ISOLATION AND CHARACTERIZATION OF A MITOGEN FROM POKEWEED (PHYTOLACCA AMERICANA)

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Previous studies[1–3] in this laboratory have shown that a lectin derived from the plant *Phytolacca americana* (pokeweed) possesses three distinct biological activities: hemagglutination, leukagglutination, and mitogenicity, i.e., the capacity to transform resting peripheral blood lymphocytes into "blast-like" cells *in vitro*. Lymphocyte transformation by the pokeweed mitogen (PWM) involved distinctive biochemical, histochemical, and fine structural changes, distinguishable from those reported for phytohemagglutinin (PHA).[1–3] A recent report[4] on the natural history of pokeweed poisoning attests to the analogous action of this mitogen *in vivo;* ingestion of this mitogen resulted in plasmacytosis.

The specific role and interrelationships of the cell types designated as lymphocytes, plasma cells, and precursor "blasts" in immunological states have been the subject of intensive investigation and controversy in recent years.[5, 6] The findings of distinctive alterations in immunocompetent cells produced by PWM *in vivo* and *in vitro* has given special impetus to its further purification and characterization.

This communication reports the isolation of PWM in homogeneous form and the characterization of its physical and chemical properties.

Methods.—Preparation of pokeweed mitogen (PWM): Roots from the plant *Phytolacca americana*, growing in the vicinity of Bethesda, Maryland, were harvested in late summer, and PWM was isolated from this source as described previously.[1] The fractionation procedure consisted essentially of saline extraction of the roots, heat coagulation of the extract, centrifugation following trichloracetic acid (TCA) precipitation of the resultant supernatant, and chromatography of the dissolved and dialyzed TCA-precipitate on a calcium phosphate (hydroxylapatite) column.

Multiphase zone electrophoresis on polyacrylamide gel: Preparative gel electrophoresis was performed using the apparatus and method essentially as described by Jovin, Chrambach, and Naughton.[7] The "Poly Prep" apparatus manufactured by Buchler Instruments, Fort Lee, N.J., was used for all preparative electrophoreses. The buffer system described by Jovin, Chrambach, and Naughton[7] was modified as described previously,[8] and both analytical and preparative electrophoresis were performed at pH 9.4 and 25°C in the absence of urea in gels with a 13% acrylamide concentration. The relative mobility of protein bands obtained by analytical acrylamide gel electrophoresis were expressed as *Rf* values which were computed from the position of each band with reference to the bromophenol blue tracking dye front. In a typical experiment, 20 mg of PWM obtained by chromatography on hydroxylapatite was dissolved in 3 ml of upper gel buffer, containing 5% (w/v) sucrose (0.046 *M* Tris, 0.032 *M* H_3PO_4, pH 6.9) and thoroughly dialyzed against this same buffer. The sample was carefully layered on top of a preparative acrylamide gel column (50 ml lower gel, 30 ml upper gel), and a trace of bromophenol blue dye was added. A constant current of 15 ma and 100 v was applied until the sample had completely entered the upper gel. The current was then increased to 60 ma and 280 v. The acrylamide concentration of the upper gel was 2.5% and that of the lower gel 13%. The elution rate was held constant at a 0.8 ml per minute; fractions were collected at 6-min intervals.

Amino acid analyses: The amino acid composition of PWM was determined using the Beckman-Spinco model 120 B analyzer modified for high sensitivity[9] and rapid elution schedules.[10] Samples were hydrolyzed *in vacuo* in 5.7 *N* HCl at 110°C for 24, 48, and 72 hr, respectively. Tryptophan was determined spectrophotometrically, using a method described by Edelhoch.[11] The cystine content of native PWM was expressed as half-cystine. Cystine was also determined

as carboxymethylcysteine after PWM was extensively reduced with 0.1 *M* dithiothreitol in the presence of 7 *M* guanidine-HCl and then was alkylated by the addition of a 1.2 molar excess of iodoacetamide relative to the sulfhydryl concentration as described by Small and Lamm.[12] Performic acid oxidation[13] of PWM permitted the evaluation of cystine as cysteic acid. Analyses for free sulfhydryl groups were made by the Ellman procedure.[14]

