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Distribution of Pz-Peptidase in Bovine Epididymal and Ejaculated Semen

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ABSTRACT

Bovine epididymal or ejaculated semen was fractionated by density gradient centrifugation in Percoll®, and seminal components recovered from the gradients were subjected to additional separation and washing steps. This procedure resulted in isolation of four major seminal constituents: particle-free extracellular fluid, washed light particulates, washed cytoplasmic droplets, and washed spermatozoa. When assayed using the Pz-peptide substrate, all the isolated seminal fractions contained substantial Pz-peptidase activity. The extracellular fluid Pz-peptidase was present in soluble form, but Triton X-100 was required for complete extraction of the Pz-peptidase activity from the spermatozoa, cytoplasmic droplets, and light particulates. The greatest Pz-peptidase activities were observed in the cytoplasmic droplet and epididymal sperm extracts, whereas the activities in extracellular fluid, extracts of light particulates, and extracts of ejaculated spermatozoa were relatively low. Most of the Pz-peptidase activity in extracts of epididymal spermatozoa was attributable to cytoplasmic droplets. The specific Pz-peptidase activities found by regression analysis were 6.1 mU/billion attached cytoplasmic droplets and 1.1 mU/billion spermatozoa. These results established that in the bovine, cytoplasmic droplets were the major source of Pz-peptidase activity in semen and that Pz-peptidase was not primarily a spermatozoal enzyme.

INTRODUCTION

The Pz-peptide is a synthetic substrate originally developed for assay of bacterial collagenases (Wünsch and Heidrich, 1963a). The Pz-peptide is cleaved rapidly by the collagenase from *Clostridium histolyticum* (EC 3.4.24.3) but is resistant to hydrolysis by many other common peptidases (Wünsch and Heidrich, 1963b; Strauch, 1970). Initially, the Pz-peptide was employed to survey mammalian tissues for collagenase activity (Strauch and Vencelj, 1967), but it was subsequently recognized that the Pz-peptide is not a specific substrate for vertebrate tissue collagenases (Heidrich et al., 1969; Harper and Gross, 1970). More recent reports have stressed the need to distinguish between authentic tissue collagenases which cleave native collagen fibers and those peptidases which hydrolyze synthetic substrates such as the Pz-peptide but are not capable of hydrolyzing native collagen (Seifter and Harper, 1970; Lazarus, 1973). Peptidases of the latter type are commonly referred to as "Pz-peptidases." Pz-peptidases have been identified in a wide variety of vertebrate tissues (Heidrich et al., 1973; Aswanikumar and Radhakrishnan, 1975; Morales and Woessner, 1977) and appear to constitute a distinct class of related peptidases (EC 3.4.99.31). Although Pz-peptidases do not hydrolyze native collagen directly, it has been proposed that they participate in normal collagenolysis by further degrading the fragments resulting from cleavage of collagen by authentic tissue collagenases (Harris and Krane, 1972; Harris et al., 1978).

Pz-peptidases were first identified in male reproductive tissues by Koren and Milković (1973), who reported that extracts of spermatozoa and testicular tissue from several mammalian species contained a "collagenase-like" peptidase capable of hydrolyzing the Pz-peptide. It has been proposed that Pz-peptidase promotes release of spermatozoa from the copulatory plug in rodents (Koren et al., 1974; Koren et al., 1975) and is responsible for the first phase of liquefaction of human ejaculates in vitro (Koren and Lukač, 1979; Lukač and Koren, 1979). However, these conclusions have been questioned by other investigators (Morton, 1977; Daunter et al., 1981), and the physiological function of Pz-peptidase in male reproductive tissues remains unclear.

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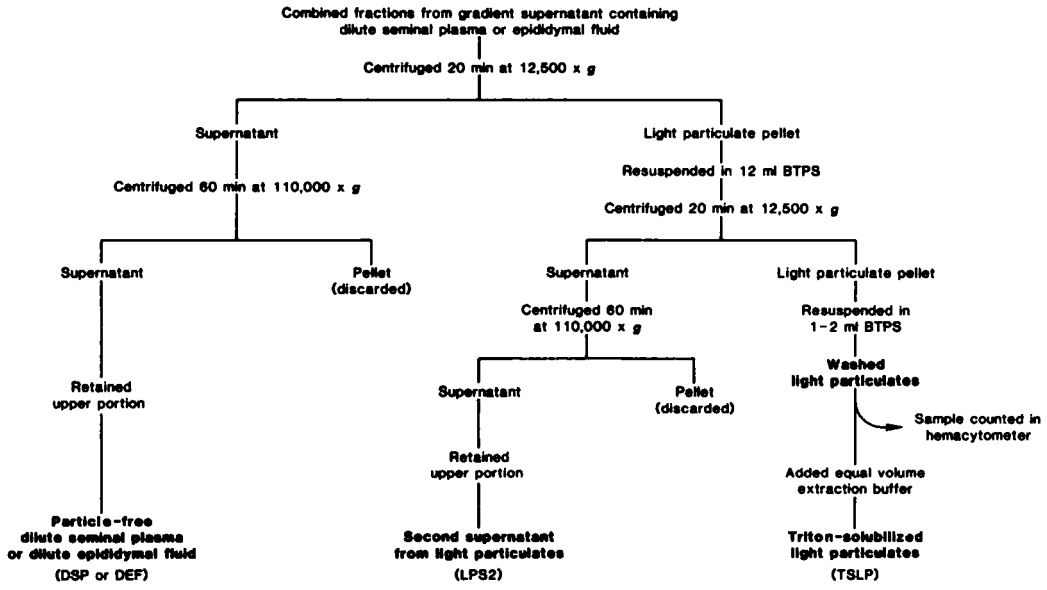

FIG. 1. Flow diagram of steps in procedure used for isolation of dilute seminal plasma or epididymal fluid and light particulates. BTPS is bis-tris propane buffered saline.

