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Identification of the endogenous cysteine-rich peptide trissin, a ligand for an orphan G protein-coupled receptor in *Drosophila*

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ABSTRACT

There are many orphan G protein-coupled receptors (GPCRs), for which ligands have not yet been identified, in both vertebrates and invertebrates, such as *Drosophila melanogaster*. Identification of their cognate ligands is critical for understanding the function and regulation of such GPCRs. Indeed, the discovery of bioactive peptides that bind GPCRs has enhanced our understanding of mechanisms underlying many physiological processes. Here, we identified an endogenous ligand of the *Drosophila* orphan GPCR, CG34381. The purified ligand is a peptide comprised of 28 amino acids with three intrachain disulfide bonds. The preprotein is coded for by gene CG14871. We designated the cysteine-rich peptide "trissin" (it means for triple S–S bonds) and characterized the structure of intrachain disulfide bonds formation in a synthetic trissin peptide. Because the expression of trissin and its receptor is reported to predominantly localize to the brain and thoracicoabdominal ganglion, trissin is expected to behave as a neuropeptide. The discovery of trissin provides an important lead to aid our understanding of cysteine-rich peptides and their functional interaction with GPCRs.

1. Introduction

G protein-coupled receptors (GPCRs) constitute a large protein superfamily, which shares a 7-transmembrane motif as a common structure. Human genome sequencing has identified several hundred orphan GPCRs, for which ligands have not yet been identified [1]. Meanwhile, the recent sequencing of the *Drosophila melanogaster* genome [2] has enabled the identification of at least 160 fly GPCRs [3]. *Drosophila* is an excellent animal model for genetic analysis, as it is a small organism with a relatively short lifecycle and can be bred easily under laboratory conditions. Combined with the possibility of manipulating the *Drosophila* genome, this offers a powerful tool for studying developmental and behavioral processes. GPCRs play crucial roles in cell-to-cell communication involved in a variety of physiological phenomena, and are the most common target of pharmaceutical drugs. Therefore, identification of endogenous ligands for orphan GPCRs will lead to clarification of novel physiological regulatory mechanisms and potentially facilitate development of new GPCR-targeted therapeutics. Recently, many bioactive molecules have been discovered or identified as endogenous ligands of orphan GPCRs by using reverse pharmacology [4]. These include nociceptin, prolactin-releasing peptide, orexin, apelin, ghrelin, metastin, and neuromedin S [5–12]. Presently, the discovery of novel endogenous ligands for orphan GPCRs in mammals is challenging. In part, this may be due to the very restricted timing of expression or distribution pattern of GPCR ligands. Structural or sequence comparison of newly discovered peptides in *Drosophila* with those in mammals may lead to the discovery of new peptide signaling modules. Here, we report the identification of trissin, a ligand for the orphan GPCR CG34381

Abbreviations: GPCRs, G protein-coupled receptors; CHO, Chinese hamster ovary; CM, carboxymethyl; ACN, acetonitrile; TFA, trifluoroacetic acid; PTH, phenyl thiohydantoin.

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in *D. melanogaster*. Trissin has 28 amino acids, three disulfide bonds, and no significant structural similarities to known endogenous peptides. Cysteine-rich peptides are known to have antimicrobial or toxicant activities, although frequently their mechanism of action is poorly understood. Our data provide an important lead to aid our understanding of cysteine-rich peptides and their functional interaction with GPCRs in both insect and mammals.

2. Materials and methods

2.1. Construction of an assay system using CG34381-expressing cells

The full-length cDNA of *Drosophila* CG34381 (GenBank Accession No. NM_135118; residues 515–2656) was obtained by RT-PCR, with *Drosophila* cDNA as the template. The sense and antisense primers were 5′-gcagcgacgtcctttattgg-3′ and 5′-aagtgggtccatccttcgat-3′, respectively. The amplified cDNA was ligated into a pcDNA3.1 vector (Invitrogen, Tokyo, Japan). The expression vector CG34381-pcDNA3.2 was transfected into Chinese hamster ovary (CHO) cells. Thereafter, stably expressing cells were selected using 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). The selected cell line CHO-CG34381-line 9–26 showed the highest expression of CG34381 mRNA. The cells were cultured in a humidified environment of 95% air:5% CO_2. Changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured using the FlexStation 3 fluorometric imaging plate reader (Molecular Devices, CA, USA). CHO-CG34381 cells (3×10^4 cells) were plated into 96-well black-wall microplates (Corning, NY, USA) 20 h before each assay. The cells were incubated with 100 μl of the Calcium 4 assay kit (Molecular Devices) for 1 h, and then 50 μl of each sample was added to the CHO-CG34381 cells. The maximum $[Ca^{2+}]_i$ change was then determined as a response.

