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Novel recombinant feline interferon carrying N-glycans with reduced allergy risk produced by a transgenic silkworm system

Sachi Minagawa^{1*} , Yuzuru Nakaso¹, Masahiro Tomita², Takenori Igarashi², Yoshio Miura¹, Hideyo Yasuda¹ and Satoshi Sekiguchi¹

Abstract

Background: The generation of recombinant proteins for commercialisation must be cost-effective. Despite the cost-effective production of recombinant feline interferon (rFeIFN) by a baculovirus expression system, this rFeIFN carries insect-type N-glycans, with core α 1,3 fucosyl residues that act as potential allergens. An alternative method of production may yield recombinant glycoproteins with reduced antigenicity.

Results: A cDNA clone encoding the fifteenth subtype of FeIFN- α (FeIFN- α 15) was isolated from a Japanese domestic cat. This clone encoded a protein of 189 amino acids with a molecular mass of 21.1 kDa. The rFeIFN- α 15 was expressed using a transgenic silkworm system, which was expected to yield an N-glycan structure with reduced antigenicity compared with the protein produced by the baculovirus system. The resulting rFeIFN- α 15 accumulated in the sericin layer of silk fibres and was easily extracted and purified by column chromatography. The N-terminal amino acid sequence of purified rFeIFN- α 15 was identical to the mature form of natural sequence. Moreover, its N-glycans did not include detectable core α 1,3 fucosyl residues. Its anti-vesicular stomatitis virus activity (2.6×10^8 units/mg protein) was comparable to that of the baculovirus-expressed rFeIFN.

Conclusions: The lower allergy risk of rFeIFN produced by the transgenic silkworm system than by the baculovirus expression system is due to the former lacking core α 1,3 fucosyl residues in its N-glycans. The rFeIFN- α 15 produced by the transgenic silkworm system may be a prospective candidate for the next generation of rFeIFN in veterinary medicine.

Keywords: Feline interferon, Transgenic, Silkworm, N-glycan, Core α 1,3 fucosyl residues, Fucose, *Bombyx mori*

Background

Recombinant proteins generated for medical use should retain as much of their original structures as possible, including amino acid sequences and attached glycans. Any structural changes in these proteins may affect their bioactivity and/or bioavailability and may also increase the side effects associated with their use [1–4]. To avoid these shortcomings, despite their high production costs, most recombinant proteins used in humans are produced using mammalian cell lines.

A cost-effective method of producing recombinant proteins for veterinary medicine involves the use of the

silkworm moth (*Bombyx mori*). Larvae or insect cell lines are infected with recombinant baculovirus [5–8], with the recombinant proteins accumulating in larval body fluid or the cultured cells. The post-translational modification pathways in insects such as silkworms are similar to those in mammalian cells, and various recombinant glycoproteins have been successfully produced. For example, commercially available recombinant FeIFN- ω (NCBI Number: E02521) was produced in silkworms using the baculovirus expression system [9–12].

Despite the cost advantages of the baculovirus expression system, there is concern with the structures of the N-glycans attached to the recombinant protein products. These are insect-type N-glycans, with a high percentage of core α 1,3 fucosyl residues. These core α 1,3 fucosyl residues have been found to induce allergic reactions

* Correspondence: s-minagawa@nippon.co.jp

¹Innovation Center, Nippon Flour Mills Co., Ltd., 5-1-3 Midorigaoka, Atsugi, Kanagawa 243-0041, Japan

Full list of author information is available at the end of the article



when administered to mammals [13]. In fact, the rFeIFN- ω produced by this system is thought to contain core α 1,3 fucosylated N-glycans [14, 15]. Although the rFeIFN- ω has over 20 years of clinical experience, eliminating the insect type N-glycan is thought to be beneficial to improve the quality of veterinary medications in terms of potential allergenicity of core α 1,3 fucosyl residues [13, 16].

For this purpose in addition to the low cost feature of silkworm systems, we decided to use a transgenic system using silkworms [17–20]. In this system, foreign DNA carried on a vector plasmid is injected into silkworm eggs and is incorporated into the silkworm genome. Recombinant proteins synthesised in the middle silk gland (MSG) are secreted into the sericin layers of silk fibres of cocoons, allowing these proteins to be easily solubilised in aqueous buffer.

The N-glycan profile of recombinant glycoproteins produced by this transgenic silkworm system was assessed using recombinant mouse IgG [20]. These experiments showed that the N-glycans attached to the recombinant IgG did not include typical insect-type oligosaccharides, paucimannosidic glycans with core α 1,3 fucosyl residues [21–23]. Because the sericultural industry requires the production of large numbers of silkworm cocoons annually, the transgenic silkworm system may become a basic technology for the cost-effective production of recombinant proteins carrying non-allergic N-glycans.

Using a cDNA clone encoding a newly discovered FeIFN α subtype, FeIFN- α 15, isolated from the blood of a Japanese domestic cat, this study evaluated the properties of rFeIFN produced by the transgenic silkworm system.

