Production of Recombinant Porcine Interleukin-2 by The Baculovirus-Insect Cell System

การผลิตริคอมบิแนนท์โปรตีนอินเตอร์ลิวคิน 2 ของสุกรในเซลล์แมลงโดยใช้แบคคูโลไวรัส

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Abstract

Interleukin-2 (IL-2) is a cytokine produced by cells of the immune system. Its activities are involved in the multiplicity and maturity of immune cells whose activities play role in cell-mediated immune response (CMI). With the aim to produce functionally folded, glycosylated porcine IL-2 that allows further development of a synthetic cytokine to induce immune response in pigs, we produced a recombinant IL-2 using the baculovirus-insect cell expression system. cDNA was isolated from porcine peripheral blood leukocytes and is predicted to encode a partial IL-2 protein of 134 amino acid with 100%

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identity to a previous reported porcine IL-2 sequence. The recombinant protein expressed in baculovirus showed an apparent mass of 21 kDa, which was consistent with the estimated molecular weight of the recombinant porcine IL-2 with a six-His tag and a few amino acids from the vector. The immunoperoxidase assay revealed that goat anti-porcine IL-2 antibody reacted with the recombinant IL-2 protein expressed in the cytoplasm of the virus-infected High-Five cells. In addition, the Western blotting assay results showed that the recombinant porcine IL-2 protein exhibited the antigenic sites and conformation necessary for specific goat anti-porcine IL-2 antibody recognition. This suggests the potential use of recombinant IL-2 proteins in development of a useful tool for enhancing cell-mediated immunity in pigs.

Key words: cloning, gene expression, interleukin-2 (IL-2), baculovirus system, recombinant protein

บทคัดย่อ

อินเตอร์ลิวคิน 2 (Interleukin-2) เป็นไซโตคายน์ชนิดหนึ่งที่ผลิตจากเซลล์ของระบบภูมิคุ้มกัน หน้าที่ของอินเตอร์ลิวคิน 2 เกี่ยวข้องกับการแบ่งตัวและเข้าสู่วุฒิภาวะของเซลล์หลายชนิดที่เกี่ยวข้องในการตอบสนองภูมิ-คุ้มกันผ่านเซลล์ (CMI) งานวิจัยนี้มีวัตถุประสงค์เพื่อผลิตโปรตีนอินเตอร์ลิวคิน 2 ของสุกรที่มีการเติมหมู่คาร์โบไฮเดรตและมีโครงสร้างที่ทำหน้าที่ได้เพื่อที่จะพัฒนานำไซโตคายน์สังเคราะห์ไปใช้กระตุ้นภูมิคุ้มกันในสุกร ด้วยการผลิตโปรตีนรีคอมบิแนนท์อินเตอร์ลิวคิน 2 ของสุกรในเซลล์แมลงโดยใช้แบคคูโลไวรัส โดยคอมพีเมนทารีดีเอนเอได้ถูกแยกสกัดจากเม็ดเลือดขาวของสุกรซึ่งคาดว่าจะถอดรหัสเป็นบางส่วนของอินเตอร์ลิวคิน 2 ที่มีขนาด 134 กรดอะมิโน และพบว่ามีความเหมือนกับลำดับกรดอะมิโนของโปรตีนอินเตอร์ลิวคิน 2 ของสุกรที่ได้มีการรายงานไว้แล้วถึง 100 เปอร์เซ็นต์ โปรตีนรีคอมบิแนนท์ที่แสดงออกในแบคคูโลไวรัสมีขนาดที่ปรากฏ 21 กิโล-ดาลตัน ซึ่งสอดคล้องกับน้ำหนักโมเลกุลที่คาดหมายของอินเตอร์ลิวคิน 2 ของสุกรรวมกับฮิสติดีนเครื่องหมาย 6 กรดอะมิโนและกรดอะมิโนบางส่วนที่ได้จากเวคเตอร์ที่ใช้จากการตรวจวัดด้วยวิธีอิมมูโนเปอร์ออกซิเดส (immunoperoxidase assay) พบว่าแอนติพอร์ไซม์อินเตอร์ลิวคิน 2 แอนติบอดีที่ผลิตในแพะ (goat anti-porcine IL-2 antibody) ได้ทำปฏิกิริยากับโปรตีนรีคอมบิแนนท์อินเตอร์ลิวคิน 2 ในไซโตพลาสซึมของไฮไฟเซลล์ (High-Five cells) ที่ได้รับรีคอมบิแนนท์แบคคูโลไวรัส อีกทั้งผลจากการตรวจเช็คด้วยเวสเทิร์นบลอต (Western blot) ยังพบว่าโปรตีนรีคอมบิแนนท์อินเตอร์ลิวคิน 2 ของสุกรมีส่วนที่เป็นจุดจับของแอนติบอดี (antigenic site) และมีรูปร่างอันจำเพาะสำหรับการจดจำของแอนติพอร์ไซม์อินเตอร์ลิวคิน 2 แอนติบอดีที่ผลิตในแพะ จากผลการทดลองที่ได้บ่งชี้ว่ามีความเป็นไปได้ที่จะนำรีคอมบิแนนท์โปรตีนอินเตอร์ลิวคิน 2 ไปพัฒนาเพื่อใช้ประโยชน์ในการกระตุ้นการสร้างภูมิคุ้มกันแบบอาศัยเซลล์ในสุกร