Gel filtration: A solution of PWM, extensively reduced and carboxamidomethylated in 7 *M* guanidine-HCl was diluted to a density equivalent to 5.2 *M* guanidine-HCl and was applied to an upward-flowing column of Sephadex G-200 equilibrated with 5 *M* guanidine-HCl and followed by ~10 ml of more dense (~5.6 *M*) guanidine-HCl.[12] Effluent fractions were monitored by absorption at 280 mμ.

Carbohydrate analyses: A chromatographic method described by Walborg, Christensson, and Gardell[15] was utilized for the separation of neutral monosaccharides. This method is based on the ion exchange of sugar-borate complexes in a boric acid glycerol buffer at neutral pH and elevated temperature. The aniline-acetic acid-*o*-phosphoric acid reagent[16] was used for quantitation of monosaccharides. Determinations of sialic acid were performed by the method of Svennerholm.[17] Hexosamine content was measured by the procedure described by Davidson.[18]

Physical measurements: The extinction coefficient for PWM was determined in distilled water and the partial specific volume ($\bar{v}$) was calculated from amino acid and carbohydrate compositions. Molecular weight determinations were made by the high-speed short-column sedimentation equilibrium method of Yphantis,[19] and calculations were done with the aid of a computer utilizing a program developed for this procedure.[12]

Results.—Isolation of pokeweed mitogen: Pokeweed mitogen was obtained in electrophoretically homogeneous form by preparative acrylamide gel electrophoresis

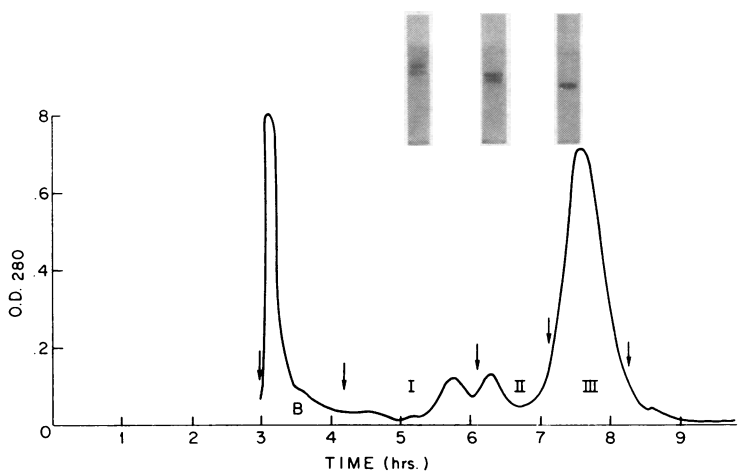

FIG. 1.—Elution pattern resulting from preparative multiphase zone electrophoresis of 20 mg of PWM obtained from hydroxylapatite chromatography. Arrows indicate how the effluent volume fractions were divided into "B" (Bromphenol blue dye front), fraction I, fraction II, and fraction III. Analytical acrylamide gel electrophoresis patterns superimposed on the effluent pattern illustrate the respective electrophoretic profile of fractions I, II, and III. Fraction III contains the purified PWM and comprises ~70% of the material applied to the column. Conditions for electrophoresis are described in the text.

of a PWM preparation which had previously been purified by chromatography on hydroxylapatite. Figure 1 depicts an elution pattern obtained after preparative gel electrophoresis on which are superimposed analytical polyacrylamide gel electrophoresis patterns of the effluent peaks. The major peak containing the electro-

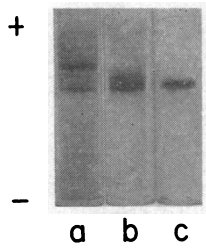

FIG. 2.—Analytical acrylamide electrophoresis patterns of (*a*) TCA-precipitate, (*b*) hydroxylapatite fraction, and (*c*) PWM purified by preparative acrylamide electrophoresis. Samples of 150 μg were applied in each case.

