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Identification of $GABA_{\beta}$ Receptor Protein and Farnesol in the Preputial Gland of Bandicoot Rat (*Bandicota indica*)

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Abstract Preputial gland is one of the prime sources of pheromones in rats. The study on pheromone identification in preputial gland is well established in laboratory rat, house rat and voles. But the study was lacking in the preputial gland of bandicoot rats. Hence, the present investigation was aimed to identify the volatile and protein profiles of preputial gland of male bandicoot rat. Gas chromatography and mass spectrometry (GC-MS) profiles revealed the presence of 47 volatile compounds in the preputial gland. More specifically, the farnesol was found to be a major compound in the preputial gland which is consistent with previous reports in the preputial gland of few other rodents. The histoarchitecture results showed that the preputial gland of male exhibited more acini cells. The protein profiles of preputial gland showed 12 prominent bands in coomassie brilliant blue stained gel. The low molecular mass protein, 19 kDa has been identified as gamma-amino butyric acid type B receptor subunit I ($GABA_{\beta}$ receptor) by MALDI-ToF analysis. Further, to the best of our knowledge, this is the first time we explored the absence of alpha 2u globulin in the 19 kDa band of preputial gland of bandicoot rat. This is in contrast to the presence of alpha 2u globulin in the preputial gland of laboratory rat (*Rattus norvegicus*) as well as house rat (*Rattus rattus*). The present study concludes that among the volatiles and protein analysis performed in the preputial gland, farnesol appears to be a prominent compound and the $GABA_{\beta}$ receptor protein was identified in 19 kDa band in bandicoot rats.

Keywords Preputial Gland, Volatile Compounds, Proteins, Bandicoot Rat

1. Introduction

Animals release their pheromones through the secretory and excretory products. The excretory products such as faeces and urine are the major pheromone sources. In several animals, the secretory sources such as preen gland [37] and uropygial glands [40] in birds, metatarsal glands in sika deer [38], paracloacal glands in crocodiles [12], suprasternal gland in opossums [14], sternal gland in old world monkey [35], labial and scrotal secretions of ringtailed lemur [34], anal gland of polecats [41], flank gland in hamsters [23], chin gland in rabbits [15], cheek glands of lesser bandicoot rats [21] and preputial gland of rat [20] and mice [26] are reported as pheromone sources.

Most of the secretory glands are modified sebaceous glands and are reported as pheromone secretors in animals. They release sebum to outside the body that acts as a transporter of pheromones [36]. Among the secretory glands, preputial gland is reported as a modified sebaceous gland and actively releases the pheromones [2, 30]. It is located in the prepuce of the male rat hence the name preputial gland. The preputial gland is also proved to be a pheromone source through Olfactory Receptor Neuron (ORN) studies [31]. It is assumed that preputial gland may release its contents through urine. Preputial gland odours play several significant functions such as mother-young interaction for the survival of the young during their prepubertal stage [7, 8, 9], for individual identification [42, 43], sex attraction [13, 20, 44] and evocation of aggression after maturity [28].

It is well known that low molecular mass proteins (17 – 20 kDa) in the pheromone sources assist the pheromone communication by binding and slow releasing of volatiles for the long term availability of pheromones from the scented sites [1]. These proteins belong to lipocalin family and called as pheromone binding / carrying protein (PBP/PCP) [5]. For instance, the PCP is known as Major Urinary Protein (MUP) in the urine of mice [17], alpha 2u globulin in the urine of rat [10], Aphrodisin in the vaginal mucus of hamster [6], apolipoprotein D in the sweat of human [39], sweat protein in horse [11] and salivary lipocalin in the salivary gland of boar [24].

There are plenty of reports available on the importance of preputial glands in pheromone communication. However, the study of pheromones and proteins in the preputial gland of bandicoot rat, *Bandicota indica* is not yet undertaken. Hence the present study was aimed to explore the volatile compounds and protein profiles in the preputial gland of bandicoot rat.

