# Characterization of a Dipeptidyl Peptidase and its Role in Motility of Rat Epididymal Maturing Spermatozoa

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**ABSTRACT:** Dipeptidyl peptidase is a highly glycosylated serine protease with a broad tissue distribution. In the present study, we investigated the alteration of the activity of dipeptidyl peptidase of spermatozoa at different maturational levels in rat epididymis and the apparent correlation of enzyme activity with sperm forward motility. The maximum activity of the enzyme was observed in spermatozoa from the corpus or middle part of the epididymis. This glycyl-proline ρ-nitroanilide hydrolyzing enzyme of epididymal spermatozoa showed significant similarities with biochemical properties of dipeptidyl peptidase (DPP) type IV. Sitagliptin, a specific inhibitor of DPPIV, leads to a more effective and complete inhibition of the peptidase in spermatozoa collected from different parts of epididymis. The complete inhibition by $Hg^{2+}$ and partial insensitivity toward $Cd^{2+}$ also confirmed the similarities with ion sensitivity of DPPIV. Treatment of mature cauda spermatozoa with sitagliptin (1 mM) showed a significant inhibition in forward thrust in motility. This result suggests that sperm forward motility may be regulated by DPPIV-like enzyme of maturing epididymal spermatozoa.

**KEYWORDS:** dipeptidyl peptidase, epididymis, maturation, spermatozoa, motility, enzyme

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## Introduction

Mammalian spermatozoa undergo numerous modifications concomitant with epididymal maturational processes that include changes in biochemical and biophysical properties of the plasma membrane during the transit through the intraluminal environment of the epididymis (caput, corpus, and cauda).[1] These processes ensure the interaction and modifications of different protein components of the epididymal lumen and the spermatozoon itself. As a result of these modifications, the spermatozoa acquire the potential to become a motile and functionally competent cell capable of undergoing capacitation and fertilization. The activity and availability of several regulatory macromolecules such as structural proteins, surface receptors, enzymes, channel proteins, hormones, and cytokines may be regulated by different protein-cleaving enzymes. Sperm surface glycoproteins are thought to play an important role in epididymal sperm maturation process.[2–6] This level of alteration in surface glycoproteins varies from species to species and differs in each epididymal region.[7] Several studies have provided evidence to suggest that the sperm plasma membrane undergoes extensive biochemical changes, including organization and modification of surface glycoprotein, as spermatozoa transit from the proximal to the distal part of the epididymis.[8]

In this article, we have focused on the activity of ectomembrane proteins of epididymal spermatozoa that cleave glycyl-proline p-nitroanilide (a synthetic substrate) in different parts of the epididymis such as the caput, corpus, and cauda. This synthetic substrate was used to assay X-prolyl dipeptidyl aminopeptidase in human serum and in homogeneous enzyme purified from the submaxillary gland.[9] X-prolyl dipeptidyl aminopeptidase (glycyl-prolyl β-napthylamidase) was discovered by Hopsu-Havu and Glenner[10] in rat liver and kidney and purified from porcine kidney as a new dipeptidyl aminopeptidase that liberates glycylproline from β-napthylamide.[10–12] Dipeptidyl peptidase-IV was previously known as dipeptidyl aminopeptidase. It is a type-II integral membrane protein[13] possessing a peptidase activity and capable of cleaving the post-proline bond of position two from the N-terminus. In the small subset of serine proteinase family, the postproline dipeptidyl aminopeptidases consist of four enzymes of the DPP (dipeptidyl peptidase) IV gene family: DPPIV, fibroblast activation protein, DPP8, DPP9, and DPPII (E.C. 3.4.14.2). DPPIV (E.C. 3.4.14.5) is a ubiquitous, multifunctional homodimeric glycoprotein with important roles in nutrition, metabolism, immune and endocrine systems, bone marrow mobilization, cancer growth, and cell adhesion.[14] Moreover, DPPIV acts as a receptor for plasminogen type 2 and adenosine deaminase and attaches with chemokine receptor CXCR4 and mannose 6-phosphate as well as insulin-like growth factor II receptor. Interaction with protein components of the extracellular matrix, such as collagen and fibronectin, indicates its role in adhesion, invasion, and metastasis of cancer cells.[15–18] It is anchored to

