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Synthesis of a Molt-Inhibiting Hormone of the American Crayfish, *Procambarus clarkii*, and Determination of the Location of Its Disulfide Linkages[1]

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A molt-inhibiting hormone (Prc-MIH) of the American crayfish, *Procambarus clarkii*, a member of the type II CHH family, was chemically synthesized and the location of its three disulfide linkages was determined. Prc-MIH consists of 75 amino acid residues and was synthesized by a thioester method. Two peptide segments, Boc-[Cys(Acm)^{7,24,27}, Lys(Boc)^{19}]-Prc-MIH(1-39)-SCH_2CH_2CO-Nle-NH_2 and H-[Cys(Acm)^{40,44,53}, Lys(Boc)^{42,51,67}]-Prc-MIH(40-75)-NH_2, were prepared using peptides obtained *via* the Boc solid-phase method. Condensation of the building blocks in the presence of silver chloride, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, and *N,N*-diisopropylethylamine, followed by removal of the protecting groups, gave the reduced form of Prc-MIH(1-75)-NH_2. This product was converted to the native form of Prc-MIH (synthetic Prc-MIH) in a buffer which contained cysteine and cystine. The synthetic Prc-MIH showed the same behavior by RP-HPLC and biological activity assays as the natural Prc-MIH. The disulfide bond between Cys7 and Cys44 was determined by isolation of a fragment from an enzymatic digest of the synthetic Prc-MIH by RP-HPLC, followed by mass analysis. The disulfide bonds between Cys24 and Cys40 and between Cys27 and Cys53 were determined by comparing the elution position of an enzymatic digest of the synthetic Prc-MIH with authentic chemically synthesized samples, which contained three types of possible disulfide linkages.

Key words: chemical synthesis, disulfide linkage, molt-inhibiting hormone, peptide thioester, *Procambarus clarkii*.

In crustaceans, the molt-inhibiting hormone (MIH), which is produced in the medulla terminalis X-organ and released from the neurohemal sinus gland in the eyestalk, is presumed to regulate molting by inhibiting the secretion of ecdysteroids from Y-organs *in vivo* (*1*). MIHs have been isolated or cloned from a variety of crustaceans, including lobster (*2*), crab (*3–7*), crayfish (*8*, *9*), prawn (*10*, *11*), and shrimp (*12*, *13*). These hormones consist of 71 to 78 amino acid residues and have similar amino acid sequences with six conserved cysteine residues. They are members of the peptide hormone family, commonly referred to as the CHH family or the CHH/MIH/VIH(GIH) family, which comprises the crustacean hyperglycemic hormone (CHH), MIH, and the vitellogenesis (gonad)-inhibiting hormone (VIH/GIH) (*1*). The CHH family is classified into type I and type II depending on its mRNA organization and the primary structures of the mature hormones (*10*, *11*, *14*). Most of the N-termini of type I hormones are pyroglutamyl residues and the C-termini are amidated. The N- and C-termini of type II hormones are free except for Prc-MIH, in which the C-terminus is amidated. One extra glycine residue is found at position 12 in type II hormones, compared with type I hormones. The mRNAs that produce the type I hormones encode a prosequence between the signal peptide and the mature hormone, although the mRNAs that produce type II hormones encode only a signal sequence and a mature hormone. The mode of disulfide linkages of several type I

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Abbreviations: Acm, acetamidomethyl; Boc, *t*-butoxycarbonyl; Boc-OSu, *N*-*t*-butoxycarbonyloxysuccinimide; Bom, benzyloxymethyl; Br-Z, 2-bromobenzyloxycarbonyl; Bzl, benzyl; CHH, crustacean hyperglycemic hormone; Cl-Z, 2-chlorobenzyloxycarbonyl; DIEA, *N,N*-diisopropylethylamine; DIPCI, 1,3-diisopropylcarbodiimide; DMSO, dimethyl sulfoxide; Fmoc, 9-fluorenylmethoxycarbonyl; cHex, cyclohexyl; HOBt, 1-hydroxybenzotriazole; HOOBt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; MALDI-TOF MS, matrix assisted laser desorption ionization/time-of-flight mass spectroscopy; MBHA, 4-methylbenzhydrylamine; MIH, molt-inhibiting hormone; NH_2-resin, MBHA resin; Nle, norleucine; NMP, 1-methylpyrolidin-2-one; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Prc, *Procambarus clarkii*; RP-HPLC, reversed-phase high performance liquid chromatography; RIA, radioimmunoassay; SPy, 2-pyridylsulfenyl; TFA, trifluoroacetic acid; Tos, tosyl; Trt, triphenylmethyl.

hormones has been already elucidated (*9*, *10*, *15–17*). However, none of complete disulfide linkages of the type II hormones has yet been determined.

The Prc-MIH from the crayfish, *Procambarus clarkii*, a member of the type II family, consists of 75 amino acid residues (*8*). The authors attempted to determine the mode of disulfide linkages by analyzing enzymatic digests. However, no useful information, except for the disulfide linkage between Cys7 and Cys44, was obtained from these studies.

In order to obtain synthetic Prc-MIH for functional studies, a complete Prc-MIH molecule was synthesized. In addition, to elucidate the location of disulfide linkages among Cys24, Cys27, Cys40, and Cys53, a set of peptides corresponding to one of the enzymatic digested peptides of the native form of Prc-MIH, was synthesized. This paper reports on the chemical synthesis and the location of the disulfide linkages of Prc-MIH.

EXPERIMENTAL PROCEDURES

General—Boc-amino acid derivatives and 4-methylbenzhydrylamine (MBHA) resin were purchased from Peptide Institute (Osaka). Fmoc-amino acid derivatives, Fmoc-Lys(Boc)-Wang resin, trifluoroacetic acid (TFA), and *N,N*-diisopropylethylamine (DIEA) were purchased from Watanabe Chemical (Hiroshima). Amino acid derivatives used were of the L-configuration. 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOObt) was commercially available and recrystallized from aqueous ethanol before use. Silver chloride was purchased from Wako Pure Chemical (Osaka). DMSO used for the segment coupling reaction was silylation grade (Pierce, Rockford, IL).

