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Effects of Protease Inhibitors on the Proteins in Two Dimensional Electrophoresis Maps of *Hoya carnosa* Mitochondria

Hoang Thi KIM HONG, Akihiro NOSE and Sakae AGARIE
(Laboratory of Tropical Crop Improvement)
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Summary

In this study, we firstly probed the protein expression on the two dimensional electrophoresis (2DE) maps of *Hoya carnosa* mitochondria during CAM phase III. Then, we investigated the effects of some protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), leupeptin (Leu), and monoiodoacetate (MIA) on 2DE maps. Mitochondrial proteins were extracted in the lysis buffer with and without one of the protease inhibitors. The mitochondrial proteins from 2DE maps were surveyed by using Image Master 2DE software (Amersham Pharmacia Biotech, USA). The results indicated that the protease inhibitors clearly affected on the number proteins in the mitochondrial 2DE maps.

Key words: *Hoya carnosa* mitochondria, Protease inhibitors, Protein spots, 2DE maps.

Introduction

The analysis and characterizations of complex protein mixtures are the central aims of proteomics. The core technology of proteomics is 2DE which simultaneously separates and displays hundreds to thousands of proteins. In the 2DE technique, the sample preparation step with high protection against proteolysis is one of the necessary steps for high quality resolution of proteins in 2DE maps.

The power of 2DE as a biochemical separation technique has virtually been recognized since its introduction. Its application, however, has significantly increased in the last few years. Due to the great diversity of protein sample types and origins, the optimal procedure for sample preparation in 2DE must be determined empirically for new sample source (Berkelman et al., 1998). In deed, some methodologies have been described to extract mitochondrial protein for 2DE; however, the sample preparations in these methods are varied depending on sample types and species. While many methodologies had been described to alleviate these problems, the definition of the optimal conditions for sample preparation from every new cell source is still somewhat of an art

Corresponding author:
e-mail: nosea@cc.saga-u.ac.jp
Tel +81-952-288723
Fax +81-952-288737

(Stasyl et al., 2001). It had been indicated that for the new materials which were initially applied to probe the protein expression by 2DE technique, the determination of the suitable conditions for mitochondrial protein extraction was necessary.

In 2DE technique, proteolysis greatly complicates analysis of the result. Proteases may be liberated upon cell disruption, thus the protein sample should be protected from proteolysis during cell disruption. When cells are lysed, proteases are often liberated or activated. Degradation of protein through protease action greatly complicates the analysis of 2DE results, so measures should be taken to avoid this problem. In fact, the 2DE technique has been widely applied to investigate the mitochondrial protein from some plants such as pea (Bardel et al., 2002), rice (Kruft et al., 2001; Millar et al., 2001; and Heazlewood et al., 2003), Zea mays (Hochholdinger et al., 2004), or nodules and root of soybean (Hoa et al., 2004), but not with Crassualacean acid metabolism (CAM) plants which are characterized by the accumulation of malate in the vacuoles at night while releasing malate into cytoplasm at the day time.

In this study, we firstly probed the protein expression on 2DE maps and effects of the protease inhibitors on 2DE maps of *H. carnosa* mitochondria during light phase. Our purpose was to find out the suitable conditions for optimizing sample preparation in the application of 2DE technique to further investigate the mitochondrial proteome of *H. carnosa*.

Materials and Methods

1. Plant material

Experimental *H. carnosa* plants were kept in a growth chamber (KG-50 HLA, Koito Industrial Co., LTD., Japan) with a photoperiod of 12 hours (h) light and 12 h dark. The temperature in the growth chamber was maintained at 35℃ during the light period and 25℃ during the dark period with photosynthetically active radiation of 420 to 450 μmol m^{-2} s^{-1} at the top of the plant. Fully expanded mature leaves of *H. carnosa* were used for mitochondrial isolation. The leaves were harvested at 6 to 7 h after the beginning of the light period.

