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# COMPARATIVE STUDY OF SEED PROTEINS IN THE GENUS *PISUM* XI. IMMUNOLOGICAL RELATIONSHIPS OF THE S2 ALBUMIN FRACTIONS[1]

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Summary. The S2 albumin fraction (MW ca 40 000) was previously reported to be responsible for the distinction of ten characteristic albumin patterns in *Pisum*, EP I — EP X. In this work the S2 fractions from the Pisum forms representing EP I — EP X were investigated by means of immunoelectrophoresis and immunoblotting, at the level of native proteins and subunits. It has been shown that the S2 fraction is serologically distinct from other components of crude albumin extracts. On the other hand, the obtained data have provided an evidence that the S2 albumin fractions giving different electrophoretic patterns are closely related with respect to antigenic properties.

A comparative electrophoretic analysis of seed albumins from about 500 Pisum accessions, representing a wide range of genetic and geographic variation, revealed 10 characteristic patterns: EP I — EP X. Differences between the distinguished EP patterns — obtained using polyacrylamide gel electrophoresis (PAGE) in an anionic buffer system, under non-denaturating conditions — were due to the occurrence of several well-defined protein bands, designated **a-f**. Data concerning distribution of the EP patterns distinguished contributed discriminating information to the taxonomy of *Pisum*. An electrophoretic analysis of seed albumins proved useful in detecting differences at the species/subspecies level and was found helpful as a guide to the geographic origin of *Pisum* accessions (Jakubek, Przybylska 1983; Przybylska et al. 1977, 1985; Przybylska, Zimniak-Przybylska 1985). The so-far performed genetic studies, covering some of the distinguished EP patterns, indicated monogenic inheritance (Blixt et al. 1980; Przybylska et al. 1984a).

After gel filtration of crude albumin extracts on Sephadex G 100, albumins corresponding to the characteristic bands were recovered in a distinct fraction with

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the approximate molecular weight 40 000. In contrast to the differentiated patterns of the native proteins, the corresponding SDS-PAGE patterns of the S2 fractions proved to be similar, showing one or two polypeptides with the molecular weight about 23 000 (Jakubek, Przybylska 1979; Przybylska et al. 1984b). The data are in agreement with the results reported recently by Croy et al. (1984) who described two closely related albumins from *Pisum sativum* seeds suggesting their dimeric structure.

The purpose of the present work was to get some knowledge about immunological relationships of the S2 albumin fractions from the Pisum accessions representing EP patterns I - X.

## MATERIAL AND METHODS

### PLANT MATERIAL

The investigated *Pisum* forms, listed according to their EP patterns, were as follows: *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. syriacum* (=*P. humile*) VIR 2521 (EP VIII), *P. elatius* Gat. 255 (EP IX), *P. fulvum* — Population 1 (EP X). The *Pisum* forms were obtained from several sources: the Pisum Gene Bank at the Weibullsholm Plant Breeding Institute, Landskrona, Sweden (W); the N. I. Vavilov All Union Research Institute of Plant Industry, Leningrad, USSR (VIR); "Zentralinstitut für Genetik und Kulturpflanzenforschung" of the Academy of Sciences of GDR, Gatersleben, GDR (Gat.); the Department of Botany of the Hebrew University of Jerusalem, Israel (*P. fulvum* accession called "Population 1").

The material used for analyses was grown and rejuvenated in a greenhouse at the Institute of Plant Genetics of the Polish Academy of Sciences in Poznań.

### ANALYTICAL TECHNIQUES

#### Extraction procedures

For the preparation of the crude albumin extracts mature pea seeds were hulled and the cotyledons milled. The meal was extracted twice with distilled water, pH 5.0, at a ratio of 100 mg meal to 1 ml water, for 1 h at 4°C. After centrifugation at 10 000 ×g for 30 min the supernatants were combined, adjusted to pH 7.0 with 0.1 N NaOH and immediately subjected to immunochemical analysis or stored at −20°C.

