Purification and Properties of a Mitogenic Lectin from *Wistaria floribunda* Seeds†

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ABSTRACT: Extracts of *Wistaria floribunda* seeds contain separable erythroagglutinating and lymphocyte mitogenic activities. Cheung et al. [Cheung, G., Haratz, A., Katar, M., Skrokov, R., & Poretz, R. D. (1979) *Biochemistry 18*, 1646] have reported a method of purifying the agglutinin. The mitogen as reported here is purified by a compatible procedure. The protein of an aqueous extract of ground seeds precipitated by ethanol contains erythroagglutinating and mitogenic activities. Following dissolution of the precipitate, the erythroagglutinin is adsorbed onto polyleucyl hog gastric mucin (allowing its purification by desorption with a saccharide inhibitor, lactose), resulting in a solution with a trace of erythroagglutinating activity and much of the mitogenic activity. After isoelectric precipitation of the residual erythroagglutinin and other contaminants, the mitogenic activity is further purified by ion-exchange chromatography. The mitogen is obtained in a homogeneous state by liquid isoelectric focusing. The purified mitogen is isoelectric at pH 7.3. It displays a molecular weight of 66 000 by gel filtration and possesses two sodium dodecyl sulfate dissociable subunits of 32 000. The purified mitogen exhibits one protein and carbohydrate staining band on both basic and acidic polyacrylamide gel electrophoresis. The mitogen is approximately 10% neutral carbohydrate by weight and has no detectable erythroagglutinating activity. It is mitogenic for murine and human lymphocytes. Binding of the mitogen to the lymphocyte cell surface is inhibited by D-mannose and thyroglobulin glycopeptides.

Mitogenic lectins serve as useful tools in the examination of the biochemical events of cellular immune recognition and initiation of lymphocyte activation and blastogenesis. Prior to their use as such reagents, it is essential to understand the chemical and physical properties of the purified mitogens. The lymphocyte mitogenic activity of extracts of *Wistaria floribunda* seeds was first described by Barker & Farnes (1967). However, these seeds contain a second, nonmitogenic hemagglutinating lectin which has been purified to chemical homogeneity (Cheung et al., 1979). In 1971, Toyoshima et al. (1971) reported the partial purification of the mitogenic lectin and its separation from the hemagglutinating lectin.

In the present paper we describe details of the purification of a potent mitogenic protein from the *W. floribunda* seeds. This procedure, employed for the isolation of the mitogenic lectin, was designed to be compatible with the simultaneous separation and purification of the erythroagglutinin of these seeds as previously described (Cheung et al., 1979).

Experimental Procedure

The initial steps for the purification of the mitogen are identical with the isolation of the agglutinin by affinity adsorption (Cheung et al., 1979). One hundred grams of ground *W. floribunda* seeds (Schumacher, MA) was suspended in 1 L of 0.01 M sodium phosphate buffer, pH 6.8, containing 0.12 M NaCl (PBS) at 4 °C. After stirring for 18 h, we filtered the suspension through cheesecloth and centrifuged it at 15000*g* for 45 min. To the supernatant (crude extract, fraction F-1) was added, at 4 °C, 1.1 volumes of 95% ethanol, and after stirring for 2 h we centrifuged the suspension at 15000*g* and 4 °C for 45 min. Following removal of the supernatant, the precipitate was suspended in 150 mL of PBS and dialyzed against 3 × 4 L of the same buffer. This was centrifuged at 25000*g* for 1 h at 4 °C, and the resulting supernatant was filtered through a glass wool plug.

The filtrate (fraction F-2) was mixed with an affinity adsorbant, polyleucyl hog gastric mucin (PLHGM) (Kaplan & Kabat, 1966; Poretz, 1973), which is capable of binding the hemagglutinin but not the mitogenic activity. After stirring for 30 min at 4 °C, we centrifuged the suspension at 400*g* and 4 °C for 15 min, producing a supernatant containing virtually all of the mitogenic activity. The hemagglutinin may be eluted from the PLHGM with a specific saccharide inhibitor, lactose (Cheung et al., 1979). The proteins not adsorbed onto the PLHGM (fraction F-3), which contain the mitogenic activity, were dialyzed against 3 × 2 L of 0.025 M sodium acetate buffer, pH 5.0, at 4 °C. The dialysate was centrifuged at 28000*g* for 45 min at 4 °C, and the resulting supernatant (fraction F-4) was filtered through a 3-μm filter (Millipore, Bedford, MA).

The mitogen was further purified from the filtrate by ion-exchange chromatography on sulfonylpropyl-Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.025 M sodium acetate buffer, pH 5.0. After application of the protein to the ion-exchange column (2.5 × 40 cm), the material was eluted (25 mL/h) first with 200 mL of the application buffer, followed by a 750-mL linear gradient of NaCl (0–0.3 M) in 0.025 M acetate buffer, pH 5.0. The protein content of fractions (1.2 mL) was determined by absorption of light at 280 nm. Appropriate fractions were pooled (see Figure 1) and dialyzed against PBS, pH 6.8.

