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Pumpkin (*Cucurbita maxima*) Seed Proteins: Sequential Extraction Processing and Fraction Characterization

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ABSTRACT: Seed proteins extracted from Tunisian pumpkin seeds (*Cucurbita maxima*) were investigated for their solubility properties and sequentially extracted according to the Osborne procedure. The solubility of pumpkin proteins from seed flour was greatly influenced by pH changes and ionic strength, with higher values in the alkaline pH regions. It also depends on the seed defatting solvent. Protein solubility was decreased by using chloroform/methanol (CM) for lipid extraction instead of pentane (P). On the basis of differential solubility fractionation and depending on the defatting method, the alkali extract (AE) was the major fraction (42.1 (P), 22.3% (CM)) compared to the salt extract (8.6 (P), 7.5% (CM)). In salt, alkali, and isopropanol extracts, all essential amino acids with the exceptions of threonine and lysine met the minimum requirements for preschool children (FAO/WHO/UNU). The denaturation temperatures were 96.6 and 93.4 °C for salt and alkali extracts, respectively. Pumpkin protein extracts with unique protein profiles and higher denaturation temperatures could impart novel characteristics when used as food ingredients.

KEYWORDS: *pumpkin, protein solubility, protein differential extraction, fractionation*

INTRODUCTION

Pumpkin (*Cucurbita* sp.) seeds are a key food source for humans because they are a very good source of proteins (24−36.5%) and oil (31.5−51%).[1−4] In the southern parts of Austria, Hungary, and Slovenia pumpkin seeds are mainly used in culinary practices.[5] However, in many African countries, especially in Tunisia, seeds are utilized directly as snacks after salting and roasting. The experiments of Nwokolo and Sim[6] showed that pumpkin seed proteins were similar to those of soybean cake in high availability of amino acids, which makes them a good candidate for the formulation of nutritious foods.[4,7] On the other hand, in addition to human nutrition, a variety of beneficial biological activities such as antidiabetic,[8] antifungal,[9] antibacterial and anti-inflammatory,[10] and antioxidant activity[11] have been reported for pumpkin seed proteins.

Given the reported health effects and potential therapeutic properties of pumpkin seeds, data derived from the characterization of protein-enriched fractions may enhance industrial utilization. This is because the structure−function relationships of plant proteins affect their behavior in food systems during preparation, processing, storage, and consumption.

In recent years, few studies have been conducted on fractionation and characterization of bitter melon[12] and watermelon seed proteins[13] belonging to same botanical family as pumpkin (Cucurbitaceae). However, to the best of our knowledge, fractionation and characterization of pumpkin seed remain fairly unexplored, particularly for North African varieties.

Our aim is consequently to evaluate in the case of Tunisian pumpkin seeds (*Curcubita maxima*) the interest of a sequential extraction procedure, in water, salt solution, alkali, and alcohol (70% ethanol), to prepare such protein-enriched fractions. According to this applied objective, the influence of the defatting conditions, NaCl concentration, and pH value on the protein solubility and fractionation of pumpkin seed proteins (*Curcubita maxima*) based on the solubility sequential procedure of Osborne was studied. Each protein fraction was characterized for its protein and amino acid composition as well as thermal properties. This may lead to the innovative utilization of North African varieties of pumpkin, not exploited yet, as protein sources for functional ingredients in different food systems.

MATERIALS AND METHODS

Materials. Pumpkin seeds (*Curcubita maxima*) were bought from a local market in Chebika region, located in southeastern Tunisia. The seeds were directly isolated, washed to remove impurities, and air-dried. All chemicals for protein extraction and fractionation and protein content determination were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals for electrophoresis were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and Euromedex (Strasbourg, France).

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Sample Preparatiosn. Prior to analysis, whole seeds were milled in a heavy grinder (Brown, Germany) to pass through an inox filter (200 mesh) to obtain a fine powder and kept in a refrigerator at 4 °C until use. The seed flour was defatted with two different solvents, namely, pentane and a mixture of chloroform/methanol (3:1 v/v). The flour/solvent slurries at a ratio 1:10 w/v were stirred for 24 h. The solvent was then removed by centrifugation, and the meal was dried at room temperature and stored in airtight sample bottles at 4 °C until use.

Protein Content. Nitrogen contents of the whole seed, defatted seed flours, and protein extracts were determined according to the Kjeldahl method. Each sample's protein content was calculated as N × 6.25 as described by the AOAC method.[14]

Influence of the Defatting Conditions, NaCl Concentration, and pH Value on the Protein Solubility. The protein solubility profile of chloroform/methanol defatted seed flour (CMDF) and pentane defatted seed flour (PDF) was determined using deionized water (DW) and salt aqueous solutions at various concentrations of NaCl (0.5, 1, 1.5 mol/L). The extraction was carried out with a magnetic stirrer at room temperature for 30 min using defatted seed flour at a flour/solvent ratio (1:100, w/v) in the pH range of 1−12. The extraction pH was adjusted by 0.5 mol/L HCl or 0.5 mol/L NaOH in acidic or alkaline pH, respectively; the pH of the slurry was maintained constant throughout the extraction experiment.[15,16] The nitrogen content of each extract was determined according to the Kjeldahl method.[14]

