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ISOLATION OF A PROFORM OF PORCINE SECRETIN BY ION-EXCHANGE AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A polypeptide with secretin-like bioactivity has been isolated from upper small intestinal porcine tissue by ion-exchange and reversed-phase high-performance liquid chromatography (HPLC). The purification was followed by determination of biological activity. Its elution position in the ion-exchange HPLC indicated that it was less basic than secretin. Amino acid analysis showed that it contained an additional glycine residue as compared to scretin. Digestions by trypsin and subtilisin established that the polypeptide was a variant form of secretin in which the previously known secretin is extended C-terminally by a glycine residue.

INTRODUCTION

Secretin is a polypeptide hormone which stimulates pancreatic secretion[1]. It was originally isolated from porcine intestine[2] and later from cow[3] and chicken[4] intestine. Secretin-like bioactivity has been found in extracts of porcine brain[5] and secretin-like immunoreactivity in both porcine and rat brain[6].

Secretin is composed of 27 amino acid residues[3,4,7] and, as in many other biologically active peptides, its C-terminal residue is amidated. The C-terminal α-amide structure is often, although not in all cases. important for full biological activity[8,9].

It has been shown for promelittin[10], and in all cases where the nucleotide sequences have been established for the precursor proteins of amidated peptides[11,12], that the residue that becomes amidated in the mature peptide is always followed by a glycine residue in the precursor protein. This was first suggested for the formation of α-melanotropin from corticotropin[13].

In this paper we describe the isolation of a secretin variant, 28 amino acids long, with a C-terminal glycine residue. The peptide was isolated from a porcine upper intestinal extract, by ion-exchange and reversed-phase high-performance liquid chromatography (HPLC), and fractions obtained were tested for biological activity.

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EXPERIMENTAL

Starting material

The starting material was obtained as a side-fraction during the routine purification of secretin by CM-cellulose chromatography[14,15], where it appeared before the main secretin fraction.

Ion-exchange HPLC

The instrument used for the ion-exchange HPLC consisted of an LKB 2150 HPLC pump, an LKB 2152 HPLC controller, an LKB 2040 gradient-mixing valve, an LKB 2154 HPLC injector and an LKB 2151 HPLC variable-wavelength detector connected to an LKB 2210 recorder. The separation was carried out on an LKB UltroPac TSK 535 CM cation-exchanger column (150 × 7.5 mm I.D.). Elution was performed with a gradient of sodium chloride (0–0.3 *M*) in a sodium phosphate buffer, pH 6.4 (1.14 g sodium hydroxide and 22.5 ml 1 *M* phosphoric acid, and water added to a final volume of 1000 ml), filtered through a Millipore MF-filter (0.22 μm) and degassed by vacuum. The flow-rate was set at 1 ml/min and the absorbance of the eluent was recorded at 215 nm. Fractions of 2 ml were collected with an LKB 2112 RediRac fraction collector.

Reversed-phase HPLC

The apparatus was from Waters Assoc. and was equipped with two Model 6000 A pumps, a 720 system controller, a U6K injector, a 440 UV detector in connection with an extended-wavelength module set at 214 nm, and a μBondapak C_{18} (300 × 3.9 mm I.D.) reversed-phase column. The solvent system used consisted of (A) 0.1% trifluoroacetic acid (sequanal grade, Pierce), and (B) 0.1% trifluoroacetic acid in acetonitrile (HPLC-grade S, Rathburn Chemicals). The aqueous phase was filtered through a Millipore MF-filter (0.22 μm) and degassed by vacuum; the organic solvent was degassed by ultrasonication. The flow-rate was 1.5 ml/min.

Bioassay

The various HPLC fractions were assayed for bioactivity on the rat fundus strip, set up as described previously[16]. Regular responses were obtained with acetylcholine (Ach) and bioactivity was monitored as the ability to inhibit the response elicited by Ach. The secretin-like bioactivity was confirmed by the secretin bioassay[17].

