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Characterization of Cauxin in the Urine of Domestic and Big Cats

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Abstract Cauxin is an abundant protein in feline urine. We have used proteomics strategies to characterize cauxin from the urine of domestic cats and a number of big cat species. Proteins were resolved by gel-based electrophoretic purification and subjected to in-gel digestion with trypsin. The resultant tryptic peptides were mass-measured by matrix-assisted laser desorption ionization time of flight mass spectrometry. Peptides were also resolved by liquid chromatography and analyzed by electrospray ionization and tandem mass spectrometry to generate fragment ion data to infer the amino acid sequence. We identified cauxin polymorphisms and corrected a sequencing artifact in cauxin from the domestic cat. The proteomics data also provided positive evidence for the presence of a cauxin homolog in the urine of big cats (Pantherinae), including the Sumatran tiger, Asiatic lion, clouded leopard, Persian leopard, and jaguar. The levels of cauxin in the urine of all big cats were substantially lower than that in the urine of intact male domestic cats.

Keywords Carboxylesterase · Cauxin · Felidae · Felinine · Gel electrophoresis · MALDI-ToF · Mass spectrometry · Pantherineae · Proteomics

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Introduction

All cat species (Felidae) use their urine for communication (Macdonald 1985; Asa 1993; Mellen 1993; Ogata and Takeuchi 2000) and have two distinct patterns of behavior when depositing fluid from the urinary tract. These are *normal urination*, during which, the animal squats on the ground and releases a stream of urine, and *spray marking*, whereby urine is ejected by the animal while it stands with its hindquarters towards the target and its tail lifted vertically. The precise direction of spray varies with the species, and adult males typically spray at a higher rate than females, although adult females of most species also spray mark (Schaller 1972; Macdonald 1985; Mellen 1993; Brahmachary and Singh 2000). In both tiger, *Panthera tigris*, and lion, *P. leo*, the fluid released comprises normal urine mixed with a whitish lipid precipitate, which is also present in the bladder. The average total lipid content of the marking fluid of tiger is 1–2 mg/ml of urine (Poddar-Sarkar 1996), and as much as 20 g of lipid a day is excreted (Hewer et al. 1948). When released during normal urination, the lipids do not leave marks on the ground. When sprayed on vertical objects, however, the lipids of tiger urine stick to the surface and in time turn into a dark viscous deposit with a distinct odor (Smith et al. 1989). Odors of tiger marking fluid may last up to 3 weeks and, when fresh, can be detected up to 3 m away (Smith et al. 1989). Marking fluid from lions does not leave the same black long-lived deposit of lipids as that from tigers (Anderson and Vulpius 1999), whereas no lipids have been found in urine from cheetah, *Acinonyx jubatus*, leopard, *P. pardus*, or puma, *Felis concolor* (Asa 1993).

Intact male domestic cats, *Felis catus*, frequently spray urine that has a characteristic species-specific aroma onto vertical objects, although the locations and rates of marking vary between studies (Macdonald 1985). Felinine, a sulfur-containing amino acid (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid), has been identified in the urine of several members of the Felidae including the domestic cat (Datta and Harris 1951, 1953; Westall 1953; Hendriks et al. 1995a) where urinary felinine concentration is positively correlated to blood plasma testosterone concentration. Intact males produce the greatest amount of urinary felinine (122 μmol kg^{-1} d^{-1}) compared with 41, 36, and 20 μmol kg^{-1} d^{-1} in castrated males, intact females, and castrated females, respectively (Hendriks et al. 1995b). Felinine is present in the urine from 6 mo of age (Tarttelin et al. 1998) and is detectable in the urine, kidney, and bladder (Hendriks et al. 2001). It is present in the blood as a precursor tripeptide, γ-glutamylfelinylglycine, which is a glutathione analog. Felinine production is gender-linked and is believed to be synthesized in tissues other than the kidney (Rutherfurd et al. 2002). The biological role of felinine is still unclear, but it is a urinary component involved in domestic cat spray marking. It is likely that it degrades to another compound or compounds that give rise to the distinctive "tom cat" urine odor (Hendriks et al. 1995a). Felinine has also been detected in the urine of ocelot, *F. pardalis*, Indian leopard cat, *F. bengalensis* (Datta and Harris 1951), and bobcat, *F. rufus* (Datta and Harris 1953). However, reports differ for leopards (Datta and Harris 1953; Roberts 1963), and felinine has not been found in the urine of other members of *Panthera* (Hendriks et al. 1995a; W. H. Hendriks, personal communication). Chemical signals among non-domestic cats appear to be more complex than those of the domestic cat. More than 50 different volatile compounds have been identified from lion urine, with no two individuals having exactly the same chemical composition (Anderson and Vulpius 1999). Seven compounds were found in most individual lions tested (male and female), whereas urine from males contained much more 2-butanone than that from females. Of the compounds found in only-male samples, none was detected in all males. Thus, it is unclear whether one or more of these compounds provides a male-specific signal.

