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IMMUNOELECTROPHORETIC STUDIES WITH BEAN PROTEINS

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Abstract—The immunoelectropherograms of twenty-four extracts from different varieties of kidney beans (*Phaseolus vulgaris*) differed from each other, revealing between eight and eleven precipitation lines. A crystalline protein and a pure globulin from black beans gave identical immunological reactions although they were different in chemical composition. The antisera from rabbits sensitized with this crystalline protein produced one or two precipitation lines with the extracts of different beans. Nineteen of the bean extracts had haemagglutinating activity. The corresponding extracts originated one or two precipitation lines which could be stained with sudan black, indicating the presence of lipoproteins; none of the haemagglutinin-free extracts originated stainable immunoprecipitates. It is concluded that the bean phytohaemagglutinins are probably lipoproteins.

INTRODUCTION

QUANTITATIVE serological methods have been applied for many years to taxonomic investigations and are useful for the differentiation of plant species since they may offer an overall picture of the similarity of the proteins.[1] The immunoelectrophoretic method offers the possibility of distinguishing different proteins without the need of previous separation and is therefore exceptionally well suited for comparison of protein patterns from different origins. The wide variations in the anatomical, chemical and physiological characteristics in different varieties of some plant species suggest that similar differences may exist in protein patterns also within a single species. Therefore, the number and electrophoretic mobility of the antigenic proteins from different varieties of kidney beans (*Phaseolus vulgaris*) were compared and the immunochemical properties of some of the homologous proteins from different seed samples were studied. Moreover, the immunopherograms of bean varieties with and without phytohaemagglutinins were compared in an attempt to identify the precipitation lines corresponding to the lectins.

RESULTS

The immunoelectropherograms produced by the NaCl solution extracts and the water-soluble proteins with the corresponding antisera revealed that more precipitation lines were obtained in the latter case than in the former. Therefore these sera were used for the comparison of the different bean samples. They produced patterns which differed from each other by the position and in some cases also the number of precipitation lines. Between eight and eleven lines could be distinguished in the reaction between the various extracts and the anti-black-bean serum. The great variety of the patterns made it difficult to detect the homologous proteins. The variations in the method of sample preparation, including washing of the ground bean seeds with hexane prior to preparing the extracts, lyophilization of the extracts, and time of holding the extracts prior to the immunological reaction were of little importance

[1] C. A. LEONE, editor, *Taxonomic Biochemistry and Serology*, The Ronald Press, New York (1964).

for the results. The use of different antisera caused more evident differences in the resulting precipitation patterns, but extracts from various bean cultivars were always distinguishable when compared with the reference sample from black beans.

Antiserum prepared from the rabbits injected with the crystalline protein F from black beans produced only one precipitation line with the total extract or the water-soluble proteins from this bean variety. An identical line appeared with the globulin E and the crystallized protein F. Sera from rabbits sensitized with the globulin E or the crystallized protein F behaved exactly in the same way, producing identical results with these two purified antigens or the bean extract.

Extracts from all the bean samples prepared with NaCl solution were tested with the anti-F serum. In twenty cases, a single precipitation line appeared and four extracts produced two lines in the corresponding experiments. The single line was very faint in one case (Fig. 1). Absorption of antiserum anti-F by black-bean meal or Saxa bean meal suppresses the formation of the corresponding precipitation line between these extracts and the antiserum. When an extract of one of the bean varieties producing two lines was tested with the absorbed serum, one line was still formed.

Staining with sudan black was applied to the immunoelectrophoresis slides of the water-soluble proteins of the different bean varieties and the antiserum of rabbits sensitized with the albumins from black beans and to the slides used for the double-diffusion test. In nineteen of the twenty-four samples tested, the presence of one or two lines stainable by this technique was observed (Fig. 2). The extract of these same seed samples showed haemagglutinating activity when assayed with rabbit erythrocytes, but those inactive as agglutinins failed to give the coloration for lipoproteins with the staining technique indicated. The isolated haemagglutinins A and B, prepared according to Jaffé and Hannig[2] from black beans, and the purified lectin of Takahashi *et al.*[3] from wax beans revealed also a precipitation line stainable with sudan black when they were analysed by the immunoelectrophoretic method using the same anti-black-bean serum. None of the fractions from beans devoid of haemagglutinating action produced immunoprecipitation under these conditions which could be stained by this method.

DISCUSSION

The differences of the immunoelectrophorograms between the seed extracts of various varieties of kidney beans observed in the present experiments demonstrate that they may contain antigenic proteins in different number and of different electrophoretic behaviour, confirming previous observations with black beans and red kidney beans.[2] The results, moreover, show that bean varieties not only differ in the patterns of the unfractionated seed extracts but also in the behaviour of homologous proteins. In several cases the proteins from the different seed samples precipitating with antiserum against the crystalline protein F from black beans exhibited remarkable differences in migration velocity, intensity of immunological reaction, and even in the number of precipitation lines (Fig. 1). It is possible that a low intensity of the immunological reaction may be attributed, at least in some cases, to low solubility rather than to low immunological affinity.

