ISOLATION, AMINO ACID COMPOSITION AND TERMINAL AMINO ACID RESIDUES OF THE VASOACTIVE OCTACOSAPEPTIDE FROM CHICKEN INTESTINE. PARTIAL PURIFICATION OF CHICKEN SECRETIN

Ann NILSSON

Medicinska Nobelinstitutet, Biokemiska Avdelningen, Karolinska Institutet, Stockholm, Sweden

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1. Introduction

Recently Said and Mutt showed that the porcine upper intestinal wall contains a vasoactive octacosapeptide [1], and the amino acid sequence of this peptide was subsequently determined [2]. The only secretin that has hitherto been isolated in pure form is prepared from the same source [3].

In this paper the isolation of the chicken vasoactive intestinal octacosapeptide and the partial purification of chicken secretin are described.

The vasoactive peptide has the composition Ala_2 Asx_4 Arg_2 Glx_1 His_1 Leu_2 Lys_3 Met_1 Phe_2 Ser_3 Thr_2 Tyr_2 Val_3. The corresponding porcine peptide differs from this by having Asx_5 Ile_1 Leu_3 Phe_1 Ser_2 Val_2. The chicken vasoactive peptide has an N-terminal histidine residue like the porcine vasoactive peptide, secretin and glucagon. A residue of threonine amide constitutes its C-terminus as compared to asparagine amide in the porcine peptide.

On comparing the biological activities of the porcine and chicken vasoactive intestinal peptides, S. I. Said (unpublished) has found both qualitative and quantitative differences. For example, as compared with the porcine variant, the chicken peptide is a relatively more potent relaxant of guinea pig tracheal than of rat gastric smooth muscle. Also the hypotensive effect, in dogs, of the chicken peptide is less marked and shorter lasting than that of the porcine peptide.

2. Materials and methods

2.1. *Materials*

Alginic acid, Sephadex G-25 (fine) and carboxymethyl cellulose were obtained from the same commercial sources and pretreated as described by Said and Mutt [4]. Porcine secretin and vasoactive intestinal octacosapeptide were the natural hormones prepared as described previously [3,4] in this laboratory. Hydrochloric acid was of Aristar quality from BDH. Tosyl–lysyl–chloromethyl ketone treated chymotrypsin was from E. Merck (Darmstadt). L-aspartic acid diamide HCl, hemihydrate was obtained from Cyclo Chemical and L-threonine amide from Fox Chemical Company Los Angeles California.

2.2. *Bioassays*

The criteria for increase in purity of the material during the various purification steps were a) the increase in secretin-like activity determined by the method of Mutt and Söderberg [5], b) vasoactivity, determined by measuring femoral arterial blood flow and aortic blood pressure as described previously [1] and c) activity on several isolated smooth muscle organs. b) and c) were tested by Professor S. I. Said, University of Texas, Dallas.

2.3. *Quantitative amino acid analyses*

Acid catalysed hydrolyses of the peptide were carried out for 22 hr at 109°C in an atmosphere or

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argon using 6 M HCl [6], containing 0.5% mercaptoethanol as recommended by Potts et al. [7]. Qualitative amino acid analyses were carried out either by two-dimensional paper chromatography by the method of Redfield [8] (except for the use of Whatman 42 paper instead of Schleicher & Schull No. 507), or by cellulose thin-layer chromatography according to Jones & Heathcote [9]. Quantitative amino acid chromatography was carried out according to Spackman, Moore and Stein [10].

2.4. *Columns for chromatography*

A column of Sephadex G-25 (fine) with the dimensions 4 X 90 cm and carboxymethyl–cellulose column with the dimensions 0.9 X 16 cm were prepared and stored under 0.2 M acetic acid containing 3‰ tricresol R and 0.1 M NaOH respectively until required for chromatography.

