



Extraction and purification of human interleukin-10 from transgenic rice seeds

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ABSTRACT

Recombinant protein production system using transgenic rice grain offers many advantages in higher accumulation, preservation, lower production cost, ease of scale up and low risk of contamination by toxic materials. We developed a transgenic rice strain whose seeds accumulate human interleukin (IL) 10, a cytokine that suppresses inflammation related immune responses. We also developed a method of extracting and purifying IL 10 from rice seeds. A biochemical crosslinking method was used to detect the biologically active noncovalent dimer of IL 10. This method was useful for developing efficient methods of refolding and purification. The purified IL 10 comprised only noncovalent dimers and showed higher activity than the commercial IL 10. The purified IL 10 had very low endotoxin contamination and is expected to have broad clinical application.

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Introduction

Production platform of recombinant proteins using transgenic plants have many benefits such as the high level of transgene expression, inexpensive initial and running cost, long stability at room temperature and ease of scale up [1]. In addition, the plant systems have a low risk of toxic contamination because they are free from animal pathogens, oncogenic DNA sequences, and endotoxins [1].

Endotoxins are lipopolysaccharides (LPS¹) derived from the cell membrane of Gram negative bacteria and potential contaminants of protein solutions originating from biologic products [2]. Endotoxins can induce pyrogenic reactions and shock in mammals upon intravenous injection at levels as low as 1 ng/ml [3]. Recombinant proteins produced from *Escherichia coli* are contaminated with endotoxins whose removal requires additional cost and time [2,4].

The transgenic rice system using a glutelin promoter is suitable for producing recombinant proteins. Glutelins are major seed proteins in rice, and recombinant protein genes under control of the glutelin promoter accumulate in the endosperm of the rice seeds [5,6]. The accumulation of recombinant protein in seeds has other

advantages as well, such as stable storage of recombinant proteins and lack of protease activity in the seeds. Many recombinant proteins have been successfully produced under the control of glutelin promoters in rice endosperm [5,7–11].

Interleukin 10 (IL 10) is a pleiotropic cytokine that stimulates innate and Th2 related immunity, but suppresses inflammation related immune responses. IL 10 is a candidate of therapeutic treatment for inflammatory and autoimmune diseases [12,13]. Intragastric administration of genetically modified *Lactococcus lactis* secreting murine IL 10 successfully improves colitis in a murine model [14]. Recent genome wide association studies demonstrated that IL 10 is a candidate therapeutic compound for ulcerative colitis in humans [15]. These results suggest that human IL 10 would be a strong therapeutic candidate for ulcerative colitis using a gastrointestinal administration protocol [16].

The active form of IL 10 is a noncovalently associated homodimeric protein [17]. This structural feature makes it rather difficult to produce recombinant IL 10 in the active form. Because the protein refolding efficiency determines the overall purification efficiency, effective refolding methods are required. The cultured cell based bioassay is a common method for measuring IL 10 activity, although it requires days to complete and is not very accurate. Therefore, a simple and accurate method to measure the activity of the refolded IL 10 must be developed to determine suitable refolding conditions. Transgenic plants producing IL 10 have already been reported [18–20]. In these studies, the aim was to produce IL 10 for oral administration without fine purification of

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¹ Abbreviations used: LPS, lipopolysaccharides; IL-10, interleukin-10; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CBB, coomassie brilliant blue; CTAB, cetyl trimethyl ammonium bromide

IL 10. Although some IL 10 activity was detected, fully active IL 10 has not yet been produced.

In the present study, we describe a system for the large scale production of human recombinant IL 10 for clinical use. We report human IL 10 production from transgenic rice seeds and efficient refolding and purification methods with a biochemical evaluation system. We produced IL 10 with high purity, high activity, and low endotoxin levels.

Materials and methods

Construction and generation of transgenic rice plants

The codon of human IL 10 cDNA (accession number: BC104252) was modified for expression in rice, and the DNA sequence for full length IL 10 with a 6× His tag and the KDEL sequence at the C terminal was synthesized by GenScript Corporation (Piscataway, NJ). KDEL sequence is an endoplasmic reticulum retention signal and increase recombinant protein accumulation in rice seed [6]. The IL 10 gene was ligated downstream of the 2.3 kb glutelin B 1 (GluB 1) promoter [21] containing a signal peptide sequence. The promoter and IL 10 gene were ligated to the binary vector pGPTV 35S HPT GluB 1term [6] (Fig. 1).

