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SCLEROTIUM ROLFSII PHOSPHATIDASE B

I. Inhibition of mung bean mitochondrial oxygen uptake by the phosphatidase[1][2]

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Abstract

Sclerotium rolfsii phosphatidase B, both crude and purified enzymes, was able to inhibit mung bean mitochondrial oxygen uptake.

Introduction

Phosphatidases (phospholipases) have been extensively studied in relation to injury of animal mitochondria. However, little attention has given to the effects of phosphatidases on plant mitochondria, particularly the enzymes produced by phytopathological organisms.

Disruption of mitochondria in intact and isolated skeletal muscles has been observed when the tissues were treated with the alpha toxin (phospholipase C) of *Clostridium welchi*. This disruption of mitochondria was attributed to the hydrolysis of the mitochondrial lipids by the action of the toxin (Slein and Logan, 1965). Several workers have shown a decrease in P/O ratio of isolated mitochondria as a result of phospholipase A action *in vitro* (Petrushka *et al.*, 1959, Rossi *et al.*, 1962) and *in vivo* (Aravindakahan and Braganca, 1959). Recently, electron microscopical study revealed that *Agkestrodon piscivorus* venom and phospholipase A produced an identical morphological alteration in mitochondria and that structural disruption accompanies respiratory decline (Augustyn *et al.*, 1970).

Previous report (Tseng and Lee, 1973) from this laboratory has shown that the purified *S. rolfsii* phosphatidase B was able to induce electrolyte leakage from mung bean hypocotyls. This paper presents the results of the effect of the phosphatidase on the mung bean mitochondrial oxygen uptake.

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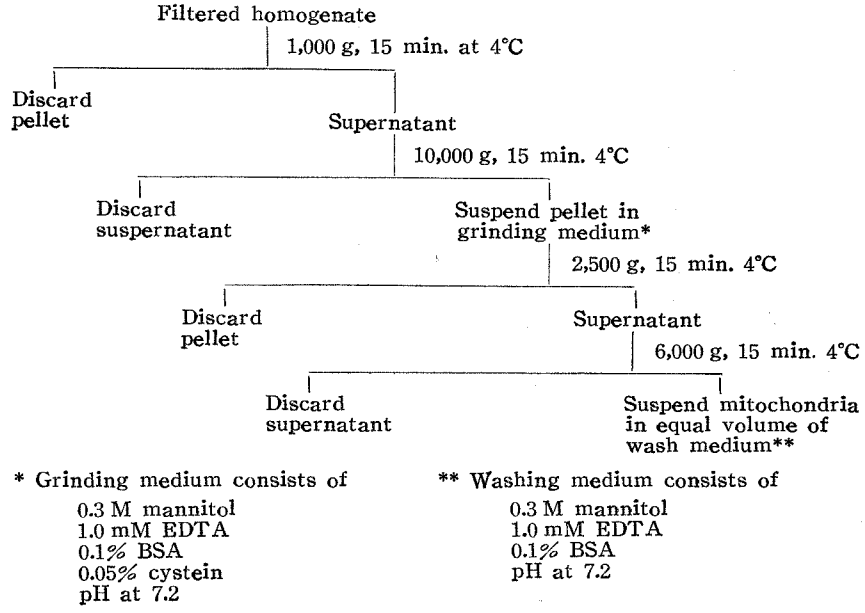
Materials and Methods

Purification of S. rolfsii phosphatidase B

S. rolfsii Sacc. (isolate 14) was used throughout this investigation. The lesion extracts of bean hypocotyls infected with the fungus were used as crude enzyme (Tseng and Bateman, 1969). The purified phosphatidase B from crude enzyme preparation was achieved by ammonium sulfate fractionation and DEAE cellulose column chromatography. This purified enzyme free from protease, *endo*-polygalacturonase, cellulase, and enzymes which attack hemicellulose was used as enzyme source (Tseng and Lee, 1973).

