STUDIES ON DIPEPTIDYL CARBOXYPEPTIDASE IN THE MALE REPRODUCTIVE ORGANS; ITS BIOLOGICAL AND PATHOLOGICAL STATUS

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There are potent kinin degrading activities in seminal plasma and testis of various mammals. The activities in boar and human seminal plasma, and testis extracts from boar, rat, guinea pig and rabbit were eluted out at a similar position as a single peak in column chromatography with Sephadex G-200 or DEAE Sephadex A-50. These enzymes degraded synthetic bradykinin and yielded angiotensin II from angiotensin I by cleaving the second peptide bond from the carboxytermini of the substrate, and it was concluded that these enzymes were dipeptidyl carboxypeptidases like kininase II or angiotensin I converting enzyme. The enzymes in male genital organs of these mammals were found to be identical with further investigation on the enzymic properties of them.

The enzyme in rat testis increased significantly in accordance with sexual maturation, and the increase was suppressed by injection of danazol or diethylstilbestrol to rats. Furthermore, the dipeptidyl carboxypeptidase content in human seminal plasma was positively correlated to the semen qualities, i.e. sperm density and motility. From these results it is supposed that dipeptidyl carboxypeptidase in male genital organ and its secretion seem to be related to the male reproductive functions.

**Keywords**—dipeptidyl carboxypeptidase; angiotensin I converting enzyme; bradykinin; testis; seminal plasma; danazol; testis maturation; semen quality

INTRODUCTION

The presence of a peptidase which yields angiotensin II (AGT II) from angiotensin I (AGT I) has been demonstrated in 1956 by Skeggs et al. This enzyme, AGT I converting enzyme (ACE), removes the carboxytermini dipeptide, His-Leu, from AGT I molecule, and is one of the peptidyl dipeptide carboxyhydralase (dipeptidyl CPase) which hydrolyse the second peptide bond from carboxytermini. Meanwhile, kinin is degraded by kininase II which releases a dipeptide, Phe-Arg, from the carboxyterminal end of bradykinin or kallidin. This enzyme is also one of dipeptidyl CPase. ACE and kiniase II were found separately but have been recognized as an identical enzyme.

There are various proteinases and peptidases in seminal plasma. Their roles in the reproductive processes are still remained to be investigated, though some of them have been said to participate in post ejaculate coagulation and liquification of semen, capacitation of sperm and fertilization, etc. The authors previously reported the purification of acrosin from boar sperm and its enzymic properties. It is a trypsin like enzyme and liberates kinin from high and low molecular weight kininogens, in vitro. While, Schill found that addition of bradykinin to human semen induced enhancement of sperm motility. These results were of interest in relation to the finding of Cushman et al. that there was an enzyme which removed His-Leu from Bz-Gly-His-Leu, a synthetic substrate for ACE, in rat testis and seminal plasma. Thus it is supposed that the biologically active peptides are involved in the reproductive function, and the peptidases in seminal plasma...
relate the activation or degradation of the active peptides.

This paper deals with a dipeptidyl CPase which is able to degrade synthetic bradykinin in seminal plasma and testis, and its relation to male sexual function.

MATERIALS AND METHODS

Chemicals — Bradykinin (BK), AGT I and II, phenylalanyl-arginine (Phe-Arg), histidyl-leucine (His-Leu), benzoyl-arginine (Bz-Gly-Arg), benzoyl-glycyl-histidyl-leucine (Bz-Gly-His-Leu), carbobenzoxy-glycyl-prolyl-leucyl-glycine (Z-Gly-Pro-Leu-Gly) and BK potentiating factor B (BPF-B) were obtained from Peptide Research Co. (Osaka). Sephadex G-200 and DEAE Sephadex A-50 were the products of Pharmacia Fine Chem. (Uppsara, Sweden). Diisopropyl fluorophosphate (DFP), soybean trypsin inhibitor (SBTI) and diethylstilbestrol were purchased from Sigma Chemical Co. (St. Louis, U.S.A.) and fluorescamine was obtained from Roche (Basel, Switzerland). Danazol (17α-pregn-4-en-20y-nol-[2,3-3]-isoxazol-17-ol) and SQ 14225 (N-3-mercaptop-2-methylpropanoyl-L-proline) were provided from Sankyo Co. and Tokyo Tanabe Co. (Tokyo), respectively. Thin-layer chromatography plate (silica gel precoated plate, 0.25 mm thick) was a product of Merck (Darmstadt, Germany). N-2-Hydroxyethyl piperazine-N’-2-ethane sulfonic acid (HEPES) and 2-N-morpholinoethanesulfonic acid (MES) were purchased from Nakarai Chemicals Co. (Tokyo).

