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# Legume α-Galactosidases Which Have Hemagglutinin Properties[1]

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## ABSTRACT

**Four legume species (four genera) were examined and found to contain hemagglutinins with properties similar to those which we have previously described for the enzymic hemagglutinin in *Vigna radiata*. Examination of extracts by gel filtration and ion exchange chromatography showed that an α-galactosidase activity exactly co-purified with a hemagglutinin activity in each of the four species. The α-galactosidase activities in the four species were virtually identical to each other with respect to substrate and inhibitor specificity as well as kinetic behavior. Additionally the hemagglutinin activities in all four species displayed very similar carbohydrate specificities. The inhibitor specificities displayed by the enzymes and the hemagglutinins were qualitatively and quantitatively very nearly identical to each other. The remarkable similarities of these proteins, both to each other and to the previously described *Vigna* enzymic hemagglutinin, suggest that each of these plants may contain a homologue from a specific class of enzymic hemagglutinin.**

In a previous report we described an α-galactosidase from *Vigna radiata* which possessed hemagglutinin properties (4). The *Vigna* hemagglutinin displayed a unique property which we refer to as "clot-dissolving activity." Apparently, under the appropriate conditions, this protein is capable of enzymically altering those erythrocyte receptors with which it interacts, resulting in a dissolution of cell aggregates. The disaggregated erythrocytes are permanently altered and are no longer agglutinable by *Vigna* hemagglutinin (although they remain agglutinable by many other legume lectins). Clot-dissolving activity is readily observed, even with crude extracts, as a disappearance (dissolution) of erythrocyte aggregates at a rate which is directly proportional to the concentration of extract used. That is, with very concentrated extracts, agglutination is rapid and complete but very quick (minutes) to disappear; with dilute extracts, full agglutination develops more slowly and then disappears only after prolonged (hours) incubation.

Due to our general interest in hemagglutinins, we wondered if legume species other than *Vigna* contained similar clot-dissolving agglutinins. We, therefore, surveyed a variety of legume seed extracts for hemagglutinin and α-galactosidase activities and, in those cases where agglutination was seen, we carefully looked to see if any clot dissolution occurred. We readily detected clot-dissolving activity in several species, including two which contained previously well characterized non-clot-dissolving hemagglutinins.

In this report we describe some general properties of extracts from four different legume species (four genera) each of which contains a clot-dissolving hemagglutinin. Our results suggest that all four species contain α-galactosidase-hemagglutinins very similar to that isolated from *V. radiata*.

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## MATERIALS AND METHODS

**Seeds.** *Pueraria thunbergiana, Thermopsis caroliniana,* and *Lupinus arboreus* were purchased from Schumacher Seed Co., Sandwich, Mass. *Phaseolus limensis* (Burpee Bush Lima) were from Burpee Seed Co., Riverside, Cal.

**Seed Extraction.** Seed flour was obtained by grinding dry seed in a Wiley mill using a 40-mesh screen. All subsequent procedures were carried out at 0–5 C. About 20 g dry flour were suspended in 60 ml of extraction buffer (50 mM Na-phosphate [pH 6.0], 0.4 M NaCl, 10 mM 2-mercaptoethanol, and 1 mM galactose) and gently stirred for 1 h. The suspension was filtered through cheesecloth and centrifuged for 15 min at 25,000*g* to obtain the crude extract. Ammonium sulfate (472 mg/ml) was slowly added to the crude extract and after 1 h the resulting precipitate was collected by centrifugation (as before) and stored at −20 C. The precipitate obtained from 20 g flour was dissolved in (20 ml) and dialyzed against extraction buffer just prior to use (70%[$NH_4$]$_2SO_4$ fraction).