Results

Isolation of a clone of FeIFN

Total mRNA was isolated from leucocytes of a domestic cat, and a FeIFN clone was isolated by PCR using primers designed from the sequences of FeIFN 7 and FeIFN 8. The open reading frame of the FeIFN clone consisted of 567 base pairs, predicted to encode a 189 amino acid protein with a molecular mass of 21.1 kDa (Fig. 1). An alignment with reported sequences (Fig. 1) showed that the amino acid sequence of this FeIFN clone was very similar to those of other FeIFNs, including type α subtypes 1–14 and type ω . Although subtypes α 5, α 7, and ω contained a five amino acid insertion, this clone did not (Fig. 2). The clone had a predicted signal peptide sequence at amino acids 1–23 (underlined) and a consensus site for asparagine-linked glycosylation (double underlined). The FeIFN is almost identical to α 3, 9, 13, and 14 with the exception of four amino acid residues. Based on its amino acid sequence, the gene encoding this clone was defined as FeIFN- α 15.

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M A L P S S F L V A L V A L G C N S V C 60
ATGGCGCTGCCTCTTCTTGGTGGCCCTGGTGGCGTGGGCTGCAACTCCGTCTGC

S L G C D L P Q T H G L L N R R A L T L 120
TCTCTGGCTGTGACCTGCCTCAGACCCACGGCTGCTGAACAGGAGGGCCTTGACGCTC

L G Q M R R L P A S S C Q K D R N D F A 180
CTGGGACAAATGAGGAGACTCCCTGCCAGCTCCTGTGAGAAGGACGAAATGACTTCGCC

F P Q D V F G G D Q S P K A Q A L S V V 240
TTCCCCAGGACGTGTTCCGGTGGAGACCAGTCCCCAAGGCCAAGCCCTCTCGTGGTGG

H V T N Q K I F H F F C T G A S S S A A 300
CACGTGACGACCAGAAGATCTTCCACTTCTTCTGCACAGGGGCGCTCCTCGTCTGTGCT

W N T T L L E E F C T G L D R Q L T R L 360
TGGAAGCACCACCTCTGGAGGAATTTGCACGGGACTTGATCGGAGCTGACCCCGCTG

E A C V L Q E V G E G E A P L T N E D S 420
GAAGCTGTGTCTCGCAGGAGGTGGGGAGGGAGAGGCTCCCTCACGAACGAGGACTCC

L L R N Y F O R L S L Y L Q E K K Y S P 480
CTCCTGAGGAAGACTTCCAAAGACTCTCCCTACTCTGCAAGAGAAGAAATACAGCCCT

C A W E I V R A E I M R S L Y Y S S T A 540
TGTGCTGGGAGATCGTCAGAGCAGAAATCATGAGATCCTTGATTATTCATCAACAGCC

L Q K R L R S E K * 600
TTGCAGAAAAGATTAAGGAGCGGAAATAA

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Fig. 1 FeIFN- α 15 cDNA and deduced amino acid sequences. Amino acids 1–23 (underlined) are thought to be a signal sequence. The double underline indicates a consensus site for asparagine-linked glycosylation

Purification of rFeIFN- α 15 from cocoons

Using transformation vectors (Fig. 3), rFeIFN- α 15 was expressed in the cocoons of transgenic silkworms. The resulting rFeIFN- α 15 protein in the cocoons was dissolved in phosphate-buffered saline (PBS) and purified by two steps of column chromatography (Table 1). Starting with approximately 100 mg cocoons, 411 μ g of crude proteins were extracted, including 267 μ g rFeIFN- α 15, making the latter 65% pure, as determined by silver staining of SDS-PAGE gels. The crude extracts were chromatographed on a Blue Sepharose column, yielding 93% pure rFeIFN- α 15; this was followed by chromatography on a ceramic hydroxyapatite column, yielding 67 μ g of 99% pure rFeIFN- α 15 (Table 1 and Fig. 4). Electrophoresis of the purified rFeIFN- α 15 on SDS-PAGE gels followed by silver staining showed two main bands, a lower band at less than 20 kDa and an upper band at around 20 kDa, both close to the expected molecular weight of mature rFeIFN- α 15 (18.9 kDa). The upper and lower protein bands are likely the glycosylated and non-glycosylated proteins, respectively. The net recovery of rFeIFN- α 15 from the crude extract was calculated to be 25%.

