

High-level expression of a codon optimized recombinant dust mite allergen, Blo t 5, in Chinese hamster ovary cells

L.H. Lim,^a H.Y. Li,^a N. Cheong,^b B.W. Lee,^a and K.Y. Chua^{a,*}

^a Department of Paediatrics, National University of Singapore, Singapore

^b Bioprocessing Technology Institute, Biomedical Science Institutes, Agency for Science Technology and Research (A*STAR), Singapore

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Abstract

Blo t 5 is a major allergen from house dust mite *Blomia tropicalis*. Purification of native Blo t 5 (nBlo t 5) from whole dust mite extract is tedious and gave low yield. In this study, we demonstrated that codon optimization facilitated high-level expression of Blo t 5 in Chinese hamster ovary (CHO)-K1 cells and thus allows production of sufficient recombinant cBlo t 5 for specific immunotherapy. A codon optimized Blo t 5 gene was synthesized by PCR and the codon optimized or wild-type Blo t 5 gene in pcDNA3.0 was transfected into CHO-K1 cells and stably selected with Geneticin (G418). Western-immunoblot analysis of spent culture media detected a positive band at 14 kDa for the codon optimized but not wild-type gene transfectants. In addition, a stable CHO-K1 clone produced up to 13 mg/L of the cBlo t 5 protein having a high correlation of human IgE reactivities and allergenicity to the native Blo t 5, thus indicating proper conformation of this recombinant form.

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Keywords: *Blomia tropicalis*; Blo t 5; House dust mite; Chinese hamster ovary cells, CHO; Codon optimization

Blo t 5, a 14 kDa major allergen from house dust mite *Blomia tropicalis*, is of clinical importance in the tropical and subtropical regions of the world [1,2]. Recombinant Blo t 5 produced in *Escherichia coli* was shown to react with 45–69% of sera from *B. tropicalis*-allergic asthmatics and induced positive immediate skin tests; Blo t 5 is implicated as a probable IgE-mediated independent cause of sensitization for allergic asthma, rhinitis, and atopic dermatitis [1]. Recently, we have shown by inhibition study that Blo t 5 and the group five homologue Der p 5 (*Dermatophagoides pteronyssinus*) exhibited low level of IgE cross-reactivity [3]. Therefore, specific allergens of clinical grade are required for specific diagnosis and immunotherapy of sensitization to this group of mite allergens.

Current diagnostic and therapeutic strategy for allergic diseases is unsatisfactory due to the lack of highly purified allergens for serodiagnosis, skin test, and immunization purposes. Major problems associated with using aqueous crude mite extract for immunotherapy

involve clinical efficacy and unwanted immune response due to irrelevant proteins present in the crude extract [4]. Whole mite culture to obtain purified allergens is not only expensive to perform, it is also time consuming and gave low yield of purified allergens. Thus, recombinant production of allergens presents an efficient way to obtain high yields for immunological and biochemical studies as well as diagnosis and treatment of IgE-mediated allergic disorders (immunotherapy). In our laboratory, recombinant proteins of Blo t 5 expressed in *E. coli* [1] and yeast [3] gave considerable yield but are unsuitable for specific immunotherapy and skin prick tests due to FDA requirements. The Chinese hamster ovary (CHO) cell line is currently the most widely used mammalian cell line for the production of recombinant DNA-derived biopharmaceuticals. Recently, Jacquet et al. [5] reported the high-level expression (5- to 10-fold) in CHO cells of a recombinant house dust mite allergen, ProDer p 1 (a precursor form of Der p 1), with optimized codon usage. Since the mammalian codon usage pattern is different from those of a variety of lower organisms, optimizing the codon usage of the heterologous gene of interest has been shown not only to increase

* Corresponding author. Fax: +65-6775-7593.

E-mail address: paecy@nus.edu.sg (K.Y. Chua).

expression level of the gene in mammalian cells but is also crucial for effective immunization [6,7].

In this study, we have shown that codon optimization increased the efficiency of Blo t 5 expression in CHO-K1 cell. In addition, this purified recombinant cBlo t 5 protein exhibited comparable IgE reactivity with the native form, nBlo t 5.

