Food Bioscience 40 (2021) 100908

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Electrophoretic characterization, amino acid composition and solubility properties of Macauba *(Acrocomia aculeata L.)* kernel globulins

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ARTICLE INFO

Keywords:
Acrocomia aculeata
Macauba
Bocaiuva
Sulfur-containing amino acids
Kernel press cake
Protein solubility

ABSTRACT

Macauba palm *(Acrocomia aculeata L.)* fruits are emerging sources to produce high-quality oils from pulp and kernels. Yet, the protein-rich press cake from the kernels remains underutilized, mainly due to lacking knowledge of protein composition and properties. Therefore, our aim was to characterize the main protein fractions of Macauba kernels. The globulins were the major protein fraction (58.5% of storage proteins), which were further separated into 11 S and 7 S globulins. The subunits of both globulins presented heterogeneity in isoelectric point (5.3–8.3), revealing genetic polymorphism. The 7 S globulins were soluble at low ionic strength (0.1 mol/L), whereas the 11 S globulins required higher salt concentration (0.50–0.75 mol/L) for solubilization, independent on selected salt types. Both globulins were rich in essential amino acids, especially methionine and cysteine, making Macauba kernel proteins a valuable source to complement diets based on pulses and oilseeds.

1. Introduction

Macauba *(Acrocomia acuelata L.)* is an oil-bearing palm member of the *Aracaceae* family, native to tropical and subtropical Americas (César et al., 2015). Macauba palm can grow in marginal soil and is suitable for intercropping and agroforestry systems (Colombo et al., 2018), while producing 2500 kg of oil per hectare and year on average, or even up to 5000 kg of oil per hectare and year for selected genotypes (Colombo et al., 2018). For those reasons, Macauba palm has recently spurred interest of scientists and agronomists in Brazil and other countries of Latin America.

Both Macauba pulp and kernels are rich in oil. After oil extraction a protein-rich press cake from Macauba kernels is left, which is currently used as low value animal feed or even discarded (Colombo et al., 2018). The amount of kernel press cake left after oil extraction is estimated to range from 814 to 1678 kg per hectare per year (own estimated calculations based on an annual fruit productivity of 25000 to 40000 kg/ha, a kernel proportion of 5–7% in Macauba fruit, and an average kernel protein content of about 16.5% in dry matter). Therefore, the residues from oil extraction of Macauba kernels might be a novel protein source to meet the demand for high valuable plant proteins. Hitherto, only a few publications dealing with some characteristics of Macauba kernel proteins are available. Hiane et al. (2006) determined the content of the Osborne fractions of Macauba kernel storage proteins and their amino acid profile. Macauba kernel protein fractions were rich in methionine, cysteine, valine and leucine, but poor in threonine, histidine, phenylalanine, tyrosine and lysine and were absent of protease inhibitors (Hiane et al., 2006). Bora and Rocha (2004) also determined the amino acid profile of Macauba (*Acrocomia intumescens*) kernel proteins, which were rich in leucine, isoleucine, phenylalanine, and threonine, but deficient in methionine, lysine, and valine. However, knowledge on the molecular properties of Macauba kernel proteins such as molecular weight (MW) distribution and isoelectric points (IP) as well as important physico-chemical properties like solubility at different conditions (pH, ionic strength, type of salt) is still scarce.

Globulins are the most widely distributed storage protein fraction in seeds of various plants, including of both mono and dicotyledonous seeds (Marcone et al., 1998). Globulins can be further fractionated into two distinct and heterogeneous families, the 11 S and 7 S globulins (Marcone, 1999). 11 S globulins are considered to be hexameric proteins

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https://doi.org/10.1016/j.fbio.2021.100908
Received 27 September 2020; Received in revised form 4 January 2021; Accepted 26 January 2021
Available online 29 January 2021

of relatively high MW ranging from 350 to 400 kDa. Each of the six subunits consists of one acidic (~40 kDa) and one basic (~20 kDa) polypeptide, linked by a disulfide bond (Derbyshire et al., 1975). The 7 S proteins are trimeric proteins of apparent MW in the range of 150–180 kDa (Fukushima, 1991). The subunits of 7 S globulins (~50 kDa) often lack interchain disulfide bridges (Fukushima, 1991). Due to their difference in structure and MW, the 7 S and 11 S globulins present considerable differences in physicochemical properties, which also affect their functional and nutritional properties (Tzitzikas et al., 2006). Moreover, the detailed characterization of the storage proteins is necessary to trace back functional properties (Marcone, 1999). In the case of Macauba kernels, the molecular composition of the globulin fraction and its physicochemical properties are still to be determined to assess its potential as new protein source for both technological and nutritional purposes. Therefore, the present paper aimed at characterizing the Macauba kernel proteins with emphasis on the globulin fraction. Thereby, the electrophoretic patterns of the Osborne fractions of de-oiled Macauba kernel flours were investigated along with the profiling of 11 S and 7 S subfractions by 2-dimensional gel electrophoresis. In addition, the amino acid composition of the 11 S and 7 S as main fractions of Macauba kernel proteins was evaluated and compared to the amino acid composition of Macauba total kernel proteins. Furthermore, the solubility of Macauba kernel proteins as a function of pH, ionic strength and type of salt was additionally investigated to correlate the profiles of the Osborne fractionation with protein solubility in aqueous media for future production of Macauba protein concentrates or isolates.

2. Materials and methods

2.1. Chemicals

Barium hydroxide, bovine serum albumin (BSA), bromophenol blue, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), coomassie blue, dithiothreitol (DTT), glycerol, iodoacetamide, sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris) and urea were purchased from Sigma-Aldrich (Steinheim, NW, Germany). Acetone, copper sulfate, hydrochloric acid, *n*-hexane, potassium chloride, sodium acetate, sodium chloride, sodium hydroxide and sodium sulfate were purchased from Synth (Diadema, SP, Brazil). Potassium sodium tartrate was purchased from Merck (Darmstadt, HE, Germany). Carrier ampholytes Pharmalyte™ broad range pH 3–10 was acquired from GE Life Sciences (Freiburg, BW, Germany). MW standard from 10 to 250 kDa (Precision Plus Protein™ Kaleidoscope™ Prestained or Unstained) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were analytical grade. Dialysis tubes with a MW cut-off of 12–14 kDa were purchased from Serva (Heidelberg, BW, Germany).

2.2. Macauba kernels

Macauba kernels (*Acrocomia aculeata* L.) from native populations of the municipality of Patos de Minas (MG, Brazil) harvested in 2017 were donated by INOCAS Soluções em Meio Ambiente S.A. (Patos de Minas, MG, Brazil). The fruits were mechanically de-hulled and pulped. The endocarps (inner shell) were broken using an endocarp breaking machine (Elipse, Campinas, SP, Brazil) to obtain the kernels, which were stored frozen at -20 °C until use.

2.3. Preparation of defatted Macauba kernel flour (MKF) for protein characterization

Macauba kernels were defrosted and partially de-oiled by pressing using an endless screw extractor ERT 60II (Scott Tech, Vinhedo, SP, Brazil). Press cake temperature was kept between 45 and 55 °C to prevent protein denaturation. De-oiling was completed in a 2.5 L laboratory Soxhlet apparatus (500 g raw material) with *n*-hexane for 24 h. The de-oiled press cake was de-solventized under a fume hood overnight and then ground using an impact mill (Ika, Koenigswinter, NW, Germany) equipped with a 2 mm sieve insert.

2.4. Composition of MKF

Dry matter and ash content were determined using a thermogravimetrical system (TGA 601, Leco Corporation, St. Joseph, MI, USA) according to § 64 LFGB methods (German Food Act, 1980) at 105 °C until constant weight and after combustion at 550 °C, respectively. Protein content of the samples was determined by means of the Dumas combustion method (AOAC, 1990a) using a nitrogen analyzer FP 528 (Leco Corporation) and a nitrogen conversion factor of N x 6.25. Total dietary fiber content was determined following the enzymatic-gravimetric method (AOAC, 2000). Residual lipid content was determined by the Soxhlet extraction method using *n*-hexane (AOAC, 1990b).

