Stallion epididymal fluid proteome: qualitative and quantitative characterization, secretion and dynamic changes of major proteins

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Stallion Epididymal Fluid Proteome: Qualitative and Quantitative Characterization; Secretion and Dynamic Changes of Major Proteins[1]

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ABSTRACT

Proteins present in and secreted into the lumen of various regions of the stallion epididymis were characterized qualitatively and quantitatively by two-dimensional electrophoresis. Using this proteomic approach, 201 proteins were found in the lumen and 117 were found that were secreted by the epithelium in various parts of the organ. Eighteen proteins made up 92.6% of the total epididymal secretory activity, lactoferrin (41.2%) and clusterin (24.8%) being the most abundant. Procathepsin D, HE1/CTP (cholesterol transfer protein), GPX (glutathione peroxidase), beta-*N*-acetyl-hexosaminidase, and PGDS (prostaglandin D2 synthase) were the other major compounds secreted. The most abundant proteins found in the luminal fluid were albumin and the secreted proteins: lactoferrin, PGDS, GPX, HE1/CTP, and hexosaminidase.

Three main secretory epididymal regions were identified from the protein pattern, i.e., regions E0–E2, E3–E5, and E6–E9. Region E0–E2 was characterized by the secretion of clusterin (53%), PGDS (44%), and GPX (6%). Region E3–E5 had the highest number of secreted proteins, the highest protein concentrations (60–80 mg/ml), and the highest spermatocrit value (85%). Lactoferrin (60% in E4), clusterin (29% in E3), hexosaminidase (10% in E3), and procathepsin D (6.9% in E4) were the most abundant proteins in this region. Region E6–E9, in which few region-specific secreted compounds were found, was characterized by a high quantity of lactoferrin in the luminal fluid (2–14 mg/ml). Comparison between the secretion of the major proteins and their concentrations in the lumen throughout the organ showed that the behavior of each protein is specific, in particular for the three isoforms of clusterin.

INTRODUCTION

Mammalian spermatozoa obtained from the testis are immotile and immature. They are unable to fertilize the oocyte. The development of fertilization ability takes place as sperm travel through the male genital tract as the result of post-testicular remodeling of the sperm membrane, such as changes in lipids, loss or modification (e.g., changes in glycosylation, proteolysis, relocalization) of preexisting (testicular) glycoproteins, and incorporation of new glycoproteins [1, 2]. Condensation of gamete DNA in the testis suggests limited biosynthetic capability of spermatozoa. Their maturation is therefore largely the consequence of their interactions with the epididymal fluid. This specific microenvironment, which is isolated from the blood by the epididymal-blood barrier, also ensures protection of the gametes until ejaculation as well as regulation of the functionality and integrity of the epididymis. The epididymal fluid is a composite of 1) rete testis fluid that enters through efferent ducts in the proximal part of the epididymis, 2) epididymal secretion and absorption, 3) proteolytic activity of preexisting proteins within the fluid, and 4) to some extent, metabolic activity of the spermatozoa.

Most of the proteins found in the epididymal fluid are secreted by the epididymal epithelium [3], and inhibition of protein secretory activity of the epididymis leads to a loss of fertilization ability of the spermatozoa [4]. The secretory activity of most epididymal compounds has previously been shown to be regionalized in all species studied. Each anatomical region (caput, corpus, and cauda) is thus characterized by its own secretory activity. Qualitative and quantitative variations in secretory activity of the epididymal epithelium have been observed throughout the organ in the human [5], rat [6], and mouse [7] and in domestic mammals, particularly the boar [8] and ram [9]. During transit through the epididymis, spermatozoa are bathed in various successive biochemical environments that are specific to each region and in which sequential interactions with their membrane occur. This leads to the ability of sperm to fertilize eggs.

Knowledge of the proteins present in the lumen of the epididymal tubule is essential to the understanding of epididymal physiology and function. Molecular biology approaches have been used to identify and study the expression of several messages in the epididymis, but the intense posttranslational and postsecretory modifications of the proteins in this organ could be accessed overall only by proteomic approaches such as two-dimensional (2D) gel electrophoresis.

As epididymal sperm maturation occurs in all mammals, comparative studies would make it possible to reveal the differences and similarities between species, conservation of the latter through evolution being presumably essential to epididymal physiology.

The present study was performed with the aim of establishing stallion epididymal proteomic activity, by identifying the epididymal characteristics of this species and subsequently to compare these characteristics with those of other domestic mammals already studied (ram and boar [8, 9]). Little information is available concerning epididymal secretory function in the stallion, except for our recent study showing the presence of prostaglandin D2 synthase in the epididymal fluid [10]. We therefore wished to extend our understanding of epididymal protein secretory activity in this species.

We have developed a proteomic approach to carry out a comprehensive study of proteins present in the epididymal fluid of the stallion. The aims of this study were 1) to characterize and identify the major fluid proteins, 2) to determine the major proteins secreted by the epididymal epithe-

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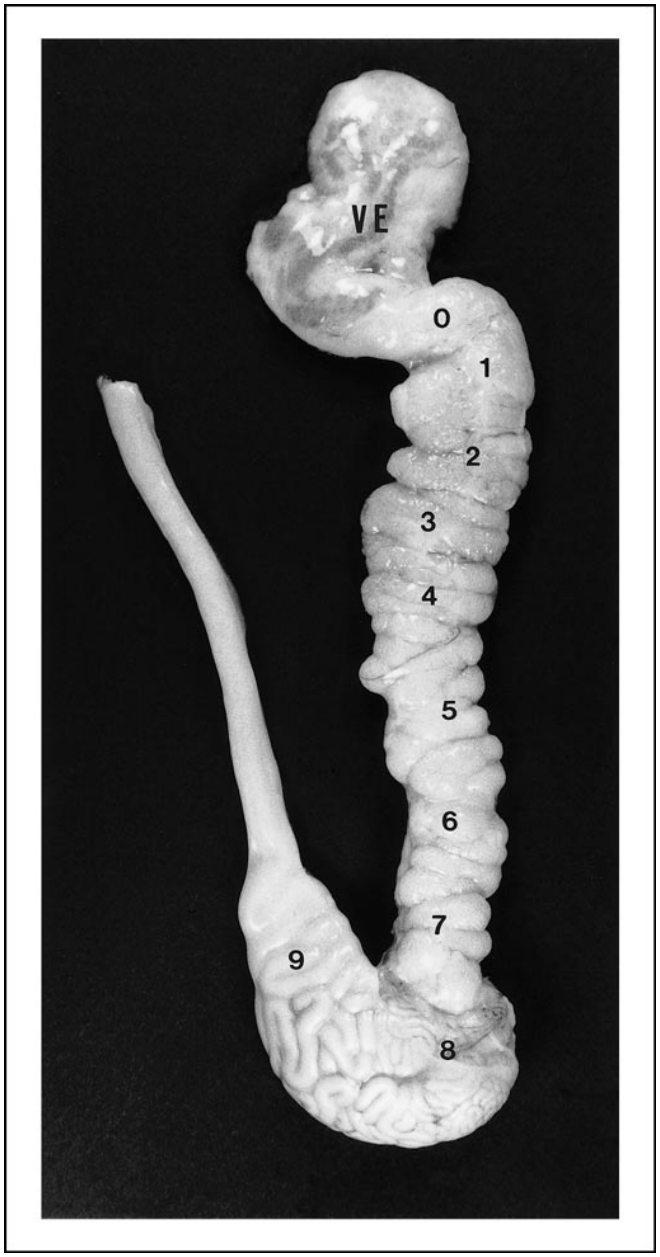

FIG. 1. Stallion vas efferens (VE) and epididymis, with the various anatomical epididymal regions: E0–E1, proximal caput; E2, middle caput; E3–E4, distal caput; E5–E6, corpus; E7–E9, cauda.

lium, and 3) to study changes in the major proteins identified in the fluid throughout the tract.

MATERIALS AND METHODS

Reagents and Chemicals

Dulbecco's modified Eagle's medium without methionine and cysteine (DMEM−), x-ray films (X-OMAT-XAR5; Eastman Kodak, Rochester, NY), CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), goat anti-rabbit IgG and anti-mouse IgG coupled to horseradish peroxidase, Hepes, carnitine, and mineral oil were purchased from Sigma Chemical Co. (St. Louis, MO). [^{35}S]Methionine and [^{35}S]cysteine (^{35}S-protein labeling mix, EXPRE^{35}S^{35}S) were purchased from NEN (Les Ulis, France); acrylamide (30% acrylamide, 0.8% *N*,*N*-methylenebisacrylamide) from Millipore (St. Quentin, France);

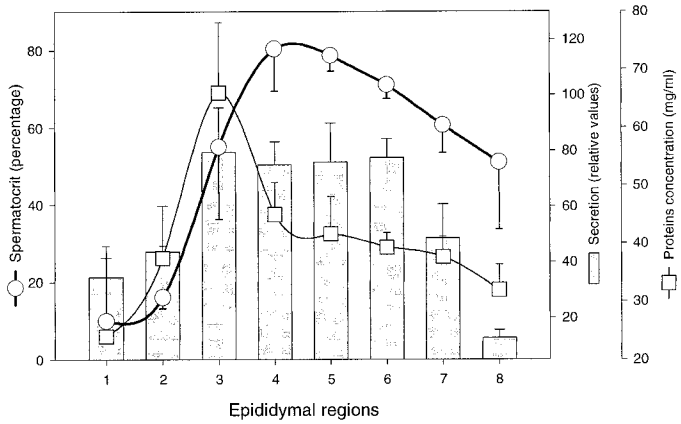

FIG. 2. Total protein concentration (mg/ml), spermatocrit (%), and protein secretion activity (% of total secretion) in the various regions of the stallion epididymis. These results were obtained from 3 stallions for protein concentration and spermatocrit (S8–S10) and for 7 animals for protein secretion (S1 to S5, S8–S9).

ampholytes pH 2–11 (servalytes) from Serva (Heidelberg, Germany); ampholytes pH 3–10 (ampholytes), Coomassie Brilliant Blue (Phastgel Blue R), and electrophoresis calibration kits (standard proteins) from Pharmacia (Saclay, France); and the Bradford assay kit from Biorad (Paris, France).