คำสำคัญ: การโคลนยีน, การแสดงออกของโปรตีน, อินเตอร์ลิวคิน 2, แบคคูโลไวรัส, รีคอมบิแนนท์โปรตีน

Introduction

Interleukins are group of cytokines produced mainly by T lymphocytes (T-cells). Some also are produced by mononuclear phagocytes, or by tissue cells. Those produced by lymphocytes especially T-cells are often called lymphokines. They have a variety of functions, but most of them involve in directing immune cells to divide and differentiate. Each interleukin acts on a specific, limited group of immune cells which expresses the correct receptor for that interleukin. Interleukin-2 previously known as T-cell growth factor, is synthesized and secreted primarily by T-helper lymphocytes that have been activated by certain mitogens or by interaction of the T-cell receptor complex with antigen/MHC complexes on the surfaces of antigen-presenting cells (Thrope, 1998).

IL-2 acts as a chemical communicator between cells that provide the critical signals for effective cell-mediated response and humoral response. IL-2 enhances MHC-restricted cytotoxic T-cell responses, B cell expansion and immunoglobulin production, as well as non specific immune response such as NK cells and lymphokine-activated killer (LAK) cells. IL-2 was first purified and subsequently cloned and made available in recombinant form.

Porcine IL-2 gene is approximately 465 bp in length and located on chromosome 8 at 8q23 (Davoli *et al*., 2002). IL-2 is synthesized as a precursor protein of 154 amino acids which the first 20 aminoterminal amino acids functioning as a hydrophobic secretary signal sequence. The size of recombinant porcine IL-2 express in *E. coli* was about 15 kDa (Choi and Yoo, 2002; Collins *et al*., 1994), while recombinant IL-2 produced in insect cells has MW of 15 and 17 kDa (Iwata *et al*., 2000).

This paper reported the success of the cloning of porcine IL-2 cDNA and the expression of recombinant porcine IL-2 in insect cells using *Autographa californica* nuclear polyhedrosis virus (AcNPV) as a vector.

Materials and Methods

Isolation of porcine peripheral blood mononuclear cells (PBMC)

Except where indicated, all of the reagents used in this study were purchased from GIBCO/BRL. Ten millilitres of whole blood was collected from jugular vein of adult pigs into a plastic tube (EUROTUBO) containing heparin (20 unit/ml). The peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep™ (Nyclomed) separation medium according to manufacturer's protocol. The purified PBMC were resuspended in RPMI 1640 supplemented with 10% fetal calf serum (Hyclone), 2mM L-glutamine, 100 μM non-essential amino acid, 1mM sodium pyruvate, 50 μM 2-mercaptoethanol and 100 unit/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml of amphotericin B (antibiotic/antimycotic solution) and 5 mg/ml Con A (Sigma®) at 37°C in 5% CO_2 incubator for 48 h.