2.2. Purification of Drosophila trissin

A total of 400 g of *D. melanogaster* flies (Canton S.) was collected on dry ice. A basic peptide fraction was prepared as described previously [13] and then fractionated on a Sephadex G-50 gel filtration column (2.9 × 142 cm; GE Healthcare, Tokyo, Japan). A portion of each fraction, equivalent to 1.16 g of flies, was subjected to the Calcium 4 assay using CHO-CG34381 cells. The active fraction was separated by carboxymethyl (CM)-ion exchange high-performance liquid chromatography (HPLC) on a TSK CM-2SW column (4.6 × 250 mm; Tosoh, Tokyo, Japan) with an ammonium acetate ($HCOONH_4$) (pH 6.5) gradient of 10 mM to 1 M in the presence of 10% acetonitrile (ACN) at a flow rate of 1 ml/min for 180 min. The active fraction was further purified by fractionation on the same column at pH 4.8. The active fractions were separated by reverse-phase (RP)-HPLC with a μBondasphere C18 column (3.9 × 150 mm; Waters, MA, USA) with a 10–60% ACN/0.1% trifluoroacetic acid (TFA) linear gradient at a flow rate of 1 ml/min for 80 min. The active fractions were further purified by RP-HPLC using a diphenyl column (2.1 × 150 mm; 219TP5125, Vydac, Hesperia, CA, USA) for 80 min under a linear gradient of 10–60% ACN/0.1% TFA at a flow rate of 0.2 ml/min. Fractions corresponding to absorption peaks were collected, and an aliquot of each fraction (2 g tissue equivalent) was assayed by the FLEX system. Approximately 5 pmol of the final purified peptides were analyzed with a protein sequencer (Model 494; Applied Biosystems, CA, USA). Approximately 0.5 pmol of the active fraction was used for molecular weight determination by matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) mass spectrometry with a Voyager-DE PRO instrument (Applied Biosystems).

2.3. Cloning of Drosophila prepro-trissin cDNA

A tblastn search of the *Drosophila* genome resources was performed using the sequence of the purified peptides, and a *D. melanogaster* mRNA sequence (CG14871; NM_142214) derived from an annotated genomic sequence was obtained. We searched for the open reading frame both upstream and downstream of the genome sequence of CG14871 by using specific primers (5′-gttcacatgcccactggagtc-3′; 5′-ctggggatatccttaggggtagtag-3′). The candidate PCR product was subcloned into the pCR-II TOPO vector and sequenced. The nucleotide sequence of the isolated cDNA fragment was determined by automated sequencing (DNA sequencer: model 3100, Applied Biosystems) according to the protocol for the BigDye terminator cycle sequencing kit (Applied Biosystems).

2.4. Peptide synthesis and structural analysis

Trissin was synthesized by Peptide Institute, Inc (Osaka, Japan). Three disulfide bonds (S–S bonds) were spontaneously formed by oxidation. Synthetic S–S bonded peptides was digested by thermolysin or endoproteinase Asp-N and analyzed with a protein sequencer or mass spectrometry.

