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ORIGINAL PAPER

Cloning, expression in *Escherichia coli*, and purification of soluble recombinant duck interleukin-2

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Abstract Interleukin-2 (IL-2) is a vital cytokine secreted by activated T lymphocytes, and plays an important role in the regulation of cellular and immunity of animals. In this study, a gene encoding duck IL-2 was cloned and the soluble recombinant duck IL-2 (rDuIL-2) was expressed in *Escherichia coli* via fusion with glutathione S-transferase (GST). The results indicated that the GST-rDuIL-2 fusion protein expressed in *E. coli* Origami (DE3) was confirmed to be of about 40 kDa molecular mass by SDS-PAGE and western blotting. In order to produce soluble rDuIL-2 in a low-cost, nontoxic and high-level expression process, lactose was used as a substitute for Isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the above recombinant strain Origami (pGEX-DuIL-2). By optimizing the expression conditions, the production of soluble GST-rDuIL-2 fusion protein was about 29% of total cellular soluble proteins, which was similar with IPTG used as inducer. The soluble GST-rDuIL-2 fusion protein was purified by one-step affinity chromatography, and GST was removed by thrombin. Then rDuIL-2 was purified by a second affinity chromatography. As a result, the 95% pure rduIL-2 was obtained, and the yield of rDuIL-2 was about 10.6 mg/l bacterial culture. The bioactivity of rduIL-2 was determined by lymphocyte proliferation assay in vitro. Our study provided a feasible and convenient approach to produce soluble and biologically active rDuIL-2, which would be used as an immunoadjuvants for enhancing vaccine efficacy.

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Keywords Duck interleukin-2 · Recombinant expression · *Escherichia coli* · Purification

Introduction

In recent years, avian influenza virus infection caused significant morbidity and mortality and had marked social and economic impacts throughout the world (Abubakar et al. 2011). Therefore, determining how to improve the immunity of the birds to reduce or avoid these viral diseases is particularly important. Interleukin-2 (IL-2) is a vital cytokine that functions to promote survival and growth of T cells, to terminally differentiate T cells for activation-induced cell death, and to induce T regulatory cells (Antony et al. 2006). IL-2 has been used as an enhancing agent in vaccines (Fei et al. 2004). Duck IL-2 (DuIL-2) also presents similar bioactivity both in vivo and in vitro (Zhou et al. 2005b). Therefore, if we could use genetic engineering method to achieve high-yield production of DulL-2, and applied it to be the immune adjuvant, the immunity of ducks would be enhanced.

IL-2 genes from chicken, ducks and geese have been cloned since 2000s (Zhou et al. 2003, 2005a, b; Wang et al. 2008). Particularly, systematic research about the high expression of chicken IL-2, antibody preparation and the application as immune strengthening agent has been done (Fei et al. 2004; Cao and Jimmy 2004; Guo et al. 2006), which provided certain ideas and methods in the research of other birds' IL-2. In 2000, a cDNA sequence of Mallard (ML) duck IL-2 (DuIL-2) was deposited in GenBank (GenBank accession no. AF294323) (Schmohl and Schultz 2000; Sreekumar et al. 2005). Recently, a cDNA sequence of Shaoxing (SX) duck IL-2 was cloned (GenBank accession no. AY173028) and recombinant duck IL-2 (rDuIL-2)

has been expressed in *Escherichia coli* (Zhou et al. 2005b), however, the most of rDuIL-2 proteins couldn't be correctly folded and deposited as insoluble inclusion bodies. During in vitro refolding process, the recovery of soluble and active rDuIL-2 was very low (Zhou et al. 2005b), which prevented use for the production of rDuIL-2 on an industrial scale. Therefore, it is necessary to express rDuIL-2 in a soluble form by *E. coli*.

In the present study, we cloned a gene encoding IL-2 from Muscovy duck, which shares 100% identity with the GenBank AY193713 sequence. Then the MV duck IL-2 was expressed soluble and biologically active recombinant duck IL-2 (rDuIL-2) in *E. coli* via fusion with glutathione S-transferase (GST) and using the trxB/gor mutant *E. coli* strain, Origami (DE3). In addition, in order to produce soluble rDuIL-2 in a low-cost, nontoxic and high-level expression process, lactose was used as a substitute for IPTG to induce the recombinant strain Origami (pGEX-DuIL-2). The biological activity of rDuIL-2 was demonstrated in vitro using lymphocyte proliferation assay. Our study provided a feasible and convenient approach to produce soluble and biologically active rDuIL-2, which would be used as an immunoadjuvants for enhancing vaccine efficacy.

