

Identification and Characterization of Molecules Having Anti-microbial Activities from Rabbit Epididymis Using Proteomic Approach

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Abstract

Antimicrobial peptides (AMPs) are the key components of the host innate immune system and form the first line of host defense against invading pathogens. In the present study attempts were made to identify and characterize AMPs that are synthesized in the epididymal epithelial cells (EPECs) of rabbit, *Oryctolagus cuniculus*. *Ex-vivo* induction of EPECs with TLR-9 ligand, CpG-ODN-2006 resulted an up-regulation of several AMPs. These molecules were identified in the acid extracts of pooled epididymal tissue homogenates using proteomic approach (Ultra-filtration, cation-exchange chromatography, RP-HPLC, Far Western blotting - FWB, 2D-PAGE and MALDI-TOF-MS). The pooled RP-HPLC fraction (peaks 1-5), named as rabbit epididymal secretory antimicrobial protein (RESAMP), showed activity against several pathogens. FWB, 2D-PAGE and MALDI-TOF-MS results revealed that of the ~19 proteins identified, four protein sequences (hemoglobin- α/β subunits, transthyretin and calreticulin) found to be reacted with HIV antigens (gp120, gp17, gp41, p24). One of the four sequences (VLSHHFGKEFTPQVQ) showed >90% homology with hemoglobin- β protein. A 29 mer peptide with VLSHHFGKEFTPQVQ sequence designated as rabbit epididymal hemoglobin beta (REHb β P). The synthesized peptide showed the highest Probability Based Mowse Score (PBMS) and demonstrated good bactericidal activity (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Candida albicans*). REHb β P did not show cytotoxicity against human endocervical cells (End1/E6E7) and rabbit erythrocytes. Immunofluorescent results revealed that REHb β P localized in the stereocilia epididymis and on the acrosome of ejaculated spermatozoa of rabbit. To the best of our knowledge, this is the first study describing the detection of AMPs in rabbit epididymis. In conclusion, the present study suggests that EPECs derived REHb β P may have therapeutic potential in the management of reproductive well being of human and animals.

Keywords: Rabbit; Epididymal epithelial cells (EPECs); REHb β P; AMPs

Introduction

Peptides with antimicrobial activities have been discovered across virtually all forms of life as a primitive component of their innate immune system and are constitutively or inducibly expressed in response to invasion by pathogens [1,2]. A number of microorganisms infect reproductive tract in humans and animals, with serious consequences for reproductive function [3]. In men, the penile urethra is the portal of entry for pathogens (eg: *Neisseria gonorrhoeae* and *Chlamydia trachomatis*) and urethritis is the most common clinical syndrome [4]. Occasionally, these pathogens can also ascend into deeper organs such as the epididymis [4]. Retrograde infection of the epididymis may also occur from microorganisms present in the vas deferens, or via the blood vessels supplying epididymis. A common result of microbial infection of the epididymis is epididymitis [5], the condition that may lead to the destruction of the epididymal duct and transient or permanent sterility in mammals [3].

Epididymal epithelial cells (EPECs) form a barrier to create an unique microenvironment in the lumen, where interactions between EPECs and spermatozoa take place via the fluid milieu [6]. Spermatozoa bind a variety of proteins as they pass through the epididymis [7]. Besides sperm maturation and fertilization, epididymis is known to have efficient self-defense mechanisms against bacterial infection [2,8]. Recent studies have shown that EPECs of rat, mice and human synthesize molecules having antimicrobial activities. Some of them are Bin-1 β [9], Eppin [8], SPAG-11 [2], HE2- α [10], β -defensins [2,11], SOB3 [12], EP2/HE2 [4], hCAP-18 [13], and cystatin-11 [14] etc. Despite the progress made in other species, the repertoire of host defense molecules also known as antimicrobial peptides (AMPs)

having microbicidal activities have not been investigated previously in the epididymis of rabbit, *Oryctolagus cuniculus*. To the best of our knowledge this is the first study dealing the identification and characterization of AMPs in the rabbit epididymis.

Rabbits are being used widely as a first choice *in vivo* model to evaluate the safety of various products before planning for expensive clinical trials [1]. Since the emergence of antibiotic-resistant pathogenic bacteria, the discovery of new AMPs to substitute for traditional antibiotics has become increasingly important [15]. Therefore, in the present study we intended to identify, isolate and characterize AMPs that are synthesized along the epididymis and secreted into the lumen.

The results of the present study demonstrated that using proteomic approach, we generated rabbit epididymal AMP repertoire and compared with the AMP repertoire obtained after *Ex-vivo* induction of rabbit epididymis by CpG-ODN-2006, a ligand for toll-like receptor-9 (TLR-9). The ligand induced the synthesis of new

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and constitutively expressed AMPs. One of the molecule having antimicrobial activity was identified and named as rabbit epididymal hemoglobin beta peptide (REHb β P). The peptide did not lyse RBCs or decrease the viability of human vaginal cells. Immunofluorescence studies reveal that REHb β P found to be localized in epididymis and spermatozoa. The study would aid in designing newer intervention strategies for the prevention of sexually transmitted infections (STI's).

Materials and Methods

Animals

Healthy and sexually mature male Belgium white rabbits (mean age 10 \pm 1 month; mean body weight, 3 \pm 0.50 kg) were maintained under standard laboratory conditions (temperature 20 \pm 1°C, relative humidity 50 \pm 10%, and 12 h light:12 h darkness cycle). Animals were housed individually in stainless steel cages, food and water were made available ad libitum.

Adult female Holtzman rats weighing ~120 gm were used for raising anti-peptide antibodies. The study was approved by the Animal Ethics Committee of the National Institute for research in Reproductive Health (NIRRH), Parel, Mumbai (IAEC # 05/08) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Reagents

The peptides viz., REHb β P, hemoglobin- α , transthyretin and calreticulin were commercially procured (USV Ltd., Mumbai, India). Goat anti-rat and goat anti-human secondary antibodies, cell culture reagents and media used in various experiments were procured from Sigma, USA. CpG-ODN-2006 was obtained from In Vitrogen Life science technology, USA For the preparation of stock solutions, all the reagents were dissolved in endotoxin-free water (Sigma). Unless otherwise stated, all other chemicals and buffers are of high grade quality were procured from local suppliers.

Bacterial strains and culture

The clinical isolates were obtained from patients attending the microbiology laboratory, King Edward Memorial Hospital, Mumbai, India. Vaginal swabs were taken and cultured in respective growth media. Identification of the organisms was done by biochemical tests, and their antibiotic sensitivity was determined by standard criteria [16]. For antibacterial testing clinical pathogens viz., *C. albicans* and Gram negative bacteria (*Escherichia coli*, and *Pseudomonas aeruginosa*), and Gram positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*) were used. The bacteria were grown at 37°C overnight in Muller Hinton Broth (MHB) or Sabouraud dextrose agar (for *C. albicans*) (Hi-Media laboratories Pvt Ltd, Mumbai, India) and then subcultured into fresh broth and raised to log phase for 4-6 h. The cultures were centrifuged at 6000 rpm (10 min, 4°C) and the pellets washed twice in sterile 10 mM sodium phosphate buffer (pH 7.4) and resuspended in the same buffer. All bacterial suspensions were calibrated and routinely standardized to an absorbance of 0.5 at 630 nm, which gave a concentration of approximately 10⁸ Colony Forming Units (CFU) /ml).

Ex-vivo induction and extraction of AMPs from rabbit epididymis

Epididymis was removed from the rabbits (N=22) at various time points during the course of the present study (June-2007 to May

2010). The tissues were cleaned from superficial fluid, fat body and blood stain by gently blotting onto Kleenex paper and immersed in 10 ml PBS. A single dose (10 μ g/gm tissue) of 25 mer peptide, CpG-ODN-2006 (5'-tcgtcgtcgttcgaacgacgttgat-3'), a pathogen associated molecular pattern (PAMP) was administered *Ex-vivo* into the caput and cauda regions of intact epididymis and incubated for 6 h at 37°C in CO₂ incubator in the presence 199 medium. Following washing in the same medium, epididymal tissue was minced with fine scissor and homogenized in PBS (pH:7.4). During collection and processing of the tissue, utmost care was taken to avoid sample contamination with blood. The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The resulting supernatant was collected, dialyzed using Amicon ultrafiltration unit with a 30 kDa cut-off membrane (Millipore, MA USA). The unit was centrifuged at 5000 g for 30 min and the filtrate was lyophilized. The lyophilized samples were dissolved in required amount of endotoxin free water (Sigma) and determined the total protein concentration [17] before subjected to purification by cation-exchange chromatography.