Animals and Organ Sampling

Eleven adult (2–4 yr old) horses (New Forest and Conemara ponies) were used in this study (S1 to S11). Epididymides and testes were obtained from animals at the time of castration. All animals presented normal and motile spermatozoa in caudal epididymal regions. The length of the epididymis varied between animals from 10 to 17 cm, with a mean length of 13.7 ± 2.2 cm. Luminal proteins for all animals were analyzed by one-dimensional (1D) gel electrophoresis. Secretory activity was analyzed after biosynthesis in vitro by 2D gel electrophoresis on seven horses (S1 to S5; S8; S9), of which two (S5 and S8) were also used for quantification of luminal proteins. Spermatocrit and protein concentrations in the luminal fluid were estimated on three animals (S8 to S10).

The epididymides, whatever their size, were subdivided into 10 regions (0–9) as previously described [10] (Fig. 1). For protein analysis, the luminal fluids of the epididymis were obtained by microperfusion as previously described [1], performed with PBS or mineral oil.

All samples were centrifuged (1500 × *g* for 15 min) to remove sperm. The supernatants were centrifuged (15 000 × *g* for 10 min) and kept at −20°C until use. In vitro incubation was performed as described below.

In Vitro Secretion of [^{35}S]Methionine-Cysteine-Labeled Proteins by Tissue Samples and Isolated Tubules

For seven animals, in vitro secretion of [^{35}S]methionine-cysteine-labeled proteins was estimated from the testes (T), vasa efferentia (VE) (minced tissues), and all epididymal regions (isolated tubules) according to previously described methods [1]. Briefly, one sample of minced tissues (T and VE) and one closed end-tubule for each epididymal region were incubated in 0.5 ml modified DMEM− (20 mM Hepes, 2 mM carnitine in DMEM−) in the presence of 100 μCi ^{35}S-protein labeling mix, under 95% O_2, 5% CO_2 at

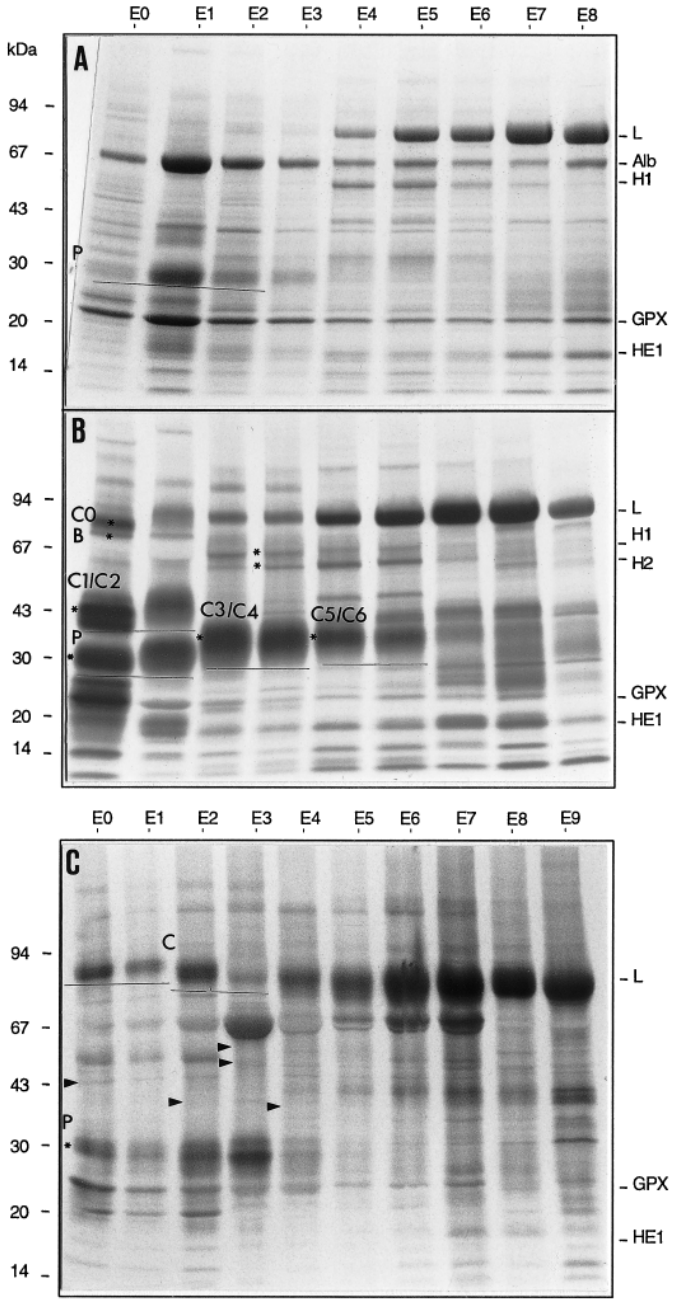

FIG. 3. 1D Electrophoresis of luminal epididymal fluid (**A**: Coomassie Blue staining) and secreted [^{35}S]methionine-labeled proteins (**B** and **C**: fluorographs) from various epididymal regions (E0–E9) of stallion S9. **A** and **B**) Under reducing conditions; **C**) under nonreducing conditions. L, Lactoferrin; Alb, albumin; H1/H2, hexosaminidase; P, prostaglandin D2 synthase; HE1 (CTP), cholesterol transfer protein; B, alpha 1-B glycoprotein; C0 to C6, different isoforms of clusterin. Asterisk indicates where the secretion of compounds began. Arrowheads, position of the disappearance of clusterin and hexosaminidase subunits under nonreducing conditions. Molecular masses are indicated on the left.

32°C. After 5 h, the incubation medium was collected, and the luminal fluid of each tubule was obtained by perfusion with DMEM. The luminal fluids were then centrifuged and the supernatants stored at −20°C until use.

Gel Electrophoresis

SDS-PAGE separation was carried out for all samples according to the method of Laemmli [11] on 6–16% linear polyacrylamide gels (14 × 16 cm or 6 × 8 cm). For each epididymal region, approximately 50 μg and 150 μg of total proteins were deposited on 1D and 2D gels, respectively.

Isoelectric focusing was performed using the O'Farrell technique [12] modified as previously described [13]. The pH gradient was obtained with 1% pH 3–10 ampholytes and 1% pH 2–11 servalytes. Isoelectric focusing was run at 20 mA, 0.1 W/tube, 700 V for a total of 10 000 V/h followed by 20 mA, 0.1 W/tube, 3000 V, and a total of 2000 V/h. The 2D separation was performed on a 6–16% acrylamide gel (1.5 mm thick) at 40 mA.

For detection of radioactive proteins, the gels were impregnated with a fluorography enhancer (Amplify; Amersham, Les Ulis, France) after silver [14] or Coomassie Blue staining. After drying, all the gels of each experiment were exposed on preflashed x-ray film at −80°C during the same period (around 3 wk), or on a phosphoimager screen (Storm; Molecular Dynamics, Paris, France).

Determination of Protein Concentrations in Epididymal Fluid and Spermatocrit

To determine the luminal protein concentrations and the spermatocrit in each epididymal region, the contents of the tubules were perfused with mineral oil and taken up in a 10-μl capillary tube. After centrifugation (5000 × *g*, 1 h), the total height, fluid height (supernatant), and spermatozoa height (pellet) were measured. For each region, the protein concentration in the fluid was determined using Bradford assay with known amounts of horse caudal epididymal freeze-dried protein as standards.

Coomassie Blue Staining and Fluorography Quantitative Analysis

Despite its low-level sensitivity of detection, Coomassie Blue staining, chosen for its property of quantitative staining between different proteins, was used to reveal the major proteins. The stained gels (before drying) and the corresponding fluorographs were digitalized with an Eikonix 1412 scanner camera (Eastman Kodak, Rochester, NY), and the quantitative analysis was performed using Kepler software (Large Scale Biology Corporation, Rockville, MD) as previously described [8]. Epididymal secretions were quantified on the fluorographs, corresponding to the 2D electrophoresis used for quantification of epididymal fluid proteins. For each region, each spot on the fluorograph and on the stained gel was characterized by a volume of secretion and staining. The total secretory activity and total staining of each region was the sum of all detected spots. All samples quantitatively analyzed by electrophoresis were obtained after perfusion of the tubule with DMEM. Thus, the dilution of the luminal fluid in these samples was unknown. To estimate the real concentration of protein in the fluids, we perfused the tubules of the ipsilateral epididymis with mineral oil. A correction factor was applied to the spot values from each of the 2D gels to obtain the values that should be obtained if an equal volume of undiluted epididymal fluid had been deposited on the 2D gels.

