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Human seminal plasma prolactin-inducible protein is an immunoglobulin G-binding protein

Will Wei-Cheng Chiu, Lawrence William Chamley*

Department of Obstetrics and Gynaecology, University of Auckland, National Women's Hospital, Claude Road, Auckland, New Zealand

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Abstract

Antisperm antibodies (ASA) are present in 20% of couples seeking treatment for infertility. Antibody-binding proteins in seminal plasma may protect sperm from ASA-induced damage. We have previously isolated several IgG-binding proteins from human seminal plasma using IgG affinity chromatography. Here, we report another such protein which we have identified by amino acid sequencing and confirmed by western blotting to be prolactin-inducible protein (PIP). PIP binds via the Fc fragment of IgG. We have determined the level of PIP in normal seminal plasma to be 3.4 mg/ml (interquartile range 2.0–4.4 mg/ml). We have found there is no difference in the mean level of PIP in seminal plasma from fertile or infertile men regardless of ASA status. PIP was shown to exist in several isoforms in seminal plasma by Western blot. There is a complex pattern of PIP isoform variability in seminal plasma from fertile and infertile men but one multimeric form of PIP was absent from the seminal plasma of men with ASA who were fertile. This may reflect consumption of PIP in these men. The physiological function of PIP remains unknown, but the ability of PIP to bind IgG–Fc suggests PIP may have an immunomodulatory role.

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* Corresponding author. Fax: +64-9-630-6858.

E-mail addresses: will.chiu@auckland.ac.nz (W.W.-C. Chiu), l.chamley@auckland.ac.nz (L.W. Chamley).

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1. Introduction

One in five couples of reproductive age will experience difficulty in achieving pregnancy (Mosher and Pratt, 1991). Nearly a quarter of these couples will have infertility of undetermined aetiologies. In these cases, immunological factors may play an important role. Antisperm antibodies directed against different sperm proteins have been characterized and some have been shown to interfere with fertilization both in humans and animals (Naz and Menge, 1994).

Human seminal plasma is known to have diverse effects on immune function both in the male and female reproductive tracts. It has immunosuppressive properties which are reported to be mediated by prostaglandins of the E series, complement inhibitors, cytokines and receptors for the Fc portion of IgG (Kelly, 1995). One other protective mechanism may be conferred by factors in seminal plasma that help counter the detrimental effects of the ASA by binding and neutralizing them. One such protein was initially discovered by the finding of that human seminal plasma prevented the binding of aggregated human IgG to bull spermatozoa. Furthermore, the binding appeared to be IgG–Fc mediated (Witkin et al., 1983). Further studies using monoclonal antibodies reactive with Fc γ RIII (CD16) resulted in the discovery of soluble proteins resembling Fc γ RIII in human seminal plasma (Thaler et al., 1989). One of these proteins has been characterised by amino acid sequence analysis and shown not to be Fc γ RIII (Liang et al., 1991). We reported recently three seminal plasma proteins that are capable of binding IgG via its Fc fragment (Chiu and Chamley, 2002). We describe here the characterisation of a further seminal plasma antibody-binding-protein (ABP). This protein has been found to be prolactin-inducible protein (PIP). PIP is known variously as gross cystic breast cyst fluid protein-15 (GCDFP-15), a marker of human primary and metastatic apocrine breast carcinomas, or gp 17/secretory actin binding protein (SABP)/extra-parotid glycoprotein (EP-GP), a secretory glycoprotein isolated from seminal vesicles, salivary glands and sweat glands (Mazoujian et al., 1989; Akiyama and Kimura, 1990).

The function of PIP in human seminal plasma is not known. It has been reported that PIP inhibits the interaction of HIV-1 gp-120 envelope protein with CD4 on sperm (Bergamo et al., 1997). In addition, the interaction of PIP with CD4 on T cells blocks CD4-mediated T cell apoptosis (Gaubin et al., 1999). These functions suggest PIP may be involved in immune regulation. The objective of this study was to delineate whether PIP has other functions, especially those related to reproductive biology

2. Methods

2.1. Patient details

Semen was collected from three men who were fertile following vasectomy reversal (Group A) and 19 men who remained infertile 1 year following vasectomy reversal (group B). Men in both groups A and B had a positive (> 80%) SpermMAR test (mixed agglutination reaction) and sperm surface antibody test (Ferti-PRO, Belgium). Semen was also collected from 61 donors with normal sperm parameters who had no ASA detectable by SpermMAR (WHO, 1999) (group C). All individuals had a sperm concentration > 10 million/ml and motility > 75%. The semen was allowed to liquefy and seminal plasma immediately separated from sperm and other cellular debris by centrifugation (13,000 × g) and stored at –80 °C until use. All samples were obtained with informed consent and after ethical approval from the regional Ethics Committee.