2. Materials and Methods

2.1. Animals

Adult male rats, *Bandicoota indica* were collected from nearby paddy fields at Bharathidasan University, Tiruchirappalli and housed separately in polypropylene cages (40x25x15 cm) with 2 cm of rice husk lining the bottom as bedding material, light on from 6.00 to 18.00 hour, temperature 24±1°C, reared with pelleted food (SaiDurga feeds and foods, Bangalore) & water *ad libitum*. The bedding material was changed twice a week.

2.2. Isolation of Preputial Gland

Six adult intact males were sacrificed by cervical dislocation. Then preputial gland was removed carefully and frozen immediately at -20°C until use.

2.3. Histology

The histology of preputial gland was performed by adopting the routine paraffin method [16]. Briefly, Preputial glands were dissected out from the bandicoot rats, fixed in Bouin's fluid fixative immediately after autopsy. After fixation the tissues were transferred to 70% alcohol. Several changes of 70% alcohol were given until the yellow color disappeared from the tissues. The tissues were then dehydrated by passing through ascending grades of alcohol, cleared in xylene, infiltrated with molten paraffin, and finally embedded in paraffin wax. Transverse and longitudinal sections with 3-5 µm thickness were obtained using a rotary microtome (Leica, Germany). The sections, thus obtained, were stained in Harris hematoxylen and eosin, dehydrated using alcohol, cleared in xylene and mounted using DPX.

2.4. Preparation of Tissue Extract

A crude extract from preputial glands was prepared by homogenization with Phosphate Buffer Saline (PBS) (7.2 pH) under ice-cold conditions, followed by centrifugation at 10000 rpm for 15 min. The clear supernatant was immediately used in the subsequent steps.

2.5. GC-MS Analysis

The GC–MS analyses were made in QP-5000 (Shimadzu, Japan).The 2 µl of extract was injected into the GC–MS on a 30 m glass capillary column with a film thickness of 0.25 µm (30 m x 0.2 mm i.d., coated with UCON HB 2000) using the following temperature programme: initial oven temperature, 40ºC for 4 min, increasing to 250ºC for 10 min. The GC–MS was under the computer control at 70eV, using ammonia as reagent gas at 95eV to perform chemical ionization. Identification of unknown compounds is by following libraries such as WILEY7, NIST05 and NIST05s [30].

2.5. SDS-Poly Acrylamide Gel Electrophoresis

The total protein concentration was determined by the method of Bradford (1976). The 12% SDS-PAGE was performed as described by Laemmli, 1970 [22] with slight modifications. 50 µg protein was loaded on to the gel. For determination of molecular mass, 4 µl of protein standard (protein molecular weight marker-medium range, Genei, Bangalore), was loaded into the gel.

2.6. MALDI-TOF Mass Spectrometry

The protein spot at 19 kDa was excised, and then subjected to in-gel tryptic digestion following the method of Armstrong *et al.* 2005[3]. After tryptic digestion, the mixture of peptides was placed in the MALDI target plate and mixed with the matrix solution. Following calibration of known peptides, the samples were processed and mono-isotopic masses of spectra from the tryptic-digested peptides were acquired for database searching. Based on the results, matching compounds and the suspected sequence of the particular sample were obtained. Statistical evaluation of the results and scoring algorithms using Mascot (Matrix Science Ltd, http://www.matrixscience.com) facilitated the identification of best match.

3. Results

A well-developed preputial gland was observed in bandicoot rat and each gland was 2.85-3.2 cm in length, 1.5 – 2.1cm in width and weighed about 3.5 – 4.32 g. The morphology of the gland appeared as pyriform (Fig. 1a & b). The histoarchitecture of this gland revealed the presence of acini cells secreting sebum (Fig. 1c). GC-MS results showed the presence of 47 compounds (Fig.2; Table 1) in the preputial gland. Among the compounds, farnesol was found to be a major compound. The protein profile of preputial gland was also observed for the presence of low molecular mass proteins (17-20 kDa). A very thin band of 19 kDa was observed in the coomassie stained gel (Fig. 3).