The proteins designated as the S2 fraction were isolated from total albumins using gel filtration on Sephadex G 100, as previously described (Jakubek, Przybylska 1979).

Polyacrylamide gel electrophoresis (PAGE)

Crude albumin preparations and the S2 fractions were subjected to PAGE on 7.5% slab gels in anionic buffer system as described by Davis (1964) and also to SDS-PAGE on 12% slab gels according to Laemmli (1970). 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".

Immunochemical procedures

Antibody preparation. — Antisera directed against the S2 fractions from *P. sativum* W 110 (EP I) and from *P. abyssinicum* W 808 (EP IV) were produced in rabbits primed and boosted three times over space of two months by subcutaneous injections with freeze-dried preparations of the appropriate protein in Freund's adjuvant. One week after the last injection bleeding was induced by puncture of the ear vein. Antisera used for immunoblotting studies were purified by ammonium sulphate precipitation followed by affinity chromatography on Protein A covalently linked to CNBr-activated Sepharose 4 B (Pharmacia, Sweden); bound immunoglobulins were eluted with 0.2 M glycine-HCl buffer, pH 2.3, containing 0.5 M NaCl (Nieden et al. 1982). The specificity of the obtained antisera was established by immunoelectrophoresis or immunoblotting using crude albumin extracts as an antigenic material. Antisera from preimmune animals served as a control.

Immunoelectrophoresis. — Immunoelectrophoresis was performed in a tris-glycine-barbital buffer system, pH 8.8 (Weeke 1973), on 1.5% agarose gels prepared according to Scheidegger (1955). After electrophoresis, for 2 h at 2 V/cm, an antiserum was added and allowed to diffuse for 24-48 h. Dried and washed gels were stained for proteins with 0.5% Coomassie Brilliant Blue G 250 (CBB). Cross reactivities of the specific S2 fraction antisera to crude albumins from different *Pisum* forms were established according to Ossermann (1960) using the S2 fractions from *P. sativum* W 110 and from *P. abyssinicum* W 808 as reference antigens.

Immunoblotting. — Proteins separated by PAGE were transferred from polyacrylamide gels to nitrocellulose sheets (Schleicher and Schüll, BA 85, 0.45 µm pore size) as described by Towbin et al. (1979) with the modification that the tris-glycine-methanol blotting buffer contained additionally 0.1% SDS. After completion of the transfer procedure, the reactive sites of the nitrocellulose were blocked with 3% bovine serum albumin (BSA) dissolved in 0.15 M NaCl buffered with 10 mM Tris-HCl, pH 7.4 (TBS). Subsequently, the blots were incubated overnight at a room temperature with the specific antibody, diluted appropriately with TBS containing 1% BSA. The blots were washed with several changes of TBS containing 0.5% Triton X-100 and then incubated for 30 min with $10^6$ cpm/ml ($^{125}$I) Protein A labelled by a chloramine T procedure with a carrier-free ($^{125}$I) sodium iodide (Ehrlich et al. 1979). After a final washing, the blots were air-dried and exposed to X-ray film

(ORWO, HS 11), in the presence of an intensifying screen (Perlux, VEB Kali-Chemie, Berlin), for 24 h at −70°C.

Affinity purification of antibodies using proteins immobilized on nitrocellulose. — The procedure according to Smith and Fisher (1984) was employed. Briefly, the S2 fraction from *P. sativum* W 110 was electrophoresed under non-denaturating conditions by PAGE and blot transferred as described above. Albumin bands on the blot were located by indirect immunoperoxidase staining using specific antiserum, horseradish peroxidase conjugated with goat anti-rabbit immunoglobulin, and 4-chloro-1-naphthol as the chromogene. The blot was rinsed in TBS-Tween 20 and the regions containing the prominent albumin bands were excised and separately washed with 5 mM glycine-HCl, pH 2.3, supplemented with 500 mM NaCl, 0.05% Tween 20 and 100 mg/ml BSA (three washes each for 30 seconds). The individual eluates were immediately neutralized with $Na_2HPO_4$. The pooled eluates of particular bands were saturated with preimmune rabbit immunoglobulins and used to probe immunoblots from crude albumin preparations according to the standard Western blotting protocol.

