Hemocyanin from the Australian Freshwater Crayfish *Cherax destructor*. Subunit Heterogeneity

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ABSTRACT: The hemocyanin of the Australian freshwater crayfish, *Cherax destructor*, is typical of that of crustaceans in that it contains components with sedimentation coefficients of 5 S, 17 S, 25 S, and 29 S, the 17S and 25S components predominating. As the pH is increased from 7.8 to 10, progressive dissociation to the 5S component occurs in the absence but not in the presence of Ca^{2+}. Gel electrophoresis and gel filtration studies show that *C. destructor* hemocyanin is composed of three different monomers in a ratio of about 7:2:1. The predominant monomer has been isolated and found to have a molecular weight of 74,700 by sedimentation equilibrium and to bind two copper atoms per mole. Estimates of the molecular weights of the other two monomers are also near 75,000. The monomer present in the least amount exists as a dimer upon dissociation of hemocyanin by sodium dodecyl sulfate unless reducing agent is present. The 17S component (hexamer) of *C. destructor* hemocyanin, which appears electrophoretically heterogeneous, contains two different monomers. In addition to these, a third monomer, that which forms dimer, appears to play a role in the formation of components of higher molecular weight than the hexamer.

Arthropod hemolymph contains hemocyanin components with sedimentation coefficients of 5 S, 16 S, 25 S, 34 S, and 60 S (Van Holde and Van Bruggen, 1971), the 5S component of molecular weight 65,000–90,000 apparently being the smallest functional subunit. In crustaceans the 16S and 25S components usually predominate, the 16S component of molecular weight 480,000 corresponding to hexamer, and the 25S component of molecular weight 938,000, to dodecamer (Ellerton *et al.*, 1970). The 34S and 60S components represent states of further aggregation.

For some time the microheterogeneity of hemocyanin components has been suspected (Di Giamberardino, 1967; Konings *et al.*, 1969; Ellerton *et al.*, 1970). Recently the 5S component of *Cancer magister* hemocyanin was shown to be composed of equal amounts of two distinct proteins (Loehr and Mason, 1973; Carpenter and Van Holde, 1973). This suggests that the microheterogeneity of the high molecular weight components of hemocyanin may arise from combination of heterogeneous monomers.

The present work reports progress so far in establishing the number and size of crayfish hemocyanin subunits with the ultimate aim of establishing their role in the formation of polymeric species and in the oxygen transport system. *Cherax destructor*, an Australian freshwater crayfish, is widely distributed in artificial dams and inland waterways of mainland Australia (Reik, 1969), and was therefore a convenient species for study.

Experimental Section

Preparation of Hemocyanin. Blood (2 or 3 ml) was collected from each crayfish by cutting off a limb at the second joint from the claw. The clotted blood was centrifuged at 18,000 rpm for 30 min in a Sorvall RC2-B centrifuge. The protein of the resulting serum was essentially pure hemocyanin. The serum was stored under toluene at 5° for up to 2 months. Hemocyanin concentrations were obtained from dry weight determinations on dialyzed serum. Protein absorption was measured on a Cary 14 recording spectrophotometer or on a Zeiss PMQ II spectrophotometer.

Analysis for Metal Ions. Serum ultrafiltrate was obtained through use of an Amicon ultrafiltration apparatus with a UM-10 Diaflo membrane. Analyses of ultrafiltrate or other protein solutions for Na, K, Ca, Mg, Cu, and Fe were performed on a Techtron A5 atomic absorption spectrometer using the flame atomization technique.

Ultracentrifugation. A Spinco Model E analytical ultracentrifuge was used. All protein solutions were dialyzed for 2 days at room temperature against two changes of 250 volumes of the appropriate buffer prior to ultracentrifugation and the dialysate was used in the solvent sector of the ultracentrifuge cell. Sedimentation velocity studies were carried out at 44,000 rpm and 20°. The resulting schlieren patterns were recorded on Ilford R-40 photographic plates which were measured on a Gaertner microcomparator. Sedimentation coefficients were evaluated from the maximum ordinates of the schlieren peaks in the usual fashion. The sedimentation equilibrium experiment was carried out at 24,000 rpm and 20° according to the high-speed method of Yphantis (1964). Rayleigh interference patterns were recorded on Kodak IIG photographic plates. Measurements and computations were exactly as described by Yphantis (1964). A value of 0.73 ml/g was used for the partial specific volume of hemocyanin (Ellerton *et al.*, 1970). A linear plot of logarithm of fringe displacement *vs.* the square of the distance from the axis of rotation was obtained for the experiment reported, indicating homogeneity with respect to molecular weight.

