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LECTIN-LIKE ACTIVITY FROM *Persea americana*

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ABSTRACT

An extract from the seeds of *Persea americana* possessed an erythro-agglutinating activity. The agglutinin was devoid of specificity for carbohydrates, but interacted readily with basic proteins or basic polyamino acids. The interaction between the agglutinin and egg-white lysozyme was not inhibited by chaotropic salts, but was sensitive to relatively low concentrations of urea. An affinity chromatographic procedure was developed in an effort to purify the agglutinin. Products from the chromatographic procedure were found not to contain higher specific agglutinating activities than the crude extract. Amino acid acid analyses of the extract showed the presence of relatively high proportions of glutamic and aspartic acids. In addition, the extract contained phosphorus and a visible chromophore. The agglutinin was resistant to detergents and denaturants, and proteases, nucleases, and other enzymes. The results suggest that, as opposed to other plant agglutinins, the active component from *Persea* is not a protein. Similarly, in contrast to many lectins, the agglutinin from *Persea* was not mitogenic for mouse lymphocytes. The agglutinin partially inhibited the mitogenesis of lymphocytes when the cells were treated with concanavalin A, or with bacterial lipopolysaccharide.

INTRODUCTION

Hemagglutinins of non-immunoglobulin structure are found in a variety of plant and animal tissues, and, generally, are termed lectins, because of their selectivity in promoting red-cell agglutination[1]. As far as is known, all lectins are proteins or glycoproteins, although reports have appeared that describe the hemagglutinating properties of ill-defined, crude extracts[2,3]. It has not been documented that the agglutinins in these extracts are, indeed, proteins. In addition, it has become traditional to consider lectins as carbohydrate-binding proteins[4–6]. Only recently have papers appeared which suggest that certain purified, plant hemagglutinins do not directly interact with carbohydrates or simple saccharides[7–9]. Goldstein and Hayes[4] have reviewed the chemistry and biology of purified lectins, Liener[5] has reviewed the

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properties of lectins isolated from plants, and Lis and Sharon[6] described current research on the effects of lectins on immune systems.

Staat *et al.*[10] observed that an extract from the seeds of *Persea americana* possessed the ability to inhibit the adherence of the glucan-producing bacterium *Streptococcus mutans* to smooth surfaces. Furthermore, the extract was found to contain hemagglutinating activity. The present work defines additional properties of the agglutinin. The results suggest that the agglutinin is not a protein, and that the agglutinin interacts with protein side-chains instead of with carbohydrates.

EXPERIMENTAL

Reagents. — Arginine, aspartic acid, glutamic acid, histidine, lysine, ornithine, and tryptophan (all L isomers) were obtained from United States Biochemical Corporation (Cleveland, OH). Poly(L-lysine), poly(L-glutamic acid), poly(L-aspartic acid), poly(DL-ornithine), poly(glycine), egg-white lysozyme, ovalbumin, fetuin from fetal-calf serum, bovine-plasma albumin, spermidine [*N*-(3-aminopropyl)-1,4-butanediamine], rabbit-liver glycogen, L-rhamnose, 2-acetamido-2-deoxy-D-galactose, *N*-acetylneuraminic acid, methyl α-D-mannopyranoside, *o*-nitrophenyl β-D-galactopyranoside, and *p*-nitrophenyl α-D-glucopyranoside were products of Sigma Chemical Company (St. Louis, MO). Calbiochem (Los Angeles, CA) provided D-mannose, L-fucose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, Pronase, wheat-germ lipase, and human umbilical-cord hyaluronic acid. D-Glucose, sodium dodecyl sulfate (SDS), lithium chloride, urea, disodium (ethylenedinitrilo)tetraacetate (EDTA), sodium iodide, and ammonium sulfate were purchased from Fisher Scientific Company (Fair Lawn, NH). D-Galactose and maltose were obtained from Pfanstiehl Laboratories, Inc. (Waukegan, IL). Mann Research Laboratories (New York, NY) provided cellobiose. Acids and solvents were purchased from J. T. Baker Chemical Company (Phillipsburg, NJ). Chlorhexidine [*N*,*N*″-bis(4-chlorophenyl)-3,12-diimino-2,4,11,13-tetraazatetradecanediimidamide] digluconate was a generous gift from ICI Americas, Inc. (Wilmington, DE). Human-blood α_1-acid glycoprotein was a gift from U. Westphal. Dermatan sulfate and chondroitin 4-sulfate were obtained from Miles Laboratories (Elkhart, IN).

Phosphate-buffered saline (PBS) was prepared by buffering 0.15M sodium chloride (Fisher Chemical Company) with 0.05M potassium phosphate (Mallinckrodt, Inc., St. Louis, MO) and adjusting to pH 7.3 with sodium hydroxide.

Extraction of the agglutinin. — *Persea americana* seeds were obtained by courtesy of James Olds, Louisville, KY. After removal of the seed coat, the seeds were freeze-dried, and pulverized to a fine powder. The powdered seed material (100 g) was suspended in either 1.000 L of water or 1.000 L of PBS. The slurry was stirred gently for 16–20 h at 4°, and the sediment was removed by centrifugation. The supernatant liquor (~800 mL) was dialyzed against water (5 changes, 5 L per change) and subsequently freeze-dried. The yield was usually 800–1200 mg.