Antibodies

Polyclonal antibodies against ram HE1/CTP (human epididymal 1/cholesterol transfer protein) were produced earlier in our laboratory [9], as were polyclonal antibodies against boar hexosaminidase [15]. Mouse monoclonal an-

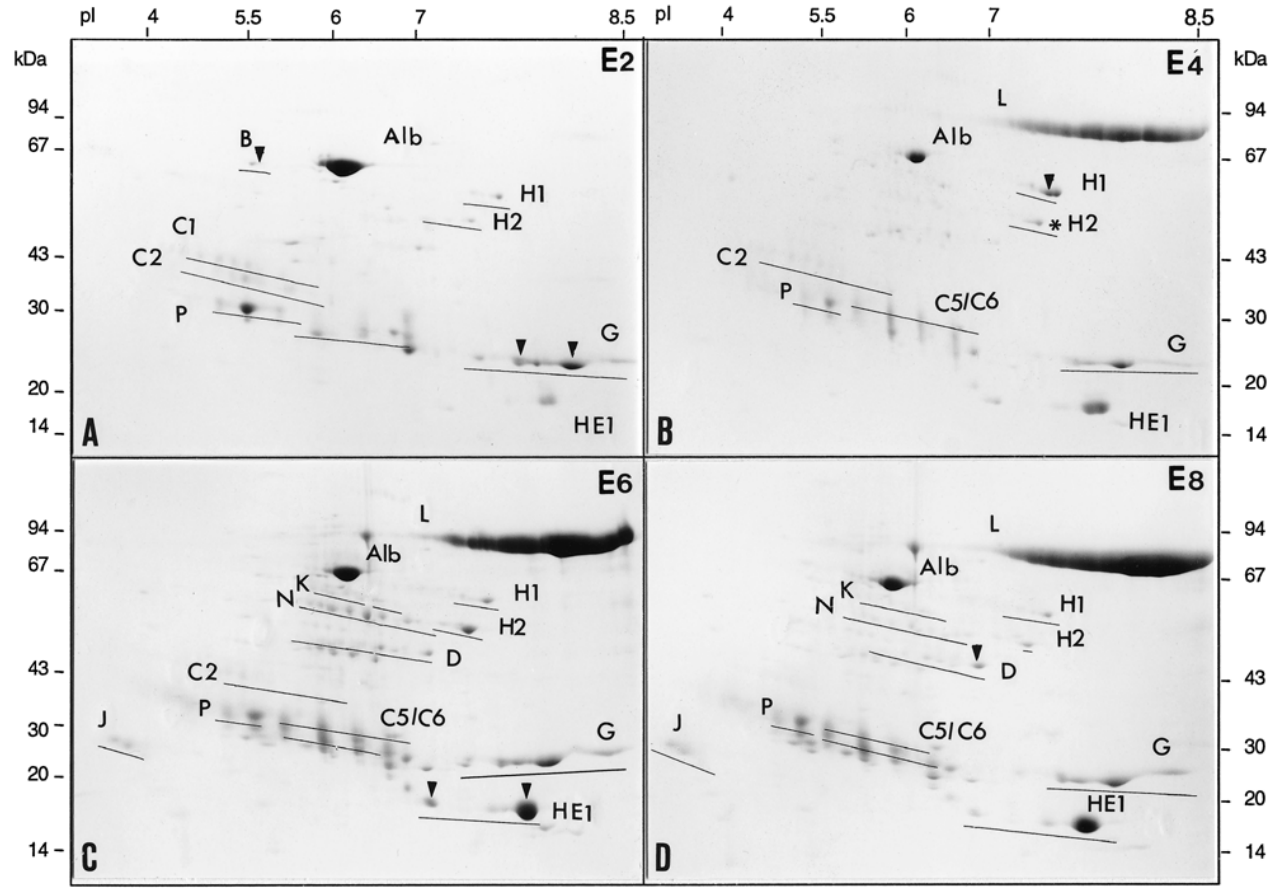

FIG. 4. Coomassie Blue-stained epididymal proteins of regions E2 (**A**), E4 (**B**), E6 (**C**), and E8 (**D**) from stallion S8 after 2D electrophoresis. The gels were scanned for quantification analysis. Arrowheads, sequenced N-terminal spots (Table 1); (*), protein with N-terminal blocked. D, Procathepsin D; G, glutathione peroxidase. J, N, and K are unidentified trains. See Figure 3 for other abbreviations. Groups of spots presumed to represent a common protein are underlined.

tibodies against ram clusterin and rabbit antiserum against human lactoferrin were kindly provided by Dr. Fritz (Cambridge, UK) and Dr. Spik (UMR CNRS 111, Lille, France), respectively.

Western Blotting and Immunodetection Antigens

Immunodetection was performed on two horses (S6 and S7). The proteins from acrylamide gels were electrotransferred (0.8 mA/cm^2 for 2 h) by a semidry technique onto 0.2-μm nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The transferred nitrocellulose membranes were stained with Ponceau Red S (Sigma) and incubated overnight at 4°C with a blocking solution (10% goat serum in Tris-buffered saline [TBS]) [10].

The primary antibody diluted in blocking buffer (5% goat serum in TBS) was applied for 2 h at room temperature, or 1 h at 37°C. The dilutions used were 1:5000 for antisera against clusterin and HE1, 1:1000 for antiserum against lactoferrin, and 1:10 000 for antiserum against hexosaminidase. The Western blots were then washed with the same buffer and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (for polyclonal antibodies) or anti-mouse IgG (for monoclonal antibodies) diluted at 1:10 000 in 5% goat serum in TBS for 30 min at 37°C. After several washes, immunoreactive proteins were detected by chemiluminescent substrate (Western Blot Chemiluminescence Reagent Plus; NEN, Boston, MA) according to the manufacturer's instructions.

N-Terminal Amino Acid Sequence Analysis

After transferring the proteins to a polyvinylidene fluoride (PVDF) membrane (Hyperbond; Porton Instruments, Tarzana, CA), the membranes were lightly stained with Coomassie Blue R-250, and the protein spots were excised. The N-terminal amino acid sequence of the proteins was determined with a Porton sequencer (Beckman, Palo Alto, CA; model LF3000) using the reagents and methods recommended by the manufacturer. Similarities between the amino acid sequences obtained and those of other known proteins were determined by comparisons within GenBank, PIR, EMBL, Genpept, PDB, and SwissProt using BLAST or FASTA software [16].

RESULTS

Protein Concentrations and Spermatocrits of Various Epididymal Fluids

The protein concentrations and spermatocrits for the various regions of the epididymis (Fig. 1) were obtained for three stallions (S8 to S10) (Fig. 2). The spermatocrit in the rete testis fluid (RTF) was less than 1%. The proximal caput of the epididymis (E0–E1) had a spermatocrit of 15%; this then increased progressively to reach a maximum value of around 85% in E4/E5. It subsequently decreased progressively to 30% in the cauda fluid.

The protein concentration of 35 mg/ml in the proximal caput was around 5 times higher than in RTF. The protein

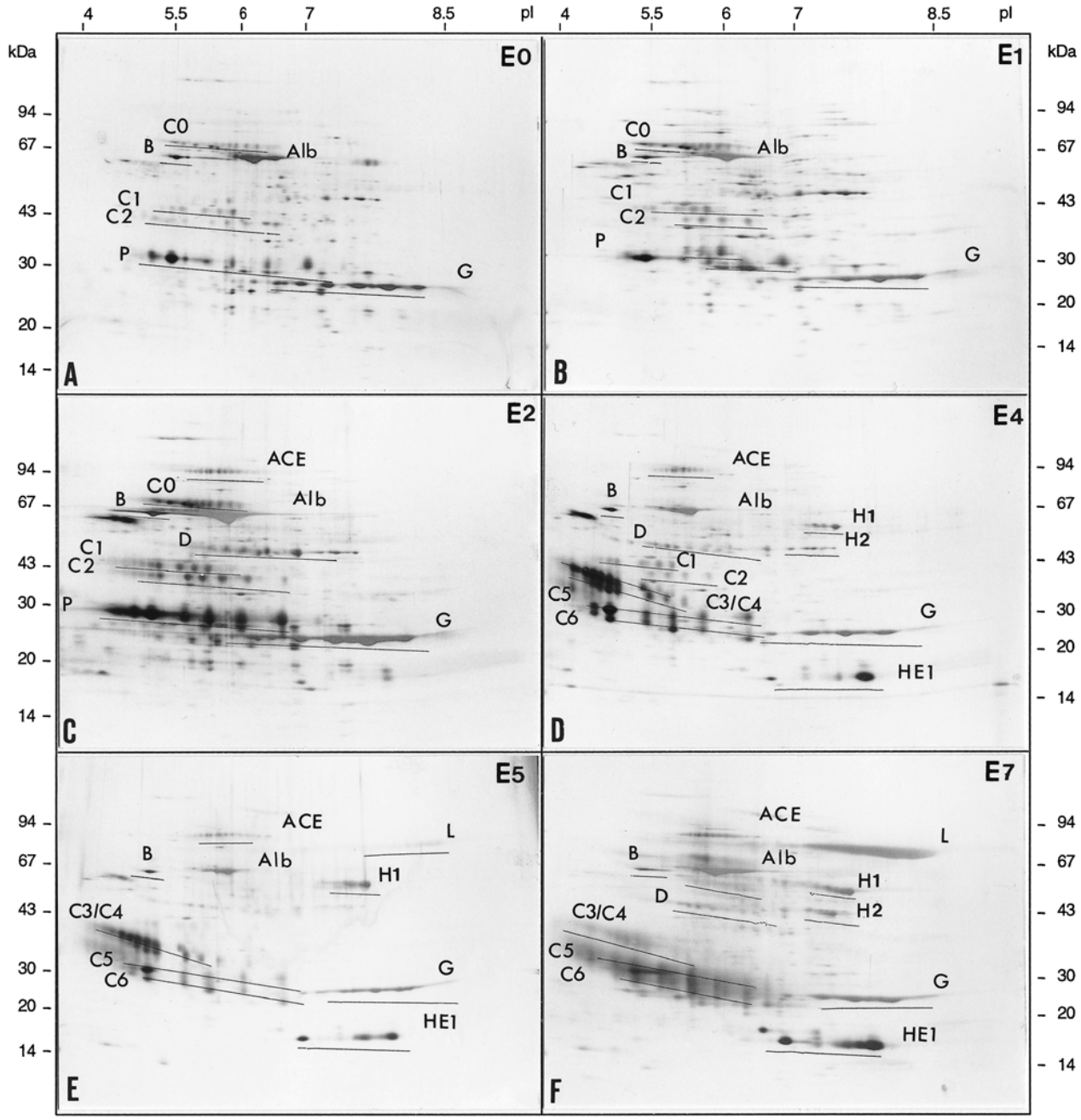

FIG. 5. Silver-stained epididymal proteins from regions E0 (**A**), E1 (**B**), E2 (**C**), E4 (**D**), E5 (**E**), and E7 (**F**) from stallion S4 after 2D electrophoresis. See Figure 3 for other abbreviations. Groups of spots presumed to represent a common protein are underlined.

concentration reached a maximum value of 60–80 mg/ml in the distal caput (E3). It then decreased progressively toward the corpus and cauda to reach 20–30 mg/ml. The highest protein concentration occurred in a more proximal epididymal segment than the highest spermatocrit value.

