UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

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ESTUDIO DE VARIOS NEUROPEPTIDOS DE *Procambarus bouvieri* (ORTMANN)

T E S I S

QUE PARA OBTENER EL GRADO DE
MAESTRIA EN CIENCIAS QUIMICAS
(BIOQUIMICA)

P R E S E N T A :

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ABREVIATURAS USADAS

ADN, ácido desoxirribonucleico

AMPc, adenosín monofosfato cíclico

AnalAA, análisis automático de aminoácidos

ARN, ácido ribonucleico

ARNm, ácido ribonucleico mensajero

CCF, cromatografía en capa fina

CHH(s), crustacean hyperglycemic hormone(s)

CHH-B, most abundant isoform of the CHH

CHH-C, less abundant isoform of the CHH

CLAP-FR, cromatografía líquida de alta presión en fase reversa

CompAA, composición de aminoácidos

CPY, carboxipeptidasa Y

kDa, kilodaltones

ExtrC, extremo carboxilo

ExtrN, extremo amino

GMPc, guanosín monofosfato cíclico

GS(s), glándula(s) sinusal(es)

HHG(s), hormona(s) hiperglucemiante(s)

HHG-B, isoforma más abundante de la HHG

HHG-C, isoforma menos abundante de la HHG

HIM, hormona inhibidora de la muda

MIH, molt inhibiting hormone

OX, órgano X ; OXMT, órgano X de la médula terminal

OX-GS, sistema neurosecretor del órgano X y la glándula sinusal

pI, punto isoeléctrico

PM, peso molecular

RP-HPLC, reversed-phase high pressure liquid chromatography

TO(s), tallo(s) ocular(es)

-TPCK,-tosyl-phenylalanyl chloromethyl ketone

Abreviaturas de aminoácidos

NOMBRE	ABREVIATURA DE 3 LETRAS	ABREVIATURA DE 1 LETRA
Acido L-aspártico	Asp	D
ácido L-glutámico	Glu	E
L-alanina	Ala	A
L-arginina	Arg	R
L-asparagina	Asn	N
L-cisteína	Cis	C
L-fenilalanina	Fen	F
L-glicina	Gli	G
L-glutamina	Gln	Q
L-histidina	His	H
L-isoleucina	Ile	I
L-leucina	Leu	L
L-lisina	Lis	K
L-metionina	Met	M
L-prolina	Pro	P
L-serina	Ser	S
L-treonina	Tre	T
L-triptofano	Trp	W
L-tirosina	Tir	Y
L-valina	Val	V
ácido L-aspártico o L-asparagina [a]	Asx	B
ácido L-glutámico o L-glutamina [a]	Glx	Z

[a] Durante la hidrólisis de una proteína, los grupos amida de los residuos de Asn y Gln se hidrolizan al mismo tiempo que los enlaces peptídicos y producen Asp y Glu. Como consecuencia de esto, no es posible saber cuántos y cuáles de estos últimos, observados en el análisis de aminoácidos, se encontraban amidados, por lo que se designan como Asx y Glx.

R E S U M E N

La glándula sinusal del tallo ocular de los crustáceos es el sitio de almacenamiento y liberación de varias neurohormonas importantes. Dos de las actividades biológicas localizadas en la glándula son: (1) la Hormona Inhibidora de la Muda (HIM), que tiene un efecto represor sobre la síntesis y secreción de ecdisteroides a partir de los órganos Y durante los periodos de intermuda y (2) la Hormona Hiperglucemiante (HHG) que aumenta los niveles de glucosa de la hemolinfa.

Durante las pruebas preliminares de la purificación de la HHG de Procambarus bouvieri (Ortmann) mediante cromatografía líquida de alta presión en fase reversa (CLAP-FR), un pico asimétrico contenía toda la actividad hiperglucemiante de los extractos de glándulas sinusales. Se obtuvieron tres péptidos hidrofóbicos al fraccionar dicho pico por medio de una segunda columna cromatográfica. El primero, que no tenía actividad hiperglucemiante, demostró ser la HIM en un bioensayo heterólogo in vitro. El segundo y el tercero tuvieron efectos hiperglucemiantes en el bioensayo y fueron nombradas HHG-B (la isoforma más abundante) y HHG-C (la menos abundante). Cada uno de estos tres neuropéptidos fue semejante a los otros en lo referente a hidrofobicidad, composición de aminoácidos y peso molecular.

El objetivo de este trabajo fue obtener información estructural fundamental acerca de los tres neuropéptidos, que permitiera definir la estrategia adecuada para la determinación de sus secuencias completas de aminoácidos. Al mismo tiempo, estos datos podrían ser útiles para descubrir la naturaleza de las semejanzas y diferencias entre las hormonas. Con este fin, se purificaron y caracterizaron bioquímicamente la HIM y las HHGs a partir de extractos de glándulas sinusales.

A pesar de que mostraron diferencias en sus grados de hidrofobicidad, punto isoeléctrico y contenido de estructura secundaria helicoidal, hay semejanzas notables entre ellas en todas las demás propiedades estudiadas.

No se encontraron diferencias importantes entre todas ellas en relación a peso molecular (6.1-6.4 kDa) y extremos amino (bloqueado) y carboxilo (Ile).

La composición de aminoácidos dio resultados interesantes: (1) las tres hormonas carecen de His, Met y Trp y doce residuos aparecen en la misma cantidad (el número de residuos amidados se desconoce). Hay sólo una diferencia de dos residuos de aminoácido entre cualquier par de hormonas.

Los mapas de los péptidos trípticos indican que: (1) hay sólo un péptido diferente entre la HHG-B y la HHG-C. Los seis pares restantes de péptidos idénticos contienen el 85% de la composición de aminoácidos de las HHGs. (2) Hay 5-6 péptidos idénticos entre la HIM y la HHG-B o la HHG-C. Los residuos contenidos en ellos constituyen el 57% de la composición de las hormonas, mientras que los péptidos que faltan son muy semejantes en composición.

Los resultados indican claramente que las tres hormonas pertenecen a una familia de neuropéptidos de crustáceos.

Las tres hormonas podrían ser el producto de un gen, mediante modificaciones postraduccionales o de tres genes originados por duplicación génica y evolución divergente a partir de un gen ancestral. Otra posibilidad es que se originen a partir de un gen por medio de empalme alternativo del ARNm primario. La respuesta a esta interrogante se tendrá cuando se conozcan las secuencias de aminoácidos completas de la HIM y de las HHGs y se correlacionen con la estructura de su(s) gen(es).

SUMMARY

The sinus gland of the crustacean eyestalk is the site of storage and release of several important neurohormones. Two of the biological activities located in the gland are: (1) the Molt-Inhibiting Hormone (MIH) which has a repressing effect on the synthesis and secretion of ecdysteroids from the Y-organs during the intermolt periods, and (2) the Crustacean Hyperglycemic Hormone (CHH) which raises the glucose level of the hemolymph.

During the early attempts for the purification of the CHH from Procambarus bouvieri (Ortmann) by means of reversed-phase high pressure liquid chromatography (RP-HPLC), an asymmetric peak did contain all of the hyperglycemic activity of the sinus gland extracts. On a second chromatography column, this peak was fractionated into three hydrophobic peptides. The first one, which does not have hyperglycemic activity, later was shown to be the MIH by means of a heterologous *in vitro* bioassay. The second and the third had hyperglycemic effects on bioassay, and were named CHH-B (the most abundant isoform) and CHH-C (the less abundant one). Each one of these three neuropeptides was similar to the other with respect to hydrophobicity, amino acid composition and molecular weight.

The aim of this work was to obtain fundamental structural information about the three neuropeptides, which would allow to determine the adequate approach for the determination of their complete amino acid sequence. At the same time, these data could be useful to uncover the nature of the resemblances and differences among the hormones. For this purpose, the MIH and the two CHHs were purified from sinus glands extracts and were partially characterized from the biochemical point of view.

Although they showed differences in their degree of hydrophobicity, isoelectric point and helical content, there are remarkable similarities among them in all of the other properties studied.

No important differences were found among all of them with respect to molecular weight (6.1-6.4 kDa), and N- (blocked) and C-terminus (Ile).

The amino acid composition leads to striking findings: (1) the three hormones lack His, Met and Trp, and twelve residues appear in the same number (the number of amidated residues is unknown). There is only a difference of two amino acid residues between any two of them.

The tryptic peptide mapping shows that: (1) there is only one different peptide between the CHH-B and the CHH-C. The remaining six pairs of identical peptides account for 85% of the CHHs' amino acid composition. (2) There are 5-6 identical peptides between the MIH and the CHH-B or the CHH-C. The residues contained in them account for at least 57% of the hormones' composition, while the rest of the fragments are very similar in composition.

The results clearly show that the three hormones belong to a crustacean neuropeptide family.

The three hormones could be the product of one gene with posttranslational modifications or of three genes, which originated by gene duplication and divergent evolution from an ancient gene. Another possibility is that they originate from one gene by alternative splicing of their mRNA. The answer to this question will be found when the complete sequences of the MIH and the CHHs are known and are correlated with the structure of their gene(s).

I N T R O D U C C I O N

I. EL CONTROL NEUROENDOCRINO EN LOS CRUSTACEOS

A) El sistema neurosecretor del órgano X y la glándula sinusal (OX-GS)

En los crustáceos, un centro de control metabólico muy importante es el constituido por el órgano X de la médula terminal y la glándula sinusal.

En las especies decápodas, este complejo se localiza en la porción neural de los tallos oculares (que son extensiones del cerebro) y consiste de: (1) un conjunto de células neurosecretoras (el órgano X) que sintetizan hormonas en sus somas, (2) un tracto formado por los axones de dichas neuronas, por medio del cual se transportan las secreciones (empaquetadas en gránulos de neurosecreción característicos) hasta (3) el órgano neurohémico (la glándula sinusal) formado por las terminales axónicas abultadas que están en contacto íntimo con la hemolinfa (Figuras 1 y 2).

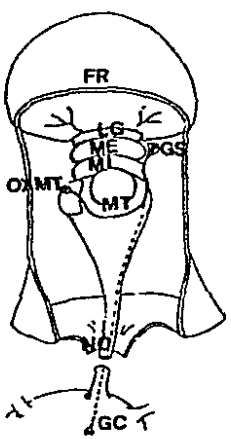


Figura 1. El tallo ocular de los crustáceos decápodos, derivado del ganglio cerebroide (GC), consiste de los fotorreceptores distales (FR) y de 4 ganglios o neurópilos: la médula terminal (MT), la médula interna (MI), la médula externa (ME) y la lámina ganglionar (LG). El sistema neurosecretor está formado por somas neurosecretores situados principalmente en la MT, denominados órgano X de la MT (OXMT) y de sus terminales axónicas ensanchadas que se agrupan y forman a la glándula sinusal (GS). Las terminales de otros somas situados en el GC llegan a la GS a través del nervio óptico (NO). Modificado de Charniaux-Cotton y Kleinholz, 1964.

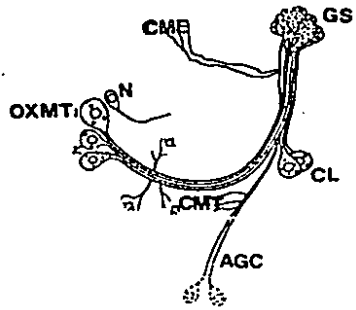


Figura 2. El sistema neurosecretor del tallo ocular. Se observa la contribución de células neurosecretoras laterales (CL) a la glándula sinusal (GS) además de la participación de los tipos celulares del órgano X de la médula terminal (OXMT). También se ven los axones de células localizadas hipotéticamente en el ganglio cerebroide (AGC), las cuales están representadas con líneas punteadas, así como colaterales que van hacia la médula externa (CME) y hacia la médula terminal (CMT) y una neurona no neurosecretora de la médula terminal (N). Las flechas indican conecciones con dendritas postsinápticas. Tomado de Gupta, 1983.

B) Actividades biológicas asociadas al sistema OX-GS

Los estudios basados en la extirpación de los tallos oculares y la sustitución de sus funciones mediante la inyección de extractos de los mismos, indican la participación del sistema neurosecretor OX-GS en una cantidad grande de procesos fisiológicos como: concentración y dispersión de los pigmentos de los cromatóforos y de los pigmentos retinianos distales de adaptación a la luz y a la oscuridad; hiperglucemia e hipoglucemia; inhibición y aceleración de la muda; estimulación y represión de las gónadas; promoción e inhibición del crecimiento de las extremidades en regeneración; aumento y disminución de la respiración mitocondrial y tisular; modulación de la actividad locomotora espontánea; etc. (véase la revisión de Kleinholz, 1985).

C) Hormonas del sistema OX-GS

Solamente se ha logrado asignar tres moléculas específicas a las actividades fisiológicas que están reguladas por las secreciones de la GS. La hormona concentradora del pigmento rojo de Pandalus borealis (Fernlund y Josefsson, 1972) y las hormonas concentradoras del pigmento retiniano distal u hormonas de adaptación a la luz de Pandalus borealis (Fernlund, 1976) y de Uca pugila-

tor (Ranga Rao et al., 1985), han sido caracterizadas en términos de la secuencia de aminoácidos.

Las otras hormonas de crustáceos mejor estudiadas son la Hormona Hiperglucemiante (HHG) y la Hormona Inhibidora de la Muda (HIM), que se conocen hasta el nivel de su composición de aminoácidos (véanse las revisiones de Keller et al., 1985; Keller y Sedlmeier, 1988; Chang y O'Connor, 1988).

II. LA MUDA EN LOS CRUSTACEOS

A) Descripción general

a) El exoesqueleto y el tegumento

El exoesqueleto o cutícula de los crustáceos tiene dos funciones principales: (1) sirve de soporte para los órganos internos y (2) es una cubierta protectora de todo el organismo. Sin embargo, debido a su rigidez, constituye un obstáculo para el crecimiento y, en algunos casos, para el desarrollo de los adultos a partir de las larvas.

Para resolver el problema del crecimiento, los crustáceos cuentan con el recurso de la muda. Esta puede definirse, en términos muy generales, como la renovación periódica del exoesqueleto e implica: (1) la desintegración parcial y reabsorción de las capas más internas de la cutícula que va a eliminarse, (2) la síntesis inicial de un exoesqueleto nuevo, debajo del anterior, (3) el desprendimiento de la cutícula antigua, (4) el aumento de volumen (crecimiento) del cuerpo y (5) la terminación de la síntesis y el endurecimiento de la cubierta recién producida (Blias, 1982).

La estructura general del exoesqueleto de los crustáceos se muestra en la Figura 3.

La capa exterior es la epicutícula, que tiene poco espesor y está compuesta por proteínas, lípidos y sales de calcio. Los estratos siguientes, la exocutícula, la endocutícula y la capa membranosa (en ese orden), están constituidas principalmente por quitina, proteínas y, excepto en la capa membranosa y en las regiones de las membranas artródicas, sales de calcio.

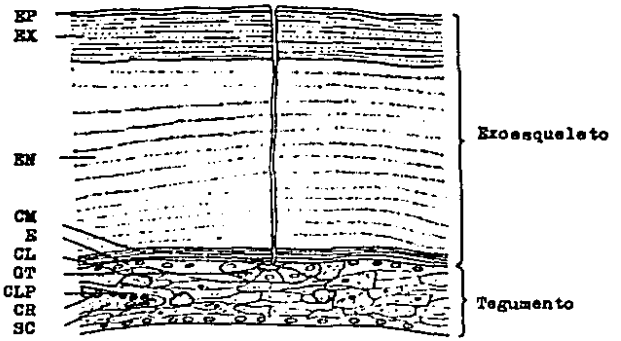


Figura 3. Esquema de un corte del tegumento y exoesqueleto de un crustáceo en la etapa de anecdisis. Las cuatro capas acelulares del exoesqueleto son: EP, epicutícula; EX, exocutícula; EN, endocutícula; CM, capa membranosa. El tejido tegumentario está compuesto de: E, epidermis; CL, células de Leydig; GT, glándula tegumentaria; CLP, células cargadas de lipoproteínas; CR, células de reserva; SC, sinusoide circulatorio. Modificado de Skinner, 1962.