2.2. Preparation of affinity chromatography columns

The preparation of IgG and IgG–Fc fragment affinity columns has been described previously (Chiu and Chamley, 2002). Briefly, an IgG affinity column was constructed to enable the purification of antibody-binding proteins from human seminal plasma. Normal human IgG (CSL, Australia) was diluted to 10 mg/ml in coupling buffer (150 mM NaCl, 100 mM Na citrate, pH 5.5) then oxidized with 0.002% (w/v) sodium periodate at room temperature in a light-proof container for 1 h. The reaction was stopped by addition of 20 mM glycerol and the oxidized IgG coupled to 3 ml of Affi-gel Hydrazide beads following the manufacturer's instructions (Affi-Gel Hz kit; BioRad, Auckland) overnight at 4 °C. The beads were packed into a chromatography column (Econo column; BioRad, Auckland) and conditioned by the passage of 10 bed volumes of 0.1 M glycine (pH 2.5) elution buffer. The column was then equilibrated in PBS (138 mM NaCl, 1.6 mM KCl, 1.4 mM KH₂PO₄, 8 mM Na₂HPO₄), pH 7.4, and stored at 4 °C until use.

IgG–Fc fragments were prepared from 15mg normal IgG (CSL, Australia) by digestion with papain (2% w/v; Sigma, Sydney), dissolved in sodium phosphate buffer (100 mM sodium acetate, pH 7.0, 0.01 M cysteine, 2 mM EDTA) overnight at 37 °C. The IgG digest was dialyzed with two changes of PBS, pH 7.4, and IgG–Fc fragments purified from any intact antibody by gel exclusion chromatography (HR 100; Pharmacia, Auckland) followed by protein A affinity chromatography (miniProtein A column; BioRad, Auck-

land). The isolated proteins were confirmed to be Fc fragment (~ 50 kDa) by Western blot probed with goat horseradish peroxidase (HRP)-conjugated anti-human IgG (Fc-specific) (Caltag; Biotek, Auckland) and Coomassie blue-stained SDS-PAGE. The purified IgG-Fc fragments were coupled to hydrazide-activated Affi-gel (BioRad, Auckland) and packed into a chromatography column and treated as described for the IgG affinity column.

2.3. Isolation of seminal plasma IgG-binding proteins

Seminal plasma samples from six men were thawed and pooled, diluted 1:1 with 0.5% (w/v) eACA (ϵ -Amino-Caproic Acid; Sigma) and filtered through a 0.22 μm syringe filter (Acrodisc; Gelman Science, Auckland), then applied to the affinity columns. The diluted seminal plasma was passed through the column at 1 ml/min, then the column was washed with PBS, pH 7.4, until no further proteins were washed off as determined by absorbance at OD 280 nm.

Bound seminal plasma proteins were eluted from the affinity column by a high salt ionic buffer (1.5 M NaCl/PBS, pH 7.4) and collected in containers at 4 °C. These high salt fractions were dialyzed against PBS, pH 7.4, and concentrated with Amicon cell (Diaflo Ultrafilters, Beverly, MA) and centrifugal concentrators (Vivaspin, Vivascience, Lincoln, UK). The concentrated proteins were stored at -80 °C until use.

The eluted proteins obtained by purification from 6 ml of seminal plasma were separated by 4–15% gradient SDS-PAGE (BioRad, Auckland), as previously described (Chiu and Chamley, 2002), and subjected to amino acid sequencing as described below.

2.4. Purification of PIP

For the purpose of obtaining purified PIP as standard for determination of PIP levels in patient seminal plasma, 12 ml of seminal plasma were subjected to the same purification process, 2 ml at a time, using the method described above. The eluted proteins were concentrated and separated using preparative 10–20% SDS-PAGE. A comparative Western blot was carried out using a portion of the gel for the identification of PIP. The dominant 16–18 kDa isoform bands were excised from a Coomassie-blue stained gel, minced and eluted from the gel by dialysis against PBS, pH 7.4, at 4 °C. The purified PIP was concentrated using centrifugal concentrators and the amount of PIP determined spectrophotometrically at 280 nm based on the assumption that a solution with an optical density of one contains approximately 1 mg/ml of protein. The purified PIP was stored at -20 °C until use.

