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Full Length Research Paper

One step purification of biological active human interleukin-2 protein produced in yeast (*Pichia Pastoris*)

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Pharmacological importance of recombinant human interleukin-2 protein has increased the demand to establish effective, reliable and cost effective chromatography method for its production and purification on large scale. One step mimetic ligand affinity chromatography method for purification of mutated human recombinant interleukin-2 (mrhIL-2), from *Pichia Pastoris* is described with higher yield then reported before. The mrhIL-2 was expressed extracellularly under methanol inducible AOX1 promoter of *P. pastoris.* Extracellular expression of mrhIL-2 in the culture supernatant was ~210 mg/L. Cell free culture supernatant containing mrhIL-2 protein was concentrated and buffer exchanged by diafiltration by tangential flow filtration system. Different mimetic legends column from sigma were screened for efficient binding of mrhIL-2 in culture supernatant. Maximum binding was observed with Mimetic Blue SA P6XL and least with Mimetic Green 1 A6XL. One step Dye ligand affinity chromatography method was developed by using Mimetic Blue SA P6XL ligand for high level purification of mutant of interleukin-2. Final yield of purified protein was 115 mg/L and purity of 97%. The interleukin-2 protein prepared by this protocol was found to be monomeric based on SDS-PAGE, western blot and HPLC analysis. Purified protein was biological active as checked by cell proliferation assay.

Key words: Interleukin-2, *Pichia pastoris*, Dye ligand affinity chromatography.

INTRODUCTION

The interleukin-2 molecule is a glycosylated 15.5 Kilo Dalton (KDa) protein involved in proliferation of T lymphocyte (Robb and Smith, 1981). Interleukin-2 is the FDA-approved treatment of metastatic renal carcinoma and the first therapy approved for the treatment of metastatic melanoma and is being tested in combination therapy with monoclonal antibodies for the treatment of non-Hodgkin's lymphoma and breast cancer (Atkin, 2002; Sarah et al., 2004).

Different multistep chromatography method has been reported to purify interleukin -2 expressed in *Echerschia coli* and *Pichia pastoris* (Welte et al., 1982; Malcolm et al., 1987; Yun et al., 1988; Liu et al., 2006; Sengupta et al., 2008). In a r ecent research paper, on column refolding and purification of interleukin-2 protein produced in *E. coli* has been reported (Najafabadi et al., 2010). Purification of recombinant protein based on multistep procedures that result in low protein yield and are not economical for large scale production. To increase the product yield, it will be ideal to develop a single-column purification scheme. Dye affinity chromatography has been used for purification of different proteins previously (Lowe et al., 1984). This chromatography has advantage of high level purification in one step. Chromatography media which are based on textile dyes are not suitable for human recombinant protein. Because textile dyes have leaky nature. Synthetic based mimetic legend resins are more suitable for such proteins. These chromatography media show specific interaction with protein because of their biomimetris structure (Lowe et al., 1992). These chromatography media are available commercially from different companies like sigma Aldrich, GE Health Care, etc. These mimetric legends are synthetic and immo-

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bilized on solid matrix by highly stable linkages. Also they do not have low quality leaky nature as compared to textile dye based media. Sensitive immunoassays confirm that these chromatography media have not shown any ligand leakage (Stewart et al., 1992; Lowe et al., 1990). Ligands are stable in pH range of 2 to 14 and biologically inert and can be sterilized chemically by treatment with 8 M urea, ionic and non ionic detergent or 1 M NaOH. Also media can be sterilized by autoclaving for 30 min at 120°C.

Human IL-2 has three Cysteine residues, namely, Cys-58, Cys-105, and Cys-125, and native IL-2 has an intramolecular disulfide bond between Cys-58 and Cys-105 (Tsuji et al., 1987). Recombinant human IL-2 with substitution of cys-125 to ser-125, expressed in *E. coli* has been found biologically active after *in vitro* refolding (Wang et al., 1984; Yun et al., 1988). *Pichia pastoris* has the ability to perform eukaryotic-specific post-translational modifications such as proteolytic processing, folding, disulfide bridge formation, and glycosylation (Eckart and Bussineau, 1996; Cregg et al., 1985, 1993).

In the present study, a mutated clone of IL-2 substituted C125/S125 was expressed in *Pichia pastoris*. Different mimetic legend chromatography media were used for high yield purification of mrhIl-2. Mimetic Blue SA P6XL gave highest recovery of purified mrhIL-2. Final yield and purity of intact rmhIl-2 was 115 mg/L and 95%, respectively. The characterization of the purified protein was done by SDS-PAGE, Western blot and HPLC analysis. However, the purified protein was biologically active as checked by the cell proliferation assay.

