# PURIFICATION AND PROPERTIES OF THE PHYTOHEMAGGLUTININ OF PHASEOLUS VULGARIS*

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The existence of hemagglutinins in the seeds of many species of plants, particularly in those of Leguminosae, has been known for many years (1–7). In a previous communication (8) the application of the phytohemagglutinin of *Phaseolus vulgaris* (red kidney beans) or *Phaseolus communis* (navy beans) for the separation of leucocytes from erythrocytes was described. The advantages of having this agglutinin available in a pure stable dry state of known titer are obvious. Partially purified products obtained from *P. vulgaris* in the past (9–12) indicated that this phytohemagglutinin is a protein, and it was thought to be an albumin.

In a preliminary report (13), we summarized a procedure for obtaining this agglutinin in the form of a mucoprotein. We have recently observed that this material, in an environment at low pH, dissociates into two parts, one a protein hemagglutinin and the other an inactive polysaccharide. In this paper we shall present first, in brief, the method of purification of the mucoprotein,[1] and then, in more detail, the purification of the active protein.

## *Materials and Methods*

Dry beans of *P. vulgaris* or *P. communis*, finely ground, were used. Low temperature ethanol fractionation and salting out procedures were applied. Electrophoretic analyses were performed with a Klett apparatus by utilizing the 11 ml. cell and buffers of ionic strength 0.1 (14). The patterns were recorded by Longsworth's scanning technique after a migration for 180

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[1] This mucoprotein in partially purified form but of high agglutination titer, prepared by a simplification of the method outlined in this paper, is now commercially available from Difco Laboratories, Inc., Detroit 1, Michigan, under the name Bacto-Phytohemagglutinin, code No. 0528.

minutes at a temperature of 1°. The sampling device (15) was used to obtain fractions from the electrophoretic cell for chemical analyses and agglutination titrations. Agglutination titers were determined by the Salk technique (16).

## EXPERIMENTAL

*Preparation of Phytohemagglutinin in Mucoprotein State*—This method was developed with information derived from studies performed on frac-

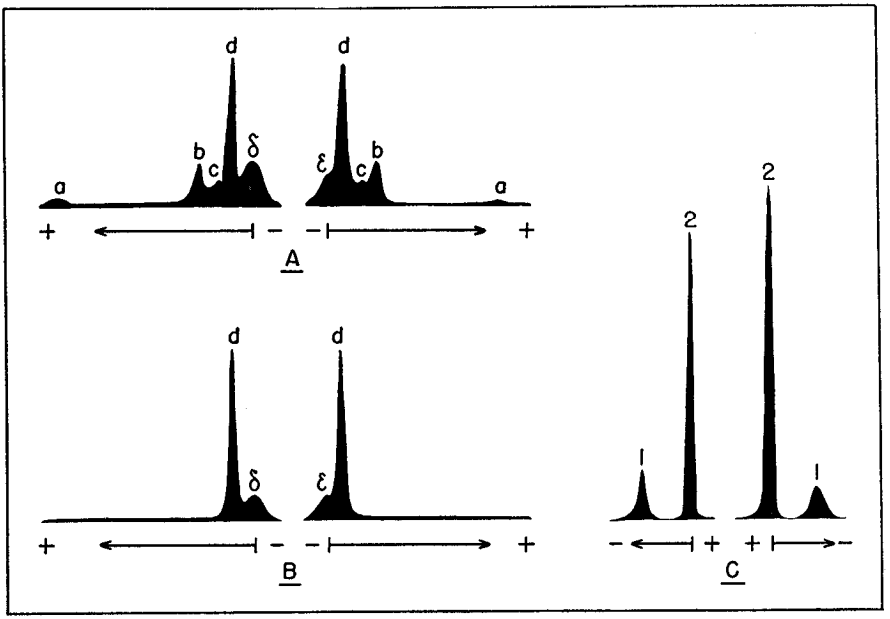


FIG. 1. Electrophoretic patterns taken after a 3 hour migration in Miller's buffers, ionic strength 0.1. *A*, partially purified product at pH 8.0. *B* and *C*, purified mucoprotein at pH 8.0 and 2.0, respectively.

