Phytoalexin Production by Isolated Soybean Protoplasts

Hannelore Mieth, Volker Speth, and Jürgen Ebel

Biologisches Institut II der Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg, Bundesrepublik Deutschland

Z. Naturforsch. **41c**, 193–201 (1986); received August 1, 1985

Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Protoplasts, Soybean Cell Suspension Cultures, Phytoalexin Production, *Phytophthora megasperma* f. sp. *glycinea,* Glucan Elicitor

Protoplasts isolated enzymatically from suspension-cultured cells of soybean (*Glycine max* L. Merr., cv. Harosoy 63) were used to study the production of the isoflavonoid-derived phytoalexin, glyceollin. A large enhancement in the *in vivo* rates of synthesis and catalytic activities of two of the enzymes associated with glyceollin biosynthesis, phenylalanine ammonia-lyase and chalcone synthase, preceded phytoalexin accumulation during early stages of culture of isolated protoplasts while cell wall regeneration occurred. A glucan elicitor from cell walls of the fungus *Phytophthora megasperma* f. sp. *glycinea,* an effective inducer of the phytoalexin response in cultured cells, was not capable of enhancing phytoalexin formation in protoplasts. Lack of responsiveness of the protoplasts to the glucan elicitor could either be associated with their stressed metabolic state in which the response system is already saturated or with the removal from cultured cells of an essential factor of the glucan elicitor-mediated phytoalexin induction during protoplast isolation. At least two components of the protoplast isolation medium, the osmoticum and the fungal endopolygalacturonase, have the potential to initiate the observed phytoalexin synthesis during protoplast isolation. Our results indicate that under the methods employed isolated soybean protoplasts display a stress response which other types of soybean cells show following microbial attack or treatment with elicitor.

Introduction

One type of defense mechanism of plants against pathogens is the accumulation of phytoalexins at the site of infection [1]. Phytoalexin synthesis is also induced by compounds termed elicitors [2]. Elicitors of biotic origin include fungal cell wall carbohydrates, fungal lipids, plant cell wall oligogalactosyluronides, and enzymes which release elicitor-active fragments from pectic polysaccharides of plant cell walls [3].

Treatment of various soybean *(Glycine max)* tissues with a 1,3-β-glucan elicitor isolated from the cell walls of the fungus *Phytophthora megasperma* f. sp. *glycinea,* a pathogen of soybean, induces the *de novo* synthesis and accumulation of isoflavonoid-derived phytoalexins [4]. Cell suspension cultures of soybean have been extensively used as a model system for studies on the elicitor-induced phytoalexin response [5–8]. Shortly after exposure to the elicitor, soybean cells stop growth and uptake of nitrate from the culture medium and show major changes in the population of translatable total RNA [5, 9]. During extended elicitor treatment of soybean cells the level of the isoflavonoid phytoalexin, glyceollin, increases from low basal values to concentrations more than one hundred-fold higher than in untreated cells [7].

Isoflavonoid phytoalexin accumulation is associated with large transient increases in the activities of the enzymes involved in phytoalexin biosynthesis [7, 8, 10–12]. The elicitor-induced transient increases in the rates of synthesis of two of the enzymes, phenylalanine ammonia-lyase (EC 4.3.1.5) and chalcone synthase, were similar when measured *in vivo* and *in vitro* and were sufficient to account for the changes in catalytic enzyme activities [6, 7]. It was recently demonstrated that the enhancement of translatable mRNA activity encoding this enzyme reflects increased mRNA levels, consistent with the hypothesis that phytoalexin synthesis in soybean cells is regulated by temporary gene activation [13] or activities of subsequent steps.

The mechanism by which the glucan elicitor and other microbial compounds [5, 7] cause the rapid and drastic alterations in the metabolism of soybean cells and hence phytoalexin formation is largely unknown. Evidence has been obtained for the existence of a host enzyme and of an endogenous oligogalacturonide elicitor from soybean cell walls both of which can induce phytoalexin synthesis in soybean tissus [14–16]. It is, however, not known whether

Abbreviation: HPLC, high-pressure liquid chromatography.

Reprint requests to Dr. J. Ebel.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0100–0193 $ 01.30/0

these or other substances participate in the induction of phytoalexin synthesis by the microbial glucan elicitor [3].

One approach to studying the mechanism of action of the microbial glucan elicitor could be to exploit protoplasts as an experimental system. Because to our knowledge protoplasts have not been used before in studies on phenylpropanoid product synthesis, we investigated whether protoplasts could be induced to produce isoflavonoid phytoalexins.

Our results show that protoplasts isolated from suspension-cultured soybean cells are capable of synthesizing significant amounts of glyceollin when cultured in growth medium. Addition of the glucan elicitor to protoplast suspensions did not cause any further increase in glyceollin accumulation above the levels observed in protoplast suspensions without elicitor treatment. Several factors are described which might initiate glyceollin synthesis during or after enzymatic release of protoplasts from cultured soybean cells. The results are discussed in relation to current views of elicitation of phytoalexin synthesis in plant cells.

