IMMUNOLOGICAL REACTIVITY OF INSULIN TO SEPHAROSE COUPLED WITH INSULIN-ANTIBODY---ITS USE FOR THE EXTRACTION OF INSULIN FROM SERUM

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Received January 26, 1970

SUMMARY

Anti-insulin serum globulin fraction coupled to agarose (Sepharose 2B) bound labeled and nonlabeled insulin at pH 8.2-8.4 by a specific antigen-antibody reaction. Insulin bound to this matrix was dissociated with 1 M acetic acid. Anti-insulin globulin-coupled agarose could be used repeatedly after regeneration of its insulin-binding capacity by washing with Tris-HCl-albumin buffer (pH 8.2-8.4). The column of this matrix appeared of practical value to extract insulin from serum.

There are several forms of specific binding between biological substances such as enzyme-substrate complexes, antigen-antibody reactions and steroid hormone-carrier protein bindings. If one of the partners of these complexes can be fixed to an insoluble matrix without loss of its specific binding properties, it will be useful not only for the study of the binding mechanism, but also for the purification of the enzymes, antigens or hormones (1). Akanuma and Glomset (2) reported a method for coupling of lipoproteins to agarose according to Porath's technique (3), and subsequently used it for the study of binding of lecithin : cholesterol acyltransferase to lipoproteins. Cuatrecases (4) prepared a column of insulin-coupled agarose, and demonstrated that it adsorbed the insulin antibody

at pH 8.8 and the adsorbed antibody was eluted with HCl. In this communication we describe the binding of insulin to the agarose coupled with anti-insulin globulin and the elution of insulin with acetic acid.

MATERIALS AND METHODS

Anti-insulin serum was obtained from guinea pigs immunized with a mixture of pork insulin and Freund's complete adjuvant. The globulin fraction was precipitated by adding $(NH_4)_2SO_4$ to the final concentration of 40% saturation. The precipitate was dissolved in distilled water, dialyzed against 0.14 M NaCl for 24 hours, and subsequently used for the coupling with agarose (Sepharose 2B, Pharmacia, Uppsala) according to the method of Porath et al (3) with some modifications. Sepharose 2B was washed with distilled water extensively and was activated with cyanogen bromide (1 ml of 25 mg/ml CNBr per 2 g wet weight Sepharose) for 6 minutes at pH 11, and then washed with ice-chilled distilled water and with 0.1 M $NaHCO_3$. The activated Sepharose was mixed with anti-insulin globulin fraction (AIG) dissolved in 0.14 M NaCl-0.1 M $NaHCO_3$ and stirred gently at 4°C for 20 hours. Finally, the gel was washed on a glass filter with 0.14 M NaCl and 0.01 M Tris-HCl buffer (pH 8.6) to remove the non-bound proteins. ^{131}I-insulin with specific activity of about 250 mc/mg was purchased from Dainabot Radioisotope Lab., purified by a column of Sephadex G-50, and diluted to approximately 60 μU/ml before use. Insulin in serum and column fractions was assayed by a double-antibody radioimmunoassay. Protein concentration was measured by Lowry-Folin's method.

RESULTS AND DISCUSSION

By a reaction of 650 mg anti-insulin globulin fraction (AIG) with 60 g (wet weight) of CNBr-activated Sepharose, 92% of AIG as the protein amount was coupled to the Sepharose. Thus, 1 g Sepharose bound 10.0 mg AIG protein.

First, the reaction of this AIG-coupled Sepharose (AIG-AGA) with ^{131}I-insulin was studied. Sepharose 2B coupled with high-density lipoprotein (HDL-AGA) was used as the control. AIG-AGA and HDL-AGA were washed thoroughly with 0.01 M Tris-

HCl buffer (pH 8.2-8.4) containing 1.0 % bovine serum albumin (BSA, Fr. V, Armour).* To 1 g of each Sepharose, 1 ml of ^{131}I-insulin solution (60 μU/ml, in 0.01 M Tris-HCl-BSA buffer) was added, stirred for three hours at room temperature, and then centrifuged. The supernatant was discarded, and the precipitate was washed with 8 ml of Tris-HCl-BSA buffer. As shown in Fig. 1, the radioactivity bound to Sepharose matrix became almost constant after 3 washings. AIG-AGA bound about 55 % of the intial radioactivity whereas the radioactivity bound to HDL-AGA was negligible.

