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STUDIES ON ACROSOMAL PROTEINASE OF RABBIT SPERMATOZOA

RICHARD STAMBAUGH AND JEAN BUCKLEY

The Division of Reproductive Biology, Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, Philadelphia, Penn. 19104 (U.S.A.)

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SUMMARY

Kinetic data were obtained to further characterize the acrosomal proteinase of epididymal, ejaculated, and capacitated rabbit spermatozoa. The studies demonstrated that the properties examined were not altered during capacitation, and there is no evidence for a direct involvement of these enzymes in capacitation. Marked differences were noted, however, between the inhibitory effects of lima bean trypsin inhibitor and *p*-aminobenzamidine on acrosomal proteinase and bovine pancreatic trypsin (EC 3.4.4.4). Lima bean trypsin inhibitor displayed partial specificity for pancreatic trypsin, while *p*-aminobenzamidine showed partial specificity for acrosomal proteinase.

INTRODUCTION

Acrosomal proteinase exists in the acrosomes of spermatozoa from numerous species, and is highly specific for the peptide bond of arginine and lysine[1–8]. This enzyme is responsible for the dissolution of a small tunnel through the zona pellucida or capsule surrounding the vitellus of the ovum, which allows the spermatozoon to reach the perivitelline space. Once penetration of this capsule has occurred, the plasma membranes of the spermatozoon and ovum fuse, and fertilization takes place forming the zygote. However, before sperm develop the ability to fertilize the ovum, they must spend a minimum amount of time in the uterus, a process referred to as capacitation[9]. The nature of this capacitation phenomenon remains obscure, and no one has been able to duplicate this biological process reproducibly *in vitro*, with the exception of golden hamster spermatozoa[10].

The work described in this article was carried out primarily to further characterize this acrosomal proteinase. However, the possibility that a change in the properties of this important enzyme might occur during capacitation was also investigated.

Abbreviations: BAEE, benzoyl-L-arginine ethyl ester; BAPNA, *N*-benzoyl-DL-arginine *p*-nitroanilide; BTEE, benzoyl-L-tyrosine ethyl ester.

EXPERIMENTAL PROCEDURE

Protein determinations

Protein concentrations were determined by the method of Lowry *et al.*[11].

Enzyme assays

Acrosomal proteinase was assayed spectrophotometrically using either benzoyl-L-arginine ethyl ester (BAEE) by the procedure of Bergmeyer[12], or on *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) by the procedure of Erlanger *et al.*[13]. Chymotrypsin-like activity was assayed spectrophotometrically using benzoyl-L-tyrosine ethyl ester (BTEE) by the procedure of Hummel[14].

Lysozyme was determined by the rate of lysis of *Micrococcus lysodeikticus* by the method of Shugar[15]. Leucine aminopeptidase (EC 3.4.1.1) activity was measured by the procedure of Mitz and Schlueter[16]. Elastase (EC 3.4.4.7) activity was determined by the procedure of Sachar *et al.*[17]. Carboxypeptidase (EC 3.4.2.1) activity was assayed using hippuryl-L-phenylalanine by the procedure of Folk and Schirmer[18].

Isolation and extraction of sperm heads

Rabbit epididymal, ejaculated, and capacitated spermatozoa were collected, washed, and fractionated subcellularly using sonication and sucrose density gradient centrifugation as we previously described[2]. Capacitated sperm were collected from the uterine horns 6.5 h after mating. Acrosomal proteinase was extracted from these isolated head fractions by sonicating the suspensions in 1.0% deoxycholate for 1.0 min at 1.0 A with cooling using a Branson Model LS-75 sonifier and a microtip for sonication. This mixture was stirred at room temperature for 15 min, centrifuged and the supernatant solution used for kinetic studies on acrosomal proteinase.

Materials

BAEE, BTEE, *p*-aminobenzamidine, and benzamidine were obtained from Sigma. Crystalline bovine pancreatic trypsin, soybean trypsin inhibitor, lima bean trypsin inhibitor, hippuryl-L-phenylalanine, elastin, *Micrococcus lysodeikticus*, and L-leucinamide were obtained from Worthington.

