Scientific Research Publishing

American Journal of Molecular Biology, 2021, 11, 29-37
https://www.scirp.org/journal/ajmb
ISSN Online: 2161-6663
ISSN Print: 2161-6620

Expression of Difficult-to-Express Proteins, Human IL-12 and IFN-γ

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How to cite this paper: Hosaka, Y., Matsutani, S., Kawate, S., Itoh, K., Miura, A., Mizoura, Y., Yamada, S., Uemura, S., Konno, H., Grave, E., Wakui, H. and Itoh, H. (2021) Expression of Difficult-to-Express Proteins, Human IL-12 and IFN-γ. *American Journal of Molecular Biology*, **11**, 29-37.
https://doi.org/10.4236/ajmb.2021.112003

Received: January 6, 2021
Accepted: March 1, 2021
Published: March 4, 2021

Abstract

It is known to be that *lactic acid bacteria* induce the IL-12. IL-12 activates NK cells and promotes the production of IFN-γ. IFN-γ activates macrophages, resulting in enhanced phagocytosis and bactericidal activity. We have been investigating fermented foods that activate the immune function. For that purpose, a specific antibody is required. We tried to express IL-12p35 by the usual method, but IL-12p35 was not expressed at all. In the present study, we constructed, purified human IL-12p35 and obtained a specific antibody against IL-12p35. We also purified human IFN-γ and obtained specific antibody against IFN-γ. We have established a method for expressing poorly expressed proteins. The method we have established can be applied to the purification of poorly expressed proteins and antibody production.

Keywords

Difficult-to-Express Proteins, IL-12, IFN-γ, Purification, Antibody Production

1. Introduction

Cytokines play an important role in maintaining the homeostasis of hematopoiesis, cell proliferation and differentiation, as well as the immune system consisting of innate and adaptive immunity in the body. Interleukin-12 (IL-12) is a member of the IL-12 family of cytokines. IL-12, which is secreted by phagocytic cells in response to pathogens, is a heterodimer protein of two subunits (p35 and

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p40) that acts primarily during the induction of Interferon-γ (IFN-γ) via the signal transducer and activator of transcription 4 (STAT4) in T and natural killer (NK) cells [1]. IL-12 is a cytokine that strongly induces the Th1 response. Activated macrophages by IFN-γ enhance phagocytosis and bactericidal activity [2].

We have been studying fermented foods that activate the immune function using IL-12 as merkmal. Since a specific antibody is required for that purpose, we tried to prepare a specific antibody against human IL-12 and IFN-γ. In a protein expression system using *Escherichia coli*, IL-12 and IFN-γ are considered to be difficult to express. Therefore, baculo-expression is generally used for the expression of expression difficult proteins. In the present study, we established a method for purifying the IL-12 and IFN-γ using *E. coli* expression system. We also obtained specific antibodies against the IL-12 and IFN-γ.

2. Material & Method

Materials

Human IL-12p70 and IFN-γ were purchased from Gibco. An antibody against human IL-12p40 and β-actin were purchased from Abcam and Sigma-Aldrich, respectively.

Gene cloning and plasmid construction of IL-12p35

The pGEX-5X-3 expression vector (Cytiva) were replaced in Factor Xa recognition sequence to TEV (Tobacco Etch Virus) protease with PCR mutagenesis method using forward primer 5'-AACCTTTATTTTCAAGGTCGTGGGATCCCCAGG-3' and reverse primer 5'-TTGAAAATAAAGGTTTTCGATCAGATCCGATTT-3' (Greiner Japan). The TEV sequence was replaced Human Rhino Virus 3C (PreScission) protease recognition sequence with PCR mutagenesis method because the Factor Xa with human IL-12p35 expressed couldn't be cut by TEV protease. This mutagenesis was in twice because this sequence is long about can't replace in once using forward primer 5'-TCCTTGAAGTCCTTTTTCAAGGTCGTGGGATCC-3' and reverse primer 5'-AAAGGACTTCAAGGATCAGATCCGATTTTGGAG-3', then second time forward primer 5'-CAAGGTCCCGGGATCCCCAGGAATTCC-3' and reverse primer 5'-GATCCCGGGACCTTGAAAAAGGACTT-3' (Greiner Japan). The PCR was conducted in the following conditions: denaturation at 98˚C for 10 sec, annealing at 55˚C for 15 sec, and extension at 72˚C for 30 sec for up to 35 cycles using a SimpliAmp Thermal Cycler (ThermoFisher Scientific). The IL-12p35 genes were amplified by PCR using forward primer 5'-GGATCCCCAGGAATTCCATGTGGCCCCCTGGGTCA-3' and reverse primer 5'-AGTCACGATGCGGCCTTAGGAAGCATTCAGATAGC-3' (Greiner Japan). PCR was conducted in the following conditions: denaturation at 94˚C for 30 sec, annealing at 59˚C for 30 sec, and extension at 72˚C for 35 sec for up to 35 cycles using a SimpliAmp Thermal Cycler (ThermoFisher Scientific). IL-12p35 was inserted PreScission recognition sequence in pGEX 5x-3-PreScission sequence using In-Fusion system (Clontech TaKaRa cellartis).

