Preparation of anti-Ractopamine Polyclonal antibody and the Development of an indirect Competitive Enzyme-Linked Immunosorbent Assay *

Jiang Jinqing, Zhang Haitang, Li Renfeng, Ma Jinyou and Wang Ziliang**

College of Animal Science, Henan Institute of Science and Technology, Xinxiang, Henan, 453003, China
**Corresponding author. E-mail: wangzl_2008@yahoo.com.cn

Abstract—Based on the polyclonal antibodies (pAbs), 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 artificial antigen of Rac-BSA and coating antigen of Rac-OVA; and two female New Zealand white rabbits were used to produce anti-Rac pAb. By the square matrix titration, the icELISA method was developed. The linear range was from 0.006 to 30 ng/mL, with LOD and IC50 value of 0.003 ng/mL and 0.44 ng/mL, respectively. Except for a moderate cross-reactivity (34.5%) to Dobutamine, negligible cross-reactivity to the other compounds was observed. After optimization, 1:10 dilution in swine muscle and 1:20 dilution in cattle muscle produced the satisfactory B0 and IC50 values, 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.

Keywords-Ractopamine; Artificial antigen; Polyclonal antibody; Indirect competitive ELISA

I. 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]. But 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.

Different conventional approaches, carried out on biological fluids were reported for the detection of Rac, including HPLC [5], LC-MS method [6-7], and GC-MS detection [8-9]. These methods are sensitive, but generally require complicated pretreatments and are also time consuming for detection of Rac.

The screening methods are often immunoassays, which provide the advantages of sensitivity, specificity and user-friendly analysis. So several screening methods for Rac have been reported [10-12]. 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 rapid screening indirect competitive ELISA method for monitoring Rac residues in swine and cattle.

II. MATERIALS AND METHODS

A. Materials and Reagents

Ractopamine was obtained from Dr. Ehrenstorfer GmbH (Aubsburg, Germany), while 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 while N-hydroxysuccinimide (NHS) was from Japan, MSDS available. O-(Carboxymethyl) hydroxylamine hemihydrochloride , Succinic anhydride, bovine serum albumin (BSA，MW67 000) and Ovoalbumin (OVA，MW 45 000) were supplied by Sigma while Dialysis bag (8000-14000 Da) was from Solarbio company. GaRIgG-HRP (whole molecule specific) was purchased from Sino-American Biotechnology Company (Shanghai, China). Transparent 96-well polystyrene microtitre plates (Boyang Experimental Equipment Factory, Jiangsu, China) were used for the colorimetric measurement... 3, 3, 5, 5-Tetramethylbenzidine (TMB), phenacetin, urea peroxide were obtained from Sigma Company. All other solvents and reagents were of analytical grade or higher, unless otherwise stated.

The buffers used were as follows: phosphate buffered saline (PBS) consisted of NaCl (137 mM), $Na_2HPO_4.12H_2O$ (10 mM), KCl (2.68 mM) and KH_2PO_4 (1.47 mM), pH 7.4; Carbonate buffer saline (CBS) contained Na_2CO_3 (15 mM), $NaHCO_3$ (35 mM), pH 9.6; Washing buffer consisted of PBS containing 0.05% Tween-20; Blocking buffer contained BSA (1%, w/v) in PBS buffer. Assay buffer consisted of adding part a (500 mL) and part B (500 mL) solutions together. Part A contained (per 1 L of water) 3.15 g of Citric acid, 6.966 g of Anhydrous sodium acetate, 0.08 g Phenacetin and 0.05 g of Urea peroxide adjusted to pH 5 with HCl. Part B contained 1.27 g Tetramethylbenzidine (TMB) dissolved in

500 mL of Methanol and 500 mL of Glycerol. The stopping solution was 2 M H_2SO_4.

B. Synthesis of Immunogen and Coating Antigen

The Rac-BSA and Rac-OVA were prepared as described previously with some modifications [13]. Briefly, Ractopamine HCl was reacted with glutarate anhydride in the presence of pyridine. The reaction was stirred for 30 h at room temperature under a nitrogen atmosphere. Antigen was formed by dissolving Rac hemiglutarate in dimethylformanide/1,4-dioxane (v/v, 1:1) and adding tributylamine. The mixture was stirred on ice for 10 min, isobutylchloroformate was added, and the reaction was brought to room temperature and stirred for 1 h. The mixture was added dropwise to an ice-cold protein solution. The antigen was finally purified by PBS (pH, 7.4), and stored at -20℃ in refrigerator.

