Expression of an interleukin-6 - interleukin-2 fusion protein (pIL-6-IL-2) in *P. pastoris*

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ABSTRACT. Interleukin-2 and interleukin-6 can stimulate the growth and proliferation of T lymphocytes and the differentiation of activated B lymphocytes respectively, and in turn enhance cellular and humoral immune responses. In this work, an expression clone using *Pichia pastoris*, a methylotrophic yeast strain, has been developed in order to produce large amounts of the functional recombinant fusion protein pIL-6-IL-2, which contains the mature porcine interleukin-6 peptide and the mature porcine interleukin-2 peptide. Two components of the fusion protein were connected by means of a flexible linker (Gly-Gly-Gly-Gly-Ser-Glu-Phe-Gly-Ser-Gly-Gly). In response to 1% methanol induction, the recombinant strain GS115/9K-IL6-IL2 secreted an exogenous protein, with a molecular weight of approximately 40 kD, into the culture medium. This was confirmed to be pIL-6-IL-2 by means of SDS-PAGE and Western Blot analysis. The protein was visible on the 2nd day following methanol induction, and peaked on the 4th day. By this time, the level had reached 50 mg/L as determined using the method of Bradford. After treatment with PNGase F and analysis of the concentration of sugar, the fusion protein pIL-6-IL-2 was further confirmed to be mainly a glycoprotein with an approximately 2 kDa sugar decoration. In addition, the IL-6 and IL-2 biological activities of the fusion protein, determined by cell proliferation assays using the IL6-dependent cell line B9 and the IL2-dependent cell line CTLL-2, reached 1×10^5 U/mg and 8×10^5 U/mg, respectively.
This report is the first description of fused porcine cytokines expressed in *P. pastoris*, which might be an interesting adjuvant product for veterinary vaccines.

Keywords: porcine interleukin-6, porcine interleukin-2, fusion protein, *Pichia pastoris*

INTRODUCTION

Interleukins (ILs), which are produced by leucocytes, are a class of cytokines that have stimulating activities for various cells. To date, 23 types of interleukins have been described. Interleukin-2 (IL-2) is a glycoprotein, which is produced by activated Tlymphocytes and NK cells. Secreted by type 1 helper T cells (Th1), IL-2 mainly stimulates cellular immune responses such as the induction of cytotoxic T cells, activation of NK and LAK cells and enhancement of production of other cytokines by T cells [1, 2]. IL-2 can also stimulate the proliferation of activated B lymphocytes and induce immunoglobulin secretion [3]. Interleukin-6 (IL-6), known as a Th2-like cytokine, is found to be associated with humoral immune responses. This cytokine is secreted by a number of different cell types and plays an essential role in the final differentiation of B lymphocytes into immunoglobulin-secreting cells, which produce large amounts of secretory IgA [4-7]. As a late-acting killer helper factor, IL-6 can also stimulate the differentiation of cytotoxic T lymphocytes [8, 9]. In view of all the actions of IL-2 and IL-6, it is not surprising that they have been studied extensively as potential vaccine adjuvants [10, 11]. Moreover, it is notable that these two pleiotropic cytokines have reciprocal enhancing activities: IL-2 can stimulate LAK cells to produce various cytokines including IFN-γ, IL-4, IL-6 [12], and IL-6 can induce IL-2R expression and enhance IL-2 production by T cells [13]. The description of their different functions in immune responses and their reciprocal collaborating activities, encouraged us to produce a fusion protein containing both IL-2 and IL-6 bioactivities as a candidate adjuvant for veterinary vaccines.

P. pastoris, a methylotrophic yeast strain, is able to utilize methanol as its sole carbon source and has been developed as a host for the high expression of heterologous proteins, with relatively low production costs [14, 15]. Identification of the strong and tightly regulated alcohol oxidase I (AOX1) promoter, construction of user-friendly integration vectors, and well-established fermentation methods, have contributed to the emergence of *P. pastoris* as a commercially useful host for the production of foreign proteins. Hence, we chose the *P. pastoris* system as the tool, with which to produce the fusion protein pIL6-IL2.

In the present study, we report the development of a recombinant *P. pastoris* clone for the expression of fusion protein pIL-6-IL-2, and the methods of production of highly expressed and biologically active pIL-6-IL-2. To our knowledge, this is the first report of expression of a fusion protein

containing porcine cytokines in *P. pastoris*. This study lays the foundations for research into cytokines as vaccine adjuvants in animals, and provides an experimental basis for potential applications in human.

