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COMPARATIVE STUDY OF SEED PROTEINS IN THE GENUS *PISUM* VIII. FURTHER INVESTIGATION ON VARIATION IN ELECTROPHORETIC ALBUMIN PATTERNS[1]

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Summary. This paper presents further data on variation in electrophoretic seed albumin patterns (EP patterns) in *Pisum*. A screening of more than 250 *Pisum* accessions for EP patterns resulted in discovering new patterns and provided additional data on distribution of the previously distinguished patterns.

Albumins of cotyledons of ungerminated seeds of *Pisum* accessions representing all the EP patterns distinguished so far in *Pisum* (EP I - EP IX) were fractionated on Sephadex G 100 and the obtained peaks were analysed electrophoretically. Like in the case of the earlier investigated EP I - EP V, the fraction with MW ca 40 000 (S2 fraction) proved to be responsible for the distinction of EP VII - EP IX. The elution profile of the *Pisum* accession representing EP VI — without major characteristic bands — showed no peak corresponding to MW ca 40 000.

A comparative electrophoretic analysis of the S2 fraction revealed a variation in the electrophoretic mobility of some of the characteristic bands considered homologous in the screening analysis. SDS gels of the S2 fraction showed one or two polypeptide components with approximate MW 23 000.

In the previous investigations comprising 37 cultivars and breeding lines of *P. sativum* two distinct patterns of seed albumins were revealed. The characteristic

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patterns, designated A and B, appeared to be controlled by two allelic genes (Przybylska et al., 1973). On extending the electrophoretic comparative analysis of seed proteins to comprise also more distant *Pisum* forms, five albumin patterns, designated EP I — EP V, were distinguished (Przybylska et al., 1977); pattern EP I corresponded to that found previously in some cultivars and designated as pattern B. The patterns EP I, EP II and the intermediate pattern EP I/II were found in 38 related *Pisum* forms classified by Lehmann (1954) as *P. elatius*, *P. humile* (=*P. syriacum*) and *P. sativum*. The EP III was found in a single line, W1490, called *P. cinereum* and classified by Lehmann as *P. sativum* var. *nigro-umbilicatum* Gov. (personal communication), obtained from the Pisum Gene Bank at Weibullsholm, Sweden. The EP IV was observed only in six accessions of *P. abyssinicum* and the EP V only in three accessions of *P. fulvum*. The patterns EP I and EP III were found to be determined by two alleles at one locus, weakly linked with the gene *pl* in chromosome 6 (Blixt et al., 1980).

Differences between the distinguished patterns were due to the occurrence of several well-defined protein bands, designated **a-f**, which under the applied conditions of electrophoretic analysis had the following *Rp* values: **a** — 0.42; **b** — 0.50; **c** — 0.56; **d** — 0.64; **e** — 0.26 and **f** — 0.34. Albumins corresponding to the characteristic bands, called "specific albumins", were recovered in the sephadex fraction with MW about 40 000 (S2 fraction); when dissociated with SDS in the presence of 2-mercaptoethanol they produced subunits with approximate MW 23 000 (Jakubek and Przybylska, 1979).

This paper presents further data on variation in electrophoretic seed albumin patterns in *Pisum*.

MATERIAL AND METHODS

PLANT MATERIAL

The material for this study was 257 *Pisum* accessions obtained from: 1) N. I. Vavilov All-Union Research Institute of Plant Industry (VIR), Leningrad, USSR (160 accessions); 2) Pisum Gene Bank at Weibullsholm Plant Breeding Institute, Landskrona, Sweden (90 accessions); 3) "Zentralinstitut für Genetik und Kulturpflanzenforschung" of German Academy of Sciences, Gatersleben, GDR (4 accessions); 4) University of Melbourne, Australia (*P. abyssinicum* MU 38); 5) Hebrew University of Jerusalem, Israel (*P. fulvum*, Population 1), and 6) VIR via Gatersleben Collection (*P. sativum* var. *fusco-umbilicatum* Gov., PIS 1395=VIR 284).