2.5. Amino acid sequence analyses of affinity purified proteins

To determine the N-terminal amino acid sequence of the seminal plasma ABP, the proteins eluted from the IgG affinity column were separated by electrophoresis as above and transferred to PVDF membrane (Applied Science, Auckland). The protein bands were excised and subjected to Edman's sequencing by the Protein Sequencing Facility of the University of Auckland, using an Applied Biosystems Procise 492 protein sequencer.

To determine internal peptide sequences, tryptic digestion of the proteins was carried out within the polyacrylamide matrix followed by passive extraction of the peptides according to Rosenfeld et al. (1992). This procedure was also performed by the Protein Sequencing Facility of the University of Auckland, New Zealand.

2.6. Western blot analysis

Proteins eluted from the IgG-affinity chromatography columns were separated by electrophoresis by 4–15% SDS-PAGE, then electroblotted onto a nitrocellulose membrane (Hybond-C extra; Amersham, Auckland) using a semi-dry blotting apparatus according to the manufacturer's instructions (Gelman Science, Life Technologies, Auckland). The membranes were then stained with Ponceau-S (0.1% (w/v) in 5% (v/v) acetic acid) to confirm the transfer and destained with PBS, pH 7.4, containing 0.05% (v/v) Tween 20 (PBS-T). The membranes were then blocked for 1 h in 5% (w/v) nonfat milk powder (Pams, Auckland) dissolved in PBS-T (blocking buffer) at room temperature. Membranes were then probed with anti-PIP monoclonal antibody (Clone 23A3; Novocastra, Auckland) diluted 1:500 in blocking buffer for one hour followed by HRP-conjugated goat anti-mouse IgG antibody (Caltag, Biotech, Auckland) diluted 1:3000 in blocking buffer for one hour. The blots were washed, exposed to ECL reagent (New England Nuclear, Life Science Technologies, Auckland) according to the manufacturer's instructions, then exposed to Hyperfilm ECL (Amersham, Auckland) in a film cassette for a varying time from 30 s to 30 min and developed in an automated film processor (Agfa Curix 60, Germany).

In order to confirm that PIP binds to the Fc portion of IgG, additional Western blots of the seminal plasma IgG-binding proteins were probed with an HRP-conjugated goat IgG F(ab)₂ or HRP-conjugated whole IgG, then developed with ECL as above.

To examine the expression of different isoforms of PIP in seminal plasma, Western blots of seminal plasma were carried out. The same volume (2 µl) of seminal plasma from each patient was resolved using precast Criterion 4–

15% gradient gels (25 wells; BioRad, Auckland) and electrotransferred as above. The membranes were treated and probed with PIP monoclonal antibody as above.

2.7. Determination of levels of PIP in seminal plasma from male partners of fertile couples by ELISA

Initially, duplicate wells of 96 well polystyrene ELISA plates (Maxisorb; Nunc, Life Technology, Auckland) were coated with 50 μ l/well of dilution series of purified PIP in PBS, pH 7.4, for the purpose of establishing a linear standard curve. At the same time, triplicate wells were coated with 50 μ l/well of seminal plasma from a man whose seminal plasma was shown in preliminary experiments to contain abundant PIP. His seminal plasma was diluted 1:200 in PBS, pH 7.4, overnight at 4 °C. The plates were washed three times with PBS-T. The plates were then blocked by the addition of blocking buffer for one hour at room temperature. The blocking solution was discarded and the wells washed three times with PBS-T, pH 7.4. Primary antibody (anti-PIP monoclonal antibody; Australian Laboratory Service, Novocastra, Auckland) diluted 1:500 in blocking solution was added to the plates and incubated for 1 h. The plates were then washed three times with PBS-T, pH 7.4 and incubated with biotinylated goat anti-mouse IgG diluted 1:3000 (Caltag, Life Technology, Auckland) in blocking solution for 1 h. The wells were washed three times with PBS-T followed by incubation with HRP-conjugated streptavidin (Sigma, Auckland) diluted 1:1000 in blocking solution. The plates were washed three times with PBS-T, pH 7.4 and the assay developed by the addition of 1 mg/ml of *o*-phenylenediamine (Sigma, Auckland) in 0.1 M citrate buffer, pH 5.0 containing 0.04% H₂O₂ for 40 min. The reaction was stopped by addition of 10% HCl and optical density determined at 490 nm using Benchmark automated ELISA Reader (Biorad, Auckland). The levels of PIP were determined by comparison to the linear range of the standard curve of purified PIP.

