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Localization of a Proteolytic Enzyme Within the Efferent and Deferent Duct Epithelial Cells of the Turkey (*Meleagris gallopavo*) Using Immunohistochemistry[1]

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ABSTRACT

Turkey seminal plasma contains a serine protease found to be distinct from the spermatozoal acrosin. However, the origin and biological roles of this enzyme are unknown. Our experimental objective was to identify the cellular source of this protease within the male reproductive tract. The enzyme was isolated from seminal plasma using benzamidine-Sepharose 6B chromatography. A synthetic substrate, Nα-benzoyl-DL-arginine *p*-nitroanilide, was used to detect fractions containing the enzyme. The affinity chromatography technique yielded a 150-fold increase in amidase activity. Analysis of the protease by SDS-PAGE revealed two protein bands with relative molecular masses of 37 000 and 61 000. Proteolytic activity was detected within the smaller band as evidenced by casein digestion. Further analysis of the purified protein revealed that the smaller protein band was glycosylated. To determine the cellular source of the protease, a panel of mouse monoclonal antibodies was then developed against the purified protease, and used in immunohistochemistry. Frozen tissue sections from the liver, testis, epididymal region, and deferent duct were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.2% (v/v) (octylphenoxy)polyethoxyethanol followed by routine immunohistochemistry procedures. Monoclonal antibodies did not bind to tissue sections from either the liver or testis, or to blood plasma proteins. Both the distal portion of the efferent duct and the deferent duct were immunoreactive. We concluded that the protease found in turkey seminal plasma is concentrated to the distal efferent duct and the deferent duct epithelial cells.

immunology, male reproductive tract, vas deferens

INTRODUCTION

Seminal plasma contains proteins that are necessary for sperm maturation, motility, and maintenance of sperm viability and fertilizing capacity [1]. In mammals, the source of these proteins is the epithelia of the reproductive tract ducts and accessory glands [2, 3]. Avian species lack accessory glands [4, 5], but do possess paired paracloacal vascular bodies that generate lymph-like fluid at the time of ejaculation [6]. Hence, most of the proteins in the seminal plasma of aves probably originate from ductal epithelia.

Some seminal plasma proteins are enzymes with proteolytic activity, and the presence of secondary sex glands in mammals has facilitated the study of these seminal plasma proteases [1, 3]. The most studied protease from the prostate gland is prostate-specific antigen, which acts on the seminal vesicle protein, semenogelin I, to liquefy the semen [7]. For poultry species, there was a general belief that proteolytic activity was limited to acrosin within spermatozoa [8]. This assumption seemed reasonable in view of the report that fowl seminal plasma contained a low molecular weight serine protease inhibitor [9] whose biological role was postulated to serve to inhibit acrosin activity from dead or moribund sperm. In fact, the seminal plasma of the domestic fowl was shown to be devoid of proteolytic activity at basic pH, but did contain protease activity if the seminal plasma was acidified [10].

Thurston et al. [11] showed that this assumption was incorrect for the domestic turkey because this species was shown to have significant seminal plasma protease activity at basic pH. This enzyme consisted of two subunits and was distinctly different from spermatozoal acrosin. Assay of fluids removed from various parts of the reproductive tract showed that the majority of the activity was confined to the deferent duct, but the source was not determined. However, the liver, testis, or excurrent ducts appear to be a likely source of this enzyme. Therefore, the experimental objective of the present research was to identify the origin of the turkey seminal plasma protease using immunohistochemical techniques.

MATERIALS AND METHODS

Affinity Chromatography

Semen was collected from Small White turkey toms (n = 100) by abdominal massage [12]. Pooled ejaculates were microcentrifuged for 2 min at 12 000 × *g*. Prior to storage at −20°C, seminal plasma supernatant was recentrifuged twice and then filtered through a 0.22-μm syringe filter to remove residual sperm.

