Involvement of human seminal plasma inhibin, a multifunctional protein, in anti-HIV activity

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Body fluids of mammals, including semen, are increasingly recognized to harbour anti-microbial proteins which play a role in host defence against a myriad of pathogens. Human seminal plasma inhibin (hSPI) is reported to be a multifunctional protein, well-studied primarily for its fertility-related effects and recently for its anti-fungal activity. Therefore, it was thought worthwhile to study whether it also possesses anti-HIV activity. Towards this objective, human seminal plasma proteins were fractionated using gel-permeation chromatography and chromatofocusing. Results demonstrated that fraction-5 (containing purified hSPI) of the chromatofocused fractions demonstrated substantial anti-HIV activity. The data suggest that hSPI possesses anti-HIV activity. In future, molecular models based on such naturally occurring proteins could be potentially employed for prevention/ therapeutics for HIV/AIDS.

Keywords: Anti-microbial proteins, host defence, innate immunity, semen.

ANTI-microbial proteins, also called host defence proteins, are known to exist amongst diverse species and are important components of the innate immune system. They are reported to be present at sites most likely to encounter pathogenic microbes, for example, on the skin and within the myeloid cells[1]. A vast majority of human pathogens initiate infections at mucosal surfaces of the gastrointestinal, respiratory and urogenital tract, thus warranting the presence of such proteins in the secretion of these tracts[2]. Most of the anti-microbials are positively charged and relatively low molecular weight proteins.

The male reproductive tract harbours a number of proteins which possess anti-microbial activities[3]. One of the proteins reported to be present abundantly in human semen is human seminal plasma inhibin (hSPI) or 'beta inhibin', a 94 amino-acid protein, first isolated by Sheth and co-workers[4–6] and reported to have an apparent molecular weight of approximately 10.4 kDa. Several studies carried out with hSPI and its synthetic peptides indicated that it played a major role in fertility[7,8]. It is reported to be a 'multifunctional protein' which modulates circulating follicle-stimulating hormone levels[5], regulates sperm function[9] and inhibits tumour growth[10]. Subsequently, other researchers designated it as β-microseminoprotein and PSP-94 (prostate secretory peptide of 94 amino-acids)[11,12]. Recently, preliminary studies using a proteomics approach revealed that β-microseminoprotein was one of the proteins identified in the human seminal plasma which was likely to possess activities against HIV[13]. Edström *et al.*[14] demonstrated its anti-fungal activity against *Candida albicans*.

In the light of these studies and with renewed interest in this protein, it was thought worthwhile to demonstrate the presence of this protein in purified fractions from human seminal plasma and characterize it for anti-HIV activity. To achieve this objective, human seminal plasma was fractionated by gel-permeation chromatography and the fractions tested for the presence of hSPI by Western blotting using specific antibodies to hSPI. The fraction containing hSPI was further purified by chromatofocusing. The fractions thus obtained were tested for HIV infectivity-inhibiting activity in TZM-bl cells and to observe whether they inhibited the binding of gp120 (HIV envelope glygoprotein) to CD4 (receptor on target cells to which HIV attaches) in an *in vitro* ELISA system.

Approval from the Institutional Ethics Committee for Clinical Studies was obtained for the use of anonymized pooled human semen and blood to obtain peripheral blood mononuclear cells (PBMCs) for the purpose of preparing viral stocks. A waiver was given by the Committee to use anonymized left-over semen samples after the completion of routine tests. Informed consent was taken from healthy, HIV-negative participants for collection of blood (10 ml each).

Semen samples were collected by masturbation after 3 days of abstinence. After the completion of routine semen analysis, anonymized samples having normal semen parameters and HIV-negative status were preserved at 4°C. Seminal plasma from pooled semen was separated from sperms by centrifugation at 1000 *g* for 10 min and stored at –80°C in batches of 10 ml until used for purification.