**Assays.** Hemagglutinin activity was assayed by mixing 25 μl samples with 25 μl of trypsinized (6) rabbit erythrocytes (2% suspension in phosphate-buffered saline) and incubating at room temperature for 60 min. The reciprocal of the highest dilution (serial two-fold, phosphate-buffered saline) of extract which gave

Table I. *α-Galactosidase and Hemagglutinin Activities of Seed Extracts*

| Plant | Crude Extract | | 70% $(NH_4)_2SO_4$ Fraction | |
|---|---|---|---|---|
| | α-Galactosidase | Hemagglutinin Titer | α-Galactosidase | Hemagglutinin Titer |
| | *units/ml* | | *units/ml* | |
| *P. thunbergiana* | 116 | 512 | 486 | 2048 |
| *T. caroliniana* | 79 | 256 | 321 | 1024 |
| *L. arboreus* | 37 | 128 | 253 | 1024 |
| *P. limensis* | 62 | 64 | 742 | 512 |

Table II. *Carbohydrate Inhibition of Hemagglutinin Activity*

| Sugar | *P. thunbergiana* | *T. caroliniana* | *L. arboreus* | *P. limensis* | *V. radiata* |
|---|---|---|---|---|---|
| | | | mM[a] | | |
| *p*-Nitrophenyl galactoside | 1.0 | 1.0 | 3.1 | 0.6 | 0.34[b] |
| Galactose | 1.5 | 0.8 | 3.1 | 0.8 | 1.0 |
| Xylose | 3.0 | 3.0 | 6.3 | 6.2 | 4.0 |
| Inositol | 25 | 25 | 25 | 31 | 20.0 |
| Galactosamine[c] | ≥50 | ≥50 | ≥50 | ≥50 | ≥50 |

[a] Minimum concentration required to totally inhibit four hemagglutinin units.

[b] Data from reference 4.

[c] Glucose, mannose, fucose, *N*-acetylgalactosamine, and *p*-nitrophenyl β-galactoside were noninhibitory at 50 mM.