Materials and methods

Generation of codon optimized Blo t 5 gene. The codon optimized Blo t 5 gene was designed based on codon preference of highly expressed human genes [5]. All PCRs were performed using the Expand High Fidelity DNA Polymerase (Boehringer). The codon optimized Blo t 5 gene was synthesized by PCR using a set of partially priming overlapping oligonucleotides [B1 (5'-CCCCCGGATCCACCATGGAGCAGGAGCACAAAGCCC-3'); B2 (5'-CAGGAGCACAAAGCCCAAGAAGACGACTTCAGGAACGAGTTCGACCACTTGCTGATCGAG-3'); B3 (5'-GACCACTTGCTGATCGAGCAGGCtAACCACGCGATC GAAAAGGGGGAGCATCAACTGCTC-3'); B4 (5'-GGGGAGCATCAACTGCTCTACCTGCAGCACCAGCTCGACGAGCTGAATGAGAACAAGAGC-3'); B5 (5'-CTGAATGAGAACAAGAGCAAGGAAGTGCAGGAGAGAAATCATCCGCGAGCTTGATGTGGTC-3'); B6 (5'-CGCGAGCTTGATGTGGTCTGCGCCATGATCGAAGGC GCCCAGGGCGCCCTGGAGCGCGAG-3'); B7 (5'-GGCGCCCTG GAGCGCGAGCTGAAGCGGACCGACCTAACATCCTGGAGCGCTTAACTAC-3'); B8 (5'-CTGGAGCGCTTAACTACGAA GAGGCCAGACTCTCTCCAAGATCCTGCTCAAGGACCTG-3'); and B9 (5'-ATCCTGTCTCAAGGACCTGAAGGAGACCGAGCAGAAAGTGAAGGACATTACAGACCCAGTGATCTAGACCCCC-3')]. Oligonucleotides B2–B9 were mixed in equal volume and 1 μ L of this mixture was used in a template-free primary PCR with the following condition: 30 cycles, denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 1 min. The codon optimized gene was amplified from the generated products by a secondary PCR using the 3' and 5' terminal primers (B1 and B9) with the same PCR condition. The resultant 366 bp DNA fragment was cloned into pGEMT-vector (Promega) and a clone, designated as pGEMT-cBlo t 5, was DNA sequenced.

A codon optimized leader peptide of the VZV-glycoprotein E (VZV) was generated using overlapping oligonucleotides V1 to V4 [V1 (5'-CCCCCGGATCCCGGGGCAACTGCGTGGTTTAAAGTGAC TATATCCCCAGGGTCGCC-3'); V2 (5'-GCCACCACAGGCTTA TCACTGTGCCATGGTGGCGTTACAGGCGACCCTGGGGA TATAG-3'); V3 (5'-AATAAGCCTGTGGTGGGCGTACTGATGG GGTTCGGAATCATCAGGCTGACTTGCCTGATA-3'); and V4 (5'-CCTTCTTGGGCTTGCTCCTGTCTGCTCTGACCGGT TGGTTATACGCAAGGTACCCGTG-3')] and subsequently tagged in-frame to the 5' end of the codon optimized Blo t 5 gene by PCR. Briefly, this gene was re-amplified from pGEMT-cBlo t 5 using primers V5 [5'-CCAACCCGGTCAGAGCAAGACAGGAGCACAAGCCC AAGAAGG-3'] and B9. Mixture of the codon optimized VZV and Blo t 5 PCR products was subjected to a single cycle PCR (denaturation 94 °C for 10 min, annealing at 52 °C for 5 min and extension at 72 °C for 10 min) followed by a secondary PCR (30 cycles; denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min) using oligonucleotides V1 and B9. The resultant codon optimized VZV-Blo t 5 gene contained a *Bam*HI and a *Xba*I site at the 5'- and 3'-end, respectively, for directed cloning into the mammalian expression vector, pcDNA3.0 (Invitrogen). Similarly, a construct containing the wild-type VZV-Blo t 5 gene was generated. Both constructs were confirmed by DNA sequencing.

