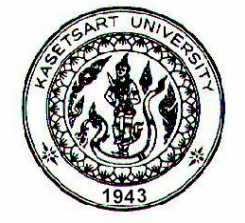

THESIS

CLONING AND EXPRESSION OF PORCINE INTERLEUKIN-2 (IL-2)

KANOKRAT SRIKIJKASEMWAT

GRADUATE SCHOOL, KASETSART UNIVERSITY

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THESIS

CLONING AND EXPRESSION OF PORCINE INTERLEUKIN-2 (IL-2)

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This study was to clone and express porcine IL-2 using insect cells. The whole porcine interleukin-2 gene encoded complementary strand DNA (cDNA) was cloned using specific primers designed from the sequence data as report in GenBank. The recombinant plasmid porcine IL-2 was sequenced and used to produce the recombinant baculovirus containing porcine IL-2 gene. The recombinant baculovirus was used to inoculate High-Five cells for producing the recombinant procine IL-2. The SDS-PAGE analysis showed the distinct band around 23 kDa which was approximately the size of porcine IL-2. The immunoperoxidase monolayer assay using goat anti-porcine IL-2 antibody of the infected High-Five cells showed the IL-2 in the cytoplasm of the cells. The dot and Western blot analysis of the recombinant porcine IL-2 using goat anti-porcine IL-2 antibody also showed the positive results. Biological activity of IL-2 was analyzed using T4 cell. The T4 cells showed proliferation after the stimulation with recombinant porcine IL-2. This assay gives 50% of maximal response induced by approximately 6.96 pg/ml of the recombinant porcine IL-2 proteins. Thus, the recombinant porcine IL-2 proteins were biologically functional and glycosylation of recombinant protein.

Student's signature

Thesis Advisor's signature

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Kanokrat Srikijkasemwat

February 2006

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LIST OF ABBREVIATIONS

µl	=	microlitre
ml	=	mililitre
µg	=	microgram
mg	=	milligram
bp	=	base pair
kDa	=	kilodalton
pmol	=	picomole
mM	=	millimolar
M	=	molar
U	=	unit
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
dNTPs	=	Deoxynucreotide
PCR	=	Polymerase Chain Reaction
EtBr	=	Ethidium Bromide
UV	=	ultraviolet
SDS-PAGE	=	Sodium Dodecyle Sulfate Polyacrylamide Gel Electrophoresis
rpm	=	round per minute
ºC	=	Degree Celsius
h.p.i.	=	hour post infection
MOI	=	multiplicity of infection
E. coli	=	*Escherichia coli*

CLONING AND EXPRESSION OF PORCINE INTERLEUKIN-2 (IL-2)

INTRODUCTION

Vaccines have traditionally consisted of live attenuated pathogens, whole inactivated organisms or inactivated toxins. In many cases, these approaches have been very successful at inducing immune protection, mainly based on antibody responses. However, to develop vaccines against more difficult pathogens, which often establish chronic infections, e.g. human immunodeficiency virus (HIV), hepatitis C virus (HCV), tuberculosis (TB) and malaria, the induction of cell-mediated immunity (CMI) is likely to be necessary. Unfortunately, non-living vaccines have generally proven ineffective at inducing CMI. In addition, although live vaccines may induce CMI, some live attenuated vaccines can cause disease in immunosuppressed individuals and some pathogens are difficult or impossible to grow in culture, e.g. HCV , making the development of inactivated vaccines impossible (Bowerstock and Martin, 1999; O'Hagan *et al*., 2001).

Several new approaches to vaccine development have emerged, these approaches include: (1) recombinant protein subunit; (2) synthetic peptide; (3) protein polysaccharide conjugates; and (4) plasmid DNA. While these new approaches may offer important safety advantages, a general problem is that these vaccines may not be cost effective for veterinary use and are often poorly immunogenic (Singh and O'Hagan, 2003).

Under some circumstances it may be desirable to enhance the normal immune response by administering an adjuvant with the antigen. Adjuvants enhance the body's immune response to the antigen. A large variety of compounds have been employed as adjuvants. Many adjuvants enhance the immune response by slowing the release of antigen into the body, however in many cases their mode of action is unknown. The major prohibition on adjuvants are that they should not be toxic and in the case of food animals, they should not adversely affect the quality of meat (Tizard, 1995). The only adjuvants currently approved by the U.S. Food and Drug Administration is aluminum based mineral salts (generally called alum). Alum has a good safety record but comparative studies show that it is a weak adjuvant for antibody induction to protein subunits and a poor adjuvant for CMI (Gupta, 1998).

In the development of vaccines for diseases in which humoral immunity is the most effective means of protection, it is not only essential to obtain adequate amounts of antibody but also important to generate a particular immunoglobulin isotype in order to ensure that the causative agent is effective eliminated (Taylor, 1995). Certain immunoglobulin isotypes are more efficient than others in activating complement or binding to receptor on monocytes. Thus, it is often desirable to employ adjuvants that will induce synthesis of isotypes with greater protective value. In general, the primary objective of increasing the antibody response is achieved be employing chemical adjuvants to boost the response. However, because of the need for obtaining antibody of an appropriate isotype, it is not surprising that the use of recombinant cytokines, rather than standard formulations, as adjuvants for vaccines is getting

considerable attention (Taylor, 1995). Some adjuvants are also developed to enhance immunologenicity of vaccine for CMI against some viral diseases.

Therefore, there is a need for the development of potent and safe adjuvants that can be used with newer generation vaccine. Interleukin-2 (IL-2) has been shown in several studies to induce Th1 response and increase cellular immunity against viral, bacteria and parasite infection (Chow *et al*., 1997; Nobiron *et al*., 2001; Gaffen and Liu, 2004). The use of IL-2 as an adjuvant might be employed as immunostimulants to augment the potency of week immunogen, stimulated immunity, decrease the doses of antigen in the vaccine and reduce costs.

OBJECTIVES

1. To clone porcine IL-2 gene and produce recombinant porcine IL-2 protein in insect cells.
2. To determine efficiency of recombinant IL-2 as an adjuvant to enhance the immune response *in vitro*.

LITERATURE REVIEW

Cytokines

Cytokines can be described as secreted regulatory proteins or glycoproteins of relatively small molecular mass (rarely more than 8-25 kDa) (Roitt *et al*., 2003). Cytokines act as chemical communicators between cells that provide the critical signals for effective humoral (antibody-mediate) and cell-mediated responses. Cytokines bind to specific receptors on the surface of target cells that are coupled to intracellular signal-transduction pathways. They regulate all the importance biological processes including cell growth, cell activation, inflammation, immunity, tissue repair, fibrosis and morphogenesis. Some cytokines are also chemotactic for specific cell types (Taylor, 1995).

$CD4^+$ T helper (Th) cells act as the center for mediating immunological activity through cytokine production. T helper cells are subdivided into Th_1 helper cells and Th_2 helper cells on the basis of their production of and responses to specific cytokines (Roitt *et al*., 2003). The Th_1 cytokines also activate macrophage's phagocytosis and promote the production of IgG subclasses which have effects on activating complement and stimulating phagocytosis. Th_1 cells produce Th_1 cytokines including IL-2, IFN-γ, lymphotoxin (LT), TNF-α and IL-12 which are involved in the cell-mediated immune and inflammatory responses. Th_2 cells produce Th_2 cytokine which indicate the humoral immune responses. The Th_2 cytokines include IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann and Sad, 1996; Romani *et al*., 1997). Th_2 type

response is associated with activation of B cells and as a result, they stimulate IgG, IgA, IgM production and enhance IL-4-induced IgE synthesis (Wong *et al*., 2002). This humoral response is generally thought to be protective against larger extracellular organism such as helminthic (worm-like) parasite (O'Sullivan *et al*., 2001).

Interleukins

Interleukins are a large group of cytokines produced mainly by T-cells, although some are also produced by mononuclear phagocytocytes, or by tissue cells (Roitt *et al*., 2003). Those produced by lymphocytes especially T-cells are often called lymphokines. Interleukins generally function as intercellular messenger molecules that evoke particular biological activities after binding to receptor on responsive target cell. The numerous physiologic responses that require interleukins involvement are development of cellular and humoral immune response, induction of the inflammatory response, regulation of hematopoiesis, control of cellular proliferation and differentiation and the healing of wounds (Goldsby *et al*, 2000). Both of cellular and humoral response produced different sets of interleukins. Interleukins that involve humoral response are IL-4, IL-5, IL-6, IL-10 and IL-13, while those of cellular immune response including IL-2, IL-3 and IL-12 (Abbas *et al*., 2000).

Interleukin-2

Interleukin-2, previously known as T-cell growth factor is synthesized and secreted primarily by T-helper lymphocytes activated by stimulation with certain mitogens such as concanavalin A (Con A) and phytohaemagglutinin (PHA) or by interaction of the T-cell receptor complex with antigen/MHC complexes on the surfaces of antigen-presenting cells (Dinarello, 1994). IL-2 is an autocrine factor, driving the expansion of the antigen-specific T cells. IL-2 also acts as a paracrine factor, influencing the activity of other cells including B lymphocytes and natural killer (NK) cells (Shibuya *et al*., 1993).

The mature human IL-2 protein consists of 133 amino acids (Robb, 1984). It is synthesized as a precursor containing 153 amino acids and subsequently 20-residue hydrophobic leader sequence (signal peptide) is cleaved to produce the mature protein prior to or during secretion (Thorpe, 1998). The molecule contains a single N-linked glycosylation site at position 3 and differences in glycosylation of this cause size and charge heterogeneity in both natural and cell line-derived IL-2 (Robb, 1984). Glycosylation is not necessary for biological activity, but is neccessry for binding of at least one monoclonal antibody. Several rDNA-derived IL-2 preparations have been produced; nonglycosylationed material prepared using prokaryotic organism is both stable and fully biologically active. The natural molecule contains three cysteine residues at positions 58, 105, and 125, two of which (58 and 105) form a disulfide bridge that is essential for the biological activity of the molecule (Wang *et al*, 1984). The cysteine at position 125 can form intramolecular disulfide bridges, leading to the

production of dimers and higher aggregates. Molecules folded with an internal disulfide bridge involving the cysteine at position 125 (i.e., between residues 58-125 and 105-125) show minimal biological activity. Although these do not seem to occur in natural IL-2 preparations, they can be present or even predominate in rDNA-derived products (Thorpe, 1998).

Natural human IL-2 occur as a group of glycoproteins with apparent M_r values between 13000 and 17500 as difined by SDS-PAGE and pI values between 6.6 and 8.2 (Robb and Smith, 1981). It is a hydrophobic molecule which is stable to moderate heat and stable at low pH (Robb *et a*l, 1983). Crystallography has shown IL-2 to consist of six-α-helical domains (A-F) which no apparent β structural regions. An antiparallel helical bundle is formed a bent loop to the rest of the molecule (Mott *et al*., 1995). The human IL-2 gene is present as a single copy on chromosome 4q bands 26-28 (Thorpe, 1998).

Porcine IL-2

Porcine IL-2 is a product of $CD4^+$ lymphocyte activation. Porcine IL-2 gene is approximately 465 bp in length and located on chromosome 8 at 8q23 (Davoli *et al*., 2002). Porcine IL-2 shares many of the properties of human IL-2 and has a similar molecular weight of 15-23 kDa. Porcine and human IL-2 molecule display a homology of approximately 72 percent (Davoli *et al*., 2002). Porcine IL-2 is synthesized as a precursor protein of 154 amino acids with the first 20 aminoterminal amino acids functioning as a hydrophobic secretary signal sequence. The porcine IL-

2 contains 134 amino acids. At the amino acid sequence level, there is approximately 60-90% similarity between species such as human, bovine, mouse and porcine. The protein contains a single disulfide bond (positions Cys58/105) essential for biological activity (Choi and Yoo, 2002). Porcine, bovine, mouse and human IL-2 were compared for activity on homologous and heterologous cells, difference were seen. Porcine, bovine and human IL-2 enhances, promote and stimulate proliferation of T cells of the homologous species at high efficiency. Porcine IL-2 was active on porcine, bovine, human 4 days Con A blasts or mouse CTLL cell line. However, human Con A blasted could only be stimulated by porcine IL-2 to approximately 45 % of human IL-2 potential (Collins *et al*., 1994). Human IL-2 also stimulates *in vitro* proliferation of mouse T cells at similar to mouse IL-2 concentrations, whereas mouse IL-2 stimulates human T cells at a lower (6 fold to 170-fold of human IL-2) efficiency than human IL-2 (Mai *et al*., 1994).

The molecular weight of native IL-2 molecules were reported to range from 15 to 32 kDa (depending on stimulatory condition and measuring methods of molecular weight) (English *et al*., 1985; Gillis *et al*., 1978). The difference in molecular weight might be attributed to glycosylation of polypeptides and/or aggregation of IL-2 molecules, N-link sugar have a molecular weight of 5 kDa while O-link is variable (Gillis *et al*., 1980; Denis and Huber, 2003). From *in vitro* studies, IL-2 transcripts was detected in phytohaemagglutinin (PHA) or concanavalin A (Con A)-stimulated spleen cells, peripheral blood mononuclear cells (PBMC) and alveolar macrophages, and its characteristics confirmed by biological and biochemical studies (Yancy *et al*.,

2001; Choi *et a*l., 2002). Porcine IL-2 has a possible N-linked glycosylation site as reported in bovine (Ceretti *et al*., 1986; Goodall *et al*., 1991; Iwata *et al*., 1993).

