PHYTOHEMAGGLUTININ MITOGENIC PROTEINS

Structural Evidence for a Family of Isomitogenic Proteins*

By J. BRUCE MILLER,‡ CLAUDIA NOYES, ROBERT HEINRIKSON,§ HENRY S. KINGDON,|| and STANLEY YACHNIN¶

(*From the Departments of Medicine and Biochemistry, University of Chicago, and the Franklin McLean Memorial Research Institute [operated by the University of Chicago for the United States Atomic Energy Commission; formerly Argonne Cancer Research Hospital], Chicago, Illinois 60637*)

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The phytohemagglutinins (PHAP)[1] obtained from the red kidney bean, *Phaseolus vulgaris*, have been shown to be a mixture of five glycoproteins that are heterogenous by several physicochemical and biological criteria (1). One of these proteins is a potent leukoagglutinin with low hemagglutinin activity (L-PHAP); a mixture of three other closely related proteins with modest leukoagglutinating activity but potent hemagglutinating properties (H-PHAP) has also been isolated (2). Both L-PHAP and H-PHAP are excellent mitogens. Of particular interest is the observation that the mitogenic activity of H-PHAP is markedly potentiated by the presence of autologous red blood cells (RBC) during in vitro human lymphocyte transformation (3, 4). In contrast, the ability of L-PHAP to transform lymphocytes is not affected by the presence of RBC. Enhancement of mitogenicity by RBC correlates well with the ability of these mitogens to effect mixed lymphocyte-RBC agglutination, a property potently displayed by H-PHAP but lacking in L-PHAP. The fifth PHAP mitogenic protein has been clearly defined by means of polyacrylamide gel electrophoresis, but detailed study of its properties has been hampered by contamination with L- and H-PHAP during preparative isolation procedures.

We have postulated previously that the five PHAP molecules consist of tetrameric isomitogens made up of varying proportions of two different subunits (2, 4). Our model hypothesizes that L-PHAP consists of four subunits,

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¶ To whom correspondence should be addressed.

[1] *Abbreviations used in this paper*: PHAP, phytohemagglutinins; H-PHAP, high titer hemagglutinating PHAP; L-PHAP, low titer hemagglutinating PHAP; SDS, sodium dodecyl sulfate.

designated L, which have high affinity for lymphocyte membrane receptors, but low or absent affinity for red cell membrane receptors. Conversely, the most positively charged of the H-PHAP mitogenic subfractions consists of four subunits, designated R, which have high affinity for red cell membrane receptors and low affinity for lymphocyte membrane receptors. Intermediate structures would include a series of 3L-1R, 2L-2R, and 1L-3R tetramers. These hybrid molecules are the mitogens capable of mixed RBC-lymphocyte agglutination and are those susceptible to potentiation during lymphocyte transformation by the presence of RBC, through a process of matrix formation on the red cell surface (4). This paper presents new physicochemical evidence supporting this hypothesis.

Materials and Methods

Isolation of L-PHAP and H-PHAP.—Starting material for the study of the mitogenic subfractions was Bacto-PHAP obtained from Difco Laboratories (Detroit, Mich.; catalog no. 3110-57). The procedure has been described previously (1). In brief, a solution of PHAP was dialyzed against starting buffer (0.01 M phosphate buffer, pH 5.5), a small amount of precipitate was discarded, and the supernatant was layered onto a 1 × 25 cm column of carboxymethyl (CM)-Sephadex C-50 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) previously equilibrated with starting buffer. After elution of the initial peak with the starting buffer, stepwise elution was performed with 0.0167 M phosphate buffer, pH 6.6, followed by 0.03 M phosphate buffer, pH 8.1. Finally, a linear gradient was developed with 0.03 M phosphate, pH 8.1, containing 0.5 M NaCl, into an equivalent volume of 0.03 M phosphate, pH 8.1. The material eluted at pH 6.6 (L-PHAP) and that eluted with the salt gradient (H-PHAP) were concentrated by pressure ultrafiltration and separated from contaminating proteins by passage through Sephadex G-150 (Pharmacia Fine Chemicals Inc.). For the isolation of a so-called "4R" H-PHAP moiety, 0.2 M NaCl was used to develop a shallow salt gradient (2).

