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Expression and Purification of Mutated Porcine Growth Hormone Binding Protein by Using Site-Directed Mutagenesis in *E. coli*

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ABSTRACT

The present study was designed to obtain porcine growth hormone binding protein (pGHBP) improved biological activation as derived mutation in binding site with growth hormone (GH). A 756 bp of fragment encoding the extracellular domain of pGHBP gene was cloned from the total RNA of porcine fat tissue by reverse transcriptase polymerase chain reaction (RT-PCR) and created mutation in positions 26 and 122 using site-directed mutagenesis method. Position 26 is one and it is near to get on five potential N-linked glycosylation sites located in the extracellular domain of porcine growth hormone receptor known to have a direct influence on combination with GH. Position 122 is known as one of conformational epitope in bovine. It was over-expressed in *E. coli* using pET-32(c) expression vector and precisely purified by S-protein agarose and enterokinase. In our results, we was obtained pmGHBP of 30 kDa. It suggests to study the effects of the pmGHBP on cell proliferation *in vitro* and growth rate in vivo after administration.
(Key words : GHBP, GH, Site-directed mutagenesis)

Ⅰ. INTRODUCTION

Growth hormone (GH) is a pituitary polypeptide hormone. It is a pleiotropic hormone demonstrated somatogenic, lactogenic, diabetogenic and immunomodulatory functions (Nicoll et al., 1986). The biological effects (Baumann, 1987) of the secreted polypeptide hormone are triggered by the binding on the specific receptor in target organ: the liver is a well known target tissue for GH action (Husman et al., 1986).

Response to GH in target tissues is regulated by the GHR (growth hormone receptor), a single-pass transmembrane protein of the cytokine receptor superfamily (John et al., 2000). Although the level of expression at the protein and mRNA levels varies widely, with the highest level of receptor being found in liver, fat and kidney, GHR has been detected in practically all tissues examined (Barnard and Waters, 1997).

At least two types of GHBP (growth hormone binding protein) exist in blood: high affinity and low affinity GHBPs (Baumann, 2001). Low affinity GHBPs are high capacity binding components in plasma that complex GH with dissociation constants in or near that micromolar range. They have been found in the plasma of human as well as animals (Tar A et al., 1990). High affinity GHBPs are soluble, circulating forms of the GH receptor extracellu-

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lar domain. The term "GHBP", when used generically, refers to the high affinity GHBP (Baumann, 2001). In serum, GH can be bound by GHBP, a circulating glycoprotein that binds the hormone with affinity comparable to that of the receptor (Barnard and Waters, 1997), and GHBP transmit GH signal to nucleus of target cell (John et al., 2000).

The GHR is a membrane bound protein of 620 amino acids containing a 246 amino acid extracellular hormone binding domain, a single 24 amino acid hydrophobic transmembrane domain, and a 350 amino acid cytoplasmic domain (Cioffi et al., 1990). In all species examined GHBP corresponds to the extracellular hormone-binding domain of GHR and is a product of the same gene (Leung et al., 1987). However, the mechanism of GHBP synthesis differs among species. The truncated protein is produced by one of two different processes, depending on the species. In mice and rats, alternative splicing of GHR precursor mRNA replaces the transmembrane and intracellular region with a very short hydrophilic tail (John, 2000). In human, bovine, and porcine GHBP is produced by proteolysis of the GHR (Martini et al., 1997).

GHBP increases the half-life of GH in the serum by decreasing the rate of clearance/degradation of GH (Baumann et al., 1988). *In vitro* studies have indicated that GHBP may reduce the biological activity of GH (Mannor et al., 1991), whereas studies in rats show increased potency of GH if the hormone is administered in conjunction with the binding protein (Chen et al., 1991). The existence of this soluble GHBP and access to both recombinant GHBP and GH has led to a detailed analysis of GH-GHBP interaction using various mutagenesis techniques (Cunningham and Wells, 1991) and has culminated in the publication of the crystal structure of the GH-GHBP complex (De Vos et al., 1992). This identified a stoichiometry of 1 : 2 for the GH -GHBP binding complex with asymmetric dimerisation of GHBP by ligated GH. Thus, it may be derived that there was a highly packing effect of GH-GHBP binding complex, if binding sites between GH and GHBP were pertinently changed. The present study was carried to establish fundamental data for porcine GHBP to improve its effect on coherence with GH.

