

# Characterization of Rat Odorant Binding Protein Variants and its Post-Translational Modifications (PTMs): LC-MS/MS analyses of Protein Eluted from 2D-Polyacrylamide Gel Electrophoresis

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## Abstract

Identification of odorant-binding protein (OBP) variants in mammalian system is of great importance to an understanding of their role in the binding of pheromones and other volatiles during chemical communication. Our previous proteomics studies have revealed the presence of OBP as well as confirming that, in the preputial gland of Indian commensal rat, the bound form of the protein associates with various farnesols. In addition to this, a recent mass spectrometry based proteomics analysis has shown that rat recombinant OBP undergoes post-translational modification (PTM) and there are a number of different variants in terms of phosphorylation. This suggests that the phosphorylation of OBP may affect its binding properties and the binding properties may change in the presence of phosphorylation. Thus, in the present investigation we have investigated these OBP variants. The variants were separated by 2DE-PAGE and protein identification was done using mass spectrometry. The results indicated that OBP has ten variants. Further, we employed anti serine phosphorylation immunoblotting in association with 2DE-PAGE to confirm that three spots were phosphorylation at a serine. In addition to immunoblotting, we also employed structural analysis, by multiple sequence alignment, secondary structural analysis, and a three dimensional analysis of the OBP's using known lipocalin members. To the best of our knowledge, this is the first report demonstrating PTM variants of an OBP from the preputial gland of the Indian commensal rat.

**Keywords:** Rat odorant-binding protein; Post-translational modification; Phosphorylation; Mass spectrometry; 2DE-PAGE; Variants

**Abbreviations:** OBP: odorant-binding protein; PTM: post-translational modification; 2D-PAGE: 2- Dimensional poly acrylamide gel electrophoresis;  $\alpha$ 2u: Alpha 2-globulin; mRNA: messenger ribonucleic acid; LC-MS/MS: Liquid chromatography mass spectrometry; IAEC: Institutional Animal Ethics Committee; PBS: Phosphate buffered saline; IPG: Immobilized pH gradient; DTT: Dithiothreitol; CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; SDS: Sodium dodecyl sulfate; PVDF: Polyvinylidene difluoride;  $\text{NH}_4\text{HCO}_3$ : Ammonium bicarbonate;  $\text{K}_3\text{Fe}(\text{CN})_6$ : potassium hexacyanoferrate (III); LTQ: Linear trap quadrupole; HPLC: High performance liquid chromatography; NCBI: National Center for Biotechnology Information; SDS-PAGE: Sodium dodecyl sulfate- poly acrylamide gel electrophoresis; RNA: ribonucleic acid; ATP: Adenosine tri phosphate; DNA: deoxy ribonucleic acid; kDa: kilo Dalton; PDB: Protein data bank; BLAST: Basic Local Alignment Search Tool

## Introduction

In rodents, the preputial gland is being considered to be the predominant organ associated with chemo-communication [1-3]. For instance, the role of pheromones in establishing and maintaining social dominance, evocation of aggression, attracting prospective mates, mark territories, mediate mother-young interaction and individual identification has been confirmed beyond the doubts [4-10]. The preputial glands have been specifically identified as a major source for aggression-promoting as well as attraction delivering pheromones in rodents [11,12]. For instance, dominant mice have been shown to have larger preputial glands than do subdominants and subordinate mice [6]. Furthermore, two of the preputial compounds,  $\alpha$ - and  $\beta$ -farnesene, have been linked to murine social status and to

aggressive behavior [6]. Similarly, farnesol has been identified from the rat preputial gland and found to be a sex-attractant [13]. However, earlier studies demonstrated volatile pheromones have been associated with soluble proteins [14]. Supporting to this report, we have recently identified the pheromone carrying protein i.e.  $\alpha$ 2u-globulin in the rat preputial gland and proteomic analysis further showed the soluble protein capable of binding with  $\alpha$  and  $\beta$  farnesols and mediates chemo-communication [13] and it belongs to the large family of lipocalin. In addition, the presence of  $\alpha$ 2u-globulin in the pub preputial gland of rat indicating that the pheromone carrying/odorant binding protein plays significant a role in adult [15]. This protein have been expressed in most of the mammalian species with the different designation for instance, major urinary protein in mice [16]  $\alpha$ 2u-globulin ( $\alpha$ 2u) in rat [17] and aphrodisin in hamster [18] salivary OBP in buffalo [19] and

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their stable compact structure encloses binding site for hydrophobic ligands [20,21].

