Garden of Medicinal Plants[1], Faculty of Pharmacy, Food Research Institute[2], Department of Botany[3], Faculty of Natural Sciences, Department of Cell and Molecular Biology[4], Faculty of Pharmacy, Comenius University; Export-Import[5], Janotova 18, Bratislava, Slovak Republic

Identification and determination of the intra- and extracellular aminopeptidase activity by synthetic L-Ala-, L-Tyr-, and L-Phe-β-napthylamide

J. Stano[1], P. Siekel[2], K. Mičieta[3], V. Blanáriková[4], M. Koreňová[1], E. Bergerová[2], P. Nemec[5]

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Dr. Peter Siekel, Food Research Institute, P.O. Box 25, Priemyselna 4, 824 75 Bratislava 26, Slovak Republic siekel@vup.sk

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A simple, rapid and straightforward procedure for identification and determination of intracellular and extracellular activity of aminopeptidases employing synthetic substrates β-naphtylamides of L-Ala, L-Phe, and L-Tyr was used. Poppy cells (*Papaver somniferum* L.) permeabilized by Tween 80 were immobilized via crosslinking by glutaraldehyde. Glutaraldehyde immobilized poppy cells lost their viability and demonstrated significantly lower aminopeptidase activities than untreated control cells probably due to a damage to the enzyme active centre. Poppy cells immobilized by pectate and alginate have retained high activity of studied aminopeptidases. The culture medium (without cells) used for the identification and determination of extracellular enzyme activities retained 20–21%, whereas intracellular activities were estimated to be 79–80% of total enzyme activity. Thus the intracellular specific activity was 1.00–1.07 higher.

1. Introduction

Opium poppy (*Papaver somniferum* L.) is one of the earliest domesticated plants. Until now it is an important and the only source of morphine and codeine besides anumber of other benzylisoquinoline alkaloids of pharmaceutical significance such a muscle relaxants papaverin and noscapine (Balážová et al. 2002; Bilková et al. 2005a, b).

The formation of opium alkaloids, e.g thebaine, during germination of poppy seedlings is well documented. In the poppy seedlings precursors of alkaloids can arise the *de novo* via shikimic pathway or by proteolysis from storage proteins and peptides. The proteolytic activities within germinating seeds and seedlings are described (Balážová et al. 1988; Benešová et al. 2002). Tyramine and dopamine play an important role in the initial steps of benzylisoquinoline biosynthesis (Bilková et al. 2006).

Decarboxylation of L-tyrosine, L-phenylalanine and L-DOPA was shown in experiments in poppy seedlings (Jindra et al. 1966). Activities of L-tyrosine and L-DOPA decarboxylases were demonstrated in poppy cell suspension cultures immobilized by glutaraldehyde (Stano et al. 1995).

Synthetic and natural substrates may be used for the identification and determination of aminopeptidase activity (Mrestani-Klaus et al. 2002; De Mester et al. 2002). Immobilization of whole cells or enzymes represents an effective way of producing highly efficient enzyme catalysis with application in many industrial processes (Trelles et al. 2004). Plant proteolytic enzymes metabolize peptides and proteins and are involved in processes such as degradation, posttranslational protein modification etc. (Guo et al. 1998; Mertová et al. 2002). Proteinases participate in the mobilisation of storage proteins to amino acids, which are indispensable for the primary and secondary metabolism of cells (Bilka et al. 2002; Benešová et al. 2002). Germination and ripening of seeds and pollen is associated with the expression of various hydrolytic enzymes (Duarte et al. 1998; Tegeder et al. 2000).

Aminopeptidases (aminoacylpeptide hydrolase EC 3.4.11) catalyse the release of the N-terminal amino acids from peptides or synthetic substrates. The determination and immobilization of aminopeptidase activity plays an important role in research activities (Siekel and Mičieta 1998; Trelles et al. 2002). The availability of a simple and rapid screening method for the detection of aminopeptidase activity moreover coupled with immobilization of those enzymes is of some importance for both research and production purposes. The synthetic substrates such as β-naphtylamides (βNA) provide an additional advantage of defined condition for enzymes study (Stano et al. 1997).