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis.—The procedure of Fairbank et al. (5) was followed. Gels were stained with both Coomassie blue and periodic acid-Schiff (PAS).

Amino (NH_2)-Terminal Amino Acid Sequence Determination.—Identification and quantitation of the NH_2-terminal amino acid residues of L-PHAP and H-PHAP were performed by a modification of the procedure of Stark and Smyth (6), in which urea was replaced by 4 M guanidine hydrochloride. Amino acid quantitation was performed on a Bio-Cal BC-200 analyzer (Bio-Cal Instrument Co., Richmond, Calif.).

The sequences of the NH_2-terminal amino acid residues of L-PHAP and H-PHAP were determined by automated Edman degradation on the Beckman protein-peptide sequencer (model 890) (Beckman Instruments, Inc., Fullerton, Calif.). For this purpose the Quadrol program (program no. D-X1) supplied by the manufacturer was used. The phenylthiohydantoin amino acids liberated after each cycle of the degradation were identified and quantitated, either as such, or as the trimethylsilyl derivatives, by gas chromatography (7) on a Beckman GC-45 unit. In some cases, identifications were confirmed after back conversion to the amino acid by hydrolysis with HI (8), followed by amino acid analysis on a Bio-Cal BC-200 analyzer. Arginine residues were detected by the phenanthrene-quinone fluorescent spot method (9).

The number of NH_2-terminal serine residues per molecule of L-PHAP (and presumably the number of subunits per L-PHAP molecule) was estimated by use of the following equation:

$$\frac{(\mu\text{moles serine}/\mu\text{mole of L-PHAP})}{\mu\text{moles NH}_2\text{-terminal serine}/\mu\text{mole L-PHAP}} = \frac{\text{No. of serine residues/L-PHAP molecule}}{\text{No. of NH}_2\text{-terminal serine residues/L-PAP molecule}}.$$

The total number of serine residues per whole molecule of L-PHAP has been found previously to be 104 (1). The amounts of total serine (total acid hydrolysis) and NH_2-terminal serine (cyanate procedure) were determined from their respective areas as found by integration under the serine peaks obtained on the Bio-Cal BC-200 amino acid analyzer. The serine value for the cyanate procedure was corrected for 80% decompositional losses incurred during the back conversion of the hydantoin to the free amino acid (6).

Isoelectric Focusing of L- and H-PHAP.—This technique was performed using the LKB 8102 (440 ml) isoelectric focusing column (LKB Produkter AB, Stockholm, Sweden) with a sucrose gradient in 8 M urea. The PHAP proteins were denatured before electrofocusing with 5.8 M guanidine hydrochloride and then transferred to 8 M urea by dialysis, as described by Weber et al. (10, 12). Both L-PHAP and H-PHAP were first electrofocused in an ampholine mixture with a pI range of 3–10, as supplied by the manufacturer. In addition, H-PHAP was electrofocused in an ampholine mixture of pI range 4–8, made by mixing equal amounts of the ampholine mixtures of pI 4–6 and pI 5–8 that are supplied by the manufacturer. Pooled protein subfractions were dialyzed against 10% acetic acid, concentrated by pressure ultrafiltration, and analyzed on the Beckman protein-peptide sequencer as described above.

RESULTS

SDS-Polyacrylamide Gel Electrophoresis.—This procedure separates proteins and/or protein subunits purely on the basis of molecular size. When the gels were stained for protein with Coomassie blue, both L-PHAP and H-PHAP displayed a single band with an R_f of 0.66, as shown in Fig. 1. Similar results were obtained after PAS staining, and no additional carbohydrate-containing bands were identified (Fig. 2). When this R_f value was plotted on a semilog scale together with the values obtained for marker proteins of known molecular