Ⅱ. MATERIALS AND METHODS

1. Cloning of the pGHBP Gene

Total RNA was extracted from porcine fat tissues by using Trizol (Gibco, NY, USA). Isolated total RNA was reversely transcribed to synthesize the first strand cDNA by using AMV Reverse Transcriptase First-strand cDNA Synthesis kit (Life Sciences, FL, USA). All primers were designed according to the published porcine mRNA for growth hormone receptor sequence (accession no. X54429), and synthesized in Bioneer Co., Korea. Primer sequences for each cDNA are as follow: pGHBP, forward primer 5'-CTAGAGGTCCTACAGGTATG-3'; reverse primer 5'-TGGTGAGGACCTGTTGATAG-3'; product size, 756 bp. Thermal cycling profiles for amplifying each cDNA were as follow: denaturation at 94℃ for 30 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 60 seconds for 30 cycles. The PCR products were eluted by Geneclean Ⅱ kit (Bio 101, CT, USA). The eluted pGHBP gene was ligated with pGEM -T-vectors (Promega, AC, USA).

2. Site-Directed Mutagenesis

Mutation of pGHBP (pmGHBP) was generated to replace glycine (Gly) residues with glutamic acid (Glu) at position 26 and isoleucine (Ile) residues with threonine (Thr) at position 122 by QuickChange™ Site-Directed Mutagenesis kit (Stratagene. Co. USA). The pmGHBP was classified into

non-mutation, mutation of position 26 (1' mutation), mutation of position 122 (2' mutation) and mutation of position both 26 and 122 (1' and 2' mutation). All primer were designed according to the published porcine mRNA GHR and pGEM-T vector sequence, and purified by polyacrylamid gel electrophoresis (PAGE) after primers were synthesised (Bioneer Co., Korea). The primer sequences for site-directed mutagenesis are as follows: 1' mutation, forward primer 5'-GAGTTCATCCAGGCCTAGGGACAAATTCTTCTGG-3'; reverse primer 5'-CCAGAATTTGTCCCTAGGCCTGGATGAACTC-3': 2' mutation, forward primer 5'-GCTGACTAGCAATGGTGGGATTGTGGATCGAAAGTG 3'; reverse primer 5'-CACTTTTGATCCACAATCCCACCATTGCTAGTCAGC-3'. Approximately 50 ng template plasmid was mutated with presented primers in reaction buffer with 5 μl of 10× reaction buffer, 1 μl (125 ng) of oligonucleotide primer (both forward and reverse primers), 1 μl of dNTP mix, 1 μl of *pfu* Turbo DNA polymerase (2.5U). The following program was used after 95℃ for 30 sec, denaturation at 95℃ for 30 sec, annealing at 55℃ for 60 sec, and extension at 68℃ for 7 min 31 sec for 12 cycles.

3. Construction of pmGHBPs Expression Vector

A pET-32(c) expression vector (Promega. AC. USA) was digested with *Sal I* and *XhO I* and eluted by Geneclean II kit (Bio 101. CT. USA). These fragments were ligated with pGHBPs gene which both ends were made *Sal I* and *Xho I* sites by pBluescript SK(+) vector (Stratagene. CO. USA). The ligation mixture was 1 μl of T4 DNA ligase 10× buffer, 50 ng of pET-32(c) vector, 50 ng of eluted pGHBP gene, 1 μl of T4 DNA ligase (Promega, WI, USA). The overnight reaction was performed on a 4℃ water bath. Then, the pmGHBPs was transformed into BL21(DE3)*pLysS* competent cells (Novagen, WI, USA) by heat shock method (Fig. 1).

4. Over-Expression of pmGHBPs in *E. coli*

1) Expression of Fusion Protein

To evaluate protein expression, pmGHBPs was transformed into BL21 (DE3)*pLysS*. For routine cell preparation of BL21 (DE3)*pLysS*/pmGHBPs, the seed culture was grown overnight and reinoculated into LB broth containing both ampicillin (5 μg/ml) and chloramphenicol (35 μg/ml). All cultures were grown until an optical density of 1.00 at 600 nm (OD_{600} = 1.00) was reached, and protein expression was induced by adding 1 mM IPTG (Sigma -Aldrich Co. USA). The cells were grown for 4.5 h at 37℃ and harvested by centrifugation. In all cases, protein synthesis was regulated by the T7 *lac* promotor expression system.

