Chemical Synthesis of a 7 kDa Insect Gonadotropic Neurohormone

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An original insect neurohormone of 65 residues was synthesized by the solid-phase methodology using t-Boc strategy and Boc-Val-PAM-resin. The purification, conducted by several steps of liquid chromatography having mass, polarity or charge as separative criteria, yielded the product with the correct molecular weight of 6922 Da determined by mass spectrometry. The synthetic peptide had both the same affinity for the anti-native neurohormone serum and the same biological activity as the native neurohormone.

Keywords: Gonadotopic hormone; peptide synthesis; biological activity; insect

INTRODUCTION

A gonadotropic neurohormone has been previously isolated from an insect, the African locust [1]. It is an original monomeric polypeptidic chain of 65 residues with one microheterogeneity at position 26 with either a serine (major peptide) or a valine residue [2]. This molecule is needed for ovarian maturation and was called Lom OMP for Ovary Maturating Parsin of Locusta migratoria [1]. Studies of structure–function relationships of this neurohormone by 3D structure determination and detailed knowledge of its physiological function show that large amounts of protein are needed. The storage site of the neurohormone (neurosecretory lobes of corpora cardiaca) contains several proteins but only 2 μg of total protein per adult mature insect. Consequently, following the successful chemical synthesis of the

three distinct hydrophilic domains of the Lom OMP [3, 4], a chemical synthesis of the Ser^{26} isoform of the entire neurohormone was attempted.

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1            10            20            30
YYEAPPDGRHLLLQPAPAAPAVAPASPASWPHQQRRQ
     40            50            60
ALDEFAAAAAAAADAQFQDEEEDGGRRV
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In the present work, we report the preparation by solid-phase methodology and the purification using three distinct types of liquid chromatography of a synthetic serine Lom OMP which has the same chemical characteristics and biological activity as the native extracted one.

MATERIALS AND METHODS

Synthesis

The chemical synthesis of the 65 amino acid sequence was automatically performed on an Applied Biosystems peptide synthesizer 431 A using the t-Boc strategy introduced by Barany and Merrifield [5]

and modified by Applied Biosystems. Nα-protected valine linked to 4(-oxymethyl)-phenylacetaminomethyl copoly(styrene-1% divinylbenzene)-Boc-Val-PAM-resin and other Nα-Boc-protected amino acids were from Novabiochem (Switzerland). *N,N7*-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazol (HOBt), 1,2-ethanedithiol (EDT), piperidine, mercaptoethanol, dimethylsulphoxyide (DMSO) from Sigma (Sigma-Chimie France), dimethylforamide (DMF), dichloromethane (DCM), *N*-methylpyrrolidone (NMP), trifluoroacetic acid (TFA), di-isopropylethylamine (DIEA), methanol (MeOH), acetic acid (AcOH) and acetonitrile (CH_3CN) were purchased from SDS (SDS-PEYPIN France); Hydrogene fluoride (HF) was obtained from UCAR (Belgium).

The *t*-Boc–Nα-amino acids were protected as follows: cyclohexyl ester (OcHex) for aspartic and glutamic acids, 2-bromocarbobenzoxy (BrZ) for tyrosine, *p*-toluenesulphonyl (Tos) for arginine, dinitrophenyl (Dnp) for histidine, formyl (CHO) for tryptophan and benzyl (Bzl) for serine.

Elongation was done on Boc-Val-PAM–resin (0.5 mmol; loading of starting resin: 0.43 mmol/g) according to the following operational cycle protocol: deblocking with 50% TFA/DCM, washing with DCM, neutralization with 10% DIEA/DCM, washing with DCM, then coupling with performed HOBt esters in NMP.

Addition of DMSO and DIEA increases the coupling efficiency by breaking peptide self-aggregation [6].