To date, there have been a limited number of reports that have described post-translational modification (PTM) of OBPs [19,22-24]. In this context, the study of Srikantan et al. [22] did not characterize the particular site of glycosylation in hamster OBP using either site directed mutagenesis or a proteomics based mass spectrometer approach; therefore the exact site of modification in pheromone binding protein present in the hamster salivary gland is unknown. Recently two reports are demonstrated the presence of phosphorylation in rat recombinant OBP. Furthermore, it is believed that protein variants and PTM play a vital role in these proteins' ligand binding specificity [24]. Until now there have been no proteomics analysis demonstrating the presence of PTM and protein variants of the OBP isolated from the preputial gland of Indian commensal rat. Protein variants may originate by alternative mRNA splicing [25] point mutation, and PTM, including proteolytic cleavage [26]. These modifications often cause a variation in the molecular mass and/or the net charge of the protein [27]. In general, very little is known about pheromone binding protein variants. However, one known case involves pheromaxein A and pheromaxein C in pig, which have  $p'$  values of 4.78 and 5.35, respectively. These two forms have different specificities with respect to several different odorant molecules [28]. Nonetheless, PTM of OBPs is still not well understood. To further advance in this field, it is of great importance to understand the PTM patterns of various OBPs and to investigate how these changes to the OBP may affect the ligand binding processes.

Considering the importance of the preputial gland, particularly with respect to pheromonal communication [29] the aim of the present investigation was to separate the OBP variants of the Indian commensal rat and to obtain detailed information on their modification pattern. It is well known that the various protein isoforms found in a sample occur because of multiple PTM events. In order to separate the various PTM variants, we used 2D-PAGE and the relatively new technique of LC-MS/MS analysis. By employing this proteomic platform, we were able to identify with high confidence ten isoforms of the OBP using 2D-PAGE and document three types of modifications. The modification sites found during the present investigation are all within the conserved regions of the lipocalin protein family (data not shown). However, notwithstanding the fact that no functional analysis was carried out on these PTMs, the results generated from the sequence alignment and structural analysis suggests that these modification may significant affect the ligand binding process and pheromonal communication.

## Material and Methods

Indian commensal rats (*Rattus rattus*) were collected from villages close to Bharathidasan University and were acclimatized to laboratory conditions for five weeks prior to the experiments. They were housed in cages in the laboratory, reared on pelleted food (Sai Durga Ltd., Bangalore) and water *ad libitum*. They were housed separately in polypropylene cages (40 x 25 x 15 cm) with 2 cm of rice husk lining the bottom as bedding material. The bedding material was changed once every 3 or 4 days to maintain hygienic conditions for the animals. All animals were maintained on a normal light and dark cycle (12:12) throughout the experimental period. The procedures strictly adhered to the guidelines for animal care produced by the Institutional Animal Ethics Committee (IAEC), India.

## Isolation and extraction of whole cell lysate of preputial gland

Sexually mature and reproductively active normal Indian

commensal rats (*Rattus rattus*) were sacrificed through cervical dislocation and the preputial glands were removed. The glands were then homogenized with the PBS ( $p^H$  7.2) and centrifuged at 4°C for 25 min at 15,000 rpm. After centrifugation the supernatant was collected and separated by 15% 2D-PAGE.

