ISOLATION OF RABBIT INSULIN

Jiří JIRÁČEK[a], Jana BARTHOVÁ[a] and Tomislav BARTH[b]

[a] *Department of Biochemistry, Charles University, 128 40 Prague 2 and*
[b] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6*

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Insulin extract has been prepared from rabbit disintegrated pancreas by means of acidic ethanol extraction in combination with ammonium acetate precipitation and purified by permeation chromatography. The enriched preparation of rabbit insulin has been fractionated further by affinity chromatography on AH-Sepharose 4B substituted with polylysine.

Porcine and rabbit insulins are structurally closest to the human insulin. These three hormones differ only in the carboxy-terminal amino acid of the B chain. In human insulin amino acid threonine is at this position, in porcine insulin it is replaced with alanine and in rabbit insulin with serine. The world production of insulin is based mainly on processing porcine pancreas, whereas negligible attention is paid to the rabbit insulin. From the times of publication of rabbit insulin structure[1], only few information concerning it has been presented about the isolation of the rabbit C-peptide[2] and transpeptidation of rabbit insulin to human insulin[3].

This work presents results of the isolation of insulin from rabbit pancreas and its purification by means of affinity chromatography.

EXPERIMENTAL

Materials

Porcine insulin was obtained from Dr T. Buchvaldek (Léčiva Prague). Sephadex G-50 Medium and AH-Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden), N,N,N′,N′-tetramethylenediamine (TEMED*) from Fluka (Buchs, Switzerland), N,N′(methylene)-bis-acrylamide from BDH-Chemicals (Pool, Great Britain). Amido-black, bromphenol blue and ammonium persulfate were supplied by Merck (Darmstadt, F.R.G.). We are obliged to Mrs H. Janešová from the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences, Department of Organic Synthesis I, for giving us the homopolymer L-polylysine with

* Abbreviations: TEMED N,N,N′N′-tetramethylenediamine, Bis N,N′(methylene)-bis-acrylamide, EDC N-ethyl-N′(3-di-methylaminopropyl)carbodiimide, R_p relative mobility, Tris tris(hydroxymethyl)aminomethane.

average molecular weight 67 000 daltons. All the other chemicals were purchased from Lachema (Brno, Czechoslovakia) and were of analytical grade.

Methods

Isolation of the crude rabbit insulin was carried out according to the method described for the isolation of rabbit C-peptide[2] at temperatures of 2—4°C, if not stated otherwise. Rabbit pancreases taken from rabbit slaughter house in Kolín (Prague Poultry Enterprise, Libuš, works Kolín IV) have been used for the preparation.

Pancreases from killed rabbits were frozen immediately in solid carbon dioxide and stored at −12°C. Organs weighing approximately 1 kg were unfrozen overnight, ground in a precooled meat-mincer and disintegrated for 1 min in a mixer with 0·5 l of 86% ethanol the pH of which was adjusted with phosphoric acid to 3·0. Further 2 l of 86% ethanol, pH 3·0 cooled to 3°C were added to the homogenized tissue. The extraction proceeded for 4 h under intermittent stirring. The suspension was filtered over gauze and the tissue was extracted again with 1·5 l of 65% ethanol acidified with phosphoric acid (pH 2·9). The extract obtained after 1 h was combined with the first extract and left for 20 h 3°C. The pH was then adjusted to 8·3 with concentrated ammonia. Precipitated proteins were filtered over silica gel and the pH of the extract was adjusted to 5·3 with 6M-HCl. An amount of 67 ml of ammonium acetate (2 mol l^{-1}, pH 5·3) were added to the extract (2·7 l; v : v = 40 : 1). Then 2 volumes of 99% ethanol, i.e. 5·5 l, and 4 volumes of diethylether (11 l) were added. After 20 h standing at 3°C, the precipitate was centrifuged (Janetzki K-24, 1 500 rpm, 20 min, 4°C). Acetic acid (25 ml, 7 mol l^{-1}) was added to the sediment (the pH was altered to 4·0) and the extract was stored at 4°C until further processing.

Gel chromatography on Sephadex G-50 Medium was employed for the separation of compounds with different molecular weights. Details are presented in Results and in texts to figures. The elution course was monitored by measuring the eluate absorption at 280 nm. Concentration of proteins was determined according to Lowry et al[4]. Electrophoresis in polyacrylamide gel was carried out either in 17% or 7·5% separation gel in alkaline buffer by the method of Maurer[5]. Porcine insulin was mostly used as a standard.