Materials and methods

Chemicals, enzymes and materials

The restriction endonucleases (*EcoR* I and *Not*I), *Taq* DNA polymerase and T4 DNA ligase were purchased from Takara (Dalian, PR China). *E. coli* DH5α, *E. coli* Origami (DE3) and the expression vector pGEX-4T-3 were stored by our laboratory. Isopropylthio-D-galactoside (IPTG), lactose, ampicillin, EDTA, thrombin, Con A, α-methyl D-mannoside, RPMI1640 Medium and fetal calf serum (FCS) were purchased from Sigma. The duck splenic mononuclear cells (SMC) were prepared as described by Zhou et al. (2005b). *Escherichia coli* strain DH5α was used for the transformation and propagation of recombinant plasmids. *Escherichia coli* strain Origami (DE3) with glutathione reductase (*gor*) and thioredoxin reductase (*trxB*) mutations, which greatly facilitated disulfide bond formation in expressed proteins, was used as host to express recombinant proteins. The Glutathione Sepharose was purchased from GE (formerly Amersham bioscience).

RNA isolation, RT-PCR and sequencing

The duck SMC were stimulated with Con A for 48 h and were harvested by centrifugation at 500× *g* for 5 min at 4°C and followed by washing three times with PBS. Total cellular RNA was extracted with Trizol reagent (Takara, Dalian, P. R. China). The cDNAs were synthesized by AMV Reverse Transcriptase (TaKaRa, Dalian, P. R. China) from total cellular RNA using oligo dT primer. According to ML DuIL-2 cDNA (GenBank accession number AF294323) and MV DuIL-2 cDNA (GenBank accession number AY193713), a pair of the specific oligonucleotide primers for amplifying the nucleotide sequence of DuIL-2 open reading frame (ORF) were designed and synthesized: forward primer 5′-ATGTGCAAAGTACTCAT-3′ (located in 94–110 bp of GenBank AF294323 and in 13–29 bp of GenBank AY193713), and reverse primer 5′-TTATTTTAGCATAGATC-3′ (located in 501–516 bp of GenBank AF294323 and in 419–435 bp of GenBank AY193713). The PCR mixture contained 2 μl cDNA (100 ng/μl), 20 pmol each primer, 5 μl 10 × PCR Buffer, 100 nmol $MgC1_2$, 1.25 U *TaKaRa Ex Taq* and 10 nmol each dNTP in 50 μl. The reaction was carried out at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s and DNA extension at 72°C for 1 min. Lastly, the reaction was carried out 72°C for 7 min. RT-PCR products were purified by TaKaRa DNA fragment recovery kit Ver.2.0 and cloned into the TA cloning site of pUCm-T (named as pUCm-T-duIL-2) for sequencing to identify whether the RT-PCR product was DuIlL-2 ORF by comparing the sequencing result with ML DuIL-2 cDNA (GenBank accession number AF294323) and MV DuIL-2 cDNA (GenBank accession number AY193713). Signal peptide of DuIL-2 protein was predicted by SignalP version 2.0 (SignalP-NN and SignalP-HMM software) of SignalP World Wide Web server, and the relate sequence coding for signal peptide was identified (located in 94–156 bp of GenBank AF294323 and in 13–75 bp of GenBank AY193713).

Construction of expression plasmid pGEX-DuIL-2

The above plasmid pUCm-T-DuIL-2 was used as template to obtain the mature DuIL-2 gene (*DuIL-2*) fragment (360 bp) by PCR. The forward primer was 5′-G C T **G A A T T C** C G C A C C T C T A T C A G A G A A A A -3′ (located in 157–174 bp of GenBank AF294323 and in 76–93 bp of GenBank AY193713), in which the restriction enzyme site *EcoR* I is represented in bold and followed by the sequence encoding the N terminus of DuIL-2. The reverse primer was 5′-T A **G C G G C C G C** T T A T T T T A G C A T A G A T C-3′ (located in 501–516 bp of GenBank AF294323 and in 419–435 bp of GenBank AY193713), which was designed to add a translation stop codon (TTA) after the C terminus of DuIL-2, and the restriction enzyme site *Not*I (in bold) was added for the cloning convenience. Both the PCR product (the mature DuIL-2 gene fragment) and the plasmid pGEX-4T-3 were

double digested by *Eco*R I and *Not* I, and then were ligated at 16°C overnight. The ligated product was transformed into the competent *E. coli* DH5α cells and transformants were screened for ampicillin resistance. Plasmid DNA from resistant colonies was confirmed by restriction enzyme digestion and checked by complete DNA sequencing of the inserted fragment. The recombinant plasmid containing the mature DuIL-2 sequence was named as pGEX-DuIL-2. The construction process of the recombinant plasmid pGEX-DuIL-2 was showed in Fig. 1.