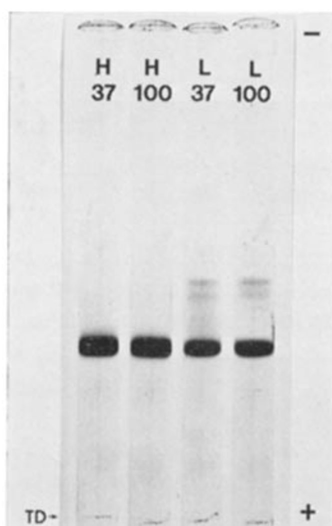

FIG. 1. SDS-polyacrylamide gel electrophoresis of L- and H-PHAP. Before electrophoresis, the proteins were denatured in SDS at either 37°C for 10 min or 100°C for 1 min (14). The gels were stained with Coomassie blue. TD = Tracker dye.

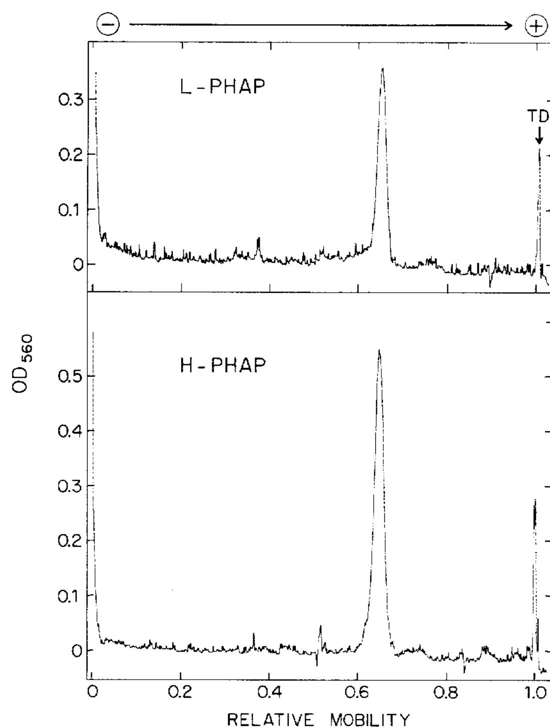

FIG. 2. PAS stain of SDS-polyacrylamide gel electrophoretic analyses of L- and H-PHAP. The gels were scanned at 560 nm (5).

size that were run simultaneously, the mol wt of the PHAP subunit was found to be 34,000 (Fig. 3).

NH_2-Terminal Amino Acid Group Analysis by the Cyanate Method.—L-PHAP yielded a single NH_2-terminal residue, serine, while H-PHAP was found to possess two different NH_2-terminal residues, serine and alanine. Simultaneous quantitative determinations of L-PHAP total serine content by total acid hydrolysis, and L-PHAP NH_2-terminal serine by the cyanate procedure, were performed. When these data were used to estimate the number of NH_2-terminal serine molecules per molecule of L-PHAP (see Materials and Methods), the number obtained was 4.4. Presumably, then, there are four subunits per L-PHAP molecule.

Sequential NH_2-Terminal Amino Acid Analysis of L-PHAP and H-PHAP.—Automated Edman degradation amino acid analysis corroborated that the NH_2-terminal residue of L-PHAP was serine. A single sequence of residues was found for L-PHAP (Table I). The recoveries of the easily quantitated derivatives were 60–65% of the yield expected on the basis of a four subunit structure with a subunit mol wt of 34,000. Repetitive yield was 93–97%.

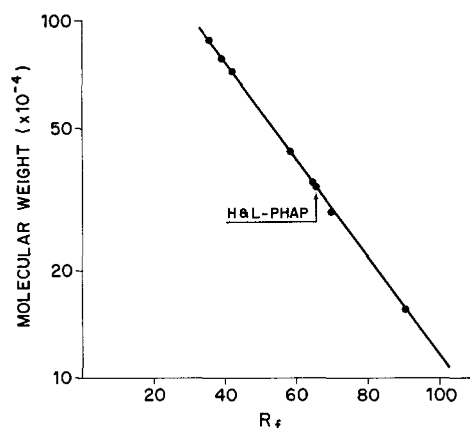

FIG. 3. Molecular weight determination of L- and H-PHAP subunits. The reference marker proteins used were red cell membrane protein constituents of known molecular weight (5).