Porcine IL-2 chemical properties could be determined in the eluted fractions corresponding to pH 5.2 to 5.8 by chromatofocusing (Iwata *et al*., 1993). The estimated pI value of porcine IL-2 was slightly higher than that of 5.0 to 5.5 as reported by English *et al*. (1985). Porcine IL-2 molecules were found to be sensitive to the proteolytic enzyme of trypsin, the acid (pH 3.2) or the basic (pH 10.5) conditions, urea (2, 4 or 8 M) and heating at 70 ^{o}C for 15, 30 or 60 min (Iwata *et al*., 1993). These properties were considered to be similar to rather feline IL-2 than human or murine IL-2 (Gillis *et al*., 1982; Goitsuka *et al*., 1986; Mochizuki *et al*., 1980).

IL-2 Receptors

IL-2 receptor contains three subunits including the alpha subunit or p55, the beta subunit or p75 and the gamma subunit or p64. The alpha subunit is specific for IL-2. The beta subunit is shared between IL-2 and IL-15 receptors. The gamma subunit, also know as common gamma chain, is shared by receptors of IL-2, IL-4, IL-7, IL-9 and IL-15. All subunits together interact with each other to effectively signal IL-2 mediated events to the cell (Minami *et al*., 1993). Only the beta and gamma subunits are capable of signal transduction. There are two types of IL-2 receptors: the αβγ high affinity receptor (affinity 10-50 pmol) and the βγ intermediate affinity receptor (affinity 1 nmol). The αβγ receptor is expressed by CD 4^{+} lymphocyte upon

antigen or mitogen stimulation, B lymphocytes and 10% of circulating NK cells, whereas the βγ receptor of IL-2 is expressed by 90% of circulating NK cells, neutrophils and monocyte/magcrophage cells (Alileche, 2003).

Interleukin-2 in cancer treatment

IL-2 is a major regulator of the immune system especially cell-mediated immune response. Thus, it has been used to stimulate the immune system against cancer. Recombinant human IL-2 molecule has been used for the treatment of cancer, including metastatic melanoma (Lode *et al*, 2000), prostate cancer, neuroblasma, renal cell carcinoma (Dillman, 1999; Margolin, 2000), hepatocellular carcinoma (Alileche, 2003). Possible mechanism of IL-2 ability to treat cancer cells is that IL-2 can inhibit T cell growth and induce cell death (Leonardo, 1991; Rafaeli and Abbas, 1998). An other feature of IL-2 is the induction of the lymphokine activated killer (LAK) phenomenon. The LAK are generated *in vitro* and *in vivo* from human peripheral blood lymphocytes and lymphocytes from the spleen, the thymus, the lymphnodes, the bone marrow and the thoracic duct, the LAK cells are able to kill freshly isolated and cultured tumor cells and not normal cells (Grimm and Rosenberg, 1982; Rayner and Lotze, 1985). The IL-2 induced LAK activity is not MHC restricted and consists of three phases: recognition and binding of the effector LAK cells to its target tumor cells, release of cytotoxic substances like perforin and death of the tumor cells. The nature of the antigens responsible for the recognition by the LAK cells are not known (Alileche, 2003).

IL-2 as an adjuvant

The use of recombinant IL-2 as adjuvants in combination with vaccine against several infectious diseases were studied, such as bovine viral diarrhea virus (BVDV) DNA vaccine (Nobiron *et al*., 2001), *Eimeria* DNA vaccine (Min *et al*., 2002), foot and mouth disease DNA vaccine (Wong *et al*., 2002). Murine IL-2 has been shown to enhance antibody titers to either vaccine antigens or inactivated viral vaccines when parenterally administered either exogenously or in liposomal formulations (Heath *et al*., 1992; Lachman *et al*., 1996; Schijns *et al*., 1994). The use of IL-2 to treat infectious diseases such as hepatitis B (Tilg *et al*., 1993) and herpes simplex virus genital infection (Weinberg *et al*., 1986) has been described. In HIV infection the numbers of $CD4^+$ cells decrease over time leading to illness and death. Production of cytokines associated with CMI (interferon-gamma, IL-2 and IL-12) fall in AIDS patients while production of cytokines associated with humoral immunity (IL-4, 6, 10) rise. In previous studies, IL-2 therapy resulted in increases in total $CD4^+$ cell numbers in some HIV-infected people. But whether this makes a difference in the number of AIDS-related illnesses a person has, or the length of life is unknown. A recent trial has shown that infusion of IL-2 in selected AIDS patients can induce substantial sustained increase in $CD4^+$ lymphocytes without associated increases in plasma HIV RNA levels (Kovacs *et al*., 1996). Ten years ago, people with AIDS were given IL-2 in much higher doses than are being tested now. The results were disastrous, people became sick and died. Low-dose IL-2 therapy seems promising in this clinical condition (Jacopsen *et al*., 1996). Rollwagen and Baqar (1996) reported successfully oral treatment for Campylobacter infection in mice, the results showed

immune response to cholera vaccine and protective immunity against Campylobacter colonization in mice were induced when inactivated bacteria whole cells vaccine were given with IL-2 at the time of vaccination.

A major function of IL-2 is acts on all subpopulations of T cells and promotes progression through the G_1 phase of cell cycle, resulting in growth of cells and increase in cell numbers. IL-2 also stimulates cytolytic activity of subsets of T lymphocytes, enhances T cell motility, and induces secretion of other cytokines such as IFN-γ, IL-4 and TNF (Thrope, 1998). Cytotoxic lymphocytes (CTLs) response was greatly enhance in the present of IL-2, ether from a direct injection of IL-2 molecules (Chan *et al*., 2001) or the expression products of IL-2 genes transfected to the cells (Progador *et al*., 1993; Ohe *et al*., 1993). It was also found that co-adminitration of plasmids encoding IL-2 with the antigen dramatically increased the Th cells proliferation (Kim *et al*., 1998). This enhancing effect was very strong for Th1 cells and relatively mild for Th2 cells (Chow *et al*., 1997). IL-2 can enhance the proliferation of B cells, thus enhancing the humoral response directly. However, IL-2 regulates the B cell response mostly through an indirect way. The effectiveness of IL-2 on infectious disease treatment involve activation of Th cell proliferation, especially Th2 cells and stimulation of the production of Th2 cytokines, including IL-4, IL-6 and so called 'professional' B cell growth factors such as IL-5 and IL-10. These Th2 cytokines production result in an increase in the growth and differentiation of activated B cells and as a result, they stimulate IgG, IgA, IgM production and enhance IL-4-induced IgE synthesis (Wong *et al*., 2002).

Adjuvants

Adjuvants have been used to augment the immune response to antigen after vaccination for more than 70 years. A large number of agents have been employed as adjuvants to augment the immunogenic potency of weak immunogens. The commercial adjuvants used include aluminum based on mineral salt (generically called alum), lipopolysaccharide (LPS), Freund's incomplete adjuvant and Freund's complete adjuvant. Freund's complete adjuvant which contains mycobacteria antigen in water in-oil emulsions is a strong activator of the Th1 cell type and also has an ability to enhance humoral responses (Linblad, 2000). Although Freund's complete adjuvant is a very effective adjuvant for production of antibodies, there are problems and hazards associated with its use. At the site of injection, Freund's complete adjuvant causes a chronic inflammatory response that may be severe and painful for the animal depending on the site as well as the quantity and quality of adjuvant injected. The inflammatory response may result in formation of chronic granulomas, sterile abscesses, and/or ulcerating tissue necrosis. Adjuvant-induced lesions may appear to be metastatic when excessive amounts of the emulsion are injected in a single site. Emulsion injected subcutaneously on the dorsal region of some species (rabbit, in particular) may migrate by fistulous tracts to the ventral region of the animal. Emulsion injected intramuscularly may spread along fascial planes to distant muscular sites or may travel to lung, liver or other organs, apparently by a hematogenous route (Broderson, 1989; Kleinman *et al*., 1993; Claassen *et al*., 1992; Stills and Bailey, 1989; Stills 1994). Freund's complete adjuvant is unacceptable in

the clinic and even Freund's incomplete-type adjuvants have been banished from human use (Audibert and Lise, 1993).

Adjuvants can be broadly divided into two classes (based on their principal mechanisms of action) vaccine delivery systems and immunostimulatory adjuvants. Vaccine delivery systems are generally particulate e.g. emulsions, microparticles, iscoms and liposomes, and mainly function to incorporate and deriver target antigens into antigen presenting cells (APC). In contrast, immunostimulatory adjuvants are predominantly molecules derived from pathogens and often represent pathogen associated molecular patterns (PAMP) for example lipopolysaccharide (LPS), monophosphoryl lipid A (MPL), CpG DNA, which activate cells of the innate immune system (O'Hagan *et al*., 2001).

Immunoadjuvants represent a number of separate compounds with a large variety of conformations and actions via different pathways. It is now recognized that their biological properties depend upon their ability to activate selectively one of two $CD4^+$ T helper cell subpopulations, Th1 or Th2 cells. This would lead to either CMI or HI (Audibert and Lise, 1993). Adjuvants have abilities to improve the immune response to vaccine antigens in several ways. Vaccine adjuvants can increase the potency of small, antigenically weak synthetic or recombinant peptides such as hepatitis B polypeptide and malaria synthetic polypeptide. They can enhance the speed, vigor and persistence of immune response to stronger antigens. For example, aluminum adjuvants used with licensed pediatric vaccine elicit early and higher antibody response after primary immunization than unadjuvanted preparations.

Adjuvants can increase the immune response to vaccines in immunologically immature, immunosuppressed or senescent individuals (Edelman, 2000).

Mechanism of adjuvants for improvent of response is exerted by preferentially stimulation either Th1 or Th2 $CD4^+$ T helper cells (reviewed in Cooper, 1994; Newman and Powell, 1995). Second, depending upon the adjuvant, the immune response can be modulated in favor of MHC class I or MHC class II response by means of fusion or disruption of cell membranes (Cooper, 1994; Newman and Powell, 1995). Aluminum salts principally stimulate the Th2 response, work by establishing a depot of Ag which is released slowly over a period of 2-3 weeks, nonspecific activation of macrophages and complement activation (Gupta and Siber, 1994), while the Th1 response is induced by many adjuvants, such as muramyl dipeptide and MPL through to interact with Toll-like receptor 4 (TLR4) on antigen presenting cells (APCs), resulting in the release of pro-inflamatory cytokines such as IL-2 and IFN-γ (Cooper, 1994; Singh and O'Hagan, 2003). To increase humoral immune response adjuvants can modulate antibody avidity, specificity, quantity, isotype and subclass against epitopes on complex immunogens (Hunter and Lal, 1994;Hui *et al*., 1991; Kenney *et al*., 1989). Vaccine adjuvants slowly release small amount of antigens in vaccines, prolonging the time for interaction between antigen and antigen-presenting cells and lymphocytes which induces lower antigen competition and carrier-specific epitope suppression.

IL-2 has also been used as an therapeutic agent for treatment of infectious disease and cancer (Farner *et al*., 1997; Lillehoj *et al*., 2000). The local

administration of IL-2 alone induces the migration of immune cells to the site of injection, increases local expression of MHC class II, and enhance skin antigen reactivity (McElrath *et al*., 1990). Activation of monocytes and dendritic cells is also improved (Murray *et al*, 1988; Murray *et al*., 1985; Bykovskaja *et al*., 1998). In vitro, mononuclear cells from AIDS patients can respond to recombinant IL-2 with enhanced IFN-γ production (Murray *et al*., 1985). Civallero (2000) found that IL-2 may act as a maturative factor rather than as a growth factor in the dendritic cell differentiation. The success of recombinant IL-2 incorporation with several vaccine model system such as vaccines for viral, bacterial and parasitic diseases have been reported. The use of rpIL-2 resulted in significantly higher virus neutralizing (VN) antibody levels and cytotoxic T lymphocyte activities on PRV IAV vaccination. The promotion of PRV-specific secretion of pIL-4 and pIFN-gamma from PBMC of pigs gave concrete evidence of the adjuvant effects of rpIL-2. In addition, the co-administration of the rpIL-2 with PRV IAV in pig also produced an improved protection to the viral challenge (PRV), demonstrated by significant reduction of the ratios of fever and viral excretion in nasal swabs (Lin *et al*., 2005).

Baculovirus Expression Vector System

The Baculovirus Expression Vector System (BVES) is one of the most powerful and versatile eukaryotic expression systems available. The BVES is a helper-independent viral system which has been used to express heterogeneous genes from many different sources, including fungi, plants bacteria and viruses, in insect cells. Baculovirus have a restricted host range, limited to specific invertebrate

species. These viruses are safer to work with the most mammalian viruses since they are noninfectious to vertebrates. Most of susceptible insect cell lines are not transformed with pathogenic or infectious viruses and can be cultured for under minimal containment conditions. Helper cell lines or helper viruses are not needed since the baculovirus genome contains the genetic information needed for propagation in a variety of cell lines or larvae from different insect species.

The baculovirus genome is replicated and transcribed in the nuclei of infected host cells where the large baculovirus DNA is packaged in to rod-shaped nucleocapsids. Since the size of this nucleocapsid is flexible, recombinant baculovirus particles can accommodate large amounts of foreign DNA. Two of common isolates baculovirus used in foreign gene expression are *Autographa californica* multiple nuclear polyhedrosis virus (*Ac*MNPV) and *Bombyx mori* (silkworm) nuclear polyhedrosis virus (*Bm*NPV). Wild-type baculoviruses have both lytic and occluded life cycles independent developing throughout three phases of virus replication as follow.

1. Early Phase or Virus synthesis phase Viruses infected host cell for viral DNA replication (*Jarvis et al.,* 1996). There are several steps included as attachment, penetration, uncoating, early viral gene expression. This step occurs 0.5 to 6 hours after infection (h.p.i.) (Ghosh *et al.,* 2002).

2. Late phase or viral structural phase Late genes that are encoded for replication of viral DNA and for the assembly of virus are expressed (6-12 hours.

after infection) The cell produces extracellular virus (EV) or so called budded virus (BV). They are contains plasma membrane envelope and glycoprotein (gp) 64, which is necessary for endocytosis. At 18-36 h.p.i, the EV is released (Ghosh *et al.,* 2002).

