The Soluble Proteome of the *Drosophila* Antenna

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Abstract

The olfactory system of *Drosophila melanogaster* is one of the best characterized chemosensory systems. Identification of proteins contained in the third antennal segment, the main olfactory organ, has previously relied primarily on immunohistochemistry, and although such studies and in situ hybridization studies are informative, they focus generally on one or few gene products at a time, and quantification is difficult. In addition, purification of native proteins from the antenna is challenging because it is small and encased in a hard cuticle. Here, we describe a simple method for the large-scale detection of soluble proteins from the *Drosophila* antenna by chromatographic separation of tryptic peptides followed by tandem mass spectrometry with femtomole detection sensitivities. Examination of the identities of these proteins indicates that they originate both from the extracellular perilymph and from the cytoplasm of disrupted cells. We identified enzymes involved with intermediary metabolism, proteins associated with regulation of gene expression, nucleic acid metabolism and protein metabolism, proteins associated with microtubular transport, 8 odorant-binding proteins, protective enzymes associated with antibacterial defense and defense against oxidative damage, cuticular proteins, and proteins of unknown function, which represented about one-third of all soluble proteins. The procedure described here opens the way for precise quantification of any target protein in the *Drosophila* antenna and should be readily applicable to antennae from other insects.

Key words: chemosensation, mass spectrometry, odorant-binding proteins, olfaction, proteomics

Introduction

The olfactory system of *Drosophila melanogaster* has emerged as one of the best characterized chemosensory systems. Odorants are recognized by sensory neurons housed in sensilla of the third antennal segment and the maxillary palps, the main olfactory organs, as well as chemosensory neurons on the tarsi, wing margins, and female reproductive organs. Olfactory sensory neurons in basiconic sensilla express odorant receptors that contain 7 transmembrane domains (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999), but differ from classical G protein–coupled receptors in membrane orientation (Benton et al. 2006) and transduction mechanism, as odorant activation results in the opening of a cation channel formed by a complex between a unique olfactory receptor and the universal Or83b receptor (Sato et al. 2008; Wicher et al. 2008). The molecular receptive fields and activation properties of a large fraction of odorant receptors have been characterized by elegant electrophysiological studies (de Bruyne et al. 1999, 2001; Hallem et al. 2004; Hallem and Carlson 2006), and projections of cells expressing defined receptors have been mapped to individual glomeruli in the antennal lobes (Vosshall et al. 2000). Olfactory sensory neurons in coeloconic sensilla express yet another family of odorant receptors that resemble ionotropic glutamate receptors with distinct ligand specificities, including responses to amines (Benton et al. 2009). In addition to odorant receptors, a large family of odorant-binding proteins (Obps) that are secreted by supporting cells into the antennal perilymph has been characterized (Galindo and Smith 2001; Hekmat-Scafe et al. 2002), and members of this family have been implicated in pheromone detection (Xu et al. 2005; Laughlin et al. 2008), host plant selection (Matsuo et al. 2007), and combinatorial recognition of general odorants (Wang et al. 2007).

Transcriptional profiling studies have shown that expression of the chemosensory repertoire of *Drosophila* is dynamic and changes under different developmental, environmental, and physiological conditions (Zhou et al. 2009). Clearly, it would be of value to correlate overall protein levels with
changes in transcript abundance. Previously, the expression of Obps and odorant receptors, as well as proteins implicated in removal of xenobiotics, including odorants, has been characterized by in situ hybridization (McKenna et al. 1994; Pikielny et al. 1994; Clyne et al. 1999; Vosshall et al. 1999; Rollmann et al. 2005), immunohistochemistry (Hekmat-Scafe et al. 1997), enhancer trap (Riesgo-Escovar et al. 1992; Anholt et al. 1996), and transgenic drivers using the GAL4-UAS binary expression system (Vosshall et al. 2000; Galindo and Smith 2001). Although such studies have been highly informative, they focus generally on one or few gene products at a time and quantification by any of these methods is difficult. Furthermore, biochemical purification of native proteins from the antenna is challenging due to the small size of the Drosophila antenna and because olfactory sensory neurons are encased in a hard cuticle.

Here, we describe a simple method for the large-scale detection of soluble proteins from the Drosophila antenna using chromatographic separation of tryptic peptides by nano-LC followed by tandem mass spectrometry (MS/MS), with femtomole detection sensitivities. Confident protein identifications are obtained when LC retention time data are combined with accurate mass measurements and MS/MS fingerprints. The procedure described here opens the way for precise quantification of any target protein in the Drosophila antenna and should be readily applicable to antennae from other insects, including disease vectors, such as mosquitoes and urban pests, such as cockroaches, which rely on olfactory input for host identification, mating, and oviposition site selection.