Materials and Methods

Materials

L-[^{35}S]methionine (1400 Ci/mmol) and [^{14}C]methylated protein mixture (high molecular weight) were purchased from Amersham-Buchler (Braunschweig); cellulase (Driselase) was from Fluka (Buchs, Switzerland); endopolygalacturonase (pectinase) was from Sigma Chemie (München); sorbitol and mannitol were from Merck (Darmstadt). Prior to use cellulase and endopolygalacturonase samples were dialyzed against water and lyophilized. Cellulase and endopolygalacturonase activity of the samples were determined according to standard procedures [17, 18]. Antisera against phenylalanine ammonia-lyase and calcone synthase were gifts from Dr. K. Hahlbrock (Köln).

Cell cultures

Cell suspension cultures of soybean (*Glycine max* L. Merr., cv. Harosoy 63) were grown in the dark and used for the experiments as described [6, 7].

Protoplast isolation

Cell suspensions were harvested under sterile conditions by filtration through a porous glass filter as described [5]. Protoplasts were prepared by suspending cell samples (10 g) in 10 ml enzyme solution consisting of 1% (w/v) cellulase, 2.4% (w/v) endopolygalacturonase, 0.2 mol/l mannitol and 0.2 mol/l sorbitol in growth medium [6]. The suspension was incubated in a plastic dish (8.5 cm diameter) at 27 °C without shaking for 3.5 h. After filtration through a nylon screen (70 μm pore size) protoplasts were collected by centrifugation at 100 × *g* for 3 min. Protoplasts were purified by resuspending the pellet three times in growth medium containing 0.2 mol/l each of mannitol and sorbitol (protoplast medium) and collected by centrifugation. Washed protoplasts were suspended in protoplast medium and were obtained at a yield of about 3×10^6 protoplasts per gram of suspension-cultured soybean cells. Protoplasts were incubated in plastic dishes (3.5 or 8.5 cm diameter) in the dark at 27 °C without shaking.

Elicitor preparation and application

Crude glucan elicitor ("Mycelial wall-released elicitor") from *Phytophthora megasperma* f. sp. *glycinea* was prepared as previously described [19]. In standard incubations heat-sterilized solutions of elicitor were applied to cell cultures at growth stage I [6] or protoplasts to give a final concentration of 50 μg of glucose equivalents per ml of medium [7]. Treatment with other compounds was done with autoclaved (mannitol, sorbitol) or filter-sterilized (enzymes) solutions of the samples at various concentrations as indicated in the text.

Tissue extraction and enzyme assays

Crude cell extracts were obtained by stirring frozen cells (2 g) or protoplasts (0.5 g) with 4 ml of 0.2 mol/l Tris-HCl buffer, pH 7.5, containing 20% glycerol, 14 mmol/l β-mercaptoethanol, and 0.2 mmol/l phenylmethylsulfonyl fluoride in the presence of 200 mg Dowex 1X2 as described [7].

Phenylalanine ammonia-lyase and chalcone synthase activity were measured in the crude extract by standard procedures [20, 21].

Protein synthesis in vivo

Cell cultures (40 ml) or unstirred protoplast suspensions (about 10^6 protoplasts per 1.5 ml protoplast medium) in plastic dishes (3.5 cm diameter) were incubated for 1 h with 10 μCi of L-[^{35}S]methionine.

Cells were harvested by filtration [5] and protoplasts by centrifugation at 100 × *g* for 1 min. Cell-free extracts were prepared from frozen cells (2 g) with 2 ml of buffer as described previously [6]. Lysis and extraction of protoplasts was achieved by mixing twice with 0.5 ml each of buffer [6] for 1 min on a Vortex mixer. The slurry was centrifuged at 10,000 × *g* for 5 min.

The incorporation of L-[^{35}S]methionine into the *in vivo* synthesized phenylalanine ammonia-lyase and chalcone synthase was determined by immunoprecipitation followed by electrophoretic separation of the enzyme subunits as described previously [6]. Enzyme subunits were identified by their molecular weight and analyzed for incorporation of radioactivity by fluorography [22]. Relative film darkness at the positions of enzyme subunits was used as the measure of enzyme synthesis and was quantitated by scanning with an LKB scanning densitometer. Relative rates of enzyme synthesis are defined as the incorporation of L-[^{35}S]methionine into the enzyme subunits as a percentage of incorporation into total protein [6].

Analysis of isoflavonoid compounds

Isoflavonoid compounds were extracted from frozen soybean cells, protoplasts, and culture media and usually analyzed by HPLC on Partisil 5 (Merck) under isocratic conditions with hexane/isopropanol (90:10, v/v) at a flow rate of 2.5 ml/min according to the method described previously [7]; retention times (min) were: glyceollin I 11.1, daidzein 15.9, glycinol 20.4. To assure product identification reversed phase HPLC on Lichrosorb RP 18 (Merck) under isocratic conditions with methanol/water (64:36, v/v) at a flow rate of 1 ml/min was applied; retention times (min) were: glycinol 3.4, daidzein 5.3, glyceollin I 12.3.

