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Phaseolus coccineus L. Storage Proteins. Extraction and Characterization†

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Abstract. *Phaseolus coccineus* storage globulins were extracted from mature cotyledons, purified and characterized. Three major proteins were separated. A component showing erythroagglutinating activity was thoroughly purified by thyroglobulin-Sepharose chromatography. The relative molecular masses of the three fractions are Mr = 330, 178, and 500 kDa as determined by polyacrylamide gel electrophoresis (PAGE). They correspond to the proteins found in other systems and classified as phytohaemagglutinin (PHA), vicilin and legumin, respectively. Electrophoretic analyses under denaturating conditions (SDS-PAGE) evidenced the major subunits for the three proteins. Isoelectrofocusing of the isolated proteins indicated a large heterogeneity for vicilin.

Storage proteins are accumulated in large quantities during the maturation of leguminous seeds; globulins are the major component which is represented by few different protein species (for a review, see HIGGINS 1984). The major reserve proteins of *Phaseolus vulgaris* are phaseolin (vicilin) and the lectin phytohaemagglutinin (PHA); both are glycosylated and contain mannose and glucosamine in high quantities among other sugars (RACUSEN and FOOTE 1971). Vicilin is a 6.9 S protein which contains 3 or 4 non identical polypeptides (HALL *et al.* 1978), PHA is a 6.4 S protein composed by a leucoagglutinating subunit with a relative molecular mass (Mr) of 34 kDa and an erythroagglutinating subunit with Mr 36 kDa (LEAVITT *et al.* 1977).

Phaseolus coccineus and *Phaseolus vulgaris* are genetically very close relatives (SMARTT 1970). Studies on *P. coccineus* lectins have been presented by several authors (NOWAKOVÁ and KOCOUREK 1974, MORGAN and MANEN 1985); elec-

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trophoretic patterns of total proteins have been described (LIOI 1987) but little work has been made on characterization of total storage proteins.

In this paper, we present the result of our investigations of the molecular composition of the major reserve proteins of *P. coccineus* seeds.

MATERIALS AND METHODS

Plant Material

Seeds of *Phaseolus coccineus* (L.) were kindly provided by the Botanical Garden, University of Pisa (Italy).

Protein Extraction, Purification and Fractionation

Protein extraction and protein purification through G50 Sephadex chromatography and DEAE-cellulose were carried out following the methods described by BOLLINI and CHRISPEELS (1978).

For the determination of S values, a sucrose linear gradient of the protein extract was perfomed according to BOLLINI and CHRISPEELS (1979).

Affinity Chromatography and Agglutination Assay

In order to identify the PHA fraction, proteins of the peaks from DEAE-cellulose chromatography were analysed by chromatography on a thyroglobulin-Sepharose 4B column (Pharmacia, Uppsala, Sweden) according to the procedure of FELSTED *et al.* (1975) and through agglutinating activity determinations (BOLLINI and CHRISPEELS 1978).

Gel Electrophoresis

Gels (7%) for PAGE were prepared according to DAVIS (1964) in a vertical slab apparatus. Electrophoresis was performed in 25 mM Tris-glycine buffer pH 8.3 at 85 V until the tracing dye reached the separating gel, afterwards the voltage was incresed to 140 V. Total run was about 5 h. Molecular mass standards (Sigma, USA: Mr 14–480 kDa) were used as markers.

SDS-PAGE gels were prepared according to LAEMMLI (1970). Protein samples were diluted 1 : 4 (v/v) with a solution containing 62.5 mM Tris-glycine pH 6.8, 10% glycerol, 2% SDS and 5% 2-mercaptoethanol, and heated at 100 °C for 5 min. Molecular mass standards (low range 10–100 kDa, Bio-Rad) were used as markers.

Abbreviations used: PAGE = native polyacrylamide gel electrophoresis; SDS-PAGE = electrophoresis in denaturating condition; PHA = phytohaemagglutinin; TCA = trichloroacetic acid; SDS = sodium dodecyl sulphate; PAS = periodic acid Schiff's reagent.

Electrophoresis was carried out in 25 mM Tris-glycine buffer pH 8.3 containing 0.1% SDS at 30 mA.

Gels from PAGE and SDS-PAGE were stained with 0.2% Coomassie brillant blue R in 50% methanol and 7% acetic acid for 1 h and unstained with a solution containing 20% iso-propanol and 7.5 % acetic acid. The gels were scanned with a Vernon densitometer. PAS (periodic acid, Shiff's reagent) was used to stain glycoproteins (DURANTE *et al.* 1974).

