

Cloning and sequencing of α -2u globulin of rat preputial gland to assess its longevity in the context of developing an effective rodent trap

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α -2u globulin, a pheromone binding protein found majorly in the preputial gland, was reported to involve in chemo-communication of rats. The biosynthesis of this pheromone binding protein is under complex multihormonal control and its regulation takes place at transcription level. Assessing and increasing the longevity of this protein may retain the volatility of pheromone. So far nearly 20 isoforms of α -2u globulin in rat have been studied. The present study was aimed to extend the longevity of pheromone compound by cloning and sequencing of the mRNA which codes for α -2u globulin in the preputial gland of *Rattus norvegicus*. Unexpectedly, this study resulted in a new isoform, which is similar in function with α -2u globulin protein with some different exons removed. Further analysis with this isoform may pave a way for rodent pest management.

Keywords: Isoform, Pheromone binding protein, Preputial gland, Rat trap

Pheromones are volatile chemical secretions which act as molecular signals and used mainly for communication with other conspecific members by changing their behavior or developmental process in animals^{1,2}. Among different mammalian species, the role of chemical communication has been best documented in rodents³. Especially in rats, the clitoral⁴, preputial⁵ and cheek glands⁶ are reported to be important scent glands involved in reproductive and social behavior. As preputial glands produce a variety of pheromone molecules they are regarded to be predominant source for pheromone communication. Pheromone Binding Proteins (PBP) binds with the odorant in the blood stream and transports them into the environment which are also considered to be very important in prolonging the period of bioavailability and modulating pheromone activity by affecting sensory organ responses⁷⁻⁹.

α -2u globulin which comes under the class of lipocalin, low molecular weight pheromone binding protein was identified in the urine and preputial glands of *Rattus norvegicus*^{10,11}. However, the highest concentration of α -2u globulin was

found in the preputial gland, a holocrine secretory organ with pheromone function. Numerous studies have explained that the synthesis of α -2uglobulin in the liver is regulated by a variety of hormones, including androgens, estrogens, glucocorticoids, growth hormone, and thyroxine¹²⁻¹⁴, and regulation appears to occur primarily at the level of transcription¹⁵. The investigation of the presence of α -2u globulin in preputial gland of *Rattus rattus* bound with farnesol 1 and 2 strongly supports for the involvement of chemo-communication in rat¹⁶.

When the α -2u globulin undergoes random mutagenesis, either the replacement of hydrophilic amino acids with the hydrophobic amino acids may occur which in turn increase the longevity and retains the volatility of the pheromone. At the same time this technique may likely to give the unexpected results also¹⁷. Hence, cloning and mutation of α -2u globulin gene result in extending the longevity of pheromone which directly increases its high binding affinity towards the pheromone compound.

Though different approaches are under process in regard to the rodent trap development^{18,19}, this particular study was planned for the same cause but based on pheromones and pheromone-carrier protein as source. Therefore, the present

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study was sketched with an idea to obtain the positive clone of α -2u globulin gene and to perform mutational analysis in order to make the protein stable for longer time as this helps in an efficient rodent-pest trap development.

Materials and Methods

Animal dissection

Male rats (*Rattus norvegicus*) of 10-12 weeks old were maintained in the animal house at $25 \pm 20^\circ\text{C}$ temperature condition with circulating aeration system. The rats were fed with the commercially available rat feed *ad libitum*. Preputial glands were collected immediately after sacrificing the rats by cervical dislocation.

RNA isolation

The total RNA was isolated from the gland using TRI reagent (Sigma Aldrich) by following the reagent protocol. The purified RNA was suspended in DEPC treated water and stored at -80°C .

Synthesis and amplification of cDNA

The first strand cDNA was synthesized from the total RNA isolated using Verso cDNA synthesis kit. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed in Gene AmpR PCR 9700 (Applied Biosystems). Cycling program for RT-PCR was: cDNA synthesis step for 30 min at 42°C and inactivation step for 2 min at 95°C . The first strand cDNA obtained was amplified by using Biolabs PCR kit and α -2u globulin gene specific primers which were designed using Primer3. The following are the two sets of gene specific primers, first was ORF and second was Partial.

