Salivary Proteome Profile of Women during Fertile Phase of Menstrual Cycle as Characterized by Mass Spectrometry

Ganesan Saibaba1,2, Durairaj Rajesh3, Subramanian Muthukumar4, Ganesan Sathiyanarayanan5, Archunan Priya Aarthys, Govindaraju Archunan*1

1Department of Animal Science, Bharathidasan University, Tiruchirappalli, 2Department of Biotechnology, School of Chemical and Biotechnology (SCBT), SASTRA Deemed University, Thanjavur, 3School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, 4Division of Obstetrics and Gynecology, Rabindra Nath Tagore Medical College, Udaipur-313001, Rajasthan, India, 5Department of Animal Science, Agricultural Research Organization, Volcani Center, Rishon LeTsiyon-7528809, Israel, 6Department of Molecular Biology and Chemical Communication, Research Institute in Semiochemistry and Applied Ethology (IRSEA), 84400 Apt, France

Abstract

Objectives: Ovulation is such a critical physiological process that its noninvasive detection based on salivary constituents has several advantages in humans. Hence, the present study is proposed to identify the ovulatory-specific proteins in saliva in order to detect ovulation phase.

Materials and Methods: Samples were collected from women volunteers. The procedure adopted was approved by the Institutional Human Ethical Committee (DM/2014/101/38), Bharathidasan University. The saliva samples were collected from thirty healthy female volunteers, with a prior written consent. One-way analysis of variance was used to calculate protein concentration and band intensity using SPSS 16 software (SPSS Inc., Cary, NC, USA). The salivary protein expression pattern during different phases of menstrual cycle was analyzed using gel-based high resolution-liquid chromatography-mass spectrometry/mass spectrometry and matrix-assisted laser desorption ionization-time of flight/time of flight. Further, bioinformatics tools were adopted to annotate the proteins identified at various phases of menstrual cycle.

Results: As many as 530 proteins showed up in the saliva during ovulatory phase, whereas there were only 251 proteins identified during postovulatory phase. The functional annotation of salivary proteins revealed that the proteins got assigned to the class of “extracellular proteins” which are concerned with regulatory functions. The 16 unique and/or differentially expressed protein spots appeared during ovulatory phase, among which Cystatin-S, Prolactin-inducible protein, Cystatin-A, Cystatin-SN, BPI fold-containing family A member 2, Alpha-tubulin N-acetyltransferase 1, Carbonic anhydrase-6, Protein LEG1 homolog, Hemoglobin subunit beta, and Pancreatic alpha-amylase were identified.

Conclusion: Total salivary proteome profile has been listed with respect to various phases of menstrual cycle. Among the protein listed, Cystatin-S offers a biomarker protein and/or indicator of ovulatory phase. However, extensive validation is required before arriving to a candidate bio-marker protein.

Keywords: Biomarker, cystatin, gel electrophoresis, liquid chromatography-mass spectrometry/mass spectrometry, ovulation, protein, saliva, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

The term saliva stands for secretions from the major (i.e., submandibular, sublingual, and parotid) and minor salivary glands, which contains molecules from blood and salivary epithelial protein combined.1 The secretion of saliva is regulated by the autonomic nervous system via signal transduction systems that couple receptor stimulation to ion transport and protein secretory mechanisms.2 Many salivary biomolecules arrive from the blood through passive intracellular diffusion and active transport or extracellular

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The levels of salivary components vary in respect to the spectrum of oral and general health. For example, low levels of lysozymes and presence of lactoferrin were observed in saliva under a condition of dental caries. In the beginning of menstruation and during ovulation, the protein content of saliva increases considerably, which turns out to be a rich source of nutrient to bacteria, the count of which may increase during menstruation and ovulation. As diagnostic fluid, saliva and urine offer many advantages over blood, which include simple, noninvasive, and less protein concentration.

