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BRIEF COMMUNICATION

A simple procedure for the detection of plant extracellular proteolytic enzymes

J. STANO, P. KOVÁCS*, I. ŠAFAŘÍK**, D. KÁKONIOVÁ***
and M. ŠAFAŘÍKOVÁ**

Garden of Medical Plants, Faculty of Pharmacy, Comenius University, Odbojárov 10, 832 32 Bratislava, Slovak Republic
*Department of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Comenius University, Odbojárov 10, 832 32 Bratislava, Slovak Republic**
*Laboratory of Biochemistry and Biotechnology, Institute of Landscape Ecology, Academy of Sciences of the Czech Republic, Na sádkách 7, 370 05 České Budějovice, Czech Republic***
*Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 842 38 Bratislava, Slovak Republic****

Abstract

A simple procedure for the detection of extracellular plant proteolytic enzymes using insoluble dye stained gelatin substrates incorporated into an appropriate culture medium is described. Extracellular proteinases produced by the tested plant cells (callus culture and cell suspension) hydrolyzed the substrates and dyed peptide fragments were released. Dyed zones around and under the proteinase producing callus cultures were formed on the agar medium. Similarly, coloration of the culture media using proteinase-producing cell suspensions was observed.

Additional key words: extracellular proteolytic activity, insoluble chromolytic substrate, callus cultures.

Spectrophotometric methods are very often used for the determination of proteolytic activity. Naturally occurring insoluble proteins such as collagen, keratin, elastin or fibrin, or soluble proteins, rendered insoluble by cross-linking with a suitable bifunctional reagent or by entrapment into an appropriate polymer matrix, may be used for the determination of proteolytic activity after labelling with a suitable dye or fluorescent marker (Wunderwald 1984, Šafařík 1988, Šafařík 1989).

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Corresponding author: J. Stano, fax: (+421) 7 5262065; e-mail: kbm@fpharm.uniba.sk

A number of screening procedures has been developed for the detection of extracellular proteolytic activity of microorganisms. Usually the casein agar (Frazier and Rupp 1928), gelatin agar (Whaley *et al.* 1982), elastin agar (Rust *et al.* 1994) or fibrin agar (Šafařík *et al.* 1983) are used. The contrast between unhydrolyzed and hydrolyzed part of the substrate in the agar plate is usually low and the detection of protease production is sometimes difficult. In some cases overlaying the agar plate with a suitable precipitating agent, such as trichloroacetic acid or saturated ammonium sulphate solution, is necessary to improve the resolution. This treatment, of course, causes the damage of the tested biological material.

To overcome these problems, new, insoluble chromolytic proteinaceous substrates have been developed and used. Many of them, *e.g.*, dye-stained gelatin microcarrier particles (Šafařík 1990) can be incorporated into nutrient agar or culture medium and serve as dyed hydrolyzable substrate (Šafařík and Šafaříková 1994). Extracellular proteinases hydrolyze the insoluble substrate and clearly visible coloured zones around and under the microbial colonies are formed on the agar plates.

In our work we have shown that insoluble chromolytic gelatin-based substrates can also be used for simple and rapid detection of extracellular plant proteinases. Dye-stained gelatin porous microcarriers (substrate I; Šafařík 1990) and another gelatin-based substrate (substrate II; Šafařík and Šafaříková, unpublished) have been used. The latter substrate has been prepared similarly as dye-stained gelatin porous microcarriers, but fine non-porous particles prepared from glutaraldehyde cross-linked gelatin have been used for the dye binding.

Long term callus culture was derived from seedlings of *Cucumis sativus* L. cv. Znojmia and was cultivated as described previously (Stano *et al.* 1995). The insoluble chromolytic substrate (concentration 0.5 % for substrate I or 1 % for substrate II; m/v) was added to the culture medium (with or without agar) in the Erlenmayer flasks and autoclaved in the usual way. Sterile culture medium with agar was cooled to 50 °C and poured into the Petri dishes. Homogeneous distribution of the insoluble substrate should be observed. Both culture media (with and without agar, respectively) were inoculated with tested gherkin cells from growing cultures and cultured for 1 - 8 d. On agar plates the plant extracellular proteolytic activity was detected by the presence of dyed zones under and around the areas of growth; the degree of extracellular proteolytic activity can be roughly assessed by dyed zones diameters. In liquid culture media the dyed gelatin fragments released by the hydrolysis of the substrates cumulate in the medium causing its coloration; the proteolytic activity can be assessed by the colour intensity of liquid culture medium (after subtracting the blank value). The absorbance of the filtrates was measured at 595 nm in glass 1-cm cuvettes.

The insoluble chromolytic substrates used in this study were easily hydrolyzed by various proteases such as trypsin, chymotrypsin, papain, bromelain and various bacterial proteinases. The substrates spontaneously release only small amounts of dye; no coloration of agar medium was observed on uninoculated parts. For studies in liquid media blank value has to be subtracted to obtain correct values of absorbances. Due to the simplicity and reproducibility of the described procedure this method can

be successfully used in plant biochemistry research for the detection of plant producers of extracellular proteolytic enzymes.

References

Frazier, W.C., Rupp, P.: Studies on the proteolytic bacteria of milk. I. A medium for the direct isolation of caseolytic milk bacteria. - J. Bacteriol. **16**: 57-63, 1928.

Rust, L., Messing, C.R., Iglewski, B.H.: Elastase assays. - Meth. Enzymol. **235**: 554-62, 1994.

Stano, J., Nemec, P., Kákoniová, D., Kovács, P., Neubert, K., Lišková, D.: Alpha-galactosidase in immobilized cells of *Cucumis sativus* L. - Biologia **50**: 279-281, 1995.

Šafařík, I.: An insoluble chromolytic substrate for the determination of proteolytic activity. - J. Biochem. biophys. Meth. **17**: 193-198, 1988.

Šafařík, I.: Spectrophotometric determination of proteolytic activity in coloured solutions. - J. Biochem. biophys. Meth. **19**: 201-206, 1989.

Šafařík, I.: Dye-stained gelatin microcarriers as insoluble chromolytic substrates for the determination of proteolytic activity. - Clin. chim. Acta **187**, 149-154, 1990.

Šafařík, I., Šafaříková, M.: A modified procedure for the detection of microbial producers of extracellular proteolytic enzymes. - Biotechnol. Techniques **8**: 627-628, 1994.

Šafařík, I., Stejskalová, R., Králová, B.: Bacterial producers of fibrinolytic enzymes isolated from soil. - Sborník VŠCHT Praha **E 55**: 75-86, 1983.

Whaley, D.N., Dowel, V.R., Jr., Wanderlinder, L.M., Lomnard, G.L.: Gelatin agar medium for detecting gelatinase production by anaerobic bacteria. - J. Clin. Microbiol. **16**: 224-229, 1982.

Wunderwald, P.: Proteinases. Proteins as substrates. - In: Bergmeyer, H.U. (ed.): Methods of Enzymatic Analysis. Vol. 5. Pp. 258-270. Verlag Chemie, Weinheim 1984.