

# Co-Expression and Purification of Recombinant Human Insulin-Like Growth Factor II and Insulin-Like Growth Factor Binding Protein-6 in *Pichia pastoris* Yeast

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**Abstract:** For the preparation of the complex of IGF-II and IGFBP-6, a co-expression vector containing two copies of human IGF-II and IGFBP-6 expression cassette was constructed with alcohol oxidase (AOX1) promoter and secretion signal sequence of  $\alpha$ -factor, and transformed to *Pichia pastoris* yeast. Through a purification procedure involving anion-exchange chromatography and gel filtration, a complex of IGF-II with IGFBP-6 was obtained. An additional C-terminal sequence of IGFBP-6 (CS-BP6) was found to be bound to this complex. Dynamic light scattering showed that this complex was very stable and homogenous in solution. Western blotting based on non-reducing Tricine-SDS-PAGE indicated that IGF-II expression coupled with IGFBP-6 might significantly avoid the mispairing of disulfide bonds compared with the IGF-II expressed alone.

**Keywords:** *Pichia pastoris*, insulin-like growth factor II, insulin-like growth factor binding protein-6, co-expression, complex.

## INTRODUCTION

Insulin-like growth factors (IGF-I and IGF-II) are members of the insulin superfamily of hormones, growth factors and neuropeptides whose biological actions are achieved through binding to cell surface receptors [1]. IGF-I and IGF-II are 67% identical single polypeptide chains of 70 and 67 amino acids, respectively.

There are six well-characterized mammalian IGF binding proteins, designated IGFBP-1 to IGFBP-6 [2-6]. They are proteins of 216-289 residues and consist of three distinct domains of approximately equal size [7]. Both the N- and C-domains are implicated in high-affinity IGF binding because the isolated domains bind IGFs although with lower affinity than full-length IGFBPs [7]. Because of their higher affinity for IGFs than the IGFs type I receptor itself, these binding proteins not only act as carriers of IGFs, but also function as modulators of IGFs availability and activity [8].

Of these IGFBPs, IGFBP-6 is distinct mainly for its 20-100-fold higher binding affinity for IGF-II over IGF-I. This means that IGFBP-6 is a relatively specific inhibitor of IGF-II actions [9]. IGFBP-6 is extensively O-glycosylated [10,11], whereas IGFBP-3 and IGFBP-4 [12] are N-glycosylated, and IGFBP-5 is O-glycosylated to a limited extent [13].

For further understanding the relationship of IGFs structure and function, knowledge of the complex of IGFs with their binding proteins or their receptors becomes increasingly important [1,14,15]. Until now, only a few structures of IGF-I complexes with IGFBP domains have been determined by X-ray diffraction. These are the N-terminal domain of IGFBP-5 with IGF-I [1], N-terminal domain of IGFBP-4 with IGF-I [15,16], N-terminal domain of IGFBP-4 and C-terminal domain of IGFBP-1 with IGF-I [16], N-terminal and C-terminal domains of IGFBP-4 with IGF-I [15,16]. Thus acquisition of the complex of IGFBP-6 with IGF-II and its crystal structure determination is both important and significant for obtaining structural information. Here, we report the preparation and purification of the complex of IGFBP-6 with IGF-II, and some of its biophysical characteristics.

## MATERIALS AND METHODS

### Strains and Vector

*Pichia Pastoris* GS115 (*his4*) and expression plasmid pAO815 were purchased from Invitrogen. The vector pUC18 was used for gene construction. All cloning steps were performed in *E.coli* strain DH5 $\alpha$ , and the transformants were selected at LB medium with 100mg/L Ampicillin.

### Yeast Culture Media

*P. pastoris* cells were cultured in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose). BMG medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4 $\times 10^{-5}$ % biotin, 1% glycerol, and 100 mM potassium phosphate, pH 6.0) and BMMY medium (1% methanol substituting glycerol in BMG) were used for the proliferation of yeast cells and protein expression, respectively. MD plate (1.34%

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yeast nitrogen base, 2% dextrose,  $4 \times 10^{-5}$ % biotin, 1.5% agar) was used to select the transformants. Competent GS115 cells were prepared according to the Invitrogen manual (EasySelect™ *Pichia* Expression Kit).