N-terminal amino acid sequence of rFeIFN- α 15

The five N-terminal amino acids of purified rFeIFN were analysed by Edman degradation (Table 2). Because the

IFN alpha 15	1	MALPSSFLVALVALGCNSVCSLGCGLPQTHGLLNRRALTLGQMRRLPASSCQKDRNDF	60
IFN alpha 1	1S.....	60
IFN alpha 2	1	60
IFN alpha 3	1	60
IFN alpha 5	1S.....	60
IFN alpha 6	1	60
IFN alpha 7	1	..S.....	60
IFN alpha 8	1S.....C.....	60
IFN alpha 9	1	60
IFN alpha 10	1	60
IFN alpha 11	1	..S.....	60
IFN alpha 12	1	..S.....R.....	60
IFN alpha 13	1	60
IFN alpha 14	1	60
IFN omega	1	60
IFN alpha 15	61	FPQDVFVGGDQSPKAQLSVMVHTNQKIFHFHFFCTGASSSAANNTLLEEFCTGLDRQLTRL	120
IFN alpha 1	61H.....E.....W.....	120
IFN alpha 2	61H.....E.....	120
IFN alpha 3	61H.....E.....E.....	120
IFN alpha 5	61H.....E.....	120
IFN alpha 6	61H.....E.....	120
IFN alpha 7	61H.....E.....	120
IFN alpha 8	61H.....E.....	120
IFN alpha 9	61H.....E.....	120
IFN alpha 10	61H.....E.....	120
IFN alpha 11	61H.....E.....	120
IFN alpha 12	61H.....E.....	120
IFN alpha 13	61H.....E.....	120
IFN alpha 14	61H.....E.....	120
IFN omega	61H.....E.....	120
IFN alpha 15	121	EACVLQEVGEGEAPLTNED----SLLRNYFQRLSLYLQEKKYSPCAWEIVRAEIMRSLY	175
IFN alpha 1	121	..M.....I.....	175
IFN alpha 2	121	..M.....I.....	175
IFN alpha 3	121	..V.....	175
IFN alpha 5	121	..V.....IHPED.I.....	180
IFN alpha 6	121	..V.....I.....	175
IFN alpha 7	121	..E.....IHPED.I.....	180
IFN alpha 8	121	..V.....I.....	175
IFN alpha 9	121	..V.....I.....	175
IFN alpha 10	121	..V.....I.....	175
IFN alpha 11	121	..V.....I.....	175
IFN alpha 12	121	..V.....I.....	175
IFN alpha 13	121	..V.....I.....	175
IFN alpha 14	121	..V.....T.....	175
IFN omega	121	..E.....IHPED.I.....	180
IFN alpha 15	176	YSSTALQKRLRSEK	189
IFN alpha 1	176	189
IFN alpha 2	176	189
IFN alpha 3	176	189
IFN alpha 5	181	194
IFN alpha 6	176	189
IFN alpha 7	181	194
IFN alpha 8	176	189
IFN alpha 9	176	189
IFN alpha 10	176	.T.....	189
IFN alpha 11	176	189
IFN alpha 12	176	189
IFN alpha 13	176	188
IFN alpha 14	176	188
IFN omega	181	194

Fig. 2 Homology of amino acid sequences of feline IFN alpha subtypes 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 and IFN omega and IFN alpha subtype 15 (this study). The GenBank accession numbers of these proteins are AY117395, AY117394, AY117393, AY117392, AY117391, AB094996, AB094997, AB094998, AB094999, AB095000, AB095001, AB095002, AB095003, and E02521, respectively [9, 23, 24]

cDNA sequence indicated that the N-terminal residue of mature FeIFN-α15 was cysteine, the first degradation cycle did not show any peak as expected. The second to fifth amino acids were found to be D, L, P, and Q, consistent with the deduced amino acid sequence from the cDNA. The signal sequence of this protein was likely processed correctly for secretion into the cocoons.

Glycosidase digestion of rFeIFN-α15

Glycosylation of purified rFeIFN-α15 was assessed by blotting with an anti-IFN antibody and concanavalin A (Con A), which recognises mannose residues (Fig. 5).

Treatment of the sample with glycosidase F in the presence of both SDS and NP-40 (Fig. 5, lane 1) resulted in an almost complete absence of the upper, 20-kDa protein band compared with the untreated control (Fig. 5, lane 3), probably because treatment with this enzyme removed all N-glycan residues and shifted its molecular weight to that of the lower, < 20-kDa protein band. This lower band was stained by both CBB (lane 1) and anti-FeIFN antibody (lane 4), but not by ConA (lane 7). In contrast, treatment with the enzyme in the absence of SDS and NP-40 or non-treatment with the enzyme resulted in both protein bands being stained by CBB (lanes 2 and 3) and

