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Pisum sativum seed globulins as biochemical markers for « wrinkled-smooth » seed character : comparison of genotypes through different protein extraction and electrophoretic procedures

Rosa RAO ⁽¹⁾ & Jean-Claude PERNOLLET

I.N.R.A., Laboratoire d'Etude des Protéines, Centre de Recherches agronomiques, Route de St-Cyr, F 78000 Versailles.

SUMMARY

*Pisum sativum,
Seed,
Storage proteins,
Legumin,
Vicilin,
Protein bodies,
Storage carbohydrate,
Wrinkled-smooth character,
Genetic markers,
Electrophoresis.*

Nine cultivars representing four genotype classes involving two genes *ra* and *rb* which are implicated in the smooth-wrinkled character of seeds and in the simple-compound character of starch granules, have been investigated through storage protein electrophoretic patterns. Proteins were alternatively extracted from flours and from protein bodies which were obtained either from cotyledons or from seed flours. Vicilin and legumin fractions were isolated from globulins originating from different sources. Their amino acid compositions were analyzed and the legumin-vicilin ratios were calculated. Sodium dodecyl sulfate polyacrylamide (homogeneous or gradient) gel electrophoresis was used to reveal the heterogeneity of the fractions. If the electrophoregrams of protein body crude extract allowed cultivar comparison, only purified vicilin ones could be interpreted in order to classify the cultivars in groups corresponding to the different genotypes.

Comparing the vicilin patterns of the four genotypes, correlation between wrinkled phenotypes and presence of a 60 kD vicilin band is evidenced. As for cereal mutants, the genes involved in the polysaccharide synthesis influence only slightly the storage protein patterns but greatly the relative amount of globulin fractions. It is concluded that the two considered genes *ra* and *rb* are involved in storage protein synthesis.

RÉSUMÉ

*Pisum sativum,
Grains,
Protéines de réserve,
Légumine,
Viciline,
Corpuscules protéiques,
Réserves glucidiques,
Caractère lisse-ridé,
Marqueurs génétiques,
Electrophorèse.*

Les globulines, protéines de réserve du pois (Pisum sativum L.), marqueurs biochimiques du caractère « lisse-ridé » des graines : comparaison de génotypes par différentes techniques d'extraction et d'électrophorèse.

Neuf cultivars, appartenant à quatre classes de génotypes impliquant les gènes *ra* et *rb* responsables du caractère lisse-ridé des grains et du caractère simple-composé des grains d'amidon, ont été étudiés par l'électrophorèse des protéines de réserve. Ces protéines ont été extraites soit de moutures, soit de corpuscules protéiques, eux-mêmes obtenus soit à partir de moutures, soit par broyage en milieu humide des cotylédons décortiqués. Les deux fractions de globulines, viciline et légumine, ont été séparées par chromatographie, leurs compositions en acides aminés déterminées, ainsi que le rapport légumine/viciline. L'électrophorèse en gel de polyacrylamide (homogène ou en gradient) en présence de dodécyl sulfate de sodium a été utilisée pour mettre en évidence l'hétérogénéité de ces fractions. Bien que les diagrammes électrophorétiques des extraits bruts de corpuscules protéiques permettent de comparer les différents cultivars, seuls les électrophorégrammes de viciline purifiée peuvent être interprétés de manière à classer les cultivars en groupes correspondant aux différents génotypes.

En comparant les diagrammes électrophorétiques de viciline des quatre génotypes, nous avons pu mettre en évidence une corrélation entre les phénotypes ridés et la présence d'une chaîne polypeptidique de viciline de 60 kD. De même que pour les mutants de céréales, les gènes impliqués dans la synthèse des polysaccharides de réserve n'influent que très légèrement sur le diagramme électrophorétique des protéines de réserve, alors qu'ils modulent très significativement les quantités relatives des deux fractions de globulines. Nous concluons que les deux gènes *ra* et *rb* sont impliqués dans la synthèse des protéines de réserve.

⁽¹⁾ Present address : Istituto di Agronomia generale e coltivazioni erbacee, Università di Napoli, Via Università 100, Portici NAPOLI (Italia).

