Further Studies on Induction of Enzymes of Phytoalexin Synthesis in Soybean and Cultured Soybean Cells

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Z. Naturforsch. 38c, 730 – 735 (1983); received June 23, 1983

Phytoalexins, Soybean, Soybean Cell Cultures, Enzyme-Induction

The glucan elicitor from cell walls of the fungal pathogen, Phytophthora megasperma f.sp. glycinea, caused a decrease in activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in wounded soybean cotyledons, whereas wounding alone led to an increase in the activity of this enzyme. A decrease of HMG-CoA reductase activity after elicitor treatment was also found in soybean hypocotyls and soybean cell cultures.

In contrast to the activity of HMG-CoA reductase, the activity of dimethylallylpyrophosphate: 3,6a,9-trihydroxypterocarpan dimethylallyltransferase increases after elicitor challenge of soybean cell cultures and after inoculation of soybean hypocotyls (cv. Amsoy 71) with mycelium of either race 1 (incompatible) or race 3 (compatible) of P. megasperma.

Introduction

The accumulation of the phytoalexin glyceollin* in soybean infected by Phytophthora megasperma f.sp. glycinea or treated with a glucan elicitor from this fungus is preceded by activity increases of a number of enzymes [2, 3]. Induction of enzymes by elicitor-treatment has also been found in soybean cell cultures [4, 5]. Synthesis of glyceollin was correlated with large increases of phenylalanine ammonia-lyase, 4-coumarate:CoA ligase and chalcone synthase. For phenylalanine ammonia-lyase and chalcone synthase it was demonstrated that increases in activity are due to an enhanced rate of their synthesis [2, 5]. It was also observed that a prenyltransferase (dimethylallylpyrophosphate: 3,6a,9-trihydroxypteroocarpan dimethylallyltransferase) which is specific for synthesis of 2- and 4-dimethylallyl-trihydroxypteroocarpan, putative precursors of the glyceollins (Fig. 1), is present only in elicitor-treated soybean cotyledons or soybean cell cultures and could not be detected in cotyledons which had only been wounded [6, 7].

Dimethylallylpyrophosphate, one of the substrates of the prenyltransferase, originates from acetate via mevalonic acid. In this pathway 3-hydroxy-3-methylglutaryl coenzyme A reductase catalyses a key regulating step in animal tissue [8] and is also considered to have an important control function in plants [9, 21]. We have therefore investigated the question whether the activity of this enzyme in

* The term glyceollin is used in this paper for the mixture of the three isomers which accumulate in challenged soybean [1].

Abbreviations: DMAPP. dimethylallylpyrophosphate; THP, (6aS,11aS)3,6a,9-trihydroxypteroocarpan; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IPP, isopentenylpyrophosphate; PAL, phenylalanine ammonia-lyase.

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0341-0382/83/0900-0730 $ 01.30/0

Fig. 1. Position of HMG-CoA reductase and prenyltransferase in the biosynthesis of glyceollins.
soybean is influenced by treatment with elicitor. Furthermore we have studied the course of induction of the prenyltransferase by infection of soybean seedlings with \textit{P. megasperma} f.sp. \textit{glycinea} or by elicitor-treatment of soybean cell cultures.

**Materials and Methods**

**Fungal culture**

\textit{Phytophthora megasperma} Drechs. f.sp. \textit{glycinea} Kuan and Erwin races 1 and 3 were obtained from B.L. Keeling (Stoneville, MS, USA) and were maintained on lima bean agar.

Cultures for inoculation of plants were grown as described \cite{10} on asparagine medium \cite{11}.

**Soybean seedlings**

Seeds of soybean (\textit{Glycine max} L. Merr., cv. Amsoy 71) were obtained from Agricultural Alumni Seed Improvement Assoc. (Romney, Ind., USA). Seedlings were grown in vermiculite as described previously \cite{10}.

**Soybean cell suspension cultures**

Soybean cell suspension cultures initiated from callus cultures of cultivar Harosoy 63 were grown as described \cite{4} in medium I containing 1 mmol l\(^{-1}\) CaCl\(_2\) \cite{12}.