2.5. *Crude polypeptide concentrate*

The chicken pancreas is composed of three lobes that occupy the space between the limbs of the duodenum. Three secretory ducts pass to the distal end of the duodenal loop and open on a common papilla with the bile duct. From broilers of the variety Hybro ranging from 1000 to 1100 g, the duodenum and the uppermost part of jejunum, together about 15 cm, were removed as soon as possible after killing the chickens. The intestines were freed of pancreatic tissue, rinsed with water and immersed for 10 min in boiling water. After mincing, the material was stored at −20°C. It was treated as for the preparation of porcine secretin according to Mutt [11] until the second treatment with alginic acid. The polypeptide material salted out from the acid eluate was collected on a suction filter and was the starting material for the purifications described in the present paper. From 100 kg intestines, from about 20 000 animals, approx. 40 g peptide concentrate (wet weight) was obtained.

3. Results

3.1. *Chromatography on the Sephadex G-25 (fine) column*

The column was washed with 0.2 M acetic acid until free of tricresol. A solution of 4 g starting material in 40 ml 0.2 M acetic acid was filtered through a Millipore filter of 0.45 μ porosity and allowed to sink into the column.

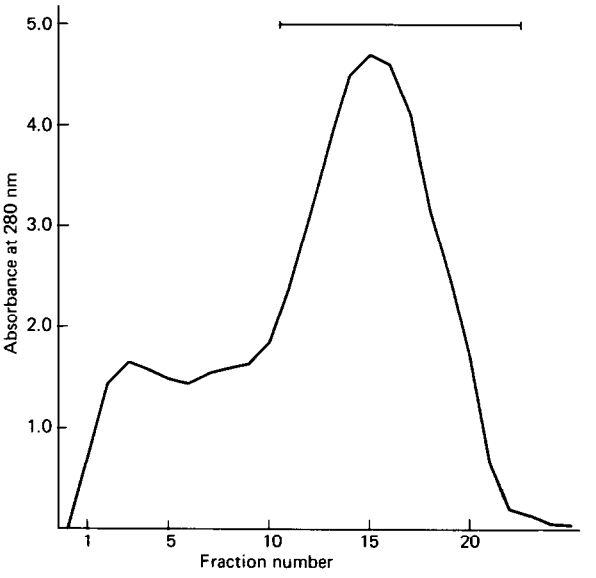

Fig. 1. Chromatography on Sephadex G-25 (fine) of 4 g starting material. Eluant: 0.2 M acetic acid. Column: 4 X 90 cm. Flow rate: 5 ml/min. Fraction volume: 10 ml. Fractions 11–22, indicated by the bar, were combined for further processing.

Elution was carried out with 0.2 M acetic acid with a flow rate of 5 ml/min. Fractions of 10 ml each were collected, starting with the introduction of the polypeptide solution into the column.

Fig. 1 shows the absorbance at 280 nm. Fractions 11–22 were combined and saturated with sodium chloride. The salted out material weighed 1.1 g. After reprecipitation at pH 4.0 it weighed 0.93 g.

3.2. *Extraction with methanol*

Salted out material, prepared as in the previous section, weighing 3.8 g was extracted with methanol, an inert fraction removed by neutralization of the extract and the active material precipitated, after acidification with HCl, with ether and dissolved in water. The solution was saturated with sodium chloride. The salted out material weighed 101 mg. The technique of the extraction into methanol was that described previously for the preparation of porcine secretin [11]. The methanol-insoluble fraction

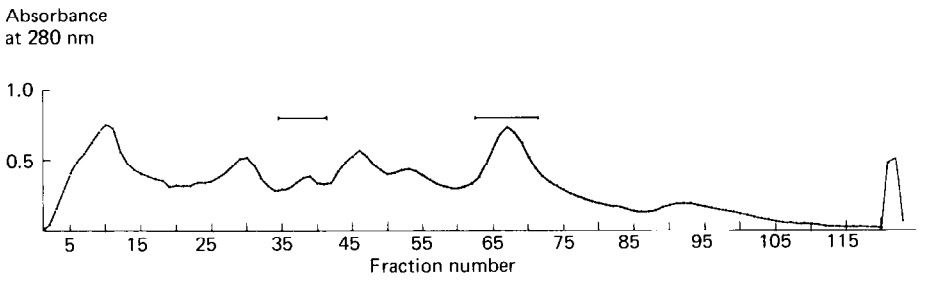

Fig. 2. Chromatography on CM-cellulose of 100 mg of the methanol soluble fraction of the peptide material from fractions 11–22 of fig. 1. Prior to chromatography the material was salted out from aqueous solution with sodium chloride. Gradient from 75 ml 0.0225 M sodium–phosphate buffer of pH 6.4 to 75 ml of the buffer 0.3 M in NaCl. Column: 0.9 × 16 cm. Flow rate: 1.5 ml/2.5 min. Fraction volume: 1.5 ml. Fraction 33–39 and 62–69 indicated by the bars were combined respectively for further processing.

weighed 1.6 g and contained cholecystokininpancreozymin activity.