Isolation of mitochondria from mung bean hypocotyls

Mung bean (*Phaseolus aureus*) was used for this purpose. Seeds were soaked in 0.5% NaClO for 10 min and in water for 2 hr. The soaked seeds were then germinated and grown on Nylon screen holders which were placed over water-filled trays in a growth chamber at 30°C in dark. Five days after, the beans were harvested and only the hypocotyls were used for mitochondrial isolation. The hypocotyls were cut into small pieces about 2–3 cm long, and were ground in "Virtis 45" homogenizer for 40 seconds then ground for 50 seconds in a cold moter with 100 ml of grinding medium. All operations were carried out in a cold room at 5°C and the ground tissues were maintained at pH 7.2. The homogenate was squeezed through two layers of cheesecloth, and the filtrate was processed to obtain mitochondria as shown in the following diagram:



Assay for oxygen uptake of isolated mitochondria from mung bean hypocotyls

The Warburg apparatus was used to determine oxygen uptake (Umbret *et al.*, 1957). Flasks containing the reaction medium were chilled before the mitochondrial suspensions were added. Equilibration time was set in 10 min and all experiments were run at 30°C. Each flask contained 1 ml of mitrochondrial suspension and 2 ml of reaction mixture at various pH values. The reaction mixture consisted of 0.1 M buffer (citrate or phosphate) containing 40 μM pi, 300 μM sucrose, 2 μM cytochrome C, and 20 μM sodium succinate. For measuring the effect of *S. rolfsii* phosphatidase on mitochondrial respiratory activity, 0.8 ml of the enzyme preparation (crude or purified enzyme) was added to the flask side arm and poured into the reaction medium after 180 min incubation. Oxygen uptake was expressed as μl/mg dry weight of mitochondria.

Results

Effect of pH on the isolated mitochondria from mung bean hypocotyls

Six milligrams of mitochondrial suspension was obtained from 1,000 g of 6 days old mung bean hypocotyls following the isolation procedures. Vital examination of the isolated mitochondria has been carried out by coloration with Janus green, which stains mitochondria greenish blue. This staining is due to the action of the cytochrome oxidase system present in mitochondria, which maintains the dye in its oxidized (colored) form. In the surrounding cytoplasm the dye is reduced to a colorless leukobase. The respiratory activities of the isolated mitochondria were assayed at various pH values. Results from Figure 1 showed that the isolated mitochondria exhibited a maximum oxygen uptake at pH 6.5 and no response was found when the pH of reaction mixture were below 4.0 and above 8.0.

Effect of S. rolfsii phosphatidase B on mung bean mitochondrial oxygen uptake

Preliminary study (Tseng and Bateman, 1969) indicated that the optimum pH for the phosphatidase activity was about pH 4.5, and no enzyme activity was detected in the enzyme source at pH values greater than pH 6.0. Since the optimum pH for isolated mitochondrial oxygen uptake (Figure 1) is quite different from the phosphatidase activity, it was decided to use pH 5.5 as a pH in the reaction mixture for measuring the effect on the isolated mitochondrial oxygen uptake. Two enzyme preparations, crude and purified enzymes, were used for this purpose. Results as shown in Figure 2, when the crude enzyme was added to the reaction medium after 180 min incubation, the following oxygen uptake of the mitochondria became less as compared with

