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Application of receptor-affinity chromatography to bioaffinity purification

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ABSTRACT

Receptor-affinity chromatography based upon the receptor–ligand interactions has been utilized for the purification of recombinant human interleukin-2 (rIL-2) from microbial and mammalian sources. The receptor-affinity purification process of rIL-2 is used as a model system to demonstrate the utility of this approach for the purification of recombinant proteins. The receptor-affinity purified biomolecule is shown to be biochemically and biologically more homogeneous than the immunoaffinity purified material.

INTRODUCTION

Affinity chromatography based upon the ability of the macromolecules in solution to bind specifically and reversibly to immobilized ligands is an ideal tool for the purification of biomolecules[1–4]. This versatile technique became well established in the mid-1970's for the purification of proteins such as enzymes, hormones, antibodies, receptors, etc. Immunoaffinity chromatography, which utilizes the specificity of the antigen–antibody interactions, realized its full potential only after the discovery of the monoclonal antibody producing hybrid cell lines by Kohler and Milstein[5] in 1975. In 1987, the development of an alternative affinity method termed receptor-affinity chromatography (RAC) was reported[6], which exploits the biochemical interactions between a matrix-bound receptor and its soluble protein ligand. In general, the receptor is chemically bonded to an inert polymer support, packed into a column and after proper equilibration, the crude ligand preparation is passed through the column. The unadsorbed materials are washed away and the specifically adsorbed ligand is eluted with mild desorbing agents.

In this paper, the receptor-affinity purification process of recombinant human interleukin-2 (rIL-2) is used as a model system to demonstrate the utility of this approach for the purification of recombinant proteins. A systematic approach to the development and optimization of receptor-affinity purification systems is discussed. The receptor-affinity purified biomolecule is shown to be biochemically and biologically more homogeneous than the immunoaffinity purified material.

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EXPERIMENTAL

Materials

Silica-based NuGel P-AF polyaldehyde, NuGel P-DE200 and NuGel P-AF poly-N-hydroxysuccinimide (PNHS) were purchased from Separation Industries (Metuchen, NJ, U.S.A.). Sephacryl S-200 and Sephadex G-50 (superfine) were obtained from Pharmacia (Piscataway, NJ, U.S.A.). Monoclonal antibody 5B1 and rIL-2 were prepared in-house at Hoffmann-La Roche. Mannitol was purchased from ICI (Wilmington, DE, U.S.A.). All other reagents used were of reagent quality.

Construction of receptor-affinity column

Production and purification of interleukin-2 receptor. A recombinantly produced soluble form of the low affinity p^{55} subunit of the human interleukin-2 receptor denoted IL-2RΔNae was engineered and expressed in Chinese hamster ovary cells (CHO) by the gene-linked co-amplification technology[7]. The IL-2RΔNae was purified by employing IL-2 ligand-affinity chromatography as described[8].

Immobilization of IL-2RΔNae. The IL-2RΔNae was immobilized to NuGel P-AF PNHS (500 Å, 40–60 μm) at a coupling density of 1.55 mg/ml gel according to the previously published protocol[6]. The coupling reaction mixture contained equal volumes of the activated gel and a 2-mg/ml IL-2R solution in 0.1 *M* potassium phosphate, pH 7.0, containing 0.1 *M* sodium chloride.

Determination of the IL-2 binding capacity of the receptor column

A known volume (0.5–1.0 ml) of the receptor gel was packed into an Amicon 10 × 1 cm column (Amicon, Danvers, MA, U.S.A.) fitted with two adapters and equilibrated with phosphate-buffered saline buffer (PBS), pH 7.4. The column was then saturated with an excess of purified or crude IL-2 at a flow-rate of 1 ml/min. After washing away the unadsorbed materials, the adsorbed IL-2 was eluted with 0.2 *M* acetic acid containing 0.2 *M* sodium chloride and its protein content determined by the method of Lowry *et al.*[9].

Factors affecting the IL-2 binding capacity of the receptor adsorbent

Effect of coupling pH. A 1-g amount (1.4 ml) of NuGel-PNHS was mixed with 2 ml, 2.5 mg/ml IL-2RΔNae made up in buffers having pH 5, 6, 7, 8 and 9. The IL-2 binding capacities of the resulting affinity sorbents were determined as described in the preceding paragraph.

