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Production of single-chain Fv–Fc fusion protein in stably transformed insect cells

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ABSTRACT

We describe the secretory production of a mouse anti-bovine ribonuclease A single-chain variable fragment (scFv) fused with the Fc region of human IgG1 in stably transformed lepidopteran insect cells. Use of the insect-derived Bip and melittin signal peptides resulted in higher yields of the secreted scFv–Fc fusion protein than use of the baculovirus gp64 signal peptide. After cotransfection with an expression vector that contains the Bip signal sequence upstream of the DNA encoding the scFv–Fc and a selection vector that carries a neomycin resistance gene, *Trichoplusia ni* BTI-TN-5B1-4 (High Five) cells were cultured in the presence of G418. Colonies of resistant cells were obtained around 2 weeks after adding G418, and clonal cells were screened by enzyme-linked immunosorbent assay (ELISA) of the culture supernatant. The yield of the scFv–Fc protein secreted from the most productive clone was around 60 mg/l in a shake-flask culture. To improve the productivity, we investigated the effect of medium supplements of sodium butyrate (NaBu), dimethyl sulfoxide (DMSO), and sericin. Supplementing culture medium with sericin increased the scFv–Fc protein yield to 82 mg/l, but productivity was not increased by either NaBu or DMSO. These results indicate that the stably transformed insect cells may allow for the efficient production of scFv–Fc and other Fc fusion proteins.

1. Introduction

In recent years various types of engineered antibody molecules have been developed [1]. Among them, the single-chain variable fragment (scFv) is one of the simplest and many scFvs have been developed for fundamental research and clinical applications [2,3]. Due to their small size and simple structure, scFvs are easily produced in bacterial expression systems, including *Escherichia coli*, which is an inexpensive and very popular system for the production of therapeutic proteins [4–6]. To augment the binding activity of scFvs, dimerization strategies have been developed. One of the most popular dimerization methods is Fc fusion technology using the Fc domain of immunoglobulin G (IgG) and there are many reports on the production of scFv–Fc fusion proteins. The Fc fusion confers several advantages, including an avidity effect, a longer half-life in serum, an effector function, and easy purification by staphylococcal protein A and protein G. In general, relatively large and complex proteins like Fc fusion proteins are produced in a costly mammalian cell system, although scFv–Fc fusion proteins have been successfully produced in the soluble and functional form using *E. coli* [7–9].

Various recombinant protein production systems, including bacteria, yeast, insect, plant, and mammalian cell systems and in vitro cell-free systems, are currently available, and selection of an appropriate system depends on the characteristics and intended application of the recombinant protein. Simple proteins that do not require glycosylation or complex folding can be produced successfully in bacteria and cell-free systems. However, expression of complex proteins through proper folding and post-translational processing and modifications often requires eukaryotic host cells. Yeast cells, one of the most popular of the eukaryotic host cells, can produce recombinant glycoproteins, but their typical sugar chain is a high mannose type that often shows immunogenicity in human and other animals. However, mammalian cells generally produce properly folded glycoproteins with complex *N*-linked glycans containing terminal sialic acids similar to those found in human proteins. Currently, many biopharmaceutical glycoproteins have been manufactured with mammalian cell systems such as CHO and NS0. Apart from these 2 eukaryotic cell systems, insect cells are now being used for the production of biologically active recombinant proteins. Cervarix, which has been approved recently as a human papillomavirus (HPV) vaccine, is produced with an insect cell line [10]. Insect cell systems are similar to mammalian systems, but insect cells are easy to culture as compared with mammalian cells. Insect cells derived from lepidopteran insects, Sf9, Sf21, and BTI-TN-5B1-4 (High Five), are commonly used in the baculovirus–insect cell system. They can be maintained at 25–28 °C without CO_2 supplementation in the atmosphere. Insect cells have traditionally been cultured in basal media supplemented

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with around 10% vertebrate serum, most usually fetal bovine serum (FBS), instead of insect hemolymph. However, insect cells are easily adaptable to serum-free medium and can be grown to high densities in suspension culture. Furthermore, insect cells provide safety advantages for the production of biopharmaceuticals because they do not support the growth of mammalian viruses or mycoplasmas. Proteins produced by insect cells are not contaminated with endotoxins. Therefore, insect cells are currently an attractive alternative platform for the production of complex recombinant proteins [11].

