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Studies on Secretin. III. Purification of Porcine Secretin by High Performance Liquid Chromatography and Comparison of the Product with Synthetic Secretin[1)]

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Natural porcine secretin was purified by high performance liquid chromatography (HPLC) in order to compare it with synthetic secretin. As a starting material we used partially purified secretin obtained by extraction from porcine duodenum followed by purification by ion-exchange chromatography and gel filtration. Preparative HPLC was performed on a reverse-phase column with a volatile eluent. The biological activity of the purified secretin was 5450 c.u./mg in the anesthetized rat, which is higher than those of other natural secretins reported so far (4000 c.u./mg), and was reasonably close to that of our synthetic secretin (5750 c.u./mg). The natural and synthetic secretins were compared by several physicochemical methods.

Keywords——synthetic secretin; natural porcine secretin; HPLC; optical rotation; CD; chemical purity; purification by HPLC; peptide; biological activity

In the preceding paper,[2)] we reported a new practical synthesis of porcine secretin that was chemically and optically pure as evaluated by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and other physicochemical methods. The synthetic secretin possessed high biological activity in stimulating exocrine pancreatic juice in the anesthetized rat. Its biological activity was 5750 clinical unit (c.u.)/mg, which is higher than those of pure natural secretins reported so far. Pure natural secretin was reported to possess the biological activity of 4000 c.u./mg in stimulating exocrine pancreatic juice in the anesthetized cat by Jorpes and Mutt[3)] and in the anesthetized rat by Tachinbana *et al.*[4)]

In order to clarify the reasons for the difference in biological activity between the synthetic and natural secretins, it was necessary to obtain highly pure natural secretin in order to compare the two. In this paper, we propose a novel procedure for the preparation of pure natural secretin by HPLC. The natural secretin was compared with the synthetic peptide in terms of biological activity and purity.

As regards the purification of secretin by HPLC, chicken secretin and bovine secretin were isolated by HPLC on a μ-Bondapak C_{18} column (7.8 × 300 mm) with 0.005 M $AcONH_4$ and 0.1% AcOH in 50% ethanol as eluents, respectively.[5)] Further application of HPLC for the purification of synthetic porcine secretin using a Nucleosil C_{18} column (4.0 × 150 mm) with methanol/water/trifluoroacetic acid (TFA) 65/35/0.1 was reported.[6)] In this case, however, details of the purity and biological activity of the purified secretin have not been presented.

As we previously reported,[2)] the application of HPLC on a Nucleosil $5C_{18}$ packed column with dilute HCl/CH_3CN as an eluent has been found to be particularly effective in purifying synthetic secretin. HPLC could separate secretin isomers which might be produced by racemization and α–β rearrangement on Asp at position 3.

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TABLE I. Purification of Porcine Secretin[4)]

	Weight	c.u./mg
Upper one meter of intestine from 10000 hogs. minced	1000 kg	
Extraction with hot 0.1 N acetic acid. Activity adsorbed on CMC, eluted with HCl. Precipitated with NaCl at saturation	3.5 kg	0.25—0.5
Extraction with isopropyl alcohol and methanol. Gel filtration with Sephadex G-25	100 g	25—50
Chromatography on CMC	4 g	25—50
Chromatography on SP-Sephadex C-25 and gel filtration with Sephadex G-25 (superfine)	200 mg	500—1000
Chromatography on QAE-Sephadex at pH 9.0 and gel filtration with Biogel	35 mg	4000

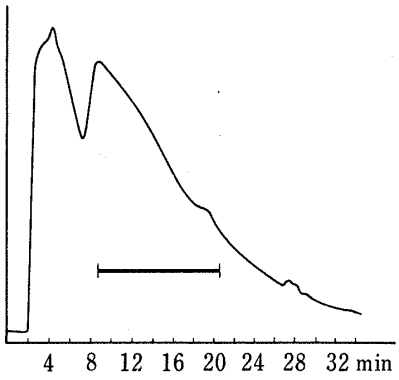

Fig. 1. First Preparative HPLC Purification

Column; Nucleosil $5C_{18}$ (2.0×25 cm). Eluent; 0.01% HCL/CH_3OH/CH_3CN (610/310/215). The secretin fraction is indicated by a bar.

