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Electrophoretic study of α-D-mannosidase and α-D-galactosidase from dry seeds of *Pisum sativum*

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ABSTRACT

Polyacrylamide gel electrophoresis in an acidic buffer system was used to study the electrophoretic behaviour of one form of α-D-mannosidase and the three forms of α-D-galactosidase from pea seeds *Pisum sativum*. Affinity electrophoresis was used to study the interaction of the studied enzymes with saccharides; water-soluble O-glycosyl polyacrylamide copolymers and polysaccharides were used for the preparation of affinity gels. Multiple forms of α-D-galactosidase were shown to inteact with immobilized α-D-galactosyl residues, whereas no interaction of α-D-mannosidase with immobilized α-D-mannosyl residues or with mannan, dextran or glycogen was observed. On the basis of the results of affinity electrophoresis of α-D-galactosidase, dissociation constants of complexes between the enzyme and immobilized α-D-galactosyl residues were calculated.

INTRODUCTION

Dry seeds of leguminous plants are known to contain several types of glycosidases, some of which are used in the solution of the structure of the saccharide moiety of glycoconjugates; α-D-mannosidase and α-D-galactosidase are hydrolytic enzymes that have been found to be localized in protein bodies together with other enzymes, storage proteins and lectins.

α-D-Galactosidase has been reported to be present in dry seeds in multiple forms [1] that were separable by molecular sieve chromatography. For dry seeds of *Vicia faba* [2], *Glycine soja* [3,4], *Vigna radiata* [5,6] and *Lens esculenta* [7], two forms of α-D-galactosidase were separated, *i.e.*, tetramer and monomer, both of which were active. In previous papers [8–10] we have shown that polyacrylamide gel electrophoresis combined with specific staining is a suitable method for the detection of multiple forms of α-D-galactosidase, as was demonstrated on examples of this enzyme from seeds of *Vicia faba* [8], *Glycine soja* [9], *Vigna radiata* [9] and *Canavalia ensiformis* [10].

α-DMannosidase is now considered as a marker activity for vacuoles or protein bodies of vacuolar origin in plant tissue [11,12]. Some properties of pea α-D-mannosidase have been described, in addition to its localization and changes in its activity during pea seed development [13–17]; recently, α-D-mannosidase was purified from cotyledons of developing pea seeds [11].

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Relatively little attention has been paid to pea α-D-galactosidase; changes in the α-D-galactosidase activity during germination [14] and localization of this enzyme in protein boedies of pea cotyledons [12] have been described.

In this paper, the electrophoretic behaviour of α-D-mannosidase and of multiple forms of α-D-galactosidase from pea seeds, their changes during germination and their saccharide binding properties are described.

EXPERIMENTAL

Seeds of *Pisum sativum* cv. Bohatýr were supplied by Sempra (Prague, Czechoslovakia). Water-soluble poly (glycosyloxyalkenylacrylamide) copolymers used for affinity electrophoresis were prepared as described earlier [18].

Ammonium sulphate fractions

A 0.15 *M* sodium chloride extract of pea seed meal was precipitated with ammonium sulphate to 50% and between 50 and 70% of saturation either directly or after acidification of the extract to pH 4.5; after dialysis against water, protein fractions were lyophilized. For the separation of multiple forms of α-D-galactosidase, gel chromatography on a Sephacryl S-200 column (Parmacia, Uppsala, Sweden) (80 × 1.5 cm I.D.) equilibrated with 0.05 *M* acetate buffer (pH 5.0) was used.

Extraction of pea seeds

Finely ground pea seed meal (50 g) was extracted with 0.15, 0.5 and 0.05 *M* NaCl, 0.05 *M* acetate buffer (pH 5.0), 0.05 *M* Tris–HCl buffer (pH 8.0), 0.15 *M* NaCl + 1% Triton X-100 or 0.5 *M* NaCl + 1% Triton X-100 (250 ml) for 18 h at 4°C. After removal of insoluble material by centrifugation, the extracts were used either directly for the enzyme activity determination and electrophoretic examination, or were dialysed against water and then lyophilized.

