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Expression of Codon Optimized β_2-Adrenergic Receptor in Sf9 Insect Cells for Multianalyte Detection of β-Agonist Residues in Pork

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β_2-adrenergic receptor (β_2-AR) was expressed efficiently using Bac-to-Bac Baculovirus Expression System in Sf9 cells as a bio-recognition element for multianalyte screening of β-agonist residues in pork. Sf9 cells were selected as the expression system, and codon optimization of wild-type nucleic acid sequence and time-dependent screening of expression conditions were then carried out for enhancing expression level and biological activity. Under optimum conditions of multiplicity of infection (MOI) = 5 and 48 h post transfection, the protein yield was up to 1.23 mg/ml. After purification by chromatographic techniques, the purified recombinant protein was applied to develop a direct competitive enzyme-linked receptor assay (ELRA) and the efficiency and reliability of the assay was determined. The IC50 values of clenbuterol, salbutamol, and ractopamine were 28.36, 50.70, and 59.57 μg/l, and clenbuterol showed 47.61% and 55.94% cross-reactivities with ractopamine and salbutamol, respectively. The limit of detection (LOD) was 3.2 μg/l and the relevant recoveries in pork samples were in the range of 73.0-91.2%, 69.4-84.6%, and 63.7-80.2%, respectively. The results showed that it had better performance compared with other present nonradioactive receptor-based assays, indicating that the genetically modified β_2-AR would have great application potential in detection of β-agonist residues.

Keywords: β_2-adrenergic receptor, Sf9 cells, codon optimization, β-agonist, receptor-based assay, pork

Introduction

The illegal abuse of β-adrenergic agonists (β-agonists) as nutrient repartitioning agents in the livestock industry to promote fat-redistribution into muscles has been widely reported recently [1, 2]. The drug residues accumulated in animal tissues may pose a potential risk for consumer health, such as muscular tremors, vomiting, palpitations, nervousness, and headache [3, 4]. Hence, no β-agonists have been licensed for food-producing animals in China [5] and many European countries [6]. Nonetheless, mixed abuse of these drugs and novel synthetic structural analogues have caused great trouble for the supervision departments. With more and more public attention on food safety, a high-throughput paralleled detection technique of β-agonists is urgently required.

At present, chromatographic analysis and immunoassays are the mainstream technologies for confirmation and screening of β-agonists in animal feed and animal products [7]. However, the various chromatographic methods reported, such as high-performance liquid chromatography (HPLC) [8, 9], gas chromatography-mass spectrometry (GC–MS) [10, 11], ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS) [12, 13] and capillary electrophoresis (CE) [14, 15] are not suitable for field analysis because of the requirement of sophisticated instruments and complicated sample pretreatment. Therefore, plenty of immunoassays based on the enzyme-linked immunosorbent assay (ELISA) [16, 17], electrochemistry [18], fluorescence [19], chemiluminescence [20] and surface-

enhanced Raman scattering [21] have been prevalent methods recently and are particularly suitable for rapid screening. However, these methods are mostly designed for detection of single or a small amount of analyte due to the complex antibody preparation procedure.

The receptor assay based on β_2-AR has gained growing attention since it is able to achieve group-specific recognition of β-agonist structural analogues. In recent years, a few radio-receptor assays have been reported using natural membrane-bound β_2-AR [22] or recombinant β_2-AR expressed in *E. coli* [23], Chinese hamster ovary cells [24], and NCB20-D1 cells [25]. But owing to the large costs and possible harmful impact of radioactive isotopes on the human body, it is imperative to develop nonradioactive detection methods at present. Our team successively established direct, competitive enzyme-linked receptor assay (ELRA) methods based on β_2-AR protein synthetized in HEK293 cells [26] and a cell-free system [27] for multianalyte detection of β-agonists. In addition, Cheng *et al.* [28] also developed an ELRA method using wild-type Syrian hamster β_2-AR expressed in Sf9 cells to determine β-agonists in animal feed. In spite of these beneficial attempts, the biggest obstacle still for their further application is getting enough recombinant protein with high-affinity.