The expression construct was introduced into the rice genome (*Oryza sativa* cv. Kita ake) using the *Agrobacterium tumefaciens* mediated transformation method, and transgenic plants were selected as described previously [5]. Transgenic plants were grown in a greenhouse (28 °C, 12 h light/dark cycle). Individual rice grain (at least four grains per individual line) was ground to fine powder using Multi beads Shocker (Yasui Kikai Corp., Osaka Japan) and protein was extracted to analyze IL 10 expression. The highest transgene expressing line was screened and then homozygous line was fixed by self crossing through a few generations.

Protein analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting were performed [22,23]. Total seed proteins were extracted with SDS PAGE sample buffer containing 8 M urea. After electrophoresis, the gels were stained with Coomassie brilliant blue (CBB) or transferred to polyvinylidene difluoride membranes (Immun Blot, Bio Rad, Hercules, CA). The membranes were incubated with the anti His tag antibodies (Roche Diagnostics GmbH, Mannheim, Germany) and then a peroxidase conjugated secondary antibody (Dako Denmark A/S, Glostrup, Denmark). Alternatively, the membranes were stained using kits based on Periodic acid Schiff (G.P. SENSOR, J Oil Mills, Inc., Tokyo, Japan) or lectin (HRP Lectin Kit, J Oil Mills) methods. The signals were visualized using an ECL Plus Western blotting detection system (GE Healthcare UK Ltd., Little Chalfont Buckinghamshire, England) and the images were captured using ChemiDoc XRS (Bio Rad).

Total protein concentration was measured using either a standard BCA protein assay kit or reducing agent compatible BCA protein assay kit (Thermo Scientific, Rockford, IL) with bovine serum albumin as the standard. IL 10 concentration was measured using a human IL 10 instant enzyme linked immunosorbent assay (Bender MedSystems, Vienna, Austria) or Western blotting.

Crosslinking experiment

Crosslinking of IL 10 was performed basically according to Syto et al. with slight modification [26]. When the buffer used for the protein samples contained chemicals with an amino group (e.g., Tris or arginine), the samples were dialyzed against phosphate buffered saline. For a denatured IL 10 control, a one tenth volume of 1 N HCl was added to the samples and incubated for 30 min at room temperature. The pH was neutralized by adding the same volume of 0.4 M sodium bicarbonate, pH 8.5. Crosslinking was performed with 1 mM bis(sulfosuccinimidyl) suberate (BS3) (Thermo Scientific) in 0.2 M sodium bicarbonate, pH 8.5 for 30 min at room temperature. The crosslinking reaction was stopped by adding a one twentieth volume of 1 M Tris pH 8.0. The crosslinked samples were mixed with SDS PAGE sample buffer and analyzed using SDS PAGE and Western blotting methods.

Extraction and purification of IL 10

Milli Q water (Millipore, Bedford, MA) was used for the purification experiment. All glassware was dry heated at 250 °C for 90 min to decompose any endotoxins. Buffers were prepared just before use with dry heated glassware and fresh Milli Q water.

