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Processing of prosecretin: Isolation of a secretin precursor from porcine intestine

(protein sequence/alternative splicing)

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ABSTRACT **A precursor to the gastrointestinal hormone secretin has been isolated. The starting material for the purification of the precursor was a peptide fraction purified from pig intestinal extracts, containing peptides with a molecular weight higher than that of secretin. The purification could be followed by measurement of secretin bioactivity (alkali secreted in the pancreatic juice of anesthetized cat). Sequence analysis of the isolated secretin precursor revealed a 71-amino acid residue polypeptide that contained the sequence of secretin N terminally, followed by a Gly-Lys-Arg sequence and a C-terminal extension of 41-amino acid residues. With the exception of an arginine residue, which occurs directly after the Gly-Lys-Arg sequence, the remainder of the C-terminal residues in this precursor are identical to the 40 C-terminal residues predicted by the recently described cDNA sequence for porcine preprosecretin. Compared to the deduced preprosecretin sequence, a stretch of 32 amino acid residues directly following the Gly-Lys-Arg sequence is missing in the now purified secretin precursor. This implies that differential splicing may occur when the secretin gene transcript is processed to mRNA.**

After its discovery in 1902 by Bayliss and Starling (1), almost 60 years elapsed before secretin (the porcine form of it) was isolated (2) and another 10 years elapsed before its primary structure was finally deduced (3). Pig secretin is a 27-amino acid residue peptide with a C-terminal valine amide. Cow and guinea pig secretin were found to be identical to that of the pig form (4, 46), whereas the human (5), dog (6), rat (7), and rabbit (8) secretin all differ slightly from each other and from the porcine/bovine/guinea pig form. Chicken secretin is different from all the mammalian forms. However, it is, like the others, composed of 27-amino acid residues and is C-terminally amidated (9). The main action of secretin is to stimulate secretion of bicarbonate-rich pancreatic juice, but it also exerts other actions (10). Although secretin immunoreactivity, as well as bioactivity, has been reported in the central nervous system (11–13), secretin has so far only been isolated from intestine, where it is known to be localized in endocrine cells, called S cells, in the duodenum and upper jejunum (14, 15).

Until recently, the structure of the secretin precursor was unknown. This was probably due to secretin being composed of amino acids with degenerated codons, making it difficult to synthesize appropriate probes. However, using the polymerase chain reaction technique, Kopin *et al.* (16) were able to isolate cDNA encoding pig and rat preprosecretin. The deduced amino acid sequence of the porcine secretin precursor (16) includes a signal peptide, an N-terminal flanking peptide (≈10 amino acid residues), secretin, a Gly-Lys-Arg cleavage and amidation sequence, and a 72-amino acid residue C-terminal flanking peptide.

Because of the difficulties in isolating the secretin precursor by conventional cDNA techniques and to investigate the biosynthetic pathway of secretin, we decided to isolate a larger prosecretin protein. We knew from earlier studies on C-terminal extended forms of secretin, secretin-Gly (17) and secretin-Gly-Lys-Arg (18), that a free C-terminal valine amide is not required for biological activity in the secretin bioassay measuring alkali secreted in the pancreatic juice of anesthetized cat. On the other hand, a free N-terminal histidine seems essential for appreciable bioactivity (19). When high molecular weight side fractions from the purification of secretin from pig upper intestine were investigated for secretin bioactivity, activity could be found in one of these fractions. The present paper reports the isolation and characterization of a secretin precursor that is C-terminally extended.

MATERIALS AND METHODS

Preparation of Starting Material. Extraction of pig intestines (upper part) was carried out as described (20). Briefly, batches (≈1000 kg) of rinsed, boiled (10 min in boiling water), frozen, and minced pig intestines were extracted in 3% acetic acid at 0°C–10°C overnight. Extracted peptides were collected from the filtrate on alginic acid, eluted with 0.2 M HCl, and NaCl precipitated, resulting in a concentrate of thermostable intestinal peptides (CTIP). The CTIP fraction usually weighs ≈1 kg. Each kilogram of CTIP was dissolved in 10 liters of water, whereafter 20 liters of ethanol was added and the pH was adjusted to 7.2 with 1 M NaOH in 66% ethanol. The precipitate that formed was removed by filtration and to the filtrate 30 liters of cold ethanol was added at −20°C. The supernatant was decanted after 48 hr at −20°C, diluted with 200 liters of water, and pH was adjusted to pH 2.7; thereafter, peptides were adsorbed to alginic acid. The peptides were eluted from alginic acid with 0.2 M HCl and were collected after precipitation with NaCl at saturation. The salt precipitate (CTIP-1) usually weighs 70–80 g. CTIP-1, ≈250 g per batch, was dissolved in 2.5 liters of 0.2 M acetic acid, loaded on a Sephadex G-25 fine column (35 × 135 cm), and gel filtrated in 0.2 M acetic acid. Fraction 2, eluted between elution volumes of 10–20 liters (calculated from the first appearance of A_{280} material), was used as starting material for the purification of the secretin precursor.