Polyacrylamide Gel Electrophoresis. All experiments were carried out using continuous buffer systems and gels 7 cm long. The acrylamide to *N,N'*-methylenebisacrylamide ratio was 40:1 and the gels were polymerized with 0.1% *N,N,N',N'*-tetramethylethylenediamine and 0.067% ammonium persulfate. For experiments in the presence of SDS,[1] whether with or without dithiothreitol, protein solutions were heated at 80° for 10 min in 1% SDS before electrophoresis. Acrylamide concen-

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: Conditions for Polyacrylamide Gel Electrophoresis.

pH	Solvent	Acrylamide Concn (%, w/v)	Current (mA/gel)	Time at Current (hr)
7.8	0.05 M Tris	4	7	2.25
7.8	0.05 M Tris, 0.1% SDS	4	7	2
10.0	0.05 M glycine, 10^{-4} M EGTA[a]	5–12	5–7	1.25–2

[a] Ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

trations used with each solvent system are given in Table I; 5–20 μl of protein solution of concentration 1 mg/ml or Bromophenol Blue was applied in 10% glycerol to each gel. Electrophoresis was performed for 14 min at 2 mA/gel, then at the current indicated in Table I on a Buchler apparatus, water-cooled at 20°. Gels were stained in 25% isopropyl alcohol, 10% acetic acid, and 0.05% Coomassie Brilliant Blue overnight and destained with a Canalco horizontal destainer. Photographs were made using transmitted light and a red filter. Quantitation of protein bands on gels was done by measuring the areas under the peaks obtained from scanning the gels at 540 nm on a Schoeffel SD3000 spectrodensitometer. When this technique was applied to electrophoresis experiments with bovine serum albumin it was found that the areas under the peaks were directly proportional to the amount of protein loaded over the range 3–100 μg. It was assumed that hemocyanin also follows this relationship between peak area and concentration and that, hemocyanin components bind dye to similar extents.

Molecular weights were determined from SDS–polyacrylamide gels by the method of Shapiro *et al.* (1967) using ovalbumin and its dimer and bovine serum albumin and its dimer, trimer, and tetramer as standards. The molecular weights from polyacrylamide gels without SDS at pH 10 were determined by making use of the Ferguson (1964) relationship

$$\log R_F = \log (R_F)_0 + K_R T$$

where R_F is the mobility of a protein relative to that of a tracking dye (Bromophenol Blue), T is the total gel concentration, K_R is the retardation coefficient and $(R_F)_0$ is the R_F value at a gel concentration of zero. By electrophoresing standard proteins (ovalbumin, bovine serum albumin, alkaline phosphatase, and transferrin) as well as hemocyanin at a number of different gel concentrations at pH 10 and plotting log R_F *vs.* T, K_R values were obtained. A plot of K_R *vs.* molecular weight for the standard proteins allowed the molecular weights of hemocyanin components to be estimated by interpolation.

Results and Discussion

Studies at pH 7.8. C. destructor serum was found to have a pH of 7.8 and to contain about 3.5 g of protein/100 ml. This value represents the mean of determinations on two pooled samples collected at different times, each sample consisting of the serum of 30–40 crayfish. Individual crayfish studied showed a variation of from 2.7 to 4.8 g of protein/100 ml of serum. After dialysis against 0.05 M Tris (pH 7.8), serum exhibited an $E_{1cm}(1\%)$ at 278 nm of 12.4 and an OD_{278}/OD_{338} of 5.3. The copper content of serum was 1.76 mol/75,000 g of protein. The concentration of metal ions in serum ultrafiltrate