Chemical analyses. — Phosphorus was determined by the method of Ames[11]

and 3-deoxy-2-octulosonate by the method of Karkhanis *et al.*[12]. The limulus assay was performed with an "E-Toxate" kit provided by Sigma Chemical Company. All amino acids were quantitated by AAA Laboratory (Mercer Island, WA). Analyses were performed on hydrolyzates (6M HCl; 24 h at 110°), corrections being made for decomposition of serine and threonine. Cystine was analyzed after hydrolysis with performic acid. Tryptophan was quantitated by the method described by Hugli and Moore[13].

Assay method for hemagglutinating activity. — Agglutinating activity for erythrocytes was determined in the following way. The freeze-dried extract was dissolved in PBS at a concentration of 1.0 mg/mL, and serial, two-fold dilutions were made with PBS as the diluent. An aliquot (50 μL) of each dilution was added to a well (in a round-bottomed, microtiter plate) containing 2% (v/v) washed erythrocytes (50 μL), the mixture was kept for 1 h at room temperature, and the titer value was determined visually. Typically, the extract titer was 1:128 or 1:256.

In inhibition assays, 1:32 titer-value of extract (20 μL), human, type A, red-blood cells (20 μL), and inhibitor (50 μL) were used.

Precipitation with macromolecules. — Precipitation of the extract with proteins, polyamino acids, or polyelectrolytes was tested visually, using 1.2 mg of protein, polyamino acid, or polyelectrolyte, and 1 mg of extract in PBS (1.5 mL, total volume). In precipitation with lysozyme and with chlorhexidine, the absorbance of the mixture at 600 nm was read over a range of concentrations of the extract. The values obtained were corrected for the absorbance values obtained with the extract alone. Inhibition of precipitation with lysozyme by sodium iodide or lithium chloride was performed in 0.1M sodium acetate buffer, pH 5.5. Inhibition by urea of precipitation was conducted in PBS. Readings of absorbance were made at 600 nm.

Mitogenesis. — Female mice, strain C57Bl/6 (20–22 g) were obtained from Laboratory Supply Company, Inc. (Indianapolis, IN). Concanavalin A (con A) was prepared according to Agrawal and Goldstein[14]. Lipopolysaccharide (LPS) from *Salmonella enteritidis* (Difco Laboratories, Detroit, MI), con A, and *P. americana* extract were serially diluted in complete RPMI-1640 medium (prepared from RPMI-1640; Gibco, Grand Island, NY) and added to 10^6 mouse-spleen lymphocytes per well in a round-bottomed, microtiter plate (Microbiological Associates, Walkersville, MD) in complete RPMI-1640 medium[15]. In order to determine the effect of the *Persea* extract on con A- and LPS-stimulated mitogenesis, another series of wells was prepared that contained serial dilutions of con A or LPS which were added to 10^6 mouse lymphocytes per well in complete RPMI-1640 medium containing 100 μg/mL of *Persea* extract. Wells contained 100 μL of con A, LPS, or extract, 100 μL of spleen cells in growth medium or extract medium, and 20 μL of ^{3}H-thymidine (^{3}H-Tdr) (1.0 μCi/well, 48.1 Ci/mmol; obtained from Amersham Corporation, Arlington Hts., IL). Cells were incubated at 37°, pulsed at 48 h, and harvested in a "MASH" cell-harvester (Valcor Engineering Corporation, Kenilworth, NJ) at 72 h. Harvesting was performed by using 0.15M saline buffer, and filter-paper strips purchased from Brandel Laboratories (Rockville, MD). Beta radiation was counted by placing the

filter-paper circles, containing the harvested cells from each well, in 5 mL of "ACS" (Aqueous Counting Scintillant, Amersham Corporation) in a Beckman LS 100C automatic scintillation spectrometer. Viability testing of the lymphocytes was performed by the Trypan Blue, dye-exclusion method[16].

RESULTS

In the search for lectins, or agglutinins, from plant extracts or from other biological sources, the traditional approach is to determine whether the extract contains red-cell-agglutinating activities[4,5]. Once an activity has been established, inhibition studies are employed to determine the specificity of the agglutination reaction. Following the establishing of an empirical specificity, the agglutinin can be purified by affinity chromatography. Solutions of *P. americana* extract in PBS were mixed with washed, red-blood cells from various animals. The results (see Table I) revealed that the extract was active against all of the red cells examined. Red cells from the rat were agglutinated only at the higher concentrations of extract. Human, type A, red cells appeared to agglutinate more readily than human, type B, or type O, erythrocytes with the extract. On the basis of the data in Table I, it is suggested that the *P. americana* extract contains a lectin or lectin-like activity.

