

## Production and Purification of Monoclonal Antibodies to Human Interleukin-2 and Their Use for Immunoaffinity Purification of Recombinant Human Interleukin-2

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**Abstract:** Monoclonal antibodies (mAbs) are a powerful immunochemical tool. Today, the availability of mAbs with desired specificity enables ligand separation based upon the immunoaffinity technique. By using the high selectivity of mAbs, immunoaffinity purification offers the possibility of isolating compounds even from complex samples with a selectivity which cannot be achieved by other methods. This paper reports the preparation and identification of monoclonal antibodies against human interleukin-2 (IL-2) and the establishment of an immunoaffinity method for purifying human IL-2 (hIL-2).

Hybridomas were generated by the fusion of NSO or FO myelomas and spleen cells from immunized mice. Hypoxanthine-aminopterin-thymidine-resistant hybridomas secreting antibodies specific for hIL-2 were assayed by an ELISA and cloned using the single cell pick-up technique. The monoclonal antibody of the isotype IgM was purified from fetal calf serum (FCS)-free supernatant of hybridoma cell culture using ammonium sulfate precipitation followed by size-exclusion chromatography. The monoclonal antibody of the isotype IgG1 was purified using protein G affinity chromatography directly from supernatant of hybridomas cultured in medium supplemented with IgG-depleted FCS. In order to purify human IL-2 by immunoaffinity chromatography, anti-human IL-2 IgG1 mAb was coupled to Cyanogen Bromide-activated Sepharose 4B gel beads.

Three monoclonal antibodies, designated CAY-IL2M, CAY-IL2Mb, and CAY-IL2G, have been produced against human interleukin-2. CAY-IL2M and CAY-IL2Mb were shown to be of the isotype IgM, and CAY-IL2G was shown to be of isotype IgG1. Hybridoma tissue culture supernatants were strongly positive by ELISA at dilution of up to 1/1000. Human recombinant IL-2 was purified by immunoaffinity chromatography on CAY-IL2G-Sepharose CL-4B (5x50 mm), with a recovery of nearly 80% at a high flow rate of 0.4ml per minute. In order to prevent substantial losses in the total antigenic activity, the elution step was effectively optimized. These results suggest that the anti-human IL-2 mAb established in this study is useful for one-step purification of recombinant human IL-2.

These mAbs have great potential for the development of an immunoassay for measuring human IL-2 and their application in immunoaffinity chromatography could offer a valuable tool for the purification of natural human IL-2 secreted from mitogen plus lectin-stimulated peripheral blood mononuclear cells. Thus, mAbs directed against other epitopes that might be present on native IL-2 could be obtained in the future.

**Key Words:** Monoclonal antibody, human interleukin-2, immunoaffinity purification.

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### Introduction

A monoclonal antibody (mAb) possesses a unique specificity and an extremely high selectivity for the epitope present on the antigen (1). Antibodies are important tools used by many investigators in their research and have led to many medical advances. The use of monoclonal antibodies in biomedical research has been and will continue to be important for the identification of proteins, carbohydrates, and nucleic acids. Their use has led to the elucidation of many molecules that control cell

replication and differentiation, advancing our knowledge of the relationship between molecular structure and function (2-4). In addition, the exquisite specificity of mAb allows them to be used in humans and animals for the diagnosis and treatment of diseases (5, 6).

Interleukin-2 plays an important role in acquired immunity (7). However, only small amounts of natural IL-2 are secreted from human peripheral blood mononuclear cells (8). Therefore, a method for an effective purification system is needed. Separation based upon immunoaffinity

is becoming more and more common in a large field of applications. By using the high selectivity of biomolecules (antibodies, receptors, specific proteins), this technique offers the possibility of isolating compounds even from complex samples (9).

Accordingly, this study describes the production, characterization, and purification of anti-human IL-2 monoclonal antibodies and their application to the purification of recombinant human IL-2 using immunoaffinity column chromatography.

## Materials and Methods

Female BALB/c mice, aged 6-8 weeks, were used for immunization, isolating resident peritoneal macrophages and preparation of thymocyte-conditioned medium (TCM).

Mouse thymocytes were obtained by removing the thymus glands from four BALB/c mice and then pressing the organs through a stainless steel mesh. The cells were then pooled, washed with RPMI-1640 (Sigma Sigma Chemical Co. USA) and resuspended at  $5 \times 10^6$  cells per ml in RPMI-1640 supplemented with 20% fetal calf serum (FCS, Seromed-Biochrom KG D-12247 Berlin, Germany). The cells were cultured in 250ml flasks (Greiner, Frickenhausen, Germany) for 6 days. The culture supernatants were then harvested, centrifuged to remove the debris and filtered through a  $0.2 \mu\text{m}$  filter (Schleicher & Schuell GmbH, Dassel, Germany). The TCM was stored at  $-80^\circ\text{C}$  in aliquots until use.