Methanol Extraction. Sephadex fraction 2 (133 g), originating from six Sephadex chromatographies was extracted 3 min in a Waring blender and 10 min with manual stirring in 25 vol (3.3 liters) of methanol containing 0.1% 2-mercaptoethanol. Undissolved material was removed by filtration and the

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Abbreviations: TFA, trifluoroacetic acid; CTIP, concentrate of thermostable intestinal peptides; AMESNI, acid methanol-soluble, neutral-insoluble material.

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filtrate was pH adjusted to pH 7.5 with 250 ml of 0.1 M NaOH in methanol. The precipitate that formed was collected by filtration, dissolved in 0.2 M acetic acid, and lyophilized, giving 10.65 g of acid methanol-soluble, neutral-insoluble material [AMESNI (21)].

CM-Cellulose Chromatographies. Two CM-cellulose chromatographies were carried out. To the first CM-cellulose column (5 × 25 cm), 10.65 g of AMESNI was divided into three batches and run in a 0.02 M phosphate buffer with a NaCl gradient of 0–0.2 M. A bioactive fraction (fraction 5) eluting with 230–305 ml of 0.015–0.025 M NaCl was desalted on Sephadex G-25 coarse and the lyophilized material (643 mg) was subjected to a second CM-cellulose chromatography. CM-cellulose fraction 5 was applied to a CM-cellulose column (2.5 × 26 cm), equilibrated with 0.01 M NH_4HCO_3, and the peptides were then eluted with 0.01, 0.02, 0.04, 0.08, and 0.2 M NH_4HCO_3 and, finally, with 0.2 M NH_3. Two fractions, fractions 10 (78.3 mg) and 11 (111.6 mg), contained bioactive material. Fraction 10 eluted with 225–350 ml of 0.04 M NH_4HCO_3 and fraction 11 eluted with 95–175 ml of 0.08 M NH_4HCO_3.

HPLC Separation. CM-cellulose fractions 10 and 11 were both subjected to preparative HPLC on a Vydac C18 column (22 × 250 mm) in a 0.1% trifluoroacetic acid (TFA)/water/acetonitrile system. Fractions were eluted with a gradient of 20–50% acetonitrile in 0.1% TFA/water over 40 min at a flow rate of 10 ml/min. The bioactive fraction was eluted at ≈40% acetonitrile. This fraction (17.2 mg originating from CM-cellulose fraction 10 and 17.1 mg originating from CM-cellulose fraction 11) was further purified on an LKB Ultropac TSK 535 CM HPLC column (7.5 × 150 mm). The chromatography system was 30–50% 1 M NH_4OAc in 0.2 M acetic acid (22) over 30 min at a flow rate of 1 ml/min. The active fraction (fraction 6) was finally purified on an LKB Ultropac TSK ODS-120T (particle size, 10 μm) reverse-phase column (7.8 × 300 mm) using a gradient of 32–40% acetonitrile in 0.1% TFA/water over 32 min at a flow rate of 1.5 ml/min. The final purification yielded ≈450 μg of secretin precursor.

Bioassay. Bioactivity of the fractions tested was measured in the secretin bioassay (23). Samples and reference (pure pig secretin) were injected intravenously into an ethchlorvynol (Placidyl)-anesthetized cat and the stimulation of pancreatic exocrine secretion was measured by titration of alkali content in the secreted pancreatic juice.

Structural Analysis. The secretin precursor was cleaved with endoproteinase Lys-C (Boehringer Mannheim). The precursor (36 μg) was treated with 1.4 μg of enzyme in 100 μl of 0.1 M NH_4HCO_3 (pH 8.5) and incubated 4 hr at 37°C. The proteolytic fragments were separated on a Vydac 218TP (particle size, 5 μm) reverse-phase column (4.6 × 250 mm) using a gradient of 25–45% acetonitrile in 0.1% TFA/water over 40 min at a flow rate of 1 ml/min.

