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Studies on Secretin. II. Synthesis of Secretin with High Activity[1)]

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Highly active synthetic secretin was obtained after deprotection of completely protected secretin with hidrogen fluoride/anisole followed by a simple two-step purification, involving ion-exchange chromatography and preparative reverse-phase high performance liquid chromatography (HPLC). The homogeneity of synthetic secretin was assessed by thin layer chromatography, HPLC, disc electrophoresis and other physicochemical methods. The synthetic secretin was found on HPLC to contain none of the diastereoisomers which might be produced during fragment condensation. Its biological activity was 5750 c.u./mg in stimulating exocrine pancreatic juice in the anesthetized rat, and this is the highest value among those of various natural and synthetic secretins (4000 clinical unit/mg). Secretin was found to be unexpectedly stable throughout the purification procedures. Our synthetic method is suitable for the large-scale production of secretin for clinical use.

Keywords——secretin purification; highly active secretin; chemical purity; optical purity; diastereoisomer; HPLC; TLC; large-scale production; clinical use; anti-ulcer

In the preceding paper,[2)] we reported the synthesis of fully functional side-chain-protected secretin which was almost chemically and optically pure. We now wish to report that highly pure synthetic secretin was obtained after deprotection with HF/anisole followed by a simple two-step purification involving ion-exchange chromatography and preparative high performance liquid chromatography (HPLC).

It has been regarded as difficult to obtain practically pure secretin, since it rapidly loses its biological activity. For instance, König *et al.* reported that the biological activity decreased in spite of the increment of chemical purity after purification by column chromatography.[3)] Voskamp *et al.* also reported that pure synthetic secretin with activity of 3782 clinical unit (c.u.)/mg was obtained after purification of the crude product by preparative HPLC, though the peptide content of 88% decreased to 62%.[4)] In addition, several investigators have described the instability of secretin.[5,6)] However, the reasons for the loss of biological activity were not clear, except for the α–β rearrangement on Asp at position 3, to which the instability of secretin has generally been attributed.[5)] On the other hand, it is known that some side reactions including α–β rearrangement may occur as a result of the treatment of protected peptides with HF[7)] and these side reactions are more suppressed at $-20\,^{\circ}C$ than $0\,^{\circ}C$.[8)]

Therefore, we removed all functional side-chain protecting groups with HF at about $-20\,^{\circ}C$ and purified the crude secretin by a simple method. The protected secretin was treated with liquid HF in the presence of anisole as a scavenger at -18 to $-19\,^{\circ}C$ for 70 min. After treatment with an ion exchanger such as Amberlite IRA-45 (AcOH form) followed by lyophilization, the crude peptide was obtained as a white powder. The crude peptide was

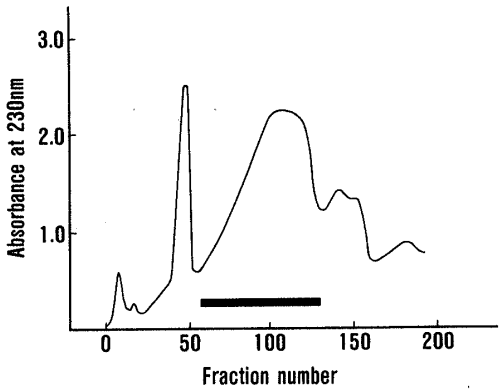

Fig. 1. Chromatography on CMC of Crude Secretin

Column; 2.6×30 cm. Eluent; a linear gradient formed from H_2O (2 l) and 0.3 M $AcONH_4$ (2 l). The secretin fraction is indicated by a bar (1.17 g).

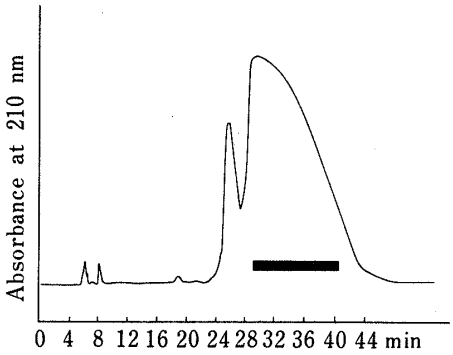

Fig. 2. Preparative HPLC of CMC-Purified Secretin

Column; Nucleosil $5C_{18}$ (2.0×250 mm). Eluent; 0.5% HCl/CH_3CN (40/17). The secretin fraction is indicated by a bar (70 mg).

purified by ion-exchange chromatography on carboxymethyl cellulose (CMC) with a linear gradient formed from H_2O and 0.3 M $AcONH_4$ as an eluent. Lyophilization of the main fraction yielded purified secretin (Fig. 1).

