[Regular Paper]

Two-dimensional gel electrophoresis of skeletal muscle nuclear proteins

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SUMMARY

In order to separate all protein components of muscle nuclei, three types of two-dimensional gel electrophoresis were tried to find out advantages and disadvantages in them. Non-equilibrium pH gradient electrophoresis gave a reasonably good pattern, but a large amount of proteins left unanalyzed on the acidic top of the gel. Isoelectric focusing of nuclear proteins extracted with SDS gave a pattern lacking proteins in acidic area, although histones were well separated. Isoelectric focusing with agarose gels in the first dimension separated almost all protein components of muscle nuclei in wide ranges of molecular size (10–400 kD) and *pI* (3.7–9.5), leaving histones in the area of *pI* over 9.5.

Key words: muscle nucleus, nuclear protein, two-dimensional gel electrophoresis.

INTRODUCTION

Differences of nuclear protein constituents between different cell types are thought to be accompanied by the difference of nuclear functions in each cell. Since the nucleus is the place where primary regulations of gene expression occur, the investigation of the changes in nuclear protein constituents during the development and the differences in the constituents between different tissues may be necessary for understanding mechanisms of differentiation and function of the cell.¹⁾ In order to find out the changes or differences in nuclear protein constituents we have to deal with whole nuclei from certain tissues, while subnuclear components, such as nuclear matrix,²⁾ nuclear lamina,³⁾ and chromatin^{4,5)} have been materials in

concern in most investigations of nuclei.

In the analysis of highly heterogeneous proteins, like the nuclear proteins, two-dimensional gel electrophoresis has been used because of its resolution and sensitivity.^{2–5)} Two-dimensional gel electrophoresis, using isoelectric focusing (IEF) in the first dimension, followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), was originally described by O'Farrell.⁶⁾ Then, various methods have been developed to make them applicable to many kinds of materials. To get around the limitation in the basic pH range in IEF, non-equilibrium pH gradient electrophoresis (NEPHGE) was developed.⁷⁾ Further, to analyze most protein constituents including high molecular ones, an improved method using the agarose gel in the first dimension was described.⁸⁾ Sample preparation for

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Abbreviations: SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

IEF is very critical, therefore it has also been modified to suit for each study. For example, SDS was used for the solubilization of membrane proteins, and the protein solution containing SDS was analyzed by IEF.⁹⁾

In this paper, we show the results of performing three types of two-dimensional gel electrophoresis in order to analyze the protein constituents of total nuclei as many as possible. Then, we conclude that IEF/SDS-PAGE with an agarose gel in the first dimension is the most preferable system for our purpose.

MATERIALS AND METHODS

Materials

White-Leghorn chickens (*Gallus domesticus* (L)) were obtained from commercial sources. The *pectoralis major* was taken from chicks on the day of hatching.

Reagents

Tris-base, sucrose, urea, thiourea, SDS, and acrylamide were purchased from Wako Pure Chemical Ind. Agarose (Agarose IEF) and ampholyte (Pharmalyte) were purchased from Pharmacia Fine Chemicals. Benzon nuclease was obtained from Merck Ltd.

Isolation of muscle nuclei

Muscle nuclei were isolated essentially as described by Mulvihill and Palmiter.¹⁰⁾ The chick muscle was minced with scissors and homogenized in a Dounce homogenizer with a Teflon pestle in 10 volumes of an ice-cold Buffer A (10 mM Tris-HCl, pH 8.0, 0.3 M sucrose, 2.5 mM magnesium acetate, 0.25% (v/v) Triton X-100, 0.5 mM DTT, 1 mM PMSF). The homogenate was filtered through two layers of cheesecloth and nuclei pelleted by centrifugation at $1,000\times g$ for 10 min. The pellet was suspended in Buffer B (10 mM Tris-HCl, pH 8.0, 2.4 M sucrose, 2.5 mM magnesium acetate, 0.1% (v/v) Triton X-100, 0.5 mM DTT, 1 mM PMSF) and centrifuged through 15 ml of Buffer B cushion at $80,000\times g$ for 2 h using a Beckman SW 28 rotor. The resulting pellet was washed with Buffer C (10 mM Tris-HCl, pH 8.0, 0.3 M

sucrose, 2.5 mM magnesium acetate, 0.5 mM DTT, 1 mM PMSF) and the nuclei were collected by centrifugation at $1,000\times g$ for 10 min.