MATERIALS AND METHODS

Construction of IL-2 expression vector pPICZαC-hIL-2

The IL-2 gene encoding the mature polypeptide chain was amplified from human placental cDNA. The sequences of forward and reverse primers for cloning purposes were (5'-gcatctcacgg-tacgcatgtagc-3') and (5'-cagcaacattggatgaaaaa-3'), respectively. PCR mixtures containing amplified interleukin-2 fragments were purified by using QIAquick® PCR Purification Kit (QIAGEN) and digested with EcoR1 enzymes. EcoR1 digested fragment was inserted into *Pichia pastoris* expression vector pPICZ alphaC to generate pPICZαIL-2 clone. Substitution of cysteine (Cys) at 125 positions with serine (Ser) was performed according to instruction manual of Quikchange site directed mutagenesis kit from QIAGEN. Sequence verified clone was designated mrhIL-2.

Expression studies

GS115 cells were transformed with *Pme1*-linearized mrhIL-2 plasmid and spread on low salt Luria bertani agar plates and incubated at 30°C for 72 h until colonies formed. Integration was confirmed by PCR from genomic DNA of selected clones by AOX1 promoter and terminator primers. High copy number transformant (Selected on zeocine selection curve) was induced as described in manufacturer's manual of Easy Select Pichia Expression Kit (In vitrogen). Cells were grown in BMGY medium until the culture reaches an OD600 of approximately 5. Harvested cells were resuspended in BMMY medium and 0.5% methanol was added every 24 h to maintain induction. Culture for expression studies were grown in baffled flasks 28°C at 300 rpm shaking in gyratory incubator shaker for ninety six h. Control strain (Strain transformed with pPICZαC vector) was induced to test the effectiveness of expression conditions. At defined time points samples were taken from the cultures for absorption measurements, SDS-PAGE and quantitative HPLC analysis.

Purification of secreted modified interleukin-2

Screening of mimetic ligends for rhIL-2 binding

Protein estimation was done by Bradford method in culture supernatant. All ten column of PIKSI kit were screened for rhIL-2 protein binding. AKTA explorer 10 was used for screening. Columns were pre equilibrated with Buffer A (Tric-HCl pH7.0, 0.01% Tween 20). 10 ml culture supernatant filtered through 0.45 filter was passed through all ten columns of kit at flow rate of 0.5 ml/min separately. Out of the ten columns, protein was bound to five columns only (Mimetic Red-3 A6XL, Green-1 A6XL, Blue-1 A6XL, Blue-2 A6XL and Mimetic Orange 3 A6XL). High yield of recovered protein was observed with Mimetic Blue SA P6XL column whereas least with Mimetic Green 1 A6XL column. For purification of mutated recombinant IL-2 protein from one liter batch culture mimetic Blue SA P6XL matrix was selected.

Diafilteration and purification

Culture supernatant was filtered by 0.45 um opticap filter (Millipore). Recombinant IL-2 protein was concentrated and buffer exchanged through Tangential flow filtrations system (TFF) from Millipore using nitrocellulose membrane cartridge with 5-KDa molecular weight cut off. Sample was passed through cartridge at flow rate of 3 ml/min by peristaltic pump. Buffer A (Tris- HCl pH 7.0, Tween 20 0.01%) was pumped into sample reservoir at flow rate of 1 ml/min. The process was completed in 5 h at room temperature. Sample was concentrated up to 25% of original volume. During the process, time to time sample was checked for pH and conductivity required for loading on to an affinity column. At the end of process, conductivity was maintained at 2 ms/cm and pH7.0. Concentrated sample was again filtered by 0.45 um opticap filter. Yield was calculated from concentrated filtered sample by bradford's method and quantitative HPLC.

For purification of rhIL-2 protein from one liter batch culture, XK16/10 column (GE Health Care Life sciences) was packed with Mimetic Blue SA P6XL as follows. Column was packed at AKTA 10 explorer system according to instruction manual of chromatography media. Concentrated filtered sample was loaded onto a Dye ligand affinity column, equilibrated with Buffer A (Tris- HCl pH 7.0, Tween 20 0.01%) at flow rate of 7 ml/min. Column was washed with buffer A for 2CV at same flow rate. mhIL-2 was eluted with buffer B (Tris-HCl pH 7.0 , NaCl 1M) with linear gradient of 15 CV at 3.0 ml/min flow rate.

SDS-PAGE western blot analyses

mrhIL-2 peak fractions from mimetic affinity chromatography were checked by 12% SDS-PAGE under denaturing conditions using Mighty small SE 260 electrophoresis system of GE Health Care Life sciences. Gel was silver stained. Purified fractions were then pooled and protein content was determined by Bradford's method.

Western blot analysis was performed by semi dry blot procedure (Bejerrum and Schafer, 1986) on semiphor system of Hoefer. After

electrophoresis molecular weight standard, samples were transferred electrophoreticaly to nitrocellulose membrane (0.45 um). For immuno detection test, the membrane was incubated in blocking buffer overnight at 4°C. In the next day, mrhIL-2 on membrane was probed with monoclonal anti Il-2 antibody (Sigma-Aldrich) for 1.5 h at room temperature. Probe was washed with Phosphate buffer saline containing 0.1% Tween 20 three times. Secondary antibody of mouse conjugated with alkaline phophatase was used from Sigma- Aldrich. NBT/BCIP tablet (Sigma-Aldrich) was used as substrate for signal detection.