Effect of IL-2R coupling density. To each of the 2-ml IL-2R solutions having concentrations of 1.0, 2.5, 5.0 and 10 mg/ml, 1 g NuGel-PNHS was added and the coupling reaction was carried out as before. The IL-2R coupling density as well as the IL-2 binding capacities of the affinity sorbents were determined as described earlier.

Recombinant IL-2 production

A synthetic gene for IL-2 was constructed and introduced into *Escherichia coli* with the plasmid RR_1/pRK 248 CI_{ts}/pRC 233 (ref. 10) and grown in appropriate medium in large fermentors. A CHO cell line transfected with the IL-2 gene was the source of mammalian glycosylated rIL-2.

IL-2 bioassay

IL-2 bioactivity was determined by the IL-2 dependent proliferation of murine CTLL cells as determined by the colorimetric determination of lactic acid produced as an end product of glucose metabolism[11]. A unit of activity is defined as the reciprocal of the dilution yielding half-maximal cell growth. A BRMP reference reagent, purified from a human T cell leukemia cell line designated Jurkat-FHCRC, was used as a reference reagent, for human IL-2.

Receptor-affinity purification procedures

The general RAC purification scheme for the production of highly pure rIL-2 from microbial and mammalian sources are given in Fig. 1. All the RAC purification steps were carried out at 2–4°C, unless otherwise noted.

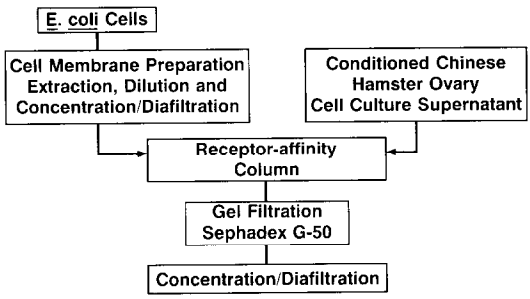

Fig. 1. The overall receptor-affinity purification scheme for rIL-2 from microbial and mammalian sources.

Extraction and solubilization of rIL-2

A 4-ml/g *E. coli* cell suspension was made in buffer A (30 m*M* Tris–HCl, pH 8.0, containing 5 m*M* EDTA). The cell suspension was passed through a Gaulin homogenizer (APV Gaulin, Everet, MA, U.S.A.), two to three times at 7000 p.s.i. The homogenized cells were centrifuged in a Sorvall RC-5 centrifuge with a GSA rotor (DuPont, Wilmington, DE, U.S.A.) at 24 000 *g* for 20 min and the pellet collected. The pellet was washed once with 4 volumes of 1.75 *M* guanidine (Gu) · HCl solution in buffer A, centrifuged and the pellet saved. The pellet was extracted with 7 *M* Gu · HCl in 0.1 *M* Tris–HCl, pH 8.0, containing 1 m*M* EDTA (4 ml/g cells) for 30–60 min and the supernatant collected by centrifugation.

rIL-2 refolding studies

The supernatant from the previous step was diluted 40-fold with PBS buffer and allowed to stand for 0–5 days. The supernatant was carefully decanted and filtered through a 0.8/0.2-μm filter (Sartorius). The clarified extract was used as the starting material for the RAC step. Aliquots of the diluted extract corresponding to 1 g cells were withdrawn each day during the five days of aging, clarified and their rIL-2 content was determined by RAC (5 × 1 cm column) as described for the determination of the saturation binding capacity of the receptor sorbents.

Receptor-affinity chromatography

An Amicon G-44 × 250 column (16.5 × 4.4 cm) was packed with 250 ml receptor gel and equilibrated with PBS buffer, pH 7.4. The clarified extract (17.5 l) derived from 100 g *E. coli* cells was applied to the receptor column at a flow-rate of 60 ml/min. The column effluents were monitored by a Gilson 111B UV detector in conjunction with a Kipp & Zonen recorder (Gilson Medical Electronics, Middletown, WI, U.S.A.). The column was washed with PBS until all the UV absorbing materials were removed. The adsorbed rIL-2 activity was eluted from the column using 1–2 bed volumes of 0.2 *M* acetic acid containing 0.2 *M* sodium chloride. The rIL-2 eluate was concentrated in a stirred-cell Amicon thin-channel concentrator fitted with a YM 5 membrane.