Efforts to establish the specificity of the interaction between the extract and red cells were conducted by use of agglutination assays in the presence of potential inhibitors. A variety of carbohydrate inhibitors, including hexoses, pentoses, 6-deoxyhexoses, hexuronic acids, disaccharides, phenyl glycosides, 2-acetamido-2-deoxyhexoses, *N*-acetylneuraminic acid, and a glycosaminoglycan, dermatan sulfate, were employed as possible inhibitors in agglutination assays (see Table II), but none of the carbohydrates or carbohydrate-containing molecules were found to inhibit the agglutination between the *P. americana* extract and human, type A, red cells, even

TABLE I

ERYTHROCYTE AGGLUTINATION BY *Persea americana* EXTRACT

Erythrocytes	*Hemagglutination titer*[a]
Human Type A	128
Human Type B	32
Human Type O	32
Mouse	128
Sheep	128
Bovine	128
Rat	8
Rabbit	64

[a]Wells contained 50 μL of extract and 50 μL of 2% washed erythrocytes in PBS. Microtiter plates were kept for 1 h at room temperature and read visually for hemagglutination end-point. Dilutions were made from an initial concentration of extract of 1.0 mg/mL.

TABLE II

NON-INHIBITORS OF *Persea americana* HEMAGGLUTININ

Class	*Compound tested*[a]
Hexoses, pentoses	D-glucose, D-galactose D-mannose, D-ribose, D-xylose
6-Deoxyhexoses	L-rhamnose, L-fucose
2-Acetamido sugars	2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-mannose
Glycosides	methyl α-D-mannopyranoside, *o*-nitrophenyl β-D-galactopyranoside *p*-nitrophenyl α-D-glucopyranoside cellobiose, maltose, lactose
Charged carbohydrates	D-glucuronic acid, 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, *N*-acetylneuraminic acid, dermatan sulfate
Amino acids or polyamine	L-arginine, L-ornithine, L-lysine, L-histidine, L-tryptophan, L-glutamic acid, L-aspartic acid, spermidine

[a]Microtiter wells contained 20 μL of 1:32 extract, 50 μL of inhibitor, and 20 μL of 2% washed human erythrocytes (type A) in PBS. Final hapten-inhibitor concentrations were 0.1M, except for *p*-nitrophenyl α-D-glucopyranoside, *o*-nitrophenyl β-D-galactopyranoside, cellobiose, and D-glucuronic acid, which were 0.2M. *N*-Acetylneuraminic acid and spermidine were assayed at 0.6, 0.3, and 0.1 mg/mL; histidine, aspartic acid, tryptophan, glutamic acid, at 1.1 mg/mL; arginine, lysine, and ornithine, at 1.1, 0.5, and 0.3 mg/mL; and dermatan sulfate, at 0.1 mg/mL.

though the kinds of carbohydrate inhibitors, and their potentially inhibitory concentrations, were so chosen as to include the known range of lectin specificities thus far examined[4]. Other substances, such as basic, hydrophobic, or acidic amino acids, as well as spermidine, were also ineffective in promoting an inhibition of the agglutination.

The work of Staat *et al.*[10] had shown that *P. americana* extracts agglutinate intact *S. mutans* cells. The extract from *P. americana* was mixed with various kinds of polymers that may be found on bacterial cell-surfaces, such as teichoic acids and dextrans, but no visible reaction occurred (see Table III). In addition, such macromolecules as glycogen, α_1-acid glycoprotein, fetuin, ovalbumin, bovine-plasma albumin, and several other polymers did not visibly interact with the extract.

In contrast, the extract readily precipitated with lysozyme, ribonuclease, trypsin, poly(L-lysine), and poly(DL-ornithine). The latter substances are characterized by the presence of relatively high contents of basic residues. These results suggest that *Persea* activity is directed primarily against the positively charged groups of macromolecules.

Fig. 1 shows that the bis(biguanide), chlorhexidine, readily precipitates with the extract. In addition, data are presented which show that the precipitation of both

TABLE III

Precipitation of Persea americana EXTRACT WITH MACROMOLECULES[a]

Polymer tested	*Precipitation*
Lysozyme (egg white)	+
Ribonuclease	—
Poly(L-lysine)	+
Poly(DL-ornithine)	+
Trypsin	+
Poly(glycine)	—
Poly(aspartic acid)	—
Poly(glutamic acid)	—
α-Acid glycoprotein	
Fetuin	+
Ovalbumin	+
Bovine-plasma albumin	—
Trypsin inhibitor (soybean)	+
Chondroitin 4-sulfate	—
Dermatan sulfate	—
Dextran (mol. wt. 227,000)	—
Rabbit-liver glycogen	—
Bacillus subtilis lipoteichoic acid	+
B. subtilis cell-wall teichoic acid	—
Hyaluronic acid	—

[a]Precipitation was tested by using 1.2 mg of the polymer and 1 mg of the extract in PBS (1.5 mL, total volume). Results were determined visually.

