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PLANT β-GALACTOSIDASES: PURIFICATION BY AFFINITY CHROMATOGRAPHY AND PROPERTIES

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Key Word Index—Plant β-galactosidase; affinity chromatography; Lactosyl-Sepharose.

Abstract—β-Galactosidases from seven plants have been extensively purified by affinity chromatography of an ammonium sulphate concentrate of the respective extracts through a column of Lactosyl-Sepharose. Five of the enzymes are composed of two subunits ranging in M_r from 42 to 45 000 and 31 to 35 000 respectively. A sixth enzyme apparently contains two M_r 34 000 subunits and a seventh is a M_r 46 000 single-subunit protein. All enzymes show charge heterogeneity containing from two to 11 enzymically active isoelectric forms. A major advantage of the purification method employed is the co-purification of all isoelectric forms of each enzyme

INTRODUCTION

Even though β-galactosidase activity is widely distributed in the plant kingdom, the precise role of the enzymes responsible is not well understood [1]. It has been suggested that they are involved in the degradation of plant cell-wall polysaccharides and more specifically of the core structure of arabinogalactan [1]. In the case of nasturtium cotyledons, convincing evidence has been presented that a β-galactosidase is responsible for the degradation of xyloglycans [2]. A necessary prerequisite for clarifying the physiological role of these enzymes is their purification and knowledge of their physical and catalytic properties. In this paper we report the purification of β-galactosidase activity from seven different plants by a convenient chromatographic method on Lactosyl-Sepharose.

Affinity chromatography employing diverse ligands and column supports has been previously used in the purification of β-galactosidases [3–7]. In most of these cases, however, the resultant preparations were of low specificity and required further purification. The procedure we describe, results in most cases in highly homogeneous enzyme preparations. A number of catalytic and physical properties of the purified enzymes were also studied and compared

RESULTS

Enzyme purification

All operations took place at 4° unless otherwise stated. β-Galactosidase activity was extracted from each tissue as described in the respective legends to Table 1 The extract was fractionally precipitated between 30 and 60% ammonium sulphate saturation. This step was mainly included to eliminate small M_r compounds and to concentrate the preparations The precipitate was dissolved in a minimum volume of 50 mM ammonium acetate buffer, pH 5. Undissolved material was removed by centrifugation. The preparation was dialysed overnight against the same buffer and was passed through a 1 × 25 cm column of Lactosyl-Sepharose previously equilibrated with 100 ml dialysis buffer. The maximum quantity of protein passed through the column was 120 mg. All plant β-galactosidase activity was retained by the column, while β-galactosidase activity originating in tissues other than plants, such as mouse liver lysosomes, bovine liver and yeast (*Kluyveromices fragilis*) was not retained. Each time the adsorbed enzyme was eluted as a single peak by means of 100 ml 50 mM lactose in 50 mM ammonium acetate pH 5, Fig. 1 shows the chromatographic profile of a typical experiment. The active fractions were pooled, dialysed against 50 mM ammonium acetate pH 5 to remove lactose and concentrated by ultrafiltration through a PM 10 Amicon filter. By this procedure the enzymes were purified from 55 to 550 times with a yield ranging between 7 and 45% (Table 1).

Physical properties of enzymes

SDS-polyacrylamide gel electrophoresis of the purified enzyme preparations showed two bands with a M_r of *ca* 45 and 35 000 in the case of jack bean, corn, barley meal and wheat germ enzymes, two major (45 and 35 000) and a minor band (29 000) in the case of the rye enzyme, a major (34 000) and two very weak (44 and 55 000 respectively) bands in the case of the spinach preparation and one band in the case of the tobacco leaves (46 000) (Fig. 2).

