ISOLATION OF CALCITONIN FROM RAT THYROID MEDULLARY CARCINOMA

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1. Introduction

The calcitonins show great differences in amino acid sequence between species. In the eight sequences so far known (human, pig, cow, sheep, eel and salmons I, II and III) only nine of the 32 residues are conserved; those at positions 1,3,4,5,6,7,9,28 and 32. Surprisingly, however, the full sequence is required for biological activity [1]. This paper describes the isolation of calcitonin from the rat as the preliminary step to determining its sequence in order to further understand the relationship between structure and function in this hormone. Studies on the structure of rat calcitonin are described in the accompanying paper [2].

The concentration of calcitonin in normal mammalian thyroid and fish or avian ultimobranchial glands is so low that large quantities of tissue must be collected. Medullary carcinoma of the thyroid in mammals is however, a tumour of the calcitonin producing C-cells and the cancers are extremely rich in the hormone [3]. Our previous isolation of human calcitonin [4,5] was only made practical by the use of such tumours in place of normal tissue. The discovery of medullary carcinomata of the thyroid in rats which contained high concentrations of calcitonin [6] prompted us to attempt the isolation of the hormone from this species for comparison with other known varieties.

2. Experimental

2.1. *Isolation of rat calcitonin*

The production of second and third generation carcinoma explants in rats has been described elsewhere [6]. First, second and third generation tumour tissue was pooled for use in this study. Finely-divided freeze dried tumours (8.38 g) were twice extracted with butanol–acetic acid–water, (150 : 15 : 42, by vol.) using 15 ml per g tissue overnight at room temperature and finally for 4 h using 10 ml solvent per g tissue. The extracts were then combined and dried by rotary evaporation. The sticky, brown solid was partitioned between 0.1 M formic acid and diethyl ether (approx. 25 ml of each phase per g original dry tissue). The ether layer was discarded and the aqueous layer taken and dried by rotary evaporation to give a brown solid (1.61 g) termed the crude extract.

For further purification the crude extract was dissolved in 0.1 M formic acid (containing 10%, v/v, propan-2-ol and 8 M urea) and chromatographed on a Bio Gel P-6 column (95 × 2.5 cm) using 0.1 M formic acid–propan-2-ol (9:1, v/v) as eluent. Pure rat calcitonin was then isolated by column partition chromatography as described by Yamashiro [7] using the solvent pair butanol–pyridine–acetic acid–water (1000 : 96 : 16 : 1000, by vol) and Bio Gel P-10 (100–200 mesh) as the support. Samples were dissolved in 400 μl of the upper phase of the solvent mixture and applied to a column (0.9 × 41.5 cm) previously equilibrated with both phases and then eluted with the upper phase collecting 500 μl fractions.

2.2. *Analytical methods*

Progress of the isolation was followed by either bioassay [8] or a radioimmunoassay for human calcitonin [9]. Quantitative estimations of protein were made by the Folin-Lowry method [12].

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Thin-layer chromatography was performed on cellulose plates (Merck, Darmstadt, GFR) using the solvent butanol–pyridine–acetic acid–water, (42 : 24 : 4 : 30 by vol). Peptide spots were located with ninhydrin, by a modified Reindel-Hoppe method [10] or with fluorescamine [11].

Amino acid analyses were performed on a JEOL 6AH instrument (JEOL Ltd., Tokyo, Japan) using a 10 mm path-length flow-cell to increase sensitivity. Samples were hydrolysed with 6 M HCl in the presence of 10 μg phenol under nitrogen in sealed tubes at 110°C for 20 h.

Dansylation, identification of dansyl amino acids and dansyl-Edman degradation were carried out as described by Hartley [13].

Enzyme digests were carried out using an estimated 5 μg hormone and 0.1 μg enzyme; for thermolysin 20 μl 0.2 M pyridine acetate pH 7.1, 16 h, 37°C, for trypsin 20 μl 0.5 M ammonium bicarbonate, 60 min, 37°C.

3. Results and discussion

Gel filtration of the crude extract of rat thyroid medullary carcinoma (fig.1) demonstrated two peaks of biological activity. They corresponded in molecular size to the monomeric and dimeric calcitonins found in extracts of human thyroid medullary carcinoma [4] and were therefore assumed to be monomeric and dimeric rat calcitonins. The dimer could be located only when samples were first allowed to stand for several hours in the assay buffer before bioassay in rats. This conversion of an inactive dimer to active

