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MAJOR PROTEINS SECRETED BY THE EPIDIDYMIS OF *LACERTA VIVIPARA*

ISOLATION AND CHARACTERIZATION BY ELECTROPHORESIS OF THE CENTRAL CORE

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Summary

Lizard epididymis produces large secretory granules (6 μm) which are discharged and mix with the spermatozoa. They consist of a dense central core and a peripheral vacuole. Central cores were prepared by two means: (1) homogenization of epididymal cells then isolation of a granular fraction by centrifugation on a discontinuous sucrose density gradient as a final step, and (2) collection of epididymal fluid containing both granules and spermatozoa, and separation of these elements by several steps of low speed centrifugations and washings. Purity of the different fractions was checked by microscopy. After complete dissolution in Triton X-100 (2.5%), the fractions containing central cores were submitted to SDS-polyacrylamide gel electrophoresis (15% acrylamide bisacrylamide). When apparently free from surrounding material, the dissolved central cores analyzed by electrophoresis showed only a main band representing a single protein (or a small group of proteins) of relative low mobility (molecular weight about 70 000). Other more mobile proteins have been identified in less purified fractions. They probably originate from the peripheral vacuole but this point is still under investigation. These two types of proteins do not originate from plasma or testis. Their androgen dependence is discussed.

Introduction

Epididymal secretions play an important part in the physiology of spermatozoa [1]. In Mammalia, certain epididymal secretions contain proteins [2,3,

4]. A glycoprotein has recently been isolated from the epididymal secretion of the rat [5]. Unlike in Mammalia, in which the secretions do not take the form of structured granules [6], the epididymis of the viviparous lizard produces large amounts of voluminous secretory granules. These are discharged into the lumen of the tubules of the epididymis where they are mixed with spermatozoa. They contain a protein constituent [7,8], and a polysaccharide constituent [8]. These secretory granules proved themselves as a convenient material from which to isolate the protein or glycoprotein constituents in order to investigate their effects on spermatozoa.

In the epididymis of the viviparous lizard the intracytoplasmic secretory granules are made up of a central spherical core 6 μm in diameter and a peripheral vacuole bounded by a membrane [9,8]. After discharge, the central cores can be seen in the lumen of the epididymal tubules, while in the histological preparations the contents of the peripheral vacuoles seem to dissolve in the epididymal fluid.

The work described here concerns the isolation of the central cores (a) by cell fractionation to separate granules still retained in the cytoplasm of the secreting cells, and (b) by fractionation of the epididymal fluid containing discharged granules. The isolated granules were analysed by electrophoresis in polyacrylamide gel.

Materials and Methods

Materials

The animals (*Lacerta vivipara* Jacquin) were collected in the Massif Central (France) in April, May and June (their reproductive period) and killed within 24 h after capture. In April, epididymes had swollen epithelia packed with secretory granules but the lumen of the tubules were empty (Fig. 1a), although in May and June numerous granules were discharged in the lumen (Fig. 1b). Each preparation was made from 20 adult animals.

Preparation of central cores

The preparations were made in a 3 mM imidazole/0.3 M sucrose buffer at pH 7.0. Each step was monitored by light or electron microscopy. A sample of each fraction was fixed using a method based on that of Castle et al. [10]. Sections of 3 μm thickness were examined under a light microscope after staining with toluidine blue. Ultrathin sections stained with uranyl acetate and lead citrate were examined under an electron microscope. Dissolution of granules was followed by light microscopy on unfixed smears stained with 1% aqueous eosin.

