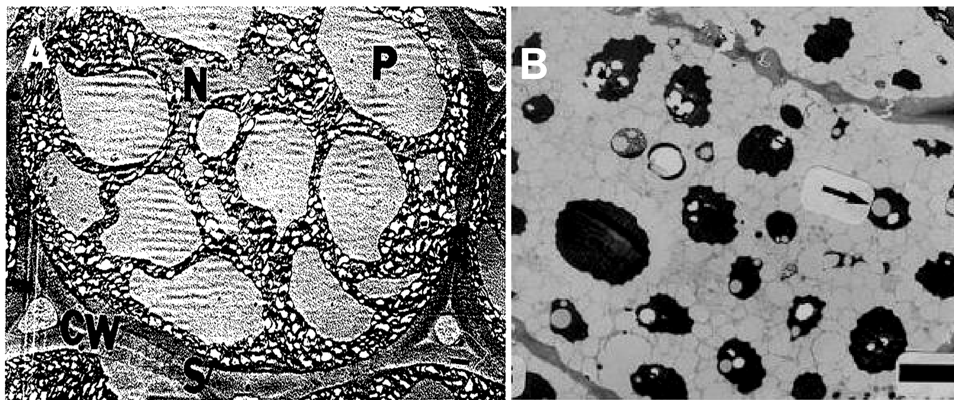

Figure 2.3 Microstructure of soya bean and peanut parenchyma cells

A: Transmission electron micrograph of a typical spongy parenchyma cell from cotyledon of a dry soybean, N-nucleus, S-spherosomes, CW-cell wall, Magnification 8000X-1micron (Martinez, 1979)

B: Transmission electron micrograph of the cross section of mid region parenchyma cells of mature peanut. Crystalline inclusions in protein bodies is indicated by an arrow (Young *et al.,* 2004)

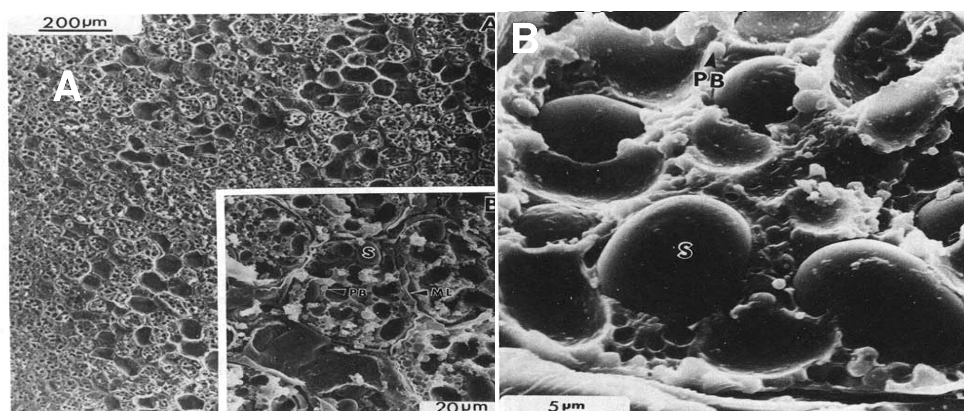

Figure 2.4 Scanning electron microscopy of cowpea parenchyma cells

A: Cross-section of cowpea, variety Adua Ayera, cotyledon sectioned after fixing in 2% glutaraldehyde

B: Cross-section of cowpea, variety Ayera cotyledon cell. S Starch granule, ML: middle lamella, PB: protein bodies (Sefa-Dedeh & Stanley, 1979)

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2.3. Protein composition of legumes

Grain legumes are important sources of plant proteins. Most proteins in legumes are present as storage proteins (Murray, 1979). These proteins are synthesised during seed development and deposited in membrane bound organelles called the protein bodies (Lott, 1981).

Indigenous legumes such as mucuna bean (Adebowale *et al.,* 2005), bambara groundnuts (Yusuf *et al*., 2008) and marama bean (Holse *et al*., 2010) have been found to be good sources of protein similar to soya bean (Mujoo *et al*., 2003) and peanuts (Ventachenka & Sathe, 2006) (Table 2.1). The following protein levels for mucuna beans: 38.6- 41.2% (Adebowale *et al.,* 2005), faba bean: 25-30%; (Mussallam *et al.,* 2004), bambara groundnuts: 20.6% (Yusuf *et al*., 2008); marama bean (*T. esculentum*): 34.1% (Amarteifio & Moholo, 1998); terapy bean: 24% (Idouraine *et al.,* 1998), beach pea legume: 29.2% (Chavan *et al.,* 2001), and lupin: 33.8% (Lqari *et al*., 2002) have been reported. When comparing the two types of legumes, oilseed legumes such as marama bean (Amarteifio & Moholo, 1998; Holse *et al.,* 2010), lupin (Lqari *et al.,* 2002) and *Bauhinia purperea* (Vijayakumari *et al.,* 1997) contain higher protein levels than most of the dry bean legumes like cowpea (Nwokolo & Oji, 1985), faba bean (Mussallam *et al.,* 2004), bambara groundnuts (Yusuf *et al*., 2008) and African yam bean (Eromosele *et al.,* 2008). Reported protein contents for marama bean and lupin were also high compared with those of sunflower (20-28%) (Dulau & Thebaudin, 1998) and some edible nuts seeds like macadamia (8.5%) and hazelnuts (14.6%) (Venkatachalam & Sathe, 2006). The variations in chemical composition of legumes may be attributed to differences in genotypes, environmental conditions and agricultural practices, as described by Young *et al.* (1974) and Salunkhe *et al*. (1985).

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Table 2.1 Chemical composition of some legumes[1]

[2]Legume types	Species	Protein	Fat	Fibre	Ash	CHO
Marama bean	*Tylosema esculentum*[d]	34.1	33.5	4.4	3.7	24.1
Mucuna bean	*Mucuna purien* [a]	41.2	9.8	1.9	2.1	43.7
Bambara groundnuts	*Vigna subterrenea*[b]	18.3	6.6	5.2	4.4	63.5
Terapy beans	*Phaseolus acutifolius*[d]	24.74	0.9	4.9	3.8	63.8
Mung beans	*Phaseolus aureus*[b]	22.3	1.1	4.8	ND	68.3
Lima beans	*Phaseolus lunatus*[b]	22.5	1.3	4.5	3.9	66.9*
Pigeon pea	*Cajanus cajan*[b]	19.4	3.2	5.5	4.1	57.2
Beach pea	*Larthyrus maritimus*[f]	29.2	1.1	12.0	3.0	57.0
Bauhinia	*Bauhinia purpurea* [g]	27.1	12.4	5.9	2.9	51.5
Cowpea	*Vigna unguiculata*[h]	22.5	1.60	5.3	3.8	56.9*
Soya bean	*Glycine max*[i]	35.9	24.9	5.0	6.6	26.8
Peanuts	*Arachis hypogaea*[j]	26.5	40.83	ND	2.8	25.4

[1] Values are reported in % dry basis, ND: Not determined, *Nitrogen free extracts, CHO: carbohydrates
[2]References: [a]Adebowale *et al*. (2005); [b]Yusuf *et al*. (2008); [d]Amarteifio & Moholo (1998); [f]Chavan *et al*. (1999); [g]Vijayakumari *et al*. (1997); [h]Nwokolo & Oji (1985); [i]Ologhobo & Fetuga (1984); [j]Onyeike & Acheru (2002)

2.3.1. Amino acid compositions

Glutamic and aspartic acid which may include glutamine and asparagine, respectively have been found to be major amino acids in most legume proteins. These amino acids account for 25-40% of the total protein in the seeds (Table 2.2) (Ventachenka & Sathe, 2006; Adebowale *et al.,* 2007; Lisiewska *et al.,* 2007).

The lysine content of marama (6 g/100 g protein) (Maruatona *et al*., 2010) is similar to that of pigeon and kidney bean (Apata & Ologhobo, 1994) and *Bauhinia spp*. (Vijayakumari *et al*., 1997). Based on reference data from FAO/WHO (1989), indigenous legumes such as marama bean (Maruatona *et al*., 2010), mucuna bean (Adebowale *et al.,* 2007) and bambara groundnuts (Vijayakumari *et al.,* 1997) are adequate sources of lysine, similar to soya bean (Bau *et al.,* 1994). These indigenous legumes including the marama bean thus offer some potential in food formulation where they could be used to complement protein from cereals, which are known to be deficient in lysine.

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Differences in some individual amino acids have been reported among legumes. Apata and Ologhobo (1994) reported significant differences in individual amino acids among bambara groundnuts, pigeon pea, jack bean and lima bean. According to these authors, jack bean protein had higher methionine content than bambara groundults, pigeon pea and lima bean. Pigeon pea was reporte to be deficient in valine and isoleucine (Ene-Obong & Carnovale, 1992).

Arginine has been reported in high amounts in legumes such as lupin (Lqari *et al.,* 2002) and faba bean (Lisiewska *et al.,* 2007) compared with soya bean (Bau *et al.,* 1994) and cowpeas (Ene-Obong & Carnovale, 1992). The arginine contents of lupin and faba bean were similar to that reported in peanuts (Ventachenka & Sathe, 2006). Green and Oram (1983) also reported similarly high arginine contents in three varieties of *Lupinus albus*. The beneficial effects of arginine in lowering cardiovascular disease, reduction oxidative load cells and possible protection against inflammation have been demonstrated (Wells *et al.,* 2005). Underutilised indigenous legumes thus possess some potential applications in nutraceuticals as described by Bhat and Karim (2009).

The tyrosine contents in marama bean species are high (Dubois *et al.*, 1995; Maruatona *et al.,* 2010) compared with most legume proteins. The tyrosine in marama protein has been reported to be almost five times that of soya bean (Ripperger-Suhler, 1983; Maruatona *et al*., 2010). High tyrosine contents have also been reported in three cultivars of *Lupinus albus* (Green & Oram, 1985) and *Mucuna utilis* (Mohan & Janardhanan, 1995) compared with soya bean. However, the levels of tyrosine in these legumes are lower than those reported for marama bean.

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Table 2.2 Amino acid composition of some legume proteins[1]

[2]Legume types	Glu	Asp	Lys	Arg	His	Ala	Ile	Leu	Met	Phe	Pro	Val	Trp	Gly	Ser	Thr	Tyr	Cys
Marama bean[a]	13.3	9.5	4.1	4.5	1.9	4.8	3.3	5.5	0.7	3.3	8	4.3	ND	9.7	7.0	3.4	7.7	0.4
Lupine [b]	22.7	10.2	4.8	11.9	2.3	2.8	4.8	8	0.7	4.2	4.3		ND	3.5	5.4	3.7	4.9	1.7
Bauhinia purpurea[c]	14.5	9.6	5.6	4.8	3.4	5.2	5.3	6.8	1.4	5.1	ND	4.8	ND	4.6	5.6	4.0	2.7	0.7
Beach pea[d]	13.1	12.4	7.7	7.9	2.6	4.3	4.1	7.7	1.1	4.7	4.2	4.8	0.3	4.2	5	4.3	3.3	1.6
Bambara groundnuts[e]	16.6	11.4	6.8	7.2	2.9	4.7	3.9	7.6	1.3	5.3	4.2	4.8	1.2	3.8	5.2	3.7	3.4	1.4
Jack bean[e]	14.8	13.5	6.6	6.9	4.6	4.9	5.1	9.1	1.5	5.9	4.1	5.8	1.0	4.6	7	4.4	4.2	0.9
Mucuna bean[f]	14.0	14.2	5.9	7.1	3.0	4.2	8	6.4	0.3	4.1	3.5	4.2	ND	6.7	4.4	4.6	5.2	1.6
Faba bean[g]	16.4	11.9	6.4	11.8	2.7	4.4	4.4	7.6	0.9	4.2	4.1	4.8	ND	4.1	4.6	3.3	2.8	1.3
Africa yam bean[h]	15.3	11.4	7.7	5.2	3.6	4.4	4.6	7.7	1.2	5.8	4.9	5.3	1.0	4.4	5.8	3.7	4.2	1.4
Pigeon pea[e]	16.5	11.0	5.9	7.2	3.2	5.5	4.8	7.4	1.4	8.1	5.0	5.2	ND	3.9	4.4	3.8	3.9	1.1
Lima bean[e]	15.9	13.1	5.9	6.2	3	6.2	5.3	8.4	1.2	6.4	5.4	6.1	1.0	5.1	6.8	4.4	4.0	0.9
Soya bean [i]	16.9	11.4	6.1	7.1	2.5	4.2	4.6	7.7	1.2	4.8	4.9	4.6	ND	4	5.7	3.8	1.2	1.7
Peanuts [j]	21.1	12.1	3.8	11.0	2.5	4.6	3.5	7.0	1.3	5.4	5.8	3.9	0.7	6.4	4.8	2.2	3.4	0.3
Cowpea[h]	18.9	12.2	6.9	6.8	2.5	4.4	4.6	7.7	1.2	5.7	3.9	5.4	1.1	4.1	5.5	3.8	3.2	1.0
[3]FAO/WHO			5.8		1.9		2.8	6.6	1.7	6.3		3.5				3.4		

[1]Amino acid values are expressed in g/100 g protein. Recalculation has been made where necessary. ND: Not determined
[2] References: [a]Mmonatau (2005); [b]Green & Oram (1983); [c]Vijayakumari *et al.,* (1997); [d]Chavan *et al.* (1999); [e]Apata & Ologhodo (1994) ; [f]Mohan & Janardhanan (1995) ; [g]Lisiewska *et al.* (2007); [h]Ene-Obong & Carnovale (1992); [i]Bau *et al.* (1994); [j]Venkatachalam & Sathe (2006)
[3] FAO/WHO recommended pattern for pre-school children age: 2-5 years

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The presence of high levels of tyrosine in underutilised legumes such as marama and lupin may influence the functionality of their proteins. Tyrosine may be involved in protein crosslinking to form either the biphenyl or ether types of tyrosine crosslinks (Fig. 2.5). Natural occurring dityrosine has been reported for structural and globular proteins (Stewart *et al.*, 1997; Ushijiama *et al.,* 1984). Rodriguez-Mateos *et al*. (2006) quantified dityrosine formation in flour and dough of six commercial types of wheat at various stages of the baking process. These authors found that dityrosine crosslinks were formed during mixing and baking due to the presence of endogenous peroxidase in flour. Dityrosine and isodityrosine have been found to occur in wheat dough and these seem to contribute to increased elasticity of the dough (Tilley *et al.,* 2001). Although, the formation of dityrosine crosslinks has been reported for wheat, the contribution of this type of crosslink to the overall viscoelastic properties of wheat dough is still subject to debate. Pĕna *et al*. (2006) reported that less than 0.1% of the tyrosine residue of wheat protein took part in crosslinking and therefore concluded that dityrosine crosslinks do not play major role in structure of wheat protein. Rodriguez-Mateos *et al.* (2006) found that there was no correlation between gluten yield of six types of wheat and their typical dityrosine concentrations and suggested that dityrosine was not a determinant factor in gluten formation.

Based on literature, it is evident that dityrosine crosslinks do occur in tyrosine-rich proteins. The formation of dityrosine crosslinks may play a major role in marama protein structure due to the fact that tyrosine residues are present in significantly high amount in this legume compared with other legumes.

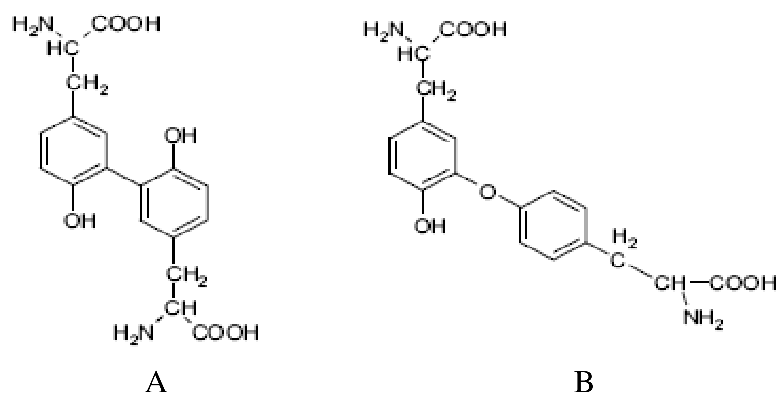

Figure 2.5 Chemical structures of dityrosine (A) and isodityrosine (B) (Joye *et al.,* 2009)

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Based on side chain properties, some differences in the proportion of hydrophobic amino acids and the relative ratios of acidic to basic amino acids have been observed (Table 2.3). Defatted marama flours from preheated and unheated beans have been found to contain high proportion of non polar amino acid compared with those of soya bean (Maruatona *et al.,* 2010). Marama bean also seems to contain high content of unpolar charged amino acids compared with legumes such as bambara groundnuts (Apata & Ologhodo, 1994), African yam bean (Ene-Obong & Carnovale, 1992) and lupine (Green & Oram, 1983) as well as soya bean (Bau *et al.,* 1994) and peanuts (Venkatachalam & Sathe, 2006). The properties of proteins are determined by the side chain characteristics of amino acids. The relative ratio of acidic to basic amino acids would determine the net charge of protein at the surface of the protein molecule, which in turn has been found to influence its solubility (Aluko & Yada, 1995; Utsumi & Kinsella, 1985).

Table 2.3 Amino acid distribution based on side chain characteristics in some legume proteins[1]

	Classification				
Legume types	Acidic	Basic	Hydrophobic	Uncharged polar	Total
Marama	22.8	10.5	29.9	28.2	91.4
Lupine	32.9	19.0	24.8	19.2	95.9
Bauhinia	24.1	13.8	28.6	17.6	84.1
Beach pea	25.5	18.2	31.2	18.4	93.3
Bambara groudnuts	28.0	16.9	33.0	17.5	95.4
Jack bean	28.3	18.1	32.4	21.1	99.9
Mucuna	28.2	16.0	30.7	22.5	97.4
Faba bean	28.3	20.9	30.4	16.1	95.7
African Yam Bean	26.7	16.5	34.9	19.5	97.6
Pigeon pea	27.5	16.3	37.4	17.1	98.3
Lima bean	29.0	15.1	32.0	21.2	97.3
Soya	28.3	15.7	32.0	16.4	92.4
Peanuts	33.2	17.3	32.2	17.1	99.8
Cowpea	31.1	16.2	34.0	17.6	98.9

[1] Values (expressed in g/100 g protein) were calculated from the amino acid data from Table 2.2

Classification was done according to Tang *et al.* (2008). Acidic (glutamic acid/glutamine, Aspartic acid /asparagine), Basic (lysine, arginine, histidine), Hydrophobic (Alanine, Isoleucine, leucine, methionine, phenylalanine, proline, valine); Uncharged polar (glycine, serine, threonine, tyrosine, cysteine/cystine)

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2.3.2. Protein types

Legume proteins have been classified as albumins (water soluble), globulins (salt soluble), prolamins (alchohol soluble) and glutelins (alkali/acid soluble) (Osborne, 1924). Although, globulins constitute the major storage protein of most legumes (Table 2.4) (Mohan & Janaharnan, 1995; Lqari *et al.*, 2002; Chavan *et al.,* 2001), some storage proteins especially among the dry beans have been found to contain albumins as their major protein fraction (Adebowale *et al.,* 2007; Idouraine *et al.,* 1994). Water-soluble albumin has been reported as the major storage protein fraction in mucuna bean species, followed by the glutelin (Adebowale *et al.,* 2007). The albumin was found to constitute 83% of the storage protein in terapy bean (Idouraine *et al.,* 1994), 73% in adzuki bean (*Vigna angularis* var Takara) and 53% in faba bean (Rahman, 1988). The navy bean showed similar proportions of albumin and globulin (approx. 35%). Albumin and globulin were found to be the predominant protein fractions in two species of *Bauhinia* including *B. purpera* and *B. vahlii,* whereas glutelin was predominant in *B. race* (Rajaram & Janardhanan, 1991). The high proportion of water-soluble albumin mainly in dry bean implies that a less complex procedure would be required for their protein extraction.

Table 2.4 Protein fractions of some legume species[1]

Legume types	Species[2]	Albumin	Globulin	Prolamin	Glutelin
Mucuna bean	*Mucuna puriens* [a]	67.0	10.1	0.12	22.1
	Mucuna cochinchinensis[a]	68.5	11.4		20.1
	Mucuna cochinchinensis[b]	13.83	58.30	7.86	19.58
	Mucuna monosperma [c]	20.7	68.6	3.3	1.5
Bambara groundnuts	*Vigna subterranae*[d]		75		
Faba bean	*Vicia faba*[e]	53.1	20.4	7.4	8.1
Terapy bean	*Phaseolus acutifolius*[f]	83.2	13.7	0.5	0.5
Beach pea	*Lathyrus maritimus*[g]	13.8	61.9	2.87	18.9
Navy bean	*Phaseolus vulgaris*[h]	36.65	35.06	0.08	0.28
Moth bean	*Vigna acontiffoli*[i]	5.06	63.93	3.17	27.83
Soya bean	*Gycine max* [j]	10	90		
Peanuts	*Arachis hypogea*[k]		73		
Cowpea	*Vigna unguiculata*[l]	24.5	53.0	1.3	21.3

[1]Values are expressed in % protein
[2]References: [a]Adebowale *et al.* (2007), [b]Ezeagu & Gowda (2006); [c]Mohan & Janadharanan (1995); [d] Odeigah & Osanyinpeju (1998); [e]Rahman (1988); [f]Idouraine *et al.* (1994); [g]Chavan *et al.* (2001); [h]Gujska & Khan (1991); [i]Sathe & Venkatachalam (2007); [j]Boulter (1977); [k]Yamada *et al.* (1979); [l]Vasconcelos *et al.* (2010)

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2.3.2.1 Globulins

Storage globulins are often composed of two major fractions, namely 7S (vicilin) and 11S (legumin). Variations in contents between the vicilin-like (7S) and legumin-like (11S) fractions of legume globulins have been reported (Meng & Ma, 2001; Adebowale *et al.*, 2007; Horax *et al.*, 2004). Both 7S and 11S have been found to be major storage protein fractions of lima bean (Chel-Guerrero *et al.,* 2007), faba bean (Kimura *et al.,* 2008) and lupin (Duranti *et al.,* 1981), similar to soya bean (Kinsella, 1979). On the other hand, the 7S globulins have been found to be abundant in mucuna bean (Adebowale *et al.*, 2007), terapy bean (Idouraine *et al.,* 1994), and red bean (*Phaseolus angularis*) (Meng & Ma, 2001), similar to cowpea (Horax *et al*., 2004). Using Size Exclusion High Performance Liquid Chromatography (SE-HPLC) and analytical ultracentrifugation, Rahman *et al.* (2000) demonstrated that 7S globulin was the major protein component in mung bean. Chavan *et al.* (2001) reported 7S as major and 11S as minor in beach pea legume protein. Protein extraction using the traditional alkaline extraction/isoelectric precipitation and micellisation, did not affect the 7S content of mung bean (Rahman *et al*., 2000). Vicilin-like proteins have been also found to be the major storage protein fraction in bambara groundnuts and pigeon pea (Benjakul *et al.,* 2000).

In addition to the two protein fractions associated with globulins, another 7S globulin designated as basic 7S has been reported in soya bean (Yamauchi, Sato & Yamagishi, 1984; Omi *et al.,* 1996). This protein is called basic 7S because of its basic isoelectric point in the pH range 9.05–9.26 (Yamauchi *et al.,* 1984) and has been found to account between 0.5-3% of the total seed protein (Omi *et al.,* 1996; Yamauchi, *et al*., 1984). The basic 7S globulin protein fraction has been found to be immunologically distinguished from 11S and 7S globulin (Sato *et al.,* 1987; Omi *et al.,* 1996). The basic 7S globulin (Bg) of soya bean, which was released by pressurisation has been found to be localized in the dermal tissue of seed cotyledons and to consist of 16 and 27 kDa subunits (Omi *et al*., 1996). Although the basic 7S globulin constitutes a minor component of seed protein, it was found to be a sulphur-rich protein and could be heat coagulated to form soluble and insoluble aggregates (Sathe *et al*., 1989; Yamuachi *et al.,* 1994).

The variation in contents between 7S and 11S of legume globulins may be attributed to differences in genotypes. The relative amount of 7S and 11S protein fractions may

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influence the functionality of proteins. For instance, Arrese *et al*. (1991) reported that the gelation characteristic of soya protein was related to the amount of 7S and 11S. High ratios of 11S to 7S in proteins led to increased textural properties of hardness, cohesiveness, and gumminess of gels (Tay & Perera, 2004). The globulin of some legumes has further been characterised to gain understanding into its functionality in food systems.

2.3.2.2 Subunit composition

One-dimensional SDS-PAGE has been employed to determine the protein profile of legume globulins. Differences have been reported in terms of number and molecular weight of constituent polypeptide subunits of legume proteins such as bambara groundnuts (Odeigah & Osanyinpeju, 1998), faba bean (Ghandorah & El-shawaf, 1993) and terapy bean (Idouraine *et al.,* 1994). Terapy bean protein extracts showed major polypeptide bands at 29, 45 and 49 kDa (Idouraine *et al.,* 1994). The globulin fraction of moth bean (*Vigna aconfitifolia* L.) protein was composed of three major polypeptides with an estimated mass of 45-55 kDa and several additional polypeptides in the range 14-32 kDa (Sathe & Venkatakchalam, 2007). Lupin (*Lupinus albus*) globulins analysed by ion exchange chromatography, gel filtration, and cellulose acetate electrophoresis revealed eight fractions, all with acidic character (Cerletti *et al.,* 1997). The seed globulin of alupin specie has been found to contain four subunits corresponding to the vicilin and two corresponding to the legumin (Duranti *et al.,* 1981).

Differences in structures and subunit composition of the two protein fractions (7S and 11S) of legume globulin have been reported. The 11S legume proteins are oligomeric, consisting of six acidic (α) and six basic (β) subunits disulphide-bonded as αβ pairs (Derbyshire, Wright & Boulter, 1976). The nomenclature of the storage soya bean globulin is shown in Figure 2.6. Many studies have confirmed the oligomeric structure of 11S protein in soya bean (Kinsella, 1979; Mujoo *et al.*, 2008; Liu *et al.*, 2007).