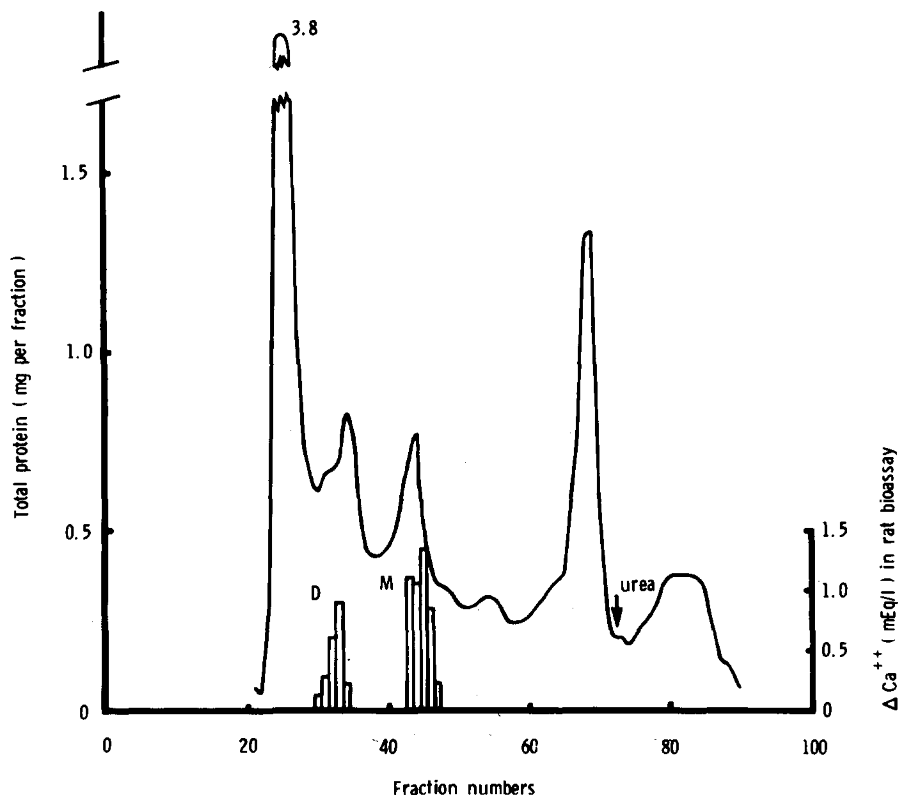

Fig.1. Gel filtration of crude extract of rat thyroid medullary carcinoma. The sample (158 mg containing 43 mg protein) was added to 3.0 g urea, 0.6 ml propan-2-ol and 1 μl mercaptoethanol, made up to 6.0 ml total volume with 0.1 M formic acid and chromatographed on a Bio Gel P-6 (200–400 mesh) (95 × 2.5 cm) column using 0.1 M formic acid–propan-2-ol, (9:1, v/v) as eluent. Fractions of 6.0 ml were collected. The continuous line shows the protein content per fraction and the histogram shows the two biologically active regions; D = dimeric, M = monomeric rat calcitonins.

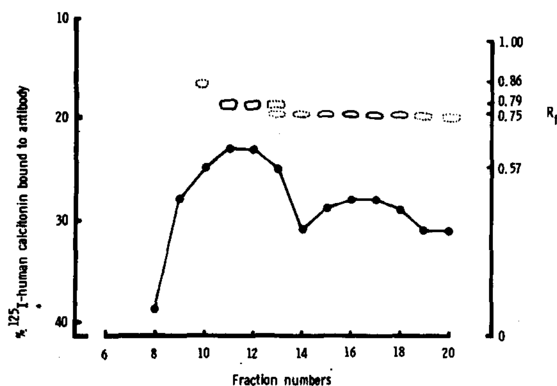

Fig.2. Partition chromatography of monomeric rat calcitonin purified by gel filtration. Two peaks of calcitonin were detected by the radioimmunoassay for human calcitonin (solid line). Superimposed on this graph is a t.l.c. analysis of a sample from each fraction. Selected R_f values are shown in the right-hand axis; on the same plate human calcitonin ran at R_f 0.75 and bovine insulin at R_f 0.57. The two peaks of immunological activity coincide with pure peptides.

Table 1
Amino acid composition of rat calcitonin

Amino acid	Residues per mole peptide (average of 3 values)	Integer value (human)
Lys	0.9	1 (1)
His	1.1	1 (1)
Arg	0	0 (0)
Asx	2.6	3 (3)
Thr	4.0	4 (5)
Ser	2.0	2 (1)
Glx	2.4	2 (2)
Pro	2.4	2 (2)
Gly	3.9	4 (4)
Ala	1.6	2 (2)
½ $(Cys)_2$	n.d.	2[a] (2)
Val	1.1	1 (1)
Met	0.8	1 (1)
Ile	1.0	1 (1)
Leu	2.9	3 (2)
Tyr	0.8	1 (1)
Phe	2.0	2 (3)

[a] N-terminal half-cystine shown by dansylation and the second residue assumed by analogy with all other calcitonins.

n.d. = not determined.

monomer is also observed with human calcitonin dimer [14] and dogfish calcitonin dimer [15] and is presumed to represent reduction by the free thiol group of bovine serum albumin present in the assay buffer. Calcitonin dimers are formed by rearrangement of the intrachenic disulphide bonds to interchain links [20].

