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Isolation of Serine, Glutamic Acid, and Glycine-Rich Nonhistone Proteins from Bovine Cerebral Cortex

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Two nonhistone proteins with molecular weights of 63000, and 53000 were isolated from bovine cerebral cortex by polyacrylamide gel electrophoresis with a urea-acetic acid system, followed by extraction from the gel. These proteins have a particularly large proportions of Ser, Gly and Glu, which represent 40% of total amino acids. Such amino acid compositions are unusual among nonhistone proteins. These proteins also contained 1.00 and 0.27% (w/w) phosphate/mg protein.

Keywords——bovine cerebral cortex chromatin; nonhistone protein; Ser-rich protein; Glu-rich protein; Gly-rich protein

Introduction

Nuclear nonhistone proteins are regarded as important regulatory proteins related to transcription and chromatin structure.[1] Many nonhistone proteins have been isolated from various tissues such as sheep thymus,[2] calf thymus,[3] dog liver,[4] rat liver,[5] Novikoff hepatoma cells,[6] and rainbow trout testis.[7] Nonhistone proteins in brain have not been purified to homogeneity yet, but were analyzed by gel electrophoresis after extraction of chromatin with urea–NaCl[8] and by chromatography on SP-Sephadex after extraction of chromatin with NaCl on a Bio-Gel A-50 column.[9] In the present study, we isolated two nonhistone proteins from bovine cerebral cortex by a simple and rapid procedure involving gel electrophoresis and extraction from the gel. The characteristics of these proteins are described.

Materials and Methods

Materials——Calf thymus deoxyribonucleic acid (DNA) (type 1), bovine serum albumin and D-ribose were obtained from Sigma Co., Ltd. Acrylamide was purchased from Kishida Chemical Co., Ltd.

Isolation of Chromatin from Bovine Cerebral Cortex——Bovine brain, obtained from a slaughterhouse, was depleted of blood vessels and meninges and then rinsed with cold 0.15 M NaCl solution. All procedures were conducted at 0—4 °C. Cerebral cortex (20 g of wet weight) was cut into pieces with scissors and homogenized with a Teflon-glass homogenizer with 10 strokes in 9 volumes of solution containing 0.25 M sucrose and 3.3 mM calcium acetate. The homogenate was filtered through 4 layers of gauze, and then centrifuged at $900 \times g$ for 20 min. This crude nuclear pellet was homogenized with a loosely fitting Teflon-glass homogenizer in 80 ml of solution containing 2.0 M sucrose and 3.3 mM calcium acetate, and then 25 ml of the suspension was layered on 5 ml of 2.0 M sucrose containing 3.3 mM calcium acetate. After centrifugation at $40000 \times g$ for 60 min, the pellets were combined and chromatin was extracted from the pellet according to the method of Shaw and Huang,[8a] with the exception that the chromatin protein was washed with 0.005 M Tris–HCl (pH 8.0). After centrifugation at $7700 \times g$ for 10 min, the pellet was suspended in 10 ml of deionized water and sheared 5 times with a Polvtron (Trnade ET 20, B. Braun Co., Ltd.) for 1 min at dial setting 20. The suspension was stirred with a magnetic stirrer overnight at 0—4 °C. The suspension was centrifuged at $10000 \times g$ for 30 min. The resultant supernatant contained chromatin and was used as a source for the isolation of nonhistone protein. The yield of chromatin was 30—44% as calculated from the DNA content of nuclei.

Protein was estimated by the method of Lowry *et al.*[10] with bovine serum albumin as a standard. DNA was extracted from chromatin or nuclei in 5% perchloric acid at 70 °C for 15 min and was determined by the method of Burton[11] with calf thymus DNA as a standard. Ribonucleic acid (RNA) in the chromatin was estimated by the orcinol method,[12] after hydrolysis in 0.3 N KOH at 37 °C for 18 h, with D-ribose as a standard.