**Chemicals**

\textit{DL-[2-\textsuperscript{3}H]}mevalonic acid lactone (745 Ci/mol), 3-hydroxy-3-methyl-[3-\textsuperscript{14}C]glutaryl-coenzyme A (26.2 Ci/mol) and \textit{[1-\textsuperscript{14}C]}isopentenylpyrophosphate, ammonium salt (53 Ci/mol) were obtained from Amersham Buchler (Braunschweig). The corresponding unlabelled compounds were obtained from Sigma (München). 3,9-Dihydroxypterocarpan, 3,6a,9-trihydroxypterocarpan and daidzein were from our laboratory collection. Biochemicals were of high quality and were obtained from several suppliers.

**Buffers**

A. 200 mmol l\(^{-1}\) K-phosphate, pH 7.3, 500 mmol l\(^{-1}\) saccharose, 10 mmol l\(^{-1}\) EDTA, 10 mmol l\(^{-1}\) mercaptoethanol.

B. 100 mmol l\(^{-1}\) Tris-HCl, pH 7.5. 14 mmol l\(^{-1}\) mercaptoethanol, 10\% (by vol.) glycerol.

C. 40 mmol l\(^{-1}\) K-phosphate, pH 7.0.

**HMG-CoA reductase preparations**

All operations were carried out at 4 °C.

a) From cotyledons

The tissue was ground in a mortar with quartz sand (0.3 g g\(^{-1}\) fr.wt.), Dowex 1 x 2 (1 g g\(^{-1}\) fr.wt.; equilibrated with buffer A), and buffer A (1.5 ml g\(^{-1}\) fr.wt.). The slurry was presssed through two layers of cheese cloth and the solution centrifuged at 5000 \(\times\) g for 10 min. The pellet was resuspended in a small volume of buffer A.

b) From cell cultures.

Cells were collected on a glass sinter funnel and were then extracted as described for cotyledons.

**Prenyltransferase preparations**

a) From cell cultures.

These were prepared as described previously \cite{7}.

b) From soybean hypocotyls.

For inoculation of the seedlings see \cite{2}.

For each enzyme assay 10 hypocotyl segments were frozen in a mortar with liquid nitrogen and ground to a fine powder. The powder was slowly stirred for 1 h with buffer B (2 ml g\(^{-1}\) fr.wt.). After centrifugation at 30000 \(\times\) g for 20 min the sediment was taken up in 0.5 ml buffer B.

**Enzyme assays**

**HMG-CoA reductase**

A combination of the methods of Suzuki \textit{et al.} \cite{13} and Brooker and Russel \cite{14} was used. The assay contained, in a total volume of 200 μl, 100–130 μl buffer C, 2 nmol dithioerythritol, 0.5 nmol EDTA, 2 nmol NADP\(^+\), 2 nmol glucose 6-phosphate, 5.8 nkat glucose 6-phosphate dehydrogenase, [3-\textsuperscript{14}C]HMG-CoA (220000 dpm) and enzyme. Before addition of [3-\textsuperscript{14}C]HMG-CoA and enzyme the mixture was preincubated for 5 min at 30 °C. After addition of enzyme and labelled substrate incubation was continued for another 30 min. 10 μl ofconc. HCl was then added and the mixture incubated for 30 min. After centrifugation at 10000 \(\times\) g for 1 min an aliquot (150 μl) was applied...
to a silica gel thin layer plate which was developed with acetone/chloroform (2:1 v/v). The zone of mevalonic acid lactone ($R_f = 0.45$) was scraped off and counted in a dioxan scintillation fluid.

**Prenyltransferase**

The assay for prenyltransferase activity was carried out as described previously [7].

**Elicitor from P. megasperma**

Cell walls from *P. megasperma* [15] were hydrolysed with 2 M trifluoroacetic acid according to the method of Valent [16]. The elicitor contained 0.15 g glucose equivalents per gram.