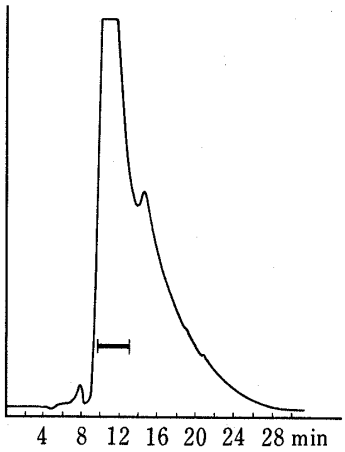

Fig. 2. Second Preparative HPLC Purification

Column; Nucleosil $5C_{18}$ (2.0×25 cm). Eluent; 0.005% HCl/CH_3CN (460/167). The secretin fraction is indicated by a bar.

Further, this preparative HPLC purification method required only lyophilization after the fractionation in order to obtain pure secretin, and no obvious problem which might cause loss of biological activity was observed during the procedures. Therefore we applied HPLC to the purification of natural secretin in the same manner as used for our synthetic secretin.

As a starting material, we used crude secretin extracted from porcine duodenum and partially purified by ion exchange chromatography and gel filtration according to the method of Tachibana[4)] (Table I). This preparation was found to contain a small amount of β-Asp^3–secretin, an α–β rearrangement product. The biological activity of crude secretin was 1660 c.u./mg in stimulating exocrine pancreatic juice in the anesthetized rat.

HPLC was conducted on Nucleosil $5C_{18}$ (2.0×25 cm) with 0.01% HCl/MeOH/CH_3CN 610/310/215 as an eluent, and with monitoring by ultraviolet (UV) at 210 nm. The crude material was subjected to HPLC, and lyophilization of the main fraction yielded a partially purified secretin (Fig. 1). Its biological activity was 3500 c.u./mg. The lyophilized peptide was again subjected to HPLC with 0.005% HCl/CH_3CN 400/167 as an eluent (Fig. 2). Lyophilized secretin hydrochloride was converted to the acetate salt by treatment with an ion exchanger such as Amberlite IRA-45 (AcOH form).

This product showed a single spot on TLC and a single band on disc electrophoresis. The amino acid composition after acidic or enzymic hydrolysis was consistent with the calculated

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TABLE II. Amino Acid Analyses of Synthetic and Natural Secretins

		HCl			AP-M	
		Natural	Synthetic		Natural	Synthetic
Asp	(2)	2.0	2.1	(2)	1.9	2.0
Thr	(2)	2.0	2.0	(2)	*a)*	*a)*
Gln				(2)	*a)*	*a)*
Ser	(4)	3.9	3.8	(4)	*a)*	*a)*
Glu	(3)	2.8	2.9	(1)	1.2	1.0
Gly	(2)	2.2	2.1	(2)	2.0	2.0
Ala	(1)	1.1	1.1	(1)	1.1	1.1
Val	(1)	1.1	1.0	(1)	1.0	1.1
Leu	(6)	5.7	6.2	(6)	6.2	6.3
Phe	(1)	1.0	1.0	(1)	1.0	1.0
His	(1)	1.1	1.0	(1)	1.1	1.0
Arg	(4)	4.0	4.1	(4)	3.8	4.0
Total	(27)	27	27			

a) Since separation between Thr, Gln, and Ser was not complete, these amino acids could not be individually quantitated.