Cleavage of the Peptide from the Resin

After the N-terminal tyrosine coupling, the Dnp groups protecting the histidine residues were removed by thiolysis. The peptide–resin was stirred in a solution of DMF containing 20% mercaptoethanol and 10% DIEA in 1 h at room temperature. After draining and thoroughly washing the resin with DMF, ethanol and finally DCM, the N-terminal Boc group was removed by the above-mentioned fashion. To deformylate the protected Trp residues prior to cleavage with HF, the peptide–resin was treated with 10% piperidine in DMF for 2 h at 0°,C, washed with DMF, DCM and ethanol and dried overnight in a vacuum desiccator. The peptide was cleaved from the resin by classical high HF treatment, with anisol and ethanedithiol as scavengers. Good splitting of the tosyl protection of arginine was achieved by allowing a reaction time of 90 min at 0°C. HF was then rapidly evaporated *in vacuo*. The peptide–resin was tritu-

rated with 50% aqueous acetic acid. After dilution with water, the cleaved deprotected peptide was lyophilized.

Amino Acid Analysis

Amino acid analysis of the peptide was determined after hydrolysis in 6M HCl (110°C, 12 h) using Millipore-Waters Pico-Tag work station.

Size-exclusion Chromatography

The crude peptide was loaded to a 2.5 × 120 cm column of Sephadex G50 fine (Pharmacia Biotech S.A., France) and eluted with 50% AcOH. Elution of the product was monitored at 220 and 280 nm. The fractions were analysed by reverse-phase HPLC and those containing the desired product pooled and lyophilized.

Analytical HPLC

The lyophilized product was dissolved in a 50% AcOH solution and centrifuged. The supernatant was analysed by HPLC on a Waters 600E System Controller and a Waters 996 Photodiode Array Detector using a C4-Delta Pak column (3.9 × 150 mm, 300 Å, 5 μm). The elution was carried out with 0.1 aqueous TFA and acetonitrile (0.08% TFA) at a flow rate of 1 ml/min, with a 10–40% acetonitrile linear gradient over 30 min.

Semi-preparative HPLC Purification

Semi-preparative HPLC was performed with a C4-Delta Pak column (7.8 × 300 mm, 300 Å, 15 μm). The chromatographic conditions were the same as for the analytical scale.

Anion Exchange Chromatography Purification

Samples of 300 μg of the lyophilized product were injected on a Mono Q HR 5/5 column (Pharmacia) connected to a Beckman gradient system (Model 333) with a variable wavelength detector (Model 165). A 33 min linear gradient from 0% to 50% of 1 M NaCl in 25 mM Tris-HCl pH 8 followed by a 2 min linear gradient from 50% to 100% were employed at a flow rate of 1 ml/min at room temperature. Peaks obtained were manually collected.

HPLC Desalting and Purification

Each peak pooled from five to nine runs of anion exchange chromatography was loaded on a reversed-phase C8 Pro RPC 5/10 column (Pharmacia) and eluted with a linear gradient from 28% to 50% of CH_3CN in 25 min at a flow rate of 0.5 ml/min. The purity and the molecular weight of each peak were monitored by a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) [7] on a Mini-Protean II dual slab gel (Bio-Rad).

Mass Spectrometry

Each peak collected after HPLC purification was dissolved in a water (1% acetic acid)/methanol 50/50 solution in order to reach a concentration of about 10 pmol/μl.

Electrospray mass spectra were performed on a VG Bio Tech BioQ mass spectrometer (VG Biotech Ltd, Altrincham, UK), with an electrostatic spray ion source operating at atmospheric pressure followed by a quadrupole mass analyser with an m/z range of 4000. The voltage cone was operated at 55 V. Calibration was performed with myoglobin.

The spectra of multiply charged ions were interpreted as previously described [8]. Since the resolution of the mass spectrometer was less than 1000, the masses measured were average masses and not monoisotopic masses.

The transformation of the spectra of the multiply charged ions into a true molecular mass scale spectra was performed by data system routines provided by the manufacturer.

Polyacrylamide Gel Electrophoresis Separation

The synthetic product was analysed by either 7.5% native PAGE according to Laemmli [9] or 12% SDS–PAGE according to Schägger and Von Jagow [7]. Electrophoreses were performed with Bio-Rad Mini-Protean II dual Slab Cell at constant voltage. Gels were stained in a fixative 10% acetic acid solution containing 50% methanol and 0.1% Coomassie Brilliant Blue R250.