Many healthy mammals exhibit an obligatory proteinuria (Monroe et al. 1989), and there is evidence that some of these proteins have a function in communication. More than 100 protein components have been identified in rat urine, including signaling proteins, enzymes, and pheromone binding proteins (Thongboonkerd et al. 2003). Cauxin (carboxylesterase-like urinary excreted protein) is a novel 70 kDa protein that is excreted in the urine of the domestic cat (Miyazaki et al. 2003), bobcat, *Lynx rufus*, and lynx, *L. lynx*, but, as yet, has not been detected in the urine of any other genus of Felidae (Miyazaki et al. 2006a). It is expressed in the epithelial cells of the straight proximal tubules and secreted from the renal tubular cells into the urine (Miyazaki et al. 2006a). Cauxin shares 40% sequence identity with a group of mammalian carboxylesterases that hydrolyze ester, thioester, and amide bonds. It is the main urinary protein of healthy male and female domestic cats, and intact males have higher levels of cauxin in their urine relative to neutered males, females, or cats of 3 mo or less (Miyazaki et al. 2003). Intact males excrete approximately 0.5 mg cauxin per milliliter of urine, decreasing by more than 90% after castration (Miyazaki et al. 2006a). Recently, Miyazaki et al. (2006b) provided evidence for the activity of this protein as a peptide hydrolase, acting on the dipeptide felinylglycine and releasing felinine into the urine. This raises the possibility that cauxin is an important component of sexual signaling in domestic cats and possibly other Felidae, functioning to control the expression of felinine in urine scents. In this study, we report the further characterization of cauxin from both domestic cats and a number of species of big cats. In marked contrast to previous studies (Miyazaki et al. 2006a), we provide proteomic-based evidence that cauxin is expressed by *Panthera* species, which are not thought to produce felinine or its precursors. This suggests that the esterase/peptidase might have other roles in the elaboration of urinary scents in this group of cats.

Methods and Materials

Urine samples from 13 mature intact domestic cats (five male and eight female; aged from 6 mo to 15 yr) were collected by cystocentesis, free catch, or by free collection from the floor. Urine samples from one individual of each of five species of mature male non-domestic cats (Asiatic lion, *Panthera leo persica*; Sumatran tiger, *P. tigris sumatrae*; Persian leopard, *P. pardus saxicolor*; jaguar, *P. onca*; and clouded leopard, *Neophilis nebulosa*) and one mature Asiatic lioness were collected by catheterization during a routine health check at Chessington World of Adventure, Surrey, UK. Samples were centrifuged to remove cell debris and stored at −20°C until required.

Urine protein content was measured using the Coomassie plus protein assay (Perbio Science UK Ltd., Tattenhall, UK). Creatinine levels were also determined to correct for urine dilution using a modification of the Jaffe method of analysis (Cook 1971). Before gel electrophoresis, samples from domestic cats were concentrated, and low molecular weight compounds were removed by acetone precipitation. To each sample, a fivefold volume of acetone was added and incubated for 1 hr at −20°C, centrifuged, 5,000×*g* for 5 min, the supernatant removed, and the pellet resuspended. Urine samples from big cats were treated with StrataClean™ Resin (Stratagene®, Hycor Biomedical Ltd., Edinburgh, UK), which binds and concentrates the protein in the sample and removes non-protein impurities. The resin was added to each sample, vortexed for 1 min then centrifuged at 5,000×*g* for 5 min. The supernatant was removed, the pellet washed with distilled, deionized, water and resuspended.

One-Dimensional Gel Electrophoresis Samples (10 μg) of the urine were electrophoresed through a 12% polyacrylamide gel according to previously documented methods (Laemmli 1970). A 12% resolving gel was prepared and allowed to polymerize before overlaying with a 4% stacking gel. Samples were incubated for 30 min at 37°C in either a reducing (Tris–HCl, 0.125 M; SDS, 0.14 M; glycerol 20% *v*/*v*; dithiothreitol, 0.2 mM and bromophenol blue, 0.03 mM) or non-reducing buffer before loading. The proteins were electrophoresed at a constant potential of 200 V until the dye front had progressed to the bottom of the gel. Gels were stained with Coomassie blue (Bio-Safe™, BioRad, CA, USA).

Two-Dimensional Gel Electrophoresis Before loading onto IPG strips, pH 3-10L (GE Healthcare UK Ltd., Little Chalfont, UK,) each sample (300 μg protein) was treated with StrataClean™ Resin. The resulting pellet was resuspended in buffer containing CHAPS (4% *w*/*v*), 7 M urea, 2 M thiourea, 20 mM dithiothreitol, and ampholytes (0.5% *v*/*v*) and incubated at room temperature for 1 hr. After centrifugation, the supernatant was separated in the first dimension on an IPGPhor unit (AP Biotech Pharmacia). In-gel rehydration (150 Vh at 30 V, 300 Vh at 60 V, 20°C) was followed by isoelectric focusing (500 Vh at 500 V, 1,000 Vh at 1,000 V, and 48,000 Vh at 8,000 V). The focused IPG strips were equilibrated in 50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% (*v*/*v*) glycerol, 2% (*w*/*v*) SDS, and a trace of bromophenol blue. Dithiothreitol (10 mg/ml) was present as a reducing agent for the initial equilibration, and iodoacetamide (25 mg/ml) was present in the second equilibration step. The proteins were electrophoresed through a linear 12.5% acrylamide gel, and the gels were stained using Coomassie blue (Bio-Safe™, BioRad).

