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MULTIPLE β-GALACTOSIDASE ACTIVITIES IN *PETUNIA HYBRIDA*: SEPARATION AND CHARACTERIZATION

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Key Word Index—*Petunia hybrida*; Solanaceae; β-galactosidase; multiple activities; isoelectrofocusing.

Abstract—Using isoelectrofocusing (IEF), multiple forms of *Petunia* β-galactosidase activity could be detected. The β-galactosidase pattern showed only minor tissue-specific differences. There were, however, species-specific differences. *Zea mays*, for instance, showed two bands which differed from the zones obtained with *Petunia* preparations. *Petunia* and corn leaves were mixed and extracted commonly. The species-specific activity patterns remained unchanged. *Petunia* preparations were inactivated by 8 M urea. Following dialysis, enzymatic activity and the *Petunia*-specific pattern were restored. The same holds true for a mixture of *Petunia* and *E. coli* β-galactosidase preparations. On refocusing isolated *Petunia* zones, untreated or inactivated by 8 M urea and reactivated by dialysis, the original mobilities were shown. Therefore, it seems highly improbable that the β-galactosidase pattern was due to artefacts. Using a *Petunia* line which was 'pure', also in respect to its β-galactosidase pattern, the four main bands were preparatively separated by IEF and characterized. They showed the same pH optimum (4.3), the same temperature optimum (55°), the same inactivation kinetics by urea, the same sensitivity against Cl^-, and closely related K_m values. In sucrose gradient centrifugation they invariably showed *S* values of 8–10. The multiple activities could not be separated by zone electrophoresis using various carrier systems, or by gel filtration. It seems possible that they represent forms which differ only in isoelectric points, not in MW.

INTRODUCTION

There is a wealth of data concerning *E. coli* β-galactosidase (EC 3.2.1.23, see [1]). On the contrary, only few and scattered reports deal with plant β-galactosidase [2–7]. Apart from its ability to split β-galactosidic linkages, its physiological functions remain widely unknown. Recently, also the plant enzyme received increased attention for its molecular aspects. For instance, plant β-galactosidase proved to be inducible like the *E. coli* enzyme [8]. Furthermore, there were claims to have succeeded in transferring the *E. coli* gene for β-galactosidase using transducing phage into plant callus [9] and cells in suspension [10]. Unfortunately, at this time the properties of the β-galactosidases of the plant species used were unknown. Consequently, there were difficulties in differentiating plant and bacterial enzymes in the apparently transformed plant material.

In later experiments with *Petunia hybrida* we used pollen as vectors for phages transducing the *E. coli* β-galactosidase gene [11]. Considering these difficulties, a first characterization of the *Petunia* β-galactosidase was carried out [7]. Based on differences in the pH- and temperature optima of the plant and bacterial enzyme, respectively, first evidence for a transfer of the bacterial gene into *Petunia* could be obtained [11]. In the course of these investigations, we detected multiple forms of *Petunia* β-galactosidase activities using IEF [7]. The study of these newly detected multiple activities could be a first step to obtain insight into the physiological functions of plant β-galactosidases.

Abbreviations used: BCIGal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside; BNGal: 6-bromo-2-naphthyl-β-D-galactoside; BNGluc: 6-bromo-2-naphthyl-β-D-glucoside; MUFGal: 4-methyl-umbelliferyl-β-D-galactoside; MUFGluc: 4-methyl-umbelliferyl-β-D-glucoside; ONPG: *o*-nitrophenyl-β-D-galactoside; PC: phosphate–citrate buffer.

RESULTS AND DISCUSSION

Multiple β-galactosidase activities in Petunia hybrida

In most investigations a pure line of *Petunia hybrida* selected from a commercial variety and called 'Cyanidintyp' [12] was used. The Cyanidintyp was kept by selfing for more than 30 generations. The first question was, whether it would be 'pure' with respect to the multiple β-galactosidase activities. Therefore, the shoot tips of 16 plants were tested for β-galactosidase activities by analytical IEF. All 16 plants showed the same pattern (Fig. 1). It proved to be identical with the pattern obtained from a mixture of 200 shoot tips. Using 4-methyl-umbelliferyl-β-glucoside as substrate, no β-glucosidic activity could be detected within the multiple β-galactosidase activities.