In order to establish the purity of the enzymes and determine their relative M_rs, all enzyme preparations were chromatographed through a Sephadex G-100 superfine column as described in the Experimental section. All enzymes were eluted from the column as a single protein and activity peak. The active fractions were pooled, concentrated and subjected to SDS-polyacrylamide gel electrophoresis. The electrophoretic patterns did not change after the gel filtration step, except for the case of the rye enzyme where the minor 29 000 band was

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Table 1 Degree of purification and yield of plant β-galactosidases following column chromatography on Lactosyl-Sepharose

Enzyme source	Extraction conditions	Specific activity† of Extract*	Specific activity† of Enzyme eluted from Lactosyl-Sepharose	Purification‡	Yield (%)
Barley meal	a	0 12	66	550	25
Corn meal	b	0 05	17 4	348	45
Jack bean meal	b	0 11	44 4	404	23
Germinated rye	c	0 25	65	260	9
TMV-infected Tobacco leaves	d	0 24	18	75	7
Spinach leaves	e	0 11	6	55	10
Wheat germ	f	0 01	3 1	310	26

a 25 g of meal were suspended in 150 ml 0 1 M ammonium acetate pH 6, homogenized and acidified to pH 5 as described in ref [8]

b 25 g of meal were suspended in 150 ml H_2O for 2 hr, and acidified to pH 5 5 as described in ref [5]

c Rye seeds were germinated for 3 5 days at 21 5 in the absence of light as described in ref [9] 190 g germinated seeds were homogenized with 285 ml 0 25 M sucrose in 5 mM Tris–HCl pH 8, 1 mM EDTA and 15 mM β-mercaptoethanol The homogenate was clarified by filtration through surgical gauze and centrifugation The low yield is mainly due to the precipitation of *ca* 50% of enzyme activity during dialysis prior to the chromatography step

d. Leaves (100 g) of TMV-infected tobacco plants were homogenized in potassium phosphate buffer treated with *n*-butanol and ultracentrifuged for the removal of TMV as described in ref [10] Following ammonium sulphate fractionation the preparation was treated with activated charcoal to remove coloured pigments By this treatment, however, 60% of activity was lost

e Spinach leaves (80 g) were homogenized for three 1 min intervals in a Waring blender with 80 ml 50 mM potassium phosphate buffer pH 7 Following passage through surgical gauze and centrifugation, the extract was acidified to pH 3 8 as described in ref [11] The enzyme is extremely labile following Lactosyl-Sepharose chromatography

f Wheat germ (25 g) was extracted with 0 1 M NaCl and brought to pH 4 as described in ref [7]

*The term 'extract' refers to the preparation resulting from the manipulations described in a f above

†*o*-NP-Gal was used as substrate

‡Ratio of the specific activity of the preparation following Lactosyl-Sepharose chromatography to the specific activity of the extract

Tissue extracts of non-plant origin were individually prepared Thus mouse liver fraction enriched in lysosomes was prepared by the method of ref [12] and extracted by blade homogenization The bovine liver preparation of Sigma (33 mg) was suspended in 30 ml sodium phosphate buffer pH 7 Yeast cells (5 g) were extracted with 50 ml 0 1 M potassium phosphate buffer pH 7 and 10^{-4} M manganese chloride, and β-galactosidase activity was precipitated with acetone as described in ref [13] In the case of the bovine and yeast enzymes the column was operated with 20 mM phosphate buffer pH 7, the pH optimum of both these enzymes

eliminated (Fig. 2B, col 5 and 6). The M_r of the native enzymes, as calculated from their migration through the Sephadex column, ranged between 63 and 70 000, except for the tobacco enzyme (46 000) M_r values for the native enzymes and their subunits are shown in Table 2

The purified enzymes were subjected to isoelectric focusing in the pH range 3–10 in polyacrylamide slab gels as described in the Experimental section. Each sample was run in duplicate and was stained for protein and enzyme activity respectively In each case multiple protein bands appeared, ranging from 2 to 11, all of which also stained for β-galactosidase activity The isoelectric forms of all enzymes are listed in Table 2 and Fig. 3 shows two representative cases.