Cation-exchange chromatography

Cation-exchange chromatography was performed as described by Yedery and Reddy [18] with following modifications. The dialyzed extract of epididymal tissue extract (5 ml) was loaded onto a 24 X 1.8 cm glass column packed with carboxy methyl cellulose-CMC (Sigma, USA), equilibrated with 0.2 M sodium acetate (pH 4.0) buffer. Unbound proteins were washed with sodium acetate until the absorbance returned to baseline. Adsorbed proteins were eluted with 0.2 M ammonium acetate buffer (pH 5.2). Fractions (1 ml) were collected and their absorbance was monitored at 280 nm using a spectrophotometer (UV-160A, Shimadzu, Japan). Protein fractions were freeze-dried, resuspended in 50 μ l of sterile deionized water containing 0.01% acetic acid and tested for antibacterial activity.

Anti-bacterial activity of peak-II fraction of cation-exchange chromatography analyzed by zone of inhibition assay

Antibacterial activity of peak-II fraction obtained on ion-exchange chromatography was assessed by a two-layer radial diffusion method described earlier by Mandar et al. [16]. Briefly, the modified protocol involves preparing a 14-ml bacterial underlay of 1% agarose in 10% MH broth supplemented with 0.02% Tween in a 12 X 12 cm Petri dish. The agar was seeded with 1X10⁶ washed bacteria. Wells of 3 mm diameter were punched into the agarose and 50 μ l of the test sample were pipetted into each well. Negative controls comprised only sterile deionized water containing 0.01% acetic acid. The plates were incubated at 4°C for 3 h and then overlaid with 14 ml vol. of sterile 1% agarose containing double strength Luria-Bertani Broth (LBB). They were incubated for a further 24 h at 35°C. Clear zones in the agar underlay were taken as indicating antibacterial activity. The diameters of zones were measured and activity was expressed as clear zone area (in mm²) minus the area of the well.

Reverse-phase HPLC (RP-HPLC)

RP-HPLC was used to determine whether or not CpG-ODN-2006 challenge lead to the induction of new AMPs proteins. The pooled and lyophilized peak-II fraction of both control and CpG-ODN administered samples obtained on cation-exchange chromatography were resuspended in 500 μ l of solvent A (0.1% trifluoroacetic acid (TFA) in water (v/v), and loaded onto a C18 RP-HPLC column using Dionex system (Hesperia, CA, USA) (Vydac., 4.6 \times 250 mm, i.d; particle

size, 5 µm). Bound proteins were eluted at a flow rate of 1 ml/min in a three-step gradient of 0.1% trifluoroacetic acid /water and 0.1% (v/v) trifluoroacetic acid in 50% acetonitrile: 0-100% (45 min) held at 100% for 5 min and brought back to 0% (100-0%). The absorbance of the eluted fractions was monitored at 220 nm using spectrophotometer (Shimadzu, UV-160, and Japan). To obtain sufficient amounts of active protein, purification was repeated at least 60-70 times and used for biotinylation and for various other studies. Pooled 1-5 peaks of control and CpG-ODN induced samples were vacuum dried (Speed vac, Christ, USA) and subjected to Tricine SDS-PAGE.

Tricine-SDS-PAGE analysis of RP-HPLC fractions

To determine the pattern of protein profiles, pooled RP-HPLC fractions of control and CpG-ODN induced epididymal samples were subjected to Tricine Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (Tricine SDS-PAGE) as per the procedure described [16]. The gels were stained with Coomassie blue (Coomassie Brilliant blue R-250; Sigma) and calibrated using molecular weight markers (Amersham Bioscience, USA).

Identification of HIV binding proteins of cation-exchange chromatography fractions by Far Western blotting approach

Far Western blotting (FWB) approach has been used in the past to study protein-protein interactions [19]. Briefly, in the protocol we used a non-antibody protein probe to detect interacting proteins immobilized on a membrane support. Since the amount of protein obtained after RP-HPLC was not sufficient enough for biotinylation, the peak-II pooled protein fraction obtained on cation-exchange chromatography was and labeled with biotin and identified molecules that are interactive to HIV proteins (gp120, gp17, gp41, p24). HIV strips (MP Diagnostics, India) were incubated with non-fat dry milk (NFDM) for 1 h at room temperature to block non specific binding sites. After two washings with Milli Q water, blots were incubated for 2 h at 37°C with biotinylated epididymal proteins. After two washings with Milli Q water, strips were incubated for 1 h with streptavidin conjugated horse redox peroxidase (HRP). After two washings, the strips were incubated with 3'-3-diaminobenzidine (DAB) and observed for binding by comparing with the standard HIV strip. The HIV protein blot to which the biotinylated proteins were bound showed a brown reaction. Each protein band corresponding to gp120, gp17, gp41, p24 was cut by keeping aside the standard HIV protein blot. The biotinylated protein (s) that bound to the HIV antigen strip were eluted with high salt buffer and dialyzed using 1 kDa dialysis membrane against Milli Q water at 4°C overnight to remove salts. The dialyzed samples were precipitated by adding chilled acetone and concentrated. To rule out whether the HIV proteins of the blot bleached along with the biotinylated proteins, the blots were reprobed with goat anti-human secondary antibody conjugated to HRP. After two washings with Milli Q water, the strips were incubated with 3'-3 DAB and compared the intensity of brown color with the fresh strips processed in the same manner. Care was taken as much as possible to preserve the native conformation and interaction conditions for the proteins under study. Though the protocol is lengthy and requires a meticulous analysis, but at the end it gave us satisfactory results. The experiment was repeated at least 25-30 times to get enough amount of the protein. The isolated protein/s was separated by 2-Dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Analysis of 2D-PAGE

Aliquots of 50 µg protein sample were diluted in 300 ml rehydration

buffer containing 8 M urea, 2% m/v CHAPS, 25 mM dithiothreitol, 0.2% Biolyte (3–10 pI range), and 0.002% bromophenol blue. The sample was then applied onto a 17 cm immobilized pH gradient strip with a linear range of pH 3–10, overlaid with mineral oil. First-dimension IEF was performed using Bio-Rad iso-electro focusing (IEF) system. The voltage gradient was standardized and set from 50 volts to 6000 volts. After 18 h run, the strips were removed and placed in equilibration buffer, with dithiothreitol (DTT) and iodoacetamide for 30 min in each reagent. After equilibrating and alkylating, the electrophoresed strips were sealed onto the top of the second-dimension gels (12% SDS- polyacrylamide gel) with 0.5% agarose. The protein spots obtained were subjected to MALDI-TOF-MS. The 2D experiment was repeated twice to confirm the spot patterns before proceeding with further analysis. Replicates were scanned with a GS-800 calibrated densitometer using standardized parameters, and gel images were processed by PDQuest 7.2.0 software. Spot densities were determined after normalization, based on the total spot volumes on the gel. Protein spots, with significant changes in densities (paired t-test, $P < 0.05$) in a consistent direction (increase or decrease), were considered to be different and selected for further identification.

In-gel tryptic digestion and peptide extraction

The Selected protein spots from the stained gel were perfectly aligned with the bands on the adjacent blots and individual protein spots of interest were manually excised from the silver-stained gels in duplicate. BSA gel plugs were used as a protein standard for each set of in-gel tryptic digestion experiment. The gel bands were destained using a 1:1 mixture of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. The protein bands were typically digested according to the protocol described earlier by Shevchenko et al. [20] with minor modifications, using sequencing grade trypsin 0.01 µg/µl (Applied Biosystems) for 16 h at 37°C. The tryptic peptides were extracted with trifluoroacetic acid (TFA). The extracts were pooled and dried completely by centrifugal lyophilization. The peptides were reconstituted in sample diluent and mixed with an equal volume of matrix (α -cyano-4-hydroxycinnamic acid, 10 mg/ml in 70% v/v ACN, 1% v/v TFA) and spotted in duplicate on a target plate and allowed to air dry. Trypsin digested β -galactosidase *Escherichia coli* (β -gal) and CHCA mixture was also spotted on MALDI plate and used as a known standard for each set of MS analysis. Peptides were analyzed by MALDI-TOF/TOF.