Quantitatively, corresponding zones showed the same activity as judged from the intensity of colouring with BCIGal. Therefore, at least gross quantitative differences in corresponding zones could be excluded. Several minor and five main bands of β-galactosidase activity were invariably found in preparations from shoot tips (Fig. 2). The Cyanidintyp therefore seemed 'pure' enough in respect to multiple β-galactosidase activities to give a reliable basis for more thorough investigations.

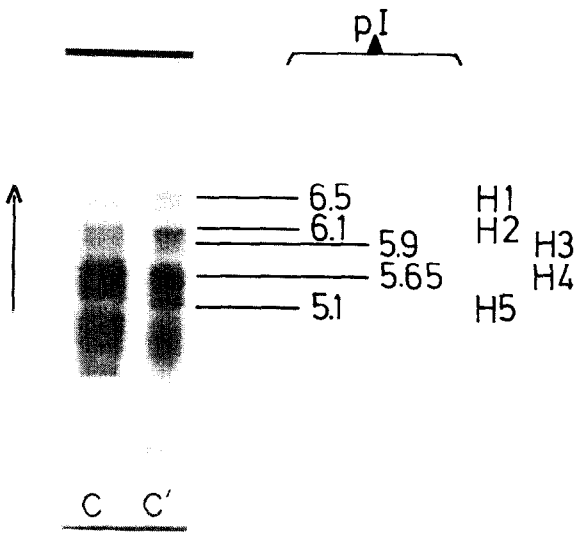

Fig. 1. Analytical isoelectrofocusing for *Petunia* β-galactosidases. Isoelectrofocusing on Ampholine PAG plates, pH 3.5–9.5 (LKB), 2 hr, 10° (for further details see [7] and the LKB manual). Thereafter staining on β-galactosidase activity using the sandwich technique and BCIGal as substrate. The five main bands (H1 H5) and their pI values are indicated. C: Cyanidintyp: C': Cyanidintyp, haploid, derived from anther cultures [29].

The pattern obtained from different plant parts showed some minor differences. In petals there was no activity at all. This was probably due to large amounts of phenolic compounds rapidly inactivating the enzyme. Old leaves showed one zone more than shoot tips. They are, however, often infected by fungi. Therefore, in all further investigations we used shoot tips.

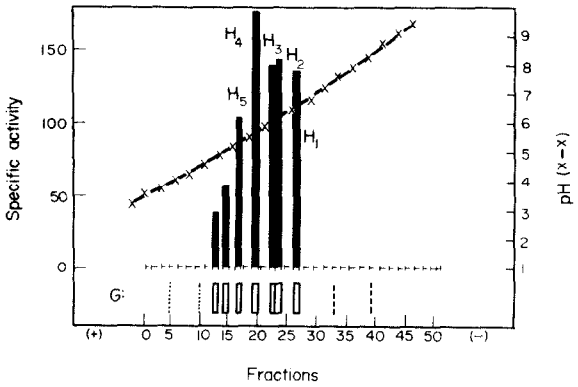

Fig. 2. Quantitative evaluation of multiple *Petunia* β-galactosidase activities following analytical isoelectrofocusing on Ampholine PAG plates, pH 3.5–9.5 (LKB). Gel sections were eluted overnight at 4° in 0.2 M PC, pH 4.3. In the eluates, β-galactosidase activity was determined using ONPG as substrate, and protein concentrations were determined by the 280/260 method. G: Reference strip, stained for β-galactosidase activity using the sandwich technique and BCIGal as substrate. H_1–H_5: main bands of β-galactosidase activity (cf. Fig. 1).