Catalytic properties of enzymes

All of the enzymes acted optimally in the acid region between pH 3 6 and 4 5 depending on the buffer used They show one order of magnitude higher affinity for the synthetic substrate *o*-nitrophenyl β-galactopyranoside than for lactose (Table 3). At this point it should be pointed out that in a recent publication [8] Simos and Georgatsos made a detailed study of the catalytic properties of the barley enzyme (substrate specificity, inhibitors, effect of anions, in addition to pH optima and catalytic constants) and it was not deemed necessary to study all parameters in the present work for all enzymes, since plant β-galactosidases, irrespective of source, proved to

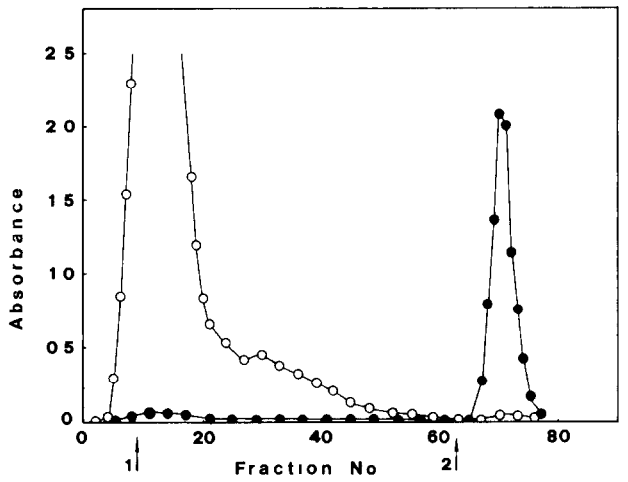

Fig 1 Lactosyl-Sepharose column elution profile of ammonium sulphate concentrate of jack bean extract The column was prepared and equilibrated as described in the text. Ammonium acetate buffer pH 5 (26 ml) containing 120 mg protein were slowly percolated through the column. At arrow 1, washing was continued with equilibration buffer. At arrow 2, 50 mM lactose was added to the eluting buffer Fraction size 4 ml Open circles *A* units of fractions at 280 nm. Closed circles: β-galactosidase activity expressed in *A* units at 410 nm (*o*-NP-Gal used as substrate)

Table 2. M_rs, subunit composition and isoelectric forms of purified plant β-galactosidases

Enzyme source	M_r ($k \times 10^{-3}$) Native enzyme*	Subunits†	Isoelectric forms (pI)
Barley	70	42 33	5 0, 5 1, 5 4, 5.7
Corn	68	45 35	4 5, 4 6
Jack bean	65	42 31	6 9, 7 7, 8 3
Rye	69	45 35	4 7, 4 9, 5.4, 6 5, 6 6, 7.1, 7 2, 7 5, 7 8, 8.0, 8 3
Tobacco	46	46	5.0, 5.25, 5 9, 6 6, 6.7, 7 1, 7 4, 7 7, 7 9, 8 0
Spinach	63	34‡	6 6, 7 3
Wheat germ	§	42 33	4 9, 5 0

*As determined by means of gel permeation chromatography through a column of Sephadex G-100 superfine.

†As determined by SDS polyacrylamide gel electrophoresis (cf. Fig. 2).

‡SDS-polyacrylamide gel electrophoresis revealed the presence of one major M_r 34 band and 2 very weak bands of 55 and 44 000 respectively (cf Fig 2). The assumption that the M_r 34 000 band is the catalytically active polypeptide is based solely on the quantitative differences of the 3 bands

§Gel permeation chromatography of the purified preparation was not performed. However, the M_r of the same native enzyme has been reported to be 75 000 by Hamazaki and Hotta [7]