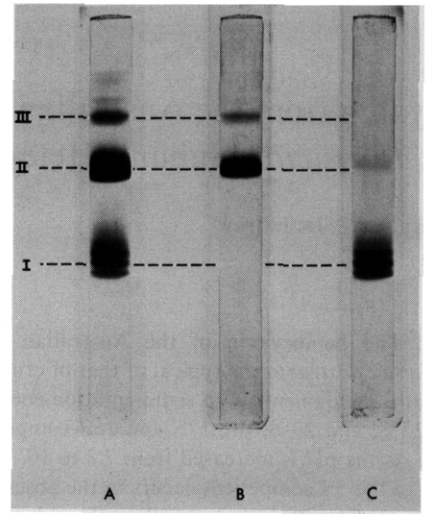

FIGURE 1: Electrophoresis of *C. destructor* serum and its Sepharose 6B fractionation products on 4% polyacrylamide gels at pH 7.8. Gel A contains serum; gel B, peak 1 material from Sepharose 6B; and gel C, peak 2 material from Sepharose 6B. Mobility was from top to bottom (cathode to anode).

were: Na, 0.088 M; K, 0.008 M; Ca, 0.028 M; Mg, 0.0013 M; Cu, nil; Fe, nil. Serum contained similar amounts of Na, K, Mg, and Fe but higher amounts of Ca and Cu due to the affinity of hemocyanin for these metal ions.

Sedimentation coefficients were measured for each peak in the schlieren profile obtained with serum, serum diluted 10 times, and serum diluted 20 times with ultrafiltrate. The results at each concentration were corrected for density and viscosity dependence to give the values corresponding to sedimentation in water at 20° and finally extrapolated to infinite dilution. These studies revealed the presence of components of $s_{20,w}°$ 5.3 S, 17.0 S, 25.5 S, and 29.3 S, which will be referred to hereafter as the 5S, 17S, 25S, and 29S components, respectively. The relative amounts of the serum components were determined by measuring the areas under the schlieren peaks obtained in sedimentation velocity experiments with samples of serum dialyzed against 0.05 M Tris, 0.03 M $CaCl_2$, and 0.077 M NaCl (pH 7.8), ionic strength 0.20, and diluted three times with dialysate. This buffer was formulated to be as close as possible to serum in its pH, ionic strength, and calcium concentration. The 17S and 25S components comprise 30–40 and 40–50%, respectively, while the 5S and 29S components each comprise 5–10% of the total protein.

Electrophoresis of serum on polyacrylamide gels at pH 7.8 gave the pattern shown in Figure 1A. The predominant bands occur in positions labeled I, II, and III. Upon scanning the gels, bands in positions I, II, and III were found to account for 32–41, 43–52, and 8–12%, respectively, of the total stain. Another band, moving much faster than those shown, is usually also present in serum. In the gel reproduced in Figure 1A the electrophoresis was continued for long enough to obtain maximum resolution of the bands in position I. As a result the rapidly moving band (about 5% of the total stain) migrated off the end of the gel. It appeared from their relative positions and amounts that positions I, II, and III corresponded respectively to the 17S, 25S, and 29S components of serum. The more rapidly moving band would correspond to the 5S component.

In order to check this assignment, serum was fractionated on Sepharose 6B and a typical elution profile is presented in Figure 2. The slowest of the resulting three peaks had its only absorption maximum at a wavelength less than 230 nm and ap-

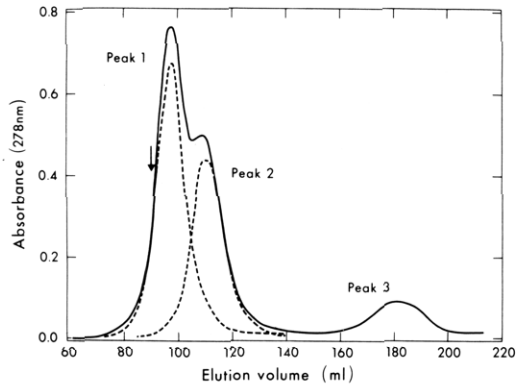

FIGURE 2: Sepharose 6B column chromatography of *C. destructor* serum; 1 ml of serum was applied to a 2.5 cm × 40 cm gel bed equilibrated with 0.05 M Tris–0.02 M $CaCl_2$ (pH 7.8), and was eluted with the same buffer at 10 ml/hr. The void volume as determined with blue dextran is indicated by the arrow. The broken lines represent rechromatography of peak 1 and 2 materials.