Sequence analysis

The amino acid sequence determination was carried out with the manual DABITC method[18] using by-products for identification by thin-layer chromatography[19].

End-group analysis

N-terminal residues were determined by the dansyl method[20] with identification on polyamide sheets in four chromatographic systems[21].

Enzyme cleavage

The methods used for the degradations of the peptides with trypsin and subtilisin have been described elsewhere[22].

TABLE I

PREPARATION OF THE STARTING MATERIAL

	Weight
Upper part of porcine intestine boiled, frozen, and minced.	3000 kg
Extraction with 0.5 *M* acetic acid. Peptides adsorbed on alginic acid. Elution with 0.2 *M* HCl. Precipitation with NaCl	3 kg
Fractionation in 66% ethanol. Soluble material adsorbed to alginic acid. Eluted with 0.2 *M* HCl. Precipitated with NaCl	550 g
Gel filtration on Sephadex G-25 (fine) in 0.2 *M* acetic acid	150 g
Extraction into methanol. Soluble peptides precipitated with ether	10 g
Gel filtration on Sephadex G-25 (fine) in 0.2 *M* acetic acid	260 mg
Ion-exchange chromatography on CM-cellulose	50 mg

Amino acid analysis

Total compositions were determined after acid hydrolysis either on a Beckman 121 M amino acid analyzer or on a Waters Assoc. amino acid analysis system using pre-column derivatization as described in [23].

RESULTS

Isolation of the secretin variant

A schematic presentation of how the starting material was prepared is shown in Table I[14,15].

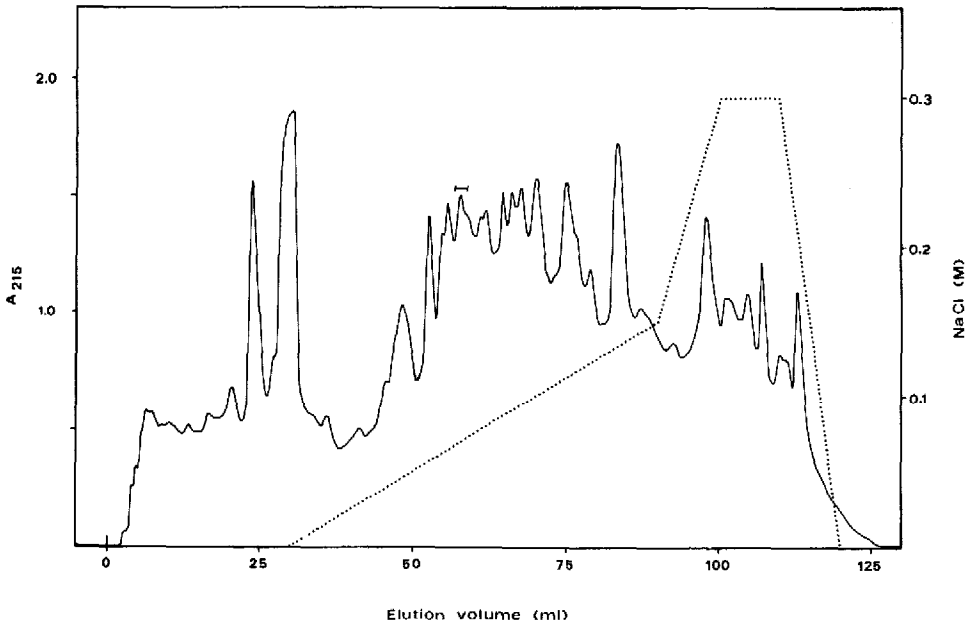

Fig. 1. Ion-exchange HPLC of the starting material. Load: 10 mg starting material. Column: TSK CM (150 × 7.5 mm I.D.). Solvent system: A = 0.02 *M* sodium phosphate buffer, pH 6.4 and B = 0.3 *M* sodium chloride in solvent A. Flow-rate: 1 ml/min. Gradient profile is shown by ·····. Fractions were collected every 2 min (2 ml). The fraction with the strongest secretin-like activity is indicated by the bar.