Electron microscopy

Fixation of cells and protoplasts was started at room temperature with 2% glutaraldehyde in the cell culture medium or the protoplast medium, respectively. After 30 min the samples were cooled to 4 °C, the fixation medium was exchanged for 2% glutaraldehyde in 100 mmol/l cacodylate buffer, pH 7.4, and fixation was continued for a further 90 min.

For thin-section electron microscopy, samples were postfixed in 1% OsO_4 in cacodylate buffer, stained with uranyl acetate, dehydrated, and embedded in Epon 812. Ultra thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined in a Zeiss electron microscope EM 10 CR (Carl Zeiss, Oberkochen).

For deep-etch electron microscopy, the glutaraldehyde-fixed samples were washed in distilled water and frozen on copper discs (Balzers AG, Liechtenstein) by injecting them into liquid propane at −185 °C with the aid of a spring-loaded device. Samples were fractured and replicated at −100 °C in a Balzers BA 360 M machine, equipped with electron-gun evaporators and quartz crystal thin film monitor. Etching time was 3 min. Replicas were cleaned with household bleach, washed with distilled water, and picked up on formvar-coated copper grids.

Determination of protein

Protein was measured by the method of Schaffner and Weissmann [23], with bovine serum albumin as reference.

Determination of radioactivity

Radioactivity was measured by scintillation spectrometry in a mixture of toluene and Triton X-100 (2:1, v/v) containing 6 g of PPO/liter.

Results

Soybean protoplasts

Protoplasts were isolated enzymatically from cultured cells of soybean at a yield of about 3 × 10^6 per gram fresh weight of cultured cells. The majority of the protoplasts (Fig. 1a) appeared viable as indicated by neutral red dye uptake. Thin-section electron micrographs of representative samples demonstrated (Fig. 1b) a good preservation of the protoplasts at the ultrastructural level during isolation. During culture of soybean protoplasts in cell culture medium [6] supplemented with 0.2 mol/l each of mannitol and sorbitol regeneration of the cell wall started as early as a few hours after isolation, but appeared to be irregular. After 24 h there were many protoplasts without any visible cell-wall material (Fig. 2a). After 65 h most protoplasts showed fibrillar material at their surface (Fig. 2b), but even after 160 h cell-wall regeneration was incomplete (Fig. 2c) when compared with the cell wall of cultured soybean cells (Fig. 2d).

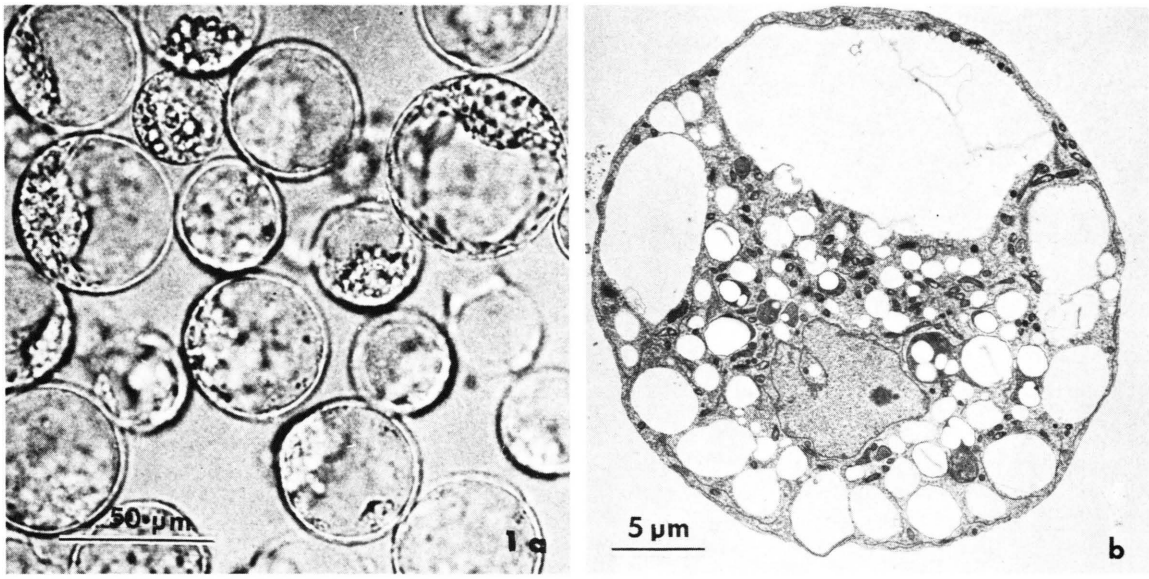

Fig. 1. Isolated soybean protoplasts visualized by light microscopy (a, magnification × 400) or by thin section electron microscopy (b, magnification × 2,500).