RP-HPLC was performed on Cosmosil $5C_{18}$ AR (10 × 250 mm) (Nacalai Tesque, Kyoto), Cosmosil $5C_{18}$ (4.6 × 250 mm), Cosmosil $5C_4$ AR-300 (4.6 × 250 mm), Shodex Asahipak C4P-50 10E (10 × 250 mm) (Showa Denko, Tokyo), YMC-Pack PolymerC18 (6 × 250 mm, 4.6 × 150 mm) (YMC, Kyoto), or Wakosil $5C_{18}$ (4.6 × 250 mm) (Wako Pure Chemical), using a linear increasing gradient of acetonitrile in 0.1% aqueous TFA, unless otherwise noted. Yields of peptides were determined by quantitative amino acid analyses on an L-8500 amino acid analyzer (Hitachi, Tokyo) after hydrolysis with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Pierce) at 110°C for 24 h in an evacuated sealed tube. Mass numbers were determined by matrix assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF MS) using a Voyager™ DE (PerSeptive Biosystems, Framingham, MA) with α-cyano-4-hydroxycinnamic acid or sinapinic acid as a matrix, and mass numbers were calculated as averages.

Preparation of Boc-[Cys(Acm)^{7,24,27}, Lys(Boc)^{19}]-MIH(1-39)-SCH$_2$CH$_2$CO-Nle-NH$_2$ (2)—Starting from Boc-Gly-SCH_2CH_2CO-Nle-NH-resin (0.89 g, Gly: 0.49 mmol) (*19*), 2.91 g of a protected peptide resin corresponding to the sequence of Prc-MIH(1-39), Boc-Arg(Tos)-Tyr(Br-Z)-Val-Phe-Glu(OBzl)-Glu(OBzl)-Cys(Acm)-Pro-Gly-Val-Met-Gly-Asn-Arg(Tos)-Ala-Val-His(Bom)-Gly-Lys(Cl-Z)-Val-Thr(Bzl)-Arg(Tos)-Val-Cys(Acm)-Glu(OBzl)-Asp(OcHex)-Cys(Acm)-Tyr(Br-Z)-Asn-Val-Phe-Arg(Tos)-Asp(OcHex)-Thr(Bzl)-Asp(OcHex)-Val-Leu-Ala-Gly-SCH_2CH_2CO-Nle-NH-resin, was prepared on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA). The 0.5 mmol standard protocol of system software version 1.40 NMP/HOBt *t*-Boc was employed. End capping with acetic anhydride was performed after each amino-acid introduction reaction. An aliquot of the resin (0.52 g) was treated with a mixture of HF (8.5 ml), anisole (0.75 ml), and 1,4-butanedithiol (0.75 ml) on an ice bath for 90 min. After evaporation of the HF, ether (10 ml) was added to the mixture, and the resulting precipitate was washed with ether (10 ml × 2), then extracted with 30% aqueous acetonitrile containing 1 ml of acetic acid (15 ml) to give 270 mg of crude freeze-dried peptide. This was purified by RP-HPLC on Cosmosil $5C_{18}$ AR (10 × 250 mm) to yield H-[Cys(Acm)^{7,24,27}]-MIH(1-39)-SCH_2CH_2CO-Nle-NH_2 (**1**) (44 mg, 8.3% yield based on Gly in the starting resin): MS (MALDI-TOF) Found: *m/z* 4,820.9. Calcd for $[M+H]^+$, 4,821.6 (average). Amino acid analysis: $Asp_{5.2}$-$Thr_{2.3}Glu_{2.6}Pro_{0.71}Gly_4Ala_{2.4}$1/2$Cys_{nd}Val_{6.6}Met_{0.79}Leu_{1.1}$-$(Tyr+Nle)_{1.6}Phe_{2.5}Lys_{1.1}His_{1.0}Arg_{3.8}$.

To a solution of peptide thioester **1** (20 mg, 3.4 μmol) in DMSO (0.60 ml), Boc-OSu (4.4 mg, 20 μmol) and DIEA (12 μl) were added. After stirring for 2 h, the peptide was isolated by RP-HPLC on Cosmosil $5C_{18}$ AR (10 × 250 mm) to give Boc-[Cys(Acm)^{7,24,27},Lys(Boc)^{19}]-MIH(1-39)-SCH_2CH_2-CO-Nle-NH_2 (**2**) (6.3 mg, 1.1 μmol, 33% yield): MS (MALDI-TOF) Found: *m/z* 5,022.8. Calcd for $[M+H]^+$, 5,021.8 (average). Amino acid analysis: $Asp_{5.0}Thr_{1.9}Glu_{3.0}Pro_{0.59}Gly_4Ala_{2.7}$-1/2$Cys_{nd}Val_{6.2}Met_{0.70}Leu_{0.92}$ $(Tyr + Nle)_{1.5}Phe_{2.0}Lys_{0.94}His_{1.0}$-$Arg_{4.2}$.