## RESULTS AND DISCUSSION

The high-titre antisera against the S2 fractions from *P. sativum* W 110 (anti-S2 -W 110 antiserum) and from *P. abyssinicum* W 808 (anti-S2-W 808 antiserum) were raised in rabbits. The specificity of the antisera was established by immunoelectrophoresis and immunoblotting using crude albumin preparations from the corresponding *Pisum* lines as antigens. The monospecificity of the antisera made it possible to use crude albumin extracts for the comparative analysis of the S2 fractions from the *Pisum* accessions representing different electrophoretic seed albumin patterns, EP I — EP X (Przybylska et al. 1984b, 1985). As the two antisera, anti-S2-W 110 and anti-S2-W 808, provided essentially the same results, the presented documentation is limited to the data obtained using anti-S2-W 110 antiserum.

### Immunoelectrophoresis

The reactivity of anti-S2-W 110 antiserum against crude albumin extracts from the investigated *Pisum* forms, exhibiting EP I — EP X, is illustrated in Fig. 1. In all forms, except the one with EP VI, distinct immunoprecipitates may be observed. The precipitation lines vary in shape and electrophoretic mobility which is consistent with the earlier reported differences in electrophoretic patterns of the S2 fractions in *Pisum*. Also, the lack of an immunoprecipitate in the *Pisum* form with EP VI is in accordance with the previous observation that the *Pisum* form with EP VI showed no peak corresponding to the S2 fraction when total seed albumins were subjected to gel filtration on sephadex (Przybylska et al. 1984). It should, however, be emphasized, that using a modified immunoelectrophoretic procedure according to Ossermann (1960) a small amount of the protein fraction precipitated by the anti-

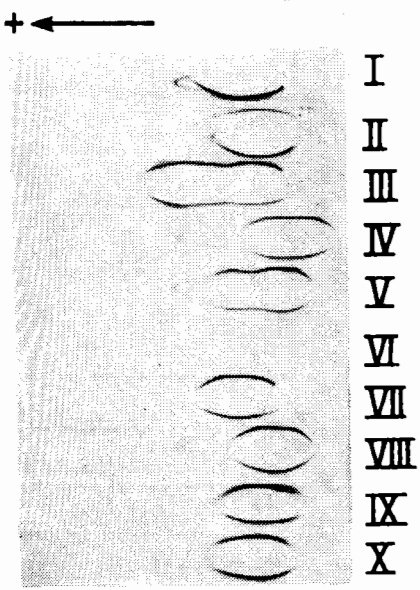


**Fig. 1.** Immunoprecipitation patterns of the crude albumin fractions from ten distant *Pisum* forms, obtained after immunoelectrophoresis using antiserum against the S2 albumin fraction from *Pisum sativum* W 110

Crude albumins were extracted from the seeds 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* Cat. 255 (EP IX), *P. fulvum* Population 1 (EP X)

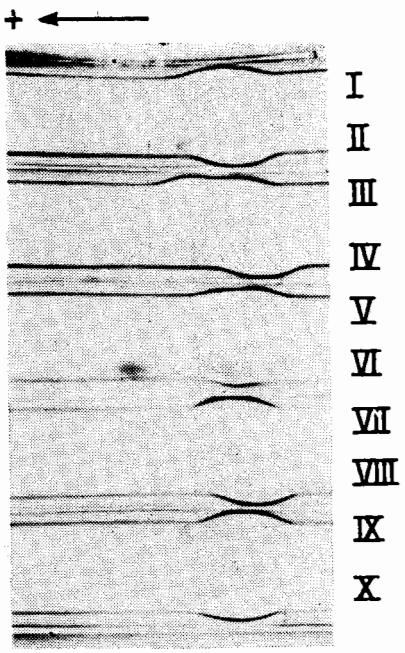


Fig. 2. Antigenic homologies between the S2 fractions from the *Pisum* accessions representing patterns EP I — EP X

