Avidin–biotin affinity chromatography: Application to the isolation of human placental insulin receptor

[Nα²B³(6-biotinylamido)hexanoylinsulin/succinoylavidin/polyethylene glycol receptor precipitation]

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ABSTRACT The ligand Nα²B³(6-biotinylamido)hexanoylinsulin was attached noncovalently to Sepharose 4B immobilized succinoylavidin to form an insulin-affinity resin. This resin was used to isolate highly purified insulin receptor from human placental tissue by a four-step process involving (i) preparation of a crude membrane fraction, (ii) solubilization with Triton X-100, (iii) wheat germ agglutinin purification, and (iv) insulin-affinity chromatography. NaDodsSO₄/PAGE of the purified 125I-labeled receptor under nonreducing conditions showed the presence of a major component with an approximate molecular weight of 350,000 and a minor component with a molecular weight of ≈166,000. Based on the assumption that the degree of labeling is comparable in both components, the material corresponding to the M₉ 350,000 peak represents ≈94% of the receptor preparation as determined by scanning the autoradiograms. The specific insulin binding capacity of the preparation is 18 ± 6 μg of 125I-labeled insulin per mg of protein as determined by the polyethylene glycol assay and analyzed by Scatchard plot. Insulin binding activity was stable at 4°C and pH 7.6 for at least 12 weeks but was destroyed by freezing and thawing. The availability of highly purified receptor afforded the opportunity to explore its precipitability by polyethylene glycol under assay conditions. Whereas trichloroacetic acid precipitated 95% of the 125I-labeled receptor, polyethylene glycol precipitated only 30%. If the specific activity of the receptor is corrected for incomplete precipitability by polyethylene glycol, the apparent specific binding would be 3.5 ± 1.2 mol of insulin per mol of receptor. These results are in disagreement with the current receptor model, which postulates that 1 mol of receptor (M₉ 350,000) binds 2 mol of insulin. Clearly, the problems associated with the method available for determining insulin binding are sufficiently serious to preclude their use in determining receptor valence.

Affinity chromatography for insulin receptor isolation was introduced by Cuatrecasas and his colleagues in 1972 (1, 2). An affinity resin was prepared by attaching unprotected insulin to Sepharose via a spacer arm. Using this resin, Jacobs et al. (3) were able to isolate a crude insulin receptor preparation from Triton X-100 solubilized rat liver membranes. The same affinity resin was used by Siegel et al. (4) for the retrieval of insulin receptor preparations from human placenta. The receptor was eluted from the affinity resin with 4.5 M urea (pH 6.0), conditions that damage the receptor. Recently, Fujita-Yamaguchi et al. (5) observed that insulin receptors with high specific insulin binding activity can be eluted from Cuatrecasas-type columns with acetate buffer, pH 5.0/1 M sodium chloride. A considerable store of information concerning the nature of insulin receptors has been accumulated by indirect methods using tracer amounts of materials (6), but methodology for isolating large quantities of receptor is not available. Finite information regarding the architecture and function of the receptor will depend on chemical structure investigations, and this requires a supply of homogeneous material.

Eleven years have passed since Cuatrecasas introduced his affinity resin, and workers in the field have shown little interest in developing different approaches to insulin receptor isolation. We have developed an affinity resin based on the avidin–biotin interaction, which makes possible the isolation of highly purified insulin receptor from human placenta in milligram amounts. The preparation of the resin, which is simple and reproducible, involves mixing the ligand Nα²B³(6-biotinylamido)hexanoylinsulin (7) (Fig. 1) with Sepharose 4B immobilized succinoylavidin. In the present paper, we review briefly the systematic development of the method and describe in detail its application to insulin receptor isolation from human placenta.