2) SDS-PAGE

SDS-PAGE was achieved in total proteins forms into *E. coli*. A 1 ml culture of IPTG induced cells was collected by centrifugation, resuspended in 1/10 culture volume of 10 mM Tris-HCl pH 8.0, 2 mM EDTA. Sample was removed and added to it equal volume of 2× SDS sample buffer for gel analysis. Samples were denatured in 70℃ for 5 min and loaded with 10 μl on an 8% SDS -polyacrylamide gels. Protein bands were visualized by staining with 0.25% brilliant coomassie blue (Reactifs IBF. France)

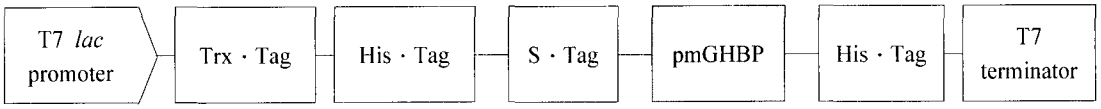


Fig. 1. Construction of pmGHBP expression vector.

or Western blotting.

3) Western Blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by electro-transfer equipment (HOEFER SCIENTIFIC INSTRUMENTS SAN FRANCISCO. USA). Membrane was incubated in blocking solution dissolved by stirring 1.25 g fat-free BSA in 25 ml 1× TBST at room temperature for 25 min to block excess protein binding sites and rinsed for 1 min in 25 ml TBST at room temperature to remove excess blocking reagent. The membrane was incubated with a 1: 5000 dilution S-protein AP conjugate in TBST for 30 min and then washed 5 times in 50 ml TBST at room temperature. For blotting, entire surface of membrane has been wetted by 1.5 ml of CDP-Star Substrate for detection and the blot was incubated in the substrate at room temperature for 1 min.

5. Purification of pmGHBPs

The pmGHBPs was purified by S · Tag Thrombin Purification kit (Novagene. WI. USA) that was designed for rapid affinity purification of S · Tag fusion protein produced. After expression of pmGHBP into BL21(DE3)*pLysS* was confirmed as SDS-PAGE, soluble or insoluble protein was bound with S-protein agarose. Then, pmGHBPs was purified as treated with thrombin. The purified pmGHBPs was confirmed by SDS-PAGE.

Ⅲ. RESULT

1. Cloning of the pGHBP Gene

The cDNAs encoding the complement of pGHR was cloned by RT-PCR confirmed that the band of 756 bp which were forecast with result of pGHBP. The amplified fragment digested with *Pst I* and *EcoR V* restriction enzyme. *Pst I* digests showed two bands at 128 and 628 bp, and *EcoR V* digests showed two bands at 229 and 527 bp (Fig. 2).

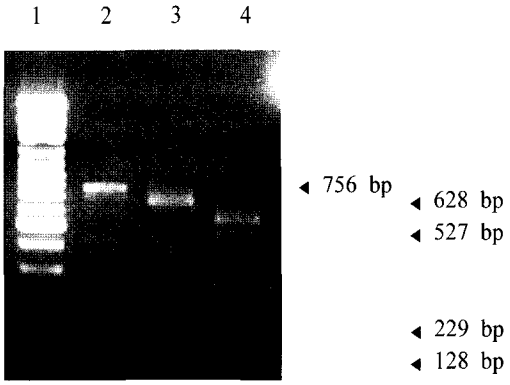


Fig. 2. PCR amplification of pGHBP cDNA and enzymatic treatment. Lane 1: 100 bp ladder, lane 2: 756 bp of PCR product, lane 3: *Pst* Ⅰ digestion of PCR product (128 and 628 bp) lane 4: *EcoR* Ⅴ of PCR product (229 and 527 bp).

2. Site-Directed Mutagenesis

A mutated pGHBPs gene (pGEM-T-pGHBPs) was generated that encoded two amino acid substitutions: Glu-26 → Gly, Thr-122 → lie as seen in Fig. 3. The pmGHBP was designed into four classes; non-mutation, mutation of position 26 (1' mutation) (* in Fig. 3), mutation of position 122 (2' mutation) (** in Fig. 3) and mutation of position both 26 and 122 (1' and 2' mutation). These changes were confirmed by sequence analysis (Macrogen Co., Korea).