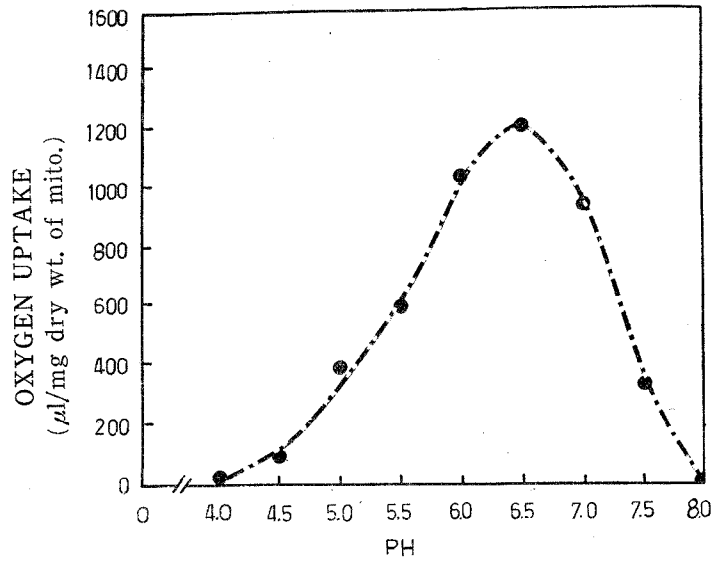


Fig. 1. Effect of pH on mitochondrial oxygen uptake. Each flask contained 1 ml of mitochondria suspension and 2 ml of reaction medium at various pH values. The reaction medium consisted of 0.1 M buffer which contained 4.0 μM Pi, 300 μM sucrose, 2 μM ATP, 0.02 μM cytochrome C, and 20 μM sodium succinate. Two buffer systems were used, 0.1 M citrate buffer for pH 4.0 to 6.5; 0.1 M phosphate buffer for pH 7.0 to 8.0. The reaction medium was carried out at 30°C for 1 hr.

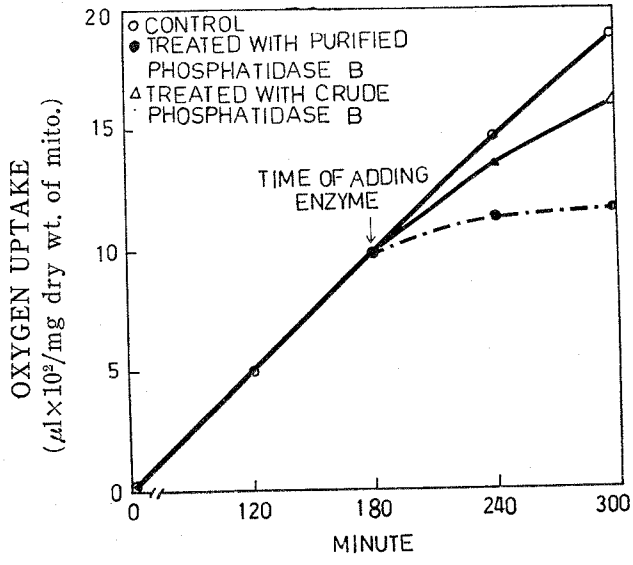


Fig. 2. Effect of *Sclerotium rolfsii* phosphatidase B on mitochondrial oxygen uptake. Each flask contained 1 ml of mitochondria and 2 ml of reaction medium at pH 5.5. The reaction medium was the same as described in Figure 1, except that 0.8 ml of the enzyme preparation (crude or purified phosphatidase B) was poured into the reaction medium after 180 min incubation. For control treatment, 0.8 ml of 0.1 M citrate buffer pH 5.5 was used.

control. Furthermore, the significant difference of oxygen uptake was found when the purified phosphatidase B was used. The rate of oxygen uptake of the enzyme treatment was 1100 μl per milligram of mitochondria as compared with 1,450 μl for control treatment after 240 min incubation.

Discussion

Studies on plant mitochondrial injury induced by phosphatidases appear to be limited as compared to that on animal systems. In 1954, Nygaard *et al.* (1954) obtained electron micrographs which suggested that morphological changes accompanied the hydrolysis of animal mitochondria by crotoxin, a purified phospholipase A from the venom of *Crotalus terrificus*. Condrea *et al.* (1965) using partially purified venom containing phospholipase A also observed these changes. Furthermore, phospholipase A has been implicated in the increase of oxygan uptake in animal mitochondria due to the uncoupling of oxidative phosphorylation (Condrea and DeVries, 1965).

On the contrary, there are little information referring to plant mitochondrial injury by phosphatidases which were produced by phytopathogens. Swelling of *Phaeolus* mitochondria by phospholipase A has been studies by Earnshaw and Truelove (1970), and they indicated that phospholipase A induced swelling of mitochondria as a result of releasing lysophospholipids and free fatty acids from the membrane. However, the enzyme which is used in this experiment was not produced by phytopathogens.