MATERIALS AND METHODS

Materials

The pPIC9K plasmid and the *P. pastoris* GS115 strain were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Recombinant plasmids were grown and identified in the *E. coli* strain DH5α, which was stored in our laboratory. All restriction endonucleases and polymerase chain reactions (PCR) kits were Takara (Japan) products. Primers were synthesized in Shenggong (Shanghai). PNGase F Kits were purchased from Biolabs (England), and MTS cell proliferation kits from Promega (USA). The commercial rpIL2 was an R&D product (652-P2), rhIL6 was from PeproTech EC Ltd. (England). The IL2-dependent cell line CTLL-2 was purchased from the cell bank of the Institute of Biochemistry and Cell Biology, SIBS, CAS and the IL6-dependent cell line B9, from the Immunology laboratory of the Fourth Military Medicine University, China.

Media

The *P. pastoris* medium formulations BMGY, BMMY, MD and YPD are described in the Invitrogen Corporation, *Pichia* Expression Kit Instruction Manual. The BMGY media contained 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6), 4×10^{-5}% biotin, 1.34% yeast nitrogen base (with ammonium sulfate and without amino acids), and 1% glycerol. The BMMY medium had the same formulation except that 0.5% methanol was substituted for the glycerol. The MD medium contained 4×10^{-5}% biotin, 1.34% yeast nitrogen base (with ammonium sulfate and without amino acids), and 2% glucose. The YPD medium contained 1% yeast extract, 2% peptone, 2% glucose.

Construction of plasmid pPIC9K-IL6-IL2

583-bp mature porcine IL6 cDNAs and 438-bp mature porcine IL2 cDNAs were amplified from SK^+-pIL6 and SK^+-pIL2 (from Pr. Yu Xingrong, University of Agriculture and Animal Science) using the procedure described previously [16] and with the following primers:

Primer A: 5'-gcGAGCTCcgggacgcctggaagaag-3'

B: 5'-cgCAATTGggaacctccacctcccattatccgaatggccctca-3'

Primer C: 5'-gcGAATTCggttctggcggtgcacctacttcaagct-3'

D: 5'-gcGTCGACcttaattatgaagtcagt-3'

In order to produce a biologically active fusion protein with the optimum spatial configuration, a linker encoding GGGGSEFGSGG was designed in primer B and primer C.

Primers A and B were used to amplify the cDNAs of the mature pIL-6 sequences, to which were added *Sac* I and *Mun* I restriction sites (underlined) at the 5' and 3' ends. In primer B, a linker with 15 bases was inserted instead of the stop the codon of the pIL-6 sequence. This PCR product was named pIL6-L.

The mature pIL-2 cDNA was amplified by primer C and D which contain *Eco* RI and *Sal* I restriction sites (underlined) respectively. Similar to primer B, primer C contained a 12-base linker after the *Eco* RI site. We named this product L-IL2.

After being inserted into the pMD18-T vector (from Takara, Japan) to generate T-IL6-L and T-L-IL2 *(figure 1)*, both PCR products were sequenced to check that their sequences were consistent with the published gene sequences (pIL2: accession X56750; pIL6: accession M86722). According to the diagram *(figure 1)*, T-IL6-L was digested with *Sac* I to produce an approximately 583bp fragment containing an IL6-linker sequence. The fragment was integrated into the plasmid pET-28a to generate 28a-IL6-L. The positive clone with the correct orientation was identified by *Bam* HI digestion. T-L-IL2 was digested with *Eco* RI and *Sal* I, and inserted into the positive clone 28a-IL6-L, which had been digested with *Mun* I and *Sal* I. The recombinant plasmid named 28a-IL6-IL2 could be transfected into BL21 for prokaryotic expression. An approximately 1000 bp fragment was generated when 28a-IL6-IL2 was digested with *Eco* RI and *Not* I, and integrated into *P. pastoris* with the plasmid pPIC-9K to produce 9K-IL6-IL2 (data not show). Each recombinant plasmid mentioned above was also transfected into the *E. coli* strain DH5 a and checked by appropriate restriction endonucleases.