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[3] T. TAKAHASHI, P. RAMACHANDRAMURTHY and I. E. LIENER, *Biochim. Biophys. Acta* **133**, 123 (1967).

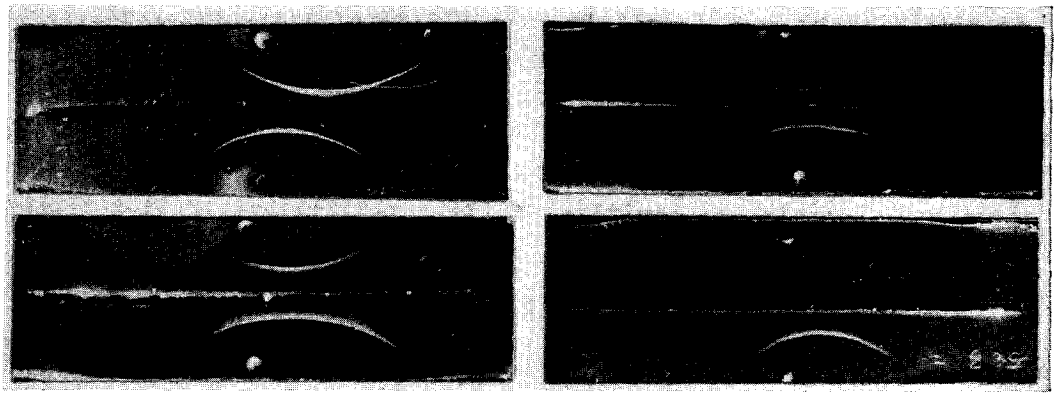

FIG. 1. IMMUNOPRECIPITATING SYSTEM PRODUCED BY ELECTROPHORESIS OF EXTRACTS FROM DIFFERENT BEAN CULTIVARS AND ANTISERUM AGAINST BEAN GLOBULIN E FROM BLACK BEANS, CULTIVAR CUBAGUA.

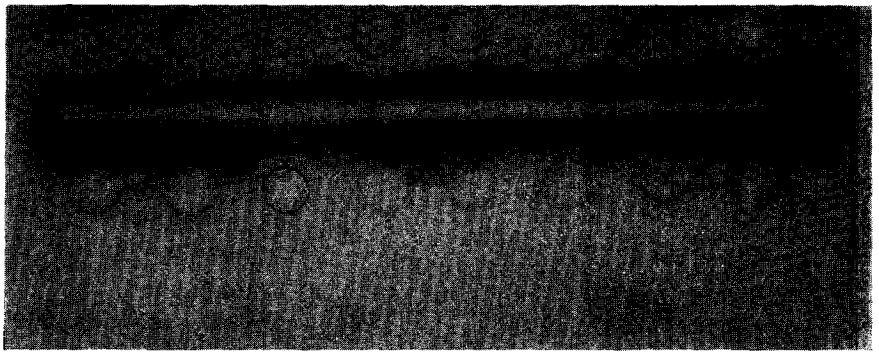

FIG. 2. PRECIPITIN SPECTRA OF BEAN LIPOPROTEINS PRODUCED BY DOUBLE-DIFFUSION TEST OF EXTRACTS FROM SIXTEEN DIFFERENT BEAN CULTIVARS.

The antiserum used was obtained by injecting the water-soluble proteins from the cultivar Balin de Albenga. Coloration with sudan black was applied. The four extracts which gave no stainable precipitation were negative in the haemagglutination test.

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It has been observed previously that the globulin E and the crystalline protein F gave identical reactions in the double-diffusion test, but differed in several chemical and physical properties.[2] Mobility of protein F in paper electrophoresis at pH 8·6 was greater than that of the globulin E; they also differed in their quantitative composition of amino acids and sugars. Recently it was found that pepsin digestion liberates eighteen peptides from the crystalline protein F and nineteen from globulin E, but only sixteen are common to both.[4]

Their identical immunological behaviour could be demonstrated in the present work not only by the double-diffusion test and the immunoelectrophoresis but also in the tests with the respective immunosera produced by sensitizing rabbits with either of these proteins. The relation between the proteins E and F from beans is comparable to that between gliadine and gluteline of wheat, for which Benhamou-Glynn *et al.*[5] have found an immunological cross-reaction, but which are different in solubility and electrophoretic mobility. The existence of some common subunit(s) would be a likely explanation for this behaviour. The detection of a great number of peptides common to both bean proteins[4] supports this hypothesis.

The formation of two lines in the experiments with the extracts of some of the bean samples and the fact that absorption of the antiserum with black-bean meal removed only one antigen indicates the presence of two proteins with different migration characteristics and which react with the antibodies of the anti-F sera. It will be interesting to compare the chemical and immunological properties of both proteins from one of these bean varieties.