tions obtained from the electrophoretic cell during the analysis of a partially purified product. The electrophoretic pattern of this product is shown in Fig. 1, *A*. It was thus possible to demonstrate that Component *d* is the phytohemagglutinin, which gives both protein and polysaccharide reactions, and that Components *a*, *b*, and *c* are inactive proteins; $\delta$ and $\epsilon$ are merely the false boundaries. It was further possible with such fractions to compare the solubility characteristics of Component *d* with those of the others. With the use of electrophoretic analysis as a test for purity, the method summarized in Scheme 1 was developed.

The material so obtained behaves as a homogeneous substance from pH 5.8 to 8.6. Fig. 1, *B* shows the electrophoretic pattern of the purified

mucoprotein obtained under conditions identical to those used for the partially purified product. The electrophoretic mobility-pH curve, when extrapolated, intercepts the zero mobility line in the neighborhood of pH 5.6. No protein or polysaccharide was found under the false boundary $\epsilon$. The product contained 6.5 per cent nitrogen (micro-Kjeldahl) and 50.4 per cent total reducing substances estimated as glucose, when the Nelson modifica-

1 kilo bean flour; extract with 4 liters 1% NaCl for 24 hours at 0°; centrifuge

↓

| | |
|---|---|
| Residue; discard | Extract; adjust pH to 5.7; add cold (−2°) ethanol to 30%; centrifuge |
| Ppt.; discard | Supernatant solution; add cold (−2°) ethanol plus 10% ethyl ether to 75% ethanol; centrifuge |
| Supernatant solution; discard | Ppt.; dry; dissolve in 600 ml. 1% NaCl; repeat ethanol fractionation as above |

↓

Ppt. (from 75% ethanol); dry; dissolve in 400 ml. 1% NaCl; repeat ethanol fractionation once more

↓

Ppt. (from 75% ethanol); dry; dissolve in 200 ml. 0.1 M phosphate buffer, pH 8.0, 0°; add ammonium sulfate to 0.4 saturation; centrifuge

↓

| | |
|---|---|
| Ppt.; discard | Supernatant solution; add ammonium sulfate to 0.7 saturation; centrifuge |
| Supernatant solution; discard | Ppt.; dissolve in water, dialyze, centrifuge, dilute to 100 ml.; repeat ammonium sulfate fraction |

↓

Ppt. (from 0.7 saturation with ammonium sulfate); dissolve in water; dialyze free of sulfate; lyophilize

↓

Phytohemagglutinin (approximately 8.0 gm.)

SCHEME 1. Diagrammatic representation of the procedure for purification of the mucoprotein phytohemagglutinin.

tion (17) is applied to the method of Somogyi (18). It is, therefore, a mucoprotein. It is very soluble in distilled water, saline, or buffer solutions, and is inactivated by being boiled. Electrophoretic analyses performed below pH 5.8 yielded patterns progressively asymmetric and, at pH 2.0, the mucoprotein completely decomposed into two components, one of which remained stationary while the other migrated as a cation (Fig. 1, *C*). Tests on fractions obtained from the electrophoretic cell showed that

Component 2 is an inactive polysaccharide; Component 1 is the hemagglutinin which is a euglobulin and can be salted out free from the polysaccharide only when the pH is maintained at 1.0. Attempts to separate them by low temperature ethanol or acetone procedures failed at all pH reactions.

*Purification of Protein Phytohemagglutinin*—All work was performed in a cold room at 1°, unless otherwise specified. A motor-driven Sharples supercentrifuge was used whenever high centrifugal force was necessary for complete sedimentation of the precipitates.

*Extraction*—1 kilo of ground beans (*Phaseolus vulgaris*) is slowly poured into 5 liters of ice-cold dilute hydrochloric acid of pH 1.0 (approximately 0.1 N) with mechanical stirring. The suspension is stirred for 24 hours and then centrifuged for 10 to 15 minutes at about 1000 × *g*. The reddish, viscous, slightly turbid extract is decanted and saved.