Expression of recombinant protein

Recombinant plasmid pGEX-DuIL-2 was transformed into *E. coli* Origami (DE3) to yield expression strains Origami (pGEX-DuIL-2). The expression strains were cultured in LB medium with 100 mg/l ampicillin at 37°C until the OD_{600} reached 0.5, at which time IPTG was added to a final concentration of 0.5 mM. Five hours later, the cells were harvested by centrifugation with 12000× *g* for 10 min at 4°C.

Lactose was used as a substitute for IPTG to induce the recombinant strain Origami (pGEX-DuIL-2) to produce soluble rDuIL-2 in a low-cost, nontoxic and high-level expression process. The culture conditions such as the lactose addition time (at the OD_{600} reached 0.8/1.5/1.9), the lactose concentration (4/8/12 mg/ml), the culture temperature (25°C/30°C/37°C) and induction durations (1/3/5/7/9/11/14/18/22 h) were optimized.

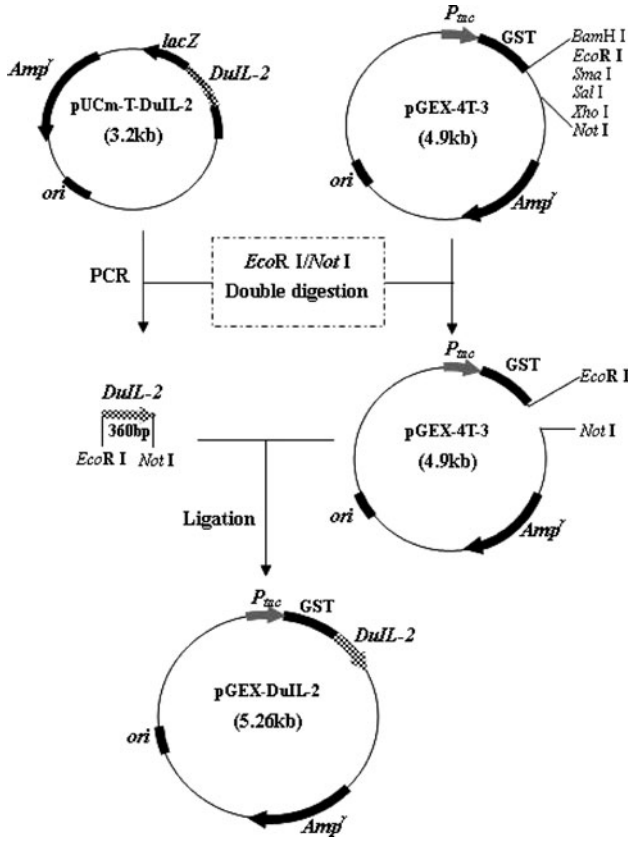

Fig. 1 The construction process of the recombinant plasmid pGEX-DuIL-2

In order to yield enough expression supernatant for purification of recombinant proteins, a single bacterial colony carrying pGEX-DuIL-2 was used to inoculate an overnight pre-culture in LB medium with 100 mg/l ampicillin at 37°C. This pre-culture was used to inoculate 1 l of fresh LB medium with 100 mg/l ampicillin. Approximately 4 h later, the OD_{600} of the bacterial broth reached 1.5. The flasks were then transferred to a 25°C incubator for 30 min, and 4 mg/ml lactose was added to initiate a 5 h induction.

The above cells were harvested by centrifugation with 12000× *g* for 10 min at 4°C and resuspended in the buffer A containing 50 mmol/l Tris–HCl, 150 mmol/l NaCl, 5 mmol/l EDTA, pH 8.0, then were disrupted by sonication, centrifuged, and separated into soluble and insoluble fractions. Both the fractions were analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The Coomassie brilliant blue-stained SDS-PAGE was scanned with an UVP White/Ultraviolet transilluminator, and analyzed with software Grab-it 2.5 and Gel-work (UVP) for estimating the purity of recombinant protein and rDuIL-2 (Du et al. 2010). The protein estimation was determined by the Bradford method using BSA (10, 20, 50, 100, 200, 500, 1000, 1500, and 2000 μg/ml) as standard (Bradford 1976).

