Isolation and Partial Structural Analysis of Insulin from the Separate Islet Tissue of *Lophius piscatorius**

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ABSTRACT: The isolation and partial structural analysis of pure insulin from anglerfish (*Lophius piscatorius*) was undertaken as a basis for studies on the biosynthesis of insulin in the separate islet tissue of this fish. A procedure is described for isolation of the insulin, first by gel filtration and then by chromatography on CM-cellulose, with a recovery of about 67% and a yield of pure insulin of about 3 mg/g of wet tissue. A new method for separation of the A and B chains following reduction and carboxymethylation is presented. Amino acid analyses show that the two chains contain the same number of residues as does insulin from beef. The A chain contains one residue each of proline and arginine and has COOH-terminal asparagine (or glutamine). Trypsin does not hydrolyze the arginyl bond in this chain. The B chain contains two residues of proline. The NH_2-terminal sequence is valyl-alanyl-prolyl and that at the COOH terminus is -prolyl-lysine. A procedure is presented for isolating the three residues of proline in three separate peptides.

The endocrine pancreatic tissue of the anglerfish (*Lophius piscatorius*) is in the form of an easily separable and relatively large organ, the Brockmann body (Bargmann, 1939). This tissue might be well suited for studies on the biosynthesis of insulin since the amount of extraneous protein to be removed would be less than with other species. Therefore the isolation and study of the partial structure of the insulin from this fish was undertaken. An earlier report dealt only with the separation of the insulin (Humbel, 1963). Although that insulin preparation was highly active (23 units/mg) and was homogeneous by paper electrophoresis, amino acid analyses later showed that it was impure.

This paper describes the purification of the insulin from anglerfish, the amino acid composition of the separated reduced and carboxymethylated (RCM)[1] chains, their NH_2- and COOH-terminal residues, and the amino acid composition of certain peptides formed by tryptic and chymotryptic hydrolyses. This information is sufficient to serve as a basis for the work on the biosynthesis of insulin described elsewhere (Humbel, 1965).

Experimental Methods

Materials. Sephadex (G-25, G-50, and G-75, medium grade, from Pharmacia, Uppsala) was washed and deaerated as described by Crestfield *et al.* (1962). CM-cellulose (medium grade, capacity 0.8 meq/g, from Carl Schleicher & Schuell Co., lot 1317) was washed according to Peterson and Sober (1962). Dowex 50-X2 (200–400 mesh, in the H^+ form, the Baker Chemical Co., lot 22051) was washed according to Moore and Stein (1951). Urea was deionized (Benesch *et al.*, 1955) prior to use by passing a 10 M solution at room temperature through a mixed-bed resin column (Amberlite MB-1, Mallinckrodt). Iodoacetic acid (Distillation Products, Inc.) was recrystallized twice from petroleum ether. Crystalline Zn-insulin from beef, lot 719106, was kindly provided through the courtesy of Dr. M. Root, Eli Lilly Co. DFP-treated carboxypeptidase A and B (lots CoA-DFP-6127 and CoB-DFP-27), trypsin (twice crystallized, salt free, lot TRL 6246), and chymotrypsin (prepared by ion-exchange chromatography, lot CDC 47) were purchased from the Worthington Biochemical Corp. All other chemicals were analytical grade reagents used without further treatment. Protein concentrations were measured either by their absorbancy at 276 mμ, when column eluates were monitored, or by Kjeldahl digestion and Nesslerization.

Isolation of Insulin from Islet Tissue. Isolated Brockmann bodies from anglerfish were obtained in the frozen state from New England Biological Associates, Narragansett, R. I. The average islet weighs about 70 mg. Islets were cleaned by removal of surrounding fibrous and exocrine pancreatic tissue and by passage through a steel screen. Each gram of strained tissue was homogenized in 10 ml of 10% (w/v) trichloroacetic acid in an all-glass homogenizer. The precipitate was washed three times with 10 ml of 5% (w/v) trichloroacetic acid and then extracted three times with about 7 ml of 74% ethanol, 0.18 N in HCl. Most of the ethanol was removed with a rotary evaporator and a solution of 1 M trisodium citrate was added to bring the aqueous residue to *p*H 3.5–4.5 according to indicator paper. Lipids were then extracted with 3 volumes of methylene chloride. Residual meth-

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[1] Abbreviation used in this work: RCM, reduced and carboxymethylated.

ylene chloride was removed *in vacuo* and a solution of 100%(w/v)trichloroacetic acid was added to a concentration of 5% (w/v). The precipitate which formed on standing for 2 hours at 4° was collected by centrifugation and taken up in 2 ml of 5 M acetic acid, 0.15 M in NaCl. Insoluble proteins were removed by centrifugation and the turbid supernatant liquid was applied to a column (50 × 2 cm) of Sephadex G-75 which had been equilibrated with the same solvent. The pooled fractions which contained the insulin were concentrated by rotary evaporation to a volume of about 2 ml and then desalted by passage through a column (30 × 0.9 cm) of Sephadex G-25 equilibrated with 50% (v/v) aqueous acetic acid.

