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The Third Intracellular Loop of Human β_2-adrenergic Receptor Expressed in *E. coli* Decreased Binding Affinity of Isoproterenol to β_2-adrenergic Receptor

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Abstract – To investigate the effect of the third intracellular loop (i3 loop) peptide of human β_2-adrenergic receptor on receptor agonist binding, we expressed third intracellular loop region of human β_2-adrenergic receptor as glutathione S-transferase fusion protein in *E. coli*. DNA fragment of the receptor gene which encodes amino acid 221-274 of human β_2-adrenergic receptor was amplified by polymerase chain reaction and subcloned into the bacterial fusion protein expression vector pGEX-CS and expressed as a form of glutathione-S-transferase (GST) fusion protein in *E. coli* DH5α. The receptor fusion protein was identified by SDS-PAGE and Western blot using monoclonal anti-GST antibody. The fusion protein expressed in this study was purified to an apparent homogeneity by glutathione Sepharose CL-4B affinity chromatography. The purified i3 loop fusion proteins at a concentration of 10 μg/ml caused right shift of the isoproterenol competition curve of [^{3}H]Dihydroalprenolol binding to hamster lung β_2-adrenergic receptor indicating lowered affinity of isoproterenol to β_2-adrenergic receptor possibly due to the uncoupling of receptor and G protein in the presence of the fusion protein. The uncoupling of receptor and G protein suggests that i3 loop region plays a critical role on β_2-adrenergic receptor G protein coupling.

Keywords□ β_2-adrenergic receptor, third intracellular loop, agonists competition, uncoupling

A family of mammalian membrane receptors with common genetic and molecular characteristics has been identified by gene cloning and sequence analysis. This family includes the β_1- (Frielle *et al.*, 1987), β_2- (Dixon *et al.*, 1986), and α_2-adrenergic receptors (Kobilka *et al.*, 1987), the M1-M4 subtypes of muscarinic receptors (Kubo *et al.*, 1986), and the receptor for the neuropeptide "substance K," all of which are coupled to guanine nucleotide-binding regulatory proteins(G proteins). This family of receptors was called as G-protein coupled receptors (Dohlman *et al.*, 1987a). Most of the genes coding for these receptors have been expressed in eukaryotic cells. These receptors probably possess a common topological organization in the plasma membrane, characterized by seven hydrophobic transmembrane segments (giving another generic family name; seven transmembrane receptors) interspersed with hydrophilic extra- and intracellular loops, a glycosylated extracellular amino-terminal region, and a cytoplasmic carboxy-terminal tail (Dohlman *et al.*, 1987b). This membrane organization is modeled on that of the light receptor rhodopsin, which was itself deduced from the structure and folding of bacteriorhodopsin.

Of the dozens of G-protein coupled rceptors, β_2-adrenergic receptors were regarded as model system for the study of the mechanism of action and regulation of receptor molecules (Lefkowitz and Caron, 1988). There have been two major fields of research for the mechanism of receptor action. One is the mechanism of receptor ligand interaction and the other is the mechanism of receptor G-protein coupling. Regarding receptor G-protein coupling, many researchers reported the importance of several intracellular hydrophilic residues of β-adrenergic receptor on the receptor G-protein coupling. Deletion and/or substitution of N-terminal region, first intracellular region, second intracellular region and C-ter-

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minal tail region of β_2-adrenergic receptor showed negligible effect if any (Dixon *et al.*, 1987a,b; Strader *et al.*, 1987a,b, 1988). Strikingly partial deletion of the third intracellular loop region of β_2-adrenergic receptor caused complete abolition of β-adrenergic agonists to stimulate adenylate cyclase activity (Strader *et al.*, 1987a) implying the importance of third intracellular loop region on β-adrenergic receptor G protein coupling. These observation implies that peptides which contain only the entire third intracellular loop may cause inhibition of receptor G-protein coupling through competition with β_2-adrenergic receptor. In this study, we expressed the third intracellular loop (i3 loop) peptide of human β_2-adrenergic receptor in *E. coli* as a glutathione S-transferase fusion protein and investigated the effects of the purified fusion protein on the agonist binding affinity to the β_2-adrenergic receptor which may decrease when receptor and G protein coupling is inhibited (Kuriyama *et al.*, 1983).