Materials and methods

Drosophila stocks and sample preparation

Isogenic D. melanogaster of the Canton S (B) strain were reared on standard cornmeal molasses agar medium at 25 °C and 70% humidity under a 12 h light:dark cycle. Antennae were dissected by hand under a stereomicroscope and immediately placed in microcentrifuge tubes on dry ice. Duplicate pools of 120 antennae from males and females were obtained separately. The antennae were subjected to osmotic lysis by 2 freeze-thaw cycles in 50-µL distilled water followed by homogenization with a small pestle and centrifugation to remove nonsoluble material. The supernatants were recovered, and 32-µL samples were evaporated to dryness, reconstituted in 25.5 µL of 5% formic acid, and filtered out introduction of detergents. For tryptic digestion of proteins, 1.5 µL of 100 mM aqueous diithiothreitol was added to each sample, and the samples were heated at 95 °C for 5 min. Upon cooling to room temperature, 3 µL of 100 mM aqueous iodoacetamide was added, and the resulting solution incubated at room temperature in the dark for 20 min. This was preceded by the addition of trypsin (1 µL of a 0.1 µg/µL trypsin solution in 1 mM HCl). Digestion was carried out at 37 °C for 3 h, following which another 1 µL of trypsin solution was added and samples were incubated at 30 °C overnight. Trypsin was quenched by addition of 1.5 µL of 5% formic acid. Samples were then evaporated to dryness, reconstituted in 100 µL of LC mobile phase A (98% H2O, 2% acetonitrile, and 0.2% formic acid), and filtered with 10 kDa molecular weight cut off filters (Millipore number 42407) prior to LC/MS/MS analyses. Serial dilutions of the sample solutions up to 1000-fold were performed prior to analysis to determine the optimum concentration for sample introduction into the instrument.

Mass spectrometry

Reversed phase high-performance liquid chromatography separation and MS detection were performed using an Eksigent nano-LC-2D system with an autosampler coupled to a hybrid LTQ-FT Ultra mass spectrometer from Thermo Scientific, Inc. The nano-LC was operated with a “continuous vented column” configuration for in-line trap and elute (Andrews et al. 2009). The analytical column was a self-packed 75-µm inner diameter (i.d.) fused silica PicoFrit capillary with 15 cm of Magic C18AQ stationary phase. The trap and dummy columns were self-packed 75 µm i.d. fused silica IntegraFrit capillaries with 5 cm and 20 cm of Magic C18AQ stationary phase, respectively. LC solvents used are mobile phase A and mobile phase B (acetonitrile/ H2O/Hformic acid [98/2/0.2% by volume]). Blank runs were performed after every sample run. Sample injections ranged from 2 to 5 µL on column. Analytical separations were run on the nanoflow pump at 500 nL/min, initially maintaining a composition of 2% B. The MS method consisted of 4 events: a precursor scan followed by 3 data-dependent tandem MS scans of the first, second, and third most abundant peaks in the ion trap. A high resolving power precursor scan of the eluted peptides was obtained using the LTQ-FT with the 3 most abundant ions selected for MS/MS in the ion trap through dynamic exclusion. The instrument was externally calibrated according to the manufacturer’s protocol.

Data analysis

The nano-LC/MS/MS data files were processed by Sequest (Bioworks, ThermoFisher Scientific, Inc.; Eng et al. 1994) and Mascot (Matrix Science; Perkins et al. 1999) for protein identifications. These algorithms apply similar general approaches in assigning peptides detected in MS/MS spectra to those in a sequence database. However, the principles behind their mathematical operations are significantly different. Mascot applies a probabilistic metric to determine the likelihood that a fragmented peptide produced an observed MS/MS spectrum. Sequest, on the other hand, applies empirical and correlation measurements to score the alignment between observed and predicted spectra, among other important differences. Batch searching of LC/MS/MS data was performed using the D. melanogaster protein database from InterPro (www.ebi.ac.uk/interpro).
**Results and discussion**

Combining the high mass measurement accuracies of the LTQ-FT mass analyzer with tandem MS fragmentation data and nano-LC retention times allows for confident protein identifications with minimal sample consumption. A representative nano-LC/MS/MS identification of an Obp (PBP2_DROME Pheromone-binding protein-related protein 2 precursor) released from a female antenna is presented in Figure 1. Tandem mass spectrometric analysis of eluted chromatographic peaks allowed for the identification of approximately 100 proteins through Sequest and Mascot database search algorithms. Sequest was able to identify 30 proteins (Table 1), whereas analysis with Mascot resulted in 49% sequence coverage (shown in red font in the middle of the figure).