3.3. *Chromatography on carboxymethyl-cellulose*

The CMC-column was equilibrated with 0.0225 M sodium–phosphate buffer of pH 6.4. 100 mg of the methanol extracted material was dissolved in 8 ml of the buffer and adjusted to pH 6.4 with 0.03 M NaOH. The solution was allowed to sink into the column and elution was carried out in a linear gradient from 75 ml of the starting buffer to 75 ml of this buffer made 0.3 M in NaCl. Fractions of 1.5 ml/2.5 min were collected. Fig. 2 shows the absorbance at 280 nm.

When tested in the cat it was found that the ability to stimulate pancreatic secretion was confined mainly to two areas of the chromatogram, fractions 33–39 and fractions 62–69. The latter showed also a strong vasoactivity when tested in the dog. Fractions 33–39 were combined and desalted on a Sephadex G-25 (coarse) column (1.5 × 90 cm) in 0.2 M acetic acid. After lyophilization the material weighed 4 mg. Fractions 62–69 when treated in the same manner yielded 7 mg. The pancreas stimulating i.e. secretin-like material prepared in this way contained approx. 50 clinical units per mg.

Attempts to purify it further by counter-current distribution under the conditions elaborated for porcine secretin [3] were inconclusive.

The pancreas stimulating and vasoactive material (from fractions 62–69) was subjected to counter-current distribution.

3.4. *Counter-current distribution*

60 mg of the material obtained as described in the preceding section was subjected to counter-current distribution. The apparatus (from H. O. Post, Inc., Middle Village, New York, USA) and the use of an inert nitrogen atmosphere were the same as used previously for secretin and the porcine vasoactive peptide [3,4]. The phase system was *n*-butanol-0.1 M NH_4HCO_3. After completion of the run, the two phases were made to coalesce by the addition of 3 ml per tube of ethanol. The absorbance of the homogeneous tube contents was determined at 215 nm and 1-cm light path. The values obtained are given in fig. 3. The contents of tubes 65–100 were combined and dissolved in 20 vol of water. The pH of the solution was brought to 2.5–2.7 with HCl and the polypeptides were recovered from solution by adsorption to alginic acid, elution with 0.2 M HCl and exchange of chloride for acetate, followed by lyophilization, as described for secretin [3]. The lyophilized material weighed 18.5 mg.

3.5. *Analytical results*

The material obtained by counter-current distribution showed only one band on polyacrylamide gel electrophoresis according to a modification [12] of the method of Johns [13]. Analysis for N-terminal amino acids by the phenylisothiocyanate method of Edman [14] revealed only one such acid, histidine, like in the porcine vasoactive peptide.

This suggested that no further purification would be necessary for the determination of the structure of the polypeptide.

Aliquots of it were hydrolyzed with 6 M HCl and the hydrolysates subjected in quatitative amino acid