Knowledge of the distribution of Pz-peptidase activity among major seminal components would provide insight into the potential functions of Pz-peptidase. The distribution of Pz-peptidase in human ejaculates was examined by Koren and Milković (1973) but the results were inconclusive. Therefore, the present study was undertaken to investigate the distribution of Pz-peptidase activity among the major components of bovine epididymal and ejaculated semen.

MATERIALS AND METHODS

Reagents

Pz-peptide (4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg) and Pz-Pro-Leu were purchased from Tridom Chemical Inc., Hauppauge, NY. Percoll was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. 1,3-*bis* [*tris* (hydroxymethyl) methylamino] propane (bis-tris propane, BTP) and [ethylene-*bis* (oxyethylenenitrile)] tetraacetic acid (EGTA), were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade and deionized distilled water was used throughout.

Collection of Epididymal and Ejaculated Semen

Ejaculated semen was collected from bulls of various breeds by electroejaculation and allowed to cool slowly to ambient temperature (22 ± 2°C). Bovine epididymides were obtained at an abattoir and processed within 2 h after slaughter. All subsequent manipulations of semen or tissues were conducted at ambient temperature. The luminal contents of distal cauda epididymides were recovered by introducing a retrograde flow of BTP-buffered saline (10 mM BTP-chloride, 150 mM sodium chloride, pH 7.5; BTPS) into the vas deferens under constant pressure (Czarnetzky and Henle, 1938). Semen samples were examined microscopically to confirm normal sperm structure and motility. The samples of ejaculated semen used were selected for containing a small proportion of spermatozoa with attached cytoplasmic droplets. Ejaculated semen was diluted 1:1 with BTPS before fractionation. Epididymal sperm suspensions were diluted with BTPS to approximately 10 times the volume of the initial portion of epididymal contents recovered. For each experiment, 3–5 ejaculates or the contents of 1–3 pairs of epididymides were combined.

Isolation of Seminal Components

The diluted sperm suspensions were separated by centrifugation in Percoll density gradients as described previously (Lessley and Garner, 1983), except that Percoll was made isotonic using 20-fold concentrated BTPS and discontinuous gradients were used. The gradients were prepared by placing 5 ml of 100% Percoll in a 50-ml polycarbonate centrifuge tube and successively layering 3 ml each of 75%, 65%, 55%, 45%, 35%, 25%, and 15% Percoll into the tube. For each experiment, 15 ml of diluted epididymal or ejaculated semen was layered over each of two Percoll gradients and centrifuged for 45 min at 1500 × *g* in a swinging bucket rotor (IEC type 279 rotor; 2400 rpm). Centrifugation and subsequent fractionation of the gradients were conducted at ambient temperature.

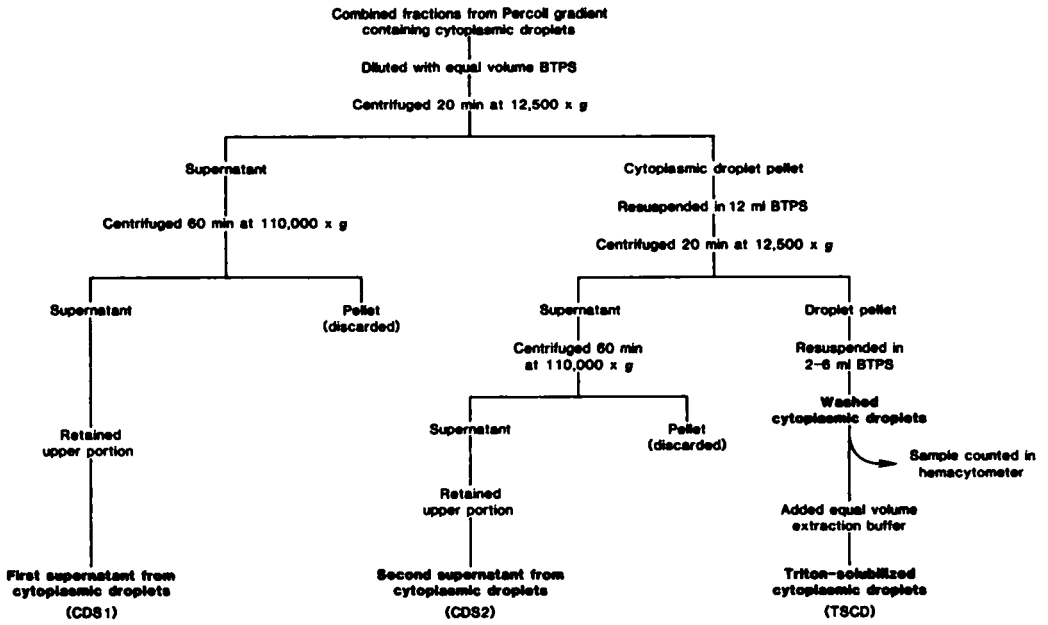

FIG. 2. Flow diagram of steps in procedure used for isolation of cytoplasmic droplets. BTPS is bis-tris propane buffered saline.

After centrifugation, the upper two-thirds of the gradients were fractionated as described previously (Lessley and Garner, 1983). The first 10–12 fractions from both gradients were combined, avoiding inclusion of fractions from the supernatant fluid-Percoll interface, and designated crude seminal plasma or epididymal fluid. The next 10–15 fractions from both gradients were individually examined microscopically, and 5–6 fractions from each gradient containing the greatest concentrations of cytoplasmic droplets with the fewest contaminating particles were combined and designated the crude cytoplasmic droplet preparation. The concentrated mass of spermatozoa located above the 100% Percoll layer was removed manually using a 5-ml adjustable pipet.

The crude preparations recovered from the Percoll gradients were subjected to further fractionation and washing procedures adapted from those described by Garbers et al. (1970). Flow diagrams containing the details of these procedures are presented in Figs. 1, 2 and 3. Briefly, the seminal plasma or epididymal fluid was recentrifuged at greater forces to recover the light particulates. The light particulates were washed once to reduce extracellular fluid contamination. Residual particles were removed from the seminal plasma or epididymal fluid by ultracentrifugation. The cytoplasmic droplets were washed twice to remove Percoll and traces of extracellular fluid. The spermatozoa were washed twice to reduce the Percoll concentration. Selected wash supernatants from the isolation procedure were retained and the residual particles removed by ultracentrifugation.

