

Characterisation of the Ability of Globulins from Legume Seeds to Produce Cocoa Specific Aroma

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Abstract: This study was carried out to extract and compare the characteristic ability of globulins from cottonseed, alfalfa seed, pea seed, mung bean and French bean with cocoa seeds to produce cocoa-specific aroma precursors. The extracted globulins were compared through SDS PAGE, amino acid and oligopeptide profiles. A very low recovery was obtained during globulin extraction from different seeds ranging from 0.5% to 2.7%. Cottonseed produced the highest total protein (13.90 mg/g), followed by cocoa seed (11.91 mg/g), whereas alfalfa seed, mung bean, pea seed and French bean produced 7.86, 4.77, 4.59 and 3.89 mg/g respectively. Two distinctive bands of 51.1 and 33.0 kDa were observed for cocoa vicilin-class globulin (VCG) from SDS PAGE. More than three bands were shown for other seed globulins. Comparative HPLC analyses of the obtained peptide mixtures revealed different and complex patterns of predominantly hydrophobic peptides. A similar high content of amides (glutamic acids-glutamine, aspartic acid-asparagine and arginine) and low concentrations of lysine were observed in all seeds globulin.

Keywords: Globulins, legume seeds, cocoa-specific aroma, peptides, amino acids

INTRODUCTION

Seed proteins are classified according to their solubilities either as water-soluble albumin, salt-soluble globulin, alcohol-soluble prolamin, and acid- or alkaline-soluble glutelin. Seed storage proteins include mainly globulins in legumes and prolamins and glutelins in cereals.

Globulins are the dominant storage proteins in legume seeds and account for 50-90 % of seed proteins. The globulins of legume seeds are classified into two types according to their sedimentation coefficient: vicilin-type 7S globulins (7.1-8.7S) and legumin-type 11S globulins (10.1-14S), differentially regulated, with the 7S globulins accumulating faster than the 11S globulins (Gatehouse *et al.*, 1986).

Cocoa beans have been reported to contain four fractions of protein, i.e. albumin,

globulin, prolamin and glutelin (Voigt *et al.*, 1993a). The globulin fractions with molecular weight of 47 and 31 kDa were shown to be the polypeptides responsible for the cocoa-flavour precursors produced by the action of endoproteinases on cocoa globulin (Biehl *et al.*, 1985). Cocoa globulin is the first seed protein known to contain only vicilin-class globulin (Spencer and Hodge, 1992).

The total proteins of cocoa bean cannot be directly extracted from its cotyledon as compared to other beans such as cowpea, acha, tepary beans, *Amaranthus hypochondriacus* seeds, mung bean, *Vicia faba* and cottonseed. This is due to the high content of phenolic compounds present in cocoa cotyledon (Voigt *et al.*, 1993a). Acetone dry powder (AcDP) must be prepared to extract storage proteins without irreversible denaturation of proteins by quinones resulting from oxidation of polyphenols during extraction. AcDP should

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contain the total protein cotyledons and proteases. Relatively little attempt has been made to study cocoa bean protein, although these are the second major constituents (15-20% dry weight) after cocoa butter which comprise 50% of the beans dry weight. The globulin from legume seeds may be added to enhance cocoa flavour if their globulin can produce cocoa aroma precursors through proteolysis. This paper describes the study of seed globulin in comparison with cocoa globulin and their ability to produce cocoa-specific aroma precursors. Globulins characteristics from legume seeds were compared through SDS PAGE, oligopeptide patterns and amino acid analyses.

MATERIALS AND METHODS

Materials

Cocoa seeds were obtained from Golden Hope Plantation, Perak, Malaysia. French bean, alfalfa, pea and mung bean seeds were obtained from the BMS organic shop, Puchong, Selangor, Malaysia. Cottonseed was bought from Khorthum, Sudan. All of the seeds were in dry form.

Preparation of Samples

Unfermented cocoa seeds were shock-frozen in liquid nitrogen and lyophilized (Labconco, Missouri, USA; -40°C, 133 x 10⁻³ bar). Seeds were crushed and finely ground in ceramic mortar with a pestle. Cottonseeds were dehulled manually using mortar and pestle and were finely ground using the multiquick system grinder (Braun ZK100, Germany). The seeds were sieved through a 450 µm mesh to obtain a uniform particle size. Alfalfa, pea, mung bean and French bean seeds were ground and sieved in the same manner. Seeds were then defatted with petroleum ether (bp 40-60°C) for 8 h in a Soxhlet apparatus (AOAC,1996).

Preparation of Acetone Dry Powder (AcDP)

The method of Kirchoff *et al.* (1989) was used to prepare the AcDP. The defatted cotyledon

powder was washed three times with 80% (v/v) cold aqueous acetone (-20°C) containing 0.1% thioglycolic acid, and subsequently four times with 70% (v/v) cold aqueous acetone. The suspensions (20 ml aqueous acetone per 1 g of seed powder) were shaken vigorously for 30 s and then stirred at 4°C in an ice bath for 1 h. The polyphenols extracts were removed by centrifugation (20 min, 10 000 x g, 4°C). After the final washing step, efficiency of polyphenol extraction was checked by heating an aliquot of the AcDP with 5 M HCL (red colour indicates the presence of residual polyphenols). After the complete extraction of polyphenols, the remnants of water were removed by washing with pure cold acetone. The resulting AcDP was evaporated under reduced pressure to remove the solvent and dried in the fume cupboard overnight. The AcDP was ground and stored at -20°C.