**Table 1** Soluble protein identifications (30 total) for Drosophila melanogaster antennae by Sequest (Obps are highlighted in bold font)

| Protein identification | Score (XC) | P       |
|------------------------|------------|---------|
| ATPB_DROME             | 101        | 6.02 x 10^{-1} |
| PBP2_DROME             | 101        | 6.02 x 10^{-1} |
| Q7K084_DROME           | 101        | 6.02 x 10^{-1} |
| SODC_DROME             | 101        | 6.02 x 10^{-1} |
| Q8SY92_DROME           | 101        | 6.02 x 10^{-1} |
| O16157_DROME           | 101        | 6.02 x 10^{-1} |
| OB10_DROME             | 101        | 6.02 x 10^{-1} |
| OB6_DROME              | 101        | 6.02 x 10^{-1} |
| Q7KUB0_DROME           | 101        | 6.02 x 10^{-1} |
| ENO_DROME              | 101        | 6.02 x 10^{-1} |
| Q9VF7_DROME            | 101        | 6.02 x 10^{-1} |
| Q9VTC3_DROME           | 101        | 6.02 x 10^{-1} |
| P91941_DROME           | 101        | 6.02 x 10^{-1} |
| PBP56D_DROME           | 101        | 6.02 x 10^{-1} |
| G3P1_DROME             | 101        | 6.02 x 10^{-1} |
| O16043_DROME           | 101        | 6.02 x 10^{-1} |
| PBP6_DROME             | 101        | 6.02 x 10^{-1} |
| PBP2_DROME             | 101        | 6.02 x 10^{-1} |
| Q7K084_DROME           | 101        | 6.02 x 10^{-1} |
| SODC_DROME             | 101        | 6.02 x 10^{-1} |
| Q8SY92_DROME           | 101        | 6.02 x 10^{-1} |
| O16157_DROME           | 101        | 6.02 x 10^{-1} |
| OB10_DROME             | 101        | 6.02 x 10^{-1} |
| OB6_DROME              | 101        | 6.02 x 10^{-1} |
| Q7KUB0_DROME           | 101        | 6.02 x 10^{-1} |
| ENO_DROME              | 101        | 6.02 x 10^{-1} |
| Q9VF7_DROME            | 101        | 6.02 x 10^{-1} |
| Q9VTC3_DROME           | 101        | 6.02 x 10^{-1} |
| P91941_DROME           | 101        | 6.02 x 10^{-1} |
| O16043_DROME           | 101        | 6.02 x 10^{-1} |

**Figure 1** nano-LC/MS/MS identification of PBP2_DROME Pheromone-binding protein-related protein 2 precursor—Drosophila melanogaster. A tryptic peptide, which eluted from the nano-LC column at a retention time (RT) of 10.29 min (chromatogram shown at the top of the figure), is identified by nano-LC/MS/MS (with database searching) to be derived from PBPRP2 precursor protein. The MS spectrum for this peptide is shown in the center of the figure. The ionized, doubly charged peptide is subjected to dynamic exclusion and fragmentation to generate an MS/MS fingerprint (lower spectrum) that conclusively verifies its molecular structure. As many as 11 peptides derived from PBPRP2 precursor protein were detected in total, providing 49% sequence coverage (shown in red font in the middle of the figure).

**XC** is the correlation score.
Table 2  Soluble protein identifications (94 total) for *Drosophila melanogaster* antennae by Mascot (Obps are highlighted in bold font; protein mass is in Daltons)

