Expression, Identification and Characterize of CD25-Binding Epitope Modified Human IL-2 in *Pichia pastoris*

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Abstract Interleukin-2 (IL-2) plays important roles in variety of immune functions and it is widely used in the medication. But in recent years it was reported that vascular leak syndrome (VLS) was induced by IL-2. Evidences showed that the interaction of IL-2 and IL-2R $\alpha\beta\gamma$ (CD25) caused VLS. Thus, this experiment modified the CD25-binding epitope in human IL-2 (hIL-2) to minimize the side effect of IL-2 in the medication. In this study, a recombinant human interleukin 2 (rhIL-2) was expressed in *Pichia* (*P.*) *pastoris*. An effective strategy was established to express rhIL-2 protein in 120 L scale and the optimal purification procedure was investigated. The purity of rhIL-2 in final product was about 98 % and the concentration of the rhIL-2 was 0.45 mg/mL. Bioactivity analysis showed that the purified rhIL-2 protein displayed high activity on proliferation of CTLL-2 cells and increased the ratio of CD4⁺/CD8⁺. It indicates that the target protein is expressed and the character of the rhIL-2 has high activity. This study provides a strategy for large-scale production of bioactive rhIL-2 protein using *P. pastoris* as an expression host.

Keywords IL-2 · Expression · Purification · Immune function

Introduction

Interleukin-2 (IL-2) was originally discovered as a growth factor for activated T cells in vitro. As an important

immunoregulatory factor, IL-2 can have both positive and negative effects on T cells when it was used to enhance T cell immunity [1]. IL-2 can rescue insulin secretion and is necessary for tregs regulatory function in the pancreas [2]. It modulates perioperative immuno-dysfunction in patients undergoing tumor nephrectomy [3]. However, high doses of IL-2 intermittent administration induced side effects in vivo with typical lymphocytic interstitial pneumonitis, nephritis and lymphocytic depletion in splenic corpuscle [4]. The main side effect was vascular leak syndrome (VLS) [5, 6]. Krieg et al. [7] found that the VLS was induced by interaction between IL-2 and IL-2R $\alpha\beta\gamma$. Thus, this experiment designed a new recombinant human IL-2 (rhIL-2) to block the binding of IL-2 and IL-2R $\alpha\beta\gamma$ and to minimize the side effect of IL-2.

Bioactivity assay of rhIL-2 in vitro was used to examine the proliferation activity of cytokine [8]. The ratio assay of CD4⁺/CD8⁺ was used to examine the cellular immune function [9]. IL-2 promotes CD8⁺ T cell growth and differentiation in vivo, but has little effect on CD4⁺ T cell function [10]. Therefore, bioactivity assay of rhIL-2 in vitro and the ratio assay of CD4⁺/CD8⁺ in vivo can be used to examine the activity of rhIL-2.

Therefore, the new rhIL-2 was designed, identified and purified. Then the character of the protein was also examined.

Materials and Methods

Strains, Plasmids and Reagents

The recombinant plasmid pGH-mhIL-2 containing synthetic mutant of human IL-2 (mhIL-2) gene was purchased from Generay biotech (shanghai, China) Co., Ltd, and propagated in *E. coli* XL-10-Gold. The methylotrophic yeast *Pichia* (*P.*) *pastoris* GS115 strain and the pPIC9 K

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plasmid were purchased from Invitrogen (CA, USA) Co. Competent cell *E. coli* C600 λ was kept in our laboratory (Institute of Special Economic Animal and Plant Science, Chinese Academy of Agricultural Sciences, Changchun, China). ^3H -TdR was purchased from China Institute of Atomic Energy (Beijing, China). HIL-2 was purchased (1.0×10^7 IU/mg) from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Construction of Expression Plasmid pPIC9 K-mhIL-2

The recombinant plasmid (pGH-mhIL-2) containing synthetic mhIL-2 gene fragments were digested with *Eco*RI and *Sna*BI, and cloned in frame at the corresponding sites of the expression vector Ppic9 k (Fig. 1). The recombinant vector with correct sequence was identified by DNA sequencing and restriction enzymes analysis. The expression vector pPIC9 K was transformed into *E. coli* C600 λ . Then the expression plasmid pPIC9 K-mhIL-2 was obtained. The plasmid pPIC9 K-mhIL-2 was verified by both *Eco*RI and *Sna*BI digestion and further sequencing (Sangon Biotech Shanghai Co., Ltd).