Transformation into **P. pastoris** *strain GS115*

The plasmid 9K-IL6-IL2 was linearized with *Stu* I and integrated into LiCl-competent GS115 as described in the Invitrogen Corporation, *Pichia* Expression Kit Instruction Manual. The transformation mixture was plated onto MD medium. After three days' incubation at 28 °C, the clones were recovered and checked for positivity with the method reported by Qiuyun Liu [17].

Expression of fusion protein IL-6-IL-2 in **P. pastoris**

The recombinant GS115 strain carrying the pIL6-linker-pIL2 cDNA, was grown in BMGY medium at 28 °C, with vigorous shaking in baffled flasks to an OD_{600} of 2-6. The cells were then pelleted by centrifugation and suspended in BMMY to an OD_{600} of 0.2, to which was added 1% methanol daily in order to induce the heterologous protein expression. After a four-day induction, supernatants containing the secreted protein were collected by centrifugation and checked on 12% SDS-PAGE and by silver staining.

Expression analysis for different induction periods

As described in section 5, the cells were incubated in BMMY for nine days. 100 µl of culture were sampled and 1% methanol added daily. The supernatants of the samples were collected by centrifugation and stored at – 20 °C until used for SDS-PAGE electrophoresis.

Western blotting analysis

Culture supernatants were electrophoresed on 12% SDS-PAGE and transferred onto nitrocellulose membranes for Western blotting analysis. Western blots were probed using either rabbit anti-porcine IL6 polyclonal antibodies, or rabbit anti-porcine IL2 polyclonal antibodies, which were raised in rabbits using the corresponding proteins expressed in *E. coli* and purified. Goat anti-rabbit IgG con-

