Journal of Chromatography, 347 (1985) 343–350
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 018

STUDIES ON LECTINS

LVIII. SUGAR-BINDING PROPERTIES, AS DETERMINED BY AFFINITY ELECTROPHORESIS, OF α-D-GALACTOSIDASES FROM *VICIA FABA* SEEDS POSSESSING ERYTHROAGGLUTINATING ACTIVITY

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(Received June 25th, 1985)

SUMMARY

The interaction of α-D-galactosidases from *Vicia faba* seeds with saccharides was studied by means of affinity electrophoresis on polyacrylamide gel in an acidic buffer system. For the preparation of affinity gels, water-soluble O-glycosyl polyacrylamide copolymers and polysaccharides were used. α-D-Galactosidases interact with immobilized O-α-D-galactosyl residues and glycogen, but no interaction was observed with immobilized O-α-D-mannosyl residues. On the basis of the results of affinity electrophoresis performed in the presence of various free sugars, dissociation constants of the various α-D-galactosidase–free sugar complexes were calculated.

INTRODUCTION

Shannon *et al.*[1] have shown that there are at least two evolutionarily-related, but distinct classes of legume proteins which possess erythroagglutinating activity: the "classic" lectins and α-D-galactosidases possessing erythroagglutinating activity. The sugar specificities of the binding sites responsible for erythroagglutinating activity of α-D-galactosidases are usually different from those of the "classic" lectins. Further, it has been found that not all legume seeds contain α-D-galactosidases with erythroagglutinating activities[1]. Dey and co-workers[2,3] have shown that *Vicia faba* seeds contain three forms of α-D-galactosidase: a high-molecular-weight (I) and two low-molecular-weight species (IIa and IIb); all forms agglutinate only trypsinized rabbit erythrocytes. Contrary to the α-D-galactosidase described by Shannon *et al.*[1], the erythroagglutinating activity of α-D-galactosidase I from *Vicia faba* seeds was inhibited by derivatives of D-mannose and D-glucose.

In our experiments, we have used affinity electrophoresis on polyacrylamide containing poly(glycosyloxyalkenyl–acrylamide) copolymers (O-glycosyl polyacrylamide copolymers)[4] to study the sugar-binding properties of the high- and low-molecular-weight forms of α-D-galactosidase from *Vicia faba* seeds.

0021-9673/85/$03.30

MATERIALS AND METHODS

Seeds of *Vicia faba*, cv. Uran were supplied by State Farm, Prague, Czechoslovakia. Water-soluble poly(glycosyloxyalkenyl–acrylamide) copolymers used for affinity electrophoresis were prepared as described earlier[4]. Mussel glycogen was purchased from Sigma (St. Louis, MO, U.S.A.). Two forms of α-D-galactosidase (α-D-galactosidase I and α-D-galactosidase II) were separated according to Dey *et al.*[2]. For the study of simultaneous interaction of both forms of α-D-galactosidase with saccharides, protein preparation obtained by the precipitation of 0.15 *M* sodium chloride extract with an ammonium sulphate fraction corresponding to 80% saturation, was used.

Affinity electrophoresis

Polyacrylamide gel electrophoresis of α-D-galactosidase was performed in an apparatus designed by Davis[5] in a discontinuous acidic buffer system[6] according to the standard procedure (omitting the large-pore gel layers).

Protein samples (15–30 μg) in 20% glycerol solution (30 μl) were applied to each tube (5 × 75 mm) and electrophoresis was run at 7 mA per tube for 70–100 min. Gels were stained specifically[7]. The migration distances of the zones of α-D-galactosidase were measured with an accuracy of ± 0.5 mm.

The dissociation constants (K_i) of the complexes of α-D-galactosidase I and II with immobilized α-D-galactosyl residues were obtained by a modification of our original method[8,9] from the dependence of $1/d_0 - d$ *vs.* $1/c_1$. The dissociation constants (K) of the complexes of α-D-galactosidase I and II and free sugars were obtained as described in our previous communications[8] from the dependence of $d/d_0 - d$ *vs.* c; c_1 = concentration of immobilized sugar, c = concentration of free sugar, d_0 = mobility on control gel containing water-soluble polyacrylamide without sugar residues, d = mobility on affinity gel at given c_i and c.

