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IDENTIFICATION OF α2U-GLOBULIN IN MARE'S URINE BY MALDI-TOF ANALYSIS

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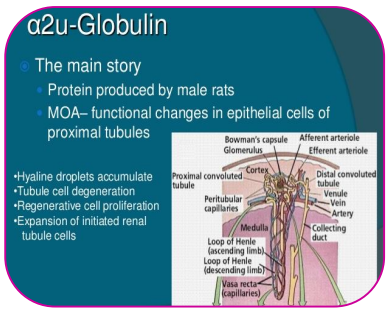
ABSTRACT



Theα2u globulin is a major urinary protein (MUP), belongs to lipocalins super family. It is synthesized in the liver and excreted through urine. It acts as a carrier protein. It has low molecular weight so it is easily bind with pheromonal compounds. The physiological role of several lipocalins has been associated with the transport of hydrophobic compounds in aqueous biological fluids. These kinds of proteins give a protection to the pheromonal compounds when they are released from the external environment. In the present investigation made identification of α2u globulin in mare's urine by MALDI-TOF analysis. Mass spectrum of 18.5 kDa protein was obtained by MALDI-TOF and the mono isotopic number of spectra were scored and analyzed with MASCOT search. Results of MASCOT search showed the presence of α2u-globulin in the first hit in the search list and the score was higher than the significant level (significant level was 51) It indicates that, the sequence search and alignment studies experimentally proved that the acquired protein sequence of mare's urine perfectly matched with α2u-globulin of mammalian origin. This concept significantly proved that the identified protein was small peptide with molecular weight of 18.5 kDa.

KEYWORDS: *Alpha2u globulin, pheromonal compounds, mammalian origin.*

INTRODUCTION

Major urinary proteins (MUPs) are lipocalins, first described in mouse, which are synthesized in the liver and excreted in the urine (Finlayson et al., 1965). The physiological role of these polypeptides remained unknown until the discovery of odorant binding proteins (OBPs) in the nasal tissue of several vertebrates provided evidence of a strong similarity between members of V1R and V2R families (Pelosi et al., 1982; Cavaggioni et al., 1987). In some cases, the V1R and V2R genes have been found to be expressed both in the nose and liver (Utsumi et al., 1999). Sequence similarity studies revealed a high similarity between mammalian pheromone carrying proteins (PCPs) and other lipocalins present in the nasal mucosa called odorant binding proteins (OBPs). It has been proposed that PCPs and OBPs play complementary roles in pheromonal communication, the first in their delivery and the second in the perception of pheromones (Pelosi, 1994; 1996; Loebel et al., 2000). The major function of pheromone- protein complex dissociated is to facilitate the slow release of odorants into the air (Hurst et al., 1998; Cavaggioni and Mucignat-Caretta, 2000). Thus, the carrier proteins play a crucial role in protecting the pheromones from being rapidly lost by evaporation or degradation and thereby extending the 'self –life' of the scent mark. In addition, these proteins are unusually resistant to drying, heating and are not likely to be quickly denatured when released into the environment.Similar to MUP, the homologous protein, i.e. α2u-globulin was first identified in rat urine, and also reported that synthesis of this proteins started only after puberty and continued until senescence. Further, very limited reports are available about role of this protein in chemical communication (Cavaggoni and MucignatCaretta, 2000). The α2u-globulin is also reported in the rat preputial gland. The literatures relating to

this suggest that presence of α2u-globulin mRNA and localization of this protein in the preputial gland of adult Sprague Dowley rats. But there is no report on functional significance of this protein. Hence, the present study was undertaken in the molecular characterization and functional significance of α2u-globulin. MALDI-TOF mass spectrometers are now commonplace and their relative ease of use means that most non-specialist labs can readily access the technology for the rapid and sensitive analysis of Biomolecules. One of the main uses of MALDI-TOF-MS is in the identification of proteins, by peptide mass fingerprinting (PMF)(Webster J, Oxley D. 2012).Protein electrophoresis is an easy,inexpensive test routinely used in clinical laboratories for screening protein abnormalities in various biological fluids.(Ramprasad et al., 2015). Electrophoresis separates proteins based on their physical properties, and the subsets of these proteins are used in interpreting the results (Theodore et al., 2005). Characteristic mass fragments of standards were investigated and compared by means of matrix assisted laser desorption time of flight mass spectrophotometry (Chen et al., 2018).