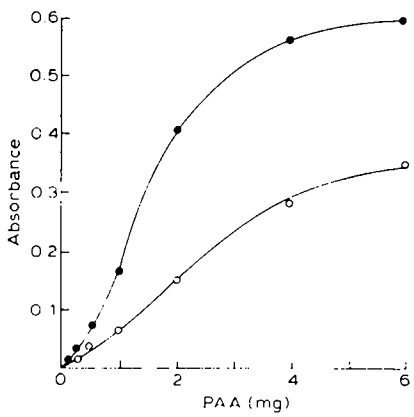

Fig. 1. Precipitation of *P. americana* extract (PAA) with lysozyme and chlorhexidine. [Lysozyme (1 mg) or chlorhexidine (1 mg) was added to increasing amounts of PAA in PBS (4.0 mL, total volume). Absorbance was read at 600 nm. Chlorhexidine (—○—); lysozyme (—●—).]

lysozyme and chlorhexidine with the extract is a function of the concentration of the extract (see Fig. 1). The "S"-shaped, precipitin profiles suggest a cooperative interaction between the precipitants.

Because the *P. americana* extract possesses many lectin-like properties, it is

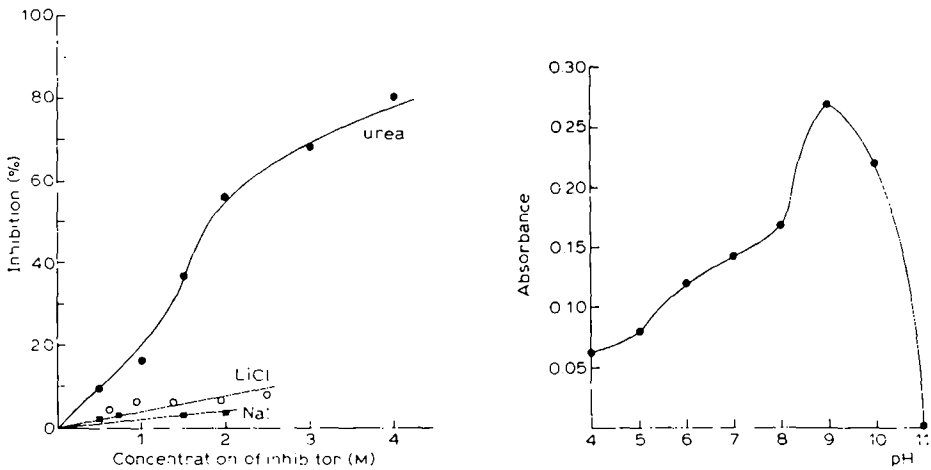

Fig. 2. Effect of inhibitors on PAA–lysozyme precipitation. [Lysozyme (1 mg) was added to 2 mg of PAA (4.0 mL, total volume) in sodium iodide or lithium chloride (0.1M sodium acetate buffer, pH 5.5) or urea (PBS buffer, pH 7.3). Absorbance was read at 600 nm, and % inhibition was calculated relative to the control absorbance of PAA–lysozyme.]

Fig. 3. Hydrogen-ion profile of PAA–lysozyme precipitation. [Lysozyme (1 mg) was added to PAA (2 mg) in 0.1M buffer (4.0 mL, total volume). Buffers used were sodium acetate (pH values 4, 5), phosphate (pH values 6, 7, and 8), and sodium carbonate (pH values 9, 10), adjusted with HCl or NaOH. Absorbance was read at 600 nm.]

tempting to call the extract a *Persea* lectin, but, as the foregoing data do not specifically show that the activity is related to a protein, it may not be entirely correct to employ a nomenclature usually reserved for carbohydrate-binding proteins[4]. Rather, the extract will subsequently be referred to as the *P. americana* agglutinin (PAA), a term that implies neither structure nor specificity.

Additional attempts were made to define further the nature of the interaction between PAA and lysozyme. It was hypothesized that the precipitation might be a result of ionic interactions, which are normally sensitive to changes in the ionic strength of the solution. Accordingly, PAA and lysozyme were allowed to interact in the presence of various concentrations of sodium iodide, and lithium chloride, and the resulting turbidities were recorded (see Fig. 2), but the salts, both of which are denaturants, were ineffective as inhibitors. In contrast, inhibition of precipitation increased with increasing concentrations of urea. These results suggest that the precipitation between PAA and lysozyme is not a function of nonspecific, salt bridges, but may involve amino groups of protein and some as-yet-unidentified, functional group on PAA.

The precipitation between PAA and lysozyme could be altered when the hydrogen-ion concentration was varied (see Fig. 3). The pH of maximal turbidity was found to be ~ 9. At pH values >9, little interaction was observed, whereas, at pH values ≤ 5, a turbidity diminished in comparison with those values obtained

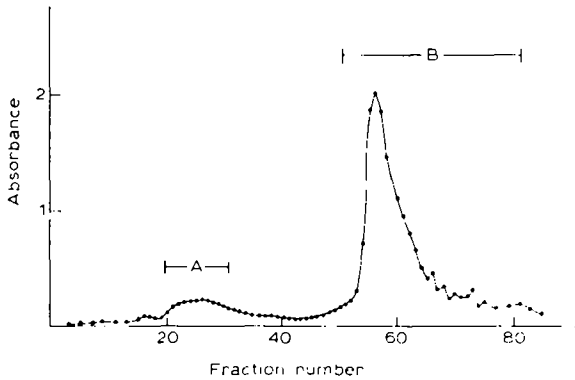

Fig. 4. Affinity-chromatography elution-profile. [Extract (25 mg), dissolved in PBS (2.5 mL) and centrifuged, was applied to the Affi-Gel-lysozyme column (6.5 × 1.8 cm) and eluted with PBS. Thirty 1-mL fractions were collected before 8M urea was substituted as the eluant. Absorbances of the fractions were read at 437 nm.]

at pH values of 5 to 9 was observed. With respect to hydrogen-ion concentration, PAA activity is qualitatively similar to those of several characterized lectins[4].

