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Inter-relationship between electrophoretic characteristics of pseudocereal and cereal proteins and their microscopic structure for possible substitution based on nutritional evaluation

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Amaranth, soybean and maize were screened for proteins and their nutritional value. Isopropanol-soluble protein and buffer-soluble protein fractions were extracted from seeds and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The comparison of the identity and differences between investigated plants was carried out by the obtained SDS-PAGE electrophoretic patterns, and their microstructure was determined by scanning electron microscopy. Electrophoretic patterns of extracted proteins have shown that the main protein subunits were concentrated between 10 and 50 kDa. Variations were found in major fractions and minor bands as well as in the fine structure. The microstructure of pseudocereal and cereal protein fractions was inter-related with the results obtained by their electrophoretic separation. Pseudocereal amaranth can be used as a nutritive substitute of cereal maize in functional foods.

Introduction

Storage proteins account for about 50% of the total protein in mature cereal grains and have important impacts on their nutritional quality for humans and livestock, and on their functional properties in food processing (Yuno-Ohto *et al*., 1994; Fernandes *et al*., 2000; Charalampopoulos *et al*., 2002; Lacroix *et al*., 2002; Cremer & Kaletunc, 2003). Current knowledge of the structures and properties of the prolamin, the main storage

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protein in cereal maize, and globulin, the main storage protein in legume soybean and pseudocereal amaranth, their mechanisms of synthesis and nutritional quality were reviewed recently (Higuchi & Fukazama, 1987; Prasanna *et al*., 2001; Rodriguez *et al*., 2001; Charalampopoulos *et al*., 2002; Krishnan, 2002; Shewry & Halford, 2002). Contrarily, the alcohol-soluble fraction (prolamin-like) in pseudocereal such as amaranth is the minor fraction in comparison with other studied plants (Bressani & Garcia-Vela, 1990; Gorinstein *et al*., 1991, 1999; Barba de la Rosa *et al*., 1992).

The role of the gliadin proteins of wheat in determining the quality of the grain for breadmaking, and how their amount and composition can be manipulated leading to changes in dough mixing properties, was also discussed (Majamaa *et al*., 1999; Saadoun-Cousin *et al*., 2002; Shewry & Halford, 2002). The effect of baking and digestion on the allergenicity of wheat flour proteins has been studied (Simonato *et al*., 2001). Pooled sera of patients suffering from a food allergy to wheat products were tested for immunoglobulin (Ig)E binding to the proteins of the wheat dough and of the breadcrumb and crust, before and after being *in vitro* digested. During *in vitro* digestion, the IgE binding protein components of the unheated dough tended to disappear, whereas a permanence of IgE recognition was evident for both the breadcrumb and crust. This indicates that the baking process increases the resistance of the potential allergens of the wheat flour to proteolytic digestion, allowing them to reach the gastrointestinal tract, where they can elicit the immunological response. Therefore, the effects of baking must be carefully considered in studying food allergies to wheat products (Majamaa *et al*., 1999; Simonato *et al*., 2001; Saadoun-Cousin *et al*., 2002).

To avoid the allergy from cereals, pseudocereals could be used as substitutes based on the relatively high amounts of amino acids in comparison with cereals (Barba de la Rosa *et al*., 1992; Abrew *et al*., 1994; Gorinstein *et al*., 2002). Calculations of age-related amino acid requirements are based on most recent estimates of human growth and of maintenance protein requirements, a tissue amino acid pattern and the new maintenance amino acid pattern. High-lysine maize supports similar weight and height growth to that of casein. Inadequate amino acid supply is not an issue with most cereal-based diets (Friedman, 1996; Escobar *et al*., 1998; Millward, 1999).

A higher amount of essential amino acids in amaranth was found in comparison with maize (Landry & Moreaux, 1970; Barba de la Rosa *et al*., 1992; Gorinstein *et al*., 2002).