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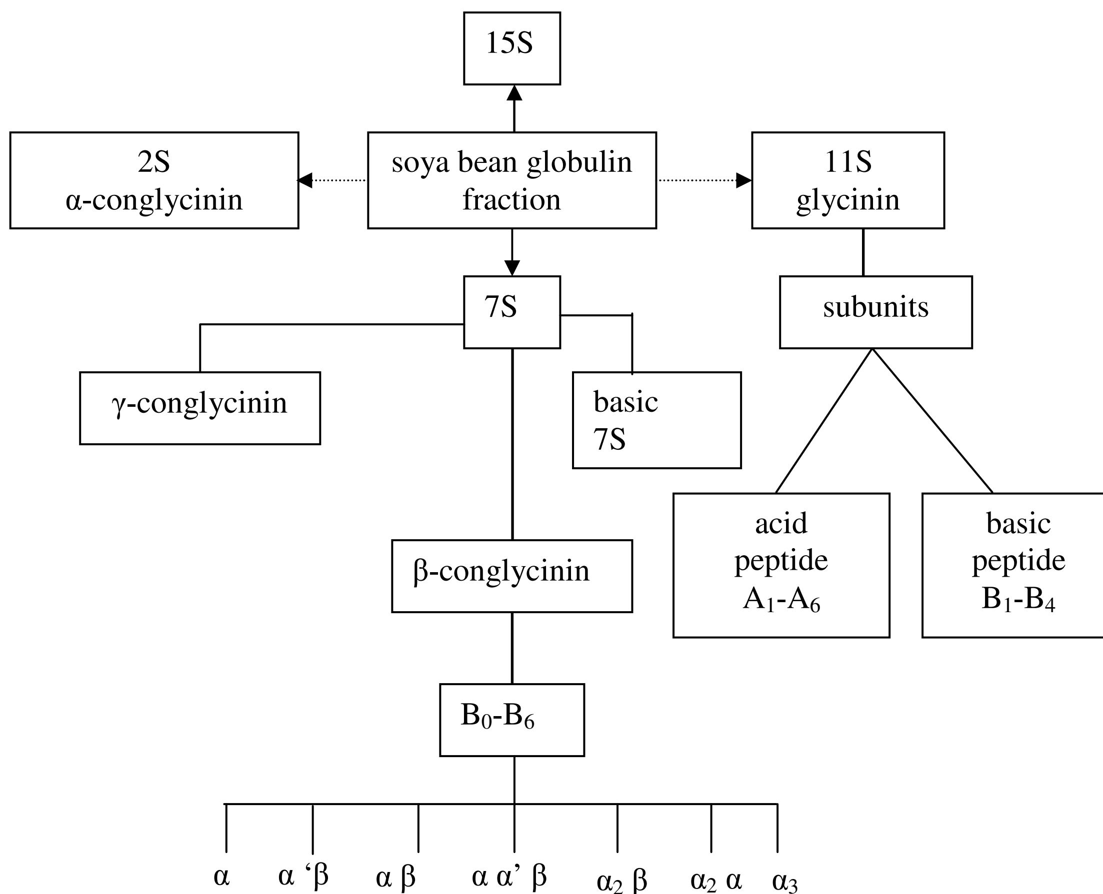

Figure 2.6 The nomenclature and composition of reserved soya bean globulins (Barac *et al*., 2004)

The protein band distribution of some legumes compared with those of soya is shown in Figure 2.7 (A-C). The vicilin (7S) of most legumes contains a variable number of subunits and these often lack disulphide bonds (Rahma *et al.,* 2000; Mujoo *et al.,* 2003; Tang, 2008). The vicilin fraction of soya protein has been found to be composed of three major subunits (Mujoo *et al.,* 2003; Liu *et al.,* 2007). Adebowale *et al.* (2007) reported two subunits with apparent molecular weight of 36 kDa and 17 kDa for the vicilin-like storage protein fraction of mucuna bean. Four subunits were separated by SDS-PAGE when the vicilin structure of broad bean (*Vicia faba)* has been analysed (Bailey & Boulter, 1972). The variation in the number of vicilin-like (7S) storage protein was attributed to post-translational proteolytic processing of the pre-proprotein and/or the differential extent of glycosylation (Muntz, 1998; Sathe, 2002).

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Similarity in protein band distribution under reducing and non-reducing conditions was reported among Bambara groundnut var. HT, bambara groundnuts var. T, cowpea and pigeon pea (Fig. 2.7) (Benjakul *et al.,* 2000), suggesting an absence of disulphide bonds. This similarity may be due to fact that these legumes have vicilin as major storage protein (Benjakul *et al.,* 2000). Rahman *et al.* (1988) analysed the subunit pattern of mung bean (*Phaseolus aureus)* by means of SE-HPLC and SDS-PAGE. These authors reported a major protein fraction of molecular weight: 54 ± 2kDa (Rt: 84.1 min), which was similar under reducing and non-reducing conditions, suggesting again that this was a vicilin-like storage protein. Based on the results of both one and two dimensional gel electrophoresis, Rahman *et al.* (2000) elucidated the absence of disulphide bonds in polypeptides of 7S vicilin of mung bean protein fraction. Horax *et al*. (2004) reported on electrophoretic characteristics of three cowpea varieties in comparison with soya. These authors found that cowpea protein isolates were concentrated at 60 and 40 kDa, whereas soya bean protein bands were at 95, 65, 60, 40, and 35 kDa. Similarity in electrophoresis bands between cowpea and soya have been found in the range 40-62 kDa (Horax *et al.,* 2004). This study confirms that vicilin (7S) is the major storage fraction in cowpea compared with soya protein, which has both vicilin (β-conglycinin) and legumin (glycinin) (Kinsella, 1979; Mujoo *et al*., 2003) as major storage protein fractions. The molecular size distribution reported for cowpea (Horax *et al*., 2004) was similar to that reported for navy bean and kidney bean (Kohnhorst *et al.,* 1990). The latter had a major band ranging from 43-47 kDa and a minor band with size 26-28 kDa (Kohnhorst *et al.,* 1990).

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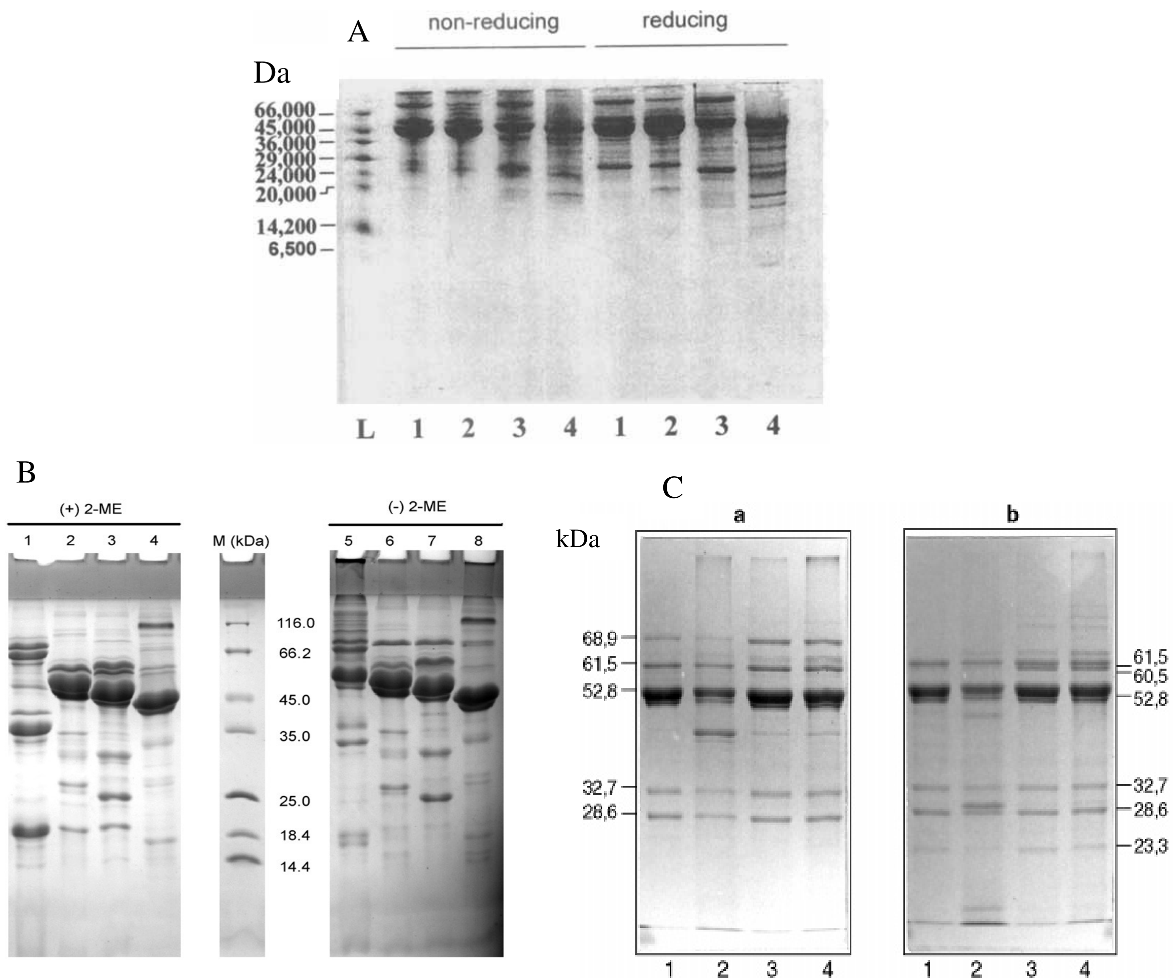

Figure 2.7 SDS-PAGE of some legume globulins

A**:** Seed protein extracts under reducing and non-reducing conditions (20 µg protein was applied). (L) The low molecular weight standards; (1) bambara groundnuts var. HT; (2) bambara groundnuts var. T; (3) cowpea; (4) pigeon pea (Benjakul *et al.,* 2000)

B: Protein isolates in absence (a) and presence (b) of dithioerythritol (DTE) as reducing agent: 1, M-PI; 2, M-PIins; 3, I-PI; 4, I-PIins. M-PI: Mung pea protein isolate produced using micellisation of the proteins from a salt-containing water extract and M-PIin: represents an insoluble fraction obtained by this procedure I-PI: Mung pea protein isolate produced using the traditional alkaline water extraction at pH 8 (Four/water 1:20 w/v) and I-PIins represents an insoluble fraction obtained by this procedure; M: Molecular weight standards (Rahman *et al*., 2000)

C: Protein isolates from red bean (RPI), kidney bean (KPI), mung bean (MPI) and soybeans (SPI) in the presence and absence of reducing agent (2-ME). Lanes 1–4: SPI, RPI, MPI and KPI, respectively, in the presence of 2-ME; Lanes 5–8: SPI, RPI, MPI and KPI, respectively, in the absence of 2-ME; M, molecular weight markers (Tang, 2008)

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Two dimensional (2D) maps of seed proteomes can be useful in both fundamental and applied research. Proteomic technologies have increasingly been applied to determine the protein profile of legumes such as soya bean (Natarajan *et al.,* 2006; Natarajan *et al*., 2007), lupin (Magni *et al*., 2007) and peanuts (Kottapalli *et al.,* 2008). A typical proteome map of soya bean is shown in Figure 2.8. Using combined 2D electrophoresis and Mass Spectrometry (MS), Natarajan *et al.* (2005) identified polypeptides such as β-conglycinin, glycinin, Kunitz trypsin inhibitor, alcohol dehydrogenase, and sucrose binding proteins in soya.

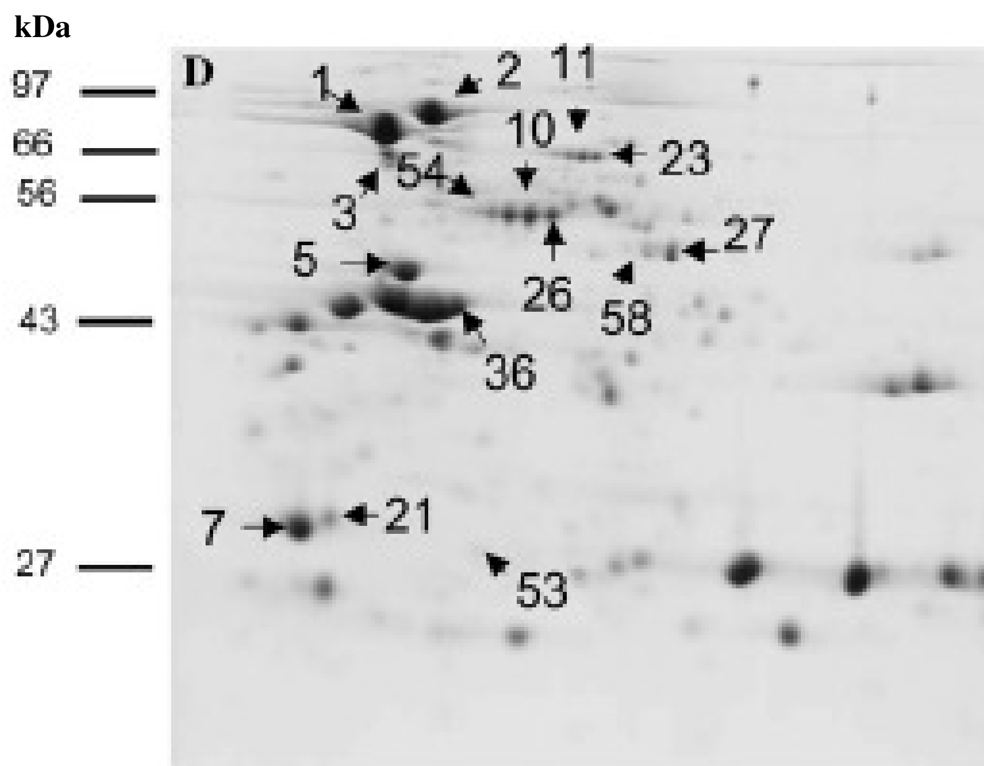

Figure 2.8 A typical proteome map of soya bean (*Glycine max*) (Natarajan *et al*., 2005)

1 & 3: α-subunit of β-conglycinin; 2: α’-subunit of β-conglycinin; 5: Glycinin A3b4 subunit homohexamer; 7: Soybean trypsin inhibitor; 10, 26 & 54: β-conglycinin β-homotrimer; 11 & 23: sucrose-binding protein precursor; 21: Kunitz-type trypsin inhibitor protein; 36: soybean proglycinin A1ab1b homotrimer; 27 & 58: alcohol dehydrogenase 1; 53: Allergen Gly m Bd 28K

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The patterns of proteome maps of globulin protein have been found to vary in their constituent polypeptides depending on legume type. Natarajan *et al.* (2007) reported significant variations in the five glycinin subunits (G1-G5) among sixteen soya bean genotypes analysed by proteomic techniques and genetic analysis. Major variation was observed among wild and cultivated genotypes rather than within the same group. The glycinin to β-conglycinin ratios were also found to vary significantly among 14 commercial soya bean cultivars (Zarkadas *et al.,* 2007). Similar variations in the ratio of glycinin to β-conglycinin have been also reported by Yagasaki *et al.* (1997); although low ratio values compared with those of Zarkadas *et al.* (2007) were found. Protein quality of soybeans varieties can be determined from their ratio of the major seed storage protein components of glycinin and β-conglycinin.

The proteome map of lupin (*Lupine albus)* revealed an intrinsically complex pattern of lupin storage proteins with several spots of same molecular weight but with different pIs. The digital image processing of the map detected 357 spots, seed protein families (Magni *et al*., 2007). The profiles of total seed proteins isolated from mature seeds of four peanut cultivars were studied using two-dimensional gel electrophoresis coupled with nano-electrospray ionization liquid chromatography tandem mass spectrometry (nESI-LC–MS/MS) (Kottapalli *et al*., 2008). The four peanut cultivars revealed distinct differential expression of storage proteins, with the number of polypeptides varying from 457-556. Abundant polypeptides identified belong to the globulin fraction consisting of Arachin (glycinin and Arah3/4) (Kottapalli *et al.,* 2008).

Chemical characteristics of storage proteins from legume seeds in terms of subunit composition and structure have been found to significantly influence the functional properties of proteins (Kinsella, 1979; Nakai, 1983). The remarkable variability in protein expression among legume storage proteins will bring about differences in their functionality. The protein expressions of many existing indigenous legumes such as the marama bean are not known and this information would be required to gain understanding of their function in foods.

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2.4. Functional properties of legume proteins

Functional properties are physicochemical properties which affect the behaviour of proteins in food systems during preparation, processing, storage and consumption (Kinsella, 1979). These properties include solubility, water and oil absorption, emulsifying and foaming properties as well as rheological properties (Kinsella, 1979). The thermal and rheological properties of legume protein are discussed in the following sections.

2.4.1. Thermal properties

The temperature of denaturation (T_d) and enthalpy (ΔH) are the two parameters commonly used to describe the thermal characteristics of legume proteins (Hermanson, 1986; Renkema *et al.,* 2001; Horax *et al.*, 2004). T_d and ΔH provide information on the type of protein structure (simple or complex). The enthalpy is the measure of heat flow into the protein. The greater the heat flow the greater the state of nativity and the more complex the protein structure (Hermanson, 1986; Sorgentini *et al.,* 1995).

A study on thermal denaturation of cowpea storage protein by Differential Scanning Calorimetry (DSC) revealed only 1 major peak corresponding to the vicilin fraction as compared to soya protein which showed two peaks (Horax *et al.*, 2004) corresponding to the β–conglycinin (7S) and glycinin (11S) protein fractions (Hermanson, 1986; Wagner & Annor, 1990). The temperatures of denaturation of 7S cowpea protein (85-88.4°C) have been found to be similar to that of 7S soya protein. But, variable denaturation enthalpies ranging from 8.4-10.33 J/g protein for 7S cowpea protein and 0.6-1.01 J/g protein for 7S soya protein have been found (Horax *et al.,* 2004). The difference in enthalpy (heat flow) may be attributed to differences in 7S contents between soya and cowpea proteins

Differences in thermal stability between vicilin (7S) and legumin (11S) fractions of legume protein have been reported (Sorgentini *et al.,* 1995; Hermanson, 1986). The denaturation temperature (96.3°C) and enthalpy (4 J/g) of glycinin (11S) were high compared to those of β-conglycinin (7S) (T_d: 82.5 and ΔH: 0.7-1.0 J/g protein) for soya bean protein. Similarly, Sorgentini *et al.* (1995) reported high thermal transition point for glycinin (92°C) compared with β–conglycinin (72°C), suggesting that

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glycinin is more thermally stable than β-conglycinin. Glycinin showed a denaturation temperature of 90°C at neutral pH and an ionic strength of 0.25, whereas ß-conglycinin already unfolded at 74°C under the same condition (Hermanson, 1986). Differences in network structure and interaction have been found to be responsible for the differences in thermal stability between 7S and 11S proteins (Barac *et al.,* 2001; Renkema *et al.,* 2001). β-conglycinin has been reported to be a trimer consisting of α', α, and β subunits (Thanh & Shabadaki, 1979; Mujoo *et al.,* 2003), while the glycinin has been found to be a hexamer consisting of pairs of acidic and basic polypeptide subunits; each pair linked by disulphide bonds (Wagner & Annor, 1990; Mujoo *et al.*, 2003).

Variation in thermal stability among dry bean legumes including kidney bean, red bean and mung bean has been reported (Tang, 2008). For instance, the T_d of kidney bean vicilin protein (90.2ºC) was high compared with those of red bean (87.1ºC) and mung bean (84.6ºC). Since T_d is the measure of thermal stability, these results suggest that kidney bean is more thermal stable than red bean and mung bean. Values of thermal properties reported for red bean by Tang (2008) were similar to those of Meng and Ma (2001).

The influence of heating temperature on thermal denaturation of legume proteins has been investigated (Sorgentini *et al.,* 1995, Remondetto *et al.,* 2001). Sorgentini *et al.* (1995) reported that heating protein at 100°C for 30 min led to the denaturation of both 7S and 11S proteins. At 80°C the 7S was totally denatured but the 11S still retained its conformational structure.

The effects of denaturing and reducing agents on legume protein thermal stability have been reported (Meng & Ma, 2001; Tang, 2008). According to Meng and Ma (2001), the decrease in enthalpy resulting from the addition of protein perturbants such as SDS, urea and ethylene glycol suggests a partial denaturation of the protein, while the similarity in thermal properties under reducing and non- reducing conditions suggests that vicilin is the major storage protein fraction of red bean globulin. A minor participation of red bean legumin in protein network was found. Disulphide bonds have been quantified in kidney bean, red bean and mung bean protein and the variations in thermal stability among these legumes have been related to their disulphide bond contents (Tang, 2008). However, the transition temperatures of these

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legumes were not significantly affected when treated with reducing agent (DTT) as compared with that of soya (Fig 2.9).

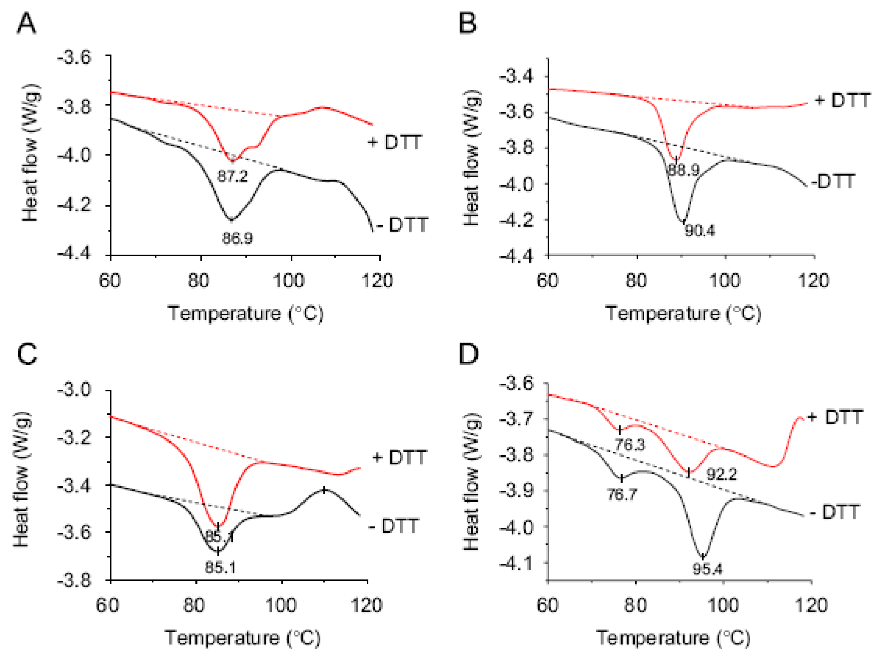

Figure 2.9 Typical DSC profiles of RPI (A), KPI (B), MPI (C) and SPI (D), in the absence (black lines) and presence (red lines) of 20 mM DTT (Tang, 2008)

RPI: red bean protein isolate, KPI: kidney bean protein isolate, SPI: soya bean protein isolate

The pH and the ionic strenght have also been found to influence the protein structure which is reflected during thermal analysis. Shand *et al.* (2007) observed some influence of salt concentration on enthalpy of denaturation of soy protein isolates. Increasing the salt concentration from 0-2% increased the enthalpy of denaturation from 0.49 – 6.6 J/g protein. In a similar way, there was an increased in the transition temperature of both the 11S and 7S soya bean protein isolates, demonstrating the stabilizing effects of NaCl on the protein. This stabilization of protein structure in the presence of salt has also been reported by several authors (Kinsella, 1979; Aluko & Yada, 1995). The addition of salt (NaCl) tends to interact with positively charged proteins, reducing electrostatic repulsion and enhancing hydrophobic interaction (Aluko and Yada, 1995). Meng and Ma (2001) observed a decrease in enthalpy and T_d of red bean globulin protein under highly acidic or alkaline pH. This was because

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high pH value increases the net negative charge on protein. The effect of this increase coupled with salting-in effect of NaCl on hydrophobic interaction would result in dissociation of protein aggregates (Utsumi & Kinsella, 1985; McWatters & Holmes, 1979). Petruccelli and Añón (1996) investigated pH-induced modifications on the thermal stability of soybean protein isolates by DSC. These authors reported that the glycinin denaturation temperature decreased by 10°C when the pH was increased from 6 to 11, while the 7S globulin denaturation temperature did not change. A 50% decrease in ΔH at pH 11 was observed for glycinin. This behaviour of the glycinin may be due to a dissociation of the hexameric form to trimeric and/or monomeric forms. The structural modification of protein at varying ionic strength has been observed by Thanh and Shibasaki (1979). β-conglycinin has been found to possess a trimeric form of 7S with an ionic strength above 0.5 and a hexameric structure at ionic strength of below 0.2. At very low ionic strengths ($\acute{\iota}$ < 0.01) and acidic pH, the R subunit dissociates from the trimer, leading to 2S and 5S forms (Thanh & Shibasaki, 1979). At pH 11, soya bean isolate gave only one endothermic representing both globulins suggesting a complete change in structure (Petruccelli & Añón, 1996). The pattern of protein association–dissociation under varying pH conditions proposed by Prakash and Narasingo Rao (1988) is shown in Figure 2.10.

Figure 2.10 Association-dissociation of protein at varying pH condition (Prakash & Narasingo Rao, 1988)

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The dependency of thermal denaturation of soy protein on water content of the protein was investigated by Kitabatake *et al.* (1990). These authors reported that heat-denaturation temperature T_d of protein in solution is often below 100°C. Denaturation temperatures found for 7S and 11S with 94% water were 76.5°C and 94°C, which agree with reported T_d values in other literature for 7S and 11S soya protein, respectively (Kinsella, 1979; Scilingo & Añón, 1996; Shand *et al.*, 2007). Based on these findings, Kitabatake *et al.* (1990) suggested that soya bean protein with a water content of 94% is an appropriate condition for thermal property evaluation of the protein. The solid concentration can also influence the degree of denaturation. According to Romondetto *et al.* (2001), higher solid concentration (11% protein) has protective effect on protein denaturation.