AH-Sepharose 4B was modified with L-polylysine[6]. An amount of 19·6 mg of L-polylysine was dissolved in 30 ml water, 1 ml of swollen AH-Sepharose was added and then solution of 10 mg carbodiimide in 1 ml water was added successively under continuous stirring. The mixture was stirred for 24 h at laboratory temperature, the modified Sepharose 4B was rinsed with water acidified with HCl to a pH value 4·5, then with 0·5M-NaCl and finally, with 0·1M Na-phosphate buffer, pH 7·5. The modified Sepharose was stored in this buffer at the temperature of 4°C.

Affinity chromatography was carried out on a column with modified AH-Sepharose (1 × 1·5 cm). Samples of porcine insulin or crude rabbit insulin were dissolved in 100 µl of 0·5M acetic acid, pH of the solution was adjusted to 7·5 with 1M-NaOH and water was added up to 1 ml. Two types of elution were employed. First, elution of the adsorbed material with 1M and 1·5M acetic acid, respectively, and second, elution with buffers of decreasing pH (for the range between 7·5 to 6·0 with 0·1M Na-phosphate buffer, for the range between 6·0 to 3·0 with 0·05M citrate buffer). The elution was carried out at laboratory temperature at the speed 0·15 ml min^{-1}. The absorbance of the eluate was monitored at 280 nm and the amount of insulin was ascertained from the calibration curve.

RESULTS

From 1 kg of rabbit pancreas 55 ml of acidic pancreatic extract was obtained by

following the process described above. This extract was separated further on the column of Sephadex G-50 Medium (Fig. 1). Fractions corresponding to individual peaks were pooled, lyophilized and concentration of proteins was determined in the samples (Table I). Fractions I and IV were analyzed by means of electrophoresis in polyacrylamide gel using commercial porcine insulin as standard (Fig. 2), as it was presumable that the mobilities of human, rabbit and porcine insulin would be alike[7]. The presence of insulin was proved mainly in fraction IV, in agreement with data

TABLE I

Summary of protein contents in fractions obtained after chromatography of rabbit pancreas extracts on the column of Sephadex G-50

Fraction	I	II	III	IV	V	VI
Protein contents mg	6·90	2·66	4·76	3·94	2·10	13·80

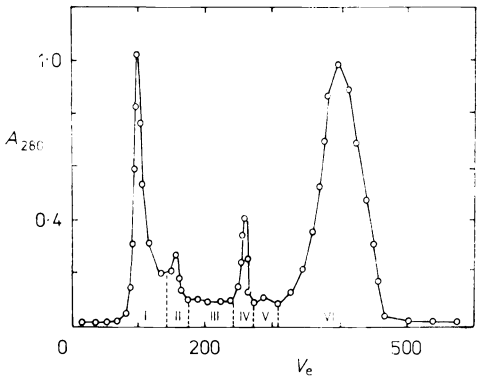

FIG. 1

Permeation chromatography of the extract from rabbit pancreases. An amount of 10 ml (40 mg of proteins) of the acidic extract were applied on a column of Sephadex G-50 Medium (3·6 × 50 cm) in 1M-CH_3. .COOH. Elution with 1M-CH_3COOH was carried out at laboratory temperature at a rate 3—4·5 ml per h. A_{280} absorbance at 280 nm, V_e eluate volume in ml

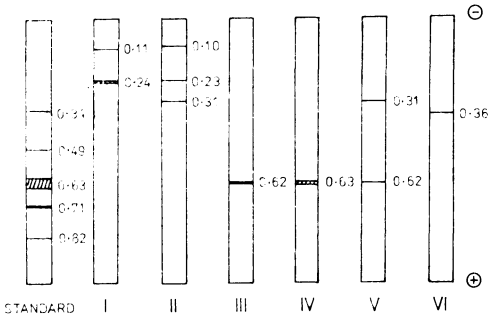

FIG. 2

Electrophoresis of fractions after permeation chromatography of the rabbit pancreas extract. The electrophoresis was carried out at pH 8·3 in 17% polyacrylamide gel. Porcine insulin was used as standard, mobility of protein bands (detected with amido black) is related to the mobility of bromphenol blue

published by Chance et al.[2], and partially also in fractions III and V. The insulin content in fraction IV gradually decreased during storage. Thus, the balance of the yield was ascertained from the chromatography of the fresh insulin extract. From combined fractions III to V 6 mg of crude insulin were obtained which corresponds to 30 to 35 mg of insulin from 1 kg of rabbit pancreas. This yield is in good agreement with yields of porcine insulin isolated by established technological processes (approximately 80 mg from 1 kg of porcine pancreas[8]).

