Expression of Porcine Interleukin-2 in *Escherichia coli*

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ABSTRACT. A mature form of porcine interleukin-2 (IL-2) protein without signal peptides was expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* using pGEX vector. Since most of GST-IL-2 fusion protein was detected in an insoluble fraction on SDS-PAGE analysis, the insoluble fusion protein was solubilized by refolding procedure using urea. The recombinant IL-2 (rIL-2) was purified by a batch method using Glutathione Sepharose 4B and factor Xa digestion and used for preparation of antisera in mice. The antisera reacted with rIL-2 expressed in baculovirus system on immunoblot analysis. In addition, the purified rIL-2 showed a high biological activity on CTLL-2 proliferative response.

KEY WORDS: bacterial expression, interleukin-2, swine.

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Interleukin-2 (IL-2) has many biological functions and plays a central role in immunological response [1, 3, 5, 8, 14]. In recent studies, IL-2 has been mainly secreted from helper T cell (Th) type 1 and considered to be useful for the classifications of helper T cells in combination with other cytokines as γ -interferon, IL-4 and IL-6 [12]. Although the concept may be applicable for immunological studies in other species, there is no information about porcine Th subsets. In pigs, there are several reports of studies such as the production and biochemical characterization of IL-2 [4, 6, 9]. Although porcine IL-2 cDNA has been cloned [7] and expressed in baculovirus system [2], less is known about its bacterial expression of porcine IL-2. Our previous report demonstrated the expression of bovine IL-2 in *Escherichia coli* (*E. coli*), however the expressed protein in bacteria was insoluble [10] as seen in ovine IL-2 expression in *E. coli* [13]. In this study, we attempted to express porcine IL-2 in *E. coli* as glutathione S-transferase (GST) fusion protein and to prepare antiserum against porcine IL-2 in mice. Furthermore, biological activity of recombinant IL-2 (rIL-2) was determined by cell proliferation assay using murine IL-2 dependent cell line, CTLL-2.

Porcine IL-2 cDNA was prepared by reverse transcriptional polymerase chain reaction (RT-PCR) method from mRNA isolated from phytohemagglutinin-P (PHA-P; Difco, MI, U.S.A.) stimulated porcine spleen lymphocytes by using QuickPrep Micro mRNA Purification Kit (Pharmacia P-L Biochemicals, Sweden). In brief, the open reading frame of porcine IL-2 was amplified by GeneAmp[®] RNA PCR kit (ABI, U.S.A.) using the following primers: 5'-CCCGGATC-CATGTAT AAGATGCAGCTCTTG-3' (sense primer) and 5'-CCCGGATCCACTAGTATTACAAAAGTTA GA-3' (antisense primer) designed from the sequence data as reported previously [7]. Amplified products were purified by electroelution, cloned into pUC118 at a *Bam*H I site, and confirmed by sequencing using dideoxynucleotide termination method. The cDNA without signal peptides encoding region was then amplified by the PCR replacing the sense

primer (5'-CGCCTCGAGGCACCTACTTCAAGC-3'), which was designed to construct a cDNA with a *Xho* I site at 5' end. The cDNA was inserted to pcDNA3 at a *Bam*H I site with TNF leader sequence which possessed *Bam*H I site at 5' end and *Xho* I site at 3' end. The cDNA was then released from the pcDNA3 by *Xho* I digestion and was ligated into *Xho* I site of pGEX-5x-3. GST-IL-2 fusion protein was expressed in bacteria by isopropyl- β -D-galactopyranoside (IPTG). Briefly, 2xYT-G medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl and 20 g/l glucose) supplemented with 100 μ g/ml of ampicillin was inoculated with a single colony of bacteria transformed with recombinant plasmid and incubated overnight at 37°C. The cultures were transferred into 20 and then 200 ml of 2xYT-G, incubated for 2 hr, and added with 0.1 mM IPTG. The cultures were centrifuged at 7,700 g for 5 min at 4°C, resuspended in 10 ml ice-cold PBS(-), and sonicated. Expressed proteins in bacteria were analyzed by SDS-PAGE. Fig. 1A shows the result of SDS-PAGE analysis for *E. coli* lysates. The band of the fusion proteins was mostly observed in the pellet of sonicates, suggesting that the IL-2 fusion protein was expressed in bacteria as insoluble proteins in inclusion body [13]. Although IL-2 proteins expressed in mammalian cells are soluble, the IL-2 fusion proteins expressed in bacteria were insoluble in spite of expressing their hydrophilic region without signal peptides as seen in bovine and ovine [10, 13]. The different folding processes between prokaryotic and eukaryotic cells might affect to the solubility of the expressed protein.

