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PARTIAL CHARACTERIZATION AND AMINO ACID COMPOSITION OF A HIGH MOLECULAR WEIGHT PEPTIDE WITH SALMON CALCITONIN IMMUNOREACTIVITY IN A CRUSTACEAN : NEPHROPS NORVEGICUS

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SUMMARY : Anti-salmon calcitonin antibodies were used to follow the purification of a high molecular weight peptide present both in the haemolymph and in the hepatopancreas of the Norway lobster Nephrops norvegicus . An apparent molecular weight of 22 kDa has been measured in electrophoresis on SDS gels and amino acid composition compared to salmon calcitonin. The amount determined by the immunoreactivity assay corresponds to about 1/40 and 1/140 of that which is based on direct protein measurement for the hepatopancreas and the haemolymph respectively. The total amount of this peptide could be estimated as 3.5 mg/g fresh weight for the hepatopancreas and 140 ug/ml for the haemolymph. The function of this peptide is still unknown.

Calcitonin, a small polypeptide hormone of approximately 3500 daltons was first purified from the thyroïd gland of rats (1) and its presence further confirmed in the thyroïd glands of other mammals as well as in the ultimobranchial glands of birds and fishes (2 - 3). Calcitonin-like peptides have been also detected in the alimentary tract of a protochordate, Ciona intestinalis , on the basis of an immunoassay (4). All the hormones so far isolated and characterized share a 32 amino acid peptide sequence exhibiting an internal disulfide bridge between the 1st and 7th position from the NH_2 terminus and a proline amide at the C-terminus. In mammals, the main biological activity is to lower the calcium concentration in the blood but additional actions on gastrointestinal glands were also described (5, 6).In Crustacea, calcitonin-like molecules which are immunoreactive with salmon calcitonin antibodies were detected in neurosecretory cells of the eyestalks (7), and their presence was also characterized by radioimmunoassay using the same antibodies in the haemolymph (8) of Palaemon serratus . Recently, high concentrations of this peptide were observed in the foregut and the hepatopancreas of Nephrops norvegicus (9). Yet, in this species, while calcitonin-like peptides with a molecular weight of 4500 Da were detected, large quantities of high molecular weight peptides were also characterized.

We report here the purification and amino-acid composition of a high molecular weight component of calcitonin in Nephrops norvegicus

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extracted from the haemolymph and the hepatopancreas. The different steps were followed by a radioimmunoassay using anti-salmon calcitonin antibodies.

MATERIAL and METHODS

Animals and sample preparation: Hepatopancreas and haemolymph were obtained from 10 kg of living Norway lobsters (Nephrops norvegicus) trawled in the vicinity of the Marine Station of Concarneau. Haemolymph serum (100 ml) was prepared after coagulation and centrifugation, lyophylised and submitted to a 5 fold concentration. Hepatopancreas were collected in liquid nitrogen, and kept frozen at - 80°C until used. The frozen glands (23 g) were homogenized in 10 volumes of 0.1 M acetic acid (Ac OH) in a Warring blendor at 4°C. The supernatant obtained after centrifugation (15,000 g, 20 mn) was lyophilized and the volume adjusted to 4 ml with 0.1 M NH_4 acetate, pH 8.5.

Radioimmunoassay: A radioimmunoassay for crustacean calcitonin, using a synthetic anti salmon calcitonin (sCT) sheep antiserum, kindly donated by Dr Moukhtar, has been previously described (8). This radioimmunoassay allowed the detection of 20 pg of salmon calcitonin. Serial dilutions of the crude extracts from the Norway lobster showed a relatively good parallelism between the standard curve of salmon calcitonin (9) and the calcitonin-like compound from this crustacean.

Purification: Preparative chromatography was performed on a Sephadex G 50 column : (Pharmacia Fine Chemicals). For the serum, 20 ml was placed twice on the column (2.5 x 100 cm) previously equilibrated with 10 mM NH4 acetate, pH 8.5. Four fractions of 10 ml were obtained called He1 - He2 - He3 - He4. Only the smaller molecular weight fractions He3 or He4 were further characterized using preparative gel electrophoresis. For the hepatopancreas, 4 ml of concentrated extract was placed on the G 50 F column and only smaller calcitonin-like peptides were further characterized on HPLC ion exchange chromatography (Mono Q Pharmacia).