Husks and bran were removed from transgenic IL 10 rice seeds using FC2 K (Otake Seisakusho, Oharu, Aichi, Japan) and Magic Mill RSKM5B (Satake, Hiroshima, Japan), respectively. The seeds were then ground using an electric stone mill (F 50, Samap S.A., Andolsheim, France). The rice powder was passed through a 355 µm sieve and then used for protein extraction. Forty grams of rice powder was mixed with 800 ml pre extraction buffer (50 mM Tris HCl pH 7.4, 0.5 M NaCl) and extracted with a magnetic stirrer at 400 rpm for more than 3 h. After centrifugation at 14,000 g for 10 min, the supernatant was discarded and the pellet was resuspended with 800 ml of extraction buffer (50 mM Tris HCl pH 7.4, 0.5 M NaCl, 1% cetyl trimethyl ammonium bromide [CTAB] and 10 mM β mercaptoethanol). IL 10 was pre extracted twice. The extract was stored on ice for several hours, and the precipitated CTAB and fine particles were removed by filtration with a nylon mesh and a glass microfiber filter (GF/A, GE Healthcare), respectively. Imidazole (pH 7.4) was mixed with the extract and adjusted to 20 mM. The extract was applied to a HisTrap FF 5 ml column on an AKTA Prime Plus System (GE Healthcare). His tagged IL 10 was eluted with 50 mM Tris HCl pH 7.4, 0.5 M NaCl, and 0.4 M imidazole. The eluate was precipitated with equal amounts of acetone, dissolved with 6 M guanidine, 20 mM Na phosphate pH 7.4, 0.5 M NaCl, 20 mM imidazole, and 10 mM β mercaptoethanol, and re applied to a HisTrap HP 1 ml column. IL 10 was eluted with the same buffer containing 0.4 M imidazole. The eluate was dialyzed overnight with 10 mM Tris HCl pH 8.0 and again precipitated with acetone.

Refolding was performed by the dilution method. IL 10 (20 mg) was dissolved with 0.5 ml denaturation buffer (6 M guanidine, 50 mM Tris HCl pH 8.5, 30 mM dithiothreitol). The solution was then diluted to 40–100 volumes of refolding buffer (50 mM Tris HCl pH 8.5, 0.4 M arginine, 1 M ammonium thiocyanate, and 0.5 mM oxidized glutathione) and incubated at 33–42 °C overnight. The refolded IL 10 was dialyzed against phosphate buffered saline at 5 °C overnight, and then dialyzed against 50 mM Tris HCl pH 9.0. The refolded IL 10 was applied to anion exchange

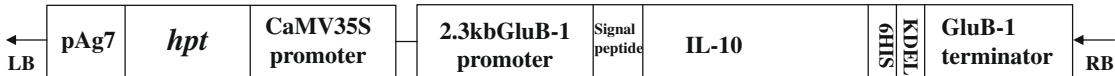


Fig. 1. Construction of the expression vector for rice transformation. hpt, hygromycin phosphotransferase gene; pAg7, 3′terminal region of agropine synthase gene; RB, right border; LB, left border; CaMV35S P, cauliflower mosaic virus 35S promoter, GluB-1 promoter, glutelin B1 promoter, GluB-1 terminator, glutelin B1 terminator; 6his, 6× histidine tag sequence.

column chromatography (HiTrap Q HP, GE Healthcare) with 50 mM Tris HCl pH 9.0, and a linear gradient from 0 to 0.5 M of NaCl was applied to elute the IL 10 dimer fraction. The eluate was dialyzed with 50 mM MES, pH 6.5 and applied to cation exchange column chromatography (HiTrap SP, GE Healthcare) and eluted with a linear gradient from 0 to 0.5 M NaCl. The eluate was applied to gel permeation column chromatography (HiPrep Sephacryl S 100HR, GE Healthcare). Throughout the purification procedure, recombinant IL 10 was monitored by Western blotting with an anti his tag antibody. After refolding, a crosslink analysis was performed to distinguish dimers and other forms of IL 10, and the fraction containing the IL 10 dimers was collected.

Endotoxin measurement

Endotoxin was measured by the *Limulus* amoebocyte lysate method using an Endospecy ES 24S kit with an EG reader SV 12 (Seikagaku Biobusiness Co., Tokyo, Japan) according to the manufacturer's instructions.

IL 10 bioassay

Bone marrow derived dendritic cells were used for the IL 10 bioassay [24]. Cells were removed from the bone marrow of BALB/c mice and cultured in the presence of granulocyte macrophage colony stimulating factor for 8 days. Differentiated dendritic cells were used for the assay. Interleukin 12p70 was produced by LPS stimulation of the dendritic cells, and IL 12p70 production was inhibited by IL 10 in a dose dependent manner. The purified IL 10 was assayed by comparing with standard commercial human and mouse IL 10. Standard IL 10 was purchased from R&D Systems Inc. (Minneapolis, MN).