This 19 kDa band was excised and subjected to in-gel trypsin lysis for MALDI-ToF analysis. After the MALDI-ToF analysis, the monoisotopic number of spectra were scored.The mascot search showed that the 19 kDa band was gamma-aminobutyric acid type B receptor ($GABA_{\beta}$) subunit I (Fig.4; Table 2). But we got sequence coverage of 13% and 9 matching peptides.

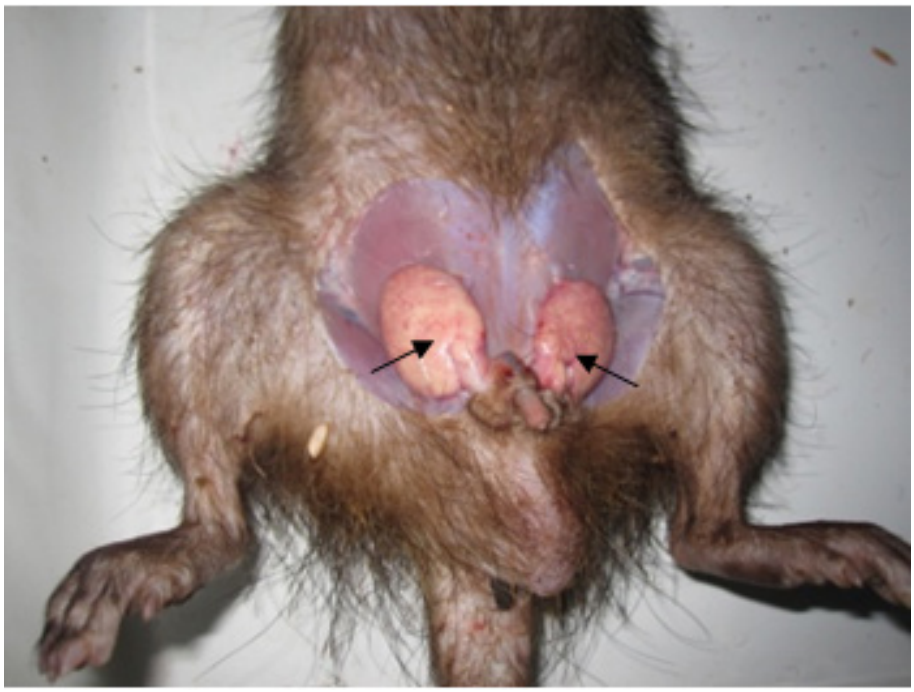


Fig. 1[a]