Materials and Methods

Materials

Taq polymerase was obtained from Perkin Elmer (Branchburg, NJ, U.S.A.). Restriction enzymes and other enzymes used in molecular cloning were purchased from Promega (Madison, WI, U.S.A.). Reagents for *E. coli* culture including yeast extract were obtained from Difco (Detroit, MI, U.S.A.). Isopropyl-β-D-thio-galactoside (IPTG) was purchased from Boehringer Mannheim (Mannheim, Germany). Prestained electrophoresis molecular weight standards (myosin, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, β-galacto globulin, lysozyme) and electrophoresis reagents were obtained from GIBCO-BRL (Gaithersberg, MD, U.S.A.) or Sigma chemicals (St. Louis, MO, U.S.A.). Horse radish peroxidase labelled goat anti-mouse IgG antibody was obtained from Pierce (Rockford, IL, U.S.A.). Nitrocellulose membrane was purchased from Amersham (Aylesbury, UK). Sepharose CL-4B was obtained from Pharmacia (Uppsala, Swedden) or from Sigma chemicals (St. Louis, MO, USA). Centricon 10 which required for sample concentration was obtained from Amicon (Beverly, MA, U.S.A.). (-)-[^{3}H]Dihydroalprenolol ([^{3}H] DHA, 76 Ci/mmol) was purchased from Amersham (Aylesbury, UK). All other chemicals were obtained from Sigma Chemicals and Pharmacia (Uppsala, Sweden) and were highest commercially available grade.

Construction of expression vector pGEXβi3

Human β_2-adrenergic receptor gene was amplified by polymerase chain reaction (PCR) and subcloned into pBS-KS resulting in a cloning vector pBSβAd. The DNA fragments which encode the third intracellular loop (i3 loop, amino acids 221-274) of the human β_2-adrenergic receptor were amplified by PCR and purified by agarose gel electrophoresis (Manniatis *et al.*, 1989). The primer sequences used in PCR were 5'-GTG GAT CCC AGG GTC TTT CAG GAC GC- 3' and 5' - GAG AAT TCA CGT CTT GAG GGC TTT GTG CT- 3'. The primer sequences were modified to generate BamHI and EcoRI restriction site and purchased from Korea Biotech, Inc. (Taejon, Korea). The PCR amplified DNA fragments were digested with BamHI and EcoRI and purified by gel electrophoresis. Expression vector pGEX-CS was digested with BamHI and EcoRI and 5'-terminal phosphate residues were removed by digestion with alkaline phosphatase. The purified receptor DNA fragment was ligated with the dephosphorylated pGEX-CS with bacteriophage T4 ligase. The resulting β_2-adrenergic receptor i3 loop expression vector was named as pGEXβi3.

Transformation and expression of the fusion protein

Competent *E. coli* DH5α was transformed with Heat Shock Method (Maniatis *et al.*, 1989). A single colony was grown overnight in 4 m*l* LB media containing 50 μg/m*l* ampicillin. Overnight cultures of transformed cells were induced with isopropyl-β-D-thio-galactoside (IPTG, 0.5 mM, 4 hours at 37°C).

Identification of the fusion protein expression

The identity of expression vector pGEXβi3 was identified by the restriction analysis. For the identification of the expression of the β-adrenergic receptor i3 loop-GST fusion protein, equivalents to 100 μl culture of *E. coli* cell membrane fractions transformed with pGEXβi3 were electrophoresed (Laemmli *et al.*, 1970), followed by staining with Coomassie brilliant blue. For Western blot analysis, the protein bands were electrically transferred to nitrocellulose membrane (Towbin *et al.*, 1979) and treated with monoclonal anti-GST antibody and probed with HRP-labelled goat anti-mouse IgG antibody for 1 hour at room temperature. After extensive washing with PBS-T (0.2% Tween 20 in phosphate buffered saline), the bands were visualized with diaminobenzidine substrate solution (0.6 mg/m*l* in 50 mM Tris, pH 7.6).

