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Isolation and Partial Characterization of a Seed Lectin from Tepary Bean that Delays Bruchid Beetle Development[1]

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ABSTRACT

Four isolectin forms of a seed lectin from mature seed of tepary bean (*Phaseolus acutifolius*) were isolated using solubility fractionation, affinity chromatography, and high performance liquid chromatography. The subunits are polypeptides with an apparent molecular mass of 30,000 daltons. The 30 kilodalton subunits are produced starting approximately 13 days after flowering and subsequently comprise a major fraction of the proteins found in the mature seed. The amino terminus of each isolectin fraction was determined to be highly homologous with that of the subunits of common bean (*Phaseolus vulgaris* L.) phytohemagglutinin (PHA). The tepary isolectin cross-reacts with both erythroagglutinating and leucoagglutinating subunits of PHA antibodies, although differential cross-reactivity was noted. A seed protein fraction enriched in tepary bean lectin was found to be toxic to bean bruchid beetles (*Acanthoscelides obtectus*), when incorporated into their diets at incremental concentrations from (1–5% w/w) above that of PHA concentrations in mature seeds of the susceptible common bean variety "Red Kidney."

The physiological functions of plant lectins have not yet been elucidated. One possible function, that of serving as a chemical defense against animal predation, was suggested by Janzen *et al.* (6). Recent investigations confirm that certain *N*-acetylgalactosamine and *N*-acetylglucosamine binding lectins from diverse plant species have antibiotic effects on the development of *Callosobruchus maculatus* (F.) (cowpea weevil) larvae (3, 13).

All domesticated *Phaseolus* species *(P. vulgaris, P. coccineus, P. lunatus, and P. acutifolius*) are known to contain seed lectins (19). These species are subject to seed predation by only a few species of the family *Bruchidae.* This observation suggests that those few species that have the ability to feed on lectin containing *Phaseolus* seeds have evolved adaptive mechanisms that allow them to cope with the different lectins found in the *Phaseolus* species. The question then arises: can certain *Phaseolus* lectin variants be found that display allelochemical properties to which the bruchid species have not yet evolved resistance mechanisms?

It was observed that when resistance to bruchid beetles was found among a few accessions of wild *P. vulgaris*, it was always associated with the presence of a particular protein. That protein was later described and named arcelin (14). The bioactivity of arcelin against an economically important seed predator has been confirmed (15). Arcelin has considerable sequence identity with PHA[4] although it displays only limited agglutinating activity. That finding supports the hypothesis that certain lectins (or lectin-like proteins) may serve an important biological function as deterrents to insect predation in nature.

The tepary bean (*P. acutifolius*) is closely related to common bean (*P. vulgaris* L.) and displays considerably better resistance than common bean to an important seed-feeding bruchid (*Acanthoscelides obtectus*) (1, 8, 21). Tepary seed are known to contain lectins (16). We report here the characterization of a lectin found in a bruchid resistant tepary accession and evidence that this lectin has bioactivity against a bruchid predator.

MATERIALS AND METHODS

Plant Materials

Tepary bean (*Phaseolus acutifolius*, PI 440–790) seeds produced in Arizona (Catalina, 1983) were used. Red Kidney bean seed were obtained from the Indiana Seed Co., Inc.

Protein Fractionation

Following a protocol to optimize the extraction of the tepary lectin fraction, prechilled SAB (0.05 M NaCl, 25 mM Na acetate [pH 4.9]) was added to fine bean flour at a solvent-to-flour ratio of 10:1. The extracts were centrifuged (20,000*g*, 10 min at 4°C) and the pellets discarded. The soluble fractions were dialyzed for 24 to 48 h at 4°C against several changes of ddH_2O. Dialysates were centrifuged (20,000*g*, 10 min at 4°C) and the supernatants frozen and lyophilized (23).

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[4] Abbreviations: PHA, phytohemagglutinin; AZ, Arizona: PBS, phosphate buffered saline (0.15 M NaCl, 10 mM sodium phosphate [pH 6.8]); SAB, sodium acetate buffer (0.05 M NaCl, 25 mM sodium acetate [pH 4.9]); RBC, red blood cell; PI 440–790, U.S. Department of Agriculture plant introduction number 440–790; PHA-E, *P. vulgaris* erythroagglutinating PHA subunit; PHA-L, *P. vulgaris* leucoagglutinating PHA subunit; ddH_2O, deionized distilled water.