For subsequent analysis, seminal plasma from patient one was used to constitute a standard curve of PIP concentrations. Intra-assay and inter-assay coefficients of variation were 10.2% (CV) and 11.9% (CV), respectively.

2.8. Two-dimensional gel electrophoresis

Seminal plasma PIP was further characterised by two-dimensional electrophoresis using immobilized polyacrylamide gel (IPG) strips (pH 3–10; BioRad, Auckland). IgG-binding proteins purified from pooled seminal plasma by IgG-affinity chromatography were suspended in 0.2% (w/v) Bio-

Lyte 3/10 (BioRad, Auckland); 40 mM Tris and 0.00001% bromophenol blue, and used to rehydrate the IPG strip for 12–16 h at room temperature. First dimensional isoelectric focusing was performed under the following conditions: prefocusing at 500 V for 15 min followed by linear voltage ramping for 2 h to 4000 V and final focusing to 40,000 V/h using an IEF cell (BioRad, Auckland) at 20 °C. The focused strips were either immediately resolved in their second dimension or stored at –80 °C until later analyses.

Second dimensional electrophoresis was carried out after the IPG strips were equilibrated with PAGE sample buffer (0.165M Tris–HCl, pH 6.8, 2% SDS, 20% glycerol) for 15 min. The IPG strips were applied to a nonreducing 13% polyacrylamide gel using a mini-Protean apparatus (Bio-Rad, Auckland). The gel was overlain with 0.5% agarose to ensure contact between the strip and the gel. Electrophoresis was carried out at 30 mA per gel. The proteins resolved on the PAGE gel were visualised with Coomassie blue stain (Coomassie R, 0.05%; Coomassie G, 0.05%; 20% acetic acid; 20% methanol).

3. Results

3.1. Identification of PIP by amino acid sequence analysis

A 16 kDa seminal plasma protein that eluted from an IgG affinity column was subjected to automated Edman N-terminal amino acid analysis. This analysis was unsuccessful, possibly indicating the protein's N-terminal was blocked. Partial N-terminal amino acid sequence was subsequently obtained from three peptides derived by tryptic digestion of the 16 kDa protein. These sequences showed identity with the amino acid sequence of PIP (SWISS-PROT-accession number P12273) (Fig. 1).

3.2. Confirmation of the identity and immunoreactivity of PIP

In order to confirm the identity of the 16 kDa protein isolated from seminal plasma, seminal plasma IgG-binding proteins were affinity-purified by IgG chromatography, separated by SDS–PAGE under nonreducing

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0-50      MRLQLLFRA SPATLLLVLCLQLGANKAQDNTRKIIKNE DIPKSVRPND
51-100    EVTAVLAVQTELKECMVVKTYLISSIPLQGFNYKYTACLCDDNPKTFYW
101-140   DFYTNRTVQIAAVVDVIRELGICPDAAVPIKNNRFYTI EILKVE

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Fig. 1. The amino acid sequence of PIP as recorded in the web-based database (SWISS-PRO-Accession code P12273). Highlighted areas are the N-terminal amino acid sequences obtained from tryptic peptides derived from a 16 kDa protein isolated from seminal plasma by IgG and IgG–Fc affinity chromatography.