MATERIALS AND METHODS

Cyanogen bromide (CNBr)-activated Sepharose was purchased from Pharmacia; Triton X-100, phenylmethylsulfonyl fluoride (PhMeSO₂F), polyethylene glycol 6000 (PEG), thyroglobulin, and GlcNAc were purchased from Sigma. Bovine γ globulin was from Miles; Na¹²⁵I and [¹⁴C]biotin were from Amersham; apoferritin was from Calbiochem; wheat germ agglutinin was from E-Y Laboratories (San Mateo, CA); Hepes was from United States Biochemical (Cleveland, OH), and crystalline bovine insulin was a gift from Eli Lilly. Nα²B³,Nα⁴B⁴(BOc)₂-insulin (BOc, tert-butoxycarbonyl) was a gift from R. Geiger (Hoechst A.G., Frankfurt am Main, Federal Republic of Germany). Molecular weight standards for PAGE, myosin (M₉, 200,000), β-galactosidase (M₉, 116,500), phosphorylase b (M₉, 94,000), bovine serum albumin (M₉, 68,000), and ovalbumin (M₉, 43,000), were from Bio-Rad, α₉-macroglobulin (M₉, 340,000) was from Boehringer Mannheim. Avidin was isolated from hen's eggs by iminobiotin–Sepharose affinity chromatography (8). Buffers were filtered through Millipore filters (type HA, 0.45 μm) prior to use.

Synthesis of Nα²B³(6-biotinylamido)hexanoylinsulin (Ligand). N-Hydroxysuccinimide (6-biotinylamido)hexanoate was initially prepared by the method of Sakakibara and Inukai (9). A more convenient route is described here. A stirred suspension of (6-biotinylamido)hexanoic acid (7) (374 mg, 1.04 mmol) and N-hydroxysuccinimide (180 mg, 1.57 mmol) in dimethylformamide (12 ml) was heated at 80°C and N,N'-(dicyclohexylcarbodiimide (322 mg, 1.56 mmol) in dimethylformamide (1.5 ml) was added. The suspension was stirred at 80°C for 30 min (clear solution after 5 min) and stirring was continued at RT for 17 hr. The suspension was cooled in an ice-bath for 2 hr, the N,N'-(dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness in vacuo. The residue was recrystallized from boiling 2-pro-

Abbreviations: PhMeSO₂F, phenylmethylsulfonyl fluoride; PEG, polyethylene glycol.

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panol; yield, 316 mg (66%); mp, 160°C-162°C; dec; Rf, 0.56; lit (7) mp, 160°C-162°C; Rf 0.56 (chloroform/methanol/water, 8:3:1). Coupling to insulin was performed as described (7).

Preparation of Wheat Germ Agglutinin Sepharose 4B. CNBr-activated Sepharose 4B (15 g) was washed with 1 mM HCl (3 liters) at RT on a Buchner funnel. The resin was sucked dry, placed in a plastic bottle, and a solution of wheat germ agglutinin (500 mg) in 0.1 M NaHCO₃ (100 ml) containing 0.5 M NaCl was added at 4°C. The suspension was rotated at 4°C for 24 hr, the resin was collected on a Buchner funnel (filtrate contains negligible amounts of ligand) and was rotated at 4°C with 50 ml of 1 M ethanolamine-HCl (pH 8.0) for 3 hr. The resin was collected and subjected to three washing cycles, each consisting of a wash with 0.1 M NaHCO₃/1.0 M NaCl, pH 8.0 buffer (1 liter) and 0.1 M NaOAc/1.0 M NaCl, pH 4.0 buffer (1 liter). The washed resin was stored at 4°C in pH 4.0 buffer to which sodium azide (0.05%) was added.

Preparation of Succinoylavidin Sepharose 4B. CNBr-activated Sepharose 4B (10 g) was washed at RT with 1 mM HCl (2 liters) and the washed resin, in a plastic bottle, was rotated at 4°C for 22 hr with a solution of avidin (100 mg) dissolved in 0.1 M NaHCO₃/50 ml containing 0.5 M NaCl. The resin was collected (filtrate contains negligible amounts of avidin), resuspended in (25 ml) 1 M ethanolamine-HCl (pH 8.0), the suspension was rotated at 4°C for 3 hr, and the resin was collected and washed with three cycles each of 600 ml of pH 8.0 and pH 4.0 buffers. The washed resin was slurried in 0.5 M Na₂CO₃, pH 9.0 (75 ml), the suspension was cooled in an ice-bath and succinic anhydride (59 mg) in dioxane (1.8 ml) was added with vigorous stirring. Stirring was continued for 1 hr at ice-bath temperature and for 4 hr at RT. The resin was collected, washed with three cycles (600 ml each) of pH 8.0 and pH 4.0 buffers, and stored in pH 4.0 buffer to which sodium azide (0.05%) was added.

