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Expression of Chicken Interleukin-2 in Insect Cells

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Abstract—Full-length chicken interleukin-2 (ChIL-2) protein was successfully expressed using the recombinant baculovirus/Sf9 insect cell system. The expressed protein was soluble and reached approximately 12 μg/ml. Similarly to native ChIL-2, baculovirus expressed ChIL-2 revealed two main bands corresponding to molecular masses of 22 and 20 kD as detected by SDS-PAGE and Western blot. Treatment of the expressed protein with N-endoglycosidase F for 2 h caused the complete disappearance of the 22 kD band, while the 20 kD band (which is close to the molecular weight predicted from the cloned cDNA sequence) remained unchanged. Together with results on native ChIL-2, it can be concluded that ChIL-2 is an N-glycosylated protein.

Key words: chicken, glycosylation, interleukin-2, monoclonal antibody

Interleukin-2 (IL-2, previously named T cell growth factor) is mainly involved in T cell differentiation and activation, B cell development, and natural killer (NK) cell stimulation. IL-2 is principally secreted by T cells under stimulations by antigens, cytokines, or mitogens. Mammalian IL-2 has been well documented, including human [1-3], rat [4], mouse [5], cow [6], and swine [7]. The functions and characteristics of IL-2 from these species have been studied extensively. IL-2 is supposed to increase the cellular immune response in the host, while change in mucosal immune response is not obvious [8].

By constructing a cDNA library, the entire sequence of chicken IL-2 (ChIL-2) was determined recently [9]. The expression of ChIL-2 in both prokaryotic and eukaryotic systems was also further studied [10]. Sequence homology comparison showed that sequences of mammalian IL-2 exhibit 58-100% of amino acid identity [11]. However, ChIL-2 shares only 24.5% identity to bovine IL-2 [9]. Though the identity between chicken and mammalian IL-2 molecules is low, their biological functions are fundamentally the same. Thus, similarly to mammalian IL-2, ChIL-2 is essential in the prevention of avian diseases by enhancing the immune responses of the host, and it is a potential adjuvant in vaccination.

More recently, we reported the purification to high homogeneity of ChIL-2 native protein using an immunoaffinity column, and its biological activity was investigated [12]. Interestingly, purified ChIL-2 revealed two protein bands corresponding to the molecular masses of 16 and 14 kD, respectively, and the upper band was hypothesized to be possibly a glycosylated form. Because of the extremely low content of native ChIL-2 in chicken lymphocytes culture medium, it is difficult to test this hypothesis using native ChIL-2. In this work, using the baculovirus/Sf9 insect system, full-length ChIL-2 was expressed, and we show evidence that ChIL-2 is an N-glycosylated protein.

MATERIALS AND METHODS

Chemicals. Anti-ChIL-2 monoclonal antibody was produced as described [12]. Iscove's Modified Dulbecco medium (IMDM), serum-free Sf-900 II medium, and fetal bovine serum (FBS) were products from GIBCO BRL (USA). N-Endoglycosidase F was from Sigma (USA). Fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse IgG and horseradish peroxidase conjugated rabbit anti-mouse IgG were purchased from DAKO (Denmark). Molecular weight marker for SDS-PAGE was a product of Bio-Rad (USA), and pre-stained molecular weight marker was from BioLabs (USA).

Cell line. The Sf9 cell line was maintained in monolayer culture at 27°C in serum-free Sf-900 II medium supplemented with 50 mg/liter streptomycin sulfate and 125 mg/liter penicillin. Cells were split every three days to maintain a density ranging from $5 \cdot 10^4$ to $7 \cdot 10^5$ cells/ml.

