A proform of secretin with high secretin-like bioactivity

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Received 9 April 1985

A variant form of the heptacosapeptide amide secretin, with C-terminal -Val-Gly-Lys-Arg instead of valine amide, has been isolated from porcine upper intestinal tissue. Unexpectedly, this triacontapeptide exhibited a substantially higher bioactivity than the heptacosapeptide amide.

Secretin *Secretin precursor* *Amino acid sequence* *Secretin bioactivity* *Porcine intestinal extract*

1. INTRODUCTION

Secretin is a hormone stimulating pancreatic secretion [1]. It was first isolated from the upper part of porcine intestine [2] and subsequently also from chicken, bovine and human intestine [3–5]. In all these cases it was found to be a polypeptide of 27 amino acid residues. The porcine and bovine secretins are identical. They differ from the human form in two positions and the chicken form in 13 positions. Bioactive and immunoreactive secretin material has also been found in brain extracts [6,7].

The C-terminal amino acid residue of secretin (valine in porcine/bovine/human, methionine in chicken) is, as in many other hormonally active peptides, amidated. However, a variant form of secretin which is not amidated at its C-terminal residue but instead has a C-terminal residue of glycine has been described [8]. This extended form of secretin is biologically active in a bioassay for secretin, but its potency has not been determined. It is known that in the precursors of amidated peptides, the amino acid that becomes amidated in the mature hormone is always followed by a glycine residue and it is the amino group of this glycine residue that gives rise to the C-terminal amide group in the enzymatic amidation reaction [9]. The glycine is, in turn, usually followed by a pair of basic amino acid residues.

We now report on the isolation of a secretin variant, 30 amino acid residues long, which is a secretin molecule extended with a glycine followed by a lysine and an arginine at its C-terminus. This secretin variant is at least as potent as secretin in stimulating the secretion of bicarbonate by the pancreas in the anesthetized cat [10].

2. MATERIALS AND METHODS

2.1. *Preparation of the starting material*

A fraction, earlier described as the starting material for the purification of VIP [11], was further purified by two CM-cellulose chromatographies. The first one was carried out in a 0.02 M phosphate buffer at pH 6.4 with a salt gradient of 0–0.3 M NaCl. The fraction, eluting at about 0.15 M NaCl, was further chromatographed on a second CM-cellulose column, eluted by stepwise increase of NH_4HCO_3 concentration (0.02, 0.04, 0.2 M). The fraction which is here referred to as the starting material for the purification of the secretin variant was eluted with 0.04 M NH_4HCO_3. Details concerning the two CM-cellulose chromatographies will be described elsewhere.

2.2. *High-performance liquid chromatography (HPLC)*

Reverse-phase HPLC was carried out on a Waters μBondapak C_{18} column (7.8 × 300 mm,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
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10 μm particle size) or an LKB Ultropac TSK ODS-120T column (4.6 × 250 mm, 5 μm particle size) with 0.12% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Ion-exchange chromatography was performed on an LKB Ultropac TSK 535 CM-column (7.5 × 150 mm) with 0.02 M sodium phosphate buffer at pH 6.4 and a gradient of NaCl (0–0.3 M). Both types of separations were carried out on an instrument from Waters Associates.

2.3. *Bioassay*

The biological activity was measured by the ability of the material to stimulate the secretion of alkali in the pancreatic juice of anesthetized cat [10]. The sample was compared to a secretin standard (3500 clinical units per mg).

2.4. *Proteolytic fragmentation*

The peptide was digested with TPCK-treated trypsin (Worthington) for 6 h or TLCK-treated chymotrypsin (Merck) for 4 h in 1% NH_4HCO_3 at room temperature. The ratio of peptide to enzyme was 50:1 by wt. The digests were separated by reverse-phase HPLC on a μBondapak C_{18} column as described above. The gradients used were, for tryptic fragments, 0–40% solvent B for 40 min at a flow rate of 1 ml/min and, for chymotryptic fragments, 10–25% solvent B for 30 min at 1 ml/min.