MALDI-TOF Mass spectrometric analysis

All the proteomics work reported in this paper was carried out at central proteomics facility of National Institute for research in Reproductive Health (NIRRH). Mass spectrometry analyses were performed using the Applied Biosystems 4700 Proteomics Analyzer (MALDI-TOF-TOF; Foster City, CA, USA) in reflector mode for positive ion detection. The laser wavelength was 355 nm. All the MS spectra resulted from accumulation of at least 1000 laser shots. MALDI target plate was internally calibrated and default updated using the [M+H]⁺ ion from 4700 proteomics analyzer calibration mixture (4700 Cal Mix, Applied Biosystems) as per the manufacturer's instructions. β gal (β -galactosidase digested), a known standard, was used to validate mass accuracy of mass spectrometer. In case of samples, the mass spectra were calibrated using the three trypsin auto digest products: fragment 92–99 ([M+H]⁺=805.416 Da), fragment 50–69 ([M+H]⁺=2163.056 Da), and fragment 70–89 ([M+H]⁺=2273.159 Da).

MALDI-TOF-MS data analysis

Combined MS and MS/MS spectra were used to search against the taxonomy Mammalia using the GPS software (version 3.5, Applied Biosystems) running Mascot search algorithm. The isoelectric point (pI) of the identified proteins/peptides was predicted using the Expert Protein Analysis System (ExpASY online software (produced by Molecular Biology of the University of Geneva service station). All peptides with scores less than the identity threshold ($p = 0.05$) or a rank less than one were not considered. The criteria used to accept identifications included the number of peptides matched, and the Probability Based Mowse Score (PBMS > 35). Proteins having origin of rabbit and molecular weight estimated from the gel slice was additional requirements for protein identification. A protein was accepted into the list if it was confidently identified in the sample with more than two highly confident peptide hits. If necessary, peptides were excluded if their masses corresponded to those for trypsin, and keratins or to other irrelevant proteins. The sequence which has highest PBMS value was selected for further characterization

Sequence alignment of hemoglobin- β subunit from different mammals

The amino acid sequence of β -hemoglobin of rabbit, bovine, human and mouse were aligned with bio-software Lasergene 6 program. The MegAlign module of the Lasergene 6 displayed the difference among these sequences. In Figure 6, we compared the amino acid sequences of Hb- β of rabbit with human, bovine and mice.

Design and synthesis of peptides and antibody production

A 29 mer linear peptide, REHb β P was synthesized commercially by standard F-moc (N-(9-fluorenyl) methoxycarbonyl) chemistry. REHb β P had an overall charge of +4. The peptide was purified via analytical RP- HPLC on C18 column (USV peptide synthesis facility, Mumbai, India). To raise anti-peptide polyclonal antibody, REHb β P was coupled with a keyhole limpet hemocyanin carrier (KLH) (Pierce, Rockford, IL, USA) according to manufacturer's instructions. Two rats were intradermally immunized with 100 μ l of PBS containing 25 μ g of the peptide emulsified in 50 μ l complete Freund's adjuvant. The first immunization was followed by two boosts at 2-week intervals with aliquots of peptide emulsified in incomplete Freund's adjuvant. Anti-serum was collected 10 days after the last injection and titers were checked by ELISA. The anti-peptide antibody was purified via affinity chromatography over a sepharose column conjugated with REHb β P used as an immunogen.

Circular dichroism

Circular Dichroism (CD) spectra were collected using a Jasco J-720 spectropolarimeter available at our Institute Central equipment facility. Spectral scan was performed from 250 to 195 nm, with step resolution of 0.1 nm and bandwidth of 1.0 nm at speed 50 nm/min [21]. A 1-mm path length quartz cuvette was used for the measurements, and values from 3 scans were averaged per sample. Freshly prepared peptide samples were measured at 40 μ m. The sodium phosphate buffer (pH 7.4) was used as a solvent for measurements, and its background was subtracted from actual sample values. The molar ellipticity was calculated using the equation, $[\theta]_{222} = -39,500 \times (1 - 2.57/n)$, where n =number of residues.

Radial diffusion assay

The antibacterial activity of cation-exchange and RP-HPLC

purified fractions were tested in radial diffusion assay (RDA) against *E. coli* and *S.aureus* at neutral pH (pH:~7) as described earlier [16].

Determination of the minimal inhibitory concentrations (MICs)

The antibacterial activity of synthesized peptide, REHb β P was determined against *Candida albicans*, gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*) was evaluated as described by Mandar et al. [16]. Briefly, overnight grown bacterial cultures in TSB were diluted with medium to obtain $\sim 1 \times 10^8$ cfu/ml and then diluted to $\sim 1 \times 10^6$ cfu/ml using MHB, and incubated in serial two fold dilutions of REHb β P (1.89 - 60.61 μ M), after which the bacteria was washed. About 10 μ l aliquots were removed at 30 min, diluted 1:1000 in growth medium and spread on MH agar plates to quantify the number of cfu survived by counting the average number of surviving cfu/ml in the presence of peptide. As a control, 10 μ l of PBS was incubated with each strain bacterial culture.

Preparation of small unilamellar phospholipid vesicles

To elucidate the ability of REHb β P to permeabilize bacterial cell membranes, a dye leakage assay with liposome was performed. Liposomes mimicking negatively charged microbial membranes were prepared as described earlier [22]. To a suspension of calcein-loaded liposomes, two fold serial dilutions of REHb β P (1.89-60.61 μ m) were added and incubated 1-5 min. Time dependent changes in fluorescence intensities were determined as a percentage of the maximal fluorescence intensity obtained after total lysis of the liposomes by 4 μ l of 0.1% (v/v) Triton X-100. Peptide induced calcein fluorescence dye leakage was monitored continuously over time at excitation and emission wavelengths of 494 nm and 520 nm respectively, on an FLX 800 microplate spectrofluorometer.

Binding studies of REHb β P to gp120

The binding affinity of REHb β P with gp120 was evaluated by ELISA. Briefly, 96 well plate was coated with gp120 (1 μ g/well) and incubate over night at 4°C. Wells were washed with PBS and nonspecific binding was blocked with blocking solution (1% BSA in PBS) by incubating for 1 hr at 37°C. After two washes with 0.1% PBS-T20, two fold serial dilutions of REHb β P (1.89-60.61 μ M) was incubate at 37°C for 2 h (wells with out peptide were also run simultaneously and treated as controls). After two washes with 0.1% PBS-T20, anti- REHb β P antibody was added at a dilution of 1/100 and incubated for 1 h at 37°C. Pre-immune serum controls were also maintained to check the nonspecific activity. After two washes with 0.1% PBS-T20 goat anti rat secondary antibody (1:500) was added and incubated at 37°C for 1hr. Plate was washed with PBS and Ortho-phenylenediamine (OPD) was added and kept in dark or 15 min. Finally the reaction stopped using 100 μ l of 4N H2SO4. The brown color was read at 490 nm using ELISA reader.