2. Preparation of mitochondria

The methods to isolate mitochondria and to check the respiratory property of *H. carnosa* mitochondria were conducted as described previously by Hong et al. (2004). The intactness of the outer membranes and inner membranes was checked by measuring the latency of both cytochrome C oxidizes (COX, EC 1.9.3.1) and malate dehydrogenase (MDH, EC 1.1.1.37) activities. COX was assayed according to Moller and Palmer (1982) and MDH was assayed as previous reported by Hong et al. (2004). Oxygen consumption was measured using an oxygen electrode (Rank Brothers England) at 25℃ in 2 mL of assay buffer. The mitochondria were preincubated with 0.16 mM ATP for 2 min to ensure full activation of succinate dehydrogenase before each assay with succinate oxidation. NADPH oxidations were investigated at pH 6.8 with 1 mM Ca^{2+}. Respiration control rate (RCR) and ADP/O ratios value were calculated according to Estabrook (1967). The O_2 concentration in air-saturated medium was taken as 258 μM. The protein content was measured following the method of Bradford (1976) using BSA as the standard.

3. Two-dimensional gel electrophoresis

Mitochondrial protein samples were mainly prepared according to Heazlewood et al. (2003) with slight modifications, and the operating steps for 2DE were according to the instructions of manufacturer (Immobiline DryStrips, Amersham Pharmacia Biotech., USA). Mitochondrial proteins were extracted in lysis buffer [40 mM Tris-HCl (pH 7.5), 50 mM DTT and 2% (w/v) Triton X-100] in the presence and absence of 0.2 mM Leu, or 0.5 mM MIA, or 1 mM PMSF to survey the effect of these protease inhibitors on mitochondrial protein expression. Amount of 500 μg mitochondrial proteins were precipitated in 80% (v/v) cold-acetone at -20℃ for 4 h, and centrifuged at 18,000*g* for 15 min. The pellets were resuspended in IEF sample buffer consisting of 6 M urea, 2 M thiourea, 2% (w/v) CHAPS, 20 mM DTT, 2% (w/v) IBG buffer, 2% (w/v) tributylphosphine (TBP) and 0.002% (w/v) bromophenol blue (BPB) and used immediately for isoelectric focusing. Aliquots of 360 μL were used to reswell dried 180 mm, pH range of 3 to 10 nonlinear immobilized pH gradient strips during 12-15 hours. The IEF was performed for 1 min at 500 V, 1 $^{1}/_{2}$ hr at 3500 V gradient, and then held at 3500 V for 5 $^{1}/_{2}$ hours at 20℃ on a flat-bed electrophoresis unit (Multiphor II system, Amersham Pharmacia Biotech, USA). Immobilized pH gradient strips were then transferred to 15 mL of an equilibration buffer consisting of 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) BPB and 150 mg DTT for the first equilibration step and 375 mg iodoacetamide for the second equilibration step. The strips were incubated for 15 min with vibration. The equilibrated strips were slotted at 15℃ into an ExcelGel SDS XL Gradient 12-14 which performed for 35 min at 600 V, 20 mA of first steps and 1 $^{1}/_{2}$ hour at 600 V, 50 mA of second step.

4. Image analysis of two-dimensional gels

The separated proteins on the 2DE gels were stained with Sypro Ruby (Bio-Rad, USA). The protein spots were detected and characterized by using a Typhoon 9000E (Amersham Bioscience Corp., U.S.A.) scanner. The protein expressions in the maps were probed by using Image Master 2DE software (Amersham Pharmacia Biotech, USA). Molecular weight and pI standards from Amersham Pharmacia Biotech were used to confirm fixed pH gradient positioning on first dimensional separation and to identify apparent molecular mass on second dimensional separation.