A 6-week-old mouse (mouse 1) was immunized with  $50 \mu\text{g}$  of recombinant human interleukin-2 (r-h-IL-2, Lot No: 20B06Y, Glaxo IMB, 46 routes des Acacias, 1211 Geneva 24-Switzerland) intraperitoneally every second week. Another mouse (mouse 2) was immunized with  $50 \mu\text{g}$  of recombinant human interleukin-2 (r-h-IL-2, Proleukin, Aldesleukin,  $18 \times 10^6$  IU/ml, Roche) intraperitoneally every month. Complete and incomplete Freund's adjuvants (Sigma) were used for the first and second inoculations respectively. The final injection of antigen was given in 0.09% NaCl. The spleen cells of the animals were collected for fusion 3 days after the final boost. Cell fusion procedure was performed as described previously (10). Briefly, all splenocytes from mouse 1 were fused with FO myeloma cells (ATCC, American Type Culture Collection, Rockville, Maryland, USA) and half of the splenocytes from mouse 2 were fused with NSO

myeloma cells (obtained from Royal Postgraduate Medical School, London) using polyethylene glycol (Wt 3000-3700, Sigma). After fusion, cells were resuspended in complete medium (CM) consisting of RPMI-1640 with L-glutamine and  $\text{NaHCO}_3$  supplemented with 20% heat-inactivated FCS, 10mM HEPES, 1mM sodium pyruvate, 10% TCM, and 4% non-essential amino acids (Sigma) and then incubated in feeder cell-seeded 24-well tissue culture plates (Greiner, Frickenhausen, Germany) at a density of  $2 \times 10^6$  cells per ml in an atmosphere of 5%  $\text{CO}_2$  in air at  $37^\circ\text{C}$ . Peritoneal resident macrophages from non-immunized BALB/c mice were used as feeder cells at a density of  $5 \times 10^4$  cells per well. After incubation for 24 h, hypoxanthine-aminopterin-thymidine (HAT, Sigma) mixture was added to the medium (11). Cultures were subsequently fed with HAT containing complete medium. Hybrid growth was assessed visually using an inverted microscope. Culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) for antibody production.

Screening for the presence of anti-h-IL-2 mAb in the culture supernatants of hybrid cells was performed with lab-made ELISA. High binding capacity 96-well flat-bottomed microtiter plates (Cat no: 3590, Costar, Corning Incorporated, Corning, NY, USA) were coated with Proleukin. To each well was added  $50 \mu\text{l}$  of IL-2 at  $2 \mu\text{g/ml}$  in phosphate buffered saline (PBS) and then they were incubated overnight at room temperature. After washing, potential binding sites were blocked with  $200 \mu\text{l}$  of 0.3% bovine serum albumin (BSA, Sigma). Culture supernatants,  $100 \mu\text{l}$  per well, were incubated for 1 h at  $37^\circ\text{C}$ . After washing five times with 0.03% Tween-20 containing PBS (PBS-T), to each well was added  $100 \mu\text{l}$  of horse radish peroxidase (HRP)-labeled Fab-specific anti-mouse IgG conjugate (Sigma Chem. Co., MO, USA), diluted to 1/6,000 in PBS-T, and then incubated for 1 h at  $37^\circ\text{C}$ . After washing five times, to each well was added  $100 \mu\text{l}$  of TMB-chromogen/ $\text{H}_2\text{O}_2$ -substrate and then they were incubated for 30 minutes at room temperature. The reaction was stopped by adding  $50 \mu\text{l}$  of 2M  $\text{H}_2\text{SO}_4$  per well and then optical density (OD) was evaluated with an ELISA reader (LP400, Diagnostics Pasteur, France) using either a 450nm or a 450/620 nm filter.

Hybrid cell clones were obtained by picking up a single cell with a thin-pointed pasteur pipette (hand-made) under inspection with an inverted microscope settled in a class II biological safety cabinet. Each hybrid cell was

transferred to a separate well of a 96-well flat-bottomed microtitre plate (Greiner, Germany). Single hybrid cells were fed with CM. Supernatants from wells where only one clone was microscopically detectable were tested for immunoreactivity. Cells from positive clones were propagated and then transferred into 25cm<sup>2</sup> flasks containing RPMI-1640 supplemented with 10% FCS.