Chromatography on columns of CM-cellulose was used for the final purification. Essentially, the method of Yamamoto *et al.* (1960) for separation of bonito insulin I and II was followed. The sample of desalted insulin was concentrated in an evacuated desiccator over NaOH pellets until 5–20 mg of protein was in 0.1–0.2 ml of approximately 50% acetic acid, 1–2 ml of 0.5 M sodium acetate buffer of *p*H 4.0 was added, and the solution was applied to a column (30 × 0.9 cm) of CM-cellulose which was equilibrated with the same buffer. Elution was at 20 ml/hr from a mixing chamber of 100-ml capacity which was initially filled with a solution 0.2 M in formic acid and 0.4 M in acetic acid and was fed from a reservoir of 1.2 M formic acid.

Biological assays were carried out by Dr. J. Steinke, Department of Medicine, Harvard Medical School, according to the *in vitro* method of Renold *et al.* (1960). An exactly determined amount of insulin in 50% acetic acid was dried *in vacuo* over NaOH at ambient temperature. For the assay, the insulin was dissolved in a small amount of 0.05 N HCl and further diluted with the buffer used for the adipose tissue assay.

Reduction and Alkylation of Insulin. The reduction and carboxymethylation of insulin was achieved by the procedure described by Crestfield *et al.* (1963) for 5–50 mg of protein, except that one-third or one-sixth of the quantities of reagents was used and the final volume was 4 or 2 ml. The RCM insulin chains were separated from reagents by passing the mixture over a column of Sephadex G-25 (40 × 2 cm or 40 × 1 cm), wrapped in aluminum foil, and equilibrated with 50% acetic acid. Elution of the peptide mixture was monitored by absorption measurements at 276 mμ. The pooled eluate was concentrated in a rotary evaporator at 25° and finally dried in an evacuated desiccator over NaOH pellets at room temperature.

Separation of RCM Chains of Insulin. The RCM A and B chains of insulin were separated on a column of Dowex 50-X2 (12 × 0.9 cm) which was equilibrated with 0.2 M ammonium acetate–acetic acid buffer, *p*H 4.5. The mixture of chains was applied to the column in 1 ml of 0.2 M ammonium acetate buffer to which had been added solid urea to 6 M concentration and formic acid to *p*H 4.5. The RCM A chain was eluted with this same buffer at a flow rate of 0.4 ml/min. As soon as the A chain was eluted, 5 ml of buffer without urea was passed through the column to rinse out the bulk of the urea, and then the RCM B chain was eluted with 1 M ammonium hydroxide. The contact of B chain with ammonium hydroxide was as short as possible, usually about 20 minutes. The eluted B chain was rapidly concentrated in a rotary evaporator at room temperature. Glacial acetic acid was added to make the solution 50% in acetic acid and the solution was further concentrated to about 2 ml. To free the preparations of the A and B chains from residual reagents the two preparations were passed through a column (30 × 0.9 cm) of Sephadex G-25 in 50% acetic acid.

Amino Acid Analyses. Hydrolysis of peptides was performed as described by Crestfield *et al.* (1963) with careful evacuation and deaeration of the hydrolysis tubes. The hydrolysate was dried rapidly in a flash evaporator, dissolved in citrate buffer (*p*H 2.2), and analyzed by means of a Spinco automatic amino acid analyzer according to the procedure described by Spackman *et al.* (1958). More recent analyses were done with an accelerated procedure (Spackman, 1963), a sample injector (Crestfield, 1963), and an experimental photometer ten times more sensitive[2] than the one used in the regular instrument.

The spectrophotometric estimation of the tyrosine-tryptophan ratios in insulin preparations was carried out by the method of Goodwin and Morton as described by Beaven and Holiday (1952).

Enzymic Hydrolyses. For digestion by carboxypeptidase A and B, 0.05 μmole of peptide was dissolved in 45 μl of 0.1 M sodium bicarbonate, *p*H 7.6 (for carboxypeptidase A), or 45 μl of 0.1 M phosphate buffer, *p*H 7.6 (for carboxypeptidase B). Enzyme (25 μg) in 5 μl of buffer was added; the substrate-enzyme ratio was 70:1. The mixture was incubated at 23° for 2 hours. The reaction was stopped by addition of 1.15 ml of citrate buffer, *p*H 2.2, and the sample was analyzed on the 60- and 15-cm columns of the amino acid analyzer.