In the present study, the production of a mouse anti-bovine ribonuclease A antibody scFv [12] fused with the Fc region of human IgG1 in stably transformed lepidopteran insect cells was investigated. The effects of different signal peptides and medium supplements were examined. The results obtained in the present study provide information that is important for the efficient production of scFv–Fc and other Fc fusion proteins in recombinant insect cells.

2. Materials and methods

2.1. Insect cell line, medium, and supplements

The *Trichoplusia ni* BTI-TN-5B1-4 insect cell line (High Five; Invitrogen, Carlsbad, CA, USA) was maintained at 27 °C in a serum-free medium (Express Five SFM; Invitrogen) supplemented with 2.41 g/l L-glutamine. Cell density was determined by microscopically counting the number of cells with a hemocytometer, while cell viability was judged by trypan blue dye exclusion. Sodium butyrate (NaBu), dimethyl sulfoxide (DMSO), and sericin were obtained from Wako Pure Chemical Industries (Osaka, Japan) and added to Express Five SFM to give the concentration indicated.

2.2. Plasmid construction

The recombinant DNA techniques and methods described by Sambrook and Russell [13] were used in the present study. Plasmid DNA was prepared by a standard alkaline lysis method. A QIAEX II gel extraction kit (Qiagen, Hilden, Germany) was used for extraction and purification of DNA from agarose gels. Nucleotide sequences were determined with a DNA sequencer (ABI PRISM 3100-Avant Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) using the dideoxy chain-termination procedure. The nucleotide sequence data were analyzed using DNASIS software (Hitachi Solutions, Tokyo, Japan).

The DNA fragment encoding 1 of 3 different signal sequences and a part of the VH–$(Gly_4Ser)_3$–VL gene of 3A21 mouse anti-bovine ribonuclease A scFv [12] was amplified from the plasmid pET22-3A21Fc [13] by polymerase chain reaction (PCR), as follows. For the *Autographa californica* nucleopolyhedrovirus (AcNPV) gp64 signal peptide sequence, the following primer pairs were used in the first and second PCR, respectively; 5′-TTGGCGGCGG-CGGCGCATTCTGCCTTTGCGATGGATGTGCAGCTTCAGGAG-3′ and 5′-GTCATCTTGATGTCTGATC-3′, and 5′-ATAAT CCATGG GATATC TAATAAATATGGTAAGCGCTATTGTTTTATATGTGCTTTTGGCGGCG-GCGGCGCATTCT-3′ (the underlined bases encode *Nco*I and *Eco*RV sites, respectively) and 5′-GTCATCTTGATGTCTGATC-3′. For the *Drosophila* binding protein (Bip) secretion signal peptide sequence, the following primer pairs were used in the first and second PCR, respectively; 5′-GTCGCCTTTGTTGGCCTCTCGCTCGGGATGGA-TGTGCAGCTTCAGGAG-3′ and 5′-GTCATCTTGATGTCTGATC-3′, and 5′-ATAAT CCATGG GATATC TAATAAATATGAAGTTATGCA-TATTACTGGCCGTCGTCGCCTTTGTTGGCCTCTCG-3′ (the underlined bases encode *Nco*I and *Eco*RV sites, respectively) and 5′-GTCATCTTGATGTCTGATC-3′. For the honeybee melittin (Mel) secretion signal peptide sequence, the following primer pairs were used in the first and second PCR, respectively; 5′-TTTATGGTCGTGTACATTTCTTACATCTATGCGATGGATGTGCAGCTT-CAGGAG -3′ and 5′-GTCATCTTGATGTCTGATC-3′, and 5′-ATAAT CCATGG GATATC TAATAAATATGAAATTCTTAGTCAACGTTGCCCT-TGTTTTTATGGTCGTGTACATTTCTTAC-3′ (the underlined bases encode *Nco*I and *Eco*RV sites, respectively) and 5′-GTCAT-CTTGATGTCTGATC-3′. The amplified fragments were digested by *Nco*I and *Eco*RI, and ligated into the *Nco*I–*Eco*RI site of the pET22-3A21Fc vector. The resultant plasmids were digested with *Eco*RV and *Not*I and the DNA fragments encoding the individual signal peptide and the 3A21 scFv–Fc were ligated into the plasmid pXINSECT-DEST38 (Invitrogen) at the *Eco*47III-*Not*I site. The pXINSECT-DEST38 utilizes the silkmoth (*Bombyx mori*) cytoplasmic actin promoter, from which foreign gene expression is remarkably stimulated with the *B. mori* NPV (BmNPV) IE-1 transactivator and the BmNPV HR3 enhancer [14]. The resultant plasmids were designated as pX-gp64-3A21Fc, pX-Bip-3A21Fc, and pX-Mel-3A21Fc. The sequences amplified by PCR were confirmed by nucleotide sequencing.