Phenylalanine ammonia-lyase and chalcone synthase activity in protoplasts

Protoplasts were prepared from suspension-cultured soybean cells and cultured for various periods of time in protoplast medium. Cell-free extracts were isolated from protoplasts and used to measure the activities of phenylalanine ammonia-lyase and chalcone synthase.

As can be seen from Fig. 3, freshly isolated protoplasts (0 h) contained low levels of activity for both enzymes. These levels were similar to those observed in cultured soybean cells [6, 7] from which the protoplasts had been obtained. Incubation of the protoplasts for 36 h in a medium which consisted of the cell culture medium [6] supplemented with 0.2 mol/l mannitol and 0.2 mol/l sorbitol as osmoticum caused large, continuous increases in the activities of both enzymes. These enzyme activities approached the values that are obtained in cultured cells only after treatment with a glucan elicitor from *P. megasperma* or with other compounds which possess similar activity [7]. The product of the chalcone synthase assay was identified as naringenin by thin-layer chromatography on cellulose with 15% acetic acid as solvent (R_f = 0.39). Formation of the flavanone was due to the presence of chalcone isomerase (EC 5.5.1.6) in the crude cell-free extract which was used in the assay.

Enhancement of the rates of synthesis of phenylalanine ammonia-lyase and chalcone synthase during culture of protoplasts

The unexpected finding of the marked increases in the enzyme activities during culture of soybean protoplasts prompted studies as to whether these enhancements were due to increased synthesis of the enzymes. For measurements of enzyme synthesis *in vivo,* 10^6 protoplasts in 1.5 ml protoplast medium were labelled for 1 h with L-[^{35}S]methionine. Following immunoprecipitation from crude cell-free extracts with a mixture of antisera against phenylalanine ammonia-lyase and chalcone synthase, separation by SDS polyacrylamide gel electrophoresis, and fluorography, two radioactive proteins were detected which migrated to the same positions as phenylalanine ammonia-lyase and chalcone synthase subunits (M_r = 78,000 and 41,000, respectively) isolated from labelled soybean cell cultures (Fig. 4A–D). It was concluded that these proteins represented the enzyme subunits synthesized in protoplasts. Changes in the rates of enzyme synthesis were determined by labelling protoplasts at various times

