

The Occurrence of Phenylalanine Ammonia-Lyase and Cinnamic Acid *p*-Hydroxylase on the Endoplasmic Reticulum of Cell Suspension Cultures of *Glycine max*

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Subfractionation of Microsomes, Cell Suspension Culture, PAL, Cinnamic Acid *p*-Hydroxylase

1. The time course of activity of soluble and microsomal phenylalanine ammonia-lyase (PAL) was studied in dark grown cell cultures of soybean (*Glycine max*). A distinct activity increase of PAL in the soluble and microsomal fraction occurred prior to the stationary phase of the cell culture. Cinnamic acid *p*-hydroxylase and NADH: cytochrome c reductase, too, exhibited maximal activity in the log phase, 5 days after the transfer of soybean cells to fresh culture medium.

2. Upon subfractionation of the once washed microsomal fraction by sedimentation velocity centrifugation on a sucrose gradient, membranes of the endoplasmic reticulum could be separated from fractions containing mainly membranes from the Golgi apparatus or plasma membranes, respectively. PAL and cinnamic acid *p*-hydroxylase were found in fractions of endoplasmic reticulum whereas no activity of either enzymes could be detected in fractions containing Golgi apparatus or plasma membranes.

3. Repeated washing of microsomal fractions led to a residual membrane-bound PAL representing about 1% of the total PAL activity of the cells. This residual membrane-bound activity could be solubilized almost completely by Triton X-100 or digitonin at concentrations of 0.5–5%.

Introduction

Several enzymes of the phenylpropanoid metabolism have been reported to occur in soybean cell suspension cultures [1–3]. Ebel *et al.* [4] demonstrated a concomitant increase and subsequent decrease in the activities of PAL, cinnamic acid *p*-hydroxylase and other enzymes. Although numerous investigations with respect to aromatic compounds are performed using soybean cell cultures, little is known about the subcellular distribution of the important enzymes of phenylpropanoid metabolism and properties of organelles in plant cell cultures.

In intact plants, cinnamic acid *p*-hydroxylase has been reported to be a microsomal enzyme [5]. Cinnamic acid *o*-hydroxylation has been found to occur predominantly in chloroplasts [6, 7]. Beyond that it is known that *o*- and *p*-hydroxylation co-occur on membranes of plants [8]. PAL, the key-enzyme of phenylpropanoid metabolism catalyzing the conversion of L-phenylalanine to cinnamic acid, is frequently attributed to the soluble fraction only, although a number of authors have found PAL to be localized, *e.g.* in microsomes [8] and chloroplasts [9].

This report describes the attempt to subfractionate microsomes into membranes of endoplasmic reticulum [10], Golgi apparatus and plasma membranes in order to localize enzymes of the phenylalanine metabolism such as PAL and cinnamic acid *p*-hydroxylase. For this investigations cell cultures of logarithmic phase were used when PAL activity reaches a maximum.

Materials and Methods

Cell cultures

Six days old suspension cultures of *Glycine max* were inoculated at a ratio of 1:10 into 200 ml of a slightly modified B5 medium [11] in 2 l Erlenmeyer flasks. The cells were propagated on a rotary shaker in the dark at 27 °C. Five days old cultures were harvested by vacuum filtration through a porous glass filter, immediately deep frozen and stored at –20 °C. The growth of the cell culture was measured by determining fresh weight [12].

Preparation and subfractionation of microsomes

All the following procedures were carried out at 4 °C. A weighted amount of frozen cells (50 g) was homogenized for 4 × 15 sec (full speed) with an 'Ultraturrax' blender (Janke and Kunkel, Staufen, Germany) in an equal volume of grinding medium.

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The grinding medium consisted of 15% sucrose (w/w), 20 mM Hepes buffer (pH 7.5), 10 mM KCl, 5 mM EDTA and 1 mM dithioerythritol. The crude homogenate was filtered through two layers of Miracloth and centrifuged for 20 min at $100000 \times g$. The pellet thus obtained is referred to as crude microsomes. As indicated in the experiments the microsomal pellet was washed 1–4 times by gently resuspending in grinding medium and pelleting at the same force. Soluble PAL was measured in supernatant of the $100000 \times g$ centrifugation.

