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COMPARATIVE STUDY OF SEED PROTEINS IN THE GENUS *PISUM* XII. ANTIGENIC SIMILARITIES OF THE S2 ALBUMIN FRACTIONS AS REVEALED BY A COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY ELISA[1]

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Summary. As previously reported, discrimination of electrophoretic seed albumin patterns in the genus *Pisum* (EP I — EP X) is based on genetic variability of the S2 albumin fraction proteins (approx. MW 40 000) that were shown to exhibit similar antigenic properties. Using the competitive ELISA the percentage of antigenic similarities of the S2 fractions from several *Pisum* forms, representing different taxa and showing different EP patterns, was determined. The experiments were performed using two monospecific antisera, one raised against the S2 fraction from *P. sativum* form with EP I, and another one — against the S2 fraction from *P. abyssinicum* form with EP IV. With the both antisera the same order of immunochemical similarities of the investigated S2 fractions was found. The obtained results are discussed with reference to *Pisum* taxonomy.

Extensive electrophoretic studies resulted in distinguishing ten electrophoretic seed albumin patterns in the genus *Pisum* (EP I — EP X) (Przybylska et al. 1977, 1984, 1985). Proteins responsible for the observed variation were found to have molecular weight ca 40 000 and to form a distinct fraction, called the S2 fraction, which may be easily separated by molecular sieving (Jakubek, Przybylska 1979, Przybylska et al. 1984). Recently, the S2 fractions from the *Pisum* forms representing EP patterns I — X were shown to have similar antigenic properties (Manteuffel, Przybylska 1987). In this work, immunochemical similarities of the S2 fractions from several *Pisum* forms, belonging to different taxa and showing different EP patterns, were quantitatively determined by using a sequential competition ELISA. According to the authors' knowledge, so far this approach has not been used in plant chemotaxonomic investigations.

MATERIALS AND METHODS

PLANT MATERIAL

The S2 albumin fractions were isolated from the following *Pisum* forms, representing different taxa and various EP patterns: *P. elatius* W 805 (EP I), *P. elatius*

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Gat. 255 (EP IX), *P. sativum* W 110 (EP I), *P. sativum* VIR 1987 (EP VII), *P. abyssinicum* W 808 (EP IV), *P. fulvum* W 1256 (EP V), *P. fulvum* — "Population 1" (EP X). The *Pisum* forms were obtained from different sources: the Pisum Gene Bank at the Weibullsholm Plant Breeding Institute, Landskrona, Sweden (W); the Vavilov All Union Research Institute of Plant Industry, Leningrad, USRR (VIR); the Central Institute of Genetics and Crop Plants Research of the Academy of Sciences of the GDR, Gatersleben (Gat.), and from the Department of Botany of the Hebrew University, Jerusalem, Israel (*P. fulvum* accession called "Population 1"). The plant material was grown and rejuvenated in a greenhouse at the Institute of Plant Genetics of the Polish Academy of Sciences in Poznań.

ANALYTICAL TECHNIQUES

Preparation of the S2 albumin fractions. The S2 albumin fractions were isolated from total albumins of cotyledons of dry seeds using gel filtration on Sephadex G 100, as previously described (Jakubek, Przybylska 1979). Protein contents in solutions were determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Antibody preparation. Antisera against the S2 fractions from *P. sativum* W 110 (anti-W 110 antiserum) and from *P. abyssinicum* W 808 (anti-W 808 antiserum) were raised in rabbits (Manteuffel, Przybylska 1987). The IgG fraction of the antisera was precipitated by ammonium sulphate, dialysed against several changes of phosphate buffered saline, pH 7.4 (PBS) and stored in aliquots at −20°C.

Enzyme linked immunosorbent assay (ELISA). A sequential competition ELISA (Zettner, Duly 1974) was adapted to determine antigenic similarities of the S2 albumin fractions from different *Pisum* forms. Two antigens, i. e. the S2 albumin fraction from *P. sativum* W 110 (10 μg/ml) and the S2 fraction from *P. abyssinicum* W 808 (1 μg/ml), were used, in separate experiments, for coating the microtitre plates (VEB Polyplast, Halberstadt, GDR). Coating was performed for one hour at 37°C and overnight at 4°C. After washing the plates with PBS-0.05% Tween 20, the remaining free binding sites were blocked by the addition of 1% BSA in PBS-0.05% Tween 20 (1h, 37°C). The antibody preparations (5 μg/ml), preincubated overnight at 4°C with different amounts of the investigated S2 fractions (six concentrations in the range of 0.01 - 50 μg/ml), were applied to the plates coated with the homologous antigen and left for two hours at 37°C.

