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Ethanol Extraction of Basic Proteins from Ejaculated Human Spermatozoa

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Abstract

A method for the extraction of basic proteins from human ejaculated spermatozoa has been developed It relies on the previously unreported observation that such basic protein is soluble in a solution containing 60% (v/v) ethanol. This unconventional method yields a high percentage of arginine-rich basic protein which is then able to be characterized on the basis of its amino acid composition. This method also allows comparisons to be made between single ejaculates by the banding pattern each displays when subjected to polyacrylamide gel electrophoresis.

Introduction

The extraction of nuclear basic proteins from ejaculated human spermatozoa has been the topic of much research since 1969 when Coelingh *et al.* first managed to obtain purified sperm histones. Their method consisted of solubilizing the sperm heads with β-mercaptoethanol (which breaks disulfide bonds) and of blocking the reverse reaction with ethylenimine. The solution was acidified with 5 M HCl and the basic proteins thus extracted were then purified by dialysis for 2 days. This method, although the basis for the current work, is unwieldy as the time taken to obtain purified sperm proteins is long when several hundred ejaculates need to be processed.

Kistler *et al.* (1973), used approximately 2×10^{10} spermatozoa for each extraction. The sperm heads were lysed with dithiothreitol and ethylenimine was added. Ethanol was used to precipitate the proteins, which were then dissolved in 0·25 M HCl. When this method was adapted for the purposes of research into the protein composition of single ejaculates, however, the yield was too small for any quantitative work to be carried out. In later work by the same authors (Kistler *et al.* 1975) β-mercaptoethanol was used in the place of dithiothreitol as the agent to lyse the sperm heads. Ethanol once again was used to precipitate the sperm proteins. However, when this method was adapted for specific work on single ejaculates the yield likewise was too low in 95% of the samples.

As our work was involved with the nuclear basic protein profile of single ejaculates (Reid *et al.* 1978) a method needed to be developed so that a large proportion of sperm protein available in a sperm head was extracted. The development of such a method was significantly advanced by the discovery that a large proportion of the sperm histones is soluble in ethanol, an observation previously unreported. This paper

aims to set out the method used to obtain consistently high yields from single ejaculates and to discuss the possibility that a new histone-like protein has been discovered by virtue of its solubility in ethanol.

Materials and Methods

Semen

Semen samples were obtained from outpatients of various infertility clinics in Sydney, Australia, and Sheffield, England.

Solubilization of the Spermatozoa

The semen from a single ejaculate was centrifuged at 10,000 *g* for 2 min in order to separate the seminal plasma. The spermatozoa were suspended in distilled water and centrifuged again, then resuspended in 5 ml distilled water and sonicated for 90 s at 75 W on a Bronwill Biosonik IV. The spermatozoa were counted in a haemocytometer and the suspension was centrifuged through a sucrose gradient solution at 700 *g* for 45 min (Silvestroni *et al.* 1976) in order to separate the heads and tails. The pellet (heads) was resuspended in 3 ml of a mixture of equal parts 0·2 M borate buffer (pH 9·2) and 10 M guanidium chloride; 0·1 ml β-mercaptoethanol was added, and nitrogen was bubbled through the solution. After 30 min 20 μl ethylenimine was added, followed 15 min later by a further 20 μl ethylenimine (Kistler *et al.* 1975). The mixture was incubated at 25°C for 60 min; 5 ml absolute ethanol was added to the mixture whilst agitating on a vortex mixer. The gel precipitated was homogenized in a Potter–Elvehjem homogenizer, the homogenate was centrifuged and the supernatant retained. Four volumes of cold acetone were added and the solution left to precipitate for 2 h at −20°C. The white, fluffy precipitate was obtained by centrifuging at 3000 *g* for 10 min and the pellet allowed to dry over anhydrous calcium chloride. The precipitate was dissolved in 0·1 M HCl.

