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Cell-Free Expression, Purification, and Characterization of the Functional β_2-Adrenergic Receptor for Multianalyte Detection of β-Agonists

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Abstract—Large-scale expression of β_2-adrenergic receptor (β_2-AR) in functional form is necessary for establishment of receptor assays for detecting illegally abused β-adrenergic agonists (β-agonists). Cell-based heterologous expression systems have many critical difficulties in synthesizing this membrane protein, such as low protein yields and aberrant folding. To overcome these challenges, the main objective of the present work was to synthesize large amounts of functional β_2-AR in a cell-free system based on *Escherichia coli* extracts. A codon-optimized porcine β_2-AR gene (codon adaptation index: 0.96) suitable for high expression in *E. coli* was synthesized and transcribed to the cell-free system, which contributed to increase the expression up to 1.1 mg/ml. After purification using Ni-affinity chromatography, the bioactivity of the purified receptor was measured by novel enzyme-linked receptor assays. It was determined that the relative affinities of the purified β2-AR for β-agonists in descending order were as follows: clenbuterol > salbutamol > ractopamine. Moreover, their IC_{50} values were 45.99, 60.38, and 78.02 μg/liter, respectively. Although activity of the cell-free system was slightly lower than activity of systems based on insect and mammalian cells, this system should allow production of β_2-AR in bulk amounts sufficient for the development of multianalyte screening methods for detecting β-agonist residues.

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Keywords: cell-free expression, β_2-adrenergic receptor, codon optimization, purification, β-agonist, receptor-based assay

β_2-Adrenergic receptor (β_2-AR) is a member of the largest family of membrane proteins of G-protein-coupled receptors (GPCRs), which can be activated by adrenaline and synthetic β-agonists [1]. The sites of binding and activation between agonists and the receptor [2] and the agonist-induced conformational changes [3, 4] have been studied by mutagenesis and biophysical methods through decades of effort by many scholars across the world. In view of the group-specific recognition between the receptor and β-agonists, recently receptor assays based on β_2-AR as an emerging and powerful alternative screening method have been developed to detect a panel of common β-agonist compounds. A screening method based on recombinant β_2-AR expressed in *Escherichia coli* was developed for detection of various β-agonists and β-blockers in bovine urine samples, and the detection limit was in the μg/liter range [5]. Danyi et al. [6] described a solubilization method of a recombinant human β_2-adrenergic receptor produced in genetically modified *E. coli*, using the detergent *n*-dodecyl-β-D-maltoside. The binding affinity of the solubilized β_2-adrenergic receptor was evaluated by a radio-receptor assay. A radioactive bioassay based on β_2-adrenergic receptor expressed in NCB20-D1 cells was developed for the detection of seven β-agonist compounds in animal feeds [7]. Cheng et al. [8] obtained the recombinant soluble Syrian hamster β_2-AR proteins from infected Sf9 cells, which were utilized to establish an enzyme-linked-

Abbreviations: β-agonists, β-adrenergic agonists; β_2-AR, β_2-adrenergic receptor; BCA, bicinchoninic acid; CAI, codon adaptation index; CBL, clenbuterol; ELRA, enzyme-linked receptor assays; GPCRs, G-protein-coupled receptors; HRP, horseradish peroxidase; RAC, ractopamine; SAL, salbutamol; Sf9 (cells), a clonal isolate of *Spodoptera frugiperda* 21 cells.

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receptor assay (ELRA) to detect three β-agonists simultaneously. Our research team also produced a recombinant porcine β_2-AR protein from the inner membrane of HEK293 cells for multianalyte detection of β-agonists in swine urine [9]. However, low-level expression and low-affinity state of the recombinant protein still severely hindered its practical application in the detection of β-agonists.