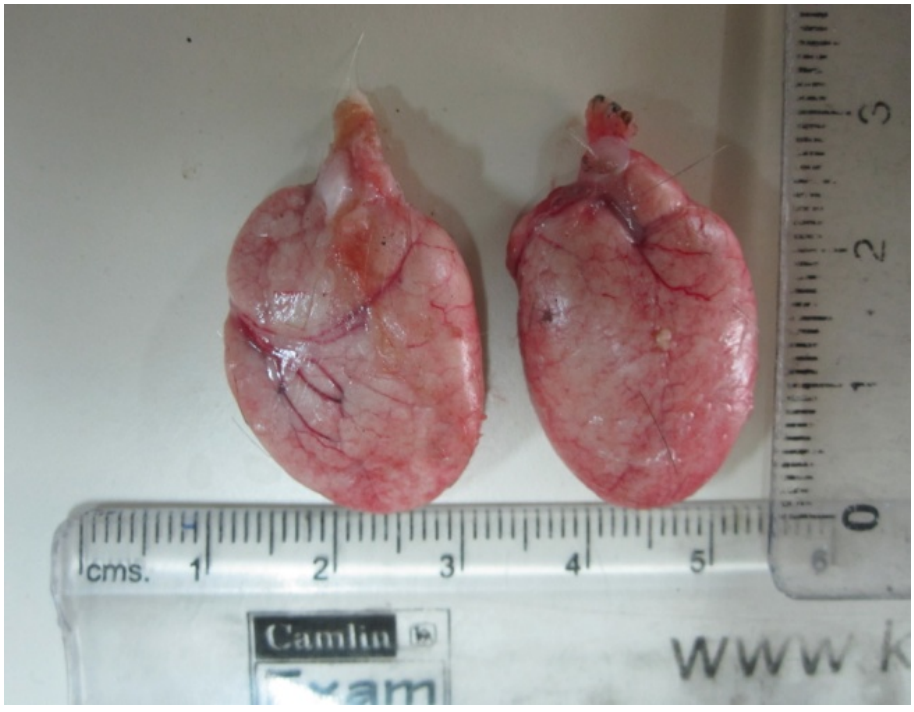
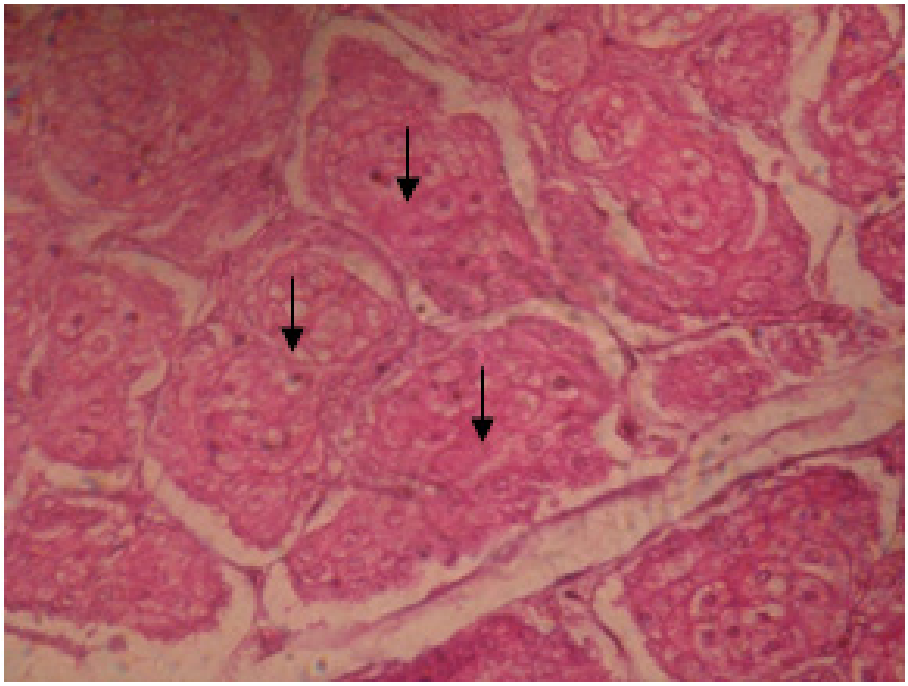


Fig. 1[b]



[c]

Figure 1. Morphology of preputial gland [a] Location of preputial gland (arrow); [b] Pear shaped preputial gland; [c] Histoarchitecture of preputial gland (arrow indicates serours acini cells) (40X)

