Plant Physiol. (1980) 66, 375-378
0032-0889/80/66/0375/04/$00.50/0

Legume

α-GALACTOSIDASE FORMS DEVOID OF HEMAGGLUTININ ACTIVITY

Received for publication November 19, 1979 and in revised form April 23, 1980

CHARLES N. HANKINS, JUANITA I. KINDINGER, AND LELAND M. SHANNON
Department of Biochemistry, University of California, Riverside, California 92521

ABSTRACT

Twenty different legume species (20 genera) were examined for α-galactosidase and hemagglutinin activities. Although all of the species contained enzyme activity, only 13 of 20 contained hemagglutinin activities and none displayed a hemagglutinin activity comparable to the previously described α-galactosidase-hemagglutinins.

The α-galactosidase activities in the 20 species possessed remarkably similar kinetic behavior and carbohydrate specificities. All were inhibited by galactose, xylose, and inositol (very similar K_i values from plant to plant) and had very similar K_m values for the substrate, *p*-nitrophenyl α-galactoside.

Gel filtration analysis of extracts from nine species suggests that legume α-galactosidase activities may frequently reside in two molecular weight forms. However, all these species contained a large molecular weight enzyme activity with a size comparable to the α-galactosidase-hemagglutinins.

Immunochemical studies reveal that the α-galactosidases in these plants are immunologically related to an α-galactosidase-hemagglutinin and, therefore, are related to one another.

These studies suggest that each of the legume species studied (and perhaps all members of this plant family) contain a homologue from a specific class of α-galactosidase. Although the previously described α-galactosidase-hemagglutinins appear to be members from this enzyme class, these proteins most frequently occur as forms devoid of hemagglutinin activity.

A large number of legume species have been shown to possess hemagglutinins, and many of these proteins have been purified and well characterized (2, 8, 10). It has been very difficult to develop meaningful generalities about hemagglutinins primarily because their physiological function(s) is totally unknown and the information required to establish the relationships between different hemagglutinins has not been available. It would be useful to know how many physiologically or evolutionarily distinct classes of protein the hemagglutinins represent, to which class each of the previously characterized hemagglutinins belong, and what (if any) relationship exists between each class. Perhaps most important, however, would be information concerning whether or not hemagglutinin activity (*i.e.* the ability to agglutinate animal erythrocytes *in vitro*) is a direct reflection of an *in vivo* function. The present studies may provide a significant first step toward answering these kinds of questions.

Recently (3) we described several legume species which appeared to contain α-galactosidase-hemagglutinins which were very similar (perhaps homologous) to that from *Vigna radiata* (5). During the search for these proteins within the legume family we noted that, although few species appeared to contain α-galactosidase-hemagglutinins (about 20%), all plants examined contained α-galactosidase activity. We questioned whether the α-galactosidase activities in these plants were the result of proteins similar to the hemagglutinating enzymes seen in certain species or if they were the result of totally distinct proteins.

Here we describe some of the physical and kinetic properties of the α-galactosidases from twenty different legume species (20 genera). The results presented reveal remarkable similarities in the properties of the enzymes from plant to plant and to the previously described hemagglutinin enzymes.

MATERIALS AND METHODS

Seeds. The legume species studied and the sources of their seeds are given in Table I.

Seed Extraction. Seed flour was obtained by grinding dry seeds in a Wiley mill with a 40-mesh screen. All remaining procedures were carried out at 0 to 5 C. About 20 g dry flour was suspended in 60 ml extraction buffer [50 mM K-phosphate (pH 6.0), 1 M NaCl, 1 mM 2-mercaptoethanol, and 1 mM galactose] and gently stirred for 1 h. The suspension was filtered through cheesecloth and then centrifuged for 15 min at 25,000*g* to obtain the crude extract. Ammonium sulfate (472 mg/ml) was added slowly to the crude extract and after 1 h the precipitate was collected by centrifugation (as before) and stored at −20 C. Just prior to use, the ammonium sulfate precipitates were suspended in and dialyzed against extraction buffer containing 0.4 M NaCl.