2.5. Characterization of MKF proteins

2.5.1. Osborne fractionation

The extractable protein fractions from MKF were determined by adapting the Osborne fractionation method (Chang et al., 2014), as illustrated in Fig. 1a. MKF was dispersed in 10 volumes of 0.5 mol/L NaCl solution at room temperature and stirred at 500 rpm for 1 h. The dispersion was centrifuged at 3300 *g* for 30 min and filtered with Whatman paper filter n° 1. This procedure was repeated until a clear extract was obtained and the supernatants containing the albumin and globulin fractions were pooled. The solid residue was extracted with 10 volumes of 70% (v/v) aqueous ethanol at room temperature under constant stirring (500 rpm) for 1 h, centrifuged and filtered as described. After three extraction cycles, the supernatants of the ethanolic extractions were combined to yield the prolamin fraction. The remaining pellet was dispersed in 0.1 mol/L NaOH solution, resulting in a pH of 12.5–13.0, at room temperature and stirred at 500 rpm for 1 h. The mixture was centrifuged and filtered as described and the extraction procedure was repeated twice. The supernatants of the alkaline extractions were combined, yielding the glutelin fraction.

The albumin and globulin fractions were further separated by dialysis against 20 volumes of deionized water for 2 days at 5 °C with the water changed twice a day. The precipitated globulins were separated from the water-soluble albumins by centrifugation at 3300 *g* for 30 min and freeze-dried in order to obtain a globulin isolate. The albumin, the prolamin and the glutelin fractions were concentrated after dialysis in a rotary evaporator operated at 40 °C to 1/10 of the original volume.

Protein content in the Osborne fractions was determined by the biuret method (Gornall et al., 1949) calibrated with BSA. The obtained protein content of the fractions was summed up and compared to the total protein content of the defatted MKF. Protein content in the final residue was also determined (AOAC, 1990a) and accounted as insoluble proteins. All fractions were stored at −20 °C until gel electrophoresis.

2.5.2. 7 S and 11 S globulins

Separation of Macauba 7 S and 11 S globulins was accomplished by sequential dialysis (Derbyshire et al., 1975), as depicted in Fig. 1b. For this, Macauba kernel globulins (MKG) and albumins were extracted as previously described. The 11 S globulins were obtained after dialysis of the liquid extract against 5 volumes of deionized water at 5 °C for two days, with the water changed twice a day. The precipitate was separated from the supernatant by centrifugation and filtration. The proteins in the solid pellet were further precipitated in 10 volumes of 10% trichloroacetic (TCA)-acetone solution overnight at −18 °C to yield a crude 11 S globulins (Natarajan et al., 2006). Further purification of the crude 11 S globulins was accomplished by filtration, washing at room temperature (three times with 10 mL of acetone) and de-solventization under the fume hood.

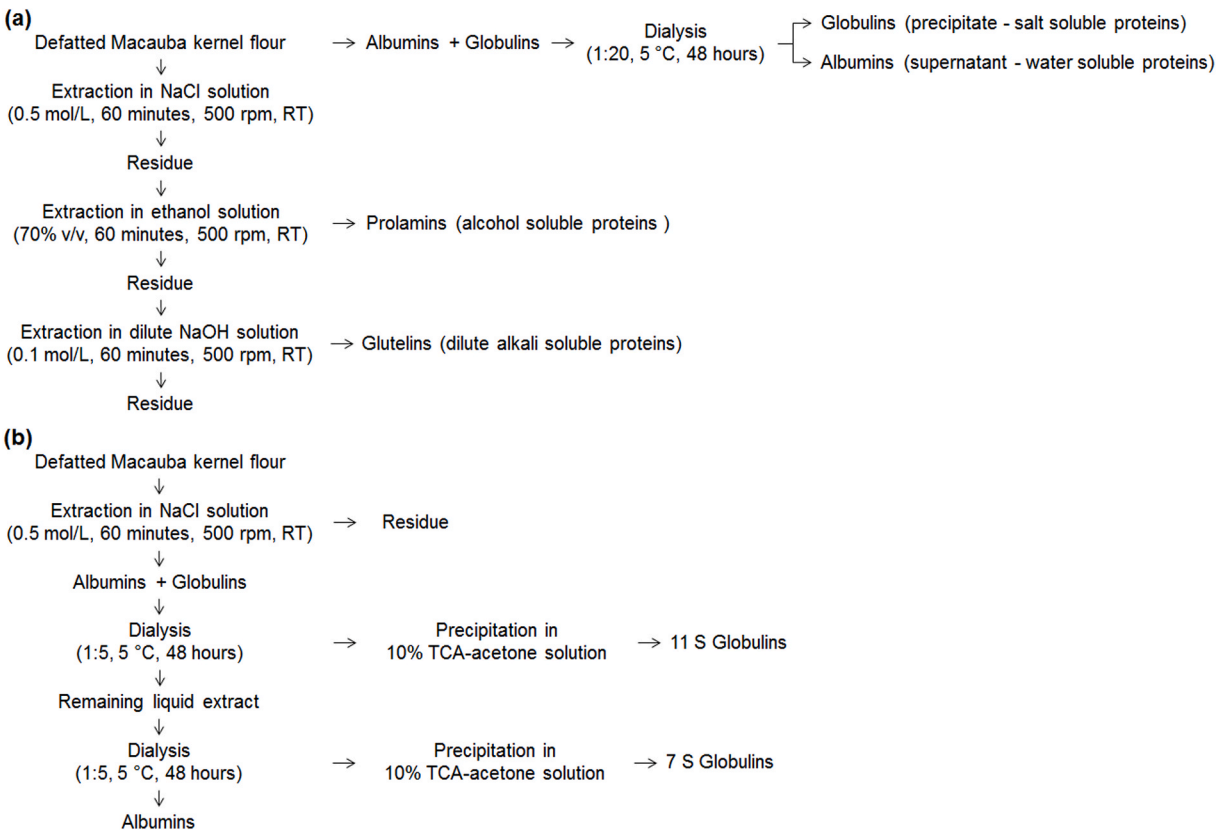

Fig. 1. Fractionation protocol of Macauba kernel (a) proteins and (b) globulins. RT: room temperature. TCA-acetone solution (trichloroacetic acid-acetone solution).

The supernatant comprising albumins and 7 S globulins was further dialyzed against 5 volumes of deionized water at 5 °C for another two days with the water changed twice a day. After centrifugation and filtration, crude 7 S globulins were obtained by precipitating the solid pellet in 10 volumes of 10% TCA-acetone solution at −18 °C overnight. The crude 7 S globulins were filtered, washed three times with 10 mL of acetone at room temperature and de-solventized. The 7 S and 11 S globulins were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis and amino acid composition.

2.5.3. Electrophoretic characterization

2.5.3.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). MKF proteins, Osborne fractions (albumins, globulins, prolamins and glutelins) as well as the 7 S and 11 S globulins were characterized regarding their MW distribution by SDS-PAGE adapted from Laemmli (1970). Prior to electrophoretic evaluation, the protein samples were treated as described by Krishnan et al. (2009) with some modifications. 10–50 mg of sample were suspended in 63 mmol/L Tris-HCl buffer (pH 6.8), 2% SDS, 10% v/v glycerol and 0.01% bromophenol blue. For reducing conditions, 100 mmol/L of DTT were added to the solubilizing solution. Then, the mixture was heated to 95 °C for 5 min and centrifuged at 11900 *g* for 2 min. The pellet was discarded, and the supernatants were diluted in the same solubilizing solution to yield a protein concentration of 5 mg/mL. Electrophoretic separation of proteins was performed by loading 50 μg of protein on pre-cast 4–20% Criterion™ TGX Stain-Free™ gels (Bio-Rad Laboratories). Electrophoretic separation was carried out at 200 V, 60 mA, and 100 W at room temperature. Visualization and documentation were performed using a Gel Doc™ EZ Imager system and Image Lab software (Bio-Rad Laboratories) for analysis of the MW distribution. The reported results are representative from 3-fold determination.