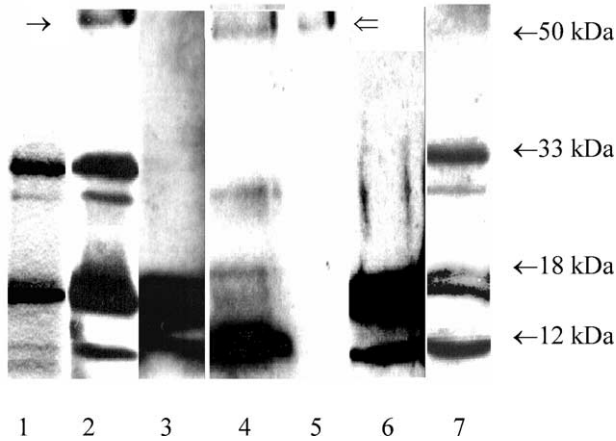


Fig. 2. Coomassie blue-stained SDS-PAGE (10–20%) of IgG-binding proteins demonstrating several protein bands (lane 1). Western blot of these proteins with a PIP monoclonal antibody identified them to be isoforms of PIP (lane 2). Western blot of the seminal plasma proteins binding to an IgG–Fc column with PIP monoclonal antibody demonstrated PIP binds to the Fc portion of IgG (lane 3). That PIP bound to IgG–Fc was confirmed by demonstrating that the IgG binding proteins have the ability to bind HRP-conjugated IgG (lane 4) but not an Fc-devoid, F(ab)₂ HRP-conjugated IgG (lane 5). A 50 kDa protein, likely to be heavy chain of IgG, bound to both conjugated antibodies (→) but is different to the 54 kDa isoform of PIP (⇐) (lanes 4 and 5). Western blot demonstrates that PIP resolves into forms of smaller molecular weights under reducing conditions (5% 2-mercaptoethanol) (lane 6). A Western blot of normal seminal plasma demonstrated the presence of all isoforms of PIP and the presence of the heavy chain (lane 7). None of the isoforms of PIP were able to bind the anti-mouse secondary antibody (lane 8).

conditions, then transferred to a Western blot and probed with a commercially available monoclonal antibody reactive with PIP. This analysis confirmed the 16 kDa protein to be PIP (Fig. 2). The analysis also showed the PIP monoclonal antibody reacted with proteins of 12, 16, 18, 25, 33 and 54 kDa, suggesting they are variants of PIP with the 16 kDa protein being the dominant form (Fig. 2). None of these proteins were bound by an isotype-matched control monoclonal antibody reactive with Fc γ RIII, confirming that this binding by the PIP antibody was antigen-specific (not shown). When these Western blots were repeated under reducing conditions, only the 12, 16 and 18 kDa variants were detected, suggesting that the 25, 33 and 54 kDa proteins are multimers of the smaller proteins or complexes of PIP with another protein (Fig. 2). The 12, 16 and 18 kDa PIP variants bound to an IgG–Fc affinity column, indicating that these isoforms bind to IgG via the Fc portion of the IgG molecule (Fig. 2, lane 3).

In order to confirm that PIP does bind to IgG via an Fc-mediated interaction, seminal proteins purified using an IgG affinity column were

separated on an SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was then exposed to HRP-conjugated whole molecule IgG or HRP-conjugated F(ab)₂ . IgG. This analysis demonstrated that the 12, 16, 18 and 25 kDa forms of PIP bound the whole IgG but not the F(ab)₂ . fragment (Fig. 2, lane 4) on three repeated occasions. A protein of 50 kDa was reactive with both of the conjugates and is likely to be the heavy chain of the IgG molecule (Fig. 2, lanes 4 and 5, arrow). Both the 50 kDa heavy chain of IgG and the 54 kDa PIP isoform were present in seminal plasma (Fig. 2, lane 7).

3.3. Two-dimensional electrophoresis of PIP

In order to further characterize seminal plasma PIP, we resolved the IgG-binding proteins purified from the IgG affinity column by two-dimensional electrophoresis, using non-reducing conditions in both the first and second dimensions. The analysis revealed that the molecular weight variants of PIP have an isoelectric point of 6.1 (Fig. 3). Interestingly, although the majority of the dominant 16 kDa variant of PIP had a pI of 6.1 small amounts of this variant were shown to have pI values of 5.6, 6.3 and 6.7 (Fig. 3). That these isoforms were all PIP was demonstrated by Western blot (data not shown).

3.4. Comparison of the levels of PIP in seminal plasma of fertile and infertile men with or without antisperm antibodies

In order to determine whether there are quantitative differences in the amount of PIP in the seminal plasma of male partners of fertile couples and

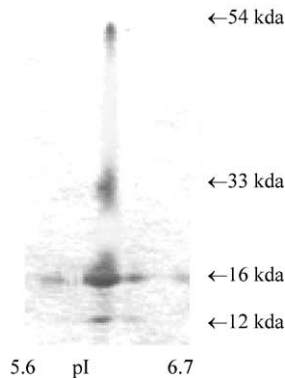


Fig. 3. Two-dimensional SDS–PAGE of PIP. Seminal plasma proteins eluting from an IgG affinity column were rehydrated into an immobilized pH gradient gel and focused using an isoelectric focusing cell. The focused proteins were resolved in the second dimension using a 13% nonreducing SDS–PAGE. The presence of different isoelectric and molecular weight isoforms was demonstrated by Coomassie blue staining.

infertile men, we devised an ELISA to quantify the levels of PIP in 57 men with normal semen parameters. The mean level of PIP in seminal plasma from these male partners of fertile couples was 3.4 mg/ml (S.D. \pm 2.19 mg/ml) and the median level of PIP was 3.3 mg/ml (interquartile range 2–4.4 mg/ml) (Fig. 4). The mean level of PIP in infertile men ($n = 19$) with ASA was 3.5 mg/ml and the mean level of PIP in seminal plasma from fertile men with ASA ($n = 3$) was 3.2 mg/ml. The differences in the levels of PIP between fertile and infertile men with ASA were not statistically significant ($p = 0.86$, t -test). The level of PIP in men with ASA ($n = 21$) was 2.38 mg/ml and the mean level of PIP in men without ASA ($n = 61$) was 3.4 mg/ml. The differences between men with and without ASA were not significant ($p = 0.86$, t -test).