This CMC-purified secretin showed a single spot on thin layer chromatography (TLC) with ninhydrin, Sakaguchi and Pauli reagents and a single band on disc electrophoresis, but showed very small amounts of contaminants on TLC with fluorescamine reagent and on analytical HPLC on Nucleosil $5C_{18}$ with 0.1% HCl/CH_3CN 65/26.

The biological activity of CMC-purified secretin was determined as 5500 c.u./mg based on Kabi secretin as a standard in stimulating exocrine pancreatic juice in the anesthetized rat.[9]

We considered that this product should be further purified for use as a clinical material. For this purpose, preparative HPLC on a reverse-phase column of Nucleosil $5C_{18}$ with dilute HCl/CH_3CN as a volatile eluent was employed.

The CMC-purified peptide was applied to the column of Nucleosil-$5C_{18}$ (2.0×25 cm), which was eluted with 0.5% HCl/CH_3CN 40/17. Lyophilization of the main fraction yielded a white powder as the hydrochloride (Fig. 2). This hydrochloride was converted to the acetate salt by treatment with an ion-exchanger such as Amberlite IRA-45 (AcOH form).

In order to confirm the homogeneity of the product, we decided not only to check the purity but also to check specifically for contamination by both β-Asp^3–secretin and diastereoisomers which might be produced by racemization during fragment condensation. Five kinds of diastereoisomers, D-Phe^6–, D-Leu^{10}–, D-Leu^{13}–, D-Ala^{17}– and D-Leu^{22}–secretin, were synthesized.[10] Analytical HPLC on Nucleosil $5C_{18}$ with 0.1% HCl/CH_3CN 65/26 was found to separate β-Asp^3–secretin and these diastereoisomers satisfactorily. The product was found to show a single peak on this analytical HPLC and on another system which was recommended for separating β-Asp^3–secretin from secretin by Schaaper *et al.*[11] (Fig. 3).

Specific rotation, $[\alpha]_D^{20}$, was $-56.4°$ ($c=0.4$, 0.1 N AcOH). The amino acid composition after acidic or enzymic hydrolysis was consistent with the calculated values for secretin (Table I). The peptide content of the product was determined as nearly 90% from the average recovery of amino acids in amino acid analysis and from the nitrogen content measured by Kjeldahl analysis. The product was estimated to be the 2 acetate salt including $11H_2O$ from elemental analysis and determination of acetic acid by gas-liquid chromatography (GLC).

Trypsin digestion of the product produced six peptide fragments T1, T2, T3, T4, T5 and

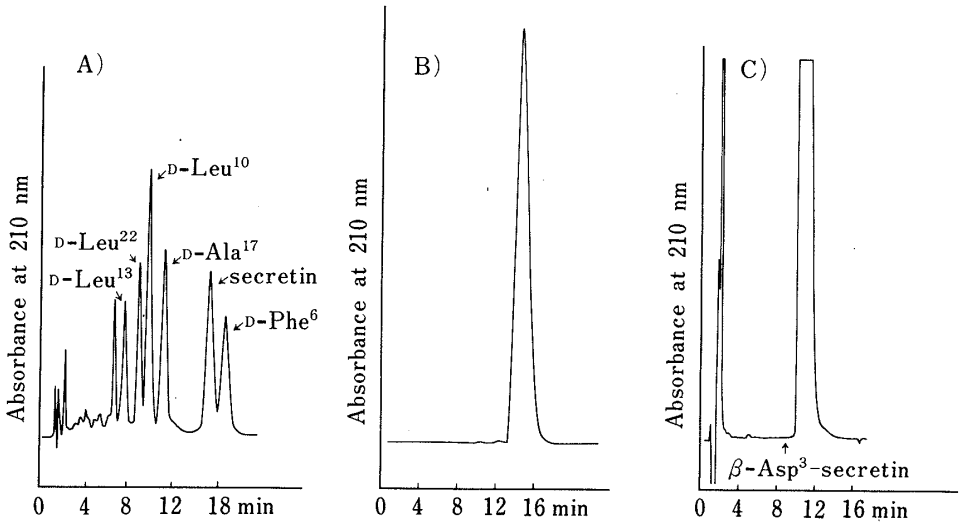

Fig. 3. HPLC of Synthetic Secretin

A) Separation of secretin diastereoisomers, B) and C) synthetic secretin.
Column; Nucleosil $5C_{18}$ (4.6 × 150 mm).
Eluent; A) and B) 0.1% HCl/CH_3CN (65/26), C) MeOH/H_2O (80/20) with 0.005 M perfluorooctanoic acid.