2.5.3.2. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE). Proteins of MKF and MKG were further characterized by means of 2D-PAGE using a Multiphor II system (GE Life Sciences). Isoelectric focusing was performed using ready-to-use Immobiline™ DryStrip immobilized pH gradients (IPG) and the horizontal SDS-PAGE step was performed using pre-cast ExcelGel™ 2-D Homogeneous 12.5 (GE Life Sciences).

50 mg of MKF and 5 mg of MKG were solubilized in a multi-chaotropic solution (8 mol/L urea, 4% CHAPS, 2% Pharmalyte™ 3–10) for 30 min at room temperature and centrifuged at 11900 *g* for 2 min. The supernatant was diluted 3-fold with rehydration solution (8 mol/L urea, 2% CHAPS, 2% Pharmalyte™ 3–10, 18 mmol/L DTT, 0.002% bromophenol blue) and an aliquot of 125 μL was used for the rehydration of the IPG strips overnight. Isoelectric focusing started at 200 V for 1 min, subsequent risen to 3500 V and kept constant for 90 min at 20 °C. The strips were equilibrated in DTT solution (50 mmol/L Tris-Cl, pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 65 mmol/L DTT) for 15 min at room temperature followed by equilibration in iodoacetamide solution (50 mmol/L Tris-Cl, pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 135 mmol/L iodoacetamide) for further 15 min. The strips were transferred to a pre-cast Excelgel™ and electrophoresis was performed at 120 V for 40 min, followed by 600 V for additional 60 min at 15 °C. The gels were rinsed with distilled water, stained with 0.02% solution of coomassie blue for 90 min, destained to remove excess staining and scanned for analysis. The Image Lab software (Bio-Rad Laboratories) was used for determination of molecular weight distribution. The reported results are representative from 3-fold determination.

2.5.4. Amino acid composition

Amino acid composition (single determination) of MKF proteins as well as of 7 S and 11 S globulins was determined by HPLC after oxidation and hydrolysis of the sample material as described by the Commission

Regulation (EC) No. 152/2009 (2009a). Tryptophan was determined by HPLC after hydrolysis in saturated barium hydroxide solution as described by the Commission Regulation (EC) No. 152/2009 (2009b). Nutritional quality of MKF proteins was evaluated by calculation of the chemical score (equation (1)).

$$CS = \left(\frac{As_i}{Ar_i}\right)\cdot 100 \quad (1)$$

where CS is the chemical score in %, As_i and Ar_i are the content of amino acid *i* in the sample and in the reference protein, both expressed in g/100 g of protein. FAO's amino acid scoring pattern for 3–10 years old children was selected for assessment of protein quality (FAO/WHO, 2013). The amino acid with the lowest chemical score was considered as the first limiting amino acid.

2.5.5. Solubility properties of MKF proteins

2.5.5.1. Effect of pH, ionic strength, and salt on MKF proteins solubility. The solubility profile of MKF proteins was determined adapting the method described by Carbonaro et al. (1997). 500 mg of MKF was dispersed in 25 mL of salt solution, the pH was adjusted to the desired value using 1.0 or 0.1 mol/L NaOH or HCl and kept constant for 60 min. Subsequently, the samples were quantitatively transferred into a 50 mL volumetric flask and the volume completed with the corresponding salt solution. The solution was centrifuged at 3300 *g* for 30 min at room temperature. The supernatant was filtered and the protein content in the liquid extract was determined by the Biuret method (Gornall et al., 1949). Protein solubility was calculated according to equation (2):

$$S[\%] = \frac{c\cdot V}{m\cdot p\cdot DM}\cdot 100 \quad (2)$$

where *S* is the protein solubility in %, *c* is the concentration of protein in the extract in mg/mL, *V* is the volume of the solution (in our case 50 mL), *m* is the mass of MKF weighed in (mg), and *p* and *DM* are the protein content of MKF determined by Dumas combustion method (AOAC, 1990a) and dry matter content determined by conventional drying at 105 °C, respectively.

The influence of pH (from 2.00 to 12.00 ± 0.01) at constant ionic strength (0.1 or 0.5 mol/L of NaCl) on the solubility of MKF proteins was evaluated. The effect of different salts, with increasing chaotropic effects of both anions (SO_4^{2-}, Cl^-, CH_3COO^-) and cations (K^+, Na^+), was investigated, according to the Hofmeister series. Therefore, the influence of sodium chloride, potassium chloride, sodium sulfate, and sodium acetate on protein solubility was determined at varying ionic strengths from 0.1 to 5.0 mol/L (or until the solubility limit) and at constant pH (7.00 ± 0.01). All experiments were performed in triplicate.

2.5.5.2. Influence of the milieu conditions on the MW distribution of solubilized proteins. MW distribution of solubilized proteins in different NaCl solutions (pH 4.0, 7.0, 8.0 and 12.0 at 0.1 mol/L NaCl, and pH 7.0 at 0.5 mol/L NaCl) was evaluated by SDS-PAGE. Additionally, MW distribution of the solubilized proteins in potassium chloride, sodium acetate and sodium sulfate solutions at pH 7.0 and ionic strengths of 0.75 and 3.0 mol/L was determined. For that, selected supernatants of the protein solubility determinations were dialyzed against 20 volumes of deionized water at 5 °C for two days (water changed twice a day) in order to extensively remove the salts. Following, the extracts were concentrated using a rotary evaporator operated at 40 °C to 1/10 of their initial volume and then subjected to SDS-PAGE as previously described.

2.6. Statistical evaluation of the data

Results are reported as mean values ± standard deviations. Average values of 2 corresponding groups were compared using Tukey test for determining significant differences ($p < 0.05$).

3. Results and discussion

3.1. Composition of MKF and Osborne fractionation

Total dietary fiber was the main constituent in MKF (48.65 ± 1.28 g/100 g DM), followed by protein (36.23 ± 0.04 g/100 g DM). Ash and residual lipid content were 5.22 ± 0.01 and 2.41 ± 0.04 g/100 g DM, respectively. Nitrogen free extractable substances, calculated by difference, accounted for 7.49 g/100 g DM. These values agree with the composition of Macauba kernels reported by other authors (Coimbra and Jorge, 2011; Hiane et al., 2006).

The Osborne fractionation revealed the globulins as the major protein fraction in MKF (58.53 ± 0.59 g BSA equivalent/g of crude protein), followed by the glutelins (22.48 ± 0.62 g BSA equivalent/100 g of crude protein). Similar, but significantly different ($p < 0.05$) contents of albumins (9.09 ± 0.09 BSA equivalent/100 g of crude protein) and prolamins (9.79 ± 0.08 g BSA equivalent/100 g of crude protein) were present in MKF. At the end of the fractionation protocol, insoluble protein in the residue accounted for 8.30 ± 0.12 g crude protein/100 g DM.

Hiane et al. (2006) also reported the globulins as the major protein fraction in Macauba kernels with 53.5% of total Macauba kernel protein. However, higher contents of glutelins (40.0%) and lower contents of albumins (5.4%) and prolamins (1.1%) were reported by those researchers. Such differences might be attributed to differences in the employed fractionation protocol. In our work, 3 extraction cycles were performed, whereas Hiane et al. (2006) reported one extraction cycle for each Osborne fraction. Differences in the content of the Osborne fractions might also relate to differences in harvest seasons/years, environmental growth conditions and genetic variability among Macauba genotypes, which might be investigated in more detail in the future. Content of insoluble protein or nitrogen-containing substances in the final residue was similar to that reported for oil palm (*Elaeis guineensis*) kernels (9.0% of total protein) (Chang et al., 2014).