| Accession numbers | Protein identification | Protein score | Protein mass | Protein matches |
|-------------------|------------------------|---------------|--------------|----------------|
| sp|O02649|CH60_DROME | 60 kDa heat shock protein, mitochondrial | 35 | 60 885 | 1 |
| sp|P07487|G3P2_DROME | Glyceralddehyde-3-phosphate dehydrogenase 2 | 212 | 35 518 | 6 |
| sp|P08171|EST6_DROME | Esterase-6 | 168 | 61 486 | 4 |
| sp|P15007-1|ENO_DROME | Isoform B of enolase | 163 | 54 561 | 4 |
| sp|P18106-3|FPS_DROME | Isoform C of tyrosine-protein kinase Fps85D | 23 | 54 963 | 1 |
| sp|P20432|GSTT1_DROME | Glutathione S-transferase 1-1 | 120 | 24 022 | 3 |
| sp|P29746|BNB_DROME | Protein bangles and beads | 305 | 45 902 | 6 |
| sp|P31409|VATB_DROME | V-type proton ATPase subunit B | 34 | 54 800 | 1 |
| sp|P34739|TF2_DROME | Transcription termination factor 2 | 37 | 118 759 | 2 |
| sp|P35381|ATPA_DROME | ATP synthase subunit alpha, mitochondrial | 183 | 59 612 | 6 |
| sp|P41073-1|PEP_DROME | Isoform B of zinc finger protein on ecdysone puffs | 22 | 78 570 | 1 |
| sp|P46863|KL61_DROME | Bipolar kinesin KRP-130 | 26 | 121 772 | 1 |
| sp|P51123-1|TAF1_DROME | Isoform B of transcription initiation factor TFIID subunit 1 | 25 | 240 307 | 1 |
| sp|P54192|PB2_DROME | Pheromone-binding protein-related protein 2 | 509 | 17 170 | 11 |
| sp|P54193|PB3_DROME | Pheromone-binding protein-related protein 3 | 80 | 17 657 | 4 |
| sp|P54195|PB5_DROME | Pheromone-binding protein-related protein 5 | 39 | 15 484 | 1 |
| sp|P55830|RS3A_DROME | 40S ribosomal protein S3a | 31 | 30 565 | 1 |
| sp|P61851|SODC_DROME | Superoxide dismutase [Cu-Zn] | 333 | 15 974 | 6 |
| sp|P62152|CALM_DROME | Calmodulin | 38 | 16 800 | 2 |
| sp|Q05825|ATPB_DROME | ATP synthase subunit beta, mitochondrial | 186 | 54 074 | 5 |
| sp|Q09101-1|HIG_DROME | Isoform 3 of locomotion-related protein hikaru genki | 26 | 108 557 | 1 |
| sp|Q23970|PB6_DROME | Pheromone-binding protein-related protein 6 | 137 | 16 500 | 6 |
| sp|Q24120|CAPU_DROME | Protein cappuccino | 23 | 114 705 | 1 |
| sp|Q24407|ATPSI_DROME | ATP synthase-coupling factor 6, mitochondrial | 68 | 11 928 | 1 |
| sp|Q27377|OB10_DROME | Putative Obp A10 | 130 | 18 111 | 2 |
| sp|Q32KD2|SETB1_DROME | Histone-lysine N-methyltransferase eggless | 38 | 143 852 | 1 |
| sp|Q85Y61|OB56D_DROME | General Obp 56d | 181 | 14 452 | 4 |
| sp|Q9NJG9-1|SUZ12_DROME | Isoform 1 of polycomb protein Su(z)12 | 34 | 101 011 | 1 |
| sp|Q93P0|PRDX1_DROME | Peroxiredoxin 1 | 118 | 21 952 | 3 |
| sp|Q9785|3BP5H_DROME | SH3 domain-binding protein 5 homolog | 22 | 54 060 | 1 |
| sp|Q97N5-1|VATC_DROME | Isoform D of V-type proton ATPase subunit C | 26 | 79 776 | 1 |
| sp|Q9655|CH60B_DROME | 60 kDa heat shock protein homolog 1, mitochondrial | 34 | 68 992 | 1 |
| sp|Q9VPS5|CH60B_DROME | Neuropeptide-like 2 | 92 | 9463 | 1 |
| sp|Q9W1C9|PEB3_DROME | Ejaculatory bulb-specific protein 3 | 73 | 14 759 | 1 |
| sp|Q9W1R5|VIR_DROME | Protein virilizer | 29 | 210 751 | 1 |
| sp|Q9V401-1|CISY_DROME | Isoform A of probable citrate synthase, mitochondrial | 34 | 51 713 | 3 |
| tr|A126K9|A126K9_DROME | CG17994-PA | 25 | 59 821 | 1 |
| tr|A127Z9|A127Z9_DROME | CG1625-PA | 26 | 129 850 | 2 |
| Accession numbers | Protein identification                          | Protein score | Protein mass | Protein matches |
|-------------------|-----------------------------------------------|---------------|--------------|----------------|
| tr|A1ZA97|A1ZA97_DROME | CG8424-PA | 80 | 62456 | 2 |
| tr|A4V3F9|A4V3F9_DROME | Fructose-bisphosphate aldolase | 193 | 39 251 | 5 |
| tr|A8DZ12|A8DZ12_DROME | CG15140-PA (Fragment) | 21 | 30 108 | 4 |
| tr|A8JV09|A8JV09_DROME | CG4532-PF, isoform F | 21 | 140 127 | 1 |
| tr|A8Q120|A8Q120_DROME | CG41561-PA (Fragment) | 31 | 40 471 | 1 |
| tr|O16043|O16043_DROME | CG2207-PA, isoform A (CG2207-PB, isoform B) (CG2207-PF, isoform F) (LD21289p) (Anon1A4) | 90 | 18 812 | 1 |
| tr|Q97102|Q97102_DROME | CG4494-PA | 82 | 10 175 | 1 |
| tr|Q0E8L0|Q0E8L0_DROME | CG8486-PC, isoform C | 26 | 305 750 | 1 |
| tr|Q4ABG9|Q4ABG9_DROME | CG33715-PE, isoform E | 22 | 1 064 061 | 2 |
| tr|Q4V516|Q4V516_DROME | IPO7112p (IP06812p) (CG14810-PA) | 26 | 22 874 | 1 |
| tr|Q59DY8|Q59DY8_DROME | CG33552-PA | 24 | 18 848 | 1 |
| tr|Q7JND6|Q7JND6_DROME | Cuticle protein ACP65A | 123 | 10 773 | 1 |
| tr|Q7K084|Q7K084_DROME | RH04549p (CG2297-PA) Obp 44a | 213 | 16 135 | 5 |
| tr|Q7K088|Q7K088_DROME | RH03850p (CG8462-PA) Obp 56e | 44 | 14 505 | 1 |
| tr|Q7K2B0|Q7K2B0_DROME | LD11455p (CG7137-PA) | 26 | 40 605 | 1 |
| tr|Q7KMR7|Q7KMR7_DROME | Thioredoxin-like protein TXL | 25 | 32 140 | 1 |
| tr|Q7KTB7|Q7KTB7_DROME | CG6214-PM, isoform M | 26 | 174 219 | 1 |
| tr|Q7KUB0|Q7KUB0_DROME | CG7176-PA, isoform A (CG7176-PE, isoform E) (CG7176-PF, isoform F) | 192 | 47 030 | 6 |
| tr|Q8I940|Q8I940_DROME | CG1271-PD, isoform D | 22 | 60 539 | 1 |
| tr|Q8IRD3|Q8IRD3_DROME | Glutathione peroxidase | 23 | 26 620 | 1 |
| tr|Q8MLS0|Q8MLS0_DROME | CG13551-PC, isoform C | 65 | 9711 | 1 |
| tr|Q8MSI2|Q8MSI2_DROME | GH15296p (CG15848-PA) | 172 | 21 919 | 4 |
| tr|Q8MSU4|Q8MSU4_DROME | CG2097-PA | 26 | 133 020 | 2 |
| tr|Q8MYW5|Q8MYW5_DROME | CG14667-PA, isoform A | 20 | 30 574 | 1 |
| tr|Q8SWW8|Q8SWW8_DROME | LD18186p (CG12489-PA) | 25 | 74 562 | 1 |
| tr|Q8SX06|Q8SX06_DROME | RH33338p (CG14141-PA) | 30 | 18 788 | 1 |
| tr|Q8SX20|Q8SX20_DROME | RE47719p | 24 | 56 222 | 1 |
| tr|Q8SY92|Q8SY92_DROME | RH21971p antennal dehydrogenase | 327 | 27 362 | 5 |
| tr|Q8T3H5|Q8T3H5_DROME | AT28279p, CG13382 | 33 | 59 050 | 1 |
| tr|Q8T3Y1|Q8T3Y1_DROME | AT26187p, CG17440 | 25 | 42 763 | 1 |
| tr|Q8T487|Q8T487_DROME | AT10439p, CG1950 | 29 | 39 778 | 2 |
| tr|Q8TSQ5|Q8TSQ5_DROME | SD05887p, CG3493 | 26 | 170 613 | 2 |
| tr|Q8T9I2|Q8T9I2_DROME | GM13608p, Bip1 | 27 | 46 380 | 1 |
| tr|Q95RB2|Q95RB2_DROME | CG8505-PA, Cpr49Ae | 94 | 14 501 | 1 |
| tr|Q95RB2|Q95RB2_DROME | CG8505-PA, Cpr49Ae | 88 | 14 501 | 1 |
| tr|Q961M4|Q961M4_DROME | GH15731p | 28 | 94 143 | 1 |
| tr|Q9NHV6|Q9NHV6_DROME | Dorsal interacting protein 2 | 37 | 26 140 | 1 |
| tr|Q9U1K3|Q9U1K3_DROME | Globin1 | 40 | 17 149 | 1 |
the identification of 92 proteins (Table 2) with high statistical confidence. Examination of the identities of these proteins indicates that they originate both from the extracellular perilymph and from the cytoplasm of disrupted cells. We classified these proteins into 8 major categories (Table 3; Figure 2). These include enzymes involved with intermediary metabolism, primarily the glycolytic pathway, tricarboxylic acid cycle, and oxidative phosphorylation; proteins associated with regulation of gene expression, nucleic acid metabolism and protein metabolism, including regulation of transcription, histone modification and mRNA splicing, protein folding, and degradation; proteins associated with microtubular transport; Obps; protective enzymes associated with antibacterial defense and defense against oxidative damage; cuticular proteins; miscellaneous proteins associated with programmed cell death (Pcs), neuropeptide hormone activity, regulation of mating and courtship behavior (Esterase-6), oxygen transport (glob1), tyrosine kinase activity (Fps85D), and cell adhesion (hig); and proteins of unknown function, which represented the largest group comprising about one-third of all soluble proteins identified (Table 3; Figure 2).

