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Coexpression of interleukin-6 and -2 from giant panda in *Escherichia coli* and the biological activity of the fusion protein

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ABSTRACT. To construct a fusion cytokine protein with more and stronger bioactivities to enhance the immunity of the cytokine alone, we expressed interleukin (IL)-6/(IL)-2 from giant panda (*Ailuropoda melanoleuca*) in *Escherichia coli* as a 59.4-kDa fusion protein. Subsequently, the inclusion bodies were solubilized with 8 M urea and applied onto a Ni-nitrilotriacetic acid column. The final production of IL-6/IL-2 reached 6 mg/L in soluble form, and the purified final product was >96% pure. In Western blot assays, the recombinant IL-6/IL-2 was recognized by polyclonal antibodies against IL-6 and IL-2 of giant panda. The results demonstrated that the protein mixture contained correctly folded IL-2 and IL-6 proteins. A 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide assay demonstrated that IL-6/IL-2 can promote lymphocyte proliferation and differentiation. These data suggest that the fusion protein could be used to develop a novel immunoadjuvant to enhance the immunity of animals against infectious diseases.

Key words: Giant panda; Interleukin-6/interleukin-2; Fusion protein; Prokaryotic expression

INTRODUCTION

The giant panda, *Ailuropoda melanoleuca*, is beloved throughout the world and considered a symbol of China, but it is close to extinction. The latest molecular census of its population size using fecal samples and 9 microsatellite loci provided an estimate of only 2500-3000 individuals (Li et al., 2010). Aside from human encroachment on the territories of giant panda, diseases are probably the greatest threats to their survival (Tan et al., 2007). To date, more than 40 diseases have been detected in this species. Although some conservation programs have been initiated, implementing further protections remains an arduous and pressing task (Peng et al., 2007). Therefore, enhancing the immune responses of giant panda to conventional attenuated and killed vaccines is critical.

Owing to their safety and effectiveness, certain cytokines have been used as adjuvants to regulate the immunity of animals. Interleukin (IL)-2 has been recognized as a key immune-regulatory protein for the proliferation of activated T cells of various types (Thompson and Staats, 2011). It is produced mainly by T helper (CD4+) lymphocytes, controls growth and differentiation of B lymphocytes, and intensifies proliferation and activity of all cytotoxic cell clones (Olejniczak and Kasprazak, 2008). IL-6 was discovered in its role as an inflammatory cytokine involved in B-cell differentiation. It is currently recognized as a highly versatile cytokine with important roles in the regulation of the immune response, inflammation, and hematopoiesis (Hirano, 1998; Spooren et al., 2011). IL-6 is synthesized by many cell types, including T cells, macrophages, and stromal cells (Su et al., 2008) and is associated with humoral immune responses. Given the actions of IL-2 and IL-6, their extensive study as potential vaccine adjuvants is not surprising (Lofthouse et al., 1996).

Fusion proteins have been reported not only to have effects in individual proteins but also to generate new properties. For instance, human IL-4/IL-6, granulocyte-macrophage colony-stimulating factor/IL-3, granulocyte-macrophage colony-stimulating factor/IL-6, and even IL-6/IL-2 have been shown to display novel and stronger bioactivities than the cytokines alone (Rock et al., 1992; Vadhan-Raj, 1994; Zhao et al., 1994; Zhang et al., 2007). Owing to the various functions in immune responses and reciprocal collaborating activities of these cytokines, we produced a fusion protein containing both IL-2 and IL-6 of giant panda for application as a therapeutic adduct and immunomodulator to enhance the response of the host to vaccination.

MATERIAL AND METHODS

Strains, vectors, and main reagents

The *Escherichia coli* strains DH5α and Rossetta (DE3) and the expression vector pET32a(+) were preserved in our laboratory. Restriction enzymes, DNA markers, T4 DNA ligase, and protein molecular weight markers were purchased from TaKaRa (Dalian, China). Plasmid Mini Kits and Gel Extraction Kits were purchased from Omega. RPMI 1640 medium and fetal bovine serum were purchased from Gibco (NY, USA) and HyClone (UT, USA), respectively.