At the present, there is no information on the effects of phosphatidases (produced by phytopathogens) on plant mitochondrial O_2 uptake, except that the purified phosphatidase C obtained from *Erwinia carotovora* has been demonstrated to be able to chang the respiratory mechanism of potato mitochondria with a decrease in oxygen uptake (Tseng, 1972). The present study also shows that *S. rolfsii* phosphatidase B was able to inhibit the mung bean mitochondrial O_2 uptake. The mechanisms of decrease of oxygen uptake in the mitochondria treated with the phosphatidases (B and C) are still not established. However, it may be explained in two ways: firstly, the enzymes may act directly on the membranes to modify mitochondrial stability or act indirectly by releasing membrane-bound enzymes which in turn directly interfere with the respiratory process, secondly, the enzymes may split phosphatide molecules which are associated with certain respiratory enzymes.

Literature Cited

ARAVINDAKSHAN, I. and B. M. BRAGANCA. 1959. Oxidative phosphorylation in brain and liver mitochondria of animals injected with cobra venom. Biochem. Biophys. Acta **31**: 463-466

AUGUSTYN, J. M., B. PARSA, and W. B. ELLIOTT. 1970. Structural and respiratory effects of *Agkistrodon piscivorus* phospholipase A on rat liver mitochondria. Biochem. Biophys. Acta **197**: 185-196.

CONDREA, E. and A. DEVRIES. 1965. Venom phospholipase A: A review. Toxicon **2**: 261-273.

CONDREA E., Y. AVI-DOR and J. MAGER. 1965. Mitochondrial swelling and phospholipid splitting induced by snake venom. Biochem. Biophys. Acta **110**: 337-347.

EARNSHAW, M. J. and B. TRUELOVE. 1970. Swelling of *phaseolus* mitochondria induced by the action of phospholipase A. Plant Physiology **45**: 322-326.

NYGAARD, A. P., M. U. DIANZANI, and G. F. BAIR. 1954. The effect of lecithinase A on the morphology of isolated mitochondria. Exptl. Cell Res. **6**: 453-458.

PETRUSHKA, E., J. H. OUASTEL, and P. G. SCHOLEFIELD. 1959. Role of phospholipids in oxidative phosphorylation and mitochondrial structure. Can. J. Biochem. Physiol. **37**: 989-998.

ROSSI, C. R., C. S. ROSSI, L. SARTORELLI, D. SILIPRANDI, and N. SILIPRANDI. 1962. Protective action of phosphorylcholine on mitochondrial oxidative phosphorylation. Arch. Biochem. Biophys. **99**: 214-221.

SLEIN, M. W., and G. F. LOGAN, Jr. 1965. Characterization of the phospholipase of *Bacillus cereus* and their effect on erythrocytes, bone, and kidney cells. J. Bacteriol. **90**: 69-81.

TSENG, T. C. and D. F. BATEMAN. 1969. A phosphatidase produced by *Sclerotium rolfsii*. Phytopath. **59**: 359-363.

TSENG, T. C. and S. L. LEE. 1973. *Sclerotium rolfsii* phosphatidase induced permeability change in mung bean (*Phaseolus aureus*) hypocotyls. Bot. Bull. Academia Sinica **14**: 41-47.

TSENG, T. C. 1972. Induction and mechanisms of cellular injury of potato and cucumber tissues by enzymes produced by a soft rot bacterium, *Erwinia carotovora* (Jones) Holland. Ph. D. Dissertation, Univ. of Mass., Amherst.

UMBREIT, W. W., R. H. BURRIS, and J. F. STAUFFER. 1957. Manometric Techniques. Burgess Publishing Co., Minneapolis, 3rd edition.

白絹病菌B型磷脂分解酵素

I. B型磷脂分解酵素對綠豆粒線體氧氣吸收的抑制作用

曾聰徹　張鈴惠

中央研究院植物研究所

白絹病菌所分泌的B型磷脂分解酵素，不論純化的或未經過純化的這種酵素，都具有抑制綠豆粒腺體吸收氧氣的功用。