Preparative electrophoresis was performed using slab gels in 10 % polyacrylamide (10). 2 gels (14 x 12 x 0.15 cm) were run in parallele (LKB unit system) after electrophoresis for 6 h at 40 mA. The gels were cut every 5 mm and extracted twice in distilled water overnight in a total volume of 50 ml. A narrow band of the gels was stained by Coomasie blue. Aliquots were taken for immunoreactivity measurement. The fractions showing calcitonin-like immunoreactivity were extensively dialysed (cut off 3000 Da) and lyophilised.

Mono Q HR 5/5 anion exchange column (Pharmacia) has been used for HPLC of calcitonin-like peptides of hepatopancreas (only fraction H_D3 was studied). The column was equilibrated in tris-HCl 10 mM, pH 7.5 and the sample applied in a 200 µl rheodyne (5 µg equivalent sCT per injection) : peptides were separated by a NaCl gradient (see Fig. 3). Aliquots of 10 ul were taken for radioimmunoassay.

Amino acid analysis: the dried fraction (1 ug) were taken up in 50 ul of 5.7 M HCl (Pierce) and hydrolysed in vacuo in sealed glass tubes for 24 h or 48 h at 110°C. Analysis was performed with a Biotronik LC 5000 analyser using BTC 270 resin with citrate/borate buffer as previously described (11).

Control of purity: The apparent molecular weight of purified calcitonin like peptide in the hepatopancreas or in the haemolymph was estimated after electrophoresis in 12 % polyacrylamide. Samples (20 ug) were boiled 2 min. before electrophoresis in mercaptoethanol, SDS and glycerol (12). Western blotting was carried out with a Biolyon apparatus using salmon calcitonin

antibody at a dilution 1/500 for 2 h at 25°C and peroxidase coupled second antibody (RASH-IgG H+L - PO NORDIC) at a dilution of 1/200, 1 h at 25°C.
Protein measurement: The protein content of each step of the purification was measured by the method of Lowry (13).

RESULTS

From 23 g hepatopancreas 2 mg equivalent sCT were extracted (Table I a). The immunoreactivity was lost by boiling and centrifugation.

Fractionation on a G-50 (Fine) column gave three fractions Hp1, Hp2, Hp3 corresponding respectively to 100 µg, 300 µg and 60 µg and to a total recovery of 23 per cent. The apparent molecular weight of the Hp3 fraction was estimated to range between 10 and 20 kDa (Fig. 1a). Hp3 was submitted to a further purification on a mono Q ion exchange column. Calcitonin-like immunoreactivity emerged mainly in 1 peak in the 0.1 M NaCl eluate (Fig. 2).

Table 1 Purification of calcitonin-like peptides from Nephrops norvegicus

A		µg sCT like	mg protein	Recovery (each step)	Specific act. % sCT like
EB		2000	4300	-	0.046
G 50	Hp 1	100			
	Hp 2	300	nd	23	-
	Hp 3	60			
Mono Q	Hp 3	20	0.8 *	33	2.5

B		µg sCT like	mg proteins	recovery (each step)	Specific act. % sCT like
EB		100	5000	-	0.002
G 50 F	He 1	6.4	2.3		0.28
	He 2	20.6	25.5	43	0.08
	He 3	6.8	2.75		0.24
	He 4	9.2	6.3		0.15
Prep. elec.	He 3	3	0.43 *	44	0.70
	He 4	2.1	nd	22	nd

A - Hepatopancreas 23 g
B - Haemolymph 100 ml

* Determined from A.A. composition
nd = not determined ; EB crude extract ; G 50 = filtration on Sephadex ; Mono Q = ion exchange HPLC ; Prep elec = preparative electrophoresis.