Isoelectric focusing of SP-Sephadex pool E (following dialysis against 0.01 M phosphate buffer, pH 6.8) was performed at 4 °C in an LKB Model 110 column (LKB Instruments, Inc., Rockville, MD) over a pH gradient of 3.5–10, physically stabilized in a sucrose concentration gradient. After 72 h, the column was drained and the pH of each 1-mL fraction was measured at 4 °C. Protein was quantified by absorption of light at 280 nm, using an arbitrary $E^{1\%}$ of 10.0. Ampholines and sucrose were removed from pooled fractions by extensive dialysis against PBS.

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Carbohydrate Analysis. Total neutral sugar content was determined by the anthrone procedure (Roe, 1955) and the phenol–sulfuric acid method (Dubois et al., 1956) using D-mannose as the reference standard.

The possible presence of sialic acid was explored by determining the effect of neuraminidase treatment [as described by Warren (1963)] of the mitogen preparation on the polyacrylamide gel electrophoresis pattern of the protein, as well as by the use of the thiobarbituric acid method with mild acid hydrolysates of the protein (Warren, 1959; Aminoff, 1961). Hexosamines were quantified during quantitative amino acid analysis.

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl in evacuated sealed tubes at 110 °C for 24, 48, and 72 h. The amino acid contents of the hydrolysates were determined with the aid of an automatic amino acid analyzer as described by Spackman et al. (1958).

Molecular Weight Estimation. Molecular weights of proteins were estimated by gel filtration on either Sephadex G-200 or Sephacryl S-200 superfine (Pharmacia Fine Chemicals, Piscataway, NJ) as described by Andrews (1965), using chymotrypsinogen (23 200), ovalbumin (43 000), bovine serum albumin (68 000), and glyceraldehyde-phosphate dehydrogenase (140 000) as standard proteins of known molecular weights.

Polyacrylamide Gel Electrophoresis. Electrophoresis in 7.5% polyacrylamide slab gels at pH 8.9 was conducted as described by Davis (1964) and at pH 4.3 was conducted according to Reisfeld et al. (1962). Electrophoresis in 15% polyacrylamide slab gels in the presence of sodium dodecyl sulfate ($NaDodSO_4$) was performed according to Laemmli & Favre (1973). Samples were boiled for 1 min in the presence of 1% $NaDodSO_4$ and 5% β-mercaptoethanol prior to electrophoresis in $NaDodSO_4$ gels. All polyacrylamide gels were fixed for 30 min in 12.5% trichloroacetic acid prior to staining for protein with 0.2% Coomassie brilliant blue R or for carbohydrate with basic fuschin by the procedure according to Zacharius & Zell (1969).

Immunochemical Procedures. Antimitogen sera were produced by footpad immunization of New Zealand white rabbits with 1.0 mL of protein (1.0 mg/mL) represented in the combined SP-Sephadex peaks C–E emulsified with an equal volume of Freund's complete adjuvant. Intramuscular booster injections of the antigen emulsified with Freund's incomplete adjuvant were given at 2-week intervals. The rabbits were bled by cardiac puncture prior to each booster injection. Double immunodiffusion was conducted in agarose as described by Ouchterlony (1948).

Hemagglutination with human erythrocytes (2% suspension in PBS) and leucoagglutination with murine lymphocytes (10×10^6 cells/mL of PBS) were conducted by the microtiter procedure as previously described (Poretz, 1973).

Lymphocyte Isolation. Human peripheral blood lymphocytes were obtained from healthy donors by Ficoll–hypaque gradient centrifugation after dextran sedimentation of erythrocytes according to Boyum (1968). Cells were then washed twice in Hanks' balanced salt solution (HBSS) and suspended to a concentration of 4×10^6 cells/mL in serum-free RPMI-1640 medium containing 100 units/mL penicillin and 100 μg/mL streptomycin.

Murine splenocytes were obtained from C3H mice. Spleens were teased to produce a suspension of single cells in HBSS containing 100 units/mL penicillin and 100 μg/mL streptomycin. After centrifugation, the cell pellet was resuspended in 1 mL of distilled water to lyse erythrocytes and then diluted with HBSS. Debris was pelleted by centrifugation at 200*g* for 1 min. The remaining cells were washed twice with HBSS and suspended to 8×10^6 cells/mL in serum-free RPMI-1640 medium containing 100 units/mL penicillin and 100 μg/mL streptomycin. Cell viability was determined by trypan blue exclusion (Hanks, 1948).

Mitogenesis Assay. Triplicate cultures containing 0.1 mL of lymphocyte suspension and 0.1 mL of various concentrations of protein in serum-free RPMI-1640 medium were incubated in flat-bottom micro test plates (Costar, Cambridge, MA) at 37 °C in an environment of 5% CO_2 for 72 h. Cultures were pulsed with 1 μCi of [^{3}H]thymidine for the last 20 h of incubation, and incorporation of tritium into DNA was measured as described elsewhere (Hartzman et al., 1971), using a multiple automatic sample harvester.