β_2-AR shares the common structural signature of GPCRs: seven membrane-spanning α-helical domains are separated by alternating intracellular and extracellular loop regions, with N- and C-terminus localized on the extracellular and intracellular side, respectively [10, 11]. Thus far, heterologous expression is the primary approach to obtain the receptor protein, because the low natural abundance and tedious purification steps make it difficult to isolate the native receptor from cell membranes. However, there are enormous difficulties in utilizing *in vitro* systems to produce the membrane protein capable of binding specific ligands, like β-agonists, etc. At present, recombinant expression was achieved in many ways, including both cell-based systems and cell-free systems. Although a limited number of previous studies have reported the receptor expression in *E. coli* [12, 13], yeast [14], insects [15], and mammalian cells [16, 17], these methods still have some critical disadvantages in synthesizing the membrane protein, such as low productivity, formation of insoluble aggregates, and misfolding. Strategies to boost the yield of the conventional cell-based methods often focus on overexpression, and thus it always leads to insufficient membrane insertion, precipitation, or even cytotoxicity.

Another attractive alternative to overcome these challenges is the emerging synthesis machinery of cell-free expression systems. The systems provide the protein translation machinery based on cell lysates gained from *E. coli*, wheat germ, rabbit reticulocytes, or *Spodoptera frugiperda* (Sf) cell lines [18]. Furthermore, several other eukaryotic cell extracts from Chinese hamster ovary cells [19], HeLa cell lines [20], as well as mouse embryonic fibroblasts [21] were used for synthesis systems.

The most distinct benefit of cell-free systems results from their open synthesis environment, which can realize the direct manipulation and optimization of a variety of additives, cofactors and enzymes, in addition to supply hydrophobic biomimetic environments for membrane protein synthesis. Very few studies have described membrane protein expression using cell-free methods [22-26], especially of the β_2-AR [27, 28]. Moreover, some problems arose in the real work, including low expression levels and aberrant folding. Choosing the appropriate hydrophobic environment is a critical point to ensure high insertion efficiency and proper protein folding, which requires time-consuming optimization of the hydrophobic mixture for each individual membrane protein.

The major objective in this work was to identify ways to improve the yields of soluble functional β_2-AR protein using the *E. coli*-based cell-free systems. Moreover, ELRA was creatively utilized to verify the affinity of β_2-AR to β-agonists instead of the radio-receptor assay, revealing its potential to determine the presence of β-agonists in biological samples.

MATERIALS AND METHODS

Materials and reagents. T4 DNA ligase and the restriction enzymes of NdeI and XhoI were purchased from Promega (USA). The vector pET-22b was supplied by Novagen (USA). The *E. coli*-based cell-free expression kits were provided by Wuhan GeneCreate Biological Engineering Technology and Service Co., Ltd. (China). MagneHis™ Ni-Particles were purchased from Promega. Horseradish peroxidase (HRP)-β-agonists and free β-agonists were gifts from Beijing Kwinbon Biotechnology Co., Ltd. (Beijing, China). Brij35, anti-His-tag mouse monoclonal antibody, and HRP-conjugated goat anti-mouse IgG were obtained from Sigma-Aldrich (USA). All chemicals were of analytical grade and used without any further purification.

Synthesis of codon-optimized porcine β_2-AR gene. The full-length cDNA of the wild-type version of the porcine β_2-AR (GenBank Accession No. KF023571.1) was obtained as described previously [9]. According to the preferential codons of *E. coli*, the optimal cDNA of the porcine β_2-AR was designed and synthesized using a successive PCR method by Sangon Biological Engineering, Technology, and Service Co., Ltd., China. Also, the sites of NdeI and XhoI were added to the two termini of it for construction of the recombinant expression plasmid.

Construction of expression plasmid. The modified β_2-AR gene was ligated to the expression vector pET-22b by T4 DNA ligase at 4°C overnight after double digestion. The ligation product was then transformed into competent cell DH5α. Finally, the positive recombinant expression plasmids were screened by blue-white spot selection and further identified by colony PCR and DNA sequence analysis.