OBJECTIVES

The Objective of the present study is Identification of α2U-Globulin in Mare's urine by using the MALDI-TOF analysis.

MATERIALS AND METHODS

Animals

Urine samples were collected from matured female horses at Singanallur horse stable near vasantha mills in Coimbatore. Urine samples were collected at a week interval for 3 months and kept at -20°C until analysis. The freshly collected sample was analysed by GC-MS.

SDS-PAGE Analysis

13% separating gel and 5% stacking gel were prepared for SDS PAGE was carried out Briefly, 50µg of protein and 4µl of molecular weight marker (PMW, Genei) with equal volume of sample buffer were loaded on the gel. Initially an electric current of 50V was applied till the dye enters the separating gel. Subsequently the electric current was increased to 100V till the tracking dye in the presence of tank buffer. After electrophoresis, the gel was removed from the glass plate and the resolved peptides were revealed by Coomassie Brilliant Blue R-250 staining solution

MALDI-TOF MASS SPECTROMETRY

Principle

Matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) permits the analysis of high molecular weight compounds with high sensitivity. MALDI is a method that allows for the ionization and transfer of a sample from a condensed phase to the gas phase. MALDI uses a solid matrix, and the ionizing beam is laser light. Ion formation in MALDI is accomplished by directing the pulsed laser beam onto a sample suspended or dissolved in a matrix.

MALDI-TOF analysis

Peptides (1µl) were mixed with matrix (1 µl) (a-cyano-4-hydroxycinnamic acid saturated Solution in a 1:1:1:1 by volume of ethanol, acetonitrile, trifluroacetic acid (0.4 %) and distilled deionized water), and resultant 2 µl was spotted on to the MALDI target. Then the samples were analyzed in reflectron mode with delay time of 90 ns and 25 Kv accelerating voltage in the positive ion mode. To improve the signal to noise ratio summation of 300 laser shots were taken for each spectrum. External calibration was done using peptide I calibration standard with masses ranging from 1046-3147 Da. Mass spectra were acquired using ULTRAFLEX-TOF mass spectrometer (Bruker Daltonics, Germany), equipped with a 337 nm pulsed nitrogen laser.

PROTEIN IDENTIFICATION BY MASCOT RESEARCH

Spectra were processed using FLEX analysis software. Monoisotopic peptide masses were assigned and used in the data base search engine (Matrix Science Limited, http://www.matrixscience.com) facilitated

the identification of best match. Scores <56 were considered to be significant ($p<0.05$) in the mascot search. Proteins with scores less than the significant level were reported as unidentified.

RESULTS AND DISCUSSION

The electro photogram of mare's urine showed four different protein fractions with molecular weight such as 65, 50 and 18.5kDa. Among these fractions, the 65 and 18.5kDa appear predominately (**Figure 2**). Therefore the 18.5kDa was excised and subjected to trypsin digestion. Mass spectrum of 18.5 kDa protein was obtained by MALDI-TOF (**Figure 2**) and the mono isotopic number of spectra were scored and analyzed with MASCOT search. Results of MASCOT search showed the presence of α2u-globulin in the first hit in the search list and the score was higher than the significant level (significant level was 51) (**Figure 3**). In addition, the protein showed matching of maximum 25 peptides with observed masses such as 47 57(1176.1340) m/z, 51-72 (2274.0210) m/z, 72-82 (997.1490) m/z, 157176 (2207.6880) m/z, 171176 (747.4190) m/z, 182189 (868.9970) m/z, 182191 (1112.1960) m/z, 190203 (491.5210) m/z, 190-203 (1494.3310) m/z, 223-230 (881.8840) m/z, 231-236 (731.4200) m/z, 251257 (833.9130) m/z, 256-263 (821.9700) m/z, 264272 (1026.1510) m/z, 264-274 (1280.4710) m/z,277281 (661.6970) m/z, 323-347 (2585.2790) m/z, 348358 (1275.4575) m/z, 403427(2668.2500) m/z, 467481 (1530.3500) m/z, 482-501 (2163.8380) m/z, 482-501 (2164.6260) m/z, 529-535 (802.9230) m/z, 529-535 (805.8770) m/z **(Table 1).**Furthermore, the sequence search and alignment studies experimentally proved that the acquired protein sequence of mare's urine perfectly matched with α2u-globulin of mammalian origin. In addition to that the occurrence of very few number of cystine molecule was noticed in the present investigation. Lower the cystine molecule lesser the protein structural complexity. This concept significantly proved that the identified protein was small peptide with molecular weight of 18.5 kDa.The MALDI-TOF results revealed the major low molecular mass protein of 18.5 kDa as α2u-globulin.