For digestion by trypsin and chymotrypsin, the enzyme was dissolved in 0.001 N HCl to give a 0.16% solution; 2 or 4 μl, respectively, was added to 0.1 μmole of peptide in 25 μl of 0.2 M ammonium bicarbonate, *p*H 8.1. Substrate-enzyme ratios were about 100:1 and 50:1 (w/w), respectively. Reactions were allowed to proceed at 23° for 90 minutes.

Electrophoretic Fractionation of Enzymic Digests. The enzymic hydrolysates were applied to dry strips of Whatman 3MM paper (6 × 45 cm) which had been soaked overnight in 50% acetic acid, and then hardened with 95% ethanol and dried in air as described by Crestfield and Allen (1957). The spots were allowed to dry between multiple applications. The strip was then thoroughly wetted from both ends with 0.2 M ammonium bicarbonate, *p*H 8.1, placed in the apparatus described by Crestfield and Allen (1955), and subjected to a potential of 800 v (30 ma) for 2–4 hours. After the strips were dried on the heated plate of the apparatus,

[2] A cell of 20-mm light path, like the one described by Crestfield (1963), replaced the 2-mm cell. Cadmium sulfide photoconductive cells (Clairex 607L-167) with batteries were used instead of the usual photovoltaic cells.

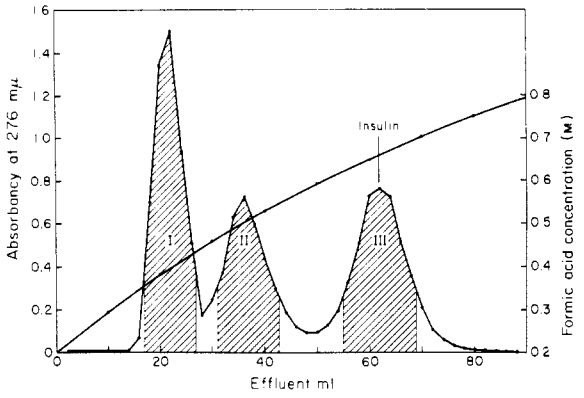

FIGURE 1: Separation of insulin from two small polypeptides by means of CM-cellulose. Protein (5–20 mg) (30 $D_{276m\mu}^{1\,cm}$ units in this case) in 1–2 ml of 0.5 N sodium acetate, acetic acid buffer of *p*H 4 applied to a column (30 × 0.9 cm) which was equilibrated with the same buffer. Elution at 20 ml/hr from a closed mixing flask of 100-ml capacity began with a solution 0.2 M in formic acid and 0.4 M in acetic acid. The reservoir contained 1.2 M formic acid. Peak III is pure insulin, obtained in a yield of about 3 mg/g of wet tissue.

they were kept for an additional 0.5 hour in an oven at 80°. The peptides were localized on a duplicate channel on the same strip by dipping in a solution of 95% alcohol containing 0.5% ninhydrin. Corresponding rectangular areas from the unstained sheet of paper were cut out and one side was trimmed to form a triangular tip. They were developed chromatographically in ascending fashion in 50% acetic acid to concentrate the peptide into the uppermost tip of paper (see Crestfield and Allen, 1956). After 2 hours the tips were dried, held in forceps, cut off from the bulk of the paper piece, and placed in 1 ml of 50% acetic acid in special hydrolysis tubes. After an additional 2 hours the paper tips were removed, the acetic acid was evaporated in a rotary evaporator, and 6 N HCl was added to the tubes for hydrolysis to amino acids.

NH_2- *and* COOH-*Terminal Residues by Chemical Methods.* Edman degradation: The modification by Guidotti *et al.* (1962) was used and the released NH_2-terminal residue was determined by the subtractive method of analyzing the residual peptide. Hydrazinolysis was kindly performed by Mr. M. Ruttenberg, by the method of Akabori *et al.* (1952).

TABLE I: Amino Acid Composition of the A and B Chains of RCM Insulin from Anglerfish.