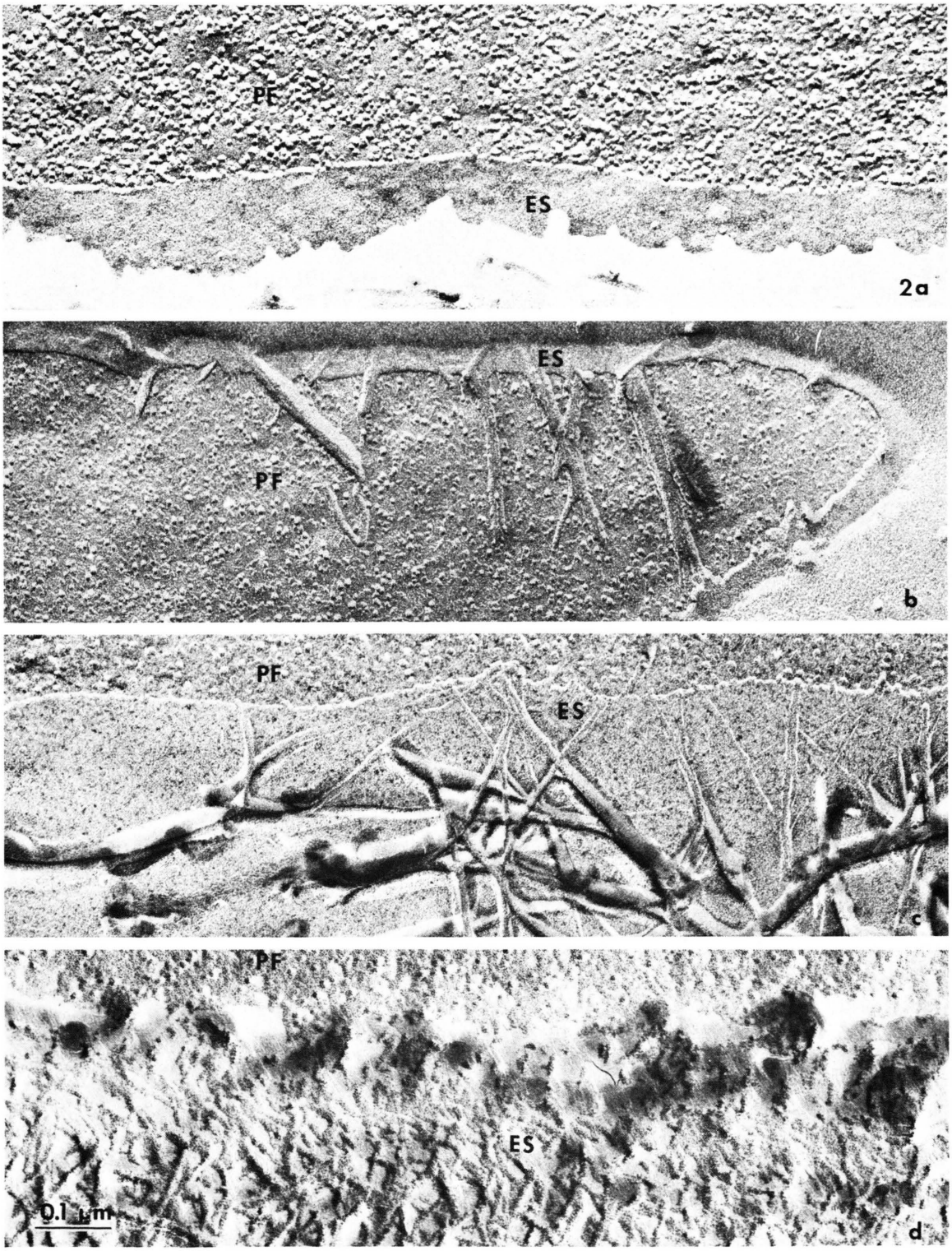

Fig. 2. Deep-etch electron micrographs of the plasma membrane of soybean protoplasts during cell-wall regeneration. Isolated protoplasts were incubated for 24 h (a), 65 h (b), or 160 h (c) in protoplast medium; d, suspension-cultured cell as control (magnification × 120,000). ES, extraplasmatic surface; PF, plasmatic fracture face.

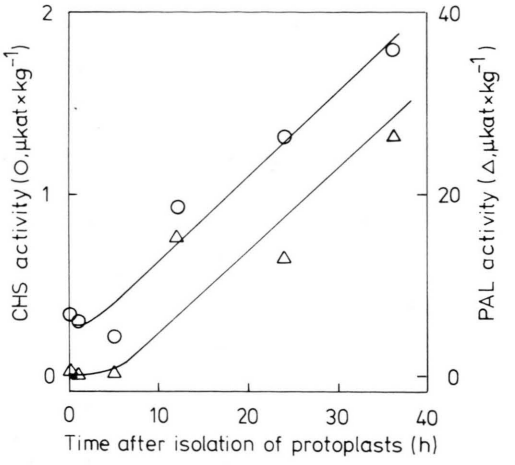

Fig. 3. Changes in phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) activity in soybean protoplasts. Protoplasts were isolated (0 h) and cultured for various periods of time in protoplast medium.

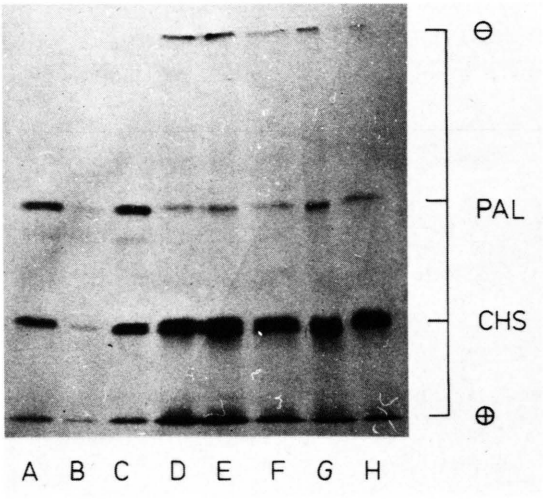

Fig. 4. Synthesis of phenylalanine ammonia-lyase and chalcone synthase in cultured soybean cells (A–C) or in isolated protoplasts (D–H). Cells or protoplasts were treated with various concentrations of the glucan elicitor for 5 h and labelled with L-[^{35}S]methionine during the last hour of elicitor treatment; A, C, 50 μg/ml elicitor; E, 13 μg/ml; F, 25 μg/ml; G, 50 μg/ml; H, 100 μg/ml; B, D, controls. After immunoprecipitation, the enzyme subunits were separated by gel electrophoresis and detected by fluorography.

after isolation. The results presented in Table I show that the enhancements in the activities of both enzymes were associated with large increases in their rates of synthesis during the culture of protoplasts for 70 h.

Comparison of enzyme synthesis in elicitor-treated and untreated protoplasts

Our earlier results had shown that the *P. megasperma* glucan elicitor induced large transient increases in the rates of synthesis *in vivo* and *in vitro* of phenylalanine ammonia-lyase and chalcone synthase in cultured soybean cells [6, 7, 9]. In the present study we investigated the effect of the glucan elicitor on the rates of enzyme synthesis *in vivo* in protoplast cultures. Protoplasts were incubated with various concentrations of the glucan elicitor at various times after protoplast isolation. In each test the protoplasts were treated with the elicitor for 5 h and labelling with L-[^{35}S]methionine was started 4 h after the beginning of the elicitor treatment. At all concentrations (Fig. 4D–H) and at all times tested after protoplast isolation (Table I) the elicitor failed to induce increases in the rates of enzyme synthesis above values already present in control samples without elicitor. By contrast it was demonstrated (Fig. 4A–C) that the same glucan elicitor induced significant increases in enzyme synthesis in soybean cells before protoplast isolation.

