Sci. Rep. Fac. Agr. OKAYAMA Univ. (59), 1 – 9 (1982)

Isolation and Partial Characterization of an Elicitor of Pisatin Production from Spore Germination Fluid of Pea Pathogen, *Mycosphaerella pinodes*[a)]

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Received November 1, 1981

Summary

An elicitor of pisatin accumulation was isolated and partially characterized from spore germination fluid, mycelial extract, and cell wall preparation of a pea pathogen, *Mycosphaerella pinodes*. The elicitor was found to be a polysaccharide or glycoprotein and the active component was composed of glucose. The approximate molecular weight was 70,000 daltons. This elicitor elicited pisatin accumulation in pea leaves at a concentration of 10 μg/ml and the maximum activity was ca. 350–400 μg/ml, but did not elicit phytoalexins in soybean, bean, and red clover. High molecular weight preparations from the mycelial extract and mycelial wall of *Mycosphaerella melonis* and *Stemphylium sarcinaeforme*, nonpathogens of pea, elicited pisatin accumulation in pea leaves.

Introduction

Molecular basis for regulation of phytoalexin biosynthesis have been called attentions to elucidate the resistant mechanism of plants against parasites, and a number of reports has indicated the presence of substances which induce phytoalexin accumulation in metabolites of fungi [1–6,8,9,11,15,18–21,26,27]. These substances were named "elicitors" by KEEN *et al* [19]. We reported recently that a pea pathogen, *Mycosphaerella pinodes* suppressed pisatin accumulation at an early stage of infection to avoid the inhibitory activity of pisatin on infection process by producing suppressors of low molecular weight peptides in spore germination fluid. The elicitor of pisatin accumulation was also contained in the spore germination fluid together with suppressors, and the suppressor counteracted the activity of elicitor [26]. After the infection of *M. pinodes* was established, the fungus induced high level of pisatin in host tissue, which we call "the second phase phytoalexin [24]".

In the present paper, we report the properties of the partially purified elicitor which was produced in the spore germination fluid of this fungus.

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a) This work was supported by a grant from the Ministry of Education, Science and Culture of Japan (No. 336006).

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Materials and Methods

Preparation of crude elicitor

Mycosphaerella pinodes (Berk et Blox.) Bestergren, IFO No. OMP-1 was cultured on V-8 juice agar medium at 25°C for 5 days. Pycnospores formed on the medium were collected by scratching colony surface with platinum loop and suspended in the deionized water to give about 4 million spores per ml. The spores in the water were allowed to germinate at 20°C for 24 hr, and filtered through Millipore filter (SMWP 02400). The filtrate was fractionated into high and low molecular weight fractions by ultrafiltration using Toyo Ultra Filter (UK 50). The concentrated high molecular weight fraction was dialyzed against deionized water at 0 − 4°C for overnight. The inner dialyzate (high molecular weight fraction) was used as the crude elicitor and subjected to further purification.

Crude elicitors were also prepared from mycelial extract and mycelial wall of *M. pinodes*, *M. melonis*, and *Stemphylium sarcinaeforme*.

These fungi were shake cultured in the modified Czapek medium (30 g glucose, 5g peptone, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 6 mg thiamine mononitrate, 2 mg pyridoxine-HCl, 2 mg riboflavin, and 3 mg calcium pantothenate in 1000 ml water), at 20° C for 7 days.

For the preparation of crude elicitor from mycelial extract, 20 g of mycelia were homogenized with 50 ml of distilled water in Waring blendor, filtered through Millipore filter (SMWP 02400) and the filtrate was treated as described above.

For the preparation of crude elicitor from mycelial wall, the homogenate of mycelia was filtered through sintered glass funnel, the residue was again homogenized three times in water, once in chloroform-methanol mixture (1 : 1 v/v), and finally in acetone. The residue (wall fraction) was dried in a dessicator under reduced pressure for 72 hr. The wall fraction was suspended in water, autoclaved for 20 min at 121°C, and the filtrate was concentrated under reduced pressure.