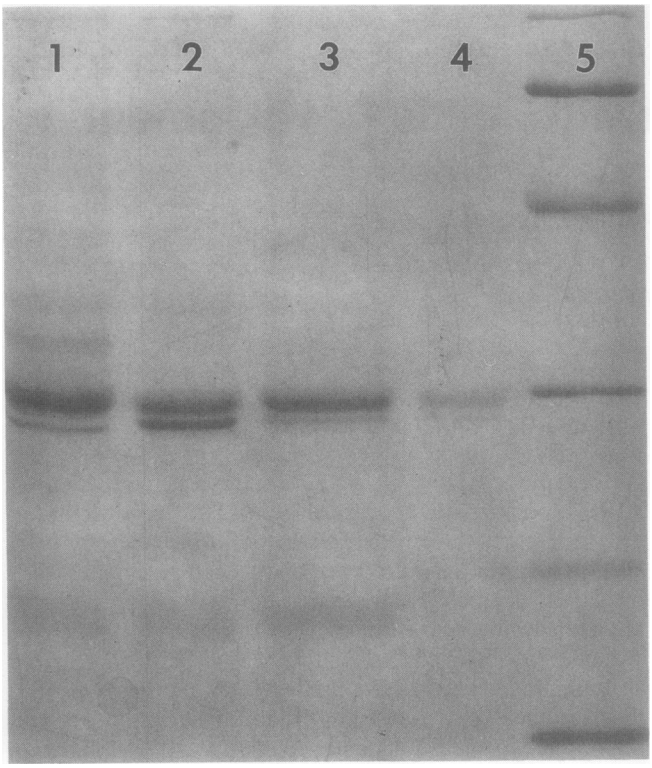

Figure 1. SDS-PAGE of tepary lectin at different stages of purification. Lanes: 1, SAB extract; 2, SAB extract after dialysis against ddH_2O; 3, dialysate after affinity chromatography purification; 4, HPLC purified fraction (fraction I only); and 5, Bio-Rad mol wt standard, respectively.

To compare the bioactivity of the tepary lectin fraction with *Phaseolus vulgaris* lectin fractions reported to be active against bruchids (6), tepary lectin was also extracted by the protocol of Honovar *et al.* (5). In summary, that protocol involved the extraction of seed proteins from finely ground bean flour using 1% (w/v) NaCl. The soluble fraction was adjusted to a pH of 4.0 (using 5 N HCl) and the proteins that precipitated upon acidification were discarded. A bentonite-celite mixture (1:1) was added to the supernatant, and the suspension was stirred overnight (at 4°C) and then filtered. The filtrate was brought to 0.75 saturation with ammonium sulfate and the precipitate redissolved in ddH_2O and dialyzed.

Con A Sepharose Column Chromatography

The lyophilisate from the SAB extract was resuspended in Con A buffer (10 mM acetate buffer [pH 5.5] containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$) and added to a prewashed Con A Sepharose column containing 8.75 mg Con A/mL (Sigma). After washing the column with two volumes of Con A buffer, the protein was eluted with 0.5 M methyl D-mannopyranoside and 0.2 M NaCl in Con A buffer. This procedure yields approximately 30 kD polypeptide(s) of approximately 85 to 90% purity (one major and one minor band on a one-dimensional SDS-polyacrylamide gel).

HPLC Ion-Exchange Chromatography

The lectin enriched fraction was extensively dialyzed with four changes of 4 L, each in 50 mM SAB (pH 5.5). The dialyzed fraction was centrifuged (10,000*g*, 20 min, at 4°C). A clear supernatant was used for further fractionation of lectins on HPLC.

The sample was applied on a cation exchange HPLC column (25 × 1 cm) containing polyamide on 17 μm Vydac silica (22) equilibrated with 50 mM acetate buffer (pH 5.5). The protein fractions were eluted with a 0 to 1.0 M NaCl gradient in 40 min at the rate of 3 mL/min. The elution profile was monitored at 280 nm and recorded on an HP integrator. Among several protein peaks, four major peaks were identified as being lectins by their agglutinating activity.