One of the 160 accessions obtained from VIR was labelled *P. sativum* subsp. *syriacum* (=*P. humile*) VIR 2521[1]. The remaining ones were labelled *P. sativum* subsp. *sativum*, *P. sativum* subsp. *transcaucasicum* and transgressive forms between the two subspecies.

Accessions from the Weibullsholm Collection (W) were labelled *P. sativum* subsp.

[1] The accession was provided by Dr Eva Klozova from the Institute of Experimental Botany, Czechoslovak Academy of Sciences, Praha, Czechoslovakia.

sativum (78 accessions), *P. sativum* subsp. *transcaucasicum* (W 1951), *P. tibetanicum* (W 611, pea from Ost-Thibet), *P. elatius* (W 1447), *P. humile* (W 936), *P. fulvum* (W 1256) and *P. abyssinicum* (7 accessions).

Accessions obtained from the Gatersleben Collection represent *P. sativum* subsp. *sativum* (3 accessions) and *P. elatius* (Gat. 255).

Most of the accessions from VIR originated from Caucasia and from the region of Saratov. Samples from the Pisum Gene Bank at Weibullsholm and from other sources were of different origin: South Europe, Africa, Asia Minor and Asia.

For the analyses, sometimes original samples were used, sometimes plant material reproduced at the Institute of Plant Genetics in Poznań, sometimes both. In the screening analysis 2 or 3 seeds were examined separately for each accession; if the seeds produced different patterns, additional 3-5 individuals were examined. For accessions having patterns EP III or EP VII 15-20 individuals were analysed.

ANALYTICAL TECHNIQUES

Extraction procedures

Crude protein extracts, with large proportions of albumins, were obtained as described previously from cotyledons of dry seeds (Przybylska et al., 1977).

Albumin extracts were also obtained as described previously (Jakubek and Przybylska, 1979) but the deionized water used for the protein extraction was adjusted to pH 7.0 with 0.1 M NaOH.

Gel filtration

Fractionation of total albumins was carried out on Sephadex G 100, as described in the previous paper (Jakubek and Przybylska, 1979). Protein content in the sephadex fractions was estimated on VSU-2P spectrophotometer. Aliquots of the top fractions of the chromatographic peaks were electrophoretically checked for the presence of "specific albumins", concentrated by dialysis in saturated solution of sucrose, and stored at —20°C

Polyacrylamide gel electrophoresis (PAGE)

Disc electrophoresis. — The electrophoretic separation of crude protein extracts was conducted in the discontinuous buffer system according to Davis (1964); as an electrode buffer 0.005 M tris-glycine buffer, pH 8.5, was used. The acrylamide (AA) concentration in separation gels was 7.5 per cent and the ratio of AA to methylene-bisacrylamide (Bis) was 37:1. The amounts of crude protein extracts loaded on gels corresponded to 4 mg of meal.

Electrophoresis in slab gels. — Electrophoresis was carried out in the discontinuous buffer system according to Davis (1964); as an electrode buffer 0.01 M

tris-glycine buffer, pH 8.5, was used. The AA concentration in separation gel was 10 per cent and the ratio of AA:Bis was 15:1. Electrophoresis was carried out at 15 V/cm for 1 hr, then at 25 V/cm for about 1.5 hr; after the dye marker (bromophenol blue) had reached the anodic edge of the gel, electrophoresis was continued for 0.5 hr at 25 V/cm. The amounts of sephadex fractions layered on gels contained about 10 μg of protein.

Protein bands on disc or slab gels were detected with 0.1 per cent Coomassie Brillant Blue G 250 in 12.5 per cent trichloroacetic acid (Diezel et al., 1972).