Partial purification of elicitor

The crude elicitor obtained from spore germination fluid, mycelial extract, or mycelial wall fraction was purified by gel filtration using Sephadex G-200 which had been equilibrated in distilled water. Two ml of crude elicitor were applied onto the Sephadex G-200 column, eluted with distilled water at the flow rate of 51 ml/hr, and 6.7 ml fractions were collected by fraction collector. Each fraction was assayed for elicitor activity, sugar, and protein content.

Determination of elicitor activity

The activity of elicitor to accumulate phytoalexins was determined in several leguminous plants which were grown in a growth chamber (3,000 lux light/day) at 24°C for about 2 weeks. The phytoalexins accumulated in respective legminous plant were analyzed spectrophotometrically after the fractionation on TLC plate (Silica gel GF_{254}, Merck), by using authentic phytoalexins as the standard. The developing solvent was benzene : ethyl acetate : isopropanol (90 : 10 : 1, v/v) for pisatin and maackiain, chloroform : ethyl acetate (95 : 5, v/v) for glyceollin, and chloroform : methyl alcohol (100 : 3, v/v) for phaseollin.

To estimate pisatin inducing ability, the lower side epidermis of pea leaves was removed and the mesophyll tissue was treated with 50 μl of sample solution per leaf. The treated leaves were incubated at 20 − 22°C for 24 hr. The leaves were then extracted with ethanol (10 ml/fresh weight) at 60°C for 30 min, concentrated and the amount of pisatin was determined by measuring the absorbancy at 240 − 330 nm

and calculated by the method described by CRUICKSHANK and PERRIN [10].

To estimate glyseollin or phaseollin inducing ability, leaves, hypocotyls and epicotyls of soybean or bean were used. Leaves were cut into 0.5 cm^2, rubbed with carborandom (400 mesh) and then treated with 50 μl of elicitor solution per 7 – 8 leaf pieces. In hypocotyls or epicotyls, the epidermis was removed by a razor blade, and the stripped hypocotyls or epicotyls were washed with distilled water and treated with elicitor solution. The amount of phytoalexin accumulated was analyzed by measuring the absorbancy at 280 nm for phaseollin and 285 nm for glyceollin.

For maackiain inducing ability, leaves of red clover were treated as in the case of pea leaves, and the amount of maackiain accumulated was analyzed by measuring the absorbancy at 310 nm.

Determination of protein

The amount of protein in each fraction was determined by the method of LOWRY *et al.* [22] using bovine serum albumin as a standard.

Determination of carbohydrate

The amount of carbohydrate in each fraction was determined by the method of DUBOIS *et al.* [14] and the concentration was expressed as glucose equivalent (μg/ml).

Separation of protein and carbohydrate in crude elicitor solution

Protein and carbohydrate were separated from each fraction by extraction with chloroform [7]. One ml of chloroform was added to 1 ml of each fraction, agitated, and the aqueous phase containing carbohydrate was separated from chloroform phase which contains protein. Each phase was concentrated *in vacuo* and submitted to the determination of elicitor activity.

Determination of molecular weight of elicitor

The molecular weight of elicitor was determined by Sephacryl S-300-gel filtration using dextran T 40, T 70, and T 500 (Pharmacia Fine Chemicals) as standards. A 2 ml portion of the elicitor was applied to the water equibrated Sephacryl column (2.2×30 cm, 114.09 ml bed volume), eluted with distilled water, and fractionated. Each fraction was assayed for carbohydrate content and elicitor activity, and the approximate molecular weight was estimated by comparing the elution profile with that of standard dextrans.

Hydrolysis and determination of sugar component of elicitor

The elicitor was hydrolyzed in 1 N HCl for 1 hr at 100 °C. The hydrolysate was concentrated *in vacuo*, added with distilled water, and concentrated again. This treatment was repeated until the pH became neutral. The hydrolysate was trimethylsilylated by the method of SWEELY *et al.* [28] and analyzed by gas chromatography (Shimadzu Model GC-4CM). The analytical conditions were as follows : Column 5 % SE-52 coated on Chromosorb W, 60/80 mesh, running temperature 115 – 250 °C, N_2 flow rate 50 ml/min, attenuation $10^2 \times 32$, inlet pressure 6 kg/cm^2, H_2 flow rate 0.5 ml/min, detector FID, injection temperature 280 °C. The retention time was compared with standard trimethylsilyated sugars.