## Separation of OBP variants using 2D-PAGE

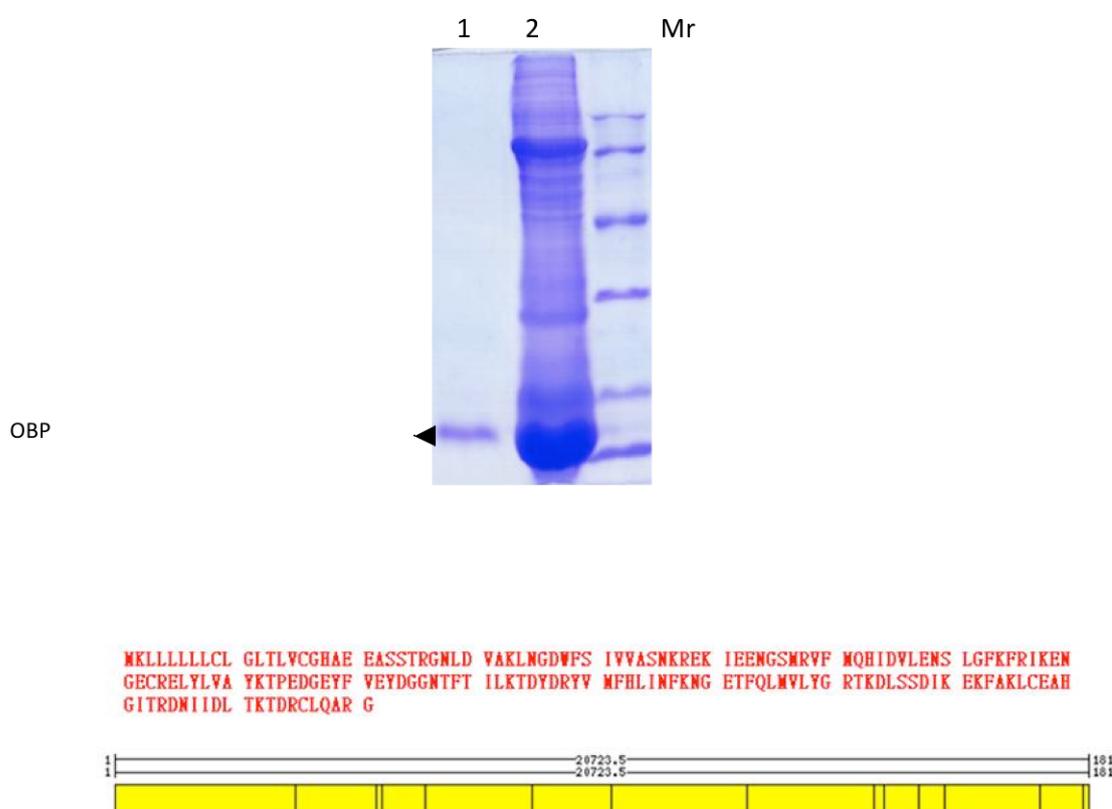
For 2D-PAGE, linear IPG strips with  $p^H$  range of 4-7 were rehydrated overnight with buffer (7M urea, 2M thiourea, 0.4% DTT, 0.5% IPG buffer, 4% CHAPS); 340  $\mu$ l of buffer was used for each 18 cm strip and the whole process was a slight modification from O'Farrell's procedure [30]. The whole cell lysate from preputial gland proteins was precipitated with chloroform-methanol to remove salts and other non-protein materials and 35  $\mu$ g of protein was mixed with sample buffer (7M urea, 2M thiourea, 0.4% DTT, 0.5% IPG buffer, 4% CHAPS, 5mM tributylphosphine). Isoelectric focusing was conducted on an IPG Phor3 manifold (GE healthcare) following cup-loading protocols (500 V, 3 h (Step-and-hold); 1000V, 7 h (Gradient); 8000V, 3 h (Gradient); 8000V, 12 h (Step-and-hold) for 18 cm strip. The strips were then re-equilibrated sequentially with 0.4% DTT in equilibration buffer (6 M urea, 30 % glycerol, 2% SDS, 50mM Tris-HCl ( $p^H$ 8.8) for 20 min each. Proteins within the equilibrated strips were resolved using 15% 2D-PAGE. The 2DE gel was fixed once for 10 min with 250 mL fixation buffer (40% acetonitrile, 10% acetic acid), and the spots were visualized by silver staining.

## Western blotting analysis of the serine phosphorylation of OBP

The purified proteins separated by 2D-PAGE were blotted onto polyvinylidene difluoride (PVDF) membrane by semi-dry-blotting at room temperature, and phosphorylation was detected using monoclonal antibody against phosphoserine (sampler kit, 1:1000; Biomol). The PVDF membrane was blocked with 1:10 dilution of Roti block buffer (Carl Roth GmbH & Co. KG Karlsruhe, Germany) at room temperature (room temperature) for 90 min under shaking. Antibody was reacted for 1 hr at room temperature and washed 4X with phosphate-buffered saline/0.1 % Tween 20; this was followed by reaction for 60 min with a 1: 10000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Invitrogen). The specific spots containing phosphoserine were detected an ECL kit and the images were capture by means of a luminescent image analyzer.

## Destaining and in-gel digestion

Protein bands from the SDS-PAGE gels were excised and destained with destaining solution containing 50mM  $NH_4HCO_3$  with 50% acetonitrile (1:1 vol/vol) for the samples from Commassie brilliant blue stained gels. The samples from 2DE-PAGE gels were excised and destained in 200  $\mu$ l of destaining solution composed of 30 mM potassium hexacyanoferrate (III) ( $K_3Fe(CN)_6$ ) and 100 mM  $Na_2S_2O_3$  in a 1:1 ratio for the silver stained gel bands. After drying in a Speed-Vac (Savant), the gel was incubated in 100  $\mu$ l of 1 %  $\beta$ -mercaptoethanol/25 mM  $NH_4HCO_3$  for 20 min at room temperature and in the dark. The same volume of 5% 4-vinylpyridine in 25 mM  $NH_4HCO_3$ /50% acetonitrile was added for cysteine alkylation. After a 20-min incubation, the gel was soaked in 1 ml of 25 mM  $NH_4HCO_3$  for 10 min, dried and then incubated with 25 mM  $NH_4HCO_3$  containing 100 ng of modified trypsin (Promega) overnight (~18 h). The tryptic digest was removed from the gel and extracted with 300  $\mu$ l of 25 mM  $NH_4HCO_3$  then 300  $\mu$ l of 25 mM  $NH_4HCO_3$ /50% acetonitrile. These two fractions were combined together and dried in a Speed-Vac at -20 °C for storage.