Cloning of porcine IL-2

Total RNA was isolated from Con-A stimulated porcine PBMC using Acid-Phenol-Guanidinium-Thiocyanate-Chloroform extraction method (Sambrook and Russell, 1998). cDNAs synthesis was performed using 1 μl of sample RNA, 10 mM dNTPs (Fermentas®), 2.5 mM Oligo-dT primers, 1X reverse transcriptase buffer (25 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl, 20 mM DTT), 5 mM $MgCl_2$, 0.4 U AMV reverse transcriptase and 0.4 U RNase inhibitor (Finzyme®) at 42°C for 50 min. The whole porcine IL-2 gene without signal sequence was amplified by PCR using a specific forward primer containing a *Xbal* restriction enzyme site 5′-CTAGTCTAGA GCACCTACTTCAAGCTCT ACA-3′ and a specific reverse primer containing a *Xhol* restriction enzyme site 5′GGGCCTCGAGTCAA GTCAGTGTTGAGTAGATG-3′. PCR reaction was performed with 1.0 U of DyNAzyme EXT™ DNA polymerase (Finzyme®) per sample in a total volume of 100 μl in reaction buffer containing 10 ml of cDNA templates, 0.25 mM dNTPs mix (Fermentas®), 10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl, 0.1% Triton X-100, 5 mM $MgCl_2$ (Finzyme®) and 1 pMol of sense and anti-sense primers. The PCR condition included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, primer annealing temperature at 62°C for 1 min and primer extension step at 74°C for 1 min, and the final extension step was performed at 74°C for 7 min. The amplified products were analyzed by electrophoresis on 1.5 % agarose gel.

Construction of expression vector

The PCR generated porcine IL-2 products were ligated to the baculovirus transfer vector, pFastBac ™HTb (Invitrogen®) at the *Xba* I restriction site and *Xho* I restriction site. The derived recombinant plasmid was used to transform *E. coli* strain DH5α™ (Invitrogen®). The transformed competent cells were plated on LB agar plates containing 100 μg/ml ampicillin and 7 μg/ml gentamicin as the selection media. The positive colonies for porcine IL-2 gene were extracted IL-2 plasmid to confirmed by PCR and restriction endonuclease assay. The sequence of the recombinant plasmid was verified using dideoxynucleotide termination method (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit) in an automatic sequencer (Applied Biosystems, Inc.). The recombinant porcine IL-2 plasmid was used to transform *E. coli* strain DH10Bac™ (Invitrogen®) for producing recombinant baculovirus DNA containing porcine IL-2. The positive colonies were identified using white-blue colony screening and PCR assay. The recombinant baculovirus DNA was purified and used to transfect insect cells.

Viruses and cells

Sf21 cell line *(Spodoptera frugiperda*; Invitrogen®) was grown at 27°C using SF900II medium (Invitgrogen®) supplemented with 4% FBS and 1× antibiotics. The recombinant baculovirus DNA was used to transfect Sf21 cells using CellFECTIN® (Invitrogen®). At 72 hour post

transfection, the recombinant baculovirus particles were collected from supernatant and viral titer was determined using plaque assay. Subsequently, the high-titer seed stock of recombinant baculovirus was produced by Sf21 insect cells at a multiplicity of infection (MOI) of 0.01 to 0.1 using Sf900 II SFM® medium (Invitrogen®) containing 4% fetal bovine serum and 1× antibiotic. High-Five™ cell line *(Trichoplusia ni*) grown in Express Five serum-free medium (Invitrogen®) supplemented with 2mM L-glutamine and 100 unit/ml penicillin G, 100 mg/ml streptomycin, 0.25 μg/ml of amphotericin B (antibiotic/antimycotic solution) was inoculated with recombinant baculoviruses for the production of recombinant IL-2 protein. After 72 h post-inoculation, the infected High-Five cells were fixed and collected for further determine by immunoperoxidase monolayer assays, SDS-PAGE analysis and Western blot analysis.

Immunoperoxidase monolayer assay (IPMA)

Both uninfected and baculovirus infected cells were incubated with 50 μl goat anti-porcine IL-2 IgG polyclonal antibody (1:50; R&D) at 37°C for an hour. Following 3 times wash in PBS-Tween (0.05%), specimen were incubated with 50 ml rabbit anti-goat IgG conjugated with horseradish peroxidase (1:500; Sigma®) at 37°C for 30 min. After 3 times washing in PBS-Tween (0.05%), specimen were incubated with Diaminobenzidine solution (Sigma®) at room temperature for 5 min. Under an inverted phase microscope, the presence of porcine IL-2 was visualized with a diamino-benzidine/hydrogen peroxidase chromogen reaction with a resultant brown color.