Transfection and selection of stable CHO-K1 cell lines producing recombinant cBlo t 5. The CHO-K1 cells were grown in a 5.0% CO₂ incubator at 37 °C, in culture flasks (NUNC) containing DMEM

(Gibco) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, and penicillin/streptomycin. The wild-type and codon optimized Blo t 5 gene in pcDNA3.0 was, respectively, transfected into CHO-K1 cells using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol. On the second day following transfection, the cells were selected with 700 μ g/mL of Geneticin (G418, Sigma Aldrich) for a total of 19 days, and media were changed every alternate 3 days. At day 13 and 19, the spent culture media were obtained for Western immunoblot analysis. Subsequently, stable and amplified recombinant clones were obtained by limiting dilution in four 96-well plates. Positive clones re-suspended in FBS with 10% DMSO (Sigma) were stored frozen in liquid nitrogen for future use.

Expression of recombinant cBlo t 5 in CHO cells. Positive CHO-K1 clones were adapted to serum-free EX-CELL 302 media (JRH Biosciences) according to manufacturer's protocol. Briefly, a stable clone from serum supplemented media were adapted to decreasing FBS concentration in EX-CELL 302 media (5%, 1%, and 0.5% FBS) by two successive passages each and finally in serum-free EX-CELL 302 media. The adapted cells were subsequently grown in 200 mL suspension culture, seeded at 2×10^5 cells/mL in 500 mL shaker flask, shaking at 60 rpm in a 5.0% CO₂ incubator at 37 °C. Spent culture media was harvested after 10 days of culture, by centrifugation at 3000 rpm for 15 min and stored at –20 °C before purification.

Affinity chromatography purification of native and recombinant Blo t 5 protein. The A47 hybridoma clone producing monoclonal antibody (mAb) against Blo t 5 was used to generate monoclonal from ascitic fluid of five Balb/c mice inoculated with the A47 hybridoma clone as previously described [8]. Subsequently, the mAb A47 was purified from the pooled ascitic fluid using the Montage antibody purification—PROSEP-A spin columns (Millipore) and purified mAb was coupled to cyanogenbromide-activated Sepharose 4B (Amersham Biosciences) according to the manufacturer's recommendations.

Whole mite extract of pure *Blomia tropicalis* culture was prepared and native nBlo t 5 was affinity chromatography purified from this crude mite extract as previously described [9,10]. The same protocol was used for purification of recombinant cBlo t 5 from CHO-K1 spent culture medium. Recombinant yBlo t 5 protein was produced and purified from the *Pichia pastoris* yeast expression system as previously described [11]. Purified native or recombinant proteins were analyzed by SDS–PAGE. Total protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad), with bovine serum albumin as standard.

SDS–PAGE and Western blot analysis. Purified nBlo t 5, yBlo t 5, and cBlo t 5 proteins were separated on a 10% Tris–Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [12]. After electrophoresis, proteins were electroblotted onto Hybond-C nitrocellulose membranes (Amersham Life Sciences, UK) using a semi-dry Transblot system (Pharmacia) at 100 mA for 1 h. The membrane was blocked in PBS-T (0.05% Tween 20) containing 3% skimmed milk for 1 h at room temperature. After overnight incubation with mAb A47 (1:10,000 dilution) in blocking buffer at 4 °C, the membrane was washed four times in PBS-T (5 min/wash) and incubated with biotinylated anti-mouse Ig (1:5000 dilution). The membrane was incubated with peroxidase conjugated ExtraAvidin (1:5000 dilution) (Sigma, St. Louis, MO) for 1 h at room temperature and subsequently developed in SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) for 5 min. Signals were detected by autoradiography using the Kodak Biomax Light ML film (Eastman Kodak Company, Rochester, NY).

N-terminal protein sequencing. A total of 2 μ g of purified nBlo t 5 and cBlo t 5 was, respectively, resolved on a 10% Tris–Tricine SDS–PAGE and electroblotted onto PVDF membranes. The membrane was rinsed in distilled H₂O, stained in 50% Methanol containing 0.1% Coomassie (2–5 min), and destained in 50% methanol. After air-drying, the respective protein bands of interest were excised for N-terminal protein sequencing.