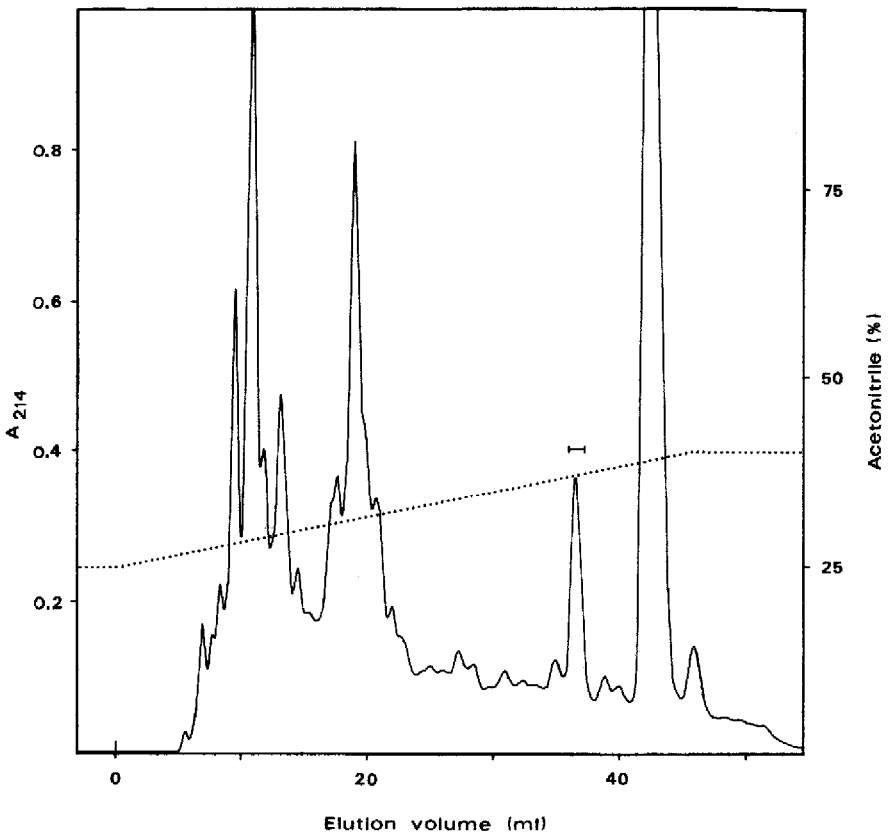

Fig. 2. Final purification of the secretin variant by reversed-phase HPLC. Load: 1/3 of the active material from the five batches prepared by ion-exchange HPLC. Column: μBondapak C_{18} (300 × 3.9 mm I.D.). Solvent system: A = 0.1% TFA and B = 0.1% TFA in acetonitrile. Flow-rate: 1.5 ml/min. Gradient profile is shown by ·····. The secretin variant is indicated by the bar.

TABLE II

AMINO ACID COMPOSITION OF THE SECRETIN VARIANT

Values are molar ratios without correction for destruction, incomplete hydrolysis, or impurity.

Amino acid	*Secretin variant*	*Secretin*
Asp	2.2 (2)	2
Thr	1.9 (2)	2
Ser	3.6 (4)	4
Glu	2.9 (3)	3
Gly	3.0 (3)	2
Ala	1.1 (1)	1
Val	1.1 (1)	1
Leu	5.9 (6)	6
Phe	1.1 (1)	1
His	0.9 (1)	1
Arg	3.9 (4)	4
Total	28	27

Portions of 10 mg starting material were dissolved in 1 ml of the sodium phosphate buffer (see Experimental) and subjected to ion-exchange HPLC (Fig. 1). Fractions of 2 ml were collected and assayed for bioactivity[16]. The fraction with the strongest secretin-like bioactivity[17] was lyophilized. A total quantity of 50 mg starting material was chromatographed on the cation-exchanger HPLC column, in five batches, showing identical elution patterns.