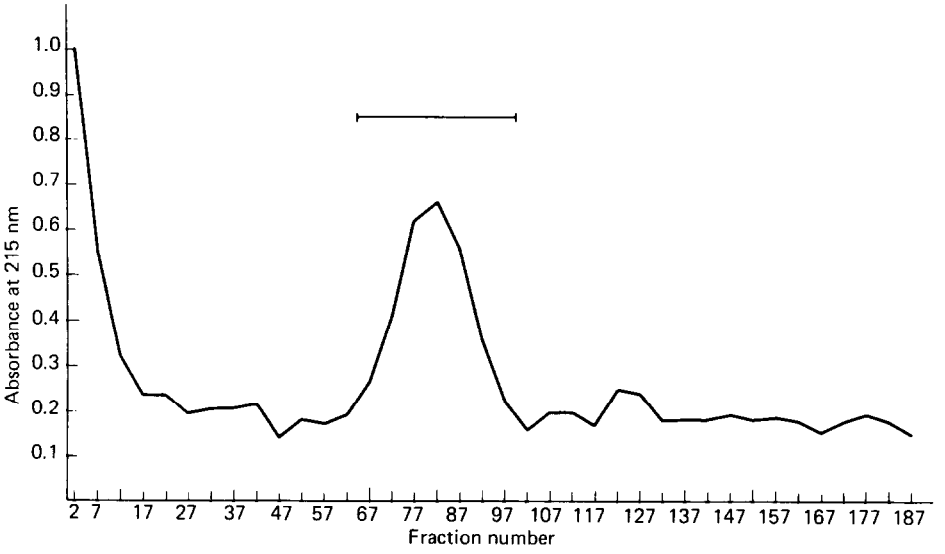

Fig. 3. Counter-current distribution of 60 mg of the material from fraction 62–69 of fig. 2. Distribution through 200 transfers during 70 hr in a nitrogen atmosphere. Solvent system: *n*-butanol - 0.1 M NH_4HCO_3. Phase volume: 10 ml. Light absorbance measured on combined phases obtained by the addition of 3 ml ethanol to each tube. Fractions 65 to 100, indicated by the bar, contained the vasoactive intestinal polypeptide in highly purified form and were combined for further processing.

Table 1
Amino acid composition of hydrolyzed chicken vasoactive intestinal peptide

Amino acid	Amount in peptide sample 240	19	Probable numer of residues in peptide
	μg	μg	
	nmol		
Alanine	110	8.9	2
Arginine	112	11.5	2
Aspartic acid	217	16.9	4
Glutamic acid	61	5.1	1
Glycine	7	0.6	0
Histidine	53	5.5	1
Isoleucine	0	0	0
Leucine	111	9.9	2
Lysine	155	16.7	3
Methionine	50	present	1
Phenylalanine	102	10.3	2
Serine	168	10.8	3
Threonine	110	8.7	2
Tyrosine	102	10.1	2
Valine	142	13.7	3
			Total 28

Hydrolysis at 109°C for 22 hr with 1 μl/μg of 6 M HCl containing 0.5 ‰ mercaptoethanol [7].

analysis by the method of Spackman et al. [10]. The results, shown in table 1, indicate that the amino acid residue composition of the vasoactive intestinal polypeptide is $Ala_2\ Asx_4\ Arg_2\ Glx_1\ His_1\ Leu_2\ Lys_3\ Met_1\ Phe_2\ Ser_3\ Thr_2\ Tyr_2\ Val_3$.

Tryptophan and cystine/cysteine were absent as shown by the Voisnet–Rhode dimethylaminobenzaldehyde reaction [15] and by analysis of material oxidized with performic acid according to Moore [16], respectively.

In order to determine the C-terminal amino acid, chymotrypsin digestion was conduction for 4 hr at 21°C in 1% NH_4HCO_3 using 0.04 mg/ml enzyme and 2 mg/ml peptide. Half the quantity of the enzyme was added to the substrate at the beginning of the degradation, and the other half after 2 hr. In parallel with this the porcine vasoactive octacosapeptide was treated in exactly the same way. The digests were lyophilized, redissolved in half the original volume of water and heated for 6 min at 100°C. They were then lyophilized in 0.2 M acetic acid and submitted to high-voltage paper electrophoresis at pH 6.4. It was found that whereas, as expected, asparagine amide could be clearly identified among the chymotryptic degradation products of the porcine polypeptide, no trace of this could be seen among the fragments from