Results

Extraction of recombinant human IL 10 from rice seeds

The *GluB 1* promoter is a powerful promoter for driving endosperm specific foreign gene expression [5,6,21]. We constructed a full length human IL 10 expression vector driven by the 2.3 kb *GluB 1* promoter containing its signal peptide (Fig. 1) and produced transgenic rice lines. We selected the highest transgene expressing homozygous line and mass cultivated it to collect rice seeds for the purification study.

A household rice polishing machine and electric stone mill were sufficient to produce rice powder for milligram scale IL 10 purification. The rice powder was passed through several different sieves and the extraction efficiency was tested. Relatively coarse powder passed through a 355 μ m sieve was sufficient for the extraction of IL 10 (data not shown).

In a preliminary experiment, IL 10 failed to be extracted under nondenaturing conditions. Further analysis revealed that reducing agents and detergent are necessary to extract IL 10 from rice powder. We tried several reducing agents and detergents, and optimized the pH, NaCl concentration, and buffer quantity to determine the optimum condition to extract IL 10 from rice powder. Extraction using a buffer without a reducing agent successfully removed unwanted rice proteins. IL 10 was extracted from the transgenic rice seeds with a buffer containing 10 mM β mercaptoethanol, 1% CTAB, 50 mM Tris pH 7.4, and 0.5 M NaCl.

Purification of recombinant IL 10

His tagged IL 10 was purified using Ni affinity column chromatography. The fact that a reducing agent was necessary for the

extraction of IL 10 means that a subsequent refolding step was also necessary for IL 10 purification because native disulfide formation is crucial for IL 10 activity [25]. Refolding can be very inefficient and lower the overall purification yield. We developed a biochemical method to evaluate the active form of IL 10, which was then used to develop a suitable refolding method. Active IL 10 is composed of noncovalent homodimers. Monomers and other forms become denatured and lose their activity [25,26]. Therefore, we treated samples with a chemical crosslinker and analyzed them using Western blotting to detect the IL 10 dimers. Using this method, we were able to analyze native form of IL 10 and test many conditions for refolding, and finally determined the suitable conditions. Without crosslinking, IL 10 was reduced, denatured and become monomer by SDS sample buffer. It is suitable for CBB staining to analyze contaminating proteins. We used the two methods to analyze protein samples during purification.

The refolded active IL 10 was applied to column chromatography and noncovalent dimers were recovered using anion exchange column chromatography (Fig. 2), cation exchange column chromatography, and gel permeation column chromatography (Fig. 3), as described in the Materials and methods. Eluted fractions were analyzed by a crosslinking method (Figs. 2 and 3) and dimer fractions were pooled.

Anion exchange column chromatography gave two major peaks on a 0–0.5 M NaCl gradient (Fig. 2A). SDS PAGE analysis of eluted fractions treated with SDS sample buffer containing β mercaptoethanol did not reveal two distinct peaks (data not shown), but the peaks were clearly distinguished when using the crosslinked samples (Fig. 2B). The first peak comprised dimers and the second