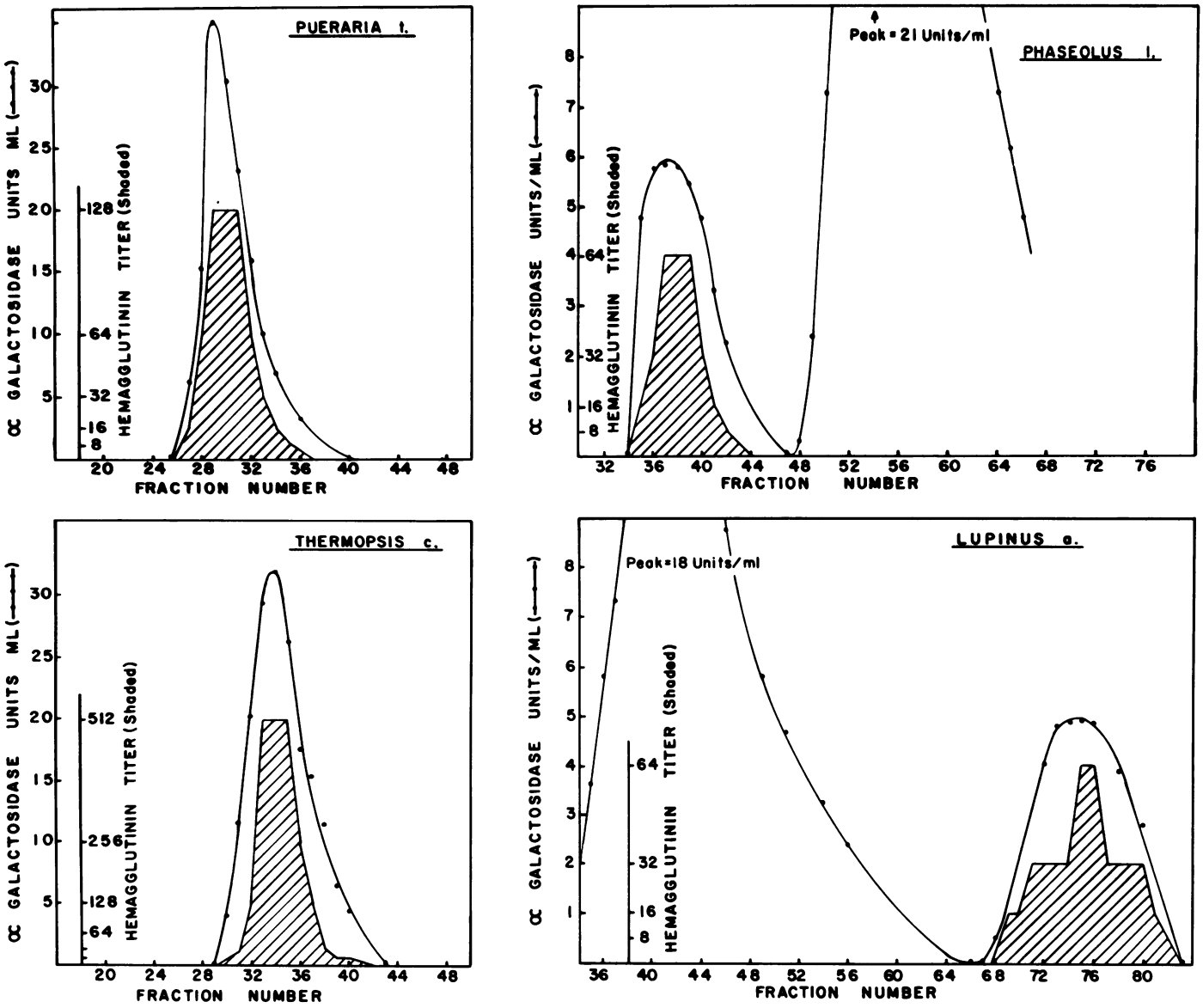


FIG. 1. Carboxymethyl cellulose chromatographic profiles.

visible agglutination (with aid of binocular microscope) of erythrocytes was recorded (titer). The immunochemical and α-galactosidase assays used were as described earlier (3, 4).

**CM-Cellulose Chromatography.** Samples of each 70% $(NH_4)_2SO_4$ fraction were exhaustively dialyzed against 10 mM Na-phosphate (pH 5.0) containing 10 mM 2-mercaptoethanol and 1 mM galactose. Dialyzed samples (5 ml) were applied to a column (2 × 20 cm) of CM-cellulose (Sigma) previously equilibrated with dialysis buffer. The column was washed with two column volumes of dialysis buffer and then eluted as follows. For *Pueraria* and *Thermopsis*, the columns were eluted with a linear increasing gradient composed of 250 ml dialysis buffer (lower limit) and 250 ml Na-phosphate, 50 mM (pH 6.0) (upper limit). The flow rate was about 1 ml/min and 8-ml fractions were collected. For *Lupinus* and *Phaseolus*, the columns were eluted with a gradient composed of 250 ml dialysis buffer (lower limit) and 250 ml of Na-phosphate, 50 mM (pH 6.0) containing 0.4 M NaCl (upper limit). The flow rate was about 1 ml/min and 5-ml fractions were collected.

**Gel Filtration.** Samples (4 ml) of each 70% $(NH_4)_2SO_4$ fraction were chromatographed on a column of Sephacryl-S200 (2 × 110 cm) (Pharmacia) previously equilibrated with extraction buffer. The column was eluted downward with extraction buffer at a flow rate of 15 ml/h and fractions about 3-ml were collected. A calibration curve for the S-200 column was obtained by plotting $V_e/V_0$ (elution volume divided by void volume) *versus* log mol wt for several standard proteins. The standards and their assumed mol wt were: blue dextran, void; alcohol dehydrogenase, 141,000; soybean agglutinin, 122,000; BSA, 68,000; and Cyt *c*, 17,500, respectively.

## RESULTS

**Activity of Crude Extracts.** Seed extracts of the four plants studied contained galactosidase activity, as assayed by *p*-nitrophenyl α-galactoside hydrolysis, and hemagglutinin activity as assayed with trypsinized rabbit erythrocytes. Both activities in each species were concentrated to about the same extent by ammonium sulfate precipitation (Table I). The hemagglutinin activity measured in lima beans is not due to the previously characterized human "A" LH[2] from the *sieva* variety of lima beans (2). LH is inhibited by *N*-acetylgalactosamine (GalNAc) which is included in our titer assays. Furthermore, pure LH is not capable of agglutinating rabbit erythrocytes (which were used in this study). Finally we have been unable to detect any significant

[2] Abbreviation: LH: specific lectin; CRM: cross-reacting material.