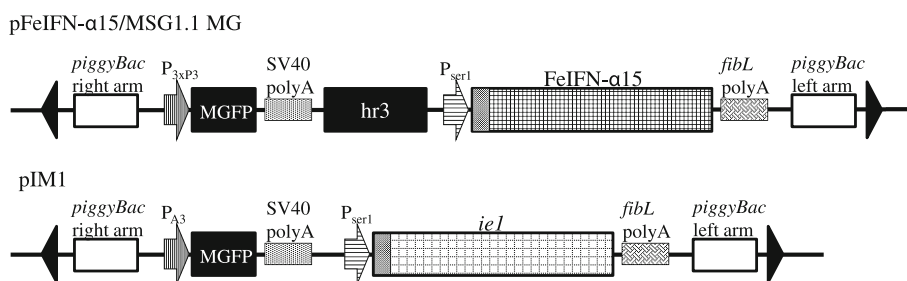


Fig. 3 Structures of the transformation vectors pFeIFN-α15/MSG1.1 MG and pIM1. Each vector consisted of expression units for a selection marker and the recombinant protein, located between the right and left arms of *piggyBac*. The selection marker *MGFP* was placed between the 3xP3 promoter (P_{3xP3}) and the SV40 polyA signal sequence (SV40 polyA) and between the *B. mori* actin promoter (P_{A3}) and SV40 polyA in pFeIFN-α15/MSG1.1MG and pIM1 vectors, respectively. The cDNA of FeIFN-α15 on pFeIFN-α15/MSG1.1 MG was placed between the ser1 promoter (P_{ser1}) and the fibroin L-chain polyA signal sequence (*fibL* polyA). The BmNPV hr3 enhancer was located upstream of P_{ser1} , and the IE1 trans activator gene (*ie1*) on pIM1 was placed between P_{ser1} and *fibL* polyA

anti-FeIFN antibody (lanes 5 and 6), although only the higher molecular weight band reacted with ConA (lanes 8 and 9). These results indicated that 20-kDa and < 20-kDa protein bands represented the N-glycosylated and non-glycosylated forms of rFeIFN-α15, respectively.

Structure of N-glycans of rFeIFN-α15

Treatment of purified rFeIFN-α15 with pepsin followed by digestion with glycosidase A resulted in the release of N-glycans. The reducing ends of the N-glycans were labelled with 2-aminopyridine (PA), with each PA-oligosaccharide identified by column chromatography and mass spectrometric analysis. The N-glycan profile of rFeIFN-α15 is summarised in Table 3. Five PA-oligosaccharide fractions were obtained by column chromatography and were shown to be Man2Man3GlcNAc2-PA (M5), GlcNAcMan3GlcNAc2-PA (GNa), $GlcNAc_{2-6}$ Man3GlcNAc2-PA (GNb), GlcNAc2Man3GlcNAc2-PA (GN2), and Man3GlcNAc2-PA (M3). The most abundant N-glycan, constituting 58.2% of the total, was M5 mannose-type, with about 30% being complex and hybrid types. About 10% of the total N-glycan was paucimannose-type (M3). There was no evidence of the existence of high-mannose-type (M6 or higher) or core-fucosylated glycans.

In vitro anti-vesicular stomatitis virus activity

The titre of rFeIFN-α15 against vesicular stomatitis virus (VSV), measured using Fcwf-4 cells, was found to be 6.2×10^7 units/mg in the crude extract and 2.6×10^8 units/

mg in the protein obtained after Blue Sepharose column chromatography (Table 4). It was reported that the anti-VSV titre of the baculovirus-produced rFeIFN-ω measured by using Fc9 cells was 2.4×10^8 units/mg [14]. Although the feline cell lines used in the measurements differed, the rFeIFN-α15 and rFeIFN-ω are likely to possess VSV activities at similar levels.

Discussion

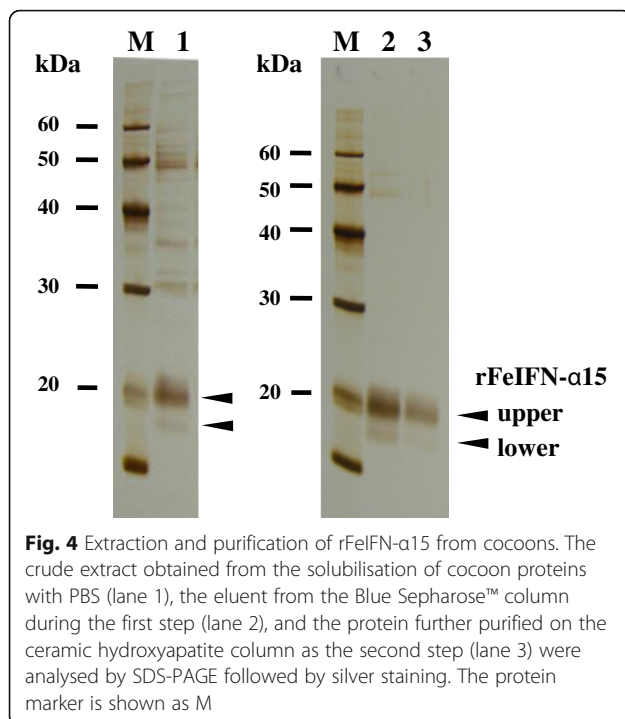
To date, 14 subtypes of FeIFN-α have been reported [24, 25]. In the present study, we cloned the fifteenth subtype of feline interferon α gene, named FeIFN-α15. We compared the protein product of this gene with rFeIFN-ω (NCBI Number: E02521), classified as a FeIFN-α [24–26], produced in silkworm larvae using the baculovirus expression system. Expression of rFeIFN-ω from its original signal sequence in silkworm larvae resulted in processing of the N-terminal peptide at a site different from the natural version [27]. Because the peptide sequence of human calreticulin has been reported to be processed at the correct site in MSG [28], we used the signal sequence of human calreticulin for the expression of FeIFN-α15 in this study. We found that this signal sequence was processed at the correct site and that the mature form of rFeIFN-α15, carrying its natural N-terminal sequence, was secreted into cocoons.