The appearance of precipitation lines stainable with sudan black in the immunoelectrophoretic analysis of bean extracts indicates lipoproteins in these seeds, an observation which confirms prior findings of Ghetie[6] and of Jaffé and Hannig.[2] Only in the bean varieties exhibiting haemagglutinating activity was this staining reaction positive (Fig. 2), pointing to the possibility that the bean haemagglutinins are lipoproteins. This conclusion is in accordance with the results of assays with purified bean agglutinins. Two of the agglutinating fractions from black beans of Jaffé and Hannig[2] and one prepared from wax beans by Takahashi *et al.*[3] produced immunoprecipitations clearly stainable with sudan black but none of the proteins without haemagglutinating activity did. These results support the conclusion that the bean agglutinins are lipoproteins. Takahashi *et al.*[3] found that only 92 per cent of the weight of the wax bean agglutinin can be accounted for on the basis of amino acids and carbohydrates and considered the possibility that the rest of the molecule may be lipid. Evidence that two other phytohaemagglutinins are also lipoproteins have been obtained, i.e. for ricin from castor beans[7] and for crepitin from *Hura crepitans*.[8]

Differences in immunochemical behaviour between the lipoproteins from different bean varieties similar to those described for the proteins E and F were observed, pointing to variations in size, shape and charge of the respective molecules which are also detectable by physicochemical methods (Fig. 2). Takahashi *et al.*[3] compared the properties of the haemagglutinin from wax beans with those described by Rigas and Osgood[9] for that of red kidney beans and by Jaffé and Hannig[2] for that of black beans. Sedimentation constants, diffusion coefficients, isolectric points, partial specific volume and carbohydrate contents were quite

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[6] V. Ghetie and L. Buzila, *Studii cercetaria Biochimie (Rumania)* **4**, 241 (1961).
[7] W. G. Jaffé, F. Wagner, P. Marcano and P. Hernández, *Acta Cient. Venezol.* **15**, 29 (1954).
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[9] D. A. Rigas and E. E. Osgood, *J. Biol. Chem.* **212**, 607 (1955).

different. Despite these differences, the molecular weights of the three proteins are all about 130,000 and all have a low content of sulphur amino acids.

More than one haemagglutinin may exist in kidney beans.[2,3] Multiple precipitation lines stainable by sudan black were observed in some bean varieties but not in all (Fig. 2). It will be interesting to determine whether multiple lectins exist only in those bean seeds which contain several lipoproteins.

The results of the present experiments revealing a great diversity in the immunological behaviour of the proteins of a single botanical species do not invalidate the taxonomic conclusions drawn from immunological studies on beans by Kloz *et al.*,[10] but suggest the need to establish the range of variability within both cultivated and wild species. Leaf proteins from different bean species differ less from each other than those from seeds.[11] Differences between the proteins of the seeds of soybean varieties have been detected by gel electrophoresis[12] but no difference between the leaf proteins of two soybean varieties studied by this method was observed.[13] The present experiments have not yet been extended to leaf proteins.

EXPERIMENTAL

Part of the seeds used were acquired at the local market and part were from the collection of this department. Samples of twenty-four different varieties of *Phaseolus vulgaris* were examined.

10 g of finely ground seeds were suspended in 100 ml of 1% NaCl solution, stirred for 2 hr at room temperature and filtered. The soluble proteins were obtained by dialysation of the salt extracts against distilled water for 3 days at 4°, centrifugation, saturation of the supernatant with $(NH_4)_2SO_4$, filtration of the precipitate, dissolution in distilled water, dialysation and lyophilization. A crystallized bean protein was prepared according to Bourdillon[14] and called fraction F. Fraction E was the more soluble bean globulin prepared by the method of Goa and Strid.[15] The isolated bean haemagglutinins used were those of Jaffé and Hannig[2] and of Takahashi *et al.*[3]

The immunosera were obtained from rabbits of about 2 kg weight injected subcutaneously with 0·5 ml of 2% protein solution in complete Freud coadjuvans (Difco); 1 week later, 0·5 ml of a 0·8% antigen solution without coadjuvans was injected intravenously and 0·3 ml subcutaneously. This treatment was repeated three times weekly until nine injections had been given. If the double-diffusion test with the blood serum was satisfactory after that time, blood was drawn by heart puncture and the serum prepared; if not, another series of six injections was applied.

The NaCl extract, the water-soluble proteins, fractions E and F from the black bean variety Cubagua and the red bean variety Saxa were used for the preparation of the corresponding antisera. The micro-method of immunoelectrophoresis analysis of Grabar and Williams[16] in veronal buffer, pH 8·6, μ 0·075, and the double-diffusion technique of Ouchterlony were used and staining with azocarmin or sudan black by the procedures as given by Crowle[17] was applied. Haemagglutinating activity was investigated with washed rabbit erythrocytes by the centrifugation technique.[2]

Absorption experiments were performed by mixing 0·3 ml of antiserum with 1 mg of bean meal, incubating for 1 hr at 37°, storing for 48 hr at 4° and centrifugation for 1 hr. This procedure was repeated three times. Three different antiserum were used in this test.

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