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Phaseolin seed variability in common bean (*Phaseolus vulgaris* L.) by capillary gel electrophoresis

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Abstract. Phaseolin, the major seed storage protein of *Phaseolus vulgaris* from forty-four wild and cultivated accessions, was studied using sodium dodecyl sulphate-capillary gel electrophoresis (SDS-CGE). In total, eleven phaseolin profiles, revealing polypeptide subunit variation in the range from 45.6 kDa to 54.4 kDa, were recorded. The number of polypeptide subunits recorded in particular profiles varied from 3 to 6; in total, eight phaseolin subunits were distinguished in the examined material. Ferguson plot analysis was used to correct non-ideal behaviour of phaseolin polypeptide subunits in capillary gel electrophoresis in the presence of SDS. The obtained results are compared to electrophoretic data received by slab polyacrylamide gel electrophoresis. The SDS-CGE method appears to provide a powerful tool for disclosure of phaseolin subunit variability.

Key words: capillary electrophoresis, common bean, CGE, genetic diversity, phaseolin, storage proteins, SDS-PAGE.

Introduction

Seeds of common bean (*Phaseolus vulgaris*) are a major source of proteins for human and animal consumption in many parts of the world (PICHICO 1989). They contain two major storage proteins: phaseolin (35-50% of total proteins) and the bean lectin phytohemagglutinin (GEPTS et al. 1992). Phaseolin belongs to the 7S or vicilin class of seed storage proteins. This protein is a trimeric glycoprotein, which consists of three types of similar subunits with molecular masses ranging from 43 kDa to 54 kDa (BROWN et al. 1981, GEPTS et al. 1992). Each subunit has two potential sites of glycosylation completely or partially glycosylated (LIOI, BOLLINI 1984). Phaseolin is encoded by six to ten tightly linked genes belonging to the α and β subfamilies of the multigene family that are clustered in a single

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complex locus (TALBOT et al. 1984, LLACA, GEPTS 1996). Members of the α subfamily have tandem direct repeat sequences of 15 bp or 21bp and/or 27 bp, whereas members of the β subfamily do not have these repeats (KAMI, GEPTS 1994). The heterogeneity of the phaseolin polypeptides is a result of a series of events both at the DNA molecular level and co- and posttranslational modifications (STURM et al. 1987, FRIGERIO et al. 1998).

Extensive polyacrylamide gel electrophoretic studies of phaseolin genetic variation in seed have allowed researchers to identify patterns of multiple domestication and a reduction in genetic diversity during domestication. Phaseolin diversity has provided evidence for a subdivision of *Ph. vulgaris* into two geographic gene pools: Central American and Andean (GEPTS et al. 1986, KOENIG et al. 1990, VELASQUEZ, GEPTS 1993, GEPTS 1998). Four phaseolin protein patterns have been identified among cultivars of common bean; the 'S', 'T', 'C' and 'B' patterns named after the cultivars 'Sanilac', 'Tendergreen', 'Contender' and 'Bonello', respectively (BROWN et al. 1981, LIOI, BOLLINI 1984, GEPTS, BLISS 1988). On the other hand, a much wider range of variability in electrophoretic patterns was observed among wild-growing common bean and eleven other electrophoretic variants have been additionally detected. The knowledge of spread of phaseolin types also contributed to the characterization of *Ph. vulgaris* gene resources.

In the last decade, capillary electrophoresis (CE) has been established as a modern, powerful separation technique, which allows rapid and efficient separation of macromolecules, such as proteins. Capillary gel electrophoresis (CGE), as one with CE modes, offers a high-resolution separation method for molecular mass determination of SDS-protein mixtures and purity check. It requires only small sample and buffer volumes, and allows detection of small differences in the molecular mass of aggregated proteins (WERNER et al. 1993, GUTTMAN, NOLAN 1994, GUTTMAN et al. 1994, SALMANOWICZ 2000).

This work presents an analysis of phaseolin polypeptide subunits from seeds of *Ph. vulgaris* by capillary gel electrophoresis. The obtained CGE profiles of the analysed samples are compared with the respective SDS-PAGE patterns of phaseolin types. The observed anomalous migration of phaseolin polypeptides in the used sieving matrix [mixture of poly(ethylene oxide) and poly(ethylene glycol)] with buffer in the presence of sodium dodecyl sulphate (SDS) by capillary gel electrophoresis was studied. The used polymer network allowed the easy preparation of the various sieving matrix concentrations required for the construction of a modern-day Ferguson plot (logarithm of relative mobility versus sieving matrix gel concentration) (FERGUSON 1964). It has been shown previously that this method enables rapid and exact estimation of the molecular masses of proteins in the capillary format (WERNER et al. 1993, GUTTMAN et al. 1994).