Extractin of vicilin (7S)-class globulin from Cocoa Seeds

Seed proteins were extracted from polyphenol-free AcDP as described by Voigt *et al.* (1993a). Ten grams of AcDP was first extracted with 1 litre of 10 mM Tris-HCl (pH 7.5) buffer containing 5 mM sodium ascorbate, 2 mM EDTA, 10 mM pepstatin A and 1 mM PMSF. The suspension was stirred for 1 h at 4°C and subsequently centrifuged for 20 min at 15,000 x g and 4°C. The pretreated AcDP was extracted with 1 litre of 20 mM Tris-HCl (pH 7.5) buffer containing 0.5 M NaCl, 5 mM sodium ascorbate, and 2 mM EDTA to obtain crude globulin fraction. The suspension was stirred at 4°C for 1 h and centrifuged for 20 min at 15,000 x g at 4°C. The extraction was repeated and the supernatants combined. Ten percent (v/v) chilled TCA was then added to the supernatants (1:100) and the solution was chilled in ice bath for 45 min and centrifuged for 15 min at 10, 000 x g at 4°C. The TCA pellets left in the tube were washed with ethanol:ether (1:1 v/v) then with distilled water. The solution was dialysed against distilled water (1:20) for 48 h in a cold room, centrifuged and the globulins obtained were

freeze dried (Labconco, Missouri, USA; -40°C, 133×10^{-3} bar).

Extraction of Globular Storage Protein from Cotton, Alfalfa, Pea, Mung Bean and French Bean Seeds

A procedure described by Wallace (1976) was used to extract the globulin from cottonseed. Ten grams of defatted seeds were washed twice with distilled water (1:15 v/v) followed by 10% NaCl (1:15 w/v), pH 7.0 for 30 min at 4°C and centrifuged. The supernatant was dialysed against distilled water overnight in a cold room (4°C) and centrifuged. The precipitate was washed with 0.3 M NaCl, pH 7.0 (1:15 w/v) for 30 min at 30°C and centrifuged. All centrifugation were carried out for 5 min at 4°C at $10,000 \times g$ (Kubota 7800, Tokyo, Japan). Once the globulin precipitate was obtained, it was resolubilized in 10% NaCl, pH 7.0 and freeze-dried (Labconco, Missouri, USA; -40°C, 133×10^{-3} bar).

The method used for the extraction of 7S and 11S globulin from broad beans (*Vicia faba*) and peas was described by Vladimir *et al.* (1990). A 100 g portion of defatted meal of alfalfa, pea, mung bean and French bean seeds was dispersed in 900 ml of distilled water and the resultant mixture was titrated with 0.5 M NaOH to pH 8.0 and then mixed at 50°C for 1 h. The suspension was centrifuged at $1,500 \times g$ for 30 min at room temperature (Eppendorf 5610, Hamburg, Germany). NaCl salt was added to the supernatant to final concentration of 0.5 M. The solution was titrated to pH 4.8 with 0.1 M HCl containing 0.5 M NaCl. The suspension obtained was centrifuged at $1,500 \times g$ for 30 min at room temperature (Eppendorf 5610, Hamburg, Germany), and supernatant was diluted with distilled water to 0.3 M NaCl concentration. After centrifugation at $1,500 \times g$ for 10 min, the supernatant was cooled to 4°C for 1 h. The precipitate obtained (fraction enriched with 7S globulin) was separated by centrifugation at $3,000 \times g$ for 15 min at 4°C (Kubota 7800, Japan). The supernatant was diluted with distilled water to 0.15 M NaCl concentration. The precipitate (7S globulin fraction) was separated by centrifugation at

$3,000 \times g$ for 15 min at 4°C and the sediment was dialysed in the same manner with cocoa seeds and centrifuged. The extracted globulins were then freeze-dried (Labconco, USA; -40°C, 133×10^{-3} bar).

Determination of Protein

Crude protein were analysed following AOAC method (1996). Protein concentrations were determined by the method of Hatree (1972) using bovine serum albumin (Sigma, St. Louis, USA) as the standard.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using modified methods of Pettipher (1990) and Laemmli (1970) on vertical mini slab gels (5 cm x 1 mm) using a Bio-Rad Mini Protean II cell (California, USA). Ten microlitres of each protein sample (approximate 10 mg protein content) was carefully loaded into the well. Electrophoresis was carried out at a constant current of 200 volt and 120 mA for double gels for 50-60 min at room temperature. The gels were stained with Coomassie brilliant blue. Molecular weight markers from Sigma Chemical Co. (St. Louis, USA) were used as standards.

Analyses of Peptides

The pattern of oligopeptides was analysed by a reverse phase HPLC as described by Voigt *et al.* (1994b), using Waters HPLC system with Ultrasphere Octadecylsilyl (ODS) C_{18} , (5 mm particle size x 6.6 mm x 250 mm) column. One gram seeds globulin was suspended in 100 ml of 0.2 M citrate-phosphate buffer, pH 3.5. The suspensions were incubated at 45°C for 8 h at 120 rpm in orbital shaker incubator (YIH DER LM510R, Taiwan). Incubation was terminated by adding 70% methanol (1:1). The mixture was stirred at room temperature for 1 h, filtered and centrifuged (Kubota 7800, Tokyo, Japan; $10,000 \times g$, 4°C, 30 min). The supernatant was collected and the methanol was removed at 40°C by means of a rotary evaporator. Finally, the aqueous solution was passed through

activated charcoal and filtered through a Whatman 0.45 mm nylon membrane filter prior to HPLC. Elution of the peptides was performed at 30°C and flow rate of 1 ml min⁻¹ with an isocratic of 100% A (0.1% (v/v) trifluoroacetic acid) for 7 min and subsequently in linear gradient of 0-50% B (80% acetonitrile containing 0.1% TFA). The eluting peptides were monitored by measuring the absorbance of the eluant at 210 nm.