Fractionation of Protein Extracts from Pumpkin Seed Flour. Pumpkin proteins were fractionated from the defatted meals (CMDF and PDF) according to the Osborne differential extraction procedure as described by Horax et al.[12] The meal/water suspensions (20 g of meal into 100 mL of deionized water) were stirred for 2 h at room temperature and were centrifuged for 30 min at 20000*g* to separate the supernatant called deionized water extract (DWE) from the pellet. These extraction/separation conditions were kept for the successive next steps of protein extraction. The water extract pellet was resuspended into 100 mL of 1 mol/L NaCl solution and stirred as mentioned above. The resulting supernatant after centrifugation was designated salt extract (SE). This resulting second pellet was then extracted in 100 mL of deionized water adjusted at pH 11 with 0.5 mol/L sodium hydroxide leading to the alkali extract (AE). Finally, this third pellet was extracted with 70% isopropanol (100 mL). After centrifugation, the resulting supernatant was identified as isopropanol extract (IE). Each extraction was repeated twice. After each extraction, the pellets were washed twice using 20 mL of solvent so as to collect the residual protein entrapped in the insoluble residues. The washings and the first extract were combined for each fraction. DWE, SE, and AE were precipitated for isolation by adjusting the pH of the obtained supernatant to the pH corresponding to the minimum of solubility (pH_{ms}) determined as described later from the turbidity experiment. The pH was adjusted by 1 mol/L HCl or 1 mol/L NaOH in acidic or alkaline pH, respectively. IE was precipitated by adding acetone as described by Horax et al.[12] After centrifugation at 15000*g* for 15 min, the isolated protein precipitates were washed twice using deionized water at their respective pH_{ms} and recentrifuged. Finally, the resulting protein fractions were resolubilized by adjusting the pH to 7.0, freeze-dried, and stored at 4 °C for further analysis.

Turbidity Profiles of Deionized Water Extract, Salt Extract, and Alkali Extract Depending on pH. The turbidity profiles of DWE, SE, and AE were determined by the absorbance at 320 nm of each protein extract over a pH range from 1.0 to 12.0, using an UV spectrophotometer (Shimadzu Co., Kyoto, Japan). Ten milliliters of the supernatants was diluted to reach a readable absorbance. The pH of the solution was then adjusted to obtain the pH ranging from 1.0 to 12.0 with an increment of 1 pH unit and a smaller increment near the pH_{ms}. The maxima of the turbidity profile corresponding to the pH values of minimum solubility (pH_{ms}) were interpreted as the average isoelectric pH region for the different protein classes.[12]

Protein Determination of Deionized Water Extract, Salt Extract, Alkali Extract, and Isopropanol Extract (IE). On the basis of the amount and protein content of the obtained protein extract fractions, yields of each protein extract were calculated as follows:

$$\text{yield}\ (\%) = (\text{wt of protein extract} \times \%\ \text{protein in the protein extract} \times 100)/(\text{wt of pumpkin seed flour} \times \%\ \text{protein of pumpkin seed flour})$$

Amino Acid Determination of Extracted Fractions. Ten milligrams of pumpkin seed protein extracts was weighed into a dry tube, and 2 mL of 6 mol/L HCl was added. The tubes were tightly corked (Teflon cork) and put in an oven at 110 °C for 24 h. These hydrolyzed peptide bonds liberated amino acids, but sulfur-containing amino acids, cysteine and methionine, are unstable and need to be stabilized by oxidation before acid hydrolysis. Tryptophan was also destroyed by acid hydrolysis and so was not detected by this method. Asparagine and glutamine were also transformed into aspartic and glutamic acids.

For sulfur amino acid determination, a solution containing formic acid/hydrogen peroxide 30% (19:1 (v/v)) was prepared, kept covered at room temperature for 1 h, and added into the dried tubes containing the pumpkin seed protein extracts (1 mL for 10 mg of protein) to oxidize cysteine and methionine into cysteic acid and methionine sulfone. After 30 min at room temperature, the resulting oxidized protein was then vacuum-dried prior to HCl hydrolysis.

After 24 h of HCl hydrolysis, samples were cooled and 50 μL of norleucine (25 μmol/mL) was added to each tube as internal standard. The hydrolysate (50 μL) was collected (in triplicate) and vacuum-dried. The samples were derived by phenylisothiocyanate (PITC) so the amino acids could be detected spectrophotometrically at 254 nm.[17] The samples were first put in a mixture of ethanol/water/triethylamine (TEA) (2:2:1 v/v/v). Twenty microliters of this mixture was then added to the tube and vacuum-dried. For derivation, 20 μL of a mixture of ethanol/water/TEA/PITC (7:1:1:1 v/v/v/v) was added into the tube. After 10 min, the tube was vacuum-dried again at room temperature for about 1 h, and the samples were dissolved in 200 μL of a solution of 95% 2 mmol/L Na_2HPO_4 adjusted at pH 7.4 with phosphoric acid (10%) and 5% acetonitrile just before analysis. A mixture of amino acid (2.5 μmol/mL, Pierce) plus norleucine, methionine sulfone, and cysteic acid (2.5 μmol/mL HCl, 10 mmol/L) was derivatized in the same conditions. Calibration was made from 250 to 1500 pmol of amino acids in the injected sample.