3. Very late phase or viral occlusion protein phase The polyhedrin and p10 genes are expressed and the occluded virus or occlusion bodies (OB) or polyhedra occlusion bodies are formed and host cell was lyzed between 24 - 96 hours after infection (Jarvis *et al.,* 1996; Ghosh *et al.,* 2002).

The general approach used to baculovirus expression vector systems present by replacing the polyhedrin protein coding region with the foreign gene of interest (Ghosh *et al.,* 2002). The polyhedrin and p10 promoters are commonly used because they are strong and provide high levels of transcription during the very late phase of infection. The recombinant proteins are processed, modified and targeted to their appropriated cellular locations, where they are functionally similar to their authentic counterparts (Jarvis *et al.,* 1996). Co-transfection of the transfer vector and NPV DNA into insect cells lines such as *Spordorptera frugiperda* (Sf) or *Trichoplusia ni* (High five) cells allows recombination between homologous sites, transferring the heterologous gene from the vector to the NPV DNA. NPV infection of insect cells resulted in the shut-off of host gene expression allowing for a high rate of recombinant mRNA and protein production. Recombinant proteins can be produced at levels ranging between 0.1 % and 50 % of the total insect cell protein (Ghosh *et al.,* 2002).

Advantages of using the Baculovirus Expression Vector System

Several unique features of the BEVS have made it the system of choice for many applications. Often, recombinant proteins expressed in bacterial systems are insoluble, aggregated and incorrectly folded. In contrast, proteins expressed in BEVS are soluble and functionally active (King and Possee, 1992).

Functional activity of the recombinant protein

The BVES typically produces over expressed recombinant proteins containing proper folding, disulfide bond formation and oligomerization. Additionally, this system is capable of performing several post-translational modification. This leads to a protein that is similar to its native counterpart, both structurally and functionally. However, in cases where the authentic protein functions as heterodimer or relies on tissue- or species-specific modifications, the recombinant baculovirus-expressed protein are may not be functionally active, unless its binding partner or modifying enzyme is co-expressed.

Post-translational modification

Several post-translation modifications have been reported to occur in BV, including N-and O-linked glycosylation, phosphorylation, acrylation, amidation, carboxymethylation, isoprenylation, signal peptide cleavage and proteolytic cleavage.

High expression levels

Compare to other higher eukaryotic expression systems, the most distinguishing feature of the BVES is its potential to achieved high levels of expression of a cloned gene. The BV system has proven particularly useful in generation of large quantities of proteins for structural analysis. The highest expression level reported is 50 % of the total cellular protein of an infected insect cell corresponding to approximately 1 g of recombinant protein per $1x10^9$ cells. However recombinant proteins are not produced at such high amounts and it is usually difficult to predict the amount of proteins expression.

Capacity for large inserts

The expandability of capsid structure of baculoviruses allows the packing and expression of very large genes. There is no known upper size limit for the insertion of foreign sequences into the BV genome.

Capacity to express unspliced genes

Insect cells have the capability to perform intron/exon splicing. However, certain virus-, tissue- or species-specific splicing patterns will not be obtained if they require the presence of particular splicing factors which are not available in infected insert cell environment. In general, for high protein expression levels, a cDNA insert rather than a genomic DNA fragment is recommended.

Simultaneous expression of multiple genes

BEVS has the capability to express two or more genes simultaneously within sigle infected insect cells. Protein complexes that depend on dimer or multidimer formation for activity can be assembled. A well known example is the formation of complete virus capsids from a variety of viruses which have been assembled in vitro, using BEVS, by co-expressing the capsid subunits simultaneously.

Localization of recombinant proteins

Baculovirus-expressed recombinant proteins are usually localized in the same sub-cellular compartment as the authentic protein. Nuclear proteins will be transported to the insect nucleus, membrane proteins will be anchored into the cell membrane, and secreted protein will be secreted by infected insect cells.

Disadvantages of using the Baculovirus Expression Vector System

Discontinuous expression

Baculovirus infection of insect cells results in death of the host and hence reinfection fresh cultures of insect cells are necessity for each round of protein synthesis. This may be inefficient for production on a commercial scale, although it is acceptable for research purposes.

Glycosylation

The process of glycosylation in insect cells is demonstrably different from the pathway in vertebrate cells. Insect cells produce glycoprotein with relatively simple, unbranched sugar side-chains with a high mannose content. The most obvious consequence of this processing is that the glycoproteins produced in insect cells have a greater mobility in denaturing polyacrylamide gels; their molecular weights are measurably different.

MATERIALS AND METHODS

1. Isolation of porcine peripheral blood mononuclear cells (PBMC)

Ten milliliter 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 (GIBCO/BRL) supplemented with 10% fetal calf serum (Hyclone), 2mM L-glutamine (GIBCO/BRL), 100 μM non-essential amino acid (GIBCO/BRL), 1mM sodium pyruvate (GIBCO/BRL), 50 μM 2-mercaptoethanol and 100 unit/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml of amphotericin B (antibiotic/antimycotic solution; GIBCO/BRL) and 5 μg/ml Con A (Sigma®) at 37 °C having 5% CO_2 for 48 hours (Collins *et al.*, 1994).

2. 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, 2001). cDNAs synthesis was performed using 1 μl of sample RNA, 10 mM dNTPs (Fermentas®), 2.5 μM 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 entire porcine IL-2 with signal sequence was amplified by PCR using a specific

forward primer containing a *XbaI* restriction enzyme site 5′-CT AGT CTA GAG CCA CCA TGT ATA AGA TGC AGC TCT TGT-3′ and a specific reverse primer containing a *XhoI* restriction enzyme site 5′-GGG CCT CGA GTC AAG TCA GTG TTG AGT AGA TG-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 µl 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 pgmol 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 (Sambrook and Russell, 2001).

3. <u>Construction of expression vector</u>

The PCR generated porcine IL-2 products were ligated to the baculovirus transfer vectors, pFastBac ^{TM}HTb (Invitrogen®) at the *Xba* I restriction site and *Xho* I restriction site. The 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 confirmed by PCR and restriction endonuclease assay. The recombinant plasmid was sequenced by the dideoxy-chain termination method using dye terminator chemistry (ABI PRISM Big

Dye Terminator Cycle Sequencing Ready Reaction Kit) in an automatic sequencer (Applied Biosystems, Inc.). Thus the result was analyzed and compared with reported porcine IL-2 (Accession x58428) using CLUSTAL W program (NCBI). 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) using CLUSTAL W program (NCBI).

The recombinant porcine IL-2 plasmid was used to transform *E. coli* strain DH10Bac™ (Invitrogen®) for producing recombinant baculovirus DNA containing porcine IL-2 gene. Recombinant bacmids are constructed by transposing a mini-Tn7 element from pFastBac ^{TM}HTb donor plasmid to the mini-*att*Tn7 attachment site on the bacmid in DH10Bac™ when the Tn7 transposition functions are provided *in trans* by a helper plasmid. The helper plasmid confers resistance to tetracycline and encode the transposase. Each vector has a baculovirus specific promoter (i.e. the polyhedron or p10 promoter from AcNPV) for expression of protein in insect cells. The mini-Tn7 in a pFastBac ^{TM} donor plasmid contain an expression cassette consisting of a Gm^r gene, a multiple cloning site, and an SV40 poly(A) signal inserted between the left and right arms of Tn7 (Invitrogen, 2002).

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 (Invitrogen, 2002).

4. The transfection of recombinant baculovirus DNA into insect cells

Five microlitre of the recombinant baculovirus DNA was used to transfect Sf21 cells using CellFECTIN® reagent (Invitrogen®). At 72 hour post transfection, the recombinant baculovirus particles were collected from supernatant and viral titer was determined using plaque assay (appendix 2). Subsequently, the high-titer seed stock of recombinant baculovirus was produced by Sf21 insect cells at a multiplicity of infection (MOI) of 0.01 using Sf900 II SFM® medium (Invitrogen®) containing 4% fetal bovine serum (GIBCO/BRL) and 1× antibiotic.

5. Viruses and cells

Sf21 cell line *(Spodoptera frugiperda*; Invitrogen®) was grown at 27°C in SF900II medium (Invitgrogen®) supplemented with 4% FBS and 1× antibiotics (GIBCO/BRL). 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 µg/ml streptomycin, 0.25 µg/ml of amphotericin B (antibiotic/antimycotic solution; GIBCO/BRL) was inoculated with recombinant baculoviruses ,at MOI of 6, for the production of recombinant IL-2 protein and inoculated with wild-type baculoviruses as negative control. After 72 hr post-inoculation, the infected High-Five cells were collected by centrifugation at 200xg for 10 min. The infected High-Five cells were resuspended in lysis buffer under native conditions for further determining the IL-2 production. The *in situ* expression of porcine IL-2 in High-Five cells was determined using the immunoperoxidase

monolayer assays. The dot blot analysis was employed for determining the structure of porcine IL-2. The size of porcine IL-2 product was showed using SDS-PAGE and Western blot analysis.

6. Immunoperoxidase monolayer assay (IPMA)

Both wild-type and recombinant baculovirus infected cells were fixed using 4 % formalin in PBS plus 0.5 % tween 80 on microscopic slide for 10 min and then incubated with 50 µl goat anti-porcine IL-2 IgG polyclonal antibody (1:50;R&D®) at 37 °C for an hour and subsequently incubated with 50 µl rabbit anti-goat IgG conjugated with peroxidase (1:500; Sigma®) at 37 °C for 30 min. Specimens were incubated with 0.006 g/ml Diaminobenzidine solution (Sigma®) at room temperature for 5 min. The presence of porcine IL-2 was determined by the reaction of peroxidase with diaminobenzidine as seen from the intracytoplasmic brownish color as observed under inverted microscope (adapted from Cochet *et al*., 1998).

7. Dot blotting analysis

The 3 µl of crude protein of both wild-type and recombinant baculovirus infectecd cells and the purified recombinant protein fraction was dotted on nitrocellulose membrane (Biorad®) and incubated with 0.5 ml goat anti-porcine IL-2 IgG polyclonal antibody (1:200) for one hour. Subsequently, the membrane was incubated with 0.5 ml rabbit anti-goat IgG conjugated with peroxidase (1:1,000; Sigma®) for 30 min. The membrane was finally incubated with 0.5 ml of 0.006 g/ml

diaminobenzidine solution (Sigma®) containing 1% H_2O_2 for 5-10 min. The positive reaction was shown as the brown dot (adapted from Cochet *et al*., 1998).

8. SDS-PAGE and Western blotting

The 10 µl of crude protein of both wild-type and recombinant baculovirus infected cells was analyzed using 12 % SDS-PAGE stained with Coomassie brilliant blue.

For Western blot analysis, proteins on SDS-PAGE were transfered onto nitrocellulose membrane using 400 mAmp at 4°C for 5 h and incubated with 5 ml goat anti-IL-2 IgG polyclonal antibody (1:200) for an hour. The membrane was then incubated with 5 ml rabbit anti-goat IgG (1:1,000) for 30 min. Finally it was incubated with 5 ml of 0.006 g/ml diaminobenzidine solution (Sigma®) containing 1% H_2O_2 for 5-10 min (adapted from Cochet *et al*., 1998).

9. Purification of the recombinant protein using Ni-NTA

Ni-NTA Agarose was used for purification of recombinant IL-2 expressed in insect cells by the 6xHis-tagged vector. The resin exhibits high affinity and selectivity for 6xHis-tagged recombinant fusion proteins. Proteins bound to the resin were eluted by competition with imidazole. In brief, After harvesting insect cells, the cell pellet was resuspended in Native Lysis Buffer (appendix 1) containing lysozyme at a concentration of 1 mg/ml. The remaining lysate was incubated on ice for 1-2 hr.

The lysate was centrifuged at 3000x for 15 min. The supernatant was collected and transferred to a 1.5 ml tube and the resin was added. The tube was shaken gently using a rotary shaker at 200 rpm for 2 hours at 4 °C. Subsequently the tube was centrifuged for 10 s at 1000 x g to pellet the resin and the supernatant was transfered to a fresh tube. The resin was washed 4 times with wash buffer (appendix 1), then the protein was eluted with elution buffer (appendix 1) (Qiagen, 2001).

10. Quantitation of recombinant protein

Quantification of recombinant proteins was done using the Modified Lowry Protein assay Kit (Pierce®). A set of protein standards (bovine serum albumin, BSA) was prepared using the elution buffer of recombinant protein as diluents. In brief, 40 µl of each standard and recombinant protein replicate were pipette into a microplate well and 200 µl of Modified Lowry Reagent were added to each well at nearly the same moment using a multi-channel pipettor. The solutions in microplate was immediately mixed on a plate mixer for 30 second. The microplate was then covered and incubated at room temperature for exactly 10 minutes. Subsequently, the fresh prepared 1x Folin-Ciocalteu Reagent 20 µl were added to each well using a multi-channel pipettor and microplate was immediately mixed on plate mixer for 30 seconds. Finally the protein standard and recombinant protein were measure the absorbance at 750 nm on a plate reader (Biorad®). Recombinant protein concentration was determined and reported with reference to standard of BSA, base on the standard curve (Pierce, 2005).