Affinity gels were prepared by the addition of an appropriate amount of the solution of O-glucosyl polyacrylamide copolymer or polysaccharide to the polymerization mixture to give a desired concentration c_i of immobilized sugar residues; c_i was used in the range $3.1 \cdot 10^{-3}$–$12.2 \cdot 10^{-3}$ *M*.

For the determination of K, solutions of free sugars were added to the polymerization mixture; the concentration range (c) of free sugars was different for various sugars according to the strength of interaction ($c = 8 \cdot 10^{-3}$–$240 \cdot 10^{-3}$ *M*).

Polyacrylamide gel electrophoresis for the detection of the presence of lectin was performed in discontinuous acidic[6] and discontinuous alkaline[5] buffer systems. Conditions of electrophoresis were the same as described for electrophoresis of α-D-galactosidase or as described by Davis[5]. Gels were stained with Amido Black. Affinity gels were prepared by the addition of O-α-D-mannosyl polyacrylamide copolymer to the polymerization mixture; c_i used was $4.7 \cdot 10^{-3}$ *M*.

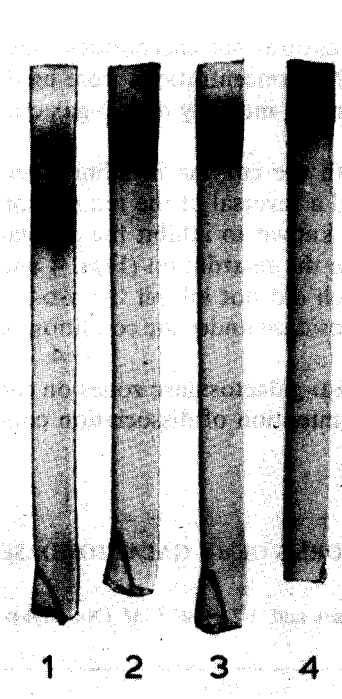
Erythroagglutinating activity

Erythroagglutinating activities of α-D-galactosidase and the lectin and inhibitory activities of sugars were assayed by a test-tube serial dilution method as described previously[10]. Human non-modified erythrocytes (A_1, A_2, B, O) and trypsinized rabbit erythrocytes were used.

RESULTS

High (I) and low molecular (II) forms of α-D-galactosidase present in *Vicia faba* seeds differ in their electrophoretic mobility on polyacrylamide gels in a discontinuous acidic buffer system (Fig. 1). They were separated by means of gel chromatography on Sephadex G-100 as described by Dey *et al.*[3] (Figs. 1 and 2). In the same purification step, the "classic" lectin was removed; it was completely bound to the dextran gel and could be eluted by a 0.2 *M* D-glucose solution. The absence of lectin in the fractions containing α-D-galactosidase was confirmed by affinity electrophoresis on polyacrylamide gels containing O-α-D-mannosyl polyacrylamide copolymer (no protein was retarded at $c_i = 4.7 \cdot 10^{-3}$ *M*) or by an erythroagglutination test with native human erythrocytes (no agglutination of human erythrocytes was observed).

In agreement with finding by Dey *et al.*[2], α-D-galactosidase I and II agglutinated trypsinized rabbit erythrocytes. Erythroagglutinating activity of both forms of α-D-galactosidase was inhibited most effectively by methyl α-D-mannoside, D-mannose and glycogen; D-galactose, methyl α-D-galactoside, D-xylose, N-acetyl-D-galac-