Expression of GST-tagged IL-12p35

The GST-tagged IL-12p35 subunit was expressed using the expression vector pGEX-5X-3 in an *E. coli* OverExpress C41 (DE3) pLysS competent cell (Lucigen). The cells were grown at 37˚C, 250 rpm in LB BROTH medium supplemented with 100 mg/ml ampicillin until the OD_{600} reached 0.6. The cells were then induced by the addition of 0.5 mM IPTG and incubated at 37˚C, 250 rpm for an additional 3 hr. The cells were harvested by centrifugation at 4˚C, 13,000 rpm for 15 min, and cell pellets were suspended in 10 mM Tris-HCl, pH 7.4.

Purification of IL-12p35

The cells were sonicated for twice, centrifuged 15,000 rpm for 15 min at 4˚C, then the precipitates were collected. The obtained insoluble fraction was dissolved in 1M arginine in 10 mM Tris-HCl, pH 7.4 solution. After centrifugation (13,000 rpm for 10 min at 4˚C), the supernatant was applied to desalting column to remove the arginine (Bio-Gel p10 gel, BioRad). Then, PreScission digestion buffer (final concentration: 50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, pH 7.0) and PreScission protease was added to to the solution and incubated for 16 hr at 4˚C. After incubation, 2× SDS Sample Buffer (0.5 M Tris-HCl pH 6.5, 2-Mercaptoethanol, 10% Glycerol, 10% SDS, 0.1% Bromophenol Blue) was added to the sample and heated for 2 min at 100˚C. The sample solution was separated with SDS-PAGE (13% gel), stained gel using CBB staining solution (0.1% Coomassie Brilliant Blue R-250, 25% isopropyl alcohol, 10% acertic acid), and destained gel using destaining solution (10% isopropyl alcohol, 10% acetic acid). The induced protein bands werer cut out from electrophoresed the SDS polyacrylamide gel and incubated in the Laemmli buffer at 4˚C for 16 hr. The gel was finely chopped using a cutter knife blade, proteins were eluted from the gels using a protein eluter MODEL BE-883 (BIO CRAFT). The IL-12p35 was purified elecrophoretically.

Gene cloning and plasmid construction of IFN-γ

pGEX-5x-3 expression vector (Cytiva) were replaced in Factor Xa recognition sequence to Tobacco Etch Virus (TEV) protease with PCR mutagenesis method using forward primer 5'-AACCTTTATTTTCAAGGTCGTGGGATCCCCAGG-3' and reverse pramer 5'-TTGAAAATAAAGGTTTTCGATCAGATCCGATTT-3' (Greiner Japan). The TEV sequence was replaced Human Rhino Virus 3C (PreScission) protease recognition sequence with PCR mutagenesis method because the Factor Xa with human IFN-γ expressed could't be cut by TEV protease. This mutagenesis was in twice because this sequence is long about can't replace in once using forward primer
5'-TCCTTGAAGTCCTTTTTCAAGGTCGTGGGATCC-3' and reverse primer 5'-AAAGGACTTCAAGGATCAGATCCGATTTTGGAG-3', then second time forward primer 5'-CAAGGTCCCGGGATCCCCAGGAATTCC-3' and reverse primer 5'-GATCCCGGGACCTTGAAAAAGGACTT-3' (Greiner Japan). PCR was conducted in the following conditions: denaturation at 98˚C for 10 seconds, annealing at 55˚C for 15 seconds, and extension at 72˚C for 30 seconds for up to 35 cycles using a SimpliAmp Thermal Cycler. IFN-γ genes were amplified by

PCR using forward primer 5'-GGATCCCCAGGAATTCCATGAAATATACAAGTTATATCT-3' and reverse primer 5'-AGTCACGATGCGCCTTACTGGGATGCTCTTCGA-3' (Greiner Japan). PCR was conducted in the following conditions: denaturation at 94˚C for 30 seconds, annealing at 59˚C for 30 seconds, and extension at 72˚C for 20 seconds for up to 35 cycles using a SimpliAmp Thermal Cycler (ThermoFisher Scientific). IFN-γ was inserted PreScission recognition sequence in pGEX 5x-3 PreScission sequence using In-Fusion system (Clontech TaKaRa cellartis).