Western Blotting Procedures

Following native or 12% SDS–PAGE, proteins were transferred onto Immobilon-P (Millipore St Quentin, Yvelines, France) using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell according to the manufacturer's procedures (25 mM Tris–glycine buffer, pH 8.3, with methanol). The transfer was carried out

at a constant voltage (100 V) and a current of 180 mA at the beginning and 250 mA at the end. The efficiency of sample transfer was evaluated by Coomassie Blue staining of the gel.

The synthetic product and the peaks obtained following anion-exchange chromatography were also dotted on nitrocellulose.

Western Blots and Dots Immunostaining

Blots and dots were treated for 30 min at room temperature in 0.2 M Tris–HCl buffer, pH 7.2, containing 3% bovine serum to saturate the fixation sites and therefore improve the specificity of the immunostaining. They were incubated in anti-Lom OMP serum diluted at 1/2000 in the previous buffer for 16 h at 4°C under stirring. Following buffer washes, blots or dots were incubated for 2 h at room temperature under stirring in 1/1000 dilution of peroxidase-labelled goat anti-Fag rabbit serum in 25 mM Tris–glycine buffer, pH 7.2. The peroxidase reaction was carried out for 5 min at room temperature with 0.05% diaminobenzidine as chromogen (DAB, Sigma St Quentin Fallavier, France) and 0.06% hydrogen peroxide as substrate in 25 mM Tris–glycine buffer, pH 7.2.

Biological Activity

The synthetic products were injected every day into adult female locusts from day 1 to day 10 to test their gonadotropic effect. Other females were injected in the same conditions with water (control) or with native extracted Lom OMP. The injection effect on the ovarian maturation was evaluated by the mean length of ten basal oocytes measured for each female at day 11. The oocyte mean length of females, treated with either synthetic molecules, native extracted Lom OMP or water, were compared by Student's t-test.

RESULTS

Synthesis

The chemical synthesis of the Lom OMP gonadotropic neurohormone was achieved using Merrifield's solid-phase peptide synthesis methodology but with a more stable PAM–resin.

No quantitative ninhydrin analysis was made after each amino acid incorporation, but in order to minimize generation of deletion sequences arising from incomplete reactions, a double coupling strat-

egy with 4 mmol amino acid per coupling, followed by a capping step with acetic anhydride, was systematically done.

Progress in synthesis was followed by amino acid analysis of hydrolysed samples of peptide–resin, and was controlled at several stages of the chain assembly using Millipore-Waters Pico-Tag workstation and the Pico-Tag column. The residue distribution made it possible to check the regular elongation of the sequence. For instance a resin sample was taken after incorporation of serine at position 29 (the first in the sequence). Amino acid analysis clearly showed the presence of serine in the hydrolysat and thus indicated the absence of extensive chain termination during the synthesis; other analyses were performed after incorporation of 52-Ala, 39-Leu and 2-Tyr. In order to allow good resin swelling, a part of it (30%) was set apart during the synthesis after incorporation of 27-proline.

After deprotection of the His and Trp residues, final cleavage of the peptide from the solid support was carried out by high HF treatment.
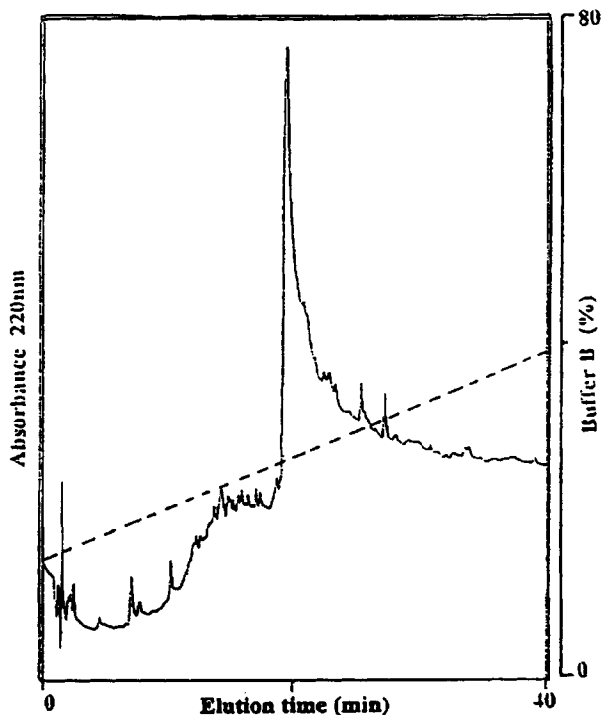