Glycan analysis. Carbohydrate analysis was performed using the DIG Glycan Differentiation Kit (Roche Diagnostics, GmbH) accord-

ing to the manufacturer's recommendations. Briefly, purified proteins (nBlo t 5, cBlo t 5, and yBlo t 5) separated on a 10% Tris–Tricine SDS–PAGE and electroblotted on nitrocellulose membranes were incubated with the different lectins (GNA, *Galanthus nivalis* agglutinin; MAA, *Maackia amurensis* agglutinin; PNA, peanut agglutinin; and DSA, *Datura stramonium* agglutinin) conjugated to digoxigenin. Complexes were detected with antidigoxigenin antibodies conjugated to alkaline.

Sandwich ELISA. Concentration of recombinant cBlo t 5 in spent culture media of CHO-K1 clones was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using antibody pairs mAb A47 and biotinylated polyclonal Guinea pig anti-Blo t 5 previously developed in the laboratory. Briefly, ELISA plates (Costar, Corning Incorporated, USA) were coated with 2 µg/mL mAb A47 in coating buffer (0.1 M NaHCO₃, pH 8.2) overnight at 4 °C. Plates were blocked with 1% bovine serum albumin (BSA, Sigma) in PBS-T for 1 h at room temperature. The spent CHO-K1 culture media (diluted at 1:500; 1:1000; 1:5000; and 1:10,000 in blocking buffer) were added in duplicate wells and recombinant Blo t 5 (0.098–100 ng/ml) was used for standard curve. Following overnight incubation at 4 °C, biotinylated polyclonal guinea pig anti-Blo t 5 (1:2000 dilution) in 1% BSA (PBS-T) was added and incubated for 1 h at room temperature. Subsequently alkaline phosphatase conjugated ExtrAvidin (1:2000 dilution) (Sigma St. Louis, Mo) was added and plates were incubated for 1 h at room temperature. Finally, colorimetric reaction was performed using *p*-nitrophenylphosphate substrate (pNPP, Sigma). Optical density at 405 nm was determined at 30 min and 1 h after addition of substrate using the Spectra (Shell) and Rainbow (Shell) Readers (Tecan Austria GmbH, Austria).

Human IgE ELISA. An ELISA was carried out as previously described [3] to compare the IgE reactivities of recombinant Blo t 5 and native nBlo t 5 in a panel of 32 sera samples consisting of sera from 22 asthma/eczema children, 4 from normal controls, and 6 cord blood samples as negative control. Briefly, ELISA plates were coated overnight at 4 °C with 5 µg/mL nBlo t 5 or cBlo t 5 in coating buffer. Plates were blocked with 1% BSA in PBS-T (1 h at room temperature) and incubated at 4 °C overnight with human sera (1:5 dilution) in blocking buffer, added in duplicate. Plates were incubated with biotinylated anti-human IgE (Pharmingen, CA) (1:2,000 dilution) and subsequently developed as described above. The IgE titer was defined as OD_{405 nm} value × serum dilution factor.

Prediction of glycosylation sites. Putative *O*- and *N*-glycosylation sites were predicted for the Blo t 5 amino acid sequence using the NetOGlyc 2.0 and NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services>).

Skin prick test. The skin prick test was performed by multiple prick technique as previously described [13]. A total of 9 individuals sera-positive for Blo t 5 were recruited for this test. Briefly, a drop of 10 µL of purified nBlo t 5, recombinant cBlo t 5 or yBlo t 5 in normal saline (25 µg/mL) was applied on the volar side of the forearm followed by a skin prick with a disposable lancet. Histamine (1 mg/mL) and normal saline were included as positive and negative controls. The diameter of the wheal and/or erythema 30 min after the skin prick was measured and recorded. A reaction of greater than 3 × 3 mm wheal and/or erythema diameter above the negative control was regarded as a positive skin reaction.

Statistical analysis. The ELISA and skin prick test values were analyzed using unpaired *t* test, a *p* value <0.05 was considered significantly different.

Results and discussions

Generation of a Blo t 5 codon optimized gene

Optimization of codon usage has been shown to increase heterologous gene expressions in mammalian cells [5–7,14–16]. Codon compositions of genes tend to be

relatively similar for all genes within each organism and matched to the population of isoaccepting tRNAs for that organism [17,18]. Increase in expression level was shown to be dependent on the availability of isoaccepting tRNAs and shortage of rare tRNAs could result in slowed elongation of the nascent peptide or premature termination of translation [19].