**Figure 1:** Proteomics analysis of preputial gland of Indian commensal rat: Lane 2. Proteins were extracted from the preputial gland and 40  $\mu$ g of protein was separated with the aid of 12 % SDS-PAGE and the total band from the whole cell extract was subjected into the mass spectrometry for proteins identifications. Lane 1 shows the purified for of  $\alpha$ 2u with the aid of gel filtration chromatography, and its purity was checked with aid of SDS-PAGE in order to identify the  $\alpha$ 2u isoforms.

Each sample was resuspended in 0.1 % formic acid immediately before use.

### Mass spectrometry analysis

All Mass spectrometry analyses were performed using an LTQ-Orbitrap (Discovery) hybrid mass spectrometer with a nano electrospray ion source (Thermo Electron, San Jose, CA) coupled to a nano flow HPLC (Agilent Technologies 1200 series). A 100 X 0.075 mm Agilent C18 column (3.5  $\mu$ m particle diameter) with mobile phases of A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) were used. The peptides were eluted using a 13.5-cm-long, 75- $\mu$ m-inner diameter tip column (YMC-Gel, Liquid Chromatography). The pump flow rate was set to 0.5  $\mu$ l/min, and peptide elution was achieved using a linear gradient of 5 to 35% B for the first 30 min followed by a rapid increase to 95% B for the next 10 min. The conventional MS spectra (survey scan) was acquired at high resolution ( $M/\Delta M$ , 60,000 full width half maximum) over the acquisition range  $m/z$  200-2000 and a series of precursor ions were selected for the MS/MS scan. The former examined the accurate mass and charge state of the selected precursor ions and the latter acquired the spectrum (CID spectrum or MS/MS spectrum) of the fragment ions generated by collision-induced dissociation.

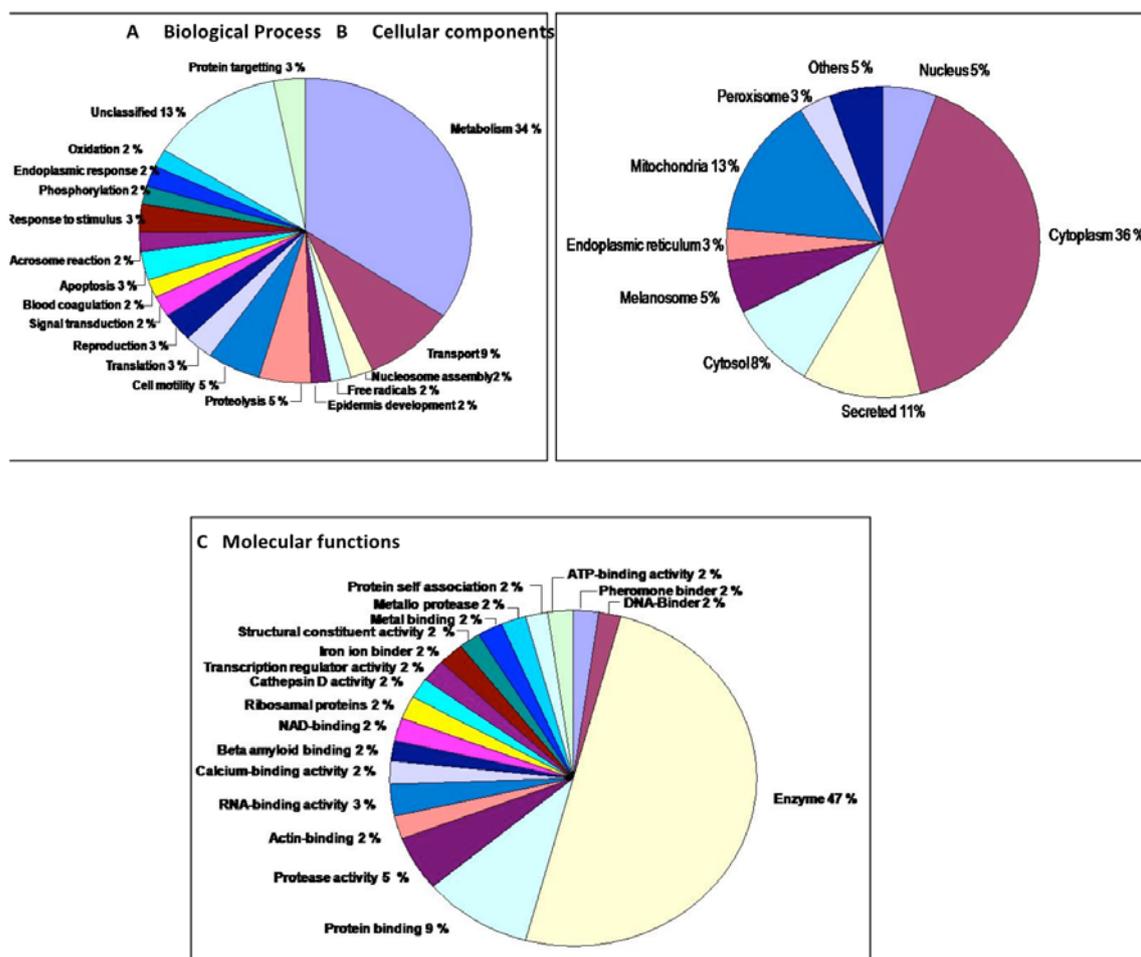
### Database Searching-SEQUEST (TURBO)