the lipid bilayer by a single hydrophobic segment situated at the n-terminus (7–29) with the cytoplasmic tail of any six amino acids. A flexible stalk links the membrane anchor with a large glycosylated region (48–324), cysteine-rich region (325–552), and the C-terminal catalytic domain (553–766).[19] DPPIV is a highly glycosylated serine protease with a broad tissue distribution, acting optimally under weakly basic conditions, but dipeptidyl peptidase present on the surface of the sperm shows an optimum activity at neutral pH. Its homodimerization seems to be essential for enzyme activity.[19,20] A recent study has shown that DPP8 and DPP9 are highly expressed in rat and bovine testis in contrast to the low levels in epididymal epithelial cells and epididymal spermatozoa, but with a significant level of DPPIV.[20] Cauda epididymal sperm cells of guinea pig showed the presence of DPPII on the apical region of acrosomal cap.[21] In horse sperm cells, it was found that the cells fuse with prostasome-like vesicles to acquire DPPIV-like proteins fused with CD26, a Glycosyl Phosphatidyl Inositol-anchoring T-cell activation antigen.[22] A similar event of binding and fusion of seminal membrane vesicle with the sperm was also reported in rabbits.[23] It has been hypothesized that prostasomes, by closely interacting with spermatozoa, can modify the sperm microenvironment and assist its fertilizing potential.[24–28]

In the present study, we investigated the alteration of activity of dipeptidyl peptidase of spermatozoa at different maturational levels in rat epididymis. The activities of this enzyme and its possible relation with forward motility of mature cauda epididymal spermatozoa were also characterized using different inhibitors and metal ions.

## Materials and Methods

Glycyl-proline p-nitroanilide hydrochloride, DPPIV inhibitor sitagliptin, cadmium acetate, phenylmethylsulfonyl fluoride (PMSF), iodoacetamide, aprotinin, and leupeptin were obtained from Sigma Chemical Co. The animal experiments were approved by the Institutional Animal Ethics Committee of the University of Kalyani.

**Isolation of rat epididymal spermatozoa.** Epididymis was removed from adult (four- to six-month-old) Swiss albino rats and kept in a sterile Petri dish containing Ringer's solution (RPS medium: 119 mM, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 16.3 mM potassium phosphate, and 50 U penicillium/mL, pH 6.9). The epididymis was then dissected into three consecutive sections, namely, caput or proximal epididymis, corpus or middle epididymis, and cauda or distal epididymis. Sperm cells were isolated separately from each of the three sections by gentle shearing of the tissues and rupturing the tubules, allowing the sperms to float into the media. The spermatozoa were then filtered through four layers of cheesecloths and sedimented by centrifugation at $500 \times g$ for five minutes and then washed in RPS medium to remove the epididymal fluid.[29]

**Dissociation of sperm heads and tails.** Sperm heads and tails were separated using equilibrium sucrose gradient centrifugation according to the modified method of Furland et al.[30] Fresh spermatozoa ($5 \times 10^6$/mL) from the cauda, corpus, and caput of the epididymis of male rats were isolated in RPS medium and washed. Then, the cells were resuspended in the original volume of RPS buffer containing 1 mM PMSF and sonicated (55 W) for 30 seconds on ice. The resulting sample was pelleted by centrifugation at $650 \times g$ at 4°C. The pellet was resuspended in 65% sucrose and loaded onto a step gradient made up of 70% and 75% sucrose, 2 mL each in RPS buffer in 6 mL Beckman's rotor ultracentrifuge tube. Then, the samples were spun at $110{,}000 \times g$ for 65 minutes at 4°C in a Beckman model optima XPN-100 ultracentrifuge. The head fraction was collected from the bottom of the tube and the tail-rich band fraction appeared in the interface layer of 65% and 70% sucrose. Highly rich (85%) head and tail fractions were further centrifuged at the same speed and then checked under the light microscope.