Expression condition of IFN-γ

The GST-tagged IFN-γ was expressed from the expression vector pGEX-5X-3 in an *E. coli* OverExpress C41 (DE3) pLysS competent cell (Lucigen). After 250 rpm in LB BROTH medium supplemented with 100 μg/ml ampicillin until the OD_{600} reached 0.6, IFN-γ expression was induced by 0.1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) for 3 hours at 37˚C. The cells were harvested by centrifugation at 4˚C, 13,000 rpm for 15 minutes, and cell pellets was preserved in 10 mM Tris-HCl pH 7.4 at −30˚C.

Purification of IFN-γ

The collected pellet was sonicated and centrifuged at 4˚C, 13,000 rpm for 15 minutes, and the insoluble fraction obtained was dissolved in 1M arginine in 10 mM Tris-HCl pH 7.4 solution. After centrifugation (4˚C, 13,000 rpm for 10 minutes), the supernatant was applied in desalting column to remove the arginine. Then, PreScission digestion buffer (final concentration: 50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, pH 7.0) and PreScission protease was added to digest PreScission protease recognition sequence in the solution at 4˚C for 16 h. After that, 2× Sample Buffer (0.5 M Tris-HCl pH 6.5, 2-Mercaptoethanol, Glycerol, 10% SDS, Bromophenol Blue) in the sample and heat shock at 100˚C for 2 min. The sample solution was separated with 15% SDS-PAGE, dye using CBB, then decolorized (10% 2-propanol, 10% acetic acid). Gel contained the proteins was cut out from electrophoresed the SDS polyacrylamide gel and incubated in the Laemmli buffer at 4˚C for 16 h. The gel was finely chopped using a cutter, after that was electrophoresed in Laemmli buffer by SDS-PAGE gel by MODEL BE-883 (BIO CRAFT). The liquid contained the proteins (contain CBB and SDS).

Antibody Production

An anti-IL-12p35 or an anti-IFN-γ antibody was produced by intramuscular injection into a rabbit of IL-12p35 or IFN-γ that was eluted from SDS-PAGE gel emulsified in complete Freund's adjuvant. Booster shots were given 3 times in the same manner as the original injection at 2-week intervals. Original injection was emulsified in complete Freund's adjuvant of 1 ml IL-12p35 or IFN-γ, booster shots was emulsified in incomplete Freund's adjuvant of 2 ml IL-12p35 or IFN-γ. The rabbit was sacrificed 10 days after the last injection. Two ml of the antiserum was dialyzed against to10 mM Tris-HCl, pH 7.4 buffer overnight at 4˚C. The dialyzed antiserum was loaded to DEAE-Cellulose column (DE52, Whatman) equilibrated in 10 mM Tris-HCl, pH 7.4 and wash the column using

the same buffer. The pass-through fractions were collected and freeze dried using Freeze Dryer (FDU-1110, EYLA, Japan). The protocols for animal experimentation described in this paper were previously approved by the Animal Research Committee, Akita University School of Medicine; the "Guidelines for Animal Experimentation" of the University were completely adhered to in all subsequent animal experiments. Specificity and titer of the anti-IL-12p35 antibody or anti-IFN-γ was analyzed by immunoblotting using human recombinant IL-12 or IFN-γ and cell culture medium.

SDS/PAGE and Immunoblotting

SDS/PAGE and Immunoblotting were according to Laemmli [3] and Towbin [4], respectively. Samples were analyzed by SDS/PAGE, followed by immunoblotting. The PVDF membrane was reacted with an IL-12p35 antibody (diluted 500 times) or IFN-γ (diluted 500 times) with an anti-rabbit AP IgG (BioRad, diluted 10,000 times) or an anti-rabbit HRP IgG (Biorad, diluted 15,000 times). Samples were treated with BCIP-NBT solution (Nacalai Tesque) or an ECL Plus Western Blotting Detection System (Cytiva). Image analysis was performed using ChemiDock XRS plus (BioRad).

