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SCLEROTIUM ROLFSII PHOSPHATIDASE INDUCED PERMEABILITY CHANGE IN MUNG BEAN (*PHASEOLUS AUREUS*) HYPOCOTYLS[1,2]

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Abstract

Sclerotium rolfsii phosphatidase B was purified 100 folds by Ammonium sulfate fractionation and DEAE cellulose chromatography. The purified enzyme was able to induce electrolyte leakage from mung bean hypocotyls.

Introduction

Sclerotium rolfsii Sacc. is known as a serious pathogen on many crops of economic importance in most of tropics and subtropics of the world (Aycock, 1966). Economic losses are substantial each year, although it is impossible to place a monetory value on losses caused by the fungus.

The rapidity with which this organism can destory susceptible host tissue is due to the fact that it is able to produce large quantity of a number of enzymes during pathogenesis (Bateman, 1968; Bateman, 1969). The ability of phytopathogens to produce phosphatidases is wide spread (Berak *et al.*, 1972; Huang and Goodman, 1970; Lumsden, 1970; Mount and Bateman, 1969; Tseng *et al.*, 1970; Tseng and Chang, 1970). Among these, *S. rolfsii* was the most active in this capacity of the pathogens tested, and it was able to produce B type of phosphatidase when cultured on autocalved bean hypocotyls or during pathogenesis (Tseng and Bateman, 1968; Tseng and Bateman, 1969). However, the role for *S. rolfsii* phosphatidase in plant pathogenesis has not been established, although the action of phosphatidases (Lecithinases) has been suggested as a possible cause of changes in the permeability of plasma memberanes of infected plant tissues (Thatcher, 1942; Tribe, 1955).

The purpose of this investigation were (i) to purified the phosphatidase produced by the fungus, and (ii) to examine the permeability changes in mung bean hypocotyls treated with the purified phosphatidase.

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Materials and Methods

Sclerotium rolfsii Sacc (isolate 14) was used for this investigation. Stock cultures was maintained on potato dextrose agar (PDA) at 25 C, and it was transferred at one month intervals. The extracts of bean tissues infected with *S. rolfsii* were served as the crude enzyme sources as previously described (Tseng and Bateman, 1969).

Enzyme assay

Since the phosphatidase produced by *S. rolfsii* has heen identified as a phosphatidase B, the enzyme activity was quantitatively assayed by measuring acyl-ester contents of the substrate in the reaction mixture using the procedures described by Snyder and Stephens (1959). Reaction mixtures contained 0.5 ml of 0.05 M citrate buffer (pH 4.5) containning 2.75 μmole soybean lecithin and 0.5 ml of the enzyme preparations. The reation mixture was incubated at 30 C for 1 hr.

Enzyme purification

Lyophilized lesion extracts of *S. rolfsii* were used as the crude enzyme source. Five grams of lyophilized extracts were dissolved in 100 ml of distilled water and dialyzed against several liters of eold distilled water for 24 hr at 4 C. The dialyzed solution was brought to 50% saturation with powdered ammonium sulfate, allowed to stand for 1 hr at 5 C, and centrifuged for 20 min at 20,000 g. The precipitate was collected and dissolved in 10 ml of cold distilled water, this procedure was repeated for ammonium sulfate saturation of 60, 70, 80 90%. The precipitate of each fraction was immidiately assayed for protein content and the phosphatidase activity. The concentrated phosphatidase fractions were combined, dialyzed and applied to a 2.5 × 40.0 cm column of diethylaminoethyl cellulose (Cl^- form) (DEAE cellulose) packed with distilled water as the liguid phase. The column was eluted with 100 ml of distilled water followed by a citrate buffer (pH 4.5) concentration gradient. Five ml fractions were collected and immediately assayed for the enzyme activity. Enzyme purification achieved by these procedures was based in increasing in specific activity (units/mg protein). Protein measurements were made by using the method of Lowry *et al.* (1951), crystalline bovine serum albumin was used as the reference protein. One unit of enzyme activity was defined as the amount of enzyme hydrolysis 1.1 microeqivalent of acyl-ester in reaction mixure at 30 C for 1 hr.

Measurement of electrolyte leakage

Eight grams of mung bean seeds (*Phaseolus aureus*) were sterilized in 0.5% sodium hypochloride solution for 20 min, rinsed with sterilized water, and germinated at 30 C in a sterilized petri-dish (13 × 13 cm) with wet cotton for keeping moisture. Three days after, hypocotyls were cut into small sec-

tions (2 × 2 × 2 mm), 500 mg of the sections were then transferred to beakers containing 5 ml of the enzyme preparations in buffer with 50 ppm streptomycin, the beakers containing the sections were placed in a water bath shaker at 30 C for a certain period of time. Conductivity of the bathing solution was measured with a YSI (Yellow Springs instrument CO.) Mode 31 conductivity bridge, using a dip type electrode cell (K=1.0). Conductance was given as μmhos per mg wet weight.