Sodium dodecyl sulphate electrophoresis (SDS-PAGE). — The applied procedure was essentially the same as described in the previous paper (Jakubek and Przybylska, 1979). Electrophoresis was performed in 12 per cent polyacrylamide slab gels in the discontinuous buffer system according to Laemmli (1970); the ratio of AA:Bis was 37:1. The gels were stained with 0.1 per cent Coomassie Brillant Blue G 250 in a methanol — acetic acid — water (5:1:4, by volume) for 1 hr at 60°C according to Lambin et al. (1976). The following standard proteins were used: bovine serum albumin (MW 67 000), ovalbumin (MW 45 000), chymotrypsinogen A (MW 25 000), and lysozyme (MW 14 000), obtained from "Serva".

RESULTS AND DISCUSSION

As reported in the previous paper, distinction of the characteristic electrophoretic seed albumin patterns (EP patterns) in *Pisum* may be based on the analysis of the total albumin fraction or the crude protein extract containing a large proportion of albumins (Przybylska et al., 1977). However, in the analysis, slow-moving characteristic bands sometimes may be partly masked by other proteins. Thus, electrophoretic examination of albumin fractions with MW about 40 000 (S2 fraction), containing "specific albumins", revealed the characteristic f-band in *P. fulvum*, which was overlooked in the analysis of crude protein extracts and total albumins (Jakubek and Przybylska, 1979). Therefore, data provided by S2 fraction analysis may be of some help in interpreting and classifying electrophoretic patterns from crude protein extract analysis, used for screening.

In this work, the screening by means of crude protein extract analysis covered 250 accessions so far not examined for EP patterns and two accessions (*P. humile* VIR 2521 and *P. fulvum*, Population 1), which were found to have "deviating" patterns (Przybylska et al., 1977). While classifying the electrophoretic patterns obtained the presence of the characteristic bands a-f (see introduction) was taken into account. The gels were also examined for the occurrence of additional characteristic bands. The results from the screening analysis are described in Section 1.

Seed albumins of the accessions representing the so far distinguished patterns EP I — EP IX were fractionated by gel filtration on sephadex. The S2 fractions were subjected to a comparative electrophoretic analysis. The results of the S2 fraction analysis, which covered both native and SDS-treated albumins, are presented in Section 2.

