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RAISING ANTIBODY AGAINST MOUSE SEX PHEROMONE BINDING PROTEIN IN RABBIT

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Abstrak: Dalam kajian ini, antibodi poliklonal dihasilkan dengan protein pengikat feromon seks (*sex pheromone binding protein*, SPBP) daripada *Mus musculus* (L) (tikus jantan Swiss) dan digunakan untuk mengesan protein pengikat feromon (*pheromone binding proteins*, PBPs) dalam sistem olfaktori (deria) tikus seperti sistem deria bau utama dan aksesori dengan ekstrak protein tisu hati dan ginjal sebagai kawalan negatif. Usaha permulaan ini telah membuktikan bahawa PBP ialah protein tunggal yang hadir dalam kalangan profil protein organ vomeronasal (VNO). Didapati bahawa PBP yang jantina-spesifik ini tidak hadir dalam semua tisu lain termasuk sistem deria bau utama.

Abstract: In the present investigation polyclonal antibody was raised against male sex pheromone binding protein (SPBP) of *Mus musculus* (L) (Swiss mice) and has been used to identify the pheromone binding proteins (PBPs) of mouse olfactory systems such as the main and accessory olfactory system with the liver and kidney tissue protein extracts were used as negative control. This maiden attempt authentically proves that the PBP is only present among the protein profiles of vomeronasal organ (VNO) and it was reported that this sex specific PBP is found not to be expressed in all other tissues including main olfactory system (MOS).

Keywords: Pheromone, Binding Protein, Rabbit, Antibody

INTRODUCTION

Pheromone perception in mammals is mediated by specific olfactory receptor neurons (Kannan 2003). The mammalian pheromones exist in the form of a small lipophilic/hydrophobic compound which precisely bind to olfactory epithelial neurons by which a chain of biochemical events is stimulated to convert the chemical signal into electric signal (Kannan *et al*. 1998; Kannan & Archunan 2001). The pheromone binding to the receptor cell membrane is apparently facilitated by soluble binding protein present in the nasal mucus (Krieger & Breer 1999; Vincent *et al*. 2001). The primary sequence of pheromone binding protein (PBP) of several insects like moth and other lepidopteron species has been well documented. The reports related to immunology of mammalian pheromone binding protein are still scanty. Therefore, the present investigation has been designed to assess the immuno reactivity of PBP.

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It is very interesting to note that the PBPs are belonging to a super family of proteins named as 'lipocalin'. In rodents, the lipocalins are third class of label molecules used as complex chemical signals (Halpern & Martinez-Marcos 2003). Several authentic scientific evidences clearly stated that the lipocalins are a large, disparate and diverse group of small proteins with the molecular weight of about 20 kDa to 40 kDa most of which are extra-cellular and display binding with high selectivity and affinity for small hydrophobic molecules (Flower 1996; Flower *et al*. 1993). All lipocalins share a common structure which is a central eight stranded, β-barrel with simple forth and back repeated topology lining a hydrophobic cavity, an N-terminal helical turn long C-terminal helix. Till now the three dimensional structure of approximately five lipocalins have been resolved by X-ray crystallographic (Bocskei *et al*. 1992) and C^{13} and N^{15} NMR spectroscopic analysis (Ferrari *et al*. 1997). Such behaviourally and physiologically important protein has been designated as a PBP in some other mammalian species. Hence in the present investigation, an attempt has been made to develop polyclonal antibodies in rabbit against the purified sex pheromone binding protein (SPBP) of *Mus musculus* (L) (Swiss mouse).

MATERIALS AND METHODS

Test Organism

Sexually mature male *Mus musculus* (L) (Swiss mice) were purchased from the King's Research Institute, Chennai and housed separately in polypropylene cages (40 cm x 25 cm x 15 cm). The bottom of the cage was lined (2 cm in height) with rice husk as bedding material. Adequate amount of pelleted food and water were supplied *ad libitum*. Animals were maintained in the animal house as per the standard guidelines of Institutional Animal Ethics Committee, Government of India.