I. INTRODUCTION

Seed storage proteins are known to be useful genetic markers (LADIZINSKY & HYMOWITZ, 1979) and may allow either to perform cultivar identification or to investigate species relationships (HYMOWITZ & KAIZUMA, 1979; JOHNSON, 1975; AUTRAN & BOURDET, 1975; DHALIWAL, 1977; LADIZINSKY, 1975, KIM *et al.*, 1979) by the use of electrophoresis. Only a few studies have dealt with *Pisum sativum* globulins, the Leguminosae storage proteins, as genetic markers; HYNES (1968) has done the first investigation of pea variants through storage proteins electrophoresis and THOMSON *et al.* (1978a, b) have turned more recently to the study of pea storage protein heterogeneity. The latter have used protein extracted from protein bodies which are membrane-bound organelles filled with storage proteins (PERNOLLET, 1978) with successful results. At last, CROY *et al.* (1979) and CASEY (1979) have shown that *Pisum sativum* legumins from different lines show light genetic variations. In cereals, during seed development the storage of sugars and of proteins is genetically programmed and controlled, with very frequent relationship between the two systems (NELSON & BURR, 1973). Considering only maize, numerous mutants in which an alteration has been shown for synthesizing starch (namely opaque 2, floury 2, shrunken 2, amylose extender) exhibit some more or less important storage protein modifications, which are mainly represented in the storage protein fraction ratios (MOSSÉ, 1966; TSAI & DALBY, 1974).

Pisum sativum seed is an interesting material for studying the relationship between glucidic and proteic metabolism involved in seed maturation. As in cereals, important differences occurring in the carbohydrate storage may be induced in pea by a single gene: cultivars are also divided into two groups, the wrinkled and the smooth seed varieties due to the classical mendelian gene *R*. KOOISTRA (1962) has later shown that the smooth or wrinkled seed character is, in fact, influenced by two genes, *Ra/ra* and *Rb/rb*, which are also involved at least in the water adsorptive power of mature seed and in the starch synthesis (amylose and amylopectin). As a consequence, two kinds of starch granules can be distinguished (simple or compound) in such a way that it is possible to individualize both genetically and biochemically four groups as concerning carbohydrates (MUCCI, 1974). Our purpose was to investigate seed storage globulins of cultivars of *Pisum sativum*, phenotypically smooth or wrinkled, but containing in their genotypes different doses of *ra* and *rb*. Modified methods suitable for genetical purpose have been preliminary set up on the basis of those described by THOMSON *et al.* (1978a).

II. MATERIALS AND METHODS

Plant material

Seeds of nine *Pisum sativum* cultivars were provided by the Station d'Amélioration des Plantes (INRA, Versailles). These varieties together with their properties and genotypes are listed in table 1. Mature dry seeds were used as a source of proteins which were extracted both from flours and from protein bodies.

TABLE 1

Samples
Echantillons

Genotypes and phenotypes	Cultivars or lines	Protein content as N × 6.25 % dry weight
<i>Group I:</i>		
<i>Ra Ra Rb Rb</i>	Frimas	26.4
(Smooth seeds)	Sommette	28.2
Simple starch granules)	Allround	22.4
	Cameor	28.2
<i>Group II:</i>		
<i>ra ra Rb Rb</i>	Midfreezer	32.8
(Wrinkled seeds)	Erygel	31.2
Compound starch granules)	E.A.	31.5
<i>Group III:</i>		
<i>Ra Ra rb rb</i>	Alaska Sweet	31.5
(Wrinkled seeds)		
Simple starch granules)		
<i>Group IV:</i>		
<i>ra ra rb rb</i>	Line 1 (*)	33.6
(Wrinkled seeds)		
Compound starch granules)		

(*) arising from a cross between "Midfreezer" and "Alaska Sweet".

Isolation of protein bodies

Protein bodies were first isolated from dehulled cotyledons dissected free from axis, according to THOMSON *et al.* (1978a) by differential centrifugations in cold distilled water. In order to improve the rapidity of the isolation they have comparatively been isolated from flours.