Results

Partial purification of elicitor from spore germination fluid

Clude elicitor which was obtained by ultra-filtration of spore germination fluid was further fractionated by gel filtration through Sephadex G-200. Sugar-, protein content,

and the pisatin inducing ability (elicitor activity) of each fraction were determined (Fig. 1).

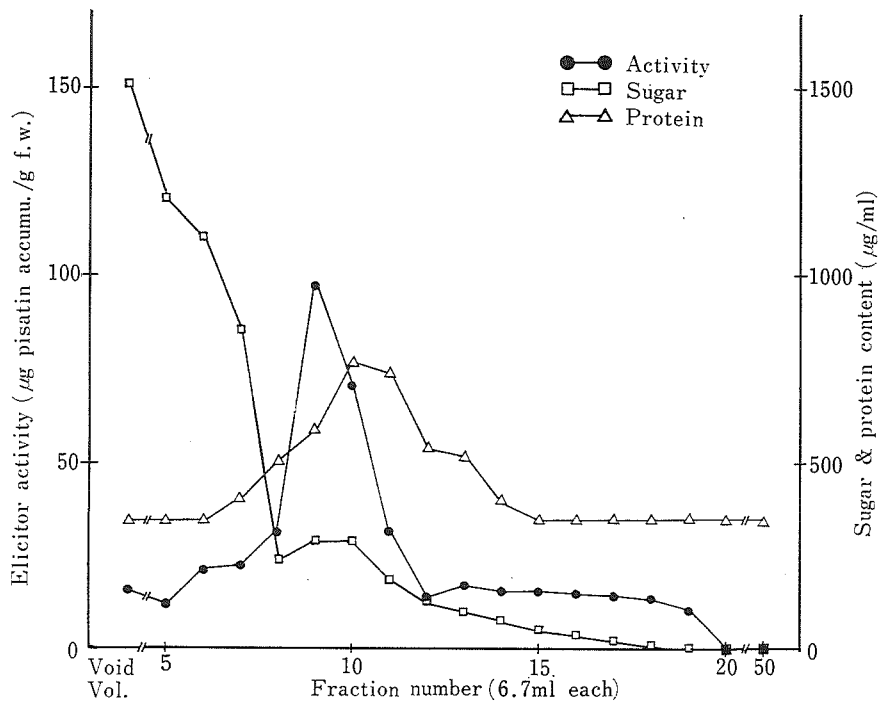

Fig. 1 Elution profile of elicitor activity, sugar, and protein of high molecular weight fraction from spore germination fluid of *M. pinodes* through Sephadex G-200 column.

As indicated in the figure, the elution profile of the elicitor activity coincided with the peak of carbohydrate rather than protein.

To confirm the above results that elicitor is mainly composed of carbohydrate, crude elicitor was extracted with chloroform, and the aqueous and the chloroform fractions were assayed for elicitor activity (Table 1).

Table 1 Effect of chloroform extraction and mild acid hydrolysis on the activity of elicitor from spore germination fluid of *M. pinodes*

Treatment of elicitor	Activity (Pisatin accumulated in μg/g fresh wt. leaves)
H_2O control	0
Non-treated original elicitor*	136.1
Aqueous phase after chloroform extraction	116.3
Chloroform phase	12.1
Hydrolysate	0

* The concentration of original elicitor was 374.7 μg glucose equivalent/ml.

The result clearly show that elicitor activity was found in aqueous phase, but not in chloroform phase. Mild hydrolysis of the crude elicitor lost the elicitor activity completely. In other words, the active component in the spore germination fluid to induce pisatin might be polysaccharide.

Approximate molecular weight of the elicitor

By comparison of elution profile through Sephacryl S-300 of the elicitor and standard dextrans, the molecular weight of the elicitor was estimated approximately 7×10^4 daltons (Fig. 2).

