‘COLLAGENASE-LIKE’ PEPTIDASE IN HUMAN, RAT AND BULL SPERMATOZOA

E. KOREN AND S. MILKOVIĆ

*Laboratory for Experimental Medicine, University of Zagreb,
Vinogradska c. 29, 41000 Zagreb, Yugoslavia*

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Summary. A peptidase capable of splitting synthetic collagenase substrate and collagen peptides, ‘collagenase-like’ peptidase, has been discovered in human semen, rat and bull epididymal spermatozoa and rat and bull testes. This enzyme is also present in human seminal plasma and sperm washings, but its total activity pattern in human semen fractions, its specific activity in washed spermatozoa and the negligible activity found in the azoöpermic ejaculate prove that it is a sperm enzyme. As shown by the thin-layer chromatography of the reaction products, ‘collagenase-like’ peptidase cleaves synthetic collagenase substrate at the same site as bacterial collagenase does. The pH optimum of the ‘collagenase-like’ peptidase is 7.5.

The enzyme has been partially purified from rat testis homogenate by ammonium sulphate saturation and gel filtration on a Sephadex G-200 column. Its molecular weight appears to be about 110,000. The possible rôle of this enzyme in fertilization is discussed.

INTRODUCTION

An increasing number of authors are coming to believe that the proteolytic enzymes in spermatozoa (Stambaugh & Buckley, 1968, 1969, 1970; Stambaugh, Brackett & Mastroianni, 1969; Allison & Hartree, 1970; Zaneveld & Williams, 1970) and seminal plasma (Moghissi & Syner, 1970; Syner & Moghissi, 1972) play a rôle in the fertilization process.

This paper presents evidence for the existence of a ‘collagenase-like’ peptidase in human, rat and bull spermatozoa as well as in rat and bull testes.

MATERIALS AND METHODS

Synthetic PZ-pentapeptide or ‘collagenase chromophore substrate’ (4-phenylazo-benzyloxy-carbonyl-L-prolyl-L-leucyl-L-glycyl-L-prolyl-D-arginine) and PZ-dipeptide (4-phenylazo-benzyloxy-carbonyl-L-prolyl-L-leucin) were purchased from Fluka, Buchs, Switzerland. Bacterial collagenase and alpha casein were obtained from Worthington Biochemical Corporation, U.S.A.; bovine serum albumin (monomer) and human serum γ -globulin from Calbiochem, U.S.A.;

and porcine insulin from Pliva, Zagreb, Yugoslavia. Acid-soluble rat skin collagen was prepared according to the method of Lapière & Gross (1965).

Fractionation of human semen

Human semen obtained from healthy donors was allowed to liquefy at room temperature and then diluted with 0.9% sodium chloride to double the original volume (usually 6 to 7 ml). Aliquots (2 ml) of the diluted semen homogenized with a Teflon homogenizer at low speed and freeze-thawed twice were used as whole semen homogenates.

Four ml of the diluted semen were centrifuged for 15 min at 3000 rev/min and the supernatant was used as diluted seminal plasma. The sperm pellet was resuspended in 0.9% sodium chloride to the starting volume (4 ml), homogenized and freeze-thawed twice. Aliquots of this preparation were used as homogenates of unwashed spermatozoa. By the same procedure, seminal plasma from an azoöpermic ejaculate was also obtained.

To obtain washed spermatozoa, the method of Allison & Hartree (1970)—four washings with hypotonic tris buffer—was strictly followed. The final washed sperm pellet was resuspended in 0.9% sodium chloride to the starting volume of the ejaculate, then homogenized and freeze-thawed twice. The washings were also homogenized and freeze-thawed.

Fractionation of spermatozoa and testis tissue of rat and bull

Rat epididymal spermatozoa, flushed from the epididymides of fifteen 6-month-old Fischer strain rats immediately after the animals had been killed, were washed according to the procedure for human spermatozoa. The tunica albuginea of both testes from the same animals was emptied by squeezing out the testicular tissue, which was first homogenized and then diluted in 0.9% sodium chloride to 1 : 10, tissue : sodium chloride, ratio. Bull epididymal spermatozoa and testis homogenates were subjected to the same procedure as rat epididymal spermatozoa and testis homogenates.