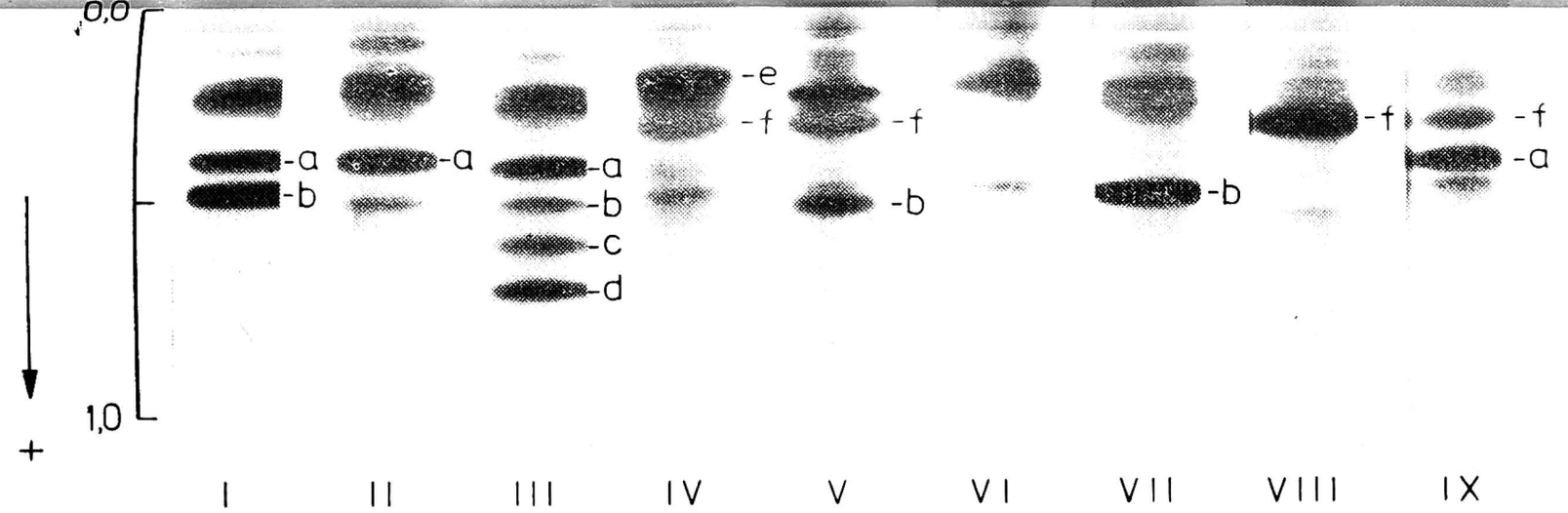

Fig. 1. Disc gels showing electrophoretic patterns EP I – EP IX

The patterns are produced by crude protein extracts of the following *Pisum* accessions; *P. sativum* W 110 (EP I); *P. humile* W 936 (EP II); *P. sativum* W 1490 (EP III); *P. abyssinicum* W 808 (EP IV); *P. fulvum* W 1256 (EP V); *P. sativum* W 1973 (EP VI); *P. sativum* VIR 1987 (EP VII); *P. humile* VIR 2521 (EP VIII); *P. elatius* Gat. 255 (EP IX).
The characteristic bands are indicated with letters a-f

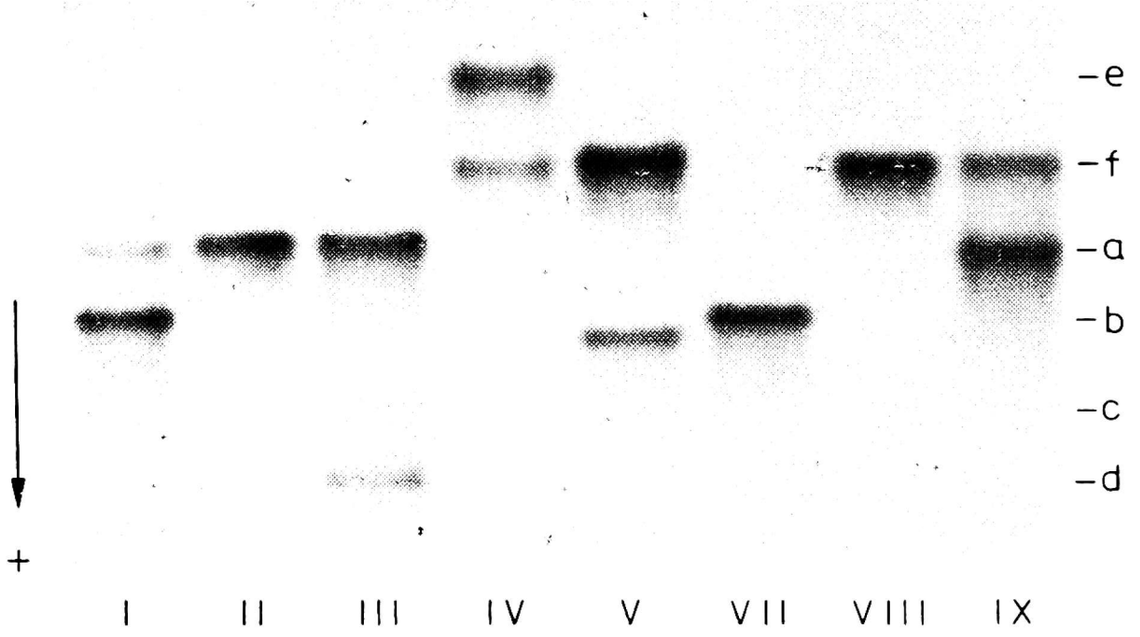

Fig. 2. Slab gel showing electrophoretic patterns of the S2 fraction from the *Pisum* accessions representing patterns EP I – EP V and EP VII – EP IX.

