FULL PAPER *Immunology*

Establishment of a Large-Scale Purification Procedure for Purified Recombinant Bovine Interferon-τ Produced by a Silkworm-Baculovirus Gene Expression System

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ABSTRACT. We developed a procedure for the large-scale purification of bovine interferon-τ (boIFN-τ) by means of a silkworm-baculovirus gene expression system. Recombinant boIFN-τ (rboIFN-τ) was efficiently produced in the silkworm infected with boIFN-τ cDNA recombinant baculovirus and accumulated in the haemolymph. To establish a purification method suitable for mass production, we tried three crude purification methods, namely, an acidification and neutralization treatment (ANT), silica gel column chromatography (SGCC), and Blue sepharose column chromatography (BSCC) with a combination of Q-sepharose (QSC) and chelating sepharose column chromatographies (CSCC). As a result, the acidification and neutralization treatment was found to be the most efficient and cost effective. With this combination, we obtained 91% pure products. To confirm the applicability of the procedure for mass production, we inoculated 100 silkworms with the recombinant virus, and recovered about 4.55 mg (1.26×10^8U/mg) of 91% pure rboIFN-τ by means of a combination of the ANT, followed by QSC and CSCC.

KEY WORDS: baculovirus, bovine, IFN-τ, purification, silkworm.

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Interferon (IFN)-τ, originally named trophoblast protein-I, is the major secretory protein of trophoblastic tissues and plays an important role in the maternal-fetal recognition of pregnancy in ruminants [1, 8, 19]. Interferon-τ has been classified as a member of the large multi-gene type I IFN family, and has about 85 and 70% overall homology to IFN-ω on the bases in the cDNA nucleotide and amino acid sequences, respectively [4, 9, 20]. As expected from the homology, IFN-τ possesses most of the major functions of IFNs, including anti-viral activity, but does not lead to a febriferous reaction, a serious side effect of the IFN therapy. Because of its biological activities, IFN-τ is applied as a useful tool in ongoing studies on the establishment of pregnancy, and is also expected to be used as an agent to improve the pregnancy rate through the control of implantation and to treat viral diseases. For these applications, it is essential to establish a mass production procedure for purified IFN-τ. Up to now, recombinant ovine and bovine IFN-τ boIFN-τ) had been expressed by baculovirus, yeast and *Escherichia coli* (*E.coli*) gene expression systems [3, 6, 16]. Among these, the baculovirus gene expression system is the most suitable system for producing recombinant IFN-τ for animal experiments and practical biological agents because of its similarity in terms of post-translational modifications, such as glycosylation, phosphorylation, conformation, antigenicity and so on [14]. As the baculovirus gene expression system, A*utographa californica* (Ac) nuclear polyhedrosis virus (NPV) with insect culture cells as the host and *Bombyx mori* (Bm) NPV with silkworm larvae as the host were popularly used. The advantage of the AcNPV-insect cell culture system is the absence of serum protein contamination in the culture fluids, since the cells can be cultured with serum-free media that makes it easy to purify the products accumulated in the fluids. On the other hand, the advantage of the silkworm-BmNPV system is its high expression efficiency and low feeding cost [11]. The concentration of recombinant product in the haemolymph is about 10 times higher than in the insect cell culture fluid, but the disadvantage of the expression systems is the difficulty of achieving product purification, because the product accumulates in the haemolymph, which contains many proteins in large quantities. Therefore, the purification method is the key to establishing large-scale production of type I IFN with the silkworm-baculovirus gene expression system. Purification with the combination of ion exchange and metal chelate has high specificity for IFN and is suited for purification from recombinant proteins produced by the silkworm-baculovirus system. Yonehara *et al.* [26] purified human lymphoblastoid interferon by a combination of salt precipitation, ion exchange chromatography, metal chelate chromatography and hydrophobic chromatography. Recombinant IFN-γ produced by *E.coli* was purified through ion exchange and Ni-chelate chromatography and size exclusion [27]. We have purified mouse IFN-β produced in the silkworm-baculovirus expression system with the method combination of salting-out, silica gel, ion exchange and copper ion chelating chromatography (unpublished data). Metal chelating sepharose is popularly utilized for the purification of IFN [7]. These methods are, however, only applicable for relatively clean materials, so that crude purification is essential

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to purify recombinant protein in the silkworm haemolymph. Feline IFN, expressed by the silkworm-baculovirus expression system, was purified with acidification and neutralization as a crude preparation [22]. Type I IFNs were also partially separated by silica gel [17] and blue dye affinity gel [5, 10] column chromatography. We therefore tried these three methods, namely acidification and neutralization treatment (ANT), silica gel column chromatography (SGCC), and blue sepharose column chromatography (BSCC), in order to assess them for use as the crude purification method for the recombinant bovine interferon-τ (rboIFN-τ) in the haemolymph, and we prepared a large quantity of purified rboIFN-τ.