Material and methods

Plant material

The study covered 44 wild and cultivated accessions of *Ph. vulgaris*. The accessions were obtained from the following sources: a = Centro International de Agricultura Tropical (CIAT), Cali, Columbia; b = Western Regional Plant Introduction Station, USDA-ARC, Pullman, USA; c = International Institute of Tropical Agriculture, Ibadan, Nigeria; d = Institut für Pflanzenbau und Pflanzenzüchtung, FAL, Brunswick, Germany; e = Department of Seed Science and Technology, Agricultural University, Poznań, Poland; f = National Botanical Garden of Belgium, Meise, Belgium. They were representative of the phaseolin diversity distinguished by 1D-SDS-PAGE and 2D-IEF-SDS-PAGE in the literature (TORO et al. 1990). The origin and identification of the seed samples are indicated in Table 1.

Table 1. Information on geographic origin and status of the investigated bean accessions

Geographic origin	Status	Number of accessions	Donors[a]
Europe	cultivar	6	d, e, f
South America	wild	16	a, b
	cultivar	4	a
Central America	wild	12	a, b, d
	cultivar	2	b, f
Africa	cultivar	2	c
Unknown	cultivar	2	a, d, f

[a] For key to the seed donors see: Plant material.

Chemicals

Chemicals and polypeptide markers used for slab gel electrophoresis were obtained from Serva Electrophoresis (Germany). All other chemicals and thyroglobulin were purchased from Sigma (St. Louis, MO, USA). All buffer solutions were prepared by using HPLC-grade water (Elgastat UHQ PS water purification system, Elga, England). Cyanogen bromide activated Sepharose 4B was obtained from Pharmacia.

Analytical techniques

Isolation and purification of phaseolin from seeds

Common bean flour in the first place was extracted twice with 10 mM NaCl (pH 2.4) to remove lectins and albumins. The extraction residue was extracted

twice with 0.5 M NaCl (1:10 w/v) using magnetic stirring for 1 hour at room temperature. The suspensions were centrifuged for 15 min at 10000 × g. The combined supernatant (named G1 fraction) was purified by affinity chromatography according to BONORDEN and SWANSON (1992). Briefly, porcine thyroglobulin (3 g) was coupled to cyanogen bromide activated Sepharose 4B (15 g) in a coupling buffer of 0.1 M sodium carbonate (pH 8.00) containing 0.5 M sodium chloride. The coupled resin was mixed with G1 fraction for 2 hours and transferred to a column. The phaseolin isoforms were washed away with 5 mM potassium phosphate (pH 7.2) in 1.0 M NaCl. The lectins were removed from the column with a 5 mM glycine-HCl buffer (pH 3.0).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were separated by SDS-PAGE in 15% (w/v) slab polyacrylamide gel in a discontinuous buffer system according to LAEMMLI (1970). After electrophoresis, proteins were stained with Coomassie Brillant Blue G 250. Relative molecular masses (M_rs) of polypeptide bands were estimated by SDS-PAGE with the following standard proteins: phosphorylase (M_r 97.4 kDa), bovine albumin (M_r 66 kDa), egg albumin (M_r 45 kDa) and carbonic anhydrase (M_r 29 kDa).

Sodium dodecyl sulphate-capillary gel electrophoresis (SDS-CGE)

All capillary electrophoresis experiments were performed on a Beckman P/ACE Model 2100 automated capillary electrophoresis system employing the "eCAP SDS 14-200" kit from Beckman-Coulter (USA). The analyses were performed in a coated eCAP SDS fused-silica capillary (37 cm × 100 μm) using SDS-polymer solution (Beckman) as a sieving matrix. 0.2 M Tris-CHES buffer (pH 8.8) was used for preparation of various concentrations of the sieving matrix. Samples were injected into the capillary by applying low pressure (3.5 kPa) for 60 s. The apparatus was operated in the reversed polarity mode (cathode at the injection side) and capillary temperature was 20°C.