A complementary effect was shown between the proteins of wheat and amaranth (Abrew *et al*., 1994), in the evaluation of the proteins in a mixture of rice and soybean (Fernandes *et al*., 2000) or a maize–soya–bean-meal diet (Camden *et al*., 2001).

There are several reports on the main proteins in cereals, legumes and preudocereals, and their nutritional value (Landry & Moreaux, 1970; Higuchi & Fukazama, 1987; Gorinstein *et al*., 1991, 1999; Friedman, 1996; Fernandes *et al*., 2000; Marcone, 2000; Landry *et al*., 2000; Prasanna *et al*., 2001; Krishnan, 2002). The multiple beneficial effects of cereals and pseudocereals can be exploited in different ways, leading to the design of novel cereal foods or cereal ingredients that can target specific populations. It could be concluded that functional foods based on cereals and pseudocereals is a challenging perspective; however, the development of new technologies of cereal processing that enhance their health potential and the acceptability of the food product are of primary importance (Millward, 1999; Shewry & Halford, 2002).

Based on reported nutritional and dietary studies and our recent research, it is important to find a substitute for cereals.

Some work was carried out on the microscopy of legumes, amaranth and maize (Konishi *et al*., 1995; Puppo & Anon, 1998; Wood *et al*., 1998; Batterman-Azcona *et al*., 1999), showing the value of microscopy as a tool for evaluating physical and chemical changes in bean components due to processing. Most of the reported research was focused on the microstructure, which determines the films of proteins from cereals and legumes (Yuno-Ohto *et al*., 1994; Puppo &

Anon, 1998; Wood *et al*., 1998). Microstructure observations showed that the mechanical characteristics of films are closely related to their microscopic structure (Batterman-Azcona *et al*., 1999; Lacroix *et al*., 2002). The extruded cereals were also characterized by microstructure (Batterman-Azcona *et al*., 1999; Cremer & Kaletunc, 2003).

The relationship between the isolated fractions (the major fraction, buffer-soluble glutelin; the minor fraction, alcohol-soluble, prolamin-like) from cereals and pseudocereals and their microstructure has not been shown until now.

Therefore, this work describes the evaluation of maize, amaranth and soybean proteins extracted by two different solvents, such as isopropanol and buffer, the electrophoretic patterns and their fine structure.

Experimental

Sample preparation

Whole mature seeds of plants such as three species of amaranth [*Amaranthus* (*A.*)*: hybridum v.* 1004, Pakistan; *hypochondriacus v.* 1023, Mexico; and *cruentus v.* R104, USA] soybean (*Glycine mar* (*L.*) *Merrill*, Brazil) and maize (*Zea mays L*., Brazil) were investigated.

Seeds were ground on a mill (Janke & Kunkel GmbH & Co. KG–IKA, Labortechnik, Staufen, Germany) through a 60-mesh screen. The meal was defatted in a Soxhlet extractor with *n*-hexane for 10 h and then was stored at 5°C after removal of hexane.

Protein extraction

Proteins were extracted stepwise according to the following methods (Landry & Moreaux, 1970; Gorinstein *et al*., 1991, 1999). The meal (1 g) was extracted with a solvent:sample ratio of 6:1 for alcohol-soluble proteins and of 3:1 for buffer-soluble glutelins (v/w), and was vigorously shaken. The extracts were separated by centrifuging at 10,000 *g* for 10 min. Each step was repeated twice. The sequence of the used solvents was as follows: isopropanol-soluble protein fraction (IPSPF) (prolamin-like), 70% (v/v) isopropanol; buffer-soluble protein fraction (BSPF) (glutelins), 0.125 M sodium borate buffer, pH 10, containing 0.5% sodium dodecyl sulfate (SDS). Extracts were combined, lyophilized, dissolved in sample buffer that contained 10% glycerol, 5% 2-mercaptoethanol (2-ME), 2% SDS in 0.125 M (Tris–HCl), pH 6.8. Then the extracts were boiled for 5 min before being loaded. The nitrogen content in each fraction was determined by the micro-Kjeldahl method combined with a colorimetric determination (Nkonge & Balance, 1982).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli (1970), using gradient (5–20% w/v) gels. The gels were 1.5 mm thick and consisted of a 2 cm stacking gel and a 10 cm running gel. Fifty micrograms of protein was applied to sample slots. Electrophoresis was carried out at 150 V for 2 h. Gels were stained with 0.25% Coomassie Brilliant Blue R in methanol:water:acetic acid (5:5:1 v/v) and distained in the same solvent. M_W standards were used to estimate protein subunit molecular weights: phosphorylase b, 94 kDa; hemoglobin, canine, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, soybean, 20 kDa; and lactalbumin, bovine milk, 14 kDa.

