Stilbene Synthase from Seedlings of *Pinus sylvestris*: Purification and Induction in Response to Fungal Infection

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The heartwood of pine trees contains considerable amounts of the stilbenes pinosylvin and pinosylvin monomethyl ether. In young plants of *Pinus sylvestris*, however, amounts of these stilbenes are hardly detectable. At the onset of stress conditions, such as UV light or fungal attack, stilbene formation is greatly increased and pinosylvin becomes the preferentially formed phenol. It is considered to be the main phytoalexin produced by pines and may be responsible for induced resistance. A unique synthase acting on cinnamoyl-CoA is the key enzyme for the formation of the stilbenes. Stilbene synthase (pinosylvin-forming) was purified to near homogeneity from 4-wk-old plants treated with UV light. The purification included affinity chromatography on red agarose, molecular sieving, and chromatofocusing. In addition to malonyl-CoA as substrate, stilbene synthase uses cinnamoyl-CoA six times more efficiently than *p*-coumaroyl-CoA. The stilbene synthase preparation was virtually free of chalcone synthase activity. The enzyme is a homodimer with a subunit molecular weight of 43,000. Separation by chromatofocusing indicates the presence of multiple forms differing in isoelectric points between pH 5.1 and 4.6. Treatment of pine seedlings with *Botrytis cinerea* leads to a 38-fold increase in stilbene synthase activity within 1 day. This is a rapid response and can be measured prior to any detectable lesion, blight, or sclerotia.

Additional keywords: induction by UV light, 5-phenylethenyl-benzene-1,3-diol.

Phenolic substances with a stilbene skeleton have been found as constituents in the cambium and heartwood of trees (Billek 1964; Hillis and Carle 1962; Kindl 1985). The special disease resistance of some tropical wood is correlated with a high concentration of hydroxylated stilbenes (Billek 1964; Grundon and King 1949; Hart and Shrimpton 1979). In this respect, the stilbenes have been classified as compounds preventing the decay of wood by microorganisms (Hart 1981; Rudman 1963; Rennerfelt 1945) or as agents against attack by bark beetles and other insects (Billek 1964; Wolcott 1953). Fungi symbiotically associated with pine beetles may also be affected by derivatives from phenylpropanoids (Bridges 1987). Pinosylvin and oxyresveratrol have been reported to have fungistatic activity (Hart 1981; Hart and Hillis 1974). An oligomeric stilbenoid, viniferin, shows a broad antibiotic action against many fungi (Ingham 1976; Langcake and Pryce 1977).

An increasing amount of data shows that some of the stilbenoid constituents may function as phytoalexins in these plants during the first few weeks of growth (Ingham 1976; Schöppner and Kindl 1979; Hart 1981; Dercks and Creasy 1989). Noteworthy examples of plants with stilbenes showing this physiological function are most species of the genus *Pinus* that synthesize pinosylvin and pinosylvin monomethyl ether (Rudloff and Jorgensen 1963; Schöppner and Kindl 1979). The formation of stilbenes can be induced in young plants of *P. sylvestris* L. by UV light and stress (Schöppner and Kindl 1979). Determining whether biotic elicitors originating from fungi also induce the enzymes responsible for stilbene formation was one of the goals in this investigation.

The pathway to hydroxystilbenes originates from L-phenylalanine and includes the activities of L-phenylalanine ammonia-lyase (PAL), an acyl-CoA synthetase, and stilbene synthase (Billek 1964; Kindl 1985; Schöppner and Kindl 1979). Comparison of the enzyme activities indicates that the stilbene synthase activity presents the limiting factor in the pathway leading to pinosylvin and its monomethyl ether. If so, the change in gene expression of stilbene synthase (F. Melchior and H. Kindl, unpublished) is the crucial point in the induced resistance of plants relying on the function of stilbenoids as putative phytoalexins.

