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# Use of Highly Purified and Mixed Antibodies for Simultaneous Detection of Multiple Protein Species Released from Mitochondria upon Induction of the Permeability Transition

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**Abstract** Concomitant with the induction of the mitochondrial permeability transition (PT), cytochrome c is released from mitochondria into the cytosol where it triggers subsequent steps of cellular apoptosis. Thus, inducers of the mitochondrial PT would become "seed compounds" of regulators of apoptosis. However, when we examine the actions of certain chemicals on the release of mitochondrial cytochrome c, the behaviors of not only cytochrome c but also multiple mitochondrial protein species must be carefully examined because the mitochondrial PT and release of proteins from mitochondria occur in diverse manners. In the present study, we examined whether it is possible to measure the behaviors of multiple protein species in a single experiment using purified and mixed antibodies. The results obtained clearly indicate that this procedure would be applicable for high-throughput screening of regulators of apoptosis. Further requirements necessary for the establishment of a useful screening system for apoptosis regulators are discussed.

**Keywords** Mitochondria · Permeability transition · Protein release · Cytochrome c · Western blotting

## Introduction

Mitochondria are well established as a major site of energy conversion. In this organelle, energy of nutrient molecules is first converted into the electrochemical gradient of $H^+$ ($\Delta\mu H^+$) across the inner mitochondrial membrane by the electron transport system; then, using this $\Delta\mu H^+$ as

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a driving force, the organelle phosphorylates ADP to make ATP. Thus, to enable effective energy conversion, the permeability of the inner mitochondrial membrane is kept very low even for tiny ions or solutes. However, under certain conditions such as in the presence of $Ca^{2+}$ and inorganic phosphate (Pi), this permeability is known to be transiently and markedly elevated. This transition is referred to as the mitochondrial permeability transition (PT). The physiological meaning of this PT was uncertain for a long time; however, recent studies revealed that the PT is involved in the regulation of cellular fate. Namely, concomitant with the induction of the mitochondrial PT, cytochrome c (Cyt c), generally located at the outer surface of the inner mitochondrial membrane, is released into the cellular cytosol. The Cyt c, thus released into the cytosol, triggers subsequent apoptotic cascades (for review, see [1–3]). Thus, inducers of the mitochondrial PT could be nice candidates as regulators of apoptosis.

In addition to $Ca^{2+}$ and inorganic phosphate, numerous other chemicals were found to cause the mitochondrial PT (for review, see [4–6]). We also discovered that it could be induced by copper-*o*-phenanthroline, cyanine dyes, metal ions or by *N*,*N*′-dicyclohexylcarbodiimide (DCCD), a coupling reagent used in peptide synthesis [7–11]. However, the mitochondrial PT induced by various chemicals is not always associated with the release of mitochondrial Cyt c. For example, the mitochondrial PT induced by DCCD does not lead to the release of Cyt c from mitochondria [10] and that induced by $Ca^{2+}$ in the absence of a respiratory substrate causes the release of adenylate kinase 2 (AK2), located in the intermembrane space of mitochondria, but not that of Cyt c [11]. These results clearly indicate the presence of diverse properties of the mitochondrial PT, especially with respect to the induction of protein release. Thus, when inducers of mitochondrial PT are considered as "seed compounds" of regulators of apoptosis, we must carefully examine whether or not they cause the selective release of mitochondrial Cyt c.

To examine the behavior of individual mitochondrial proteins, we must carry out numerous Western blotting analyses. For this reason, the application of these technologies for high-throughput screening of apoptosis-regulating agents would seem to be very difficult. If the behavior of multiple proteins could be analyzed in a single assay, high-throughput screening of such agents could be much more easily achieved. In the present study, as a first step toward this goal, we examined whether it would be possible to evaluate the proportions of proteins released from mitochondria for three well-known mitochondrial proteins in a single assay using highly purified mixed antibodies.