Scanning electron microscopy

Scanning electron microscopy was performed on dried protein samples, which were mounted on aluminum stubs with double sticky tape and coated with 20 nm gold. Photographs were taken using the scanning device JEOL (JEOL, USA, Inc., Peabody, MA, USA) at accelerating voltage of 30 kV. The current for scanning was 2×10^{-10} A (Enamuthu *et al*., 1993; Konishi *et al*., 1995).

Results and Discussion

Electrophoretic separation

Total proteins (% nitrogen) in amaranth, soybean and maize were 15, 40 and 10, respectively. The relative proportions of nitrogen content between IPSPF and BSPF in amaranth, soybean and maize were about

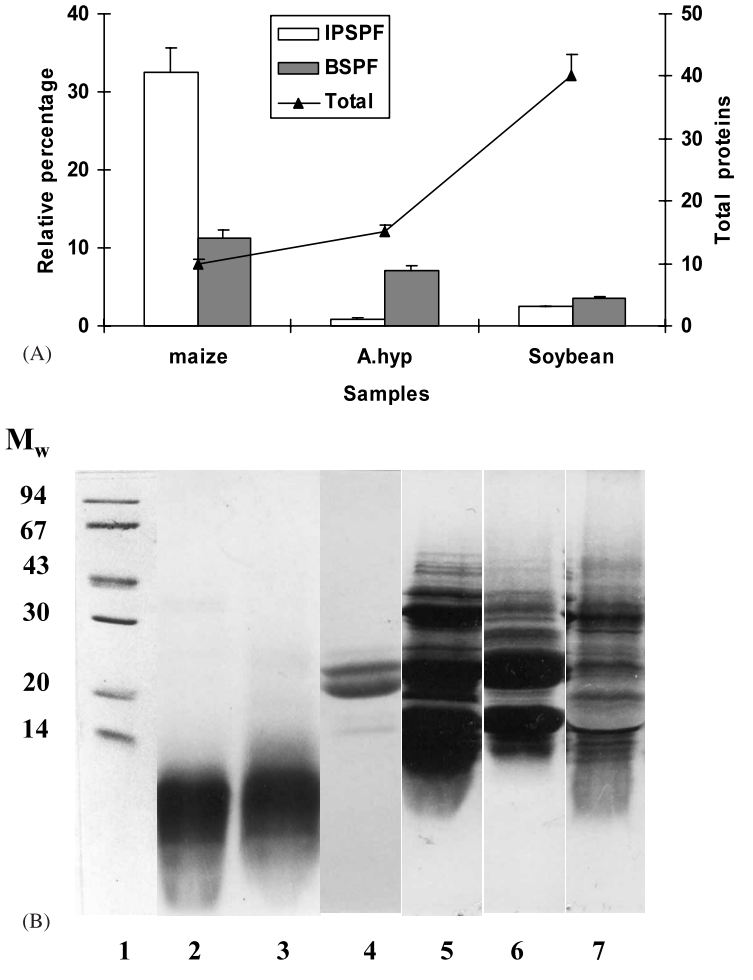

Figure 1. (A) Total protein content (% nitrogen) and protein fractions distribution (relative percentage) of isopropanol-soluble protein fractions (IPSPF) and buffer-soluble protein fractions (BSPF) in amaranth, soybean and maize samples. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 5–20% polyacrylamide gel of IPSPF: lane 1, molecular marker (94 kDa, phosphorylase b; 67 kDa, hemoglobin, canine; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, trypsin inhibitor, soybean; and 14 kDa, lactalbumin, bovine milk); lane 2, amaranth; lane 3, soybean; lane 4, maize. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 5–20% polyacrylamide gel of BSPF: lane 5, amaranth; lane 6, soybean; lane 7, maize. IPSPF were extracted with 70% (v/v) isopropanol; BSPF with 0.125 M sodium borate buffer, pH 10, plus 0.5% sodium dodecyl sulfate (w/v).

0.9:7.1, 2.4:3.5 and 32.4:11.2, respectively (Figure 1A). The three amaranth species [*Amaranthus* (*A.*)*: hybridum v.* 1004; *hypochondriacus v.* 1023 and *cruentus v* R104] studied in this report have shown similar results in the amount of extracted fractions as well as in the electrophoretic patterns. Therefore, only one of the investigated species (*hypochondriacus v.* 1023) is shown in our results.

The IPSPF from *A. hypochondriacus* and soybean (Figure 1B, lanes 2 and 3) contained nearly 85–95% of polypeptides of 8–14 kDa, which have shown unseparated material. Unseparated subfractions are the result of aggregation connected with S–S bridges as well as with the presence of membrane proteins. This can be explained by the extraction of these proteins, where isopropanol was used without reductant 2-ME. The

rest of the amaranth and soybean proteins were minor fractions and were not detected. The protein subunits in the region of 8–14 kDa, which have concentrated the major unseparated material in amaranth and soybean, differ completely from those of maize.