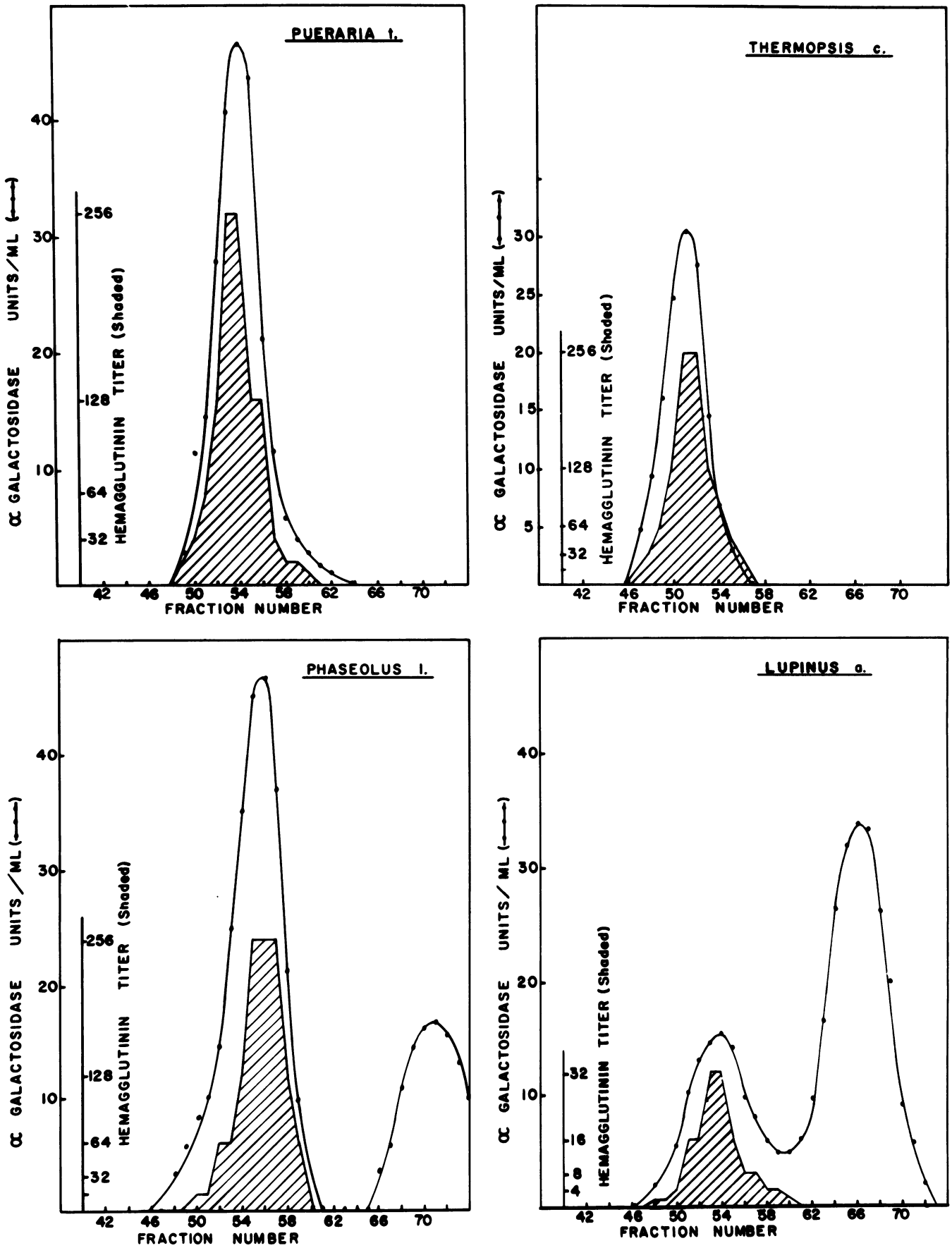


FIG. 2. Sephacryl S-200 gel filtration profiles.

quantity of human "A" erythrocyte specific hemagglutinin activity in the bush variety of lima beans used in this study.

**Hemagglutinin Specificities.** The carbohydrate specificities of the hemagglutinins were examined in detail and the results are shown in Table II. The four hemagglutinin activities are remarkably similar to each other with respect to both the kind and quantities of carbohydrate which inhibit hemagglutination. Note that the carbohydrate specificities displayed by these four hemagglutinins are virtually indistinguishable from those observed previously (4) with the highly purified enzymic hemagglutinin from *V. radiata*.

**Ion Exchange Chromatography.** Samples from each species were chromatographed on a column of carboxymethyl cellulose. The elution profiles of each species is depicted in Fig. 1. In every case a single peak of hemagglutinin activity was observed which exactly chromatographed with a peak of α-galactosidase activity. Note that with *Lupinus* and *Phaseolus*, a second peak of enzyme activity was observed.