Sequential amino acid analysis of H-PHAP revealed two different sequences for six of the first seven residues (Table I). Since one sequence was 3.7 times as plentiful as the other, the two amino acids found at position 1–5 and 7 could be differentiated and easily assigned to the major or the minor sequence. The minor sequence (approximately 20% of the total yield, as noted above) was identical with that of L-PHAP as determined previously. The major sequence (80% of the total yield) differed in six of the first seven amino acids. It is probable that phenylalanine at the sixth position is common to both the major and the minor sequence since it is found in the L-PHAP sequence, and the yields obtained (63%) clearly identify it as part of the major sequence as well. After the seventh residue, the sequences of L-PHAP and H-PHAP were identical through the 24th residue, the last that could be clearly identified. It should be noted that several sequential amino acid analyses of both L-PHAP and H-PHAP have uniformly failed to yield an identifiable residue for the 12th position.

Amino acid sequencing of the most positively charged H-PHAP subfraction, the so-called 4R H-PHAP, revealed the same major and minor sequences for the first seven residues as those seen for the mixture of all three H-PHAP subfractions ("bulk" H-PHAP). However, whereas the ratio of the major to minor sequences of bulk H-PHAP was 3.7:1, the same ratio for 4R H-PHAP was 9:1 (Table II).

Isoelectric Focusing of PHAP Subfractions in 8 M Urea.—When L-PHAP was subjected to isoelectric focusing in 8 M urea over a pH range of 3–10, a single protein peak with a pI of 5.25 was obtained (Fig. 4 *A*). Sequential amino acid analysis of the protein from this peak revealed the previously described sequence of L-PHAP with the additional identification of serine at

TABLE I

Results of Sequential Amino Acid Analysis of the Various Phytohemagglutinin Proteins (L-PHAP, "Bulk" H-PHAP, and "4R" H-PHAP)

Amino acid residue no.	L-PHAP 7.8 mg (230 nmol)*			H-PHAP‡ (a) 10.3 mg (303 nmol)* (b) 9.6 mg (283 nmol)*						4R H-PHAP 4.1 mg (120 nmol)*					
				Minor sequence			Major sequence			Minor sequence			Major sequence		
	Residue	Nmoles	Yield*	Residue	Nmoles	Yield*	Residue	Nmoles	Yield*	Residue	Nmoles	Yield*	Residue	Nmoles	Yield*
			%		(a) (b)	% (a) (b)		(a) (b)	% (a) (b)			%			%
1	Ser§			Ser			Ala	194; 158	64; 56	Ser			Ala	45	38
2	Asn			Asn			Ser			Asn			Ser		
3	Asp	116	51	Asp	34; 39	11; 14	Gln			Asp	4	3	Gln		
4	Ile	90	39	Ile	65; 31	21; 11	Thr			Ile	7	6	Thr		
5	Tyr	135	59	Tyr	40; 73	13; 26	Ser			Tyr	4	3	Ser		
6	Phe	150	65	(Phe)			Phe	175; 193	58; 68	(Phe)			Phe	72	60
7	Asn			Asn			Ser			—			Ser		
8	Phe	180	78	—			Phe	177; 167	58; 59	—			Phe	66	55
9	Gln			—			Gln			—			Gln		
10	Arg			—			Arg			—			Arg		
11	Phe	148	64	—			Phe	133	44	—			Phe	57	48
12	—			—			—			—			—		
13	Glu	65	28	—			Glu	48	16	—			Glu	28	23
14	Thr			—			Thr			—			Thr		
15	Asn			—			Asn			—			Asn		
16	Leu	85	37	—			Leu	96	32	—			Leu		
17	Ile	92	40	—			Ile	90	30	—			Ile	30	25
18	Leu	90	39	—			Leu	88; 115	29; 41	—			Leu		
19	Gln			—			Gln			—			Gln		
20	Arg			—			Arg			—			Arg		
21	Asp	24	10	—			Asp	10; 24	3; 8	—			Asp	12	10
22	Ala	57	25	—			Ala	33; 42	11; 15	—			Ala		
23	(Ser)‖			—			Ser			—			—		
24	Val	47	20	—			Val	32	11	—			Val	16	13

Only those residues that are easily quantified have been assigned numerical yields. The other residues were clearly identified, but not quantified.