3. Construction of pmGHBP Expression Vector

Before constructing an expression vector, a pGEM -T vectors with pmGHBPs gene and a subcloning vector, pBluescript SK(+), were digested with the two restriction enzymes, *Sal I* and *Sac II*, because both vectors had compatible ligation site. After extention of enzyme sites for *Sal I* and *Xho I* present in pBluescript SK(+), an expression vector pET-32(c) was digested with *Sal I* and *Xho I* to

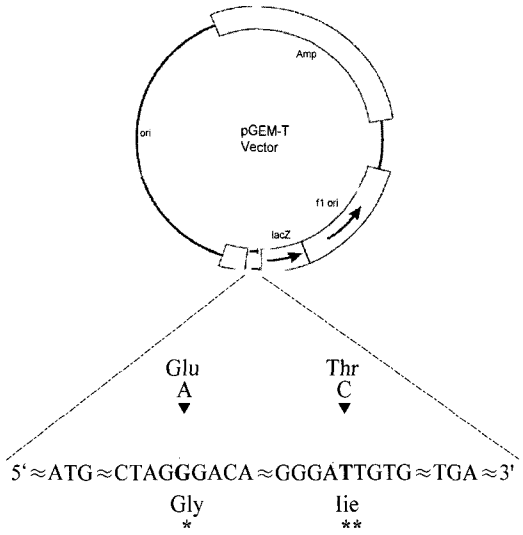


Fig. 3. General strategy of site-directed mutagenesis. It changed the A of 149 bp with the G and to induce the Gly, and the C of 422 bp with the T and the Iie it changed it induced. * mutation of position 26 (1' mutation), ** mutation of position 122 (2' mutation).

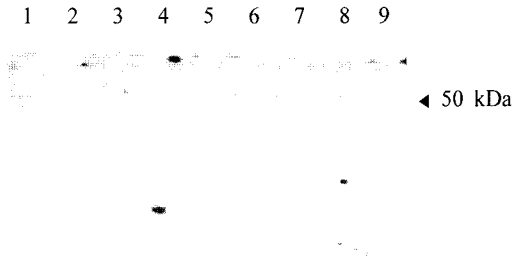


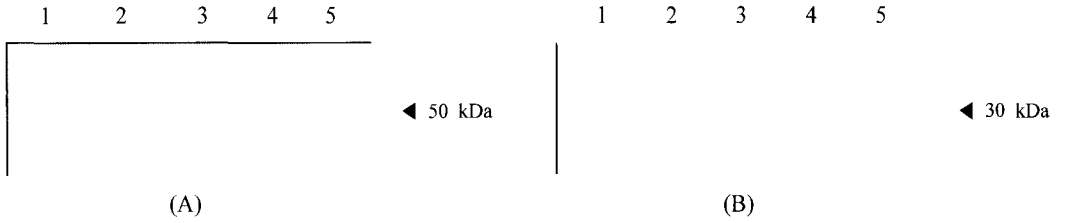
Fig. 4. Western blot analysis of solubility and insolubility protein bound S-protein agarose. Lane 1: Prefect Protein Western marker, lanes 2-5: solubility protein bound S-protein agarose, lanes 6-9: insolubility protein bound S-protein agarose of approximately 50 kDa, Lanes 2 and 6: non-mutated pGHBP bound S-protein agarose; Lanes 3 and 7: 1' mutated pGHBP bound S-protein agarose; Lanes 4 and 8: 2' mutated pGHBP bound S-protein agarose; Lanes 5 and 9: 1' and 2' mutated pGHBP bound S-protein agarose.

ligate to the pmGHBPs fragment isolation from pBluescript SK(+).

4. Over-Expression of pmGHBP in *E. coli*

The recombinant pmGHBPs gene inserted inside a pET-32(c) expression vector was transformed into *E. cloli* strain BL21(DE3)*pLysS*. The transformed BL21 (DE3)*pLysS* expressed the recombinant proteins upon induction with 1 mM IPTG.

Solubility and insolubility proteins were subjected to a 8% SDS-PAGE gel electrophoresis following isolation from *E. coli*. Protein bands were visu-



(A) (B)

Fig. 5. SDS-PAGE of Trx · Taq fused pmGHBPs and purified pmGHBPs. (A) Trx · Taq fused pmGHBPs of approximately 50 kDa. Lanes 1: non-mutated pGHBP, lane 2: 1' mutated pGHBP, lane 3: 2' mutated pGHBP, lane 4: 1' and 2' mutated pGHBP, lane 5: Prefect Protein Western marker. (B) Purified pmGHBPs approximately 30 kDa. Lanes 1: non-mutated pGHBP, lane 2: 1' mutated pGHBP, lane 3: 2' mutated pGHBP, lane 4: 1' and 2' mutated pGHBP, lane 5: Prefect Protein Western marker.

alized by Western blotting (Fig. 4). In the case of insolubility pmGHBPs, a thick band was observed in the area of about 50 kDa. The protein band indicates the fused pmGHBPs with Trx · Taq protein of producted pET-32(c) expression vector of 20 kDa.