Preparation of chicken lymphocytes. Specific pathogen free (SPF) chickens aged 1-3 months were used in the experiment. Spleens were collected under aseptic

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conditions and homogeneous cell suspension in phosphate buffered saline (PBS, pH 7.4) containing penicillin (100 μg/ml) and streptomycin (100 μg/ml) was obtained by gently pressing through a fine stainless steel mesh. The cells were centrifuged at 300*g* for 10 min. The cell pellet was resuspended in PBS and centrifuged two more times. The resulting pellet was then resuspended in PBS and layered carefully over an equal volume of Lymphoprep (Nyegarrd, Norway). The sample was centrifuged at 800*g* for 20 min and the interphase was collected. The cells were further washed with PBS three times and finally suspended in IMDM containing 10% FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cell concentration was adjusted to $1 \cdot 10^7$ cells/ml, and cell viability as checked by Trypan blue dye was >95%.

Cloning and expression of recombinant chicken IL-2 in baculovirus. Total RNA was isolated from chicken lymphocytes stimulated by ConA (10 μg/ml) for 20 h using TRIzol (Invitrogen, The Netherlands). cDNA was synthesized from 1 μg of total RNA using oligo$(dT)_{16}$ adaptor primer (Roche Diagnostics, Germany). The cDNA containing chicken IL-2 coding sequences was amplified by rapid amplification of cDNA ends polymerase reaction (3′-RACE-PCR). The primers used for 3′-RACE-PCR were designed on the basis of chicken IL-2 as reported [9] as follows: sense primer, 5′-CGCGGATCCATGATGTGCAAAGTACTGATCTTTG-3′ and antisense primer: 5′-ACGCGTCGACTTATTTTTGCAGATATCTCACAAAG-3′. The cleavage sites of *Bam*HI and *Sal*I are underlined. Amplification was performed in the thermal cycler Geneamp 9700 (Perkin Elmer, USA) for 30 cycles at 94°C for 30 sec, 59°C for 45 sec, and 72°C for 2 min. A single band at approximately 450 bp was obtained and purified from the gel.

Purified PCR product was digested with *Bam*HI and *Sal*I and then inserted into the *Bam*HI and *Sal*I sites of the pBlueBacHis2 baculovirus vector (Invitrogen) according to the manufacturer's instruction. The inserted ChIL-2 fragment was confirmed by DNA sequencing. The recombinant baculovirus containing full length ChIL-2 recombinant plasmid was co-transfected into Sf9 insect cells and cultured in Sf-900 II SFM medium. Recombinant virus stocks were propagated in Sf9 cells in tissue culture flasks at 27°C, and the virus titer was determined by end-point dilution. For the production of full-length ChIL-2, Sf-9 cells were infected by recombinant virus for 48 h, sonicated in PBS, and centrifuged at 1000*g* for 10 min to remove cell debris. The supernatant was used to run SDS-PAGE followed by Western blot.

Optimization of recombinant ChIL-2 expression. Sf9 cells in 25 ml flasks were infected with recombinant viruses at a multiplicity of infection (MOI) from 1 to 10. Sf9 cells were harvested after infection for 24, 48, and 72 h, and the protein expression was analyzed by SDS-PAGE followed by Western Blot.

SDS-PAGE and Western blot. SDS-PAGE was performed under reducing conditions according to the method of Laemmli [13] using 15% gel. Western blot was carried out as described [14]. Briefly, ChIL-2 was subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with 3% of bovine serum albumin (BSA) in PBS containing 0.05% Tween-20 (PBST). After washing, the membrane was incubated with anti-ChIL-2 monoclonal antibody in 1% BSA for 2 h at room temperature and washed with PBST. After incubation with rabbit anti-mouse IgG coupled to horseradish peroxidase in 1 : 20,000 dilution, the membrane was further extensively washed with PBST. Immunodetection was carried out using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) following the manufacturer's instruction.

Immunofluorescence assay (IFA). Sf9 cells were fixed with 3.7% formaldehyde 24 h post-infection. Cells were incubated with anti-ChIL-2 monoclonal antibody (1 : 5000) at 37°C for 30 min and washed three times with PBS. After incubation with FITC labeled rabbit anti-mouse IgG antibody (1 : 200) for 30 min, fixed cells were extensively washed and visualized under a fluorescence microscope IX51 (Olympus, Japan) equipped with appropriate filter sets. Instead of using recombinant virus infected cells, control test was performed using Sf9 cells without infection under the same conditions.