Preparation of total globulins (globulins A) from flour

10 g flour were stirred for 3 hours with 100 ml 0.5 N NaCl, 1 mM EDTA in borate buffer pH 8.5 (buffer A) at room temperature. After centrifugation (15 min, 20 000 × g) in a Sorval RC2B centrifuge at 5 °C, proteins in the supernatant fraction were exhaustively dialyzed against distilled water at 5 °C. The precipitated globulins, collected by centrifugation (20 min, 20 000 × g), were washed with cold distilled water and immediately dissolved in 20 ml McIlvaine buffer pH 7.0 (824 ml 0.2 M Na₂HPO₄ 2H₂O, 176 ml 0.1 M citric acid) containing 0.15 N NaCl for zone precipitation. Globulins not immediately used were kept at 2 °C.

Preparation of total globulins (globulins B) from protein bodies

Proteins were immediately extracted from protein bodies in buffer A at room temperature with constant magnetic stirring for 30 min, centrifuged and dialyzed. Further treatments were performed as in the previous paragraph.

Vicilin and legumin purification

10 ml of total globulins (A or B) solutions were subjected to zone precipitation as described by SCHOLZ *et al.* (1974)

with the only difference that the buffers used here contained 0.15 M NaCl. Vicilin-containing fractions were rechromatographed in the same conditions, dialyzed against distilled water, and freeze-dried. Legumin-containing fractions were isoelectrically precipitated by dialysis against McIlvaine buffer pH 4.8 (493 ml 0.2 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 507 ml 0.1 M citric acid), 0.15 N NaCl, dialyzed against distilled water, and freeze-dried.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE)

Crude protein extracts from flours, globulins A, globulins B, vicilins and legumins (50-70 μg) were examined on SDS-PAGE in the presence of 1 p. 100 (w/v) SDS, carried out in slab gels ($85 \times 85 \times 2.5$ mm) with a 12 p. 100 running gel and a 4 p. 100 stacking gel (1 cm) using the Laemmli discontinuous buffer system. Electrophoreses were performed on a Pharmacia gel electrophoresis apparatus GE-4II, for 20 min at 120 V (« run-in » electrophoresis) then for 70 min at 200 V. Lyophilized samples were dissolved in 10 mM Tris HCl pH 8.0, 1 mM EDTA, 1 p. 100 SDS, 10 p. 100 sucrose with or without 1 p. 100 2-mercaptoethanol (2-ME) while crude protein extracts from flours and globulins B were diluted with the same buffer to a final concentration of 5-6 mg of protein/ml. Aliquots of globulins A were precipitated in 70 p. 100 ethanol and redissolved in the same buffer. Sample reduction was performed by heating at 100°C with 2-ME for 5 min. Gels were stained with Coomassie Blue R250 as described by SOAVE *et al.* (1977) and electrically destained in a Pharmacia gel destainer GD-4 with 5 p. 100 (v/v) methanol, 7.5 p. 100 (v/v) acetic acid in water.

A calibration graph of these gels was prepared by electrophoresing the Pharmacia fine chemicals calibration kits according to Pharmacia procedure. Apparent molecular weights were determined by reference to the mobilities of the markers and the values given in kilodalton (kD) are intended to facilitate identification of bands or groups of bands rather than to establish precise values for the molecular weight of each component.

Legumins and vicilins were also analyzed on gradient PAGE (PAA 4-30 p. 100 Pharmacia) as described by THOMSON *et al.* (1978a).

Polyacrylamide gel electrophoresis (PAGE) at acidic pH

Disc electrophoresis of vicilins and legumins were also performed at acidic pH as described by SCHOLZ *et al.* (1974) in β -alanine acetic acid buffer pH 4.5.

Protein and nitrogen assays

Samples of various fractions were treated with ethanol (80 p. 100 v/v) to precipitate protein for analysis. The precipitates were washed once with 80 p. 100 ethanol, then redissolved in 0.25 M NaCl for determination of proteins by the Biuret reaction (GORNALL *et al.*, 1949) using bovine serum albumin (Sigma fraction V) as a standard. Nitrogen determinations were made by the micro Kjeldhal procedure. The legumin-vicilin ratios were determined on a dry weight basis.

