BIOCHEMICAL DETECTION AND ACTIVATION OF AN INACTIVE FORM OF A TRYPSIN-LIKE ENZYME IN RABBIT TESTES

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(Received 31st May 1972, accepted 17th July 1972)

The acrosomal trypsin-like enzyme of rabbit spermatozoa has been reported to be involved in sperm penetration of the zona pellucida (Stambaugh, Brackett & Mastroianni, 1969). It has also been suggested that seminal plasma trypsin inhibitors inhibit mammalian sperm trypsin-like activity until removed from spermatozoa during capacitation (Zaneveld, Srivastava & Williams, 1969). The present report describes the biochemical detection and activation of an inactive form of a trypsin-like enzyme in rabbit testis.

Trypsin-like activity was assayed by the spectrofluorometric detection of β -naphthylamine released from α -benzoyl-DL-arginine- β -naphthylamide $\cdot$ HCl (BANA, Schwarz, Mann Company), which is a specific substrate for trypsin and trypsin-like enzymes (Riedel & Wunsch, 1959). A Hitachi-Perkin-Elmer 2A spectrofluorometer was utilized for the assays which were carried out at 25° C with an excitation wavelength of 330 m μ and an emission wavelength of 405 m μ (Greenberg, 1962). Assay solutions consisted of (a) 3.6 ml of 0.05 M-tris-HCl, 0.05 M-CaCl₂, pH 8, (b) 0.05 ml of 50 μ M-BANA dissolved in dimethylformamide, and (c) 0.05 ml to 0.1 ml of enzyme solution. Assays were carried out with amounts of enzyme which produced linear rate responses and which never consumed more than 10% of the substrate. Enzyme units were defined as the amount necessary to hydrolyse 1 nmol BANA/min.

Protein content was assayed by the biuret method B of Chaykin (1966).

Following their removal from six fertile New Zealand white rabbits (killed by injection of air into an ear vein), the testes were placed in an ice-cold 0.9% NaCl solution, and the microdissection technique of Christensen & Mason (1965) was used to isolate the seminiferous tubules. These tubules were almost free of visible blood contamination, but microscopic observation of tubule sections fixed in formalin and stained with H & E revealed some contamination by interstitial cells. Tubule suspensions were centrifuged at 2000 *g* for 15 min at 0° C, the supernatant was discarded, and the pelleted tubules were stored at –20° C.

The procedures used to extract the enzyme from the seminiferous tubules and to remove some contaminants were similar to those used during the isolation of human pancreatic trypsinogen (Travis & Roberts, 1969). All of these procedures were carried out at 0° C. The seminiferous tubules from one

or two rabbits were homogenized in 0.25 N-H₂SO₄ (1 g wet weight tubules/2 ml H₂SO₄), using a Virtis 60K homogenizer (three 30-sec bursts at 12,000 rev/min). The homogenate was kept for 1 hr and then centrifuged at 23,500 g for 30 min. The supernatant was then brought to 20% ammonium sulphate saturation and centrifuged as above. The 20% supernatant was brought to 80% ammonium sulphate saturation and centrifuged, and the 80% supernatant was brought to 100% saturation and centrifuged. The ammonium sulphate precipitates were dissolved in 0.001 N-HCl (0.2 ml/g wet weight of tubules), and dialysed against two changes of 1000 vols of 0.001 N-HCl for 18 hr and then centrifuged at 100,000 g for 1 hr. Dialysis tubing (Union Carbide) with an average pore radius permeability of 24 Å was used after moistening with distilled H₂O.

TABLE 1
TRYPSIN-LIKE ACTIVITY OF RABBIT SEMINIFEROUS TUBULE PREPARATIONS
BEFORE AND AFTER ACTIVATION WITH BOVINE PANCREATIC TRYPSIN

<i>Preparation*</i>	<i>Total units before activation</i>	<i>Total units after† activation</i>	<i>Specific activity (units/mg protein) after activation</i>
H ₂ SO ₄ homogenate 23,500 g supernatant before dialysis (I)	1.0	—‡	—
H ₂ SO ₄ homogenate 23,500 g supernatant after dialysis (II)	1.25	6.0	6.5
0% to 20% ammonium sulphate fraction after dialysis (IIIa)	0	1.7	0.425
20% to 80% ammonium sulphate fraction after dialysis (IIIb)	1.20	24.7	80.0
80% to 100% ammonium sulphate fraction after dialysis (IIIc)	0	0	0

* Prepared from 1.8 g seminiferous tubules.