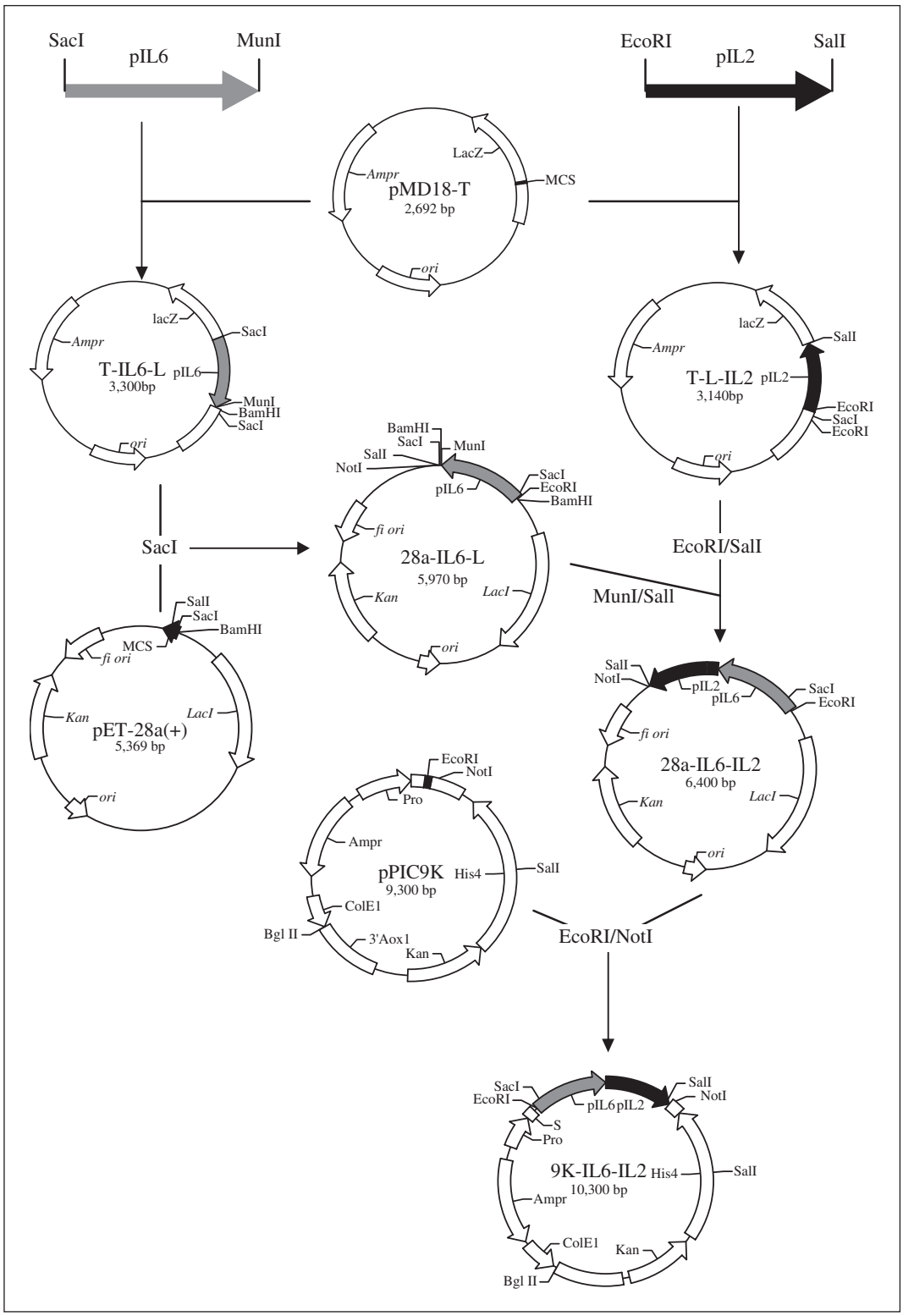

Figure 1

Construction of *P. pastoris* expression plasmid pPIC9K-IL6-IL2. The two fragments were amplified from SK^+-pIL6 and SK^+-pIL2 and a partial linker was added between the end of pIL6 and the front of pIL2. Both of them were cloned into the pMD18-T vector. Then, 28a-IL6-L, 28a-IL6-IL2 and 9K-IL6-IL2 were constructed sequentially by relevant enzyme digestion. The mature pIL6 and pIL2 cDNAs of 9K-IL6-IL2 were linked by 33bp cDNA linker, translated as GGGGSEFGSGG.

jugated to horseradish peroxidase was used as secondary antibody. Finally, blots were developed with 0.05% diaminobenzidine tetrahydrochloride in the presence of H_2O_2.

N-glycosylation analysis by PNGase F

162 µl of supernatant containing recombinant protein pIL-6-IL-2 were collected, and 18 µl of 10 × denaturing buffer

were added. The solution was thoroughly mixed and heated for 10min at 100° C. The solution was then mixed with 20 µl of 10 × GT buffer, 20 µl of 10% NP-40 and 1 µl PNGase, and incubated overnight at 37° C to ensure a complete reaction. The supernatant of the recombinant *P. pastoris* strain GS115/9K was analyzed as a negative control. Samples were checked by SDS-PAGE.

Sugar content analysis of the recombinant protein

The sugar content analysis of the recombinant protein was performed as described by Hongjun [18]. An aliquot of 40 µl of supernatant was mixed with 1 ml of 75% sulfuric acid, 200 µl of 6 mg/ml resorcinol, and 160 µl of distilled water. The solution was heated for 30 min at 90° C followed by immediate immersion in a water bath at room temperature and protected from light for 30 min to ensure a complete reaction. The reactive solution was observed for color developments and values of mannose (OD_{430}) and galactose (OD_{415}) were monitored by spectrophotometry. Mannose (40 µl of 50 µg/ml) and galactose (40 µl of 50 µg/ml) were used as standards. The supernatant of the recombinant GS115 strain with the 9K plasmid was used as a blank control, and 40 µl of recombinant IL-6-IL-2 expressed in *E. coli* were used as a non-glycosylated negative control.

Bioactivity assay of the recombinant protein

IL-2 and IL-6 bioactivity of the recombinant fusion protein IL-6-IL-2 was examined by the cell proliferation assay using the murine IL2-dependent cell line CTLL-2, and the murine IL-6-dependent cell line B9 as described previously [19]. Firstly, the supernatants of recombinant strains GS115/pPIC-9K-IL6-IL2, or GS115/pPIC-9K at a 1/500-dilution with an approximate concentration of 0.1 µg/ml, were diluted from 2^1 to 2^8 in 96-well, flat-bottomed microtiter plates. CTLL-2 or B9 cells were washed twice, suspended in RPMI 1640, and seeded at 1×10^4 cells/well in microtiter plates. The cells were cultured in the presence of the diluted samples for 20hr (for CTLL-2 cells) or 68hr (for B9 cells) at 37 °C in a humidified 5% CO_2 atmosphere. Then, 20 µl MTS were added to each well, and incubated for a further four hours. At the end of the incubation period, the plates were read in an ELISA plate reader, at a wavelength of 492 nm. The commercial rpIL-2 and rhIL-6 (with a 10 IU/ml starting concentration) as positive controls, were diluted (two fold dilutions) to make a standard curve. At the same time, a blank control (200 µl of medium) and a negative control (culture supernatant of 200 µl of cells without stimulator) were also analyzed. Results of the proliferation assays (OD at 492 nm) were analyzed using SPSS 10.0 software.