The aim of present study was to show that following synthetic substrates L-Ala-βNA, L-Tyr-βNA, L-Phe-βNA can be employed for the identification and determination of the activity of intra-and extracellular plant aminopeptidase in a simple and rapid array.

2. Investigations, results and discussion

2.1. Identification and determination of poppy aminopeptidases

The synthetic substrates β-naphtylamides of L-Ala, L-Phe, and L-Tyr were used in this study to determine the intracellular and extracellular activity of aminopeptidases. The

Table 1: Distribution of L-alanine, L-phenylalanine and L-tyrosine aminopeptidases in cell culture and culture medium of opium poppy

Fraction	Volume (ml)	Protein (nkat/g fresh mass)	Activity (mg/g fresh mass)			Specific activity (nkat/mg protein)		
			L-Ala-AP	L-Phe-AP	L-Tyr-AP	L-Ala-AP	L-Phe-AP	L-Tyr-AP
Intracellular activity (Homogenate of isolated cells)	1	0.82	1.8 ± 0.06	1.5 ± 0.06	1.6 ± 0.07	2.19	1.83	1.95
Extracellular activity (Culture medium without cells)	5	0.22	0.45 ± 0.02	0.40 ± 0.02	0.42 ± 0.02	2.04	1.82	1.91

* Corresponding to the amount of isolated cells
Values are averages ± SD from five experiments

activity of extracelluar aminopeptidase was detected as redish bright zone beneath and around the cell area on the agar plates. In the case of 3–4 day old seedling root tips and hairy roots the redish bright zone was visible after 30–90 min. The stained zone was not present when the plant material was thermally treated (100 °C, 10 min).

The intra- and extracellular aminopeptidase activity assay of homogenized cell suspension cultures and medium used for they 12 days cultivation was performed (Stano et al. 1997). The data from above mentioned synthetic substrates demonstrate that the 79–80% were of intacellular and 20–21% of extracellular activity. The intacellular specific activity was 1.00–1.07 higher. It was shown that intra- and extracellular activity of aminopeptidase and invertase is similar (Stano et al. 2004). Contrary to that the extracelluar activity of α-galactosidase and β-galactosidase (Mičieta et al. 2002; Neubert et al. 2004) is 3–4 fold higher than that of invertase (Stano et al. 2004).

The production of extracellular aminopeptidases as well as other hydrolases released from plant cells and/or microorganisms might be of some importance for taxonomical purposes (Križo and Liška 1999), biotechnological applications and elucidation of the study of biologically active compounds (Mučaji et al. 1999).

The formation of the opium alkaloid thebaine during germination of opium poppy seedlings is well documented (Facchini and Park 2003). In studied seedlings the precursors of alkaloids can arise either *de novo* via shikimic pathway or by proteolysis from reserve proteins (Stano et al. 1995). The influence of cycloheximide, chloramphenicol and phenylmethylsulfonyl fluoride resp. upon the growth and development of poppy seedlings and on the protease activity was studied by Benešová et al. (2002). The inhibition of studied endopeptidase (caseinolytic) activity by these effectors indicates *de novo* formation of this enzyme during poppy seedlings development. Phenylmethylsulfonyl fluoride (inhibitor of serine proteases) did not affect enzyme activity and the growth and development of poppy seedlings.

2.2. Activity of aminopeptidase in immobilized opium poppy cells

Immobilization techniques have had an evident impact on biotechnological research and applications (Klibanov 1983; Hulst and Tamper 1989; Hansen et al. 1989, Gill and Ballestros 2000).

In this work immobilization by pectate, alginate and glutaraldehyde were used. Glutaraldehyde immobilized poppy cells differed significantly from the cells in suspension as they lost their viability as proved by vital staining and respiration rate. Pectate and/or alginate immobilized cells from culture suspensions remained viable as demonstrated by utilization of glucose (Fig.).