Stilbene synthase activities have been determined for *Rheum rhaponticum* L. (Rupprich and Kindl 1978), peanut (Schöppner and Kindl 1984), and various Vitaceae (Fritzemeier and Kindl 1981). A preliminary report on the occurrence of a pinosylvin-forming stilbene synthase in seedlings from *P. sylvestris* has been given (Schöppner and Kindl 1979). Investigations on the different steps of gene expression (Vornam et al. 1988) of a resveratrol-forming stilbene synthase from cell suspension cultures of *Arachis hypogaea* L. have shown that the elicitor-dependent increase of gene expression can also be studied in cell cultures. Work on the genomic structure (Schröder et al. 1988) of the resveratrol-forming stilbene synthase indicated a close similarity of this enzyme with the chalcone synthase from various sources.

We describe here the purification and partial characterization of a new stilbene synthase whose activity is dramatically increased upon stress. Some of the stress conditions used, especially the biotic signals originating from fungal cells, mimic situations of physiological relevance under environmental conditions.

MATERIALS AND METHODS

Materials. Coenzyme A esters of various cinnamic acids were synthesized as described previously (Stöckigt and Zenk 1975; Fritzemeier and Kindl 1983) and purified as given previously (Fritzemeier and Kindl 1983; Brodie and Porter 1960). [2-^{14}C]Malonyl-CoA (1.9 GBq/mmol), [1-^{14}C]acetyl-CoA (2.1 GBq/mmol), and L-[1-^{14}C]phenylalanine (1.8 GBq/mmol) were obtained from Amersham-Buchler, Braunschweig, Federal Republic of Germany. Polyclar AT was purchased from Serva, Heidelberg, Federal Republic of Germany. Reactive Red 120-agarose (type 3000) came from Sigma Chemical Co., St. Louis, MO, and other column materials including Polybuffer were from Pharmacia, Freiburg, Federal Republic of Germany.

Culture conditions and treatments. Seeds of *P. sylvestris* were obtained from Staatdarre Wolfgang, Hanau, Federal Republic of Germany, and the seedlings were grown and maintained for 3–6 wk on vermiculite in sterilized tap water at 20° C and under a regime of 12 hr of light at 22° C and 12 hr of dark at 18° C.

After the growth period indicated, the intact seedlings were harvested; wounded on needles, stems, and roots with a scalpel; and treated with 255 nm UV light for 30 min using a Camag inspection lamp without a filter at a distance of 10 cm. Subsequently, the plants were kept at 100% humidity for the time indicated. Alternatively, the needles of intact seedlings were dipped into a suspension of the conidia of *Botrytis cinerea* Pers.:Fr. in 0.5% (w/v) sucrose for 2 min. The suspension contained 1×10^4 conidia per milliliter. Then the plants were kept at 100% humidity and 20° C for 24 hr. The induced plant material was frozen under liquid nitrogen, ground, and stored.

B. cinerea was obtained from E. Schloesser, Institute of Plant Pathology, Giessen, Federal Republic of Germany, and grown on potato-dextrose agar medium (Booth 1971).

Isolation of stilbenes and other phenols. Solvent systems were used for thin-layer chromatographic separations according to Fritzemeier and Kindl (1983).

Determination of stilbene content. Samples of 1 g were extracted three times with 5 ml of ethyl acetate each. The clear extract was concentrated *in vacuo*, and a two-phase separation between 1% (w/w) sodium bicarbonate and ethyl acetate was used to remove carboxylic acids. The organic phase was reduced to 150 μl, and the phenols were measured by HPLC using a reversed-phase column (Pharmacia Pep-S5μm, 4 × 250 mm). A linear gradient starting with 20% methanol, 80% 10 mM phosphoric acid and ending with 100% methanol was applied at a flow rate of 1.0 ml/min for 1 hr. The phenol peaks of the eluant were monitored at 280 nm by a variable wavelength monitor (LKB 2141) in connection with an integrator (LKB 2221). Authentic samples of pinosylvin and pinosylvin monomethyl ether were used for quantitation of the peak heights.