RESULTS

Expression of the recombinant fusion protein

The fusion protein pIL-6-IL-2 was successfully secreted into the culture medium when the recombinant *P. pastoris* strain GS115 was induced by 1% methanol for four days. SDS-PAGE showed that the pIL-6-IL-2 expression strain, GS115/pPIC9K-IL6-IL2, secreted a major protein of approximately 40-kDa, also in addition to a minor protein of 38-kDa, into the culture medium. These proteins were visible after two days of culture with 1% methanol, and peaked after four days *(figure 2)*. The recombinant protein secreted from *P. pastoris* in the four-day culture supernantant reached 50 mg/L as estimated by the method of Bradford. By seven days, these protein bands had slowly faded. It is worth noting that during the course of the culture, while the bands of the 40-kDa and 38-kDa proteins became weak, the 19 kDa and 26 kDa bands increased in intensity. Those latter bands displayed similar molecular weights to pIL-2 and IL-6 expressed in *P. pastoris*. Western blot analysis showed that these proteins were recognized specifically by rabbit anti-pIL-2 and rabbit anti-pIL-6 antibodies (data not shown), thus demonstrating that the protein mixture did contain correctly folded IL-2 and IL-6 proteins.

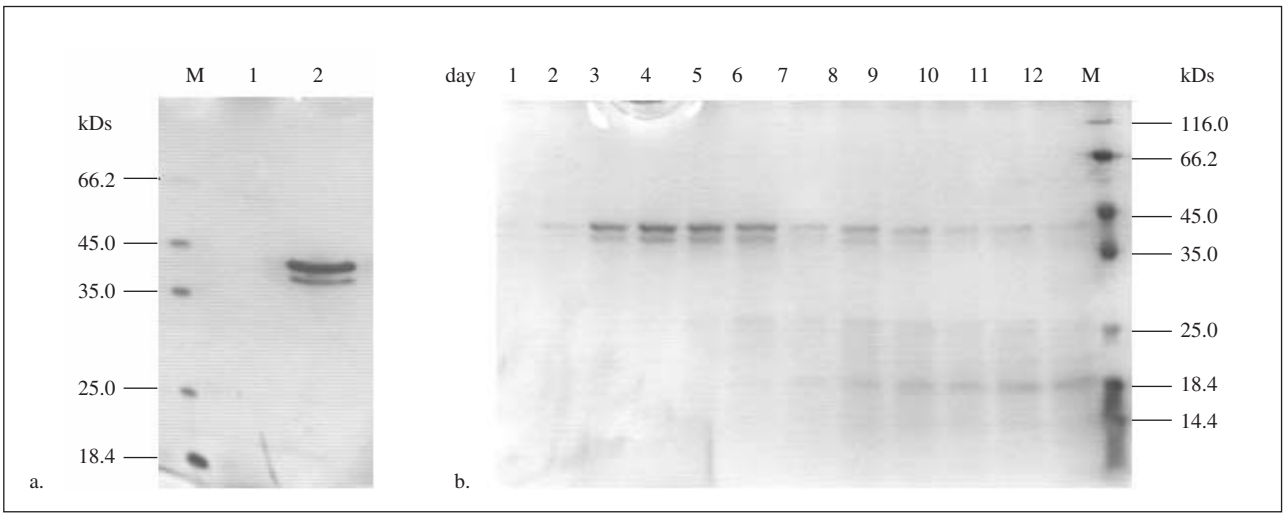

Figure 2

SDS-PAGE analysis of recombinant protein in *P. pastoris* strain GS115 supernatants were applied to a 12%SDS-PAGE and silver stained. (a), recombinant *P. pastoris* strain was induced by 1% methanol in 4d. Lane 1, supernatant of control transformant GS115/pPIC9K; lane 2, supernatant of recombinant GS115/pPIC9K-IL6-IL2; M: protein molecular weight markers