For subfractionation, the microsomal pellet was resuspended in grinding medium and layered onto a 25 ml linear gradient from 20 to 45% (w/w) sucrose. All sucrose solutions contained 5 mM EDTA and 1 mM dithioerythritol. The gradients were centrifuged at 21000 rpm for 4 hours in a Beckman L2-65B ultracentrifuge with a SW-27 rotor, following which 1.2 ml fractions were collected.

Analytical methods

PAL (E.C. 4.3.1.5) and cinnamic acid hydroxylase activities were measured according to established methods [13]. All other enzymes were tested spectrophotometrically as described for NADH-cytochrome c reductase [14] (E.C. 1.6.99.3), inosine diphosphatase [15] (E.C. 3.6.1.6), and catalase [16] (E.C. 1.11.1.6). The position of plasma membranes in sucrose gradients was determined by studying the binding of naphthylphthalamic acid (NPA) following the procedures given by Hertel *et al.* [17] and Jesaitis *et al.* [18].

Protein was measured by the method of Lowry *et al.* [19] with bovine serum albumine as standard. Sucrose concentrations were determined refractometrically.

Radioactive compounds

L-[U- ^{14}C]Phenylalanine (450 mCi/mmol) and [3- ^{14}C]cinnamic acid (50 mCi/mmol) were purchased from the Radiochemical Centre, Amersham and Schwarz Chemicals, respectively. [^{14}C]Naphthylphthalamic acid was prepared from [$\text{CO-}^{14}\text{C}$]phthalic anhydride and α -naphthyl amine; [$\text{CO-}^{14}\text{C}$]phthalic anhydride was obtained from CIS (Isotopen Dienst West, Sprenglingen). [U- ^{14}C]p-Coumaric acid was prepared from [U- ^{14}C]L-tyrosine by means of tyrosine ammonia-lyase [13].

Results

Time course of changes in enzyme activities in soybean cell cultures

The growth curves obtained for a soybean cell culture were basically the same as those described by other authors [4, 20]. As shown by changes in cell fresh weight in Fig. 1 a they include a log phase of about 2 days, a subsequent period of rapid growth for about 5 days and a stationary phase which was reached after 7 days. A maximum of microsomal protein was measured shortly before the culture reached a maximum in cell fresh weight; there was, however, no comparable rise in the protein content of the soluble fraction. As to the specific activity of PAL, the maxima were found to be greatly dependent upon the growth stage of the cell culture. Fig. 1 b demonstrates that relatively high

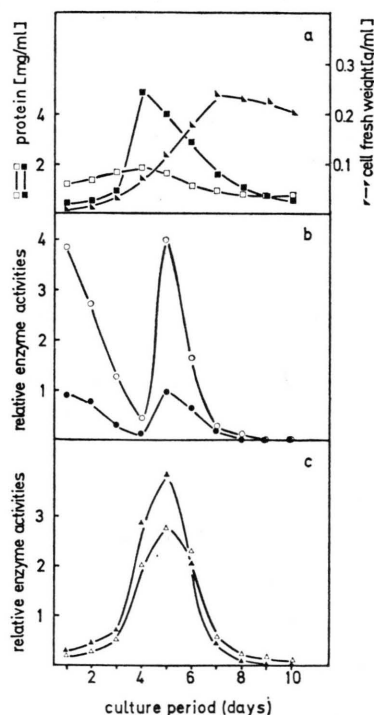


Fig. 1. Profiles of cell fresh weight, protein and enzyme activities during the growth of soybean cultures. (a) Fresh weight (\blacktriangle — \blacktriangle), total protein (\square — \square), microsomal protein (\blacksquare — \blacksquare). (b) Specific activities of soluble PAL (\circ — \circ) and microsomal PAL (\bullet — \bullet). (c) Specific activities of cinnamic acid *p*-hydroxylase (\blacktriangle — \blacktriangle) and NADH-cytochrome c reductase (\triangle — \triangle). Activities of microsomal enzymes were determined using $100\,000 \times g$ pellets which were washed twice.

specific activities of PAL of the soluble as well as of the microsomal fraction were present either during the first hours of induction, and, then, shortly before the stationary phase was reached. The fact that a very high value of PAL activity was observed shortly after inoculation is noteworthy but hardly to explain.

The distinct activity peak of the PAL at the 5th day of growth culture coincides with a maximal specific activity of NADH-cytochrome c reductase, a marker-enzyme for membranes of the endoplasmic reticulum (Fig. 1c). At this stage, a maximum of *p*-coumaric acid formation is also observed.

