Isolation of Nonhistone Proteins from Pig Thymus for Large Scale Preparation*

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SUMMARY

A method is described for the large scale isolation of nonhistone proteins from salt-urea dissociated pig thymus chromatin. After precipitating DNA in a dissociated chromatin solution with LaCl_3 at pH 7.9, the chromosomal proteins in the supernatant were precipitated by raising the pH of the supernatant to 8.6. The chromosomal proteins in the precipitate were dissolved and separated into nonhistone proteins and histones by chromatography on SP-Sephadex C-25 using a sodium chloride stepwise elution. Almost all of the total nonhistone proteins in chromatin was eluted from the column at 0.25M and 0.3M NaCl.

Key words : isolation, nonhistone proteins, pig thymus.

Introduction

The nonhistone proteins (NHP) have been studied extensively¹⁻⁴⁾, partly because of their intrinsic interest^{5,6)}, and partly because of the virtually universal expectation that some of them must function as specific regulators of gene transcription in eukaryotic organisms⁷⁻¹⁰⁾. To develop more detailed studies on the action of NHP in the expression of specific genes, it is quite desirable to establish a practical method to fractionate NHP and obtain the NHP fractions in enough quantity to be able to carry out protein-chemical or physico-chemical works in addition to biochemical ones. Van den

Broek et al. have reported¹¹⁾ a method for the isolation of NHP. They dissociated the chromatin in 3M NaCl-10mM Tris-HCl (pH 8.0) and removed DNA by gel filtration on Bio-Gel A-50m. Bhorjee & Pederson have also reported¹²⁾ a method for the isolation of chromatin proteins. They dissociated the chromatin in guanidine HCl-urea and removed DNA by ultracentrifugation. The removal of DNA from the dissociated chromatin solution by centrifugation or gel filtration is not always suitable for the large scale isolation of NHP, owing to the requirements of long-time centrifugation or a large volume of gel materials. On the other hand, Goodwin et al. have reported a method for the

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isolation of loosely bound NHP. They extracted the loosely bound NHP from the chromatin with 0.35M NaCl¹³⁻¹⁵.

In our laboratory, we have taken a new approach to the preparation of chromosomal proteins, using lanthanum chloride as a selective precipitant of free DNA from dissociated chromatin in 5M urea-2M NaCl-Tris-HCl buffer (pH 7.9), leaving the chromosomal proteins in the supernatant solution^{16,17}. This method, however, has a disadvantage in that the concentration of the chromosomal protein obtained is too low to submit to further fractionation procedures in large scale experiment.

In this report, an attempt is made to overcome this disadvantage by precipitating, at a higher pH, the chromosomal proteins as their lanthanum salts with a yield of around 70% of total chromosomal proteins. Thus, the LaCl₃ method can be used for large-scale preparation of NHP under relatively mild conditions.

Materials and Methods

Materials

Sephadex G-15 and SP-Sephadex C-25 (40-120 μ , 2.3 $\pm$ 0.3 meq/g) were purchased from Pharmacia Fine Chemicals, 2-mercaptoethanol from Nakarai Chemicals, LaCl₃·7H₂O from Mitsuwa's Pure Chemicals. Specially prepared reagents, acrylamide (monomer), N, N, N', N'-tetramethylethylenediamine and N,N'-methylenebisacrylamide were obtained from Wako Pure Chemical Industries. Coomassie brilliant blue R was obtained from Sigma Chemical Company. Urea was purchased from Wako Pure Chemical Industries, and deionized with Amberlite MB-3 just before use. All other reagents were of the purest grade commercially available.

Preparation of Chromatin from Pig Thymus

The preparation of chromatin from fresh pig thymus was carried out by the method described previously¹⁸ with a slight modification. All steps throughout the fractionation of nonhistone proteins

were carried out at 0-4°C. Minced pig thymus was homogenized in 6 volumes of 0.14M NaCl, 0.01M sodium citrate and 1mM phenylmethylsulfonylfluoride (PMSF) (pH7.0) with a Waring blender and filtered through two layers of gauze. The filtrate was centrifuged at 2,500 $\times$ g for 15 min. The sediment was then washed five times with the NaCl-sodium citrate solution in the same way. The precipitate was next resuspended in 0.05 M Tris-HCl (pH 7.6) (3ml/g fresh weight of pig thymus), and centrifuged at 6,000 $\times$ g for 20 min. The washing procedure was repeated once again in the same manner. Then, the precipitate was homogenized with two liters of 1mM PMSF solution using a Waring blender. The chromatin suspension obtained was poured into an equal volume of 4M NaCl by rapid stirring. The viscous solution of chromatin was sheared by a Potter-Elvehjem homogenizer and centrifuged at 35,000 $\times$ g for 60 min to remove unsheared chromatin. The supernatant was used for the isolation of whole chromosomal proteins.