Assays. Hemagglutinin assays, α-galactosidase assays and Ouchterlony double diffusion were carried out exactly as described (4–6). Assays for β-glucosidase, β-galactosidase, and α-mannosidase were performed using *p*-nitrophenyl derivatives (Sigma) as described by Agrawal and Bahl (1). A unit of enzyme activity is defined as 1 nmol of substrate hydrolyzed/min.

Immunochemical. Rabbit antisera to pure *Vigna* α-galactosidase-hemagglutinin was prepared as described (5). IgG[1] was purified from both anti-*Vigna* sera and preimmune rabbit sera by using Protein A Sepharose (Pharmacia) and the manufacturers suggested methods. IgG was coupled to Sepharose 4B (Pharmacia) by the cyanogen bromide method of Porath *et al.* (8). Approximately 1.2 mg of IgG was added to each ml moderately activated Sepharose, which resulted in preparations with about 1 mg IgG coupled/ml of packed Sepharose.

The IgG-Sepharose was used as follows. Two columns (1.4 × 10 cm), one containing preimmune IgG-Sepharose and one containing anti-*Vigna* IgG-Sepharose were prepared and equilibrated with extraction buffer at room temperature. Three ml crude seed extract were loaded onto each column and allowed to run into the resin, and then the column flow was stopped for 30 min. After the incubation period, the columns were eluted with extraction buffer and fractions were collected. The enzyme activities (α-galactosidase, β-glucosidase, β-galactosidase, and α-mannosidase) of the

[1] Abbreviations: IgG, immunoglobulin G; CRM, cross reactive material.

material loaded and in the effluent fractions were measured. Also the total protein (A_{280}) applied and in the effluent was recorded. After the column was washed with 5 column volumes of elution buffer, 1 ml K-phosphate (0.1 M, pH 7.5) containing 1 mol *p*-nitrophenyl α-galactoside was layered and run into the column. The presence of enzyme activity bound to the column was revealed by the rapid production of visible yellow color.

The results of the immunoadsorption experiments are expressed as per cent specific binding which is defined as the percentage of applied activity recovered in the effluent from the preimmune (control) column minus the percentage recovered from the anti-*Vigna* (specific) column.

RESULTS

Extracts from the seeds of 20 different legume species (20 genera) were examined for α-galactosidase and hemagglutinin activities, as well as for CRM immunologically related to the *Vigna* enzymic hemagglutinin. All twenty plants contained α-galactosidase activity (Table I) and most appeared to contain *Vigna* CRM. Thirteen of the 20 species contained significant hemagglutinin activity (*i.e.* seven species appeared to be totally devoid of hemagglutinin activity). All of the species which displayed hemagglutinin activity are species from which a hemagglutinin has previously been characterized. None of the 20 plants appeared to contain a hemagglutinin activity with properties comparable to the α-galactosidase-hemagglutinins (*i.e.* none displayed clot-dissolving activity, none contained hemagglutinin activity which was inhibitable by xylose or inositol and all hemagglutinin activities that were seen were inhibited by carbohydrates which do not inhibit the α-galactosidase-hemagglutinins).

Since the α-galactosidase activities measured in these crude preparations could be the result of more than one specific class of enzyme, additional tests were designed to show whether or not these plants contained α-galactosidases with physical and kinetic properties in common with the α-galactosidase-hemagglutinins.

