

Fluorescence Two-dimensional Differential Gel Electrophoresis

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ABSTRACT

To detect the proteome differences between two samples, the 2D-PAGE methodology uses the comparison of at least two gel images. But due to many reasons, no two gels images are superimposable directly. "Average gel" from several gels has to be made before they are compared. In order to increase the accuracy and the reproducibility of 2D-PAGE, a new methodology Fluorescence Differential Gel Electrophoresis is used. Two protein samples are labeled with two cyanine dyes: cy3 and cy5 separately before they are mixed and run on one same 2D gel. By scanning the gel with two different lasers, two gel pictures from the same 2D picture can be made and they are perfectly superimposed.

Compared to the conventional "one-sample-per-gel" 2D-PAGE, Fluorescence Differential Gel Electrophoresis works much better in comparing two protein samples. Using the method described, the proteome changes of yeasts that are grown in medium containing 16 kinds of metal salts have been determined. The results are significant.

INTRODUCTION

To improve the accuracy of the 2-D SDS PAGE gels and quickly identify the proteome differences between two protein samples, the new method termed Fluorescence Differential Gel Electrophoresis (DIGE) is used. Two fluorescence probes (Cy3 and Cy5) were used to label two different proteins samples respectively. The labeled protein samples were loaded into same 2-D SDS PAGE gel and run. By scanning the gel using lasers of different wavelength, the global proteome image of both the two samples can be easily obtained.

The binding of the fluorescence probes is covalent. The N-hydroxy-succinimidyl (NHS) esters of the Cy3 and Cy5 molecules undergo nucleophilic substitution reactions with the free amine group on the lysine, arginine and asparagines residues to form an amide (Ünlü, Morgan and Minden, 1997). Therefore the peptides with these residues will be labeled by the probe and later can be detected on the 2-D SDS PAGE gel by the laser with the corresponding wavelength. Due to this high similarity of the pI value and molecular weights (13 Dalton) of the two probes, the difference between the alteration of pI and molecular weight by Cy3 and Cy5 binding can be consider as negligible

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during gel image analysis. Therefore both Cy3 labeled and Cy5 labeled proteins with same expression level in the two samples will be perfectly superimposed and the color of the spots will appear to be orange. The proteins with different expression levels will appear either bluer or redder on the gel. Those special proteins are identified by mass spectrometry. Normally only the highly up regulated or down regulated proteins spots are cleaved from the gel and sequenced. The MS results will be used to search the available protein sequence database on the Internet. And the proteins of interest can be identified.

Using the DIGE method, a series of experiments were carried out to study the heavy metal toxicities.

Heavy metal toxicity is one of the major environmental hazards. Some heavy metals, for example cadmium, are believed to be very toxic and carcinogenic at low concentration. But the biological basis of the metal toxicity is yet not known. It is reported that the toxicity of cadmium is due to the generation of toxic lipid peroxides and by affecting the cellular thiol redox balance. It is also reported that the induction of heavy metals resulted in a displacement of zinc and ferrous ion in the intracellular proteins (Vido *et al*, 2001). The release of these metal ions in the cell causes the generation of very active hydroxyl radicals and gives the cell an oxidative stress (Vido *et al*, 2001).. It is also believed that the induction of heavy metals stimulates the biosynthesis of GSH and cysteine in yeast.

The yeast strain S288C was incubated in the medium containing 16 different metal salts, including, Potassium, Lithium, Rubidium, Chromium, Ferric, Ferrous, Nickel, Zinc, Sodium, Magnesium, Manganese, Calcium, Copper, Silver, Cadmium and Cobalt. The crude protein extracts of all the yeast cells were analyzed using DIGE method. Some proteome alteration was detected rapidly using the DIGE method.

METHODS AND MATERIALS

Studies are performed with the yeast strain S288C. Cells were grown in liquid YPD medium containing metal salts at 30°C till the OD₆₀₀ reaches 0.5.

Cells were collected by centrifugation at 5000 rpm for 15 minutes in a Beckman centrifuge. Before the pellets were lysed by sonication, protease inhibitor cocktail solution was added. The crude protein was dissolved in the dissolving buffer, containing urea, thiourea and CHAPS. The concentration of the protein extract was determined using the Bradford method (Protein assay kit I, Biorad).

