Demonstration of lactase activity in culture medium of melon cells

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ABSTRACT: Lactase activity was detected in a culture medium of the cell suspension culture of watermelon (*Citrullus vulgaris* L.). A simple, rapid and reproducible procedure for identification of extracellular lactase is described using callus cultures of seedlings from the tested plant, hairy roots of 2.5 days old seedlings of watermelon germinating on agar plates as well as cell suspension cultures derived from callus cultures. For the determination of intracellular activities of lactase, 6-bromo-2-naphthyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside were used as synthetic substrates. The extracellular lactase activity was determined by evaluating the day-zone in agar medium. The enzyme from watermelon callus cultures and seedling roots, cultivated on agar plates supplemented with 6-bromo-2-naphthyl-2-bromo-β-D-galactopyranoside, hydrolyzed this substrate releasing 6-bromo-naphthyl. By simultaneous coupling with hexazonium *p*-rosaniline or Fast Blue BB the corresponding azo dye was formed. The parallel extracellular and intracellular activities were determined in cell suspension cultures derived from callus cultures. The results show a 43.8% intracellular and 54.2% extracellular distribution of lactase activity. The described agar plate method enables a rapid, simple and specific detection of plant processes of extracellular lactase.

Keywords: intracellular lactase; extracellular lactase; watermelon

Plant glycosidases that catalyse the hydrolysis of aryl and alkyl glycosides are involved in a wide variety of biological processes. In the last decades, several methods for determining the activity of α-galactosidase have been developed. Various naturally occurring or synthetic substrates may be used for these purposes (SIMONS et al. 1989; CHEN et al. 1993; MACHOVÁ 1994).

Lactase (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 (SINGH, KNOX 1985; BUDÍK 1992; DE VEAU et al. 1993; BILISICS et al. 1994; SAWICKA, KACPERSKA 1995). Lactase hydrolyses lactose into glucose and galactose and has recently aroused considerable interest because of its application in food industry, nutrition and medicine (GOSSRAU 1991; ROGALSKI, LOBARZEWSKI 1995; SZCZODRAC 1999; ASANO et al. 2000). Therefore simple and rapid screening methods for the detection of lactase 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 to determine the activity of the enzyme under study (SIMONS et al. 1989; ROGALSKI, LOBARZEWSKI 1995; SZCZODRAC 1999). 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 lactase, are advantageous (BUDÍK 1992; GOSSRAU 1991; ASANO et al. 2000).

The aim of this paper is 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 lactase.

MATERIALS AND METHODS

Tissue cultures

Long-term tissue cultures and cell suspension were derived from seedlings of watermelon *Citrullus vulgaris* cv. Samara as previously described by TILEMANN et al. (2003).

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 (LOJDA et al. 1979; STOWARD, PEARSE 1991; MACHOVÁ 1994) was used.

6-Bromo-2-naphthyl-β-D-galactopyranoside (10 mg) was dissolved in 0.5 ml of dimethylformamide and 10 ml of McIlvaine buffer containing Fast Blue BB (10 mg) pH 5.1 or 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 a usual way (BUDÍK 1992; DIXON 1991). Agar plates were then inoculated with cells from growing callus cultures or 2–4 day old seedlings of watermelon and were cultivated for 20–120 min.

Hexazonium *p*-rosaniline was prepared according to LOJDA et al. (1979).

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

Determination of intracellular and extracellular activity of lactase

Enzyme preparation

Cell suspension cultures were used to determine the intracellular lactase 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 at 4°C. The homogenate was squeezed through two layers of a nylon cloth and centrifuged at 15,000 g for 15 min at 4°C.

For the determination of the extracellular enzyme activity, the cultivation medium (without agar) was centrifuged at 15,000 g 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 (PNG) as the substrate. The reaction mixture contained suitable amounts of enzyme preparation and 0.5 mg PNG in 2 ml of McIlvaine buffer (pH 5.4). 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 M 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 (1976) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Synthetic substrates *p*-nitrophenyl-β-D-galactopyranoside and 6-bromo-2-naphthyl-β-D-galactopyranoside were used in this study to determine the intracellular activities of lactase. Culture media (agar plates with and without the substrate 6-bromo-2-naphthyl-β-D-galactopyranoside and hexazonium *p*-rosaniline or Fast Blue BB (LOJDA et al. 1979; GOSSRAU 1991; STOWARD, PEARSE 1991; MACHOVÁ 1994) were incubated with cells from growing callus cultures and then cultivated for 0.5–2 h. The activity of extracellular lactase was detected by the appearance of stained reddish-brown zones beneath and around the areas of the cells on the agar plates. Extracellular lactase was also to be present when reddish-brown or reddish-violet zone staining occurred after 20–60 min in zones around the root tips of 2.5 day-old seedlings of watermelon 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).