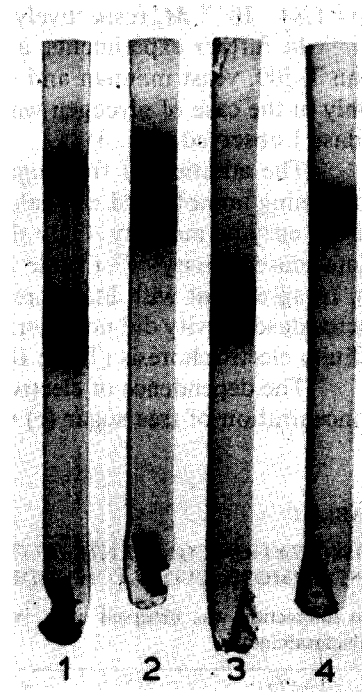


Fig. 1. Affinity electrophoresis of α-D-galactosidase I. 1 = Ammonium sulphate fraction, 2–4 = α-D-galactosidase I, 1, 2 = control gels, 3 = affinity gel containing O-α-D-mannosyl polyacrylamide copolymer ($c_i = 1.9 \cdot 10^{-2}$ *M*), 4 = affinity gel containing O-α-D-galactosyl polyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*).

Fig. 2. Affinity electrophoresis of α-D-galactosidase II. 1, 2 = Ammonium sulphate fraction, 3, 4 = α-D-galactosidase II, 1, 3 = control gels, 2, 4 = affinity gels containing O-α-D-galactosyl polyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*).

tosamine and *myo*-inositol did not affect the erythroagglutinating activity. Contrary to α-D-galactosidase from *Vigna radiata*[1], α-D-galactosidase from *Vicia faba* seeds does not possess the so-called "clot dissolving activity". A clot of agglutinated erythrocytes was observable even after standing for 12 h at laboratory temperature.

Affinity electrophoresis

The addition of O-α-D-galactosyl polyacrylamide copolymer to polyacrylamide gels caused a decrease in the electrophoretic mobility of both forms I and II of α-D-galactosidase (Figs. 1 and 2). The decrease of electrophoretic mobility is dependent on the concentration of immobilized D-galactosyl residues in the polyacrylamide gels. The control gels were prepared by the addition of water-soluble polyacrylamide (containing no saccharide residues) in the same concentration as the O-α-D-galactosyl polyacrylamide copolymer.

From the dependence of electrophoretic mobility on the concentration of immobilized sugar (c_i), the dissociation constant of the complex enzyme-immobilized D-galactosyl residue was calculated (Table I). The electrophoretic mobility of both forms of α-D-galactosidase was not affected by the presence of N-acetyl-O-α-D-galactosaminyl and O-α-D-mannosyl polyacrylamide copolymers at $c_i = 1.98 \cdot 10^{-2}$ *M* and $1.84 \cdot 10^{-2}$ *M*, respectively.

In further experiments, affinity gels containing natural polysaccharides (dextran T-500, yeast mannan and glycogen) in 1% and 2% concentrations were used. Only in the case of glycogen was a marked decrease in the mobility of α-D-galactosidase I observed (Fig. 3).

The addition of free sugars, which interact with the enzyme in affinity gels containing immobilized α-D-galactosyl residues, caused a reversal of the retardation of the enzyme mobility. Only those sugars which are known to inhibit the α-D-galactosidase activity[11,12] (Table II) were able to reverse the retardation (Figs. 4 and 5). In agreement with literature data[10,11], sugars which did not inhibit the α-D-galactosidase activity did not interact with the α-D-galactosidase under the condition of affinity electrophoresis (Table II).

The dependence of electrophoretic mobility of α-D-galactosidase zones on the concentration of free sugar (c) was used for the determination of dissociation con-

TABLE I

DISSOCIATION CONSTANTS OF COMPLEXES OF BOTH FORMS OF α-D-GALACTOSIDASE AND IMMOBILIZED SACCHARIDE

No interaction was observed at $c_i = 1.9 \cdot 10^{-2}$ *M* (D-mannose) and $1.7 \cdot 10^{-2}$ *M* (N-acetyl-D-galactosamine).

Immobilized saccharide	K_i *(M)*	
	α-D-Galactosidase I	*α-D-Galactosidase II*
D-Galactose	$11.5 \cdot 10^{-3}$	$6.9 \cdot 10^{-3}$
D-Mannose	–	–
N-Acetyl-D-galactosamine	–	–

Fig. 3. Affinity electrophoresis of α-D-galactosidase on affinity gel containing glycogen. 1–3 = Ammonium sulphate fraction, 1 = control gel, 2 = affinity gel containing 1% glycogen, 3 = affinity gel containing 1% glycogen and $1.1 \cdot 10^{-1}$ *M* methyl α-D-mannopyranoside.