The analysis was done with reverse phase high-performance liquid chromatography HPLC (Alliance HT system, module 2795, Waters, Milford, MA, USA) on a Picotag C18 column (4 mm × 15 cm, Waters). Detection was done at 254 nm with an absorbance detector (Waters model 2487). The column was equilibrated at 1 mL/min and 40 °C by solvent A (sodium acetate 0.14 mol/L, TEA 0.05% adjusted to pH 6.4 with glacial acetic acid, 94%/acetonitrile 6%). Four microliters of the derivatized sample was injected into the HPLC column. The elution was performed by an exponential gradient from 0 to 40% of solvent B (40% H_2O, 60% acetonitrile) in 12 min. The results represent the means of the three injections.

Electrophoretic Patterns of Extracted Fractions. SDS-PAGE was performed according to the procedure of Laemmli[18] on a slab gel (4% stacking gel and 15% separating gel) in an SDS−Tris−glycine discontinuous buffer system. Protein solutions (2 μg protein/μL) were prepared in reducing (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, and 0.05% 2-β-mercaptoethanol) and nonreducing buffer solutions (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.05% bromophenol blue). Ten microliters of the solution was loaded onto the gel. Electrophoresis was performed at a constant current of 25 mA per gel for approximately 90 min. The gel was stained by 0.1% Coomassie Blue in acetic acid/ethanol/water solution (10:40:50), v/v/v) and destained in acetic acid/ethanol/water solution (10:20:70, v/v/v). Approximate molecular sizes of the proteins were determined by using Euromedex molecular size standards.

Characterization of Salt and Alkali Extracts by Differential Scanning Calorimetry (DSC). The thermal properties of SE and AE

were determined using a differential scanning calorimeter model (DSC Q100, TA Instruments, Newcastle, DE, USA) equipped with a thermal analysis software (Analysis 2000). Protein−water slurries were prepared in DW at the respective protein concentration of 20 and 30 mg mL^{-1} for SE and AE, respectively. The slurries were left for 30 min at room temperature to reach water−protein equilibrium before analysis. Four milligrams of the protein slurry was accurately weighed into a stainless steel pan and hermetically sealed. The sealed pan was scanned using the calorimeter from 25 to 140 °C at a rate of 3 °C/min. An empty pan was used as a reference. The instrument was calibrated using indium. Peak temperature and enthalpy were computed from thermograms by the data processing software.

Statistical Analysis. All extractions and calculations were conducted in triplicate. Data were expressed as the mean ± standard deviation (SD). The means were compared by using one-way analysis variance (ANOVA) followed by Tukey's HSD test. The differences between individual means were deemed to be significant at $p < 0.05$. All analyses were performed by the Statistica v 5.1 software.[19]

RESULTS AND DISCUSSION

Protein Content of Whole Seeds and Defatted Seed Flours. The protein content of the whole seed flours was found at 33.9%, whereas after defatting it was increased at 40.9 and 43.1% for pentane and chloroform/methanol extracted meals, respectively. The higher protein content value obtained for CM meals is due to the higher efficiency of chloroform/methanol in extracting polar lipids.

Influence of the Defatting Conditions, NaCl Concentration, and pH Value on the Protein Solubility. The effects of the pH on the protein solubility profile of defatted flours in (DW and salt solutions at various concentrations are illustrated in Figure 1. For both meals, extracted by either chloroform/methanol or pentane, the protein solubility is very low (<20%) in the acidic pH region (pH <5). It drastically increased above pH 6. Addition of salt improves this solubility, the maximum values being obtained for 1 mol/L NaCl above pH 7 (70−80%). However, above pH 10, the ionic strength effect is rather limited. Generally, protein solubility is known to increase with moderately increasing salt concentrations due to the salting-in effect, whereas, at higher salt concentrations, the protein solubility decreased due to salting-out mechanisms.[20] Above pH 10 and for higher salt concentration (1.5 mol/L), this salting-out effect was observed.