We detected substantially fewer proteins in male antennal extracts than in antennal extracts from females (see Supplementary Tables 1–4). This is most likely due to greater resistance to cell disruption under the conditions used in antennae from males than females, for unknown reasons. However, 6 of 8 Obps and 4 of 8 proteins associated with biotic and abiotic defense were found both in male and female antennae, suggesting that extracellular proteins in the perilymph are released effectively from male antennae and that detection of Obp44a and Obp36e in female but not male samples may reflect true sexual dimorphism in the expression of Obps, in line with previous observations (Anholt et al. 2003). Pbprp2 (aka Obp19d), Pbprp3 (aka Os-F, Obp83a), Pbprp5 (aka Obp28a), and Pbprp6 (aka Os-E, Obp83b) have been localized previously to antennae (Pikielny et al. 1994; Hekmat-Scafe et al. 1997; Shandbag et al. 2001). In these initial experiments, we identified only 8 members of the Obp family, which comprises more than 50 Obp genes (Hekmat-Scafe et al. 2002). Notably, absent was the Lush protein (Kim et al. 1998), which mediates recognition of the male courtship pheromone 11-cis-vaccenylacetate in T1 trichoid sensilla of the antenna (Xu et al. 2005; Laughlin et al. 2008). It is possible that the amounts of Lush are below our detection limit. The same may be true for other members of the Obp family. Indeed, when the amount of tissue was increased from 120 to 500 antennae, we were able to detect a larger number of proteins with 10 additional Obps, including Lush, A5, Pbprp1 (aka Obp69a), Pbprp4 (aka Obp84a),