In order to determine the isotypes of the monoclonal antibodies produced by the hybridoma clones, a commercially available isotyping kit (ISO-1, ImmunoType Mouse Antibody Isotyping Kit, Sigma Chemical Co) was used and studied according to the manufacturer's instructions.

Hybridoma cells were cultured at 37°C with 5% CO<sub>2</sub> and 95% air and under fully humidified conditions. The culture medium was RPMI-1640 supplemented with 10% FCS. Hybridoma cells were seeded at 1x10<sup>4</sup> cells per ml in 24-well plates (Greiner, Germany) on day 0.

Ascitic fluid was obtained as described previously (10). The IgM content of diluted and glass wool-passed ascitic fluid was obtained using 30% saturated ammonium sulfate (SAS) precipitation (12). A protein pellet was resuspended in PBS and dialyzed against PBS. Dialysate was passed through a Sephadex G-200 packed column attached to a high-performance liquid chromatographic (HPLC) system (Waters 650 Advanced Protein Purification System-600E System controller, Division of Millipore, Milford, MA, USA) equipped with a programmable multiwavelength detector (Waters 490E) and an interface module. A personal computer was used to control the apparatus and the standard HPLC software (Maxima 820 chromatography workstation) was used to evaluate chromatographic runs. IgM containing the leader peak was pooled and then any contaminant IgG was completely removed by passing through a protein G Sepharose High Performance (1 ml MAbTrap G II Kit, Supelco Bellefonte, PA, USA) affinity column as described previously (13). Protein concentrations in the samples were determined by the method described elsewhere (14) using bovine serum albumin (BSA, Sigma Chem. Co., MO, USA) as a standard.

CAy-IL2Mb was purified from 50 ml supernatant of hybridomas cultured in FCS-free RPMI-1640. The initial cell concentration was adjusted to 5x10<sup>6</sup> cells per ml and then incubated for 48h. After a 50% SAS precipitation, the protein pellet was resuspended in PBS. For desalting,

it was passed through Sephadex G-50 fine (Sigma, packed in a 10x50mm glass column).

CAy-IL2G mAb was purified as described previously (15). In brief, anti-human IL-2 IgG1 mAb secreting hybridomas were cultured in RPMI-1640 supplemented with 10% IgG-depleted FCS for 48 h. The initial cell concentration was adjusted to 5x10<sup>6</sup> cells per ml. Nearly 50ml of supernatant was passed through 1ml protein G column and the bound fraction was eluted with 2ml of 0.1M citric acid pH 2.6. The fraction eluted was neutralized with 1M Trisma base.

Human IL-2 concentrations were measured with a commercially available ELISA kit (BioSource International, 820 Flynn Road, Camarillo, California, USA). ELISA was performed according to the manufacturer's instructions.

CAy-IL2G was immobilized on Cyanogen Bromide-activated Sepharose 4B gel beads as described elsewhere (16). MAb-bound gel matrix was packed in a glass column of 5x50mm (Tosohaas, Zettachring 6, 7000 Stuttgart 80, Germany). The column was equilibrated with run buffer (RB; 10mM PO<sub>4</sub> buffer pH 7.2 containing 9g NaCl per L). Then 2ml of Proleukin solution (40µg/ml) was applied to the HPLC system-attached CAy-IL2G column at a flow rate of 0.4ml/min. The matrix-bound fraction was eluted with 2ml of 0.1M Glycine-HCl pH 2 at a flow rate of 1ml/min and 3ml of the peak fraction was neutralized with 0.5ml of 1M Trisma base.

## Results

Three hybridoma cell lines were established. CAy-IL-2M and CAy-IL-2Mb hybridomas were obtained by fusing splenocytes with FO myeloma. CAy-IL-2G was obtained by fusing splenocytes with NSO myeloma. The isotypes of the monoclonal antibodies were found to be IgM for both CAy-IL-2M and CAy-IL-2Mb hybridomas and IgG1 for CAy-IL-2G hybridoma (Table 1).

Culture supernatants of CAy-IL-2G and CAy-IL-2Mb hybridomas demonstrated a strong reaction against proleukin-coated solid phase. However, it was not prominent with CAy-IL2M. When hybridomas were cultured for various periods of time, mAb concentrations in the culture supernatants increased in a time dependent manner (Figure 1).