3.2. Electrophoretic characterization

The MW distributions of MKF proteins, their Osborne fractions (with exception of glutelins) and 11 S and 7 S globulins are presented in Table 1.

MKF proteins were resolved into 6 and 7 bands (10.5–50.6 kDa) under non-reducing and reducing conditions, respectively. Similar results were obtained for storage proteins from other members of the *Aracaceae* family such as coconuts (7 protein bands: 14–52 kDa) (Kwon et al., 1996) and oil palm kernels (6 protein bands: 20.7–51.5 kDa) (Zheng et al., 2015).

SDS-PAGE profile of storage proteins from legume and oilseeds generally presents a higher number of protein bands within a broader range of MW. In peas (Tzitzikas et al., 2006), soybeans (Krishnan et al., 2009) and sunflower (González-Pérez et al., 2002) over 10 protein bands were observed with MW ranging from 4.5 to 97.4 kDa. Heterogeneity of protein subunits and extensive post translational processing often result in complex SDS-PAGE profile of storage proteins. The limited diversity of MKF proteins electrophoretic pattern could be due to the presence of similar MW patterns in the Osborne fractions or the relative abundance of globulins, in a way that protein bands from other Osborne fractions like albumins and prolamins present in low amounts might not be visible. Furthermore, limited diversity of MKF proteins SDS-PAGE band patterns could be a result of non-extensive proteolytic processing *in vivo*.

The albumin fraction presented 4 and 9 protein subunits under non-reducing and reducing conditions, respectively, with MW in the range of 10.9–58.9 kDa. Usually rich in cystine, those storage albumins, are commonly referred to as 2 S albumins (Martínez et al., 1997) and are present in a variety of different kernels, like in soybeans, peas, coconuts, sunflower and rapeseed (Dalgalarrondo et al., 1986; Fukushima, 1991; González-Pérez et al., 2002; Marcone, 1999). This corroborates our

Table 1
Molecular weight distribution of Macauba kernel total proteins, their Osborne fractions and 11 S and 7 S globulins.

Molecular weight (kDa)	Total proteins (%)		Albumins (%)		Total Globulins (%)		11 S globulins (%)		7 S globulins (%)		Prolamins (%)	
	NR	R	NR	R	NR	R	NR	R	NR	R	NR	R
58–60	–	–	–	9.8	–	–	–	–	–		–	–
52–57	–	–	21.6	23.8	–	–	14.0	23.1	100.0	69.4	–	44.6
49–51	30.2	42.0	53.0	–	23.7	33.8	–	–	–	–	50.5	–
43–48	23.4	–	–	7.2	74.3	–	17.4	–	–	–	45.0	–
34–36	–	–	19.4	8.0	–	–	–	–	–	–	–	–
31–33	–	–	–	9.7	–	–	–	15.2	–	–	–	15.9
28–30	09.3	9.4	–	1.9	–	22.1	12.3	4.0	–	–	–	3.8
25–27	–	–	–	–	–	–	–	–	–	7.7	–	–
19–21	–	–	–	–	2.0	41.9	56.3	57.7	–	–	4.5	35.8
16–18	30.1	38.5	6.0	20.2	–	–	–	–	–	8.7	–	–
13–15	3.2	2.7	–	9.2	–	–	–	–	–	–	–	–
9–12	3.9	7.3	–	10.2	–	2.2	–	–	–	14.1	–	–
Number of protein bands	6	7	4	9	4	5	4	4	1	4	3	4

NR: Non-reduced condition (without dithiothreitol); R: Reduced condition (with dithiothreitol). No bands either under reducing or non-reducing conditions were obtained for the glutelin fraction, which is therefore not being displayed here. The relative contents were determined by software-based evaluation of bitmaps of SDS-PAGE electropherograms. For each sample, the total area of all protein bands was set to 100%.

findings of the increasing numbers of protein bands due to cleavage of disulfide bonds observed under reducing conditions. Furthermore, these findings are also consistent with the amino acid composition of the albumin fraction determined by Hiane et al. (2006), revealing the highest content of sulfur-containing amino acids (Cys + Met) of 4.2 g/100 g.

At non-reducing conditions, 74.3% of MKG presented MW of 43.6 kDa. This protein band was not detected at reducing conditions, with two new protein bands with MW of 19.2 kDa (41.9%) and 27.6 kDa (22.1%) detected, probably subunits of the aforementioned protein (43.6 kDa) linked by a disulfide bond. The 11 S globulins presented 3 protein bands with MW of 52.7, 30.6 and 19.4 kDa under reducing conditions. As shown in Table 1, the protein bands of 30.6 and 19.4 kDa accounted for 76.9% of the MKG resolved protein bands, showing that the 11 S subunits were predominant. Those protein bands were also present in non-reducing SDS-PAGE. This could be due to partial disassembling of the 11 S subunits during extraction and precipitation. Disassembling of subunits was also reported to occur in isolated 11 S globulins from rapeseed (Dalgalarrondo et al., 1986) and coconuts (Garcia et al., 2005).

The 7 S globulins presented one major protein subunit, with MW of 53.1 kDa under both non-reducing and reducing conditions, corresponding to 69.4% of the bands resolved by SDS-PAGE. Faint bands with MW of 25, 17 and 12 kDa were also present in the reducing SDS-PAGE, which could be part of albumins or basic 7 S globulins (Duranti et al., 2008; Mendoza et al., 2001; Sato et al., 1987).

The prolamin fraction presented 3 and 4 bands under non-reducing and reducing conditions, respectively, with MW ranging from 19.5 to 54.7 kDa. Proteins of the prolamin family are highly polymorphic with subunit MW ranging from 30 to 50 kDa. Different from cereals and pseudocereals, the prolamins from Macauba presented limited number of bands, which was comparable to prolamins from coconut and oil palm kernel with the latter resolving into 3 bands of 27, 33 and 50 kDa (Chang et al., 2014) and the former into 4 bands (17–56 kDa) (Kwon et al., 1996). Prolamins might have the ability to trigger immunotoxicity in people within the gluten-sensitive spectrum, so a gluten threshold of 20 mg/kg is the standard for gluten-free food (Cebolla et al., 2018). Considering the relative high content of prolamins in MKF (compared to e.g. soybeans and peas), future research on Macauba proteins should assess potential homology of this fraction to wheat glutenins.

No protein bands were observed under reducing and non-reducing conditions in the glutelins electropherograms, with large amounts of proteins not loaded into the resolving gel. Similar results were obtained for proteins of coconut milk press cake extracted at pH 11.0 and 50 °C (Chambal et al., 2012) and for oil palm kernel glutelins (Chang et al., 2014). Treatment of proteins under alkaline pH as applied during glutelin extraction might result in intra- and intermolecular cross-linking of the polypeptides, resulting in increased apparent MW via lysinoalanine, lanthionine and histidinoalanine formation (Friedman, 1999; Friedman et al., 1984). Macauba kernel glutelins might similarly be susceptible to intermolecular crosslinking at alkaline treatment, impairing its resolution by SDS-PAGE. In contrast to our findings, Hiane et al. (2006) displayed MW of Macauba kernel glutelins of 14–45 kDa. These differences might be attributed to the use of other Macauba genotypes or to the applied extraction procedure, which lasted for only 1 h at high pH compared to 5 h in our fractionation protocol (comprising 3 extraction cycles). This increased extraction time might have led to formation of intermolecular crosslinking of the glutelins.

