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Abstract

Mass spectrometric analysis of a prion protein (PrP)-containing complex isolated from ram cauda epididymal fluid revealed a protein that showed homology to a carboxylesterase-like protein previously identified in cat urine (cauxin). Using anticauxin antibodies, immunoreactive bands were detected in corpus and cauda epididymal fluid from all mammals tested (ram, boar, mouse, and cat). In the ram, the protein was also present in seminal fluid but not found to be associated with sperm. The bands reacting with the anti-cauxin antibody coincided with those having esterase activity in a zymographic assay and its levels paralleled the esterase activity of native epididymal fluids. A partial nucleotide sequence of 1143 bp, corresponding to 380 amino acids, was obtained by RT-PCR amplification from total RNA from the corpus epididymis (zone 6). The deduced protein sequence shows a high degree of homology (up to 90%) with the different cauxin proteins found in databases but only up to 60% with other known carboxylesterases. By PCR, strong mRNA expression was found in the corpus and cauda epididymis, while the testis, kidney, and caput epididymis had low expression. No mRNA was detected in the lung, heart, or liver. These data demonstrate that an epididymal form of the cauxin enzyme is secreted into mammalian epididymal fluid. In the ram, it is associated with a high molecular-weight PrP-associated complex and may be responsible for the majority of the esterase activity in the cauda epididymal fluid of this species.

Keywords

like, form, enzyme, fluids, present, reproductive, male, mammalian, active, cauxin, carboxylesterase, epididymal, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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An Epididymal Form of Cauxin, a Carboxylesterase-Like Enzyme, Is Present and Active in Mammalian Male Reproductive Fluids¹

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ABSTRACT

Mass spectrometric analysis of a prion protein (PrP)-containing complex isolated from ram cauda epididymal fluid revealed a protein that showed homology to a carboxylesterase-like protein previously identified in cat urine (cauxin). Using anti-cauxin antibodies, immunoreactive bands were detected in corpus and cauda epididymal fluid from all mammals tested (ram, boar, mouse, and cat). In the ram, the protein was also present in seminal fluid but not found to be associated with sperm. The bands reacting with the anti-cauxin antibody coincided with those having esterase activity in a zymographic assay and its levels paralleled the esterase activity of native epididymal fluids. A partial nucleotide sequence of 1143 bp, corresponding to 380 amino acids, was obtained by RT-PCR amplification from total RNA from the corpus epididymis (zone 6). The deduced protein sequence shows a high degree of homology (up to 90%) with the different cauxin proteins found in databases but only up to 60% with other known carboxylesterases. By PCR, strong mRNA expression was found in the corpus and cauda epididymis, while the testis, kidney, and caput epididymis had low expression. No mRNA was detected in the lung, heart, or liver. These data demonstrate that an epididymal form of the cauxin enzyme is secreted into mammalian epididymal fluid. In the ram, it is associated with a high molecular-weight PrP-associated complex and may be responsible for the majority of the esterase activity in the cauda epididymal fluid of this species.

epididymis, male reproductive tract, sperm maturation

INTRODUCTION

In mammalian species, maturation of sperm in the epididymis is essential in order for them to gain the ability to exhibit coordinated motility and to fuse with and fertilize an egg. The epididymal duct can be subdivided into three gross morphological regions: the caput, corpus, and cauda epididymis. The caput and corpus epididymis are the key regions for the posttesticular changes that enable this maturation. These

physiological changes are linked to the sequential interaction of the sperm with its surrounding media and, in particular, the proteins present in the epididymal lumen [1–3]. Proteomic analyses of the epididymal fluid from different mammals (including the mouse, boar, ram, rat, and stallion) have demonstrated that many of the main proteins are present in each of these species; however, they have a different distribution pattern along the organ [4–7]. Once epididymal maturation is complete, sperm are stored in a quiescent state in the cauda epididymis, before being ejaculated. The cauda fluid is particularly well equipped to protect these sperm from different types of physical (such as bacteria) and biochemical (such as oxidation) aggressions [8].

Recently, we have demonstrated that a soluble form of the prion protein (PrP) is present in the cauda epididymal fluid of rams and, in an attempt to purify this protein, we found that it was part of a high molecular-weight complex [9]. The biochemical analysis of the proteins present in this complex indicated that the majority (including the prion protein) had hydrophobic properties and one of the main proteins showed sequence similarities and immuno-cross-reactivity to a recently described carboxylesterase named cauxin (for carboxylesterase-like urinary excreted protein; gene symbol *CES7*) [9, 10]. Cauxin, a novel member of the carboxyl/cholinesterase multi-gene family, is secreted by the kidneys and is present in large quantities in the urine of male cats [10]. It appears to be a member of a new group of carboxylesterases present in body fluids. Here we demonstrate that this enzyme is not restricted to the kidney but is also present and enzymatically active in the epididymal fluid of various mammals, including the cat. Possible roles for this protein and its association with a PrP-associated high molecular-weight complex in the male reproductive fluid are discussed.

MATERIALS AND METHODS

Reagents and Antibodies

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Sigma, Saint Quentin Fallavier, France). The anti-*CES7* antibodies used were raised against the full-length protein purified from cat urine (anti-*CES7*) and against a C-terminal linear peptide of the deduced cat amino acid sequence (anti-*CES7*-CT), as described previously [10].

Biological Samples

Experiments on animals were conducted according to the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction. Epididymal tissues sections were collected from adult Ile-de-France and Romanov rams, boars, black-56 mice, and castrated cats. The zonation of the ram and boar epididymis used in this study was the same as that described previously [4, 11, 12] i.e., zones 0–4 (caput region), zones 4/5–7 (corpus region), and zones 7–9 (cauda region). The

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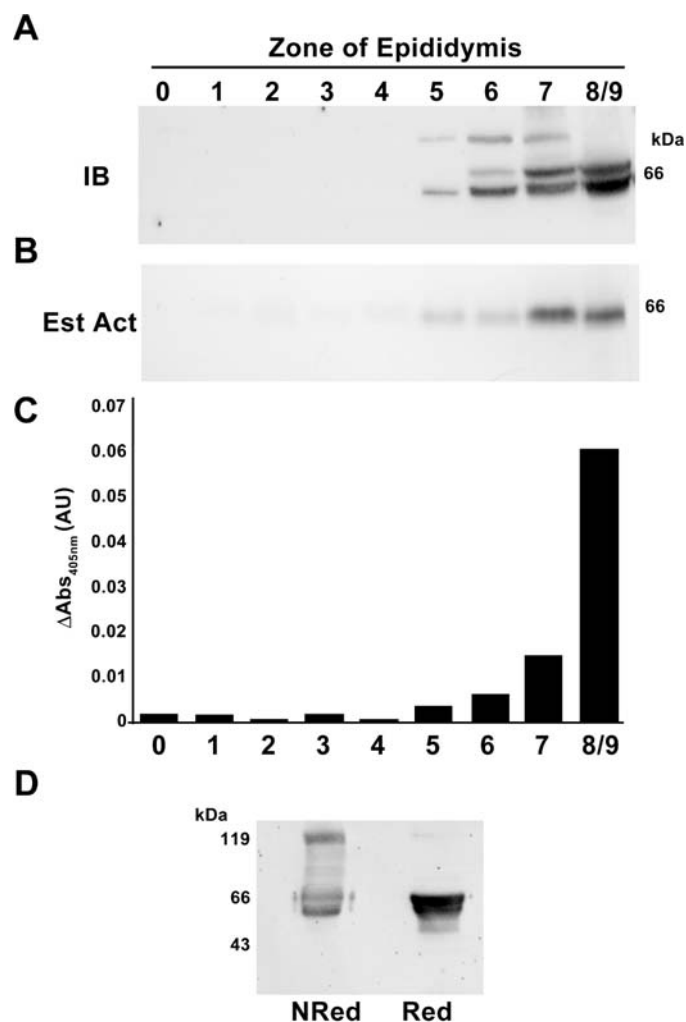


