Biochimica et Biophysica Acta, 657 (1981) 364—373
© Elsevier/North-Holland Biomedical Press

BBA 69193

ISOLATION, PROPERTIES, IMMUNOLOGICAL SPECIFICITY AND LOCALIZATION OF MOUSE TESTICULAR HYALURONIDASE

G.S. GUPTA [a,b,*] and E. GOLDBERG [a]

[a] *Department of Biological Sciences, Northwestern University, Evanston, IL (U.S.A.) and*
[b] *Department of Biophysics, Panjab University, Chandigarh 160014 (India)*

(Received June 6th, 1980)
(Revised manuscript received September 16th, 1980)

Key words: Hyaluronidase; Localization; Immunological specificity (Mouse testis)

Summary

Hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) was purified from mouse testes by ion-exchange chromatography, Sephadex G-200 filtration and Con A-agarose affinity chromatography. The final preparation had 94-fold purity and 12.2 units spec. act. of the enzyme (unit of specific activity = μmol *N*-acetylglucosamine released/h per mg protein at 37°C and pH 4.5). Hyaluronidase is relatively heat stable and loses 10—20% of its activity at 50—55°C for 10 min. E_a for heat denaturation of enzyme is 42—45 kcal between 45 and 63°C. The Michaelis constant of mouse testicular hyaluronidase is 1.1 mg/ml hyaluronic acid.

Antibodies to the purified enzyme were produced in rabbits and showed a single precipitin line by Ouchterlony gel diffusion. Antiserum to hyaluronidase inhibited enzyme activity by 25%. Immunologically, mouse testicular hyaluronidase is species specific. Tissue extracts of mouse vital organs, except testes and epididymis did not react with the antisera, though nonspecific precipitation occurred between intestinal extracts and anti-hyaluronidase serum.

Hyaluronidase was localized in testis sections by indirect immunofluorescence. A specific dark green fluorescence was localized on cell boundaries extending from spermatogonia to spermatids and appeared on the sperm acrosome. Cytoplasm of spermatogonia and spermatocytes showed light green fluorescence, whereas interstitial tissue was devoid of fluorescence.

* To whom correspondence should be addressed at: Department of Biophysics, Panjab University, Chandigarh 160014, India.

Introduction

The mammalian testes and semen are the richest sources of hyaluronidase (hyalyronate 4-glycanohydrolase, EC 3.2.1.35). Sperm hyaluronidase which is localised in the sperm acrosome is known to disperse the cells of the cumulus oophorous [1] and, thus, plays an important role in fertilization by depolymerizing the hyaluronic acid of the cumulus [2—8]. From an immunological point of view, hyaluronidase has been reported to be cell (spermatozoa) and probably species specific [3,4]. Furthermore, it seems to be iso- and possibly autoantigenic though antibodies against highly purified hyaluronidase have not been tested [4]. This communication describes the purification of mouse testicular hyaluronidase by affinity chromatography, its immuno-specificity, immunocytochemical localization in mouse testis and some of its biochemical properties.

Materials and Methods

Enzyme assays. Under standard conditions, hyaluronidase activity was determined by method of Aronson and Davidson [9]. 0.5 ml reaction mixture comprising of 0.1 ml hyaluronic acid (sodium salt, 6 mg/ml unless stated)/0.1 ml acetate buffer (pH 4.5, 0.1 M)/0.1 M NaCl (2 M)/0.1 ml testicular hyaluronidase were incubated for 1 h at 37°C. The reaction was terminated by adding 0.1 ml 0.8 M potassium tetraborate buffer (pH 9.1) and 0.01 ml of 4 M NaOH. The mixture was tested for release of *N*-acetylglucosamine. 1 unit of enzyme is defined as that amount of protein that causes the release of 1 μmol *N*-acetylglucosamine in 1 h at 37°C. Specific activity of enzyme is defined for 1 mg of protein. *N*-Acetyl-glucosaminidase and β-glucuronidase activities were determined by methods reported elsewhere [10,11]. Protein was measured by absorbance as described by Layne [12].

