Preparation of a polyclonal antibody based heterologous indirect competitive ELISA for detecting ractopamine residue

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Abstract

Based on the polyclonal antibody (pAb), an indirect competitive ELISA (icELISA) has been developed for the determination of ractopamine (Rac) residue in swine and cattle. Mixed anhydride method was employed to synthesize the immunogen while 1,4-butanediol diglycidyl ether was used to synthesize the coating antigen, thus producing the heterologous sensitivity; and two female New Zealand white rabbits were used to produce anti-Rac pAb. By the square matrix titration, an icELISA standard curve was established. The linear range was from 0.006 to 30 ng/mL, with LOD and IC50 value of 0.003 and 0.44 ng/mL, respectively. Except for a moderate cross-reactivity (34.5%) to dobutamine, negligible cross-reactivity to other compounds tested was observed. After optimization, 1:10 dilution in swine muscle and 1:20 dilution in cattle muscle produced the satisfactory B0 value of 0.003 and 0.44 ng/mL, respectively. When applied in real sample tests, the correlation coefficients (R2) of the concentration spiked and concentration determined were 0.9411 in swine and 0.9623 in cattle. Therefore, this assay has the potential for the rapid screening of Rac residue in food.

Key words: Ractopamine, artificial antigen, polyclonal antibody, indirect competitive ELISA, heterology.

Introduction

β-Adrenergic agonists are repartitioning agents used to increase feeding efficiency and carcass leanness, and also promote animal growth 1. Ractopamine (Rac), which acts as nutrient repartitioning agent in livestock by diverting nutrients from fat deposition in animals to the production of muscle tissues, is an effective β agonist that had been widely used before. Although Rac has been licensed for use in the United States, the use of it for growth-promoting purposes in cattle has been banned in the European Union (EU) due to health concerns 2. It is reported that Rac residues can become high enough to cause acute toxic effects, such as heart palpitations, muscle tremors, tetany, and severe migraines in consumers 3. To ensure compliance with regulations banning the use of Rac, the EU suggested a cutoff concentration of 1 ng/mL for Rac 4. In China and other Asian countries, the government requires that Rac must not be present at all in food (zero tolerance), therefore, sensitive analytical techniques are needed to detect Rac residue in animal food.

There have been an increasing number of physico-chemical methods to monitor Rac residue in animal urine, feeds and tissues, such as liquid chromatography-mass spectrometry (LC-MS) 2,4 and gas chromatography-mass spectrometry (GC-MS) detection 5,6. However, these analytical approaches, which use several clean-up procedures (liquid-liquid extraction) and solid-phase extraction, are quite complicated, time-consuming, and expensive. Furthermore, it is a currently demand for faster onsite (farmhouses) and/or online (slaughterhouses) test systems. Immunoassays as screening method can rapidly detect low amounts of residues in many samples. So several screening methods for Rac have been reported 7-9, but to some degree, these immunoassays are not sensitive enough to perform the zero tolerance policy in Asia.

As a result, it is necessary to develop more sensitive methods for the determination of Rac residues in animal edible tissues. In this study, we have aimed to prepare the artificial antigen of Rac and produce anti-Rac polyclonal antibody (pAb). We have also developed a heterologous rapid screening immunoassay for monitoring Rac residues in swine and cattle.

Materials and Methods

Materials and chemicals: Ractopamine (Rac) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany), clenbuterol, dobutamine, terbutaline, isoproterenol, and salbutamol were purchased from Sigma (St. Louis, Mo.), 1-Ethyl-3-(3-dimethylaminopropy) carbodiimide (EDC), Freund’s complete adjuvant (FCA) and Freund’s incomplete adjuvant (FIA) were obtained from Pierce, and N-hydroxysuccinimide (NHS) was from Japan, MSDS available. O-(carboxymethyl) hydroxylamine hemihydrochloride, succinic anhydride, bovine serum albumin (BSA) and ovalbumin (OVA) were supplied by Sigma, and Dialysis bag (8000-14000 Da) was from Solarbio Company, GaRlgG-HRP was purchased from Sino-American Biotechnology Company (Shanghai, China). Transparent 96-well polystyrene microtine plates (Boyang Experimental Equipment Factory, Jiangsu, China) were used for the colorimetric measurement, and 3,3,5,5-tetramethylbenzidine (TMB), phenacetin and urea peroxide were obtained from Sigma Company. All other solvents and reagents were of analytical grade or higher, unless otherwise stated.