For amino acid analysis, peptides were hydrolyzed at 110°C for 22 hr in evacuated tubes containing 6 M HCl with 0.5% phenol. The hydrolysates were analyzed in a Beckman 121M amino acid analyzer.

The amino acid sequence of the secretin precursor was determined in an Applied Biosystems 470A protein sequencer with identification of phenylthiohydantoin derivatives by reverse-phase HPLC as described (24) and in a Milligen Prosequencer 6600 instrument using carboxyl group coupling of the peptide to an arylamine filter.

RESULTS

Isolation of the Secretin Precursor. In the search for larger secretin precursors, high molecular weight side fractions from the purification of secretin were tested for secretin bioactivity in the secretin bioassay. In Table 1, the preparations of CTIP and CTIP-1 are common for the purification of secretin and the secretin precursor. In the third purification step, a Sephadex G-25 fine chromatography in 0.2 M acetic acid on a column (35 × 135 cm), secretin activity was detected in fraction 2 and in fractions 4–6. The activity in the last three fractions corresponds to secretin, while the activity found in fraction 2, which only corresponds to a few percent of the total activity, originates from material with a higher molecular weight than secretin. This material was used as the starting material for the purification of the secretin precursor. The purification is outlined in Table 1 and after each purification step the fractions were tested for bioactivity in the secretin bioassay.

Table 1. Recovery data for purification of the secretin precursor

	Secretin activity, unit(s)/mg
CTIP (17.4 kg)	
CTIP-1 (1320 g)	
Sephadex fraction 2 (133 g)	0.15
AMESNI (10.65 g)	0.7
Fraction 5 (643 mg of CM-cellulose) (pH 6.4)	10
Fractions 10 + 11 (189.9 mg of CM-cellulose) (pH 8)	30
Fraction 5 (34.3 mg) (RP-HPLC)	250
Fraction 7 (weight ND) (CM HPLC)	ND
RP-HPLC-purified secretin precursor (450 μg)	3350

Data are from 16,500 kg of pig intestine (originating from ≈230,000 animals). Pure pig secretin used as standard has an activity of 3500 units/mg. The values of secretin bioactivity are, especially in the earlier steps, approximate (*i*) due to the crudeness of the material, and (*ii*) because they were calculated from single activity measurements. ND, not determined; RP, reverse phase.

The starting material, Sephadex fraction 2, was methanol extracted. All secretin activity was retained in a fraction that was methanol soluble at acidic pH but was insoluble at neutral pH. This fraction was further purified by two consecutive CM-cellulose chromatographies. In the first chromatographic procedure, the peptides were eluted with a NaCl gradient (0–0.2 M NaCl) in a phosphate buffer (pH 6.4). Most of the secretin activity was found in fraction 5 (eluted with 0.015–0.025 M NaCl), which was further chromatographed on a second CM-cellulose column at pH 8 and eluted with increasing concentrations of ammonium bicarbonate. Secretin activity was found in two fractions—10 and 11. These fractions were subjected separately to preparative reverse-phase HPLC using identical separation conditions for the two fractions. The secretin activity was found to elute at the same position in the chromatograms of both fractions 10 and 11. It was concluded that these two fractions contained the same bioactive peptide and, after the reverse-phase chromatography, the active fractions were pooled together. Fig. 1 shows the active reverse-phase fraction chromatographed on a CM HPLC column in an acetic acid/ammonium acetate system. All the secretin activity was found in fraction 6, which was essentially pure as shown in the last purification step, a reverse-phase chromatography (Fig. 2).

The secretin activity of the purified secretin precursor compared to a secretin standard is outlined in Table 2. The activity of the precursor appears to be ≈2 times stronger than that of secretin on a molar basis.

Structural Analysis. The intact peptide was degraded in a protein sequencer and the amino acid sequence could be read up to ≈40 cycles. However, the amino acid composition suggested that the peptide was larger. It was therefore cleaved with endoproteinase Lys-C, yielding two fragments. The C-terminal fragment was identified by its absorption at 280 nm and by its amino acid composition. The amino acid sequence of the C-terminal fragment was determined in another sequence analysis in a Milligen protein sequencer