Extracellular lactase was also considered to be present when reddish-brown or reddish-violet staining occurred after 20–60 min in zones around the root tips and hairy roots of 2.5-day-old seedlings of watermelon on agar plates.

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

Homogenized cell suspension cultures and culture medium alone after 10 days cultivation were used for assaying the activity of intracellular and extracellular lactase, 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 Table 1.

The data indicated lactase activity distributed as 43.8% intracellular and 54.2% extracellular, the extracellular specific enzyme activity being 7.6 times higher.

The seeds of watermelon were germinated in distilled water. Lactase activity in the culture medium of cell

Table 1. The lactase activity in cell culture and culture medium of watermelon

Fraction	Volume (ml)	Protein (mg/g fresh weight)	Activity (nkat/g fresh weight)	Specific activity (nkat/mg protein)
Intracellular activity (homogenate of isolated cells)	5.0	2.90	98.4	33.9
Extracellular activity (culture medium without cells)*	4.5	0.49	126.3	257.8

*Corresponding to the amount of isolated cells

suspension was very high, but in water there was only a minute concentration of the studied enzyme.

Lactose is poorly soluble in water, insufficiently sweet and may also have a mild laxative effect when consumed in large quantities. Cow's 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 lactase to glucose and galactose constitutes a potential route for decreasing its intolerance for human beings. Several microbial sources of lactase have been used for this purpose, but new sources of this enzyme are still of great value (ROGALSKI, LOBARZEWSKI 1995; SZCZODRAC 1999).

It is now well recognized that galactosidase inhibitors such as many mono- and bicyclic polyhydroxylated pyrrolides, piperides and azepines (referred to as iminosugars or azasugars) have the potential as antiviral, anticancer and antidiabetic agents. Some of these inhibitors have already been put on the market for treatment of diabetes. The mechanism of these effects has 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 (ASANO et al. 2000; EL ASHRY et al. 2000a,b,c).

Lactase as well as proteolytic enzymes (STANO et al. 1997/1998; ANDRIAMAINTY et al. 2000; MERTOVÁ et al. 2002) of plant origin can be applied in future in biotransformation processes of food as well as in the pharmaceutically important compounds; their application in the structure of these studied compounds is another possible field of their practical use (PAEK et al. 1998; MULINAMI, DEVENDRA 1999; WATSON et al. 2001; MATSUURA et al. 2004).

The production of extracellular lactase as well as proteolytic enzymes (STANO et al. 1997/1998; ANDRIAMAINTY et al. 2000; MERTOVÁ et al. 2002) which are released from plant cells might be of some importance for biotechnological applications in the food and pharmaceutical research and industry (PAEK et al. 1998; MULINAMI, DEVENDRA 1999; ASANO et al. 2000; EL ASHRY 2000a,b,c; WATSON et al. 2001; MATSUURA et al. 2004). These enzymes as well as lactase and invertase (SIMONS et al. 1989; MACHOVÁ 1994; BARTH et al. 2002; NEUBERT et al. 2002) are generally present in plants. Until now they have not been used in biotechnological processes (STANO et al. 1997/1998; PAEK et al. 1998; MIČIETA et al. 1999; MULINAMI, DEVENDRA 1999; BILKA et al. 2002).

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

References

ANDRIAMAINTY F., STANO J., MIČIETA K., BARTH A., BARTHOVÁ H., ČIŽMÁRIK J., KOREŇOVÁ M., 2000. Identification and determination of plant extracellular α-galactosidase. Hort. Sci., *27*: 131–134.