Binding Assay. Binding of the mitogen to the lymphocyte membrane was examined by immunofluorescence microscopy. All reagents were used at 4 °C and were 3 mM in sodium azide. Splenocytes were suspended in PBS, pH 6.8, at a concentration of 10×10^6 cells/mL. The cell suspension (1 mL) was mixed with an equal volume of the mitogen solution in PBS and was allowed to stand for 30 min at 4 °C with occasional mixing. After centrifugation at 4 °C and washing with 2×1 mL of cold PBS, we resuspended the cells in 1 mL of diluted rabbit antimitogen antisera. After incubation for 30 min at 4 °C with occasional mixing, the cells were sedimented by centrifugation and washed with cold PBS. The cells were resuspended in 1 mL of diluted fluorescein isothiocyanate labeled globulin fraction from goat antirabbit globulin (F-GARG) and incubated 30 min at 4 °C with occasional mixing. Following centrifugation, the cell pellet was washed with cold PBS and resuspended in 0.2 mL of PBS–glycerol (1:1). Fluorescence was visualized by epillumination by using a Leitz Ortholux microscope equipped with a Leitz MPV compact microscope photometer. The cell-bound fluorescence was quantified by measuring the amount of light emitted by individual lymphocytes identified under phase-contrast illumination. At least 25 separate measurements were made for each sample. Average values were calculated with an average variation from the mean of 30%.

Inhibition of binding of mitogen to cells was performed by preincubation for 30 min at 4 °C of 0.5 mL of the mitogen solution with 0.5 mL of a solution containing a potential inhibitor. One milliliter of the lymphocyte suspension was then added to the mixture. After centrifugation, the cells were prepared for fluorescence microscopy and quantitation of cell-bound fluorescence as described above. The results were compared to the fluorescence of cells treated in a like manner but without added inhibitor.

Results

The characteristics of the fractions obtained during the purification of the *W. floribunda* mitogen are summarized in Table I. The procedure results in a 50-fold purification yielding 10% of the initial mitogenic activity. Although the material which did not adsorb to the PLHGM (fraction F-3) contains a substantial amount of hemagglutinating activity, dialysis of this solution against acetate buffer, pH 5.0, causes the precipitation of most of the residual activity. Further fractionation of the proteins by ion-exchange chromatography (Figure 1) results in pool E, with a 12-fold increase in specific mitogenic activity and 12% of the mitogenic activity as compared to the crude seed extract. Isoelectric focusing of pool E yields 30 mg of protein (IEF pool 3), homogeneous by polyacrylamide gel electrophoresis (see later), that represents only 0.2% of the extractable seed protein and 10% of the

Table I: Characteristics of the Fractions Obtained during the Purification of the *W. floribunda* Mitogen[a]

fraction	total protein (mg)	sp hemagglutination titer[b]	sp mitogenic act.[c]	mitogenic units ($\times 10^{-5}$)[d]	% act. recovered
crude extract (F-1)	12.2×10^3	1.0	16.5	7.4	100
supernatant of redissolved ethanol precipitate (F-2)	1.33×10^3	4.8	2.7	5.0	68
PLHGM, nonabsorbable protein (F-3)	1.13×10^3	0.8	2.5	4.5	61
nonprecipitable protein at pH 5.0 (F-4)	565	0.4	2.0	2.8	38
SP-Sephadex pool B	100	0.3	8.5	0.12	1.6
SP-Sephadex pool C	115	0.4	6.0	0.19	2.6
SP-Sephadex pool D	80	0.2	3.0	0.27	3.6
SP-Sephadex pool E	130	0.2	1.4	0.93	12.6
IEF pool 1	15	ND[e]	5.4	0.03	0.4
IEF pool 2	24	ND[e]	0.8	0.30	4.0
IEF pool 3	30	NA[f]	0.4	0.75	10.0

[a] From 100 g of seeds. [b] Titer with 0 erythrocytes/protein concentration. [c] Protein concentration (micrograms per milliliter) needed to induce maximum incorporation of [^{3}H]thymidine into the DNA of human lymphocytes. [d] Total protein/protein concentration required for maximum stimulation of [^{3}H]thymidine incorporation. [e] ND, not determined. [f] NA, no activity.