Gel permeation chromatography

A Pharmacia K50/100 column was packed with Sephadex G-50, superfine, to a height of 90 cm. The mobile phase used was 50 m*M* sodium acetate, pH 3.5, containing 200 m*M* sodium chloride and 5 mg/ml mannitol. An amount of 50 ml concentrated rIL-2 was applied to the column at a flow-rate of 1 ml/min. Five-minute fractions were collected on an LKB Ultra RAC-7000 fraction collector (LKB, Bromma, Sweden) and the column effluents were monitored as in the RAC step. The fractions containing the rIL-2 activity were pooled and concentrated to 5–10 mg/ml, sterile filtered with a 0.2-μm filter and stored at 4°C under aseptic conditions.

Purification of mammalian rIL-2

The conditioned CHO medium (18 l) was filtered through a 0.8/0.2-μm filter and applied to the receptor column. The receptor-affinity purified material was also subjected to gel permeation chromatography as described earlier.

Protein determination

The protein content of the rIL-2 samples was determined by the method of Lowry *et al.*[9] and confirmed by quantitative amino acid analysis using a post-column fluorescamine amino acid analyzer[12].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis

The rIL-2 samples were analyzed by sodium dodecyl (lauryl) sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions according to the method of Laemmli[13].

Determination of amino acid composition

Amino acid composition was determined using a post-column fluorescamine amino acid analyzer[12]. Samples were hydrolyzed in 6 *M* hydrochloric acid containing 4% thioglycolic acid at 110°C *in vacuo* for 20–24 h. Proline and cysteine values were determined after performic acid oxidation.

Immunoaffinity purification of microbial rIL-2

A murine monoclonal antibody to rIL-2 designated 5B1 was immobilized to NuGel-PNHS at an antibody loading of 12.8 mg/ml gel, as described for IL-2R. The rIL-2 binding capacity of the immunoadsorbent was determined as described for the

RAC column. The rIL-2 from *E. coli* was immunoaffinity purified and subjected to gel permeation chromatography using the same conditions used in the RAC purification scheme.

RESULTS AND DISCUSSION

IL-2R production, purification and immobilization

The soluble form of the low affinity p^{55} subunit of the human interleukin-2 receptor designated IL-2RΔNae lacks 28 amino acids at the carboxyl terminus and contains the naturally occuring N- and O-linked glycosylation sites[7]. These deletions result in the removal of the presumptive transmembrane and cytoplasmic domains in the IL-2R, thus allowing it to be secreted into the medium by the transfected CHO cells. The IL-2RΔNae was purified from conditioned CHO medium employing IL-2 ligand affinity chromatography[8]. The purified IL-2RΔNae was chemically bonded to the commercially available high-flow, low-pressure, silica-based NuGel-PNHS at a coupling density of 1.55 mg/ml gel with a coupling efficiency of 78%. At this IL-2R loading, the affinity adsorbent had an IL-2 binding capacity of 1.3 mg/ml gel or 68% of the theoretical binding capacity (see Table VI).

Factors affecting the IL-2 binding capacity of the receptor sorbent

Coupling conditions such as pH, activated group density on the matrix and the receptor coupling density affect the coupling efficiency and IL-2 binding capacity of the receptor bead. The IL-2 binding capacities of the receptor adsorbents prepared at various coupling pHs are given in Table I.

Both the coupling efficiency and the IL-2 binding capacity were optimized when the coupling pH was around 7–8. The best coupling efficiency and IL-2 binding capacities were observed when the activated group density was 15–30 μmol/ml gel (data not shown).

The effect of IL-2R coupling density on the IL-2 binding capacity of the receptor-affinity sorbents are summarized in Table II.

As observed for antibodies by Comoglio *et al.*[14], high IL-2R loadings resulted in

TABLE I

EFFECT OF COUPLING pH

Binding capacities were calculated taking into account the molecular weights of IL-2RΔNae (encoded for the polypeptide chain) and rIL-2 as 25 000 and 15 500 dalton, respectively.