There are many strategies to enhance the expression level, such as codon optimization, site-directed mutagenesis, optimization of expression conditions and the use of different tags and strong promoters. Although Sf9 insect cells were considered one of the most efficient expression systems [29], the minor codons in β_2-AR gene still affected severely the yield and activity of expression products. The aim of this study was to establish high-level expression of β_2-AR using the Bac-to-Bac Baculovirus Expression System in Sf9 cells by codon optimization and screening of the optimal expression conditions. Following this, the purified receptor was then utilized to develop an ELRA for detecting the multi-residue β-agonists in pork samples.

Materials and Methods

Materials and Reagents

Spodoptera frugiperda insect cell line Sf9 was purchased from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Sf-900 II SFM media was from Hyclone (USA). Enzymes used for manipulating DNA, such as Pfu DNA Polymerase, T4 DNA ligase, EcoRI and XbaI were purchased from NEB (USA). The Bac-to-Bac Baculovirus Expression System Kit, including the pFastBac1 vector, the *E. coli* competent cell DH10 Bac and the transfection reagent Cellfectin II were from Invitrogen (USA). Nickel–nitrilotriacetic acid (Ni–NTA) His Bindresin was provided by Qiagen (Germany). HRP-conjugated goat anti-mouse IgG and anti-His monoclonal antibody were obtained from Sigma-Aldrich (USA). Ractopamine (RAC), clenbuterol (CBL) and salbutamol (SAL) were also purchased from Sigma-Aldrich. HRP-β-agonists were gifts from Beijing Kwinbon Biotechnology Co., Ltd. (China). All chemicals were of analytical grade.

Pork Samples

Pork samples (tenderloin) collected from a supermarket in Hebei Province of China were ascertained to be free of β-agonists by LC-MS/MS using a method of the Chinese Ministry of Agriculture in announcement No. 1063. The sample processing steps were as follows: After the fat and fascia tissue were removed, 2 ± 0.02 g of homogeneous sample was weighed accurately in a 50-ml centrifuge tube followed by adding 6 ml of solution containing 4% NaCl and 0.1 mol/l HCl. Then the above mixture was stirred thoroughly for 3 min and stood at room temperature for 20 min. After that, it was centrifuged at 5,000 ×*g* for 10 min and the pH value of the resulting supernatant (10 ml) was adjusted to be between 5.5 and 6.0 with about 20 µl of 1 mol/l NaOH. The sample solution was spiked with 1, 10, 50, and 100 µg/l of each type of β-agonist, respectively. The mixture was centrifuged at 5,000 ×*g* for 10 min at 4°C. The supernatant was transferred into another centrifuge tube and evaporated under a stream of nitrogen in a water bath at 55°C. The extract was then reconstituted using 1 mL PBS solution for the ELRA.

Codon Optimization and Synthesis of Porcine β_2-AR Gene

According to the codon usage bias of insect cells, the wild-type porcine β_2-AR gene (GenBank Acc. No. KF023571.1.) was codon optimized by the tool Protparam (http://www.expasy.org/tools/protparam.html) and the restriction enzyme sites of EcoRI and XbaI were respectively arranged at the 5′ and 3′ ends of it. The modified gene with the name β_2-AR′ was then synthesized by Wuhan GeneCreate Biological Engineering Technology and Service Co., Ltd., China.

Construction of Recombinant Vector

In order to construct the recombinant plasmid, the modified gene β_2-AR′ was ligated into the vector pFastBac1 by incubation overnight at 4°C with T4 DNA ligase after double digestion with restriction enzymes EcoRI and XbaI. The transfer vector pFastBac1-β_2AR′ was then transformed into competent *E. coli* DH5α cells, which were then spread on blue/white selective LB agar plates. The recombinant plasmid was extracted from DH5α cells and confirmed by restriction endonuclease digestion and sequencing (service provided by Shanghai Sangon Biological Engineering, Technology & Service Co., Ltd., China).