Note that the accession with EP VI is not included as having no S2 fraction (see the text). The characteristic bands are indicated with letters a-f (compare with Fig. 1)

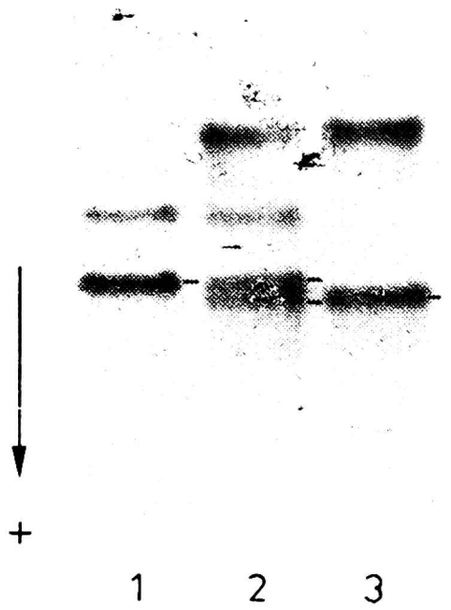

Fig. 3. Distinction of bands $\mathbf{b}_1$ and $\mathbf{b}_2$ by their electrophoretic mobility

Tracks 1 and 3: fractions S2 from *P. sativum* W 110 ($\mathbf{b}_2$) and *P. fulvum* W 1256 ($\mathbf{b}_1$), respectively. Track 2: mixture of the S2 fractions from the two accessions. Bands $\mathbf{b}_1$ and $\mathbf{b}_2$ are marked with dots

