AFFINITY CHROMATOGRAPHY OF INSULIN ANTIBODIES

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Dedicated to Professor F. Šantavý on the occasion of his 60th birthday.

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The following copolymers were prepared for affinity chromatography of insulin antibodies: Sepharose 4B-insulin, Spheron P-300-insulin, Sepharose 4 B-NH$(CH_2)_6$NH-insulin, Spheron P-300-NH$(CH_2)_6$NH-insulin, and Enzacryl AH-insulin. Except for Enzacryl AH-insulin, whose specific binding capacity for insulin antibodies was negligible, the immunosorbents prepared specifically bound insulin antibodies. These immunosorbents can be classified in order of decreasing binding ability as follows: Spheron P-300-NH$(CH_2)_6$NH-insulin, Sepharose 4 B-insulin, Spheron P-300-insulin, and Sepharose 4 B-NH$(CH_2)_6$NH-insulin. The antibodies adsorbed were displaced from the sorbents by 0·02M citrate buffer at pH 3·2 in 80–90% yield.

Different types of copolymers of insulin with insoluble supports have been employed successfully for the isolation of antibodies and cellular insulin receptors[1,2]. The copolymers were obtained by coupling covalently insulin through primary amino groups (Phe, Gly, Lys) and tyrosine and histidine residues of its peptide chain to the functional groups of the activated support.

In this paper we report on the preparation of some new types of specific adsorbents of insulin antibodies. Their properties are compared with the characteristics of adsorbents derived from Sepharose 4B, Enzacryl AH, Spheron P-300, and aminohexamethylene-Spheron P-300 were used for the preparation of the new types of adsorbents. Enzacryl AH activated by nitrous acid reacts with the primary amino groups of insulin and an amide bond is formed. Spheron P-300, a new type of support based on a methacrylate copolymer and containing hydroxyls as functional groups[3], binds after activation by cyanogen bromide (similarly to Sepharose) insulin through its amino groups. Spheron P-300 and Sepharose 4B to which a spacer (hexamethylenediamine) had beed coupled by the cyanogen bromide method, were used as supports for the binding of insulin through its carboxyl groups. The reaction was catalyzed by a water soluble carbodiimide. All insulin copolymers were tested for their ability to adsorb specifically guinea pig insulin antibodies.

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EXPERIMENTAL

Materials

Enzacryl AH was purchased from Koch-Light, Sepharose 4 B was from Pharmacia, Uppsala. Spheron P 300 and hexamethylene diamine were products of Lachema, Brno. A standard sample of Spheron P-300 was kindly supplied by Dr J. Čoupek, Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences. The Zn-salt of mixed insulin (porcine-bovine) was a product of Spofa; insulin used for immunization was from Novo, Denmark. 1-Ethyl-3-(3-dimethylamino)--propylcarbodiimide was purchased from Fluka, Switzerland. Zn-Insulin was converted into its hydrochloride[4] before being coupled to the support.

Methods

Enzacryl AH-insulin. Activated Enzacryl AH(100 mg, ref.[5]) in 10 ml of 0·05M borate buffer at pH 8·8 was treated with 1 ml of insulin solution (10 mg of insulin . HCl in 1 ml of 0·001M-NaOH) and the total volume was made up to 100 ml with the borate buffer. The mixture was slowly stirred 24 h at 2—3°C, the gel was centrifuged off, washed several times with the borate buffer containing 1M-NaCl, and finally with the buffer only. The gel suspension with attached insulin was stored at 7°C.

Sepharose 4 B-insulin. Sepharose 4 B (1 ml) was diluted with 1 ml of water, 1 ml of 10% CNBr was subsequently added, and the pH of the solution was kept at 11 by the addition of 2M-NaOH with constant stirring. After a period of 12—15 min the gel was washed with 30 ml of cold borate buffer and immediately mixed with a solution of 10 mg of insulin. HCl in 1 ml of buffer. The mixture was stirred 24 h at 2—3°C and then washed with 30 ml of the buffer containing 1M-NaCl and subsequently with the same volume of the buffer only.

Spheron P-300-insulin. This insulin derivative was prepared as described above. One ml of Spheron P-300 (c. 250 mg of dry matter) was used for the reaction. Spheron P-300-insulin was kept in borate buffer at 4°C.