Preparative Gel Electrophoresis——The lyophilized chromatin was dissolved in a solution containing 0.9 N acetic acid, 10 M urea and 1% 2-mercaptoethanol, and the solution was stirred with a magnetic stirrer for 18 h at 25 °C. After centrifugation at 34000 × *g* for 30 min, the supernatant (3 mg/ml, 2 ml) was electrophoresed according to the method of Panyim and Chalkley.[13] The slab gel (2 × 100 × 100 mm) containing 15% acrylamide, 6.25 M urea, 0.9 N acetic acid, 0.1% *N,N'*-methylenebisacrylamide, 0.5% *N,N,N',N'*-tetramethylethylenediamine and 0.125% ammonium persulfate was preelectrophoresed for 4 h at 16 mA. The sample was layered on the slab gel and run at 16 mA for 8 h. After electrophoresis, the gel was cut transversely into 2.5 mm slices from the top. A part of the cut gel was stained with Coomassie blue and destained with 0.9 N acetic acid. The gel strip confirmed to contain protein was buried in 15% acrylamide gel and a slot (2 × 40 × 10 mm) was made along the buried gel as depicted in Fig. 2. The gel was electrophoresed at 20 mA for 5 min and the protein was eluted into the slot, which was filled with 0.9 N acetic acid. The solution was dialyzed against deionized water for 18 h and lyophilized. The protein content was determined by amino acid analysis.

Analytical Electrophoresis——The molecular weights of proteins were determined by sodium dodecyl sulfate (SDS)–urea/phosphate gel electrophoresis according to the method of Olson *et al.*[14] Bovine serum albumin (68000), catalase (240000, subunit 60000), aldolase (158000, subunit 39500) and chymotrypsinogen (25000) were used as standards for molecular weight estimation.

Amino Acid Analysis——Amino acids were analyzed with an amino acid analyzer (KLA-5, Hitachi Co., Ltd.) after hydrolysis of the samples in 6.0 N HCl for 22 h at 110 °C.

Phosphate Estimation——Phosphate of proteins was determined by the method of Chen *et al.*,[15] after hydrolysis in 70% perchloric acid containing a few drops of concentrated H_2SO_4 at 100 °C for 3 h.

Results

Chemical Composition and Characteristics of Chromatin

The RNA/DNA and protein/DNA ratios of chromatin prepared from bovine cerebral cortex were 0.10 ± 0.02 and 2.14 ± 0.22, respectively (six experiments). The A_{230}/A_{260} and A_{260}/A_{320} ratios of the chromatin preparation were 1.02 and 20.58, respectively. These values coincide well with those reported for pig cellebellar and pituitary chromatins.[8a] Although over 400 nonhistone proteins have been reported, only a few of these proteins are selectively solubilized in medium containing 10 M urea, 0.9 N acetic acid and 0.5 M 2-mercaptoethanol and can selectively penetrate into the gel in a urea–acetic acid system. When the chromosomal proteins solubilized in the above medium were analyzed by one-dimensional gel electrophoresis using the urea–acetic acid system, they were separated into 9 bands of nonhistone proteins and 3 bands of histone proteins, as shown in Fig. 1-A and 1-B. Each band was numbered (bands 1 to 9 for nonhistone proteins and H1, H3, H2B, H2A and H4 for histone proteins), as shown in Fig. 1. These histones were identified by electrophoresing calf thymus histones under the same conditions as in Fig. 1. After two-dimensional gel electrophoresis, protein from each band except bands 3 and 4 was separated into two or three spots (Fig. 1-C). In contrast, protein from bands 3 and 4 was electrophoresed as a single spot in each case.

TABLE I. The Solubility of Chromatin in Various Solvents

Solvent	Solubility
2% SDS	96—99%
5 M guanidine–HCl	95—97%
6 M urea–0.4 M guanidine–HCl	90—95%
6 M urea–4 M NaCl	90—95%
10 M urea, 0.9 N acetic acid, 0.5 M 2-mercaptoethanol	40—60%

Bovine braine chromatin was solubilized in the various solvents and each solution was centrifuged at 34000 × *g* for 10 min. Protein in the supernatant was determined by the method of Lowry *et al.*[10]