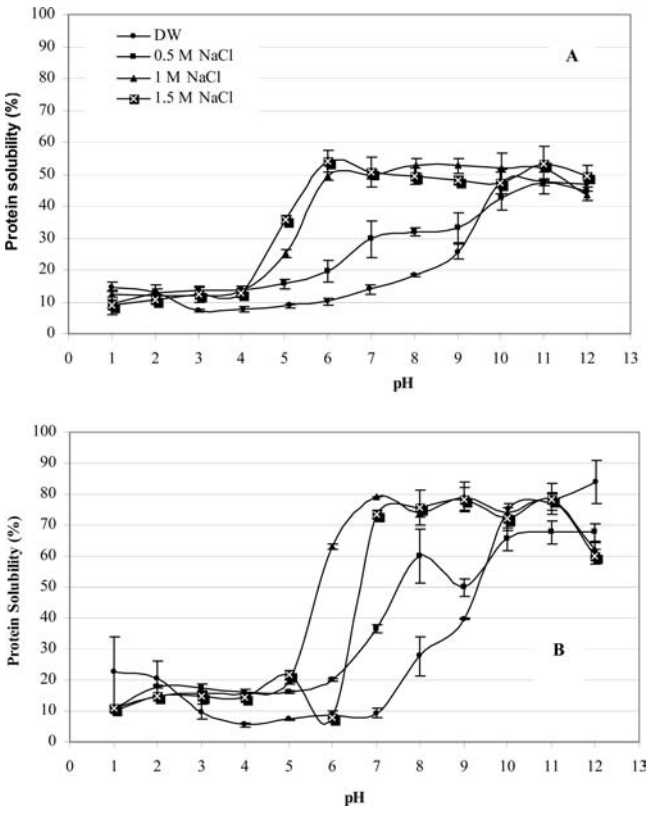

Figure 1. Effect of pH on the solubility of pumpkin defatted flour in deionized water and different salt (NaCl) concentrations: (A) CMDF; (B) PDF. Bars show standard deviation.

A plateau solubility profile is observed in the alkaline pH region (7 < pH < 10). In these conditions, 80% solubility is reached for pentane defatted meal, whereas only 55% is obtained for chloroform/methanol defatted meal. Proteins could be partly denatured by this polar solvent in alkaline conditions.

Because of its rather high oil content (31.5%),[4] pumpkin seed could be considered as an oleaginous seed. From our data, it can be deduced that the oil extraction processing, at both laboratory and industrial scales, has to be preferably performed by apolar solvents such as pentane or hexane to avoid eventual protein denaturation.

For protein extraction, higher yields could be reached either in DW above pH 10 or in salt solutions (1 mol/L NaCl) around pH 8−9. These results are consistent with previous data obtained on watermelon (*Citrullus vulgaris*) [21] and bitter melon (*Momordica charantia*) seeds,[22] which also belong to the Cucurbitaceae family. Horax et al.[22] concluded that optimum conditions for protein extraction from bitter melon seeds were obtained at pH 9 and 1.3 mol/L NaCl.

As the scaling up of salt extraction is rather difficult due to salt recycling, we may recommend water extraction in alkali conditions (pH 9.5−10, protein extraction yield ∼60−70%) for industrial processes at room temperature to limit amino acid derivatives formation, and mild salt extractions for laboratory studies (0.5 mol/L NaCl, protein extraction yield ∼80%).

Fractionation of Protein Extracts from Pumpkin Seed Flour According to Turbidity Experiments. The procedure for sequential extraction was established according to the solubility profile previously described expecting to extract albumin, globulin, and glutelin classes, respectively.

As about 20% of the proteins remained in the pellet of the pentane defatted meal (Figure 1), after these three sequential extractions, a complementary isopropanol extraction was performed as a final step.

To optimize the pH values for protein precipitation, the turbidity profile was established, depending on the pH for each extract. DWE, SE, and AE from CMDF (Figure 2A) showed turbidity maxima at pH 3.4, 5, and 5.3, respectively. For the corresponding PDF extracts, thes maxima were at pH 3, 5, and 5, respectively (Figure 2B). These pH values corresponded to the average isoelectric pH region for the different protein classes. For isopropanol extract, proteins were precipitated by acetone.

The turbidity profiles of DWE showed a plateau in the range pH 3−12, with a weak maximum at pH 3 in both cases, pentane and chloroform/methanol defatted meal. On the other hand, many maxima were observed for both meals, for salt and alkali extracts.

As an example, for the salt extract from pentane defatted meal, at least three maxima appeared at pH 2.2, 5, and 7, meaning that the extract should be composed of many protein