In further experiments the Tris–HCl extract (pH 8.0) was dialysed for 24–72 h against 0.05 *M* acetate buffer (pH 5.0) and *vice versa*. The dialysed extracts were used for further analysis.

Extracts of pea seedlings

Pea seeds were germinated on wet filter paper for 0–6 days; after 1–2 day periods, the separated cotyledons were homogenized in either 0.05 *M* acetate buffer (pH 5.0) or 0.05 *M* Tris–HCl buffer (pH 8.0)(10 ml).

Determination of protein and glycosidase activities

Glycosidase activities were assayed using *p*-nitrophenyl glycosides as substrates as described [19]. For the determination of the α-D-mannosidase activity, 0.05 *M* acetate buffer (pH 5.0) containing 1 m*M* zinc chloride was used. Protein was determined using the method described by Bradford [20] and bovine serum albumin as a standard. The unit of the enzyme activity is 1 μmol of released *p*-nitrophenol per minute.

Electrophoresis

Polyacrylamide gel electrophoresis of α-D-galactosidase was performed in a

discontinuous acidic buffer system [21]. Specific staining of gels and the conditions for the affinity electrophoresis of α-D-galactosidase were as described earlier [8,9,22].

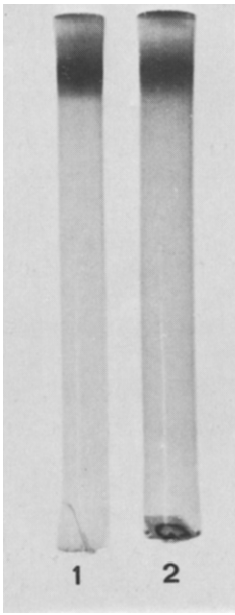
Polyacrylamide gel electrophoresis of α-D-mannosidase was performed in an alkaline buffer system [23]. The conditions for specific staining and for affinity electrophoresis were as described previously [10].

RESULTS

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis combined with specific staining showed that dry pea seeds contain one form of α-D-mannosidase (Fig. 1) and three active form of α-D-galactosidase (α-D-galactosidase I, II and III) (Fig. 2); the zone with the lowest mobility does not yield a typical colour for the α-D-galactosidase activity. Three active forms of α-D-galactosidases were detected not only in ammonium sulphate fractions (as shown in Fig. 2), but also in extracts directly applied to polyacrylamide gels. As is shown in Fig. 2, multiple forms of α-D-galactosidase can be partially separated by the ammonium sulphate fractionation of extracts of pea seeds.

α-D-Galactosidase I and α-D-galactosidase II differ in their molecular weight, as they can be separated by molecular sieve chromatography on the Sephacryl S-200 column; however, the presence of the third form, α-D-galactosidase III, was detected in the preparations of both α-D-galactosidase I and II (Fig. 3).



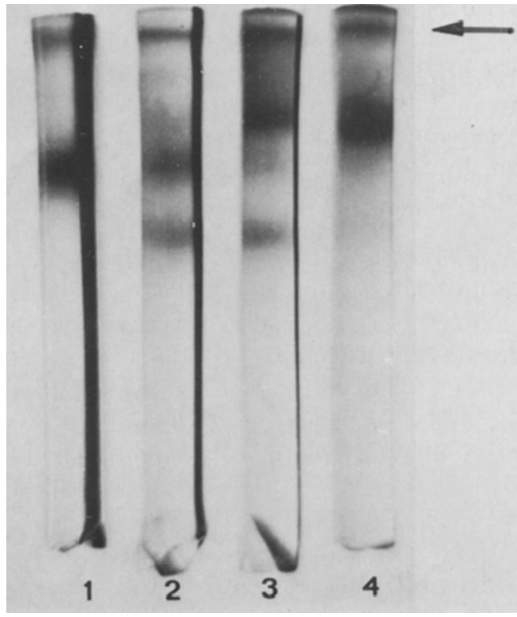


Fig. 1. Polyacrylamide gel electrophoresis of pea α-D-mannosidase. 1 = Ammonium sulphate fraction precipitating at 0–50% saturation; 2 = ammonium sulphate fraction precipitating at 50–70% saturation.

