Salivary proteome profile of women during fertile phase of menstrual cycle as characterized by mass spectrometry

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Abstract

Background: Human saliva contains several biomolecules, especially proteins, some of which have been found to serve as biomarkers of different physiological statuses and/or pathological conditions. Saliva is a much superior biological material for investigation over the other body fluids. Ovulation is such a critical physiological process that its non-invasive detection based on salivary protein biomarkers has several advantages in the human. Therefore, it was hypothesized that saliva would potentially contain non-invasive predictor(s)/detector(s) of ovulation. Methods: Samples were collected from women volunteers. The procedure adopted was approved by the Institutional Ethical Committee (DM/2014/101/38), Bharathidasan University. The saliva samples were collected between 8.00 to 9.00 AM from 30 healthy female volunteers (age, mean = 24, range = 19 - 30), with a prior written consent. The protein expression pattern during different phases of menstrual cycle was analyzed using gel-based HR-LC-MS/MS and MALDI TOF/TOF. Results: As many as 530 proteins showed up in the saliva during ovulation phase whereas there were only 251 proteins during post-ovulation 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/differentially expressed protein spots appeared during ovulation 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, Pancreatic alpha-amylase were identified. Conclusions: These ten proteins that were highly expressed during ovulation phase would serve as indicator(s) of ovulation, but extensive validation is required before arriving at a conclusion.

Introduction

Saliva is a clear oral fluid composed of 98% water and 2% other compounds such as mixture of proteins, electrolytes and small molecular weight organic compounds. The term saliva stands for secretions from the major (submandibular, sublingual and parotid) and minor salivary glands. Normally, human salivary glands secrete about 1.0-1.5 L saliva every day, which contains molecules from blood and salivary 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]. In recent years, saliva has been recognized as a diagnostic tool and general health indicator. Many salivary biomolecules arrive from the blood through passive intracellular diffusion and active transport or extracellular ultrafiltration [3]. The levels of salivary components vary in respect of the spectrum of oral and general health. For example, low levels of lysozymes [4] and presence of lactoferrin [5] 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 [6]. The concentration of analytes in saliva is 100- to 1000-fold lower compared to blood [7]. As diagnostic fluid saliva and urine offer many advantages over blood, which include (i) simple, cost-effective and non-invasive nature of sample

collection, (ii) no need of skilled personal to collect saliva, and (iii) the protein concentration of saliva is lesser than plasma. The third attribute renders saliva easier to investigate for diagnostic purposes [7].

Most of the animals have limited and short fertile period (estrus) and the external indications and attractiveness synchronize with ovulation and maximize the chance for fertilization [8, 9]. 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 [10]. The time of ovulation in human is associated with the fertile phase of menstrual cycle. In the mammals, it is generally accepted that many reproductive processes such as ovulation, menstruation, implantation and parturition are linked with inflammation [11]. As the consequence of these processes there is upregulation of a host of inflammatory mediators, which include cytokines, growth factors and lipid mediators which influence the growth and function of the immune and vascular compartments [12]. Similarly, in response to inflammation in an ovary, there are specific pro- or anti-inflammatory cytokine/protein expressions in the body fluids. This can as well be utilized to predict the time of ovulation [13]. Still there is not yet a reliable non-invasive 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 indicting important events in the reproductive cycle. Thus, we adopted a proteome-based approach to detect salivary biomarkers for fertile phase [13]. The complex protein mixtures were analyzed qualitatively by SDS-PAGE [14], which suggested that SDS-PAGE is an adequate technique to assess the protein composition of the human saliva [15]. However, in the recent times, 2-D SDS-PAGE is used to separate complex protein mixtures of saliva based on the different modifications and isoforms of the same protein and recent advancements in mass spectrometry techniques are adopted to better facilitate protein biomarker identification in saliva [3]. In our previous study [16], the ovulation-specific proteins in saliva were mapped, and validated. Hence, it was thought that the identification of an ovulation-specific protein in saliva would do well for future application. 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 (**Figure S1**) to discern the various proteins, and map the ovulation-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 Ethical Committee (DM/2014/101/38), Bharathidasan University. The saliva samples were collected between 8.00 to 9.00 AM from 30 healthy female volunteers (age, mean = 24, range = 19 - 30), with a prior written consent [16]. 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 collection of saliva so as to prevent microbial contamination.

Sample collection and process

The saliva was collected by spitting method. The duration of collection of saliva was about 10 min and the saliva secretion over the first minute 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 x g for 15 min to remove insoluble materials and cells, if any. The samples were stored at -80˚C until further use. The saliva samples were assigned among three phases, viz., pre-ovulatory (day 6 to 12), ovulatory (day 13 and 14) and post-ovulatory (day 15 to 26), according to the pattern of salivary hormones and fern pattern analysis, as was done in our previous study[16].

Protein precipitation and estimation

The salivary proteins were precipitated by trichloroacetic acid (TCA)-acetone precipitation method [17]. The samples were mixed with TCA:acetone (TCA-20% W/V; Acetone-90% V/V) in 1:1 ratio and 20 mM dithiothreitol (DTT) and incubated overnight at -20 °C. After incubation, the samples were centrifuged at 5000 x g at 4 °C for 30 min. The pellets were washed twice with cold acetone and centrifuged at 5000 x g at 4 °C for 30 min. Finally, the pellets were air-dried and re-suspended in UTC (6M urea, 3M thiourea, and 8% CHAPS) buffer. The protein concentration was determined adopting the modified protocol of Bradford [18].

1D – 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 [19]. Standard medium-range molecular weight marker (Low-Range SDS-PAGE Standards, Bio-Rad, Hercules CA) was used as reference and it consisted of phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). 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% bromophenol blue, and 100 mM β-mercaptoethanol] and kept for 1 min at 100 °C for complete denaturation of proteins, after which the sample was loaded onto the gel. A constant current of 50 V was applied for electrophoresis and the entire setup was maintained at room temperature.

Iso-electric focusing

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%b blue. De-streak TM reagent (GE Healthcare) was used for better resolution. The strips were then focused in IPGphor III after 16 hr of passive rehydration. The programme used for focusing 11 cm (3-10 pH) IPG strips was as follows: 200 V-3 hrs (Step and Hold); 500 V-2 hrs (Step and Hold); 2000 V-1 hr (Gradient); 4000 V- 2 hrs (Gradient); 6000 V – 2 hrs (Gradient); 8000 V – 6 hrs (Step and Hold). The focused strips were stored at -80 ºC until further analysis.

2D – Gel electrophoresis

The frozen strips were brought to room temperature and 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 (14 cm x 14 cm x 1 mm) and sealed with an overlay of 0.5% agarose solution. In the electrophoresis apparatus, the upper tank contained 2x tris-glycine buffer (0.6 % tris, 2.88% glycine, and 0.2 % SDS) and the lower tank was filled with 1x buffer. The electrophoresis conditions were 0.5 W for 45 min and 2 W for 5 to 6 hrs until the tracking dye reached the bottom of the gel plate.