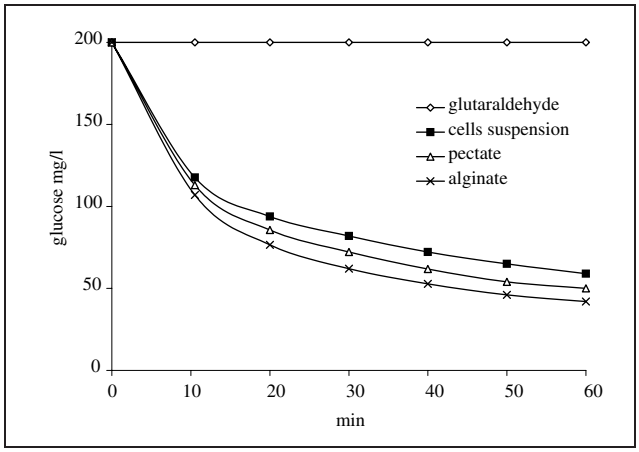

Fig.: Time course of glucose utilization in cells immobilized by glutaraldehyde, pectate and alginate

The enzyme activity of cells cross-linked by glutaraldehyde showed a considerable decrease (Table 2). This was in agreement with previous results for plant proteases (Báleš et al. 1987; Elcin and Sacak 1996). It was concluded that crosslinking with glutaradehyde may damage the active centre of an enzyme and consequently decrease its activity. Contrary to these results the glutaraldehyde immobilization of many plant cells was shown to be a convenient method for long-term preservation of different catalysts as are α-galactosidase and β-galactosidase, invertase, L-tyrosine decarboxylase and L-DOPA decarboxylase (Hansen et al. 1998; Stano et al. 1995; Weissová et al. 2001).

An alternative of the plant cells immobilization is an employment of alginate or other hydrogels (Brodelius et al. 1979; Furuya et al. 1984; Shoichet et al. 1996). Poppy cells immobilized this way appeared to be convenient for several enzymes (Báleš et al. 1987) (Table 2). The cells entrapment in beds facilitate the continuous flow-through arrangements, improves separation of products, prolongs biocatalyst half-live, protects cells from shear forces, prevents cell aggregation, stimulates secondary metabolism and other features, too (Berlin et al. 1998; Trelles et al. 2004).

Biotransformation using free or immobilized biocatalysts not only provides and alternative and efficient solution to the synthesis of several compounds, but also offers environmentally friendly technologies that profit from mild reaction conditions (Trelles et al. 2004).

Chemical synthesis of opium poppy alkaloids is very complicated and expensive. Cell suspension cultures of opium poppy produce relatively higher amount of benzophenanthridine alkaloid, sanguinarin upon elicitation (Bilková et al. 2005a). The absence of 1,2-dehydroreticuline reductase (DRR) activity in opium poppy cell cultures is likely

Table 2: Activity of L-alanine, L-phenylalanine and L-tyrosine aminopeptidases in cell suspension of glutaraldehyde, pectate and alginate immobilized poppy cells

Cells	Protein (mg/g dry mass)	Activity (nkat/g dry mass)			Specific activity (nkat/mg protein)		
		L-Ala-AP	L-Phe-AP	L-Tyr-AP	L-Ala-AP	L-Phe-AP	L-Tyr-AP
Suspension	18.9 ± 0.26	39.6 ± 0.29	33.2 ± 0.26	35.2 ± 0.27	2.09	1.76	1.09
Tween 80 permeabilized	6.0 ± 0.23	41.3 ± 0.28	35.4 ± 0.25	37.1 ± 0.25	6.88	5.90	6.18
Glutaraldehyde immobilized*	5.9 ± 0.24	2.1 ± 0.18	2.1 ± 0.18	2.1 ± 0.18	0.36	0.31	0.32
Pectate immobilized**	18.9 ± 0.24	14.4 ± 0.26	1414 ± 0.25	13.9 ± 0.24	0.76	0.75	0.74
Alginate immobilized**	18.8 ± 0.23	14.0 ± 0.23	13.8 ± 0.23	13.7 ± 0.24	0.74	0.73	0.73