Construction of the plasmid expressing IL-6/IL-2

The overall scheme for the construction of IL-6/IL-2 fusion proteins was carried out in several steps. Mature IL-6 cDNAs without stop codons and mature porcine IL-2 cDNAs were amplified from truncated (T)-IL-6 and T-IL-2 (containing the complete open reading frames of

IL-2 and IL-6, which were preserved in our laboratory) using the following primers: p6-1: 5'-*GAATTC*ATGCTCCCTACCCCGGGACACCTGGGAA-3'; p6-2: 5'-*GTCGAC*AAGCTT*GGATCC*ACTTCCACTTCCCATGACCCGCACAGCCCTC-3'; p2-1: 5'-*GGATCC*GGAAGTGGTAGTGGAAGTATGGCACCTACTCCTTCAAGCCCT-3'; p2-2: 5'-*GTCGAC*TTAAGTCAGTGTTGAGAAGAT-3'. To produce a biologically active fusion protein with the optimum spatial configuration, a linker encoding $(GlySer)_3$ was designed in primers p6-2 and p2-1, which were used to amplify the cDNAs of IL-6 sequences. The enzyme sites for *Eco*RI, *Bam*HI, and *Sal*I (underlined above) were added to the ends. The mature IL-2 cDNA was amplified with primers 2-1 and 2-2, which contained *Bam*HI and *Sal*I restriction sites (underlined above), respectively. Both fragments were inserted into the pMD19-T vector (TaKaRa) to generate T-IL-6-L and T-L-IL-2 (L represents linker). Subsequently, T-L-IL-2 was digested with *Bam*HI and *Sal*I to produce an approximately 441-bp fragment containing an IL-2-L sequence. The fragment was integrated into the plasmid T-IL-6-L, which was also digested with *Bam*HI and *Sal*I to generate T-IL-6-L-IL-2. Finally, T-IL-6-L-IL-2 was digested by *Eco*RI and *Sal*I, and the fusion gene was inserted into the similarly digested prokaryotic expression plasmid pET32a(+). The structure of the resultant plasmid was confirmed through restriction enzyme digestion and sequencing (data not shown).

Expression of the fusion protein

Expression plasmids were transformed into *E. coli* strain Rossetta (DE3) and grown overnight on Luria-Bertani plates containing 100 µg/mL ampicillin. Single-colony cultures were grown overnight at 37°C and inoculated into 3 mL Luria-Bertani medium with ampicillin at a final concentration of 100 µg/mL. To induce protein expression, we added isopropyl-beta-D-thiogalactopyranoside (IPTG) until the cultures reached the exponential phase. Preparation of cell lysates was performed according to manufacturer instructions.

To optimize the production of insoluble material, we tested various expression conditions, including various temperatures (25°, 30°, and 37°C), bacterial cell densities (optical density $(OD)_{600}$ of 0.4-1.0), concentrations of IPTG (0.1-1.0 mM), and incubation times (1-6 h) after induction (data not shown). Cells were harvested via centrifugation at 8000 rpm at 4°C for 10 min. The pellets were stored at -20°C until further analysis with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The Bandscan5.0 software was applied to assess the expression of the fusion protein.

Purification and renaturation of the fusion protein

The cell pellet was resuspended in 15 mL 50 mM Tris, pH 8.0, 5 mM ethylenediaminetetraacetic acid, 150 mM NaCl, and 0.1% Triton X-100 (lysis buffer) containing 2 mg lysozyme and incubated at room temperature for 30 min. Then, the cell lysate was sonicated and centrifuged at 12,000 rpm for 10 min at 4°C to collect the pellet containing the inclusion bodies. The pellet was washed twice with 10 mL 2 M urea in lysis buffer for preliminary purification and resuspended in 8 M urea. The preliminarily protein mixture was further purified on a Ni-nitrilotriacetic acid column according to manufacturer instructions. Refolding was carried out with chaotropic agents' concentration gradient dialysis. The solution of denatured protein was dialyzed against 2 L freshly made 6, 4.5, 3, 1.5, and 0 M urea containing 50 mM Tris, 1 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 1 mM glutathione, 0.1 mM glutathione disulfide, 5% glycerol, and 5% glucose. With each concentration, the protein was

dialyzed for 12 h at 4°C (Sorensen et al., 2003; Wang et al., 2005). Protein concentration was determined using a method described by Bradford (1976) with bovine serum albumin as a protein standard.

Production of polyclonal antibodies

Polyclonal antibodies were prepared in rabbits against IL-6 and IL-2 of giant panda using the corresponding proteins expressed in *E. coli* and purified. The purified protein was mixed with an equal volume of Freund's complete adjuvant (Sigma, Shanghai, China) and intradermally injected into rabbits at a dose of 0.5 mg protein/rabbit (100 µL/site). Two more booster immunization was given on days 15 and 29 with Ag mixed with incomplete Freund's adjuvant. Ten days after the final injection, antiserum was harvested from the carotid artery. Immunoglobulin G was purified from the antisera with a DEAE-Sepharose column (Bio-Rad, Shanghai, China) and stored at -70°C until further use.