Synthesis of immunogen and coating antigen: The immunogen of Rac-BSA was prepared as the method described previously with some modifications 10. Briefly, Rac (85 mg) and glutaric anhydride (32 mg) were added to 2 mL of pyridine. The mixture
was stirred overnight at room temperature and dried under a nitrogen atmosphere. A total of 5 mL dimethylformamide:dioxane:triethylamine (v/v/v, 40:40:1) was added and stirred for 15 min at 0°C. Isobutyl chloroformate (36 µL) was added and stirred for 1.5 h at room temperature. A total of 5 mL BSA ice-cold solution (66 mg of BSA dissolved in 5 mL sodium tetraborate solution, pH 7.4) was added and stirred overnight at room temperature. The antigen was finally purified by PBS (pH 7.4) and stored at -20°C in refrigerator. The coating antigen of Rac-OVA was synthesized according to Li et al. 9.

**Immunization schedule:** Rac-BSA was used to raise anti-Rac polyclonal antibodies. Two female New Zealand white rabbits were subcutaneously immunized at multiple sites on the back. The initial immunization was injected with 500 µg of conjugate in 1 mL of PBS and 1 mL of FCA. Subsequent boosters with FIA as emulsion were performed at 3 week intervals. On the seventh day after each boost, blood samples were taken from the marginal vein of the ear to check the titers of antisera. Ten days after the fifth boost, both rabbits were exsanguinated by heart puncture. The blood sample was allowed to coagulate overnight at 4°C and the serum was separated by centrifugation at 3000 r/min. Saturated ammonium sulfate (SAS) precipitation method was used to purify the crude serum. The purified serum was then aliquotted and stored at -70°C.

**Generic ELISA procedures:** The microplates were coated with coating antigen in PBS (100 µL/well) by overnight incubation at 4°C. Plates were washed with PBST three times and unbound active sites were blocked with 250 µL/well of blocking buffer, followed by incubation for 2 h at room temperature. The solution was discarded, and plates were washed three times with washing solution. Then, 50 µL/well of antibody was added, and the plates were incubated for 15 min at 37°C. After another washing procedure, GaR1gG-HRP (50 µL/well) was added, followed by incubation for 25 min at 37°C. The final washing procedure was followed by a color development, which was initiated by adding 20 µL/well of freshly prepared TMB substrate solution. After incubating at room temperature for 15 min, the enzymatic reaction was stopped using 2 M sulfuric acid (100 µL/well). The absorbance was measured at 450 nm and the antibody titer was defined as the reciprocal of the dilution that resulted in an absorbance of 0.1. The antibody titer was calculated using the IC₅₀ of the antibody.

**Titrations of the coating antigen and antibody:** Sensitivity was evaluated according to the inhibition rate, and the data were calculated using the IC₅₀ values, which represented the concentration of Rac that produced 50% inhibition of antisera binding to the hapten conjugate. The limit of detection (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition 11. The dynamic range for the icELISA was calculated as the concentration of the analyte providing a 20-80% inhibition rate (IC₂₀-IC₈₀ values) of the maximum signal. Specificity was defined as the ability of structurally related chemicals to bind to the specific antibody. The cross-reactivity was calculated as: (IC₅₀ of Rac)/(IC₅₀ of competitors) x100.

**Matrix effects and spiking experiments:** It is commonly acknowledged that immunoassay performance is often affected by chemical parameters such as ionic strength, pH values, organic solvent concentration, and other substances in the sample matrix 12. The effects of these parameters were estimated by the maximum absorbance (Bₒ, the absorbance value at zero concentration of Rac) and half-maximum inhibition concentration (IC₅₀, the value represents the concentration of Rac that produce 50% inhibition of antibody binding to the hapten).

In order to evaluate the matrix effects, muscle samples from swine and cattle (Xinxiang, China) were homogenized with 20 mL of acetonitrile. The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was extracted into 50 mL glass centrifuge tubes and 20 mL of n-hexane was added. Then the sample was shaken on a shaker for 10 min, and the lower layer was collected for detection. Extracted muscle samples were diluted in PBS (total 2, 5, 10 and 20 fold dilution) before they were applied to the microtiter plate. Bₒ and IC₅₀ values from each diluted curve were compared with that generated from the PBS buffer to determine the appropriate dilution schedule.

Under the optimal dilution programme, the recovery was calculated by interpolation of the mean absorbance values on a standard curve constructed by icELISA in PBS, and accuracy was expressed as the recovery data of the estimated concentration.

**Results and Discussion**

**Antigen synthesis and heterologous detection:** Heterologous system in competitive ELISA is termed to indicate the differences in hapten structure, linker attachment site or bridge character, which usually results in weaker recognition of antibodies to coating antigen compared to target compound, allowing analyte to compete with coating antigen at low concentrations. Therefore, heterology is a proper strategy for the improvement of assay sensitivity in immunoassays. In our study, mixed anhydride method was employed to synthesize the immunogen while 1,4-butanediol diglycidyl ether was used to synthesize the coating antigen, thus produce the heterologous sensitivity. The synthesis procedures are shown in Figs 1 and 2.