Protein Extraction and PAGE Analysis

The main olfactory system (MOS) and vomeronasal organ (VNO) of male mice were removed and used as source of PBP. Tissues were separately homogenized at 4°C for 3 minutes with a glass homogenizer in 20 mM Tris HCl buffer (pH 7.4) in presence of protease inhibitor (1.5 mM phenyl methyl sulphonyl fluoride). Homogenates were centrifuged at 12,000 rpm for 15 minutes (Plasto Craft, India) to pellet down the debris and the supernatant was subjected to native PAGE (10%) in poly-acrylamide gels using a Bangalore Genei electrophoretic apparatus (India) and a discontinuous buffer system according to Pevsner *et al*. (1990). Proteins were stained in the Coomassie Brilliant Blue R-250. Pheromone binding protein was removed by following sonication extraction method of Ramoni *et al*. (2001) with minor modifications in extraction buffer concentrations. The excised PBP band was sliced into small pieces (2–4 mm in size) with a sterile razor blade and washed with 0.2 ml of 250 mM Tris buffer/250 mM Ethylene Diamine Tetra Acetic Acid (EDTA), pH 7.4 in a microfuge tube (1.5 ml) followed by three rinses of 20 seconds duration each with distilled water. The distilled water was then removed with a Pasteur pipette and the gel slices were

chopped finely (or) minced with autoclaved stainless steel spatula (pieces of 2–4 mm in size). The sliced gel pieces were dissolved in 1 ml of 20 mM Tris buffer (pH 7.4) containing 1% Sodium Dodsyl Sulfate (SDS) (w/v). The samples were sonicated for 3 minutes under ice cold condition (five to six passes of 45 seconds).

Chromatographic Purification of PBP

In order to isolate the PBP from the extraction buffer, 1.5 ml of collected sample was applied to Sephadex G-50 mini-column with the capacity of 5 ml (5 ml glass syringe) filled to 1 ml Sephadex G-50 resin, equilibrated with 20 mM Tris buffer (pH 7.4) and centrifuged for 10 minutes at the speed of 5,000 rpm under 4°C to obtain a final volume of 1 ml protein, free of gel matrix. One milliliter of tissue xtract was loaded to the pre-equilibrated Sephadex G-50 packed column (with 20 mM Sodium acetate) and finally eluted with 0.5 M NaCl.

Mouse PBP Immunization and Collection of Antiserum

Approximately 1 mg of purified SPBP was dissolved in 1 ml of deionized sterile water and then injected into thigh muscle of rabbit. The sterile needle was inserted from the rear at right angles to the skin surface, at a point half way along the femur, so that its point lied within the muscle. The inoculation was then made, needle was withdrawn and finally the injected site was gently massaged. The booster dose was administered after 15th and 25th day of immunization program. For collecting the antiserum, the rabbit was bled from the marginal vein of the ear. About 5 ml to 10 ml of blood was collected in a single bleed from the immunized healthy rabbit with the time interval of 0, 15th, 20th and 30th days of immunization. To separate the plasma from collected blood in presence of anti-coagulant, 50 mM Citrate buffer prepared with 10% $CaCl_2$ in 3.8% Sodium Citrate and 2% EDTA was directly centrifuged at 2,000 rpm for 5 minutes at 4°C. The serum was used for further studies (which was considered as anti pheromone binding protein source).

ELISA for PBP

Immuno-chemicals were obtained from Sigma Chemical Ltd. (St. Louis, MO, USA). Standards for cross-reactivity were purchased from Aldrich Chemical Ltd. (Milwaukee, WI, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) at a concentration of 1 mM. The coating antigens were stored freeze-dried at –40°C. Working aliquots with the necessary amounts to prepare two microtitre plates were prepared in Eppendorf tubes and stored at 4°C. Unless otherwise indicated, phosphate buffered saline (PBS) concentration used is 0.01 M phosphate buffer and 0.8% saline solution (pH 7.2.) Phosphate Buffer Saline with Tween20 (PBST) used for blotting contained PBS with 0.05% Tween 20; PBST-EtOH (Ethyl Alcohol) (PBS with 10% ethanol and 0.01% Tween 20; pH adjusted to 8.6); Coating buffer (0.05 M carbonate and bicarbonate buffer, pH 9.6); Citrate buffer (0.04 M solution of sodium citrate, pH 5.4); substrate solution (0.01% tetramethylbenzidine and 0.004% H_2O_2 in citrate buffer).