TZM-bl cells, HIV viruses (HIV-1 93 IN 101 and HIV-1 93 IN 105), HIV-1 gp120 and monoclonal antibodies to gp120 were kind gifts from NIH AIDS Research and Reference Reagent Program (Bethesda; Maryland, USA). TZM-bl cells were maintained in DMEM (Gibco InVitrogen, India) supplemented with 10% FBS (HiMedia India Ltd) and penicillin/streptomycin (Gibco Invitrogen) and the cells were passaged and frozen at –80°C until use. Viral stocks were prepared by infecting normal human PBMCs, passed through 0.22 μm filters and stored at –80°C until further use.

The TZM-bl indicator cell line is a HeLa cell derivative that expresses high levels of CD4, CCR5 and CXCR4. Cells contain HIV long terminal repeat (LTR)-driven β-galactosidase (β-gal) and firefly luciferase reporter cassettes that are activated by HIV infection and subsequent Tat protein expression. HIV-infected cells

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expressing β-galactosidase can thus be quantified and compared with uninfected cells.

Human seminal plasma proteins were purified using gel-permeation chromatography (GPC; Vernekar *et al.*). In brief, human seminal plasma proteins were clarified by centrifugation at 3000 *g* for 10 min. The supernatant thus obtained was diluted 1 : 3 (volume/volume) with 0.02 M phosphate buffered saline pH 7.2 buffer (PBS) and 40 mg/ml/run was fractionated using HiLoad™ 16/60 Superdex-200 column (GE Healthcare, India) on AKTA Explorer protein purification system (GE Healthcare, Uppsala Sweden) using the same buffer. The eluted fractions were dialysed against distilled water (DW) to remove excessive salt and lyophilized. The concentration of the proteins was estimated by Bradford's method[15] and the fractions visualized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), as described by Laemmli[16] on a 12% gel.

The presence of hSPI was ascertained in the purified fractions by Western blotting using specific antibodies previously raised in rabbit[8] and preserved in a lyophilized format –80°C. Proteins in the different fractions were separated on 17% SDS–PAGE and Western blot was performed as described. In brief, proteins separated on SDS–PAGE were transferred onto a nitrocellulose (NC) membrane and blocked overnight with a mixture of 5% skimmed milk powder and 1% bovine serum albumin (BSA) dissolved in 0.01 M PBS (blocking buffer). Next day, the blots were washed thrice with 0.05% Tween-20 dissolved in PBS (T-PBS) and incubated in antibodies to hSPI (1 : 600) for 1.5 h. The blots were further washed thrice with T-PBS before incubating for 2 h with HRP-labelled secondary antibody (1 : 2500; Bangalore Genei, India). After washing thrice with T-PBS, the blots were subsequently visualized by electro-chemoluminiscence (ECL Plus kit, obtained from GE Healthcare, India) using X-ray films in accordance with the manufacturer's instructions.

As fraction-5 purified by GPC revealed the presence of hSPI by Western blotting, it was further fractionated by chromatofocusing (CF) technique using pre-packed column (Mono-P™ 5/50, GE Healthcare, India) according to the manufacturers' protocol.

The fractionation was performed by HPLC using AKTA Explorer protein purification system. In brief, the column was first equilibrated using 0.075 M Tris-acetate buffer, pH 9.2 (start buffer). A pre-gradient volume (2 ml) of elution buffer (polybuffer 96; obtained from GE Healthcare, diluted in distilled water 1 : 10, pH 6.3) was passed through the column. Fraction-5 obtained by GPC was diluted 1 : 5 with start buffer and 1 ml (containing approximately 3.5 mg of protein) per run was loaded onto the column and eluted out with the elution buffer. Next 1 ml of the fractions was collected per tube at a flow rate of 1 ml/min. The column was then re-equilibrated with 5 column volumes of start buffer until the UV absorbance and pH/conductivity values were stable before performing the next run. Four similar runs were performed and the individual fractions from each run were pooled together. The fractions were dialysed against DW and lyophilyzed and designated as Fr.CF-1, Fr.CF-2, Fr.CF-3, Fr.CF-4 and Fr.CF-5. The fractions were then run on a 12% gel and the presence of hSPI was demonstrated by Western blotting as described above.