Coupling pH	*IL-2R coupled*		*IL-2 binding capacity*		
	mg/ml gel	*nmol/ml gel*	*Calculated (nmol/ml)*[a]	*Observed (nmol/ml)*	*Residual (%)*
5.0	0.900	36	72	14	19
6.0	1.150	46	92	36	39
7.0	1.505	60	120	80	67
8.0	1.575	63	126	88	70
9.0	1.625	65	130	84	65

[a] Two IL-2 binding sites (equivalents) were assumed for the IL-2RΔNae (unpublished observation).

TABLE II

EFFECT OF COUPLING DENSITY

The IL-2R coupling and the IL-2 binding capacity determinations were carried out as described in the text.

IL-2R coupled		*IL-2 binding capacity*		
mg/ml gel	*nmol/ml gel*	*Calculated (nmol/ml)*	*Observed (nmol/ml)*	*Residual (%)*
0.830	33	66	45	68
1.505	60	120	80	67
3.610	144	288	128	44
7.780	311	622	174	28

lower binding capacities, possibly due to steric hindrance. The maximal IL-2 binding capacities were observed when the receptor coupling density was 1–2 mg/ml gel.

Receptor-affinity purification procedures

Extraction, solubilization and renaturation of rIL-2. The rIL-2 was expressed in high concentrations in *E. coli* in an insoluble form within the inclusion bodies. The 1.75 *M* Gu · HCl wash of the pellet, prior to 7 *M* Gu · HCl extraction, removed soluble cellular materials. The extraction of the rIL-2 from inclusion bodies required strong denaturants like 7 *M* Gu · HCl. The solubilized rIL-2 under these conditions was denatured. It was diluted 40-fold and allowed to age for varying periods of time. The results of such a refolding study are shown in Table III. Approximately 3–4 days of aging were needed to renature the Gu · HCl extracted rIL-2.

In the case of conditioned CHO medium, in general, no special treatments were necessary before application to the receptor column.

Adsorption, washing and elution. The adsorption is one of the critical aspects of RAC. The 40-fold dilution of the 7 *M* Gu · HCl extract rendered the Gu · HCl concentration low enough to allow efficient adsorption of the rIL-2 to the receptor column. In order to take advantage of the ability of the receptor sorbent to

TABLE III

rIL-2 REFOLDING STUDIES

The diluted extract was aged for varying periods of time. See text for details.

Age of extract (days)	*Amount of rIL-2 recovered (mg/g cells)*
0	1.536
1	1.918
2	2.300
3	2.480
4	2.604
5	2.572

preferentially bind the fully renatured soluble form of rIL-2 from a heterogeneous population, the column was operated at or above saturating binding conditions. Up to 20 ml/min (maximum flow-rate tried), the capture efficiency was unaffected and no pressure increase or compression of the column bed was observed. The high-flow NuGel affinity support allowed the processing of large volumes of diluted extract and conditioned medium in a short period of time.

Immediately after adsorption, the column was washed extensively with PBS to remove unadsorbed and non-specifically adsorbed materials from within and surrounding the beads. The efficient elution of the adsorbed rIL-2 from the receptor column was achieved by dissociating the receptor–ligand complex with the low pH buffer (pH 2.8) of 0.2 *M* acetic acid containing 0.2 *M* sodium chloride.

Sizing-column, concentration and storage. Gel permeation chromatography was a final step in the purification scheme. It was a convenient way of preparing the rIL-2 free of high-molecular-weight contaminants such as oligomers, trace unwanted proteins of microbial or mammalian origin, pyrogens, etc., as well as low-molecular-weight contaminants like fragments and buffer reagents. This step also exchanged the rIL-2 into its final storage buffer. When kept under aseptic conditions in the acetate–mannitol buffer at pH 3.5, the rIL-2 did not lose any activity, at least for two years.