Binding of insulin through COOH-groups. Sepharose 4 B and Spheron P-300 to be used for covalent binding of insulin through carboxyl groups were first modified by the attachment of a spacer, hexamethylenediamine. Supports activated by CNBr as described above were used for the reaction. The binding sites for insulin were thus sterically separated from the compact matrix of the supports.

Preparation of aminohexamethylene-Sepharose 4 B and aminohexamethylene-Spheron P-300. The supports were activated by CNBr as described above. After washing with borate buffer, 1 ml of the support was mixed with 1 ml of buffer and treated with 2 mmol of hexamethylenediamine dissolved in 1 ml of water. The pH of the solution was adjusted first to 10 by 6M-HCl. The suspension was stirred slowly 16 h at 4°C and washed subsequently with 0·1M carbonate buffer at pH 9. The modified product obtained served for binding insulin through its carboxyl groups.

Hexamethylene-Sepharose 4 B-insulin. A suspension of aminohexamethylene-Sepharose 4 B was washed with 0·05M borate buffer at pH 8·9 and finally mixed with an equal volume of the buffer. One ml of the suspension was mixed with a solution of 10 mg of insulin. HCl dissolved in 2 ml of water and then treated dropwise with 200 mg of carbodiimide dissolved in 1 ml of H_2O. The mixture was stirred 20 h at room temperature. The suspension was subsequently washed with 1M-NaCl solution in the borate buffer and finally with the buffer only. The modified product was kept at 2—3°C.

Hexamethylene-Spheron P-300-insulin was prepared as described for the analogous Sepharose derivative.

Preparation of antiinsulin serum. Guinea pigs were immunized with insulin Novo. The immunization has been described in detail elsewhere[6]. The titer of the serum was determined by the agglutination method and by the radioimmunoassay according to Sirakov and Ditzov[7]. ^{125}I-insulin was also prepared from insulin Novo (ten times recrystallized) by the method of Greenwood and coworkers[8].

Isolation of antibodies from serum. A 0·5 ml portion of each individual gel was washed with 20 ml of H_2O, 3-times with 6 ml of 0·04M Na-phosphate buffer at pH 7 containing 0·1% sodium azide, and finally with 1 ml of phosphate buffer containing 0·25 mg of human blood serum albumin. The supports were mixed with 1 ml of the antiserum (either concentrated or diluted 20-times); the mixture was incubated 20 h at 20°C. The immunosorbents were packed into columns, washed 5-times with 3 ml of 0·04M phosphate buffer, and eluted twice with 1 ml of 0·02M citrate buffer at pH 3·2. The eluates were immediately dialyzed and concentrated by dialysis against 15% dextran. The presence of antibodies in samples from the column was tested by the radioimmunological assay[6]. After the completion of the elution the columns were washed with 50 ml of H_2O and stored at 2°C before being used again.

Test of nonspecific adsorption of insulin antibodies. Enzacryl AH, aminohexamethylene-Sepharose 4 B, and aminohexamethylene-Spheron P-300 were tested as described above as such and after contact with an insulin solution (0·5 ml of the support and 1 ml of 0·04M phosphate buffer containing 2 mg of insulin) for their ability to bind insulin antibodies.

Expression of results. The size of binding ability for insulin was determined in serum samples before contact with the support and in every fraction of washings or effluents. An aliquot of the fraction was incubated with ^{125}I-insulin, the mixture was analyzed electrophoretically and the

TABLE I

Adsorption of Insulin Antibodies to Immunosorbents

The results are expressed in % of total binding capacity of serum before contact with the sorbent.

Sorbent	Quantity of antibodies adsorbed to the sorbents	
	undiluted serum	serum diluted 20-times
Hexamethylene Spheron P-300-insulin	97	98
Spheron P-300-insulin	75	98
Hexamethylene Sepharose 4B-insulin	40	90
Sepharose 4B-insulin	90	90
Enzacryl AH-insulin	0—5	99
		88

distribution of free and antibody-bound insulin was determined. The results were expressed as the number of counts per minute (see details in paper[7]).

The concentration of proteins was determined by the method of Lowry and coworkers[9,10].

RESULTS

The data on the adsorption of insulin antibodies obtained in experiments with specific immunosorbents were compared with the data on nonspecific adsorption of plasmatic antibodies by unmodified supports. Insulin can be bound to the support also by electrostatic forces only. This phenomenon will increase the binding ability of the adsorbents for antibodies on the one hand, it will, however, unfavorably affect to a certain degree the results on the other. Electrostatically bound insulin is released during washing and elution and competes with ^{125}I-insulin for the binding site for antibodies in the process of testing.