Colloidal Coomassie blue staining

After electrophoresis, the gels were rinsed with distilled water and fixed with fixative solution (10% acetic acid, 40% ethanol, and 50% distilled water), following which gels were stained with colloidal Coomassie blue stain (0.02% CBB G-250, 5% ammonium sulfate, 10% ethanol, and 2% orthophosphoric acid) solution according to Dyballa and Metzger [20].

Gel analysis

Digital images of 2D-gels were acquired using ChemiDoc™ XRS imaging system (Bio-Rad) with internal calibration. The acquisition parameters were 300 dpi and epi white illumination. Gel analysis was performed by adopting PDQuest software (Bio-Rad) for spot detection, according to manufacturer's protocol. Spot volume normalization, in the various 2-DE maps, was carried out using the relative spot volumes (% Vol). Initially, automated spot detection was performed, followed by manual editing for spot splitting and noise removal. The gels containing the largest number of protein spots for each phase were chosen as the reference gels. All other gels were matched with the reference gel by placing user

landmarks on approximately 10% of major and minor protein spots, which were visualized to assist in automatic matching. Finally, all matches were checked for errors and edited manually.

HR-LC-MS/MS

The 1D protein spots were analyzed using 6550 i-Funnel 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). For MS measurements, we employed the positive-ion mode with the mass range of up to *m/z* 4000 with the resolution setting 60,000 at *m/z* 400. The proteins were identified by comparison with the SWISS-PROT database entries. Search parameters for MS data were, species: *Homo sapiens*; Protein Mass: 0–500 kDa; Protein pI: 3–10; Enzyme: trypsin; Misscleavage: 1; Mass type: monoisotopic; Charge state: MH+; precursor and product mass tolerance +/-50 and +/-100ppm, respectively; Fixed modification: carbamidomethylation of Cystine (C); and Variable modifications: oxidation of methionine (M). 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 ovulation **(File.S1)** and the post-ovulation protein datasets **(File.S2)** were obtained and listed.

MALDI TOF/TOF analysis

2D protein spot was processed using an automated gel cutter and processor (Shimadzu, Xcise™). The gel spots were washed and destained with 50% ACN and 50 mM NH4HCO3 (Solvent 1), and subjected to in-gel digestion with 30 µL of solvent 7 (50 µLof trypsin stock solution in 4 ml of 50 mM of NH_4HCO_3) for 2 hr at 37 ºC. ZipTips (C18) were wetted and conditioned with 50% ACN and 0.05% TFA (Solvent 5) and 0.1% TFA (Solvent 3), respectively. Cleaved peptides bound to the C-18 resin were desalted using 0.1% TFA (Solvent 3). The peptides were then eluted and spotted with 2.5 µL of Solvent 4 (5mg/mL of CHCA in 50% ACN and 5mM of NH_4CHO_3) onto a 384-well MALDI plate. Finally, samples were identified using MALDI TOF/TOF (AB Sciex 4800).

Data processing

The acquired mass spectra were processed using DataExplorer® software, and the mono-isotopic peptide masses were assigned and used in the database search. The protein identification was analysed against *Homo sapiens* protein sequence in MASCOT database search (http//www.matrixscience.com) using SWISS-PROT database entries. Modification of cysteine by carbamidomethylation and methionine by oxidation was allowed. The precursor and product mass tolerance were set as +/-50 and +/-100 ppm,

respectively. Two or more unique peptides for each protein were taken for confirmation of the protein present in the sample.

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 (http://www.bumc.bu.edu/cardiovascularproteomics/cpctools/strap/)[22].

Molecular functional ontology

The prominent salivary proteins showing up during the ovulation and post-ovulation phases were further classified based on their cellular component, biological process, and molecular function in the UniProt database (http://www.uniprot.org/). 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 (http://revigo.irb.hr). The scatter plotting was carried out with semantic x- and y- axes corresponding to the log size value and log10 P value, respectively.

Statistical analysis

The protein concentration and band intensity values corresponding to ovulation, pre-ovulation and post-ovulation phases were represented as mean ± SD and analysed using one-way analysis of variance (ANOVA) using SPSS 16 software (SPSS Inc., Cary, NC, USA).

Results

Validation of ovulation

It is necessary to assess the ovulation phase after appropriate screening of the menstrual cycle. Not less than 5 cycles were observed in order to confirm the length of the reproductive cycle of the volunteers prior to sample collection. Those women who exhibited normal 28 day cycle length through the 5 or more cycles were chosen to be the volunteers, and the saliva sample was collected. The ovulatory phase was confirmed from direct fertility marker viz., status of the follicle adopting ultrasonography, and biophysical fertility markers such as basal body temperature and fern pattern in saliva **(Figure.S2)** [23].**1D – Gel electrophoresis**

The protein concentration in saliva differed slightly between ovulation and post-ovulation phases. The total proteome was fractionated by 12% SDS-PAGE **(Figure 1A)**. The protein profile of ovulatory phase saliva was compared to that of post-ovulatory phase. Put together, during the two phases, a total of 12 distinct protein bands appeared in the Coomassie brilliant blue-stained gels, and their molecular mass ranged from 14 to 97 kDa. Further, the protein pattern was verified in the gradient gel also **(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 ovulation phase compared to post-ovulatory phase.

HR-LC-MS/MS 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 ovulation and post-ovulation phases of menstrual cycle. During ovulation phase 530 proteins were found, whereas 251 proteins were found during post-ovulation phase. These results clearly indicate that the expression of more number of proteins during ovulation phase than post-ovulation phase. Among these proteins, 35 were present during both ovulation and post-ovulation phases. The schematic diagram (Venn) developed by Oliverios (http://bioinfogp.cnb.es/tools/venny), revealed the protein identities in each phase **(Figure 1C)**.

Ovulation-specific proteins

Several functionally important ovulation phase-specific proteins were identified and listed. **Table 1** shows the list of 30 functionally important proteins related to reproduction during ovulation phase. The biological functions of protein were deduced from STRAP; the theoretical pIs and monoisotopic molecular weights were calculated adopting the procedure in Swiss-Prot – ExPASy website (http://web.expasy.org/compute_pi). 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 unknown, uncharacterized and repeated proteins were removed. The GO entries were predicted using GI number of proteins in UNIPROT database. After refinement of datasets it was seen that saliva of ovulation and post-ovulation phases contained 154 and 117 GO entries, respectively. The gene ontology clearly revealed more UNIPROT entries and greater percentage of annotation during the ovulation phase than the other phases.

Functional annotation

The salivary proteins identified during the ovulation and post-ovulation phases were subjected to functional annotation using STRAP online database. The GO terms of proteins were classified based on the biological processes in which they participate, the cellular location and the molecular function. The results revealed that the ovulation phase had a great number of GO terms and greater percentage of annotations than during the post-ovulation phase **(Figure S3)**.