Amino acid analysis

Amino acid ion exchange chromatography was performed on a modified model K 8000 B Phoenix amino acid analyzer

by the method of SPACKMAN (1963): proteins were hydrolyzed with 6 N HCl in sealed glass tubes at 115°C for 16, 24 and 72 h. The performic acid oxidation method was used for sulphur amino acid determination (MOORE, 1963). Tryptophan determinations were performed by adsorption chromatography on Sephadex G 10 after barytic hydrolysis in absence of oxygen (modified from SLUMP and SCHREUDER, 1969).

III. RESULTS

A. Comparison of proteins extracted from flours and from protein bodies

1. Crude extracts, total globulins

Crude proteins extracted from flours vary from 70 to 85 p. 100. Using the protein body isolation step, the protein yield is only 15 to 20 p. 100 as regard to the total nitrogen of the seed. Nevertheless the nitrogen extraction yield from protein bodies is about 75-80 p. 100.

Electrophoretic patterns of crude protein extracts from flour are shown in figure 1. The resolution of this technique is good enough to evidence slight differences among cultivars even with crude extracts. Nevertheless the total globulin patterns obtained from flour (fig. 2) are still partially contaminated with albumins (shown by arrows) but are simpler to interpret. The protein body crude extract patterns are almost devoid of albumins and allow the easiest comparison (fig. 3a). When comparing electrophoregrams obtained from different protein body extractions (fig. 3b) it is obvious that proteins extracted from protein bodies obtained directly from flours exhibit no differences as regard to protein bodies isolated from dehulled cotyledons after embryonic axis removal.

Figure 1

Electrophoretic patterns of crude proteins extracted from flour: A) Frimas; B) Alaska Sweet; C) Midfreezer; D) Line 1; E) Cameor; F) All round; G) Erygel; H) E.A.; I) Sommette. The arrows indicate albumins. Protein concentration varies from one sample to another.

Diagrammes électrophorétiques des protéines brutes extraites de farines: A) Frimas; B) Alaska Sweet; C) Midfreezer; D) Ligne 1; E) Cameor; F) All round; G) Erygel; H) E.A.; I) Sommette. Les flèches indiquent les albumines. La concentration des échantillons varie d'un échantillon à l'autre.

Figure 2

Electrophoretic patterns of flour total globulins: A) Frimas; B) Alaska Sweet; C) Line 1; D) Cameor; E) All round; F) Erygel. The arrows indicate albumin contaminations.

Diagrammes électrophorétiques des globulines totales de farines: A) Frimas; B) Alaska Sweet; C) Ligne 1; D) Cameor; E) All round; F) Erygel. Les flèches indiquent les albumines contaminantes.

Figure 3

Electrophoregrams of protein body total globulins. a) proteins extracted from protein bodies isolated from flours. A) Midfreezer; B) Line 1; C) Cameor; D) All round; E) Erygel; F) E.A. b) Cultivar Frimas, proteins extracted from protein bodies isolated from dehulled cotyledons (PB) and from flours (M).

Diagrammes électrophorétiques des globulines totales extraites de corpuscules protéiques. a) Protéines extraites de corpuscules protéiques isolés à partir de farines. A) Midfreezer; B) Ligne 1; C) Cameor; D) All round; E) Erygel; F) E.A. b) Cultivar Frimas, protéines extraites de corpuscules protéiques isolés de cotyledons décortiqués (PB) et de farines (M).

2. Vicilin and legumin separation

SDS-PAGE of vicilin and legumin from flours and from protein bodies are shown in figure 4. Vicilins show a great heterogeneity which, for same samples, seems to be emphasized in heavier vicilin bands ranging about 75, 70 and 60 kD extracted from protein bodies. Among common bands, the

Figure 4

SDS-PAGE of vicilins (V) and legumins (L) obtained from flour (a) and from protein bodies (b): A) Alaska Sweet; B) Line 1; C) E.A. Apparent molecular weights are indicated in kD.