Cell Culture, Transfection, and Expression

Sf9 insect cells from *Spodoptera frugiperda* were semi-adherently cultured at 27°C in Sf-900 II SFM medium, and passaged once

every 3 or 4 days. Sf9 cells were placed in six-well plates and 9×10^5 cells in exponential phase were seeded in each well containing 2 ml medium, and incubated at 27°C for 2 h. Then the monolayer of Sf9 cells was transfected with the recombinant bacmid pFastBac1-β_2AR′ using the transfection reagent Cellfectin II, according to the manufacturer's instructions in the Bac-to-Bac Baculovirus Expression System Kit (Invitrogen). The transfected cells were then incubated at 27°C with daily observation of cytopathic effect (CPE) caused by virus. The virus from the cultured Sf9 cells was harvested at 72 h post-transfection by centrifuging at 3,000 ×*g* for 5 min. The prepared P1 virus was amplified to generate high-titer P2 virus.

In order to improve the expression level of β_2AR receptor protein, two key factors of multiplicity of infection (MOI) and incubation time post-transfection were explored. Virus from the P2 stock was added into each well seeded with 8×10^5 Sf9 cells beforehand at the MOI of 1, 5, and 10, respectively. After 48 h and 72 h following transfection, the infected cells were precipitated respectively by centrifugation at 3,000 ×*g* for 15 min and washed three times with phosphate-buffered saline (PBS, pH 7.4). The Sf9 crude membranes were prepared as previously described [30]. The obtained supernatant was analyzed by western blot with anti-His monoclonal antibody for further purification, and the negative controls were prepared by the same procedures using Sf9 cell lysate.

Purification of Recombinant β_2-AR

The purification of the recombinant β_2-AR protein was carried out by Ni-NTA-affinity chromatography (Germany) according to the manufacturer's instructions. The supernatant was applied to a Ni-NTA column (10 ml, 1.6 × 5 cm) with a flow rate of 1 ml/min. After washing the column with washing buffer 1 (PBS, pH 7.4), washing buffer 2 (30 mmol/l imidazole, PBS, pH 7.4) and washing buffer 3 (50 mmol/l imidazole, PBS, pH 7.4) in turn, the recombinant β_2-AR was eluted with a stepwise imidazole of 200-400 mM in PBS (pH 7.4). The eluted fractions were collected and their purity was evaluated by western blot analysis. The concentration of the purified β_2-AR was determined by bicinchoninic acid (BCA) assay.

Binding Assay and Analytical Procedures

The binding affinity of β-agonist to the purified β_2-AR protein was verified first by a direct ELRA as described in our previous literature [27]. Then a competitive ELRA format was adopted for further analyzing β_2-AR binding and detection of β-agonists in pork. The ELRA procedures were as follows: (1) The 96-well microtiter plate was precoated with 100 µl/well receptor solution overnight at 4°C. (2) The plate was washed 3 times using 0.2% PBS-T (10 mmol/l PBS containing 0.2% Tween 20, pH 7.4) and then blocked with 1% bovine serum albumin (BSA) at 37°C for 2 h. (3) After washing the microplate 3 times, 50 µl of standard solution (1, 10, 50, 100, 500, 1,000 µg/l) or sample was incubated in triplicate with 50 µl of HRP-CBL solution (recommended working concentration 1:1000) at 37°C for 30 min. (4) 100 µl of a chromogen [3, 3′ 5, 5′-tetramethylbenzidine (TMB)] solution was added to each well. (5) After incubation for 15 min at room temperature, 50 µl of 2 mol/l H_2SO_4 was used to stop the reaction and the absorbance was measured at 450 nm.