RESULTS

Specificity

All of the sperm head extracts were specific for BAEE and BAPNA. No activity could be detected on BTEE, L-leucinamide, hippuryl-L-phenylalanine, *Micrococcus lysodeikticus*, or elastin. This was in contrast to the specificity of commercial crystalline bovine pancreatic trypsin, which did show a low level of activity on BTEE.

pH optimum and K_m

Using $1.5 \cdot 10^{-4}$ M BAEE as a substrate, a sharp pH optimum of 8.2 was found for acrosomal proteinase extracted from epididymal as well as for ejaculated and capacitated spermatozoa (Table I). Using a pH of 8.2, K_m values were determined by double reciprocal plots of the initial reaction velocity against the concentration of BAEE. Essentially identical K_m values were found for epididymal, ejaculated, and

TABLE I

SOME PROPERTIES OF RABBIT ACROSOMAL PROTEINASE AND PANCREATIC TRYPSIN

Property	*Rabbit acrosomal proteinase*			*Bovine pancreatic trypsin*
	Epididymal	*Ejaculated*	*Capacitated*	
pH optimum (BAEE)	8.2	8.2	8.2	8.2
K_m (BAEE)	$5.2 \cdot 10^{-6}$ M	$5.2 \cdot 10^{-6}$ M	$5.1 \cdot 10^{-6}$ M	$5.76 \cdot 10^{-6}$ M
*p*H optimum (BAPNA)	8.2	8.2	8.2	8.2
K_m (BAPNA)	$10.9 \cdot 10^{-3}$ M	$10.0 \cdot 10^{-3}$ M	$10.7 \cdot 10^{-3}$ M	$6.57 \cdot 10^{-3}$ M
K_i (soybean inhibitor)	$1.0 \cdot 10^{-10}$ M	$1.0 \cdot 10^{-10}$ M	$1.0 \cdot 10^{-10}$ M	$2.1 \cdot 10^{-10}$ M
K_i (lima bean inhibitor)	$2.0 \cdot 10^{-5}$ M	$1.8 \cdot 10^{-5}$ M	$2.0 \cdot 10^{-5}$ M	$1.0 \cdot 10^{-8}$ M
K_i (*p*-aminobenzamidine)	$8.0 \cdot 10^{-6}$ M	$6.0 \cdot 10^{-6}$ M	$8.5 \cdot 10^{-6}$ M	$3.7 \cdot 10^{-5}$ M
K_i (benzamidine)	$2.6 \cdot 10^{-5}$ M	$2.3 \cdot 10^{-5}$ M	$2.3 \cdot 10^{-5}$ M	$1.8 \cdot 10^{-5}$ M
Half life (50 °C, pH 7.5)	1.5 min	1.6 min	1.6 min	4.1 min

capacitated sperm acrosomal proteinase, as well as for bovine pancreatic trypsin (Table I).

With $0.96 \cdot 10^{-3}$ M DL-BAPNA as a substrate, a sharp pH optimum of 8.2 was again found for acrosomal proteinase extracted from epididymal as well as from ejaculated and capacitated spermatozoa (Table I). Using a pH of 8.2, K_m values were determined by double reciprocal plots of the initial reaction velocity against the substrate concentration. Again, the K_m values for epididymal, ejaculated, and capacitated sperm appeared to be within experimental error, but bovine pancreatic trypsin displayed a somewhat lower value (Table I).

K_i values for inhibitors

Using BAEE as a substrate, K_i values for soybean and lima bean trypsin inhibitors were determined on acrosomal proteinase using Dixon plots (Table I). The