SDS-PAGE de vicilines (V) et de légumine (L) extraites de farines (a) et de corpuscules protéiques (b): A) Alaska Sweet; B) Ligne 1; C) E.A. Les masses molaires apparentes sont exprimées en kD.

vicilin 60 kD band is always in common with legumin, but the SDS-PAGE of reduced vicilins and legumins (fig. 5) shows that this band does not represent legumin contamination as it remains unaltered after reduction, while reduced legumins give rise to two major bands corresponding to 40 and 20 kD. Moreover when analyzed at acidic pH without

Figure 5

SDS-PAGE of reduced vicilins (RV) and legumins (RL) obtained from flour and from protein bodies: A) Alaska Sweet, vicilin from flour (left) and from protein bodies (right). B) Line 1; vicilin from flour (left) and from protein bodies (right). C) Alaska Sweet, Legumin from flour (left) and from protein bodies (right). D) Line 1, legumin from protein bodies (left) and unreduced legumin (right). Apparent molecular weights are indicated in kD.

SDS-PAGE de vicilines (RV) et de légumine (RL) extraites de farines et de corpuscules protéiques: A) Alaska Sweet, vicilines extraites de farine (à gauche) et de corpuscules protéiques (à droite). B) Ligne 1, vicilines extraites de farine (à gauche) et de corpuscules protéiques (à droite). C) Alaska Sweet, légumine extraite de farine (à gauche) et de corpuscules protéiques (à droite). D) Ligne 1, légumine extraite de corpuscules protéiques (à gauche) et légumine non réduite (à droite). Les masses molaires apparentes sont exprimées en kD.

detergent, vicilins show only one major band which excludes legumin contamination (fig. 6). At last the amino acid composition of the two fractions (shown in table 2) are quite different especially for tryptophane, cysteine and methionine which are nearly absent in vicilin and also, but less evident, for other amino acids (lysine, arginine, threonine, serine, glycine, alanine, isoleucine, leucine and phenylalanine).

Legumin electrophoregrams from flours and from protein bodies are shown in figure 7. Legumin fraction prepared from flour shows at least two major bands displayed respectively at about 75 and 60 kD beside faint bands of

Figure 6
Disc electrophoresis of vicilin from Line 1 protein bodies at pH 4.5.
Electrophorèse en tube de viciline extraite de corpuscules protéiques de Ligne 1 à pH 4.5.

TABLE 2

Amino acid composition of legumin and vicilin fractions as number of residues per thousand recovered ones

Composition en acides aminés des fractions de légumine et de vicilines en nombre de résidus pour 1 000 résidus retrouvés

Amino acids	Alaska Sweet		Line 1	
	Legumin	Vicilin	Legumin	Vicilin
Gly	78	52	75	53
Ala	59	45	60	44
Val	58	57	57	56
Leu	83	103	81	102
Ile	45	55	41	55
Ser	63	78	64	77
Thr	37	30	35	27
Tyr	22	23	24	22
Phe	38	50	37	50
Trp	6.6	0.5	6.7	0.2
Pro	51	44	44	43
Met	7.8	1.3	6.9	1.1
Cys	6.6	0.5	3.5	0.5
Lys	55	72	53	74
His	21	16	22	16
Arg	77	57	86	59
Asx	117	134	117	134
Glx	176	182	189	187

lower molecular weight. In the homologous fraction prepared from protein bodies, the corresponding band of 75 kD is fainter and the major bands are all displayed at about 60 kD.

Figure 7
Electrophoregrams of legumins : A) Midfreezer, legumin extracted from protein bodies (PB) and from flour (M). B) All round, Legumin extracted from protein bodies (PB) and from flour (M). Apparent molecular weights are indicated in kD.

Electrophorégrammes de légumine : A) Midfreezer, PB : légumine extraite de corpuscules protéiques ; M : de farine. Les masses molaires apparentes sont exprimées en kD.

B. Comparison of vicilins belonging to different genotypes

Figure 8 illustrates the vicilin electrophoretic patterns obtained with globulins extracted from protein body. The patterns are gathered in respect to the genotype classes which are given in table 1. Comparing these patterns, it appears that, in each class, bands are qualitatively identical and quantitatively quite similar. Only slight differences can be seen within the classes : in the first one (genotype *Ra Ra Rb Rb*), « Frimas » (A) and « Sommette » (B) cultivars exhibit only one 40 kD band whereas a common doublet (shown by arrow) is shown by « Allround » (C) and « Cameor » (D) cultivars. In the second class (genotype *ra ra Rb Rb*) a 35 kD faint band (shown by arrow) characterizes « Midfreezer » (E). On the contrary, vicilins belonging to the first three classes, exhibit more different patterns : only the first class is deprived of a band corresponding to 60 kD, while cultivar named « Alaska Sweet » (I) (third class) and line 1 (H) (fourth class) are characterized by a doublet in the region of 50 kD.

