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IDENTIFICATION AND DETERMINATION OF AMINOPEPTIDASE ACTIVITIES SECRETED BY LEMON BALM

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Using synthetic substrates, an uncomplicated and sensitive procedure for the identification and determination of extracellular aminopeptidase was developed. The β-naphthylamides of the amino acids were applied for the identification of extracellular aminopeptidase, whereas the 4-(phenylazo) phenylamides of the amino acids were used for the determination of intra- and extracellular aminopeptidase activity. The results show a 81.8–88.9% intracellular and 11.1–18.2% extracellular distribution of the studied enzyme activity.

Key words: identification, determination, aminopeptidase, lemon balm.

Plant proteolytic enzymes play many roles in metabolism such as peptide and protein degradation, posttranslation protein modification, and other processes as well [1, 2]. Proteolysis is ubiquitous in biological systems, providing a means for cells to change their protein content during development and adaptation to altered environmental conditions. Proteinases participate in the mobilization of storage proteins by transforming stored polypeptides through oligopeptides to free amino acids, which are indispensable for the development and primary and secondary metabolism of cells [3, 4]. Germination and ripening of seeds and pollen is associated with the expression of various hydrolytic enzymes [5, 6].

In the last years, several methods for the identification and determination of the activity of aminopeptidases have been developed [5, 7]. Naturally occurring or synthetic substrates may be used for these purposes [8, 9].

Aminopeptidases (aminoacylpeptide hydrolase EC3.4.11) catalyze the release of N-terminal amino acids from peptides or synthetic substrates. The determination of aminopeptidase activities plays an important role in many fields of basic and applied research [1, 10].

The availability of a simple and rapid screening method for the detection of aminopeptidase activity is of great importance for both scientific and production purposes. One advantage is the use of synthetic substrates such as 4-(phenylazo) phenylamides (PAP-amides) and β-naphthyl amides (βNA) of the amino acids [5, 11]. The aim of this work was to show that the synthetic substrates L-Arg-βNA, L-Phe-βNA, and L-Leu-βNA could be employed for the identification of the activity of extracellular plant aminopeptidases in an uncomplicated and rapid procedure, whereas L-Arg-PAP amide, L-Phe-PAP-amide, and *L*-Leu-PAP-amide could be employed for its determination.

The synthetic substrates L-Arg-PAP amide, L-Phe-PAP amide, and L-Leu-PAP amide were used in this study to determine the intracellular and extracellular activities of aminopeptidase. The results presented are mean values ± SD of five experiments. Culture media (agar plates) with and without the substrates L-Arg-βNA, L-Phe-βNA, L-Leu-βNA, and GBC salt [12, 13] were inoculated with cells from growing callus cultures and then incubated for 30–90 min. The corresponding azo dye was formed by simultaneous azocoupling of the β-naphthylamine released by the enzyme activity with Fast Garnet GBC salt (GBC salt).

The activities of extracellular aminopeptidase were detected by the presence of stained bright reddish zones beneath and around the areas of the cells on the agar plates. Extracellular aminopeptidase was also considered to be present in cases where bright reddish staining occurred after 30–60 min in zones around the root tips of 3–5 days old lemon balm seedlings on the agar plates.

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TABLE 1. Arginine, Phenylalanine, and Leucine Aminopeptidase Activity in the Cell Culture and Culture Medium of Lemon Balm

Aminopeptidase	Intracellular activity (homogenate of isolated cells), 2 mL*		Extracellular activity (culture medium without cells), 8 mL**	
	activity	specific	activity	specific
Arginine	1.60±0.07	1.28	0.20±0.01	0.31
Phenylalanine	1.78±0.08	1.42	0.22±0.01	0.34
Leucine	1.70±0.08	1.36	0.21±0.02	0.32

*Protein 1.25±0.13 mg/g f.w.

**Protein 0.65±0.15 mg/g f.w., corresponding to the amount of isolated cells.

Staining did not occur on the agar medium or plant materials after the inoculation with heat-treated calluses or seedlings (100°C, 10 min).