Results

1. Integrity and function of *H. carnosa* purified mitochondria

In this study, we examined the integrity and function of the purified mitochondria to ensure the quality and function of the mitochondria. The intactness of the outer membrane from purified mitochondria was estimated by comparing the specific mitochondrial activity of COX before and after added external cytochrome C. And, the intactness of the inner membrane was estimated by comparing the MDH activities in purified mitochondria before and after lysis with Triton X-100. In *H. carnosa*, the COX activity was 19 times higher in the mitochondria with cytochrome C than that of without cytochrome C. The MDH activity before lysis with Triton X-100 was approximately 6% of that after lysis (Table 1). Furthermore, *H. carnosa* mitochondria oxidized succinate and NADPH at high rates and coupling in which succinate oxidation showed much higher rate

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.

Enzyme	COX activities (nmol / mg pro / min)		MDH activities (mmol / mg pro / min)
Without Cyt C	25 ± 5	Without Triton	1.53 ± 0.08
With Cyt C	421 ± 33	With Triton	26.54 ± 4.21

Table 2. Respiratory properties of *H. carnosa* mitochondria
Oxygen uptake was measured as described in "Materials and Methods". Concentrations used were: 10 mM succinate, 1 mM NADPH, 160 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
	State 3	State 4		
Succinate	168 ± 15	71 ± 13	2.48 ± 0.41	1.51 ± 0.12
NADPH	102 ± 12	51 ± 10	1.97 ± 0.46	1.46 ± 0.14

than that of NADPH oxidation. The respiration rates and ADP/O ratios in these oxidations were typical similar to mitochondria of other CAM plants (Table 2). These results indicated that the intactness of the inner and outer mitochondrial membranes were acceptable, and the mitochondria showed typical respiratory property similar to that of the other CAM plants (Hong et al., 2004, 2005)

2. Protein expression with 2DE assay

To probe the protein expression in *H. carnosa* mitochondria, the proteins were separated in a gel arrays using the apparent standard pH range from 3 to 10 and a standard molecular mass range from 14.4 to 97.4 kDa. The gels were stained with Sypro ruby and scanned by a Typhoon 9000E scanner. The new software of Image master 2DE platinum was used to detect the protein spots in the gels and to illustrate some of the dominant protein spots under 3D view. Figure 1A showed one of the typical Sypro ruby-stained 2DE maps of mitochondrial proteins from *H. carnosa* leaves under these assays. Mitochondrial proteins were dissolved in lysis buffer with a protease inhibitor of MIA. The proteome maps of mitochondrial soluble proteins showed less vertical streaking indicating that well proteins were transferred from strips into the gel. The map revealed more than three hundreds of protein spots and most protein spots were focused on a gel area of pH range from 4.0 to 8.5 and molecular mass range from 14 kDa to 80 kDa. Among the total protein spots of the maps, about 60% protein spots were distributed in a range of the molecular weight from 18 kDa to 55 kDa. Enlarged form of a small gel area from this map indicated that the overall quality of proteins was superior (Fig. 1B) and the protein spot resolution under 3D view was sharp and clear (Fig. 1C).

In this study, three protease inhibitors of PMSF, Leu and MIA were added in the lysis buffer to probe their effect on mitochondrial protein expression. Four 2DE maps of mitochondrial protein were examined and compared based on the number of spots. These maps were obtained from different preparation of mitochondrial protein by direct dissolving of mitochondrial proteins