Data Analysis

The methodology of the established ELRA for detection of β-agonist residues was evaluated with a series of tests. Various parameters were represented as mean ± SD and needed graphs were processed using Microsoft Excel 2010 and GraphPad Prizm 7.05.

Results and Discussion

Gene Reformation and Plasmid Construction

Codon bias was one of the most important factors influencing heterogenous gene expression. The nucleic acid sequence of the codon-optimized porcine β_2-AR and its

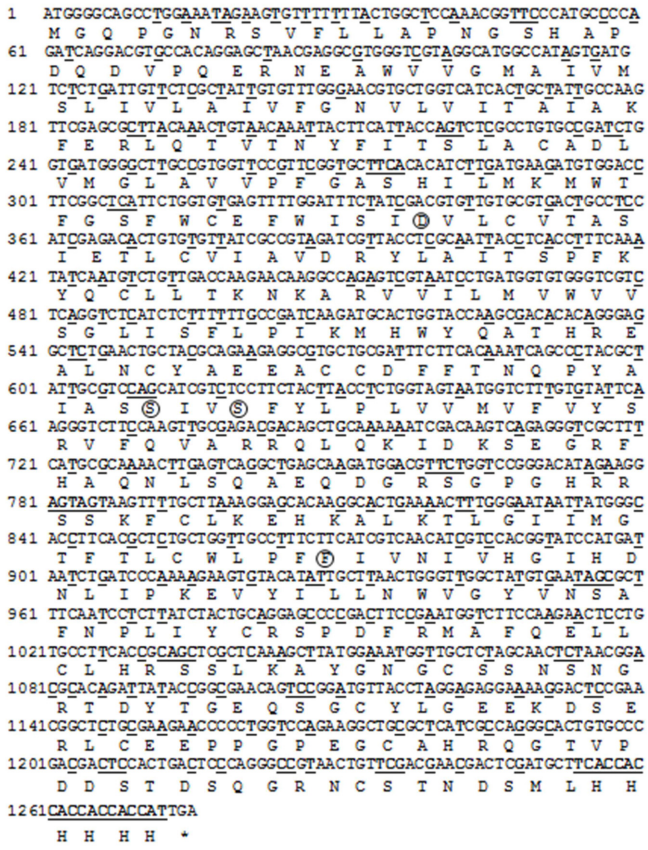

Fig. 1. The codon-optimized nucleotide sequences and the coded amino acid sequence of β_2-AR.

The base replacements comparing with the wild-type β_2-AR gene were underlined, and the ligand bound amino acids (D113, S204, S207, and F290) were circled.

coded amino acid sequence were shown in Fig. 1. Through analyzing by multiple sequence alignments, it could be found that the nucleotide homology was 77.09% between the modified and wild-type genes. There were several characteristics of the optimized gene revealing its better adaptability to insect cells. The codon adaptation index (CAI) value of the nucleotide sequence was increased from 0.80 to 0.95 and the rare or low-usage codons for insect cell sharply declined to zero. Besides, the GC content of the optimized nucleic acid was decreased from 57% to 49%. These changes in the indicators showed that the efficiency of transcription and translation would be enhanced.

The codon-optimized, full-length β_2-AR gene containing His-tag at the C-terminal region was cloned as a fusion cassette into the baculovirus transfer vector (pFastBac 1). The recombinant plasmids were identified by restriction enzyme (EcoRI and XbaI) digestion. As seen in Fig. 2, the bands of pFastBac 1 vector and target gene appeared respectively in 4.8 kb and 1.3 kb on 1% agarose gel as expected. By further sequence analysis, the positive plasmids were named pFastBac1-β_2AR′.