Purification results

The purification results are summarized in Table IV. During RAC, 63% or 246 mg of the rIL-2 activity in the starting material adsorbed to the column and 39% of the activity was found in the flow-through material. The unadsorbed rIL-2 activity was partly due to the operation of the column above its saturation binding capacity and might be also due to the presence of not fully renatured rIL-2 molecules which might not bind to the RAC column. In the case of mammalian glycosylated rIL-2, 90% or 86 mg bound to the column. After the receptor-affinity step, both forms of rIL-2 had a specific activity of $1.8 \cdot 10^7$ U/mg. The final recoveries for the microbial and mammalian rIL-2s after the gel filtration step were 58 and 88%, respectively. Both forms of rIL-2 had a specific activity of $2.0 \cdot 10^7$ U/mg.

TABLE IV

rIL-2 PURIFICATION RESULTS

To a 250-ml receptor column, 17.5 l diluted extract derived from 100 g *E. coli* cells or 18 l conditioned CHO medium were applied at a flow-rate of 20 ml/min. See the text for further details.

Purification step	*Microbial rIL-2*				*Mammalian rIL-2*			
	Protein (mg)	*Activity ($\cdot 10^7$ U)*	*Recovery (%)*	*Specific activity ($\cdot 10^7$ U/mg)*	*Protein (mg)*	*Activity ($\cdot 10^7$ U)*	*Recovery (%)*	*Specific activity ($\cdot 10^7$ U/mg)*
Extract	1925	703	100	–	81 000	171	100	–
Flow-through	1500	274	39	–	80 900	5	3	–
RAC	246	443	63	1.8	86	154	90	1.8
Sephadex G-50	204	408	58	2.0	75	150	88	2.0

Characterization of the RAC purified rIL-2s

The SDS-PAGE profiles of the microbial and mammalian rIL-2s are shown in Fig. 2. Microbial rIL-2 showed a single band (lane 1), whereas the mammalian rIL-2 (lane 2) contained a major slow moving glycosylated form and a minor fast moving non-glycosylated form, identified by Western blotting with appropriate monoclonal antibodies (data not shown).

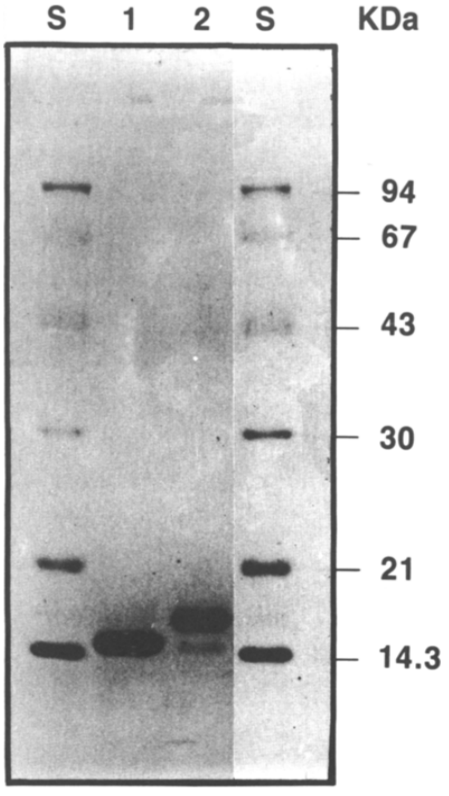

Fig. 2. The SDS-PAGE analysis of receptor-affinity purified rIL-2 from microbial and mammalian sources under non-reducing conditions. Lanes: S = standard molecular weight marker proteins (molecular weights indicated in kilodalton, KDa); 1 = microbial rIL-2; 2 = mammalian glycosylated rIL-2.

The amino acid composition of the both forms of rIL-2 was in good agreement with the expected values (Table V). Structural analysis confirmed the identity of the rIL-2s and the existence of a disulfide linkage between the cysteine residues at positions 58 and 105 (data not shown). The microbial rIL-2 also contained two polypeptide chains, one with an N-terminal Met residue (position 0) and one without, in the ratio of around 9:1 (data not shown).

Comparison of receptor and immunoaffinity purification methods

The rIL-2 binding capacities of the receptor and immunoaffinity adsorbents are given in Table VI.

On a weight-to-weight basis, nine times more antibody was needed per unit

TABLE V

AMINO ACID COMPOSITION OF rIL-2s

Amino acid analyses were performed on a post-column fluorescamine amino acid analyser as described in the text.