11. T lymphocyte proliferation assay

The biological activity of recombinant porcine IL-2 was determined using the T-lymphocyte proliferation assay (adapted from Iwata *et al*., 1993). The assay was conducted on the T4 cells, human T cell line (kindly provided by Department of Medical Sciences, Ministry of Public Health, Thailand) in 96-well microplate. The proliferation was revealed by the use of Cell Proliferation Reagent WST-1 (Roche) (Iwata *et al*., 1993), which was a colorimetric assay for the quantification of cell proliferation and cell viability, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Quantification of formazan dye produced by metabotically active cells was measured by a scanning multiwell spectrophotometer (ELISA reader). T4 cells were seeded at a concentration of $1x10^4$ cells/well in 100 µl RPMI 1640 plus 5 µg/ml Con A. Each well contained various amounts of IL-2 protein (final concentration between 0.2-1.6 ng/ml) of two sources; one from recombinant baculovirus transfected High-Five cells and the other from the commercial porcine IL-2 (R&D®). Three experiments were conducted and each had three replication. Each IL-2 concentration was repeated three times. After incubated cells for 24 h at 37°C and 5% CO_2, Cell Proliferation Reagent WST-1 10 µl/well were added and incubated for 4 h at 37°C and 5% CO_2. The samples were finally measured the optical density (OD) of each well against a background control as blank using a microplate reader (Biorad®) at the absorbance of 450 nm according to manufacture's protocol.

12. Statistic analysis

Statistical analysis was performed using the computer software NCSS 2000 (East Kaysville, Utah, USA). In the T-lymphocyte proliferation assay, percentage of proliferation was calculated using a following equation (adapted from Bounous *et al*., 1992).

$$\% \text{ proliferation} = \frac{\text{mean OD of IL-2 stimulated cell-mean OD of IL-2 unstimulated cell}}{\text{mean OD of wild-type infected cell's purified protein stimulated cell}} \times 100$$

The mean percentage of proliferation from insect cell-produced recombinant porcine IL-2 stimulated cells and the mean from bacteria -produced recombinant porcine IL-2 (R&D®) stimulated cells were compared using the ONE-WAY ANOVA analysis. The difference was indicated as significant as p value ≤ 0.05. To determine the doses of recombinant protein that gave 50 % of maximal proliferation rate, regression of percentage of proliferation of insect cell-produced recombinant porcine IL-2 stimulated cells was performed using analysis of variance for linear regression response. The correlation coefficient (r) and the coefficient of determination (r^2) were also calculated.

Results

1. Cloning of the gene encoding of porcine interleukin-2 protein

Porcine cDNA was prepared by RT-PCR method from mRNA isolated from Con-A stimulated porcine PBMC. The whole porcine IL-2 gene was cloned using the specific primers designed from the sequence data as reported in GenBank. The PCR product of porcine IL-2 mRNA was approximately 491 bp in size (Fig.1), including 6 bp *XbaI* restriction site, 6 bp Kosak sequence and 4 bp overhang at 5' end and 6 bp *XhoI* restriction site, 4 bp overhang at 3' end.

The PCR generated porcine IL-2 products were ligated to the baculovirus transfer vector, pFastBac™HTb at the *XbaI* restriction site and *XhoI* restriction site. The derived recombinant plasmid were used to transform to competent cell of DH5α™. Afterwards, the transformed competent cells were plated on LB agar plates containing 100 μg/ml ampicillin and 7 μg/ml gentamicin as the selective media. Numerous colonies of DH5α™ were recovered on the selective media. The transformant was primarily checked for the possession of the recombinant plasmid by using PCR assay. The result showed that the clone has possessed the gene encoding porcine IL-2 protein in recombinant pFastBac™HTb plasmid by giving the amplified products of 491 bp in length. Subsequently, the restriction endonuclease assay was conducted to confirm the correctness of the gene encoding for IL-2 protein by the using of *DraI* restriction endonuclease, as analyzed by DNASIS which *DraI* cut the fragments of IL-2 cDNA at position 217 bp. The result of the restriction

endonuclease assay showed 217 bp and 274 bp fragments (Fig. 2). The recombinant plasmids were isolated and used to do restriction endonuclease assay using *XbaI* and *XhoI*. As expected, the restriction endonuclease assay showed two fragments of 491 bp and 4778 bp.

The DNA sequencing of recombinant IL-2 gene was done by the automatic DNA sequencer (ABI PRISM™) using dideoxynucleotide termination method. The PCR product was analyzed by CLUSTAL W program and compared with the reported porcine IL-2 (Accession X58428). The results showed nucleotide sequences of recombinant plasmid have 100 % homology with the porcine IL-2 sequence (Fig. 3).

The nucleotide sequences of recombinant plasmid were then translated to amino acid sequence by the DNASIS program when was compared with the reported porcine IL-2 protein (Accession X58428) by CLUSTAL W program. Comparison of amino acid sequences of the recombinant IL-2 with the reported porcine IL-2 reference sequence showed 100 % homology (Fig. 4).

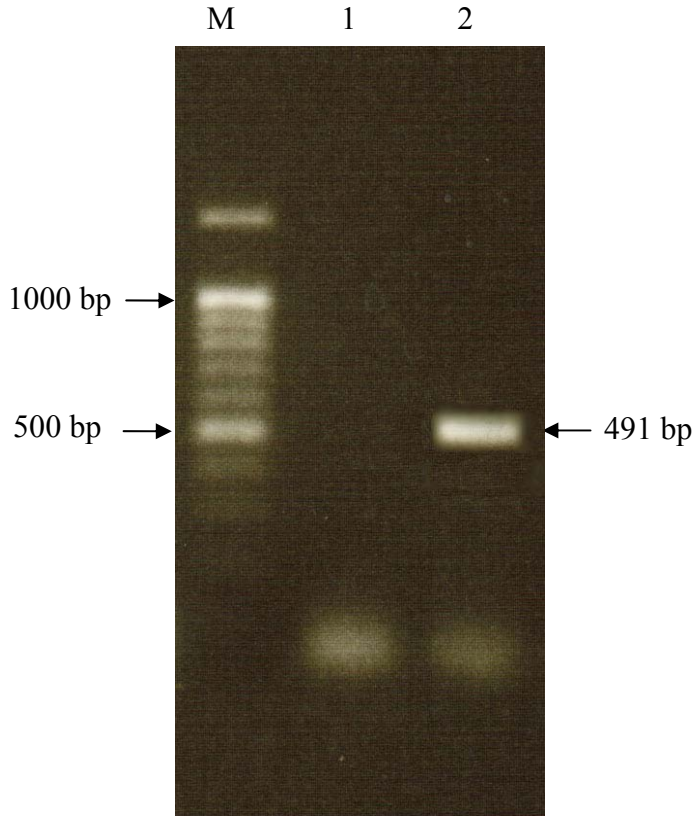

Figure 1 PCR product of porcine IL-2 using 1.5 % agarose gel electrophoresis. M=100 bp DNA marker; Lane 1 = negative control; Lane 2 = IL-2 cDNA showing a distinct band at 491 bp.

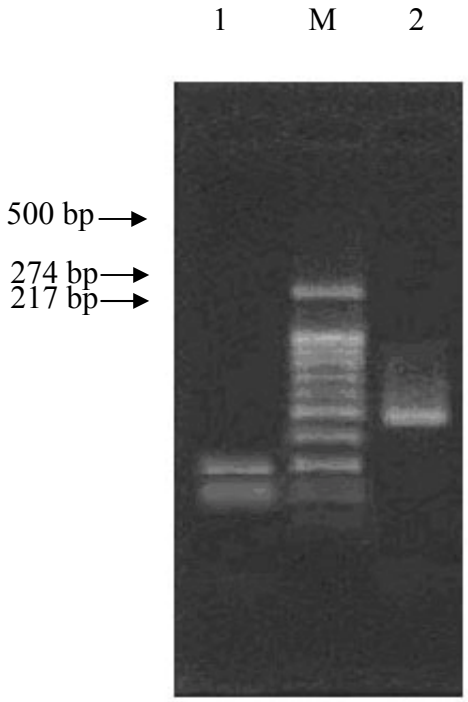

Figure 2 Restriction endonuclease analysis of PCR product of recombinant porcine IL-2. M=100 bp DNA marker; Lane 1 = the PCR products after cut by *DraI*, separated to DNA fragments of approximately 217 and 274 bp; Lane 2 = the PCR product of recombinant IL-2 gene, approximately 491 bp.

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CLUSTAL W (1.82) multiple sequence alignment

rIL-2           ATGTATAAGATGCAGCTCTTGTGTTGCATTGCACTAACCCTTGCACTCATGGCAAACGGT 60
x58428          ATGTATAAGATGCAGCTCTTGTGTTGCATTGCACTAACCCTTGCACTCATGGCAAACGGT 60
                ************************************************************

rIL-2           GCACCTACTTCAAGCTCTACAAAGAACACAAAGAAACAACTGGAGCCATTGCTGCTGGAT 120
x58428          GCACCTACTTCAAGCTCTACAAAGAACACAAAGAAACAACTGGAGCCATTGCTGCTGGAT 120
                ************************************************************

rIL-2           TTACAGTTGCTTTTGAAGGAAGTTAAGAATTACGAGAATGCTGATCTCTCCAGGATGCTC 180
x58428          TTACAGTTGCTTTTGAAGGAAGTTAAGAATTACGAGAATGCTGATCTCTCCAGGATGCTC 180
                ************************************************************

rIL-2           ACATTTAAATTTTACATGCCCAAGCAGGCTACAGAATTGAAACACCTTCAGTGTTTAGTA 240
x58428          ACATTTAAATTTTACATGCCCAAGCAGGCTACAGAATTGAAACACCTTCAGTGTTTAGTA 240
                ************************************************************

rIL-2           GAAGAACTCAAAGCTCTGGAGGGAGTGCTAAATTTAGGTCAAAGCAAAAACTCTGACTCA 300
x58428          GAAGAACTCAAAGCTCTGGAGGGAGTGCTAAATTTAGGTCAAAGCAAAAACTCTGACTCA 300
                ************************************************************

rIL-2           GCAAATATCAAGGAATCAATGAACAATATCAACGTAACAGTTTTGGAACTAAAGGGATCT 360
x58428          GCAAATATCAAGGAATCAATGAACAATATCAACGTAACAGTTTTGGAACTAAAGGGATCT 360
                ************************************************************

rIL-2           GAAACAAGTTTCAAATGTGAATATGATGATGAGACAGTAACTGCTGTTGAATTTCTGAAC 420
x58428          GAAACAAGTTTCAAATGTGAATATGATGATGAGACAGTAACTGCTGTTGAATTTCTGAAC 420
                ************************************************************

rIL-2           AAATGGATTACCTTTTGTCAAAGCATCTACTCAACACTGACTTGA 465
x58428          AAATGGATTACCTTTTGTCAAAGCATCTACTCAACACTGACTTGA 465
                *********************************************
```

Figure 3 Nucleotide sequences comparison between cloned porcine rIL-2 and reported porcine IL-2 (Accession X58428) using CLUSTAL W program. The results showed 100 % homology between the cloned porcine rIL-2 and the reported porcine IL-2.

```
CLUSTAL W (1.82) multiple sequence alignment


rIL-2           MYKMQLLCCIALTLALMANGAPTSSSTKNTKKQLEPLLLDLQLLLKEVKNYENADLSRML 60
x58428          MYKMQLLCCIALTLALMANGAPTSSSTKNTKKQLEPLLLDLQLLLKEVKNYENADLSRML 60
                ************************************************************

rIL-2           TFKFYMPKQATELKHLQCLVEELKALEGVLNLGQSKNSDSANIKESMNNINVTVLELKGS 120
x58428          TFKFYMPKQATELKHLQCLVEELKALEGVLNLGQSKNSDSANIKESMNNINVTVLELKGS 120
                ************************************************************

rIL-2           ETSFKCEYDDETVTAVEFLNKWITFCQSIYSTLT 154
x58428          ETSFKCEYDDETVTAVEFLNKWITFCQSIYSTLT 154
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Figure 4 Amino acid sequences comparison between cloned porcine rIL-2 and reported porcine IL-2 (Accession X58428) using the CLUSTAL W program. The results showed 100 % homology between the cloned porcine rIL-2 and the reported porcine IL-2.

2. Purification and determination of the recombinant IL-2 protein

The recombinant porcine IL-2 plasmid was used to transform *E. coli* strain DH10Bac™ for producing recombinant baculovirus DNA containing porcine IL-2. The recombinant porcine IL-2 plasmid was analysed using the PCR to verify the success of recombinant baculovirus production. The recombinant baculovirus DNA was used to transfect into the Sf21 cells using CellFECTIN® reagent. At 72 hour post transfection, the recombinant baculovirus particles were collected from the supernatant. Determination of infectious potency of the porcine IL-2 gene contained baculovirus was accomplished by plaque formation in immobilized monolayer culture. The result showed the viral stock titers were $1x10^6$ pfu/ml.

The High-Five cell line was inoculated with either recombinant baculovirus or wild-type baculovirus as the control, at MOI of 6, for the production of recombinant IL-2 protein. After 72 hr post-inoculation, the infected High-Five cells were collected for further determination of presence of recombinant porcine IL-2 proteins using immunoperoxidase monolayer assays (IPMA), dot blot analysis, SDS-PAGE, and Western blot analysis.

During IPMA, both wild-type and recombinant baculovirus infected High-Five cells were fixed on microscopic slide and incubated with goat anti-porcine IL-2 IgG polyclonal antibody and subsequently incubated with rabbit anti-goat IgG conjugated with peroxidase. Finally, the specimens were incubated with Diaminobenzidine solution (Sigma®). The presence of recombinant porcine IL-2 was

determined by the reaction of peroxidase with diaminobenzidine which showed the brownish red intracytoplasmic staining as observed under an inverted microscope (Fig 5a). The High-Five cells transfected with recombinant baculovirus show the positive reaction, however the wild-type infected cells were negative (Fig 5b). This result indicated the presence of the porcine IL-2 in the High-Five infected cell.

