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Protein in Various Legume Seeds: Electrophoretic Comparison after Optimized Extraction

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ABSTRACT

Seed proteins of seven legume genera (of twelve cultivars) were compared within one gel under different conditions. Meal preparation using a centrifuge mill and dry ice and extraction using ultrasonication proved to be most effective. Also the conversion of protein to monomers by treatment with SDS in an ultrasonic bath at low temperature yielded somewhat sharper bands compared to the usual boiling procedure.

Extraction was by water, buffer or directly by SDS. The water extract differentiated between genera and cultivars better than the other two extractants. Standard PAGE for crude water extracts showed patterns, characteristic for genera, but not for cultivars. Clear differentiation between genera and cultivars was achieved by PAGIF in thin-layer gel after dialysis or after loading with SDS. PAGIF gave the best resolution.

ABBREVIATIONS USED

PAGE = Polyacrylamide gel electrophoresis.
PAGIF = Polyacrylamide gel isoelectric focusing.
SDS = Sodium dodecyl sulfate.
ME = Mercaptoethanol.
MW = Molecular weight.
PGGE = Pore gradient gel electrophoresis.

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INTRODUCTION

Legumes are a major source of proteins and calories for human nutrition, especially in developing countries. Their protein content and distribution have been thoroughly investigated. Electrophoretic patterns of different species and cultivars or fractions of identical seeds were compared. SDS-PAGE is commonly used, which includes the estimation of apparent MW of subunits (protomers). A review by Derbyshire *et al.* (1976) gives details on the structure, distribution and electrophoretic patterns of legumin and vicilin of legume seeds. Water-soluble proteins (albumin) of eight edible legume species were separated by SDS-PAGE (Bhatty, 1982). Miége (1982) summarized recent studies on legumin, vicilin and convicilin, as well as on other globulins in seeds of some leguminosae and emphasized the important role of SDS-PAGE.

This work describes a fast and effective milling with intensive cooling, an efficient extraction by sonication and a buffer system which allows for the comparison of seed proteins from various legumes by SDS-PAGE in one gel. Fractions were obtained by extraction with water, followed by buffer or directly with solutions of SDS and ME. Legume and cereal proteins, separated by SDS-PAGE, were compared at different pH-values; namely, pH 7·1 and 8·9, since the estimation of apparent MW is dependent on the buffer system used (Stegemann, 1979). The water extracts were further compared by isoelectric focusing in thin-layer gels.

MATERIALS AND METHODS

Seeds from Egypt

Dry mature seeds of faba bean (*Vicia faba*) cv. Giza 1; chickpea (*Cicer arietinum*) cv. Giza 2 and Family 2; Lentils (*Lens esculenta*) cv. Giza 9; *Lupinus termis*; and fenugreek (*Trigonella foenum graecum*) cv. Giza 2 and Giza 30 were obtained in April, 1982 from the Food Legume Section, Field Crop Institute, Agricultural Research Center, Giza.

Seeds from Germany

Faba bean cv. Diana; Pea (*Pisum sativum*) cv. Kleine Rheinänderin and cv. Wunder v. Kelvedon; French bean (*Phaseolus vulgaris*) cv. Dopp. Holl. Prinzess were supplied by the Bundessortenamt, D-3000 Hannover 61. Seeds of lupins (*Lupinus albus*) were received from the Bundesanstalt für Fettforschung, Institut für Biochemie und Technologie, D-4400 Münster.

Preparation of meal

The seeds were first manually decoated (except those of fenugreek), then mixed with dry ice (1:10, w/w) and milled in a precooled centrifuge mill (Retsch type ZM 1), speed set at 2, sieved with 0·2 mm pore size. Also, the decoated seeds of *Vicia faba* were milled by a 'Rotating-Blade'-mill (Type 10 S, Janke and Kunkel, D-7813 Staufen/Breisgau) jacketed for cooling. The distribution of particle size was determined by passing the meal through sieves of decreasing mesh size (90, 45 and 25 μm). The meals were defatted (twice, 15 min with 10 vol of acetone), dried on filter paper in an air stream and stored at -20°C.