Protein Estimation

Protein concentrations were estimated by the method of Udenfried *et al.* (1972); 50 μl of the protein solution was added to 1·45 ml of 0·2 M borate buffer (pH 9·2) and 0·5 ml of fluorescamine (Fluram, Roche) was mixed in whilst agitating. Solutions of histone type II-A (Sigma) were used as standards.

Polyacrylamide Gel Electrophoresis

Electrophoresis on 15% (w/v) acrylamide gels was performed at pH 2·7 as described by Panyim and Chalkley (1969). The gel tubes were 8 cm in length with an internal diameter of 6 mm. A tracking dye (0·1% aqueous solution of methyl green) was used to monitor the time of electrophoresis. The gels were removed from the tubes and stained in a solution containing 0·15% (w/v) amido black, 20% (v/v) ethanol and 7% (v/v) acetic acid for 2 h. The gels were de-stained by diffusion in 6% (v/v) acetic acid. A standard amount (250 μg) of sperm basic protein was added to each gel.

Column Chromatography

Bio-Gel P6 was prepared by hydrating the beads in 0·01 M HCl overnight. The slurry was packed into a 2·5 cm by 30 cm column; 0·01 M HCl was used as the eluant with a flow rate of 0·75 ml/min. 135 2-ml fractions were collected, and were assayed at 280 nm on a spectrophotometer.

Amino Acid Composition

The separated peaks from the column chromatography were hydrolysed with 6 M HCl *in vacuo* for 24 h at 110°C. The amino acid composition was obtained using a Beckman amino acid analyser. The analyses were performed according to the method of Moore and Stein (1963). Three samples of each peak were analysed separately and the mean values obtained.

Results

Protein Extraction

When acetone is added to the alcohol solution, the mixture becomes cloudy and after 2 h at −20°C a substantial precipitate forms. When alcohol is not used, the amount of precipitate obtained is considerably less.

Protein Estimation

In a batch of 100 single ejaculates the mean sperm count was $128{\cdot}4 \times 10^6$ sperm, and the mean protein yield was 2·41 mg. The dry weight of a single human sperm has been estimated at 23 pg; thus the total dry weight available for extraction was 2·953 mg. Borenfreund *et al.* (1961), have estimated that 15% of the weight of a human sperm is DNA; thus the maximum total protein available was 2·51 mg. The extraction thus represented a yield of 96% of all available protein.

Gel Electrophoresis

The band of poly-L-arginine is distributed over a wide area of the polyacrylamide gel as would be expected for such a heterogeneous mixture of molecules. However, the electrophoretic mobility of poly-L-arginine is similar to that of the heavy band of highest electrophoretic mobility in the extracted sperm proteins (Fig. 1). The other marker used, histone type II-A, migrated as two discrete bands and their electrophoretic mobility closely approximated that of some protein bands in the extracted sperm proteins. When compared with a series of standard histones from calf thymus (type II-A, III, III-S, and VIII-S) the sperm proteins showed a similar electrophoretic mobility, with the exception of the heavy band of highest electrophoretic mobility (Fig. 2). This band migrated much further than the standard histones, indicating a significant difference in molecular weight. It is, however, difficult to generalize as each single ejaculate gave a different electrophoretic banding pattern (Reid *et al.* 1978).

Bio-gel P6 Chromatography

The basic proteins were further characterized by exclusion chromatography. Four peaks (Fig. 3) were obtained, two of which, peaks A and B, were eluted in the void volume, indicating molecular weights greater than 6000. Peaks C and D were eluted much later, indicating molecular weights of less than 6000 and on gel electrophoresis, showed the same mobility and so were combined for convenience into fraction C. The electrophoretic mobility of this peak was similar to that of poly-L-arginine on polacrylamide gel electrophoresis.