Conditions for centrifugation were as follows: 350 X *g*, 1200 rpm in an IEC type 279 swinging bucket rotor; 12,500 X *g*, 9000 rpm in a Sorvall HB-4 swinging bucket rotor; 110,000 X *g*, 35,000 rpm in a Beckman type 50 Ti angle rotor. Centrifugation at 350 X *g* was performed at ambient temperature; all other centrifugation procedures were performed at 5°C. The centrifugation steps employed for isolation of light particulates and cytoplasmic droplets were performed simultaneously.

Extraction of Spermatozoa and Particles

Pz-peptidase was extracted from the spermatozoa, cytoplasmic droplets, and light particulates using either direct detergent solubilization or a combination of physical disruption and detergent solubilization (see Figs. 1, 2 and 3). All extraction procedures were conducted at 5°C. The extraction buffer contained 50 mM BTP-chloride, 50 mM sodium chloride, 50 mM benzamidine, 1 mM calcium chloride, 1 mM dithiothreitol, and 2% (v/v) Triton X-100; the pH was 7.6. The washed light particulates and the washed cytoplasmic droplets were solubilized by adding an equal volume of extraction buffer (final Triton concentration was 1%). The resulting extracts were designated the Triton-solubilized light particulates (TSLP) and the Triton-solubilized cytoplasmic droplets (TSCD).

Spermatozoa were first disrupted by nitrogen decompression (Hunter and Commerford, 1961). The washed spermatozoa were loaded into a 250-ml Yeda cell press (LINCA Lamon Instrumentation Co., Ltd., Tel-Aviv, Israel) and pressurized to 2000 lb/in^2 with nitrogen. After equilibration with stirring for 30 min, the contents of the cell press were expressed through the needle valve. The resulting sperm homogenate was diluted with an equal volume of extraction buffer (final Triton concentration of 1%) and mixed oc-

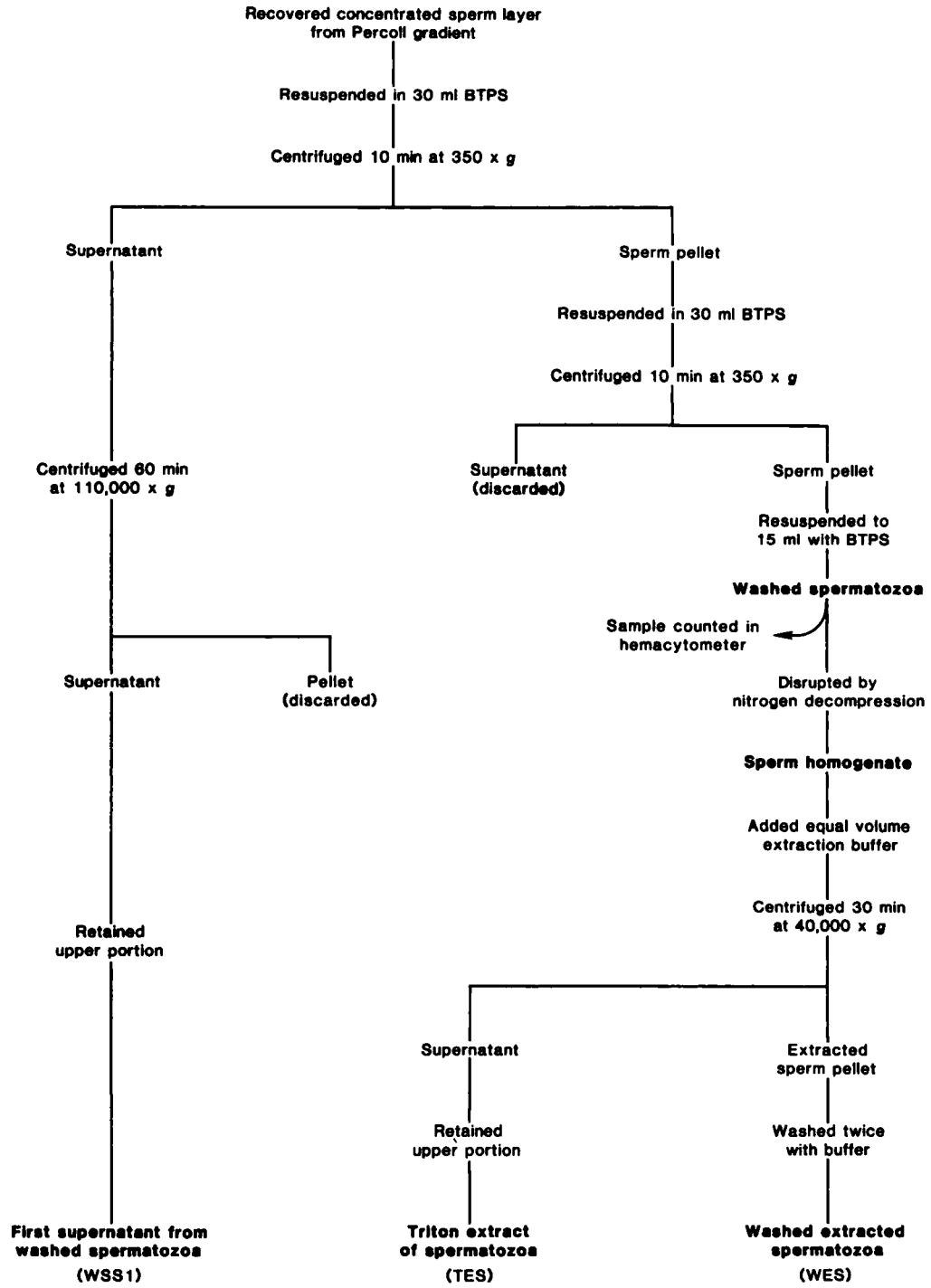

FIG. 3. Flow diagram of steps in procedure used for isolation and extraction of spermatozoa. BTPS is bis-tris propane buffered saline.

casionally for 15 min. The sperm homogenate was then centrifuged for 30 min at 40,000 X *g* in an angle rotor (Sorvall SS-34; 18,000 rpm). The supernatant was designated the Triton extract of spermatozoa (TES). The sperm pellet was washed twice in extraction buffer from which Triton X-100 had been omitted. The final sperm pellet was resuspended in 15 ml extraction buffer without Triton and designated the washed extracted spermatozoa (WES).

