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A SIMPLE AND RAPID METHOD OF IDENTIFICATION OF EXTRACELLULAR PLANT GALACTOSIDASES

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A simple, rapid, and sensitive procedure for the identification of plant extracellular α- and β-galactosidase is described using callus cultures of seedlings from the tested plant, roots of 4-6 day old seedlings of Californian poppy germinating on agar plates, and cell suspension cultures cultivated from callus cultures. Synthetic substrates were used for the determination of the intra- and extracellular activities of α- and β-galactosidase. Many imino- or azasugars are strong glycosidase inhibitors, and some of them show promising chemotherapeutic effects against viral disease and are potential antidiabetic as well as antitumor agents. This fact aroused our interest for a rapid and sensitive assay method of the activity of α- and β-galactosidases of plant origin. The results presented here show the perspective usefulness of extracellular galactosidases of plant origin in inhibitory and/or biotechnological studies.

Key word: identification, determination, α- and β-galactosidase, biotransformation.

The study of natural inhibitors of glycosidases was started in 1993 by Chrzaszcz and Janicki [1] using several cereals. Glycosidases are enzymes that are involved in several important biological processes such as digestion, biosynthesis of glycoproteins, and catabolism of glycoconjugates. Glycosidase inhibitors have the potential to produce a number of beneficial therapeutic effects and are biotechnologically relevant [2]. They stimulate interest for the potential treatment of metabolic disorders [3] and lysosomal storage diseases [4]. Some inhibitors of glycosidases have been used also as antitumour agents [5], antiobesity drugs, fungistatic compounds [6], antivirals [7], and immune modulators [8].

Inhibitory studies of glycosidases were done chiefly by using glucosidases, galactosidases, mannosidases, trehalases, and rhamnosidases. The chemotherapeutic value of glycosidase inhibitors was recently discussed in a series of papers by El Ashry [9-11].

α-Galactosidase (α-D-galactoside galactohydrolase EC.3.2.1.22) and β-galactosidase (β-D-galactoside galactohydrolase EC.3.2.1.23) lactase catalyze the hydrolysis of terminal α- or β-galactose, respectively. The enzymes are widely distributed in various plant tissues. It has been suggested that they are involved in the mobilization of reserve sugars, cell growth, fruit ripening, and seed germination [12]. These enzymes play an important role in many fields of basic and applied research [13]. To enable a broader study of the application possibility of glycosidases of plant origin for the evaluation of therapeutically effectically effective substances, we present a convenient method for the determination of the activities of α- and β-galactosidase. The aim of this work was to show that the synthetic substrates 6-bromo-2-naphthyl-α-D-galactopyranoside, 6-bromo-2-naphthyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, and *p*-nitrophenyl-β-D-galactopyranoside can be used in a simple and rapid method for the detection and determination of extra- and intracellular plant galactosidases.

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TABLE 1. Galactosidase Activities in Cell Culture Medium of Californian Poppy after 6 Days of Cultivation

Fraction	Volume (ml)	Protein (mg/g f.w.)	Activity (nkat/g f.w.)		Specific activity (nkat/mg protein)	
			α	β	α	β
Homogenate of isolated cells	8	1.68±0.42	38.93±2.8	88.26±4.6	23.17	52.53
Culture medium without cells*	4.3	0.40±0.36	16.28±2.6	138.21±5.3	40.70	345.52

The synthetic substrates *p*-nitrophenyl-α-D-galactopyranoside, 6-bromo-2-naphthyl-α-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, and 6-bromo-2-naphthyl-β-D-galactopyranoside used in this study proved to be suitable for studying the intracellular and extracellular activity of α- and β-galactosidase.

Culture media (agar plates with and without substrates 6-bromo-2-naphthyl-α-D-galactopyranoside and 6-bromo-2-naphthyl-β-D-galactopyranoside) and Fast Blue BB [24-26] were inoculated with cells from growing callus cultures and then cultivated for 1-2 h. The activity of extracellular α- and β-galactosidase was detected by the presence of stained violetred (azo-dye) zones beneath and around the areas of the cells on the agar plates. Azo-dye was formed by simultaneous azo coupling of 6-bromo-2-naphthol and Fast Blue BB.

Extracellular α- and β-galactosidase were considered to be present also in cases where on the agar plates after 20-60 min violet-red staining appared in zones around the root tips and hairy roots of 2-4 days old seedlings of cucumber, poppy, Californian poppy, pea, tomano, and *Amsonia tabernaemontana* Walt. on the agar plates (unpublished results).