Western blot

Western blot analysis was performed using either rabbit anti-panda IL-6 polyclonal antibodies or rabbit anti-panda IL-2 polyclonal antibodies following a standard procedure (Towbin et al., 1979). The protein was separated using 12% SDS-PAGE and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, USA) with a semi-dry transfer cell (BioRad) at 20 V for 1 h. The membranes were blocked at room temperature for 2 h with blocking solution (3% bovine serum albumin in Tris-buffered saline, pH 7.4) and then incubated for 2 h at room temperature with the first antibody. After washes in Tris-buffered saline, pH 8.0, containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (Bio-Rad). Diaminobenzidine substrate buffer was used to visualize the reaction.

3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) cell-proliferation assay

The bioactivity of the recombinant IL-6/IL-2 protein was determined by its capability for provoking the proliferation of lymphoblasts stimulated with concanavalin A through MTT colorimetry (Tsang et al., 2007). Briefly, the spleen was aseptically removed from a BALB/c mouse. A suspension of single spleen cells was prepared by lysing the red blood cells and collecting them into cell culture media that contained RPMI 1640, 2 mM glutamine, 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 U/mL penicillin. Triplicate cultures were grown on 96-well U-bottom plates at 5 x 10^6 cells/well. Various concentrations (0.05-20 µg/mL) of IL-6/IL-2 were added to the cell suspension and the splenocytes were incubated for 6 h in the presence of 5 mg/mL concanavalin A at 37°C in a humidified incubator with 5% CO_2. Seventy-two hours later, 20 µL 5 mg/mL MTT solution was added to each well and mixed. The cells were further incubated for 3 h. Dimethyl sulfoxide (100 µL) was added to dissolve the formazan for 10 min. Absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) plate reader with a test wavelength of 570 nm.

Statistical analysis

Each treatment was analyzed in triplicate and data are reported as means ± standard deviation. The significance of differences between treatments was assessed with analysis of variance using the SPSS 16.0 software (SPSS Inc., Chicago, USA). Differences were considered to be significant at $P < 0.05$.

RESULTS

Construction of prokaryotic expression plasmid

Using the T-IL-6 and T-IL-2 plasmids as templates, we amplified the fragments IL-6-L and IL-2-L. The electrophoretic analysis results of the amplified products showed that the sizes of the 2 genes were as expected (Figure 1). The recombinant plasmids were then constructed following the cloning strategy described above. The analysis by restriction endonuclease digestion proved that the insertion of the fusion gene in the recombinant plasmid was correct (Figure 2). The DNA sequence of the target gene was also confirmed.

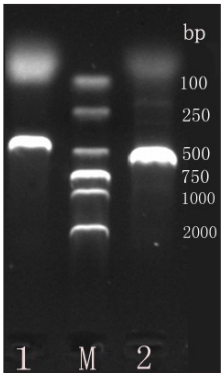

Figure 1. PCR amplification of IL-2 and IL-6 genes. *Lane 1* = IL-2; *lane M* = DNA marker; *lane 2* = IL-6.

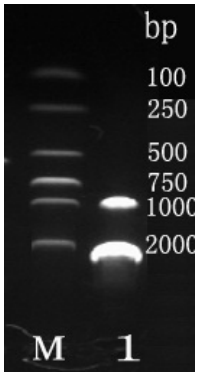

Figure 2. Identification of the recombination vector pET32a (+)-IL-6/-2 by restriction enzyme digestion. *Lane M* = DNA marker; *lane 1* = recombination plasmid pET32a (+)-IL-6/-2 digested with two restriction enzymes *Eco*RI and *Sal*I.

Protein expression and purification

Once optimized, the highest yield of insoluble IL-6/IL-2 material was obtained with 0.1 mM IPTG after reaching an OD_{600} of 0.6 and incubating for 4 h at 37°C after induction. An obvious band (Figure 3; lane 3) with a molecular weight of approximately 59.4 kDa was observed, corresponding to the expected size of the histidine (His)-tagged IL-6/IL-2 protein. Furthermore, the SDS-PAGE analysis of the soluble and insoluble fractions (see Figure 3; lanes 4 and 5) showed that the majority of the induced protein was found in the insoluble fraction, suggesting that the His-tagged IL-6/IL-2 protein was insoluble in the form of inclusion bodies. Analysis using BandScan 5.0 detected a relatively high expression level of the recombinant protein, which constituted approximately 25.8% of the total proteins. The His-tagged IL-6/IL-2 protein was then purified as described above. SDS-PAGE results verified successful purification, because only one clear band was observed (see Figure 2; lane 6). After purification, the IL-6/IL-2 protein was dialyzed with refolding buffer to produce IL-6/IL-2 in amounts up to 6 mg/L in the soluble form. The proteins were >96% pure.