Competitive indirect ELISA was performed according to the method described below. The microtitre plates were coated overnight (12 ± 1 hours) at 4°C with the coating antigen (mouse pheromone) appropriately diluted in coating buffer (100 µL/well). Next day, plates were washed four times with PBST and 12 serial dilutions of PBP (10,000 nM to 1 pM, in PBST, 50 µL/well) were added to the coated plates followed by antisera appropriately diluted in PBST (50 µL/well).The mixture was incubated for 45 minutes at room temperature (28 ± 4°C) and the plates were washed for about five times using PBST. A solution of goat anti-rabbit IgG conjugated to horseradish peroxidase (anti-IgG-HRP, 1:6000 dilution in PBST) was added (100 µL/well) and incubated for 45 minutes at room temperature. The plates were washed again using PBST washing buffer, and the substrate solution was added to the wells (100 µL/well). Colourimetric reaction was stopped after 30 minutes of incubation using stop solution (5N H_2SO_4 (50 µL/well)), and the absorbance was read at 450 nm.

The standard curve was plotted to a four parameter logistic equation according to the following formula

$$Y = \frac{(A - B)}{1 - (X/C)^{D}} + B$$

Where

A = maximal absorbance
B = minimal absorbance
C = concentration producing 50% of the maximal absorbance
D = the slope at the inflation point of the sigmoid curve
X = absorbance value

Western Blot Analysis of PBP

After electrophoretic separation, proteins were electro-transferred onto nitrocellulose membrane following western blotting procedure of Ramoni *et al.* (2001) with minor modification in buffer concentration. First, nitrocellulose membrane was incubated with 1.2% bovine serum albumin in PBP buffer containing 0.7% Tween 20 for 45 minutes at 25 ± 3°C. After overnight incubation with PBP antiserum at a dilution of 1:4000, bound antibodies were detected with goat anti-rabbit IgG labelled with HRP-horseradish peroxidase (Bangalore Genei, Pvt. Ltd., India) at 1:2500 dilution and was detected using 4-chloro-1-napthol as substrate. Simultaneously, control tissues like liver and kidney of the same test mice were processed for using as negative control.

RESULTS AND DISCUSSIONS

Several developments during the last two decades profoundly affected our understanding of the molecular biology of VNO of vertebrates (Halpern & Martinez-Marcos 2003). The VNO receptor cells contain a diverse group of proteins with the molecular weight ranging from 19 kDa to 200 kDa. These proteins collectively known as olfactory marker or receptor proteins (Weiler *et al.*

1999). Antibodies raised against olfactory receptor proteins (ORP) of ferret is used to confirm that the ORPs are only expressing in the anterior part of VNO rather than in posterior end (Shapiro *et al.* 1997). Interestingly, no reports are available to identify the expression of SPBPs in mouse VNO although the role of SPBP in chemosensory transduction had already been proved in our laboratory (Kannan & Krishnan 2006). In the present investigation it is clearly noticed that the SPBP of mouse VNO elicit immune response even in the absence of adjuvants. This may be due to the antigenic property of SPBP in rabbit system. Moreover, a similar phenomenon has been observed in the olfactory and Vomeronasal Neuron (VN) receptor cells of *Mustel putorious* (ferret) (Shnayder *et al.* 1993). Further, the Odorant Marker Protein (OMP) immunoreactivity is well proved by Western blot as well as immuno-histochemical studies (Weiler *et al.* 1999).

The cumulative observation of this experiment clearly showed that the mouse SPBP was expressed in the Accessory Olfactory System (AOS), purified by column chromatography and the purity was checked in 10% SDS-PAGE analysis (Fig. 1). Kannan and Krishnan (2006) authentically proved that the identified 191 kDa protein of AOS effectively bond to the sex pheromone of mouse. Further they emphasized that the biometric assay (volatile-odourant binding protein) for identified SPBP and the sex pheromone, 2-Octanamine (2-1, methyl heptyl) were observed to be bound to each other with 1:1 ratio. Uptake of airborne odorants in nearly physiological conditions enhances the role of PBP as volatile hydrophobic pheromone carriers in the VNO of Swiss mouse. It was also found that SPBP was the immunizing antigen rendering the highest amount of competitive immunoassays with acceptable parameters such as A_{max} 0.5–1.5 units of absorbance (Fig. 2). This result has been clearly addressed by the greatest exposure of well-defined immune system to the pheromonal signalling hydrophobic molecules. The high-resolution capacity of the chromatographic techniques leads to a high level of purity of the proteins and consequently to a very high specificity appeared while purification of protein using affinity column.