Expression of β_2-AR in Sf9 Cells

Sf9 cells have been generally considered to be the first choice for efficient expression of β_2-AR and other G-protein-coupled receptors (GPCRs) [29]. In addition, the culture conditions of Sf9 cells are very important for high-level expression of β_2-AR. Sf9 cells were infected with the recombinant bacmid of pFastBac1-β_2AR′ and then incubated for 72 h at 27°C. Cell size, cell morphology and cell viability were monitored every 24 h out of 72 h. The results showed that cell bodies gradually became larger and more circular in shape 24 h post-transfection; the cells stopped growing along with an obviously enlarged nucleus 24-72 h post-transfection; intracellular particulate matter increased and partial adherent cells fell off and even died 72 h post-transfection (Fig. 3b). These cell morphology changes indicated that the recombinant plasmid was transfected into Sf9 cells successfully. The plaque assays showed that P1 viral stock had plaque forming units (pfu) of 2×10^6 per ml, and the corresponding datum was 1.8×10^7 pfu/ml in the P2 viral stock.

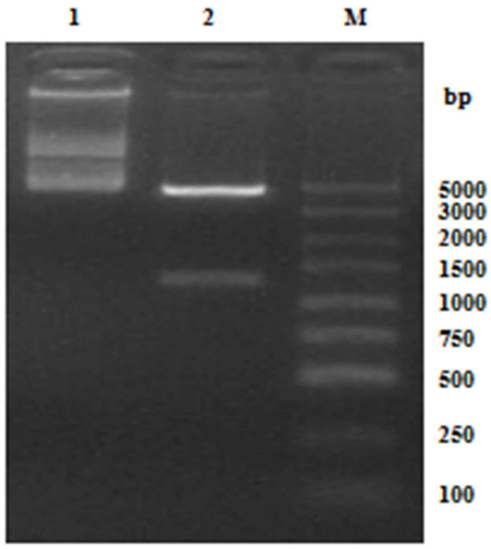

Fig. 2. Identification of recombinant plasmids by double restriction enzyme digestion.

The recombinant plasmids were digested with EcoRI and XbaI. Restricted DNA products were separated by 1% agarose gel electrophoresis. Lane 1, the plasmid pFastBac1-β_2AR′. Lane 2, digested products with two bands of 4.8 kb and 1.3 kb, which were the same size of target gene and vector sequences, respectively. M, DNA marker.

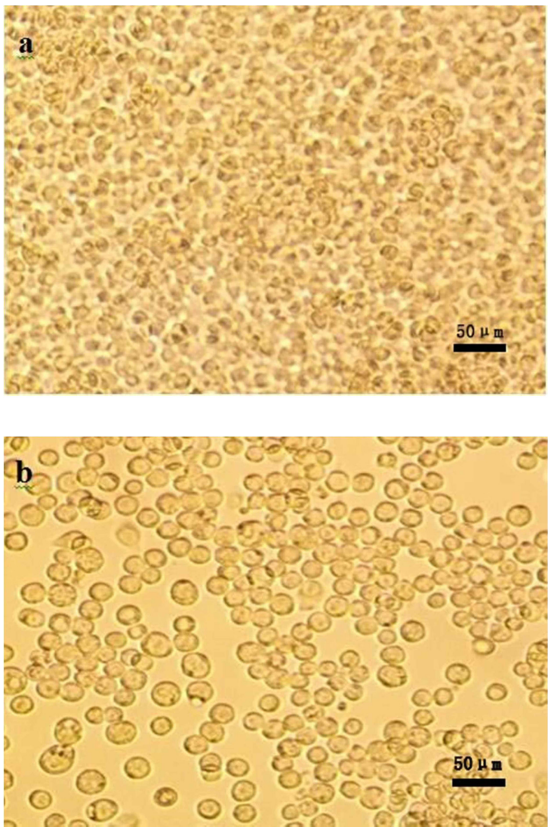

Fig. 3. Microscope photographs of normal Sf9 cells and Sf9 cells at 72 h post transfection (× 400).

(a), (b), photomicrographs at 400 times magnification of normal Sf9 cells and the transfected cells after 72 h. Cell morphology changes before and after transfection were compared and analyzed in detail in the thesis.