Preparation of Insulin Affinity Resin. Freshly prepared succinoylavidin Sepharose 4B (5.5 ml of settled resin) was poured into a column (0.9 × 7 cm), washed with 15 vol of 50 mM Hepes/1 M NaCl/0.1% Triton X-100, pH 7.6, and suspended in 6 vol of Hepes, salt, Triton X-100 in a plastic bottle. Resin that was stored for some time was washed with pH 8.0 and 4.0 buffers in the manner described prior to washing with Hepes/salt/Triton X-100 buffer. To the suspension was added a solution of N⁴ᵗʰ(6-biotinylamido)hexanoylinsulin (ligand) (700 µg in 0.1 M HCl added to 6 vol of Hepes/salt/Triton X-100) and the suspension was rotated at RT for 1 hr. The suspension was poured into a column (0.9 × 7 cm) and the effluent was collected. The column was washed with 100 ml of Hepes/salt/Triton X-100, and the washing was added to the effluent. This was followed by a wash with the same buffer (1 liter), which was discarded. To determine the amount of bound ligand, the ligand concentration in the effluent and first washing was determined with fluoroscene (10), using ligand as the standard.

Bioassays and Protein Determinations. The insulin binding capacity of receptor at various stages of purification was determined by PEG assay (1) at an insulin concentration of 0.16-33 nM. Wheat germ agglutinin column eluates were assayed at a protein concentration of 10-20 µg; eluates from the insulin affinity resin were assayed at 0.1-0.2 µg. Charcoal assays were performed according to Williams and Turtle (11) at an insulin concentration of 0.33 nM. For protein determinations, concentrated HCl (0.5 ml) was added to receptor solutions (0.5 ml) and the mixture was heated at 110°C in vacuum reaction tubes (Pierce) for 20 hr. The hydrolysates were lyophilized and the residue was dissolved in water (2 ml) for amino nitrogen determination with fluorescamine (12).

Labeling the Receptor. Purified receptor (1 µg) was added to a 1.5-ml microfuge tube containing 0.3 M sodium phosphate (pH 7.4) (35 µl) and Na¹⁵¹I (0.5 mCi; 1 Ci = 37 GBq). This was followed by chloramine T (0.5 nmol) and the reaction mixture (60 µl total volume) was kept at RT for 5 min. Sodium metabisulfite (1 nmol) was added and unreacted iodine was separated from the protein on a column of Sephadex G25 (5 ml) in 50 mM Hepes/0.1% Triton X-100, pH 7.6. Receptor Isolation. All operations except solubilization were carried out at ice-bath temperature. Highly purified insulin receptor was isolated from fresh human placental tissue in a process that involves (i) preparation of a crude membrane fraction (4), (ii) solubilization with Triton X-100 (1), (iii) wheat germ agglutinin affinity chromatography (13), and (iv) insulin affinity chromatography. The wheat germ agglutinin eluate from step iii was thawed and adjusted to 1 M with NaCl and to 0.1 M with PhMeSO₄F solution. This solution, at 4°C, was cycled over a column of insulin affinity resin (0.9 × 7 cm) at a flow rate of 0.2 ml/min for 12 hr (estimated number of cycles, 8). The flow-through was retained for assay and reaplication and the resin was washed with 1.2 liters of Hepes/salt/Triton X-100 buffer at a flow rate of 50 ml/hr. The column was then eluted with 50 mM sodium acetate, pH 5.0/1 M NaCl/0.1% Triton X-100/0.1 mM PhMeSO₄F at a flow rate of 4.5 ml/min. Eluate fractions (2 ml each) were collected into 1 ml of 0.5 M Hepes, pH 7.6/0.1 mM PhMeSO₄F as described (5). The location of insulin binding material was determined by charcoal assay and suitable fractions were pooled, assayed for protein by fluoroscene (12) and for binding activity by Scatchard analysis (14), and stored at 4°C with addition of 0.05% sodium azide. For processing larger amounts of receptor (10 placenta columns of 30 ml of affinity resin were used and operated in the manner described. The columns were then washed with Hepes/salt/Triton X-100 buffer and were ready to use.

RESULTS
For the wheat germ agglutinin purification, a pooled sample of crude solubilized membranes from 40 placentas was divided into 20 identical portions. On the basis of this series of experiments, we found that the average yield of wheat germ agglutinin purified receptor is 44 ± 10% with an average specific activity of 39 ± 14 pmol/mg.

