

Isolation and identification of a diuretic hormone from the mealworm *Tenebrio molitor*

(water balance/corticotropin-releasing factor/cyclic AMP/Malpighian tubules)

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ABSTRACT A diuretic hormone of unusual structure was isolated from extracts of whole heads of the mealworm *Tenebrio molitor*. The hormone is a 37-aa peptide of 4371 Da, with the sequence SPTISITAPIDVLRKTWEQERARKQM-VKNREFLSLN. This peptide increases cAMP production in Malpighian tubules of *T. molitor*. The amino acid sequence reveals that this peptide is a member of the family of sauvagine/corticotropin-releasing factor/urotensin I-related insect diuretic hormones. The C-terminal sequence of this peptide is quite different from other members of this family, which have a hydrophobic C terminus (isoleucinamide or valinamide). When aligned comparably, *T. molitor* diuretic hormone has a more hydrophilic C terminus, leucylasparagine (free acid). In contrast to all other known diuretic hormones of this family, this peptide has exceptionally low stimulatory activity on cAMP production in Malpighian tubules of *Manduca sexta*. However, at nanomolar concentrations it stimulates cAMP production in Malpighian tubules of *T. molitor*. Diuretic hormones of this family have been isolated previously from Lepidoptera, Orthoptera, Dictyoptera, and Diptera. This appears to be the first diuretic hormone isolated from a coleopteran insect.

The regulation of water balance in insects has been heavily studied (1). With a variety of approaches, biologically active factors which promote urine production have been described from a large number of insect species (2). Recently, a number of insect diuretic hormones (DHs) have been isolated and characterized (3–9) which are homologous to a family of peptides including sauvagine, corticotropin-releasing factor (CRF), and urotensin. For simplicity, these DHs are referred to as the CRF-related DHs; all appear to act on the Malpighian tubules (Mt) via cAMP as second messenger (10). The leucokinin (11) and achetakinins (12), which were originally isolated as myotropic peptides, have been shown to stimulate fluid secretion from Mt (13), although this effect is not mediated via cAMP (14). Serotonin may also function as a DH; it stimulates fluid secretion by Mt of many insect species (14–18) and causes elevation of cAMP levels in Mt of some, but not all, of the stimulated species (14, 16, 18–20).

CRF-related DHs have now been isolated from four orders of insects, Lepidoptera, Orthoptera, Dictyoptera, and Diptera. Head extracts of the coleopteran insect *Tenebrio molitor*, the yellow mealworm, stimulate fluid secretion from Mt of *T. molitor* and elevate intracellular cAMP levels in Mt of *T. molitor* (21). This observation suggests that a CRF-related DH may occur in the head of *T. molitor*.

We have isolated a CRF-related DH from extracts of whole heads of *T. molitor* pupae. To screen fractionated samples, we used an assay measuring the release of cAMP from Mt of *T. molitor*. Here we report the isolation and identification of this

coleopteran DH and preliminary results on its unusual biological activity.§

MATERIALS AND METHODS

Experimental Insects. *T. molitor* were kept under crowded conditions at 27°C on a 14 hr/10 hr light/dark cycle and were fed on bran and raw potatoes. Pupae were decapitated 24–48 hr after pupation. The heads were collected in liquid nitrogen and stored at –80°C until extraction. For the cAMP bioassay, Mt were taken from newly emerged adults of both sexes 3–12 hr after adult eclosion.

Bioassay. DH activity in fractions from all purification steps was detected by the increase of cAMP in isolated Mt dissected from newly emerged adult *T. molitor*. Single Mt of *T. molitor* were incubated as described (5, 21), with modifications. For each fraction from liquid chromatography (LC), an aliquot representing 20 head equivalents was sampled, and 50 µg of bovine serum albumin (BSA; Sigma) in 0.1% (vol/vol) trifluoroacetic acid (TFA) was added to each aliquot to avoid adsorption of peptides to the 1.5-ml polypropylene tubes. The sample with BSA was dried in a vacuum centrifuge (Savant), dissolved in 400 µl of Nicolson's *T. molitor* saline (21) modified by addition of 0.5 mM 3-isobutyl-1-methylxanthine, and split into four 5-head-equivalent aliquots in 100 µl of saline. A single Mt was added and incubated for 1 hr; for each set of four replicates, tubules were from different animals. The cAMP released into 50 µl of incubated medium was quantified by the Gilman assay (22) with modifications (23). Each Mt had been preincubated for 1 hr in 100 µl of saline before use; assay of a 50-µl aliquot of the preincubation medium gave a control value. The BSA had no effect on the cAMP assay.