ASANO N., NASH R., MOLYNEUX R., FLEET G.W.J., 2000. Sugar mimic glycosidase inhibitors: natural occurrence, biological activity and prospects for therapeutic application. Tetrahedron Asymmetry, *11*: 1645–1680.

BARTH A., JANINA M.M., NEUBERT K., MIČIETA K., TILEMANN I., STANO J., BILISICS L., 2002. Sucrose biotransformation by carrot immobilized cells. Acta Facult. Pharm. Univ. Comenianae, *49*: 17–25.

BILISICS L., LIŠKOVÁ D., KUBAČKOVÁ M., AUXTOVÁ O., KÁKONIOVÁ D., 1994. On the possible participation of UDP-D-glucose 4-epimerase and some NADP-dependent dehydrogenases in spruce tissue organization. Biologia, *49*: 911–915.

BILKA F., KĽÚČOVSKÁ J., BILKOVÁ A., BENEŠOVÁ M., 2002. Aminopeptidases in opium poppy latex. Biologia, *57*: 761–764.

BRADFORD M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem., *72*: 248–254.

BUDÍK D., 1992. α-Galactosidase in poppy cell suspension (*Papaver somniferum* L.). [Diploma Thesis.] Bratislava, Comenius University, Faculty of Pharmacy: 21–38.

CHEN CH.S., CHEN Y.F., YEN A., 1993. Activities and some properties of glycosidases from germinating mung bean. J. Chin. Biochem. Soc., *22*: 1–8.

DE VEAU E.L., GROSS K.C., HUBER D.J., WATADA A.E., 1993. Degradation of pectin by β-galactosidases purified from avocado mesocarp. Physiol. Plant., *87*: 279–285.

DIXON R.A., 1991. Isolation and maintenance of callus and cell suspension cultures. In: DIXON R.A. (ed.), Plant Cell Culture. A Practical Approach. Oxford, Washington, IRL Press: 1–20.

EL ASHRY E.S., RASHED N., SHOBIER A.H.S., 2000a. Glycosidase inhibitors and their chemotherapeutic value, part 1. Pharmazie, *55*: 251–262.

EL ASHRY E.S., RASHED N., SHOBIER A.H.S., 2000b. Glycosidase inhibitors and their chemotherapeutic value, part 2. Pharmazie, *55*: 331–348.

EL ASHRY E.S., RASHED N., SHOBIER A.H.S., 2000c. Glycosidase inhibitors and their chemotherapeutic value, part 3. Pharmazie, *55*: 403–415.

GOSSRAU R., 1991. Catalytic histochemistry of acid and neutral hydrolases in plant seedlings. Histochem. J., *23*: 483–489.

LOJDA Z., GOSSRAU R., SCHIEBLER T.H., 1979. Enzyme Histochemistry. A Laboratory Manual. Berlin, Heidelberg, New York, Springer Verlag: 122–128.

MACHOVÁ B., 1994. Study of some properties of α-galactosidase in gherkin seedlings (*Cucumis sativus* L.). [Diploma Thesis.] Bratislava, Comenius University, Faculty of Pharmacy: 35–57.

MATSUURA H., MIYAZAKI H., ASAKAWA CH., AMANO M., YOSHIHARA T., MIZUTANI J., 2004. Isolation of α-glucosidase inhibitors from hyssop (*Hyssopus officinalis*). Phytochemistry, *65*: 91–97.

MERTOVÁ J., ALMÁSIOVÁ M., PEREČKO D., BILKA F., BENEŠOVÁ M., BEZÁKOVÁ L., PŠENÁK M., KUTEJO-

VÁ E., 2002. ATP-dependent Lon protease from maize mitochondria-comparison with the other lon proteases. Biologia, *57*: 739–745.

MIČIETA K., STANO J., BLANÁRIKOVÁ V., HAVRÁNEK E., ANDRIAMAINTY F., BIRJUKOVA N., IGNATOVA S., ŠAFAŘÍK I., 1999. Activity of invertase in immobilized cells of carrot. Bull. Food Res., *38*: 153–161.

MULINAMI V.H., DEVENDRA S., 1999. Effect of soaking cooking and crude α-galactosidase treatment on the oligosaccharide content of red gram flour. Food Chem., *61*: 475–479.

