ISOLATION OF INSULIN FROM THE FISH, LOPHIUS PISCATORIUS, BY GEL FILTRATION

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Previous methods for purification of insulin from isolated islets of fishes* entailed the use of acid-ethanol extraction in combination with paper chromatography (Bauer and Lazarow, 1961; Humbel et al., 1961; Taylor et al., 1961), isoelectric precipitation and CM- and DEAE-cellulose chromatography (Yamamoto et al., 1960; Humbel, 1963). The more simple procedures provide insufficient purification, and with the more elaborate methods there is a relatively low yield because of small losses in each of several steps.

In aqueous solution near neutral pH insulin is present as aggregates which are multiples of the molecular weight unit of 5750. It can be converted to the monomer using extremes of pH, solvents of low dielectric constant, or concentrated guanidine chloride (cf. Yphantis and Waugh, 1957). The low molecular weight of the insulin monomer, compared to that of most other tissue proteins, suggested the use of gel filtration for separating insulin from tissue proteins of higher molecular weight. This paper describes a simple procedure based on Sephadex gel filtration, which allows the preparation of pure insulin in high yield.

While this work was in progress, a report by Davoren (1962) and one by Epstein and Anfinsen (1963) appeared on isolation of insulin from mammalian pancreas using Sephadex gel filtration.

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* In certain teleost fishes the islets of Langerhans form one separate organ, called the principal islet or Brockmann body, which, in the case of Lophius piscatorius, is free of pancreatic exocrine tissue (cf. Bargmann, 1939).

METHODS

Anglerfish (Goosefish, Lophius piscatorius) were obtained from a fisherman in Narragansett, R.I., and from New England Biological Associates, Narragansett, R.I. The islets of freshly caught fish were immediately excised, frozen and shipped in the frozen state. After slow thawing, they were carefully cleaned of the surrounding pancreatic exocrine and fibrous tissue, pressed through a steel mesh and homogenized in water at 4°C. The proteins were precipitated in 10% TCA. The precipitate was washed three times with 5% TCA and then extracted three times with acid-ethanol (.18N HCl in 74% ethanol (Jephcott, 1931)) at room temperature. The alcohol of the pooled extracts was removed in a rotary evaporator and the aqueous residue brought to pH 4 with sodium-citrate buffer. Lipids were then extracted by shaking three times with equal volumes of methylene chloride at 4°C. The remaining methylene chloride traces were evaporated and the aqueous residue precipitated in 5% TCA. The precipitate was washed with ether, dried and redissolved in 1 ml 5M acetic acid/.15M NaCl. The insoluble proteins were centrifuged off and the supernate applied on a column (40 x 1 cm) of Sephadex (G-75, medium, Pharmacia Uppsala), which had been previously equilibrated with the same solvent. The eluate was collected in .5 ml portions, the insulin peak pooled, concentrated to .5 ml and run a second time over the same column. The final protein was precipitated in 5% TCA, washed with ether and dried.

Oxidation of insulin was performed according to Sanger (1949).

Paper electrophoresis of the purified insulin and its chains was carried out on Schleicher and Schuell paper # 2043 in 5M acetic acid pH 2.8 at a potential gradient of 8 V/cm. The proteins were stained with Pauly's reagent according to Brown et al. (1955).

Protein determinations were made according to Lowry et al. (1951) using a mixture of bonito and tuna insulin (Shimizu Seiyaku Co., Japan) as standard. Crystalline beef insulin was a gift of Dr. M. Root, Eli Lilly Co., Indianapolis (batch # 711906).

Determinations of the biological activity of the different protein fractions was kindly performed by Dr. J. Steinke, Harvard Medical School, by the method of Renold et al. (1960).

RESULTS

The elution pattern of the defatted acid-ethanol extract of islet tissue from the Sephadex column is shown in fig. 1.

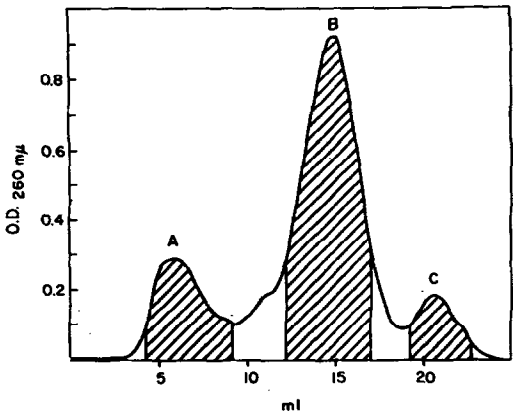

Fig. 1. Elution pattern of acid-ethanol extract of islet tissue from a Sephadex column in 5M acetic acid/.15M NaCl.