Determination of Pz-peptidase Activity

Pz-peptidase activity was assayed by a procedure incorporating several modifications of the original method described by Wünsch and Heidrich (1963b). The substrate solution contained 2.5 mM Pz-peptide dissolved directly in assay buffer containing 50 mM BTP-chloride, 1 mM calcium chloride, and 0.1 mM dithiothreitol; the pH was 7.5 at 30°C. For each assay, 4 ml of substrate solution was prewarmed to 30°C. To initiate the assay, 1.0 ml of enzyme solution was added to the substrate solution and the mixture was incubated at 30°C. At eight intervals during the incubation, 0.5 ml of the assay medium was withdrawn and added to 0.5 ml of acidification reagent to stop the reaction. Sampling intervals ranged from 10 to 30 min, depending on the activity. For samples containing Triton X-100, the reagent used to acidify the assay medium was 0.2 M phosphoric acid; for samples without detergent, the acidification reagent contained 0.2 M phosphoric acid and 0.2% (v/v) Triton X-100. All portions of assay medium contained 0.1% Triton X-100 after acidification. Following completion of the incubation, the hydrolysis product was extracted from the acidified portions of assay medium by shaking with 5.0 ml of butyl acetate in sealed tubes. To promote separation of the phases after extraction, the tubes were cooled at 0°C and centrifuged for 10 min at 250 X *g* in a swinging bucket rotor (IEC type 240 rotor; 1000 rpm). After warming to ambient temperature, the absorbance of the butyl acetate phase was determined at 322 nm using stoppered cuvettes. The concentration of hydrolysis product in the butyl acetate phase was calculated from the absorbance using a molar extinction coefficient for Pz-Pro-Leu of 20,200 M^{-1} cm^{-1}. This value was determined experimentally from the absorbance of Pz-Pro-Leu in butyl acetate at 322 nm and was similar to the extinction coefficient of Pz-Pro-Leu in ethyl acetate at 320 nm (Wünsch and Heidrich, 1963b).

For calculation of activity, the amount of Pz-Pro-Leu produced at each interval during the assay was plotted versus time. The linear portion of the rate plot was analyzed by least squares regression, and the slope used to calculate the initial rate of hydrolysis. One unit of Pz-peptidase activity was defined as that amount of enzyme which produced one micromole of Pz-Pro-Leu min^{-1} ml^{-1} at 30°C. Pz-peptidase activity was routinely expressed as milliunits/ml (mU/ml), equivalent to nanomoles of Pz-Pro-Leu produced min^{-1} ml^{-1}.

Other Procedures

Protein concentration was determined by the method of Bradford (1976) using bovine gamma globulin as a standard. For samples containing Triton X-100, blanks containing equivalent amounts of Triton X-100 were used to compensate for the absorbance attributable to the detergent.

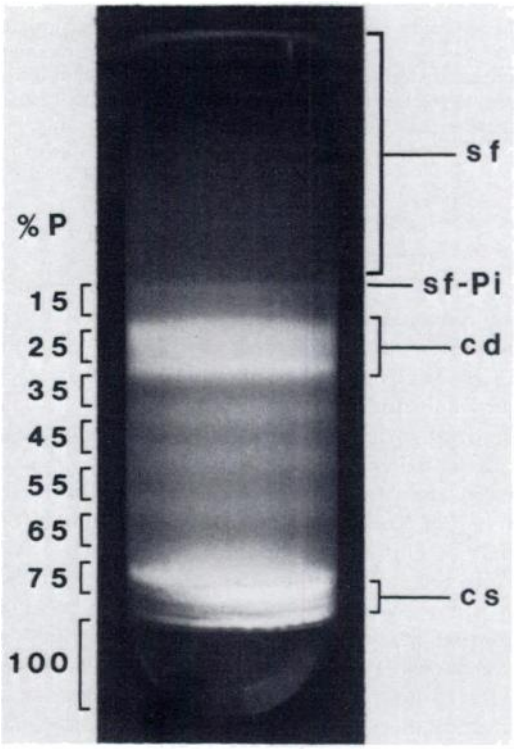

FIG. 4. Appearance of gradient after fractionation of semen by density gradient centrifugation in Percoll. Fifteen ml of diluted bovine ejaculated semen containing a total of 6.5 billion spermatozoa was layered over a discontinuous Percoll gradient and centrifuged for 45 min at 1500 X *g*. The resulting distribution of seminal constituents within the gradient was photographed against a dark background using indirect lighting. The figures labeled *%P* to the left of the photograph refer to the percentage of Percoll in each layer of the gradient. Abbreviations used: *sf*—supernatant fluid; *sf-Pi*—supernatant fluid-Percoll interface; *cd*—cytoplasmic droplets; *cs*—concentrated spermatozoa. The faint bands visible between the cytoplasmic droplet layer and the concentrated sperm layer were due to retention of particles and spermatozoa at the interfaces of adjacent Percoll layers.

The concentrations of spermatozoa and cytoplasmic droplets were determined by counting in a hemacytometer chamber using phase-contrast microscopy. Samples were withdrawn from the unfractionated semen, washed light particulates, washed cytoplasmic droplets, and washed spermatozoa during the isolation procedure and fixed immediately by dilution in phosphate-buffered saline containing 37 mM sodium cacodylate and 0.2% glutaraldehyde (Johnson et al., 1976). Three dilutions were prepared from each sample and duplicate counts were performed on each dilution. In the light particulate and cytoplasmic droplet preparations, only those particles clearly

recognizable as intact cytoplasmic droplets were counted as such. For unfractionated semen and washed sperm suspensions, the number of cytoplasmic droplets included both detached droplets and those droplets attached to spermatozoa.