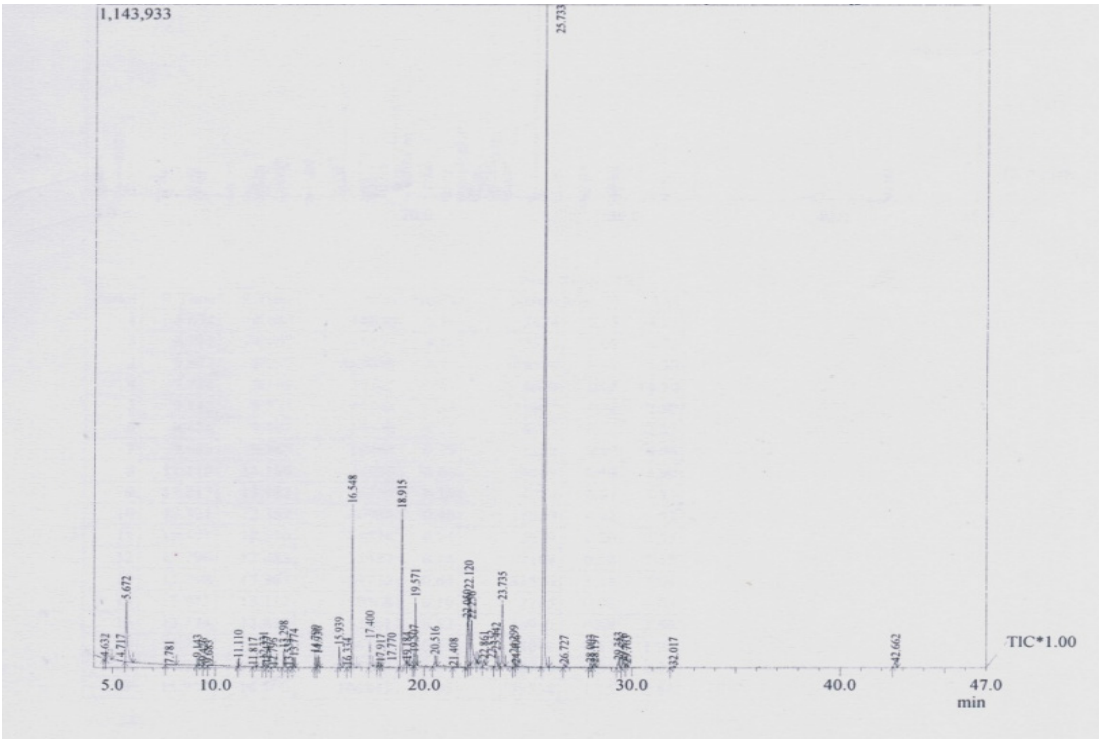


Figure 2. Gas chromatogram of the preputial gland

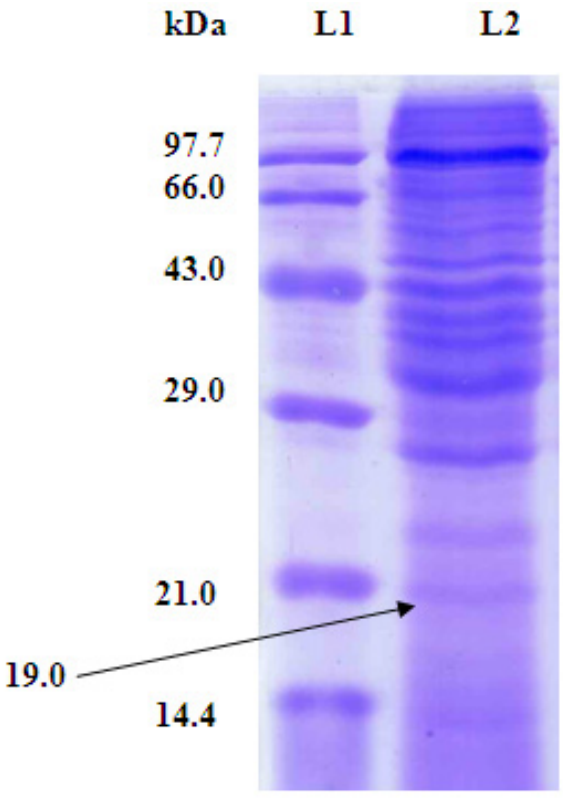


Figure 3. Protein profile of preputial gland of bandicoot rat [L1- molecular weight marker; L2- preputial gland]