TABLE II

DISSOCIATION CONSTANTS OF COMPLEXES OF BOTH FORMS OF α-D-GALACTOSIDASE WITH FREE SUGARS

No interaction was observed at the concentration of free saccharide, $c = 0.25$ *M*.

Saccharide	*K (M)*	
	α-D-Galactosidase I	*α-D-Galactosidase II*
D-Galactose	$11.1 \cdot 10^{-3}$	$8.8 \cdot 10^{-3}$
Methyl α-D-galactoside	$8.5 \cdot 10^{-3}$	$4.8 \cdot 10^{-3}$
Ethyl α-D-galactoside	$7.1 \cdot 10^{-3}$	$4.3 \cdot 10^{-3}$
D-Xylose	$31.2 \cdot 10^{-3}$	$30.4 \cdot 10^{-3}$
myo-Inositol	$66.1 \cdot 10^{-3}$	$12.9 \cdot 10^{-3}$
L-Arabinose	–	$73.6 \cdot 10^{-3}$
D-Fucose	–	$64.9 \cdot 10^{-3}$
2-Deoxy-D-galactose	–	–
4-O-Methyl-D-galactose	–	–
N-Acetyl-D-galactosamine	–	–
N-Acetyl-D-glucosamine	–	–
D-Mannose	–	–
L-Mannose	–	–
L-Galactose	–	–
L-Rhamnose	–	–
L-Fucose	–	–
D-Fructose	–	–
Lactose	–	–

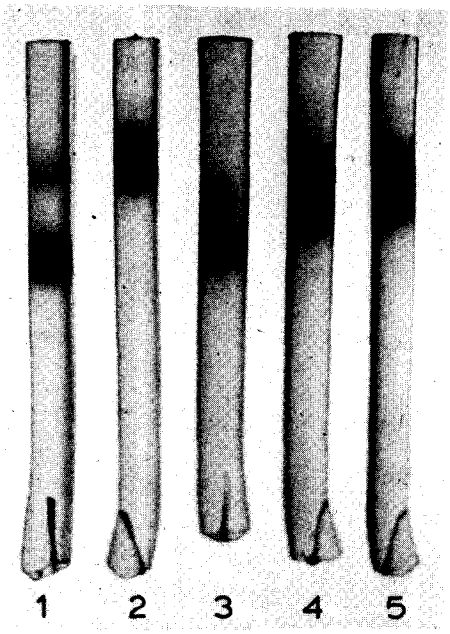


Fig. 4. Affinity electrophoresis of α-D-galactosidase in the presence of free D-galactose. 1–5 = Ammonium sulphate fraction, 1 = control gel, 2–5 = affinity gels containing O-α-D-galactosyl polyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*), 3–5 = affinity gels containing immobilized α-D-galactosyl residues and decreasing concentration of free D-galactose ($c = 7.0 \cdot 10^{-2}$ *M*, $2.0 \cdot 10^{-2}$ and $8.0 \cdot 10^{-3}$ *M*, respectively).

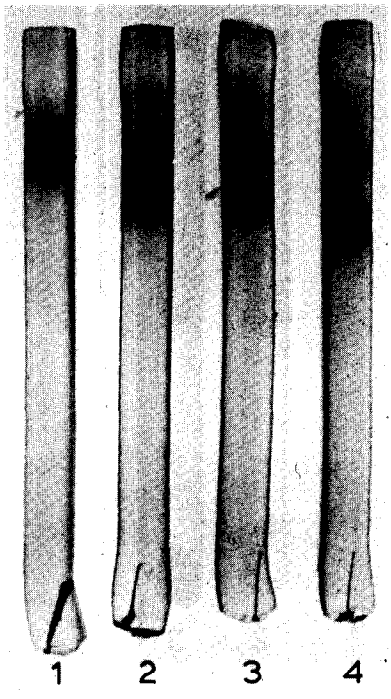


Fig. 5. Affinity electrophoresis of α-D-galactosidase in the presence of D-xylose. 1–4 = Ammonium sulphate fraction, 1–4 = affinity gels containing O-α-D-galactosyl polyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*), 2–4 = affinity gels containing immobilized α-D-galactosyl residues and increasing concentration of free D-xylose ($c = 4.5 \cdot 10^{-2}$ *M*, $9.0 \cdot 10^{-2}$ *M*, $2.4 \cdot 10^{-1}$ *M*, respectively).

