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W.S Chen Expression of rhIFN-γ in baculovirus-insect/larval system

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Expression of recombinant human interferon-γ with antiviral activity in bi-cistronic baculovirus-insect/larval system

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List of Abbreviations

5’ UTR IRES - 5’ untranslated region internal ribosome entry site

AcMNPV - *Autographa californica* multiple polyhedrosis virus

BV- budded virus

dpi - day post-infection

EGFP - enhanced green fluorescent protein

Ni-NTA – Nickel (II) nitrilotriacetic acid

P_{PH} - polyhedrin promoter gene

rhIFN-γ - Recombinant human interferon- gamma

RhPV - *Rhopalosiphum padi* virus

Sf 21 - *Spodoptera frugiferda* 21 cells

$TCID_{50}$ - 50% tissue culture infectious dose

TN - tunicamycin

Abstract

A bi-cistronic baculovirus-insect/larval system containing a *polyhedron* promoter, an internal ribosome entry site (IRES), and an *egfp* gene was developed as a cost-effective platform for the production of recombinant human interferon gamma (rhIFN-γ). There is no significant difference between the amounts of rhIFN-γ produced in the baculovirus - infected Sf21 cells grown in serum-free medium and the serum supplemented medium. While the *Trichoplusia ni* (*T. ni*) and *Spodoptera exigua* (*S. exigua*) larvae afforded rhIFN-γ amounting to 1.08 ± 0.04 and 9.74 ± 0.35 μg/mg protein, respectively. The presence of non-glycosylated and glycosylated rhIFN-γ was confirmed by immunoblot and lectin blot. The immunological activity of purified rhIFN-γ (with 96% purity by Ni-NTA affinity chromatography) is similar to the commercially available. Moreover, the rhIFN-γ protein from *T. ni* has more potent antiviral activity. These findings suggest that this IRES-based expression system is a simple and inexpensive alternative for large-scale protein production in anti-viral research.

Keywords: baculovirus; bi-cistronic; *Spodoptera exigua* larvae; human interferon gamma; antiviral activity

Introduction

From the time Smith and colleagues successfully used the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) as an expression vector for the production of human β-interferon in insect cells,[1] the baculovirus - insect expression system was heralded a powerful tool in the field of recombinant protein expression technology. Interferons are cytokines that possess many biological functions including modulation of the immune and inflammatory responses, promotion of tissue repair and antiviral activity. Specifically, interferon-γ (IFN- γ) is regarded to have various biological activities that include its potent antiviral activity.[2-4] In humans, interferon-γ (hIFN-γ) functions as a lymphokine normally secreted by antigen-sensitized T lymphocytes which stimulates major-histocompatibility-complex Class II expression during an immune response.[5, 6] Efforts to clone and express this gene had been undertaken in various systems such as the *Escherichia coli*,[7] mammalian cells,[8] yeast[9] and the transgenic plant cells.[10, 11] Moreover, most papers would only deal with its over-expression (i.e. *E. coli*) and the efficient purification scheme of rhIFN-γ but overlooking its biological activity.[12, 13]

Rhopalosiphum padi virus (RhPV) which is a member of the *Dicistrviridae* family infects the insect cells that commonly affect rice crops. The viruses impart the physicochemical properties due to the members of the *Picornaviridae*, a family of virus identified foremost to exhibit the cap-independent mechanism involving the 5' untranslated region (5'UTR).[14] Amongst the papers written about the proteins via the RhPV 5'UTR IRES element to efficiently mediate cap-independent translation in

baculovirus-infected insect cells such as *Spodoptera frugiferda* 9 (Sf9) and *Spodoptera frugiferda* 21(Sf21) cells,[15, 16] still an approach that directly attaches the RhPV 5'UTR IRES into a conventional baculovirus strong very late promoter (e.g. polyhedrin or p10 promoter) -based transfer vector needs to be given further attention and compare to *in vitro* translation system and T7 promoter-based expression systems. [16] Our laboratory recently reported the hIFN-γ protein and EGFP could be co-expressed simultaneously in single recombinant bi-cistroniv baculovirus containing the genes for hIFN-γ and *egfp* flanked between the RhPV 5'UTR IRES element in the same recombinant baculovirus-infected Sf21 cells. [18]

Another route that proved to be a very powerful tool to increase the amount of recombinant protein is the use of baculovirus-infected larvae. The system is economical and searched literatures clearly depict that foreign proteins often gives post-translational modifications that are typical in the native protein of an organism. During the last decade, a number of recombinant protein have been produced in the insect larvae, including cytokines, immunogens, and enzymes. The larval system depends on the simultaneous infection and harvesting when the expression of the target protein is at its optimum,[17] albeit the characteristic on the expression of rhIFN-γ in larvae is yet undiscovered. In addition, we as well demonstrated that AcMNPV can efficiently infect *Trichoplusia ni* larvae by aerosol routing through the spiracle[19].

Herein, to obtain rhIFN-γ with high purity, the release of EGFP could facilitate the determination of the lysis of the infected cells. Thus, we got 96% pure rhIFN-γ produced in the baculovirus -infected Sf21 cells grown in serum-free medium purified by Ni-NTA affinity chromatography. The immunological activity is similar to the

commercially available hIFN-γ. Furthermore, to obtain the high level rhIFN-γ protein, both *Trichoplusia ni* and *Spodoptera exigua* larvae were infected; the yield and activity were compared. To our knowledge, this is the first report on the evaluation of the inhibitory effect of rhIFN-γ produced in the baculovirus-insect/larval expression system against dengue serotype PL046 as the challenge virus on human adenocarcinomic human alveolar basal epithelial (A549) cells. The approach on using the baculovirus insect/larval establishes a relatively inexpensive and easy to handle mode of producing a biologically active recombinant human interferon-γ that could be of potential use in the development of antiviral therapeutics.

Materials and Methods

Insect Cell line, larva and media

The insect cell lines used were derived from species belonging to the genus *Spodoptera* namely *S. frugiperda* (Sf2 IPBL), *S. exigua* and *S. litura*. The medium used for routine maintenance was TNM-FH insect medium containing 8% heat-inactivated fetal bovine serum.[20] The individual insect cell monolayer was kept at 27^{o}C in T-flask in a non-humidified incubator for use in virus propagation; all viral stocks were prepared and titers were determined according to the standard protocol described by O'Reilly.[21] For experiments requiring a serum-free medium, EX-CELL^{TM} 420 (SAFC Bioscience) was used instead and the cells were acclimatized prior to experiments.