**Determination of dipeptidyl aminopeptidase activity.** Enzymatic activities were determined by the modified method of Nagatsu et al[9] using glycyl-proline ρ-nitroanilide-HCl as a substrate in a final volume of 1.05 mL for 30 minutes at 37°C by measuring the OD at 385 nm using a spectrophotometer. The assay volume contained $1.5 \times 10^6$ cells in 1.5 µmol glycyl-proline ρ-nitroanilide-HCl dissolved in 0.002% Triton X-100 and 75 µmol of glycine-NaOH buffer at pH 7.1. The assay mixture without cells was taken as the control system. The reaction was stopped by adding 3.0 mL of 1M acetate buffer at pH 4.2. One unit of enzymatic activity was defined as the change of OD of 0.1 at 385 nm in the presence of substrate.

**Effect of pH and temperature on enzymatic activities.** Effect of pH and temperature on enzymatic activities was tested with 75 µM glycine-NaOH buffer from pH 4.8 to pH 9.4. All buffers contained 0.002% Triton X-100. The pH stability was determined at 37°C for 30 minutes. The optimum temperature for DPPIV activity was determined to be between 25°C and 65°C.

**Kinetic analysis and effect of specific and nonspecific inhibitors on enzyme activity.** The initial rates of enzyme activity were determined with increasing concentrations of the substrate (glycyl-proline p-nitroanilide). $K_m$ and $V_{max}$ were determined by adjusting the experimental data to Michaelis–Menten and Lineweaver–Burk plot.[31] Effects of inhibitors were determined by quantifying the decreased enzymatic activity in the assay mixture containing sperm cells ($1.5 \times 10^6$ cells) preincubated with different effectors (0.5, 1.0, and 10 mM) for 30 minutes at 37°C in glycine-NaOH buffer using 1.5 µmol glycyl-proline ρ-nitroanilide-HCl as the substrate. The relative enzyme activity was measured at 385 nm.

The effect of various inhibitors such as protease inhibitors, namely, PMSF, iodoacetamide, β-mercaptoethanol, leupeptin, diprotin-A, and sitagliptin (a specific inhibitor of DPPIV), was determined. To study the effect of sitagliptin, the epididymis was dissected into the three sections, caput epididymis, corpus epididymis, and cauda epididymis. Sperm cells were isolated

separately from each of the three sections by gentle shearing of the tissues and rupturing the tubules, allowing the sperm cells to float into RPS medium (pH 6.9). The assay medium contained 75 µM glycine-NaOH buffer, 1.5 µmol glycyl-proline ρ-nitroanilide-HCl dissolved in 0.002% Triton X-100, $0.2 \times 10^6$ cells, and 1 mM sitagliptin at pH 7.1, and the total volume was made up to 200 µL with water. The reaction was stopped by sodium acetate buffer (pH 4.2).

**Effect of various metal ions on enzyme activity.** The effect of metal ions, $Zn^{+2}$, $Cd^{+2}$, $Ca^{+2}$, $Mg^{+2}$, $Al^{+3}$, $Cu^{+2}$, $Hg^{+2}$, $Fe^{+3}$, $Pb^{+2}$, and $Mn^{2+}$ (5, 10, and 15 mM), and a chelator of divalent cations, ethylenediaminetetraacetic acid (EDTA), was determined by adding them to the reaction mixture. The relative enzyme activity was measured at 385 nm at pH 7.1.

**Assessment on forward motility of cauda epididymal spermatozoa in the presence of sitagliptin.** The forward motility of spermatozoa was quantified by spectrophotometric method,[32,33] which consists of layering 50 µL of freshly extracted cauda epididymal spermatozoa ($200 \times 10^6$/mL) mixed with 10% Ficoll in a total volume of 0.5 mL RPS medium using a Hamilton syringe at the bottom of a standard cuvette containing 1.3 mL of RPS medium, which was sufficient to cover the entire width of the light beam. Vigorously motile spermatozoa that moved upward in the light beam at any particular time were registered continuously as an increase of absorbance at 545 nm with a Gilford spectrophotometer equipped with a recorder. After reaching the maximum absorbance ($A_{eq}$), the contents of the cuvette were mixed and the absorbance for all the cells was noted ($A_t$). The percentage of cells that showed vigorous forward motility was calculated as $A_{eq}/A_t \times 100$. The change of velocity after treatment with sitagliptin (1 mM) was measured according to the change of forward motility activity. One unit of forward motility activity of the most vigorous group of spermatozoa was defined as an initial linear increase of absorbance of 0.01/minute under the standard assay condition. The specific activity of sperm forward motility was expressed as units of forward progression per $10^7$ spermatozoa.