Qualitative and Quantitative Analysis of Proteins Present in Epididymal Fluid

Characterization, regional localization, and identification of major proteins. Proteins from each epididymal fluid were characterized by 1D and 2D gel electrophoresis after staining either with Coomassie Blue (Figs. 3A and 4) or with silver (Fig. 5). Representative results for six stallions studied by 2D electrophoresis are illustrated.

A total of 324 different spots distributed throughout the epididymis were detected after 2D electrophoresis using Kepler software. They corresponded to 201 isolated spots or trains of different proteins (Table 1). Most epididymal proteins appeared to be polymorphic, and they occurred as trains of several spots corresponding to isoforms of differing molecular mass and/or isoelectric points (pIs). Identification of major epididymal proteins was carried out by microsequencing the spots on PVDF membranes obtained with fluids E1, E3, and E8 from two stallions (Fig. 6). Some proteins were also identified on fluids from one stallion by immunodetection with specific

TABLE 1. Proteins present in the epididymal fluid of the various regions (expressed as % of luminal protein for each region).

Parameter	Regions of epididymis							
	E0/1	E2	E3	E4	E5	E6	E7	E8
Total number of spots observed (n = 324 for whole epididymis)	120	73	67	76	149	140	160	114
Total number of proteins observed (n = 201 along length of epididymis)	97	47	42	44	85	90	71	71
Luminal proteins not detected in secretion	71	23	12	23	55	61	46	46
Protein (%)								
Lactoferrin	—	1.7	6.6	53.8	26.5	36.8	43.8	40.7
Clusterin	1.5	6.0	7.4	6.7	13.5	11.2	7.5	9.2
Procathepsine D	—	—	—	—	0.2	0.1	0.1	0.2
HE1/CTP	—	3.9	6.8	9.8	11.9	9.1	7.9	9.5
Hexosaminidase	—	1.5	15.3	4.2	1.6	1.2	1.3	0.9
PGDS	20.8	18.9	4.5	4.0	3.2	4.3	5.5	4.8
K	—	—	0.3	0.2	0.8	1.1	0.4	0.7
GPX	15.6	18.6	14.3	6.6	12.2	8.0	6.0	6.6
J	—	—	—	—	0.8	1.1	0.9	1.6
N	—	—	—	—	3	3.1	1.5	1.2
W	—	—	—	0.2	0.8	1.1	1.0	1.5
Z	—	—	1.5	0.3	0.5	0.3	—	—
Albumin	33.0	31.4	18.6	5.5	8.9	4.9	6.9	5.1
Others	29.1	18	24.7	8.7	16.1	17.7	17.2	18

antisera (Fig. 7). The major compounds of each region were quantified after Coomassie Blue staining of the 2D gels, and their staining intensity was expressed as a percentage of the total staining intensity of all the spots detected from the same epididymal region (Table 1). Minor compounds, not stained by Coomassie Blue, were visualized by silver staining (Fig. 5).

The major Coomassie Blue-stained fluid proteins that occurred in various regions of the epididymis are described below.

Proximal caput region (E0–E1). Regions E0 and E1 had very similar fluid proteins (Figs. 3A and 5). We have previously found prostaglandin D2 synthase (PGDS) (30 kDa, pI 4.7–7.2) in this region [10]. In the present study we also found clusterin by using a specific antiserum in 1D and 2D immunoblotting (Figs. 7 and 8), as well as glutathione peroxidase (GPX) (25.5 kDa, pI 7.3–8.5) by N-terminal microsequencing (Fig. 6). Of the 97 proteins detected in regions E0-E1, PGDS, GPX, and clusterin together with albumin represented 70% of the total luminal fluid protein (LF) with 20.8%, 15.6%, 1.5%, and 33%, respectively (Table 1). Two isoforms of clusterin were present: C0 (69 kDa, pI 5.6–6.2), which was not visible in the Coomassie-stained gels but detected by silver staining and after immunodetection, and Ca, which was composed of two main subunits, C1/C2 (38–42 kDa, pI 5–7) (Figs. 5, 7, and 8).

Albumin, GPX, and PGDS were detected throughout the duct in progressively decreasing amounts, whereas clusterin increased from regions E0 to E5, then decreased toward the cauda (Table 1).

Spot B (67.7 kDa, pI 5.5) stained lightly after Coomassie Blue staining but was clearly visible until the cauda after silver staining (Fig. 5). This protein was identified as alpha 1B-glycoprotein (Fig. 6).

Middle caput region (E2). As in the proximal caput, the 4 major proteins among the 47 proteins present in the fluid of this region were albumin (31.4%), PGDS (18.9%), GPX (18.6%), and the Ca form of clusterin (6%) (Figs. 3A, 4, 5 and Table 1). Two major proteins first appeared in this region: 1) HE1/CTP (18 kDa, pI 7.8) and 2) beta-*N*-acetyl-hexosaminidase. The former (3.9%), which appeared as a single spot, was identified by microsequencing (Fig. 6) and immunodetection (Fig. 7C) whereas the second (1.5%), which was also identified by microsequencing (Fig. 6) and immunodetection (Fig. 7B), was composed of two forms, H1 (58.5 kDa, pI 7.4) and H2 (47.2 kDa, pI 7.2). These 6 proteins (albumin, PGDS, GPX, clusterin Ca, HE1/CTP, beta-*N*-acetyl-hexosaminidase) made up around 80% of the proteins in the LF.

Another protein, the angiotensin-converting enzyme (ACE) (94 kDa, pI 5.4–6.5) identified in a previous study [17], was detectable after silver staining (Fig. 5).

Distal caput region (E3–E4). The major protein among the 43 proteins detected in region E3–E4 was revealed by immunodetection (Fig. 7D) to be lactoferrin (81 kDa; pI 7–8.5). It first appeared in region E2 and increased to 53.8% of the total protein of E4 fluid (Table 1). Trains Z (1.5%; 96 kDa, pI 6.2–7.2), K (0.3%; 65 kDa, pI 5.8–6.3), and W (0.2%; 20–22 kDa, pI 5.7–6.3) also first appeared in these regions, but these 3 proteins were not identified.

Two new isoforms of clusterin were detected in this region. Isoform Cb, composed of subunits C3/C4 (32–40 kDa, pI 4–5.6), was detected in region E3 after silver staining (Fig. 5) and by immunodetection (Figs. 7E and 8), and isoform Cc, which is composed of two subunits C5/C6 (29–33 kDa, pI 4.5–6.5), was also detected in this region. These isoforms of clusterin represented around 7% of LF. Ca became undetectable between regions E3 and E4.

Among the proteins described above, the H1/H2 forms of beta-*N*-acetyl-hexosaminidase represented 15.3% of the total proteins in E3, decreasing in E4 to 4.2%. Albumin and GPX, respectively 18.6% and 14.3% in region E3, had decreased in region E4 to 5.5% and 6.6%, respectively. PGDS represented 4% of E3-E4 proteins; and HE1/CTP, which appeared now as a train of pI 7–7.8 (Fig. 4), increased from 6.8% in E3 to 9.8% in region E4 (Table 1). Lactoferrin, albumin, PGDS, GPX, clusterins, HE1/CTP, and hexosaminidase together represented around 90% of the total proteins in this fluid.

Corpus region (E5–E6). Among the 85–90 proteins present in the LF in these regions, most of those detected in the more proximal part of the epididymis were still present (Figs. 3A, 4, and 5). Three new compounds, trains N (50 kDa, pI 5.7–6.4), J (26 kDa, pI 3.8–4), and D (45 kDa, pI

Name (1)	N terminal sequences and comparison with sequences in databases	Identity
Cathepsin D precursor	10 XVRIPLYKFKSIRRTI :||||:|| ||||: P07339 VIRIPLHKFTSIRRTMSE 10	73.3% identity in 15 aa overlap
Hexosaminidase	10 XFYTGLXPLPLSVEMSP | |||||:|:| A31250 VSAKPGPALWPLPLSVKMTPNLLHLAS 50 60 70	75.0% identity in 12 aa overlap
HE1/CTP	10 20 NPVHFKDCGSKXXXVKXENNNX ||:|||||| :| | I38365 QAEPVQFKDCGSVDGVIKEVNVSPC 20 30 40	55.6% identity in 18 aa overlap
Glutathione peroxidase	10 20 ENNAEKMKTDCYKDVK-TIYEYSAV |||| ||||||| |||:| |: A47367 VQTTPRPEKMKMDCYKDVKGTIYDYEALSL 20 30 40	76.2% identity in 21 aa overlap
Alpha 1B glycoprotein (Spot B)	10 AVVFDPKPAL ||||| ||| PL0028 AVVFDPPPALWA 10	90.0% identity in 10 aa overlap

(1) The spots sequenced are indicated with an arrowhead in Fig. 4

FIG. 6. Identification of major stallion epididymal proteins by N-terminal sequencing.

5.8–7), appeared in this region. Train D (0.2% of LF) was identified by N-terminal microsequencing as procathepsin D (Fig. 6). Only the last spot of the train was sequenced; the other spots of this train appeared in the same region. Proteins N and J were not identified. Thirteen major compounds, representing 83% of luminal corpus fluid (Table 1), occurred in this region. They included lactoferrin (36.8% in region E6), all isoforms of clusterin (13.5% in region E5), GPX (12.2% in region E5), HE1 (11.9% in region E5), albumin (9.8% in region E5), and PGDS (4.3% in region E6).