Purification of the fusion proteins

IPTG induced culture of *E. coli* (250 m*l*) was harvested (4,000×g, 10 min) and washed with 10 m*l* of STE buffer (150 mM NaCl, Tris.HCl, 1 mM EDTA, pH 8.0) containing 5 mM dithiothreitol (DTT), 10 μg/m*l* trasylol, 10 μg/m*l* leupeptin, 1 mM phenylmethylsulphonylfluoride (PMSF). The cells were resuspended in 10 m*l* of PBS and the cells were lysed by Branson probe sonifier (set at ×6, 50% duty cycle with three 15 sec bursts). After sonication, the solution was centrifuged for 10 minutes at 40,000×g and the resulting supernatants were applied to glutathione Sepharose CL-4B affinity column (1×10 cm) which was preequilibrated with PBS at a flow rate of 10 m*l*/hour. After loading, the column was extensively washed with PBS until A280 values returned basal level. The fusion proteins were eluted with PBS containing 10 mM reduced glutathione. The fractions were analyzed by SDS-PAGE and fusion protein containing fractions were pooled and concentrated with Centricon 10 (Amicon, NJ) to a protein concentration of 2 mg/m*l*. The identity and the purity of purified proteins were verified by SDS-PAGE and Western blot using monoclonal anti-GST antibody. The purified fusion proteins were aliquoted and freezed (-70°C) until required.

Preparation of the membrane particulate fraction from hamster lung

Hamster lung crude membrane preparation was prepared by the modification of the previously reported procedures (Benovic *et al.*, 1984). Hamster lung (1 g of tissue in 10 m*l* of buffer) was thoroughly minced and disrupted with a Tekmar Tissumizer (Cincinnati, OH) at a maximum speed (three 10-sec bursts on ice interrupted by two 20-sec rests on ice). The buffer containing 50 mM Tris, 5 mM EDTA, pH 7.4 (pH determined at 25°C), 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/m*l*), trasylol (10 μg/m*l*) and pepstatin (5 μg/m*l*). The homogenate was centrifuged at 48,000×g in a Ti 45 rotor in a Beckman L-80 centrifuge (High Wycombe, UK) for 20 minutes at 4°C. The pellet was resuspended in 10 volume of buffer using motor driven teflon glass homogenizer and the homogenate was filtered through 2 sheets of cheese cloth. The filtrate was centrifuged and washed three times in 10 volume of buffer by successive resuspension and centrifugation step. Finally, the pellet was resuspended by Teflon-glass homogenizer in 10 volume of buffer and used receptor ligand competition binding assay.

Ligand binding assay

For the determination of the effects of β_2-adrenergic receptor intracellular third loop - GST fusion proteins on agonist binding affinity, competition ligand binding assay was performed. The assay was performed in triplicate, in a volume of 0.5 m*l* containing hamster lung crude membrane fraction (5 mg of wet tissue) in 50 mM Tris, 5 mM EDTA buffer (pH 7.4 at 25°C) containing 1 mM ascorbate, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 βg/m*l*), trasylol (10 βg/m*l*) and pepstatin (5 βg/m*l*). The concentration of radioligand [^{3}H]Dihydroalprenolol was fixed to 2 nM (Kd value) and the concentration of β-adrenergic agonist l-isoproterenol was varied from 10^{-10} to 10^{-3} M to obtain full range competition curve. Purified GST or 2-adrenergic receptor i3 loop-GST fusion proteins (10 βg/m*l*) were added into the binding mixture and the reaction goes on for 1 hour at 23°C with vigorous shaking. After which receptor-radioligand binding complex was separated from free radioligands through 0.3% polyethyleneimine pretreated Whatman GF/B filters by the vacuum filtration procedure. The filters containing the receptor-radioligand complex were dried and added into 10 m*l* of toluene based liquid scintillation cocktail and counted by liquid scintillation counter. Nonspecific binding was defined as the counting value not inhibited by 10 μM *dl*-propranolol. The competition binding assay data were analyzed by iterative curve fitting computer program 'LIGAND' (Munson *et al.*, 1980) and the K*i* value of isoproterenol in the presence or the absence of β_2-adrenergic receptor i3 loop-GST fusion proteins was calculated by Cheng and Prusoff's procedures (Yamamura *et al.*, 1990).