We have previously shown that the wild-type Der p 2 gene in pEE6 vector expressed poorly in CHO cells [20]. The codon usage of house dust mites including that of *Blomia tropicalis* in general shows different preference for synonymous codon usage compared to that of highly expressed human genes (Fig. 1). With this in mind, our criteria to generate a codon optimized Blo t 5 gene for expression in CHO-K1 cells were based on the codon preference of highly expressed human genes and the availability of rare tRNAs. In addition, we also consider the fact that mammalian genes characterized by high transcriptional activity are generally more GC-rich and that codon with third base GC composition is more common in codons specifying amino acids critical for gene replication and cell growth [21–23]. To generate a codon optimized Blo t 5 gene, the nucleic acid sequence of the codons was replaced without changing the amino acid sequence in order to achieve a closer percentage

Ala	H	N	S	Gly	H	N	S	Pro	H	N	S
GCG	17	0	17	GGG	24	0	33	CCG	17	0	0
A	13	17	0	A	14	100	0	A	16	100	0
T	17	50	17	T	12	0	0	T	19	0	0
C	53	33	67	C	50	0	67	C	48	0	100
Arg	H	N	S	His	H	N	S	Ser	H	N	S
AGG	18	0	20	CAT	21	40	20	AGT	10	0	0
A	10	0	0	C	79	60	80	C	34	100	50
CGG	21	0	20	Ile	H	N	S	TCG	9	0	0
A	6	80	0	ATA	5	0	0	A	5	0	0
T	7	20	0	T	18	38	12	T	13	0	0
C	37	0	60	C	77	62	88	C	28	0	50
Asn	H	N	S	Leu	H	N	S	Thr	H	N	S
AAT	21	17	17	TTG	6	65	6	ACG	15	0	25
C	78	83	83	A	2	0	0	A	14	0	0
Asp	H	N	S	CTG	58	0	59	T	14	50	25
GAT	25	88	12	A	3	0	0	C	57	50	50
C	75	12	88	T	5	24	6	Tyr	H	N	S
Cys	H	N	S	C	26	12	29	TAT	21	0	0
TGT	32	0	0	Lys	H	N	S	C	78	100	100
C	68	100	100	AAG	82	85	85	Val	H	N	S
Gln	H	N	S	A	18	15	15	GTG	64	33	67
CAG	88	9	91	Phe	H	N	S	A	5	0	0
A	12	91	9	TTT	20	0	33	T	7	67	0
Pro	H	N	S	C	80	100	67	C	25	0	33
Glu	H	N	S					GAG	75	17	78
								A	25	83	22

Fig. 1. Comparison of the codon usage of wild-type Blo t 5 (N), codon optimized Blo t 5 (S), and that of the highly expressed human genes (H). The codon usage pattern is considered to be related with the translational efficiency of the Blo t 5 gene but does not affect the nature of the protein synthesized. The numbers indicate percentage frequencies of individual codons for each corresponding amino acid. The most prevalent codon is underlined.

frequency for individual codons to that of highly expressed human genes. The codon optimized Blo t 5 gene fragment with a codon optimized VZV leader peptide at the N-terminal was generated by PCR using overlapping oligonucleotides. A construct containing the wild-type VZVBlo t 5 gene was also generated.

Transfections and selection of stable CHO-K1 cell lines producing recombinant cBlo t 5

A successful high-titer expression of recombinant proteins is dependent on cell-line, proper construction of expression plasmid (promoter/regulatory sequences), efficiency of transfection (electroporation or cationic lipids), and integration at highly transcription active site within the genome by selection and amplification [24,25]. In this paper, a high-level expression of codon optimized Blo t 5 gene was achieved in CHO-K1 cells via a conventional mammalian expression vector, pcDNA 3.0. Gene expression was driven by a cytomegalovirus promoter and a Kozak sequence was also engineered preceding the AUG initiation codon to facilitate initiation of mRNA translation [26].