The prolamin electrophoretic patterns from maize (Figure 1B, lane 4) showed three characteristic bands of 16 (very weak), 22 and 25 kDa. The maize prolamin, the main storage protein, did not show any electrophoretic relationship with amaranth and soybean alcohol-soluble fractions. The electrophoretic data proved that isopropanol-soluble proteins could not represent the main protein fraction in cereal-like plant such as *A. hypochondriacus* and soybean (Gorinstein *et al*., 1991, 1999; Landry & Moreaux, 1970).

The main subunits of 18, 22, 32 and 50 kDa were common in all glutelin fractions from amaranth, soybean and maize (Figure 1B, lanes 5–7). Subunits of unseparated material at 10–14, 18, 20, and 28 kDa were presented in glutelins (Figure 1B, lane 5) extracted from amaranth. Maize (Figure 1B, lane 7) showed 14, 20, 33 and 49 kDa in the main subunits of glutelins. Unseparated material in the region between 20 and 28 kDa in the major fraction was typical for glutelins from the blends of amaranth with soybean and maize (results of the electrophoretic patterns not shown). Protein subunits of glutelins for *A. hypochondriacus* with maize and for *A. hypochondriacus* with soybean displayed similar patterns, with major subunits at 20 and 40 kDa and the average subunits at 48 and 55 kDa. Glutelins of *A. hypochondriacus* presented less protein material in the region of 40 and 65 kDa. All samples and their blends share some common subfractions (14, 20, 30, and 50 kDa) and showed identity between glutelin fractions. The results with blends of cereals and pseudocereals were in accordance with others (Abrew *et al*., 1994).

Wheat flour and amaranth in three mixtures (wheat flour:amaranth, 0.87:0.13, 0.75:0.25 and 0.64:0.36 on a protein basis) were assayed for amino acid scores (AAS) and protein value (PV) in rats. Lysine and leucine were the limiting amino acids for wheat and amaranth proteins, with an AAS of 50% and 61%, respectively. The AAS of the combinations wheat flour:amaranth 0.87:0.13, 0.75:0.25 and 0.64:0.36 were 12%, 24% and 34%, respectively, and are higher than those of wheat proteins. The PV of amaranth seeds (4.09) was two times higher than that of wheat (1.94). The combinations wheat flour:amaranth 0.75:0.25 and 0.64:0.36 showed a PV 45% higher than that of the 0.87:0.13 combination, which in turn was similar to that of the wheat proteins. It is concluded that the supplementation of wheat flour with amaranth up to 20% could improve the protein quality up to 45%.

