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Capillary electrophoresis of seed 2S albumins from *Lupinus* species

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Abstract

Two modes of capillary electrophoresis (CE) — free-solution capillary zone electrophoresis (CZE) and sodium dodecyl sulfate capillary electrophoresis (SDS-CE) using a non-gel sieving matrix — have been developed for comparative analysis of low-molecular-mass 2S albumin isoforms from lupins. The albumin fraction and 2S albumins were separated in uncoated fused-silica capillary by CZE with 0.02 *M* phosphate buffer, pH 7.3, containing the sodium salt of phytic acid. The use of phytic acid (0.025 *M*) as buffer modifier and ion-pairing agent improved migration reproducibility, peak shape and separation efficiency. The reduced 2S albumins were separated by SDS-CE using a high concentration (0.3–0.5 *M*) mixture of tris(hydroxymethyl)aminomethane and borate buffers in uncoated fused-silica capillary. Of the various polymers used as non-gel sieving matrix, SDS-CE with a 10% dextran solution was found to be suitable for separation of 2S albumin polypeptides with molecular masses of 4000–7000 and 8000–11 000. The addition of glycerol or ethylene glycol to the SDS separating buffer improved the resolution of polypeptides. The examined *Lupinus* species showed species-specific CZE and SDS-CE migration profiles of the 2S albumins.

Keywords: *Lupinus* spp.; Ion-pairing reagents; Buffer composition; Albumins; Proteins

1. Introduction

Lupins, in comparison to other legumes, have a particularly high seed protein content (30–50%). As in other legumes, globulins represent the main protein fraction, and albumins constitute about one-fourth of the protein. Among albumins, low-molecular-mass 2S albumins constitute one of the main components [1]. The 2S albumins are synthesized and stored in developing seeds and are principally utilized by the plant as a source of nutrients during subsequent germination and seedling growth. They were previously classified as globulins and named conglutin δ [2]. Since 2S albumins are sulfur-rich (~nine residues percent 1/2 cysteine), they make a very important contribution to the nutritional value of lupin seeds (about 70% of the seed sulfur) [3]. The 2S albumin isoforms are encoded by small multigene families. The detection of genetic polymorphism of seed 2S albumins from Old World *Lupinus* species has been achieved through polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sufate (SDS)-PAGE [1] and reversed-phase HPLC [4]. In *Lupinus* species the 2S albumin class consists of four to eight isoforms, which are composed of two subunits of ≈3000–6000 and 8000–12 000 linked by four disulfide bridges [1,4]. Most of the recent interest in 2S albumins has focused on their exploitation in genetic engineering as candidates for use in correcting the deficiency of the sulfur amino acids, methionine and cysteine, in legumes and most vegetable proteins [5].

During the past few years, capillary electrophoresis (CE) has been proved to be a powerful technique for the rapid and high-resolution separation of seed proteins, especially cereal proteins, with full

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automation and quantitative digital data [6–11]. In the analysis of proteins, CE presents a method complementary to HPLC and potentially an alternative to PAGE. However, limitations of the application of CE to protein separation are protein–capillary wall electrostatic interactions. Positively charged residues of proteins interact with negatively charged silanol groups of fused-silica surface, which often leads to peak broadening, loss in efficiency, poor reproducibility of migration time and low recovery of protein during the separation [12,13]. Various methods have been used to minimize or control the effects of silanols on protein separation. These include capillary wall modifications, control of the pH and ionic strength and use of isoelectric, acidic buffers or different additives [12–15]. Some of the capillary wall modifications have demonstrated remarkable stability and migration time reproducibility [14]. Nonetheless, addition of buffer modifiers remains attractive because of their versatility, ease of use and low cost.

The use of a dodecasodium salt of phytic acid or phytic acid as a buffer additive in buffers of neutral pH was reported to improve separation efficiency and resolution of proteins and peptides [15–19]. A reduced inner wall adsorption of proteins using phytic acid as buffer modifier was observed for buffers with pH values >6.0 [18]. In addition, the polyanionic character of the sodium salt of phytic acid enables ion-pair formation with basic proteins or peptides, thus suppressing the Coulombic interaction between positively charged proteins or peptides and the negatively charged silanol groups of the fused-silica surface [16,17].

SDS-CE using a non-gel sieving matrix has gained popularity as an alternative to SDS-PAGE for the separation of seed proteins with M_r values of 14 000–200 000 [20–22]. Several commercial sieving buffers for SDS-proteins are available. In the case of lupins, SDS-CE was used for separation of seed storage proteins from different species [22,23]. Also, the non-gel sieving SDS-CE method for the separation of standard polypeptides (M_r range of 2500–17 000), as an alternative for separation of polypeptides by SDS-PAGE, has been described [24].

This study was aimed at developing two modes of CE — capillary zone electrophoresis (CZE) and sodium dodecyl sulfate capillary electrophoresis (SDS-CE) using a non-gel sieving matrix — for comparative analysis of albumin fraction and isolated 2S albumins. The sodium salt of phytic acid is used as a buffer modifier and an ion-pairing agent to improve peak shape, efficiency and migration reproducibility during separation of albumin by CZE. Three polymers are used as a sieving matrix in SDS-CE method for separation of reduced low-molecular-mass 2S albumins (M_r 4000–12 000). Finally, the 2S albumins from six *Lupinus* species are analyzed to show the potential of two modes of CE methods in characterization of this class of proteins and taxonomic studies. The CZE and SDS-CE migration profiles of analyzed samples are compared to respective published data of RP-HPLC elution profiles and PAGE or SDS-PAGE patterns.

2. Experimental

2.1. Chemicals

Sodium dihydrogenphosphate and disodium hydrogenphosphate (ultrapure bioreagent) were purchased from J.T. Baker (Deventer, Netherlands). All other chemicals for preparing buffer solutions were obtained from Sigma (St. Louis, MO, USA). All buffer solutions were prepared by using HPLC-grade water (Elgastat UHQ PS water purification system, Elga, UK). Phytic acid (inositolhexaphosphoric acid dodecasodium salt), N-ethylmaleimide and hydroxypropylmethylcellulose (HPMC) (M_r ~86 000) were obtained from Aldrich (Milwaukee, WI, USA). Dextran (M_r 2 000 000), poly(ethylene glycol) (PEG) (M_r 35 000), ethylene glycol, glycerol and peptide markers MW-SDS-17S (M_r 2510–16 950) were delivered by Sigma. SDS was purchased from Fluka (Buchs, Switzerland).

2.2. Sample preparation

Seed samples of *Lupinus albus*, *L. angustifolius* and *L. hispanicus* subsp. *hispanicus* were obtained from the Plant Experimental Station (Wiatrowo, Poland); *L. cosentinii* and *L. subcarnosus* from the

Institut für Pflazenbau und Pflanzenzüchtung der FAL (Brunswick, Germany) and seed samples of *L. mutabilis* were received from the Western Regional Plant Introduction Station, USDA-ARC (Pullman, USA). Cotyledons of mature seeds of the original samples were analyzed.