2.4.2. Rheological properties

The rheological properties of legume storage proteins have been found to be significantly associated with protein composition (Nagano *et al.*, 1996; Tang *et al.,* 2008). The contents of β-conglycinin and glycinin of soya protein have been found to influence the elastic properties (G') of soya protein isolates (Nagano *et al.,* 1996). Riblett *et al.* (2001) found an increase in storage modulus G' with a high percentage of hydrophobic residue while investigating the characteristics of β-conglycinin and glycinin soya protein from four genotypes.

The effect of heating on glycinin and β-conglycinin fractions of soya bean protein has been investigated (Shimada & Matsushita, 1980; Nakamru *et al.,* 1986). Heating soya protein above 70°C causes dissociation of the quaternary structure; denatures their subunits and promotes the formation of protein aggregates. Some studies have shown that the relative contribution of β-conglycinin and glycinin to soya protein gel properties was dependent on the heating rate (Shimada & Matsushita 1980; Nakamru *et al.* (1986). Heating at 80°C for 30 min in water (pH 7.0) revealed that β-conglycinin play a major role since the gel formed was harder than that of glycinin (Shimada and Matsushita, 1980). On the other hand, Nakamru *et al.* (1986) observed that glycinin formed a harder gel than β-conglycincin when the protein solution was heated at 100°C (pH 7.6 ionic strength 0.5). Tang *et al.* (2009) found that heat-induced aggregation varied with the types of protein isolate when comparing the thermal properties of vicilin-rich legumes including red bean, kidney bean and mung

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bean. The variation in heat-induced aggregation among these legumes may be attributed to difference in heat stability, as was described in previous study by Tang, (2008).

Factors such as pH and protein concentration as well as processing history significantly influence the viscoleastic properties of soya protein isolates (Torrezan *et al.,* 2007; Shand *et al.,* 2008). A model (hexamers sandwich model) used to explain the change in conformation behaviour (association-dissociation, dissociation-association, dissociation denaturation) of protein is shown in Figure 2.11. Minor variation in pH ionic strength, nature of ions, presence of denaturing agent, and temperature could result in disruption of stabilizing forces of proteins (Prakash & Narasingo Rao, 1988). The influence of pH on gel forming properties of soya proteins isolate and purified glycinin in relation to denaturation and aggregation were investigated by Renkema *et al.* (2001). Gels at pH 3.8 had higher G' values than G' values at pH 7.6 for both glycinin and soya protein isolate (Renkema *et al.*, 2001).

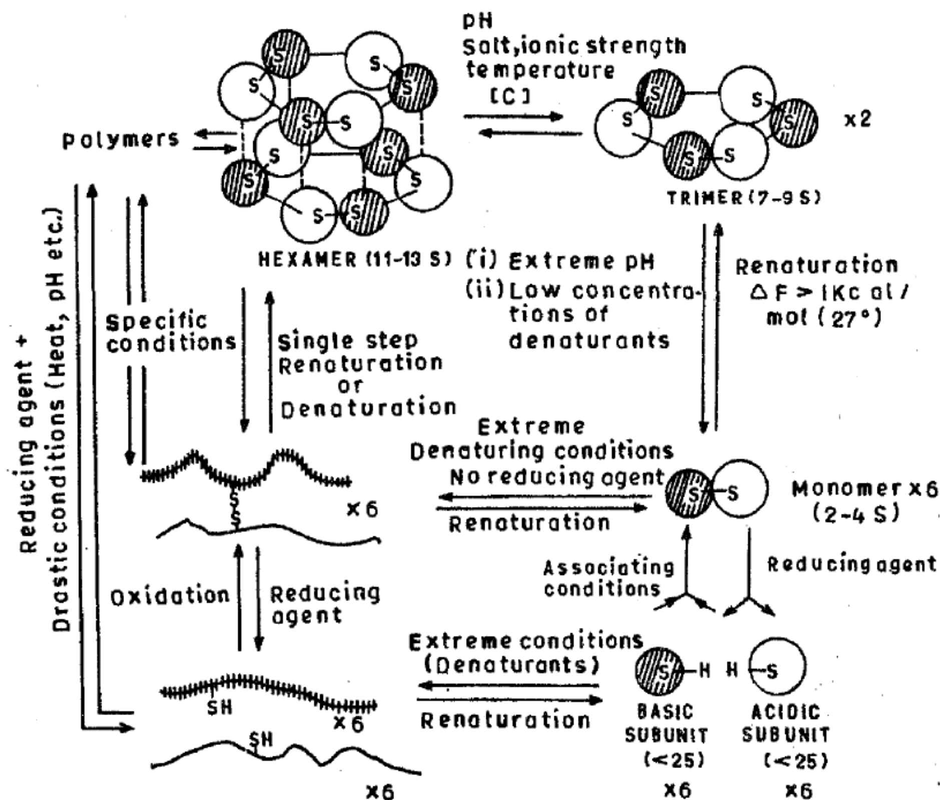

Figure 2.11 Proposed model for the association-dissociation, denaturation and reassociation of the high molecular weight protein fractions from various oilseeds Dashed lines indicate weak non-covalent interaction, solid lines indicate strong noncovalent interactions, S-S, disulphide bridge (Prakash & Narasingo Rao, 1988)

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2.5. Conclusions

The protein compositions of legumes are variable depending on legume type, genotype and growing conditions. The proteome maps of most indigenous legumes including the marama bean have not been established. In addition, the protein body structure and protein composition in terms of protein type and constituent polypeptides as well as the functional properties with respect to thermal and rheological properties are not known for marama. This information is required to determine the potential usage of marama protein in food systems.

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2.6. Hypotheses

1) The protein bodies in marama bean will consist of spherical protein bodies similar to those of soya bean. Microstucturally, the protein bodies of oilseed legumes such as soya and peanuts have been found to consist of spherical bodies surround by a network of lipid bodies (Young *et al.,* 2004; Martinez, 1979; Lott & Buttrose, 1978).
2) The major storage protein in marama will be globulin, which will be composed of the vicilin-like (7S) and legumin-like (11S) protein fractions similar to soya bean. However, differences may be observed in terms of number and molecular weight of constituent polypeptide subunits between marama and soya globulins. Differences in the number of vicilin (7S) subunits have been reported for some legume species (Bailey & Boulter, 1972; Mujoo *et al.,* 2003; Liu *et al.,* 2007; Adebowale *et al.,* 2007). These differences were attributed to post-translational proteolytic processing of the pre-proprotein and/or the differential extent of glycosylation (Muntz, 1998; Sathe, 2002).
3) Marama storage protein will have high elastic properties as compared to that of soya bean. The high tyrosine residues in marama proteins may lead to increased structural stability since tyrosine is involved in cross-linking polypeptides (Takasaki *et al*., 2005; Tilley *et al*., 2001).

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2.7. Objectives

1) To determine the microstructure of protein bodies in marama beans and compare this with that of soya beans
2) To determine the physicochemical properties of marama storage protein in comparison with soya bean protein
3) To determine the thermal and rheological properties of marama storage protein in comparison with soya bean protein

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3. RESEARCH

This chapter is organised into three sections based on specific objectives of this study. These sections are presented as follows:

3.1 Microstructure of protein bodies in marama bean species

3.2 Marama bean protein composition

3.3 Thermal and rheological properties of marama protein

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3.1. MICROSTRUCTURE OF PROTEIN BODIES IN MARAMA BEAN SPECIES[1]

Abstract

Marama bean is an underutilised indigenous legume from Southern Africa. The understanding of the microstructure of marama protein bodies, the organelles of protein storage, is an important step towards the characterisation and utilisation of its protein. The protein body structures of two species of marama beans (*Tylosema esculentum* and *T. fassoglense*) were determined in comparison with soya bean (*Glycine max). T. fassoglense* seemed to have higher protein content than soya. Marama beans showed clustered spherical protein bodies surrounded by lipid bodies, similar to soya beans. *T. esculentum* seemed to contain smaller sized (4 ± 2 μm) protein bodies per cell as compared with *T. fassoglense* (7 ± 4 μm). Marama protein bodies contained spherical globoid and druse crystal inclusions, which were absent in soya. P, K, Mg and Ca were the major minerals in marama, which probably originated mainly from storage protein sites. The protein body structure of marama is similar to soya in terms of spherical shape and localisation within the parenchyma cells.

Key words: marama bean, soya bean, protein bodies, microstructure

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3.1.1. Introduction

Marama bean is an underutilised indigenous legume from Southern Africa. *Tylosema esculentum and T. fassoglense* are the two species of marama bean that occur in South Africa and Namibia (Coetzer & Ross, 1976). Marama bean is found to be a good source of protein (Amarteifio & Moholo, 1998; Holse *et al*., 2010) similar to oilseed legumes such as peanuts (Venkatachalam & Sathe, 2006) and soya beans (Garcia *et al*., 1998) However, the use of marama bean remains mainly domestic. Traditionally, marama beans are roasted and consumed as a snack. Matured roasted seeds are also ground or pounded for making porridge or a cocoa-like beverage (Keegan & van Staden, 1981). Marama oil has reportedly been used for cooking (Ketshajwang *et al.,* 1998). Marama bean could become a valuable crop and a potential source of income in its production zones if its major component parts like the protein and fat are adequately utilised.

Understanding of the microstructure of protein bodies, the organelles of protein storage is an important step towards the isolation, characterisation and utilisation of food proteins. In particular, the knowledge of the seed microstructure may be important in commercial processing and utilisation of legume protein. When isolating proteins from peas and soya bean, residual lipids in extracted isolates were attributed to not only differences in lipid compositions but also on the physical location of lipids in the seeds (Shand *et al*., 2007). The physical location of storage protein in the seed may affect its protein digestibility (Aguilera, 2005). Furthermore, the microstructure of seeds has been found to influence their physical properties such as seed hardness (Aguilera & Stanley, 1999).

The structure of protein bodies in marama beans is not known. The objective of this study was to determine the protein body structure of two species of marama beans (*T. esculentum* and *T. fassoglense*) in comparison with soya beans (*Glycine max*).

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3.1.2. Materials and methods

3.1.2.1. Materials

Two species of marama beans *T. esculentum* (Burch) A. Schreib and *T. fassoglense* (Schweinf) Torre and Hillc and soya bean (*Glycine max L. Merr.*) were used. *T. esculentum* was gathered from two different locations, Rooidraaitrust, South Africa (voucher specimen deposited at H.G.W.J. SCHWEICKERDT (PRU), Accession number: 113873) and Gantsi, Botswana in 2008 (voucher specimen deposited at PRU, Accession number: 113872). *T. fassoglense* was gathered near Kruger Park, South Africa in 2007 (voucher specimen deposited at PRU, Accession number: 113870). Species were identified based on the seed morphology (Fig. 3.1.1) and leaves. Soya bean was obtained from AGRICOL, Pretoria, South Africa.

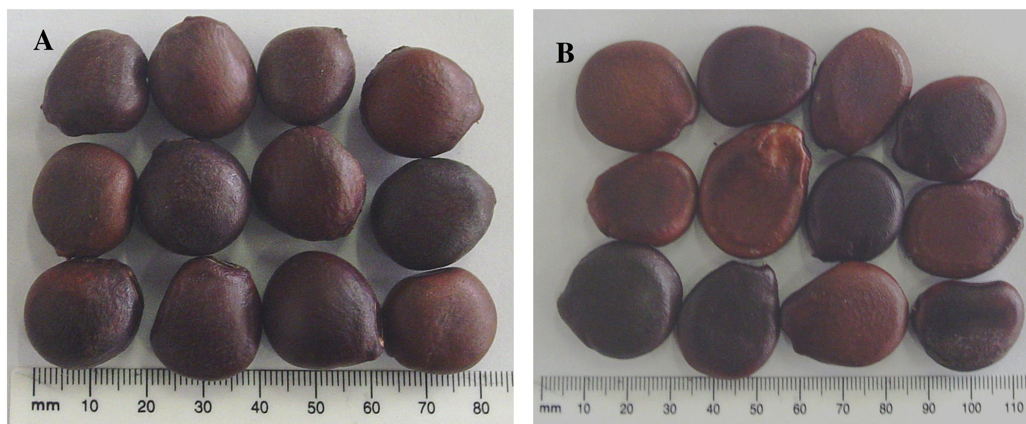

Figure 3.1.1 Marama bean *T. esculentum* (A) and *T. fassoglense* (B)

3.1.2.2. Sample preparation

Marama beans were dehulled using a cracking device (WMC Sheet Metal Works, Tzaneen, South Africa). Soya beans were dehulled with a Tangential Abrasive Dehulling Device (TADD) (Shepherd, 1979). Dehulled marama and soya beans were ground into flours using a laboratory attrition mill coupled with 0.5 mm screen sieve (Ika Werke, Staufen, Germany). Processed bean flours were stored at 4°C until analysed.

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3.1.2.3. Proximate composition

Bean flours were analysed for moisture content (AOAC method no. 934.01), fat (AOAC method no. 920.39), ash (AOAC method no. 942.05), crude fibre (AOAC method no. 962.09) and crude protein (N x 5.71) by combustion analysis (AOAC method no. 968.06) (AOAC, 2000). Total carbohydrate was obtained by difference.

3.1.2.4 Mineral composition

Bean flours (0.5 g) were acid digested with 5 ml nitric/perchloric acid (2:1) mixture. Mineral content was analysed by AOAC method no. 6.1.2 (AOAC, 1984), using Inductively Coupled Plasma (ICP) Spectroscopy.

3.1.2.5. Light Microscopy (LM), Transmission Electron Microscopy (TEM) and Confocal Laser Scanning Microscopy (CLSM)

Sample fixation for LM and TEM was by the method of Young *et al*. (2004), Pattee, Schadel, and Sanders (2004) with slight modifications. Modifications included fixing of the tissue blocks (1 mm^3), cut from the inner surface of cotyledons, in 0.075 M phosphate buffer, pH 7.4 containing 2.5% (w/v) glutaraldehyde for 48 h at room temperature.

For LM, sections of 1 µm were cut after fixation using an ultramicrotome. These sections were stained with Coomassie Brilliant Blue R 250 (Gahan, 1984). Stained specimens were mounted in immersion oil.

For TEM, ultrathin sections were cut using an ultramicrotome. The sections were placed on copper grids and contrasted in 4% aqueous uranyl acetate for 10 min, followed by 2 min in Reynolds lead acetate.

For CLSM, tissue blocks (1mm^3) cut from the cotyledon inner surface were fixed in 2.5% (w/v) formaldehyde overnight. Fixed tissues were rinsed three times for 10 min in 0.075 M phosphate buffer and dehydrated at room temperature at 10 min intervals in a graded series of aqueous ethanol (50%, 70%, 90%, 100%, 100%). Dehydrated samples were infiltrated with 50% (v/v) LR White resin in ethanol for 1 h and 100% LR White resin overnight before polymerisation at 60°C for 39 h. Sections with

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thickness of 1.5 µm were cut and viewed under a CLSM. Excitation was at 405 nm. Fluorescence protein was detected after passing through a 420 long passing filter, with a pinhole set at 55 µm.

3.1.2.6. Proteinase K digestion of marama bean and soya bean cotyledons

Tissue blocks (1 mm^3) were cut from the inner surface of the cotyledons and digested with Proteinase K (Sigma P 2308) following the procedure of Miflin and Burgess (1982) with modifications. Sectioned tissue blocks were incubated in Proteinase K (500 µg/ml) in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM calcium chloride for 45 min at 37°C. Control samples were incubated in the same buffer without Proteinase K. Incubated tissue samples were then fixed in glutaraldehyde as described above and viewed with the TEM.

3.1.2.7. Statistical analyses

Chemical analyses were done on duplicate samples. Mean diameter of protein bodies and the distribution of protein bodies per cell (N=50 cells) were determined. One way analysis of variance was conducted on proximate and mineral data and the means compared using Fisher's Least Significant Difference Test at $p<0.05$.

3.1.3. Results and discussion

3.1.3.1. Chemical composition of marama bean

Marama *T. fassoglense* seemed to have a higher protein content compared with marama *T. esculentum* and soya (Table 3.1.1). The protein content of *T. esculentum* is within the range previously reported for this specie (Hosle *et al*., 2010; Maruatona *et al*., 2010). The protein content of marama bean appeared to be higher compared with peanuts (Wu *et al.,* 1997) and other oilseeds such as hazelnut and macadamia nut (Venkatachalam & Sathe, 2006). Marama bean also contained almost twice as much fat as soya (Table 3.1.1). The fat content of marama beans is in agreement with previous reports (Amarteifio & Moholo, 1998; Ketshajwang, Holmback & Yeboah, 1998). Marama and soya had similar crude fibre contents. However, the total carbohydrate and ash contents were different between these legumes. The carbohydrates contents of marama from this study are lower than the values previously reported by Amarteifio and Moholo (1998). Changes in chemical

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composition may occur in seed as a result of the changes in environmental conditions, type of soil and agricultural practices such as the use of fertiliser in growing areas.

Table 3.1.1 Proximate composition of marama bean and soya bean (g/100 g)[1]

Samples	Moisture	Protein	Fat	Crude fibre	Ash	Total [2] carbohydrates
T. esculentum (SA)	$7.0^{b} \pm 0.2$	$30.6^{a} \pm 0.2$	$32.9^{c} \pm 0.5$	$7.9^{a} \pm 0.1$	$3.3^{a} \pm 0.1$	$18.3^{a} \pm 0.7$
		(32.9)	(35.4)	(8.5)	(3.5)	(12.7)
T. esculentum (BW)	$5.1^{a} \pm 0.7$	$30.6^{a} \pm 0.3$	$36.2^{d} \pm 0.3$	$8.1^{a} \pm 0.2$	$3.2^{a} \pm 0.7$	$16.8^{a} \pm 0.3$
		(32.2)	(38.1)	(8.5)	(3.3)	(12.8)
T. fassoglense (SA)	$7.1^{b} \pm 0.1$	$37.3^{c} \pm 0.2$	$29.2^{b} \pm 0.7$	$8.4^{a} \pm 0.2$	$3.4^{a} \pm 0.4$	$14.9^{a} \pm 0.5$
		(40.2)	(31.4)	(9.0)	(3.6)	(8.7)
Soya bean	$7.9^{b} \pm 0.8$	$32.5^{b} \pm 0.2$	$14.9^{a} \pm 0.1$	$8.6^{a} \pm 0.2$	$4.8^{b} \pm 0.7$	$31.3^{b} \pm 1.3$
		(34.9)	(16.2)	(9.3)	(5.2)	(26.3)

SA: South Africa, BW: Botswana

[1] Mean ± SD. Mean values with different superscript letters in columns are significantly different ($p<0.05$).

Values in parentheses are mean values expressed on a dry matter basis

[2]Total carbohydrates by difference

Phosphorus, potassium, calcium, magnesium and sulphur were the major minerals in both species of marama (Table 3.1.2). Soya bean seemed to contain higher concentrations of K and S than marama beans. However, Ca and Mg were higher in marama compared to soya. The concentration of the various mineral in marama is in agreement with previous reports (Amarteifio & Moholo, 1998; Mmonatau, 2005). The mineral composition of marama bean species were also similar to mineral profiles reported for oilseed legumes such as peanut (Wu *et al*., 1997) and soya bean (Garcia *et al.,* 1998).

Legume storage proteins are found in specialised organelles, the protein bodies (Lott, 1981). The protein bodies also act as compartments for mineral reserves in seed. These mineral reserves occur as phytate, which is stored in globoid inclusions within the protein bodies (Lott & Buttrose, 1978). The structure of protein bodies in marama was determined in comparison with soya using LM and TEM.

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Table 3.1.2 Mineral composition of marama bean and soya bean (mg/100 g flour)[1]

Elements	*T. esculentum* (*SA*)	*T. esculentum* (*BW*)	*T. fassoglense* (*SA*)	*Soya bean*
P	1065 [b] ± 108	796 [a] ± 82	1123 [c] ± 100	1199[d] ± 130
Ca	157 [b] ± 13	200 [c] ± 6	156 [b] ± 19	133 [a] ± 2
K	634[b] ± 14	500[a] ± 51	615 [b] ± 16	1159[c] ± 73
Mg	358[b] ± 26	319 [b] ± 12	402[b] ± 29	272[a] ± 10
S	297[a] ± 37	270[a] ± 60	340[a] ± 45	409[b] ± 50
Na	6.0[c] ± 0.5	3.4 [a] ± 1.1	6.9 [c] ± 0.2	4.8 [b] ± 2.1
Cu	1.8 [a] ± 0.2	0.4 [a] ± 0.2	0.9 [a] ± 0.1	0.6 [a] ± 0.2
Fe	1.7 [a] ± 1.6	1.3 [a] ± 1.0	1.4 ± 1.1[a]	4.2[a] ± 2.2
Mn	2.9[a] ± 1.0	2.1 [a] ± 2.0	1.3 [a] ± 0.9	1.7 [a] ± 1.1
Zn	3.7 [a] ± 0.7	3.2 [a] ± 0.5	4.1 [a] ± 1.0	4.4 [a] ± 0.8

SA: South Africa, BW: Botswana

[1] Mean ± SD is reported on a dry matter basis

Mean values with different superscript letters in rows are significantly different ($p<0.05$)

3.1.3.2. Microscopy of protein bodies in marama bean

Pre-fixed sections of marama and soya beans were stained with Coomassie Brilliant Blue and viewed by LM. The protein bodies appeared as distinct bodies, circular in cross-section and stained blue within the cells while the remaining space of the cytoplasm was not stained (Fig. 3.1.2). Coomassie Brilliant Blue dye is frequently used to stain protein as it binds to it via physical adsorption to arginine, aromatic amino acids and histidine (Hafiz, 2005). From LM, it is evident that the parenchyma cells of marama bean contain circular organelles, protein bodies similar to soya bean.

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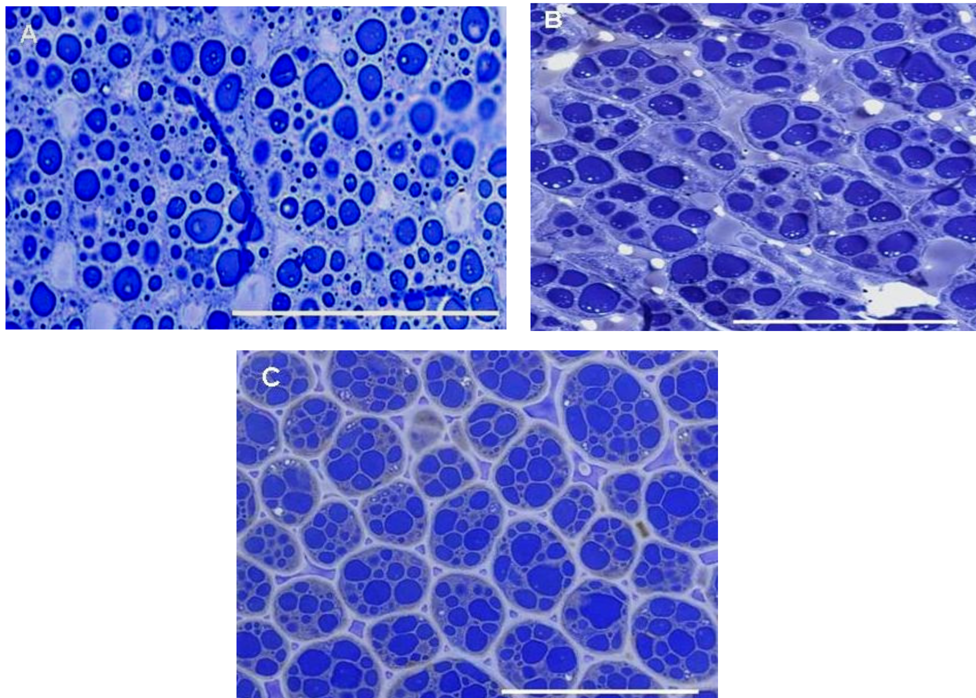

Figure 3.1.2 Light microscopy of marama and soya bean parenchyma cells stained with Coomassie Brilliant Blue R 250
A: Marama bean (*T. esculentum*) from Botswana; B: Marama bean (*T. fassoglense*) from South Africa, and C: Soya bean. Bar: 100 µm. Protein bodies are stained blue in parenchyma cells

With TEM, marama bean parenchyma cells showed circular, electron-dense protein bodies surrounded by lipid bodies (Fig. 3.1.3 A, B). The protein bodies were similar to those in the soya bean parenchyma cells (Fig. 3.1.3 C). Structurally, the protein bodies in legumes, including peanuts, hazelnuts and soya beans consist of homogenous proteinaceous matrix material surrounded by a network of lipid bodies (Lott & Buttrose, 1978; Young *et al.,* 2004). The organisational structure of marama protein bodies is therefore similar to microstructure of protein bodies in most legumes including soya.