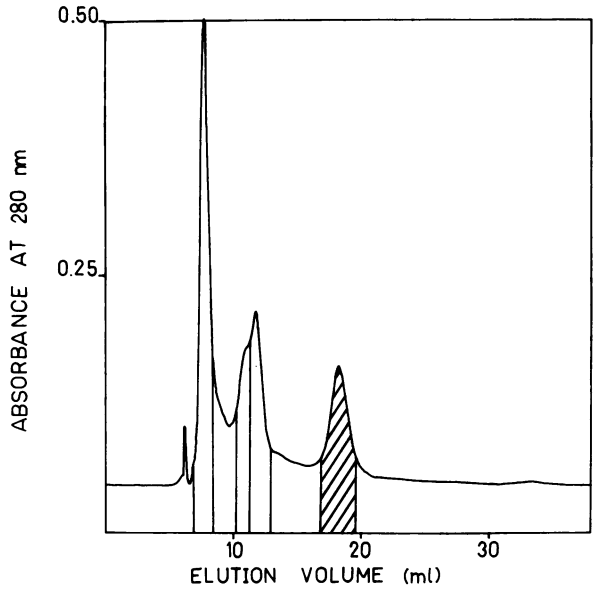

FIG. 1. CM HPLC chromatography. Batches (2 mg) of a secretin bioactive reverse-phase HPLC fraction were loaded on the CM HPLC column. Fractions were eluted with a gradient of 30–50% 1 M NH_4OAc in 0.2 M acetic acid over 30 min at a flow rate of 1 ml/min. Secretin bioactive material is indicated by hatched area.

instrument. The sequence of the entire fragment could be determined this way with the exception of position 38 (position 67 in the whole peptide), which was unclear but was identified as a serine residue based on the amino acid composition of the fragment. Comparison to the published preprosecretin sequence (16) also predicts serine at this position. The deduced sequence of the entire secretin precursor is shown in Fig. 3.

DISCUSSION

A secretin precursor has been isolated from pig upper intestine. This precursor consists of 71 amino acid residues. It includes secretin at the N terminus, beginning with the N-terminal histidine of secretin, and has a C-terminal extension of 44 residues, including the cleavage and amidation signal Gly-Lys-Arg directly following the secretin sequence.

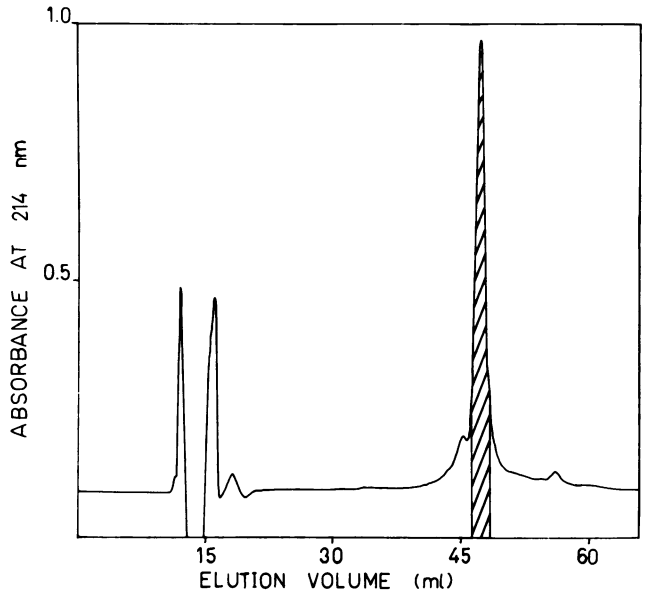

FIG. 2. Final purification of the secretin precursor on a reverse-phase C18 HPLC column. The elution system was a gradient of 32–40% acetonitrile in 0.1% TFA/water over 32 min at a flow rate of 1.5 ml/min. Hatched area represents the fraction containing the secretin precursor.

Table 2. Secretin bioactivity of purified secretin precursor measured in the secretin bioassay

	Secretin, pmol	Secretin activity
Cat 1		
Secretin standard (1 unit)	80	280
Secretin standard (0.5 unit)	40	176
Secretin precursor	45	312
Secretin standard (0.5 unit)	40	158
Secretin precursor	22.5	200
Secretin standard (1 unit)	80	260
Secretin standard (0.5 unit)	40	162
Cat 2		
Secretin standard (1 unit)	80	224
Secretin standard (0.5 unit)	40	124
Secretin precursor	36	230
Secretin precursor	18	116
Secretin standard (0.5 unit)	40	144

Secretin activity is expressed as microequivalents of alkali secreted. Activity was determined in separate experiments in two animals.

