INSULIN-SEPHAROSE: IMMUNOREACTIVITY AND USE IN THE PURIFICATION OF ANTIBODY

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Received April 14, 1969

In recent years much interest has centered on the covalent attachment of biologically active compounds (i.e., enzymes, antibodies, and antigens) to insoluble polymers (Silman and Katchalski, 1966). These derivatives have found special use as immunosorbents for the purification of antibodies. A highly porous derivative of agarose, Sepharose, has recently been described as a suitable matrix for the purification of enzymes (Cuatrecasas et al., 1968), and avidin (Cuatrecasas and Wilchek, 1968), by affinity chromatography.

Porcine insulin can be covalently attached to Sepharose predominantly through a single amino acid, phe B1 or lys 29, depending on the reaction conditions (Cuatrecasas, 1969a). Such derivatives can modify glucose utilization and lipolysis in isolated fat cells in concentrations nearly as low as those of native insulin, and their maximal responses are unimpaired. It was of interest to compare the biological effectiveness of these derivatives with their capacity to bind insulin antibody, and to explore the possible use of these derivatives as immunosorbents for antibody purification.

Materials and Methods. Crystalline pork zinc-insulin (2FR27L, 24.4 units/mg) was purchased from Eli Lilly. Anti-porcine insulin guinea pig serum was kindly provided by Dr. T. E. Prout. The antiserum was diluted 100-fold in 0.1 M sodium barbital buffer, pH 8.6, containing fasted guinea pig serum (1%, v/v) and human albumin (1%, w/v). One ml

of this solution could neutralize 20 Munits of insulin.

The preparation of the insulin-Sepharose derivatives is described elsewhere (Cuatrecasas, 1969a). Insulin-lys-Sepharose and insulin-phe-Sepharose are terms used to indicate the predominant residue through which insulin was linked in the Sepharose derivative; lys is residue B29, phe is residue B1. Acetylinsulin-Sepharose is the derivative of insulin having the α-amino groups of both N-terminal residues completely acetylated, and linked to Sepharose by lys B29.

The radioimmunoassay used in these studies was a modification of the paper wick chromatography method described by Ørskov et al. (1968). The usual reaction mixture consisted of 0.15 ml of sodium phosphate buffer, pH 8.0, containing bovine albumin (1%), 10 mμg of ^{3}H-insulin (specific activity 8.6 mc/μmole, Cuatrecasas, 1969b), 10 μl of the anti-insulin serum, and standard or unknown amounts of nonradioactive insulin. The mixtures were incubated for 24 hours at 4°. A 30 μl aliquot was spotted near the bottom of a 12 x 1.3 cm strip of Whatman 3 MC paper which was then placed into a test tube containing 0.5 ml of 0.05 M phosphate buffer, pH 8.0, 1% albumin. When the ascending solvent front reached the top of the paper wick, the strips were cut into sections. These soaked paper strips were placed in scintillation vials containing 1 ml of 0.1N HCl, 10 ml of TLA toluene fluoralloy (Beckman), 2 ml of Bio-Solv Solubilizer BBS-3 (Beckman), and 3 drops of 4% $SnCl_2$ in 0.1N HCl. The vials were shaken at room temperature for 20 hours; radioactivity was then determined at 22% efficiency in a liquid scintillation spectrometer. Free insulin remained at the point of application on the paper wicks, while the insulin-antibody complex moved with the solvent front. Nonimmune guinea pig serum was used in the controls to correct for incubation damage, which usually represented less than 10% of the total labeled insulin.