Dissociation of Chromatin

In order to dissociate the chromatin into DNA and chromosomal proteins, solid NaCl, 8M deionized urea, 0.5M Tris-HCl (pH 7.9) and 2-mercaptoethanol were added to the chromatin solution obtained above to give final concentrations of 5M urea, 2M NaCl, 0.05M Tris-HCl and 2mM 2-mercaptoethanol (pH 7.9). The DNA concentration of dissociated chromatin solution was adjusted to 400 μ g per ml. The solution was allowed to stand at 4°C for 24h. with occasional shaking.

Precipitation of DNA by LaCl₃ and Concentration of Whole Chromosomal Proteins

Selective precipitation of DNA from the dissociated chromatin solution and concentration of whole chromosomal proteins in the supernatant were carried out as described in "Results".

Fractionation of the Chromosomal Proteins on SP-Sephadex C-25

About 500mg of the chromosomal protein fractions (pH8.6 Ppt. or pH8.6 Sup., see Table 1) dial-

alyzed against 0.02M Na-acetate buffer (pH5.2) containing 5M urea, 0.25M NaCl, 3mM 2-mercaptoethanol and 1.5mM EDTA were applied to a SP-Sephadex C-25 column (4.7×35cm) preequilibrated with the same Na-acetate buffer and eluted stepwise with 0.25, 0.3, 0.4 and 1.0M NaCl, successively, in 0.02M Na-acetate buffer (pH5.2) containing 5M urea, 3mM 2-mercaptoethanol and 1.5mM EDTA. The flow rate was about 30ml per h. The fractions collected from the column were combined into seven pools. After the determination of protein contents, each protein pool was dialyzed extensively against water, and lyophilized.

Gel Electrophoresis of Protein

Sodium dodecyl sulfate (SDS) gel electrophoresis was performed as described previously¹⁸⁾. The proteins were incubated at 37°C overnight in 0.01M Na-phosphate buffer (pH7.0)-1% SDS-1% 2-mercaptoethanol-8M urea. The running gels (5 × 85mm) were made up from 0.1M sodium phosphate buffer (pH7.0), 10% acrylamide, 0.27% N,N'-methylenebisacrylamide, 0.15% N,N,N',N'-tetramethylethylenediamine, 0.075% ammonium persulfate and 4 M urea. The samples were run at a constant current of 7 mA per tube for about 5 h, and were then stained with 0.25% Coomassie brilliant blue in 7% acetic acid-45% methanol for 2 h, and destained by diffusion in 7% acetic acid-5% methanol.

General Methods

Pig thymus whole histone was separated by the procedure described previously¹⁹⁾. DNA was determined by the diphenylamine method²⁰⁾, with pig thymus DNA as a standard. In some experiments, the concentration of DNA in chromatin was determined by measuring the absorbance, assuming that a 22.0 optical-density unit at 260nm was equivalent to 1 mg of DNA per ml. Protein was determined according to the method of Lowry et al²¹⁾, with bovine serum albumin as a standard.

Results

Effect of LaCl_3 Concentration on the Precipitation of DNA

It was ascertained that in the solution of 5M urea, 2M NaCl, 0.05M Tris-HCl and 2mM 2-mercaptoethanol (pH7.9), about 93% of chromosomal proteins were dissociated from DNA by gel filtration on Sephadex G-200 (data not shown). To precipitate the DAN selectively from the dissociated chromatin solution, the LaCl_3 method previously described in

Fig.1. Effect of concentration of LaCl_3 on the precipitation of DNA. For the preparation of dissociated chromatin, solid NaCl, 8M deionized urea, 0.5M Tris-HCl (pH7.9) and 2-mercaptoethanol were added to the chromatin solution to give final concentrations of 5M urea, 2M NaCl, 0.05M Tris-HCl and 2mM 2-mercaptoethanol (pH7.9). The DNA concentration of the dissociated chromatin solution was adjusted to 400 μg per ml. Nine ml of dissociated chromatin solutions were taken into test tubes and one ml of $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ solution, previously dissolved in the Tris-HCl buffer described above, was added to each dissociated chromatin solution to make the concentration of $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ as indicated in the figure. After standing for 1h at 0~4°C, the solution was centrifuged at 40,000×g for 1h. An aliquot of the supernatant was used for the determination of DNA and protein.

the case of calf thymus chromatin was used¹⁶⁾ with a slight modification. As shown in Fig. 1, at concentrations higher than 0.3% of $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$, about 98% of total DNA was precipitated as lanthanum salt. In this condition, however, chromosomal proteins were hardly precipitated. Thus, we adopted a concentration of 0.7% $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ in the present experiment.