FIG. 1. The expression and activity of CES7 in the epididymal fluid of the ram. **A)** Fluids from different zones of the epididymis (0–9) were collected, the proteins resolved by SDS-PAGE under nonreducing conditions, and immunoblotted using the anti-CES7 full-length antibody. **B)** The in-gel esterase activity against 1-naphthylacetate of these same proteins resolved by SDS-PAGE under nonreducing conditions. **C)** The relative esterase activity of native fluids collected from each zone of the epididymis was tested by using *p*-nitrophenylacetate. The change in absorbance at 405 nm was monitored for 3 min and is reported in arbitrary units (AU). **D)** Fluid from zone 7 was collected, the proteins resolved under nonreducing (NRed) or reducing (Red) SDS-PAGE conditions, and then immunoblotted using the anti-CES7 full-length antibody. The results shown are from the one animal and are representative of three independent experiments. IB, Immunoblot; Est Act, esterase activity assay.

mouse and cat epididymidis were divided into the three gross morphological regions; the caput, corpus, and cauda epididymis. For the ram epididymal tissue homogenates, sections from each zone were prepared by first flushing them free of the luminal contents (sperm and epididymal fluid) by microperfusion of the tubule (see below). These ram tissues and nonflushed mouse and cat epididymal tissues were then minced in lysis buffer (2% w/v SDS, 10 mM Tris-HCl, pH 7.4) containing a protease inhibitor cocktail (Sigma, Saint Quentin Fallavier, France), and 2 mM phenylmethyl sulfonyl fluoride (PMSF). The samples were centrifuged at $45\,000 \times g$ for 20 min and the supernatant collected, centrifuged again at $45\,000 \times g$, and then stored at -20°C . Samples were mixed with an equal volume of reduced gel sample buffer, such that the final concentration of 2-mercaptoethanol was 2.5% (v/v), and then heated (95°C , 5 min) before loading onto gels.

Epididymal fluid was collected from rams, boars, mice, and cats by microperfusion, as described previously [13]. Ram-ejaculated sperm and seminal plasma were collected with an artificial vagina. Spermatozoa were separated from the fluid by centrifugation ($5000 \times g$, 10 min, 4°C), the fluid collected and centrifuged again ($15\,000 \times g$, 10 min, 4°C). The fluids were then

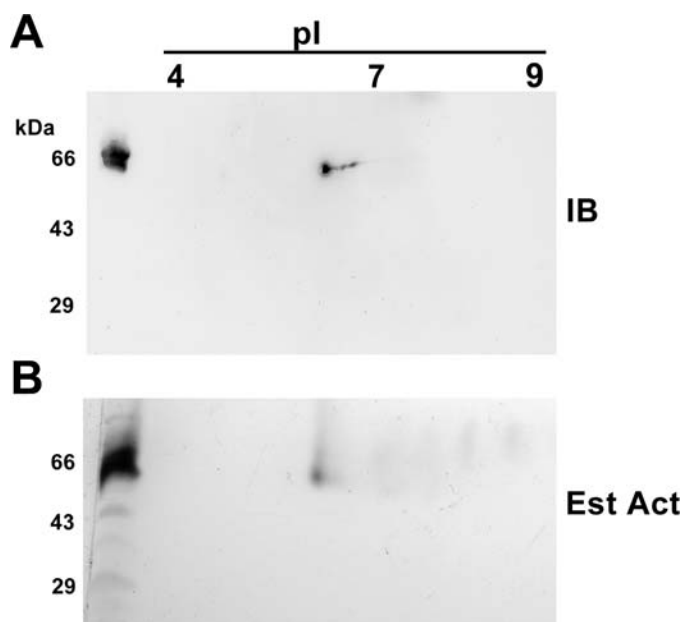


FIG. 2. Two-dimensional PAGE of ram cauda epididymal fluid performed under nonreducing conditions. Following separation, the gels were either immunoblotted against the anti-CES7 full-length antibody (**A**) or the esterase activity of the proteins assessed using 1-naphthylacetate (**B**). The left-hand side of each gel shows a one-dimensional SDS-PAGE separation of the fluid proteins under nonreducing conditions. IB, Immunoblot; Est Act, esterase activity assay.

stored at -20°C . In some experiments, ram cauda epididymal fluid (zones 8 and 9) and seminal plasma was ultracentrifuged at $45\,000 \times g$ for 2 h at 4°C to pellet the membrane vesicles (epididymal and seminal plasma exosomes, EPS and SMS, respectively), as already described [14, 15]. Samples were mixed with an equal volume of reducing sample buffer, such that the final concentration of 2-mercaptoethanol was 2.5% (v/v), and then heated (95°C , 5 min) before loading onto gels.

Gel Electrophoresis and Immunoblotting

The methods of isoelectric focusing and SDS-PAGE were the same as those described previously [4]. Before loading onto gels, samples were mixed with nonreducing or reducing sample buffer. SDS-PAGE was conducted on 6–16% gradient gels and proteins semidry transferred to nitrocellulose over 2 h at 0.8 mA/cm^2 . All membranes were stained with Ponceau-S to visualize the proteins on the membrane and ensure their equal loading, rinsed in TBS containing 0.05% Tween (TBS-T), and then blocked with 3% BSA for 1 h at room temperature. The membranes were rinsed in TBS-T and incubated in TBS-T containing 1% BSA and primary antibody (anti-CES7 and anti-CES7-CT: 1:5000) overnight at 4°C . The blots were washed four times (10 min) with TBS-T, incubated in goat anti-rabbit secondary antibody (1:5000, Sigma) conjugated to peroxidase, and then washed again in TBS-T. The labeled proteins were detected using a chemiluminescent substrate according to the manufacturer's instructions (West-dura, Pierce, Rockford, IL) and the images recorded on an image analysis station or exposed to film. No reactive bands were found when the membranes were incubated with the secondary antibodies alone (data not shown).

Enzyme Assays

Esterase-based enzyme assays were performed on the fluid collected from various zones along the epididymis from a number of different species. Esterase activity assays against *p*-nitrophenylacetate were conducted in 50 mM Tris-HCl (pH 8.0), as previously described [16]. Ten microliters of native fluid were used for each sample and the change in absorbance at 405 nm monitored every 30 sec for 3 min. The esterase activity was corrected for total protein content (determined using the Bio-Rad protein assay kit) and is reported in arbitrary units. In-gel esterase activity was assayed by separating the fluids on SDS-PAGE gels under nonreducing conditions. For zymography, nonreduced samples were not heated. After electrophoresis, the gels were washed one time

for 1 h in PBS (pH 6.5) containing 1% Triton X-100, and then twice for 20 min in PBS (pH 6.5). The gels were then placed into the same buffer containing 1-naphthylacetate (5 mM) and Fast Blue RR (0.4 mg/ml). Proteins with esterase activity were visualized through the formation of a black, insoluble complex consisting of 1-naphthol and diazotized Fast Blue RR [17].

Protein Deglycosylation

The proteins present in reducing sample buffer were deglycosylated by adding 2% (w/v) CHAPS, 5 mM EDTA, and 5 U of N-glycosidase F and incubating for 2 h at 37°C. The reaction was stopped by heating the sample for 5 min at 95°C. Negative controls were conducted by replacing the N-glycosidase F in the reaction mix with an equal volume of H₂O.

Mass Spectrometry Amino Acid Sequence Analysis

The identification of protein spots from two-dimensional gels were obtained by mass spectrometry. The Coomassie blue-stained spots were cut with a sterile scalpel blade into small blocks. The blocks were rinsed, then reduced and alkylated with iodoacetamide and incubated overnight at 37°C in a microtube with 12.5 ng/μl of trypsin (sequencing grade; Roche, Meylan, France) in 25 mM NH₄HCO₃. The tryptic fragments were extracted, dried, reconstituted with 0.1% formic acid, and sonicated for 10 min. Tryptic peptides were analyzed either directly by MALDI (M@LDI-L/R; Waters Micromass, Manchester, U.K.) or sequenced by nano-LC-MS/MS (Q-TOF-Global equipped with a nano-ESI source; Waters Micromass) in automatic mode. The peptides were loaded on a C18 column (Atlantis dC18 3 μm 75 μm × 150 mm NanoEase; Waters) and eluted with a 5–60% linear gradient with water/acetonitrile 98/2 (v/v) containing 0.1% formic acid in buffer A and water/acetonitrile 20/80 (v/v) containing 0.1% formic acid in buffer B in 30 min at a flow rate of 180 nl/min. The peptide masses and sequences obtained were used to manually blast the current databases (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_blast.html).