Most of the animals have limited and short fertile period, but the external indications and attractiveness synchronize with ovulation maximize the chance of successful fertilization. In human, females do not show corresponding cyclical changes that would be an indicator of ovulation, therefore it was felt essential to develop a method to identify the time of ovulation during the reproductive cycle. The time of ovulation in humans is associated with the fertile phase of menstrual cycle. In mammals, it is generally accepted that many reproductive processes such as ovulation, menstruation, implantation, and parturition are linked with inflammation. As the consequence of these processes, there is upregulation of inflammatory mediators, which include cytokines, growth factors, and lipid mediators. Similarly, in response to inflammation in an ovary, there are specific pro- or anti-inflammatory cytokine/protein expressions in the body fluids. Still there is not yet a reliable noninvasive modality to detect the time of ovulation. Hence, a method for accurate prediction or detection of the fertile phase during menstrual cycle has enormous significance in promoting or controlling fertility. Saliva, for the reasons mentioned above, has long been speculated to possess one or more biomarkers indicating important events in the reproductive cycle. Thus, we adopted a proteome-based approach to detect salivary biomarkers for fertile phase. The complex protein mixtures were analyzed qualitatively by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which suggested that SDS-PAGE is an adequate technique to segregate the protein composition from the human saliva. However, in the recent decade, two-dimensional (2D) gel-electrophoresis is used to separate complex protein mixtures of saliva based on different modifications and isoforms of the particular protein and recent advancements in mass spectrometry techniques are adopted to better facilitate protein biomarker identification in saliva. In our preliminary study, the ovulatory-specific proteins in saliva have been mapped. Hence, it was thought that the identification of an ovulatory-specific protein in saliva would help for future application in regard to ovulation detection. Therefore, the present study was directed towards identifying the proteins in human saliva in relation to phases in the menstrual cycle by adopting 1D and 2D gel electrophoresis followed by mass spectrometry to discern the various proteins, and to map the ovulatory-specific proteins.

Materials and Methods

Volunteers’ information and ethical statement

Samples were collected from women volunteers, and the procedure adopted was approved by the Institutional Human Ethics Committee (DM/2014/101/38), Bharathidasan University, Tiruchirappalli, India. The saliva samples were collected between 8.00 and 9.00 AM from 30 healthy female volunteers (age, mean = 24, range = 19–30), with a prior written consent. The volunteers were instructed not to consume food and/or soft drink for 10 h before the sample collection. The volunteers were also asked to brush the teeth 30 min before sample collection of saliva so as to prevent contamination.

Sample collection and process

The saliva sample was collected by spitting method. The duration of collection of saliva was about 10 min and the saliva secretion over the 1st min was discarded. The collected samples were kept in an ice box and brought to the laboratory without any time delay. The samples were centrifuged at 16000 × g for 15 min to remove insoluble materials and cells, if any. The samples were stored at −80°C until further analysis. The saliva samples were segregated among three phases, viz., preovulatory (day 6–12), ovulatory (day 13 and 14) and postovulatory (day 15–26), according to the pattern of salivary hormones and fern pattern analysis, as was done in our previous study.

Protein precipitation and estimation

The salivary proteins were concentrated by trichloroacetic acid (TCA)-acetone precipitation method. The samples were mixed with TCA: Acetone (TCA-20% W/V; Acetone-90% V/V) in 1:1 ratio and 20 mM dithiothreitol (DTT) then incubated overnight at −20°C. After incubation, the samples were centrifuged at 5000 × g at 4°C for 30 min. The pellets were washed twice with ice cold acetone by centrifugation at 5000 × g at 4°C for 30 min. Finally, the pellets were air-dried and re-suspended in UTC (6 M urea, 3 M thiourea, and 8% CHAPS) buffer. The protein concentration was determined adopting the modified protocol of Bradford.

One-dimensional – Gel electrophoresis

To resolve the salivary proteins, SDS-PAGE was carried out on 12% gel and 5%–15% gradient gel (Bio-Rad) by adopting the modified method of Laemmli. The salivary protein preparation from each volunteer (30 µg) was thoroughly mixed with 1x sample buffer (50 mM Tris-Cl [pH 6.8], 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 100 mM β-mercaptoethanol) and kept for 1 min at 100°C for complete denaturation of proteins.
**Two-dimensional – Gel electrophoresis**