Caudal region (E7–E8). The proteins of the caudal and corpus regions were very similar, the major proteins being lactoferrin (40.7%), HE1 (9.5%), clusterin (9.2%), GPX (6.6%), albumin (5.1%), and PGDS (4.8%) among the total of 71 proteins detected in region E8 (Table 1).

Changes in concentration of the major proteins. The concentration of each stained compound was calculated from the total protein concentration, and the percentage of each protein concentration was determined (Fig. 2 and Table 1). Among the 201 proteins characterized, only 13 reached 1 mg/ml or more in any particular epididymal region. The highest concentrations were found for albumin and lactoferrin (Fig. 9 for lactoferrin). Albumin decreased from 12 mg/ml in the caput to 1 mg/ml in the cauda. Lactoferrin reached its highest concentrations between regions E4 and E6 (not shown). In the results shown in Figures 4 and 9, lactoferrin reached 25 mg/ml in region E4 and remained around 15 mg/ml in the posterior regions.

The highest concentrations of GPX, HE1, hexosaminidase, and PGDS were observed in regions E3, E4, E3, and E2, with concentrations of 8.4, 4.5, 9, and 7.3 mg/ml, respectively (Fig. 9). The concentrations of these 4 proteins decreased along the duct. The greatest decrease observed was in hexosaminidase, which was at the limit of detection in the cauda.

For the clusterins, the highest concentration for isoforms Ca/Cb (3.9 mg/ml) was found in regions E2–E3. These clusterins had decreased in region E3 and could not be detected by Coomassie Blue staining beyond region E6, although they could be visualized in this region by silver staining (Fig. 5) and immunodetection (Fig. 7). For clus-

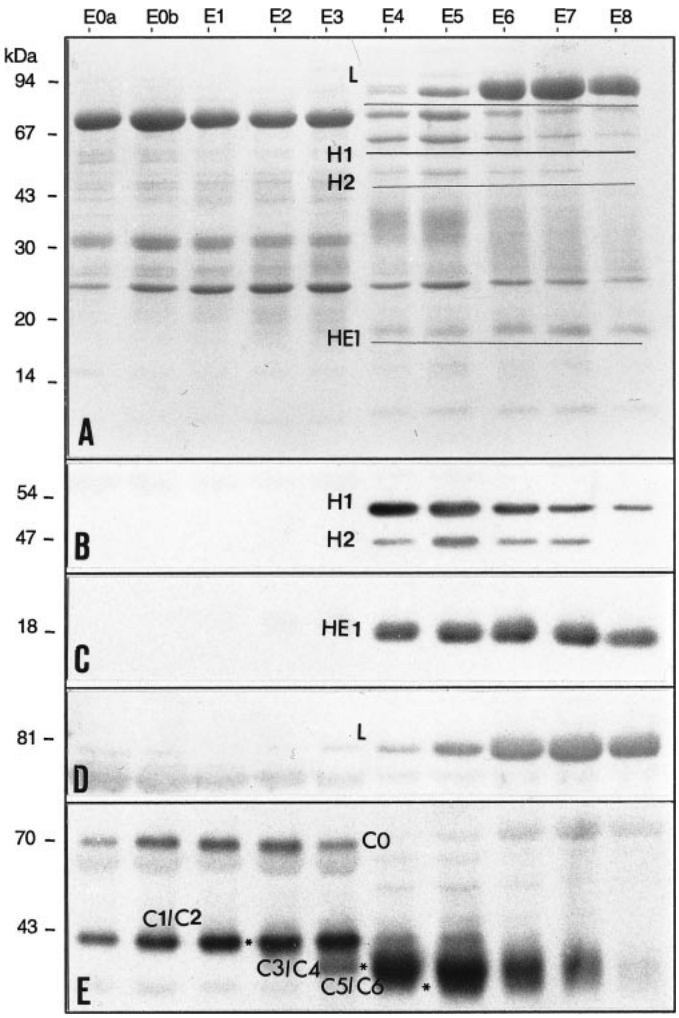

FIG. 7. 1D Electrophoresis of epididymal fluid proteins (stallion S6) from regions E0 (E0a/E0b) to E8 after Coomassie Blue staining (**A**) and immunodetection with specific antisera against hexosaminidase (**B**); HE1 (**C**); lactoferrin (**D**); clusterin (**E**). See Figure 3 for abbreviations.

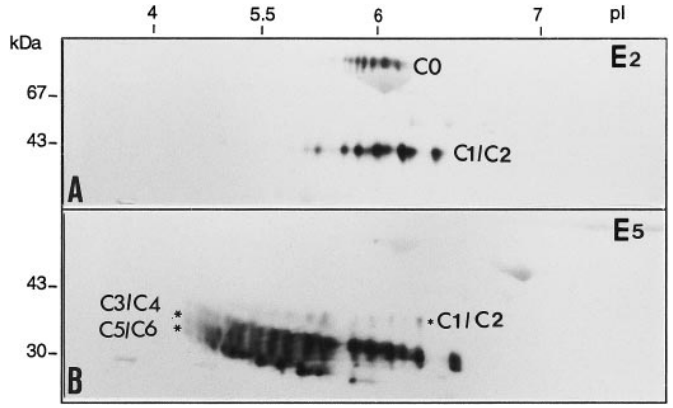

FIG. 8. Immunodetection after 2D electrophoresis of clusterin in fluid from regions E2 (**A**) and E5 (**B**) by the specific antiserum against ram clusterin (stallion S6); C0 to C6: different isoforms of clusterin.

terin Cc, the maximum concentration was found in region E5 (4.8 mg/ml) (Fig. 10).

Characterization and Quantification of Proteins Secreted by the Testis, Efferent Ducts, and Epididymal Epithelium

Proteins secreted by the testis, efferent ducts (Fig. 11), and luminal epididymal epithelium were obtained after in vitro incubation with [^{35}S]methionine-cysteine. Epididymal proteins were then separated both by SDS-PAGE under reducing (Fig. 3B) and nonreducing conditions (Fig. 3C) and by 2D electrophoresis (Fig. 12). Under nonreducing conditions, the nondissociated subunits of clusterin presented a molecular mass of 80 kDa (Fig. 3C), and the two forms of beta-*N*-acetyl-hexosaminidase were no longer detected. The proteins secreted in each region were quantified and compared to those present in the fluid.

Proteins secreted by the testis and efferent duct tissues. The 5 major polymorphic proteins secreted by testicular tissue (Fig. 11A) were an isoform of clusterin (35–43 kDa, pI 4–5.6), an isoform of PGDS [10] (30 kDa, pI 5.5–6), train T1 (67–80 kDa, pI 4–6.2), train T2 (94 kDa, pI 6.5), and train T3 (43 kDa, 6.5–7.8). Trains T1, T2, and T3 were specific to the testis; they were not found in any of the efferent duct samples (Fig. 11B), nor were they secreted by the epididymis (Fig. 12).

The secretory activity of the VE was characterized by three major trains, one of which was specific to the VE (V1), whereas the other two clusterins and PGDS were also present in the testis. In the VE, the isoforms of clusterin were more basic (pI 5.5–6.2) than those in the testis. Different isoforms of PGDS were also secreted by this tissue (30 kDa, pI 5.6–7).

Proteins secreted by the epididymis. The caput (E0–E4) was the most active region, accounting for 73% of total epididymal secretion. The corpus was the next most active, secreting 20.5%, followed by the cauda, 6.5% (Table 2 and Fig. 2).

A total of 326 spots corresponding to at least 117 proteins were detected along the epididymis (Table 2).

Secretion of only 18 of these proteins corresponded to 92.6% of the total secretory activity (Table 2). Lactoferrin and clusterin (Fig. 13) were the most abundant proteins, making up 41.2% and 24.8% of the total epididymal secretion, respectively. Procathepsin D, CTP/HE1, hexosaminidase, PGDS, and GPX were the other major compounds secreted and represented 5.2%, 3%, 2.9%, 2.3%, and 1.4% of the total epididymal secretion, respectively (Fig. 13).

Three main secretory epididymal regions (E0–E2, E3–E5, and E6–E9) were evident from the secretion patterns of these major proteins.

Regions E0–E2. In the proximal and middle caput, 44 different proteins were detected, of which 22 were specific to this region. Moreover, 10 represented 88% of the secretory activity of these regions, of which 3, namely PGDS, GPX, and clusterin, were maximally secreted (Figs. 12, A–C, and 13). In region E1, PGDS represented 44% of the secretion; in region E2, clusterin represented 53% and GPX 5%.

Clusterins, PGDS, and GPX had the same isoforms as those described in the fluid, except for PGDS, which was secreted under its most basic isoforms [10]. For clusterin, two isoforms were secreted, Ca from E1 and Cb from E2.

Trains Z (96 kDa, pI 6.2–7.2) and S (67 kDa, pI 4.5–5.8), which were unidentified proteins, appeared in region E2 (Fig. 12) and represented 0.8% and 1.9% of the entire epididymal secretion, respectively (Table 2 and Fig. 13).