The dry cotyledons were milled and the resulting meal was defatted with *n*-hexane. The albumin fraction was extracted twice from the defatted meal by homogenization in water (pH adjusted to 5.0 with HCl) containing 0.05 m*M* N-ethylmaleimide (NEM) to block accessible sulphydryl groups [1]. The suspension was stirred for 2 h at room temperature and the insoluble material removed by centrifugation. The supernatants were combined and dialyzed against water at pH 5.0, for 48 h, at 4°C. The water-soluble fraction was filtered through a 0.45-μm membrane (Millipore, Milford, MA, USA).

The 2S albumins were recovered from the albumin fraction by solid-phase extraction (reversed-phase chromatography) using a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) [1]. The 2S albumins were eluted with 42% acetonitrile in 0.1% trifluoroacetic acid (TFA) and dried under vacuum in a SpeedVac concentrator (Savant, Farmingdale, NY, USA). The proteins were dissolved in separation buffer and filtered through a 0.22-μm membrane (Schleicher and Schuell, Dassel, Germany).

2.3. Instruments

A Model P/ACE system 2100 CE unit with UV detector (Beckman, Fullerton, CA, USA) was used. Data were collected and integrated using the software SYSTEM GOLD, VER. 8.11 (Beckman). Fused-silica capillaries CElect FS, 50 or 75 μm I.D. and 375 μm O.D. with polyimide cladding, were obtained from Supelco (Bellefonte, PA, USA). The liquid cooling system of the capillary was set to 25°C (CZE) or 20°C (SDS-CE). The buffers were filtered through a 0.2-μm filter (Gelman Sciences, Ann Arbor, MI, USA) and degassed for use in an ultrasonic bath. All rinsing stages were carried out with 137.8 kPa (20 p.s.i.), using nitrogen pressure. Injections were performed hydrodynamically at 3.4 kPa (0.5 p.s.i.). In all experiments the absorbance was monitored at 214 nm.

2.4. Capillary zone electrophoresis

The electrophoresis was carried out with 20 m*M* phosphoric buffer (pH 6.8–8.4) containing from 0 to 40 m*M* sodium salt of phytic acid on a capillary of total length 47 cm (effective length 40 cm)×50 μm I.D. Capillaries were preconditioned, prior to their first use, by rinsing with 1 *M* phosphoric acid for 30 min, next rinsed with 0.5% HPCM for 30 min. Prior to each protein sample the capillary was rinsed for 2 min with 0.1 *M* NaOH and then for 1 min with deionized water for cleaning purposes. Afterwards the capillary was reconditioned by rinsing for 1 min with separation buffer containing 0.2 *M* SDS, then for 2 min with water and for 6 min with separation buffer taken from buffer vial other than that used during separation. Prior to storage the capillary was flushed for 10 min with 0.1 *M* phosphoric acid, for 5 min with water and then with air–nitrogen. Separation was carried out by applying a constant voltage of 160 V/cm with normal polarity.

2.5. Sodium dodecyl sulfate capillary electrophoresis

Four different polymer systems (a commercial polymer from eCAP SDS 14–200 kit from Beckman; PEG, dextran and a mixture of the last two) were evaluated for use in SDS-CE separation of reduced 2S albumins. Dextran and PEG polymer–buffer systems were optimized by manipulating the buffer composition and polymer concentration and through the use of glycerol or ethylene glycol to slow the diffusion of polypeptides.

In the SDS-CE studies the injection was carried out at the cathodic side. Polymer solutions were degassed by centrifugation to fill the capillary at 137.8 kPa. The mixture of protein and sample buffer was heated at 95°C for 15 min and after cooling the mixture was injected for 60 s into the capillary filled with polymer solution. The molecular masses of the protein samples were estimated by using the computer software MOLECULAR WEIGHT DETERMINATION, VER. 8.11 from the SYSTEM GOLD software package (Beckman). For reliable peak assignment, the migration time relative to orange G was calculated. The resolution (R_s) was calculated according to the following formula: $R_s = 2(t_{m2} - t_{m1})/(w_2 + w_1)$,

where 1 and 2 refer to any two protein peaks of interest, t_m is migration time, and w is peak width at base.

2.5.1. Commercial mixture of poly(ethylene oxide) and PEG as sieving matrix

The samples were prepared and analyzed using an eCAP SDS 14–200 kit from Beckman. In general, 100 μl of the sample buffer (120 m*M* Tris–HCl, 1% SDS, pH 6.6) was mixed with 85 μl of the sample, 5 μl of 2-mercaptoethanol and 10 μl of the internal standard, orange G (OG). The analyses were performed on an eCAP neutral coated capillary (47 cm×100 μm I.D.) in 100 m*M* Tris–HCl buffer (pH 8.8), containing 0.1% SDS. The separations were performed at constant voltage of 11.5 kV for 26 min. SDS molecular mass standard mixture consisted of α-lactoalbumin (14 200), soybean trypsin inhibitor (20 100), carbonic anhydrase (29 000), ovalbumin (45 000), bovine serum albumin (66 000), phosphorylase *b* (97 400) and β-galactosidase (116 000).

2.5.2. Dextran or PEG as non-gel sieving matrix

The separating buffer solution was 0.3–0.5 *M* Tris–borate (pH 8.4), 0.1% SDS and glycerol or ethylene glycol (5–15%) unless stated otherwise. The polymers were dissolved in the separating buffer to give the specified concentration. The 2S albumins resolved in 75 m*M* acetic buffer (pH 4.6) (100 μl) were mixed with 100 μl of 100 m*M* Tris buffer (pH 9.2) containing 1% (w/v) SDS, 10 μl 2-mercaptoethanol and 8 μl of 0.1% (w/v) OG. Separations were carried out in a fused-silica capillary (27 cm× 75 μm I.D.). Prior to the first run of the day or of a new set of conditions, the uncoated capillary was prepared for use by successive rinsing with: 0.1 *M* NaOH for 10 min, 0.1 *M* SDS solution for 3 min, water for 2 min, 1.0 *M* HCl for 5 min, and SDS run buffer from Beckman kit for 60 min. After every run the capillary was flushed with 0.1 *M* HCl for 3 min and with the separating buffer for 6 min. The reduced 2S albumins were separated at constant voltage of 8.5 kV (current ≈35–38 μA). The correlation between the relative migration time (RMT) and log M_r was evaluated using co-analyzed SDS peptide markers MW-SDS-17S (six peptides obtained by enzymatic cleavage of horse-heart myoglobin in the range of 2500–17 000, plus bovine glucagon — 3500).