Collection of Semen — Freshly ejaculated boar semen was obtained from Kanagawa Life Stock Improvement Association (Kanagawa). Human semen was supplied by the Department of Obstetrics and Gynecology, School of Medicine, Keio University. The ejaculates were obtained after 4 days sexual abstinence. Sperm density was counted by hemocytometer and the percent of motile sperm was recorded.

Preparation of Seminal Plasma and Testis Extract — Seminal plasma was separated by centrifugation (1000×g, 30 min, 4°C). The testis with epididymis was minced and extracted twice with equal volume (w/v) of 0.05 M Tris-HCl containing 0.2 M NaCl, pH 8.0. Insoluble materials were removed by centrifugation (7000×g, 30 min, 4°C). The seminal plasma and the testis extract were recentrifuged (80000×g, 1 hr, 4°C) preceding column chromatography.

Determination of Kinin Degrading Activity — The activity was determined by measurement of degradation of synthetic BK. For rough estimation of the activity, the mixture of 100 µl of an assay sample and 10 ng BK (100 ng BK/ml, 0.05 M Tris-HCl-0.2M NaCl, pH 8.0) was incubated for 10 min at 30°C, then the reaction mixture was poured on an isolated rat uterus segment which was suspended in a siliconized organ bath containing 5.0 ml of aerated de Jalon’s solution (30°C). The activity was estimated by comparing the contractile response with that of 10 ng BK. A precise determination of the activity was carried out with an isolated guinea pig ileum segment. The ileum segment was suspended in 10 ml of aerated Mg2+-free Tyrode’s solution (30°C). A mixture of 100 µl each BK (1.0 µg/ml, 0.05 M Tris-HCl-0.2 M NaCl, pH 8.0) and a sample was incubated for defined periods at 30°C, and its contractile response was recorded isometrically. The remaining BK in a incubated mixture was estimated from the calibration curve made by 10—100 ng BK. When a sample inactivated more than 70 ng BK, it was diluted to give at least 30 ng BK remaining after incubation. One unit of the activity was defined as an amount of enzyme which degraded 1 μg of synthetic BK in 1 min at 30°C.

Fluorometric Determination of Peptide Degradation — The substrate peptide was dissolved in small amount of dimethyl sulfoxide, and 10 mM HEPES-NaOH containing 0.1 M NaCl, pH 7.0 was added to make 2.0 mM of final concentration of the substrate. A sample (100 µl) which was dialysed against the above buffer at 4°C overnight, and 1.9 ml of the substrate solution were incubated together for 30 min at 30°C, and 0.5 ml of 2 M NaHCO3 (pH 8.2) was added to adjust pH for convenience of fluorescamine reaction. Subsequently 0.5 ml of fluorescamine (0.6 mg/ml dioxane) was added under stirring. A blank was pro-
FIG. 1. Chromatographic Fractionation of Dipeptidyl CPase from Seminal Plasma and Testis Extract on Sephadex G-200 and DEAE Sephadex A-50

(A) Sephadex G-200: The column (3.5 x 60 cm) was equilibrated and eluted with 0.05 M Tris-HCl containing 0.2 M NaCl, pH 8.0.

(B) DEAE Sephadex A-50: The active fraction from Sephadex G-200 was dialysed and applied on DEAE Sephadex A-50 column (2.0 x 90 cm) which was equilibrated with 0.05 M Tris-HCl, pH 8.0. The column was washed with the equilibrium buffer until the absorbance at 280 nm became negligible. The elution was performed by linear gradient from 0 to 0.5 M NaCl in the equilibrium buffer. The concentration of NaCl was expressed by conductivity (mΩ /cm).

TABLE I. Decomposition of Synthetic Peptides by Dipeptidyl CPase in Boar and Human Seminal Plasma and Boar Testis

<table>
<thead>
<tr>
<th></th>
<th>Boar Seminal plasma</th>
<th>Boar Testis</th>
<th>Human Seminal plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bz-Gly-Arg</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Bz-Gly-His-Leu</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Z-Gly-Pro-Leu-Gly</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Z-Leu-Gly-Gly</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ : decomposed, − : not decomposed.
vided by the same procedures except incubation. The intensity of fluorescence was measured with Shimadzu Fluorometer Type Rf 502 (excitation: 390 nm, emission: 480 nm).