Because the enzyme of interest was a serine protease, it was purified by affinity chromatography using a serine protease-specific inhibitor attached to Sepharose, benzamidine-Sepharose 6B. Fifteen milliliters of filtered seminal plasma was dialyzed against the column buffer, 25 mM imidazole-HCl (pH 6.8) in 100 mM NaCl, overnight at 4°C within a 7000 molecular weight cutoff dialysis tubing. Dialyzed seminal plasma was loaded onto a 7-ml benzamidine-Sepharose 6B (Amersham Pharmacia Biotech Inc., Piscataway, NJ) column. The column was washed with 3 bed volumes of buffer, and the protein was eluted with 10 mM ammonium formate (pH 3) in 1.0 M NaCl. The eluate was collected in 2-ml fractions, and all fractions were assayed for enzyme activity as described previously [11]. Briefly, a 100-μl aliquot of each fraction was mixed with 1 ml of 250 mM triethanolamine-HCl (pH 8) and 0.5 ml of the substrate 2.3 mM Nα-benzoyl-DL-arginine *p*-nitroanilide-HCl (BAPNA). Enzyme activity was measured at 25°C by an increase in absorbance at 405 nm. Fractions with enzyme activity were combined, and the protein solution was dialyzed against deionized distilled water overnight at 4°C. This dialysate was lyophilized and stored at −20°C.

Characterization of Isolated Protein

Discontinuous SDS-PAGE of the purified protease was performed with a 12% polyacrylamide gel (Ready Gels, Bio-Rad Laboratories, Hercules,

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CA) using the buffer system of Laemmli [13]. Protein solutions were diluted 1:2 in Laemmli sample buffer containing 5% (v/v) β-mercaptoethanol and then heat-denatured at 100°C for 5 min. Denatured proteins were electrophoresed at 200 V for 35 min in a Mini Protean II Dual-Slab Cell apparatus (Bio-Rad). After electrophoresis, gels were either stained with the Silver Stain Plus Kit (Bio-Rad) to detect protein, or thymol-sulfuric acid to detect glycosylation [14]. Proteolytic activity of the purified enzyme was confirmed after electrophoresis under nonreducing conditions using the casein Ready Gel Zymogram assay kit (Bio-Rad) according to the manufacturer's directions.

Monoclonal Antibody Production

Lyophilized protease was resuspended in PBS (1 mg/ml), and one half of the solution was denatured for 5 min in a boiling water bath. A mixture containing equal concentrations of native and heat-denatured protease (50 μg each) was used as an immunogen. Two female RBF/DnJ mice (Jackson Laboratory, Bar Harbor, ME) were immunized on Days 1, 14, 35, and 56. Each initial subcutaneous immunization of 0.2 ml was performed in the inguinal region. The injections contained 100 μg of the protease mixture in a 1:2 dilution of protease solution and TiterMax Gold adjuvant (CytRx Corp., Norcross, GA). For all subsequent injections, the immunogen was suspended in PBS. Five days after the last immunization, spleen cells were fused with FOX-NY myeloma cells (American Type Culture Collection, Manassas, VA) in the presence of 50% (w/v) polyethylene glycol 4000 (EM Science, Gibbstown, NJ). Hybridomas were grown in adenine-aminopterin-thymidine-RPMI selective media for 10 days before screening for immunoreactivity against the protease using ELISA.

ELISA assays were performed at room temperature unless specified. Immulon-4 flat bottom 96-well plates (Dynatek Technologies, Inc., Chantilly, VA) were coated with 100 μl/well of a 1:10 dilution of poly-L-lysine, covered with plastic wrap, and then incubated overnight at 4°C. The following morning the contents were flicked out and the plate was allowed to air dry before 100 μl of a 25-μg/ml protease solution was aliquoted into each well. Plates were covered and incubated overnight at 4°C. Three washes containing 0.1% (v/v) Tween-20 in PBS were performed between each step. Nonspecific binding was blocked for 1 h with 3% (w/v) BSA in PBS using 100 μl/well. Either 100 μl of undiluted hybridoma supernatants or PBS was aliquoted into each well and incubated for 1 h. A 100-μl volume of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin heavy and light chain specific (Ig H&L; Sigma, St. Louis, MO) diluted 1:1500 in PBS was added to each well, and then the plate was incubated for 1 h. Wells containing bound antibody were identified using 100 μl of a 1-mg/ml solution of *p*-nitrophenyl phosphate in diethanolamine buffer, pH 9.8. Positive hybridomas were cloned by limiting dilution and cultured for further analysis using slot blotting and Western blotting procedures as described below.