Results and Discussion. The shapes of radioimmunoassay curves

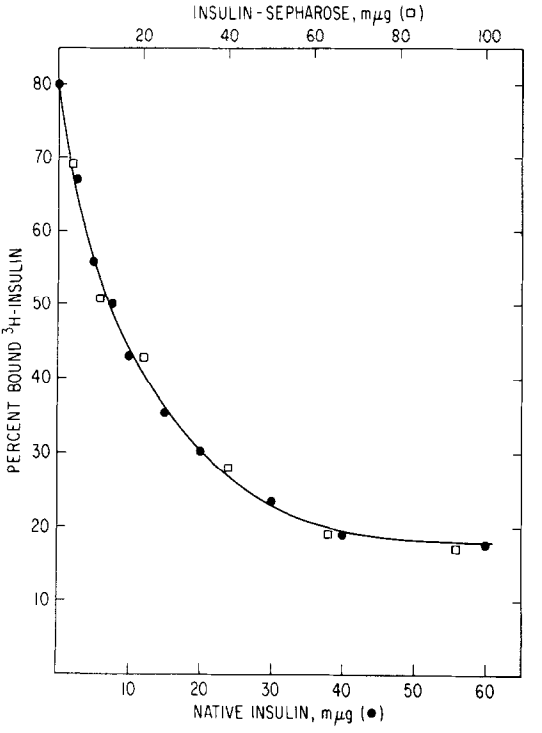

Figure 1. Radioimmunoassay of native insulin and insulin-lys-Sepharose. The amount of insulin coupled was determined by acid hydrolysis and amino acid analysis of the Sepharose derivative.

indicate that insulin-Sepharose displaces labeled insulin from its antibody in a manner identical to the displacement caused by native insulin (Figure 1). The amount of insulin bound to the three different Sepharose preparations was measured by such immunoassays, and compared to the amount of chemically bound insulin present in these derivatives, as measured by acid hydrolysis and amino acid analysis (Table I). Although a considerable proportion of the total insulin in all three derivatives is capable of binding antibody, it appears that the most effective binding results with the derivative bound exclusively by lys B29 (acetylinsulin-lys-Sepharose). The somewhat lower capacity of insulin-lys-Sepharose may be due to the presence in this preparation of a small proportion of molecules which are probably bound by phe B1 (Cuatrecasas, 1969a).

Table I

Insulin-Sepharose preparation[a]	% of Sepharose-bound insulin which is immunoreactive[b]
Insulin-phe-Sepharose	38
Insulin-lys-Sepharose	59
Acetylinsulin-lys-Sepharose	72

a. Refers to the predominant amino acid residue through which insulin is linked to Sepharose, as described in Methods.
b. Determined by radioimmunoassay, as illustrated in Figure 1.

Although the differences are small, the relative immunochemical effectiveness of these three preparations do not exactly parallel their relative biological activities as measured with the isolated fat cell system (Cuatrecasas, 1969a). In these studies, the most active derivative was insulin-lys-Sepharose, while the least active was acetylinsulin-lys-Sepharose.

Since it is very likely that the insulin is bound to Sepharose in a monomeric state (Cuatrecasas, 1969a), it appears that the prominent property of insulin for intermolecular association is not a necessary feature of its specific interaction with antibody. The capacity of insulin rigidly attached to an insoluble carrier to bind antibody nearly as well as native insulin in solution probably is a reflection of the predominantly univalent nature of the insulin-antibody interaction, which ordinarily results in the formation of soluble complexes.

Arquilla and Coblence (1960) and Arquilla and Finn (1963) demonstrated that insulin antibodies could be adsorbed to an insoluble red blood cell stroma-cellulose mixture to which insulin was coupled with bis-diazobenzidine. In view of the efficient interaction of Sepharose-bound insulin with antibody, and the favorable properties of Sepharose as an insoluble support for the purification of enzymes by affinity chromatography

(Cuatrecasas et al., 1968), it appeared that insulin-Sepharose derivatives would be ideally suited for use as immunosorbents. This idea was tested with the use of columns containing insulin-Sepharose. These columns were found to completely remove anti-insulin antibodies from crude mixtures containing the antibody in very low concentrations (Figures 2 and 3). The antibody which was bound to the column could be eluted by lowering the pH of the buffer. The very strong binding of antibody by this column is reflected by the need to use strong acid conditions (1N HCl) to achieve elution of the major fraction of the antibody. No destruction of antibody was detected with concentrations of HCl as high as 3N, provided the fractions were neutralized immediately. Recovery of the anti-insulin material applied to these columns approached 100%.