Debajo del exoesqueleto se localiza el tejido tegumentario, formado por células especializadas diversas (de Leydig, pigmentarias, de reserva, cargadas de lipoproteínas, de tejido conectivo, etc.) y la epidermis. Esta última consiste en un estrato simple de células caracterizadas por una actividad secretora intensa: producen la epicutícula, la exocutícula, la endocutícula y el fluido de la muda. En algunas regiones y debajo de la epidermis, se localizan las glándulas tegumentarias, que contienen fenoles y una oxidasa de fenoles que pueden utilizarse en la cutícula para su endurecimiento y otras funciones como adherencia de huevos y fijación del crustáceo a un sustrato; también hay en ellas mucopolisacáridos, posiblemente para lubricación (véanse las revisiones de Stevenson, 1985 y Skinner, 1985).

b) El ciclo de la muda

Ya que la muda es un proceso que se produce periódicamente en la vida de los crustáceos, se describe, técnicamente, como un ciclo que consta de las etapas siguientes: proecdisis o premuda, ecdisis o muda, metecdisis o postmuda y anecdisis o intermuda. Brevemente: (1) la proecdisis es una etapa de preparación para la muda que incluye sucesos como la acumulación de agua; la disolución, mediante el fluido de la muda, de la capa membranosa y la región

más interna de la endocutícula y su reabsorción por la epidermis; la apolisis o separación de la epidermis de la endocutícula degradada; el almacenamiento de calcio, en forma concentrada o gastrolitos, a partir del medio y de la cutícula disuelta parcialmente; y la síntesis inicial del exoesqueleto nuevo. (2) La ecdisis consiste de la apertura de la cutícula vieja en la zona de las membranas artródicas; la expansión del cuerpo por absorción de agua; y la eliminación del exoesqueleto antiguo, que se inicia en el cefalotórax y termina en el abdomen en la mayoría de los crustáceos, incluyendo a los decápodos. (3) la metecdisis consiste en la terminación de la síntesis de la cutícula nueva y su endurecimiento por calcificación. (4) la anecdisis es un periodo de reposo en lo referente a los cambios metabólicos, fisiológicos y morfológicos, relacionados con la muda (véase la revisión de Stevenson, 1985).

La Figura 4 muestra los cambios en el exoesqueleto y tegumento de un crustáceo durante el ciclo de la muda.

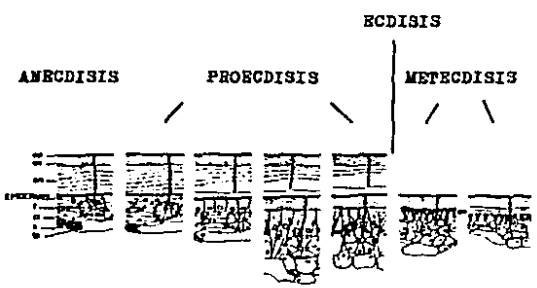


Figura 4. Cambios en el tejido tegumentario de un crustáceo, durante el ciclo de muda. ep, epicutícula; ex, exocutícula; en, endocutícula; t, glándula tegumentaria; cl, célula de Leydig; s, sinusoide circulatorio; lp, célula cargada de lipoproteína. Modificado de Skinner, 1962.

B) Descripción bioquímica

a) Cambios metabólicos en la muda

De la discusión anterior, muy simplificada, del fenómeno de la muda, es fácil darse cuenta, sin embargo, de que tal proceso implica una actividad metabólica muy grande, antes, durante y después de la ecdisis. Asimismo, no es difícil percibir que los sucesos moleculares involucrados en la muda misma y en su regulación son numerosos y muy complejos; de hecho, sólo se conocen parcialmente.

(1) Glucógeno

Durante la anecdisis y la procdisis se almacena glucógeno en la epidermis, músculo, tejido conectivo subepidérmico, etc. La glucosa se utilizará en la síntesis de la quitina de la exocutícula y la endocutícula del exoesqueleto nuevo (véase la revisión de Stevenson, 1985).

(2) Proteínas

Se sabe que el patrón de síntesis de las proteínas tegumentarias cambia durante el ciclo de la muda. Estudios in vitro han mostrado que es posible clasificarlas en cinco grupos de acuerdo a lo anterior. Uno de ellos (grupo I), que incluye péptidos de pesos moleculares muy variados, se produce con una velocidad casi constante, por lo que sus miembros fueron considerados como proteínas de mantenimiento. Algunos péptidos del grupo anterior, que aumentan notablemente en la procdisis, cuando hay formación de epicutícula y exocutícula del exoesqueleto nuevo, constituyen el grupo II. La tercera categoría (grupo III) se produce intermitentemente a partir de la procdisis temprana, cuando se inicia la degradación de la cutícula antigua y continúa hasta el final de dicha etapa. Los miembros de los grupos IV y V se consideran componentes del exoesqueleto nuevo; el grupo IV se genera cuando hay síntesis de epicutícula y exocutícula y consta de especies de pesos moleculares muy diversos; en la categoría V, donde hay proteínas muy grandes, la síntesis ocurre durante la metecdisis, contemporáneamente con la producción de endocutícula para la terminación del exoesqueleto nuevo (Stringfellow y Skinner, 1988).

Se han descrito actividades enzimáticas definidas que aumentan durante alguna etapa del ciclo de la muda. La quitinasa y la quitobiasa (véase la revisión de Stevenson, 1985) así como las proteasas ácidas (O'Brien y Skinner, 1988) y las proteasas alcalinas de cisteína, que degradan proteínas de la ca-

pa membranosa (O'Brien y Skinner, 1987) corresponden al grupo III mencionado antes, mientras que las enzimas que intervienen en la síntesis de la quitina de la cutícula nueva, así como las proteínas exocuticulares y endocuticulares, estarían dentro de los grupos II, IV y V.

Durante la intermuda se produce un péptido inhibidor de cinasas de proteínas dependientes de AMPc y GMPc, las cuales son activas durante la premuda y postmuda; la transcripción y la traducción del gen de dicho péptido se reprimen durante la premuda (Christ y Sedlmeier, 1987). Es posible que las cinasas mencionadas regulen a enzimas involucradas en la degradación de la cutícula vieja o en la síntesis inicial del exoesqueleto nuevo, o a ambos tipos de enzimas.

(3) Lípidos

El tejido conectivo subepidérmico contiene células cargadas de lipoproteínas que parecen sintetizar y proveer lípidos a la cutícula en desarrollo, durante la procdisis y la metecdisis (véase la revisión de Stevenson, 1985).

(4) ADN

Durante el inicio de la procdisis se ha encontrado un aumento considerable en la síntesis de ADN en el tegumento. Esto se correlaciona con la fase de división celular que ocurre en la epidermis, la cual tendrá que incrementar su actividad metabólica durante la procdisis y la metecdisis, según se ha mencionado ya (véase la revisión de Stevenson, 1985).

(5) ARN

La síntesis de ARN varía durante el ciclo de la muda. En general, aumenta en la procdisis y disminuye a partir de la metecdisis (véase la revisión de Stevenson, 1985). Esto se relaciona íntimamente con los cambios en la síntesis de proteínas.

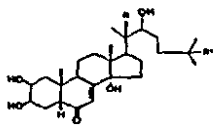
(6) Transporte hidroelectrolítico

También hay que considerar el gasto energético invertido en la absorción del agua que se usará para la expansión del cuerpo durante la ecdisis, así como el que se hace para captar iones de calcio y concentrarlos para su utilización posterior en el endurecimiento del exoesqueleto nuevo.

b) Regulación del ciclo de la muda

(1) Hormona de la muda

A pesar de diferencias pequeñas entre las especies de crustáceos, en general, se ha podido distinguir una variación característica de los niveles de ecdisteroides (las estructuras de algunos de ellos se muestran en la Figura 5) en la hemolinfa, durante el ciclo de la muda (Figura 6a).



ecdisona : R=H, R'=OH
20-hidroxiecdisona: R=OH, R'=OH
ponasterona A: R=OH, R'=H

Figura 5. Estructuras químicas de la ecdisona, 20-hidroxiecdisona y ponasterona A.

También se ha encontrado una relación directa entre las concentraciones circulantes de ecdisteroides y la actividad sintética y secretora de estos compuestos a partir de los órganos Y (Figura 6b); la presencia de estos últimos es un requisito indispensable (salvo en algunos casos muy especiales, todavía no entendidos) para que pueda llevarse a cabo la muda. Asimismo, en general, la administración de ecdisteroides exógenos en dosis bajas, frecuentes y de liberación lenta, produce mudas anticipadas. Todas estas observaciones han generado el concepto del órgano Y como la fuente de la hormona de la muda (véase la revisión de Chang y O'Connor, 1988).

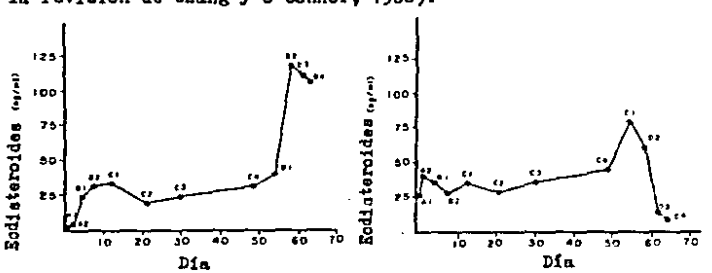


Figura 6. (a) Niveles de ecdisteroides durante el ciclo de la muda de un crustáceo adulto. La concentración de ecdisteroides se determinó por medio de radioinmunoanálisis en muestras de hemolinfa.
(b) Actividad secretora de ecdisteroides de los órganos Y in vitro de un crustáceo adulto. Modificado de Chang y O'Connor, 1988.

El producto de secreción de los órganos Y, la ecdisona (α-ecdisona), no es realmente la forma activa de la hormona de la muda. La actividad promotora se adquiere cuando la α-ecdisona es convertida a 20-hidroxiecdisona (β-ecdisona) en el hepatopáncreas, epidermis, hemolinfa y otros tejidos periféricos. De hecho, un aumento en los niveles de esta última (desencadenado por factores ambientales como temperatura, fotoperiodo, etc. y fisiológicos como actividad reproductora, tensión, etc., todavía no entendidos) se produce durante la premuda y desaparece justo antes de la ecdisis.

Sin embargo, existe un equilibrio entre la α- y la β-ecdisona en cada etapa del ciclo de la muda. Hay evidencia de que la α-ecdisona puede actuar como hormona (se han encontrado receptores para ella en la hipodermis); durante la anecdisis, la α-ecdisona predomina sobre la β-ecdisona (véase la revisión de Huberman, 1989).

Los ecdisteroides circulan en la hemolinfa sin unirse a proteínas y ejercen sus acciones en los tejidos blanco (epidermis, glándulas tegumentarias, etc.) al parecer mediante receptores citoplásmicos y nucleares (véase la revisión de Chang y O'Connor, 1988), afectando la activación y represión de genes específicos (como sucede en otros organismos como insectos y mamíferos) que codifican a las enzimas y proteínas que intervienen en el fenómeno de la muda (Bielefeld *et al.*, 1986).

(2) Hormona inhibidora de la muda

La idea de un control neurohormonal de la muda surgió de la observación, en muchas especies de crustáceos, principalmente decápodos, de que la extirpación de los tallos oculares producía una aceleración en el ciclo de la muda. En el tallo ocular se localiza un sistema neurosecretor constituido por las neuronas peptidérgicas del órgano X y sus terminales axónicas, las cuales forman la llamada glándula sinusal, que es un órgano neurohémico a partir del cual las secreciones son descargadas en la hemolinfa.

Otros experimentos apoyaron este concepto: (1) la eliminación de los tallos oculares producía aumentos notables en los niveles de ecdisteroides circulantes y los animales entraban en la etapa de premuda; (2) la inyección de extractos de tallos oculares en ejemplares destallizados conducía a concentraciones bajas de ecdisteroides en la hemolinfa; (3) la secreción de ecdisona a partir de los órganos Y *in vitro* se inhibía en un medio de cultivo que había sido incubado con glándulas sinusales; (4) los órganos Y de animales a los que

se había inyectado extractos de glándulas sinusales mostraron, en cultivo, una producción disminuída de ecdisona (véase la revisión de Chang y O'Connor, 1988).

Estos resultados han llevado a postular la existencia de una Hormona Inhibidora de la Muda (HIM) en la glándula sinusal. Se han aislado varias moléculas peptídicas que poseen dicha actividad.

Hasta la fecha, la HIM de Carcinus maenas (Webster y Keller, 1986) y Homarus americanus (Chang et al., 1987) se han caracterizado en términos de sus pesos moleculares, composiciones de aminoácidos y extremos amino. Se sospecha una semejanza entre la HHG y la HIM de Carcinus maenas.

La liberación de la HIM, que ocurre durante la mayor parte del ciclo de la muda, excepto en la proecdisis, está mediada por la 5-hidroxitriptamina. Además, está sujeta a retroalimentación por los niveles de ecdisteroides; las concentraciones elevadas de α-ecdisona favorecen la secreción de la HIM, mientras que el aumento de la β-ecdisona la inhibe (Mattson y Spaziani, 1986a; véase la revisión de Huberman, 1989).

La HIM se une a sus receptores en los órganos Y y activa la producción de AMPc intracelular. Se ha encontrado que, tanto in vitro como in vivo, los extractos de tallos oculares inhiben la captación de colesterol (que es el núcleo para la síntesis de los ecdiateroides) por parte de los órganos Y. Asimismo, algunas fracciones de extractos de glándulas sinusales disminuyen la síntesis de ARN y proteínas; esta última es necesaria para la elaboración de la ecdisona (Mattson y Spaziani, 1986b).

El efecto inhibidor de la HIM sobre los órganos Y se da en función de la dosis aplicada, es fisiológicamente directo, reversible, específico en cuanto al tejido blanco, no específico en lo referente a la especie de crustáceo y se manifiesta a concentraciones subpicomolares del neuropéptido (véase la revisión de Huberman, 1989).

III. LA GLUCEMIA EN LOS CRUSTACEOS

A) Descripción general

a) Los niveles de glucosa en los crustáceos

Ya que los crustáceos son animales nocturnos, no es extraño encontrar que su glucemia es baja durante el día y alcanza su máximo valor en la madrugada (Figura 7). Sin embargo, los crustáceos tienen niveles de glucosa circulante muy bajos en relación con los animales superiores.

Por otra parte, se han encontrado curvas de hiperglucemia experimental parecidas a la del hombre en cuanto al aumento y caída bruscos y a las oscilaciones leves del nivel basal de glucosa después de la inyección de una dosis gran

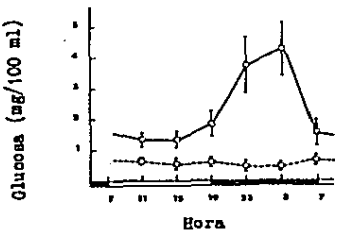


Figura 7. Concentración de glucosa de la hemolinfa de un crustáceo durante el día, en animales intactos (línea continua) y destallizados (línea de guiones). Tomado de Hamann, 1974.

de de ésta. Este hallazgo llevó a la conclusión de que debía existir un mecanismo regulador que mantenía casi constante la concentración de glucosa de la hemolinfa, el cual podía actuar mediante el depósito de la glucosa en el glucógeno o en alguna otra forma de almacenamiento de carbohidratos (Hemmingsen, 1924).

b) Condiciones que afectan la glucemia

Varias formas de tensión fisiológica (manipulación prolongada, temperatura elevada, asfixia, amontonamiento, etc.) y algunas sustancias (glucagon, adrenalina, serotonina, etc.) producen hiperglucemia en los crustáceos intactos pero no en los destallizados. Esto indica que su acción es indirecta y está mediada por algún factor localizado y liberado en el tallo ocular (Abramowitz et al., 1944; Kleinholz et al., 1950; Leinen y Mc Whinie, 1971; Berdeja, 1972; Teldford, 1974).

B) Descripción bioquímica

a) Regulación neuroendócrina de la glucemia

La extirpación de los tallos oculares (TOs) (Kleinholz y Keller, 1973; Huberman et al., datos no publicados) y de las glándulas sinusales (GSs) (Hamann, 1974) conduce a hipoglucemia.

Abramowitz et al.(1944) descubrieron que la inyección de extractos cru-

dos de TOs de Callinectes sapidus aumentaba las sustancias reductoras totales de la hemolinfa; el compuesto responsable del efecto fue denominado "factor diabetógeno". Los mismos autores demostraron que la molécula activa se localizaba en la GS.