### Gene Construction

The plasmids of pAO815-2×IGFBP-6 and pAO815-2×IGF-II were constructed as described [17, 18] with fusion of  $\alpha$ -factor signal. The double digestion product from the pAO815-2×IGFBP-6 with *Bam*HI and *Bg*III, 2× $\alpha$ -factor-IGFBP-6 sequence, was ligated to the *Bam*HI site of dephosphorylated pAO815-2×IGF-II. The plasmid with right orientation, 2×IGF-II-2×IGFBP-6, was selected using the double digestion with *Bam*HI and *Bg*III.

### Protein Expression and Purification

Electrocompetent *P. pastoris* GS115 cells were prepared according to the Invitrogen manual. Prior to the yeast transformation, pAO815-2×IGF-II-2×IGFBP-6 was linearized by *Sa*II. The transformation was performed by electroporation on Biorad's Gene Pulser II System with parameters of 2.5 kV, 25  $\mu$ F and 200 Ohms. The recombinant yeast clones were selected directly on MD plates. For screening of IGF-II and IGFBP-6 coexpressing transformants, positive clones were grown in the same volume of BMG medium overnight to  $OD_{600nm} \approx 10$ , and then collected the cells, resuspended in BMMY medium. After induced 72 hr with 1% methanol at 30°C under shaking of 200 rpm, the parallel culture supernatants were checked using Tricine-SDS-PAGE. The most efficient protein-secreting transformant was identified by the color intensity of bands stained. The recombinant GS115 selected with high expression ability was grown and induced according to the screening method.

The expression supernatant was diluted ten times of its initial volume using 62 mM NaCl solution, and then was performed on S-Sepharose (Pharmacia) column which was pre-equilibrated with the same solution. 1 M NaCl elution from S-Sepharose was sampled on the other S-Sepharose (Pharmacia) column with a linear gradient elution profile from 62 mM NaCl to 1 M NaCl. The elution from this S-Sepharose column was concentrated to no more than 1.0 ml before performed on Superdex 75 prep grade (Amersham Biosciences), which was pre-equilibrated with 200 mM NaCl solution. Each eluted peak from Superdex 75 was collected and concentrated with Amicon Centrifugal Filter (Millipore). All solutions used here were 50 mM NaAc-HAc buffer containing 3mM EDTA (pH 5.0) with different NaCl concentrations.

### Dynamic Light Scattering Analysis

Dynamic light scattering (DLS) signals can provide quantitative information about the behavior of protein in solution [19]. The DLS experiment here was performed on a DynaPro DLS instrument with a 781.8 nm wavelength laser (Protein Solutions) as described [20].

### Tricine-SDS-PAGE

0.9 ml culture supernatants were precipitated by 0.1 ml 100% trichloroacetic acid (Sigma) on ice for about 30min or

overnight. After centrifugation at 12,000 g for 10 min, the supernatants were discarded thoroughly, and the precipitants were washed by 100% alcohol. Repeating the centrifuge process, and then, the precipitants were resolved in sample buffer after getting rid of the residual alcohol. The supernatant samples and purified proteins were assayed by Tricine-SDS-PAGE. Negative cathode buffer was 0.1 M Tris-HCl (pH 8.45), 0.1 M Tricine and 0.1% SDS, while positive cathode buffer was 0.2 M Tris-HCl (pH 8.9). The running gel buffer was 3 M Tris-HCl (pH 8.45) and 0.3% SDS.

### Western Blotting

Proteins in the gel were transferred onto nitrocellulose membrane. This membrane was blocked for 2 hrs at room temperature or at 4°C overnight in blocking buffer (5% (w/v) skimmed milk powder in PBS, 0.1% Tween 20). Then, the membrane was incubated with polyclonal antibody against human IGFBP-6 or IGF-II for 1 hr at room temperature. The membrane was washed with PBS-T (0.1% Tween 20 in PBS) for three times and incubated with the secondary antibody, HRP-labelled rabbit anti-goat IgG (1:1000) at room temperature for 1 hr. Finally, the membrane was washed with PBS-T for three times, developed by 0.06% 3,3'-diaminobenzidine tetrahydrochloride (DAB) containing 0.03% H<sub>2</sub>O<sub>2</sub> in 10 mM Tris-HCl (pH 7.6).