RNA Extraction, Reverse Transcription, and PCR Amplification

Total RNA was prepared from 200 mg of frozen tissue using RNeasy according to the manufacturer's instructions (Eurobio, Les Ulis, France). The RNA quality and concentrations were assessed using agarose gel electrophoresis and spectrophotometric readings. First-strand cDNA synthesis was performed on 2 μg RNA that had been primed with 50 μM oligo-dT in the presence of 10 mM dithiothreitol, 1 mM dNTP, 0.5 U RNasin, and 50 U Superscript-RT enzyme (Invitrogen, Cergy Pontoise, France) for 1 h at 42°C. PCR amplification was conducted on the cDNA product with 200 μM dNTP, 100 pmoles of 5' primer, 100 pmoles 3' primer, and 0.2 U Taq DNA-polymerase (Invitrogen). PCR cycles of 94°C for 40 sec, 55–60°C for 40 sec (depending on the primers used), 72°C for 40 sec were performed, with a final extension at 72°C for 5 min. Primers used in these PCR reactions were CES71: F; 5'-GTGGCCATCCTGCCTTTA-3' and R; 5'-CCGGTCCGAGCAAAGTT-3'. CES72: F; 5'-CGAAGACTGCCTGTACCTTA-3' and R; 5'-CCACTCTCATGATGGCTTT-3'. CES73: F; 5'-CGCTTCTCTTCTCTGTGG-3' and R; 5'-CGAGTCAGCAGGAGGATA-3'. They were designed from the conserved carboxylesterase motif found in the CES7 sequence already present in the database (EDCYL) [10] and the translation of two amino acid peptide sequences obtained by mass spectrometry that matched the CES7 protein sequence [9] (see Fig. 4). Using combinations of these primers, RT-PCR was performed on mRNA extracted from zone 6 of the epididymis, as this epididymal zone was found to be highly immunoreactive with the anti-CES7 antibody (see Results). PCR products were either directly sequenced or sequenced after amplification and selection by cloning in PCR2.1 vector (Invitrogen). Two nucleotide sequence fragments were obtained (405 and 155 bp). Using these fragments, we designed a new set of specific primers to amplify the bridging mRNA sequence. PCR using these primers produced a 778-bp product, and these primers were used to investigate the expression of CES7 mRNAs in other tissues.

For the comparison of mRNA expression in different tissues, the amplification (20 PCR cycles) of a 232-bp product of actin (gene symbol *ACTB*) was performed and the quantity of cDNA added for the PCR reaction adjusted such that equal levels of cDNA were amplified for all samples (*ACTB* primers: F; 5'-GGACTTCGAGCAGGAGATGG-3' and R; 5'-GCACCGTGTGGCGTAGAGG-3'). The RT-PCR products were subjected to electrophoresis in 1.5% agarose gels and the specificity of the fragment amplified with the primers was confirmed by DNA sequencing (not shown). Both the no-template and no-reverse transcriptase controls were performed on the RNA extracted from zone 6 of the epididymis.

GAA GAC TGC CTG TAC CTT AAC ATC TAT GCC CGG GCC CAT GCA GAA ACC GGC TCC AAG CTC	60
E D C L Y L N I Y A P A H A E T G S K L	20
CGG GTC ATG GTG TGG TTC CCC GGC GGC GCC TTC GAG ACT GGC TCA GCA TCC ATC TTC GAT	120
P V M V W F P G G A F E T G S A S I F D	40
GGG TCC GCC CTG GCT TCC TAT GAG AAT GTG CTG GTT GTG ACC ATC CAG TAC CGA CTA GGA	180
G S A L A S Y E N V L V V T I Q Y R L G	60
ATA TTT GGC TTC TTC AAC ACA GGG GAC GAG CAC GCG CGG GGG AAC TGG GCC TTC ATG GAC	240
I F G F F N T G D E H A R G N W A F M D	80
CAG GTG GCC GCT CTG GTC TGG GTC CAG GAA AAC ATT GAG TTC TTC GGT GGG GAC CCA CGC	300
Q V A A L V W V Q E N I E F F G G D P R	100
TGC GTG ACC ATC TTC GGC GAG TCA GCA GGA GCG ATA AGT GTT TCC AGC CTT ATT CTG TCC	360
C V T I F G E S A G A I S V S S L I L S	120
CCC ATG ACC AAA GGC TTA TTC CAC AAA GCC ATC ATG GCG AGT GGG GTG GCC ATC ATC CCT	420
P M T K G L F H K A I M A S G V A I I P	140
TAC CTG AAG GCC TCT GAT TAC GAG AGG AAT GAT GAT TTG CAG ACA ATT GCA AGT ATT TGT	480
Y L K A S D Y E R N D L L Q T I A S I C	160
GAC TGC AAC GCA TCA GAC TCC GTG GCC CTG CTG CAG TGC CTG CGG GCA AAG TCC TCC GAG	540
D C N A S D S V A L Q C L R A K S S E	180
GAG CTG CTG AGC ATC AGC CAG AAA ACC AAG TCT TTC ACT CGA GTG GTT GAT GGC CTT TTC	600
E L L S I S Q K T K S F T R V V D G L F	200
TTT CCT AAT GAG CTG CTA GAC CTG TTG GCT CAA AAA TTA TTT CAT CTG GTT CCT TCC ATC	660
F P N E L L D L L A Q K L F H L V P C S I	220
ATC GGA GTC AAT AAT CAC GAG TGT GGC TTC CTG CTA CGG ATG AAG GAG TTT CCT GAG ATC	720
I G V N N H E C G F L L P M K E F P E I	240
CTC GGG GGC TCC AAC AAG TCC CTT GCC CTG CAA CTG ATA CAC TCA GTC CTG CAC ATC CCT	780
L G G S N K S L A L Q L I H S V L H I P	260
GTC CAG TAT TCA TAC CTT GTG GCT GAT GAA TAT TTC CAC AAC AAG CAC TCT CTG CTT GAT	840
V Q Y L V A D E Y F H N K H S L L D	280
ATA CGA AAT CGC TTC CTG GAT TTA CTT GGA GAT GTG TTC TTT GTG GTC CCT GGG CTG GTC	900
I R N R F L D L L G D V F F V V P G L V	300
ACA GCT CAA TAT CAC ACA GAT GCT GGT GCC CCT GTC TAC TTC TAT GAG TTT CAG CAT CGG	960
T A Q Y H T D A G A G V Y E F Q H R	320
CCC CAG TGC TTG AAG GAC AGG AAG CCA CCT TTT GTC AAA GCT GAT CAC ACT GAT GAA ATC	1020
P Q C L K D R K P P F V K A D H T D E I	340
CGC TGT GTC TTT GGA GGT GCC TTC CTG AAG GGC AAC ATT GTC ATG TTT GAA GAA GCC ACA	1080
R F V F G G A F L K G N I V M F E E A T	360
GAG GAA GAG AAA GCG CTG AGC AGG AAG ATG ATG AGA TAC TGG GCT AAC TTT GCT CGG ACC	1140
E E E K A L S R K M R Y W A N F A R T	380

FIG. 3. Partial nucleotide and deduced amino acid sequence of the ovine epididymal CES7. The carboxylesterase family protein motif (EDCLY) and conserved GX SXG active site motif are underlined. Sequences corresponding to the specific primers used to generate this partial nucleotide sequence are underlined.