Estudios posteriores demostraron que el "factor diabetógeno" producía una hiperglucemia verdadera. Esta consiste en un aumento rápido del nivel de glucosa en 10 a 15 min y un máximo en 1 a 2 h, después de la inyección de los extractos crudos; luego, la glucemia disminuye rápidamente y al cabo de unas 6 h se vuelven a alcanzar los niveles basales (Kleinholz et al., 1950; Keller y Andrew, 1973; Skorkowski et al., 1977). En Procambarus bouvieri, el efecto es más pronunciado en los animales intactos que en los destallizados (Berdeja, 1972). En 1967 se sugirió el nombre de Hormona Hiperglucemiante (HHG) para la sustancia responsable de la hiperglucemia (Kleinholz et al., 1967).

De hecho, se han purificado y caracterizado moléculas proteínicas que tienen la actividad biológica de la HHG (Huberman y Aguilar, 1986).

La HHG de varias especies se conoce parcialmente. Se trata de un péptido (Kleinholz et al., 1967) y se sabe que es específica del grupo taxonómico (Keller, 1969; Kleinholz y Keller, 1973; Martin et al., 1984; Leuven et al., 1982; Keller, 1977) y que hay varias formas moleculares de ella en una misma especie (Kleinholz et al., 1967; Keller, 1969; Kleinholz y Keller, 1973; Keller, 1977; Huberman et al., 1974; Newcomb, 1983; Skorkowski et al., 1977; Trausch y Bauchau, 1982; Andrew y Saleuddin, 1979; Stuenkel, 1983, 1986; Van Wormhoudt et al., 1984; Keller y Segel, 1984; Kallen et al., 1986; Van Deijnen, 1986). Se tienen algunos datos estructurales de las hormonas de Carcinus maenas (Keller y Wunderer, 1978), Orconectes limosus (Keller, 1981), Cancer magister (Kleinholz, 1975), Porcellio dilatatus (Martin et al., 1984) y Procambarus bouvieri (Huberman y Aguilar, 1986). El último estudio es el más completo en la actualidad e incluye: peso molecular mínimo, punto isoeléctrico, composición de aminoácidos y extremos amino y carboxilo. Sin embargo, no se ha determinado todavía la secuencia de aminoácidos de ninguna HHG.

b) Acciones de la hormona hiperglucemiante

La forma de las curvas de hiperglucemia causada por inyección de extractos crudos de TOs o de GSs sugiere que el fenómeno es causado por una movilización rápida de glucosa a partir de reservas de carbohidratos o por transferencia desde depósitos de monosacáridos u oligosacáridos en las células blanco y no por cambios de flujos entre las vías metabólicas principales.

(1) Metabolismo del glucógeno

La HHG podría producir su efecto mediante una acción glucogenolítica en los tejidos blanco. Evidencia que apoya esta idea es la observación de que la inyección de la hormona conduce a una disminución en el glucógeno del músculo abdominal y de las gónadas de Orconectes limosus y de las branquias y tegumento de Uca pugilator (Keller y Andrew, 1973). También se ha encontrado aumento en la liberación de glucosa radiactiva a partir del músculo abdominal de Cambarus robustus marcado previamente con ^{14}C -glucosa y tratado in vitro con extracto de TOs (Teldford, 1975); lo mismo se observó en un experimento semejante hecho con el hepatopáncreas de Orconectes limosus incubado con HHG purificada (Sedlmeier, 1987).

Por otra parte, se han visto aumentos en el contenido de glucógeno en el músculo abdominal y tegumento de Panulirus japonicus (Schwabe et al., 1952) y en el músculo abdominal de Ocypode platytarsis (Parvathy, 1972) como consecuencia de la extirpación de los TOs.

Con un desarrollo temporal comparable al de la hiperglucemia, se ha encontrado, in vivo, activación de la fosforilasa de glucógeno del músculo abdominal de Orconectes limosus (Keller, 1965), por inyección de extractos de TOs; resultados similares se obtuvieron con el músculo de Carcinus maenas y Eriocher sinensis, in vitro (Bauchau et al., 1968). El efecto opuesto, es decir, la inactivación de la fosforilasa, se ha observado después de la eliminación de los TOs de Orconectes limosus (Keller, 1965) y Hemigrapsus (Ramamurthi et al., 1968).

Como se esperaba, la actividad de la sintetasa de glucógeno se ha encontrado aumentada después de la extirpación de los tallos oculares, en el músculo abdominal de Orconectes limosus (Keller, 1966; Sedlmeier, 1982) y Cancer (Ramamurthi et al., 1968). En ambos casos, el efecto fue anulado por la inyección de extractos de TOs o de HHG purificada.

En cuanto al mecanismo de acción, se ha encontrado que los nucleótidos cíclicos, AMPc y GMPc, intervienen como mensajeros secundarios de la HHG. En estudios in vivo, varios tejidos (principalmente corazón, hepatopáncreas y músculo abdominal, pero también tegumento y glándula antenaria) mostraron un aumento de dichos mensajeros intracelulares después de la inyección de la HHG (Sedlmeier y Keller, 1981).

Experimentos in vivo e in vitro han mostrado que el aumento intracelular de los nucleótidos cíclicos, producido por la HHG en los tejidos blanco, se debe a un incremento de la actividad de las ciclasas respectivas que los producen y no a una inhibición de la fosfodiesterasa que los degrada (Dieberg y Sedlmeier, 1984; Sedlmeier, 1985).

Como sucede en los organismos superiores, en los crustáceos los nucleótidos cíclicos se unen a cinasas de proteínas y las activan. Se ha encontrado en el músculo abdominal de Orconectes limosus un aumento en el cociente de actividad de dichas cinasas, que es paralelo al incremento de los nucleótidos cíclicos; en este sistema, parece existir una sola clase de cinasa de proteínas que fija por igual al AMPc y al GMPc (Christ, 1984).

En el músculo abdominal de Orconectes limosus se ha encontrado que las ciclasas de adenilato y de guanilato están reguladas por iones de calcio y calmodulina (Sedlmeier y Dieberg, 1983). La HHG podría afectar a las enzimas de la síntesis y degradación del glucógeno por medio de la liberación de iones de calcio a partir del retículo endoplásmico por la vía del fosfatidilinositol.

En resumen, el mecanismo de acción de la HHG podría ser el siguiente: (1) la hormona se une a receptores específicos en los tejidos blanco; (2) por medio de estos receptores activa a las cinasas de adenilato y de guanilato y como resultado del aumento de los niveles intracelulares de los nucleótidos cíclicos se produce estimulación de cinasas de proteínas; asimismo, activa a la fosfolipasa C y el inositol trifosfato producido se une a receptores específicos en el retículo endoplásmico y favorece el aumento de la concentración de iones de calcio citoplasmáticos, que en combinación con la calmodulina activan a las cinasas de adenilato y guanilato; (3) las cinasas de proteínas dependientes de nucleótidos cíclicos inhiben, por fosforilación, a la sintetasa de glucógeno y activan a la fosforilasa de glucógeno; (4) el efecto combinado sobre estas dos enzimas produce un aumento de glucosa en la hemolinfa, al secretarse aquella desde las células blanco.

(2) Digestión

Se ha encontrado que la HHG puede intervenir en el metabolismo de los carbohidratos mediante la regulación de enzimas extracelulares. El hepatopáncreas, que posee receptores para la hormona, interviene en la secreción de fluido digestivo, en la absorción del alimento digerido y en el almacenamiento de lípidos y carbohidratos (véase la revisión de Chang y O'Connor, 1988).

Se han observado cambios en las actividades amilolítica y proteolítica del hepatopáncreas por inyección de extractos de TOs, inclusive en animales en ayuno (Fingerman et al., 1967) y por extirpación de los TOs, así como durante las diferentes etapas del ciclo de la muda (Van Wormhoudt et al., 1976). La HHO purificada aumenta notablemente la secreción hepatopancreática de amilasa (Sedlmeier, 1988).

Hasta la fecha, se cree que la secreción de la amilasa está mediada por aumento de los iones de calcio intracelulares y que es posible la intervención indirecta de nucleótidos cíclicos generados por ciclasas dependientes de calcio y calmodulina (véase la revisión de Chang y O'Connor, 1988).

A pesar de que tanto la degradación muscular de glucógeno, como la secreción hepatopancreática de amilasa producen aumento en la glucemia, no se ha comprendido todavía el significado fisiológico de la acción de la HHO sobre la actividad amilolítica del hepatopáncreas.

O B J E T I V O

El estudio de la HHG tiene un interés bioquímico general. El conocimiento de la estructura de las HHGs de varias especies permitiría compararlas entre sí, en busca de homologías, alteraciones evolutivas y del sitio activo. Su comparación con hormonas de organismos superiores que tienen actividad hiperglucemiante (ya sea primaria o secundaria) conduciría a la obtención de información acerca de la relación estructura-función en proteínas y en hormonas, en particular.

Por otra parte, resultará interesante descubrir el mecanismo que ha conducido a la existencia de varias HHGs en una misma especie y su significado fisiológico. Hay varias posibilidades que no se excluyen mutuamente: duplicación génica y evolución divergente, empalme alternativo del ARNm primario y modificación postraduccional.

Asimismo, el estudio de su mecanismo de acción y de su regulación podría tener implicaciones en la fisiología humana, particularmente en lo que se refiere a la comprensión de los mecanismos fisiopatológicos de la diabetes.

El conocimiento de la estructura de la HIM también aportaría información básica muy valiosa en los campos de la evolución y de las relaciones estructura-función de proteínas y en especial, de hormonas de invertebrados.

El objetivo de esta Tesis de Maestría es hacer una caracterización inicial (peso molecular, punto isoeléctrico, composición de aminoácidos, extremos amino y carboxilo y contenido de estructuras secundarias) de las HHGs y de la HIM de Procambarus bouvieri (Ortmann), así como un estudio comparativo mediante mapas peptídicos, para obtener información que será fundamental para definir la estrategia que se usará en la determinación de las estructuras completas de las tres hormonas y para explorar la naturaleza de las semejanzas y diferencias entre estos neuropéptidos de la glándula sinusal del tallo ocular de los crustáceos.

MATERIALES, METODOLOGIA Y RESULTADOS

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A NEUROSECRETORY HYPERGLYCEMIC HORMONE FROM THE SINUS GLAND OF THE MEXICAN CRAYFISH *PROCAMBARUS BOUVIERI* (ORTMANN)—II. STRUCTURAL COMPARISON OF TWO ISOFORMS OF THE HORMONE*

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Abstract—1. The hyperglycemic activity of a crude extract of *Procambarus bouvieri* (Ortmann) sinus glands was resolved into two UV-absorbing peaks by means of a single step of reverse-phase high performance liquid chromatography (RP-HPLC) on a μ-Bondapak-Phenyl column. These peaks have been designated Crustacean Hyperglycemic Hormones CHH-B and CHH-C in the order of elution.

2. The ratio CHH-B/CHH-C was approximately 3:1, both in area under the curve and in protein content.

3. A structural comparison of the two isoforms of the CHH showed a substantial homology manifested in molecular weight (6000–6200), pI (4.79), number of residues (52–53), number of cysteines (4), number of acid residues, including their amides (12), number of basic residues (8), missing amino acids (methionine, histidine and tryptophane), amino end (blocked) and carboxyl end (isoleucine).

4. The only clear difference between the two isoforms of the CHH is their degree of hydrophobicity which might be due to minor differences in the number of neutral hydrophobic residues and/or postranslational modifications of the type amidation deamidation of acid residues which cannot be detected in acid hydrolysates.

INTRODUCTION

It has frequently been observed that the Crustacean Hyperglycemic Hormone (CHH) isolated from crude extracts of sinus glands of different decapods, consists of more than one molecular species and thus presents an almost constant degree of heterogeneity.

Thus, Kleinholz and Keller (1973) found in *Cancer magister* and in *Pandalus jordani*, by means of gel filtration and ion-exchange chromatography, at least two peaks of 6000–7000 MW with hyperglycemic activity. Keller (1977) has found, by means of PAGE, that sinus gland extracts from *Pacifastacus leniusculus* and *Orconectes limosus* show two bands of hyperglycemic activity, while in *Carcinus maenas* only one band was observed, all of them of 6000–7000 MW. Skorkowski *et al.* (1977) found by means of gel filtration of an eyestalk extract of *Crangon crangon*, two peaks of hyperglycemic activity, but of very different MW, 20,500 and 7300, respectively. Andrew and Saleuddin (1979) found, by means of two-dimensional PAGE in a *Callinectes sapidus* eyestalk extract, three peptides of 7000 MW with hyperglycemic activity. Newcomb (1983) and Stuenkel (1983, 1986) have found in *Cardisoma carnifex*, by means of RP-HPLC of a sinus gland extract, two peptides of 6000 MW with hyperglycemic activity. Van Wormhoudt *et al.* (1984) have found in *Palaemonetes serratus* three peptides of 20,000, 8000 and 2000 MW with hyperglycemic activity. Keller and Kegel (1984) have shown by means of RP-HPLC of sinus gland extracts of *Carcinus maenas*, *Orconectes limosus* and *Eriocheir sinensis* two peaks of hyperglycemic activity of 6000–7000 MW. Kallen *et al.* (1986) have shown, by means of RP-HPLC of a sinus gland extract of *Astacus leptodactylus*, two peptides of 7000 MW with hyperglycemic activity. Van Deijnen (1986) has found, by means of RP-HPLC of an extract of *Homarus americanus* sinus glands, two peptides of 7000 MW with hyperglycemic activity.

We have shown in a previous work (Huberman and Aguilar, 1986), by means of RP-HPLC, that in a sinus gland extract of *Procambarus bouvieri*, there are two u.v.-absorbing peaks that have hyperglycemic activity and that these peptides are of 6000–6500 MW.

On the other hand, Martin *et al.* (1984), while analysing a sinus gland extract of the isopod *Porcellio dilatatus* by means of RP-HPLC, could see only one peak of hyperglycemic activity.

In view of the overwhelming evidence in favor of more than one peptide with hyperglycemic activity in each crustacean species hitherto investigated, we decided to further characterize the two hyperglycemic peptides of *Procambarus bouvieri* which we have designated as CHH-B and CHH-C in the order of elution from a μ-Bondapak-Phenyl column. Our aim is to elucidate if these are the products of one gene, with a postranslational modification of a fraction to

*Preliminary reports comprising part of this work were presented at the XXX National Congress of Physiological Sciences (Xalapa, July 1987) and at the XXII Mexican Congress of Pure and Applied Chemistry (Veracruz, Sept. 1987).

give two hyperglycemic hormones, or if they are the products of two genes.

MATERIALS AND METHODS

Animals

Adult crayfishes of both sexes were obtained from local fishermen in Uruapan, State of Michoacán. Body size: 7 ± 1 cm; weight: 20 ± 5 g. They were kept in plastic containers with tap water at room temperature, through which compressed air was bubbled continuously. They were fed daily with crayfish meat.

Sinus glands

Animals were cold-anesthetized and their eyestalks (ES) cut with sharp scissors. The ES were kept at −70°C until used. The sinus glands (SG) were dissected under ice-cold crustacean saline (van Harreveld, 1936) and kept in a small volume of ice-cold distilled water (1 μl/SG).

Crude extract

The protocol described by Huberman and Aguilar (1986) was followed with slight improvements. Briefly, 2000 SG in water were disintegrated with one 5–10 sec burst of the Kontes Microultrasonic Cell Disrupter followed by centrifugation at 107,000 *g* (average) for 15 min at 2°C in a Beckman TL-100 Tabletop Ultracentrifuge provided with a TLA-100.2 rotor. After decanting the supernatant, the precipitate was resuspended in 100 μl of water and the process repeated twice. Finally, the three supernatants were mixed, concentrated to 0.5 μl SG, centrifuged at 356,000 *g* (average) for 30 min at 2°C. The supernatant was filtered through a centrifugal microfiltration device provided with a 0.2 μm regenerated cellulose filter (Model MF-1, Bioanalytical Systems, Inc.), at 1800 *g* and 4°C for 10 min.

Protein determination

Samples were hydrolysed in 5.7 M HCl at 145°C under N_2, for 1 hr in calcinated flame-sealed Pyrex tubes. The micromethod of Hazra *et al.* (1984) was employed.