RESULTS AND DISCUSSION

Interleukin-2 (IL-2) has been isolated and characterized from different animal sources such as human [1, 15], bovine [16], and mouse [17]. For the purification of IL-2, the most efficient way was by using an immunoaffinity column. An immunoaffinity column purifying ChIL-2 revealed two main bands corresponding to the molecular mass of 16 and 14 kD on SDS-PAGE [12]. A similar result was obtained for human IL-2, and a different degree or mode of the O-glycosylation was presumed to be responsible for molecular mass heterogeneity [3]. Because ChIL-2 was presumed to be a protein that can undergo N-glycosylation based on its sequence analysis [9], this was further confirmed in the present study. Although using native ChIL-2 should be ideal for this study, because the content of native protein in tissue culture supernatant is extremely low, we used the baculovirus expression system to express the protein instead. Compared with the prokaryotic expression system, baculovirus and insect cell expression system is a convenient and versatile eukaryotic system. Furthermore, it can provide correct folding of recombinant protein and other important post-translational modifications and form glycosylation similar to that of mammalian cells.

After isolating total RNA from chicken lymphocytes, cDNA sequences encoding full-length ChIL-2 were

cloned. The cloned full-length gene has one nucleotide difference from that reported by Sundick and Gill-Dixon [9]. The predicted protein sequences were the same (data not shown). Recombinant baculovirus encoding the full length ChIL-2 gene was obtained. Sf9 insect cells were then infected with the recombinant virus and the expression condition optimized by varying the MOI and infection time. Maximum expression was achieved at MOI of 5 and infection time of 48 h. Expressed proteins were detected both by immunofluorescence assay (IFA) and Western blot using anti-ChIL-2 mAb. The IFA result showed that ChIL-2 was mainly expressed in the cytoplasm of Sf9 cells (Fig. 1). On SDS-PAGE, compared with the control band where Sf-9 cell only was used, two new protein bands with molecular masses of 22 and 20 kD, respectively, were identified (Fig. 2a). Because baculovirus expressed ChIL-2 is a fusion protein containing the full-length ChIL-2 and a pBlueBacHis2 signal peptide, the predicated size of recombinant ChIL-2 is around 20 kD, and this prediction is in accordance with the lower band (20 kD). The strong immunological reaction of the upper band (22 kD) with anti-ChIL-2 monoclonal antibody (Fig. 2b) suggested that it is quite possibly the glycosylated form of ChIL-2. This result is in good agreement with our previous result of purified native ChIL-2 where two protein bands with sizes of 16 and 14 kD were identified [12].

The full-length ChIL-2 encodes 143 amino acid residues including a signal peptide consisting of 22 amino acid residues. Residues from 109 to 111 consist of Asn-His-Thr, which is a peptide sequence generally regarded as a signal for potential N-glycosylation on the asparagine residue [9]. To confirm if ChIL-2 is a glycoprotein, baculovirus expressed recombinant ChIL-2 was incubated with N-endoglycosidase F for 0 and 2 h at 37°C, and the reaction mixture was checked by Western blot. As shown in Fig. 3, on comparison with the undigested sample,

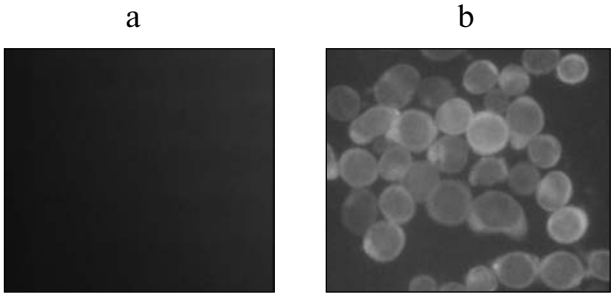

Fig. 1. Immunofluorescence assay of Sf9 cells infected with recombinant baculovirus. Sf9 cells infected with recombinant baculovirus for 24 h were fixed and analyzed by indirect immunofluorescence detection using anti-ChIL-2 monoclonal antibody followed by reaction with FITC-labeled rabbit anti-mouse IgG. a) Control, Sf9 cells without infection; b) Sf9 cells 24 h post-infection by recombinant virus.