Determination of 'collagenase-like' peptidase activity

The activity of 'collagenase-like' peptidase (CLP) was determined with PZ-pentapeptide (Wünsch & Heidrich, 1963). Samples (1 ml) for analysis were incubated at 37°C with 1 ml 0.15 M-tris-acetate buffer, pH 7.5, containing 0.2 mg PZ-pentapeptide. After 60 min, the reaction was stopped by lowering the pH to 3 with 0.5 ml 0.47 M-citric acid, and the released PZ-Pro-Leu was extracted with 3 ml ethyl acetate. Undegraded PZ-pentapeptide remained in the watery phase because it was not soluble in ethyl acetate at this pH. The concentration of PZ-Pro-Leu was determined from the extinction at 320 m μ (Strauch & Vencelj, 1967).

The unit of enzyme activity was defined as the amount of enzyme that releases 1 μ g of PZ-Pro-Leu under the above conditions. Total activities were calculated back to 1 ml of semen and specific activities were expressed as units per mg protein.

The optimal pH of CLP was determined in the pH 4 to 9 range by incubating 1-ml aliquots of various samples for 60 min at 37°C with 2 ml of 0.15 M-tris-

acetic acid buffer containing 0.2 mg of PZ-Pro-Leu-Gly-Pro-D-Arg. The pH of each incubation mixture was checked at the beginning and end of incubation.

The site of cleavage of the PZ-Pro-Leu-Gly-Pro-D-Arg substrate was determined by thin-layer chromatography of the ethyl acetate extracted reaction products on silica gel G with butanol : acetic acid : water—6 : 2 : 2 (Heidrich, Prokopova & Hannig, 1969). The chromatograms were sprayed with concentrated H_2SO_4 .

Total protein concentration was determined by the spectrophotometric method of Waddell (1956), and caseinolytic activity in the rat testis homogenate and the purified CLP preparation was determined according to the method of Kunitz (1947).

Purification of CLP from rat testis tissue

The testis homogenate prepared from thirty-three testes of 6-month-old rats (about 30 g) was diluted with 0.9% NaCl (buffered with 0.5 M-tris to pH 7.5) to 300 ml and clarified by centrifugation (12,000 g for 10 min at 4°C). The supernatant was fractionated with ammonium sulphate saturation (Dixon & Webb, 1961) to give 20, 40, 50, 60 and 70% saturation precipitates. Each was dissolved in 10 ml 0.15 M-tris-acetate buffer, clarified by centrifugation and then passed through a Sephadex G-25 coarse column (1.5 × 40 cm) to remove the ammonium sulphate. Protein concentration and CLP activity were determined.

Ten ml of the pooled 50 to 60% and 60 to 70% saturation fractions, which showed the highest specific CLP activity, were transferred to a Sephadex G-200 column (89 × 2.5 cm). The column was equilibrated and eluted with 0.1 M-tris-HCl buffer, pH 8, containing 1 M-NaCl, at a rate of 15 ml/hr. The eluate was monitored at 280 m μ . Five-ml fractions were collected and the CLP activity was determined in each fraction. Fractions 32 and 33 which showed the highest activity were pooled, dialysed against distilled water and freeze-dried. The pooled fractions were used in further experiments as purified rat CLP.

The molecular weight of the enzyme was approximated by the V_e/V_o -log molecular weight plot. The column was calibrated with dextran blue (mol. wt 2×10^6), human serum γ -globulin (mol. wt 1.4×10^5), bovine serum albumin (mol. wt 6.7×10^4) and $\text{Fe}(\text{CN}_6)^{3-}$ (mol. wt 2.12×10^2).

The effect of purified rat CLP on rat skin collagen was observed by polyacrylamide disc electrophoresis (Reisfeld, Lewis & Williams, 1962) using Shandon apparatus and the Kipp and Zonen (Holland) densitometer DD2 with BD5 micrograph and BCI integrator. Two incubation mixtures were prepared: one contained 4 mg heat-denatured collagen and the other, 4 mg native rat collagen, in addition to 15 mg purified rat CLP dissolved in 2 ml 0.15 M-Krebs-Ringer phosphate buffer, pH 7.5. From both incubation mixtures, 30- μ l samples were taken after 0, 1 and 2 hr of incubation and immediately mixed with the sample gel. The action of CLP was stopped by urea, the concentration of which in the sample gel was 8 M.

By the above procedure and densitometry of gels, an attempt was made to follow the action of purified rat CLP on bovine serum albumin and porcine insulin after 1, 2 and 4 hr of incubation at 37°C and pH 7.5.

RESULTS

The distribution of CLP activity in human semen fractions is presented in Tables 1 and 2.

In the seminal plasma of an azoöspemic ejaculate, the total CLP activity amounted to 0.75 units. Microscopic examination revealed some spermatozoa in all four washings, but cytoplasmic droplets could not be demonstrated in washed sperm preparations.

