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A simple method for the identification and assay of extracellular plant β-galactosidase

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Dedicated to Professor Jozef Tomko and Associated Professor Jaroslav Kresánek, Bratislava, on the occasion of their 80th birthdays

A simple, rapid and reproducible procedure for the identification of extracellular Californian poppy (*Eschscholzia californica* Cham.) β-galactosidase is described using callus cultures of seedlings from the tested plant, roots of 4-days-old seedlings of Californian poppy germinating on agar plates and cell suspension cultures cultivated from callus cultures. 6-Bromo-2-naphthyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside were used as substrates for the determination of the intracellular and extracellular activities of β-galactosidase.
The extracellular β-galactosidase activity was identified by evaluating the dye-zones in an agar medium. The enzyme from Californian poppy callus cultures or from seedling roots cultivated on agar plates supplemented with 6-bromo-2-naphthyl-galactopyranoside hydrolyzed this substrate releasing 6-bromo-2-naphthol. By simultaneous coupling with hexazonium *p*-rosaniline the corresponding (reddish-brown) azo-dye was formed. The agar plate method described permits rapid, simple and specific detection of plant producers of extracellular β-galactosidase.

1. Introduction

In the last years several methods for determination of the activity of β-galactosidase have been developed. Naturally occurring or synthetic substrates may be used for these purposes [1–3].
β-Galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23) catalyses the hydrolysis of the terminal β-galactose in glycosides. The enzyme is widely distributed in plant tissues, but its precise role is not well understood. It has been suggested that this enzyme is involved in the degradation of plant cell-wall polysaccharides in relation to cell growth, fruit ripening and seed and pollen germination [1, 4–7]. β-Galactosidase hydrolyses lactose into glucose and galactose and has recently aroused considerable interest because of its application in food industry, nutrition and medicine [3, 8–10]. Therefore simple and rapid screening methods for the detection of β-galactosidase activity are of great importance both for scientific and production purposes. Naturally occurring substrates such as lactose (as well as synthetic substrates) may be used for determination of the activity of the enzyme under study [2, 8, 9]. For this purpose, the synthetic substrates *p*-nitrophenyl-β-D-galactopyranoside and also 6-bromo-2-naphthyl-β-D-galactopyranoside, which have been used for the biochemical localization and determination of β-galactosidase, are advantageous [2, 3, 10].
The aim of this work was to show that the synthetic substrate 6-bromo-2-naphthyl-β-D-galactopyranoside can be used in a simple and rapid method for the detection of extracellular plant β-galactosidase.

2. Investigations, results and discussion

p-Nitrophenyl-β-D-galactopyranoside and 6-bromo-2-naphthyl-β-D-galactopyranoside were used in this study to determine the intracellular activities of β-galactosidase.
Culture media (agar plates with and without the substrate 6-bromo-2-naphthyl-β-D-galactopyranoside and hexazonium *p*-rosaniline [10–13]) were incubated with cells from growing callus cultures and then cultivated for 0.5–2 h. The activity of extracellular β-galactosidase was detected by the appearance of stained reddish-brown zones beneath and around the areas of the cells on the agar plates. Extracellular β-glactosidase was also to be present in cases when reddish-brown staining occurred after 20–60 min in zones around the root tips of 2–4 day-old seedlings of pea, Californian poppy or *Amsonia tabernaemontana* Walt., respectively on the agar plates. No coloration of the agar medium or plant materials was observed after inoculation with heat inactivated callus (100 °C, 10 min).
Homogenised cell suspension cultures and culture medium alone after 14 days cultivation were used for assaying the activity of intracellular and extracellular β-galactosidase, respectively. In both cases *p*-nitrophenyl-β-D-galactopyranoside was used as a substrate. The distribution of intra- and extracellular enzyme activity is shown in they Table. The data indicated β-galactosidase activity distributed 38.2% intracelluar und 61.8% extracellular, the extracellular specific enzyme activity being 6.5 times higher.
Lactose is poorly soluble in water, insufficiently sweet and may also have a mild laxative effect when consumed in

Table: β-Galactosidase activity in cell culture and culture medium of Californian poppy