2.3. Transient expression

High Five cells were inoculated into 6-well plates with 2 ml of Express Five SFM at a cell density of 4×10^5 cells/ml 24 h before transfection. The cells were transfected with 1 μg of plasmid using 4 μl of HilyMax transfection reagent (Dojindo Laboratories, Kumamoto, Japan). Two days after transfection, culture supernatant was removed to analyze the production of the 3A21 scFv–Fc.

2.4. Stable transformation and cell culture

High Five cells were inoculated into 6-well plates with 2 ml of Express Five SFM at a cell density of 4×10^5 cells/ml 24 h before transfection. The cells were transfected with 1 μg of pX-Bip-3A21Fc and 10 ng of pBmA:neo (Invitrogen), which contains a neomycin resistance gene, using 4 μl of HilyMax transfection reagent. Four hours after transfection, the culture medium was replaced with fresh Express Five SFM. Two days after transfection, the cells were removed from the plates and inoculated into 100-mm plastic culture dishes with 15 ml of Express Five SFM containing 1.0 mg/ml G418 (Nacalai tesque, Kyoto, Japan) to select stable expression cells. G418, also known as geneticin, is an aminoglycoside antibiotic similar in structure to gentamicin and neomycin and is commonly used in the selection of eukaryotic cells stably transfected with neomycin resistance genes. The selective medium was replaced every 4 days until colonies of resistant cells were formed. Cells were isolated from each colony into 96-well plates with 100 μl of medium containing 0.8 mg/ml G418. After the cells had grown to confluence in the presence of G418, the culture supernatant was removed to assay the 3A21 scFv–Fc. Cells with a high yield of the 3A21 scFv–Fc were expanded in T-flasks with medium containing 0.8 mg/ml G418.

Cells in the exponential growth phase were collected and suspended at a density of 5×10^5 cells/ml in fresh medium containing 0.8 mg/ml G418. Five ml of cell suspension was transferred into T25-flasks and 15 ml of cell suspension was transferred into 125-ml Erlenmeyer flasks. The cells in the T-flasks were statically cultivated, while the cells in the Erlenmeyer flasks were cultivated at 27 °C on a reciprocal shaker (100 oscillations/min) and aliquots of the cell suspension were sampled every day.

2.5. SDS-PAGE and Western blot analysis

SDS-PAGE was carried out according to a method established by Laemmli [15], which uses 10–20% polyacrylamide gels under reducing conditions. Reduced samples were prepared by treatment with 2.5% 2-mercaptoethanol at 95 °C for 5 min. Ten microliter