Western blot

The IL-6- and IL-2-specific antibody responses were determined using an indirect ELISA with purified recombinant IL-6 and IL-2 proteins as the antigens. The ELISA results showed that the titer of both antibodies was 1:160. Western blot was carried out using rabbit anti-IL-2 and anti-IL-6 antibodies. Specific blotting bands were detected at the corresponding positions (Figure 4), thus demonstrating that the protein mixture contained correctly folded IL-2 and IL-6 proteins.

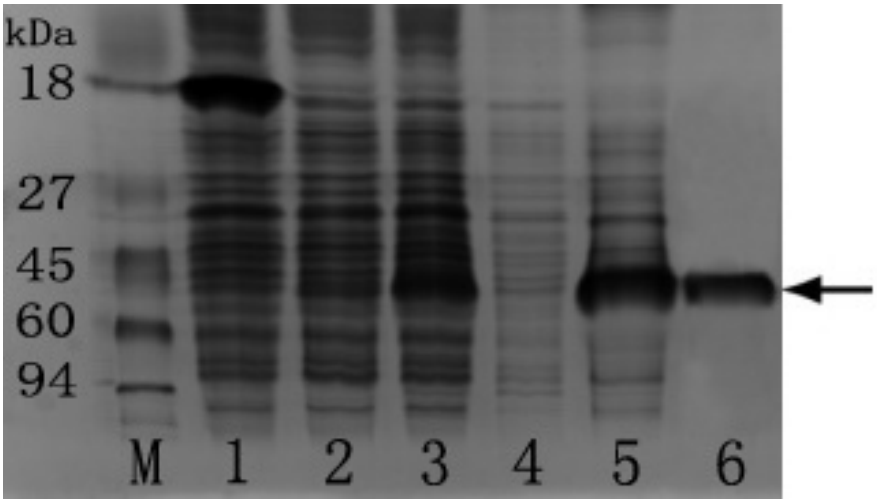

Figure 3. Expression and purification of the His-tagged IL-6/-2 protein. *Lane M* = protein maker; *lane 1* = plasmid pET32a induced with IPTG; *lane 2* = uninduced expressing strain with pET32a-IL-6/-2; *lane 3* = total protein from *Escherichia coli* Rossetta (pET32a-IL-6/-2; *lane 4* = supernatant of induced pET32a-IL-6/-2 after sonication; *lane 5* = inclusion bodies of IL-6/-2 by rough extraction; *lane 6* = purified His-tagged IL-6/-2 protein after dialysis. Arrowhead indicates the position of the His-tagged IL-6/-2 protein.

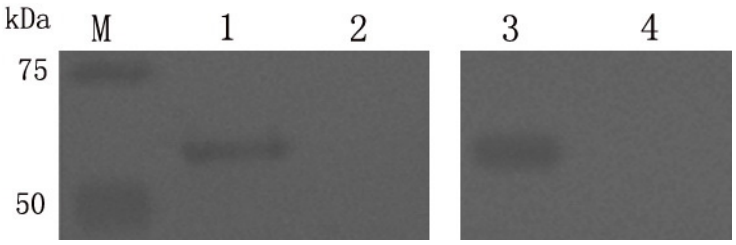

Figure 4. Western blot analysis of IL-6/-2. *Lane M* = protein maker; *lane 1* = IL-6/-2 protein incubated with polyclonal anti-IL-6 antibodies; *lane 3* = IL-6/-2 protein incubated with polyclonal anti-IL-2 antibodies; *lanes 2* and *4* = the negative control.

Bioactivity assay of the fusion IL-6/IL-2 protein *in vitro*

To address the specific biological activity of the purified IL-6/IL-2, we conducted an MTT assay. Various concentrations of the purified IL-6/IL-2 (0.05-20 μg/mL) were added to lymphocytes (5 x 10^6) grown on 96-well plates. After 72 h of incubation, cell proliferation was determined (see Material and Methods). As Table 1 shows, the addition of as little as 0.05 μg/mL IL-6/IL-2 stimulated the growth of the lymphocytes and was most significant at a dose of 0.1 μg/mL. When the concentration reached 20 μg/mL, proliferation was inhibited.