Results

Purification of S. rolfsii phosphatidase B

When the dialyzed crude enzyme preparations were subjected to ammonium sulfate fractionation, the phosphatidase was mainly found in the precipitate obtained from the 60 to 70% ammonium sulfate fraction. This was fraction used for further purification by DEAE cellulose column chromatography. Only one phosphatidase B peak was obtained and it was eluted between 330 and 440 ml, and the most active fraction was obtained around 380 ml (Fig. 1).

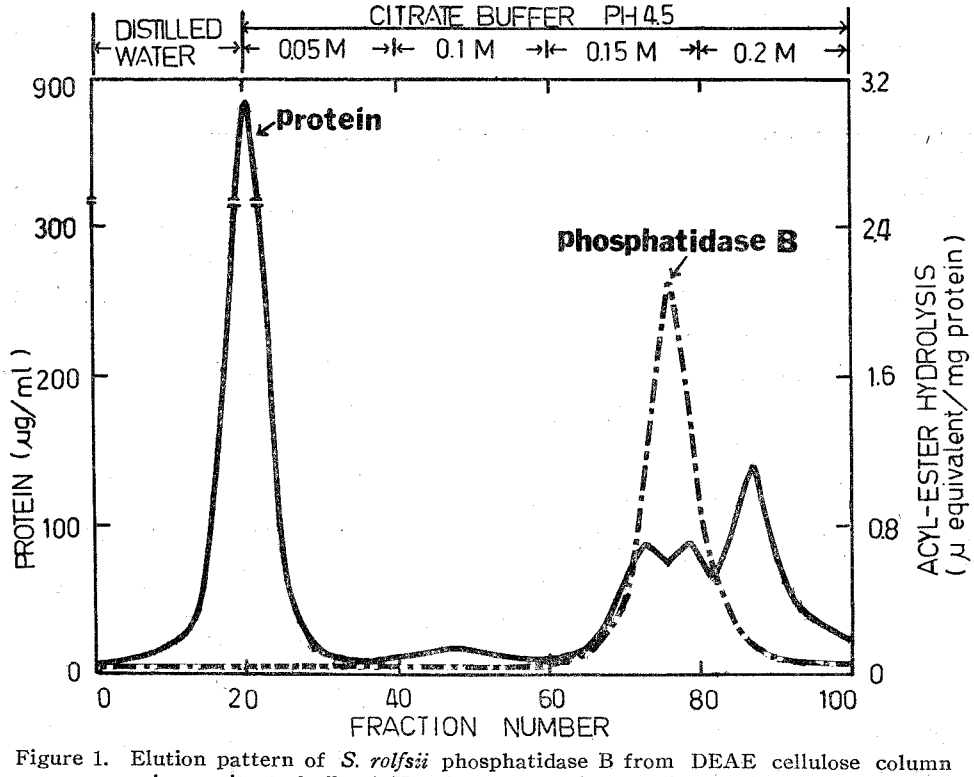

Figure 1. Elution pattern of *S. rolfsii* phosphatidase B from DEAE cellulose column using a citrate buffer (pH 4.5) concentration gradient. Five ml of dialyzed enzyme from 60-70% ammonium sulfate fraction were applied to a DEAE cellulose column (2.5 x 40.0 cm) at 5 C. Five ml fractions were collected and the phosphatidase was immediately assayed by acyl-ester method (Snyder and Stephens, 1959).

The purification achieved by ammonium sulfate fractionation and DEAE column chromatography was 100 folds (Table I). The partially purified enzyme was further checked for proteinase, cellulolytic and pectic enzyme activities. The results indicated that the enzyme preparation free from proteinase, endopolygalacturonase, cellulase, and enzymes which attack carboxylmethyl cellulose, araban, galactan, galactomanan, and xylan at pH 4.5.

Table 1. *Purification of* ***Sclerotium rolfsii*** *phosphatidase B*

The enzyme activity was estimated by acyl-ester method (Snyder and Stephens, 1959). The crude enzyme consisted of 5.0 g of lyophilyzed lesion extract in 100 ml cold water and dialyzed against several liters of distilled water at 5 C for 24 hr. Five ml of the 70% ammonium sulfate fraction was layered on the DEAE cellulose column and eluted with a citrate buffer (pH 4.5) concentration gradient. All of the enzyme was eluted in one peak consisted of 110 ml solution.