This α2u-globulin is reported to belong to lipocalin family like that of pheromone carrying proteins with the molecular weight of 17-20 kDa in the urine of mouse (Cavaggioni and Mucignat-Caretta, 2000) rat , saliva of pig, vaginal fluid of hamster and preputial gland of house rat . Interestingly, odorant binding proteins (OBPs) of vertebrates have the molecular weight of around 20 kDa (Pelosi, 1994).The α2u-globulin is the major urinary protein excreted by adult male rats, belongs to lipocalin family, supposed to act as a pheromone carrier. The electrophoretic mobility is similar to that of serum α2-globulin; it was named as α2u-globulin with the subscript "u" denoting its origin in urine. Nevertheless, the α2u-globulin is synthesized in the liver, secreted into the blood and excreted in the urine (Roy et al., 1976). Itrepresents 30% of the total urinary protein and is produced in adult male rats under androgen control (Roy *et al*., 1974). However, a very similar protein in urine from female rats has been reported to occur but at a much lower concentration; approximately 120 times less (Vandoren *etal*., 1983). It is well known that the α2u-globulin is homologous to MUPs of mouse. In this regard, it has been suggested that MUPs act as a carrier for urinary pheromones (Cavaggioni *etal*., 1990). The organic extracts from MUPs are having four female specific pheromonal compounds such as Cyclohexane, 1-Propene, 2-Methyl-3-Propoxy, Hydroperoxide and 1, 2, 6- Hexanetriol. Together with their bound urinary ligands, urinary proteins may elicit various effects in rodents, like attraction of males, oestrus synchronization etc. we found oestrus dependent volatile compounds in the mouse urine (Achiraman, 2010).Together with their bound urinary ligands, urinary proteins may elicit various effects in rodents, like attraction of males, oestrus synchronization etc. The report on identification of α2u-globulin in female oestrus mouse urine is very scanty. Circulating alpha 2u-globulin is expressed by the liver. It is removed from the plasma by the kidney and excreted. Rodent studies have employed alpha 2u-globulin nephropathy as an experimental model for the development of kidney tumors (Edward A. Lock Gordon C. Hard 2010). These substances appear to act by binding to a protein present in large quantities in male rat urine called α_{2u}-globulin (Cohen, 2018).Characteristic mass fragments of standards were investigated and compared by means of matrix assisted laser desorption time of flight mass spectrophotometry (Chen et al., 2018).

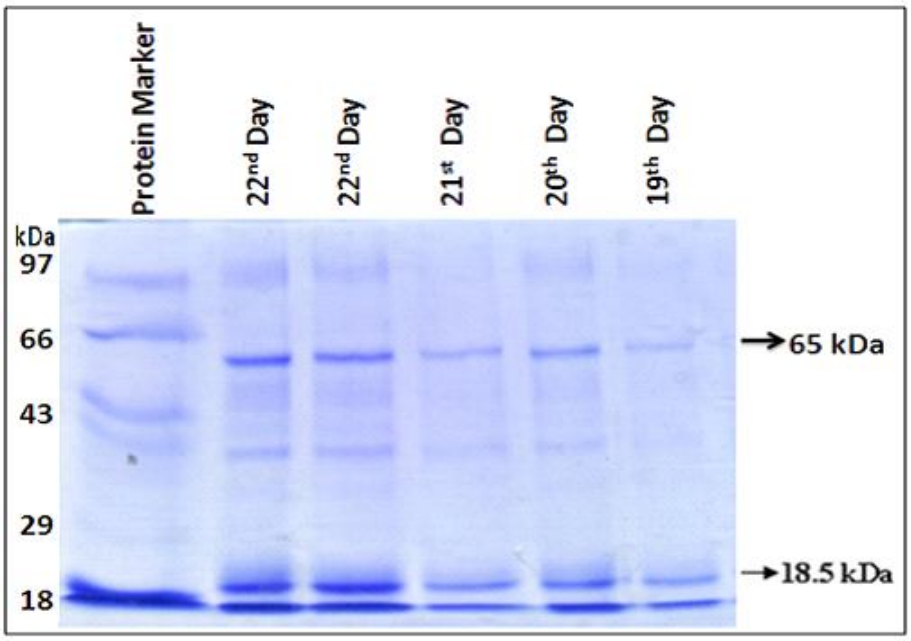


Figure 1: Oestrus mare's urinary protein analyzed by 13% SDS-PAGE. The concentration of MUP was found to be higher at heat period when compare to rest of the reproductive phase. In addition to the 65kDa was also reported to be reproductive stage dependent.