Preparation of H-[Cys(Acm)^{40,44,53},Lys(Boc)^{42,51,67}]-MIH(40-75)-NH$_2$ (4)—Starting from an MBHA resin (1.13 g, NH_2: 0.41 mmol/g, 0.46 mmol), 2.82 g of a protected peptide resin corresponding to the sequence of Prc-MIH(40-75), Fmoc-Cys(Acm)-Arg(Tos)-Lys(Cl-Z)-Gly-Cys(Acm)-Phe-Ser(Bzl)-Ser(Bzl)-Glu(OBzl)-Met-Phe-Lys(Cl-Z)-Leu-Cys(Acm)-Leu-Leu-Ala-Met-Glu(OBzl)-Arg(Tos)-Val-Glu(OBzl)-Glu(OBzl)-Phe-Pro-Asp(OcHex)-Phe-Lys(Cl-Z)-Arg(Tos)-Trp(CHO)-Ile-Gly-Ile-Leu-Asn-Ala-NH-resin, was prepared on a 430A peptide synthesizer except for the N-terminal Fmoc-Cys(Acm) residue, which was introduced manually. The 0.5 mmol standard protocol of system software version 1.40 NMP/HOBt *t*-Boc was employed. End capping with acetic anhydride was performed after each amino-acid introduction reaction. An aliquot of the resin (0.36 g) was treated with mixture of HF (8.5 ml), anisole (0.75 ml), and 1,4-butanedithiol (0.75 ml) on an ice bath for 90 min to give crude peptide (184 mg). This was purified by RP-HPLC on Cosmosil $5C_{18}$ AR (10 × 250 mm) to yield Fmoc-[Cys(Acm)^{40,44,53}]-MIH(40-75)-NH_2 (**3**) (26 mg, 3.7 μmol, 6.3% yield based on amino group in the starting resin): MS (MALDI-TOF) Found: *m/z* 4,703.8. Calcd for $[M+H]^+$, 4,704.6 (average). Amino acid analysis: $Asp_{2.3}Ser_{1.8}$-$Glu_{3.9}Pro_{0.72}Gly_{2.0}Ala_{3.3}$1/2$Cys_{nd}Val_{0.73}Met_{1.2}Ile_{2.1}Leu_4$-$Phe_{4.1}Lys_{3.1}Trp_{0.73}Arg_{3.1}$.

To a solution of peptide **3** (9.9 mg, 1.6 μmol) in DMSO (0.40 ml) were added Boc-OSu (3.9 mg, 18 μmol) and DIEA (10 μl). After stirring for 2 h, ether was added and the resulting precipitate was washed with ether (2 ml × 3), then dissolved in DMSO (0.40 ml). Piperidine (40 μl) was added to this solution and, after 1 h, acetic acid (40 μl) was added, and the peptide was isolated by RP-HPLC on Cosmosil $5C_{18}$ AR (10 × 250 mm) to give H-[Cys(Acm)^{40,44,53}, Lys(Boc)^{42,51,67}]-MIH(40-75)-NH_2 (**4**) (5.0 mg, 0.85 μmol, 53% yield): MS (MALDI-TOF) Found: *m/z* 4,784.6. Calcd for $[M+H]^+$, 4,782.7 (average). Amino acid analysis: $Asp_{2.0}$-$Ser_{1.6}Glu_{3.3}Pro_{0.65}Gly_2Ala_{2.8}$1/2$Cys_{nd}Val_{0.67}Met_{1.4}Ile_{1.8}Leu_{3.3}$-

$Phe_{4.1}Lys_{2.7}Trp_{0.73}Arg_{2.9}$.

Preparation of the Reduced Form of Prc-MIH—To a solution of peptide thioester **2** (2.9 mg, 0.53 μmol), peptide **4** (2.4 mg, 0.41 μmol), HOOBt (2.6 mg, 16 μmol), and DIEA (1.9 μl, 11 μmol) in DMSO (100 μl) was added AgCl (0.3 mg, 2 μmol). After stirring for 24 h, dithiothreitol (DTT) (5 mg) was added, then the mixture was washed with ether (3 × 1 ml) to give a crude peptide, Boc-[Cys(Acm)[7,24,27,40,44,53], Lys(Boc)[19,42,51,67]]-MIH(1-75)-NH_2 (**5**). The crude peptide **5** was treated with TFA containing 5% 1,4-butanedithiol (100 μl) for 1 h. The product was precipitated with ether, washed with ether (1 ml × 3), then lyophilized from aqueous acetonitrile. After purification by RP-HPLC on Cosmosil $5C_4$ AR-300 (4.6 × 250 mm), a peptide, H-[Cys(Acm)[7,24,27,40,44,53]]-MIH(1-75)-NH_2 (**6**) was obtained (2.3 mg, 0.20 μmol, 49% yield): MS (MALDI-TOF) Found: *m/z* 9,084.1. Calcd for $[M+H]^+$, 9,084.6. Amino acid analysis: $Asp_{7.2}Thr_{1.8}Ser_{2.2}Glu_{7.1}Pro_{1.4}Gly_6Ala_{4.5}$1/2$Cys_{nd}Val_{5.9}Met_{2.1}$-$Ile_{2.1}Leu_{4.9}Tyr_{1.2}Phe_{5.4}Lys_{4.1}His_{1.0}$ $Trp_{1.0}Arg_{6.5}$.

Peptide **6** (2.3 mg, 0.20 μmol) was dissolved in 90% acetic acid (400 μl). To the solution was added AgOAc (0.6 mg, 4 μmol), and after stirring for 4 h, DTT (6 mg) was added, followed by 1 M hydrochloric acid (50 μl). After stirring for 1 h, the product was extracted with TFA and purified by RP-HPLC on Cosmosil $5C_4$ AR-300 (4.6 × 250 mm) to give the reduced form of Prc-MIH (1.6 mg, 0.11 μmol, 55% yield): MS (MALDI-TOF) Found: *m/z* 8,658.5. Calcd for $[M+H]^+$: 8,658.7 (average). Amino acid analysis: $Asp_{7.3}Thr_{1.9}Ser_{2.1}$-$Glu_{6.6}Pro_{2.9}Gly_6Ala_{4.8}$1/2$Cys_{nd}Val_{7.1}Met_{1.9}Ile_{2.0}Leu_{4.9}Tyr_{1.7}$-$Phe_{5.8}Lys_{4.2}His_{0.93}$ $Trp_{0.74}Arg_{7.6}$.