Extraction and Preliminary Purification (Step 1). For lipid removal, collected heads (fresh weight, ≈47 g) from ≈8400 *T. molitor* pupae were homogenized in 300 ml of cold (4°C) methylene chloride with a Polytron with PTA 20TS saw teeth (Brinkmann). After centrifugation at 15,000 × g for 20 min at 4°C, the solid residue was extracted with 300 ml of 1 M acetic acid/20 mM H₂SO₄/0.1 mM phenylmethanesulfonyl fluoride/0.01 mM pepstatin A and centrifuged at 15,000 × g for 20 min at 4°C. After reextraction of the pellet with 300 ml of this solution, and recentrifugation, the combined supernatants were applied to 10 g of Vydac C₄ reversed-phase packing material (20- to 30-µm particles, contained in a 75-ml polypropylene syringe barrel with a polyethylene frit) equilibrated with 0.1% TFA. Bound material was eluted with 100 ml each of 0.1% TFA and 20%, 45%, and 60% (vol/vol) CH₃CN in

Abbreviations: DH, diuretic hormone; DP, diuretic peptide; Mt, Malpighian tubule(s); TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; CRF, corticotropin-releasing factor; Tem-DH1, *Tenebrio molitor* DH1; Mas-DH, *Manduca sexta* DH; BSA, bovine serum albumin; ESI, electrospray ionization.

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§The sequence reported in this paper has been deposited in the PIR data base (accession no. A57127).

0.1% TFA. To each fraction, 0.1% (vol/vol) 2-(methylthio)-ethanol was added as an antioxidant.

Liquid Chromatographic Purification (Steps 2–9). An eight-step purification by LC was performed with two instruments. For semipreparative and analytical purifications (steps 2–4 and purification of synthetic peptide), a Perkin–Elmer model 410 Bio pump, a Rheodyne 7125 loop injector, and a Perkin–Elmer model 235 detector set at 220 and 280 nm were used. The pump was modified so that water-diluted fractions could be pumped into the column (24). Column temperature was ambient. For purification with narrow-bore columns (steps 5–9, all enzymatic digestions, and the verification of retention times of the natural peptide and synthetic peptide), a Michrom ultrafast microprotein analyzer was used with monitoring at 220 nm and a column temperature of 40°C. Elution rates were 200 μ l/min for 2.1-mm (inner diameter) columns, and 50 μ l/min for 1.0-mm columns; fractions were collected manually.

Vydac C₄ semipreparative LC. (Step 2) Half of the biologically active fraction from step 1 was diluted with 550 ml of 0.1% TFA and pumped onto a Vydac C₄ (10 μ m) semipreparative column (10 mm \times 250 mm) equilibrated with 0.1% TFA. Bound material was eluted with a gradient of 0–20% CH₃CN in 0.1% TFA for 10 min, 20–50% for 60 min, and 50–60% for 5 min at 5 ml/min, and 10-ml fractions were collected. Biologically active fractions from two runs were pooled.

Analytical-scale LC purification steps. (Step 3) Active fractions from step 2 were diluted with 240 ml of 0.1% (vol/vol) heptafluorobutyric acid (HFBA) and pumped onto a Vydac C₁₈ (5 μ m) column (4.6 mm \times 150 mm) that was fitted with a guard column (RP-18 Newguard, Applied Biosystems) and equilibrated with 0.1% HFBA. Elution was with a linear gradient of CH₃CN increasing at 0.5%/min in 0.1% HFBA at 1 ml/min. Two-milliliter fractions were collected.

(Step 4) The active fraction from step 3 was diluted with 36 ml of 0.1% TFA and pumped onto a PLRP-S (8 μ m) column (30-nm pores, 4.6 mm \times 150 mm) equilibrated with 1% (vol/vol) 1-propanol in 0.1% TFA. Elution was with a linear gradient of 1-propanol increasing at 0.167%/min in 0.1% TFA at 1 ml/min, and 1-ml fractions were collected.