The larvae of *Trichoplusia ni* and *Spodoptera exigua* were provided and maintained in the Agricultural Chemicals and Toxic Substances Research Institute (Wufeng

Taichung, Taiwan). The insects were reared in a non- sterile climatic chamber at 26±1°C and were fed with artificial insect diet as described by Jinn and co-workers.[19)]

Baculovirus infection on insect cells and larvae

The recombinant baculovirus, vAcIFN-γ-Rhir-E was generated as reported in our previous study.[18)] Briefly, each insect cell type (2 x 10^5 cells) was co-transfected with linearized viral DNA Bac-N-Blue® (Invitrogen) and the AcMNPV vector (0.8 μg) pBAcIFN-γ-Rhir-EGFP by 4 μL of cellfectin. Transfection was monitored by the green fluorescence emitted by the cells viewed under a fluorescence microscope following a two round end-point dilution. The resulting virus was named as vAcIFN-γ-Rhir-E. The virus titer was determined through end-point dilution and EGFP fluorescence detection done in a 96-well plate, the viral titer was obtained according to the 50% tissue culture infectious dose (TCID50) method.[22)]

Haemocoelic injection of the budded virus (BV) containing the recombinant human interferon- γ to each larva of *T. ni* (35 larva/trial) and *S. exigua* (35 larva/trial) at a titer of 10^8 pfu/mL and a volume of 4μL/larva was done by using a microinjector (Burkard Manufacturing); those larvae that emitted green fluorescence were collected and frozen for protein extraction.

Determination of recombinant human interferon-γ protein expression

Post-infection, an aliquot from supernatant media of either the serum-free or serum supplemented baculovirus infected-insect cell culture was collected daily (7 days) to monitor the expression of the recombinant human IFN-γ and EGFP, where the release of

the latter to the medium is indicative of cell lysis. Samples were kept at -20^{o}C until used. Inhibition of *N*-glycosylation was was done by incubating baculovirus-infected Sf21 cells with tunicamycin (1 μg/mL). Supernatant medium of the tunicamycin treated baculovirus-infected Sf21 cells was collected and was kept frozen at -20^{o}C until used for Western blot analysis with anti-hIFN-γ polyclonal antibody and visualized using an X-ray film (Kodak®) .

The frozen larvae (see Section 3.2) of *T. ni* and *S. exigua* were thawed and homogenized in phosphate buffered saline at a proportion of 1:10 (weight of larva: volume of PBS). The homogenate was centrifuged at 4^{o}C removing large cell debris. The supernatant was collected and about 1.5 mL aliquot was pipetted into an Eppendorf tube and was kept at -20^{o}C until analyzed.

For small-scale protein purification, a hexa-Histidine tag was introduced at the C-terminus of hIFN-γ gene. Purification of rhIFN-γ was achieved by subjecting 40 mL of the supernatant from infected Sf21 cells to Ni-immobilized His-Binding affinity column chromatography (Ni-NTA, Proteus IMAC) using the elution buffer containing 50 mM $Na_2H_2PO_4$, 300 mM NaCl, 300 mM imidazole, pH 7.5); eluate was concentrated by Centricon Plus-20 (MWCO = 8 kDa, Millipore) and dialyzed thrice with 50 mM Tris buffer (pH 7.5) with 0.02% protease inhibitor cocktail (Sigma). The purified protein was stored at -20^{o}C until used for Lectin blot analysis.

Protein concentration was determined by Coomassie ® reagent (Pierce). Western and Lectin Blot analyses were carried out on the protein extracts. Briefly, separation was done by SDS-PAGE on a mini Protean III system (Bio-Rad) followed with

electroblotting onto a polyvinylidene difluoride membrane (PVDF) membrane. The resulting membrane was blocked with either 5% non-fat dry milk in Tris-buffered saline [TTBS; 100 mM Tris (pH 7.4), 100 mM NaCl and 0.1 Tween 20] or commercial blocking buffer (Roche) at room temperature for 1 hour with shaking. The membrane was then incubated with either monoclonal antibodies namely (1:2000) anti-EGFP [Clonetech], (1:1000) anti-IFN-γ [Merck] in TBS with 5% non-fat dry milk or digoxigenylated lectin [Roche, GNA- *Galanthus nivalis* agglutinin recognizing terminal mannose linked (1-3), (1-6) or (1-2) to mannose] in a buffer with 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1mM $CaCl_2$, pH 7.5. Then, the membrane was incubated with either 1:2500-diluted horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugated secondary antibodies followed by enhanced chemiluminiscence detection (Pierce) and staining using NBT/BCIP (Roche, 4-nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl-phosphate) as substrate, respectively. Indirect ELISA (Endogen™ Human IFN-γ ELISA kit, Pierce Biotechnology) was used to determine the level of protein expression in the supernatant liquids from either baculovirus infected Sf21 cells or infected-larval (*S. exigua* and *T. ni*) homogenates. The absorbance of samples and controls was measured at 450 nm (OD_{450}) in an ELISA microplate reader (SpectraMax Plus) where the standard curve for EIA with recombinant human IFN-γ (rhIFN-γ) as control was linear ranging from 10 to 2500 pg/mL.

Recombinant human interferon-γ antiviral protection assay

Supernatants containing the human interferon-γ generated from the following: screened infected insect cell lines in serum supplemented medium, infected Sf21 cells

cultured in serum-free (Ex420 medium) medium; purified rhIFN-γ by Ni–NTA affinity column and the larval homogenates were used for antiviral assay with commercial INF-γ (Bio-Rad) as positive control. Samples were diluted tenfold and were used for the anti-viral assays against dengue virus serotype PL046 infection on human A549 cells. The A549 cells (100,000 cells/well) were pre-treated with serum and serum-free fractions, purified fraction, supernatant from larval homogenates and commercially available IFN-γ at various protein concentrations overnight then the cells were infected with dengue virus at multiplicity of infection (MOI) 0.1. The A549 cells were replenished with fresh medium, added with test samples and incubated for another 2 days.