Inhibition of protein–protein interaction of gp120 to CD4 by fractions purified by chromatofocusing was studied using an ELISA system and a modified method as described by Chams *et al.*[17]. Wells of a 96-well plate (NuncNalge, Denmark) were coated with 75 ng of soluble CD4 dissolved in bicarbonate buffer, pH 9.6 and incubated overnight at 4°C. Next day, the wells were blocked for 2 h at room temperature (RT) in PBS containing 2% gelatin and 0.2 normal rabbit serum. Then 75 ng/50 µl of gp120 was incubated with 50 µl of different concentrations of the fraction in glass test-tubes for 4 h at RT. The contents of the tubes were then added to the blocked wells and the plate incubated overnight at 4°C. Wells coated with CD4 to which only gp120 was added served as negative controls. The following day, wells were washed once with PBS and antibodies to gp120 (1 : 100 in PBS) were added to each well and incubation carried further for 1.5 h at 37°C. Thereafter, the plate was washed thrice with T-PBS and 1 : 4000 horse radish peroxidase (HRP)-labelled secondary antibody was added. After 2 h of incubation at 37°C, the plate was washed thrice with T-PBS. Colour was developed and optical density (OD) at 490 nm was measured on an ELISA reader.

The HIV-infectivity inhibition test was performed using a modified method as described by Wei *et al.*[18]. TZM-bl cells (2×10^4)/200 µl/well were cultured for 24 h in 96-well culture plates (Nunc, Denmark) in a medium containing DMEM, 10% FBS, pencillin and streptomycin (10,00 U/ml and 100 µg/ml respectively; designated as 'complete medium') at 5% CO_2 at 37°C. Next day 50 µl (approx. 200 pg as determined by p24 assay carried out by an ELISA kit, XpressBio Life Sciences products, USA) of the virus in complete medium containing 0.1% diethylaminoethyl (DEAE)-dextran (Sigma-Aldrich, USA), at a multiplicity of infection (m.o.i.) of 0.15, was added to the wells with different concentrations of the fractions and incubated further for 24 h. PBS was added as vehicle control instead of the fractions in 'virus-only control' wells.

β-Galactosidase assay was performed using the mammalian β-galactosidase quantitative assay kit (obtained from Pierce Thermo Scientific, USA) in accordance with the manufacturers' instructions. In brief, the wells were washed once with PBS, pH 7.2 and 100 µl of the β-gal assay reagent provided was added to the wells, the plate was covered and incubated for 30 min at 37°C. The reaction was stopped by adding 100 µl stop solution and absorbance was measured at 405 nm.

Results are represented as mean or mean ± SD from three independent estimates. Data were analysed using