Fraction	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg protein)	Purification (fold)
Crude enzyme	100	0.30	1.16	0.51	—
Ammonium sulfate fraction (70%)	5	0.58	0.43	2.70	5.30
DEAE cellulose column	20	1.00	0.02	50.00	100.00

Effect of purified phosphatidase B on electrolyte leakage from mung bean hypocotyls

The phosphatidase free from other enzmes was used for this purpose. When 500 mg of mung bean hypocotyls were treated with the purified enzyme preparations, only the enzyme prepared in 0.1 M citrate buffer (pH 4.5) was able to induce electrolyte leakage into the bathing solution (Table 2.). The

Table 2. *Effect of purified phosphatidase B on electrolyte leakage from mung bean hypocotyls*

Each treatment contained 5 ml of buffer or purified phosphatidase B preparations with 50 ppm streptomycin to prevent bacteria contamination. The amount of streptomycin in bathing solution does not interfer the phosphatidase activity. Active enzymes were prepared in 0.1 M of citrate (pH 4.5) or phosphate (pH 7.0 and 8.0) buffer. Electrolyte leakage was measured by a conductivity bridge as described in the text.

Treatment	Conductance change in bathing solution (μmhos/mg wet weight)					
	Time of incubation (hour)					
	0	1	2	6	12	20
Citrate buffer (pH 4.5)	100	101	101	101	101	101
Active enzyme (pH 4.5)	104	164	185	190	200	200
Active enzyme (pH 7.0)	110	114	115	115	116	117
Active enzyme (pH 8.0)	113	114	114	115	115	116
Autoclaved enzyme	101	101	102	103	103	104

drastic change of conductance has been observed after 1 hr incubation. There were no difference in the rates of electrolyte leakage in the other active enzyme treatments. Since the phosphatidase B has an optimum pH at acidic range (Tseng and Bateman, 1968), the leakage effect of the enzyme preparation was apparently due to its enzyme activity.

Discussion

Increased loss of electrolytes by tissues invaded by plant pathogens has been observed in group of diseases (Hancock, 1968; Lai *et al.*, 1968; Thatcher, 1942; William and keen, 1967). There is evidence that these permeability changes can be attributed to enzymes of pathogen origin in diseases involving such pathogens as *Rhizoctonia solani* (Lai *et al.* 1968), *Botrytis cinera* and *Scleatium sclerotorium* (Thatcher, 1942). The types of enzymes responsible for this alternation of suscept membrane has not been eluciated. Phosphatidase B in *S. rolfsii* infected bean tissues was considered to be of fungal origin, and it was suggested the phosphatidase produced by *S. rolfsii* may be associated with disease development (Tseng and Bateman, 1969).

The presence of phospatidase in infected tissue early in disease development suggests that it may play a role in pathogenesis (Lumsden, 1970). Beraka *et al.* revealed that avirulent mutant of *Erwinia carotovora* was associated with no phosphatidase activity and low levels of polygalacturonase, pectate lyase, and cellulase. Studies with phosphatidase from snake venom (Condea and Devries, 1965) and *Clostridum welchii* (Ansell and Spanner, 1965) demonstrated that the enzyme is quite toxic to animal tissue. This toxicity is believed to be associated with the destruction of phosphatides in biological membrane (Selein and Logan, 1965). Current studies with the purified phosphatidase B from *S. rolfsii* showed that this enzyme was able to induce electrolyte leakage from mung bean hypocotyls. The fact that the rapid change of conductance in bathing solution after one hour incubation is of interesting. It may be the results of alternation of suscept membranes by the B type phosphatidase produced by *S. rolfsii.* Recent studies by Tseng and Mount (1973) demonstrated that both purified phosphatidase C and protease caused lysis of the protoplast. Leakage of neutral red from the protoplast was evident before bursting of phosphatidase C and protease treated protoplasts indicating a modification of cellular membranes of host tissue. The current need is to isolate protoplasts from host tissue, and to treat them with the *S. rolfsii* phosphatidase. Since the enzyme acts on protoplasts without cell wall, one can eliminate the cell wall as the enzyme barrier, thus the role of the phosphatidase in plant pathogenesis would be better understood.

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白絹病菌產生的磷脂分解酵素對於綠豆苗半透性膜的影響

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白絹病菌所產生的B型磷脂分解酵素，經硫酸氨分劃和纖維素色層分析法，純化步驟，將原來酵素純化100倍。用這種酵素去處理綠豆苗的結果，發現它有改變綠豆苗組織半透性率的能力。