The culture supernatants were harvested to determine titers of dengue virus. Plaque assays, were performed by adding various virus dilutions to 80% confluent BHK-21 cells (baby hamster kidney cells) followed by incubation at 37^{o}C for 1 h. After viral absorption, cells were washed and overlaid with 1% agarose (Sea Plaque, FMC Bio-Products, Rockland, USA) containing RPMI-1640 mammalian cell culture medium supplemented with 10% FCS (fetal calf serum, GIBCO). After seven days of incubation, cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet.

Results

Expression and purification of recombinant human interferon-γ in baculovirus – insect cells

A recombinant baculovirus containing the RhPV 5'UTR IRES that simultaneously expresses two genes, under the control of the polyhedrin promoter (*polh* promoter) is

generated. This reporter construct contains human interferon-γ and enhanced green fluorescent protein genes flanking the RhPV IRES as shown in Figure 1A. Co-expression of two proteins, one being a molecular marker and the other the target gene has been a valuable tool for monitoring protein translation.[23-25] Several studies demonstrated that the enhanced green fluorescent protein can efficiently be used as a reporter protein for fast selection of recombinant baculoviruses and viral titer measurements [26, 27] as depicted in Figures 1B and 1C. Screening with insect cell lines for rhIFN-γ expression was carried out in a serum supplemented medium (Figure 1D) and results showed that among the cell lines used, the baculovirus-infected Sf21 cells gave a significantly high of rhIFN-γ expression. This finding led to the use of Sf21 cells for in further experimentations. Furthermore, the baculovirus-infected Sf21 cells were grown in serum free-medium and findings revealed a slight decrease in the amount of rhIFN-γ generated.

<Insert Figures 1A, 1B, 1C and 1D here>

Characterization of the rhIFN-γ obtained from the vAcIFN-γ-Rhir-E -infected Sf21 cells was done. Supernatants from the baculovirus infected Sf21 cell culture in EX-CELL™ 420 serum-free medium were subjected to Western blot analysis. Results revealed the presence of a band at MW ~27kDa signifying that the EGFP was released to the medium starting on the 6th day post-infection (dpi) as demonstrated by the faint band on the PVDF membrane and an even darker band on the 7th dpi (Figure 2A) that is indicative of cell lysis releasing the EGFP to the medium. This observation correlates

with the results of the ELISA experiment done to quantify the amount of rhIFN-γ where the amount of secreted rhIFN-γ continues to increase up to the 6th dpi but no further increase was observed on the 7th dpi as revealed in Figure 2B. This finding was also confirmed by western blot analysis (*data not shown*). Therefore, supernatant from the fifth day post-infection was chosen and collected for further purification of the secreted rhIFN-γ in the culture supernatant by subjecting to Ni-immobilized His-Binding affinity column chromatography and yielded about 39 ±1 μg that is 46.5% of the total protein recovered from the virus-infected cell culture medium ***(refer to Supplementary data 1-Table 1)***. This observation is true at conditions when the baculovirus *polh* promoter controls the transcription initiation, the recombinant proteins are efficiently expressed in infected insect cells; generally, it ranges from 30% to 50% of the total insect protein in late stages of infection.[28)]

<Insert Figures 2A, 2B, and 2C here>

Expression of glycosylated human interferon-gamma

Baculovirus-infected Sf21 insect cells

During the production of recombinant proteins, the influence of a host cell is of prime importance. Various cell types have different capacity to carryout post-translational modifications particularly on glycosylation that can greatly affect the biological function of the protein such as immunogenicity.[8)] The supernatant collected from the serum-free culture of baculovirus-infected Sf21 cells on 5th day post-infection containing the rhIFN-γ was purified and separated by SDS-PAGE and subject to being

detected by Western blot analysis with anti-IFN-γ antibody. Four distinct bands were seen at ~17.79, 19.41, 21.44 and 22.61 kDa on the Coomassie blue stained gel (Figure 2C, lane CCB) as well as on the PVDF membrane (Figure 2C, lane WB) respectively; these findings suggest that four forms of secreted recombinant human interferon gamma were expressed by the baculovirus-insect cell system. Lectin blotting further confirms that the interferon gamma produced are glycosylated (Figure 2C, lane GNA) coinciding with the absence of the four bands at the same molecular weight region on the membrane as observed on the Western Blot membrane after incubating the cells with tunicamycin (Figure 2C, lane TN), a known inhibitor of *N*-glycosylation.

The baculovirus-infected Sf21 cells were grown in either TNM-FH insect medium containing 8% heat-inactivated fetal bovine serum or EX-CELL^{TM} 420 insect serum-free medium to assess and to compare the effect of serum supplementation on the level of expression of the recombinant hIFN-γ as analyzed by Western blot with anti-human IFN-γ. It was also observed that there is no significant difference upon comparing qualitatively the relative intensities of the four distinct bands with molecular weights in between 17-24 kDA corresponding to the glycosylated rhIFN-γ produced from serum supplemented and serum- free insect cell culture. Similar result was obtained when the purified rhIFN-γ from serum free baculovirus-infected Sf21 cell culture was subjected to Western Blot analysis. The findings corroborate with indirect ELISA measurement of rhIFN-γ in which on the 5th day post infection, 2.413µg/mL was found to be present in supernatant of the serum supplemented while 2.132µg/mL in the serum free baculovirus-infected Sf21 cells *(data not shown)*.

Baculovirus infected larva of Trichoplusia ni and Spodoptera exigua

When the recombinant baculovirus, vAcIFN-γ-Rhir-E containing the human interferon-γ and *egfp* gene was inoculated to either *T. ni* or *S. exigua* larvae, the EGFP was expressed efficiently in the *T. ni* larvae as evidenced by the emission of green fluorescence when the larvae were observed under longwave ultraviolet radiation (Figure 3A). This result suggest that the *T. ni* larvae could effectively generate the recombinant target protein *in vivo*. However, the fluorescence was not observed on *S. exigua* that may be due to its thick and dark exoskeleton. Interestingly, upon infecting the *Spodotera exigua* larvae with the vAcIFN-γ-Rhir-E and with subsequent rhIFN-γ quantification of the larval homogenate by ELISA; there is a approximately 10-fold increase in the amount of rhIFN-γ observed compared to *T. ni* larval expression system, respectively as depicted in Figure 3B.