Insulin affinity resins prepared by methods illustrated in Fig. 2, initially the eluate was divided into two portions, corresponding to fractions 3-11 and 12-20. Aliquots of these fractions were iodinated and subjected to NaDodsSO₄/PAGE. Autoradi-
grams of the gels indicated that the samples of both portions are indistinguishable (data not shown).

The binding capacity of the immobilized succinylavadin columns was determined with \(^{14}C\)biotin (15), and the amount of ligand added was \(\approx 20\%\) of this figure. We have not systematically investigated the concentration of ligand necessary for optimal receptor binding and elution. Table 1 provides information regarding the operation of the insulin affinity columns. On the average, 1 ml of affinity resin bound 149 \(\pm\) 23 pmol of receptor. The unbound receptor can be reapplied to a fresh resin bed for rebinding. The amount of receptor bound to the column is calculated as the difference between applied and unadsorbed receptor. The yield of receptor in the pH 5 eluates based on the amount bound to the resin is 29\% \(\pm\) 14\%. Experiments 6 and 7, performed using a 30-ml resin bed, gave similar results, indicating that considerably larger amounts of wheat germ agglutinin eluate can be processed conveniently.

We have determined that at pH 5.0, the insulin binding activity of the purified receptor remains unchanged for at least 24 hr at 4\(^\circ\)C. Although a 2-fold higher yield of binding activity could be obtained by eluting the affinity resin with a pH 4.0 buffer, the binding activity decayed with an approximate \(t_{1/2}\) of 67 min at 4\(^\circ\)C.

The apparent specific activity of the receptor, determined by Scatchard plots, was 18 \(\pm\) 6 \(\mu\)g (3000 pmol) of \(^{125}I\)-labeled insulin bound per mg of protein. The Scatchard plot as illustrated in Fig. 3 is curvilinear. The binding data have been replotted according to Klotz (16) to ascertain that saturation has been reached.

Isolated receptor was iodinated with \(^{125}I\) and its precipitability with trichloroacetic acid and PEG was measured. The results of these experiments are presented in Table 2. Trichloroacetic acid precipitates the radioactivity nearly quantitatively, but under the standard conditions of the PEG precipitation assay (1) only \(\approx 30\%\) of the radioactivity is precipitated. Identical results were obtained when the radiolabeled receptor was mixed with unlabeled purified receptor, with wheat germ agglutinin eluate, or with saturating amounts of unlabeled insulin.

The elution profile obtained by Sepharose 6B gel filtration of the radiolabeled receptor (Fig. 4) contained a major radioactive peak that accounted for 95\% of the radioactivity.

A representative autoradiogram of a NaDodSO\(_4\)/PAGE of

![Fig. 2. Typical elution profile of insulin receptor from insulin affinity resin. \(^{125}I\)-labeled insulin binding fmol per 50 \(\mu\)l was measured by charcoal assay (see text for details).](image)

![Fig. 3. Scatchard and Klotz plots of a typical binding assay with highly purified insulin receptor. B, bound; F, free.](image)

<table>
<thead>
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<th>Table 1. Efficiency of affinity resin</th>
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The affinity column contained 0.13 mg of ligand per ml of settled resin. For experiments 1–5, a resin bed of 5.5 ml was used; experiments 6 and 7 were conducted with 30 ml of resin. The average binding capacity of the column was 149 \(\pm\) 23 pmol of receptor per ml of settled resin. Numbers in parentheses are % of bound receptor. Sp. act., specific activity (\(\mu\)g of \(^{125}I\)-labeled insulin bound per mg of protein).

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<th>Table 2. Precipitability of (^{125}I)-labeled purified insulin receptor with trichloroacetic acid and PEG</th>
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Each sample contained \(^{125}I\)-labeled purified receptor (=95,000 cpm). For trichloroacetic acid (TCA) precipitation, 10% TCA (0.5 ml) was added to 0.1% bovine serum albumin in 0.03 M phosphate buffer (pH 7.4) (0.5 ml) containing the appropriate sample. A 250-\(\mu\)l aliquot was withdrawn, centrifuged in a microfuge for 1 min, and the pellet was counted in a \(\gamma\) counter. For PEG precipitation, the appropriate sample was dissolved in 50 mM Tris-HCl, pH 7.4/0.1% bovine serum albumin and precipitated with PEG.