parently is not protein. Material from peaks 1 and 2 was rechromatographed twice and in each case gave a single, symmetrical peak eluting at the same position as in the original sample. Peak 1 and peak 2 materials were found to have sedimentation coefficients of 25 S and 17 S, respectively. Figure 1B shows that the major part (about 85%) of peak 1 material moves in position II on polyacrylamide gel electrophoresis at pH 7.8, while Figure 1C shows that the major part (about 95%) of peak 2 material moves in position I. Thus the bands in positions I, II, and III (Figure 1A) can be identified as the 17S, 25S, and 29S components, respectively, of hemocyanin. As Figures 1A and C show, the 17S component gives rise to three or four bands on gel electrophoresis. It is not known at present whether these bands represent size, charge, or conformational isomers.

The Effect of pH on Sedimentation Properties. In general, arthropod hemocyanins dissociate at elevated pH values (Van Holde and Van Bruggen, 1971). The marked differences in the sedimentation velocity profiles of *C. destructor* hemocyanin at pH 7.8 and at pH 10 in the absence of Ca^{2+} showed that the species is typical in this respect. As summarized in Table II, increasing the pH above 7.8 in the absence of Ca^{2+} causes a progressive dissociation until at pH 10.6 none of the high molecular weight components remain. The presence of Ca^{2+} has no detectable influence on the sedimentation velocity profile at pH 7.8. However, at pH 10 Ca^{2+} prevents the dissociation of *C. destructor* hemocyanin (Table II). The stabilization of the polymeric forms by Ca^{2+} is also characteristic of arthropod hemocyanins (Van Holde and Van Bruggen, 1971).

Studies at pH 10. The sedimentation velocity studies reported above show that *C. destructor* hemocyanin dissociates to a large extent at pH 10 in the absence of Ca^{2+}. Electrophoresis of the serum on polyacrylamide gels at pH 10 yields several protein bands, the predominant three of which will be referred to as bands M_1, M_2, and M_3' (Figure 3A). Bands M_1, M_2, and M_3' account for 40, 12, and 10%, respectively, of the total stain. Molecular weights were determined from such gels as described in the Experimental Section and plots of log R_F *vs.* gel concentration for bands M_1, M_2 and M_3' are shown in Figure 4A. Figure 4B shows the relationship between protein retardation coefficients and molecular weights. The molecular weights of hemocyanin dissociation products corresponding to bands M_1, M_2, and M_3' determined in this way are 70,000, 72,000, and 132,000, respectively. The important point here is that under these conditions it can be seen that two types of 5S

TABLE II: The Effect of pH and Ca^{2+} on the Dissociation of *C. destructor* Hemocyanin.[a]

		Percentage of Total Protein			
pH	Ca^{2+} Present	Less than 17 S	17 S	25 S	Above 25 S
7.78	−	10	32	48	9
8.76	−	22	22	49	7
9.98	−	62	18	13	7
10.60	−	100	0	0	0
7.80	+	10	32	51	7
9.94	+	8	34	50	9

[a] The buffer used was either 0.05 M Tris (pH 7.7–8.8) or 0.05 M glycine (pH 9.9–10.6). Ca^{2+}-free solvents (−) contained 0.002 M EDTA. Ca^{2+}-containing solvents (+) contained 0.03 M $CaCl_2$. The ionic strength was maintained at 0.20 with NaCl.

component or monomer exist which have very similar molecular weights but which vary considerably in charge. Band M_3' is of appropriate molecular weight to be a dimer, but further comment on this will be made later.