SDS-PAGE and Western blotting

The crude protein of both uninfected and baculovirus infected cells was analyzed using 15 % SDS-PAGE stained with Coomassie brilliant blue. For Western blot analysis, proteins on SDS-PAGE were transferred onto nitrocellulose membrane using 400 mAmp at 4°C for 5 h. The membrane was first treated with blocking buffer (5% powder milk in PBS) and then incubated with goat anti-IL-2 IgG polyclonal antibody (1:200) for an hour. Following 3 times wash in PBS-Tween (0.05%), the membrane was then incubated with rabbit anti-goat IgG (1:1,000) conjugated with horseradish peroxidase (1:300) for an hour. Finally, after 3 times washing in PBS-Tween (0.05%) it was incubated with diaminobenzidine solution (Sigma®) containing 1% H_2O_2 for 5-10 min.

Results

Cloning of the cDNA encoding porcine interleukin-2

The PCR product of porcine IL-2 gene without signal sequence was approximately 425 bp in size which included 20 bp of restriction enzyme site and overhang at 5′ end and 3′ end. To confirm the IL-2 gene was corrected, the sequence of recombinant plasmid was verified using dideoxynucleotide termination method by automatic

DNA sequencer (ABI PRISM™). The nucleotide sequences of recombinant plasmid were then translated to amino acid sequence by DNASIS program and compared with reported porcine IL-2 (Accession X58428) by CLUSTAL W program. Comparison of amino acid sequences of the recombinant IL-2 with the reported porcine IL-2 sequence in GenBank showed 100 % homology (Fig. 1).

Expression of the recombinant protein

Using IPMA the recombinant baculovirus infected High-Five cells showed brownish red intracytoplasmic staining (Fig 2a) whereas, the uninfected cells did not (Fig 2b). This result indicated the presence of IL-2 in the High-Five infected cell. The SDS-PAGE analysis of the crude protein from the recombinant baculovirus infected cells revealed

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CLUSTAL W (1.82) multiple sequence alignment

x58428          APTSSSTKNTKKQLEPLLLDLQLLLKEVKNYENADLSRMLTFKFYMPKQATELKHLQCLV 60
rIL-2           APTSSSTKNTKKQLEPLLLDLQLLLKEVKNYENADLSRMLTFKFYMPKQATELKHLQCLV 60
                ************************************************************

x58428          EELKALEGVLNLGQSKNSDSANIKESMNNINVTVLELKGSETSFKCEYDDETVTAVEFLN 120
rIL-2           EELKALEGVLNLGQSKNSDSANIKESMNNINVTVLELKGSETSFKCEYDDETVTAVEFLN 120
                ************************************************************

x58428          KWITFCQSIYSTLT 134
rIL-2           KWITFCQSIYSTLT 134
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Figure 1 Comparison of deduced amino acid sequences for the recombinant porcine IL-2 (rIL-2) with the known porcine IL-2 (GenBank accession number X58428). Consensus line; asterisks (*) indicate identical amino acid residues.

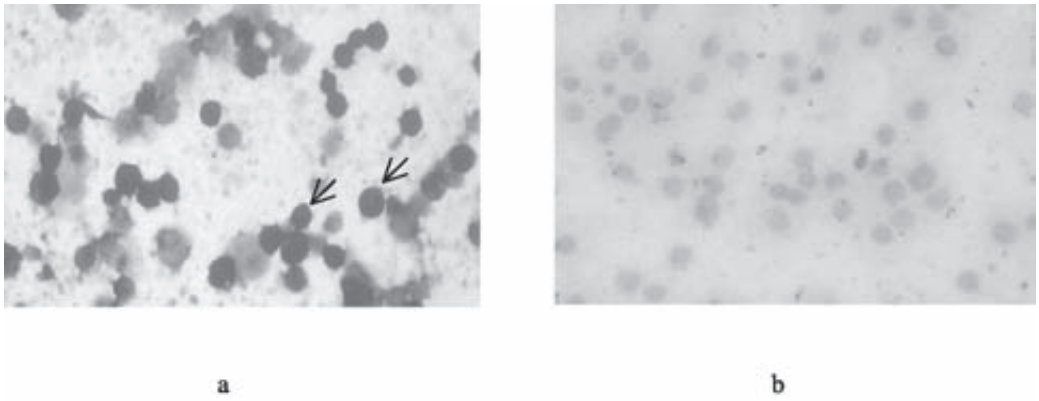

Figure 2 Immunoperoxidase staining of High-Five cells infected with the recombinant baculovirus (a) or with wild-type baculovirus (b). The red- brown staining represents cellular localization of the recombinant IL-2 protein (arrows). Magnification 200x.

a distinct band of relative molecular mass of 21 kDa fusion protein which included 5 kDa of 6xhis and a few amino acids from the vector when compared to wild-type baculovirus infected cells (Fig.3). Western blotting analysis of total lysate showed the immunological reaction between the anti-porcine IL-2 polyclonal antibody and recombinant protein of 21 kDa and negative reaction in wild-type infected High-Five cells lysate (Fig.4).