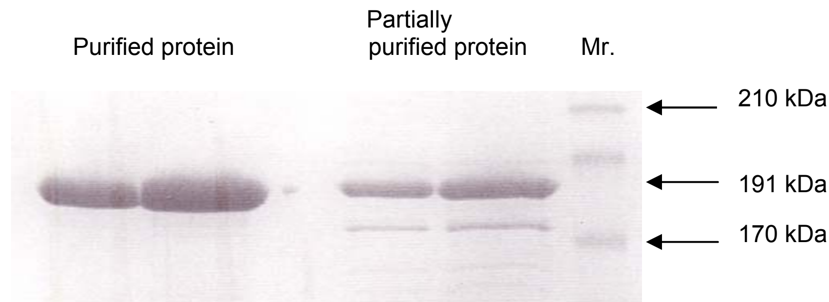

Figure 1: The 10% SDS–PAGE analysis confirms the level of purity of the mouse SPBP to be used as immunogen to raise antibody in rabbit. The protein bands were visualized with Coomassie brilliant blue staining technique. Mr., Molecular weight marker.

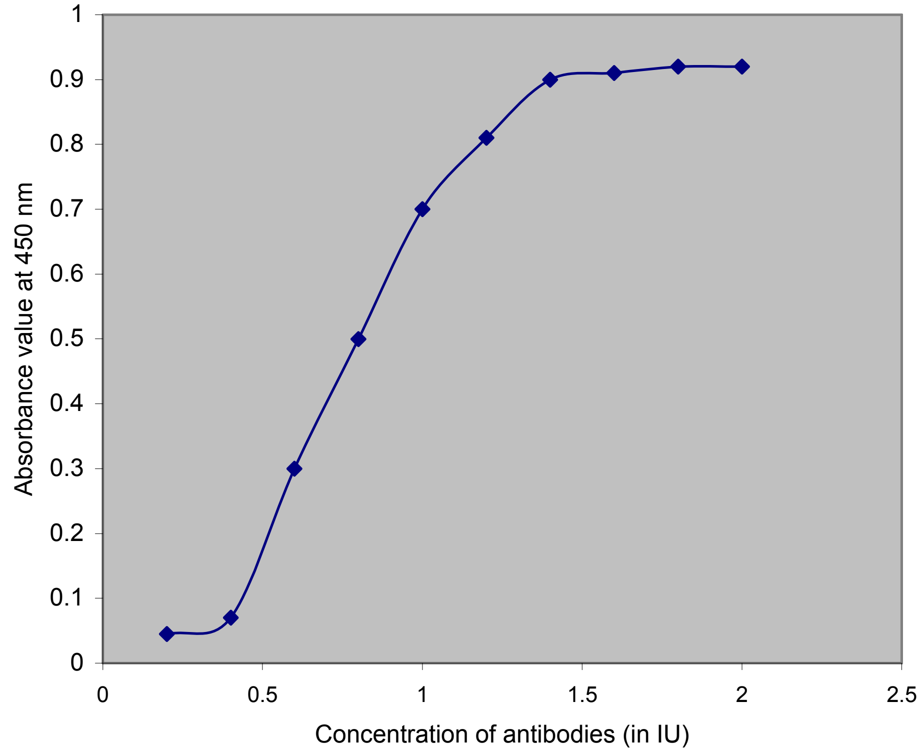

Figure 2: the competitive immunoassay was performed against the SPBP with antiserum raised against it in rabbit. This graphical representation shows that the antigen antibody reaction is at the maximum of 1.5 International Units (IU) of antibody.