To investigate the MKF proteins, the MKG as well as the 7 S and 11 S globulins in more detail, 2-dimensional gel electrophoresis was performed, and the protein mapping is presented in Fig. 2. This technique resolves the protein fractions according to their molecular weight and isoelectric points, allowing the evaluation of the degree of heterogeneity in protein families. To our knowledge, this is the first time that Macauba kernel proteins and globulins are characterized in such detail. Protein mapping of MKF proteins showed 53 protein spots with IPs ranging from pH 3.6–9.6. For a clearer overview, the single protein fractions were grouped in 7 different clusters (Fig. 2a_{II}) according to their MW and IP. MKF proteins were resolved predominantly in the pH range of 5.3–8.3 with MW of 11.0–56.5 kDa, comprising clusters 1, 2, 3 and 7. Thereby, cluster 3 is the most variable cluster as shown by the great number of individual spots being related to MW of 13–18 kDa with IP at neutral to slightly alkaline pH. Protein spots present in cluster 1, 2, 3 and 4 were also present in the MKG, but might also comprise the albumins and glutelins. The remaining clusters (5, 6 and 7) could not be assigned based on literature data.

Protein spots from the kernels of date (*Phoenix dactylifera*) (Sekhar & Demason, 1988a) and *Washingtonia* (Sekhar & Demason, 1988b) palms were focused within a narrower pH range (6.2–7.3) and presented MW of 45–66 kDa. On the other hand, protein spots from coconuts were focused predominantly in the pH range of 5.5–8.5 (Demason & Sekhar, 1990), similar to our results. Therefore, peptide expression profiles are likely to be different even among closely related species. Additionally, genotype and variety can also influence the expression of proteins as reported for wild (*Glycine soja*) and cultivated (*G. max*) soybean genotypes (Natarajan et al., 2006). As the sample used in the present study relates to wild species of Macauba, future research might address the influence of factors such as genotypes, maturity, and environmental conditions on protein expression profile based on our results.

Protein mapping of MKG is presented in Fig. 2b_{II}. The 16 protein spots from MKG were arranged in similar clusters as for the MKF proteins. The protein spots in clusters 2 and 3 (Fig. 2b_{II}) in relation to the results presented in Table 1 comprise the 11 S globulins. Those could be

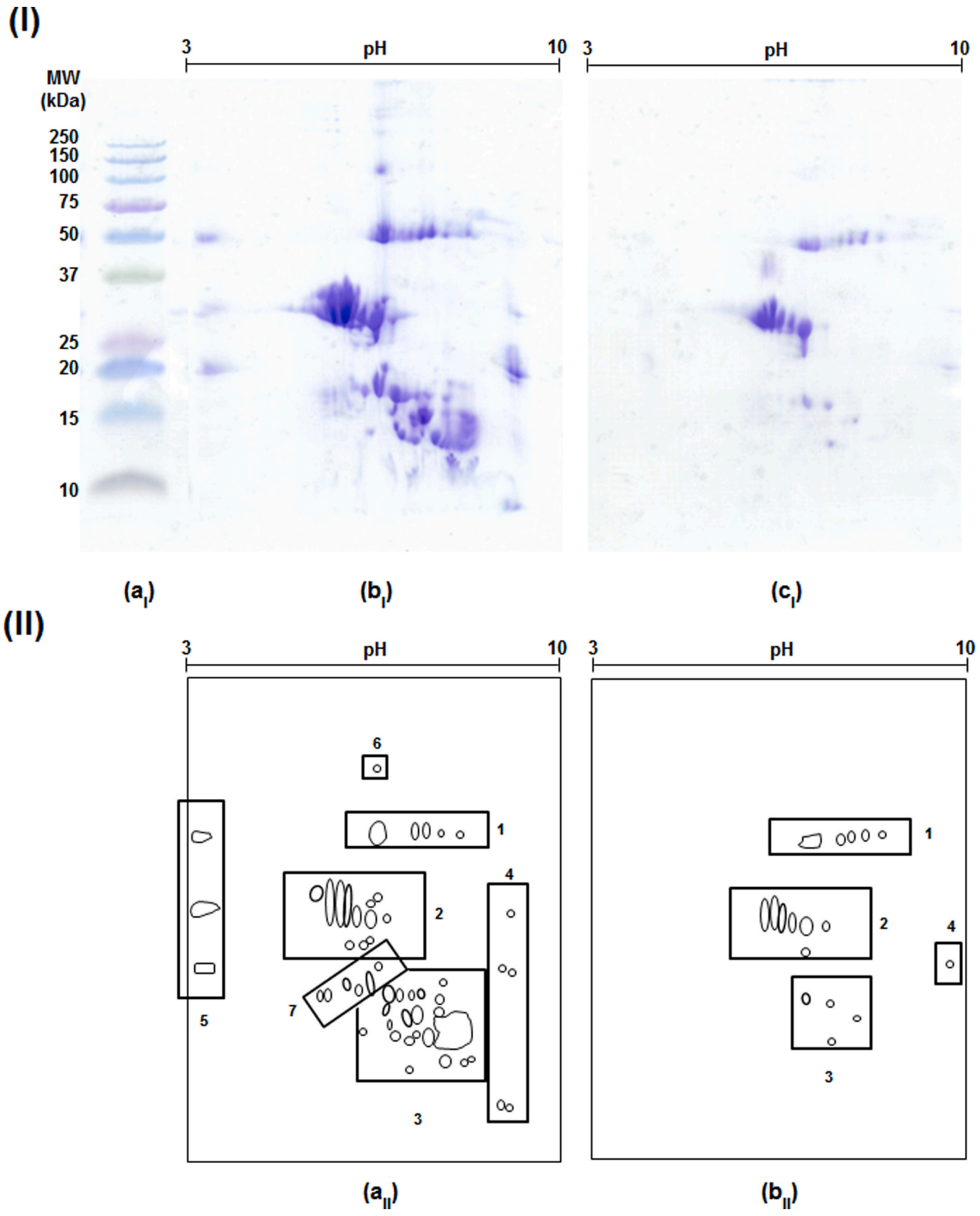

Fig. 2. Two-dimensional electrophoresis of Macauba kernel proteins. (I) 2D PAGE gel: (a_I) molecular weight (MW) standard, (b_I) total proteins and (c_I) globulins. (II) Protein mapping: (a_{II}) total proteins and (b_{II}) globulins. Numbers in Fig. 2a_{II} and 2b_{II} refer to grouping of protein spots in clusters according to their MW and isoelectric points.

attributed to a polypeptide subunit with MW of 43.6–50.2 kDa composed of a heavier polypeptide (26.1–30.6 kDa) with slightly acidic IP (6.1–7.4) and a lighter peptide (14.1–19.2 kDa) with neutral to alkaline IP (6.8–8.0), linked by a disulfide bond. The acidic and basic subunits of rapeseed cruciferin presented IP in similar range as determined for Macauba 11 S subunits (Dalgalarrondo et al., 1986). Similar MW distributions were also observed for 11 S globulins from pulses, pseudocereals, oilseeds and palm fruits such as mungbean (Mendoza et al., 2001), fava bean (Kimura et al., 2008), cowpea (Kimura et al., 2008), amaranth (Martínez et al., 1997), sesame (Tai et al., 1999) and coconuts (Garcia et al., 2005). Despite similar MW and IP patterns being recognized as universal characteristics of 11 S globulins (Marcone, 1999), high degree of variability is found in their subunits. In soybeans, 5 different subunit pairs were reported with MW in the range of 52.2–61.2 kDa. From those, 6 different acidic polypeptides (MW of 37–42 kDa and IP of 4.75–5.40) and 5 basic polypeptides (MW 20 kDa and IP 8.0–8.5) were isolated (Staswick et al., 1981), whereas in peas, the composition of legumin subunits (MW 44–66 kDa) is highly heterogeneous with 22 acidic polypeptides (24.5–43.0 kDa, IP 4.8–6.1) and 11 basic polypeptides (20.7–22.7 kDa, IP 6.2–8.0) being previously identified (Matta et al., 1981).