**Statistical analysis.** Results depicted in Tables 1–4 are shown as representative values of six similar experiments. Data were analyzed using analysis of variance (Tukey's multiple comparison method). Values of $P < 0.05$ were considered as the level of statistical significance. Statistical analysis was performed using the SPSS statistical software.

## Results

**Enzyme activity.** Preliminary experiments were carried out to determine the enzyme activity in the sperm cells at different maturational stages in the epididymis. A significant alteration in glycyl-proline ρ-nitroanilide cleaving enzyme was observed in the whole sperm from different parts of the epididymis. Based on the sperm concentration, a significant increase in DPPIV activity was observed in the corpus epididymal spermatozoa in relation to the caput that declined in the cauda sperm to a value that was still greater than that of caput sperm (Fig. 1).

**Characterization of glycyl-proline p-nitroanilide cleaving enzyme in whole cell.**

I. Kinetic analysis of enzyme activity on sperm head and tail of cauda epididymal spermatozoa

Head-rich fraction collected from the bottom of the tube was found to show the highest amount of Gly-pro p-nitroanilide–cleaving enzyme activity. The tail's fraction showed an insignificant enzyme activity. This may be due to the minor contamination of tail fractions with heads (Table 1). A loss of total activity of ~10%–14% was observed due to sonication of spermatozoa.

II. Kinetic analysis and effect of pH and temperature on enzymatic activity

The approximate $K_m$ value of this enzyme for the substrate is 1.062 mM, with an average $V_{max}$ of 0.625/mL/minute (Fig. 2A). The enzymatic activity was linear upto 30 minutes under the standard assay condition (Fig. 2B). The optimum pH for the hydrolysis of glycyl-proline ρ-nitroanilide by the enzyme was pH 7.1. The enzyme activity was stable at pH 6.6–7.23, which is in the range of pH of epididymal fluid of rat.[34] A strong reduction in activity was found below pH 6 and above pH 8.5 (Fig. 2C).

The enzyme activity of DPPIV was determined at different temperatures ranging from 25°C to 65°C. The optimum temperature recorded was 37°C (Fig. 2D). The activity drastically declined at temperature beyond 45°C.

III. Effect of protease inhibitors and a chelator on glycyl-proline p-nitroanilide–cleaving enzyme in whole cell

The effect of different inhibitors on the enzyme activity of whole sperm DPPIV is shown in Table 2. Of all the inhibitors tested, PMSF was able to inhibit only 69% and 73% at 5 and 10 mM concentrations, respectively, whereas diprotin-A was able to inhibit 57% of the enzyme activity at 10 mM. Other protease inhibitors such as iodoacetamide, leupeptin, and aprotinin did not show any considerable effect on the enzyme activity.

**Table 1.** Enzyme activity on sperm heads and tails.

| DIFFERENT PARTS OF EPIDIDYMIS | WHOLE CELLS (% OF ACTIVITY) | HEADS (% OF ACTIVITY) | TAILS (% OF ACTIVITY) |
|---|---|---|---|
| Cauda sperm | 100 | 89.50 | 1.24 |
| Corpus sperm | 100 | 86.44 | 1.045 |
| Caput sperm | 100 | 84.91 | 1.11 |

**Table 2.** Effect of various protease inhibitors and a chelator on Gly-pro p-nitroanilide–cleaving enzyme activity.

| PROTEASE INHIBITORS | CONCENTRATION (mM) | REMAINING ACTIVITY (%) |
|---|---|---|
| Diprotin-A | 0.5 | 73.5 |
| | 1.0 | 58 |
| | 10 | 43 |
| Control | 0 | 100 |
| PMSF | 5 | 31 |
| | 10 | 27 |
| Control | 0 | 100 |
| | 0.1 | 86 |
| DFP | 1.0 | 51 |
| | 5.0 | 32 |
| Aprotinin | 5 | 96 |
| | 10 | 94 |
| Control | 0 | 100 |
| Leupeptin | 5 | 98 |
| | 10 | 96 |
| Control | 0 | 100 |
| Iodoacetamide | 5 | 73 |
| | 10 | 71 |
| | 15 | 70 |
| Control | 0 | 100 |
| β-Mercaptoethanol | 5 | 100 |
| | 10 | 100 |
| | 15 | 100 |
| Control | 0 | 100 |
| EDTA | 5 | 71 |
| | 10 | 70 |
| | 15 | 70 |
| Control | 0 | 100 |