Fig. 2. Polyacrylamide gel electrophoresis of pea α-D-galactosidase. 1, 2 = Ammonium sulphate fraction precipitating at 0–50% saturation; 3, 4 = ammonium sulphate fraction precipitating at 50–70% saturation; 1, 3 = protein fraction soluble after dialysis against water; 2, 4 = protein fraction insoluble after dialysis against water. Arrow: this zone does not yield the colour typical of the α-D-galactosidase activity.

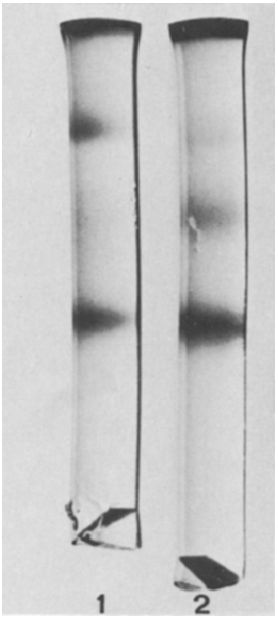


Fig. 3. Polyacrylamide gel electrophoresis of pea α-D-galactosidase after separation on Sephacryl S-200. 1 = α-D-Galactosidase with higher molecular weight; 2 = α-D-galactosidase with lower molecular weight.

Extraction of α-D-mannosidase and α-D-galactosidase from pea seeds under different conditions

The extractibility of α-D-mannosidase and α-D-galactosidase under different conditions from ground dry seeds is given in Table I. Extraction media with higher ionic strength and the presence of a detergent (Triton X-100) in the solution increased

TABLE I

EXTRACTION OF α-D-GALACTOSIDASE AND α-D-MANNOSIDASE FROM DRY PEA SEEDS UNDER DIFFERENT CONDITIONS

Extraction medium	Specific activity of extract (units mg^{-1})	
	α-D-Galactosidase	α-D-Mannosidase
0.15 *M* NaCl	1.70	0.72
0.5 *M* NaCl	2.23	1.00
0.05 *M* Acetate buffer (pH 5.0)	1.46	0.72
0.05 *M* Tris–HCl buffer (pH 8.0)	1.59	1.03
0.15 *M* NaCl + 1% Triton X-100	2.18	1.00
0.5 *M* NaCl + 1% Triton X-100	2.57	0.80

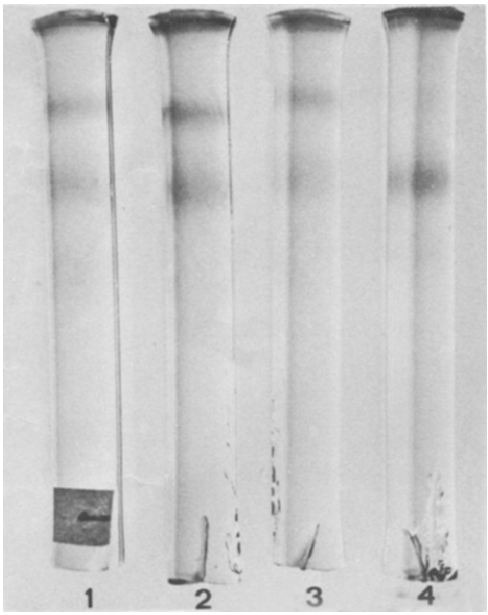


Fig. 4. Polyacrylamide gel electrophoresis of pea α-D-galactosidase extracted under different conditions. Extraction with: 1 = 0.15 *M* NaCl; 2 = 0.5 *M* NaCl; 3 = 0.05 *M* acetate buffer (pH 5.0); 4 = 0.05 *M* Tris–HCl buffer (pH 8.0).

the recovery of the α-D-galactosidase activity, whereas they affected the extraction of α-D-mannosidase only slightly.