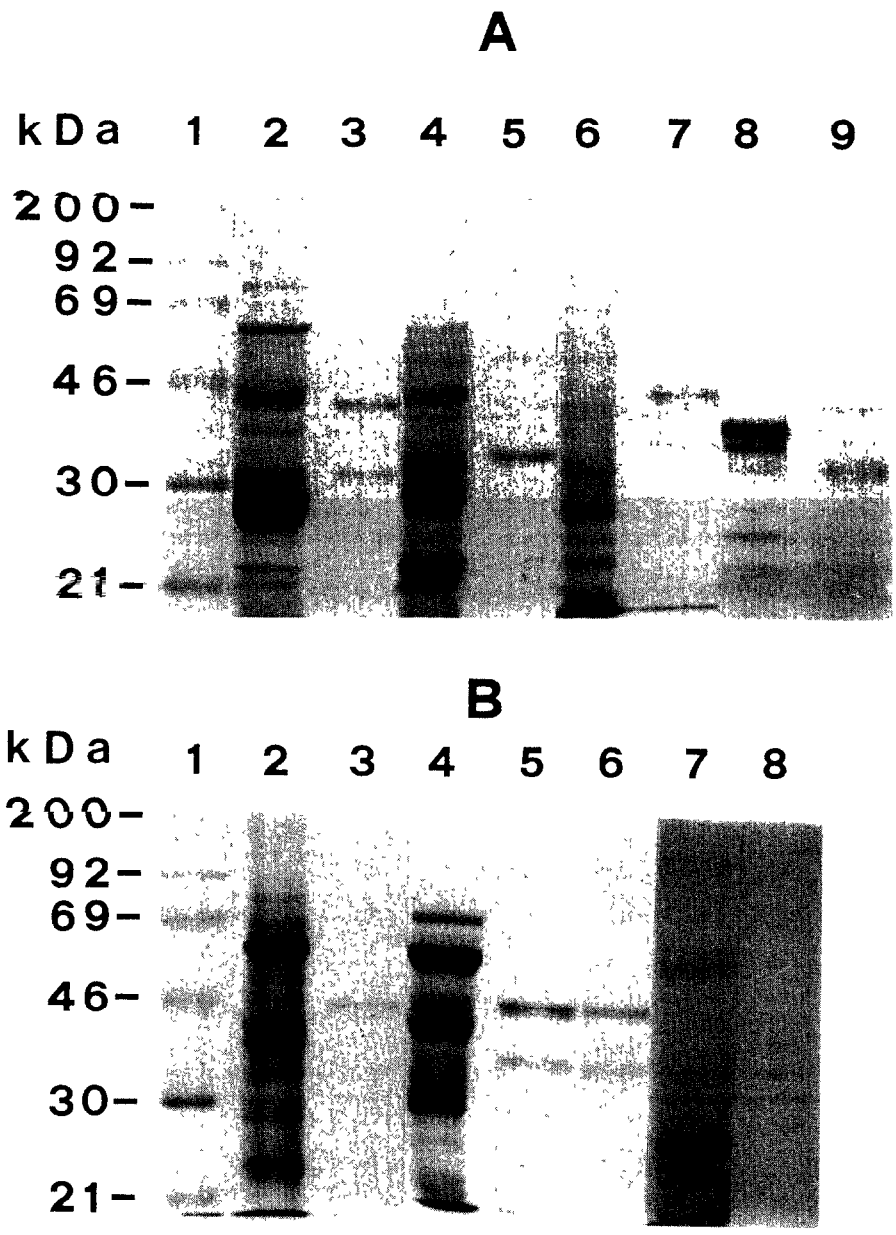

Fig 2 SDS-polyacrylamide gel electrophoresis of $(NH_4)_2SO_4$ fractions and enzyme preparations following Lactosyl-Sepharose chromatography A 1-M_r markers, 2,4,6 and 8 $(NH_4)_2SO_4$ fractions of jack bean meal, spinach leaves, corn meal and barley meal respectively, 3,5,7 and 9 the respective preparations following Lactosyl-Sepharose chromatography B 1-M_r markers, 2,4,7 $(NH_4)_2SO_4$ fractions of tobacco leaves, germinated rye seeds and wheat germ respectively, 3,5,8 the respective preparations following Lactosyl-Sepharose chromatography, 6 is preparation 5 following gel permeation chromatography through a column of Sephadex G-100 superfine Gels were stained with Coomassie blue R-250

be very similar to the barley enzyme in physical properties and in three major catalytic properties (pH optima, V_{max} and K_m values)

DISCUSSION

Lactosyl-Sepharose has been used in the purification of β-galactoside binding lectin [14]. In the present work it is shown that it is also a very useful agent in the purification of plant β-galactosidases The inability of Lactosyl-Sepharose to retain β-galactosidase of other than plant origin, such as yeast and mammalian tissues, is interesting and points to the existence of a specific structural feature in higher plant β-galactosidases that leads to strong interaction with the lactose ligand One structural characteristic that differentiates the plants from the yeast and