Attempts were made to purify the hemagglutinating activity of PAA. The extract was passed through columns of O-(2-diethylaminoethyl)cellulose and *O*-(carboxymethyl)cellulose, which are, respectively, basic and acidic ion-exchange resins, and eluted with salts. The activity appeared to adsorb to the resins, and it

TABLE IV

EFFECTS ON *Persea americana* HEMAGGLUTININ OF PHYSICAL AND CHEMICAL TREATMENTS

Treatment	*Hemagglutination titer*
Control (freeze-dried preparation)	128
Saturated ammonium sulfate (precipitate)[a]	128
Saturated ammonium sulfate (supernatant liquor)	128
Autoclaving[b] at 20 lb.in.$^{-2}$ for 15 min at 115°	128
SDS (3% w/v) for 30 min at 100°	128
Dialysis *vs.* 3% SDS[c]	128
Dialysis *vs.* 8M urea	256
Dialysis *vs.* 5M lithium chloride	256
Dialysis *vs.* 0.1M NaOH	32
Dialysis *vs.* 1.0M hydroxylamine (pH 8.0)	256
Dialysis *vs.* 40mM EDTA (pH 7.3)	256
Dialysis *vs.* 0.1M HCl	256

[a]PAA (100 mg) was added to 50 mL of saturated ammonium sulfate solution and stirred overnight at 4°. Soluble and insoluble fractions were separated by centrifugation. [b]Extract was dissolved in PBS before autoclaving. [c]Dialysis was against 500 mL of reagent in each of the seven treatments for 2 h, followed by dialysis against water. Following dialysis against water, all samples were freeze-dried.

could not be eluted with concentrated sodium chloride. In addition, PAA was passed through a column of agarose (Bio-Gel A-15m) and a column of poly(acrylamide) beads (Bio-Gel P-4). When eluted with PBS, each column showed a "smearing" of hemagglutinating activity. Fractions collected, dialyzed, and freeze-dried showed specific activities (titers 1:128) similar to that of the crude extract; thus, neither column served to provide any enrichment. Finally, a lysozyme–agarose conjugate[17] was synthesized, and poured into a column; the extract was added to the top of the column, and fractions were collected (see Fig. 4). The ability of urea to reverse PAA-lysozyme precipitation was utilized in this technique. Buffer (PBS) eluted a small amount of absorbing material (fractions 19–31). Addition of 8M urea caused elution of an additional fraction (fractions 50–81). Material that was eluted from each peak was dialyzed against water, lyophilized, and tested for hemagglutinating titer. The first peak (A) had a titer of 1:4, and the second (B), a titer of 1:64. Both titers were lower than the control titer of 1:128. Thus, the lysozyme affinity-column afforded no apparent enrichment of hemagglutinating activity. Additional attempts to purify the agglutinating factor from the extract were made by fractional precipitation with ammonium sulfate (see Table IV). Approximately half of the material was insoluble in saturated ammonium sulfate, but the soluble and insoluble fractions possessed the same titer against human, type A red-cells.

In Table IV are presented additional data that show the effects of various physical and chemical agents on the agglutinin. Denaturants, such as heat, SDS, concentrated lithium chloride, urea, and strong acid failed to cause any inactivation of the agglutinin. Moreover, EDTA, and hydroxylamine, were without effect on the active component(s). In contrast, dilute base (0.1M sodium hydroxide) gave rise to a

TABLE V

EFFECT OF ENZYMES ON THE HEMAGGLUTINATION OF *Persea americana* WITH HUMAN RED-CELLS

Enzyme tested[a]	*Hemagglutination titer*
Control (freeze-dried preparation)	128
DNAase (bovine pancreas)	128
RNAase (bovine pancreas)	128
Trypsin	128
Pronase	128
Pepsin	128
Lipase (wheat germ)	128
Neuraminidase	128

[a]Enzyme treatments were performed in test tubes by adding 20 mg of extract to 10 mL of enzyme solution. Enzyme concentrations were 100 μg/mL (Pronase, pepsin, lipase, DNAase, trypsin), 76 μg/mL (RNAase), and 19 μg/mL (neuraminidase). All enzyme solutions were prepared in PBS, except pepsin, which was prepared in 0.05M HCl. Enzyme-extract solutions were incubated for 16 h at 37°. Following incubation, any insoluble material was removed by centrifugation. The supernatant liquor was dialyzed against water, and freeze-dried.

product that contained lessened activity. Such solvents as acetone, 1-butanol, ethanol, ethyl ether, and hexane failed either to solubilize the extract, or lower the hemagglutinating ability of the extract (data not shown). Based on the foregoing, it is suggested that the agglutinin is highly polar, and is probably neither a pure polypeptide having a nonrandom structure, nor an ester-containing molecule.