Discussion

In this study, the recombinant porcine IL-2 was successfully cloned and expressed. Amino acid sequences of the recombinant porcine IL-2 in the present study had 100 % homology with the known porcine IL-2 sequence (GenBank Accession X58428). The SDS-PAGE analysis of recombinant porcine IL-2 produced by the insect cells had the size approximately 21 kDa, which included about 5 kDa of 6xhis and a few amino acids from the vector. Porcine IL-2 is a 134 amino acid protein with a molecular mass varying from 15 to 18 kDa, according to the degree of post-translation modification (Danis and Hubber, 2003). From in vitro culture lymphocytes were able to produce porcine IL-2 molecules of 15 to 23 kDa (English *et al*., 1985; Gasbarre *et al*., 1984). Similarly, polypeptides from cDNA of porcine IL-2 had molecular weight of 15 kDa in *E. coli* (Choi and Yoo, 2002; Collin *et al*., 1994; Iwata *et al*., 2000), and about 15 and 17 kDa in insect cells (Iwata

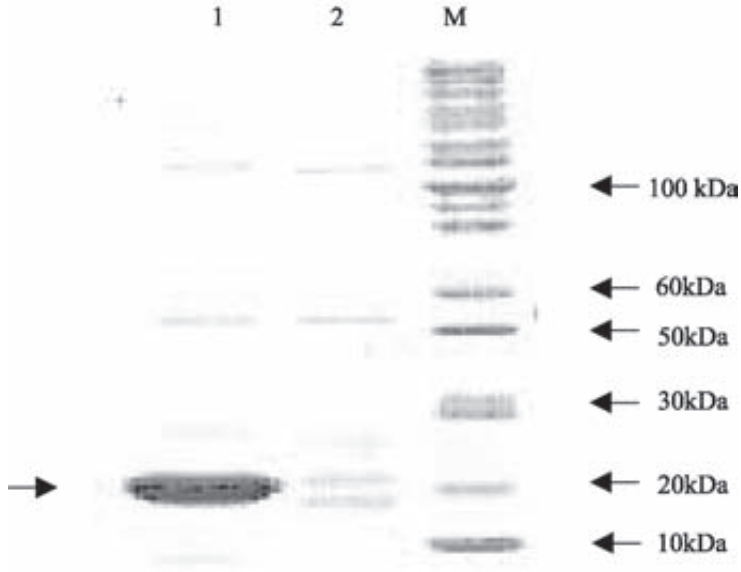

Figure 3 SDS-PAGE analysis of protein in cell lysates from High-Five cells infected with the recombinant baculovirus (1) or wild-type baculovirus (2). The SDS-PAGE was stained with coomassie brilliant blue. Molecular mass (lane M) is indicated on the right. The arrow indicates a single protein band with a molecular weight of 21 kDa.

Figure 4 Western blot analysis of the porcine IL-2 protein in cell lysates from High-Five cells infected with wild-type baculovirus (lane 1) or the recombinant baculovirus (lane 2). The Western blot were stained with anti-porcine IL-2 polyclonal antibody.

et al., 2000), when determine by SDS-PAGE. This finding might be attributed to glycosylation of polypeptides and/or aggregation of IL-2 molecules (Gills *et al*., 1982; Danis and Hubber, 2003). The function significance of glycosylation of IL-2 is not known, but it is likely that it enhances solubility in aqueous environments (Gaffen and Liu, 2004).

According to the expression analysis using IPMA and Western blot analysis, the recombinant porcine IL-2 reacting with goat anti-porcine IL-2 antibody indicated that the recombinant porcine IL-2 may display a conformation similar to the native protein, and be valuable for further investigation on its biological active. At the present, the determination of biological activity of this molecule is underway. IL-2 has been shown in several studies to induce Th1 response and increasing cellular immunity (Giedlin, 2000). Thus, the use of recombinant porcine IL-2 as an immunostimulant adjuvant will be a very useful tool to augment the potency of immunogen and stimulate immune response.

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