Crude albumins were separated by the Ossermann technique using antiserum against the S2 albumin fraction from *Pisum sativum* W 110 and the S2 fraction from *Pisum sativum* W 110 as a reference antigen

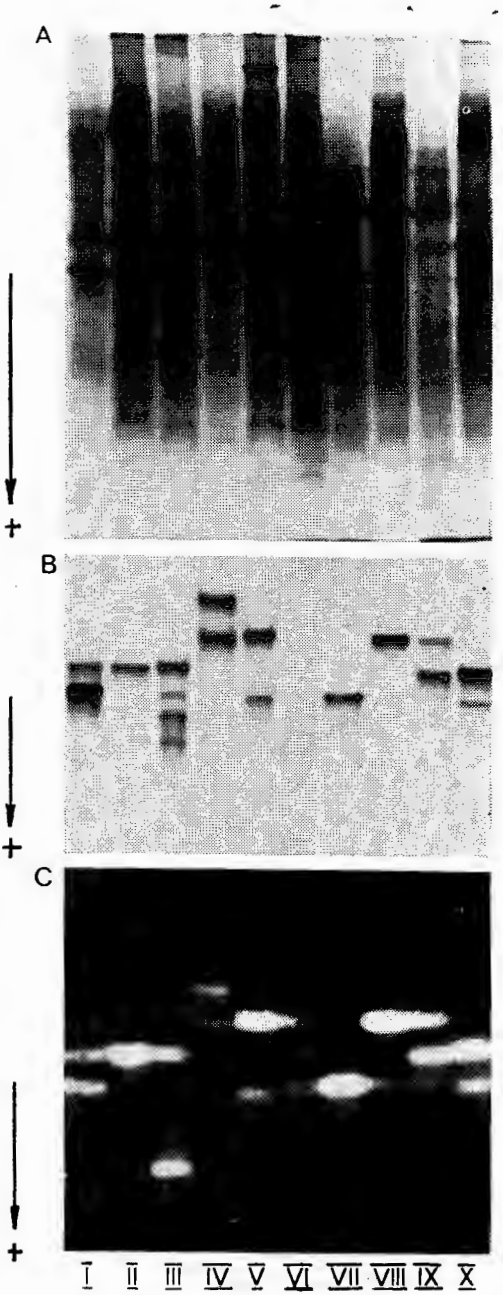


Fig. 3. Immunoblotting analysis of the S2 fractions from the *Pisum* forms representing EP I — EP X

A — CBB-stained PAGE patterns of crude albumin extracts, B — CBB-stained PAGE patterns of the S2 fractions, C — Labelling patterns of crude albumin extracts after PAGE and immunoblotting

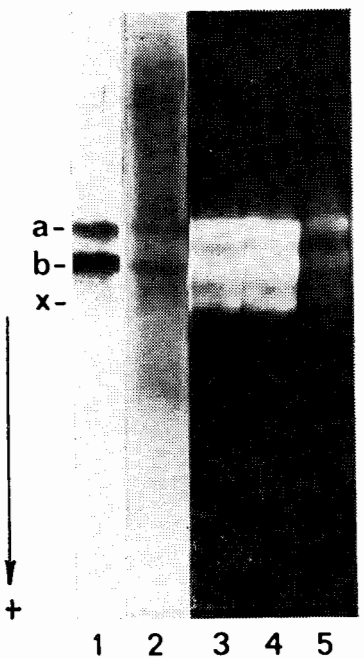


Fig. 4. Detection of immunological similarities between the components of the S2 fraction from *P. sativum* W 110 by immunoblotting using affinity-purified antibodies