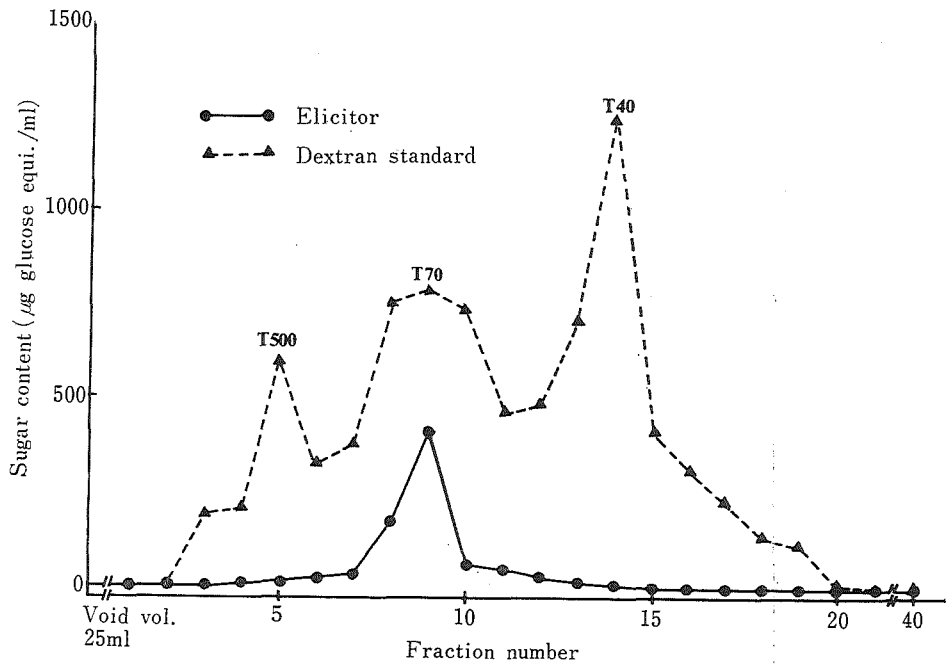

Fig. 2 Determination of approximate molecular weight of elicitor from spore germination fluid of *M. pinodes* by Sephacryl S-300 gel filtration.

Sugar constituent of the elicitor

Crude- and partially purified elicitors were hydrolyzed with 1 N HCl for 1 hr, and the hydrolyzates were analyzed by gas liquid chromatography after trimethylsilylation.

Table 2 shows the retention time of the samples and the trimethylsilylated authentic

Table 2 Retention time of the trimethylsilylated hydrolysate of elicitor and authentic monosaccharides

Sample (trimethylsilylated)	Retention time (min)			
Glucose	18.25	20.25		
Galactose	17.5	18.5	20.5	
Mannose	16.25	18.25		
Hydrolysate of crude elicitor	16.25	17.5	18.25	20.25
Hydrolysate of partially purified elicitor	18.25	20.25		

monosaccharides. Thus, the elicitor was found to be composed mainly of glucose.

Table 3 Composition of elicitor from spore germination fluid of *M. pinodes*

Elicitor analyzed	Percentage of			
	Glucose *	Galactose *	Mannose *	Protein
Clude elicitor	5.62	—	0.59	82.34
Partially purified elicitor	61.03	—	—	34.54

* Sugar content of elicitor was determined by gas chromatography after trimethylsilylation of the hydrolyzate, using authentic sugars as standards.

As shown in Table 3, partially purified elicitor contains about 34% of protein, hence, it is not clear whether the pure elicitor is polysaccharide or glycoprotein at present, though the protein content reduced through purification without losing elicitor activity.

Biological activity of elicitor

The minimum concentration of elicitor for the effective induction of pisatin was 10 μg glucose equivalents/ml, and the optimum seemed to be 350−400 μg/ml (Fig. 3).