ORF clone

Forward primer: 5'-ATG AAG CTG TTG CTG CTG-3'

Reverse primer: 5'-TCA ACC TTG GGC CTG GAG-3'

Partial clone

Forward primer: 5'-CGA GAG AGG GAA CGT CGA-3'

Reverse primer: 5'-ATC AGC TGG AAG GTT TCC-3'

Agarose electrophoresis was carried out using 1.5% agarose concentration. The gel extraction was carried out using Macherey-Nagel Nucleospin gel kit.

DNA ligation

The gel extracted PCR products were ligated by using TA cloning vector, pTZ57R/T (Fig. 1) plasmid vector obtained from Fermentas. Linearized pTZ57R vector with 3'-ddT overhangs was generally utilized for TA cloning of PCR products with blue/white screening.

Selection of transformed colonies

The gene of interest was transformed into DH5 - α competent cells (*Escherichia coli*) which was prepared by CaCl_2 method. The transformed cells were cultured on LB-ampicillin plate by adding 30 μL X-gal (5 bromo-4-chloro-3-indolyl- β -D-thiogalactoside) to enable blue-white screening. After overnight incubation at 37°C , recombinant colonies appear white and non-recombinant colonies appear blue in color.

Colony PCR

Colony PCR was performed with the obtained white colonies (transformants) to check for amplification of gene of interest and the blue colonies acted as negative control. M13 forward and reverse primers were used. The steps implemented in colony PCR were tabulated in (Table 1).

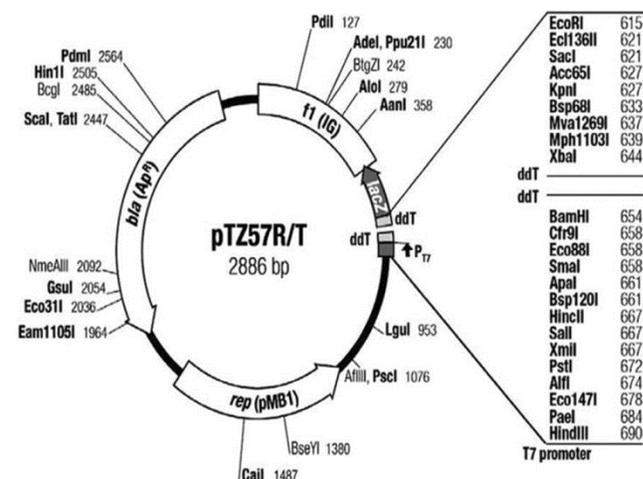


Fig. 1 — pTZ57R/T vector map from Fermentas

Table 1 — Colony PCR cycling program

Steps	Temperature ($^\circ\text{C}$)	Duration (in mins)	No. of Cycles
Cell lysis	94	10	1
Denaturation	94	30	35
Annealing	50	45	35
Extension	72	1	35
Final extension	72	1	1

Plasmid Isolation

Plasmids were prepared from positive colonies using Qiagen Plasmid Purification kit. The concentration of plasmid DNA was 174.3 ng/ μ L and the A260/280 ratio was 1.92. The plasmids were sequenced using ABI prism.

Blast analysis

From the sequenced plasmids, only the insert sequence was retrieved using Laser gene software. After retrieving, BLAST analysis was performed for the insert sequence in order to find homology with other isoforms of α -2u globulin.

Results

Isolated RNA

The purity of RNA was assessed by checking the absorbance (A260/280) ratio using NanoDrop

spectrophotometer which was 1.93 (1.8 to 2.0) indicating that the RNA was pure. Further the presence of three bands in the 1.5% agarose gel denotes the size of isolated RNA with 28S, 18S and 5.8S bands (Fig. 2A).

PCR

The gel image (Fig. 2B) showed that the Partial (471 bp) and Full length ORF (542 bp) fragments of α -2u globulin gene (543 bp) were amplified successfully in RT-PCR. Partial amplicon in lane number 5 added further confirmation of the sequence of interest.