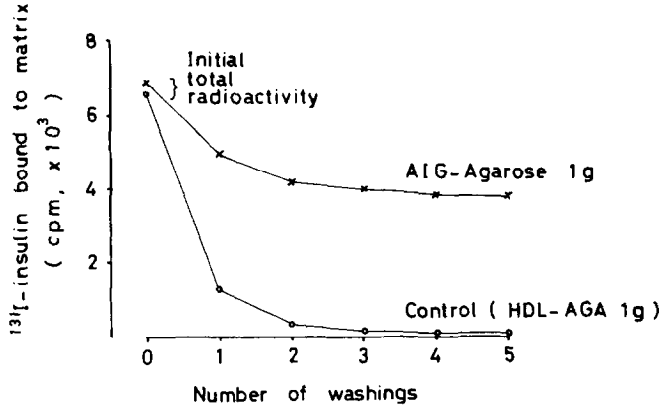

Fig. 1 Binding of ^{131}I-insulin to anti-insulin globulin (AIG)-coupled agarose and high density lipoprotein (HDL)-coupled agarose. 60 μU of ^{131}I-insulin was mixed with 1 g of each agarose and washed repeatedly with 8 ml Tris-HCl-BSA buffer. AIG-agarose bound about 55 % of the initial radioactivity while HDL-agarose adsorbed very little radioactivity. Bound radioactivity became nearly constant after the third washing.

In the second experiment, the dissociation of ^{131}I- insulin bound to AIG-AGA was studied with the use of 1 M acetic acid (Table 1). 0.7 g of AIG-AGA bound a significant amount of ^{131}I-insulin, while the CNBr-activated control agarose did not bind radioactivity

*Addition of serum albumin was necessary, because ommission of it from the buffer caused a significant nonspecific binding of labeled insulin to the control agarose.

to any appreciable extent. After three washings with 1 M acetic acid, about 90 % of ^{131}I-insulin was dissociated, leaving 10 % of radioactivity. In order to test whether AIG-AGA retains its specific binding capacity for insulin after acid treatment, AIG-AGA was washed three times with 1 M acetic acid, subsequently three times with 0.01 M Tris-HCl-BSA buffer (pH 8.2-8.4), and then reacted with ^{131}I-insulin.

As shown in Table 1, the acid washing did not cause any loss of insulin bindings capacity of AIG-AGA. Even after two repetitions of this washing treatment, AIG-AGA bound nearly the same amount of ^{131}I-insulin.

Table 1 The dissociation of ^{131}I-insulin bound to anti-insulin globulin coupled agarose (AIG-AGA) with 1 M acetic acid, and the effect of washings with acetic acid and Tris-HCl-BSA buffer on the insulin binding capacity of AIG-AGA.

Matrix	Radioactivity bound to matrix (cpm)*
Anti-insulin globulin-coupled agarose	
before acid washing	11414
remaining radioactivity after 3 washings with 1 M acetic acid	1019
after 1 cycle of washing treatment**	11819
after 2 cycles of washing treatment**	12067
CNBr-activated agarose	33

* Total radioactivity applied was 62480 cpm.

**One cycle of washing treatment consisted of three washings with 1 M acetic acid followed by three washings with 0.01 M Tris-HCl-BSA buffer (pH 8.2-8.4).

Next, AIG-AGA was packed to a column (1.5 x 13 cm) filled with Tris-HCl-BSA buffer. On this column, 0.5 ml of ^{131}I-insulin solution (60 μU/ml in Tris-HCl-BSA buffer) was applied, and the column was washed with about 100 ml Tris-HCl-BSA buffer. Bound

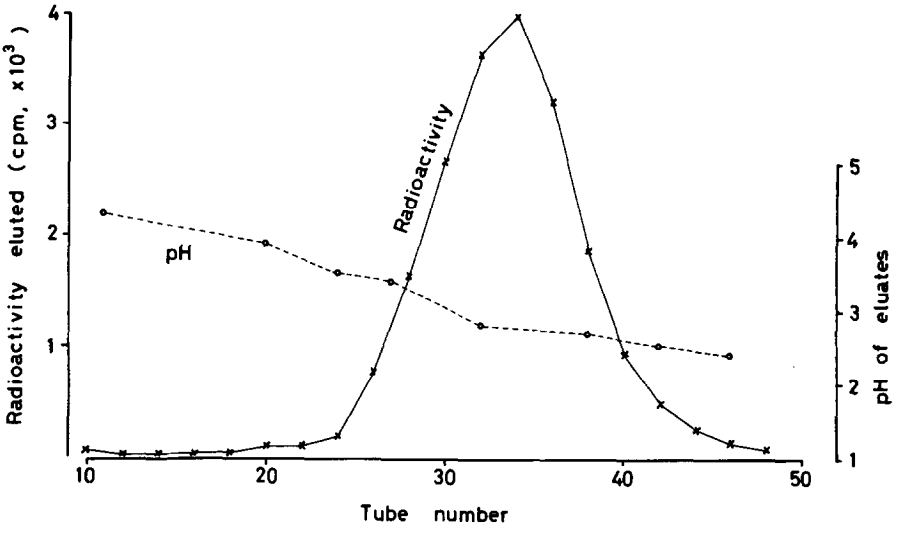

Fig. 2 Elution of ^{131}I-insulin from a column (1.5 x 13 cm) of AIG-AGA by the gradual addition of 1 M acetic acid. 30 μU of ^{131}I-insulin was applied on the column equilibrated with Tris-HCl-BSA buffer (pH 8.2-8.4). Bound ^{131}I-insulin was eluted at the pH between 3.4 and 2.6. Each tube contained 5-ml aliquot.