Generation of the Native Form of Prc-MIH—The reduced form of Prc-MIH (1 mg, 60 nmol) was dissolved in ethylene glycol (1.2 ml), then the solution was diluted with 0.25 M Tris-HCl (pH 8.4) containing 5 mM cysteine, 0.5 mM cystine, and 1.5 M urea (10 ml). After 24 h, the mixture was acidified to pH 5 with acetic acid, and the product was purified by RP-HPLC on Cosmosil $5C_4$ AR-300 (4.6 × 250 mm) to yield the native form of Prc-MIH (0.3 mg, 23 nmol, 38% yield): MS (MALDI-TOF) Found: *m/z* 8,651.5. Calcd for $[M+H]^+$: 8,652.1 (average). Amino acid analysis: $Asp_{6.6}$-$Thr_{1.9}Ser_{2.1}Glu_{6.5}Pro_{1.9}Gly_6Ala_{4.9}$1/2$Cys_{3.9}Val_{6.8}Met_{2.4}Ile_{1.9}$-$Leu_{4.6}Tyr_{2.0}Phe_{5.2}Lys_{4.1}His_{1.2}$ $Trp_{0.63}Arg_{7.1}$.

Preparation of a Prc-MIH Fragment That Contains Two Disulfide Linkages—Starting from Fmoc-Lys(Boc)-Wang resin (0.10 mmol), fully protected peptide resins, corresponding to Prc-MIH(20-42), Val-Thr('Bu)-Arg(Pmc)-Val-Cys(X)-Glu(O'Bu)-Asp(O'Bu)-Cys(X)-Tyr('Bu)-Asn(Trt)-Val-Phe-Arg(Pmc)-Asp(O'Bu)-Thr('Bu)-Asp(O'Bu)-Val-Leu-Ala-Gly-Cys(X)-Arg(Pmc)-Lys(Boc)-Wang resin, in which X represents Acm or Trt, and Prc-MIH(52-67), Leu-Cys(Trt)-Leu-Leu-Ala-Met-Glu(O'Bu)-Arg(Pmc)-Val-Glu(O'Bu)-Glu(O'Bu)-Phe-Pro-Asp(O'Bu)-Phe-Lys(Boc)-Wang resin, were prepared by an Fmoc solid-phase method on a multiple organic synthesizer, 440Ω MOS (Advanced ChemTech, Louisville, Kentucky). Standard Fmoc chemistry protocol was employed; deprotection: 20% piperidine in 1-methylpyrolidin-2-one (NMP) (5, 10, and 10 min), coupling: 5 equivalents of Fmoc-amino acid derivatives with 1,3-diisopropylcarbodiimide (DIPCI) and 1-hydroxybenzotriazole (HOBt) in NMP (1.5 h), capping: 10% acetic anhydride and 5% DIEA in NMP (10 min). The obtained fully protected peptide resins were treated with reagent K (*23*) for 3 h, and after precipitation of the peptides with ether, the products were purified by RP-HPLC on Shodex Asahipak C4P-50 10E (10 × 250 mm) to give the fragments of Prc-MIH, [Cys(Acm)[27,40]]-Prc-MIH(20-42) (**F1**), [Cys(Acm)[24,40]]-Prc-MIH(20-42) (**F2**), [Cys(Acm)[24,27]]-Prc-MIH(20-42) (**F3**), and Prc-MIH(52-67) (**7**). **F1**: MS (MALDI-TOF) Found: *m/z* 2,806.9. Calcd for $[M+H]^+$: 2,806.2 (average). **F2**: MS (MALDI-TOF) Found: *m/z* 2,807.6. **F3**: MS (MALDI-TOF) Found: *m/z* 2,806.7. **7**: MS (MALDI-TOF) Found: *m/z* 1,941.8. Calcd for $[M+H]^+$: 1,941.3 (average).

Peptide **7** (5.0 mg) was treated with 2,2'-dithiodipyridine (5.5 mg) in 2-propanol–2 M acetic acid (2:5, 0.70 ml), and the product was immediately isolated by RP-HPLC on YMC-Pack PolymerC18 (6 × 250 mm) (*24*) to give [Cys(SPy)]-Prc-MIH(52-67) (**8**) (4.3 mg): MS (MALDI-TOF) Found: *m/z* 2,050.7. Calcd for $[M+H]^+$: 2,050.5 (average).

Peptide **8** was coupled with **F1**, **F2**, and **F3**, respectively (*24*). In a typical experiment, a solution of **8** (1.5 mg) in 1 M acetic acid (0.50 ml) and 2-propanol (0.050 ml), and a solution of **F2** (1.5 mg) in 1 M acetic acid (0.50 ml) were mixed, and the pH of the solution was adjusted to 6.5 with 28% NH_3 aq. After 1 h, acetic acid was added to adjust the pH to 3, then the product was isolated by RP-HPLC on YMC-Pack PolymerC18 (6 × 250 mm) to give [Cys(Acm)[24,40]]-Prc-MIH(20-42,52-67) (**F2-8**) (1.8 mg): MS (MALDI-TOF) Found: *m/z* 4,743.6. Calcd for $[M+H]^+$: 4,744.5 (average). Based on the same procedure, [Cys(Acm)[27,40]]-Prc-MIH(20-42,52-67) (**F1-8**) (2.4 mg) {MS (MALDI-TOF) Found: *m/z* 4,743.8}, and [Cys(Acm)[24,27]]-Prc-MIH(20-42,52-67) (**F3-8**) (1.6 mg) {MS (MALDI-TOF) Found: *m/z* 4,744.2}, were obtained.

Peptides **F1-8**, **F2-8**, and **F3-8** were each treated with iodine. To a solution of a peptide (0.2 mg) in 75% acetic acid (50 μl) was added 1 μl of a solution of 10% iodine in methanol, and after 20 min of stirring, a saturated solution of ascorbic acid was added. The respective products **L3-1**, **L3-2**, and **L3-3** were isolated by RP-HPLC on YMC-Pack PolymerC18 (6 × 250). **L3-1**: MS (MALDI-TOF) Found: *m/z* 4,603.5. Calcd for $[M+H]^+$: 4,600.3 (average). **L3-2**: MS (MALDI-TOF) Found: *m/z* 4,601.2. **L3-3**: MS (MALDI-TOF) Found: *m/z* 4,600.5.