Purification of recombinant protein

The soluble GST-rDuIL-2 fusion protein was purified by affinity chromatography. The above soluble fraction containing recombinant fusion protein was applied at 1 ml/min to a glutathione Sepharose column(10 × 100 mm) and equilibrated with buffer A. The protein fractions were eluted at 1 ml/min with 20 mmol/l reduced glutathione buffer, and protein contents in eluted fractions were measured by absorbance at A_{280} with an on-line preparative UV detector. Fractions containing recombinant protein (GST-rDuIL-2 fusion protein) were pooled for the next purification step. After purification, the GST-rDuIL-2 fusion protein was digested by thrombin, and was applied to the above glutathione Sepharose column(10 × 100 mm)that had been equilibrated with buffer A. Un-adsorbed fraction was collected to obtain non-fusion rDuIL-2. The column then was regenerated sequentially with 20 mmol/l reduced glutathione buffer and 0.1 mol/l NaOH.

Western blot analysis

The expressed proteins and purified proteins were subjected to SDS-PAGE and then blotted to a nitrocellulose membrane (Amersham Pharmacia Biotech, New Jersey,

USA) as described (Zhou et al. 2004). The membrane was blocked for 1 h at room temperature with TBST (25 mM Tris–HCl, 125 mM NaCl, 0.1% Tween 20, pH 8.0) containing 5% skimmed milk. After washing for five times with TBST, the blots were incubated with anti-DuIL-2 polyclonal antibody (1:1000, produced by our laboratory). Antiserum against rduIL-2 (pAb) was generated by immunization of KM mice, the purified recombinant GST-DuIL-2 fusion protein emulsion (100 μg fusion protein per mouse). Immunized mice were bled 4 weeks after last injection and the serum was collected. After washing three times for 5 min with TBST, the membranes were incubated with Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG2a (1:5000, Southern Biotechnology Associates Inc., Birmingham, USA) in TBST containing 5% skimmed milk. DAB (Promega, New Jersey, USA) was added to corresponding reaction at color development period.

RDuIL-2 bioactivity assay

The bioactivity of rDuIL-2 was determined by lymphocyte proliferation assay in vitro using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay (Liang et al. 2010). Briefly, the duck SMC stimulated by ConA for 24 h were collected by centrifugation with 500× *g* for 10 min at room temperature. After washing cells twice with RPMI 1640 medium containing 0.1 M α-methyl D-mannoside (α-MM), the viability of the lymphocytes was evaluated with trypan-blue exclusive staining. The enriched viable lymphocytes were resuspended in RPMI 1640 containing 10% FCS and cultured at 2×10^6 cell/ml with the supernatant containing different concentrations of rDuIL-2 protein in triplicate in 96-well microtiter plates (100 μl/well). Cells containing RPMI 1640–FCS only were used as a negative control, and RPMI 1640–FCS only was used as blank. After incubation at 40°C under 5% CO_2 for 48 h, 20 μl WST-1 was added to each well, and the samples were incubated for 4 h at 40°C under 5% CO_2 and placed at room temperature for 20 min. The optical density value (OD) was measured at 450 nm with a Biorad Microplate Reader 3550. The stimulation index (SI) of lymphocytes = $(OD_{rDuIL\text{-}2} - OD_{blank})/(OD_{negative} - OD_{blank})$. The SI value of 1.5 or above was defined as the positive criterion of the rDuIL-2 bioactivity.