Table I. Rates of synthesis *in vivo* of phenylalanine ammonia-lyase and chalcone synthase in isolated soybean protoplasts. Soybean protoplasts were prepared enzymatically from cultured cells (0 h) and labelled with L-[^{35}S]-methionine at various times after isolation in the absence or the presence of the glucan elicitor. Elicitor treatment was for 5 h and labelling was during the last hour of treatment. Following immunoprecipitation, the enzyme subunits were separated by gel electrophoresis, and incorporation of radioactivity was analyzed by fluorography.

Treatment	Culture age	Enzyme synthesis	
		Phenylalanine ammonia-lyase	Chalcone synthase
	[h]	% of maximum	
None	5.5	0	22
Elicitor		0	20
None	24	28	46
Elicitor		35	97
None	70	100	100
Elicitor		96	88

Accumulation of glyceollin during culture of protoplasts

Following culture of protoplasts for various lengths of time, the culture medium was analyzed for

accumulation of the isoflavonoid phytoalexin, glyceollin. Isoflavonoid compounds were extracted from the culture medium into ethyl acetate and separated by HPLC on Partisil 5. Glyceollin I was the most prominent compound in the column effluent (Fig. 5). Production of glyceollin I by soybean protoplasts was unequivocally verified by using a radioimmunoassay (results not shown) which was previously described to be highly specific for this compound [24]. Glyceollins II and III and glycinol which were tentatively identified by co-chromatography using reference compounds were found in smaller amounts (<20%). Significantly higher levels of all compounds were measured after culture of protoplasts for 72 h (Fig. 5B) as compared to 27 h (Fig. 5A). A rough time course of glyceollin I accumulation in the protoplast medium is shown in Table II. As for the synthesis of phenylalanine ammonia-lyase and chalcone synthase (Table I), no differences detectable within the variability of the material in the levels of glyceollin I were observed in elicitor-treated versus untreated protoplast cultures (Table II). Although the absolute amounts of glyceollin I per mg protoplast protein differed between several experiments (Table II for example), the general pattern of the accumulated compounds was very similar.

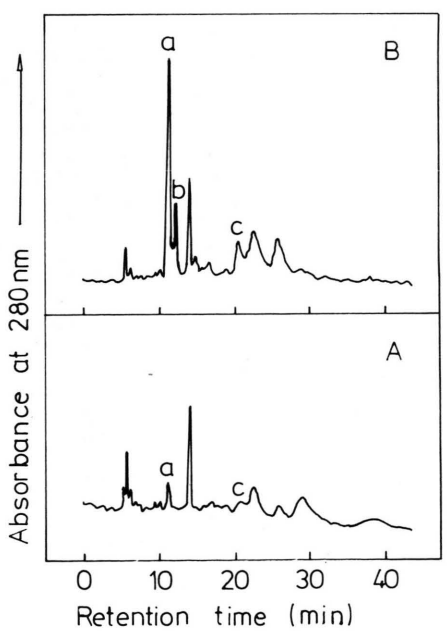

Fig. 5. Chromatography of an extract prepared from the protoplast medium after 27 h (A) or 72 h (B) of culture of isolated soybean protoplasts. HPLC on a Partisil 5 column at ambient temperature in hexane/isopropanol (90:10, v/v) permitted the separation of glyceollin isomers I (a) and II plus III (b) and glycinol (c).

Table II. Accumulation of glyceollin during culture of isolated soybean protoplasts. Following culture of protoplasts for various lengths of time in the absence or presence of the glucan elicitor, glyceollin was extracted from the culture medium and analyzed by HPLC. Data in brackets are results from a separate experiment.

Treatment	Culture age [h]	Amount of glyceollin [nmol (mg protein)$^{-1}$]
None	22	30 (150)
Elicitor		20 (70)
None	66	90 (310)
Elicitor		40 (210)
None	144	190 (450)
Elicitor		300 (480)

Production of isoflavonoids in soybean cells in response to substances used for protoplast isolation

The substances used for protoplast isolation, mannitol, sorbitol, endopolygalacturonase, and cellulase, were tested separately for their ability to induce the production of glyceollin I and related isoflavonoids in soybean cell cultures. The total amounts of the compounds which accumulated in the cells and the culture medium after treatment with the various substances for 120 h are summarized in Table III. Markedly enhanced glyceollin I levels similar to those induced by the glucan elicitor were found after treatment with all of the substances except cellulase. The glyceollin I content of untreated control cells was low. An enhancement, although to a different extent, was also observed in the levels of daidzein and glycinol, two putative precursors of glyceollin. The lowest effective concentrations of the osmoticum (mannitol plus sorbitol) and endopolygalacturonase were about 100 mmol/l and 5 nkat/ml, respectively (results not shown). Concentrations of the substances much higher than those reported in Table III did not result in a further increase in isoflavonoid accumulation but, rather, caused severe damaging to the cells. This was especially the case for cellulase at concentrations higher than 0.1 nkat/ml. Incubation of soybean cells with a mixture of the glucan elicitor and endopolygalacturonase resulted in glyceollin I levels which were considerably higher than those observed after treatment with any of the substances separately (Table III).