Narrow-bore LC purification steps. (Step 5) The active fraction from step 4 was diluted with 12 ml of 0.1% TFA and injected into a PLRP-S (5 μ m) column (10-nm pores, 2.1 mm \times 150 mm). The column was equilibrated with 3.8% CH₃CN in 0.1% TFA, and bound material was eluted with a linear gradient increasing the concentration of CH₃CN at 0.5%/min in 0.1% TFA. Fractions corresponding to UV-absorbing peaks were collected.

(Step 6) Half of the biologically active fraction from step 5 was added to 50 μ l of 0.8 M triethylammonium phosphate (TEAP) (0.8 M H₃PO₄ adjusted to pH 2.8 with triethylamine) and CH₃CN was added to a final 85%. This was injected onto a PolyHydroxyethyl A (5 μ m) column (2.1 mm \times 150 mm) equilibrated with 82% CH₃CN in 10 mM TEAP. Elution was with a 30-min linear gradient of 82–11.6% CH₃CN in 10 mM TEAP. Fractions corresponding to UV-absorbing peaks were collected. Biologically active fractions from two runs were pooled.

(Step 7) Active fractions from step 6 were diluted with 1.5 ml of 0.1% TFA and injected into a Vydac C₁₈ (5 μ m) column (1.0 mm \times 150 mm). The column was equilibrated with 3.8% CH₃CN in 0.1% TFA. A linear gradient of 3.8–61% CH₃CN in 0.1% TFA over 60 min was used. Fractions corresponding to UV-absorbing peaks were collected.

(Step 8) The active fraction from step 7 was diluted with 0.8 ml of 4% CH₃CN in 0.1% TFA and loaded on a PLRP-S (8 μ m) column (30-nm pores, 1.0 mm \times 150 mm). The column was equilibrated with 3.8% CH₃CN in 0.1% TFA. A linear gradient of 3.8–61% CH₃CN in 0.1% TFA over 60 min was used. The single UV-absorbing peak was collected.

(Step 9) After addition of 0.8 ml of 2% 1-propanol in 0.1% HFBA, the active fraction from step 8 was injected on the same column as in step 7 equilibrated with 1.9% 1-propanol in 0.1% HFBA. Elution was with a linear gradient of 1.9–36.2% 1-propanol in 0.1% HFBA for 60 min. The single UV-absorbing peak was collected.

Enzymatic Digestions. Purified peptide from *T. molitor* (\approx 150 pmol) was incubated with 0.36 μ g of endoproteinase Asp-N (Boehringer Mannheim) in 100 μ l of 50 mM phosphate buffer (pH 8.0) for 18 hr at 37°C. For Lys-C digestion, peptide (\approx 80 pmol) was incubated with 1.6 pmol of lysyl endopeptidase (Wako) in 100 μ l of 0.2 M Tris-HCl (pH 9.0) for 14 hr at 30°C. For Glu-C digestion, peptide (\approx 60 pmol) was incubated with 0.08 μ g of endoproteinase Glu-C (Boehringer Mannheim) in 100 μ l of 0.1 M Tris-HCl (pH 8.0) for 30 hr at 25°C. After incubation, digests were purified with a Vydac C₁₈ column (1.0 mm \times 150 mm), for Asp-N and Lys-C digests, or a Reliasil C₁₈ (5 μ m) column (1.0 mm \times 150 mm), for Glu-C digests; fragment peptides were eluted with a linear gradient of 3.8–61% CH₃CN in 0.1% TFA over 45 min.

Structural Analysis. Purified peptide from *T. molitor* and peptide fragments from enzymatic digests were sequenced with a Porton Instruments PI 2090 gas-phase sequencer with an integral phenylthiohydantoin amino acid analyzer. Sample solutions from LC were loaded onto peptide supports (Beckman) pretreated with 1.1 M NaCl. The C-terminal amino acid of the peptide was not detected upon sequencing of Lys-C- or Glu-C-digested C-terminal fragments (K-3 and E-3 in Fig. 3) with the Porton sequencer; the C-terminal amino acid washed off the support. Consequently, half of the E-3 was analyzed with an Applied Biosystems Procise sequencer, which showed asparagine to be the C-terminal amino acid.