Transformation into *P. pastoris* GS115 and Selection of Positive Transformant

After linearization with *Sal*I, the plasmid pPIC9 K-mhIL-2 was transformed into the *P. pastoris* GS115 cells by the method of LiCl according to Lei et al. [11]. The transformants harboring the AOX1 promoter-controlled mhIL-2 expression cassette were plated on the MGY plates containing zeocin (0.10 mg/mL)

to select His⁺Mut⁺ positive transformants. The positive transformant was further tested for the presence of integrated plasmid using the following primers by PCR amplification.

primer 5':5'-GACTGGTTCCAATTGACAAGC-3';
primer 3':5'-GCAAATGGCATTCTGACATCC-3'.

Expression of mhIL-2

The expression of rhIL-2 in *P. pastoris* was performed according to Lei et al. [11]. The selected transformant was expanded at 28–30 °C in 100 mL of BMGY medium. After the density reaching an OD_{600nm} of 2–3, cells were collected by centrifugation at 4000 rpm for 10 min, then were suspended in 20 mL of BMMY (PH 6.0) medium. During the induction period, methanol (100 %) was added into the culture every 24 h to concentration of 0.5 % v/v. The culture that induced for 96 h was recovered, and the supernatants were tested by SDS-PAGE and western blot according to the method described previously [12].

Purification and Identification of rhIL-2

After 96 h of inducing expression, the culture was centrifuged at 4,000 rpm for 10 min. The supernatant was directly loaded onto a 5 mL Sephadex G-25 column (GE Healthcare) operated with ÄKTA-FPLC (GE Healthcare). The impurities were removed and proteins were dissolved in sodium acetate solution (20 mM, PH 4.7). The rhIL-2 was purified by cation exchange column (Hitrap SP, GE Healthcare) with 20 mM sodium acetate buffer solution (pH 4.7) as the elution buffer. Proteins were eluted with a linear NaCl gradient (0–1 M). After identification by SDS-PAGE, the target fraction was further purified through a 20 mM Tris–HCl (pH 8.0) equilibrated sephacryl S100 HR column (GE Healthcare). The purity of rhIL-2 was identified by SDS-PAGE. A Micro BCA kit (Pierce Co., OR, USA) and densitometric analysis of SDS-PAGE were used to quantify the yield of purified protein.

The Assay of Bioactivity of rhIL-2

Bioactivity assay of rhIL-2 in vitro was done according to Lei et al. [11]. This experiment includes 3 groups: experiment group, positive group and negative group, 5 replicates in each group. 50 ng of mhIL-2 was added in the experiment group; hIL-2 was added in the positive group and the termination concentration was 500 IU/mL (50 ng); the same volume of RPMI-1640 medium was added in the negative group. Then the cell was incubated at 37 °C in the 5 % carbon dioxide incubator (Stik. Shanghai, Co., Ltd). After 30 h ^3H -TdR 1μCi was added into each group and continue to culture to 40 h. The culture was transferred into glass fiber filter membrane. The saline, 5 % trichloroacetic