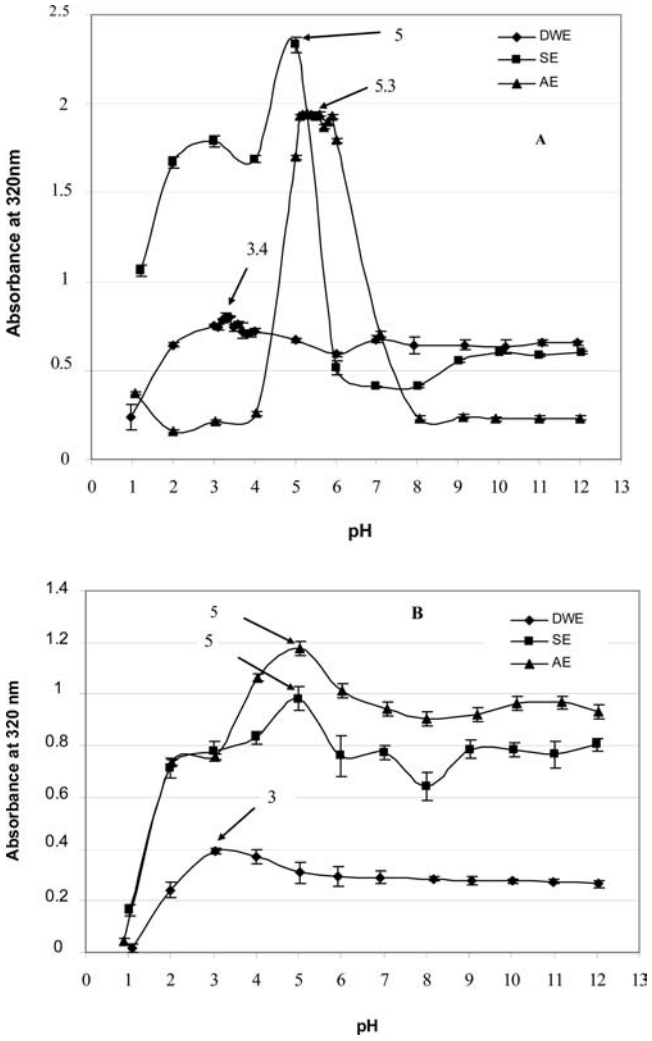

Figure 2. Absorbance of pumpkin seed protein extracts (DWE, SE, and AE) at various pH values: (A) pumpkin seed protein extracts defatted with chloroform/methanol mixture; (B) pumpkin seed protein extracts defatted with pentane. Bars show standard deviation.

classes. For the alkali extract, two maxima were obtained at pH 2.2 and 5 (Figure 2B). For chloroform/methanol extracts, similar complexities of turbidity profiles were also obtained. The maximum at pH 2.2 for the salt extract is surprising and could result from conformational changes induced by very acidic conditions. Consequently, we decided to recover the proteins from these extracts by precipitation at the pH value corresponding to the higher values of turbidity, that is, 3, 5, and 5 for DWE, SE, and AE, respectively, in the case of pentane defatted meal. For the chloroform/methanol meal, the corresponding values were 3.4, 5, and 5.3.

Adding the various extracts, a total of 31.9% of protein was recovered from CMDF compared with 55.2% for PDF (Table 1).

This confirmed the lower protein solubility for chloroform/methanol defatted flour. For PDF, the major recovered fraction is obtained for the alkali extract (42.1%); on the other hand, rather low yields were observed for water and salt extracts. These results did not confirm the previous data obtained for bitter melon (*Momordica charantia*).[12] The recovery yields obtained by these authors were 49.2 and 29.4% for water-soluble proteins (albumin) and salt-soluble proteins (globulins), respectively, with only 3.1% for alkali-extracted protein (glutelin). Our data are more consistent with regard to those obtained on *Cucumis melo*[23] and *Cucurbita pepo*,[24] which have been reported to have a pattern different from the *Citrullus taxa* in having glutelins instead of albumins as the second larger fraction.[25]

Table 1. Protein Contents and Yields of Extracted Pumpkin Seed Protein (DWE, SE, AE, and IE)[a]

protein extract	precipitation condition	protein content (%)	yield of recovered protein after precipitation[b] (%)
		CMDF	
DWE	pH 3.4	62.1 ± 7.0 b (a)	1.3 ± 0.1 c (b)
SE	pH 5	71.2 ± 3.4 ab (a)	7.5 ± 1.5 b (a)
AE	pH 5.3	75.4 ± 4.2 a (a)	22.3 ± 3.5 a (b)
IE	acetone	71.2 ± 0.2 ab (b)	0.8 ± 0.1 c (a)
		PDF	
DWE	pH 3	31.4 ± 0.9 b (b)	3.7 ± 0.6 bc (a)
SE	pH 5	72.9 ± 5.4 a (a)	8.6 ± 5.0 b (a)
AE	pH 5	80.6 ± 6.2 a (a)	42.1 ± 1.7 a (a)
IE	acetone	79.2 ± 2.9 a (a)	0.8 ± 0.2 c (a)

[a]Values are means ± SD of three determinations. For each defatted flour, mean values with different letters in the same column are significantly different. Mean values with different letters in parentheses in the same column are significantly different and concern differences between each protein extract in both defatted flours ($P < 0.05$). [b]Yield of each protein extract was calculated as follows: yield (%) = weight of protein extract × % protein of protein extract × 100/weight of pumpkin sample flour × % protein of pumpkin sample flour.

All of these studies showed very different protein solubility profiles depending on the species, even if they all belong to the Cucurbitaceae family. From the phylogeny trees, it was, however, observed that pumpkin (*Cucurbita maxima*) belongs to the Cucurbitae tribe as does *Cucurbita pepo*, whereas watermelon (*Citrullus*) is a member of the Benincaseae tribe.[26] This might explain why the protein profile of our sample (*Curcubita maxima*) is closer to that of *Cucurbita pepo* [24] than to those of other Cucurbitaceae.