Results

Cloning and sequencing analysis of duck IL-2 cDNA

A specific DNA fragment (about 400 bp) was cloned from duck ConA-stimulated lymphocytes by RT-PCR (Fig. 2). The recombinant cloning plasmid pUCm-T-duIL-2 was

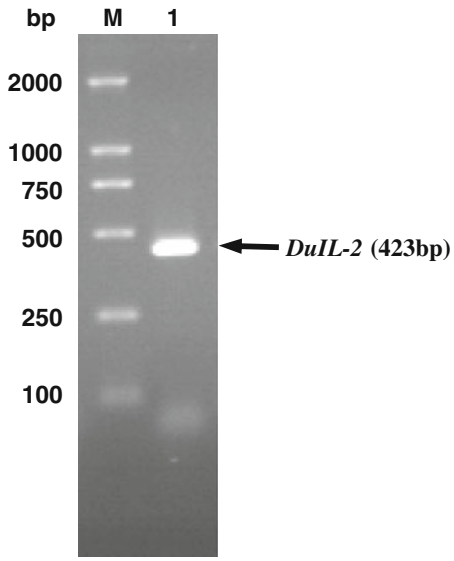

Fig. 2 Agarose gel (1%) electrophoresis analysis of the PCR product. *Lane M*, DL2000 DNA Marker; *lane 1*, the PCR product

constructed for sequencing. Sequencing results showed that the sequence of the insert was 423 bp (located in 94–516 bp of nucleotide sequence of ML DuIL-2 and in 13–435 bp of nucleotide sequence of MV DuIL-2) encoding a polypeptide of 140 amino acid residues (included the signal peptide) with a predicted molecular mass of 16.06 kDa and the theoretical pI of 4.60, and shares 100 and 98% identity with MV DuIL-2 and ML DuIL-2, respectively. Signal peptide analysis revealed that the DuIL-2 precursor peptide comprised a signal peptide with 21 amino acid residues with the potential cleavage on between *Gly*21 and *Ala*22, which was similar in size to that of mammalian animals (20 amino acid residues) and other birds (22 amino acid residues) from the data acquired from GenBank (Zhou et al. 2005a, b). The predicted molecular weight for mature DuIL-2 was 13.76 kDa and the theoretical pI was 4.52.

Construction of plasmid pGEX-DuIL-2

After mature DuIL-2 gene (360 bp) was sub-cloned into the pGEX-4T-3 vector, the recombinant plasmid pGEX-DuIL-2 was identified by enzyme digestion as shown in Fig. 3. The sequencing result showed that the ORF encoding a polypeptide of 119 amino acid residues and nucleic acid sequence of mature DuIL-2 gene were correct.

Expression of recombinant proteins

Escherichia coli strain Origami (DE3) was used as host for the expression of recombinant proteins. The host strains, harboring the plasmid pGEX-DuIL-2, were induced with 0.5 mM IPTG at 25°C for 9 h. The results were showed in

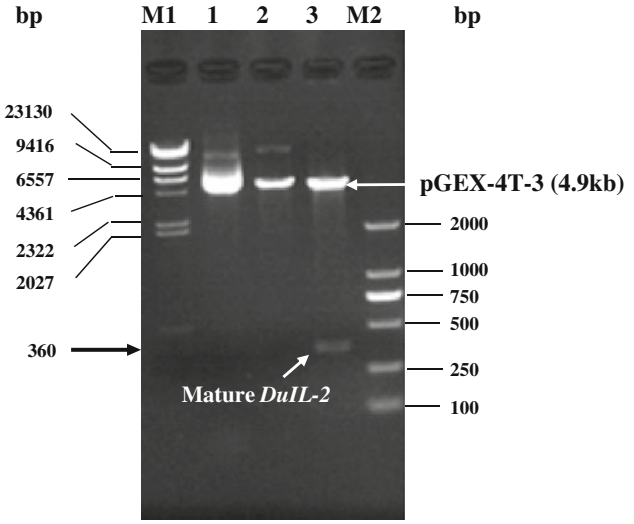

Fig. 3 Identification of recombinant plasmid pGEX-DuIL-2 by restriction enzyme digestion. *Lane M1*, *λ-Hind* III digest DNA Marker; *lane 1*, plasmid pGEX-DuIL-2; *lane 2*, plasmid pGEX-DuIL-2 digested by *Eco*R I; *lane 3*, plasmid pGEX-DuIL-2 double digested by *Eco*R I and *Not* I; *lane M2*, DL2000 DNA Marker