Regions E3–E5. In regions E3–E5, 88 different proteins were secreted, 67 of which were new compounds (23 in E3, 20 in E4, 24 in E5) and 30 of which were not detected

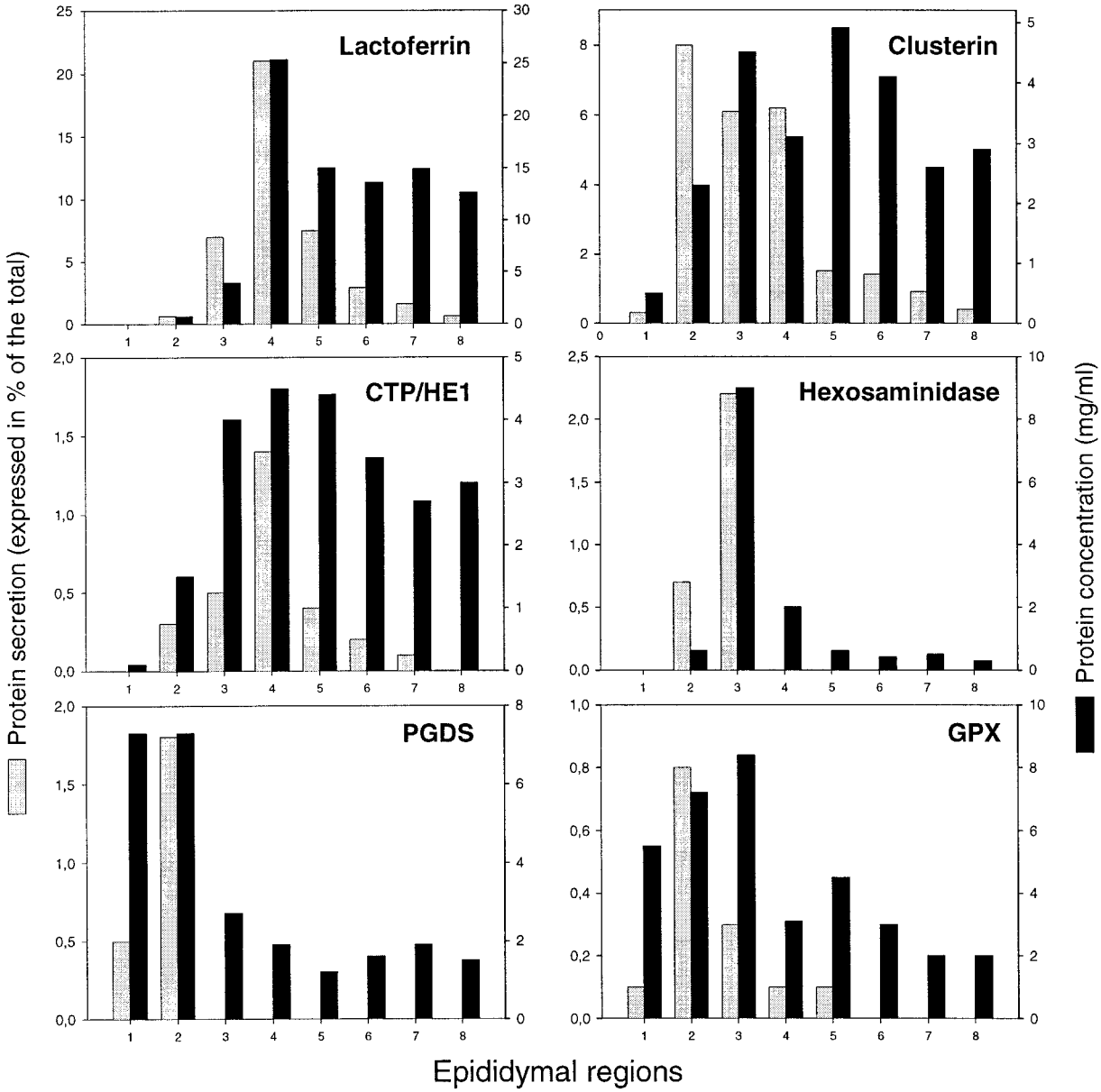

FIG. 9. Protein concentration (mg/ml) and secretion level (% of total secretion) of lactoferrin, clusterin, CTP/HE1, hexosaminidase 1 and 2, PGDS, and GPX. The results presented represent the analysis performed on two stallions (S5 and S8).

in other regions. Among the proteins secreted, 18 represented 65.3% of the total secretory activity (Table 2).

Large amounts of lactoferrin, CTP/HE1, hexosaminidase, clusterin, and procathepsin D were secreted in these regions and together represented 88% of secretory activity of these regions.

Clusterin, hexosaminidase, and train T (not visualized in stained gels; 67 kDa, pI 5.5–5.8) secretions represented 28.9%, 10%, and 3.4% of secretory activity in region E3, respectively. In region E4, procathepsin D and HE1/CTP represented 6.9% and 4%, but lactoferrin represented 60% of the secretory activity of this region. Among the animals analyzed, the epididymal localization of the maximum secretory activity of lactoferrin was found in region E4 for one animal, in region E5 for two, and in region E6 for two others (Fig. 14).

The third clusterin isoform, Cc, appeared in region E3 (Figs. 11D, 12D, and 10).

The other major compounds (representing more than 0.5% of the whole epididymal secretory activity) that appeared in these regions included trains E (44 kDa, pI 5.8–6.3), F (66 kDa, pI 6.8–7.2), and M (55 kDa, pI 5.8–6.2) in region E3, and trains I (26 kDa, pI 7.5), V (69 kDa, pI 6–6.2), and W (20–22 kDa, pI 5.7–6.3) in region E4 (Table 2).

Regions E6–E9. In these regions, only 6 new proteins appeared among the 42 proteins secreted, of which train J, which was secreted only in regions E5 to E7 (Fig. 12), represented less than 0.5% of the whole epididymal secretory activity.

Twenty-five compounds were not found beyond these regions, the major proteins that were still being secreted in this region being lactoferrin and clusterin together with trains S, K, I, T, and V (Table 2).

DISCUSSION

The present study is the first proteomic investigation of the stallion epididymal fluid. The analysis shows that more

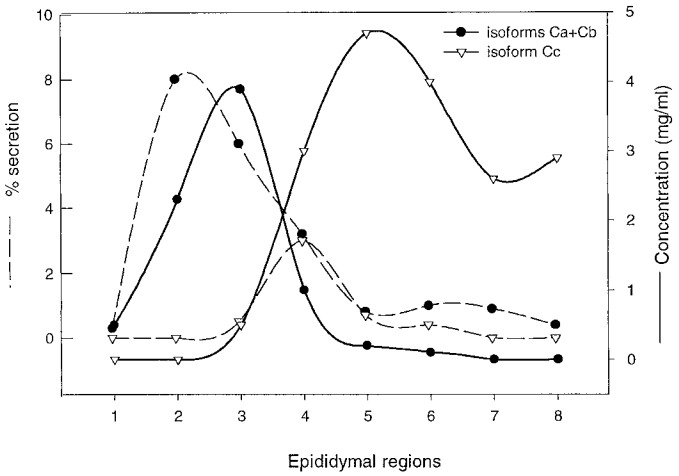

FIG. 10. Secretion and concentrations in the fluid of isoforms of clusterin (stallion S8): isoforms Ca and Cb, secreted in caput; isoform Cc, secreted in corpus.

than 250 different proteins can be characterized in the fluid by 2D gel analysis. Most of these proteins are differentially distributed throughout the epididymis. Such complexity has been previously shown for the boar [8], ram [9], and laboratory rat [18, 19].

In this study, the protein composition of the fluid and the proteins secreted by the surrounding epithelium could be directly compared because the analyses were performed on the same samples. The relationship between the number of proteins present in the fluid and those secreted by the epithelium lining varied according to the region of the tract. Many of the proteins shown to be secreted could not be detected in the fluid, probably because the Coomassie Blue staining used for protein quantification of the fluid was not sensitive enough to detect the minor proteins that could, however, be visualized with silver staining. Conversely, many of the proteins found in the fluid could not be detected in the secretion of the tubule (Fig. 15). For instance, in region E1, most (73%) of the proteins found in the fluid were not apparently secreted by that region of the duct. They no doubt originated from fluid further up the tract, probably from the Sertoli cells of seminiferous tubules, rete testis, and/or the efferent ducts; this is probably the case for the alpha 1 B-glycoprotein, a metalloproteinase inhibitor [20], or albumin, not secreted by epididymis but detected throughout the tract. Most of the proteins found in region E1 had disappeared by region E3, probably due to selective reabsorption, as occurs in other species [2]. In regions E4 to E8 of the epididymis, the number of proteins present in the fluid that could not be detected as secreted proteins had increased. These have several possible origins: 1) they may result from processing of particular sperm proteins such as ACE [17]; 2) they may represent low levels of secretion of compounds that had accumulated in the LF and that were not detectable in our assays; and/or 3) they may result from the processing of proteins after secretion, causing a change in their electrophoretic mobility characteristic and thus appearing as new compounds, e.g., PGDS in the stallion and HE1/CTP in the boar [21].

The regions with the most active secretory activity and the highest number of proteins secreted were regions E3–E5 (Table 2 and Figs. 2 and 15). Here the volume of LF around the sperm was the lowest and the protein concentration the highest (Fig. 2). This high secretory activity of the caput has also been found in the boar [8]. The highest protein concentration in the distal caput was previously found in the boar [22], stallion [23], and ram (unpublished results).

Although 117 proteins were secreted, only about 20 were abundant; they represented 98% of the total secretory activity for the whole epididymis. This marked variation in the expression of the protein genes has previously been found in the ram [9] and boar [8]. The major proteins that were secreted also corresponded to the major proteins present in the LF. In the stallion, we found lactoferrin, clusterin, procathepsin D, CTP/HE1, GPX, hexosaminidase, and PGDS to be the most abundant proteins. Among these, hexosaminidase and the mRNA of CTP/HE1 have previously been described in the epididymis of the stallion [24, 25]. Most were also abundant in the epididymis of the ram [9], boar [8], and bull (unpublished results). These 7 proteins represented around 80% of the total secretory activity in the stallion.