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 ovulation phase are essentially those with binding property and catalytic activity. Particularly, the ovulation phase salivary proteins **(Figure 2A)** showed up higher number of binding proteins (41.6%) and metal ion binding proteins (16.1%) compared to other phases **(Figure 2B)**. Further, the molecular functional network was constructed using GO terms of salivary proteins. The integrated network map revealed the proteins identified during ovulation phase to have glycoprotein binding, ion binding, and immunoglobulin binding properties, also with receptor activity. Additionally, molecular network analysis revealed the 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 **(Figure S4)**. The GO terms were used to analyse the enrichment with log size by adopting REViGO to predict the scatter plot of binding proteins of each phase of menstrual cycle. Scatter plotting was carried out with semantic X and Y axes corresponding to log size value and log10 p-value, respectively. The proteins were segregated and grouped according to their functions, and displayed in different colors. The cluster of yellow bubbles during ovulation phase corresponds, to the receptor-, protein complex-, peptide-, and GABA-binding and MAP kinase activities. 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 3A)**. Toll-like receptor-, fatty acid-, and lipid-binding proteins, and protein transporter activity proteins during post-ovulation phase are shown as separate clusters **(Figure 3B)**. These proteins might be having some functional significance during ovulation phase.

2D – Gel electrophoresis

The salivary protein expression profiles were assessed during pre-ovulation, ovulation, and post-ovulation phases of menstrual cycle. In Coomassie-stained 2D gels, protein spots were present in the pI range 4-7 and the molecular weight between 14-97 kDa **(Figure 4A-C)**. The analyses carried out during different phases using PDQuest software (Bio-Rad) showed nearly 50 spots in each phase. Particularly, there were more spots during ovulation 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 to 21 kDa spot region of ovulatory phase proteins compared to the other phases. It was noticed that a few low molecular weight protein spots were specifically expressed during the ovulation phase **(Figure 5A).**

Differential expression of salivary protein

Based on the visual assessment and multi-channel image analysis from PDQuest (Bio-Rad) protein spot positions during pre- and post-ovulation phases were unchanged. Though the ovulation phase had a different protein expression compared to pre- and post-ovulation phases, 16 spots were identified as unique and highly expressed during this phase. These protein spot expressions were also validated in Melanie 3D viewer **(Figure 5B)**.

Identification of proteins from selected spots

Sixteen spots with significant difference in intensities i.e., 2-fold increase, were excised from the 2D gel of ovulation phase salivary proteins. The spots were subjected to trypsin digestion and subjected to mass spectrometry. The monoisotopic mass were searched in the protein database of human from MASCOT search to identify the full length sequence. Spot 1 closely matched with Cystatin-S family protein with 71% sequence coverage and eight matched peptides (**Figure 6A & B, Table 2**). The differentially expressed spots were identified 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, 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 (**Table. 3**).

Discussion

Saliva is an important diagnostic biological fluid, which reflects the physiological as well as biochemical changes in the body [24]. Human saliva has been subjected to proteomic analysis rather extensively adopting proteomic technique viz., two-dimensional electrophoresis followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and quadrupole time-of-flight mass spectrometry (Q-TOF/MS)[25] as well as comparative proteomic analysis on intra- and inter-person variability of whole saliva using LC/ESI-TOF/MS[26]. Detailed salivary proteomic studies during

menstrual cycle adopting the 2D-based mass spectrometry have not been conducted earlier. However, recently studies have been carried out to comprehensively catalog the salivary proteome with regard to cellular localization, biological processes and molecular 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 ovulation phase-specific protein. Analysis of this band revealed that the Cystatin-S expression was significantly higher during the ovulation 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 the three major phases of menstrual cycle. Among, 495 proteins are ovulation-specific, and 216 proteins are post-ovulation-specific. Many proteins reported in human saliva has been previously identified in humans under different physiological conditions. The functionally important proteins associated with reproductive mechanisms 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 ovulation 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 secretion of specific peptides/proteins are different in the pediatric age compared to adults [29].

The functional annotation of the identified proteins obtained from STRAP database 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 period [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]. It clearly indicates that there is some specific role for the binding proteins during ovulation. The occurrence of large number of binding proteins during ovulation phase, assigning to molecular function, is an aspect of defence response and immunological process. The binding proteins may have a role in increasing the stability of other proteins [32].

Several salivary biomarkers such as Lactoferrin, Beta-2-microglobulin and Cystatin for Sjögren's syndrome, C-erbB-2, and Epidermal growth factor (EGF) for breast cancer, and Lactoferrin for periodontitis and type 2 diabetes mellitus have been listed using MS-based proteomic techniques [33, 34, 35]. The 2D gel electrophoresis (2-DE) is capable of providing for better biomarker separation (based on both charge and mass) and, therefore, expounds a higher number of 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 4-7. 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 ovulation phase of menstrual cycle.

Since the composition of salivary proteins is influenced by physiological and environmental factors, they have the potential to access and monitor the diseases [38, 24]. Indeed, the differences in protein expression have been well documented in pathological conditions such as cystic fibrosis, dental caries, and periodontitis [39, 40]. So far, only limited proteomics studies have been carried out on human saliva with reference to variations in the different phases of menstrual cycle. On the other hand, proteomic analyses of saliva in ruminants are well established concerning estrous cycle. Electrophoretic separation revealed many distinct protein bands in sheep and goat parotid secretion [41, 42]. A comparative salivary proteomic study was undertaken between goats and sheep [28], and goats and cattle [43]. 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-based marker from saliva for the detection of ovulation time in the human.

In order to make an in-depth salivary proteome analysis the following steps were followed: i) avoid protein loss and get enough protein concentration, ii) a good protein separation to avoid complexity of proteins and to remove abundant proteins, and iii) high mass accuracy peptides sequencing with resolution in scanning[44]. As reported previously, the total concentration of salivary proteins is low, and does not exceed approximately 2.5 mg/mL [45]. Generally, the concentration of proteins can be assessed by several techniques such as dialysis, lyophilization, reverse-phase separation, ultra-filtration, and enrichment by affinity columns or beads; the precipitation with TCA acetone or ammonium sulphate is also advisable [46, 47, 48]. The salivary proteins were efficiently extracted using TCA-acetone method followed by the method of Gehrke[17].Our previous study showed the 14.5 kDa protein specifically expounded to be the dominant protein in saliva [16]. Similarly, the present study also confirms that the proteins in low molecular weight range are highly expressed in ovulation phase compared to other phases.