Table 1. Total CLP activity, total protein content and specific CLP activity in various fractions of human semen

<i>Human semen fractions</i>	<i>Total CLP activity (units/ml semen)</i>	<i>Total protein content (mg/ml semen)</i>	<i>Specific CLP activity (units/mg protein)</i>
Whole ejaculate homogenate	36.02 ± 2.56* (17)	65.92 ± 5.17 (9)	0.54
Seminal plasma	12.47 ± 1.68 (20)	50.54 ± 3.80 (9)	0.24
Unwashed sperm homogenate	21.15 ± 3.01 (20)	19.17 ± 2.15 (9)	1.10

* Mean ± S.E. CLP, 'Collagenase-like' peptidase. () number of estimations.

Table 2. Total CLP activity, total protein content and specific CLP activity in four sperm washings and washed human spermatozoa

<i>Material</i>	<i>Total CLP activity (units/ml semen)</i>	<i>Total protein content (mg/ml semen)</i>	<i>Specific CLP activity (units/mg protein)</i>
W ₁	22.39 ± 3.30* (11)	50.62 ± 3.76 (9)	0.44
W ₂	2.03 ± 0.25 (12)	3.49 ± 0.19 (9)	0.58
W ₃	1.36 ± 0.17 (12)	2.23 ± 0.13 (9)	0.60
W ₄	0.68 ± 0.10 (12)	1.60 ± 0.07 (9)	0.42
Washed spermatozoa	6.01 ± 0.39 (16)	1.61 ± 0.22 (9)	3.73

* Mean ± S.E. CLP, 'Collagenase-like' peptidase. W₁, W₂, W₃, W₄, four sperm washings. () number of estimations.

Table 3. Specific CLP activity in rat and bull testis homogenates and washed rat and bull epididymal spermatozoa

<i>Material</i>	<i>Specific CLP activity (units/mg protein)</i>	<i>No. of estimations</i>
Rat testis homogenate	3.25	11
Bull testis homogenate	2.36	4
Washed rat epididymal spermatozoa	3.66	11
Washed bull epididymal spermatozoa	4.24	4

CLP, 'collagenase-like' peptidase.

Table 3 shows CLP activities in rat and bull testis and epididymal spermatozoa. The optimal pH for CLP activity in rat testis homogenate, rat epididymal sperm homogenate, human washed sperm homogenate, bovine epididymal sperm homogenate, and for purified rat testis CLP was 7.5.

Thin-layer chromatography showed that all these preparations, as well as human seminal plasma and bacterial collagenase, split PZ-Pro-Leu-Gly-Pro-D-Arg between Leu and Gly. In all cases, ethyl acetate extracts of the incubation

mixtures gave only one yellow spot on the chromatogram which had the same R_F , (0.86) as the PZ-Pro-Leu test substance.

By gel filtration of 10 ml of pooled 50 to 60% and 60 to 70% ammonium

TEXT-FIG. 1. Gel filtration of 10 ml pooled 50 to 60% and 60 to 70% $(NH_4)_2SO_4$ fractions of the rat testis homogenate on Sephadex G-200 column (89×2.5 cm). —, Protein, extinction at $280 m\mu$; O, CLP activity, extinction at $320 m\mu$.

TEXT-FIG. 2. Determination of the molecular weight of rat testis CLP. A calibration curve of known proteins on a Sephadex G-200 column (89×2.5 cm). alb. = bovine serum albumin; glob. = human serum γ -globulin. The arrow points to the elution position of CLP. Void volume = 133 ml, elution volume = 232 ml for rat testis CLP.

sulphate saturation fractions of the rat testis homogenate, four protein peaks and one CLP activity peak were obtained. Total CLP activity in the eluate was recovered at a 60% rate (Text-fig. 1).

During the purification procedure of the rat testis CLP, a decrease in the ratio of caseinolytic to CLP activity was obtained (Table 4).

The molecular weight of the rat testis CLP was found to be about 110,000 (Text-fig. 2).

Table 4. Specific CLP and caseinolytic activities during the purification process of rat testis CLP

<i>Rat testis fractions</i>	<i>Specific CLP activity (units/mg protein)</i>	<i>Specific caseinolytic activity (μg tyrosine/mg total proteins)</i>
Rat testis homogenate	2.77	19.10
50 to 60% $(\text{NH}_4)_2\text{SO}_4$ fraction	7.61	Not estimated
60 to 70% $(\text{NH}_4)_2\text{SO}_4$ fraction	9.20	Not estimated
Purified CLP (fractions 32 and 33)	19.79	8.20

CLP, 'collagenase-like' peptidase.