Determination of Free Amino Acids

Free amino acids were extracted from the seeds globulins using modified methods as described by Cohen *et al.* (1990). Seven hundred milligram of seeds globulin was mixed with 1.4 g polyvinyl pyrrolidone, stirred for 1 h at 0°C in 15 ml deionised water and adjusted to pH 2.5 with acetic acid glacial. The mixture was centrifuged at 15,000 x g 15 min at 4°C (Kubota 7800, Tokyo, Japan), filtered and made up to 50 ml with deionised water. Three millilitres of filtrate was added with 0.5 ml alpha-amino-n-butyric acid (1:6 v/v) as internal standard and 12 ml of acetone (4:1 v/v), kept at room temperature for 30 min, and then centrifuged (Kubota 7800, Tokyo, Japan; 15,000 x g, 15 min, 4°C). Acetone was removed by nitrogen gas streaming. The free amino acids were separated by reverse phase HPLC using the method of Cohen *et al.* (1990) and modified by Puziah *et al.* (1998). The free amino acids were detected using Waters 486 Tunable Absorbance Detector at 254 nm. The mobile phase A of the gradient elution consisted of 940 ml of sodium acetate buffer containing 0.05% triethylamine (TEA), pH 5.7 with 60 ml acetonitrile (HPLC Grade) and mobile phase B consisted of acetonitrile: water (60:40 v/v). Waters Pico-Tag free amino acid column, 3.9 x 300 mm was used for the analysis. The temperature was set at 37°C and injection volume was 20 mL.

Statistical Analyses

Data were subjected to Analysis of Variance (ANOVA) and Duncan's Multiple Range Test using Statistical Analysis System (SAS, Institute Inc., Cary, NY, 1985).

RESULTS AND DISCUSSION

Partial Purification of Vicilin-Class Globulins from Cocoa Seeds

Percent recovery of globulin extraction, total and crude proteins is summarized in Table 1. Low recovery of cocoa globulin was obtained from cocoa AcDP at 1.2%. This can be explained by the presence of phenolic compounds in cocoa seed which may have caused difficulties in the proteins extraction due to the occurrence of phenolic-protein interaction (Voigt *et al.*, 1993a). It has also been reported that the influence of protein-polyphenol interaction will produce impure protein extracts (Seow *et al.*, 1995). To extract the protein from unfermented cocoa without the interference from polyphenols, cold aqueous acetone extraction in the presence of thioglycollic acid have shown to be successful (Biehl *et al.*, 1985; Kirchoff *et al.*, 1989). Thioglycollic acid inhibits cocoa phenolase and quinone tanning of proteins, thus protecting the protein from denaturation (Biehl *et al.*, 1982). AcDP free from polyphenols is a prerequisite for a reliable protection of proteins against denaturations by quinones during the extraction procedure. Extraction of VCG from cocoa beans is best achieved using salt buffer (0.5M NaCl) at pH 7.5 and dialyzed against sodium acetate at pH 5.0 (Voigt *et al.*, 1994b). The salt extraction procedure is a mild process found to cause little or no denaturation to protein (Arntfield and Murray, 1981). The removal of salt have caused the globulin to become insoluble in distilled water, further precipitation occurred in sodium acetate, with pH 5 because it is near to the isoelectric point of cocoa vicilin-class globulin. However, it is difficult to further purify the VCG of cocoa beans since it tends to aggregate or to form complexes (MacDonald *et al.*, 1994). This may be a result of its insolubility and protein's high hydrophobicity.

Table 1: Percent recovery of globulin extraction, total protein and crude protein of cocoa, cotton, alfalfa, pea, mung bean and French bean seeds

Sample	Recovery of globulin extraction (%)	Total protein (mg/g)	Crude protein (%)
Cocoa AcDP	1.2 (± 0.4) ^{bc}	11.9 (± 0.5) ^b	10.8 (± 1.0) ^c
Cottonseed	2.0 (± 0.5) ^a	13.9 (± 0.6) ^a	24.7 (± 2.5) ^a
Alfalfa seed	1.3 (± 0.30) ^{bc}	7.9 (± 0.2) ^c	25.6 (± 3.9) ^a
Pea seed	1.4 (± 0.26) ^{bc}	4.6 (± 0.3) ^d	20.6 (± 1.2) ^b
Mung bean seed	1.7 (± 0.47) ^{ab}	4.8 (± 0.2) ^d	19.5 (± 0.5) ^b
French bean seed	1.1 (± 0.46) ^{ab}	3.9 (± 0.1) ^e	18.0 (± 0.2) ^{ab}

Means with the same letter within the same column are not significantly different ($p>0.05$). Means are from three replicates for total protein and % crude protein and five replicates for % recovery of globulin extraction. The numbers in parentheses are standard deviation.

Isolation of Globulins from Cotton, Alfalfa, Pea, Mung Bean and French Bean Seeds

Low percentage recovery of globulin of between 0.6 to 2.7% was obtained from each seed (Table 1). It is well known that total extraction of globulin from high globulin containing meals, i.e. greater than 50%, is very difficult (Derbyshire *et al.*, 1976) and may explain the lower amounts of extractable globulin obtained in this study. In legume seeds, globulins (7S vicilins and 11S legumins) account for 30-80% of the total seed protein, while albumins constitute the remainder (Derbyshire *et al.*, 1976). In the common bean, *Phaseolus vulgaris*, globulins account for 50 to 75% of the total protein (Maria *et al.*, 1998). It was observed that precipitation for 24 h gave higher globulin recovery. More 0.1 M HCl containing 0.5 M NaCl was titrated to alfalfa seed extraction solution to reach the desired pH 4.8. This may be due to the low pH of alfalfa seed and more 0.5 M NaOH needed to reach pH 8.0 during the first titration and made the solution more alkaline compared to the other seeds. The vicilin: legumin ratio (7S:11S) explains some of the difference between the physicochemical properties of the protein, as a whole, of the different seed legumes. Results obtained by Gatehouse *et al.* (1980) and Mortensen (1980) showed that the vicilin: legumin ratio can also vary to a large extent

from one cultivar to another. Nevertheless, legumin were not collected in this study.