Effect of pH on the Precipitation of DNA by LaCl_3

The precipitation of DNA by LaCl_3 was sensitively dependent on the pH of the dissociated chromatin solution. In a pH range from 7.8 to 8.0, a gelatinous precipitate of DNA appeared. In a pH range higher than 8.0, however, chromosomal proteins as well as

Fig.2. Effect of pH on the precipitation of DNA. For preparation of dissociated chromatin, solid NaCl, 8M deionized urea, 2-mercaptoethanol and 0.5M Tris-HCl of various pH values, as indicated in the figure, were added to the chromatin solution to give final concentrations of 5M urea, 2M NaCl, 0.05M Tris-HCl and 2mM 2-mercaptoethanol. The DNA concentration of the dissociated chromatin solution was adjusted to 400 μg per ml. Nine ml of dissociated chromatin solutions were taken into test tubes and one ml of $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ solution previously dissolved in the Tris-HCl buffer described above was added to each dissociated chromatin to make a final concentration of 0.7%. After standing for 1h at 0~4°C, the solution was centrifuged at 40,000 $\times g$ for 1h. DNA and protein in the supernatant were determined as described in Fig.1.

DNA were precipitated by LaCl_3 (Fig. 2). Usually, a buffer solution of intermediate pH (7.9) was employed. The gelatinous precipitate of DNA was removed by centrifugation at 40,000 $\times g$ for 1h. The supernatant contained about 91% of whole chromosomal protein and 2% of total DNA.

Precipitation of Chromosomal Proteins at a higher pH

The results of Fig. 2 indicates that fair amount, if not most of chromosomal proteins in the supernatant are precipitated by increasing pH above 8.1. Thus, an optimal pH for recovering the chromosomal proteins from the supernatant was searched for. Ten ml protein solutions of the supernatant were taken into test tubes with pH's adjusted from 8.0 to 9.6 with 2 N NaOH at intervals of 0.2. After standing at 0°C for 2 h, the resulting precipitates were recovered by centrifugation at 10,000 $\times g$ for 15min. As shown in Fig. 3, the precipitation of

Fig.3. Effect of pH on the precipitation of chromosomal proteins. Ten ml of chromosomal protein solutions after precipitation of DNA were taken into test tubes and pH's were adjusted from 8.0 to 9.6 with 2N NaOH at intervals of 0.2. After standing at 0°C for 2h, the resulting precipitates were recovered by centrifugation at 10,000 $\times g$ for 15min. The protein content in the precipitate was determined after dissolving into 1N NaOH.

Table I. Fractionation of pig thymus chromosomal proteins by LaCl_3 and pH treatments

Fraction	Volume (ml)	Protein Conc. ($\mu\text{g}/\text{ml}$)	Total Protein (g)	Recovery (%)
Dissociated chromatin	36000	611	22.0	100
LaCl_3 Sup. Fraction	39000	513	20.0	91
pH 8.6 ppt. Fraction	600	25333	15.2	69
pH 8.6 Sup. Fraction	38400	120	4.8	22

chromosomal proteins increased gradually, and the maximum recovery of the proteins was attained at pH 9.2 with a yield of about 88%. In the present experiment, however, the pH of the solution in the large scale experiment was settled at 8.6 to minimize the danger of peptide-bond hydrolysis. In this pH condition, 75% of the chromosomal proteins was precipitated.

The precipitate of chromosomal proteins at pH 8.6 (pH8.6 Ppt. fraction), dissolved in 0.02M CH_3COOH containing 0.25M NaCl, 3mM 2-mercaptoethanol

and 1.5mM EDTA was subjected to gel filtration on Sephadex G-15 to remove LaCl_3 . The flow-through protein fraction from Sephadex G-15 was subjected to further fractionation. On the other hand, the supernatant fraction from the precipitation at pH 8.6 (pH8.6 Sup. fraction) was concentrated to about 5 mg protein per ml on an Amicon Ultrafiltration Membrane UM-2. The concentrated solution was also applied to gel filtration on Sephadex G-15 as described above. The recovery of chromosomal proteins is summarized in Table I.