The N-glycan structure of rFeIFN-α15 was assessed using glycosidase F, an enzyme that can cleave N-glycan linkages in the presence of both SDS and NP-40 and when the α 1,3 fucose is not attached to the core N-acetylglucosamine (GlcNAc). The results obtained indicated that N-glycans attached to the rFeIFN-α15 did not contain core α 1,3 fucosyl residues. Furthermore, comparison of the N-glycan structures of the rFeIFN-α15 produced by the transgenic silkworm system during expression in MSG and of the ω type produced by the baculovirus expression system in entire silkworm larvae [10] showed noticeable differences between them. The N-glycan profile of rFeIFN-α15 by the transgenic silkworm system in MSG consisted of about 30% hybrid-

Table 1 Purification profile of rFeIFN-α15 from 100 mg of cocoon

Purification Step	Total Protein (μg)	Amount of rFeIFN-α15(μg)	Purity of rFeIFN-α15(%)
Crude Extract	411	267	65
Blue Sepharose™	107	100	93
Ceramic Hydroxyapatite	68	67	99

The amounts (middle) and purities (right) of rFeIFN-α15 relative to total protein (left) during each purification step are described



and complex-types, 58% M5, and 10% paucimannose-type. No high-mannose-type (M6 or higher) nor core α 1,3 fucosylated glycans were detected. This profile differs markedly from the typical insect profile. Although the precise N-glycan structures of rFeIFN- ω have not been determined, the sugar chains were reported to consist of mannose, GlcNAc, and fucose, in a 2: 2: 1 ratio [14]. These data suggest that the N-glycans of rFeIFN- ω had a typical insect-type profile, with FM2 (Man₂GlcNAc₂-Fuc) thought to be a main N-glycan [14]. The N-glycan structures of recombinant mouse IFN- β produced by the baculovirus expression system were reported to consist of 20% high-mannose-type (more than M6), 51% paucimannose-type, and 12% core α 1,3-fucosylated glycans [29]. Thus, the N-glycan pattern of rFeIFN- α 15 synthesised in silk glands in this study differed from those

Table 2 N-terminal amino acid sequence of purified rFeIFN- α 15

Cycle no.	Amino acid	pmol
1	ND	–
2	D	26.1
3	L	18.9
4	P	10.5
5	Q	15.3

Purified rFeIFN- α 15 was separated on a SDS-PAGE gel, and its N-terminal amino acid sequence was determined by five cycles of automated Edman degradation. The N-terminal sequences of the two main bands on SDS-PAGE were determined. Results obtained from analysis of the larger, 20-kDa band are shown; results obtained with the < 20-kDa band were identical. ND: No amino acid was detected during the first cycle of degradation, suggesting that it was cysteine

of previously reported rIFNs synthesised in the baculovirus expression system, in which recombinant proteins were expressed in baculovirus-infected organs, such as fat bodies or haemolymph except silk glands.

These findings and those regarding IgG production in MSG [20] suggest that the mechanisms of N-glycosylation, especially core α 1,3 fucosylation, in MSG differ from those in other organs. The silk gland is specialised to produce proteins required for weaving cocoons, and MSG is involved in the production of sericin, which covers the fibroin core. This specific functional feature of MSG may be related to the specificity of N-glycan structure. Although detailed analysis of the glycosylation mechanism of MSG is necessary, it is thought that the specificity of the N-glycan structure in MSG provides a significant benefit with regard to the production of safe recombinant proteins with insects.

The anti-VSV titres of rFeIFN- α 7 and ω , both of which had a five amino acid insertion, were approximately 10 times higher than the titres of rFeIFN- α 8 and - α 10 without the insertion [30]. As these rIFNs were produced in *E. coli* without any N-glycans on them, the high anti-VSV titres of rFeIFN- α 7 and - ω have been attributed to the five amino acid insertion. However, our study showed that the anti-VSV titre of rFeIFN- α 15 (without the insertion) was comparable to that of rFeIFN- ω (with the insertion), suggesting that anti-VSV activity may be affected by both the N-glycan profile and amino acid insertions.

In this study, the anti-viral activity of the recombinant rFeIFN- α 15 was evaluated by in vitro assay with a single kind of virus, VSV. To demonstrate the clinical usability of the rFeIFN- α 15, in vivo studies for anti-viral activities to several kinds of feline infective viruses should be evaluated.

