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研究报告

Evaluation of the Hydroxynitrile Lyase Activity in Cell Cultures of Capulin (*Prunus serotina*)

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Abstract: Enzymatic preparations obtained from young plants and cell cultures of capulin were screened for hydroxynitrile lyase activity. The threeweek old plants, grown under sterile conditions, were used to establish a solid cell culture. Crude preparations obtained from this plant material were evaluated for the transformation of benzaldehyde to the corresponding cyanohydrin (mandelonitrile). The results show that the crude material from roots, stalks, and leaves of young plants and calli of roots, stalks, internodes and petioles biocatalyzed the addition of hydrogen cyanide (HCN) to benzaldehyde with a modest to excellent enantioselectivity.

Keywords: cyanohydrins, hydroxynitrile lyase, *Prunus serotina*, cell culture

Plants are a source of secondary metabolites such as terpenoids, coumarins, antraquinones, flavonoids, etc, which are known as "natural products". Those compounds are used as drugs, flavors, fragrances, pigments and agrochemicals[1]. The plant kingdom contains some unique enzymes which produce a great variety of chemicals[2]. *In vitro* plant cell and organ culture and plant enzymes act as suitable biocatalysts to perform complex reactions, including oxidations, reductions, hydroxylations, methylations, acetylations, isomerizations, glycosylations and esterfications[1]. Hydroxynitrile lyases (HNLs) are involved in the cyanogenic process and there are numerous reports of screening for HNLs in seeds, fruits and plants[3], a novel screening assay being recently described for this purpose[4]. Nowadays HNLs are used for the preparation of chiral cyanohydrins from aldehydes and as a suitable source of HCN in organic solvents[5]. This process has become a fundamental tool for many investigations related to the preparation of enantiopure cyanohydrins[6]. In recent years, several modifications to the original procedure introduced by Effenberger for the preparation of (*R*) and (*S*)-cyanohydrins[7] have been developed, opening the possibility of using crude preparations obtained, for example, from almond[8], apple[9] and mamey seeds[10] in the case of (*R*)-HNLs, and sorghum shoots[11] or guanabana seeds[12] for (*S*)-HNLs, instead of the isolated enzymes.

As part of our search for alternative sources of HNLs and with the aim of developing a protocol to establish a homogeneous plant material free of environmental influences, we report herein the results of evaluating HNL activity in solid cell cultures and young plants of *Prunus serotina* (capulin).

1 Materials and methods

Reagents and solvents were purchased from Organic Research, Baker or Aldrich and were used without any further purification. ^{1}H-NMR spectra were recorded on a 400 MHz Varian instrument in $CDCl_3$, using tetramethylsilane (TMS) as an internal reference. The optical purity was determined using a CHIRACEL OD column (Eluent: *n*-hexane/isopropanol mixtures) in a Hewlett Packard 1100 instrument, equipped with a diode array detector.

1.1 *Prunus serotina* with source of biocatalysts

For the plant organ biocatalyst: *Prunus serotina* seeds were obtained from the fruit of a garden tree. For germination, the seeds were scarified using a razor blade and surface-sterilized by submerging them for 10 min in a 20% aqueous solution of commercial bleach, followed

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by five washings with sterile water. The seeds were germinated in Murashige and Skoog (MS)[13] medium at 25°C with a photoperiod of 16 h of light and 8 h of dark for 15 days. Young plants, three weeks old, were dissected into roots, stalks and leaves, and each part was used for the preparation of the crude biocatalyst.

For the cell culture biocatalyst: Some young plants, obtained with the above procedure, were cut in 1 cm sections and these explants were introduced in MS medium complemented with sucrose (3%), 2, 4-D (2 mg/L), vitamins of MS, ascorbic acid (5 mg/L)-citric acid (5 mg/L), and phytagel (2.5 g/L), at pH 5.7 to develop the calli. The conditions of incubation were similar to those described above. The first subculture took place after three weeks; the calli were maintained on solid MS medium and subcultured every three weeks. The HNL activity of the calli was then evaluated after two or three weeks of growth after of subculture.

1.2 Preparation of the crude biocatalysts of *Prunus serotina*

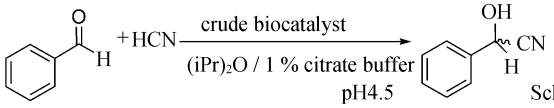
The calli of roots, stalks, petioles and internodes or plant sections were frozen with liquid nitrogen and crushed in a mortar. The solid material was blended sequentially three times with enough acetone to completely cover the biological material. After filtering and discarding the solvent each time, the resulting powder was air-dried in the fume hood and stored in tight closed jars at 5°C until use.