Simple Michaelis-Menten kinetic analysis of the enzyme activities were performed with extracts from all twenty species. The results (a typical example is shown in Fig. 1) revealed that galactose, xylose, and inositol acted as competitive inhibitors of *p*-nitrophenyl α-galactoside hydrolysis in every case. A summary of the kinetic parameters extrapolated from these studies is given in Table II. It can be seen that the relative inhibitory power of the three carbohydrates tested is identical for all species, in fact, the apparent K_i for each inhibitor is remarkably similar from plant to plant. Also the apparent K_m for *p*-nitrophenyl α-galactoside varies

Table I. *Galactosidase and Hemagglutinin Activities of Several Legume Extracts*

Plant	Source	α-Galactosidase	Hemagglutinin Present	CRM to *Vigna* Lectin
		units/g dry seed		
Amorpha fruticosa	1[a]	22	–[b]	–[c]
Bandeiraea simplicifolia	2	20	+	–
Bauhinia purpurea alba	1	72	+	+
Caragana arborescens	1	200	+	+
Cercis siliquastrum	1	39	–	+
Colutea arborescens	1	104	–	+
Conavalia ensiformis	3	225	+	–
Cytisus multiflorus	1	36	+	+
Dolichos biflorus	1	60	+	+
Genista monosperma	1	450	–	+
Laburnum alpinum	1	250	+	+
Lathyrus latifolia	1	300	+	+
Lens culinaris	3	175	+	–
Lespedeza bicolor	1	300	–	–
Mimosa pudica	1	75	–	+
Phaseolus vulgaris	3	60	+	+
Sophora japonica	1	140	+	+
Spartium junceum	1	50	–	+
Ulex europaeus	1	225	+	+
Wistaria sinensis	1	180	+	+

[a] Sources: 1: F. W. Schumacher Co. (Sandwich, Mass.); 2: Sigma (St. Louis, Mo.); 3: Burpee Seed Co. (Riverside, Calif.).

[b] As determined by the visual agglutination of trypsinized rabbit or human (A, B, or O) erythrocytes.

[c] As determined by Ouchterlony double diffusion of extracts with antisera raised against pure *Vigna* α-galactosidase-hemagglutinin.

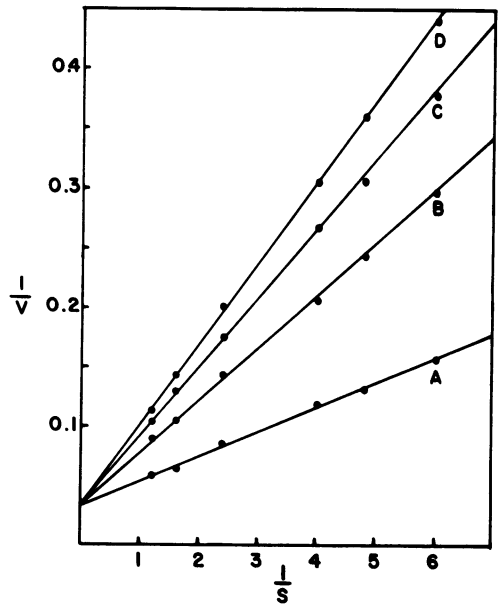

FIG. 1. Substrate titration curves for *Sophora* α-galactosidase. A: no inhibitor; B: 10 mM inositol; C: 0.75 mM galactose; D: 5 mM xylose. V: nmol/min; S: mM concentration of *p*-nitrophenyl α-galactoside.

Table II. *Kinetic Parameters of the α-Galactosidases in Several Legumes*

Plant	K_m *p*-Nitrophenyl α-Galactosidase	K_i		
		Galactose	Xylose	Inositol
	mM		mM	
A. fruticosa	1.25	0.53	2.95	12.6
B. simplicifolia	1.11	0.62	4.45	14.7
B. purpurea alba	1.00	0.50	3.10	15.8
C. arborescens	0.80	0.61	3.85	9.5
C. siliquastrum	1.82	0.47	3.40	9.0
C. arborescens	0.44	0.37	3.20	17.3
C. ensiformis	1.18	0.22	1.75	9.5
C. multiflorus	1.00	0.30	1.85	6.1
D. biflorus	1.18	0.51	3.60	
G. monosperma	0.58	0.35	1.60	11.0
L. alpinum	1.43	0.53	2.75	14.7
L. latifolia	1.33	0.61	3.50	8.7
L. culinaris	1.21	1.19	3.30	6.9
L. bicolor	0.47	0.35	2.70	11.7
M. pudica	0.65	0.38	2.20	11.6
P. vulgaris	0.71	0.50	3.15	12.8
S. japonica	0.57	0.39	2.25	9.0
S. junceum	1.66	0.45	3.55	11.4
U. europaeus	0.67	0.37	1.75	10.0
W. sinensis	0.65	0.58	3.65	17.8