Homogenized cell suspension cultures and culture medium alone after 8 days of cultivation were applied for assaying the activity of both intracellular and extracellular aminopeptidases. In both cases, the given PAP amides (L-Arg, L-Phe, L-Leu) as described in the characterization of the aminopeptidase activity in lemon balm seedlings [6] were used as the substrates. The distribution of intra- and extracellular activity is presented in Table 1. The data demonstrate that the L-Arg-AP, L-Phe-AP, and L-Leu-AP activities are distributed by 81.8–88.9% intracellularly and by 11.1–18.2% extracellularly, the intracellular specific enzyme activity being 3.89–4.14 times higher.

The distribution of intra- and extracellular activity of invertase and aminopeptidase is similar [14]. It is very interesting that the activity of extracellular α-galactosidase and β-galactosidase [15] is 3- to 4-fold higher than the activity of invertase [14] and aminopeptidase studied. The production of extracellular aminopeptidases as well as other hydrolases, which are released from plant cells, might be of some importance for biotechnological applications in food and pharmaceutical research as well as in industry [16, 17]. Biotransformations using free or immobilized biocatalysts not only provide an alternative and efficient solution to the synthesis of many compounds, but also offer environmentally clean technologies that profit from very mild reaction conditions [18, 19]. These enzymes are generally present in plants. Until now they have not been used in biotechnological processes [14, 20, 21].

Due to its simplicity and reproducibility the method presented here could be very useful for the identification and determination of the activity of aminopeptidases in plants.

EXPERIMENTAL

Callus and Suspension Cultures. Long-term callus cultures were derived from steam of *Melissa officinalis* L. and continuously subcultured every two weeks on Murashige-Skoog (1962) medium [22, 23]. For cell suspension culture induction the callus culture (2 g) was inoculated into liquid Philips-Collins (1979) [24] medium, and cell suspension cultures were grown on the rotatory shaker (110 r.p.m.) in 250 mL flasks, containing 100 mL medium at 24°C under 16 h light period (45–60 $\mu M/m^2 s$). The suspension was subcultured every two weeks. Seedlings of *Melissa officinalis* L. were cultivated from sterilized seeds under aseptic conditions [25].

Identification of Extracellular Enzyme Activity. L-Arg-βNA, L-Phe-βNA, and L-Leu-βNA were used for the identification of extracellular aminopeptidase. The coresponding azo dye was formed by coupling β-naphthylamine released by enzyme activity with Fast Garnet GBC salt (GBC salt) under the optimum condition for this reaction [12, 13]. 2 mg L-Arg-βNA, L-Phe-βNA, or L-Leu-βNA were dissolved in 0.5 mL dimethyl formamide 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) was added to the above mixture and the whole autoclaved in the usual way. Agar plates inoculated with cells from callus cultures (growing) or 3–5 days old seedlings of lemon balm were cultivated 30–90 min.

Enzyme Preparation. Cell suspension cultures were used to determine the intracellular aminopeptidase activity. The cells (12 g) were filtered off and washed with 3 liters 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 150.000 m/s^2 for 15 min at 4°C.

For the determination of the extracellular enzyme activity, the cultivation medium was centrifuged at 10.000 m/s^2 for 15 min at 4°C.

Enzyme Assay. The activity assay of aminopeptidase was performed using the modified method of Stano et al. [1] employing L-Arg-PAP amide, L-Phe-PAP amide, or L-Leu-PAP amide as chromogenic substrates (prepared by K. Neubert, Institute of Biochemistry, M. Luther University in Halle, Germany). As was experimentally estimated in our previous work [2], the reaction mixture contained 1.5 mL Theorell-Stenhagen buffer (0.1 M H_3PO_4–NaOH) of pH 7.9, 6.7 or 8.1 and 0.3 mL substrate solution in an optimum concentration of 1.6 mM L-Arg PAP amide, 2.5 mM L-Phe PAP amide, and 1.9 mM L-Leu-PAP amide, as well as a suitable amount of enzyme preparation (0.1–0.5 mL). The control contained heat-inactivated enzyme (100°C, 5 min). Both mixtures were kept for 30 min at 30°C and adding 0.5 mL 40% TCA stopped the reaction. The concentration of the liberated 4-(phenylazo) aniline was determined spectrophotometrically at 500 nm [6]. The enzyme activity was expressed in katal. Protein contents were determined by the method of Doumas [26] using bovine serum albumin as the standard protein.

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