A Finnigan MAT SSO 710 mass spectrometer with an electrospray ionization (ESI) interface (Analytica, Branford, CT) was used to acquire positive-ion ESI spectra of the samples. Fractions were infused into the ESI ion source at 1 μ l/min with a syringe pump or analyzed by on-line LC/ESI-MS with a Michrom ultrafast microprotein analyzer with 1.0-mm columns and a homemade splitter with 50:1 split ratio. 2-Methoxyethanol/2-propanol, 1:1 (vol/vol), was used at 2 μ l/min as sheath liquid, with N₂ heated to 250°C as the drying gas. The ESI needle assembly was cooled to 50°C with a refrigerated 1/8-inch (outer diameter) copper tube.

Peptide Synthesis. The *T. molitor* diuretic hormone, referred to as *T. molitor* DH1 (Tem-DH1), was synthesized by solid-phase methods with an Applied Biosystems 431A synthesizer on 0.1 mmol of *p*-hydroxymethylphenoxy-derivatized resin. 1-Hydroxybenzotriazole in 1-methyl-2-pyrrolidinone in the presence of dicyclohexylcarbodiimide was used for fluoren-9-ylmethoxycarbonylamino acid activation. User-devised 2-hr single coupling cycles with 10-fold molar excess of acylating species were employed. Protecting groups were Arg(2,2,5,7,8-pentamethylchromansulfonyl), Asn(trityl), and Gln(trityl). Dry resin-peptide (200 mg, 38%) was cleaved with reagent K (25). After precipitation with ether, washing with ether, and drying, 101.5 mg (0.023 mmol) of crude peptide was recovered (61% yield). Hexapeptide with the sequence Phe-Leu-Asn-Ser-Leu-Asn-NH₂ was synthesized by Research Genetics.

Crude Tem-DH1 was purified by LC on a Vydac C₄ semipreparative column with a multisegment linear gradient of CH₃CN in 0.1% TFA at 5 ml/min. A hydrolysate of purified peptide was converted to amino acid aminoquinoline carbamate derivatives (26) and analyzed with a Hewlett–Packard 1090 chromatograph. The purity and molecular mass of the synthetic peptide were determined by ESI-MS as described above. Native peptide (\approx 50 pmol) and synthetic peptide (\approx 50 pmol) were mixed, injected on a Reliasil C₁₈ column (1.0 mm \times 150 mm), and eluted with a linear gradient of 3.8–61% CH₃CN in 0.1% HFBA for 60 min at 50 μ l/min; a single peak was observed. All analyses were consistent with the synthetic

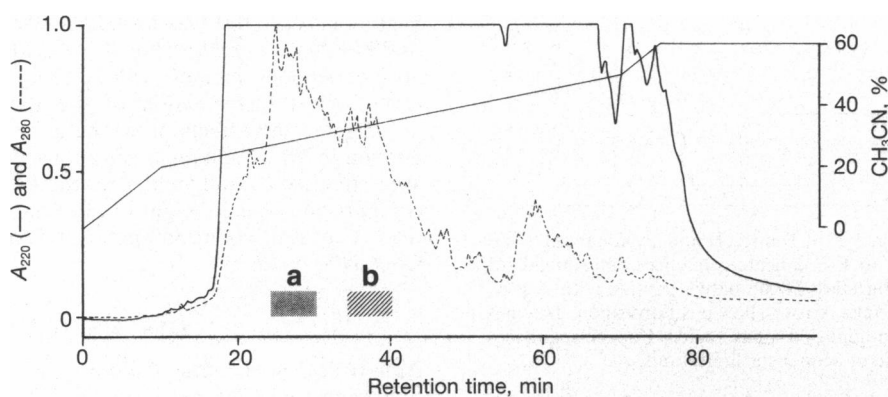


FIG. 1. Vydac C₄ semipreparative LC (step 2; see *Materials and Methods*). Two fractions eluted at 26–30 min (a) and 36–40 min (b) stimulated cAMP production in Mt of *T. molitor*. The material in fraction a, which had higher biological activity, was further purified.

peptide having the desired structure; the latter analysis suggested that it was identical to the natural peptide.