1. Separation of intracytoplasmic granules by cell fractionation. Epididymes from animals collected in April (Fig. 1a) were cut with a razor blade in buffer. In each experiment about 1 g of tissue was homogenized using a Dounce homogenizer. After sedimentation under gravity, a first supernatant fraction was removed. The sediment was resuspended in the buffer, allowed to settle out again, and a second supernatant fraction removed and combined with the first. The combined fractions were centrifuged for 5 min at $50 \times g$ to eliminate remaining intact cells and cell fragments. The supernatant fraction thus ob-

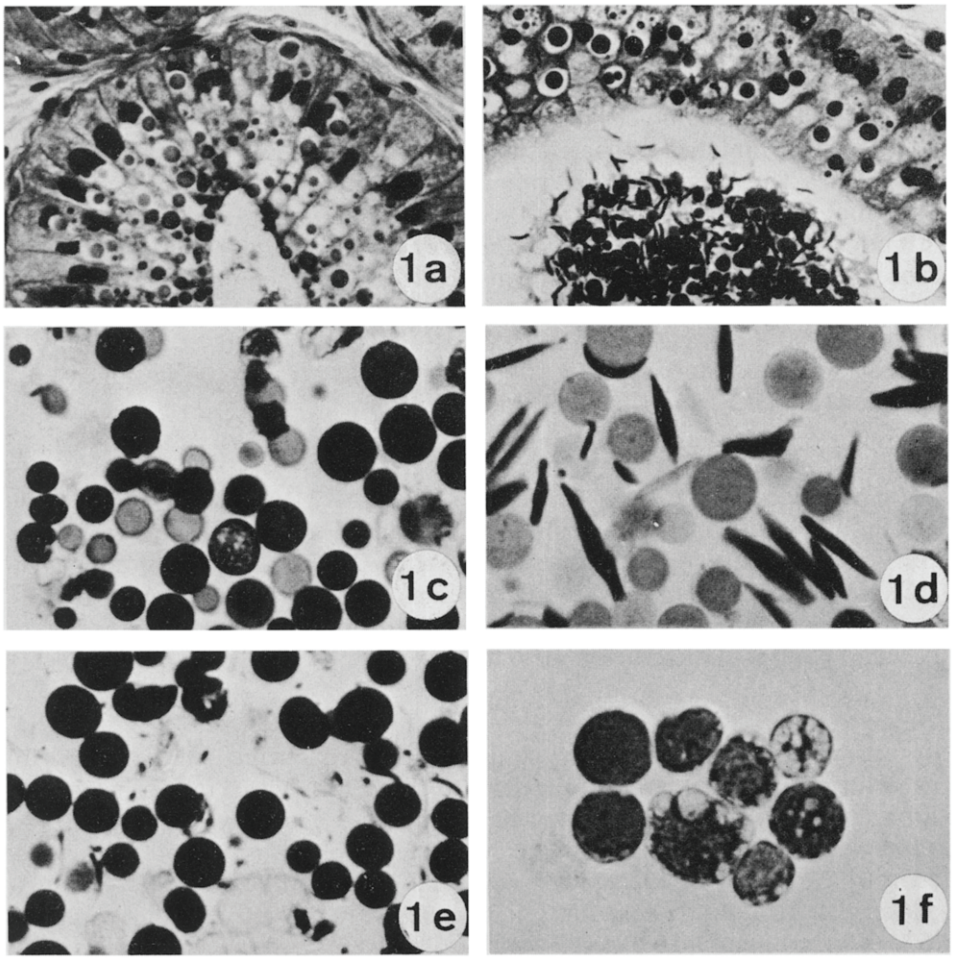

Fig. 1. (a) Tubule of lizard epididymis in April. Epithelial cells are tall and crowded with numerous secretory granules but these latter have not yet been discharged. Central cores are dense spherical bodies although peripheral vacuoles seem to be empty. Material from the peripheral vacuole and limiting membrane are only preserved in preparations for electron microscopy (magnification ×400). (b) Tubules of lizard epididymis in May. The lumen of the tubule contains numerous discharged granules whose central cores are mixed with spermatozoa. Undischarged granules are still present in the cytoplasm (×400). (c) Histological aspect of a C_2 pellet obtained by cellular fractionation. Section (3 μm) stained with toluidine blue. Contamination by other cellular organelles is very low (×1000). (d) Pellet obtained from crude epididymal fluid containing central cores and spermatozoa. Section (3 μm) stained with toluidine blue (×1000). (e) Histological aspect of a LC_3 fraction obtained by centrifugations of the epididymal fluid. Section (3 μm) stained with toluidine blue. This granule-rich fraction is impure, some tails of spermatozoa being visible in the section (×1000). (f) Histological aspect of central cores treated for 5 min with 2.5% Triton X-100. Smear stained with 1% eosine. Vacuoles appear at the periphery of several granules (×1000).