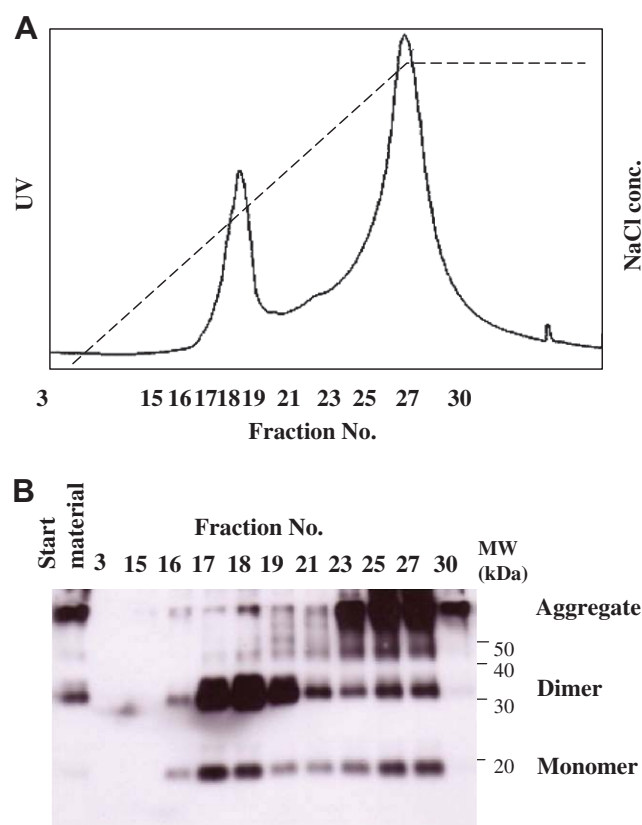


Fig. 2. Purification by Anion Exchange Chromatography. Refolded IL-10 was dialyzed against 50 mM Tris pH 9.0 and applied to an anion exchange column, HiTrap Q HP 5 ml. IL-10 was eluted with a linear gradient of 0–0.5 M NaCl. (A) UV absorbance (solid line) and NaCl concentration (dashed line). (B) The protein samples in each fraction were crosslinked and subjected to SDS–PAGE and Western blotting using anti-6 $\times$ his antibody.

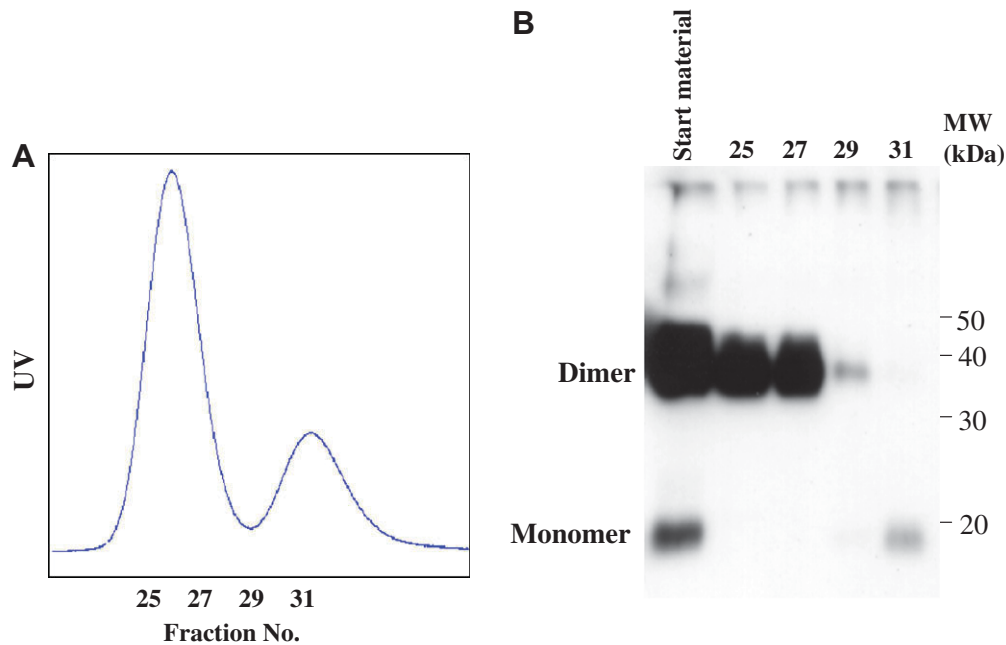


Fig. 3. Purification by gel permeation chromatography. The eluate from a cation exchange column was applied to a gel filtration column, HiPrep 16/60 Sephacryl S-100 HR. Sodium phosphate (50 mM), pH 7.4, NaCl (150 mM) was used as the buffer. (A) UV absorbance (solid line). Dimers and monomers were separated. (B) The eluted fractions were crosslinked and analyzed by Western blotting.

peak comprised high molecular aggregates. Gel permeation column chromatography effectively separated the dimers and monomers (Fig. 3) and pure active IL 10 noncovalent dimers were obtained. A summary of typical purification data is shown in Fig. 4A and Table 1. From 40 g of rice powder, we obtained 2 mg of pure IL 10.

Analysis of the purified recombinant IL 10

The crosslinking method showed that the purified recombinant IL 10 contained only the dimer form (Fig. 4B). Both noncovalent

dimers as well as inactive covalent dimers may be contained in the dimer band. Using the crosslinking method, we attempted to distinguish active noncovalent dimers from inactive covalent dimers, which are produced under some refolding conditions. Acid treatment denatures and dissociates noncovalent dimers [26], and therefore the dimer band remaining after acid treatment can be considered to comprise inactive covalent dimers. The analysis also showed that our refolding method produced few covalent dimers (Fig. 4B). Periodic acid Schiff and lectin blotting experiments showed that recombinant human IL 10 was not glycosylated (data not shown), and was the same as natural human IL 10 [27].