Protein samples were mixed with an equal volume of UTC buffer (6M urea, 3M thiourea, 8% CHAPS, 100 mM DTT, and 2% IPG buffer (GE, Amersham), and incubated for 30 min in ice. The content was then diluted to the required volume using rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 0.5% ampholytes, 50 mM DTT, 1% IPG buffer (GE, Amersham), and 0.004% bromophenol blue). The strips (IPG Strips-pH 3-10NL) were then focused in IPGphor III after 16 h of passive rehydration. Consecutively, the strips were subjected to reduction and alkylation. For reduction, the strips were incubated in SDS-equilibration buffer I (6 M urea, 50 mM Tris-Cl, 30% glycerol, 2% SDS, 0.004% bromophenol blue, and 1% DTT) for 15 min in a gel rocker. For alkylation, the strips were incubated in SDS-equilibration buffer II (6 M urea, 50 mM Tris-Cl, 30% glycerol, 2% SDS, 0.004% bromophenol blue, and 2.5% iodoacetamide) for 15 min in a gel rocker. The strips were then placed on top of 12% polyacrylamide gel and sealed with overlay of 0.5% agarose solution. The electrophoresis conditions were 0.5 W for 45 min and 2 W for 5–6 h until the tracking dye reached the bottom of the gel plate. After electrophoresis, the gels were stained according to Dyballa and Metzger.[20] Digital images of 2D-gels were acquired using ChemiDoc™ XRS imaging system (Bio-Rad) with internal calibration.

**High-resolution-liquid chromatography-mass spectrometry/mass spectrometry**

The 1D protein spots were analyzed using 6550 i-Funnel quadrupole-time-of-flight liquid chromatography-mass spectrometry/mass spectrometry (QTOF-LC-MS/MS) coupled with 1260 Infinity Nano pump and 1260 Cap pump along with 1260 Chip-cube (Agilent Technologies). The peptides were fractionated along with Solvent A (0.1% formic acid in milliQ water) and Solvent B (90% acetonitrile +0.1% formic acid +10% milliQ water). The proteins were identified by mass comparison with the SWISS-PROT database entries. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[21] with the dataset identifier PXD004511. The article has been registered as preprint at Research Square (10.21203/rs.2.13524/v2).

**Matrix-assisted laser desorption ionization-time of flight/time of flight analysis**

2D protein spots were processed using an automated gel cutter and processor (Shimadzu, Xcise™). The gel spots were washed and destained with 50% ACN and 50 mM NH4HCO3, and subjected to in-gel digestion with 30 μL of trypsin solution for 2 h at 37°C. ZipTips (C18) were wetted and conditioned with 50% ACN and 0.05% TFA and 0.1% TFA, respectively. Cleaved peptides bound to the C-18 resin were desalted using 0.1% TFA. The peptides were then eluted and spotted with 2.5 μL of Solvent (5 mg/mL of CHCA in 50% ACN and 5 mM of NH4HCO3) onto a 384-well matrix-assisted laser desorption ionization (MALDI) plate. Finally, samples were identified using MALDI-time of flight/time of flight (MALDI TOF/TOF) (AB Sciex 4800). The acquired mass spectra were processed using DataExplorer® software, and the mono-isotopic peptide masses were assigned and utilized for the database search. The proteins identification was searched against Homo sapiens protein sequence in MASCOT database.

**Functional annotation**

The salivary proteins of the ovulatory phase were further analyzed to decipher their cellular location, molecular function and biological process by STRAP 1.5 online database.[22]

**Molecular functional ontology**

The prominent salivary proteins showing up during the ovulatory and postovulatory phases were further classified based on their cellular component, biological process, and molecular function in the UniProt database. The GO entries were used to depict the percentage of proteins through Interproscan analysis in BLAST2GO. The retrieved GO ID’s of protein entries, with their enrichment values, were used to generate a scatter plot by adopting Reduce Visualize Gene Ontology (REViGO) web server.

**Statistical analysis**

The protein concentration and band intensity values corresponding to ovulatory, preovulatory, and postovulatory phases were represented as mean ± standard deviation and analysed using one-way analysis of variance using SPSS 16 software (SPSS Inc., Cary, NC, USA).