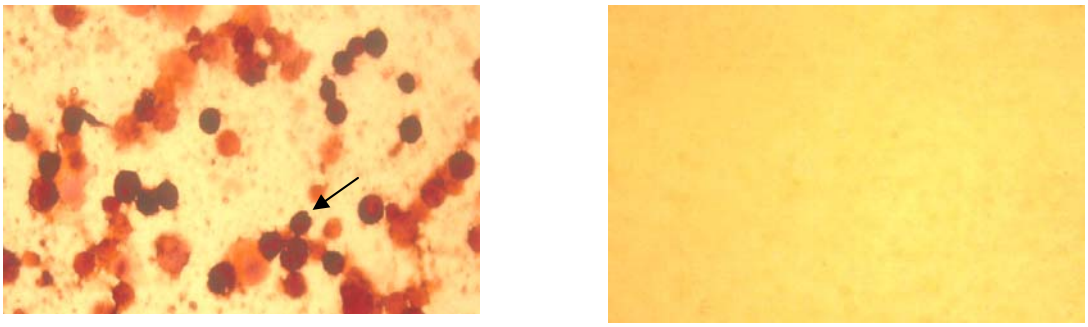
The recombinant protein was determined for its solubility. In brief, the cell pellet was resuspended in native lysis buffer including lysozyme (1mg/ml) and cells were incubated on ice for 1 hr. The lysate was centrifuged then the supernatant was harvested and used to determine the recombinant protein by dot blot analysis. The dot blot analysis of crude protein from the recombinant baculovirus infected cells using goat anti-porcine IL-2 polyclonal antibody showed the positive results. But the crude protein from wild-type baculovirus infected cells was negative (Fig.6).

The recombinant protein was purified under native condition using Ni-NTA Agarose from the 6xHis-tagged vector. The resin exhibits high affinity and selectivity for 6xHis-tagged recombinant fusion proteins. During the purification step, the non-specific proteins were washed out and the recombinant protein was left out as purified protein fraction. Each protein fraction from the purification process has been performed by dot blot analysis (Fig.7) to determine IL-2 in each fraction. The result showed positive reaction in 8 fractions. This result indicated the presence of recombinant porcineIL-2 in the 8 elution fractions.

Quantification of recombinant proteins in transfected cells was done using the Modified Lowry Protein assay Kit (Pierce®). Recombinant protein concentration was determined and compared with reference to the standard concentration of bovine serum albumin. The result showed cells at a density of $1x10^6$ cells/ml infected with viral stock at titer of 10^6 pfu/ml can produce more than 0.1 μg recombinant protein.

Size of the crude protein of both wild-type and recombinant baculovirus infected cells was analyzed using electrophoresis technique in 12 % SDS-PAGE stained with Coomassie brilliant blue. The crude protein from the recombinant baculovirus infected cells was revealed as a distinct band of relative molecular mass of 23 kDa which included 6xhis and a few amino acids from the vector when compared to wild-type baculovirus infected cells (Fig.8).

For Western blot analysis, proteins on SDS-PAGE were transferred onto nitrocellulose membrane. Subsequently, the nitrocellulose membrane was treated with anti-porcine IL-2 polyclonal antibody. The Western blotting analysis of total lysate showed the immunological reaction between the anti-porcine IL-2 polyclonal antibody and recombinant protein at 23 kDa whereas the protein from wild-type infected High-five cell lysate was negative (Fig.9).



a b

Figure 5 IPMA results of recombinant baculovirus infected in the High-Five insect cells (a) and wild-type infected High-Five cells (b). The positive cytoplasmic staining with red-brown color indicated the presence of IL-2 in the cells (arrow).

a b

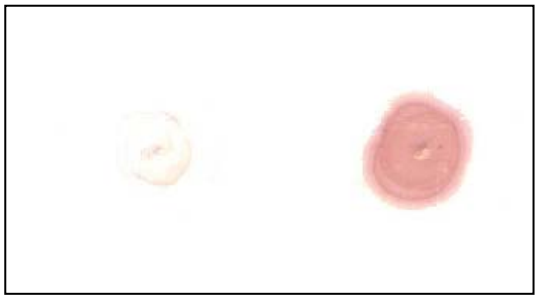


Figure 6 Dot blotting analysis of total cell lysate from wild-type infected (a) and recombinant baculovirus infected High-Five cells (b) reacting with anti-porcine IL-2 polyclonal antibody. The cell lysate from recombinant baculovirus infected cells was positive. The cell lysate from wild-type infected cells was negative.

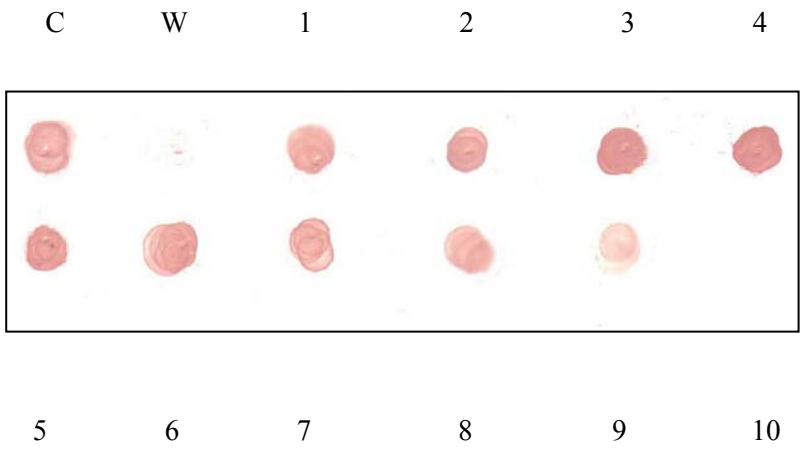


Figure 7 The dot blot analysis showed the presence of IL-2 protein in each fraction of the purified protein along the purification step of porcine IL-2 protein. C=crude protein in native buffer; W=Wash fraction after the protein bind to Ni-NTA; 1-10=elute fractions from elution buffer. The positive staining of red-brown color indicated the presence of IL-2 in each fraction.

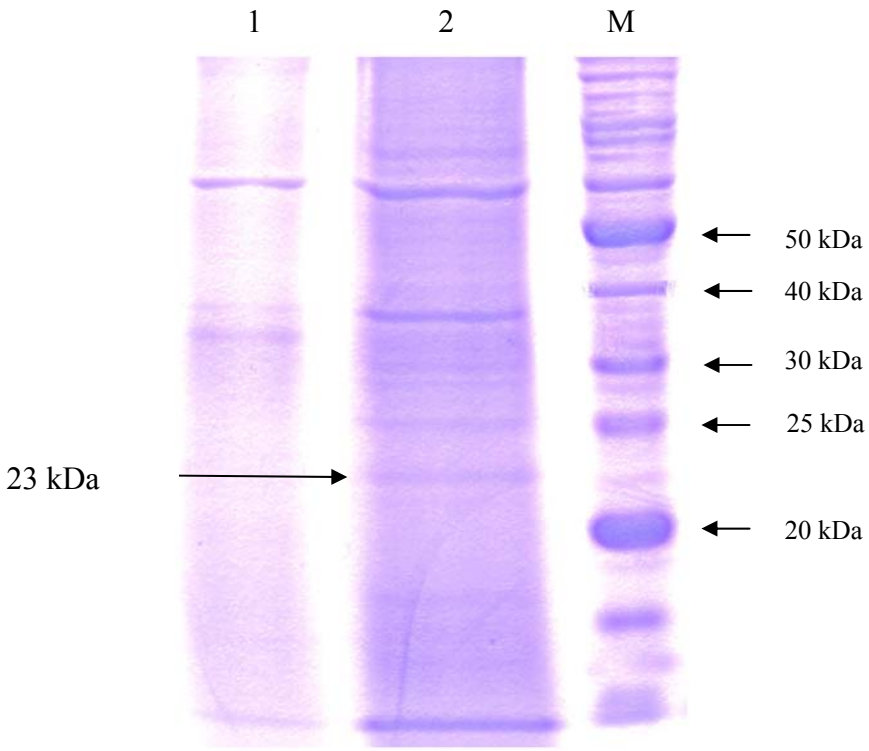


Figure 8 SDS-PAGE analysis of crude protein from wild-type baculovirus infected High-Five cells (1) and from recombinant baculovirus infected High-Five cells (2) and protein marker (M). Crude protein from recombinant baculovirus infected High-Five cells gave a distinctive band approximately 23 kDa in size.

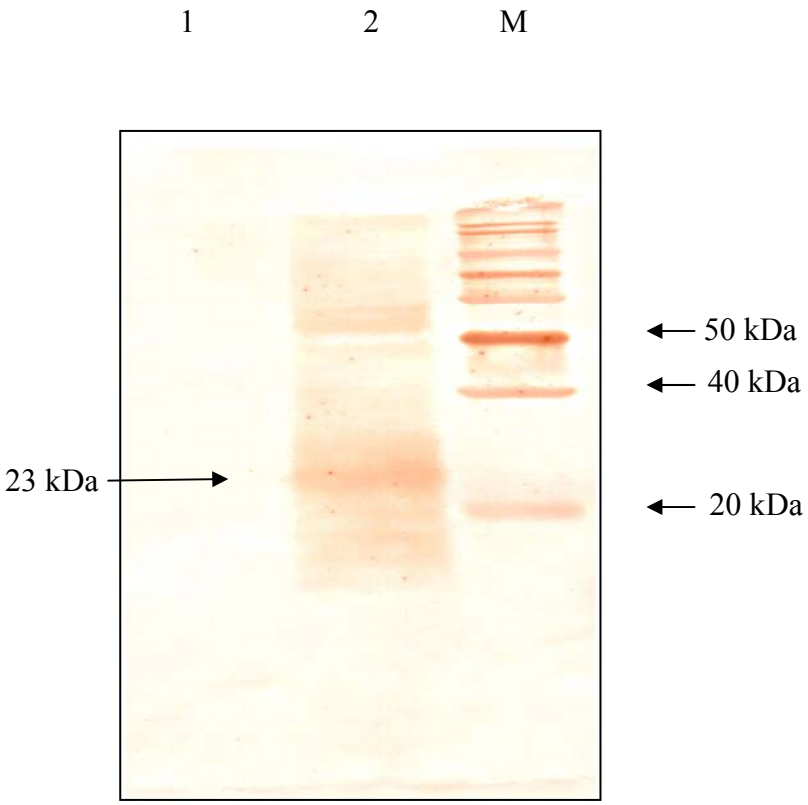


Figure 9 Western blotting analysis of total lysate total cell lysate from wild-type infected (1) and recombinant baculovirus infected High-Five cells (2) reacting with anti-porcine IL-2 polyclonal antibody. The cell lysate from recombinant baculovirus infected cells gave a positive band approximately 23 kDa in size. The cell lysate from wild-type infected cells was negative.

3. Biological activity of recombinant porcine IL-2

Biological activity of IL-2 was analyzed with proliferation of the human T cell line (T4 cell). The *in vitro* proliferation assays have become available for analyzing the number of viable cell by the cleavage of the tetrazolium salt WST-1 added in the culture medium by mitochondrial dehydrogenases in viable cells. An expansion in the number of viable cell results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity lead to an increase in the amount of formazan dye formed, which directly correlates to the numbers of metabolically active cells in the culture.

T4 cells were seeded at a concentration of $1x10^4$ cells/well. Each well was added with various amounts of IL-2. After incubated cells for 24 h, Cell Proliferation Reagent WST-1 were added and finally measured the absorbance using a microplate (ELISA) reader at 450 nm.

The T4 cell line showed an increased proliferation response after the stimulation with recombinant porcine IL-2 in dose-dependent manner. The similar result was found in those treated with commercial porcine IL-2 (table 1). The recombinant porcine IL-2 induced significantly higher percentage of proliferation when compared to those stimulated with commercial porcine IL-2 (R&D®) ($p<0.05$) (Table1). The linear regression showed that the doses of recombinant porcine IL-2 producing from insect cells between 0.2 ng/ml to 1.6 ng/ml would produce percentages of proliferation range from 75.35 to 188.72 while the commercial porcine

IL-2 would produce percentages of proliferation range from 7.49 to 109.86 (Table1, Fig. 10).

To determine the doses of recombinant protein that gave 50 % proliferation of insect cell-produced recombinant porcine IL-2 stimulated cells the correlation coefficient (r) and the coefficient of determination (r^2) between doses of IL-2 and percentage of proliferation were also calculated. The linear regression showed that the doses of recombinant porcine IL-2 producing from insect cells between 0.05 pg/ml to 10 pg/ml would produce percentages of proliferation range from 42.54 to 53.91 and the dose that gave 50 % proliferation was 6.96 pg/ml (table 3, Fig. 11).

Table 1 Comparison [A] of the T4 cell proliferation (%) of insect cell-produced recombinant porcine IL-2 and the commercial porcine IL-2 at various concentrations (ng/ml).

Concentration (ng/ml)	Proliferation (%)[B]	
	Insect cell-produced pIL-2	Commercial pIL-2
0.2	75.35[a]	7.49[b]
0.4	89.38 [a]	10.82 [b]
0.6	107.82 [a]	27.42 [b]
0.8	133.13 [a]	52.16 [b]
1.0	148.35 [a]	63.77 [b]
1.2	166.26 [a]	74.33 [b]
1.4	178.96 [a]	94.34 [b]
1.6	188.72 [a]	109.86 [b]

[A] Result from insect cell-produced pIL-2 and bacteria-produced pIL-2 were significantly different. The difference of OD ratio of both pIL-2 was analyzed using the ONE-WAY ANOVA analysis.

[B] % proliferation was calculated by following equation;

$$\frac{\text{(mean OD of IL-2 stimulated cell-mean OD of IL-2 unstimulated cell)}}{\text{OD wild-type infected's purified protein stimulated cells}} \times 100$$

Table 2 The difference of OD ratio between insect cell-produced recombinant porcine IL-2 and the commercial porcine IL-2 was analyzed using the ONE-WAY ANOVA analysis (NCSS 2000 program; East Kaysville, Utah, USA).