Recent proteomic platforms have showed about 3000 differentially expressed proteins and peptides in human saliva, many of which are of microbial origin [49]. 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 ovulation phase. Among these proteins, 14.5 kDa Cystatin-S proteins were highly expressed as five isoforms falling in different pI ranges during ovulation phase. Further, the expression of Cystatin-S affirms the previous studies [16]. Likewise, Cystatin-A and cystatin isoforms were reported in gingival crevicular fluid in periodontal patients and chicken egg white during embryogenesis, respectively [50, 51]. It is suggested that salivary glands respond to inflammation stimuli to secrete more Cystatin-C into saliva [50] and it has been considered as a biomarker candidate of renal function [52]. Alterations of serum Cystatin C was considered as early marker for hyperthyroidism, cancer, renal function in diabetic patients, and cardiovascular diseases [53-56]. Cystatin S is a promising tumor biomarker for early cancer diagnosis and treatment evaluation [57]. In many body fluids, Carbonic anhydrase (CA) appears to regulate acid-base balance [58], and possible expression of isoenzymes is found in human and animal reproductive organs [59]. Remarkably, CA is

present during all phases of the estrous cycle of animals including cattle, camel and goat [28]. Additionally, the salivary proteins and microbiota serve as biomarkers to assess the early childhood caries risks [60]. In the present study, the variations in protein expression indicate that physiological changes influence the protein secretion in saliva. Hence, the specific expression of Cystatin-S in saliva during the ovulation phase would lead to bring up an important biomarker for ovulation detection in human.

Conclusion

The protein expression pattern in human saliva was characterized during different phases of menstrual cycle. The total salivary proteome profile is listed, and as many as 530 proteins appeared during ovulation phase as compared to 251 proteins of the post-ovulation phase. Functional annotation of the identified salivary proteins revealed them as extracellular proteins associated with regulatory function. The unique and differentially expressed protein spots during ovulation phase were identified and confirmed. Of these proteins 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 ovulation phase. Among the protein listed, Cystatin-S offers promise as a biomarker protein indicator of ovulation. Further study to characterize Cystatin-S to bring up a suitable indicator to detect ovulatory phase in human has been initiated.

List Of Abbreviations

HR-LC-MS/MS	-High Resolution Liquid Chromatograph Mass Spectrometer
MALDI TOF/TOF	-Matrix Assisted Laser Desorption/Ionisation - Time of Flight
SDS-PAGE	-Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
TCA	-Trichloroacetic acid
DTT	-Dithiothreitol
PRIDE	-PRoteomics IDEntifications
STARP	-Software Tool for Rapid Annotation of Proteins
REViGO	- Reduce and Visualize Gene Ontology

Declarations

Ethics approval and consent to participate

All the procedure adopted was approved by the Institutional Ethical Committee (DM/2014/101/38), Bharathidasan University, India. The saliva samples were collected from healthy female volunteers, with prior written consent.

Consent to publish

Not applicable

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD004511.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors' contributions

GS (lead author) and GA involved in conceiving the idea; GS (lead author), SM,DR and GA designed the study; GS (lead author), DR, SM, GS, APA performed experimental and statiscal analysis; GS, APA, PP, BG, MAA and GA contributed to interpretation of data, and provided critical revision; GS (lead author), GA, MAA, SM, DR, BG, PP involved in writing manuscript and managing the overall progress of the study. The final manuscript was read and approved by all authors.

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Tables

Table 1. List of functionally important salivary proteins identified during ovulatory phase of menstrual cycle.

Swiss-Prot acc. no.[a]	Name	Function[b]	pI[c]	MW[d]	Length[e]
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
Q9UIA9	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
Q5FYB1	Arylsulfatase I	Displays arylsulfatase activity at neutral pH	8.82	64.03	569
P02768	Serum albumin	Binding capacity for water, Ca(2+), Na(+), K(+), fatty acids, hormones, bilirubin and drugs	5.92	69.36	609
Q5XXA6	Anoctamin-1	Chloride conductance	8.76	114.07	986
Q9Y5E6	Protocadherin beta-3	Potential calcium-dependent cell- adhesion protein	4.88	86.77	796
P28566	5-hydroxytryptamine receptor	G-protein coupled receptor	9.09	41.68	365
Q496J9	Synaptic vesicle glycoprotein 2C	Positively regulates vesicle fusion	4.92	82.34	727
Q8WXI7	Mucin-16	Protective, lubricating barrier	-	-	22152
Q969D9	Thymic stromal lymphopoietin	Antimicrobial peptide in the oral cavity	9.75	18.14	159
Q8TE04	Pantothenate kinase 1	Role in the physiological regulation	7.51	64.33	598
P50453	Serpin B9	Protease inhibitor	5.61	42.40	376
Q8TD43	Transient receptor (TRPM4)	Mediates transport of monovalent cations	8.49	134.3	1214
Q3YEC7	Rab-like protein 6	May enhance cellular proliferation	5.11	79.54	729
Q13733	Sodium/potassium-transporting ATPase subunit alpha-4	Hydrolysis of ATP coupled with the exchange of sodium and potassium ions	6.23	114.1	1029
Q8N587	Zinc finger protein 561	Transcriptional regulation	9.09	55.19	486
Q02790	Peptidyl-prolyl cis-trans isomerase FKBP4	Interaction with heat-shock protein 90 (HSP90)	5.35	51.80	459
P28325	Cystatin-D	Cysteine proteinase inhibitor	6.71	16.08	142
Q8WYA1	Aryl hydrocarbon receptor nuclear translocator-like protein 2	Component of the circadian clock Transcriptional activator which is required for	7.01	70.88	636

O75177	Calcium-responsive transactivator	calcium-dependent dendritic growth	5.96	42.99	396
Q9H6R6	Palmitoyltransferase ZDHHC6	Palmitoylates calnexin (CALX), which is required for its association with the ribosome translocon complex and efficient folding of glycosylated proteins.	8.81	47.66	413
Q05JX5	Apolipoprotein B	Ion binding	8.41	46.48	384
P16112	Aggrecan core protein	Resist compression in cartilage	4.10	250.1	2399
Q5EK51	Lactotransferrin	Anti microbial response	8.56	78.32	711
Q8N4F0	Bactericidal/permeability-increasing protein-like 1	Lipid binding	8.82	49.17	458
O00154	Acyl-CoA thioesterase 7	Hydrolysis of acyl-CoAs	8.85	41.79	380
P12273	Prolactin-induced protein	Protein binding	8.26	16.57	146
P23284	Peptidylprolyl isomerase B	Accelerate the folding of proteins	9.42	23.74	216
P23280	Carbonic anhydrase 6	Unknown	6.51	35.36	308
P49895	Deiodinase	Hormone biosynthetic process	8.90	28.92	249
Q59FP8	Neogenin	Unknown	6.56	123.9	1130

[a,e] Proteins having at least one identified peptide in ovulation phase saliva are listed with their Swiss-Prot/TrEmbl accession numbers and length.

[b] Functions were retrieved using the STRAP online database bioinformatics resource.

[c,d] Theoretical pIs and monoisotopic molecular weights were calculated using the Swiss-Prot website.

Table 2. Mass values of cystatin-S. Observed and expected masses (M+H) of cystatin-S protein and tryptic digested peptide sequence map by MALDI-TOF/MS, which was retrieved from Mascot database. The matched peptides are bolded in black.