**RESULTS**

**Validation of ovulatory phase**

It is necessary to assess the ovulatory phase after appropriate screening of the menstrual cycle. Those women who exhibited normal 28–30 day cycle length through the 5 or more cycles were chosen to be the volunteers, and then saliva sample was collected. The ovulatory phase was confirmed from direct fertility marker, namely, status of the follicle adopting ultrasonography [Supplementary Figure 1a], and biophysical fertility markers such as basal body temperature and fern pattern in saliva [Supplementary Figure 1b].[23]

**One-dimensional – Gel electrophoresis**

The total proteome was fractionated by 12% SDS-PAGE [Figure 1a]. The protein profile of ovulatory phase saliva was compared to that of postovulatory phase. Put together,
during the two phases, a total of 12 distinct protein bands appeared in the CBB-stained gels. Further, the proteins pattern was verified in the gradient gel [Figure 1b] and it was found to be similar to that revealed in 12% SDS-PAGE. Among the various bands 66, 43, and 14.5 kDa were in the highest intensity during the ovulatory phase compared to postovulatory phase.

**High-resolution-liquid chromatography-mass spectrometry/mass spectrometry analysis**

In order to identify the salivary proteins, the SDS-PAGE protein profiles of each phase were excised equally into six separate pieces, which were individually subjected to trypsin digestion followed by mass spectrometry analysis. In total, 781 proteins were identified combining ovulatory and postovulatory phases of menstrual cycle. During ovulatory phase 530 proteins were found, whereas 251 proteins were found during postovulatory phase. Among these proteins, 35 were common to both ovulatory and postovulatory phases [Figure 1c].

**Ovulatory-specific proteins**

Several functionally important ovulatory phase-specific proteins were identified and listed. Table 1 shows the list of thirty functionally important proteins related to reproduction during ovulatory phase. Cystatin-S, Disintegrin, Metalloproteinase domain-containing protein 7, TANK-binding kinase 1 and Exportin 7 appeared to be predominant, having more number of peptide identifications [Table 1].

**Gene ontology**

The GO entries were mapped using GI number of proteins in UNIPROT database. After refinement of datasets, it was seen that saliva of ovulatory and postovulatory phases contained 154 and 117 GO entries, respectively. The gene ontology clearly revealed more UNIPROT entries and greater percentage of annotation during the ovulatory phase than the other phases.

**Functional annotation**

The salivary proteins identified during the ovulatory and postovulatory phases were subjected to functional annotation using STRAP online database. The results revealed that the ovulation phase had more number of GO terms and greater percentage of annotations than during the post-ovulation phase [Supplementary Figure 2].

**Molecular functional ontology**

The GO entries were used to depict the percentage of proteins at the molecular functional level through Interproscan analysis in BLAST2GO. The cloud tag image confirmed that the proteins identified in saliva of ovulatory phase are essentially those with binding property and catalytic activity. Particularly, the ovulatory phase salivary proteins showed up higher number of binding proteins (41.6%) and metal ion binding proteins (16.1%) compared to other phases [Supplementary Figure 3]. Further, the molecular functional network was constructed using GO terms of salivary proteins. The integrated network map revealed the proteins identified during ovulatory phase have roles on glycoprotein binding, ion binding, and immunoglobulin binding, and receptor activity [Figure 2a]. Additionally, molecular network analysis revealed the major interaction between the identified proteins.

**Scatter plot for binding proteins**

Overall, molecular function analysis expounded most of the proteins thus identified to possess binding property. The cluster of proteins during ovulatory phase corresponds, to the receptor-, protein complex-, peptide-, and GABA-binding and MAP kinase activities as depicted in yellow bubbles. The GPCR-, heat shock-, hyaluronic acid-, lipid phosphatase-, and ligase-binding, and motor activity proteins are shown in linear green bubbles. GABA-A receptor activity and receptor activity proteins are denoted in orange bubbles [Figure 2b]. These proteins might be having some functional significance during ovulatory phase. Toll-like receptor-, fatty acid-, and lipid-binding proteins, and protein transporter activity proteins during postovulatory phase are shown as separate clusters [Figure 2c].