Figure 1 Analytical reversed-phase HPLC of crude Lom OMP. The peptides were applied on a Delta Pak C4, 5 μm, 300 Å column (3.9 × 150 mm), buffer A was 0.1% TFA in water, buffer B was 0.08% TFA in CH_3CN. Elution was done by linear gradient from 10% to 40% B for 30 min at a flow rate of 1 ml/min. Detection was monitored by absorbance at 220 nm.
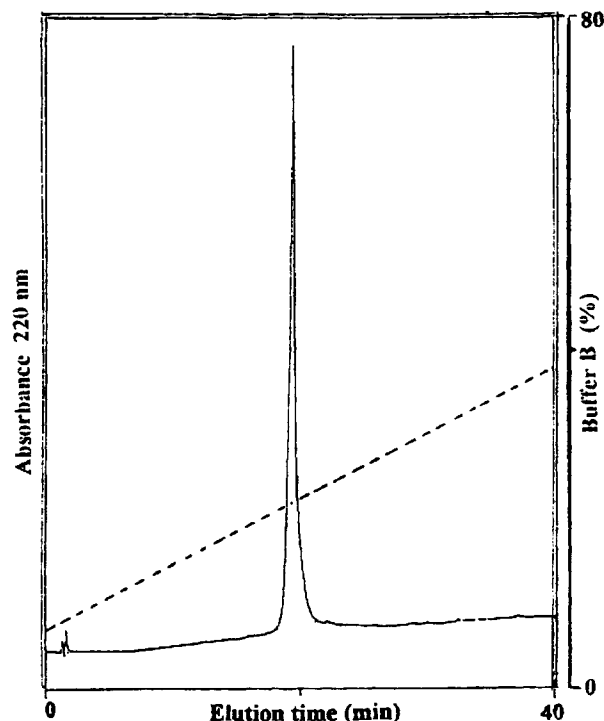

Figure 2 Analytical reversed-phase HPLC of synthetic peptide obtained after semi-preparative HPLC purification, same conditions as in Figure 1.

Purification

The crude product was initially submitted to gel filtration on a Sephadex G 50 column in order to separate the target product from the truncated sequences, issued from acetylation capping of unreacted amino groups during the synthesis. The fractions were lyophilized, identified by amino acid analysis, and the synthetic hormone was analysed by RP-HPLC on an analytical column (Figure 1).

The second step of purification was a semipreparative RP-HPLC with the same conditions as described earlier. It led to a large increase in purity of the synthesized sequence (Figure 2), but did not yield a homogeneous product, as it was shown by ion exchange chromatography and mass spectrometry.