Start-End	Observed mass	Expected mass	Calculated mass	Peptide sequence
1-24			2488.3579	MARPLCTLLLLMATLAGALASSSK
25-28			546.2398	EENR
29-46	2074.6160	2073.6087	2073.0167	IIPGGIYDADLNDEWVQR
47-57	1293.0520	1292.0447	1291.6561	ALHFAISEYNK
58-65	1046.8020	1045.7947	1045.4352	ATEDEYYR
66-72	881.8430	880.8357	880.5607	RPLQVLR
73-74			245.1488	AR
75-91	1964.5230	1963.5157	1962.9112	EQTFGGVNYFFDVEVGR
92-96			621.3156	TICTK
97-114	2142.5570	2141.5497	2140.9848	SQPNLDTCAFHEQPELQK
115-115			146.1055	K
116-130	1971.2200	1970.2127	1969.8880	QLCSFEIYEVPWEDR
131-137			805.4116	MSLVNSR
131-137	823.3630	822.3557	821.4065	MSLVNSR + Oxidation (M)
138-141			506.1795	CQEA

Table 3. List of highly and specifically expressed proteins in saliva during ovulation phase.

Spot no	Protein Name	Accession code[a]	pI[b]	MW (kDa)[c]
1	Cystatin-S	P01036	4.95	16.21
2	Prolactin-inducible protein	P12273	8.26	16.57
3	Cystatin-S	P01036	4.95	16.21
4	Cystatin-S	P01036	4.95	16.21
5	Cystatin-A	P01040	5.38	11.00
6	Cystatin-A	P01040	5.38	11.00
7	Cystatin-S	P01036	4.95	16.21
8	Cystatin-SN	P01037	6.73	16.38
9	BPI fold-containing family A member 2	Q96DR5	5.35	27.01
10	Cystatin-S	P01036	4.95	16.21
11	Alpha-tubulin N-acetyltransferase 1	Q5SQI0	5.78	37.92
12	Carbonic anhydrase 6	P23280	6.51	35.36
13	Protein LEG1 homolog	Q6P5S2	5.78	37.90
14	Hemoglobin subunit beta	P18984	7.24	16.06
15	Carbonic anhydrase 6	P23280	6.51	35.36
16	Pancreatic alpha-amylase	P00690	6.52	50.07

[a] Proteins having identified peptides in ovulation period saliva are listed with their Swiss-Prot/Uniprot accession numbers.

[b,c] Theoretical pIs and monoisotopic molecular weights were calculated using the Swiss-Prot website (http://web.expasy.org/compute_pi/)

Additional File Legends

Figure S1. The schematic representation of work flow of the present study.

Figure S2. Confirmation of ovulation. A) An ultrasound image of the dominant follicle size about ~ 20 mm of Left Ovary (LO). B) Salivary Fern Pattern.

Figure S3. 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.

Figure S4. Molecular function analysis. Most of the proteins thus identified to possess binding property

File.S1. The protein dataset of ovulation.

File.S2. The protein dataset of post-ovulation.

Figures

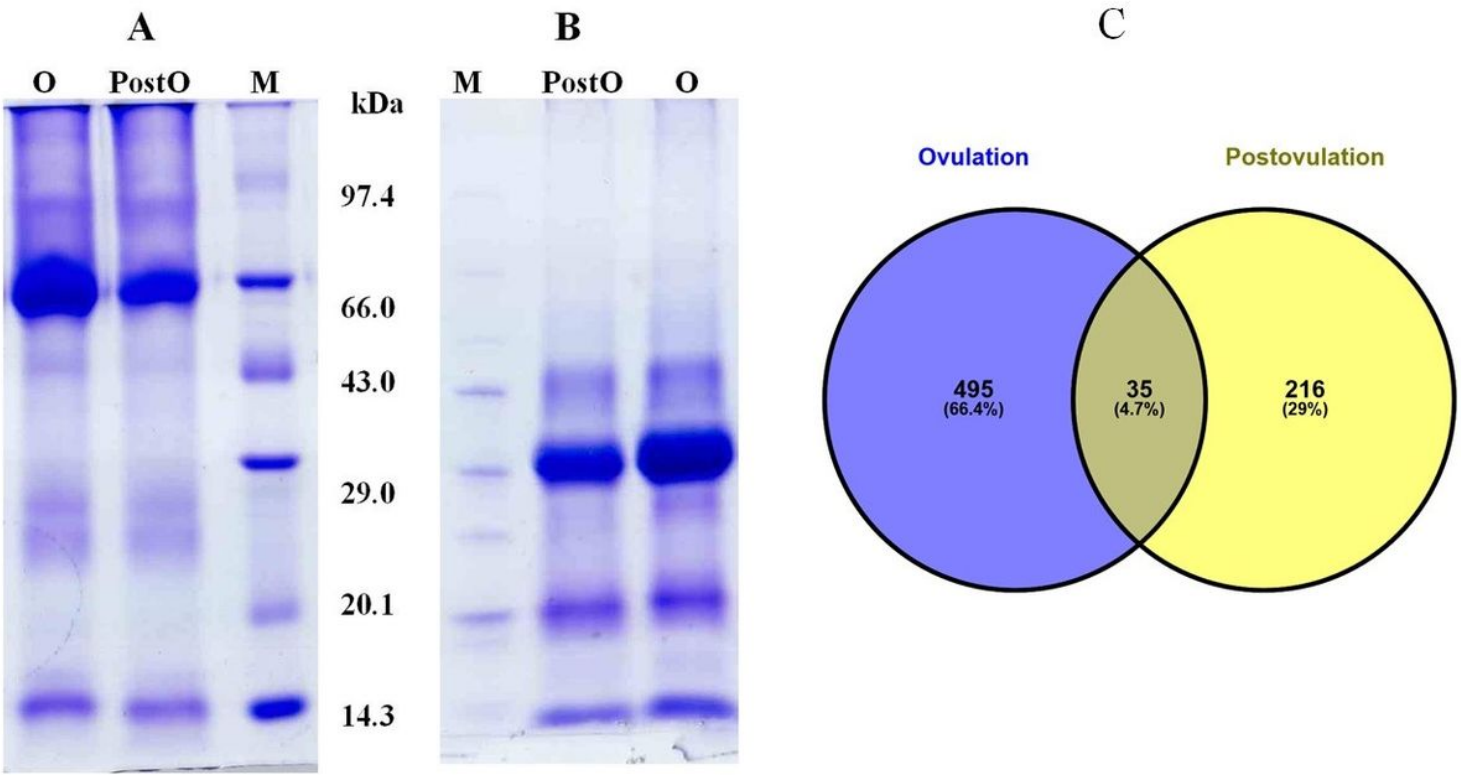


Figure 1

Figure 1. A) Salivary protein profile in 12% SDS-PAGE. O- Ovulation phase, PostO- Post-ovulation phase, M- Protein marker. B) Salivary protein profile in gradient gel (5-15%). O-Ovulation phase, PostO- Post-ovulation phase, M- Protein marker. C) Venn diagram for overall identified salivary proteins. In ovulation phase 495 proteins showed up whereas 216 proteins were observed during post-ovulation phase.