After reduction with 4 M mercaptoethanol of all dimeric hormone to the monomeric form and its subsequent rechromatography, all monomeric rat calcitonin was pooled and fractionated by partition chromatography (fig.2). The fractions from the column were analysed by radioimmunoassay and t.l.c. but protein estimations were not made in order to conserve material. It is evident that two immunoreactive peptides were eluted from this column both of which appeared homogenous on t.l.c. In further examination both peptides gave the same amino acid composition after acid hydrolysis and the same tryptic and thermolytic peptide patterns on one-dimensional t.l.c. It is likely, therefore, that they both have the same sequence but the modification responsible for the difference in polarity remains unknown.

The amino acid composition of rat calcitonin is given in table 1. Except for cystine, which was not determined, the residue numbers are very similar to those for human calcitonin. The provisional values reported by Burford et al. [16] are also similar to our present values. However, the inability of these workers to assign whole residue numbers for some amino acids must reflect a degree of impurity in their preparation.

Following oxidation with performic acid, dansylation revealed cysteic acid as the amino terminal residue. This is characteristic of all other known calcitonins.

The biological potency of rat calcitonin was not determined rigourously but estimated to be of the order of a few hundred MRC units per mg in common with other mammalian species; Burford et al. reported 400 MRC U/mg [16]. The biological activity was destroyed on mild oxidation with hydrogen peroxide as is the case with human calcitonin where the methionine residue at position 8 is oxidised to its sulphoxide. Pig and salmon calcitonin are not affected in this way.

Several publications have reported the cross-reaction of rat calcitonin in radioimmunoassays for human calcitonin [16,17,18]. We confirm this and

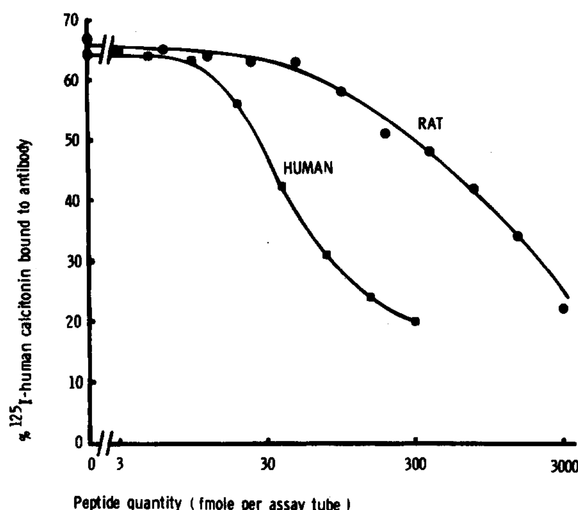

Fig.3. Comparison on a molar basis of the reaction of rat (•) and human (▪) calcitonins with an anti-human calcitonin antiserum (336/6) at a final dilution of 1:24 000) using ^{125}I-human calcitonin as tracer. Human calcitonin was the MRC synthetic standard 70/50 and rat calcitonin was quantitated by amino acid analysis.

have been able to use the human calcitonin radioimmunoassay to follow the progress of isolation of the rat hormone. A direct comparison on a molar basis for the reaction of pure rat and human calcitonins with antiserum 336/6 at a final dilution of 1 : 24 000 is shown in fig.3. Rat calcitonin appears to reach the same maximum displacement of ^{125}I-human calcitonin from antibody but only when present in approx. 10-fold higher molar concentration. This degree of cross-reaction clearly demonstrates a large degree of sequence homology between rat and human calcitonins, at least in the region of the binding site which is at the C-terminus for this particular antiserum [19].

This work shows the importance of medullary carcinoma of the thyroid as a general source tissue in the isolation of calcitonins. The concern that peptides from tumours may not have the natural sequence is to some extent supported by the fact that rat calcitonin and human calcitonin (both isolated from tumours) are similar to each other but dissimilar to all other known calcitonins. However the provisional amino acid composition reported by Burford et al. [16] for rat calcitonin from normal thyroid tissue is broadly in agreement with our results for tumour-derived rat calcitonin. It seems likely, therefore, that 'normal' and 'tumour' rat calcitonin are identical.

Acknowledgements

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