MATERIALS AND METHODS

Recombinant baculoviruses and expression of recombinant bovine IFN-τ: Bovine IFN-τ cDNA was prepared as reported by Takahashi *et al.* [24]. The cDNA was excised and inserted into the baculovirus transfer vector (pBm050) to obtain the recombinant transfer vector (pBm boIFN-τ). This recombinant transfer vector was co-transfected with BmNPV (Cpd strain, [23]) DNA into the Bm cell line (BmN). Recombinant viruses were screened by the end-point dilution method on 96-well micro plates [13].

The recombinant virus was injected into the body cavities of silkworm larvae (8.0×10^5 p.f.u./head) at the early stage of the fifth instar. The haemolymph was collected at 24-hr intervals after injection, and 100 mM dithiothreitol (DTT) solution was added to a final concentration of 1 mM. The recovered haemolymph samples were centrifuged at 12,000 × g, for 20 min and the supernatants were stored at –80°C.

Purification of the rboIFN-τ: The haemolymph was collected from the recombinant-virus-infected silkworm larvae at 5 days after injection into 9 volumes of 0.05 M Tris-HCl (pH8.0) containing 1mM of DTT. The ANT was performed as follows. The pH of the haemolymph was adjusted to 2.0 with HCl and maintained for 2 hr. The solution was then neutralized with NaOH to pH8.0 and centrifuged at 12,000 × g for 20 min. The SGCC was performed as follows. Fifty grams of Kasei gel (TOKYO KASEI KOGYO CO., LTD. Tokyo, Japan) was equilibrated with 0.05 M Tris-HCl, pH8.0, and packed in a 200 m*l* column. The diluted haemolymph was applied to this column and the rboIFN-τ was eluted with 0.05 M Tris-HCl containing 30% ethyleneglycol. The BSCC was performed as follows. The Blue sepharose FF (Amersham Biosciences Corp, NJ, U.S.A.), equilibrated with 0.05 M Tris-HCl, was poured into a 1.5 × 10 column and washed with the same buffer. The diluted haemolymph was applied to column chromatography, and rboIFN-τ was eluted by a step-wise gradient of NaCl at 0.05 M, 0.1 M, 0.3 M, 0.5 M and 1 M. The QSCC was performed as follows. The supernatant containing boIFN-τ was loaded onto a Q-sepharose FF (Amersham Biosciences Corp, NJ, U.S.A.) 70 m*l* column equilibrated with 0.05 M Tris-HCl. After washing with Tris-HCl, rboIFN-τ was eluted by a step-wise gradient of NaCl at concentrations of 0.05 M, 0.1 M, 0.3 M, 0.5 M and 1 M. The CSCC was performed as follows. The chelating sepharose FF gel (Amersham Biosciences) was packed in a 30 m*l* column and washed with distilled water (D.W.). Then the column was charged with copper ions and washed with D.W. to remove excess copper ions. The rboIFN-τ fraction of the anion-exchange column was directly applied to the chelating column followed by washing with 0.05 M Tris-HCl, containing 0.5 M NaCl. The rboIFN-τ was eluted by reducing the pH with an elution buffer 0.05 M acetic acid, pH4.0, containing 0.5 M NaCl. All chromatographies were performed at 4°C.

Sodium dodecyl sulfate polyacrycamide gel electrophoresis (SDS-PAGE) and Western blotting: SDS-PAGE was done by the Laemmli method [12], and visualized by silver staining or Coomassie Brilliant Blue (CBB) staining. The molecular mass was calculated based on the molecular mass standards (Amersham Biosciences). The rboIFN-τ was monitored by Western blot analysis with the anti-human IFN-α polyclonal antibody (CHEMICON INTERNATIONAL INC., Temecula, California, U.S.A.) or boIFN-τ monoclonal antibody, generated by the purified rboIFN-τ as in this report.