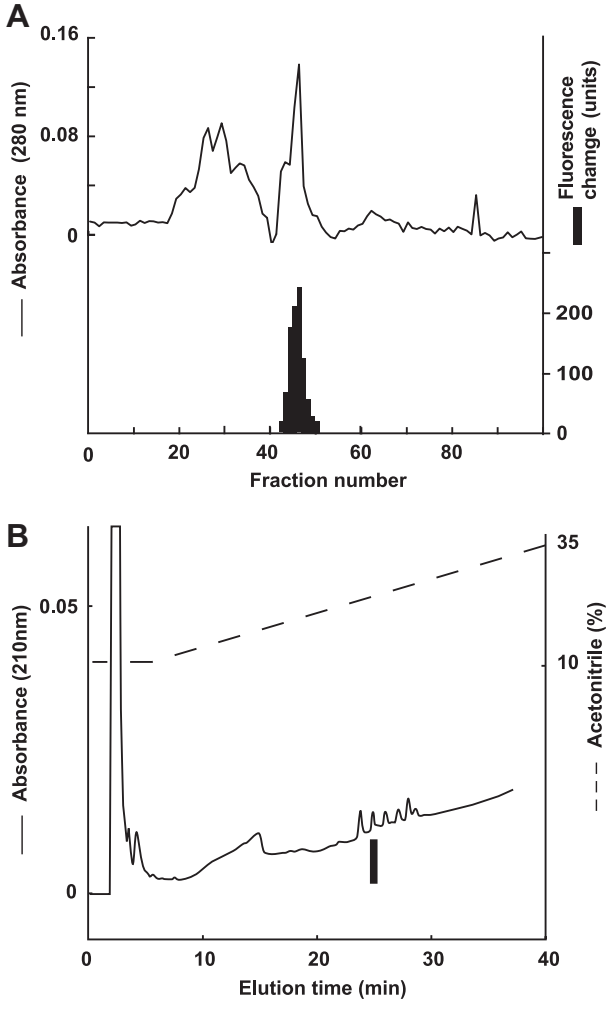

Fig. 1. Purification of trissin from fly extracts. *Black bars* indicate fluorescence signal changes of $[Ca^{2+}]_i$ in CHO-CG34381 cells. (A) G-50 gel filtration of the basic peptide fraction of fly extracts. Active fraction was subjected to two steps of CM-ion exchange HPLC and 2 steps of RP-HPLC. (B) Final purification of the active fraction by RP-HPLC.

3. Results and discussion

The first $[Ca^{2+}]_i$ assays were performed using the gel filtration samples as part of the effort to isolate the endogenous ligand of CG34381 (Fig. 1A). Nine sequential fractions exhibited activity (numbers 43–51). Fractions 46–47, which contained particularly high activity, were further separated by CM-ion exchange HPLC at pH 6.5 and pH 4.8. The active fraction was purified as a single peak in the final RP-HPLC (Fig. 1B). The final yield of the purified peptide was approximately 5.5 pmol. A partial N-terminal amino acid sequence of the purified peptide was determined to be IKXDTXGKEXASAXGTKHFRTXXFNY, using a protein sequencer (where X is a position that was not identified). For elucidating the complete amino acid sequence of the peptide, a *Drosophila* cDNA encoding the purified peptide was isolated by RT-PCR. The cDNA encoded a 108-residue protein (CG14871) that contained