DNA Ligation

The size of pET 23b expression vector is ~ 3666 bp and the size of interest sequence is approximately ~ 543 bp. Hence, it is to be noted that vector with

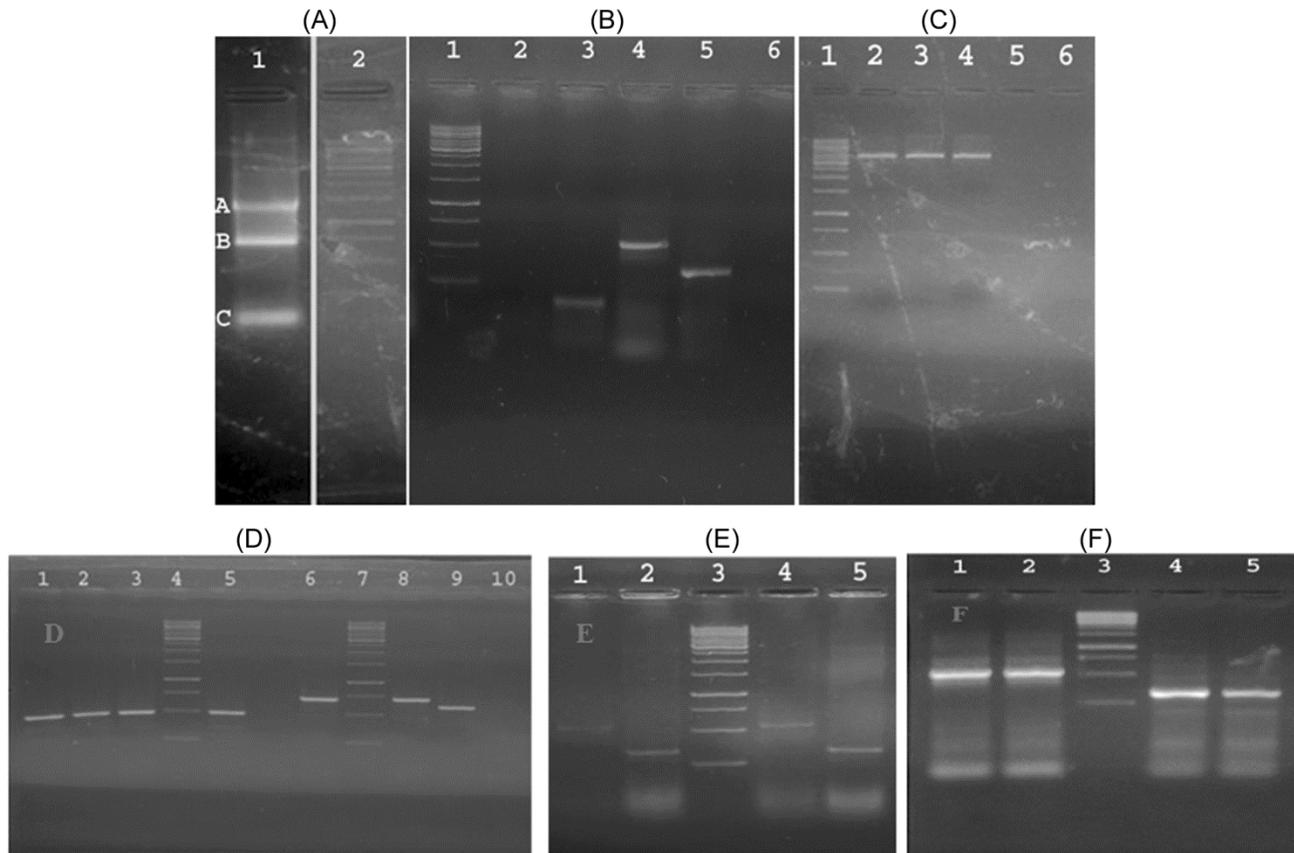


Fig. 2 — (A) 1.5% agarose gel showing the size of total RNA. (Lane 1 – Whole RNA isolated from rat preputial gland with (1) 28S; (2) 18S; and (3) 5.8S, Lane 2 – 1 Kb ladder); (B) Gel picture displaying amplicons generated using α -2u globulin gene. (Lane 1 – 1Kb DNA ladder; Lane 2 – β -actin- app.42 kDa (Positive control); Lane 3 – GAPDH-app. 36 kDa; Lane 4 – ORF amplicon; and Lane 5 – Partial amplicon); (C) Gel picture showing the vector carrying α -2u globulin gene. (Lane 1 – Marker (1Kb DNA Ladder); Lane 2, 3 and 4 – pET vector carrying the insert sequence with ~4209 bp); (D) 1.5% agarose gel showing amplicons of colony PCR (Lanes 1, 2, 3 and 5 - Partial amplicons; Lanes 6 and 8 - ORF amplicons, Lane 4 and 7 – 1 Kb); (E) 1.5% agarose gel showing selected amplicons of colony PCR with the specific primer. (Lanes 1 and 4 – ORF amplicon; Lane 2 and 5 – Partial amplicon; Lane 3 – 1 Kb DNA ladder); and (F) 1.5% agarose gel showing full length and partial amplicons of preputial gland (Lanes 1 and 2 – Full length amplicon of preputial gland; Lane 3 – 1 Kb DNA ladder; Lanes 4 and 5 – Partial amplicon of preputial gland)

insert will be around 4209 bp as displayed in (Fig. 2C) indicating the successful ligation.