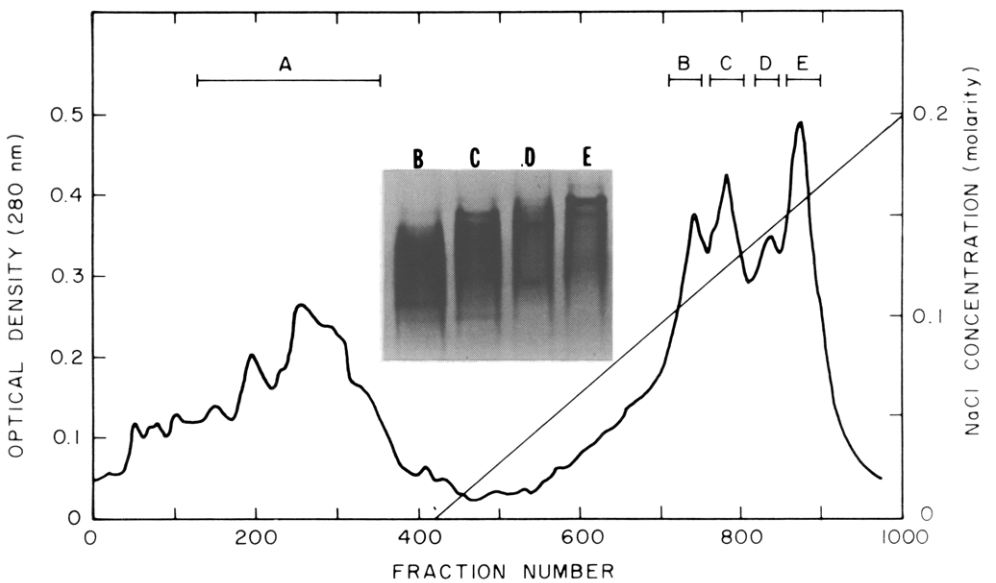

FIGURE 1: SP-Sephadex chromatogram of fraction F-4. Fractions were pooled as indicated. Inset: polyacrylamide gel electrophoresis at pH 8.9 of individual pools (80 μg of protein).

original mitogenic activity. This purified mitogen lacks any detectable hemagglutinating activity and shows a specific mitogenic activity 41 times greater than that of the crude extract.

Upon polyacrylamide slab gel electrophoresis at pH 8.9, each pooled peak from the SP-Sephadex column is seen (Figure 1, inset) to consist of a collection of protein staining species. Periodate–fuschin staining of the gel for carbohydrate results in an identical pattern, all protein staining species also stain for carbohydrate, and no component is selectively stained for protein or carbohydrate. The streaking of these glycoproteins upon electrophoresis could not be prevented by variations in the sample buffer pH or composition and is uneffected by age of the samples or the duration of electrophoresis. The mobility of the species is uneffected by prior treatment of the samples with neuraminidase. However, each pool elutes from a Sephadex G-200 filtration column as a single symmetrical peak; furthermore, the protein of each pool has the same elution volume. Polyacrylamide gel electrophoresis in $NaDodSO_4$ of β-mercaptoethanol-treated SP-Sephadex pools yields nearly identical protein staining patterns with differences in the content of minor bands. The electrophoretogram in Figure 2 shows two major bands of approximately 32 000 and 31 000 g/mol. Staining for carbohydrate produces patterns identical with those developed by

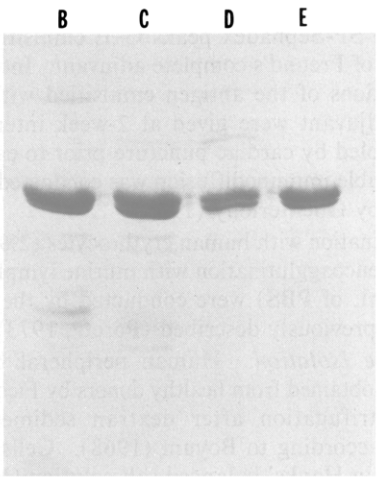

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of SP-Sephadex pools B–E following reduction with 2-mercaptoethanol (40 μg of protein).

stains for protein. Double immunodiffusion analysis of the SP-Sephadex B–E pools with rabbit antisera raised against the combined pools C–E yields one precipitin arc for each pool. The precipitin arcs appear to fuse, indicating immunochemical identity of the components of these pooled fractions.

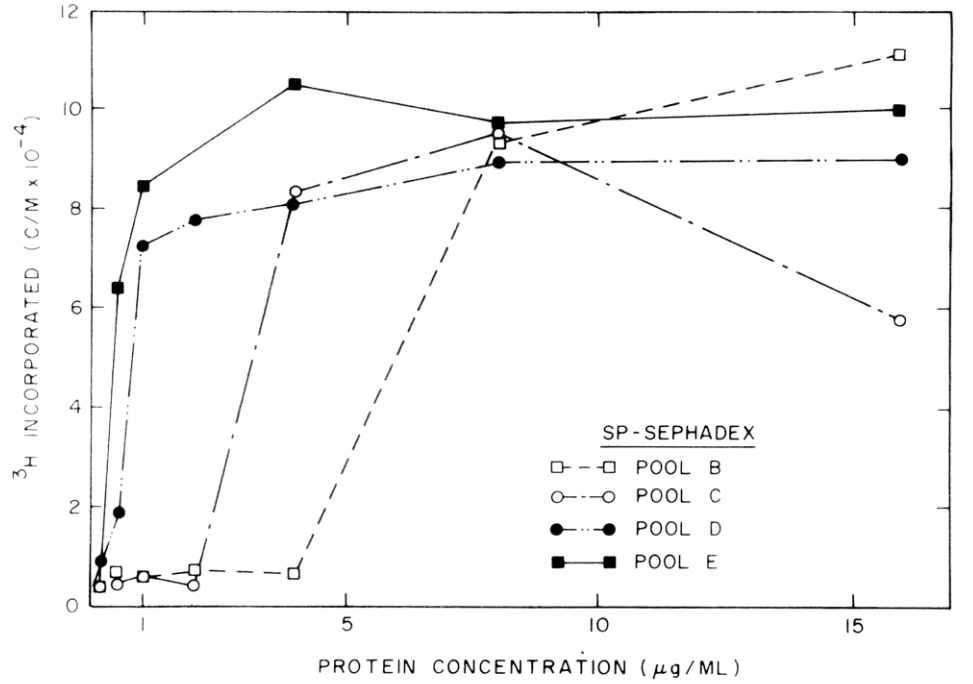

FIGURE 3: Stimulation of incorporation of [^{3}H]thymidine into DNA of human lymphocytes by SP-Sephadex pools B–E. Results are mean values of triplicate cultures with an average variation of 12%.