RESULTS

Cauxin Is Present and Active in the Epididymal Fluid of the Ram

Our previous study [9] employed mass spectrometry to identify proteins associated with the soluble prion protein in the ram cauda epididymal fluid. It revealed several peptides that matched with CES7, a carboxylesterase-like protein recently found in high quantities in cat urine [10]. We further investigated the presence of this protein in ram epididymal fluid using the anti-CES7 antibody raised against the cat urinary protein. We observed that, under nonreducing conditions, immunoreactive bands were present from the mid-corpus to the cauda epididymis (zones 5–9), the latter having the highest levels of immunoreactivity suggesting an accumulation in this zone (Fig. 1A). The pattern of bands detected varied between these fluids with a protein of 60 kDa being detected in zone 5, an additional 70-kDa band being detected in zones 6–9, and a band at >120-kDa being also detected in zones 5–7. A zymographic esterase assay showed that, in fluid from zones 5–9, only 1 major protein at 60 kDa had esterase activity capable of hydrolyzing 1-naphthylacetate (Fig. 1B). This esterase activity was confirmed by in vitro assay using *p*-nitrophenylacetate and native fluids from the ram epididymis (Fig. 1C), where the highest levels of activity were found in zones 7–9, lower levels being measured in zones 5 and 6, and little or no activity being detected in more proximal sites. When reducing SDS-PAGE was conducted on the ram cauda epididymal fluid (zone 7), only the 70- and 60-kDa bands were

dog	1	MSGEWGHLGQTLIWAIVWLAAATEGPA-ADAPVRSTRLGWVRGKQATVLGSTMVNVFLGIPFAAPPLGPLRFKRPKPA
cat	1	MSGMWVHFGRTLIWALWVLAIVIKGPA-ADAPVRSTRLGWVRGKQATVLGSTVPVNMFLGIPFAAPPLGPLRFKRPKPA
bovine	1	MSGEWVHFGQTLIWAIVWLAAATKGPATADAPARHTNLGWVQGTQASVLGNDMLVNVFLGVFPFAAPPVGLPFRFAKPELL
sheep	1	-----
human	1	MSGNWWHFGQILIAIIVWLAAPTKGPS-AEGPQRNTRLGWLQGGKQVTVLGSVPVNVFLGVFPFAAPPLGSLRFTNBPQAS
mouse	1	MSGDWVRPGQALIWVIFGAIIEGSV-TEEPHRYTKLGWVQGGKQATVLGRLEPVPNVFLGIPFAAPPLGPLRFKRPKPI
rat	1	MSGDWVHTGQALIWVWLIFPAIIRGSV-TEEPHRYTRLGWVQGGKQATVLGRLEPVPNVFLGIPFAAPPLGPLRFKRPKPI
consensus	1	msgewvhpgq liwavvlla kgp adap r trlgwvqgkqatvlg mpvvnvflgipfaapplgplr p p l
dog	80	LWNDSRDATSYPKLCLQNSVWLLSDQHFLKVHYPNLEVSEDCLYLNIYAPAHANTGSKLPVMVWVFPFGAFETGSASIFDG
cat	80	PGNDFRNATSYPKLCFQDLEWLVSYQHVLKVRYPKLEASEDCLYLNIYAPAHADNGSNLPVMVWVFPFGAFKMGSSSFDG
bovine	81	PWDGFLNATSYPKLCFQNSEWLFDTDQHILKVHYPKFRVSEDCLYLNIYAPAHAEFGSKLPVMVWVFPFGAFETGSASIFDG
sheep	1	-----EDCLYLNIYAPAHAEFGSKLPVMVWVFPFGAFETGSASIFDG
human	80	PWDNLREATSYPNLCLQNSEWLLLDQHMLKVHYPKFGVSEDCLYLNIYAPAHADTGSKLPMVWVFPFGAFKMGSSSFDG
mouse	80	PWDNLREATSYPNLCFQDLEWLFYQNLKVSYPILGSEDCLYLNIYAPAHADNGSNLPVMVWVFPFGGFGTGSASIFDG
rat	80	PWDHLREATSYPNVCFQDLEWLFYQNLKVHYPKLGVSEDCLYLNIYAPAHADNGSNLPVMVWVFPFGGFGTGSASIFDG
consensus	81	pw rdatsyp lcfqn ewl qhvlkvhyphkl vsEDCLYLNIYAPAHAdtGSkLPvmvWfPFGaFetGSASiFDG
dog	160	SALAAYEDVLIVTTQYRLGIFGFFKTDGDHAPGNWAFLDQLAALTWVQENIEFFGGDPHSVTIFGESAGAISVSGLWLSP
cat	160	SALAAYEDVLIVTTQYRLGIFGFFDTGDEHARGNWALLDQVAALTWVRDNIIEFFGGDPRSVTIFGESAGAISVSGLWLSP
bovine	161	SALASYEDVLVTTIYRLGIFGFFNTGDEHARGNWAFMDQVAALIWVQENIEFFGGDPRCVTIFGESAGAISVSGLWLSP
sheep	42	SALASYENVLVTTIYRLGIFGFFNTGDEHARGNWAFMDQVAALWVQENIEFFGGDPRCVTIFGESAGAISVSGLWLSP
human	160	SALAAYEDVLVTVVQYRLGIFGFFTTWQDHPGNWAFMDQVAALSWVQKNIEFFGGDPRSVTIFGESAGAISVSGLWLSP
mouse	160	SALAYEDVLVTTIYRLGIFGFFTTQNHAPGNWAFMDQLAALLWVRENINIEFFGGNPDSTVIFGNSAGAISVSGLWLSP
rat	160	SALAAYEDVLVTTIYRLGIFGFFNTQNHAPGNWAFMDQLAALQWVRENINIEFFGGNPDSTVIFGNSAGAISVSGLWLSP
consensus	161	SALAaYedVLvvtiQYRLGIFGFF TgdqHapGNWafldQvAAL WvqenIefFGGdPhsVTIFGeSAGAIsvSsLiLSP
dog	240	MASGLFHKAIMESGVAIIP-FLRAPDDERNEEDLOVIARICGCNVSDSVALLOCLRAKSSSEELLdINKKTSFTRVVDGFF
cat	240	IANGLFHKAIMESGVAIIP-ELLMRPPGDERKDLQVLARICGCHASDSAAALLQCLRAKSPSEELMDISKKLTFSIPVDDFF
bovine	241	MTKGLFHKAIMESGVAIIP-YLKAPDYERNDDLOTIASICDCASDSIALLOCLRAKSSKELLSTINKKTSFTRVVDGLF
sheep	122	MTKGLFHKAIMESGVAIIP-YLKASDYERNDDLOTIASICDCASDSVALLOCLRAKSSKELLSTINKKTSFTRVVDGLF
human	240	MAKGLFHKAIMESGVAIIP-YLEAHDYEKSEDLQVVAHFCGNASDSEALLRCLRTKPSKELLTISQKTSFTRVVDGAF
mouse	240	LSADLFHRAIMOSGVAIIP-SLKSSDNDLKHDLQVVAHFCGNVSDSKALLCLREKSSLELLSLSQKAKSFTRVVDGSF
rat	240	LSAGLFHRAIMOSGVAIIP-SLKNFDDLEKHGLQVVAHFCGNVSDSKVLLKCLREKSSLELLSLGQKAKSFTRVVDGSF
consensus	241	mt gLFHkAIMeSGVAiIp flka d er edLQviA iC cnaSDS aLLqCLRaKsS ELIsisqKtksftrVvDg F
dog	319	FPDEPLDLLTEKTFNSIPSVIGVNNHECGFLPMKEFPEILGSGNKSALHLIHRVLHIPNOYLVLVADQYFYFNKHSVPVE
cat	320	FPDEPVALLTQKAFNSVPSIIGVNNHECGFLPST-EFSEILGGSNRSLALYLHVTFLNIPTQYLHLVADHYFYFNKHSVPVE
bovine	320	FPNEPLDLLAQKLFHLVPSIIGVNNHECGFLPMKEFPEILWGSNKSALQLIHSVLHIPVOYLVLVANEYFHNKHSVLVD
sheep	201	FPNEPLDLLAQKLFHLVPSIIGVNNHECGFLPMKEFPEILGSGNKSALQLIHSVLHIPVOYSLVLADEYFHNKHSLLD
human	319	FPNEPLDLLSQKAFKAIPSIIGVNNHECGFLPMKEAPEILGSGNKSALHLITQNLHIPPOYLHLVANEYFHDKHSLTE
mouse	319	FSEEPLELLSQKTLKIVPSIIGVNNQECGYLIPVRDTPAILGSESTALTILHTLLHIPTQHYLIVTKEYFHKHSPTD
rat	319	FPEEPMELLSQKTFKTVPISIIGVNNQECGYLIPMREAPEILGSGNESTALTILHVLHIHIPQYMHIVAKDYFHKHSITD
consensus	321	FpdEpldLLtqk fh vPSiIGVNNHECgflPmkefPeIL GSNkslAl LihtvLhIP QylYlVa eYFhnKHSlvd
dog	399	IRDSFLDLLGDVFFVVPGLVTARYHRDAGAPVYFYEFQHPPECLKDRPAFVKADHSDAIRFVFGGAFLKGNIVMFEAT
cat	399	IRDSFLDLLGDVLFVVPGLVTARYHRDAGAPVYFYEFQHPQCLNDTRPAFVKADHSDAIRFVFGGAFLKGDIVMFEAT
bovine	400	IRNRFLDLLGDVFFVPLGLVTAQYHTDAGAPVYFYEFQHRPQCLKDRKPSFVKADHTDEIRFVFGGAFLKGNIVMFEAT
sheep	281	IRNRFLDLLGDVFFVVPGLVTAQYHTDAGAPVYFYEFQHRPQCLKDRKPPFVKADHTDEIRFVFGGAFLKGNIVMFEAT
human	399	IRDSFLDLLGDVFFVVPGLVTARYHRDAGAPVYFYEFQHRPQCFEDTKPAFVKADHDAEVRFVFGGAFLKGDIVMFEAT
mouse	399	IRDTLLDLLGDVFFVVPGLVTARYHRDAGPVYFYEFQHRPQCFQNSRPAFVKADHTDEIRFVFGGFLEKGDVVMFEAT
rat	399	IRDTLLDLLGDVFFVVPGLVTARNHRDAGPVYFYEFQHRPQCFQNTSRPAFVKADHTDEIRFVFGGFLEKGDVVMFEAT
consensus	401	IRdsfLdLLGDVffVvPglvTArYHrDagaPVYFYEFqHrPqCl dtrPaFvKADhtDeiRFVFGGaFLkGdiVMFEaT
dog	479	EEKLLSRKMMRYWANFARTGDPNGEGLPLWPAYSQSEQYLKLDLNISSVGQKLKEQEVFWSDTPLIMSMSTAPPGPV
cat	479	EEKLLSRKMMRYWANFARTGDPNGEGLPLWPAYTQSEQYLKLDLSVSVGQKLKEQEVFWMNTIIP-----
bovine	480	EEKGLSRKMMRYWANFARTGNPNKGGLPLWPAYRQSEYLLQLDLNISSVGQRLKELELKFWTETPLIMMTSSGATLASLS
sheep	361	EEKALSRKMMRYWANFART-----
human	479	EEKLLSRKMMRYWANFARTGNPNNGNDLSLWPAYNLTEQYLLQLDLNMSSGQRLKEPRVDFWSTSTPLILSASDMLHSPLS
mouse	479	EEKLLSRKMMRYWANFARSGDPNGADLPWPVYDENEQYLEDLVNISTGRRLKDRVEFWTDPLPLILSASKALLSPTF
rat	479	EDEKLLSRKMMRYWANFARSGDPNGDDLPWPAYDQNESYLLKLDVNISTGRRLKDRRVEFWTDPLPLIMSASKALLSPTF
consensus	481	EeKlLSRKMMrYwAnFartgdpng lplwpay qseqyl ldlnisvgqrLke vefwtdtPlims s al p
dog	559	PLLSLSVLPLFLFSSAP
cat	-----	-----
bovine	560	SSTFLFLPLFLFIFSFAP
sheep	-----	-----
human	559	SLTFLSLLQFFFFFCAP
mouse	559	SLILLSLLSPVLLSAAS
rat	559	PLILFSLPLPSLLSIAS
consensus	561	l lsll p l s a