Cell-free synthesis of β_2-AR. *Escherichia coli*-based cell-free expression kits from Gene Create (China) were used to synthesize β_2-AR protein referring to the technical manual of S30 T7 High-Yield Protein Expression System (Promega). The reagents R_1 to R_6 were introduced into a DNase- and RNase-free 1.5-ml centrifuge tube in sequence, closely followed by 1 μg of the recombinant plasmid. To provide a hydrophobic environment to avoid the formation of insoluble aggregates, the non-ionic detergent Brij35 was added directly to the reactions at a concentration of 0.2% w/v. Then the above 50-μl reaction mixture was mingled thoroughly by vortexing gently and incubated at 180 rpm for 6 h at 30°C. The con-

centrations of the various components were repeatedly adjusted to achieve optimal protein yield. Especially, the Mg^{2+} concentration in the expression system was carefully optimized in the range from 14 to 22 mM under the fixed K^+ concentration of 300 mM. When the expression conditions were optimized, large amounts of the receptors were synthesized by this cell-free system. After cell-free synthesis, the reaction mixtures were centrifuged at 10,000 rpm for 10 min. The supernatant containing the solubilized protein was obtained for Western blot analysis. The supernatant was prepared and loaded in 10% SDS-PAGE gels. For blotting, the gel-resolved proteins were transferred to nitrocellulose membranes. Then the membranes were blocked with 5% skim milk (30 min at room temperature) and embathed in TBST two times. After that, the membranes were incubated with the anti-His monoclonal primary antibody (0.5 mg/ml in TBST, 2 h at room temperature), and then with the goat-anti-mouse HRP-conjugated secondary antibody (1 : 5000 in TBST, 1 h at room temperature). Finally, the chromogenic reaction was performed using ECL Western Blotting Substrate (Pierce, USA) according to the manufacturer's instructions. Final reaction volumes of 0.5-5 ml were applied to produce protein, which was purified for binding analysis of β-agonists.

Purification of recombinant β_2-AR. The purification of His-tagged recombinant β_2-AR protein was performed using the MagneHis™ Ni-Particles (Promega) following the manufacturer's direction. The products were separated by SDS-PAGE, and their protein concentrations were measured by the bicinchoninic acid (BCA) method. The final receptor protein was frozen in liquid nitrogen and stored at –80°C.

Ligand-binding assay. A direct ELRA was used to verify the binding affinity between purified receptors and β-agonists instead of radio-receptor assay. To microwells, 100 μl of the receptor solution in 10-fold dilution was coated and the plate (Thermo Fisher Nunc, USA) was incubated at 4°C overnight. After emptied completely and washed once with 0.2% PBS-T (PBS containing 0.2% Tween 20), additional binding sites were blocked with 5% skimmed milk powder at 37°C for 2 h. Then 100 μl of four dilutions of HRP-β-agonists in PBS (1 : 500, 1 : 1000, 1 : 2000, 1 : 4000) were added to each well at 37°C for 30 min after washing the plates three times. A quantity of 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma) chromogen solution was added to each well followed by incubation at 37°C for 15 min. The reaction was terminated with 50 μl of stop reagent (2 M sulfuric acid), and the light absorption at 450 nm was measured using a iMark microplate reader (Bio-Rad, USA). Non-coated microwells, which were only blocked with 5% skimmed milk powder, were used as negative controls.

To further characterize that significant binding occurred, competitive binding assays were also carried out in the presence of HRP-CBL plus multiple concentrations (1, 10, 50, 100, 500, 1000 μg/liter) of unlabeled β-agonists of clenbuterol (CBL), salbutamol (SAL), and ractopamine (RAC). In addition, the detailed operation procedures were described as above.

All tests were repeated three times. Statistical analysis of the data and competition curve fitting were processed using Microsoft Excel 2010 and GraphPad Prizm 6.01 (GraphPad Software, USA), respectively.

RESULTS AND DISCUSSION

Codon optimization and construction of expression plasmid. Figure 1 shows the modified gene and amino acid sequence of β_2-AR. Through the analysis of DNAMAN (Lynnon Co., USA), it could be found that the identity of nucleotides between before and after optimization was 74.86%. The optimized gene sequence was more suitable for *E. coli* and mainly reflected in the following several aspects. (i) The codon adaptation index (CAI) value of the gene sequence was raised from 0.70 to 0.96. Generally, CAI = 1 was considered to be the optimal value of the gene in an expression system, so codon optimization in this study increased the CAI value close to 1, thus improving the expression level in the *E. coli* system. (ii) After codon optimization, the minor codon usage frequency for *E. coli* fell sharply to zero, which could greatly increase the efficiency of translation. (iii) The GC content of the optimized gene was decreased to 46.17% from the original value of 58%, which could enhance the efficiency of transcription and translation. Recombinant plasmid of pET-22b-β_2-AR was confirmed to be successfully constructed as expected by colony PCR and sequencing.