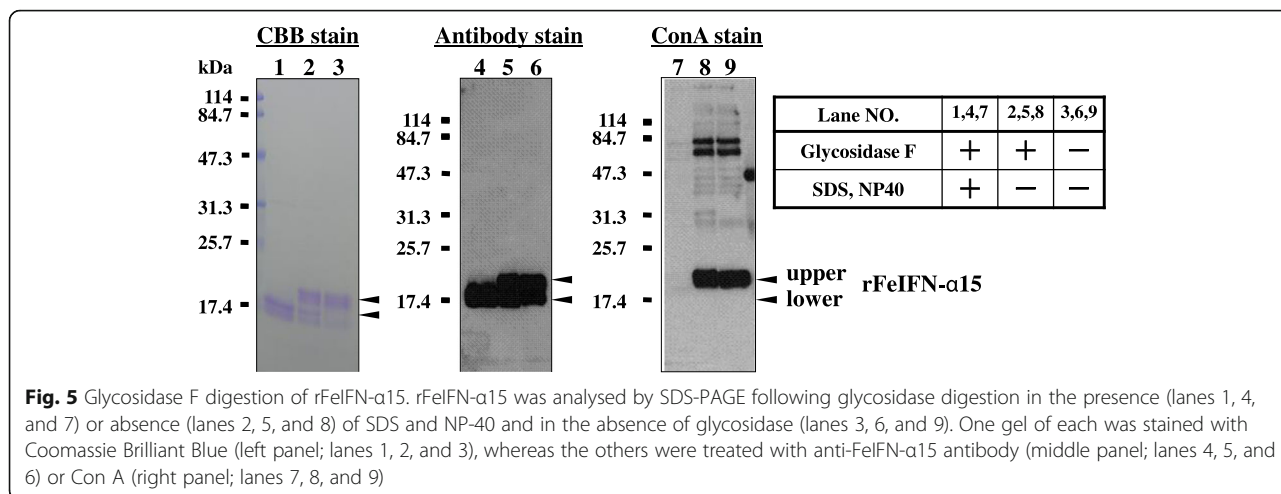
Conclusion

This study showed that the rFeIFN- α 15 produced by the transgenic silkworm system had the N-terminal amino acid sequence identical to the natural one and had no core α 1,3 fucosyl residues in its N-glycans. Additionally, the anti-VSV activity of the rFeIFN- α 15 assessed in vitro was comparable to that of rFeIFN- ω produced by the baculovirus expression system in silkworms. These results indicate that rFeIFN- α 15 may be useful in veterinary medication, but further studies including an assessment of in vivo anti-viral activity would be required to verify its availability.

Methods

Experimental animals

B. mori strain w1-pnd was obtained from the National Institute of Agrobiological Science (Tsukuba, Japan). The larvae were reared at 25 °C on an artificial diet (Silk MatePM; Nosan Corp, Kanagawa, Japan). Feline blood was obtained from a Japanese domestic cat



acting as a donor for blood transfusion. Fcwf-4 cells, the cell line derived from feline embryo fibroblasts, were purchased from the ATCC (ATCC no. CRL-2787).

Cloning of FeIFN-α and vector construction

Leukocytes were isolated from feline blood, and total RNA was extracted from the cells using TRIzol® LS Reagent (Thermo Fisher Scientific K.K., Yokohama, Japan). IFN-α cDNA was synthesised using a One-Step RT-PCR Kit (Invitrogen, Carlsbad, USA) with the two amplification primers, 5'- ATGGCGCTGCCCTCTTCCTTCTTGG-3' (forward) and 5'-TTTCTCGCTCCTTAATCTTTTCTGTC-3' (reversed) derived from the common sequences of IFNA7

and IFNA8 (GenBank Accession Nos. NM_001009197 and NM_001009193, respectively). The cDNA fragments obtained by reverse transcription polymerase chain reaction (RT-PCR) were sequenced. The cDNAs encoding the open reading frames of full-length FeIFN-α (IFNα family) were cloned. FeIFN-α15, a novel type of FeIFN-α, was also isolated (GenBank Accession No. AB608218). The predicted signal sequence region of FeIFN-α15 was replaced with that of human calreticulin, as this signal sequence has been reported to be processed at the correct site in MSG [28]. The PCR product amplified using the following primer sets was inserted into a cloning vector. The 5'-untranslated region (UTR) sequence of *Bombyx mori* nuclear polyhedrosis

Table 3 Structure of N-glycans attached to the rFeIFN-α15 produced by the transgenic silkworm system. The ratio (%) of each glycan was calculated from the corresponding peak area on the chromatogram obtained by HPLC

Glycan structure	Abbreviation	Peak area
$\begin{array}{l} \text{Man}\alpha 6 \\ \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagdown \\ \diagup \end{array} \begin{array}{l} \text{Man}\alpha 6 \\ \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagup \\ \diagdown \end{array} \text{Man}\beta 4 \text{GlcNAc}\beta 4 \text{GlcNAc-PA}$	M5	58.2 %
$\begin{array}{l} \text{GlcNAc}\beta 2 \text{Man}\alpha 6 \\ \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagdown \\ \diagup \end{array} \begin{array}{l} \text{Man}\alpha 6 \\ \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagup \\ \diagdown \end{array} \text{Man}\beta 4 \text{GlcNAc}\beta 4 \text{GlcNAc-PA}$	GNa	6.7%
$\begin{array}{l} \text{Man}\alpha 6 \\ \text{GlcNAc}\beta 2 \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagdown \\ \diagup \end{array} \begin{array}{l} \text{Man}\alpha 6 \\ \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagup \\ \diagdown \end{array} \text{Man}\beta 4 \text{GlcNAc}\beta 4 \text{GlcNAc-PA}$	GNb	7.9%
$\begin{array}{l} \text{GlcNAc}\beta 2 \text{Man}\alpha 6 \\ \text{GlcNAc}\beta 2 \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagdown \\ \diagup \end{array} \begin{array}{l} \text{Man}\alpha 6 \\ \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagup \\ \diagdown \end{array} \text{Man}\beta 4 \text{GlcNAc}\beta 4 \text{GlcNAc-PA}$	GN2	14.9%
$\begin{array}{l} \text{Man}\alpha 6 \\ \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagdown \\ \diagup \end{array} \begin{array}{l} \text{Man}\alpha 6 \\ \text{Man}\alpha 3 \end{array} \begin{array}{l} \diagup \\ \diagdown \end{array} \text{Man}\beta 4 \text{GlcNAc}\beta 4 \text{GlcNAc-PA}$	M3	10.4%