Extraction

Aliquots of 250 mg defatted meal of different particle size (see 'Results and Discussion') were mixed in a centrifuge tube with 5 ml water containing 0·01% sodium azide and 0·2 ml of a mixture of $Na_2SO_3/Na_2S_2O_5$ (5 g/4 g in 100 ml water) and then sonicated for 30 min in an ultrasonic apparatus (Sonorex RK 102 H, Bandelin, D-1000 Berlin) using an ice bath. The mixtures were centrifuged (20 min, 18 000 *g*, 4°C) and the sediments from the first water extract were further extracted with 0·125M Tris-borate buffer (pH 8·9) in the same manner. The same patterns were found whether the meal was extracted once or three times with water before the buffer extraction.

Treatment with SDS for SDS-PAGE was done by mixing one part of the extract with one part of 4% SDS/2% ME-solution and sonication at 0–5°C for 30 min, at 20°C for 30 min and at 99°C for 15 min; by shaking at 22°C for 30 min in an electric shaker (Type RO S1, C. Gerhardt, Bornheimer Straße 100, 5300 Bonn 1) or by boiling for 15 min, respectively.

Electrophoresis was performed in vertical polyacrylamide gels (PANTAPHOR or MONO-PHOR, Labor Müller KG, D-3510 Hann, Münden). The gels for PAGE were prepared with 5·7% acrylamide and 0·43% methylene-bis-acrylamide in Tris-borate buffer pH 8·9 (Stegemann *et al.*, 1973). The SDS-PAGE was carried out in 0·01M Tris-borate buffer of pH 7·1 or 8·9 (Koenig *et al.*, 1970; Stegemann *et al.*, 1983). Water or buffer extracts were used either directly or after loading with SDS. The gels were stained overnight with 0·025% Supranolcyanin 6B (trichloroacetic acid/water/methanol/60% acetic acid (30:750:200:116)) and destained with a mixture of methanol/water/60% acetic acid (12:27:33).

Isoelectric focusing was carried out either in gel cylinders (8 cm long, 5 mm in diameter, or in slabs (10 × 10 cm, 0·2 mm thick). In the first case, glass tubes, 10 cm long, were filled with 1·6 ml of 4·32% acrylamide, 0·12% methylene-bis-acrylamide in 1% Servalyt T (pH 2–11 or 4–9) with or without 8M urea

(Stegemann *et al.*, 1983). The anolyte was 0·1% phosphoric acid and the catholyte 0·15% 1-dimethyl-amino-propan-2-ol. Thin-layer isoelectric focusing was performed in 5% PAA and 3% Servalyt T (pH 4–9). Strips of cellulose paper (2 mm thick) were used as bridges for solutions, soaked in a mixture of 0·025M aspartic and 0·025M glutamic acids for the anode and in 2M methylenediamine for the cathode. Carbon electrodes were used as an attachment for the MONO-PHOR.

RESULTS AND DISCUSSION

Effect of milling methods

Meals obtained by the 'Rotating-blade'-mill had 43% of the particles between 25 and 90 μm, while 29% were smaller than 25 μm and 24% were above 90 μm. The corresponding figures for the centrifuge mill were 20·5% between 25 and 90 μm, 74% below 25 μm and only 1% above 90 μm.

The extractability of meals produced by these two milling methods was compared by PAGE. Qualitatively, the same electrophoretic patterns were obtained either for the water or the buffer extract (after exhaustive water extraction) or for the extract done directly with SDS. This is an indication that the particle size did not noticeably affect the ratio of the extracted proteins but their amounts were about twice as high for the fine meal obtained in the centrifuge mill with dry ice. However, when the centrifuge-milling was done without cooling, higher aggregates were formed in SDS-PAGE (Stegemann & Pietsch, 1983). Also, the centrifuge milling resulted in a complete disintegration of particles hard to mill, e.g. seed coats of *Vicia faba*.