*Fractionation with Ammonium Sulfate*—To the extract an equal volume of ice-cold saturated ammonium sulfate solution[2] of pH 1.0 is slowly added with constant stirring, giving a final concentration of 25.4 gm. of ammonium sulfate per 100 ml. of solution. The suspension may be kept overnight and then centrifuged at 13,000 × *g*, with a sufficiently low rate of flow so that the pink effluent solution shows only slight turbidity.

To the clarified solution an equal volume of the saturated ammonium sulfate of pH 1.0 is slowly added with mechanical stirring, thus raising the final concentration to 38.1 gm. per 100 ml. The suspension should stand for 24 hours, after which any clear supernatant fluid is carefully siphoned off and discarded. The rest is centrifuged at 13,000 × *g*, the rate of flow being adjusted so that almost complete clarification occurs. The pinkish precipitate, finely dispersed in approximately twice its volume of distilled water, is transferred to thin wall, small diameter cellophane tubes[3] and dialyzed against water until the pH has risen to 3.9 and the volume has increased about 50 per cent. This can be accomplished by dialysis against running water at 14° for 36 hours, followed by dialysis at 1° against 40 times the original volume of distilled water for another 36 hours. The suspension is then centrifuged, and the clear, straw-colored supernatant solution is saved.

The pH is adjusted to 5.8 with 1.0 N NaOH. The precipitate is allowed to settle for 24 hours, and the clear, yellowish supernatant solution is recovered by siphoning or centrifugation.

The protein phytohemagglutinin is now precipitated in pure form by a

[2] The ammonium sulfate solution used in this step can be prepared by dissolving 508 gm. of crystalline ammonium sulfate in 600 ml. of distilled water to which 80.5 ml. of concentrated HCl have been added, diluted to 1000 ml. with distilled water.

[3] Cellophane sausage casing, size 27/32, obtainable from the Visking Corporation, Chicago, Illinois, was used.

10-fold dilution with ice-cold distilled water, centrifuged, and lyophilized. The yield is approximately 2 gm. per kilo of beans. A flow sheet summarizing this method is presented in Scheme 2.

1 kilo bean flour; extract with 5 liters dilute HCl (approximately 0.1 N), pH 1.0, at 1° for 24 hrs.; centrifuge

↓

| | |
|---|---|
| Residue; discard | Extract; add equal volume of cold (1°) concentrated ammonium sulfate solution, pH 1.0, to obtain final salt concentration of 25.4 gm. per 100 ml.; centrifuge |
| | ↓ |
| Ppt.; discard | Clarified solution; add equal volume of same ammonium sulfate solution to obtain final salt concentration of 38.1 gm. per 100 ml.; centrifuge |
| | ↓ |
| Clarified fluid; discard | Ppt.; suspend in twice its volume distilled water; dialyze against running tap water, followed by distilled water, until pH of 3.9 and 50% volume increase is reached; centrifuge |
| | ↓ |
| Residue; discard | Supernatant solution; adjust pH to 5.8 by dropwise addition of 1.0 N NaOH; centrifuge |
| | ↓ |
| Ppt.; discard | Supernatant solution; dilute 10-fold with cold distilled water; centrifuge |
| | ↓ |
| Supernatant fluid; discard | Ppt.; lyophilize |

↓

Protein phytohemagglutinin (approximately 2.0 gm.)

SCHEME 2. Diagrammatic representation of purification procedure of protein phytohemagglutinin.