* Prior glutaraldehyde immobilization the cells were permeabilized by Tween 80
** The cells immobilized by pectate and alginate entrapment resp. were not permeabilized
Values are averages ± SD from five experiments

a primary reason for the lack of morphinane alkaloid biosynthesis differentiated tissues (De Eknamkul and Zenk 1992). The opium poppy plants still remain important as the only source for the analgetic and antitussive drugs, morphine and codeine, in addition to a number of other benzylisoquinoline alkaloids of pharmaceutical significance such as papaverine and noscapine (Bilka et al. 2003, 2004).

Results of this study indicate that cell immobilization by glutaraldehyde crosslinking causes significant decrease in aminopeptidases and proteases activities. The immobilization of cells by alginate and/or pectate protects most of studied enzymatic ativity and clearly showed suitability for research purposes. A sensitive and straightforward method for detection of extracellular aminopeptidases was developed by employment of synthetic substrates.

3. Experimental

3.1. Plant material

Long-term callus cultures were derived from opium poppy seedlings (*Papaver somniferum L. cv. "Amarín"*) and were cultivated as previously desribed (Stano et al. 1995). Seedlings of *Papaver somniferum* L. *cv. "Amarín"* were cultivated from sterilized seeds under aseptic conditions (Dixon 1991).

3.2. Extracellular enzyme activity

L-Ala-βNA, L-Tyr-βNA and L-Phe-βNA were used for the identification of extracellular aminopeptidase. The corresponding azo-dye was formed by coupling β-naphtylamine released by enzymatic activity with Fast Garnet GBC salt (GBC salt) as described (Stoward and Pearse 1991; Lojda et al. 1979).

2 mg L-Ala-βNA, L-Arg-βNA, L-Phe-βNA, or L-Tyr-βNA resp. were dissolved in 0.5 ml dimethylformamide and 4.5 ml of 0.1 M Na-phosphate buffer pH 6.5 containing 10 mg GBC salt. 5 ml of 2% agar in 0.1 M Na-phosphate buffer (pH 6.5) were added to the above mixture and autoclaved in the usual way. Agar plates were then inoculated with cells from growing callus cultures or 4–5 days old seedlings of opium poppy and were than cultivated 30–90 min.

3.3. Determination of intracellular and extracellular activity of aminopeptidase

3.3.1. Enzyme preparation

Cell suspension cultures were used to determine the intracellular aminopeptidase activity. The cells (12 g) were filtered off and washed with 3 l of distilled water. Soluble proteins were extracted by grinding the cells in a precooled mortar using 1 : 1 (g/ml) cells and 0.1 M Na-phosphate buffer pH 7.0 at 4 °C. The homogenate was squeezed through two layers of nylon cloth and centrifuged at 15000 × g for 15 min at 4 °C. For determination of the extracellular enzyme activity, the cultivation medium was centrifuged at 1000 × g for 15 min at 4 °C.