Enzyme assays. Stilbene synthase activity was determined with cinnamoyl-CoA and [2-^{14}C]malonyl-CoA as substrates as previously described (Rupprich and Kindl 1978). PAL activity was measured with L-[1-^{14}C]phenylalanine according to Kindl (1970).

Routine enzyme test. One gram of powdered plant material and 0.15 g of Polyclar AT were suspended in 2.5 ml of 20 mM HEPES-NaOH, pH 7.5, containing 2 mM mercaptoethanol. Homogenization was with an Ultraturrax for 3 × 10 sec at full speed. The homogenate was centrifuged at 100,000 × g for 1 hr at 4° C. The supernatant (0.8 ml) was passed through a Sephadex G-50 column equilibrated with the same buffer as given above. The void volume was discarded, and 1.2 ml eluted after the void volume was collected. Forty microliters of this solution was used for the enzyme assay.

Enzyme purification. Four-week-old plants were treated with UV light as described above. Twenty grams of induced plant material was transferred into a mortar, covered with liquid nitrogen, and finely powdered with a pestle. The powder was mixed with 3 g of Polyclar AT and extensively blended with 100 ml of buffer containing 10 mM phosphate, pH 6.4, and 2 mM mercaptoethanol using an Ultraturrax for 3 × 30 sec. The clear extract was prepared by centrifugation at 7,000 × g for 30 min. Recentrifugation of the supernatant at 130,000 × g for 180 min was performed to pellet the cytoplasmic membranes and high molecular weight proteins. The supernatant of the second centrifugation was slowly pumped during 3.5 hr onto a small column filled with 6.5 ml of Reactive Red 120-agarose. The column was washed with 50 ml of the buffer containing 10 mM phosphate, pH 6.4, and 2 mM mercaptoethanol at a rate of 1 ml/min. Stilbene synthase activity was developed with 250 mM phosphate, pH 8.0, and 2 mM mercaptoethanol. The 5.4 ml of eluate with the peak activity was applied to an Ultrogel AcA-44 column (1.65 × 94 cm) equilibrated with 20 mM HEPES-NaOH buffer, pH 7.5, containing 2 mM mercaptoethanol. Stilbene synthase was eluted with the same buffer (0.15 ml/min flow rate; 3-ml fractions).

For further purification and separation into differently charged forms, the peak fractions obtained after molecular sieving were pumped, at a flow rate of 1.5 ml/hr, onto a column for chromatofocusing with PBE-94 (Pharmacia) previously equilibrated with 25 mM histidine-HCl, pH 6.2, and 2 mM mercaptoethanol. The fractionation was by elution with Polybuffer 74, pH 4.0, diluted 1:8 with 2 mM mercaptoethanol. The flow rate was 0.34 ml/min, and fractions of 1.36 ml were collected.

Other methods. Sodium dodecyl sulfate-PAGE was used to estimate the degree of enrichment of the stilbene synthase. Electrophoresis was done with 10% polyacrylamide gels according to Köller et al. (1979). Protein bands were detected by staining with Coomassie Brilliant Blue R 250. The molecular mass of native enzyme was determined by gel permeation chromatography. Protein content was determined according to Bradford (1976) using bovine serum albumin as the protein standard. Radioactivity was determined by liquid scintillation counting. Absolute radioactivity was calculated by a calibration program based on the channels ratio method.

RESULTS

The pinosylvin content in unchallenged 4-wk-old pine seedlings was less than 0.2 μg/g fresh weight. Stilbene synthase activity was found to be lower than 0.1 pkat/g fresh weight. Treatment with UV light, however, led to an increase in enzyme activity to 3 pkat/g within 24 hr.