Isoelectrofocusing (IEF) and Two-Dimensional Electrophoresis (2-DE)

Proteins were separated by charge in first dimension according to the method described by OSTERMAN (1984) in a polyacrylamide gel containing 2% preblended ampholine pH 5.0–8.0 (LKB) using a LKB 2117 Multiphor apparatus. Protein samples were dissolved in 1% glycine and IEF was at 25 W for 2 h 30 min. Thereafter the proteins were separated in second dimension by molecular mass according to O'FARREL (1975) and the gel was stained with Silver Stain (Bio-Rad). For determinations of pI values the Sigma IEF-Mixer 3.5–9.3 markers were used.

PAGE and Electroelution of the Proteins

Sample of proteins (2.5 mg) was applied on a polyacrylamide gel slab (6 mm thick) and PAGE was performed under the conditions described for native PAGE. A developed strip of gel was stained to locate the position of the separated bands. Thereafter strips, each containing one type of band, were cut in horizontal, laid along the separating gel and overlaid with a solution of stacking gel. A second PAGE was run with the aim to eliminate contamination between the different proteins. After localization, the protein-containing gel zones were excised and placed on a dialysis membrane above the concentration wells of an ISCO electrophoretic concentrator with 25 mM Tris-glycine buffer pH 8.3. Electroelution was carried out for a period of 3 h at 3 W and at a temperature of 4 °C.

RESULTS

After DEAE-cellulose chromatography the proteins were distributed in three peaks: the first eluted without NaCl, the second with NaCl 0.2 M and the third with NaCl 0.3 M (Fig. 1). The elution profile from DEAE-cellulose chromatography suggested that peaks 2 and 3 may represent vicilin-like and legumin-like proteins similar to the two main storage proteins found in *Leguminosae* species. Only the protein fraction from peak 1 bound to the Thyroglobulin-Sepharose column (data not shown) presented non-specific erythroagglutinating activity when tested through the erythroagglutinating assay with ABO types of human erythrocytes (Fig. 1).

The apparent molecular masses of the three separated proteins resolved by native PAGE were: 178 kDa for vicilin, 500 kDa for legumin and 330 kDa for PHA.

Moreover, the sedimentation coefficient of the most representative protein (vicilin) was 6.9 S as determined through a linear 5–20% sucrose gradient in 25 mM phosphate buffer pH 7.0 with 0.3 M NaCl.

The molecular masses of the native proteins were confirmed after preparative PAGE (see Materials and Methods) of the total extract and electroelution of the bands (Fig. 2). Only the protein with Mr 330 kDa showed erythroagglutinating activity.

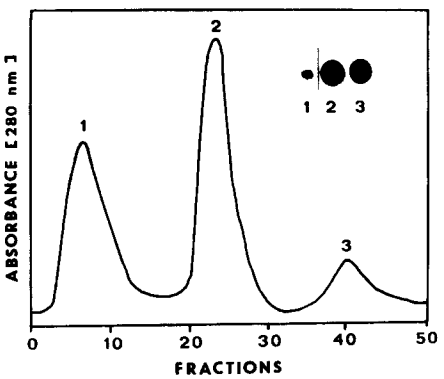

Fig. 1. Elution tracing of *P. coccineus* seed globulins from DEAE-cellulose. Fractions 1, 2, 3 were eluted with linear gradient of NaCl (from 0.0 to 0.4 M). The peaks represent phytohaemagglutinin, vicilin and legumin, respectively. The photograph represents the erythroagglutination test for the three fractions.

The purified proteins were resolved into their constituent subunits by SDS-PAGE and the results are reported in Fig. 3, which shows the presence of three major subunits for PHA with 34, 32, 30 kDa and for legumin (40, 37, 20 kDa), while three major components (48, 46.5, 44 kDa) and three minor components (28.3, 24.4, 17 kDa) were found for vicilin.

Gels derived from PAGE and SDS-PAGE of proteins separated and purified through electroelution were PAS stained in order to evidence the presence of carbohydrates bound to polypeptides. After PAGE, PHA and vicilin were PAS positive, whereas legumin did not show any reaction with the stain. After SDS-PAGE, only the two heavier polypeptides of vicilin appeared to be stained, while all major PHA components showed glycosilation.

Further fractionation of total extract and of the separated proteins was carried out by isoelectrofocusing in a pH 5.0–8.0 gradient. As shown in Fig. 4, the IEF profile of the total extract displayed numerous components between pI 5.4–7.37. Isoelectrofocusing of the isolated proteins (Fig. 4) indicated a great heterogeneity for vicilin and PHA (several bands at pI 5.9–5.1 and pI 7.37–5.4, respectively), while for legumin two major bands at pI 6.1 and 5.9 were present.