The electrophoretic analysis revealed that the antibody against PBP was shown to be gradually increased with increasing incubation period and a distinct electrophoretic migration as compared with other proteins present in the test samples namely MOS and AOS of male mice (Fig. 3) and positive immuno reactivity was noticed with AOS/VNO of male mice [Fig. 4(a)]. While in both olfactory systems, more than ten protein bands have been observed and some of them had more or less similar electrophoretic mobility but did not show immunological response with the anti-serum (anti-PBP of mice i.e., IgG) in the case of MOS. Further, we tested the tissue specificity of the antiserum using Western staining method. This attempt revealed that the immuno-reactive protein was only observed in homogenates of VNO and not from MOS, liver and kidney tissues of male mice [Fig. 4(b)].

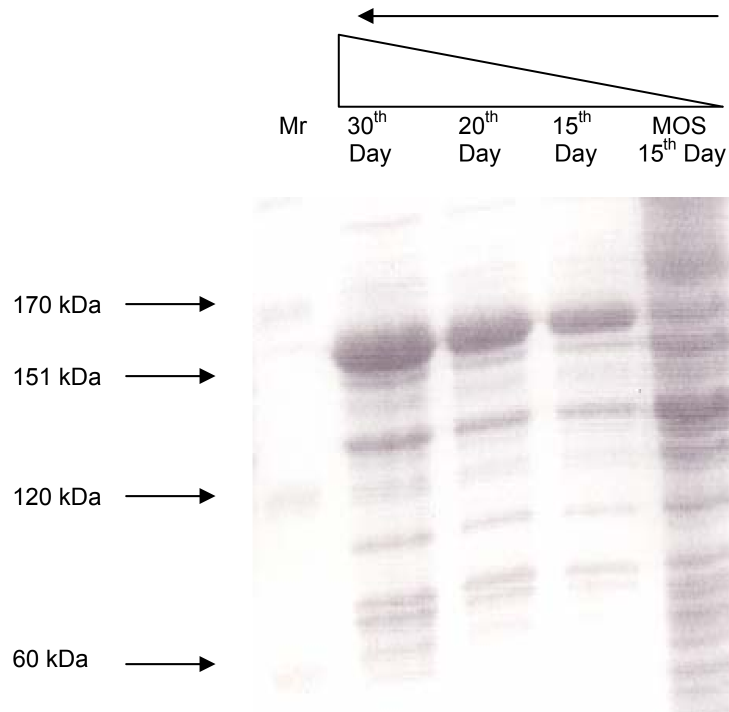

Figure 3: Protein profile of serum collected from rabbit after immunizing with SPBP with different time intervals. The level of antibody production against the PBP is increased with increasing time duration after the appropriate booster dose (as per the arrow indicates).

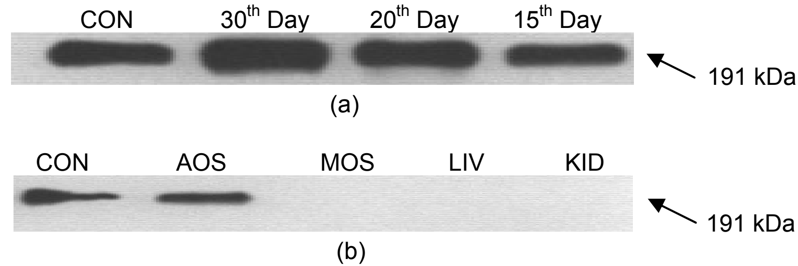

Figure 4: Shows the cross reaction between SPBP and anti-pheromone binding protein (Anti serum raised in rabbit). (a) Describes the level of antibody production is increased with increasing the time duration with appropriate booster dose, (b) Western blot explains that the sex pheromone binding protein is tissue specific and only expressed in the VNO but not in the other selected tissues such as, main olfactory system (MOS), Liver (LIV) and Kidney (KID). CON, Control.

An attempt has been made to describe a method for production of antibodies against the PBP without addition of adjuvants and DMSO. In addition, it is reported as a simple, rapid, reliable and reproducible protocol for raising antibody against mouse olfactory receptor protein.