In general, 90 μl of phaseolin sample was combined with 100 μl of buffer (0.12 M buffer TRIS-HCL, pH 6.6, containing 1% SDS), 5 μl of 2-mercaptoethanol and 5 μl of internal standard, orange G. The mixtures thus prepared were boiled for 5 min and cooled on ice for 3 min. The capillary was first washed with 0.1 M HCl, filled with separation gel buffer containing 0.1% SDS and conditioned for 5 min. The separations were performed with an applied constant voltage 300 V/cm. In all experiments the absorbance was monitored at 214 nm. To generate Ferguson plots, the phaseolin samples and standard proteins were analysed at four different concentrations of the separation gel buffer (100%, 75%, 67% and 50%).

The molecular masses of the SDS-protein mixture were estimated using the computer software MOLECULAR WEIGHT DETERMINATION, ver. 8.11 from the SYSTEM GOLD software package (Beckman-Coulter). The standard mixture of SDS molecular masses consisted of α-lactoalbumin (14.2 kDa), soybean

trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and phosphorylase b (97.4 kDa). The relative migration time (RMT) was calculated by dividing the migration time of the protein by the migration time of internal standard, orange G. The Ferguson plots were constructed by plotting the logarithm of 1/RTM of individual proteins in different polymer solutions as a function of the polymer concentration. Linear regression provides the slope, which is a negative value of the retardation coefficient (K_R). The standard curve was constructed by plotting the logarithm of M_w for standard proteins as a function of the square root of the retardation coefficients and then used to determine the molecular masses of the particular phaseolin polypeptides.

Results and discussion

Phaseolin isolated by salt extraction from the seeds of common been and then purified by affinity chromatography showed a high degree of purity. SDS-electrophoretic analysis of purified phaseolin samples (data not shown) revealed the existence of only polypeptide bands in the range from 42 kDa to 52 kDa, which conform to M_rs of phaseolin subunits (BROWN et al. 1981, GEPTS et al. 1992). The detected SDS-PAGE patterns of phaseolin polypeptide subunits from the analysed accessions were represented by eleven phaseolin types ('T', 'S', 'C', 'Sb', 'Sd', 'A', 'B', 'CH', 'H', 'J', 'M') characterized in the literature (BROWN et al. 1981, GEPTS, BLISS 1988, GEPTS et al. 1986, KOENIG et al. 1990, ZEVEN et al. 1999). Only four out of 44 examined accessions proved to be heterogeneous with respect to electrophoretic phaseolin patterns.

A typical SDS-CGE electrophoregram indicating separation of molecular mass protein standards as well as the tracking dye of orange G in the coated fused silica capillary filled with SDS-polymer solution, are shown in Figure 1. A linear relationship ($r^2 = 0.996$) existed when molecular mass of protein standards, ranging from approximately 14 to 97 kDa, was plotted against their peak migration times on a logarithm (log-log) scale. Precision of the protein peak migration times was determined by repeatedly injecting (n = 6) a standard protein solution at approximately 1 mg protein per ml separation buffer. Relative standard deviations (R.S.D.) for the peak migration times of subunits were in the range of 0.36-0.64%.

The separation of phaseolin polypeptide subunits by SDS-CGE revealed a good separation efficiency for all subunits and high repeatability in migration times (R.S.D. in the range of 0.48-1.16%). SDS-CGE profiles for most electrophoretic phaseolin types showed marked qualitative differences. Besides, significant quantitative differences were observed in polypeptide subunit composition. In total, eight polypeptide subunit peaks of phaseolin in the analysed material were distinguished. Figure 2A-D presents four major SDS-CGE peak patterns of phaseolin distinguished in cultivated common bean. They showed from 3 to 6 polypeptide subunits with similar molecular masses. This is in accordance with