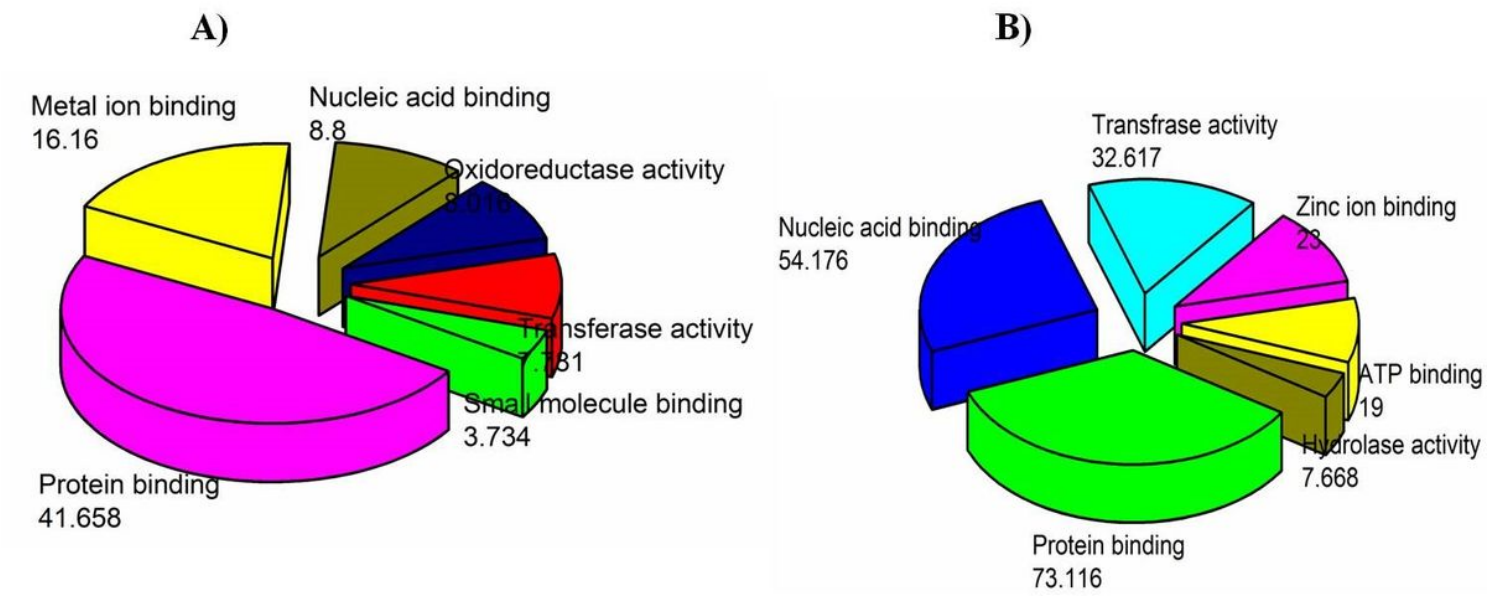


Figure 2

Figure 2. The score distribution according to molecular functional ontology. A) Ovulation phase, B) Postovulation phase. Protein domain entries used to depict the percentage of proteins through Interproscan analysis in BLAST2GO.

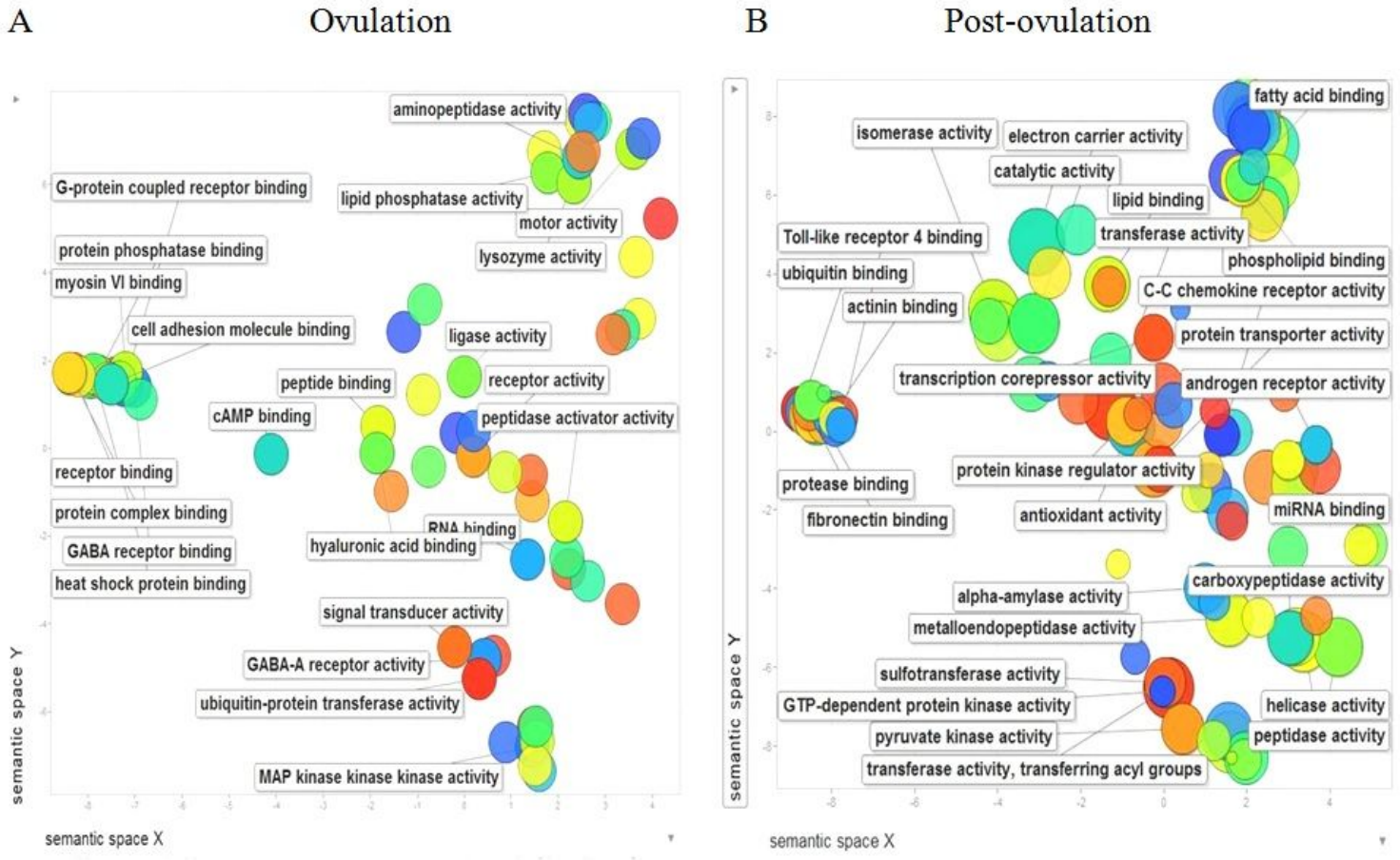


Figure 3

Scatter plot of molecular functions of proteins. The plots show the clusters of protein and activity in A) ovulation and B) post-ovulation phases. The ovulation plot has the higher number of binding and lesser activity protein compared to post-ovulation stage.