Crude and Total Protein from Different Seeds

The total protein extracted from cocoa AcDP, cottonseed, alfalfa seed, pea seed, mung bean seed and French bean seed ranged from 13.90 mg/g for cottonseed to 3.89 mg/g for French bean seed. It has been reported that poyphenol-free AcDP contained about 30% (w/w) of total proteins (Biehl *et al.*, 1982). The difference in results of total protein of cocoa AcDP to the value observed in this study may be due to the difference in the quantity of residual polyphenols in the cotyledon. Besides, the genotype used in this study may be different from the one used for studies reported in the literature and also the condition of the AcDP preparation may have some effect of the absorbance reading. The seed storage proteins are laid down at a specific stage during seed development, principally to act as a store of nitrogen when the seedling germinates (Bewley and Black, 1994). Proteins within the albumin class are more diverse, both structurally and functionally. From the result shown in Table 1, alfalfa seed was observed to contain the highest crude protein content with a value of 26%, while cocoa displayed the least percentage (11%) and was significantly different ($p<0.05$) from the others. Values

obtained were found to be in general agreement with results reported by Massimo *et al.* (1987). While cereals rarely exceed 15% protein (w/w dry weight), legumes and oilseeds usually contain over 20% protein and substantially more (Krochko and Bewley, 1988).

Polypeptide Bands of Cocoa and Seeds Globulin on SDS PAGE

The patterns of polypeptide bands of seeds globulin are shown in Figure 1. Three distinct polypeptide bands were visible on the 12.5% (w/v) SDS PAGE for the cocoa VCG. The apparent molecular weights of the bands were calculated as 51.1 (band I), 33.0 (band II) and 10.2 kDa (band IV) respectively. There was another band observed mildly at 27.8 kDa (band III). The first two bands (band I and II) representing the subunits of the native VCG were believed to represent vacuolar storage proteins (Biehl *et al.*, 1982). These polypeptide bands were similar to those obtained by several researchers (Voigt *et al.*, 1993a; Spencer and Hodge, 1992; Biehl *et al.*, 1982; MacDonald *et al.*, 1994; Pettipher, 1990). The apparent molecular weights of band I (51.1 kDa) and band II (33.0 kDa) were slightly higher than those reported previously (Biehl *et al.*, 1982; Voigt *et al.*, 1993a) as 47 kDa and 31 kDa. Amin *et al.* (2002a) obtained three distinct bands of 47.0-48.8, 31.3-32.9 and 21.9-22.4 kDa respectively for all cocoa genotypes studied. Band III with apparent molecular weight of 27.8 kDa might be the albumin as it was proven that 22 kDa is the storage albumin in cocoa cotyledon (Amin *et al.*, 2003; Voigt *et al.*, 1993a; MacDonald *et al.*, 1994). Although the storage protein albumin represents 25% of total protein in cocoa cotyledon (Voigt *et al.*, 1993a), the band appeared to be less distinct than those of VCG (band I and II). Band IV (10.2 kDa) could be glutenin fraction consisting of residual globulin. An additional polypeptide band with apparent molecular weight of 14.5 kDa has been reported to belong to VCG subunits (Voigt *et al.*, 1993a). However, there is

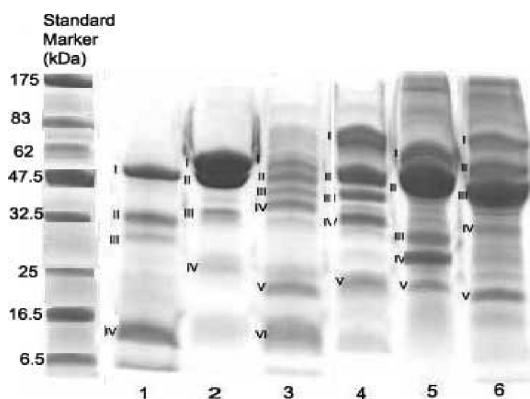


Figure 1: SDS PAGE patterns of cocoa and other seeds globulin. Lanes: 1, cocoa VCG; 2, cottonseed; 3, alfalfa seed; 4, pea seed; 5, mung bean seed; 6, French bean seed

no clear explanation about the *in-vivo* proteolytic degradation of cocoa cotyledon proteins (Biehl *et al.*, 1996). Biehl *et al.* (1982) found 11 polypeptide bands in their electrophoretic patterns of cocoa cotyledon protein, of which two (44 and 26 kDa) were tentatively identified as storage proteins.