	RCM Insulin[a]			
	A Chain[b]		B Chain[b]	
Amino Acid	Found	Nearest Integer	Found	Nearest Integer
Aspartic acid	4.4	4	3.1	3
Glutamic acid	3.0	3	1.1	1
Glycine	1.0	1	3.0	3
Alanine	0	0	3.0	3
Valine	0.5[c]	1	2.9	3
Leucine	1.0	1	4.0	4
Isoleucine	1.3[c]	2	0	0
Serine[d]	0	0	0.9	1
Threonine	0	0	0	0
Methionine	0	0	0	0
S-Carboxymethylcysteine	4.1	4	2.0	2
Proline	1.0	1	2.1	2
Phenylalanine	0.9	1	2.0	2
Tyrosine[d]	1.0	1	2.0	2
Lysine	0	0	1.0	1
Arginine	1.0	1	1.0	1
Histidine	1.0	1	2.0	2
Ammonia[e]	4.4	4	2.1	2
Tryptophan[f]	0	0	0	0
Total number of residues		21		30

[a] Each column represents the results of a single analysis by ion-exchange chromatography of a 22-hour hydrolysate. The results are expressed as the number of amino acid residues per molecule, assuming there is one residue of arginine in each chain. [b] Amounts of amino acids less than 0.001 μmole are called zero. At the loads used with the special photometer this amount represents less than 0.1 residue. [c] The isoleucyl-valyl bond is only partly hydrolyzed in 22 hours with beef insulin. [d] Not corrected for loss during hydrolysis. [e] Amide NH_3 is that remaining after correction for free NH_3 in the intact peptide preparation and for adventitious NH_3 produced by acid hydrolysis from reagents and glassware. [f] Tryptophan was determined spectrophotometrically (Beaven and Holiday, 1952).

Results and Discussion

Chromatography by the procedure of Yamamoto *et al.* (1960) of that insulin preparation from anglerfish which is obtained by gel filtration in 30% acetic acid (Humbel, 1963) yields three peaks as shown in Figure 1. Fractions I and II contained tryptophan and methionine, and in amino acid composition resembled glucagon, as isolated from pork (Behrens and Bromer, 1958). These fractions also resembled glucagon in size, as indicated by their retention volumes on this column, and they showed less than 0.5% of the biological activity of insulin. Fraction III is pure insulin which showed in two sets of triplicate determinations a biological potency of 26 and 27 units/mg with an index of precision of 0.1008, and 95% confidence limits of 18–33 and 20–37, respectively. Different preparations of beef insulin show activities in the range of 22–27 units/mg. The total yield of pure insulin was about 3.2 mg/g of wet tissue, cor-

TABLE II: Amino Acid Composition of Tryptic and Chymotryptic Peptides from the RCM B Chain of Insulin.[a]

	Peptide[b]							
	Bt_1		Bt_2		Bt_1c_1		Bt_1c_2	
Amino Acid	Found	Theory	Found	Theory	Found	Theory	Found	Theory
Aspartic acid	2.0	2	1.0	1	0	0	2.3	2
Glutamic acid	1.1	1	0	0	1.0	1	0	0
Glycine	2.0	2	1.0	1	0	0	2.4	2
Alanine	3.0	3	0	0	2.0	2	1.4	1
Valine	2.9	3	0	0	1.1	1	1.9	2
Leucine	4.1	4	0	0	1.0	1	3.1	3
Serine[c]	0.7	1	0	0	0	0	0.8	1
S-Carboxymethyl-cysteine	2.0	2	0	0	0	0	1.3	2
Proline	1.0	1	1.0	1	1.0	1	0	0
Phenylalanine	0	0	1.9	2	0	0	0	0
Tyrosine[c]	0.9	1	0.9	1	0	0	0.7	1
Lysine	0	0	1.0	1			0	0
Arginine	1.0	1	0	0			1.0	1
Histidine	1.9	2	0	0			0.9	1

[a] These results are from 22-hour hydrolysates. The numbers are expressed as the number of amino acid residues per molecule, assuming there to be 4, 1, or 3 residues of leucine in peptides Bt_1, Bt_1c_1, and Bt_1c_2, respectively, and assuming there to be 1 residue of glycine in peptide Bt_2. [b,c] See footnotes *b* and *d*, Table I.

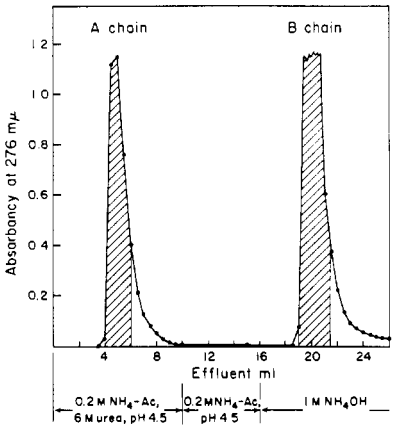

FIGURE 2: Isolation of RCM A chain and RCM B chain from anglerfish insulin by chromatography on Dowex 50-X2. RCM protein (10 mg) was dissolved in 1 ml of eluent and applied to a column of Dowex 50-X2 (12 × 0.9 cm). Rate of flow was 0.4 ml/min for the first buffer and 0.6 ml/min for the 1 M ammonia.