To ensure that the multiple activities were not artefacts, the following experiments were performed:

(a) In *Zea mays*, there are only two zones of β-galactosidase activity which differed from the *Petunia* bands (pI 4.95 and 4.80, respectively). Leaves of *Zea mays* and shoot tips of *Petunia* were mixed in a 1:1 ratio and commonly extracted for β-galactosidase. Following IEF, the species-specific patterns remained unchanged.

(b) A *Petunia* preparation, not subject to IEF, was mixed with *E. coli* β-galactosidase in a 1:1 ratio (25 units β-galactosidase activity each). The mixture was denatured by urea (see below) and renatured. Following IEF, *E. coli* β-galactosidase as well as the *Petunia*-specific pattern was obtained. Hybrid bands could not be detected.

(c) Isolated *Petunia* bands, denatured by urea, renatured and subjected to IEF showed the original pI values. These results make it unlikely that we were dealing with artefacts.

Preparative IEF and characterization of the multiple β-galactosidase activities

Several systems were tested for preparative IEF. The best results were obtained using preparative polyamide gels, pH 3.5–9.5 (LKB) and granulated gels, pH 5–7 (Serva). Separation on polyamide gels was somewhat sharper and the sandwich technique was easier to perform. Nevertheless, for routine work we used the much cheaper granulated gels and omitted the activity at pI 5.1, which could not be separated satisfactorily.

Elution and refocusing of the four remaining main activities showed bands with the same mobility. Only refocusing of closely adjacent zones gave the main zone and additionally portions of the neighbouring bands. Therefore, the separation on preparative granulated gels seems to be sharp enough to enable tests of individual zones.

The main zones at pI 6.5, 6.1, 5.9 and 5.65 were investigated for pH optima, temperature optima, and K_m values. All the activities showed the same pH optimum (4.3) and equally the same temperature optimum at 55° (Table 1). In their K_m values the first three activities were close together, the zone at 5.65 showed a somewhat higher K_m value. pH optima, temperature optima and K_m values are in the range known for other plant β-galactosidases

Table 1. Properties of the multiple β-galactosidas activities of *Petunia hybrida* 'Cyanidintyp'. The pH optimum and optimum temperature for each was 4.3 and 55°, respectively

Activity	pI	K_m value (mol/l.)
H1	6.5	1.05×10^{-3}
H2	6.1	1.33×10^{-3}
H3	5.9	0.95×10^{-3}
H4	5.65	1.82×10^{-4}
H1–4†	—	1.25×10^{-4}
Extract‡	—	0.83×10^{-3}

* Substrate ONPG, pH 4.3, 55°. Lineweaver–Burk [26].

† Mixture of the isolated activities (1:1:1:1, units).

‡ Enzyme preparation not subject to isoelectrofocusing (see Experimental).

(see [7]). In none of these species, however, could multiple activities be compared hitherto.

Using sucrose gradient centrifugation [14], the activities pI 6.5, 6.1, 5.9 and 5.65 showed the same behavior. Differentiation was not possible. The *S* values of the four activities, of a 1:1:1:1 mixture of the four activities, and of *Petunia* preparations not subject to IEF were invariably 8–10 compared with *E. coli* β-galactosidase added as marker (16 S, see [15]).

In animal systems, a β-galactosidase$_1$ could be activated and a β-galactosidase$_2$ inactivated by Cl^- [16, 17]. In *Petunia hybrida*, three activities (the activity 5.65 was not available) showed nearly the same sensitivity against NaCl: at 10 mM NaCl no influence, a slight stimulation at 25 mM NaCl, at 250 mM slight inhibition, and at 500 mM 60–70% inactivation. Furthermore, a separation of the multiple activities using gel filtration was not possible. Using Sephadex G 200, there was just one peak of β-galactosidase activity.