*Properties*—The protein phytohemagglutinin thus obtained is an amorphous product, insoluble in distilled water, soluble in 0.85 per cent saline, and all the buffer solutions (14) that have been tried. In solution, it is inactivated at boiling temperature but is quite stable at 1°, even at a pH as low as 1.0 (a solution stored in the refrigerator for 1 month at pH 1.0 retained its original agglutination titer). It contains 14.6 ± 0.05 per cent nitrogen, 3.4 ± 0.25 per cent total reducing substances, and 7.25 per cent tyrosine determined by the Folin-Ciocalteu method (19). Electrophoretic analyses between pH 2.0 and 8.0 showed it to be homogeneous, with an isoelectric point at pH 6.5. Fig. 2, *I*, depicts the patterns obtained at pH

2.0, 6.47, and 8.0. An analysis[4] of a 0.7 per cent solution in Miller's buffer, pH 7.0, ionic strength 0.1, with a Spinco analytical ultracentrifuge at 52,600 r.p.m., temperature 26°, revealed a large homogeneous component having a sedimentation constant of 7.2 S, contaminated by only a small amount of material sedimenting ahead of it (Fig. 2, *II*).

The insolubility of this protein in distilled water and its low electrophoretic mobility[5] indicate that it is a euglobulin.

*Titration and Use of Phytohemagglutinin for Leucocyte Separations*—We have found the small tube method described by Salk (16) the most suitable

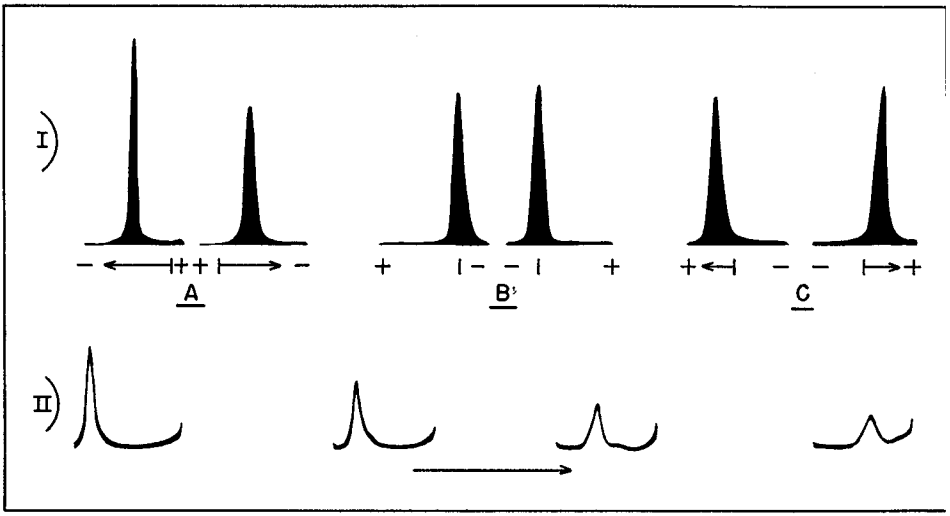


FIG. 2. *I*, electrophoretic patterns of the purified protein-phytohemagglutinin taken after a 2 hour migration in Miller's buffers, ionic strength 0.1. *A*, pH 2.0; *B*, pH 6.47; and *C*, pH 8.0. *II*, ultracentrifugal patterns of the same preparation taken with a Spinco ultracentrifuge rotor speed 52,640 r.p.m., temperature 26°, time after beginning of sedimentation 16, 32, 56, and 80 minutes.

for titration of the phytohemagglutinin, since it gives a clear, objective, and reproducible end-point. Such titrations of both the mucoprotein and the protein are shown in Table I, Columns 2 and 3. The final concentration of the phytohemagglutinin in the titration tubes is shown in Column 1. The mucoprotein gives a positive agglutination down to 10 $\gamma$ per ml., and the protein down to 0.1 $\gamma$ per ml.

For routine leucocyte separations, it is preferable to use the titration

[4] We are greatly indebted to Dr. John W. Gofman and Dr. Frank T. Lindgren of the Donner Laboratory, University of California, Berkeley, for carrying out the ultracentrifugal analysis.