RESULTS

Isolation and Characterization of Seminal Constituents

After centrifugation, the major seminal components were distributed among three well-defined zones within the Percoll gradient (Fig. 4). The diluted seminal plasma or epididymal fluid remained above the Percoll gradient. This supernatant fluid still contained substantial amounts of particulate matter which were later recovered in the light particulate fraction. The cytoplasmic droplets sedimented only a short distance into the gradient and collected within the 25% Percoll layer. The spermatozoa sedimented nearly the full length of the gradient and were concentrated into a compact band above the 100% Percoll layer. The procedures employed for subsequent fractionation and washing of the crude preparations recovered from the Percoll gradient produced four major seminal fractions: particle-free seminal plasma or epididymal fluid, washed light particulates, washed cytoplasmic droplets, and washed spermatozoa. A large proportion of the washed spermatozoa displayed normal progressive motility.

The microscopic appearances of the isolated seminal fractions are shown in Fig. 5. Washed ejaculated spermatozoa (Fig. 5a) appeared structurally intact and were essentially free of contamination by detached droplets or other particles. Washed epididymal spermatozoa (Fig. 5b) were similar except that a large proportion of the spermatozoa possessed an attached droplet. The washed cytoplasmic droplet fraction from ejaculated semen (Fig. 5c) contained primarily droplets, but substantial quantities of other unidentified particles were also present. Many of the cytoplasmic droplets from ejaculated semen appeared wrinkled or irregular in outline. In contrast, the washed cytoplasmic droplets from epididymal samples (Fig. 5d) were more homogeneous in appearance; almost all the particles were clearly identifiable as intact droplets. The washed light particulates from ejaculated semen consisted mostly of small irregular particles (Fig. 5e). The washed light particulates from epididymal semen (not shown) were similar in appearance.

Homogeneity of the major seminal fractions was evaluated using the sperm and cytoplasmic droplet concentrations determined with the hemacytometer. The results for representative samples of epididymal and ejaculated semen are presented in Table 1. Most of the seminal fractions were recovered with minimum contamination by other constituents. One exception was the washed epididymal sperm preparation, which contained a large proportion of spermatozoa with attached cytoplasmic droplets. Also, the cytoplasmic droplet preparations from ejaculated semen contained substantial amounts of contamination by other types of particles which were not reflected in these counts.

Modified Pz-peptidase Assay

The modifications incorporated into the Pz-peptidase assay resulted in reduced blank absorbances caused by extraction of unhydrolyzed Pz-peptide into the solvent phase (Lessley and Garner, unpublished data) and allowed the use of greater substrate concentrations than were previously practical. The rate of hydrolysis of the Pz-peptide was linear for only a limited period even under the modified assay conditions. The problems associated with nonlinearity were circumvented by sampling the assay mixture at multiple intervals to generate an approximation of a continuous rate assay. The variation between duplicate assays was commonly 1–2% and rarely exceeded 5%. The lower limit of sensitivity for the assay was approximately 0.04 mU/ml.

Solubilization of Pz-peptidase from Spermatozoa and Particles

Preliminary experiments indicated that the Pz-peptidase was not completely solubilized from spermatozoa by mechanical disruption alone. Although almost all spermatozoa were effectively disrupted by homogenization in the Yeda press (Fig. 5f), approximately one-third of the total Pz-peptidase activity in the homogenate was recovered in the washed extracted sperm pellet. Addition of 1.0 M sodium chloride, 1.0 M magnesium chloride, or 2.5 mM EGTA to the extraction medium did not decrease the residual Pz-peptidase activity of the washed extracted spermatozoa. Inclusion of Triton X-100 in the extraction medium, however, reduced the Pz-peptidase activity of the washed extracted sperm pellet below detect-