The IEF separated bands corresponding to legumin and vicilin showed in

2-DE/SDS-PAGE the polypeptide composition typical of these proteins, while PHA band did not show a uniform polypeptide composition. In fact, the pI 7.37 band contains all three polypeptides, the pI 6.0 band contains only the 32 kDa subunit, while all the other bands contain two polypeptides with Mr 34 and 30 kDa (Fig. 5). These results clearly demonstrated that a great microheterogeneity exists in the reserve protein subunits.

DISCUSSION

The storage proteins of *Phaseolus coccineus* were analyzed on the basis of their solubility in NaCl through DEAE-cellulose chromatography and three major proteins were identified corresponding to PHA, vicilin and legumin found in other *Leguminosae* species. The molecular masses, polypeptide composition and microheterogeneity of the three proteins were investigated using electrophoretic techniques. PHA showed one diffuse PAS-positive band in native PAGE with the apparent molecular mass of 330 kDa and three major subunits with Mr 34, 32, 30 kDa in SDS-PAGE. According to MORGAN and MANEN (1985), most of the cultivars and wild race varieties of *P. coccineus* examined by them can be classified in three groups represented by 5, 3, 1 lectin bands, while only the cultivar Alubia showed two lectin bands. In SDS-PAGE these lectins were separated in three very close bands with Mr 29.5 kDa. The cv. Alubia was previously studied by NOWAKOVÁ and KOCOUREK (1974): these authors evidenced the presence of two lectins (PHA I and PHA II) with Mr 180 kDa, whereas SDS-PAGE analysis showed only one band (Mr 34 kDa).

The PHA isolated from our accession showed erythroagglutinating capability without blood group specificity as evidenced for the lectins studied by the above mentioned authors. The different molecular masses and polypeptide composition can be explained both with the different extraction and purification techniques used and with the existence of genetic differences between varieties and accessions. When analyzed through IEF followed by two-dimensional SDS-PAGE, PHA showed a large charge heterogeneity.

The most representative storage protein in *P. coccineus* is vicilin. The molecular mass of the native protein, the sedimentation coefficient and polypeptide composition are similar to those reported in several cultivars of *P. vulgaris* by several authors (DERBYSHIRE *et al.* 1976, BOLLINI and CHRISPEELS 1978).

P. coccineus vicilin is a glycoprotein: in fact, the band in native PAGE is PAS-positive, but the PAS staining of the subunits after SDS-PAGE revealed that only the two heavy polypeptides contained carbohydrates, whereas the lighter one had little or none. This result is in agreement with that reported by ERICSON *et al.* (1973) in *P. aureus*.

Vicilin also shows in IEF charge heterogeneity and each protein band separated by IEF contains all the three components of vicilin as evidenced by 2-DE. This result is

in agreement with that reported by BROWN *et al.* (1980) in *P. vulgaris* cv. Tendergreen.

The third protein separated by DEAE-cellulose with the higher concentration of NaCl corresponds to legumin-like fraction. Legumin is soluble in more concentrated salt solution than vicilin and it is a protein with Mr around 400 kDa and sedimentation coefficient around 12 S (DANIELSSON 1949). Legumin is the major storage protein in *Pisum sativum* (CROY *et al.* 1980), *Vicia faba* (WRIGHT and BOULTER 1974) and *Glycine max* (BADLEY *et al.* 1975), whereas the more representative storage protein in the genus *Phaseolus* is vicilin. Detectable legumin amount occurs in *P. coccineus* (present results) and in *P. aureus* (ERICSON and CHRISPEELS 1973). The elution profile of DEAE-cellulose column of *P. vulgaris* globulins does not show a peak at high NaCl concentration (BOLLINI and CHRISPEELS 1978), although DERBYSHIRE *et al.* (1976) have reported its presence.

The native PAGE of legumin purified through the electroelution method showed a unique PAS-negative band whose apparent Mr is quite different from that reported for *P. vulgaris* legumin (340 kDa) (DERBYSHIRE *et al.* 1976). Further analyses are necessary in order to determine if the structure of *P. coccineus* legumin can be assimilated to the currently accepted molecular model of legumin presented for *Vicia faba* by WRIGHT and BOULTER (1974) and extended to *Glycine max* (BADLEY *et al.* 1975) and *Pisum* (CROY *et al.* 1980).