## Materials and Methods

### Materials

*Escherichia coli* host strain BL21(DE3)pLysS and expression vectors pET-3a and pColdIII were purchased from Novagen Inc. (Madison, MI) and TaKaRa Bio Inc. (Ohtsu, Japan), respectively. ECL kit (code RPN2106) and anti-rabbit IgG conjugated to peroxidase (code NA934V) were obtained from GE Healthcare UK Ltd. (Bucks, UK). 2-Fluoro-1-methylpyridinium toluene-4-sulfonate (FMP)-activated Cellulofine (code 810167) came from Seikagaku Kogyo (Tokyo, Japan).

### Preparation of Antisera Against Cyt c, AK2, and $F_1\beta$ and Their Affinity Purification

Antibodies against three mitochondrial proteins, Cyt c, AK2, and β-subunit of $F_1$-ATPase ($F_1\beta$) were raised by injecting synthetic peptides with amino acid sequences of

HTVEKGGKHKTGPNLHGLFC, TVKQAEMLDDLMDKRKEKLDC, and VPADDLTDP APATTFAHLDATTC, respectively, into New Zealand white rabbits, as described previously [12–14].

For the affinity purification of these antisera, we first prepared affinity columns bearing the respective synthetic peptides. For this, individual synthesized peptides (1 mg) were covalently coupled to FMP-activated Cellulofine according to the instructions from the supplier. Next, each antiserum was partially purified using ammonium sulfate precipitation. Briefly, ammonium sulfate was added to the antiserum to 50% saturation. After a 1-h incubation at room temperature, the antibody was precipitated by centrifugation at 10,000 rpm for 20 min at 4 °C. The pelleted antibody was then dissolved in 2 ml of phosphate-buffered saline, and the residual ammonium sulfate was removed by dialysis. This partially purified antibody was incubated with 3 ml of the peptide-FMP Cellulofine affinity resin, pre-equilibrated with absorption buffer (100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4) overnight at 4 °C. The resin was then washed with the absorption buffer, and antibody bound to the resin was eluted with 2 ml of 10 mM acetic acid containing 100 mM NaCl and immediately neutralized with 0.2 ml of 1 M Tris–Cl buffer, pH 8.5.

The protein concentration of the purified antibodies thus obtained was in the range of 10–15 mg/ml, and the antibodies were used at a 1,000-fold (antibody against Cyt c) or 4,000-fold (antibodies against $F_1\beta$ or AK2) dilution.

## Construction of Expression Vectors for Cyt C, AK2, and B-Subunit of $F_1$-Atpase

For the construction of expression vectors, cDNA fragments of Cyt c, AK2, and $F_1\beta$ were prepared by RT-PCR using first-strand cDNA prepared from rat liver. The cDNA fragments corresponding to the entire open reading frames of Cyt c (NM_012839.2) and AK2 (NM_030986) were amplified using amplimer sets GE1409 (5′-TTTCATATGGGT GATGTTGAAAAAGGC) and GE2039 (5′-GGATCCTTATTCATTAGTAGCCTTTTTAA GATAAGC) for Cyt c and GE1091 (5′-CATATGGCTCCCAACGCGTT) and GE1092 (5′-GGATCCCAAACCCAACATTAG) for AK2. The cDNA encoding the mature form of $F_1\beta$ (i.e., that encoding a peptide lacking the N-terminal 46 amino acids, but supplemented with an artificial N-terminal Met residue for initiation of translation) was prepared using amplimers GE2028 (5′-GTCCATCCTGCCAGACATATGGCGGC) and GE2001 (5′-GGATCCTCACGACCCATGC). After amplification, the cDNAs of Cyt c and AK2 were digested with *Nde*I and *Bam*HI and subcloned into pET-3a, whereas the cDNA encoding mature $F_1\beta$ was digested with *Nde*I and *Hin*dIII and subcloned into pColdIII. By transforming *E. coli* cells of the BL21(DE3)pLysS strain with these expression vectors, we thus established expression systems for these three mitochondrial proteins.