*Purified receptor (0.12 pmol).
\(^r\)Wheat germ eluate containing 0.13 pmol of receptor.
\(^s\)Purified receptor (0.12 pmol) containing 10 pmol of insulin.
DISCUSSION

In 1976 we suggested (18) that suitably biotinylated peptide hormones could be attached noncovalently to carrier immobilized avidin to form affinity resins useful for the isolation of hormone receptors. The high affinity of avidin for biotin (Kd, 10^{-15} M) prompted this reasoning. We recognized that the biotinylated hormones had to be as active as their parent to be useful for this purpose, because this would ensure that their affinity for the receptor was unimpaired. We selected the insulin receptor as the test object for experimental study, because this receptor is reasonably stable and has been investigated in many laboratories.

We synthesized N-^6^B-biotinylinsulin (biotinylinsulin) (19) and found that its ability to stimulate lipogenesis in rat epididymal adipocytes was identical to that of insulin. Thus, biotinylation of insulin on the N-^6^B position does not interfere with receptor binding. As was expected, biotinylinsulin bound to avidin.

In adapting the avidin-biotin approach to receptor isolation, it was of importance to eliminate, as much as possible, nonspecific interactions between the affinity resin and constituents in the receptor extracts. In addressing this problem (20), we discovered that avidin, a strongly basic protein, binds avidly and nonspecifically to rat liver plasma membranes. Succinylation markedly reduces this interaction; consequently, we use succinoylavidin in our studies.

For biotinyl derivatives of insulin to be useful as bifunctional reagents for affinity chromatography of receptors, they must be capable of binding simultaneously to succinoylavidin and receptor. To discover such derivatives, we synthesized a series of biotinyl-X insulins (7, 21), in which the length of the spacer X was systematically increased, and we measured the rate of dissociation of their complexes with succinoylavidin. The t_{1/2} for dissociation of the biotin succinoylavidin complex is 127 days (20). The t_{1/2} for dissociation of the biotinylinsulin complex is 3 hr, that of N-^6^B-(6-biotinylamido)hexanoylinsulin is 76 days. It follows that the proximity of insulin to biotin in biotinylinsulin reduces drastically its affinity for avidin. The interposition of a single 6-aminohexanoyl molecule as in N-^6^B-(6-biotinylamido)hexanoylinsulin provided a biotinylated insulin with good affinity for succinoylavidin. This and other biotin-X insulins are active insulin in stimulating glucose oxidation in rat epididymal adipocytes (22), demonstrating that they are capable of binding to the insulin receptor of these cells.

To explore their ability to bind simultaneously to both succinoylavidin and insulin receptors, we investigated the ability of the biotin-X insulins to stimulate glucose oxidation in fat cells in the presence of a large excess of succinoylavidin (22). With the exception of biotinylinsulin (X = 0), which is completely inhibited, the compounds retain 20%–30% of their biological activity even in the presence of a 100:1 molar ratio of succinoylavidin to analog. We interpret this result to mean that, indeed, these compounds are able to bind simultaneously to both the receptors on the adipocyte and to the succinoylavidin.

N-^6^B-(6-biotinylamido)hexanoylinsulin (Fig. 1), the simplest representative of our series, was selected as the ligand for receptor isolation studies. When this ligand is mixed with Sepharose 4B immobilized succinoylavidin, an affinity column is generated that binds solubilized insulin receptor. It could be argued that the affinity resin binds the receptor via hydrophobic interactions, because it contains a long stretch of aliphatic hydrocarbon chain. To eliminate this possibility, we conducted experiments with columns containing (6-biotinylamido)hexanoic acid (7) attached to the Sepharose 4B immobilized succinoylavidin and found that they failed to bind significant amounts of receptor (experiments not shown).
The efficiency of the affinity columns is summarized in Table 1. On the average, 72% of the added wheat germ agglutinin purified receptor adsorbs to the column and highly purified receptor is eluted under the conditions described (5). The adsorbed receptor is retrieved with a yield of 29% ± 14% with a specific binding activity of 18 ± 6 μg of 125I-labeled insulin per mg of protein. For the reasons discussed below, these figures are subject to serious error.

