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Fractionation of basic nuclear proteins of human sperm by zinc chelate affinity chromatography

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ABSTRACT

Immobilized metal affinity chromatography was investigated for the fractionation of basic nuclear proteins of human sperm. Human sperm nuclei essentially contain two classes of protamines: a protamine of type P1 (HP1), rich in cysteine but with only one histidine, and three protamines of type P2 (HP2, HP3, HP4), rich in cysteine and histidine (nine in protamine HP2), potential ligands for transition metal ions. The critical conditions for metal affinity chromatography were defined: choice of metal, protein material and buffer, type of elution and sample loading. Chromatography of nuclear proteins, without histones and with cysteine residues alkylated by iodoacetamide, was optimum on zinc Chelating Sepharose in a Tris–acetate buffer and elution with an increasing concentration gradient of imidazole. Under these conditions, the two classes of protamines were completely separated. The intermediate basic proteins were further purified by reversed-phase high-performance liquid chromatography. Heterogeneity of binding to zinc of protamine HP1 was demonstrated. The proposed method is simple and reproducible and the recovery of proteins is high. It may be applied to study the expression and function of P1 and P2 protamines, *e.g.*, in the case of infertile men.

INTRODUCTION

Metal chelate affinity chromatography of proteins was first described by Porath *et al.* [1]. This method allows the separation of proteins on the basis of their different affinities to chelated metal ions. The principles of the binding have been presented in detail by Porath's group [1–4]: exposed histidine, cysteine and tryptophan residues coordinate to many transition metals such as zinc, copper, nickel and cobalt etc. Of these amino acids, histidine seems to be the predominant ligand for zinc and copper [4]. The topography of histidine residues at the surface of the molecule is a critical factor for metal chelate affinity chromatography [4–6].

The nucleus of human sperm contains four different protamines, together with a small proportion of histones and of minor intermediate basic proteins [7]. The protamines may be divided into two groups: HP1 (P1 protamine), rich in cysteine but with only one histidine [8], and HP2, HP3 and HP4 (P2 protamines), which are structurally related and very rich in histidine (*e.g.*, nine in HP2 and eight in HP3), and containing also cysteine (five in HP2 and HP3) [9,10]. The intermediate basic proteins HPI1, HPI2, HPS1, HPS2 are now known as precursors of protamines HP2 and HP3 [11], and these proteins are also rich in histidine and cysteine. On the basis of these

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structural differences, metal chelate affinity chromatography could be expected to effect a fractionation of human protamines. In this paper, a procedure using chromatography on zinc Chelating Sepharose is described as a simple and convenient method for the separation of HP1 (a P1 protamine) from HP2, HP3 and HP4 (P2 protamines) together with their precursors.

EXPERIMENTAL

Isolation of nuclei and extraction of basic nuclear proteins

Sperm from human healthy, fertile donors was obtained from the CECOS (Lille, France). Nuclei were purified as described by Ammer *et al.* [10]. Extraction of the basic nuclear proteins was performed according to Gusse *et al.* [7], with slight modifications. Briefly, a pellet of nuclei was incubated for 1 h at 4°C under nitrogen in 0.05 *M* Tris–HCl–0.002 *M* EDTA–0.01 *M* dithiothreitol (pH 8.8). Alkylation of the thiols was effected with iodoacetamide (final concentration 0.03 *M*) for 1 h at 4°C. Reduced and alkylated proteins were extracted with 0.25 *M* hydrochloric acid, then precipitated with 20% trichloroacetic acid, washed with acidified acetone and acetone, dissolved in water and freeze-dried. A variation of this procedure was used in which nuclei were reduced with dithiothreitol as described above, but the alkylation step with iodoacetamide was omitted.

Metal chelate affinity chromatography

Chelating Sepharose 6B (Pharmacia) was thoroughly washed with water and the suspension (in water) was poured in a 3 × 1 cm I.D. column (volume of the gel = 0.5 ml). The gel was charged with 0.6 ml of 3 mg/ml zinc chloride solution, (*i.e.*, *ca.* 13.5 μmol of $ZnCl_2$).