Disc gel electrophoresis revealed that purified rat testis CLP degraded heat-denatured rat skin collagen but not native collagen (Pl. 1, Figs 1 and 2).

No effect of the purified rat CLP on bovine serum albumin and porcine insulin could be demonstrated by this method.

DISCUSSION

In human semen, rat and bull epididymal spermatozoa and rat and bull testes, an enzyme has been found which is capable of degrading synthetic collagenase substrate and collagen peptides but not native collagen molecules. We have called the enzyme a 'collagenase-like' peptidase, as proposed by Sakai and colleagues (1967) for a similar enzyme in fibrotic liver. The total activity pattern of CLP in the human semen fractions (Table 1), its greatest specific activity in washed spermatozoa (Table 2) of the three species mentioned above and its presence in the epididymal spermatozoa and testis homogenate (Table 3) constitute evidence showing that it is a spermatozoal enzyme.

The CLP content of seminal plasma is probably due to release of the enzyme from the spermatozoa, since azoöspemic seminal plasma has negligible CLP activity. However, the results of the sperm washing experiment indicate that the enzyme might also come from the cytoplasmic droplets, which are known to possess hydrolytic enzymes (Dott & Dingle, 1968).

The pH optima and site of cleavage of PZ-Pro-Leu-Gly-Pro-D-Arg were the same in all cases. This proves that in testes, epididymal spermatozoa, washed

EXPLANATION OF PLATE 1

FIG. 1. Disc electropherograms of an incubation mixture containing heat-denatured rat skin collagen and purified rat testis CLP after (A) 0 hr, (B) 1 hr and (C) 2 hr of incubation. The disappearance of the collagen bands is visible.

FIG. 2. Disc electropherograms of an incubation mixture containing native rat skin collagen and purified rat testis CLP, after (A) 0 hr, (B) 1 hr and (C) 2 hr of incubation. No significant change in the collagen bands can be seen. s.g. = spacer gel; coll. = collagen peptides; CLP = purified CLP bands.

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spermatozoa and seminal plasma, the same enzyme is responsible for the cleavage of PZ-Pro-Leu-Gly-Pro-D-Arg.

The peptidase (or peptidases) which at physiological pH cleave synthetic bacterial collagenase substrates with the amino acid sequence -Pro-x-Gly-Pro-y characteristic of the apolar regions of the collagen molecule have been found in tissue cultures of He-La cells (Strauch & Vencelj, 1967), a variety of tissues taken from rabbits (Espey & Rondell, 1967), fibrotic liver (Sakai *et al.*, 1967), mouse fibroblast cells, liver, bone and skin of the chick embryo (Strauch, Vencelj & Hannig, 1968), tadpole tail fin (Harper & Gross, 1970), calf costal cartilage (Rabadjija, Koren & Pende, 1971) and in Graafian follicles (Espey & Rondell, 1965). Although these enzymes cannot be considered true collagenases because they do not degrade native collagen, they do possess specificity for -Pro-x-Gly-Pro-y sequence according to some authors (Strauch, 1970; Woessner, 1970) and probably participate in the final stages of collagen degradation (Harper & Gross, 1970). The PZ-Pro-Leu-Gly-Pro-D-Arg sequence possessing C-terminal D-arginine is resistant to non-specific proteases such as trypsin, chymotrypsin, carboxypeptidase A and B (Wünsch & Heidrich, 1963). To a certain extent, the results of our experiments have also shown the specificity of sperm CLP because: (1) the site of cleavage of PZ-Pro-Leu-Gly-Pro-D-Arg is between -Leu and -Gly, which is characteristic of bacterial collagenase; (2) purification of CLP from the rat testis homogenate shows a decrease in the ratio of caseinolytic to CLP activity (Table 4); (3) purified CLP does not degrade bovine serum albumin or porcine insulin.

Sperm proteases have been described in several publications (Buruiana, 1956; Stambaugh & Buckley, 1968, 1969; Ho & Meizel, 1970; Allison & Hartree, 1970) and have even been detected in the acrosomes. Their biological rôle is believed to be the cleavage of the zona pellucida (Stambaugh & Buckley, 1969) or corona radiata proteins (Zaneveld & Williams, 1970).

Since the specificity requirements and the true biological substrate of CLP have not been clearly determined so far, the rôle of such an enzyme in the spermatozoa is uncertain, but it is logical to suppose that among the substances encountered by spermatozoa (cervical mucus, uterine mucus, oviduct mucus, cumulus oophorus and zona pellucida) there is also a substrate for CLP. By the degradation of this substrate, CLP might facilitate sperm motility or penetration into the egg.

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