* Based on a presumed four subunit structure; subunit mol wt = 34,000.

‡ Values from two different runs are included.

§ A small amount of alanine (10 nmol) was also found.

‖ Determined by analysis of L subunits isolated by isoelectric focusing (see text).

TABLE II

Molar Ratios of L-(H_2N-Ser) and R-(H_2N-Ala) Subunits of Various Phytohemagglutinin Subfractions as Calculated from Sequential Amino Acid Analysis Data

PHAP subfraction	Ser:Ala (L:R subunit) content*
L-PHAP	11.4:1
"Bulk" H-PHAP	1:3.7
"4R" H-PHAP	1:9

* See Table I. The molar ratios of L:R subunit were obtained by calculation from the averaged yields of Asp, Ile, and Tyr (positions 3–5) for the L subunit, and the yield of Ala (position 1) for the R subunit.

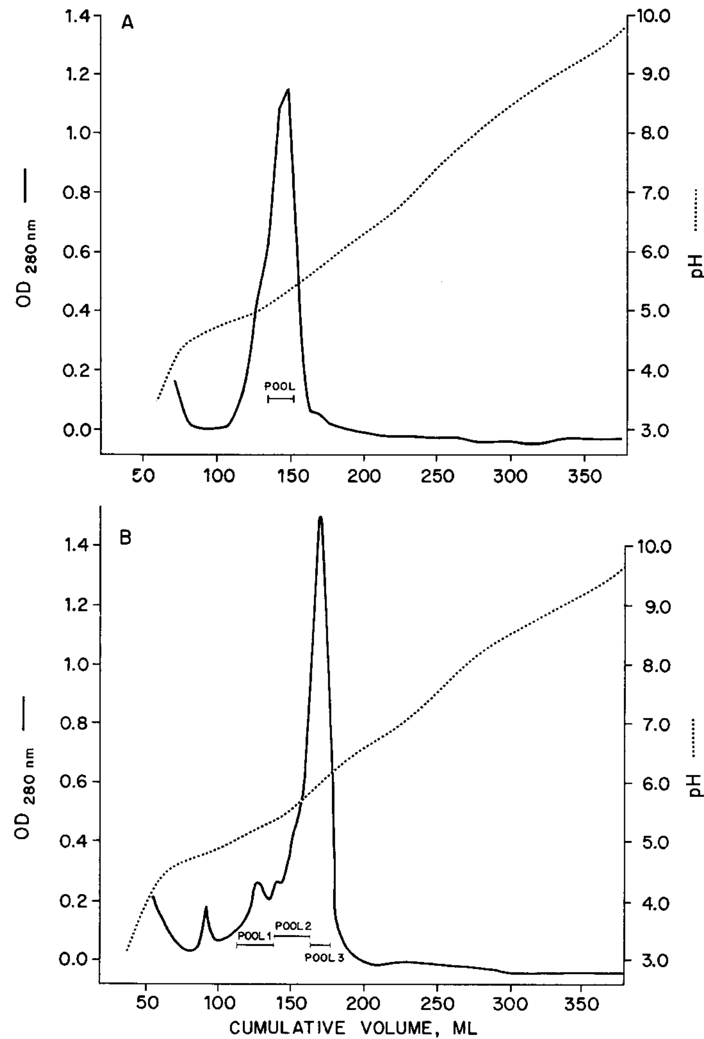

FIG. 4. Isoelectric focusing of L-PHAP (A) and H-PHAP (B) subunits in 8 M urea with an ampholine mixture of pH range 3–10. —, OD 280 nm, —, pH.