The mass spectrometry data were analyzed using Xcalibur software (version 2.0 SR1). Product ion scans obtained from tandem mass spectrometry experiments were examined by the database search

software SEQUEST (TURBO). Peak lists were generated from the products in the scan data (threshold set to 10,000) and these were searched against the rat protein sequence database (obtained from National Center for Biotechnology Information, NCBI). For proteolytic cleavage, only tryptic cleavage was allowed and the maximum number of missed cleavage sites was set to two. Modification at cysteines by carboxymethylation and methionine by oxidation was allowed. The mass tolerance for the precursor peptide ions was set to 3.5 and the fragment ion tolerance was set to 1. Briefly, all accepted results had a DelCN (delta Cn) of 0.1 or greater, a value known to lead to high confidence in a SEQUEST result. Cross-correlation (Xcorr) scores of singly-charged, doubly-charged and triply-charged peptides had to be higher than 1.9, 2.2 and 3.75, respectively. The above criteria were used in the database search in order to give high confidence in terms of protein identification. When a protein was identified by three or more unique peptides possessing SEQUEST scores that passed the above mentioned criteria, no visual assessment of the spectra was conducted and the protein was considered present in the sample.

### Molecular modeling of OBP

The multiple sequence alignment was carried out using the online bioinformatics tool at <http://workbench.sdsc.edu>. The molecular modeling was done using SWISS-MODEL and the figures were prepared using PyMOL. SWISS-MODEL is a freely web-based database that can help users to build protein homology models easily. In the present study, the amino acid sequence of rat  $\alpha$ 2u from *Rattus*



**Figure 2:** Panel A Shows the distribution of the identified proteins based on biological process. The pie chart depicts the distribution of the biological processes among the 64 unique proteins that were identified. Panel B shows distribution of the identified proteins based on cellular component. The pie chart depicts the distribution of the cellular component among the identified proteins. Panel C shows the distribution of the identified proteins based on molecular function. The pie chart depicts the distribution of the molecular function among the identified proteins.

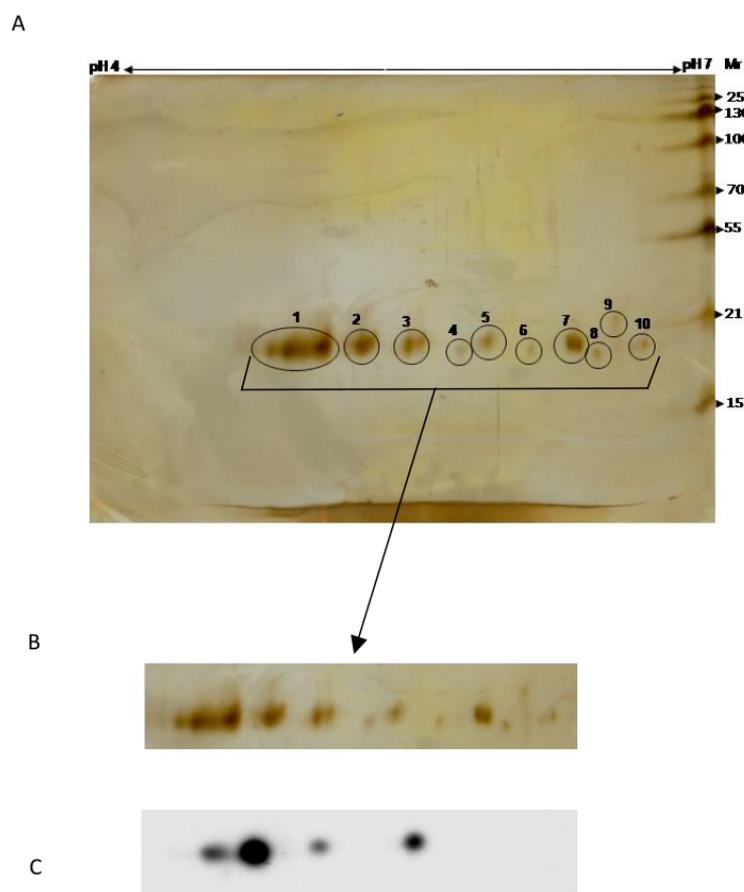
*rattus* was found to display high identity to *Rattus norvegicus*  $\alpha 2u$  [14] in terms of peptides, namely about 98% (E value  $8.9 \times 10^{-81}$ ). SWISS-MODEL with *Rattus norvegicus*  $\alpha 2u$  as the structural template (PDB record 1g85), was used to model the 3D structure of  $\alpha 2u$  from *Rattus rattus*.