Testes extract and purification of hyaluronidase. Testes were homogenized in phosphate buffer (pH 6.0, 0.1 M) and the pellet obtained at 16 000 × *g* was suspended in an equal volume of 0.1 M acetic acid overnight at 5°C. The suspension was sonicated and centrifuged at 5000 × *g* for 20 min. The sediment was further extracted with a mixture of Triton-X and hyamine (0.1%), at 37°C for 2 h. The supernatants at 5000 × *g* from both extractions were combined, dialysed and the dialysate was saturated to 30% with $(NH_4)_2SO_4$ and the precipitate discarded. The supernatant was saturated with $(NH_4)_2SO_4$ to 60%. The precipitate protein obtained at 10 000 × *g* was dissolved in water and dialysed against water for 20 h (Step 1). The dialysed protein in phosphate buffer, (pH 7.3, 0.05 M) was passed through a DEAE-cellulose column (40 × 2.5 cm) and eluted with phosphate buffer and the activities for hyaluronidase, *N*-acetylglucosaminidase and glucuronidase were tested. The active fractions for hyaluronidase (Fig. 1) were pooled and dialysed against water and the dialysed protein was lyophylized or concentrated by Amicon filtration (Step 2). The concentrated hyaluronidase dissolved in phosphate buffer (pH 6.0, final molarity 0.02 M) containing NaCl (0.15 M) was applied to a Sephadex G-200 column (100 × 2.5 cm). The active fractions (Fig. 2) were concentrated by Amicon filtration and dialysed at 4°C (Step 3).

Hyaluronidase from Step 3 was dissolved in Tris-HCl buffer (0.02 M, pH 6.95) containing NaCl and $MnCl_2$ ($1 \cdot 10^{-3}$ M) and applied over the Con A-agarose (Sigma) column (10 × 1 cm) equilibrated with Tris-HCl buffer (0.02 M, pH 6.9)/1 M NaCl/$1 \cdot 10^{-3}$ M $CaCl_2$/$1 \cdot 10^{-3}$ M $MnCl_2$, as reported by Yang and Srivastava [13]. Hyaluronidase was absorbed under these conditions [13] and then eluted by a stepwise gradient of 0.0—0.6 M α-methyl-D-glucoside in Tris-HCl buffer (0.02 M at pH 6.9 containing 1 M NaCl). Active fractions were concentrated, dialysed against water and frozen (Step 4).

Disc electrophoresis. Acrylamide gel electrophoresis was performed at pH 4.5 according to the method of Brewer and Ashworth at a current density of 1.5 mA [14] and stained with Coomassie brilliant blue R.

Biochemical properties. (i) Thermal stability: Thermal stability of mouse testicular hyaluronidase was determined by incubating the enzyme solution in a water bath for 5—25 min at 50—55° C. The tubes were then cooled in ice-cold water and hyaluronidase activity determined. In one set of experiments, hyaluronidase was incubated at 25—72° C for 10 min and residual activity was determined after cooling the tubes in water.

(ii) Effects of substrate: The effect of hyaluronic acid concentration, varying between 0.4 and 2.0 mg/ml reaction mixture, was determined at pH 4.4 in the presence of 0.4 M NaCl at 37°C.

Immune response and detection of antibodies. Antisera to purified hyaluronidase were raised in rabbits by giving a primary injection (intradermal route) of 150 μg antigen emulsified in complete Freunds adjuvant, followed by a booster (intravenous route) injection of 100 μg antigen 23 days after the primary dose. Control sera were drawn before the primary injection.

Hyaluronidase antibodies were detected by enzyme inhibition assay in the presence of serial dilutions of control sera and antisera and by Ouchterlony gel diffusion. Precipitin lines were stained with Coomassie brilliant blue R after fixing the gels in 12% trichloroacetic acid.

Tissue specificity. 10% tissue extracts of mouse liver, kidney, brain, intestine, pancreas, testes, epididymis, skin, heart and muscle were made in 0.1% Triton-X and hyamine as previously described and centrifuged at 5000 × *g*. Supernatants were tested for cross reaction by placing 20 μl control sera and antisera in the center wells and 20 μl of each tissue extract in the side wells. Cross reaction of antihyaluronidase serum was also examined by reacting with highly purified bovine hyaluronidase (Worthington), human seminal plasma, monkey and rat testicular extracts and with control sera from mice and rabbits.