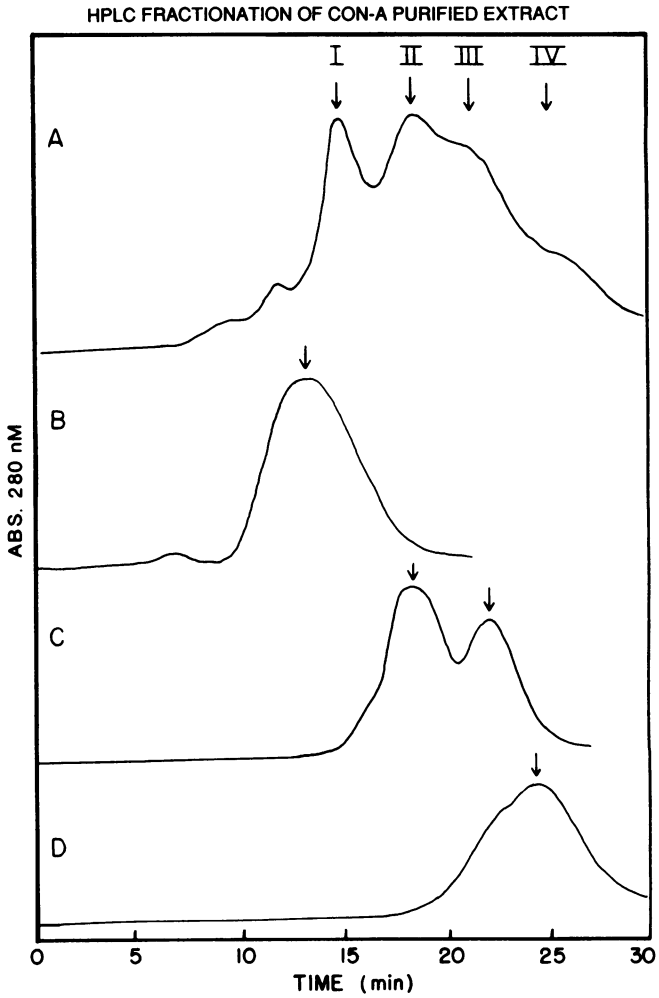

Figure 2. Elution profile of protein monitored at 280 nm from a preparative SCX-polyamide HPLC column as described in "Materials and Methods." The protein was extracted from seeds with SAB and prepurified on Con A Sepharose chromatography before HPLC. Following the first run (A) the conditions were optimized for the separation of the tepary isolectins I, II, III, and IV by a second run on the same column. Isolectin peaks are marked with arrows.

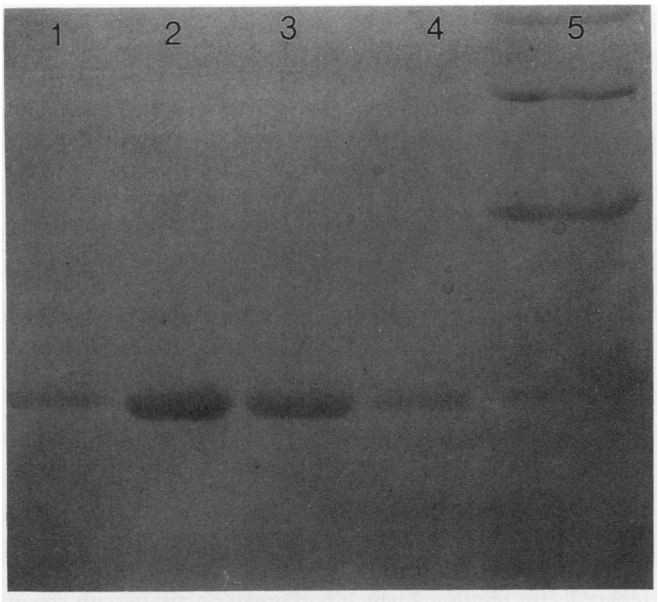

Figure 3. SDS-PAGE of tepary lectin subunits. Lanes 1 through 5 represent HPLC fractions I, II, III, and IV and mol wt. markers, respectively.

Thyroglobulin-Agarose Affinity Chromatography

Thyroglobulin-agarose (E-Y Labs, Sigma Chemicals) was washed in several changes of ddH_2O and loaded onto four 5 mL disposable plastic columns, each column containing 1 mL packed volume thyroglobulin-agarose beads. Columns were washed with 10 mL of 5 mM potassium phosphate buffer (pH 7.4), containing 150 mM NaCl (wash buffer). Four lectin fractions (I–IV) which were eluted from HPLC were applied to these columns at a very slow flow rate (0.2 mL/min). At a higher flow rate, part of the lectin fractions could not be retained on the column. Columns were washed with 2 mL wash buffer and protein was eluted from the column with 50 mM Tris-glycine (pH 3.0), containing 0.5 M NaCl. The pooled eluates from each column were separately dialyzed and lyophilized.