phoretically homogeneous mitogen represents approximately 70 per cent of the material originally applied to the electrophoresis column. The progress of purification from TCA precipitate through hydroxylapatite eluate to homogeneous PWM is illustrated in Figure 2 which compares the acrylamide gel electrophoresis patterns of the product yielded by each of these purification steps. The TCA-precipitated fraction contains at least 12 electrophoretic components which upon hydroxylapatite chromatography are reduced to 4 and finally, after preparative acrylamide gel electrophoresis, yield a single electrophoretic component. When cut out and re-electrophoresed at pH 9.4, this component again moved as a single band with essentially the same *Rf* value (0.42) as the original preparation. Single bands were also obtained at three different acrylamide gel concentrations (7.5, 15, and 20%), i.e., when the pore size of the gel narrowed to obtain more sieving. The yield of mitogen from 100 lb of roots at the three purification stages was approximately 2.5 gm of TCA powder which, in turn, yielded 1.5 gm of hydroxylapatite fraction from which was obtained approximately 1 gm of homogeneous mitogen.

Molecular weight of PWM: The extinction coefficient $E^{1\%}_{1\,cm}$ of PWM was determined in distilled water at approximately pH 6.0 and was found to be 18.5. The

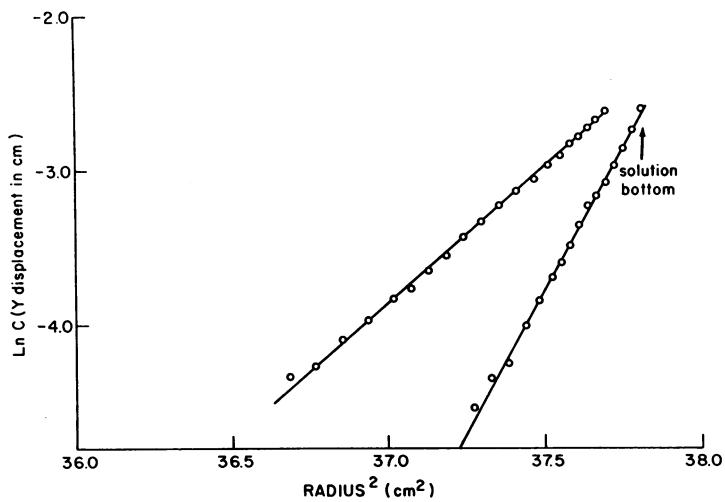

FIG. 3.—Plot of the logarithm of the vertical displacement from the base line of a fringe measured in centimeters (ln *C*) (*Y* displacement in centimeters) vs. distance from the center of rotation squared (radius²) from the sedimentation equilibrium experiments. Arrow indicates the bottom of the liquid column. The lines represent least square fit of the points.

partial specific volume ($\bar{v}$) was calculated from amino acid and carbohydrate compositions as 0.620. This is the value used in the calculation of molecular weights. The weight average molecular weights obtained at three different initial protein

concentrations and at two different speeds of centrifugation are summarized in Table 1. Figure 3 shows the ln C vs. (radius^2) plots of the experiment in which the molecular weight of PWM was determined by high-speed short-column sedimentation.

TABLE 1

MOLECULAR WEIGHTS $\times 10^{-2}$ OF POKEWEED MITOGEN

Speed (rpm)	Starting Concentration (mg/ml) 0.17	0.33 Weight Average	0.50	Av. ± SE*
25,980	318	322	323	320 ± 1.5
37,020	325	316	319	

* Standard error.