Table 1. Purification of pine stilbene synthase

Fraction	Total protein (mg)	Total activity (pkat)[a]	Specific activity (pkat/mg)	Recovery of activity (%)[b]	Purification (-fold)
Crude extract	124.0	6.3	0.05	...	1
130,000 × g supernatant	65.0	9.4	0.14	...	3
Red agarose eluate	36.0	22.5	0.62	74	12
Ultrogel AcA-44[c]	2.5	19.0	7.6	62	152
Chromatofocusing[d]	0.07	1.4	20.0	5	400

[a] Enzyme activities are based on katal (kat).
[b] Since the extremely strong inhibitory effects in the crude extract did not allow us to relate the yields to the values of crude extracts, we have arbitrarily chosen the sum of activities observed during the chromatography on red agarose as 100%. Thus, 30.6 pkat was set at 100%; 6.8 pkat (20%) was found in the fraction with proteins not absorbed by the red agarose, and 1.3 pkat (4%) was detected in the buffer of the subsequent washing.
[c] In fractions 35 to 39 of the column.
[d] In fractions 32 to 36 of the column.

Thus, this kind of induction was used to provide the starting material for enzyme purification. Induction by UV light of needles from 4- or 20-yr-old pines led, under the same conditions, to an enzyme activity of 0.9 pkat/g fresh weight.

Purification of stilbene synthase from 4-wk-old plants of *P. sylvestris*. Due to the inhibition by phenols, including pinosylvin, it is not advisable to determine stilbene synthase activity in crude extracts, even in the presence of Polyclar AT. The removal of low molecular weight material is decisive both for any routine enzyme test and during the first step of enzyme purification. For this reason, the purification procedure summarized in Table 1 could not be related to the activity measured in crude extracts. Without marked loss in activity, we were able to purify the enzyme 150-fold in two quick and simple steps.

Binding of stilbene synthase to red agarose is the most crucial step in the purification of stilbene synthase. It allows the concentration of the protein and the removal of strongly inhibitory material. To avoid overloading this affinity material, the large amounts of ribulose bisphosphate carboxylase present in the extracts had to be eliminated before the binding to red agarose. This is easily achieved by sedimenting the bulk of ribulose bisphosphate carboxylase during an ultracentrifugation step according to Gezelius and Widell (1986). Monitoring this step by gel electrophoresis showed that 90% of the 54- and 17-kDa subunits belonging to ribulose bisphosphate carboxylase were removed by sedimentation (data not shown). Binding of stilbene synthase to red agarose and subsequent elution with small volumes of 250 mM phosphate, pH 8.0, is the most efficient step in concentrating diluted stilbene synthase solutions. All other standard concentrating procedures, such as precipitation with ammonium sulfate or ultrafiltration, were paralleled by great losses in activity.