<Insert Figure 3A and 3B here>

Inhibitory Activity of rhIFN-γ against Dengue Virus

The ability of the rhIFN-γ to protect human cells (A549 cells) from the infection of dengue virus serotype PL046 was evaluated. It was reflected in Figure 4A that the rhIFN-γ produced from the vAcIFN-γ-Rhir-E -infected Sf21 cells grown in serum-free medium has the most potent activity in inhibiting the dengue virus from infecting the A549 cells compared to the rhIFN-γ generated from the screened cell lines. However, by comparing the activity of the rhIFN-γ generated from the three infected cell lines

used, it was found that the rhIFN-γ has a greater ability in inhibiting the dengue virus from infecting the A549 cells.

The Ni-NTA affinity column purified rhIFN-γ; the supernatants from serum supplemented and serum-free media of the vAcIFN-γ-Rhir-E-infected Sf21 cell; the larval homogenates from *T. ni* and *S. exigua* were used in the antiviral assay. It is clearly exhibited in Figure 4B that there is a comparable activity between the Ni-NTA purified rhIFN-γ and the commercially available one. At a multiplicity of infection equal to 0.1 (M.O.I. = 0.1), it was seen that a pronounced decrease in the viral titer was observed when the rhIFN-γ from the serum free culture medium was incubated with the dengue virus as judged against the serum supplemented and the Ni-NTA affinity column purified recombinant hIFN-γ *(data not shown)*. It is noteworthy to mention that though the amount of human interferon gamma produced from the serum-free culture is slightly lower as mentioned in the preceding section; still it can effectively inhibit 90% of the virus proliferation at a dose of 400 pg/mL than the rhIFN-γ from serum supplemented culture and the Ni-NTA purified.

In addition to this, the recombinant human interferon-γ present in the *T. ni* and *S.exigua* larval homogenate acted *at par* with the rhIFN-γ from serum supplemented and the Ni-NTA purified (data not shown) against the dengue virus. However among the samples tested against the activity of the dengue virus, the *T. ni* larval homogenate is considered to be most potent (Figure 4C).

<Insert Figure 4-A, B and C here>

Discussion

Successful expression of the human interferon gamma was achieved in this study owing to the advantages offered by the baculovirus expression system such as expression of high levels of foreign genes, carrying out of various post-translational modifications and the ability to efficiently scale up the target protein product.[29, 30] The RhPV 5'UTR virus IRES-based bicistronic baculovirus transfer vector used in this study provided a simple way to isolate a recombinant virus in which ease was brought about by the coupling of the enhanced green fluorescent protein with the hIFN-γ gene in the bicistronic vector. Not only the rhIFN-γ as a secreted protein is present in abundance in the culture medium but also EGFP served as a molecular marker to rule out whether contaminations may be present in the culture medium since EGFP is a cytosolic protein. Hence, the EGFP was used to monitor the effective secretion of the rhIFN-γ protein until the 5th day post-infection in the vAcIFN-γ-Rhir-E –infected Sf21 cell culture.

Typically, culturing of insect cells is done in basal media supplemented with about 10% verterbrate serum (*i.e.* fetal bovine serum) that supports cell growth, baculovirus infection and recombinant protein production. In this study, serum-free medium, EX-CELL™, was used for the expression of the rhIFN-γ in the insect cell culture. There is a very slight decrease in the amount of human interferon-γ produced in the serum-free medium (2.13 μg/mL) than in the serum supplemented (2.41 μg/mL) as observed in the Western blot analysis and ELISA, even so, the baculovirus expression system was able to efficiently express the targeted secreted protein- human interferon gamma. Based on literature survey, during the baculovirus infection cycle polyhedron is expressed in the

very late stage and the polyhedrin promoter is switched on about 24 hours after infection.[31] The comparable level of generation of recombinant hIFN-γ therefore suggests that serum is required to stimulate recombinant protein production pending the activation of polyhedrin promoter. However, the precise mechanism on how serum put forth a promoting effect on the baculovirus expression system for protein production remains elusive since it was observed that both cell lines from *S. exigua* and *S. litura* grown in serum supplemented medium gave a decreased expression rhIFN-γ.

Assessment of the antiviral activity of the vAcIFN-γ-Rhir-E-infected insect culture supernatants from serum-free, serum supplemented and Ni-NTA purified was performed and the findings indicated that the serum free medium was more active than the other samples assayed against the dengue virus. This denotes that during the post-translational modifications, the serum in the medium somehow affected the integrity and the bioactivity of the generated protein given that the addition of serum to culture media imposes several disadvantages such as the potential contamination by disease-causing agents and proteinaceous infectious molecules; the difficulty in the downstream processing and purification of the targeted protein product.[32]

In insect cells such as Sf9 cells, processing of recombinant INF-γ appeared to contain components with variable Asparagine (Asn) sites. Studies have shown that the glycosylation in Sf9 cells forms oligomannose type *N*-glycans.[8, 33] In this study, the Western blot analysis of the generated rhIFN-γ was found to be glycosylated as confirmed by the Lectin blot analysis.[34] The molecular weights (~17.79, 19.41, 21.44 and 22.61 kDa) obtained from this study corresponding to the non-glycosylated

(17.79kDa) and the glycosylated (19.41, 21.44 and 22.61 kDa) recombinant hIFN-γ are consistent with those reported.[35] Tunicamycin (TN) is a known inhibitor of glycosylation. TN changes the composition of the cell surface glycoproteins by preventing the bonding of *N*-acetylglucosamine 1- phosphate to the intermediate lipid carrier, dolichol phosphate. The use of tunicamycin verified further the presence of the carbohydrate moiety in the recombinant hIFN-γ at MW ~19.41, 21.44 and 22.61 kDa, since upon subjecting the tunicamycin treated supernatant to Western blot analysis with anti-hIFN-γ antibody, only a single bond at ~17.79 kDa was detected implying the presence of only the non-glycosylated recombinant hIFN-γ.

The larva-based baculovirus system employed in this study is a cost-efficient tool for the production of recombinant hIFN-γ. This system offers a potential for utilization in the large scale expression of the target protein since the vAcIFN-γ-Rhir-E- infected *Spodoptera exigua* larva was able to generate the recombinant hIFN-γ at about 9. 74 μg/mg larva which is 10x higher compared to that produced from the vAcIFN-γ-Rhir-E-infected- *Trichoplusi ni* (1.08 μg/mg larva). On the other hand, the recombinant human interferon-γ formed from the vAcIFN-γ-Rhir-E-infected *T. ni* larvae is more potent in protecting the human cells from the infection of the dengue virus serotype PL046. The antiviral activity of rhIFN-γ may have been affected by the heterogeneity of the recombinant protein and the host cell in which the target protein is expressed[3, 36, 37] and the half-life of the recombinant protein resulting from different *N*-glycan processing. The non-uniformity in the structure of the human interferon-γ from different expression systems is variable on the following: (a) proteolysis; (b) Asn site occupancy and (c) *N*-

glycan processing with emphasis on the latter as the most host specific.[8] Relying on our basic knowledge and understanding about the molecular requisites for the INF-γ bioactivity, the action of this lymphokine may be predicted since an intact C- and N-terminus is essential for the complete activity of INF-γ.[38] It can be hypothesized that the increased antiviral activity of could have been a result of a different *N*-glycan processing in the *T. ni* larva.