When the extract was treated with proteases, nucleases, a lipase, or a neuraminidase, no diminution in agglutination was observed (see Table V). These results further support the premise that the agglutinin is not a protein. Furthermore, it is unlikely that the agglutinin is a nucleic acid, or that it depends on sialic acid or a lipid for reactivity.

In Fig. 5 are shown the ultraviolet and visible spectra of the extract. The spectra are characterized by maxima at 277, 437, and 485 nm. In other attempts to determine the nature of the agglutinin, it was found that the extract did not contain 3-deoxy-D-*manno*-octulosonic acid, nor was it capable of eliciting a positive, limulus test. Thus, it is unlikely that a lipopolysaccharide contaminant contributes to the properties of the extract. The material contained 0.69 μmol of phosphorus per mg (dry weight). The proportion of phosphorus in the preparations was not dependent on whether the seeds were extracted with PBS or with water. Assays for protein and carbohydrate were inconclusive, because of the presence of the visible-absorption chromophores. When the extract was stored as a solution in PBS for long periods at 4°, slow loss of activity was observed (see Table VI). On standing, a small proportion of insoluble material appeared; any insoluble material was removed by centrifugation prior to the titer determinations.

The extract was subjected to hydrolysis and analyzed for amino acids. The results (see Table VII) show the presence of relatively high proportions of glycine,

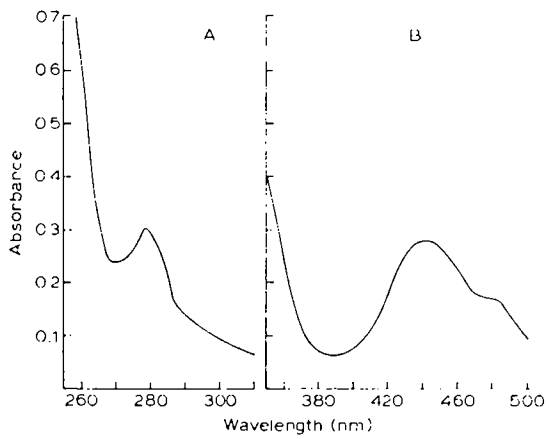

Fig. 5. Ultraviolet and visible-light spectra of PAA. [In the ultraviolet spectrum (A), the concentration of PAA was 0.075 mg/mL. A path-length of 1.0 cm was employed for both spectra. The solvent was PBS. The visible spectrum (B) was obtained by use of 0.75 mg of PAA/mL in PBS.]

TABLE VI

STABILITY OF THE *Persea americana* AGGLUTININ IN BUFFER[a]

Time (days)	*Hemagglutination titer*
0	128
7	128
14	64
21	32
28	32
35	32
46	8
53	4

[a]PAA (1 mg/mL in PBS) was stored at 4°, and tested for hemagglutinating activity after removing any precipitate by centrifugation. Human, type A cells were used in the assays.

TABLE VII

AMINO ACID COMPOSITION OF THE *Persea americana* CRUDE EXTRACT

Amino acid (L)	*Proportion (nmol/mg)*
Alanine	13.2
Arginine	4.0
Aspartic acid	22.5
Cystine/2	3.2
Glutamic acid	60.6
Glycine	54.7
Histidine	2.7
Isoleucine	4.4
Leucine	7.1
Lysine	5.9
Methionine	7.3
Phenylalanine	2.9
Proline	7.2
Serine	16.2
Threonine	9.4
Tryptophan	0.3
Tyrosine	1.4
Valine	5.8

and glutamic and aspartic acids. The low content of hydrophobic and basic amino acids, compared to the acidic residues, is striking.

Lectins have been widely used in research dealing with immune phenomena. Because of the extensive literature on the effects of lectins on lymphocyte mitogenesis, and on other immune-related processes, such as immediate or delayed hypersensitivity,