Gel electrophoresis analysis revealed the occurrence of some glutelins as the major proteins in the seeds of cereals, pseudocereals and legumes. Based on our experimental and literature data, it can be concluded that not only the globulin fraction is the main storage protein, but also the glutelin fraction can be suggested as one of the main proteins in the pseudocereals and legumes (Bressani & Garcia-Vela, 1990; Gorinstein *et al*., 1991, 1999, 2002; Barba de la Rosa *et al*., 1992). Upon electrophoresis, prolamins were made up of fewer and less abundant components in amaranth and soybean. As it was shown the prolamin-like and prolamin fractions were completely different (Figure 1B, lanes 2–4), but the glutelin fractions showed some similarity in the same plants (Figure 1B, lanes 5–7).

Based on electrophoretic patterns of glutelins, it can be decided that amaranth plant can be used as a substitute for several cereals or as part of a mixture for food nutrients. Our data are in accordance with the results of investigations of different species of pseudocereals (Bressani & Garcia-Vela, 1990; Barba de la Rosa *et al*., 1992; Marcone, 2000). These authors also show that amaranth is a valuable resource, containing a higher level of protein of better quality than cereals like maize may play an important role in human diets (Bressani & Garcia-Vela, 1990).

Scanning electron microscopy

The nutritional value or quality of structurally different proteins varies and is governed by amino acid composition, ratios of essen-

tial amino acids, susceptibility to hydrolysis during digestion, source, and the effects of processing (Higuchi & Fukazama, 1987; Gorinstein *et al*., 1991, 1999; Puppo & Anon, 1998; Millward, 1999).

Cereal-based foods are derived from grains that have a well-organized microstructure (Batterman-Azcona *et al*., 1999; Enamuthu *et al*., 1993; Cremer & Kaletunc, 2003). The microstructure determines the appearance and texture of protein fractions and the stability of the final product. Therefore, it was important to search the identity and the differences between identically extracted protein fractions from pseudocereal (amaranth), legume (soybean) and cereal (maize) in order to find a substitute for a cereal.

The IPSPF has a different morphology observed by scanning electron microscopy (Figure 2). However, the fine structures of IPSPF from *A. hypochondriacus* and soybean extracted by the same solvent system were similar (Figure 2A–D). These results are in correspondence with others that amaranthus seed protein bodies are morphologically similar to type of legumes (Konishi *et al*., 1995; Puppo & Anon, 1998). Maize (Figure 2E, F) differed from the pictures shown for