### Periodic acid Schiff Analysis

After Tricine-SDS-PAGE, the gel was soaped in fixative of 25% isopropanol and 10% acetic acid overnight. Then, the gel was washed in turn by 10% isopropanol and 10% acetic acid for 2 hrs, 0.5% periodic acid for 2 hrs, 0.5% sodium arsenite and 5% acetic acid for 30-60 min, 0.1% sodium arsenite and 5% acetic acid for 20 min at each of two times, and glacial acetic acid for 10-20 min. The gel was then stained with Schiff reagent for overnight, followed by destained with the solution of 0.1% sodium pyrosulfite and 0.01 M HCl.

## RESULTS AND DISCUSSION

### The Identification and Purification of Expressed Proteins

With the induction of 1% methanol, the recombinant human IGF-II (rhIGF-II) and IGFBP-6 (rhIGFBP-6) were expressed and secreted to the medium under the direction of  $\alpha$ -factor secretion signal peptide. The expression supernatant was purified as described in Materials and Methods. The gradient elution from S-Sepharose (Fig. 1A) was concentrated and performed on size exclusion chromatography. Then, two well-separated eluted peaks (Fig. 1B), peak 1 and peak 2, appeared and were collected separately. As shown in Fig. 2A, peak 2 was mainly composed of 18kDa (lane 6); while peak 1 (lane 5) held three bands similar to the expression supernatant (lane 3) and gradient elution (lane 4). These three bands with different molecular weights appeared simultaneously through gel filtration which suggested that they should bind together to form one protein.

The concentrated peak 1 was further analyzed using Western blotting and Schiff acid analysis. As shown in Fig. 2B, the 30kDa and 18kDa bands had immune activity with

IGFBP-6 antibody, and the 7kDa band had immune reaction with IGF-II antibody. Schiff analysis (Fig. 2C) further showed that, just as the IGFBP-6 expressed alone [17], the co-expressed IGFBP-6 was also glycosylated, resulting in a larger molecular weight than expected 23kDa or so, which was similar to those from human serum [21, 22]. In addition, 18kDa band occurred like it in single-expressed system of IGFBP-6, which had been identified as the C-terminal sequence from of IGFBP-6 (residues109-213, CS-BP6) [17].

All of these implicated that rhIGF-II and rhIGFBP-6 were successfully expressed in *Pichia pastoris* GS115, and existed as a complex.

### The Characteristics of IGF-II Complex with IGFBP-6

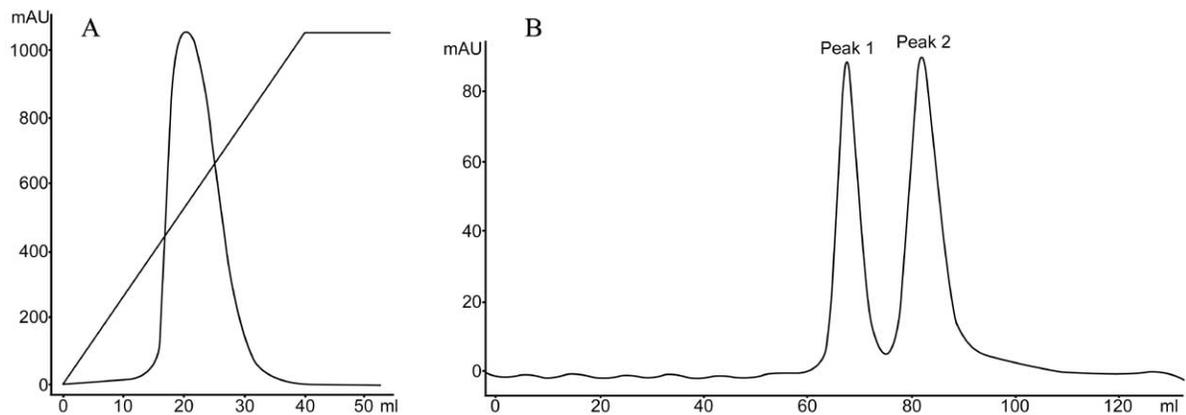
The complex of IGF-II with IGFBP-6 obtained here was analyzed by dynamic light scattering (DLS). Fig. 3A showed that this protein preparation only had a sharp intensity peak with a radius of about 3.7nm, which perhaps indicated that this complex had been purified to a good homogeneity. When it was laid on 4°C for two weeks, there was no obvi-

ous change on its DLS result (Fig. 3B). That is, no aggregation or degradation happened, which suggested that this complex was very stable in solution of 50 mM NaAc-HAc (pH 5.0) containing 200 mM NaCl at 4°C.