3.3.2. Enzyme assay

The activity was estimated by using L-Ala-βNA, L-Phe-βNA, and L-Tyr-βNA as substrates. The incubation mixture contained 0.5 ml 0.1 M Tris-HCl buffer solutions having pH values of 8.1, 7.5, and 6.7 respectively, 0.5 ml of 2 mM L-Ala-βNA, 2.2 mM L-Tyr-βNA and 2.5 mM L-Phe-βNA respectively, and appropriate amounts of enzyme (0.3–0.5 ml) or enzyme incubated with 1 mM diisopropylfluorophosphate (DFP). The enzyme reaction was terminated by addition of 0.5 ml of 20% $HClO_4$. 1 ml of the supernatant was centrifugated for 10 min at 2000 × g supplemented by 1 ml of 0.2% $NaNO_2$, maintained at 4 °C for 10 min, than 1 ml of 0.5% ammonium sulphamate was added. After addition of 2 ml of 0.1% methanolic solution of N,N′-(naphtyl)ethylendiamine hydrochloride and incubation at 37 °C for 30 min the azo-dye was formed. Its intensity was determined spectrophotometrically at 578 nm against a control sample of DFP inactivated enzyme (Schaper et al. 1990). The enzyme activity is expressed in katals. Proteins were determined by the method of Doumas et al. (1981) using bovine serum albumine as a standard. The enzyme data were measured in pentaplicate and the average ± SD was determined.

3.4. Cell permeabilization by Tween 80 and immobilization by glutaraldehyde

Cell suspensions were filtred through a nylon cloth, and 10 g of fresh mass was suspended in 50 ml of 5% Tween 80 in 0.15 mol · l^{-1} NaCl solution. Permeabilization was carried out for 3 h under moderate stirring at room temperature. The cells were filtered off and washed with 3 l of distilled water and 3 l of 0.15 M NaCl solution and separated by filtration. The permeabilized cells were immediatelly suspended 50 ml of 0.15 M NaCl solution. Then 5 ml of 25% glutaraldehyde was slowly added with gentle stirring and left for 3 h at room temperature. The immobilized cells were then separated and washed with 3 l of distilled water and 3 l of 0.15 M NaCl solution.

3.5. Cell immobilization by pectate and/or alginate

Cell suspensions were filtred through anylon cloth, and then immobilized by pectate and/or alginate. In total, 5 g of fresh mass of cells suspension was resuspended in 20 ml of 5% pectate and alginate respectively and then dropped into solution of 0.05 M $CaCl_2$ (100 ml). The spherical gel particles produced were fairly homogenous having diameters of approximatelly 4 mm. The gel beds (100 beds contained 1 g cells) with immobilized cells were collected from the $CaCl_2$ solution. Beds (3 g) were washed, then added to the 20 ml growth medium and cultured in 100 ml flasks on a rotatory shaker (80–90 r.p.m) (Furuya et al. 1984; Shoichet et al. 1996).

3.6. Determination of fresh and dry mass

Fresh and dry mass of cell suspensions were determined gravimetrically, the samples were dried to constant weight at 105 °C.

3.7. Glucose utilization

The immobilized cells and cell suspensions were exposed to an initial glucose concentration of 250 mg/l in cultivation media (Furuya et al. 1984; Stano et al. 1998) without the presence of sucrose. The concentration of glucose was determined by the method of Trinder (1969).

3.8. Cell viability

The cell viability was determined by a method of Dixon (1991) with 2,3,5-triphenylterazolium chloride (TTC), fluorescendiacetate and with oxygen electrode resp.

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References

Balážová A, Pšenák M (1998) Biosynthesis of morphinans: Enzymological and molecular-biological aspects. Chem Listy 92: 1006–1015.

Balážová A, Bilka F, Blanáriková V, Pšenák M (2002) Changes in sanguinarine content and polyphenoloxidase activity due to a fungal elicitor in suspension cultures of the opium poppy plant *Papaver somniferum* L. Czech Slov Pharm 51: 182–185.

Báleš V, Gemeiner P, Kuniak Ľ, Rexová-Benková Ľ, Vojtíšek V, Zemek J (Eds.) (1987) Enzyme engineering. Bratislava, Alfa, pp. 147–149.

Benešová M, Bilka F, Pšenák M, Kovács P (2002) The effects of inhibitors of proteosynthesis on the growth and protease activity on the developing poppy seedlings (*Papaver somniferum* L.). Acta Fac Pharm Univ Comeniane 49: 37–45.

Berlin J, Martin B, Nowak J, White L, WrayV, Strack D (1989) Effect of permeabilization on the biotransformation of phenylalanine by immobilized tobacco cell cultures. Z Naturforsch 44c: 249–254.

