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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*

ALBERTO HUBERMAN* and MANUEL B. AGUILAR
Department of Biochemistry, Instituto Nacional de la Nutrición "Salvador Zubirán", Vasco de Quiroga 15, Tlalpan, 14000 México, D.F. (México)

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

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residues, 8; missing amino acids, methionine, histidine and tryptophan; amino terminus, blocked; carboxyl terminus, isoleucine; and p*I*, 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 p*I* 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[6] 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 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 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

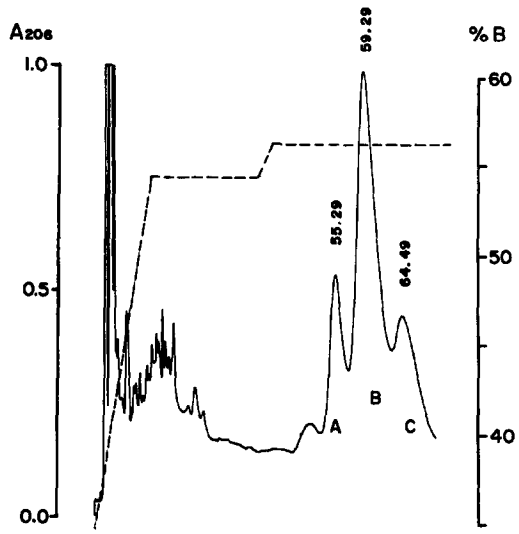

Fig. 1. One-step purification of the two isoforms of the crustacean hyperglycaemic hormone, CHH-B and CHH-C. Crude extract from 500 sinus glands of *Procambarus bouvieri* (250 μl) was fractionated on a μBondapak-Phenyl column (300 × 3.9 mm I.D., 10-μm particle size) without a pre-column. Solution A was 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-

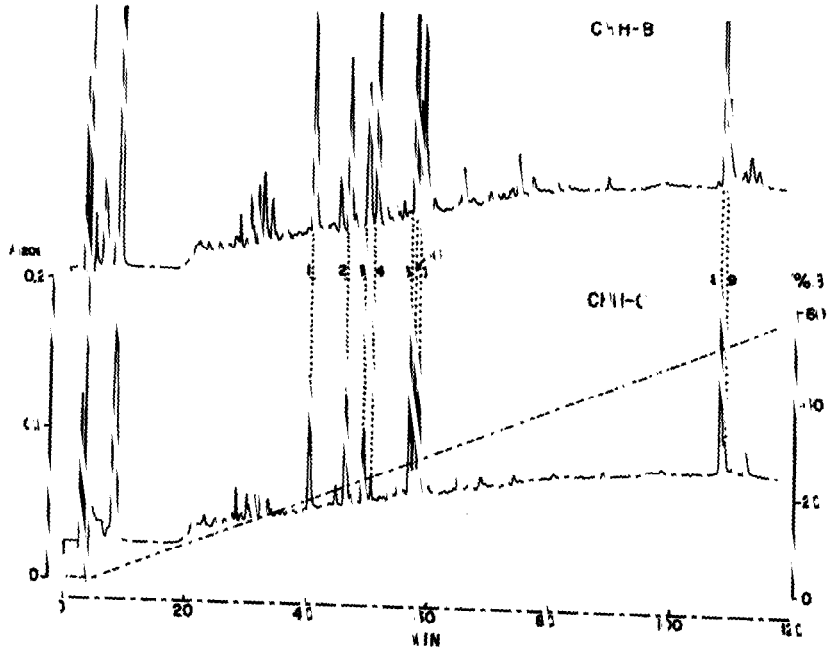

Fig. 2. Comparative tryptic peptide maps of isoforms CHH-B and CHH-C. CHH-B (103.2 μg = 17.2 nmol) and CHH-C (51.2 μg = 10.2 nmol) were reduced and carboxymethylated. After purification, they were digested with trypsin–TPCK, evaporated to dryness, resuspended in 250 μl of solution A and injected directly into an Ultrasphere-ODS column (25 × 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 arising 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×), his, 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

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[5]. 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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