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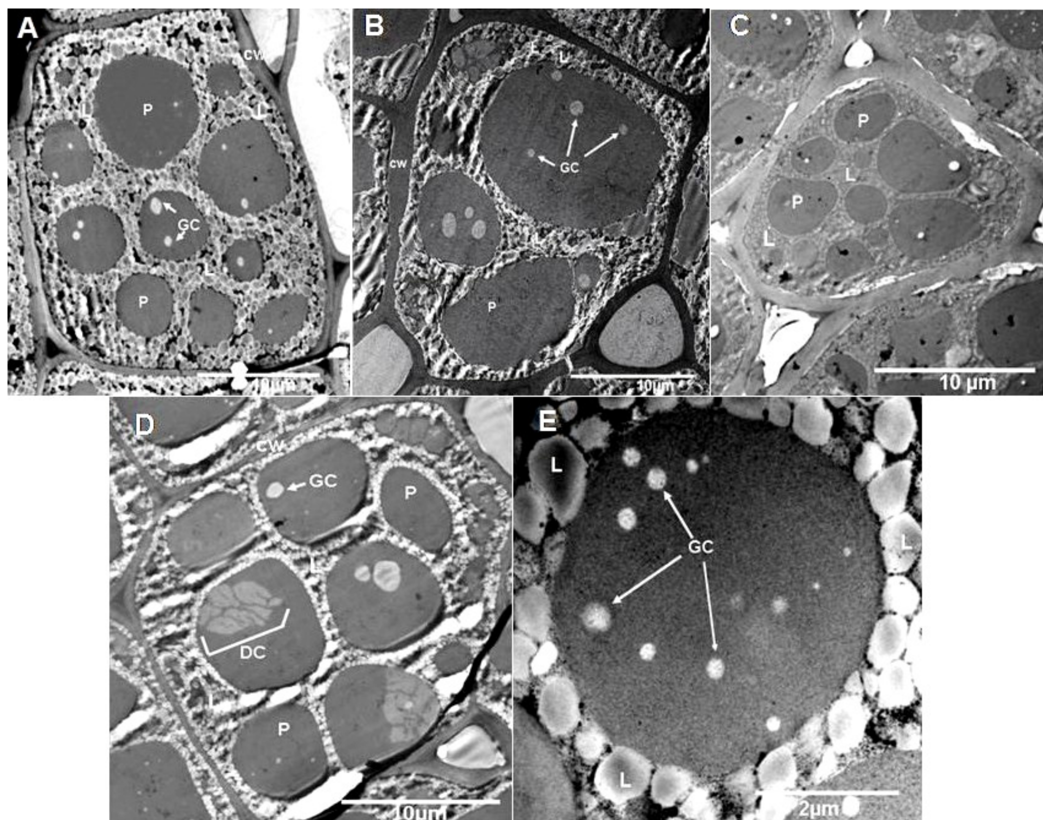

Figure 3.1.3 Transmission electron microscopy of protein bodies in marama and soya bean parenchyma cells

A: Marama bean (*T. esculentum*) from Botswana, B: Marama bean (*T. fassoglense*) from South Africa, C: Soya bean, D: Marama bean (*T. esculentum*) from Botswana with druse crystal (DC) type of inclusion in protein body, E: Protein body of marama bean (*T. esculentum*) from South Africa with spherical Globoid Crystals (GC)

P: Protein bodies, L: Lipid bodies

The dimensions of marama protein bodies, which varied between 2-12 µm in diameter are similar to sizes reported for oilseed legumes, including soya bean (Martinez, 1979) and peanuts (Young *et al.*, 2004). Differences in protein body size and distribution within the parenchyma cells were observed between the two species of marama bean. The parenchyma cells of *T. esculentum* from both South Africa and Botswana seemed to contain more smaller sized protein bodies (16 ± 9 per cell; 4 ± 2 µm) per parenchyma cell compared to *T. fassoglense* (7 ± 4 per cell; protein body size: 7 ± 4 µm). Protein bodies of *T. esculentum* and soya bean seemed to be similar in terms of protein body size and distribution per cell. The variations in protein body size and number between *T. esculentum* and *T. fassoglense* may be related to species.

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The higher protein content of T. *fassoglense* (Table 3.1.1) may be related to the relatively larger size of its protein bodies.

TEM of marama protein bodies also showed the presence of globoid inclusions within the protein bodies (Fig. 3.1.3 D, E). It appeared that spherical globoids and druse crystals (Lott & Buttrose, 1978) were the two types of inclusion in protein bodies of marama bean. Globoids constitute storage sites for seed phosphorus deposited as insoluble phytate in protein bodies (Martinez, 1979). Spherical globoids are the most common inclusion reported in protein bodies of legumes like peanuts (Young *et al.*, 2004), walnuts and hazelnuts (Lott & Buttrose, 1978). Marama bean is thus similar to most legumes in terms of spherical globoid inclusions in its protein bodies.

Elemental composition analysis of spherical globoids from many legumes showed that they are rich sources of P, K, Ca and Mg (Lott & Buttrose, 1978; Lott, 1981). These minerals, which were found in high concentration in marama beans (Table 3.1.2), probably originated mainly from the globoid sites. Most soya protein bodies did not contain globoid inclusions. According to Prattley and Stanley (1982), phytic acid in soya is likely in the form of soluble protein-phytate salt with 10-15% of phytate specifically deposited in globoids in an insoluble form. The lack of globoids in protein bodies of soya may be attributed to the soluble form of its phytic acid. Druse crystals (one per protein body) were observed only in protein bodies from *T. esculentum* (Fig. 3.1.3 D). Druse crystals consist of cluster of small crystals in arrangement called a druse or rosette (Lott, 1981). This type of inclusion has been reported in some protein bodies of hazelnuts (Lott & Buttrose, 1978). The norm is that where druse crystals are found, only one occurs per protein body (Lott, 1981).

Compared to soya, marama appeared to have a relatively higher calcium content (Table 3.1.2), which is probably due to druse crystals in its protein bodies (Fig. 3.1.3). Druse crystals have been found to be rich in calcium (Lott & Buttrose, 1978). Thus, the mineral composition of marama bean seems to be related to the structure of its protein bodies, the organelle of protein storage.

Marama bean parenchyma cells showed clusters of spherical protein bodies with CLSM (Fig. 3.1. 4). Small spherical bodies were also observed within the protein bodies that did not fluoresce. These bodies seemed to represent the globoid inclusions

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that were observed with TEM (Fig. 3). CLSM of marama bean thus confirms that its protein bodies contain globoid inclusions.

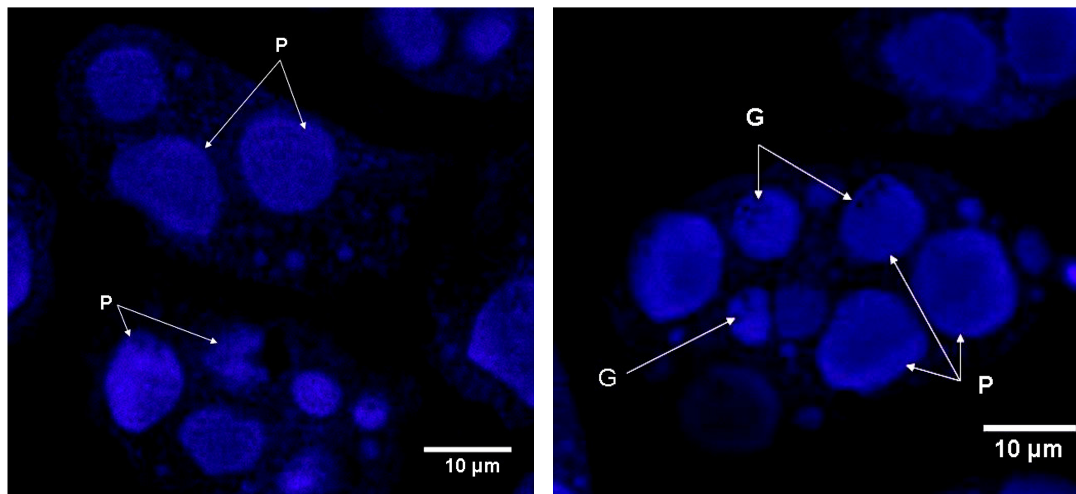

Figure 3.1.4 Confocal laser scanning microscopy of protein bodies in marama bean
P: Protein bodies, G: Globoids

To elucidate marama bean protein body structure, tissue sections were incubated with Proteinase K prior to fixation with glutaraldehyde. The protein bodies in marama and soya that were incubated in Tris-HCl buffer (pH 8) without Proteinase K maintained their structure and did not show any sign of digestion (Fig. 3.1.5 A, B, C), whereas protein bodies treated with Proteinase K (Fig. 3.1.5 D, E, F) showed disruption of the cell structure with digested protein bodies within the parenchyma cells. The TEM of the control samples (Fig. 3.1.5 A, B, C) seemed to be slightly different from the initial TEM (Fig. 3.1.3). This is possibly due to some components being leached out from the parenchyma cells during the incubation of sample in Proteinase K buffer. Proteinase K is a stable serine protease that digests native protein effectively (Ebeling *et al*., 1974). This enzyme has been used to digest protein bodies in maize, wheat and peas (Miflin & Burgess, 1982). Treatment of marama with Proteinase K confirms that digested organelles are protein bodies. The outer layer of the protein bodies that resisted the digestion with Proteinase K is likely to be the protein body membrane (Fig. 3.1.5 D, E, F).

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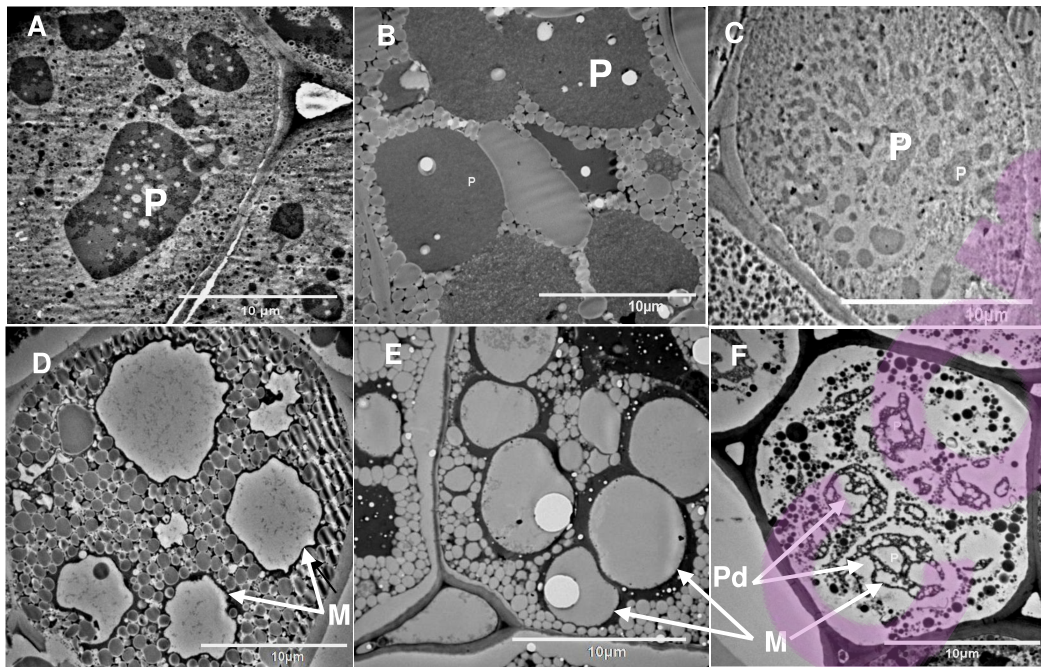

Figure 3.1.5 Transmission electron microscopy of marama and soya beans protein bodies treated with Proteinase K (P: protein bodies, M: Protein body membranes, P_d: Pockets of digestion)
A & D: Marama bean (*T. esculentum*), B & E: Marama bean (*T. fassoglense*) C & F: Soya bean, A, B & C: untreated

Various organelles in the parenchyma cells including the protein bodies are membrane bound (Pusztai *et al*., 1979). Protein body membranes mainly consist of phospholipids and glycosylated proteins (lectins) (Pusztai *et al.*, 1979; Hafiz, 2005). These membrane proteins are insoluble and disruption of the membrane and extraction with organic solvents is required to solubilise them (Hafiz, 2005). The insoluble nature of protein body membranes reduces the interaction with the enzyme and the membranes will remain indigested within the cell. However, differences were observed in the manner in which protein bodies were digested within the parenchyma cells between marama and soya. Marama protein bodies were clearly digested from the inside to the outer part as compared to those of soya bean, which showed pockets of digestion throughout the cytoplasmic network (Fig. 3.1.5 F). This difference in the manner of digestion between marama and soya beans may be due to differences in solubility behaviour of their proteins in Proteinase K buffer.

Protein and fat constituted major components of marama bean. The physical location of protein bodies relative to fat has been found to be similar to that of soya bean.

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These finding suggest that the extraction of marama protein may be done in the same way as soya. The presence of crystalline inclusion in marama bean, suggests that phytic acid in marama may be predominantly in an insoluble protein-phytate complex form. This may influence the protein nutrition by reducing its digestibility when compared with soya in which the phytic acid is likely in the form of soluble protein-phytate (Prattley & Stanley, 1982). The presence of these inclusions in marama protein may explain its lower protein digestibility compared to soya from previous studies (Maruatona *et al.*, 2010). It may be necessary to determine the elemental composition of these inclusions in marama protein for further characterisation.

3.1.4. Conclusions

Marama beans are rich in protein and fat. Potassium, phosphorus, calcium magnesium and sulphur are the major minerals in marama beans. The protein body structure of marama is similar to soya in terms of spherical shape and localisation within the parenchyma cells. However, marama bean protein bodies contain spherical globoid and druse crystal inclusions. Due to similarity in seed microstructure, marama protein can be extracted in the same way as soya protein.

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3.2. COMPOSITION OF MARAMA BEAN PROTEIN

Abstract

The protein composition of marama beans, an indigenous African oilseed legume, was determined in comparison with soya beans. Marama bean protein contained a substantial amount of tyrosine compared with soya bean protein. It was slightly richer in proline than soya. By SDS-PAGE, marama protein contained fewer protein bands compared with soya. The patterns of these bands in marama under non-reducing and reducing conditions were similar, suggesting an absence of disulphide bonds. The vicilin (7S) and acidic 11S subunits seemed to be absent in marama protein. This is most unusual in legume proteins. Only a major basic legumin (11S) (20 kDa), medium (63 kDa) and high (148 kDa) molecular weight protein bands were separated for marama protein. Most polypeptides in the marama proteome map are basic compared with soya. Only one polypeptide match was tentatively identified compared with soya. Marama protein composition is very different from that of soya.

Key words: marama bean, soya bean, 11S and 7S proteins, tyrosine

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3.2.1 Introduction

Marama bean is a protein-rich indigenous oilseed legume, similar to soya bean. In the previous research (Section 3.1), marama protein bodies were found to be similar to those of soya in terms of shape and localisation within the parenchyma cells. Marama and soya also had similar protein contents (approx. 35%). However, very limited information is available on the protein composition of marama bean (Ripperger-Suhler, 1983; Maruatona *et al.,* 2010). Marama flour contains much higher tyrosine content (6.2 g/100 g flour) than soya bean flour (1.8 g/100 g flour) (Maruatona *et al.,* 2010).

This study compares the chemical characteristics of marama protein in terms of constituent polypeptides and their structure with those in soya. This information is required to facilitate the effective use of marama protein as a functional ingredient in food systems.

3.2.2 Materials and methods

3.2.2.1. Materials

Marama bean specie *Tylosema esculentum* (Burch) A. Schreib was used. This was gathered from Rooidraaitrust, Gauteng Province, South Africa in 2008 (voucher specimen deposited at H.G.W.J. SCHWEICKERDT (PRU), Accession number: 113873). Soya bean (*Glycine max L.* Merr.) was obtained from AGRICOL, Pretoria, South Africa.

3.2.2.2. Chemicals

The following chemicals were used: Immobilized pH gradient (IPG) strips pH 3-11 NL and pH 6-11 and carrier ampholytes corresponding to each pH range; acrylamide, bisacrylamide; sodium dodecyl sulphate (SDS); N,N,N,N-tetramethyl-Ethylenediamine (TEMED); ammonium persulfate (APS); thiourea; urea; dithiothreitol (DTT); iodoacetaminde and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) from GE Healthcare (Uppsala, Sweden); methanol, *n*-hexane, 2-mercaptoethanol (2-ME), trichloroacetic acid (TCA), acetone and glycerol from Sigma-Aldrich (St. Louis, MO).

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3.2.2.3. Protein preparations

Marama beans were dehulled using a cracker (WMC Sheet Metal Works, Tzaneen, South Africa). Soya beans were dehulled with a Tangential Abrasive Dehulling Device (TADD) (Shepherd, 1979). Dehulled marama and soya beans were coarse milled in a food blender. The bowl (sample holder) and blade of the blender were pre-chilled at -20°C for 10 min to prevent heating during milling.

Defatted flours

Coarse milled flours were then defatted four times with *n*-hexane, flour: hexane ratio 1:5, for 90 min at room temperature. Defatted flours were placed in a fume hood overnight to remove the remaining hexane. The flours were milled in a laboratory attrition mill (Ika Werke, Staufen, Germany) and sieved through 0.5 mm opening screen. The protein contents of the defatted marama and soya flours were 55.7 and 50.0% (N x 5.71), respectively.

Purified protein extracts

Proteins were extracted from defatted marama and soya flours with 30 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, at a flour to solvent ratio of 1:20 (Aluko & Yada, 1995). Protein extraction was done at 40°C for 1 h in a shaking water bath. Crude protein contents (N x 5.71) of bean flours and protein extracts were determined by combustion analysis (AOAC method no. 968.06) (AOAC, 2000).

Total protein preparation

Total protein extraction was performed by the TCA/acetone precipitation method of Natarajan *et al*. (2005), except that DTT was included. The protein precipitate was suspended in 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer without DTT. Protein solutions were kept at -20°C until analysed. Protein concentrations were determined by the Bradford method (Bradford, 1976).

3.2.2.4. Amino acid analysis

The amino acid contents of bean flour and protein extracts were determined using the PICO.TAG-Method (Bidlingmeyer *et al*., 1984). This method is based on the principle of reverse phase chromatography with pre-column derivatization, following acid digestion.

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3.2.2.5. SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Taylor *et al*., 2007. Ten microlitre of aliquots containing 10, 15 and 20 µg protein was loaded onto a gradient gel of 4–12% polyacrylamide. Gels were stained with Coomassie Brilliant Blue R-250.

3.2.2.6. Two-dimensional gel electrophoresis

The Isoelectric Focusing (IEF) was performed using 13 cm IPG strips (pH 3–11 Non Linear, NL) and pH 6-11). The strips were focused on a gradient at 150 V for 2 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 3 h using the Ettan IPGphor II system (Amersham Biosciences, Uppsala, Sweden). After IEF, the strips were first equilibrated in equilibration buffer with DTT and then in buffer with iodoacetamide each for 15 min.

SDS-PAGE was carried out in a Hoefer SE 600 Ruby electrophoresis unit (Amersham Biosciences) at 30 mA/gels, voltage set at maximum. The 2-DE gels were stained with Coomassie Blue.

Gel images were acquired using a Versa Doc Documentation system (Bio-Rad, Hercules, Canada) and analysed with PDQuest 2 D Image Software. Master gels were generated for each sample. The 2-D electrophoresis soya protein spots were identified by reference to Natarajan *et al.* (2005) and Zarkadas *et al.* (2007) as well as maps from a database (Hajduch *et al*., 2005). It was attempted to identify the marama protein spots based on these.

3.2.2.7. Statistical analysis

The experiment was repeated twice. Chemical analyses were thus done on two separate protein extracts. One way analysis of variance was performed for protein content and amino acid composition and the means compared using Fisher's Least Significant Different Test at $p \leq 0.05$.

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3.2.3 Results and discussion

3.2.3.1. Protein contents and yields of marama protein extract

The protein content of the marama protein extract was high (86%) and similar to that of soya (Table 3.2.1). The protein yield in the marama extract (63%) was similar to that of soya (65%). The method used for preparing the extracts was a standard procedure for soya (Aluko & Yada, 1995). Therefore for the purpose of this study, the protein yields of the respective extracts were considered to be sufficiently representative.

Table 3.2.1 Protein content (dry basis) and protein yield of marama and soya protein extracts[a]

Samples	Protein content (N x 5.71) (g/100 g)	Protein yield (%)
Marama extract	$86.1^a \pm 1.5$	$62.8^a \pm 1.4$
Soya extract	$85.6^a \pm 1.3$	$65.3^b \pm 1.6$

[a]Mean ± SD (n = 4) is reported;

Mean values with different superscript letters in columns are significantly different ($p<0.05$)

3.2.3.2. Amino acid profile

Glutamic and aspartic acid, which may include glutamine and asparagine, respectively, were the major amino acids in the purified marama protein extract (Table 3.2.2) as well as in the flour (Maruatona *et al*., 2010). These are also the major amino acids in soya (Table 3.2.2). The lysine content of marama protein extract (5.7 g/100 g protein) was also similar to that of soya. Based on the FAO/WHO (1989) recommended pattern, marama flour and protein extract are adequate sources of lysine for pre-school children.

The tyrosine contents of marama flour and purified protein extract were very high (11 and 9 g/100 g protein, respectively), almost 3 times those of soya, thus confirming other reports on marama protein composition (Maruatona *et al*., 2010; Ripperger-Suhler, 1983). In fact, the tyrosine content of marama protein was substantially higher than that in other legumes, including peanuts (Venkatachalam & Sathe, 2006) and lupin (Lqari, 2002). Since tyrosine is involved in polypeptide crosslinking (Takasaki

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et al., 2005), the high tyrosine content in marama may contribute to the structural stability of its protein as suggested for gluten (Tilley *et al.*, 2001). The proline content of marama was also slightly higher than that of soya, which may affect its protein structure (Levit, 1981). Proline can impart a rigid structure to protein by participating in protein folding and unfolding (Levit, 1981).

Table 3.2.2 Amino acid composition (g/100 g protein) of marama and soya flours and their respective protein extracts[1]

	Flours		Protein extracts		FAO/WHO[2] recommended pattern	
Amino acid (AA)	Marama	Soya bean	Marama	Soya bean	Pre-school children (2-5 years)	Adult
Lysine	5.7[a]	6.3[b]	6.6[c]	6.9[c]	5.8	1.6
Isoleucine	4.3[a]	4.7[b]	5.0[c]	4.9[c]	2.8	1.3
Leucine	7.9[a]	9.8[c]	7.8[a]	9.4[b]	6.6	1.9
Methionine	1.0[a]	1.5[b]	1.2[a]	1.8[c]	2.5	1.7
Cysteine	0.1[a]	0.2[b]	0.2[b]	0.5[c]		
Phenylalanine	3.7[a]	4.0[b]	4.1[b]	4.9[c]	6.3	1.9
Tyrosine	11.4[c]	3.9[a]	9.1[b]	4.0[a]		
Valine	4.8[a]	5.0[b]	5.2[b]	4.9[a]	3.5	1.3
Threonine	3.2[a]	4.0[b]	3.2[a]	3.9[b]	3.4	0.9
Histidine	2.7[a]	3.1[b]	3.0[b]	2.7[a]	1.9	1.6
Subtotal Essential AA	*44.8*	*42.5*	*45.4*	*43.9*		
Aspartic acid/ Asparagine	9.4[a]	10.2[b]	9.5[a]	9.9[b]		
Glutamic acid/ Glutamine	15.2[b]	18.9[c]	13.2[a]	19.0[c]		
Serine	5.5[a]	5.6[a]	5.9	5.2		
Glycine	5.9[b]	4.3[a]	6.7[c]	4.4[a]		
Arginine	8.0[c]	7.8[b]	7.1[a]	7.6[b]		
Alanine	3.5[a]	4.5[b]	3.6[a]	4.4[b]		
Proline	7.2[b]	5.5[a]	8.3[c]	5.2[a]		
Subtotal Non-essential AA	*54.7*	*56.8*	*54.3*	*55.7*		
Recovery	*99.5*	*99.3*	*99.7*	*99.6*		

[1]Mean (n = 2) values with different superscript letters in rows are significantly different ($p<0.05$)

[2]FAO/WHO (1989) recommended pattern (Pre-school children age 2-5 years; Adults)

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3.2.3.3. Protein subunit composition

SDS-PAGE revealed differences in protein composition between marama and soya (Figs. 3.2.1 & 3.2.2). The protein profiles from defatted marama and soya flours (Fig. 3.2.1) were similar to those of their respective protein extracts (Fig. 3.2.2). This indicates that these protein extracts were representative of the storage proteins of each of the respective seeds. By SDS-PAGE, marama protein contained fewer major protein bands (three) compared with soya (approx. eight) (Mujoo *et al.,* 2003).

The patterns of these bands in marama under non-reducing and reducing conditions were similar, suggesting an absence of disulphide bonds. This was unlike the situation in soya where 11S storage proteins were separated under reducing conditions (lane 2), due to the fact that the 11S acidic and basic units are linked by disulphide bonds (Mujoo *et al.,* 2003; Kinsella*,* 1979). Further, in comparison with soya bean, the acidic 11S legumin subunits were absent in marama. Only a major broad band (around 20 kDa), was present in marama, corresponding to a basic 11S legumin was present.

Two major higher molecular weight protein bands were also observed in marama. There was a medium intensity protein band of approx. 63 kDa. Under non-reducing conditions, this appeared to match a major band in soya. However, while this band remained in marama under reducing conditions, it disappeared in soya, probably forming the 11S sub-units when separated under reducing conditions. The other protein band was a very high molecular weight band (148 kDa), which did not match any bands in soya. This protein band was also not reduced by mercaptoethanol, suggesting that its constituent polypeptides are not linked together by disulphide bonds. Since marama protein contains such a high level of tyrosine compared with soya (Maruatona *et al*., 2010) and tyrosine can participate in polypeptide crosslinking (Tilley *et al.,* 2001; Takasaki *et al*., 2005), it is suggested that dityrosine type of crosslinks may be responsible for the stability of this high molecular weight protein in marama.

Marama protein was further analysed using proteomic type 2-D electrophoresis to attempt to identify its constituent polypeptides by comparison with soya.