3. Results and discussion

3.1. Capillary zone electrophoresis

3.1.1. CZE separation of albumins

Seed water-soluble proteins from *Lupinus* species are a complex and heterogeneous group of proteins [1,4]. The separation of seed albumins from *Lupinus* species in the uncoated fused-silica capillary filled with phosphate buffer at pH 6.8–8.4 was not successful and resulted in a low resolution of most of albumins and in a considerable broadening of protein peaks (Fig. 1A). Moreover, the migration times of all analytes in the uncoated capillary tended to increase with consecutive separations, despite flushing of the capillary between the successive runs, because of irreversible adsorption of some albumins onto the capillary inner wall [6,12,13]. The addition of a relatively small amount of sodium salt of phytic acid (0.5–5%) to the separation buffer significantly improves the performance of separation (Fig. 1B and C). CZE electropherograms showed greatly improved shapes of the peaks, resolution and migration reproducibility, due to the fact that adsorption of proteins onto the inner wall of the capillary in the presence of phytic acid is strongly reduced. Similar behavior has been observed by other researches [16–19]. In the presence of this polyanionic species the CZE patterns showed at least 11 and 31 protein peaks in the water-soluble fraction from the seeds of *L. albus* and *L. mutabilis*, respectively. The application of the phytic acid allowed the separation of particularly low-molecular-mass 2S albumin isoforms, which constitute one of the main components of the albumin fraction (peaks 9–12 in Fig. 1).

3.1.2. Effect of phytic acid concentration on separation of 2S albumins

The influence of the phytic acid concentration in the buffer on the separation of albumins was investigated in uncoated capillary filled with 20 m*M* phosphate buffer, pH 7.3. With the addition of 5–50 m*M* phytic acid (10 m*M*≈0.92%) to the phosphate buffer, prominent changes in the resolution, sepa-

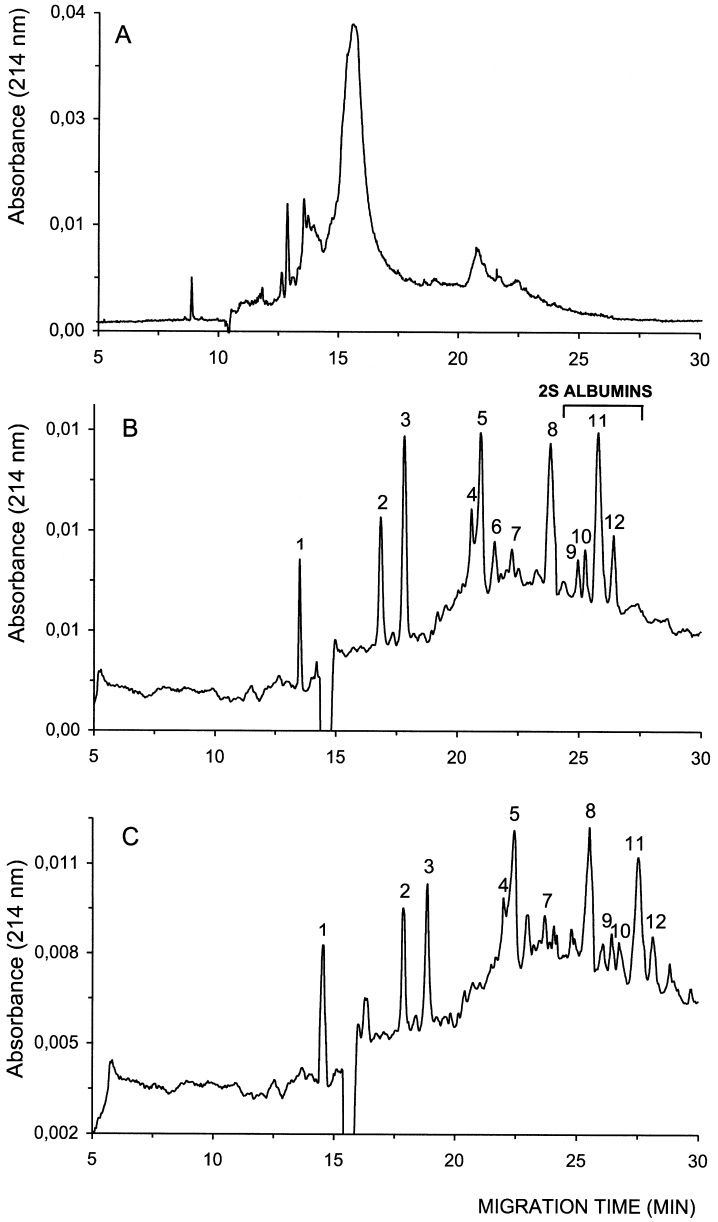

Fig. 1. CZE patterns of seed albumin fraction from *L. albus* separated at pH 7.3 in a 20 m*M* phosphate buffer in the absence (A) and presence (B) and (C) of the sodium salt of phytic acid (25 m*M* and 40 m*M*, respectively). The albumins were separated at 160 V/cm in uncoated capillary (47 cm×50 μm I.D.) at 25°C.

ration efficiencies of albumins and migration times of peaks, in particular the 2S albumins, were observed (Fig. 1B and C). The separation time increases with concentration increase of the sodium salt of phytic acid as a consequence of the decreased ζ potential and reduced electroosmotic flow, because of the increase of the ionic strength of the background electrolyte. In addition, the migration of part of the proteins was slowed because the proteins overall have become less positive due to interaction

with phytic acid. With 5–20 m*M* phytic acid in the buffer, the peak efficiency was significantly improved, but the migration reproducibility was still poor. When 20–40 m*M* sodium salt of phytic acid was added, the separation efficiency was satisfactory and high reproducibility was obtained (Fig. 1B and C). The reduction of protein adsorption onto the capillary wall was the predominant factor limiting the peak broadening. The best separation in terms of efficiency and resolution were obtained for 25 m*M* phytic acid. The efficiency for major protein peaks ranged within $1.2 \cdot 10^5$–$3 \cdot 10^5$ plates/m and reproducibility of migration time [RSD ($n=8$)] for five major protein peaks (peak numbers 1, 3, 5, 8 and 11 in Fig. 1B) was $<1.6\%$. A further increase in the concentration of phytic acid in the separation buffer (above 40 m*M*) gave a considerably longer separation time and decreased migration reproducibility. This may be explained by the occurrence of a thermal effect due to considerable current increase during separation in the presence of a high concentration of phytic acid in the separation buffer.

3.1.3. Effect of buffer pH on separation of albumins

The influence of the buffer pH (6.8–8.6) on the separation of the albumin fraction and 2S albumins was significant. When buffer pH was changed from 7.3 to 8.0 (20 m*M* phosphate buffer), the efficiencies of major protein peaks were reduced to $1.0 \cdot 10^5$–$1.8 \cdot 10^5$ plates/m and the average reproducibility of migration times was decreased (RSD$>2.5\%$). An increase in pH value above 8.0 resulted in a large decrease of the separation efficiency. At the same time poorly reproducible protein migration time was also observed (RSD of migration time $>3.5\%$) because of the increase in the number of negative charges on the proteins. Optimum resolution was obtained using phosphate buffer, pH 7.3, containing 25 m*M* phytic acid. The separation of proteins and peptides at neutral pH in phosphate buffer containing phytic acid as buffer modifier was also recommend by other authors [18,19].