A similar result was obtained from globulin of cotton, alfalfa, pea, mung bean and French bean seed, in which the smaller molecular weight polypeptide is believed to be a degradation product of the larger subunit of VCG (Baumgartner and Chrispeel, 1977). Osborne and Campbell (1988) reported that the smaller polypeptides (12.0 -34.0 kDa) of pea cotyledon vicilin are formed by proteolytic processing of the larger polypeptides in protein bodies. The first two bands (band I and II) in cottonseed globulin were seen to have high density globulin subunits while the latter two (band III and IV) were slightly shown. Globulin A dissociated into two subunits of approximately 80 kDa as reported by Wallace (1976). Reduction and alkylation of the subunits split them into further smaller components of 40 kDa molecular weight. Six slight bands in alfalfa globulin correlate with findings of Krochko and Bewley (1988) who found that alfin, globulin of alfalfa composed

of polypeptides ranging from 14 to 50 kDa. Pea globulin which showed five polypeptide bands in SDS PAGE are in agreement with Utsumi *et al.* (1980) who separated three major bands in SDS PAGE having MW of 61.7, 59.8 and 48 kDa. In reduction conditions, six major polypeptide bands were seen with MW of 51, 49, 36, 23, 20.5 and 19 kDa. Gwiazda *et al.* (1980) reported that pea vicilin had MW of 180 kDa (6.4S) with four subunits. Both mung bean and French bean seeds globulin showed five polypeptide bands. Mung bean vicilin contains four different polypeptides with molecular weights of 63,500, 50,000, 29,500, and 24,000 in the molar ratios of 1:5:1:1 (Ericson and Chrispeel, 1976). Two main subunits for French bean globulin, MW 50,000 and 47,000 in approximate ratio of 2-3:1, but four other prominent components, MW 60,000, 32,000, 23,000 and 20,000 are also present (Sun and Hall, 1975). From comparisons through the polypeptide patterns shown by individual seeds globulin, it can be seen that all seeds show major bands at molecular weight of 35 to 55 kDa. Seeds globulin from cotton, mung bean, and French bean seeds showed high-density polypeptide bands at molecular weights ranging from 33.0 to 50.0 kDa, especially for cotton at 54.6 kDa (band I) and 48.1 kDa (band II), mung bean at 48.6 kDa (band II) and French bean globulin at 48.2 kDa (band III). All the seeds globulin also showed subunits quite similar to cocoa VCG first band (51.1 kDa). Thus, it may be assumed that all the seeds have the potential to produce cocoa-specific aroma in terms of similarity of the globulin subunits to cocoa.

Oligopeptide Patterns of Cocoa and Other Seeds Globulin

The oligopeptide patterns of cocoa seed, cottonseed, alfalfa seed, pea seed, mung bean and French bean globulin are shown in Figures 2(a), (b), (c), (d), (e) and (f) respectively. The globulin from each seed was not subject to any autolysis or incubation with any enzyme at certain temperatures. Peptide profiles from VCG showed a complex pattern of

hydrophobic and hydrophilic peaks (Figure 2a). The predominant peaks were eluted within 20 to 50 min. Two high peaks were observed at 21 min (Peak 1) and 41 min (Peak 2), similar to Amin *et al.* (2002b). The peptides represent hydrophobic and hydrophilic chemical compounds which are responsible for the formation of cocoa aroma (Biehl *et al.*, 1982). The globulin and albumin storage protein represented 23 and 14%, respectively of the total seed protein and indicated the presence of other unknown abundant polypeptides (Derbyshire *et al.*, 1976). Cottonseed globulin formed slight peaks of peptide pattern (Figure 2b). Two high peaks were at 18.5 min (Peak 1) and 22 min (Peak 2). The dominant peaks were at 18 to 22 min while other minor peaks were formed at 24 to 26 min and 34 to 45 min. A very high peak was observed at 17 min in alfalfa seed peptide profile (Figure 2c). Two peaks which stood near to each other at 21 and 22 min were also observed. Another peak (Peak 4) at 37 min was also formed. The dominant peaks were observed from 17 to 22 min. One peak at 17 min (Peak 1) was observed in peptide profile of pea seed (Figure 2d) with the dominant ones formed at 17 to 22 min while other three formed scarcely thereafter at 27, 35 and 37 min (Peak 2, 3 and 4). Most peaks were seen to be formed at the latter part of the elution time for mung bean profile (Figure 2e). At 22, 29, 36 and 38 min, higher peaks were observed. For French bean seed globulin (Figure 2f), the peptide was observed to form at the middle part at 16 to 28 min with two major peaks at 17 and 27 min (peak 1 and 2).

Free Amino Acids Composition of Cocoa and Other Seeds Globulin

The composition of free amino acids from different seeds is shown in Table 2. Examination of the amino acid composition of seed globulins showed a similar high content of amides (glutamic acids-glutamine, aspartic acid- asparagine and arginine) indicating that they have a storage role and may be equivalent