The fractions showing secretin bioactivity were combined and dissolved in 3 ml water. Samples of 1 ml were applied to the reversed-phase column. Fig. 2 shows the elution profile for such an experiment (see Fig. 2 legend for details). Peaks were collected manually, lyophilized and tested for secretin-like bioactivity.

With this purification procedure a total quantity of about 80 μg of the peptide showing secretin-like bioactivity was obtained.

Structure determination

The amino acid determination of the secretin variant (Table II) shows that it contains an additional glycine residue as compared to authentic secretin.

About 10 nmol of the purified secretin variant was subjected to the manual DABITC method[18,19]. The first fifteen amino acid residues were determined and all were identical to those in the corresponding positions in secretin.

The secretin variant (15 nmol) was digested with trypsin, and fragments were separated by reversed-phase HPLC (Fig. 3; conditions are given in the legend). Total

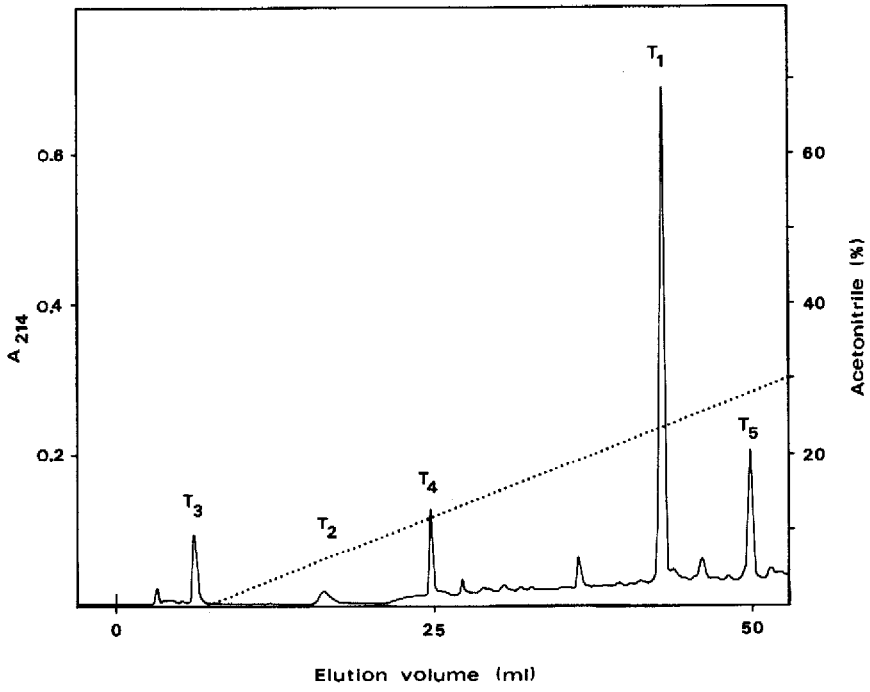

Fig. 3. Separation of the secretin variant tryptic peptides by reversed-phase HPLC. Load: 12 nmol trypsin-digested secretin variant. Column: μBondapak C_{18} (300 × 3.9 mm I.D.). Solvent system: A = 0.1% TFA and B = 0.1% TFA in acetonitrile. Flow-rate: 1.5 ml/min. Gradient profile is shown by ·····. T_1-T_5 indicates the tryptic fragments.

TABLE III

AMINO ACID COMPOSITIONS AND N-TERMINAL RESIDUES OF THE TRYPTIC FRAGMENTS T_1–T_5

Values are molar ratios without correction for destruction, incomplete hydrolysis, or impurity.