**Two-dimensional – Gel electrophoresis**

The salivary protein expression profiles were analysed during preovulatory, ovulatory, and postovulatory phases of menstrual cycle by 2D gel electrophoresis. The protein spots were present in the pI range 4–7 and the molecular weight between 14 and 97 kDa [Figure 3a-c]. The gel analyses carried out during different phases using PDQuest software (Bio-Rad) showed nearly 50 spots in each phase. Particularly, there were more spots during ovulatory phase, and many of them had higher densities compared to the other phases. Additionally, we found that the quantitative differences in the spots were high in 14.5–21 kDa region of ovulatory phase proteins compared to the other phases. Though the ovulatory phase had a different protein expression compared

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*Figure 1: (a) Salivary protein profile in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. O – Ovulatory phase, PostO – postovulatory phase, M – Protein reference markers. (b) Salivary protein profile in gradient gel (5%–15%). O – Ovulatory phase, PostO – Post-ovulatory phase, M – Protein reference markers. (c) Total identified salivary proteins. In ovulatory phase 530 proteins showed up whereas 251 proteins were observed during postovulatory phase.*

*Figure 3: Scatter plot for binding proteins.*
Table 1. List of functionally important salivary proteins identified during ovulatory phase of menstrual cycle.

| Swiss-Prot acc. no. | Name | Function | pI | MW | Length |
|-------------------|-----|---------|----|----|--------|
| P01036            | Cystatin-S | Strong inhibitor | 4.83 | 14.18 | 141   |
| Q9H2U9            | Disintegrin and metalloproteinase domain-containing protein 7 | Role in Reproduction | 5.92 | 65.09 | 754   |
| Q9UHD2            | TANK-binding kinase 1 | Regulating inflammatory responses | 6.32 | 83.64 | 729   |
| Q9ULIA9           | Exportin 7 | Export of proteins | 5.91 | 123.9 | 1087  |
| P29122            | Proprotein convertase subtilisin/kexin type 6 | Endoprotease activity | 7.96 | 106.4 | 969   |
| Q14031            | Collagen alpha-6(IV) chain | Major structural component | 9.31 | 163.8 | 1691  |
| P29122            | Proprotein convertase subtilisin/kexin type 6 | Endoprotease activity | 7.96 | 106.4 | 969   |
| Q9UHD2            | TANK-binding kinase 1 | Regulating inflammatory responses | 6.32 | 83.64 | 729   |
| P29122            | Proprotein convertase subtilisin/kexin type 6 | Endoprotease activity | 7.96 | 106.4 | 969   |
| Q5XXA6            | Anoctamin-1 | Chloride conductance | 8.76 | 114.07 | 986   |
| Q9Y5E6            | Protocadherin beta-3 | Potential calcium-dependent cell-adhesion protein | 4.88 | 86.77 | 598   |
| P28566            | 5-hydroxytryptamine receptor G-protein coupled receptor | Mediates transport of monovalent cations | 8.49 | 123.4 | 1121  |
| Q9XXA6            | Anoctamin-1 | Chloride conductance | 8.76 | 114.07 | 986   |
| Q9Y5E6            | Protocadherin beta-3 | Potential calcium-dependent cell-adhesion protein | 4.88 | 86.77 | 598   |
| P28566            | 5-hydroxytryptamine receptor G-protein coupled receptor | Mediates transport of monovalent cations | 8.49 | 123.4 | 1121  |
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| P28566            | 5-hydroxytryptamine receptor G-protein coupled receptor | Mediates transport of monovalent cations | 8.49 | 123.4 | 1121  |

Proteins having at least one identified peptide in ovulation phase saliva are listed with their Swiss-Prot/TrEMBL accession numbers and length. Functions were retrieved using the STRAP online database bioinformatics resource. Theoretical pIs and monoisotopic molecular weights were calculated using the Swiss-Prot website.

Identification of proteins from selected spots

The differentially expressed 16 spots were identified as: Cystatin-S, Prolactin-inducible protein, Cystatin-A, Cystatin-SN, BPI fold-containing family A member 2, etc.
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Alpha-tubulin N-acetyltransferase 1, Carbonic anhydrase 6, Protein LEG1 homolog, Hemoglobin subunit beta, and Pancreatic alpha-amylase. The spots 1, 3, 4, 7, and 10 were identified as Cystatin-S and these spots might be isoforms/variants of Cystatin-S proteins. Notably, spot 1 identified as cystatin-S family protein having 71% sequence coverage with eight peptides matches [Figure 3d and e].