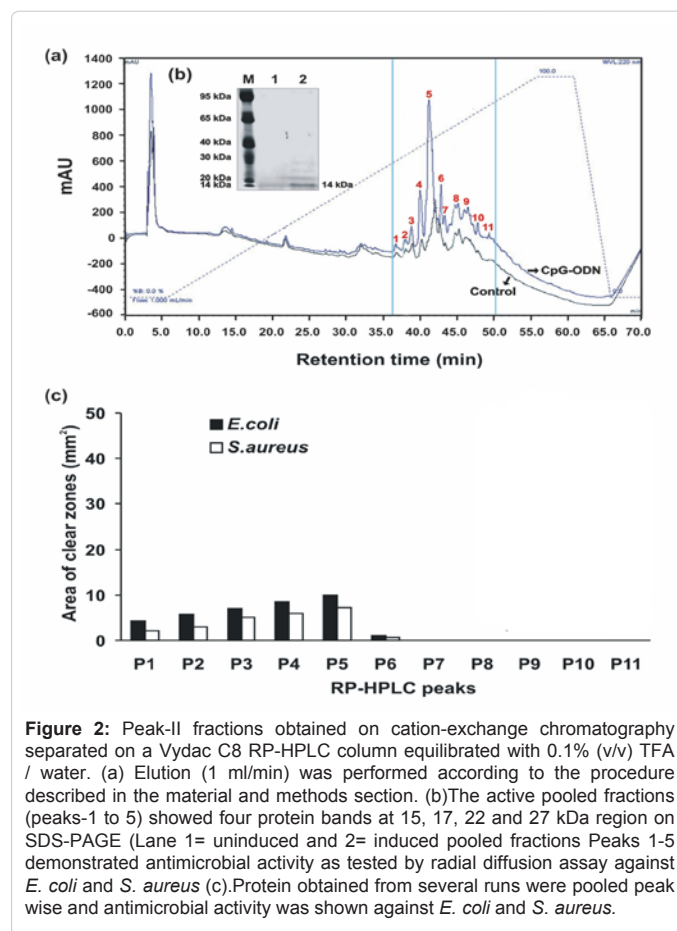
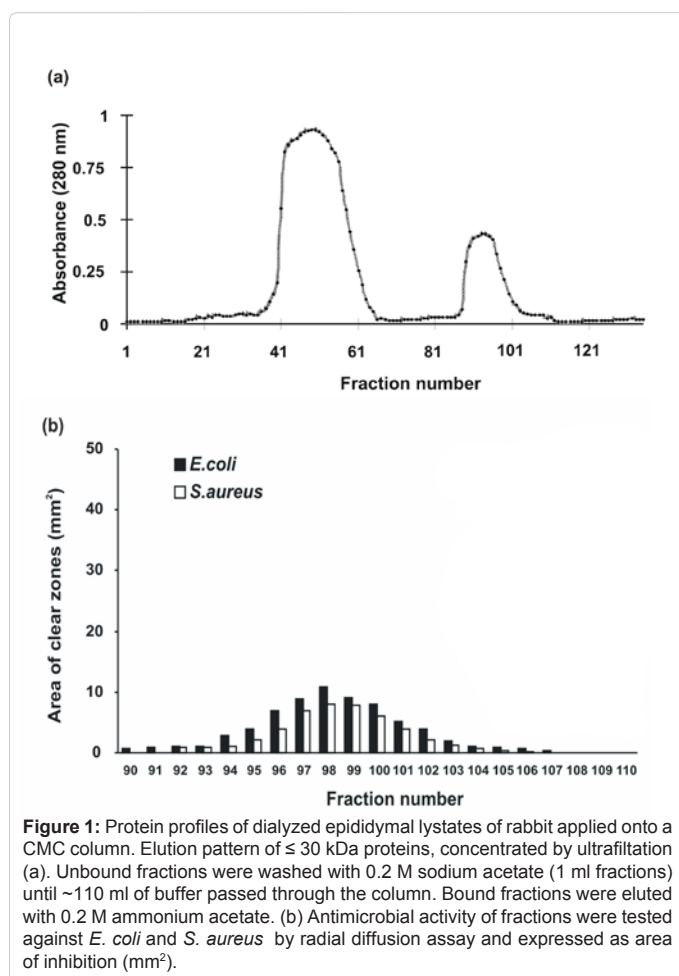
Cell-based luciferase reporter gene fusion assay

HeLa-derived HL2/3 (effector) cells, which express the HIV-1 Env on surface and Tat protein in their cytoplasm were co-cultured with TZM-bl cells (Target/indicator) at a 1:1 cell density ratio (2.5×10^4 cells/well) in order to examine whether the peptide interfered with the binding process of HIV-1 Env and the CD4 receptor as described earlier [23]. Briefly, co-cultures were incubated for 48 h in the absence or presence of two-fold serial dilutions of REHb β P (1.89-60.61 μ M) and trimeris (0.312-10 μ g) of (N-acetyl-YTSLIHSLEESQNQQEKNEQ ELLELDKWASLWNWF-CONH2) (+ve control for inhibition). Upon

fusion of both cell lines, the Tat protein from HL2/3 cells activates luciferase reporter gene expression in TZM-bl-CD4-LTR-LUC cells. Efficiency of fusion was determined by measuring luciferase activity in the wells following the instructions of the manufacturer using the bright-glow luciferase determination kit (Promega, Madison, USA). The percentage of inhibition of HL2/3-TZM-bl-CD4 cell fusion was calculated with respect to the positive control of untreated cells. Light intensity was measured using Luminometer. Background fluorescence in TZM-bl cells was obtained without the addition of HL2/3 cells.

Indirect Immunofluorescence (IIF)

Tissue samples were obtained from rabbit epididymis, and snap frozen with Tissue-Tek optimal cutting temperature (OCT) compound (Sigma). The cauda epididymis was cut into 5 mm thick frozen sections. Rabbit semen was collected by artificial vagina as described earlier [24] and smeared onto clean glass slides. Tissue sections and sperm slides were fixed in ice-cold acetone for 30 min, and then blocked with 5% BSA in PBS for 30 min at room temperature, and later incubated either with anti-rat REHbβP antibody (1:100 dilution) in PBS overnight at 4°C or with normal rat IgG (1:500 dilution) as control. Samples were washed with PBS and incubated with a secondary goat anti-rat antibody linked with fluorescein isothiocyanate (1:500 dilution; Sigma) for 1 h at 37°C. Digital photographs of fluorescent sections were viewed under a laser scanning confocal microscope (Carl Zeiss LSM-510; Carl Zeiss



Vision, Germany). This study was carried out at Central Equipment Facility of NIRRH, Mumbai, India.

Hemolysis assay

The effect of REHbβP on RBCs was determined using freshly isolated rabbit erythrocytes [24]. Briefly, aliquots of erythrocyte suspension (10⁶ cells/microfuge tube) were incubated at 37°C for 60 min along with twofold serial dilutions of peptides (0.47-60.61 μm). The hemoglobin release was monitored by measuring absorbance at A450 on a microplate reader (Bio-Tek Instruments Inc., Winooski, VT). 100% hemolysis was determined with 4μl of 0.1% Triton X-100.

Cell viability assay

Cytotoxic effects of REHbβP on immortalized human endocervical cells (End1/E6E7 cells) (Gift from Dr. Raina Fichorova, Harvard Medical school, Boston, USA) and rabbit erythrocytes were determined using KineticBlue reagent (Krishgen Biosystems, India). End1/E6E7 cells were grown in keratinocyte serum medium (Invitrogen, USA). Exponentially growing End1/E6E7 cells were seeded into a sterile tissue culture 96-well plates at a density of 10⁶ cells/well and incubated for 24hrs h at 37°C prior to exposure to two fold serial dilutions of peptide in DMEM medium (1.89-60.61 μm). Plates were incubated for 3 hrs before adding KineticBlue reagent as described [24].

Statistical analysis

The antimicrobial assays were performed using the same batch of REHbβP. Data analysis for each microbe was carried out using one-

way ANOVA. Zones of growth inhibition were measured and the width of the well subtracted prior to calculations. CFU/ml for each sample was calculated by multiplying the number of colonies counted by the dilution factor. Data points in all experiments were performed in triplicate to obtain valid statistical evaluation of the results.

Results

Identification of AMPs

To identify the molecules having antimicrobial activities, we intended to isolate AMPs from the epididymis of rabbit that are expressed along the tissue and secreted into the lumen. Our initial efforts in the identification of AMPs could not yield satisfactory results. Therefore, in order to induce the constitutively expressed proteins and/or synthesis of new defense molecules, rabbit epididymis were challenged under *ex vivo* with CpG ODN-2006. The schematic presentation of purification protocol is depicted in supplemented data (Figure 1).