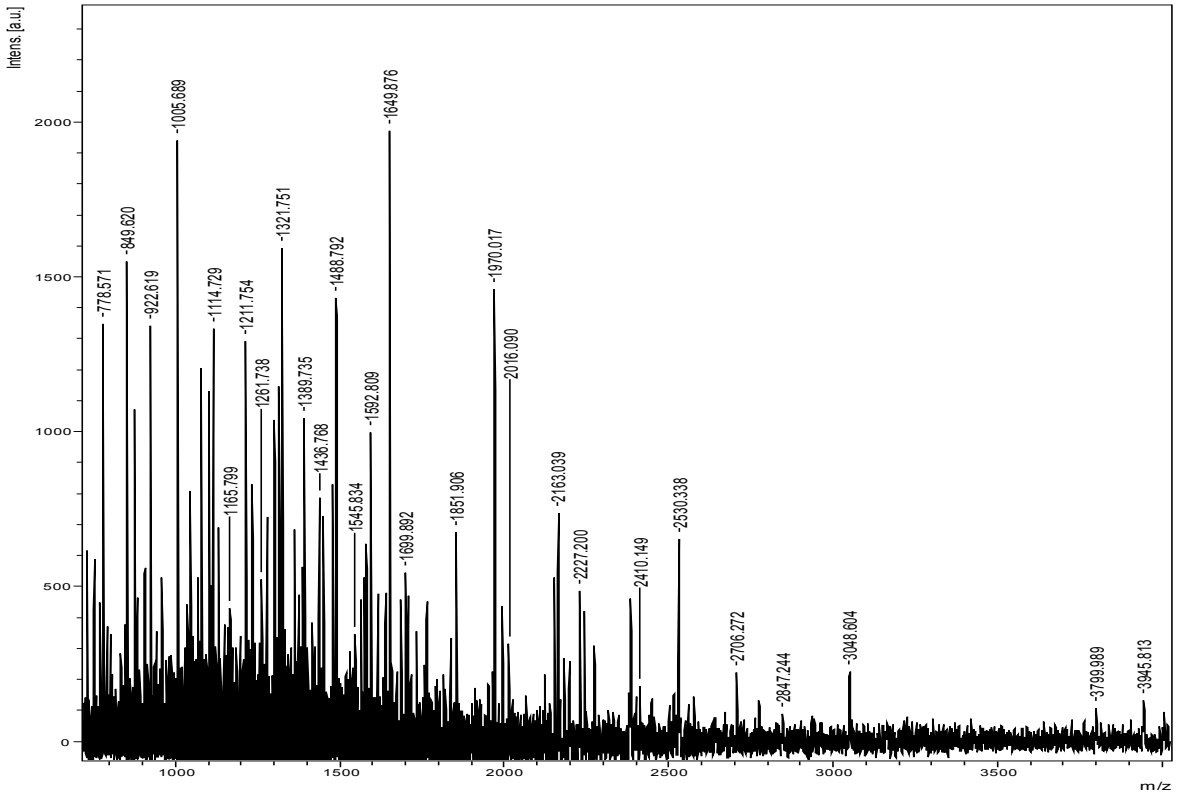


Figure 4. MALDI-TOF mass spectrum of 19kDa protein band

Table 1. List of compounds identified in preputial gland of bandicoot rat

S.No.	Retention time	Compounds
1	4.633	1 Chloro 1-Buten-3-yne
2	4.717	1,2,3 Butatriene
3	5.667	1,4 Dichlorobenzene
4	7.783	1,1”-Tertricylohexane
5	9.150	Cyclopropane
6	9.433	3-Aminoheptane
7	9.683	3-Heptanone
8	11.117	2 Methyl octane
9	11.817	Diaziridine
10	12.333	3,4,Dihydropyrane
11	12.483	2,Decyloxyethanol
12	12.800	Pyrrolidine
13	13.300	Octanoic acid
14	13.533	Difluoromethyldifluoromethanesulphonate
15	13.767	Octyne
16	14.800	Hexanenitrile
17	14.933	2-Undecanone
18	15.933	Nonanoic acid
19	16.333	6-Heptan-3-one
20	16.550	Octadecanal
21	17.400	1 Hexadecanol
22	17.767	2-propenyloxy ethanol
23	17.917	2,6,10,11,11-Pentamethyl-2,6,9-dodecatriene
24	18.917	Hexadecanoic acid
25	19.183	4-Butoxy-1 butanol
26	19.500	1-dodecanol
27	19.567	Hexadecanal
28	20.517	Stenol
29	21.400	2-None-1-ol
30	22.050	Silane
31	22.117	9-Octadecenoic acid
32	22.250	1,10-Decanediol
33	22.533	2-Propyldecan-1-ol
34	22.867	8-Methyl 2- decene
35	23.450	1,6-Heptadiene
36	23.733	Limoneneoxide
37	24.300	3-Methyl2-buten-1-ol
38	24.400	2-Propenoic acid
39	25.733	Farnesol
40	26.733	2-Methyltetradecane
41	28.000	1,3-Benzodioxol-2-one
42	28.200	Aziridine
43	29.350	4-Penten-1-ol
44	29.583	Cyclopentanemethanol
45	29.767	1,4-Pentadiene
46	32.017	2-Nitro-1-octanol