Peak A has a biological activity of less than .12 U/mg protein, whereas Peak B has a activity of 18 U/mg. Peak C contains no protein. It consists probably of lipid material, since its size varies inversely with the completeness of the previous lipid extraction with methylene chloride. Electrophoresis of Peak B revealed a band moving like bonito-tuna insulin, but probably slightly contaminated with other proteins.

Upon refiltration of Peak B on the same column, the elution pattern of fig. 2 was obtained.

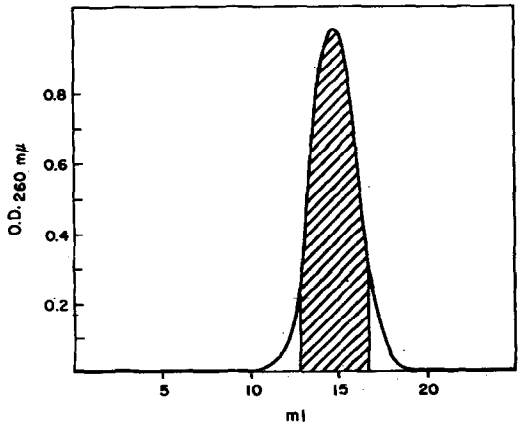

Fig. 2. Elution pattern of refiltrated Peak B of fig. 1 on Sephadex in 5M acetic acid/.15M NaCl.

The final protein has a biological activity of 23 U/mg (average of two determinations in triplicate each, λ = .10, 95% confidence limits 70 - 150%). Electrophoresis in 5M acetic acid showed one single band corresponding to bonito-tuna insulin (fig. 3a). Upon oxidation, two bands were obtained corresponding to the A and B chain, resp., of oxidized bonito-tuna insulin (fig. 3b). No other bands were detectable*.

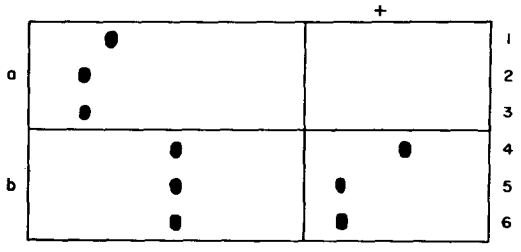

Fig. 3. Schematic representation of paper electrophoresis in 5M acetic acid of
a) intact insulin of beef (1), bonito-tuna (2) and anglerfish (3)
b) oxidized insulin of beef (4), bonito-tuna (5) and anglerfish (6).

Similar results were obtained in 20% formic acid pH 1.2.

The defatted acid-ethanol extract contains proteins insoluble in 5M acetic acid/.15M NaCl. These were probably denatured by the methylene chloride treatment. When these acetic acid-insoluble proteins were applied as a suspension on the Sephadex column, part of them formed a crust on top of the column, and another part emerged at the position of peak A in fig. 1.

Recovery of purified bonito-tuna insulin after Sephadex filtration was 97%, determined by protein measurement, and 102%, determined by measurement of biological activity.

The yield of insulin was consistently around 80% of the total insulin in the original homogenate, as determined by measurement of biological activities. The loss of 20% can wholly be accounted for by the cutting of the eluted insulin peak from the Sephadex columns. The yield

* Previously four bands were obtained upon electrophoresis of oxidized bonito-tuna or toadfish insulin, but not of beef insulin (Humbel, 1963). This, however, represents an artifact due to local overheating of the applied material at the start of the run.

of insulin is approximately 4 mg/g wet weight tissue (equivalent to about 10 islets from as many fish) or 80 mg/g total islet tissue protein.

DISCUSSION

While investigating the biosynthesis of insulin it became desirable to modify the purification procedure previously worked out for insulin (Humbel, 1963), since the yield and the biological activity of the final product were not entirely satisfactory.

Yphantis and Waugh (1957) have studied the conditions required for complete dissociation of insulin into its monomers. From their studies it would appear that under the conditions used by Davoren (1M acetic acid), insulin is not completely dissociated. The concomitant presence of insulin monomers, dimers and tetramers is possibly responsible for the incomplete separation of insulin from other proteins as obtained by this author by gel filtration.

Epstein and Anfinsen (1963) used .2M NH_4HCO_3 pH 7.8 as solvent, conditions which favour aggregation of insulin to units of 36,000 - 48,000 molecular weight. Under these conditions the separation of insulin from other proteins seems to be less satisfactory than the one obtained with insulin monomers.

The procedure described here is relatively simple and appears to be superior to the ones used by Davoren and by Epstein and Anfinsen, both with respect to yield and purity of the final product, as assessed by the determination of its biological activity and by paper electrophoresis of the intact and oxidized protein. However, it should be mentioned that the isolation of insulin from mammalian pancreas is a more difficult task than its isolation from the separate islet tissue of fishes.

The determination of the amino acid composition and of the terminal amino acid residues of this particular fish insulin is now in progress.

ACKNOWLEDGMENTS

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