Fraction	Volume (ml)	Protein (mg/g fresh weight)	Activity (nkat/g fresh weight)	Specific Activity (nkat/mg protein)
Intracellular activity (Homogenate of isolated cells)	10	1.72	86.32	50.18
Extracellular activity (Culture medium without cells)*	5	0.43	139.64	324.74

* Corresponding to the amount of isolated cells

large quantities. Cows milk contains more than 4.8% of this sugar. Hence, the production of low-lactose milk may be of great economic value. Another related industrial problem concerns whey utilization. The enzymatic hydrolysis of lactose by β-galactosidase to glucose and galactose constitutes a potential route for decreasing its intolerance for human beings. Several microbial sources of β-galactosidase have been used for this purpose, but new sources of these enzyme are still of great value [8, 9].

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) have potential 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 [3, 21–23].

The production of extracellular α- and β-galactosidases as well as proteolytic enzymes [14, 24] which are released from plant cells, might be of some importance for biotechnological applications in the food and pharmaceutical research and industry [3, 14–17, 21–23, 25, 26]. These enzymes as well as α-galactosidase and invertase [1–3, 11, 18–20, 24] are generally present in plants. Until now they have not been used in biotechnological processes [14–20, 26].

Due to its simplicity and reproducibility the method presented here could be useful for the detection of galactosidase in plants and their enzymatic improvement.

3. Experimental

3.1. Plant material

Long term callus cultures were derived from seedlings of *Eschscholzia californica* Cham. by Dr. V. Blanáriková (Department of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Comenius University, Bratislava) and were cultivated as described previously [27].

Seedlings of Californian poppy, pea and *Amsonia tabernaemontana* Walt. were cultivated from sterilized seeds under aseptic conditions according to Dixon [28].

3.2. Identification of extracellular enzyme activity

6-Bromo-2-naphthyl-β-D-galactopyranoside was used for the identification of extracellular β-galactosidase. β-Galactosidase hydrolyzed the substrate releasing 6-bromo-2-naphthol. By coupling 6-bromo-2-naphthol with hexazonium *p*-rosaniline, the corresponding azo dye was formed. A modified method for its biochemical study [11–13] was used.

6-Bromo-2-naphthyl-β-D-galactopyranoside (10 mg) was dissolved in 0.5 ml dimethylformamide and 10 ml of buffered hexazonium *p*-rosaniline solution were added (9.4 ml McIlvaine buffer pH 6.0 and 0.6 ml hexazonium *p*-rosaniline solution, final pH 5.0 adjusted with 0.1 N NaOH). 10 ml of 2% agar in McIlvaine buffer (pH 5.0) were added to the above mixture and autoclaved in the usual way [2, 29]. Agar plates were then inoculated with cells from growing callus cultures or 2–4 day old seedlings of pea, Californian poppy or *Amsonia tabernaemontana* Walt. respectively and were cultivated 20–120 min.

Hexazonium *p*-rosaniline was prepared according to Lojda et al. [12].

Solution A: 400 mg of *p*-rosaniline were dissolved in 8 ml of distilled H_2O and 2 ml of conc. HCl were added. Solution B: 4% sodium nitrate. Solution A and B were mixed in equal volumes.

3.3. Determination of intracellular and extracellular activity of β-galactosidase

3.3.1. Enzyme preparation

Cell suspension cultures were used to determine the intracellular activity of β-galactosidase. The cells (10 g) were filtered off and washed with 3 l of distilled H_2O. Soluble proteins were extracted by grinding the cells in a precooled mortar using a 1 : 1 (g/ml) ratio of cells and McIlvaine buffer of 4.9 at 4 °C. The homogenate was squeezed through two layers of nylon cloth and centrifuged at 15 000 × g for 15 min at 4 °C.

3.3.2. Enzyme assay

The enzyme assay was performed by a modified method [19, 30] using *p*-nitrophenyl-β-D-galactopyranoside (PNG) as the substrate. The reaction mixture contained a suitable amount of enzyme (0.1–0.3 ml) and 3 mM of PNG in 2 ml of McIlvaine buffer (pH 4.0). The control contained a boiled (100 °C) enzyme preparation. Both mixtures were kept for a period of 20 min at 30 °C and the reaction was stopped by adding 2 ml of 1 M Na_2CO_3. The *p*-nitrophenol released 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 [31] using bovine serum albumin as the standard.

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