RESULTS

Extraction and Preliminary Purification (Step 1). The fractions from the Vydac C₄ reversed-phase cartridge were assayed for their ability to stimulate cAMP production in Mt of *T. molitor*. Diuretic activity was found in the fraction that was eluted from the cartridge during loading of extracts and in the 45% CH₃CN fraction. Aliquots representing five heads produced 68.1 ± 9.8 and 93.5 ± 2.0 pmol of cAMP, respectively, compared with 1.9 ± 0.5 pmol of cAMP for the 60% CH₃CN fraction (an inactive control) and ≈ 110 pmol for a maximally stimulated sample (these values cannot be used for calculating total activity; for this a full dose–response curve would have had to be measured).

Purification by LC (Steps 2–9). The 45% CH₃CN fraction from the Vydac C₄ reversed-phase cartridge was diluted with 12 volumes of 0.1% TFA and then loaded onto a Vydac C₄ semipreparative column (step 2) directly through the pump (24). This method diminishes loss of biological activity caused by adsorption to containers when fractions are evaporated. Two zones were found to increase cAMP production in Mt of *T. molitor*, eluted at 26–30 min and 36–40 min and producing 69.7 ± 18 and 53.2 ± 4.5 pmol of cAMP per five-head aliquot, respectively (Fig. 1), with 0.8 ± 0.2 pmol for a control (see above for comments on total activity). The more active fractions (26–30 min) were pooled and chromatographed on a

Vydac C₁₈ analytical column (step 3). The DH activity was eluted between 40 and 44 min. This fraction was further purified on a PLRP-S analytical column (step 4); an active fraction was eluted at 79–81 min. The latter was purified on a PLRP-S (10-nm pore) 2.1-mm column (step 5); an active fraction, eluted at 21–23 min, was further fractionated by normal-phase (hydrophilic interaction) LC on a PolyHydroxyethyl A 2.1-mm column (step 6). This proved to be a very effective purification (Fig. 2); the biological activity was found in a small peak at 24 min. The pooled active fractions from the normal-phase column were chromatographed on a Vydac C₁₈ 1.0-mm column (step 7); an active peak was eluted at 26 min. This biologically active material was injected onto a PLRP-S (30-nm pore) 1.0-mm column (step 8). A major single symmetric peak was eluted with a retention time of 25 min. This peak material was rerun on a Vydac C₁₈ 1.0-mm column with a different solvent system (step 9). The pure Tem-DH1 was recovered in a peak at 46.2 min.

The ESI mass spectrum of the active peak from step 8 indicated that two major components (4371 and 4409 Da) existed in the fraction (data not shown). These components could correspond to a peptide plus its K⁺ adduct or an impurity. This fraction was further purified (step 9) but still contained a small peak at 4409 Da. As K⁺ adducts were observed in other ESI spectra of enzymatically digested fragments of Tem-DH1, step 9 was probably unnecessary.

Structural Analysis. The material from the final purification was analyzed by ESI-MS and found to have purity acceptable for characterization with a molecular mass of 4370.6 ± 0.7 Da.

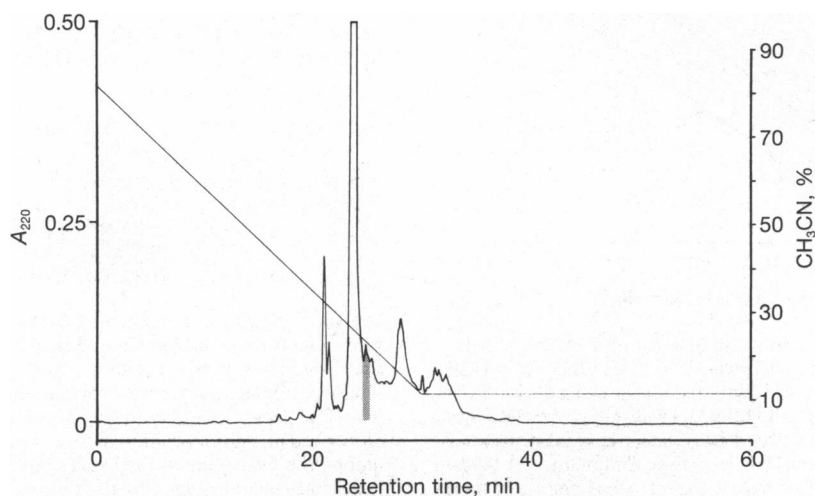


FIG. 2. PolyHydroxyethyl A narrow-bore LC (step 6; see *Materials and Methods*). A fraction stimulating cAMP production was eluted at 24 min (shaded).