From the labeled-methionine findings we report two active regions of the stallion epididymis in which characteristic proteins were synthesized and maximal secretion of such major proteins took place. Regions E0–E2 were char-

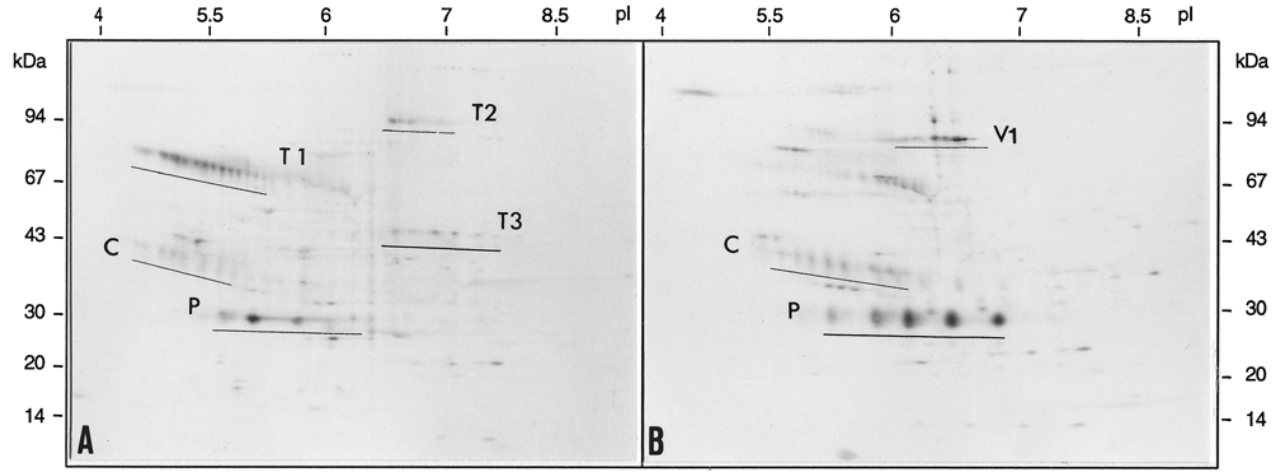

FIG. 11. 2D Electrophoresis fluorographs of proteins synthesized in vitro by testis (**A**) and vas efferens (**B**) tissues from a normal adult stallion. The molecular masses are indicated on the left and on the right and the pI values at the top. C, Clusterin; P, PGDS; T1, T2, T3, and V1 are unidentified trains.

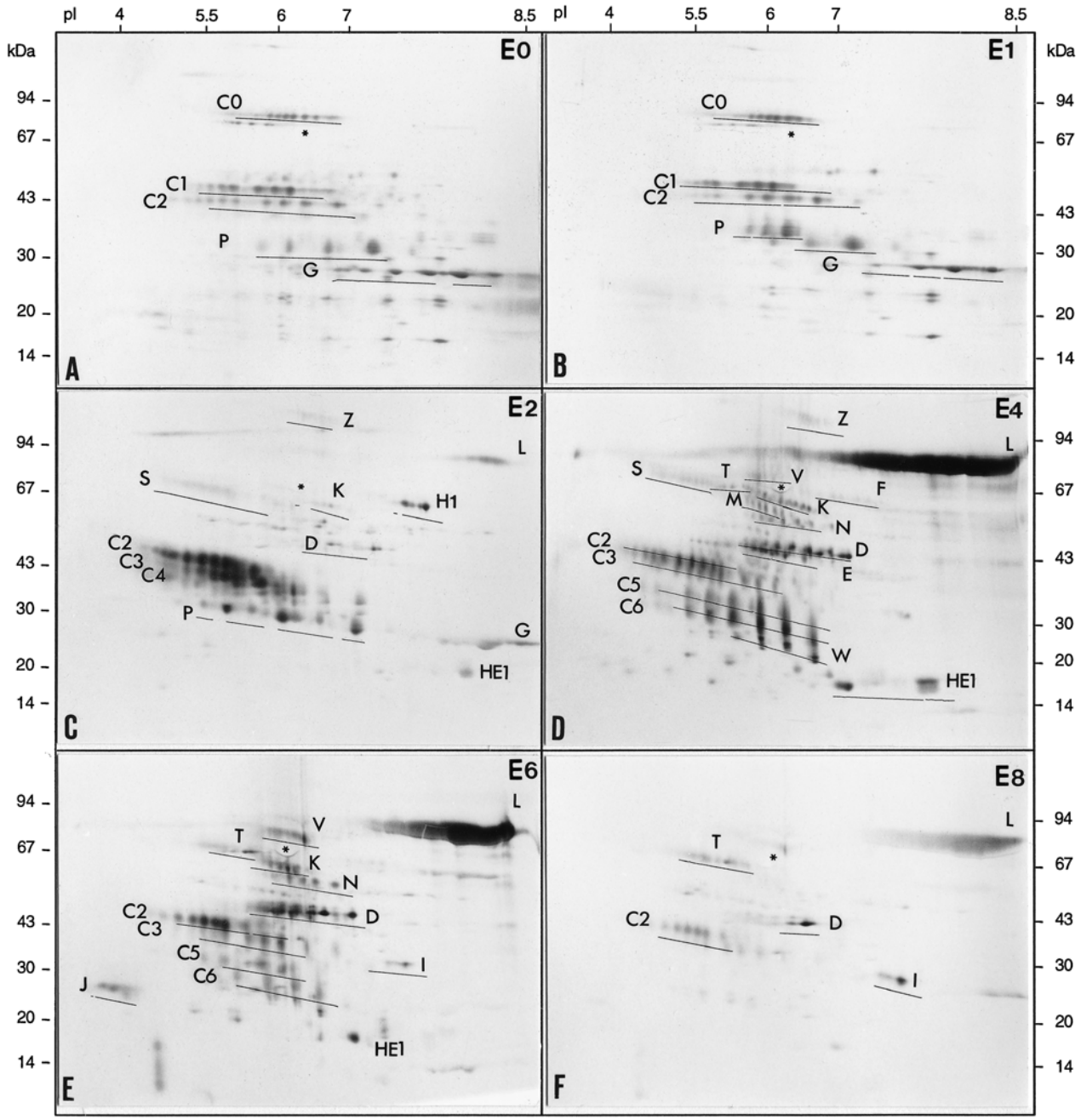

FIG. 12. Epididymal proteins secreted in vitro by isolated tubules and detected in 2D fluorographs of LF from E0 (**A**), E1 (**B**), E2 (**C**), E4 (**D**), E6 (**E**), and E8 (**F**) (E0 and E1 from one stallion [S4] and E2 to E8 from another [S8]). The molecular masses are indicated on the left and on the right and the pI values at the top. (*), Position of albumin (not secreted). For abbreviations, see Figure 3. Trains F, I, J, M, N, S, T, V, and W represent unidentified proteins.

acterized by the secretion of 23 specific compounds and by the highest secretion of PGDS, hexosaminidase, GPX, and clusterin, whereas regions E3–E5 was characterized by 30 specific compounds and by the highest secretion of procathepsin D, lactoferrin, and CTP/HE1. The distal segment of the epididymis, regions E6–E9, had very limited secretory activity, as in other species [8, 9].

This epididymal biochemical regionalization in the stallion is comparable with that of the boar and ram, where the two most biochemically active regions occur in the proximal and distal caput [8, 9].

In the stallion, the most active regions (E0 to E5) may be functionally similar to the caput of the boar and ram, where gross anatomy varies somewhat in being curved in the region of the mid-caput. This is also supported by the fact that in the stallion, the migration of the cytoplasmic droplet along the sperm tail, which may be used as a physiological marker of distal caput function, occurs in regions E4–E5 (Fouchécourt et al., unpublished results). Region E5 therefore belongs functionally to the epididymal caput, although it is anatomically described as the first region of the corpus (Fig. 1).

There does not appear to be a direct relationship for a given protein between the intensity of secretion of the epithelium and the accumulation of the material in the LF. The quantity of protein in the fluid at a particular location appeared to be the result, not only of its secretion, but also of its resorption, degradation, passive concentration, and/or

TABLE 2. Proteins secreted in the various regions of the epididymis and distribution of the major proteins[a] (expressed as percentage of total secretion).

Parameter	Regions of the epididymis								
	E1	E2	E3	E4	E5	E6	E7	E8	Total
Total number of spots (n = 326 for whole epididymis)	41	120	101	161	171	132	81	66	—
Total number of proteins (n = 117 for whole epididymis)	21	32	44	40	60	49	30	26	—
Number of specific proteins[b]	12	10	18	5	7	2	1	0	55
Secretion/region (% of total secretion)	1.2	15.0	21.2	35.6	13.0	7.5	4.6	1.9	100
Protein (%)									
Lactoferrin	—	0.6	7.0	21.0	7.5	2.9	1.6	0.6	41.2
Clusterin	0.3	8.0	6.1	6.2	1.5	1.4	0.9	0.4	24.8
Procathepsine D	—	1.3	2.4	6.9	5.6	8.2	12.9	—	5.2
HE1/CTP	—	0.3	0.5	1.4	0.4	0.2	0.1	—	3.0
Hexosaminidase	—	0.7	2.2	—	—	—	—	—	2.9
PGDS	0.5	1.8	—	—	—	—	—	—	2.3
S	—	0.6	0.1	0.6	0.1	0.1	0.2	0.1	1.9
K	—	0.2	0.4	0.5	0.2	0.3	0.1	0.1	1.8
GPX	0.1	0.8	0.3	0.1	0.1	—	—	—	1.4
I	—	—	—	0.1	0.4	0.3	0.4	0.1	1.3
T	—	—	0.7	0.2	0.1	0.1	0.1	0.1	1.3
N	—	—	0.1	0.4	0.4	0.2	—	—	1.1
E	—	—	0.2	0.3	0.2	0.2	0.1	—	1.1
W	—	—	—	0.4	0.2	0.1	0.1	—	0.8
Z	—	0.2	0.3	0.2	—	—	—	—	0.8
V	—	—	—	0.1	0.2	0.2	0.1	0.1	0.7
F	—	—	0.1	0.2	0.1	0.1	—	—	0.5
M	—	—	0.1	0.4	—	—	—	—	0.5

[a] Protein representing more than 0.5% of the sum of the luminal epididymal secretion.
[b] Restricted to one region.

dilution as a result of water influx across the epididymal blood barrier. The latter was estimated in the present study by determining the spermatocrit for various regions of the duct. It was found that the sperm became 5.5 times more concentrated between regions E0 and E4 and then 3.6 times more diluted between regions E5 and E9.