Characterization of PWM on Sephadex G-200: To determine the molecular weight of PWM by a different method and to ascertain whether the molecule consists of one or several polypeptide chains, an extensively reduced and alkylated preparation was subjected to gel filtration on Sephadex G-200 in 5 M guanidine-HCl. Since shape factors are minimized due to extensive reduction and alkylation

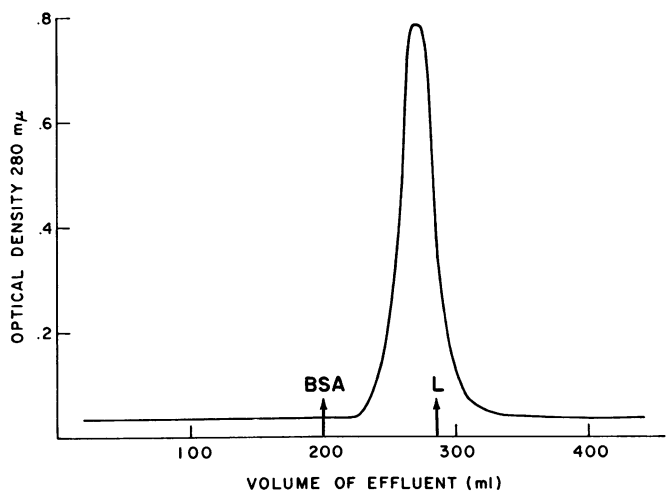

FIG. 4.—Gel filtration of PWM on a column of Sephadex G-200 (120 × 2.5 cm) in the presence of 5 M guanidine-HCl. The arrows show the position of the peak for the following extensively reduced and alkylated materials when passed through this same column. *BSA*, bovine serum albumin (mol wt 68,000). *L*, light polypeptide chain of rabbit γG-immunoglobulin (mol wt 23,000).

and sieving in the presence of 5 M guanidine, it was anticipated that the elution position of PWM would permit a fair estimate of its mass. The resulting elution diagram shown in Figure 4 is a single symmetrical peak with a K_D-value of 0.406. In order to approximate the mass of PWM, a plot of $\sqrt{K_D}$ vs. $\sqrt{\text{mol wt}}$ was utilized which had been constructed from previous column calibrations[20] with compounds of known molecular weights. Thus the K_D value of 0.406 indicated a molecular weight of 31,000 ± 1,000 which is in agreement with the value 32,000 ± 1,000 determined by sedimentation equilibrium analyses.

TABLE 2

AMINO ACID COMPOSITION OF PWM

	24 Hr	48 Hr	72 Hr	Average or extrapolated	Mole fraction	Observed residues per mole*	Mole integer
	μMole Amino Acid						
Lysine	0.014	0.014	0.015	0.014	0.029	8.4	8
Histidine	0.013	0.013	0.013	0.013	0.027	7.8	8
Arginine	0.023	0.023	0.024	0.023	0.049	14.1	14
Aspartic acid	0.062	0.061	0.065	0.063	0.133	38.4	38
Threonine	0.017	0.017	0.020	0.018	0.038	11.0	11
Serine	0.034	0.031	0.033	0.033	0.070	20.1	20
Glutamic acid	0.058	0.056	0.060	0.058	0.123	35.7	36
Proline	0.012	0.012	0.012	0.012	0.025	7.2	7
Glycine	0.071	0.083	0.071	0.075	0.159	46.2	46
Alanine	0.015	0.012	0.014	0.014	0.029	8.4	8
Half-cystine	0.085	0.092	0.093	0.090	0.190	56.2	56†
Valine	0.014	0.018	0.019	0.019	0.040	11.2	11
Methionine‡	—	—	—	—	—	—	—
Isoleucine	0.002	0.002	0.002	0.002	0.004	1.2	1
Leucine	0.016	0.016	0.016	0.016	0.034	9.9	10
Tyrosine	0.011	0.011	0.011	0.011	0.023	6.7	7
Phenylalanine	0.013	0.012	0.012	0.012	0.025	7.2	7
Tryptophan§	—	—	—	—	—	9.0	9

* Observed residues/mole were calculated as follows: (Residue mol wt/Av. residue mol wt) (mole fraction) (observed mol wt/residue mol wt). Mole fraction was calculated by dividing average μmoles of each amino acid (col. 4) by the total of μmoles of all amino acids. The residue molecular weight of each amino acid multiplied by its respective mole fraction gave the average residue molecular weight. The observed molecular weight was 32,000 from which the weight due to carbohydrate (1,500) was subtracted to arrive at a corrected value of 30,500 used for these calculations.