position 23, and the absence of any alanine at the NH_2-terminus. Isoelectric focusing of H-PHAP over a similar pH range revealed the pattern seen in Fig. 4 *B*. A minor peak at pH 5.25 was evident, whereas a much larger peak at pH 5.95 was seen. The total protein yield from the pooled and concentrated major peak was approximately 3.5 times as great as the protein pool yield of the minor peak. When the proteins from these two peaks and the material present in the trough between them were studied on the sequential amino acid analyzer, the ratios of major to minor H-PHAP sequences differed markedly from that of the

starting material, as shown in Table III. However, the ratio of the sums of the major and minor sequences obtained when the three pooled fractions were analyzed individually was 3.8:1, a figure consistent with that obtained by analysis of undissociated H-PHAP. These results suggest that the minor H-PHAP sequence subunit possesses a pI of 5.25, whereas a second subunit with the amino acid sequence of the major H-PHAP component migrates to pH 5.95. The failure to obtain complete separation of L (minor) and R (major) subunits is presumably due to the substantial overlap through the trough. In an attempt at better separation of the major and minor sequence subunits, isoelectric focusing over a narrower pH range was carried out.

Electrofocusing of H-PHAP in 8 M urea over a pH range of 4–8 again revealed minor and major protein peaks at pH 5.25 and 5.95, respectively (Fig. 5). As expected, however, there was considerably better separation of the major and minor H-PHAP subunits. The major to minor sequence ratio of the protein peak at pH 5.25 was 1:4, whereas *no* identifiable minor sequence residues, i.e. isoleucine at position 4 or tyrosine at position 5, were identified during amino acid sequence analysis of the protein peak at pH 5.95. Thus, successful

TABLE III

Subunit Content as Determined by NH_2-Terminal Sequential Amino Acid Analysis of Pooled Fractions Obtained by Isoelectric Focusing of H-PHAP in 8 M Urea

	Total protein	R subunit content	L subunit content	R:L subunit ratios
	OD units at 280 nm	*nmol*	*nmol*	
Pool 1*	5.9	18	27	1:1.5
Pool 2*	6.4	105	27	3.9:1
Pool 3*	14.3	236	11	21:1

* See Fig. 4 *B*.

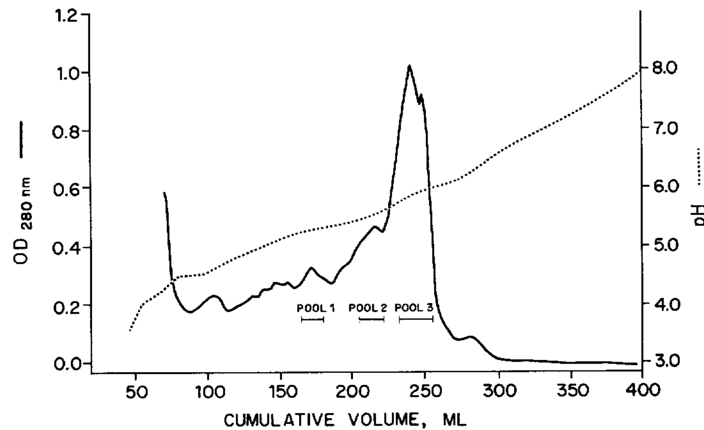

FIG. 5. Isoelectric focusing of H-PHAP subunits in 8 M urea with an ampholine mixture of pH range 4–8. —, OD 280 nm, ---, pH.

isolation of the R subunit by this technique eliminated any ambiguity concerning the amino acid sequence of its first 24 NH_2-terminal amino acid residues.