Immuno Slot Blot Assay

Lectin fractions I–IV eluted from the HPLC column were adsorbed onto nitrocellulose membranes (BA85, 0.45 μm) using a Minifold II slot blot apparatus. An equal amount of protein (0.5 μg in 25 μL 10 mM potassium phosphate buffer [pH 6.5] containing 150 mM NaCl) was applied in each slot. Duplicate slot blots were made, and the blots were incubated with TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Triton X-100) containing 0.2% (w/v) nonfat dry milk powder for 30 min to block the free protein binding sites on the nitrocellulose. These blots wre then challenged with PHA-L and PHA-E antibodies (E-Y Labs), diluted (1:200) in TBST containing 0.1% milk powder in separate containers for 3 h. The blots were washed five times in TBST and incubated for 2 h with protein A radioiodinated with ^{125}I by the method of Markwell (10). The blots were washed five times in TBST and exposed to x-ray films at −70°C after drying.

N-Terminal Amino Acid Sequencing

Purified lectin fractions I–IV were dialyzed in 8% formic acid and lyophilized prior to amino acid sequencing. The amino acid sequences of the NH_2-termini of the tepary isolectins were determined by automated Edman degradation on a Beckman spinning-cup peptide sequencer as described by Singh *et al.* (22).

Hemagglutination Assay

Purified tepary polypeptide fractions (I–IV plus pre-I–IV eluate) and commercial common bean lectin (Sigma Type V PHA) were tested for their ability to agglutinate mammalian erythrocytes. Hemagglutination assays were conducted using flexible, round-bottom polyvinyl chloride microtiter plates (Flow Labs). Lyophilized, glutaraldehyde fixed, human (type O), and sheep erythrocytes (Sigma) were reconstituted using fresh PBS, vortexed, allowed to stand about 30 min, and then washed once again with PBS. A 50 μL drop of PBS was placed into each well. Fifty μL of 80 μg/mL tepary protein sample was added to give a starting concentration of 40 μg/mL. Twofold serial dilutions were then made using a 50 μL serial diluting apparatus. The starting concentration of PHA was 400 μg/mL. A drop of 50 μL blood suspension was added to each well. The microtiter plate was then covered with polyethylene wrap to prevent desiccation. After 1 h at room temperature, the end point was expressed as the concentration capable of causing an obvious agglutination of the erythrocytes.

Artificial Seed Bioassay

A lectin enriched fraction was isolated from PI 440-790 seeds by the methods of Honovar *et al.* (5). This fraction, which was approximately 80% or greater pure lectin (Fig. 6), was incorporated into a bean artificial seed system according to the methods of Shade *et al.* (20). Finely ground flour from decorticated cotyledons of the susceptible common bean va-

Table I. *Agglutinating Activity of Tepary and Common Bean Protein Fractions with Human and Sheep Erythrocytes*

Sample	Erythrocyte Source	
	Sheep	Human Type 0
Tepary Proteins		
Nonlectin	ND[a]	ND
Fraction I	1[b]	40
Fraction II	1	1
Fraction III	3	1
Fraction IV	5	3
Common bean protein		
PHA	25	25

[a] No detectable agglutinating activity at 40 μg/mL. [b] Numbers indicate approximate concentration (μg/mL) of greatest dilution with hemagglutinating activity.

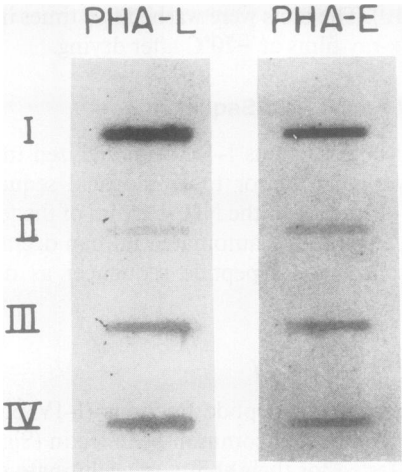

Figure 4. Immunoslot blot of tepary lectin fractions (I–IV) challenged with PHA-L and PHA-E antibodies followed by I^{125} labeled protein A.

riety Red Kidney was mixed with the lectin enriched protein fraction (solubilized in ddH_2O). The mixture was then extruded into teflon molds with a disposable syringe. The molds were immediately frozen with dry ice, and subsequently with liquid nitrogen. Frozen molds were then lyophilized to dryness, and the seeds removed and equilibrated to ambient conditions in the culture room before feeding studies began. Artificial seed were then challenged with *A. obtectus*, an important predator of *Phaseolus* seed.