The following buffers were used for equilibration: (A) 0.05 *M* sodium acetate–0.5 *M* NaCl (pH 7.0) and (B) 0.05 *M* Tris–acetate–0.5 *M* NaCl (pH 7.0). The column was equilibrated overnight with either buffer A or buffer B at a flow-rate 6 ml/h.

The elution procedures were as follows: (1) stepwise elution with buffer A or B of pH 7.0, 6.0, 5.0, 4.0 and 3.0 (14 ml of each buffer); (2) washing of the column with buffer A or B (pH 7.0) (14 ml), then stepwise elution with buffer A or B (pH 6.0) containing 0.1 *M* histidine (14 ml), then 0.5 *M* histidine (14 ml); (3) washing with buffer A or B (pH 7.0) (14 ml), then elution with a gradient of imidazole produced by mixing two identical cylindrical chambers containing for the first 40 ml of buffer A or B and for the second 40 ml of buffer A or B containing 0.25 *M* imidazole–HCl. The actual profile of the imidazole gradient was established by monitoring the absorbance at 220 nm (buffer B) or 240 nm (buffer A) of the effluent in several separate runs where no sample was applied to the column. Stepwise elution with imidazole in buffer B was also used as follows: washing with buffer B (14 ml) and elution with 0.05, 0.1 and 0.2 *M* imidazole in buffer B (14 ml of each solution).

In all the experiments, fractions of 2 ml were collected and the protein concentration in each fraction determined by the Coomassie blue method (see below). Fractions containing proteins were precipitated three times with 20% trichloroacetic acid and washed with acidified acetone and acetone. The pellet was stored dried at −20°C or dissolved at a final concentration 1 mg/ml in 0.01 *M* hydrochloric acid–8 *M* urea–0.5 *M* mercaptoethanol prior to polyacrylamide gel electrophoresis.

After chromatography, elution was performed with 0.05 *M* EDTA (pH 8)–0.05 *M* NH_4HCO_3 (pH 10.5)–water (50 ml of each) [12]. Fractions of 2 ml were again collected and the protein contents were determined. The column was then recharged with zinc chloride as described above.

The various elution procedures were also performed with Chelating Sepharose Fast Flow (Pharmacia). The experimental conditions for the preparation of the column and for elution were identical with those described above except that a flow-rate of 30 ml/h was used.

Reversed-phase high-performance liquid chromatography

Fractions containing P2 protamines together with precursors were loaded on a Deltapak C_{18} HPLC column (Millipore–Waters) (300 mm × 3.6 mm I.D.) equilibrated in 0.1% trifluoroacetic acid (TFA)–10% acetonitrile (flow-rate 1.5 ml/min). About 0.1 mg of protein material in 0.1 ml of 0.1% TFA was loaded on the column. A linear gradient from 10 to 35% acetonitrile in 0.1% TFA was developed in 60 min. Fractions of 1 ml were collected.

Protein determination

The protein concentrations in the solution loaded onto the zinc Sepharose column and in the fractions from chromatography were determined by Coomassie Blue protein dye-binding assay according to Read and Northcote [13] with an adaptation to a microtitration plate, and reading on a enzyme immunoassay (EIA) reader. A 10-μl volume of the protein solution was mixed in a well of a microtitration plate (96-well flat bottomed, microtitration plate, Falcon) with 40 μl of distilled water and 150 μl of Coomassie Brilliant Blue solution prepared as described [13]. The mixture was stirred to effect homogenization on a stir plate. The plate was read on an EIA reader (Dynatech MR 610) equipped with an interference filter at 600 nm. A standard of salmon protamine (Sigma, grade IV) was used at concentrations ranging from 5 to 100 μg/ml.

Electrophoretic controls

Proteins were analysed by electrophoresis in 17% acrylamide–6.25 *M* urea (pH 3.2) [14] on slab gels as described previously [7].

RESULTS

Protein material

The first experiments were performed with proteins extracted from human sperm nuclei according to Gusse *et al.* [7]. The material contains histones, together with protamines and intermediate basic proteins. Histones from sperm nuclei could not be resolved from one of the protamines, HP1, on a zinc Sepharose column whatever the procedure used. Further, this separation also could not be achieved on a copper Sepharose column (data not shown). Therefore, the simple preparation procedure of Ammer *et al.* [10] was used. This gives a protein material devoid of histones but containing all the protamines and intermediate basic proteins.