Further purification of stilbene synthase was achieved by chromatofocusing. Figure 1 summarizes the profiles of this separation in combination with the gel electrophoretic analyses of the respective fractions. This last step in the isolation procedure, leading to a 400-fold purification, was paralleled by great loss of activity, partly due to the fact that the activity was split into several peaks. The separation was according to the charge of proteins and showed the occurrence of at least three multiple forms. The highest specific activity and total activity were in fraction 34. This form, characterized by an isoelectric point of pH 5.1, was purified to near homogeneity. The other peaks correspond-
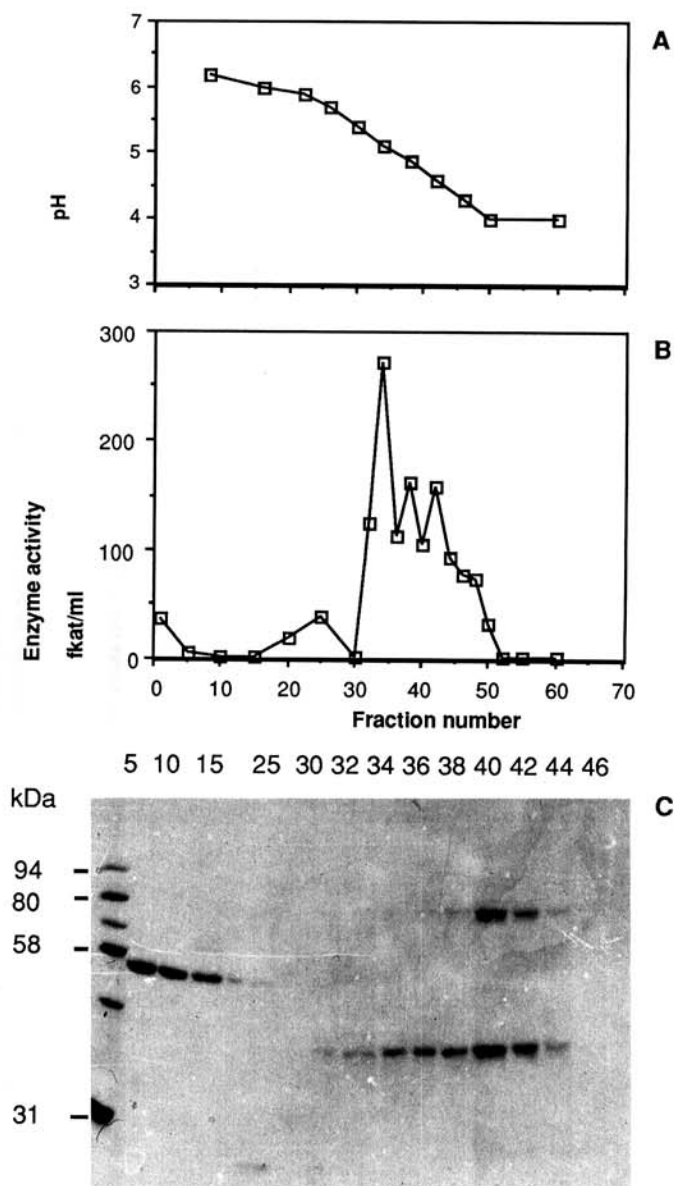
Fig. 1. Separation of stilbene synthase isoenzymes by chromatofocusing (last step in purification). **A**, pH gradient as determined in the fractions collected. The profile of stilbene synthase enzyme activity (**B**) and the electrophoretic analysis of proteins in the fractions obtained (**C**) are compared.

ing to a pH of 4.9 (fraction 38) and 4.6 (fraction 42) contained major contaminations by a monomeric protein showing an M_r of 83,000–86,000.

Properties of purified pine stilbene synthase. Product analysis showed that the stilbene synthase preparation was essentially free of chalcone synthase activity. Furthermore, we were unable to detect significant amounts of 3,5-dihydroxystilbene 2-carboxylic acid, a possible intermediate in the formation of 3,5-dihydroxystilbene. The enzyme showed absolute specificity for malonyl-CoA as the substrate used for chain lengthening. Radioactive acetyl-CoA at a concentration of 2–100 μM yielded no detectable conversion into pinosylvin (series of data not shown). The selectivity toward the second substrate, cinnamoyl-CoA, is not absolute as depicted in Table 2.