We employed affinity chromatography for further purification of rabbit insulin. On the basis of findings of Lutz[9-13] that insulin forms complexes with basic peptides, we utilized synthetic L-polylysine as the ligand. L-Polylysine was bound covalently by its carboxy group to the amino group of AH-Sepharose 4B by means of soluble carbodiimide. First, conditions for the adsorption and elution from this modified carrier were studied for commercial porcine insulin.

In Table II are presented results of insulin adsorption to the modified carrier and its elution (column III and IV). AH-Sepharose 4B (column V), AH-Sepharose 4B with L-polylysine bound only by adsorption (column I) and finally, carrier which was for 24 h in contact with carbodiimide alone (column II), served as controls.

AH-Sepharose 4B with bound L-polylysine in the amount 19·52 mg ml^{-1} (Table II, column III) proved to be the most favorable carrier for binding insulin. Control experiments (column II and I) show that other mechanisms take place too during the interaction of insulin with AH-Sepharose 4B.

AH-Sepharose·4B with bound L-polylysine (19·53 mg ml^{-1}) was finally employed for the purification of crude rabbit insulin. Elution of the adsorbed insulin was carried out first with Na-phosphate buffer (pH values decreasing from 7·5 to 6·0) and then with citrate buffer (pH decreasing from 6·0 to 3·0 – Fig. 3). Fractions corresponding to individual peaks were pooled, dialyzed shortly (3 exchanges of medium during 120 min), lyophilized and analyzed by means of electrophoresis in 7·5 polyacrylamide gel. Figure 4 shows electrophoretic mobilities of the two major components of porcine insulin- the faster component is deamidoinsulin, the slower one is insulin. Results of the electrophoretic analysis proved affinity chromatography to be a favorable method for the separation of both components in the commercial insulin preparation.

The method of separation on AH-Sepharose 4B with bound polylysine was employed for the purification of combined fractions of rabbit insulin (fractions III to V) obtained after permeation chromatography on Sephadex G-50 Medium. Electrophoretic analysis of fractions III–V (Fig. 5) revealed heterogeneity of the material with significant portion of insulin (R_p 0·62) and probably proinsulin (R_p 0·30).

An amount of 1·25 mg of the sample containing rabbit insulin were applied to a column of AH-Sepharose 4B with bound L-polylysine. Non-adsorbed material was lyophilized after short dialyzation and subjected to electrophoretic analysis together, with fractions eluted from the column with the buffer solution with a decreasing

TABLE II

Chromatography of porcine insulin on derivatives of AH-Sepharose 4B. I AH-Sepharose 4B with adsorbed L-polylysine (8·65 mg ml^{-1}), II AH-Sepharose 4B modified with EDC, III AH-Sepharose 4B with bound L-polylysine (19·53 mg ml^{-1}), IV AH-Sepharose 4B with bound L-polylysine (10·0 mg ml^{-1}), V AH-Sepharose 4B

Fraction	Carrier				
	I	II	III	IV	V
Insulin applied, mg	0·56	0·56	0·56	0·56	0·56
Insulin adsorbed, mg	0·33	0·35	0·40	0·37	0·16
Insulin eluted with 1M-CH_3COOH, mg	0·33	0·07	0·40	0·37	0·04
Insulin eluted with 1·5M-CH_3COOH, mg	0·00	0·29	0·00	0·00	0·11

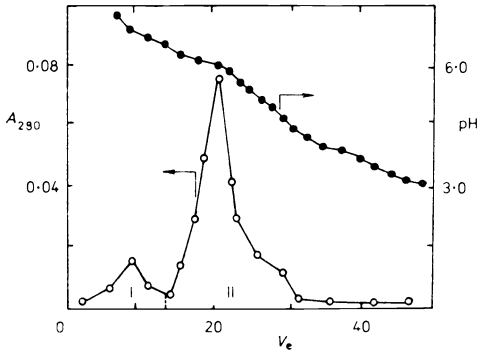

FIG. 3

Elution of porcine insulin adsorbed on a column of AH-Sepharose 4B with bound L-polylysine with solution of linearly decreasing pH. Porcine insulin (1 mg) was applied to the column and eluted with Na-phosphate buffer (pH 7·5—6·0) and citrate buffer (pH 6·0—3·0). Details are described in Methods. A_{280} absorbance at 280 nm, V_e eluate volume in ml

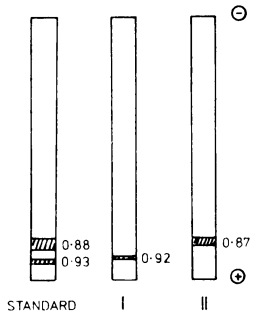

FIG. 4

Electrophoretic analysis of fractions after chromatography of porcine insulin on AH-Sepharose 4B with bound L-polylysine. Electrophoresis was carried out in 7·5% gel, mobility of insulin components are related to that of bromphenol blue

