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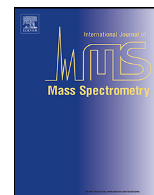

Mass spectrometry for structural analysis and quantification of the Major Urinary Proteins of the house mouse

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ABSTRACT

The Major Urinary Proteins (MUPs) of the house mouse, *Mus musculus domesticus*, are 18–19 kDa beta-barrel lipocalins that are involved in chemical communication between individuals. Many of them are excreted in urine where they play multiple roles, including coding of owner identity and transport, and slow release of bound volatile pheromones. One of them, darcin, is a pheromone in its own right and induces long-term memory for the identity and location of the scent mark owner. We have shown that mass spectrometric analysis of intact proteins, and their ion mobility behaviour, is capable of dissecting subtle structural differences between the members of this class of proteins. Moreover, mass spectrometric analysis of the intact proteins can contribute towards molecular phenotyping of MUPs. However, whilst allowing relative quantification, the ionisation propensity or gas phase properties of the individual MUPs may compromise absolute quantification. To solve the challenge of absolute quantification of MUP expression, we have designed and constructed a QconCAT built from endopeptidase LysC peptides that is capable of quantifying MUPs found in laboratory animal strains and some MUPs from wild caught individuals.

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1. Introduction

1.1. Major urinary proteins: structure and function

Chemical communication among individual house mice (*Mus musculus domesticus*) is mediated in part through urine, and achieved by a combination of low molecular weight volatile compounds and proteins. Mouse urine is highly proteinaceous, which sets it apart from many other mammals; protein concentrations can reach as high as 70 mg/mL [1,2]. The protein content is predominantly (99%+) attributable to a family of 18–19 kDa, eight stranded beta-barrel lipocalins known as major urinary proteins, or MUPs. The MUPs are encoded on mouse chromosome 4, and reference to this region of the genome of the C57BL/6J laboratory mouse (Fig. 1) has revealed at least 21 protein coding genes (as well as multiple pseudogenes) [3,4]. All are synthesised as pre-proteins, but the signal peptide is removed precisely to reveal a conserved N-terminus. Mouse MUPs contain a single disulphide bond but only two have consensus sites for N-linked glycosylation (MUP3 and MUP21) of which one has been proven to be glycosylated [3]. Of these 21 genes, the central 15 show very high sequence homology, and the peripheral six are more variable in their sequence. Some of the peripheral MUPs are not expressed in urine but are expressed in nasal tissue (for example, MUP4) or salivary (MUP5, MUP6) or lachrymal glands (MUP4). The central MUPs are predominantly expressed in urine, with considerable variation in expression between individuals such that these MUPs confer an individual 'bar code' or scent mark ownership on the scent mark [5–7]. Some are male-specific, notably the central MUP7 and peripheral MUP20. Recently, we have focused our studies towards one protein, the male-specific MUP20, or 'darcin'. Darcin (named after Mr Darcy from 'Pride and Prejudice') is a peripheral MUP that is responsible

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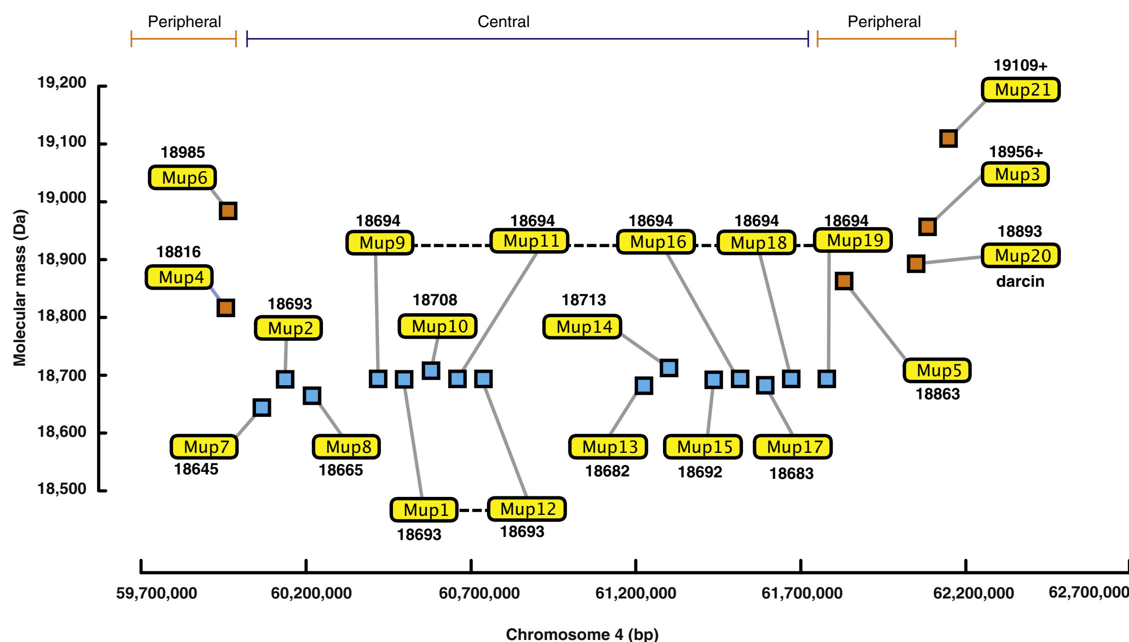