† This is a minimum value (see Table 3).

‡ Methionine was not detected as methionine sulfone after hydrolysis of a performic acid oxidized sample.

§ Determined spectrophotometrically.

The amino acid composition of PWM: The amino acids found in PWM are listed in Table 2. Methionine was the only amino acid absent in PWM. The amino acid composition of PWM is unique since the native mitogen has an extraordinarily large number of half-cystine residues. Reaction of native PWM with 5,5 dithiobis (2-nitrobenzoic acid)[14] in the presence of guanidine did not reveal any free sulfhydryl groups.

Since it is not feasible to obtain accurate half-cystine values due to partial destruction during hydrolysis, cystine was also determined as carboxymethylcysteine following extensive reduction and carboxamidomethylation in 7 *M* guanidine-HCl and as cysteic acid after performic acid oxidation of PWM. The data of these analyses shown in Table 3 indicate that PWM has 66 half-cystine residues in the form of 33 disulfide bridges.

TABLE 3

CYSTINE CONTENT OF PWM

	Untreated	Reduced and alkylated*	Oxidized†
Half-cystine	56	3	—
S-carboxymethylcysteine	—	60	—
Cysteic acid	—	1	66

* Extensively reduced and carboxamidomethylated in 7 *M* guanidine-HCl. A second reduction and alkylation of the sample with 0.3 *M* dithiothreitol and 0.66 *M* iodoacetamide at 40°C gave the same values.

† Oxidized with performic acid.

It is not possible to utilize N-terminal analyses to establish whether PWM consists of a single polypeptide chain since preliminary determinations revealed the absence of a free amino terminal end group.

The carbohydrate composition of PWM: The monosaccharide composition of

PWM as ascertained by chromatographic methods is shown in Table 4. The molecule was found to contain three residues of hexosamine (1.4% by weight) and 3.26 per cent by weight of monosaccharides consisting of four residues of mannose and one residue each of glucose and fucose; sialic acid was not present.

TABLE 4

CARBOHYDRATE COMPOSITION OF PWM

Carbohydrate	μMoles/mg	Per cent by weight	Residues per 32,000
Mannose	0.124	2.01	3.97
Fucose	0.041	0.60	1.31
Glucose	0.040	0.65	1.28
Hexosamine	0.086	1.40	2.76
Sialic acid		Not detected	

Biological activity of PWM: Employing the assay system for mitogenic activity previously described,[1] we found that the end point for maximal stimulation of 3×10^6 lymphocytes in 5-ml cultures was obtained at the following levels: crude saline extract, 50–100 μg protein N/ml; TCA-extracted material, 0.25 μg protein N/ml; electrophoretically homogeneous PWM, 0.05 μg protein N/ml. There was thus achieved a 2000-fold increase in mitogenic activity. It is noteworthy that this large increase in mitogenic activity was not paralleled by either a large increase in hemagglutinin or leukagglutinating activity; the former proved to be only four- to fivefold, while the latter is difficult to quantitate.

Discussion.—A study of the physical and chemical properties of pokeweed mitogen has shown that the material obtained by preparative acrylamide gel electrophoresis is an electrophoretically homogeneous glycoprotein with a molecular weight of 32,000 ± 1,000. *S*-carboxymethyl cysteine equivalent to only 30 reduced disulfide bridges was observed despite double reduction and alkylation in the presence of 7 *M* guanidine. It seems highly probable that the molecule consists of a single polypeptide chain containing 33 intrachain disulfide bridges, since it is most likely that the three disulfide bridges which could not be cleaved reductively are intrachain rather than interchain bridges. The latter are known to have greater reactivity and are therefore considered to be far more susceptible to reductive cleavage.[21] An alternate possibility is that the data indicate simply reduction of all bonds with 90 per cent efficiency. However, experimental proof of complete reduction to monomeric units is still incomplete since the data allow for as many as three intact interchain disulfide bridges.