The electrophoretic mobility of α-D-mannosidase present in extracts obtained with different extraction media is identical with that present in ammonium sulphate fractions (Fig. 1). The content of the three individual forms of α-D-galactosidase in the extract obtained with Tris–HCl buffer (pH 8.0) differed from that obtained with the other extraction media; whereas the extract obtained with 0.15 and 0.5 *M* NaCl in either the presence or absence of 1% Triton contained all three forms of α-D-galactosidase (in slightly different proportions), the Tris–HCl buffer extract contained only α-D-galactosidase II and III (Fig. 4). This situation did not change after dialysis of the Tris–HCl extract (pH 8.0) against acetate buffer (pH 5.0) and *vice versa*. Lyophilization of different extracts (after dialysis) resulted in a decrease in the specific activity of α-D-galactosidase (by 30–40%) but had no significant effect on the proportions of the individual forms of α-D-galactosidase. Dialysis and lyophilization of the extracts decreased the α-D-mannosidase specific activity more than the α-D-galactosidase activity.

Changes in the α-D-galactosidase and α-D-mannosidase activity during germination

Changes in the specific activity of α-D-galactosidase and α-D-mannosidase in cotyledons of germinating pea in the first phases (0–6 days) of germination were determined. For the extraction either 0.05 *M* acetate (pH 5.0) or 0.05 *M* Tris–HCl buffer (pH 8.0) was used. In the first phases of germination (the phase of testas

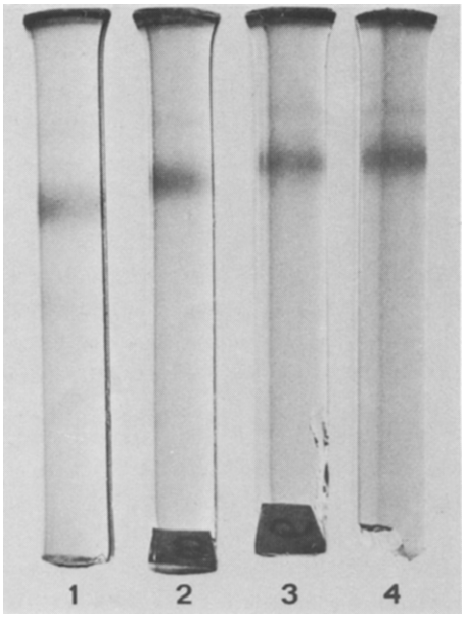


Fig. 5. Affinity electrophoresis of pea α-D-galactosidase II. 1 = Control gel; 2–4 = affinity gels containing O-α-D-galactosylpolyacrylamide copolymer (concentration of immobilized sugar, c_i = $1.0 \cdot 10^{-3}$, $2.0 \cdot 10^{-3}$ and $2.5 \cdot 10^{-3}$ M, respectively).

splitting), the specific activity of both enzymes increased in comparison with that of dry seeds, and then it slowly decreased.

The electrophoretic study showed that during the period studied, no changes in the electrophoretic behaviour of α-D-mannosidase or in the proportions of three forms of α-D-galactosidase occurred; acetate buffer extracts of seedlings germinated for 0–6 days contained α-D-galactosidase I and II and traces of α-D-galactosidase III, whereas the Tris–HCl extract only α-D-galactosidase II and III.