Developmental Study

To characterize the presence of the 30 kD polypeptide(s) during seed maturation a study was performed using tepary plants (PI 440–790) grown in the greenhouse under a short-day photoperiod regime (8 h light/16 h dark). Flowers were tagged at anthesis, during 1 month, and pods were harvested over a subsequent 4-week period. Ovules were dissected and homogenized in −20°C acetone.

Electrophoresis

Preparation of samples and PAGE was carried out as previously described (17). The approximate protein content of the samples was determined by colorimetric assay using Bio-Rad protein reagent. Samples were made 10 mg/mL in cracking buffer (0.065 M Tris-HCl [pH 6.8], 2 mM EDTA, 2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, 1 mM PMSF, 0.1% [w/v] bromophenol blue) and placed in a boiling water bath for 3 min before electrophoresis.

RESULTS

Purification of Isolectins

The 30 kD polypeptide(s) from tepary seed were highly enriched (over 70%) in the SAB fraction (Fig. 1). The SAB fraction was further purified by affinity chromatography on Con A-Sepharose (lane 3, Fig. 1). When Con A purified protein was subjected to ion exchange HPLC chromatography, it could be separated into four major peaks (Figs. 2, 3) suggesting that the native lectin likely consists of tetrameric forms differing in their charges, as has been established for *Phaseolus vulgaris* (3). In addition, some other proteins either did not bind to the column or eluted earlier during chromatography. One of these earlier fractions was used in the agglutination assay with the four main fractions (Table I). Further purification of the polypeptides was attempted with thyroglobulin-agarose affinity chromatography. Each of the four lectin fractions bound to the column very weakly, and there were no apparent differences between these fractions with respect to their thyroglobulin binding affinity.

Hemagglutinating Activity

Each of the four major polypeptides designated as fractions I to IV was capable of agglutinating both human O and sheep erythrocytes. Tepary lectins generally displayed greater activity for agglutination than did PHA V, with the exception of tepary lectin fraction I which had greater activity with sheep erythrocytes only (Table I). These results also suggest that there may be some functional (agglutination) differences between these isolectins, besides the differences in their apparent charge properties.

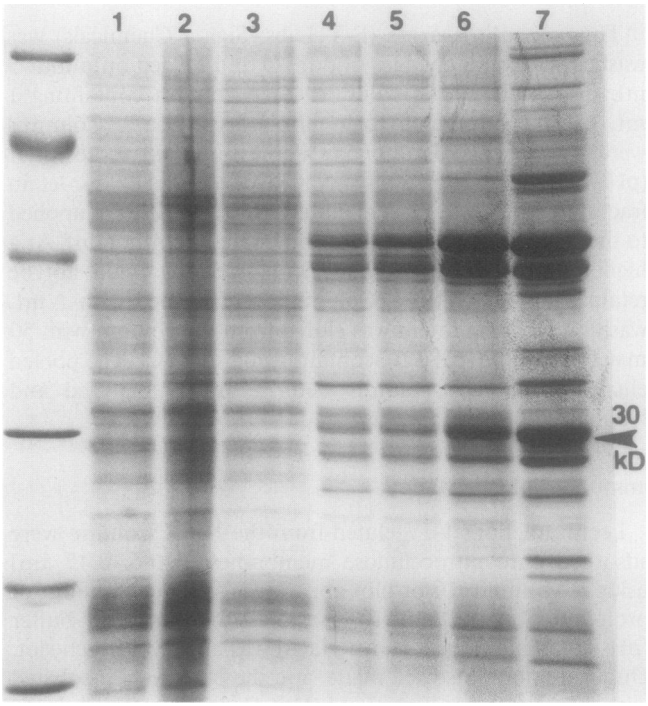

Figure 5. Tepary seed proteins during ovule development. SDS-PAGE (10–16% gradient) of tepary bean (PI 440-790) seed proteins during ovule development. Lanes 1 to 7 represent 3, 5, 9, 13, 17, 21, 30 DAF. Molecular mass standards are shown on the left. Position of lectin corresponds with carbonic anhydrase molecular mass = 30,000 D. Protein from equal amounts of acetone dry powder from ovules was added to each lane.