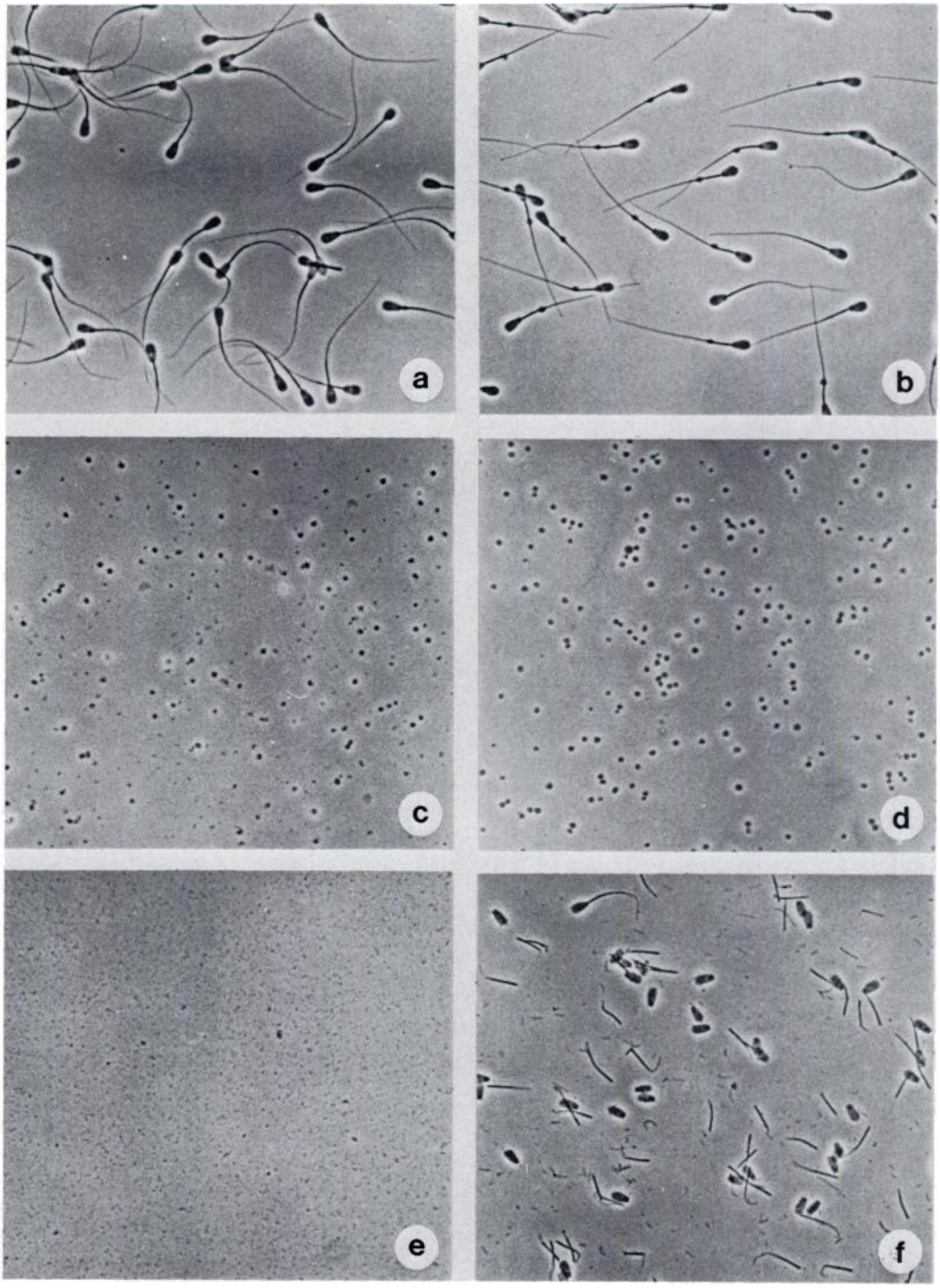

FIG. 5. Microscopic appearance of major seminal fractions from the isolation procedure. Glutaraldehyde fixed samples of: *a*) washed ejaculated spermatozoa; *b*) washed epididymal spermatozoa; *c*) washed ejaculated cytoplasmic droplets, *d*) washed epididymal cytoplasmic droplets; *e*) washed ejaculated light particulates; *f*) Yeda press homogenate of washed ejaculated spermatozoa. Photographed using phase contrast microscopy. ×290.

TABLE 1. Homogeneity of major seminal fractions from representative isolation procedures.

Preparation analyzed	Spermatozoa million/ml		Cytoplasmic droplets million/ml		Droplet/sperm ratio	
	Ejaculated	Epididymal	Ejaculated	Epididymal	Ejaculated	Epididymal
Diluted semen before fractionation	356 ± 28[a]	668 ± 94	198 ± 35	600 ± 61	0.56	0.90
Washed spermatozoa	574 ± 54	913 ± 133	3 ± 5	658 ± 83	<0.01	0.72
Washed cytoplasmic droplets	1 ± 2	3 ± 3	480 ± 40	1002 ± 32	480	334
Washed light particulates	1 ± 1	1 ± 1	2 ± 1	1 ± 1	---	---

[a]Each value represents the mean ± the standard deviation of six replicate counts conducted using a hemacytometer. One experiment with epididymal semen and one with ejaculated semen were selected as being representative of the overall results. For each source, the data recorded were the results of counts performed on the fractions obtained from a single isolation procedure. Therefore, the standard deviation reflects the variation of the replicate counts within a particular fraction.

ability and resulted in a corresponding increase in the activity of the sperm extract. Similar results were obtained with cytoplasmic droplets and light particulates; addition of Triton X-100 solubilized these particles almost completely and was effective in releasing the Pz-peptidase activity.

The extraction medium contained several compounds which could have affected the Pz-peptidase activity. Benzamidine, dithiothreitol, and Triton X-100 were tested individually for their effects on epididymal cytoplasmic droplet Pz-peptidase at concentrations equivalent to those present during assay of the extracts. The Pz-peptidase activities relative to untreated controls were 124% for benzamidine, 91% for dithiothreitol, and 93% for Triton X-100. It was also found that the presence of Triton X-100 had no adverse effect on the efficiency of extraction of Pz-Pro-Leu from the assay medium.

Pz-peptidase Activity of Isolated Seminal Fractions

The Pz-peptidase activities of the major seminal fractions isolated from both epididymal and ejaculated semen are summarized in Table 2. Substantial quantities of Pz-peptidase activity were consistently found associated with all of the seminal constituents. However, cytoplasmic droplets were the richest source of Pz-peptidase activity. This was apparent from the high activities present in extracts of cytoplasmic droplets from both epididymal and ejaculated semen as well as the large difference in activities of ejaculated and epididymal sperm extracts. Extracts of ejaculated spermatozoa, only a few percentage of which had attached cytoplasmic droplets, contained one of the lowest levels of Pz-peptidase activity among the major seminal constituents.

The Pz-peptidase activities of most of the wash supernatants saved from the isolation procedure (see Figs. 1, 2 and 3) were negligible compared to the activities of the major seminal fractions. The activity of the LPS2 (ejaculated and epididymal) and the WSS1 from ejaculated spermatozoa were usually below the limit of detection. However, substantial amounts of Pz-peptidase activity ranging from 0.1–0.3 mU/ml were consistently found in the CDS1, CDS2 (ejaculated and epididymal), and somewhat less activity in the WSS1 from epididymal spermatozoa.

TABLE 2. Pz-peptidase activity of major seminal fractions from isolation procedure.