(b), recombinant strain GS115/pPIC9K-IL6-IL2 were induced on different days. Lane 1-12, the supernatant collected following induction from 1st to 12th day; M: protein molecular weight markers

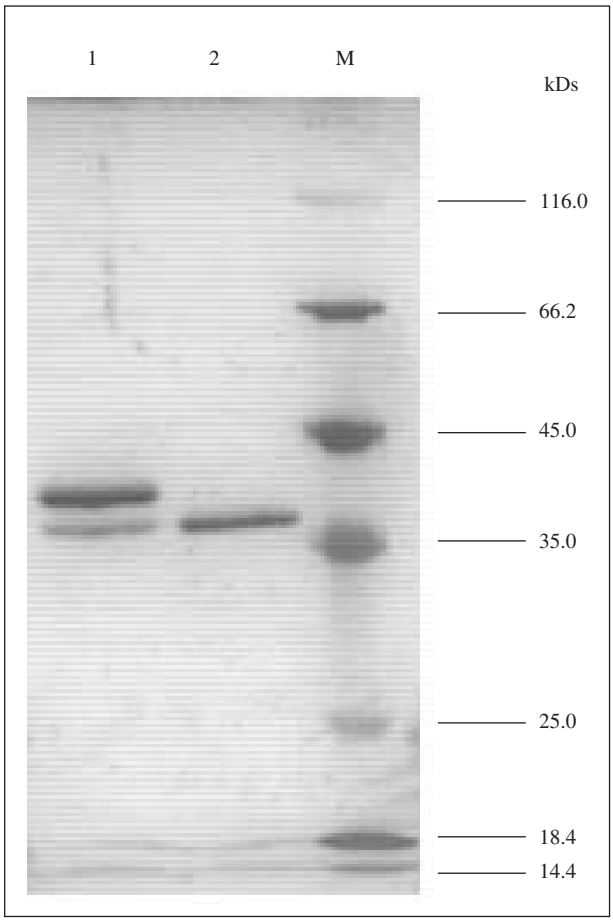

Figure 3
SDS-PAGE analysis of products treated with PNGase F
1: *P. pastoris* product pIL6-IL2 undigested by PNGase F
2: *P. pastoris* product pIL6-IL2 digested by PNGase F
M: Protein Molecular Weight Marker

N-glycosylation analysis by PNGase F

Digestion of the pIL-6-IL-2 preparation with pNGase F resulted in the disappearance of the 40-kDa band and the increased intensity of the 38-kDa band *(figure 3)*. This indicated that the recombinant *P. pastoris* strain secreted fusion protein pIL-6-IL-2 as both the N-glycosylated and non-glycosylated forms, with N-sugar representing 2-kDa in the former.

Sugar content analysis of the recombinant protein

An orange color is seen when glycoprotein reacts with resorcinol after treatment with sulfuric acid. The intensity of the color is proportional to the sugar content of the protein. A color change was observed with recombinant protein pIL-6-IL-2 secreted from *P. pastoris*. In contrast, no color change was observed with the recombinant pIL-6-IL-2 expressed by *E. coli.* or with that found in the supernatant of recombinant GS115 strain with the 9K plasmid. OD_{430} and OD_{415} reading values indicated that the pIL-6-IL-2 from *P. pastoris* contained both mannose and galactose components *(Table 1)*.

Bioactivity assay

CTLL-2 (or B9) cells cannot proliferate in culture without IL-2 (or IL-6). When the medium containing the recombinant protein induces CTLL-2 and B9 cell proliferation, it means that pIL-6-IL-2 possesses both IL-2 and IL-6 activities. Live CTLL-2 and B9 cells reacting with MTS give OD492nm values, which are proportional to the number of live cells in the culture well. Therefore, bioactivity units of pIL-6-IL-2 could be calculated from the OD492nm values at 24 (CTLL-2) or 72 hour (B9) culture times respectively. As shown in *figure 4*, the fusion protein pIL-6-IL-2 secreted from *P. pastoris* demonstrated IL-6 and IL-2 activities simultaneously. In the IL-6 bioactivity assay, the pIL-6-IL-2 in dilution (initial concentration of approximately 0.1 μg/ml) gave similar values to that of standard rhIL6 (initial concentration of 10 IU/ml). The specific IL-6 activity of the recombinant protein pIL-6-IL-2 was then calculated as 1×10^5 U/mg. In the same way, data for the IL2 bioactivity assay showed that pIL-6-IL-2 in dilution (initial concentration of approximately 0.1 μg/ml) had to be diluted by 8-fold compared to the standard rpIL2 (initial concentration of 10 IU/ml) in order to obtain the same absorption values at OD492 nm. The specific IL-2 activity of the recombinant protein pIL-6-IL-2 was then calculated as 8×10^5 U/mg.