Bilka F, Kľúčovská J, Bilková A, Benešová M (2002) Aminopeptidases in opium poppy latex. Biologia (Bratislava) 57: 761–764.

Bilka F, Balážová A, Bilková A, Šubr Z, Pšenák M (2003/4) Characterization of polyphenol oxidase from opium poppy latex. Biologia Plantarum 47: 11–15.

Bilková A, Bezáková L, Bilka F, Pšenák M (2005a) An amine oxidase in seedlings of *Papaver somniferum* L.. Biologia Plantarum 49: 389–394.

Bilková A, Bilka F, Bezáková L (2005b) Enzymology of production of benzylisoquinoline alkaloids. Czech Slov Pharm 54: 17–22.

Bilková A, Balážová A, Obložinský M, Bilka F (2006) Biochemical aspects of production of secondary metabolites in opium poppy plants. Farm Obzor 75: 152–158.

Brodelius P, Deus B, Mosbach K, Zenk MH (1979) Immobilized plant cells for the production and transformation of natural products. FEBS Lett 103: 93–97.

De-Eknamkul W, Zenk MH (1992) Purification and properties of 1,2-dehydroreticuline reductase from *Papaver somniferum* seedlings. Phytochemistry 31: 813–821.

De Meester I, Durinx C, Proost P, Scharpé S, Lambier AM (2002) Natural substances of medical importance. In Langer J, Ansorge S (eds) Endopeptidases, Kluwer Academic Plenum Publishers, New York, pp. 223–258.

Dixon RA, (1991) Plant cell culture. A practical approach. Press Oxford, Washington DC, pp. 1–20.

Doumas BT, Bayse DD, Carter RJ, Peters T, Schaffer R (1981) A candidate reference method for determination of total protein serum. I. Development and validation. Clin Chem 27, 1642–1650.

Duarde IC, Ricardo CPP, Dugue-Magalhaes MC (1993) Peptide hydrolases in cotyledons germinating lupin. Phytochemistry 33: 35–40.

Elcin YM, Sacak M (1996) Acrylamide grafted poly (polyethylene tereftalate) fibers activated by glutaraldehyde as support for urease. Appl Biochem Biotechnol 60: 19–32.

Facchini PJ, Park SU (2003) Developmental and inducible accumulation of gene transcripts involved in alkaloid biosynthesis in opium poppy. Phytochemistry 64: 177–186.

Furuya T, Yoshikawa T, Taira M (1984) Biotransformation codeinone to codeine by immobilized cells of *Papaver somniferum* L. Phytochemistry 23: 909–101.

Gill I, Ballesteros A (2000) Bioencapsulation within synthetic polymers. (Part 1): sol-gel encapsulated biologicals. Trends Biotechnology 18: 282–296.

Guo ZJ, Lamb C, Dixon RA (1998) A serine protease from suspension cultured soybean cells. Phytochemistry 47: 547–553.

Hansen EW, Olafsen K, Klaveness TO, Kvemberg PO (1998) Probing the gelation of polyvinylalcohol-water-glutaraldehyde within a porous material by H n.m.r. – A preliminary investigation. Polymer 39: 1279–1284.

Hulst AA, Tramper J (1989) Immobilized plant cells. A literature survey. Enzyme Microbiol Technol 11: 546–558.

Jindra A, Kovács P, Pittnerová Z, Pšenák M (1966) Biochemical aspects of the biosynthesis of opium alkaloids. Phytochemistry 5: 1303–1315.

Klibanov AM (1983) Immobilized enzymes and cells as practical catalysts. Science 219: 722–727.

Križo M, Liška I (1999) Amino acids in the pollen of *Larix decidua* Mill. Biológia (Bratislava) 38: 97–104.

Lojda Z, Gossrau R, Schiebler TH (1979) Enzyme histochemistry. A laboratory manual. Springer, Berlin, Germany, pp. 171–180.