Preparation assayed	Pz-peptidase activity mU/ml		Specific activity based on protein mU/mg protein		Specific activity based on counts mU/billion particles	
	Ejaculated	Epididymal	Ejaculated	Epididymal	Ejaculated	Epididymal
Dilute seminal plasma or epididymal fluid	0.47 ± 0.17[a]	0.79 ± 0.42	0.01 ± 0.001	0.31 ± 0.13	1.22 ± 0.59[b]	1.24 ± 0.29[b]
Triton-solubilized light particulates	0.31 ± 0.28	0.17 ± 0.07	0.45 ± 0.29	0.45 ± 0.30	---	---
Triton-solubilized cytoplasmic droplets	1.62 ± 0.30	1.48 ± 0.77	1.33 ± 0.14	0.99 ± 0.36	7.40 ± 2.23[c]	3.65 ± 0.67[c]
Triton extract of spermatozoa	0.31 ± 0.08	1.92 ± 0.52	0.19 ± 0.03	0.64 ± 0.18	1.19 ± 0.25[d]	5.20 ± 1.02[d]

[a]Each value represents the mean ± the standard deviation of the results from three individual isolation procedures.

[b]Expressed as activity per billion spermatozoa present in the diluted semen before fractionation.

[c]Expressed as activity per billion cytoplasmic droplets present in the washed cytoplasmic droplet fraction before solubilization.

[d]Expressed as activity per billion spermatozoa present in the washed spermatozoa fraction before extraction.

Specific Activities and Distribution of Pz-peptidase

When the Pz-peptidase activities of the major seminal preparations were expressed as specific activities based on protein content or concentration of cells and particles (Table 2), cytoplasmic droplets still had the greatest specific activity. The count-based specific activities of the cytoplasmic droplets from ejaculated and epididymal sources were significantly different ($P<0.05$), but there was no significant difference ($P>0.10$) between the specific activities of ejaculated and epididymal cytoplasmic droplets when based on protein content.

The Pz-peptidase activities of dilute epididymal fluid and seminal plasma were not significantly different ($P>0.20$) when expressed as mU/billion spermatozoa present in the corresponding unfractionated samples (Table 2).

Independent estimates for the Pz-peptidase activity of spermatozoa and attached cytoplasmic droplets were obtained by plotting the Pz-peptidase activity of Triton extracts of spermatozoa versus the proportion of spermatozoa with attached cytoplasmic droplets in the corresponding washed sperm preparations (Fig. 6). Least squares analysis of the data indicated a strong correlation ($r=0.98$) between the proportion of spermatozoa with attached cytoplasmic droplets and the Pz-peptidase activity in the sperm extract. The *y*-intercept of the regression line (1.11 ± 0.13 SD) represented the Pz-peptidase activity attributable to spermatozoa alone expressed in mU/billion spermatozoa, whereas the slope (6.14 ± 0.30 SD) represented the Pz-peptidase activity of attached cytoplasmic droplets in mU/billion droplets. This estimate for the specific activity of attached epididymal cytoplasmic droplets was significantly greater ($P<0.01$) than the corresponding estimate for detached epididymal cytoplasmic droplets (see Table 2). When the data from epididymal samples only were analyzed by regression, the estimate for Pz-peptidase activity attributable to spermatozoa alone was 0.90 ± 0.47 mU/billion spermatozoa. This value was not significantly different ($P>0.10$) from the estimate obtained with the combined data, indicating that no difference existed between the Pz-peptidase activities of ejaculated and epididymal spermatozoa when corrected for the contribution of cytoplasmic droplets.

The overall distribution of Pz-peptidase was estimated by adjusting the specific activities of the seminal constituents to reflect the contribution of each to 1 ml of unfractionated sperm suspension. These calculations indicated that cytoplasmic droplets contibuted approximately two-thirds of the total activity, whereas the remainder was almost evenly divided between the extracellular fluid and spermatozoa. The contribution of light particulates was less than 1% of the total activity of the other fractions. Similar results were obtained for both epididymal and ejcaulated semen.

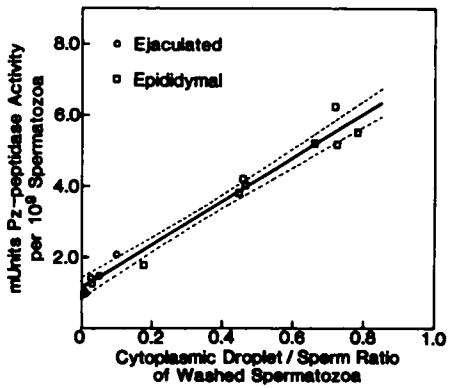

FIG. 6. Relationship between Pz-peptidase activity of sperm extract and proportion of washed spermatozoa with attached cytoplasmic droplets. The data from 14 separate isolation experiments were analyzed by least squares regression. The caluclated regression line is represented by the *solid line*; the 95% confidence limits for the regression line are represented by *broken lines*. The regression line equation was $y=6.14x + 1.11$. The correlation coefficient was 0.98.

DISCUSSION

The peptidase from male reproductive tissues responsible for hydrolysis of the Pz-peptide, a substrate originally developed for assay of collagenases, was initially designated a "collagenase-like" peptidase (Koren and Milković, 1973). The more general term, Pz-peptidase, has been used throughout the present report to indicate solely that the seminal peptidase under consideration is capable of hydrolyzing the Pz-peptide.

Fractionation of semen by centrifugation in Percoll gradients proved very useful as a preliminary step in isolation of the major seminal components, facilitating simultaneous separation of extracellular fluid, cytoplasmic droplets, and spermatozoa under conditions involving a

minimum of osmotic and mechanical stress (Pertoft et al., 1978). This is an important consideration, as damage to spermatozoa or cytoplasmic droplets during isolation could result in redistribution of enzyme activity (Dott and Dingle, 1968; Garbers et al., 1970). The retention of motility by the spermatozoa, maintenance of structural integrity of the spermatozoa and cytoplasmic droplets, the relatively low activity in most wash supernatants, and the incomplete solubilization of Pz-peptidase by physical disruption alone all suggested that the activities of the isolated seminal fractions were representative of the distribution of Pz-peptidase in unfractionated semen. Some soluble Pz-peptidase was released from cytoplasmic droplets during isolation, but partial loss of activity from the droplets would not invalidate the major conclusions of this study. It is difficult to evaluate the possible influence of naturally occurring inhibitors and activators, zymogen precursors, or multiple forms of Pz-peptidase on the apparent distribution of Pz-peptidase activity in semen, as the seminal Pz-peptidase has not been well characterized.