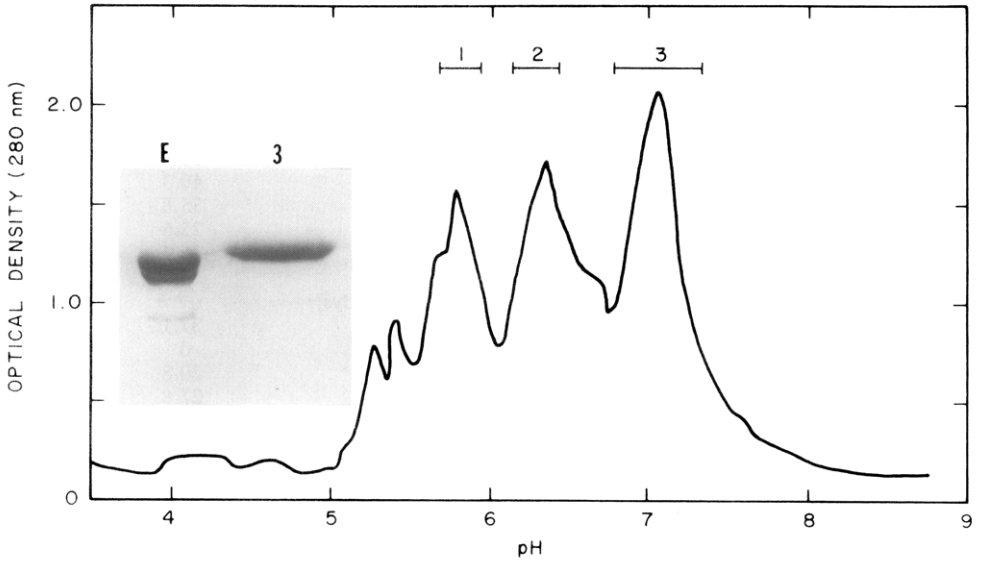

FIGURE 4: Elution profile of column isoelectric focusing of SP-Sephadex pool E. Fractions were combined as indicated. Inset: $NaDodSO_4$–polyacrylamide gel electrophoresis of SP-Sephadex pool E (40 μg) and IEF pool 3 (20 μg).

SP-Sephadex pools B–E differ greatly in their mitogenic activities. As is apparent from the dose profiles in Figure 3, pool E has the greatest specific activity, with maximum stimulation of [^{3}H]thymidine incorporation typically occurring at 1–3 μg of protein per mL. Pools D, C, and B have decreasing activities with an extreme requirement of 10 μg of protein per mL of pool B for maximum stimulation. Pool A displays no detectable activity. As shown in Figure 1, pool E, which has the greatest mitogenic activity, possesses a component of slowest electrophoretic mobility not seen in pools B–D. To further purify this protein, we conducted isoelectric focusing (IEF) with pool E over a pH range of 3.5–10. Figure 4 illustrates that the fractions eluted from the IEF column were combined into three separate pools (IEF-1, -2, and -3). It is apparent from Figure 5 that IEF-3 contains the greatest specific mitogenic activity of the pooled fractions obtained from the isoelectric focusing column. Polyacrylamide gel electrophoresis at pH 8.9 (Figure 6) demonstrates that pool 3 consists entirely of the protein staining band of slowest electrophoretic mobility identified in the electrophoretogram of SP-Sephadex pool E (Figure 1). IEF pools 1 and 2 consist of the faster migrating bands observed in polyacrylamide gel electrophoresis of SP-Sephadex pool E and contain decreasing amounts of the band of slowest mobility, isolated in pool 3. The relationship of the electrophoretic characteristics of the purified mitogen and the electrophoretic pattern of the proteins not adsorbed onto PLHGM obtained during the purification process is also shown in Figure 6.

The molecular weight of the purified mitogen (IEF pool 3) was estimated by gel filtration through Sephacryl S-200 superfine. An approximate molecular weight of 66 000 was calculated from its elution volume when compared with the elution volumes of chymotrypsinogen, ovalbumin, bovine serum albumin, and glyceraldehyde-3-phosphate dehydrogenase. The