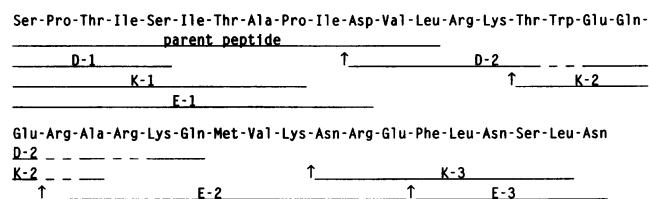


FIG. 3. Amino acid sequence of Tem-DH1 and fragments. D-1 and D-2, K-1 to K-3, and E-1 to E-3 indicate sequences determined for peptide fragments from digestion of the native peptide with Asp-N, Lys-C, and Glu-C, respectively. Arrows show positions where enzymes cut. Broken lines show unidentified amino acids. Longer sequences were not obtained because of sequencer limitations.

Approximately 40 pmol of purified peptide was analyzed with a Porton sequencer; only residues 1-13 could be assigned (Fig. 3), because of inherent limitations of this sequencer. Digestion of the purified peptide with Asp-N, Lys-C, and Glu-C gave two (D-1 and D-2), three (K-1 to K-3), and three (E-1 to E-3) fragments, respectively. Each digest was separated by 1.0-mm column LC coupled to on-line ESI-MS, allowing determination of the molecular mass of each fragment (data not shown). All fragments were sequenced with a Porton sequencer, and half of the E-3 sample was sequenced with an Applied Biosystems sequencer. All sequences are shown in Fig. 3; these results show Tem-DH1 to be a 37-residue peptide. The LC retention time of E-3 and a synthetic hexapeptide (Phe-Leu-Asn-Ser-Leu-Asn-NH₂) prepared with the amidated C terminus common to other CRF-related DHs were found to differ. Infusion ESI-MS of both peptides indicated that E-3 had 1-Da higher molecular mass (706.4 ± 0.2 Da) than the synthetic amidated peptide (705.4 ± 0.2 Da). This indicates that the C terminus of Tem-DH1 is the free acid form. Therefore, the complete structure of Tem-DH1 is as shown in Fig. 3. The calculated molecular mass of Tem-DH1 is 4370.71 Da, agreeing with the 4370.6 ± 0.7 Da observed for the native peptide.

Synthesis and Biological Properties of Tem-DH1. We synthesized Tem-DH1 by automated solid-phase methods. The synthetic peptide was analyzed by ESI-MS (calculated, 4370.71 Da; found, 4370.5 ± 0.2 Da) after purification. The retention time of the synthetic peptide was compared with that of the natural Tem-DH1 by analyzing a mixed sample on a 1.0-mm Reliasil C₁₈ column. A single peak was eluted, and the width at half-height of this peak was almost the same as those of peaks in separate analyses of each peptide (data not shown).

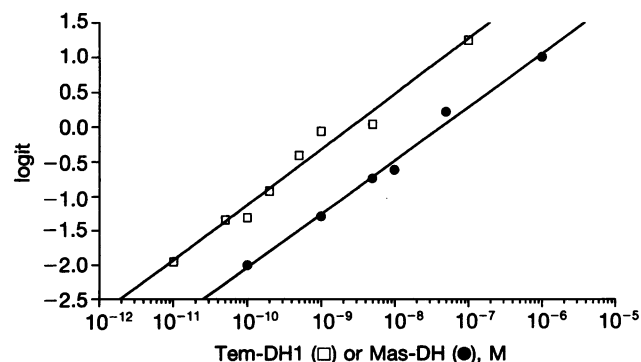


FIG. 4. Logit-transformed dose-response data for effects of solutions of Tem-DH1 and *Manduca sexta* DH (Mas-DH) on cAMP production by Mt of *T. molitor*. All dilutions were prepared with 0.1% BSA; activity of both Tem-DH1 and Mas-DH was reduced ≈100 times when solutions were prepared without BSA, because of adsorption of hormones to containers. □, Tem-DH1 (*n* = 3-6; for line fit, *r*² = 0.98), logit 1 corresponds to 101 pmol of cAMP, logit 0 to 55.5 pmol, and logit -1 to 10.1 pmol; ●, Mas-DH (*n* = 3; for line fit, *r*² = 0.99), logit 1 corresponds to 104 pmol of cAMP, logit 0 to 57 pmol, and logit -1 to 10.4 pmol.