CpG-ODN induces antimicrobial defense molecules in rabbit epididymis

Freshly prepared rabbit epididymal tissue homogenates from both control and CpG-ODN induced epididymal tissue were mixed separately with 0.01% acetic acid and fractionated on a CMC cation exchange resin. Fractions (1 ml) were eluted in 0.2 M ammonium acetate. Two peaks were obtained. The peak-I was the non-cationic peak, while the peak-II was the cationic. Fractions of peak-I exhibited no antibacterial ability (data not shown). Fractions 94 -104 of peak-II were found to contain detectable growth inhibiting activity against *E. coli* and *S.aureus* as demonstrated by radial diffusion assay (supplemented

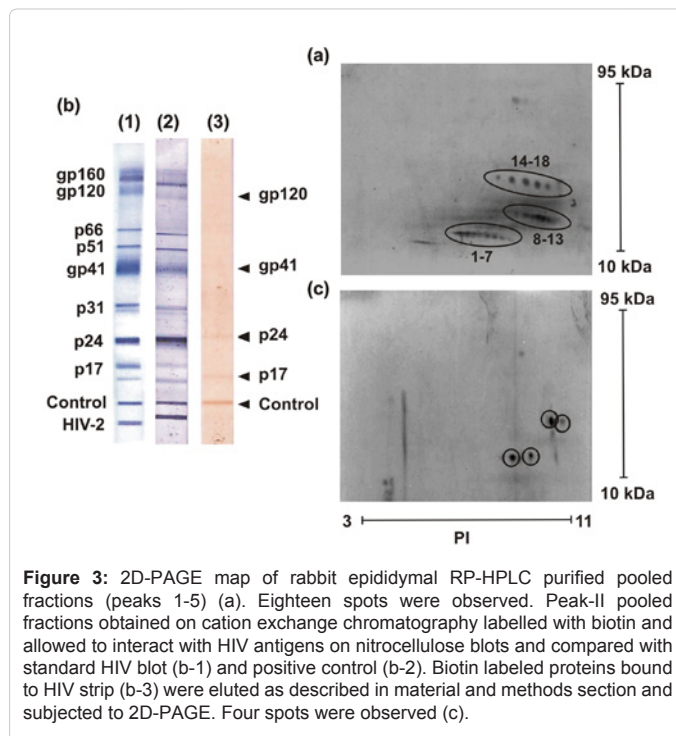


Figure 3: 2D-PAGE map of rabbit epididymal RP-HPLC purified pooled fractions (peaks 1-5) (a). Eighteen spots were observed. Peak-II pooled fractions obtained on cation exchange chromatography labelled with biotin and allowed to interact with HIV antigens on nitrocellulose blots and compared with standard HIV blot (b-1) and positive control (b-2). Biotin labeled proteins bound to HIV strip (b-3) were eluted as described in material and methods section and subjected to 2D-PAGE. Four spots were observed (c).

Figure 2a, b). These fractions were pooled, lyophilized, reconstituted with 0.1% TFA and further purified by RP-HPLC using C_8 column.

Reverse-phase HPLC purification of AMPs

RP-HPLC purification of peak-II protein fraction obtained on cation-exchange chromatography from CpG-ODN2006 challenged (10 μ g/gm tissue) rabbit epididymis showed, stimulation could induce the synthesis of few new proteins as well as up-regulation of some of the constitutively expressed proteins as compared to the control. The proteins were eluted in a three-step gradient as mentioned in materials and methods. The purification was repeated at least 60-70 times to get enough amount of the protein. Based on the retention time, eleven protein peaks (P1-P11) were observed (35, 36, 37, 38, 40, 42, 43, 45, 46, 48 and 50). All the peaks were collected, lyophilized and tested against *E.coli* and *S.aureus* by radial diffusion assay. Peaks 1 to 5 demonstrated detectable amounts of antibacterial activity (supplemented figure 3a,b) were pooled and named as rabbit epididymal secretory anti-microbial protein (RESAMP). The RP-HPLC results were in agreement with SDS-PAGE data, where we observed the induction of few low molecular weight proteins (< 30 kDa) with a mobility corresponding to ~15 to 27 kDa as compared to the non-induced control. The silver stained gel showed the presence of a typical RP-HPLC elution pattern of control and stimulated samples.

Separation of proteins by 2D-PAGE analysis

RP-HPLC fractions from CpG-ODN induced and control epididymal samples were pooled and subjected to 2D-PAGE analysis. Eighteen polypeptide spots < 30 kDa were visualized on 2D gel (Fig: 3a). These spots were cored and subjected to MALDI-TOF analysis to determine their identity. Peptide mass fingerprinting was performed with the Mascot search engine against the NCBI non-redundant protein data base for the species rabbit. Some of the proteins identified by MALDI-TOF showed identity with several known proteins (Table

Sr. No.	Far Western blotting (Saline administered)	Far Western blotting (CpG-ODN administered)
1	β -defensin-5	β -defensin-5 *
2	Lysozyme	Lysozyme
3	Triose phosphate dehydrogenase-1	Triose phosphate dehydrogenase-1
4	Hemoglobin α -subunit	Hemoglobin α -subunit *
5	Calreticulin	Calreticulin *
6	Calgranulin	Calgranulin
7	Calcyclin	Calcyclin
8	Calpain	-
9	Histone-H4	Histone-H4 *
10	Transthyretin	Transthyretin
11	Hemoglobin β -subunit	Hemoglobin β -subunit *
12	Lactate dehydrogenase	Lactate dehydrogenase
13	Epididymal secretory protein-1	Epididymal secretory protein-1
14	Laminin	Laminin
15	Keratin-10	Keratin-10
16	Trypsin	Trypsin
17	Epididymal secretory protein-1	-
18	Myelin basic protein (MBP)	Myelin basic protein (MBP)
19	Fetuin	Fetuin
20	Tropomyosin- α chain	-
21	3-phosphoglycerate dehydrogenase (PGD)	3-phosphoglycerate dehydrogenase (PGD)

Table 1: Comparative Proteome Profiling of rabbit epididymal lysates analyzed by Conventional chromatographic and Far Western blotting methods before and after CpG-ODN-2006 challenged (* = Proteins activated after CpG -ODN stimulation).

1). At this point of time it was difficult for us to know which of the identified molecules which specifically interact with HIV antigens, hence we employed Far western blotting (FWB) technique.

Far Western blotting method detected HIV antigens

The results of FWB reveal that some of the biotinylated proteins interacted with HIV antigens such as gp120, p24, p41 and p17 (Figure 3b). Four spots < 30 kDa were visualized on 2D gel (Figure 3c).

MALDI-TOF analysis

When variations of densities were more than three times, four polypeptide spots were then successfully identified using MALDI-TOF/TOF mass spectrometry with MS/MS followed by database searching (Swiss-Prot protein database). We were able to identify several proteins with high confidence. However, four protein spots of 2D gel analyzed by MALDI-TOF/TOF representing seven proteins. Of the four spots, one spot had two protein matches (2 proteins i.e hemoglobin- α and β peptides) while 1 spot gave a single match (1 protein i.e 3-phosphoglycerate dehydrogenase), and the remaining two spots gave two protein matches each (4 proteins) viz transthyretin, Triose phosphate dehydrogenase-1 and Lactate dehydrogenase- β and calreticulin. As summarized in Table 2, each of these proteins is characterized by its predicated MW, pI, mean fold, Swiss-Prot accession number, and regional abundance. Single entry search was performed to get more information about the identified proteins from National Center for Biotechnology Information (NCBI) database, Universal Protein Resource (UniProt) and Expert Protein Analysis System (ExPasy) proteomics server of the Swiss Institute of Bioinformatics (SIB). The results suggests that, of the 21 peptide sequences identified by MALDI-TOF (Table 1), only four sequences belongs to hemoglobin- α and β subunits, transthyretin and calreticulin protein respectively were reacted with HIV proteins (gp120, gp41, gp17 and p24). The proteins were tested against *E.coli* and *S.aureus* using MIC assay. Of the four proteins, Hb- β sub unit showed better activity against *E.coli* and *S. aureus* than the other three proteins (data not shown). Using bio-informatic tools, a domain interactive to gp120 has been identified within the Hb- β . Interestingly, the domain is the part of the Hb- β peptide sequence identified by the MALDI-TOF. Therefore, all the studies reported here were carried out with the 29 mer peptide of Hb- β sub unit.