Immunocytochemical localization. Immunocytochemical localization of hyaluronidase was performed on paraffin sections of testes which were fixed in Bouins fluid. The procedure followed is described by Hintz and Goldberg [15,16]. For microscopy, IgG-conjugated fluorescein isothiocyanate was layered over the sections and the slides were incubated at room temperature for 30 min in the dark. After rinsing in phosphate-buffered saline (pH 7.4) (twice) and water, the slides were counter-stained with 0.01% Evans blue for 5 min, washed in buffered saline (twice) and water and mounted in 90% glycerine prepared in 10% buffered saline. The slides were examined immediately.

Results and Discussion

The elution profile of the crude hyaluronidase preparation from the DEAE-cellulose column is presented in Fig. 1. Most of the protein runs through the column in two sharp peaks within the first few fractions. The second peak contained the hyaluronidase activity, as well as *N*-acetylglucosaminidase and β-glucuronidase. Most of the hyaluronidase activity was restricted to fractions 14—15 (Fig. 1).

The three overlapping enzymes were then separated partially by Sephadex G-200 gel filtration (Fig. 2). Though β-glucuronidase could be resolved from hyaluronidase, the *N*-acetylglucosaminidase activity peak still overlapped with hyaluronidase. Yang and Srivastava [13] suggested that elution of the Sephadex G-200 column with buffer containing NaCl is essential to obtain the separation of these three enzymes; probably NaCl prevents protein-protein interactions. Isolation of hyaluronidase following Sephadex filtration resulted in a 25-fold purification of this enzyme (Table I).

Hyaluronidase obtained after gel filtration was further purified by Con A-agarose chromatography. Hyaluronidase eluted in a separate peak between 0.16 and 0.32 M of α-methyl-D-glucoside. Void volume protein did not show hyaluronidase activity (Fig. 3). The elution by α-methyl-D-glucoside at low temperature (5°C) gave 10% yield of the enzyme applied over the column. At room temperature, however, more than 90% of the enzyme could be eluted from an affinity column resulting in a total recovery of 19% and enrichment of 94-fold with respect to the acetic acid extract. An earlier report [13] suggested that hyaluronidase from bull seminal plasma could be eluted from a Con A-agarose column at low temperature. However, several variables appear to be important

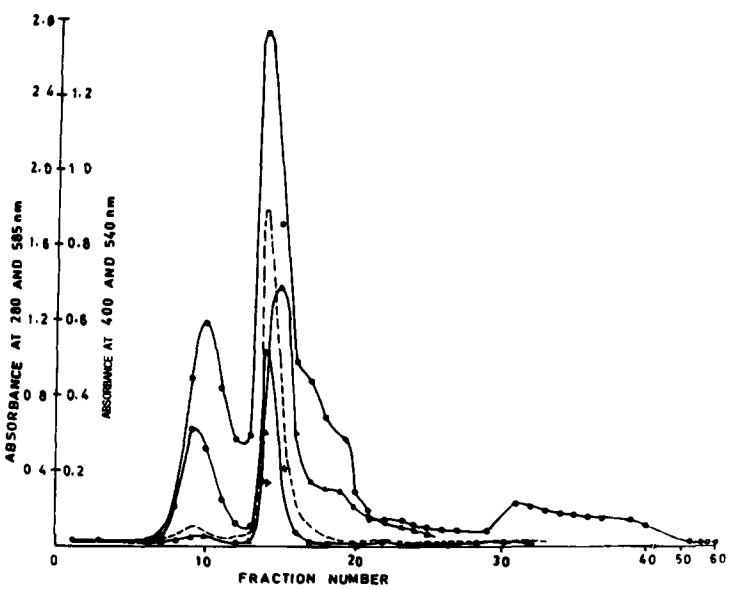


Fig. 1. Elution profile of active fractions from $(NH_4)_2SO_4$ fractionations by DEAE-cellulose chromatography (Step 2). The column was eluted with 0.05 M sodium phosphate buffer (pH 7.3). ○———○, 280 nm; ------, hyaluronidase (585 nm); ▲———▲, β-glucuronidase (540 nm); ●———●, *N*-acetylglucosamine (400 nm).