5. Purification of pmGHBP

The pmGHBPs was purified by S · Tag Thrombin Purification kit (Novagene. WI. USA). It was found that pmGHBPs expressed insoluble protein forms in BL21(DE3)*pLysS* (Fig. 4). A purified pmGHBPs of 30 kDa by the S · Tag Thrombin Purification kit was confirmed by SDS-PAGE (Fig. 5).

Ⅳ. DISCUSSION

This study describes mutation of binding site with GH by using site-directed mutagenesis and the expression of a pmGHBP in *E. coli* expression system.

Mutation was derived at positions 26 and 122. Position 26 was replaced by glycine (Gly) residues with glutamic acid (Glu) and position 122 was replaced by isoleucine (Ile) residues with threonine (Thr). Position 26 is one and it is near to get on five potential N-linked glycosylation sites located in the extracellular domain of pGHR known to have a direct influence on combination with GH (Harding et al., 1990). Position 122 is known as one of conformational epitope in bovine (Beattie et al., 1996). Then, it was replaced by amino acid of the bovine epitope with an identical one thought to be compared to position 122 of porcine and bovine.

The initial step in GH signaling is the dimerization of two GHRs. Two different binding sites on the GH molecule are known to exist (Chen et al., 1991). The data indicated that there are two overlapping binding sites on the GHBP for GH and that there are two distinct binding determinants on GH for GHBP (Cunningham et al., 1991). Also, the binding from one GHBP molecule binds to site 1 on GH, then a second GHBP binds to site 2 on GH. The second GHBP can bind only if the first is already bound (no intermediates of GHBP bound to site 2 on GH have been observed (Cunningham et al., 1991). Receptor dimerization appears to be crucial for signal transduction since GH analogues that cannot induce receptor dimerization are inactive (Cunningham et al., 1991). Consequently, the pGHRP gene was mutated at positions 26 and 122 which have known important parts in GH -GHBP complex.

Administration of pmGHBP in differentiated cell or grown animal to investigate biological activity may yield two different effects either increase or decrease of growth. The growth increases the thing which will operate with the pmGHBP like original operation of the GHBP. Contrary to this, the growth decreases the thing which will operate with obstructed material passage of pmGHBP.

Protein expression has a nonglycosylated form in *E. coli* expression system, but Fuh et al. (1990) produced recombinant rat and human GHBP in the *E. coli* expression system. Thordarson et al. (1996) reported that prokaryotic proteins expressed in a eukaryotic gene expression system are more likely to retain posttranslational modifications similar to those of the native protein. However, in case of using *E. coli* expression system, transformed foreign gene may be operated as toxicoid inside *E. coli* (Dclare et al., 2001). It may be like this reason that we failed in expression of pmGHBP using *E. coli*: BL21(DE3) but succeed in expression of pmGHBP using *E. coli*: BL21(DE3)*pLysS*. BL21 (DE3)*pLysS* are used to suppress basal expression of T7 RNA polymerase prior to induction and thus stabilize pET recombinants encoding target proteins that affect cell growth and viability (Derman et al., 1993).

Therefore, this study suggests to investigate coherence of pmGHBPs and GH, and to study the effects of the pmGHBP on cell proliferation *in vitro* and growth rate *in vivo*.

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요 약

Site-Directed Mutagenesis를 이용하여 변이된 돼지 성장 호르몬 결합 단백질의 대장균 내 발현과 정제

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본 연구는 돼지에서 성장호르몬과 결합되는 부위에 변이를 유도하여 결합력이 향상된 성장호르몬 결합단백질을 획득하기 위하여 수행되었다. 돼지의 지방으로부터 얻은 성장호르몬 수용체 RNA 내 성장호르몬 결합단백질 부분을 756 bp의 cDNA로 전향하고 클로닝한 후 site-directed mutagenesis 방법을 이용하여 26과 122번째 아미노산을 변이시켰다. 26번째 아미노산은 성장 호르몬과의 결합에 관련이 있다고 알려져 있는 돼지 성장호르몬 수용체 외막에 존재하는 다섯 군데의 N-linked glycosylation 부위와 가까이 위치한 부분이고, 122번째 아미노산은 소에서의 결합부위로 알려져 있다. 이렇게 변이를 유도한 성장호르몬 결합 단백질을 pET-32(c) 발현벡터에 삽입시키고 과발현시켰고 이를 정제하여 30 kDa의 변이를 유도한 성장호르몬 결합 단백질을 얻었다. 이러한 방법으로 성장호르몬 결합 단백질을 성장기에 있는 세포나 동물에 주입한다면 보다 향상된 성장을 볼 수 있을 것으로 사료된다.

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