Qualitative and quatitative HPLC

RP-HPLC was carried out using source 5 RPC (4.6 x 15 cm) column on schemadzu LC 20 system with UV/Vis detector. Absorbance wavelength was set at 280. A step gradient was used for elution of mrhIl-2. Mobile phase A was 0.1 % TFA in water and mobile phase B consisted of 65% acetonitrile in 0.1 % TFA. Flow rate of 0.5 ml/min was maintained. A calibration curve was plotted for rhIL-2 standard for quantification of mrhIL-2 protein estimation in culture supernatant.

In vitro Biological activity studies

MrhIL-2 test samples and standard rhIL-2 (Sigma-Aldrich) were prepared as follows. Two fold serial dilutions in 96-well flat-bottomed micro plate (tissue culture grade) starting with 10 ng/ml in a volume of 100 µl was prepared. Culture medium was used as a negative control. Lymphoblast cell line CTLL-2 cells (ATCC # 214) were harvested and washed three times by centrifugation in culture medium without hIL-2. 100 ul cells were introduced into each well of test plate to revealing a final concentration of 4×10^3 cells/well. Microplates were incubated for 48 hours at 37°C and 5% CO_2. After the incubation cells were labeled with dye by using MTT kit (Promega) Microplate was incubated for another 6 h at 37°C and 5% CO_2. Absorbance of the proliferative cells was measured at a wavelength of 495 nm by using a microplate reader (Spectra Max). A curve was prepared to determine biological activity of standard and mutants of rhIL-2.

RESULTS AND DISCUSSION

Human recombinant interleukin-2 protein is FDA approved drug for metastatic renal cell carcinoma and melanoma. Increased therapeutic value of IL-2 protein in cancer, AIDS and other auto immune diseases shows great need to develop highly efficient, cost effective procedure to produce this drug at commercial scale.

PCR amplified interleukin-2 gene from human placenta cDNA was amplified (Figure 1). PCR amplified fragment was sub cloned in pPICZalpha vector under AOX1 promoter for extracellular expression (Figure 2). Interleukin-2 variant with cysteine 125 subsitution with serine has been studied in *E. coli* (Wang et al., 2004; Yun et al., 1988). Liu et al. (2006) have developed an IL-2 variant clone with three substitutions. In present study single mutation C125/S125 was generated by site directed mutagenesis kit to check the extracellular expression for single step purification of rhIL-2 variant.

For secretory expression, culture conditions were optimized. It was observed that expression of interleukin-2 was maximal before 70 h and it started declining immediately afterwards. To avoid the factor of protein degradation by proteolyses of host cell, culture was grown for 65 h and harvested for purification of interleukin-2. Figure 3 represents expression condition for extracellular expression of mrhIL-2.

Interleukin-2 was first purified from lymphocyte cells to apparent homogeneity with 19% yield by four chromatography steps Anion exchange, gel filteration and two dye affinity chromatography steps (Welte et al., 1982). A two step gel permeation chromatography procedure for purification of interleukin-2 protein produced in *E. coli* with 22% yield and purity of 95% has been reported (Wier and Sparks, 1987). Gel filteration method with yield of 5% and purity of 99.5% from *E. coli* source has been studied (Sangupta et al., 2008). Interleukin-2 protein produced in *P pastoris* was purified by cation exchange and gel filteration chromatography with 20.2% yield (Liu et al., 2006) In comparison to different reported methods for interleukin-2 protein purification, which involved multitude of chromatography steps as discussed above, a simple and easily scalable one step protocol for high level purifycation of extracellularly expressed mrhIL-2 in *P. pastoris* was developed.

To maintain pH 7.0 and conductivity of 2.0 ms, the sample was buffer exchanged by diafilteration. The recovery of purified mrhIL-2 after concentration and buffer exchange was 90%.

The diafiltered and clarified mrhIL-2 sample was passed through mimetic blue A6XL column at 7 ml /min flow rate pre equilibrated with 50 mM Tric-HCL, pH 7.0, 0.01% Tween 20). We used Tween 20 in Buffer A to avoid protein aggregation. Without Tween 20 almost 80% protein was aggregated as analyzed by non reducing SDS-PAGE.

Column was washed with Buffer A for 2 CV and a linear gradient of 0 to 1 M NaCl was established using AKTA 10 explorer system as described earlier. Figure 4 shows elution profile of mrhIl-2 purified by mimetic affinity column. Few of early fractions contain some impurities while the rest contain only homogenous IL-2 protein purified fractions from dye legend affinity chromatography were subjected to SDS-PAGE and visualized by silver staining (Figure 5). Final yield as described in Table 1 was 120 mg/l, which is higher then previously reported for secretion production of interleukin-2 in *P. patoris*. Qualitative analyses reveal that purity of protein was more than 97% as analyzed by RP-HPLC (Figure 6) and by SDS-PAGE (Figure 7). The above results also indicated that protein was purified to apparent homogeneity with monomeric form of interleukin-2 protein. Further monomeric form of purified protein was assessed by Western blot analyses (Figure 8).