The molecular properties of pine stilbene synthase were found to be similar to stilbene synthases converting p-coumaroyl-CoA instead of cinnamoyl-CoA. Determination of the molecular weight by analytical chromatography on Ultrogel AcA-22 afforded a value of 95,000 (data not shown). This agrees with the properties observed with other
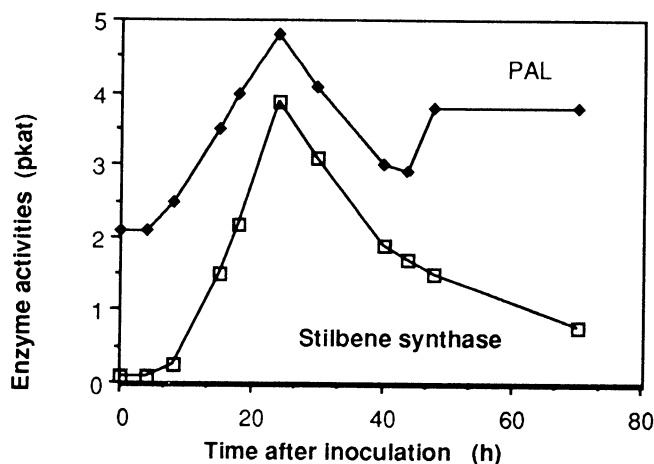
stilbene synthases. The subunit molecular weight estimated from migration on polyacrylamide gels is 43,000. The isoelectric point is in agreement with the occurrence of multiple forms in the range of pH 4.5–5.3.
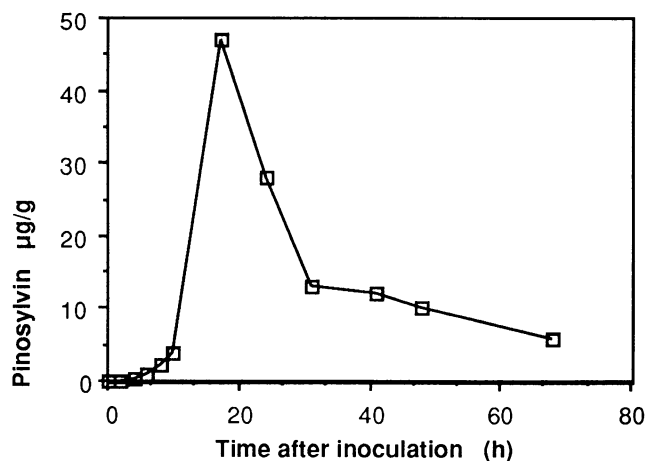
Increase in enzyme activity in plants treated with B. cinerea. During normal growth of pine seedlings, stilbene synthase activity is detectable only with very sensitive methods. UV light activates the formation of stilbenes and stilbene synthase. Even more pronounced was the induction of stilbene synthase when infection with B. cinerea was used as a biotic stimulus. Figure 2 shows the profiles of enzyme activities of stilbene synthase and PAL during the time period after inoculation with B. cinerea. The data show that the increase of stilbene synthase activity is dramatic even after 10 hr and may be utilized as a rapid and very sensitive indicator of biotic stress. It is especially remarkable that the first quick response of pine seedlings upon contact with the fungus takes place before the formation of lesions or any damage detectable by light microscopy. The induction of stilbene synthase activity was 38-fold (Fig. 2) while that of PAL was much less.

In intact plants and without artificial wounding, the fungus stimulated stilbene formation in a rather rapid mode (Table 3). The profile of pinosylvin content shows a transient course (Fig. 3). This is in part due to the fact that

Table 2. Comparison of conversions of potential substrates catalyzed by pine stilbene synthase

Expt.	Substrate[a]	Product	Activity (nkat)
1	Cinnamoyl-CoA	Pinosylvin	40
2	Cinnamoyl-CoA[b]	Pinosylvin	ND[c]
3	p-Coumaroyl-CoA	Resveratrol	6
4	Feruloyl-CoA	Rhapontigenin	2
5	Caffeoyl-CoA	Piceatannol	2
6	Dihydrocinnamoyl-CoA	Dihydropinosylvin	11

[a] The various cinnamoyl-CoA esters were assayed at a final concentration of 10 μM. The concentrations of [2-¹⁴C]malonyl-CoA were 20 μM. In each experiment, 80 μl of an enzyme preparation purified through the molecular sieving step was used.
[b] In experiment 2, radioactive malonyl-CoA was substituted with 20 μM [1-¹⁴C]acetyl-CoA.
[c] ND, not detectable.

Table 3. Increase of pinosylvin and pinosylvin monomethyl ether in pine seedlings following treatment with Botrytis cinerea

	Products formed[a] (μg)	
Hours after inoculation	Pinosylvin	Monomethyl ether
0.0	0.10	0.15
2.0	0.15	0.15
4.0	0.25	0.15
6.0	0.35	0.20
10.0	3.1	1.4
20.0	46.0	20.0
24.0	28.0	36.0
30.0	14.0	42.0
50.0	12.0	47.0
72.0	9.0	42.0

[a] One gram of plant material was used in each experiment.