The nucleic acid content was determined as follows. A part of the isolated nuclei was washed two times with ice-cold 5% perchloric acid and once with ice-cold 95% ethanol. The nuclei were suspended in 5% perchloric acid, incubated at 90°C for 15 min, and centrifuged at $10,000\times g$ for 10 min. The nucleic acid content of the supernatant was measured by u.v. absorbance at 260 nm.

Microscopy

A part of the isolated nuclei was put on the slide glass and stained with 0.1% Azur C. Micrograph was taken at a magnification of 400 $\times$.

Extraction of nuclear proteins

The isolated nuclei were suspended in a digestion buffer (50 mM Tris-HCl, pH 8.5, 4 M urea, 1% (v/v) Nonidet P-40, 1 mM magnesium chloride, 1 mM PMSF) to bring the concentration of 50 A_{260} units per ml, and sheared through a needle of size 25 G. After the addition of Benzon nuclease of 500 units per ml, the suspension was incubated at 37°C for 30 min and dialysed for 3 h against three changes of 5 M urea, 1 M thiourea, 10 mM tetrasodium pyrophosphate, and 0.13% (v/v) 2-mercaptoethanol. The dialysed solution was centrifuged at $10,000\times g$ for 20 min, and 100 μ l of the supernatant was used for two-dimensional IEF/SDS-PAGE and NEPHGE/SDS-PAGE.

The extraction of nuclear proteins with urea, thiourea, and SDS was carried out as follows. After nuclease digestion of the nuclei as described above, urea, thiourea, SDS, and 2-mercaptoethanol were added to bring the concentrations to 5 M, 1 M, 1.5% (w/v), and 0.13% (v/v), respectively. Then, the mixture was centrifuged at $10,000\times g$ for 20 min, and 100 μ l of the supernatant was used for the two-dimensional IEF/SDS-PAGE.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out according to the method of Xie et al.¹¹⁾ with some modifications as follows: The nuclear extract

was loaded on the basic side (IEF) or acidic side (NEPHGE) of a cylindrical agarose gel (1% Agarose IEF) in the presence of 1% (v/v) Nonidet P-40. After first-dimension electrophoresis at 500 V for 25h (IEF) or 17.5h (NEPHGE) at 4°C, the agarose gel was immersed in 10% trichloroacetic acid (TCA) and 5% sulfosalicylic acid (SSA) for 1h, washed with circulating 1% TCA and 0.5% SSA for 30 min, and used for the second-dimension SDS-PAGE with a 15–20% polyacrylamide separating gel. The polyacrylamide gel was fixed with a solution of 45% methanol and 10% acid, stained with 0.5% Coomassie Brilliant Blue R in 0.08M Napicrate, 9% methanol, and 2% acetic acid, and then destained with distilled water.¹²⁾

RESULTS AND DISCUSSION

Isolation of muscle nuclei

The nuclei were isolated from chick muscle (*pectoralis major*) and prepared for microscopic observation as described in MATERIALS AND METHODS. Purity of the nuclei on the basis of a micrograph (Fig. 1) and an RNA/DNA ratio of 0.17 indicates that the sample is reasonably free from myofibrillar or cytoplasmic contamination¹³⁾ for the work with muscle tissue.

Two-dimensional NEPHGE/SDS-PAGE

Nuclear proteins were extracted from the muscle nuclei essentially with urea, and thiourea, after nuclease digestion. Since NEPHGE was improved in order to analyze basic proteins, using this method in the first dimension was considered to be appropriate to investigating the nuclei which were abundant in basic proteins. As shown in Fig. 2, most basic proteins as well as acidic ones could be separated fairly well. But a large amount of proteins was remained on the top of the first-dimension gel. Such proteins remained on the top may be caused by the denaturation of proteins under the acidic condition of the NEPHGE. The arrowheads indicate the positions of histone subunits on the second-dimension SDS-PAGE as determined by relative mobilities reported in the previous report.⁴⁾ Each arrowhead points

Fig. 1. Nuclei isolated from chick skeletal muscle.