Bound antibodies were detected with anti-rabbit IgG covalently linked to alkaline phosphatase. The plates were incubated with the enzyme labelled antibodies (2h, 37°C) and then *p*-nitrophenylphosphate was added as the enzyme substrate. After 30 min, the reaction was stopped with 3M NaOH and the absorption values were recorded at 405 nm with a spectrocolorimeter SPEKOL (Zeiss, Jena GDR).

The 50% inhibition concentrations, within the linear range of the curve, were calculated and in each case compared with the homologous antigen-antibody

reaction (with both antisera). The S-shaped curves were fitted by non-linear regression analysis on a KRS 4201 calculator (VEB Robotron, GDR) (Helbing, Meister — in preparation for the print) and the concentrations of particular antigens (S2 fractions) necessary for 50% inhibition of antibody binding to the solid phase were printed. The results were calculated as follows:

$$\% \text{ cross-reactivity} = \frac{\text{concn. of the ref. antigen* at 50\% inhib.}}{\text{concn. of a competitor at 50\% inhib.}} \times 100$$

RESULTS

Figure 1 shows a dose-response curve obtained in the competitive ELISA, with the use of antibodies against the S2 fraction from *P. sativum* W 110 (W 110 antibodies) and different concentrations of the S2 fraction from *P. sativum* W 110 (W 110 antigen). The curve has a typical sigmoidal shape. The higher the concentration of the competitor the more antibody is prevented from binding to the solid phase and the lower are the measured absorption values. The coefficient of variation among triplicate wells was 5%, typical of most ELISA procedures. The antigen concentration required to inhibit antibody binding by 50% was 0.04 μg/ml.

The competition ELISA was used to test the ability of the S2 fractions from different *Pisum* forms to bind to anti-W 110 and anti-W 808 antibodies. Results obtained with both antibody preparations are presented in Table 1. The 50% inhibition values ranged from 0.042 to 0.971 μg/ml with anti-W 110 antibodies and from 1.14 to 23.1 μg/ml with anti W 808 antibodies, indicating a higher titre

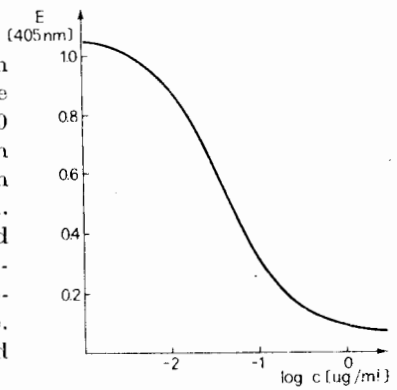

Fig. 1. Competitive ELISA for the S2 fraction from *Pisum sativum* W 110. The wells of a microtitre plate were coated with the S2 fraction from *P. sativum* W 110 (W 110 antigen). Aliquots of anti-W 110 preparation (5 μg/ml) were preincubated with a range of antigen W 110 concentrations from 0.01 μg/ml to 50 μg/ml. The supernatants were applied to the antigen-coated plate and bound antibodies were detected by anti-rabbit IgG labelled with alkaline phosphatase and subsequent enzymatic cleavage of *p*-nitrophenylphosphate. Colour development was terminated by NaOH and monitored at 405 nm

for the W 808 antiserum than for the anti-W 110 antibody preparation. Apparently the W 110 antigen was more effective than W 808 antigen in the competition with

* the reference antigen, i. e. the S2 fraction from *P. sativum* W 110 or from *P. abyssinicum* W 808, depending on the antiserum used.

anti-W 808 antibodies. No antigenic difference could be found between the S2 fraction from *P. sativum* W 110 (EP I) and *P. elatius* W 805 (EP I). However, in the case of other fractions distinct differences were noted. It should also be stressed that similar relationships were observed in the experiments with different antisera. With both antisera the order of immunochemical similarities of the S2 fractions investigated, in terms of decreasing cross-reactivities, was as follows: *P. elatius* W 805 (EP I) and *P. sativum* W 110 (EP I) → *P. elatius* Gat. 255 (EP IX) → *P. abyssinicum* W 808 (EP IV) → *P. sativum* VIR 1987 (EP VII) → *P. fulvum* W 1256 (EP V) → *P. fulvum*, "Population" 1 (EP X). A graphical presentation of the results obtained with the use of anti-W 110 antiserum is given in Figure 2.