For Western blots, samples were electrophoresed by SDS-PAGE as described above, then transferred onto a 0.45-μm nitrocellulose membrane. Membranes and gels were equilibrated in transfer buffer containing 25 mM Tris, 190 mM glycine, and 20% (v/v) methanol pH 8.2, for 15 min before electrophoretic elution. Transfer of the protease to the nitrocellulose was performed in a Mini-Transblot Electrophoretic Transfer cell (Bio-Rad) run at 30 V overnight at 4°C according to the manufacturer's instructions. After transfer, the nitrocellulose was submerged in 5% (w/v) nonfat dry milk in PBS for 1 h with moderate shaking. Slot blot analysis was performed using 0.45-μm nitrocellulose membranes presoaked for 15 min in transfer buffer before being assembled into a Bio-Dot SF microfiltration apparatus (Bio-Rad). Slight vacuum was applied to remove excess transfer buffer, and 200 μl of 25 μg protease/ml PBS was aliquoted into each well. The apparatus was covered with plastic wrap and incubated overnight at 4°C. For both immunoblotting procedures the membranes were washed three times with 0.1% (v/v) Tween-20 in PBS for 5 min with agitation between each step. Hybridoma supernatants were incubated for 90 min. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (H&L) diluted 1:1500 in PBS was applied to the nitrocellulose for 1 h with slow agitation. Detection of bound antibody was measured using 5-bromo-4-chloro-3-indoyl-phosphate/nitro-blue tetrazolium in alkaline phosphatase buffer containing 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris pH 9.5. Immunoglobulins from cloned hybridomas were isotyped by ELISA using the SBA Clonotyping System/AP (Southern Biotechnology Associates, Inc., Birmingham, AL).

Immunohistochemistry

Turkeys (n = 5) were killed with an injection of FatalPlus before tissue samples were removed. Tissue samples were excised from the liver, testis, epididymis, and the deferent duct and placed into sterile PBS until small blocks from each tissue could be cut and trimmed. Tissue sections were frozen, fixed, and stained according to modified protocols of Harlow and Lane [15]. Once trimmed, each sample was placed onto a piece of cardboard and snap-frozen in liquid nitrogen [15]. Frozen tissues were stored at −80°C prior to sectioning. Frozen tissue blocks were equilibrated to −20°C inside a cryostat microtome, and 8-μm tissue sections were taken for immunohistochemistry. Each 8-μm section was placed on a poly-L-lysine coated slide. Tissue fixation was performed with 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature, then sections were washed 3 times with PBS. Thereafter, cells were permeabilized using 0.2% (v/v) (octylphenoxy)polyethoxyethanol (IGEPAL CA-630; Sigma) in PBS for 5 min. Tissues were blocked with 5% (w/v) BSA in PBS for 30 min, then washed twice in PBS for 5 min per wash. Excess fluid was blotted off the slide before sections were covered for 1 h with monoclonal hybridoma supernatants from confluent cultures. A 3% (w/v) solution of BSA in PBS was used as a negative control. Tissue sections were washed three times in PBS for 5 min per wash, then incubated for 1 h with a 1:50 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (H&L) in PBS containing 1% (w/v) BSA. After this incubation, sections were washed twice in PBS for 5 min per wash in order to remove unbound secondary antibody. Sections were then covered for approximately 5 min with a horseradish peroxidase substrate solution containing 3,3′-diaminobenzidine (DAB) according to the instructions for the DAB Substrate Kit (Vector Laboratories, Inc., Burlingame, CA). Sections were then counterstained with 0.2% (w/v) methylene blue for 10 sec in order to visualize any unlabeled cellular details.