Amino Acid Composition

The amino acid composition of the three peaks obtained from column chromatography is shown in Table 1. It will be noted that fraction C has the highest percentage

Fig.1

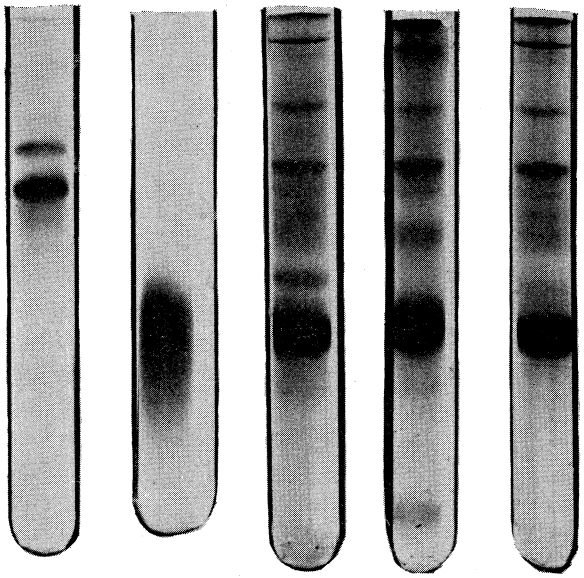

Fig. 2

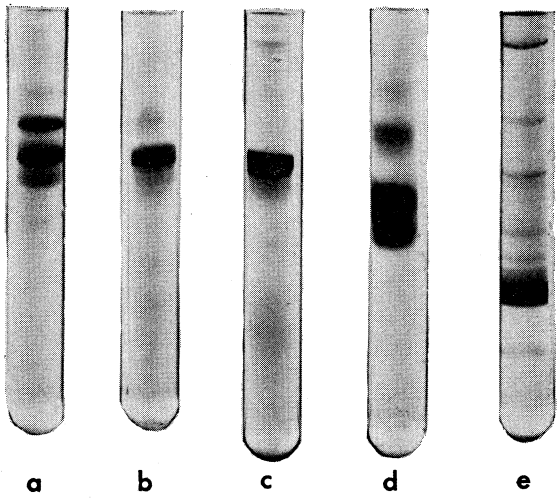

Fig. 1. Polyacrylamide gel electrophoresis of (*a*) histone type II-A standard; (*b*) poly-L-arginine standard; (*c*)–(*e*) sperm basic proteins extracted from single ejaculates from three different males.

Fig. 2. Comparison of standard histones from calf thymus and extracted sperm basic proteins on 15% (w/v) polyacrylamide gel electrophoresis. (*a*)–(*d*) Histone types FII-A, FIII, FIII-S, FVIII-S respectively. (*e*) Sperm basic proteins.

of arginine in agreement with the previous evidence from polyacrylamide gel electrophoresis.

Discussion

The basis of the above method was the extraction procedure of Coelingh *et al.* (1969), who isolated and purified nuclear basic protein on the basis of reductive cleavage of disulphide bonds by β-mercaptoethanol. This reagent has been used extensively for the lysis of sperm heads (Panyim *et al.* 1970). The effectiveness of β-mercaptoethanol is due to the disruptive effect on sulfydryl bonds. These bonds are responsible for the extraordinary structural stability of human spermatozoa (Calvin and Bedford 1971). Dithiothreitol is another solvent that has been used to lyse sperm heads (Kolk and Samuel 1975; Puwaravutipanich and Panyim 1975). It was found to be ineffective under the conditions used, as microscopic examination of the solution 60 min after addition of dithiothreitol showed many intact sperm heads. No intact sperm heads were found when β-mercaptoethanol was used.

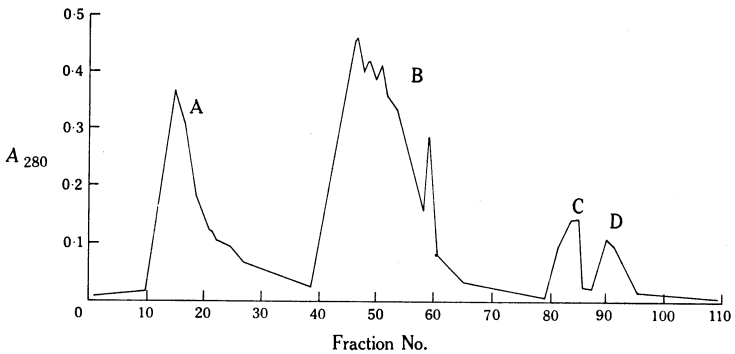

Fig. 3. Bio-Gel P6 column chromatography of a solution of sperm basic proteins. The elution was carried out in 0·1 M HCl. Each fraction contained 2 ml of solution. Peaks C and D were combined to give fraction C; peaks A and B gave fractions A and B respectively.