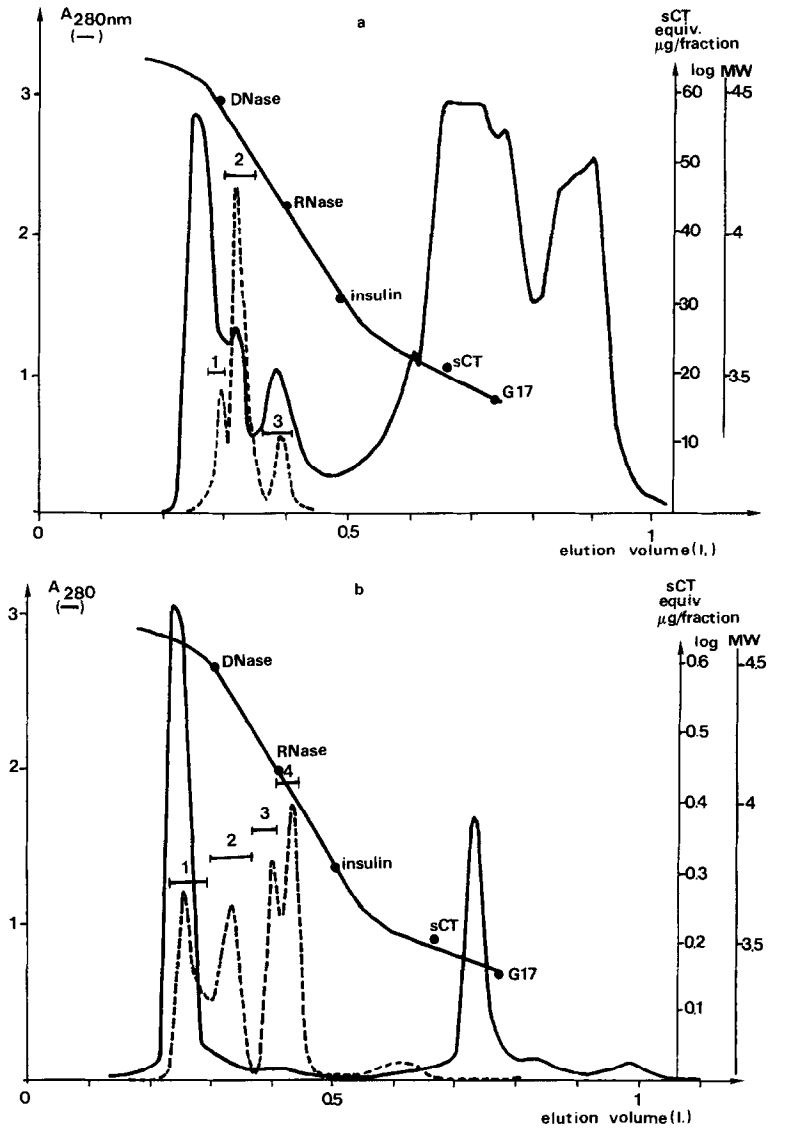


Fig. 1. Gel filtration of hepatopancreas calcitonin-like peptides (a) and haemolymph (b) in Nephrops norvegicus . Hepatopancreas was extracted in acetic-acid 0.1 M.

From 20 ml of concentrated haemolymph a total recovery of 43 per cent was found for immunoreactivity (Tabl. Ib). Four fractions were obtained, called He1, He2, He3, He4 (Fig. 1b) which eluted in the range of 10-15 kDa. Only the smaller weight fractions He3 and He4 with an apparent molecular weight 10-12 kDa comparable to hepatopancreas fraction were purified using preparative electrophoresis in 10 % polyacrylamide. In both cases, immunoreactivity emerged in one fraction which stained intensively with Coomasie blue (Fig. 3). After dialysis and lyophilization, recovery of immunoreactivity was about 44 % for He3 and 22 % for He4.