Statistical analysis software

We analyzed the statistical processing using Excel (Office 16) software.

3. Results

Expression and purification of IL-12p35

To obtain the specific antibody against the IL-12p35 subunit, we constructed, expressed, and purified the protein. We first attempted to express IL-12p35 by the conventional method using a pET-15b expression vector and an OverExpress C41 pLysS competent cell. However, IL-12p35 was not expressed at all by the expression method (**Figure 1(a)**). Therefore, we attempted to express GST-tagged IL-12p35. As shown in **Figure 1(b)**, GST-tagged IL-12p35 was expressed in an insoluble fraction as an inclusion body. The insoluble fractions were dissolved in arginine as described in the "Materials and Methods". We digested the GST-tagged IL-12p35 using PreScission protease. The protease cut each IL-12p35 and GST (**Figure 1(c)**). Finaly, we electrophoretically purified IL-12p35 (**Figure 1(d)**).

An antibody against IL-12p35

We immunized the purified IL-12p35 to rabbit and obtained the anti-IL-12p35 antibody. The commercially available IL-12p40 antibody also cross-reacted to human recombinant IL-12p70 (**Figure 2(a)**). The antibody against human IL-12p35 cross-reacted with the human recombinant IL-12p70 in a dose dependent manner (**Figure 2(b)**). The degradation of the proteins was also detected for the antibody. We made a calibration curve based on **Figure 2(b)**. The coefficient of determination (R^2) was 0.9992 (**Figure 2(c)**). Thus, the anti-IL-12p35 antibody produced in our laboratory was able to recognize the human recombinant IL-12p70 the same as the commercially available IL-12p40 antibody.