The present study will then examine the physicochemical properties of these protein fractions extracted from the pentane defatted flour because the corresponding extractability yield is higher, leading to fewer damaged proteins compared to chloroform/methanol defatted flour.

Amino Acid Composition. The amino acid composition of the pumpkin seed protein extracts is shown in Table 2. The amino acid composition of the DWE fraction differs from those of the other extracts and is characterized by a less hydrophobic amino acid. This characteristic may explain its water extractability.

The salt, alkali, and isopropanol extracts showed rather close amino acid compositions even if the salt extract presented a higher content in sulfur amino acids.

Arg and Glx are the major amino acid components of the protein extracts. These results were similar to previously reported data for watermelon[27] and for bitter melon[12] seed protein fractions.

On the grounds of the above data, the FAO/WHO/UNU[28] recommended that preschool children (2−5 years, a safe level for all age groups) should have protein diets containing at least 3.4, 3.5, 2.5, 2.8, 6.6, 6.3, and 5.8 mg/100 mg protein for Thr, Val, (Met + Cys), Ile, Leu, (Phe +Tyr), and Lys, respectively. DWE was the most limiting of Thr, Ile, Phe + Tyr, and Lys. However, in SE, AE, and IE all essential amino acids with the

Table 2. Amino Acid Composition[a] of Deionized Water Protein Extract (DWE), Salt Protein Extract (SE), Alkali Protein Extract (AE), and Isopropanol Protein Extract (IE) of Pumpkin Seed Protein[b]

amino acid[c]	DWE	SE	AE	IE
Cys	6.3 ± 0.8 a	6.4 ± 0.1 a	3.4 ± 0.1 b	4.2 ± 0.1 b
Met	0.5 ± 0.2 b	2.1 ± 0.0 a	2.0 ± 0.0 a	0.2 ± 0.1 c
Thr	2.0 ± 0.3 c	3.3 ± 0.2 a	3.4 ± 0.1 a	2.6 ± 0.1 b
Ser	3.1 ± 0.5 c	7.4 ± 0.0 a	6.8 ± 0.1 a	6 ± 0.0 b
Glx	21 ± 0.5 c	27.8 ± 0.3 a	26.1 ± 0.2 b	18.5 ± 0.2 d
Asx	3.2 ± 0.3 c	7.3 ± 0.1 a	4.4 ± 0.1 b	4.6 ± 0.1 b
Gly	4.9 ± 0.3 c	6.8 ± 0.1 a	6.1 ± 0.1 b	5.8 ± 0.0 b
Ala	4.0 ± 0.3 d	6.9 ± 0.0 a	5.8 ± 0.1 b	5.2 ± 0.1 c
Val	4.0 ± 0.4 c	6.7 ± 0.0 a	5.4 ± 0.2 b	5.4 ± 0.2 b
Ile	1.9 ± 0.7 c	4.9 ± 0.0 a	3.9 ± 0.1 b	4.2 ± 0.1 ab
Leu	6.7 ± 0.5 d	12.2 ± 0.3 a	8.9 ± 0.1 b	7.8 ± 0.2 c
Phe	2.3 ± 0.0 c	8.2 ± 0.2 a	6.3 ± 0.3 b	6.4 ± 0.0 b
Tyr	0.4 ± 0.0 c	4.3 ± 0.2 a	3.1 ± 0.1 b	2.7 ± 0.3 b
Pro	4.2 ± 0.6 ab	5.0 ± 0.0 a	4.0 ± 0.2 b	5.0 ± 0.1 a
His	0.3 ± 0.0 d	3.0 ± 0.0 a	2.6 ± 0.1 b	1.4 ± 0.0 c
Lys	4.0 ± 0.2 a	3.8 ± 0.0 a	3.4 ± 0.0 b	2.6 ± 0.0 c
Arg	21.2 ± 0.6 b	23.1 ± 0.1 a	22.0 ± 0.3 b	18.3 ± 0.2 c
SAA	6.8 ± 0.8 b	8.6 ± 0.1 a	5.4 ± 0.2 c	4.4 ± 0.0 c
HAA	28.3 ± 1.2 c	51.0 ± 0.4 a	40.7 ± 0.6 b	40.0 ± 0.2 b
BAA	25.7 ± 0.4 c	30.1 ± 0.1 a	28.1 ± 0.3 b	22.4 ± 0.1 d

[a]In mg/100 mg protein. [b]Values are means of three determinations. Mean values with different letters in the same row are significantly different (P value <0.05). [c]SAA, sulfur amino acids; HAA, hydrophobic amino acids; BAA, basic amino acids.