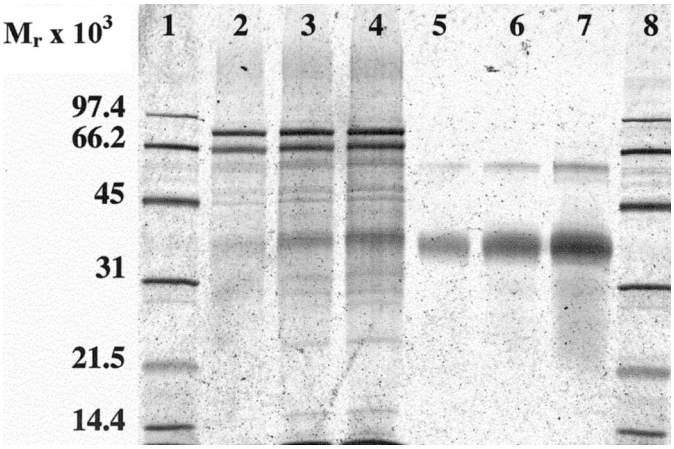

FIG. 1. Electrophoretic gel containing seminal plasma proteins (lanes 2–4; 1.0, 2.0, and 3.0 μg of protein, respectively) and the protease eluted from a benzamidine-Sepharose 6B affinity column (lanes 5–7; 0.5, 1.0, and 2.0 μg of protein, respectively). Electrophoresis was performed on 12% SDS-PAGE followed by silver staining of proteins. Lanes 1 and 8 were loaded with the following SDS-PAGE low-range standards: phosphorylase b (M_r 97 400), BSA (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400).

RESULTS

Purified Protein

Fifty replicate chromatography purifications were performed in order to procure adequate protease. On average, a 150-fold purification was achieved with a recovery of 0.2% of the total protein applied to the column. As shown in lanes 5–7 in Figure 1, 2 major protein bands were observed following SDS-PAGE. Their relative M_r were 37 000 and 61 000. The 37 000 M_r band was glycosylated on the basis of staining with thymol-sulfuric acid (data not shown). As shown in Figure 2, the 37 000 M_r protein exhibited proteolytic activity. Therefore, affinity chromatography was effective in isolating a serine protease from turkey seminal plasma.

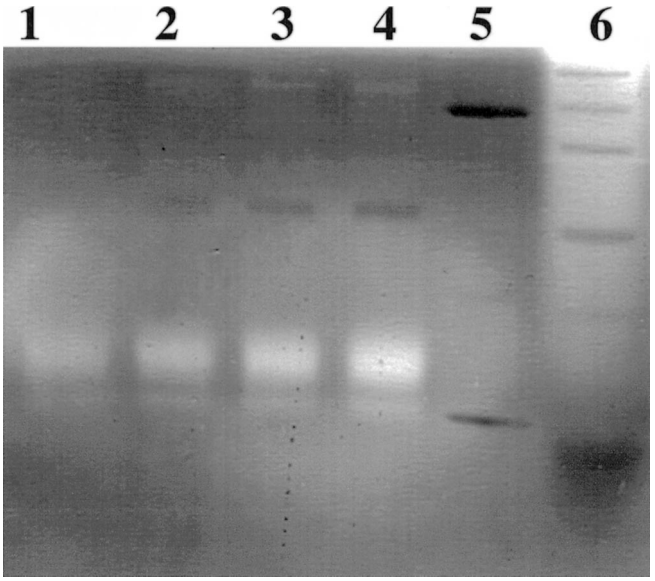

FIG. 2. Proteolytic digestion of casein following electrophoresis of the protease eluted from a benzamidine-Sepharose 6B column. After electrophoresis the gel was incubated in a renaturation buffer to restore enzyme activity. The dark background denotes casein stained with Coomassie blue R250. In contrast, clear bands in lanes 1–4 denote hydrolysis of casein. Lanes 1–4 were loaded with 0.5, 1.0, 1.5, and 2.0 μg of protease, respectively. Lanes 5 and 6 are Bio-Rad SDS-PAGE low range and Bio-Rad Precision molecular weight standards, respectively.

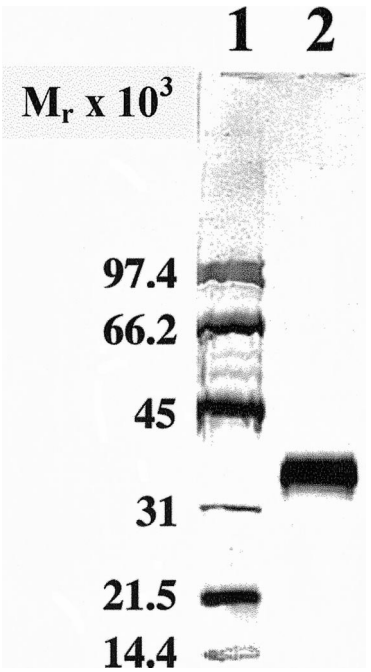

FIG. 3. Reactivity of monoclonal antibody following Western blot analysis. Lane 1 contains biotinylated SDS-PAGE low range standards. In lane 2, the B3F6 monoclonal antibody recognized the 37 000 M_r band of the protease that contained proteolytic activity. Western blot analysis was performed following 12% SDS-PAGE, and the protein was blotted onto nitrocellulose. Bands were visualized using alkaline phosphatase BCIP/NBT color development.