The DMSO solubilized nitrocellulose paper containing pheromone-binding protein was immunized into rabbit (n = 6) without any adjuvant. The collected serum was used for competitive immunoassay (ELISA). Hence, this method serves as a fast and would possibly performs as better than as other antibody production methods. Mean-while, retaining all these advantages of the antibody production protocol, it would be more useful to detect the male specific PBP. Thus, the generation of sex specific mouse PBP antibody will serve as a novel olfactory marker system to identify mouse species among the members of class rodentia. Besides, it has highly advantageous property to postulate a classical hypothesis to design an immuno-contraceptive inhaler method to block the binding of reproductive pheromones in the olfactory system where, the pheromones regulate reproductive-endocrine function.

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REFERENCES

Bocskei Z, Groom C R, Flower D R, Wright C E, Phillips S E V, Cavaggioni A, Findly J B C and North A C T. (1992). Odourant Binding Proteins of insects. *Nature* 360: 186–188.

Brennan P A and Keverne E B. (2004). Something in the air? New insights into mammalian pheromones. *Curr. Biol.* 14: R81–R85.

Briand L, Nespoulous C, Huet J C, Takahashi M and Ernollet J C. (2001). Ligand binding and physico-chemical properties of ASP2, a recombinant odour-binding protein from honeybee (*Apis mellifera*). *Eur. J. Biochem.* 268: 1–10.

Ferrari E, Lodi T, Sorbi R T, Trindelli R, Cavaggioni A and Spisni A. (1997). Expression of a lipocalin in *P. pastoris*: Secretion, purification and binding assay of a recombinant mouse urinary protein. *FEBS Lett.* 401: 73–77.

Flower D R. (1996). The lipocalin protein family: Structure and function. *Biochem. J.* 318: 1–14.

Flower D R, North A C T and Attwood T K. (1993). Structure and sequence relationships in the lipocalins and related proteins. *Protein Sci.* 2: 386–391.

Halpern M and Martinez-Marcos A. (2003). Structure and function of the vomeronasal system: An update. *Neurobiol.* 70: 245–318.

Kannan S. 2003). Preputial gland of male house rat: Chemical identification and its biochemical activity. *Jurnal Biosains* 14: 1–6.

Kannan S and Archunan G. (2001). Chemistry of clitoral gland secretions of the laboratory rat: Assessment of behavioural response to identified compound. *J. Biosci.* 26: 247–252.

Kannan S and Krishnan M. (2006). Partial purification and sequence determination of mouse sex pheromone binding protein (Unpublished data).

Kannan S, Rameshkumar K and Archunan G. (1998). Sex attactant in male preputial gland: Chemical identification and assessment of their role in reproductive behaviour in rats. *Curr. Sci.* 74: 689–691.

Krieger J and Breer H. (1999). Olfactory reception in vertebrates. *Science* 28: 720–723.

Novotny M V. (2003). Pheromones, binding proteins and receptor responses in rodents. *Biochem. Soc. Trans.* 31: 17–22.

Pevsner J, Hou V, Snowman A M and Snyder S H. (1990). Odorant-binding protein: Characterization of ligand binding *J. Biol. Chem.* 265: 6118–6125.

Ramoni R, Vincent F, Groli S, Conti V, Malsse C, Boyer F D, Nagnan-le meillour P, Spinelli S, Cambillau C and Tegoni M. (2001). The Insect attractant 1–octen–3–ol is the natural ligand of bovine odorant-binding protein. *J. Biol Chem.* 276: 7150–7155.

Shapiro L S, Roland R M and Halpern M. (1997). Development of olfactory marker protein and N-CAM expression in chemosensory system of the opposum. *J. Morphol.* 234: 109–129.

Shnayder L, Schwanzel-Fukuda M and Halpern M. (1993). Differential OMP expression in opossum accessory olfactory bulb. *NeuroReport* 5: 193–196.

Steinbrecht R A. (1998). Odourant binding proteins: Expression and function. *Ann. New York Acad. Sci.* 855: 323–332.

Vincent F, Lobel D, Brown K, Spinelli S, Grote P, Breer H, Cambillau C and Tegoni M. (2001). Crystal structure of aphrodisin, a sex pheromone from female hamster. *J. Mol. Biol.* 305: 459–469.

Weiler E, Apfelbach R and Farbman A I. (1999). The Vomero nasal organ of the male ferret. *Chem. Senses* 24: 127–136.