Effect of Inhibitors on Dipeptidyl CPase — Equal volume of a sample and inhibitor (10 mM HEPES-NaOH-0.1 mM NaCl, pH 7.0) were mixed and preincubated for 10 min at 30°C. The remaining activity was assessed by the bioassay and the fluorometric determination as described above.

Determination of Protein Concentration — Protein concentration was estimated spectrophotometrically from the absorbance at 280 nm (Hitachi Spectrophotometer Type 181).

RESULTS
Chromatographic Analysis of Dipeptidyl CPase in Seminal Plasma and Testis of Various Mammals
Seminal plasma of human and boar, and the extracts from testes of boar, guinea pig, rat and rabbit were fractionated by column chromatographies with Sephadex G-200 and DEAE Sephadex A-50. Seminal plasma (20 ml) or testes extract (corresponded to 10 g of minced testes) were applied on Sephadex G-200 column, and BK degrading activity of each fraction was determined. The elution profiles of the activity formed one peak behind the void volume, and more than 80% of the activity of each specimen was recovered in the active fraction. The active fraction from Sephadex G-200 was dialysed against 0.05 M Tris-HCl, pH 8.0, and chromatographed with DEAE Sephadex A-50. All the activity in each sample was completely adsorbed to DEAE Sephadex A-50 and eluted out in 15 ml fractions with recovery of 70—80%. The elution profiles of human seminal plasma and rat testis were given in Fig. 1. The results of the other specimens were essentially the same in both chromatographies.

Enzymic Properties of Dipeptidyl CPase
Partially purified preparations from boar seminal plasma (74.0 U/μl), human seminal plasma (20.0 U/μl) and boar testes (26.7 U/μl) by DEAE Sephadex A-50 were diluted to make 15 U/ml, and these three preparations were em-

FIG. 2. pH Optimums of Dipeptidyl CPase from Boar and Human Seminal Plasma and Boar Testis
The samples were dialysed against 0.01 M HEPES-NaOH containing 0.1 M NaCl, pH 7.0 and adjusted to 15 U/ml. Bz-Gly-His-Leu was dissolved in 0.1 M acetate buffer (pH 4.5—5.5), 0.1 M MES-NaOH (pH 6.0—7.0) and 0.1 M Tris-HCl (pH 7.5—9.0) to make 2.0 mM substrate. All the buffers contained 0.1 M NaCl. The activity was measured by fluorometric method and shown by % of the maximum activity.


n-butyl alcohol : acetic acid : H₂O (50 : 1 : 50)

bradykinin

angiotensin I

FIG. 3. Thin Layer Chromatograms of the Hydroly-sates of Bradykinin and Angiotensin I by Dipeptidyl CPase in Human Seminal Plasma
ployed in the following experiments.

They were incubated with various synthetic peptides and decomposition of peptides were assessed fluorometrically with fluorescamine. All samples hydrolysed BZ-Gly-His-Leu and Z-Leu-Gly-Gly, but not others (Table I). In hydrolysis of Bz-Gly-His-Leu, the optimum pH of them were found at pH 7.0 (Fig. 2).

To investigate the sites of action of the enzyme, BK or AGT I (5 μg/50 μl) and 50 μl of the enzyme preparations (15 U/ml phosphate buffered saline) were incubated for 2 hr at 30°C. Each reaction mixture was labeled with fluorescamine, and developed on silica gel plate with Arg, Phe-Arg and BK for BK hydrolysis, or AGT I, II and His-Leu for AGT I hydrolysis. Each preparation gave a similar result in thin layer chromatography, and typical chromatograms obtained by the preparation from human seminal plasma were shown in Fig. 3. The hydrolysate of BK gave two spots, and one of them coincided with Phe-Arg. AGT I treated with this enzyme preparation also gave two spots which corresponded with AGT II and with His-Leu, respectively. Thus it was demonstrated that the peptidase in seminal plasma and testis cleaved not only BK but also AGT I at the site of the second peptide bond from carboxy-termini.

The enzymes in boar and human seminal plasma, and boar testis on BK and Bz-Gly-His-Leu hydrolysis were inhibited by Hg²⁺, Ni²⁺, Cu²⁺, Fe²⁺, chelating agents, BPF-B⁹ and SQ, 14225¹⁰ natural and synthetic inhibitors for dipeptidyl CPase (Table II).