Fig. 2. Profiles of enzyme activities of stilbene synthase and L-phenylalanine ammonia-lyase (PAL) during the time period following infection with Botrytis cinerea. The fungus was applied to the needles of 4-wk-old pine seedlings that were then maintained at 20° C in a moist atmosphere for the time indicated. (More details on the processing of plant samples are given in the text.)

Fig. 3. Profile of pinosylvin content during the time period following infection with Botrytis cinerea. Inoculation was on the needles, while whole plants were analyzed after the time indicated.

pinosylvin is converted into pinosylvin monomethyl ether. Thirty hours after inoculation, however, the reason for the decrease in pinosylvin synthesis is mainly because of the greatly reduced activity of stilbene synthase. At that time, the first microscopic necrotic lesions became visible under the microscope.

DISCUSSION

Pinosylvin formation is characteristic of the genus *Pinus*. Induced formation of pinosylvin is especially pronounced in young plants. To study the key enzyme in stilbene formation, that is stilbene synthase, well-induced plant material is required. Sensitivity to induction of stilbene synthase is less prominent in needles from older plants. However, the occurrence of pinosylvin is generally indicative of biotic stress in needles from trees of different age. Pinosylvin formation is utilized as a very early biochemical indicator after exposure of pine to 0.5 ppm of ozone (Sandermann *et al.* 1989).

Since the young seedlings of *P. sylvestris* are extremely responsive to the induction of stilbene synthase (R. Gehlert and H. Kindl, unpublished results), they are highly suitable for the study of very early responses in the interaction of plants and fungi. In this respect, pine seedlings are clearly distinguishable from spruce seedlings that also form hydroxystilbenes but lack the prompt response in activating the genes coding for stilbene synthase (Rolfs and Kindl 1984). At present, we do not know what ecological consequences these marked differences between two gymnosperms may have on their behavior toward microorganisms. Our study provides data required for further investigations in the field of gymnosperm-fungus interaction. The amino acid sequences, derived from cDNA sequences and not further modified by posttranslational reactions, show a net charge of -5 for the grapevine enzyme subunit (Melchior and Kindl 1990) and -3 for the peanut enzyme subunit (Schröder *et al.* 1988). Therefore, an anionic structure is also likely for the pine stilbene synthase. Since stilbene synthases are encoded by a small gene family in the case of grapevine (F. Melchior and H. Kindl, unpublished), it is likely that the three forms observed upon separation according to charge (Fig. 1) actually represent isoenzymes rather than multiple forms.

To focus on the molecular mechanisms of the pine seedling in reacting upon contact with the fungus rather than on the specific resistance mechanisms in the interaction between plant and fungus, we used *B. cinerea*, which shows a broad host specificity compared to *Lophodermium pinastri* (Schrad.:Fr.) Chev. (Schuett 1971). *B. cinerea* is active at low temperatures, and its spores seldom penetrate actively growing tissue directly. It may, therefore, be applicable in a model system where the signals originating from a fungus reach the plant cell prior to damage and can be transmitted, after transformation at the plasma membrane, into the plant nucleus. The early response of the plant compared to the time required for the manifestation of an infection indicates an undefined stress situation. We are currently studying the possibility that pine seedlings are able to recognize a prepenetration signal coming from the fungus.

Work with stilbene synthases can be rather tedious and difficult. The establishment of simple and highly reproducible procedures is an important step toward achieving further progress. To this end, we established a rather quick but highly reproducible procedure leading to good enrichment within two steps. This simple and reliable methodology was the main goal of our investigations. By applying a further purification step, that is chromatofocusing, the procedure leads to an almost pure protein with a subunit M_r of 43,000. This value agrees well with the M_r of 43,000 found for the subunit of the resveratrol-forming stilbene synthase from grapevine (Liswidowati and H. Kindl, unpublished), the peptide deduced from the sequence of a full-length cDNA (Melchior and Kindl 1990), and with the M_r of 47,000 that characterizes the subunits of isoenzymes of resveratrol-forming stilbene synthases in peanut (Schöppner and Kindl 1984). In all cases, the active enzyme is a dimer.

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