Concentration (ng/ml)	F(df)	*P*
0.2	2456.48 (1,16)	0.000000
0.4	528.90(1,16)	0.000000
0.6	266.64(1,16)	0.000000
0.8	577.04(1,16)	0.000000
1.0	520.62(1,16)	0.000000
1.2	710.94(1,16)	0.000000
1.4	778.10(1,16)	0.000000
1.8	1085.00(1,16)	0.000000

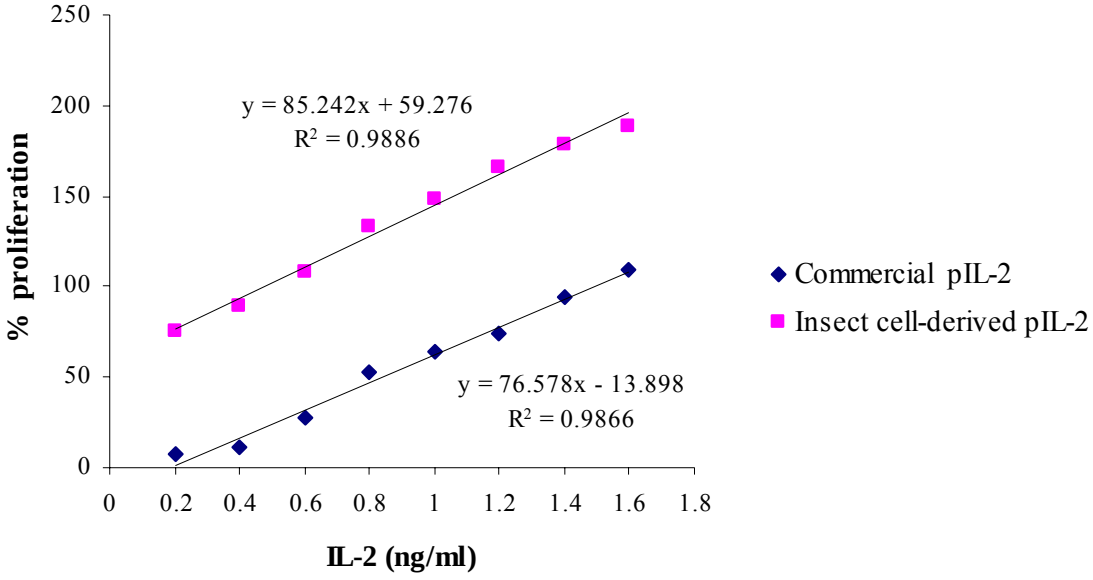


Figure 10 Comparison of T4 human cell response to porcine IL-2 (pIL-2).

T4 human cells proliferated after stimulation with pIL-2P purified from insect cell (▫) and with commercial porcine IL-2 (as control) (◆).

Table 3 Analytical data of the T4 cell proliferation (%) of recombinant porcine IL-2 produced from insect cells (pg/ml).

Concentration (pg/ml)	Proliferation (%)
0.05	42.54
0.1	42.82
0.5	42.85
1.0	43.38
5.0	46.72
10	53.91

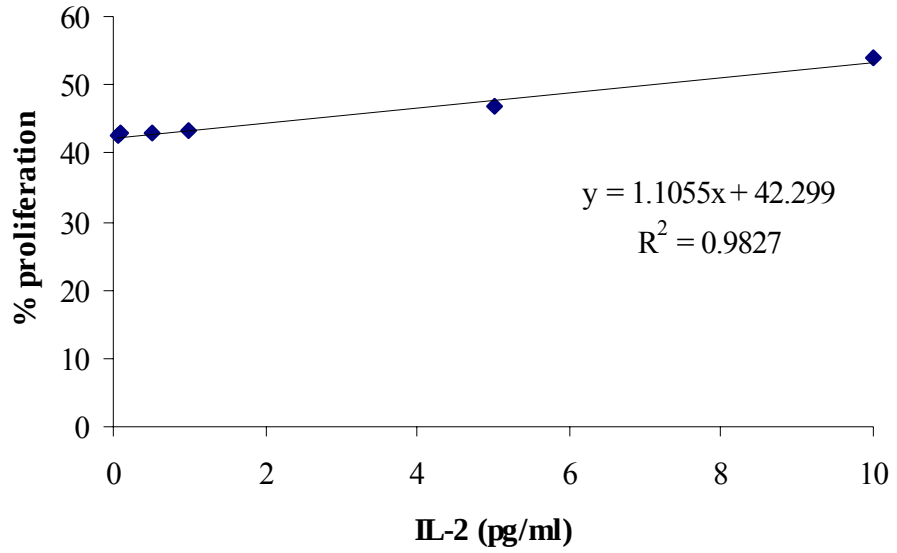


Figure 11 Activity of porcine IL-2 on T4 human cells. Responses are expressed as a percentage of maximum seen for T4 human cells.

DISCUSSION

Cloning of porcine IL-2 gene

In this study, the recombinant porcine IL-2 gene was successfully cloned and expressed in High-Five insect cells. The restriction endonuclease assay was conducted to confirm the correctness of the cloned IL-2 gene and the result showed 217 bp and 274 bp fragments as analyze by DNASIS program. Afterwards, the recombinant gene was sequenced and compared with reported porcine IL-2. The results showed nucleotide sequences of recombinant plasmid have 100 % homology with the porcine IL-2 sequence reported in the GenBank. This result confirmed that the cloned recombinant porcine IL-2 gene have correct sequence.

The reported porcine IL-2 gene is approximately 465 bp in length (Davoli *et al*., 2002). The recombinant porcine IL-2 gene produced in this study contains 491 bp. The extra 26 bp sequenced cloned IL-2 gene included 6 bp *XbaI* restriction site, 6 bp Kosak sequence for efficient translation initiation and 4 bp overhang at 5' end, 6 bp *XhoI* restriction site and 4 bp overhang at 3' end. The Kosak sequence, a short sequence of nucleotides found at the beginning of most mRNA transcripts plays a role in enhancing binding of ribosomal subunits to the mRNA from a gene increasing the rate of protein translation (Kosak, 1987). Moreira and Noren (1995) studied the efficiency of digestion of polylinker regions with a variety of enzymes and found that, the addition of 2-6 extra bases upstream of an engineered restriction site in a PCR

primer are to increase the efficiency of digestion of the amplification product, that the restriction site close to the end of 3' end.

Comparison of amino acid sequences of the recombinant porcine IL-2 with the reported porcine IL-2 sequence in GenBank showed 100 % homology. This result confirmed that the recombinant porcine IL-2 gene produced in insect cells can be translated to porcine IL-2 protein. The recombinant IL-2 protein produced in this system has a correct amino acid sequence. Recently, complementary DNA of IL-2 of several species have been cloned such as human, bovine, ovine and chicken (Taniguchi *et al*., 1983; Kashima *et al*., 1985; Ceretti *et al*., 1986; Goodall *et al*., 1990; Goodall *et al*., 1991; Collins *et al*., 1994; Seow *et al*., 1997; Iwata *et al*., 2000; Choi and Lillehoj, 2000; Denis and Huber, 2003; Cao *et al*, 2005). The full-length human IL-2 encodes 153 amino acid residues including a signal peptide consisting of 20 amino acid residues while chicken IL-2 encodes 143 amino acid residues including a signal peptide consisting of 22 amino acid residues. The mature human IL-2 occur as a group of glycoproteins with apparent MW between 13 and 17.5 kDa as difined by SDS-PAGE (Robb and Smith, 1981). The purified chicken IL-2 was revealed to protein band corresponding the MW of 16 and 14 kDa on SDS-PAGE (Cao *et al*., 2005). The baculovirus-expressed recombinant bovine IL-2 had an observed MW 15kDa compared with the predicted MW of 15.5 kDa (Collin *et al*., 1994). Porcine IL-2 is synthesized as a precursor protein of 154 amino acids with the first 20 amino terminal amino acids functioning as a hydrophobic secretory signal sequence (Choi and Yoo, 2002).

The recombinant IL-2 expression system

IL-2 is naturally secreted in low level by activated T lymphocytes (Walsh and Headon, 1994). Although the culture of other type of cell lines, such as tumor and recombinant cells, can result in higher IL-2 concentrations but it seem not enough for application. Both prokaryotic and eukaryotic host/vector systems are available for the production of a large quantities of proteins from cloned target genes. For many applications such as vaccine production and IL-2 production as an adjuvant, it may be desirable or even necessary that a recombinant gene product must be identical to the natural protein made in *in vivo*. Therefore, not only must a cloned gene be transcribed faithfully and the massage translated, but the product must be properly modified, through such processes as glycosylation, cleavage of a signal peptide, disulfide-bridge formation, and proper folding (Smith *et a*l., 1985).

The bacterial expression systems have difficulties in producing biologically active molecules. Usually this is due to incorrect folding of the protein during processing within the prokaryotic cells (Collins *et al*., 1994). The amino acid sequence of human IL-2 made in *E. coli* is not identical to natural IL-2 because of the addition of a bacterial formylmethionine at the N terminus (Rosenberg *et al*., 1984). In bacterial vectors, it is usually necessary to delete the coding region for signal peptide to express the mature form of eukaryotic genes for protein, like IL-2, with cleavable signal sequence (Smith *et al*., 1985). To avoid such problems for the expression of porcine IL-2 a eukaryotic expression system was sought. A number of eukaryotic system are available, however the insect cell system was chosen as

recombinant proteins synthesized in this system are report to be expressed at high concentrations and usually retain the biological properties of the natural protein (Lucklow and Summers, 1988).

In this research describe the production of recombinant porcine IL-2 in insect cells, using AcNPV as an expression vector. For transfection and amplification of recombinant virus, Sf 21 cell line were use. Sf 21 cell line are the traditional cell lines used with baculovirus and originated from the IPLBF-21 cell line, derived from the pupal ovarian tissue of the fall army worm, *Spordoptera frugiperda* (O'Reilly *et al*, 1992; Vaughn *et al*., 1977). Sf 21 cell line are regular in size, easy to manipulate, and form good monolayers for plaque assays. While, High-Five cell line were used for expression of recombinant protein. The High-Five cell line was originated from the ovarian cell. High-Five cell line can be grown in serum-free medium, adaptable to suspension culture, and produce high level of recombinant protein (Davis *et al*., 1992). The recombinant IL-2 in several species has been expressed at high levels of IL-2 activity in Sf 21 cell line such as human, bovine and porcine (Smith *et al*., 1985; Collin *et al*., 1994). Recombinant human IL-2 was expressed at high levels of IL-2 activity in *S. frugiperda* (IPLB-Sf21-AE) cell line at a cell density of 1×10^{7} cells per ml, which had IL-2 activity approximately 1×10^{-5} units/ml (Smith *et al*., 1985). The Sf 9 cell line were also use to expressed recombinant porcine IL-2 and recombinant chicken IL-2 (Iwata *et al*., 2000; Cao *et al.*, 2004).

Expression of porcine IL-2 protein in insect cells.

In this experiment the result showed the presence of porcine IL-2 protein in the nine purified recombinant protein fractions, but not in wash fraction. Size of the recombinant protein was analyzed using SDS-PAGE. The crude protein from recombinant baculovirus infected insect cells was revealed that the size of recombinant protein was around 23 kDa as shown in the Western blot analysis. This result indicated the recombinant porcine IL-2 have 23 kDa in size and confirmed that the recombinant porcine IL-2 produced in this experiment possesses correct structure.

The molecular weight of native IL-2 molecules were reported to range from 13 to 32 kDa in relation to stimulatory conditions, cell types and methods of molecular weight estimation (English *et al*., 1985; Gillis *et al*., 1978). Natural IL-2 occurs as a group of glycoproteins with apparent M_r values between 13 and 17.5 kDa (as defined by SDS-PAGE) (Robb and smith, 1991). The variation of molecular weight of IL-2 might be attributed to glycosylation of polypeptides and/or aggregation of IL-2 molecules (Gillis *et al*., 1980; Denis and Huber, 2003). The cysteine at position 125 can form intramolecular disulfide bridge, leading to the production of dimers and higher aggregates. Molecules folded with an internal disulfide bridge involving the cystein at position 125 (i.e. between residues 58-125 and 105-125) can be present or even predominate in recombinant DNA Jurkat cells-derived products (Thrope, 1998). The functional significance of glycosylation of IL-2 is not known, but it is likely that it enhances solubility in aqueous environments (Gaffen and Liu, 2004). Compared with the prokaryotic expression system, baculovirus and insect cell expression system

is convenient and versatile eukaryotic system. Furthermore, it can provide correct folding of recombinant protein and other important post translational modifications and form glycosylation similar to that of mammalian cells (Coa *et al*., 2005), while IL-2 produce from *E. coli* is not glycosylated, has no N- terminal alanine, and cysteine at position 125 is substituted with serine. The size of recombinant porcine IL-2 express in *E. coli* was about 15 kDa (Choi and Yoo, 2002; Collins *et al*., 1994; Iwata *et al*., 2000). The recombinant porcine IL-2 produced in High-Five cells contained molecular weight which includes 5 kDa of 39 amino acid and 6x His from vector. Our result was parallel to the report by Iwata and coolegues (2000) that recombinant IL-2 produced in insect cells has MW of 15 and 17 kDa.

Recombinant porcine IL-2 protein concentration was determined. The result showed the infected High-Five cells at a initial density of $1x10^6$ cells/ml can produce more than 0.1 μg recombinant protein. The doses of recombinant human IL-2 range from 1μg/kg to1 mg/kg, with the lower doses being administered over 3-5 day following antigen (Giedlin, 2000). Kawashima and Platt (1989) described the use of human IL-2 as a Pseudorabies vaccine adjuvant in swine, with the IL-2 dose of $6x10^5$ IU/kg, can enhanced serum antibody.