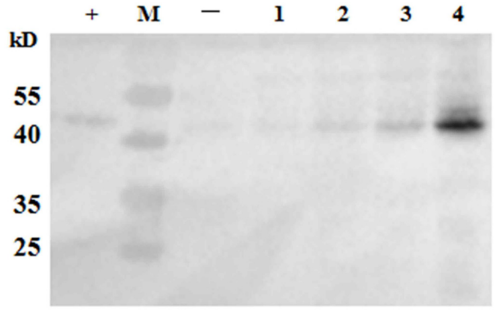

Fig. 4. Western blot analysis of lysates from Sf9 cells before and after transfection.

The extracts of the expression products from four recombinant bacmids were separated by gel electrophoresis, and western blotted with anti-His monoclonal antibodies. Lane 1-lane 4, the protein extracts of the Sf9 cells from different bacmids (1#-4#). A 47 kDa specific band appeared respectively in lane 1-lane 4 as expected. Also, the band in lane 4 (4# bacmid) was the most clearest among them. Lane (+), His-tag-fused proteins as positive control. Lane (-), the nontransfected Sf9 cells as negative control. Lane M, molecular weight standard.

The baculovirus expression system has many obvious advantages over the prokaryocyte expression system, because it allows the expression of functional eukaryotic proteins in native folding state and post-translational modifications including phosphorylation, amidation, acetylation, etc. Expression of β_2AR in Sf9 cells was verified by western blot as shown in Fig. 4. The apparent molecular weights of around 47 kDa were consistent with the predicted values.

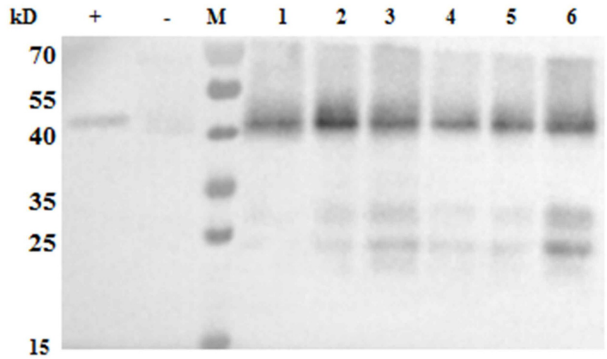

Fig. 5. Western blot analysis of the synthesized β_2-AR in Sf9 cells with different MOI and incubation time.

Lane 1-3, the products obtained at MOI=1, 5, 10 respectively 48h post transfection. Lane 4-6, the products obtained at MOI=1, 5, 10 respectively 72 h post transfection. It was evident that the optimal conditions were MOI=5 and 48 h post transfection (lane 2), because of its distinctive target band. Lane (+) , lane (-) and lane M were identical to those described in Fig. 4.

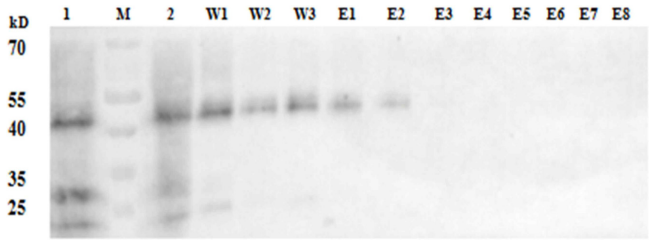

Fig. 6. Western blot analysis of the purified protein by Ni-NTA-affinity chromatography.

Lane 1, the protein sample before purification. Lane 2, the flow-through solution after purification. Lane W1-W3, washing buffer passed across the column. Lane E1-E8, eluent passed across the column. Lane M, molecular weight standard. A single and clear band with apparent molecular weight of 47 kDa was detected respectively in lane E1 and E2, so both of them were collected together for activity identification and construction of detection method.