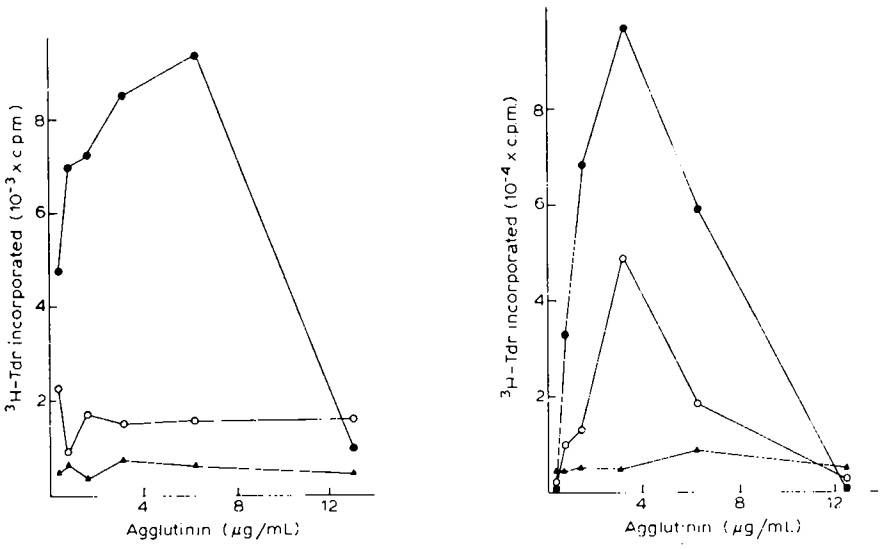

Fig. 6. Effect of PAA on mitogenesis of mouse lymphocytes induced by LPS, and mitogenic effect of PAA on mouse lymphocytes. [Serial dilutions of LPS were added to 10^6 lymphocytes/well, in complete RPMI-1640 medium (—●—), or complete RPMI-1640 medium containing 100 μg of PAA/mL (—○—). Wells contained 100 μL of LPS and 100 μL of lymphocytes (10^7 lymphocytes/mL of medium). In a separate set of wells, serial dilutions of PAA were added to 10^6 lymphocytes/well, in complete RPMI-1640 medium (—▲—). All cells were incubated at 37°, pulsed at 48 h with ^{3}H-thymidine, and harvested at 72 h. Background radiation (control) was 1594 c.p.m./10^6 lymphocytes (LPS + PAA), 1126 c.p.m./10^6 lymphocytes (LPS), and 3849 c.p.m./10^6 lymphocytes (PAA)].

Fig. 7. Effect of PAA on mitogenesis of mouse lymphocytes induced by con A, and mitogenic effect of PAA on mouse lymphocytes. [Serial dilutions of con A were added to 10^6 lymphocytes/well, in complete RPMI-1640 medium (—●—), or complete RPMI-1640 medium containing 100 μg/mL PAA (—○—). Wells contained 100 μL of con A and 100 μL of lymphocytes (10^7 lymphocytes/mL of medium). In a separate set of wells, serial dilutions of PAA were added to 10^6 lymphocytes/well, in complete RPMI-1640 medium (—▲—). Wells contained 100 μL of PAA and 100 μL of lymphocytes (10^7 lymphocytes/mL of medium). All cells were incubated at 37°, pulsed at 48 h with ^{3}H-thymidine, and harvested at 72 h. Background radiation (control) was 1480 c.p.m./10^6 lymphocytes (con A + PAA), 1619 c.p.m./10^6 lymphocytes (con A), and 3849 c.p.m./10^6 lymphocytes (PAA).]

it was deemed appropriate to examine the effects of PAA on selected, cellular, biological systems. Therefore, PAA was used in experiments designed to assess the mitogenic stimulation of T and B lymphocytes. The effect of PAA on LPS- or con A-induced mitogenesis of mouse lymphocytes is diagrammed in Figs. 6 and 7. PAA inhibited mitogenesis of lymphocytes by ~50% in con A-stimulated cells, and by >90% in LPS-stimulated cells. The extract was not itself mitogenic (see Figs. 6 and 7), nor was it toxic to the lymphocytes, as shown by viabilities paralleling those of control cells (cells were ~80% viable, as determined by the dye-exclusion test[16]).

DISCUSSION

The results of the present study suggest that, although it has certain, lectin-like

properties, *Persea americana* agglutinin does not generally conform with the characteristics expected of molecules having polypeptide structures. All lectins studied to date have been pure proteins, glycoproteins, or metalloproteins[4]. Some evidence indicating that PAA is not a pure polypeptide is shown in Tables IV and V. The agglutinin withstands the effects of heat, SDS, strong acid, urea, and lithium chloride. Precipitation with ammonium sulfate does not bring about additional purification, as the supernatant and the precipitate fractions had equivalent, hemagglutinating activities. The extract was completely soluble in 20% trichloroacetic acid.

Attempts to purify the agglutinin by using such dependable, protein-separation techniques as gel exclusion and affinity and ion-exchange chromatography were unsuccessful. Furthermore, a variety of protein-degrading enzymes, such as pepsin, trypsin, and Pronase, were without effect on the potency of PAA. In addition, treatments specific for other classes of molecules, such as attempted solubilization in organic solvents, and treatment with lipase, neuraminidase, or nucleases failed to lessen the hemagglutination ability. Although these data do not provide the exact, chemical nature of the agglutinin, they suggest that PAA is non-protein and highly polar, and that its activity is not related to ester, sialic acid, lipid, or nucleic acid components. The amino acid analyses yielded information suggesting the presence of highly acidic polypeptides (see Table VII). Acidic polypeptides, as well as phosphates, could readily interact with basic groups on cell surfaces, or with basic side-chains of proteins. Such interactions would account for the apparent affinity of the agglutinin for basic substances.

Although a wide range of carbohydrates and a number of amino acids and polyamines were tested, none of them were found inhibitory to agglutination, and therefore none were considered likely to be responsible for PAA specificity. *Persea americana* "lectin" is not unique in its lack of carbohydrate specificity. The findings of Shiomi *et al.*[7] with *Agardhiella tenera* lectin showed that the red alga lectin is not inhibited by simple sugars. Similarly, Pacák and Kocourek[8] studied the *Evonymus europeaea* L. lectin, and reported no inhibition of agglutination by the sugars tested. Sugar specificity is also lacking in the *Arion emporicum* lectin, according to Habets *et al.*[9]. Although the hapten-inhibition assay failed to demonstrate an affinity of PAA towards any of the molecules tested, PAA was observed to precipitate with certain macromolecules.