Determination of protein

Content of protein was estimated by the procedure of Bradford (1976), using Bovine Serum Albumin as the standard.

Results

For the expression of β_2-adrenergic receptor i3 loop-GST fusion proteins in *E. coli*, a DNA fragment encoding only β_2-adrenergic receptor i3 loop regions was generated by PCR and subcloned into pGEX-CS and resulting expression plasmid was named as pGEXβi3. The identity of expression vector pGEXβi3 was first verified by restriction analysis. After induction with 0.5 mM

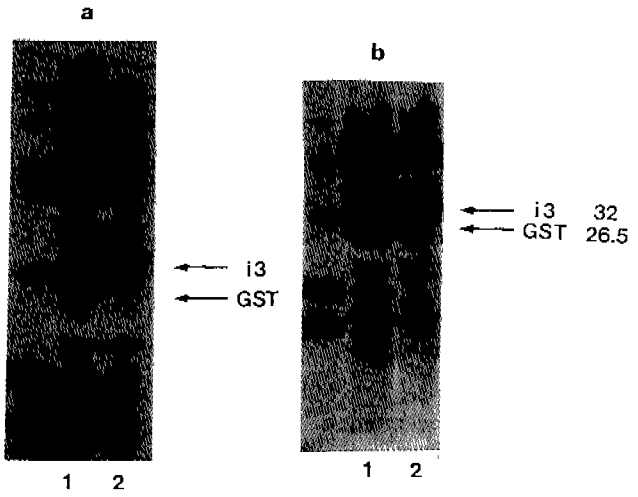

Fig. 1. Identification of third intracellular loop of human β_2-adrenergic receptor -GST fusion protein expression by SDS-PAGE ; *E. coli* DH5α transformed with pGEX-CS or pGEXβi3 were grown in LB broth containing 50 μg/m*l* ampicillin until O.D. 250 of 0.5 was reached. The culture was induced with 0.5 mM IPTG for 4 hours at 37°C. A 100 μl equivalent of induced cell was treated with 2 X SDS sample treatment buffer and electrophoresed. The gel was stained with Coomassie Brilliant Blue. (a) 12% gel (b) 12% gel. lane 1; pGEX-CS, lane 2 ; pGEXβi3. GST (26.5 kDa) and i3 loop - GST fusion protein (32 kDa) were indicated by arrows