47	42.667	1-Chloro-3-methylbutane

Table 2. Sequence coverage and peptide masses of gamma-aminobutyric acid type B receptor subunit 1[Sequence coverage of 13% and 9 matching peptides of gamma-aminobutyric acid type B receptor subunit 1 using MALDI-MS data]

Matched peptides shown in Bold Red

1 MLLLLLVPLF LRPLGAGGAQ TPNATSEGCQ IIHPPWEGGI RYRGLTRDQV
51 KAINFLPVDY EIEYVCRGER EVVGPKVRKC LANGSWTDMD TPSRCVRICS
101 KSYLTLENGK VFLTGGDLPA LDGARVEFRC DPDFHLVGSS RSVCSQGQWS
151 TPKPHCQVNR TPHSERRAVY IGALFPMSGG WPGGQACQPA VEMALEDVNS
201 RRDILPDYEL KLIHHDSKCD PGQATKYLYE LLYNDPIKII LMPGCSSVST
251 LVAEAARMWN LIVLSYGSSS PALSNRQRFP TFFRTHPSAT LHNPTRVKLF
301 EKWGWKKIAT IQQTTEVFTS TLDDLEERVK EAGIEITFRQ SFFSDPAVPV
351 KNLKRQDARI IVGLFYETEA RKVFCEVYKE RLFGKKYVWF LIGWYADNWF
401 KTYDPSINCT VEEMTEAVEG HITTEIVMLN PANTRSISNM TSQEFVEKLT
451 KRLKRHPEET GGFQEAPLAY DAIWALALAL NKTSGGGGRS GVRLEDFNYN
501 NQTITDQIYR AMNSSSFEGV SGHVVFDASG SRMAWTLIEQ LQGGSYKKIG
551 YYDSTKDDLS WSKTDKWIGG SPPADQTLVI KTFRFLSQKL FISVSVLSSL
601 GIVLAVVCLS FNIYNSHVRY IQNSQPNLNN LTAVGCSLAL AAVFPLGLDG
651 YHIGRSQFPF VCQARLWLLG LGFSLGYGSM FTKIWWVHTV FTKKEEKKEW
701 RKTLEPWKLY ATVGLLVGMD VLTLAIWQIV DPLHRTIETF AKEEPKEDID
751 VSILPQLEHC SSKKMNTWLG IFYGYKGLLL LLGIFLAYET KSVSTEKIND
801 HRAVGMAIYN VAVLCLITAP VTMILSSQQD AAFAFASLAI VFSSYITLVV
851 LFVPKMRRLI TRGEWQSETQ DTMKTGSSTN NNEEEKSRLL EKENRELEKI
901 IAEKEERVSE LRHQLQSRQQ LRSRRHPPTP PDPSGGLPRG PSEPPDRLSC
951 DGSRVHLLYK