C. Gradient gel electrophoresis of protein body extracted legumins and vicilins

When analyzed on gradient polyacrylamide gel in presence of SDS and after reduction with 2-mercaptoethanol legumins give mostly two bands corresponding to 40 and 20 kD, while vicilins give at least 8 bands (results not shown).

Figure 8

Vicilins obtained from protein bodies of different genotypes: A) Frimas; B) Sommette; C) All round; D) Cameor; E) Midfreezer; F) Erygel; G) E.A.; H) Line 1; I) Alaska Sweet. Apparent molecular weights are indicated in kD. The arrows show bands which vary within each genotype class.

Vicilins extraites de corpuscules protéiques de génotypes différents: A) Frimas; B) Sommette; C) All round; D) Cameor; E) Midfreezer; F) Erygel; G) E.A.; H) Ligne 1; I) Alaska Sweet. Les masses molaires apparentes sont exprimées en kD. Les flèches indiquent les bandes qui varient à l'intérieur de chaque classe de génotypes.

D. Legumin-vicilin ratios

As shown in table 3 different flour legumin-vicilin ratios can be observed among different classes of genotypes. It is worth noticing that line 1, the result of the cross between « Alaska Sweet » and « Midfreezer », shows a ratio which is intermediate between the two very different ones of the parent lines. The presence of the dominant gene *Ra* is correlated with a higher legumin-vicilin ratio while the contrary happens with the dominant gene *Rb*.

TABLE 3
Legumin/vicilin ratios as function of genotypes
Rapport légumine/viciline en fonction des génotypes

	<i>Ra Ra</i>	<i>ra ra</i>
<i>Rb Rb</i>	1.1	0.5
<i>rb rb</i>	2.9	0.7

IV. DISCUSSION

From the results presented, it is likely that the differences in crude protein yields obtained from flours and from protein bodies originate in the fact that the protein body isolation is not exhaustive as has already been mentioned by THOMSON *et al.* (1978a). Nevertheless, since total globulin electrophoretic patterns are indistinguishable whatever the procedure may be, these differences in extraction level are without importance for our aim. It is worth noticing that all electrophoretically detected globulins are shown to belong

to protein bodies. As a consequence, the previous isolation of these organelles does not deprive the geneticist of information. In contrast to MURRAY (1979) who has suggested that albumins might be stored in special protein bodies, we have observed only globulins in our crude protein body isolates. This could be due to the absence of exhaustivity in our protein body preparations.

The SDS-PAGE technique we used is very accurate and reproducible and allows the comparison of many cultivars through their electrophoretic patterns obtained in different runs. THOMSON *et al.* (1978a) have observed two legumin bands by use of gradient gel polyacrylamide electrophoresis in presence of SDS and after reduction. We have obtained the same results as these authors; but using homogeneous gels we have found that, after reduction (fig. 5), many faint bands can be seen and that the 40 kD band seems to be a doublet.

The separation methods used in order to isolate vicilins from legumins is reproducible and effective. If, at first view, some legumin components seem to contaminate vicilins, a more detailed investigation by the use of reducing agent, of polyacrylamide gel electrophoresis at acidic pH, and finally of amino acid analysis checks the quality of the separation. A similar situation has been reported by BAILEY & BOULTER (1972) for *Vicia faba* in which one vicilin band corresponding to 56 kD is common with legumin. The amino acid compositions that we have obtained from our two peculiar genotype classes (table 2) are in good agreement with those published by BOULTER (1977). As a matter of fact, beside others, tryptophan and sulphur amino acids, which are known to characterize legumins in respect to vicilins (DAVIES, 1976; BOULTER, 1977), are a good index for the effectiveness of legumin-vicilin separation.