All the experiments reported below were performed with proteins extracted after reduction of nuclei with dithiothreitol and alkylation of the thiols with iodoacetamide. In order to determine the contribution of thiols to interaction with zinc Sepha-

rose, an extraction of nuclear proteins was performed without alkylation of the thiols with iodoacetamide. However, such a protein material, either in sodium acetate buffer (buffer A) or in Tris–acetate buffer (buffer B), yielded a substantial precipitate. The precipitate was analysed by polyacrylamide gel electrophoresis in 6.25 *M* urea–2-mercaptoethanol–0.9 *M* acetic acid (pH 3.2). All the protamines (together with minor intermediate basic proteins) were observed (data not shown). It was concluded that at neutral pH, reduced thiol groups are reoxidized with the formation of intermolecular bridges. Such a material was unsuitable for metal chelate affinity chromatography.

Buffers

Metal chelate affinity chromatography was first expected to be performed in a buffer with no chelating properties and a good buffering capacity at neutral pH, *i.e.*, phosphate buffer. Nevertheless, basic nuclear proteins of human sperm precipitate in 0.05 *M* sodium phosphate–0.5 *M* NaCl buffer (pH 7.0), probably because of the neutralization of guanidinium groups of arginine residues by phosphate ions. Protein determination in the supernatant indicated that less than 10% was soluble in phosphate buffer.

In order to study the effects on zinc affinity chromatography of buffers with or without chelating properties, two buffers were selected: 0.05 *M* sodium acetate–0.5 *M* NaCl buffer (buffer A) with no chelating activity and 0.05 *M* Tris–acetate–0.5 *M* NaCl (buffer B), where Tris is known to have low chelating properties [2,15,16]. The disadvantage of buffer A is its low buffering capacity at pH 7.0. In addition, sperm basic nuclear proteins are poorly soluble in buffer A (about 40–50%), whereas their solubility in buffer B is good (about 80–90%).

Elution by a pH gradient

Fig. 1 shows the elution profile with (a) buffer A and (b) buffer B at pH 7.0, 6.0, 5.0, 4.0 and 3.0. No protein is eluted with equilibration buffer of pH 7.0. With buffer A, two peaks are observed, at pH 5.0 (about 19%) and pH 4.0. The total yield of proteins recovered by chromatography is about 45%, starting with 0.5 mg of material. This low recovery is probably due to the poor solubility of sperm nuclear proteins in sodium acetate buffer (pH 7.0). Elution with buffer B also shows two peaks, one at pH 6.0 (12%) and the other at pH 4.0. The yield of protein is about 90%. With both buffers, no additional material is recovered by sequential treatment of the column with EDTA, ammonium carbonate and water. The first peak (eluted at pH 5.0 with buffer A and pH 6.0 with buffer B) represents protamine HP1 (Fig. 2); HP1a is the monophosphorylated form and HP1b the dephosphorylated protamine [7]. The second peak contains several proteins: the minor intermediate basic proteins, mainly HPS2, and the P2 protamines HP2, HP3 and HP4, but also a significant amount of protamine HP1, fraction HP1b being predominant (Fig. 2).

Elution with competitor ligand

Histidine. Fig. 3 shows the elution profile of a stepwise gradient with 0.1 and 0.5 *M* histidine in buffer B. The same results were obtained with buffer A (not shown). Also a gradient from 0 to 0.5 *M* histidine (2 × 40 ml) gave a similar profile. Protamine HP1 is recovered in the first peak (Fig. 4), and represents 25% of the total