Table III. Accumulation of isoflavonoids in suspension-cultured soybean cells in response to substances used for protoplast isolation and to the glucan elicitor. Cells were treated for 120 h with the diverse substances, the isoflavonoids extracted from the cells and the culture medium, and analyzed by HPLC.

Treatment	Concentration of substances		Amount of isoflavonoids		
			Daidzein	Glycinol	Glyceollin
	[μg ml^{-1}]		[nmol (g cells)$^{-1}$]		
None (control)	0		0.3	0.4	<0.1
Glucan elicitor	50		24	10	256
	[mmol l^{-1}]				
Mannitol	400		18	113	264
Sorbitol	400		14	139	278
Mannitol + Sorbitol	400		15	163	272
	[nkat ml^{-1}	μg ml^{-1}]			
Endopolygalacturonase	84		99	8	30
Endopolygalacturonase + Glucan elicitor	84	50	26	5	679
Cellulase	0.1		16	4	<0.1
Cellulase + Glucan elicitor	0.1	50	14	11	187

Discussion

Plant protoplasts have gained increasing popularity as a tool for various physiological and biochemical studies [25]. One of the advantages of exploiting protoplasts is their reduced complexity when compared with cells of differentiated tissue from intact plants or cultured plant cells. It should be noted, however, that during the generation of protoplasts the cells are removed from their original environment and brought into an entirely different physicochemical situation which alters much of their metabolism compared with normal plant cells.

Our interest in using protoplasts as an experimental system for metabolic studies was twofold. First, we wished to determine the activities of enzymes and the levels of products associated with phenylpropanoid metabolism in soybean protoplasts and second, to examine the possibility of the induction of isoflavonoid phytoalexin synthesis upon treatment of protoplast suspensions with a glucan elicitor from *P. megasperma*. We have found that soybean protoplasts during a phase of extensive cell wall regeneration exhibit large increases in the rates of synthesis and catalytic activities of two of the enzymes of phenylpropanoid pathways, phenylalanine ammonia-lyase and chalcone synthase. These increases are associated with a marked accumulation of glyceollin and probably also glycinol. It, therefore, appears that the metabolic state of the protoplasts is to some degree similar to suspension-cultured cells following treatment with the *P. megasperma* glucan elicitor or soybean seedling tissue upon infection with the fungus, *P. megasperma* [4]. Treatment of soybean protoplasts at various culture stages with the glucan elicitor at concentrations which are sufficient to induce the phytoalexin response in cultured cells did not result in any further enhancement of phytoalexin accumulation and synthesis of enzymes of the biosynthetic pathway.

There could be at least two reasons for the lack of responsiveness of the protoplasts to the glucan elicitor. One possibility is that the protoplasts are in a stressed metabolic state and the response system is saturated. Therefore, protoplasts might not be capable of responding to an additional stress signal by the elicitor. A second is that the protoplasts cannot perceive the signal evoked by the elicitor because an important factor of the signal chain is absent. This could be a component of the plant cell wall such as an endogenous elicitor [3] or of the plasma membrane which might have been altered during enzymatic wall degradation.

Our results are consistent with the assumption that the phytoalexin response is already induced during enzymatic release of soybean protoplasts from cultured cells and is maintained during culture of iso-

lated protoplasts. Substances which might contribute to the induction are the osmoticum and the endopolygalacturonase. The effect of the osmoticum on cultured cells is apparent at concentrations of higher than 100 mmol/l and is not confined to mannitol or sorbitol because sucrose is similarly active (results not shown). Fungal endopolygalacturonase is thought to act as an elicitor in castor bean *(R. communis)* by producing pectic fragments of the plant cell wall, suspected obligate intermediates in the induction of the castor bean phytoalexin casbene [26]. Endopolygalacturonase has previously been shown to induce phytoalexin synthesis in soybean cells [7]. The enzyme concentration used in this study to give a phytoalexin response in cultured cells (Table III) is considerably lower (1/24) than that employed for protoplast isolation. The lack of response of the soybean cells to fungal cellulase could be because the enzyme preparation causes large deleterious effects on soybean cells at concentrations far below those used for protoplast isolation when the osmoticum as stabilizing agent is absent.