In-Gel Trypsin Digest Gel plugs containing protein from areas of interest were excised, and the proteins were subjected to in-gel tryptic digestion and peptide extraction using a MassPrep™ digestion robot (Waters, Manchester, UK). Briefly, excised gel plugs were placed in distilled deionized water (50 μl) before addition of ammonium bicarbonate (50 μl, 100 mM) and acetonitrile (50 μl). This liquid was removed, and the gel piece was treated with destain solution [10 μl of 50% (*v*/*v*) acetonitrile, 50% (*v*/*v*) 100 mM ammonium bicarbonate]. The protocol included a second cycle of reduction and alkylation. The gel plug was dehydrated in acetonitrile and then rehydrated with 25 μl of trypsin (3 ng/μl) in buffer. After digestion (5 hr), tryptic peptides were extracted from the gel matrix by addition of 30 μl of 2% (*v*/*v*) acetonitrile/1% (*v*/*v*) formic acid. Peptides (1 μl) were mixed with matrix (1 μl) [α-cyano-4-hydroxycinnamic acid saturated solution in a 1:1:1:1 by volume of ethanol, acetonitrile, trifluoroacetic acid (0.4%), and distilled deionized water], and the resultant 2 μl was spotted onto the MALDI target. ACTH (adrenocorticotropic hormone, M=2,464.20), 1 μl of 1 μg/ml stock was used as an external lockmass standard.

MALDI-ToF Mass Spectrometry Peptides were analyzed by using a matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-ToF MS) mass spectrometer (M@LDI™, Waters) over the *m/z* range of 1,000 to 3,500 thompsons. The mass spectrometer was calibrated with 2.5 pmol each of bradykinin (MW=903.47), neurotensin (MW=1,671.92), ACTH (MW=2,464.20), and insulin β-chain (MW=3,493.65). Typically, a residual error of ±0.05 ppm was obtained. The mass accuracy of spectra could be further improved by internal standardization with the major trypsin autolysis peak ($[M+H]^+$=2163.057 thompsons). Proteins were identified from their peptide mass fingerprint by manual searching using a locally implemented MASCOT server (ver 1.9, http://www.matrixscience.co.uk) against the Swiss-Prot http://expasy.org/sprot/) or MSDB http://csc-fserve.hh.med.ic.ac.uk/msdb.html/) databases. The initial search parameters

allowed a single missed tryptic cleavage, obligatory (fixed) carbamidomethyl modification of cysteine residues, variable oxidation of methionine, and an *m/z* error of ±250 ppm. The taxonomic search space was restricted to Mammalia. Before MALDI-ToF MS analysis, residual contaminants were removed if necessary from exotic cat samples using ZipTip® C18 tips (Millipore, Hertfordshire, UK).

Electrospray Ionization Mass Spectrometry Electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS) were performed on a Q-ToF Micro instrument (Waters-Micromass) fitted with a nanospray source. The electrospray was created from a silver-coated glass capillary with a 10-μm orifice (Presearch, Hitchin, UK) held at a potential of +2,000 V relative to the sampler cone. Before ESI-MS/MS, peptides were separated by reversed phase high-performance liquid chromatography on a Dionex Ultimate system. The unit was fitted with a PepMap C18 column (LC packings), 15 cm×75 μm, bead size 3 μm and pore size 100 Å. Before separation, aliquots (10–20 μl) of sample digest were taken up into the injection loop of the Dionex Famos autosampler and desalted in-line using a Dionex Switchos apparatus fitted with a 5 mm× 300 μm C18 pre-column. The pre-column was initially equilibrated in 0.2% (*v*/*v*) formic acid at 30 μl/min. Peptides were then loaded and washed for 3 min at the same rate, after which, the trap and downstream PepMap column were developed with 90% acetonitrile/0.2% formic acid, introduced as a linear gradient of 0–50% in 30 min at 0.2 μl/min. The column eluant was monitored for UV absorbance at 214 nm in an in-line flow cell before being introduced into the mass spectrometer. The initial quadrapole analyzer was set to allow the transmission of selected precursors into the gas cell. The masses of the resulting fragment ions were then measured by the ToF analyzer. Precursor spectra were acquired between *m/z* 400 and 1,500 at a scan/interscan speed of 2.4/0.1 sec. Product ion spectra were acquired between m/m 100 and 2,000 at a scan speed of 1.0/0.1 sec. Raw product ion spectra were deconvoluted using the MaxENT algorithm within the Mass Lynx software. Interpretation of product ion spectra and the determination of peptide sequences were facilitated by the PepSeq module within Mass Lynx. Peptide sequences were searched using the National Center for Biotechnology Information basic local alignment search tool (BLAST). The search was limited to Mammalia; no other parameters were limited.