HPLC

A Beckman/Altex system was used. It consisted of a Mod. 420 Microprocessor Controller/Programmer, two Mod. 110A pumps, a manual injection valve Mod. 210 and a Mod. CRIA Recorder/Data Processor. The detector was a LKB Uvicord-S with an 8 μl cell. The column was μ-Bondapak-Phenyl (3.9 × 300 mm, 10 μm particles, Waters). The effluent was monitored at 206 nm. Solution A was 0.1% aqueous trifluoroacetic acid (TFA) and solution B was 60% aqueous CH_3CN containing 0.1% TFA. The column was equilibrated with 35% B and an aliquot of 400 SG injected in a volume of 250 μl. A linear gradient from 35% B to 56% B in 10 min was followed by an isocratic elution at 56% B for 1 hr. Selected peaks were collected manually, avoiding carefully the valleys between adjacent peaks, and were concentrated in a Savant centrifugal evaporator.

Amino acid analysis

Peptide CHH-B (110 μg; 18.0 nmoles) and 40 μg (6.7 nmoles) of peptide CHH-C were divided into five lots of 22 μg (3.6 nmol) and 8 μg (1.3 nmol), respectively. The first three lots were hydrolysed in 5.7 M HCl + 0.16% (w/v) phenol, at 110°C for 24, 48 and 72 hr. The fourth lot was oxidized with performic acid (Moore, 1963) and then hydrolysed as above. The fifth lot was hydrolysed in 5.7 M HCl containing 3% thioglycolic acid (Matsubara, 1969). Samples were taken to dryness in the Savant centrifugal evaporator and resuspended in 200 μl of Beckman's High Performance Amino Acid Sample Dilution Buffer (Na-System). Fifty microliters were injected into a Beckman 6300 Amino Acid Analyser. Norleucine (1250 pmol/50 μl) was used as internal standard.

End-group analysis

Two nmol of each peptide was dansylated according to Gray and Hartley (1963) as described by Allen (1981). They were hydrolysed and chromatographed on polyamide 3 × 3 cm sheets. Carboxypeptidase Y was used for the determination of the carboxyl-terminal amino acid on 1.5 nmol of reduced and carboxymethylated peptide. The product was dansylated and chromatographed as above.

Isoelectric focusing

This was performed on an LKB Multiphor I apparatus with a 0.5 mm preformed polyacrylamide gel containing a wide gradient of Ampholine (pH 3.5–9.5). Twenty microliter samples were applied in filter paper pads 1 cm from the cathode. Focusing was performed at 4°C with a power limit of 8 W and a maximum voltage set at 1500 V for 1.5 hr. The pH gradient was determined by cutting 2 mm segments of a parallel strip of gel, leaching out the Ampholines at room temperature in 2 ml of water in sequentially numbered tubes and measuring the pH of each tube after 18 hr.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The method described by Schägger *et al.* (1985), utilizing SDS-polyacrylamide/glycerol gels was used with slight modifications. Horizontal slab gels were 11 × 24.7 cm and 1 mm thick with preformed 5 μl wells. The acrylamide concentration of the sample gel was 4% and that of the separating gel was 16%. In both, the ratio acrylamide/bisacrylamide was 30:1. The height of the separating gel was 5.5 cm. An LKB Multiphor II apparatus provided with a Macrodrive 5 Power Supply was used at 30 V for 2 hr and at 180 V for the following 6 hr, at 20°C. CHH-B and CHH-C were applied at a concentration of 1.5 μg in 5 μl of sample buffer. A standard of aprotinin (6.5 kDa) was simultaneously run. The gel was stained with Coomassie Brilliant Blue G-250.

Reagents and glassware

All reagents were of analytical grade. HPLC solvents were from Fisher. Carboxypeptidase Y was from Sigma. Double-distilled water was further purified by passing through a Sybron-Barnstead NANOpure-A system and only water with a resistivity above 10 MΩ cm was used. Glassware was calcinated at 550°C for 4 hr.

RESULTS

As can be seen in Fig. 1, peptides CHH-B (lane 2) and CHH-C (lane 3) migrated exactly the same distance during the SDS-PAGE/glycerol electrophoresis. Lane 1 corresponds to peptide A (Huberman and Aguilar, 1986) which is purified in the same column, but as it does not have hyperglycemic activity it will be the subject of another paper. By comparison with the band of aprotinin (6.5 kDa), we extend for the three peptides the previous conclusion arrived at by us that the major hyperglycemic hormone (CHH-B) has an MW between 6000 and 6500. Moreover, they are shown to be free from any contaminant.

The amino acid analysis of peptides CHH-B and CHH-C show a remarkable similarity (Table 1). While there is coincidence in the number of residues per molecule of 6.0 kDa in the case of 16 amino acids, only two, leucine and phenylalanine, show a slight discrepancy. The total number of residues is 52–53 in both cases. Glycine is taken as 1–2 on account of

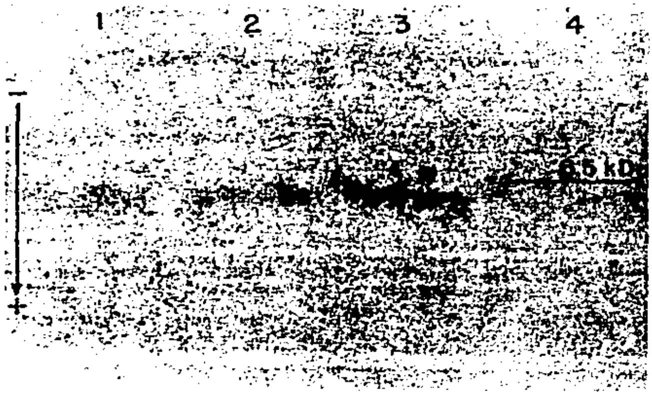

Fig. 1. SDS-PAGE/glycerol electrophoresis of peptides A, CHH-B and CHH-C. Horizontal slab gels (11 × 24.7 × 0.1 cm) with preformed 5 μl wells were used. Acrylamide concentration of the sample gel was 4% and of the separating gel was 16%. The acrylamide/bisacrylamide ratio was 30:1. The height of the separating gel was 8.5 cm. Peptide A (lane 1), peptide CHH-B (lane 2) and peptide CHH-C (lane 3) were applied in a concentration of 1.5 μg per 5 μl. The position of aprotinin (6.5 kDa, 2 μg/5 μl) is indicated. Run conditions were 30 V for 2 hr and 180 V for the following 6 hr, at 20°C. Stained with Coomassie Brilliant Blue G-250.

the high frequency of contamination of reagents and water with this amino acid. Both peptides lack the same amino acids: methionine, histidine and tryptophane. These latter data were confirmed by acid hydrolysis in 5.7 M HCl in the presence of 3% thioglycolic acid and by dansylation and thin layer chromatography on polyamide sheets (Matsubara and Sasaki, 1969). The number of aspartic acids + asparagines (ASX) and of glutamic acids + glutamines (GLX) is the same in both peptides, but as these values were obtained by acid hydrolysis, we ignore them if the number of amidated residues is the same or not in both cases. From the above data, the calculated MW of both peptides is 6000–6200, which is in agreement with the gel electrophoresis results. The pI obtained by isoelectric focusing was the same for both peptides, 4.79.

A free amino end was not found and both peptides are considered to be blocked. The carboxyl end was found to be isoleucine in both peptides, but as the enzyme used, carboxypeptidase Y, has amidase activity, it is not possible to state at present if the carboxyl terminal amino acid is amidated or not. All the structural data found for peptides CHH-B and CHH-C is given in Table 2.

Table 1. Comparative amino acid composition of the major (CHH-B) and the minor (CHH-C) hyperglycemic hormones from the sinus gland of *Procambarus bouvieri* (Ortmann)

Amino acid	CHH-B Average[a]	CHH-B Residues[b]	CHH-C Average[a]	CHH-C Residues[b]
ASX[c]	7.37	7	7.11	7
THR	1.88	2	1.86	2
SER	1.74	2	1.99	2
GLX[c]	5.33	5	5.35	5
PRO	0.87	1	1.00	1
GLY[d]	1.77	1–2	1.97	1–2
ALA	3.08	3	3.10	3
CYS[e]	4.49	4	4.27	4
VAL[f]	5.29	6	5.24	6
MET[g]	—	0	—	0
ILE	3.31	3	2.74	3
LEU	4.59	5	3.90	4
TYR	3.07	3	2.84	3
PHE	2.33	2	2.81	3
HIS[g]	—	0	—	0
LYS	2.75	3	3.34	3
ARG	4.70	5	5.11	5
TRP[g]	—	0	—	0
		52–53		52–53

[a]Average values obtained after acid hydrolysis in 5.7 M HCl + 0.16% (w/v) phenol, for 24, 48 and 72 hr.
[b]Number of residues per molecule of 6 kDa.
[c]ASX = ASP + ASN, GLX = GLU + GLN.
[d]Taken as 1–2 on account of high frequency of contamination of reagents and water with glycine.
[e]24 hr acid hydrolysis (as above) after oxidizing the sample with performic acid.
[f]Next higher integer of the 72 hr acid hydrolysate.
[g]Verified by acid hydrolysis in 5.7 M HCl + 3% thioglycolic acid, dansylation of an aliquot of the hydrolysate and thin-layer chromatography on polyamide sheets.

DISCUSSION

We have recently shown that it is possible to isolate in one step from a crude extract of the sinus glands of the Mexican crayfish *Procambarus bouvieri* (Ort-

Table 2. Structural comparison of the major (CHH-B) and the minor (CHH-C) hyperglycemic hormones from the sinus glands of *Procambarus bouvieri* (Ortmann)

	CHH-B	CHH-C
Molecular weight	6000–6200	6000–6200
Number of residues	52–53	52–53
Number of acid residues*	12	12
Number of basic residues	8	8
Number of cysteines	4	4
Missing amino acids	MET-HIS-TRP	MET-HIS-TRP
Amino end	blocked	blocked
Carboxyl end	ILE	ILE
Isoelectric point	4.79	4.79

*ASX + GLX. The number of amidated acid residues is unknown.

mann) two peptides with hyperglycemic activity which we have designated CHH-B and CHH-C in the order in which they are eluted from an RP-HPLC μ-Bondapak-Phenyl column. A third peptide, termed A, which elutes ahead of CHH-B and CHH-C, is devoid of hyperglycemic activity and will not be dealt with in this paper.

We have endeavored in the present work to do a comparative parallel study of both peptides CHH-B and CHH-C. The results shown here point toward a remarkable homology between them in molecular weight (6000–6200), number of residues (52–53), number of cysteines (4), number of acid residues, including their amides (12), number of basic residues (8), missing amino acids (methionine, histidine and tryptophane), blocked amino end, isoleucine as C-terminal amino acid and isoelectric point (4.79). Even in a bioassay, both peptides have a very similar specific hyperglycemic activity (Huberman and Aguilar, 1986).

The only clear difference between them is their relative degree of hydrophobicity, peptide CHH-C being more hydrophobic than peptide CHH-B. This could be due to a difference in the number of hydrophobic neutral amino acids and/or in the number of amidated acid residues.

Newcomb (1983) has also found an extensive similarity in the amino acid composition of two peptides with hyperglycemic activity isolated from the sinus glands of *Cardisoma carnifex*.

We do not know at present what is the physiological significance of having two hyperglycemic hormones in the same animal but we are sure this is not an accidental finding because: (a) we have always found them in the same approximate ratio of 3:1 in favor of CHH-B; (b) regardless of the method of extraction or preparation, by us or by other authors (see Introduction), there are almost always two peptides with hyperglycemic activity in practically all decapods studied in this respect; (c) rechromatography of the pure peptide produces the same peptide with no interconversion.

In the few cases in which only one hyperglycemic peptide was found, it could be due to imperfect separation, low sensitivity of the bioassay and/or poor recovery of the minor peptide.

The two hyperglycemic hormones could be the product of one gene, with a posttranslational modification of a fraction, or the products of two genes arisen by gene duplication. In the first case, a double reciprocal modification of the type amidation/deamidation could take place without it being detected in an acid hydrolysate. This would be necessary in order to maintain the pI invariable, but a change of glutamic acid to glutamine would render a peptide more hydrophobic than an amidation of aspartic acid to asparagine. This reasoning could also apply to the second case without precluding a difference in the number of neutral hydrophobic amino acids.

It is obvious that an answer to these questions will be found only when the two peptides are completely sequenced. In preliminary comparisons of the tryptic peptides of CHH-B and CHH-C we have found only one peptide with a marked change in hydrophobicity in peptide CHH-C (unpublished).

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SINGLE-STEP PURIFICATION OF TWO HYPERGLYCAEMIC NEUROHORMONES FROM THE SINUS GLAND OF *PROCAMBARUS BOUVIERI*

COMPARATIVE PEPTIDE MAPPING BY MEANS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A crude aqueous extract from 2000 sinus glands of the Mexican crayfish *Procambarus bouvieri* (Ortmann) was fractionated on a μBondapak-Phenyl column. Two isoforms of the crustacean hyperglycaemic hormone, designated CHH-B and CHH-C in order of elution, were isolated in pure form. Their biochemical characterization showed a remarkable degree of homology. A tryptic digest of each isohormone was fractionated on an Ultrasphere-ODS column. Only one tryptic peptide in CHH-C was eluted later than its homologous peptide in CHH-B. On acid hydrolysis both tryptic peptides had the same composition but, as they contain Asp and Glu, we suspect that the difference resides in a double reciprocal amidation–deamidation of two acidic residues.

INTRODUCTION

Abramowitz *et al.*[1] first described the presence of a "diabetogenic" factor in the eyestalk of the crab *Callinectes*, which, when injected into destalked animals, produced a substantial hyperglycaemic effect. This factor, now named the crustacean hyperglycaemic hormone (CHH), is synthesized by a group of peptidergic neurons in the medulla terminalis X-organ. In the form of neurosecretory granules, it is accumulated in the sinus gland, the most conspicuous and accessible neurohaemal organ of crustaceans, from which it is secreted directly into the haemolymph by a process of exocytosis[2,3].

We have isolated[4] from aqueous extracts of 2000 sinus glands two neuropeptides with hyperglycaemic activity (designated CHH-B and CHH-C), which show a remarkable degree of homology when subjected to a partial biochemical characterization[5]: molecular weight, 6000–6200; number of residues, 52–53; number of cysteines, 4; number of acidic residues (including their amides), 12; number of basic

* Preliminary reports comprising part of this work were presented at the XXX National Congress of Physiological Sciences (Xalapa, July 1987) and at the XXII Mexican Congress of Pure and Applied Chemistry (Veracruz, September, 1987).

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residues, 8; missing amino acids, methionine, histidine and tryptophan; amino terminus, blocked; carboxyl terminus, isoleucine; and *pI*, 4.79.

As the only clear difference between the two isohormones is their relative degree of hydrophobicity, we decided to digest them with trypsin [treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)] and purify the tryptic peptides on an Ultrasphere-ODS column in order to search for peptides with different mobilities. Of a total of nine peptides for each isoform, only one peptide in CHH-C was eluted later than its corresponding peptide in CHH-B. Both had the same composition on acid hydrolysis and a blocked amino terminus. We suspect that the difference in hydrophobicity between the two peptides resides in a reciprocal amidation-deamidation of a pair of acidic residues because they both contain Asp and Glu and there is no difference between the *pI* values of CHH-B and CHH-C.

EXPERIMENTAL

Animals

Adult crayfish of both sexes were obtained from local fishermen in Uruapan, State of Michoacán. The body size was 7 ± 1 cm and the weight 20 ± 5 g. They were kept in plastic containers filled with tap water at room temperature, through which compressed air was bubbled continuously. They were fed daily with crayfish meat.

Reagents and glassware

All reagents were of analytical-reagent grade. Solvents for high-performance liquid chromatography (HPLC) were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Trypsin-TPCK was purchased from Sigma (St. Louis, MO, U.S.A.). Doubly distilled water was further purified by passing it through a Sybron-Barnstead (Boston, MA, U.S.A.) NANOpure-A system, and only water with a resistivity above 10 MΩ/cm was used. Glassware was calcined at 550°C for 4 h.