Table II. Recovery of the chromosomal proteins from a SP-Sephadex C-25 column

Fraction	pH 8.6 Ppt.		Fraction	pH 8.6 Sup.	
	mg	%		mg	%
Total Protein Applied	500	100	Total Protein Applied	500	100
A	37.9	8.3	a	17.4	3.9
B	32.4	7.1	b	28.5	6.4
C	13.2	2.9	c	25.8	5.8
D	42.0	9.2	d	43.6	9.8
E	94.0	20.6	e	81.4	18.3
F	158.9	34.8	f	88.1	19.8
G	27.8	6.1	g	70.8	15.9
Total Recovery (%)		91.3	Total Recovery (%)		89.1

Fractionation of the Chromosomal Proteins on SP-Sephadex C-25

The Sephadex fractions obtained above were dialyzed against 0.02M sodium acetate buffer (pH5.2) containing 5M urea, 0.25M NaCl, 3mM 2-mercaptoethanol and 1.5mM EDTA and applied to a

Fig.4. Elution profiles from a SP- Sephadex C-25 column of pH8.6 Sup. (A) and pH8.6 Ppt. (B). About 500mg of the chromosomal protein fractions (pH8.6 Sup. or pH8.6 Ppt.) dialyzed against 0.02M Na-acetate buffer (pH5.2) containing 5M urea, 0.25M NaCl, 3mM 2-mercaptoethanol and 1.5mM EDTA were applied to a SP-Sephadex C-25 column (4.7×35cm) preequilibrated with the same Na-acetate buffer and eluted stepwise with 0.25, 0.3, 0.4 and 1.0M NaCl, successively, in 0.02M Na-acetate buffer (pH5.2) containing 5M urea, 3mM 2-mercaptoethanol and 1.5mM EDTA. The flow rate was about 30ml per h. The arrows indicate the position where the different salt concentrations were applied to the column. The fractions combined into seven pools are indicated by bars above the column profiles.

SP-Sephadex C-25 column preequilibrated in the same buffer. Fig 4 A and 4 B show the chromatographic profiles of chromosomal proteins from a SP-Sephadex C-25 column by stepwise elution with buffers of increasing NaCl concentration. The eluates were combined into seven pools as indicated in the Figures, dialyzed against cold distilled water and lyophilized.

The sample applied to the column and each of the seven pools was analyzed for protein content to determine the recovery from the column. As shown in Table II, the average recoveries were 91% for pH8.6 Ppt. fraction and 89% in pH8.6 Sup. fraction. **Analysis of the SP-Sephadex C-25 Column Fractions with SDS Gel Electrophoresis**

The protein fractions obtained from the SP-Sephadex column were analyzed with SDS gel electrophoresis. Fig. 5A and 5B show the electrophoretic patterns of pH8.6 Ppt. and pH8.6 Sup. fractions, respectively. The results indicate that the two fractions were composed of virtually identical protein components. The passed-through fractions, A and a, were shown to be composed of a large number of NHP with a high degree of heterogeneity and wide distribution of molecular weights. The molecular weight of the smallest NHP was found to be ca. 5,000. The largest NHP had molecular weight greater than 150,000. The second fractions, B and b, which were eluted from the column at 0.3M NaCl, revealed four bands of NHP. Further electrophoretic examinations of these fractions under various conditions such as acid-urea gel system described by Panyim and Chalkley²²⁾ and two-dimensional gel system described by Yeoman, L.C. et al.²³⁾ indicated that histones were absent in this fraction (data not shown). The molecular weights of each band were estimated to be 24,000 for B-1 and b-1 ; 21,000 for B-2 and b-2 ; 18,000 for B-3 and b-3 and 16,000 for B-4 and b-4. The third fractions, C and c, which were also eluted from the column at 0.3M NaCl, contained histone H2B (C-1 and c-1), H2A (C-2 and c-2) and one of the major components of NHP (C-3 and c-3). The molecular weights of C

Fig.5A. SDS gel electrophoresis patterns of pH8.6 Sup. fraction. The electrophoresis was conducted as described in "Materials and Methods". The run was from the top to the bottom.

Fig.5B. SDS gel electrophoresis patterns of pH8.6 Ppt. fraction. The electrophoresis was conducted as described in "Materials and Methods". The run was from the top to the bottom.

-3 and c-3 were estimated to be about 15,000. The fourth fractions, which were eluted from the column at 0.4M NaCl (D and d), contained histone H2B (D-1 and d-1), H2A (D-2 and d-2) and H4 (D-4 and d-4) and few NHP (D-3 and d-3). The fifth fractions, which were also eluted from the column at 0.4M NaCl (E and e), contained only histone H3 (E-1 and e-1) and H2B (E-2 and e-2). The sixth fractions, which were eluted from the column at 0.4M NaCl (F and f), contained histone H2B (F-2 and f-2), H3 (F-1 and f-1) and H4 (F-3 and f-3).