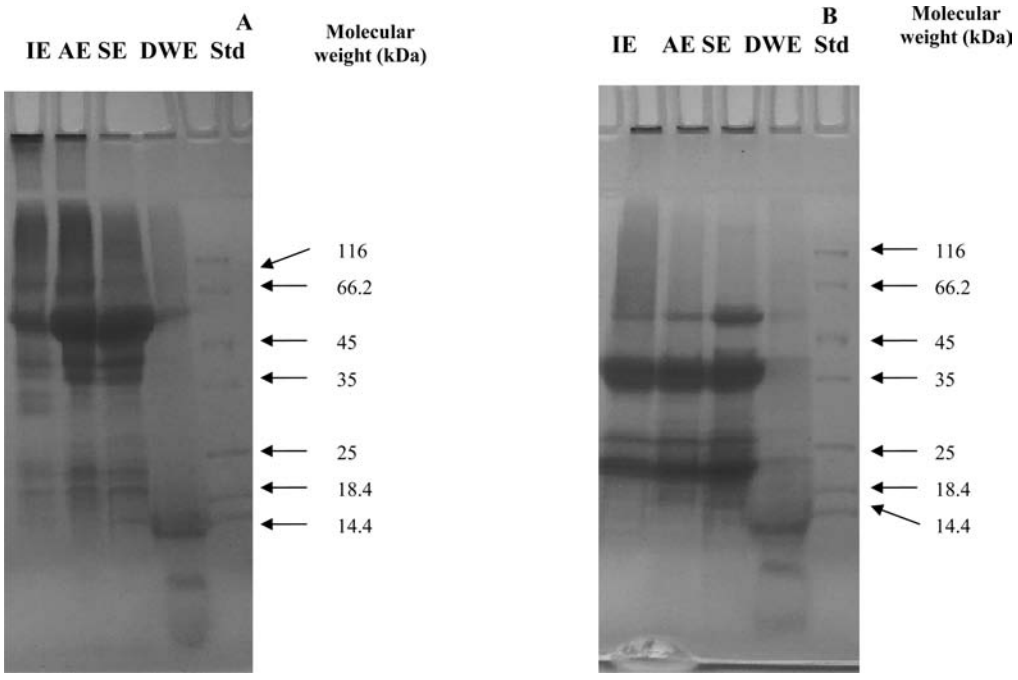

Figure 3. Electrophoregrams of pumpkin seed protein extracts (DWE, SE, AE, and IE) under nonreducing (A) and reducing conditions (B). Molecular sizes of the protein standards range from 14.4 to 116 kDa (β-galactosidase, 116 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; lactate dehydrogenase, 35 kDa; REase Bsp881, 25 kDa; β-lactoglobulin; and lysozyme 14.4 kDa).

exception of Thr and Lys met the minimum requirements. The low level of lysine coincides with a previous result on melon seed protein.[29]

Electrophoretic Patterns of Pumpkin Protein Extracts. Figure 3 shows the electrophoretic patterns of DWE, SE, AE, and IE in nonreducing and reducing conditions. Under nonreducing conditions, the DWE resolved into three major bands at 63.6, 14.3, and 13.3 kDa and a few minor bands in the range of 7−10 kDa. After reduction, the 63.6 kDa pattern is cleared into three polypeptide classes, one migrating at 60.6 kDa, the second between 36.8 and 44.3 kDa, and the third between 21.9 and 23.5 kDa.

The lower molecular weight components remained unchanged. The 14.3 kDa compound should correspond to 2S albumin with ribonuclease activity.[30] King and Onuora[29] reported only one major band with a molecular weight of 12 kDa for melon (*Colocynthis citrullus* Linn.) seed proteins. However, in bitter melon (*Momordica charantia*) belonging to

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the same botanical family (Cucurbitaceae), the electrophoretic pattern of albumin fraction in nonreducing conditions showed a protein with a dense band of about 55 kDa with two minor proteins with molecular sizes of about 40 and 7 kDa. In reducing conditions, this fraction showed major bands of about 20, 25, and 35 kDa.[12] The albumin fraction in watermelon (*Citrullus lanatus*) was found to contain polypeptides of molecular weights 22−194 kDa in nonreducing conditions in the Mateera cultivar.[27] Differences in the molecular weight of the albumin fraction's polypeptides of these species could be due to their belonging to different tribes even if they all belong to the Cucurbitaceae family.[26]

SDS-PAGE of the salt extract under nonreducing conditions revealed a protein with major bands in the range of 63.6−53.5 kDa. Minor bands of higher and lower molecular weight components were also seen. Under reducing conditions and according to the coloration intensity, the major bands appeared to be reduced into two polypeptide classes, one migrating between 44.5 and 34.8 and the other between 25.1 and 17.9 kDa. This profile under reducing and nonreducing conditions might correspond to the characteristic pattern of an 11S globulin with six subunits around 50−60 kDa, composed for each of two polypeptides, one acidic at about 40 kDa and one basic at about 20 kDa, linked by disulfide bridges. A well-defined single band at 57.6 kDa was also detected after reduction, which could correspond to one of the major bands migrating in the same region in nonreducing conditions. After reduction, the higher molecular weight polypeptides (81.6−134.1 kDa) also disappeared. Characterization studies on pumpkin (*Cucurbita* sp.) seed proteins have shown that their major protein fraction is represented by an 11S globulin, homologous to those reported in legume seeds. Called cucurbitin in *Cucurbita pepo*, it is a hexameric globular protein with a molecular weight of 54 kDa for each subunit, further consisting of a large, acidic subunit of 33 kDa disulfide-bonded to a small, basic subunit of 22 kDa.[25] These data are rather consistent with those found in the present paper for the alkali extracted proteins in the ranges of 44.5−34.8 and 25.1−17.9 kDa.