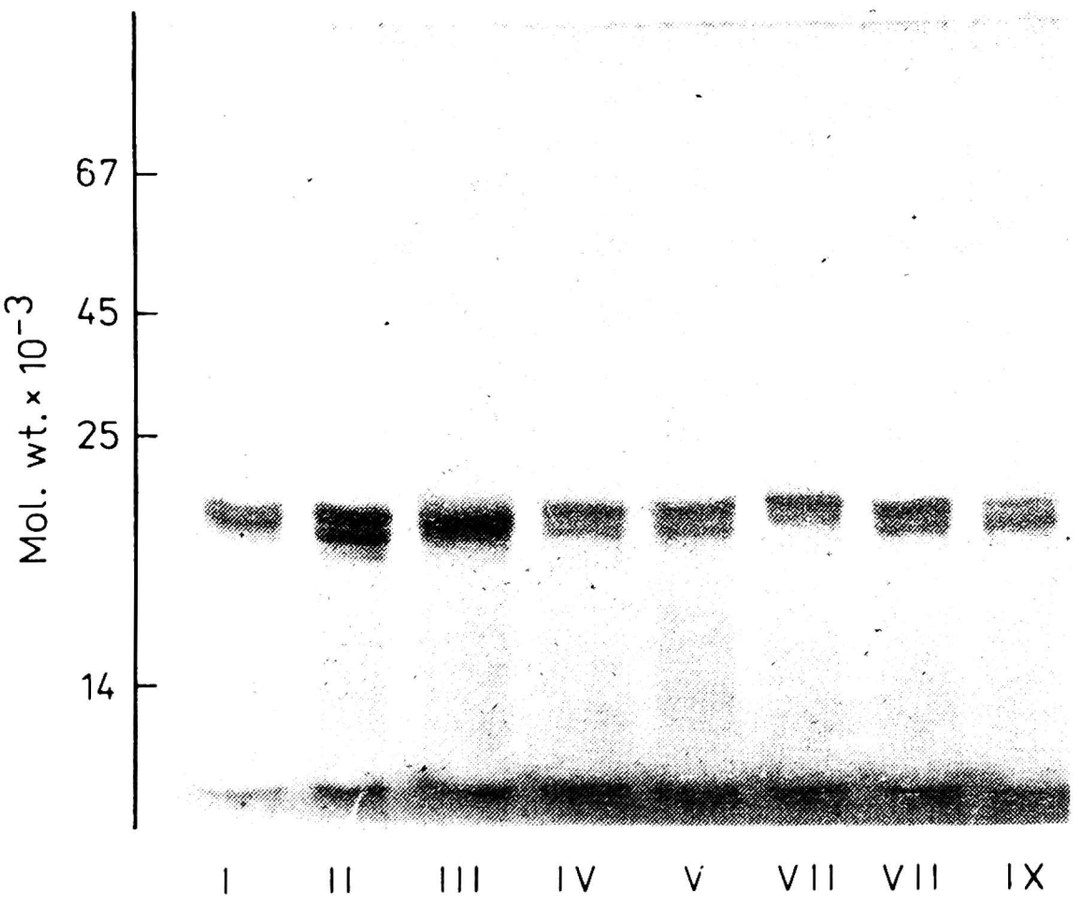

Fig. 4. SDS-gel (reducing conditions) showing the polypeptide patterns of the S2 fraction from the *Pisum* accessions representing EP I — EP V and EP VII — EP IX

1. SCREENING ANALYSIS FOR EP PATTERNS

A screening analysis of crude protein extracts was performed by means of disc electrophoresis. The electrophoresis was carried out until the dye marker migrated about 40 mm into the separation gel. The electrophoretic patterns obtained were examined using as references patterns of crude protein extracts representing the previously distinguished EP I — EP V (Jakubek and Przybylska, 1979; Przybylska et al., 1977) and pattern A (Przybylska et al., 1973) designated now as EP VI. If a given pattern could not be assigned to any of the previously distinguished classes, a new EP pattern was defined. In the investigated material, no new characteristic bands were observed. The new patterns distinguished were due to differences in the number and combination of the previously known bands **a-f**. The so far distinguished patterns EP I — EP IX show the following characteristic bands or sets of bands:

EP I — **a**, **b** (band **b** more intensive)
EP II — **a**
EP III — **a**, **b**, **c**, **d** (band **b** may be split)
EP IV — **e**, **f**
EP V — **b**, **f** (band **f** may be accompanied by a relatively intense band migrating a little slower)
EP VI — no characteristic bands
EP VII — **b**
EP VIII — **f**
EP IX — **a**, **f**.

The patterns are presented in Fig. 1.

In the present investigation eight EP patterns, EP I — EP IV and EP VI — EP IX, were represented. No accession representing pattern V was included in the present material.

Five EP patterns were found within *P. sativum* accessions: EP I, EP II, EP III, EP VI and EP VII. The EP I occurred commonly (195 accessions), which is consistent with the earlier data. The EP II was found in 8 accessions. The EP III, previously reported for a single accession called *P. cinereum* W 1490, was found in 7 out of 30 accessions originating from the Saratov region and obtained from VIR. The EP VI was found in 14 accessions. The EP VII was observed in 59 out of 124 accessions originating from Caucasia and obtained from VIR. Some accessions proved to be heterogenous with respect to EP pattern; e.g., in the accessions with EP III and EP VII (for which up to 20 individual seeds were examined) individuals with EP I were often found.

Of 2 accessions representing *P. elatius*, the accession *P. elatius* W 1447 had EP II, and *P. elatius* Gat. 255 had a new pattern, EP IX.

The pattern of *P. humile* VIR 2521, previously reported as deviating pattern has now, after the analysis of the S2 fraction, been redefined as a new pattern, EP VIII.

The presently investigated accessions of *P. abyssinicum*, like those examined earlier, all showed EP IV.

The pattern of *P. fulvum*, Population 1, has been previously reported as deviating. It was similar to EP II. It should be emphasized that other *P. fulvum* accessions examined so far had EP V. Analysis of the S2 fraction of *P. fulvum*, Population 1 will be performed.