DISCUSSION

Evidence previously reported and our present results provide considerable support for our PHAP subunit hypothesis. SDS-polyacrylamide gel electrophoresis of L-PHAP and H-PHAP reveals a single band for both, with identical R_f values indicating a subunit mol wt of 34,000. Since this is approximately one-fourth the previously reported values for the intact PHAP molecule (1, 10–12), both L-PHAP and H-PHAP may be visualized as consisting of four noncovalently bound subunits of equal molecular weight. This finding is corroborated by Weber et al. (10, 13), who studied leukoagglutinin and erythroagglutinin fractions of PHAP both by ultracentrifugation in 6 M guanidine hydrochloride and by SDS-polyacrylamide gel electrophoresis. Both mitogenic fractions were found to be composed of subunits with mol wt of 34,000–38,000. On the other hand, Allen and Crumpton found that PHAP "lymphoagglutinin" separated into two different glycoproteins with mol wt of 29,000 and 33,000 (14) during SDS-polyacrylamide gel electrophoresis. These two glycoproteins differed in carbohydrate content, and the possibility that this property affected their electrophoretic mobilities (the basis of their being assigned different molecular weights) was recognized.

Support for the postulate that there are two different protein subunits is derived from biological and physicochemical studies. The properties of the various PHAP subfractions as isolated by polyacrylamide gel electrophoresis and CM-Sephadex column chromatography are summarized in Table IV (1–4).

TABLE IV

*Summary of Various Biological and Physicochemical Properties of L-PHAP and H-PHAP**

Biological and physical properties	L-PHAP	H-PHAP
Lymphocyte transformation	4+	3–4+
Hemagglutination	±	4+
Binding to RBC	–	4+
Binding to platelets and leukocytes	+	+
Leukoagglutination	4+	2+
Mixed agglutination (lymphocytes and RBC)	–	4+
Potentiation of transformation by RBC	No	Yes
Nonspecific precipitation with serum proteins	–	+
Homogeneous by acrylamide gel electrophoresis and CM-Sephadex chromatography	Yes	No (3 bands)
mol wt of subunits by SDS-acrylamide gel electrophoresis	34,000	34,000
pI of subunits	5.25	5.25, 5.95
NH_2-terminal amino acid residue	Serine	Alanine (major) Serine (minor)

* See Refs. 1–4.

Of particular interest is the finding of three separate H-PHAP subfractions when bulk H-PHAP is subjected to either polyacrylamide gel electrophoresis or elution from CM-Sephadex using a shallow linear salt gradient (2). With increasing positive charge, the H-PHAP proteins become progressively more potent as hemagglutinins and progressively less potent as mitogens. The most positively charged H-PHAP subfraction was designated the 4R H-PHAP (2).

Our studies on the primary structure of L-PHAP and H-PHAP provide further evidence for the two-subunit hypothesis. The NH_2-terminal amino acid sequence of L-PHAP and the minor component of H-PHAP are identical, while the sequence of the major component of H-PHAP differs from them for six of the first seven amino acids. If the L and R subunits were randomly distributed amongst all the PHAP isomitogens, then bulk H-PHAP, consisting of 2L-2R, 1L-3R, and 4R isomitogenic molecules, would have a major to minor sequence ratio of 3:1, a figure close to the value of 3.7:1 as actually determined. Material enriched in the 4R H-PHAP subfraction yields a major to minor sequence ratio of 9:1. From these data we conclude that our postulated L subunit is that component identified by the L-PHAP and the minor H-PHAP sequence, and that the major H-PHAP sequence identifies the R subunit. Isoelectric focusing of L-PHAP and H-PHAP reveals the L subunit to have a pI of 5.25; the pI of the R subunit is 5.95. These results are similar to the findings of Weber et al. (10, 13). Their leukoagglutinin subunit showed a pI of 5.0, whereas the erythroagglutinin exhibited a minor protein peak with a pI of 5.0 and a major peak with a pI of 6.5.

Our failure to identify the amino acid residue at position 12 in either L- or H-PHAP may be explained by attachment of a carbohydrate moiety at that site, since glycosylated residues are not recovered by the procedure of Edman degradation as performed on the sequential analyzer. The 12th, 13th, and 14th residues of PHAP are an unidentified amino acid residue, glutamic acid, and threonine, respectively (Table I). In several glycoproteins, glycosylated L-asparagines have been shown to occur when they are separated by one amino acid residue from an L-serine or L-threonine residue on their C-terminal side (15). Apparently there is no structural restriction on the identity of the middle amino acid residue. We are currently investigating the possibility that the 12th residue is a glycosylated asparagine.