Table 1. Lymphocyte proliferation activity of recombinant IL-6/-2.

Final concentration	Mean OD_{570} ± SD
0.05 μg/mL IL-6/-2	0.4160 ± 0.00244^{c}
0.1 μg/mL IL-6/-2	0.4503 ± 0.00958^{b}
0.2 μg/mL IL-6/-2	0.4244 ± 0.00524^{c}
0.4 μg/mL IL-6/-2	0.3191 ± 0.0666^{d}
2 μg/mL IL-6/-2	0.2420 ± 0.0646^{e}
20 μg/mL IL-6/-2	0.2161 ± 0.0828^{f}
Normal saline	0.2180 ± 0.00185^{f}
5 mg/mL ConA	0.5301 ± 0.1467^{a}

Data in the same column with different superscript letters are significantly different ($P < 0.05$), and vice versa ($P > 0.05$). ConA = concanavalin A.

DISCUSSION

IL-2 and IL-6 can stimulate T-lymphocyte proliferation and the secretion of immunoglobulins by B lymphocytes, enhancing the cellular and humoral immune responses of animals. The synergistic interaction between IL-2 and IL-6 extends to the induction of cytotoxic T-lymphocyte responses as well. Therefore, 2 cDNAs of the mature IL-6 and IL-2 of giant panda were linked and cloned into *E. coli* expression plasmid pET-32a. Protein expression using *E. coli* is a widely used system to make proteins in large quantities (Hannig and Makrides, 1998; Sorensen and Mortensen, 2005). Like many other recombinant proteins, IL-6/IL-2 forms inclusion bodies when expressed in *E. coli*. Although some strategies, such as the use of a fusion protein, can be successfully applied to produce soluble protein (Baneyx, 1999), attempts to make IL-6/IL-2 in a soluble form in *E. coli* have failed. This failure probably occurs

because IL-6/IL-2 contains 106 hydrophobic amino acid residues (31.27% of the sequence). So the fusion protein requires denaturation and renaturation before further use.

The refolding of proteins from inclusion bodies is affected by several factors, including solubilization of inclusion bodies by denaturants, removal of the denaturant, and assistance in refolding by small molecule additives (Tsumoto et al., 2003). Wang et al. (2005) have demonstrated that the yield of refolded proteins obtained from urea dialysis is 20 times higher than that from guanidine hydrochloride. So we choose urea as the denaturant, and the denatured protein was refolded with dialysis in gradient urea. In the process of purification and renaturation of the fusion protein, Triton X-100 was added to the inclusion body wash and lysis buffers, which helped to solubilize hydrophobic proteins, reduce interchain disulfide bonds, and minimize nonspecific binding of proteins to the Ni column (Zhao et al., 2005). Glycerol and glucose were included in the refolding buffer. As powerful cosolvents, they enhance native protein stability. In addition, a redox couple of 1 mM reduced and 0.1 mM oxidized glutathione was used to encourage correct disulfide bond formation during protein refolding.

A flexible linker is necessary to connect the 2 parts of the fusion protein to obtain a correct, three-dimensional structure displaying efficient co-bioactivity. Amino acids with hydrophobicity and low electric charges were selected as linker peptides: Gly, Ser, Pro, Ala, Thr (especially Gly and Ser) (Lin et al., 2004). In this study, we designed the linker Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser. The SOPMA software analysis of the secondary structure revealed that the 2 parts of the fusion protein expanded correctly. GGATCC (*Bam*HI) encoding for Gly-Ser was contained in the linker sequence, and we used it to connect IL-6 and IL-2. To a certain extent, it simplified the construction process.

The fusion protein IL-6/IL-2 produced with the pET32a system was also biologically active, as measured by a mice lymphocyte proliferation assay. After a series of preliminary tests, we observed that several concentrations (0.05, 0.1, 0.2, 0.4, and 2 μg/mL) of the fusion protein display enhanced *in vitro* proliferation activity, most significantly at a dose of 0.1 μg/mL. However, when the concentration reached 20 μg/mL, proliferation was inhibited. Selecting an appropriate dose in clinical application is important.

In this study, we highly expressed the recombinant IL-6/IL-2 protein in *E. coli*. All data have demonstrated that the recombinant denatured IL-6/IL-2 fusion protein, like other proteins, can be refolded effectively. Production of IL-6/IL-2 provides a useful tool in the study of giant panda immunology in general, as well as a potential vaccine-enhancing agent. Further studies with recombinant IL-6/IL-2 will reveal its usefulness in these and other applications.

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