Purified mrhhIL-2 was assayed for the activity on proliferation of CTLL-2 cells, while rhIL-2 protein from Sigma was used as positive control. Both exerted potent

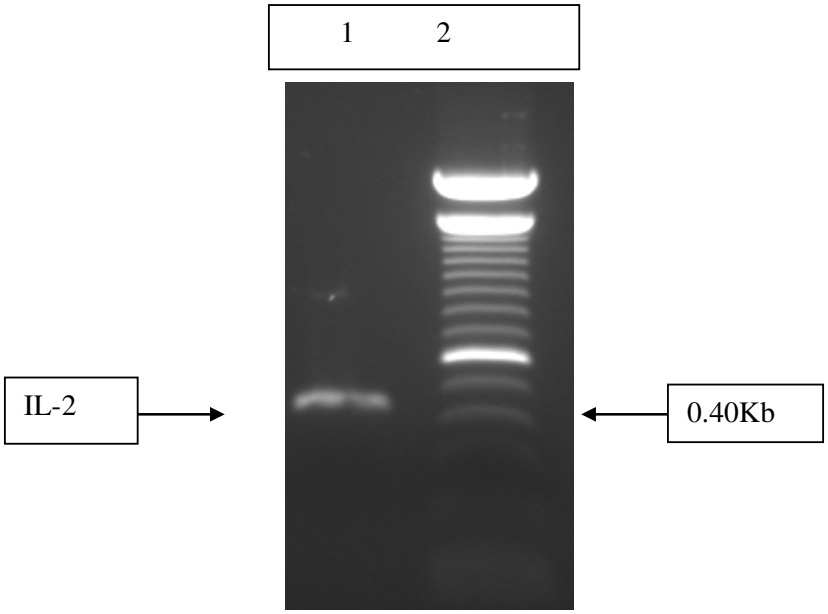

Figure 1. Lane 1 PCR amplified hIL-2 gene, and lane 2 100 bp DNA ladder.

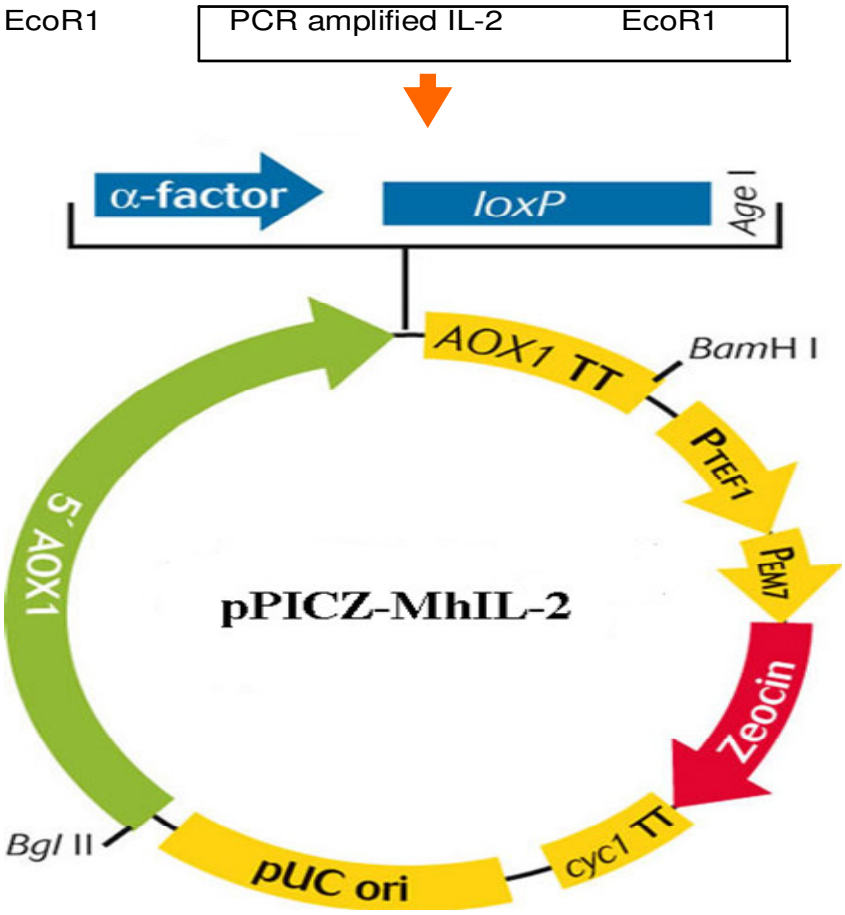

Figure 2. Construction of expression cassette. PCR amplified interleukin-2 gene was cloned in pPICZαC vector at *Eco*R1 site.