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REFERENCES

BADLEY, A. R., ATKINSON, D., HAUSER, H., OLDANI, D., GREEN, J. P., STUBBS, J. M.: The structure, physical and chemical properties of the soybean protein glycinin. – Biochim. Biophys. Acta **412** : 214–228, 1975.

BOLLINI, R., CHRISPEELS, M. J.: Characterization and subcellular localization of vicilin and phytohemagglutinin the two major reserve proteins of *Phaseolus vulgaris* L. – Planta **142** : 291–298, 1978.

BOLLINI, R., CHRISPEELS, M. J.: The rough endoplasmic reticulum is the site of reserve-protein synthesis in the developing *Phaseolus vulgaris* cotyledons. – Planta **146** : 487–501, 1979.

BROWN, J. W. S., BLISS, F. A., HALL, T. C.: Microheterogeneity of G1 storage protein from French bean with isoelectrofocusing. – Plant Physiol. **66** : 838–840, 1980.

CROY, R. R. D., DERBYSHIRE, E., KRISAND, T. G., BOULTER, D.: Legumin in *Pisum sativum* and *Vicia faba*. – New Phytol. **83** : 29–35, 1980.

DANIELSSON, C. E.: Seed globulins of the gramineae and leguminosae. – Biochem. J. **44** : 387–400, 1949.

DAVIS, B. J.: Disc electrophoresis – II Method and application to human serum proteins. – Ann. N. Y. Acad. Sci. **121** : 404–427, 1964.

DERBYSHIRE, E., WRIGHT, D. J., BOULTER, D.: Legumin and vicilin, storage proteins of legume seeds. – Phytochemistry **15** : 3–24, 1976.

DURANTE, M., COVI, A., BARSANTI, L.: Microsomal rat liver B-glucuronidase: characterization and behaviour. – Ital. J. Biochem. **23** : 292–305, 1974.

ERICSON, M. C., CHRISPEELS, M. J.: Isolation and characterization of glucosamine-containing storage glycoproteins from the cotyledons of *Phaseolus aureus*. – Plant Physiol. **52** : 98–104, 1973.

FELSTED, R. D., LEAVITT, R. D., BACHUR, N. R.: Purification of the phytohemagglutinin family of proteins from red kidney beans (*Phaseolus vulgaris*) by affinity chromatography. – Biochim. Biophys. Acta **405** : 72–81, 1975.

HALL, T. C., MA, Y., BUCHBINDER, B. U., PYNE, J. W., SUN, S. M., BLISS, F. A.: Messenger RNA for G1 protein of French bean seeds: cell-free translation and product characterization. – Proc. nat. Acad. Sci. **75** : 3196–3200, 1978.

HIGGINS, T. J. V.: Synthesis and regulation of major proteins in seeds. – Annu. Rev. Plant Physiol. **35** : 191–221, 1984.

LAEMMLI, U. K.: Cleavage of structural proteins during the assembly of the head bacteriophage T4. – Nature **227** : 680–689, 1970.

LEAVITT, R. D., FELSTED, R. L., BACHUR, N. R.: Biological and biochemical properties of *Phaseolus vulgaris* isolectins. – J. biol. Chem. **252** : 2961–2980, 1977.

LIOI, L.: Species relationships in *Phaseolus*: electrophoretic analysis of seed storage proteins. – Biol. Plant. **29** : 49–53, 1987.

MORGAN, M. R., MANEN, J. F.: Lectin variability in *Phaseolus coccineus*. – Phytochemistry **24** : 1981–1985, 1985.

NOWAKOVÁ, N., KOCOUREK, J.: Isolation and characterization of hemagglutinin from scarlet runner seeds (*Phaseolus coccineus* L.) – Biochim. Biophys. Acta **359** : 320–332, 1974.

O'FARREL, P. H.: High resolution two-dimensional electrophoresis of proteins. – J. biol. Chem. **250** : 4007–4021, 1975.

OSTERMAN, L. A.: Methods of Protein and Nucleic Acid Research, Vol. 1. – Springer-Verlag, Berlin-Heidelberg-New York-Tokyo 1984.

RACUSEN, D., FOOTE, M.: The major glycoprotein in germinating bean seeds. – Can. J. Bot. **49** : 2107–2111, 1971.

SMARTT, J.: Interspecific hybridization between cultivated American species of the genus *Phaseolus*. – Euphytica **19** : 480–489, 1970.

WRIGHT, D. J., BOULTER, D.: Purification and subunit structure of legumin of *Vicia faba* L. – Biochem. J. **141** : 413–425, 1974.

Figs. 2–5 at the end of the issue.