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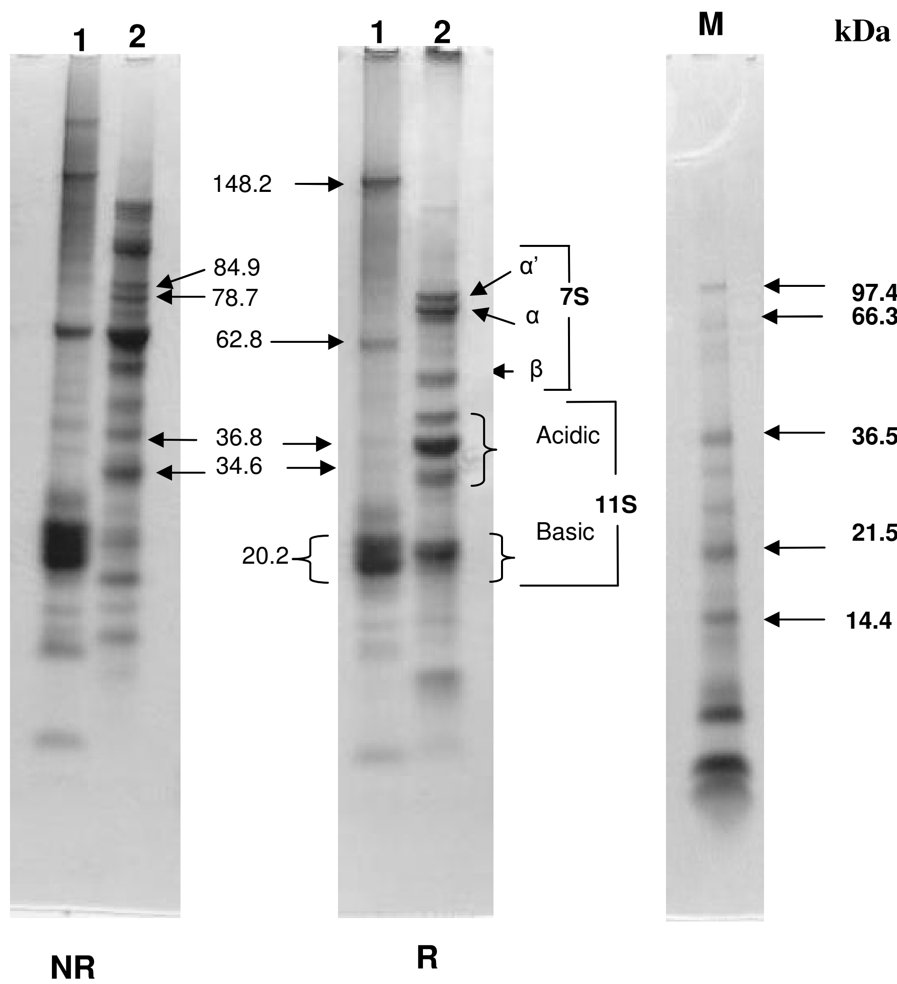

Figure 3.2.1 SDS-PAGE profile of defatted marama and soya bean flours under non- reducing (NR) and reducing (R) conditions, Lane 1: Marama, Lane 2: Soya, Lane M: Molecular weight standards

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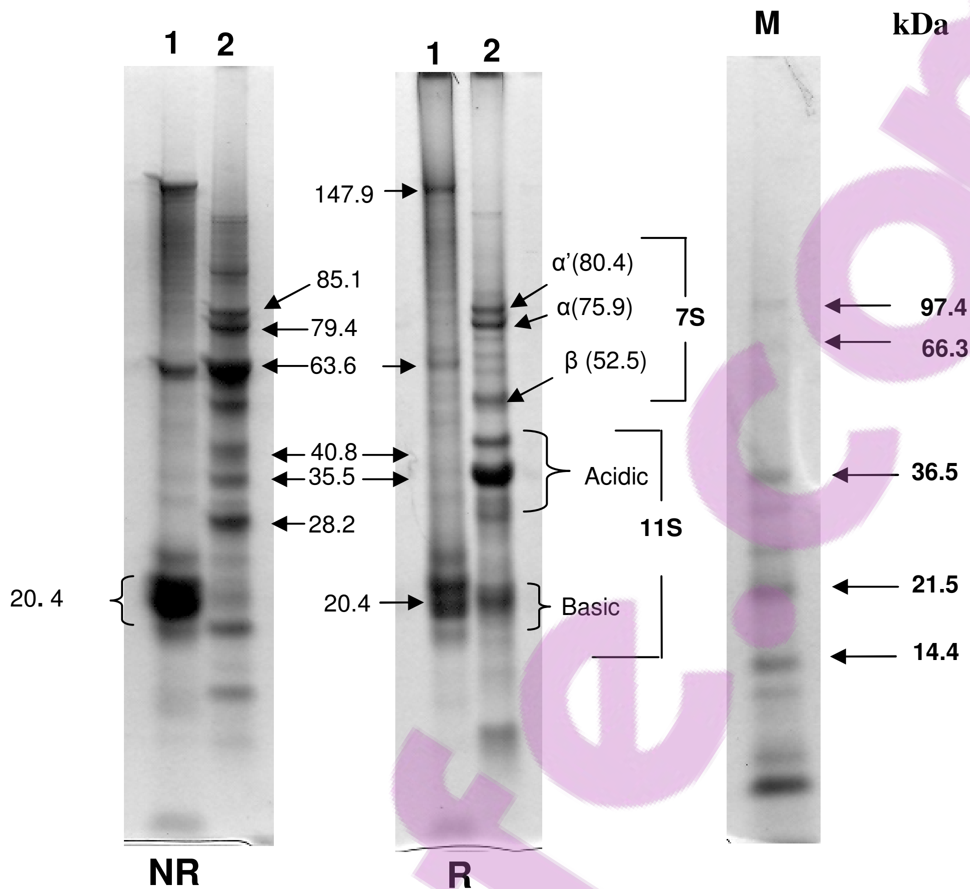

Figure 3.2.2 SDS-PAGE profile of extracted marama and soya bean proteins under non-reducing (NR) and reducing (R) conditions, Lane 1: Marama, Lane 2: Soya, Lane M: Molecular weight standards

3.2.3.4. Two-dimensional gel electrophoresis

Marama appeared to contain a similar number of polypeptides to soya, as indicated by protein spots (Fig. 3.2.3, Table 3.2.3). However, the total protein of marama and its purified protein extract contained more basic polypeptides than those of soya (Fig. 3.2.4 and Table 3.2.3).

As indicated by the proteome map, the vicilin (7S) subunits were apparently absent in marama, unlike soya (Fig. 3.2.3). The absence of 7S subunits in legumes is most unusual, if not unique. Variations in the major storage protein in terms of vicilin (7S) and legumin (11S) contents (Kinsella, 1979; Horax *et al.*, 2004) have been reported in the literature for some legume species (Kinsella, 1979; Horax *et al.*, 2004; Chel-

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Guerrero *et al*., 2007). For instance both 7S and 11S have been found to be major storage protein fractions of *Lupinus albus* (Duranti *et al.,* 1981) and soya bean (Kinsella, 1979), whereas the 7S has been found to be the major protein fraction of dry bean legumes such as mucuna bean (Adebowale *et al*., 2007) and cowpeas (Horax *et al.,* 2004).

Table 3.2.3 Polypeptide distribution and matches between marama and soya proteins[1]

	pH range			
	pH 3-11		pH 6-11	
Samples	Marama	Soya	Marama	Soya
Protein spots	276 ± 13	286 ± 21	153 ± 6	96 ± 5
Tentative spot matches	52 ± 5		26 ± 7	

[1]Mean ± SD number of protein spots in gels from two separate protein extracts

The protein band of 63 kDa from SDS-PAGE (Figs. 3.2.1 & 3.2.2) most likely corresponds to polypeptides with pI in the range of 6.4-7.5 and molecular weight of about 64 kDa on the marama proteome map (Fig. 3.2.3).

To provide a better separation of basic proteins of marama, proteomic analysis was performed on 13 cm strips, pH 6-11 (Fig. 3.2.3 A, B). The proteome map of marama that was obtained with IPG strip pH 6-11 (Fig. 3.2.4 A-B) further confirmed differences in proteome patterns between marama and soya with respect to the high number of basic polypeptides in marama. Compared with other legumes, the protein profile of marama seemed to be different from those of peanuts (Kottapalli *et al.* 2008) and also protein-rich oilseeds like canola (Uruakpa & Arntfield, 2006) and sunflower (Sammour *et al.,* 1995). Based on amino acid composition data of purified protein from this study and those published by Maruatona *et al.* (2010), marama protein has a lower acidic amino acid content than soya. The proteome pattern of marama thus seems to be related to its amino composition.

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Only one polypeptide in marama was tentatively identified by comparison with soya. This was spot 5 (Figs. 3.2.3 & 3.2.4), which appear to correspond with Glycinin G2: *A2B1,* a subunit of the 11S basic soya proteins. This single match with the 11S soya proteins and the apparent absence of 7S proteins indicates the storage proteins of marama are very different from those of soya.

Storage protein compositions have been found to significantly influence the functionality of proteins (German *et al.,* 1982; Tolstoguzov 1993; Yuan *et al.,* 2009). The absence of vicilin (7S) and the presence of more 11S basic protein in marama may increase the stability of protein to heat and extreme pH's, as suggested for 11S basic soya glycinin (Yuan *et al*., 2009). The stability of basic 11S of soya protein was associated with its high hydrophobicity compared with the acidic subunits and the total glycinin (11S) (German *et al.,* 1982; Yuan *et al.,* 2009). Marama protein has higher aromatic amino acid content than soya protein. These together with the aliphatic amino acids may increase the hydrophobicity and stability of marama protein in comparison with soya. Further, the high tyrosine in marama may also contribute to the stability of its protein structure as described previously.

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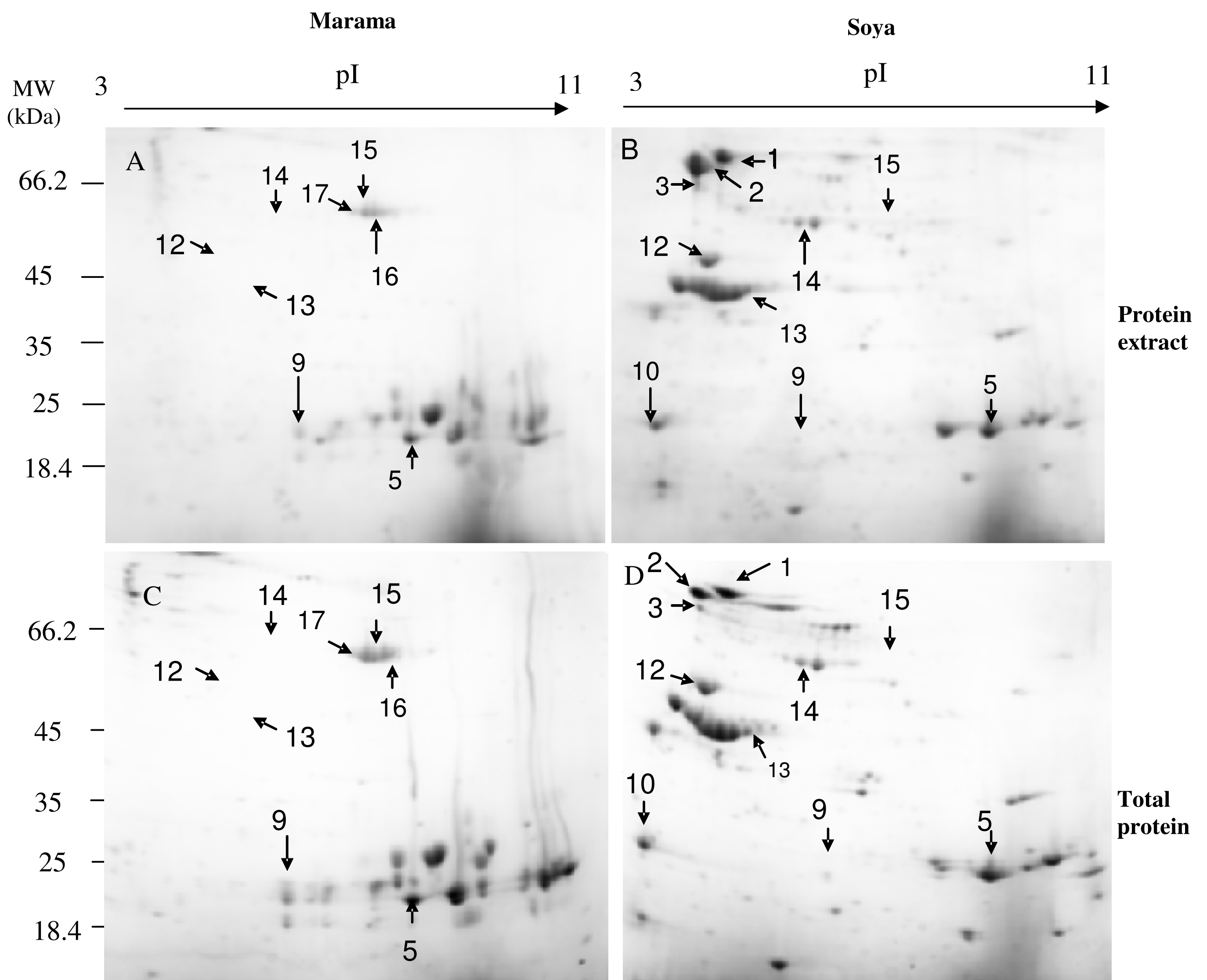

Figure 3.2.3 Two-dimensional maps of marama (A & C) and soya (B & D) proteins using Immobilized pH gradient (IPG) strips (3-11 Non Linear, NL)

A: marama protein extract, C: total marama protein, B: soya protein extract, D: total soya protein 1: α'-subunit of β conglycinin, 2 & 3: α - subunit of β conglycinin, 5: glycinin G2 A2B1, 10: soybean trypsin inhibitor, 12: Glycinin A3b4 subunit homohexamers, 13: Soybean proglycinin A1ab1b homotrimer, 14: β-Conglycinin β-homotrimer (Natarajan *et al.*. 2005; Hajduch *et al.*, 2005; Zarkadas *et al.*, 2007;), the same numbers on maps indicate spot matches, spots 9, 15, 16, 17 not identified

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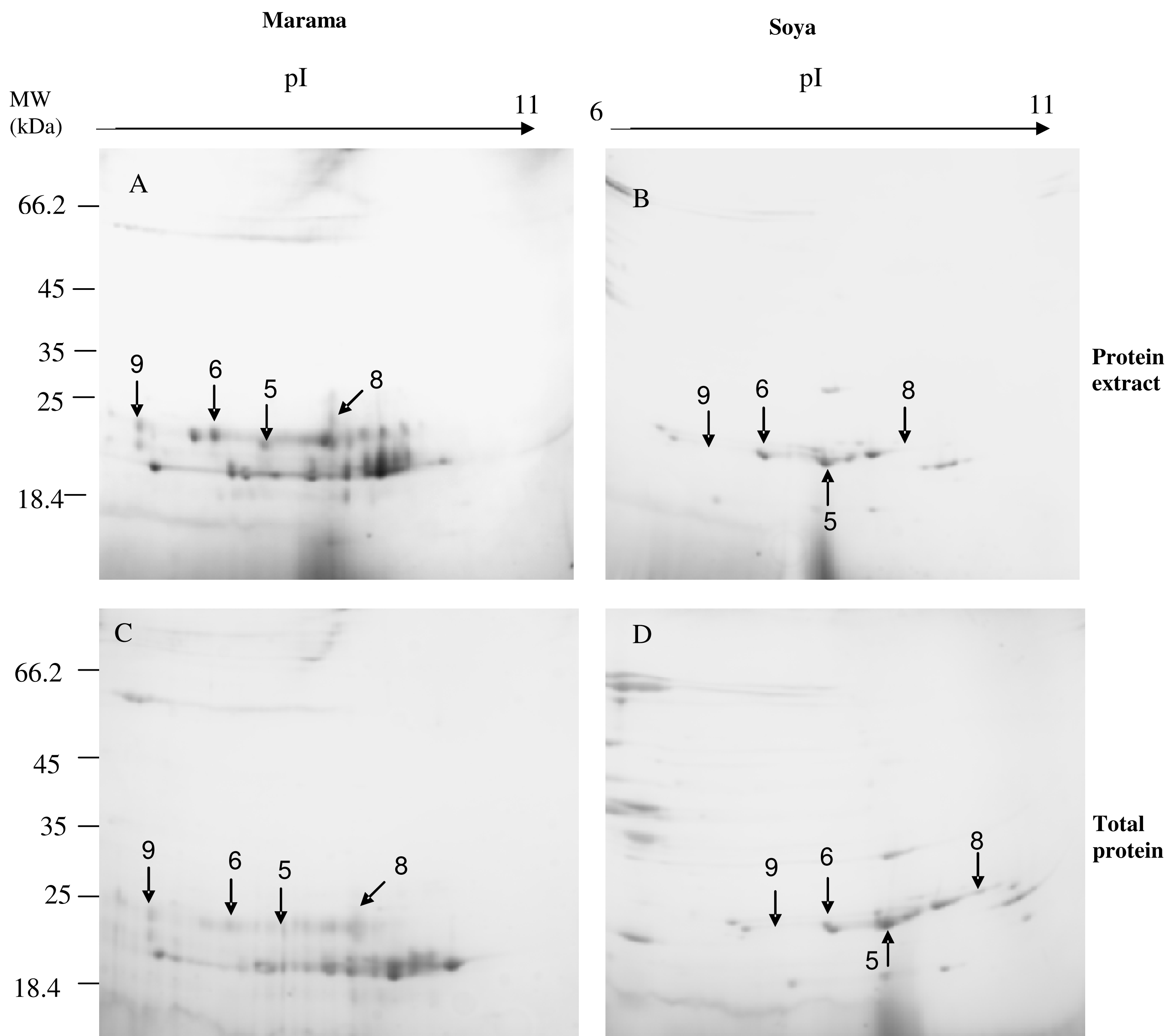

Figure 3.2.4 Two-dimensional proteome maps of marama (A & C) and soya (B & D) proteins using Immobilized pH gradient (IPG) strips (6- 11) A: marama protein extract, C: total marama protein, B: soya protein extract, D: total soya protein 5: Glycinin G2 A2B1 (Natarajan *et al.*, 2005; Hajduch *et al.*, 2005; Zarkadas *et al.*, 2007;), the same numbers on maps are used to indicate polypeptide matches, spots 6, 8, 9 not identified

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3.2.4 Conclusions

The protein composition of marama bean is very different from that of soya bean. The vicilin (7S) and the acidic 11S subunits seem to be absent in marama protein. The only major proteins in marama are basic legumin (11S) and two additional proteins of relatively higher molecular weight. Also, marama protein seems to lack disulphide bonds. These findings suggest that marama protein may have increased heat stability compared with soya, due to greater hydrophobic interactions, which are associated with the basic 11S. Since tyrosine is involved in polypeptide crosslinking, the high level of tyrosine in marama may also contribute to the structural stability of its protein.

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3.3. THERMAL AND RHEOLOGICAL PROPERTIES OF MARAMA BEAN PROTEIN

Abstract

Marama bean is an indigenous southern Africa oilseed legume. In this study, thermal and rheological properties of marama protein were determined in comparison with soya. Marama protein was characterised by one major endothermal transition (96°C) as compared with soya protein which had two. At approximately 38% water content, the extensibility of marama protein was high (60 mm), about twice that of soya protein and gluten. Marama protein was highly adhesive compared with gluten. Dynamic oscillatory data indicated that marama protein has a less stable protein structure than gluten. With added peroxidase, the storage modulus (G') of marama protein dough increased with time, suggesting the formation of strong protein networks. SDS-PAGE and HPLC/MS data from incubated doughs suggest that dityrosine crosslinks may be important in increasing the structural stability of marama protein. The stability of marama protein to heat is high compared with that of soya. Marama protein is characterised by viscous behaviour and extensibility.

Key words: marama protein, extensibility, soya protein, gluten, endothermal transition

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3.3.1. Introduction

Marama bean is an indigenous Southern African oilseed legume (Coetzer & Ross, 1976). Its protein content (approx. 35%) is similar to soya (Mujoo *et al*., 2003) and peanuts (Venkatachalam & Sathe, 2006). In previous research (Section 3.2), marama protein composition has been found to be very different from that of soya. The tyrosine content of marama protein is high (11 g/100 g protein); almost 3 times that of soya. Marama protein is also slightly richer in proline compared to soya protein. The vicilin (7S) and acidic legumin (11S) subunits seem to be absent in marama. Only basic legumin (11S) is present. Protein composition and structure of legume proteins have been found to influence their functionality (German *et al.,* 1982; Tolstoguzov, 1993; Yuan *et al*., 2009). In this study, thermal and rheological properties of marama protein were determined in comparison with soya protein. .

3.3.2. Materials and methods

Marama protein was extracted as described in research Section 3.2. Soya protein extracted in the same way as marama protein was used as reference. Vital gluten (commercial) was also included in the study for comparison.

3.3.2.1. Chemical composition of protein preparations

Dry matter, ash and protein contents of protein preparations were determined as described in research Section 3.1.

3.3.2.2. Thermal properties

These were determined by Differential Scanning Calorimetry (DSC) (Horax *et al.*, 2004). Protein dispersions (10% w/v) were prepared with 0.05 M phosphate buffer (pH 7.0). Well mixed dispersions (15 mg) were weighed into 40 µl aluminium pans and hermetically sealed. These were allowed to equilibrate for 2 h and scanning was performed from 20-120°C at 10°C/min. A sealed empty pan was used as reference. Calorimetric measurements were done in triplicate using a Mettler DSC 1 (Toledo, Columbus, OH).

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3.3.2.3. Rheological properties

Description of protein dough preparation for rheological measurements

Protein doughs at varying water contents (37.8, 44.8 and 52.3%) were prepared by mixing freeze dried protein extracts with water using a spatula and kneaded by hand until a homogenous dough was obtained (Schober *et al*., 2008).

Extensibility of protein dough

The extensibility of protein dough was measured with a TA-XT2 Texture Analyser (Stable Micro Systems Ltd, Godalming, England). Dough (3 g) was placed on a lubricated lower plate of a mould and compressed with a lubricated top plate. Olive oil was used as the lubricant. The dough was left to rest for 15 min and then cut into a strip (60 mm x 0.5 mm) using a lubricated razor blade to prevent sticking. A hook to lift the strip of dough was attached to the Texture Analyser equipped with 5 kg load cell (Selinheimo *et al*., 2007). Dough strips were placed in the sample holder having a gap of 23 mm and held firmly in place on each side of the holder by double-sided adhesive tape. Measurement was carried out within 1 min to prevent moisture loss. The test was carried out in a tension mode. TA-XT2 settings were: Pre-test: 2.0 mm/s, Test speed 3.3 mm/s, Post-test speed: 10 mm/s, Distance 120 mm, Trigger force: auto- 5 g. The test was repeated at least 3 times per sample. The peak force or resistance to extension and the distance of dough extension at which the peak force occurred were measured.

Stickiness of protein dough

Dough stickiness was measured with a TA-XT2 Texture Analyser using SMS/Chen-Hoseney dough stickiness rig (A/DSC) and a 25 mm perpex cylinder probe (P/25P) (Grausgruber *et al.,* 2003). Dough (1g) formed into a ball was allowed to rest for 15 min and placed in the sample chamber. The internal screw was rotated to extrude a small amount of the dough through the extrusion hole. This first extrudate was removed with a spatula from the stickiness rig. The perspex cap was placed on the extrudate lid and approximately 1 mm dough was extruded by rotating the screw again. The screw was rotated slightly backwards to reduce pressure and to ensure that the dough was not extruded further under tension. After resting for 30 s, the dough stickiness cell was placed directly under the texture analyser probe. The perspex cap was removed and the test was started. The Texture Analyser settings for these

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measurements were as follows: Test option: adhesive test, Pre-test speed: 0.5 mm/s, Test speed: 0.5 mm/s, Post-test speed: 10 mm/s, Distance: 4 mm, Force: 40 g, Trigger-type: auto-5g, Data acquisition: 500 pps. The stickiness test was done in triplicate for each sample.

Dynamic small oscillatory measurement

Dynamic small oscillatory experiments were performed using Physica MCR 301 Rheometer (Anton Paar, Ostfildern, Germany) equipped with two parallel plates (d= 25 mm). The gap between the plates was set at 2 mm for all experiments. Dough (3 g) was loaded on a temperature controlled (25°C) lower plate and the upper plate was lowered onto the dough. Excess dough was trimmed off using a razor blade and the periphery of the sample was covered with a thin layer of paraffin oil to prevent dehydration (Oom *et al.*, 2008). The dough was allowed to equilibrate for 15 min before the start of each test. To determine the linear viscoelastic range, a series of amplitude sweep tests was conducted over strain amplitude range of 0.01-100% at a constant angular frequency of 6.28 rad/s.

The mechanical spectra of protein doughs were determined by performing an oscillatory shear tests over a frequency range of 0.01 to 100 rad/s, at a constant strain amplitude of 0.5%, which was within the linear viscoelastic region. Storage modulus G', loss modulus G" and tan δ were continuously monitored during. All tests were repeated at least twice per sample.

3.3.2.4. Rheological properties of peroxidase treated protein dough

The effect of peroxidase on dough rheological properties was monitored by performing a time sweeps test. 85 U of horseradish peroxidase (236 units/mg) (Sigma, P8250) and 100 µl of 30% hydrogen peroxide were added to 3 g of dough at 44.8% water content (flour basis). Dough was incubated in-situ in a temperature controlled rheometer where plates were set at 37°C. The dough was allowed to equilibrate for 15 min and the time scan was conducted over a period of 10800 s at a constant angular frequency of 6.28 rad/s and 0.5 % strain amplitude (within the linear viscoelastic range). The reference dough sample was incubated in the same manner but without peroxidase and hydrogen peroxide. Storage modulus, loss modulus and tan & were recorded every 60s. The test was repeated twice per sample. At the end of the time

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scan, the dough was recovered, flash frozen in liquid nitrogen and kept at -20°C. Frozen dough samples were freeze dried and used in subsequent experiments.

SDS-PAGE

Dough samples were analysed by SDS-PAGE under reducing and non-reducing conditions (on a gradient gel containing 4–12% polyacrylamide (Taylor *et al.,* 2007). Gels were stained overnight with Coomassie Brilliant Blue R-250.

HPLC/MS

Tyrosine and dityrosine in protein extracts and dough samples were determined by Reverse-phase HPLC and Mass Spectrometry (MS) (Michon *et al*., 1999). Proteins were hydrolysed in 6 N HCl (0.05 µg protein/µl) containing 0.1% phenol under vacuum for 24 h at 110°C (Tilley *et al.*, 2001). Hydrolysed protein samples were dried under vacuum in a rotary evaporator at 50°C. The dried sample was solubilised with water containing 0.06% Trifluoroacetic acid (TFA) using syringe driven filter unit (Millex, LH, 0.45 µm, Nihon Millipore, Tokyo, Japan). The detection of these aromatic compounds was done at 283 nm (Michon *et al*., 1999). The amino acids were separated on Reverse phase C_{18} columns (250 X 4.6 mm I.D., 5 µm, 12 nm, YMC Co. Ldt, Kyoto, Japan), flow rate 1 ml/ min, and sample size of 20 µl. Mobile phases consisting of 75% acetonitrile containing 0.04% TFA (medium B) and water containing 0.06% TFA were used. After 5 min wash, a 5% to 25% gradient was applied over 8 min (Michon *et al*., 1999).

The amino acid fractions recovered by HPLC were further analysed with a Linear Ion Trap Triple Quadrupole Mass Spectrometer (4000 Q trap AB, Applied Biosystems, Carlsbad, CA, USA). The positive mode was used for ionisation. The mass scan was performed in the following ranges: 180-185 m/z for tyrosine and 350-370 m/z for dityrosine (Takasaki *et al.,* 2005).