Mertová J, Almásiová M., Perečko D, Bilka F, Benešová M, Bezáková L, Pšenák M, Kutejová E (2002) ATP-dependent Lon protease from maize mitochondria-comparison with the other Lon proteases. Biologia (Bratislava) 57: 739–745.

Mičieta K, Tokhtaeva E, Stano J, Koreňová M, Neubert K, Ulbrich-Hoffmann R, Blanáriková V (2002) A simple and rapid method for identification of extracellular plant galactosidases. Chem Nat Comp 38: 284–287.

Mrestani-Klaus C, Lorey S, Faust J, Brühling F, Neubert K (2002) Detection of the activity of the ectopeptidases DPP IV and APN using sensitive fluorogenic substrates. In: In Langer J, Ansorge S (eds) Endopeptidases, Kluwer Academic Plenum Publishers, New York, pp. 1–4.

Mučaji P, Grančai D. Nagy M, Buděšínsky M, Ubík K (1999) Triterpenoid saponins from *Cynara cardunculus* L. Pharmazie 54: 714–716.

Neubert K, Stano J, Mičieta K, Koreňová M, Blanáriková V (2004) Study of extracellular plant lactase. Eng. Life Sci 4: 281–283.

Sharper K, Demuth HU, Baumgrass R, Fischer G, Neubert K, Barth A (1990) Development of enzyme-activated inhibitors for DPP IV. Beiträge zur Wirkstoff-Forschung 38: 7–12.

Shoichet MS, Li RH, White ML, Winn SR (1996) Stability of hydrogels used in cell encapsulation. An in vitro comparison of alginate and agarose. Biotechnol Bioeng 50: 374–381.

Siekel P, Mičieta K (1996) Demonstration of proteolytic activity secreted from plant cells. Biologia 53: 791–793.

Stano J, Nemec P, Weissová K, Kovács P, Kákoniová D, Lišková D (1995) Decarboxylation of L-tyrosine and L-DOPA by immobilized cells of *Papaver somniferum*. Phytochemistry 38: 859–860.

Stano J, Kovács P, Pšenák M, Gajdoš J, Erdelský K, Kákoniová D, Neubert K (1997) Distribution of dipeptidyl peptidase IV in organs and in tissue cultures of poppy plants *Papaver somniferum* L. cv. Amarin. Pharmazie 52: 319–321.

Stano J, Nemec P, Bezáková L, Kákoniová D, Kovács P, Neubert K, Lišková D, Mičieta K, Andriamainty FH (1998) β-Galactosidase in immobilized cells of gherkin *Cucumis sativus*, Acta Biochimica Polonica 45: 621–626.

Stano J, Mičieta K, Tintemann H, Kovács P (2004) Identification and determination of invertase secreted by tomato cells. Pharmazie 59: 323–324.

Stoward PJ, Pearse AGE (1991) Histochemistry. Theoretical and applied. Vol. 3. Churchill Livingstone, Edinburgh, UK, pp. 639–652.

Tegeder M, Offler ChE, Frommer WB, Patrick JW (2000) Amino acids transporters are localized to transfer cells of developing pea seeds. Plant Physiol 122: 319–324.

Trelles JA, Bentancor L, Schoijet A, Porro S, Lewkowicz ES, Sinisterra J, Iribarren AM (2004) Immobilized *Escherichia coli* BL 21 as acatalyst for synthesis of adenine and hypoxanthine nucleosides. Chem Biodiv 1: 280–288.

Trinder P (1969) Determination of blood glucose using an oxidase-peroxidase system with anon-carcinogenic chromogen. Ann Clin Biochem 6: 1969, 24–32.

Weissová K, Stano J, Neubert K, Kákoniová D, Kovács P, Mičieta K, Lišková D (2001) Immobilized plant cells in the biotransformation of some precursors of poppy alkaloids and glycosides. Hort Sci 28: 151–155.