The desalting of 50% SAS precipitated CAy-IL2Mb mAb was effectively performed with Sephadex G-50 size-

Hybridoma cell	Myeloma partner used for fusion	Secreted mAb specific for	Isotype of mAb secreted
CAy-IL2M	FO	human IL-2	IgM
CAy-IL2Mb	FO	human IL-2	IgM
CAy-IL2G	NSO	human IL-2	IgG1

Table 1. Characteristics of the hybridoma cell lines established.

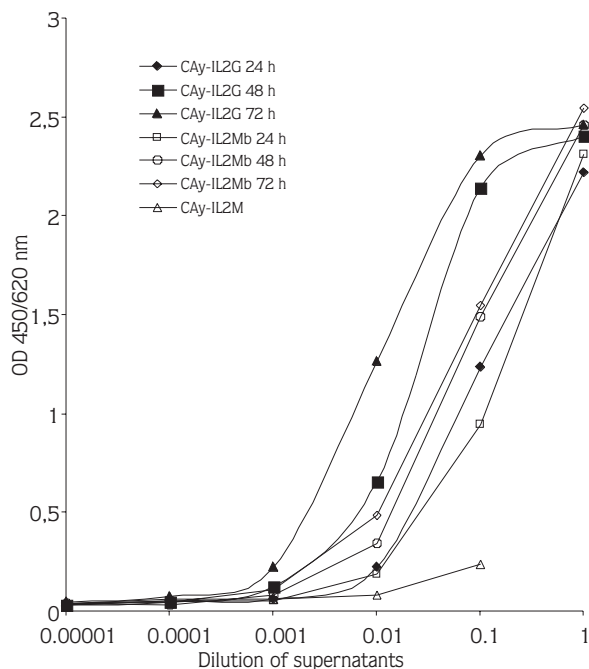


Figure 1. Anti-human IL-2 secretion capacities of hybridoma cells. Hybridomas were cultured in RPMI-1640 supplemented with 10% FCS for various time periods. Hybridomas were initially seeded at a concentration of  $1 \times 10^4$  cell per ml. Interleukin-2 level, represented as OD450/620 nm at serial dilution, was determined using proleukin-coated solid phase and HRP-labeled Fab-specific conjugate. Data are represented as the mean of duplicate wells.

exclusion chromatography (Figure 2). As shown in Table 2, nearly all of the CAy-IL2Mb was recovered from FCS-free culture supernatant by 50% SAS precipitation followed by desalting.

During each application, the IgG content of 5 ml of FCS was almost completely removed by 1 ml protein G column. When IgG-depleted FCS (G-FCS) was re-loaded on protein G, almost no binding occurred (data not shown). Thus, the mAb purified from the supernatant of hybridomas cultured in G-FCS-supplemented medium demonstrated high purity. As shown in Figure 3, 0.1M citric acid pH 2 buffer eluted the protein G-bound IgG1. Almost all of the IgG1 was purified from the culture supernatant by protein G affinity chromatography (Table 3).

In preliminary experiments it was determined that 0.1M Glycine-HCl pH 2 was an effective elution buffer for releasing bound Proleukin from CAy-IL2-Sepharose (Figure 4). At a flow rate of 0.4ml per minute, the CAy-IL2G-Sepharose immunoaffinity column bound most of the human recombinant IL-2 and the antigenic property was maintained when the IL-2-containing peak fraction was immediately neutralized with 1M Tris base (Table 4).