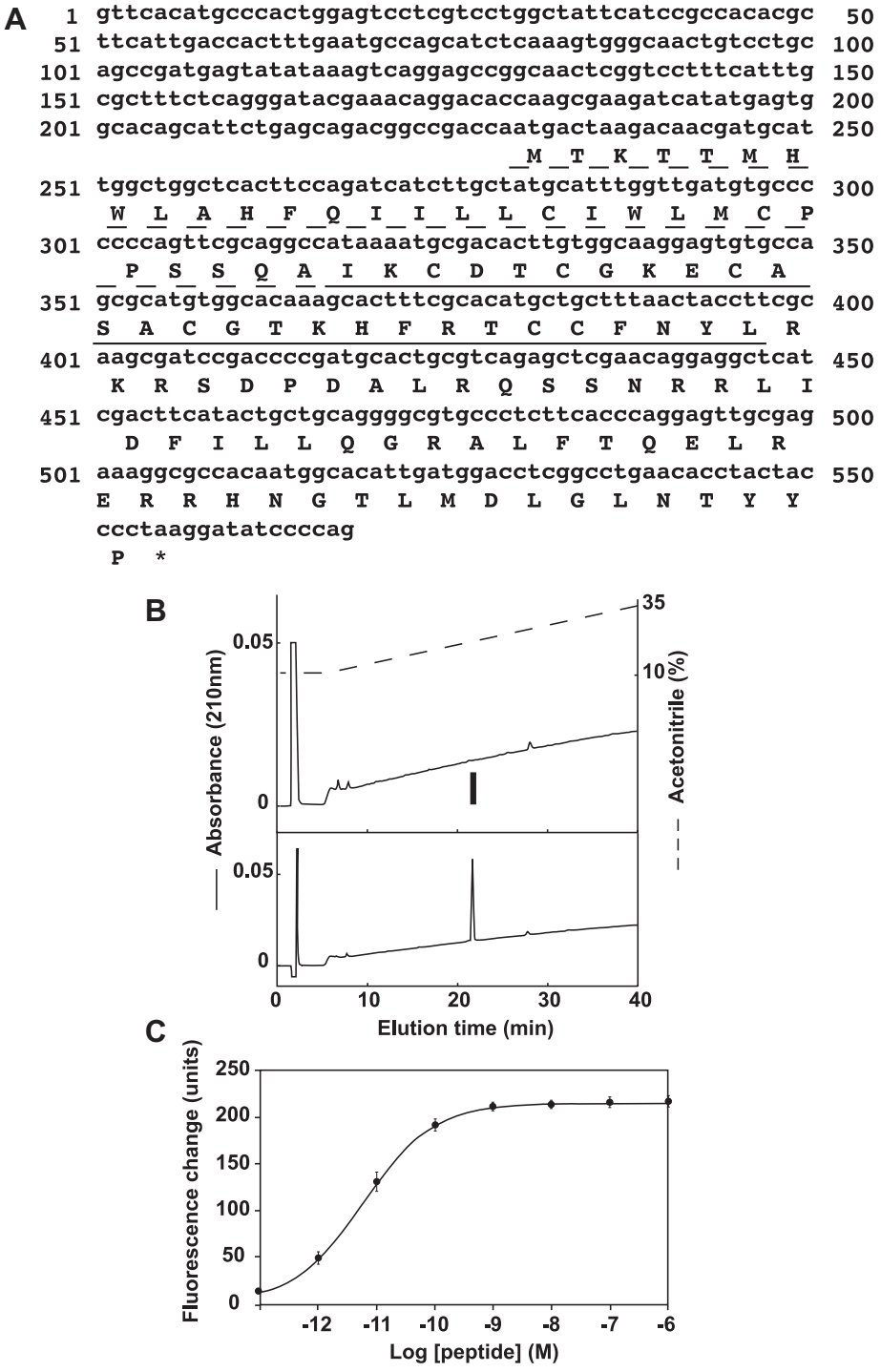

Fig. 2. (A) Nucleotide sequence and deduced amino acid sequence of trissin. The trissin cDNA encodes a 108-residue protein. The dotted line denotes the predicted signal peptide, which preceded the sequence of trissin itself (continuous underline). (B) Chromatographic comparison by RP-HPLC of natural trissin (upper panel) and synthetic trissin (lower panel). *Black bars* indicate the fluorescence signal changes of $[Ca^{2+}]_i$ in CHO-CG34381 cells (upper panel). Each peptide was applied to a symmetry C18 column with a linear gradient elution for 80 min. (C) Dose–response relationship of changes in $[Ca^{2+}]_i$ for synthetic trissin in CHO-CG34381 cells. Each symbol on the line graph represents the mean ± S.E.M. of data from six replicates for each dose.