These results indicated that the enzymes with kinin degrading activity in seminal plasma and testis were dipeptidyl CPase, and they were identical with kininase II or ACE.¹¹ Then the properties of dipeptidyl CPase in seminal plasma and testis of various mammals were similar to each

### TABLE II. Inhibition of Bz-Gly-His-Leu Hydrolysis and Bradykinin Inactivation by Dipeptidyl CPase from Boar Seminal Plasma

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Substrate</th>
<th>Bz-Gly-His-Leu&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BK&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>20</td>
<td></td>
<td>++</td>
<td>N.D.</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>20</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>20</td>
<td></td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>20</td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>20</td>
<td></td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>Pb²⁺</td>
<td>20</td>
<td></td>
<td>++</td>
<td>N.D.</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>20</td>
<td></td>
<td>++</td>
<td>N.D.</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>20</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>20</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>EDTA</td>
<td>20</td>
<td></td>
<td>++</td>
<td>N.D.</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>20</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>DFP</td>
<td>1</td>
<td></td>
<td></td>
<td>N.D.</td>
</tr>
<tr>
<td>SBTI</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPF-B</td>
<td>10</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SQ 14225</td>
<td>10</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>


<sup>a</sup> Fluorometric method.  <sup>b</sup> Bioassay with guinea pig ileum.
other in the substrate specificity, pH optimum, inhibition spectra and chromatographic behaviours.

Change of Dipeptidyl CPase Content in Rat Testis during Sex Maturation

Homogenized rat testes of 2–14 weeks old were centrifuged (1000 × g, 30 min, 4°C), and BK degrading activity in the supernatants were determined with guinea pig ileum. The activity in rat testis increased significantly during 4–7 weeks old, and in this period the rate of increase in the activity was in parallel with that of testis weight. The activity reached plateaus at 7 weeks old, and it was about 4 times higher than that of infant (Fig. 4).

Effects of Danazol and Diethyl Stilbestrol on the Content of Dipeptidyl CPase in Rat Testis

For further investigation of the relationship between dipeptidyl CPase and male sexual function, danazol, a synthetic anti-FSH and -LH agent (3 mg/0.2 ml of corn seed oil/day) or diethylstilbestrol (10 μg/0.2 ml of corn seed oil/day) were injected subcutaneously every day to 4 weeks old rats for 3 weeks. Control rats were given 0.2 ml of corn seed oil. The rats were sacrificed 1 day after the last injection and kinin degrading activity in the testes extracts which were prepared as described in “Methods” were determined with guinea pig ileum. As shown in Table III, the contents of dipeptidyl CPase in the testes of danazol- or diethylstilbestrol-administered rats were significantly lower than that of control group. The wet weights of testes of these experimental groups were also lighter than the control, but there was no difference in the body weights of danazol and control groups.

The Correlation between Semen Quality and the Content of Dipeptidyl CPase in Human Seminal Plasma

The fresh human semen from 60 persons were liquefied and determined for the contents of dipeptidyl CPase by measuring kinin degrading activity with guinea pig ileum. As shown in Fig. 5, the enzyme activities in seminal plasma were significantly lower (p < 0.05) in either oligozoospermia (less than 4 × 10^7 sperm/ml) or asthenozoospermia (motility less than 40%) than normozoospermia (sperm density > 5 × 10^7/ml, motility > 60%). The means of enzyme activities in oligozoospermia and normozoospermia were 31.4 ± 6.4 and 53.5 ± 11.3 U/ml, and those of asthenozoospermia and normal ones were 30.0 ± 4.5 and 49.5 ± 10.2 U/ml, respectively. There were positive correlations between dipeptidyl CPase activity and sperm density (correlation coefficient; r = 0.73) or motility (r = 0.68).

DISCUSSION

Cushman et al. mentioned that there was BzGly-His-Leu splitting activity in rat seminal plasma and testis, and that the activity in matured testis was higher than that of infant.8) The present investigation demonstrated the presence of dipeptidyl CPase in the male genital system. The activity increased rapidly from 4 to 7 weeks old and it reached adult level in 7 weeks old. Sexual maturation of rat occurs in 4–8 weeks old, i.e., testis maturation and spermatogenesis initiate in 4–5 weeks old and matured sperm appear in the cauda epididymis in 7–8 weeks old.13) These

![Graph](image-url)