Monoclonal Antibodies

After fusion, cell suspensions were aliquoted in four 96-well plates per mouse. Each of 768 hybridoma supernatants was tested for immunoreactivity to native or heat-denatured protease by ELISA after 10 days of culture. A total of 28 primary hybridoma supernatants tested positive by ELISA, with 13 colonies containing antibodies specific to native protease and 15 colonies containing antibodies specific to heat-denatured protease. None of the primary hybridomas produced antibodies that recognized both native and heat-denatured protease. Further analysis of these 28 immunoreactive cultures by slot blotting resulted in 18 hybridoma supernatants positive for the native protease. In contrast, Western blots revealed 5 cultures that produced antibody specific to denatured protease. Four primary hybridoma cultures were selected for limiting dilution and cloned based on their antibody affinity for either native or denatured protease as evidenced by immunoblotting procedures. This choice afforded 4 cloned hybridomas producing B1B12 and B4D11 monoclonal antibodies, and A4C6 and B3F6 monoclonal antibodies against native and denatured protease, respectively. Of the 2 monoclonal antibodies that recognized denatured protease, A4C6 bound to the 61 000 M_r protein, whereas the B3F6 monoclonal antibody recognized the 37 000 M_r protein as evidenced by Western blotting (Fig. 3). All four monoclonal hybridomas produced antibodies of the immunoglobulin G_1, κ isotype.

Immunohistochemistry

Western blot analysis was performed prior to immunohistochemistry in order to determine whether monoclonal antibodies recognized any blood serum protein. Each monoclonal antibody tested negative (data not shown). In all tissues evaluated, no positive reaction (brown color development) was observed within the negative control sections. Tissue sections from the liver and testis were evaluated for antibody binding. Neither the liver nor the testis tested positive for the protease as evidence by color development with horseradish peroxidase-labeled secondary antibody and DAB (data not shown). In contrast, the 3 monoclonal antibodies, B1B12, B4D11, and B3F6 bound to antigen associated with the excurrent ducts of the testis. The A4C6 monoclonal antibody against the 61 000 M_r protein shown in Figure 1 did not recognize antigen in fixed tissue. Using the anatomical criteria identified by Hess et al. [16], antibody binding was most pronounced in the distal efferent duct and the deferent duct (Figs. 4 and 5, respectively). Specifically, monoclonal antibody binding was associated with the epithelial layer, which could not be confined to any particular cell type. Of the 3 monoclonal antibodies that bound to epithelial cells 2 of the 3 recognized native protein (B1B12 and B4D11), whereas the third monoclonal antibody, B3F6, recognized the 37 000 M_r proteolytic protein band (Figs. 1 and 2). Sperm within the lumen of the ducts did not bind any of the 4 antibodies.

DISCUSSION

Much of the data compiled on seminal plasma serine proteases are often limited to their isolation and purification. Serine proteases that have been isolated from seminal plasma include prostate-specific antigen [7, 17, 18], basic arginine esterase [3, 19–22], kallikrein [23–26], tissue plasminogen activator [3, 27–29], prostasin [30], basic carboxypeptidase [31], elastase [3, 32], and a thrombin-like enzyme [33]. Understanding an enzyme's properties unquestionably assists in its purification. However, enzyme purification does not necessarily provide information relative to a biological role for the protease.