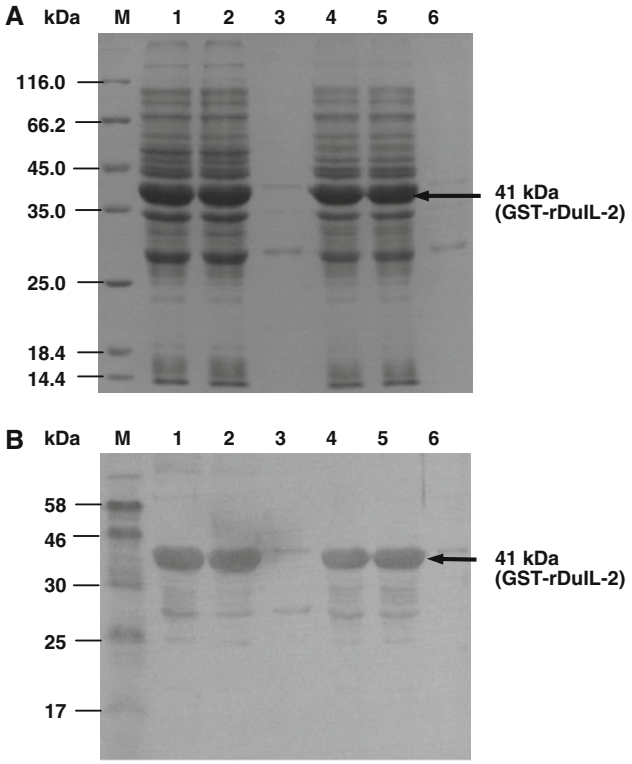

Fig. 4 Analysis of GST-rDuIL-2 fusion protein expressed in *E. Coli.* The expression was induced with 0.5 mM IPTG at 25°C for 9 h or with 4 mg/ml lactose at 25°C for 5 h. The expressing cells were separated into soluble (supernatant) and insoluble (precipitation) fractions and analyzed by SDS-PAGE (**a**) or western bolt (**b**). **a** SDS-PAGE (12%) analysis. *Lane M*, protein molecular marker; *lane 1*, total proteins from crushed cells of Origami (pGEX-rDuIL-2) induced with IPTG; *lane 2*, supernatant from crushed cells of Origami (pGEX-rDuIL-2) induced with IPTG; *lane 3*, precipitation from crushed cells of Origami (pGEX-rDuIL-2) induced with IPTG; *lane 4*, total proteins from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose; *lane 5*, supernatant from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose; *lane 6*, precipitation from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose. (B) Western bolt analysis. *Lane M*, prestained protein molecular marker; *lane 1*, total proteins from crushed cells of Origami (pGEX-rDuIL-2) induced with IPTG; *lane 2*, supernatant from crushed cells of Origami (pGEX-rDuIL-2) induced with IPTG; *lane 3*, precipitation from crushed cells of Origami (pGEX-rDuIL-2) induced with IPTG; *lane 4*, total proteins from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose; *lane 5*, supernatant from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose; *lane 6*, precipitation from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose

Fig. 4. Figure 4a was SDS-PAGE analysis of GST-rDuIL-2 fusion protein expressed in *E. coli* and Fig. 4b was the western bolt analysis. In Fig. 4, lane 1 was total proteins from crushed cells of Origami (pGEX-rDuIL-2) induced with IPTG, lane 2 was supernatant (containing rDuIL-2 expressed in the soluble form) from crushed cells of Origami (pGEX-rDuIL-2) induced with IPTG, and lane 3 was precipitation (containing rDuIL-2 expressed in inclusion bodies form) from crushed cells of Origami (pGEX-rDuIL-2) induced with IPTG. The above results revealed that GST-rDuIL-2 fusion protein of about 41 kDa was expressed in the soluble form, which was consistent with the predicted molecular mass of rDuIL-2 with about 14 kDa, GST with 26 kDa molecular mass and linker between GST and rDuIL-2 with about 1 kDa.

In order to produce soluble rDuIL-2 in a low-cost, nontoxic and high-level expression process, lactose was used as a substitute for IPTG to induce the above recombinant strain Origami (pGEX-DuIL-2). By optimizing the expression conditions, the most of GST-rDuIL-2 fusion proteins were expressed in the soluble forms when the Origami (pGEX-rDuIL-2) strains ($OD_{600} = 1.5$) were induced with 4 mg/ml lactose at 25°C for 5 h. The results were showed in Fig. 4. In Fig. 4, lane 4 was total proteins from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose, lane 5 was supernatant (containing rDuIL-2 expressed in the soluble form) from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose, and lane 6 was precipitation (containing rDuIL-2 expressed in inclusion bodies form) from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose. The Coomassie brilliant blue-stained SDS-PAGE (Fig. 4a) was scanned with an UVP White/Ultraviolet transilluminator, and analyzed with software Grab-it 2.5 and Gel-work (UVP) for estimating the purity of GST-rDuIL-2 fusion protein. The results revealed that the production of soluble GST-rDuIL-2 fusion protein was about 29% of total cellular soluble proteins when the Origami (pGEX-rDuIL-2) strains were induced with lactose, which was similar with IPTG used as inducer.