Sephadex G-200 column chromatography was used to fractionate the dissociation products of hemocyanin at pH 10 and the resulting elution profile is shown in Figure 5. Peak 1 eluted at the void volume and was not studied further, while peaks 2 and 3 were each rechromatographed and found to elute as single, symmetrical peaks at the same positions as in the original sample (Figure 5). Polyacrylamide gel electrophoresis at pH 10 shows that rechromatographed peak 2 material corresponds to band M_3' (Figure 3B) while peak 3 material yields band M_1 and often a small amount of band M_2. Peak 3 material yielding only electrophoretic band M_1 (Figure 3C) has been used in all experiments.

The molecular weight of monomer M_1 was found by the high speed sedimentation equilibrium technique to be 74,700.

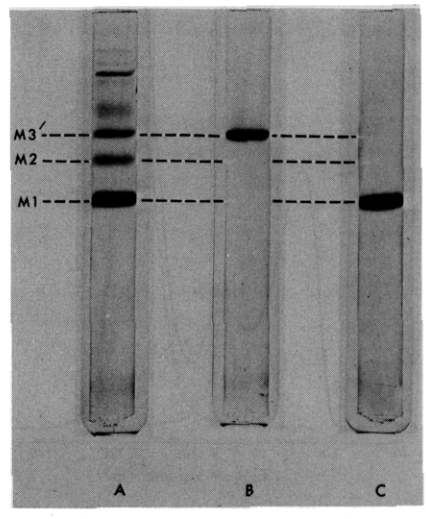

FIGURE 3: Electrophoresis of *C. destructor* serum and its Sephadex G-200 fractionation products on 8% polyacrylamide gels at pH 10. Gel A contains serum; gel B, peak 2 material from Sephadex G-200; and gel C, peak 3 material from Sephadex G-200. Mobility was from top to bottom (cathode to anode). Significant bands were labeled M_1, M_2, and M_3' for convenience of discussion in the text.

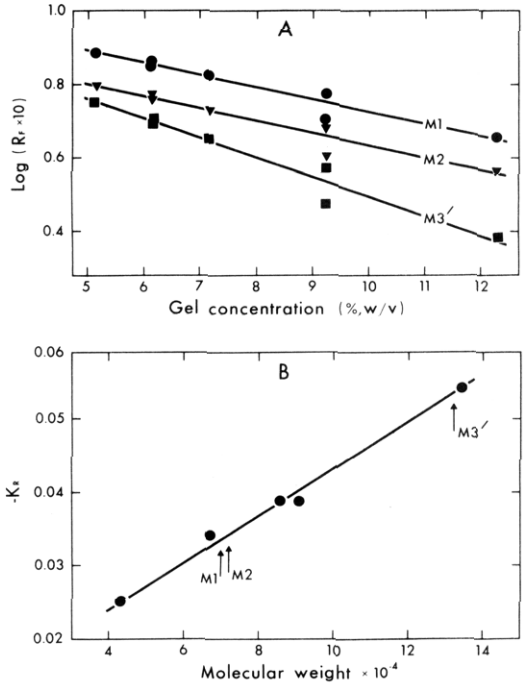

FIGURE 4: Determination of the molecular weights of hemocyanin dissociation products by polyacrylamide gel electrophoresis at pH 10. (A) The relationship between R_F values and gel concentration for proteins corresponding to bands M_1 (●), M_2 (▼), and M_3' (■) of Figure 3. (B) Plots of K_R *vs.* molecular weight for standard proteins (ovalbumin, serum albumin, alkaline phosphatase, and transferrin). K_R values and thus molecular weights of proteins M_1, M_2, and M_3' are indicated by arrows.

This monomer bound 1.95 mol of copper/74,700 g of protein under these conditions. The copper analysis and the spectrum indicate that at pH 10 copper and oxygen binding to monomer M_1 occur.