**Table 3.** Effect of various metal ions on Gly-pro p-nitroanilide–cleaving enzyme activity.

| METAL IONS | CONCENTRATION (mM) | REMAINING ACTIVITY (%) |
|---|---|---|
| $Cd(CH_3COO)_2 \cdot H_2O$ | 5 | 65 |
| | 10 | 49 |
| Control | 0 | 100 |
| $ZnSO_4$ | 5 | 62.5 |
| | 10 | 28 |
| Control | 0 | 100 |
| $CaCl_2$ | 5 | 100 |
| | 10 | 100 |
| | 15 | 100 |
| Control | 0 | 100 |
| $MgSO_4$ | 5 | 124 |
| | 10 | 126 |
| | 15 | 126 |
| Control | 0 | 100 |
| $HgCl_2$ | 5 | 6 |
| | 10 | 0 |
| | 15 | 0 |
| Control | 0 | 100 |
| $AlCl_3$ | 5 | 9.7 |
| | 10 | 0 |
| | 15 | 0 |
| Control | 0 | 100 |
| $MnCl_2$ | 5 | 100 |
| | 10 | 100 |
| | 15 | 100 |
| Control | 0 | 100 |
| $CuSO_4$ | 5 | 98 |
| | 10 | 96.7 |
| | 15 | 96 |
| Control | 0 | 100 |
| $Pb(CH_3COO)_2$ | 5 | 78 |
| | 10 | 73.8 |
| Control | 0 | 100 |

β-Mercaptoethanol and EDTA did not show any significant effect on the activity of this enzyme.

IV. Effect of metals ions

Among the ions tested, $Hg^{2+}$, $Al^{3+}$, $Zn^{2+}$, and $Cd^{2+}$ significantly inhibited the enzyme activity (Table 3). Mercury (5 mM) and aluminum (5 mM) were remarkably strong inhibitors of dipeptidyl peptidase-IV than cadmium and zinc. Other metal ions such as $Cu^{2+}$, $Ca^{2+}$, $Pb^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ did not show any appreciable effect on enzyme activity. Mercury and aluminum exerted the same degree of inhibition on enzyme activity.

V. Effect of sitagliptin on glycyl-proline p-nitroanilide–cleaving enzyme in whole cell

Sitagliptin, a highly selective DPPIV inhibitor, was evaluated in clinical trials as a monotherapeutic drug and antidiabetic agent. Sitagliptin at a concentration of 0.1 mM inhibited ~50% of enzyme activity, whereas at 1 mM concentration, the activities were totally lost (Fig. 3). This result showed that the glycyl-proline p-nitroanilide–cleaving enzyme on the outer surface of epididymal spermatozoa may be DPPIV. The effect of sitagliptin on DPPIV of the sperm isolated from different parts of the epididymis (caput, corpus, and cauda) was also studied. It was observed that sitagliptin (1 mM) was a strong inhibitor of enzymatic activity in the maturing epididymal spermatozoa (Table 4).

**Assessment of forward thrust of cauda epididymal spermatozoa.** The movement of the cells in the vertical plane

**Table 4.** Effect of a selective inhibitor (sitagliptin) on Gly-pro p-nitroanilide–cleaving enzyme activity.

| PARTS OF EPIDIDYMIS | CONCENTRATION OF SITAGLIPTIN (mM) | ENZYME ACTIVITY WITHOUT INHIBITOR | REMAINING ACTIVITY (%) |
|---|---|---|---|
| Caput | 1 | 5.05 | 0 |
| Corpus | 1 | 6.21 | 0 |
| Cauda | 1 | 5.68 | 0 |

was measured using the automated computerized spectrophotometric system,[33] as all the motility analyzers till date, including CASA, never considered the importance of motility in the vertical direction. The computerized spectrophotometric assay method showed a drastic inhibition in the percentage of forward motility with time when treated with sitagliptin (1 mM) as compared to the control sperm. The forward motility percentage decreased to about 30%, 50%, and 80% after 30 minutes, one hour, and 2 hours of incubation, respectively, in the presence of extracellular sitagliptin (Fig. 4).