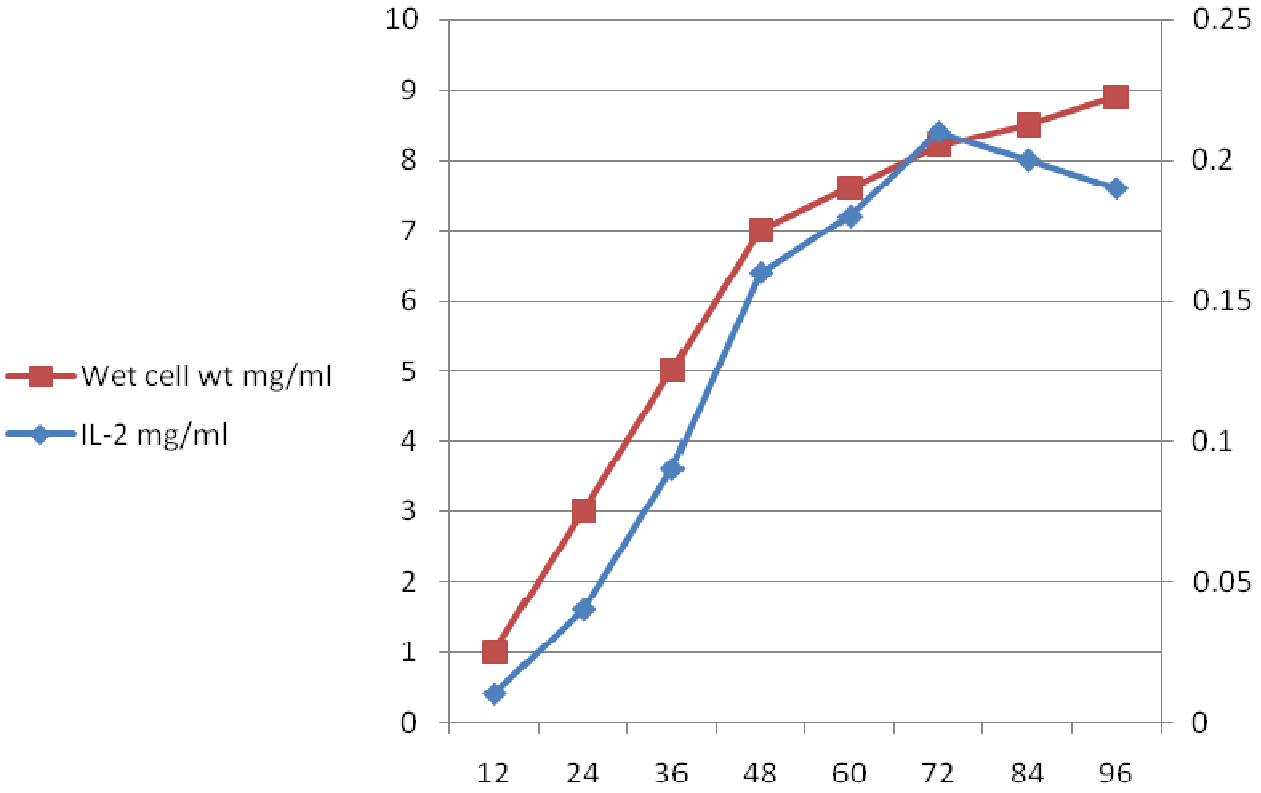

Figure 3. Expression studies of human interleukin-2. X-axis shows time in h, Y- right axis explain wet cell weight in mg/ml. Y-left axis indicates expression of mrhIL-2 protein in mg/ml; It was observed that expression was almost negligible after 15 h and peak at 72 h. But after 65h some degradation was also observed as analyzed by SDS-page (Data not shown). Wet cell weight grows exponentially up to 80 h.