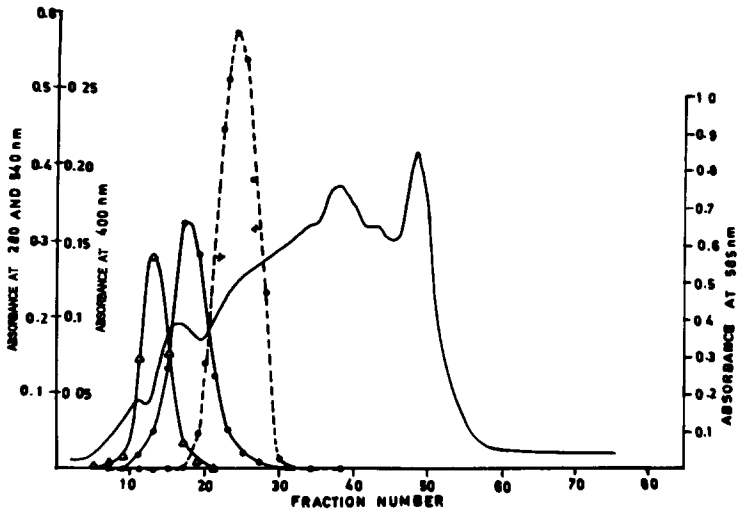


Fig. 2. Elution profile of Sephadex G-200 column chromatography (Step 3). The column was developed with 0.02 M phosphate buffer/0.15 M NaCl (pH 6.0). ○———○, β-glucuronidase (540 nm); ●- - - - -●, hyaluronidase (585 nm); ●———●, *N*-acetylglucosamine; ———, protein at 280 nm.

in binding of glycoproteins to lectins and their elution by glycoside. Fast elution of several enzymes from concanavalin A, at higher temperature has been reviewed by Dulaney [17]. Purified hyaluronidase did not show the activity of β-*N*-acetylglucosaminidase and β-glucuronidase and had 12.2 units of specific activity of hyaluronidase, which is unusually low. Though at alkaline pH, hyalurunidase did not migrate in disc electrophoresis, however at pH 4.5 it revealed one major protein band and two minor components in agreement with other observations [18]. Whether two minor bands are due to contaminant proteins or to subunit dissociation remains to be established. Nonetheless, Khorlin et al. [18] and other investigators [3] suggested that hyaluronidase is made up of two subunits which could form as many as three isozymes. Morton [3] suggested that three isozymes are identical immunologically and give one precipitin line on Ouchterlony plates.

TABLE I

SUMMARY OF PURIFICATION OF HYALURONIDASE FROM MOUSE TESTES

Unit = that amount of protein which releases 1 μmol *N*-acetylglucosamine at 37°C during 1 h.

Step	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg protein)	Relative activity	Yield
1. Initial activity in pellet extract after detergent treatment.	2387	316	0.13	1	100
2. $(NH_4)_2SO_4$ fractionation	400	166	0.41	3	63
3. DEAE-cellulose chromatography	109	118	1.02	8	37
4. Sephadex G-200 filtration	21	68	3.24	25	22
5. Concanavalin A chromatography	5	61	12.2	94	19