There was some difficulty in obtaining accurate estimates of the specific Pz-peptidase activity of cytoplasmic droplets. The count-based specific activity of droplets isolated from ejaculated semen was significantly greater than the corresponding value for epididymal droplets. As there was no significant difference between the specific activities of ejaculated and epididymal cytoplasmic droplets when based on protein content, this discrepancy may have been related to the other particles present in the ejaculated cytoplasmic droplet preparation. Some of the particles not counted as cytoplasmic droplets could have been derived from degenerating droplets and possessed substantial amounts of Pz-peptidase activity. Consequently, the specific activity for ejaculated cytoplasmic droplets may have been overestimated, and the value obtained for epididymal droplets was probably the more reliable estimate of Pz-peptidase activity per billion detached cytoplasmic droplets.

If the Pz-peptidases in all seminal fractions are identical as suggested by the results of Koren and Milković (1973), it is possible that the cytoplasmic droplets were the original source of Pz-peptidase activity in all the isolated seminal fractions. It has been postulated that the boundary membrane of the cytoplasmic droplet may temporarily rupture during detachment from the sperm midpiece, resulting in a partial loss of droplet contents into the extracellular medium (Garbers et al., 1970). The Pz-peptidase activity of detached epididymal cytoplasmic droplets was significantly less than the activity of an equivalent number of attached droplets, providing support for this suggestion. Also, the droplet/sperm ratio of the ejaculated samples was consistently lower than that of the epididymal samples in the present study, suggesting that a substantial fraction of the cytoplasmic droplets originally present in the cauda epididymidis had completely disintegrated. Garbers et al. (1970) observed that the light particulate fraction isolated by their procedure contained primarily membranous vesicles, some of which were similar to the vesicles present inside intact cytoplasmic droplets. The presence of substantial Pz-peptidase activity in the supernatants from isolation of the cytoplasmic droplets provided evidence that soluble Pz-peptidase could be released from cytoplasmic droplets. Therefore, the soluble Pz-peptidase in epididymal fluid and seminal plasma, as well as the Pz-peptidase associated with epididymal and ejaculated light particulates, may have been derived from cytoplasmic droplets. Alternately, some of the particles in the light particulate fraction may originate from the epididymal epithelium (Garbers et al., 1970), and a portion of the light particulate Pz-peptidase could have come from this source. The Pz-peptidase activity present in the sperm extracts may have also originated from the cytoplasmic droplets. It is conceivable that the process involved in detachment of droplets from the spermatozoa does not result in complete segregation, and some remnant of droplet cytoplasm may remain associated with the sperm midpiece. The observation that detergent was required for complete solubilization of Pz-peptidase from the spermatozoa, cytoplasmic droplets, and light particulates suggested that a portion of the Pz-peptidase may have been intracellular or bound to membranes.

Because the present investigation utilized epididymal as well as ejaculated semen, some inferences could be made regarding the distribution of Pz-peptidase within the male reproductive system. The similarity of the Pz-peptidase activity in epididymal fluid and seminal plasma, when expressed as a function of sperm concentration in the unfractionated samples, suggested that most of the soluble

Pz-peptidase present in the seminal plasma could be accounted for by the epididymal contribution to the ejaculate. Thus, it appeared unlikely that the accessory gland secretions were a major source of soluble Pz-peptidase in the bovine ejaculate. It could not be determined whether any of the Pz-peptidase associated with the light particulates originated from accessory gland secretions.

Overall, these investigations clearly established that cytoplasmic droplets were the major source of bovine seminal Pz-peptidase. Based on equivalent numbers, cytoplasmic droplets contained approximately six times as much Pz-peptidase activity as spermatozoa. This result was somewhat unexpected as proteinase activity has only rarely been reported in cytoplasmic droplets (Dott and Dingle, 1968). However, a wide variety of other hydrolytic enzyme activities have been detected in cytoplasmic droplets (Dott and Dingle, 1968; Garbers et al., 1970; Moniem and Glover, 1972; Farooqui and Srivastava, 1979). Although Pz-peptidase activity was found in extracts of bovine spermatozoa in the present study, it was not certain that the Pz-peptidase actually originated from the spermatozoa. The relatively small amount of activity attributable to the spermatozoa could have been due to retention of traces of droplet cytoplasm or secondary adsorption of Pz-peptidase to the plasma membrane of spermatozoa. It was difficult to differentiate among these alternatives on the basis of available data, and further investigations will be required to determine whether spermatozoa contain intrinsic Pz-peptidase and, if so, to establish its subcellular location. In the sense that mature ejaculated bovine spermatozoa no longer have an attached cytoplasmic droplet, it appeared that the Pz-peptidase was not primarily a spermatozoal enzyme. This conclusion is contrary to that reported for the origin of Pz-peptidase in human and rat semen (Koren and Milković, 1973; Koren et al., 1975). Therefore, it is possible that the distribution of Pz-peptidase among the seminal constituents of other species is different from that of bovine.

The function of the Pz-peptidase in male reproductive tissues remains uncertain. It seems unlikely that the seminal Pz-peptidase is involved in processes related to collagenolysis, and the physiological substrate of this peptidase in reproductive tissues has not been determined. Identification of cytoplasmic droplets as the major source of seminal Pz-peptidase and the presence of substantial activity in cauda epididymal fluid indicated that the function of the Pz-peptidase could be related to epididymal maturation of spermatozoa. Further experimentation will be required to clarify the distribution of Pz-peptidase in other portions of the epididymis. Without conclusive evidence that the Pz-peptidase activity present in sperm extracts actually represents an intrinsic spermatozoal enzyme, it is uncertain whether the Pz-peptidase might participate in some spermatozoal function associated with fertilization. The results of this investigation suggested that the physiological function of the seminal Pz-peptidase may not be limited to those proposed previously and that the potential role of Pz-peptidases in reproduction needs to be reevaluated.

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