The amount of the purified recombinant hIFN-γ (39 μg ~ 46.5 % of the total protein) from the serum-free medium acquired in this study is as good as those yields reported when the hIFN-γ was expressed in different systems like the *E. coli* where 32% purified bioactive hIFN-γ was recovered[39] and the high density cultivation technique by Khalilzadeh and co-workers[13] where 0.35 ± 0.02 g rhIFN-γ/ g dry *E. coli* cell weight was generated; about 699.79 ng/g cell of secretory and intracellular bioactive human interferon gamma against dengue virus rhIFN-γ was afforded from the transgenic rice suspension cells.[10]

In conclusion, the present study demonstrates the applicability of the RhPV 5'UTR IRES-based baculovirus insect/larval expression system as a valuable instrument for a simple and high level production of recombinant human interferon-γ with innate bioactivity. The effect of serum in the quality of the protein produced should be taken into greater consideration so as to ensure that the efficacy of the protein product is not compromised. Moreover, the slightly diminished activity of the rhIFN-γ from the *S. exigua* exhibits the potential tradeoff between product quantity and quality that must be evaluated further in the use of larval expression system after all these observable facts

may have an implication toward the development of new therapeutic avenues in the field of vaccine and antiviral research.

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List of Figures

Figure 1

Analysis of the yield of rhIFN-γ expression in insect cells infected with vAcIFN-γ-Rhir-E .

(A) The bi-cistronic baculovirus construct, vAcIFN-γ-Rhir-E. P_H, polyhedrin promoter; hIFN- γ, human interferon-gamma gene; RhPV-IRES, element of RhPV 5' IRES; EGFP, enhance green fluorescence protein gene.

(B) Recombinant baculovirus, vAcIFN-γ-Rhir-E was generated and propagated in Sf21 cells. The cells were infected with vAcIFN-γ-Rhir-E (at an MOI of 1); the progeny of the virus could easily be observed by fluorescence microscopy. Pictures were taken at the same field under phase contrast (B) or with a conventional FITC channel with a 450/490 filter set (C). Picture was taken at an exposure time of 264ms. Scale bar, 30 μm.

(C) Screening of *Spodoptera frugiperda* 21 (Sf21), *Spodoptera exigua (S. exigua)* and *Spodoptera litura* (*S. litura*) insect cell lines infected with vAcIFN-γ-Rhir-E for production of IFN-γ protein and comparison of the expression levels of infected Sf21 cells between serum-free and serum-supplemented medium.

Figure 2

Time course experiment on EGFP and rhIFN-γ production in infected Sf21 cells and the expression pattern of purified rhIFN-γ protein.

(A) The lysis of infected cells was determined by the release of EGFP. Sf21 cells were infected with vAcIFN-γ-Rhir-E and the supernatants were collected at different time for western blot analysis with anti-EGFP polyclonal antibody. (B) The expression of rhIFN-γ protein was quantified by indirect ELISA.

(C) The purification procedure was performed as described in "Materials and Methods." Purified rhIFN-γ protein was separated utilizing SDS-PAGE and analyzed by Coomassie blue staining (CCB), western blot (WB) with the polyclonal antibody against IFN-γ and lectin blot (GNA) with the digoxigenylated lectin against terminal mannose. Tunicamycin (TN) was added to the serum-free medium, only the non-glycosylated form of IFN-γ is seen.

Figure 3

Analysis of the amount of rhIFN-γ expression in infected insect larvae.

(A) The *Trichoplusia ni* (*T. ni*) larvae were inoculated with vAcIFN-γ-Rhir-E and emitted green fluorescence at 4 dpi under UV illumination, scale bar, 1 mm.

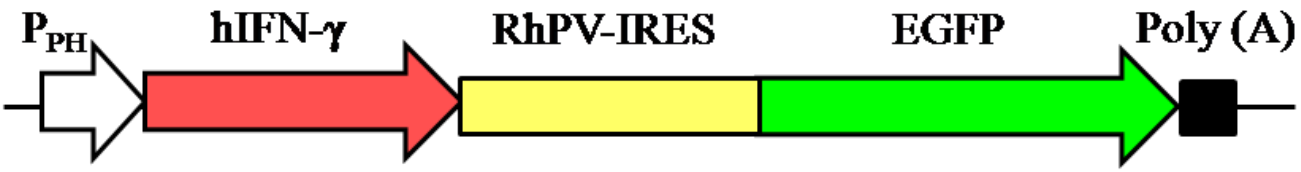
(B) The *S. exigua* and *T. ni* larvae were injected with vAcIFN-γ-Rhir-E. The homogenates were collected and quantified by indirect ELISA for the expression of rhIFN-γ protein.

Figure 4

Anti-viral activity of rhIFN-γ produced in infected insect cells or larvae.

Human A549 cells were pre-treated with (A) crude supernatants of infected *S. exigua* and *S. litura* cells as well as Sf21 cells with serum-free or serum-supplemented medium , (B) the purified IFN-γ available and the commercially IFN-γ standard and (C) the homogenates of infected *S. exigua* and *T. ni* larvae. Samples were diluted into various concentrations, then infected with dengue virus (MOI = 0.1). After virus adsorption, cells were replenished with medium, plus test samples, and incubated for 2 days, The virus titers, plaque forming units (PFU)/ml, were determined by plaque assay as described in “Materials and methods.”