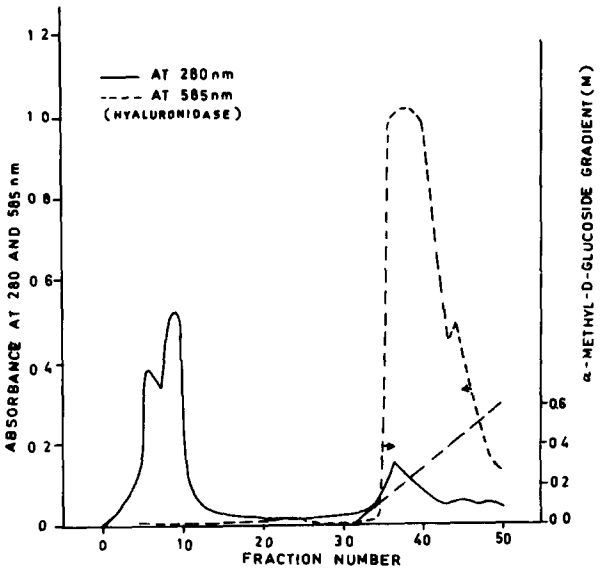


Fig. 3. Elution profile of active fractions from Sephadex G-200 column by Con A-agarose affinity chromatography (Step 4). ———, hyaluronidase (280 nm); - - - - - -, hyaluronidase (585 nm).

Mouse testicular enzyme appears to be relatively heat resistant at 50—55°C which is evident from Fig. 4. 8—10% enzyme activity is lost after 5 min heating under these conditions. But a temperature of 60°C for 10 min destroyed virtually all the mouse testicular hyaluronidase. The rate of denaturation (D) obtained from semi-log plot is $0.2—4.7 \cdot 10^{-3}$ per s. Arrhenius plot for D revealed that the denaturation of hyaluronidase requires E_a of 42—45 kcal (Fig. 5). The Lineweaver-Burk plot for the effects of substrate concentration on mouse testicular hyaluronidase revealed the Michaelis constant (K_m) around 1.1 mg/ml hyaluronic acid. Some of the biochemical features of mouse testicular hyaluronidase have been described earlier [19]. There are significant differences in the kinetic properties between mouse and bovine testicular hyaluronidases [19] and between bovine and monkey testicular hyaluronidases [20]. However, the Michaelis constant with purified mouse hyaluronidase is very close to the value obtained with partially purified enzyme [19].

The formation of antibodies in a rabbit to mouse hyaluronidase was first detected on Ouchterlony diffusion plates on day 23, after the primary injection. 10 days after a booster injection, 10 μl undiluted serum inhibited hyaluronidase activity by 25% (Fig. 6). A similar inhibition of enzyme activity in the presence of higher concentration of antiserum suggested low equivalence point of inhibitory antibodies in anti-serum. These antibodies reacted with crude testicular extracts giving rise to a single precipitin line of identity indicating that the enzyme was an immunologically homogeneous preparation. 5 μl undiluted serum did not produce visible precipitin band. Therefore, the bands obtained by gel electrophoresis probably represent isozymes of hyaluronidase [4,16]. Nonetheless, Dunbar et al. [21] have reported isoantibodies of partially purified hyaluronidase, whereas Morton [3] showed complete identity of three multiple forms of rat sperm hyaluronidase on gel diffusion by immunological

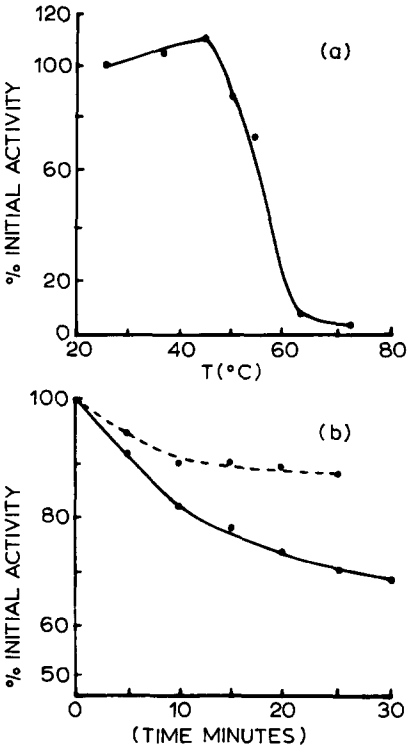
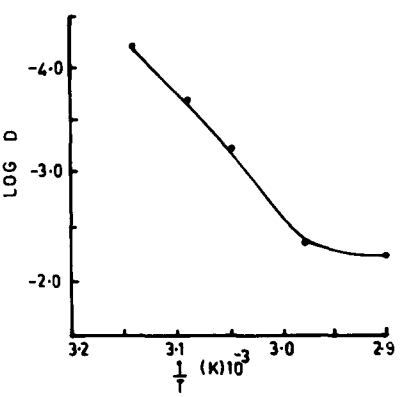