Table 4 Anti-VSV activity of rFeIFN- α 15 as measured using CPE assay

Sample	unit/mg
Crude extract	6.2×10^7
Blue Sepharose™ column elute	2.6×10^8

The inhibitory activity of rFeIFN- α against VSV infection of Fcwf-4 cells was determined by a 50% reduction in CPE. Commercially available rFeIFN- ω was used as a standard

virus (BmNPV) polyhedrin [31, 32] was inserted upstream of the cDNAs by this PCR amplification using the primers 5'-CACCCCGGGGAAGTATTTTACTGTTTTTCGTAACA GTTTTGTAATAAAAAACCTATAAATATGCTGCTAT CCGTGCCGTTGCTGCTCGGCCTCCTCGGCCTGGCC GTCGCCTGTGACCTGCCTCAGACCCACG-3' (forward) and 5'-CCCGGGTTATTTCTCGCTCCTTAATCT TTTCTGC-3' (reverse), where the bold letters indicate a restriction site for the enzyme *Sma*I. The resulting fragment was inserted into the pENTR_D-TOPO cloning vector (Invitrogen), released from the vector by digesting with *Sma*I, and inserted into the plasmid pMSG1.1MG to yield pFeIFN- α 15/MSG1.1MG for generation of transgenic silkworms [28, 32].

Generation of a TG silkworm line expressing rFeIFN- α 15

pFeIFN- α 15/MSG1.1MG was injected, along with the helper vector pHA3PIG [33], into pre-blastoderm silkworm embryos as described [33]. The hatched G0 larvae were reared to moths at 25 °C. G1 embryos were generated by mating siblings or w1-pnd and screened for ocular expression of monster green fluorescent protein (MGFP), with those showing positive expression defined as transgenic silkworms bearing the FeIFN- α 15 gene. These transgenic silkworms were subsequently mated with transgenic silkworms expressing the trans-activator IE1 in the MSG, as described [28] (Fig. 3), yielding transgenic silkworms expressing higher levels of rFeIFN- α 15.

Extraction and purification of rFeIFN- α 15 from cocoons

Sheared cocoons were immersed in PBS, pH 7.4, at 20 mg/ml and maintained at 4 °C for 24 h. The crude extract was centrifuged, with the supernatant dialysed against 10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl and applied to a Blue Sepharose™ column (HiTrap™ Blue, GE Healthcare, Tokyo, Japan). rFeIFN was eluted with a linear gradient concentration of buffer containing 1.0 M NaCl. The fractionated eluent from the Blue Sepharose™ column was analysed by SDS-PAGE. Fractions containing rFeIFN- α 15 were collected, dialysed against 5 mM potassium phosphate buffer, pH 7.0, 100 mM NaCl, and loaded onto a ceramic hydroxyapatite column. rFeIFN was eluted with a linear gradient concentration of buffer containing 500 mM NaCl. The purified rFeIFN- α 15 was used for various assays except for N-glycan analysis. For N-glycan

analysis of rFeIFN- α 15, the protein eluted from the Blue Sepharose™ column was dialysed against 5 mM potassium phosphate buffer, pH 7.0, 150 mM NaCl and purified on a Superdex 75 gel filtration column (GE Healthcare).

Total protein content in the extract and purified materials was quantified using a micro bicinchoninic acid protein assay kit (Thermo Fisher Scientific K.K.). The ratio of rFeIFN- α 15 to total protein was analysed by SDS-PAGE and silver staining, with the gel images scanned with ChemiDoc XRS (Bio-Rad, Tokyo, Japan). The amount of purified rFeIFN- α 15 was calculated from the total protein content and the ratio of rFeIFN- α 15 (Table 1).

N-terminal amino acid sequence of rFeIFN- α 15

The N-terminal sequence of purified rFeIFN- α 15 was determined with a protein sequencer, HP G1005A (Agilent Technologies International Japan, Tokyo, Japan), using a standard Edman degradation protocol.