3.5. Variation in the isoforms of PIP in seminal plasma

As there are a number of isoforms of PIP, we extended the above study to examine whether the molecular weight isoforms of PIP differed between male partners of fertile couples and men with ASA. We probed Western blots of seminal plasma from 61 male partners of fertile couples and 22 men who have ASA following vasectomy reversal, three of whom were fertile, with a PIP monoclonal antibody (Fig. 5). This analysis showed a considerable variation in isoforms among the men. Several features were noted from this study. Firstly, the dominant isoform in all individuals was the 16 kDa variant and all men studied have this isoform. Secondly, the 54 kDa isoform was absent in all men who were fertile following vasectomy reversal but had ASA (Fig. 5, lanes 1–3). The 54 kDa isoform was also absent in 16 of the men who were infertile following vasectomy reversal and had ASA (Fig. 5, lanes 4–23), as well as in all of the male partners of fertile couples with no ASA (Fig. 5, lanes 24–85). Thirdly, the 33 kDa isoform was not detected in 12 out of 61 (Fig. 5).

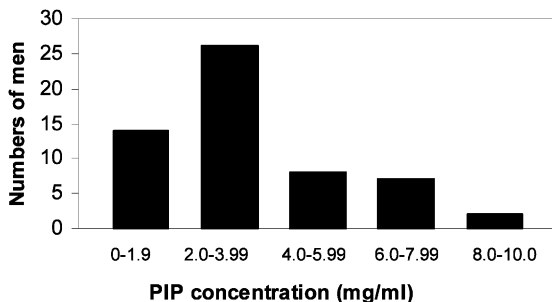


Fig. 4. Graph of distribution of seminal plasma PIP levels in males of fertile couples determined by ELISA. The levels of PIP were determined in 61 males of fertile couples without ASA by ELISA. Their PIP levels were grouped into categories of 5 mg/ml.