The inactivation kinetics in increasing concentrations of urea (12 molarities from 0 to 8 M were tested) were nearly the same with the four isolated activities, with a 1:1:1:1 mixture of them, and with an enzyme preparation before IEF. As with *E. coli* β-galactosidase [18], the activity of the isolated main bands, as well as of a preparation not subject to IEF, was completely lost in the presence of 8 M urea (60 min dialysis against 0.01 M PC containing 8 M urea, pH 4.3), and restored following dialysis against 0.01 M PC containing 1 mM $MgSO_4$ (16 hr dialysis, pH 4.3). This is consistent with the denaturation of the quaternary structures into their components, followed by renaturation. Due to the minute amounts of the single activities, however, we were unable to follow the suspected components during denaturation and renaturation and therefore have no proof of quaternary structures. Isotopic labelling would be helpful in clarifying this point.

Summarizing, we have to state that we were dealing with at least four β-galactosidase activities which showed identity or close relationship in all the properties tested, including preliminary data concerning their MW (one peak on Sephadex G 200, no differences in sucrose gradients detectable). However, they could be easily separated using IEF. A comparable situation found with mouse β-galactosidase could be explained by the existence of enzyme forms differing in charge but not in MW. They were derived from a common precursor protein by post-translational modifications [19, 20]. Further investigations are needed to show whether we were dealing with comparable enzyme forms in *Petunia*.

From the physiological point of view it seems necessary to take into consideration the existence of multiple β-galactosidase activities, whatever their physical state. As already mentioned, we detected multiple β-galactosidase activities in *Zea mays* as well. We have to add that this is a unique property of all plant species tested so far [13]. Therefore, in discussing the physiological function of β-galactosidase, for instance in cell enlargement [21, 22], one now has to look for the behaviour of single activities and activity patterns, and no longer for 'β-galactosidase' generally.

EXPERIMENTAL

Plant material. Petunia hybrida 'Cyanidintyp' [12].

Enzyme preparation. Extraction, acetone precipitation, and concn by ultrafiltration using the Amicon system, tests on β-galactosidase activity using ONPG as substrate, and protein determinations remained unchanged from the previous work [7]. The prepn obtained was used directly for enzyme tests, and, following a further concn using a Minicon B-15 cell, for IEF.

Analytical IEF. This was as described earlier [7], using Ampholine PAG plates pH 3.5–9.5 (LKB) and the LKB Multiphorese system according to the procedure described by the manufacturer. Following IEF, part of the gels was fractionated and eluted. pH gradients in the gels were determined either by using marker proteins (protein test mixture T8, Serva/Heidelberg) or by measuring the pH of eluates using a microelectrode. Part of the gels was stained for proteins (Coomassie Brilliant Blue G 250 [23]) or for enzyme activity. β-Galactosidase activity was detected using the incubation method described earlier [7] or a newly developed sandwich technique [24]. Filter paper was soaked with 0.2 M Pi–citrate buffer of the desired pH and dried. Thereafter it was imbibed with the substrate (for testing β-galactosidases with 100 mg BCIGal in DMF and rolled onto the surface of the gel. The resulting 'sandwich' was incubated for 1–2 hr at the temp. desired. Several substrates (BNGal, MUFGal, ONPG, BCIGal for β-galactosidases, and BNGluc, MUFGluc for β-glucosidases) were compared in the incubation and in the sandwich technique. Optimal resolution and staining for β-galactosidase was obtained using the sandwich technique and BCIGal as substrate.

Preparative IEF. Enzyme prepn concd in the Minicon (see above) was separated on Ampholine PAG plates (LKB) or on granulated gels (Pheragel containing 1.25% servalytes, Serva/Heidelberg) and eluted as described by LKB [25]. Enzyme tests and protein determinations in the eluates were carried out as described earlier [7]. For determination of pH gradients, see analytical IEF. Gel filtration on Sephadex G 200 was as described earlier [7], and sucrose gradient centrifugation according to ref. [14].

Inactivation by urea. The methods worked out for *E. coli* β-galactosidase [18, 27, 28] were adapted for *Petunia* β-galactosidase.

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