Sinus glands and crude extract

Animals were cold-anaesthesized and their eyestalks (ES) cut with sharp scissors. The ES were kept at −70°C until used. The sinus glands (SG) were dissected under the microscope while covered with ice-cold crustacean saline[9] and kept in a small volume of ice-cold water (1 μl per SG). For the preparation of the crude extract, the protocol described earlier[4] with subsequent improvements[5] was followed. Briefly, 2000 SG in water were disintegrated with one 5–10-s burst of a Kontes (Vineland, NJ, U.S.A.) Micro-Ultrasonic Cell Disrupter, followed by centrifugation at 107 000 *g* (average) for 15 min at 2°C in a Beckman (Palo Alto, CA, U.S.A.) TL-100 table-top ultracentrifuge, provided with a TLA-100.2 rotor. After decanting the supernatant, the precipitate was resuspended in 100 μl of water and the process repeated (twice). Finally, the three supernatants were mixed, centrifuged at 356 000 *g* (average) for 30 min at 2°C and concentrated to 0.5 μl per SG. The concentrate was centrifuged at 12 000 *g* (Microfuge 12, Beckman) for 5 min at room temperature. The supernatant was filtered through a centrifugal microfiltration device provided with a 0.2-μm regenerated cellulose filter (Model MF-1; Bioanalytical Systems, West Lafayette, IN, U.S.A.) at 1800 *g* at 4°C for 10 min.

Protein determination

Samples were hydrolyzed in 5.7 *M* hydrochloric acid at 145°C under nitrogen for 1 h in calcined flame-sealed Pyrex tubes. The micro-method of Hazra *et al.*[7] was employed.

HPLC

A Beckman/Altex (San Ramon, CA, U.S.A.) system consisting of a Model 420 Microprocessor Controller/Programmer, two Model 110A pumps, a Model 210 manual injection valve and a Model CR1A Recorder/Data Processor was used. The detector was an LKB (Bromma, Sweden) Uvicord-S with an 8-μl cell. The column used for hormone purification was μBondapak-Phenyl (300 × 3.9 mm I.D., 10-μm particle size; Waters Assoc., Milford, MA, U.S.A.). The effluent was monitored at 206 nm. Solution A was 0.1% aqueous trifluoroacetic acid (TFA) and solution B was 60% aqueous acetonitrile containing 0.1% TFA. For the purification of the tryptic peptides an Ultrasphere-ODS column (250 × 4.6 mm I.D., 5-μm particle size; Beckman/Altex) was used. Solution A was 0.1% aqueous TFA and solution B was acetonitrile containing 0.1% TFA. Details of the elution programme are provided in the legends to Figs. 1 and 2.

Trypsin digestion

A 103.2-μg (17.7-nmol) amount of peptide CHH-B and 61.2 μg (10.2 nmol) of peptide CHH-C were reduced and carboxymethylated according to Allen[8] and then repurified in a Novapak-C_{18} column (150 × 3.9 mm I.D.; Waters Assoc.). Solution A was 0.1% aqueous TFA and solution B was 60% aqueous acetonitrile containing 0.1% TFA. A linear gradient from 35% to 100% B was run for 70 min. Peptide-containing peaks were collected and evaporated to dryness in 1.5-ml Eppendorf tubes (Thomas Scientific, Swedesboro, NJ, U.S.A.) and the residue was resuspended in 100 μl of hydrogencarbonate buffer (0.1 *M* ammonium hydrogencarbonate, 0.1 *M* calcium chloride, 0.02% 2,2'-thiodiethanol, pH 8.7). Trypsin-TPCK was dissolved at 1 mg/ml in 0.1 *M* hydrochloric acid and was added to the peptides in a ratio of *ca.* 1:50. Peptide CHH-B received 2 μl and peptide CHH-C 1 μl of the enzyme solution. After incubation at 37°C for 2 h, the same amount of enzyme was added and the incubation was continued for a further 2 h. The incubation mixtures were evaporated to dryness, 50 μl of water were added and the solutions were evaporated to dryness again. They were then resuspended in 250 μl of solution A and injected directly into the Ultrasphere-ODS column. In order to compensate for the large difference in hormone content in each SG, the amount of CHH-C corresponded to 3000 glands (from two preparations).

Amino terminus analysis

A 500-pmol amount of each CHH-B tryptic peptide and 420 pmol of each CHH-C tryptic peptide were dansylated as described by Allen[8]. The peptides were hydrolyzed in 5.7 *M* hydrochloric acid containing 0.16% phenol and chromatographed on 3 × 3 cm polyamide plates.

Amino acid analysis

A 500-pmol amount of each CHH-B tryptic peptide and 420 pmol of each

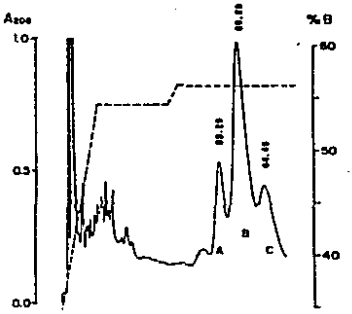

Fig. 1. One-step purification of the two isoforms of the crustacean hyperglycaemic hormone, CHH-B and CHH-C. Crude extract from 500 sinus glands of *Procambarus bouvieri* (250 μl) was fractionated on a μBondapak-Phenyl column (300 × 3.9 mm I.D., 10-μm particle size) without a pre-column. Solution A was 0.1% (w/v) aqueous TFA and solution B was 60% aqueous acetonitrile containing 0.1% (w/v) TFA. The effluent was monitored at 206 nm. The column was equilibrated with 35% B and, after injection, a linear gradient from 35% to 54.5% B was developed in 15 min, followed by isocratic elution at 54.5% B for 30 min. The concentration of B was increased to 56.4% in 2 min and continued isocratically at this level for 25 min. The peaks with hyperglycaemic activity in a bioassay are B (59.29 min) and C (64.49 min). The time scale (abscissa) has been compressed (chart speed: 1 mm/min for 45 min, 2 mm/min for 25 min). Flow-rate, 1 ml/min.

CHH-C tryptic peptide were hydrolyzed as above, evaporated to dryness in a Savant (Farmingdale, NY, U.S.A.) centrifugal evaporator, resuspended in 200 μl of Beckman High-Performance Amino Acid Sample Dilution Buffer (Na^+ system) and 50 μl were injected into a Beckman 6300 Amino Acid Analyzer. Norleucine (125 pmol per 50 μl) was used as an internal standard.

RESULTS

Fig. 1 shows the result of the one-step fractionation of a crude extract of 500 SG from *Procambarus bouvieri* on a μBondapak-Phenyl column. A linear gradient from 35% to 54.5% B in 15 min eliminated most of the hydrophilic components of the extract and the remainder was eluted during an isocratic 30-min run at 54.5% B. In order to speed up the elution of the hydrophobic peptides, the concentration of B was increased to 56.4% in 2 min and continued isocratically for 25 min. Three peptides were eluted at 55.29, 59.29 and 64.49 min, and these were designated peptides A, B and C, respectively. When tested in a bioassay[4], only peptides B and C had hyperglycaemic activity and they were then redesignated CHH-B and CHH-C. Peptide A did not have any hyperglycaemic activity and will be dealt with separately[9]. It is noteworthy that the CHH-B to CHH-C ratio is almost constant at 3:1, both in areas under the curve and in protein content, and the specific hyperglycaemic activ-

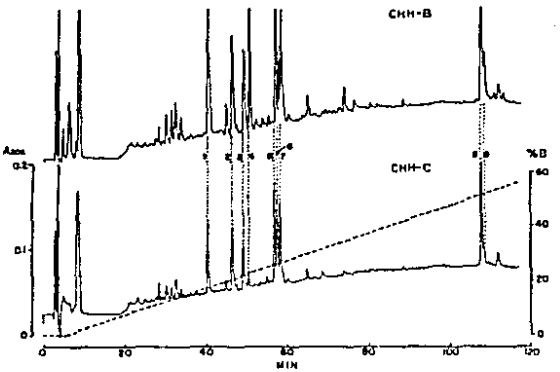

Fig. 2. Comparative tryptic peptide maps of isohormones CHH-B and CHH-C. CHH-B (103.2 μg = 17.2 nmol) and CHH-C (61.2 μg = 10.2 nmol) were reduced and carboxymethylated. After purification, they were digested with trypsin–TPCK, evaporated to dryness, resuspended in 250 μl of solution A and injected directly into an Ultrasphere-ODS column (250 × 4.6 mm I.D., 5-μm particle size), without a pre-column. Solution A was 0.1% (w/v) aqueous TFA and solution B was acetonitrile containing 0.1% TFA. After injection, solution A was passed through for 5 min, then a slow linear gradient from 0% to 60% B was developed in 120 min at a flow-rate of 1 ml/min. The effluent was monitored at 206 nm. For clearness, the gradient and scales are represented only on the lower chromatogram. Only the peaks that on acid hydrolysis and analysis were shown to be peptides have been numbered in order of elution.

ities of the two peptides are very similar[4]. In order to ensure that there was no cross-contamination of the peptides, due to the limited separation obtained between them, they were concentrated and chromatographed again in the same μBondapak-Phenyl column and the small amounts of contaminants eliminated by manual collection of the main peaks (not shown).

The remarkable similarity of the biochemical characteristics of both peptides CHH-B and CHH-C (see Introduction) while manifesting an obvious difference in hydrophobicity led us to search for a homologous peptide or peptides with different mobilities in the two neurohormones. Fig. 2 shows the results of fractionating on an Ultrasphere-ODS column the tryptic peptides of 103.2 μg (17.2 nmol) of CHH-B from 2000 SG and 61.2 μg (10.2 nmol) of CHH-C from 3000 SG. Only peaks which on acid hydrolysis were shown to be peptides are numbered in order of elution. Taking into account that there is 40% more peptide material in CHH-B than in CHH-C, it can readily be seen that there is a perfect correlation between them in the elution time of each pair and in the relative proportions between them. It is notable, however, that peptide CHH-B-4 is missing from hormone CHH-C. When the composition of all peptides was completed, we realized that peptide CHH-C-6 had the same composition as CHH-B-4 on acid hydrolysis, *i.e.*, Ala, Cys, Asp, Glu (2×), Phe, Val, Lys. Both of them have a blocked amino terminus. As they both contain Asp and Glu, we conclude that the difference in mobility is probably due to a re-

ciprocal amidation-deamidation of two acidic residues in CHH-C-6, rendering it more hydrophobic than its homologous peptide CHH-B-4. The amidation of Glu would render a peptide more hydrophobic than the amidation of *Asp*.

DISCUSSION

The CHH is polymorphic in almost all crustacean where its purification has been attempted[3]. Moreover, it is usually composed of a major and a minor peak. With *Procambarus bouvieri* there is an almost constant ratio of 3:1 between CHH-B and CHH-C, both in the area under the curve and in protein content. We do not know yet the physiological significance of having two isohormones with hyperglycaemic activity in the same organism.

In an attempt to establish whether the two isoforms of the *Procambarus bouvieri* CHH are the products of one gene with a postranslational modification of a fraction of the initial product to give two isohormones or whether they are the products of two genes, probably evolved by gene duplication to give two products with a great deal of homology, we have continued to characterize them.

We have succeeded in applying our one-step purification of the two isohormones[4] with an improvement in the yield from 30 to 55 ng per SG for CHH-B and from 16.5 to 20 ng per SG for CHH-C. From each fractionation of 500 SG we recovered approximately 30 μg of CHH-B and 10 μg of CHH-C, which allowed us to carry out extensive comparative work on the microscale.

Apparently, there is one peptide missing in CHH-C compared with CHH-B, eight instead of nine, but careful comparison of the two peptide maps leads us to the conclusion that the position of CHH-C-6 is occupied also by a very small amount of a homologous peptide to CHH-B-6 but which is overlapped by the former.

The completion of the primary structure of both isohormones of the crustacean hyperglycaemic hormone will answer many of the questions raised here.

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A NEUROPEPTIDE WITH MOLT-INHIBITING HORMONE ACTIVITY FROM THE SINUS GLAND OF THE MEXICAN CRAYFISH *PROCAMBARUS BOUVIERI* (ORTMANN)

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Abstract—1. By means of reverse-phase high-performance liquid chromatography (RP-HPLC) on a μBondapak-Phenyl column, a neuropeptide with molt-inhibiting hormone (MIH) activity was isolated from a crude extract of 2000 sinus glands from the crayfish *Procambarus bouvieri* (Ortmann) with a yield of 25 ng/SG.

2. The activity was tested in a heterologous *in vitro* assay as the depression of ecdysteroid biosynthesis by cultured *Orconectes limosus* Y-organs.

3. On SDS-PAGE, the peptide migrated as a single band of approximately 6000 Da, and on IEF, it migrated as a single band to a pI of 5.50.

4. The *N*-terminus was shown to be blocked by means of the dansyl-Cl method, while the *C*-terminus was shown to be isoleucine by means of carboxypeptidase-Y digestion.

5. The following amino acid average composition of MIH was obtained: $(Asx)_{3-4}$, $(Val)_1$, $(Glx, Leu, Arg)_{3-4}$, $(Cys, Lys)_{1-2}$, $(Ala, Ile, Tyr, Phe)_{2-3}$, $(Ser)_3$, $(Thr, Gly)_{1-2}$, $(Pro)_1$. MIH has 53–55 amino acid residues and its minimal molecular mass is calculated as 6243–6402 Da. It does not contain Trp, His nor Met.

6. 37.7 μg (6.3 nmol) of the molt-inhibiting hormone (MIH) and 29.7 μg (11.8 nmol) of the major peak of the crustacean hyperglycemic hormone (CHH-B) were reduced, carboxymethylated and digested with trypsin. The tryptic peptides of each of the hormones were purified on an Ultrasphere-ODS column and the peptide maps compared with respect to retention times and the composition of each peak.

7. 57.4% of the total amount of amino acid residues were accounted for in five peptides which coincided in elution time and composition.

8. As we have found a clear-cut difference of only two residues between MIH and CHH-B (without taking into account the amides of aspartic and glutamic acids), the present results confirm that the two hormones belong to a family of neuropeptides comprising at least one molt-inhibiting hormone and two hyperglycemic hormones.

9. Evidence is presented for the existence of microheterogeneity in the structure of each of these hormones.

INTRODUCTION

The process of molting in crustaceans is considered to be regulated by neurohormonal factors in the eyestalk (ES) since Zeleny (1905) and Megusar (1912) described that ES removal results in an accelerated molt cycle. These findings were rediscovered and confirmed by Hanström (1939) and by Abramowitz and Abramowitz (1940).

The role of the X-organ and of the sinus gland (SG) in molt control was established by Passano (1953) who also showed that the factor was inhibitory. Since then it has been termed the molt-inhibiting hormone (MIH).

Proecdysis and molting are positively correlated with increased levels of circulating ecdysteroids (Skinner, 1985) which are synthesized in the Y-organs mainly as α-ecdysone (Chang and O'Connor, 1977) that is later converted to 20-OH-ecdysone (β-ecdysone) in the peripheral target tissues (King and Siddall, 1969; Chang and O'Connor, 1977; Jegla *et al.*, 1983).

The SG of decapods contains a factor which can repress the rise in circulating ecdysteroids after ES ablation in intermolt animals (Keller and O'Connor, 1982; Bruce and Chang, 1984). This rise in ecdysteroid titres does not occur in Y-organ deficient animals. On the other hand, it has been shown *in vitro* (Soumoff and O'Connor, 1982; Mattsen and Spaziani, 1985) that an extract of brachyuran SG will repress ecdysteroid biosynthesis by cultured Y-organs.

Webster and Keller (1986) have isolated, purified and characterized a peptide with molt-inhibiting activity from the SG of *Carcinus maenas* which is 61 amino acid residues long and has a minimum molecular mass of 7200 Da. Chang *et al.* (1987) have purified and characterized two closely related peptides with molt-inhibiting activity from the SG of the lobster *Homarus americanus* of approximately 70–80 amino acid residues and a minimum molecular mass of 8700 ± 1000 Da.

While studying the crustacean hyperglycemic hormones (CHH) of *Procambarus bouvieri*, we isolated three hydrophobic peptides from an SG extract (Huberman and Aguilar, 1986). Two of the peptides, termed CHH-B and CHH-C (the major and the minor peaks of CHH, respectively), had hyper-

glycemic activity when tested *in vivo* in the same animal and were shown to be structurally closely-related (Huberman and Aguilar, 1988a; Huberman and Aguilar, 1988b). A third peptide, termed A, which did not have hyperglycemic activity has been shown to have molt-inhibiting hormone activity (measured *in vitro* as the depression of ecdysteroid biosynthesis by cultured Y-organs of *Oreonectes limosus*).

We report here the isolation, purification and partial biochemical characterization of the putative molt-inhibiting hormone of *Procambarus bouvieri*, and we present a comparison of the tryptic peptide maps of MIH and CHH-B. The composition of the peptides that coincide in retention times point towards the conclusion that these two neuropeptides belong to the same family of crustacean neurohormones.