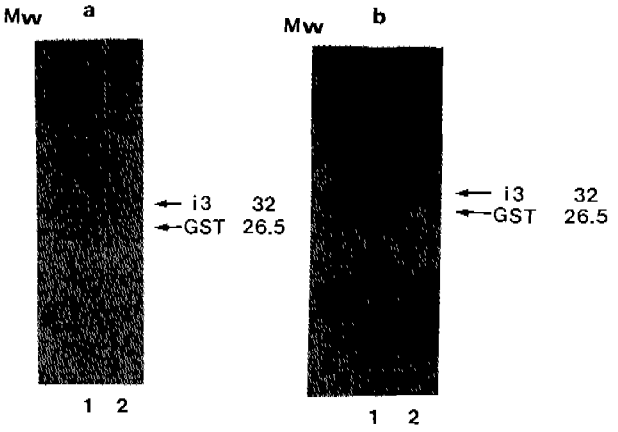

Fig. 2. Identification of third intracellular loop of human β2-adrenergic receptor -GST fusion protein expression by Western blot; *E. coli* DH5 transformed with pGEX-CS or pGEXβi3 were grown in LB broth containing 50 μg/m*l* ampicillin until O.D. 250 of 0.5 was reached. The culture was induced with 0.5 mM IPTG for 4 hours at 37°C. The control (pGEX-CS) and fusion protein expressing cell (pGEXβi3) were electrophoresed and the protein bands were electrically transferred to nitrocellulose membrane. The strips were incubated with monoclonal anti-GST antibody for 1 hours at room temperature and probed with HRP-labelled goat anti-mouse IgG. lane 1; pGEX-CS, lane 2; pGEXβi3. a) 12% gel b) 14% gel. GST (26.5 kDa) and i3 loop - GST fusion protein (32 kDa) were indicated by arrows.

IPTG, the *E. coli* culture which was transformed with pGEXβi3 displayed new major protein bands migrating more slowly than the *E. coli* culture transformed with pGEXβi3 which expressed GST (Fig. 1). The molecular weight of the new protein band was 32 kDa which was higher than that of GST alone (26.5 kDa). The increment of molecular weight was exactly matched with the calculated value for the β_2-adrenergic receptor i3 loop region which consists of 54 amino acids. For the identification whether this new protein band was really GST-fusion protein, Western blot was performed using monoclonal anti-GST antibody. As shown in Fig. 2, the 32 kDa protein band was intensely stained while control cultures revealed only 26.5 kDa band of GST indicating this new protein band was really GST fused form of protein. This fusion proteins were purified to an apparent homogeneity by glutathione Sepharose CL-4B affinity chromatography (Franzioni and Neel, 1993). After elution with 10 mM glutathione, only proteins with molecular weight of 32 kDa were purified from pGEXβi3 transformed *E. coli* while in case of *E.coli* transformed with pGEXCS only GST protein with molecular weight of 26.5 kDa was purified (Fig. 3). The GST fused nature of the purified 32

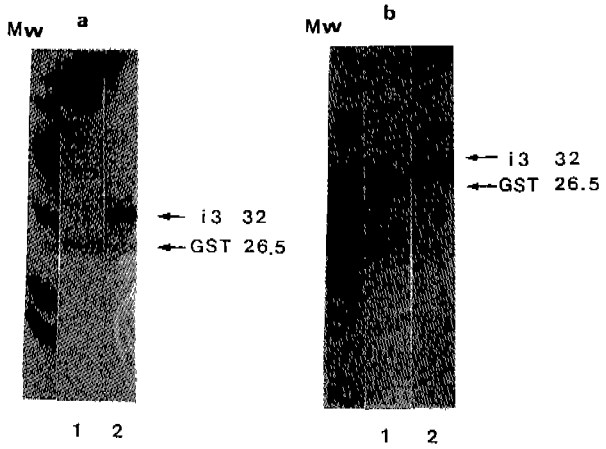

Fig. 3. Purification of human β_2-adrenergic receptor-GST fusion protein. Crude membrane fraction of *E. coli* culture transformed by pGEXβi3 or pGEX-CS was solubilized by sonication. The soluble fraction was loaded onto glutathione-agarose affinity column (1×10 cm) at a flow rate of 10 m*l*/hour. The column was washed extensively and the bound protein was eluted with 10 mM reduced glutathione. Peak fraction was combined and concentrated at a concentration of 2 mg/m*l* with Centricon 10. A 2 μg portion of purified GST (lane 1) and i3 loop fusion protein (lane 2) were treated with 2 X SDS sample buffer and electrophoresed. The band was visualized by Coomassie staining. a) 12% gel b) 14% gel. GST (26.5 kDa) and i3 loop - GST fusion protein (32 kDa) were indicated by arrows.