Table 1

Preparation of starting material

The upper part of porcine intestine was boiled, frozen and minced.
Extraction with 0.5 M acetic acid. Peptides adsorbed on alginic acid. Elution with 0.2 M HCl and precipitation with NaCl.
Gel filtration on Sephadex G-25 (fine) in 0.2 M acetic acid.
Extraction with methanol. Soluble material precipitated with ether.
Gel filtration on Sephadex G-25 (fine) in 0.2 M acetic acid.
Ion-exchange chromatography on CM-cellulose. Elution with 12.5 mM phosphate buffer, pH 8.
Ion-exchange chromatography on CM-cellulose in 0.02 M sodium phosphate buffer, pH 6.4. Elution with a gradient of NaCl (0–0.3 M).
Ion-exchange chromatography on CM-cellulose with a stepwise increase of NH_4HCO_3 concentration (0.02, 0.04, 0.2 M). The 'starting material' was eluted with 0.04 M NH_4HCO_3.

2.5. *Amino acid composition analysis*

Hydrolysis was carried out at 110°C for 24 h in evacuated tubes with 6 M HCl containing 0.1% β-mercaptoethanol. The tryptic digest hydrolysates were analyzed in a Waters amino acid analysis system using precolumn derivatization with *o*-phthalaldehyde [12]. All other hydrolysates were analyzed in a Beckman 121 amino acid analyzer.

2.6. *Identification of free arginine*

The tryptic digest was treated with PITC [13] to form the phenylthiocarbamyl derivatives of the

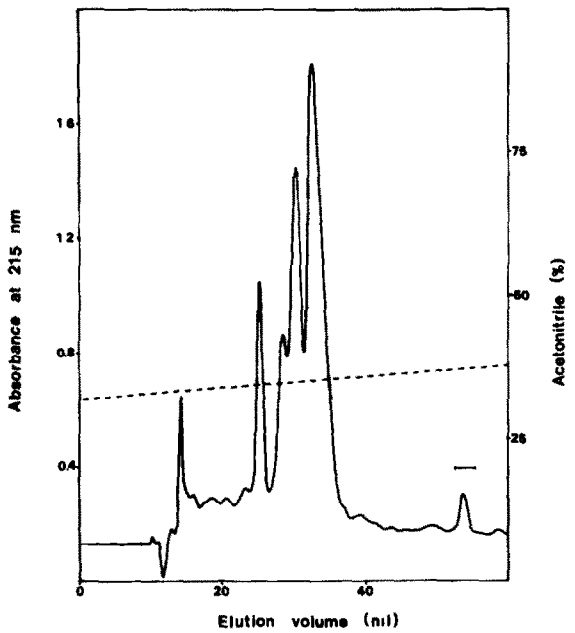

Fig.1. Purification of the starting material by reverse-phase HPLC. Sample: about 1.8 mg/run of the fraction defined as the starting material in section 2. Column: Waters μBondapak C_{18} (7.8 × 300 mm, 10 μm particle size). Mobile phases: A, 0.12% trifluoroacetic acid (TFA)/water; B, 0.1% TFA/acetonitrile. Flow rate: 2 ml/min. Gradient: 32–38% B, 30 min, indicated by the dotted line. The secretin bioactivity is indicated by the bar.

fragments. These were separated by HPLC on a Spherisorb ODS 2 column (4.6 × 100 mm, 3 μm particle size) using a system described in [14] but at pH 6.0. In this system, free amino acids were identified as PTC-derivatives.

3. RESULTS AND DISCUSSION

During the purification of variant forms of VIP [15] a certain fraction was observed to have a secretin- rather than a VIP-like bioactivity. Since this active fraction originated from material eluting later than secretin on a CM-cellulose chromatography, the activity could be assumed to be due to a peptide with a more alkaline isoelectric point than that of secretin, rather than to secretin itself. It was therefore of interest to isolate this material, and to characterize it.