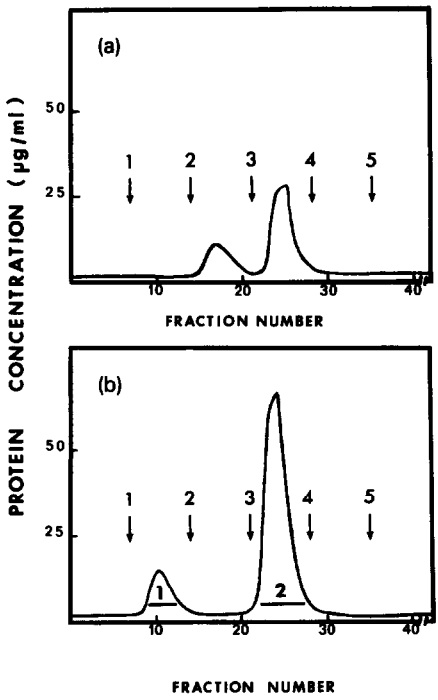

Fig. 1. Elution profile of human sperm basic nuclear proteins (0.5 mg) on a zinc Chelating Sepharose column (3 × 1 cm I.D.), equilibrated either in (a) buffer A (0.05 *M* sodium acetate–0.5 *M* NaCl, pH 7.0) or (b) in buffer B (0.05 *M* Tris–acetate–0.5 *M* NaCl, pH 7.0). Elution with equilibration buffer, then with buffer of pH (1) 6.0, (2) 5.0, (3) 4.0 and (4) 3.0, followed by (5) 0.05 *M* EDTA (pH 8.0) [Elutions with 0.05 *M* NH_4HCO_3 (pH 10.5) and water are not shown]. Volume of gel, 0.5 ml; flow-rate, 6 ml/h; volume of fractions, 2 ml. Protein concentration was determined by the Coomassie Brilliant Blue protein dye assay [13].

protein eluted from the column. The fraction eluted with 0.5 *M* histidine contains P2 protamines together with intermediate basic proteins and a small amount of protamine HP1b (Fig. 4). No protein was eluted either with the washing buffer or, at the end of chromatography, with EDTA, ammonium carbonate and water applied sequentially.

Imidazole. Fig. 5 shows the elution profile obtained with a gradient of imidazole from 0 to 0.25 *M* in buffer B. Two main peaks are obtained, but the second is preceded by a shoulder. It may be noted that the first fraction is eluted in a part of the gradient where the concentration of imidazole in the effluent increases weakly (from 0 to 0.02 *M*). This can be interpreted as binding of imidazole to available zinc ions. In the second part of the gradient, the concentration of imidazole increases sharply, from 0.02 to 0.1 *M*, corresponding to elution of the shoulder (fraction 2); thereafter, the gradient is linear from 0.1 to 0.25 *M* (elution of fraction 3). Electrophoretic controls (Fig. 6) showed that fraction 1 (about 29% of the total) contains HP1a and HP1b, fraction 2 (8% of the total) corresponds to HP1b and fraction 3 (63% of the total) contains P2 protamines, HP2, HP3 and HP4, together with intermediate basic

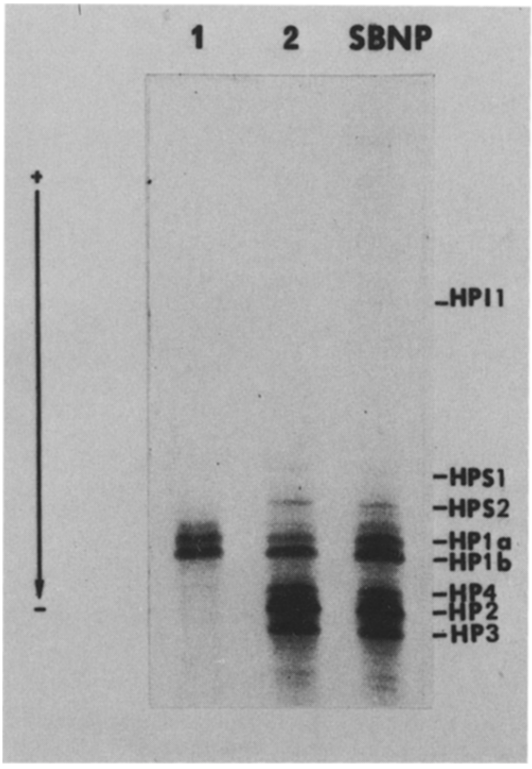

Fig. 2. Controls of fractions 1 and 2 from Fig. 1b by polyacrylamide slab gel electrophoresis (17% acrylamide) in 0.9 *M* acetic acid–6.25 *M* urea (pH 3.2). SBNP = sperm basic nuclear proteins loaded onto to the zinc Chelating Sepharose column.