Cell-free expression of β_2-AR. The *Escherichia coli*-based cell-free expression system was successfully applied to express the codon-optimized porcine β_2-AR. To achieve ideal expression level, the Mg^{2+} concentration in the expression system was carefully optimized. In Fig. 2a, Western blot analysis using anti-His tag monoclonal antibody showed that the cell-free systems at different Mg^{2+} concentrations could synthesize the receptor proteins with the same molecular mass of approximately 47 kDa, whereas, the yield of the produced protein varied depending on the Mg^{2+} concentration (Fig. 2b). The optimal Mg^{2+} concentration was finally chosen to be 22 mM for its maximum protein expression of 1.1 mg/ml comparing with the yields at other concentrations. After the precipitate was removed by centrifugation, the concentration of the soluble protein in the supernatant was 0.99 mg/ml when Mg^{2+} concentration was 22 mM. In addition, the proportions of the soluble protein were all very large in all samples at different Mg^{2+} concentrations (Fig. 2b), which were 96.17, 95.23, 92.05, 95.45, and 90.27%, because the detergent Brij35 could avoid the aggregation of the proteins.

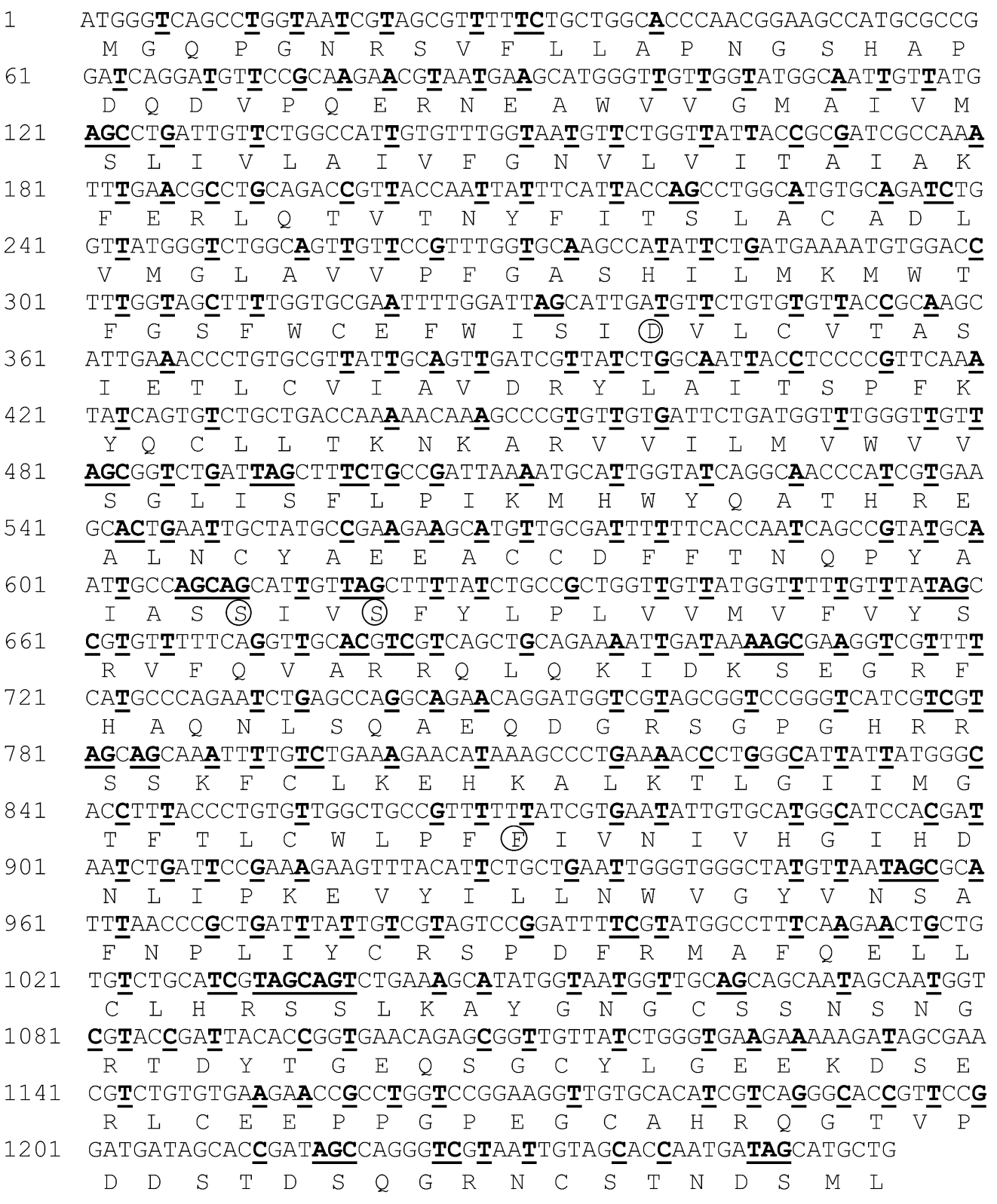

Fig. 1. Nucleotide and amino acid sequences of the modified β_2-AR. Compared with the wild-type β_2-AR sequence, the changes in the nucleotide sequence of the optimized gene are underlined, and the amino acids for ligand binding (D113, S204, S207, and F290) are circled.

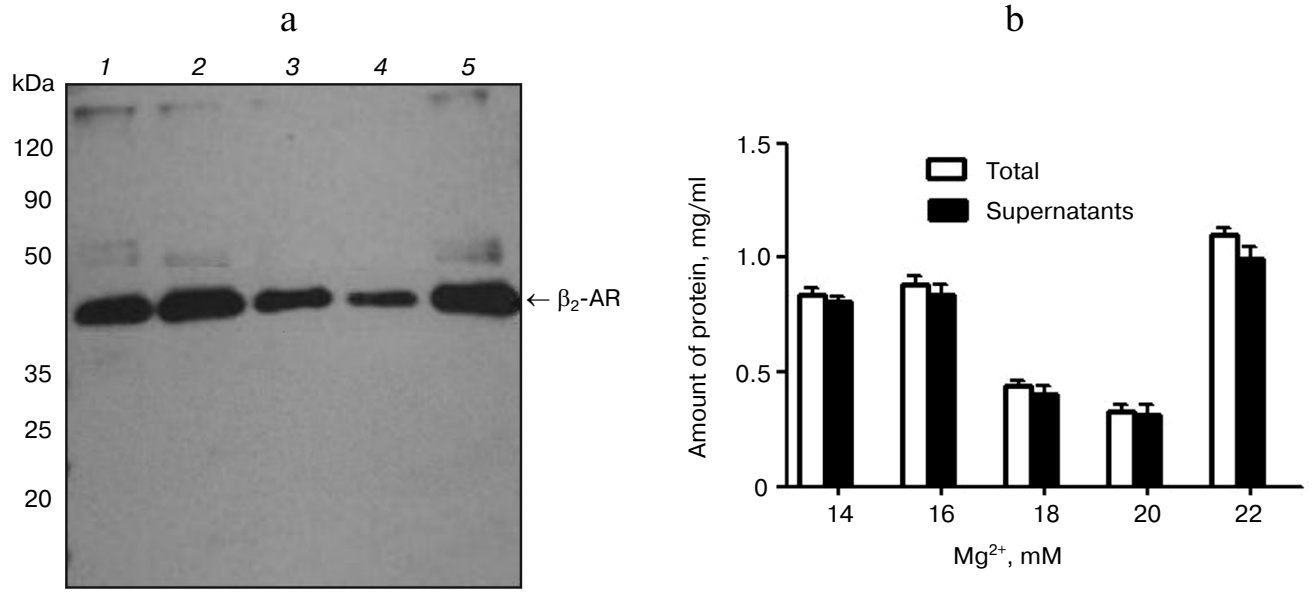

Fig. 2. Western blot analysis of the synthesized β_2-AR from cell-free system with different Mg^{2+} concentrations. a) In Western blot analysis, the specific proteins were detected by the anti-His monoclonal antibody. To achieve optimal protein yield, Mg^{2+} concentration was optimized at five levels with a fixed K^+ concentration (300 mM): 14 (lane *1*), 16 (lane *2*), 18 (lane *3*), 20 (lane *4*), and 22 mM (lane *5*). b) Total synthesized protein concentrations are shown as closed bars, and the supernatants appear as open bars at different Mg^{2+} concentrations for a statistical comparison.