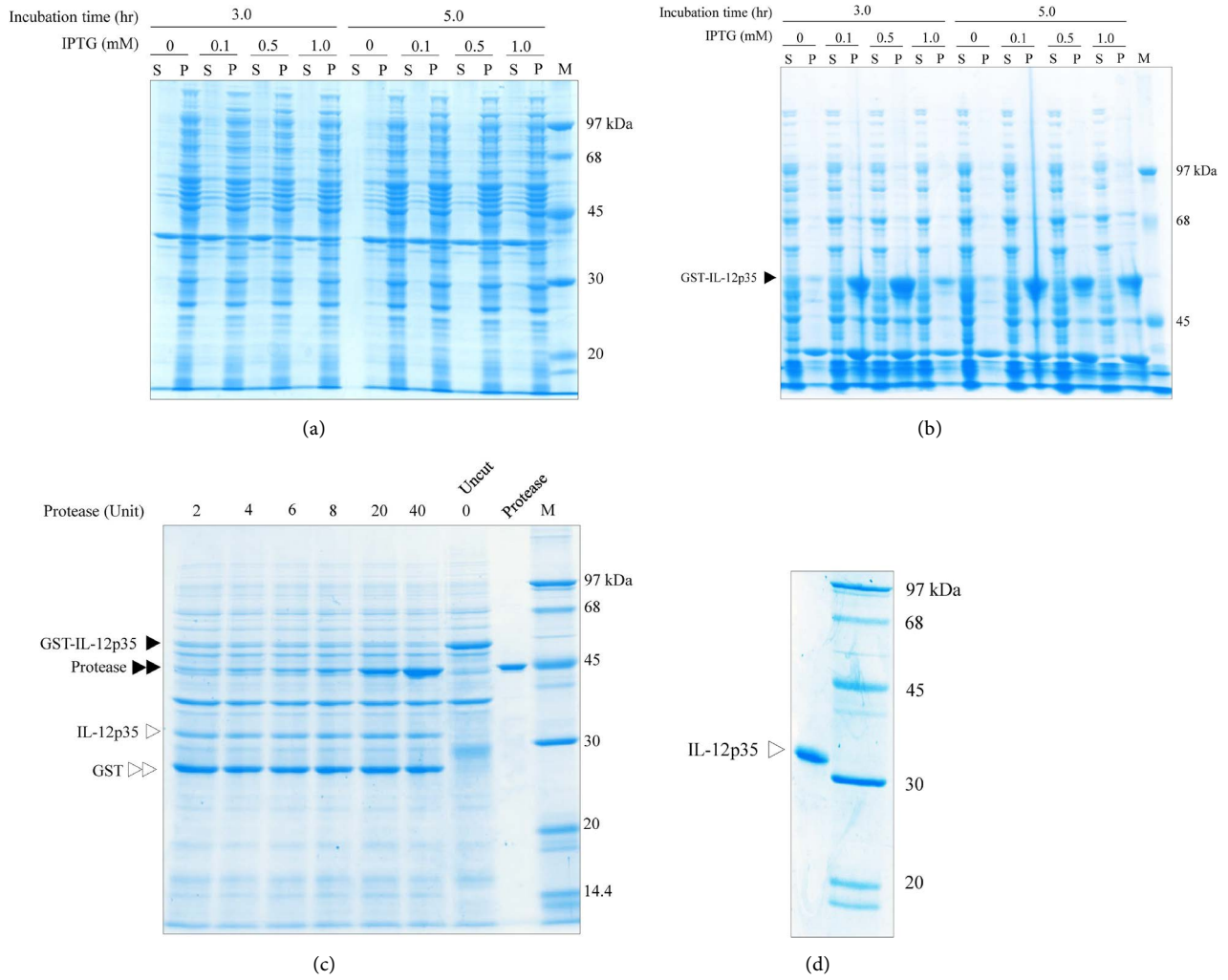

Figure 1. Induction and purification of IL-12p35. (a) Plasmid pET-15b-IL-12p35 was transformed into BL21 (DE3) and OverExpress C41 (DE3) pLysS competent cells. Protein expression was induced by 0, 0.1, 0.5, 1.0 mM IPTG and incubated for 3 hr or 5 hr at 37˚C. After expression, the cells were harvested by centrifugation at 4˚C, 15,000 rpm for 15 min, and the cell pellets were suspended in 10 mM Tris-HCl buffer (pH 7.4). The cells were sonicated, centrifuged at 4˚C, 15,000 rpm for 15 min and the soluble fraction (S) and insoluble fraction (P) were collected. The proteins were separated by SDS-PAGE (7% gel) and stained with CBB; (b) Plasmid pGEX-5x-3-IL-12p35 was transformed into BL21 (DE3) and OverExpress C41 (DE3) pLysS competent cells. Protein expression was induced by 0, 0.1, 0.5, 1.0 mM IPTG and incubated for 3 hr or 5 hr at 37˚C. After expression, the cells were harvested by centrifugation at 4˚C, 15,000 rpm for 15 min, and the cell pellets were suspended in 10 mM Tris-HCl buffer (pH 7.4). The cells were sonicated, centrifuged at 4˚C, 15,000 rpm for 15 min and the soluble fraction (S) and insoluble fraction (P) were collected. The proteins were separated by SDS-PAGE (7% gel) and stained with CBB; (c) Induced GST-IL-12p35 in the precipitate was dissolved in 1 M arginine and digested using PreScission protease (0, 2, 4, 6, 8, 20, 40 Units). The digested proteins were analyzed by SDS-PAGE (12% gel); (d) IL-12p35 protein bands were cut from the SDS gel (b) and proteins were electrophoretically eluted from the gels. The purified IL-12p35 was analyzed by SDS-PAGE (9% gel).

Expression and purification of IFN-γ

As shown in **Figure 3(a)**, GST-tagged IFN-γ was mainly expressed in an insoluble fraction. The insoluble fractions were dissolved in arginine and digested the GST-tagged IFN-γ using PreScission protease. The protease cut each IFN-γ and GST (**Figure 3(b)**). Finally, we electrophoretically purified IFN-γ.