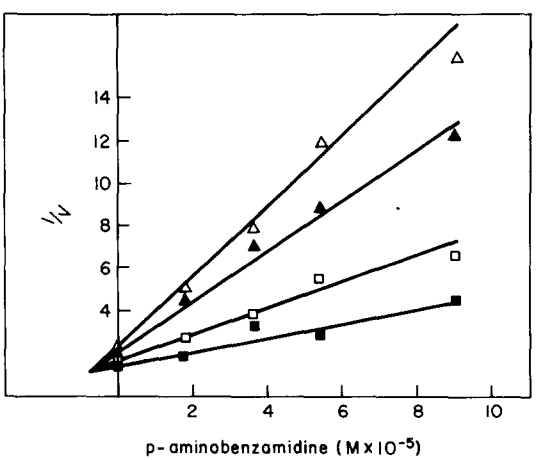

Fig. 1. Dixon plot of *p*-aminobenzamidine inhibition of acrosomal proteinase from rabbit epididymal sperm. The reactions were carried out in 0.05 M Tris buffer (pH 8.2) at the indicated inhibitor concentrations. BAEE concentrations were 0.938 mM (△), 0.469 mM (▲), 0.235 mM (□), and 0.177 mM (■). The reciprocal of the initial reaction velocity in △ A/10 min at 254 nm is plotted against the concentration of *p*-aminobenzamidine.

Dixon plots demonstrated a competitive type of inhibition for all three types of acrosomal proteinase preparations using either the soybean or lima bean trypsin inhibitors. K_i values for soybean trypsin inhibitor on acrosomal proteinase were identical for epididymal, ejaculated, and capacitated spermatozoa (Table I). This value was slightly lower than that observed for bovine pancreatic trypsin. Using lima bean trypsin inhibitor, similar K_i values were again observed for epididymal, ejaculated, and capacitated sperm, respectively. These values were considerably higher than the $1.0 \cdot 10^{-8}$ value observed using pancreatic trypsin (Table I).

Two synthetic inhibitors for trypsin were also tested, *p*-aminobenzamidine and benzamidine. Inhibition by *p*-aminobenzamidine was of the competitive type for acrosomal proteinase, and the K_i values for extracts of epididymal, ejaculated, and capacitated sperm heads are probably within experimental error of each other, but the K_i value using bovine pancreatic trypsin is considerably lower (Table I and Fig. 1). Thus, *p*-aminobenzamidine displays some specificity for acrosomal proteinase.

Inhibition of acrosomal proteinase by benzamidine was also of the competitive type, and very similar K_i values were observed for epididymal, ejaculated, and capacitated sperm extract, as well as for benzamidine inhibition of crystalline bovine pancreatic trypsin (Table I).

DISCUSSION

Previous reports from our laboratory demonstrated that a trypsin-like enzyme localized in the acrosome was the enzyme effecting penetration of the zona pellucida by its dissolution action on this capsule[1–3]. Subsequently, we confirmed its biological importance for fertilization by inhibiting *in vitro* fertilization of rabbit ova[4]. The presence of this acrosomal proteinase has now been confirmed for spermatozoa in rhesus monkeys[4], humans[4], bulls[6], rabbits[5,7], and in fowl[8].

The primary objective of this study was to further characterize this enzyme, with a secondary objective of ascertaining whether any kinetic properties of this proteinase are altered during the capacitation process. It has been 21 years since the discovery that spermatozoa require a period of some hours in the female reproductive tract before they develop the ability to penetrate the ovum[9], and the changes which occur in sperm during this period are still obscure. The kinetic studies presented here have further characterized this important enzyme, but no changes in properties of acrosomal proteinase were found as a result of capacitation. The enzyme possessed the same K_m value for BAEE and BAPNA, the same pH optimum, and the same K_i values for soybean and lima bean trypsin inhibitors as well as for *p*-aminobenzamidine and benzamidine both before and after capacitation. Also, no change in the heat stability of acrosomal proteinase was detectable by capacitation. However, we should mention here that evidence for inhibition of acrosomal proteinase by seminal plasma inhibitors, and deinhibition during capacitation has been presented[5,18], although we have been unable to confirm their observations[2,4]. The partial specificity of *p*-aminobenzamidine for acrosomal proteinase reported here has been utilized with limited success for contraceptive experiments in the rabbit[20], but the toxicity of this compound would seem to preclude any use of this inhibitor for human contraception. This study, then, has further characterized the acrosomal proteinase of spermatozoa and demonstrated that its enzymic properties do not change during capacitation.

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