| Accession numbers | Protein identification | Protein score | Protein mass | Protein matches |
|-------------------|------------------------|---------------|--------------|----------------|
| tr|Q9U3Y5|Q9U3Y5_DROME | Dynnein heavy chain | 25 | 529 605 | 1 |
| tr|Q9V3H9|Q9V3H9_DROME | BcDNA.LD27873 | 26 | 113 870 | 1 |
| tr|Q9V677|Q9V677_DROME | CG6954-PA, CG6954 | 29 | 181 921 | 1 |
| tr|Q9VE81|Q9VE81_DROME | CG7998-PA (IP09655p) | 122 | 35 524 | 2 |
| tr|Q9VF51|Q9VF51_DROME | F104488p | 89 | 139 567 | 3 |
| tr|Q9VGD4|Q9VGD4_DROME | CG17471-PA | 23 | 193 892 | 1 |
| tr|Q9V847|Q9V847_DROME | CG9492-PA | 33 | 539 599 | 1 |
| tr|Q9V917|Q9V917_DROME | CG9318-PA (LD44273p) | 24 | 75 732 | 2 |
| tr|Q9V9F7|Q9V9F7_DROME | CG9894-PA, isoform A (CG9894-PB, isoform B) (RE38782p) | 180 | 15 508 | 3 |
| tr|Q9VQT8|Q9VQT8_DROME | CG16712-PA (RH38008p) (RH05411p) | 75 | 9144 | 1 |
| tr|Q9VS81|Q9VS81_DROME | CG4022-PA | 29 | 57 600 | 1 |
| tr|Q9VTC3|Q9VTC3_DROME | CG6409-PA (GH07049p) | 86 | 40 356 | 4 |
| tr|Q9V1A9|Q9V1A9_DROME | CG11290-PA | 27 | 257 089 | 2 |
| tr|Q9W246|Q9W246_DROME | CG4554-PA | 27 | 313 818 | 1 |
| tr|Q9W306|Q9W306_DROME | CG9691-PA, isoform A (CG9691-PB, isoform B) (RH12290p) | 57 | 13 081 | 1 |
| tr|Q9W321|Q9W321_DROME | CG15319-PB | 37 | 343 477 | 2 |
| tr|Q9W383|Q9W383_DROME | CG1885-PA (GH17465p) | 35 | 31 218 | 2 |
| tr|Q9W4C3|Q9W4C3_DROME | Ubiquitin carboxyl-terminal hydrolase, CG4165 | 38 | 122 838 | 3 |
Obp19a, Obp47b, Obp56a, Obp59a, Obp99b, and Obp99c. Furthermore, some Obps may not be expressed in antennae. For example, some Obps are expressed in the tarsi (Galindo and Smith 2001; Matsuo et al. 2007), fat body (Fujii and Amrein 2002), or male accessory gland (Takemori and Yamamoto 2009) and therefore would not be detected in the antenna.