features characteristic of an N-terminal signal peptide immediately preceding the purified peptide sequence. It also indicated that every X residue was cysteine, while the rest of the sequence was identical to that determined by peptide sequencing (Fig. 2A). Sequencing resulted in a very low yield of phenyl thiohydantoin (PTH) at the steps involving X, suggesting that all these cysteines might form disulfide bonds (S–S bonds). This preproprotein contained a potential processing site at the C-terminal end of the purified peptide sequence. We therefore deduced the primary structure of the newly purified 28-residue peptide to be IKCDTCGKECASACGTKHFRTCCFNYL. Mass spectrometric analysis revealed that the observed monoisotopic *m/z* value of the purified peptide (2996.0182) was very close to the theoretically predicted value for this peptide (2996.2446), which has three intrachain disulfide bonds. Since the exact positions of S–S bonds in purified peptide were unknown, we generated a synthetic peptide and subjected it to oxidizing conditions. This lead to the formation of three S–S bonds that were confirmed by amino acid analysis and mass spectrometry. The synthetic peptide had an identical retention time to the natural peptide according to RP-HPLC (Fig. 2B). We also examined the interaction of the synthetic peptide with the GPCR CG34381. This peptide induced dose-dependent, robust increases in $[Ca^{2+}]_i$ in CHO-CG34381, with half-maximal response concentrations (EC_{50}) of 5.73×10^{-12} M (Fig. 2C). The natural peptide and synthetic peptide showed comparable potency and efficacy for this receptor. Neither peptide induced a response in CHO cells transfected with vector alone (data not shown). These results suggest that the structure of the synthetic peptide is nearly identical to that of the natural peptide. To identify the structure of three intrachain disulfide bonds, we first digested the synthetic peptide with thermolysin. Three digested segments (TH1, TH2, and TH3) were obtained and each segment was analyzed by mass spectrometry (Fig. 3A). In addition, TH1 was analyzed with a protein sequencer. Because of sequences information, there are three segments which were bridged by three S–S bonds in TH1. We then digested the TH1 segment with endoproteinase Asp-N. Two digested segments (TH1/D1 and TH1/D2) were obtained and each segment was analyzed by mass spectrometry (Fig. 3B). These data suggest that TH1/D1 has an S–S bond (Cys3–Cys14) and TH1/D2 has two potential S–S bonds, although the mode of disulfide pairings in the latter peptide remained unclear. Theoretically, two types of S–S modes could be considered for TH1/D2, i.e. A-type and B-type as illustrated in Fig. 3B. Sequence analysis confirmed that TH1/D2 has the A type S–S bonds (Cys6–Cys23 and Cys10–Cys22), since $PTH(Cys)_2$ was observed at the fifth and seventh cycles. Therefore, we propose that the structure of this peptide is IKCDTCGKECAS ACGTKHFRTCCFNYL (Cys3–Cys14, Cys6–Cys23, Cys10–Cys22) and designate it 'trissin' (Fig. 3C). We have not found this disulfide linkage type, C^{I}–C^{IV}, C^{II}–C^{VI}, C^{III}–C^{V}, in other cysteine-rich peptides. Trissin shares high sequence similarity with peptides from various insects (Fig. 4). Kaplan et al. reported raalin as a predicted toxin-like peptide from *in silico* studies of *Apis mellifera* [14]. The amino acid sequence of raalin is DQCGRKCANICGTQQFPACCFN, and it therefore shares some similarity with trissin. However, the deduced sequence of raalin has only five cysteines, and characteristic N-terminal signal peptide or C-terminal processing sites were not identified. Therefore, we suggest that raalin may be derived from a longer polypeptide homologous to the *Drosophila* trissin preproprotein. Further studies are required to determine whether the structure of honeybee raalin is similar to that of trissin.

Cysteine-rich peptides have diverse physiological functions, and further studies are now required to place trissin within this polypeptide class. The cysteine-rich defensin family exhibits antimicrobial activity in vertebrates, invertebrates, and plants [15,16], whereas scorpion toxins and conus snail venoms have toxicant activities [17,18]. In addition, LUREs attract competent pollen tubes