Track 1: pattern of the S 2 fraction from *P. sativum* W 110 after PAGE, blotting on nitrocellulose and indirect immunoperoxidase staining. Blot fragments showing bands **a**, **b** and **x** were excised the bound antibodies separately eluted and used to reprobe Western blot of crude albumins (Track 3 - 5)

Track 2: pattern of the crude albumin extracts from *P. sativum* W 110 after PAGE, blotting on nitrocellulose and staining with CBB

Track 3 - 5: patterns of the crude albumin extracts from *P. sativum* W 110 after PAGE and immunoblotting using antibodies affinity-purified on immobilized bands **a** (3), **b** (4) and **x** (5) and ($^{125}$I) Protein A as label

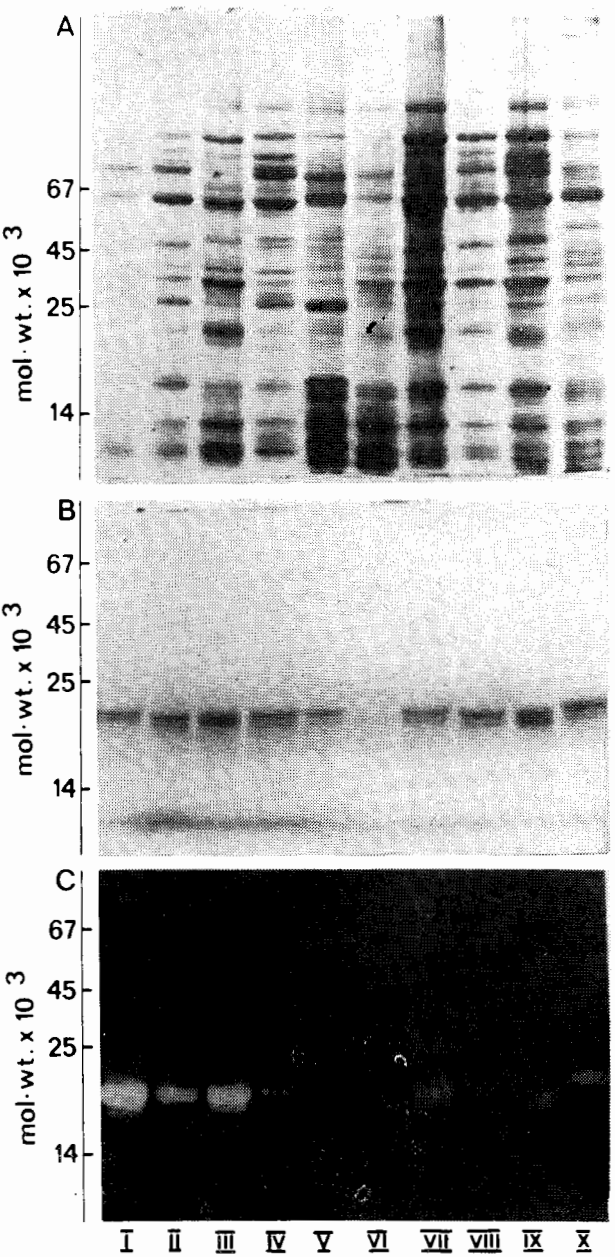


Fig. 5. Immunoblotting analysis of subunits of the S2 fractions from the *Pisum* forms representing EP I — EP X

A — CBB-stained SDS-PAGE patterns of crude albumin extracts, B — CBB-stained SDS-PAGE patterns of the S2 fractions, C — Labelling patterns of crude albumin extracts after SDS-PAGE and immunoblotting

-S2-W 110 antiserum could be detected in the *Pisum* form with EP VI (Fig. 2). The results obtained by means of the above technique provided a supporting evidence that the S2 fractions from all the investigated *Pisum* forms are antigenically identical or at least very similar.

## Immunoblotting

The immunoblotting analysis was used to compare the S2 fractions at the level of native proteins and subunits.