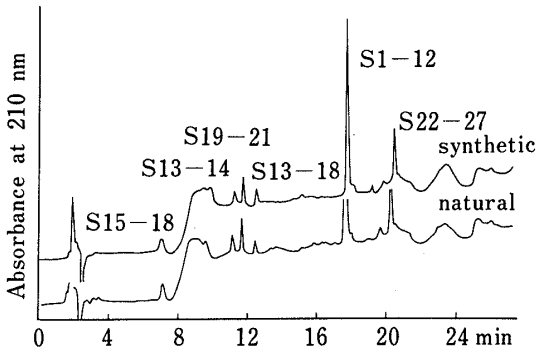

Fig. 3. Separation of Tryptic Fragments from Secretin by HPLC

Column; Nucleosil $5C_{18}$ (4.6 × 150 mm). Eluent; 0.0086% TFA, 0—60% CH_3CN.

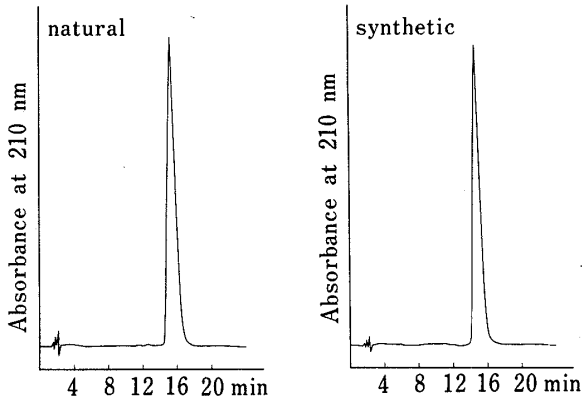

Fig. 4. HPLC of Synthetic and Natural Secretins

Column; Nucleosil $5C_{18}$ (4.6 × 150 mm). Eluent; 0.1% HCl/CH_3CN (65/26).

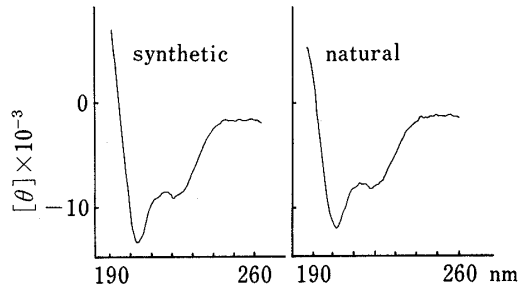

Fig. 5. CD spectra of Synthetic and Natural Secretins

Solvent; 0.1 M phosphate (pH 4.0).

values (Table II). Tryptic digestion of the product produced six peptide fragments T1, T2, T3, T4, T5 and T6, which were theoretically expected to be formed (Fig. 3).

The specific rotation, $[\alpha]_D^{20}$, of the product was $-51.9°$ ($c=0.4$, 0.1 N-AcOH), which is approximately consistent with that of our synthetic secretin[2] (Table III).

Analytical HPLC of the product showed a single peak under several conditions including

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Table III. Optical Rotation of Synthetic and Natural Secretins

	$[\alpha]_D^{20}$ ($c=0.4$ 0.1 N AcOH)
Synthetic secretin	−56.4
Natural secretin	−51.9

Table IV. Purification of Porcine Secretin by HPLC

	Weight	c.u./mg
Crude secretin	118 mg	1660
First preparative HPLC purification	46.6 mg	3500
Second preparative HPLC purification	14.5 mg	5450
Synthetic secretin		5750

Table V. Comparison of Various Secretin Preparations

	Biological activity (c.u./mg lyophilized salt)[a]	Peptide content (%)[b]	Biological activity (c.u./mg dry free base)[c]
Natural secretin			
Karolinska[c]	4770	80	6280
HPLC-purified	5450	86.0	6340
Synthetic secretin	5750	89.0	6460

a) Determined based on Kabi natural secretin as a standard. *b*) Calculated from the average recovery in amino acid analysis. *c*) A generous gift from Dr. V. Mutt.

a procedure which was recommended by Schaaper *et al.*[7] as effective to detect β-Asp[3]–secretin and aspartoyl secretin (Fig. 4). The circular dichroism (CD) and optical rotatory dispersion (ORD) spectra of the product were found to be identical with those of synthetic secretin (Fig. 5). All analytical data thus obtained agreed with those of our synthetic secretin.