The effect of the buffer pH on the separation of 2S albumin isoforms is shown in Fig. 2. In the range of pH 6.8–7.8 an increase of migration time of 2S albumin isoforms was observed, whereas above pH 8.0 a decrease of migration time was noted. At the same time a variation in the migration order of separated isoforms was revealed. At pH 6.8 and 7.3 all four isoforms were well separated but in different sequence (Fig. 2A and B), whereas at pH 8.4 two isoforms (peaks 3 and 4) were not separated. Phytic acid influenced the mobility of the particular isoforms through forming ion-pairs with positive charges of 2S albumin isoforms, which are rich in arginine residues (nine to eleven per molecule) and partly in lysine residues [25,26]. This variation in migration times probably causes differentiation in participation of arginine and lysine residues in particular isoforms of 2S albumin. It was previously found that these two residues have an essential influence on ion-pair formation [16]. A similar effect was observed in the separation of aprotinin [17] and lysozyme [18], which are also rich in arginine and lysine residues. The buffer pH had also considerable influence on the shape of peaks of 2S albumin isoforms (Fig. 2A–C). The highest number of theoretical plates for lupin 2S albumin peaks was obtained at pH 6.8, where ion-pairing was expected to be the strongest (Fig. 2A).

3.1.4. Comparison of CZE patterns of 2S albumins from different Lupinus species

CZE electropherograms of seed albumin fraction from three *L. mutabilis* accessions show significant quantitative differences in the albumin composition of this fraction (Fig. 3). The first two electropherograms (Fig. 3A and B), in the migration time between 16 and 28 min, showed 31 components each, which differed considerably in the peak heights and the peak areas. Whereas the electropherogram of the third accession (Fig. 3C), besides clear differences in the heights of some peaks in comparison to the respective peaks of the first electropherograms, showed no protein peaks number 10 and 12. Each of these accessions of *L. mutabilis* contained eight 2S albumin isoforms (peaks 22–29), which differed significantly with respect to peak heights.

The electrophoretic mobilities of structurally very similar 2S albumin isoforms from different *Lupinus* species were found to be species-specific. Each species distinguished by its specific CZE profile. Fig. 4A–C presents CZE profiles of 2S albumin isoforms of other three examined *Lupinus* species (*L. angustifolius, L. hispanicus* and *L. subcarnosus*). The total

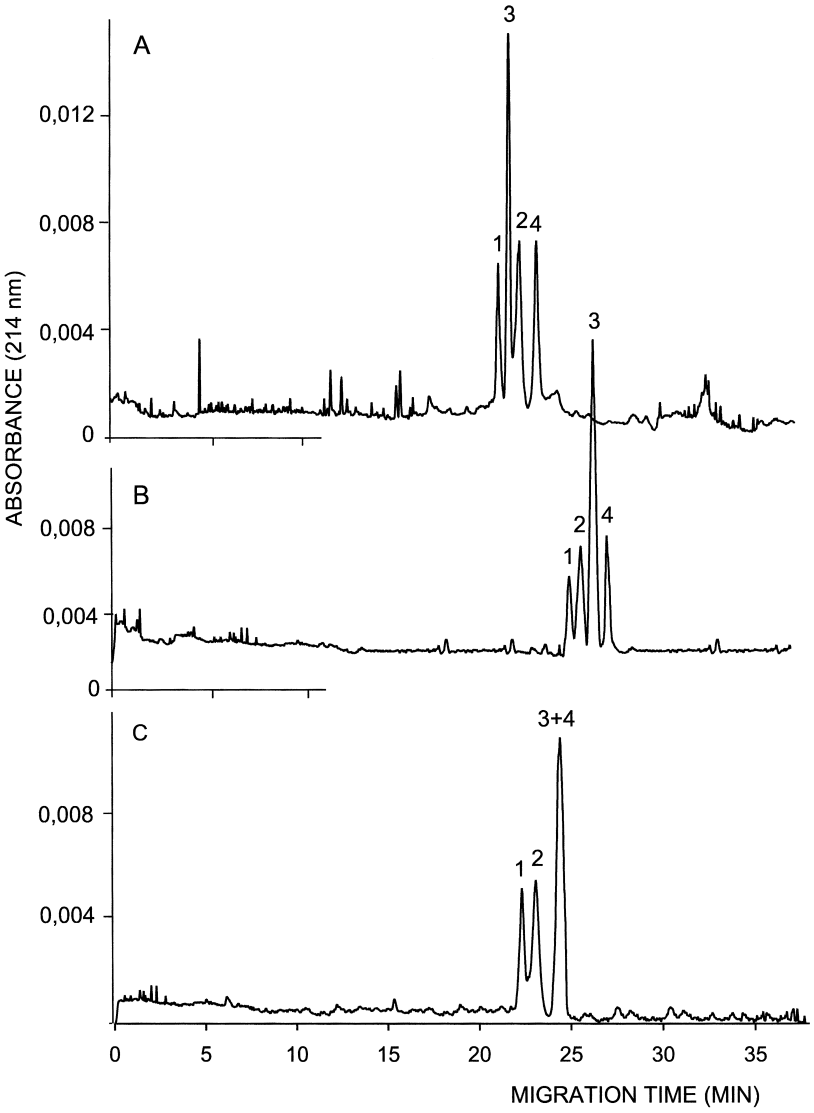

Fig. 2. Effect of pH buffer on the separation of 2S albumin isoforms from *L. albus*. Separation buffer: 20 m*M* phosphate buffer–25 m*M* sodium salt of phytic acid at pH 6.8 (A), pH 7.3 (B) and pH 8.4 (C). Voltage and capillary parameters are as in Fig. 1.

numbers of seed 2S albumin isoforms from the albumin fractions of *L. albus, L. angustifolius, L. hispanicus, L. cosentinii*, *L. mutabilis* and *L. subcarnosus* were 4, 7, 7, 4, 8 and 7, respectively. The last two New World species have an isoform number similar to those of the remaining Euro–African (Old World) species. The CZE analysis of 2S albumins, proved to have a much higher resolution power as compared to PAGE [1], but comparable to RP-HPLC [4].