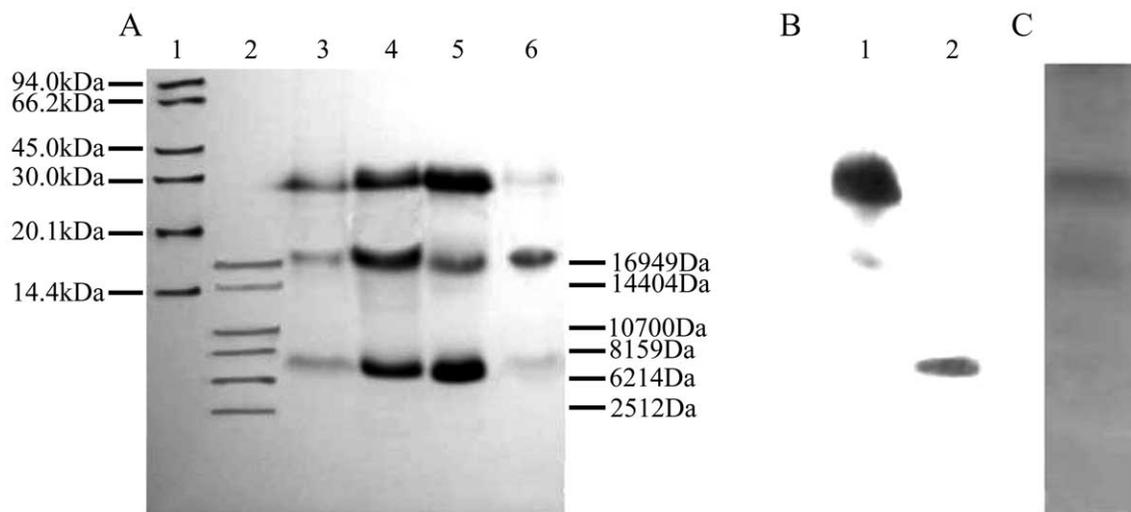
It was noticed that the purified complex still contained a fragment of IGFBP-6 (CS-BP6) which could not be separated from the complex of IGF-II with IGFBP-6 by size exclusion chromatography. However, the DLS results indicated that the three components, IGF-II, IGFBP-6 and CS-BP6, were organically integrated together to form a ternary complex structure. Although there have some reports of several complexes [15, 16, 23-26], the complex of IGF-II with IGFBP-6 is still little known. Further crystallographic studies of this complex will expand our knowledge of correlation between IGFs and IGFBPs.

### The Possible Function of IGFBP-6 on the Folding of IGF-II

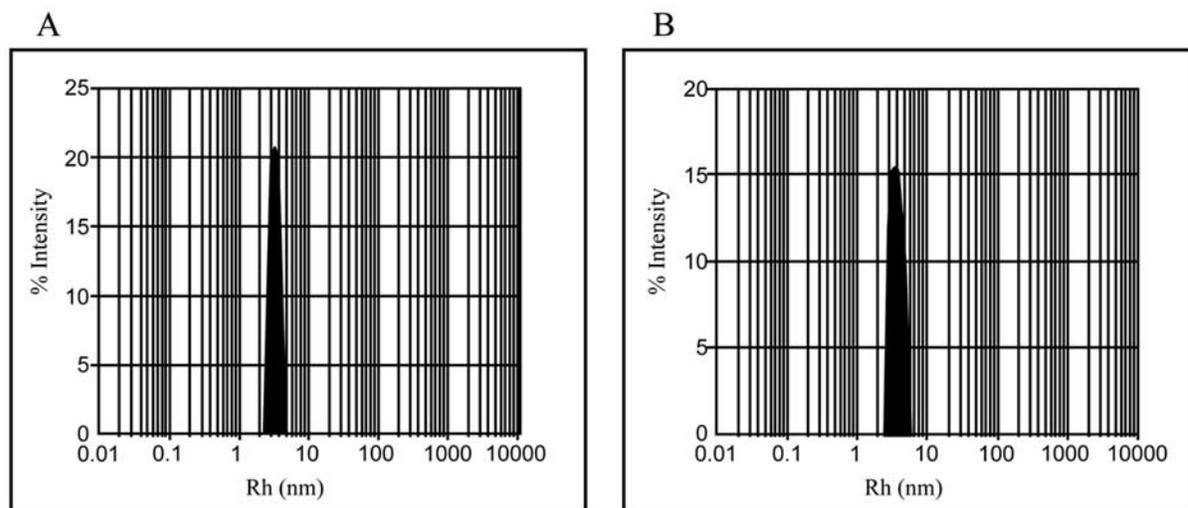
When analyzing the complex with non-reducing Tricine-SDS-PAGE, it was found that IGF-II migration on gel was