| Table 3 | The soluble proteome of Drosophila melanogaster antennae |
|---------|----------------------------------------------------------|
| **Intermediary metabolism (17.4%)** | |
| Glyceraldehyde phosphate dehydrogenase | |
| Glycerol kinase | |
| **Aldolase** | |
| Enolase | |
| l-Malate dehydrogenase | |
| Citrate synthase | |
| Isocitrate dehydrogenase | |
| ATP synthase uncoupling factor 2 | |
| Vha44 | |
| Vha55 | |
| **Blw, ATP synthase** | |
| ATP synthase subunit B | |
| CG6054 | |
| CG14741 | |
| CG1885 | |
| Calmodulin | |
| **Regulation of gene expression, nucleic acid metabolism, and protein metabolism (17.3%)** | |
| Taf1 | |
| Lds | |
| Pep | |
| Histone-lysine N-methyltransferase | |
| Su(2)12 | |
| Cg11290 | |
| Vir | |
| Nej | |
| CG2207 | |
| CG33715 | |
| **Hsp60** | |
| Ribosomal protein S3A | |
| CG4494 | |
| CG2097 | |
| CG1950 (ubiquitin thiolesterase) | |
| CG4165 (ubiquitin thiolesterase) | |
| **Cytoskeletal organization/microtubule movement (5.5%)** | |
| Capu | |
| CG4532 | |
| Klp61F | |
| **CG9492** | |
| Dynnein heavy chain KI-5 | |
| **Obps (8.7%)** | |
| Pbprp2 | |
| Pbprp3 | |
| Pbprp5 | |
| **Pbprp6** | |
| A10 | |
| Obp44a | |
| Obp56d | |
| Obp56e | |
| **Oxidative enzymes and defense mechanisms (8.7%)** | |
| Peroxiredoxin 1 | |
| Superoxide dismutase | |
| GsbD1 | |
| Tfl | |
| CG6214 | |
| Glutathione peroxidase | |
| CG13551 | |