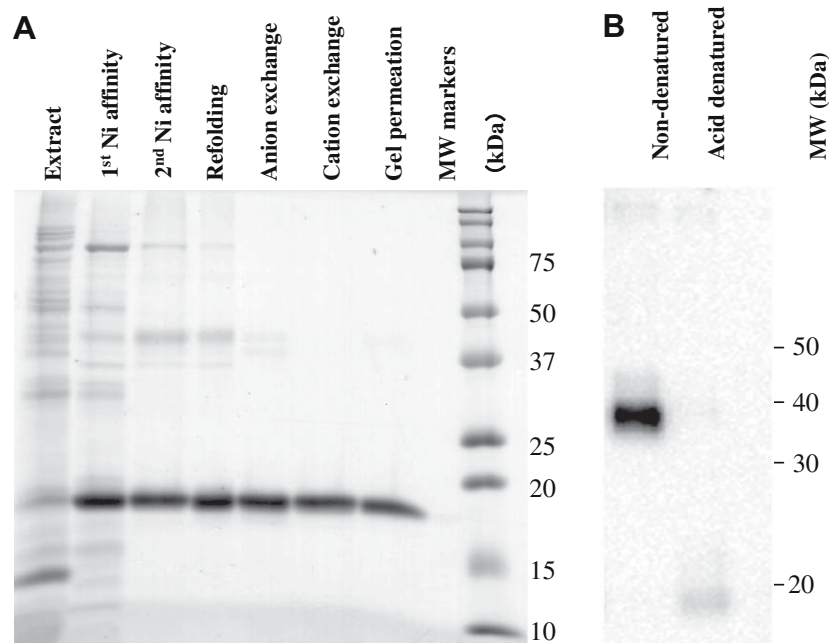


Fig. 4. Analysis of protein samples from each purification step and the purified IL-10. (A) Protein samples from each purification step were analyzed by SDS-PAGE. The gel was stained with Coomassie brilliant blue. (B) Purified IL-10 and acid denatured IL-10 were crosslinked and analyzed by Western blotting.

Table 1
Summary of recombinant human IL-10 purification.

	Total protein (mg)	IL-10 (mg)	Recovery (%)	Purity (%)
Extract	1040.8	48.2	100	4.6
1st Ni affinity	71.2	46.4	96.3	65.2
2nd Ni affinity	31.6	23.7	49.2	75.0
Refolding	26.3	19.6	40.6	74.5
Anion exchange	7.3	6.3	13.1	86.3
Cation exchange	3.4	3.3	6.8	97.1
Gel permeation	2.1	2.1	4.3	100

Note: 40 g of rice seeds were used.

Endotoxin concentrations of three independently purified IL 10 samples were measured and 0.000024 ± 0.000014 EU/ μ g of endotoxin was detected.

The biologic activity of the purified IL 10 was measured using mouse bone marrow dendritic cells (Fig. 5). IL 10 activity was measured as the quantitative inhibition of LPS induced IL 12 production. Human IL 10 standards (2 ng/ml) and mouse IL 10 standards (0.2 ng/ml) almost fully suppressed IL 12 production. The three independently purified IL 10 samples induced full suppression at 0.2 ng/ml and some suppression at even 0.02 ng/ml. The purified recombinant IL 10 had at least 10 times higher activity than did commercially available human IL 10.

Discussion

In the present study, we produced a transgenic rice line that expresses human IL 10 and developed methods to extract and purify the IL 10. The purified IL 10 had high purity, high activity, and very low endotoxin levels.

A preliminary extraction experiment using a buffer without a reducing agent failed to extract IL 10. IL 10 is considered to exist in rice seed in the aggregated form by binding to itself or other proteins, e.g., prolamins or glutelins that have many cysteine residues, via disulfide bonds [28]. Based on this tendency, we succeeded in removing unwanted rice seed proteins, mainly globulin and albumin [29], using a buffer without a reducing agent.