The synthetic product eluted in one peak from the semi-preparative HPLC was split into several peaks, more or less individualized, by anion-exchange chromatography (Figure 3). The last peak (P3) was eluted at the same NaCl concentration as the native extracted Lom OMP. All peaks gave only one peak following desalting.
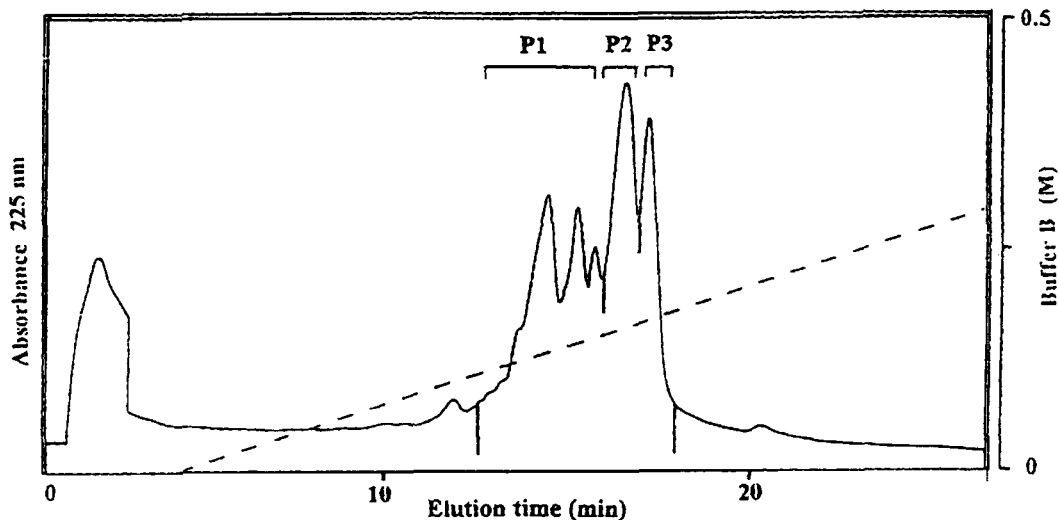
Figure 3 Anion-exchange separation of the peptide eluted from the last semi-preparative HPLC. The peptide was applied on to a Pharmacia Mono Q, HR 5/5 column, 10 μm, buffer A was 25 mM Tris-HCl pH 8, buffer B was 1 M NaCl in buffer A. Elution was done by linear gradient from 0 to 0.5 M NaCl for 33 min at a flow rate of 1 ml/min. Detection was monitored by absorbance at 225 nm.
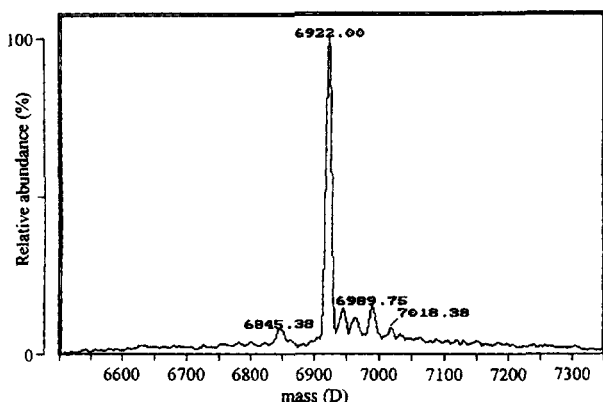

Figure 4 Mass spectrum of P3. Spectrum of molecular masses obtained by the transformation of the multiply charged ions' spectrum plotted versus m/z.

Biochemical Characterization

Mass spectrometry analysis of P3 (Figure 4) gave only one major component of an M_r value (6922 Da) identical to that of the native extracted Lom OMP (6922.5 Da). Mass spectrometry analysis of the other peaks (P1 and P2) gave several components from 6071 to 7420 Da including a major peak of 6991 Da, higher than Lom OMP M_r, present both in P1 and P2 (Figure 5).

Non-denaturing electrophoresis analysis of the synthetic product eluted from the semi-preparative HPLC (prepurified synthetic product) confirmed the product complexity (three major brands). The three

peaks (P1, P2 and P3) analysed on native PAGE showed from P1 to P3 a concentration increase of the most anodic band which displays the same electrophoretic mobility as Lom OMP (Figure 6). Following 12% SDS–PAGE, the prepurified synthetic product was resolved into one band having the same molecular weight as the native extracted Lom OMP and sometimes another band slightly lower.

Comparison of Coomassie Blue staining intensities with either native Lom OMP extracted from graded number of storage organs or graded doses of prepurified synthetic product indicated that one storage organ could contain about 80 ng of the neurohormone.

Immunochemical Characterization

The prepurified synthetic product and all its components separated either by anion-exchange chromatography (dots) or native PAGE (Western blots) were bound to the anti-Lom OMP serum as the native extracted Lom OMP (Figure 7).