^{131}I-insulin was eluted as a sharp peak when the pH of the effluent fell to 3.4-2.6 by the gradual addition of 1 M acetic acid (Fig. 2). Of the radioactivity applied, 83 % was bound to the column and 75 % was eluted. When 0.5 mμg of ^{125}I-growth hormone was applied to the same column, at pH 8.2-8.4, 94 % of radioactivity passed through the column and no significant radioactivity was recovered by elution with 1 M acetic acid. This result and the failure of binding of ^{131}I-insulin to the control Sepharose (Fig. 1 and Table 1) indicate that the binding of ^{131}I-insulin to AIG-AGA is based on the specific antigen-antibody reaction. In another experiment in which nonlabeled insulin was used instead of ^{131}I-insulin, it was demonstrated by radioimmunoassay that this column could bind at least 10 mU of crystalline insulin.

Finally, the possible use of this column for the separation of insulin in serum was studied with the use of the pancreatic vein serum of a dog which contained about 1.35 mU/

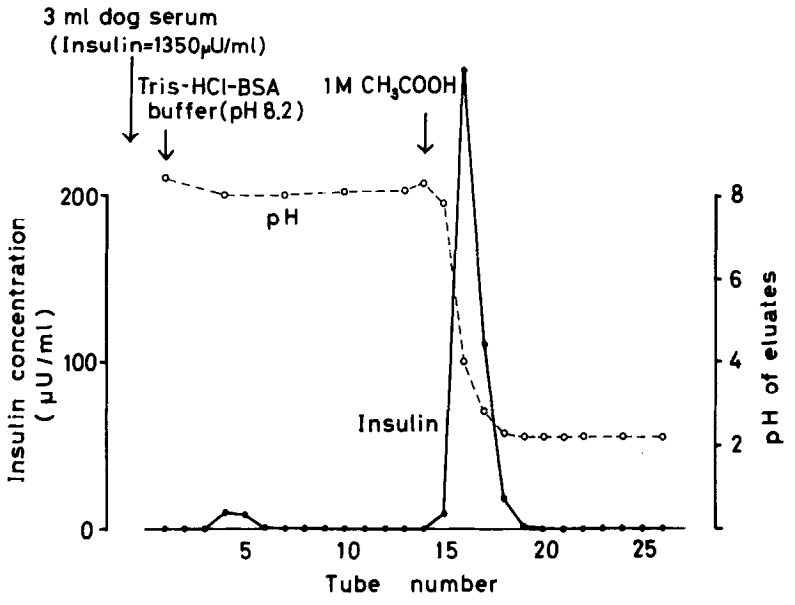

Fig. 3 Binding of serum insulin by a column (1.5 x 13 cm) of AIG-AGA and its subsequen elution by acetic acid. Three ml of dog pancreatic vein serum containing 1.35 mU/ml insulin was applied. Serum proteins were eluted in tubes #3-6. At pH 8.2-8.4, most of serum insulin was adsorbed to the column. Bound insulin was eluted sharply with 1 M acetic acid. Each tube contained 5-ml aliquot.

ml of insulin. Three ml of this serum was applied to the column, washed with Tris-HCl-BSA buffer, and then eluted with 1 M acetic acid. Insulin concentration of the eluates was assayed directly for the fractions eluted with Tris-HCl-BSA buffer. The fractions eluted with acetic acid were evaporated, redissolved in 1 ml of 0.01 N HCl and mixed with 4 ml of 0.02 M Tris-HCl-BSA buffer before the assay of insulin. As shown in Fig. 3, only a minute amount of insulin was detected in the effluent at the pH above 8.0 suggesting that serum insulin was mostly adsorbed to the column at this pH range. Bound insulin was eluted sharply with 1 M acetic acid.

Our present study demonstrated that anti-insulin globulin fraction coupled to CNBr-activated agarose could bind labeled or nonlabeled insulin by specific antigen-antibody

reaction. The bound insulin was dissociated with 1 M acetic acid, and AIG-AGA could bind insulin repeatedly after re-equilibration of the matrix with Tris-HCl-BSA buffer. The experiment with dog serum suggests that the AIG-AGA column method provides a new efficient technique for the extraction of insulin from the various biological fluids. Also, it may be useful to remove nearly all the insulin from the serum. Such insulin-free serum would be of practical use for the immunoassay of serum insulin. Furthermore, this method of antibody-AGA column appears to be of a wider potential usefulness, because it is not only confined to insulin, but seems applicable to various peptide hormones or macromolecules, whenever their antibodies are available.

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