Results and Discussion

Urine samples from a range of domestic cats each contained multiple proteins distributed between 5 and 300 kDa (Fig. 1a). The overall pattern of bands from one-dimensional gel electrophoresis was similar among individuals, and there was no pronounced difference between males and females. Prominent bands were evident at a mobility corresponding to a molecular weight of approximately 66 kDa, but other bands were also present. To determine the identity of these protein bands, small plugs of gel (1 mm^2) corresponding to each band were recovered, instilled with trypsin, and the resultant tryptic peptides were recovered and analyzed by MALDI-ToF mass spectrometry (peptide mass fingerprinting, results not shown) and electrospray ionization mass spectrometry. This permitted the identification of several of the bands. Predominant urinary proteins included uromodulin (Tamm-Horsfall glycoprotein) present as a diffuse band at 120 kDa and transferrin at approximately 75 kDa. The most intense band in almost every sample migrated at approximately 67 kDa, and might have been anticipated to be serum albumin—urinary

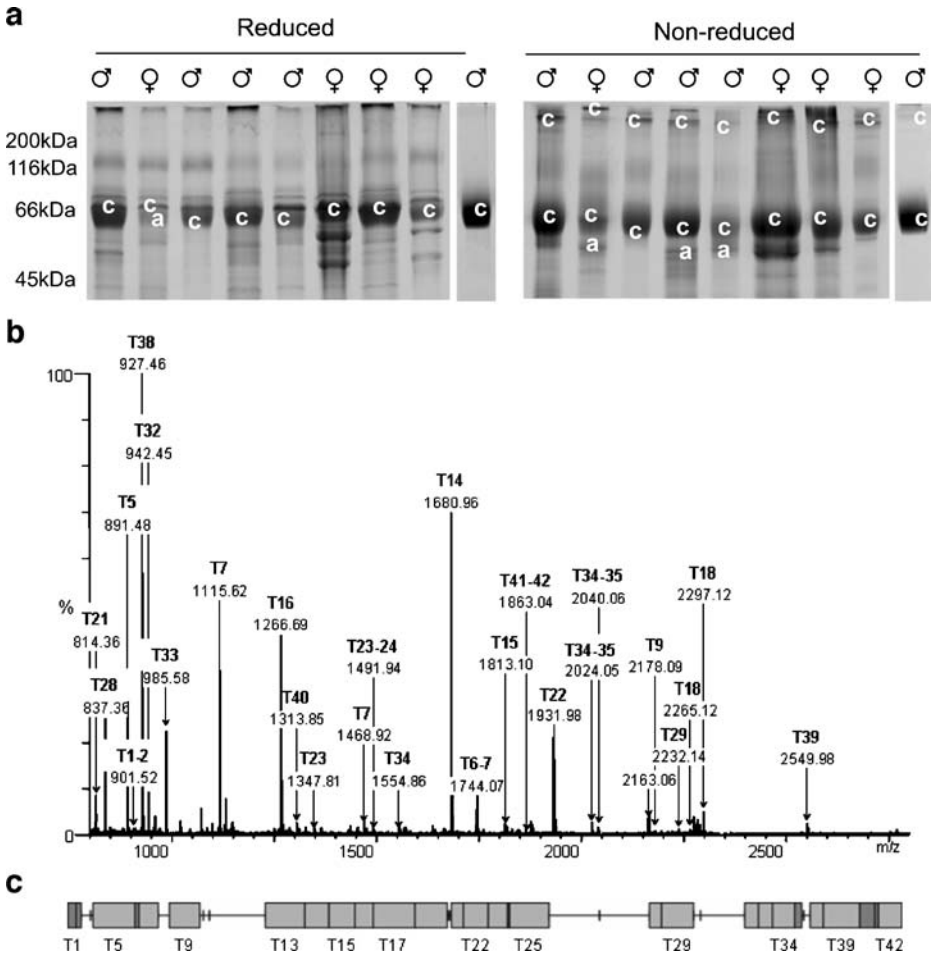

Fig. 1 Analysis and identification of urinary proteins from the domestic cat, *Felis catus*. Proteins in urine samples from adult male and female domestic short hair cats were resolved by reducing and non-reducing one-dimensional SDS-PAGE (**a**). Protein bands in the highly stained region of 60 kDa were excised and subjected to gel digestion before MALDI-ToF mass spectrometry. The proteins identified as cauxin (*white c*) and albumin (*white a*) are indicated on the gel. The mass spectrum of the band identified as cauxin is presented (**b**), and each tryptic peptide from the MALDI-ToF spectrum is highlighted in the peptide map (**c**)

serum albumin is a common observation in renal dysfunction. However, peptide mass fingerprinting revealed a complex series of tryptic peptides that matched with a very high score to the protein cauxin (Entrez Protein ref. BAC22577). Some peptides were also consistent with the presence of feline serum albumin, but it is likely that most of the protein in this band is cauxin. Cauxin is a single polypeptide chain 67 kDa, but exists in part as disulfide-linked multimers. When urine samples were resolved in non-reduced gels, a new series of bands migrating from 300 to 350 kDa was resolved. MALDI-ToF analysis of these bands confirmed that each of them contained tryptic peptides from cauxin. In contrast, albumin, which does not exist as disulfide-linked multimers, remained at a position on the gel approximating 60 kDa. The multiple high-mass cauxin bands might reflect N-linked glycosylation, as the sequence contains four potential glycosylation motifs

(Asn-X-Ser/Thr). Alternatively, the different bands could reflect disulfide-linked association between differentially proteolyzed subunits. Further evidence for cauxin being the predominant protein at the 66 kDa region of the gel was derived from two-dimensional gel electrophoresis (results not shown). After separation, a major protein staining area, smeared over the pI region from 3.5 to 5.8, was identified as cauxin. A smaller, more intense discrete spot, at pI 4.7, was identified by peptide mass fingerprinting as serum albumin.