Ni affinity column chromatography effectively purified His tagged IL 10, and refolding was performed. A preliminary experiment showed that published refolding methods of IL 10 were not very effective, and a conventional bioassay method using cultured cells was too time consuming and inaccurate. We used a crosslinking method to detect active noncovalent dimers of IL 10. This strategy was efficient and many conditions could be quickly evaluated. Using the crosslinking method, we were also able to evaluate purified fractions (Figs. 2 and 3). This method revealed that the dimer form of IL 10 was isolated effectively from aggregates using anion exchange column chromatography (Fig. 2), and from monomers using gel permeation chromatography (Fig. 3).

The noncovalent dimer is the only active form of IL 10 [26]. Monomers and other forms lost their activity and were irreversibly denatured. Therefore, recombinant IL 10 production requires synthesis of the noncovalent form; otherwise, the denatured form of IL 10 must be refolded. Menassa et al. and Bortesi et al. reported that transgenic plants produce active IL 10 [18–20], although they reported that the IL 10 'dimer' was analyzed by nonreduced SDS PAGE. SDS dissociates noncovalent IL 10 dimers to monomers [30], so their IL 10 samples are considered to contain a considerable amount of covalent dimers.

Using mouse dendritic cells, IL 10 activity was measured as the quantitative inhibition of LPS induced IL 12 production (Fig. 5). The IL 10 produced from rice had 10 times higher activity than standard IL 10. Fig. 4B shows that most of the IL 10 from purified rice is in the dimer form, and our study also showed that commercial IL 10 contains a large amount of monomers, covalent dimers, and aggregates (data not shown).

The purified samples contain much lower endotoxin levels compared to recombinant protein produced by bacteria (0.1–1 EU/ μ g). The removal of endotoxins is troublesome and costly. Production of recombinant proteins by *E. coli* inevitably produces contamination with a considerable amount of endotoxins [2,4]. Low endotoxin levels in recombinant protein are a great advantage of this transgenic plant system. The removal of endotoxins is unnecessary if the extraction and purification procedures are performed carefully to prevent contamination from the water used for extraction and purification. Using this rice system, IL 10 is inexpensive and safe. This method can be used to increase the use of IL 10 for clinical applications that require relatively large amount of IL 10, such as nasal or oral applications in the treatment of allergies.

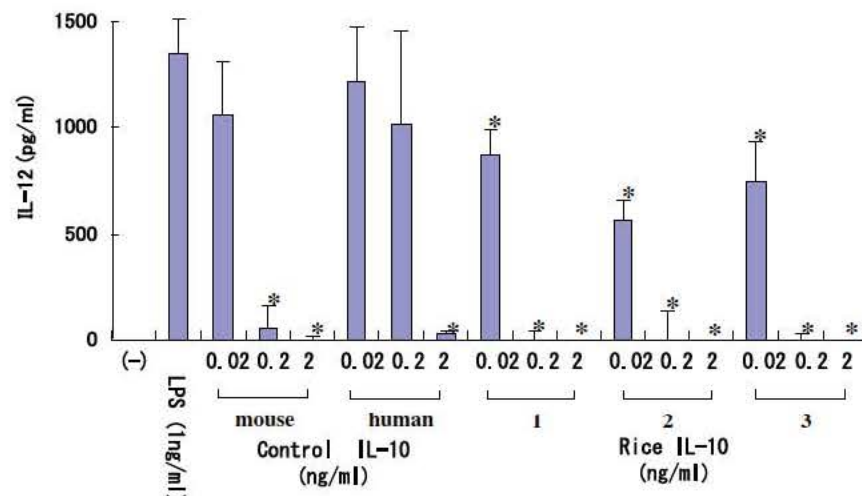


Fig. 5. Bioassay of recombinant human IL-10 using dendritic cells. IL-10 activity was measured using dendritic cells that were differentiated from cultured mouse bone marrow cells. IL-10 activity levels were determined by the inhibition of IL-12p70 production stimulated by LPS. Results are expressed as mean $\pm$ standard deviation from three experiments. Asterisks represent statistical significance compared to LPS induction control (* P < 0.05).

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