3.2. Sodium dodecyl sulfate capillary electrophoresis

3.2.1. SDS-CE separation of albumins

Fig. 5 shows the separation of the seed albumin

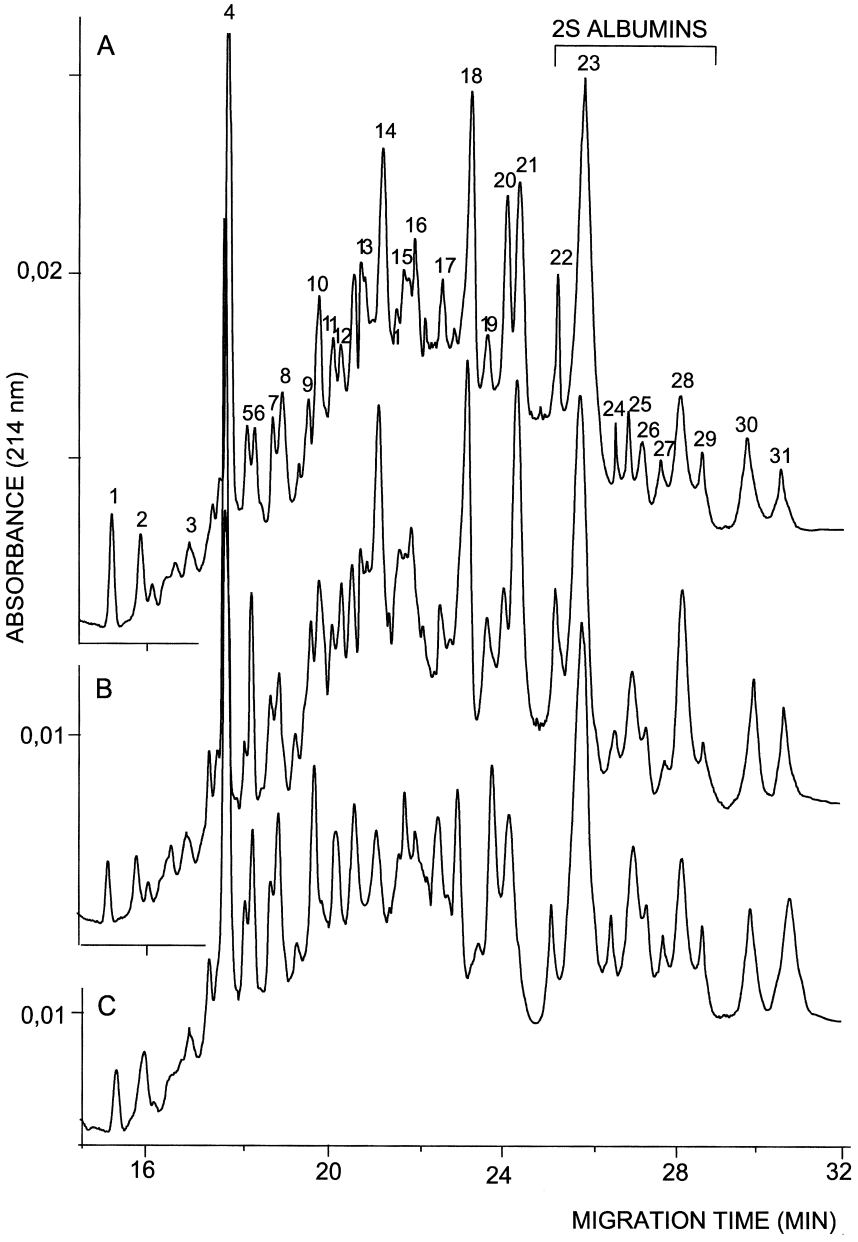

Fig. 3. Electrophoretic profiles of albumin fraction and 2S albumins from three accessions of *L. mutabilis*. Separation buffer: 20 m*M* phosphate buffer–25 m*M* sodium salt of phytic acid, pH 7.3. Voltage and capillary parameters are the same as in Fig. 1.

fraction from *L. albus* as well as the tracking dye of orange G by SDS-CE using a commercial mixture of 10% poly(ethylene oxide) (PEO) and PEG (eCAP SDS 14–200 kit) as a sieving matrix. The separation of denatured SDS albumins in the presence of reducing agent (2-mercaptoethanol) was successfully

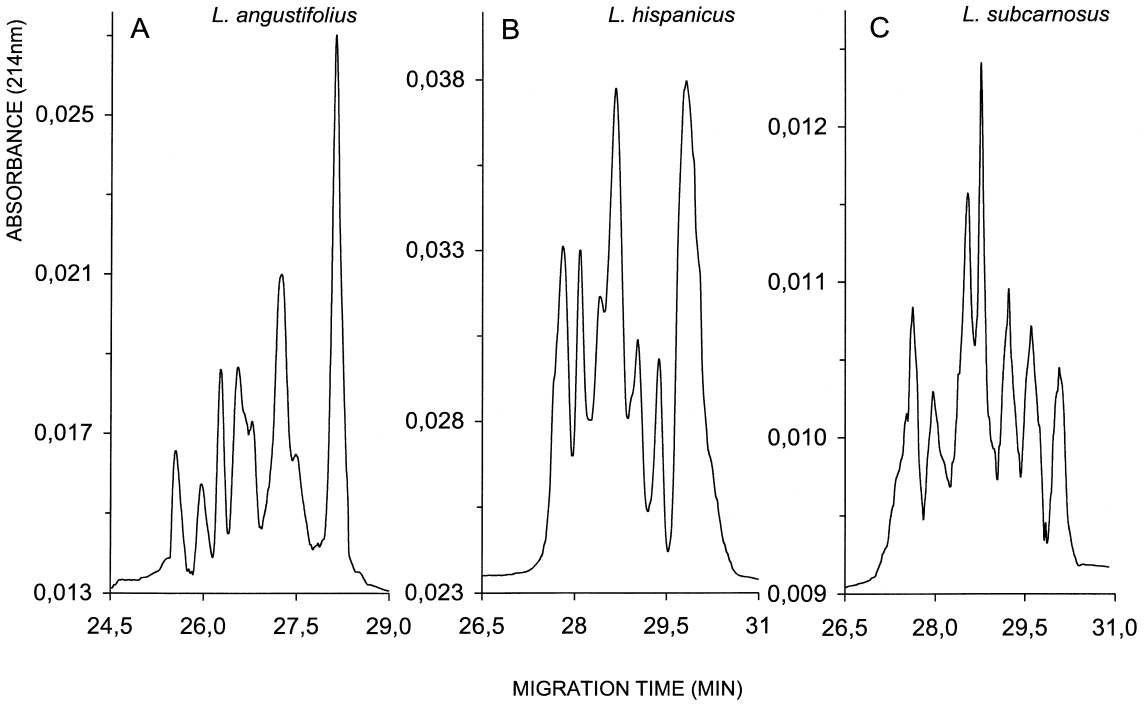

Fig. 4. Electrophoretic profiles of seed 2S albumin isoforms from different *Lupinus* species. (A) *L. angustifolius*, (B) *L. hispanicus* (C) *L. subcarnosus*. Separation buffer: 20 m*M* phosphate buffer–25 m*M* sodium salt of phytic acid, pH 7.3. Voltage and capillary parameters as in Fig. 1.

performed over 22 min. Values of molecular masses of the major albumin polypeptides below 50 000 estimated from the calibration curve for the SDS protein markers were a little higher in comparison to those estimated from traditional SDS-PAGE [1] and were equal to 26 000, 33 000, 39 000 and 48 000. However, the M_r values of high-molecular-mass albumins estimated by SDS-CE were higher in comparison to those estimated by SDS-PAGE, and were equal to 91 000 and 96 000. No marked interspecific differences were found in subunit composition of albumins with M_r above 18 000 for the studied species. Contrary to SDS-PAGE patterns, SDS-CE electropherograms revealed a considerable quantity of polypeptides with M_r below 18 000 in the albumin fraction. That provides evidence for the elution of low-molecular-mass polypeptides from polyacrylamide gels during their staining and then destaining steps. A low resolution of 2S albumin polypeptides with $M_r < 14\,000$ by SDS-CE using the PEO–PEG mixture made impossible accurate estimation of molecular masses of the both polypeptide subunits of lupin 2S albumins.