The tryptic peptide fragments were subjected to *de novo* peptide sequencing (Fig. 2). Peptides were resolved by C18 reversed phase capillary chromatography, and specific peptides were fragmented by collision-induced decomposition. The product ion spectra were then used to direct manual peptide sequencing. Thirteen peptides were sequenced this way. These peptide sequences provide unambiguous confirmation that the protein is cauxin. One tryptic peptide in particular was readily and repeatedly sequenced ($[M+H]^+$=1,115.6 *m/z*) as PALPWNDFR, a sequence that explains all of the mass of the peptide (predicted $[M+H]^+$=1,115.56). This sequence was significant for two reasons. First, the equivalent tryptic peptide expected from cDNA sequencing was *QPK*PALP*G*NDFR (Miyazaki et al. 2003). The glycine residue (G) in this cDNA sequence is at variance with other reported cauxin-like sequences from several species, from which, the consensus *P*x*P*xxx*W* is evident (Ecroyd et al. 2006), and the peptide sequence obtained here corrects a previously reported anomaly possibly because of a cDNA sequencing error. The DNA sequence used to derived the previously published cat sequence was CCC[GGG]AATGACTTCCGA; for tryptophan (W) to be coded for in the same sequence, the DNA sequence would have to be CCC [GGA]AATGACTTCCGA, a single base shift near a region of potential GC compression. The second significant feature of this peptide is that it has nominally been generated by tryptic proteolysis, from the primary sequence context—KQPK[*palPWNDFR*]NAT—trypsin is not generally considered to be capable of cleaving peptide bonds in which the amino acid C-terminal to the scissile bond is proline (Olsen et al. 2004). Thus, the most likely explanation is that cauxin is trimmed by additional N-terminal processing possibly related to the secretion into urine. The proximal tubule of the kidney is replete with exo- and

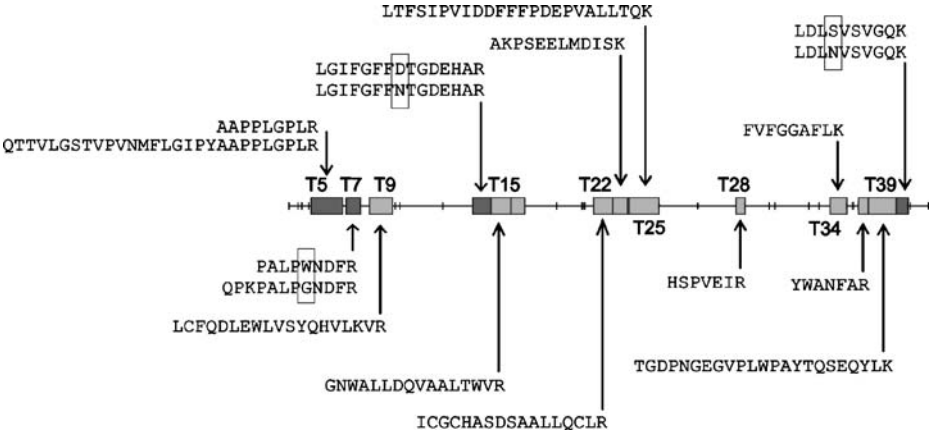

Fig. 2 *De novo* peptide sequencing of tryptic peptides of cauxin from the domestic cat, *F. catus*. Tryptic peptides from an in-gel digest of a band identified by peptide mass fingerprinting as cauxin were resolved using nanoflow reversed phase chromatography, fragmented by tandem mass spectrometry, and the product ion spectra were interpreted manually to obtain the peptide sequence data. The peptides for which sequence data were obtained are highlighted on the peptide map, and peptides that differed from the published sequence are highlighted (this study, top line, previously published sequence, accession number BAC22577)

endopeptidases that could be responsible for this processing. This processing must be partial, however, as we also found clear sequence data for an additional peptide that was derived from cauxin and that was N-terminal to the sequence PALPWNDFR. The multiple bands on non-reducing gels are also indicative of mass heterogeneity in cauxin subunits.

Cauxin has the signature motifs and overall sequence that places it in the class of carboxylesterases (Miyazaki et al. 2003). Although the function of cauxin is not known, it has been suggested that it is responsible for the elaboration of the strong scent of feline in urine used in territorial marking (Miyazaki et al. 2006b). Urinary scent marking is a feature of many members of the Felidae, and accordingly, we examined urine samples from a number of species of big cats for the presence of cauxin, including Asiatic lion, Sumatran tiger, jaguar, Persian leopard, and clouded leopard. In all instances, urine samples were obtained from intact adult males. In all of the male cats, a band corresponding in mass to cauxin was evident, although in several of the samples, the presence of urinary serum albumin migrating at approximately the same position on the gel made identification of cauxin more difficult (Fig. 3). However, when the gels were run under non-reducing conditions, high-mass complexes were evident (Fig. 3a), and these yielded tryptic peptides that could be sequenced readily by tandem mass spectrometry. Multiple peptides were analyzable, which, interestingly, were the peptides cognate to those seen in the domestic