(A)



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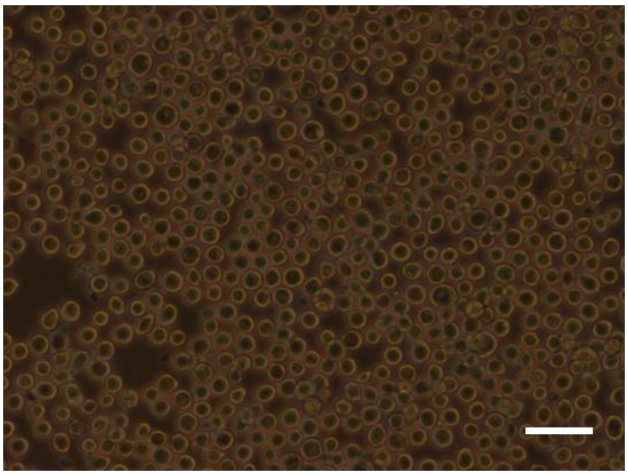

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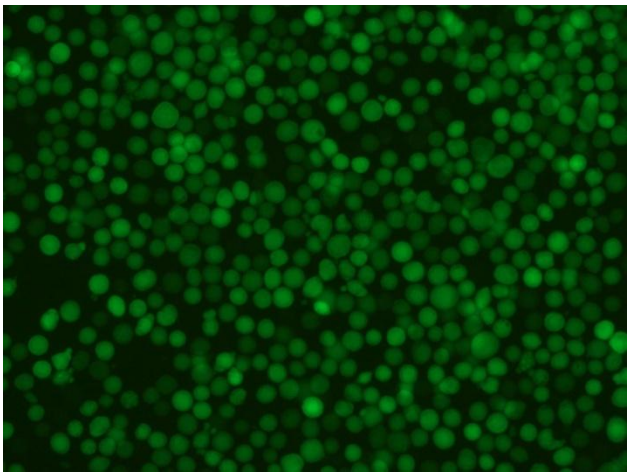

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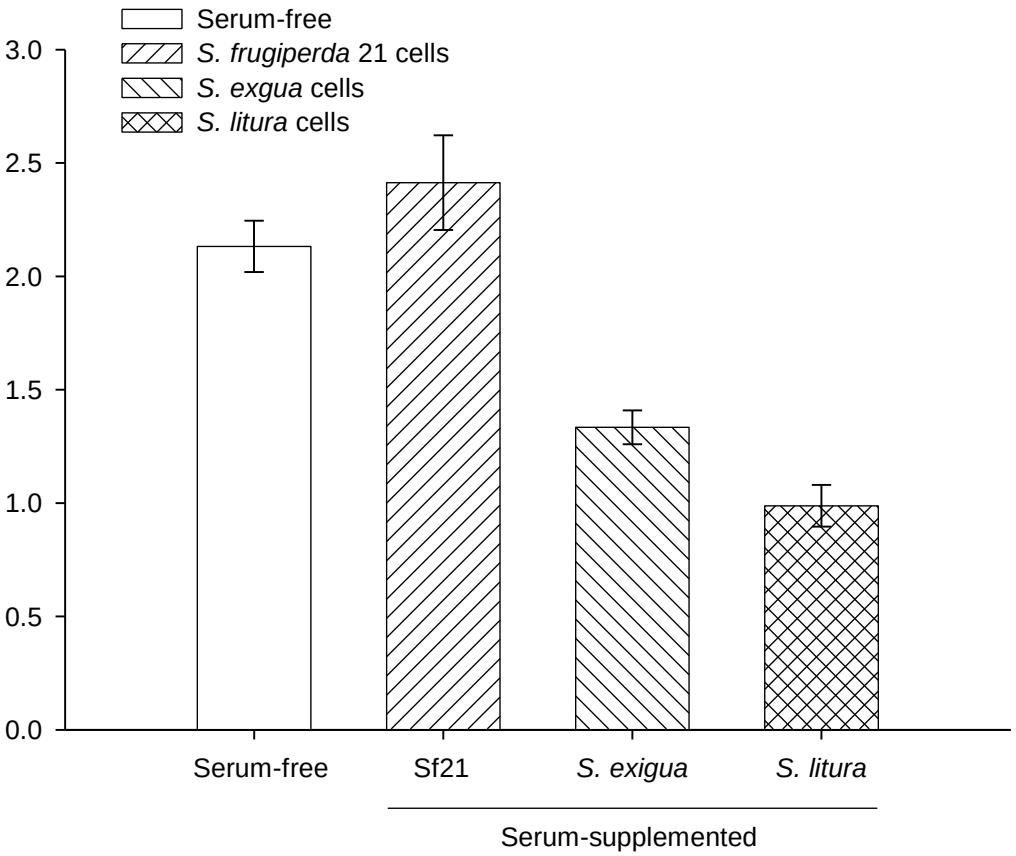