Enzymatic Digestion of Synthetic Prc-MIH—The synthetic Prc-MIH (10 μg, 1 nmol) was digested with lysyl endopeptidase (Wako Pure Chemical) (0.7 μg, 2 mAU) in 25 mM ammonium hydrogencarbonate buffer (100 μl) at 10°C for 2 days. The resulting mixture of peptides was separated by RP-HPLC on YMC Polymer C_{18} (4.6 × 150 mm), and the mass number of the peptide in each fraction was measured by use of a MALDI-TOF MS spectrometer.

RP-HPLC of Prc-MIH—A sinus gland extract, which was prepared using 30% acetonitrile containing 0.3% NaCl (*8*, *25*), and the synthetic Prc-MIH were analyzed by RP-HPLC using Cosmosil $5C_{18}$ (4.6 × 250 mm) or Wakosil $5C_{18}$ (4.6 × 250 mm) with a 65-min linear gradient of acetonitrile from 0 to 65% containing 0.05% TFA at a flow rate of 1 ml/min (40°C). The eluate was monitored at 225 nm. Fractions were collected at 0.5 min intervals. A peak at 225 nm or a pooled zone of materials in HPLC was dried, re-dissolved in culture medium, and subjected to a bioassay for MIH.

Bioassay—American crayfish, *Procambarus clarkii*, carapace width 20–23 mm, was used for the *in vitro* bioassay of Prc-MIH. Y-organs of the crayfish were dissected out 4 days after eyestalk removal and rinsed thoroughly in Harrev-

eld's saline. They were then placed in glass dishes which contained 0.5 ml of culture medium (*26*) in such a way that each dish contained one of a bilateral pair of Y-organs: one organ served as a control, and the other as the experimental organ. After incubation on a rotary shaker at 25°C for 6 h, the amount of ecdysteroids in the culture medium was determined by radioimmunoassay (RIA). The MIH activity was expressed as the rate of inhibition of ecdysteroid secretion by comparison with the control (*25*).

RESULTS

Synthesis of Prc-MIH—The amino acid sequence of Prc-MIH is shown in Fig. 1, in which an arrow indicates the site of segment condensation. The Prc-MIH molecule was synthesized according to the reaction sequence shown in Scheme 1. An Cys(Acm)-containing peptide thioester was prepared using a previously reported procedure (*19*). The peptide chain was elongated by an automated peptide synthesizer using a Boc standard protocol, starting from Boc-Gly-SCH_2CH_2CO-Nle-NH-resin. After HF treatment and purification by RP-HPLC, a peptide thioester, H-[Cys(Acm)^{7,24,27}]-MIH(1-39)-SCH_2CH_2CO-Nle-NH_2 (**1**), was obtained in a 8.3% yield based on the Gly residue in the starting resin. Peptide thioester **1** was converted to Boc-[Cys(Acm)^{7,24,27},Lys(Boc)^{19}]-MIH(1-39)-SCH_2CH_2CO-Nle-NH_2 (**2**) by treatment with Boc-OSu. A C-terminal peptide segment, H-[Cys(Acm)^{40,44,53},Lys(Boc)^{42,51,67}]-MIH(40-75)-NH_2 (**4**), was also prepared based on a Boc solid-phase method, starting from an MBHA resin. The N-terminal Cys(Acm) residue was introduced using Fmoc-Cys(Acm). After HF treatment followed by purification, Fmoc-[Cys(Acm)^{40,44,53}]-MIH(40-75)-NH_2 (**3**) was obtained in 6.3% yield based on the amino groups in the starting resin. After introduction of the Boc groups to the side chain amino groups, followed by removal of the N-terminal Fmoc group with piperidine, H-[Cys(Acm)^{40,44,53},Lys(Boc)^{42,51,67}]-MIH(40-75)-NH_2 (**4**) was obtained in 53% yield.

Peptides **2** and **4** were condensed in DMSO in the presence of silver chloride as an activator for the thioester. HOObt was used as an active ester component, and DIEA as a base (*20, 21*). The reaction was nearly complete after 24 h, as evidenced by the RP-HPLC elution profile of the reaction mixture, which is shown in Fig. 2. The desired product, Boc-[Cys(Acm)^{7,24,27,40,44,53},Lys(Boc)^{19,42,51,67}]-MIH(1-75)-NH_2 (**5**), was separated as the main peak, and no des-Acm product was detected by RP-HPLC and MALDI-TOF

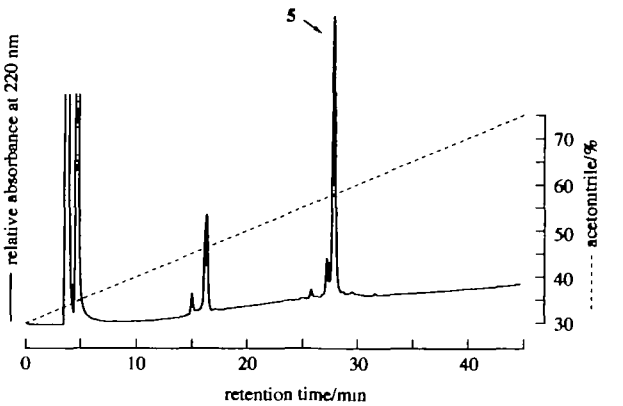


Fig. 2. **RP-HPLC elution profile of the reaction mixture of Boc-[Cys(Acm)^{7,24,27,40,44,53},Lys(Boc)^{19,42,51,67}]-MIH(1-75)-NH_2 (5).** Peptide segments **2** and **4** were condensed in DMSO in the presence of AgCl, HOObt, and DIEA. After 24 h, the reaction mixture was analyzed on Cosmosil 5C_4 AR-300 (4.6 × 250 mm) with a linear gradient of acetonitrile concentration in 0.1% TFA aqueous solution at a flow rate of 1.0 ml/min.