Colony PCR

Following ligation, the transformed cells were confirmed by blue-white screening and also by the bands observed in lanes 6 and 8 of (Fig. 2D) with colony PCR. Further, the amplicons from colony PCR were selected with α -2u globulin gene specific primers designed earlier which was witnessed in (Fig. 2E).

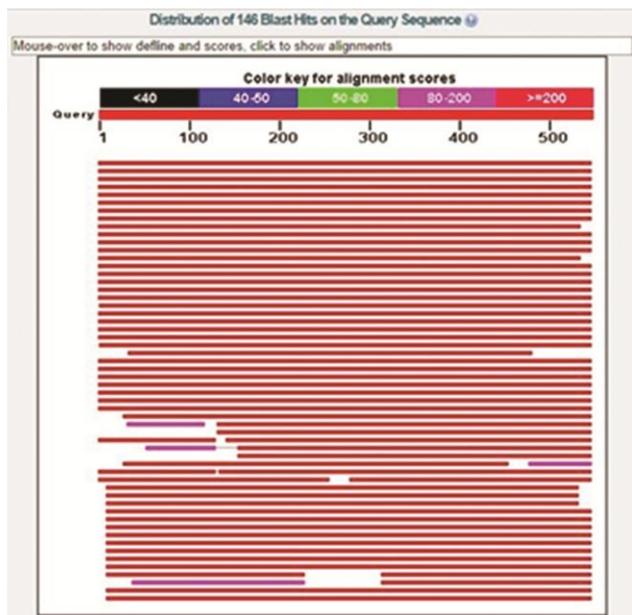


Fig. 3 — Picture displaying the BLAST analysis performed to check homology with other isoforms of α -2u globulin

Confirmation of cloned gene

Isolation and amplification of full length and partial amplicon of preputial gland (Fig. 2F) showed similar base pair with that of cloned gene sequence. It was finally confirmed that amplified fragment was from genomic DNA but not from total RNA or first stranded cDNA.

Blast analysis

Amplicons obtained after colony PCR were subjected to sequencing followed by the BLAST analysis (Fig. 3). The image (Fig 4) denotes the highest percentage of similarity *i.e.* 99% with that of other isoforms of α -2u globulin of *Rattus norvegicus*.

Discussion

The new isoform of α -2u globulin of rat was identified through blast analysis. Cloning and sequencing of α -2u globulin protein²⁰ paved a way to further analyze the major pheromone binding protein. Cloning of mRNA coding for the α -2u globulin protein was performed in which it undergoes random mutagenesis. The clones obtained were subjected to sequence analysis, where it was concluded that the transformed gene was a new isoform of α -2u globulin. Till date, nearly 20 different isoforms of α -2u globulin has been reported²¹⁻²³. Such isoform varies in their amino acid sequence with other isoforms due to variation in DNA. Multiple major urinary protein isoforms coded by different gene in mouse were demonstrated²⁴. It has also been reported that α -2u globulin with 20 isoforms were identified from