Effect of extraction methods

When the two extraction processes, shaking (Stegemann *et al.*, 1980) and ultrasonic, were evaluated, the water-soluble proteins had identical patterns in standard PAGE. The ultrasonic method led to more buffer-extracted soluble proteins, especially those separating in the cathodic (upper) part of the gel. This indicates fractions of higher MW but not more basic proteins since both methods of extraction showed the same pattern when focused in tubes (PAGIF). It was found that sonication can be used also for dissociating proteins to monomer with SDS at 0°C or 20°C giving sharper but identical bands compared with the conventional boiling with SDS. Hence, sonication for uncoiling proteins by SDS is to be preferred.

The extraction by sonication was applied to twelve different samples representing seven leguminous genera: (i) with water, (ii) with buffer after

water extraction, (iii) from seed meal directly extracted by SDS/ME. All extracts were then reduced with ME and treated with SDS to dissociate proteins to monomers. No attempt was made to investigate 'pure' albumins by extracting the whole meal by buffer and precipitation of the 'albumins' by dialysis against water since in no case could a distinct pattern be found for 'albumins' and the procedure was more cumbersome. Furthermore, loss of proteins and changes in patterns due to prolonged handling was substantial.

Evaluation

Each extraction process and all samples were compared on the same SDS-gel (Fig. 1). The genera could be differentiated but not all cultivars in any type of extract. Only with the water-soluble proteins, converted to monomers, were patterns characteristic for all cultivars of *Vicia faba*, of *Pisum sativum* and of *Trigonella foenum graecum* obtained. Buffer-soluble proteins showed some differentiation in SDS-gels for fenugreek. Patterns of bean and pea proteins extracted directly with SDS (similar to patterns of type II extract) showed no characteristic differences dependent upon cultivars but the differences between genera remained. In the case of *Phaseolus vulgaris* all extracts gave similar patterns.

Boulter (1979) correlated sedimentation constants of storage protein fractions with their apparent MW in SDS-PAGE. Matta *et al.* (1981) reported three fractions in globulin storage proteins, characterized by their MW and identified as legumin (MW 35 000 to 53 000), vicilin (MW > 50 000) and convicilin (MW 70 000). In our work, the electrophoretic patterns of the three extracts, (i), (ii) and (iii), contained mainly two vicilin fractions of MW 50 000 and 33 000 and four fractions of legumin having MWs between 20 000 and 40 000. The different fractions varied with the tested genera. The estimation of MW by SDS-PAGE may be quite arbitrary, since they depended on the buffer used and eight different estimates were obtained in eight buffer systems (Stegemann, 1979). Therefore, extracts (i) and (ii) of legume seeds were compared in two different buffers, together with proteins extracted in the same fashion from cereal seeds. A better resolution and somewhat sharper bands were obtained in the SDS/Tris-borate buffer, pH 7·1 (Fig. 2), monomers ranging between MW 14 000 and 97 000. Bhatty (1982), dealing with albumins of mung beans, lentils, faba beans, vetch, beans, peas, lathyrus and chickpeas, found MWs ranging between 10 000 and 100 000.

The structure and subunit composition of proteins of *Pisum sativum*, *Phaseolus vulgaris* and *vicia faba* have been compared by methods including SDS-PAGE (review by Ersland *et al.*, 1983). Similarities between legumin subunits from *vicia faba* and *Pisum sativum* have been reported by Jackson