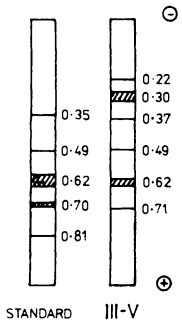

FIG. 5

Electrophoretic analysis of fractions III—V containing rabbit insulin. The electrophoresis was carried out with 60 μg of the mixture containing rabbit insulin in 17% polyacrylamide gel. The mobility of individual components was related to that of bromphenol blue. Porcine insulin was used as standard

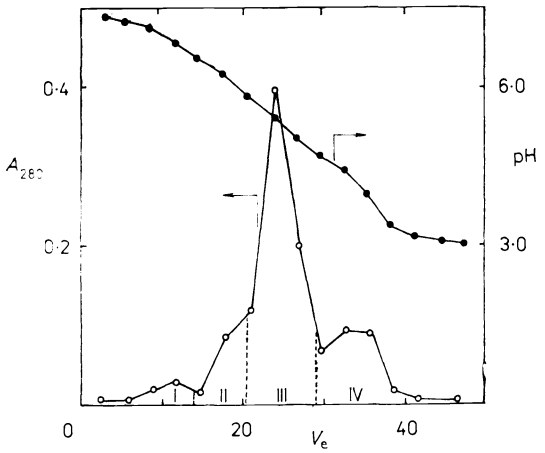

FIG. 6

Elution of crude rabbit insulin adsorbed on a column of AH-Sepharose 4B with bound L-polylysine with a solution with decreasing pH. Experimental conditions are given in Methods. A_{280} absorbance at 280 nm, V_e eluate volume in ml

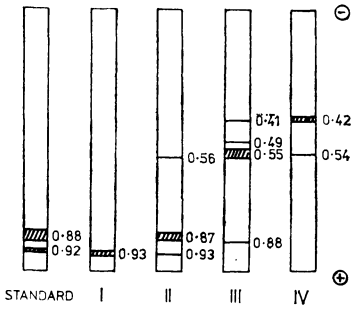

FIG. 7

Electrophoretic analysis of fractions of rabbit insulin obtained by chromatography on AH-Sepharose 4B with bound L-polylysine. Standard — porcine insulin standard; fractions I—IV were separated in 7·5% polyacrylamide gel; the mobility of components was related to that of bromphenol blue

pH (7·5–3·0). Figure 6 depicts the course of the elution, individual peaks being denoted I–V. Figure 7 shows the results of electrophoretic analysis of individual fractions. Rabbit insulin was eluted in fraction II and it was detected also in fraction III constituted mainly of proinsulin.

DISCUSSION

Almost negligible attention paid so far to the isolation of rabbit insulin can be attributed not only to the market coverage with porcine insulin but also to difficulties connected with the collection of rabbit pancreases. This situation is illustrated also in the work of Soviet authors who did not isolate rabbit insulin from pancreas for the transpeptidation into the human insulin[3] but prepared it by means of transpeptidation of porcine insulin[14]. For the isolation of insulin from rabbit pancreas we employed first the method developed for the isolation of porcine insulin, however without great success. Thus, we adopted the method suggested for the isolation of rabbit C-peptide[2]. The enriched rabbit pancreas extract was purified on the column of Sephadex G-50. The content of insulin in the extract decreased gradually during storage at −12°C, until it disappeared completely. On the basis of findings of Lutz[9–13] on the interaction of basic peptides with insulin, we prepared AH-Sepharose 4B with bound L-polylysine for affinity chromatography of insulin. We verified the method first by using it for the purification of porcine insulin and then we employed it successfully for the preparation of rabbit insulin. In the course of elaboration of the method, some difficulties occurred caused by nonspecific electrostatic binding of L-polylysine to the carrier. From Table II it is evident that polylysine adsorbed on the AH-Sepharose 4B binds a part of insulin in a similar manner as L-polylysine bound covalently to the carrier. Thus, the adsorbed L-polylysine had to be removed completely from the carrier before the chromatography of porcine or rabbit insulin, as the adsorbed L-polylysine would be eluted in a complex with insulin and its presence in the eluate would result in altered electrophoretic mobilities of insulins. The adsorbed L-polylysine was removed by rinsing of the modified carrier with 1 M-NaCl in Na-phosphate buffer pH 8·0 and 5·5. The carrier was equilibrated in 0·1 M Na-phosphate buffer, pH 7·5 and thus prepared for the affinity chromatography. The polyacrylamide electrophoresis revealed the possibility for employing affinity chromatography for purification of rabbit insulin, as well as for the separation of some impurities from commercial porcine insulin.

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