Affinity electrophoresis

Affinity electrophoresis on polyacrylamide gel containing O-glycosylpolyacrylamide copolymers [18] was used to study the sugar binding properties of α-D-mannosidase and different forms of α-D-galactosidase from pea seeds. The following O-glycosylpolyacrylamide copolymers were used: O-α-D-galactosyl-, O-α-D-mannosyl-, N-acetyl-O-α-D-galactosaminyl- and β-lactosyl- (the concentration of immobilized saccharide was $1.5 \cdot 10^{-3}$–$2.0 \cdot 10^{-2}$ M). Of the O-glycosyl copolymers used, only the addition of O-α-D-galactosylpolyacrylamide copolymer to polyacrylamide gel caused a decrease in the electrophoretic mobility of all three forms of α-D-galactosidase (Fig. 5). From the dependence of the electrophoretic mobility on the concentration of immobilized saccharide residues, the dissociation constant (K_i) of the complex between the enzyme and the immobilized D-galactosyl residues was calculated as follows: α-D-galactosidase I, $K_i = 8.5 \cdot 10^{-3}$ M; α-D-galactosidase II, $K_i = 5.5 \cdot 10^{-3}$ M. The retarded zone corresponding tot α-D-galactosidase III on affinity gels could not be identified.

In contrast to α-D-galactosidase, no interaction of α-D-mannosidase with immobilized α-D-mannosyl residues in polyacrylamide gels was observed, or with the other O-glycosylpolyacrylamide copolymers used (see above). The same results were obtained with affinity gels containing yeast mannan, dextran or glycogen; pea α-D-mannosidase did not interact with any of the polysaccharides used.

DISCUSSION

Seeds of leguminous plants are characterized by the presence of multiple forms of α-D-galactosidase [1]. In some of them, the presence of two forms of this enzyme (tetrameric and monomeric) has been proved [2–7]. In previous papers [8–10] we have shown that these forms differ in their electrophoretic mobilities. In contrast, the presence of three active, electrophoretically different forms of α-D-galactosidase in dry seeds of pea was detected (α-D-galactosidase I, II and III). Using molecular sieve chromatography, it was possible to separate α-D-galactosidase I and II, as with multiple forms from seeds of *Vicia faba* [2], *Glycine soja* [3] and *Vigna radiata* [6], but in contrast to these examples, both preparations of this enzyme contained the third form, α-D-galactosidase III, with the highest mobility. As the presence of α-D-galactosidase III was detected in crude extracts, applied directly to the polyacrylamide gel, this form does not arise as a result of the ammonium sulphate precipitation or acidification of the extract prior the precipitation. On storage of lyophilized preparations and repeated lyophilization, the relative proportion of α-D-galactosidase III increased.

It has been shown that interconversion of α-D-galactosidase I and II is not pH dependent, as was described for α-D-galactosidase from *Vigna radiata* [5,6] and *Glycine soja* [3,4].

The electrophoretic behaviour of the pea α-D-galactosidase I and II on affinity gels containing O-α-D-galactosylpolyacrylamide copolymer was very similar to that of the enzymes from *Vicia faba* [8], *Glycine soja* and *Vigna radiata* [9]. The zone corresponding to the third form of α-D-galactosidase on affinity gels could not be detected, even at very low concentrations of immobilized D-galactosyl residues. The values of the dissociation constants of complexes of α-D-galactosidase I and II with immobilized D-galactosyl residues were very similar to those obtained for the two forms of α-D-galactosidase from *Vicia faba*, *Glycine soja* and *Vigna radiata* seeds [8,9].

As far as pea α-D-mannosidase is concerned, its electrophoretic behaviour is very similar to that of α-D-mannosidase from *Canavalia ensiformis* seeds [10]. Similarly, α-D-mannosidase from pea seeds did not interact with immobilized α-D-mannosyl residues or polysaccharides (dextran, glycogen, mannan); the inability to interact with yeast mannan under conditions of affinity electrophoresis was in agreement with the findings of Krishna and Murray [11]; they did not succeed in using immobilized mannan for isolation of pea α-D-mannosidase.

Our experiments showed that the electrophoretic behaviour of multiple forms of α-D-galactosidase during the first phases of germination did not change. However, the proportions of these forms were significantly affected by different conditions of extraction. A similar effect has been described for multiple forms of α-D-galactosidase from *Vicia faba* [24] an *Cyamopsis tetragonolobus* [25].

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