FIG. 4. Sequence alignment between the partial fragment of sheep CES7 and the corresponding fragment from other CES7s. The full-length proteins from human, bovine, dog, cat, mouse, rat, and the partial sequence of sheep CES7 were aligned using clustalw [19] and represented using the box-shade program. The proteins were clustered from their phylogenetic distances.

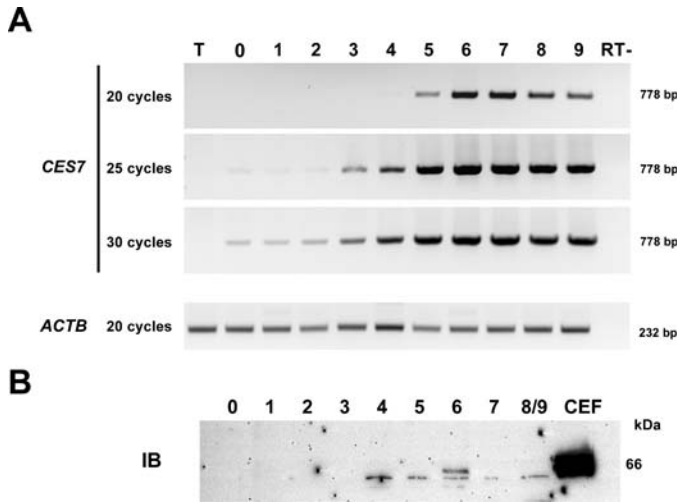


FIG. 5. The expression of *CES7* mRNA and protein in reproductive tissues of the ram. **A**) RT-PCR amplification of mRNA isolated from the testis (T) and epididymal zones 0–9 using *CES7* specific primers for 20, 25, and 30 PCR cycles, and using beta-*ACTB*-specific primers for 20 PCR cycles. RT–, Reverse transcriptase enzyme was omitted from the reaction mix. **B**) Proteins from tissues collected from the different zones of the epididymis (0–9), after flushing them free of luminal contents, were extracted in SDS-lysis buffer, separated by SDS-PAGE, and immunoblotted with an anti-*CES7* antibody. The proteins from cauda epididymal fluid (CEF) were used as a positive control. IB, Immunoblot.

found to be immunoreactive against the anti-*CES7* antibody (Fig. 1D). The >120-kDa band was not detected, suggesting that the protein can form dimers and/or multimers through disulfide bonds.

To confirm the identity of the protein, two-dimensional SDS-PAGE of ram cauda epididymal fluid was performed under nonreducing conditions. Immunoreactivity with the anti-*CES7* antibody (Fig. 2A) superimposed exactly to the same region of the gel that displayed esterase activity against 1-naphthylacetate (60 kDa and pI of approx. 6.5; Fig. 2B). Mass spectrometric analysis showed that tryptic fragments obtained from this spot (60 kDa and pI of approx. 6.5) and another with a more basic pI >9 (not seen on this gel), contained sequences matching either cat *CES7* (Accession NP_001009188) or related *CES7* sequences (human; AAH69501, bovine; XP_591772, mouse; NP_001003951, dog; NP_001003969). These sequences were VSEDCLYLNIYAPAAHAETGSK, LGIFGFFDTGDTGDEHAR, AIMASGVAIIPYLK, SSEELL-SISQK, FVFGGAFLLK, GNIVMFEEATEEEK, YWANFART, LKELELK, ADHTDEIR (see also Fig. 4).