**Induction of plant material**

Five day old soybean cotyledons were treated with the Pmg elicitor as described [3]. The induction of soybean cell cultures has also been described [7]. In this case the elicitor was dissolved directly in the culture medium to a final concentration of 1 mg ml$^{-1}$.

**Results**

1. **3-Hydroxy-3-methylglutaryl-coenzyme A reductase**

**Detection of HMG-CoA reductase in soybean**

This enzyme was assayed by formation of $[^{14}C]$mevalonic acid lactone from $[^{1-14}C]HMG$-CoA [13, 14] with a NADPH regenerating system using glucose 6-phosphate and glucose 6-phosphate dehydrogenase. Higher reductase activity in extracts from cotyledons or hypocotyls of 5-day-old soybean seedlings was obtained when phenolic substances were bound by addition of Dowex 1 x 2 rather than by addition of polyvinylpyrrolidone. The optimal amount of Dowex was 1 g per g fresh weight of tissue. With such extracts the enzyme assay was linear with time up to 45 min and linear with protein concentration up to 1 mg protein/ml.

The distribution of HMG-CoA reductase activity in different parts of the seedlings is shown in Table I. The apical part contains a considerably higher activity than cotyledons, hypocotyl, or roots. About 90% of the total activity was present in the 100000 x $g$ fraction.

<table>
<thead>
<tr>
<th>Part of seedling</th>
<th>HMG-CoA reductase$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[μkat/kg]</td>
</tr>
<tr>
<td>Apical part</td>
<td>5.01</td>
</tr>
<tr>
<td>Cotyledons</td>
<td>0.09</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>0.57</td>
</tr>
<tr>
<td>Root</td>
<td>0.36</td>
</tr>
</tbody>
</table>

$^a$ Data represent the average of 2 determinations.

**Elicitor-induction of soybean cotyledons**

Since at the time when these experiments were carried out soybean cotyledons had been used for investigation of enzyme induction by a glucan elicitor from *P. megasperma* [3, 6], cotyledons were also used to investigate the change in HMG-CoA reductase activity after challenge with this elicitor. For comparison the activity of PAL and the absorption of the wound droplets at 285 nm as a measure for phytoalexin accumulation were determined. The results are shown in Fig. 2. While the observation that PAL and phytoalexins are induced by elicitor are in agreement with earlier observations [3], the results with HMG-CoA reductase were unexpected. This enzyme showed a large increase in activity in wounded cotyledons, but a small decrease in activity between 0 and 18 h after elicitor application to the wounded cotyledons. Not until 24 h after elicitor application was the HMG-CoA reductase activity somewhat higher than at the beginning of the experiment, but it was still much lower than in the only wounded cotyledons, where a second rise in enzyme activity occurred.

To investigate the possibility that formation of an inhibitor of HMG-CoA reductase was induced by elicitor treatment, experiments with mixed enzyme preparations were carried out. No inhibition of enzyme activity was found when extracts from wounded and elicitor treated cotyledons were added to extracts from only wounded cotyledons.

A decrease of HMG-CoA reductase activity after elicitor treatment was also found in hypocotyls of 5-day-old soybean seedlings. Whereas controls contained $1.82 \pm 0.38 \mu$kat/kg, elicitor-treated hypocotyls showed an activity of $1.35 \pm 0.22 \mu$kat/kg after elicitor application.
II. Dimethylallylpyrophosphate: 3,6a,9-trihydroxypterocarpan dimethylallyltransferase (Prenyltransferase)

Induction of prenyltransferase in soybean seedlings

Five-day-old soybean seedlings were inoculated in the hypocotyls [2] with mycelium from either

HMG-CoA reductase in soybean cell cultures

Enzyme activity was determined during growth of the soybean cell suspension culture. As shown in Fig. 3, HMG-CoA reductase activity rises sharply 2 days after transfer of the cells to fresh medium, reaches a maximum after about 4 days, and subsequently undergoes a steep decline. When 5-day-old cell cultures in which HMG-CoA reductase activity was low again (0.16 ± 0.02 μkat/kg) were treated with elicitor, not an increase, but again a decrease to 0.02 ± 0.007 μkat/kg enzyme activity was found. In agreement with previous reports these cells did accumulate glycine upon elicitor treatment (6 μmol 1⁻¹ glycine) [4].