Analysis of immunoreactivity intensities of either native Lom OMP extracted from graded number of storage organs or graded doses of synthetic product confirmed the evaluation of the neurohormone content of one storage organ.

Biological Characterization

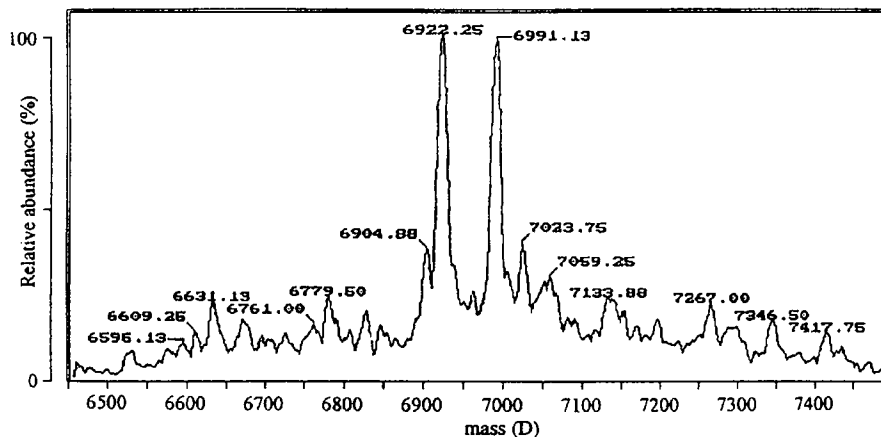To compare the biological activity of both the pure synthetic molecule and the native extracted Lom

Figure 5 Mass spectrum of P2. Spectrum of molecular masses obtained by the transformation of the multiply charged ions' spectrum plotted against m/z.
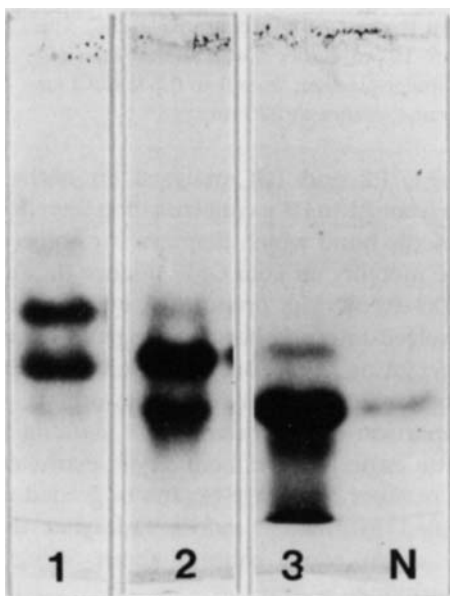

Figure 6 Photography of 7.5% PAGE of P1, P2, P3 and native Lom OMP. Coomassie Blue staining.
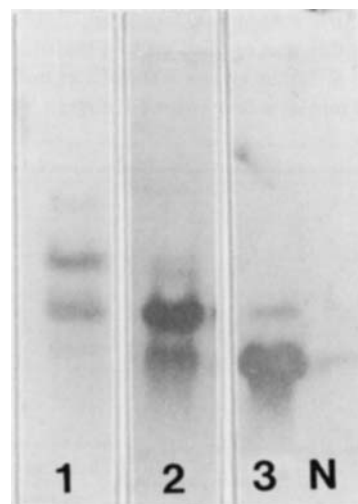

Figure 7 Photography of Western blot following 7.5% PAGE of P1, P2, P3 and native Lom OMP immunostained with the anti-Lom OMP immune serum at 1/2000. Secondary antibody labelled with peroxidase, diaminobenzidine as chromogen.

OMP, we chose peaks (P3 and the Lom OMP peak obtained from crude extracts of the storage organ) of the same surface area eluted from the anion-exchange chromatography column in order to have about the same quality of material.

Injections of peak 3 to young adult females stimulated ovarian maturation as the native extracted Lom OMP did at a daily dose of two storage organ equivalents. At day 11, the oocyte mean lengths of females treated by either the native Lom OMP or the synthetic pure Lom OMP were comparable (p > 0.05) and significantly higher (p < 0.001) than the oocyte mean lengths of control females (Figure 8).