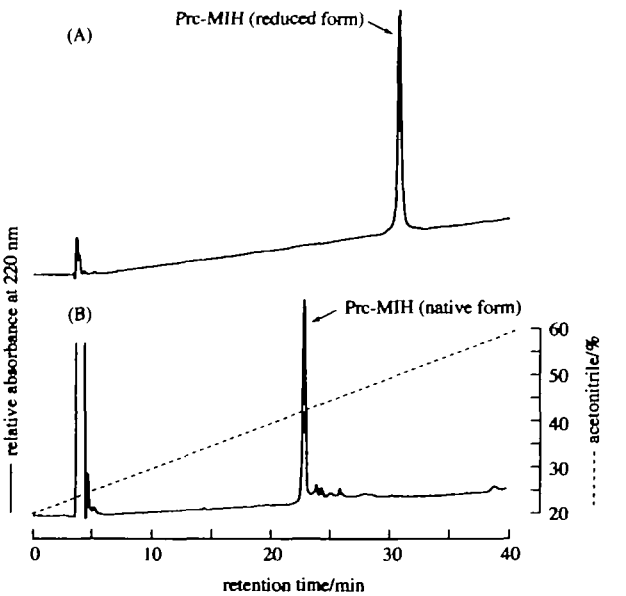


Fig. 3. **RP-HPLC elution profiles of the reduced and native forms of Prc-MIH.** (A) Reduced form of Prc-MIH. (B) Product of the folding reaction of the reduced form of Prc-MIH in 0.22 M Tris-HCl (pH 8.4) containing 10% ethylene glycol and 1.3 M urea in the presence of 4.3 mM cysteine and 0.43 mM cystine. After a 4 h reaction, the resulting mixture was analyzed on Cosmosil 5C_4 AR-300 (4.6 × 250 mm) with a linear gradient of acetonitrile concentration in 0.1% TFA aqueous solution at a flow rate of 1.0 ml/min.

1 ↓ 50
RYVFEECPGV MGNRAVHGKV TRVCEDCYNV FRDTDVLAGC RKGCFSSEMF
75
KLCLLAMERV EEFPDFKRWI GILNA-NH_2

Fig. 1. **Amino acid sequence of Prc-MIH.** The arrow indicates the site of segment condensation.

Boc-[Cys(Acm)^{7,24,27}, Lys(Boc)^{19}]-MIH(1—39)-SCH_2CH_2CO-Nle-NH_2 (2)
+
H-[Cys(Acm)^{40,44,53}, Lys(Boc)^{42,51,67}]-MIH(40—75)-NH_2 (4)
↓ AgCl, HOObt, DIEA, DMSO
Boc-[Cys(Acm)^{7,24,27,40,44,53}, Lys(Boc)^{19,42,51,67}]-MIH(1—75)-NH_2 (5)
↓ 1) TFA treatment
2) RP-HPLC
H-[Cys(Acm)^{7,24,27,40,44,53}]-MIH(1—75)-NH_2 (6)
↓ 1) AgOAc, 90% AcOH
2) DTT, HCl
3) RP-HPLC
Prc-MIH (reduced form)
↓ 1) 0.22 M Tris-HCl (pH 8.4) containing 10% ethylene glycol, 1.3 M urea 4.3 mM cysteine and 0.43 mM cystine
2) RP-HPLC
Prc-MIH (native form)

Scheme 1. **Scheme for the synthesis of Prc-MIH.**

MS analyses. After the addition of DTT to inactivate excess silver ions, the reaction product was precipitated with ether, then the resulting peptide was treated with TFA containing 5% 1,4-butanedithiol to give H-[Cys-(Acm)^{7,24,27,40,44,53}]-MIH(1-75)-NH_2 (**6**) in 49% yield based on peptide **4**. The Acm groups in peptide **6** were removed by treatment with silver acetate in 90% acetic acid for 4 h. After removal of silver ions by the addition of DTT and hydrochloric acid, the reduced form of Prc-MIH was extracted from the precipitate with TFA and isolated by RP-HPLC in 55% yield.

Folding of Prc-MIH—The reduced form of Prc-MIH was converted to the native form in 0.22 M Tris-HCl buffer (pH 8.4) containing 10% ethylene glycol and 1.3 M urea in the presence of 4.3 mM cysteine and 0.43 mM cystine. The RP-HPLC elution profiles of the reduced and native forms of Prc-MIHs are shown in Fig. 3. The folding of the reduced form of Prc-MIH was complete within 4 h, and the product was isolated by RP-HPLC in 38% yield.

Biochemical Characterization of Synthetic Prc-MIH—The behavior of the synthetic Prc-MIH and MIH-active fraction obtained from sinus glands in the American crayfish was compared by RP-HPLC. As shown in Fig. 4, A and B, the synthetic Prc-MIH was eluted as a single sharp peak at 39.3 min. The retention time corresponded to that of the MIH-active fraction obtained from the sinus gland extract. As shown in Fig. 5, the synthetic peptide inhibited *in vitro*

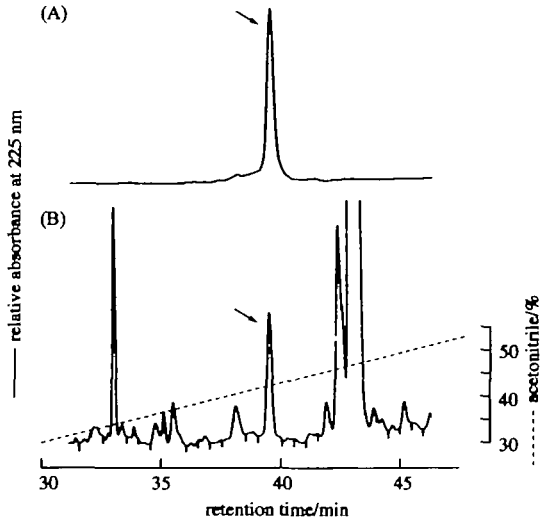


Fig. 4. **Comparison of retention times of the synthetic Prc-MIH and natural Prc-MIH.** The native form of the synthetic Prc-MIH (A) and a sinus gland extract (B) were applied to the Wakosil $5C_{18}$ column (4.6 × 250 mm), and eluted with a 65-min linear gradient of 0–65% acetonitrile in 0.05% TFA aqueous solution at a flow rate of 1 ml/min. The arrows indicate the MIH activity.