Fig. 4. Thermal stability of mouse testicular hyaluronidase; (a) % residual activity at various temperatures corresponding to room temperature (25°C); (b) % residual activity after heating at 50 (- - - - - -) and 55°C (———) for various time intervals.

methods. Serum and crude extracts of most mouse tissues did not react with antihyaluronidase serum. However, large and small intestine extracts in addition to testis formed a distinct nonspecific precipitin line with the antiserum,



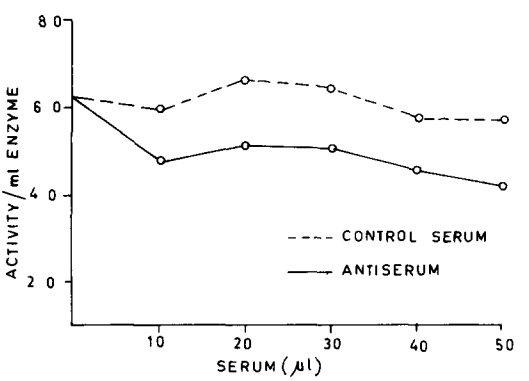


Fig. 5. Arrhenius plot between rate of denaturation (D) on log scale vs. (temperature)$^{-1}$ (Kelvin scale) for mouse testicular hyaluronidase.

Fig. 6. Inhibition of mouse hyaluronidase in presence of increasing volumes of control and anti-hyaluronidase sera.

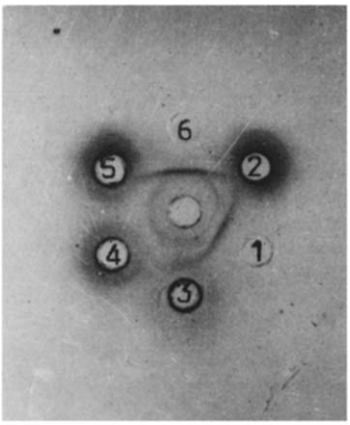


Fig. 7. Ouchterlony gel diffusion test for mouse testicular hyaluronidase (1, $(NH_4)_2SO_4$ testis extract; 2, large intestine; 3, detergent testis extract; 4 and 5, small intestine; 6, purified hyaluronidase).

which was washed out with normal saline. On the other hand, bovine testicular hyaluronidase, human semen and monkey and rat testicular extracts did not cross-react with anti-mouse-testicular hyaluronidase. It appears from these studies that mouse testicular hyaluronidase is probably species specific (Fig. 7, Table II).

Hyaluronidase was localized in the testis as dark yellowish green fluorescence observed at the junctions of membranes of spermatogenic cells. This characteristic localization was observed through the complete spermatogenic cycle from spermatogonia to spermatozoa. The head region of the spermatozoan was particularly fluorescent. No fluorescence due to hyaluronidase could be seen in interstitial cells. Control sections incubated under similar conditions did not show any fluorescence (Fig. 8).

The concentration of hyaluronidase in testes and its relation to the presence

TABLE II

OUCHTERLONY DIFFUSION TEST BETWEEN ANTITESTICULAR HYALURONIDASE SERUM AND TISSUE EXTRACTS AND FLUIDS

(—) indicates no precipitation, (+) indicates precipitation and (?) indicates nonspecific precipitation.

Tissue	Diffusion test	Tissue	Diffusion test
Mouse tissue extracts			
Brain	—	Heart	—
Spleen	—	Epididymis	+
Muscle	—	Testes	+
Liver	—	Seminal Vesicles	—
Kidney	—	Prostate	—
Pancreas	—	Bovine testicular hyaluronidase	—
Small intestine	?	Monkey testicular extracts	—
Large intestine	?	Rat testicular extracts	—
Skin	—	Serum (Mouse)	—
Lungs	—	Serum (Rabbit)	—
		Human Semen	—

