Two Dimensional Proteome Map in Purified Mitochondria Isolated from Leaves of *Hoya carnosa*

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Hoya carnosa から精製したミトコンドリアタンパクの二次元電気泳動解析

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[Introduction]

In this study, we firstly used leaves of *H. carnosa* to isolate mitochondria, and check the integrity and function of the obtained mitochondria. Mitochondrial proteins were extracted in lysis buffer [40 mM Tris-HCl (pH 7.5), 50 mM DTT and 2% (w/v) Triton X-100)] with and without the protease inhibitors of Phenylmethylsulfonyl fluoride (FMSF), Leupeptin, and Monoiodoacetate (MIA) for observing their effects on the protein expression in two dimensions electrophoresis (2 DE) maps of *H. carnosa* mitochondria during light phase. Our purpose is to find out the suitable conditions for optimizing sample preparation in the application of 2 DE techniques to further investigate the mitochondria proteome of *H. carnosa*. The 2 DE proteome map of *H. carnosa* mitochondria in this study is the first step for investigating the CAM specific characteristics through mitochondrial proteome.

[Materials and methods]

The methods to isolate mitochondria, to check the integrity and respiratory property of *H. carnosa* mitochondria were as previous describe (Hong et al., 2004). Mitochondrial protein expression was investigated by using 2 DE techniques according to 2 DE manual (Amersham Biosciences). The separated proteins on the 2 DE gels were stained with Sypro Ruby (Bio-Rad, USA). The protein spots were detected and characterized by using Typhoon 9000E (Amersham Bioscience Corp., U.S.A.). The protein expression in the maps was probed by using Image Master 2D software (Amersham Pharmacia Biotech, USA).

[Results and Discussion]

H. carnosa mitochondria oxidized succinate and NADH with high rates and coupling in which succinate was oxidized with much higher rate than NADH and NADPH. In *H. carnosa* mitochondria, the COX activity was 19 times higher in the exogenously added cytochrome c than in the absence cytochrome c. The MDH activity before lysis with Triton X-100 was approximately 6% of that after lysis with Triton X-100 (Table 1). These results indicated that the intactness of the inner and outer mitochondrial membrane was acceptable. Figure 1 showed one of the typical Sypro ruby-stained 2DE maps of *H. carnosa* mitochondrial proteins. The map revealed more than three hundreds of protein sports and most protein sports were focused on a region of pH range from 4.0 to 8.5 and molecular mass range from 14 kDa to 80 kDa. The supplying Leupeptin in lysis buffer increased significantly the number of protein sports. This map yielded 397 spots, compared with 351 sports for adding PMSF or 348 sports for adding MIA or 327 sports for no treatment of the inhibitor (Table. 3). Some proteins sports were unclear or disappeared in the absence of the protease inhibitors. Detail of the effect of protease inhibitors on mitochondrial protein expression were illustrated in a dominant region of pI between 5.3 and 6.2, with molecular mass between 23.1 and 33.1 kDa from four different 2 DE maps of *H. carnosa* mitochondria (Fig. 2). Five protein spots numbered by 1, 2, 3, 4 and 5 were easily observed in this region of the map which was no treated any protease inhibitors (Fig. 2A). Except sport 1 which was unclear or disappeared, four other sports were clearly detected in this region of the maps which were treated by the inhibitors (Fig. 2B, 2C and 2D). Four different sports numbered by 6, 7, 8 and 9 were easily observed in the map which was treated by 1 mM PMSF (Fig. 2B). Three other sports numbered by 10, 11 and 12 were further detected in the map which was treated by 0.2 mM Leupeptin while sport 8 became unclear in this map (Fig. 2C). Among 12 detected spots in these maps, except sports 1 and 10 which were not detected and sports 7 and 8 which were unclear, 8 other sports were clearly observed in the map which was treated by 0.5 mM MIA (Fig. 2D). The putative functional categorization of these 12 protein sports in same region of these maps was presented in Table. 4. The isoelectric points and molecular mass of these proteins were conjectured with dataset from ExPASy by TagIdent tool.

Our results suggested that the protease inhibitors as PMSF, MIA and Leupepin clearly effected on the number proteins in the map of *H. carnosa* mitochondria. Further study on the individual and combination of these inhibitors should be conducted to discover the role of these protease inhibitors on *H. carnosa* mitochondrial proteome.

Table 1. The activities of cytochrome c oxidize (COX) and malate dehydrogenase (MDH) in *H. carnosa* mitochondria. Results shown are means ± SE (n= 4-5) of separate preparations.

COX activities (nmol mg^{-1}protein min^{1})		MDH activities (μmol mg^{-1}protein min^{-1})	
+ Cyt c:	22 ± 5	- Triton:	1.53 ± 0.08
- Cyt c:	421 ± 33	+ Triton :	26.54 ± 4.2

Table 3. Effect of protease inhibitors on the number of protein sports detected in 2DE gel

Protein preparation	Sports number
1. Protein extracted without inhibitor	327
2. Protein extracted with PMSF	351
3. Protein extracted with Leup.	397
4. Protein extracted with MIA	348

Table 2. Respiratory properties of *H. carnosa* mitochondria
Concentrations used were: 10 mM succinate, 2 mM NADH, 2 mM NADPH, 320 nmol ADP. Each value was the average of four or five independent experiments.