Injections to young females of either 80 or 160 ng (corresponding to the putative neurohormone content of one and two storage organs respectively) of the prepurified synthetic product stimulated the oocyte growth equally (p > 0.05) as the pure synthetic Lom OMP (Figure 8). Because P3 corresponded to less than 30% of the components of the prepurified synthetic product, these results strongly suggest that not only P3, but also the other major components, have a gonadotropic effect. This deduction was supported by the gonadotropic effect analysis of the 6991 Da peak.
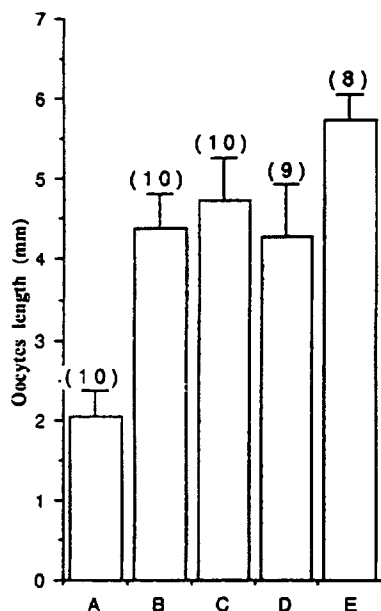
Figure 8 Effects of the injection of 5 μl of water (A), two storage organ equivalents of pure native Lom OMP in 5 μl of water (B), two storage organ equivalents of pure synthetic Lom OMP in 5 μl of water (C), 80 ng of prepurified synthetic Lom OMP (D) and 160 ng of prepurified synthetic Lom OMP (E). ( ) number of animals.

DISCUSSION

We succeeded in the chemical synthesis of the Lom OMP, the first entirely sequenced gonadotropic neurohormone of insect, isolated and purified from the African locust. As discussed recently by Mutter *et al.* [10], Bayer [11] and Bianchi *et al.* [12], it is necessary to adopt a multidimensional chromatographic approach in order to get homogeneous material. Therefore we successively used techniques based on molecular size, hydrophobic interaction and charge. Following three steps of liquid chromatography having three distinct separative criteria (mass, polarity and charge), we obtained a polypeptide chain of 65 residues which had the same charge, the same mass, the same immunoreactivity and the same gonadotropic activity as the native extracted one.

This pure product will be used in the next future for its conformation determination, by spectroscopic techniques (CD, RMN, X-ray), since this molecule has no sequence similitude with other known proteins [2].

Moreover, the prepurified synthetic product, obtained following only two liquid chromatography

steps (separation by mass, then by polarity), contains other components with masses different from the native extracted neurohormone. Some have a lower mass than the native Lom OMP and probably result from truncated sequences. Most of them have higher masses than the native Lom OMP and probably result from incomplete cleavage during the different deprotection steps. However, no easy explanation can be given for a major peak of 6991 Da, the mass difference (69 Da) from the mass of the native neurohormone is lower than the smallest mass of protecting groups. A trypsic cleavage of this peptide, followed by mass evaluation of the three resulting fragments, indicated that the overloaded mass was in the N-terminal 1–9 fragment.

The prepurified synthetic product, composed with the synthetic equivalent of Lom OMP and other components, stimulated the oocyte growth at the same concentration and with the same intensity as the native extracted Lom OMP. Consequently the prepurified synthetic product can be used directly for biological studies. The Lom OMP biological activity is triggered by two of the three hydrophilic domains indicated by the hydropathy profile of the molecule [2]. These domains, at the N (Y1–D7) and the C (A49–V65) terminal extremities of the molecule, induced the same stimulation of the oocyte growth as the entire native or synthetic Lom OMP, but needed a 100 times higher concentration [3, 4].

Owing to the successful chemical synthesis of this original 7 kDa molecule, it is now possible to begin studies needing large quantities of material such as structure determination and receptor characterization using synthetic Lom OMP biotinylated on the N-terminal tyrosine at the end of the synthesis.

Acknowledgement

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