C. Immunization

Immunogenic emulsions were injected subcutaneously into four sites on the Female New Zealand white rabbits. FCA was employed in the first immunization and FIA was used in the subsequent boost injections. Rabbits were immunized every 28 days with 500 μg of immunogen, and blood samples were taken from the marginal vein of the ear 10 days after each immunization (from the third immunization onward).

D. Generic ELISA Procedures

The microplates were coated with coating antigen in CBS (100 μL/well) by overnight incubation at 4 ℃. 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 ℃. After another washing procedure, GaRIgG-HRP (50 μL/well) was added, followed by incubation for 25 min at 37 ℃. The final washing procedure was followed by a color development, which was initiated by adding 60 μL/well of freshly prepared HRP substrate solution. After incubating at room temperature for 25 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 value that was twice higher than that of the background.

The icELISA procedure was the same as the indirect ELISA described above except that after blocking, a competition step was introduced by adding 50 μL/well of analyte, followed by 50 μL/well of appropriate concentration of antibody. With the icELISA format, analytes that do not react with the antibody would produce absorbance near 100%; conversely, analytes that do react with the antibody would decrease in percentage of absorbance. All samples were run in triplicates, and competition curves were obtained by plotting absorbance against the logarithm of analyte concentrations, which were fitted to a four-parameter logistic equation.

E. Establishment of icELISA Standard Curve

Titration Checkerboard tests were performed to determine optimal dilution of the coating antigen and the antibody. Sensitivity was evaluated according to the inhibition rate, and the data were calculated using the IC_{50} values, which represented the concentration of Rac that produced 50% inhibition of antiserum binding to the hapten conjugate. The detection of limit (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition [14]. The dynamic range for the icELISA was calculated as the concentration of the analyte providing a 20–80% inhibition rate (IC_{20}–IC_{80} 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: (IC_{50} of Rac)/ (IC_{50} of competitors) ×100.

F. 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. The effects of these parameters were estimated by the maximum absorbance (B0, the absorbance value at zero concentration of Rac) and half-maximum inhibition concentration (IC_{50}, 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. B0 and IC_{50} 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 recoveries were 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.

III. RESULTS AND DISCUSSIONS

A. Immunogen Synthesis

With a molecular mass of 337.85, Rac is not able to stimulate the immune response for anti-Rac antibody production. To make it immunogenic, it must be conjugated to a carrier protein before immunization. The synthetic scheme is shown in **Fig.1**. As can be seen, through mixed anhydride method, the immunogen and coating antigen can be prepared by conjugation of the carboxylic acid group and an amino group of a carrier protein, thus produce a 5-C bridge. With this advantage, the single amide linkage produces satisfactory immunogenic effects.
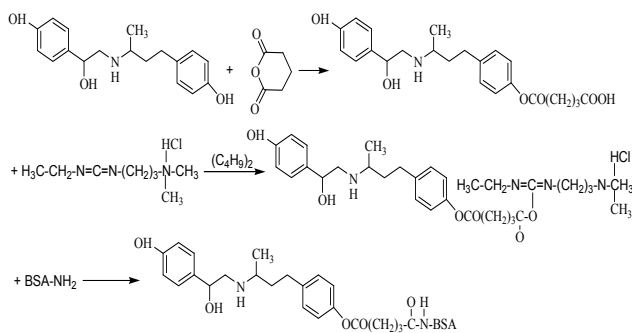
Figure 1. Synthesis procedure for Rac immunogen through mixed anhydride method.

B. Standard Curve of icELISA

After three subsequent injections, two of the rabbits produced antisera with significant anti-Rac activities (data not shown). Checkerboard titrations were performed, taking into account the optimal dilutions. The optimal reagent concentrations were determined when the maximum absorbance (A_{max}) was around 1.0, and the dose-response curve of inhibition ratio versus the logarithm of Rac concentration pursued the lowest IC_{50} values. From the 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. 2) allowed the detection of Rac (20-80% inhibition of color development) from 0.006 to 30 ng/mL, with an IC_{50} value of 0.44 ng/mL. The limit of detection (LOD) of the assay, which is represented by IC_{15} value, was 0.003 ng/mL.
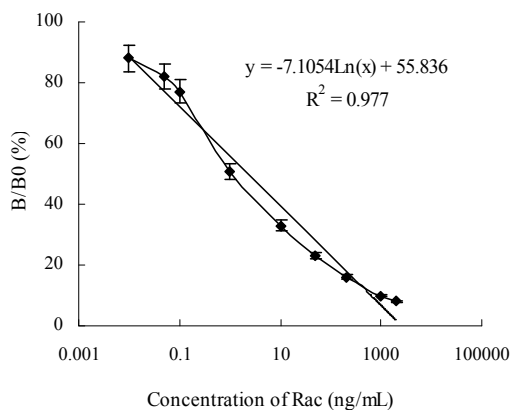