Apart from prostate-specific antigen, the biological roles

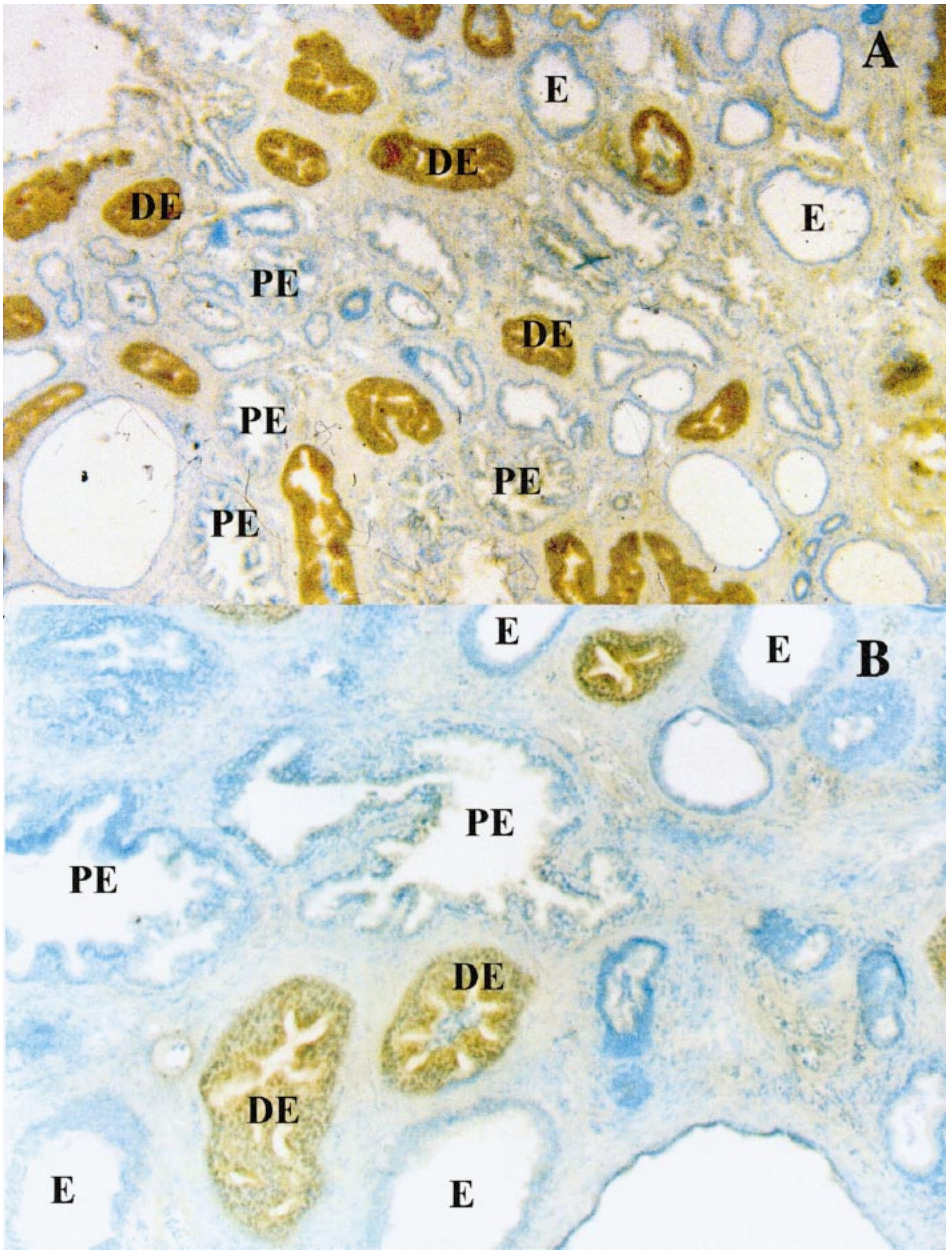

FIG. 4. Photomicrographs of tissue from the turkey epididymal region of the testis incubated with the B3F6 monoclonal antibody made against a protease found in turkey seminal plasma. A horseradish peroxidase-conjugated secondary antibody and a DAB substrate produced a brown coloration, indicative of a positive reaction. Tissue was counterstained with methylene blue. Cross-section magnification ×54 (**A**) and ×134 (**B**) of the epididymal region of the testis containing the proximal efferent duct (PE), distal efferent duct (DE), and the epididymal duct (E). Note the pronounced brown labeling of the distal efferent duct epithelia by the B3F6 monoclonal antibody specific for the proteolytic band of the enzyme.

of other seminal plasma serine proteases are highly speculative. For example, basic arginine esterase is believed to be involved with the removal of fibronectin from canine prostate epithelium [34], and yet it is hypothesized that it also has a role in the early events associated with the acrosome reaction and fertilization of human spermatozoa [22]. Similarly, a precise role for seminal plasma kallikrein is still unknown. In rats and humans, kallikrein was found to increase sperm motility, increase Sertoli cell function, increase testicular tissue metabolism, and up-regulate the incorporation of thymidine in testicular tissue [24]. In some cases these experimental endpoints have been based on 1 or more presumed functions. An inherent limitation of this type of research has been the tendency to extrapolate information gained from an enzyme's isolation and activity relative to arbitrary synthetic or unnatural substrates to identify function, but apart from prostate-specific antigen, endogenous substrates for most other proteases are essentially unknown.