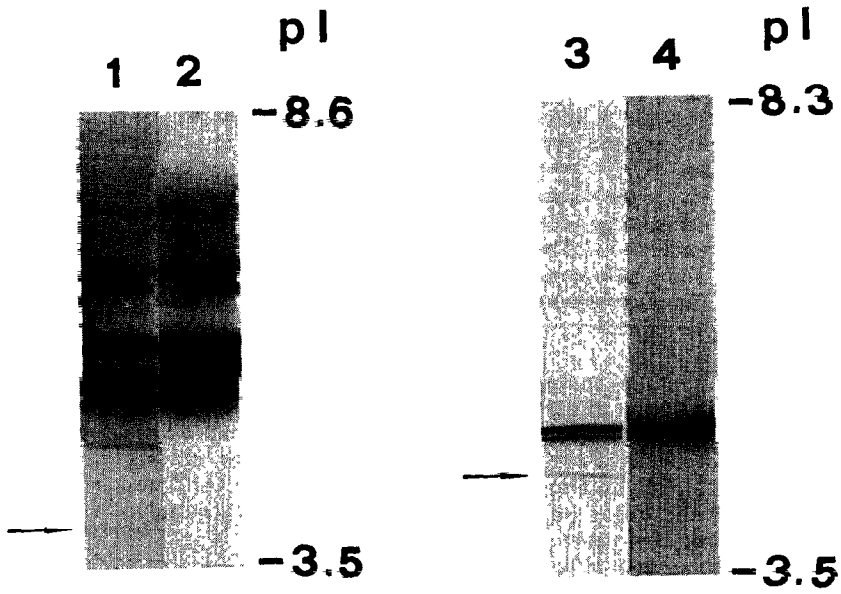

Fig. 3 Polyacrylamide gel isoelectric focusing of β-galactosidases following Lactosyl-Sepharose chromatography of germinated rye (1 and 2) and corn meal (3 and 4). 1 and 3 were stained for protein with silver nitrate, 2 and 4 were stained for β-galactosidase activity. In column 2 the isoforms with pI 8.0 and 8.3 are not visible on the picture. The arrow points to the methyl red dye used as marker.

Table 3. Catalytic properties of plant β-galactosidases

Enzyme source	pH Optimum	K_m (mM)		V_{max} (μ/mg)		V_{max}/K_m	
		o-NP-gal	Lactose	o-NP-gal	Lactose	o-NP-gal	Lactose
Barley	4.0*†	1.35	27	61.8	17.4	46	0 6
Corn	4.0* 3 6†	0 77	13.5	13.5	5.9	18	0.4
Jack bean	4.0* 3.6†	0.53	38	36	22.4	68	0 6
Rye	4.0* 4.4†	1.16	21	63	16.7	54	0.8
Tobacco	4.0*†	1.11	15.7	22	13.8	20	0.9
Spinach	4.0* 4.4†	0.61	39.1	6.3	2.2	10	0.1
Wheat germ	4.5*†	8.4	18.7	3	1.5	0.4	0.1

*Ammonium acetate buffer.
†Sodium citrate buffer.

liver enzymes is the relative simplicity of the former. Thus, according to the present work, with one exception, plant β-galactosidases are composed of two subunits of *ca* M_r 45 and 35000 respectively. The spinach enzyme is most probably composed of two M_r 34000 subunits. The one exception is the enzyme of tobacco leaves that has one 46000 subunit. On the contrary, the yeast enzyme has a M_r of 201000 [13] while the liver enzyme contains a 65000 subunit that aggregates into dimeric and higher M_r forms, ranging from 150 to 800000 [15].

The β-galactosidases of barley, jack bean meal and spinach have been previously purified by other more laborious methods ([8], [5] and [11] respectively). In some of these cases, as well as in others, affinity steps have been included. Thus the jack bean meal enzyme has been partially purified by chromatography on Sepharose columns carrying either galactonate-benzidine [4] or 2-{6-aminohexamido-hexanamido} ethyl-1-β-thio-D-galactopyranoside [5] or even 6-*N*-β-(4-aminophenyl)-ethylamino-3-*O*-β-galactopyranosyl-6-deoxy-L-glucitol [6] as ligands. β-Galactosidase of *Escherichia coli* has been purified on columns of agarose bead absorbents containing *p*-aminophenyl-β-D-thio-galactopyranoside [3]. In these procedures, non-specific or at best weak interactions apparently took place as elution required relatively high pH (9.2–10) [3, 4, 6] and in one case the enzyme was just retarded on the column [5]. Wheat germ β-galactosidase has been purified by Hamazaki and