Table 1
Mannose and galactose content analysis of pIL6-IL2 from *P. Pastoris.* Mannose and galactose concentrations of 50 μg/ml were used as positive controls at OD_{415} and OD_{430} respectively, and pIL6-IL2 from E. coli as a non-glycosylated negative control has low absorbency at both OD_{415} or OD_{430}. Data for pIL-6-IL-2 from P. pastoris indicated that the protein contained mannose and galactose components

	OD_{415}	OD_{430}
mannose	-----	0.762
galactose	0.971	-----
pIL6-IL2 from*E. coli*	0.025	0.017
pIL6-IL2 from*P. pastoris*	0.367	0.339

DISCUSSION

With the development of DNA recombinant technology, there is more choice of heterogenous gene expression systems. Classical *E. coli* expression systems have the advantages of short time growth and high production yields, but suffer from purification difficulties and low bioactivity levels. Furthermore, clinical application of bacteria-derived products may be affected by the potential presence of endotoxins that sometimes contaminate *E. coli*-expressed proteins. Unlike prokaryotic systems, eukaryotic cells are able to carry out many of the post-translational folding, processing and modifications, which have positive effects on the biological activity of the recombinant protein produced. However, protein production in insect cells with baculovirus expression systems need expensive and carefully controlled culture conditions. When it comes to mammalian expression systems, the low production by mammalian cells is frequently a problem. Since the 1990s, the methylotrophic yeast *Pichia pastoris*, a new and efficient host permitting heterologous expression has been getting more and more attention in genetic engineering fields [20, 21]. The *P. pastoris* expression system has not only the advantage of both prokaryotic and eukaryotic expression systems, but also has none of their disadvantages: as a eukaryotic host, *P. pastoris* can per-

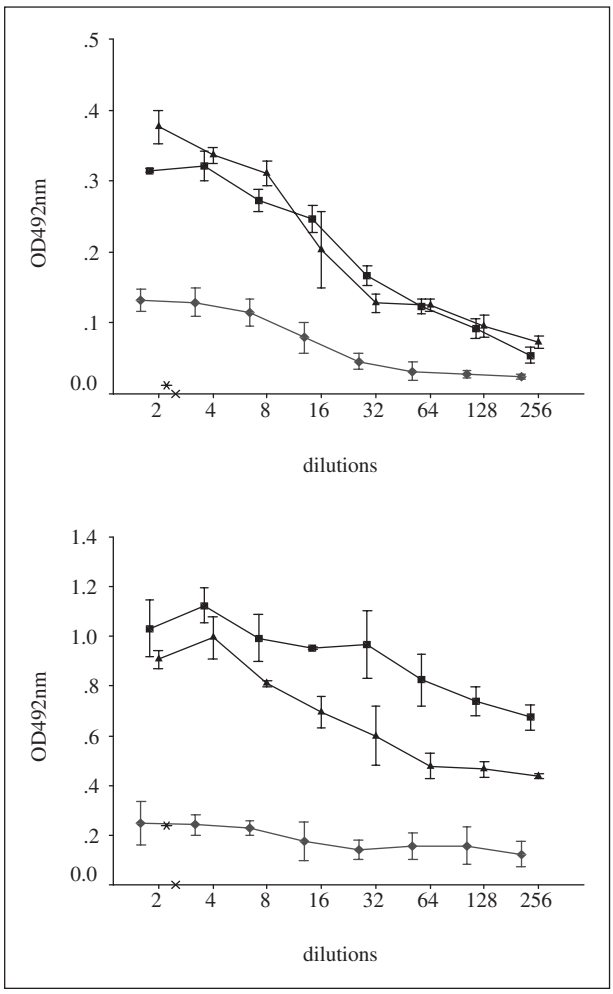

Figure 4
Cell proliferation assays of B9 (a) and CTLL-2 (b). (**SYMBOLE**) Standard IL2 and standard IL6 at the initial concentration of 10 IU/ml, (|) Supernatant of recombinant strain GS115/pPIC-9K-IL6-IL2 with an initial concentration of approximately 0.1 µg/ml, 1/500-dilution), (**SYMBOLE**) supernatant of recombinant strain GS115/pPIC-9K at a 1/500-dilution, (**SYMBOLE**) cell control and (×) negative control. Each point represents the mean ± SD of triplicate assay.