[5] The studies of the electrophoretic mobility-pH curve, as well as of other physicochemical constants of this protein, will be presented in a subsequent paper.

method of Li and Osgood (8). A solution should be prepared in 0.85 per cent saline of 0.1 gm. per 100 ml. of the mucoprotein, or 0.01 gm. per 100 ml. of the protein phytohemagglutinin. The solution should be sterilized by filtration through a Seitz filter, and a series of 2-fold dilutions with 0.85 per cent saline should then be prepared. An aliquot of 0.1 ml. of the dilution to be tested is introduced into a centrifuge tube containing 10 ml. of blood with sufficient anticoagulant, and the directions given by Li and Osgood (8) followed. The optimal amount is the one which gives the maximal yield of leucocytes with a minimal admixture of erythrocytes. This

TABLE I

*Agglutination Titers (Salk Method) of Purified Phytohemagglutinin*

| Final concentration phytohemagglutinin, γ per ml. | Mucoprotein phytohemagglutinin | Protein phytohemagglutinin | Supernatant solution from protein phytohemagglutinin titration tubes | Preparation made by Goddard-Mendel method |
|---|---|---|---|---|
| 2000 | + | + | | + |
| 1000 | + | + | + | ± |
| 500 | + | + | + | − |
| 200 | + | + | + | − |
| 100 | + | + | + | − |
| 50 | + | + | + | − |
| 20 | + | + | + | − |
| 10 | + | + | + | − |
| 5 | ± | + | + | − |
| 2 | − | + | + | − |
| 1 | − | + | + | − |
| 0.5 | − | + | − | − |
| 0.2 | − | + | − | − |
| 0.1 | − | + | − | − |
| 0.05 | − | ± | − | − |
| 0.025 | − | − | − | − |

optimum varies somewhat between different individuals, from 0.1 to 0.25 ml. of the sterilized solution per 10 ml. of blood.

*Mode of Action of Phytohemagglutinin*—Whether this phytohemagglutinin agglutinates the erythrocytes by actually combining with them, or by altering their membrane through an enzyme-like action, was investigated by a second titration of the supernatant solution obtained from each tube of the titration shown in Column 3, Table I. The results are recorded in Column 4. It is apparent that the agglutination titer has been diminished to one-tenth. An enzymatic action being thus unlikely, it is concluded that human erythrocytes can adsorb or otherwise inactivate about 10 times the amount of phytohemagglutinin necessary to give a positive agglutination. The sites of action on the erythrocyte surface apparently are

different from the A, B, and Rh sites, since all types of human erythrocytes, including type O and Rh-negative, are equally agglutinated by the phytohemagglutinin within the accuracy of the method.

## DISCUSSION

During the development of these procedures, the methods described by Goddard and Mendel (10) and by Bourdillon (20) were also tried. A product prepared by the Goddard-Mendel method had a very low titer compared with either the mucoprotein or the protein phytohemagglutinin (Table I), and on electrophoresis showed a small amount of mucoprotein, heavily contaminated with inert proteins. This fact, we believe, led to the erroneous classification of this substance as an albumin. We were unable to prepare any agglutinating material by the method of Bourdillon, who describes this substance as a lipide, extractable by organic solvents.

The fact that the titer of the protein phytohemagglutinin is of the order of 100 times that of the mucoprotein cannot be explained entirely by the elimination of the polysaccharide part. From the nitrogen content of the mucoprotein it appears that the protein part accounts for 40 per cent of its weight; thus one would expect only a 2.5-fold increase of the agglutination titer. It is possible that the elimination of the polysaccharide unveils more active sites of the protein which may have been sterically hindered, or may be participating in bonds with the polysaccharide. In the latter event, it would appear probable that this phytohemagglutinin may agglutinate the erythrocytes by combining with a polysaccharide of the stroma possessing groups similar to those of the mucoprotein polysaccharide. Work is now in progress designed to elucidate the nature of this bond and its relationship to erythrocyte agglutination.

This phytohemagglutinin has been found to be non-toxic (11, 21), in contrast to the highly toxic *ricin* (1). It is a panagglutinin, agglutinating equally human erythrocytes of all blood types tested in our laboratory, although lower titers for erythrocytes of type A have been reported (7, 22). It also agglutinates the erythrocytes of horse, pig, dog, cat, rabbit, chicken, and frog (9, 23). Since it does not agglutinate human leucocytes and nucleated erythrocytes, it is suitable for separation of nucleated cells of blood or marrow from erythrocytes for culture or metabolic studies. The application of the phytohemagglutinin method yields leucocytes in a greater state of purity than ever before, with a minimal amount of time, effort, and expense and also increases the reliability of chemical and metabolic studies of leucocytes.