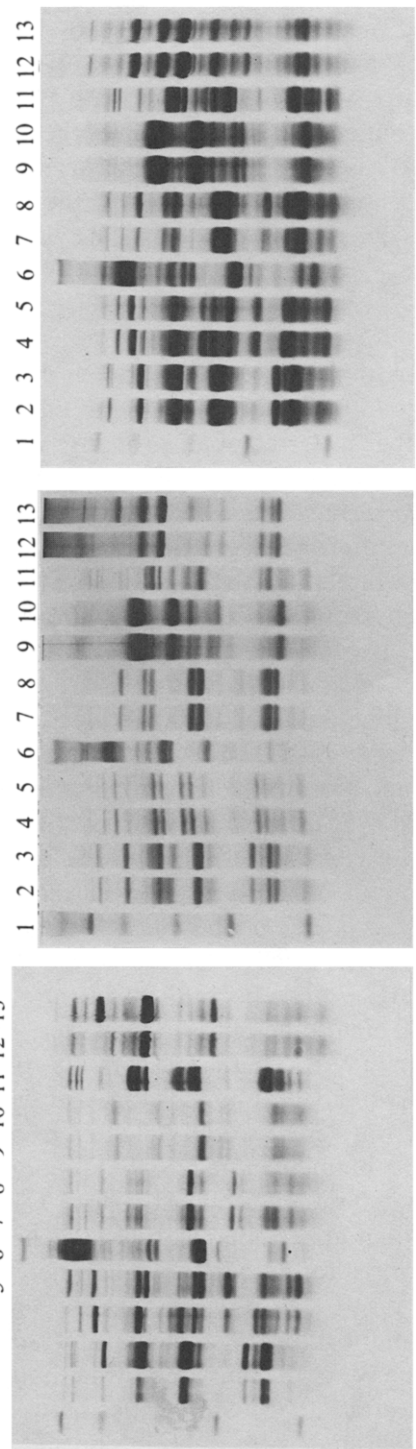

Fig. 1. Performance of a continuous buffer for separation of seed protein from very different legume genera and varieties on SDS-PAGE. SDS-PAGE is done in 6% PPA and Tris-borate buffer (pH 7·1) with 0·1% SDS. Shown are the SDS-protomers of protein extracted by: (a) water; (b) buffer after three successive extractions by water; (c) directly by 4% SDS and 2% ME in water. 1, Marker proteins; 2, *Vicia faba* cv. Diana; 3, *Vicia faba* cv. Giza 1; 4, *Pisum sativum* cv. Kl. Rheinländerin; 5, *Pisum sativum* cv. WU von Kelvadon; 6, *Phaseolus vulgaris* cv. Dopp. Holl. Prinzess; 7, *Cicer arietinum* cv. Giza 2; 8, *Cicer arietinum* cv. Family 2; 9, *Lupinus albus*; 10, *Lupinus termis*; 11, *Lens esculenta* cv. Giza 9; 12, *Trigonella foenum-graecum* cv. Giza 2; 13, *Trigonella foenum-graecum* cv. Giza 30. Protomers produced from the water-soluble proteins showed clear varietal and genera differences.