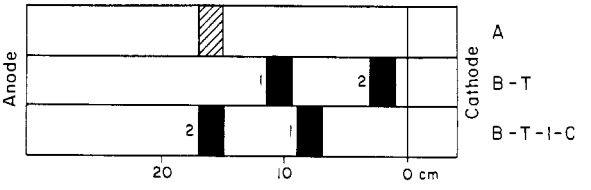

FIGURE 3: Paper electrophoretic separation of the RCM A chain and of tryptic and chymotryptic hydrolysates of the RCM B chain of insulin. NH_4HCO_3 (0.2 M) was used on Whatman 3MM paper. Peptides were detected with ninhydrin. Each sample was separated in 3 hours at 800 v across paper of 45 cm length.

responding to a recovery of about 64% based upon bioassays of homogenates of islet tissue.

The methods of separation of the A and B chains from RCM insulin of beef which was described by Crestfield *et al.* (1963) could not be applied to this insulin since separation was unsatisfactory. Small-scale tests showed that at *p*H 4.5 neither chain would dissolve in the absence of urea, whereas at about *p*H 8 the B chain did dissolve without addition of urea. Therefore a convenient preparative procedure was devised in which Dowex 50-X2 is employed, first with a buffer containing urea to elute the A chain and then rapidly with 1 M ammonia to elute the B chain. A typical separation is shown in Figure 2. The separated chains were pure, as seen in Table I from the absence of lysine in the A chain and the absence of isoleucine in the B chain. The total number of residues is 21 in the A chain and 30 in the B chain. These values are the same as for beef insulin.

The two chains yielded the following results upon application of the carboxypeptidase and Edman procedures for NH_2- and COOH-terminal residues: *RCM A chain:* Carboxypeptidase A released 0.92 residue of asparagine (or glutamine) and traces of CM-cysteine, tyrosine, and aspartic acid. The COOH-terminal residue is therefore likely to be asparagine, since all other insulins studied thus far have this amino acid in that

position.[3] *RCM B chain:* Carboxypeptidase A showed no reaction. Carboxypeptidase B released 0.76 residue of lysine, showing that the COOH terminus is lysine, which in other insulins is usually found second from this end. Edman degradation established the NH_2-terminal sequence as valyl-alanyl-prolyl with the following analyses:

	Val	Ala	Pro
B chain	2.92	2.90	2.00
Stage 1	*1.96*	2.82	1.69
Stage 2	1.90	*2.07*	1.80
Stage 3	1.95	1.90	*1.19*

Tryptic and chymotryptic hydrolyses of the two chains were carried out in order to localize, approximately, the 3 residues of proline, since it was incorporation of this amino acid which was to be studied in the biosynthesis of insulin. Only very limited amounts of the peptides were available, but the added sensitivity of the experimental amino acid analyzer encouraged a trial of paper electrophoresis for isolation of the enzymically produced fragments. Trypsin failed to hydrolyze the arginyl bond in the A chain. This is found to be the case also with other fish insulin (Kotaki, 1963). Tryptic hydrolysis of the B chain yielded two peptides, as seen in Figure 3. The analyses shown in Table II identify these as a peptide (Bt_1) containing 23 residues, including 1 residue of arginine, and a peptide (Bt_2) containing 7 residues, among them 1 proline and the single lysine residue from the COOH terminus of the B chain. Chymotryptic hydrolysis of peptide Bt_1 yielded the two peptides shown in Figure 3. Their analyses shown in Table II identify them as a peptide (Bt_1c_1) containing 6 residues, including proline *3*, near the NH_2 terminus of the B chain and a peptide (Bt_1c_2) of 16 residues. In order to establish the position of the second proline in the B chain, the COOH-terminal lysine was removed from peptide Bt_2 by carboxypeptidase B. The remaining hexapeptide was then subjected to hydrazinolysis. Proline was found in 30% yield, thereby establishing it to be residue 29, adjacent to the terminal lysine. Hence, by a combination of chromatographic and electrophoretic procedures, three peptides are easily resolved, each one containing a different one of the 3 residues of proline in this insulin.

[3] L. F. Smith, personal communication.

Acknowledgments

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