The protein spots of cluster 1 had MW of 47.2–53.2 kDa and IP of 6.4–8.1. Protein bands with similar MW and no interchain disulfide bond were present in the SDS-PAGE profile. This suggests that these proteins can be assigned to the group of the 7 S globulins. Similarly, the 7 S globulins from oil palm kernels had MW of 45–65 kDa and alkaline IP of approximately 8.0 (Morcillo et al., 1997), whereas the 7 S globulins of coconut endosperm presented a broader MW range of 16–55 kDa (Garcia et al., 2005). High degree of heterogeneity is also observed among the 7 S globulins of soybean and peas. Soybean β-conglycinin is composed of 3 non-identical subunits, namely α, α' and β with MW in the range of 52–72 kDa and IP of 4.9–6.0 (Krishnan et al., 2009), whereas the electrophoretic pattern of pea vicilin revealed subunits with MW in the range of 12.5–50.0 kDa and IP between pH 5.0 and 6.0 (Gatehouse et al., 1981). Proteolytic cleavage during seed maturation is supposed to result in the extremely heterogeneous pattern of polypeptide composition of pea vicilin (Tzitzikas et al., 2006).

Macauba 7 S fraction also presented a minor component stabilized by disulfide bonds, evidenced by SDS-PAGE (Table 1). However, not all of those subunits were detected in the 2D PAGE, but are potentially part of cluster 4. As previously discussed, those protein fractions could be part of the basic 7 S globulin family, which is also present in lupins (Duranti et al., 2008) and soybeans (Sato et al., 1987). Therefore, Macauba kernel 7 S fraction is most likely a mixture of different proteins with similar subunit MW, but variable IP. Thus, further characterization of the 7 S fraction is necessary to clarify their composition and biological role.

The inherent heterogeneity of the 7 S and 11 S globulins is due to alternate splicing of the same gene, products of multigene families and extent of co- and post-translational modifications (Dalgalarrondo et al., 1986). Genetic polymorphism was also observed in MKG in our work for the first time. Therefore, the present investigation paves the way for an in-depth characterization of Macauba kernel proteins in relation to genotype, growth conditions and stages of maturity, which might also influence the functionality profile of the proteins. This might be addressed in future to better understand those differences.

3.3. Amino acid composition

The amino acid composition of MKF proteins, 11 S and 7 S globulins is presented along with its nutritional characteristics in Table 2.

High content of Glu and Arg were found in 11 S and 7 S globulins as well as in MKF. This highlights the storage function of these proteins (Marcone et al., 1998). Amino acid composition of MKF was similar to that of coconut endosperm proteins (Kwon et al., 1996), but different from oil palm kernel (Zheng et al., 2015). In terms of nutritional quality, MKF was rich in Cys and Met, which are often limiting amino acids in a wide range of pulses and oilseeds (1.7–3.4 g/100 g) (Carbonaro et al., 1997; Iqbal et al., 2006; Raymond et al., 1991). MKF was also richer in sulfur-containing amino acids compared to the proteins of Macauba kernels (*Acrocomia intumescens*) (Bora & Rocha, 2004), highlighting the higher nutritional potential of Macauba (*Acrocomia aculeata*) kernel proteins.

Table 2
Amino acid composition and nutritional quality parameters of Macauba kernel total proteins, 11S and 7S globulins.

Amino acid	Content (g/100 g)		
	Total proteins	11S globulins	7S globulins
Essential			
Isoleucine	3.5	4.0	2.0
Leucine	6.2	6.9	4.6
Lysine	4.7	3.9	6.0
Methionine	2.3	2.8	1.2
Phenylalanine	4.7	5.2	3.1
Threonine	3.1	3.4	2.1
Tryptophan	1.0	1.0	0.7
Valine	4.8	5.4	3.0
Arginine	15.5	15.0	19.0
Histidine	2.3	2.2	2.1
Non-essential			
Alanine	4.0	4.3	2.4
Aspartic Acid	8.7	9.3	6.4
Cysteine	2.0	1.2	3.7
Glutamic acid	21.7	19.3	31.0
Glycine	4.8	4.6	4.1
Proline	3.7	3.6	3.2
Serine	4.4	4.9	2.7
Tyrosine	2.6	3.0	2.5
Nutritional quality parameter			
Essential-to-total amino acid ratio (%)	35.0	36.8	28.9
Chemical score (%)	98.1	82.2	67.0
Limiting amino acid*	Lys	Lys	Ile

*As reference the following FAO amino acid scoring pattern was used for calculations: His = 1.6 g/100 g, Ile = 3.0 g/100 g, Leu = 6.1 g/100 g, Lys = 4.8 g/100 g, Cys + Met = 2.3 g/100 g, Tyr + Phe = 4.1 g/100 g, Thr = 2.5 g/100 g, Trp = 0.66 g/100 g and Val = 4.0 g/100 g of protein.

Lys was the first limiting amino acid in MKF as determined by the chemical score calculation. However, the overall chemical score of MKF was 98.1%, being in a similar range as for soybeans and superior to peas, chickpeas, and lentils (Iqbal et al., 2006), and oil palm kernel proteins (Chang et al., 2014). As pulse proteins are generally high in Lys, Glu, Asp and Arg, but are often deficient in Trp and Cys (Boye et al., 2010; Iqbal et al., 2006), MKF proteins can complement or enrich diets in which pulses are the main protein source. Macauba palms might contribute to enhance the consumption of vegetable proteins as 214.5–442.2 kg of kernel protein per hectare per year is estimated to be available from intensive exploitation of Macauba palms (own estimated calculations). Therefore, the proteins from Macauba are highly valuable due to the high content of essential amino acids and thus, a high potential for future food uses and human nutrition can be expected.

Besides proteins, Macauba kernel press cake is also rich in dietary fibers (48.7 ± 1.3 g of total dietary fibers/100 g DM), which were considerably higher than values reported in literature with 20.3 and 35.8% of total dietary fibers in Macauba kernels and kernel press cake, respectively (Coimbra and Jorge, 2011; Andrade et al., 2020). Additionally, MKF also presented low residual lipid content (2.41 ± 0.04 g/100 g DM). According to Coimbra and Jorge (2012), Macauba kernel oil is also rich in total polyphenols (4.4 mg of gallic acid equivalent/g), higher than the content of polyphenols found in the oil of blueberries, blackberries, and raspberries seeds (Coimbra & Jorge, 2012). Furthermore, Macauba kernels are rich in minerals like manganese, copper, and zinc with content of 24.3, 11.1, and 30.9 μg/g, respectively (Hiane et al., 2006). Therefore, Macauba kernels present high nutritional value due to

their valuable proteins and other macro and micronutrients. This underlines the potential of Macauba kernel press cake as an alternative in the pursuit to overcome undernourishment and hunger.

In contrast to the findings of MKF, Macauba 11 S and 7 S globulins revealed significantly lower chemical scores of 82.2 and 67.0%, respectively. This is related to the greater deficiency of the two fractions in the limiting amino acids, which were Lys for the 11 S globulins and Ile for the 7 S globulins.

Furthermore, the 7 S globulins were richer in charged amino acids (Arg, Lys and Glu), whereas higher contents of non-polar amino acids (Ile, Leu, Val and Phe) are present in the 11 S globulins. This difference in amino acid composition is consistent with the difference observed between glycinin and β-conglycinin from soybeans (Riblett et al., 2001), legumin and vicilin from peas (Fukushima, 1991) as well as 11 S and 7 S globulins from fava beans (Derbyshire et al., 1975).

Compared to other 11 S globulins, Macauba had higher contents of Arg and Met (Dalgalarrondo et al., 1986; Derbyshire et al., 1975; Fukushima, 1991; Riblett et al., 2001). In turn, Macauba 7 S globulins are characterized by higher amount of Cys, but lower content of branched chain and aromatic amino acids compared to 7 S globulins from pulses (Derbyshire et al., 1975; Thanh & Shibasaki, 1978). As previously discussed, the presence of albumins and basic 7 S globulins could contribute to increase the content of Cys (Martínez et al., 1997; Mendoza et al., 2001; Sato et al., 1987) in the 7 S globulins of Macauba kernel. However, as the findings are most likely influenced by genotype of Macauba, the present investigation is the basis for further research.