Amino acid	*Residues*		
	Microbial	*Mammalian*	*Expected*
Aspartic acid	12.6	11.7	12
Threonine	12.5	12.3	13
Serine	7.2	7.3	8
Glutamic acid	18.5	17.2	18
Proline	5.1	3.9	5
Glycine	2.3	3.0	2
Alanine	5.5	4.7	5
Cysteine	3.0	3.4	3
Valine	4.0	3.7	4
Methionine	4.5	3.9	4 or 5
Isoleucine	8.0	8.1	9
Leucine	22.1	23.4	22
Tyrosine	3.0	3.7	3
Phenylalanine	6.9	6.5	6
Histidine	3.1	3.1	3
Lysine	10.3	11.3	11
Arginine	4.0	4.2	4
Tryptophan	0.9	1.3	1
Total	134	133	133 or 134

volume of immunosorbent to obtain the same amount of IL-2 binding capacity retained by the receptor column. This was partly due to the difference in the molecular weight of the two molecules and also due to the steric hindrance caused by the higher protein loading in the immunosorbent.

As previously shown, the RAC purified rIL-2 was essentially monomeric,

TABLE VI

BINDING CAPACITIES OF RECEPTOR AND IMMUNOSORBENTS

The rIL-2 binding capacities of the receptor and immunosorbents were determined as described in the text. Two binding sites were assumed for the receptor (unpublished observation) and antibody. The molecular weights of the receptor, antibody and rIL-2 were taken as 25 000, 158 000 and 15 500 dalton, respectively.

Adsorbent	*Receptor or antibody coupled (mg/ml gel)*	*IL-2 binding capacity*			
		Calculated (nmol/ml gel)	*Observed*		*Residual (%)*
			mg/ml gel	*nmol/ml gel*	
Receptor	1.55	124	1.3	84	68
5B1 Antibody	12.80	162	1.2	77	48

whereas the immunoaffinity purified material contained significant amounts of oligomeric or aggregated forms of rIL-2, which eluted as a high-molecular-weight fraction during gel permeation[6] along with the desired soluble monomeric form. The specific activities of the rIL-2 purified by both methods are compared in Table VII.

TABLE VII

SPECIFIC ACTIVITIES OF RECEPTOR AND IMMUNOAFFINITY PURIFIED rIL-2s

See the text for experimental details. NA = Not applicable.

Experiment	*Specific activity (· 10^7 U/mg)*	
	RAC	*Immunoaffinity*
Affinity step	1.8	0.8
G-50 Peak 1	NA	0.2
G-50 Peak 2	2.0	2.3

Prior to gel permeation chromatography, the specific activities of the RAC and immunoaffinity purified materials were 1.8 and 0.8 · 10^7 U/mg, respectively. The specific activity of the high-molecular-weight fraction was only one-tenth of that of the soluble monomeric form and caused a two-fold reduction in the specific activity of the immunoaffinity purified total IL-2. These results indicated that in immunoaffinity chromatography, various molecular forms of rIL-2 with varying degrees of biological activity and renaturation state might bind to the antibody column. In contrast, the RAC purified rIL-2 contained essentially the soluble monomeric form.

In summary, RAC has been demonstrated as a viable purification method for rIL-2. The versatility of RAC is such that it is also capable of purifying various rIL-2 muteins, rIL-2 homologues and IL-2 fusion proteins[15,16]. The receptor-affinity sorbents are also stable and performed satisfactorily for at least 500 cycles of operation with no significant impairment in the functionality (data not shown).

The two major areas of concern are the cost-effectiveness and the stringent U.S. FDA regulations[17,18], regarding the quality control requirements for producing recombinant proteins for medicinal use in humans. The advances made in the recombinant DNA technology should enable the large-scale production of soluble receptors economically (*e.g.*, IL-2, IL-1, etc.). The quality control aspects of using RAC for purifying protein biotherapeutics for human use still has to be resolved.

Since biotechnology is on the verge of producing soluble receptors of biomolecules such as IL-1 (ref. 19), γ-interferon, tumor necrosis factor, etc., we strongly believe that it is only a matter of time before RAC becomes an established method for the purification of high-value recombinant proteins.

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