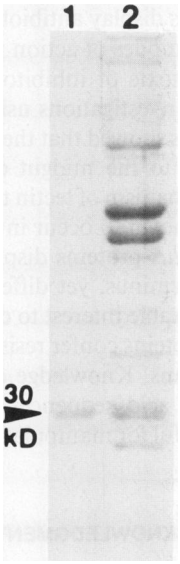

Figure 6. Purified lectin fraction used in artificial seed feeding experiment. Lane 1 is the partially purified fraction used in feeding experiment (see Fig. 8), that was prepared according to the method of Honovar *et al.* (5). Lane 2 is initial extract (1% NaCl soluble protein) from mature dry seed; see "Materials and Methods."

Immunological Cross-Reactivity

Each of the four lectin fractions cross-reacts with both anti-PHA-L (leucoagglutinating lectin) and anti-PHA-E (erythroagglutinating lectin). However, tepary fraction I lectin has higher immunological cross-reactivity to both anti-PHA-L and anti-PHA-E as compared with fractions III and IV (Fig. 4). Fraction II lectin has the weakest cross-reactivity with both antibodies. This further indicates that structural differences exist between the different tepary isolectins.

Developmental Expression

Total SDS-extractable proteins from developing ovules were examined after increasing times following the day of flowering. The 30 kD polypeptide appears approximately 13 DAF and increases in abundance until maturation has been reached (Fig. 5). Western blotting of these proteins with anti-PHA revealed that anti-PHA cross-reacts with the 30 kD polypeptide plus a few other proteins from the ovules (data not shown). A cross-reacting 30 kD polypeptide was not detected with PHA antibody until d 13 further indicating that it does not accumulate in the ovules before this time. Since the 30 kD polypeptide appears to increase in quantity after its first appearance, it would suggest that either the message for the lectin sub-units is very stable or transcription of the message continues.

Bioactivity Against Bruchids

Incorporation of the lectin enriched protein fraction obtained by the protocol of Honovar *et al.* (Fig. 6) (5) into the diets of bruchid larvae significantly increased the time required for larval development within the artificial seed. A linear increase in developmental time of approximately 2 d was attributed to each 1% (w/w) incremental increase of the protein (Fig. 7). It should also be noted that these incremental increases are in addition to the existing concentrations of Red Kidney bean lectin. Mortality levels did not increase with increasing dosages of the lectin enriched fraction (data not presented).

Amino Acid Sequence Homology with Common Bean Lectins

The N-terminal amino acid sequences of tepary isolectins were identical. The amino acid sequence of tepary lectin and its similarity with common bean lectin is shown in Figure 8. Tepary isolectins have a very high amino acid sequence identity (87%) with the PHA-L lectin-subunit and with the PHA-E subunit from common bean (77%). The high similarity between the N-terminal sequences of these polypeptides indicates that differences in the amino terminal sequences of PHA-L and PHA-E subunits may not account for the differential agglutinating activity of the respective subunits as was suggested previously (11).

DISCUSSION

Previous studies (2, 6) have suggested that PHA is an antibiosis factor that can deter insect predation. Other inves-

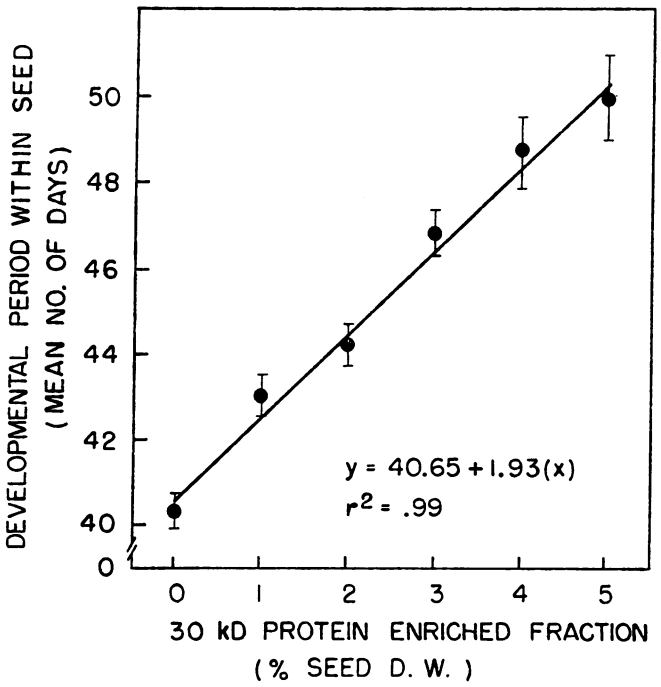

Figure 7. Developmental response of *A. obtectus* to tepary bean lectin enriched fraction incorporated into artificial seed. Feeding experiments were conducted using 10 separate seeds (2 replications, 5 observations/rep) per amount of lectin used. Twelve eggs were included with each seed. Shown is the average developmental time for adult emergence ± SE as a function of lectin concentration.