Assays for anti-viral activity, protein concentration and purity: The anti-viral activity of the rboIFN-τ was determined by measuring the inhibition of the cytopathogenic effect of the *Sindbis* virus with the MDBK cell line [2, 18]. The assay was calibrated against a human IFN-α reference standard (Interferon alpha 2b, human, 2nd International Standard 1999) provided by the National Institute for Biological Standards and Control (NIBSC, U.K.). The protein concentration was measured with Coomassie Protein Assay Reagent (PIERCE Co., Rockford, Illinois, U.S.A.) with bovine serum albumin as a standard. Purified boIFN-τ was applied on SDS-PAGE and its purity was measured densitometrically. The data were analyzed with PC software NIH Image 1.55 (National Institutes of Health, U.S.A.).

RESULTS

Recombinant virus preparation and rboIFN-τ expression: The recombinant virus was obtained by the co-transfection of the recombinant transfer vector DNA and baculovirus DNA, as described in Materials and Methods. The recombinant virus was inoculated into fifth instar silkworm larvae at 8.0×10^5 p.f.u., and the accumulation of rboIFN-τ in the haemolymph was monitored by anti-viral activity, SDS-PAGE, and Western blotting. About 10^5 U/m*l* of the anti-viral activity was detected as early as the day after infection, then the activity was increased to about 10^9 U/m*l* at day 5, but the haemolymph was hardly recovered by day 7 post inoculation (PI), because most of the larvae were dead. The rboIFN-τ accumulation was also detected by SDS-PAGE and Western blotting (Fig. 1). About 22 kDa and 19.5 kDa bands were detected with the anti-human IFN-α antibody at day 3 (PI). The 22 kDa protein was continuously accumulated to day 5 (PI).

Purification of rboIFN-τ: We purified the rboIFN-τ accu-

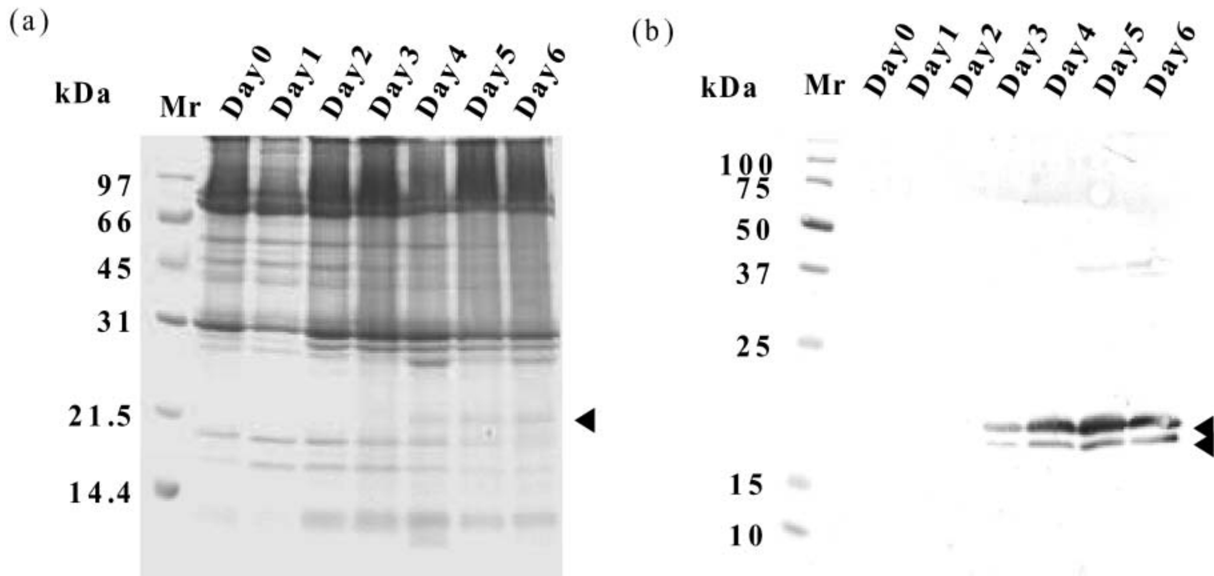

Fig. 1. Accumulation of rboIFN-τ in the recombinant-baculovirus-inoculated larvae haemolymph. SDS-PAGE (a) and Western blotting with anti-human IFN-α polyclonal antibody (b). Arrowheads indicate the rboIFN-τ bands. Each fraction was separated by 12.5% gel SDS-PAGE. This gel was stained with CBB.