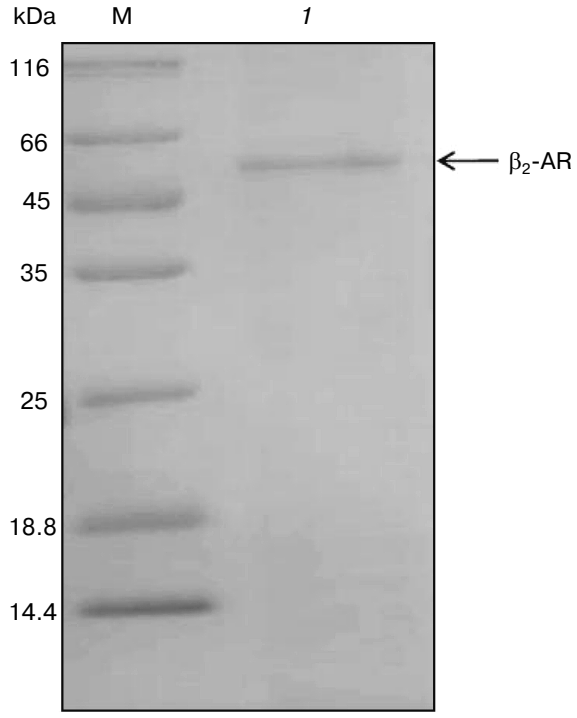

Fig. 3. SDS-PAGE of the purified His-tagged β_2-AR protein made by *in vitro* translation. The expression product was purified using MagneHis™ Ni-Particles, which was processed as described earlier in methods; then it was analyzed by SDS-PAGE. The single band with an apparent molecular weight of 47 kDa appeared as expected on the electrophoregrams (lane *1*); M, molecular mass markers.

Previously, the expression levels of β_2-AR in yeast and baculovirus systems ranging from 1.2 to 20 mg/liter of crude membrane protein have been reported by different groups [15, 29]. In contrast, the yield of the cell-free synthesized β_2-AR protein reached up to 1.1 mg/ml, which demonstrated obviously higher expression level than those of cell-based systems. Besides, the yield was also slightly above the result of another cell-free translational system as described by Ishihara [28]. Perhaps codon optimization made the enormous contribution to the small increment of the expression. The data fully demonstrated that this system could produce the membrane protein β_2-AR at more than 1 mg/ml in 6 h. In addition, the yield could meet the basic needs for the follow-up purification and development of new technologies to detect β-agonists.

The recombinant protein was purified using MagneHis™ Ni-Particles. Compared with the common Ni-NTA-affinity chromatography, the technology provided a simple and rapid means to accomplish protein purification in 20 min. The purified β_2-AR receptor was found to be >90% pure as determined by 10% SDS-PAGE (Fig. 3). We also found that it migrated as single distinct band with a molecular mass of around 47 kDa on the electropherogram. The purified receptors were stored at −80°C at a concentration of 800 μg/ml.

Binding properties of the recombinant β_2-AR. The ligand binding activities of the purified β_2-AR proteins were assessed by measuring OD_{450} values in ELRA. The table shows the specific binding of the cell-free products

Detection of activity of the purified β_2-AR synthesized *in vitro* by ELRA

Dilution	HRP-CBL (OD_{450})	HRP-RAC (OD_{450})	HRP-SAL (OD_{450})	Negative control
1 : 500	1.117 ± 0.021	0.524 ± 0.024	0.671 ± 0.025	0.071 ± 0.012
1 : 1000	0.974 ± 0.016	0.347 ± 0.013	0.428 ± 0.009	0.058 ± 0.013
1 : 2000	0.536 ± 0.015	0.166 ± 0.010	0.293 ± 0.011	0.035 ± 0.013
1 : 4000	0.318 ± 0.018	0.092 ± 0.015	0.125 ± 0.010	0.027 ± 0.009

to the three HRP-β-agonists by comparing the results of negative controls, and OD_{450} values were decreasing in the order HRP-CBL, HRP-SAL, and HRP-RAC. Hence, HRP-CBL was chosen for use in the following competitive binding reactions, with the optimal working 1000-fold dilution. The OD_{450} values at the corresponding dilution ratios were slightly lower than what were observed with the receptor protein produced from HEK293 cells previously [9]. We speculate that only part of the total proteins obtained maintained their proper active structures.