Biological activity of IL-2

IL-2 plays an important role in the initiation of antigen-specific immune responses, as well as their maintenance after the responses are established (Wong *et al.,* 2002). IL-2 is acts on all subpopulation of T cells to promoting the proliferation,

survival and apoptosis of T cells (Lenardo, 1991; Refaeli *et al*., 1998). IL-2 promotes progressing through the G1 phase of cell cycle, resulting in growth of cells and increase in cell numbers. IL-2 also stimulates cytotoxic activity of subsets of T lymphocytes, enhances T cell motility, and induces secretion of other cytokines such as IFN-γ, IL-4 and TNF (Thrope, 1998). Cytotoxic lymphocytes (CTLs) response was greatly enhance in the present of IL-2 (Chan *et al*., 2001). In addition to its effects on T cells, IL-2 is also acts as an activating factor for natural killer (NK) cell (Yu *et al*., 2000), lymphokine-activated killer (LAK) cells, monocytes, macrophages and immunoglobulin (Ig) synthesis by B cell (Gold and DeFranco, 1994).

The biological activity of insect cell-produce recombinant porcine IL-2 was determine using a cell proliferation assay using human T cell line (T4 cell). Recombinant porcine IL-2 has been shown to be able to activate the proliferation of human T lymphocyte (Collins *et al*., 1994). It is probably due to the homology of porcine and human IL-2 which is approximately 72 % (Goodall, 1991; Davoli *et al*., 2002). The IL-2Rα subunit is responsible for conferring species specificity in IL-2 binding (Gaffen and Liu, 2004).

The T4 cell line showed proliferation response after the stimulation with recombinant porcine IL-2 in dose-dependent manner. It suggested that recombinant pIL-2 produced in High Five cells was biologically active. IL-2 stimulates dramatic proliferation of activated (antigen or lectin) T lymphocytes. It acts on all subpopulation of T cells and promotes progression through the G1 phase of cell cycle, resulting in growth of cells and increase the cell numbers (Thorpe, 1998). The dose

of recombinant protein that gave 50 % of maximal response was 6.96 pg/ml. The recombinant porcine IL-2 statically significant induced higher percentage of proliferation when compared to the stimulated with bacteria-produced porcine IL-2. It suggests that the recombinant protein from insect cell line were provided correct folding, functionally and the binding to IL-2 receptor were higher affinities than bacteria-produced porcine IL-2. Compared with the prokaryotic expression system, baculovirus and insect cell expression system is convenient and versatile eukaryotic system. Furthermore, it can provide correct folding of recombinant protein and other important post-translational modifications and form glycosylation similar to that of mammalian cells (Cao *et al*., 2005). On the basis of bioassay, the total units and units/mg of purified recombinant protein from insect cell preparation were higher than those of bacteria (Nuntaprasert *et al*., 2005). Iwata *et al*. (2000) was cloned and expressed porcine IL-2 cDNA in *E. coli* and examined a biological activity using murine dependent cell line, CTLL-2, the result showed pIL-2 concentration was 2.4 ng/ml enhanced the proliferation of CTLL-2 at 50 % maximal CTLL-2 response.

This study thus showed that porcine IL-2 can also be produced in a biologically active form in insect cell and further developed and tested as adjuvants to be used in immunizations with vaccines aimed at porcine. The use of IL-2 as an adjuvant might be employed as immunostimulants to augment the potency of week immunogen, stimulated immunity, decrease the doses of antigen in the vaccine and reduce costs.

CONCLUSION

In this study, the recombinant porcine IL-2 was successfully cloned and expressed in High-Five insect cells. Both nucleotide and amino acid sequences of recombinant pIL-2 showed 100 % homology with the porcine IL-2 sequence reported in GenBank (Accession X58428). The SDS-PAGE analysis of recombinant porcine IL-2 produced by the insect cells had the size approximately 23 kDa which included 6xhis and a few amino acids from the vector. According to the expression of pIL-2 analyzed with IPMA, dot and Western blot analysis, the recombinant porcine IL-2 had positive result to goat anti-porcine IL-2 antibody suggested that the recombinant porcine IL-2 produced in insect cells may have correct post-translational modification. Biological activity of IL-2 on T4 cell was determined. The human T4 cells showed proliferation after the stimulation with recombinant porcine IL-2. This assay gives 50% of maximal response induced by 6.96 pg/ml of the recombinant porcine IL-2 proteins. Thus, the recombinant porcine IL-2 proteins produced in insect cells were biologically functional and contained glycosylation of recombinant protein. The recombinant porcine IL-2 will be a very effective tool as an immunostimulant adjuvant to augment the immune response to vaccination.

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APPENDIX

APPENDIX 1

Chemical Reagents and Substances

1. Bacterial Media and Solution

- Luria Bertani (LB) medium (broth)

  10 g/litre tryptone

  5 g/litre yeast extract

  10 g/litre NaCI

- LB agar

  LB medium containing 15 g/litre agar

- Ampicillin stock solution

  100 mg/ml in H_2O, sterile filter, store in aliquots at -20 °C

- Kanamycin stock solution

  25 mg/ml in H_2O, sterile filter, store in aliquots at -20 °C

- IPTG (1 M) 238 mg/ml in H_2O, sterile filter, store in aliquots at -20 °C

2. Plasmid Isolation Reagents

- Solution I

    25 mM Tris-HC1 pH 8.0

    10 mM EDTA

    50 mM glucose

- Solution II

    0.2 N NaOH

    1% SDS

- Solution III

    60 ml of 5 M Potassium acetate

    11.5 ml of glacial acetic acid

    Distilled water 28.5 ml

    3 M Sodium acetate pH 5.2

    99.5% ethanol

- Tris-EDTA (TE pH 8.0)

    10 mM Tris HCI

    1 mM EDTA

    adjust pH to 8.0

- Phenol / chloroform / isoamyl alcohol (25:25:1) total volume 204 ml

    100 ml TE-saturated phenol

    100 ml chloroform

    4 ml isoamyl alcohol

3. Buffer for Agarose Gel Electrophoresis

- 20X TAE buffer pH 8.3 (1 litre)

  0.8 M Tris ; 96.9 g

  0.4 M Sodium acetate; 32.8 g of $NaOAc\text{-}3H_2O$

  0.04 M Na_2EDTA; 14.9 g

  Adjust pH with glacial acetic acid to pH 8.3 and bring to 1 litre with distilled water.

- 10X loading buffer / dye

  20% glycerol

  0.01% Bromphenol blue

  add TE to final volume

- 5 mg/ml ethidium bromide (EtBr)

  500 mg EtBr

  add distilled water to 100 ml

4. Buffer for DNA Extraction from Agarose Gel

- lysis buffer pH 7.0 (100 ml)

  4M Guanidine Thiocyanate; 47.28 g

  50mM TrisCl ; 0.6055 g

  20mM EDTA; 0.80894 9

  adjust pH to 7.0

- Washing buffer pH 7.0 (100 ml)

50% ethanol . 50 ml

200mM NaCI ; 1.1688 g

10mM EDTA; 0.40497 g

50mM TrisCl; 0.6055 g

adjust pH to 7.0

5. SDS-PAGE Reagents

- 2x SDS-PAGE sample buffer

2.5 ml of 4xTris Cl / SDS, pH 6.8 (250 mM Tris Cl)

2.0 ml of glycerol (20% glycerol)

0.4 g of SDS or 4 ml of 1 0%SDS (4% SDS)

0.2 ml of beta- mercaptoethanol (2% of 2-ME)

0.006% bromophonol blue

- 4x Tris Cl/SDS pH 6.8, buffer for stacking gel (250 ml)

0.5M TrisCl ; 15 g TrisCl

0.4% SDS; 1 g SDS

200 ml Distilled water

Adjust pH using concentrated HCl , then add distilled water to 250 ml total volume

- 4x Tris Cl/SDS pH 8.8. buffer for separating get (500 ml)

1.5M TrisCl; 91 g Tris Base

0.4% SDS; 2 g SDS

400 ml Distilled water

Adjust pH using concentrated HCI then add distilled water to 500 ml total volume

-30% Acrylamide / Bis-acrylamide (bis-acrylamide acrylamide =1:36) to prepare 513.5 ml of solution:

acrylamide 150 g

Bis-acrylamide 4.1 g

Add distilled water to 513.5 ml sterilize by filter and store at 4°C

- 10% Ammonium persulfate

100 mg Ammonium persulfate

1 ml distilled water

- TEMED (N,N,N',N'-tetramethylethylenediamine) store protected from light at40°C

- Glycine buffer

192 mM glycine

25 mM Tris base

0.1% SDS

- Coomasie Brilliant Blue stain (2 litres)

2 g Coomasie brilliant blue powder

1 litre methanol

200 ml acetic acid

800 ml distilled water

Stir for minimum 2 hours and filter through Whatman filter disc

- Destainning solution (100 ml)

225 ml methanol

10 ml acetic acid

225 ml distilled water

6. Immunoblotting Reagents

-PBS buffer, pH 7.4 (1 litre)

NaCI 8.0 g

$K_2H_2PO_4$ 0.2 g

KCl 0.2 g

Na_2HPO_4 1.15 g

Distilled water adjust to 1000 ml

- PBS-Tween buffer

add to final concentration of 0.5% Tween 20 (Sigma®)

- Blocking agent

5% skim milk in PBS- 0.5 %Tween buffer

- Serum diluting agent

2% skim milk in PBS- 0.5 %Tween buffer

- DAB (Sigma®) substrate

6 mg of DAB

10 ul of H_2O_2

990 ul of Sterilize water

-Transfer buffer (1 litre)

25mM Tris; 3 g Tris base

190mM glycine; 14.4 g glycine

20% Methanol ; 200 ml Conc. Methanol (water adjust to 1,000 ml)

7. <u>Insect cells medium</u>

- Sf 21 cell medium (100l)
  - 4% Fetal Bovine Serum 4 ml
  - Antibiotic(Penicillin,Streptomycin) 1 ml
  - Sf 900II SFM adjust to 100 ml
- High Five cell medium (150 ml)
  - Antibiotic(Penicillin,Streptomycin) 1.5 ml
  - L-glutamine 13.5 ml
  - Express Five SFM adjust to 150 ml

8. Buffer for purification under native condition.

- Lysis buffer (1 liter)

50 nM NaH2PO4

300 mM NaCl

10 mM imidazole

adjust pH to 8.0 using NaOH

- Wash buffer (1 liter)

50 nM NaH2PO4

300 mM NaCl

20 mM imidazole

adjust pH to 8.0 using NaOH

- Elution buffer (1 liter)

50 nM NaH2PO4

300 mM NaCl

250 mM imidazole

adjust pH to 8.0 using NaOH

APPENDIX 2

The standard methods

1. Preparation of Ultra-Competent Cells for Transformation (Inoue *et al.*, 1990)

1. Culture cells on LB agar plate at 37 °C overnight. In case of M15 strain *E. coli* LB agar contains 25 µg/ml kanamycin.

2. Pick up a large colony and culture in 1 ml of LB broth at 37°C overnight with vigorous shaking (~ 250 rpm).

3. From 500 µl of overnight culture, subculture to 100 ml of SOB medium containing 25 µg/ml kanamycin, incubate at 37°C until OD600 is 0.4 - 0.8 (approximately 3 – 4 hours).

4. Store the culture on ice for 10 minutes.

5. Centrifuge at 4 °C, for 10 minutes at 3,000 rpm, discard the supernatant.

6. Gently resuspend the pellet in 33 ml of ice-cold TB and store on ice for additional 10 mintues

7. Centrifuge at 4°C, for 10 minutes at 3,000 rpm, discard the supernatant.

8. Gently resuspend the pellet with 2 ml of ice-cold TB, then add 7% DMSO (150 µl)

9. Aliquot the cell to ependorf tube each 200 ul and store at -70 °C until use for transformation.

2. Transformation

1. Thaw the competent cell on ice, for 30 minutes.

2. Mix the constructed plasmid from ligation to the competent cell, stand on ice for 30 minutes.

3. Heat shock the cell at 42°C for 90 second in the heat block, and immediately place tube on ice for 5 minutes.

4. Add the L.B. broth 900 µl and incubate with shaking at 37°C for 1 hour.

5. Centrifuge the culture at 6,000 rpm for 1 minute.

6. Spread the cells on the prewarmed LB plate containing 100 ug/ml ampicillin and 7 µg/ml Getamycin, air dry plate, and incubate for 1-126 hours at 37°C

7. The recovery clone of *E. coli* with recombinant plasmid was determined by PCR assay, restriction endonuclease, and DNA sequencing.

4. Ligation (adapted from Sambrook and Russell, 2001)

The following reaction conditions are for ligation of DNA inserts with cohesive ends to DNA vectors with complementary cohesive ends to produce circular recombinant molecules. A molar ratio of 5:1 insert:vector was used.

1. Add the following component to the 0.5 ml microtube

- 10x T4 ligase buffer 2 µl
- vector DNA : insert DNA (1:5)
- autoclaved distilled water to 20 µl

2. Add 1.0 µl (0.1 unit) of T4 DNA ligase (Promega®, Inc.). Mix gently and then breifly spin down to bring the content to the bottom of the tube.

3. Incubate at 4°C, 48 hours.

4. The ligation reaction was used in transformation to competent cell of *E. coli* 7 µl each reaction.

4. Kit QIA prep® spin Method for Plasmid Isolation

1. From overnight cultures of *E. coli* in LB broth, pour 1.5 ml into 1.5 ml microtube.

2. Centrifuge for 5 minutes, at 13,000 rpm and carefully aspirate off the medium.

3. Add 250 µl of P1 buffer with RNAse A and resuspend by vortexing until the suspension become homogenous.

4. Add 250 µl of P2 buffer and gently inverse the tube up side down. The cells should lyse and turn somewhat clear and viscous.

5. Let it stand for 3 minutes (should not more than 5 minutes) and then add 350 µl of P3 buffer. Mix again by inversion until a white clot of DNA/protein/SDS form.