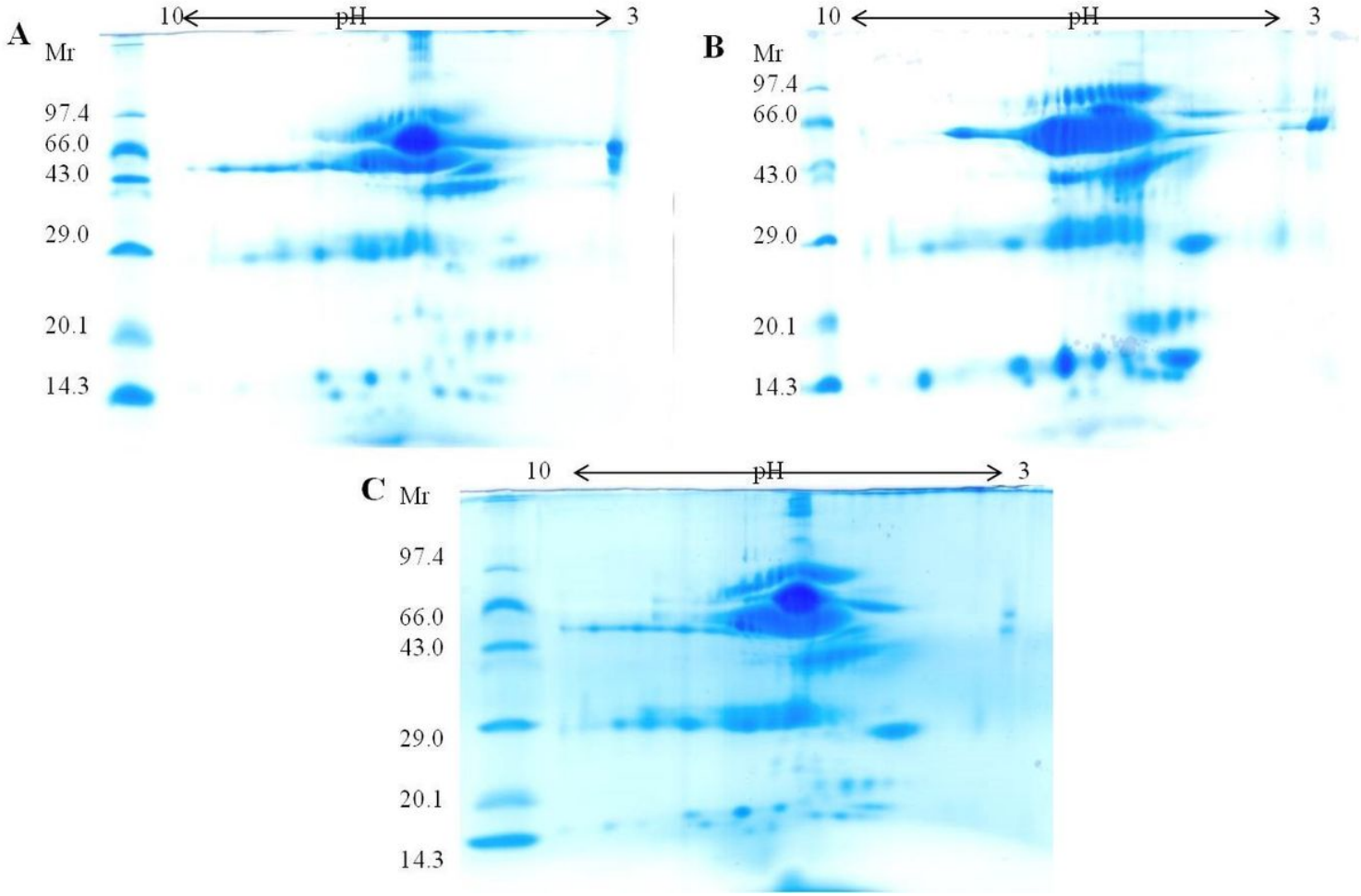


Figure 4

Figure 4. The 2D gel electropherogram of salivary proteins round the menstrual cycle. A) Pre-ovulation phase, B) Ovulation phase, C) Post-ovulation phase. The 11 cm IPG strips were used with 3-10 pH range and gel run with the 12% SDS-PAGE.