Synthetic Tem-DH1 was tested for effects on cAMP production in Mt of *T. molitor* (Fig. 4). The Mt were stimulated in a dose-dependent manner, with an EC₅₀ of 2.6 nM determined from logit transformation of the data (Fig. 4). We also investigated the effects of synthetic Mas-DH on cAMP production in Mt of *T. molitor*; its approximate EC₅₀ was 43 nM. We estimated overall yield after the final purification step by comparison of peak height of both native and synthetic peptide. A total of about 380 pmol of Tem-DH1 was recovered from 8400 heads.

DISCUSSION

Amino acid sequences of known insect CRF-related DHs, sauvagine, and CRF are shown in Fig. 5. These peptides are homologous to a family of peptides isolated from amphibians, mammals, and fish, which include sauvagine, CRF, and urotensin I, respectively (28-36). These releasing factors trigger production of corticotropin, endorphins, and melanocyte-stimulating hormone or their analogues. The sequence of Tem-DH1 identifies it as a CRF-related DH. In the sequence alignment shown in Fig. 5, Tem-DH1 has 43% sequence identity with *Periplaneta americana* DP and *Musca domestica* DP, 41% with Mas-DH and *Acheta domesticus* DP, 38% with *Locusta migratoria* DH (Lom-DH), 30% with *M. sexta* diuretic peptide II (Mas-DP_{II}), 32% with sauvagine, and 27% with human and rat CRF. [Coast's group cautiously named several members of this family "diuretic peptides" because of insufficient evidence for their hormonal nature. Recently they have presented "unequivocal evidence for a hormonal function" of Lom-DH (37).] However, Tem-DH1 is distinct from all other characterized CRF-related DH in both its C terminus and its biological activity. While all other members of this family have a hydrophobic amide-containing C terminus (isoleucinamide or valinamide), Tem-DH1 in optimal alignment terminates in the much more hydrophilic leucylasparagine-OH. Unlike the other CRF-related DHs, Tem-DH1 contains tryptophan, which should aid in biophysical studies.

All other known CRF-related DHs stimulate Mt of *M. sexta* (10) and bind to the expressed, recombinant *M. sexta* receptor with IC₅₀ values ranging from ≈1 to ≈12 nM (38). However, solutions of synthetic Tem-DH1 up to 0.1 mM elicit only ≈80% of the maximal stimulation of cAMP production caused by Mas-DH in Mt of *M. sexta*. Thus, we could not determine an EC₅₀ value for Tem-DH1 on *M. sexta* Mt, but we estimate that it is about 4 orders of magnitude higher than the EC₅₀ value of Mas-DH on adult Mt [EC₅₀ ≈ 1.5-3.5 nM (K.F., unpublished work) or 6.9 nM (10)]. This is not surprising, as changing the C terminus of Mas-DH from isoleucinamide to isoleucine reduces biological activity ≈1000 times in *M. sexta* (3, 10). Synthetic Tem-DH1 and Mas-DH were tested for their effects

	1	10	20	30	40	48
Tem-DH1	...SPTISIT	APIDVLRKTW	EQERARKQMV	K.....	N	REELNSLN-OH
Mas-DP _{II}	...SFSVN	PAVDILOHRY	MEKVA.....	QNN	BNELNRY-NH ₂
Mas-DH	...RMPLSID	LPMSVLRQKL	SLEKERK...	...VHALRAAN	BNELNDI-	NH ₂
Lom-DH	MGMGPSLSIV	NPMQVLRQRL	LLEIARRRLR	D.AEEQIKAN	KDFEQQI-	NH ₂
Pea-DP	TGSGPSLSIV	NPLDVLRLR	LLEIARRRMR	Q.SODQIQAN	REILOQI-	NH ₂
Mud-DP	...NKPSLSIV	NPLDVLRLR	LLEIARRRMR	E.NTROVELN	BAIKKNV-	NH ₂
Acid-DP	.TGAGLSIV	APLDVLRRL	MNELNRRLR	ELOGSRIOQN	ROLLTSL-	NH ₂
sauv	...QGPISID	LSLELLRKM	EIEKO.....	EKEQQAANN	RLLDQTI-	NH ₂
h,rCRF	.SEEPISLD	LTFFLLREVL	EMARA.....	EOLAQQAHSN	BKLMEII-	NH ₂