mulated in the recombinant-virus-infected silkworm haemolymph by a combination of crude purification and two-step column chromatography. We tried three different methods of crude purification, namely, acidification and neutralization treatment (ANT), silica gel column chromatography (SGCC), and blue sepharose column chromatography (BSCC). As two-step column chromatography, we used Q-sepharose column chromatography (QSCC) and metal-chelating column chromatography (CSCC). First we tried the ANT with HCl and NaOH. Under this treatment, most of the protein bands in the haemolymph were precipitated and removed by centrifugation (Fig. 2(a) lane 4), but the rboIFN-τ bands remained in the supernatant fraction throughout the treatment (Fig. 2(b) lane 4). The rboIFN-τ band was clearly identified on the silver stained gel after the treatment, because the proteins, which masked the rboIFN-τ bands, were removed (Fig. 2(a) lane 4).

For the next purification step, we selected the QSCC, because the isoelectric point of boIFN-τ calculated from the amino acid sequence data was 5.95. The supernatant of the ANT was applied on the column. This column was then washed with Tris-HCl buffer pH8.0, and the adsorbed proteins were eluted with an NaCl step-wise gradient. As shown in Fig. 3, the rboIFN-τ was retained in 0.05 M NaCl (lane 4), but gradually eluted by 0.1 M NaCl (lane 5), and mostly eluted by the 0.3 M fraction (lane 6). In this process, the proteins aside from rboIFN-τ around 30 to 50 kDa were fractionated in flow-through and 0.05 M NaCl fractions, whereas other proteins, including rboIFN-τ, were fractionated in a 0.3 M NaCl fraction.

The 0.3 M NaCl fraction containing rboIFN-τ through the QSCC was applied to the CSCC saturated with copper ions. All proteins in the fraction including rboIFN-τ were adsorbed by the column. Only rboIFN-τ was eluted when the pH of the elution buffer was reduced to 4 (Fig. 3 lane 7). The purity of the rboIFN-τ was as high as 91.1%.

Next we tried the SGCC. The rboIFN-τ in the haemolymph tightly bound to the silica gel, but the majority of 30–85 kDa haemolymph proteins passed through the column (Fig. 4 lane 3). The rboIFN-τ was then eluted out with 0.05 M Tris-HCl buffer containing 30% ethylene glycol (Fig. 4 lane4). The eluate from the SGCC was then applied on the QSCC and the CSCC (Fig. 4 lane 5). The rboIFN- τ was purified to 91.8 % through the process. This result indicates that silica gel column chromatography is also an effective method for improving the purity as a crude preparation.

Then we tried the BSCC (data not shown). The rboIFN-τ in the haemolymph tightly bound to the blue dye matrix, but almost all of the haemolymph proteins passed through the column. The rboIFN-τ bound to the matrix was then eluted out with 0.05 M Tris-HCl buffer containing 0.1 M NaCl. Next, the eluate from the BSCC was applied in the CSCC, but a few contaminant proteins were not removed.

By these experiments, we concluded that the combination of the QSCC and the CSCC saturated with the copper ions was suitable for the purification of rboIFN-τ in the silkworm haemolymph after crude purification by either the ANT the SGCC.

To examine whether this purification procedure is applicable for mass production, 50 m*l* of haemolymph obtained from 100 heads of recombinant virus infected silkworm larvae were processed through the series of purification meth-