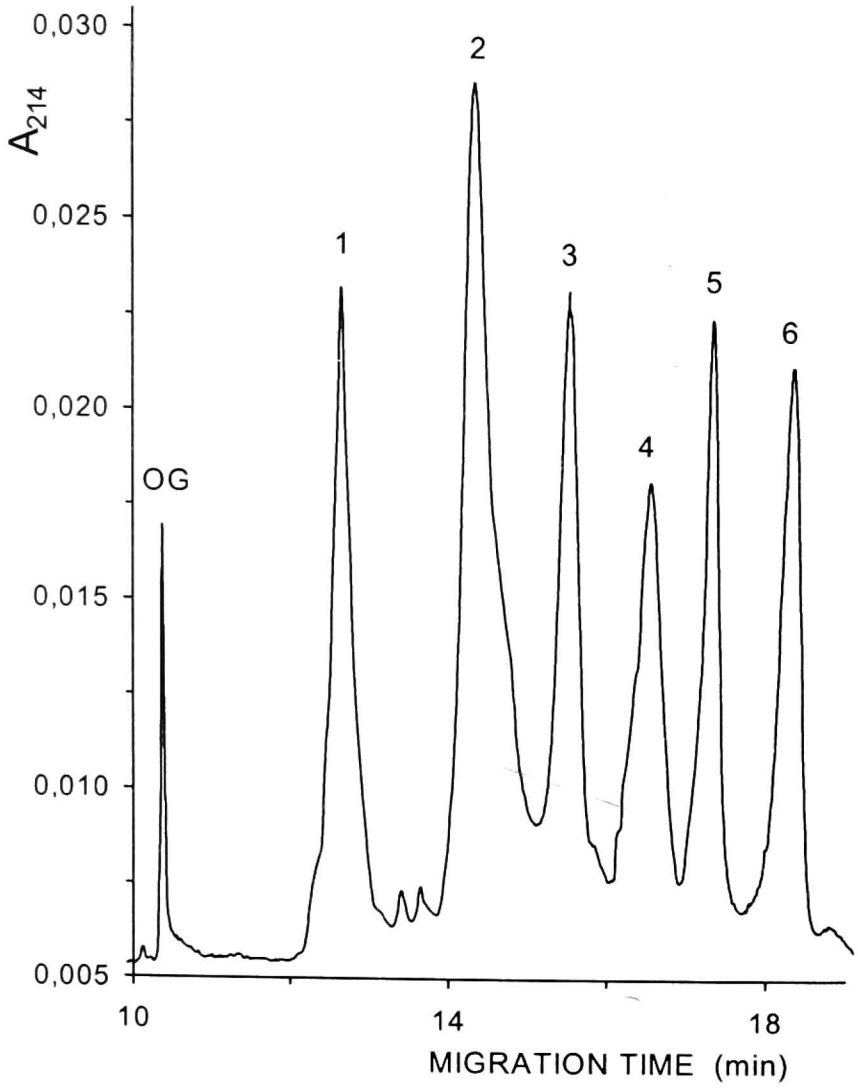

Figure 1. SDS-CGE pattern of the standard proteins. Peaks: 1 = α-lactoalbumin (14.2 kDa); 2 = soybean trypsin inhibitor (20.1 kDa); 3 = carbonic anhydrase (29 kDa); 4 = ovalbumin (45 kDa); 5 = bovine serum albumin (66 kDa); 6 = phosphorylase b (97.4 kDa). A tracking dye orange-G (OG) was added to the sample as a marker.

electrophoretic data, where 1D-SDS-PAGE has also revealed from 3 to 6 polypeptide bands in particular phaseolin variants (BROWN et al. 1981, LIOLI, BOLLINI 1984). Besides, two subtypes differing by the presence or absence of polypeptide peak with M_r 49.1 kDa can be distinguished within 'C' phaseolin type (Table 2). Wild-growing forms of common bean, as compared to cultivated material, exhibit a greater variation in electrophoretic phaseolin patterns (GEPTS et al. 1986, KOENIG et al. 1990). On the basis of SDS-CGE, six additional phaseolin profiles were recorded among wild accessions (Table 2). It is also important to note that division of SDS-CGE phaseolin profiles in the wild accessions not always fully coincided with that of SDS-PAGE patterns; some electrophoretic phaseolin types showed similarity to other phaseolin types. For example, capillary electrophoretic 'Sb' and 'Sd' phaseolin patterns resembled the 'S' phaseolin type