tained (S_0), was centrifuged at 3000 × *g* for 15 min and gave a supernatant S_1 and a pellet C_1. C_1 was suspended in 1 ml of buffer and loaded onto a discontinuous sucrose gradient: four stacked layers of 0.8 ml 3 mM imidazole buffer pH 7.0 with 3, 2.5, 2.0 and 1.5 M sucrose, respectively. A 108 000 × *g* centrifugation for 60 min gave five fractions which are called from the top to the

bottom: B_1, B_2, B_3, B_4 and C_2. C_2 was treated with 1% SDS and centrifuged (15 000 × *g* for 5 min). The resulting pellet of undissolved granules was called C_2'.

2. Separation of discharged granules by fractionation of epididymal fluid. Epididymes from animals collected in May and June (Fig. 1b) were roughly cut in buffer. The liquid obtained contained numerous central cores mixed with spermatozoa (Fig. 1d), but also some complete granules. After sedimentation under gravity, the supernatant fraction LS containing granules and spermatozoa was centrifuged for 10 min at 700 × *g* giving a supernatant LS_1 and a pellet LC_1. The pellet LC_1 was purified by two successive washes and centrifugations (700 × *g* for 10 min). The final pellet was called LC_3. The supernatant LS_2 was separated by a 15 000 × *g* centrifugation for 5 min, in a spermatozoa fraction CLS_1 and a soluble fraction SLS_1.

Dissolution of central cores

Treatment with SDS (2% in Tris-HCl buffer, pH 7.0) brought about only partial, though uniform, dissolution of the central cores, judging from the lowering of their affinity for eosin. Only Triton X-100 (2.5% final concn. in Tris-HCL buffer, pH 7.0) enabled the granules to be dissolved (during or following treatment with SDS). Onset of vacuolisation was observed after 5 min (Fig. 1f) and complete dissolution after 20 min *.

Electrophoresis

This was performed in 15% acrylamide gel in Tris buffer, pH 8.8 (4.5% in concentration gel, 15% in migration gel). The characteristics of buffer, gel stock solutions and conditions of preparations are those given by Laemmli [11]. Migration at 3 mA/gel took about 7 h. After electrophoresis, the proteins were treated with the following mixture of stain and fixative: 0.25% Coomassie blue, 50% methanol, 10% acetic acid for 2 h. The gels were destained with a solution of 4.5% (v/v) methanol/10% acetic acid/2.5% glycerol. After destaining, the absorbance of the gels was measured at 580 nm on a Vernon spectrophotometer. For comparison, parallel runs were carried out using reference solutions of proteins which had been subjected to the same treatment as the samples (cytochrome *c*, lactoglobulin, chymotrypsinogen, ovalbumin and bovine serum albumin (2 mg/ml)).

Results

During cell fractionation, the nuclei had settled out in the first pellet. Fraction S_1, B_1 and B_2 contained no granules. Morphological criteria showed S_1 to be a microsomal fraction, B_1 to be a fraction of membrane vesicles and B_2 to be a mitochondrial fraction. Fractions B_3 and B_4 contained a mixture of spermatozoa, mitochondria and some central cores. Fraction C_2 was rich in central cores, apparently uncontaminated with organelles (Fig. 1c).

* Since a histochemical study had already shown the granules to contain proteins [7], the low solubility of the granules was surprising. Enzymic digestion was attempted in order to confirm the protein nature of the cores. Brief treatment (4—5 min) with trypsin at room temperature brought about dissolution of the peripheral part of the central cores.