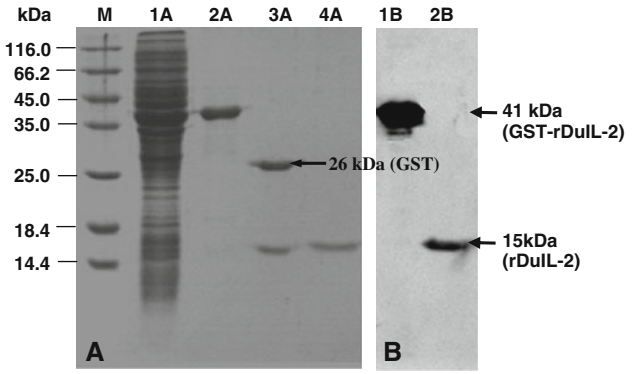

Fig. 5 Purification of the recombinant duck interleukin-2 (rDuIL-2). **a** SDS-PAGE (15%) analysis. *Lane M*, protein molecular marker; *lane 1A*, supernatant from crushed cells of Origami (pGEX-rDuIL-2) induced with lactose; *lane 2A*, purified GST-rDuIL-2 fusion protein; *lane 3A*, the GST-rDuIL-2 fusion protein digested by thrombin; *lane 4A*, purified rDuIL-2 protein. **b** Western boltting analysis. *Lane 1B*, purified GST-rDuIL-2 fusion protein; *lane 2B*, purified rDuIL-2 protein

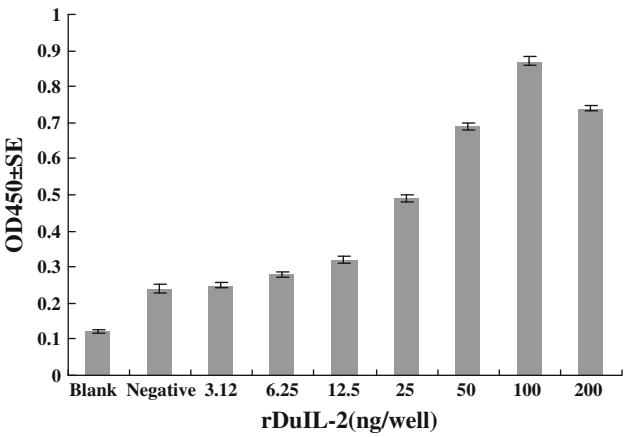

Fig. 6 In vitro lymphocyte proliferation assay of the recombinant duck interleukin-2 (rDuIL-2). Cells containing RPMI 1640–FCS only were used as a negative control, and RPMI 1640–FCS only was used as blank. The stimulation index (SI) of lymphocytes = ($OD_{rDuIL\text{-}2}$-OD_{blank})/($OD_{negative}$–OD_{blank}). SI value of 1.5 or above was defined as the positive criteria of the rduIL-2 bioactivity

Table 1 Purification of recombinant duck interleukin-2 (rDuIL-2) from 1 l *E. coli* culture

Purification steps	Total protein (mg)	Purity of fusion protein (%)	Purity of rDuIL-2 (%)
Cell extract (supernatant)	234.5	23.8	
Glutathione affinity chromatography (first)	36.2	95.2	
Thrombin digestion	36.2		35.1
Glutathione affinity chromatography (second)	10.6		95.8

Purification of the rDuIL-2

After induction, the cell pellet was sonicated and the supernatant was applied to a glutathione affinity column. Proteins without GST tag were removed from the glutathione Sepharose 4B resin by washing with PBS, and the fusion protein with GST tag was then eluted with 50 mM Tris–HCl containing 20 mM reduced glutathione. The eluted fraction was detected by SDS-PAGE and the purity of GST-rDuIL-2 fusion protein was about 95% (Fig. 5, lane 2A).