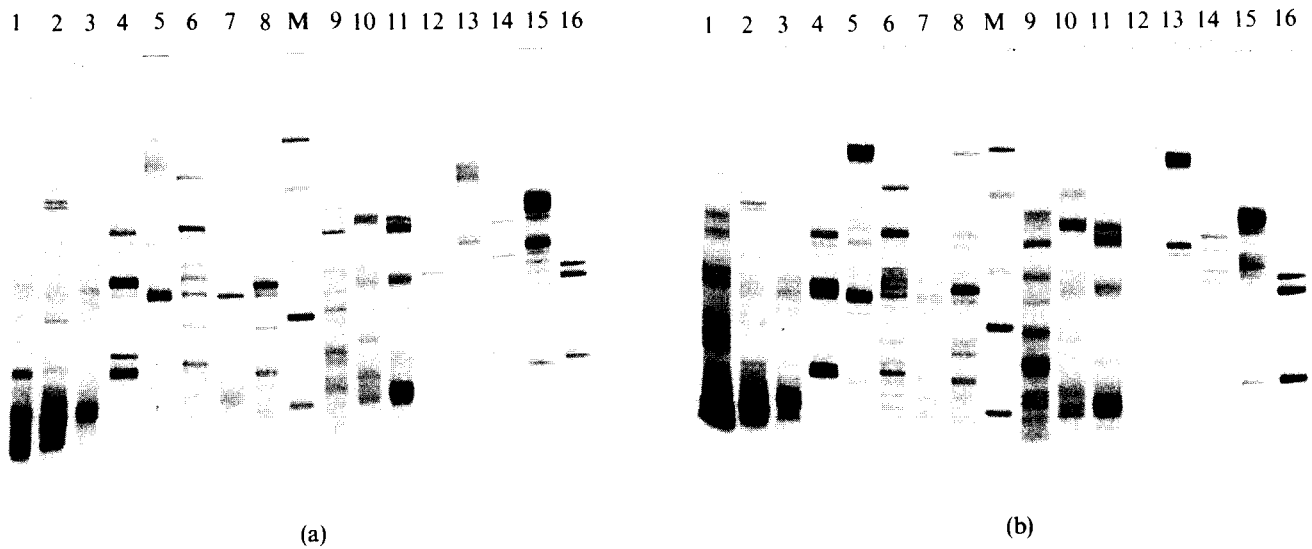

Fig. 2. Protomers of cereal and legume seeds as compared in SDS-PAGE. SDS–PAGE was made in Tris-borate buffer; (a) pH 7·1; (b) pH 8·9. Water extracts are shown on the left hand side (slots 1–8): buffer extracts (Trisborate, pH 8·9 after water extraction) on the right-hand side (slot 9–16); markers (M) show the molecular weight in the middle with 14·3, 25·7, 37, 97·4 kD, respectively. Different cereal and legume cultivars are analysed: 1,9, *Zea mays*, cv. 1807 Waxy; 2,10, *Triticum aestivum* cv. Sacha 8; 3,11, *Oryza sativa* cv. S 253; 4,12, *Vicia faba* cv. Express; 5,13, *Phaseolus vulgaris* cv. Doop. Holl. Prinzess; 6,14, *Pisum sativum* cv. Kleine Rheinlanderin; 7,15, *Lupinus albus* (commercial cultivar); 8,16, *Cicer arietinum* cv. Giza 2.