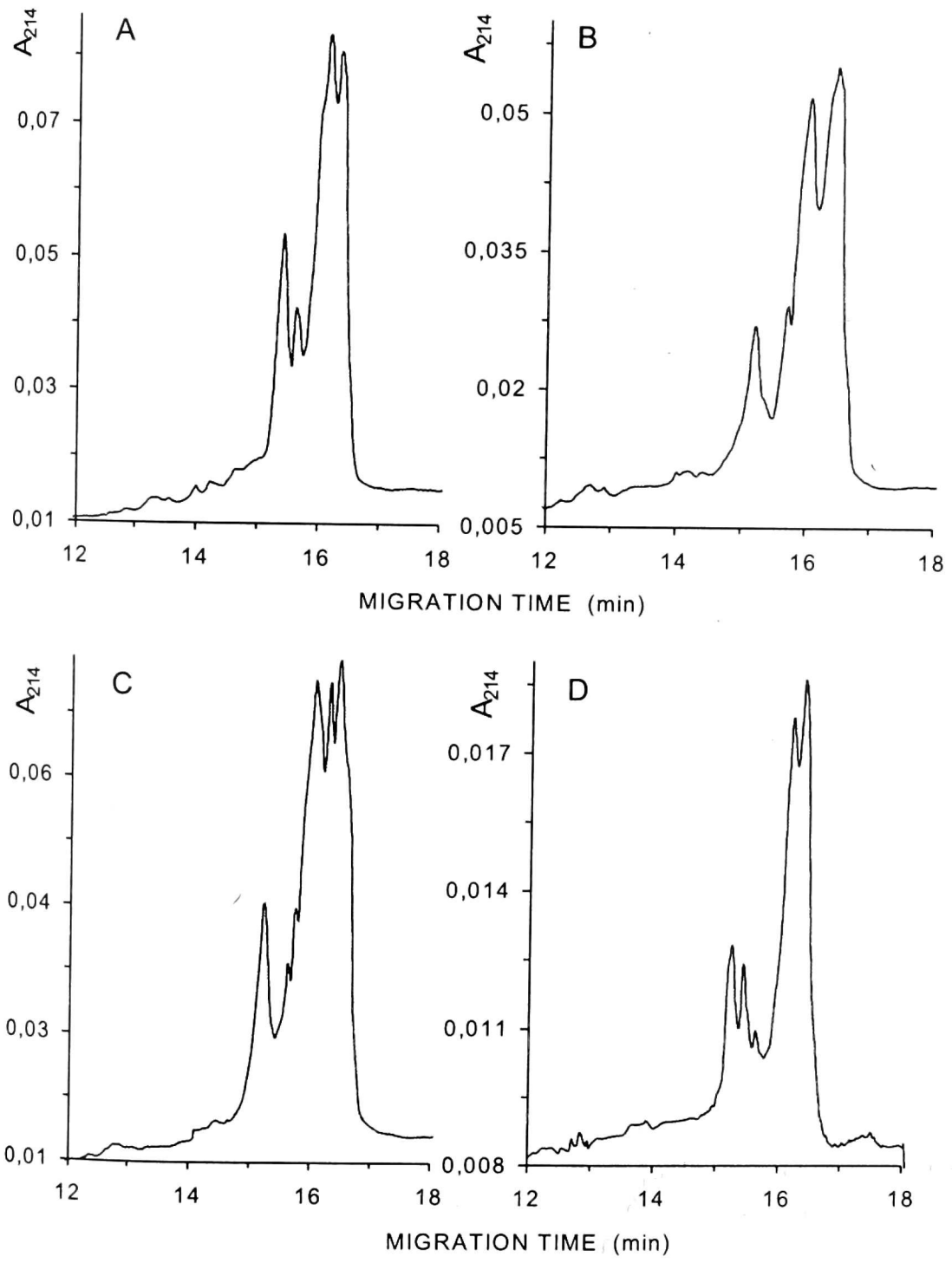

Figure 2. SDS-CGE patterns of phaseolin subunits from the cultivated common bean. A = 'S' phaseolin type, B = 'T' phaseolin type, C = 'C' phaseolin type, and D = 'B' phaseolin type.

that occurred frequently in cultivated bean, as in the case of separation by 1D-SDS-PAGE (KOENIG et al. 1990, GEPTS et al. 1992). On the other hand, electrophoretic 'CH' phaseolin pattern from two analysed accessions produced a specific SDS-CGE 'CH' pattern, while two other accessions showed similarity to the SDS-CGE 'J' pattern (Table 2). In total, eleven SDS-CGE phaseolin profiles were revealed in the analysed material.