Nuclei were isolated from chick skeletal muscle as described in the MATERIALS AND METHODS section. Nuclei were suspended and stained in a 0.1% Azur C solution. The bar indicates the length of 50 μm.

Fig. 2. Two-dimensional pattern of muscle nuclear proteins separated by NEPHGE/SDS-PAGE.

The first-dimension NEPHGE was performed from right (acidic) to left (basic) at 500 V for 17.5h at 4°C. The second-dimension SDS-PAGE was carried out essentially by Laemmli's method with a gradient polyacrylamide gel (15–20%). The arrow indicates the protein which is observed in the basic region in the pattern of two-dimensional IEF/SDS-PAGE (Fig. 3). Arrowheads indicate histone subunits H₁, H₃, H₂B, H₂A, and H₄ in the order of mobility on the second-dimension SDS-PAGE.

the location of histone H₁, H₃, H₂B, H₂A, and H₄ in the descending order. Most of histone H₂B and nearly half of histone H₂A formed spots in the basic region, while most of histone H₁, H₃ and H₄ were remained on the acidic top of the first-dimension gel

Another two-dimensional gel electrophoresis system, using an acetic acid-urea-Triton (AUT) gel in the first dimension, was reported to provide a good resolution of basic proteins.¹⁴⁾ Using the two-dimensional AUT/SDS-PAGE, we obtained good spotting of not only basic proteins but also acidic proteins of around pI 5 (data not shown). But most of nuclear proteins became insoluble in the acidic solution and were not analyzed by this system, so that the system did not suit to our purpose of analyzing all nuclear proteins.

Two-dimensional IEF/SDS-PAGE

A two-dimensional IEF/SDS-PAGE pattern of muscle nuclear proteins extracted essentially with urea and thiourea is shown in Fig. 3. Almost all proteins including large molecules of over 400kD could be analyzed by this system. Although the pH gradient covered approximately 3.7 to 9.5,¹⁵⁾ histones (arrowheads) were mostly remained on the top of the first dimension gel. As far as IEF in a wide range of pH is concerned, this pattern seemed to be rather good since a basic protein (arrow in Fig. 2) could be focused at the most basic area (arrow in Fig. 3). However, it was curious that histones which should have been out of pH range got into the gel of the first dimension. Probably this was due to comigration of histones with nucleic acid which was detected by ethidium bromide staining in the gel immediately after IEF (data not shown). Furthermore, when histones extracted with sulfuric acid to remove nucleic acid was charged, no histone migrated into the gel of IEF. These observations suggest that the migration of histones in Fig. 3 was caused with nucleic acid which somehow formed complexes with histones even the presence of urea and thiourea.

Two-dimensional SDS-IEF/SDS-PAGE

Several papers reported that proteins solubilized with SDS could be analyzed in IEF.^{9,16)} In these experiments, SDS sticking to proteins could be dissociated from protein molecules while the proteins were migrating to their native pI s.⁹⁾ Then we dissolved muscle nuclei in a solution containing urea, thiourea, and SDS to charge onto IEF gels

Fig. 3. Two-dimensional pattern of muscle nuclear proteins separated by IEF/SDS-PAGE.

The first-dimension IEF was performed from left (basic) to right (acidic) at 500 V for 25 h at 4°C.

The second-dimension SDS-PAGE was performed with a gradient concentration of acrylamide (15-20%). Arrow indicates the basic protein which is indicated by the arrow in Fig. 2. Arrowheads indicate histone subunits as shown in Fig. 2.

Fig. 4. Two-dimensional pattern of muscle nuclear proteins separated by SDS-IEF/SDS-PAGE.

Muscle nuclear proteins were extracted with the solution including 1.5% SDS, and analyzed by two-dimensional gel electrophoresis which was carried out by the same procedure as that of two-dimensional IEF/SDS-PAGE. Arrowheads indicate histone subunits as shown in Fig. 2.