Insoluble porcine IL-2 fusion proteins were attempted to be refolded for solubilization as reported previously [10]. In brief, the pellet after centrifugation of bacterial sonicates was resolved in 10 ml of 8 M urea solution containing 50 mM Tris-HCl pH 8.0, 1 mM DTT and 1 mM EDTA and incubated for 1 hr at room temperature. The supernatant was dialyzed against 100 ml of 4 and then 2 M urea solution for 1 hr at 4°C twice. After dialysis against 100 ml of 50 mM Tris-HCl pH 8.0 with 1 mM DTT for 1 hr at 4°C twice, dialysis was continued overnight against another 100 ml of the same

Fig. 1. SDS-PAGE analysis of porcine IL-2 expressed in *E. coli*. MW marker (lanes 1 and 4), and insoluble (lane 2) and soluble (lane 3) fractions in sonicated *E. coli* lysate are shown. Fig. 1B shows the purification steps as GST fusion protein purified by using GS4B (lane 5), GST (26 kDa) and IL-2 (15 kDa) proteins after digestion of the purified GST fusion protein by Factor Xa (lane 6), and purified IL-2 with 15 kDa released from GST-IL-2 bound to GS4B by Factor Xa digestion (lane 7). Arrows indicate GST-IL-2 fusion proteins at a position of about 41 kDa and an arrow head indicates purified porcine IL-2 at about 15 kDa.

buffer at 4°C. The dialyzed sample was centrifuged and refolded GST-IL-2 in supernatant was recovered. Solubilized IL-2 fusion proteins were purified by glutathione Sepharose 4B (GS4B) according to manufacturer's instructions (Pharmacia, Sweden). Briefly, the supernatants were added with GS4B, followed by incubation with gentle agitation at room temperature for 30 min. After GS4B were washed with PBS(-), GST-fusion proteins were eluted from the matrix by reduced glutathione and analyzed on SDS-PAGE. As shown in Fig. 1B (lane 5), the IL-2 fusion proteins were purified by this method. Porcine rIL-2 protein was attempted to be cleaved off from GST fusion proteins by factor Xa (Pharmacia) digestion. Briefly, GST-IL-2 bound to GS4B as described above was washed with cleavage buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM CaCl₂), and added with 50 µg of factor Xa in 1 ml of cleavage buffer for each ml of GS4B bed volume. Reactions were performed overnight at 4°C, and the proteins in eluates were recovered and analyzed by SDS-PAGE as shown in Fig. 1B. The result indicated that IL-2 was purified as a mature form with a molecular weight (MW) of 15 kDa by this method (Fig. 1B, lane 7). The yield of the IL-2 fusion protein in this method was estimated at about 20 mg/l bacterial culture determined by the Lowry method using DC protein assay (Bio-Rad, U.S.A.). Seow *et al.* reported that ovine IL-2 expressed in *E. coli* using pT7-7 and pGEX vectors was also insoluble and solubilized by using nonionic detergent and their yield were

10 and 1 mg/l, respectively [13]. They did not use urea and guanidine-HCl because of protein precipitation during refolding process. However, we could efficiently solubilize insoluble forms of porcine IL-2 by refolding using urea without protein precipitation and the yield might be improved when compared to that of ovine IL-2.