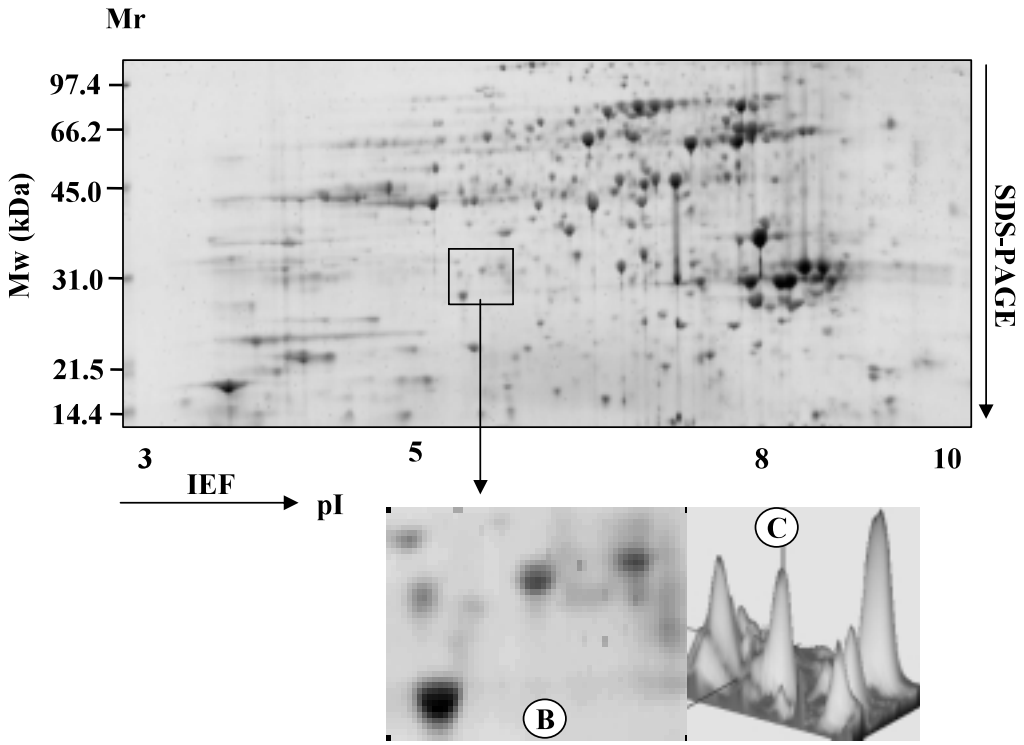

Fig. 1. The Sypro ruby-stained 2DE map of mitochondrial proteins in the leaves of *H. carnosa* (A); Enlarged form of a small gel area of this 2DE map (B); 3D view of protein spots from a small gel area cut from this 2DE map by using the Image master 2D platinum software (C).

in lysis buffer without any protease inhibitors (A); dissolved of mitochondrial protein in lysis buffer with 1 mM PMSF (B); with 0.2 mM Leu (C); and with 0.5 mM MIA (D). Spots resolution and number was consistently better when mitochondrial protein was extracted in lysis buffer supplied with the protease inhibitors. The results showed that supplying Leu in lysis buffer increased significantly the number of protein spots. This map yielded 397 spots, compared with 351 spots for adding PMSF or 348 spots for adding MIA or 327 spots for no treatment of the inhibitor (Table. 3). Some proteins spots were unclear or disappeared in the absence of the protease inhibitors.

To easily observe and compare the effect of the protease inhibitors on mitochondrial protein expression in these maps, we selected a dominant region of pI between 5.3 and 6.2, with Mw be-

Table 3. Effect of the dissolved protein in lysis buffer with and without the protease inhibitors on the number of protein spots detected in 2-DE gels

Mitochondrial protein preparation	Number of Sypro ruby-stained spots*
1. Dissolved protein buffer without inhibitor	327
2. Dissolved protein buffer with 1 mM PMSF	351
3. Dissolved protein buffer with 0.2 mM Leu	397
4. Dissolved protein buffer with 0.5 mM MIA	348

Protein sport were detected with use of Image Master 2D software (Amersham Pharmacia Biotech)