(Fig. 4). Certainly, the amount of remained proteins on the top of the gel decreased drastically, and histones migrated into the IEF gel to form elongated spots. This pattern presented two peculiar points: One is that the pH gradient at acidic area between pH 6 to 4 disappeared (cf Fig. 3) and almost all proteins in this area run to the acidic end of the gel. The other point is that histones which have basic pI over 9.5 migrated to around pH 7.5 and formed spots. This suggests a possibility of analyzing basic proteins which have native pI out of pH gradient made up with an

ampholine. But the former point is a serious drawback to our purpose of analyzing all nuclear proteins in a two-dimensional pattern. Therefore, the SDS-IEF/SDS-PAGE is useful only when we want to analyze basic proteins like histones by a system similar to IEF, but still we consider that NEPHGE is more useful than SDS-IEF in its reliability.

Separation of all protein components of one organism, one tissue, or one cell on a single two-dimensional gel is one of the most useful techniques to describe the biochemical construction of materials obtained from various biological sources. However, this is very difficult, since proteins have a wide variety of characters corresponding to various activities of life. In this report we have tried and compared three different types of the first dimension for two-dimensional gel electrophoresis, NEPHGE, IEF, and SDS-IEF. In NEPHGE, separation of each protein is very good, but a large amount of proteins is remained as denatured form on the acidic gel top. In SDS-IEF, proteins in acidic area are lost. In conclusion, IEF/SDS-PAGE with an agarose gel in the first dimension is the most preferable system for separation of most protein components of muscle nuclei, even if there are some very basic proteins left unresolved.

REFERENCES

- 1) Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 1992; 116: 201-11.
- 2) Dworetzky SI, Fey EG, Penman S, Lian JB, Stein JL, Stein GS. Progressive changes in the protein composition of the nuclear matrix during rat osteoblast differentiation. *Proc Natl Acad Sci USA* 1990; 87: 4605-9.
- 3) Worman HJ, Lazaridis I, Georgatos SD. Nuclear lamina heterogeneity in mammalian cells. *J Biol Chem* 1988; 263: 12135-41.
- 4) Chan PK, Liew CC. Identification of specific phosphoproteins in nuclease-digested chromatin subunits. *Can J Biochem* 1977; 55: 847-55.
- 5) Jackowski G, Liew CC. Changes in non-histone nuclear proteins during postnatal myocardial development. *Biochem J* 1983; 210: 175-82.
- 6) O'Farrell P. High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 1975; 250: 4007-21.
- 7) O'Farrell PZ, Goodman HM, O'Farrell PH. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* 1977; 12: 1133-42.
- 8) Hirabayashi T. Two-dimensional gel electrophoresis of chicken skeletal muscle proteins with agarose gels in the first dimension. *Anal Biochem* 1981; 117: 443-51.
- 9) Ames GFL, Nikaido K. Two-dimensional gel electrophoresis of membrane proteins. *Biochemistry* 1976; 15: 616-23.
- 10) Mulvihill ER, Palmiter RD. Relationship of nuclear estrogen receptor levels to induction of ovalbumin and conalbumin mRNA in chick oviduct. *J Biol Chem* 1977; 252: 2060-8.
- 11) Xie L, Hirabayashi T, Miyazaki JI. Histological distribution and developmental changes of tropomyosin isoforms in three chicken digestive organs. *Cell Tissue Res* 1992; 269: 391-401.
- 12) Stephano JL, Gould M, Rojas-Galicia L. Advantages of picrate fixation for staining polypeptides in polyacrylamide gels. *Anal Biochem* 1986; 152: 308-13.
- 13) Jackowski G, Liew CC. Fractionation of rat ventricular nuclei. *Biochem J* 1980; 188: 363-73.
- 14) Bonner WM, West MHP, Stedman JD. Two-dimensional gel analysis of histones in acid extracts of nuclei, cells, and tissues. *Eur J Biochem* 1980; 109: 17-23.
- 15) Nakamura M, Imai H, Hirabayashi T. Coordinate accumulation of troponin subunits in chicken breast muscle. *Dev Biol* 1989; 132: 389-97.
- 16) Anderson L, Anderson NG. High resolution two-dimensional electrophoresis of human proteins. *Proc Natl Acad Sci USA* 1977; 74: 5421-5.