form certain post-translational modifications during expression, such as proteins processing, folding, disulfide bond formation, and glycosylation, which have positive effects on keeping protein structure stability and biological activity. Moreover, as a result of insertion into the *P. pastoris* genome, unlike insertion of the plasmid into an *E. coli* host, the heterogenous protein can be secreted stably and continually from the *P. pastoris* strain. In addition, heterogenous protein can be secreted into the culture medium by signal peptides from the expression vector (such as pPIC9K), making the purification process easier [22]. Simply put, the *P. pastoris* expression system offers the potential for really high protein expression levels at relatively low production costs [14, 23]. To date, many recombinant cytokines have been successfully expressed in *P. pastoris*, for example MIC-1, hIL-17, mEGF, hTNF [24, 25, 26, 27]. In this study, the high-level production of pIL-6-IL-2 in *P. pastoris* adds another example to this growing list.

A flexible linker is necessary to connect the two parts of the fusion protein in order to obtain a correct, three-dimensional structure displaying efficient co-bioactivity. Amino acids with hydrophobicity and low electric charges were selected as linker peptides. These were Gly, Ser, Pro, Ala, Thr, and especially Gly and Ser [28]. In this report, we designed the linker: Gly-Gly-Gly-Gly-Ser-Glu-Phe-Gly-Ser-Gly-Gly. An analysis with the SWISS 2DPAGE software in www.Expasy.com revealed that the two parts of the fusion protein was able to expand correctly. In order to simplify the construction process, we synthesized the linker sequence in two primers (Primer B and C) so that the linker between pIL6 and pIL2 cDNAs could be added easily by PCR amplification. The subsequent results of bioassays of the fusion protein showed that the linker was effective without adversely affecting expression.

The recombinant expression plasmid pPIC9K-IL6-IL2 was transfected and integrated into the *P. pastoris* strain GS115. After induction by 1% methanol, the recombinant *P. pastoris* strain secreted high levels of pIL-6-IL-2 into the culture medium, which peaked at 50 mg/L. Such levels are as high as those obtained with several other heterologous proteins, such as hTNF, rTNF- α [27, 29]. The biological activity of pIL-6-IL-2, as evaluated by the cell proliferation assay using the IL-6-dependent cell line B9 and IL2-dependent cell line CTLL-2, reached 1×10^5 U/mg and 8×10^5 U/mg respectively. We also showed that the fusion protein pIL-6-IL-2 was secreted in *P. pastoris* both as non-glycosylated and glycosylated forms. For the latter form, the glycosylation represented 2-kDa and was composed of significant amounts of both mannose and galactose sugars. Of interest was the observation that, during the culture incubation period, while the 40-38 kDa bands of the fusion proteins became weaker, we observed an increased expression of 19 kDa and 26 kDa bands, which had precisely the molecular weights of pIL-2 and pIL-6 expressed singly in *P. pastoris*. Perhaps the peptide sequences of the linker were correctly identified and cut by proteases in the host.

In conclusion, an engineered strain of yeast, which secreted fusion protein pIL-6-IL-2 was constructed. The optimal induction conditions, the large quantities of products secreted and their efficient biological activities, were determined. This report, as the first description of expression of two fusedporcine cytokines in *P. pastoris*, provides a low-cost method for obtaining milligram quantities of recombinant protein co-displaying high pIL-6 and pIL-2 biological activities. Such a method is not only useful for studying the structure and function of the fusion protein pIL-6-IL-2, but it can also provide us with the basis for a scaling-up to fermenter-scale expression of pIL-6-IL-2 at levels making commercial production a viable proposition. Further studies will be carried out in animal models to demonstrate the adjuvant effects of the fusion protein pIL-6-IL-2 for use in veterinary vaccines

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