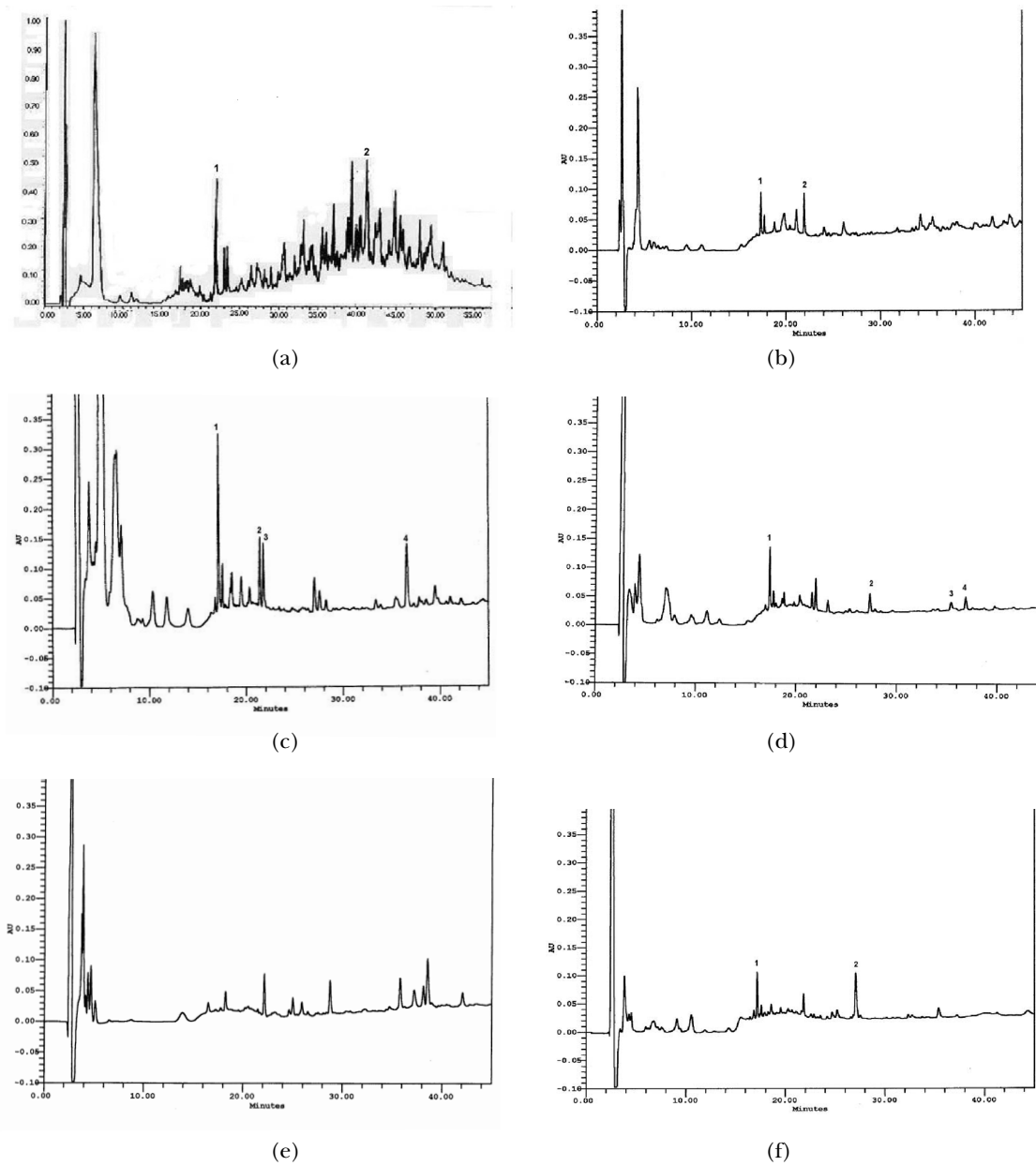


Figure 2: Chromatographic profile of peptide mixtures from (a) cocoa, (b) cotton, (c) alfalfa, (d) pea, (e) mung bean and (f) French bean

proteins (i.e. have similar function, structure etc) (Massimo *et al.*, 1987). A comparable level of other amino acids was also found. The total amino acids were separated into two categories which are hydrophobic and hydrophilic. All the seed globulins including cocoa VCG are significantly different ($p < 0.05$) from each other for all amino acids detected.

In comparison, seeds globulin from cotton, alfalfa, pea, mung bean and French bean are significantly different ($p < 0.05$) from cocoa VCG in total free amino acids with 25.7%, 54.6%, 11.8%, 12.5% and 16.8% respectively. Cocoa VCG contained a significant concentration of total hydrophobic: hydrophilic compounds at 45%: 55%. Puziah *et al.* (1998) reported that unfermented cocoa beans contained significant concentrations of total acidic, hydrophobic and other free amino acids at 30%: 18%:52% respectively. The ratio of hydrophobic:acidic:other amino acids of unfermented cocoa bean from different origins were 33%:30%:35% and that of the unfermented Malaysian cocoa bean was 41%:26%:33% (Kirchoff *et al.*, 1989). The difference in the free amino acids concentration could be due to maturity, harvesting season, transportation time, variety and origin of cocoa beans (Pettipher, 1990). Hydrophobic peptides and amino acids are responsible for the contribution to specific-aroma in cocoa seeds (Kirchoff *et al.*, 1989). All the seeds showed a higher proportion of hydrophilic to hydrophobic amino acids except for alfalfa seeds which gave a ratio of 1.20 (Table 2).

Low concentrations of amino acid Lys were observed in all seeds globulin. This correlates with the fact that although cereal and legumes are major components of the human diet, the seed storage proteins in both are generally deficient in essential amino acids, especially Lys (Krishnan and Puppke, 1993). While a number of seed proteins rich in sulphur containing

amino acid are available (Coulter and Bewley, 1990), not many lysine rich seed storage proteins have been identified so far.

CONCLUSION

The seeds were shown to be rich sources of proteins. The recovery of globulins extracted from each seed sample was very low, ranging from 1 to 2%. The extractions were repeated several times until a sufficient amount was obtained for further analyses. SDS PAGE from each seed globulin showed that several polypeptide bands correlate to their molecular weights of globulin subunits. Complex patterns of oligopeptide were observed from seeds globulins. Cocoa VCG showed the highest value of total free amino acid compared to other seeds globulin. Comparing the polypeptide patterns through SDS PAGE, it can be assumed that cotton seed have the most similar characteristics with cocoa VCG but with some limitations. The globulins are to be subjected to proteolysis by cocoa proteases and the characteristics should be compared before and after proteolysis in order to obtain sufficient data to further improve on the findings of this research.