Description	Max score	Total score	Query cover	E value	Ident	Accession
<input type="checkbox"/> <i>Rattus norvegicus</i> alpha-2u globulin PGCL1 mRNA (cDNA clone MGC:108577 IMAGE:7322607), complete cds	989	989	99%	0.0	99%	BC088109.1
<input type="checkbox"/> <i>Rattus norvegicus</i> alpha-2u globulin PGCL1 mRNA (cDNA clone MGC:124935 IMAGE:7367233), complete cds	989	989	99%	0.0	99%	BC105816.1
<input type="checkbox"/> <i>Rattus norvegicus</i> alpha-2u globulin PGCL1 (LOC259246), mRNA	989	989	99%	0.0	99%	NM_147214.2
<input type="checkbox"/> <i>Rattus norvegicus</i> alpha-2u globulin mRNA, complete cds	989	989	99%	0.0	99%	U31287.1
<input type="checkbox"/> <i>Rattus norvegicus</i> mRNA for alpha-2u globulin, complete cds, clone PGCL1	989	989	99%	0.0	99%	AB039822.1
<input type="checkbox"/> Rat alpha-2u-globulin (L type) mRNA, complete cds, clone pA8	989	989	99%	0.0	99%	M26835.1
<input type="checkbox"/> Rat alpha-2u-globulin (S type) mRNA, complete cds, clone pA120	989	989	99%	0.0	99%	M26837.1
<input type="checkbox"/> <i>Rattus norvegicus</i> similar to alpha-2u-globulin (RGD1566134), mRNA	961	961	99%	0.0	98%	NM_203512.2
<input type="checkbox"/> Rat hepatic alpha-2u globulin mRNA, 5' end	961	961	97%	0.0	99%	J02737.1
<input type="checkbox"/> <i>Rattus norvegicus</i> alpha-2u globulin PGCL2 mRNA (cDNA clone MGC:108578 IMAGE:7372252), complete cds	955	955	99%	0.0	98%	BC088943.1
<input type="checkbox"/> Rat alpha-2u-globulin (S type) mRNA, complete cds, clone pA108	955	955	99%	0.0	98%	M26838.1
<input type="checkbox"/> <i>Rattus norvegicus</i> alpha-2u-globulin (L type) (LOC298116), mRNA	950	950	99%	0.0	98%	NM_001003409.1
<input type="checkbox"/> Rat alpha-2-u globulin mRNA	922	922	97%	0.0	98%	M27434.1
<input type="checkbox"/> PREDICTED: <i>Rattus norvegicus</i> similar to alpha-2u globulin PGCL2 (LOC500473), transcript variant X2, mRNA	905	905	99%	0.0	97%	XM_006238221.2
<input type="checkbox"/> PREDICTED: <i>Rattus norvegicus</i> similar to alpha2u globulin (LOC688489), transcript variant X1, mRNA	905	905	99%	0.0	97%	XM_006225336.1
<input type="checkbox"/> <i>Rattus norvegicus</i> mRNA for alpha-2u globulin, complete cds, clone PGCL7	856	856	99%	0.0	95%	AB039828.1
<input type="checkbox"/> <i>Rattus norvegicus</i> alpha-2u globulin PGCL3 (LOC259244), mRNA	856	856	99%	0.0	95%	NM_147212.1
<input type="checkbox"/> PREDICTED: <i>Rattus norvegicus</i> major urinary protein-like (LOC102548083), transcript variant X2, mRNA	845	845	99%	0.0	95%	XM_008763747.1
<input type="checkbox"/> <i>Rattus norvegicus</i> alpha2u globulin (LOC298111), mRNA	843	843	99%	0.0	95%	NM_001024248.1
<input type="checkbox"/> PREDICTED: <i>Rattus norvegicus</i> alpha2u globulin (LOC298111), transcript variant X1, mRNA	839	839	99%	0.0	94%	XM_008763616.1

Fig. 4 — Picture displaying the percentage of similarity in BLAST analysis

various glands of rat through Iso-electric focusing method²⁵. It is to be noted in previous studies that most of the isoforms were identified through iso-electric focusing method; while performing cloning we obtained a new isoform. Pheromone carrier protein of lipocalin family in goat was recently analyzed in goat²⁶ and such proteomic analysis is being carried out for prolonging the rate of release of volatile compounds. The identification of the new isoform of α -2u globulin in our study is consistent with earlier report and this might be useful for extending the activity of pheromone in the preputial gland. By extending the longevity of pheromone, it is possible to manage the wild rat population through integrated pest management. Further, the new isoform can be structurally aligned for identification of evolutionarily related protein and sequentially distant protein which might be used for prolonging the pheromone activity.

Conclusion

In the present study, a new isoform was identified in rat preputial gland and in future, the identified isoform will be analyzed for its stability in eukaryotic system followed by behavioral study. Also, it is strongly believed that mutation in gene increases the longevity and binding affinity towards pheromone compound. Regarding that our identification will surely assist in achieving the prolongation of pheromone activity. Further, it aids in ecological friendly pheromone based efficient biological trap development for rodent pest management.

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