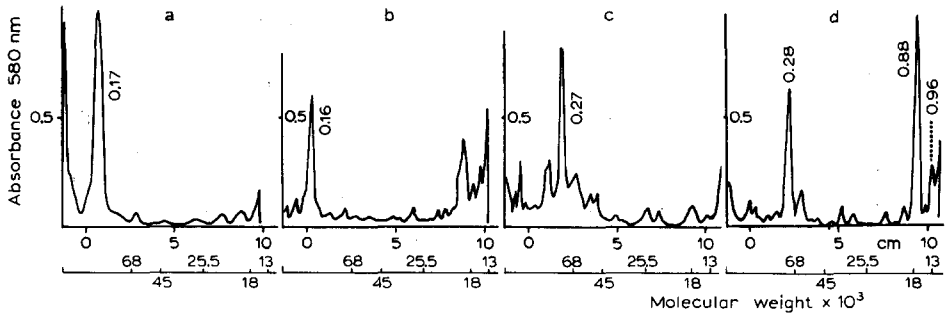

Fig. 2. Analysis of protein secretions in the lizard by 15% polyacrylamide gel electrophoresis. Preparation of samples and buffers was carried out according to Laemmli [11]. Running conditions were 3 mA/gel for 7 h. Absorbance spectra of the gels were recorded on a Vernon spectrophotometer at 580 nm. The scale at the bottom of the figure indicates the pattern of migration for reference proteins of known molecular weights. (a) Purified granule fraction (C_2') obtained from whole cells (undischarged granules). 2.5% Triton X-100-soluble proteins. (b) Purified granule fraction (LC_3) obtained from discharged material. 2% SDS plus 2.5% Triton X-100-soluble proteins. (c) Soluble proteins of testicular fluid: supernatant obtained after a 5 min 15 000 × *g* centrifugation of the testicular fluid (testis roughly fragmented in imidazole buffer). (d) Soluble proteins of epididymal fluid SLS_1 fraction: supernatant obtained after a 5 min 15 000 × *g* centrifugation of the epididymal fluid (epididymis roughly fragmented in imidazole buffer).

During fractionation of the epididymal fluid, central cores had settled out in fraction LC_3. Fraction SLS_1 contained the soluble part of epididymal fluid, the pellet CLS_1 contained spermatozoa.

The pellet C_2' completely dissolved in Triton X-100 was subjected to electrophoresis. The simple profile in Fig. 2a was obtained. A main band appeared at R_F 0.17 which could be due to a protein, or a tight group of proteins of high molecular weight (about 70 000 by comparison with reference proteins). The fraction LC_3 obtained from discharged granules dissolved directly in SDS plus Triton X-100 and, analysed in the Laemmli system, gave a rather more complex profile (Fig. 2b). The same band (R_F 0.16) was found at the beginning of the gel, and at the other end of the gel a group of low molecular weight proteins (about 16 000 by comparison with reference proteins) was located. The fraction containing central cores obtained from the epididymal fluid could have been contaminated by proteins of testicular origin (proteins from testicular fluid, e.g. of an ABP* type). The possible presence of such proteins was checked as follows: testes collected at the same period were minced in imidazole/sucrose buffer and centrifuged directly at 12 000 × *g* for 5 min; the supernatant liquid both treated and untreated with Triton X-100 underwent electrophoresis, and a principal protein was separated at R_F 0.27. Similarly a spermatozoa pellet (CLS_1) was completely dissolved in 2% SDS and underwent electrophoresis. Neither protein from testis nor from spermatozoa correlated with anything found in fraction LC_3, thus contamination by testicular proteins is unlikely. The epididymal fluid used to prepare the fraction LC_3 must also contain a significant amount of peripheral vacuole constituents. In order to locate these, the supernatant fraction, free of visible structure, SLS_1, was examined. The electrophorogram (Fig. 2d) showed two characteristic bands: a protein (or

* ABP, androgen-binding protein.

group) of high molecular weight at R_F 0.38 appeared to be of testicular origin; a group of low molecular weight proteins included a main element at R_F 0.88 and a smaller band at R_F 0.96. This same group of low molecular weight proteins was also found to have contaminated certain preparations of central cores obtained by cell fractionation (C_2). It seems likely that these are proteins from the peripheral vacuole. However, as these vacuoles have not yet been isolated in pure state, and as certain plasma proteins migrate in the same region (unpublished results) this question remains open and will be the subject of further work.