## Bacterial Preparations of Standard Proteins

Cyt c, AK2, and β-subunit of $F_1$-ATPase of rat were prepared by the use of the aforementioned bacterial expression systems. Briefly, individual transformants were cultured in Luria–Bertani medium supplemented with ampicillin and chloramphenicol at 37 °C until their OD at 600 nm reached 0.4. The expression of Cyt c and AK2 was induced by the addition of isopropyl thio-D-galactoside (IPTG) to a final concentration of 1 mM and that of $F_1\beta$ by cooling down the culture suspension to 15 °C. After a subsequent 12-h culture period, the bacterial cells were harvested by centrifugation. The pelleted cells were resuspended in TE medium and then disrupted by sonication. The expressed standard proteins were recovered as inclusion bodies and used without further purification.

### Preparation of Liver Mitochondria and Sample Preparation of Proteins Released from Mitochondria

Liver mitochondria were prepared from 10-week-old male Wistar rats according to the procedure described previously [10, 11]. The protein concentration of mitochondrial suspensions was determined by the Biuret method using bovine serum albumin as a standard.

Samples of proteins released from the mitochondria into the incubation medium or retained in the mitochondria concomitant with the induction of permeability transition were collected and used for the subsequent assays. Briefly, for the induction of the PT, mitochondria were suspended in + Pi medium (200 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4) kept at 25 °C to a final protein concentration of 0.7 mg protein/ml. $Ca^{2+}$ was added to the mitochondrial suspension to a final concentration of 100 μM. When a respiratory substrate was used, 10 mM succinate (plus 0.5 μg rotenone/mg mitochondrial protein) was added to the incubation medium. Induction of the PT was monitored by measuring the optical density of the mitochondrial suspension at 600 nm using a Shimadzu spectrophotometer, model UV-3000. After a 5-min incubation, the mitochondria were centrifuged and the resulting supernatant and pelleted mitochondria were used as samples of proteins released from the mitochondria into the incubation medium and those retained in the mitochondria, respectively. Samples of protein released from the mitochondria (10.5 μg of protein) and protein retained in the mitochondria (6.3 μg protein) were subjected to SDS-PAGE and subsequent Western analysis.

### SDS-PAGE and Western Blotting

SDS-PAGE and Western blotting were performed as described previously [13]. Immunoreactive protein bands were detected using an ECL kit and visualized by exposure to X-ray film. Signal intensities of individual immunoreactive protein bands were measured by the use of an ATTO image analyzer, model AE-6900.

## Results and Discussion

### Preparation of Bacterially Expressed Protein Samples as Positive Controls for Immunodetection

To establish the evaluation method for the simultaneous detection of protein release from mitochondria, in the present study, we selected three mitochondrial proteins, Cyt c, AK2, and $F_1\beta$. AK2 is a water-soluble protein present in the intermembrane space of the mitochondria, and $F_1$-ATPase is the catalytic subunit of $F_oF_1$-ATPase, which is present in the inner mitochondrial membrane. To enable specific detection of these three mitochondrial proteins, we raised antibodies against these individual proteins by injecting their synthetic peptides corresponding to a certain part of each protein into rabbits using one animal for each protein (for details, see "Materials and Methods"). Furthermore, to obtain "standard proteins" of these three mitochondrial proteins, we constructed expression vectors of these proteins in the pET-3a or pColdIII vector and then transformed *E. coli* cells (BL21 (DE3)pLysS) with them, thus establishing expression systems for Cyt c, AK2, and $F_1\beta$. Bacterial expression of these proteins was induced by the addition of IPTG or by reducing the culture temperature to 15 °C. When lysates of bacterial cells having expression vectors

of Cyt c, AK2, and $F_1\beta$ were subjected to SDS-PAGE and subsequent Coomassie staining, protein bands with apparent molecular sizes of 13, 27, and 50 kDa, respectively, showed stronger staining intensities than lysates of bacterial cells having the sham vector (data not shown), indicating that these three mitochondrial proteins had been successfully expressed.