**DISCUSSION**

Saliva is an important diagnostic biological fluid, which reflects the physiological as well as biochemical changes in the body. Human saliva has been subjected to proteomic analysis by extensive proteomic technique, namely, 2D gel-electrophoresis followed by MALDI-TOF/MS and Q-TOF/MS as well as comparative proteomic analysis on intra-and inter-person variability of whole saliva using LC/ESI-TOF/MS. Even though, detailed salivary proteomic studies during menstrual cycle adopting the 2D-based mass spectrometry have not been conducted. However, very recently studies have been carried out to comprehensively catalog the salivary proteome with regard to cellular localization, biological processes and molecular
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functions.[27] In our previous study, we have reported that the 14.5 kDa protein band is consistently present and we projected it as the ovulatory phase-specific protein. Analysis of this band revealed that the Cystatin-S expression was significantly higher during the ovulatory phase, which was further validated adopting immunoblotting, confirming that Cystatin-S is the predominant protein during ovulation.[16]

In the present study, a complete proteomic catalogue on human saliva has been mapped with as much as 781 proteins during various phases of menstrual cycle. Among, 495 proteins are ovulatory-specific, and 216 proteins are postovulatory-specific, and 35 proteins were common in both phases. Many proteins reported in human saliva have been previously identified in human in different physiological conditions. The functionally important proteins associated with reproduction such as Cystatin-S, Disintegrin and metalloproteinase domain-containing protein 7 (ADAM7), TANK-binding kinase 1, Anoctamin-1, Carbonic anhydrase 6, and so on, appeared during ovulatory phase. More specifically, Carbonic anhydrase is present during all phases of animals exhibiting estrous cycle. Indeed, there is a significant expression of Carbonic anhydrase in cattle, camel, and goat.[28] In the human, recent studies have confirmed that secretions of specific peptides/proteins are differentially expressed in the pediatrics compared to adults.[29]

The functional annotation of the identified proteins showed that these proteins are associated with binding property and regulatory function. In addition, these extracellular proteins would play an important role during ovulation. It is to be noted that proteins having binding property are widely present along with volatiles in body fluids and facilitate chemical communication during the estrus phase.[30,31] A more recent report showed that the binding proteins are abundant during estrus compared to the other phases in estrous cycling mammals.[32] The binding proteins may have a significant role in increasing the stability of other proteins.[33]

Several salivary biomarkers such as lactoferrin, beta-2-microglobulin, and cystatin for Sjögren’s syndrome, C-erbB-2, and epidermal growth factor for breast cancer,[34] and Lactoferrin for periodontitis and type 2 diabetes mellitus[35] have been listed using MS-based proteomic techniques. The 2D gel electrophoresis (2-DE) is capable of providing for better proteins separation (based on both charge and mass) and therefore, expounds a candidate biomarkers. In most of the studies, 2DE is used as the first step for protein separation, followed by tandem MS (MS/MS). The whole saliva separated by isoelectric focusing showed most distinctive proteins at different pIs.[36] The outcome of the present study strongly agrees with the previous reports for the IEF separation between the pI 4 and 7. Recently, the 2DE analysis in periodontitis patients evidenced 15 altered spots out of 128.[37] Likewise, the present study revealed that 16 spots out of 62 were altered during the ovulatory phase of menstrual cycle. Since the composition of salivary proteins is

Figure 3: The two-dimensional gel electrophoresis of salivary proteins (a) preovulatory phase, (b) ovulatory phase, (c) postovulatory phase. The 11 cm IEF strips were used with 3–10 pH range, and gel run with the 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. (d) Two-dimensional protein spot 1 was subjected to in-gel tryptic digestion, and the spectrum was collected form matrix-assisted laser desorption ionization-time of flight mass spectrometry/mass spectrometry. The number of the mass spectrum gives precise m/z (M + H) values for detected peptide ion signals. (e) Single letter coded of cystatin-S sequence was obtained from mascot search. The matched 71% sequence coverage is highlighted in bold red color.
influenced by physiological and environmental factors, they have the potential to access and monitor the diseases and physiological status.\textsuperscript{[24,38]} Indeed, the differences in protein expression have been well documented in pathological conditions such as cystic fibrosis, dental caries, and periodontitis.\textsuperscript{[39,40]} A comparative salivary proteomic study was undertaken between goats and sheep,\textsuperscript{[28]} and goats and cattle.\textsuperscript{[41]} Likewise, salivary proteomics is a promising tool for the discovery of biomarkers for various diseases. Currently, researchers are interested in developing biochemical-based and/or protein-, peptide-based marker from saliva for the detection of ovulatory phase in the human.