In vivo, the phenylpropane derivative *p*-coumaric acid is immediately further metabolized, towards lignin as demonstrated by Nimz *et al.* [21], and towards *p*-hydroxybenzoic acid. We found that [U-¹⁴C]*p*-coumaric acid was converted into [¹⁴C]*p*-hydroxybenzoic acid at a rate of 0.2 nmol/min × mg protein when suspension cultures of *Glycine max* were used.

Subfractionation of microsomes

Since the phenomenon of enzymes being located in one common compartment may become prominent in the interpretation of metabolic sequences, our emphasis was on the exact location of PAL and cinnamic acid *p*-hydroxylase within the microsomal preparations. The subfractionation of microsomal membranes was performed by means of zonal centrifugation whereby the bands of lighter membranes do not reach their equilibrium densities exactly. A centrifugation for 4 h at 21000 rpm led to a suitable separation of the submicrosomal fractions as outlined in Fig. 2. According to the markers used – NADH-cytochrome c reductase for endoplasmic reticulum ($d = 1.12 \text{ g/cm}^3$), inosinediphosphatase for Golgi apparatus ($d = 1.15 \text{ g/cm}^3$), and NPA-binding for plasma membrane ($d = 1.17 \text{ g/cm}^3$) – the distribution patterns of microsomal PAL and cinnamic acid *p*-hydroxylase are best reconciled by attributing these enzymes to the membranes of endoplasmic reticulum. For both enzymes, there was no activity detectable in the fractions containing dictyosomes or plasma membranes. In agreement with the observation of Benveniste *et al.* [22] we also found that in the presence of Mg^{2+} in the gradient no segregation of cinnamic acid *p*-hydroxylase and NADH-cytochrome c reductase activities is encountered. Furthermore, we could demonstrate (data not

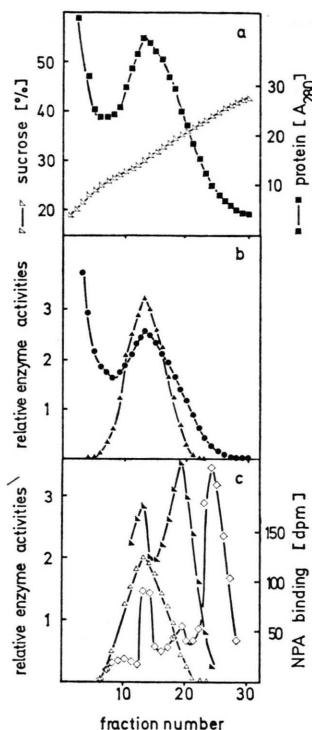


Fig. 2. Subfractionation of microsomes prepared from cells at the 5th day of growth. (a) Protein profile; (b) activity profiles of PAL (●—●) and cinnamic acid *p*-hydroxylase (▲—▲); (c) profiles of NPA-binding (◇—◇), inosine diphosphatase (◄—◄), and NADH-cytochrome c reductase (△—△).

shown) that, under these conditions, the microsomal PAL also banded at the same density ($d = 1.16 \text{ g/cm}^3$). The peak fraction of endoplasmic reticulum exhibited cinnamic acid *o*-hydroxylase activity at twice the value measured for the *p*-hydroxylase.

Solubilising PAL from the microsomal membranes

While it is accepted that cinnamic acid *p*-hydroxylase is a membrane bound enzyme, it is not yet clear whether some PAL might artificially become attached to the endoplasmic reticulum. To test this possibility, microsomal pellets were subjected to repeated washing procedures. They were resuspended in grinding medium and recentrifuged at 21000 rpm for 45 min. After each operation, the washed pellet and the corresponding supernatant were assayed for PAL activity. Fig. 3 shows that after the third washing the level of PAL activity on the membrane remained constant but decreased continuously in the respective supernatant. If the supernatant obtained after the first resuspending is mixed

with the pellet resulting from the 5th washing operation, additional PAL activity is again found on the membrane but removed by three washing operations. Therefore, we attained the same level of membrane-bound activity as was observed before the addition of solubilized PAL.

The fact that PAL activity on the membrane is virtually completely restored when the washing of the first operation is added back to the pellet after the 5th operation, clearly indicates that the PAL removed from the crude microsomal fraction can be re-associated with the membrane. This proportion of the membrane PAL activity can again be removed by three washing operations.