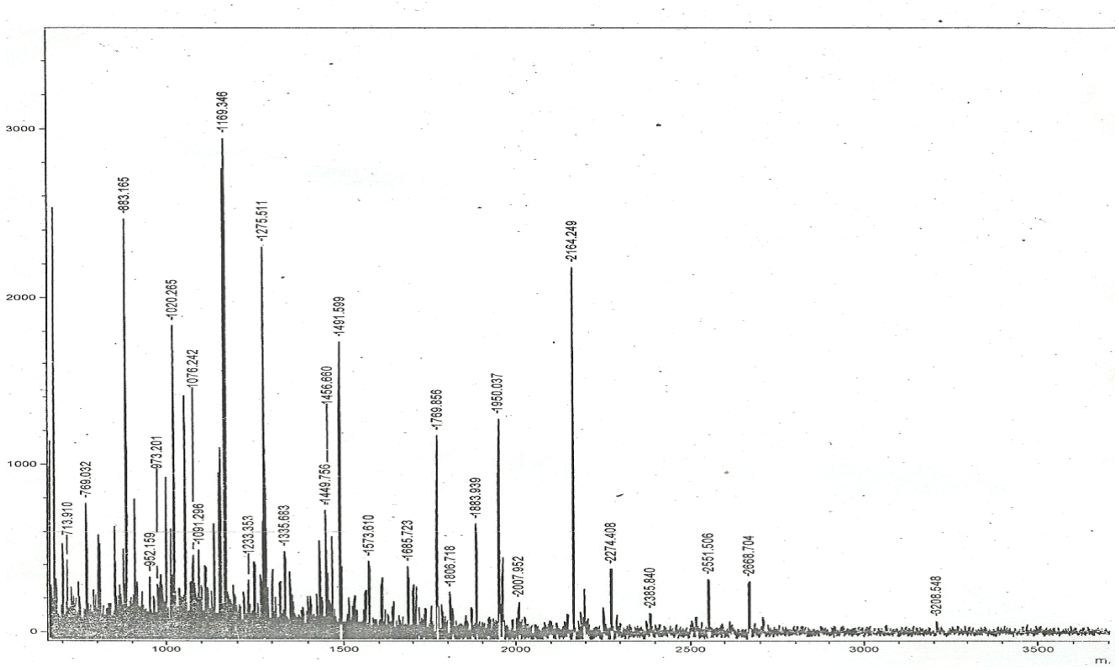


Figure 2: MALDI-TOF mass spectrum of oestrus mare's urinary carrier protein

Figure 3: Sequence coverage and peptide mass of α2u globulin Sequence coverage 41%

Matched peptides shown in Bold:

1 MASLSLAPVN IFKAGADEER AETARLTSFI GAIAIGDLVK STLGPK**GMDK**
51 ILLSSGRDAS LMVTNDGATI LKNIGVDNPA AKVLVDMSRV QDDEVGDGTT
101 SVTVLAAELL REAESLIAKK IHPQTIIAGW REATKAAREA LLSSAVDHGS
151 DEVKFR**QDLM NIAGTTLSSK LLTHHK**DHFT K**LAVEAVLRL KGSGNLEAIH**
201 IIKKLGGSLA DSYLDEGFLL DK**KIGVNQPK RIENAK**ILIA NTGMDTDKIK
251 IFGSRVRVDS TAKVAEIEHA EKEKMK**EKVE R**ILKHGINCF INRQLIYNYP
301 EQLFGAAGVM AIEHADFAGV ER**LALVTGGE IASTFDHPEL VKLGSCKLIE**
351 EVMIGEDKLI HFSGVALGEA CTIVLRGATQ QILDEAERSL HDALCVLAQT
401 VK**DSRTVYGG GCSEMLMAHA VTQLANR**TPG KEAVAMESYA KALRMLPTII
451 ADNAGYDSAD LVAQLR**AAHS EGNTTAGLDM REGTIGDMAI LGITESFQVK**
501 RQVLLSAAEA AEVILRVDNI IKAAPRKR**VP DHHP**