Substrates	Respiration rate (nmol O_2 $min^{-1}mg^{-1}$ protein)		RCR	ADP/O
	+ADP	-ADP		
Succinate	168 ± 15	71 ± 13	2.48 ± 0.41	1.51 ± 0.12
NADH	121 ± 11	58 ± 9	2.07 ± 0.50	1.48 ± 0.16
NADPH	102 ± 12	51 ± 10	1.98 ± 0.46	1.46 ± 0.14

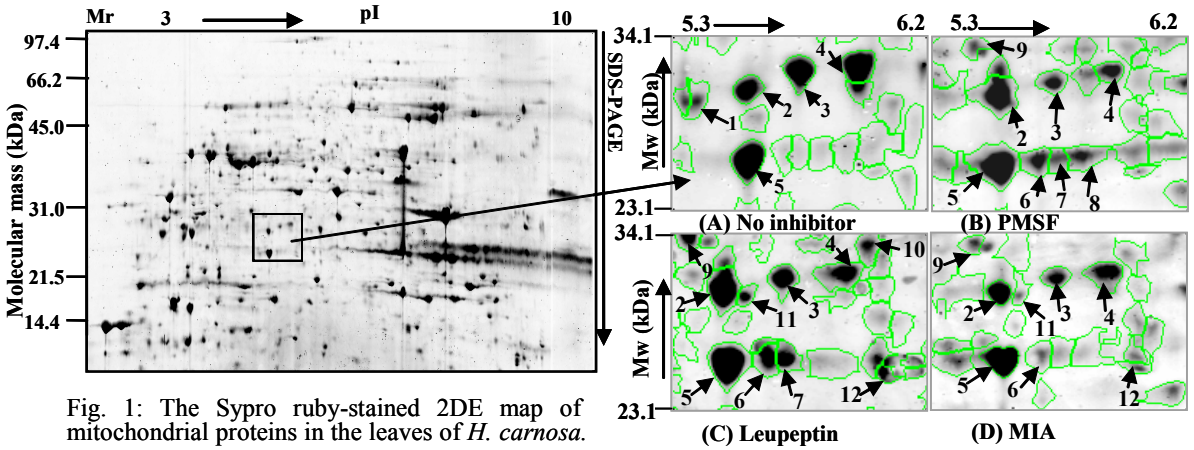

Fig. 1: The Sypro ruby-stained 2DE map of mitochondrial proteins in the leaves of *H. carnosa*.

Fig. 2. Effect of the protease inhibitors on the protein expressions in a same area of the different proteome maps from *H. carnosa* mitochondria. Mitochondrial protein were extracted in lysis buffer with and with out the protease inhibitors, where (A): with out inhibitor (B) with 1 mM PMSF, (C): with 0.2 mM Leupeptin and (D): with 0.5 mM MIA.

Table 4. The putative functional categorization of mitochondrial proteins of *H. carnosa*. Using Image master 2D platinum software together with dataset from ExPASy by TagIdent tool

No	Locus	Description	pI	Mw
1	COX2-PEA (P08744)	Cytochrome c oxidase subunit 2 (EC 1.9.3.1)	5.50	30096
2	COX2-BETVU (P98012)	Cytochrome c oxidase subunit 2 (EC 1.9.3.1)	5.57	29908
3	COX2-DAUCA (P27168)	Cytochrome c oxidase subunit 2 (EC 1.9.3.1)	5.74	30474
4	AOX1-TOBAC (Q41224)	Alternative oxidase 1, mitochondrial precursor (EC 1.-.-.-)	5.91	30873
5	CY12-SOLTU (P29610)	Cytochrome c1, heme protein, mitochondrial precursor	5.59	26028
6	PSA2B-ARATH (Q8L4A7)	Proteasome subunit alpha type 2-B (EC 3.4.25.1)	5.70	26395
7	PSA6A-ARATH (O81146)	Proteasome subunit alpha type 6-A (EC 3.4.25.1)	5.76	26397
8	PSA3-ARATH (O23715)	Proteasome subunit alpha type 3 (EC 3.4.25.1)	5.81	26397
9	MDHM2-ARATH (Q9LKA3)	Malate dehydrogenase 2, mitochondrial precursor	5.52	33052
10	AOX1C-ARATH (O22048)	Alternative oxidase 1c, mitochondrial precursor (EC 1.-.-.-)	5.95	31635
11	PSA6B-ARATH (O81147)	Proteasome subunit alpha type 6-B (EC 3.4.25.1)	5.62	28629
12	PAS6A-RATH (081146)	Proteasome subunit alpha type 6-A (EC 3.4.25.1	5.83	26395

Data were collected from ExPASy by TagIdent tool (http://tw.expasy.org/tools/tagident.html). Search was conducted in a range of pI ± 0.05 and molecular weigh ± 5 %.

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