**Standard curve of icELISA:** After three subsequent injections, two of the rabbits produced antisera with significant anti-Rac activities. Checkerboard titrations were performed, taking into account the optimal dilutions. The optimal reagent concentrations were determined when the maximum absorbance (A₅₀) was around 1.0 and the dose-response curve of inhibition ratio versus the Rac concentration pursued the lowest IC₅₀ values. From the
Figure 1. Synthesis procedure for Rac immunogen through mixed anhydride method.

**Figure 2.** Synthesis procedure for Rac coating antigen through 1, 4-butanediol diglycidyl ether.

checkerboard assays (data not shown), the optimum concentrations of coating antigen was 0.5 μg/mL and pAb was 1:10,000 dilution. The competitive curve obtained with the icELISA (Fig. 3) allowed the detection of Rac (20-80% inhibition of color development) from 0.006 to 30 ng/mL, with an IC₅₀ value of 0.44 ng/mL. The limit of detection (LOD) of the assay, which is represented by IC₁₅ value, was 0.003 ng/mL.

**Figure 3.** Optimized heterologous icELISA standard curve for Rac. Data were obtained by averaging three independent curves, each run in triplicate.

**Specificity:** Cross-reactions can affect analytical results by either false positives or by elevating the predicted concentration of the target compound when both the target and one or more structurally similar compounds are present. Therefore, the specificity of the antibody toward a compound and its most probable crossreactants should be determined. In this work, the study was undertaken by adding various competitors of functional related analogues. The cross-reactivity rate for each compound is presented in Fig. 4. It can be seen that the established icELISA method was highly specific for Rac and showed negligible cross-reactivity to the other compounds, except for a moderate cross-reactivity (34.5%) to dobutamine.

**Matrix effects and assay parameter determinations:** To determine the matrix effects, swine and cattle muscle were tested. The test was carried out in triplicate with a single batch, and sample values were calculated from the standard curve. Fig. 5 presents effects of the different dilutions of the meat solution on the ELISA.

In swine, the mean Bₜ values for muscle dilutions 1:2, 1:5, 1:10, and 1:20 had absorbance of 0.6832, 0.5217, 0.9845 and 0.9726 (n = 6 per dilution), respectively, compared to 0.9869 for antibody in PBST. The IC₅₀ values, with Rac as the competitor, were 1.36, 0.92, 0.48, and 0.46 ng/mL compared with 0.44 ng/mL in buffer. Because 1:10 dilution caused only a small effect on the assay based on the deviation of the IC₅₀ from PBST, it was recommended to generate the accuracy data. Although the 1:20 dilution showed somewhat less of a matrix effect, the loss of sensitivity caused by this dilution makes it unattractive.

In cattle, the Bₜ values for 1:2, 1:5, 1:10 and 1:20 dilution were 0.7503, 0.8219, 0.9236, and 0.9745, respectively, compared to 0.9816

**Figure 4.** Cross-reactivity of functional related analogues in the Rac immunoassay.

**Figure 5.** Effects of dilution in meat samples: (A) in swine, (B) in cattle. Each solid symbol represents the mean of six replicates. Insets indicate the fluctuation of IC₅₀ values.
for that in PBST. The IC50 were 1.05, 0.77, 0.59, and 0.46 ng/mL compared with 0.44 ng/mL in buffer. Based on the results, 20-fold dilution in cattle was used for the following study.

Validation of the icELISA method: Under the 10-fold dilution in swine and 20-fold dilution in cattle, the accuracy of the analysis was studied by the comparative detection of fortified Rac samples at different concentrations, and measurement correlations were shown in Fig. 6. We can find that the data spots were nearly distributed on both sides of the trendline, the regression equation for this assay in swine was \( y = 0.9831x + 0.3207 \), with a correlation coefficient \( R^2 = 0.9411 \). In cattle, it was \( y = 0.9532x + 1.6268 \) \( R^2 = 0.9623 \). The results demonstrate this ELISA can be used as a screening method for detecting veterinary Rac residues in foodstuffs.

![Concentration vs. Concentration Determined](image)

**Figure 6.** Correlations between concentration spiked and concentration determined in swine samples (A) and in cattle samples (B).

**Conclusions**

In summary, we have prepared a high-quality polyclonal antibody with high specificity for Rac. In all papers describing the production of antibody, the authors have the same general view that selection of the right artificial antigen is the key step for the whole method. Therefore, two different artificial antigen synthesis procedures were employed to expect the heterologous sensitivity. The feasibility to apply this antibody in a competitive ELISA to detect the residues of Rac has been explored and the method is inexpensive, accurate, fast, and simple. It can be concluded that this icELISA has been shown to be capable of detecting Rac residue in muscle, and it also be potentially applied to the analysis of Rac contents in other matrices.

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**References**