Table 2. Identification of phaseolin types among common bean accessions by sodium dodecyl sulphate-capillary gel electrophoresis (SDS-CGE)

SDS-CGE phaseolin type	Molecular masses of phaseolin subunits[1/], kDa								No. of analysed accessions representing individual SDS-CGE phaseolin types	Electrophoretic phaseolin types by 1D-SDS-PAGE or 2D-IEF-SDS-CGE[2/]
	45.6	47.8	49.1	50.3	51.6	52.1	53.8	54.4		
1	+*			t	+*			+	10	T
2	+			t	+*		+	+*	7	C
3	+		+	t	+*		+	+*	2	C
4		+*	+			+	+*		14	S, Sb, Sd
5			t	+*				+*	1	A
6	+*	+	t			+	+*		2	B, M
7		+		+		+*	+*		1	CH
8		+				+*	+*		3	CH, J
9			+		+	+*		+*	1	H
10		+	+		+*		+*		2	M
11		+		t	+*		+	+	1	M

[1/] Molecular masses of phaseolin polypeptide subunits were estimated by Ferguson method.
[2/] Data from BROWN et al. (1981), GEPTS, BLISS (1988), GEPTS et al. (1986), KOENIG et al. (1990).
*Major polypeptide subunits, t = trace amount of phaseolin polypeptide subunit.

Computer-estimated values of molecular masses of phaseolin polypeptide subunits by SDS-CGE in 100% SDS-polymer solution were in the range 48-59 kDa. The values were higher than values determined by SDS-PAGE (BROWN et al. 1981, GEPTS, BLISS 1988, KOENIG et al. 1990) or nucleotide sequences (KAMI, GEPTS 1994, KAMI et al. 1995). The difference varied from about 10% for smaller phaseolin polypeptide subunits to about 15% for larger subunits. This unusual behaviour can be attributed to special groups on the polypeptide chains, such as carbohydrate side chains (glycosylation) or lipids (lipoproteins) (WERNER et al. 1993, GUTTMAN, NOLAN 1994). These groups may cause an increase in the migration of proteins in the capillary filled with SDS-polymer solution, as a result of an unusually large viscosity radius of macromolecules. However, in SDS-PAGE analysis of phaseolin, this anomaly was not observed. The phenomenon suggests that the mechanism of migration in SDS-CGE is slightly different from that in SDS-PAGE. In these cases it has been recommended to use an additional classical Ferguson plot to obtain molecular mass data with a higher precision (WERNER et al. 1993, GUTTMAN, NOLAN 1994).

The Ferguson plot in Figure 3 shows a comparison of the phaseolin 'J' type to standard proteins. The relative migration times used for the construction