Fig. 1 Recombinant expression plasmid pPIC9 K-mhIL-2

Table 1 The treatment of the rats in different groups

Item	The first treatment	The second treatment
1	None	50 μ L of 1 mg/mL recombination human IL-2 was subcutaneously injected at the first, third, fifth and seventh days
2	None	50 μ L of 1 mg/mL recombination rhIL-2 was subcutaneously injected at the first, third, fifth and seventh days
3	None	50 μ L of 150 mM PBS was subcutaneously injected at the first, third, fifth and seventh days
4	50 μ L of Salmonella was intraperitoneally injected at the first day	50 μ L of 1 mg/mL recombination human IL-2 was subcutaneously injected at the first, third, fifth and seventh days
5	50 μ L of Salmonella was intraperitoneally injected at the first day	50 μ L of 1 mg/mL recombination rhIL-2 was subcutaneously injected at the first, third, fifth and seventh days
6	50 μ L of Salmonella was intraperitoneally injected at the first day	50 μ L of 150 mM PBS was subcutaneously injected at the first, third, fifth and seventh days

acid and absolute ethyl alcohol were passed through the filter membrane. After the filter membrane was dried it was put into the measuring bottle with 5 mL scintillation solution. The cpm value was measured by β liquid flash calculating instrument (Zhongjianweikang, Beijing, Co., Ltd).

Ratio Assay of CD4⁺/CD8⁺ T Cells

The treatment of the rats was done according to Table 1. The CD4⁺ and CD8⁺ T cells isolated from the rats in different group were used to detect the biological activity of rhIL-2. The proliferation of cells was determined by [³H] thymidine incorporation. After cultured with rhIL-2 or IL-2 for 3 days, the cells (1×10^5 cells/well) were plated in a 96-well round-bottomed plate after several washes. [³H] thymidine (1 μ Ci/well) was added into each well simultaneously. 30 h later, the cells were harvested on fiber glass filter mats to measure the nuclear bound radioactive [³H] thymidine using a liquid scintillation counter.

Statistical Analysis

Statistical analysis were done using SPSS 13.0 package programmer (SPSS Inc., Chicago, IL, USA) with one-way analysis of variance followed by Student's *t* test. Data were shown

Fig. 2 SDS-PAGE and Western blot analysis of rhIL-2. Lane 1: expression supernatant; lane 2: western blot hybridization; lane M: protein molecular weight markers

Fig. 3 The SDS-PAGE of the purified rhIL-2. Lane 1: purified rhIL-2; lane M: protein molecular weight markers

as means and standard deviation (SD). The different left symbol means markedly difference ($P < 0.01$); the different right symbol means significant difference ($P < 0.05$); the same right symbol means no significant difference ($P > 0.05$).

Results

Expression and Identification of rhIL-2

SDS-PAGE analysis showed that the rhIL-2 protein was highly secretory expressed by *P. pastoris* GS115 in the

Table 2 Purification summary for rhIL-2

Purification step	Amount of mhIL-2 (g/L)	Purity (%)
Expression supernatant	1.35	64
Sephadex G-25 column	0.87	72
Cation exchange column	0.76	87
Sephacryl S100 HR column	0.45	98

Fig. 4 The CPM of the rhIL-2. The *different left* symbol means markedly difference ($P < 0.01$); the *different right* symbol means significant difference ($P < 0.05$); the *same right* symbol means no significant difference ($P > 0.05$)

induction period. Western blot analysis showed that the expected major protein band was 15 kDa and it was rhIL-2 in the supernatant (Fig. 2).

Purification of rhIL-2

The rhIL-2 protein was efficiently purified to 98 % purity through a Sephadex G-25 column chromatography, cation exchange column chromatography and sephacryl S100 HR column chromatography. The purification summary is presented in Table 2 and the purified rhIL-2 protein had an apparent molecular weight of 15 kDa (Fig. 3).

The Concentration and Activity of rhIL-2

The concentration of rhIL-2 was 0.45 mg/mL (Table 3). The CPM in the experiment group was significant higher than that in the negative group ($P < 0.01$), The CPM in positive group was significant higher than that in the negative group ($P < 0.01$). There is no significant difference between the experiment group and positive group (Fig. 4).