These results convey the idea that such washing patterns might be typical of any soluble or solubilized enzyme. To assess this possibility, we mixed microsomal pellets with catalase and then applied the same procedures. As shown in Fig. 3, two washes only were necessary to remove all catalase activity from the microsomal pellet. Mixing this pellet with the supernatant in an analogous way indicates that under these artificial conditions, catalase was less tightly associated with the membranes than the PAL removed from the microsomes some operations before.

The PAL activity which could not be removed from the membrane by mechanical desintegration (*i.e.* 1% of total PAL) also resisted solubilization by ultrasonic treatment. Applying a non-ionic detergent (Triton X-100) or digitonin, however, resulted in a loss of PAL activity in the pellet and in a

corresponding increase of the enzyme activity in the supernatant. Fig. 4 summarizes the effect of increasing concentrations of detergent. Besides solubilization, these operations led to an increase of the total enzyme activity as calculated from the amount of PAL activity in pellet and supernatant (upper part of Fig. 4).

PAL, solubilized in this way, did not differ from soluble PAL as judged by molecular weight, K_m value for L-phenylalanine, pH-optimum and the inhibition by cinnamic acid and several of its derivatives.

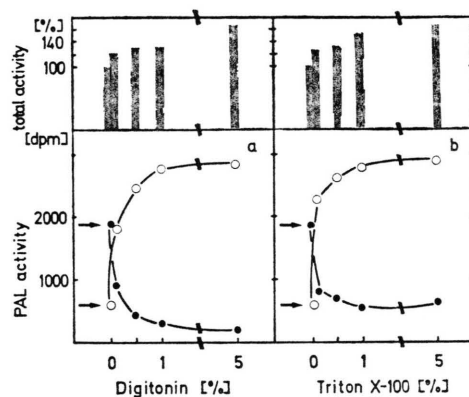


Fig. 4. Solubilization of microsomal PAL by increasing concentrations of digitonin and Triton X-100, respectively. Microsomal preparations which had been washed already 3-times were subjected to detergent treatment. ○—○, PAL activity in the supernatant; ●—●, PAL activity in the corresponding pellet. The upper part indicates the rise in total activity upon treatment with detergent.

Discussion

As numerous investigations with respect to aromatic compounds have been performed using soybean cell cultures, it was desirable to characterize some of the compartments which come into consideration. The endoplasmic reticulum which was found to be one of these compartments is most active on the 5th day of growth and represents then the dominant membrane species within the microsomal fraction.

The synthesis of *p*-coumaric acid is strictly correlated with the most active period of endoplasmic reticulum and, thus, paralleled by biosynthesis of cell wall and mitochondria. For that, the synthesis of *p*-coumaric acid is essential as it provides for precursors of lignin synthesis and of the mitochondrial pathway to *p*-hydroxybenzoic acid and ubiquinone.

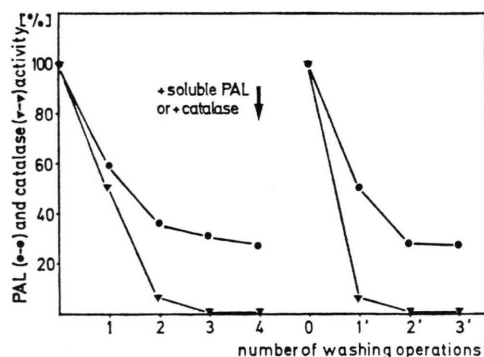


Fig. 3. Partial solubilization of microsomal PAL. During the washing procedure the behaviour of microsomal PAL (●—●) and catalase (▼—▼), externally supplied to the membrane fraction, was compared. After the 4th washing, the pellet was resuspended in the supernatant which resulted from the 1st operation.

The interrelationship between PAL and endoplasmic reticulum is characterized by pronounced selectivities. Thus, PAL is only associable with endoplasmic reticulum and not with other cellular membranes, and on the other hand, endoplasmic reticulum binds PAL by preference and not a number of other cytoplasmic enzymes or enzymes which eventually come into contact with endoplasmic reticulum, *e.g.* catalase. These facts can be reconciled by assuming that a high percentage of soluble PAL is very loosely associated with endoplasmic

reticulum *in vivo*, while *in vitro* due to the preparation methods most of this coat of associated PAL is found to be stripped off and only minor amounts of PAL remain adherent to the membranes; any further purification of the microsomal membranes implies a further loss of PAL. Finally, only a very tightly bound proportion of PAL is found on the membrane.

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