It appears from legumin-vicilin ratios (table 3) that large variations are shown to occur among different genotypes, which is in agreement with results of THOMSON *et al.* (1978a) and of DAVIES & BEDFORD (1980). The latter have mentioned that the *ra* locus has an influence on the legumin amount of storage proteins and that the genotypes *Ra Ra* have a higher legumin content than the genotype *ra ra*; our results confirm these ones but it is important to emphasize that the cultivar corresponding to the genotype class *Ra Ra rb rb*, phenotypically wrinkled, has a legumin-vicilin ratio much higher than the cultivars corresponding to the genotype *Ra Ra Rb Rb*, phenotypically smooth. This could suggest an influence of both locus, *ra* and *rb*, on the synthesis of these proteins. Similar to the case for cereal mutants (TSAI & DALBY, 1974) the observed *Pisum sativum* cultivars differ only slightly in their storage proteins as regard to the globulin polypeptide chain composition, on a qualitative point of view, but, on the contrary, the relative amount of the two major protein groups undergoes important variations in relation to the polysaccharides. This allows us to suggest a difference in amino acid composition between wrinkled and smooth seed peas.

The differences in the electrophoregrams observed between legumins and vicilins prepared from flours and the homologous proteins prepared from protein bodies could be due to different conditions in which the storage proteins have been solubilized. Obviously the protein body membrane prevents interaction between the proteins stored in these organelles with cytoplasmic components which may alter their structure. The reproducibility of electrophoretic patterns of different cultivars supports the evidence that the different polymers of vicilins are biologically associated forms rather than artifactual preparation aggregates.

The heterogeneity of *Pisum sativum* vicilin has already

been reported in literature. PRZYBYLSKA *et al.* (1979) have found three distinct subunits falling within approximate molecular weight range of 49 000-56 000, 35 000-42 000 and 17 000-22 000, which is in good agreement with other results (DERBYSHIRE *et al.*, 1976). On the other hand, THOMSON *et al.* (1978a, b) have characterized seven major vicilin subunits with molecular weight from 75 000 to 12 000. SDS-PAGE patterns we obtained include from nine to ten major vicilin subunits corresponding respectively to seeds phenotypically smooth and wrinkled: a band of about 60 kD seems to characterize the vicilin pattern of wrinkled seeds. Besides, among phenotypically wrinkled seeds the cultivar named « Alaska Sweet » (genotype *Ra Ra rb rb*) shows a doublet of about 50 kD instead of a single band. It is worth noticing that the same doublet appears in line 1, the double recessive coming from a cross between « Midfreezer » and « Alaska Sweet ». Variant systems similar to those observed for the 40 kD (genotype class *Ra Ra Rb Rb*), and the 50 kD (genotype class *Ra Ra rb rb*), have already been reported by THOMSON *et al.* (1978b) for vicilin subunits corresponding to 75, 30, 14 and 12 kD; the authors have shown that these variants are controlled by alternative alleles to single loci. At the genetic level, the observed complexity of vicilin could be explained by duplication(s) of original gene(s) with subsequent independent development. As duplications of genes and part of genes seem to have occurred frequently during seed storage protein evolution (THOMSON & DOLL, 1979), one can hypothesize that the process of storage protein gene duplication could have a selective advantage for plants.

As legumins and vicilins exhibit some polypeptide chains of the same molecular weight, we have shown that, if crude

proteins electrophoretic patterns may be used for cultivar identification, the isolation of vicilins is a necessary step in order to interpret wrinkled character-protein electrophoregram correlation. As a matter of fact it has already been shown that in a fraction of wheat storage proteins, namely β -gliadin, one single band may hide a more subtle heterogeneity (TERCÉ-LAFORGUE *et al.* 1980). In *Pisum sativum* the use of protein bodies as a source of globulins may be a way to discard this kind of ambiguity.

Our results, together with the results of DAVIES & BREDFORD (1980) and of SHIA & SLINKARD (1977) who have found a pleiotropic effect of the gene(s) for smooth vs. wrinkled seed on seed yield, seed weight and percentage of proteins, suggest the possible implication of both loci *ra* and *rb* on storage protein synthesis. Nevertheless our conclusions will need more numerous samples to be confirmed.

On the other hand if the differences in the form of the starch grains and in water holding capacity of the seed and consequently in the form of the seed surface result from the divergence in chemical composition, as already supposed by KOOISTRA (1962), the gene action can be reduced to the effect the gene(s) has on the metabolism of the seed.

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