Amino acid	T_1		T_2		T_3		T_4		T_5	
	Secretin variant	*Secretin*	*Secretin variant*	*Secretin*	*Secretin variant*	*Secretin*	*Secretin variant*	*Secretin*	*Secretin variant*	*Secretin*
Asp	1.1 (1)	1	–	–	1.1 (1)	1	–	–	–	–
Thr	1.9 (2)	2	–	–	–	–	–	–	–	–
Ser	2.7 (3)	3	–	–	0.9 (1)	1	–	–	–	–
Glu	1.1 (1)	1	–	–	–	–	1.0 (1)	1	1.0 (1)	1
Gly	1.1 (1)	1	–	–	–	–	–	–	2.1 (2)	1
Ala	–	–	–	–	1.0 (1)	1	–	–	–	–
Val	–	–	–	–	–	–	–	–	1.0 (1)	1
Leu	1.0 (1)	1	1.0 (1)	1	–	–	1.0 (1)	1	2.9 (3)	3
Phe	1.0 (1)	1	–	–	–	–	–	–	–	–
His	1.0 (1)	1	–	–	–	–	–	–	–	–
Arg	1.0 (1)	1	1.0 (1)	1	1.0 (1)	1	1.0 (1)	1	–	–
Total	12	12	2	2	4	4	3	3	7	6
N-terminus	His	His	Leu	Leu	Asp	Asp	Leu	Leu	Leu	Leu

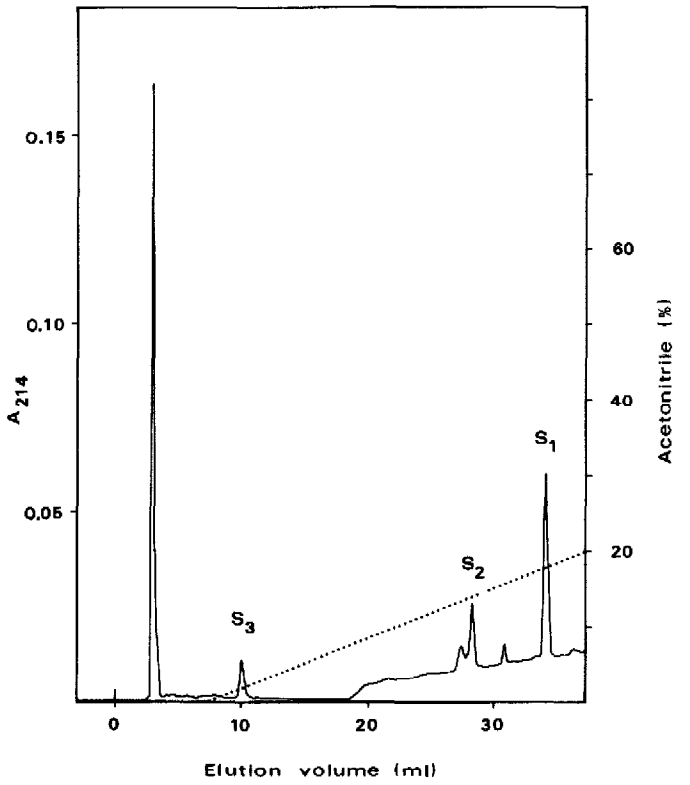

Fig. 4. Separation of the peptides obtained by digestion of tryptic peptide T_5 with subtilisin by reversed-phase HPLC. Load: 7 nmol of T_5 digested with subtilisin. Column: μBondapak C_{18} (300 × 3.9 mm I.D.). Solvent system: A = 0.1% TFA and B = 0.1% TFA in acetonitrile. Flow-rate: 1.5 ml/min. Gradient profile is shown by ·····. S_1–S_3 indicates the fragments obtained by subtilisin digestion.

TABLE IV

AMINO ACID COMPOSITIONS AND N-TERMINAL RESIDUES OF PEPTIDES S_1–S_3

Values are molar ratios without correction for destruction, incomplete hydrolysis, or impurity.