A schematic presentation of how the starting material was obtained is shown in table 1. The final purification of the secretin variant from the starting material was obtained by 3 HPLC steps. The first step was a reverse-phase chromatography on a μBondapak C_{18} column using a linear gradient, described in the legend to fig.1. The active fraction was further subjected to an ion-exchange step on a TSK-CM column eluted with a gradient of 0.12–0.3 M NaCl in 0.02 M phosphate buffer, pH 6.4, for 15 min and isocratic elution at 0.3 M NaCl in the same buffer for another 15 min. The flow rate was 1 ml/min. Finally the material was desalted by reverse-phase HPLC in the system described in the legend to fig.2. This step also gave some purification (fig.2) and the isolated peptide was considered pure after this step. Active fractions were identified with the bioassay for secretin (described in section 2) throughout the purification procedure.

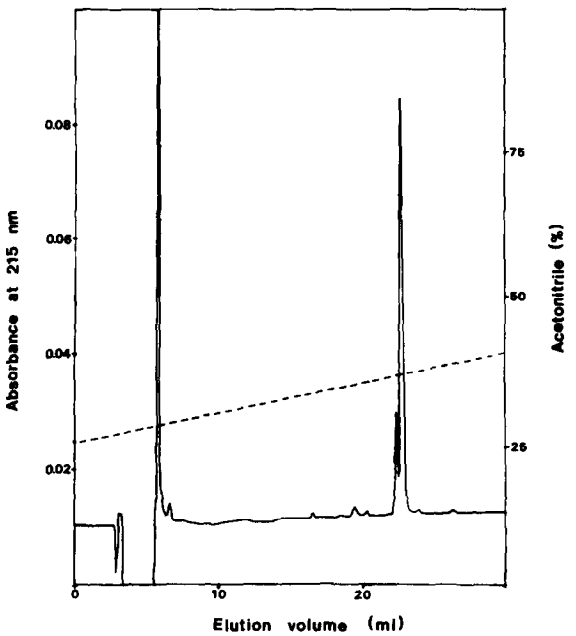

Fig.2. Final purification of secretin-Gly-Lys-Arg by reverse-phase HPLC. Sample: 65 μg of the bioactive fraction from the CM-HPLC step. Column: LKB Ultropact TSK ODS-120T (4.6 × 250 mm, 5 μm particle size). A and B as in legend to fig.1. Flow rate: 1 ml/min. Gradient: 25–40% B, indicated by the dotted line. The larger peak corresponds to the secretin variant.

The amino acid composition of the intact peptide is shown in table 2. Comparison of this amino acid composition to that of secretin indicates that the isolated peptide differs from porcine secretin in containing a Lys residue and one additional residue each of Arg and Gly. To ascertain whether the purified peptide is a variant form of secretin, tryptic digestion was carried out and the fragments obtained were separated by reverse-phase HPLC.

Table 2

Amino acid composition of the secretin variant

Amino acid	Secretin variant	Secretin
Lys	1.1 (1)	–
His	1.3 (1)	1
Arg	4.9 (5)	4
Asx	2.1 (2)	2
Thr	1.8 (2)	2
Ser	3.3 (4)	4
Glx	3.0 (3)	3
Gly	3.0 (3)	2
Ala	1.2 (1)	1
Val	1.0 (1)	1
Leu	6.0 (6)	6
Phe	1.0 (1)	1
Total	30	27

Values are molar ratios without correction for destruction, incomplete hydrolysis or impurity. Figures within parentheses represent the predicted number of each amino acid in the secretin variant