3.3.2.5. Statistical analysis

Means were calculated and Analysis of Variance was performed on the data where appropriate. LSD (Fisher's test) was used for mean separation at a 5% level of significance. Statistica for Windows (Statsoft, Tulsa, OK, USA) package was used for data analysis.

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3.3.3. Results and discussion

3.3.3.1. Chemical composition of protein preparations

Marama and soya protein preparations had similar protein contents, which were higher than that of wheat gluten (Table 3.3.1). Moisture and ash contents were similar among the three protein samples. The protein content of the soya protein preparation is agreement with previous work (Hua *et al*., 2005). Labat *et al*. (2001) also reported a similar protein content for gluten. Chemical composition data for the marama protein preparation are in agreement with results from research Section 3.2.

Table 3.3.1 Chemical composition of marama protein, soya protein and wheat gluten preparations[1]

Samples	Moisture (g/100 g)	Protein (N x 5.7) (g/100 g)	Ash (g/100 g)
Marama protein	$4.5^{b} \pm 0.4$	$85.2^{b} \pm 1.0$	$1.1^{b} \pm 0.1$
Soya protein	$4.6^{b} \pm 0.5$	$84.7^{b} \pm 1.3$	$1.5^{c} \pm 0.2$
Wheat gluten	$3.7^{a} \pm 0.4$	$73.4^{a} \pm 1.2$	$0.6^{a} \pm 0.2$

[1]*Mean* (n = 4) ± *SD. Protein and ash contents are expressed on dry basis.*

3.3.3.2. Thermal properties of marama protein

Marama protein showed one major endothermal peak at around 96°C in contrast to soya protein which showed two peaks at around 76°C and 91°C, respectively (Fig. 3.3.1). These peaks in soya correspond to thermal denaturation temperatures of β-conglycinin and glycinin, respectively (Renkema *et al.*, 2001; Shand *et al.,* 2007). Differences in network structure and interaction have been found to be responsible for differences in thermal behaviour between the two soya protein fractions (German *et al.*, 1982; Renkema *et al.,* 2001). The single transition observed in marama was very broad. The enthalpy (8.5 ± 0.4 J/g) of marama protein was slightly higher than that of soya glycinin (11S) (5.0 ± 0.6 J/g). This indicates that marama protein has greater heat stability compared to soya glycinin (11S).

In soya bean, disulphide bonds between pairs of acid and basic polypeptides subunits of glycinin (11S) have been found to be mainly responsible for its stability (Kinsella, 1979; Mujoo *et al*., 2003). In research Section 3.2, most polypeptides in marama were

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found to be basic legumin (11S). The acidic 11S subunits were absent and there was also no evidence of disulphide bonds. Unlike hydrogen bonds and electrostatic interactions, hydrophobic interactions are endothermic. As a result, they are stabilised as temperature increases (Creighton, 1993). The thermal stability of marama protein may therefore be attributed to hydrophobic protein interactions as suggested for the basic soya glycinin (German *et al*., 1982). The high tyrosine content in marama protein (Table 3.2.2) may also contribute in a way to the structural stability of its protein structure. This is because tyrosine is involved in protein crosslinking (Takasaki *et al*., 2005).

Furthermore, since no transition corresponding to vicilin (7S) was observed in marama protein compared to soya protein, DSC data seem be in agreement with protein composition results (Figs 3.2.2 & 3.2.3), thus confirming the absence of vicilin (7S) fraction in marama protein. .

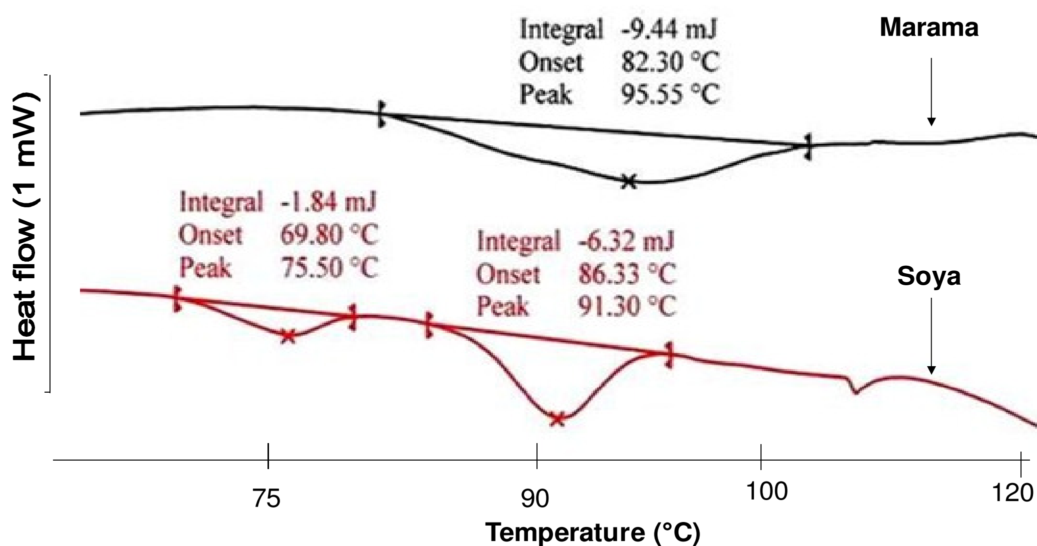
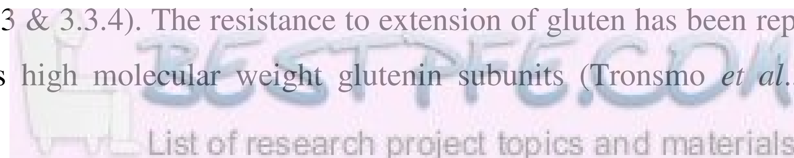


Figure 3.3.1 Typical DSC thermograms of marama and soya protein preparations

3.3.3.3. Rheological properties of marama protein

Marama protein was more extensible than the wheat gluten (Fig. 3.3.2). However, gluten showed more resistance to extension compared to marama protein. Soya protein had a significantly reduced extensibility when compared with marama protein (Figs. 3.3.2, 3.3.3 & 3.3.4). The resistance to extension of gluten has been reported to be related to its high molecular weight glutenin subunits (Tronsmo *et al*., 2003),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which are mainly stabilised by disulphide bonds (Kinsella, 1979; Song & Zheng, 2007). The extensibility of both marama protein and gluten was increased with increasing dough water content. This is probably due to the plasticising effect of water. At approximately 38% water content, the extensibility of marama protein (60 ± 2.4 mm) was about twice that of gluten. Marama protein was extremely extensible (> 120 mm) when the dough water content was increased from 38 to 45% (Fig. 3.3.3). At 52% water content, marama protein appeared more like a viscous fluid. This is unlike the situation with gluten where it was possible to form dough with well defined shape at all the water contents.

Due to the difficulties in forming dough with soya protein and the lack of the repeatability of results, soya was not included in subsequent rheological measurements.

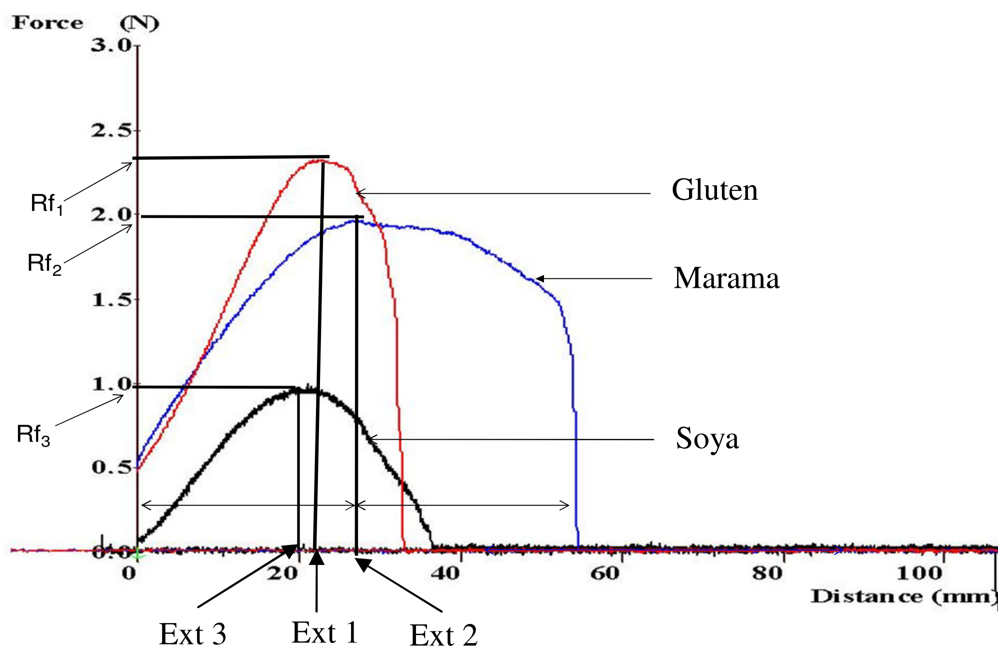

Figure 3.3.2 Typical deformation curves for marama protein, soya protein and gluten at 37.8% water content (flour basis)
Rf: Maximum resistance force to extension, Ext: extensibility at break

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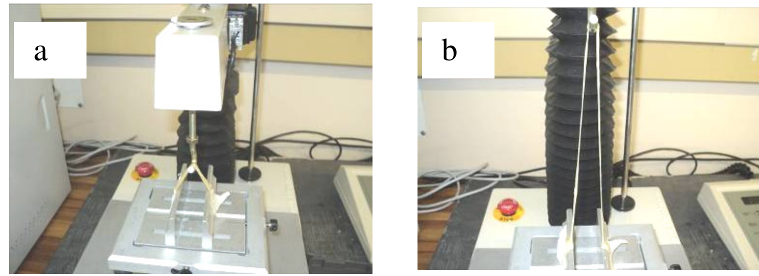

Figure 3.3.3 Extensibility of marama protein at 45% water content (flour basis) using a TA-XT2 Texture Analyser
a: Start, b: Extension

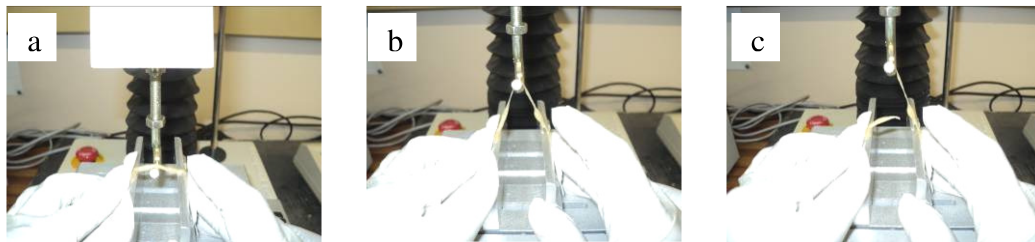

Figure 3.3.4 Extensibility of soya protein using a TA-XT2 Texture Analyser
a: Start, b: Extension, c: Break

The adhesiveness of marama protein at varying water contents was further compared with that of gluten. This was estimated from the stickiness analysis (Figure 3.3.5). The maximum force (287 ± 15; 271 ± 23 and 264 ± 17 g at approximately 38, 45 and 52% water contents, respectively) attained before marama dough separated itself from the texture analyser probe was high, more than twice that of gluten. This force (also referred to as degree of stickiness) may be considered as force of adhesion (Adhikari *et al.,* 2001), holding the protein dough to the surface of the texture analyser probe. Marama protein thus formed a highly adhesive (sticky) dough compared with gluten. This is further indicated by high relaxation gradient of marama stickiness curves compared with gluten (Figure 3.3.6). Similarly, Dobraszczyk (1997) reported greater relaxation gradients for highly sticky dough. According to Huang and Hoseney (1999), sticky dough is a viscous dough that will flow and not overcome the adhesive forces.

Dynamic rheological measurements were then determined to elucidate the rheological behaviour of marama protein

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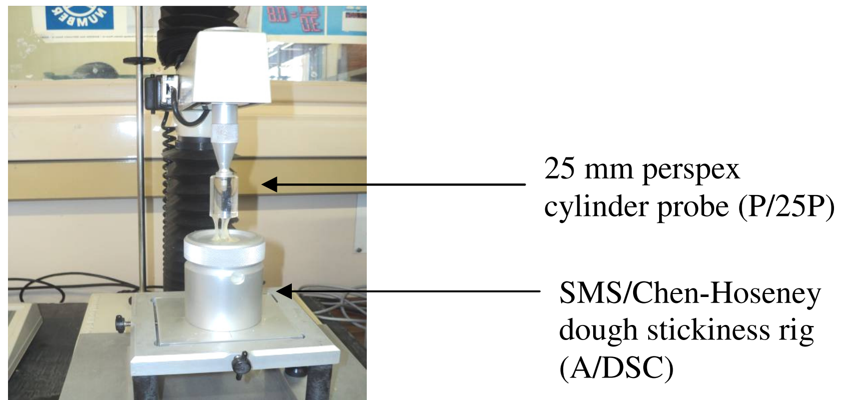

Figure 3.3.5 Measuring the stickiness of marama dough with a Texture Analyser using SMS/Chen-Hoseney dough stickiness rig (A/DSC) and a 25 mm perspex cylinder probe (P/25P)

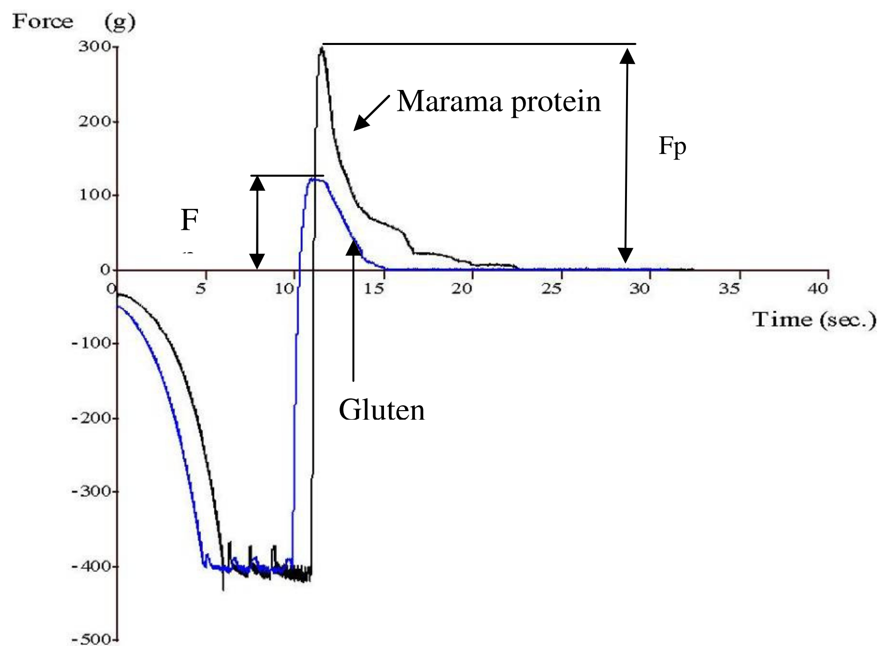

Figure 3.3.6 Typical stickiness curve of marama protein and wheat gluten dough at 37.8% water content (flour basis)

Fp: maximum peak force at which the dough separates itself from the probe

The linear viscoelastic range (LVR) of marama protein was low, within 1% strain at approximately 38% water in comparison with gluten (15%). Unlike gluten, the LRV of marama increased from 1 to 5% strain when dough water contents were increased

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from 38 to 52%. LVR of gluten in this study was within the range reported by Letang, Piau and Verdier (1999). The shorter LVR of marama compared to gluten suggests that it has a less stable protein structure.

Small oscillatory deformation performed within this linear range revealed the frequency dependency of storage (G') and loss (G") moduli for marama protein to be similar to that of gluten. Both G'and G" seemed to increase with increasing frequency (0.1 – 100 Hz) (Fig. 3.3.7). However, G' and G" decreased with increasing dough water contents (Table 3.3.2). This is because water has been reported to behave like a plasticizer reducing both the viscous and elastic behaviour of proteins (Masi *et al*., 1998). Similar reductions in G' and G" with increasing water content have been reported for wheat flour dough (Letang *et al*., 1999). The loss tangent (Tan δ) values were low (0.2-0.5) for marama protein and these were similar to those of gluten (Table 3.3.2). Tan δ, defined as the ratio of G" over G', reflects the balance between the viscous and the elastic character of a viscoelastic material. Small dynamic deformation analysis is non-destructive and thus provides some information on the types of molecular bonding that may be responsible for the structural stability of proteins. A low tan δ value is often associated with a high degree of crosslinking (Tsiami *et al*., 1997; Mezger, 2006) and a stronger protein network. Marama protein thus appeared to have some structural stability, but only when small deformations were applied.

The difference in rheological behaviour between marama protein and gluten may be attributed to differences in types of molecular bonding responsible for the structural stability of the two proteins. However, marama protein may be similar to wheat gliadin when considering its rheological behaviour. The gliadin in gluten has been found to act like a plasticizer promoting gluten viscous behaviour and extensibility (Orth & Bushuk, 1972; Zaidel *et al*., 2010). In wheat gluten, subunits can associate through hydrogen bonds (Belton, 1999). Unlike covalent disulphide bonds, hydrogen bonds can interchange easily (Belitz *et al*., 1986), thus promoting viscous flow. Hydrogen bonding can form between the amide group of amino acids and glutamine. From research Section 3.2, there was no evidence of disulphide bonds in marama protein. Glutamic acid, which may include glutamine, is the most abundant amino acid in marama protein, accounting for about 15% of all amino acids. The viscous behavior and extensibility of marama protein may be attributed to hydrogen bonds

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forming among the various subunits. Furthermore, in addition to non polar amino acids, marama protein has a higher content of aromatic amino acids compared to soya protein, which can also impart its hydrophobicity. Hydrophobic interactions may also contribute to the high stickiness (that is cohesion-adhesion properties) of marama protein. Possible covalent crosslinking between the tyrosine residues in marama protein may also play role.

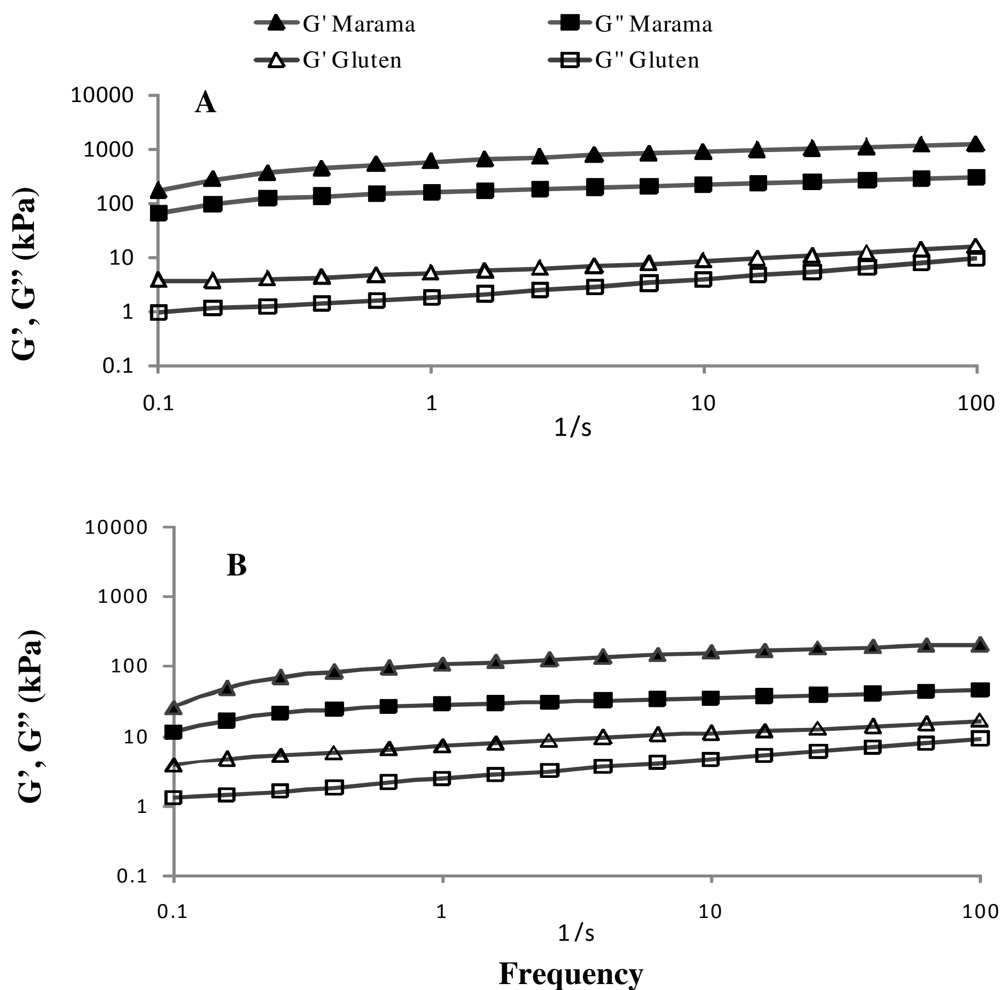

Figure 3.3.7 Typical mechanical spectra of marama protein and gluten preparations as a function of frequency

A: 45% water content, B: 52% water content (flour basis)

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Table 3.3.2 Rheological properties of marama protein and gluten preparations[1]

Sample	ω (1/s)	G' [kPa]	G" [kPa]	Tan & [1]	ŋ* [kPa.s]
Dough at 45% water (flour basis)					
Marama	1	319.3 ± 38.9	86.7 ± 10.6	0.3 ± 0.0	330.5 ± 40.4
	10	487.8 ± 57.9	119.6 ± 14.6	0.3 ± 0.0	50.2 ± 6.1
	63.1	648.1 ± 79.4	157.5 ± 18.7	0.2 ± 0.0	10.6 ± 1.3
	100	681.0 ± 83.2	167.5 ± 19.5	0.3 ± 0.0	7.0 ± 0.8
Gluten	1	6.4 ± 1.8	2.3 ± 0.7	0.4 ± 0.0	6.8 ± 1.9
	10	10.8 ± 3.0	4.9 ± 1.2	0.4 ± 0.0	1.2 ± 0.3
	63.1	17.9 ± 5.1	10.0 ± 2.8	0.5 ± 0.0	3.3 ± 0.1
	100	20.5 ± 5.8	12.1 ± 3.4	0.5 ± 0.0	2.4 ± 0.1
Dough at 52% water (flour basis)					
Marama	1	90.8 ± 24.3	24.3 ± 6.2	0.3 ± 0.0	93.6 ± 2.5
	10	137.5 ± 31.8	31.4 ± 6.1	0.2 ± 0.0	14.1 ± 3.2
	63.1	178.5 ± 34.6	39.4 ± 6.2	0.2 ± 0.0	2.9 ± 0.6
	100	187.0 ± 31.1	42.2 ± 5.7	0.2 ± 0.0	1.9 ± 0.3
Gluten	1	7.2 ± 0.1	2.5 ± 0.0	0.3 ± 0.0	7.6 ± 0.1
	10	11.4 ± 0.3	4.9 ± 0.2	0.4 ± 0.0	1.2 ± 0.0
	63.1	16.8 ± 0.2	8.7 ± 0.9	0.5 ± 0.0	0.3 ± 0.0
	100	18.8 ± 0.2	10.2 ± 0.1	0.5 ± 0.0	0.2 ± 0.0

[1]Mean ± SD

3.3.3.4. Rheological properties of peroxidase treated marama protein dough

Changes in rheological properties of marama protein dough treated with peroxidase (POX) and untreated (no POX) were monitored over time. This was done to see whether the high tyrosine content in marama protein could contribute to its structural stability. With added peroxidase, the storage (G') modulus of marama protein dough increased with time (Fig. 3.3.8). Although a similar pattern was recorded for the untreated marama protein dough, the values of G' were lower than those of the treated dough. The rheological behaviour of marama protein dough was different from that of gluten dough (Fig. 3.3.8). Only a slight increase of G' was observed for treated gluten dough with time, while the untreated gluten dough remained almost unchanged during the time of incubation. The increase in G' for POX treated marama protein dough indicates that new protein networks were formed. These new networks probably resulted from tyrosine oxidation and crosslinking. Takasaki *et al.* (2005)

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reported the formation of dityrosine crosslinks in wheat flour dough when incubated with POX and hydrogen peroxide. The difference in dough response to POX between marama protein dough and gluten dough may be attributed to differences in tyrosine contents between the two proteins and to some extent to the accessibility of POX enzyme to tyrosine. The increase in G' for the untreated marama protein dough may also be due to tyrosine oxidation caused by reaction with atmospheric oxygen and /or endogenous enzymes present in the dough.

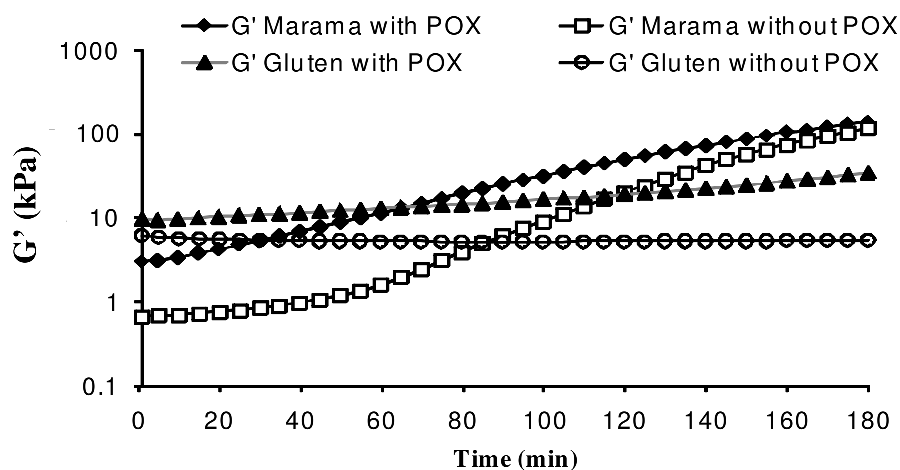

Figure 3.3.8 Storage modulus (G') of marama protein and gluten treated with (POX) as a function of time
(Mean of duplicate samples is plotted)

SDS-PAGE of marama protein doughs collected at the end of incubation was performed to determine the types of protein crosslinks that were formed. Unlike gluten dough, SDS-PAGE of marama protein dough revealed the presence of high molecular protein bands under reducing condition indicated by the arrows in lane 1, Fig. 3.3.9. The absence of high molecular weight proteins in gluten under reducing condition is due to the fact that disulphide types of linkages are responsible for its structural stability (Song & Zheng, 2007). The high molecular weight proteins in marama protein are most likely stabilised by dityrosine types of crosslinks formed during tyrosine oxidation in the presence of POX.