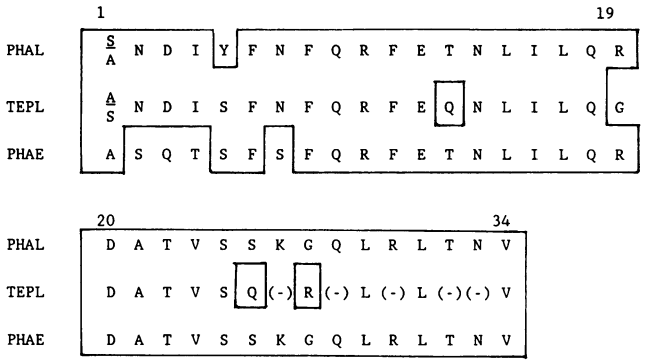

Figure 8. Amino acid sequences of tepary lectin fraction II from Figure 4 (TEPL), PHA-E (PHAE), and PHA-L (PHAL) amino termini. Identical amino acids are shown in blocks, and positions which are unidentified are represented by (–). PHA-E and PHA-L sequences were deduced from DNA sequences (4).

tigators (15) have recently demonstrated that particular variants of a novel seed protein (arcelin), discovered in a wild common bean accession (PI 325–690), can act as a chemical defense against bruchid beetles (*Acanthocelides obtectus*, and/or *Zabrotes subfaciatus*) which are natural predators of *Phaseolus* beans. Those findings, and our own, suggest that members of a family of related proteins in the genus *Phaseolus* characterized by lectin or lectin-like properties have evolved substantial bioactivity against major seed predatory insect species. Moreno and Chrispeels (12) discovered that an α-amylase inhibitor from common bean that inhibits insect and mammalian α-amylases is also a product of a lectin gene. They proposed that members of the PHA gene family, that may encode either carbohydrate binding or noncarbohydrate binding proteins, all are involved in plant defense against insects.

Considerable differences in the degree of bruchid resistance exist among *P. acutifolius* accessions (21). However, it is not known if the degree of resistance can be correlated with the amount or type of lectin present in the different accessions.

Considerable variability has been noted among the tepary lectins thus far described (9, 19, 24). Manen (9) proposed that the electrophoretic banding patterns of lectin subunits extracted from a cultivated tepary accession were like those that gave rise to the tetrameric EELL form in common bean. This tepary lectin bound to a thyroglobulin affinity column whereas lectin extracted from a wild tepary accession did not (9). Based on SDS-PAGE and double diffusion patterns when challenged with E *versus* L. subunit antisera, Pusztai *et al.* (19) suggested that one tepary accession that they examined was related to the EELL common bean tetrameric form whereas another resembled the EEEE form. A tepary lectin consisting of four 21,000 D subunits each with 7.4% total carbohydrate also has been described by Vargas-Albores *et al.* (24). The tepary lectin subunits described in our study show both a high degree of homology and apparent mol wt similarities with the PHA-L subunits from common bean. However, they retain considerable agglutinating activity for RBCs as do the PHA-E subunits from common bean.

Within the genus *Phaseolus* it appears that certain lectins and lectin-like proteins display antibiotic activity against bruchid beetles. Possible modes of action for these proteins thus far proposed include toxic or inhibitory properties. Indirect immunofluorescence investigations using monospecific antisera for globulin lectins showed that the lectins, when ingested by the larvae, bound to the midgut epithelial cells. It was suggested that the mechanism of lectin toxicity in this instance is analogous to that known to occur in the rat (7, 18).

All of these *Phaseolus* proteins display considerable similarity of the amino-terminus, yet differ in other respects. It will now be of considerable interest to determine which properties of the related proteins confer resistance to insects which feed on *Phaseolus* beans. Knowledge of these protein properties and the amino acid sequences that determine them should prove quite useful for manipulation of insect resistance by genetic engineering.

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