Characterization of RESAMP fraction

MALDI-TOF-MS results of the RESAMP demonstrates that one of the proteins of the RESAMP identified showed highest Probability

Based Mowse Score (PBMS) of 99, which corresponds to110-124 (LVIVLSHHFGKEFTP) residues of the Hb- β chain. Using AMP Database (<http://aps.unmc.edu/AP/main.php>) and bioinformatics tools, we synthesized a 29 mer peptide i.e REHb β P with a net positive charge of +4, hydrophobicity of 45 % and pI value of 8.1 (supplemented figure. 4). The β and α regions, and Kyte-doo-little plot of the Hb were depicted by the Protean module of the Lasergene 6 (supplemented figs. 5a & b). Approximately 50-60 amino acid in rabbit Hb- α / β were associated with microbicidal activity and form α -helical and β -sheet structures. Based on the predicted analysis of the β -chain of the rabbit Hb, the peptide is in the highest hydrophobic region. These indicate that hydrophobicity of the peptide is related to its antibacterial activity.

Tricine-SDS-PAGE of RESAMP and REHb β P

The RESAMP that affected the growth of *E. coli* and *S.aureus* was resolved on Tricine-SDS-PAGE and determined the molecular weights. RESAMP showed 4 protein bands (<30 kDa) corresponding to 15, 17, 22 and 27 kDa (Figure 2b).

Circular Dichroism (CD)

Circular dichroism (CD) was used to determine the secondary structure of the REHb β P and to predict whether the peptide exhibit antibacterial activity. Results showed that REHb β P is largely a α -helical and β -sheet structure in sodium phosphate buffer (pH 7.4), which proved to be essential for its antimicrobial activity (data not shown). These results are in conformity with the prediction analysis obtained using Protean module Lasergene-6 software.

Binding of REHb β P with gp120

The interaction of REHb β P with gp120 was confirmed by gp120 binding assay using ELISA. The results revealed that REHb β P bound to gp120 in a dose dependent fashion, suggesting the peptide might be having anti-HIV properties (Figure 4).

Luciferase reporter fusion assay to determine the anti-HIV potential of REHb β P

A cell-based fusion assay was used to mimic the gp120-CD4-mediated fusion process of HIV-1 to the host cells. Fusion efficiency between HL2/3 and TZM-bl cells was monitored based on activation of HIV-LTR-driven luciferase cassette in TZM-bl cells by HIV-1 Tat from the HL2/3 cells. The results indicated that TZM-bl -CD4-LTR-Luc cells fused as the result of the gp120-CD4 interaction, and the

Peptide count	Protein ID	Name	Mol. Wt. (kDa)	PI	Mowse Score	Function
7	HBA_RABIT	Hemoglobin subunit α -1/2	15.51	8.07	71	Oxygen Transport and antimicrobial
10	HBB_RABIT	Hemoglobin subunit β -1/2	15.95	8.06	99	Oxygen Transport and antimicrobial
5	I46521	Titin	110	7.3	44	Muscle formation and elasticity
5	CALR_RABIT	Calreticulin	7.900	4.33	60	Epididymal sperm maturation and anti-bacterial
4	JC1462	Neutrophil peptide - N5	3.551	9.01	78	Anti-bacterial*
2	TTHY_RABIT	Transthyretin	13.66	5.79	45	Thyroid hormone-binding protein
2	LDHA_RABIT	Lactate dehydrogenase A	36.56	8.17	37	Spermatogenesis and sperm maturation
4	TPM1_RABIT	Tropomyosin α -1 chain	32.68	4.690	39	cytoskeleton interacting proteins binds to muscle actin filaments

Table 2: Comparative Proteome Profiling of rabbit epididymal protein extracts analyzed by proteomic approach consisting of 2D, Far Western blotting and MALDI-TOF-MS.

amount of fused cells were measured with the Luciferase reporter gene fusion assay. In the presence of REHbβP, the fusion of HL2/3 with TZM-bl cells efficiently blocked ($P < 0.001$) between both cells in a dose-dependent manner, suggesting REHbβP may have anti-HIV potential in *in vitro* (Figure 5). However, these observations being confirmed by p24 antigen assay.

REHbβP Inhibits the growth of various clinical pathogens

The results of antibacterial activity of REHbβP against gram negative bacteria (*E. coli* and *P. aeruginosa*) and gram positive organisms (*S. aureus* and *S. pyogenes*) demonstrates an increased susceptibility of these organisms to the peptide and the effect was found to be dose dependent. *E. coli* and *P. aeruginosa* seem to be more susceptible to the peptide than *S. aureus* and *S. pyogenes* (Figure 6). The higher susceptibility of gram negative bacteria as compared to the gram positive bacteria clearly indicates the possibility of REHbβP being target specific (e.g. against lipopolysaccharides) in respect to its antimicrobial activity. Similar experiments need to be performed with a broader panel of microorganisms including filamentous fungi, yeast and virus including HIV.

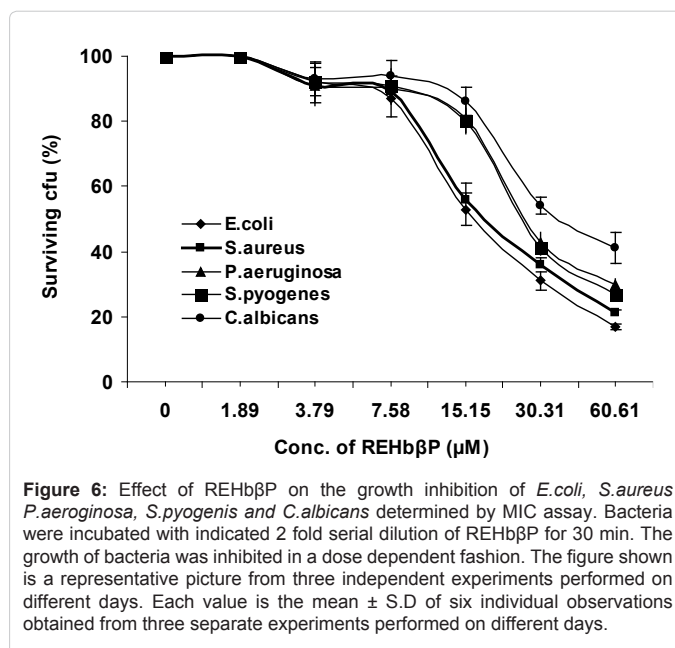


Figure 6: Effect of REHbβP on the growth inhibition of *E. coli*, *S. aureus*, *P. aeruginosa*, *S. pyogenes* and *C. albicans* determined by MIC assay. Bacteria were incubated with indicated 2 fold serial dilution of REHbβP for 30 min. The growth of bacteria was inhibited in a dose dependent fashion. The figure shown is a representative picture from three independent experiments performed on different days. Each value is the mean \pm S.D. of six individual observations obtained from three separate experiments performed on different days.

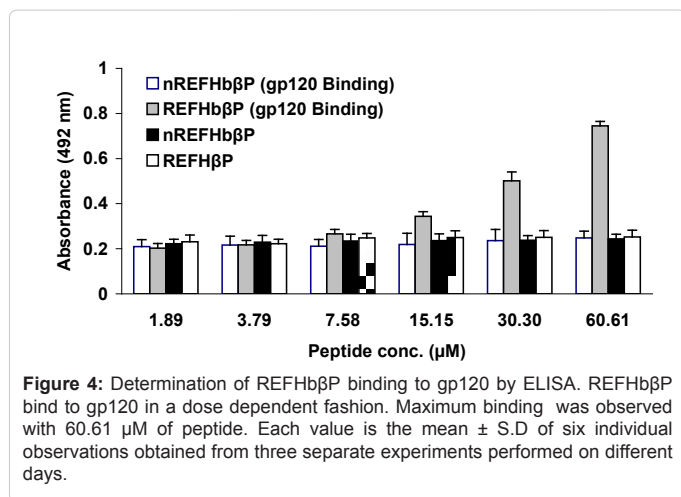


Figure 4: Determination of REHbβP binding to gp120 by ELISA. REHbβP bind to gp120 in a dose dependent fashion. Maximum binding was observed with 60.61 μM of peptide. Each value is the mean \pm S.D. of six individual observations obtained from three separate experiments performed on different days.