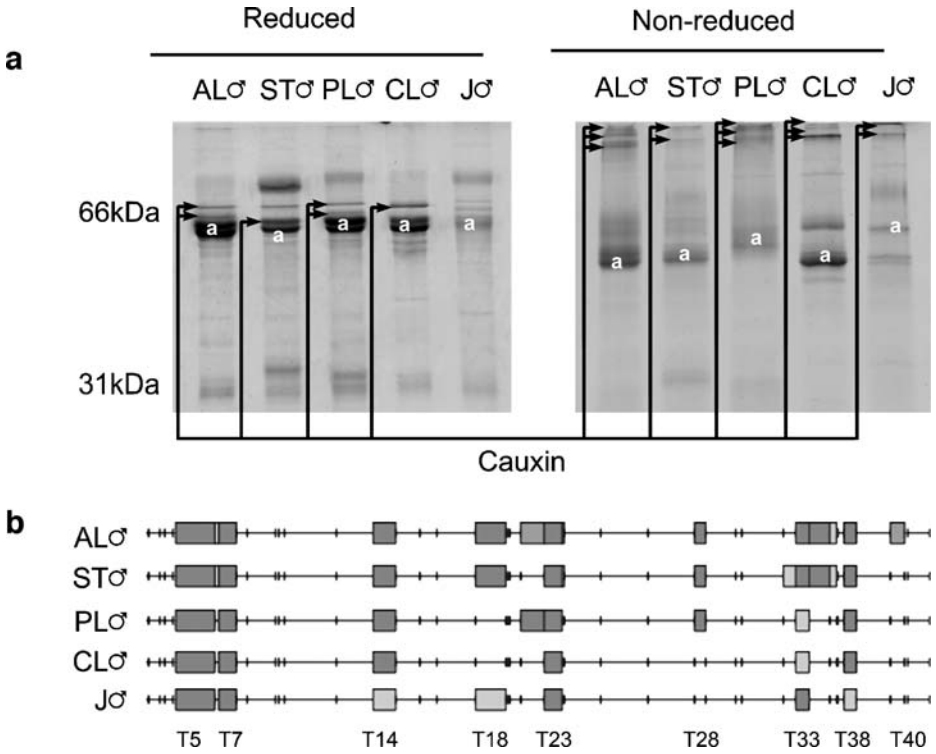

Fig. 3 Analysis and identification of male urinary proteins from the Asiatic lion (*AL*), Sumatran tiger (*ST*), Persian leopard (*PL*), clouded leopard (*CL*), and jaguar (*J*). Proteins in urine samples were resolved by reducing and non-reducing one-dimensional SDS-PAGE (**a**). The proteins identified as cauxin and albumin (*white a*) are indicated on the gel. Major protein bands were excised and subjected to in-gel digestion before peptide mass fingerprinting and the peptides observed by mass spectrometry are highlighted (**b**)

cat. This implies that these were similar peptides that yielded strong ion currents and good fragmentation data irrespective of the source (Fig. 4). Although multiple peptides were sequenced for each of the big cats, there were remarkably few amino acid changes within the big cats, or indeed, when compared to the domestic cat, from which, we infer a high degree of conservation of structure, activity, and function for this protein (Fig. 5). Equally intriguing, the unusual tryptic peptide PALPWNDFR, previously discussed, was present in all big cat samples, implying a similar pattern of N-terminal processing or unusual tryptic cleavage in different species (Fig. 5). The sequence of cauxin from each of the big cats is noticeably similar to that of the domestic cat. We compared this degree of sequence similarity with that obtained for serum albumin also present in urine samples (Fig. 6). For