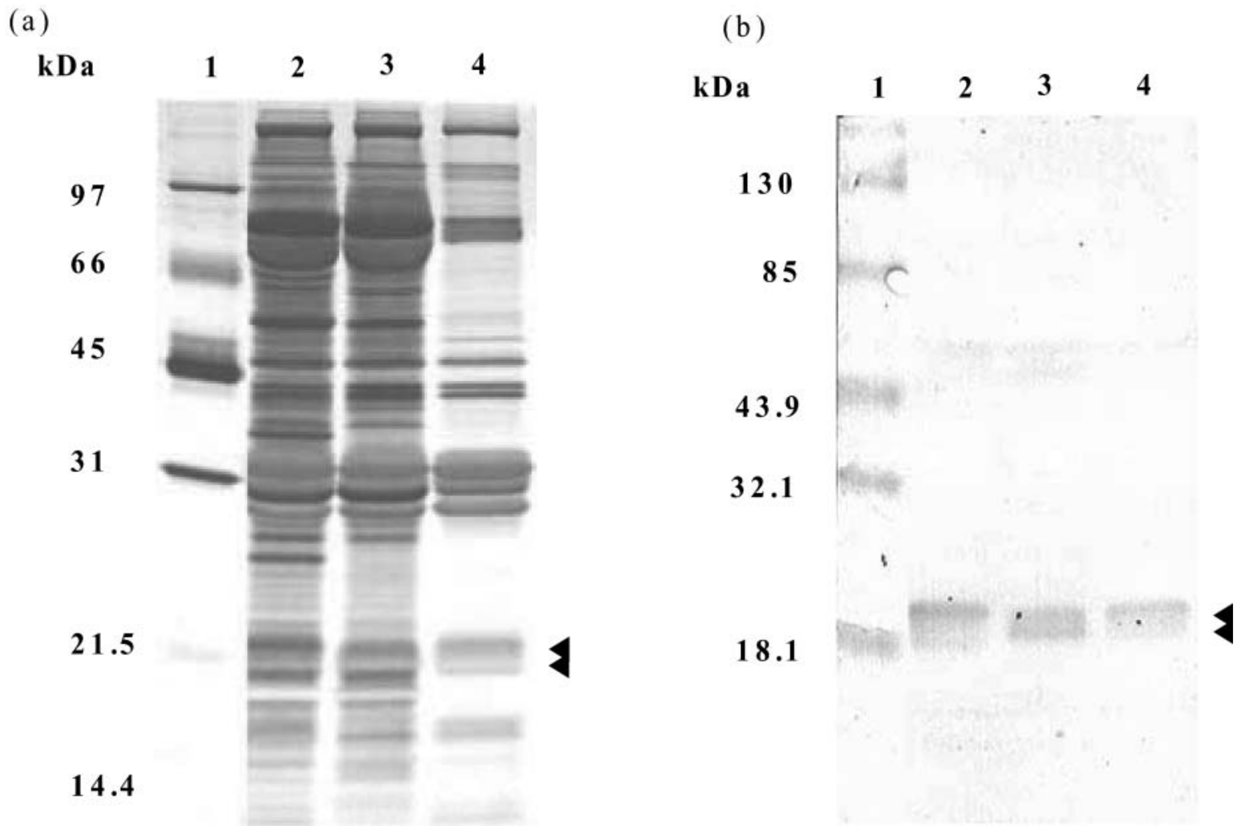

Fig. 2. Crude purification of rboIFN-τ in the recombinant-baculovirus-infected larvae haemolymph by acidification and neutralization treatment. Silver-stained SDS-PAGE (a) and Western blotting with anti-human IFN-α polyclonal antibody (b). Lane 1, molecular mass markers; Lane 2, Starting material; Lane 3, Acidified haemolymph with HCl; Lane 4, Supernatant of centrifugation after neutralization with NaOH. Arrowheads indicate the rboIFN-τ bands. Each fraction was separated by 12.5% gel SDS-PAGE.

ods. The results are summarized in Table 1. The collected haemolymph contained 5.6×10^9 U rboIFN-τ altogether. After crude purification as the acidification and neutralization treatment, the recovery of boIFN-τ was 46.25%, and the specific activity was 9.64×10^6 U/mg. As determined through the two-step column chromatography, the final elute fraction containing the purified rboIFN-τ was 1.26×10^8 U/mg protein, the purification factor of which was about 37 times higher than that of the original haemolymph. The total amount of purified rboIFN-τ was 4.55 mg, and the overall recovery was 10.3%. The purified rboIFN-τ appeared as two bands of 22 and 19.5 kDa on SDS-PAGE. The purity, measured by densitometry, was 91% (data not shown).

DISCUSSION

In this research we studied methods for purifying rboIFN-τ suitable for large-scale production. The silkworm-baculovirus gene expression system was able to efficiently produce rboIFN-τ. In the haemolymph of the silkworm, rboIFN-τ was accumulated to 1×10^9 U/m*l*. On the other hand, by means of the yeast (*Pichia pastoris*) expression system, bovine IFN-ω1 was accumulated to 1-5.5 × 10^6 U/m*l* [15], and recombinant ovine IFN-τ in the recombinant-baculovirus-infected insect cell (Sf21) culture fluid was accumulated to 5×10^5 U/m*l* [3]. Recently Watanabe *et al.* [25] established an insect cell (BTI TN5B1-4) -baculovirus rboIFN-τ expression system. By this system, rboIFN-τ was accumulated to 1×10^8 U/m*l* (unpublished data). The silkworm-baculovirus expression system we described in this report was, therefore, at least 10 times higher than other expression systems.