The biological activity of our natural secretin was 5450 c.u./mg based on Kabi secretin as a standard in stimulating exocrine pancreatic juice in the anesthetized rat. This value is reasonably close to that of our synthetic secretin (Table IV).

We also compared the biological activities in terms of the dry free base.[8] The peptide content of the purified natural secretin was estimated as 86% from the average recovery of amino acids in amino acid analysis. Therefore the biological activity of purified natural secretin was found to be 6340 c.u./mg in the form of the dry free base, by calculation based on the peptide content. In the same manner, the biological activity of our synthetic secretin was found to be 6460 c.u./mg in the form of the dry free base (Table V).

This result indicates that the biological activity of our natural secretin is closely equivalent to that of our synthetic secretin both as the lyophilized secretin salt and as the dry free secretin base.

On the other hand, the biological activity of Karolinska natural secretin (acetate), a generous gift from Dr. V. Mutt, Karolinska Institute, Stockholm, Sweden, was 4770 c.u./mg when determined in the same manner as that used for our secretin.[2] The peptide content and purity of this secretin were more than 80% and 95%, respectively.[9] Therefore the biological activity, by calculation based on the peptide content and purity, was about 6280 c.u./mg in the form of the dry free secretin base (Table V).

These results indicate that the biological activity of our synthetic secretin is essentially identical with that of Karolinska natural secretin as the dry free secretin base, and with that of our natural secretin in the form of both the dry secretin base and the lyophilized salt.

We can therefore conclude that our synthetic secretin is identical with natural secretin. It

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appears that our new procedure using HPLC for the purification of natural secretin is very effective.

Experimental

Analysis of secretin by reverse-phase HPLC was performed on a column (0.46 × 15 cm) packed with Nucleosil-$5C_{18}$. TLC was performed on silica gel plates (Merck F254, butanol/pyridine/acetic acid/water 15 : 5 : 5 : 8). The spots were made visible by using the fluorescamine reagent. Amino acid compositions of acid and enzyme hydrolysates were determined with a Hitachi model 835 amino acid analyzer. Samples were hydrolyzed with 6 N HCl at 110 °C for 24 h or with aminopeptidase M. (Protein Research Foundation) at 37 °C for 24 h. Trypsin digestions were carried out as described in the preceding paper.[2] The concentration was 1 mg/ml for peptides and 0.1 mg/ml for trypsin. Assay of biological activity was carried out as described in the preceding paper.[2]

Partially Purified Secretin——Crude natural secretin (30 mg, 1660 c.u./mg), obtained according to the method of Tachibana,[4] was dissolved in 2.0 ml of eluent (0.01% HCl/MeOH/CH_3CN 610 : 310 : 215). The solution was chromatographed in two runs at RT on a column (1.0 × 30 cm) packed with Nucleosil $5C_{18}$. The flow rate was set at 3.0 ml/min. The main fractions, identified by UV detection at 210 nm, were collected, combined and lyophilized (Fig. 1). Yield: 11.8 mg. Biological activity: 3600 c.u./mg.

Highly Purified Secretin——Partially purified secretin (46.6 mg, 1660 c.u./mg) was dissolved in 6 ml of eluent (0.005% HCl/CH_3CN 400 : 167). The solution was chromatographed in eight runs at RT on a column (2.0 × 25 cm) packed with Nucleosil $5C_{18}$. The main fractions, identified by UV detection at 210 nm, were collected, combined and lyophilized (Fig. 2). The product was dissolved in 5 ml of water, applied to a column (1.5 × 18 cm) of Amberlite IRA-93 (acetate form), and eluted with 80 ml of water. The eluate was lyophilized. Yield: 14.5 mg. HPLC: see Fig. 5. Biological activity: 5450 c.u./mg.

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References and Notes

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