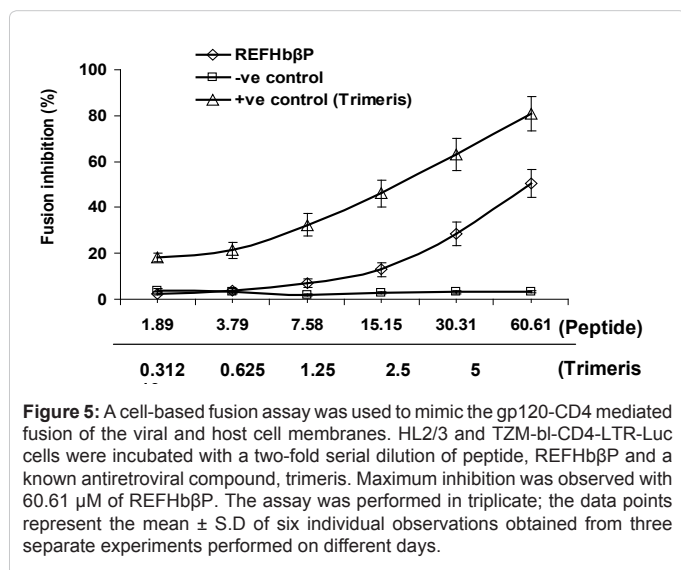


Figure 5: A cell-based fusion assay was used to mimic the gp120-CD4 mediated fusion of the viral and host cell membranes. HL2/3 and TZM-bl-CD4-LTR-Luc cells were incubated with a two-fold serial dilution of peptide, REHbβP and a known antiretroviral compound, trimeris. Maximum inhibition was observed with 60.61 μM of REHbβP. The assay was performed in triplicate; the data points represent the mean \pm S.D. of six individual observations obtained from three separate experiments performed on different days.

REHbβP induces liposome membrane permeabilization

The effect of REHbβP on the integrity of liposomes was studied using calcein-loaded liposomes. The release of calcein was measured as the 'dequenching' of the calcein fluorescence and monitored continuously. Following addition of REHbβP (1.89 – 60.61 μM) an immediate increase in fluorescence was observed which was dose and time dependent. While a concentration of 30.30 and 60.61 μM was able to cause maximum leakage (~70 %) after 5 min of liposomes, lower concentrations of peptide demonstrated a leakage about 37 % after 5 min of incubation. Addition of 0.1% Triton X-100 to the liposome suspension served as a positive control as it caused total (100%) calcein release as determined from the fluorescence signal (Figure 7).

REHbβP did not damage rabbit erythrocytes

Most natural peptides are cationic, and hence their interaction with anionic membrane phospholipids provides a ready explanation for their specificity for bacterial membranes. However, some AMPs exhibit hemolytic activities, and delivery of such peptides becomes problematic, and hence hemolytic activity of REHbβP was carried out. Rabbit erythrocyte suspension was incubated in the presence of two fold serial dilution of the peptide (0.47 - 60.61 μM) for 60 min. The peptide did not cause erythrocyte hemolysis at concentrations upto 30.31 μM. However, only a minor hemolytic activity (8-10 %) was observed at 60.61 μM concentration (Data not shown).

REHbβP did not reduce human endocervical cell viability

Human endocervical cells (End1/E6E7) were used to determine relative sensitivity to REHbβP. Human endocervical cells were seeded overnight in a 96-well plate at a density of 1×10^5 cells/well. After exposure to REHbβP (60.61 μM) for 24 h, cells were evaluated microscopically for any change in the cell morphology. Light microscopy results revealed that cells treated with REHbβP showed smooth surface, firm anchorage to the substratum and no visibly evident loss in cell number and morphology. These results were further

confirmed by cell ELISA, which indicate no significant loss in the cell viability of End1/E6E7 cells as against >90% cell death in Triton X-100 treated cells (Figure 8).

REHbβP expressed by rabbit epididymal tissue and spermatozoa

Secreted epididymal proteins are known to bind to spermatozoa with varying tenacities. To elucidate the nature of the binding of the REHbβP to the epididymal tissue and sperm plasma membrane, indirect immunofluorescent staining was performed. Confocal laser scanning microscopy results revealed that REHbβP was detected on stereocilia in the lumen of cauda epididymal epithelium and on the sperm surface covering the acrosome region in freshly ejaculated sperm (Figure 9).

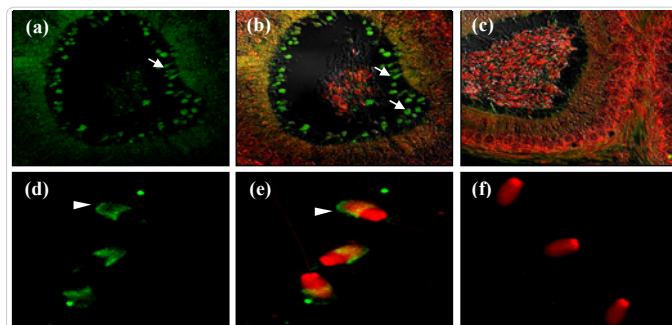


Figure 9: Images showing the expression of REHbβP in the cauda epididymis (Mag X 40) (a-c) and spermatozoa (d-f) (Mag X 100) (a,d= FITC; b,e= PI, FITC merge and c,f= pre-immune controls). Stereocilia of epididymis and spermatozoa are positive for REHbβP. The protein localized on the acrosome of the sperm (PSCE= Pseudo-stratified columnar epithelium). The figures shown are the representative pictures from three independent experiments .

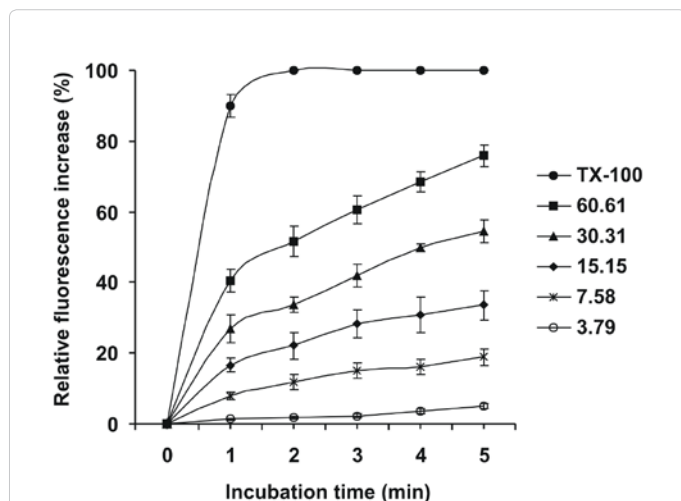


Figure 7: Dye leakage pattern induced by REHbβP in calcein enclosed liposomes. Liposomes mimicking microbial membranes (containing PC and PS) were incubated with indicated concentrations of REHbβP and intensity of calcein leakage at different time points was monitored. Maximum leakage was observed with 60.61 μM and 30.31 μM while considerably less leakage was observed with 50 μM and 25 μM when compared with leakage induced by 0.1% Triton X-100. Each value is the mean ± S.D of six individual observations obtained from three separate experiments performed on different days.