TABLE I. Amino Acid Analysis of Synthetic Secretin

		HCl		AP-M[a]
Asp	(2)	2.1	(2)	2.0
Thr	(2)	2.0	(2)	*b)*
Gln			(2)	*b)*
Ser	(4)	3.8	(4)	*b)*
Glu	(3)	2.9	(1)	1.0
Gly	(2)	2.1	(2)	2.0
Ala	(1)	1.1	(1)	1.1
Val	(1)	1.0	(1)	1.1
Leu	(6)	6.2	(6)	6.3
Phe	(1)	1.0	(1)	1.0
His	(1)	1.0	(1)	1.0
Arg	(4)	4.1	(4)	4.0
Total	27		27	

Average recovery 89.8%

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

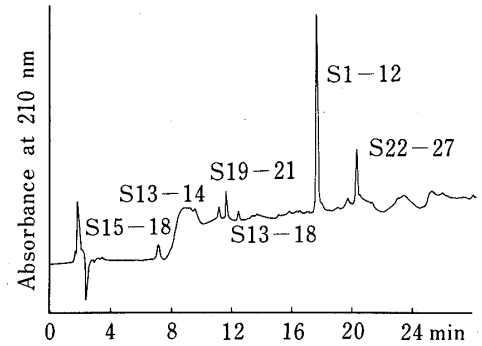

Fig. 4. Separation of Tryptic Fragments from Secretin by HPLC

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

TABLE II. Data for Tryptic Fragments of Synthetic Secretin

Composition	1—12	13—14	15—18	13—18	19—21	22—27
Asp	1.1 (1)		0.9 (1)	1.0 (1)		
Thr	2.0 (2)					
Ser	2.7 (3)		0.9 (1)	0.9 (1)		
Glx	1.0 (1)				1.0 (1)	1.0 (1)
Gly	1.0 (1)					
Ala			1.1 (1)	1.1 (1)		1.0 (1)
Val						1.1 (1)
Leu	1.1 (1)	1.2 (1)		1.0 (1)	1.0 (1)	2.9 (3)
Phe	1.0 (1)					
His	1.0 (1)					
Arg	1.1 (1)	0.8 (1)	1.1 (1)	2.0 (2)	1.0 (1)	
Total	(12)	(2)	(4)	(6)	(3)	(6)

TABLE III. Biological Activity of Synthetic Secretin and Its Diastereoisomers

	Biological activity[a] (c.u./mg)
Crude secretin (HF treated peptide)	3750
Partially purified secretin (CMC)	5500
Purified secretin (HPLC)	5750
[D-Phe^{6}]–secretin	40
[D-Leu^{10}]–secretin	193
[D-Leu^{13}]–secretin	488
[D-Ala^{17}]–secretin	318
[D-Leu^{22}]–secretin	113

a) Stimulating exocrine pancreatic juice in anesthetized rats.

T6, which were theoretically expected to be formed (Fig. 4 and Table II). The optical rotatory dispersion (ORD) and circular dichroism (CD) spectra of the product were similar to those reported by Bodanszky *et al.*[12]

The biological activity of the product was determined as 5750 c.u./mg based on Kabi secretin as a standard in the anesthetized rat, and this was a little higher than that of the CMC-purified peptide (Table III). The biological activity of pure natural secretin has been reported as 4000 c.u./mg and those of other synthetic secretins were reported to be approximately consistent with that of pure natural secretin.[13] Therefore, Karolinska natural secretin, a generous gift from Dr. V. Mutt, was tested for biological activity in the same manner as that used for our synthetic secretin. As its activity was found to be 4770 c.u./mg, our synthetic secretin was 20% more active than Karolinska secretin.