## Results

### Categorization and functional annotation analysis of the preputial gland proteins of the Indian commensal rat

Total preputial gland proteins were separated by 12% SDS-PAGE, as shown in Figure 1, lane 1. To characterize these proteins, the protein bands from the SDS-PAGE were excised, divided into 15 gel pieces, digested with trypsin and identified using highly accurate LC-MS/MS. Furthermore, the identified proteins were classified based on their cellular location, biological process, and molecular function. Functional annotation of the identified proteins from the preputial gland is presented in Figure 2 panels A, B, and C. The categories are based primarily on the protein's associated biological process, its localization, and its molecular function. Overall, 34% of the proteins were found to be involved in metabolism, 9% were found to be

involved in transport, 5% were found to be involved in proteolysis and 5% were found to be involved in cell motility, Finally, 3% were found to be involved in translation, reproduction, apoptosis and responses to stimulus, while 2% were found to be involved in nucleosome assembly, free radicals metabolism, epidermis development, signal transduction, blood coagulation, apoptosis, acrosome reaction, phosphorylation, and endoplasmic reticulum. These findings are presented in Figure 2, panel A. In terms of cellular localization, 36 % of the proteins were found to be from the cytoplasm, 11% were found to be from secreted, 13% were found to be from the mitochondria, 8% were found to be from the cytosol, 5% were found to be from the melanosome, and 3% were found to be from the endoplasmic reticulum and peroxisome, as is shown in Figure 2, panel B. Interestingly, 47% of the proteins are enzymes when we classify the identified proteins based on their function. Of these, 9% are protein binding proteins, 5% exhibit protease activity, 3% exhibit RNA binding activity, % exhibit actin binding, 2% exhibit calcium binding activity, 2% exhibit beta amyloid binding, 25 exhibit NAD binding, 2% are ribosomal proteins, 2% show cathepsin D activity, 2% show transcription regulator activity 2% are iron binding proteins, 2% have structural constituent activity, 2% are metal binding proteins, 2% are metallo proteases, 2% have protein self association,



**Figure 3:** Analysis of isoforms of  $\alpha$ 2u-globulin by 2D-PAGE and 2D-PAGE immunoblot. Panel A demonstrated the 2D-PAGE isoform distribution of  $\alpha$ 2u, it shows 10 spots with different pI ranges between 4.5 to 5. Panel B and C shows the separated 2D-PAGE isoforms were further confirmed with serine antiphosphorylation, which conclude that four spots were confirmed as phosphorylated.

2% have ATP binding activity, 2% are DNA binding proteins and 2% are pheromone binding proteins; these are shown in Figure 2, panel C. The list of proteins with their reference numbers and the localization of the proteins, as generated from the NCBI, are summarized (Table 1 (included as supplementary data)).

#### Purification of OBP using size exclusion chromatography

We have previously reported the presence of OBP in Indian commensal rats [14] nonetheless the distribution of OBP variants is unknown for the Indian commensal rat. To answer this question, we purified OBP from the preputial gland. Total preputial glandular proteins were separated using SDS-PAGE (Figure 1, lane 1) and the protein identified as OBP was purified from the preputial gland based on Rajkumar et al. [14]. The OBP protein is by far the most abundant OBP in the rat preputial gland and exists as a single band when separated by electrophoresis under denaturing conditions (12 % SDS-PAGE); it migrates with the apparent mass of 18.54 kDa.

#### Separation of OBP variants using 2DE-PAGE

We have studied the presence of OBP and its bound form to volatiles in the preputial gland of Indian commensal rat using mass spectrometry [15]. The presence of volatiles, namely  $\alpha$ -farnesene and  $\beta$ -farnesene, has been found to be involved dominance advertisement

and attractiveness to females in mice [31]. In order to further explore this topic, we investigated the presence of OBP isoforms and their PTM patterns using a proteomics platform. To the best of our knowledge, there has been no previous report demonstrating the presence of PTM in OBP from the preputial gland of Indian commensal rat; nevertheless, it is believed that PTM plays a crucial role in determining the functions of this protein. It is well known that the proteins variants result from multiple PTM events and this type of event can only be studied using proteomics tools. Various types of PTMs have been demonstrated in other studies, including phosphorylation, and glycosylation [26,32]. PTM is an important process that is able to directly affect the functioning, stability, conformation, and binding interactions of a protein, all of which may be important to the binding of volatile molecules when the protein is an OBP [24]. Our 2DE-PAGE results gave a cluster of polypeptide spots (about ten spots) with wide pI values from 4.6 to 6.7. These were visible after silver staining and four of the spots were highly abundant with a mass of 18.54 kDa (Figure 3, panel A). These spots were identified as OBP variants with higher confidence based on sequence coverage (Figure 3 and panel C). Although the molecular weight of the proteins was very similar, they varied dramatically in pI, which suggest that these proteins only differ in terms of post-translational modification.