very little from plant to plant. These kinetic data are virtually identical both qualitatively and quantitatively to those observed with the α-galactosidase-hemagglutinins described previously (4).

These twenty plants appear to contain enzymes which are very similar to the α-galactosidase-hemagglutinins, yet none displayed a comparable hemagglutinin activity and seven plants appeared to contain no hemagglutinin activity whatsoever. Previous studies (2, 7) have shown that different mol wt forms of α-galactosidase exist in legumes, and we have observed small mol wt nonagglutinating and large mol wt agglutinating forms of this enzyme in the same plant (4). Evidence has been presented (2, 7) which suggests that the large mol wt legume α-galactosidases may be aggregated (multimeric) forms of a smaller size enzyme. We question if the α-galactosidases in these 20 species might not all be small mol wt forms (monomeric?) which would not be expected to display hemagglutinin activity. Therefore, gel filtration studies were performed to determine the mol wt distributions of the enzymes in several of the plants. Nine different species were examined with similar results obtained in most cases. Figure 2 depicts a typical result. All nine plants contained a large mol wt form of α-galactosidase and most (seven) also contained a small mol wt form. The relative quantities of the two forms varied considerably from plant to plant. The estimated mol wt for the activities observed are summarized in Table III.

Although all the plants examined contained α-galactosidases very similar to the *Vigna* enzyme and most also contained *Vigna* CRM, one cannot assume from these studies that the enzymes are, in fact, *Vigna* CRMs. If, however, these proteins are immunologically related to *Vigna* α-galactosidase (responsible for at least some of the CRM), then anti-*Vigna* antibodies should be capable of adsorbing enzyme activity from extracts. With this assumption in mind, immunoadsorption experiments were performed with rabbit IgG immobilized on Sepharose 4B. The experiments were carried out as described under "Materials and Methods" and the results are summarized in Table IV.

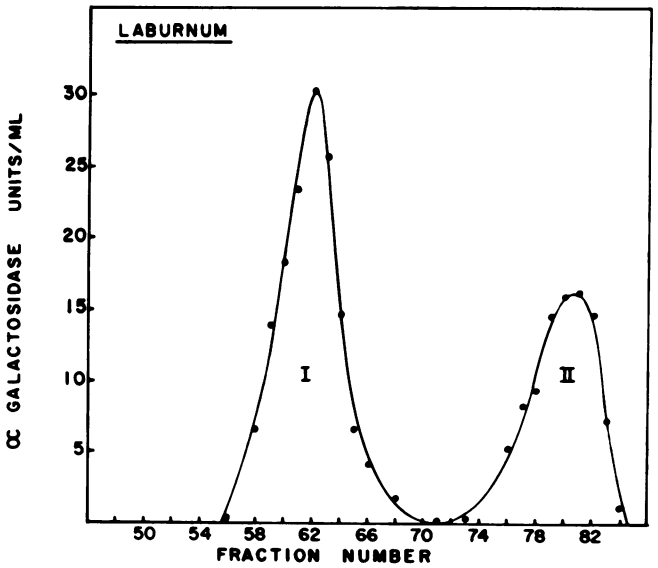

FIG. 2. Sephacryl S-200 gel filtration profile for *Laburnum* extract.