Selection of CHO-K1 transfectants with 700 µg/ml G418 started a day after transfection and on day five, most of the untransfected cells were dead and colonies became distinct on day ten. These colonies were allowed to expand for additional 9 days in culture with a total of three changes of fresh media (with G418). Western analysis of spent culture media obtained on day 13 and 19 showed a positive band at 14 kDa for the codon optimized but not for the wild-type Blo t 5 gene transfectants (result not shown). Subsequently, stable production cell lines of cBlo t 5 were obtained by the cloning of homogeneous cell populations from heterogeneous cell pools by limiting dilution and four clones with different expression levels of cBlo t 5 were obtained. One clone, E5, when adapted to serum-free EX-CELL 302 media and grown in 200 mL suspension culture for 10 days, produced 13 mg/L of recombinant cBlo t 5 protein in culture media, assayed by sandwich ELISA. Clone E5 stably produced recombinant cBlo t 5 in the absence of G418, indicating that selection of transfectant cells via G418 led to eventual integration in the genome. The cBlo t 5 purified using A47 affinity column showed a purity of greater than 90% as determined on SDS-PAGE and Coomassie staining (Figs. 2 and 3).

Biochemical characterization of cBlo t 5

The N-terminal amino acid sequencing of purified recombinant cBlo t 5 protein indicated that its N-terminal starts at residue Gln₁ (Gln Glu His Lys Pro Lys Lys Asp Asp Phe Arg Asn Glu Phe Asp His), corresponds to the expected putative N-terminal sequence, indicating that cleavage of the VZV signal peptide pro-

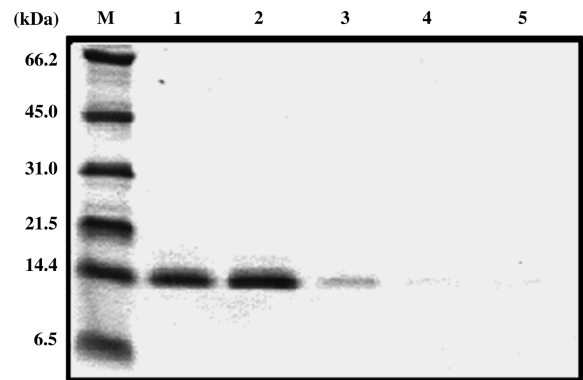


Fig. 2. Affinity chromatography purification of cBlo t 5. Approximately 200 mL of spent culture media was purified through the mAb A47 affinity column. The cBlo t 5 recombinant protein was eluted in the first three 0.5 mL fractions of the elution buffer (pH 2.6, 0.1 M glycine, 0.5 M NaCl) as analyzed on a 10% Tris-Tricine SDS-PAGE and by Coomassie staining. Lanes: M, protein molecular weight markers (Bio-Rad), (1–5) are 20 µL of the respective 1–5 fractions.

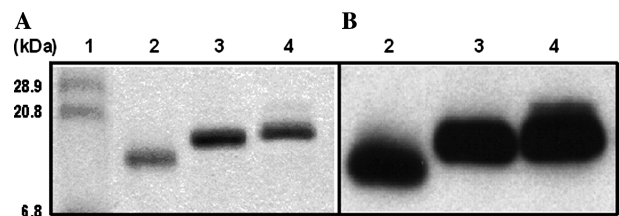


Fig. 3. SDS-PAGE (A) and Western blot (B) analysis of purified nBlo t 5, yBlo t 5, and cBlo t 5. A total of 1 µg of protein/lane was run on a 10% Tris-Tricine SDS-PAGE for Coomassie staining and 200 ng for Western analysis. The immunoblot was probed with mAb A47 (1:10,000 dilution) overnight at 4 °C, followed by 1 h incubation at room temperature with biotinylated anti-mouse Ig (1:5000 dilution) and detection with ExtrAvidin peroxidase conjugated (1:5000 dilution), respectively. Lanes: (1) protein molecular weight marker (Bio-Rad), (2) nBlo t 5, (3) yBlo t 5, and (4) cBlo t 5.

ceeded efficiently. On the contrary, the N-terminal residue of nBlo t 5 starts at E₁₃ (Glu Phe Asp His Leu Glu Ile Glu Gln Ala Asn His Ala Ile). Thus, the shift in SDS-PAGE gel of cBlo t 5 and yBlo t 5 when compared to the native protein (Fig. 3) was likely caused by the truncation of nBlo t 5 as shown by N-terminal sequencing data indicating deletion of 12 amino acid residues at the N-terminal.