Antiserum against porcine IL-2 was prepared in mice by GST-fusion protein as antigen. Fifty µg of purified GST-IL-2 was injected intraperitoneally into 10-weeks-old male ddy mice with Freund's complete adjuvant and then with Freund's incomplete adjuvant was injected twice at 2 week intervals. After 1 week from the last injections, blood was collected and centrifuged to obtain antisera. Porcine rIL-2 was also expressed in insect *Sf9* cells infected with recombinant baculoviruses according to the method as reported previously [2, 10]. As shown in Fig. 2A, porcine rIL-2 in the insect cells was weakly stained by coomassie brilliant blue as indicated by arrows at positions of about 15 and 17 kDa (lane 4), but not in the culture supernatant (lane 6). On immunoblot analysis (Fig. 2B), the antiserum reacted with the bacterial rIL-2 protein (lane 2), but detected an extra band with an MW of about 26 kDa which was considered to be GST proteins contaminated to the purified rIL-2 fraction. However, the antiserum reacted with two bands with MWs of 15 and 17 kDa expressed in insect cells (lane 4) infected with recombinant baculovirus and its culture supernatant (lane 6) on immunoblot analysis. Although the band with a lower

Fig. 2. SDS-PAGE analysis of the expressed porcine IL-2 proteins stained by coomassie brilliant blue (A) and immunoblot analysis using mouse antiserum against GST-IL-2 bacterial fusion protein (B). MW marker (lane 1), purified rIL-2 expressed in *E. coli* (lane 2), expressed proteins in insect cells uninfected (lane 3) and infected (lane 4) with recombinant baculovirus, and culture supernatants of insect cells uninfected (lane 5) and infected (lane 6) with recombinant baculovirus are shown. Arrows indicate two protein band of porcine rIL-2 in insect cells with MWs of 15 and 17 kDa considered to be mature and immature forms, respectively.

MW was considered to be a mature form of porcine IL-2, the band with a higher MW might be an immature form of porcine IL-2 with signal peptides as reported by Collins *et al.* [2]. The result suggested that the antiserum prepared in this study might be useful for detection of porcine IL-2 in eukaryotic cells.

A biological activity of porcine rIL-2 was examined by the cell proliferative assay using murine IL-2 dependent cell line, CTLL-2 according to the method described previously [9, 11]. Briefly, CTLL-2 cells were washed twice and suspended in RPMI1640 supplemented with 10% FCS, 25 mM HEPES, 5×10^{-5} M 2-ME and then seeded at 1×10^4 cells/well in a 96-well flat-bottomed microtiter plate. The cells were cultured in the presence of diluted samples for 20 hr at 37°C in a humidified 5% CO₂ atmosphere. Then, 10 μ l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma, U.S.A.) in PBS(-) was added to each well and incubated for further 4 hr. At the end of incubation, the dark blue formazan was dissolved by adding 100 μ l of 0.04 N HCl in isopropanol. The plates were read by ELISA plate reader (Bio-Rad) at a test wavelength of 570 nm and a reference wavelength of 630 nm. As shown in Fig. 3, porcine rIL-2, of which protein concentration was 0.24 mg/ml, enhanced the proliferation of CTLL-2 in a dose-dependent manner and the maximal response was observed in the presence of diluted samples at more than 1:1,000, suggesting that porcine rIL-2 expressed in *E. coli* also possessed a biological activity as those in eukaryotic cells. The specific IL-2 activity of the recombinant protein expressed as a reciprocal of dilution which showed 50% maximal CTLL-2

Fig. 3. Cell proliferative response of CTLL-2 with the culture supernatant of rat spleen cells stimulated with concanavalin A (○) and recombinant porcine IL-2 by bacterial expression (●). Each point represents the mean $\pm$ SD of triplicate assay.

response was calculated at 4×10^6 U/mg, which was comparable to that of ovine rIL-2 expressed in bacteria [13].

As stated above, porcine rIL-2 was expressed in *E. coli* and the insoluble protein was efficiently solubilized by using urea. The rIL-2 had a high bioactivity of 4×10^6 U/mg comparable to ovine IL-2 as reported previously. In addition, mouse antiserum against porcine IL-2 was obtained, which

reacted with rIL-2 expressed in insect cells infected with the recombinant baculovirus. These results suggested that the bacterial expressed proteins might be available as antigens for preparation of polyclonal and monoclonal antibodies and some immunoassays and the antiserum obtained might be also useful for detection of porcine IL-2 expressed in eukaryotic cells.

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