The P2 viral stock amplified from 4# bacmid (lane 4) was applied to optimize the expression condition. As seen from Fig. 5, the most distinctive band was obtained in lane 2 (MOI = 5 and 48 h post-transfection). Under these conditions, it reached the highest expression level of 1.23 mg/ml of crude protein.

Purification of Recombinant β_2-AR

The purified protein was confirmed as recombinant β_2-AR by staining with anti-His antibody on western blot (Fig. 6). The washing buffer after passing across column (lane W1-W3) presented as one major band with an apparent molecular mass of 47 kD accompanied by some impure proteins. It revealed that some proteins of interest might be eluted during the washing process. In contrast, the eluents E1 and E2 (lane E1 and E2) whose purity was over 95% appeared as clear single target bands, respectively. Finally, they were collected together and blended for activity identification.

Binding Assay

A direct ELRA was applied to determine the binding affinity of the purified β_2AR proteins to the β-agonists. As shown in Table 1, the purified product presented specific binding to all three HRP-β-agonists of HRP-CBL, HRP-SAL, and HRP-RAC. Moreover, their average optical density (OD) values were 1.005 ± 0.023, 0.489 ± 0.033, and 0.394 ± 0.024 at the optimal working concentration of 1:1000 dilution which preceded the results of Cheng *et al.* [28]. The results indicated that the purification process had little influence on the binding activity of β_2-AR protein.

Table 1. Determination of the binding characteristics of the purified β_2-AR expressed in Sf9 cells by ELRA.

Dilution	HRP-CBL (OD_{450})	HRP-RAC (OD_{450})	HRP-SAL (OD_{450})	Negative control
1:500	1.241 ± 0.050	0.600 ± 0.016	0.706 ± 0.032	0.063 ± 0.006
1:1000	1.005 ± 0.023	0.394 ± 0.024	0.489 ± 0.033	0.064 ± 0.011
1:2000	0.697 ± 0.034	0.216 ± 0.025	0.351 ± 0.025	0.038 ± 0.011
1:4000	0.428 ± 0.038	0.101 ± 0.017	0.175 ± 0.015	0.029 ± 0.010

Calibration Curve, Precision and Accuracy of Assays

A four-parameter curve-fitting model was introduced to draw the calibration curves by plotting [1-(B/B0)]×100% against the logarithm of β-agonist concentration. From the graph (Fig. 7), it is evident that the binding ratio decreased gradually with the increase in concentration of β-agonists. The IC_{50} values, the concentrations of CBL, SAL, and RAC needed to inhibit 50% of β_2-AR activity, were 28.36, 50.70, and 59.57 μg/l, respectively. The data were slightly superior to those of the other two reports based on the heterogenous receptor proteins expressed from wild-type β_2-AR gene of Syrian hamster (44.93, 65.94, and 76.06 μg/l) [28] and swine (34, 53, and 63 μg/l) [26]. The results suggested that codon optimization in our study had no adverse effects on binding affinity of the synthesized protein under the premise of high-yield expression.

Cross-reactivity was determined to estimate the selectivity of the method for multianalyte assay. The results showed that the cross-reactivity of CBL with RAC was 47.61% and with SAL was 55.94%, and RAC with SAL was 85.11%. It revealed that β_2-AR had the capacity to bind a panel of well-known β-agonist compounds, and their binding affinities were variable due to the different chemical structures. The limit of detection (LOD) was evaluated by analyzing 20 randomly collected pork samples certified free of β-agonist from Hebei Province. The LOD calculated as the mean + 3 SD was 3.2 μg/l which was lower than that of the reported ELRA [28]. Although there were some differences in sensitivity of the outcomes between this approach and commercial ELISA kits, the receptor showed good potential application in rapid detection of structurally different β-agonists.