When proteins in these bacterial lysates were transferred onto a nitrocellulose membrane and the latter was incubated with crude antisera obtained from individual rabbits, in addition to the strong immunoreactive protein bands with apparent molecular sizes of 13, 27, and 50 kDa, a number of weak protein bands, possibly due to non-specific binding, were observed (data not shown). Thus, to eliminate the immunoreactive protein bands due to non-specific binding, we affinity-purified the individual antisera (for details, see "Materials and Methods"). When the aforementioned nitrocellulose membranes were incubated with individual affinity-purified antibodies against Cyt c, AK2, and $F_1\beta$, the weak bands were not present, except for the weak band corresponding to a molecular size of 26 kDa in the case of the Cyt c-containing lysate (data not shown). Essentially the same results were obtained when all three purified antibodies were mixed together and applied to the nitrocellulose membrane (Fig. 1, left three lanes). By using high-resolution mass spectrometric analysis, we concluded the above 26-kDa protein band to be a dimer of Cyt c (data not shown), possibly formed by improper folding of the expressed protein in the absence of heme [15]. For this reason, elimination of this immunoreactive protein band was difficult, even by affinity purification.

## Application of Mixed Antibody for Simultaneous Detection of Mitochondrial Proteins

The above results clearly indicate that the specificity of each purified antibody was sufficiently high. Thus, we next examined their usefulness in the analysis of proteins released from the mitochondria using actual protein samples. The addition of $Ca^{2+}$ to the mitochondria suspended in medium containing Pi and respiratory substrate is well known to cause the mitochondrial PT and concomitant release of Cyt c and AK2 [16]. In contrast, we recently reported that the addition of $Ca^{2+}$ to the mitochondria suspended in medium containing Pi but no respiratory substrate also induces the PT [11]. Under these conditions, however, the release of AK2 present in the intermembrane space of mitochondria occurred, but that of Cyt c did not. Thus, in addition to the control mitochondrial suspension made in medium containing Pi and respiratory substrate but without added $Ca^{2+}$, we also prepared suspensions of mitochondria treated with $Ca^{2+}$ in the absence or presence of succinate. By centrifugation, these three types of suspensions were separated into mitochondrial pellet (P) and supernatant (S). These six protein samples were then subjected to SDS-PAGE, and the separated proteins were transferred onto a nitrocellulose membrane and analyzed by the Western blot method. For the primary antibody, a mixture of the aforementioned three affinity-purified antibodies against Cyt c, AK2, and $F_1\beta$ was used, as in the case of detection of the proteins in the bacterial lysate. The obtained luminogram is shown in Fig. 1. From the signal intensities of the immunoreactive protein bands observed for the P and S fractions, the proportion of each protein released from the mitochondria could be quantified, as shown in Fig. 2. When the protein of the P sample obtained by the centrifugation of a mitochondrial suspension incubated in the presence of respiratory substrate and absence of $Ca^{2+}$ (+ Succ, − $Ca^{2+}$) was examined, all three protein bands were detected. These three protein bands were not detected in the S sample prepared from this mitochondrial suspension, indicating that none of the mitochondrial proteins was spontaneously released from the mitochondria incubated in the absence of $Ca^{2+}$. In the case of the protein samples prepared from a mitochondrial suspension incubated in the