The protein concentration in region E4 never reached the level calculated from the reabsorption and increase in secretory activity, since the increase in total protein between regions E1 and E3 was less than 2-fold despite the occurrence of a 3-fold decrease in LF volume (Figs. 2 and 9). This discrepancy may be due to selective disappearance of these proteins, e.g., the testicular PGDS isoforms [10]. This could either be due to specific absorption by the epithelium, as has been shown for clusterin in the ram [26], or to the binding of specific proteins to the plasma membrane of the spermatozoa, e.g., GPX [27], clusterin [28], or lactoferrin [29], or to modification(s) of the electrophoretic characteristics of the protein.

The concentration of protein in the corpus and cauda

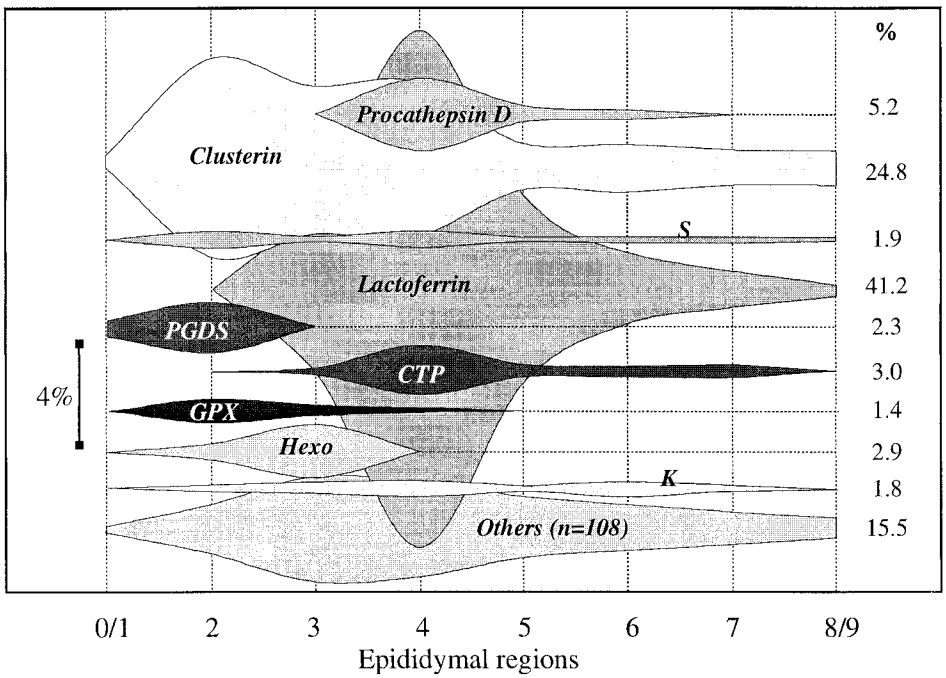

FIG. 13. Distribution of the major secreted stallion proteins according to epididymal region and labeling intensity, expressed as a percentage of total labeled epididymal secretion (values given in Table 2). The percentage values represent the total secretion for a protein in the epididymis. The bar corresponds to 4% of secretion. Each surface represents the total secretion of each compound.

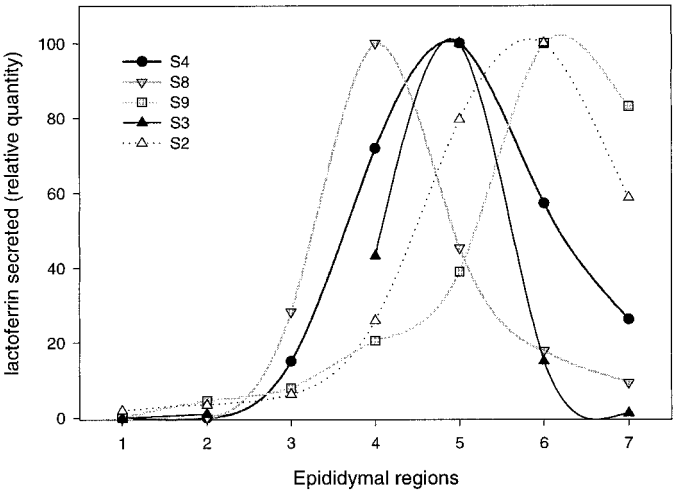

FIG. 14. Distribution of lactoferrin secretion (expressed in relative amounts) according to epididymal region for 5 different stallions analyzed (S2, S3, S4, S8, S9). Maximum secretion occurred between E4 and E6.

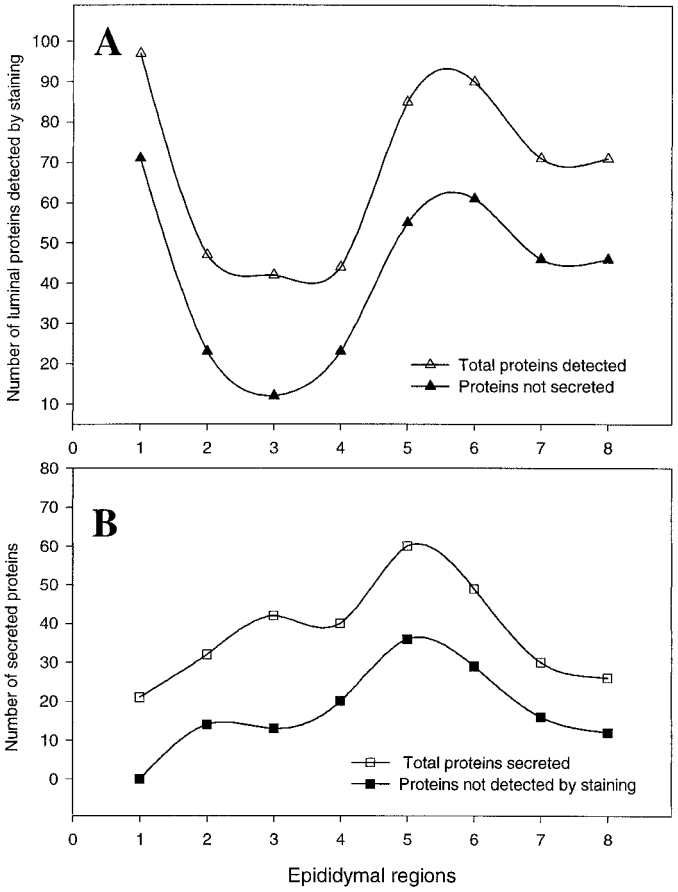

FIG. 15. Number of proteins secreted and stained as detected in the various regions of the epididymis. The results presented were obtained from the analysis of stallion S8. Similar results were obtained with 6 other stallions (S1, S2, S3, S4, S5, S9).

epididymidis and the site of secretion varied for each particular protein. For the proteins secreted only in the caput (e.g., PGDS, hexosaminidase), the progressive decrease in concentration in the fluid may be due either to its disappearance and/or to dilution of the intraluminal milieu. The proteins secreted in the corpus and cauda (e.g., lactoferrin, clusterin, CTP/HE1) only exhibited a small decrease in concentration, which suggests that they are not secreted in high enough concentrations in the distal part of the epididymis to compensate for their passive dilution by water influx. The concentration of clusterin varies according to the isoforms secreted, the Ca and Cb isoforms being exclusively secreted in the anterior caput. Their decrease in the fluid coincides with the reduction in their secretory activity. However, Cc, the third isoform, secreted in the distal caput (E4), has a concentration similar to those of Ca and Cb, even though there is less than half the secretory activity compared to that for the other forms (Fig. 10).

The proteomic analysis performed in this study determined several groups of proteins presenting similar distributions along the tract: 1) proteins present around the spermatozoa as soon as they are formed in the testis and during epididymis transit, such as clusterin, PGDS, albumin; 2) proteins present in the fluid throughout the organ but whose secretion is restricted to a very limited segment of the epididymis, such as hexosaminidase and GPX; 3) proteins secreted through almost all the epididymis, for example lactoferrin and HE1/CTP. This distribution is fairly similar in all the species studied. The relationship between the distribution of these proteins and their roles in sperm maturation and/or epididymal physiology is not yet understood.

The biochemical function of some identified epididymal proteins might suggest a potential role in epididymal physiology [30]. Some of the proteins found in the fluid might be involved in sperm preservation and storage (e.g., GPX and lactoferrin, both of which have potential antioxidant roles); they may therefore protect the plasma membrane of the spermatozoon from the damaging effects of reacting oxygen species [27, 29]. In the stallion, the high concentration of lactoferrin found in the fluid of regions E4 to E8 (2–12 mg/ml) suggests the possibility that this compound may be more important in this species than in some others. Varying concentrations and sites of secretion between animals seemed not to be related to age of animals or size of the epididymis. Such variability has to be researched in the ejaculate and related to sperm quality and fertility of the animals.

Other major proteins might be involved in transporting or binding of hydrophobic compounds, e.g., clusterin [31], HE1/CTP [21], and PGDS [32]. The significance of such functions for sperm maturation and on epididymal regulation is, however, not clear. Of the proteins with enzyme activity (hexosaminidase, procathepsin D, and PGDS), the specific substrate has not yet been identified and the in vivo enzymatic activity has not yet been clearly established (as it has for PGDS [33, 34]).

It is perhaps surprising that the secretion of several minor and major proteins is restricted to a very limited region of the duct. For the majority, this is in the anterior region of the epididymis where significant modification of the sperm occurs. However, few of these proteins have been sequenced. More information is needed concerning these several hundred proteins before informed speculation can be offered on their importance and function in relation to sperm maturation.

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