The purified GST-rDuIl-2 fusion protein was cleaved with thrombin to release rDuIL-2. The result of SDS-PAGE showed that the most of fusion protein was cleaved within 16 h at 37°C (Fig. 5, lane 3A). The reaction mixture was then reloaded onto the glutathione affinity resin for purification. The rDuIL-2 (about 15 kDa) was obtained in the flow-through, whereas the GST tag was retained by the resin. The purity of rDuIL-2 determined by optical density scanning in SDS-PAGE gel was about 95% (Fig. 5, lane 4A). The summary of purification is presented in Table 1. The final yield of purified rDuIL-2 was about 10.6 mg/l bacterial culture. Further characterization using western blot analysis showed that both of the purified GST-rDuIl-2 fusion protein and rDuIL-2 could be recognized by an anti-DuIL-2 polyclonal antibody (Fig. 5, lane 1B and 2B).

In vitro proliferation of lymphocytes stimulated by rDuIL-2

The biological activity of rDuIL-2 was tested by in vitro proliferation of duck spleen cells. After the activated duck spleen cells co-cultured with the serial two-folds dilutions of the protein preparations containing rDuIL-2 protein, the culture mixtures reacted for 4 h with WST-1. The result showed that the rDuIL-2 stimulated proliferation of duck lymphocytes from 12.5 ng rDuIL-2/well (SI = 1.67), and it maximally stimulated the proliferation at 100 ng rDuIL-2/well (Fig. 6). The results showed that the rDuIL-2 proteins possessed biological activity.

Discussion

Recombinant proteins expressed in *E. coli* are often produced as aggregates called inclusion bodies. But in many applications, it is desirable to express target proteins in their soluble active forms. In a previous report, the rDuIL-2 expressed in *E. coli* was in the form of inclusion bodies, and only about 4.375 mg rDuIL-2/l bacterial culture was obtained (Zhou et al. 2005b), which limited its further purification and resulted in its costly preparation.

In the present study, we have shown that rDuIL-2 with biological activity can be expressed in soluble form by *E. coli*. Firstly, GST was chosen as carrier fused to the N-terminus of rDuIL-2. The result showed that GST tag could enhance solubility of rDuIL-2, and greater than 90% of the expressed protein was soluble by comparing lane 2 with lane 3 in Fig. 4. Secondly, the host used in the study was the *trxB*/*gor* mutant *E. coli* Origami (DE3), which greatly facilitates disulfide bond formation in expressed proteins (Du et al. 2010). It was reported that the *trxB*/*gor* mutant strains have the potential to enhance disulfide bond formation and ultimately solubility and activity to a greater degree than the *trxB*-only mutants (Bessette et al. 1999). Thirdly, in order to produce soluble rDuIL-2 in a low-cost, nontoxic and high-level expression process, lactose was used as a substitute for IPTG to induce the above recombinant strain Origami (pGEX-DuIL-2). By optimizing the expression conditions, the production of soluble GST-rDuIL-2 fusion protein was about 29% of total cellular soluble proteins, which was similar with IPTG used as inducer (Fig. 4a, lane 2 and lane 5).

We also developed a simple, economical purification process for rDuIL-2 isolation. GST fused to the N-terminus of rDuIL-2 was used as an affinity purification tag, so the recombinant protein was directly purified by glutathione affinity chromatography to get an electrophoresis-level fusion protein GST-DuIL-2 with the purity about 95% (see Fig. 5). The purified GST-rDuIL-2 fusion protein was cleaved with thrombin to release rDuIl-2. After second glutathione affinity chromatography, the un-adsorbed fraction was collected to obtain non-fusion rDuIL-2. As a result, about 10.6 mg of rDuIL-2 with purity by SDS–PAGE of 95% was finally obtained from 1 l flask culture (Table 1).

The biological activity of rDuIL-2 was usually tested by MTT colorimetric method (Zhou et al. 2005b). But the formazan which had been generated from MTT was water insoluble, it had to be dissolved by the specific solution, sometimes it could not be completely dissolved, which led to error in experimental results. Therefore, in this study, we improved the MTT method, using WST-1 cell proliferation detection kit instead of the traditional MTT colorimetric method. WST-1 is a compound similar to MTT, and it can be reduced to generate a water-soluble orange formazan by the dehydrogenase in the mitochondria (the solution has a maximum absorption peak at 450 nm), which could reduce error in the experiments (Liang et al. 2010).

In summary, the *E. coli* expression system described here could provide a feasible and convenient approach to produce soluble recombinant duck IL-2 with biological activity in a low-cost, nontoxic and high-level expression process, which lays the foundation for development of novel immunoadjuvants for enhancing vaccine efficacy.

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