The synthetic diastereoisomers were found to possess weak biological activity (Table III). This result implies that if these diastereoisomers were included in the final product, the biological activity would be decreased.

As mentioned above, highly pure and active synthetic secretin was obtained without any obvious problems after removal of all the side-chain protecting groups followed by ion-exchange chromatography and preparative HPLC. These results suggested that the protected secretin was highly pure and that the formation of by-products was well suppressed during the HF deprotection. Secretin was found to be unexpectedly stable throughout this purification. These results show that our synthetic method should be adaptable to the large-scale production of secretin, which would then be available for clinical use. Confirmation of

the identity of synthetic secretin with the natural product is presented in the following paper.

Experimental

Optical rotations were determined with a JASCO model DIP-140 digital polarimeter. The amino acid compositions of acid and enzyme hydrolysates were determined with a Hitachi model KLA-5 amino acid analyzer. TLC was performed on silica gel (Kieselgel 60 F254, butanol/pyridine/acetic acid/water 15/5/5/8). The spots were made visible by using ninhydrin, Sakaguchi, Pauly and fluorescamine reagents. HPLC was performed with an ALTEX model 110A pump, a RHEODYNE model 7125 injector and a JASCO model UVIDEC 100A ultraviolet (UV) detector. Determination of acetic acid was performed by gas chromatography on a Chromosorb 101 (60—80 mesh) column (3 mm × 2 m). Nitrogen content was determined by the Kjeldahl method. Secretin activity was determined in Wistar–Imamichi rats by a twin-crossover method as described by Tachibana[9] using Kabi (GIH) standard secretin (batch Nos. 162—904, 178—61, 178—111 and 178—121). The secretins were dissolved in saline containing bovine serum albumin in order to avoid the adsorption of secretin on glass surfaces, *etc.*

Deprotection of Protected Secretin with Liquid HF——Protected secretin (2.32 g) was treated for 70 min at −18 to −19 °C with anhydrous liquid HF (50 ml) in the presence of anisole (4.5 ml). After removal of the HF and anisole at the same temperature *in vacuo*, the crude reaction product was dissolved in H_2O, and the fluoride ions were exchanged by treatment with Amberlite IRA-45, acetate form. The mixture was stirred for 25 min at 0 to 5 °C, then the resin was removed by filtration, and the filtrate was lyophilized. Yield: 1.53 g (88%) crude secretin.

Purification of Crude Secretin by Ion Exchange Chromatography——Crude secretin (1.83 g) was purified on a column (2.6 × 30 cm) packed with CMC. The product was dissolved in water (100 ml), applied to the column, and eluted with a linear gradient formed from H_2O (2 l) and 0.3 M $AcONH_4$ (2 l). The flow rate was 2.5 ml/min. Fractions of 25 ml were collected. UV detection was performed at 230 nm. The fraction (fractions 55—129) indicated by a bar (Fig. 1) was lyophilized, followed by lyophilization from H_2O. Yield: 1.17 g. TLC: one major spot, $Rf=0.42$. Biological activity: 5500 c.u./mg.

Purification of CMC-Purified Secretin by Preparative HPLC——CMC-purified secretin (100 mg) was dissolved in 2 ml of the eluent (0.5% HCl/CH_3CN 40/17). It was chromatographed at room temperature on a column (2.0 × 25 cm) packed with Nucleosil $5C_{18}$. The flow rate was set at 9.0 ml/min. The fractions containing the main product were lyophilized (Fig. 2). Lyophilized secretin hydrochloride was exchanged by treatment with Amberlite IRA-45, acetate form. The mixture was stirred for 25 min, then the resin was removed by filtration, and the filtrate was lyophilized. Yield: 70 mg. HPLC: see Fig. 3. Amino acid analysis: see Table I. Biological activity: 5750 c.u./mg. *Anal.* Calcd for $C_{130}H_{219}N_{44}O_{41}\cdot 2AcOH\cdot 11H_2O$: C, 47.34; H, 7.50; N, 18.40. Found: C, 47.80; H, 7.06; N, 18.41. Acetic acid: 4.1% H_2O: 5.3%.