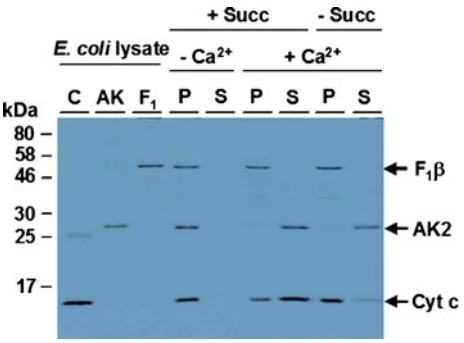


**Fig. 1** Results of Western analysis using mixed antibodies for the detection of the three mitochondrial proteins. Bacterially expressed Cyt c (*C*), AK2 (*AK*), and $F_1\beta$ (*$F_1$*) were used as positive controls for Western analysis (*E. coli* lysates, *left three lanes*). They were not purified samples but just lysates of *E. coli* cells in which one of the three mitochondrial proteins was expressed. In addition to these standard protein samples, supernatant (*S*) and pellet (*P*) obtained by centrifugation of three different mitochondrial suspensions were also applied to the same acrylamide gel. These three mitochondrial suspensions were incubated (1) in the presence of succinate and the absence of $Ca^{2+}$ (+ Succ, − $Ca^{2+}$), (2) in the presence of succinate and $Ca^{2+}$ (+ Succ, + $Ca^{2+}$), and (3) in the presence of $Ca^{2+}$ and the absence of succinate (− Succ, + $Ca^{2+}$). After separation of these proteins by SDS-PAGE, they were transferred onto a nitrocellulose membrane. The obtained membrane was first soaked in a blocking solution, then incubated in the "mixture" of the affinity-purified antibodies against Cyt c, AK2, and $F_1\beta$. It should be emphasized that the whole membrane was incubated in the single solution containing all three antibodies. Immunoreactive protein bands were detected by secondary antibody conjugated with horseradish peroxidase and visualized by the use of an ECL kit. One typical result of more than three independent runs is shown

presence of $Ca^{2+}$ and a respiratory substrate (+ Succ, + $Ca^{2+}$), which is the ordinary condition to cause the mitochondrial PT, $F_1\beta$ and AK2 were observed exclusively in P and S, respectively, and approximately 75% of the Cyt c was detected in the supernatant. On the contrary, in the case of protein samples prepared from a mitochondrial suspension incubated in the presence of $Ca^{2+}$ and absence of respiratory substrate (− Succ, + $Ca^{2+}$), the specific distributions of $F_1\beta$ and AK2 in the P and S samples were the same as those in the case of "+ Succ, + $Ca^{2+}$," but more than 80% of the Cyt c was retained in the mitochondria. These obtained results accord well with the results obtained in previous studies [11] in which individual proteins were detected in separate experiments, indicating that the experimental procedure established in this study is reliable.

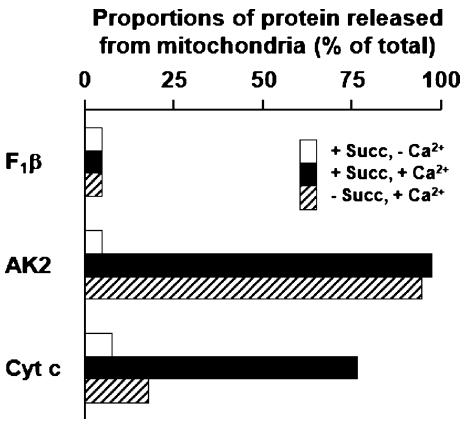


**Fig. 2** Proportion of $F_1\beta$, AK2, and Cyt c proteins released from mitochondria in the three mitochondrial suspensions The proportion of each protein, i.e., $F_1\beta$, AK2, and Cyt c, released from the mitochondria was calculated by determining the signal intensities of their immunoreactive protein bands in pellet (*P*) and supernatant (*S*) samples, with the results expressed as S/(P+S)%. *Open*, *closed*, and *hatched columns* are defined in the figure

In the present study, we mainly examined the usefulness of "mixed" purified antibodies as the primary antibodies for the simultaneous detection of three mitochondrial proteins by ordinary Western blot analysis, with horseradish peroxidase-conjugated antibody as the second antibody. Because the use of the mixed antibodies did not have any serious influence on the detection and quantification of individual proteins, we concluded this trial to be successful.