To establish an efficient purification method for rboIFN-τ, suitable for the mass purification from silkworm haemolymph, first we compared the ANT, the SGCC and the BSCC as crude purification methods. Among them, the ANT has an advantage, because this treatment not only efficiently removed the contaminating proteins but also completely inactivated the recombinant baculovirus in the haemolymph. This method is also easy, quick and economical. The SGCC was also efficient for removing contami-

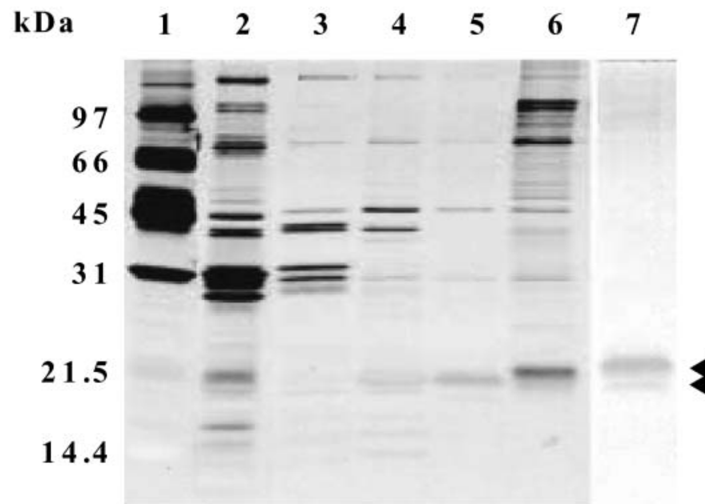

Fig. 3. Purification of rboIFN-τ by Q-sepharose column and metal chelate sepharose column chromatographies. Lane 1, molecular mass markers; Lane 2, the acidification and neutralization treated haemolymph; Lane 3, Q-sepharose column flow through fraction; Lane 4, 0.05 MNaCl fraction; Lane 5, 0.1 M NaCl fraction; Lane 6, 0.3 M NaCl fraction; Lane 7, Metal chelate sepharose column pH 4.0 fraction. Each fraction was separated by 12.5% gel SDS-PAGE. SDS-PAGE gel was stained with silver. Arrowheads indicate the migration positions of rboIFN-τ.

nating proteins, but it is time consuming and laborious, especially when treating large amounts. By the BSCC, the majority of haemolymph proteins were efficiently removed, but a few contaminant proteins in the BSCC eluate were not removed by subsequent chromatographies.

By the combination of the ANT, followed by the QSCC and the CSCC, we produced 4.55 mg of 91% pure rboIFN-τ with 5.75×10^8 units of anti-viral activity with 100 silkworms. The recovery was 10.3%, and the specific activity of purified rboIFN-τ was 1.26×10^8 U/mg.

Silkworms are very domesticated, and therefore the feeding techniques for silkworms are highly developed. We are able to feed more than ten thousand germ-free inbred silkworms in all seasons without raw leaves in a containment facility. So theoretically we can prepare more than 6×10^{10} units (400 mg) of purified rboIFN-τ as one lot. It is also easier to expand the feeding size than to expand the culture volume of a bio-reactor. The system we reported is therefore a suitable one for rboIFN-τ mass purification.

By recombinant baculovirus infection, 19.5 kDa and 22 kDa proteins were accumulated in the recombinant-virus-infected silkworm haemolymph. As shown by Western blotting, both proteins were rboIFN-τ, because both proteins were reacted with the same mAb. Watanabe *et al.* [25] reported that 19.5 kDa and 22 kDa proteins were accumulated in insect cell culture medium by rboIFN-τ cDNA recombinant baculovirus infection. In this case, only a 19.5 kDa molecule was detected by N-glycosylation inhibition with tunicamycin. It is likely that the same event occurred in the silkworm body, and therefore the 22 kDa molecule must be N-glycosylated, and the 19.5 kDa molecule must be in a non-N-glycosylated form. In the bovine trophoblast protein-1 (boIFN-τ), the glycosylation may prolong biological half-life and stability, and also enhance its affinity for its own receptors [21]. Therefore, posttranslational glycosylation is a very important process and can influence the biological and pharmacokinetic properties of synthesized glycoproteins when they are used *in vivo*, such as in clinical applications. From this point of view, the silkworm-baculovirus system was suitable, since the major product was glycosylated 22 kDa protein. This easy and low-cost procedure for the mass production of purified rboIFN-τ, described in this report will accelerate animal experiments with cattle and will lead to the development of novel methods to improve the pregnancy rate by control of implantation and of novel methods to treat viral diseases.