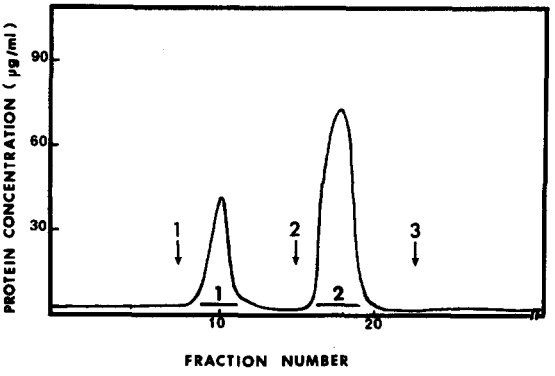

Fig. 3. Elution profile of human sperm basic nuclear proteins (0.4 mg) on zinc Chelating Sepharose (0.5 ml of gel) equilibrated in buffer B (0.05 *M* Tris–acetate–0.5 *M* NaCl, pH 7.0). Elution with (1) 0.1 *M* histidine, (2) 0.5 *M* histidine in buffer B (pH 6.0) followed by (3) 0.05 *M* EDTA (pH 8.0). Flow-rate, volume of fractions and protein determination as in Fig. 1.

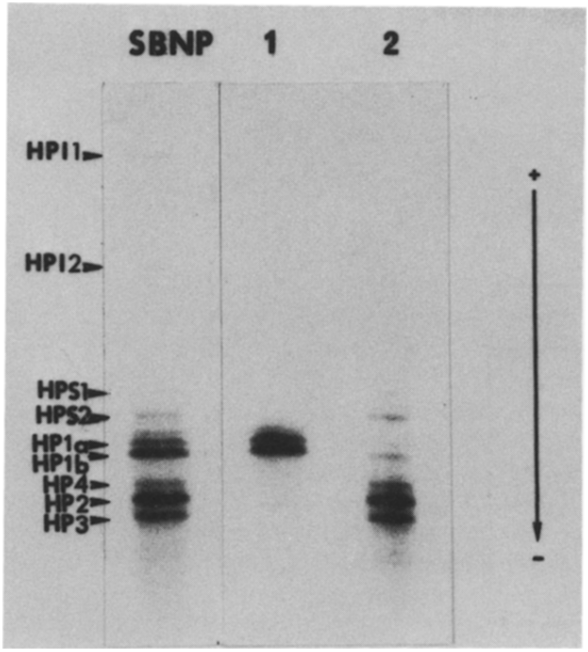

Fig. 4. Polyacrylamide slab gel electrophoresis of fractions 1 and 2 from Fig. 3. Experimental conditions as in Fig. 2. SBNP = Sperm basic nuclear proteins used as starting material.

proteins, mainly HPS2. The P2 protamines and the main minor protein HPS2 were resolved by reversed-phase high-performance liquid chromatography (HPLC) (Fig. 5b) with a linear gradient from 10 to 35% acetonitrile. Fraction 3a corresponds to P2 protamines and fraction 3b to protein HPS2 (Fig. 6). The other minor proteins were present in too small amounts to be detected.

It is important to note that a gradient of imidazole in sodium acetate buffer (buffer A) gave similar results, and that no protein material was detected either by elution with the washing buffer or with subsequent treatments for regeneration of the column. In addition, we observed that stepwise elution with buffer B and buffer B with 0.05, 0.1 and 0.2 *M* imidazole was able to separate P1 protamine (elution with 0.05 *M* imidazole) from P2 protamines and intermediate basic proteins (elution with 0.2 *M* imidazole). In all the experiments, the yield of protein recovered from the column was between 80 and 90%.