For continuing experiments, it is of utmost importance that the preparation of high-quality antibodies be reproducible. We could obtain such antibodies in a highly reproducible manner, even in separate preparations (data not shown). Possibly, one of the reasons for this high reproducibility in the preparation of purified antibodies is the use of a synthetic peptide as the immunogen. The use of monoclonal antibody would also be expected to be useful, especially with respect to the reproducibility of experiments.

To enable actual high-throughput screening of seed compounds as apoptosis inducers, the separation step of proteins by SDS-PAGE should be omitted. For this purpose, the formation of individual antigen/antibody complexes should be separately detected. If labeling of individual primary antibodies with distinct fluorescent probes is done, the detection of individual antigen/antibody complexes would be possible.

In the present trial study, three mitochondrial proteins, i.e., Cyt c, AK2, and $F_1\beta$, were selected as target proteins for analysis. Because AK2 is present in the intermembrane space of mitochondria, by measuring its behavior, the status of the outer mitochondrial membrane could be monitored. To monitor the status of the inner mitochondrial membrane, the behavior of proteins located in the matrix space of the mitochondria should also be measured, in addition to Cyt c and $F_1\beta$. Furthermore, the behavior of other mitochondrial proteins such as apoptosis-inducing factor or Smac/DIABLO should also be analyzed as they, like Cyt c, are known to be associated with the regulation of apoptosis.

## References


1. Crompton, M. (1999). *Biochemical Journal, 341*, 233–249.
2. Kroemer, G., & Reed, J. C. (2000). *Nature Medicine, 6*, 513–519.
3. Bernardi, P., Petronilli, V., Di Lisa, F., & Forte, M. (2001). *Trends in Biochemical Sciences, 26*, 112–117.
4. Gunter, T. E., & Pfeiffer, D. R. (1990). *American Journal of Physiology: Cell Physiology, 258*, C755–C786.
5. Zoratti, M., & Szabò, I. (1995). *Biochimica et Biophysica Acta, 1241*, 139–176.
6. Bernardi, P., Colonna, R., Costantini, P., Eriksson, O., Fontaine, E., Ichas, F., et al. (1998). *Biofactors, 8*, 273–281.
7. Shinohara, Y., Bandou, S., Kora, S., Kitamura, S., Inazumi, S., & Terada, H. (1998). *FEBS Letters, 428*, 89–92.
8. Almofti, M. R., Ichikawa, T., Yamashita, K., Terada, H., & Shinohara, Y. (2003). *Journal of Biochemistry, 134*, 43–49.
9. Yamamoto, T., Tachikawa, A., Terauchi, S., Yamashita, K., Kataoka, M., Terada, H., et al. (2004). *European Journal of Biochemistry, 271*, 3573–3579.
10. Yamamoto, T., Terauchi, S., Tachikawa, A., Yamashita, K., Kataoka, M., Terada, H., et al. (2005). *Journal of Bioenergetics and Biomembranes, 37*, 299–306.
11. Yamamoto, T., Yoshimura, Y., Yamada, A., Gouda, S., Yamashita, K., Yamazaki, N., et al. (2008). *Journal of Bioenergetics and Biomembranes, 40*, 619–623.
12. Shinohara, Y., Almofti, M. R., Yamamoto, T., Ishida, T., Kita, F., Kanzaki, H., et al. (2002). *European Journal of Biochemistry, 269*, 5224–5230.
13. Yamada, A., Yamamoto, T., Yamazaki, N., Yamashita, K., Kataoka, M., Nagata, T., et al. (2009). *Molecular & Cellular Proteomics, 8*, 1265–1277.
14. Terauchi, S., Yamamoto, T., Yamashita, K., Kataoka, M., Terada, H., & Shinohara, Y. (2005). *Mitochondrion, 5*, 248–254.
15. Tzul, F. O., Kurchan, E., Roder, H., & Bowler, B. E. (2009). *Biochemistry, 48*, 481–491.
16. Scarlett, J. L., & Murphy, M. P. (1997). *FEBS Letters, 418*, 282–286.