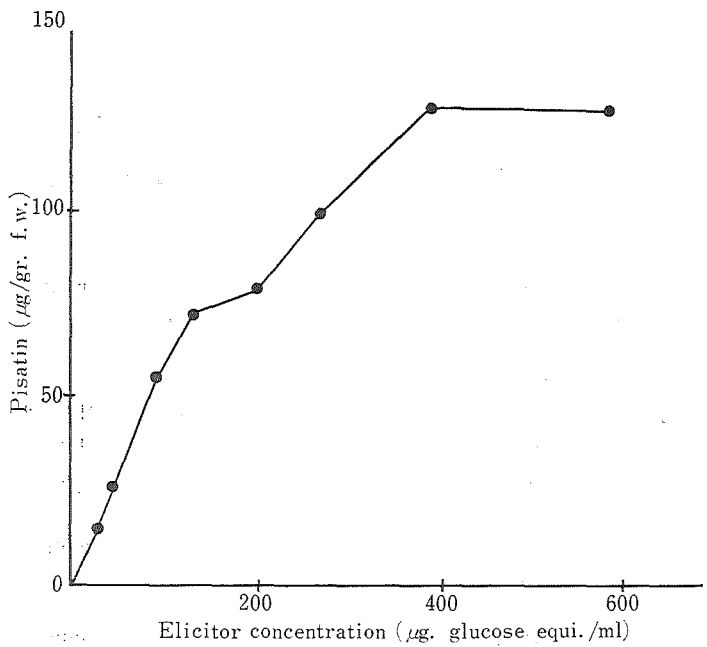

Fig. 3 Dosage response curve of elicitor to pisatin accumulation.

The elicitor isolated from spore germination fluid of *M. pinodes* was tested for induction of phytoalexin in several non-host plants.

The serial dilutions of elicitor were applied to leaves, hypocotyls or epicotyls of legminous plants, pea, soybean, bean, and red clover, and the accumulation of each phytoalexin was determined by the method described above (Table 4).

Table 4 Activity of elicitor from spore germination fluid of *M. pinodes* to accumulate phytoalexins on several leguminous plants

Plant species tested	Phytoalexins *	Accumulation of phytoalexins (μg/g f.w.) Dilution of elicitor (times)					
		H_2O(Cont.)	1**	2	3	4	5
Pisum sativum L.	Pisatin	−	138.4	97.3	54.0	31.6	16.0
Glycine max L.	Glyceollin	−	−	−	−	−	−
Phaseolus vnlgaris L.	Phaseollin	−	−	−	−	−	−
Trifolium pratense L.	Maackiain	−	−	−	−	−	−

* Phytoalexins were determined 24 hr after incubation at 20°C.

** The concentration of original elicitor was 394 μg glucose equivalents/ml.

The elicitor elicited only pisatin on pea. Glyceollin, phaseollin, or maackiain were not elicited in respective host plant.

Evidence for the presence of the same elicitor in mycelial extract and wall fractions of M. pinodes

Crude elicitor was also prepared from mycelial extract and mycelial wall fractions as described above, and examined for elicitor activity and approximate molecular weight. As the results, it was found that the same elicitor as that from spore germination fluid was present in both fractions of mycelia. That is, the approximate molecular weight of elicitors from mycelial origin was ca. 7×10^4 daltons, and elicited only pisatin on pea.

Induction of pisatin in pea leaves by elicitors of the other fungal origins

High molecular weight fractions were prepared from spore germination fluids, mycelial extracts, and wall fractions of *M. melonis* and *S. sarcinaeforme*, nonparasitic on pea, by the same method as in *M. pinodes*, and tested for induction of pisatin in pea leaves (Table 5).

Pisatin was found to be elicited by all fractions of these fungi.

Table 5 Elicitor activity of high molecular weight fractions (HMF) from several plant pathogenic fungi to accumulate pisatin in pea leaves

Fungal species	Host plant	HMF obtained	Pisatin accumulated (μg/g f.w. leaves)	Sugar content in HMF (μg glucose equi./ml)
Mycosphaerella pinodes	pea	Spore germ. fl.	16.0	100.0
		Mycelial extract	95.9	134.7
		Mycelial wall	70.5	41.3
Stemphylium sarcinaeforme	Red clover	Spore germ. fl.	74.6	94.7
		Mycelial extract	188.4	100.3
		Mycelial wall	130.7	70.3
Mycosphaerella melonis	Melon	Spore germ. fl.	257.7	88.0
		Mycelial extract	236.2	94.7
		Mycelial wall	114.4	154.7

