Effects of alamethicin on the substrate oxidations in mitochondria isolated from Crassulacean acid metabolism (CAM) plant, *Hoya carnosa*

Hoang Thi Kim Hong*, Akihiro Nose, Sakae Agarie and Takayuki Yoshida
(Faculty of Agriculture, Saga University)

CAM 植物 *Hoya carnosa* から単離したミトコンドリアにおける基質酸化特性に及ぼす alamethicin の影響

Hoang Thi Kim Hong*，野瀬昭博，東江栄，吉田隆之
(佐賀大学部 農学部)

[Introduction]

This study probes the permeability of alamethicin (AlaM) in mitochondrial membrane and effects of AlaM on respiratory property in *H. carnosa* mitochondria during light time. Malate dehydrogenase (MDH) and cytochrome c oxidase (COX) activities were measured in both intact and Triton X-100 disrupted mitochondria to confirm the permeability of AlaM inside mitochondrial matrix. AlaM (5-80 μg ml^{-1}) was directly added on state 3 in the oxidations of succinate, NADH and NADPH to investigate their effects on these substrate oxidations. The aim of our study was probed the AlaM permeabilization inside mitochondrial membrane for further investigating the respiratory property, characteristics and contributions of the internal dehydrogenases enzymes operating the substrate oxidations in the matrix of *H. carnosa* mitochondria.

[Materials and methods]

Plants were transferred to a growth chamber (KG-50 HLA, Koito Industrial Co., LTD., Japan) with a photoperiod of 12 h light and 12 h dark. The temperature in the growth chamber was maintained at 35^{o}C during the light period and 25^{o}C during the dark period with photosynthetically active radiation of 420 to 450 μmol m^{-2} s^{-1} at the mid-plant height, and a relative humidity of 70%. The methods to isolate mitochondria, to check the integrity and respiratory property of *H. carnosa* mitochondria were as previous describe (Hong et al., 2004). The oxygen consumption was measured using an oxygen electrode (Hansatech, CA1D, Japan). To permeabilize the mitochondrial preparations, AlaM (0-80 μg ml^{-1}) was added to the oxygraph champer containing 2 ml reaction medium with 0.4 mg protein ml^{-1} intact mitochondria. The reaction medium with AlaM was incubated for 3 min and three substrates as succinate, NADH and NADPH were used to initiate the reaction.

[Results and Discussion]

H. carnosa mitochondria showed about 91 and 94% intactness of inner and outer membrane, respectively (Fig. 1 and Fig. 2.). The mitochondria oxidized succinate, NADH and NADPH with high rates and coupling in which succinate was oxidized with much higher rate than NADH and NADPH (Table 1). AlaM was rapidly permeabilized in inner membrane, allowing the quantification of mitochondrial MDH. In the absence of Triton X-100, incubating the mitochondria with varied AlaM concentrations from 0 to 80 μg ml^{-1} clearly increased the MDH activities (Fig. 1). Incubating of the intact mitochondria with 5 μg ml^{-1} AlaM increased the rate from 2.41 ± 0.79 to 16.74 ± 1.49 μmol $min^{-1}mg^{-1}$, these rates were further increased via the increasing of AlaM concentrations and reached a maximum rate of 20.56 ± 1.43 μmol min^{-1} mg^{-1} at 20 μg ml^{-1} AlaM (data was collected from Fig. 1). In the presence of the detergent Triton X-100 which releases all the MDH to the assay mixture, the MDH activity remained unaffected by the AlaM treatment (Fig. 1). All of these data indicated that AlaM made the inner mitochondrial membrane permeable to NADH, and permeabilize NADH was oxidized by MDH enzyme inside of *H. carnossa* mitochondria. In the absence of Triton X-100, AlaM did not cause a significant effect on COX activity by intact mitochondria. However, addition AlaM with various concentrations from 5 to 80 μg ml^{-1} lead to a decrease in the COX activity of Triton X-100 disrupted mitochondria (Fig. 2). These data indicated that AlaM did not allow outer membrane permeable to reduced cytochrome c, but AlaM permeabilized mitochondria inhibited complex IV in the electron transport chain of *H. carnossa* mitochondria. Direct addition AlaM on state 3 were stimulated NADH and NADPH oxidations but inhibited succinate oxidation (Fig. 3). Incubation AlaM in the reaction medium at 25°C for 3 minutes lead to a full AlaM permeabilization into the matrix of *H. carnosa* mitochondria (Fig. 4).

Our results suggests that AlaM rapidly accessed into inner mitochondrial membrane and the AlaM permeabilization provides a valuable tool for measurement of the specific internal dehydrogenases activities which were hidden within the *H. carnosa* mitochondrial matrix.

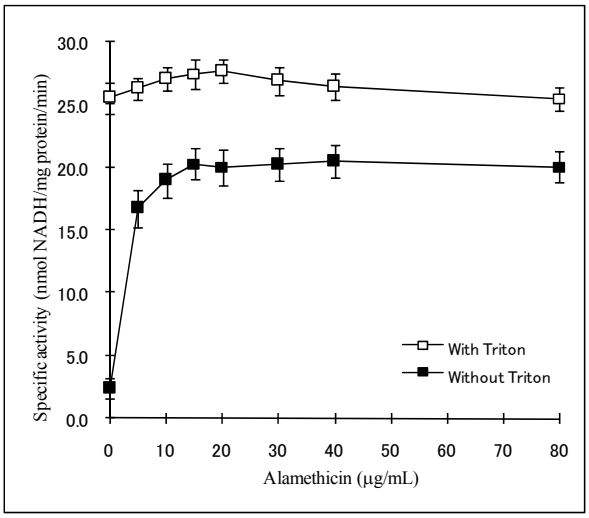

Fig. 1. Effect of ALaM concentration on NADH-dependent MDH activity in *H. carnosa* mitochondria. Mitochondria (5 μg protein ml^{-1}) were incubated with AlaM at 25° C for 3 min in reaction medium.