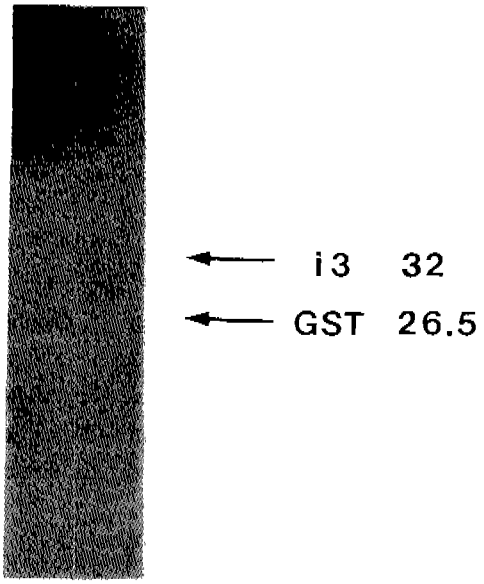

Fig. 4. Western blot of purified i3 loop - GST fusion protein with monoclonal anti-GST antibody. An aliquot (2 μg protein) of purified GST (lane 1) and i3 loop - GST fusion protein (lane 2) were electrophoresed and the protein bands were electrically transferred to nitrocellulose membrane. The strips were incubated with monoclonal anti-GST antibody for 1 hours at room temperature. After thorough washing with 0.2% Tween 20 in PBS the strips were probed with HRP-labelled goat anti-mouse IgG. GST (26.5 kDa) and i3 loop - GST fusion protein (32 kDa) were indicated by arrows.

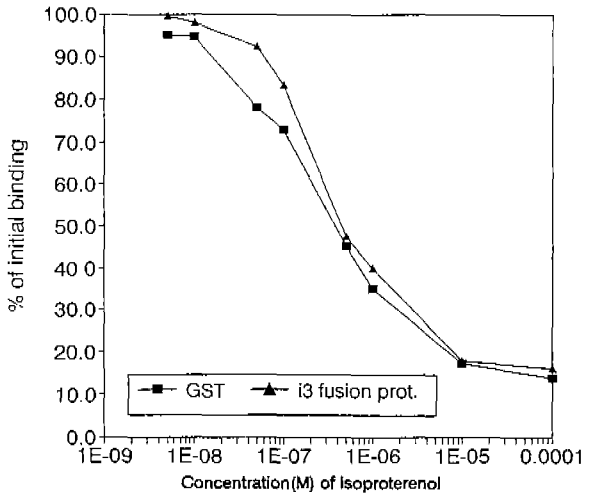

Fig. 5. Effects of purified β_2-adrenergic receptor i3 loop - GST fusion protein on binding affinity of isoproterenol to β-adrenergic receptor. Crude membrane preparation of hamster lung (5 mg wet tissue) was incubated with 2 nM [^{3}H]DHA and various concentration of isoproterenol. Purified i3 loop fusion protein, purified GST were added to this mixture and incubated for 30 minutes at 23°C. After incubation, the bound and the free form of the radioligand were separated by rapid vacuum filtration. The competition data were analyzed with 'Ligand' program. Inclusion of i3 loop fusion protein caused right shift of the competition curve. The experiments were done in triplicate and two other experiments gave similar results

kDa β_2-adrenergic receptor i3 loop fusion protein was again verified by Western blot using monoclonal anti-GST antibody (Fig. 4). For the determination of the effects of the β_2-adrenergic receptor i3 loop-GST fusion protein on β_2-adrenergic receptor agonist (*l*-isoproterenol) binding affinty, the purified i3 loop fusion protein was included in the hamster lung β_2-adrenergic receptor- isoproterenol competition binding assay mixtures. As shown in Fig. 5, the purified i3 loop fusion proteins at a concentration of 10 μg/ml caused right shift of the isoproterenol competition curve indicating lowered affinity of isoproterenol to hamster lung β_2-adrenergic receptor in the presence of the fusion protein while purified GST alone showed virtually no such effect.