Discussion

Pea seedling responds generally to incompatible or some of the nonpathogenic fungi at an early stage of infection by accumulating the 1st phase phytoalexin, but the

compatible pathogen, after the infection was established[23]. In the previous paper[26], we reported that a pea pathogen, *M. pinodes* produced in the spore germination fluid both elicitor and suppressor of pisatin accumulation, and suppressor plays a role in the disappearance of pisatin at the 1st phase in this host-parasite combination. Treatment of pea leaves with the suppressor inhibited the accumulation of the 1st phase pisatin by inoculation with incompatible fungi and rendered the treated leaves susceptible to nonpathogen, *Stemphylium sarcinaeforme* and *Alternaria kikuchiana*[25]. Thus, it seemed to be probable that the host specificity of this fungus might be determined by the pathogen-produced suppressor of defense reaction of the host.

The suppressors of pisatin production produced by *M. pinodes* were reported to be low molecular weight peptides[26].

The present paper shows that the elicitor of pisatin produced by *M. pinodes* in the spore germination fluid is polysaccharide or glycoprotein of which main sugar constituent is glucose. The same elicitor had been found in the mycelial extract and mycelial wall fraction of this fungus.

Although several phytoalexin elicitors have been isolated from mycelia of pathogenic and nonpathogenic fungi and characterized as glucans, most of them was extracted by means of drastic experimental conditions such as homogenization of mycelia, repeated washing, and finally autoclaving[4,16,17]. These elicitors were demonstrated to have activity to elicit phytoalexins, but no evidence has been obtained yet that these elicitors actually play roles in defense reaction of the host-parasite interfaces.

Our elicitor was extracted from spore germination fluid of *M. pinodes* together with the suppressor of low molecular weight peptides. In our experiments, suppressor was active and elicitor was inactive when the leaf surface of pea was treated without artificial injury. The elicitor activity was found to be active when leaf surface was rubbed with carborandom or applied to the mesophyll tissue directly on the epidermis -eliminated leaves. These results indicate that, in the host-parasite interface, the low molecular weight suppressor secreted by spores reached into plant cells faster than the elicitor, and suppressed the elicitation of defense reaction by high molecular weight elicitor.

KEEN[18] reffered the race-specificity of *Phytophthora megersperma* var. *sojae* on soybean to the specificity of elicitor activity on host cultivars. However, glucans isolated from the same fungus which have elicitor activity were reported to be non -race-specific[5]. DOKE reported that some cell wall components of late blight fungus of potato elicited hypersensitive cell death and rishitin accumulation to all varieties of potato tested[12], and hypersensitive necrosis to several plants belonging to Solanaceae, Leguminosae, and Liliaceae[13].

Our elicitor was active only to pea and did not elicit phytoalexins in bean, soybean and red clover, but the host specificity of *M. pinodes* seemed to be determined by the suppressor of defense reaction, which is secreted into the spore germination fluid[25].

Acknowledgement

Financial support from Sankyo Co., Ltd., is acknowledged..

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エンドウ褐紋病菌の胞子発芽液に含まれるピサチン蓄積誘導物質の分離と性質

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エンドウにピサチンの蓄積を誘導する物質を，エンドウ褐紋病菌の胞子発芽液，菌糸抽出液，菌糸細胞壁分画から分離し，その性質についてしらべた．誘導物質はブドウ糖を構成単糖とする多糖類あるいは糖蛋白であり，分子量は約70,000ダルトンである．本物質は10 μg/mlでエンドウにピサチン蓄積を誘導し，最大活性は350〜400 μg/mlである．しかし，ダイズ，インゲン，赤クローバーにはファイトアレキシンの蓄積を誘導しない．他植物の病原菌，*Mycosphaerella melonis*, *Stemphylium sarcinaeforme* の菌糸抽出液，菌糸細胞壁分画から抽出した高分子分画はエンドウに対しピサチン蓄積を誘導する．

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