Although the L and R subunit amino acid sequences differ in six of the first seven NH_2-terminal amino acid residues, only one of these differences, that at position 3, results in a difference in charge. Thus, the L subunit, with aspartic acid at position 3, would be negatively charged by comparison with the R subunit that contains glutamine at position 3. Supposing that this were the only difference in charge to be found between the L and R subunits, it would still be sufficient to account for the progressive increase in net positive charge displayed by the 3L-1R, 2L-2R, and 1L-3R tetramers, culminating at last in the 4R H-PHAP, which would have a net charge of +4 compared with

L-PHAP (containing four L subunits). Thus, if net charge differences only are considered, no further differences between the L subunit and R subunit amino acid sequences are required to account for the observed progression of five mitogenic protein bands as defined by polyacrylamide gel electrophoresis with 4R H-PHAP migrating most rapidly towards the cathode. It is thus conceivable, although not necessarily likely, that the amino acid sequences of the L and R subunits may be identical from the eighth position through their C-terminus. This would be of interest, since it would mean that the differences in biological behavior that they exhibit in regard to binding to cell membranes and serum glycoproteins would depend on highly restricted differences in primary structure over a short segment of their NH_2 termini. Experiments leading to further definition of the remainder of the primary structure of the L and R subunits are being actively pursued.

We conclude that PHAP mitogenic proteins consist of a family of five isomitogenic proteins, each member of which contains varying proportions of the L subunits and R subunits (Fig. 6), in a manner analogous to the enzyme lactic dehydrogenase (16). These subunits can be distinguished by their NH_2-terminal amino acid sequences, their isoelectric points, and their biological properties. L-PHAP consists of four identical subunits, all of which have the NH_2-terminal amino acid residue serine, a pI of 5.25, and strong affinity for lymphocyte membrane receptors but little for those of red cells. The 4R H-PHAP isomitogen contains four identical subunits with an NH_2-terminal amino acid sequence beginning with alanine and differing from that of the L subunit for six of the first seven residues, a pI of 5.95, and a strong affinity for RBC membrane receptors and similar receptors on serum glycoproteins. The three intermediate isomitogenic proteins between these two extremes consist of tetramers containing varying proportions of the two different subunits; this hybrid structure serves to explain their special properties of causing mixed RBC-lymphocyte agglutination and exhibiting potentiation of their mitogenic activity by the presence of RBC.

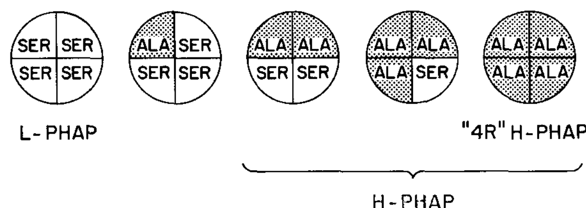

FIG. 6. Schematic representation of the tetrameric structure of the five phytohemagglutinin isomitogens, consisting of varying proportions of the L (NH_2-terminus Ser) and R (NH_2-terminus Ala) subunits.

SUMMARY

The phytohemagglutinin (PHAP) glycoproteins derived from *Phaseolus vulgaris* consist of five isomitogens that are tetrameric structures made up of two different glycoprotein subunits. Although identical in size (mol wt = 34,000), the subunits differ in their isoelectric points and amino acid sequences for six of the first seven amino-terminal residues, but are identical in primary structure from the 8th through the 24th amino acid residue. The isomitogen containing four L subunits (L-PHAP) is a potent leukoagglutinin and mitogen that lacks hemagglutinating properties. The isomitogen made up of four R subunits (4R H-PHAP) is a potent hemagglutinin. The hybrid isomitogens consisting of varying proportions of the two subunits (3L-1R, 2L-2R, 1L-3R) are capable of causing mixed erythrocyte-lymphocyte agglutination. These studies provide a structural basis for explaining the differences in biological activities of the various PHAP isomitogens.

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