## SUMMARY

The methods for purification of the phytohemagglutinin of *Phaseolus vulgaris* as a mucoprotein and as a protein have been described.

The mucoprotein behaves as a homogeneous substance on electrophoresis over the pH range of 5.8 to 8.6, and its electrophoretic mobility curve, when extrapolated, intercepts the zero mobility line at about pH 5.6. Below pH 5.8 it dissociates into a protein phytohemagglutinin and an inactive polysaccharide. This mucoprotein contains 6.5 per cent nitrogen, 50.4 per cent total reducing substances estimated as glucose, it is heat-labile, and very soluble in distilled water.

The protein behaves as a homogeneous substance on electrophoresis over the pH range of 2.0 to 8.0, and its isoelectric point is 6.5. The nitrogen content of this protein is 14.6 per cent and its total reducing substances 3.4 per cent. It is a heat-labile euglobulin, insoluble in distilled water, but very soluble in saline, or buffer solutions.

This phytohemagglutinin, in either the mucoprotein or the protein form, is a non-toxic, powerful hemagglutinin of all types of human erythrocytes, and those of the horse, pig, dog, cat, rabbit, chicken, and frog. Directions are given for its use in the separation of human leucocytes from erythrocytes.

## BIBLIOGRAPHY


1. Osborne, T. B., Mendel, L. B., and Harris, I. F., *Am. J. Physiol.*, **14,** 259 (1905).
2. Landsteiner, K., and Raubitschek, H., *Centr. Bakt.*, **45,** 660 (1907).
3. Dorset, M., and Henley, R. R., *J. Agr. Res.*, **6,** 333 (1916).
4. Dorset, M., and Henley, R. R., *J. Am. Vet. Med. Assn.*, **50,** 699 (1917).
5. Osborne, T. B., The vegetable proteins, Monographs on biochemistry, London and New York, 2nd edition (1924).
6. Murphy, R. A., Hall, A. R., and Goddard, V. R., *Proc. Soc. Exp. Biol. and Med.*, **30,** 178 (1932).
7. Boyd, W. C., and Reguera, R. M., *J. Immunol.*, **62,** 333 (1949).
8. Li, J. G., and Osgood, E. E., *Blood*, **4,** 670 (1949).
9. Wienhaus, O., *Biochem. Z.*, **18,** 228 (1909).
10. Goddard, V. R., and Mendel, L. B., *J. Biol. Chem.*, **82,** 447 (1929).
11. de Souza, A. H., *Bol. Ministério agr. Brazil*, **33,** 15 (1944).
12. de Souza, A. H., *Bol. divulgação inst. óleos*, No. 2, 47 (1944).
13. Rigas, D. A., Li, J. G., and Osgood, E. E., Abstracts, Western Society for Clinical Research, Seattle, Jan. 26–27 (1951); *Am. J. Med.*, **10,** 776 (1951).
14. Miller, G. L., and Golder, R. H., *Arch. Biochem.*, **29,** 420 (1950).
15. Rigas, D. A., Maupin, T. J., and Heller, C. G., *J. Lab. and Clin. Med.*, **39,** 492 (1952).
16. Salk, J. E., *J. Immunol.*, **49,** 87 (1944).
17. Nelson, N., *J. Biol. Chem.*, **153,** 375 (1944).
18. Somogyi, M., *J. Biol. Chem.*, **160,** 61 (1945).
19. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73,** 627 (1927).
20. Bourdillon, J., *Bull. Soc. chim. biol.*, **31,** 83 (1949).
21. Assmann, F., *Arch. ges. Physiol.*, **137,** 489 (1911).
22. Cazal, P., and Lalaurie, M., *Acta haematologica*, **8,** 73 (1952).
23. Nungester, W. J., and Van Halsema, G., *Proc. Soc. Exp. Biol. and Med.*, **83,** 863 (1953).