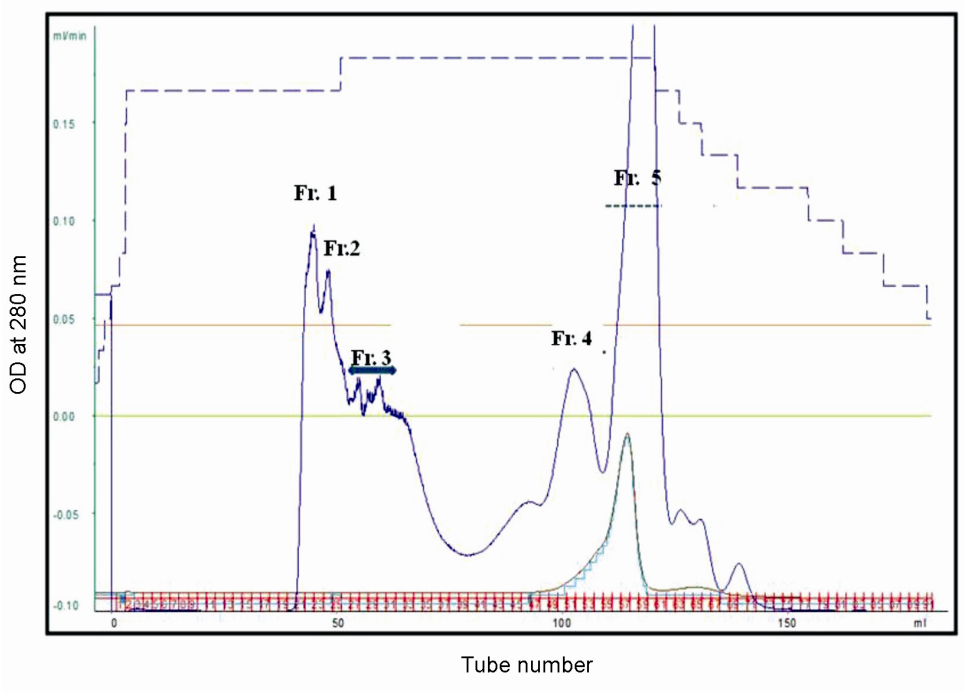

Figure 1. Chromatogram of human seminal plasma proteins purified by gel-permeation chromatography.

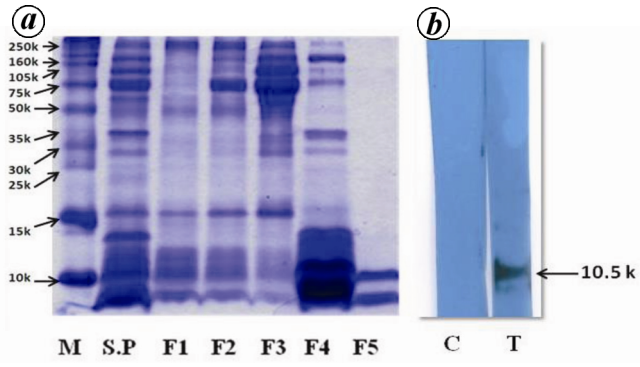

Figure 2. ***a***, Protein profile of the fractions separated by GPC on 12% SDS–PAGE (SP, seminal plasma). ***b***, Presence of hSPI in fraction-5 (F5) by Western blot (C, Blot probed with pre-immune serum; T, F5 probed with specific antibodies of hSPI).

GraphPad Prism version 4.0 (La Jolla, CA). Difference between test groups was examined statistically by one-way ANOVA and Dunnett's multiple post-test comparison and P value <0.05 was considered to be significant. Next 50% inhibitory concentration (IC_{50}) was calculated by plotting the effect versus concentration curves using nonlinear regression curve.

Seminal plasma proteins were separated by GPC using a SuperdexG-200 pre-packed column by HPLC. Five major fractions were obtained as depicted in the chromatogram (Figure 1). The protein profile of the fractions as revealed by SDS–PAGE is shown in Figure 2 *a*. A band of approx. 10.5 kDa was demonstrated in the fifth fraction (Fr.5) of the GPC-purified fractions by Western blot analysis using specific antibodies to hSPI (Figure 2 *b*).

The fifth fraction isolated by GPC showed the presence of hSPI. Since this fraction contained two discrete fractions, further purification was carried out using chromatofocusing technique on a Mono-P column. The chromatogram of the five fractions obtained by chromatofocusing is shown in Figure 3 *a*. Figure 3 *c* shows the presence of hSPI in the fifth fraction (Fr.CF-5) when probed with specific antibodies to the protein. There were no bands detected with pre-immune serum (negative control; Figure 3 *b*). The yield of hSPI was 120 µg/mg of the GPC purified fraction-5 of seminal plasma proteins.

The chromatofocused fractions were tested for their ability to inhibit the binding of gp120 to CD4 in an ELISA system. A representative graph of percentage inhibition of binding with 5 µg/ml of the chromatofocused fractions is shown in Figure 4. Significant inhibition of binding of gp120 to CD4 was observed with fraction CF-4 and Fr.CF-5 (containing hSPI) compared to negative control (gp120–CD4 only without any addition of fractions).