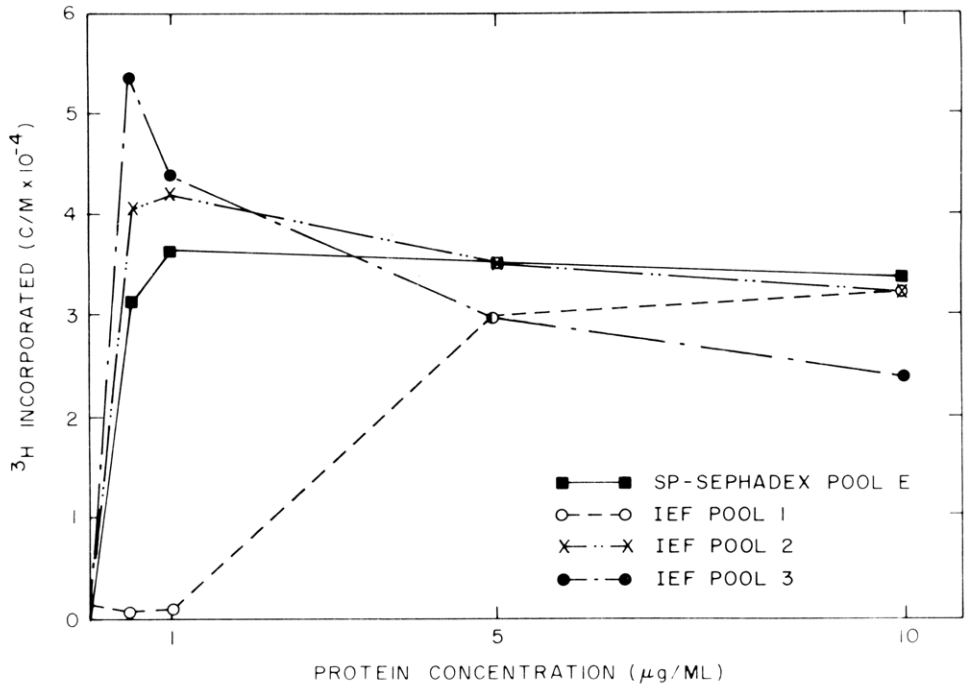

FIGURE 5: Stimulation of incorporation of [^{3}H]thymidine into DNA of human lymphocytes by IEF pools 1–3. Results are mean values of triplicate cultures with an average variation of 12%.

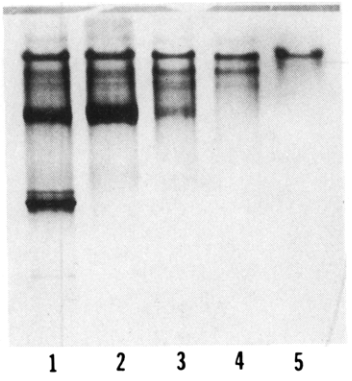

FIGURE 6: Polyacrylamide gel electrophoresis at pH 8.9 of fractions obtained during the purification of the *W. floribunda* mitogen. (1) F-2 (140 μg); (2) F-3 (120 μg); (3) F-4 (100 μg); (4) SP-Sephadex pool E (80 μg); (5) IEF pool 3 (30 μg).

molecular weight obtained for IEF pool 3 is identical with those determined for SP-Sephadex pools B–E. Furthermore, IEF pools 1–3 yield one precipitin arc upon double immunodiffusion against antisera raised to combined SP-Sephadex pools C–E. These precipitin arcs appear to fuse with each other, as well as with the arcs of SP-Sephadex pools B–E. To determine the reactivity of the antibody with the mitogenic protein of IEF pool 3, we added the immunoglobulin fraction of the antiserum to IEF pool 3 to reach immunochemical equivalence. The supernatant of such a precipitin reaction when tested for mitogenicity in RPMI-1640 medium containing 5% serum exhibits no detectable activity even at concentrations which originally contained 20 times more lectin than needed for maximum lymphocyte stimulation by IEF pool 3. $NaDodSO_4$–polyacrylamide gel electrophoresis of the resulting immunoprecipitate shows protein staining bands derived only from the immunoglobulin fraction and the major band of IEF pool 3 (see Figure 4). These results demonstrate that the antiserum is capable of neutralizing and precipitating the mitogenic protein of IEF pool 3.

The neutral sugar content of the purified *W. floribunda* mitogen is 10.3% by using D-mannose as a standard in the anthrone procedure. SP-Sephadex column pools B–E were also found to have similar quantities of neutral sugar when analyzed by the anthrone and phenol–sulfuric methods. Hexosamines were detected during amino acid analysis of IEF pools 1–3. The purified mitogen, IEF pool 3, contains approximately eight hexosamine residues per 66 000 g of protein (see Table II). No sialic acid can be detected, even at levels which would represent one residue per polypeptide chain. This is consistent with the lack of any apparent effect of sialidase treatment on the electrophoretic mobility of the mitogen.

Table II: Amino Acid Composition of IEF Pools 1, 2, and 3

residue	residues/dimer		
	pool 1	pool 2	pool 3
Asp	56.4	54.0	55.0
Thr	35.6	35.0	34.5
Ser	40.3	40.5	42.8
Glu	35.6	35.3	34.2
Pro	23.6	22.5	22.4
Gly	36.7	37.0	36.7
Ala	37.9	38.4	40.3
1/2-Cys	trace	trace	trace
Val	37.1	38.8	38.2
Met	0	0	0
Ile	20.8	21.5	20.8
Leu	27.8	27.6	27.9
Tyr	15.3	16.0	17.1
Phe	34.0	34.8	36.0
Lys	17.3	18.7	16.9
His	9.4	9.6	10.4
Arg	16.4	16.0	16.7
hexosamines	7.9	8.0	8.5

Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ demonstrates that the purified mitogen possesses a detergent-solubilized subunit with a mobility expected for a polypeptide of 32 000 g/mol when compared to the mobilities of $NaDodSO_4$-solubilized lysozyme, chymotrypsinogen, ovalbumin, bovine serum albumin, and glyceraldehyde-3-phosphate dehydrogenase.