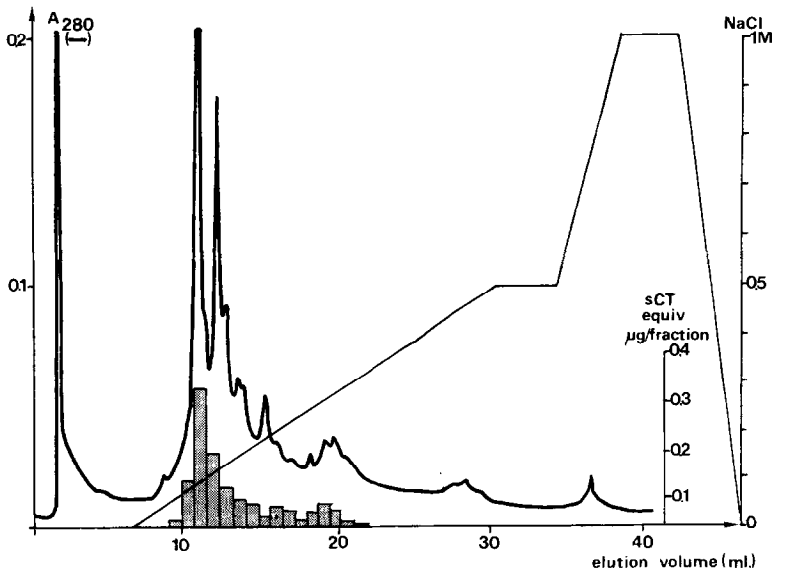
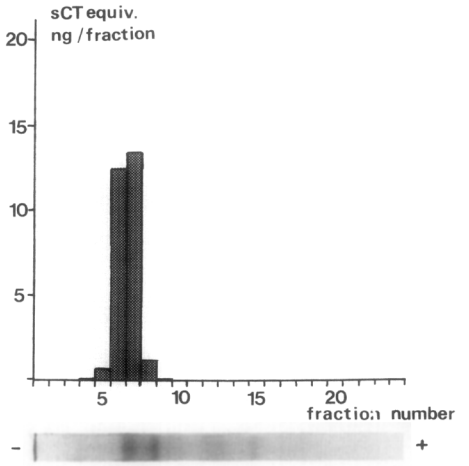


Fig. 2. Ion exchange chromatography on Mono R (Pharmacia) of hepatopancreas sCT fraction 3 from G 50 F.

In haemolymph extract (He 3) direct measurement of protein after preparative electrophoresis gave a value of 430 µg, while only 3 µg of salmon equivalent sCT could be found. The same apparent discrepancy was



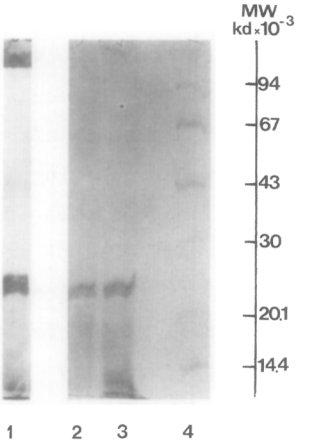


Fig. 3. Preparative electrophoresis of haemolymph sCT-like from Nephrops norvegicus . About 100 ng sCT equivalent were laid on the gel. Fractions of 5 mm were cut, extracted in 1 ml H_2O and aliquots of 50 to 200 ul were quantified by RIA.

Fig. 4. SDS characterization of a calcitonin-like peptide from Nephrops norvegicus .

1 : Hepatopancreas (fraction 3 G 50 F)
2 : Haemolymph, fraction 3 G 50 F
3 : Haemolymph, fraction 4 G 50 F
4 : Low molecular weight standards (Biorad).