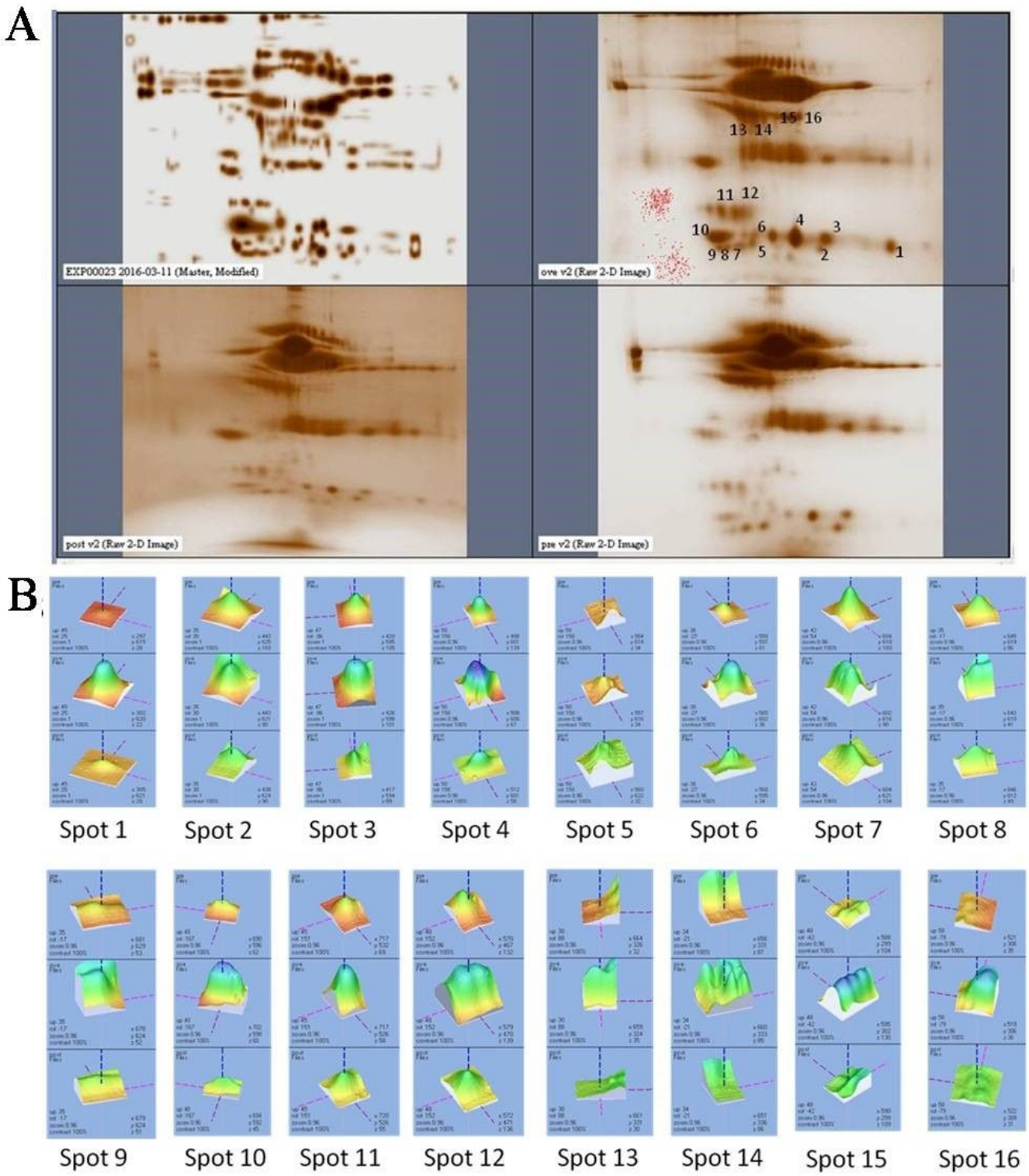
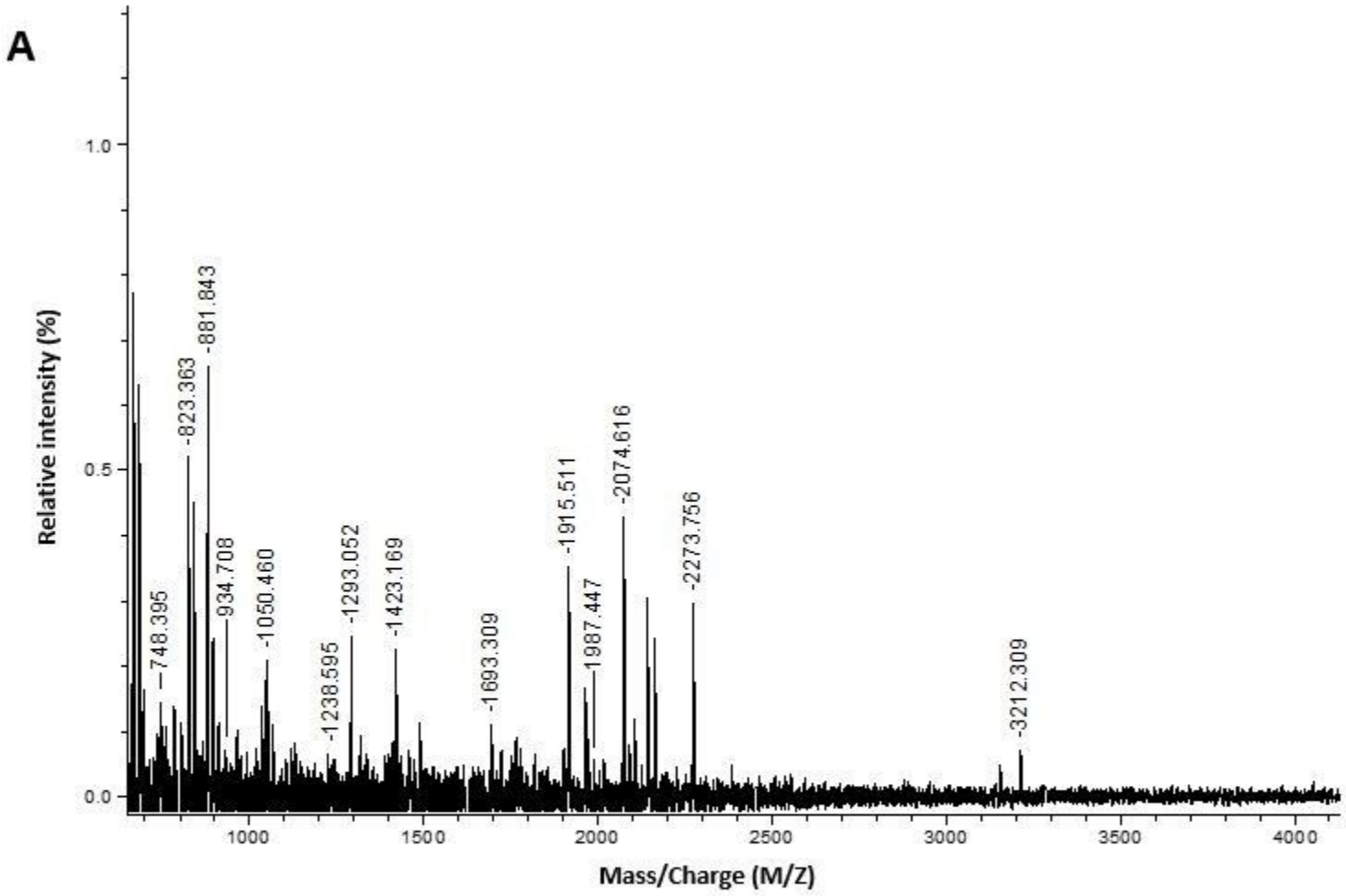


Figure 5

Figure 5. Multi-channel image of 2D gel. A) The unique protein spots during ovulation phase. B) The expression level of spot 1 in pre-ovulation, ovulation and post-ovulation phases from the top respectively, by Melanie 3D viewer.



B

1	MARPLCTLLL	LMATLAGALA	SSSKEENRII	PGGIYDADLN	DEWVQRALHF
51	AISEYNKATE	DEYYRRPLQV	LRAREQTFGG	VNYFFDVEVG	RTICTKSQPN
101	LDTCAFHEQP	ELQKKQLCSF	EIYEVPWEDR	MSLVNSRCQE	A

Figure 6

Figure 6. MALDI-mass spectrum and Sequence coverage of cystatin-S. (a) 2D Protein spot 1 was undergone for in-gel tryptic digestion and the spectra was collected form MALDI-MS. Number in the mass spectrum gives precise m/z (M+H) values for detected peptide ion signals. (b) Single letter coded protein sequence was obtained from mascot search. The matched 71% sequence coverage was highlighted in red colour.

Supplementary Files

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