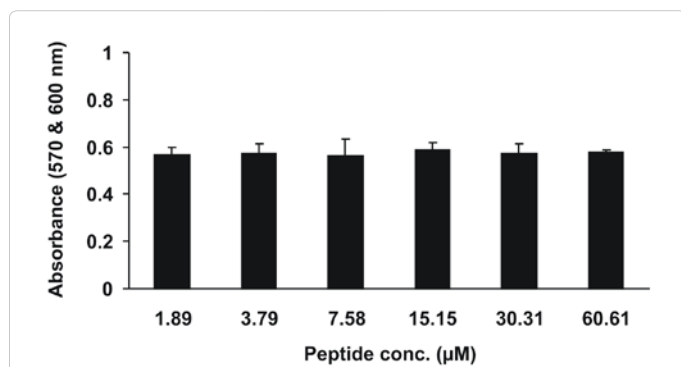


Figure 8: *In vitro* effect of REHbβP on the viability of human endocervical cells (End1/E6E7) evaluated by KineticBlue assay. The peptide non-toxic to End1/E6E7 cells. Each value is a mean ± S.D of six individual observations obtained from three different experiments performed on different days. Each value is the mean ± S.D of six individual observations obtained from three separate experiments performed on different days.

Discussion

Sexually transmitted infections (STIs) remain a public health problem of major significance in most parts of the world. The reproductive tract invaded by microorganisms, and may contribute to infertility in humans and animals, if left untreated they predispose to secondary infections [16,25]. In addition to all these problems, the levels of resistance to antibacterial agents are increasing at an alarming rate as antibiotics are progressively demonstrating decreased efficacy. Gonorrhoeae and syphilis, once easily cured, have become resistant to many of the older traditional antibiotics [24]. Therefore it is an urgent need to identify molecules having antibacterial properties for the prevention of STIs.

In the present study we demonstrated that, EPECs of rabbit respond to CpG-ODN stimulation, and synthesize an array of AMPs. MALDI-TOF-MS results of the RP-HPLC pooled fractions (P1-P5) named as RESAMP, demonstrate that of the four proteins (hemoglobin-α and β peptides, transthyretin and calreticulin that were reacted with HIV antigens (gp120, gp41, gp17 and p24), hemoglobin- β (Hb-β). The 15 amino acid fragment of the rabbit Hb-β, corresponding to the region of 110-124 (LVIVLSHHFGKEFTP) was further characterized as it showed the highest PBMS score of 99 as compared to other three proteins (Table 2 and supplemented figure 4). Using AMP Database and bioinformatics tools, we synthesized a 29 mer peptide, REHbβP (RLGNVLVIVLSHHFGKEFTPQVQAAYQK), which demonstrated significant antibacterial activity against several infectious pathogens. The results indicated that the presence of Hb derived peptides in rabbit epididymis is surprising, since globin genes are normally expressed by erythroid cell lineages. However, few studies have indicated the expression Hb-β gene in cell lineages of non-erythroid origin (human fibroblasts, macrophages and HeLa cells) after stimulation with LPS [26]. Two elegant studies from Cornelia et al. [27] and Du et al. [28] revealed the entire hemoglobin molecule might not be an antibacterial, but the hemoglobin fragments released from RBCs can be microbicidal.

Epididymis of rat and human are rich source of AMPs [2,9]. However it remains to be investigated in the rabbits. AMPs from hemoglobin were first reported in 1999 [29]. At that time this property was dismissed as not being biologically relevant. Although Hb was first identified as oxygen transporter, recent studies have clearly demonstrated that peptides originated from Hb of humans and other species possess strong activity against Gram negative and Gram positive bacteria and participate in host defense against pathogens [25,27,30].

Recent studies have clearly indicated that Hb acts as a precursor for AMP production [28,31] and Hb derived peptides recognize PAMPs like lipopolysaccharide (LPS) [26,32]. The proteolytically generated AMPs (hemocidins) identified in human uterine secretions, cervical mucous and menstrual blood were belong to Hb family [33]. Therefore, the present study strongly suggests that Hb derived peptide fragments may be conserved evolutionarily across a wide range of mammals, and REHb β P could be one such fragment produced by EPECs during bacterial infection. Although speculative, we postulate that Hb derived peptide could be synthesized by EPECs and released into the lumen as fragments of cellular Hb. In any case, the functional significance of such extra-erythrocytically expressed Hb derived proteins in rabbit epididymis has been unclear at present and warrant further studies.

Hydropathy plot of deduced amino acid sequence of REHb β P indicated that it could be a hydrophobic protein and hydrophobicity may be attributed to the probable association of REHb β P with LPS in the bacterial cell membrane. Analysis of the REHb β P sequence using the Kyte Doolittle plot demonstrated a significant presence of hydrophobic amino acids concentrated in the N-terminal region of the protein (Supplemented figure 5a & 5b). This clearly suggests that the amino acids of the REHb β P are the part of α -helix and might be able to span across a lipid bilayer membrane. The 29 amino acid peptide of the Hb- β subunit of rabbit, could be associated with antimicrobial activity and tended to form α -helical structures as reported for other AMPs [30]. Based on the theoretical analysis of β -chain of rabbit hemoglobin, the protein we isolated is in the highest hydrophobic region of the β -chain of rabbit hemoglobin. This shows that the hydrophobicity of the protein is related to its anti-microbial activity. Higher hydrophobicity is required for binding to the LPS in the outer membrane of gram negative bacteria.

Indirect Immunofluorescence studies revealed that REHb β P was localized in the cytoplasm of epididymal epithelia demonstrated that REHb β P perhaps related to secretory protein absorbed by spermatozoa, may play a vital role in sperm protection besides maturation during passage in the epididymis and post ejaculation in the vagina. However, it is not clear at present whether REHb β P fragment present in a fully soluble form or absorbed and stored in luminal vesicles such as stereocilia and are then transferred by unknown mechanisms to the sperm plasma membrane. These studies are supported by various other studies which showed presence of various AMPs viz., sperm associated antigen (SPAG11) also known as epididymal protein 2 (EP2) in monkey, human epididymis 2 (HE2) and rat spag 11 are AMPs attached on the sperm surface [2,8]. These evidences also suggest that epididymal proteins are secreted into the luminal fluid in a soluble form and bind to specific regions of the sperm surface. Given the antimicrobial function of REHb β P, it may have a putative role in protecting epididymal sperm from bacterial infections. However, several studies are required to answer certain queries. First, the mechanism of secretion of REHb β P by EPECs is still contentious. Second, the specificity and tenacity with which REHb β P bind to the sperm plasma membrane, and ultimately their structure within it, remain problematic. Third, what evolutionary forces drove the selection and maintenance of so many AMPs in this important organ of sperm maturation? We believe that one possibility could be that each AMP evolved to control a different species of microorganisms. The environment in each organ may be conducive to growth of different microbial species. We speculate the infection would be much worse if Hb had no antimicrobial activity. Meanwhile, the present findings suggest that similar to human and rabbit vaginal fluids [16,25,31], rabbit epididymal fluid may also serve as an AMP bank

which participate in defending the host against infections.

To gain further insight into the antibacterial mechanism of REHb β P, we next investigated its ability to interact with bacterial cell membrane. The results reveal that anti-bacterial activity of REHb β P could be attributed to its net positive charge and hydrophobic ratio. To evaluate the mode of interaction of REHb β P with bacterial membranes, liposomes were prepared using Phosphatidylcholine (PC) and Phosphatidylserine (PS), which serves as a model for the negatively charged cytoplasmic membranes of bacteria. The fact that REHb β P induced maximum calcein leakage from liposomes, at a concentration of 60.61 μ M, is in concurrence with the MIC results obtained with a similar dose. These studies strongly suggest that REHb β P could be one among the key antimicrobial factors produced in the epididymis when the EPECs sense any microbial threat.

Our study marks for the first time in the evaluation of antimicrobial profiles in the rabbit epididymis, which lead to the identification of cationic AMP of Hb origin, which we demonstrated to be one of the components responsible for the microbicidal activity. From the present findings, it may be envisaged that rabbit epididymal fluid is an anti-infectious medium and REHb β P may be involved protection against potentially harmful microbes. This peptide appears to be an attractive candidate for therapeutic agents due to its specific interactions with pathogens and low toxicity to erythrocytes and human endocervical cells. Establishing the role of REHb β P in epididymal innate immunity may further unravel the intricacies in epididymal defense in this species, and is the current focus of the research in our laboratory. Taken together, this discovery may have significant impact not only in the basic antimicrobial biology of hemoglobin, but also in understanding the pathogenesis associated with microbial infection in epididymis.

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