Table 3 Concentration of rhIL-2

Concentration	Standard sample						mhIL-2
	15 pg/mL	50 pg/mL	100 pg/mL	250 pg/mL	500 pg/mL	1,000 pg/mL	
OD	2.222	2.039	1.761	1.234	0.495	0.164	0.634

The Ratio of CD4⁺/CD8⁺

The ratio of CD4⁺/CD8⁺ in the first group was markedly different from that in the second and fifth groups ($P < 0.01$). The ratio of CD4⁺/CD8⁺ in the first group was significant different from that in the sixth group ($P < 0.05$). The ratio of CD4⁺/CD8⁺ did not have significant differences with that in fourth and fifth group. The ratio of CD4⁺/CD8⁺ in the second group was markedly different from that in the third, fourth and fifth group ($P < 0.01$). No significant differences were observed between the first group and the sixth group. The ratio of CD4⁺/CD8⁺ in the third group was markedly different from that in the fifth and sixth group ($P < 0.01$). No significant differences were observed between the third group and the fourth group. The ratio of CD4⁺/CD8⁺ in the fourth group was markedly significant from that in the fifth and sixth groups ($P < 0.01$). Markedly differences were observed between the fifth and sixth groups ($P < 0.01$) (Fig. 5).

Discussion

IL-2 plays important roles in a variety of immune functions. However, High dose of IL-2 used in the medication shows side effects. The most common manifestation of IL-2 toxicity is capillary leak syndrome, resulting in a hypovolemic state and fluid accumulation in the extravascular space [13]. Krieg et al. [7] discovered that VLS were mainly caused by IL-2R $\alpha\beta\gamma$ non-immune cells activated by IL-2 in vivo. As IL-2-mediated VLS mainly depends on IL-2R $\alpha\beta\gamma$ non-immune cells. Moreover, IL-2 binding to IL-2R $\alpha\beta\gamma$ non-immune cells heavily depends on CD25. So in this experiment CD25-binding epitope in the IL-2 was modified. The rhIL-2 lost the affinity with IL-2R $\alpha\beta\gamma$ (CD25) and the VLS will not be occurred in the treatment with rhIL-2.

Secretory expression of the target protein usually facilitates subsequent purification process [14, 15]. However, degradation of target protein may occur during secretory expression especially in high-cell density culture [16]. Moreover, the degradation fragments often hinder effective purification of target protein [17]. In this experiment, the method of purification process was explored and finally the rhIL-2 was highly expressed by *P. pastoris*, a powerful eukaryotic expression system, reaching 1.35 g/L. Due to the characteristics of *P.*

Fig. 5 The ratio of CD4⁺/CD8⁺. The *different left* symbol means markedly difference ($P < 0.01$); the *different right* symbol means significant difference ($P < 0.05$); the *same right* symbol means no significant difference ($P > 0.05$)

pastoris, rhIL-2 was expressed into the culture and impurities were less in the culture, and it was beneficial to purify target protein rhIL-2. The rhIL-2 was purified through cation exchange column chromatography and sephacryl S100 HR column chromatography. The purity of rhIL-2 in final product was about 98 % and the concentration of the rhIL-2 was 0.45 mg/mL. Therefore, the optimized purification method of rhIL-2 was constructed.

Subsequently, the bioactivity of rhIL-2 in vitro showed that the purified rhIL-2 protein displayed high activity on proliferation in CTLL-2 cells and increasing the ratio of CD4⁺/CD8⁺. It indicates the rhIL-2 has the same activity as hIL-2. Therefore, we constructed an efficient refolding procedure of rhIL-2. But the effects of rhIL-2 on VLS in the medication would be examined in the future.

In conclusion, the rhIL-2 protein was successfully expressed and purified. The bioactivity of the rhIL-2 increases the proliferation of the CTLL-2 cells and the ratio of CD4⁺/CD8⁺. This study provides a strategy for large-scale production of bioactive rhIL-2 protein using *P. pastoris* as an expression host.

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