3.2.2. Effect of polymers on separation of 2S albumins

The uselessness of commercial non-gel sieving matrix for the separation of low-molecular-mass subunits of 2S albumin polypeptides in the M_r ranges of 3000–6000 and 9000–12 000 required modification of until now used procedures for separation of proteins. Without polymers (CZE separation), all polypeptides of 2S albumins and SDS peptide markers migrated within 4–6 min as overlapping peaks. Three low-viscosity polymer/buffer systems (dextran with M_r 2 000 000, PEG with M_r 35 000 or mixtures of the both polymers in high concentration Tris–borate buffer) were employed for SDS-CE

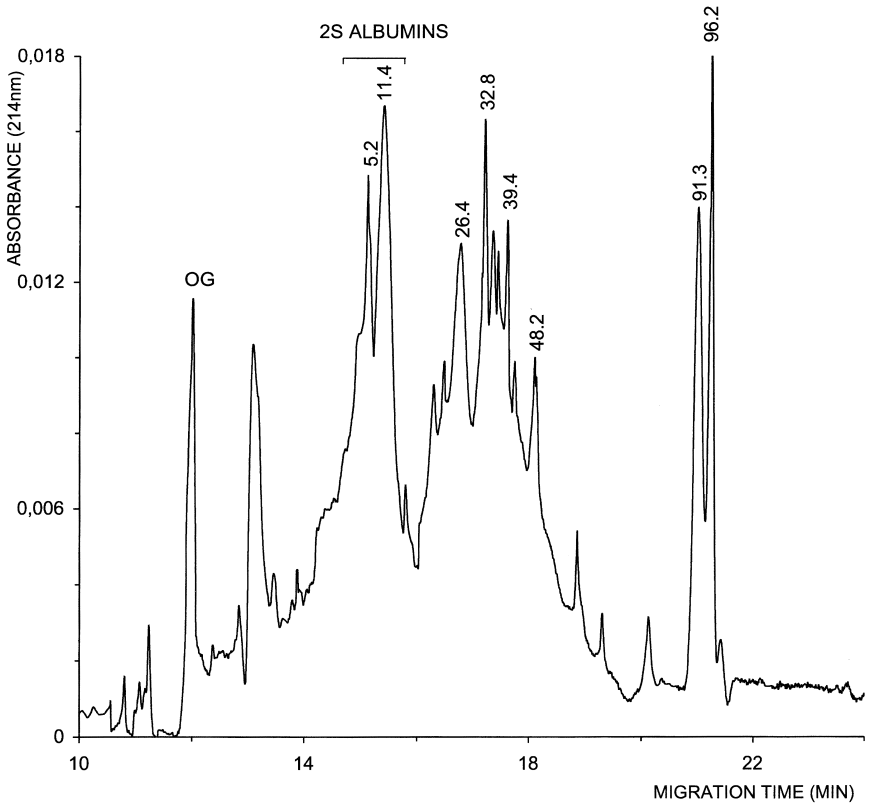

Fig. 5. SDS-CE patterns of the albumin fraction from seeds of *L. albus* separated on eCAP SDS 14–200 kit. Conditions: coated eCAP SDS capillary (47 cm×100 μm I.D.); run temperature, 20°C; field strength, 11.5 kV; negative polarity. Marker: orange G (OG).

separations of low-molecular-mass 2S albumins from *Lupinus*. These polymers and Tris–borate buffer were chosen on the basis of published data concerning their use in uncoated capillary [24–27]. Each polymer–buffer system was optimized by manipulating the polymer concentration (5–15%), buffer concentration (0.2–0.5 *M*) and through organic modifiers such as glycerol and ethylene glycol (0–15%). Only PEG (up to 10% because of its low solubility in high concentration buffer) or in the mixture with dextran, successfully used as sieving matrix for protein separations [25–27], appeared to be unsuitable for separations of small polypeptides. Fig. 6A presents SDS-CE pattern of peptide markers separated by a 10% mixture of equal amounts of PEG and dextran. Because of the low resolution obtained using this mixture, as in the separation by eCAP SDS 14–200 kit, for seven peptide markers only three peaks were revealed. In the case of using dextran as a sieving matrix, the concentration of polymer in the high molarity separation buffer was the most effective factor in the separation of polypeptides with $M_r < 12\,000$. The resolution of peaks improved with the increase of polymer concentration and molarity buffer, though a considerable increase of migration time was also observed. The obtained results for dextran were in agreement with those obtained by Zhang et al. [24]. The addition of glycerol (8–15%) to the separation buffer as a modifier improved the resolution of the separations, the shapes of the peaks and the separation efficiencies, despite dilution of the sieving polymers and longer separation time. Also during the separation of peptides by SDS-PAGE, according to Schagger and

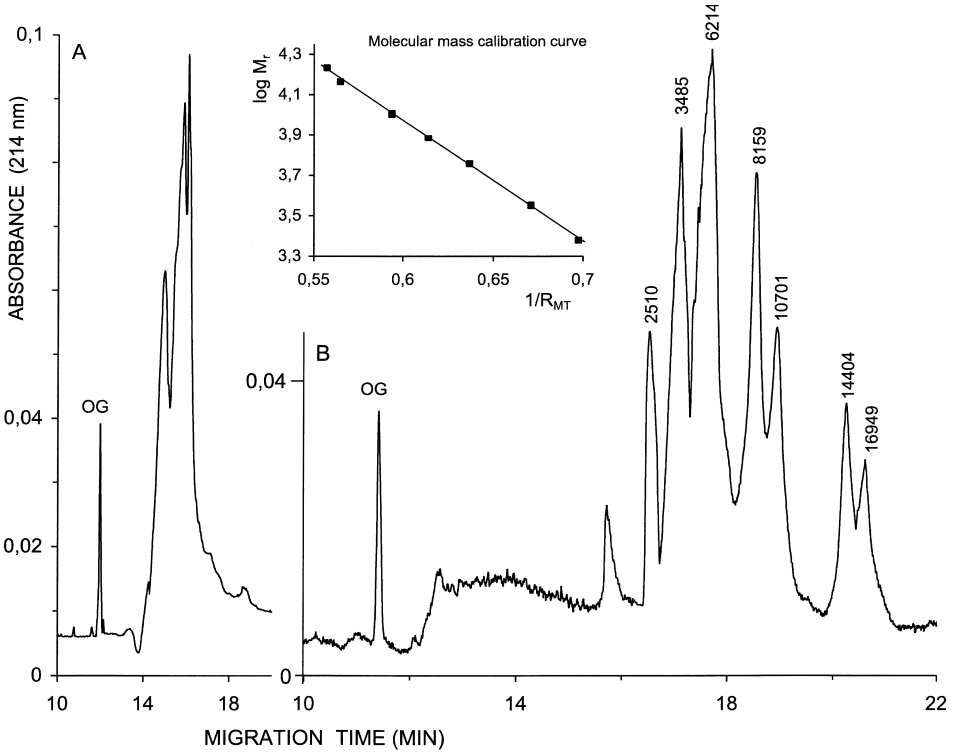

Fig. 6. Effect of polymer species as non-gel sieving matrix on separation of SDS peptide markers (M_r 2500–17 000). (A) A 10% mixture of equal amounts of poly(ethylene glycol) and dextran, (B) dextran (M_r 2 000 000). Run buffer: 0.3 *M* Tris–borate, pH 8.4, 0.1% SDS, 10% polymer, 12% glycerol. Conditions: uncoated capillary (27 cm×75 μm I.D.), run temperature, 20°C, field strength 8.5 kV, negative polarity.