Effect of protein load

This effect was only studied in the case of stepwise elution with imidazole in buffer B. A column of 0.5 ml was loaded, in separate runs, with 0.25, 0.5, 1 or 2 mg of protein. The results were reproducible when the loading of the column was from 0.25 to 1 mg of protein (Table I). With 2 mg, part of the protein material was eluted with the washing buffer and corresponded to protamine HP1 (the remaining HP1 was eluted with 0.05 *M* imidazole). P2 protamines together with intermediate basic proteins were eluted with both 0.1 and 0.2 *M* imidazole (Table I).

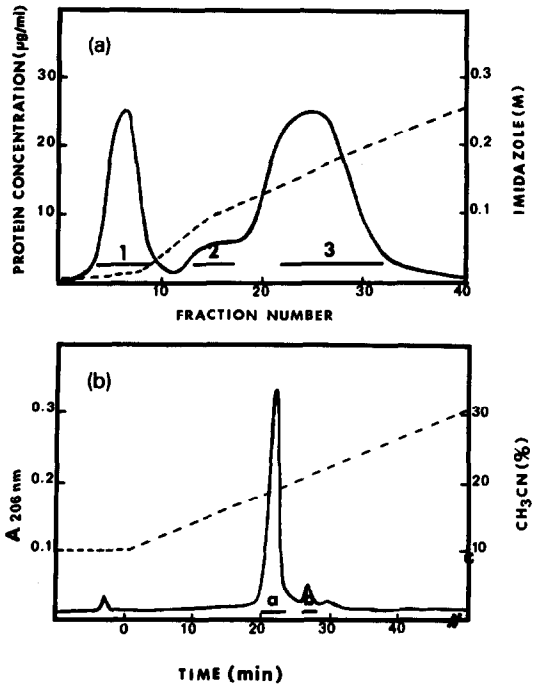

Fig. 5. (a) Fractionation of human sperm basic nuclear proteins (0.5 mg) on zinc Chelating Sepharose (0.5 ml of gel) equilibrated in buffer B (0.05 *M* Tris–acetate–0.5 *M* NaCl, pH 7.0). After washing the column with 15 ml of buffer B (not shown), an increasing concentration gradient of imidazole was applied to the column (0 to 0.25 *M*, produced by mixing two cylindrical chambers containing in the first 40 ml of buffer B and in the second 40 ml of 0.25 *M* imidazole in buffer B). The actual profile of the imidazole gradient was monitored by measuring the absorbance at 220 nm of the effluent in separate runs where no sample was applied to the column. Flow-rate, volume of fractions and protein determination as in Fig. 1. (b) Reversed-phase HPLC separation of fraction 3 from Fig. 5a. Column: Deltapak C_{18} (300 × 3.6 mm I.D.). Elution with 10% acetonitrile in 0.1% trifluoroacetic acid (TFA) for 10 min, followed by a linear gradient from 10 to 35% acetonitrile in 0.1% TFA for 60 min at room temperature and flow-rate 1.5 ml/min. Sample load, 0.12 mg. The elution profile was monitored by determination of the absorbance at 206 nm ($A_{206\ nm}$).

DISCUSSION

The purpose of this work was to evaluate metal chelate affinity chromatography as a method for the separation of human sperm basic nuclear proteins. Sperm chromatin contains essentially two classes of protamines: one P1 protamine (HP1) containing six cysteine residues but only one histidine, and three P2 protamines (HP2, HP3 and HP4) with five cysteine residues, but also very rich in histidine (*e.g.*, nine in HP2). Cysteine residues are present in the nucleus essentially as disulphide bridges. Hence extraction of the protamines (and intermediate basic proteins) requires the reduction of cystine by dithiothreitol and blocking of the thiols by an alkylating agent, such as iodoacetamide. We have tried to evaluate the role of cysteine in metal chelate affinity chromatography by performing a reduction of nuclear proteins with dithiothreitol without alkylation with iodoacetamide. However, the proteins extract-