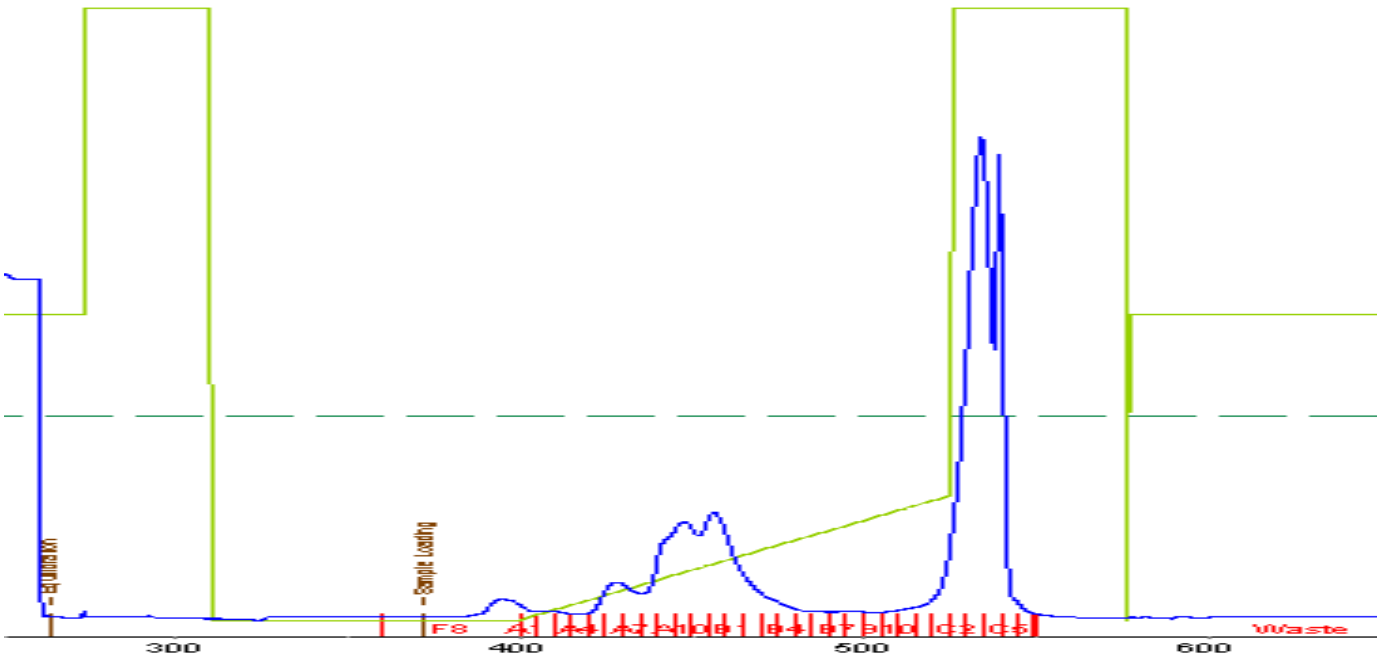

Figure 4. Dye legend affinity chromatography of mutant of human interleukin-2 produced in yeast. Concentrated Culture supernatant was loaded on a self packed XK 16/10 column of Mimetic Blue SA P6XL. Column was equilibrated with Tris-HCl buffer at pH 7.0. Protein was eluted with linear gradient of 0 to 1M NaCl in Tris-HCl buffer at pH 7.0.

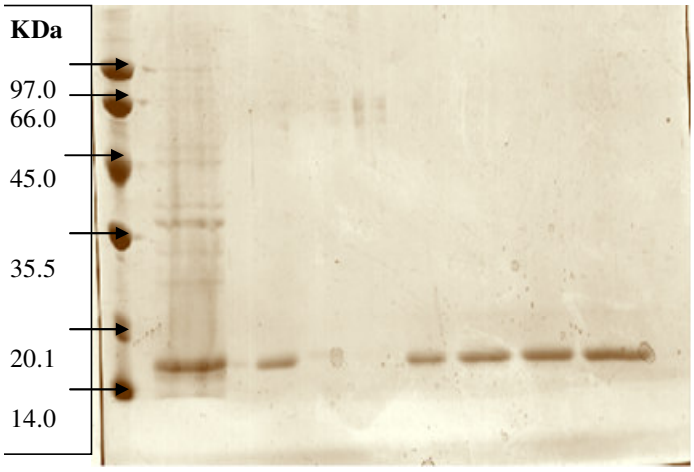

Figure 5. SDS-PAGE analysis of IL-2 protein purified by dye legend affinity chromatography. Lane 1: LMW (97.0, 66.0, 45.0, 35.5, 20.1, 14.0) KDa.l, lane 2: Crude mrhIL-2 in culture supernatant of *Pichia pastoris*, lane 3: Refrence standard (sigma Aldrich), lanes 4 to 9: Fractions of IL-2 from from dye legend affinity chromatography

Table 1. Purification of mrhIl-2 from *Pichia pastoris* shakes flask culture, **(**a)Total protein was determined by Bradford's method, **(**b) mrhIL-2 in culture supernatant was measured by Quantitative RP- HPLC, (c) Purity was assessed by RP-HPLC and SDS-PAGE, (d) Step yield was calculated based on difference in out/input step, **(**e)Overall yield was measured by difference in output of corresponding step and input of initial step.

Step	Total volume (ml)	Total [a] (mg)	mrhIL-2[b] (mg)	Purity[c] (%)	Step yield[d] (%)	Overall yield[c] (%)
Culture supernatant	500	105	85	70	100	100
Diafilteration	125	100	77	75	90	73
Dye legend affinity chromatography	10	60	58.9	97	76	56