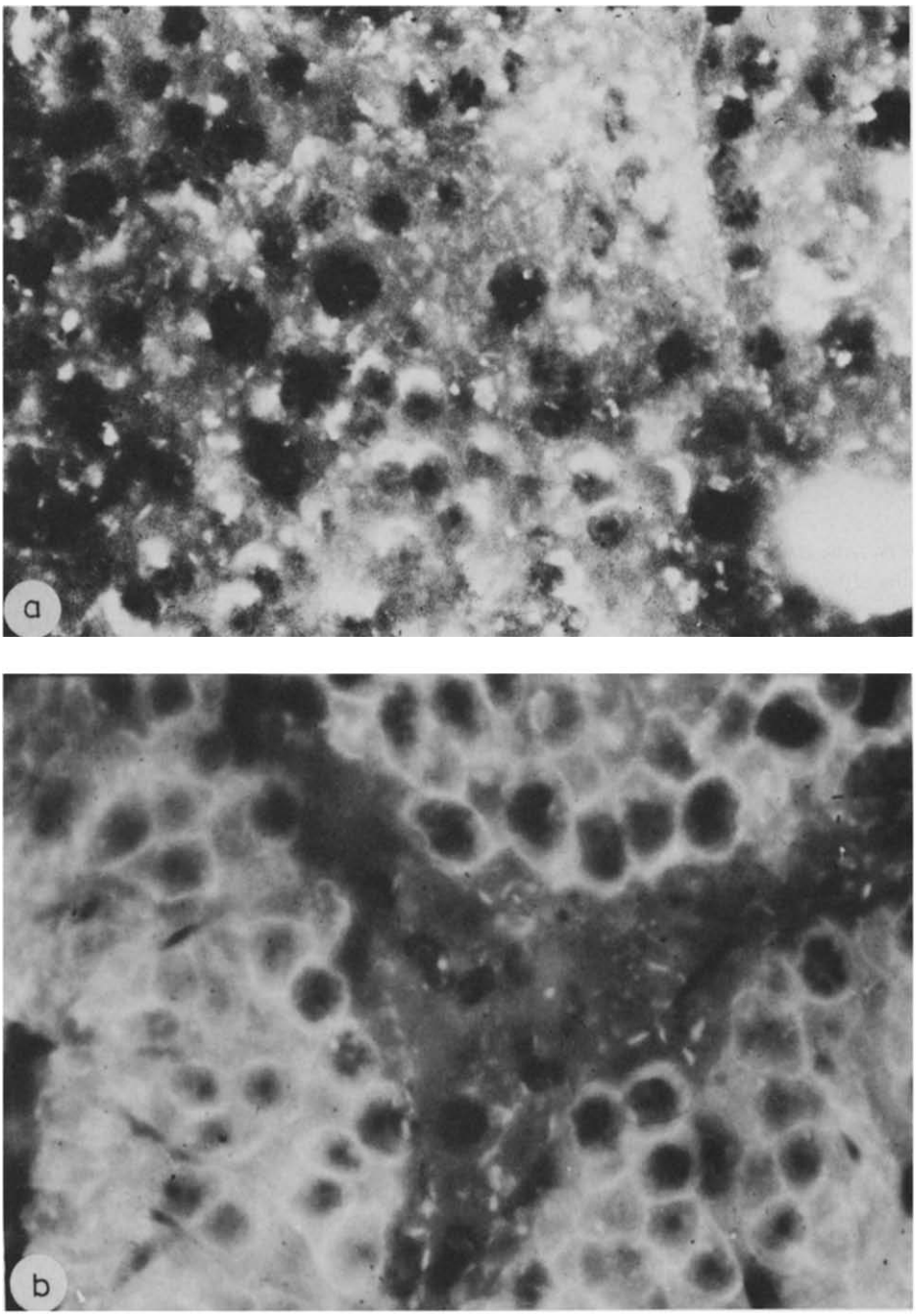


Fig. 8. Immunofluorescence localization of mouse testicular hyaluronidase, (a) control section, (b) green specific fluorescence of hyaluronidase at the junction of cell membranes (×600).