Although not studied in greater detail so far our results also indicate that a combination of two potential inducers such as the endopolygalacturonase and the glucan elicitor result in a phytoalexin response which is more than additive when compared with the results from separate treatments with the substances (Table III). A synergistic effect similar to this has previously been observed between the glucan elicitor of *P. megasperma* and the endogenous elicitor of soybean tissues [3] and between β-glucan and fatty acid elicitors in potato [27]. In conclusion, our results indicate that the initial conditions during isolation of soybean protoplasts produce a stress which other types of soybean cells show upon microbial attack or treatment with elicitors. Our results resemble other observations [25, 28–30] in that isolated plant protoplasts behave as plant cells following exposure to environmental stress.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 206) and by BASF (Ludwigshafen, FRG). We gratefully acknowledge a gift from Prof. K. Hahlbrock (Köln) of antisera. We thank S. Holmes for help during the initial stages of these studies and Prof. W. Briggs for valuable suggestions during the preparation of this manuscript.

[1] J. W. Mansfield, in: Phytoalexins (J. A. Bailey and J. W. Mansfield, eds.), pp. 253–288, Blackie, Glasgow 1982.
[2] N. T. Keen, J. E. Partridge, and A. I. Zaki, Phytopathology **62,** 768 (1972).
[3] A. G. Darvill and P. Albersheim, Annu. Rev. Plant Physiol. **35,** 243–275 (1984).
[4] J. Ebel, in: Bioregulators, Chemistry and Uses (R. L. Ory and F. R. Rittig, eds.), Amer. Chem. Soc. Symposium Series **257,** 257–271 (1984).
[5] J. Ebel, A. R. Ayers, and P. Albersheim, Plant Physiol. **57,** 775–779 (1976).
[6] A. Hille, C. Purwin, and J. Ebel, Plant Cell Reports **1,** 123–127 (1982).
[7] J. Ebel, W. E. Schmidt, and R. Loyal, Arch. Biochem. Biophys. **232,** 240–248 (1984).
[8] J. Ebel, M. R. Stäb, and W. E. Schmidt, in: Primary and Secondary Metabolism of Plant Cell Cultures (K.-H. Neumann, W. Barz, E. Reinhard, eds.), pp. 247–254, Springer, Berlin 1985.
[9] D. Grab, R. Loyal, and J. Ebel, Arch. Biochem. Biophys., in press.
[10] A. Leube and H. Grisebach, Z. Naturforsch. **38c,** 730–735 (1983).
[11] M.-L. Hagmann, W. Heller, and H. Grisebach, Eur. J. Biochem. **142,** 127–131 (1984).
[12] M. Hagmann and H. Grisebach, FEBS Lett. **175,** 199–202 (1984).
[13] E. Schmelzer, H. Börner, H. Grisebach, J. Ebel, and K. Hahlbrock, FEBS Lett. **172,** 59–63 (1984).
[14] G. D. Lyon and P. Albersheim, Plant Physiol. **70,** 406–409 (1982).
[15] M. G. Hahn, A. L. Darvill, and P. Albersheim, Plant Physiol. **68,** 1161–1169 (1981).
[16] E. A. Nothnagel, M. McNeil, P. Albersheim, and A. Dell, Plant Physiol. **71,** 916–926 (1983).
[17] N. Nelson, J. Biol. Chem. **153,** 375–380 (1944).
[18] M. Somogyi, J. Biol. Chem. **195,** 19–23 (1952).
[19] A. R. Ayers, J. Ebel, B. Valent, and P. Albersheim, Plant Physiol. **57,** 760–765 (1976).
[20] A. Zimmermann and K. Hahlbrock, Arch. Biochem. Biophys. **166,** 54–62 (1975).
[21] J. Schröder, W. Heller, and K. Hahlbrock, Plant Sci. Lett. **14,** 281–286 (1979).
[22] W. M. Bonner and R. A. Laskey, Eur. J. Biochem. **46,** 83–88 (1974).
[23] W. Schaffner and C. Weissmann, Anal. Biochem. **56,** 502–514 (1973).
[24] P. Moesta, M. G. Hahn, and H. Grisebach, Plant Physiol. **73,** 233–237 (1983).
[25] E. Galun, Annu. Rev. Plant Physiol. **32,** 237–266 (1981).
[26] D. F. Jin and C. A. West, Plant Physiol. **74,** 989–992 (1984).
[27] C. L. Preisig and J. A. Kuć, Arch. Biochem. Biophys. **236,** 379–389 (1985).
[28] D. E. Cress, Plant Cell Reports **1,** 186–188 (1982).
[29] J. Fleck, A. Durr, C. Fritsch, T. Vernet, and L. Hirth, Plant Sci. Lett. **26,** 159–165 (1982).
[30] G. S. R. L. Rao, J. H. M. Willison, and W. M. N. Ratnayake, Plant Physiol. **75,** 716–719 (1984).