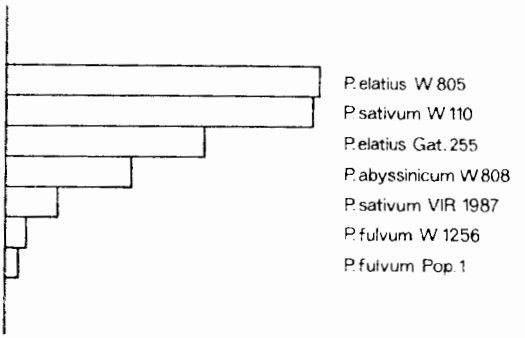

Fig. 2. Graphical presentation of immunochemical similarities of the S2 fractions from different *Pisum* forms. The percentage similarities were determined by the competitive ELISA using the antiserum against the S2 fraction from *P. sativum* W 110 (compare Table 1)

Table 1. Comparison of the S2 fractions from different *Pisum* forms with respect to the ability of binding to the antibodies against the S2 fraction from *P. sativum* W 110 and from *P. abyssinicum* W 808

Source of S2 fraction	Anti-W 110*		Anti-W 808*	
	50% inhibition concentration (µg/ml)	% of the reference antigen reactivity**	50% inhibition concentration (µg/ml)	% of the reference antigen reactivity**
P. elatius, W 805 (EP I)	0.042	102.1±0.01	1.14	157.2±13.30
P. elatius, Gat. 255 (EP IX)	0.066	64.8±0.03	1.67	107.5±3.30
P. sativum, W 110 (EP I)	0.043	100.0±0.067	1.18	152.0±6.36
P. sativum, VIR 1987 (EP VII)	0.252	17.0±0.31	5.40	33.3±3.79
P. abyssinicum, W 808 (EP IV)	0.104	41.2±0.01	1.80	100.0±1.86
P. fulvum, W 1256 (EP V)	0.607	7.1±0.75	9.50	18.9±1.19
P. fulvum, "Population 1" (EP X)	0.971	4.4±0.39	23.10	7.8±0.68

* antibody concentration was 5 µg/ml

** coefficient of variation of the 50% inhibition concentration

DISCUSSION

Systematic classification of the genus *Pisum* is disputable. As pointed out by Blixt (1972) and Marx (1979), systematists considering mainly morphological differences are inclined to distinguish more species than those taking into account genetic compatibility. Lampreeht (1974), who was a genetist, regarded *Pisum* as a monospecific genus. On the other hand, in the classification system worked out by Lehmann (1954), based on morphological characteristics, five annual species are recorded: *P. elatius* (M. B.) Stev., *P. humile* Boiss. et Noe = *P. syriacum* (Berger) Lehm., *P. sativum* L., *P. abyssinicum* A. Braun, *P. fulvum* Sibth. et Smith. Recently most authorities argue that Pisum contains only one or two species. Distinctness of *P. fulvum*, showing a relatively great genetic and cytogenetic divergence, is an argument for distinguishing this taxon as a separate species (Ben-Ze'ev, Zohary 1973, Makasheva 1979). All other *Pisum* forms, cytogenetically closely related, are regarded as a single species for which the name of *P. sativum* is proposed.

Extensive electrophoretic investigations of different protein systems in the genus *Pisum*, performed by one of the authors (J. P.) and coworkers, contributed discriminating information to the taxonomy of *Pisum* (see the reviews: Przybylska 1986, 1986a). The obtained data provided an evidence confirming distinctness of *P. fulvum* and showing some individuality of *P. abyssinicum*. Moreover, within the group of forms representing the *elatius-humile-sativum* complex some accessions having unique electrophoretic protein phenotypes could be indicated.

Electrophoretic analysis of seed albumins was found especially useful in detecting differences at the species/ecotype level. This is the reason that the S2 fraction (MW ca 40 000), comprising „specific albumins" responsible for the recorded variation, became an object of different investigations. In the light of the so far obtained genetic, biochemical and immunochemical data, differences in electrophoretic seed albumin patterns (EP patterns) in the genus *Pisum* may be due to allelic variation, and "specific albumins" showing different electrophoretic mobilities have a similar molecular structure (Blixt et al. 1980, Przybylska et al. 1984, Zimniak-Przybylska et al. 1985, Manteuffel, Przybylska 1987).

This paper reports the percentage immunochemical similarities of the S2 fractions from several *Pisum* forms, chosen to represent different taxa and various EP patterns. It is interesting to relate the reported data to *Pisum* taxonomy.

No difference in the percentage antigenic similarity was found between the S2 fractions from two *Pisum* forms with EP I, i. e. *P. elatius* W 805 and *P. sativum* W 110. This result is not surprising as also electrophoretic data, coming from examination of different protein systems, indicated the forms to be very similar. As mentioned above, *P. elatius* and *P. sativum* are closely related taxa.