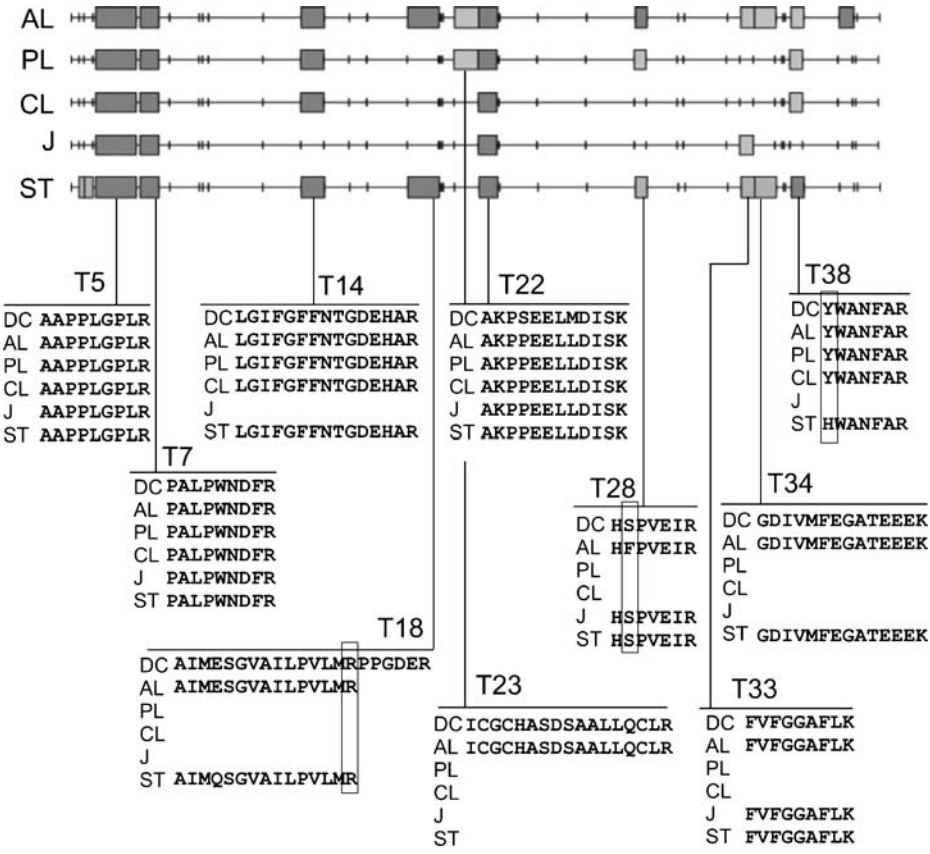

Fig. 4 De novo peptide sequencing of tryptic peptides of cauxin from big cats. Urine samples from five big cat species were resolved on SDS-PAGE (abbreviations as Fig. 3; *DC* domestic cat). Tryptic peptides from an in-gel digest of the band identified by peptide mass fingerprinting as cauxin were resolved using nanoflow reversed phase chromatography, fragmented by tandem mass spectrometry, and the product ion spectra were manually interpreted to obtained peptide sequence data. The peptides for which sequence data were obtained are highlighted on the peptide map. Most peptides had the same sequence as those for the domestic cat, but where peptide sequences differed from the domestic cat in at least one of the big cat species, the amino acid differences are *boxed*. For tryptic peptide T18 from the Asiatic lion (*AL*) and the Sumatran tiger (*ST*), the termination of the sequence at the arginine residue, which in domestic cat is extended to include a non cleavable ArgPro sequence, is presumptive evidence for an amino acid change at that position

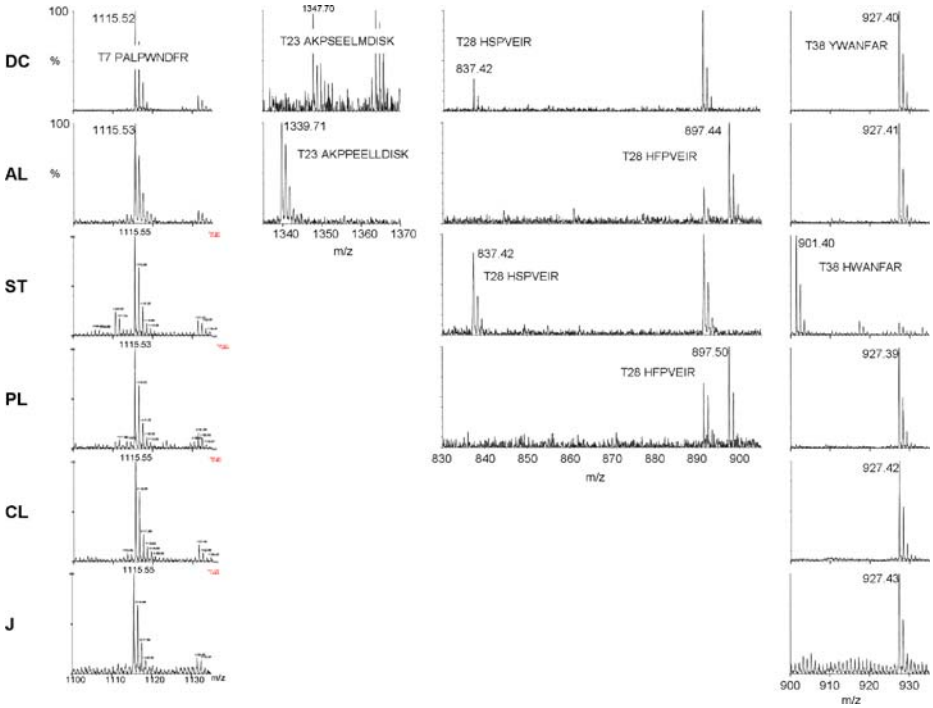

Fig. 5 Identification of polymorphic peptides in cauxin species for big cat species. Urine samples from five big cat species were resolved on SDS-PAGE (abbreviations as Fig. 3, *DC* domestic cat). Tryptic peptides from an in-gel digest were sequenced by tandem mass spectrometry, and for selected peptides where different sequences were obtained for big cats, the corresponding $[M+H]^+$ ion from MALDI-ToF mass spectrometry is shown, together with the corresponding ion that confirms the amino acid change. Additionally, the atypical tryptic fragment PALPWNDFR was observed in all six cat species, and the MALDI-ToF mass spectrum for that specific $[M+H]^+$ ion is included in the *left hand* column

domestic cat, a total of 50 tryptic peptides were identified, covering approximately 66% of the sequence. For the Asiatic lion, 34 tryptic peptides derived from albumin were identical to the domestic cat (covering 58% of the sequence); the corresponding figures for the clouded leopard and the jaguar were 38 (63% coverage) and 36 (51% coverage), respectively. In both the Sumatran tiger and the Persian leopard, we identified 43 (64% coverage) and 39 (59% coverage) tryptic peptides, respectively, but for two peptides (Sumatran tiger) and three peptides (Persian leopard), single amino acid changes were observed. The degree of sequence conversation in serum albumin is high and reflects the relatedness of these species. It is, therefore, not surprising that cauxin has a similar degree of conservation, but this also implies that mechanisms of sexual selection are not driving species-specific evolution of this protein.