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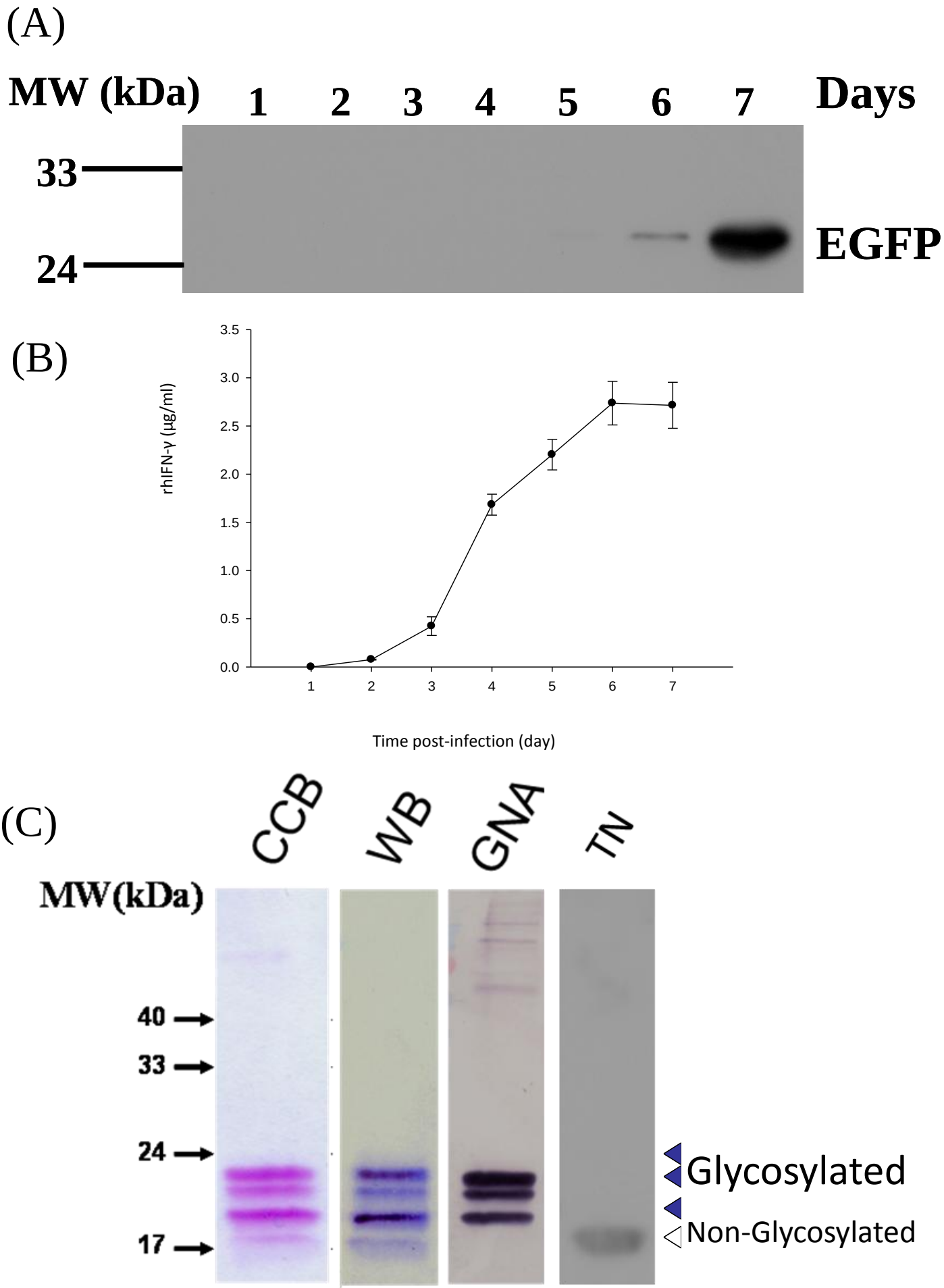

Fig. 2 W.S. Chen *et al.*

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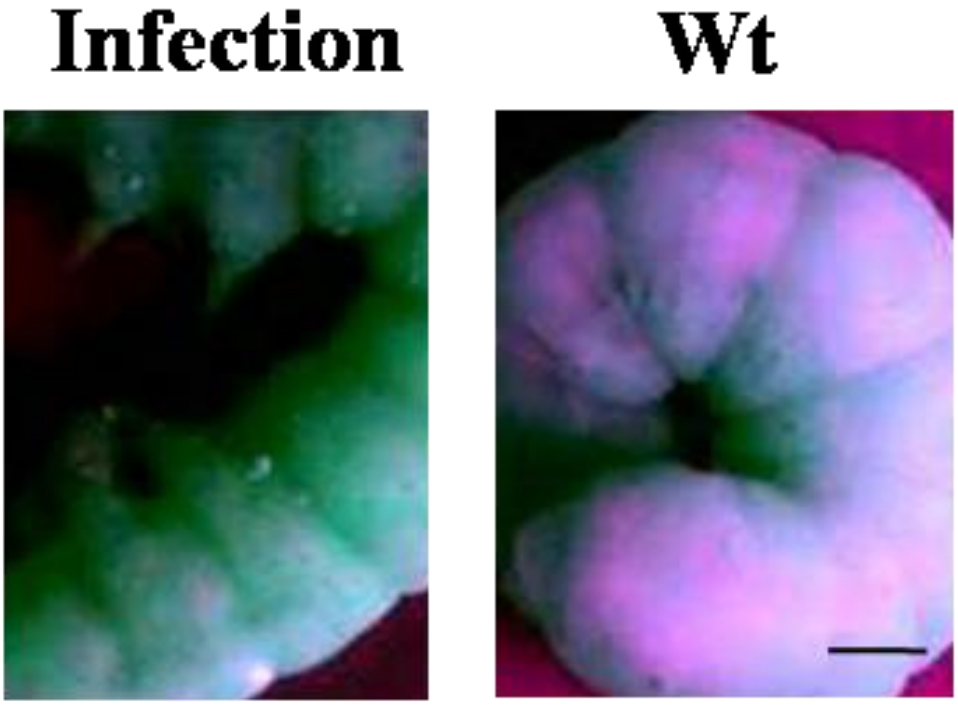

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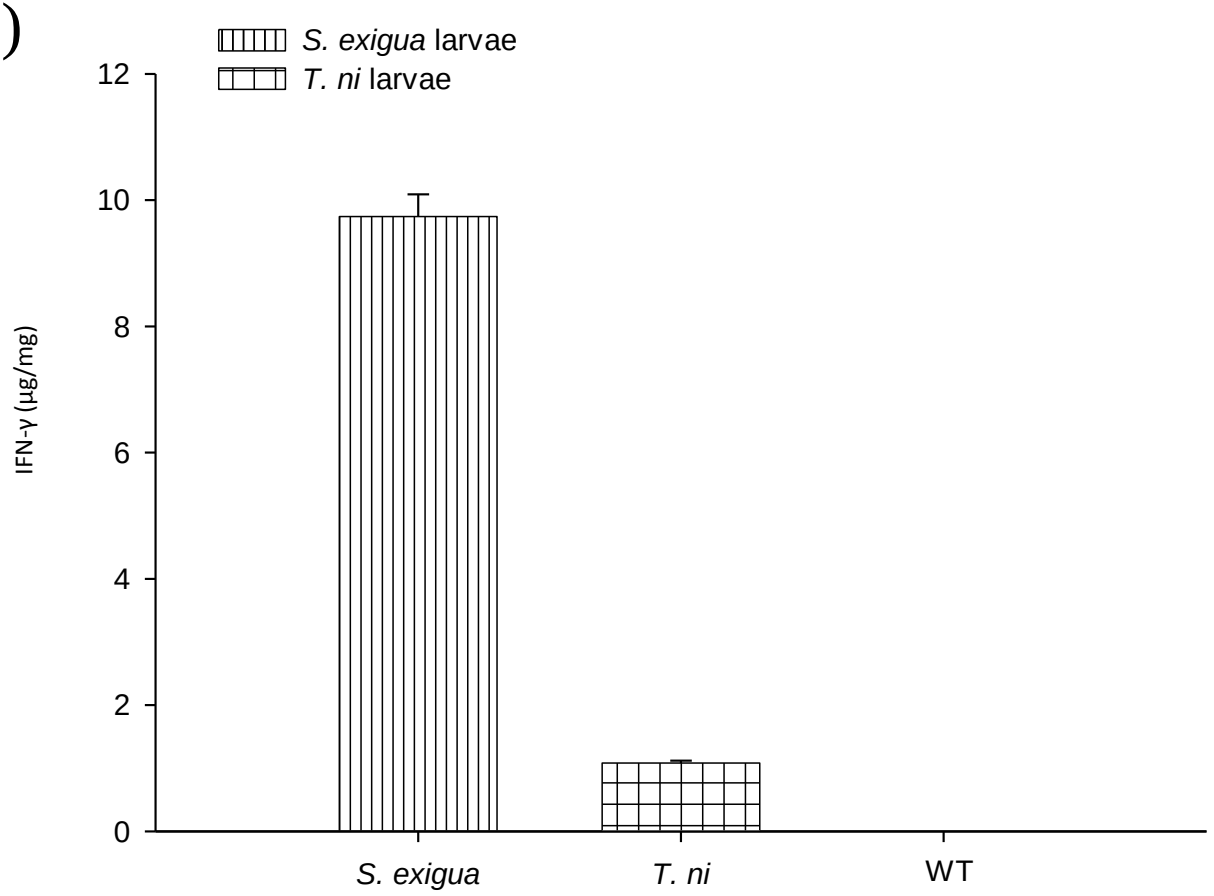