6. Centrifuge at 13,000 rpm for 10 minutes.

7. Pour off the supernatant (~400 µl) into a QIA prep column 1.5 ml microtube.

8. Centrifuge at 13,000 rpm for 2 minutes.

9. Discard the supernatant

10. Wash QIA prep with 750 µl of PE buffer

11. Centrifuge at 13,000 rpm for 1 minute.

12. Change new tube and add 30 µl of EB Buffer, incubate at room temperature 1 minute.

13. Centrifuge at 13,000 rpm for 1 minute, and keep elution buffer at -20 °C

5. <u>Restriction Endonuclease Assay</u> (recommented by Fermentus®)

This procedure is used for cutting the target DNA with two enzymes (Double digestion method).

1. Isolate the plasmid from *E. coli* or prepared the target DNA.

2. Add the following components to 1.5 ml microtube;

- target DNA 10 µl (in TE & distilled water), the volume depends on the concentration of DNA.
- Y+Tango® 1X buffer for 2 µl
- 2-fold of Hind III and 1-fold of XbaI restriction enzyme add distilled water to desirable volume (30 µl)

3. Incubate at 37 °C for 3 hours to complete enzyme reaction.

4. Purify the target DNA by agarose gel electrophoresis and DNA extraction from agarose gel.

6. QIA quick Gel Extraction Kit Protocol

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl)

3. Incubate at 50°C for 10 minutes. To help dissolve gel, mix by vortexing the tube every 2-3 minutes during the incubate.

4. After the gel slice has dissolve completely, check that color of the mixture is yellow

5. Add 1 gel volume of isopropanol to the sample and mix.

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 minute.

8. Discard flow-through and place QIAquick column back in the same collection tube.

9. To wash, add 0.75 ml of PE buffer to QIAquick colume and centrifuge for 1 minute.

10.Discard flow-through and place QIAquick column an additional 1 minute at 13,000 rpm.

11.Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

12.To elute DNA, add 50 ul of EB buffer (10 mM Tris-Cl, pH 8.5) to the center of QIAquick column membrane, let the column stand for 1 minute, and centrifuge for 1 minute.

7. Phenol-Chloroform Extraction of RNA and Ethanol Precipitation (Sambrook and Russell, 2001)

1. RNA was extracted from 100 µl of Con A blast cells. It was mixed with 500 µl denature solution and 50 µl 2M NaAc by shaken for 5-10 minutes.

2. Add 150 µl RNA phenol and 150 µl chloroform , shaken for 5 minutes.

3. Centrifuge the sample at 13,000 rpm for 5 minutes to separate the phases.

4. Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous-phenol interface. At this stage the aqueous phase can be extracted a second time with same procedure.

5. Repeat step 2-4 again.

6. Remove about 90% of the upper, aqueous layer to a clean tube, add 550 µl isopropanol and 0.5 µl glycogen (20ng/ml), invert gently up side down and keep in –80°C for 40 minutes

7. Centrifuge at 13,000 rpm for 10 - 15 minutes. Carefully decant the supernatant.

8. To wash the RNA pellet with 75% ethanol. Centrifuge at 13.000 rpm for 5 minutes. Decant the supernatant, and dry the pellet by air.

8. Agarose Gel Electrophoresis

1. Prepare an agarose gel, according to recipes listed below, by combining the agarose (low gel temperature agarose may also be used) and water in a 250 ml flask.

2. Pour the gel onto a taped plate with casting combs in place. Allow 20 - 30 minutes for solidification.

3. Carefully remove the tape and the gel casting combs and place the get in a horizontal electrophoresis apparatus. Add 1x TAE electrophoresis buffer to the reservoirs until the buffer just covers the agarose gel.

4. Add at least one- tenth volume of 5x agarose gel loading dye to each DNA sample, mix, and load into the wells. Electrophoresis the gel at 50 - 100 V/cm until the required separation has been achieved.

5. Incubate the agarose gel in EtBr tank for 15-20 minutes.

6. Visualize the DNA fragments on a long wave UV light box.

9. <u>SDS-Polyacrylamide Get Electrophoresis (SDS-PAGE)</u> (Cochet *et al.*, 1998)

9.1 Prepare the gel by the recipe as follow for 2 gels

- Separating gel (12%)

H_2O	2.8 ml
4x Tris-CI/SDS pH 8.8.	2.0 ml
30% Acry/0.8%bis-Acryl	3.2 ml
10% APS	26.6 ul
TEMED	5.3 ul

- Stacking get

H_2O	3.05 ml
4x TrisHCI/SDS pH 6.8	1.25 ml
30% Acryl/0.8%bis-Acryl	0.65 ml
10% APS	25 ul
TEMED	5 ul

9.2. If not already in electrophoresis sample buffer, add an equal volume of 2X sample buffer to all samples and boil for 5 minutes.

9.3. Apply 20-25 µl (1 - 10ug total protein) of cell lysate to each well of a 0.75-1.0 mm thick gel.

9.4. Run electrophoresis (100 volt, 70 minutes, constant ampere)

10. <u>Western Transfer and Blotting</u> (Cochet *et al.*, 1998)

1. Cut the nitrocellulose membrane with sharp blade to the expected size

2. Soak the gel (from SDS-PAGE), the nitrocellulose membrane, the Whatmann papers, and sponges in cool transfer buffer for 10 minutes.

3. Set the transfer apparatus, then transfer proteins from gel to nitrocellulose membrane at 400 mAmp for 6 hours in transfer buffer (25mM Tris base, 190mM glycine, 20% MeOH). The transfer set should be placed in ice box to control the temperature.

4. Remove the blot from the transfer apparatus immediately place into blocking buffer (5% skim milk in 1x PBS+ 0.5 % Tween 20) and incubate for 1 hour at room temperature with gently agitation (optional 2 hour at 37°C /overnight at 4°C).

5. Dilute the primary antibody (goat-anti H5N1 avian influenza virus hyperimmune sera absorb for 3 hours at 37°C with supernatant of virus wild-type (1:50) or mouse IgG anti-histidine monoclonal antibody (1:3,000). Decant the blocking buffer from the blot, add the primary antibody solution, and incubate with agitation for 90 minutes at room temperature.

6. Wash 3 times with agitation in PBS-0.5% Tween for 5 - 10 minutes each.

7. Dilute the secondary antibody with 2% skimmilk in PBS-0.5% Tween (rabbit anti-goat IgG (1:1,000) or goat anti-mouse IgG (1:500) conjugated with peroxidase). Decant the primary antibody from the blot, add the secondary antibody solution, and incubate with agitation for 45-60 hours at room temperature

8. Decant the secondary antibody solution. Wash 3 times with agitation in PBS 0.5 % Tween for 5 - 10 minutes each.

9. Add DAB substrate and incubate for 5 - 15 minutes.

10. To stop enzyme-substrate reaction, place the blot in distilled water.

11. <u>Viral Plaque Assay</u> (Invitrogen, 2002)

1. Under sterile conditions dispense 2 ml of cell suspension per well.

2. Allow cells to settle to bottom of plate and incubated, covered at room temperature for 1 hour.

3. Place the bottle of methyl agarose gel in the 70 ^{o}C water bath.

4. Following a 1 hour incubation of the plates at room temperature, observe monolayer under the inverted microscope to confirm cell attachment and 50 % confluence.

5. Produce an eight-log serial dilution of the harvested viral supernatant by sequentially diluting 0.5 ml of the previous dilution in 4.5 ml of Sf-900 II SFM in 12-ml disposable tubes.

6. Move the six well plates and the tubes of diluted virus to the hood.

7. Sequentially remove the supernatant from each well, discard and immediately replace with 1 ml of the respective virus dilution to each duplicate well. Incubate for 1 hour at room temperature.

8. Sequentially remove the virus inoculum from the wells and replace with 2 ml of the diluted methyl agarose. Work quickly to avoid desiccation of the monolayer.

9. Allow gel to harden for 10 to 20 min before moving.

10. Incubated at 27 ^{o}C in a humidified incubator for 4 to 10 days.

11. Monitor plates daily until the plaque counts does not change for two consecutive days.

12. To determine the titer of the inoculum employed, an optimal range to count is 3 to 20 plauqe per well of six well plate. The titer (pfu/ml) may be calculated by the following formula:

pfu/ml(of original stock)=1/dilution factor x no.of plaque x1/(ml of inoculum/plate).

12. Nucleotide Base Abbreviations Used in Nucleic Acid Sequences

A	adenosine
C	cytidine
G	guanine
T	thymidine
U	uridine
R	G A (purine)
Y	T C (pyrimidine)
M	A C (amino)
S	G C (strong)
W	A T (weak)
B	G T C

12. <u>Abbreviation of amino acid</u>

Phenylalanine	F
Leucine	L
Serine	S
Tyrosine	Y
Stop	X
Cysteine	C
Tryptophan	W
Proline	P
Histidine	H
Glutamine	Q
Isoleucine	I
Methionine	M
Threonine	T
Asparagine	N
Lysine	K
Arginine	R
Valine	V
Alanine	A
Aspartate	D
Glutamate	E
Glycine	G

APPENDIX 3

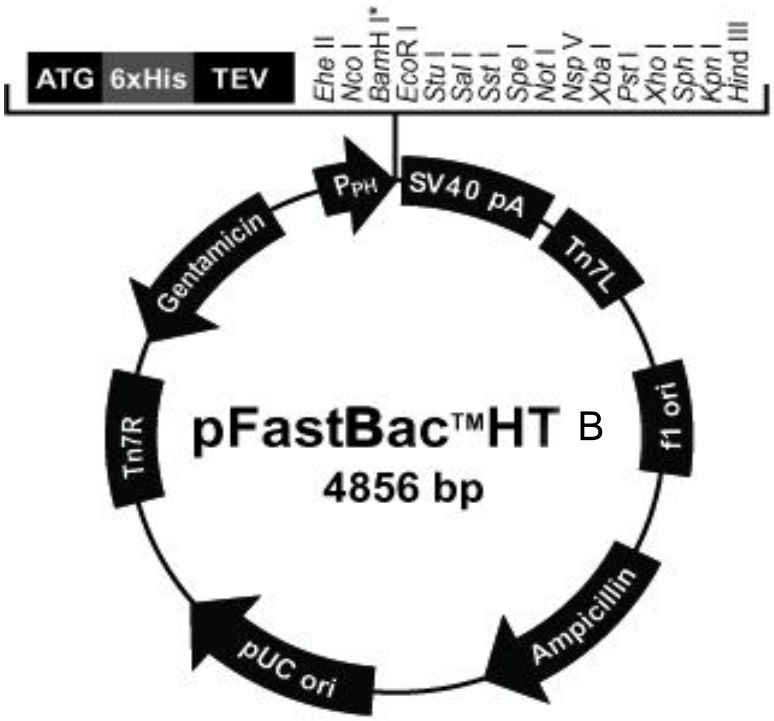

Figure 1 The pFastBac^{TM} HT B plasmid (Invitrogen$^{®}$) circle map and sequence reference points. This map show of start and 6xHistidine tag, this is used for check recombinant neuraminidase protein.

Source: Invitrogen$^{®}$

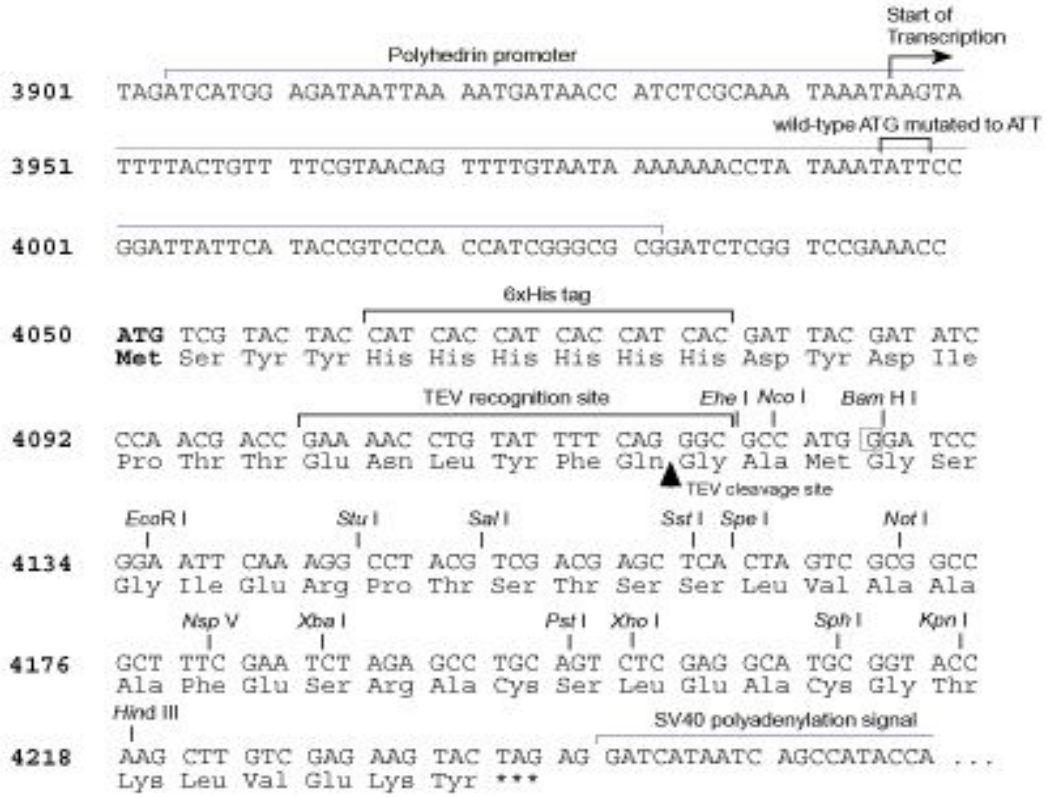

Figure 2 This picture show a baculovirus promoter and multiple cloning sequence of pFastBac™ HT b plasmid (Invitrogen®).

Source: Invitrogen®

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