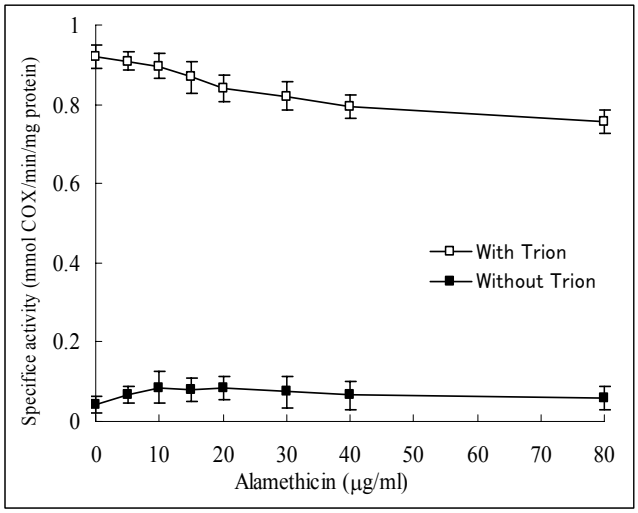

Fig. 2. Effect of AlaM concentrations on cytochrome c oxidize (COX) activity by *H. carnosa* mitochondria. Mitochondria (5 μg protein ml^{-1}) were incubated with AlaM at 25° C for 3 min in reaction medium.

Table 1. Individual and simultaneous substrate oxidation in *H. carnosa* mitochondria. Assay conditions were 10 mM succinate, 1 mM NADH, 2 mM NADPH, and 0.16 mM ADP. NADH and NADPH were oxidized in the presence of 1 mM Ca^{2+}, at pH 6.8. State 3 refers to the respiration rate of O_2 uptake in the presence of ADP; state 4 refers to the rate upon depletion of ADP. Respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 rates. Means ± SD (n = 4 - 5) of separate preparations.

Substrates	Respiration rate ($nmol\ O_2\ min^{-1} mg^{-1} protein$) State 3	State 4	RCR	ADP/O
Succinate	231 ± 35	93 ± 13	2.48 ± 0.41	1.51 ± 0.17
NADPH	147 ± 21	71 ± 9	2.07 ± 0.50	1.48 ± 0.26
NADH	187 ± 24	94 ± 10	1.98 ± 0.46	1.46 ± 0.14
NADH + NADPH	244 ± 21	99 ± 15	2.16 ± 0.15	1.21 ± 0.18
Suc + NADH	293 ± 37	113 ± 18	2.59 ± 0.23	1.33 ± 0.11
Suc + NADPH	276 ± 25	135 ± 23	2.03 ± 0.27	1.47 ± 0.15

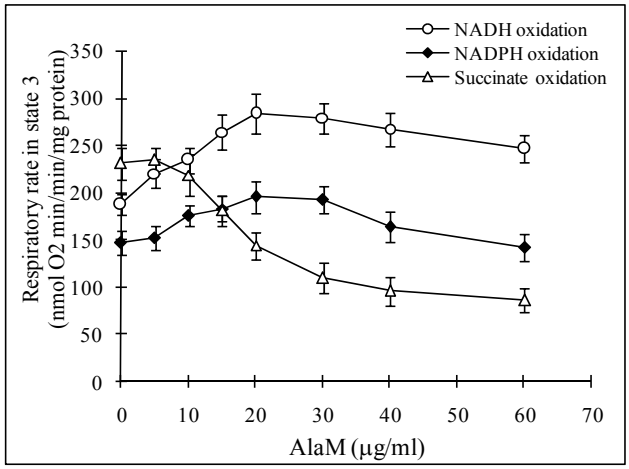

Fig. 3. Effect of the AlaM concentrations on the respiration rates of state 3 in NADH, NADPH and succinate oxidations by *H. carnosa* mitochondria.

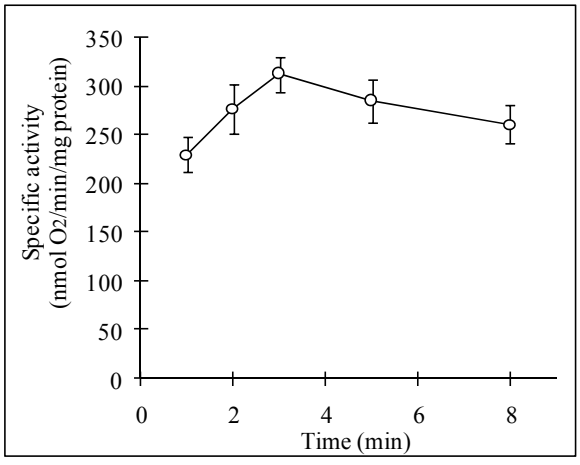

Fig. 4. Effect of the incubated time of the *H. carnosa* mitochondria with AlaM on the NADH oxidation.

[Reference] Hong HTK, Nose A, Agarie S. 2004. Respiratory properties and malate metabolism in Percoll-purified mitochondria isolated from pineapple, *Ananas comosus* (L.) Merr. cv. Smooth cayenne. Journal of Experimental Botanny. 55: 2201-2211.