The findings show that PWM does not consist of a number of noncovalently linked polypeptide chains since gel filtration in the presence of 5 *M* guanidine-HCl did not show any evidence of dissociation with a resultant change in K_D-value, indicating a lower molecular weight than was obtained from sedimentation equilibrium analysis.

The presence of the unusually large number of intrachain disulfide bridges strongly suggests that the PWM molecule, or at least certain regions of it, are relatively inflexible. This is of singular interest inasmuch as PWM with a molecular weight of 32,000 and 33 disulfide bridges contrasts markedly with phytohemagglutinin (PHA), the other plant lectin, which has a molecular weight of 128,000 and only two disulfide bridges;[22] despite these structural differences both molecules display a broadly similar range of mitogenic, erythro-, and leukagglutinating activ-

ities. From these data, as well as from the amino acid and carbohydrate composition of PHA,[22] it is apparent that the two lectins, although possessing generally similar biologic properties, differ markedly in their over-all structure. In seeking to relate chemical structure to biological function, it will be important to ascertain whether PWM and PHA are primarily protein molecules which bear a common reactive site or whether these molecules have only certain structural configurations or chemical moieties in common, e.g., carbohydrate groups which are specific for biologic effects.

The isolation and characterization of PWM was undertaken as the initial phase of an investigation to explain the role of this molecule in lymphocyte transformation, hemagglutination, and leukagglutination. Consequently it was important to determine any possible relationship of PWM to other molecules with similar activities such as PHA and immunoglobulins. It was considered significant that PWM, although possessing these three biologic activities, was chemically quite distinct from PHA and immunoglobulins. During the purification of PWM a 2000-fold increase in mitogenic activity was attained, whereas hemagglutinin activity increased only four- to fivefold. If it is assumed that all three biologic activities are present on the same molecule, a parallel increase in all of them would be expected. However, it is difficult to make a comparison between the increase in hemagglutinating and mitogenic activities achieved during the purification of PWM since several discrete hemagglutinins are present in the crude TCA extract. This was clearly shown upon assay of a number of individual components obtained by acrylamide gel electrophoresis. However, assay of these same components for mitogenic activity showed that virtually all detectable mitogenicity was confined to one component ($Rf = 0.42$) which was subsequently purified and chemically characterized. This electrophoretically homogeneous component also contains both erythro- and leukagglutinating activities.

Previously we reported[1] that hemagglutinating activity could be selectively removed from PWM purified by hydroxylapatite chromatography by absorption with red cells or stroma without affecting leukagglutinating and mitogenic activities. In the present work it was shown that this hydroxylapatite fraction could be purified further with retention of all of its three biologic activities. Further work will be required to determine whether hemagglutinating activity can be selectively removed from this electrophoretically homogeneous material, and it remains to be seen whether all three biologic activities are located on a single molecular species. Whether these activities are attributable to the same chemical groupings or determinants on this particular molecule remains to be established. At present there is no evidence which requires that biologic activity is elicited only by the intact PWM molecule. It is conceivable that subfractions of PWM can be derived with the capability of expressing one or another or even all of these biological activities.

Summary.—A mitogen (PWM) isolated from the roots of pokeweed (*Phytolacca americana*) was shown to be a homogeneous glycoprotein of 32,000 molecular weight, containing 3.2 per cent monosaccharides, 1.4 per cent hexosamine, and an unusually large amount of cystine, i.e., 19 mole per cent. Although PWM differs markedly from another plant lectin, phytohemagglutinin (PHA), it has a generally similar range of mitogenic, erythro-, and leukagglutinating activities.

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