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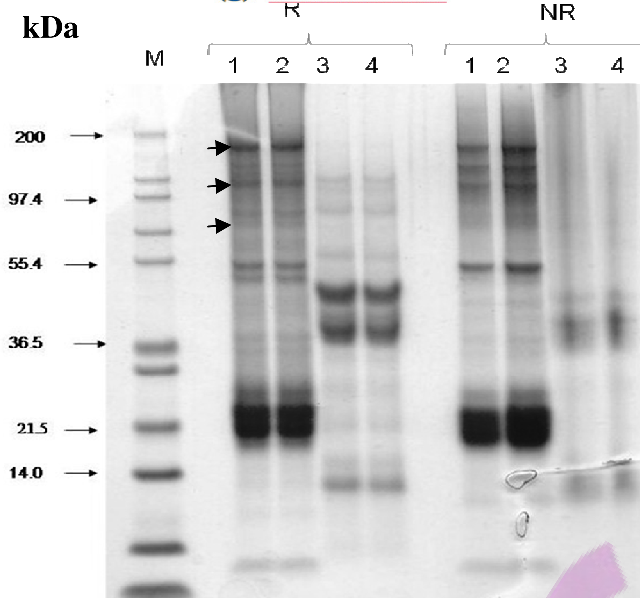

Figure 3.3.9 SDS-PAGE of marama protein and gluten doughs treated with POX under reducing (R) and non-reducing (NR) conditions, Lane M: Molecular standards, Lane 1: Marama with POX, Lane 2: Marama without POX, Lane 3: Gluten with POX, Lane 4: Gluten without POX

To further elucidate the types of crosslinks responsible for the structural stability of marama protein, marama protein extract and incubated dough samples were analysed by HPLC. Tyrosine eluted at about 12 min in marama and soya samples (Figs. 3.3.10 & 3.3.11). Spiking of protein hydrolysates with tyrosine standard was used to confirm the tyrosine peak. In addition to tyrosine peak, POX treated marama protein dough had about 10 additional peaks, similar to gluten (Figs. 3.3.12 & 3.3.13). The concentrations of these peaks were low in untreated proteins compared to those treated with POX. Among these peaks, those eluting at around 16.5, 17.5, 18, and 22.3 min were tentatively identified as tyrosine crosslinks by reference to Michon *et al*. (1999), Tilley *et al.* (2001), Peña *et al.* (2006).

To confirm the identity of the eluted compounds, they were analysed by Quadrupole mass spectrometry. A compound with m/z of 182.2 corresponding to tyrosine (Tilley *et al*., 2001; Peña *et al*., 2006) was detected in the compound eluting at about 12 min (Fig. 3.3.12). Among the remaining peaks, only those recovered at 16.5, 17.5, 23.2 min revealed compound with m/z 360.5 or 361 (Fig. 3.3.14), which corresponds to dityrosine crosslinks (Peña *et al.,* 2006; Takasaki *et al.,* 2005). Possibly, different types of tyrosine crosslinks including di, tri and a more complex form have been obtained. Marama protein seems to contain some dityrosine crosslinks with additional

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similar crosslinks forming during incubation of dough with POX. Dityrosine crosslinks may therefore be important in structural stability of marama protein.

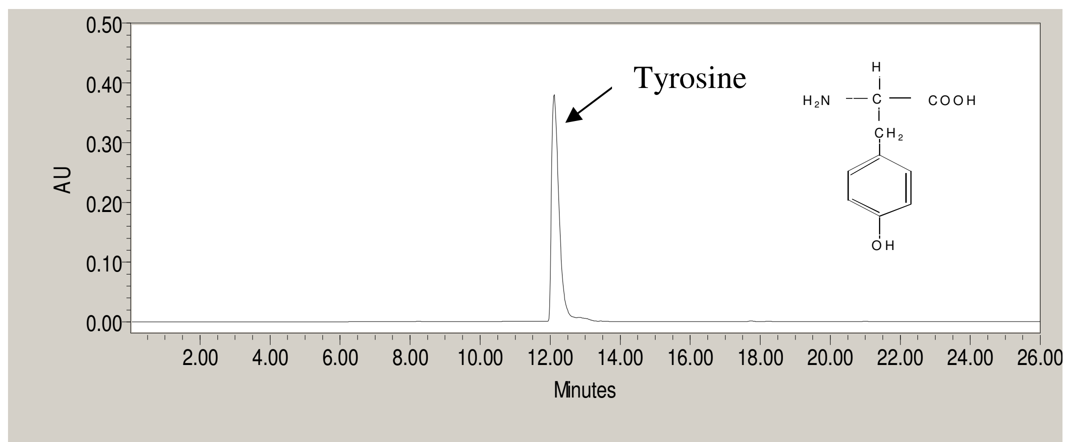

Figure 3.3.10 Reverse - phase HPLC of tyrosine standard

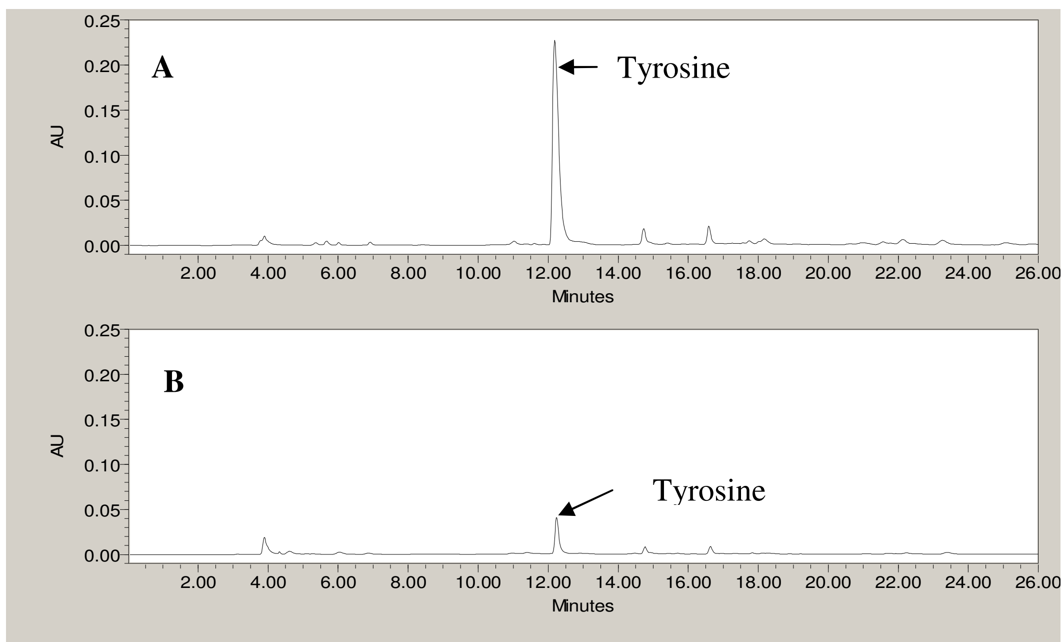

Figure 3.3.11 Reverse - phase HPLC of hydrolysed marama and soya proteins
A: marama, B: soya

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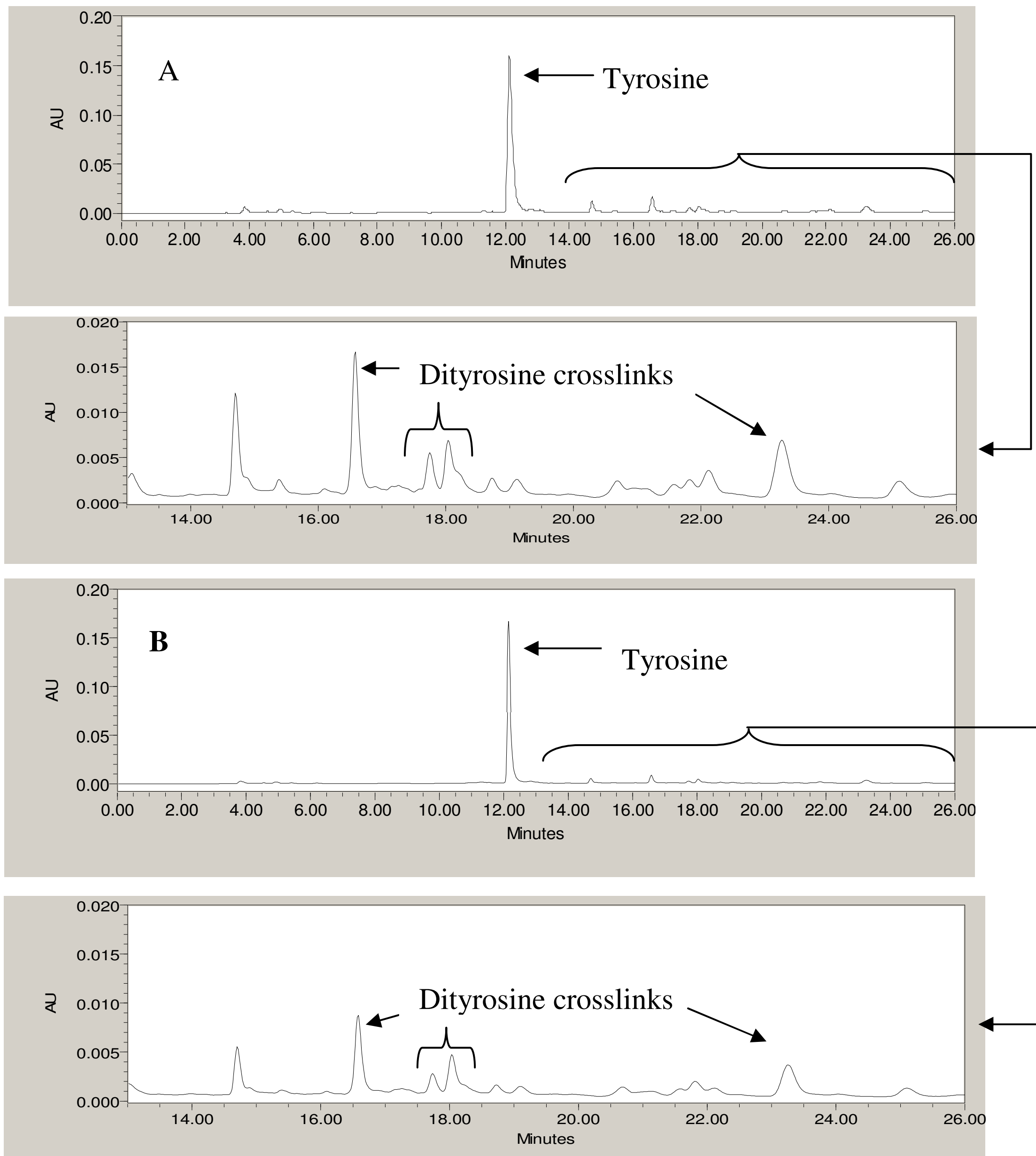

Figure 3.3.12 Reverse - phase HPLC of hydrolysed marama protein dough treated with POX

A: marama with POX, B: marama without POX

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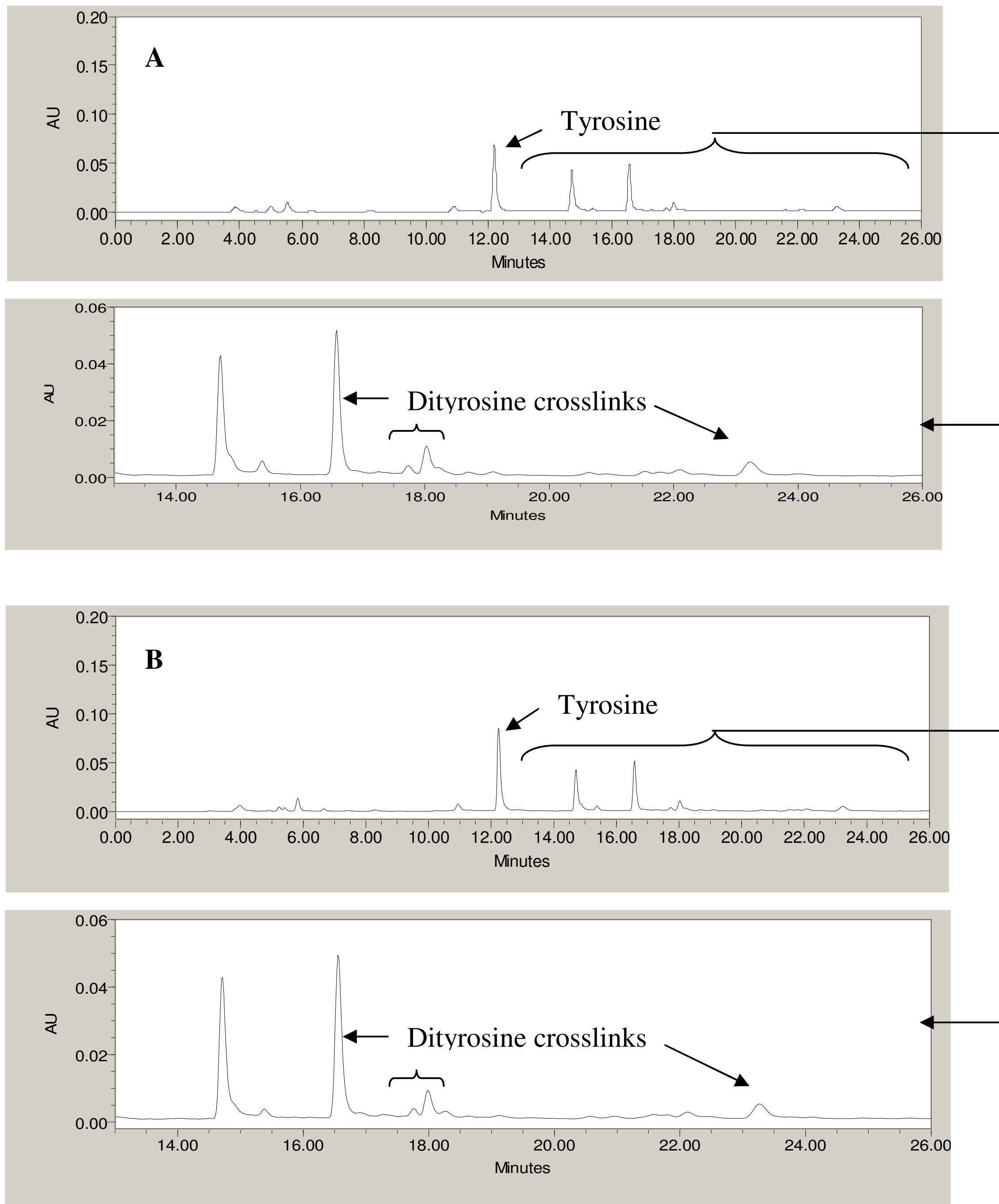

Figure 3.3.13 Reverse - phase HPLC of hydrolysed gluten dough treated with POX A: gluten with POX, B: gluten without POX

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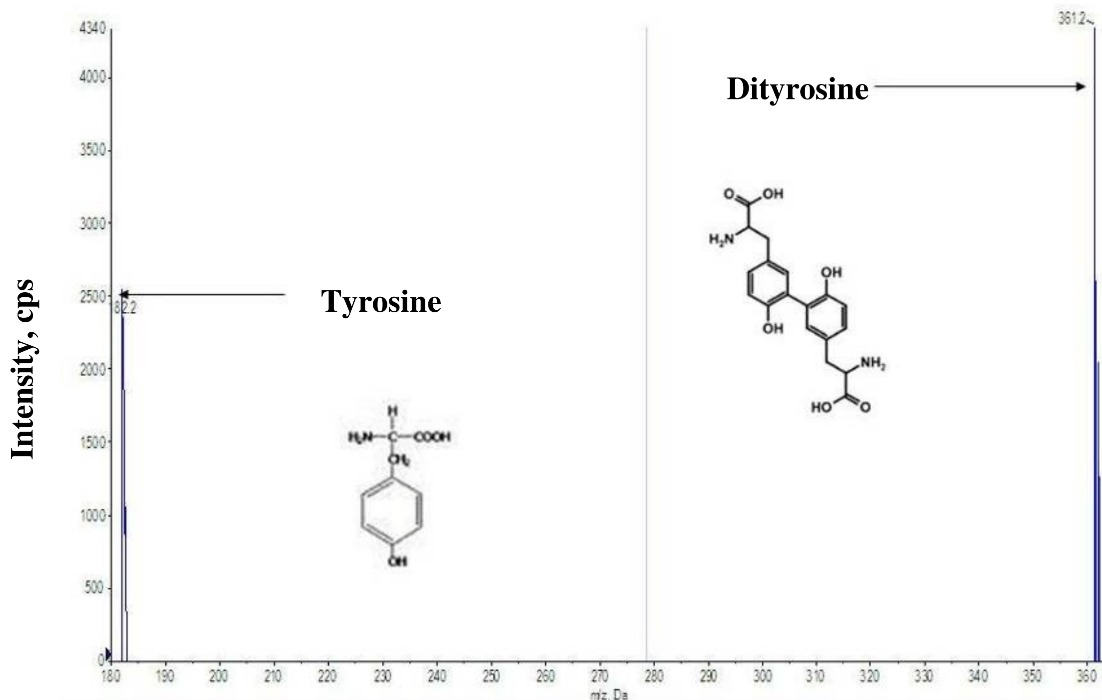

Figure 3.3.14 Typical tyrosine and dityrosine MS peaks for marama protein dough incubated with POX

Positive mode was used for ionisation

3.3.4. Conclusions

Marama protein seems to be characterised by one major endothermal transition compared to soya protein, suggesting differences in protein structure. The heat stability of marama protein is slightly higher than that of soya glycinin (11S). Marama protein is characterised by highly viscous behaviour and extensibility compared to soya and even gluten. It also forms a highly adhesive (sticky) dough. Marama has a less stable protein structure compared with gluten. Dityrosine may be important in the structural stability of marama protein through tyrosine oxidation with oxidative enzyme and crosslinking. This may be of interest in food and non-food applications.

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4. GENERAL DISCUSSION

The first part of the general discussion is a critical review of methodology. It mainly discusses how the different methods were used and applied. The second part discusses the relationship between marama protein composition and its functionality. The third part is a brief discussion on the potential applications of marama protein in food and non-food systems.

4.1 Critical review of methodology

For the microscopy study, tissue sections were fixed in 2.5% glutaraldehyde for 48 h, post-fixed in osmium and subsequently dehydrated in a graded series ethanol solutions according to Young *et al*., 2004. The constituents of protein storage organelles: phytin and some associated K^+ could be lost in the aqueous solution of glutaraldehyde and osmium (Lott & Buttrose, 1978). Some protein body organelles, which have been referred to as globoid inclusions (Lott & Buttrose, 1978; Young *et al*., 2004) in this study may correspond to regions that may have lost their cations during fixation. However, according to Chrispeels *et al.* (1976) the protein bodies could be preserved under the fixation conditions used in this study. Lott *et al.* (1984) used reduced time of fixation to minimise the exposure of tissues to water and prevent extraction of the water-soluble storage protein organelles. The method of fixation that was used in this study is a standard method that has been used for soya (Boatright & Kim, 2000) and peanuts (Young *et al*., 2000). The reduction of the time of fixation would have been a useful approach if the main focus of this study was on the characterisation of the storage protein organelles.

From this study, it appeared that most protein bodies in marama bean were spherical, which is in agreement with the reported microstructure of protein bodies in oilseed legumes (Lott & Buttrose, 1978; Young *et al*., 2004). However, some protein bodies in marama had distorted and/or elongated shapes. This variation in shape could also be due to the angle of sections or artefacts. Differences in the stage of maturation state of the protein bodies may also have led to shape distortion when dehydration takes place as described by Lewicki and Pawlak (2003). Enzymatic treatment with Proteinase K was useful to elucidate the protein bodies of an unknown oilseed legume like the marama bean. The use of Proteinase K revealed that the protein bodies in

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marama are membrane bound, which is in agreement with previous reports on microstructure of protein bodies in legumes (Lott & Buttrose, 1978; Lott, 1981). Proteinase K is a stable serine protease (Ebeling *et al.*, (1974). It is active at pH 7-11 and has a broad range of substrate specificities (Rao *et al.*, 1998). This enzyme has been used to hydrolyse protein bodies in maize, wheat and peas (Miflin & Burgess, 1982). Since it is practically impossible to avoid the generation of artefacts during sample preparation for microscopy, multi-imaging techniques are recommended for microstructure analysis for better interpretation of results. In this study, imaging techniques based on light microscopy, transmission electron microscopy and confocal laser scanning microscopy were therefore used for better understanding and interpretation of marama bean microstructure in comparison with soya bean.

In this study, the solubilisation method based on Tris-buffer (pH 8.0) containing 0.5 M NaCl followed by dialysis (Aluko & Yada, 1995) was used to extract storage proteins. Some authors have used the traditional method of alkaline solubilisation followed by acid precipitation to extract storage protein from legumes such as cowpea (Horax *et al.*, 2004), lupin (Lqari *et al.*, 2002) and beach pea (Chavan *et al.*, 2001). Unlike the dialysis method, acid precipitation may cause partial denaturation of proteins (Owusu-apenten, 2005). Globulins are the major storage of proteins of most legumes (Bailey and Boulter, 1972; Chavan *et al.*, 2001; Liu *et al.,* 2007; Adebowale *et al.,* 2007). But, other protein fractions like the glutelins and prolamins are sometimes present in minor quantities. For instance, prolamins have been reported in minor quantities in beach pea protein, a leguminous plant found in Canada and Japan (Chavan *et al.*, 2001) and some species of mucuna bean (Adebowale *et al.*, 2007). These protein fractions if present in marama seed may have eluded from the analysis due to the method of solubilisation that was used in this study. However, extracted proteins from marama have been considered to be sufficiently representative due to the similarity observed in SDS-PAGE patterns of proteins from the marama protein extract and defatted marama flour.

Furthermore, marama plants currently grow in the wild and efforts to cultivate the crop have only just been initiated. As a result, the first most common specie of marama *T. esculentum* that was available during the period of the study was used in comparison with soya bean. Five species of marama bean have currently been

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identified (Castro *et al.,* 2005). The second most common species of marama bean *T. fassoglense* may be included in future studies for a better comparison.

Molecular and structural characterisations of marama protein were determined using one-dimensional (1D) SDS-PAGE and a two-dimensional (2D) SDS-PAGE-IEF proteomic technique. Visual quantification based on staining density was used to quantify the proteins. Considering the fact that thousands of proteins may be resolved, it is possible to have many proteins co-migrating at the same spot (Gygi *et al*., 2000). The relative quantities of protein spots in the 2D gels may have been influenced if co-migration of proteins occurred with marama. Coomassie Brilliant Blue R-250 was used to stain the gels. Low-abundance proteins, if present in marama, may have eluded the analysis due to the low sensitivity of this dye compared with silver staining (Posh *et al.*, 2006). Silver staining is a better alternative to Coomassie due to its higher sensitivity. The silver stain was used on 2D gels at the initial stages of this study. However, poor quality gels were obtained. Spots in the gel could not be differentiated and identified due the abundance of basic proteins in marama.

Some alternative approaches have been employed to address the technical problems associated with co-migration of proteins, repeatability, and recovery of hydrophobic and low-abundance proteins in proteomics. These include pre-electrophoretic fractionation to simplify the protein mixture (Butt *et al*., 2001) and the use of fluorescence dye to label the proteins (Tonge *et al.* 2001). The fluorescence dye method, known as Difference Gel Electrophoresis (DIGE) relies on pre-electrophoretic labelling of the sample with three spectra distinct fluorescence, Cyanine -2 (Cy2), Cyanine-3 (Cyn3) or Cyanine -5 (Cy 5) (Butt *et al*., 2001). Labelling takes place via lysine residues and it is carried out stoichiometrically such that only a small portion of the protein is labelled. This makes the procedure compatible with in-gel digestion and mass spectrometry. The most up to date method for complex proteins analysis is based on mass spectrometry. Peptides derived from 1D and 2D electrophoresis are proteolytically cleaved into small peptides, which are analysed by Matrix Assisted Laser Desorption Ionisation Time-of-flight- Mass Spectrometry (MALDI-TOF/MS) and/or Liquid Chromatography-Mass Spectrometry (LC-MS) (Tilley *et al*., 2001; Hanft & Koehler, 2005). Mass spectrometry techniques have been used to identity constituent polypeptides of storage protein in legumes such as in soya bean (Natarajan *et al*., 2005), lupin (Magni *et al*., 2007) and peanuts

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(Kottapalli *et al*., 2008). These techniques would have been appropriate to use in the study of the marama proteome. However, due to equipment limitations and financial constraints, it was not possible to do mass spectrometry analysis of marama protein. Nevertheless, the initial separation using 2D proteomic technique proved useful to understand the pattern of the marama proteome map and to compare this with soya bean protein.

High Liquid Performance Chromatography (HPLC) was used to separate and quantify the individual amino acid residues in marama protein. One important step in amino acid determination is the complete hydrolysis prior to chromatographic analysis. For a successful hydrolysis, the reagent must be capable of cleaving the peptide and the peptide must be accessibility to the hydrolytic reagent. The most common method of acid hydrolysis (in liquid phase) was used in this study. This method of hydrolysis causes complete lost or partial destruction of certain amino acids (Bartolomeo & Maisano, 2006; Foutoulakis & Lahm, 1998). For instance, tryptophan is completely lost while cysteine cannot be recovered completely. Asparagine and glutamine are completely hydrolysed into aspartic and glutamic acid, respectively. Tyrosine may be partially lost, while methionine may be oxidised in the presence of oxygen. Some protective measures were taken in this study to prevent loss. These measures consisted of hydrolysing proteins under vacuum and adding the additive phenol according to Tilley *et al.* (2001). Phenol serves as scavenger to substantially reduce the loss of tyrosine (Lipton & Bodwell, 1976; Pickering & Newton, 1990). Methods have been reported in the literature for more accurate quantification of amino acids such as tryptophan (Wu & Hojilla-Evangelista, 2005), methionine and cyteine (Wathelet, 1999; Wu & Hojilla-Evangelista, 2005), which are not preserved by acid hydrolysis. The knowledge of the complete profile of amino acids in marama bean may be important from a nutritional point of view and to better explain its protein functionality.