3.4. Solubility properties of MKF proteins and its relation to solubilized protein fractions

The solubility properties of storage proteins play a key role in protein process design. An appropriate fractionation procedure, involving protein extraction, isolation, or dry fractionation, can be set based on solubility data of Macauba kernel proteins and will be used for further investigations. The influence of pH and ionic strength at 0.1 and 0.5 mol/L of NaCl on the solubility of MKF proteins are displayed in Fig. 3a and b. The MW distribution of the solubilized proteins at pH 4.0, 7.0, 8.0 and 12.0 at ionic strength of 0.1 mol/L and at pH 7.0 and ionic strength of 0.5 mol/L are presented in Fig. 3c and d.

Solubility of Macauba kernel proteins was dependent on both pH and ionic strength. At low ionic strength (0.1 mol/L of NaCl), protein solubility was the lowest at pH 4.0 and increased at both sides of the pH scale until maximum solubility values of 82% at pH 12. Solubilization of the glutelins contributed to the increase in protein solubility at this high pH, as those fractions account for approximately 22.5% of Macauba kernel storage proteins. The remaining non-solubilized proteins, i.e. 18%, is in good agreement to the prolamins (9.1%) and insoluble proteins (8.3%) determined to be present in MKF by the Osborne fractionation. At pH 12 only faint protein bands were observed in the electropherogram (Fig. 3c and d), and a high load of proteins remained in the stacking gel, which again could be attributed to protein complexation via formation of lysinoalanine and lantathionine crosslinks as previously discussed.

At pH 4.0 and 0.1 mol/L NaCl, only two and three protein bands were observed under non-reducing and reducing conditions by SDS-PAGE (Fig. 3c and d), which can be attributed to the albumins. At pH 7.0

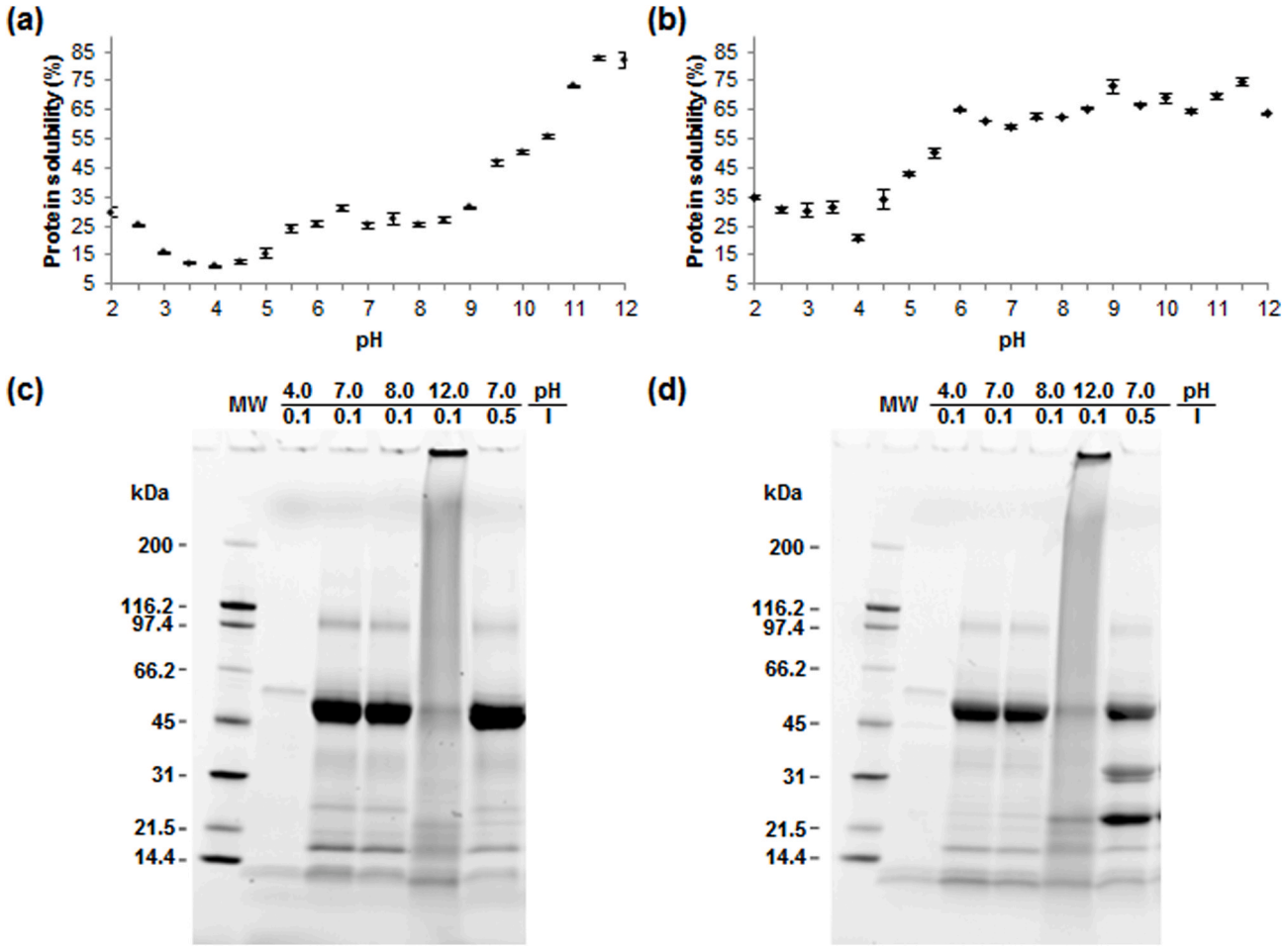

Fig. 3. Macauba kernel protein solubility and molecular weight (MW) distribution in different NaCl solutions: protein solubility as a function of pH and ionic strength of (a) 0.1 mol/L and (b) 0.5 mol/L; MW distribution by (c) non-reducing (without DTT) and (d) reducing (with DTT) SDS-PAGE. I: Ionic stregnth.

and 8.0 and 0.1 mol/L NaCl, the MW distribution was comparable to that of albumins and 7 S globulins. At those conditions, no bands corresponding to the subunits of 11 S globulins were detected in both non-reducing and reducing SDS-PAGE. This indicates the need of increasing the ionic strength for solubilization of 11 S globulins from Macauba kernels. An increase in ionic strength to 0.5 mol/L NaCl resulted in an overall increase in protein solubility at all pH values, with exception at pH 12, which could relate to the solubilization of the 11 S globulins (relative to MW of 27.5, 26.1 and 19.2 kDa) (Fig. 3d).

Furthermore, the influence of different salts and ionic strength ranging from 0.1 mol/L to 4.5 mol/L for Na_2SO_4 and CH_3COONa, from 0.1 to 4.0 mol/L for KCl and from 0.1 to 5.0 mol/L for NaCl on MKF proteins solubility was evaluated (Fig. 4a-d). The MW distribution (reducing SDS-PAGE) at ionic strengths of 0.75 and 3.0 mol/L and pH 7.0 is shown in parallel (Fig. 4e).