Table 1: Sequence coverage and peptide mass of α2u globulin Sequence coverage 41%

Start-End	Observed	Mr (expt)	Mr (calc)	Delta	Mis	Sequence
47 – 57	1176.130	1176.10	1175.6332	0.5008	1	K.GMDKILLSSGR.D
51 – 72	2274.020	2274.00	2274.2253	0.203	1	K.ILLSSGRDASLMVTNDGATILN
73 – 82	997.1490	997.140	997.5192	0.3702	0	K.NIGVDNPAAK.V
157 –176	2207.680	2207.60	2207.1732	0.5148	1	R.QDLMNIAGTTLSSKLLTHHK.D
171 –176	747.4190	747.410	747.4391	0.0201	0	K.LLTHHK.D
182 –189	868.9970	868.990	869.5334	0.5364	0	K.LAVEAVLR.L
182 –191	1112.190	1112.16	1110.7124	1.4836	1	K.LAVEAVLRLK.G
190 –203	1491.520	1491.50	1491.8773	0.3563	1	R.LKGSGNLEAIHIIKK
190 –203	1494.330	1494.30	1491.8773	2.4537	1	R.LKGSGNLEAIHIIKK
223 –230	881.8840	881.880	882.5287	0.6447	1	K.KIGVNQPK.R
223 –230	883.0880	883.080	883.0880	0.5593	1	K.KIGVNQPK.R
231 –236	731.4200	731.420	729.4133	2.0067	1	K.RIENAK.I
251 –257	833.9130	833.910	833.4872	0.4258	1	K.IFGSRVR.V
256-263	874.9700	874.970	874.4872	0.4828	1	R.VRVDSTAK.V
264 –272	1026.150	1026.10	1024.5189	1.6321	0	K.VAEIEHAEK.E
264 –274	1280.470	1280.10	1281.6564	1.1854	1	K.VAEIEHAEKEK.M
277 –281	661.6970	661.690	659.3602	2.3368	1	K.EKVER.I
323 –347	2585.270	2585.20	2584.3571	0.9219	1	R.LALVTGGEIASTFDHPELVKLGSCK.L
	1275.450	1275.40	1274.648	0.812	0	K.LIEEVMIGEDK.L
403 –427	2668.250	2668.20	2666.2363	2.0137	1	K.DSRTVYGGGCSEMLMAHAVTQLANR T
467 –481	1530.350	1530.30	1529.6892	0.6608	0	R.AAHSEGNTTAGLDMR.E
482 –501	2163.830	2163.80	2164.1198	0.2818	0	R.EGTIGDMAILGITESFQVKR.Q
482 –501	2164.626	2164.60	2164.1198	2164.1198	1	R.EGTIGDMAILGITESFQVKR.Q
529 –535	802.9230	802.920	803.3385	0.4155	0	R.VPDHHPC
529 –535	805.8770	805.870	803.3385	2.5385	0	R.VPDHHPC

CONCLUSION

The present study concluded that mare's urine is composed of oestrus dependent volatile compounds bond with low molecular weight protein. The identification of α2u-globulin was performed in 18.5 kDa of

electrophoretic band of oestrus mare urine by MALDI-TOF mass spectrometry. These results revealed that low molecular mass protein i.e., 18.5 kDa of urine of horse is α2uglobulin. The findings provided the additional report that the synthesis of this protein in the liver of horse and excreted into the urine i.e. the same protein was also detected in the 18.5 kDa urine of horse by MALDI-TOF mass spectrometry. The purified α2u-globulin still retained four ligands tightly bound. This kind of study provides an application to the reproductive management.