Cross-reactivity of the S2 fraction from *P. elatius* Gat. 255 (EP IX) was only 65 - 70% of that found for the S2 fractions of *P. elatius* W 805 and *P. sativum* W 110. The difference seems to be in good agreement with the electrophoretic protein data. Of over 600 *Pisum* accessions examined, the wild growing *P. elatius* accession

Gat. 255, originating from Spain, was the only form which had EP IX. Moreover, *P. elatius* Gat. 255 proved to have unique electrophoretic protein patterns of other protein systems (Przybylska 1986a).

The reactivity of the S2 fraction from *P. abyssinicum* W 808 (EP IV) is more or less similar to that of *P. elatius* Gat. 255. Some distinctness of *P. abyssinicum* was also revealed in electrophoretic protein studies and was partly attributed to seasonal incompatibility due to an earlier flowering of *P. abyssinicum* and to a certain amount of geographic isolation (Przybylska et al. 1979).

The cross-reactivity of the S2 fraction from *P. sativum* VIR 1987 (EP VII) was relatively weak. In this connection it should be mentioned that the accession VIR 1987, labelled *P. sativum* ssp. *transcaucasicum*, is a primitive form originating from Caucasia. It is also worth mentioning that distribution of EP pattern VII seems to be restricted to this geographic region (Przybylska, Zimniak-Przybylska 1985). It may be assumed that EP pattern VII is characteristic of *P. sativum* forms endemic to Caucasia.

Finally, marked taxonomic distinctness of *P. fulvum* seems to be reflected in a very weak cross-reactivity of the S2 fractions from two *P. fulvum* accessions, W 1256 (EP V) and "Population 1" (EP X).

Altogether, the percentage immunochemical similarities of the S2 fractions seem to be compatible with the existing *Pisum* taxonomy. This indicates that sequential competition ELISA may be a promising approach in plant chemotaxonomic investigations.

Both the results of the immunoblotting analysis (Manteuffel, Przybylska 1987) and the presently reported data obtained using the sequential competitive ELISA have provided an evidence that a part of the structure of seed albumins forming the S2 fraction in *Pisum* is conservative.

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BADANIA PORÓWNAWCZE BIAŁEK NASION RODZAJU *PISUM* XII. ANTYGENOWE PODOBIEŃSTWA FRAKCJI ALBUMINOWYCH S2 OZNACZONE PRZY ZASTOSOWANIU KOMPETYTYWNEJ ANALIZY IMMUNOENZYMATYCZNEJ (ELISA)

Streszczenie

Wcześniejsze badania wykazały, że zróżnicowanie elektroforetycznych obrazów białek albuminowych nasion u rodzaju *Pisum* (EP I — EP X) wynika z genetycznie uwarunkowanej zmienności białek frakcji S2 (c. częst. około 40 000) i że białka te mają podobne właściwości antygenowe. Stosując kompetytywną analizę immunoenzymatyczną ELISA oznaczono procentowe antygenowe podobieństwo frakcji S2 z kilku form *Pisum*, reprezentujących różne

taksony i różne typy EP. Badania prowadzono przy użyciu dwóch monospecyficznych surowic odpornościowych, przeciw frakcji S2 z formy *P. sativum* o typie EP I i przeciw frakcji S2 z formy *P. abyssinicum* o typie EP IV. Uszeregowanie immunochemicznych podobieństw badanych frakcji S2 było niezależne od stosowanej surowicy odpornościowej. Uzyskane wyniki omówiono w nawiązaniu do taksonomii rodzaju *Pisum*.

СРАВНИТЕЛЬНЫЕ ИССЛЕДОВАНИЯ БЕЛКОВ СЕМЯН РОДА *PISUM* XII. АНТИГЕННЫЕ СХОДСТВА АЛЬБУМИНОВЫХ ФРАКЦИЙ S2, ОПРЕДЁЛЁННЫХ С ПОМОЩЬЮ КОМПЕТИТИВНОГО ИММУНОЭНЗИМАТИЧЕСКОГО АНАЛИЗА ELISA

Резюме

Ранние исследования показали, что дифференциация электрофоретических спектров альбуминовых белков семян у рода *Pisum* (EP I - EP X) возникает в результате генетически обусловленной изменчивости белков фракции S2 (молекулярный вес около 40 000) и что белки эти обладают подобными антигенными свойствами. Применяя компетитивный иммуноэнзиматический анализ ELISA, было определено процентное антигенное сходство фракций S2 у нескольких форм *Pisum*, представляющих разные таксоны и разные типы EP. Исследования проводились при использовании двух моноспецифических иммунных сывороток против фракции S2 из формы *P. sativum* типа EP I и против фракции S2 из формы *P. abyssinicum* типа EP IV. Классификация иммуннохимических сходств исследуемых фракций не зависила от используемой иммунной сыворотки. Полученные результаты рассмотрены согласно таксономии рода *Pisum*.