At 0.1 mol/L, the protein solubility was lowest (around 35%) for all evaluated salts. Maximum protein solubility in NaCl, KCl and Na_2SO_4 solutions was obtained at 0.75 mol/L, with 64.8 ± 0.8% for NaCl, 60.4 ± 0.9% for KCl and 63.8 ± 0.8% for Na_2SO_4. At 0.75 mol/L, the 7 S and the 11 S globulins were the main solubilized proteins, as their protein bands were predominant (Fig. 4e). Different from the other salts, maximum protein solubility in aqueous sodium acetate solutions was reached at 2.0 mol/L with a protein solubility of 57%, which was not significantly ($p > 0.05$) altered by further increase in ionic strength. Therefore, salting in of MKF proteins was attained at a broader range of ionic strengths in sodium acetate solutions compared to the other investigated salts. The methyl group from acetate could have acted as a weak detergent, preventing the salting out of non-polar moieties of Macauba kernel proteins at higher concentrations (Retailleau et al., 1997). In contrast to the other evaluated salts, the subunits of 11 S globulins were only observed in 3.0 mol/L sodium acetate, but not at 0.75 mol/L.

In particular, our findings demonstrate that Macauba kernel 11 S globulins require higher ionic strengths to be solubilized than the 7 S globulins, which was also previously shown for coconuts (Garcia et al., 2005), mung beans (Mendoza et al., 2001), soybeans and peas (Kimura et al., 2008).

Based on solubility and storage protein composition data, protein extraction processes might be designed for producing protein concentrates or isolates from Macauba kernels. Furthermore, protein concentrates and isolates enriched in 7 S might be obtained by using low ionic strength solutions, whereas enrichment in 11 S globulins could be attained by increasing the ionic strength to 0.5 mol/L or by using higher salt concentration on a sequential extraction process. This might offer

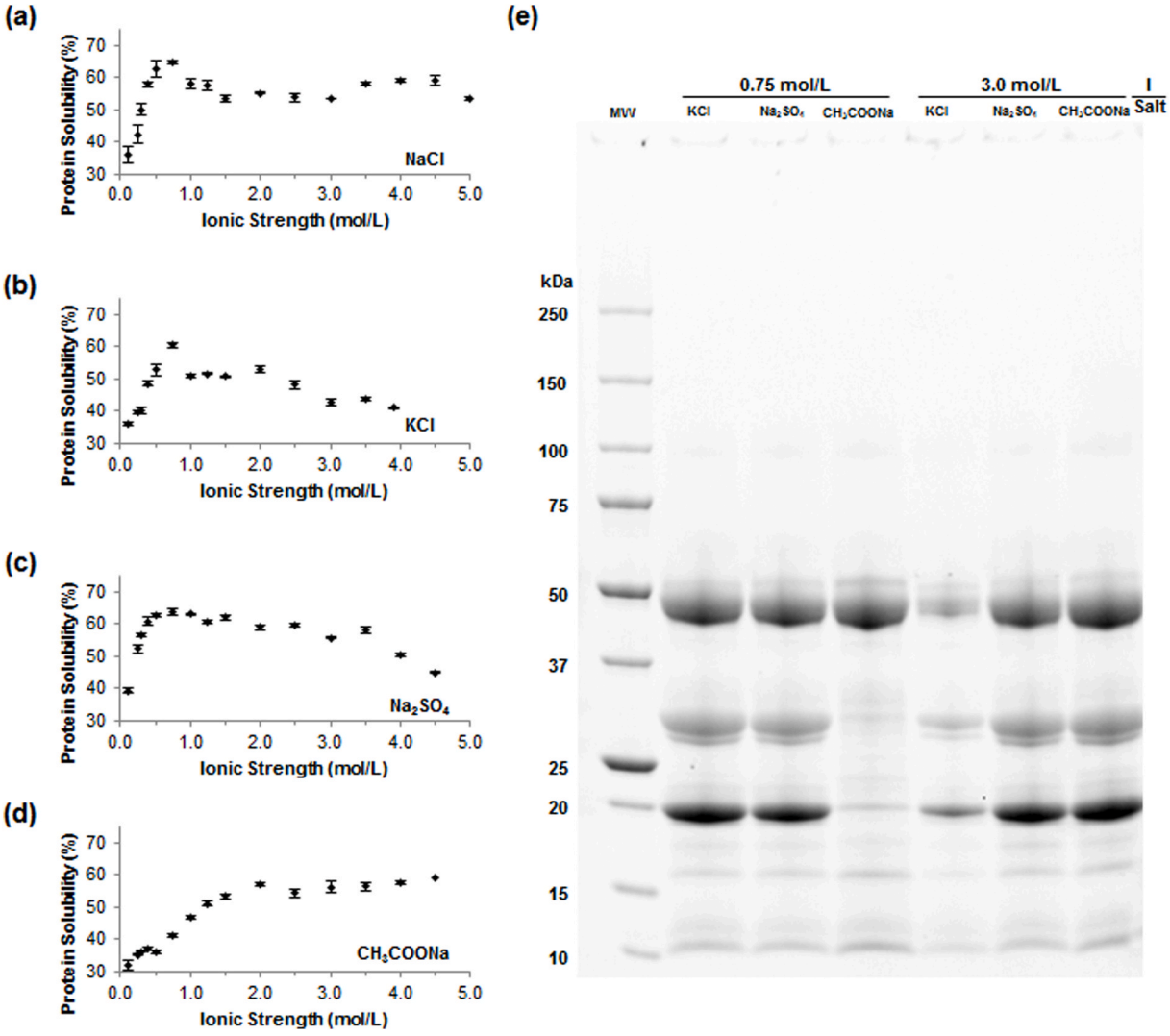

Fig. 4. Influence of ionic strength and type of salt on Macauba kernel protein solubility in (a) NaCl, (b) KCl, (c) Na_2SO_4, and (d) CH_3COONa; and (e) MW distribution of Macauba kernel protein in different salt solutions at 0.75 and 3.0 mol/L under reducing conditions (with DTT). Protein solubility measured at constant pH (7.00 ± 0.01). I: Ionic strength.

the possibility of tuning the functional properties of the obtained product by balancing the relative proportions of 7 S and 11 S globulins. Isoelectric precipitation of the proteins might be performed at pH 4.0. However, minimum protein solubility occurred on a wide range of pH values from 3.5 to 4.5, and thus, no distinct solubility minimum was determined.

Protein extraction might also be compromised owing to the high level of insolubilized protein, as the soluble protein, i.e. the globulins and albumins, accounts for only 65% of total protein. Taking this into account, further fractionation of Macauba kernel proteins can be attained by employing dry fractionation techniques, such as sieving and/or air classification. The use of such techniques can aid in overcoming solubility drawbacks with less expenditure of resources and less generation of residues.

4. Conclusion

The globulins are the main storage protein fraction of Macauba kernel flour, which showed inherent heterogeneity in protein composition and subunit properties. Macauba kernel globulins could be separated into two main fractions. One fraction soluble only at higher ionic strength (>0.5 mol/L) correlated well in electrophoretic pattern, isoelectric point of the subunits, amino acid composition and solubility to the 11 S globulins of different crops. The other fraction also resembles the properties of the 7 S globulins including vicilin-like and basic 7 S globulins. Further characterization of Macauba kernel protein fractions is recommended and might also aid in understanding evolutionary relationships among mono and dicotyledonous plants. Our findings also demonstrated that dilute solutions (0.1 mol/L) of chaotropic salts (NaCl, KCl and Na_2SO_4) can be used to solubilize the 7 S globulins. Finally, Macauba kernel proteins can complement diets in which legume kernels are the major source of proteins due to the high contents of sulfur-containing amino acids.

Author statement

Sérgio Henrique TOLEDO E SILVA designed the study, performed the experiments and collected the data, interpreted the results, and drafted the manuscript. Stephanie BADER-MITTERMAIER, Lidiane Bataglia da SILVA, Gabriele DOER and Peter EISNER assisted in interpreting the results and critically reviewed the manuscript for relevant intellectual content. Sérgio Henrique TOLEDO E SILVA finalized the manuscript, and all authors have read and agreed to the current version of the manuscript.

Funding sources

This research was financially supported by the German Federal Ministry of Education and Research (BMBF) under the program Bioeconomy International [Grant No. 031B0282].

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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