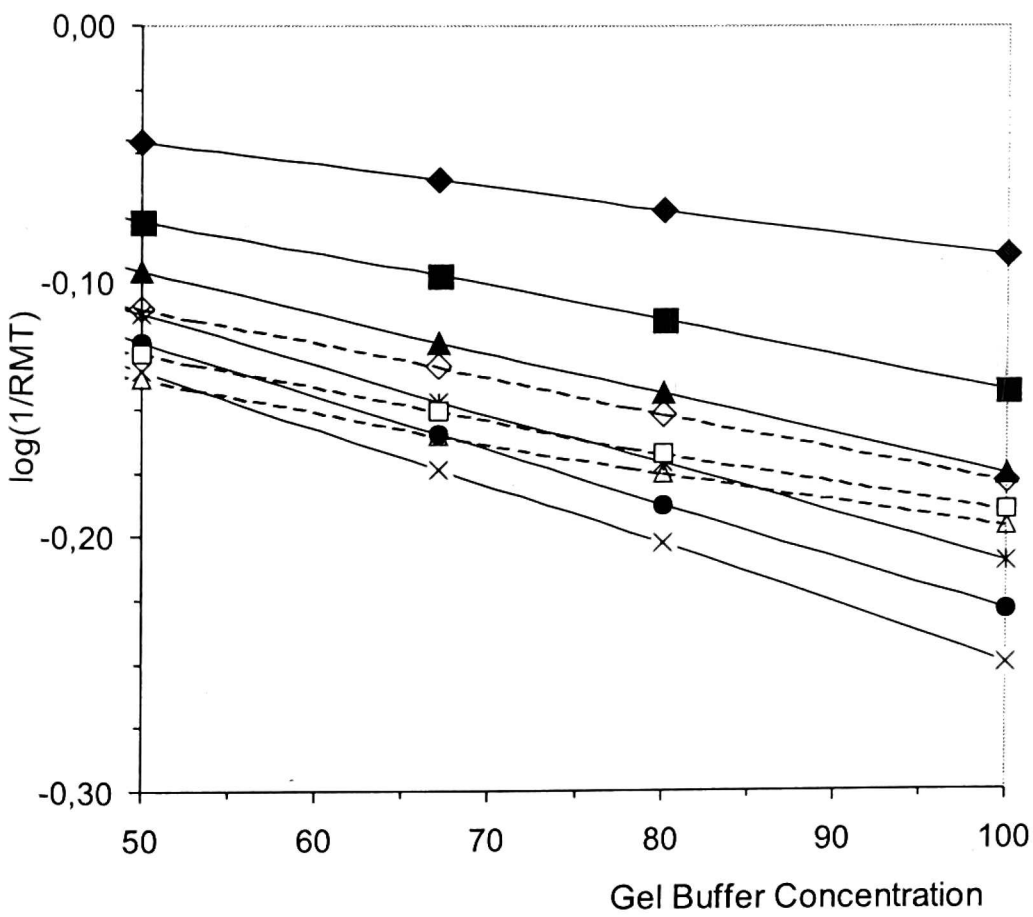

Figure 3. Ferguson plots for the standard proteins of Figure 1 and for the phaseolin subunits of 'J' phaseolin type. The logarithm of relative migrations for the standard proteins (continuous lines) and phaseolin subunits (broken lines) determined at four different concentrations of sieving matrix versus the relative concentration of the separation gel.

of the Ferguson plot represent the average relative migration times derived from three injections of each sample at four concentrations (100%, 80%, 67% and 50%) of gel separation buffer. The analysis provides a parameter called retardation coefficient (K_R), which is proportional to the square of the protein's radius, and shape, but not protein charge. It was interesting to note that on the Ferguson plot phaseolin subunits intersected the y-axis at values lower than those of the protein standards. This could indicate that the anomalous migration of the phaseolin subunits in SDS-CGC separation gel is due to high-mannose and complex oligosaccharide side chains of phaseolin subunits (STURM et al. 1987). Figure 4 shows a standard curve of the log of the molecular mass of each protein standard against the square roots of the corresponding K_R value. On this plot, the square root of the K_R's for the phaseolin polypeptide subunits of 'J' type were superimposed. The other eleven SDS-CE phaseolin types were similarly analysed, and the average molecular masses (derived from three separate Ferguson plots) are listed in Table 2. The phaseolin subunits have molecular masses in the range from 45.6 kDa to 54.4 kDa. This range is insignificantly higher than that typically seen in the conventional SDS-PAGE method (44-52 kDa) (BROWN et al. 1981, GEPTS et al. 1992) and derived from amino acid sequences (45-48 kDa) (KAMI,