FIG. 4. The Changes of Dipeptidyl CPase Activity in Rat Testis, and Body and Testis Weights after Birth. Vertical bars represented. s.e. (n = 6).
processes are regulated mainly by FSH and testosterone.\textsuperscript{13} The concentrations of these hormones in serum also start to increase in 4–5 weeks old.\textsuperscript{13} In this investigation, danazol and diethylstilbestrol induced remarkable atrophy of testis and significant decrease of the content of dipeptidyl CPase. These results indicate that dipeptidyl CPase seems to be controlled by sex hormones and relates to testis functions, such as spermatogenesis. Increment of the enzymes in rat testis during maturation were also observed in acid phosphatase,\textsuperscript{14} creatinine acetyltransferase,\textsuperscript{15} lactate dehydrogenase\textsuperscript{16} and aminopeptidase.\textsuperscript{17} Go \textit{et al.} reported that creatinine acetyltransferase in testis decreased rapidly following hypophysectomy, and the activity was restored by injection of FSH, LH or testosterone to the rat.\textsuperscript{8} Hebert \textit{et al.} found that the conversion of AGT I was twice as high in ewe as in fetal or newborn lamb.\textsuperscript{19} In the lung of rat embryo ACE also increased up to the birth and approximately doubled by the second day after birth.\textsuperscript{20} We observed sexual maturation accompanied with elevation of dipeptidyl CPase activity in rat

### TABLE III. Effects of Danazol and Diethyl Stilbestrol on Dipeptidyl CPase Activity in Rat Testis

<table>
<thead>
<tr>
<th></th>
<th>Weight (g)</th>
<th>Dipeptidyl CPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body</td>
<td>Testis</td>
</tr>
<tr>
<td>Control</td>
<td>173±4.4</td>
<td>1.14±0.03</td>
</tr>
<tr>
<td>Danazol</td>
<td>177±7.6</td>
<td>0.51±0.04*</td>
</tr>
<tr>
<td>Diethyl stilbestrol</td>
<td>134±5.9*</td>
<td>0.48±0.06*</td>
</tr>
</tbody>
</table>

*The values are mean ± s.e. (n = 6).

*: Significant difference (p < 0.05).

![Graph](Asthenozoospermia_Normozoospermia_Bradykinin.png)

**FIG. 5. Relationship between Dipeptidyl CPase Activity in Human Seminal Plasma and Sperm Density or Motility**
testis, and speculated that this enzyme seemed to relate to the functional development of the organs.

The pathogenesis of oligozoospermia and asthenozoospermia have not yet been explained, however, male infertility is ultimately able to recognize as the atrophy of testicular functions. Fructose\textsuperscript{21} and glutamate-oxaloacetate transferase\textsuperscript{22} in seminal plasma were the only parameters which correlated positively to the semen quality such as sperm density and motility. Dipeptidyl CPase in human seminal plasma was found to correlate to sperm motility and density in this investigation. The alteration of dipeptidyl CPase was also observed in some of the clinical disorders. Studd et al. reported that ACE activity which was determined by hydrolysis of Bz-Gly-His-Leu was significantly higher in sarcoidosis than normal individuals and was restored by treatment of steroid hormones.\textsuperscript{23} To the contrary, Arregui et al. mentioned that ACE in corpus striatum of human brain was remarkably reduced in patients of serious Huntington's disease from normal level.\textsuperscript{24} The results obtained in this investigation indicated that the level of dipeptidyl CPase in testis and seminal plasma remains low in the immaturity or disorders of male sexual functions, and this activity in seminal plasma seems to be useful as an index to assess the testicular function and semen quality.

SQ 14225 was newly synthesized thiol derivate of proline, and it was designed as highly specific inhibitor of ACE.\textsuperscript{10} We found that SQ 14225 induced complete inhibition of partially fractionated bradykinin degrading activity in seminal plasma and testis extract of various mammals (Table II). These preparations cleaved BK and AGT I at second peptide bond from the carboxy-termini of these molecules (Fig. 3), and these facts suggested that dipeptidyl CPase in seminal plasma and testis are identical with ACE and kininase II which have been found in other organs. Furthermore, the findings that the enzymic properties and chromatographic behaviours (Fig. 1) of dipeptidyl CPase from various mammals were almost the same sustained the conclusion that this enzyme was only enzyme which was capable of degrading bradykinin. This enzyme seems to be related to the male sexual functions as shown in Fig. 4, 5 and Table III. Schill\textsuperscript{7} found that kinin enhanced sperm motility and Fritz\textsuperscript{25} speculated on the contribution of kalikrein-kinin system or its mimic system to sperm metabolism. Our observation is of interest relating these papers.

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Studies on Dipeptidyl Carboxypeptidase