Fig. 1. The MUP gene cluster of the house mouse. The 21 known protein-coding genes within the MUP cluster are displayed according to the molecular mass (in Da) of the mature protein (including disulphide bond formation), their position in the gene cluster and their annotation as central (blue) or peripheral (orange). Note that five MUPs (9, 11, 16, 18 and 19) have identical mature sequences and thus, masses. The same is true for MUP1 and MUP12. Finally, one MUP (2) has the same mass as MUPs 1 and 12, albeit from a different primary sequence. Two MUPs (MUP3, MUP21) have a potential glycosylation (NXS/T) site and of these, MUP3 has been demonstrated to be glycosylated in urine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for the innate attraction of a female to a male urinary scent mark [8] and has unique structural properties [9,10]. It binds one of the most abundant volatile pheromones in male mouse urine (2-*sec*-butyl-4,5-dihydrothiazole) and elicits a slow, time-dependent release of this volatile [11], ensuring a long-term and remote attraction to the scent mark. Once the female encounters the scent mark, darcin stimulates a long-term memory for the physical location of the scent mark [12], and for the volatile odour of that specific male [8,13]. Both of these responses strongly promote recognition and attraction to the scent mark donor in the future. Darcin also stimulates hippocampal neurogenesis, consistent with a role in the deposition of a long-term memory [14]. A notable feature of darcin that sets it aside from other MUPs is the pronounced stability and resistance to denaturants [10] – the rigidity of the structure might explain the slow release of the volatile pheromone.

1.2. Mass spectrometry of MUPs

Being small proteins, and being readily available, the MUPs have been subject to analysis by electrospray ionisation mass spectrometry (ESI-MS) for some time. Indeed, one of the first papers reporting the use of maximum entropy deconvolution of electrospray ionisation mass spectra demonstrated the capability of high-resolution mass spectrometry for effective resolution of the MUPs as intact proteins [15]. Subsequently, when one of us (RJB) moved to UMIST in 1993, the same year as Simon Gaskell took up the role of Director of the Michael Barber Centre for Mass Spectrometry, we were able to develop a long-term collaborative study of the MUPs, both in terms of their semiochemistry, with Jane Hurst at Liverpool, but also in terms of their analysis by mass spectrometry (*e.g.* see [16–20]). Since then, a recurrent feature of our research on MUPs has been their characterisation by high-resolution mass spectrometry, whether as intact proteins or after proteolytic digestion. This has led to the discovery of new MUPs, the mapping of amino acid changes in polymorphic or allelic variants and the discovery of related urinary lipocalins. The ability to quickly survey the profile of similarly sized proteins by intact mass spectrometry has driven fundamental insights into the function of this group of proteins. One protein, darcin, is evident in ESI-MS of MUPs, but has previously been observed as a relatively minor constituent of the mass spectrum, disproportionately lower in intensity than the corresponding densitometry analysis would suggest. Moreover, under low pH conditions, there has been some evidence for a degree of undercharging [10].

1.3. Quantification of MUPs in mouse urine by QconCAT

We have explored the value of intact mass profiling for precise quantitative analysis of MUP profiles. Some mature MUPs differ in mass by 1 Da, and independent analysis of these is difficult, even for high-resolution instruments. MUPs pose a challenging example of the 'isoform problem' – the need to develop isoform-specific quantitative assays [21]. Because the diagnostic sequence signatures reside in relatively few amino acids, a selected reaction monitoring (SRM)-based analysis that requires multiple, isoform-specific standards could be ideally delivered by a QconCAT approach. We (Beynon and Gaskell) conceived of QconCATs in the first half of 2004 [22] and these novel, synthetic and non-native protein standards have now been deployed in multiple studies (at the time of writing, there are over 50 QconCAT-related publications in PubMed). QconCATs are the heterologous products of artificial genes that are designed and constructed to encode a series of concatenated quantotypic peptides (Q-peptides), each of which can act as a quantification standard for different proteins [23–25]. The QconCAT protein is expressed and labelled heterologously *in vivo* with stable isotope labelled precursors, purified by virtue of gene encoded affinity tags and quantified. The purified QconCAT is then mixed with the analyte protein mixture, and the entire mixture is proteolysed with the appropriate endopeptidase, usually trypsin. Subsequent analysis of the limit peptides allows measurement of

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