stants of the complexes of α-D-galactosidase I and II and enzyme-free sugars (*K*) (Table II).

DISCUSSION

The method of affinity electrophoresis on polyacrylamide gel has been used to study the interaction of several enzymes, such as amylase, phosphorylase, various dehydrogenases, trypsin, RNAase and D-galactose oxidase, with the respective substrates or inhibitors[13]; but, to our knowledge, this method has not yet been employed to investigate binding properties of glycosidases. The method makes it possible to evaluate simultaneously the strength of interaction of all isoenzymes (as was described in the case of lactate dehydrogenase[14]), as well as all multiple forms of an enzyme, differing in their electrophoretic mobilities (as has been shown in this article).

Vicia faba seeds have been reported to contain multiple forms of α-D-galactosidase: high-molecular-weight I and low-molecular-weight IIa and IIb forms, which

differ in their electrophoretic mobilities[3]. In our experiments, we have observed only the presence of one low-molecular-weight (denoted II) and one high-molecular-weight form (denoted I).

Dey *et al.*[2] have described the isolation and properties of α-D-galactosidase I from *Vicia faba* seeds which possess erythroagglutinating activity. This activity is inhibited by D-glucose and D-mannose derivatives, which are quite ineffective in inhibiting the α-D-galactosidase activity. α-D-Galactosidases from *Vicia faba* seeds differ in erythroagglutinating activity from α-D-galactosidases from *Vigna radiata*[1,15], *Pueraria thunbergiana, Thermopsis caroliniana, Lupinus arboreus* and *Phaseolus lunatus*[1,16] and soybean (*Glycine soja*)[17]. The erythroagglutinating activities of these enzymes are inhibited by the same sugars as those for α-D-galactosidase activity, *e.g.* by derivatives of D-galactose, D-xylose and *myo*-inositol[15–17].

Thus, α-D-galactosidases from *Vicia faba* seeds should possess two types of binding sites for saccharides: one for derivatives of D-galactose and the other for derivatives of D-mannose and D-glucose. In our experiments we have used affinity electrophoresis on gels containing water-soluble O-glycosyl polyacrylamide copolymers to study the interaction of α-D-galactosidases with both types of saccharides. Our results have shown that both forms of α-D-galactosidase from *Vicia faba* seeds interact under the conditions of affinity electrophoresis with immobilized α-D-galactosyl residues, while no interaction was observed in the case of O-α-D-mannosyl residues, even though the erythroagglutinating activity was inhibited by D-mannose and methyl α-D-mannopyranoside (see Dey *et al.*[2] and our results).

An interesting phenomenon has been observed with affinity gels containing polysaccharides; in the case of glycogen, the zone corresponding to α-D-galactosidase I was fully retarded on the affinity gel, but this retardation could not be reversed by addition of methyl α-D-mannopyranoside to the affinity gel; probably much higher concentration or more complex saccharide is needed to inhibit the interaction. The observed interaction of α-D-galactosidase with glycogen under the conditions of affinity electrophoresis is in good agreement with the data described by Dey *et al.*[2], who observed the precipitation of α-D-galactosidase I in the presence of glycogen.

Affinity electrophoresis in the absence and in the presence of free saccharides was used for the determination of dissociation constants of the complexes of α-D-galactosidase with immobilized and free sugars. The determined values are in good agreement with inhibition constants determined kinetically[11,12]. Values of dissociation constants determined separately for individual forms of α-D-galactosidase, or simultaneously in a mixture, did not differ.

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