**Gel Filtration.** Each seed extract was chromatographed on a calibrated column of Sephacryl S-200. The results shown in Figure 2 reveal that, as with ion exchange chromatography, a single peak of hemagglutinin activity chromatographed with a peak of α-galactosidase. *Lupinus* and *Phaseolus* again displayed two peaks of α-galactosidase activity, one of which was not associated with hemagglutinin activity. Mol wt profiles very similar to those seen with *Phaseolus* and *Lupinus* have previously been observed with the galactosidases in *Vicia* (1), although no association with hemagglutinins was reported. We have little information concerning a relationship, if any, between the two mol wt forms of α-galactosidase detected in *Lupinus* and *Phaseolus*. Work with the *Vicia* system, however, suggests that the larger mol wt α-galactosidase represents an aggregated form of the smaller.

The estimated mol wt of the enzyme activities detected in these four legume species are summarized in Table III. Note the close similarity in the sizes of the larger α-galactosidases from each plant. The mol wt of the tetrameric *Vigna* α-galactosidase-hemagglutinin was about 160,000 (4).

**Immunochemical Studies.** Extracts from all four species gave precipitin bands (CRM) in Ouchterlony double diffusion assays when challenged with antisera raised against the pure *Vigna* α-galactosidase-hemagglutinin. These results indicate that each species contains at least one protein which is evolutionarily closely related to the *Vigna* protein. With *Pueraria* and *Thermopsis* it was possible to detect *Vigna* CRM in S-200 fractions. With *Pueraria*, *Vigna* CRM was also detected in CM-cellulose fractions. In each case the CRM appeared only in those fractions which contained α-galacosidase and hemagglutinin activity. Thus, *Vigna* CRM also appears to co-purify with these activities.

**Kinetic Studies.** Simple Michaelis-Menten kinetic analyses of α-galactosidases were performed with extracts from the four plant species. A typical example of the kinetic patterns seen (in all four cases) is that for *Thermopsis* (Fig. 3). The apparent Michaelis contants ($K_m$) for *p*-nitrophenyl α-galactoside extrapolated from these studies were very similar among the species (Table IV). The sugar inhibition data (apparent $K_i$ values) for the enzymes are also summarized in Table IV. Comparison of the apparent $K_i$ values for the enzymes (Table IV) with the inhibition data for the hemagglutinins (Table II) makes it quite clear that the enzymic and hemagglutinin activites have virtually identical carbohydrate specificities.

Our data collectively reveal that with respect to specificity and kinetic behavior the four large mol wt α-galactosidases under study are very nearly identical, both to one another and to the *Vigna* α-galactosidase-hemagglutinin. Preliminary kinetic analysis of the small mol wt α-galactosidases (*Lupinus* and *Phaseolus*) indicates that these forms display kinetic behavior indistinguishable from the large mol wt enzyme. These results suggest that the larger sized enzymes may be aggregated forms of the smaller. This possibility would be consistent with the observed lack of hemagglutinin activity associated with the smaller form. The large and small mol wt α-galactosidases described in *Vicia* (1) and in *Glycine* (5) have been shown to be the same proteins in different aggregation states.

Table III. *Mol Wt of α-Galactosidases Estimated from Gel Filtration*

| | Galactosidase | |
|---|---|---|
| | I | II |
| | *daltons* | |
| *P. thunbergiana* | 150,000 | |
| *T. caroliniana* | 180,000 | |
| *L. arboreus* | 190,000 | 50,000 |
| *P. limensis* | 180,000 | 39,000 |
| *V. radiata*[a] | 160,000 | |

[a] From reference 4.