tween 23.1 and 33.1 kDa from the different gels to illustrate. This dominant region possesses main protein spots of electron transport chain (ETC) such as COX and alternative oxidize (AOX) which played an important role in cytochrome respiration and alternative respiration in ETC of *H. carnosa* mitochondria. And, we just focused on the dominant protein spots which were easily observed by eyes on the same map region. Detail of the effects of the protease inhibitors on mitochondrial protein expression in this dominant region from four different 2DE maps of *H. carnosa* mitochondria was shown in Fig. 2. Five protein spots numbered by 1, 2, 3, 4 and 5 were easily observed in this region of the map which was not treated with any protease inhibitors (Fig. 2A). Except spot 1 which was unclear or disappeared, four other spots were clearly detected in this region of the maps which were treated by the inhibitors (Fig. 2B, 2C and 2D). Four different spots numbered 6, 7, 8 and 9 were easily observed in the map which was treated by 1 mM PMSF (Fig. 2B). Three other spots numbered 10, 11 and 12 were further detected on the map which was treated by 0.2 mM Leu while spot 8 became unclear in this map (Fig. 2C). Among 12 detected spots in these maps, except spots 1 and 10 which were not detected and spots 7 and 8 which were unclear, 8 other spots were clearly observed in the map which was treated by 0.5 mM MIA (Fig. 2D).

The putative functional categorization and characterization of 12 of these protein spots of *H carnosa* mitochondria were estimated as shown in Table 4. The isoelectric points and molecular masses of protein in these maps were conjectured with the known of pI and Mw in mitochondria of other plants. Data were collected from ExPASy by TagIdent tool. Search was conducted in a range of pI $\pm$ 0.05 and molecular weigh $\pm$ 5% (http://tw.expasy.org/tools/tagident.html).

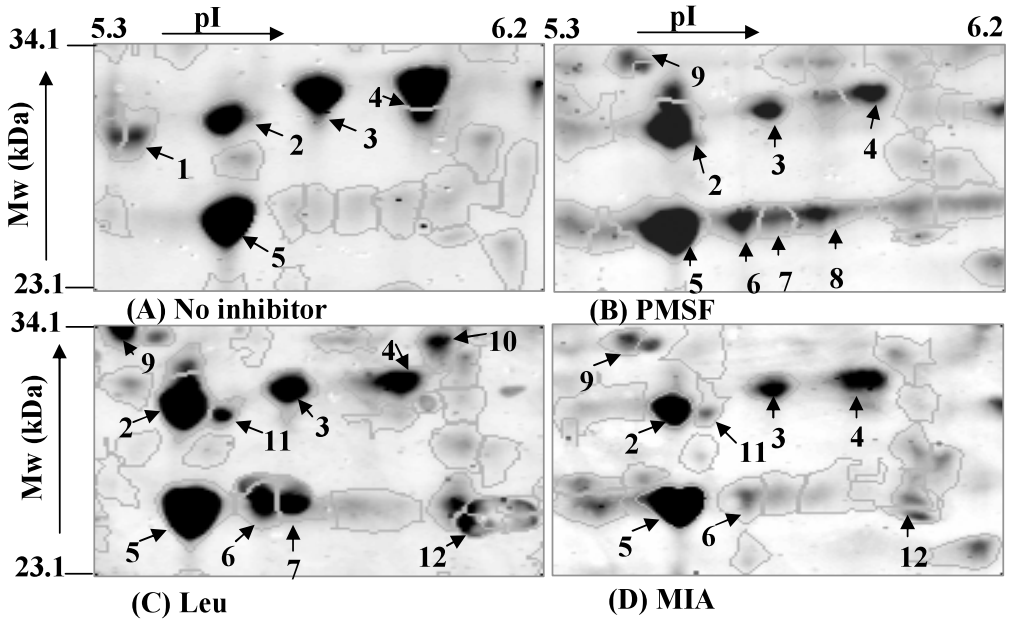

Fig. 2. Effect of the protease inhibitors on the protein expressions of *H. carnosa* mitochondria. Proteins were dissolved in lysis buffer without inhibitor (A); with 1 mM PMSF (B); with 0.2 mM Leu (C); and with 0.5 mM MIA (D). Numbers in the gel areas indicated the protein spots which were easily distinguished by eyes.

Table 4. The putative functional categorization of mitochondrial proteins of *H. carnosa*. Using Image master 2D platinum software and 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 (EC 1.1.1.37)	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	PSA6A-ARATH (O81146)	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 $\pm$ 0.05 and molecular weigh $\pm$ 5%.