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Table 2: Free amino acid composition from different seeds globulin^a

Hydrophobic/ Hydrophilic	Amino acid	Cocoa seed	Cotton seed	Alfalfa seed	Pea seed	Mung bean	French bean
Hydrophobic	Ala	52.37 ^a	24.68 ^c	12.58 ^d	40.39 ^b	11.23 ^e	6.21 ^f
	Ile	20.24 ^d	21.63 ^c	19.24 ^c	25.43 ^a	16.54 ^f	22.35 ^b
	Leu	26.07 ^e	27.58 ^d	9.13 ^f	39.89 ^a	34.95 ^c	35.40 ^b
	Met	63.25 ^a	9.67 ^d	3.93 ^f	25.21 ^c	30.49 ^b	4.35 ^e
	Phe	39.23 ^f	97.62 ^d	135.73 ^b	90.25 ^e	97.82 ^c	182.71 ^a
	Val	24.33 ^c	70.54 ^a	9.57 ^f	29.51 ^b	16.96 ^d	11.33 ^e
	Gly	29.19 ^a	17.05 ^d	6.82 ^f	14.36 ^e	23.20 ^b	18.67 ^c
	Tyr	117.28 ^a	16.14 ^d	11.67 ^f	22.41	24.64 ^b	14.53 ^e
Total Hydrophobic (mg/g)		381.96 ^a	284.91 ^d	208.67 ^f	287.45 ^c	255.83 ^e	295.55 ^b
	Arg	74.65 ^c	76.37 ^b	37.34 ^f	72.51 ^d	80.46 ^a	45.25 ^e
	Asp	66.26 ^c	40.12 ^e	28.30 ^f	89.27 ^a	83.81 ^b	51.00 ^d
	Glu	114.21 ^b	90.05 ^d	51.36 ^f	108.22 ^c	120.13 ^a	78.59 ^e
	Lys	30.44 ^c	20.03 ^f	20.65 ^e	50.83 ^a	25.90 ^d	50.63 ^b
	Cys	65.81 ^c	57.87 ^d	9.18 ^f	31.65 ^e	67.00 ^b	111.75 ^a
	Ser	44.63 ^a	21.16 ^e	12.25 ^f	35.91 ^c	43.48 ^b	24.96 ^d
	Thr	39.61 ^c	22.66 ^e	11.44 ^f	45.51 ^a	42.29 ^b	27.26 ^d
	His	26.82 ¹	13.67 ^e	3.38 ^f	25.22 ^b	18.81 ^c	16.73 ^d
Total Hydrophilic (mg/g)		461.43 ^b	341.93 ^e	173.90 ^f	456.12 ^c	481.88 ^a	406.17 ^d
Hydrophobi/Hydrophilic Ratio		0.82 ^b	0.83 ^c	1.20 ^a	0.63 ^c	0.53 ^f	0.72 ^d
Total Free Amino Acid (mg/g)		843.39 ^a	626.84 ^e	382.57 ^f	743.57 ^b	737.71 ^c	701.72 ^d

^aThe globulins were prepared from cocoa and other seeds as described in 'Materials and Methods' section. Free amino acid present in the proteolysis products were analysed by reversed-phase HPLC of the OPA derivatives (Kirchoff *et al.*, 1989). Mean values for seeds globulin followed by different letters within the same row are significantly different at $p < 0.05$.

REFERENCES

- Amin, I., Jinap, S., Jamilah, B., Harikrisna, K. and Biehl, B. 2002a. Analysis of vicilin (7S)-class globulin in cocoa cotyledons from various genetic origins. *Journal of Food Science and Agriculture*, 82: 728-732.
- Amin, I., Jinap, S., Jamilah, B., Harikrisna, K. and Biehl, B. 2002b. Oligopeptide patterns produced from *Theobroma cacao* L of various genetic origins. *Journal of Food Science and Agriculture*, 82: 733-737.
- Amin, I., Jinap, S., Jamilah, B. and Harikrisna, K. 2003. Analysis of cocoa cotyledon albumin. *Asian Journal of Plant Sciences*, 2(13): 958-962.
- AOAC 1996. Official methods of analysis. 17th edn. Airlington, USA: Association of Analytical Chemist.
- Arntfield, S.D. and Murray, E.D. 1981. The influence of processing parameters on food protein functionality. I. Differential scanning calorimetric as an indicator of protein denaturation. *Canadian Institute of Food Science and Technology Journal*, 14(4): 289-294.