A four-parameter curve-fitting model was applied to draw the affinity competitive curves using HRP-CBL as the enzyme-labeled ligand, and three β-agonists as the unlabeled competitors. As can be seen from the standard competitive curves (Fig. 4), the percentages of binding gradually decreased with increasing amounts of added β-agonists. The IC_{50} values, midpoints of calibration curves of CBL, SAL, and RAC were 45.99 ± 2.84, 60.38 ± 4.37, and 78.02 ± 3.70 μg/liter, respectively. The data were similar to those previously reported with the synthesized receptor proteins from Sf9 cells (44.93, 65.94, and 76.06 μg/liter) [8] and HEK293 cells (34, 53, and 63 μg/liter) [9]. The various bioactivities of different types of the recombinant receptors could be attributed to various reasons. The most likely reason is the different expression systems, followed by the various origins of β_2-AR and optimization of expression conditions.

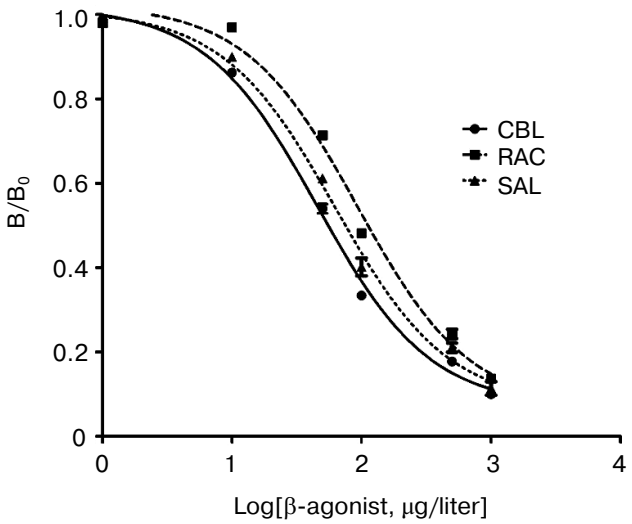

Fig. 4. Competitive displacement of specific HRP-CBL binding to the purified β_2-AR from cell-free reactions by agonists. The affinity competitive curves were established by plotting the logarithm values of concentrations of unlabeled β-agonists against the percentages of binding (B/B_0). B and B_0 were the absorbances of the β-agonists at the standard point and at zero concentration of the β-agonists, respectively. The data represent the mean values of independent experiments performed in triplicate.

Based on the results of the above direct and competitive ligand binding assays, it suggests that the synthesized receptor protein is capable of binding β-agonists. Moreover, it would have great potential for wide-scale use in in preliminary screening of β-agonists because of its high-level expression compared with other cell-based expression systems.

These results suggest that the *E. coli*-based cell-free expression system could be successfully used for abundant synthesis of porcine β_2-AR in functional form, which was a very difficult-to-express yet valuable membrane protein. Furthermore, codon optimization provided a helpful means to increase the expression up to 1.1 mg/ml in 6 h, which was the highest yield of β_2-AR in cell-free contexts reported. Novel ligand binding assays based on a direct ELRA and a competitive ELRA were introduced to certify the binding affinity of the cell-free synthesized receptor. It was proved that the purified β_2-AR specially recognized structurally different β-agonists including CBL, RAC, and SAL. In addition, their IC_{50} values were 45.99, 78.02, and 60.38 μg/liter, respectively. The data show the potential of the receptor protein to detect new compounds with agonistic activity. To this end, more valuable explorations and attempts should be done, such as the application of wheat germ-based cell-free expression system as well as further optimization of the reaction system.

The results obtained in this study demonstrate that production of functional β_2-AR in milligram amounts by a cell-free system is feasible. The synthesized receptor as a potential biocomponent could be used to develop non-radioactive multianalyte screening methods in analysis of a broad range of β-agonists residues.

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