Characterization of the mRNA and the Deduced Amino Acid Sequence of Ovine Epididymal Cauxin

Using PCR primers designed from the conserved carboxylesterase motif and internal amino acid peptide sequences obtained by mass spectrometry, a 1143-bp nucleotide cDNA sequence was amplified from epididymal zone 6 (Fig. 3) (ovine carboxylesterase-like urinary excreted protein; AM075621). After translation, a 380-amino-acid sequence was obtained (Fig. 3). The deduced amino-acid sequence contained well-defined esterase domains, including the carboxylesterase family protein motif (EDCLY) associated with a disulfide bridge present in serine esterases (Fig. 3, underlined) and a conserved GX₂SG active site motif (GESAG) associated with serine hydrolase family members (Fig. 3, underlined). When blasted against other

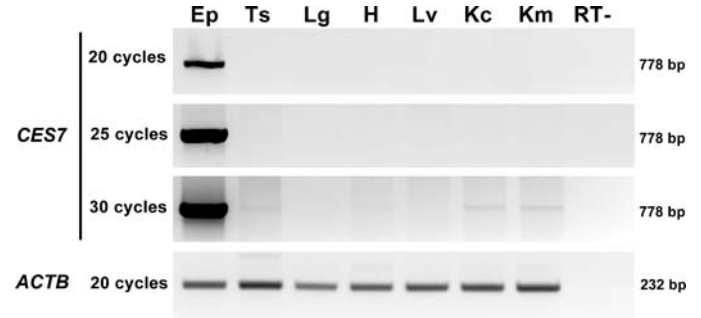


FIG. 6. The expression of *CES7* mRNA in other tissues of the ram. RT-PCR amplification of mRNA isolated from the epididymis (Ep-zone 6), testis (Ts), lung (Lg), heart (H), liver (Lv), kidney cortex (Kc), and kidney medulla (Km) using *CES7*-specific primers for 20, 25, and 30 PCR cycles, and using beta-*ACTB* specific primers for 20 PCR cycles. RT–, Reverse transcriptase enzyme was omitted from the reaction mix.

protein sequences present in the databases [18], this sequence was found to have 95% identity with the bovine *CES7* protein (98% homology), 83% identity with the dog *CES7* protein (91% homology), 80% identity (89% homology) with a putative human sequence, 76% identity (85% homology) with cat *CES7*, and 73% and 72% identity (85% and 83% homology, respectively) with the mouse and rat *CES7* proteins, respectively. The alignment of these sequences [19] is shown in Figure 4, as well

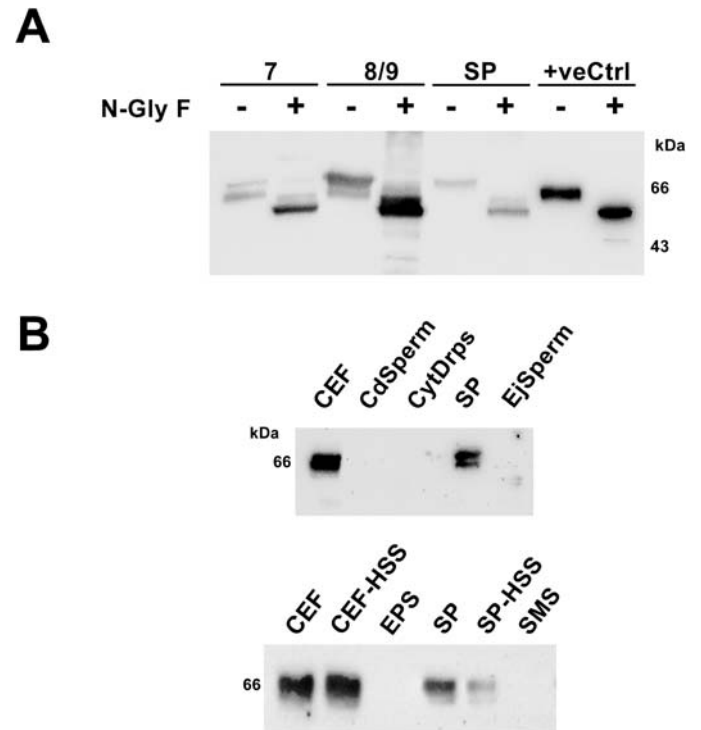


FIG. 7. Biochemical characterization of E-*CES7* in the ram. **A**) E-*CES7* exists in multiple glycosylated states. Fluid proteins from epididymal zones 7–9, seminal plasma (SP), and cat urine (+ve Ctrl) were deglycosylated by treating them with N-glycosidase F. **B**) E-*CES7* is compartmentalized within this fluid. CEF, Cauda epididymal fluid; SP, seminal plasma; CdSperm, cauda epididymal sperm; CytDrps, sperm cytoplasmic droplets; Ejsperm, ejaculated sperm; CEF-HSS and SP-HSS, cauda epididymal fluid and seminal plasma high speed supernatants; EPS and SMS, epididymal and seminal plasma exosomes. For all experiments, the proteins were separated via SDS-PAGE and immunoblotted with the anti-*CES7* full-length antibody.

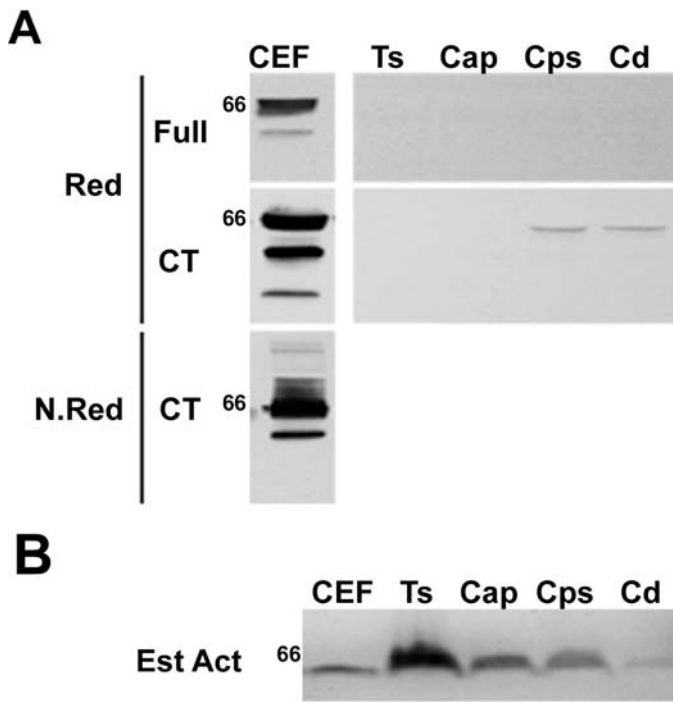


FIG. 8. The expression and activity of CES7 in the epididymal fluid of the cat. **A**) Fluid from the cauda epididymis (CEF) and tissue extracts from the testis (Ts), caput (Cap), corpus (Cps), and cauda (Cd) epididymis were collected, the proteins resolved by SDS-PAGE under reducing (Red) or nonreducing (N.Red) conditions, and immunoblotted using the anti-CES7 full length (Full) or C-terminal (CT) antibody. **B**) The in-gel esterase activity against 1-naphthylacetate of these same proteins. Est Act; esterase activity assay.

as the position of the different peptides obtained from mass spectrometry from the ram epididymal fluid that matched these different protein sequences (Fig. 4, underlined). All these CES7 sequences present the specific features of carboxylesterases such as the S-E-H catalytic triad and the two glycines involved in hydrolysis (S227, E347, H456, and G147-G148 in the consensus sequence, respectively; Fig. 4). The protein blast search also showed that the sheep epididymal protein has high homology (86%) with a crab-eating macaque hypothetical protein (BAB46884) but only 50% identity with other known carboxylesterases. Due to the high level of homology between the protein identified here and the CES7 proteins identified in other species, our data suggest that this protein is the ovine homologue of CES7. To distinguish the epididymal form of the protein from that present in the urine, we refer to the epididymal form as E-CES7.