The size of the adsorption ability of the adsorbents was determined for the most part from differences between the binding ability before and after contact with the adsorbent. The data on the binding ability of the individual adsorbents are summarized in Table I. The highest binding capacity for insulin antibodies shows hexamethylene-Spheron P-300-insulin (*cf.* column of values for undiluted serum) and Sepharose 4B-insulin. The binding capacity of the next two adsorbents, Spheron P-300 and hexamethylene-Sepharose 4B-insulin, is somewhat decreased. The value of adsorption of antibodies to Enzacryl AH-insulin is relatively low. One must not, however,

TABLE II

Nonspecific Binding of Insulin Antibodies to Various Sorbents

The results are expressed in % of binding capacity of undiluted serum before contact with the sorbent.

Sorbent	Quantity of adsorbed antibodies
Aminohexamethylene Spheron P-300	5
Aminohexamethylene Spheron P-300 after contact with insulin	25
Aminohexamethylene Sepharose 4B	20
Aminohexamethylene Sepharose 4B after contact with insulin	71
Enzacryl AH	0—5

neglect the fact that almost a 100% adsorption of insulin antibodies takes place when a smaller quantity of serum (5% of that given in the first column) is used. A role will play here also nonspecific adsorption since even adsorbents which have not been in contact with insulin possess, obviously because of their ion-exchange properties, an ability to retain insulin antibodies (aminohexamethylene-Spheron P-300-5%, aminohexamethylene-Sepharose 4B-20%, and Enzacryl AH up to 5%, *cf.* Table II).

A check-up of the recovery of insulin antibodies showed that 80–90% of the antibodies adsorbed were eluted. The elution profiles of antibodies differ with the individual adsorbents. A molecular sieve effect was observed with adsorbents derived from Sepharose 4B yet not with adsorbents derived from Spheron P-300.

DISCUSSION

The aim of this study was *a*) to examine the possible use of Spheron P-300 as specific adsorbent of insulin antibodies and *b*) to determine the binding ability of copolymers of insulin with aminohexamethylene Sepharose 4B or aminohexamethylene Spheron P 300 to which insulin had been attached through its carboxyl groups.

In view of the aim of this study, *i.e.* the comparison of a new type of Spheron P-300 and the determination of the adsorption properties of the new type of copolymer (insulin bound through its carboxyls) the stoichiometry of the reactions, that is of the attachment of insulin, was not assayed. These processes may give rise to products of side reactions (insulin contains both free amino groups and also carboxyls), to an uneven distribution of the individual amino groups and carboxyls of insulin in the condensation reactions. This uneven distribution then results in a heterogeneous character of the adsorbents prepared (as regards the participation of individual amino groups or carboxyls). The measurement of the adsorption of insulin antibodies with regard to nonspecific adsorption caused by the character of the individual supports (charge, sieving effect) served as a criterion of the suitability of the adsorbent. If we compare supports containing insulin either separated by a spacer from the support matrix or attached through its carboxyl groups, we can observe that both supports act as immunosorbents. In view of nonspecific adsorption observed with aminohexamethylene-Sepharose 4B, we cannot eliminate the possibility that a certain part of the antibody is bound *via* insulin electrostatically attached to the support. The fact that electrostatically attached insulin can considerably contribute to the quantity of antibodies bound, is obvious from a comparison of values of binding to aminohexamethylene-Sepharose 4B and aminohexamethylene-Sepharose 4B-insulin (Table I and II).

Supports with insulin attached through NH_2-groups have often been described in the literature. Sepharose 4B-insulin has been used as a reference immunosorbent in comparison experiments with two other immunosorbents, Spheron P-300-insulin and Enzacryl AH-insulin. Enzacryl AH was little effective in the isolation of anti-

bodies under the experimental conditions chosen. The reason for this is, more likely than a sterical interference preventing the antibody from contact with insulin, obviously the fact that more than one NH_2-group of insulin participates on the reaction. This most probably causes a change in the three-dimensional arrangement of the insulin molecule and thus also the loss of the ability to bind the antibody. Spheron P-300-insulin has approximately the same capacity as Sepharose 4B-insulin. Both supports do not increase markedly the value of nonspecific adsorption of andibodies after contact with free insulin.

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