$$y = -7.1054 \text{Ln}(x) + 55.836$$
$$R^2 = 0.977$$

Figure 2. Standard inhibition curve of the optimized icELISA for Rac. Coating antigen, 0.5 μg/mL; pAb produced from the immunogen of Rac-BSA, 1:10,000 dilution; goat anti-rabbit IgG (GaRIgG-HRP), 1:1000. The standard curve was obtained by normalized absorbance values (B/B$_0$%), where B_0 is the absorbance at zero concentration of Rac and B is the absorbance of Rac. Data represent the means of three determinations.

A. Specificity

Crossreactions 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. 3. 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.
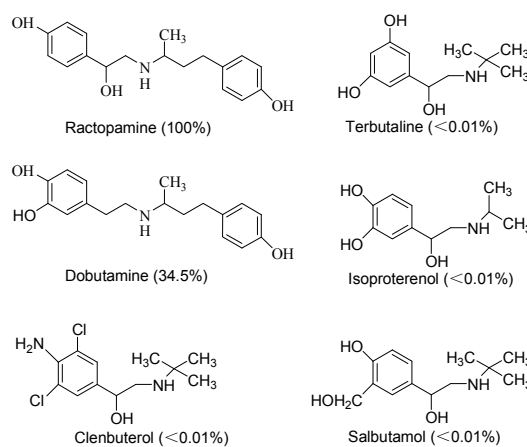

Figure 3. Cross-reactivity of functional related analogues in the Rac immunoassay.

B. 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. 4 presents the effects of the different dilutions of the meat solution on the ELISA.

In swine, the mean B0 values for muscle dilutions 1:2, 1:5, 1:10, and 1:20 had absorbances of 0.6832, 0.5217, 0.9845, and 0.9726 (n=6 per dilution), respectively, compared to 0.9869 for antibody in PBST. The IC_{50} 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 IC_{50} 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 B0 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 for that in PBST. The IC_{50} 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.
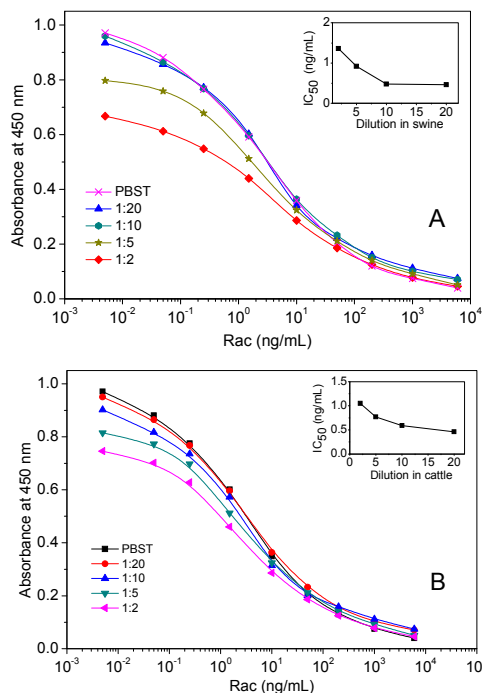
Figure 4. Effects of dilution in meat samples (A) in swine; (B) in cattle. Each solid symbol represents the mean of six replicates. Insets indicate the fluctuation of IC_{50} values.