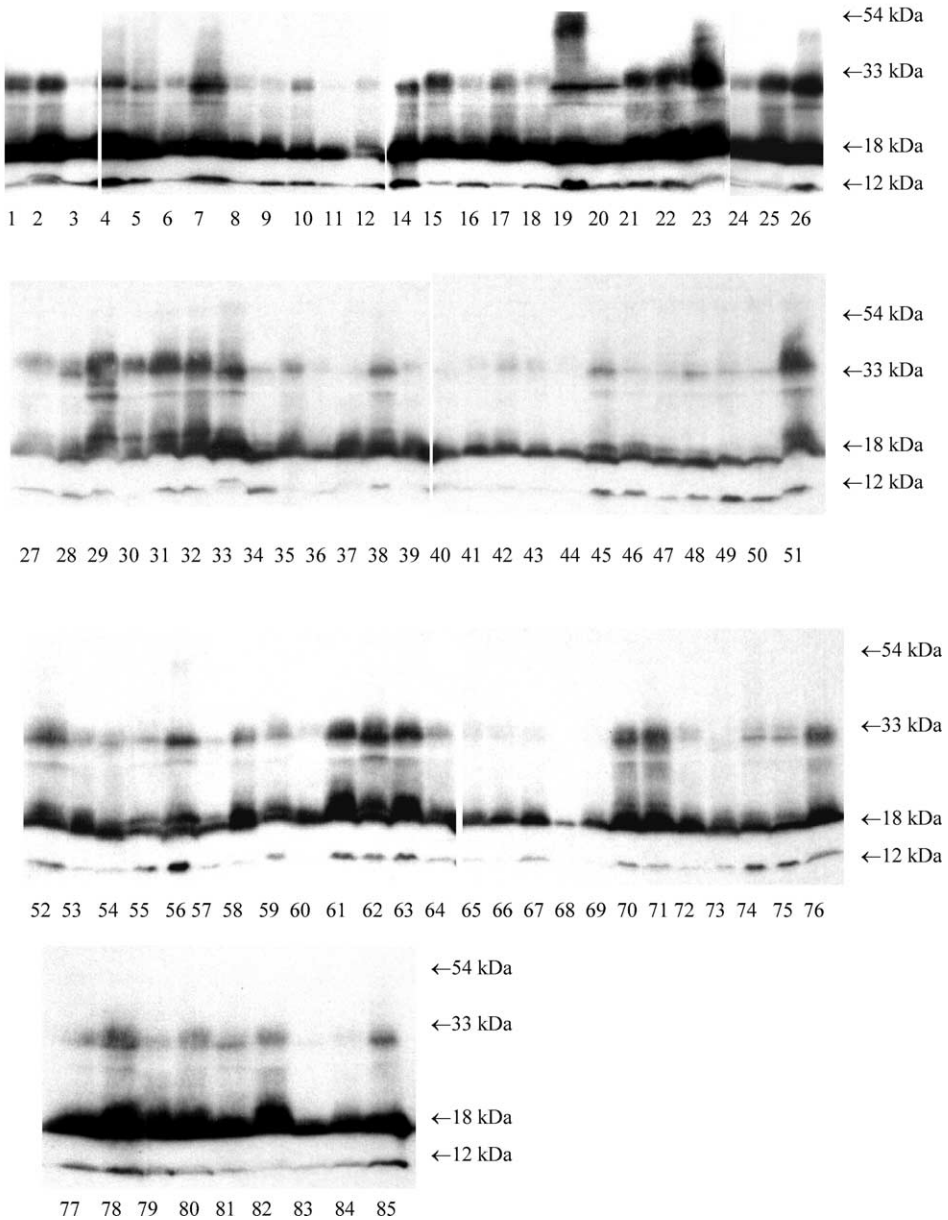


Fig. 5. Differences in the expression of PIP isoforms in seminal plasma. Two microliters of seminal plasma from three fertile men with ASA following vasectomy reversal (lanes 1–3), 19 infertile men with ASA following vasectomy reversal (lanes 4–23) and 61 males of fertile couples without ASA (lanes 24–85) were resolved on a 10–15% SDS–PAGE and transferred to a nitrocellulose membrane. The Western blots were then probed with a PIP monoclonal antibody to identify the differences in expression of PIP in the seminal plasma from these individuals.

Fourthly, the 25 kDa isoform was absent in one man from group A, 6 men from group B and 38 men from group C (Fig. 5). Fifthly, the 18 kDa isoform was greatly reduced in 16 of the male partners of fertile couples (Fig. 5). Sixthly, the 12 kDa isoform was absent in 16 male partners of fertile couples (Fig. 5). Finally, one man from group B had particularly high levels of most isoforms but a disproportionately low amount of the 33 kDa isoform (Fig. 5).

4. Discussion

Antisperm antibodies binding to specific sperm proteins can make sperm susceptible to effector cells of the immune system. Clinically, the presence of ASA may account for low in vitro fertilization rates which may result in poor cumulative conception rates (Bronson, 1999).

Human seminal plasma is known to have immunomodulating properties (Kelly, 1995). One such property may be conferred by presence of proteins capable of binding ASA, which could otherwise inflict damage on sperm (Thaler et al., 1989; Chiu and Chamley, 2002). Activities of these antibody-binding proteins may be demonstrated by the ability of seminal plasma to inhibit ASA reactivities or by the ability of proteins to bind to the Fc of IgG (Lu and Zha, 2000). We report the identification of one such antibody-binding protein from human seminal plasma as Prolactin-Inducible Protein.

In normal human adult tissues, PIP is expressed in all apocrine, lacrimal and ceruminous glands, and Moll's gland and in numerous serous cells of the submandibular, sublingual and minor salivary glands (Haagensen et al., 1990). PIP is also found in acinous cells of nasal, tracheal and bronchial glands (Haagensen et al., 1990). PIP is present in the cystic fluid of breast gross cystic disease, a common premenopausal disorder, and the presence of PIP is regarded as a highly specific and sensitive marker for metastatic breast cancer (Haagensen et al., 1990). Studies conducted using breast cancer cell lines have indicated that PIP can be induced by prolactin and androgens; on the other hand, PIP may be down-regulated by estrogen (Haagensen et al., 1990).