An antibody against IFN-γ

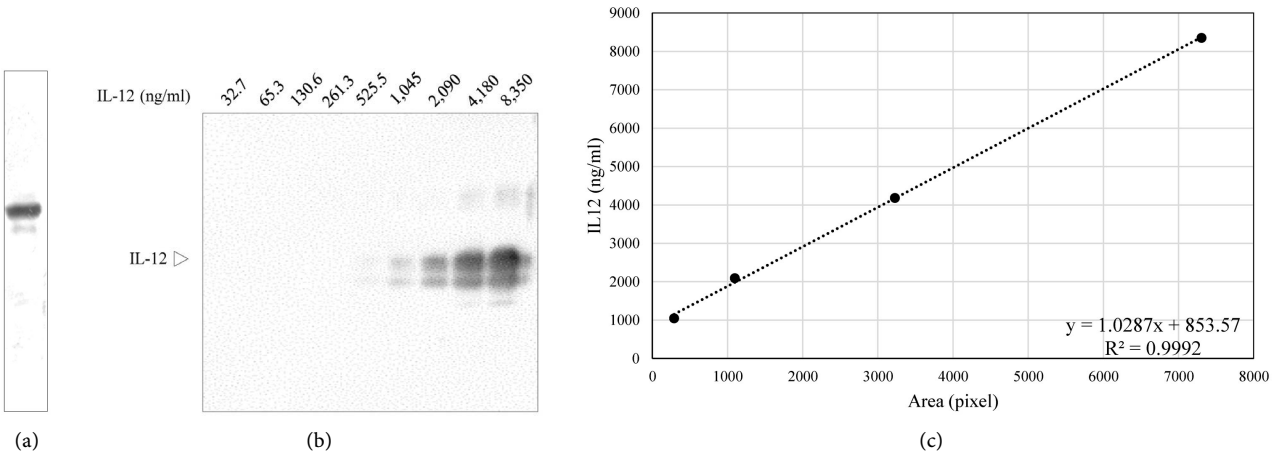

Figure 2. An antibody against IL-12. (a) Human recombinant IL-12p70 was electrophoresed on SDS-PAGE, followed by immunoblotting with an antibody against IL-12p40; (b) Human recombinant IL-12p70 (0, 32.7, 65.3, 130.6, 261.3, 525.5, 1045, 2090, 4180, and 8350 ng/ml) was electrophoresed by SDS-Page, followed by immunoblotting with antibody against IL12p35; (c) The calibration curve was prepared based on the immunoblot (b).