The alkali and isopropanol extracts showed very similar electrophoretic patterns compared to the salt extract with major bands and high molecular weight polypeptides in the same region. However, on the basis of the coloration intensity, the components in the range of 81.6−134.1 kDa appeared to be present in a higher proportion. Moreover, for these extracts, some proteins did not penetrate the gel. The rather similar subunit compositions of AE and IE compared to SE mean that these protein fractions may have close protein composition, as previously deduced from amino acid analysis, but with protein components characterized by different structural conformations with a strong tendency to aggregate.

Characterization of Salt and Alkali Extracts by Differential Scanning Calorimetry. The DSC thermograms of the SE and AE of pumpkin seed are shown in Figure 4, and corresponding values are reported in Table 3.

Surprisingly, the DSC profiles did not reveal many classes of proteins characterized by different denaturation temperatures.

The SE and AE denaturation temperatures corresponding to the peak of each thermogram were 96.6 and 93.4 °C, respectively. The thermal denaturation temperature of the SE protein was within the range reported for globulins from melon (*Colocynthis citrullus* Linn.) seed (90 °C),[31] but very different from those obtained for bitter melon globulin and glutelin

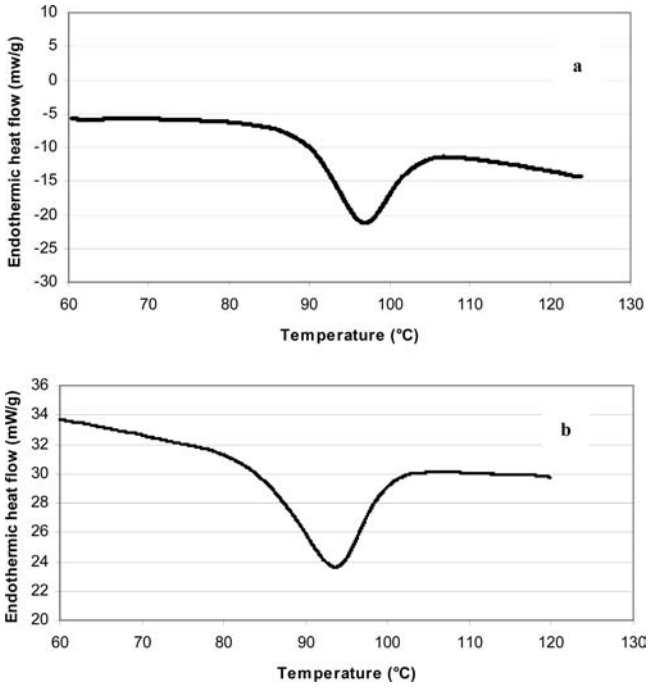

Figure 4. DSC thermograms of the salt (a) and alkali (b) protein extracts of pumpkin seed.

Table 3. Thermal Properties of Salt Extract (SE) and Alkali Extract (AE) of Pumpkin Seed Protein[a]

physical property	SE	AE
onset (°C)	89.2 ± 0.4 a	83.0 ± 0.7 b
end (°C)	103.6 ± 0.6 a	100.2 ± 0.3 b
peak (°C)	96.6 ± 0.3 a	93.4 ± 0.2 b
ΔH (J/g)	12.6 ± 2.5 a	5.1 ± 0.4 b

[a]Values are means ± SD of three determinations. Mean values with different letters in the same row are significantly different (P value <0.05).

reported around 117.3 and 133.6 °C, respectively.[12] ΔH or enthalpy values also give appropriate information about the energy required to unfold or denature the protein structure. ΔH values for salt and alkali protein extracts were 12.6 and 5.1 J/g, respectively. The differences in ΔH values for both protein extracts could be attributed to differences in protein conformations for both fractions despite their rather similar electrophoretic patterns. The enthalpy value of the SE is rather close to that reported for globulins from melon (*Colocynthis citrullus* Linn.) seed,[31] but lower than that obtained for bitter melon globulin, reported around 27.6 J/g.[12] The rather lower enthalpy value of the AE might be due to a change in the structural conformation of the protein during the extraction process.

Conclusions. Our results demonstrate that after defatting by pentane, the resulting flour could be used for industrial protein extraction, by water in alkali conditions. However, complementary studies are needed to optimize the recovery yield and evaluate the functional and nutritional properties of these proteins.

Considering the amino acid composition of the salt, alkali, and isopropanol protein extracts, all of the essential amino acids met the minimum FAO/WHO/UNU requirements for pre-school children with the exception of Thr and Lys.

On the basis of their higher denaturation temperature, salt and alkali extracts probably are suitable in specific products for which the native form is needed, because they can resist higher temperature during processing. In addition, this information is probably useful to provide a basis for functional and structural studies of protein from pumpkin seeds.

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Notes

The authors declare no competing financial interest.

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