REFERENCES

- Achiraman, S., 2010. 1-iodo-2-methylundecane (1i2mu): An Estrogen dependent urinary sexpheromone of female mouse. ***Theriogenology.* 24(6), 721-728.**
- Cavaggioni, A., Findlay, J.B., Tirindelli, R., 1990. Ligand binding characteristics of homologous rat and mouse urinary proteins and pyrazine-binding protein of calf. ***Comp. Biochem. Physiol.* 96(3),** 513-520.
- Cavaggioni, A., Mucignat-Caretta, C., (2000). Major urinary proteins, alpha (2U)-globulins and aphrodisin. *Biochim. Biophys. Acta.* 1482,218-228.
- Cavaggioni, A., Sorbi, R.T., Keen, J.N., Pappin, D.J., Findlay, J.B., (1987). Homology between the pyrazine-binding protein from nasal mucosa and major urinary proteins. *FEBS Lett*. 212(2), 225-238.
- Chen Bo-Hong, Ju-Tsung Liu, Hung-Ming Chen, Wen-Xiong Chen, Cheng-Huang Lin, (2018). Comparison of the characteristic mass fragmentations of Phenethylamines and Tryptamines by Electron Ionization Gas Chromatography Mass Spectrometry,Electrospray, and Matrix-Assisted laser Desorption Ionization Mass Spectrometry. Appl.Sci. (8) 1022: 1-10.
- Cohen.M.S. (2018). The Role of Cell Proliferation in the Etiology of Neoplasia Comprehensive Toxicology, Volume 7, 2018, Pages 226-247.
- Edward A. Lock Gordon C. Hard (2010). $Alpha_{2U}$-Globulin Nephropathy and Chronic Progressive Nephropathy as Modes of Action for Renal Tubule Tumor Induction in Rats, and Their Possible Interaction. Book Editor(s): Ching-Hung Hsu Todd StedefordFirst published: 05 August 2010.
- Finlayson, J.S., Asofsky, R., Potter, M., Runner, C.C., (1965). Major urinary protein complex of normal mice: origin. *Science.* 149**,** 981–982.
- Hurst, J., L., Rich, T., J., (1998). Scent marks as reliable signals of the competitive ability of mates. Anim. Behav. 56:727-735.
- Loebel, D., Scaloni, A., Paolini, S., Fini, C., Ferrara, L., Breer, H., Pelosi, P., (2000). Cloning post-translational modifications, heterologous expression and ligand-binding of boar salivary lipocalin. *Biochem. J.* 350,369–379.
- Pelosi, P., (1994) Odorant-binding proteins. *Crit. Rev. Biochem. Mol. Biol. 29*:199-228.
- Pelosi, P., (1996) Peri receptor events in olfaction. *J. Neurobiol*., *30*:3-19.
- Pelosi, P., Baldaccini, N.E, Pisanelli, A.M., 1982. Identification of a specific olfactory receptorfor 2-isobutyl-3-methoxypyrazine. ***Biochem. J.* 201:**245–248.
- Pelosi, P., Maremanni, C., Muratorio, A., (1982). Purification of an odorant binding protein from human nasal mucosa. Chemo.Infor.Process 39:125-130.
- Ramprasad, Kowsalya, Sidappa, Sujatha, (2015). A review of electrophoretic patterns from a tertiary care nephrourology referral centre. Jou.Canc.Therapy.,3(6): 72-76.
- Roy, A.K, Schiop, M.J, Dowbenko, D.J. (1976). Regulation of the hepatic synthesis of alpha 2u globulin and its corresponding messenger RNA in maturing male rats**.** *FEBS Lett*., 70(1):137-40.
- Roy, A.K., Milin, B.S., McMinn,D.M. 1974. Androgen receptor in rat liver: hormonal anddevelopmental regulation of the cytoplasmic receptor and its correlation with theandrogen-dependent synthesis of alpha2u-globulin. ***BiochimBiophysActa.*, 4; 354(2):**213-32.
- Theodore,X., O'Connell, Timothy, M.D., Horita, J., Kasravi, Barsam, (2005). Understanding and interpreting serum protein electrophoresis. Amer.fam. physi., 71(1):105-112.
- Utsumi, M., Ohno, K., Kawasaki, Y., Tamura, M., Kubo, T., Tohyama, M**.** (1999). Expression of major urinary protein genes in the nasal glands associated with general olfaction. *J.Neurobiol*., 39:227–236.

- Vandoren, G., Mertens, B., Heyns, W., Van Baelen, H., Rombauts, W., Verhoeven, G. 1983. Different forms of 2u-globulin in male and female rat urine. ***Eur. J. Biochem*.., 134:**175-181
- Webster J, Oxley D.(2012) Protein identification by MALDI-TOF mass spectrometry. Methods Mol Biol. 2012;800:227-40.