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
95 - 101	922.6190	921.6117	921.4524	0.1593	1	R.CVRICSK.S 2 (C)
130 - 141	1389.7350	1388.7277	1388.6143	0.1134	0	R.CDPDFHLVGSSR.S (C)
202 - 211	1261.7380	1260.7307	1260.6714	0.0594	1	R.RDILPDYELK.L
212 - 218	849.6200	848.6127	848.4504	0.1623	0	K.LIHHDSK.C
212 - 226	1649.8760	1648.8687	1648.7991	0.0696	1	K.LIHHDSKCDPGQATK.Y
308 - 328	2410.1490	2409.1417	2409.1911	-0.0494	0	K.IATIQQTTEVFTSTLDDLEER.V
340 - 351	1321.7510	1320.7437	1320.6714	0.0723	0	R.QSFFSDPAVPVK.N
620 - 655	3799.9890	3798.9817	3798.9515	0.0302	0	R.YIQNSQPNLNNLTAVGCSLA-LAAVFPLGLDGYHIGR.S
747 - 763	1970.0170	1969.0097	1968.9462	0.0635	0	K.EDIDVSILPQLEHCSSK.K (C)

4. Discussion

In the present study, volatile and protein profiles of preputial gland of bandicoot rat were studied. In this rat, the well-developed pyrifiorm preputial glands were observed. Earlier reports showed that the weight of preputial gland of male laboratory rat was below one gram and it is testosterone dependent [30]. The well-developed preputial gland in bandicoot rat indicates that it may need higher secretion of pheromonal substances from preputial gland for the maintenance of reproductive and dominance status, to attractopposite sex in the open field. It is consistent with the structure of pyriform (pear-shaped) appearance of preputial gland in rats [27]. The pyriform structure of preputial gland may be convenient to store more sebum containing volatiles and proteins.

It is well known that preputial glands are modified sebaceous glands and having the features of broad differentiating cell layer and the continuous maturation of small to large lipid droplets therein [4]. After maturation the acinar cells rupture and release the substances [25]. The sebum released through terminal urethra may get mixed with urine by which the urine acquires preputial originated pheromones and also retains the same on the genitalia.

The identified major compound, farnesol in the preputial gland has already been reported as a bee's sex pheromone in the spider orchid [33]. Similarly, the compounds E-Eα farnesene and E-β farnesene are analogues to farnesol, reported in mice preputial gland as sex attractant towards female and evoke inter-male aggression [26]. In the preputial gland of house rat (*Rattus rattus*), the same compound is reported as bound form volatile along with purified alpha 2u globulin [32]. Based on the present finding and previous reports it is strongly believed that farnesol could be a common volatile produced by preputial gland of rats and mice, therefore, this compound can be considered as male specific preputial originated compound.

The protein profiles of preputial gland of bandicoot rat revealed the appearance of very thin band of 19 kDa protein. It is contrast with high intensity of 19 kDa molecular mass protein reported in laboratory rat [30] and house rat preputial gland [18, 32]. It is interesting to note that the morphology

and histoarchitecture of preputial gland of bandicoot rat is consistent with other rats (laboratory and house rats) but the total number of volatiles and protein profile are found to be different while comparing the other reports available in rodent species.

In the present study, the low molecular protein 19 kDa was identified as gamma-aminobutyric acid type B receptor subunit. This protein belongs to G-protein coupled receptor (GPCR) subfamily. Further, the expected α_{2u}-globulin, which has been previously reported in preputial gland of laboratory rat (*Rattus norvegicus*) and house rat (*Rattus rattus*), was not identified in the preputial gland of bandicoot rat. Therefore, it is a notable report in rodent biology. The results of present study suggest that the presence of α_{2u}-globulin in the preputial and clitoral gland of rats may be used as a marker to distinguish from other rodent genus *Bandicot* sp. However, additional work on proteome analysis among rodent species would give more interesting information. Further analysis of major compound, farnesol is required for developing a bio-trap for pest management programme.

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