The chromatograms of the tryptic fragments of the purified peptide and of secretin are shown in figs 3 and 4, respectively. Only one fragment eluted at different positions. This fragment corresponds to the C-terminal fragment T_5 as shown by the amino acid composition. Each tryptic peptide of the isolated peptide was hydrolyzed and subjected to amino acid analysis. The amino acid compositions of the tryptic fragments are shown in table 3. The C-terminal fragment of the isolated peptide, T_5, contains an additional Gly and a Lys residue as compared to the corresponding T_5 fragment of secretin, while the other fragments (T_1–T_4) are identical with the secretin fragments. Chymotryptic degradation of secretin and of the isolated peptide was carried out in parallel and led in both cases to numerous fragments. With one exception each fragment of the peptide appeared to be identical to a fragment of secretin and vice versa. The amino acid compositions of the two nonidentical fragments were determined (table 3). That of the secretin fragment was Gly_1, Glx_1, Leu_1, Val_1, unequivocally showing that it represented the C-terminal Gln-Gly-Leu-Val-NH_2 sequence of secretin. That of the variant fragment was Arg_1, Gly_2, Glx_1, Leu_1, Lys_1, Val_1, suggesting the sequence Gln-Gly-Leu-Val-Gly-Lys-Arg. Consequently, the findings suggested that the isolated peptide consisted of secretin extended C-terminally with Gly-Lys-Arg. If so, free arginine should be split from it on tryptic degradation and this was also shown to be the case. A tryptic digest was derivatized with PITC [13] and applied to an HPLC system for the detection of PTC-amino acids. All 5 tryptic peptide derivatives and free PTC-arginine were indeed detected.

Taken together, the above data show that the isolated polypeptide really is a variant form of secretin with a C-terminal extension by Gly-Lys-Arg. This is in good agreement with the knowledge that a glycine residue followed by paired basic residues usually represents amidation and cleavage signals in peptide hormone precursors. It may be mentioned that the precursors of 3 other members of the secretin family, VIP, PHI and glucagon, also have the double basic amino acid combination Lys-Arg following the glycine residue involved in the amidation [16,17]. However, in a fourth member, somatocrinin (GRF), the glycine residue precedes a single basic residue, arginine [18].

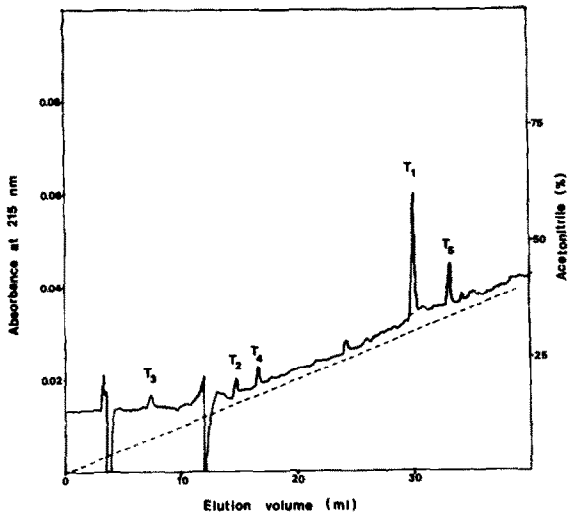

Fig.3. Separation of the tryptic fragments of the secretin variant by reverse-phase HPLC. Sample: about 4 μg trypsin-degraded secretin variant. Column: the column described in legend to fig.2. Mobile phases: A and B as in legend to fig.1. Flow rate: 1 ml/min. Gradient: 0–40% B, 40 min, indicated by the dotted line.

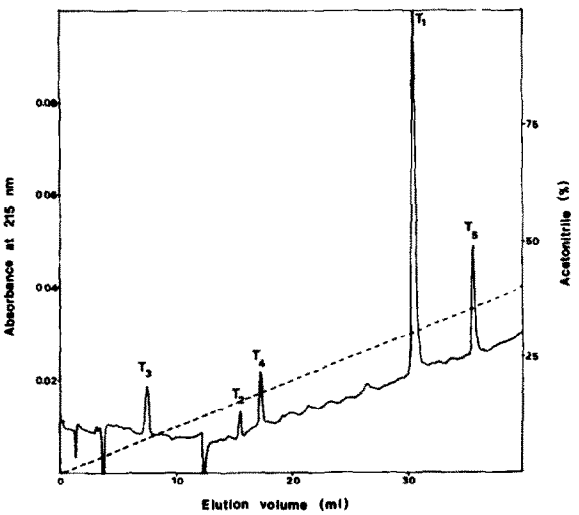

Fig.4. Separation of the tryptic fragments of secretin (10 μg trypsin-degraded secretin) by reverse-phase HPLC using the same conditions as described in legend to fig.3.