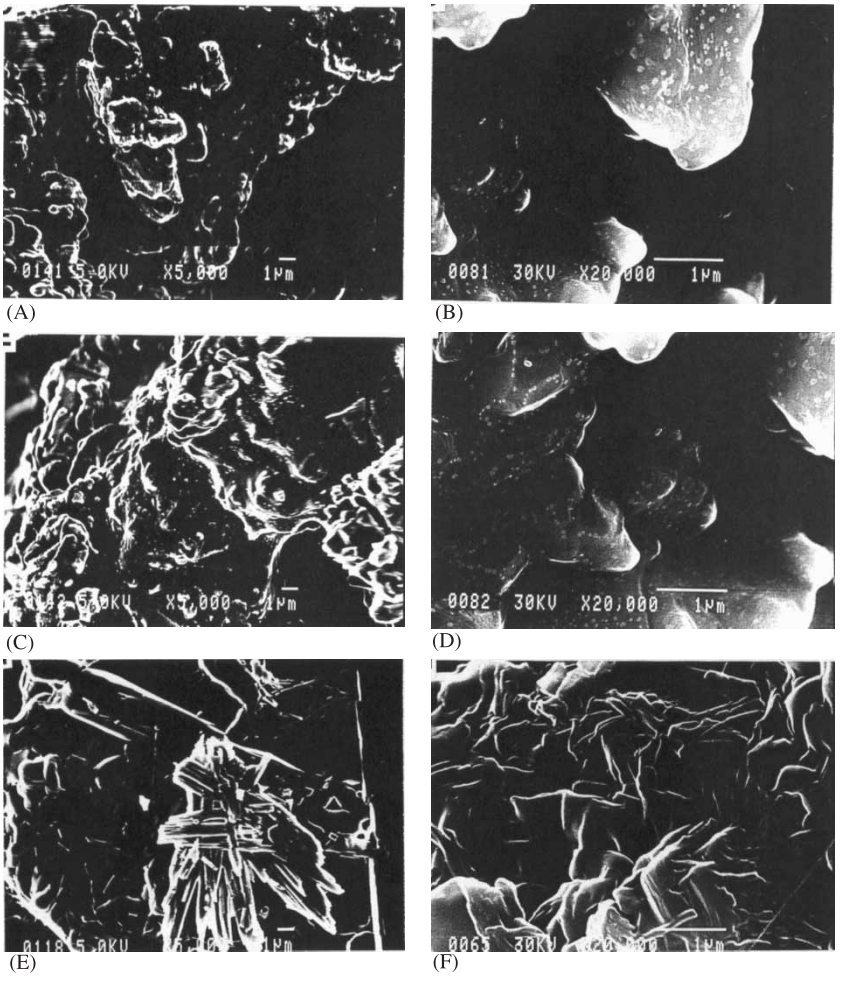

Figure 2. Microstructure of isopropanol-soluble proteins from (A) and (B) amaranth, (C) and (D) soybean, and (E) and (F) maize (prolamins). A, C and E, magnification × 5000; B, D and F, magnification × 20,000.