Fig. 3 presents the results obtained when the S2 fractions were subjected to PAGE under non-denaturating conditions. Fig. 3A and 3B show, respectively, CBB-stained PAGE patterns of crude albumin extracts and of the S2 fractions from the *Pisum* forms representing EP patterns I-X. The labelling patterns of crude albumins after immunoblotting are shown in Fig. 3C. Marked similarities between the PAGE patterns of the S2 fractions and the immunoblotting patterns may be observed. It means that of the numerous proteins present in the crude albumin extracts (Fig. 3A) only the albumins constituting the S2 fractions were specifically detected by the anti-S2-W 110 antiserum. Regarding the *Pisum* form with EP VI, it is worth noticing that its cross-reactivity pattern is similar to that observed in the *Pisum* form with EP I. In this connection it should be mentioned that EP patterns I and VI were found in the closely related *P. sativum* forms (Przybylska et al. 1977, Przybylska et al. 1984b).

To recognize immunological relationships between the components of the S2 fraction from *P. sativum* W 110 a blot-affinity purification of the respective antibodies was applied as described in "Materials and methods". The blot-affinity-purified antibodies were then used to reprobe a Western blot of the crude albumin fraction from *P. sativum* W 110. As shown in Fig. 4, labelling patterns obtained with the antibodies affinity-purified on the immobilized electrophoretic albumin variants **a**, **b**, and **x** are practically undistinguishable (tracks 3, 4, 5), though band **x** was detectable only at high sample loadings. It should be noted here that bands **a** and **b** are the characteristic bands of EP I while the additional band **x** seems to be an artefact. The appearance of additional prominent bands on storage of albumin preparations was observed in our earlier investigations (Zimniak-Przybylska et al. 1985). It was also reported by Croy et al. (1984) who ascribed it to deamidations.

Regarding the above described experiment, it is worth emphasizing that affinity-purification of antibodies provides a convenient approach to investigating immunological relationships between proteins or peptides of complex antigens used for production of antisera. The antigen remains irreversibly bound to the nitrocellulose throughout the probing and elution procedure and sufficient quantities of the purified antibodies are being recovered for reprobing Western blots. Moreover, it is possible to reuse the washed nitrocellulose for a subsequent affinity-purification.

Antigenic similarities at a subunit level were investigated using immunoblots

derived from SDS-PAGE patterns of the crude albumin extracts. Figures 5A and 5B show, respectively, CBB-stained SDS-PAGE patterns of crude albumin extracts and of the S2 fractions from the *Pisum* forms with EP patterns I — X. Fig. 5C presents the corresponding SDS-PAGE patterns of crude albumins probed by immunoblotting. The obtained results clearly indicate that subunits of the S2 fractions from different *Pisum* forms are selectively recognized with the anti-S2-W 110 antiserum. Two closely migrating subunits observed in the CBB-stained gel of the S2 fractions (Fig. 5B) appear as a single polypeptide in immunoblots (Fig. 5C) which indicates that both subunit species were recognized by the antibodies. The observed variation in the intensity of the radioactive signals, noticed also in the analysis of the native proteins, may be due to different amounts of the S2 fraction and/or to different availability of its antigenic sites in various *Pisum* forms. However, the observed cross-reaction clearly indicates that the S2 fraction subunits from all the examined *Pisum* forms are closely related with respect to antigenic properties.

The presented results of immunochemical investigations provide some information on the nature of the electrophoretically detected heterogeneity and variation of the S2 albumin fraction in *Pisum*. The antigenic similarity of the albumin components with different electrophoretic mobilities is a clear indication of their structural relatedness. This is consistent with the data provided by the structural analysis of the S2 fraction from the *Pisum* form with EP III, showing several well-separated characteristic bands (Zimniak-Przybylska et al. 1985). The amino acid sequence analysis showed this fraction to represent a single class of protein, composed of polypeptide chains containing about 200 residues, in mainly hydrophilic structures, with few methionine and cysteine/half-cystine residues. The structural analysis suggested the presence of single positions with microheterogeneities which might create charge differences observed in the banding pattern of the native protein. As pointed out by Casey and Domoney (1985), single amino acid substitutions can have large effect on electrophoretic mobility.