MATERIALS AND METHODS

Animals, sinus glands and crude extract

Adult crayfish (not selected for sex) were obtained from local fishermen in Uruapan, State of Michoacán. They were anesthetized with crushed ice and their eyestalks (ES) were cut and kept frozen at −70 C until used. The sinus glands (SG) were dissected under the microscope while covered with ice-cold crustacean saline (van Harreveld, 1936) and kept in a small volume of ice-cold water (1 μl/SG). For the preparation of the crude extract (CE), the improved protocol described by us (Huberman and Aguilar, 1988a) was followed. Briefly, 2000 SG in water were disintegrated with one 5-10 sec burst of a Kontes Micro-Ultrasonic Disrupter, followed by centrifugation at 108,000 *g* (average) for 15 min at 2 C in a Beckman TL-100 tabletop ultracentrifuge, provided with a TLA-100.2 rotor. After decanting the supernatant, the precipitate was resuspended in 100 μl of water and the process repeated (twice). Finally, the three supernatants were mixed, centrifuged at 356,000 *g* (average) for 30 min at 2°C and concentrated to 0.5 μl/SG. The concentrate was centrifuged at 12,000 *g* in a Beckman Microfuge 12, for 10 min at room temperature and the last ultracentrifugation repeated. The supernatant was filtered through a centrifugal microfiltration device provided with a 0.2 μm regenerated cellulose filter (Model MF-1, Bioanalytical Systems, Inc.) at 1800 *g*, 4°C, for 10 min.

HPLC

A Beckman/Altex system consisting of a Model 420 Microprocessor Controller/Programmer, two Model 110A pumps, a Model 210 manual injection valve and a Model CRIA Recorder/Data Processor was used. The detector was an LKB Uvicord-S provided with an 8 μl cell. The column used for hormone purification was μBondapak-Phenyl (300 × 3.9 mm i.d., 10 μm particle size; Waters). The effluent was monitored at 206 nm. Solution A was 0.1% aqueous trifluoroacetic acid (TFA) and solution B was 60% aqueous acetonitrile containing 0.1% TFA. The column was equilibrated with 35% of solution B. A linear gradient from 35% B to 58% B in 10 min was followed by isocratic elution at 58% B for 50 min.

The reduced and carboxymethylated hormones were purified in a Novapak-C_{18} column (150 × 3.9 mm i.d., 5 μm particle size; Waters), using the same solutions as above. A linear gradient from 35% B to 100% B was run for 70 min.

For the purification of the tryptic peptides, an Ultrasphere-ODS column (250 × 4.6 mm i.d., 5-μm particle size; Beckman/Altex) was used. Solution A was 0.1% aqueous TFA and solution B was acetonitrile containing 0.1% TFA. Details of the elution program are provided in the legend to Fig. 3.

Trypsin digestion

A 37.7 μg (6.3 nmol) amount of MIH and 70.7 μg (11.8 nmol) of CHH-B were reduced and carboxymethylated according to Allen (1981) and then repurified in a Novapak-C_{18} column. Peptide-containing peaks were collected and evaporated to dryness in 1.5 ml Eppendorf tubes and the residue was resuspended in 100 μl of hydrogen carbonate buffer (0.1 ammonium hydrogen carbonate, 0.1 M calcium chloride, 0.02% 2,2 thiodiethanol, pH 8.7). Trypsin-TPCK (L-1-tosylamide-2-phenethyl chloromethyl ketone) was dissolved at 1 mg/ml in 0.1 M HCl and was added to the peptides in a ratio of *ca* 1:50. Each MIH peptide received 1 μl of the enzyme solution, while each CHH-B peptide received 2 μl of the enzyme solution. After incubation at 37°C for 2 hr, the same amount of enzyme was added and the incubation was continued for a further 2 hr. The incubation mixtures were evaporated to dryness. Fifty microlitres of water were added and the solutions were evaporated to dryness again. They were then resuspended in 250 μl of solution A and injected directly into the Ultrasphere-ODS column.

Protein determination

Samples were hydrolyzed in 5.7 M HCl at 145°C under nitrogen for 1 hr in calibrated flame-sealed Pyrex tubes. The micro-method of Hazra *et al.* (1984) was employed.

Amino acid analysis

For the analysis of MIH, 36 μg (6 nmol) were divided into 5 lots of 7.2 μg (1.2 nmol) each. Three lots were hydrolyzed in 5.7 M HCl containing 0.16% (w/v) phenol, at 110 C for 24, 48 and 72 hr. Another lot was oxidized with performic acid (Moore, 1963) and then hydrolyzed as above. The fifth lot was hydrolyzed in 5.7 M HCl containing 3% thioglycolic acid (Matsubara and Sasaki, 1969). Samples of the first four hydrolysates were taken to dryness in the Savant centrifugal evaporator and resuspended in 250 μl of Beckman's High-Performance Amino Acid Sample Solution Buffer (Na-System). Fifty microlitres were injected into a Beckman 6300 Amino Acid Analyzer. Norleucine (1250 pmol/50 μl) was used as internal standard. The fifth hydrolysate was dansylated and chromatographed as explained below.

For the analysis of the tryptic peptides, 437 pmol of each MIH peptide and 400 pmol of each CHH-B peptide were hydrolyzed in 5.7 M HCl + 0.16% (w/v) phenol as described above, evaporated to dryness in a Savant centrifugal evaporator and resuspended in 200 μl of Beckman's High Performance Amino Acid Sample Dilution Buffer (Na-System). Fifty microlitres were injected into a Beckman 6300 Amino Acid Analyzer. Norleucine (125 pmol/50 μl) was used as an internal standard. Additionally, 377 pmol of each MIH tryptic peptide and 500 pmol of each CHH-B tryptic peptide were hydrolyzed in 5.7 M HCl + 3% thioglycolic acid. After evaporating to dryness, they were dansylated as described by Allen (1981), and chromatographed on 3 × 3 cm polyamide plates.

End-group analysis

MIH was reduced and carboxymethylated according to Allen (1981). For *N*-terminal amino acid analysis, 12 μg (2 nmol) of peptide were submitted to the Gray and Hartley (1963) dansyl-Cl method as described by Allen (1981). Carboxypeptidase-Y was used for the carboxyl-terminal amino acid on 9 μg (1.5 nmol) of MIH. For identification, the dansyl-amino acids were chromatographed on 3 × 3 cm polyamide sheets, employing solvent system I (1.5% v/v aqueous formic acid, II (toluene/acetic acid 10:1, v/v), III (ethyl acetate/methanol/acetic acid, 20:1:1, by vol) (Woods and Wang, 1967). When basic amino acids were suspected, solvent system IV was used after the previous three (50 mM Na_2PO_4 in 25% aqueous ethanol) (Hartley, 1970).

For the determination of each *N*-terminal amino acid, 377 pmol of each MIH tryptic peptide and 200 pmol of each

CHH-B tryptic peptide were dansylated and then hydrolyzed as above. The same chromatographic procedures were used for identification.

Isoelectric focusing (IEF)

It was performed on a LKB Multiphor I apparatus with a 1 mm preformed polyacrylamide gel containing a wide gradient of Ampholine (pH 3.5-9.5). A sample of 5 μg (0.83 nmol) in 20 μl was applied in a filter paper pad at 1 cm from the cathode. Focusing was performed at 4 C, with a power limit of 7 W and a maximum voltage set at 1500 V, for 1.5 hr. The pH gradient was determined by cutting 5 mm segments of a parallel strip of gel, leaching out the Ampholine at room temperature in 2 ml of water in sequentially numbered tubes and measuring the pH of each tube after 18 hr. Localization of the peptide was done by conventional Coomassie Blue staining.

Bioassay

It was performed essentially as described by Webster (1986), but as modified by Renate Fenrich for assay in *Orconectes limosus* (Fenrich, 1987). The incubation medium contained per 100 ml: 10 ml of Medium 199 (Serva, 10× concentrated), 477 mg HEPES, 224 mg NaCl, 238 mg $CaCl_2 \cdot 2H_2O$, 39.8 mg $MgCl_2 \cdot 6H_2O$ (440 mOsmol). The pH was adjusted to 7.5 with 1 N NaOH and 1 ml of Gibco's antibiotic-antimycotic cocktail was added per 100 ml. The medium was sterile filtered through a 0.22 μm Millipore filter. Y-organs were incubated in 200 μl of medium and the contralateral Y-organ of each animal served as control. Incubation was for 20 hr at 12°C in 100% humidity. The medium was used directly for RIA. To the experimental Y-organ, 0.1 sinus gland equivalent of *Procambarus bouvieri* peptide A was added. RIA for ecdysteroids was performed according to Chang and O'Connor (1979). Ecdysteroid secretion by Y-organs was normalized against protein content of the organ and the percentage inhibition of secretion calculated according to Soumoff and O'Connor (1982). Further details can be found in Webster and Keller (1986).

Reagents and glassware

All reagents were of analytical grade. HPLC solvents were from Fisher Scientific. Double-distilled water was further purified by passing through a Sybron-Barnstead NANOpure-A system, and only water with a resistivity above 10 MΩ/cm was used. Glassware was calcined at 550°C for 4 hr.

RESULTS

Single-step purification

Following the protocol developed for the purification of the crustacean hyperglycemic hormones (CHH) (Huberman and Aguilar, 1986) with slight modifications, an aliquot of the crude extract of 468 SG in 250 μl of water were chromatographed on a μBondapak-Phenyl column, equilibrated with 35% of Solution B. A linear gradient from 35% to 58% B in 10 min was followed by isocratic elution, at 58% B for 50 min. In this way (Fig. 1), three highly hydrophobic peptides, designated A, B, and C in the order of elution, are separated in a single chromatographic step. Peptides B and C correspond to the major and minor peaks of the CHH, respectively. Peptide A is devoid of hyperglycemic activity. It was collected manually, avoiding the valley between peptides A and B. In order to verify that it was not contaminated with peptide B, it was concentrated and chromatographed on the same column and under the same conditions as before (Fig. 2). A symmetric peak was obtained in the position of peptide A (41.5 min) without apparent contaminants from adjoining peaks.

Bioassay

This was performed in the laboratory of Prof. Rainer Keller, University of Bonn, by Renate Fenrich. Peptide A inhibited the synthesis of ecdysteroids by Y-organs of *Orconectes limosus* cultured *in vitro* as described in Materials and Methods. 0.1 SG equivalent of peptide A from *Procambarus*

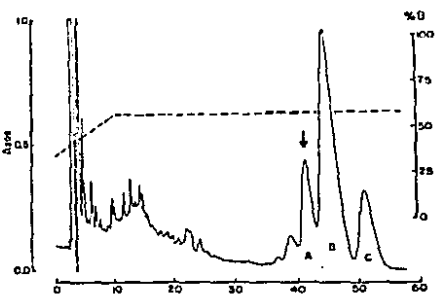

Fig. 1. Purification of MIH by RP-HPLC. Elution profile of a crude extract from 468 SG. Column: μBondapak-Phenyl (300 × 3.9 mm i.d., 10 μm particle size, Waters). Solvent A: 0.1% aqueous TFA. Solvent B: 0.1% TFA in 60% aqueous MeCN. The column was equilibrated with 35% B, and after injecting the sample a linear gradient from 35% B to 58% B was developed in 10 min. Elution was continued isocratically at 58% B for another 50 min. Flow rate: 1 ml/min. Peak A (black arrow) was shown by [illegible] to inhibit ecdysteroid biosynthesis in *Orconectes limosus* Y-organs cultured *in vitro*.

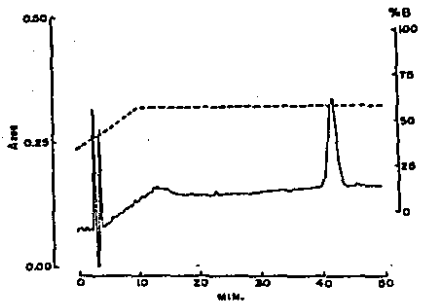

Fig. 2. Rechromatography of peak A from Fig. 1. Elution profile of 274 SG equivalents after collecting and concentrating peak A. Elution effected under exactly the same conditions and in the same column as in Fig. 1. The absence of adjoining contaminants is evident.

bouvieri inhibited the synthesis of ecdysteroids by Y-organs from *Oreoneetes* by 37% ($N = 8$) which is indicative of molt-inhibiting hormone activity. It should be mentioned here that in homologous tests, the highest inhibition of ecdysteroid synthesis obtained has been 60% (Prof. R. Keller, personal communication). In view of this result, peak A will be described as the molt-inhibiting hormone (MIH) of *Procambarus bouvieri*.

Protein determination

One hundred SG equivalents of peak A were used for quantification. The content of MIH was found to be 25 ng/SG, without correcting for losses during isolation and purification.

Isoelectric focusing

An aliquot of 5 μg of peptide A was focused for 1.5 hr. After staining (Coomassie) and destaining, only one band was seen at a position corresponding to a pI of 5.50. This procedure also served the purpose of verifying the purity of the peptide, because peptide B would separate at a pI of 4.79 [confirmed with 5 μg of peptide B (CHH-B) run simultaneously].

Amino acid analysis

The average values obtained from the acid hydrolysis of 3 lots of peptide of 7.2 μg each, during 24, 48 and 72 hr, respectively, are given in Table 1. In the same table are included the results obtained with another lot of 7.2 μg of peptide A, oxidized with performic acid and hydrolyzed for 24 hr, for the determination of cysteine as cysteic acid, and also the result of hydrolyzing another lot of 7.2 μg of peptide A in 5.7 M HCl containing 3% thioglycolic acid for 24 hr, for the determination of tryptophan (as dansyl-tryptophan) by thin-layer chromatography on polyamide sheets.

In the same table, a comparison is made with the composition of the major peak of the *Procambarus bouvieri* hyperglycemic hormone (CHH-B).

End-group analysis

Upon dansylation of 12 μg of peptide A, only 2 spots were observed after acid hydrolysis and polyamide chromatography, that corresponded to O-dansyl-tyrosine and ϵ-dansyl-lysine. This result is

Table 1. Amino acid composition of MIH (peak A) from *Procambarus bouvieri* and comparison with composition of the major peak of CHH (CHH-B)*

Amino acid	MIH Average†	MIH Residues‡	CHH-B Residues§
Asx¶	7.16	7	7
Thr	1.36	1–2	2
Ser	1.59	2	2
Glx¶	5.16	5	5
Pro	0.77	1	1
Gly**	1.92	1–2	1–2
Ala	2.93	3	2
Cys††	4.24	4	4
Val‡‡	5.37	6	6
Met§§	0.00	0	0
Ile	2.83	3	3
Leu	4.99	5	5
Tyr	3.19	3	3
Phe	2.90	3	2
Trp¶¶	0.00	0	0
His‖	0.00	0	0
Lys	4.02	4	3
Arg	4.84	5	5
Total		53–55	52–53
Mol. wt††		6243–6402	6068–6126

*Huberman and Aguilar (1986).

†Average values of residues per molecule obtained after acid hydrolysis in 5.7 M HCl + 0.16% (w/v) phenol, for 24, 48 and 72 hr, based on a mol. wt of 6000.

‡Number of residues per molecule of mol. wt 6000.

§ASX = ASP + ASN; GLX = GLU + GLU.

¶Taken as 1–2 on account of high frequency of contamination of reagents and water with glycine.

**Twenty-four hr acid hydrolysis (as above) after oxidizing the sample with performic acid.

††Next higher integer of the 72 hr acid hydrolysate.

‡‡Verified by acid hydrolysis in 5.7 M HCl + 3% thioglycolic acid, dansylation of an aliquot of the hydrolysate and thin-layer chromatography on polyamide plates.

§§Minimal mol. wt calculated from amino acid analysis.

interpreted as the presence of a blocked amino acid residue at the *N*-terminus of peptide A.

When 7.2 μg of reduced and carboxymethylated peptide A were digested with carboxypeptidase-Y and the product dansylated and subjected to polyamide chromatography, dansyl-isoleucine was detected and that establishes isoleucine as the *C*-terminal residue of the peptide. It is not possible to know if this terminus is amidated because carboxypeptidase-Y has amidase activity.

Molecular weight

As seen in Table 1, we have calculated from the amino acid composition analysis a minimum molecular weight of 6243–6402 for peptide A. This was confirmed by SDS-polyacrylamide gel electrophoresis in the presence of glycerol (Hubermen and Aguilar, 1988a).