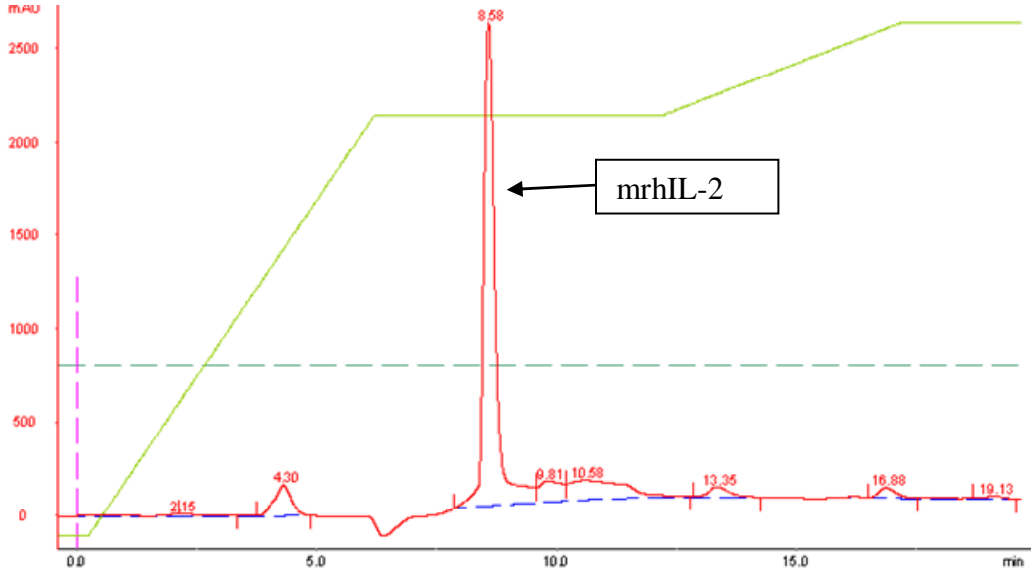

Figure 6. RP-HPLC analysis of purified mrhIL-2 was perfomed on 4.6 cm x 0.15 cm source 5RPC column with Schemadzu LC 20 system, t = 20 min. HPLC. Conditions: solvent A, 0.1% TFA in water; B, 60% acetonitrile/0.1% TFA. Gradient: 0 to 65% B, 6 min; 82%B, 14 min; 82 to 100%B, 20 min; the flow rate was 1 ml/min. Il-2 protein elute at 82%B at Retention time T of 8.58.

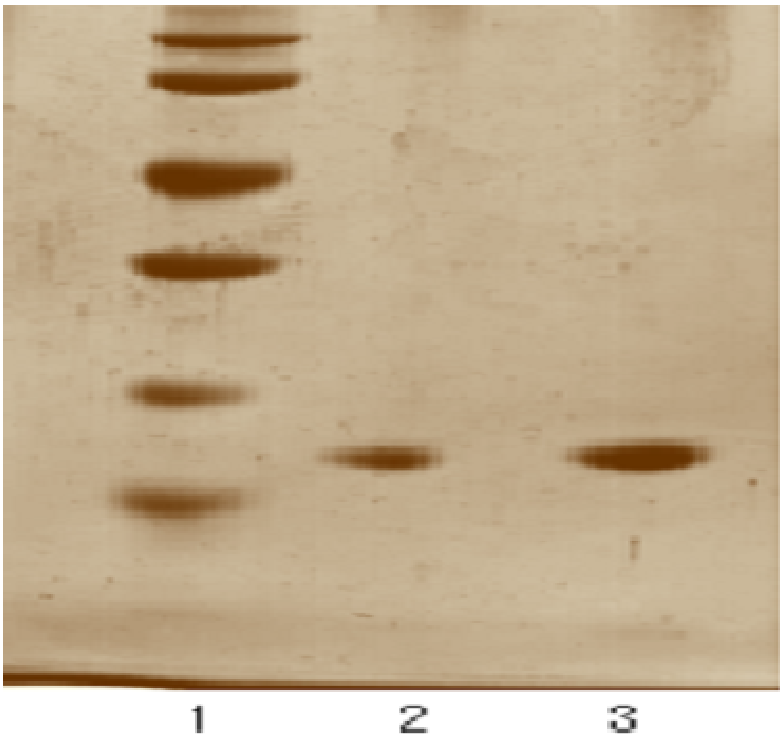

Figure 7. Reducing and non reducing SDS-PAGE analysis of mrhIL-2 purified. Lane 1: LMW (97.0, 66.0, 45.0, 35.5, 20.1, 14.0) KDa. Lane 2: mrhIL-2 with β mercapto ethanol. Lane 3: mrhIL-2 with out β mercapotoethanol. Result indicates that purified protein is essentially monomeric and homogeneous.