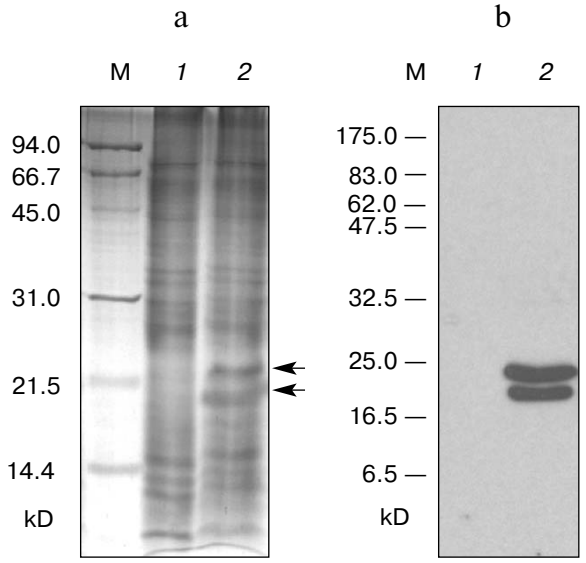

Fig. 2. SDS-PAGE and Western blot analysis of ChIL-2. a) SDS-PAGE of recombinant virus infected Sf9 cells: M) molecular weight marker; *1*) Sf9 cells without infection; *2*) Sf9 cells infected by recombinant viruses. Arrowheads indicate the position of expressed protein. b) Western blot of recombinant ChIL-2 using anti-ChIL-2 monoclonal antibody: M) pre-stained molecular weight marker; *1*) Sf9 cells without infection; *2*) Sf9 cells 48 h post-infection by recombinant virus. All samples were applied to 15% polyacrylamide gels.

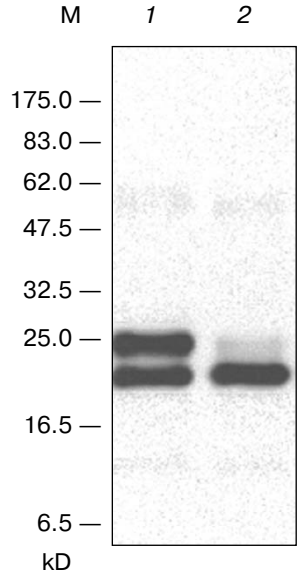

Fig. 3. N-Endoglycosidase F treatment of baculovirus expressed full-length ChIL-2. Baculovirus expressed ChIL-2 was reacted with N-endoglycosidase F for 0 and 2 h. After SDS-treatment, samples were applied to a 15% polyacrylamide gel followed by Western blot using anti-ChIL-2 monoclonal antibody. M) Pre-stained molecular weight marker; *1*) 0 h treatment; *2*) 2 h treatment.

treatment of baculovirus expressed ChIL-2 with N-endoglycosidase F resulted in the complete disappearance of the upper band (22 kD), while the lower band (20 kD) remained unchanged. This result strongly suggested ChIL-2 is a glycoprotein containing N-linked glycan.

Interleukin-2 is an important cytokine that mainly enhances the cellular mediated immune response in the host [8, 18]. The importance of glycosylation in the immune system might be ascribed to antigen recognition, providing protease protection and restriction of nonspecific lateral protein–protein interaction [19]. As ChIL-2 is a key molecule involved in the immune system of the chicken, the glycosylation of this protein may have similar important physiological functions that need further elucidation.

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