Habets *et al.*[9] showed that *Arium emporicum* lectin precipitates with serum proteins. Similarly, PAA precipitates with certain proteins and polyamines rich in amino groups, such as lysozyme, ribonuclease, poly(L-lysine), poly(DL-ornithine), and trypsin. *Persea* extract also precipitates with chlorhexidine, another basic compound. It is here postulated that the agglutinin is, therefore, specific for basic residues found in proteins, with which PAA interacts to produce the easily visible, precipitation reaction. The interaction is not simply ionic in nature, as is shown by the lack of inhibition by the salts sodium iodide and lithium chloride. Rather, the precipitation is the result of firm binding, which is, nevertheless, reversible by urea, a molecule which, although too small to cause precipitation of PAA itself, markedly decreases

the PAA–lysozyme precipitation, even at molarities <1.0 (see Fig. 2). Urea not only reverses the precipitation, but also blocks the reaction when added, before the lysozyme, to the PAA solution; this observation suggests that urea competitively inhibits the binding of lysozyme to PAA by virtue of its free amino groups. Larger, positively charged molecules would obscure the reaction by precipitating with the agglutinin. Urea is, thus, a "hapten" inhibitor of the precipitation reaction of PAA with lysozyme. The reaction is sensitive to the hydrogen-ion concentration, as shown in Fig. 3. The greatest absorbance value was obtained at pH 9, the approximate pK value of an amino group.

Cell agglutination by PAA includes representative strains of *S. mutans* from each Bratthall serotype[10], and hemagglutination of a variety of mammalian, red-blood cells. Although the agglutinin is nonspecific, in that it agglutinates all of the erythrocytes tested, the hemagglutination occurs in a differential manner (see Table I). There are several factors that determine the extent to which agglutination will occur in a cell-agglutinin system; among these are the geometry and density of the cell receptors. On some cells, the receptors may be geometrically inaccessible to binding by PAA, whereas, on others, a low density of receptors may impede formation of multiple interactions and cross-linking, preventing cell agglutination by lectin bridges. Certain cells have a greater membrane fluidity than others, owing to the various proportions of different fatty acids in the lipid bilayer, allowing mobility of the lectin-binding receptors. Cell-charge, repulsive forces also affect the extent of binding of a polar, or charged, lectin molecule.

Mitogenesis of lymphocytes by various lectins has received a great deal of attention in recent years. The nonspecific triggering of large numbers of cells to divide has enabled detailed study of the mechanism of growth activation and proliferation. The mechanism by which lectins exert their stimulatory effects is obscure, but it is generally accepted that the initial step is binding to a cell-surface sugar; this step presumably generates a signal to the cell interior, resulting in a biochemical signal to grow and divide. Binding alone is not, however, sufficient, as it is not always followed by activation of cell division. Thus, wheat-germ agglutinin binds to cell-surface sugars, and yet it is not mitogenic[18]. *Persea americana* agglutinin is not mitogenic (see Figs. 6 and 7). Factors that determine whether an agglutinin can act as an effective mitogen include the valency of the agglutinin, the affinity for receptor sites, and the kinds of receptors available on the cell type. Some agglutinins, such as con A, appear to require multivalency for mitogenesis[19]. Affinity for the kinds of receptors available on the cell surface is necessary, as binding is a prerequisite for mitogenic stimulation. Apparently, PAA (and a host of other agglutinins) does not fulfil one or more of the requirements necessary for it to be mitogenic for lymphocytes. Additionally, PAA is a suppressor of con A- and LPS-induced mitogenesis, as shown in Figs. 6 and 7. Horowitz *et al.*[19] reported blocking by the non-mitogenic, "monovalent" con A of tetravalent con A-induced mitogenesis of lymphocytes, possibly due to competition for receptor sites, but this does not appear to be the mechanism involved here, as PAA is not specific for D-mannose or D-glucose derivatives. How-

ever, a number of other possibilities include blocking of con A- or LPS-binding by adhering of the highly polar, PAA molecule to positively charged groups on the cell surface. The cascade of events preceding, and interrelated with, lymphocyte stimulation is susceptible to modifying effects. It may be speculated that macrophages no longer furnish a required signal, or are incapable of presenting antigen to the lymphocytes, in the presence of PAA. Alternatively, PAA could modify lymphokine production by lowering helper factor, or increasing production of suppressor factor by lymphocytes, or by causing an increased generation of suppressor cells.

Although the structure of the agglutinin from *P. americana* remains obscure, we suggest that the lectin-like activity associated with seed extracts of the plant offers a new kind of probe for cell surfaces. In addition to its interesting effects on T- and B-cell mitogenesis, the effects of the agglutinin on *S. mutans*, smooth-surface interactions enabled us to propose a new mechanism for the adherence of the bacterium to saliva-coated hydroxylapatite[20].

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