The Expression of Ovine Cauxin mRNA and Protein in the Ram Reproductive Tract

The expression of CES7 mRNA in the ram reproductive tract was analyzed using RT-PCR. Figure 5A shows that, after 20 cycles of PCR, the 778-bp fragment corresponding to CES7 cDNA was detected in zones 5–9 of the epididymis, with the highest levels in zone 6. Following 25 cycles of PCR, this amplicon was also detected in zones 3 and 4, and after 30 PCR cycles, it was found in all the epididymal zones, although with low intensity in the caput epididymis (zones 0–4) (Fig. 5A). No PCR product was detected when the reverse transcriptase enzyme was omitted from the reaction mix (RT-), or in the no-template control (data not shown). An ACTB amplicon (232 bp),

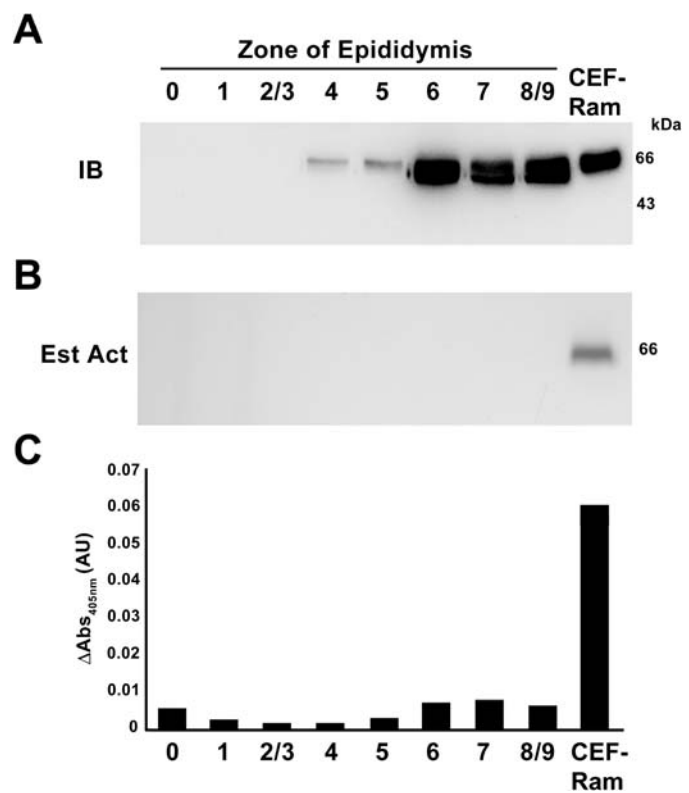


FIG. 9. The expression and activity of CES7 in the epididymal fluid of the boar. **A**) Fluids from different zones of the boar epididymis (0–9) and from ram cauda fluid (CEF-Ram) were collected, the proteins resolved by SDS-PAGE under nonreducing conditions, and immunoblotted using the anti-CES7 full-length antibody. **B**) The in-gel esterase activity against 1-naphthylacetate of these same proteins. **C**) The relative esterase activity of native fluids collected from each zone of the boar epididymis (0–9) and from native fluid from the ram cauda epididymis (CEF-Ram) was tested by using *p*-nitrophenylacetate. The change in absorbance at 405 nm was monitored for 3 min and is reported in arbitrary units (AU). Shown is a representative result from two independent experiments. IB, Immunoblot; Est Act, esterase activity assay.

used as a positive control, demonstrated that nearly equal levels of cDNA were present in the tissue samples. The presence of CES7 protein in the epididymal tissues extracted after micro-perfusion (to remove sperm and fluids) was similar to that found for the mRNA expression (Fig. 5B). A protein band immunoreactive against the anti-CES7 antibody was detected at 55 kDa in zones 4–9. In zone 6, a second band of 60 kDa was also detected. No reactive bands were detected in the more proximal epididymal zones (0–3).

The distribution of ovine CES7 mRNA in other tissues was also investigated. After 30 PCR cycles, the 778-bp mRNA amplicon was readily detected in the epididymal tissue (zone 6), only faintly detected in the kidney and testis, and not detected in the lung, heart, or liver (Fig. 6). Amplification of the ACTB amplicon demonstrated that nearly equal levels of cDNA were present in all the tissue samples.

Biochemical Properties of E-Cauxin

Deglycosylation of the reduced proteins from zones 7, 8/9, and seminal plasma resulted in a reduction in the apparent molecular weight of the bands (Fig. 7A). The main unglycosylated isoform was at 55 kDa and was similar to that present in cat urine (+ve Ctrl). Another unglycosylated isoform (60 kDa) was also detected in each of the reproductive fluids

but not in cat urine. Experiments aimed at determining the compartmentalization of E-CES7 in the male reproductive fluid showed that it is present in the cauda epididymal fluid and in seminal plasma, but it is not associated with sperm or cytoplasmic droplets from these fluids (Fig. 7B) or sperm taken from more proximal zones along the epididymis (data not shown). In both the cauda epididymal and seminal plasma, the protein partitioned into the soluble bulk phase of the fluid and was not associated with the membranous vesicles (EPS and SMS, respectively) present in these fluids (Fig. 7B).

Cauxin Is Present in the Epididymal Fluid of the Cat, Boar, and Mouse

We tested whether CES7 was also present in the male reproductive tract of the cat. Following SDS-PAGE under reducing conditions and immunoblotting with the anti-CES7 antibody, bands of 65 and 50 kDa were detected in the cat cauda epididymal fluid (Fig. 8A). We confirmed the identities of these two proteins by conducting two-dimensional SDS-PAGE on this fluid and excising spots matching to those immunoreactive against this full-length antibody. The protein spots were subjected to mass spectrometric analysis that produced peptide sequences that all matched 100% to the CES7 sequence. The anti-CES7-CT antibody also detected these two proteins in this fluid, as well as another of 40 kDa (Fig. 8A). In addition, this antibody was able to detect low levels of the 65-kDa band in tissue extracts from the corpus (Cps) and cauda (Cd) epididymis. When the cat cauda epididymal fluid was run under nonreducing conditions, the major immunoreactive bands against the anti-CES7-CT antibody were detected at 65 and 50 kDa. A minor band >120 kDa was also immunoreactive against this antibody (Fig. 8A). This anti-CES7-CT antibody did not detect any proteins from fluid collected from sites along the ram or porcine epididymis (data not shown). The in-gel esterase assay showed that cat cauda fluid contains one main protein of 65 kDa capable of hydrolyzing 1-naphthylacetate (Fig. 8B). The testis and epididymal tissue extracts also contained one main protein (>60 kDa) with esterase activity against 1-naphthylacetate, and its levels decreased distally along the tract.

Immunoblotting with the anti-CES7 full-length antibody against nonreduced porcine epididymal fluid resulted in immunoreactive bands being detected in zones 4–9 (Fig. 9A). A single band of 60 kDa was present in zones 4 and 5, and two bands (50 and 60 kDa) were detected in zones 6–9. In contrast with the ram cauda fluid, no in-gel-esterase activity against 1-naphthylacetate was detected in boar epididymal fluid (Fig. 9B). Low levels of esterase activity were detected against *p*-nitrophenylacetate in porcine epididymal fluids, these being at least six times lower than those in ram cauda fluid (Fig. 9C).

We extended these studies to a laboratory rodent. Non-reduced tissue extracts and cauda epididymal fluid from the mouse were probed with the anti-CES7 full-length antibody (Fig. 10A). Faint immunoreactive bands were detected in the corpus and cauda epididymal tissue extracts at 50 and about 70 kDa, respectively. Immunoreactive bands were also detected in the mouse cauda fluid in forms similar to those in the ram cauda fluid (60 and 70 kDa). The in-gel esterase assay showed that mouse cauda fluid contains two proteins of 60 kDa and 70 kDa capable of hydrolyzing 1-naphthylacetate (Fig. 10B). The relative esterase activity against *p*-nitrophenylacetate of native fluid from the cauda epididymis of the mouse was about two times lower than in the ram (0.034 ± 0.001 versus 0.060 ± 0.008 AU, respectively; mean \pm SEM, $n = 3$).