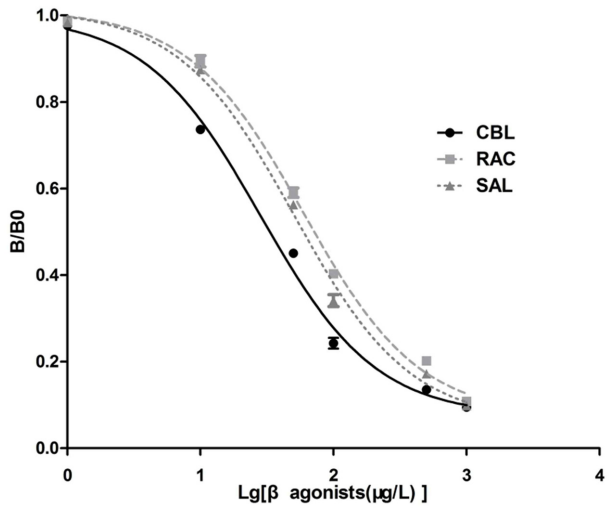

Fig. 7. Inhibition of HRP-CBL binding to the purified β_2-AR from Sf9 cells by agonists.

Data were representative of the mean values of three or more independent experiments with each point measured in triplicate.

Analysis of Spiked Pork Samples

In order to confirm the reliability of the proposed method, it was actually applied for determination of β-agonists in pork samples. The pork samples spiked with 1, 10, 50, and 100 μg/l of each type of β-agonist were detected by the assay to analyze the recoveries. The measurement results were listed in Table 2. The recoveries of CBL, RAC, and SAL were observed in ranges of 73.0-91.2%, 63.7-80.2%, 69.4-84.6%, respectively, which were lower than those of the previous reports [26, 28]. All coefficients of variation (CV) were within 15% demonstrating a good repeatability. The results indicated that the matrix effect of pork had less influence on the assay.

Our approach achieved the high-level expression of recombinant β_2-AR up to 1.23 mg/ml in Sf9 cells through these effective techniques of codon optimization and screening of appropriate MOI and incubation time post-transfection. The yield was obviously higher than the previous report involving the wild-type β_2-AR gene. The receptor protein purified using Ni-NTA-affinity chromatography still maintained the binding affinity to structurally different β-agonists. For the three β-agonists, the IC_{50} values were 28.36, 50.70, and 59.57 μg/l, and the recoveries within the range of 63.7-91.2%.

Most of the Chinese national standard methods for determination of β-agonists are LC-MS/MS and GC-MS whose LODs are in the range of 0.1–10 ng/l. Besides, the LODs of the commercial ELISA kits for β-agonists are

Table 2. Recoveries of β-agonists from the spiked pork samples determined by ELRA.

β-agonist	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Recovery (%)	Coefficient of variation (CV) (%)
CBL	1	0.73 ± 0.08	73.0	9.6
	10	7.88 ± 0.47	78.8	11.2
	50	42.55 ± 3.59	85.1	10.7
	100	91.19 ± 8.42	91.2	13.6
RAC	1	0.64 ± 0.06	63.7	10.3
	10	6.64 ± 0.31	66.4	13.5
	50	38.20 ± 3.12	76.4	12.6
	100	80.22 ± 6.67	80.2	14.7
SAL	1	0.69 ± 0.06	69.4	8.3
	10	7.18 ± 0.59	71.8	11.4
	50	38.65 ± 3.88	77.3	13.7
	100	84.63 ± 7.47	84.6	13.2

ranged from 0.025 μg/l to 0.1 μg/l. By comparison, the receptor analysis has certain inherent disparity in detection sensitivity. There may be a number of possible reasons to influence the detection results, such as maintaining the activity of receptor protein in the detection process, designing and optimizing the detection procedure, etc. However, the method showed the potential of the receptor to bind a panel of structurally different β-agonists and new compounds with agonistic activity undetectable by the previous methods. Moreover, the established competitive ELRA exhibited better performance than the current ELRA methods [25–28], providing a great application prospect for multianalyte screening of abused and illegal β-agonist drugs in pork and other related animal products.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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