ELECTROPHORETIC DETECTION OF MULTIPLE FORMS OF TRYPSIN-LIKE ACTIVITY IN SPERMATOOA OF THE DOMESTIC FOWL

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Buruiana (1956) reported the presence of trypsin activity in the spermatozoa of several species, including the cock. Recently Stambaugh & Buckley (1969) demonstrated that the trypsin-like enzymatic activity of rabbit sperm acrosomes could remove the zona pellucida of rabbit ova.

The present study is concerned with the electrophoretic detection of multiple forms of trypsin-like enzymatic activity present in ejaculated spermatozoa of the domestic fowl, *Gallus domesticus*.

Single-combed White Leghorn and Black Australorp cock semen was collected by abdominal massage, pooled, diluted with an equal volume of phosphate buffer (Wilcox, 1958), and centrifuged at 1200 *g* for 10 min. The sperm deposit was washed three times with phosphate buffer, re-suspended in an equal volume of water containing glass beads and sonicated for 10 min with a model 185D sonifier (Heat Systems, Inc.) at an output setting of seven. The sonicated suspension was centrifuged at 48,000 *g* for 1 hr.

Electrophoresis of 48,000 *g* supernatants and seminal plasma was performed with the Ortec Model 4200 flat-bed acrylamide electrophoresis system and Ortec Model 4100 pulsed constant power supply (Ortec, Inc.). Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The technique of gradient gel preparation was similar to that described by Allen (1969). The gel consisted of successive layers of 4.5%, 6% and 8% acrylamide made up in 0.375 M-tris-sulphate buffer, pH 9. The upper and lower tank buffer was 0.0625 M-tris-borate, pH 9. Electrophoresis of 1.5 mg of protein was carried out for a total of 2½ hr at 4° C. The pulse rates utilized were 75 pulses/sec (pps) for 5 min, 150 pps for 75 min and 225 pps for the remainder of the 2½ hr.

After electrophoresis, gels were incubated twice for 20 min at room temperature in 0.1 M-succinic acid-tris buffer, pH 6, containing 1 mg/ml of Fast Blue Salt B (Matheson, Coleman and Bell Chemicals) and 0.3 mg/ml of one of the following naphthylamide substrates (Fox Chemical Co.): α -benzoyl-D,L-arginine- β -naphthylamide hydrochloride (BANA), L-lysyl- β -naphthylamide carbonate (L-Lys- β -NA), L-arginyl- β -naphthylamide hydrochloride (L-Arg- β -NA), L-lysyl-L-lysine- β -naphthylamide carbonate (L-Lys-Lys- β -NA) or *N*-

carbobenzoxy-L-arginine- β -naphthylamide hydrochloride (Cbz-L-Arg- β -NA). Stock substrates were solubilized (4 mg/ml) in dimethylformamide or 0.001 N-HCl plus dimethylformamide.

Enzymatic activity was detected on the gel by observing the formation of the orange-red colour produced by the coupling of the diazonium salt, Fast Blue Salt B with β -naphthylamine, a product of the enzymatic hydrolysis of the naphthylamide substrates.

The enzymatic hydrolysis of BANA, carried out in the presence of diazonium salts, has been previously utilized in colorimetric assays (Riedel & Wünsch, 1959), histochemical localization (Hopsu & Glenner, 1963) and the electrophoretic detection (Riekkinen, Ekfors & Hopsu, 1966) of tissue trypsin-like activity. In the present paper, using BANA as the substrate, three distinct zones of sperm enzymatic activity (a, b, c, Plate 1) were detected towards the anode in the 8% gel, while sperm enzymatic activity which had no electrophoretic mobility was localized at the origin (d, Plate 1). No activity was detected after the electrophoresis of seminal plasma. The same four zones of enzymatic activity were detected after electrophoresis of frozen and fresh sperm preparations obtained from five different pooled semen samples and from semen of each strain treated separately. In all the sperm samples studied, zone c was the most intensely stained. Incubation of gels before staining for 20 min in buffer at pH 6 containing 1 mg/ml soybean, lima bean or ovomucoid trypsin inhibitors (Nutritional Biochemical Co.) completely inhibited the appearance of enzymatic activity during the first 20 min of staining, but not during the second 20 min. When trypsin inhibitors were also present in the staining solutions, no enzymatic activity was detected during the first or second incubation with staining solution. Incubation of gels with crystallized bovine albumin before and during staining did not inhibit enzymatic activity. Trypsin inhibitors purified from natural sources can contain inhibitors of chymotrypsin, but chymotrypsin does not hydrolyse BANA (Riedel & Wünsch, 1959).

The same four zones of activity, which were detected when using BANA as the substrate, were also observed when Cbz-L-Arg- β -NA was the substrate. As in the case of BANA, these four zones were also completely inhibited by soybean trypsin inhibitor. Seminal plasma did not hydrolyse Cbz-L-Arg- β -NA.

No hydrolysis of L-Lys-Lys- β -NA either by spermatozoa or seminal plasma was detected on the gels, but both hydrolysed L-Arg- β -NA and L-Lys- β -NA. Hydrolysis of these substrates occurred only at the gel origin. The seminal plasma activity was much stronger than that of the spermatozoa. Soybean trypsin inhibitor did not inhibit seminal plasma and sperm hydrolysis of L-Lys- β -NA or L-Arg- β -NA. Thus, this enzymatic activity was different from that which hydrolysed BANA and Cbz-L-Arg- β -NA at zone d. The zones of activity seen in Plate 1 may represent isozymes of trypsin-like activity. They all hydrolysed BANA and Cbz-L-Arg- β -NA, did not hydrolyse three other substrates and were inhibited by three different trypsin inhibitors. The term, 'isozymes', describes multiple molecular forms of enzymes within an organism which can be resolved by a variety of techniques and which can catalyse the same reactions (Markert, 1968). It is a broad operational definition rather than a description of a specific molecular relationship. Isozymes may include

PLATE 1

Hydrolysis of BANA by sperm homogenates was detected on the gel at a, b, c and d. There was no hydrolysis of BANA by seminal plasma.

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proteins which differ in primary structure, size, conformation, or in other ways (Markert & Whitt, 1968). Further studies are being undertaken in order to elucidate the nature of the multiple forms of trypsin-like activity detectable in cock spermatozoa.

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