TZM-bl cells were used to study the HIV-infectivity inhibition activity of the fractions. Figure 5 is a representative graph of the percentage inhibition of HIV infectivity in TZM-bl cells by the five chromatofocused fractions. hSPI present in Fr.CF-5 significantly inhibited the HIV-infectivity of the target cells, the IC_{50} being 0.75 µg/ml. In addition, Fr.CF-4 also demonstrated significant inhibition of HIV-infectivity.

Human semen is known to harbour a number of antimicrobial proteins. Unlike the host defence activities of the vaginal secretions, the anti-HIV activities of human seminal plasma proteins have not been in detail[19]. The family of host defence molecules has recently been

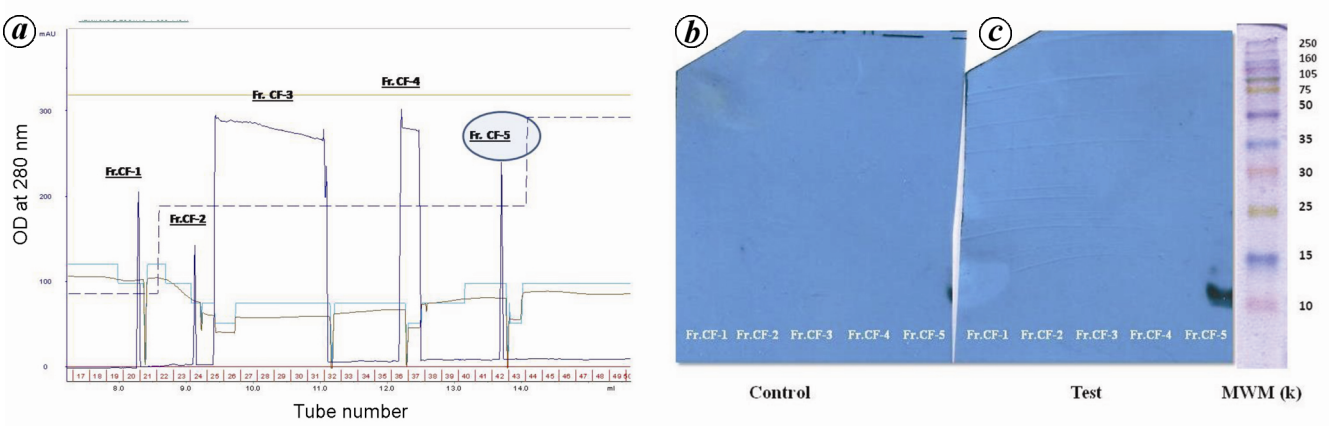

Figure 3. Chromatogram of the chromatofocused proteins of GPC-purified fraction-5. ***a***, The five discrete fractions obtained. ***b***, Negative control (blot probed with pre-immune serum). ***c***, Presence of hSPI in Fr.CF-5 when probed with specific antibodies to the protein.

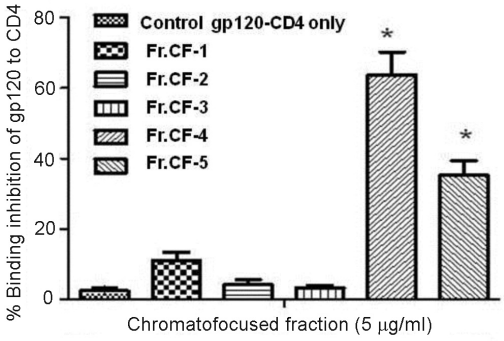

Figure 4. Graph representing comparative inhibition of the binding of CD4 to gp120 with 5 μg/ml concentrations of the fractions (*$P < 0.001$ compared to control).