Table III. *Summary of Gel Filtration Mol Wt Determinations*

Plant	α-Galactosidase	
	I	II
C. arborescens	135,000	34,000
C. multiflorus	190,000	40,000
G. monosperma	160,000	37,000
L. alpinum	155,000	30,000
L. latifolia	160,000	
L. bicolor	120,000	31,000
S. japonica	150,000	
S. junceum	160,000	30,000
U. europaeus	160,000	42,000

In every extract tested, α-galactosidase activity was adsorbed onto anti-*Vigna* IgG columns in an enzymically active form. Using preimmune IgG-Sepharose as a control, enzyme activity was never detected on the washed columns, although occasionally less than 100% (minimum was 89%) of the applied activity was recovered in the effluent. The total amount of protein (as estimated by A_{280}) that bound to these columns was negligible in all cases, which is expected if nonspecific adsorption is very low and the α-galactosidases represent only a very minor component of the total protein.

These experiments clearly show that only α-galactosidase (of the several glycosidases tested) is specifically adsorbed by anti-*Vigna* IgG, indicating that α-galactosidase activities in every plant tested are immunologically related to the *Vigna* enzyme and, thus, to each other. Although all 20 plants were not tested by this method, a representative sample was included. The eight extracts studied included four species that did not show *Vigna* CRM on Ouchterlony double diffusion, three species which did not appear to contain hemagglutinins, species which contained only large mol wt α-galactosidase and species which contained both large and small mol wt enzymes (Table I).

The lack of visible CRM in double-diffusion experiments can be explained by low antigen concentration in crude extracts as well as by a weak immunological relationship. The adsorption of enzyme from these plant extracts by anti-*Vigna* IgG shows that an immunological relationship exists, even though it might not be readily detected by double-diffusion tests.

The immunoadsorption experiments described were done under conditions that gave from 40 to 95% adsorption of the applied enzyme activity. With prolonged incubation (several hours) or by using a greater ratio of adsorbant to extract, virtually 100% of the enzyme activity can be adsorbed from these extracts. Since several of the extracts contained both large and small mol wt α-galactosidase activities, these observations support the proposal (2, 7) that the two forms of enzyme are closely related.

DISCUSSION

We have adopted the nomenclature suggested by Dey and Pridham (2) and refer to the large mol wt form of enzyme as α-galactosidase I and to the smaller as α-galactosidase II. Although most species examined contained both forms of enzyme, it is significant that every species contained an α-galactosidase I with a mol wt comparable to the hemagglutinating forms which have been described. Additionally α-galactosidase I always represented

Table IV. *Binding of Enzyme Activities in Crude Extracts to Anti-Vigna IgG-Sepharose*

Plant	% Specific Binding[a]				
	Protein	α-Galactosidase	β-Galactosidase	β-Glucosidase	α-Mannosidase
S. japonica	0	95	2	0	0
L. bicolor	0	70	9	0	0
A. fruticosa	0	68	0	0	0
C. arborescens	0	66	0	0	0
U. europaeus	0	71	0	0	0
C. arborescens	0	60	0	0	0
L. culinaris	0	80	0	0	0
C. ensiformis	0	40	0	0	0

[a] See "Materials and Methods."

a significant (not trace or minor) fraction of the total enzyme activity. If aggregation state alone determined whether or not legume α-galactosidases possess hemagglutinin activity, then all these species should display such an activity.

From the observation that the seeds of every legume species examined contained an α-galactosidase I with very similar physical and kinetic properties, it is probable that these proteins represent homologues of a specific functional class of enzymes which are most likely ubiquitous in the legume family. This contention is strongly supported by the finding that the α-galactosidase activities in a variety of legume species are all immunologically related to the *Vigna* α-galactosidase-hemagglutinin and, thus, are related to one another.

Although these enzymes are referred to as α-galactosidases, we do not wish to imply that their physiological function(s) is defined. However, due to the similarities between this class of proteins and the α-galactosidases from *Vicia faba*, described by Pridham and Dey (9), it is possible that many of these authors' suggestions concerning the function of plant α-galactosidases may be generally applicable. Regardless of the detailed nature of the physiological role(s) performed by this class of proteins, it is reasonable to assume that their function is enzymic in character.