Trypsin Digestion——The synthetic product (111 μg) was dissolved in 1% NH_4HCO_3 (0.9 ml), and trypsin solution (100 μl, 0.1 mg/ml 1% NH_4HCO_3, Millipore Corporation Trypsin-TPCK, 230 u/mg) was added. This solution was incubated at 30 °C for 8.5 h. The solution was lyophilized then the residue was taken up in H_2O (0.2 ml) and kept on a boiling water bath for 6 min. The trypsin digestion mixture (10 μl) was subjected to HPLC. The fragments were separated by reverse-phase HPLC on a column (0.46 × 15 cm) packed with Nucleosil $5C_{18}$, with a linear gradient formed from 0 and 60% CH_3CN in 0.065% trifluoroacetic acid (TFA). UV detection was performed at 210 nm (Fig. 4). Peaks were collected and lyophilized. The structure of each fragment was determined by amino acid analysis (Table IV).

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References and Notes

1) A part of this work was presented at the 102nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April 1982 and the 21st Symposium on Peptide Chemistry, Tsukuba, October 1983.
2) Part I; M. Uchiyama, T. Sato, H. Yoshino, Y. Tsuchiya, T. Tsuda, M. Konishi, M. Tsujii, Y. Hisatake, and A. Koiwa, *Chem. Pharm. Bull.*, **33**, 1990 (1985).
3) W. König, M. Bickel, R. Geiger, R. Obermeier, V. Teetz, R. Uhmann, and H. Wissmann, "First International Symposium on Hormonal Receptors in Digestive Tract Physiology, INSERM Symposium No. 3," ed. by Bonfils *et al.*, Elsevier/North-Holland Biomedical Press, 1977, p. 29.
4) D. Voskamp, C. Olieman, and H. C. Beyerman, *J. Royal Netherlands Chem. Soc.*, **99**, 105 (1980).
5) H. C. Beyerman, M. I. Grossman, T. Scracherd, T. E. Solomon, and D. Voskamp, *Life Sci.*, **29**, 885 (1981).
6) M. I. Groddman, *Gastroenterology*, **57**, 767 (1969); E. Jaeger, S. Knof, R. Scharf, P. Lehnert, I. Schulz, and E. Wunsch, *Scan. J. Gastroenterol.*, 93, (1973).

7) D. Yamashiro and C. H. Li, *J. Org. Chem.*, **38**, 591 (1973); B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3750 (1973); S. Sano and S. Kawanishi, *ibid.*, **97**, 3480 (1975); R. S. Merrifield, *ibid.*, **97**, 3485 (1975).
8) Y. Ogata, K. Igano, K. Inoue, and S. Sakakibara, "Peptide Chemistry 1974," ed. by H. Yajima, Protein Research Foundation, Osaka, 1975, p. 35.
9) S. Tachibana, *Jpn. J. Pharmacol.*, **21**, 325 (1971).
10) A report on the syntheses of the diastereoisomers is in preparation.
11) W. M. M. Schaaper, D. Voskamp, and C. Olieman, *J. Chromatogr.*, **195**, 181 (1980).
12) A. Bodanszky, M. A. Ondetti, V. Mutt, and M. Bodanszky, *J. Am. Chem. Soc.*, **91**, 944 (1969).
13) M. Bodanszky, M. A. Ondetti, S. D. Levine, and N. J. Williams, *J. Am. Chem. Soc.*, **89**, 6753 (1967); M. A. Ondetti, V. L. Naraganan, M. Saltza, J. T. Sheehan, E. F. Sabo, and M. Bodanszky, *ibid.*, **90**, 4711 (1968); E. Wünsch, *Naturwissenschaften*, **59**, 239 (1972); G. Jäger, W. König, H. Wissman, and R. Geiger, *Chem. Ber.*, **107**, 215 (1974); A van Zon and H. C. Beyerman, *Helv. Chim. Acta*, **59**, 1112 (1976); B. Hemmasi and E. Bayer, *Int. J. Peptide Protein Res.*, **9**, 63 (1977); N. Yanaihara, M. Kubota, M. Sakagami, H. Sato, T. Mochizuki, N. Sakura, T. Hashimoto, C. Yanaihara, K. Yamaguchi, F. Zeze, and K. Abe, *J. Med. Chem.*, **20**, 648 (1977); D. Voskamp, C. Olieman, and H. C. Beyerman, *J. Royal Netherlands Chem. Soc.*, **99**, 105 (1980); H. C. Beyerman, P. Kranenburg, W. M. M. Schaaper, and D. Voskamp, *Int. J. Peptide Protein Res.*, **18**, 276 (1981).