Tryptic peptides

Figure 3 depicts the chromatographic separation of the tryptic peptides of MIH and CHH-B on an Ultrasphere-ODS column. Peptides which coincide in elution time are connected with dashed lines and numbered 1 to 5 in the order of elution (they will be referred to as T1 to T5, respectively). Other tryptic peptides which do not coincide in elution time but which have very similar but not identical compositions have been designated a, b, c, and d, and will be referred to as Ta, Tb, Tc, and Td.

The first group, peptides T1 to T5, comprise 31 residues or 57.4% of the total amount of residues of MIH (average of 54). In the second group, which includes the differences found before in amino acid composition between MIH and CHH-B (Table 1), the rest of the amino acid residues or 42.6% of the total are found. It should be noted that the amides corresponding to aspartic and glutamic acids are not known because all the analytical steps include an acid hydrolysis of the peptides.

Composition of coincident tryptic peptides

Four hundred and thirty-seven pmol of each MIH peptide and 400 pmol of each CHH-B peptide were hydrolyzed in 5.7 M HCl + 0.16% phenol and the composition determined in an amino acid analyzer. Additionally, 377 pmol of each MIH peptide and 500 pmol of each CHH-B peptide were hydrolyzed in 5.7 M HCl + 3% thioglycolic acid. In this case, dansylation was followed by thin-layer chromatography on polyamide plates.

Table 2 shows the composition of peptides T1 to

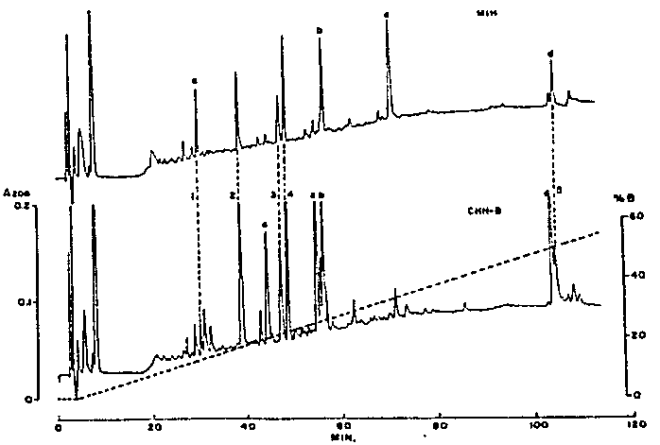

Fig. 3. Comparative tryptic peptide maps of MIH and CHH-B. 37.7 μg (6.3 nmol) of MIH and 70.7 μg (11.8 nmol) of CHH-B were reduced, carboxymethylated and repurified in a Novapack-C_{18} column. After digestion with trypsin-TPCK (as described in Materials and Methods) the dried peptides were resuspended in 250 μl of Solution A and injected directly into an Ultrasphere-ODS column (250 × 4.6 mm i.d., 5 μm particle size, Beckman) without a precolumn. Solution A was 0.1% aqueous TFA and Solution B was 0.1% TFA in MeCN. After injection, solution A was run for 5 min and then a slow linear gradient from 0% to 60% B was developed in 120 min at a flow rate of 1 ml/min. The effluent was monitored at 206 nm. For clearness, the gradient and scales are represented only on the lower chromatogram. Only the peaks that on acid hydrolysis and re-injection were shown to be peptides have been numbered in the order of elution.

Table 2. Composition of tryptic peptides from MIH and CHH-B which coincide in elution time

Number*	Composition†
T1	[T, T, A, V, C, R]
T2	[I, D, Y, G, R]
T3	[I, A, F, K]
T4	[E, E, C, A, D, F, V, K]
T5	[E, L, L, V, Y, D, G, I]

*As number in Fig. 3, in the order of elution. (T has been added to designate tryptic peptides.)
†One-letter symbol for each amino acid.
‡Underlined residue may be a contaminant.

T5 which are coincident in elution time. Peptide T5 corresponds to the *C*-terminus because it does not contain a basic amino acid residue and it includes ILE which we have shown to be the *C*-terminal amino acid residue by means of carboxypeptidase-Y digestion (Huberman and Aguilar, 1986).

Unfortunately, some of the peptides obtained by tryptic digestion have become blocked at the *N*-terminus. This can be due to cyclization of glutamine or of carboxymethylcysteine residues if they happen to be in this position, but the overall result is an added difficulty in sequencing these hormones which are originally blocked at the *N*-terminus.

Microheterogeneity

The partial coincidence in composition of peptides T5 [E, L, L, V, Y, D, G, I] and T4 [E, L, L, V, Y, D, D, G, S, I], (underlined residues could be contaminants), induces us to think that MIH and CHH-B (and most probably also CHH-C) show some degree of microheterogeneity. It is interesting to note that we have always found two *C*-terminal peptides in each hormone, with a slightly different composition in each case, and very different relative concentrations. For example, in MIH, peptide T5 is more abundant, while in CHH-B (and also in CHH-C), peptide T4 is the most abundant of the two.

DISCUSSION

Arthropods are obliged to shed periodically their confining exoskeleton in order to grow, and this process is hormonally controlled. The mediator of the molting process is the hormone 20-OH-ecdysone, synthesized as a precursor by the steroidogenic Y-organ or molting gland. It has been postulated that the rate of secretion of ecdysteroids by the Y-organ is under negative control by a molt-inhibiting hormone secreted by the sinus gland.

We have isolated in a single HPLC step, from a crude extract of 2000 SG of the Mexican crayfish *Procambarus bouvieri*, a peptide that is capable of inhibiting ecdysteroid biosynthesis (Mattson and Spaziani, 1985, 1986) by cultured Y-organs in an heterologous *in vitro* assay developed by Webster and Keller (Webster, 1986; Webster and Keller, 1986).

The MIH of *P. bouvieri* is an acidic, hydrophobic peptide of 6243-6402 mol. wt, with 53–55 amino acid residues. Its *N*-terminus is blocked and its *C*-terminus is isoleucine. It has 4 cysteine residues, probably forming two disulphide bonds. It does not contain the amino acids tryptophan, methionine nor histidine.

It is most interesting that the composition of MIH bears a great resemblance to that of the crustacean hyperglycemic hormones isolated from the same organism (Huberman and Aguilar, 1986). As can be seen in Table 1, there are only 2 differences in the composition of phenylalanine and lysine, and a difference in pI (5.50 and 4.79 for MIH and CHH, respectively). The structural similarity between the two hormones also manifests itself in a comparative tryptic map and in immunological cross-reactivity (to be published).

Webster and Keller (1986) have also found that in *Carcinus maenas*, MIH and CHH are structurally related. There is also a great similarity between the composition of these hormones and those of *P. bouvieri*. On the other hand, Chang *et al.* (1987) have recently isolated from the SG of *Homarus americanus* a peptide with molt-inhibiting activity, but there is a discrepancy between its apparent mol. wt determined by SDS-PAGE (6800) and its minimum mol. wt calculated from the amino acid composition (8790 ± 1000). A thorough review of the chemical nature of various MIH can be found in Chang and O'Connor (1988).

The MIH from *P. bouvieri* is not entirely species-specific because it exerted its inhibition of ecdysteroid synthesis on the Y-organs of *Orconectes limosus*. Davis and Costlow (1974) have shown that an eyestalk extract from *Uca* will prolong proecdysis in *Balanus improvisus*. In a previous paper (Huberman and Aguilar, 1988b), we have shown that a comparative tryptic peptide map of both hyperglycemic hormones of *P. bouvieri* uncovers only one peptide with different mobility in CHH-C with respect to CHH-B.

In this paper we compare the tryptic peptide maps of MIH and CHH-B and find that more than half of the total amount of residues are contained in peptides which have the same elution time and composition.

It is obvious that CHH and MIH comprise a family of hormones in *P. bouvieri* (and most probably in other crustaceans as well) as evidenced by the amino acid composition, by the tryptic peptide maps and by immunological cross-reactivity. The differences may be more important at the secondary and tertiary structural levels.

There is the possibility that the three hormones are the products of one gene with postranslational modifications that determine their specificity, or that there has been gene duplication and divergent evolution of an ancient primitive gene, giving rise to a closely-related family of peptides. It is also known that a mechanism that generates families of closely-related proteins is alternative splicing (Andreadis *et al.*, 1987). The answer to these possibilities will come from the knowledge of the complete amino acid sequence of MIH and CHH and of the structure of their gene(s).

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SECONDARY STRUCTURE OF A CRUSTACEAN NEUROPEPTIDE HORMONE FAMILY BY MEANS OF CD

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ABSTRACT

HUBERMAN, A., A. HERNANDEZ-ARANA, M. B. AGUILAR AND A. ROJO-DOMINGUEZ. Secondary structure of a crustacean neuropeptide hormone family by means of CD. PEPTIDES. Three hormonal neuropeptides have been purified from the sinus gland of the Mexican crayfish Procambarus bouvieri by means of a single-step HPLC method: The Molt-Inhibiting Hormone (MIH) and two isoforms of the Crustacean Hyperglycemic Hormone (CHH-B and CHH-C). Compositional analysis and partial characterization of the three neuropeptides revealed such a high degree of homology that we consider them to be members of a family. Circular dichroic spectra of the three neuropeptides showed that the secondary structure of both isoforms of the CHH are very similar, but that there are important differences in secondary structure between MIH and the CHHs, especially in helix content and in disordered regions.

KEY WORDS:

neuropeptide hormones
crustacean
peptide family
sinus gland
CHH
MIH
CD

We have described recently a single-step HPLC method for the simultaneous purification of three neuropeptide hormones from a crude extract of sinus glands of the Mexican crayfish Procambarus bouvieri [7,8]. One of the peptides (peptide A) has Molt-Inhibiting Hormone activity [10], as shown in vitro by the inhibition of ecdysteroid synthesis and secretion in incubated Y-organs of heterologous crustaceans, and the other two peptides are isoforms of the Crustacean Hyperglycemic Hormone (CHH-B and CHH-C), as shown in an in vivo test consisting of the injection of pure hormone to eyestalk-less animals of the same species (P. bouvieri) and quantitating the increase of glucose in the hemolymph vs time [7]. While characterizing these neuropeptides, we realized that there was a high degree of homology among them with respect to amino acid composition (only two differences between any two of them, without taking into account the amides of aspartic and glutamic acids), molecular masses, blocked amino ends, Ile as carboxyl ends and tryptic peptide maps [9,10]. In order to understand the difference in physiological activity between MIH and the two CHHs, and their affinity for different receptors, we studied their conformational characteristics by means of circular dichroism (CD).

METHOD

Adult crayfish were anesthetized with crushed ice (30 min) and their eyestalks were cut and kept frozen at -70°C. The sinus glands (SG) were dissected under the microscope while covered with ice-cold crustacean saline [11] and were kept in a small volume of ice-cold water (1 µl/SG). For the preparation of the crude extract [8], 2000 SG in water were disintegrated with one 5-10 sec burst of a Kontes Micro-Ultrasonic Disrupter, followed by centrifugation at 108,000 x g (average) for 15 min at 2°C in a Beckman TL-100 tabletop ultracentrifuge, provided with a TLA-100.2 rotor. After decanting the supernatant, the precipitate was resuspended in 100 µl of water and the process repeated twice. Finally the three supernatants were mixed, centrifuged at 356,000 x g (average) for 30 min at 2°C and concentrated to 0.5 µl/SG. The concentrate was centrifuged at 12000 x g in a

Beckman Microfuge 12 for 10 min at room temperature and the last ultracentrifugation repeated. The supernatant was filtered through a centrifugal microfiltration device with a 0.2 µm regenerated cellulose filter (Model MF-1), Bioanalytical Systems, Inc.) at 1800 x g, 4°C, for 10 min. For the purification of the three neuropeptides, a Beckman/Altex HPLC system was used. It consisted of a Model 420 Microprocessor Controller/Programmer, two Model 110A pumps, a Model 210 manual injection valve and a Model CR1A Recorder/Data Processor. The detector was a LKB Uvicord-S provided with a 8 µl flow cell. The column was µBondapak-Phenyl (300 x 3.9 mm I.D., 10-µm particle size; Waters). The effluent was monitored at 206 nm. Solution A was 0.1% aqueous trifluoroacetic acid (TFA) and solution B was 60% aqueous acetonitrile containing 0.1% TFA. The column was equilibrated with 35% of solution B. After injecting the sample, a linear gradient from 35% B to 58% B in 10 min was followed by an isocratic elution at 58% B for 50 min. The three peaks corresponding to the Molt-Inhibiting Hormone (peak A), the major isoform of the Crustacean Hyperglycemic Hormone (peak B) and the minor isoform of the CHH (peak C) were collected manually and concentrated. The purity of each fraction was ascertained by means of HPLC [7] and polyacrylamide gel electrophoresis [8]. For protein determination, the micromethod of Hazra *et al.* [4] was applied to samples hydrolyzed in 5.7 M HCl at 145°C under N_2 for 1 h in calcined flame-sealed Pyrex tubes. Amino acid analysis was performed in 5 lots of each hormone as described [7,8]. CD spectra were obtained in a Jasco J500A spectropolarimeter at room temperature with a 1.0 cm cell. Hormones were dissolved in deionized and neutralized water.

RESULTS AND DISCUSSION

The comparative analysis of amino acid compositions of the three neuropeptides MIH, CHH-B and CHH-C shows a high degree of homology among them. Twelve residues appear in the same number in all (the number of amidated residues is not known yet and are listed as Asx and Glx) and they all lack His, Met and Trp. Each has 4 Cys, probably forming two disulfide bonds. Their molecular masses lie between 6.1 and 6.4 kDa, and they all behave as acidic peptides.

Their amino ends are blocked and all have Ile as carboxyl end. When the tryptic peptide maps of the two isoforms of the CHH are compared, only one peptide is found to migrate differently on HPLC [9]. When the tryptic peptide maps of the major peak of the CHH (CHH-B) and the MIH are compared in the same fashion, it is found that 5 peptides that coincide in elution time and composition, account for more than half the total amount of amino acid residues of the two neuropeptides [10]. When the composition of the three neuropeptides are compared, there is only a difference of two amino acid residues between any two of them, without taking into account amidated residues. The main differences among them are the isoelectric points (4.79 for both CHHs and 5.50 for MIH), their relative hydrophobicities, and their biological activity. If we sum up all the previous data, we come to the conclusion that the three neuropeptides belong to a new family of hormones. As the high specificity of a peptide hormone for its receptor is due in part to its conformation, which is also a determinant of its biological activity, we decided to study the three neuropeptides by means of CD in order to visualize the differences in their secondary structure. Fig. 1 shows the corresponding CD spectra recorded at room temperature. The negative bands at 220 and 208 nm, together with the positive band around 190 nm reveal the presence of helical structure in these hormones [2]. However, the larger magnitude of the mean residue ellipticity indicates a greater content of helices for peptides CHH-B and CHH-C in comparison with MIH. In order to estimate the structural differences among the three hormones, we analyzed their CD spectra by a least-squares fitting to the basis spectra of Hennessey and Johnson [5], using a modified version of the program REGRES [1] adapted to run on a Hewlett-Packard HP-3000 computer. The analysis was done with and without the restriction of a total sum of structures equal to 100%. In the first case, the restriction was introduced through a Lagrangian multiplier, as suggested in [6]. For CHH-B and CHH-C, both methods of analysis gave very similar computed structure contents (Table 1), which adds confidence to

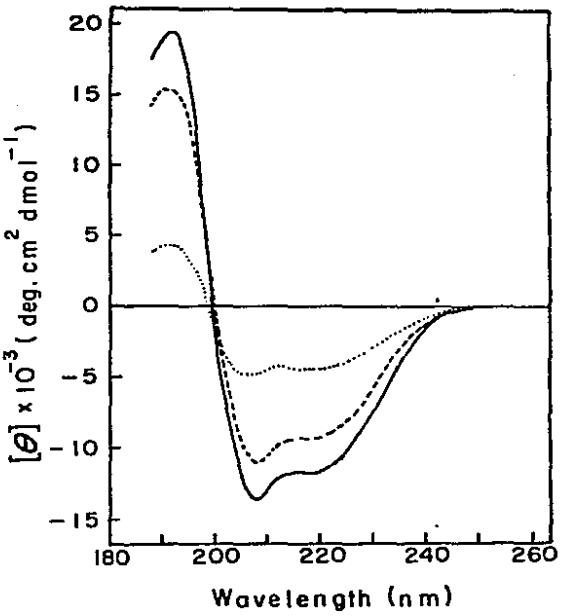

Fig. 1. CD spectra of MIH (....), CHH-B (----) and CHH-C (———). Spectra were recorded in a Jasco J500A spectropolarimeter at room temperature and aqueous solutions at neutral pH, in a 1.0 cm cell. Concentrations of the hormones were (μg/ml): MIH - 10.44; CHH-B - 29.87; CHH-C - 25.60. Mean residue ellipticity [θ], was calculated using a molecular weight of 116.73 per mean amino acid residue.