| Table 3 | Continued |
|---------|------------|
| **Dnr1** | |
| **Unknown function (33.7%)** | |
| CG17994 | |
| CG1625 | |
| CG8424 | |
| CG41561 | |
| CG8486 | |
| CG14667 | |
| Brnb (gliogenesis) | |
| Pebill | |
| CG14810 | |
| CG33552 | |
| CG7137 | |
| CG14141 | |
| Antdh | |
| CG13382 | |
| CG17440 | |
| CG15296 | |
| CG3493 | |
| **GH15731p** | |
| BcDNA-LD27873 | |
| CG60954 | |
| Dip2 | |
| CG9318 | |
| CG9894 | |
| CG16712 | |
| CG6409 | |
| CG4554 | |
| CG9691 | |
| CG15140 | |
| Smid | |
| Bip1 | |
| CG4022 | |
| **Structural proteins of the cuticle (2.2%)** | |
| Acp65Aa | |
| Cpr49Ae | |
| **Miscellaneous (6.5%)** | |
| Pcs (programmed cell death) | |
| **Nplp2 (neuropeptide hormone activity)** | |
| Esterase-6 (regulation of receptivity, sperm competition, mating, pheromone synthesis, courtship behavior) | |
| Globin 1 (oxygen transport) | |
| Pfs85D (protein tyrosine kinase activity; photoreceptor cell morphogenesis) | |
| Hig (cell adhesion) | |

*aInformation in this table is derived from Tables 2 and 3. Underlined entries designate identification in female antennae only; italic font indicates identification in male antennal extracts only; and bold font indicates identification in both male and female antennal extracts.*
The vast majority of proteins we detected are widely expressed in many or all cells. Nevertheless, their functions in different cells are essential for enabling distinct physiological functions. For example, intermediary metabolism is necessary to provide energy for olfactory signal transduction, and cytoskeletal organization is essential for maintaining dendritic and axonal structure and function. Two of the categories of soluble proteins indicated in Table 3 are specifically relevant to chemosensation: Obps, which are essential for the transport of hydrophobic odorants in the perilymph, and oxidative enzymes and defense mechanisms which likely contribute to cytochrome P450-mediated inactivation or degradation of odorants and environmental toxins. Obps have been defined based on their structure, including the characteristic positions of disulfide bonds (Hekmat-Scafe et al. 2002). However, olfactory functions have been ascribed to only few members of this family and other possible functions, such as a carrier function for molecules transmitted between males and females during mating have also been noted. Altered regulation of expression of Obp genes has been observed following mating (McGraw et al. 2004; Zhou et al. 2009) and after exposure to starvation stress (Harbison et al. 2005) or alcohol intoxication (Morozova et al. 2006). In addition, changes in expression levels of Obp genes occur as a correlated response to artificial selection for divergent levels of copulation latency (Mackay et al. 2005) and aggression (Edwards et al. 2006), and expression levels change during social crowding and as a result of ageing (Zhou et al. 2009). Systems genetics analyses of 6 X chromosome linked Obp genes showed that their transcripts form part of diverse transcriptional network niches, associated with olfactory behavior, synaptic transmission, detection of signals regulating tissue development and apoptosis, postmating behavior and oviposition, and nutrient sensing (Arya GH, Weber AL, Wang P, Magwire MM, Serrano Negron YL, Mackay TFC, Anholt RRH, unpublished data). Our proteomics analysis has identified Obps that are expressed in the antenna and thus are candidates for contributing directly to chemosensation.

Although the second antennal segment might also contribute to the proteomics profile, the fact that Obps make up a substantial fraction of the soluble proteome (Figure 2) suggests that the major contribution to the mass spectrometric profile is derived from the third antennal segment.

An additional caveat is the notion that protein detection could be limited by masking of peptide fragments in the nano-LC/MS/MS analyses. Comigration on the LC might...
also have prevented the detection of Lush or other Obps. This problem is likely to be more severe with increasing complexity of the chromatogram and more likely to impact proteins that yield few tryptic fragments, as well as those present in very low concentrations, relative to others. In addition, variations in ionization efficiencies of different peptides can lead to diminished representation in the nano-LC/MS/MS run. Furthermore, it should be noted that the proteins presented in Tables 1 and 2 represent only those that attain a high confidence score with Sequest and Mascot, respectively. Increasing the number of antennae in each sample greatly increases the number of proteins that can be interrogated by nano-LC/MS/MS approaches. This improves the chances for unambiguous identification of proteins such as Obp99d (Figure 3)—previously implicated in responses to benzaldehyde (Wang et al. 2007)—which, while detected by Sequest, did not produce a very confident identification and therefore is not included in Table 1, and Mascot did not allow for the identification of this protein. Nevertheless, information about the presence of Obp99d, which is present in low abundance, can still be extracted from the chromatogram (Figure 3).

Finally, whereas integration of chromatographic peaks can establish relative amounts of proteins represented in the sample, precise quantitative determination requires stable isotope labeled internal standard peptides. This approach is now feasible for targeted determination of specific soluble proteins of the antenna.

**Supplementary material**

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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