Fig. 3 W. S. Chen *et al.*

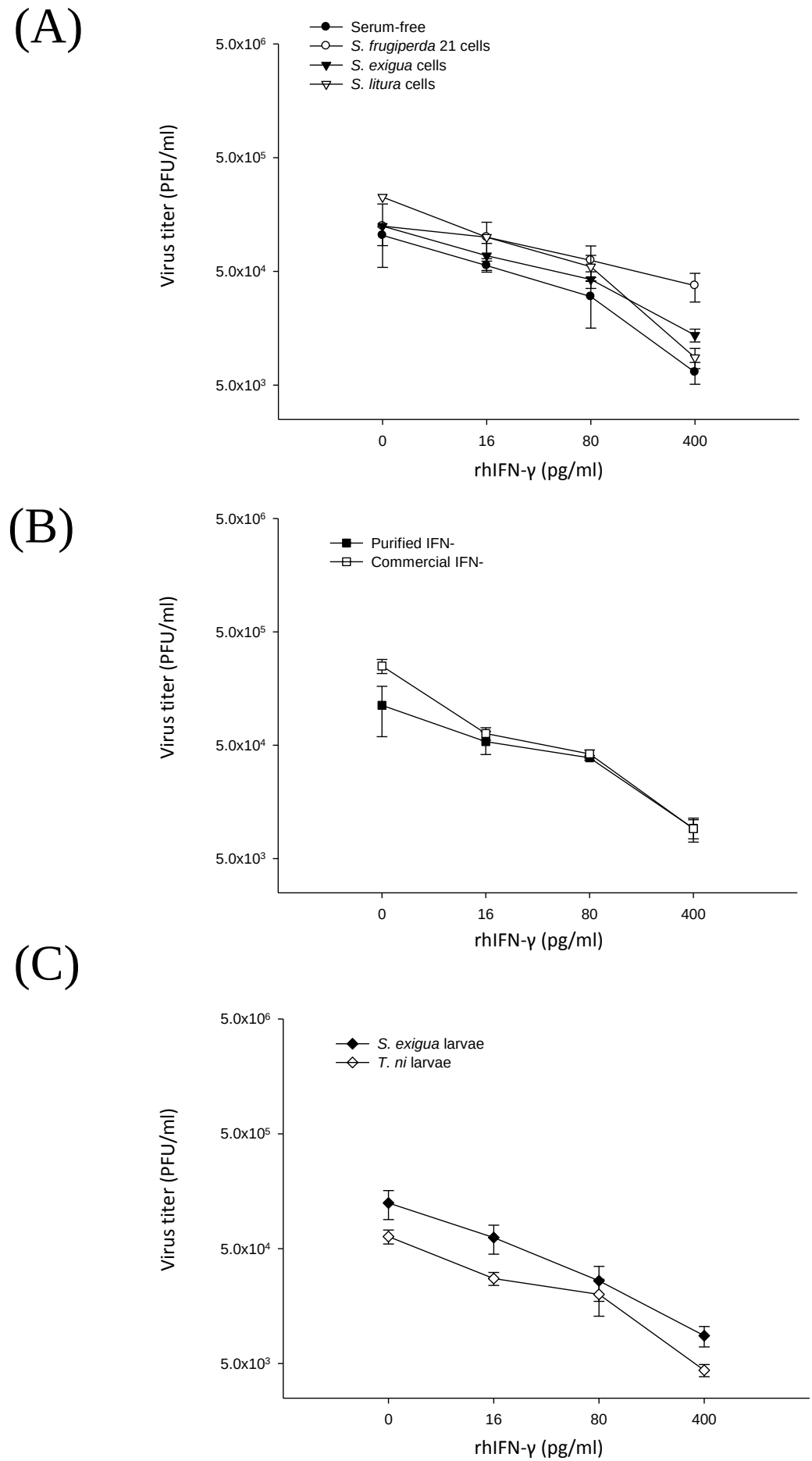

Fig. 4 W.S. Chen *et al.*

Supplementary Data 1

Table 1.

Purification Summary for r rhIFN-γ Produced by vAcIFN-γ-Rhir-E-infected Sf21 Insect Cells[a]

Steps	Total protein (mg)[b]	rhIFN-γ (μg)[c]	Specific Activity (μg/mg)[d]	Recovery yield (%)	Purification (fold)
Conditioned media[e]	1.8±0.2	84±4	47±3	100	1.0
Histag affinity	0.038±0.002	39±1	1027.5±27.5	46.5±8.5	21.9

[a]Data are mean ± S.D. values from three experiments.
[b]Total protein mass was estimated by protein assay kit (Pierce) with BSA as a standard.
[c] rhIFN-γ protein mass was determined by quantity analysis of ELISA.
[d] Specific activity = (rhIFN-γ) / (Total protein).
[e] 40 ml of culture media was used for the purification of rhIFN-γ.