Jagow [28], glycerol is used for a slower diffusion of polypeptides in the gel. However, the addition of ethylene glycol as an organic modifier improved the resolution of the separations only for polypeptide subunits with $M_r > 10\,000$. The best separations (higher separation efficiency and reproducibility of migration time) of SDS peptide markers (Fig. 6B) and reduced 2S albumins from different lupins (Fig. 7) were obtained when Tris–borate buffer concentration was 0.3 *M* and buffer contained 10% dextran and 12% glycerol. The inset in Fig. 6B shows the calibration curve for the molecular mass estimation, which was constructed by plotting the logarithms of molecular masses of SDS peptide markers versus the reciprocals of the RMT for SDS peptide markers. The increase in the concentration of dextran in the separation buffer above 12% made deformation of linear relationship log M_r as a function of 1/RMT for peptide standards with $M_r < 7000$. Similar divergences were found in the case of separation of very low-molecular-mass polypeptides in traditional polyacrylamide gel electrophoresis [24,29].

3.2.3. Migration time reproducibility

To obtain satisfactory reproducibility of migration times in SDS-CE separations of low-molecular-mass polypeptide subunits in the uncoated capillary suppression of EOF was necessary. That required appropriate preparation of the capillary (see Experimental) through its longest (for 60 min) washing using Beckman SDS separation buffer, which contained among other constituents, PEO. During separation of DNA and proteins it was revealed, that initial coating of fused-silica inner wall of the capillary by PEO solution considerably suppressed EOF [30]. On the other hand, for repeated usage of this fresh state of

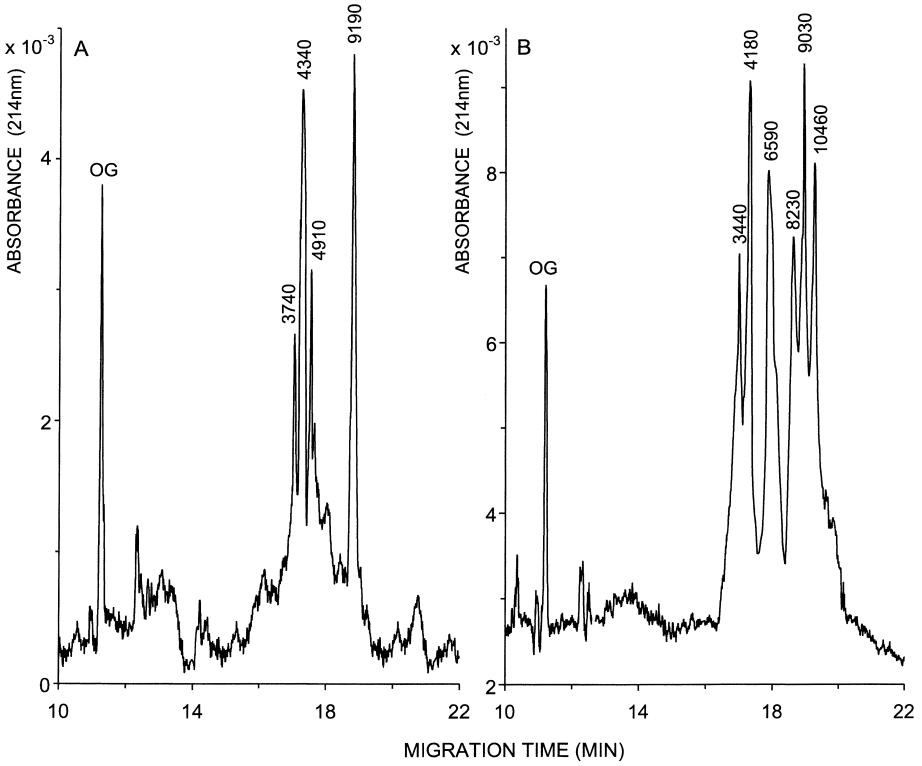

Fig. 7. SDS-CE patterns of seed 2S albumins from *L. albus* (A) and *L. cosentinii* (B) separated using 10% dextran as non-gel sieving matrix. Other conditions as in Fig. 6.

the capillary surface can be regenerated by flushing with 0.1 *M* HCl. The adsorption of PEO on the capillary surface seems to prevent the dissociation of silanol groups under higher pH. The average RSD of migration times for seven peptide markers was 0.68%. But washing capillaries with 0.1 *M* NaOH solution, as suggested by Zhang et al. [24] made additional dissociation of silanol surface to produce negative charges at pH>6.0 causing a considerable decrease in reproducibility of migration times.

To create SDS–2S albumin complexes, taking into account highly globular structure of these albumins it was necessary to use higher temperature and longer time for dissociation of these proteins. It is recommended to boil (100°C) 2S protein samples with a sample buffer containing 1% SDS and sufficient amount of reducing agent (5% 2-mercaptoethanol) for at least 15 min. When the 2S albumin samples were heated up to 90°C for 10 min, the peak efficiencies were ~35% lower as compared to those immersed in boiling water for 10 min.

3.2.4. Comparison of SDS-CE patterns of 2S albumins from different Lupinus species

SDS-CE comparative analysis of reduced 2S albumins, using dextran as a non-gel sieving matrix, covered twelve *Lupinus* accessions: three accessions of each *L. albus* and *L. mutabilis,* two accessions of each *L. angustifolius* and *L. cosentinii*, and one accession of each *L. hispanicus* subsp. *hispanicus* and *L. subcarnosus.* This analysis was performed to determine the suitability of developed SDS-CE procedure in taxonomic study. Obtained high resolution and reproducibility of migration times for polypeptide subunits of lupin albumin 2S and the possibility of a rapid and accurate estimation of molecular masses by SDS-CE have confirmed the suitability of this method. Accessions within *L. albus* and *L.*

mutabilis produced similar SDS-CE patterns of 2S albumin preparations for each species. On the other hand, the 2S albumin patterns of different taxa showed marked interspecific differences (Fig. 7). Each *Lupinus* species had a characteristic set of the both polypeptide subunits with specified molecular masses (Table 1). Sets of subunits of seed albumin 2S lupins from South America (*L. mutabilis*) and North America (*L. subcarnosus)* distinctly differ from the sets of polypeptide subunits of the remaining Euro–African lupins.

As a result of separations by SDS-CE of 2S albumin subunits, mainly with $M_r < 8000$, were found to occur in a greater number, than during the conventional SDS-PAGE method. The SDS-CE patterns of reduced 2S albumins of the investigated six *Lupinus* species revealed the presence of one to three large polypeptide subunits with M_r in the range of 8200–10 600 and two to five small subunits with M_r in the range of 3400–6800. The previous study of primary structure of 2S albumins from different *Lupinus* species displayed heterogeneity of the small subunit and also a considerable heterogeneity of the large subunit, except *L. albus* [31]. M_r values of small subunits determined by SDS-CE are very similar to those of M_r determined from amino acid sequences. However, the M_r values of large subunits determined by SDS-CE are 10% higher than M_r values estimated from amino acid sequence, whereas M_r values determined by SDS-PAGE are higher no less than 40%. In the case of small subunits ($<10\,000$) the intrinsic charge of the amino acid chain may significantly affect the overall charge of the polypeptide–SDS complex, disrupting the desirable constant mass-to-charge ratio [32]. Nevertheless precise values of molecular masses of 2S albumin subunits will be estimated from the calibration curve (log M_r as a function of the reciprocal of RMT) plotted for major polypeptide subunits of 2S albumins with known M_r estimated on the basis of sequence analysis of these proteins.