### Serine phosphorylation of rat odorant binding protein

Protein post-translational modification particularly phosphorylation is crucial to many biological functions such as cell proliferation, cell differentiation, and enzyme activity [27]. In order to further understand the PTM of OBP, we employed 2DE-PAGE based anti serine phosphorylation immunoblotting and this showed that three OBP spots with different pIs reacted strongly; these had pI values from 4.5 to 6.7 (Figure 3 B), which is higher than that of the native OBP by calculation [24]. These results suggest that the different pI values of these spots may be due to partial phosphorylated that gives multiple variant forms. Based on this finding, the identification of other types of modification using mass spectrometry became important.

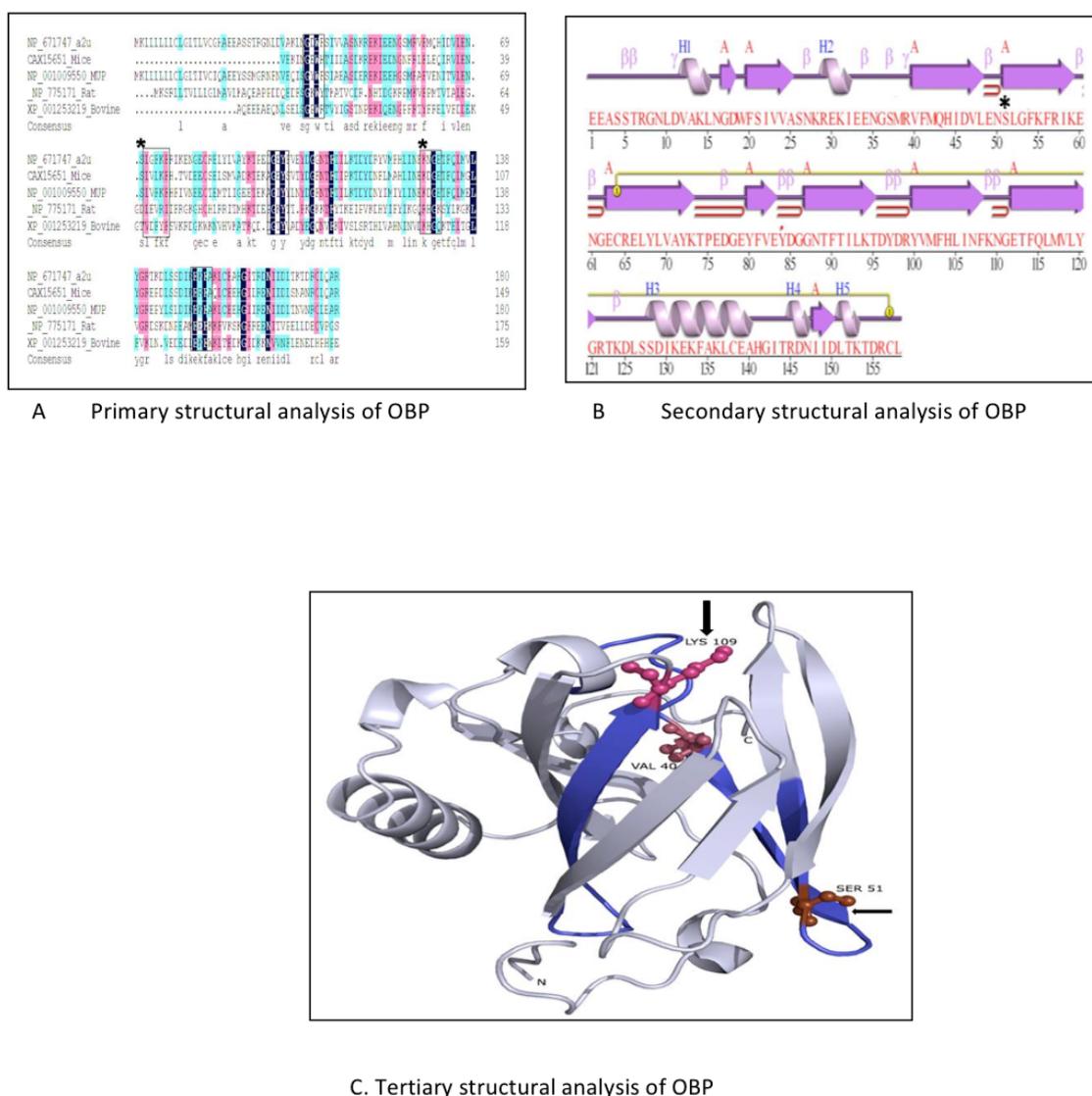
### Molecular modeling of OBP

On performing multiple sequence alignment of OBP with known lipocalin members we were able to identify the presence of rat OBP