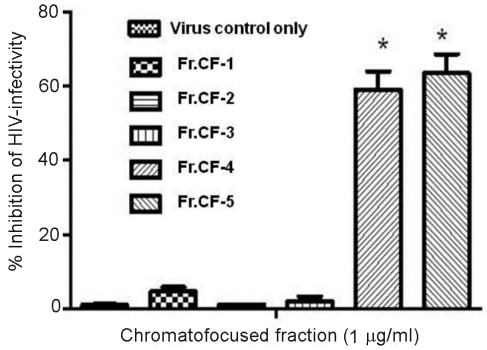

Figure 5. Representative graph depicting comparative percentage inhibition of HIV infectivity by the five chromatofocused fractions. There is a significant reduction with Fr.CF-5 which contains hSPI (*$P < 0.001$ compared to virus only control).

shown to encompass not only 'classic' anti-microbial peptides (AMPs), but also various bioactive peptides and proteins. One interesting observation is that proteins, protein variants, or protein fragments that are known principally for other bioactivities are known to be 'repurposed' to function as natural antibiotics in the innate immune system[20]. Classical examples include the beta-defensins which, apart from their immunomodulatory actions, are also involved in fertility, development, wound healing and cancer[21]. Human seminal plasma inhibin is mainly a prostatic protein, first isolated in our laboratory in the early 1980s; its role in fertility was well established by the nineties. It is alternatively named as PSP-94 and β-microseminoprotein and is reported to be a multifunctional protein[22,23]. Recent studies indicated that this protein also had anti-fungal activities against *Candida albicans*[14]. Therefore, it was considered worthwhile to evaluate its bio-activity against HIV.

We have used two steps, namely gel-permeation chromatography followed by chromatofocusing for purifying hSPI from seminal plasma. Previous researchers[4] have used tedious methods (2–4 steps); gel permeation followed by zonal electrophoresis and subsequently ion-exchange chromatography on DEAE-cellulose for purification of this protein. Jagtap *et al.*[24] have used ammonium sulphate precipitation; phenyl-sepharose chromatography followed by reverse phase RP-HPLC (reverse phase-high performance liquid chromatography) for purification. RP-HPLC is known to inactivate the biological activity of some proteins. Therefore, to maximize yield to retain the bio-activity of the purified protein, too many purification steps were avoided. Since hSPI was detected in the fraction-5 containing two discrete bands upon purification of seminal proteins by GPC, this fraction was further purified using the CF technique. Chromatofocusing was used as a polishing step in our purification strategy in order to achieve final purity by removing closely related proteins, thus leading to the isolation of homogeneous proteins.

CF-5 in which hSPI was detected by Western blotting technique, inhibited infectivity by HIV-1 virus of TZM-bl cells, thus indicating that it possessed anti-HIV activity. Inhibition of binding of gp120 to CD4 by hSPI further revealed that the mode of action could potentially be via fusion inhibition of the virus to the target cells, thus acting as a fusion inhibitor. Fr.CF-4 also demonstrated anti-HIV activities similar to Fr.CF-5, but it did not contain hSPI as observed by Western blotting, thus indicat-

ing that some other unknown protective factors against HIV may be present on this fraction when fractionated from the GPC-purified Fr.5 (Figure 2).

In the last few decades, several proteins have been reported to have 'moonlighting' properties, in that they are 'reused' for some additional purposes other than their primary function[25]. One of the best recognized examples of such proteins is lactoferrin (lactotransferrin). This is a globular glycoprotein, produced by epithelial exocrine cells and is also present in specific granules of neutrophils[26]. Besides its iron-transporting function, it also possesses anti-bacterial properties[27] and has been shown to have anti-viral effects on a broad spectrum of viruses[28]. Several such proteins implicated in host defence are found on the sperm surface, including hCAP18 and HE2 (ref. 3). hSPI is also known to be present on the sperm surface as a sperm coating antigen and is implicated to aid sperm–egg interaction[9]. The presence on sperm reinforces the notion that these host defence proteins may also be involved in the production of fertilization-competent sperms which may play a role in their survival in the female reproductive tract[29].

In conclusion, hSPI may indeed be a classical example of a protein which plays a 'multi-dimensional' role in the male genital tract, and which besides having a host of other functions, may also be acting as a protective factor against HIV. Molecular models based on this protein may provide leads in the prevention/treatment of sexually transmitted infections, including HIV/AIDS.

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