## Discussion

Several biologically active peptides contain conserved proline residue as a controlling element of proteolytic processes; therefore, proline-cleaving peptidases are the regulatory enzymes in these metabolic check points. In these cases, DPPIV are important regulators in the proteolytic inactivation or activation status quo of regulatory proteins. Over the past three decades, a complete array of different functional DPPs has been identified and characterized in immune, nervous, and endocrine systems.[35] In addition, several molecules with DPPIV-like activity were also cited. They are homologous or nonhomologous with DPPIV[13,35,36] or highly homologous to the protein, but with no enzymatic activity.[37,38] These molecular and structural complexities of DPPIV could partly explain its physiological role as a regulatory molecule.

In this study, a synthetic substrate, glycyl-proline ρ-nitroanilide, was used to identify the activity of dipeptidyl peptidase in spermatozoa from different parts of epididymis. This substrate was first synthesized to assay X-prolyl dipeptidyl aminopeptidase, which liberates p-nitroanilide, and the reaction was measured spectrophotometrically.[9] Previous studies have shown that bovine cauda epididymal tissue contained higher DPPIV activity than DPPII.[39] Dubois et al found extremely high DPPIV activities in bovine prostasomes, similar to those observed in humans.[20] In bulls, the seminal vesicles are the predominant source of DPPIV,[40] whereas it is the prostate gland in humans.[41] This suggests a species specificity in the sex accessory gland as a source of secretary DPPIV. A high DPPIV activity was found in the epididymis of rat and bovine compared to that in the testis.[20] The present study shows for the first time that there is a marked alteration of dipeptidyl peptidase that has postproline-cleaving activities on viable spermatozoa from different parts of epididymis, and the maximum enzyme activity was observed in cells from the corpus or the middle part of rat epididymis. Biochemical characterization of this endogenous enzyme showed a significant (75%) inhibition of activities in the presence of an irreversible inhibitor, PMSF, which is a weak inhibitor, according to De Meester et al.[42] Diprotin A, until recently known as a specific reversible DPPIV inhibitor and now considered as a competitive substrate,[43,44] partially inhibited the enzyme activity at 10 mM concentration (Table 2). Although diprotin A inhibits DPPIV activity, the inhibition is moderate. Sitagliptin, a specific inhibitor of DPPIV, leads to a more effective and complete inhibition (Fig. 3 and Table 4) of the peptidase in spermatozoa collected from different parts of epididymis. This result indicated that all the glycyl-proline ρ-nitroanilide–hydrolyzing enzymes on the surface of epididymal spermatozoa have some similarities with the biochemical properties of DPPIV-like enzyme. Insensitivity or insignificant sensitivity of the enzyme toward different nonspecific protease inhibitors such as leupeptin (10 mM), aprotinin (10 mM), iodoacetamide, and β-mercaptoethanol also confirmed the specificity of the enzyme. The complete sensitivity toward $Hg^{2+}$ and partial insensitivity toward $Cd^{2+}$ (Table 3) indicated the similarity with DPPIV.[42] But complete sensitivity toward $Al^{2+}$ and partial inhibition by $Zn^{2+}$ contradict the characterization of this peptidase as DPPIV.

Several proteins are present in the cell coat of epididymal spermatozoa, which probably behave independently during maturation to keep pace with the dynamic membrane fluid. Our study has shown the accumulation of maximum enzyme activity on the head part of spermatozoa in all the maturational

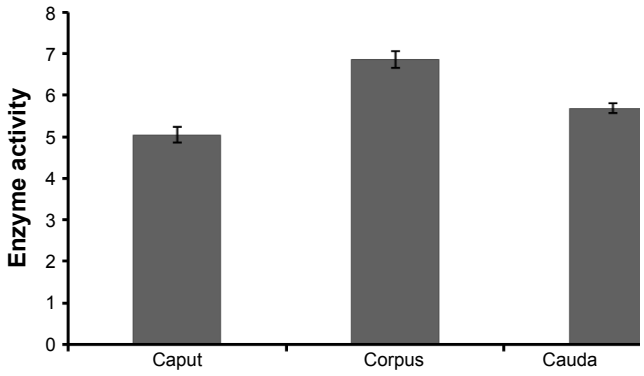


**Figure 1.** Alteration of enzyme activity in different parts of rat epididymis. One unit of enzymatic activity = 0.1 OD change at 385 nm. All data are expressed as mean ± SD of six similar results. Each bar represents the enzyme activity in the respective epididymal parts.