Ethylenimine was used to convert the free sulfydryl groups resulting from cleavage with β-mercaptoethanol to 5-β-aminoethyl derivatives (Kistler *et al.* 1973, 1975). The protein yield obtained when ethylenimine was added was five times that obtained without ethylenimine (as measured by fluorimetry). Mercury ions and iodoacetamide, potential disulfide bond reducing agents, were also used but with less satisfactory results than those obtained when using ethylenimine.

The basis of the extraction procedure is the discovery that basic proteins from the sperm head are soluble in 60% ethanol. This previously unreported fact was used to quantitatively extract milligram quantities of nucleoprotein from single ejaculates. Other proteins that are soluble in 60% ethanol include histone type II-A (from calf thymus) and poly-L-arginine.

Although this method is unique in its use of ethanol as a protein solvent, the compositions of the proteins extracted is in agreement with those of other workers. On the basis of molecular weight, the results from exclusion chromtography are similar to those obtained by Puwaravutipanich and Panyim (1975) and Kolk and

Samuel (1975). This indicates that fractions A and B may be considered as histones and fraction C as a protamine. Table 1 indicates the amino acid composition of the three peaks extracted. Fraction C has a very high proportion of arginine residues (40%) and this compares with the results of Puwaravutipanich and Panyim (1975) for their peak II. Kolk and Samuel (1975) also have reported the isolation of two protamines with an arginine content of 46%. It would appear therefore that fraction C is the same protamine that has been extracted by other techniques.

Table 1. Amino and composition of human sperm basic proteins
Fractions A, B and C are the protein peaks from Bio-Gel P-6 column chromatography. The values are moles per 100 moles of amino acids recovered after hydrolysis

Amino Acid	Fraction A	Fraction B	Fraction C
Lysine	trace	1·0	4·3
Histidine	10·78	1·0	7·5
Arginine	30·00	38·25	40·0
Aspartic acid	9·6	12·5	3·0
Threonine	2·8	4·5	1·0
Serine	18·2	11·0	5·0
Glutamic acid	trace	17·5	6·0
Proline	trace	trace	trace
Glycine	8·2	1·8	trace
Alanine	3·2	1·0	4·8
½Cystine	3·2	4·8	3·8
Valine	trace	trace	trace
Methionine	1·8	1·8	5·0
Isoleucine	1·3	2·8	trace
Leucine	2·7	3·5	trace
Tyrosine	1·7	—	9·0
Phenylalanine	1·0	4·8	trace

An important difference between the above results and other published results occurs in peaks A and B. The content of arginine is considerable (greater than 30%) and those peaks have been shown to co-migrate with the histone standard on polyacrylamide gels. For similar electrophoretic mobilities, Puwaravutipanich and Panyim (1975) have reported a much smaller amount of arginine (6%) in the protein. However, Pongsawadi and Svasti (1976) used trichloroacetic acid to extract nucleoproteins from dialysed solutions of sperm and β-mercaptoethanol and obtained four 'pools' of proteins each of which contained a concentration of arginine similar to that reported above.

It would appear, therefore, that the use of ethanol selectively extracts those basic nucleoproteins in the sperm head which are rich in arginine. However, the quantity extracted from single ejaculates (96%) indicates that these are the majority of nucleoproteins present. The results after treating 200 single ejaculates by the method described indicate that the banding pattern of extracted basic protein from human sperm heads varies significantly between males (Reid *et al.* 1978). The advantages of this method for examining single ejaculates are, firstly, the high yield obtained and, secondly, the speed of extraction.

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