The results of this investigation are also in agreement with the report of Croy et al. (1984) describing purification and some properties of two major pea seed albumins, which apparently are equivalent to the characteristic albumins **a** and **b** in the S2 fraction from *Pisum* forms with EP I. The quoted investigators showed the proteins to be dimers, the larger protein being built of the subunits with MW 25 000 and the smaller one consisting of the subunits with MW 24 000. The component subunits of the two examined proteins were found to display close homology, as judged by immunological cross-reaction, amino acid composition, N-terminal amino acids, triptic peptide maps and CNBr-cleavage products.

The molecular basis of heterogeneity and variation of the major seed albumin fraction in *Pisum*, called here the S2 albumin fraction, is not clear yet. In the case of storage pea proteins, existence of small gene families, and also post-translational modifications and storage artefacts were considered the sources of the electrophoretically detected heterogeneity and variation of the polypeptide subunits (Casey, Domoney 1985; Gatehouse et al. 1984). The biochemical basis of variation of the S2 albumin fraction may be similar. It should be stressed, however, that the distinc-

tion of EP patterns I — X cannot be attributed to storage artefacts. Monogenic inheritance of the EP patterns as well as taxonomic and geographic relationships concerning their distribution (see the introduction) indicate that the EP patterns distinguished reflect genetically controlled differences.

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## BADANIA PORÓWNAWCZE BIAŁEK NASION RODZAJU *PISUM* XI. IMMUNOLOGICZNE POWINOWACTWO FRAKCJI ALBUMINOWYCH S2

### Streszczenie

We wcześniejszych badaniach wyróżniono w obrębie rodzaju *Pisum* 10 typów elektroforetycznego spektrum białek albuminowych nasion (EP I — EP X). Jak stwierdzono, białka odpowiedzialne za wykryte różnice mają ciężar cząsteczkowy około 40 000 i można je stosunkowo łatwo wydzielić metodą sączenia żelowego jako tzw. frakcję S2.

Niniejsza praca przedstawia wyniki immunochemicznej analizy porównawczej frakcji S2 wydzielonych z form *Pisum* reprezentujących EP I — EP X. Analizowano białka natywne i ich podjednostki, przy zastosowaniu immunoelektroforezy i „immunoblotting". Wykazano, że frakcja S2 jest serologicznie różna od pozostałych składników surowych ekstraktów albuminowych nasion. Stwierdzono równocześnie, że frakcje S2 wyraźnie różniące się obrazem elektroforetycznym wykazują podobne właściwości antygenowe.

## СРАВНИТЕЛЬНЫЕ ИССЛЕДОВАНИЯ БЕЛКОВ СЕМЯН РОДА *PISUM* XI. ИММУНОЛОГИЧЕСКОЕ СРОДСТВО АЛЬБУМИНОВЫХ ФРАКЦИЙ S2

### Резюме

В предыдущих исследованиях в пределах рода *Pisum* было выделено 10 типов электрофоретического спектра альбуминовых белков семян (EP I - X). Как было установлено, белки, ответственные за обнаруженные разницы, имеют молекулярный вес около 40 000 и относительно легко можно их выделить методом гелевой фильтрации как так-называемую фракцию S2.

В настоящей работе представлены результаты сравнительного иммунологического анализа фракций S2, выделенных из форм *Pisum*, представляющих EP I - X. Анализировались местные белки и их поединицы при применении иммуноэлектрофорезы и „иммунофильтрации". Обнаружено, что фракция S2 серологически отличается от остальных компонентов сырых альбуминовых экстрактов семян. Одновременно было установлено, что фракции S2, явно отличающиеся своим электрофоретическим спектром, проявляют одинаковые антигенные свойства.