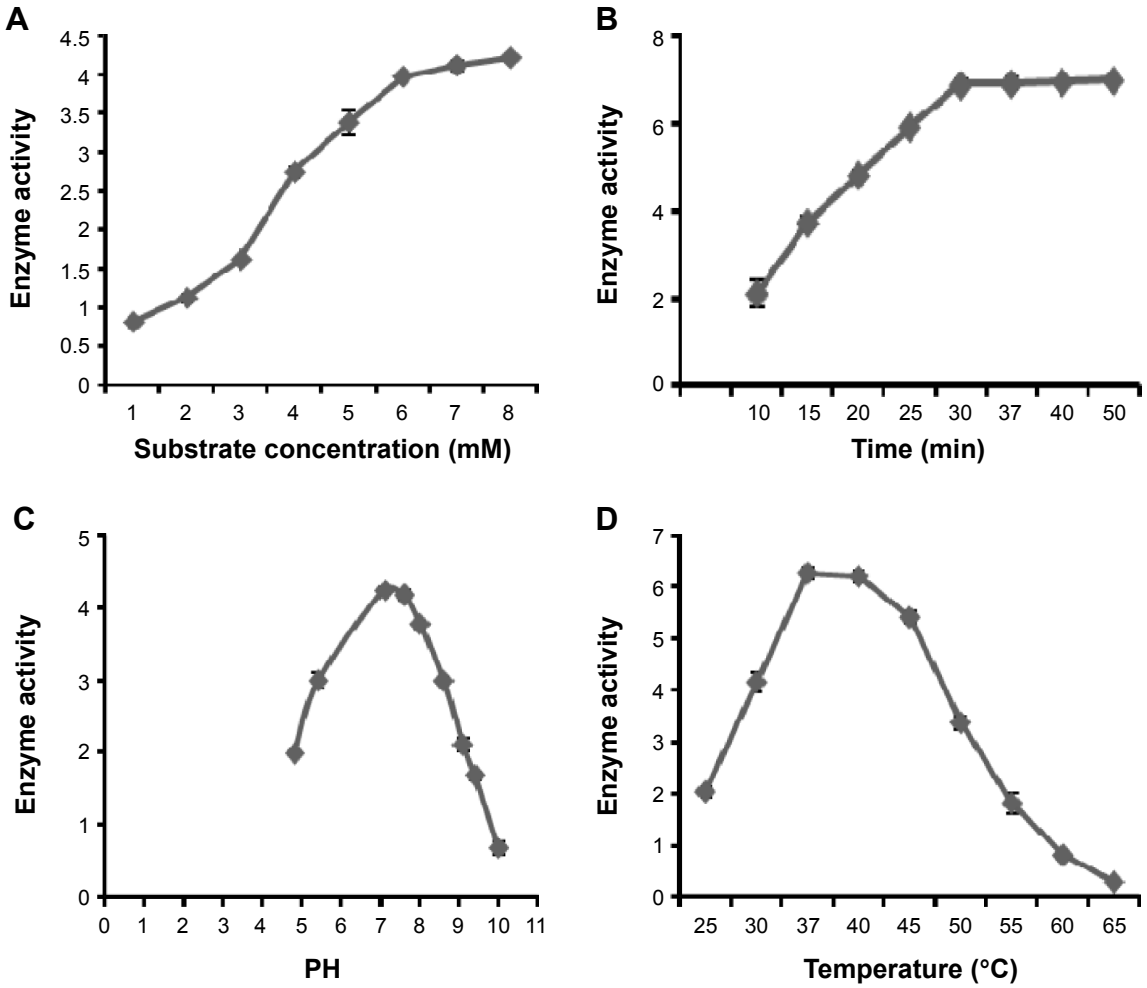


**Figure 2. A**: Enzyme kinetics depending on different physical parameters. The data are representative of six such experiments. All data are expressed as mean ± SD. **B**: Time course of hydrolysis of Gly-pro p-nitroanilide by DPP enzyme of the epididymal sperm plasma membrane in 75 μM glycine-NaOH buffer at pH 7.1. **C**: Effect of pH on the hydrolysis of glycyl-proline ρ-nitroanilide. 1.5 μmol glycyl-proline ρ-nitroanilide was dissolved in Triton X-100, 75 μmol of glycine-NaOH was added to make the assay volume, and the reaction was stopped using 1M acetate buffer at pH 4.2. **D**: Effect of temperature on the hydrolysis of glycyl-proline ρ-nitroanilide. The data are representative of five such experiments.. Data are mean ± SD; $P < 0.05$ (Tukey's test).

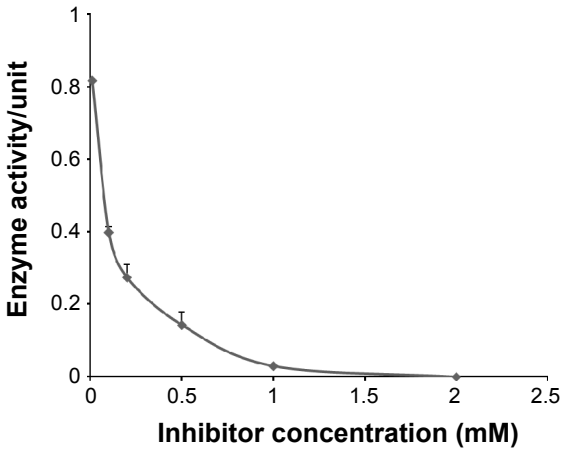


**Figure 3.** Effect of different concentrations (0.1–2.0 mM) of a specific inhibitor (sitagliptin) on the enzyme activity. Data are representative of five such experiments. Data are mean ± SD; $P < 0.05$.

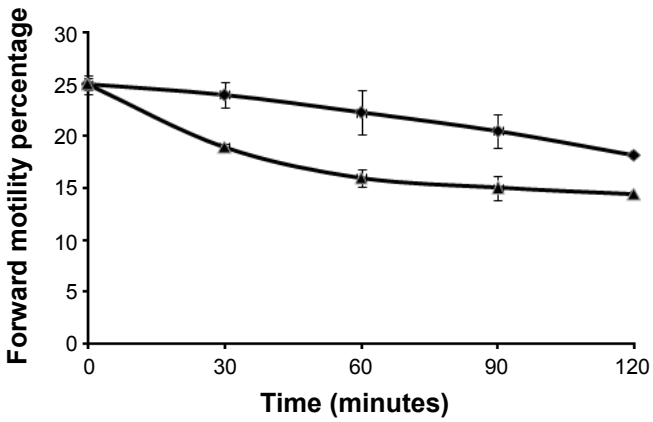


**Figure 4.** Effect of sitagliptin (1 mM) on the forward motility of mature cauda epididymal spermatozoa. The data are representative of six such experiments. ●, control cell motility; ▲, sitagliptin-treated cell motility. Data are mean ± SD.

stages of epididymis (Table 1). We also observed that sitagliptin, a specific inhibitor of DPPIV, has a significant effect on the forward motility of mature caudal spermatozoa. As indicated in previous studies,[45,46] this result indicates the involvement of this peptidase in controlling sperm movement, but the mechanism is still unknown. Although secretion of epididymal fluid is important for the survival and maturation of spermatozoa, the actual role of the proteins in the fluid is yet to be determined.[47] Based on this study, it can be concluded that dipeptidyl peptidase may have the endogenous substrate either on the surface of spermatozoa or in the epididymal fluid microenvironment, and the enzyme hydrolyzes the transition of proteins to their functional/nonfunctional state so that the spermatozoa can achieve the potential maturational status. Further studies are required to better understand the participation of this enzyme in the maturation process of spermatozoa in the epididymal lumen.

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## Author Contributions

Conceived and designed the experiments: MS and DN. Analyzed the data: MS and DN. Wrote the first draft of the manuscript: MS and DN. Contributed to the writing of the manuscript: MS and DN. Agreed with manuscript results and conclusions: MS and DN. Jointly developed the structure and arguments for the paper: MS and DN. Made critical revisions and approved the final version: MS and DN. All the authors reviewed and approved the final manuscript.

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