The α-galactosidase-hemagglutinins which have been described are clearly members of the enzyme class described herein. This information allows one to define a specific class of legume hemagglutinins, *i.e.* hemagglutinating forms of legume α-galactosidase I. Little is known about the differences between the hemagglutinating and nonhemagglutinating forms of α-galactosidase I. Both forms have comparable mol wt and display virtually indistinguishable enzymic properties; the differences between them must be relatively subtle.

It is reasonable to assume that these enzymes are generally important in legumes because they appear to be ubiquitous in the family and display a strong conservation of physical and enzymic properties. The same cannot be assumed about the "hemagglutinating activity" displayed by some forms, however, because they are relatively rare (six examples of this form have been observed from a total of 26 legumes studied). Of what importance to legumes, then, are the hemagglutinating forms of α-galactosidase I? Obviously these forms may have specific, nonenzymic functions in those legume species where they are found, but these functions, if they exist, are probably not important to legumes in general. Since the majority of the α-galactosidase I forms which have been examined do not display hemagglutinin properties *in vitro*, this property may not be a genuine reflection of any required *in vivo* activity; *i.e.* these proteins most likely function exclusively as enzymes but may occasionally display hemagglutinin activity *in vitro* because they *are* carbohydrate binding proteins and probably generally exist in forms with multiple active sites (multimeric proteins).

It is now clear that, even though they are immunologically related to other legume hemagglutinins (5), the α-galactosidase-hemagglutinins represent a class distinct from some (if not all) of the previously described (non-enzymic?) legume hemagglutinins (4). However, by analogy to the α-galactosidases, there appears to be no reason why other hemagglutinins might not also be members of a few classes of protein which are ubiquitous in legumes and the functions of which do not necessarily demand that they display "hemagglutinin activity."

Acknowledgment—We wish to acknowledge the excellent technical assistance of Mr. Keven Mielke.

LITERATURE CITED

1. AGRAWAL KML, OP BAHL 1972 α-Galactosidase, β-galactosidase, β-glucosidase, β-*N*-acetylglucosaminidase, and α-mannosidase from pinto beans. Methods Enzymol 28: 720–728
2. DEY PM, JB PRIDHAM 1969 Purification and properties of α-galactosidases from *Vicia faba* seeds. Biochem J 113: 49–55
3. GOLDSTEIN IJ, CE HAYES 1978 The lectins: carbohydrate-binding protein of plants and animals. Adv Carbohydr Chem Biochem 35: 127–340
4. HANKINS CN, JI KINDINGER, LM SHANNON 1980 α-Galactosidases which have hemagglutinin properties. Plant Physiol. *In press*
5. HANKINS CN, JI KINDINGER, LM SHANNON 1979 Legume lectins. I. Immunological cross-reactions between the enzymic lectin from mung beans and other well characterized legume lectins. Plant Physiol 64: 104–107
6. HANKINS CN, LM SHANNON 1978 The physical and enzymatic properties of a phytohemagglutinin from mung beans. J Biol Chem 253: 7791–7797
7. HARPAZ N, HM FLOWERS, N SHARON 1977 α-D-galactosidase from soybeans destroying blood-group B antigens. Eur J Biochem 77: 419–426
8. PORATH J, K ASPBERG, H DREVIN, R AXEN 1973 Preparation of cyanogen bromide-activated agarose gels. J Chromatogr 86: 53–56
9. PRIDHAM JB, PM DEY 1974 The nature and function of higher plant α-galactosidases. Annu Proc Phytochem Soc 10: 82–96
10. TOMS GC 1971 Phytohemagglutinins. *In* JB Harbourne, D Boulter, BL Turner, eds, Chemotaxonomy of the Leguminoseae. Academic Press, New York, pp 367–462