No coloration of the agar medium or plant materials after inoculation with heatinactivated calluses (100°C, 10 min) was observed.

Homogenates of the cell suspension culture medium alone after 6 days of cultivation were used for assaying the the activity of intracellular and extracellular α- and β-galactosidase. In both cases *p*-nitrophenyl-α-*D*-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside were used as substrates. The distribution of the intra- and extracellular enzyme activity is shown in Table 1. The data show a 70.5% and 39.0% intracellular and a 29.5% and 61.0% extracellular distribution of α- and β-galactosidase activity, respectively, the extracellular specific enzyme activity (α- and β-galactosidase) being 1.75 and 6.57 times higher, respectively.

It is now well recognized that glycosidase inhibitors such as many mono- and bicyclic polyhydroxylated pyrrolides, piperides, and azepines (referred to as iminosugars or azasugars) are promising as antiviral, anticancer, and antidiabetic agents. Some of these inhibitors have already been put on the marked for treatment of diabetes. The mechanisms of these effects have been studied extensively. Some of these compounds are naturally occurring and, owing to the pronounced biological activity of this class of compounds, various synthetic routes have been designed for the synthesis of many of them [9–11].

The production of extracellular α- and β-galactosidase as well as proteolytic enzymes [14] that are released from plant cells might be of importance for biotechnological application in the food and pharmaceutical industry and analyses [15, 16]. These enzymes as well as α- and β-galactosidase and invertase are generally present in plants; they have not been used in biotechnological processes until now [13, 17, 18]. The relatively high activity of the extracellular β-galactosidase points to prospects of applying this enzyme, first of all, to the testing of its inhibitors as well as in the biotransformation of lactose.

Due to the simplicity and reproducibility of the method presented, it could be useful for the detection of plant producers of extracellular α- and β-galactosidase.

EXPERIMENTAL

Plant Material. Long-term callus cultures and cell suspensions were derived from Californian poppy seedlings (*Eschscholtzia californica* Cham.) as was previously described [19].

Identification of Extracellular Ezyme Activity. 6-Bromo-2-naphthyl-α-*D*-galactopyranoside and 6-bromo-2-naphthyl- β-D-galactopyranoside were used for the identification of extracellular α-galactosidase or β-galactosidase. α-Galactosidase or β-galactosidase hydrolyzed the substrate and 6-bromo-2-naphthol was released. By the coupling of 6-bromo-2-naphthol with Fast Blue BB (4-amino-2,5-diethoxybenzanilide) an azodye was formed. A modified method for histochemical studies given by [20, 21] was used.

Ten milligrams of 6-bromo-2-naphthyl-α-D-galactopyranoside or 6-bromo-2-naphthyl-β-D-galactopyranoside was dissolved in 0.5 ml dimethylformamide, and 10 ml of buffered Fast Blue BB (10 mg) solution was added (10 ml McIlvaine buffer), pH 5.0.

Ten milliliters of 1% agar in McIlvaine buffer, pH 5.0, was added to the above mixture and it was autoclaved in the usual way [22].

Determination of Intracellular and Extracellular α- and β-Galactosidase Activities. Enzyme Preparation. Cell suspension cultures were used to determine the intracellulare α- and β-galactosidase activity. The cells (10 g) were filtered off and washed with 3.000 ml of distilled water. Soluble proteins were extracted by grinding the cells in a precooled mortar using a ratio of 1:1 (g/ml) of cells and McIlvaine buffer of pH 5.4 (for α-galactosidase) and 4.8 (for β-galactosidase) at 4°C. The homogenate was squeezed through two layers of nylon cloth and centrifuged at 150.000 $m.s^{-2}$ for 15 min at 4°C.

Enzyme Assay. The enzyme assay was performed by the modified method of Simons et al. (1989) using *p*-nitrophenyl-α-D-galactopyranoside (α-PNG) and *p*-nitrophenyl-β-D-galactopyranoside (β- PNG) as the substrate. The reaction mixture contained suitable amounts of enzyme preparation and 0.5 mg α-PNG and β-PNG in 2 ml McIlvaine buffer (pH 5.3 and 4.8, resp.). The control contained boiled (100°C) enzyme preparation. The reaction mixture was kept for 20 min at 30°C and the reaction was stopped by adding 2 ml of 2 $mol.liter^{-1}$ Na_2CO_3. The released *p*-nitrophenol was determined by measuring the absorbance at 420 nm against the control. The enzyme activity was expressed in katals.Protein content was determined by the method of Bradford [23] using bovine serum albumin as the standard.

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