Gel Electrophoresis in SDS. Following dissociation by 1% SDS in the absence of reducing agent and gel electrophoresis in the presence of 0.1% SDS, *C. destructor* hemocyanin yielded three bands as shown in Figure 6A. Peaks 2 and 3 from Sephadex G-200 chromatography have been shown to correspond to electrophoretic bands M_3' and M_1, respectively, at pH 10 (Figure 2). By electrophoresis of peak 2 and peak 3 materials in the presence of SDS it was therefore possible to identify the bands corresponding to M_3' and M_1 on the SDS gels (Figure 6B and C). The remaining band obtained by dissociation of hemocyanin by SDS was identified with the remaining monomer band M_2 resulting from dissociation at pH 10. Bands M_2, M_1, and M_3' of Figure 6A represent approximately 20, 70, and 10%, respectively, of the total stained protein and were found from the gel electrophoresis in SDS to have molecular weights of 79,000, 86,000 and 190,000, respectively.

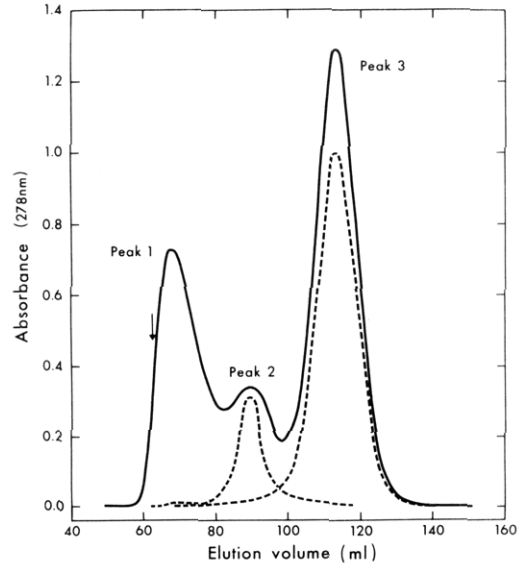

FIGURE 5: Fractionation of *C. destructor* hemocyanin by Sephadex G-200 column chromatography at pH 10; 1 ml of serum was dialyzed against 0.05 M glycine–0.002 M EDTA (pH 10) and applied to a 2.0 cm × 40 cm gel bed equilibrated with the same buffer. Elution was at the rate of 10 ml/hr. The void volume position as determined with blue dextran is shown by the arrow. Rechromatography is indicated by the broken lines.

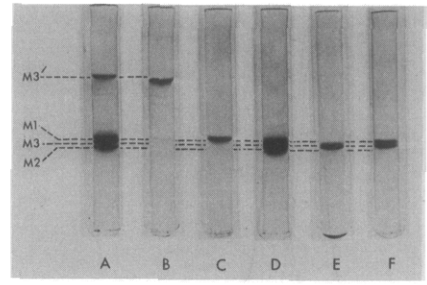

FIGURE 6: Electrophoresis of hemocyanin and some of its dissociation products on 4% polyacrylamide gels in SDS at pH 7.8. The samples loaded on the gels were as follows: gels A and D, serum; B and E, peak 2 material from Sephadex G-200; C, peak 3 material from Sephadex G-200; and F, a mixture of peak 2 and 3 materials from Sephadex G-200. Gels A, B, and C were incubated in 1% SDS prior to electrophoresis, while gels D, E, and F were incubated in 1% SDS and 10^{-3} M dithiothreitol. Mobility was from top to bottom (cathode to anode). The identification of the bands is described in the text.

Inclusion of dithiothreitol during the heating in SDS of serum or peak 2 material from Sephadex G-200 resulted in the disappearance of band M_3' from its usual gel position (Figure 6A and B) and the appearance of a new band M_3 of molecular weight 84,000 between bands M_1 and M_2 (Figure 6D and E). In order to verify the position of band M_3, a mixture of peak 2 and peak 3 proteins from Sephadex G-200 which had been incubated in the presence of 1% SDS, 10^{-3} M dithiothreitol was run on gels (Figure 6F). The result strengthened the conclusion that the effect of SDS and reducing agent on serum is the production of three monomers of very similar molecular weights. Monomer M_3 is produced from a dimer M_3' which is probably stabilized by disulfide bonds.