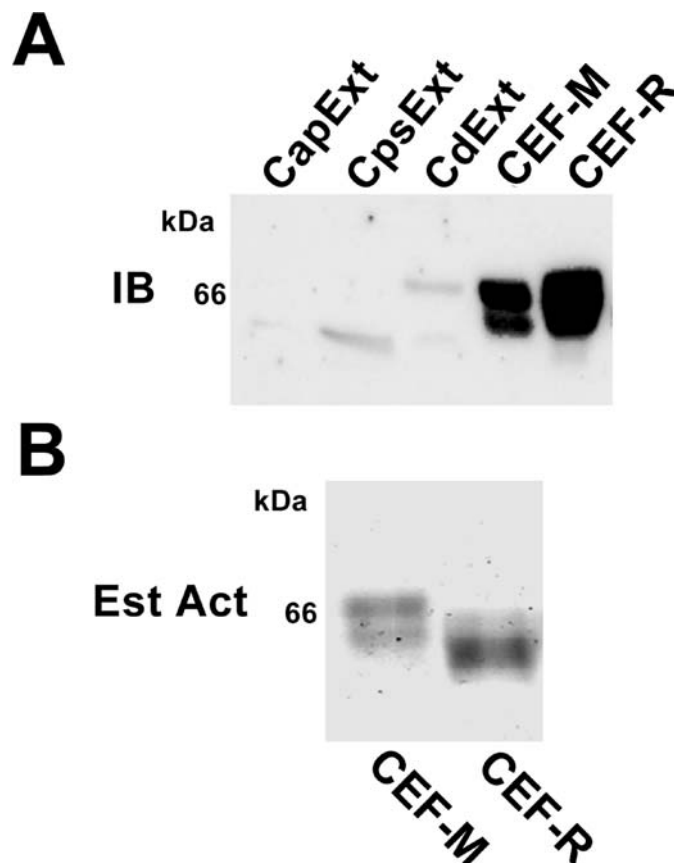


FIG. 10. The expression and activity of CES7 in the epididymis of the mouse. **A**) Proteins from tissues collected from the caput (CapExt), corpus (CpsExt), and cauda (CdExt) and from cauda epididymal fluid of the mouse (CEF-M) and ram (CEF-R) were separated by SDS-PAGE and immunoblotted with an anti-CES7 full-length antibody. **B**) The in-gel esterase activity against 1-naphthylacetate of the proteins in the cauda epididymal fluid. IB, Immunoblot; Est Act, esterase activity assay.

DISCUSSION

Carboxylesterase enzymes, which are members of the serine hydrolase superfamily (carboxyl-ester hydrolases; EC 3.1.1.1), constitute a large group of diverse proteins capable of hydrolyzing xenobiotics containing ester, thioester, or amide groups (for review, see [20]). Most of them are localized to the endoplasmic reticulum and often show tissue-specific and/or hormonal-dependent expression. These enzymes are often glycosylated and may exist in different isoforms, including splice variants. In this article, we have identified and characterized the presence of a carboxylesterase-like protein, which is secreted into the reproductive fluid of the male. We found this enzyme to be a homologue of the CES7 protein previously described in the urine of cats [10]. We refer to the epididymal form of the protein as E-CES7 to distinguish it from the form found in urine. In the ram, the presence of a protein immunoreactive against the anti-CES7 antibody coincided with the detection of esterase activity in the epididymal fluid, and both the immunoreactive protein and in-gel esterase activity against 1-naphthylacetate were located in the same region of a two-dimensional SDS-PAGE gel. Together, these data suggest that E-CES7 is responsible for the majority of the epididymal fluid esterase activity in this species.

The protein described here shares a very high degree of sequence homology with CES7s now identified in the bull,

cat, dog, rodent, crab-eating macaque, and human genomes. This strongly suggests that CES7 and CES7-like proteins are well conserved among mammals and, from our data, are highly expressed in the reproductive tissues. In the human genome, a *CES7* cDNA has been sequenced and the corresponding gene (*CES7*) localized to chromosome 16, in a carboxylesterase cluster with human carboxylesterase 1 (*CES1*) and a gene for a carboxylesterase 4 (*CES4*). However, a putative gene in chromosome 22 (KB-1269D1.1) [21] also corresponds to a carboxylesterase with more than 90% homology to the *CES7* gene, although with an apparently shorter sequence. Whether this second *CES7* gene is translated remains to be demonstrated. As already remarked [10], all these *CES7* protein sequences lack the C-terminal KDEL-like peptide that would localize them to the endoplasmic reticulum membrane and, hence, are likely to be secreted proteins, in agreement with our observations. Analysis of the sequence data also demonstrates that *CES7* proteins form a new, mammalian-specific, carboxylesterase family in the phylogenetic tree of carboxylesterases (see ESTHER data base at http://bioweb.ensam.inra.fr/ESTHER/epic?name=ESTHER_Carboxylesterase&class=Tree) [22].

Cauxin was originally identified in the urine of cats, and its mRNA was found only in the kidney [10]. However, the male reproductive-tract tissues were not included in this study. It is of note that this earlier study found that *CES7* is not expressed in immature cats, indicating that its expression could be related to sexual maturity and possibly hormonally regulated, a property shared by some carboxylesterases [20]. Here we demonstrate that *CES7* is also present within the cauda epididymal fluid of mature cats and is likely to arise due to its synthesis and secretion by the distal epididymal tissue. In the ram, we also found very low levels of *CES7* mRNA expressed in the kidneys; however, we could not detect the protein via immunoblot. This may be because the protein is not expressed or, if so, at very low levels, in the kidney of the ram. The expression of *CES7* proteins in the kidney and epididymis may be explained by their common embryonic origin (i.e., the mesonephric duct). Alternatively, two closely related genes (as in the human) might give rise to two closely related proteins under different regulation.

In the ram, our data strongly suggest that expression of *CES7* is tissue specific because the full-length anti-*CES7* antibody only reacted with one major protein band in tissue extracts from the ram distal epididymis (Fig. 5). It did not react with extracts from other tissues, including the heart, liver, and lung (data not shown), all of which are known to contain various other carboxylesterase enzymes. Because we loaded equal quantities of protein from each epididymal zone, our results indicate that *CES7* accumulates in the cauda epididymis. Our previous studies [6, 23] have found that epididymal spermatocrit and total protein levels are maximal in zones 4–5 and then gradually decrease distally along the duct. It is therefore interpreted that the increase in the luminal concentration of E-CES7 is due to its expression and secretion by the cauda epididymal epithelium.

Epididymal *CES7*s were also identified, by immunoblotting, in the cauda fluids of the boar, stallion (data not shown), and mouse. In regard to the mouse, previous reports have shown that some esterases are testis associated [24] and others are present in the epididymal fluid [25, 26]. At least 1 carboxylesterase (esterase-28) has been reported as showing strict tissue specificity for the mouse epididymis [27] and may be similar to mouse E-CES7 because they both show restricted activity to the cauda epididymis, have similar profiles after SDS-PAGE (i.e., a broad zone of esterase activity and

molecular mass of approximately 60 kDa), and have strong esterase activity toward chromogenic esters.

In the different species studied, we tried to match the zymographic esterase activity of the epididymal fluid with the presence of E-CES7. However, this can be difficult due to the fact that the majority of carboxylesterases have a similar molecular weight of 60–70 kDa [20]. For example, in the cat, although proteins with esterase activity were detected by zymographic assay in the tissue extracts of the testis and epididymis, only the cauda part of this activity may be attributable, at least in part, to E-CES7. No immunoreactivity against the anti-*CES7* antibody was detected in the testis and caput epididymis and, therefore, most likely arises from other carboxylesterase enzymes in these tissues (Fig. 8). Only in the ram can we attribute all the esterase activity of the cauda fluid to E-CES7, as demonstrated by two-dimensional-gel zymography. We also found that the total esterase activity of the cauda epididymal fluid varies substantially between species (from high in the ram to very low in the boar). Differences in activity may arise due to variations in cauda fluid components (i.e., the presence of inhibitors of carboxylesterases) or changes in the way the enzyme is processed before and/or after secretion. It is of note that similar differences have also been observed with microsomal carboxylesterases obtained from the liver of different species [20].

The physiological function of carboxylesterases is yet to be fully established because they typically have broad-range substrate specificities. Some of them specifically hydrolyze palmitoyl CoA, acyl-carnitine, and mono- and diacylglycerols, and are involved in the regulation of cellular lipids [20] and hydrophobic vitamins, such as retinol [28]. The idea that E-CES7 may play a role in the transfer of lipids is in agreement with our previous findings showing that it is associated with a high molecular-weight complex that we hypothesize to be similar to lipoprotein vesicles or chylomicrons [9].

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