With regard to the turkey seminal plasma protease, the problem is more complicated because this enzyme appears to be unique to this species, because other galliforms (i.e., chicken and guinea fowl) show no protease activity within their seminal plasma at basic pH [11]. Why would only the turkey need this enzyme to satisfy an important physiological function when other species with a similar reproductive system and physiology do well without it?

Due to the difficulty encountered when attempting to identify endogenous substrates, an alternative experimental approach has been to identify the source of an enzyme, which would serve further to elucidate its biological role. Our experimental objective was to identify the cellular source of the seminal plasma serine protease identified by Thurston et al. [11]. On the basis of inhibition with benzamidine, we hypothesized that the protease could be isolated from seminal plasma by affinity chromatography, which was substantiated by the experimental results. Thus, affinity chromatography provided an efficient method for isolating the serine seminal plasma protease.

A panel of monoclonal antibodies specific to native and

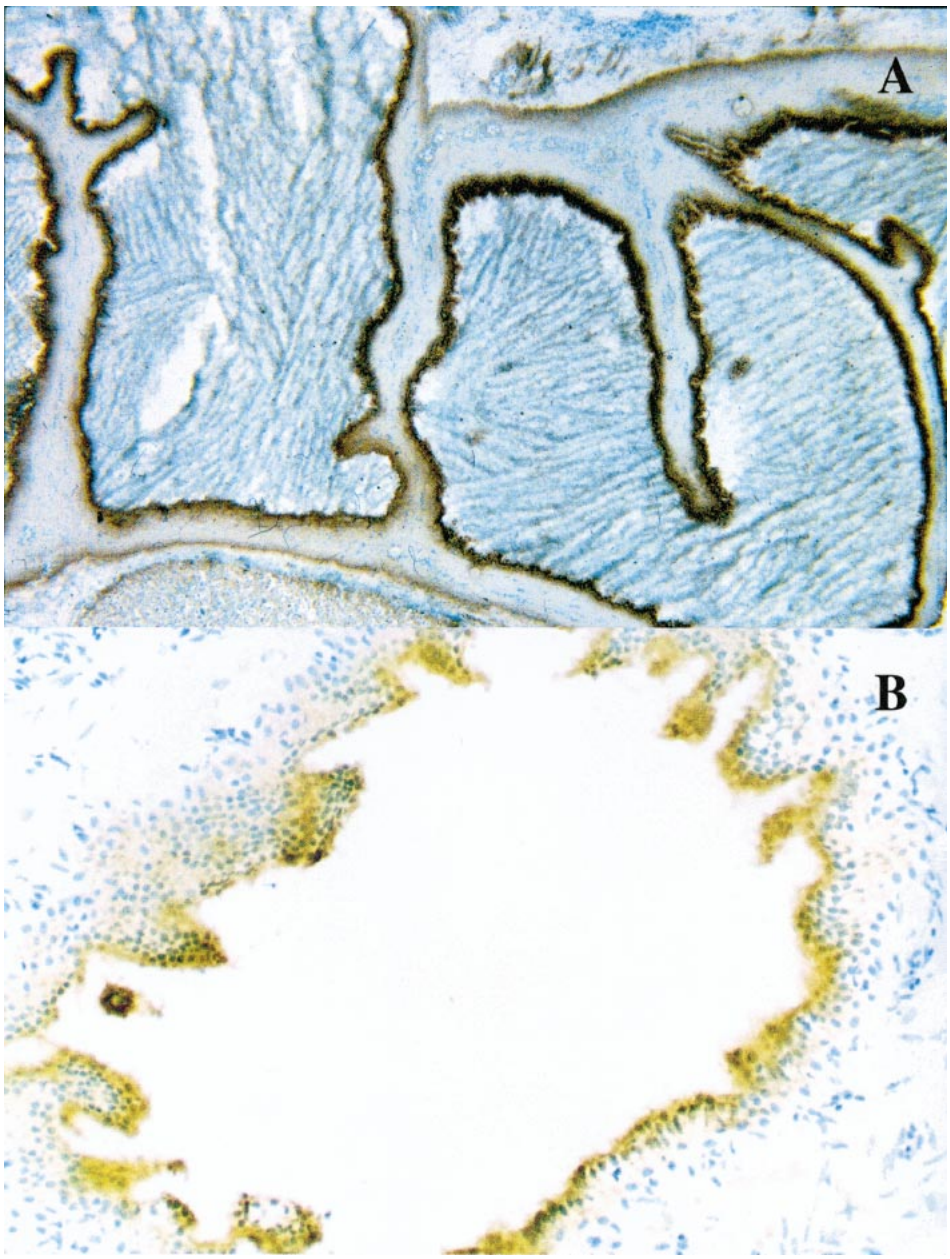

FIG. 5. Photomicrographs of the turkey deferent duct tissue incubated with the B3F6 monoclonal antibody made against a protease found in turkey seminal plasma. A positive staining denoted by a brown coloration identifies the presence of enzyme. Tissue was counterstained with methylene blue. Cross-section magnification ×54 (**A**) and ×269 (**B**) of a portion of the epithelia within the distal deferent duct. Note the presence of enzyme within the epithelia of the deferent duct, whereas no labeling of the spermatozoa within the lumen was detected.

denatured seminal plasma serine protease was produced and used in immunohistochemistry. Of the 4 specific monoclonal antibodies produced, only 1 monoclonal antibody, B3F6, met the following criteria. First, as evidenced by ELISA, this monoclonal antibody recognized denatured protease adsorbed to microtiter plate wells. Second, as evidenced by Western blotting, the B3F6 antibody bound to the 37 000 M_r proteolytic band of the protein (Fig. 3). Third, this antibody was determined to be an immunoglobulin G_1 isotype, which did not bind blood serum proteins, liver tissue, or testicular tissue. However, the B3F6 antibody did bind to both the distal efferent duct and the deferent duct epithelial cells (Fig. 4 and Fig. 5, respectively). The finding of the protease within the efferent and deferent ducts of the reproductive tract is consistent with the observation that reproductive tract BAPNA amidase activity