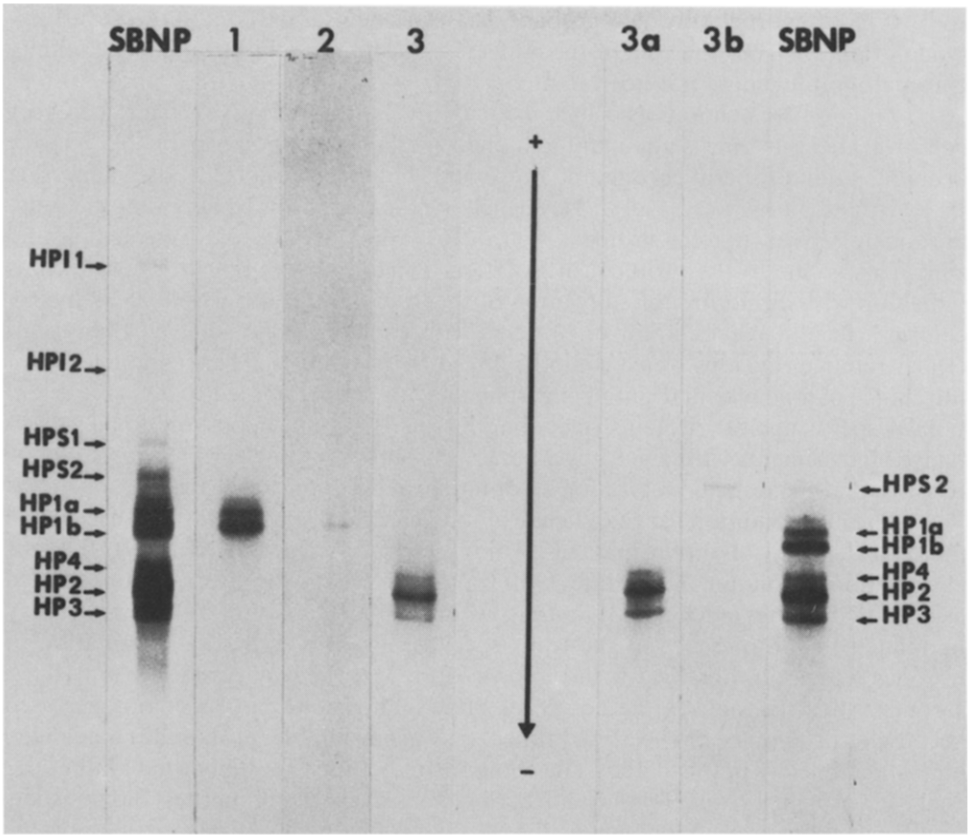

Fig. 6. Electrophoretic controls of fractions 1, 2 and 3 from Fig. 5a and fractions 3a and 3b from Fig. 5b by polyacrylamide gel electrophoresis. Experimental conditions as in Fig. 2. SBNP = sperm basic nuclear proteins used as starting material.

TABLE I

EFFECT OF PROTEIN LOAD ON ZINC CHELATING SEPHAROSE CHROMATOGRAPHY

A column (0.5 ml of gel) equilibrated in 0.05 *M* Tris–acetate–0.5 *M* NaCl (pH 7.0) (buffer B) was used. Results are recoveries of total protein (%).

Eluent	Amount of protein applied (mg)			
	0.25	0.5	1	2
Buffer B	0	0	0	10
Buffer B with 0.05 *M* imidazole	38	37	36	28
Buffer B with 0.1 *M* imidazole	0	2	2	27
Buffer B with 0.2 *M* imidazole	62	61	62	35

ed by 0.25 *M* hydrochloric acid probably make, at neutral pH, intermolecular disulphide bridges. Hence the role of the cysteine of protamines in metal chelate affinity chromatography could not be evaluated.

Zinc ion was chosen as a ligand for the following reasons. First, it has been demonstrated that zinc is abundant in human sperm nuclei and probably plays a role in stabilization of sperm chromatin; a reversible binding to thiols has been suggested by Kvist and co-workers [17,18]. Nevertheless, it is important to note that the preparations of sperm nuclear proteins used in this study are devoid of protein-bound zinc. This is due to the protocol of isolation, which involves several steps, such as reduction with dithiothreitol, alkylation of the thiols, extraction with 0.25 *M* hydrochloric acid and precipitation of proteins with trichloroacetic acid; all these steps clearly remove zinc ions which could be linked to protamines. The second reason for the choice of zinc was that initial experiments with copper ion led to a strong retention of sperm nuclear proteins (including histones), so no separation could be obtained. Experiments with zinc showed that a resolution of nuclear proteins could be possible and the critical factors for an optimum separation were studied.