Glycosidase digestion and blotting assays

Purified rFeIFN- α 15 was digested with glycosidase F (Takara Bio, Shiga, Japan) at 37 °C for 17 h in the presence or absence of 0.5% SDS and 1.0% NP-40. The samples were separated on SDS-PAGE, followed by blotting to Immobilon-P membranes (Merck Millipore, Tokyo, Japan). For immunoblotting, the membranes were incubated with primary anti-FeIFN- α 15 polyclonal rabbit antibody, generated by us, and secondary horseradish peroxidase (HRP)-conjugated anti-rabbit goat IgG (Sigma-Aldrich, Tokyo, Japan), with HRP detected with chemiluminescent reagents (ECL Western Blotting Detection System) (GE Healthcare). For lectin-blotting, the membranes were treated with biotinylated Con A (Seikagaku Corporation, Tokyo, Japan) and the resulting biotin residues reacted with streptavidin HRP conjugate (Sigma-Aldrich). HRP was detected with 3,3-diaminobenzidine (Wako Chemicals, Tokyo, Japan).

N-glycan analysis of rFeIFN- α 15

N-glycan analysis of purified rFeIFN- α 15, including glycosidase digestion of the protein, pyridylamination of the released glycans, and separation of PA-oligosaccharides by column chromatography, was performed as previously described [34].

Mass spectrometric analyses of PA-oligosaccharides

PA-oligosaccharides were subjected to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometric (MALDI-TOF MS) analysis, as previously described [35].

Antiviral activity assay of rFeIFN- α 15

The antiviral activity of rFeIFN- α 15 was determined in vitro using a standard VSV assay [36]. Fcwf-4 cells were inoculated into 96-well plates at a density of 2×10^4 cells

per well and cultured in Dulbecco's Modified Eagle's medium supplemented with 5% foetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin and streptomycin (Thermo Fisher Scientific K.K.) for 16 h in an atmosphere containing 5% CO₂. The crude extract or proteins eluted from the Blue Sepharose™ column were serially diluted with the media supplemented with 1% FBS, and each 100 μL of the media was added into the Fcwf-4 cell-containing wells. They were cultured for 20 h. Intercat1 (Toray Industries, Tokyo, Japan), a commercially available rFeIFN-ω, was used as a positive control. After each supernatant was removed, the plates were further incubated for 48 h at 37 °C, followed by infection with 100 μL of VSV (VR-158) (Summit Pharmaceuticals International Corporation, Tokyo, Japan) at a concentration of 1000 TC ID₅₀/ml. When the cytopathic effect (CPE) was greater than 95% of the control cells (without FeIFN), all the plates were stirred gently, and the cells were washed three times with PBS, fixed in 10% (v/v) formalin and stained with 0.1% (w/v) crystal violet. The stained cells were washed with deionised water and allowed to dry. The crystal violet was eluted with 100 μL of methanol, and the absorbance of each well was measured at 595 nm with a micro-titre plate reader (Spectra Max 250; Molecular Devices Japan, Tokyo, Japan). Titres of the samples were corrected by the titres of Intercat1.

Abbreviations

BmNPV: *Bombyx mori* nuclear polyhedrosis virus; Con A: Concanavalin A; CPE: Cytopathic effect; FBS: Foetal bovine serum; FeIFN: Feline interferon; fibL polyA: fibroin L-chain polyA signal sequence; FM2: Man2GlcNAc2Fuc; GlcNAc: N-acetylglucosamine; GN2: GlcNAc2Man3GlcNAc2-PA; GN3: GlcNAcMan3GlcNAc2-PA; GNb: GlcNAc₆Man3GlcNAc2-PA; HPLC: High-performance liquid chromatography; hr3: hr3 enhancer; ie1: IE1 trans activator gene; M3: Man3GlcNAc2-PA; M5: Man2Man3GlcNAc2-PA; MALDI-TOF MS: Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometric; MGFP: Monster green fluorescent protein; MSG: Middle silk gland; P3xP3: 3xP3 promoter; PA: aminopyridine; PAGE: Polyacrylamide gel electrophoresis; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; Pser1: Ser1 promoter; SDS: Sodium dodecyl sulphate; SV40 polyA: SV40 polyA signal sequence; TG: Transgenic; UTR: Untranslated region; VSV: Vesicular stomatitis virus

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MS and NY carried out the in vitro experiments. TM and IT designed and generated TG silkworm and checked the expression level of the protein from the TG line. MY prepared the polyclonal antibody against FeIFN-α15. YH coordinated the collaboration and advised on the study. MS and SS designed

the study, analysed the results, and drafted the paper. SS supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was carried out using a part of blood which had been collected for transfusion at the Veterinary Hospital. The blood was obtained with the consent of the Doctor of Veterinary Medicine who owned the cat. As stored blood was used, no separate ethical approval was required for the study. All efforts were made to minimize the discomfort of the animals during sampling.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author details

¹Innovation Center, Nippon Flour Mills Co., Ltd., 5-1-3 Midorigaoka, Atsugi, Kanagawa 243-0041, Japan. ²Transgenic Silkworm Department, Immuno-Biological Laboratories Co., Ltd., 1091-1 Naka, Fujioka, Gunma 375-0005, Japan.

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