of mature germ cells has been extensively investigated. This enzyme increases in concentration with maturation of testes and decreases with experimentally induced atrophy of the germinal epithelium [22]. Yaesger and Anderson [23]

and Mancini et al. [24] used fluorescent antibody techniques to localize the site of cellular accumulation of the enzyme in bull testes. These authors [23] suggested that spermatogonia and spermatocytes produce the enzyme. Mancini et al. [24] reported specific fluorescence in nuclei of spermatids, in the acrosome of spermatozoa and, with less certainty in the cytoplasm of other cells tentatively identified as spermatocytes. Nonspecific fluorescence could be localized in the basal cell line of seminiferous tubules [24]. In the present study, nonspecific fluorescence could not be localized in the basal cell line. However, the antigen used in this study differs in state of purity with earlier preparations. At the same time, hyaluronidase is suggested to be localized in plasma membranes of germinal cells [25].

Acknowledgements

The authors wish to express thanks to Dr Thomas E. Wheat for giving active support in this work from time to time. The financial assistance for this work was received from The World Health Organization, Geneva.

References

1 Leonard, S.L. and Kurzrok, R. (1945) Endocrinology 37, 171—176
2 Swyer, G.I.M. (1947) Biochem. J. 41, 413—417
3 Morton, D.B. (1976) in Immunology of Gametes. (Edidin, M. and Johnson, M.H., eds.), pp. 115—156, Alden Press, Oxford
4 Voisin, G.A., Toullet, F. and d'Almeida, M. (1974) Acta Endocrinol. 78, 173—201
5 Menge, A.C. (1968) Proc. Soc. Expt. Biol. Med. (N.Y.) 127, 1271—1275
6 Menge, A.C. (1970) Proc. Soc. Expt. Biol. Med. (N.Y.) 135, 108—112
7 Metz, C.B., Seiguer, A.C. and Castro, A.E. (1972) Proc. Soc. Expt. Biol. Med. (N.Y.) 140, 776—780
8 Metz, C.B. (1972) Biol. Reprod. 6, 358—383
9 Aronson, N.N., Jr. and Davidson, E.A. (1967) J. Biol. Chem. 242, 441—444
10 Tarentio, A.I. and Malley, F. (1972) Methods Enzymol. 28, 772
11 Fishman, W.H., Springer, B. and Brunette, R. (1948) J. Biol. Chem. 173, 449—456
12 Layne, E. (1955) Methods Enzymol. 3, 447
13 Yang, C.H. and Srivastava, P.N. (1975) Biochim. Biophys. Acta 391, 382—387
14 Brewer, J.M. and Ashworth, R.B. (1969) J. Chem. Educ. 46, 41—45
15 Goldberg, E. (1977) in Isozymes: Current topics in Biological and Medical Research (Rattazzi, M.C., Scandaliss, J.G. and Whitt, G.S., eds.), Vol. 1, pp. 80—124, Alan R. Liss, Inc., New York
16 Hintz, M. and Goldberg, E. (1977) Develop. Biol. 57, 375—384
17 Dulaney, J.T. (1978) J. Mol. Cell. Biochem. 21, 43—63
18 Khorlin, A.Y., Vikha, I.V. and Milishnikov, A.N. (1973) FEBS Lett. 31, 107—110
19 Gupta, G.S. and Goldberg, E. (1981) Ind. J. Biochem. Biophys., in the press
20 Bansal, M.R., Kanwar, K.C. and Gupta, G.S. (1981) Ind. J. Biochem. Biophys., in the press
21 Dunbar, B.S., Munoz, M.G., Cordle, C.Y. and Metz, C.B. (1976) J. Reprod. Fert. 47, 381—384
22 Steinberger, E. and Nelson, W.D. (1957) Endocrinology 60, 105—117
23 Yaesger, J.A. and Anderson, T.O. (1959) Acta Anat. 39, 189
24 Mancini, R.E., Alonso, A., Barquet, J., Alvarez, B. and Nemirovsky, M. (1964) J. Reprod. Fert. 8, 325—330
25 McRorie, R.A. and Williams, W.L. (1974) Ann. Rev. Biochem. 43, 777—804