Discussion

In the present study, the third intracellular loop region of β_2-adrenergic receptor was expressed in large quantities in *E. coli* as GST- fusion protein. The GST fusion protein expression system has been used for several years as a method of choice for the efficient large scale expression of eukaryotic protein (Frankel *et al.*, 1991; Guan and Dixon, 1991; Hakes and Dixon, 1992). Several researchers adopted this strategy for large scale preparation of specific receptor regions (Levey *et al.*, 1990; Hersch *et al.*, 1994). To identify the expression of β-adrenergic receptor third intracellular loop-GST fusion protein, SDS-PAGE and western blot were performed. In the both experiments a slowly migrating new protein band was observed. The molecular weight of this new protein band was exactly same with the calculated value of molecular weight of i3 loop-GST fusion protein. Also, monoclonal anti-GST antibody immunoreacted with this new protein band which again indicates the GST-fused nature of this protein.

For large scale purification of the fusion protein we performed affinity chromatography with glutathione Sepharose CL-4B affinity matrix. One major drawback of GST fusion protein expression system is that occasionally the expressed fusion protein exists as insoluble inclusion body which requires additional steps of purification such as solubilization of the inclusion body

(Frankel *et al.*, 1991) and decreases the overall yield of purification. Levey *et al.* (1990) expressed third intracellular loop of muscarinic acetylcholine receptor using pGEX system and reported the formation of inclusion body. But, in this study, the fusion protein was soluble in cytosol so, simple lysis of *E. coli* by sonication liberated the fusion proteins in the supernatants which made it possible to directly use the lysed prepation for the purification after removal of unlysed cells or debris. The descrepancy may be explained by relatively small size of β_2-adrenergic receptor i3 loop. In comparison to phospholipase C coupled receptor such as muscarinic acetylcholine receptor, adenylate cyclase coupled receptor such as β_2-adrenergic receptor has small i3 loop; about half the size that of PLC coupled receptor (Dixon *et al.*, 1988; Dohlman *et al.*, 1991). In case that the size of fusion moiety is small (in this case β-receptor i3 loop), the overall nature of the fusion protein follows that of the carrier moiety GST which is normally soluble in cytosol (Smith and Johnson, 1988).

As shown in figure 3, single step glutathione affinity chromatography yielded apparently homogeneous population of the fusion protein and the yields of purified fusion protein were about 3 mg proteins from 250 m*l* IPTG induced culture. The high level expression and ease of purification could greatly aid other research purpose regarding effects of β_2-adrenergic receptor i3 loop on G protein coupling and receptor regulation such as second messenger analysis (Fraser *et al.*, 1988), *in vitro* phosphorylation (Stadel *et al.*, 1986; Benovic *et al.*, 1986) and monoclonal antibody generation (Bahouth *et al.*, 1991; Wang *et al.*, 1989).

To investigate the effects of the purified fusion protein on the agonist binding affinity to the β_2-adrenergic receptor, we performed isoproterenol competition binding assay with or without i3 loop-GST fusion protein. The inclusion of i3 loop-GST fusion protein caused right shift of isoproterenol competition curve which means decreased affinity of isoproterenol to β_2-adrenergic receptor. In contrast to antagonists, agonists caused receptor G-protein coupling and the affinity of the agonists to G protein coupled receptor was higher than the affinity to the uncoupled receptor (Kent *et al.*, 1980). So, the decrease of affinity of agonist to the receptor might be used as an useful indicator of receptor G-protein uncoupling. In contrast to the results obtained with i3-GST fusion protein, addition of GST did not cause any effect on isoproterenol competition curve. So, the decrease of affinity of isoproterenol to the β-receptor which means uncoupling of β_2-receptor and G protein might be attributed to i3 loop moiety not to GST carrier moiety.

In summary, we expressed i3 loop peptide of β_2-adrenergic receptor in *E. coli* as a GST fusion protein. The purified i3 loop fusion protein caused decrease of affinity of isoproterenol to β_2-adrenergic receptor which means uncoupling of receptor and G protein indicating the importance of i3 loop peptide on β_2-adrenergic receptor G protein coupling. Investigation of the effects of i3 loop fusion protein on the second messenger system (*i. e.* adenylate cyclase activity) is currently under way.

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