Discussion

Unlike in most other species, particularly in mammalia, the epididymis of the viviparous lizard produces structured secretory granules. They consist of a highly insoluble compact central core and a peripheral vacuole containing a polysaccharide (identified histochemically) which could be related to the glycoprotein recently identified in the rat epididymis [5]. Fractions containing the insoluble central core were prepared in different ways. Each preparation was analysed by electrophoresis in polyacrylamide gel in the presence of SDS. Another system of SDS-polyacrylamide gel electrophoresis [12] gave the same results (unpublished), viz. a single band representing a protein or group of proteins. Estimation of the molecular weight (70 000), based on comparison with reference proteins in control experiments in two types of gel [11,12] is only approximate according to criteria defined by Rodbard and Chrambach [13]. This protein or group of proteins is neither a plasma protein, nor a protein of testicular origin (spermatozoal protein or protein from testicular fluid). Using other methods of separation, it remains to be discovered, whether there is one or several protein(s) in the band. The protein secretions of rat epididymis are androgen-dependent [3], and the same is true of those of the viviparous lizard [17]. However, the principal protein(s) from a 20 000 × g supernatant fraction, prepared from an epididymal homogenate which strongly incorporate(s) radioactive leucine [7], are lighter and thus are not identical to the protein solubilised from central cores unless they are their precursors. These light proteins do, however, seem to originate from the soluble parts of the peripheral vacuole. This possibility is to be investigated in a further study. The timing of the appearance of granules, as monitored by microscopy, leaves little doubt as to their androgen-dependence [9,7]. The difficulties met with when attempting to solubilize the protein of the central core explain perhaps why [^{3}H]leucine-incorporation in such a protein has not yet been clearly demonstrated. The highly insoluble nature of the central core of the secretory granules make determination of its physiological role difficult. The accessory glands of the genital tract of Reptilia are different from those of mammals. In Squamata (lizards and snakes) only two organs participate in sperm formation: (a) the epididymis, whose embryological origin suggests a role analogous to that of both the mammalian epididymis and the mammalian seminal vesicles, and (b) the sexual segment of the kidney, which has been compared to the prostate [14]. However, it is possible that the insoluble granule cores of the lizard epididymis have no homologue in the mammalian epididymis.

Acknowledgments

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References

1 Orgebin-Crist, M.C., Danzo, B.J. and Davies, J. (1975) in Handbook of Physiology (Greep, R.O. and Astwood, E.B., eds.), Vol. 5, Sect. 7, pp. 319—338, American Physiological Society, Washington, DC
2 Koskimies, A.I. and Kormano, M. (1975) J. Reprod. Fert. 43, 345—348
3 Cameo, M.S. and Blaquier, J.A. (1975) J. Endocrinol. 69, 47—55
4 Huang, H.E.S. and Johnson, A.D. (1975) Comp. Biochem. Physiol. 50B, 359—362
5 Lea, O.A., Petrusz, P. and French, F.S. (1978) Int. J. Androl., Suppl. 2, 592—605
6 Hamilton, D.W. (1975) in Handbook of Physiology (Greep, R.O. and Astwood, E.B., eds.), Vol. 5, Sect. 7, pp. 259—301, American Physiological Society, Washington, DC
7 Gigon-Depeiges, A. and Dufaure, J.P. (1977) Gen. Comp. Endocrinol. 33, 473—479
8 Depeiges, A. and Dufaure, J.P. (1978) Biol. Cell. 32, 4a
9 Dufaure, J.P. and Gigon, A. (1975) Gen. Comp. Endocrinol. 25, 112—120
10 Castle, J.D., Jamieson, J.D. and Palade, G.E. (1975) J. Cell Biol. 64, 182—210
11 Laemmli, U.K. (1970) Nature 227, 680—685
12 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2602—2617
13 Rodbard, D. and Chrambach, A. (1971) Anal. Biochem. 40, 95—134
14 Price, D. and Williams-Ashman, H.G. (1961) in Sex and Internal Secretions (Young, W.C., ed.), pp. 366—448, Williams and Wilkins Comp., Baltimore, MD