Peak 1 and 2 materials from Sepharose 6B (Figure 2) gave different patterns on SDS–polyacrylamide gels. Peak 1 from Sepharose 6B gave a profile similar to that of serum after treatment with SDS in the absence (Figure 6A) and the presence (Figure 6D) of dithiothreitol. Peak 2 material from Sepharose 6B showed the presence of only bands M_1 and M_2 (Figure 6) in about the same proportions as in serum. Peak 1 material from Sepharose 6B has been shown to be essentially the 25S component of serum while peak 2 is the 17S component. It appears then that the 25S component contains three different monomers (M_1, M_2, and M_3); however, since peak 1 material contains also about 15% of the 29S component we have not as yet been able to eliminate the possibility that all or part of the monomer present in least amount (M_3) arises from the latter component. The 17S component contains only two monomers (M_1 and M_2). The role, if any, of monomer M_3 (or dimer M_3') in the formation of the 25S and 29S components is under study, as are the possible interactions leading to the apparent microheterogeneity of the 17S component.

Acknowledgments

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Isolation, Characterization, and Biological Activities of Five Mitogens from Pokeweed†

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ABSTRACT: Salt extracts of *Phytolacca americana,* pokeweed or pigeon berry, have been fractionated by a new procedure to yield five separate proteins, designated Pa-1 through Pa-5. These proteins have distinct physicochemical properties, different biological activities, and appear at different times of the year. Pa-1 is the most potent hemagglutinin, a potent mitogen, and differs considerably from the other proteins in amino acid composition and in that it appears to be a polymer of 22,000 mol wt subunits. The four other proteins appear to be monomers and are unusual in that half-cystine, Asx, Glx, and glycine account for about 60% of their amino acid residues. The predominant proteins, Pa-2 and Pa-4, contain 25 and 20 disulfide bonds, respectively, bind to the same receptors on erythrocytes, and are both mitogenic for murine spleen cells. However, Pa-2 is a hemagglutinin while Pa-4 is not. Pa-3 is a minor component resembling Pa-2. Pa-5 is also a minor component during most of the year and resembles Pa-4. All of these proteins are mitogenic over an unusually wide range of protein concentrations.

A variety of plant proteins have proven to be useful tools for the study of cell surfaces and the induction of mitosis in lymphoid cells (Sharon and Lis, 1972). Many of these proteins have been detected by their ability to induce mitosis or cause hemagglutination, but only a few have been purified or described in detail. One of the most potent plant mitogens has been isolated from *Phytolacca americana* (pokeweed or pigeon berry). The juice of berries from this plant has been found to cause mitosis of lymphoid cells *in vivo* after ingestion or systemic exposure (Barker *et al.,* 1966) and extracts of other portions of the plant were also found to induce mitosis *in vivo* and *in vitro* (Farnes *et al.,* 1964; Barker *et al.,* 1965).

Extracts of pokeweed have been fractionated previously and a single protein with both hemagglutinating and mitotic activities has been isolated (Borjeson *et al.,* 1966; Reisfeld *et al.,* 1967; Chessin *et al.,* 1966; Douglas *et al.,* 1967). In contrast to other soluble mitogens, such as concanavalin A and phytohemagglutinin which are mitogenic for only the T (thymus dependent) class of lymphocytes (Greaves and Roitt, 1968; Janossy and Greaves, 1971), extracts of *Phytolacca americana* have been reported to be mitogenic for both thymus-dependent (T) and thymus-independent (B) lymphocytes (Janossy and Greaves, 1971, 1972; Stockman *et al.,* 1971; Greaves and Bauminger, 1972; Greaves *et al.,* 1972).

In view of the biological importance of these activities, extracts of pokeweed roots have been fractionated by other methods and not one, but five proteins with different mitogenic activities have been found. This paper describes the isolation, characterization, and biological activities of these five proteins. It is evident from these and other studies (Sharon and Lis, 1972) that a single plant species may contain a variety of lectins with both large and small differences in their physicochemical properties and biological activities.

Materials and Methods

Pokeweed roots were collected periodically throughout the year from a single group of plants in Montgomery County, Md. Roots were also harvested from other sites in Montgomery County and in Westchester County, N. Y.

Ground roots were extracted with phosphate-buffered saline[1] (PBS, 310 mosm, pH 7.4) and the extract was dialyzed

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[1] Abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BSS, Hanks balanced salt solution.