The homogeneity of insulin receptor preparations has, in the past, been assessed on the basis of their 125I-labeled insulin binding capacity determined by Scatchard plots (14). The presently accepted model for the architecture of the insulin receptor postulates the presence of two insulin binding sites per molecule of Mr 350,000. The theoretically expected insulin binding capacity based on this model is 34 μg of insulin per mg of protein. In agreement with the results of others, we find that our receptor preparations bind 125I-labeled insulin, affording a curvilinear Scatchard plot (Fig. 3). However, accurate assignment of the specific binding activity of receptor preparations is difficult. One of the reasons for this difficulty is the inaccuracy involved in determining the receptor number based on curvilinear Scatchard plots. Klotz (16) has recently pointed out that Scatchard plots are particularly deceptive, because near the point at which the curve approaches the abscissa, large differences in free ligand concentration cause little change in the bound/free ratio and give the impression that an extrapolated value for receptor number is accurate when, in fact, it may be grossly in error.

The precipitation of 125I-labeled insulin bound to receptor at or near saturation has been routinely used to assess receptor content of preparations during the course of purification. PEG is used in this assay because of its ability to precipitate high molecular weight proteins at concentrations at which it fails to precipitate low molecular weight molecules such as unbound insulin. Since both the specific activity of receptor preparations and the yield of purified receptor depend critically on this assay, it is important to establish that the PEG method quantitatively precipitates the receptor. The availability of highly purified receptor preparations enabled us to explore this important aspect of the PEG method. We have compared the precipitation of 125I-labeled receptor by PEG and trichloroacetic acid, because both methods should result in the precipitation of all the radioactivity attached to high molecular weight proteins. From the results presented in Table 2, it can be seen that PEG precipitates only ≈30% of the trichloroacetic acid-precipitable material of labeled receptor preparations, although 95% of the radioactivity is attached to high molecular weight material (Fig. 4). Despite the problems inherent in the PEG method, we have used this assay to arrive at the specific activity of our preparations for comparison with those obtained in other laboratories.

The PEG assay has been of immeasurable importance as a tool in insulin receptor isolation, but it must be stressed that the problems with this assay are sufficiently serious to preclude its use to establish the specific activity of receptor preparations and, hence, to verify the proposed model. We have recalculated the specific activity of our receptor preparations, assuming that only 30% of the insulin-receptor complex is detected by PEG precipitation and estimate that they contain 60 ± 20 μg of 125I-labeled insulin binding activity per mg of receptor. This figure corresponds to 3.5 ± 1.2 mol of insulin bound per mol of receptor. The reason for the curvilinear Scatchard plots characteristic of insulin-receptor interactions has been the subject of considerable debate. It is tempting to speculate that binding multiple insulin molecules per molecule of receptor may be responsible for this behavior. Clearly, better methods will be needed to determine the valency of the insulin receptor.

In the absence of a reliable binding assay, we have elected to assess the homogeneity of our preparations based on chemical criteria. We label the purified receptor with 125I and subject the labeled material to NaDodSO4/PAGE and autoradiography without resorting to immunoprecipitation. A scan of the autoradiogram (Fig. 5) shows two clearly discernible peaks corresponding to materials with molecular weights of 350,000 and 166,000. We have integrated the areas of each of the peaks and, assuming that the degree of labeling in the two materials is comparable, estimate that the material of Mr 350,000 accounts for 94% of the preparation. NaDodSO4/PAGE of the reduced receptor produces the characteristic subunit pattern (23).

The avidin-biotin affinity chromatography approach offers the following advantages: (i) Attachment of biotin to insulin is targeted and thus provides a uniform site for receptor binding. (ii) Insulin ligands are prepared by simple well-defined solution methods that can be monitored accurately. (iii) Formation of the affinity resin is highly specific (avidin–biotin interaction) and can be achieved at large quantities by simply mixing the components. (iv) The amount of ligand on the column can be varied at will to achieve optimal operation because the interaction of the components is quantitative. (v) The technique can be readily scaled up for production of larger quantities of receptors. (vi) Once the technique is worked out for a particular situation, it is highly reproducible. (vii) In theory, the technique is applicable to any receptor whose acceptor can be biotinylated without destruction of biological function.

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