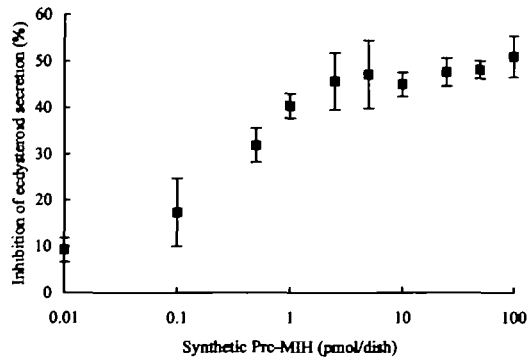
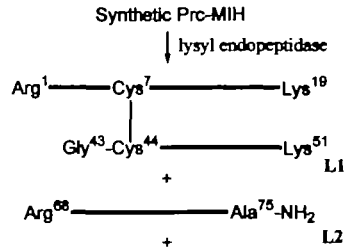


Fig. 5. **Dose-response effects of the synthetic Prc-MIH on ecdysteroid secretion by the crayfish Y-organs.** The Y-organs were incubated in glass dishes containing 0.5 ml of culture medium with the indicated amounts of the native form of the synthetic Prc-MIH for 6 h. Ecdysteroids in the medium were quantified by RIA. The MIH activity was expressed as the percent of inhibition of ecdysteroid secretion relative to untreated control cultures. Points represent the mean ± SE of three to five independent bioassays.



Scheme 2. **Digestion of synthetic Prc-MIH with lysyl endopeptidase.**

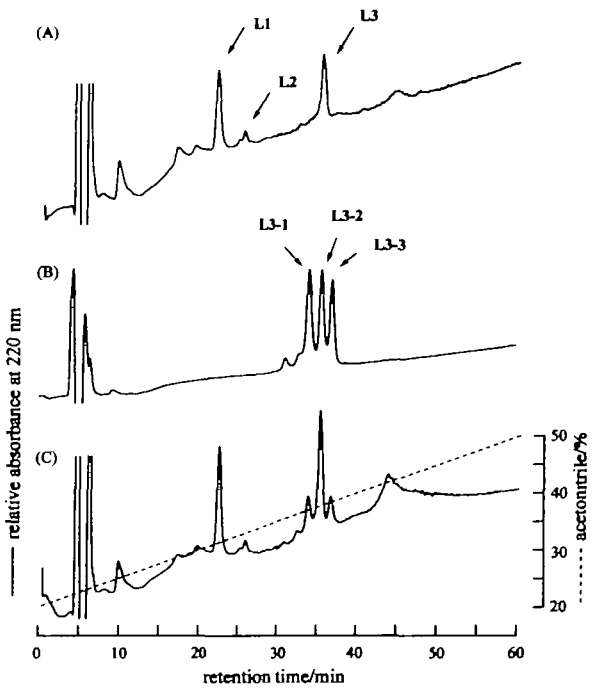
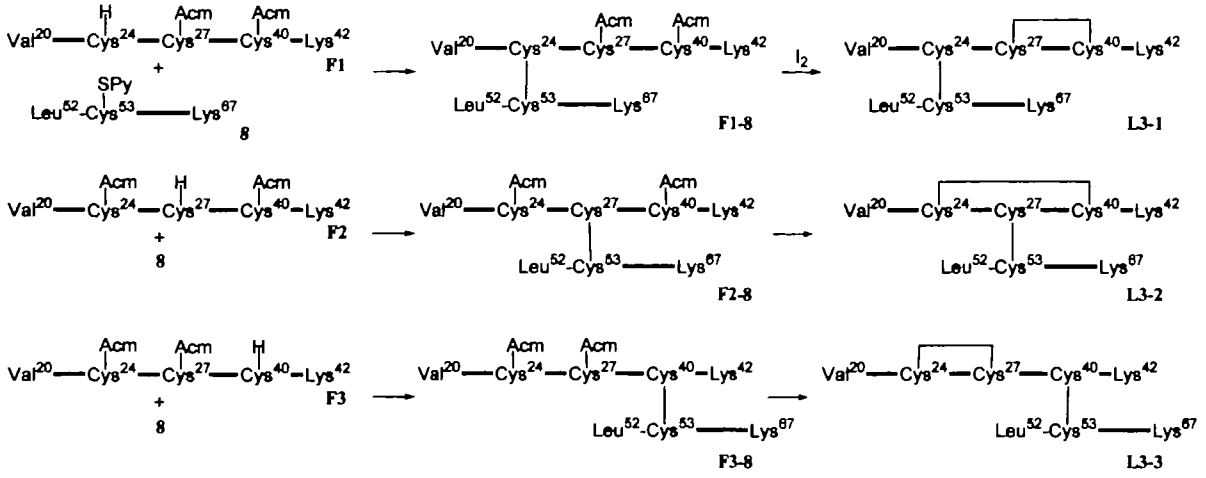


Fig. 6. **RP-HPLC elution profiles of the enzymatic digest of the synthetic Prc-MIH.** (A) An enzymatic digest of the native form of the synthetic Prc-MIH. The native form of the synthetic Prc-MIH (10 μg, 1 nmol) was digested with lysyl endopeptidase (0.7 μg, 2 mAU) in 25 mM ammonium hydrogen carbonate buffer (100 μl) at 10°C for 2 d. (B) Authentic samples of three different disulfide linkages of Prc-MIH fragments, **L3-1**, **L3-2**, and **L3-3**, which consist of Prc-MIH(20-42) and Prc-MIH(52-67). (C) Co-elution of nearly equal amounts of the digestion mixture of synthetic Prc-MIH and the authentic samples, **L3-1**, **L3-2**, and **L3-3**. Samples were analyzed on a YMC-Pack PolymerC18 (4.6 × 150 mm) by elution with a linear gradient of acetonitrile concentration in 0.1% TFA aqueous solution at a flow rate of 0.5 ml/min.