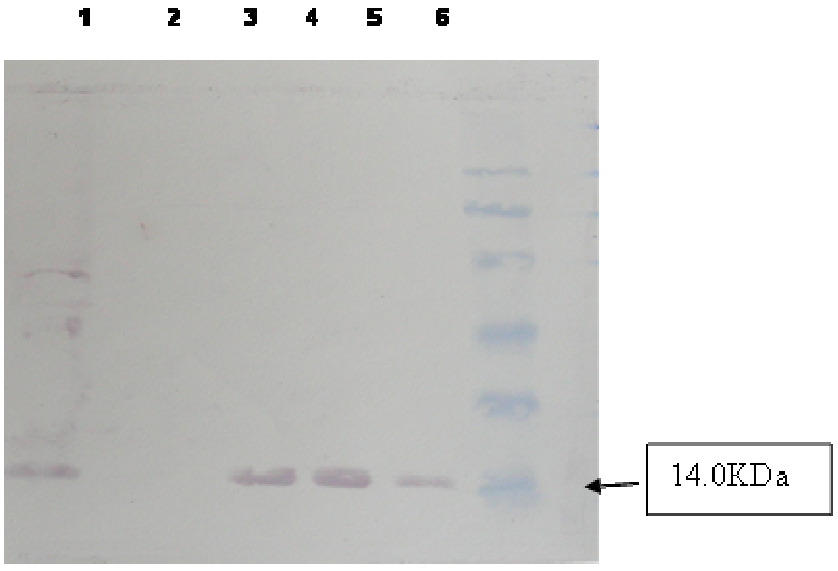

Figure 8. Western blot of homogeneous mrhIL-2. A monomeric form of rhIL-2 was observed when transferred to a nitrocellulose membrane and detected using a monoclonal Il-2 antibody. Lane 1: Culture supernatant of host cells transformed with mrhIL-2; lane 2: Cultur supernatant of host cells transformed with pPICZaplhaC (control); lane 3 Purified mrhIL-2 with β mercapto ethanol; lane 4 mrhIL-2 without β mercapto ethanol; lane 5: Reference standard (Sigma Aldrich); and lane 6: protein molecular mass markers (Invitrogen).

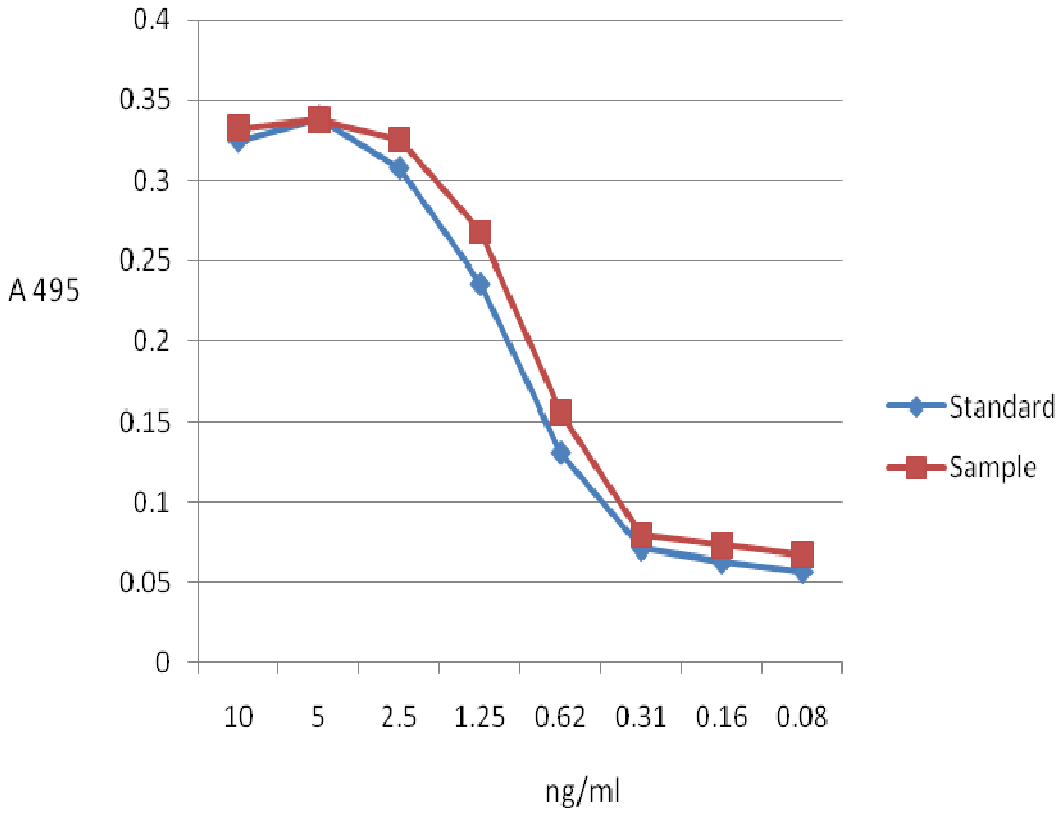

Figure 9. Biological activity determination of rhIL-2. Both sample as well as standard was biological active in cell proliferation assay.

stimulating effects on the proliferation of CTLL-2 cells in a dose-dependent manner. Substitution of serine for cysteine 125 did not affect the biological activity of rIL-2 (Figure 9). In the present study, an efficient one step chroma-tography method to get highly yield and purity of mrhIL-2 protein expressed in *P. pastoris* was established. Mimetic legend affinity chromatography media can be used for many times, because it can withstand with chemical and heat sterilization. The process described above is simple, economical in nature, cost effective and can be used for downstream process development of therapeutic human recombinant secretion products in *P. Pastoris.*

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