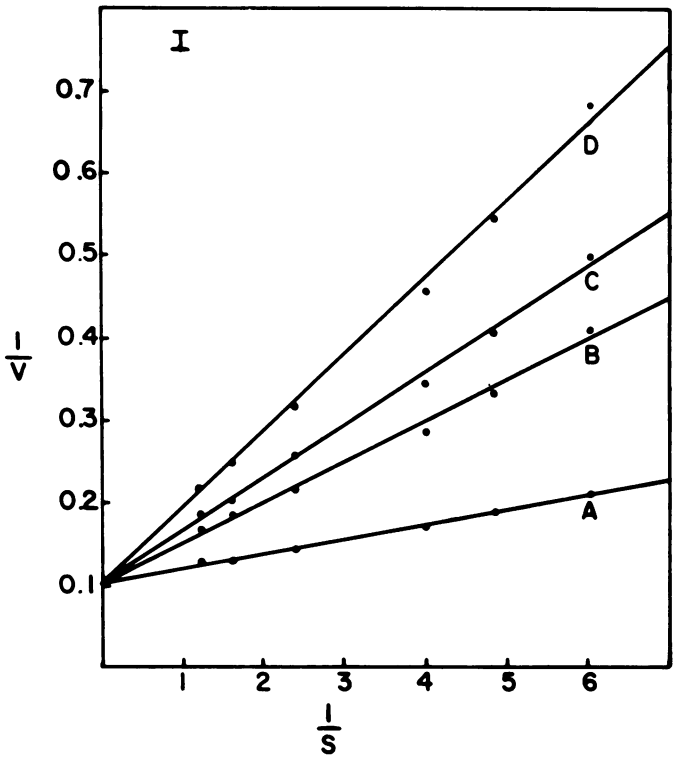


FIG. 3. Substrate titration curves with *Thermopsis* extract. A: no inhibitor; B: 0.5 mM galactose; C: 20 mM inositol; D: 7.5 mM xylose.

Table IV. *Summary of Kinetic Studies Using p-Nitrophenyl α-Galactoside as Substrate*

| Plant | $K_m$ | Inhibiting Sugars[a] ($K_i$) | | |
|---|---|---|---|---|
| | | Galactose | Xylose | Inositol |
| | mM | | mM | |
| *P. thunbergiana* | 0.42 | 0.9 | 2.9 | 8.8 |
| *T. caroliniana* | 0.20 | 0.4 | 1.6 | 8.8 |
| *L. arboreus* | 0.39 | 0.3 | 1.5 | 7.5 |
| *P. limensis* | 0.38 | 0.33 | 2.0 | 15.0 |
| *V. radiata*[b] | 0.20 | 0.75 | 5.2 | 20.0 |

[a] Carbohydrates which were noninhibitory in all four cases at 100 mM included galactosamine, *N*-acetylgalactosamine, raffinose, stachyose, glucose, mannose, and fucose.

[b] From reference 4.

## DISCUSSION

The results presented in this report provide additional evidence for the existence in legume seeds of carbohydrate utilizing enzymes which possess hemagglutinin properties. Each of the four plants studied contained a hemagglutinin which agglutinated trypsinized rabbit erythrocytes, but not human (A, B, or 0) erythrocytes. These hemagglutinins possessed very similar mol wt by gel filtration and displayed virtually identical carbohydrate inhibitor specificities. All four of these hemagglutinins also appear to be associated with a clot-dissolving activity comparable to that described in *Vigna* (4). Each of these hemagglutinins exactly co-purified with an α-galactosidase activity by both ion exchange chromatography and gel filtration. The α-galactosidase activities from all four plants displayed virtually identical kinetic behavior and inhibitor specificities. Additionally, the inhibitor specificities of the enzyme and hemagglutinin activities are directly comparable, both qualitatively and quantitatively. These results suggest that both enzyme and hemagglutinin activities result from a single protein species. This assertion is strongly supported by the observation that each of these proteins is remarkably similar (with respect to physical, enzymic and hemagglutinin properties) to the previously described α-galactosidase-hemagglutinin from *V. radiata* (4). Since these activities also appear to co-purify with material immunologically related to the *Vigna* protein, we believe it is reasonable to offer the possibility that each of the plants described herein, contain an α-galactosidase-hemagglutinin homologous to that seen in *Vigna*. Proof of this assertion must await a detailed comparison of the primary structure of these hemagglutinins.

In addition to the species discussed above we have also found an α-galactosidase which appears to have hemagglutinin properties in extracts of soybeans (*Glycine max.*) The soybean enzyme is also similar in most properties to those discussed herein. The α-galactosidase-hemagglutinins in soybeans and lima beans can be readily separated from and seem to be immunologically unrelated to the previously characterized *N*-acetylgalactosamine specific lectins in these plants (2, 5). These observations lead to the important conclusion that at least two distinct (nonhomologous, but perhaps evolutionarily related) classes of proteins with hemagglutinin activity exist in legume seeds.

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