Hotta [7] on a column similar to the one used in the present work. The absorbent was lactose attached to a hydrazide derivative of polyacrylamide in the form of Bio-gel P-300. Elution was accomplished by means of galactosyl-β-(1-4)-glucitol. In this case 50% of enzyme activity was not retained on the column and an additional column of gel filtration was required to obtain further purification. In our case the totality of the β-galactosidase activity was adsorbed on the Lactosyl-Sepharose column and the eluted enzyme was homogeneous The purified wheat germ enzyme consists of two polypeptides of 42 and 33 000 respectively, which is in agreement with the 75 000 value for the native enzyme reported by Hamazaki and Hotta [7]

A major advantage of the Lactosyl-Sepharose column is the selective adsorption and elution of all isoelectric forms of a particular β-galactosidase. As Hatton *et al.* [16] pointed out for the case of sialylated plasma glycoproteins, ion exchange chromatography frequently results in the isolation of only the major isoform in a population of charge-heterogeneous proteins In the case of plant β-galactosidases which are mostly composed of two subunits (Table 2), there is widespread charge heterogeneity ranging from two to 11 isoelectric forms. The cause of this heterogeneity is not known at present. Different degrees of sialylation, may be responsible, though this is not the case for the barley enzyme [8]. Glycosylation by different sugars to varying extent and/or different amino acid composition of one or both subunits may also account for the charge differences.

The structural similarities of plant-β-galactosidases apparently extend to their functional properties, as shown by the similarities in catalytic constants. It should be mentioned that our findings for the jack bean and the spinach enzymes are in agreement with results reported in previous publications ([5] and [11] respectively).

EXPERIMENTAL

Biochemical reagents and Sepharose 4B were purchased from Sigma. Glucose oxidase-peroxidase reagents in the form of a kit were purchased from Sclavo S p A, Fiorentina, Italy Seeds of the following plants were a generous gift of the Cereal Institute, Thessaloniki, Greece barley (*Hordeum vulgare* var Georgia), corn (*Zea mays*), rye (*Secale cereale*) Jack bean meal was a product of Sigma Tobacco mosaic virus-infected tobacco leaves were kindly provided by the Tobacco Institute, Drama, Greece Spinach leaves were purchased from the local market. Wheat germ was obtained from a local mill Livers were excised from ether-anesthetized mice of the pure imbred strain C3Hb Crude β-galactosidase from bovine liver and dried cells of *Kluyveromices fragilis*, a rich source of lactase, were purchased from Sigma. Lactose was attached to Sepharose 4B using divinylsulphone as the coupling agent [14] After each use the column was regenerated by washing with 6 M urea During operation no detectable glucose appears in the elution fractions Protein was determined by the method of ref [17] as modified in ref. [18] β-Galactosidase was assayed at pH 4 using either 6 mM *o*-nitrophenyl-β-galactopyranoside (*o*-NP-Gal) or 270 mM lactose as substrates. In the first case the reaction was followed by determining spectrophotometrically the release of *o*-nitrophenol in an alkaline medium (A_{410}) In the case of the disaccharide the reaction was followed by determining spectrophotometrically the amount of glucose released by the glucose oxidase-peroxidase reaction according to the instructions of the kit's manufacturer (the chromogen absorbs at 510 nm) A unit of enzyme activity is the amount of enzyme that hydrolyses 1 μmol substrate per min Sp act is units per mg protein SDS-polyacrylamide gel electrophoresis in slabs of 10% acrylamide was performed as described in ref [19] Gels were stained for protein with Coomassie blue R-250 or with $AgNO_3$ [20] Polyacrylamide gel isoelectric focusing in slab gels and visualization of protein and enzyme bands were carried out as described in ref [8] Gel permeation chromatography for the estimation of the M_r of native enzymes was carried out on a 1.5 × 120 cm column of Sephadex G-100 superfine, equilibrated with 50 mM NH_4OAc pH 5 and standardized with M_r markers

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