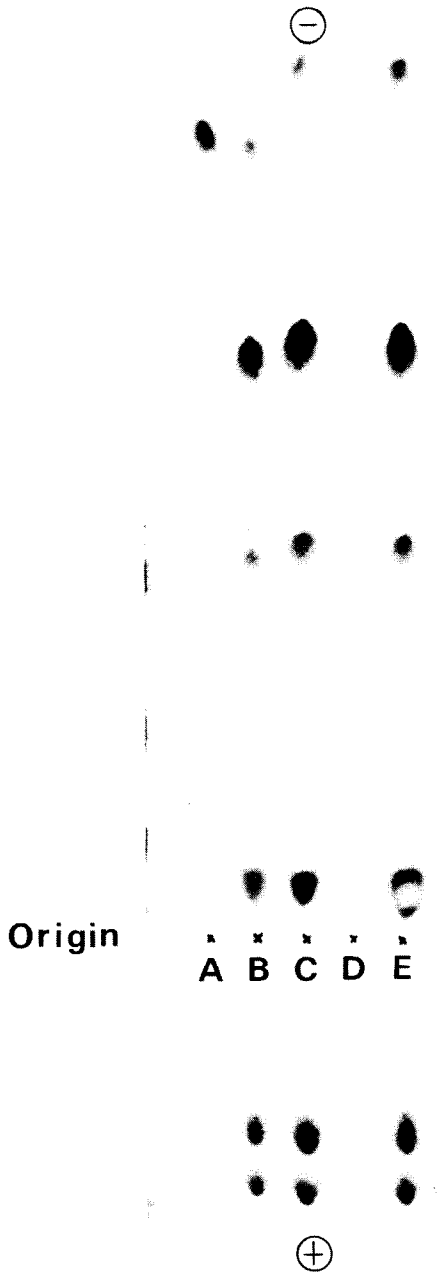

Fig. 4. Paper electrophoresis of chymotrypsin digested porcine and chicken vasoactive intestinal peptides, together with L-aspartic acid di-amide and L-threonine amide. A) 30 nmol L-aspartic acid di-amide. B) 80 μg chymotrypsin-digested porcine vasoactive peptide. C) 80 μg chymotrypsin-digested chicken vasoactive peptide. D) 30 nmol L-threonine amide. E) 80 μg chymotrypsin-digested chicken vasoactive peptide together with 30 nmol threonine amide. Electrophoresis was performed for 90 min at 50 V/cm in pyridine–acetic acid–water (300:11.5:2700, by vol) at pH 6.4 using Whatman 3 MM paper. Staining with the cadmium-ninhydrin reagent of Barrolier et al. [3]. The threonine amide in lead D gave an intense canary yellow colour with the ninhydrin reagent; this was indistinguishable in tone from that given by the material that had migrated with the same velocity in leads C and E.

the chicken material. Instead there was a fragment with higher cathodic mobility and giving a canary-yellow colour with the cadmium nin-hydrin reagent of Barrollier et al. [6]. This yellow colour was indicative of either a glycyl or a threonyl peptide.

Since the amino acid analysis had shown that the chicken, like the porcine, material lacked glycine, the chymotryptic degradation products of the chicken polypeptide were run in parallel, and mixed, with threonine amide. The results, shown in fig. 4, confirmed that the C-terminal amino acid in chicken vasoactive octacosapeptide is threonine amide.

4. Discussion

The vasoactive intestinal octacosapeptide is the first intestinal hormone to be isolated from the chicken and indeed from any species other than the hog.

Gastrin has been isolated in chemically pure form from several mammalian, but not from non-mammalian species [17]. Secretin, too, has been isolated from the hog only. Pancreatic glucagon on the other hand has been isolated not only from several mammalian species but also from the turkey [18] and the duck [19]. While no difference has been found in the amino acid sequences among the hitherto isolated mammalian glucagons, the avian hormones differ from the common mammalian variant by the replacement of one amino acid residue, asparagine – 28 by serine, (turkey), or by two residues, asparagine – 28 by serine, and serine – 16 by threonine (duck).

Like in the porcine vasoactive peptide, secretin and glucagon, the N-terminal amino acid of the chicken vasoactive octacosapeptide is histidine. The C-terminus is threonine amide.

The isolation of the chicken vasoactive intestinal octacosapeptide did not present any particular difficulties. This was, however, not true of chicken secretin. When submitted to counter-current distribution under conditions which lead to purification of porcine secretin the material obtained from the carboxymethylcellulose column (which contained approx. 50 clinical units per mg) was not further purified to any significant degree.

Dockray [20] found the porcine vasoactive peptide to be a strong, and porcine secretin a very weak stimulant of pancreatic flow in the turkey. This indicates that the porcine vasoactive peptide might resemble avian, more closely than porcine secretin.

Differences between the biological activities of the chicken and porcine vasoactive intestinal octacosapeptides have already been mentioned in the Discussion.

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