**Figure 1.** Elution profile of cation-exchange chromatography on 15 ml S-Sepharose column (A) and size exclusion chromatography on 120 ml Superdex-75 column (B).

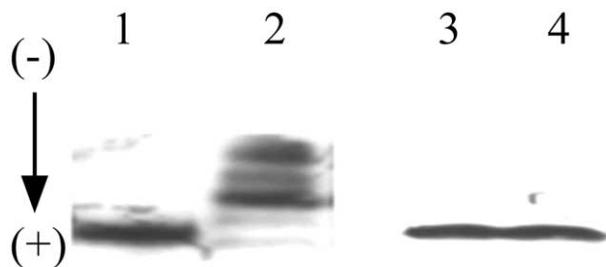


**Figure 2.** Analyses of proteins expression from recombinant GS115. A) Tricine-SDS-PAGE on 15% separating gel: lane 1 and lane 2, protein molecular weight standards; lane 3, supernatant of induced cells; lane 4, fraction of gradient elution from S-Sepharose; lane 5, peak 1 on Superdex-75; lane 6, peak 2 on Superdex-75. B) Western blotting of purified complex after Tricine-SDS-PAGE: lane 1, reaction with the antibody of goat against human IGFBP-6; lane 2, reaction with the antibody of goat against human IGF-II. C) Schiff acid analysis of purified complex after Tricine-SDS-PAGE.



**Figure 3.** DLS regularization histogram of concentrated peak 1 on Superdex-75. Panel A was the fresh sample; Panel B was the same sample laid on 4°C for two weeks.

different from that of single-expressed IGF-II by the same expression vector and strain. The Western blotting (Fig. 4) results clearly show that single-expressed IGF-II have several bands (lane 2 of Fig. 4) under the non-reducing condition, with higher molecular weights than that (lane 1 of Fig. 4) given by the co-expressed IGF-II under the same condition. In contrast, there was only one band for the two IGF-II samples under the reducing condition (lane 3 and 4 of Fig. 4).



**Figure 4.** Results of Western blotting with polyclonal antibody against human IGF-II. Lane 1 and lane 3, supernatants of GS115-2xIGF-II-2xIGFBP-6 treated by 4xSDS-PAGE sample buffer (0.2M Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 0.04% bromophenol blue) with or without the present of 20% 2-mercaptoethanol, respectively; lane 2 and lane 4, supernatants of GS115-2xIGF II treated by the sample buffer with or without 2-mercaptoethanol in term of lane 1 and lane 3, respectively.

It is well known that 2-mercaptoethanol is a reducing reagent generally used in SDS-PAGE to break disulfide bonds (-S-S-) within proteins. The differences between the single-expressed and co-expressed IGF-II shown on the gel without 2-mercaptoethanol implies that different -S-S- formations might occur for the two IGF-II expressed conditions. Since there are 7 cysteine residues and three pairs of intramolecular -S-S- for native IGF-II, there is a clear possibility of different -S-S- pairs formation occurring during *in vitro* expression. The abnormal migration of single-expressed IGF-II indicated that, for preparation IGF-II *in*

*vitro*, the single-expressed condition had a higher risk of mispairing of disulfide bonds than the co-expressed condition.

According to our data, it could be speculated that IGFBP-6 might have a function for correct -S-S- formation and folding of IGF-II. This will merit further study. Our previous experiments showed that recombinant human IGF-II with biological activity was very difficult to obtain because it inclined to form inclusion bodies in *E. coli* and also to misfold in *P. pastoris*. These implies a possible need for a molecular chaperon during the folding of IGF II. Therefore, co-expression of recombinant IGFBP-6 and IGF-II in *yeast* may provide a useful method to prepare IGF-II with biological activity.

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