Human PIP is a single chain polypeptide that consists of 146 amino acid residues and has a theoretical molecular weight of 16.572 kDa and theoretical isoelectric point of 5.47 (Murphy et al., 1987). PIP is a protein that has many isoforms (Akiyama and Kimura, 1990). Affinity purification of PIP using an IgG affinity column has enabled us to identify isoforms including several previously undocumented isoforms. The 12 kDa isoform is presumably a proteolytic fragment of PIP since it is smaller than the theoretical molecular weight. The higher molecular weight isoforms (25, 33 and 54 kDa) that we have identified in seminal plasma appear to be multimeric forms of PIP

because they degrade into smaller isoforms when exposed to reducing conditions. Alternatively, it is possible that the apparent isoforms are complexes of PIP with another protein or proteins. We cannot distinguish between these possibilities, but the former seems more likely as no bands appear on SDS–PAGE of PIP run under reducing conditions that do not react with a PIP antibody on Western blots.

The diverse nature of PIP was further demonstrated by its isoelectric variants. Human salivary PIP has been shown to have several glycosylated isoforms (Rathman et al., 1989); however, there is no data on the isoform profile of seminal plasma PIP. Variations in the isoelectric points of PIP isoforms have been previously suggested to be due to tissue-specific translational modifications including different glycosylated modifications (Caputo et al., 1998). Our finding of the several isoelectric variants of PIP is compatible with diverse post-translational modification.

The function of PIP is not known but it has the ability to prevent interaction of molecules with certain regions of CD4 (Autiero et al., 1995) and the envelope protein gp-120 of HIV-1 (Autiero et al., 1997). Others reported that PIP interfered with HIV envelope protein/CD4 binding by inhibiting syncytium formation between transfected cells (Autiero et al., 1997). The binding of PIP to CD4 also inhibits T-lymphocyte apoptosis, a mechanism that is mediated via CD4 cross-linking and T-cell receptor activation (Caputo et al., 1999). Bergamo et al. (1997) found that PIP binds to the post-acrosomal region of sperm and further observed the same region was co-localized with CD4 antibody. Taken together, these lines of evidence suggest that PIP acts as an agent of immune modulation.

The postulated role of PIP in immunity is further accentuated by its predominance in mucosal-type tissues. Salivary fluid PIP was reported to bind to hydroxyapatite, a major component of tooth enamel (Rathman et al., 1989), and to bind selectively to several species of oral and non-oral bacteria (Schenkels et al., 1997) resulting in the inhibition of bacterial growth (Mirels et al., 1998). Its predominance in mucosal-type tissues, as well as its presence in saliva, tears, submucosal glands of the bronchi, and apocrine glands of the skin, suggests that PIP may play an important role in mucosal immunity (Mirels et al., 1998). Our finding of the ability of PIP to bind IgG further suggests its role as an immunomodulatory protein.

Since we have shown PIP binds to the Fc fragment of IgG, we question whether a reduced level of PIP in seminal plasma might be associated with infertility, especially in men with ASA. We established the level of PIP of seminal plasma from males of fertile couples to be 3.4mg/ml, which is similar to that previously reported (1.06 ± 0.27 mg/ml) (Osawa et al., 1996). The plasma level of PIP is reported to be 7–85 ng/ml (Haagensen et al., 1979)

while the concentration of PIP in saliva is a thousand fold higher (10 to 70 micrograms/ml) (Haagensen et al., 1980). Both our own estimate and that of Osawa et al. (1996) suggest that the concentration of PIP in seminal plasma is substantially higher than in other body fluids and that PIP constitutes a significant proportion of the total protein content of seminal plasma.

We determined the range of PIP levels in seminal plasma of 61 males of fertile couples but found that infertile men did not have significantly lower levels of PIP than males of fertile couples. As we have demonstrated multiple isoforms of PIP in seminal plasma, we also examined, by Western blot, whether the level of expression of particular isoforms of PIP correlated with reduced fertility. The levels of PIP are varied among the subjects. Although there does not appear to be a quantitative difference of the protein related to fertility or ASA status, some individuals do have relatively lower levels of PIP in their seminal plasma. This analysis suggests that the levels of total immunoreactive PIP in seminal plasma did not correlate with fertility status, but we have not examined the ability of the expressed PIP to bind to IgG in each individual.

We investigated further whether the levels of PIP isoform expression between fertile and infertile men. There was considerable variation in the expression of isoforms, but these variations did not appear to correlate with fertility status regardless of ASA status with the notable exception that the 54 kDa is absent in all three men who were fertile but have ASA and in all males of fertile couples.

The finding that seminal plasma PIP binds to IgG has enhanced our understanding of the function of PIP. However, it is not known whether all its isoforms perform identical functions as antibody-binding proteins.

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