It is worth noticing, that out of the six characteristic bands (a-f) responsible for differentiation of the distinguished EP patterns, the bands e and f were not observed in *P. sativum*. Only in *P. abyssinicum* accessions both bands were found. Band f alone was found in a single accession of *P. humile* (VIR 2521) and together with band a in a single accession of *P. elatius* (Gat. 255). Band f occurs also in *P. fulvum* accessions, except Population 1.

2. ELECTROPHORETIC EXAMINATION OF THE ALBUMIN FRACTION S2

Gel filtration profiles of seed albumins from accessions representing EP VI – EP IX showed 4-5 peaks. As in the case of EP I – EP V (Jakubek and Przybylska 1979), the peak corresponding to MW ca 40 000 (S2 fraction) proved to be the one responsible for the patterns EP VII – EP IX. In the accession having EP VI, with no well-defined characteristic bands, no distinct peak corresponding to MW ca 40 000 was observed. In view of the above, this accession is taken meanwhile as having no fraction S2.

Electrophoretic patterns of fraction S2 from accessions representing EP I – EP V and EP VII – EP IX, are presented in Fig. 2. When comparing Figures 1 and 2 one may notice that the characteristic bands recorded in the analysis of crude protein extracts are recovered in the S2 fraction and are here relatively well separated from other proteins. The only exception is band b from *P. sativum* W 1490 (=*P. cinereum*) representing EP III. Already in the previous investigations it was found that the band b from the mentioned accession was not present on electropherogram of fraction S2. It should be pointed out, however, that accessions representing other b-containing EP patterns (EP I, EP V, EP VII) showed the b-band on electropherograms of the chromatographic fraction.

The above observation indicated that some bands, which were considered homologous represent different proteins. It was expected that more careful examination of the S2 fraction will reveal further differences between such apparently homologous bands. It should be mentioned here that with the disc electrophoretic technique, used in the previous investigations and in the presently reported screening analysis, it is difficult to detect slight differences in electrophoretic mobility. To detect possible differences between the bands considered homologous the conditions of electrophoretic analysis were changed. Slab gels were used instead of disc gels, and the concentration of the separation gel was increased from 7.5 per cent to 10 per cent. The use of slab gels made possible side-by-side comparison of the investigated samples. The increased gel concentration resulted in a somewhat better resolution of the characteristic bands.

Electrophoretic data, obtained from the analysis of S2 fractions, show that the

band **b** from *P. sativum* W 110 (EP I) migrates slower than the band **b** from *P. fulvum* W 1256 (EP V). The difference was quite evident when the mixture of the respective fractions was subjected to electrophoresis (Fig. 3). Consequently, the **b**-band from *P. fulvum* W 1256 has been called $\mathbf{b_1}$ and **b**-band from *P. sativum* W 110 — $\mathbf{b_2}$. The **b**-band from *P. sativum* VIR 1987 (EP VII) corresponds to $\mathbf{b_2}$.

Also in the case of the bands **a** and **f** slight differences in the electrophoretic mobility were noticed but could not be proved by the analysis of the respective mixtures. E.g., the band **a** from *P. elatius* Gat. 255 (EP IX) seems to move faster than **a**-bands from *P. sativum* W 110 (EP I), *P. humile* W 936 (EP II) and *P. sativum* W 1490 (EP III).

Figure 4 presents SDS electrophoretic patterns of the S2 fraction from the *Pisum* accessions representing EP I — EP V and EP VII — EP IX. In contrast to the greatly differentiated electrophoretic patterns of native proteins, the polypeptide spectra are similar; they show one or two components with MW ca 23 000. The two polypeptides observed in several *Pisum* accessions seem to correspond to two non-storage protein components with approximate MW 22 000 reported by Spencer et al. (1980) for the cotyledons of *P. sativum* cv. Greenfeast.