As seen in Figure 4, the mitogen displays a single $NaDodSO_4$-dissociable electrophoretic component. This band

stains for both protein and carbohydrate. IEF pools 1 and 2 possess an additional minor band in $NaDodSO_4$–polyacrylamide gel electrophoresis with a mobility corresponding to approximately 31 000 g/mol.

The amino acid compositions of the protein in IEF pools 1–3, as shown in Table II, appear nearly identical, with high levels of acidic and hydroxy amino acids and low levels of sulfur-containing amino acids.

The *W. floribunda* mitogen is a potent mitogen for both murine and human lymphocytes. Maximum stimulation of murine lymphocytes is generally accomplished with concentrations of less than 0.05 μg/mL of the purified mitogen (IEF pool 3). Maximum stimulation of human lymphocytes occurs over a range of mitogen concentrations (1–5 μg/mL); however, the peak of maximum stimulation with murine lymphocytes is much sharper, the response dropping to control levels at approximately 2 μg of mitogen per mL of culture.

Attempts to investigate the carbohydrate binding specificity of the mitogen by sugar inhibition of lectin-induced mitogenicity gave results difficult to interpret. Although D-mannose tended to cause inhibition of mitogenicity, *N*-acetyl-D-galactosamine potentiated the mitogenic activity of the lectin. A similar potentiating effect of D-galactose of the mitogenic lectin from *Axinella polypoides* was reported by Phillips et al. (1976). Therefore, the carbohydrate binding specificity of the mitogen was examined by inhibition of its binding to murine splenocytes as detected by quantitative immunofluorescence. Surface-bound mitogen was visualized by use of the double-antibody procedure employing rabbit antimitogen immunoglobulin and fluorescein-labeled immunoglobulin G fraction from goat antirabbit serum. Maximum immunofluorescence was obtained by incubating the cells in media containing 10 μg of mitogen per mL. Background fluorescence of cells incubated with antisera without prior incubation with mitogen was negligible. Although 0.1 M concentrations of D-galactose, *N*-acetyl-D-galactosamine, D-glucose, *N*-acetyl-D-glucosamine, and L-fucose had no detectable effect on the binding of mitogen (5 μg/mL) to the cells, 0.1 M D-mannose caused a 75% inhibition of cellular fluorescence. Thyroglobulin at 250 μg/mL caused a 50% and at 1000 μg/mL a 97% decrease in fluorescence. Thyroglobulin glycopeptides were prepared by Pronase digestion of the thyroglobulin (Fukuda & Egami, 1971). The unfractionated glycopeptides (500 μg/mL), separated from the Pronase and peptides, caused a 90% inhibition of cell surface fluorescence. Similarly, the thyroglobulin glycopeptides (300 μg/mL) caused a 65% inhibition of lectin-induced mitosis of lymphocytes.

Discussion

A *W. floribunda* mitogen purified as described in this paper is essentially homogeneous. The purity of the mitogen was demonstrated by gel filtration, double immunodiffusion, and polyacrylamide gel electrophoresis at pH 8.9, at pH 4.3, and under dissociating conditions. The mitogen lacks detectable erythroagglutinating and leucoagglutinating activity and is a potent mitogen for human and murine lymphocytes, stimulating maximal incorporation of [^{3}H]thymidine into cellular DNA at concentrations of typically less than 1 μg of protein per mL in the culture suspension. The purified mitogen contains relatively large amounts of carbohydrate but apparently lacks sialic acid. On the basis of the behavior of the reduced protein in $NaDodSO_4$–polyacrylamide gel electrophoresis and the behavior of the native protein during gel filtration, this lectin appears to exist as a dimer with two identical subunit polypeptide chains of 32 000 g/mol. The mitogenic protein is apparently a lectin, being inhibited by D-mannose and glycopeptides of thyroglobulin.

The purified mitogen is strikingly similar in structure and properties to the protein components of pools B–E isolated by cation-exchange chromatography. The proteins of pools B–E appear homogeneous and indistinguishable from the purified mitogen by double immunodiffusion analysis using an antiserum to the combined pools which is capable of precipitating the mitogenic protein, polyacrylamide gel electrophoresis at pH 4.3, and analytical gel filtration. Furthermore, rechromatography of pool E under conditions identical with those described in Figure 1 results in the elution of one symmetrical peak, thus indicating that the components of pool E remain distinct and do not give rise to the components of pools B–D under the conditions of the column elution. $NaDodSO_4$–polyacrylamide gel electrophoresis of SP-Sephadex pools B–E produces nearly identical electrophoresis patterns which differ from that of the purified mitogen only in the presence of one additional component. This band appears to be a polypeptide of approximately 1000 g/mol smaller than the mitogen subunit. However, polyacrylamide gel electrophoresis at pH 8.9 reveals patterns of considerable differences. The purified mitogen migrates as a single component while SP-Sephadex pools B–E each yield a pattern characteristic of a mixture of proteins. The various species appear to be discrete components and are not interconvertible. (Each pool retains its distinct electrophoretic identity even when maintained for up to several weeks at 4 °C.)