The first condition for zinc chelate affinity chromatography was to use a protein material depleted of sperm histones. This fraction has a weak affinity to the zinc chelate affinity column and coelutes with protamine HP1. This result was unexpected as somatic histones (such as calf thymus histones) do not bind to the column. It may be related to the presence of the testis specific variant TH2B [19]. Elimination of histones was easily obtained with the procedure described by Ammer *et al.* [10]. A second critical factor was the choice of buffer. Phosphate buffer, commonly used because of its absence of chelating properties, was unsuitable; protamines precipitate almost completely in this buffer. The comparison between sodium acetate buffer (no chelating properties) and Tris–acetate buffer (weak chelating properties) did not show significant differences. More especially, all the protein material was retained on the column at neutral pH. Hence Tris–acetate (buffer B) is to be preferred as protamines are almost completely soluble in this buffer, whereas this is not the case with sodium acetate.

The optimum separation of sperm nuclear proteins was obtained by the use of a competitor ligand. Imidazole gave the best resolution: the two classes of protamines P1 and P2 (the intermediate basic proteins being related to P2 protamines) are well separated, either by a gradient from 0 to 0.25 *M* imidazole or by stepwise elution. The latter method is simpler and is now routinely used in our laboratory.

Chelating Sepharose Fast Flow is preferred to Chelating Sepharose, as the separation is fast (2 h). The weak affinity of protamine HP1 and the high affinity of P2 protamines (together with their precursors HPS1, HPS2, HPI1 and HPI2) seems to be clearly related to their different contents of histidine residues. Nevertheless, two points deserve comment. First, the affinity of P2 protamines is very high (in comparison with other proteins or large peptides previously described). This may be due to the large number of histidine residues and also to the good accessibility of this potential ligand; protamines are small and very hydrophilic molecules. A second point observed in chromatographic elution was unexpected, namely the presence of two fractions related to HP1. The second minor fraction has the electrophoretic mobility of protamine HP1b (dephosphorylated HP1). The origin of such heterogeneity is unknown. It may be due to the existence of protamine variants. Gusse *et al.* [7] also

observed different fractions corresponding to HP1 when purification was achieved by reversed-phase HPLC after chromatography on CM-cellulose.

Zinc chelate affinity chromatography of human sperm nuclear proteins is a simple and efficient method for the purification of P1 and P2 protamines. The minor intermediate basic proteins may be recovered (essentially protein HPS2) by a second step of reversed-phase HPLC. The method is reproducible if the protein loading is correctly adjusted to the size of the column. A ratio of 1 mg of protein to 0.5 ml of gel seems optimum. Larger amounts probably induce a displacement of P1 protamine by P2 protamines, which have higher affinity for zinc ions. Such a result has been reported previously for serum proteins by Porath and Olin [2].

Other procedures for the isolation of human sperm nuclear proteins have been described previously. Reversed-phase HPLC was proposed by Ammer *et al.* [10]. This technique can only be applied to small amounts of material (a few hundred micrograms). Ion-exchange chromatography on CM-cellulose has the advantage of very good resolution [7,9]. All the protamines may be separated by this method [7], but it is difficult to use CM-cellulose chromatography for small amounts of material such as a few milligrams, and the recovery is low (about 30–40%).

Zinc chelate affinity chromatography has the advantage of a very good recovery of protein material (about 80–90%). Hence this method will be very useful for studying the expression and function of P1 and P2 protamines. For example, Balhorn *et al.* [20] have suggested that expression of P2 protamines is reduced in some infertile men. Zinc chelate affinity chromatography could be applied to the isolation of protamines from small amounts of material, such as individual ejaculates from infertile patients. In addition, the procedure may also be applied to study the expression of nuclear proteins during spermatogenesis, or to other species which possess P1 and P2 protamines such as the mouse and hamster [21].

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