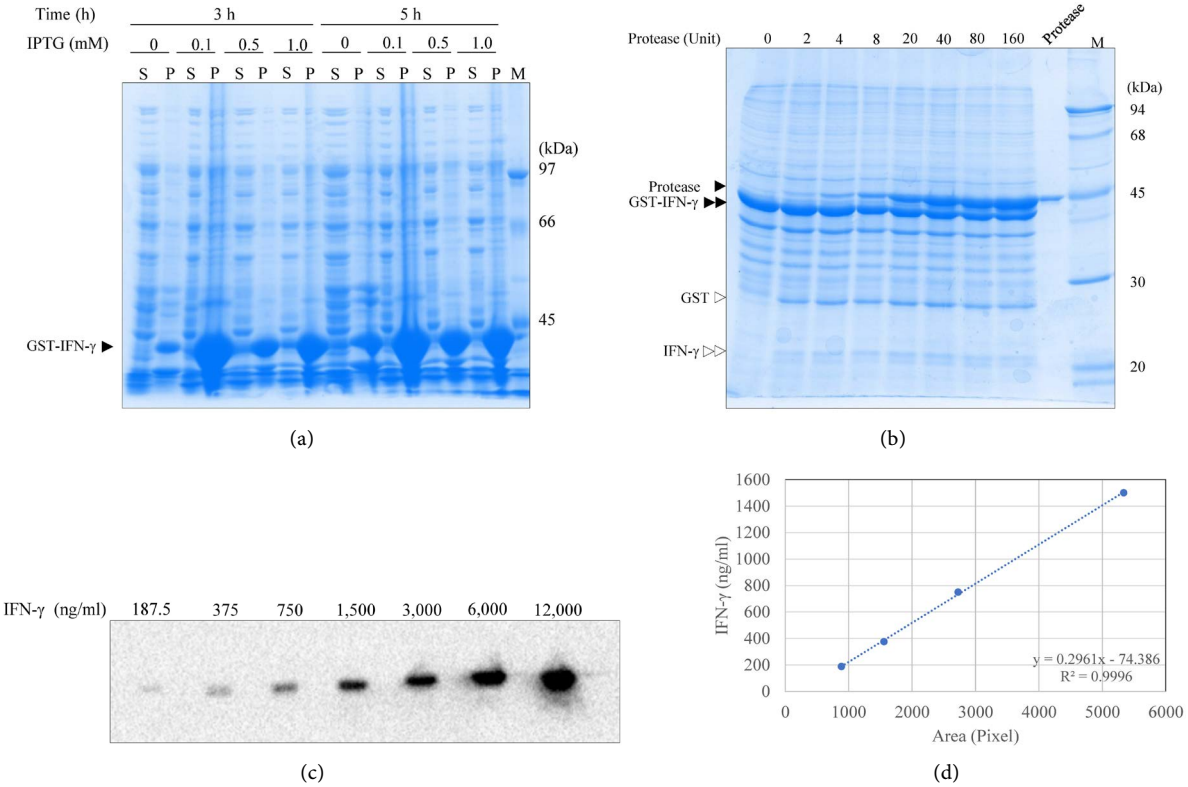

Figure 3. Purification of IFN-γ and antibody production. (a) Plasmid pGEX-5x-3 IFN-γ was transformed into BL21 (DE3) and OverExpress C41 (DE3) pLysS competent cells. Protein expression was induced by 0, 0.1, 0.5, 1.0 mM IPTG and incubated for 3 hr or 5 hr at 37˚C. After expression, the cells were harvested by centrifugation at 4˚C, 15,000 rpm for 15 min, and the cell pellets were suspended in 10 mM Tris-HCl buffer (pH 7.4). The cells were sonicated, centrifuged at 4˚C, 15,000 rpm for 15 min and the soluble fraction (S) and insoluble fraction (P) were collected. The proteins were separated by SDS-PAGE (7% gel) and stained with CBB; (b) Induced GST-IL IFN-γ in the precipitate was dissolved in 1 M Arginine and digested using PreScission protease (0, 2, 4, 8, 20, 40, 80, and 160 Units). The digested proteins were analyzed by SDS-PAGE (12% gel); (c) Human recombinant IFN-γ (0, 187.5, 375, 750, 1500, 3000, 6000, and 12,000 ng/ml) was electrophoresed by SDS-PAGE, followed by immunoblotting with antibody against IFN-γ; (d) The calibration curve was prepared based on the immunoblot (c).

We obtained the anti-IFN-γ antibody. The antibody cross-reacted with the human recombinant IFN-γ in a dose dependent manner (**Figure 3(c)**). We made a calibration curve based on **Figure 3(c)**. The coefficient of determination (R^2) was 0.9996 (**Figure 3(d)**).

4. Discussion

We searched for IL-12-specific antibodies to analyze the IL-12 inducibility, but basically only antibodies against IL-12p40. When we try to measure the IL-12 inducibility using the anti-IL-12p40 antibody, not only IL-12 but also IL-23 will be quantified, so that accurate quantification of the IL-12 inducibility is not to be expected. For the IL-12 measurement, we considered using the IL-12p35 antibody rather than IL-12p40 antibody.

We tried to express IL-12p35 using pET-15b expression vector and OverExpress C41 pLysS competent cell. Almost no IL-12p35 was expressed in this system. Proteins that are toxic to *E. coli*, species conservation, proteins related to biological defense, etc. are not expressed at all or expressed very little in the *E. coli* expression system. In that case, the baculovirus expression system is generally used, or synthetic peptides are used to prepare antibodies.

We attempted to express the GST-IL-12p35 fusion protein. As a result, although the protein was expressed in the insoluble fraction, IL-12p35 could be purified by protease treatment. We also immunized rabbits with the purified protein and we were able to obtain specific antibodies. The commercially available antibody against IL-12p40 was also cross-reacted to the 40 kDa of human IL-12p70 (p35/p40) and its degradation (**Figure 2(a)**). The antibody against IL-12p35 also cross-reacted to the commercially available human recombinant IL-12p70 (p35/p40) with its degradation (**Figure 2(b)**). However, the antibody against IL-12-p35 detected 35-kDa proteins and prepared calibration curve was obtained (**Figure 2(c)**).

In the same way, we were able to express and purify IFN-γ to obtain a specific antibody. This protein expression method is expected to exert its power in the expression and purification of other poorly expressed proteins like a full-length Arylhydrocarbon receprot (AhR) [5]. The method we have developed to this work can express proteins that were difficult to say until now. Validity can be a particularly useful method for antibody production.

Acknowledgements

We special thanks to Dr. Tomoya Okamoto for helpful comments to the work.

Conflicts of Interest

The authors have no conflict of interest. The authors declare no competing financial interests.

Author Information

Y. H., S. M., H. K., and H. I. designed the experiments. Y. H., S. M., S. K., A. M.,

Y. M., and S. Y., S. U. prepared the figures. Y. H., S. M., E. G., H. W., and H. I. wrote the main manuscript. Corresponding Author: itohh@g.ecc.u-tokyo.ac.jp

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