Table 2 A.A. composition of high molecular weight calcitonin-like peptides in Nephrops nervegicus

	Hepatopancreas sCT like	Haemolymph sCT like	sCT
Asx	10.6	10.8	6.25
Thr	6	5.7	15.62
Ser	11	11.1	12.5
Glu	10.6	13.5	9.37
Pro	2.8	3.2	6.25
Gly	11.6	13.5	9.37
Ala	11.6	10.8	0.0
1/2 Cyst	2	tr	6.25
Val	7	5.9	3.12
Met	tr	1.3	0.0
Ile	3.8	3.5	0.0
Leu	4.3	4.5	15.62
Tyr	5.2	1.2	3.12
Phe	3.5	3.3	0.0
Try	nd	nd	0.0
His	4.1	6	3.12
Lys	3.6	3.2	6.25
Arg	2.65	2.70	3.12

Results were expressed as % residue per mole.
For the hepatopancreas average of 4 analysis was determined.
Value for Thr and Ser vere obtained by linear extrapolation to zero time. Ile, Val, Leu were determined after 72 h hydrolysis. Cyst was not oxyded and underestimed.

observed with hepatopancreas where 800 ug protein were measured against only 20 µg salmon equivalent CT. SDS-PAGE electrophoresis, carried out with the equivalent of 20 µg protein gave mainly 1 fraction with an apparent molecular weight of 22 kDa (Fig. 4). This fraction cross-reacted with anti-calcitonin after western blotting. Some contamination, with a compound having an apparent molecular weight of 44 kDa was also observed in some cases and corresponded to incomplete denaturation of the peptide. Mercaptoethanol did not affect the migration.

Amino acid composition showed some analogies with that of sCT (Tabl. II). Percentages of basic and acidic amino acids are comparable. Hydrophobicity was higher due to a large amount of alanine which was absent in sCT. The threonine content was lower in sCT.

DISCUSSION

The loss of immunoreactivity after boiling the crude extracts for 10 min or submitting them to organic solvant extraction and centrifugation confirms the existence of large peptide aggregates. In contrast, denaturation of the peptide with SDS and boiling for brief periods does not affect immunoreactivity after electrophoresis suggesting that secondary or tertiary structures of the immunoreactive molecule are not essential for the antigen to bind with the antibody. Electrophoresis in the presence of

SDS is generally considered as providing an accurate estimate of the true molecular weight. The apparent discrepancy between the molecular weight (around 10-20 kDa) of the Nephrops calcitonin-like peptide obtained using a G 50 F column and using SDS (about 22 kDa) is difficult to explain. Dimerisation or aggregation of smaller peptides should not be excluded on SDS gels but it is also possible that the calcitonin-like peptides exhibit an affinity for Sephadex gel due perhaps to the interaction of aromatic acid chains of the protein with dextran, which would reduce the apparent molecular weight. Using these G-50 SF column Fouchereau-Peron et al. (9) were able to isolate from the stomach of Nephrops a compound having a molecular weight of 4.5 kDa which may be the active form of the molecule . In this case, however, extractions were made in 0.1 N hydrochloric acid followed by lyophilisation. As a result some partial hydrolysis of the molecule, should not be excluded (14). This hypothesis has to be confirmed for Nephrops calcitonin.

Another possible explanation for the existence of such large peptides could be that it results from the incomplete processing of precursors prior to secretion in vivo . This is known to occur in vertebrates (15) especially in human patients with bronchial carcinoma (16, 17). Concerning the size of these precursors in vertebrates, some disagreement exists (18-19). Values of approximately 15 kDa (20), 21 kDa (21) or 65 kDa (22) have previously been reported. Using an in vitro translation system, Arlot-Bonnemains et al. (in prep) were able to detect in the oesophagus of Norway lobsters , specific peptides having a molecular weight of 24 kDa which are immunoprecipitated with salmon calcitonin antibody. Taking into account the parallelism of the dilution curves in the radioimmunoassay as well as in crude extracts (9) or in purified fraction (data not shown), about 1/50 for the hepatopancreas and 1/140 for the haemolymph calcitonin-like molecule were measured by the salmon radioimmunoassay compared to protein content determined by amino acid analysis, suggesting a great difference on the antigenic site between Norway lobster and salmon calcitonin.

Differences are also detected after amino acid analysis but it should be noticed that among the calcitonin studied a great diversity has already been reported not only by the specifity of the immunoreaction (23) but also by amino acid sequences comparison (24).

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