Fig. 2. Upper part. Changes in phenylalanine ammonia-lyase activity in soybean cotyledons after elicitor treatment (■) and in wound controls (□) as well as increase in $A_{355}$ ($\triangle$) after challenge with elicitor. Lower part. Changes in HMG-CoA reductase activity in elicitor treated cotyledons (●) and in wound controls (○). Bars represent standard deviations.

Fig. 3. Changes in HMG-CoA reductase activity (■) after transfer of soybean cells to fresh medium in relation to cell fresh wt ($\triangle$) and conductivity (○) of the culture medium.

Fig. 4. Changes in dimethylallyltransferase activity in soybean hypocotyls after inoculation with mycelium of P. megasperma race 1 (●) or race 3 ($\triangle$) and in wound controls (○).
Induction of prenyltransferase in soybean cell cultures

The changes in the inducibility by the elicitor of the prenyltransferase during growth of a batch culture of soybean cells is shown in Fig. 5. After transfer of the cells to fresh medium, two peaks for the inducibility of the transferase were found. The first peak occurred at about 1 day after transfer (stage I) and the second peak at about 6 days after transfer, when the cultures had reached the end of the linear growth phase (stage II).

The time-course of prenyltransferase activity after elicitor-induction of a 6-day-old soybean culture is shown in Fig. 6. Enzyme activity is rapidly induced and declines again.

Discussion

I. HMG-CoA reductase

After infection of root tissue of sweet potato (Ipomoea batatas) by spores of the fungus Ceratocystis fimbriata, an increase and subsequent decrease in activity of HMG-CoA reductase was followed by an increase of furanoterpenoids [13]. In contrast, we found only a decrease of the activity of this enzyme in soybean cotyledons (Fig. 2), hypocotyls and soybean cell cultures challenged with a glucan elicitor from P. megasperma. The reason for this unexpected result is not clear. Experiments with mixed enzyme preparations showed that no inhibitor for the reductase was formed in the challenged tissue. HMG-CoA reductase activity from rat liver is known to be modulated by a phosphorylation-dephosphorylation mechanism [17], and such a modulation has recently also been reported for the enzyme from pea seedlings [18]. Whether elicitor-stimulated phosphorylation of HMG-CoA reductase could be responsible for the decrease in enzyme activity was not investigated so far.

Enzymes from two different pathways, nitrate reduction and general phenylpropanoid metabolism, are known to exhibit drastic activity changes following the transfer of cells from cell cultures of the late growth stage into fresh medium [19]. In soybean cell cultures PAL, cinnamate 4-hydroxylase, and 4 hydroxycinnamate: CoA ligase show a sharp maximum of activity shortly before the stationary phase of the culture [20]. In comparison the maximum for HMG-CoA reductase activity was found to appear during the logarithmic growth phase (Fig. 3).
II. Dimethylallyltransferase

The increase in prenyltransferase activity after inoculation of soybean hypocotyls with mycelium of *P. megasperma* (Fig. 4) occurs with a longer lag phase than the increase of PAL and chalcone synthase activities, for which apparent lag phases of about 2.5 h were found [2]. As in the case of PAL, a higher prenyltransferase activity was induced after inoculation with race 1 than with race 3 of *P. megasperma*.

The 2 maxima observed for the inducibility of the prenyltransferase by elicitor at different growth stages of soybean cell cultures are similar to the changes in inducibility found for PAL and chalcone synthase in such cultures [5].

In summary, our results lead to the conclusion that the dimethylallyltransferase is involved together with other enzymes like PAL and chalcone synthase in the regulation of glyceollin biosynthesis, whereas no clear evidence for a control function of HMG-CoA reductase could be derived from our results.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft (SFB 46) and by Fonds der Chemischen Industrie. We thank Dr. U. Zähringer, H. Börner, and A. Hille for help and valuable suggestions.