The amino acid sequences of pig and rat preprosecretin were recently deduced from isolated cDNAs from the two species (16). The porcine secretin precursor consists of a signal peptide, a short N-terminal flanking peptide (≈10 residues), the secretin sequence, a Gly-Lys-Arg cleavage and amidation site, and a 72-residue C-terminal flanking peptide. The 44-residue C-terminal extension of the now isolated secretin precursor corresponds to the Gly-Lys-Arg sequence, an additional arginine residue, and the 40 C-terminal amino acids of the published porcine preprosecretin. The isolated precursor thus lacks a stretch of 32 amino acid residues directly following the Gly-Lys-Arg sequence in the preprosecretin sequence deduced from pig cDNA. The most probable explanation for this is that the isolated secretin precursor originates from a mRNA species different from the mRNA used by Kopin *et al.* (16) for cDNA construction. This implies a differential splicing mechanism for the secretin mRNA. The arginine residue at position 31 in the isolated secretin precursor would then arise at a splicing site as a result of the splicing event. According to the consensus sequences for splice junctions (25), the exon donor site is usually AG, which is also the case for the 2 nucleotides following the Gly-Lys-Arg codons in the preprosecretin cDNA. At the acceptor site, G(G/T) is a common sequence; in the preprosecretin cDNA, GA is found at the exon side of the putative splicing acceptor site. The codon that arises after

H S D G T F T S E L S R L R D S A R L Q
10 20
R L L Q G L V G K R R M P M K P P V D Q
30 40
A W S P W L P P G L R A G A L V S E P A
50 60
I P A A E G S P M P P
70

FIG. 3. Amino acid sequence of the isolated secretin precursor deduced from sequence analysis of the intact peptide [residues 1–38, repetitive yield (glycine 4–28): 97.8%] and a proteinase Lys-C-generated C-terminal fragment [residues 30–71, repetitive yield (methionine 32–69): 95.1%]. The secretin sequence is boxed and the cleavage and amidation sequence Gly-Lys-Arg is underlined. Residue 67 was determined from amino acid composition data.

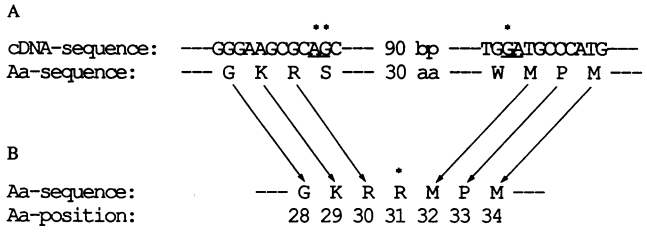

FIG. 4. The putative splicing site. (*A*) cDNA and deduced amino acid (aa) sequence from porcine preprosecretin [from Kopin *et al.* (16)]. bp, Base pairs. (*B*) Residues 28–34 of the isolated secretin precursor. Arrows indicate corresponding amino acids in the cDNA-deduced amino acid sequence and the amino acid sequence of the isolated secretin precursor. Underlined bases represent the putative exon donor and acceptor sites. Bases indicated with asterisks encode the arginine residue, also indicated by an asterisk.

the splicing event (AGG) encodes arginine and the remaining A at the splicing acceptor site corresponds to the first nucleotide in the methionine codon following the arginine codon (Fig. 4).

Differential splicing has been reported—e.g., for calcitonin–calcitonin gene-related peptide (26), the tachykinins (27–29), gastrin-releasing peptide (30, 31), and the kininogens (32). In the glucagon–secretin family of peptides, all precursor protein sequences are now known. There is no evidence for differential splicing reported for the other members of the family [i.e., glucagon (33, 34), vasoactive intestinal peptide (VIP) peptide with N-terminal histidine, C-terminal isoleucine amide (PHI) (35), gastric inhibitory peptide (36), growth hormone-releasing factor (37, 38), and pituitary adenylate cyclase-activating peptide (39)]. This does not rule out the possibility that such mechanisms occur, since the isolated cDNAs originate from single mRNA species and other mRNAs may thus be present but are not detected. In the case of glucagon and VIP/PHI, the genes have also been cloned. Preproglucagon is encoded by six exons (40, 41) and the VIP/PHI precursor is encoded by five exons (42, 43). In both cases, each functional domain of the precursor is encoded by a separate exon. In theory, these exons could be spliced in several ways, giving rise to different mRNA transcripts.

The structure of the isolated secretin precursor also suggests that during its processing the removal of the N-terminal flanking peptide (a cleavage at a single arginine residue) is the first processing event of prosecretin. It occurs before the C-terminal extension of the precursor is cleaved off to create the earlier isolated proform of secretin—secretin-Gly-Lys-Arg (18)—which in turn is the substrate for some carboxypeptidase B-like enzyme and the amidation enzyme petidylglycine α-amidating monooxygenase (44, 45).

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