C. 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 the measurement correlations were shown in Fig. 5. 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 = 0.9831 x + 0.3207, with a correlation coefficient (R^2) of 0.9411. In cattle, it was y = 0.9532 x + 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.
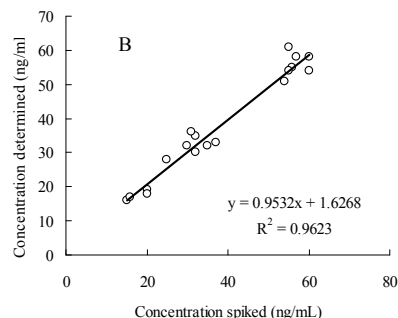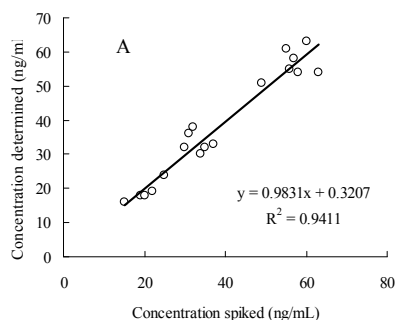

Figure 5. Correlations between concentration spiked and concentration determined in swine samples (A); in cattle samples (B).

REFERENCES

[1] P. Zuo, Y. Zhang, J. Liu and B. C. Ye, Determination of beta-adrenergic agonists by hapten microarray. *Talanta*, 2010, 82(1):61-66.

[2] L. C. Dickson, J. D. Macneil, S. Lee and A. C. Fesser, Determination of beta-agonist residues in bovine urine using liquid chromatography-tandem mass spectrometry. *J AOAC Int*, 2005, 88(1):46-56.

[3] G. A. Mitchell and G. Dunnavan, Illegal use of beta-adrenergic agonists in the United States. *J Anim Sci*, 1998, 76(1):208-211.

[4] X. Liu, X. He, C. Moore, G. Wang and C. Coulter, Highly sensitive and specific liquid chromatography-tandem mass spectrometry method for testing ractopamine in cow and sheep urine. *J Anal Toxicol*, 2009, 33(6):289-193.

[5] D. J. Smith, V. J. Feil, J. K. Huwe and G. D. Paulson, Metabolism and disposition of ractopamine hydrochloride by turkey poults. *Drug Metab Dispos*, 1993, 21(4):624-633.

[6] M. P. Turberg, T. D. Macy, J. J. Lewis and M. R. Coleman, Determination of ractopamine hydrochloride in swine, cattle, and turkey feeds by liquid chromatography with coulometric detection. *J AOAC Int*, 1994, 77(4):840-847.

[7] T. Sakai, T. Hitomi, K. Sugaya, S. Kai, M. Murayama and T. Maitani, Determination method for ractopamine in swine and cattle tissues using LC/MS. *Shokuhin Eiseigaku Zasshi*, 2007, 48(5):144-147.

[8] J. P. Wang, X. W. Li, W. Zhang and J. Z. Shen, Development of immunoaffinity sample-purification for GC-MS analysis of ractopamine in swine tissue. *Chromatographic*, 2006, 64(9):613-617.

[9] B. Bocca, M. Fiori, C. Cartoni and G. Brambilla, Simultaneous determination of Zilpaterol and other beta agonists in calf eye by gas chromatography/tandem mass spectrometry. *J AOAC Int*, 2003, 86(1):8-14.

[10] W. L. Shelver, D. J. Smith and E. S. Berry, Production and characterization of a monoclonal antibody against the beta-adrenergic agonist ractopamine. *J Agric Food Chem*, 2000, 48(9):4020-4026.

[11] W. L. Shelver and D. J. Smith, Application of a monoclonal antibody-based enzyme-linked immunosorbent assay for the determination of ractopamine in incurred samples from food animals. *J Agric Food Chem*, 2002, 50(10):2742-2747.

[12] M. Z. Zhang, M. Z. Wang, Z. L. Chen, J. H. Fang, M. M. Fang, J. Liu and X. P. Yu, Development of a colloidal gold-based lateral-flow immunoassay for the rapid simultaneous detection of clenbuterol and ractopamine in swine urine. *Anal Bioanal Chem*, 2009, 395(8):2591-2599.

[13] X. Li, G. Zhang, R. Deng, Y. Yang, Q. Liu, Z. Xiao, et al., Development of rapid immunoassays for the detection of ractopamine in swine urine. *Food Addit Contam*, 2010, 27(8):1096-1103.

[14] L. Wang, Y. Zhang, X. Gao, Z. Duan and S. Wang, Determination of chloramphenicol residues in milk by enzyme-linked immunosorbent assay: improvement by biotin-streptavidin-amplified system. *J Agric Food Chem*, 2010, 58(6):3265-3270.