Table 3

Amino acid compositions of the tryptic fragments and the C-terminal chymotryptic fragment of the secretin variant

Amino acid	T_1	T_2	T_3	T_4	T_5	Chymotryptic fragment
Lys	–	–	–	–	1.0 (–)	0.9 (–)
His	0.8 (1)	–	–	–	–	–
Arg	0.8 (1)	0.9 (1)	0.8 (1)	0.8 (1)	–	0.9 (–)
Asx	1.3 (1)	–	1.3 (1)	–	–	–
Thr	2.0 (2)	–	–	–	–	–
Ser	2.8 (3)	–	0.9 (1)	–	–	–
Glx	1.4 (1)	–	–	1.2 (1)	1.2 (1)	1.2 (1)
Gly	1.0 (1)	–	–	–	1.5 (1)	2.0 (1)
Ala	–	–	1.0 (1)	–	–	–
Val	–	–	–	–	1.3 (1)	0.9 (1)
Leu	1.0 (1)	1.1 (1)	–	1.0 (1)	3.0 (3)	1.1 (1)
Phe	0.9 (1)	–	–	–	–	–
	12	2	4	3	6	4

Values are molar ratios without correction for destruction, incomplete hydrolysis or impurity. Figures within parentheses are the numbers of amino acids in the corresponding secretin fragment

The pure secretin variant was tested for bioactivity in the secretin bioassay described above. As shown in fig.5, secretin-Gly-Lys-Arg is more potent than secretin itself. It has been observed [8] that secretin-Gly is active in the secretin bioassay but the activity has never been quantitated. It is interesting to note that two immediate proforms to secretin, secretin-Gly and secretin-Gly-Lys-Arg, have now been isolated and found to be biologically active. It is important to stress that in both these studies the secretin variants were isolated from side fractions during secretin purification and that it cannot be excluded that some other fraction(s) around the starting materials may also contain these and possibly other variant forms. Therefore, it is impossible at this stage to know if the C-terminally extended forms of secretin are just short-lived precursors to the amidated secretin or if they exist in such amounts that their physiological activity is of importance.

The biological activity of secretin-Gly-Lys-Arg is also worth noting from another point of view. In attempts to produce peptide hormones by hybrid DNA techniques, it has always been a problem to modify the C-terminal amino acid to the amidated form. Since the C-terminal amidation does not seem to be a requisite for bioactivity in the case of secretin, one can produce the natural occurring proform to secretin, secretin-Gly-Lys-Arg. A secretin analog terminating in a valine instead of a

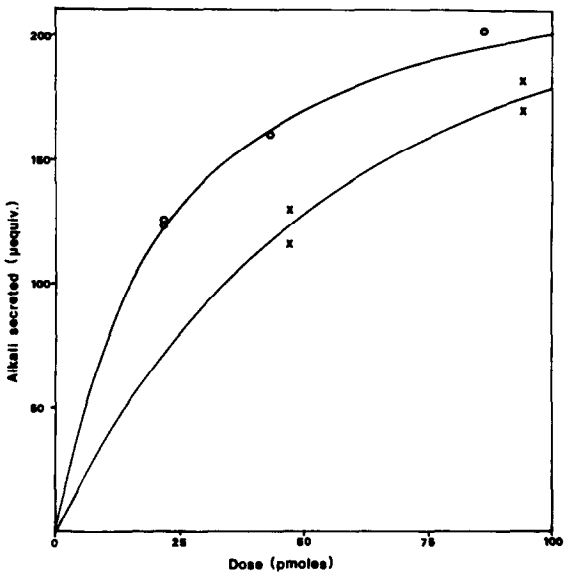

Fig.5. Dose-response curves for secretin and secretin-Gly-Lys-Arg. The assay was the bioassay for secretin described in section 2. (×) Secretin, (○) secretin-Gly-Lys-Arg.

valine amide residue has already been produced by such methodology [19].

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Medical Research Council (Project no.13X-01010) and the Karolinska Institute Fund.

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