After hydrolysis, constituent amino acids must be derivatised for detection. O-phtalaldehyde (OPA) may be used for both pre and post column derivatisation. OPA reacts with primary amines in an aqueous basic medium (pH 9-11) and in the presence of a mercaptan (RSH such as 2-mercaptoethanol), to form a fluorescent isoindole derivative (Fig. 4.1) (Roth, 1971). In this study, the Pico-tag method based on pre-column derivatisation following the protein hydrolysis was used for amino acid

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detection. This involves the derivatisation of amino acids in the hydrolysates and separation is by Reverse-phase chromatography. Pre-colunm derivatisation method implies extensive sample manipulation before analysis. This method is also affected by limited stability of the preformed derivative (Mengerink *et al.*, 2002) compared with the classical technique based on ion-exchange separation coupled with post-column derivatisation, which was found to be more precise (Anders, 2002).

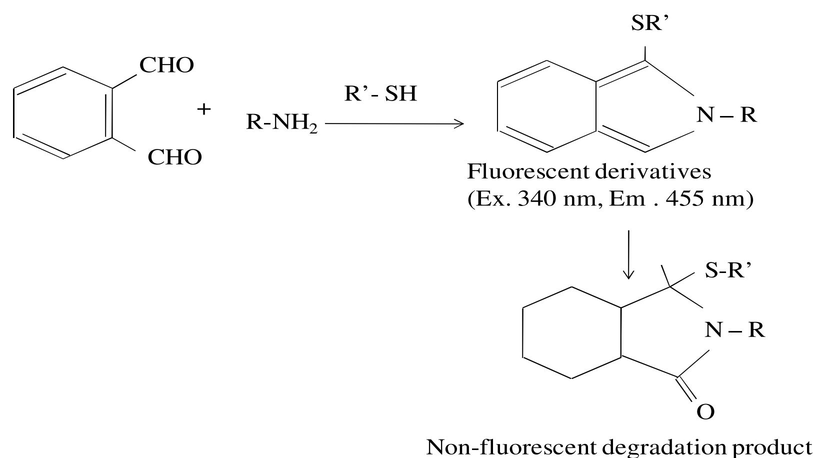

Figure 4.2 Reaction of OPA with a primary amine (Ex: excitation wavelength, Em: Emission wavelength) (Roth, 1971)

A reverse-phase chromatographic method was also used to separate tyrosine and dityrosine in marama protein. Although existing literature was useful in the understanding and interpretation of rheological data, it would have been more appropriate to have included internal standards in the experiment. Takasaki *et al.* (2005) introduced internal tyrosine and dityrosine standards when studying the effect of peroxidase and hydrogen peroxide on the dityrosine formation in wheat flour dough. The production of these internal standards involves a very complex procedure, which consists of synthesising, purification and confirmation of identity (Kikugawa *et al.*, 1991; Takasaki *et al.*, 2005). The production of these standards was beyond the scope of this study.

In this study, Differential Scanning Calorimetry (DSC) was used to evaluate the thermal behaviour of marama protein. DSC is based on a two pan arrangement consisting of a reference pan and a sample pan in a temperature controlled enclosure (Schawe, 1995). The change of the difference in the heat flow rate to the sample and to the reference sample is measured during the DSC analysis. DSC has been used to determine T_d of many legume proteins and to provide useful information on protein

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structures and compositions (Meng & Ma, 2001; Horax *et al.,* 2004). Water content of protein may influence measured parameters including T_d and ΔH of proteins (Kitabatake *et al*., 1990). Alternatively, the use of high pressure DSC has been reported by Zhu *et al.* (2004). This enables the characterisation of thermal behaviour of protein under pressure, thus preventing water evaporating that would have occurred with the normal DSC.

Information on the rheological properties of proteins may be obtained by performing empirical tests (Weipert *et al*., 1990; Mezger, 2006). These tests are based on large deformations of materials and are performed using equipment such as the extensograph, TA-XT2 Texture Analyser and Kieffer extensibility rig (Zaidel *et al.,* 2010). The extensibility and stickiness of marama protein were determined using a TA-XT2 Texture Analyser. Many factors including the mixing conditions and dough development (Mirsaeedghazi *et al*., 2008), hydration / water content (Gras *et al*., 2000), protein content, composition and interaction with non-protein material (Ortiz *et al*., 2004) may have influenced these properties. In this study, dough samples were manually prepared by mixing with a spatula until a homogeneous dough was obtained. Mechanical mixing may have been more efficient than the manual mixing in terms of network formation and dough development. Over-mixing or under-mixing has been found to affect the pattern and network formation and dough quality (Zaidel *et al.,* 2010; Mirsaeedghazi *et al.*, 2008). Gluten showed a low complex modulus (G*) when the mixing time was shorter than the optimum (Zaidel *et al*., 2010).

Frequency sweep experiments were performed to determine the mechanical spectra of marama protein using a rheometer. This is a dynamic oscillation test and is performed without altering the internal network structure to determine the viscoelastic properties of proteins (Mezger, 2006). The principle of small oscillatory deformation measurement is based on the use of two parallel plates, one rotating in sinusoidal motion and the other is stationary. Rheological information such storage modulus (G') (representing the elastic component) and loss modulus (G") (representing the viscous component) of the material being measured are automatically generated (Mezger, 2006). Dehydration and material slippage between the plates are problems that may arise during analysis. In this study, paraffin oil as used by Oom *et al*. (2008) was employed to prevent dehydration of the dough. To prevent possible occurrence of slippage of samples between the plates, some authors have placed sandpaper at the

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bottom plate before the sample, e.g. Zang *et al.* (1991). However, many authors have also obtained useful information on viscoelastic characteristics of both cereal and legume proteins without placing the sample on a rough surfaces (Oom *et al.*, 2008; Ortiz *et al.*, 2004). Although no visible slippage was observed during analysis, the use of rough surface may be considered in future studies.

4.2 Relationship between marama protein composition and functionality

The composition of seed protein has been found to influence its functionality (Tolstoguzov, 1993; Horax *et al.,* 2004; Yuan *et al.*, 2009). According to Damodaran (1997), the physicochemical properties that influence functional behaviour of proteins in food include their amino acid composition and sequence, hydrophobicity, structures (secondary, tertiary and quaternary) as well as molecular flexibility/rigidity in response to external environment (pH, temperature, salt concentration) or interaction with other food constituents.

Marama protein was characterised by greater heat stability compared to soya glycinin (11S). Thermal stability of proteins has been found to significant correlate with protein composition (Horax *et al.,* 2004) and structure (Ortiz *et al.*, 2004). Marama protein contains a high proportion of aromatic amino acids compared to soya protein. These amino acids in addition to non polar aliphatic ones can impart a hydrophobic character (Nakai *et al.*, 1986), which can stabilise the protein during thermal analysis.

In terms of protein structure, the ß-sheet is reported to be more stable than α-helix, thus displaying high denaturation temperature (Damodaran, 1988). The high thermal denaturation temperatures of ß-lactoglobulin and soya glycinin (11S) have been associated with their high proportions of ß-sheet structure, respectively 51% and 64% (Damodaran 1988; Damodaran, 1989). The thermal stability of marama protein may therefore be related to its protein structure. Possibly, it contains a high proportion of ß-sheet structure, similar to 11S soya glycinin (Hou & Chang, 2004; Zhao *et al.*, 2008).

Marama proteins were found to be composed of low (LMW) and higher (HMW) molecular weight proteins (Fig. 4.2). By comparison with soya protein, the LMW proteins represent the basic 11S legumin (monomers). The basic monomers in

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marama protein are most likely stabilised by hydrophobic interactions, as suggested for the basic soya glycinin (11S) (Mo *et al.,* 2006; Yuan *et al*., 2009). The HMW proteins in marama may be classified into two groups of 63 kDa and 148 kDa based on their relative mobility in SDS-PAGE. As already discussed in research Section 2.3, these proteins are most likely stabilised by dityrosine bonds since they were not reduced by a reducing agent.

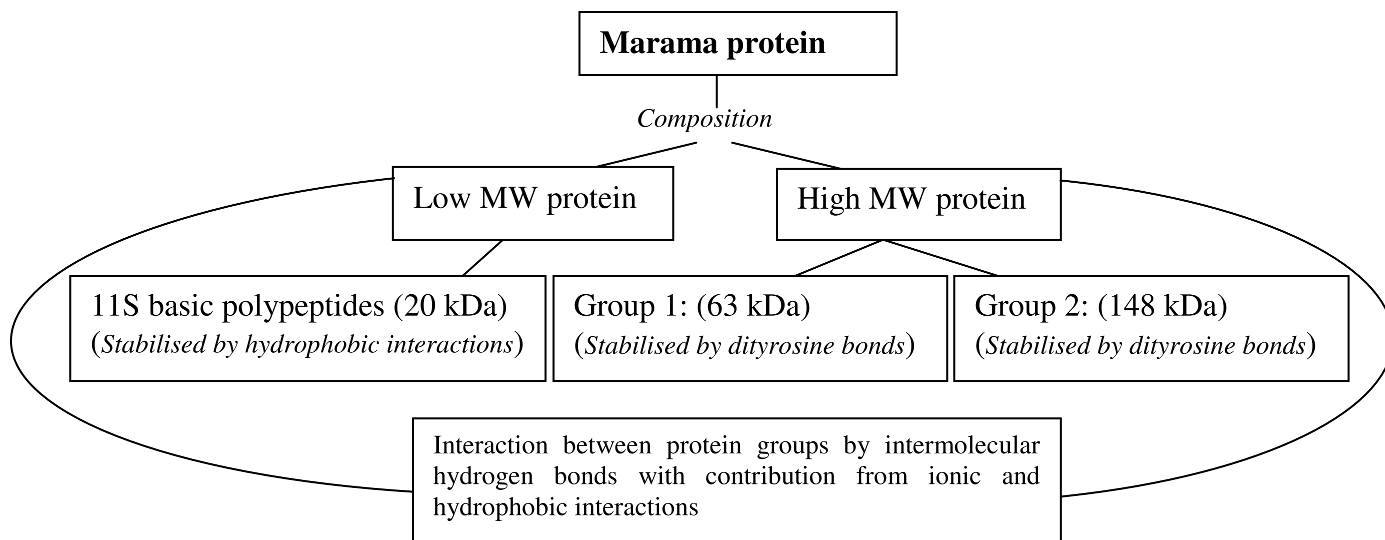

Figure 4.3 Summary diagram of proposed marama protein composition

In the proposed model to explain the molecular basis of the rheological properties of marama protein (Fig. 4.3), the HMW proteins in marama are considered to form linear chain of polymers linked together by intramolecular tyrosine bonds and may represent the backbone for the viscous flow behaviour and extensibility of marama protein (Fig. 4.3 a). Belton (1999) proposed a "loop and train model" to explain gluten's elasticity. According to his model, gluten dough development involves glutenin polypeptide interaction with each other by hydrogen bonds. In this study, there was no evidence of disulphide bonds in marama protein. Therefore, the linear chains of polymers in marama most likely interact with each other mainly by intermolecular hydrogen bonding. Hydrogen bonds can be formed between glutamine and hydroxyl residues in gluten (Belitz *et al*., 1986; Damodaran, 1996). Glutamic acid, which accounts for about 15% in marama, may be present in the form of glutamine, favouring the formation of hydrogen bonding as suggested for gluten (Belton *et al*., 1995). The highly viscous character and extensibility of marama protein suggests that molecular movement of linear polypeptide chains relative to one another is taking place. Unlike covalent bonds, hydrogen bonds interchange easily (Belitz *et al*., 1986). They can

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separate and reform during deformation. Marama is also richer in proline compared to soya. This amino acid in combination with glutamine can enhance the viscous properties of dough, as suggested for gluten of certain soft winter wheats (Fermin *et al*., 2005).

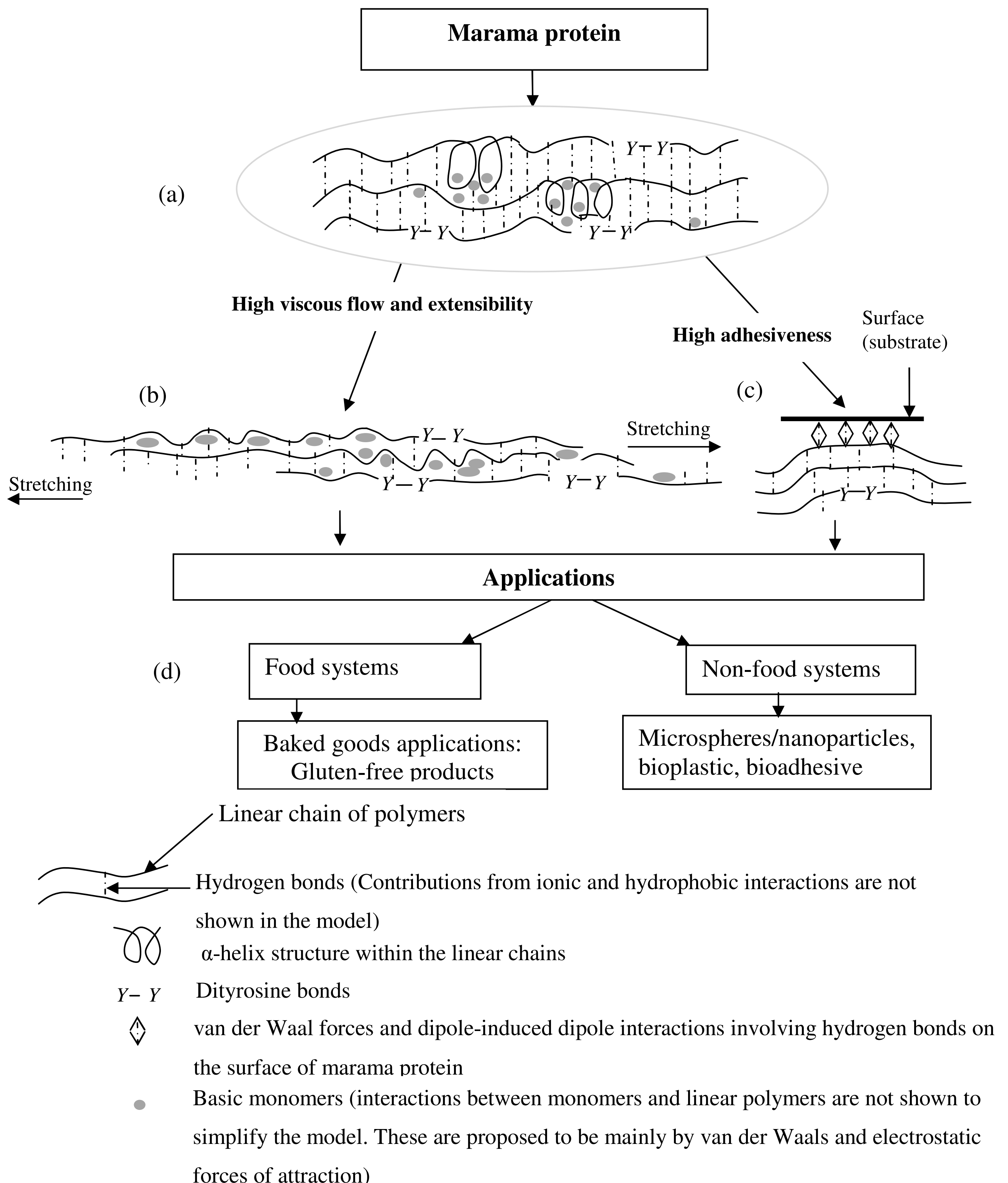

Figure 4.4 Proposed model for marama protein composition-functionality relationship and applications

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As suggested above, the protein in marama may contain a high proportion of ß-sheet structure, similar to soya 11S glycinin. It may also contain a low proportion of the α-helix structure since both structures (ß-sheet and α-helix) have been reported in soya protein fractions (Hou & Chang, 2004; Zhao *et al.*, 2008). Most LMW proteins, which represent the basic monomers in marama protein, are proposed to fit in the interior coil of the α-helix since these are small molecules (Fig. 4.3). These monomers are assumed to be interacting with the linear polymers by van der Waals forces of attraction and to some extent by electrostatic interactions with positively charged polypeptides. A section showing different linear polypeptide species of marama protein is shown in the model (Fig. 4.3 a). During extension/stretching, these linear polymers are proposed to slide over one another resulting into high extensibility (Fig. 4.3 b). The α-helix within the linear chains and possibly other types of structures like the ß-turns, which could be present, will also open up, thus contributing to the extensibility of marama protein.

Marama protein formed a highly adhesive dough compared with gluten. Several theories have been proposed to the theory of adhesion. The most widely accepted theory of adhesion is the adsorption theory (Kinlock, 1987). This theory proposes that material will adhere because of interatomic and intermolecular forces (mainly Van der Waals forces), which are established between atoms and molecules at the surface of the adhesive and the substrate. Hydrogen bonds can also form abnormally strong dipole-dipole attractions involving -OH and -NH groups (Sun, 2005). Attraction between an adhesive polymer such as marama protein and a substrate may thus involve van der Waals forces and hydrogen bonding (Sun, 2005). In gluten, hydrogen bonding between amide and hydroxyl groups contributes to the cohesion-adhesion properties of its dough (Damodaran, 1996). Polar side chains on the surface of marama protein, which are hydrogen bonded with water could induce a dipole in non polar molecules at the surface of the substrate (surface) (Fig. 4.3 c).

High viscous flow and extensibility as observed for marama protein suggests that it lacks strength and elasticity. With POX, rheological data indicated that new and stronger protein networks have been formed in marama protein. These new networks probably resulted from tyrosine oxidation and crosslinking (Fig. 4.4). Takasaki *et al.* (2005) proposed a mechanism of POX catalysed oxidation of tyrosine (Fig. 4.5). Horseradish POX, which was used in this study, binds to the substrate (tyrosine) via

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contact catalysis to oxidise it (Takasaki *et al.,* 2005). This study suggests that the structural stability of marama protein and dough strength may be improved by the addition of crosslinking enzymes in dough during preparation.

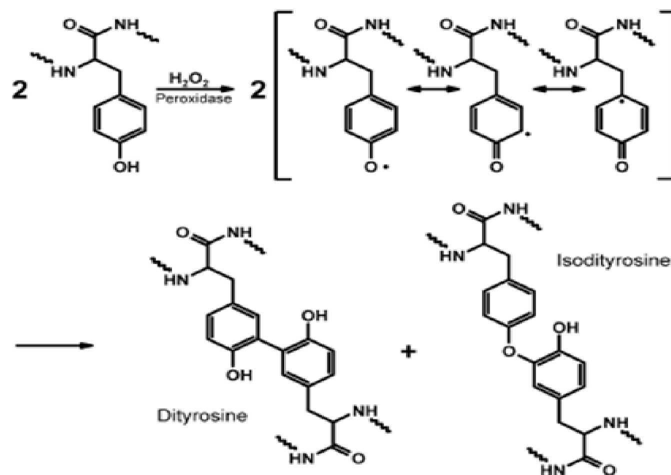

Figure 4.5 Formation of protein-bound tyrosine and dityrosine by a radical mechanism (Hanft & Koehler, 2005)

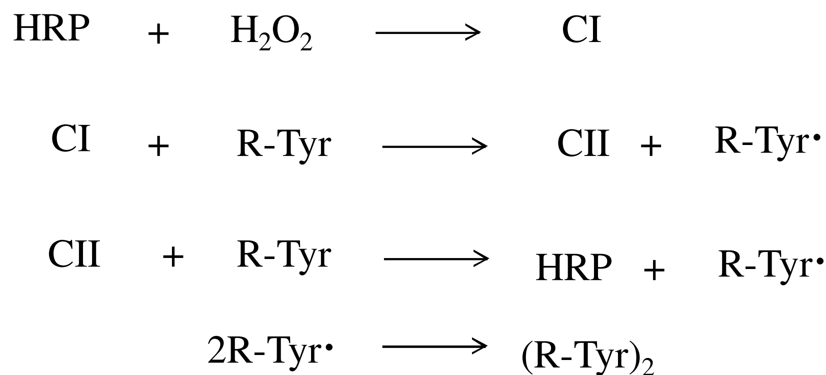

Figure 4.6 Mechanism of horseradish POX (HRP) catalysed oxidation of tyrosine (Michon *et al*., 1999)

R-tyr: tyrosine residue (tyr) in a polypeptide (R), R-Tyr•: Tyrosyl (tyr•) in a polypeptide (R); CI: compound I (Ferryl species (Fe (IV) = O + porphyrin radical cation), CII: compound II (intermediate enzyme retaining the heme in Ferryl (Fe (IV) = O) state) (Rodriguez-Lopez *et al*., 2001)

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4.3 Potential applications

Marama protein may have some unique applications in food and non-food systems compared to soya protein (Fig. 4.3 d). In baked goods applications, factors to be considered to produce good quality bread from dough have been described by Sliwinski *et al*. (2004). According to these authors, dough must have high viscosity to prevent gas cells rising and should be extensible at high level to prevent sudden breakage in gas cell membranes. Marama protein may be useful for gas retention in bread dough due to its high extensibility (Fig. 4.3 d). However, some degree of elasticity and dough strength will be required to derive the full utilisation of marama protein, especially in the production of gluten-free products. A crosslinking enzyme such as POX may be employed to improve the viscoelastic properties of marama dough in terms of elasticity.

In non-food applications, marama protein may have some potential as a bioadhesive due to its high adhesiveness compared to soya protein and even gluten. The bioadhesive potential of gliadin nanoparticles has been explored in literature (Ezpeleta *et al*., 1996; Arangoa *et al*., 2000). Since marama protein is also characterised by viscous flow behaviour and extensibility, similar to wheat gliadin, its protein just like gliadin may be therefore explored in microspheres/nanoparticles. Microspheres may be employed in the production of biodegradable films for packaging (Krochta & Mulder-Johnston, 1997). Bioadhesives from protein microspheres /nanoparticles may be useful as drug carrier to increase the drug residence time and therapeutic efficiency in pharmaceutical (Kramer, 1974; Arangoa *et al*., 2000). Protein nanoparticles have been reported to be incorporated into a variety of drugs in a non–specific manner (Kramer, 1974). Therefore their use as drug carriers in drug delivery has an advantage over other carriers from synthetic polymers. Proteins possess functional groups which can easily be adsorbed or covalently bonded to molecules such as antibodies (Alasaka *et al.,* 1988) and lectins (Woodley & Naibett, 1988), which are capable of modifying the properties of nanoparticles. Irach *et al*. (1995) have been able to produce nanoparticles from legumin (11S) storage protein of peas (*Pisium sativa*). The production of nanoparticles from legume protein such as marama bean is thus feasible.

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5. CONCLUSIONS AND RECOMMENDATIONS

The protein body microstructure of marama is similar to soya in terms of spherical shape and localisation within the parenchyma cells. However, marama bean protein bodies contain spherical globoid and druse crystal inclusions, which are absent in soya bean protein bodies.

The protein composition of marama bean is very different from that of soya bean and apparently for that matter different from other legumes. Marama protein contains a substantial amount of tyrosine. It is also slightly richer in proline compared to soya. Since tyrosine is involved in polypeptide crosslinking, the tyrosine in marama may contribute to the structural stability of its protein. The vicilin (7S) and acidic 11S subunits present in soya protein seem to be absent in marama protein. Only a major basic legumin (11S) (20 kDa) and two additional proteins of about 63 kDa and 148 kDa are found in marama. Also, disulphide bonds seem to be absent in marama protein. The pI's of most polypeptides in the marama proteome map are between 6-10, indicating that marama is a more basic protein compared to soya protein.

The functionality of marama protein in terms of thermal and rheological properties is different from that of soya protein. Marama protein is characterised by one major endothermal transition compared with soya protein, which has two. Marama protein seems to be more thermally stable compared to soya glycinin (11S). The thermal stability of marama protein may be attributed to hydrophobic interactions among its basic polypeptide subunits as suggested for the basic soya glycinin (11S). Marama protein has very high viscous flow and extensibility behaviour compared with soya protein and even gluten. It is also very adhesive compared with gluten. Non-covalent bonding between the polymers chains, namely hydrogen bonds are most likely responsible for the rheological behaviour of marama protein.

Further research is obviously required to elucidate the molecular basis of marama protein functionality and explore the unique functionality (e.g. high viscous flow and extensibility, high adhesiveness) of marama protein in food and non-food systems. It may be important to:

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i. Explore marama protein in the preparation and characterisation of microspheres and nanoparticles and determine the functionality of these particles in bioadhesives and bioplastic films.

ii. Explore the functionality of marama protein in the production of gluten-free products.

iii. Determine the functionality of marama protein in biodegradable chewing gum.

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7. PUBLICATION, PRESENTATIONS AND CONFERENCES ATTENDED BASED ON THIS WORK.

1. Amonsou, E., Taylor, J., & Minnaar, A. (2011). Microstructure of protein bodies in marama bean species. *Lebensmittel-Wissenschaft und-Technologie, 37,* 439-445.

2. Amonsou, E. O., Taylor, J. R. N., & Minnaar, A. Protein composition and protein body structure of marama bean. *CST-SA – ICC International Grains Symposium,* University of Pretoria, Pretoria, South Africa, 3-5 February, 2010.

3. Amonsou, E. O., Taylor, J. R. N., & Minnaar, A. Marama protein composition and functionality. *Final consortium meeting for the MARAMA II Project,* Cape Town, South Africa, 20-21 August, 2010.

4. Amonsou, E. O., Taylor, J. R. N., Beukes, M., & Minnaar, A. Composition of marama bean protein. *15th World Congress of Food Science & Technology, Cape Town, South Africa,* 22-26 August, 2010.

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