Detection of specific albumins in *Pisum* raised the question of their physiological function. Accumulation of relatively large amounts of the components in mature seeds suggested their possible role in nitrogen storage. However, results obtained in this laboratory seem to make such a possibility much less likely. Specific albumins were shown to be located in cotyledon cell cytoplasm, not in protein bodies (Jakubek, 1982), and to persist during 3 weeks of germination (Jakubek and Przybylska, 1983).

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BADANIA PORÓWNAWCZE BIAŁEK NASION RODZAJU *PISUM* VIII. DALSZE BADANIA NAD ZMIENNOŚCIĄ ELEKTROFORETYCZNEGO OBRAZU ALBUMIN

Streszczenie

Prezentowana praca przedstawia dalsze badania nad zmiennością elektroforetycznego obrazu białek albuminowych nasion u rodzaju *Pisum*. W wyniku „screeningowej" analizy ponad 250 form *Pisum* wyodrębniono nowe typy elektroforetycznego obrazu albumin oraz uzyskano dodatkowe informacje o rozpowszechnieniu wcześniej wyróżnionych typów.

Albuminy liścieni dojrzałych nasion 9 form *Pisum*, reprezentujących wyróżnione dotychczas typy elektroforetyczne (EP I — EP IX), frakcjonowano na żelu Sephadex G 100, a otrzymane frakcje sefadeksowe analizowano elektroforetycznie. Jak stwierdzono wcześniej dla typów EP I — EP V, zróżnicowanie typów EP VII — EP IX wynika ze specyficzności frakcji o ciężarze cząsteczkowym około 40 000 (frakcja S2). Profil elucji formy *Pisum* reprezentującej EP VI, bez dobrze zdefiniowanych charakterystycznych prążków, nie wykazał piku odpowiadającego ciężarowi cząsteczkowemu około 40 000.

Elektroforetyczna analiza porównawcza frakcji S2 ujawniła różnice w ruchliwości elektroforetycznej niektórych charakterystycznych prążków, które — w świetle wyników analizy „screeningowej" — uważano za homologiczne. Porównawcza elektroforetyczna analiza białek frakcji S2 w obecności siarczanu dodecylosodowego wykazała występowanie jednej lub dwóch podjednostek polipeptydowych o ciężarze cząsteczkowym około 23 000.

СРАВНИТЕЛЬНЫЕ ИССЛЕДОВАНИЯ БЕЛКОВ СЕМЯН РОДА *PISUM* VIII. ДАЛЬНЕЙШИЕ ИССЛЕДОВАНИЯ ИЗМЕНЧИВОСТИ ЭЛЕКТРОФОРЕТИЧЕСКОГО СПЕКТРА АЛЬБУМИН

Резюме

В настоящей работе представлены дальнейшие исследования изменчивости электрофоретического спектра альбуминовых белков семян у рода *Pisum*. В результате анализа „screening" около 250 форм *Pisum* были выделены новые типы электрофоретического спектра альбумин, а также получена дополнительная информация о распространении ранее выделенных типов.

Альбумины семядолей зрелых семян 9 форм *Pisum*, представляющих выделенные до сих пор электрофоретические типы (EP I — EP IX) фракционировались на геле Sephadex G 100, а полученные сефадоксовые фракции подвергались электрофоретическому анализу. Как обнаружено ранее для типов EP I — EP V, дифференциация типов EP VII — EP IX является результатом специфичности фракции с молекулярной массой около 40 000 (фракция S2). Профиль элюции формы *Pisum*, представляющей EP VI, без хорошо определенных характерных полос, не обнаружил пика соответствующего молекулярной массе около 40 000.

Сравнительный электрофоретический анализ фракции S2 обнаружил разницу в электрофоретической мобильности некоторых характерных полос, которые в свете результатов анализа „screening" приняты были за гомологические. Сравнительный электрофоретический анализ белков фракции S2 в присутствии додецилосернистого натрия показал появление одной или двух поединиц полипептидных с молекулярной массой около 23 000.