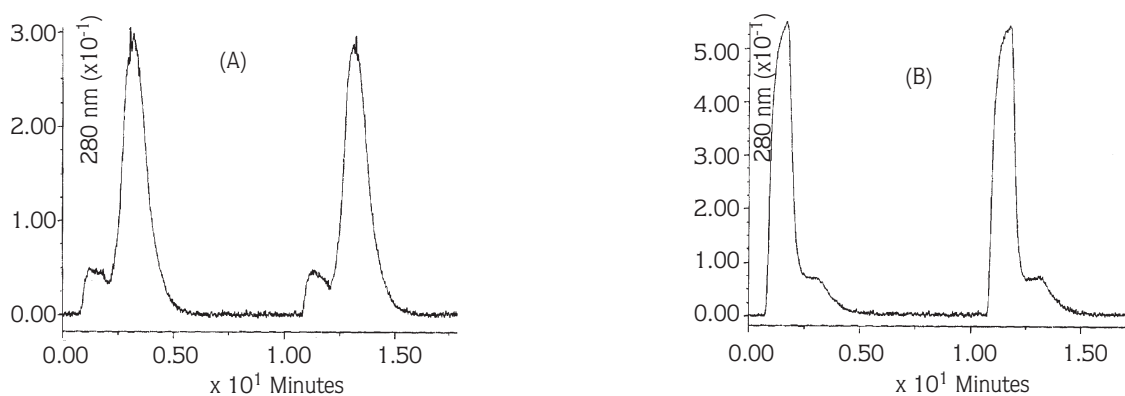


Figure 2. Purification of CAy-IL2Mb from culture supernatant without FCS using 50% SAS precipitation followed by desalting through Sephadex G-50. After centrifugation of 50% SAS-containing culture supernatant, upper layer (A) demonstrated a high content of ammonium sulfate (second peak on A) and minute amounts of CAy-IL2Mb (compare Table 2) represented as the first peak on A. Sephadex G-50 size-exclusion chromatography of the precipitate (B) demonstrated a high content of CAy-IL2Mb (compare Table 2), represented as the first peak on B, and minute amounts of ammonium sulfate (second peak on B).

Table 2. Effectiveness of 50% SAS precipitation for purification of IgM mAb from FCS-free culture supernatant.

Dilution <sup>a</sup>	OD450 nm value of the samples				
	Culture supernatant (50ml) (50 ml)	SAS Supernatant (100 ml) <sup>b</sup>	SAS Precipitate (2 ml) <sup>c</sup>	Sephadex G-50 First peak (2x1.5 ml) <sup>d</sup>	Sephadex G-50 Second peak (2x2.75 ml) <sup>e</sup>
10 <sup>0</sup>	2.824	0.996	1.836	1.680	0.173
10 <sup>-1</sup>	0.337	0.070	1.255	1.097	0.096
10 <sup>-2</sup>	0.125	0.068	0.524	0.460	0.086
10 <sup>-3</sup>	0.078	0.070	0.168	0.125	0.072
10 <sup>-4</sup>	0.070	0.062	0.082	0.081	0.068
10 <sup>-5</sup>	0.069	0.068	0.068	0.073	0.070

- a. Dilution was made in RPMI-1640 supplemented with 10% FCS  
b. Samples were taken from the supernatant of overnight-incubated 50% SAS containing CAy-IL2Mb culture supernatant after centrifugation at 1500g for 60 minutes.  
c. Samples were taken after dissolving the pellet obtained by SAS precipitation as described above.  
d. Pooled fractions (from 50<sup>th</sup> second to 135<sup>th</sup> second) obtained from Sephadex G-50 chromatographic study as depicted in Figure 2.  
e. Pooled fractions (from 135<sup>th</sup> second to 300<sup>th</sup> second) obtained from Sephadex G-50 chromatographic study as depicted in Figure 2.

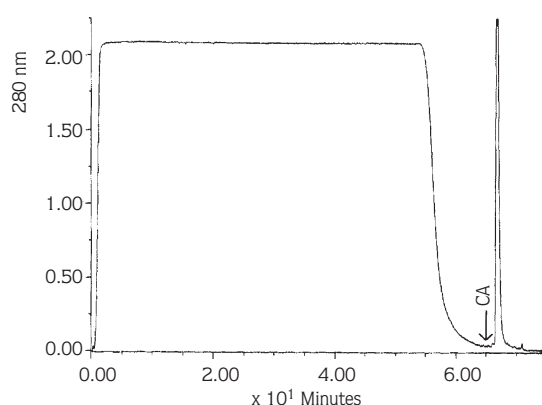


Figure 3. Purification of CAy-IL2G from G-FCS-supplemented culture supernatant using protein G affinity chromatography. Nearly all of the CAy-IL2G was recovered from 55 ml of supernatant using 1ml protein G column (compare Table 3). Single peak on the chromatogram demonstrated that protein G-bound CAy-IL2G was effectively eluted after application of 2ml of 0.1M citric acid pH 2 (CA).

## Discussion

Monoclonal antibodies are extensively used in basic biomedical research, and in the diagnosis, and treatment of illnesses such as infections and cancer. However, mAb production technology has not been developed satisfactorily in Turkey. Although several studies relating to the production of mAbs have appeared in Turkish journals during the past few years (10, 17-21), its level is still far below what it should be.

Before the advent of the hybridoma method, investigators could produce only polyclonal serum antibodies, and this required large numbers of immunized animals for a sufficient amount of antibodies. Development of the hybridoma technology provided numerous advantages such as obtaining huge amounts of specific antibodies and purification of the specific ligand in a concentrated form with high purity.