- Baumgartner, B. and Chrispeels, M.J. 1977. Purification and characteristics of vicilin peptidohydrolase, the major endopeptidase in the cotyledon of Mung bean seedlings. *European Journal of Biochemistry*, 77: 223-233.
- Bewley, J.D. and Black, M. 1994. In Bewley, J.D. and Black, M. (Eds). *Seeds: Physiology of Development and Germination*, p. 29-83. New York: Premium Press.
- Biehl, B., Heinrich, H., Voigt, J., Voigt, G., Bytof, G. and Serrano, P. 1996. Nature of proteases and their action on storage proteins in cocoa seeds during germination as compared with fermentation. *Proceedings of the 12th International Cocoa Conference*, 20-21 October, Bahia, Brazil.
- Biehl, B., Brunner, E., Passern, D., Quesnel, V.C. and Adomako, D. 1985. Acidification, proteolysis and flavour potential in fermenting cocoa beans. *Journal of Science Food Agriculture*, 36: 583-598.
- Biehl, B., Wewetzer, C. and Passern, D. 1982a. Vacuolar (storage) protein of cocoa seeds and their degradation during germination and fermentation. *Journal of Science Food Agriculture*, 33: 1291-1304.
- Cohen, S.A., Meys, M. and Tarvin, T.L. 1990. The Pico-tag method in a manual of advanced techniques for amino acid analysis.
- Coulter, K.M. and Bewley, J.D. 1990. Characterization of a small sulphur-rich storage albumin in seeds of alfalfa (*Medicago sativa* L.). *Journal of Experimental Botany*, 41: 1541-1547.
- Derbyshire, E., Wright, D.J. and Boulter, D. 1976. Review – Legumin and vicilin storage protein of legume seed. *Phytochemistry*, 15: 3-24.
- Ericson, M.C. and Chrispeel, M.J. 1976. The carbohydrate moiety of Mung bean vicilin. *Australian Journal of Plant Physiology*, 3: 763-769.
- Gatehouse, J.A., Evans, I.M., Croy, R.R.D. and Boulter, D. 1986. Differential expression of genes during legume seed development. *Philos. Trans. R. Soc. London, Ser. B*, 314: 367-384.
- Gatehouse, J.A., Croy, R.R.D., McIntosh, R., Paul, C. and Boulter, D. 1980. Quantitative and qualitative variation in the storage proteins of material from the EEC point field bean test. In Bond, D.A. (Ed). *Vicia faba: Feeding Value, Processing and Viruses*, p. 173-190. New York: Academic Press.
- Gwiazda, S., Schwenke, K.D. and Rutkowski, A. 1980. Isolation and partial characterization of proteins from pea (*P.sativum* L.). *Nahrung*, 24: 939.
- Hatree, E.F. 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Analytical Biochemistry*, 48: 422-427.
- Kirchoff, P.M., Biehl, B., Ziegleder-Berghausen, H., Hammoor, H. and Lieberei, R. 1989. Kinetics of the formation of free amino acids in cocoa seeds during fermentation. *Food Chemistry*, 34: 161-179.
- Konishi, Y., Fumita, Y., Ikeda, K., Okuno, K. and Fuwa, H. 1985. Isolation and characterization of globulin from seeds of *Amaranth hypochondriacus* L. *Agricultural and Biological Chemistry*, 49: 1453-1459.
- Krishnan, H.B. and Puppke, S.G. 1993. Biochemical and differentiation of globulins from dicotyledon plants. *Biochemical and Biophysics Research Communications*, 193: 460.
- Krochko, J.E. and Bewley, J.D. 1988. Use of electrophoretic technique in determining the composition of seed storage protein in alfalfa. *Electrophoresis*, 9: 751-763.
- Laemmli, U. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
- MacDonald, H.M., Masters, K.E. and Pettipher, G.L. 1994. Partial purification of cocoa seed protein and studies into the degradation of cocoa storage protein. *Caf  Cacao The*, 38: 119-124.

- Massimo, F.M., Kakuda, Y. and Rickey, Y.Y. 1987. Salt soluble seed globulin of various dicotyledon and monocotyledon plants. I - Isolation, purification and characterization. *Food Chemistry*, 62(1): 27-47.
- Mortenssen, P. 1980 Variation in legumin: vicilin ratio between and within cultivars of *Vicia faba* variety minor. In Bond, D.A.(Ed). *Vicia faba: Feeding, Value, and Viruses*, p. 159-172. New York: Allured Press.
- Osborne, T.B. and Campbell, G.F. 1988. Proteins of the pea. *Journal of American Chemists Society*, 20: 348-362.
- Pettipher, G. L. 1990. The extraction and partial purification of cocoa storage proteins. *Caf  Cacao The*, 1: 23-26.
- Puziah, H., Jinap, S., Sharifah, K.S.M., and Asbi, A. 1998. Changes of free amino acid, peptide-N, sugar and pyrazine concentration during cocoa fermentation. *Journal of Science of Food and Agriculture*, 78: 535-542.
- SAS. 1985. Statistical analysis system. Cary, NY, USA: SAS Institute Inc.
- Seow, T.K., Jinap, S., Lee, K.H. and Pat, M.L. 1995. Partial purification of cocoa protease. *Asean Food Journal*, 10: 160-162.
- Spencer, M.E. and Hodge, R. 1992. Cloning and sequencing of the cDNA encoding the major albumin of *Theobroma cacao*. *Planta*, 183: 528-535.
- Sun, S.M. and Hall, T.C. 1975. Solubility characteristics of globulins from Phaseolus seeds in regards to their isolation and characterization. *Journal of Agricultural and Food Chemistry*, 23: 184-189.
- Utsumi, S., Yokohama, Z. and Mori, T. 1980. Purification and properties of storage proteins of broad bean. *Agricultural and Biological Chemistry*, 43: 577-583.
- Vladimir, V.S., Irina, A.P., Valeriy, Y.G. and Vladimir, B.T. 1990. Isolation and purification of 7S and 11S globulin from broad beans and peas. *Journal of Agricultural and Food Chemistry*, 38: 92-95.
- Voigt, J., Biehl, B. and Kamaruddin, S. 1993a. The major seed proteins of *Theobroma cacao* L. *Food Chemistry*, 47: 145-151.
- Voigt, J., Biehl, B. and Heinrichs, H. 1993b. Proteolytic formation of cocoa flavour precursors. In Schreier, P. (Ed). *Progress in Flavour Precursor Studies*, p. 213-216. Wheaton: Allured Publishing Corporation.
- Voigt, J., Biehl, B., Heinrichs, H., Kamaruddin, S., Gaim Marsoner, G. and Hugi, A. 1994a. In-vitro formation of cocoa-specific aroma precursors: aroma-related peptides generated from cocoa seed proteins by co-operation of an aspartic endoprotease and a carboxypeptidase. *Food Chemistry*, 49: 173-180.
- Voigt, J., Biehl, B., Voigt, G. and Heinrichs, H. 1994b. Cocoa-specific aroma precursors are generated by proteolytic digestion of the vicilin-like globulin of the cocoa seeds. *Food Chemistry*, 50: 177-184.
- Wallace, R.W. 1976. Isolation and partial characterization of the Acalin A and B fractions of cottonseed globulins. USA: Texas A and M University, Ph.D Thesis.
- Wright, D.J. and Boulter, D. 1973. A comparison of acid extracted globulin fractions and vicilin and legumin of *Vicia faba*. *Phytochemistry*, 12:79-84.