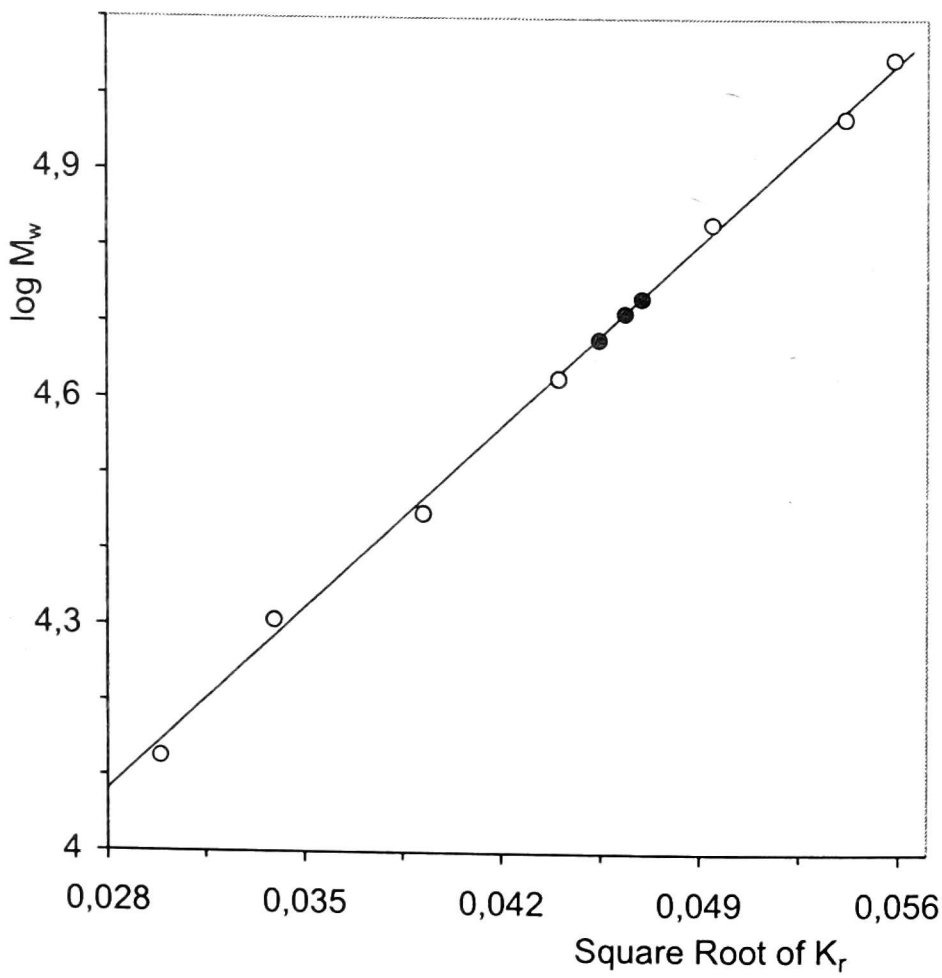

Figure 4. Standard curve for molecular mass determination. The square root of retardation coefficients determined for the standard proteins (o) and for the phaseolin subunits of 'J' phaseolin type (•) versus the logarithm of molecular masses of the standard proteins of Figure 1.

GEPTS 1994). As observed by SINGH et al. (1991), the lager seed size of the 'T', 'C', 'H', and 'A' phaseolin types was correlated with the presence of higher molecular masses phaseolin of polypeptide subunits (M_r 54.4 kDa, Table 2).

An earlier polyacrylamide gel electrophoretic study of genetic variation at the phaseolin locus in the common bean has provided evidence for subdivision of this species into two major gene pools according to the centres of origin: Central American centre and Andean centre (GEPTS et al. 1986, KOENIG et al. 1990, GEPTS 1998). Wild beans of the Central American centre show primarily the 'S' phaseolin type, whereas Andean wild beans show frequently the 'T' phaseolin type or other types depending on the accession (GEPTS et al. 1986, VELASQUEZ, GEPTS 1994). Like in SDS-PAGE, most accessions (ten of the twelve wild forms and two cultivated bean variants) from Central America analysed by SDS-CGE contained three major phaseolin subunits with M_r 47.8, 52.1 and 53.8 kDa (Table 2), which conform to major subunits revealed by SDS-PAGE in the 'S' phaseolin type. On the other hand, five wild accessions from South America have the phaseolin profiles with characteristic major subunits with M_r 51.6 and 54.4 kDa, which conform to the major subunits presented in SDS-PAGE 'T' phaseolin patterns. The occurrence of other SDS-CGE profiles, complying with the electrophoretic C-type pattern with phaseolin subunit composition intermediate between 'S' and 'T' types and other phaseolin types conforming to electrophoretic (1D-SDS-PAGE or 2D-IEF-SDS-PAGE) 'A', 'B', 'J', 'H', and 'M' phaseolin types, has also been found (Table 2). Most of European and African cultivated bean variants exhibited two SDS-CGE phaseolin profiles with major subunits with 45.6, 51.6 and 54.4 kDa, conforming to electrophoretic 'T' and 'C' types. This supports the Andean origin of most bean cultivars on these continents and agrees with results obtained by traditional electrophoretic analysis (GEPTS, BLISS 1988, LIMONGELLI et al. 1996, GEPTS 1998, ZEVEN et al. 1999).

Concluding remarks

The presented data indicate that capillary gel electrophoresis seed phaseolin profiles may be useful markers in studies of genetic diversity in *Ph. vulgaris*. The used method affords a faster time of analysis (20 min) than slab-gel electrophoresis and has a potential for analysing both qualitative and quantitative relationships. The use of Ferguson plots yields better molecular mass estimates for the phaseolin subunits than does SDS-CGE at a single gel concentration. However, the SDS-CGE method, like 1D-SDS-PAGE, does not permit to distinguish phaseolin subunits slightly differing in their amino acid composition and to detect amounts of posttranslational modifications (carbohydrates). To reveal full phaseolin variability in common bean seeds it is necessary to perform an additional analysis of this class of protein by capillary isoelectrofocusing.

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