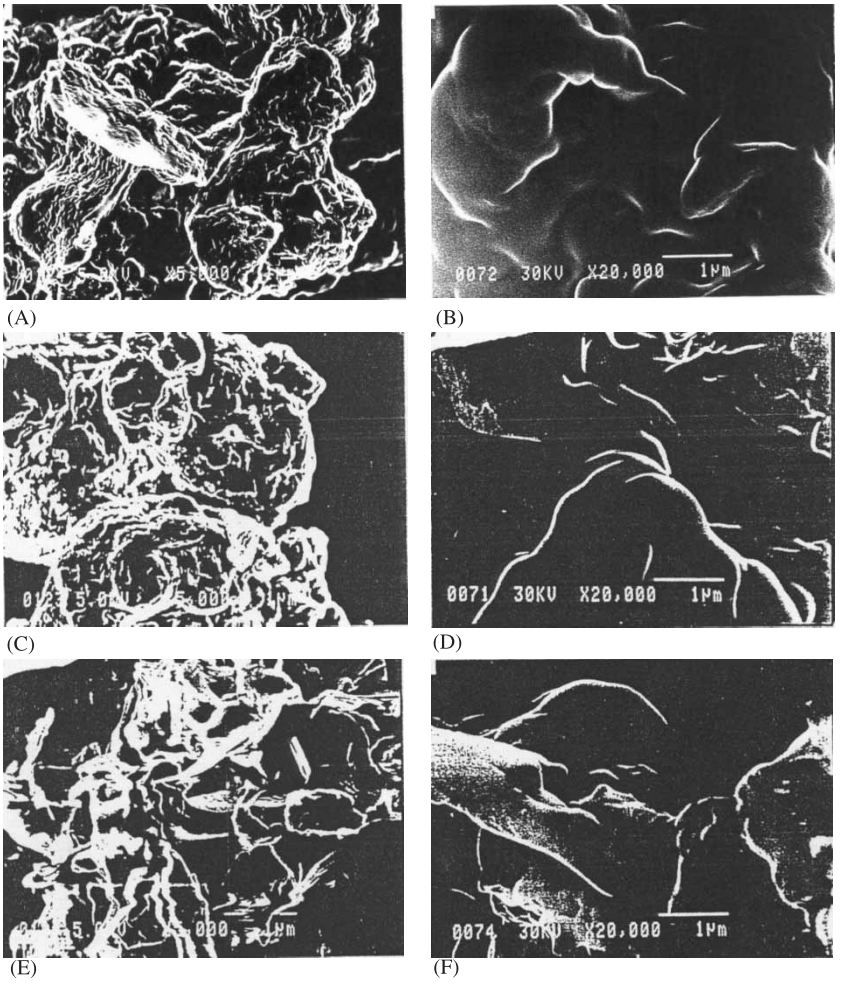

Figure 3. Microstructure of glutelins from (A) and (B) amaranth, (C) and (D) soybean, and (E) and (F) maize. A, C and E, magnification × 5000; B, D and F, magnification × 20,000.

amaranth and soybean, which consisted of large particles, irregular in shape with a rough surface texture. The morphology of maize prolamins (Figure 2E, F) differs from previous proteins. Maize powder contains small particles of regular shape with a smooth surface. It is known that processing, such as milling, causes microstructural changes in proteins and influences the fine microstructure. Therefore, the studied samples were ground and then extracted under the same experimental conditions.

In our previous research, different solvent systems were used to extract the alcohol-soluble proteins (Gorinstein *et al*., 1991, 1999). The most optimal extraction was achieved with 55% isopropanol containing 4–5% reductant (2-ME). In this research the samples of investigated plants were prepared using solvents without reductant. Our results are in agreement with others, showing that the conventional method used in this study is a less efficient procedure and reveals lower prolamin and glutelin contents than with the combination of alcohol solvent with the reductant. Proteins extracted with 55% 2-isopropanol plus reductant were made up of alpha-zeins, beta-zeins, gamma-zeins, and delta-zeins (Landry *et al*., 2000), in comparison with the use of other solvents where,

after extraction, prolamins were found as a minor fraction.

The effect of extraction of proteins without 2-ME on the morphology of total alcohol-soluble proteins (Figure 2) is in correspondence with other studies, where the density and the size of particles decreased as the result of destruction of disulfide bonds. These results are in accordance with the data on gels from plant proteins that demonstrated reduction in gel hardness upon action of 2-ME (Yuno-Ohto *et al*., 1994).

BSPF extracted without 2-ME from *A. hypochondriacus* (Figure 3A, B) soybean (Figure 3C, D) and maize (Figure 3E, F) demonstrates similarity in the morphology, containing large plates of irregular form with a smooth surface.

Electrophoretic and functional properties indicated a significant correlation between soluble protein fractions from soybean and amaranth. The protein fractions shared some common electrophoretic bands as well as a similar amino acid composition (Gorinstein *et al*., 2002). In this report we have shown that the similarity in pseudocereal and legume was also found in other protein fractions (isopropanol-soluble and buffer-soluble). True glutelins from maize were extracted in the same way as for legumes and pseudocereal (Landry & Moreaux, 1970). Our results are in accordance with others that amaranth is a valuable resource, containing a higher level of protein of better or similar quality than cereals like maize, and may play an important role in human diets (Bressani & Garcia-Vela, 1990; Escobar *et al*., 1998).

In conclusion, alcohol-soluble and buffer-soluble protein fractions from cereal, pseudocereal and legumes were characterized by electrophoretic and microscopic analyses. Apparently, correlation between electrophoretic patterns and the microstructure of proteins exists, and a closed identity between amaranth and soybean was found. Based on the present comparative data, pseudocereal amaranth and legume soybean can be used as nutritive substitutes of cereal maize in functional foods.

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