Prediction of the glycosylation sites for Blo t 5 protein performed via the NetOGlyc 2.0 and NetNGlyc 1.0 Server indicated absence of *O*-glycosylation site and the presence of a single more probable *N*-glycosylation site at residue 46–50 (..NKS..). However, carbohydrate analysis of yBlo t 5 and cBlo t 5 (with nBlo t 5 as negative control) using the glycan recognition kit did not give a positive reactivity to any lectins, indicating that the yBlo t 5 and cBlo t 5 may not be glycosylated (data not shown).

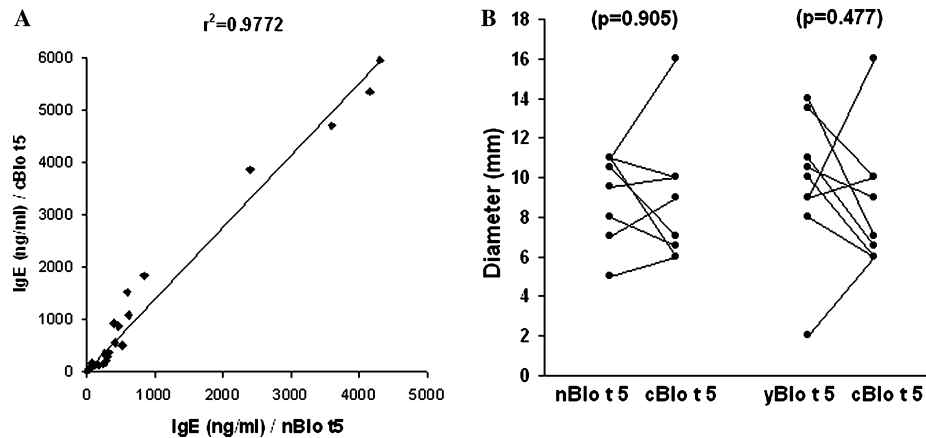


Fig. 4. (A) Correlation between the IgE reactivities of recombinant cBlo t 5 and native nBlo t 5 and (B) comparison of the allergenicity of cBlo t 5 to nBlo t 5 and yBlo t 5. A total of 32 sera samples (22 sera from asthma/eczema children, 4 normal controls, and 6 cord blood samples as negative control) were used in the direct human IgE ELISA. The in vivo skin prick test was expressed as wheal sizes observed on 9 individuals who are sera IgE positive for Blo t 5. Statistical analysis of data showed that cBlo t 5 and nBlo t 5 gave high correlation of IgE reactivities ($r^2 = 0.9772$) and there are no significant difference in allergenicity of cBlo t 5, nBlo t 5, and yBlo t 5.

IgE reactivities and skin reactivity to cBlo t 5

A close correlation of IgE reactivities was observed for recombinant cBlo t 5 compared to nBlo t 5 ($r^2 = 0.9772$) in a direct human IgE ELISA of a total of 32 sera samples (Fig. 4A). This indicated that recombinant cBlo t 5 and nBlo t 5 exhibit similar IgE-binding characteristics as well as epitopes. Since cBlo t 5 protein can be quantitated by a sandwich ELISA using a pair of mAb A47 and polyclonal anti-Blo t 5, it may display similar IgG binding epitopes as its native counterpart.

In addition, in vivo allergenicity of recombinant cBlo t 5, yBlo t 5, and native nBlo t 5 was evaluated by skin prick tests on a total of 9 individuals who are sera IgE positive for Blo t 5. All were positive for histamine (1 mg/mL) and negative for saline control. In addition, all showed positive skin reactivity to nBlo t 5, yBlo t 5, and cBlo t 5 as indicated by a wheal and/or erythema reaction of greater than 3 mm in diameter after 30 min. There are no significant differences in allergenicity of cBlo t 5, nBlo t 5, and yBlo t 5 as indicated by unpaired *t* test analysis of wheal sizes induced (Fig. 4B).

In conclusion, with codon optimization strategy, we have successfully produced high-level expression of allergenic recombinant Blo t 5 in CHO cells, which can be used for specific immunotherapy.

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