was essentially limited to fluid recovered from the excurrent ducts of the testis [11]. This discovery was enigmatic for two reasons. As reviewed by Kirby and Froman [35], these portions of the excurrent ducts of the testis arise from 2 different embryonic origins. Likewise, the efferent ducts and the deferent duct have distinct physiological functions. Specifically, fluid reabsorption and cell phagocytosis are major functions of the efferent duct epithelial cells [36, 37]. In contrast, the deferent duct appears to be merely a conduit for sperm transit from the epididymis to the receptaculum, a bean-shaped storage site for semen in birds [38].

Although immunolocalization of the protease only provides the evidence for the presence of a particular protein, it seemed unlikely that the seminal plasma protease could originate at high enough concentrations upstream from the efferent and deferent ducts to saturate the epithelia of these ducts, and still evade biochemical or immunohistochemical detection. Ongoing research in our laboratory has isolated the seminal plasma protease mRNA from the deferent duct (data unpublished), thus determining protease production within this tissue. In contrast, mRNA could not easily be extracted specifically from the distal efferent duct due to the relatively small amount of efferent ductal tissue surrounded by the many other ducts within the epididymal

region of the testis. Thus, without further experimentation, no conclusion can be discerned as to whether the epithelium of the distal efferent duct produces the protease or has absorbed it from retrograde fluid movement within the reproductive tract.

As a rule of thumb, mammalian seminal plasma proteases play a role in semen liquefaction, or coagulation [3]. Neither of these processes occurs in birds. Although sperm maturation is known to occur during sperm passage through the excurrent ducts of the testis [35], it is unlikely that the protease plays a significant role in the maturation of galliform sperm because to date it has only been found in turkey seminal plasma [11]. Another possible biological role might be that of an antimicrobial agent such as seminalplasmin found in bull seminal plasma [39]. Even though it is well known that proteolytic enzymes play a significant role in extracellular signal cascades [40], it is difficult to envision such a role in this instance. In conclusion, having identified the epithelia of the distal efferent duct and the deferent duct as the most likely source of the turkey seminal plasma serine protease, the elucidation of its biological role will require additional studies.

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