The only apparent difference in the gel patterns between the pH 8.6 Ppt. and pH8.6 Sup. fractions was that the 1.0M NaCl eluent from pH 8.6 Ppt. fraction (Band G-1 and G-2) contained histone H3 and H4, while the 1.0M NaCl eluent from pH8.6 Sup. contained only histone H 1 (Band g-1).

These results indicate that almost all of the total NHP of chromatin was eluted from the column at 0.25M and 0.3M NaCl. The remaining NHP not eluted

by 0.25M and 0.3M NaCl were partially recovered in the 0.4M NaCl eluent fraction.

Discussion

The procedure presented here provides a simple and rapid method for preparing NHP on a preparative scale, that is, even in gram amounts, with a recovery of about 70% of the original amount of NHP present in the chromatin. For good recovery of the protein, it is especially important to dissociate fully the chromosomal proteins from the DNA prior to the addition of LaCl_3 , and maintain the protein solution at pH7.8 to 8.0. At a higher pH than 8.0, the protein begins to be precipitated by LaCl_3 together with DNA (Fig. 2).

The SDS gel electrophoresis patterns of pH8.6 Ppt. fraction are essentially the same as for that of pH8.6 Sup. fraction except for the G and g fractions. The g fraction shows distinct bands of histone H 1, while the G fraction shows two bands of histone H

3 and H 4. Thus, we can say that the proteins in the pH8.6 Ppt. fraction represent actually almost all

species of NHP originally contained in the chromatin preparation. In addition, the recoveries of protein were also reasonable, as described in "Results". In this method, we could prepare several grams of each NHP fraction from pig thymus.

Goodwin et al. have reported¹³⁻¹⁵⁾ a method for the isolation and fractionation of NHP's. That is, proteins loosely bound to chromatin were extracted with a low concentration of salt (0.35M NaCl). The proteins were further separated by precipitation with 2% and 10% trichloroacetic acid and by ion exchange chromatography on CM-Sephadex. By the use of these procedures, NHP's, loosely bound to chromatin, were well separated and characterized^{24,25)}, however, NHP's tightly bound to chromatin (NHP's not extractable with 0.35M NaCl, but extractable with 5M urea and 2M NaCl) or NHP's rigidly bound to chromatin (NHP's not extractable with 5M urea and 2M NaCl) were not obtained. Moreover, in these procedures nearly half of the 2% trichloroacetic acid precipitable fraction in loosely chromatin bound NHP, which contains a large number of acidic and neutral components, was virtually insoluble in usual buffer solutions²⁶⁾. On the other hand, in the presence of 5 M urea and 2M NaCl, NHP's tightly bound to chromatin were extracted together with loosely bound NHP's, and almost all the extracted chromosomal proteins were soluble in usual buffer solutions. Therefore, we decided to use the solution of 5M urea-2M NaCl-0.05M Tris-HCl and 2mM 2-mercaptoethanol (pH 7.9) for the extraction of the chromosomal proteins.

Thus, this method can be used for the purpose of large scale isolation of loosely and tightly bound NHP's under a relatively mild conditions.

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要 旨

遺伝情報発現に重要な役割をはたすと考えられている非ヒストン蛋白質 (NHP) の構造と機能を明らかにする目的の第1歩として豚胸腺から NHP を大量分離する為の条件と回収された核蛋白質の SDS 電気泳動による分析を行った。豚胸腺からクロマチンを単離後、5 M Urea, 2 M NaCl, 0.05M Tris-HCl, 2 mM 2-mercaptoethanol 中で93%の核蛋白質を DNA から解離させた。これに塩化ランタンを加えて DNA を選択的に沈殿させる条件を検討した結果、0.7%塩化ランタン、pH7.9の条件で98%の DNA が沈殿として、また、91%の核蛋白質が上清に回収された。上清に残った核蛋白質は pH を8.6に上げるのみで75%が沈殿と

して回収できた。pH を8.6に上昇させた後の上清 (pH8.6 Sup.) と沈殿 (pH8.6 Ppt) に含まれる蛋白質分子種の異同を知る目的で、両者を SP-Sephadex C-25 による NaCl 濃度の段階的上昇により溶出させたのち各溶出画分を SDS 電気泳動で分析した所、大部分の NHP は SP-Sephadex C-25 の0.25Mおよび0.3M NaCl 溶出画分に溶出すること、pH8.6 Sup と pH8.6 Ppt 画分で異なるのはヒストン画分のみであることが明らかとなった。これにより、NHP の分離の目的には pH8.6 Ppt.画分を使用しうること、さらに、これまで、その性質が明らかとなっていない DNA に強固に結合した NHP を分離する方法としても本法が使用できることがわかった。