(Figure 4, panel A). Furthermore, an amino sequence (NP\_671747) associated with the rat OBP was subjected to BLAST search against all available sequences in the Protein Data Bank (PDB). As a result, the crystal structure of OBP A and its complexes were found to highly match (100% identity) one protein NP\_671747. Based on this, we used the crystal structure from the source *Rattus norvegicus* (2a2g), which has a resolution of 2.90 Å, for further structural analysis of amino acid substitution. Then, using the visualization tool PYMOL, the pattern of amino acid was added to the three-dimensional structural of 2a2g (Figure 4, panel B).

### Discussion

Our previous proteomics analysis of the preputial gland of the Indian commensal rat identified the presence of OBP and we have also shown that it can accommodate various pheromonal compounds such as the α and β forms of farnasols [13]. Interestingly, earlier reports have



**Figure 4:** Structural Analysis of Indian commensal rat α2u-globulin serine phosphorylation. Panel A shows the multiple sequence alignment of α2u with deduced sequence of lipocalin members. Panel B shows the secondary structural analysis of α2u, the serine phosphorylation was pinpointed with asterisk at serine 51. Panel C demonstrated the phosphorylation of α2u at serine<sup>51</sup>.

demonstrated the role of farnesols in relation to pheromone binding proteins as well as their relationship with inter-male aggression and territorial marking [6]. However, binding of these pheromonal molecules may change when the protein undergoes post-translational modification, particularly phosphorylation, and this hypothesis was extensively tested using rat recombinant odorant binding protein [23,24]. As shown by us earlier [15] rat OBP has the potential to binds pheromonal molecules, but as yet no comprehensive analysis of OBP variants in terms of post-translational modification has been reported.

In the present study, using 2D-PAGE, LC-MS/MS analysis and bioinformatics tools, we were able to separate ten spots containing OBO over a *pI* range of 4.5 to 6.7 (Figure 3). These results are in good agreement with the results for pig SAL, where it was found that there were two major bands and a few minor bands covering the *pI* range of 4.2 to 4.8 [33]. Based on reports, rat has two distinct genes encoding non-allelic VEG, probably due to gene duplication [34]. Similar findings have also been documented for porcine SALs [35]. LC-MS/MS analysis identified serine phosphorylation, the most abundant OBP variants (data not shown). However, there seem to be many more OBP variant proteins present than can be simply explained by these modifications. Recently, mass spectrometry based analysis showed the existence of phosphorylation variants in rat recombinant odorant binding protein and it has been suggested that differences in binding specificity are driven by phosphorylation [23,25]. Previous reports have identified N-linked glycan modification on Asn53, in pig SALs and phosphorylation of recombinant rat OBPs. All together ten OBP variants were identified by 2D-PAGE and phosphorylation of these variants was investigated by Western blotting. The results revealed that the three most abundant OBP spots were phosphorylated. These findings are commensurate with the recent study of rat recombinant odorant binding protein [25]. The ten OBP spots had various *pI* values slightly higher than the expected *pI* value of  $\alpha 2u$  [36] but with the same molecular mass [14] which supports modification of OBP via phosphorylation or another similar modification.

The primary structure alignment analysis revealed high sequence similarity with known lipocalin family members. In the alignment, eight residues are conserved in all the lipocalin members and they are pinpointed with block boxing (Figure 4, panel A). In addition to the sequence alignment, we also generated a secondary structure model of rat OBP, which showed that there were no changes to the hydrophobic pocket within the  $\beta$ -barrel across the two rat species (Figure 4, panel B and C). These results are in agreement with the comparison of rat and mouse major urinary protein [14,37].

## Concluding Remarks

It is well known that the protein variants detected by gel electrophoresis are in many cases caused by post translational modification of a single protein product and that they are important in terms of protein function, stability, confirmation and localization [38]. It seems likely that the presence of phosphorylation in the rat recombinant odorant binding protein may have a specific role in determining the specificity of odorant molecule bindings [23,24]. Using a proteomics strategy we identified ten OBP variants and three spots were found to be phosphorylated using anti serine phosphorylation Western blotting. In addition, mass spectrometry and our in-house PTM finder program were able to detect phosphorylation at serine-52 (unpublished data). Our 3D modeling structural analysis showed that these modifications are in conserved regions. It seems likely that these modifications may

make significant contributions to OBP binding specificity with respect to odorant molecules. However, it should be interesting to investigate their special role in binding of pheromones, using for instance specific pheromones have different binding properties between the variants.

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