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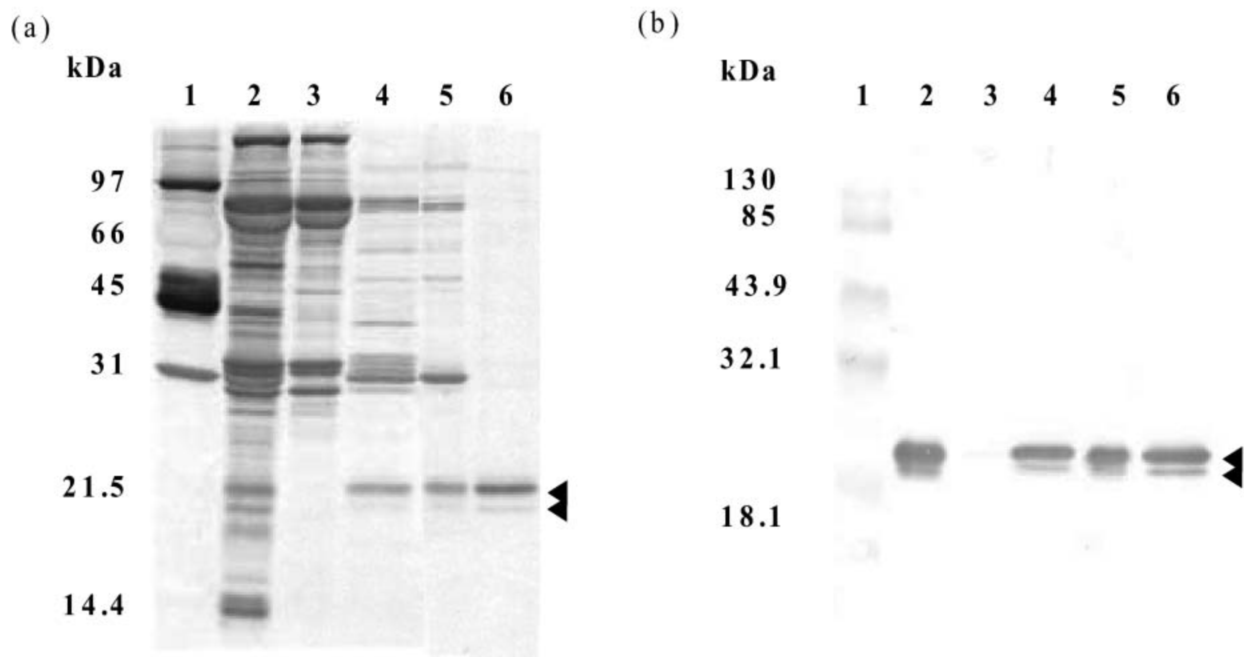

Fig. 4. Crude purification of rboIFN-τ by silica gel column chromatography and subsequent purification by Q-sepharose and chelating sepharose column chromatographies. Silver-stained SDS-PAGE (a) and Western blotting with anti-boIFN-τ mAb (b). Lane 1, molecular mass markers; Lane 2, Starting material; Lane 3, Flow through fraction of the silica gel column; Lane 4, Eluate of silica gel column with ethyleneglycol; Lane 5, Eluate of Q-sepharose column with 0.3 M NaCl buffer; Lane 6, Eluate of metal chelating sepharose FF with pH 4.0 buffer. Arrowheads indicate the rboIFN-τ bands. SDS-PAGE gel was stained with silver reagent.

Table 1. Large-scale purification of rboIFN-τ accumulated in recombinant-virus-infected silkworm haemolymph

Purification step	volume (m*l*)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification factor[a)]
Haemolymph	50	1642.5	5.6×10^9	3.41×10^6		1
Acidification and neutralization treatment	500	268.8	2.59×10^9	9.64×10^6	46.25	2.83
Q-sepharose column chromatography	180	70.5	1.06×10^9	1.50×10^7	18.9	4.4
Metal-chelating sepharose column chromatography	50	4.55	5.75×10^8	1.26×10^8	10.3	36.95

a) Purification factor is calculated so that the specific activity at each step is divided by 3.41×10^6, the specific activity of the haemolymph.

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