4. Conclusions

This study showed that separation of seed albumins, especially 2S albumin class proteins, from *Lupinus* species are possible in the capillary format. Both the used methods (CZE and SDS-CE) afford a faster time of analysis than slab-gel electrophoresis, and like RP-HPLC, they have a potential for analyzing both qualitative and quantitative relationships.

The peak broadening and low resolution observed in 2S albumins separation in uncoated capillary by CZE can be effectively eliminated by the addition of a sufficient amount of the sodium salt of phytic acid to the separation buffer. The use of phytic acid as a modifier buffer and an ion-pairing agent simultaneously results in pronounced differences between the various albumins, especially 2S albumin isoforms.

SDS-CE with the use of dextran as a non-gel sieving matrix in high molarity buffer can be a high-resolution and highly reproducible method for the size separation of low-molecular-mass (4000–

Table 1
Molecular masses of large (L) and small (S) polypeptide subunits of 2S albumins from different *Lupinus* species estimated from SDS-CE and SDS-PAGE patterns or from amino acid sequence analysis of the proteins

Species	M_r					
	SDS-CE		SDS-PAGE [1]		Amino acid sequences [3,31]	
	L	S	L	S	L[a]	S[a]
L. albus	9190	3700; **4340**; 4910	11 500	4500	8827	4404
L. angustifolius	**9640**; 10 310	3810; **4550**; 4880	**11 600;** 13 600	4800	9401	4597
L. hispanicus	**9070;** 9480; 10 060	**4670;** 5050	**11 400**; 13 100	4900	–	–
L. cosentinii	8230; **9030**; 10 460	3440; **4180**; 6590	9800; **11 200**; 14 400	3900; **4600**; 6600	8627	4233
L. mutabilis	**9110**; 9790; 10 420	3880; **4750**; 5060	**11 300**; 11 800; 13 900	5000	8708	4739
L. subcarnosus	**8780;** 9610; 10 630	3810; **4400;** 4860; 6770	**11 000**: 11 500; 14 300	**4600**; 6400	–	–

[a] Determined for major large and small subunits; boldface type indicates the M_r value for the major polypeptide subunit.

11 000) SDS–polypeptide complexes of 2S albumins from seeds of lupins. Contrary to SDS-PAGE method, this method makes possible recognition of heterogeneity of the both large and small polypeptide subunits of 2S albumins, together with a precise estimation of their molecular masses.

Both the discussed methods (CZE and SDS-CE) appeared to be useful in the detection of genetic polymorphism of seed albumins, especially the 2S-albumin class of proteins from *Lupinus* species. CZE and SDS-CE migration profiles of 2S albumins can be helpful in the determination of the taxonomic relationships between species of *Lupinus* and in the explanation of the still controversial phylogeny of this genus.

References

[1] B.P. Salmanowicz, J. Przybylska, Pl. Syst. Evol. 192 (1994) 67.
[2] G.G. Lilley, J. Sci. Food Agric. 37 (1986) 20.
[3] G.G. Lilley, A.S. Inglis, FEBS Lett. 195 (1986) 235.
[4] B.P. Salmanowicz, Pl. Syst. Evol. 195 (1995) 77.
[5] S.S.M. Sun, W. Zuo, H.M. Tuans, L. Xiong, Ann. NY Acad. Sci. 792 (1996) 37.
[6] B.P. Salmanowicz, Chromatographia 41 (1995) 99.
[7] S.M. Basha, J. Agric. Food Chem. 45 (1997) 400.
[8] K.H. Sutton, J.A. Bietz, J. Cereal Sci. 25 (1997) 9.
[9] S.R. Bean, G.L. Lookhart, Electrophoresis 19 (1998) 3190.
[10] P.G. Righetti, E. Olivieri, A. Viotti, Electrophoresis 19 (1998) 1738.
[11] B. Deng, J. Lu, J. Yan, J. Chromatogr. A 803 (1998) 321.
[12] H. Wätzig, M. Degenhardt, A. Kunkel, Electrophoresis 19 (1998) 2695.
[13] D. Corradinii, J. Chromatogr. B 699 (1997) 221.
[14] A. Cifuentes, M.A. Rodriquez, F.J. Garcia-Montelongo, J. Chromatogr. A 742 (1996) 257.
[15] L. Capelli, F. Forlani, F. Perini, N. Guerrieri, P. Cerletti, P.G. Righetti, Electrophoresis 19 (1998) 311.
[16] G.N. Okafo, H.C. Birrell, M. Greenaway, M. Hutan, P. Camilleri, Anal. Biochem. 219 (1994) 201.
[17] G.N. Okafo, A. Vinther, T. Kornfelt, P. Camilleri, Electrophoresis 16 (1995) 1917.
[18] J.R. Veraart, Y. Schouten, C. Gooijer, H. Lingeman, J. Chromatogr. A 768 (1997) 307.
[19] T. Kornfelt, A. Vinther, G.N. Okafo, P. Camilleri, J. Chromatogr. A 726 (1996) 223.
[20] P.L. Weegels, R.J. Hamer, J.D. Schofield, J. Cereal Sci. 22 (1995) 211.
[21] N. Parris, L. Dickey, J. Craig, Cereal Chem. 74 (1997) 766.
[22] N.J. Pollard, C.W. Wrigley, F. Bekes, A. Aumatell, F. MacRitchie, Electrophoresis 17 (1996) 221.
[23] N.J. Pollard, C.W. Wrigley, F. Bekes, F. MacRitchie, Plant Variet. Seeds 11 (1998) 187.
[24] Y. Zhang, H.K. Lee, S.F.Y. Li, J. Chromatogr. A 744 (1996) 249.
[25] M. Zhu, D.L. Hansen, S. Burd, F. Gannon, J. Chromatogr. 480 (1989) 311.
[26] K. Ganzler, K.S. Greve, A.S. Cohen, B.L. Karger, A. Guttman, N.C. Cooke, Anal. Chem. 64 (1992) 2669.
[27] T. Takagi, M.R. Karim, Electrophoresis 16 (1995) 1463.
[28] H. Schagger, G. von Jagow, Anal. Biochem. 166 (1987) 368.
[29] S.-H. Chiou, S.-H. Wu, Anal. Chim. Acta 386 (1999) 47.
[30] N. Iki, E.S. Yeung, J. Chromatogr. A 731 (1996) 272.
[31] J.K.P. Weder, B.P. Salmanowicz, P. Köhler, Z. Lebensm. Unters. Forsch. A 205 (1997) 452.
[32] A. Guttman, Electrophoresis 17 (1996) 1333.