Dilution <sup>a</sup>	OD450 nm value of the samples		
	Culture supernatant (55 ml)	Flow-through (60 ml) <sup>b</sup>	Peak (mAb+Trisma) (2.25+0.9 ml) <sup>c</sup>
100	2.824	0.156	2.638
10-1	2.854	0.083	2.620
10-2	1.987	0.084	2.854
10-3	0.357	0.069	2.456
10-4	0.100	0.074	0.692
10-5	0.097	0.074	0.375
10-6	0.074	0.077	0.233

Table 3. Effectiveness of protein G affinity chromatography for purification of IgG1 from G-FCS-supplemented culture supernatant.

- a. Dilution was made in RPMI-1640 supplemented with 10% FCS  
b. CAy-IL2G culture supernatant passed through protein G column.  
c. Protein G-bound CAy-IL2G mAb eluted with 3 ml of 0.1M citric acid pH 2.6. In order to neutralize citric acid-eluted 2.25 ml of mAb peak, 0.9 ml of 1M Trisma base was added.

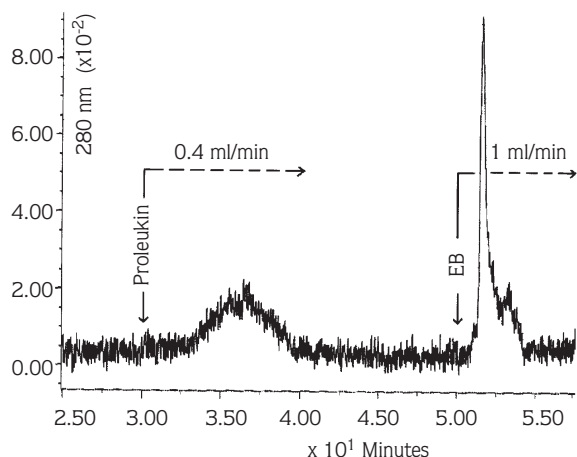


Figure 4. Purification of recombinant human IL-2 using CAY-IL2G-Sepharose 4B immunoaffinity chromatography. Most of the recombinant human IL-2 was captured by CAY-IL2G coupled Sepharose 4B even at a high flow rate of 0.4ml per min (compare Table 4). Recombinant human IL-2 was effectively eluted from the matrix by 0.1M Glycine HCl pH 2 (EB). Proleukin-containing peak reserved its antigenic property when immediately neutralized with Trisma base (compare Table 4).

This study was performed to produce anti-human IL-2 mAbs for multi-purpose usage. Among the purposes was the purification of human IL-2 by immunoaffinity chromatography. Immunoaffinity separations can be used

for any material to which a specific antibody can be raised, making this procedure applicable to a wide range of molecules of biomedical interest (22-24).

Three hybridomas (CAY-IL2M, CAY-IL2Mb, and CAY-IL2G) were established. CAY-IL2M and CAY-IL2Mb were found to secrete the mAb of the IgM isotype and CAY-IL2G secreted the mAb of IgG1 isotype (Table 1). For producing medium-scale (in micrograms) amounts of IgM and IgG antibodies, the above-mentioned methods seemed to be effective (Figures 2 and 3).

CAY-IL2G monoclonal antibody coupled to Sepharose 4B absorbed recombinant IL 2, confirming that it reacts directly with IL-2 (Figure 4). The absorbed IL-2 could be eluted by using 0.1M Glycine-HCl pH2, thus providing a potential means for further immunoaffinity purification of natural IL-2 from crude culture supernatant in future studies.

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Dilution <sup>a</sup>	OD450 nm value of the samples		
	Loaded sample (2 ml) <sup>b</sup>	Flow-through (4 ml) <sup>c</sup>	Peak (mAb+Trisma) (3+0.5 ml) <sup>d</sup>
10 <sup>-3</sup>	Overflow	0.326	0.911
10 <sup>-4</sup>	0.201	0.118	0.152
10 <sup>-5</sup>	0.126	0.103	0.110

Table 4 Effectiveness of CAY-IL2G-Sepharose immunoaffinity chromatography for purification of recombinant human IL-2.

- a. Dilution was made in sample diluent buffer of the kit.
- b. Proleukin at 40µg/ml in PBS.
- c. Pooled fractions (from 34<sup>th</sup> second to 44<sup>th</sup> second at a flow rate of 0.4ml/min) obtained from the study depicted in Figure 4.
- d. Pooled fractions (from 51<sup>th</sup> second to 54<sup>th</sup> second at a flow rate of 1ml/min) obtained from the study depicted in Figure 4.

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