NEUBERT K., STANO J., MIČIETA K., KOVÁCS P., TINTEMANN H., 2002. Invertase in immobilized cells of *Eschscholtzia californica*. Biol. Plant., *45*: 307–310.

PAEK N.S., KANAG O.L., LEE H.S., LEE J.J., CHOI J.J., KIM T.M., KIM J.J., 1998. Enzymatic synthesis of 6-O-α-galactopyranosyl-1-deoxynojirimycin using α-galactosidase from green coffee beans. Biosci. Biotechnol. Biochem., *62*: 588–598.

ROGALSKI J., LOBARZEWSKI J., 1995. The purification and immobilization of *Penicillium notatum* α -galactosidase. Acta Biotechnol., *15*: 211–222.

SAWICKA T., KACPERSKA A., 1995. Soluble and cell wall-associated β-galactosidases from cold-grown winter rape (*Brassica napus* L. var. *oleifera* L.). J. Plant. Physiol., *145*: 357–362.

SIMONS G., GIANNAKOUROS T., GEORGATSOS J.G., 1989. Plant β-galactosidases: Purification by affinity chromatography and properties. Phytochemistry, *28*: 2587–2592.

SINGH M.B., KNOX R.B., 1985. β-Galactosidases of *Lilium pollen*. Phytochemistry, *24*: 1639–1643.

STANO J., KOVÁCS P., ŠAFAŘÍK I., KÁKONIOVÁ D., ŠAFAŘÍKOVÁ M., 1997/1998. A simple procedure for the detection of plant extracellular proteolytic enzymes. Biol. Plant., *40*: 475–477.

STOWARD P.J., PEARSE A.G.E., 1991. Theoretical and Applied. Vol. 3. Enzyme Histochemistry. Edinburgh, London, Melbourne, New York, Tokyo, Churchill Livingstone: 624–627.

SZCZODRAC J., 1999. Hydrolysis of lactose in whey permeate by immobilized β-galactosidase from *Penicillium notatum*. Acta Biotechnol., *19*: 235–250.

TILEMANN I., TOKHTAEVA E., SEDLÁROVÁ E., BARTH A., VALENT A., SIEKEL P., ĎURÍČEK M., 2003. Lactase in immobilized cells of watermelon. Chem. Nat. Comp., *39*: 394–398.

WATSON A.A., FLEET G.W.J., ASANO N., MOLYNEUX R.J., NASH R.J., 2001. Polyhydroxylated alkaloids-natural occurence and therapeutic applications. Phytochemistry, *56*: 265–295.

Received for publication June 17, 2004
Accepted after corrections September 29, 2004

Dôkaz aktivity laktázy v kultivačnom médiu bunkami dyne červenej

ABSTRAKT: Prezentujeme jednoduchú, rýchlu a reprodukovateľnú metódu identifikácie extracelulárnej laktázy v kultivačnom médiu suspenznej kultúry, odvodenej z kalusu klíčnych rastlín dyne červenej a tiež v 60 hodín starých koreňových vláskoch testovaných rastlín. Pri stanovení intracelulárnej a extracelulárnej aktivity laktázy sme použili syntetické substráty: 6-bróm-2--naftyl-β-D-galaktopyranozid a *p*-nitrofenyl-β-D-galaktopyranozid resp. Extracelulárna aktivita laktázy sa vyhodnotila pomocou farebnej zóny v agarovom médiu. Enzým z kalusových kultúr a korienkov dyne červenej kultivovanej na agarovej platni s 6-bróm-2-naftyl-β-D-galaktopyranozidom hydrolyzuje tento substrát a uvoľňuje 6-bromo-2-naftol. Simultánnou azokopuláciou s hexazotovaným *p*-rozanilínom alebo Fast Blue BB resp. sa vytvára korešpondujúce azofarbivo. Intracelulárne a extracelulárne aktivity sa stanovili paralelne v suspenzných kultúrach odvodených z kalusových kultúr. Výsledky distribúcie ukázali 43,8 % intracelulárnej a 54,2 % extracelulárnej aktivity laktázy. Popísaná metóda na platni agaru umožňuje rýchlu, jednoduchú a špecifickú metódu detekcie extracelulárnej laktázy.

Kľúčové slová: intracelulárna laktáza; extracelulárna laktáza; dyňa červená

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