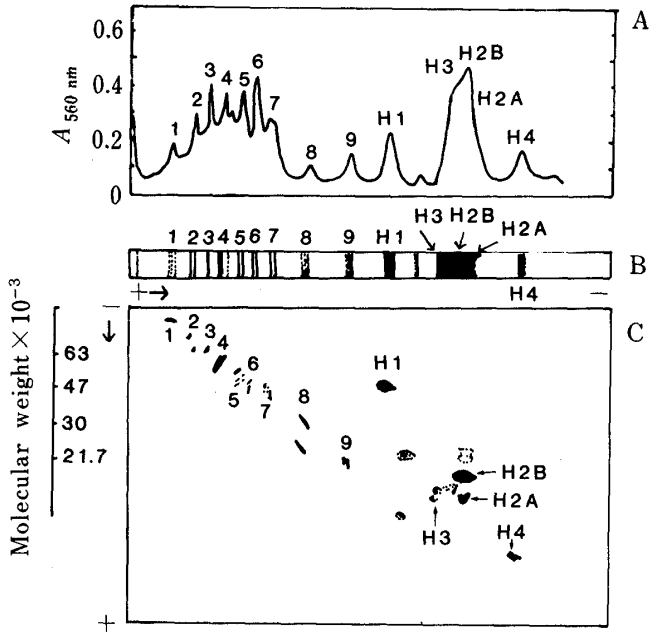

Fig. 1. Diagrammatic Representation of One-Dimensional Gel (B) and Two-Dimensional Gel (C) Stained with Coomassie Blue

Chromatin was solubilized in a solution containing 10 M urea, 0.9 N acetic acid and 1% 2-mercaptoethanol and then centrifuged at 34000 × *g* for 30 min. The supernatant was layered on polyacrylamide gel (5 × 100 mm) containing 15% polyacrylamide, 6.25 M urea, 0.9 N acetic acid according to the method of Panyim and Chalkley.[13] The gel was electrophoresed in the first dimension at 2 mA for 4 h, then stained with Coomassie blue (B) and scanned with a densitometer at 560 nm (A). Unstained gel underwent the second-dimensional gel electrophoresis as described in the text.

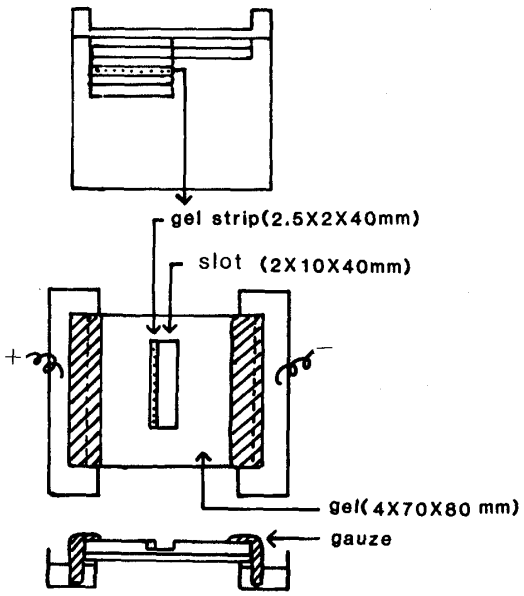

Fig. 2. Extraction Method of Non-histone Proteins from Slab Gel

After slab gel electrophoresis of chromatin proteins in the urea–acetic acid system, the slab gel was cut transversely into 2.5 mm slices from the top. Then the band containing protein was put in urea–acetic acid gel, and extracted as described in Materials and Methods.

Therefore, we extracted the proteins from bands 3 and 4 in the one-dimensional slab gel. Bands 3 and 4 proteins were estimated to amount to 4.6% and 5.0%, respectively, of total chromatin proteins that entered the urea–acetic acid gel as calculated from densitometric scanning of the Coomassie blue-stained gel.