The expression of cauxin by several *Panthera* spp. was unexpected, as a previous study had reported that cauxin was not present in urine samples (Miyazaki et al. 2006a). By contrast with the intact domestic male cat (Fig. 1, and Miyazaki et al. 2003); the band intensity of cauxin in these big cats was substantially lower. The protein-based evidence for the existence of cauxin as a urinary component is substantial: identification based on peptide mass fingerprinting, which requires a high degree of sequence conservation among the species under investigation and a model species for which a database entry already

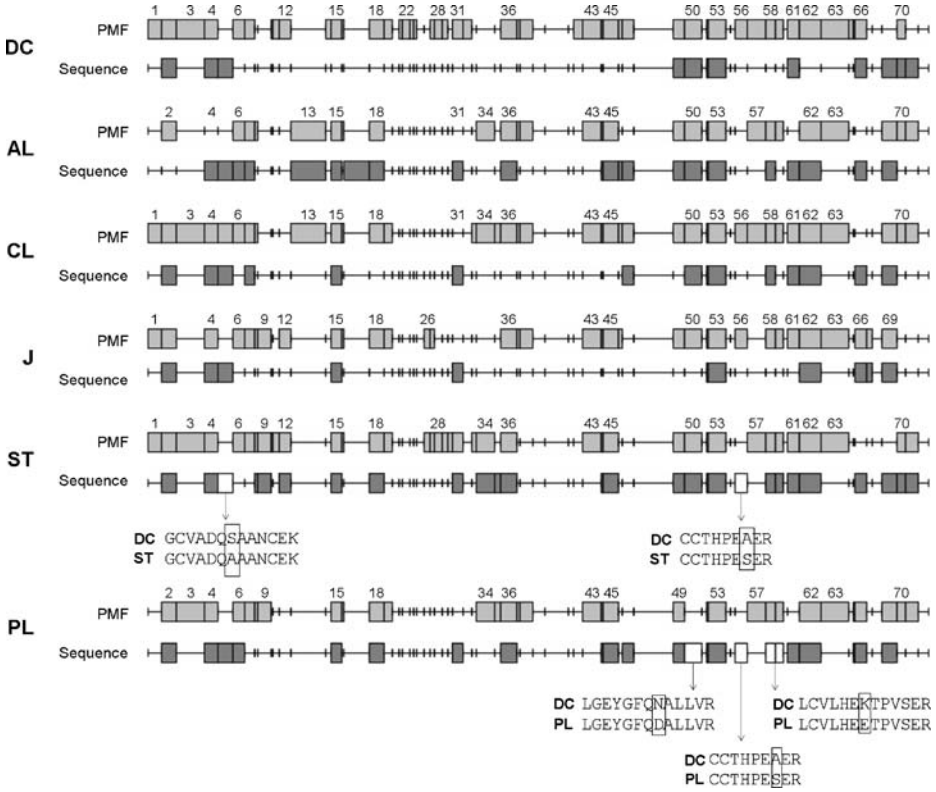

Fig. 6 Sequence homology of albumin in domestic and big cat species. Urine samples from five big cat species were resolved on SDS-PAGE. Tryptic peptides from an in-gel digest of a band putatively identified as serum albumin were used to confirm the identity of this protein. Peptides matched by mass alone (peptide mass fingerprinting) are highlighted as *boxes* in the PMF row for each species (abbreviations as Fig. 3). Additionally, selected peptides were resolved by nanoflow reversed phase chromatography, fragmented by tandem mass spectrometry, and the product ion spectra were manually interpreted to obtained peptide sequence data (row marked 'Sequence'). Where peptide sequences were identical to those for the domestic cat, no additional information is provided, but where sequence differences were observed, the changed sequence and the corresponding sequence for albumin from the domestic cat are *aligned*, with differences *boxed*

exists, and *de novo* peptide sequencing, which can be used as a probe in BLAST searches. In contrast, the previous investigation was based on Western blotting, with an antibody to a C-terminal peptide of cauxin in the domestic cat (SVGQKLKEQEVEFWMNTIVP, spanning tryptic peptides T40 to T42). It is possible that this peptide might not be so highly conserved in the big cat species, and thus, Western blotting might yield weaker signals, which would be exacerbated by the much lower level of expression of cauxin in the big cats.

It is likely that the role of cauxin in the domestic cat is not to act as a carboxylesterase, but as an amidase that catalyzes the hydrolysis of the dipeptide 3-methybutanol-cysteinylglycine to glycine and the cat-specific amino acid felinine. Felinine and its degradation products are putative pheromones (Miyazaki et al. 2007), and the role of cauxin in elaboration of the pheromone from a precursor peptide is enigmatic. What then of the expression of the homologous enzyme in big cats? Neither felinine nor its precursors are present in urine of the Pantherinae (Hendriks et al. 1995a; W.H. Hendriks, personal

communication), and the role of cauxin in these species, albeit in reduced amounts, remains enigmatic. It is possible that the cauxin homolog acts as an amidase, but with different precursor molecules. However, some cats express urine that is replete with complex fatty acyl esters, and alternative substrates should be considered. It is clear that the pheromone/enzymology axis of cats is at the beginning of a new and fascinating phase of development.

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