The exact relationship of the individual proteins present in IEF pools 1 and 2 to each other and to the purified mitogen remains a mystery. One may speculate that they are mitogen-like structures composed of two dissimilar polypeptide chains differing either in small changes in sequence of the polypeptide chains or in the composition of the relatively large amount of carbohydrate. Alternatively, one polypeptide chain may be a cleavage product of the polypeptide chain present in the purified mitogen. The literature (Carter & Etzler, 1975; Rice, 1976; Howard et al., 1971; Trowbridge, 1974) contains descriptions of such differences for other lectins. *Phaseolus vulgaris* has been shown by Leavitt et al. (1977) to contain a mixture of mitogenic and erythroagglutinating lectins that are composed of polypeptide chains displaying a high degree of sequence homology. Concanavalin A has been isolated as a mixture of tetrameric proteins containing polypeptide subunits which are cleavage products of the parent polypeptide subunit (Olson & Liener, 1967; Wang et al., 1971).

Now, with the isolation of a single homogeneous mitogenic lectin from the seeds of *W. floribunda*, we are in a position to determine the structural relationships of the various mitogen-like proteins from these seeds.

Acknowledgments

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Rapid Stimulation by Insulin of Ribosome Synthesis in Cultured Chick Embryo Fibroblasts†

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ABSTRACT: The effect of insulin on the synthesis of cytoplasmic ribosomes was studied in primary cultures of resting chick embryo fibroblasts. For obviation of problems with precursor nucleotide pools, ribosome formation was measured by the incorporation of [^{3}H]leucine into ribosomal core proteins and the results have been expressed as the quotients of the specific activities of the core and total cell proteins. The following observations were made. (1) Insulin (5 μg/mL) raised the incorporation of [^{3}H]leucine into total chick cell protein by 1.3–1.5 times whereas ribosome production was stimulated by almost fourfold. (2) The ratios of the specific activities of the core and total cell proteins rose gradually as the concentration of insulin was raised from 0 to 1 μg/mL. (3) Insulin also elevated the incorporation of [^{3}H]adenine into the 28S ribonucleic acid (RNA) of cytoplasmic ribosomes. A constant relationship was maintained between the increased labeling of the 28S RNA and the proteins of the 60S-derived cores at all the levels of the hormone tested, suggesting that insulin did not influence the specific activity of the adenosine 5′-triphosphate that served as a precursor for preribosomal RNA synthesis. (4) Insulin increased the uptake of [^{3}H]adenine into 41S preribosomal RNA by two- to threefold. (5) The hormone did not, however, affect either the speed with which the 41S rRNA was processed or the time that labeled 60S subribosomes first appeared in the cytoplasm. (6) The stimulation by insulin of the labeling of 41S preribosomal RNA and of 60S subribosomes was rapid and appeared to begin within 10 min after the resting cells were treated with the hormone. (7) Insulin increased the labeling of 41S preribosomal RNA in cells that had no exogenous source of glucose.

Most investigations on the actions of insulin have been concerned with carbohydrate, fat, and protein metabolism. The hormone is known, however, to enhance the incorporation of labeled precursors into the rRNAs of muscle (Wool, 1963), liver (Steiner & King, 1966), and cultured chick embryo fibroblasts (Baseman et al., 1974; Baseman & Hayes, 1975).

Some of these studies have taken into account possible effects of insulin on the labeling of the precursor nucleotide in the cell (Wool, 1963; Baseman et al., 1974). In these cases, however, it has been assumed that the specific activity of the appropriate nucleoside triphosphate in whole-cell extracts gives a valid picture of the status of the immediate RNA precursor. This assumption is open to question. The nucleus and the cytoplasm may have different nucleotide pools (Plagemann, 1971, 1972; Goody & Ellem, 1975; Khym et al., 1978), and there is even evidence for compartmentalization within the nucleus itself (Dämmgen & Scholtissek, 1975).

To avoid problems with precursor pools, we have measured the effect of insulin on ribosome production in resting cultures of chick embryo fibroblasts by the incorporation of [^{3}H]leucine into ribosomal proteins. The data are expressed as the quotients of the specific activities of ribosomal core and total cell proteins. The validity of the results is not influenced by the compartmentalization of free amino acids in the cell (Ward & Mortimore, 1978) but depends only on the reasonable assumption that identical pools of leucyl-tRNAs are used to make ribosomal and nonribosomal proteins.

Materials and Methods

Materials. Crystalline bovine insulin (~25 IU/mg) was from Sigma. Basal medium that lacked glucose was prepared from an MEM Select-amine Kit (Grand Island Biological Co).

Cultures. Primary cultures of chick embryo fibroblasts were made from trypsinized minces of 11- or 12-day-old embryos. The cells were grown at 38 °C in a CO_2–air atmosphere in 50-mm glass dishes in 4 mL of basal medium (Minimal Essential, Eagle) supplemented with 4% calf serum. Confluency was generally reached by the third day, and the cultures were used 2 or 3 days later. At this time, each culture

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