The two fluorescence probes used in the experiments were both synthesized by my colleague in the chemistry department. One is Cy3- N-hydroxy-succinimidyl esters (Cy3-NHS) and Cy5- N-hydroxy-succinimidyl esters (Cy5-NHS). After labeling reaction each of the reaction was quenched with 1µl 1.5M hydroxylamine in order to remove the excess fluorescence probes in the sample and avoid cross labeling after the reaction solutions were mixed.

The IEF protocol is based on the 2-D Electrophoresis Hand Book (Amersham Biosciences, UK).

After IEF was finished, two steps of equilibrations were performed immediately using Equilibration Buffer 1, 1.5M Tris-Cl pH 8.8, 6M urea, 30% Glycerol (v/v) 2%

SDS(w/v), 1% DTT(w/v) and Equilibration Buffer 2 1.5M Tris-Cl pH 8.8, 6M urea, 30% Glycerol (v/v) 2% SDS(w/v), 1.25% Iodoacetamide (w/v). Each step took 15 minutes.

The electrophoresis condition was referred to the 2-D Electrophoresis Hand Book

The whole process of the 2-D SDS PAGE will take around 24 hours. After the electrophoresis was done the gel was scanned using Typhoon 9200 laser scanner (Amersham Biosciences, UK). the scanning PMT was 650 with normal sensitivity. For some gels, which the intensity of the spots was very low, higher PMT (up to 750) was used for scanning.

From each 2-D SDS PAGE gel, two fluorescence images can be obtained by Typhoon 9200 Laser Scanner. The following analysis steps are performed by Ettan Progenesis 2-D analysis software (Amersham Biosciences, UK). The analysis steps were: steps detection, background subtraction, warping and matching.

RESULTS AND DISCUSSION

Proteome Analysis

The control yeasts protein extract was used as the protein internal standard in the 2-D gels. Because the protein standard was introduced into the gels, a direct comparison of the two channels of one gel was able to determine the proteome difference between the metal treated and the control cells.

For each gel image, ten modeling spots, in which the expression level was greatly altered, were reported. There were still around two thousand detected spots on these gels. The data analysis of these spots will not be included in this report.

Interestingly, though the growth of the FeCl₃ treated yeasts was enhanced, the protein matched protein production decreased indicating that the proteome pattern changed greatly. Many new proteins might be newly produced to overcome the toxicity stress.

From the result listed above, it is observed that the effects of the metal salts on yeast proteome were different among different metals. The effects of zinc and ferrous were similar while the effects of manganese and silver were similar. Further study needs to be done after the proteins are identified. Only then can we look into the biological pathways and find out how exactly the metal salts toxicity takes place.

Summary on DIGE

The greatest advantage of DIGE method is that it includes a protein standard inside the 2-D SDS PAGE gel, so that the proteome differences are very easy to detect.

Secondly, DIGE is a high throughput method for proteome analysis. No average gels are needed so that the number of false positive results is greatly reduced. For example, using conventional 2-D method when I want to compare the protein extracts from silver treated yeast and the control yeast, five gels containing silver treated yeast protein have to be run and scanned. And an average gel image has to be generated using 2-D analysis software. The same thing has to be done on control cells. Therefore, at least 160 gels and 160 times of scanning have to be finished for the yeast project. But if the DIGE method is used, only one gel and two times of scanning are needed for one

protein sample. Only 16 gels and 32 times of scanning are needed. Meantime, no staining is needed to visualize the proteins spots. More work is saved.

In summary, compared to conventional 2-D gel electrophoresis the advantages of using DIGE are:

1. The internal control of Cy3-labeled proteins allows high reproducibility of 2D pattern and accurate quantification of the protein spots.

2. Using the specialized software for 2D-DIGE, the 2D profiles in multiple gels are compared rapidly and automatically.

3. High throughput analysis. Analysis of more than 20 gels can be finished in one day.

DIGE is a new method. All the steps, like labeling reaction, electrophoresis running and data analysis need to be further optimized.

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