FIG. 5. Sequence alignment of the known CRF-related insect DHs, sauvagine (sauv), and human and rat CRF (h,rCRF) determined with the Genetics Computer Group program. Underlined residues are identical among members of this family in this sequence alignment, allowing gaps. The only residues underlined are those absolutely conserved at a given position in three or more peptides; with identities among the CRF-related insect DHs given priority. We used for each factor the name assigned by the discoverer of the DH/DP, using the abbreviated nomenclature of Raina and Gäde (27): Lom-DH, *Locusta migratoria* DH; Pea-DP, *Periplaneta americana* DP; Acid-DP, *Acheta domesticus* DP; Mud-DP, *Musca domestica* DP.

on cAMP production in Mt of *T. molitor* at a number of dilutions (Fig. 4). Tem-DH1 has an EC₅₀ value of 2.6 nM, only about 17 times more potent on these tubules than Mas-DH, a remarkable contrast to the inverse cross-species data.

ELISAs utilizing antibody raised against Mas-DH were used successfully in the isolation of Lom-DH (4), and we found in initial purification attempts cross reaction of this antibody with proteins in *T. molitor* extracts. However, the immunoreactivity was spread over many fractions of the liquid chromatogram, suggesting that this ELISA was not specific for a diuretic factor in *T. molitor*. Since assays measuring release of cAMP from Mt of the species under study seem to be universally reliable in isolating CRF-related DHs, we tested LC fractions of *T. molitor* head extracts with this assay. A cAMP assay using Mt of *T. molitor* showed responses only for specific LC fractions.

Interestingly, in the preliminary purification (step 1), not only the 45% CH₃CN eluate from the Vydac C₄ cartridge, but also the fraction that was eluted from the cartridge during the loading of head extracts, had a strong diuretic activity toward both *T. molitor* and *M. sexta* Mt as measured with the cAMP assay. We suspected that the activity in the loading fraction was caused by serotonin, a biogenic amine known to stimulate cAMP production in Mt of *Rhodnius prolixus* (18–20). Serotonin is extremely hydrophilic compared with the CRF-related DHs; we found that it was not retained on the reversed-phase cartridge (K.F., unpublished work). We then determined that serotonin stimulated cAMP production by *M. sexta* Mt with an EC₅₀ value of ≈0.14 μM but had essentially no effect on cAMP production by Mt of *T. molitor* even at 10 mM (K.F., data not shown). The stimulation of *M. sexta* Mt by this hydrophilic fraction is most likely due to serotonin, as immunohistochemical studies have shown a serotonin-like material in the brain (39, 40), ventral nerve cord (41), and subesophageal ganglion (42) of *T. molitor*. There may be another biogenic amine present in *T. molitor* extracts that is responsible for the stimulation of cAMP secretion by *T. molitor* Mt.

During the first LC purification step using a semipreparative column, two relatively hydrophobic fractions were observed which stimulated cAMP production in Mt of *T. molitor* (Fig. 1). We isolated the more hydrophilic of these factors, which showed greater activity. *M. sexta* is the only species from which two CRF-related DHs, Mas-DH (3) and Mas-DPII (8), have been isolated to date. Mas-DPII has 30 aa whereas Mas-DH has 41 aa (Fig. 5); both peptides stimulate cAMP production in Mt of *M. sexta* (10). It seems likely that two CRF-related DHs occur in *T. molitor*, neither of which stimulates *M. sexta* Mt.

To our knowledge, this study represents the first identification of any diuretic factor from coleopteran insects. This beetle can live in very dry conditions; Nicolson (43) has suggested that in such beetles DH may serve as a "clearance hormone," providing fluid to the midgut to moisten the dry food to aid digestion, the fluid being reabsorbed by the cryptonephridial complex. However, we have named this peptide Tem-DH1 because of its effects on Mt and according to a nomenclature convention (27). The availability of synthetic Tem-DH1 will greatly assist physiological studies on diuresis in the beetle.

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