Characterization of the Isolated Non-histone Proteins

The amino acid compositions of the isolated nonhistone proteins are shown in Table II. Surprisingly, each protein was rich in Gly and Ser, which represented about 36.5—44.2% of the total amino acids. Neither Met nor half-cysteine was detected. The ratios of acidic amino acids (Glu plus Asp) to basic amino acids (His plus Lys plus Arg) ranged from 1.67 and 1.72. The amounts of proteins extracted from bands 3 and 4 starting from 2.79 mg of chromatin proteins were 216 μg and 260 μg, respectively, as determined by amino acid analysis. The molecular weight of each protein estimated by two-dimensional electrophoresis using the SDS-urea system[14] was 63000 for band 3 and 53000 for band 4 protein. Determination of the molecular weights of these proteins by one-dimensional SDS gel electrophoresis was unsuccessful because the extracted proteins aggregated in the gel. Since nonhistone proteins are known to be phosphorylated in nuclei, the phosphate contents of proteins in bands 3 and 4 were determined as described in Materials and Methods. The protein in band 3 was found to be highly phosphorylated. The phosphate content of band 3 protein was 1.00% (w/w), which means that about 20 amino acid residues in the molecule were phosphorylated based on a molecular weight of 63000. The phosphate content of band 4 protein was 0.27%.

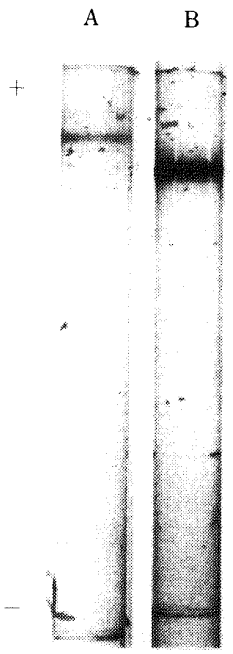

Fig. 3. Coomassie Blue-Stained Pattern of Isolated Bands 3 and 4

Each protein was extracted from the acrylamide gel, dialyzed against deionized water and lyophilized. After being solubilized in 0.9 N acetic acid, 10 M urea and 1% 2-mercaptoethanol, each protein was layered on urea–acetic acid gel[13] and electrophoresed, stained and destained. A, band 3; B, band 4.

TABLE II. Amino Acid Compositions and Pi Contents of Two Nonhistone Proteins Isolated from Bovine Cerebral Cortex

Amino acid	Band 3 (mol%)	Band 4 (mol%)
Lys	8.5	9.3
His	2.6	2.6
Arg	n.d.[a]	1.0
Asp	6.7	7.0
Thr	3.9	3.7
Ser	19.6	15.1
Glu	11.8	15.2
Pro	3.0	5.0
Gly	24.6	21.4
Ala	9.9	8.0
Cys/2	n.d.[a]	n.d.[a]
Val	4.0	4.3
Met	n.d.[a]	n.d.[a]
Ile	2.2	3.5
Leu	3.2	2.6
Tyr	n.d.[a]	n.d.[a]
Phe	n.d.[a]	1.2
Gul+Asp/ Lys+Arg+His	1.667	1.721
Pi % (w/w)	1.00	0.27

Each protein was hydrolyzed in 6 N HCl at 110 °C for 22 h, and then analyzed with an amino acid analyzer. Phosphate content was determined as described in the text. *a*) n.d. means "not detected".

Discussion

Peterson and McConkey[16] reported that there are over 400 nonhistone proteins in Hela cell nuclei. The success of the present study in isolating 2 non-histone proteins may be due to the selective solubility of these proteins in urea–acetic acid solution. The bands 3 and 4 proteins extracted from the gel both had particularly high contents of Ser, Gly and Glu and practically no Met or half-cysteine. In order to ascertain that this unusual amino acid composition is not due to contamination from the acrylamide gel, bovine serum albumin was electrophoresed in the same gel system and extracted from the gel by the same procedure. Albumin thus obtained had practically the same amino acid composition as described in the literature. It is interesting to note that the amino acid compositions of bands 3 and 4 proteins are very similar to that of the non-histone chromatin protein with a molecular weight of 13000 isolated from calf thymus[17] in that all these proteins are rich in Glu, Ser and Gly and have no Met or half-cysteine. The other interesting characteristic of this chromatin protein from calf thymus is that it is specifically phosphorylated by a cyclic adenosine monophosphate (cAMP)-independent protein kinase from nuclei of mouse spleen cells.[17] The proteins isolated in the present study also contained phosphate in the molecule. Hacha and Rredericq[18] also isolated strongly acidic nonhistone proteins from calf thymus nucleohistone by preparative gel electrophoresis with a nondenatured gel system. The proteins isolated by them, however, have somewhat different amino acid compositions in that significant amounts of Met were present.

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