† Corrected by subtraction of pancreatic trypsin activity (e.g. 2.5 units in the case of activation of 20% to 80% fraction).

‡ Activation of Preparation I could not be measured due to precipitation during assay.

After centrifugation, aliquots of the supernatants were activated with crystalline bovine pancreatic trypsin (Worthington Biochemical Company). The activation procedure consisted of incubating equal volumes of a 100,000 g supernatant and 0.05 M-tris-HCl, 0.05 M-CaCl₂, pH 8, containing bovine trypsin (protein ratio, bovine trypsin:dialysed preparation, 1:70) for 1 hr at 25° C (incubation for 2 hr gave identical qualitative and quantitative results).

Typical results are shown in Table 1. Before activation with pancreatic trypsin, only low levels of trypsin-like activity (or no activity) were detectable in all preparations. After activation, all preparations except IIIc showed increases in activity. The greatest amount of testicular trypsin-like activity was present in Preparation IIIb after activation. The ammonium sulphate fractionation steps were necessary for optimal yield of trypsin-like activity. Higher concentrations of pancreatic trypsin did not give rise to further activation.

It is important to mention here that the pH optimum of the testicular trypsin-

like activity in Preparation IIIb was 8, and the activity was inhibited to varying degrees by soybean and limabean trypsin inhibitors. Thus, the testicular activity resembles bovine pancreatic trypsin and rabbit sperm trypsin-like enzyme (Stambaugh & Buckley, 1969). It seems reasonable to assume that much, if not all, of the testicular trypsin-like activity is present in one or more stages of spermatogenic cells and/or in spermatozoa.

In other experiments, incubation of Preparation IIIb at pH 8 and 25° C for 24 hr without added trypsin or for 1 hr with crystalline bovine pancreatic α -chymotrypsin (Nutritional Biochemical Corp.) did not cause activation.

The results in Table 1 indicate that dialysis of Preparation I for 18 hr did not appreciably increase the trypsin-like activity before activation. In another experiment, continuation of dialysis for a further 36 hr with two more changes of 0.001 N-HCl resulted in a fivefold increase in trypsin-like activity. Storage of Preparation II for 36 hr did not result in activation. Activation after dialysis could have been due to the removal of a high molecular weight, slowly dialysable inhibitor.

In another experiment, activated Preparation IIIb was incubated with different quantities of unactivated Preparation IIIb for 30 min at pH 8 and 25° C (the final total incubation volume was kept constant). The purpose of this experiment was to determine whether an unactivated preparation could inhibit the trypsin-like activity of an activated preparation. While there was no inhibition of trypsin-like activity when the protein ratio of activated: unactivated preparation was 2.5:1, a ratio of 1:1 resulted in 40% inhibition, and a ratio of 1:2.5 resulted in complete inhibition. If the last mixture of preparations was then incubated with pancreatic trypsin, the trypsin-like activity detected was equal to the total that would be obtained if both fractions were activated. Thus, the inhibition of activated activity by the inactive preparation appears to be reversible.

The following tentative conclusions can be drawn from these results: (1) most of the trypsin-like enzyme in mature rabbit testes is present as an inactive form, (2) the ammonium sulphate fraction containing the inactive form may also contain a trypsin inhibitor.

The present results do not explain whether the initial inactivity of the enzyme in the various preparations was due to the presence of the trypsin inhibitor or to the enzyme being present as a zymogen (e.g. trypsinogen or plasminogen; Perlmann & Lorand, 1970). Bovine pancreatic trypsin could have activated the inactive preparations by cleaving a peptide bond in a zymogen or by successfully competing for an inhibitor's binding sites.

Further studies will be undertaken to elucidate the biological significance of this inactive form of trypsin-like enzyme. These studies will include efforts to determine the molecular basis of the enzyme's inactivity, the stages of rabbit spermatogenesis during which the inactive form is present, whether this inactive form is also present in rabbit non-testicular spermatozoa, and whether a similar inactive form is present in the testes and non-testicular spermatozoa of other mammals.

The excellent technical assistance of Mr Ching-Yin Chang during part of

this work is gratefully acknowledged. This research was supported by Health Sciences Advancement Award USPHS No. RR06138 and by a University of California grant.

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