1.3 General procedure for the biotransformation of benzaldehyde into mandelonitrile

In a typical experiment, 1.5 mL of a 1.0 mol/L buffer solution of KCN/citric acid (pH 4.5) was extracted three times with diisopropyl ether (1.5 mL each time). The organic extracts containing HCN were combined and added to a reaction vessel containing the crude biocatalyst from young plant sections or calli (150 mg). Then 45 L of citrate buffer (0.1 mol/L, pH 4.5) was added, followed by the addition of benzaldehyde (0.1 mmol). The resulting mixture was magnetically stirred at room temperature for 24 h, followed by the addition of anhydrous Na_2SO_4. The reaction mixture was then filtrated and evaporated under reduced pressure. The conversion percentage was determined by ^{1}H-NMR (comparing the integral area of the aldehyde at 9.95 ppm with the integral area of the singlet at 5.54 ppm, corresponding to HCCNOH) and the optical purities were determined by chiral HPLC of the crude product.

2 Results and discussion

We selected the synthesis of mandelonitrile from benzaldehyde (Scheme 1) for the evaluation of the hydroxynitrile lyase activity of the crude biocatalysts prepared from calli and young plant parts. A crude enzymatic preparation of leaves from an adult garden tree of *Prunus serotina* was used as a comparative reference for the HNL activity[4], since it is a completely differentiated tissue the results are shown in Tables 1 and 2.



Scheme 1

According to these results, the crude biocatalysts of roots, stalks and leaves of young capulin plants showed HNL activity (Table 1). The conversions and enantioselectivities were similar to those obtained with the crude biocatalyst from the adult tree leaves. The results also suggest that HNL activity is not affected by environmental factors. Furthermore, the age of the plants was not observed to have any significant impact on HNL activity.

Table 1 Results from the evaluation of crude biocatalysts from organs of young plants grown under sterile conditions

Part of the young plant	Weeks of growth	Conversion[a](%)	ee[b](%)
Root	3	90	91
Stalk	3	93	>99
Leaves	3	91	>99
Adult tree leaves[c]	–	95	99

a: determined by NMR^{1}H; b: determined by chiral HPLC on a CHIRACEL OD column with hexane-isopropanol 92:8; c: crude biocatalyst from leaves of an adult capulín tree, used as reference for HNL activity

A kinetic study on the callus development showed that the stationary phase in solid medium was reached after 3 weeks, so we selected this stage for the enzymatic activity evaluation.

The results from the evaluation of the crude biocatalysts from root, internode, petiole and stalk calli with a growth of three weeks after three consecutive maintenance subcultures are shown in Table 2. The crude biocatalysts from the calli resulted in enantioselectivities ranging from 10 to 55% ee and low conversions (11%~39%). It is clear that these crude biocatalysts showed HNL activity, but the quantitative parameters were lower than those obtained from the preparation of adult tree leaves used as a comparative reference. The previous results (Table 1) suggest that the notorious decrease in enantioselectivity and conversion for the HNL activity may be associated with the fact that callus cells are non-differentiated, in contrast with plant organ cells (leaves, roots and stalks).

We then evaluated HNL activity in the crude

enzymatic preparation of three-week-old calli at the end of the stationary phase after seven maintenance subcultures. Calli of stalks and roots showed a further decrease in enantioselectivity, and calli of internodes and petioles showed a decrease in the conversion. When the crude biocatalysts were evaluated at two weeks of growth in the early stationary phase after seven subcultures, the enantioselectivity for stalks and roots was a little higher than in three-week-old calli after the same number of subcultures.

Table 2 Results of the evaluation of HNL activity of crude biocatalysts prepared from calli

Explant	Weeks of growth[a]	Conversion (%)[b]	ee (%)[c]	Number of subcultures
Stalk	3	11	55	3
Root	3	36	10	3
Petiole	3	24	22	3
Internode	3	39	10	3
Stalk	3	17	12	7
Root	3	31	6	7
Petiole	3	12	32	7
Internode	3	13	11	7
Stalk	2	24	28	7
Root	2	10	10	7
Leaves of adult tree[d]	–	95	99	

a: week of growth after subculturing; b: determined by ^{1}H NMR; c: determined by chiral HPLC on a CHIRACEL OD column, with hexane-isopropanol 92:8 eluant; d: leaves of an adult tree of capulin used as reference for HNL activity

3 Conclusions

We conclude that HNL activity was observed in the crude biocatalysts prepared from roots, stalks, internodes and petioles of young plants of *Prunus serotina*, as demonstrated by the transformation of benzaldehyde into mandelonitrile with excellent enantioselectivity. In addition, crude biocatalysts of calli derived from roots, stalks, petioles and internodes of the same plant also presented HNL activity with modest enantioselectivity and this activity was influenced by the weeks of growth and number of subcultures. This enzymatic activity was strongly influenced by the degree of differentiation of the tissue under study.

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