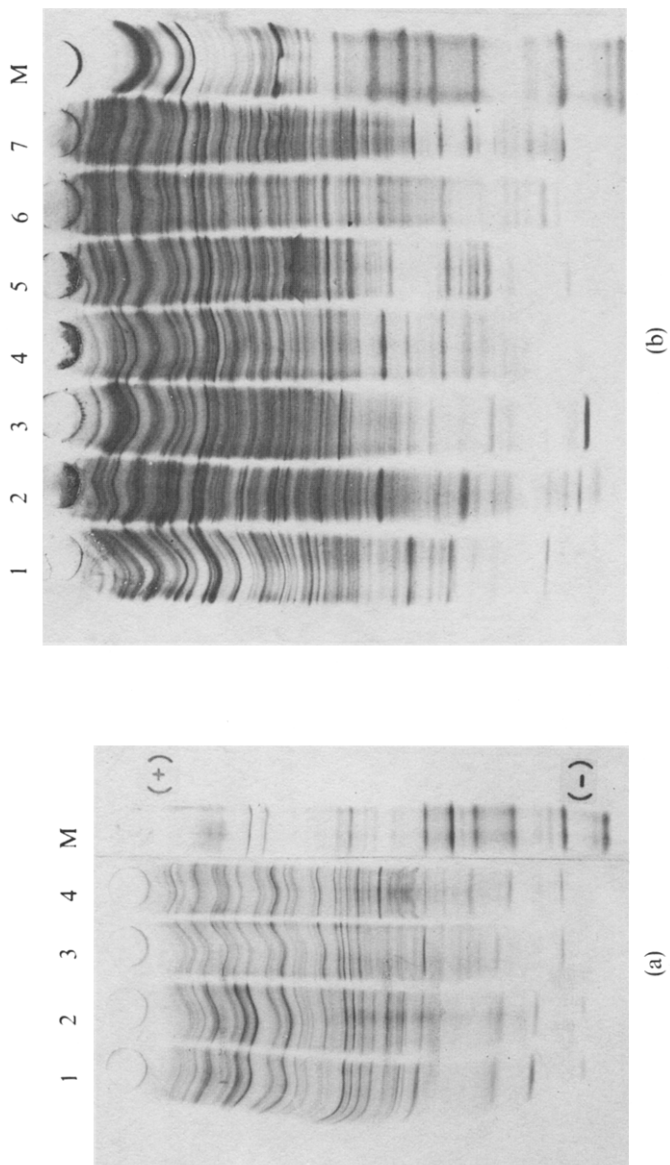

Fig. 3. (a) PAGIF in thin-layer 0·1 mm thick gel of seed proteins of two leguminous genera and four cultivars: 1, *Pisum sativum* cv. Kleine Rheinländerin; 2, *Pisum sativum* cv. Wunder v. Kelvadon; 3, *Vicia faba* cv. Diana; 4, *Vicia faba* cv. Express; M, marker proteins. (b) PAGIF proteins of seven different genera. PAGIF was done in thin-layers of 0·18 mm thick gel with 5% PAA and 3% Servalyt T pH 4–9: 1, *Vicia faba* cv. Diana; 2, *Pisum sativum* cv. Kleine Rheinländerin; 3, *Phaseolus vulgaris* cv. Dopp. Holl. Prinzess; 4, *Cicer arietinum* cv. Giza 2; 5, *Lens esculenta* cv. Giza 9; 6, *Lupinus termis* (*commercial cultivar*); 7, *Trigonella foenum-graecum* cv. Giza 2; M, marker proteins. Basic proteins in the lower part of the gel indicated better differences among genera and cultivars.

et al. (1968), Croy *et al.* (1979) and Matta *et al.* (1981). Legumin subunit MWs, for *Pisum vicilin* and *Phaseolus phaseolin* (G1) were similar (Gatehouse *et al.*, 1981). Results obtained in this work agreed with the estimated MWs found by the above authors and with the legumin subunits of chickpea (Ganesh Kumar & Venkataraman, 1978; 1980), lupin seeds (Cerletti *et al.*, 1978; Duranti *et al.*, 1981; Restani *et al.*, 1981; Sgarbieri & Galeazzi, 1978) and fenugreek (Ladizinsky 1977). Croy *et al.* (1979) gave a MW of 39 000 for *pisum* and 34 000 for *vicia* legumin subunits. Other studies compared different protein fractions of various legumes (McLeester *et al.*, 1973; Gilroy *et al.*, 1979; Weber *et al.*, 1981).

Thin-layer isoelectric focusing of the dialysed water extracts showed clear differences between varieties (Fig. 3a) and between genera (Fig. 3b). Although these differences were noticed for both acidic and basic proteins, the differences were more pronounced in the basic region.

Generally, standard PAGE for the proteins of the crude water extracts gave patterns characteristic for genera but not for cultivars. SDS-PAGE for reduced-SDS treated water extracts showed characteristic patterns for genera and cultivars. PAGIF showed good differentiation for the dialysed water extracts indicating that the charge distribution can serve for cultivar and genera differentiation. In thin-layer gels excellent resolution and a high number of bands were obtained. The crude water extracts were not suited for the differentiation among genera, species and cultivars, since obvious and unforeseeable distortions of bands were encountered.

Evaluation of protein patterns according to a single parameter (size or change) did not necessarily give a clear distinction of all cultivars tested; this was particularly misleading in spite of numerous sharp bands (Stegemann, 1979). For differentiation between closely related species and for detecting small variations in a protein pattern, separations should be based on different parameters such as size (SDS-PAGE) and charge (PAGIF).

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