Table 1. Secondary structure of MIH, CHH-B and CHH-C, estimated from circular dichroism*.

Hormone		H	A	P	T	O	Total	rms
MIH	(1)	13	11	2	10	19	55	416
	(2)	13	23	9	18	37	100	521
CHH-B	(1)	27	19	3	16	22	87	528
	(2)	27	22	5	18	28	100	534
CHH-C	(1)	33	19	1	17	23	93	600
	(2)	33	21	2	18	26	100	604

* The content of each structural type is expressed as per cent.

(1) From an unconstrained analysis.

(2) From an analysis in which the sum of structure contents was constrained to 100%.

Abbreviations: H, helix; A, antiparallel β-sheet; P, parallel β-sheet; T, β-turns; O, other or irregular structures; rms, root mean square error (in deg cm^2 $dmol^{-1}$).

the results obtained. Furthermore, the root mean square (rms) errors of the best-fit analyses were within the range reported for several proteins [5]. It is evident that these hormones differ only slightly in their secondary conformations. It is also remarkable the high content of helices in these peptides, which is comparable to that found in glucagon in basic concentrated solution [3]. On the other hand, the spectrum of MIH was poorly analyzed, since in this case the results were markedly different with and without the Lagrangian multiplier. However, the helical content was similar by both methods, suggesting that the estimated value for this structural type is accurate. Although rms values in Table 1 apparently indicate that the fitting of the MIH spectrum was as good as those of CHH-B and CHH-C, it should be remembered that this criterion only shows that a particular CD curve was adequately expressed as a linear combination of the basis spectra, irrespective of the physical meaning the results may have. In any case, our results indicate that, from the point of view of secondary conformation, MIH differs importantly from CHH-B and CHH-C. The present results could explain the different biological activities of MIH and of the CHHs regardless of their compositional and sequential similarities.

Work now in progress also shows that the sequences of the tryptic peptides of the three neurohormones have an important amount of homology, which reinforces the concept of a family of peptides (to be published).

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DISCUSION

UNA FAMILIA DE NEUROPEPTIDOS DE CRUSTACEOS

A) Antecedentes

Durante los intentos iniciales de purificación de la hormona hiperglucemiante (HHG) de Procambarus bouvieri (Ortmann) por medio de cromatografía líquida de alta presión en fase reversa (CLAP-FR), se encontró un pico hidrofóbico y asimétrico que contenía toda la actividad hiperglucemiante de los extractos crudos de la glándula sinusal; con otra columna analítica, se pudo dividir el pico anterior en tres fracciones bien separadas (picos A, B y C, según el orden de elución), cuyas purezas fueron confirmadas por métodos cromatográficos, electroforéticos y químicos. El bioensayo correspondiente demostró que únicamente los péptidos B y C tenían actividad hiperglucemiante.

Dada la predominancia de una de las hormonas hiperglucemiantes (pico B, HHG-B), se prosiguió con su caracterización bioquímica en cuanto a peso molecular (PM), punto isoeléctrico (pI), composición de aminoácidos (CompAA) y extremos amino y carboxilo (ExtrN y ExtrC, respectivamente). Debido a sus parecidos con la HHG-B, en cuanto a hidrofobicidad y CompAA preliminar, los picos A y C (HHG-C) fueron purificados simultáneamente y acumulados para su estudio posterior (Aguilar, 1986; Huberman y Aguilar, 1986).

B) Resultados

Con los antecedentes de la heterogeneidad de la HHG en varias especies de crustáceos (Huberman y Aguilar, 1988a, introducción) y de la existencia de otros neuropéptidos hormonales en la glándula sinusal (véase la revisión de Kleinholz, 1985), se procedió a caracterizar en paralelo a los péptidos A, B y C. Un bioensayo heterólogo in vitro mostró que el péptido A tiene la actividad biológica correspondiente a la hormona inhibidora de la muda (HIM). La finalidad del estudio fue caracterizar bioquímicamente a los tres neuropéptidos para obtener la información estructural necesaria con el fin de elegir la estrategia adecuada para la determinación de la secuencia de aminoácidos de las hormonas y al mismo tiempo, obtener datos que revelaran la naturaleza del polimorfismo de la HHG y de su relación con la HIM.

Estos trabajos mostraron semejanzas muy notables entre los tres neuropéptidos:

(1) La electroforesis en gel de poliacrilamida desnaturalizante indicó el mis

mo peso molecular para la HIM, la HHG-B y la HHG-C, comprendido entre 6.1 y 6.4 kDa (Huberman y Aguilar, 1988a, Fig. 1).

(2) El análisis automático de aminoácidos (AnalAA) reveló que 12 tipos de residuos se encuentran en la misma cantidad en las tres hormonas (el número de residuos amidados en cada hormona se desconoce) y que todas ellas carecen de His, Met y Trp. En más, entre los miembros de cualquier par de hormonas, la diferencia en el AnalAA está en 2 residuos solamente, sin tomar en cuenta las posibles diferencias en los residuos amidados (Huberman y Aguilar, 1988a, Tablas 1 y 2; 1989 , Tabla 1).

(3) La dansilación, seguida de hidrólisis ácida y cromatografía en capa fina de poliamida (CCF), mostró que los tres neuropéptidos tienen bloqueado el extremo amino; la identidad de dicho residuo se ignora. La digestión con carboxipeptidasa Y (CPY), seguida de dansilación y CCF indica que Ile es el ExtrC de las tres hormonas; no se sabe si este residuo está amidado o esterificado (como sucede en muchas hormonas), ya que la CPY posee actividades amidásica y esterásica (Huberman y Aguilar, 1988a, Tabla 2; 1989).

(4) El isoelectroenfoque en geles de poliacrilamida con gradiente de anfolinas demostró que las tres hormonas son péptidos ácidos. Las HHGs tienen un pI de 4.79, mientras que la HIM posee un pI de 5.50 (Huberman y Aguilar, 1988a, Tabla 2; 1989).

Los datos anteriores indican claramente que estas tres neurohormonas constituyen una familia de neuropéptidos muy parecidos entre sí.

Para localizar las diferencias pequeñas esperadas, se compararon los mapas peptídicos en CLAP-FR de la digestión de cada hormona con tripsina-TPCK. Un solo péptido tríptico tuvo movilidad diferente en las dos HHGs; el AnalAA indicó la misma CompAA para estos dos péptidos, uno de cada hormona (Huberman y Aguilar, 1988b, Fig. 2). Al comparar los mapas de la HIM y la HHG-B se encontró que hay 5 péptidos tripticos que coinciden en movilidad y CompAA (Huberman y Aguilar, 1989, Fig. 3 y Tabla 2); en los demás fragmentos, para cada uno de ellos en la HIM hay otro en la HHG-B con una CompAA muy semejante, a pesar de diferencias variables en los tiempos de retención; la cantidad limitada de los péptidos no permitió, en algunos casos, determinar en forma totalmente confiable las proporciones relativas de algunos residuos.

De los estudios de CompAA y de los mapas tripticos, puede verse fácilmente que la homología entre los miembros de esta familia de neuropéptidos es muy alta. Los seis péptidos tripticos (PTs) que coincidieron en el par HHG-B/HHG-C (Fig. 2, Huberman y Aguilar, 1988b) contienen el 85% del total de residuos de cualquiera de esas dos hormonas; si tomamos en cuenta que el AnalAA indicó que los PTs de HHG-B y HHG-C que no coincidieron en tiempo de retención tienen la misma CompAA, entonces el porcentaje de homología es todavía mayor. En la pareja HIM/HHG-B los cinco fragmentos coincidentes (Fig. 3, Huberman y Aguilar 1989) constituyen el 57%; como en este caso también hay pares de PTs (uno de cada hormona) que se comportan diferente en CLAP-FR, pero que tienen una CompAA muy parecida, es claro que la homología real en secuencia es mayor al 57%.

Es importante mencionar que algunos estudios preliminares de la secuencia de aminoácidos han confirmado que los PTs que coincidieron en tiempo de retención son, de hecho, idénticos.

En cuanto a las diferencias entre las tres neurohormonas, las principales están en la hidrofobicidad, el punto isoeléctrico y la estructura secundaria.

Las diferencias en comportamiento cromatográfico y en punto isoeléctrico se podrían explicar por las discrepancias pequeñas en sus CompAA, es decir, en los tipos y proporciones relativas de sus residuos básicos, ácidos, amidados o hidrofóbicos. En el par HHG-B/HHG-C, la diferencia en hidrofobicidad debe estar dada por los residuos amidados o hidrofóbicos o por alguna modificación postraduccional neutra o por ambas causas, ya que el punto isoeléctrico es el mismo. Las CompAA parecen indicar que la hidrofobicidad mayor de la HHG-C se debe a que posee un residuo de Fen en lugar de uno de Leu en la HHG-B. Sin embargo, ya que el AnalAA de los únicos PTs de las HHGs con tiempo de retención diferente (Huberman y Aguilar, 1988b; Fig. 2: péptidos 4 en HHG-B y 6 en HHG-C) indicó la misma CompAA y dado que dichos fragmentos contienen residuos de Asp/Asn y de Glu/Gln, la mayor hidrofobicidad de la HHG-C puede explicarse por una sustitución de una Gln en la HHG-C en lugar de una Asn en la HHG-B.

Existen varias opciones para explicar la diferencia en el pI de la HIM y las HHGs. La CompAA indica que podría ser consecuencia de un residuo adicional de Lis en la HIM en relación a las HHGs; esto a su vez volvería más hidrofílica a la HIM. Otra alternativa es una diferencia en la proporción de los

residuos ácidos/amidados entre ambos tipos de hormonas. Aquí, también es posible que las modificaciones postraduccionales y una combinación de las tres posibilidades contribuyan a la diferencia de pI.

El estudio de dicroismo circular (Fig. 1 y Tabla 1, Huberman et al., 1989, enviado para publicación) indica que, en cuanto a la estructura secundaria, las dos isoformas de la HHG son muy semejantes y que hay diferencias importantes entre este par y la HIM (sobre todo en el contenido de hélice y estructuras irregulares). Este resultado explica la actividad biológica diferente en la HIM y las HHGs, independientemente de las semejanzas y diferencias en CompAA y secuencia de aminoácidos. El hallazgo fue más bien el esperado, ya que se sabe que la actividad fisiológica de una hormona depende de su interacción con receptores específicos y ésta última es consecuencia parcial de la conformación; los tipos y proporciones de las estructuras secundarias determinan a su vez la conformación molecular. En el caso de las HHGs se esperaba encontrar que sus estructuras secundarias fueran muy parecidas ya que las actividades hiperglucemiantes son iguales prácticamente (Aguilar, 1986; Huberman y Aguilar, 1986) y la homología composicional y secuencial es mayor al 85% (Huberman y Aguilar, 1988a; 1988b). En cuanto a la comparación de las HHGs con la HIM el resultado tampoco es inesperado, ya que a pesar de diferir aparentemente en sólo dos residuos de aminoácido y de existir una homología secuencial muy alta (mayor del 57%) (Huberman y Aguilar, 1989), un cambio mínimo en la secuencia de aminoácidos de moléculas relativamente pequeñas puede ocasionar una diferencia considerable en las estructuras secundaria y terciaria y en la actividad biológica misma.

C) Perspectivas

Hay varias posibilidades para el origen de esta familia de neuropéptidos: podría ser que las tres hormonas sean el producto de un solo gen, modificado postranscripcionalmente por empalme alternativo del ARNm primario (véase la revisión de Breitbart et al., 1987; Hunt et al., 1989) o postraduccionalmente (véase la revisión de Huberman, 19). Otra opción sería la de duplicación o triplicación génica (véanse las revisiones de Acher, 1981 y de Niall, 1982), evolución divergente y expresión génica diferencial (Kakita et al., 1982). Ya que esta familia consta de tres miembros por lo menos, también es factible que su origen sea la consecuencia de una combinación de la posibilidades enunciadas anteriormente.

En lo referente al polimorfismo de la HHG, específicamente, hay varias alternativas posibles: (1) a expresión de una u otra de las isoformas puede estar relacionada al sexo. La purificación de las hormonas a partir de extractos de OSs de machos y hembras por separado contestaría esta interrogante. (2) as dos isoformas pueden ser el producto de genes alelos, dominantes o codominantes. La proporción de la isoforma más abundante (HHG-B) en relación a la otra (HHG-C) es siempre de 3:1 cuando se obtiene el extracto crudo de muchos animales; esto concuerda con la frecuencia esperada para genes alelos dominantes. Para aclarar esta posibilidad sería necesario obtener las hormonas de un solo animal y ver si contiene las dos HHGs; aún en el caso de que fueran alelos codominantes podrían observarse animales con una sola isohormona. (3) Si las isoformas son el producto de dos genes, surgen preguntas con respecto a la localización cromosómica de dichos genes y a su regulación.

Hasta el momento, la caracterización estructural de la HIM y de las HHGs de Procambarus bouvieri no permite saber cuál de las posibilidades acerca del mecanismo que originó la familia de neuropéptidos es la verdadera. La respuesta para esta interrogante se tendrá cuando se hayan determinado las estructuras primarias completas de las hormonas; tal vez sea necesario compararlas con las secuencias deducidas a partir de su(s) gen(es).

El paso siguiente hacia la dilucidación de la estructura primaria será caracterizar completamente a los PTs (CompAA, secuencia); en el caso de los fragmentos bloqueados, será necesario generar (por métodos químicos o enzimáticos) un ExtrN que permita la secuenciación manual o automática. Posteriormente, habrá que producir (química o enzimáticamente) otro juego diferente de péptidos y caracterizarlos en los mismos términos que a los anteriores, con el fin de encontrar secuencias traslapadas y poder establecer el orden de los PTs en las hormonas y con ello la secuencia de aminoácidos completa.

Otra forma de determinar el orden de los péptidos trípticos en las hormonas intactas consiste en limitar la hidrólisis tríptica mediante el bloqueo de las lisinas (con anhídrido succínico, por ejemplo) o de las argininas (con 1,2-ciclohexanodiona) antes de la digestión con la tripsina.

Hasta la actualidad se ha determinado la secuencia completa, manualmente, en sólo 3 de los 7 péptidos trípticos de la HHG-B. El impedimento para conocer la estructura de los péptidos restantes ha sido el bloqueo de sus extremos amino, al parecer por un residuo de ácido piroglutámico; éste puede producirse cuando una glutamina queda en la posición amino terminal como consecuencia de una ruptura química o enzimática de la molécula intacta. Existen métodos químicos y enzimáticos para "abrir" o cortar el residuo cíclico de ácido piroglutámico (algunos de los cuales han sido probados ya en el laboratorio) que se aplicarán a la molécula intacta o a sus fragmentos trípticos.

Como ya se mencionó, se pueden utilizar otras enzimas en este estudio, ya sea para aplicarlas a la HHG directamente o a sus fragmentos químicos o enzimáticos, con el fin de obtener péptidos que sean susceptibles de someterse a la secuenciación manual o automática. Dadas las composiciones de aminoácidos de los péptidos trípticos, podrían usarse la quimotripsina, la pepsina, la termolisina, la proteasa V8 de estafilococo, la endoproteasa Arg-C de la glándula submaxilar del ratón, etc.

Queda también el recurso de la espectrometría de masas, que si bien en el estado actual de la tecnología no es capaz de resolver estructuras del tamaño de la HHG, puede aplicarse al estudio de fragmentos químicos o enzimáticos de ésta. Ha sido especialmente útil en el caso de péptidos bloqueados en el extremo amino (Fernlund, 1972; Scarborough et al., 1984).

Recientemente, se ha determinado ya la secuencia completa de la HHG de Carcinus maenas (Kegel et al., comunicación personal). Con los datos parciales de la hormona de Procambarus bouvieri puede verse claramente una homología considerable en la secuencia general y en la posición de las cisteínas, a pesar de que la hormona del animal europeo contiene cerca de una docena más de residuos en el extremo carboxilo.

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