Recent proteomic platforms have showed about 3000 differentially expressed proteins and peptides in human saliva, many of which are of microbial origin.\textsuperscript{[42]} Similarly, in the present study most of the proteins present in saliva are antimicrobial and defensive proteins. The proteins around 14.5 kDa, such as Cystatin-S, Prolactin-inducible protein, Cystatin-A, Cystatin-SN, BPI fold-containing family A member 2, Alpha-tubulin N-acetyltransferase 1, Carbonic anhydrase-6, Protein LEG1 homolog, Hemoglobin subunit beta, Pancreatic alpha-amylase are express at high levels during ovulatory phase. Among these proteins, Cystatin-S protein was highly expressed as five isoforms falling in different pI ranges during ovulatory phase. Further, the expression of Cystatin-S affirms the previous studies.\textsuperscript{[16]} Likewise, Cystatin-A and cystatin isoforms were reported in gingival crevicular fluid in periodontal patients and chicken egg white during embryogenesis, respectively.\textsuperscript{[43,44]} It is suggested that salivary glands respond to inflammation stimuli to secrete more Cystatin-C into saliva\textsuperscript{[45]} and it has been considered as a biomarker candidate of renal function.\textsuperscript{[45]} Alterations of serum Cystatin C was considered as early marker for hyperthyroidism, cancer, renal function in diabetic patients, and cardiovascular diseases.\textsuperscript{[46-49]} Cystatin S is a promising tumor biomarker for early cancer diagnosis and treatment evaluation.\textsuperscript{[50]} In the present study, the variations in cystatin-S expression indicate that physiological changes influence the protein secretion in saliva. Hence, the specific expression of Cystatin-S in saliva during the ovulatory phase would lead to bring up an important biomarker for ovulation detection in human.

Conclusions

The protein expression patterns in human saliva were analysed during different phases of menstrual cycle. Total salivary proteome profile is listed, and as many as 530 proteins appeared during ovulatory phase as compared to 251 proteins of the postovulatory phase. The unique and differentially expressed protein spots during ovulatory phase were identified as follows: Cystatin-S, Prolactin-inducible protein, Cystatin-A, Cystatin-SN, BPI fold-containing family A member 2, Alpha-tubulin N-acetyltransferase 1, Carbonic anhydrase-6, Protein LEG1 homolog, Hemoglobin subunit beta, Pancreatic alpha-amylase are highly expressed during ovulatory phase. Among the protein listed, Cystatin-S offers as a biomarker protein and/or indicator of ovulatory phase. Further study to characterize Cystatin-S to bring up a suitable indicator to detect ovulatory phase in human has been initiated.

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Conflicts of interest
There are no conflicts of interest.

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Supplementary Figure 1: Confirmation of ovulation. a) An ultrasound image of the dominant follicle size about ~ 20 mm of Left Ovary (LO).
b) Salivary Fern Pattern

Supplementary Figure 2: Functional annotation of the salivary proteins. a) Biological process, b) Cellular component, c) Molecular function. Functional annotation was carried out by using STRAP 1.5 online database and found most of the identified proteins to be associated with binding property and regulatory function

Supplementary Figure 3: Molecular functional ontology. a) Ovulation phase, b) Post-ovulation phase. Protein domain entries are used to depict the percentage of proteins through Interproscan analysis in BLAST2GO
Supplementary Figure 4: Multi-channel image of 2D gel. a) The unique protein spots during ovulation phase. b) The differential expression level of 16 protein spots during pre-ovulation, ovulation and post-ovulation phases from the top respectively, by a Melanie 3D viewer.