Scheme 3. **Synthesis of all possible fragments of L3.**

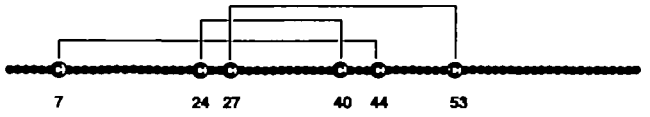


Fig. 7. **Schematic drawing of the location of disulfide linkages in the synthetic Prc-MIH.**

ecdysteroid secretion by Y-organs in a dose-dependent manner at levels between 0.2 and 5 nM. The inhibition reached a plateau (approximately 50% inhibition) at concentrations in excess of 10 nM. The Y-organs responded appreciably to a concentration of the synthetic Prc-MIH as low as 0.2 nM, indicating that the peptide was effective at physiological concentrations.

Determination of the Location of the Disulfide Linkages— The synthetic Prc-MIH was digested with lysyl endopeptidase (Scheme 2) and analyzed by RP-HPLC (Fig. 6A). Three major peaks, **L1**, **L2**, and **L3**, were observed, and the fractions were subjected to MALDI-TOF MS analysis. The mass number of **L1** was *m/z* 3,184.1. This value is in agreement with that of Prc-MIH(1-19,43-51) {Calcd for $[M+H]^+$ 3,183.7 (average)}. The mass numbers of **L2** and **L3** are in agreement with those of Prc-MIH(68-75)-NH_2 {Calcd for $[M+H]^+$ 942.1 (average), found *m/z* 942.9} and Prc-MIH (20-42,52-67) {Calcd for $[M+H]^+$ 4,600.3 (average), found *m/z* 4,600.7}, respectively. At this point one disulfide bond was determined: one cystine residue is contained in the Prc-MIH (1-19,43-51), and thus a disulfide linkage is formed between Cys7 and Cys44. In Prc-MIH-(20-42,52-67), two cystine residues can be found; therefore three standard peptides, **L3-1**, **L3-2**, and **L3-3** of Prc-MIH-(20-42,52-67), which have three possible modes of disulfide linkages, were chemically synthesized (Scheme 3). These three peptides were eluted at different retention times (Fig. 6B). On RP-HPLC, the digested peptide **L3** of Prc-MIH was eluted at the same retention time as that of the authentic peptide **L3-2**, in which disulfide linkages are formed between Cys24 and Cys40 and between Cys27 and Cys53 (Fig. 6C). Thus, the three disulfide linkages, as shown in Fig. 7, can be confirmed.

DISCUSSION

In this study, we synthesized Prc-MIH having identical biological and analytical features to natural Prc-MIH and determined the location of the disulfide linkages by comparing enzymatic digests with authentic synthesized samples.

We have been developing an efficient method for polypeptide synthesis using peptide thioesters as building blocks (*18–22*). Recently the basic conditions that permit the syntheses of cysteine-containing polypeptides have been developed, in which Cys(Acm)-containing peptide segments, which are easily prepared by a Boc solid-phase method, are used as building blocks. The Cys(Acm)-containing peptide segments are condensed without decomposition of the Acm groups when silver chloride is used as an activator for the thioester moieties. The utility of this method has been demonstrated by synthesizing peptides with one or two cysteine residues, such as adrenomedullin (*19*), reaper protein (*20*) and a partial peptide of Cre BP1 (*21*). Prc-MIH, which consists of 75 amino acid residues including six cysteine residues, was an attractive target to examine how the thioester method works in the synthesis of a multiple cysteine-containing peptide. The Acm groups, used for the protection of thiol groups, which tend to be partially decomposed by silver ions, were quite stable under segment condensation conditions in the presence of silver chloride, which is barely soluble in DMSO. On the other hand, the Acm groups were removed by silver acetate in aqueous acetic acid solution. Therefore, it is possible to control the stability of the Acm groups by appropriate choice of reaction conditions, even in the presence of silver ions. The Acm group can also be removed by treatment with base under basic conditions in the presence of silver ions and water (*19–21*). This method was applied to the synthesis of Prc-MIH.

Folding of the reduced form of Prc-MIH was accomplished in a Tris-HCl buffer containing ethylene glycol and urea. Folding in this buffer without ethylene glycol or urea resulted in a poor yield, and a considerable amount of precipitate appeared in the reaction mixture. The addition of ethylene glycol improved the reaction, although the precipitate was still observed. The addition of guanidine hydro-

chloride improved the solubility, although mis-folded peaks were prominent on the chromatogram. In contrast, the addition of urea improved the yield and reduced the amount of precipitate (*27*). Under these conditions, the desired product was observed as an almost single and symmetrical peak on the chromatogram (Fig. 3B), although the isolated yield was still low. This low yield must have been due to precipitation during folding of the reduced form of Prc-MIH, and adsorption of the reduced and native forms of Prc-MIH during folding and isolation processes.

It has been demonstrated in the American crayfish that ecdysteroids in the hemolymph, which are secreted by Y-organs, change quantitatively in a molt-stage-specific manner during the molt cycle (*28*). This change is presumed to be regulated by MIH *in vivo* (*1*, *25–28*). The synthetic Prc-MIH inhibited ecdysteroid secretion by *in vitro* Y-organs in a dose-dependent manner, as shown in Fig. 5. These results indicate that chemical synthesis is a useful alternative means to prepare small proteins, such as Prc-MIH, that contain 100 amino acid residues or so. The inhibition data are closely comparable to those of the natural Prc-MIH extracted from sinus glands in the American crayfish (Sonobe *et al.*, submitted). Using synthetic Prc-MIH, it was possible to determine the location of the disulfide linkages. Prc-MIH, which belongs to type II CHH family, was revealed to have the same disulfide linkages as type I CHHs. Once a set of peptides with a different type of disulfide linkage is prepared, it is very easy to determine the mode of disulfide linkages. A synthetic approach is a definitive route to confirm the location of disulfide linkages that are determined with difficulty based on enzymatic digestion strategy. Structural and functional studies of Prc-MIH will be carried out based upon the synthetic peptide.

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