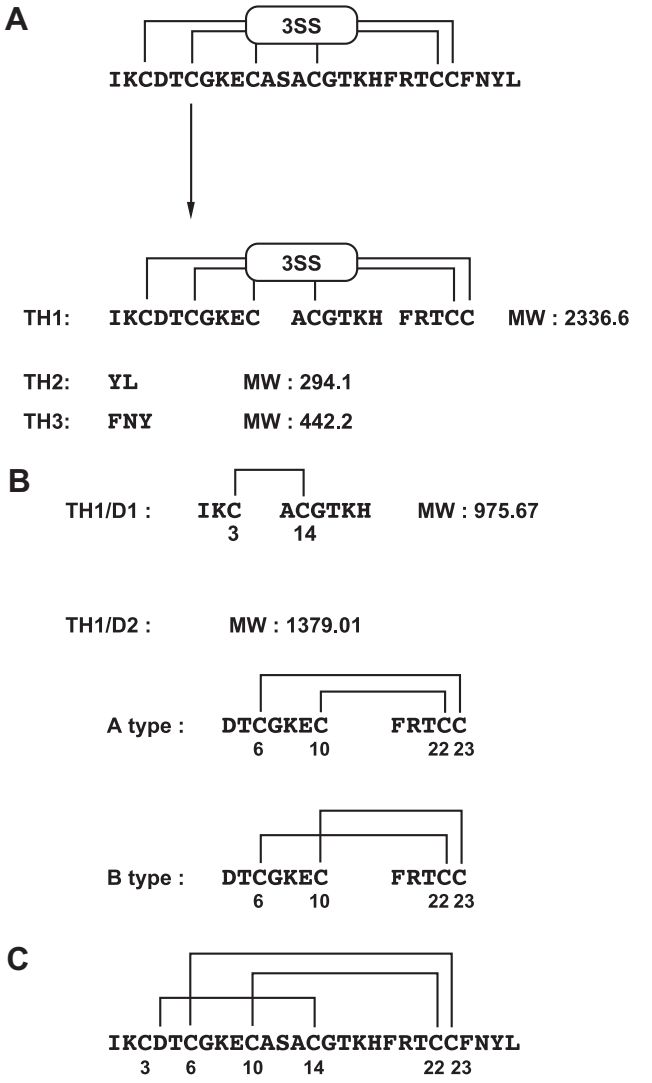

Fig. 3. Structure determination of three intrachain disulfide bonds in trissin. (A) Structures of the peptides obtained by digesting the synthetic trissin with thermolysin. (B) Structures of TH1/D1 and TH1/D2 obtained by digesting TH1 with endoproteinase Asp-N. Two possible disulfide structures, i.e. A-type and B-type could be considered for TH1/D2. (C) Structure of trissin.

in plants [19]. The activities of defensins and LUREs are not mediated by GPCRs. Rather, defensins act as effectors of immunity via a direct interaction with the microbial membrane that leads to disruption or lesion formation, or their translocation into the cytoplasm, where they can reach intramolecular targets [20]. On the other hand, most receptors recognized by scorpion toxins and conus snail venoms are ion channels [20,21]. Prokineticin-1 and -2, which are mammalian homologs of the black mamba peptide VPRA/MIT1 and frog skin peptide Bv8, contain 10 cysteine residues. These polypeptides are the cognate ligands for two closely related GPCRs that couple either to G*i* or G*q* [22–24]. The distribution of defensins is consistent with their defensive role, as they are mainly found in immunocompetent cells or surface epithelia [20]. Prokineticins are expressed not only in peripheral tissues, but also in the central nervous system [25]. Intracerebroventricular injection of prokineticin-2 controls the circadian rhythm from the SCN [26]. Like prokineticins, we show here that trissin is a cognate GPCR ligand. However, the physiological role of trissin remains to be determined. Both trissin (CG14871) and the trissin receptor (CG34381) are expressed predominantly in the brain and thoracicoabdominal

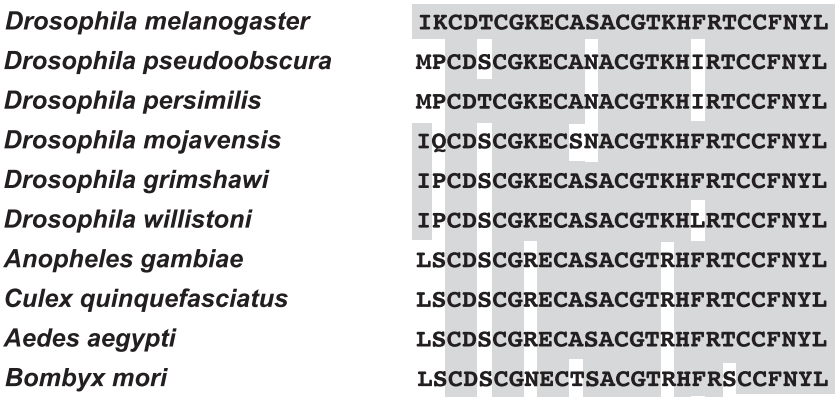

Fig. 4. Sequence comparison of trissin between insects. *Drosophila melanogaster*, *D. pseudoobscura*, *D. persimilis*, *D. mojavensis*, *D. grimshawi*, *D. willistoni*, *Anopheles gambiae*, *Culexquinque fasciatus*, *Aedes aegypti*, and *Bombyx mori* sequences are aligned. Residues identical between peptides are shaded.

ganglion (by FlyAtlas; http://www.flyatlas.org/; University of Glasgow). These distributions raise the possibility that trissin functions as a neuropeptide. Thus, future functional genetic studies of the role of trissin and its receptor at these sites in *Drosophila* and in mammalian systems will provide greater insight into its biological role, and into GPCR modulation in general.

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