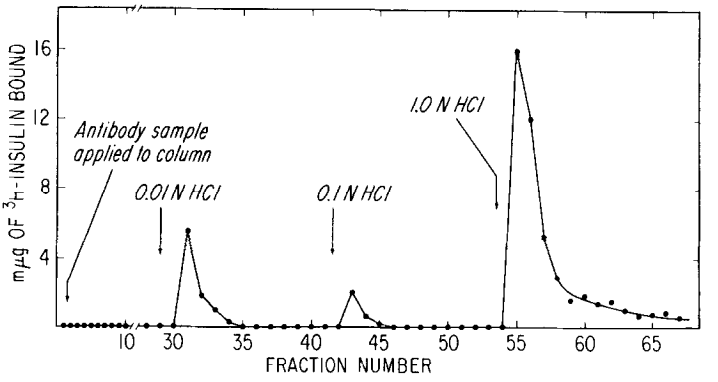

Figure 2. Affinity adsorption chromatography of guinea pig anti-porcine insulin serum on an insulin-Sepharose column. A 0.5 x 3.0 cm column was packed with insulin-lys-Sepharose and washed with 30 ml of 0.05 M phosphate buffer, pH 8.0, containing 0.08 M NaCl. A 2 ml sample of anti-insulin in 0.1 M sodium barbital buffer, pH 8.8, 3% albumin (capable of neutralizing 20 Munits of insulin per ml) was applied to the column. After washing with 20 ml of buffer, elution was attempted with HCl at various concentrations (arrows) containing bovine serum albumin (0.2%). The fractions (0.5 ml), collected in tubes containing 7 μl of phenol red, were immediately neutralized to end-point with NaOH. A 20 μl sample from each fraction was tested for its capacity to bind ^{3}H-insulin. Another small peak of antibody can be eluted with 3N HCl (not shown in figure).

It is notable that several populations of antibody, differing in their avidity for insulin, were present in the anti-insulin serum used;

these could be separated by stepwise acid elution (Figure 2). Even on addition of a single solution of strong acid (1N HCl) separate antibody containing peaks emerged (Figure 3). Such columns should be of considerable help in studies of the heterogeneity of anti-insulin antibodies, since they may permit "immunochromatographic" fractionation of antibodies with varying affinities. Furthermore, it should be possible to purify antibodies completely and in one step with insulin-Sepharose, since there is very little nonspecific adsorption of proteins to the uncharged Sepharose

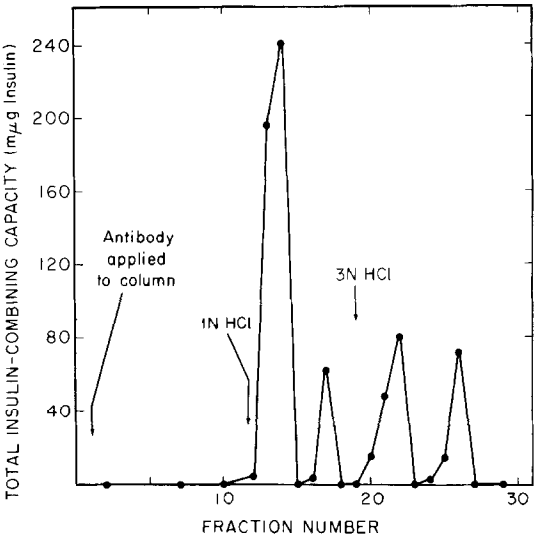

Figure 3. Pattern of elution of insulin antibody from an insulin-lys-Sepharose column using strong acid solutions. Column size and buffers were described in Figure 2. The sample applied was 3 ml of a 1:100 dilution of anti-insulin serum, containing a total insulin-binding capacity of 467 mμg of insulin. The total amount of antibody recovered was capable of combining with 484 mμg of insulin.

polymers, especially in the pH range below 3 (Cuatrecasas et al., 1968).

Other properties which characterize insulin-Sepharose as a useful immunosorbent, and which suggest that similar procedures are applicable to other polypeptide hormones, are its high capacity (6-25 mg of antibody/ml of packed Sepharose, assuming a molecular weight of 150,000 for antibody and a one-to-one binding of insulin), high yield on elution, stability to storage and to extremes of pH, good flow properties,

suitability for repeated use, and ready separation from mixtures by filtration or centrifugation. It should be possible, furthermore, to attach the antibodies purified by these techniques to Sepharose, and to use these derivatives to isolate and characterize small amounts of insulin which are present in biological tissues and serum.

Acknowledgement. The invaluable discussions with Dr. Dean Lockwood and Dr. Sheldon Schlaff, and the technical assistance of Mrs. Juanita Eldridge, are gratefully acknowledged.

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