Amino acid	*S_1*		*S_2*		*S_3*	
	Secretin variant	*Secretin*	*Secretin variant*	*Secretin*	*Secretin variant*	*Secretin*
Glu	1.2 (1)	1	–	–	–	–
Gly	–	–	0.9 (1)	1	0.9 (1)	–
Val	–	–	–	–	1.1 (1)	1
Leu	1.8 (2)	2	1.1 (1)	1	–	–
Total	3	3	2	2	2	1
N-terminus	Leu	Leu	Gly	Gly	Val	Val

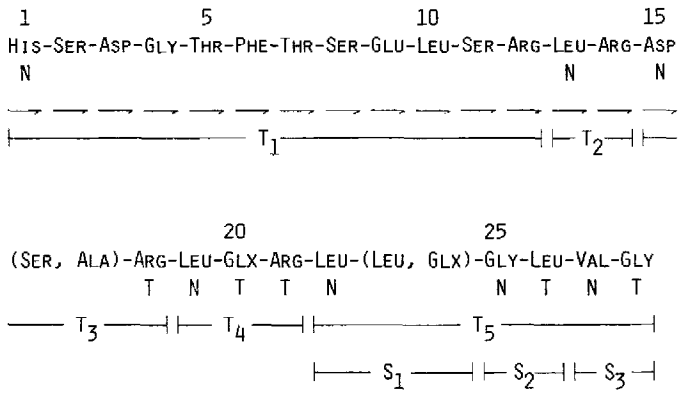

Fig. 5. Amino acid sequence of the secretin variant. Manual sequence analysis is shown by —. The tryptic peptides are T_1–T_5 and fragments obtained by subtilisin digestion S_1–S_3. N-terminal determination, N; position given by total composition, T. Peptides S_2 and S_3 are positioned on the basis of homology to porcine secretin.

compositions and N-terminal amino acid residues are shown in Table III, together with the corresponding tryptic peptides from porcine secretin. From these results it is evident that the additional glycine residue is located in the C-terminal tryptic fragment T_5. An aliquot (2.5 nmol) of T_5 was further digested with carboxypeptidase Y[24], but no amino acids were liberated in detectable amounts. When 7 nmol of T_5 were further digested with subtilisin, three new peptides were formed which were also separated by reversed-phase HPLC (Fig. 4). Total compositions and N-terminal residues for these peptides are shown in Table IV.

All these results show that the additional glycine residue is the C-terminal amino acid in this variant form of secretin (Fig. 5). In other words, this variant is a form of secretin C-terminally extended by a glycine residue.

DISCUSSION

From 50 mg starting material approximately 25 nmol of the secretin-Gly were obtained. Since only one of the fractions (the starting material) from the CM-cellulose chromatography was subjected to ion-exchange HPLC it is possible that some fraction(s) around the starting material could contain more of this secretin variant. Nevertheless, it was possible to isolate the secretin variant from the starting material, as described in this paper.

In some cases it might be preferable not to lyophilize the biologically active material in the presence of non-volatile buffer as used in the ion-exchange HPLC, as this could lead to loss of biological activity, but to apply the active fraction directly to the reversed-phase column. In this study, however, lyophilization was carried out to reduce the volume, and no adverse effect was found.

When using non-volatile buffer systems in ion-exchange HPLC, it is convenient to follow the latter by reversed-phase HPLC with a volatile system, which further purifies as well as desalts the material.

The elution position of the secretin variant in the ion-exchange HPLC indicated that it was less basic than secretin, which could be explained by the fact that

the variant does not contain a C-terminal α-amide structure, but instead, a glycine residue with a free carboxyl group as the C-terminal residue.

The reason why no detectable amounts of any amino acids were liberated by the carboxypeptidase digestion was probably the very slow release of the C-terminal glycine residue by this enzyme.

The finding of a secretin variant in which the previously known secretin is extended C-terminally by a glycine residue is in full agreement with previous reports concerning other polypeptide hormone proforms[10,11,12]. It also strongly supports the idea that this type of C-terminally extended peptide is the true immediate precursor of the amidated peptide[25].

It shows, too, that this type of unprocessed proform exists in intestinal extracts.

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