Discussion

The optimal conditions for sample preparation in 2DE technique from every new cell source are difficult. One of the trouble signs in this technique is that poor representation of higher molecular weight protein appears in the gels. Proteolysis of sample is well-known as a possible cause of this symptom; therefore, preparation of samples in a manner that limits proteolysis or use of protease inhibitor to overcome this problem is necessary.

Among the proteases inhibitors, PMSF is most common to use to protect samples from proteolysis. PMSF is an inhibitor that inactivates serine protease and some cysteine protease; however, it should be noted that this inhibitor does not inhibit all serine proteases. PMSF enables vanadate to catalyze the oxidation of NADH and it is widely used to protect enzymes from proteolytic cleavage when they are purified (Takeda and Kawamura, 1987). Unfortunately, PMSF, like several other protease inhibitors commonly used during protein purifications, inhibits irreversibly. Different from PMSF, Leu is one of the peptide protease inhibitors and it is a reversible inhibitor. Leu inhibits serine, plasmin, porcine kallikrein and cysteine proteinases (papain and cathepsin B), but it does not inhibit chymotrypsin and thrombin (Salminen, 1984). MIA is one of other peptide protease inhibitors which are mostly used for the modification of cysteine residues in proteins (http://www.sigmaaldrich.com).

In this study, among the protein spots which were observed in all of the gels, we found many different protein spots in the 2DE gels which had inhibitors while they were not found in the gel with no inhibitor. And, although using the protease inhibitors in sample preparation significant increased the number of protein spots in 2DE gels than that with no inhibitor, some protein spots observed from the gels with no inhibitors were not observed in the inhibitor treated gels (Table 3). For example, in a dominant region of pI between 5.3 and 6.2, with Mw between 23.1 and 33.1 kDa from the obtained 2DE gels of *H. carnosa* mitochondria in this study, spot number 1 was observed in the gel with no inhibitor, but it was not detected in the gels with the inhibitors (Fig. 2).

Four protein spots number 2, 3, 4 and 5 were detected in all gels while spots number 6 and 9 were only observed in the gels with the inhibitors but not in the gel with no inhibitor. Some proteins such as spot number 8 and 10 were only observed in the gels with PMSF and Leu, respectively, but other gels did not. Some other proteins such as spots number 11 and 12 were observed in both gels with Leu and MIA while not in other gels. These results suggest that protease inhibitors clearly effected the protein detection in 2DE gels of *H. carnosa* mitochondria, but individual proteases were only active against specific classes of protease, so using a combination of protease inhibitors is necessary to sufficiently protect the sample against proteolysis. At present, we are continuously conducting further experiments to better understand the effect of individual and combination of protease inhibitors during application of the 2DE technique as well as the function and characteristics of specific protein spots in mitochondrial proteomic of *H. carnosa*.

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Hoya carnosa ミトコンドリア二次元電気泳動マップのタンパク質同定に及ぼすタンパク質分解酵素阻害剤の影響

ホアン ティ キム ホン・野瀬　昭博・東江　栄
（熱帯作物改良学研究室）

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摘　　要

本研究ではまず，CAM フェーズⅢにおけるサクララン（Hoya carnosa）ミトコンドリアタンパク質の発現を二次元電気泳動で調べた．ついで，フェニルメチルスルフォニルフルオライド（PMSF），ロイペプチン（Leu），モノヨードアセテート（MIA）といったタンパク質分解酵素阻害剤の二次元電気泳動マップに及ぼす影響を調べた．上記の阻害剤を単独に含む Tris 緩衝液と含まない緩衝液にミトコンドリア膜を可溶化してタンパク質を抽出し，二次元電気泳動マップを Image Master 2DE software（Amersham Pharmacia Biotech, USA）で解析した．その結果，タンパク質分解酵素阻害剤は明らかにミトコンドリア二次元電気泳動マップにおけるタンパク質の数に影響した．