Preparation and Identification of anti-Ractopamine Monoclonal antibodies *

Jiang Jinqing, Zhang Haitang 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: This study aimed to prepare high-sensitivity monoclonal antibodies against Ractopamine (Rac), which provided a solid foundation for icELISA kit. Mixed anhydride method was employed to synthesize the immunogen of Rac-BSA and 1,4-butanediol diglycidyl ether was used to prepare the coating antigen of Rac-OVA, thus pursue the heterologous sensitivity. Through cell fusion technology, four Hybridoma cell lines named R1-B5, R2-B3, R2-C6, and R4-C8 were screened out, their corresponding mAbs were of the IgG1 isotype with k light chain, and the Kafs of all mAbs were between 2.7 and 4.8×10^9 L/mol. Based on the R1-B5, a heterologous icELISA method was developed. The working range was from 0.013 to 33.7 ng/mL, with LOD and IC_{50} value of 0.007 ng/mL and 0.67 ng/mL, respectively. Except for a higher cross-reactivity (42.7%) to Dobutamine, negligible cross-reactivity to the other compounds was observed. The recoveries of Rac were in the range of 98.2-109.5%, 85.7-110.5% and 97.4-101.8% for cattle muscle, liver and kidney, respectively, with coefficients of variation (CV) values all < 10%. When applied in real samples, the correlation coefficient (R2) of the ELISA and LC-MS was 0.9373 in cattle muscle. Therefore, this assay can be used for detecting Rac residue in animal products.

Keywords-Ractopamine; Artificial antigen; Monoclonal antibody; Indirect competitive ELISA; Heterologous

I. INTRODUCTION

Ractopamine (Rac) is a phenethanolamine β- agonist that enhances animal growth by inhibiting fat synthesis, stimulating lipolysis, increasing protein synthesis, and reducing protein breakdown in muscle [1]. Recently, Rac has been approved as a feed additive for swine by the US FDA [2], but in other regions such as the European Union and China, the drug has been officially banned for use in animals as growth-promoting agents.

Therefore, there have been an increasing number of analytical methods reported to monitor Rac residue in animal urine, feeds, and tissues such as instrument methods like HPLC [3], LC-MS method [4-5], and GC-MS detection [6-7]. 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 [1, 8-10]. 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 monoclonal antibodies for the determination of Rac residues in edible animal foods, including chicken muscle. In our study, high-sensitivity monoclonal antibodies displaying excellent affinity and specificity towards Rac were produced, and an indirect competitive ELISA protocol based on selected monoclonal antibody has been developed. This work potentially optimizes the pre-treatment procedures for LC-MS and GC-MS detection, lay a solid foundation for Rac-kit and test strip development.

II. MATERIALS AND METHODS

A. Materials and Chemicals

Ractopamine was obtained from Dr. Ehrenstorfer GmbH (Aubsburg, Germany), while Clenbuterol, Dobutamine, Terbutaline, Isoproterenol, and Salbutamol were purchased from Sigma (St. Louis, Mo.). FCA and FIA were obtained from Pierce. GaMIgG-HRP 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. HAT and HT were obtained from Sigma-Aldrich (USA). RPMI-1640 with L-glutamine was obtained from Gibco. Polyethylene glycol 1500 (PEG 1500, 50%) was from Roche Diagnostics Corporation (Indianapolis, USA). Fetal bovine serum (FBS) was from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China). Cell culture plates were obtained from Costar Inc. (Bethesda, MD, USA). A mouse monoclonal antibody isotyping kit was purchased from Pierce Biotechnology, Inc. (Rockford, II, USA). TMB, phenacetin, urea peroxide were obtained from Sigma Company. All other solvents and reagents were of analytical grade or higher, unless otherwise stated.

B. Instruments

A spectrophotometric microtitre reader (MULTISKAN MK3, Thermo company, USA), provided with a 450 nm filter, was used for absorbance measurements. A GS15R high speed refrigerated centrifuge were supplied by Thermo Company (USA). CO_2 incubator from RS-Biotech (Galaxy S+, UK) was used for cell cultivation. SW-CJ-2FD Superclean Bench was purchased from Suzhou purification equipment Co., Ltd (Suzhou, China). Inverted microscope (TS100-F, Nikon Company, Japan) was used for cell observation.

C. Synthesis of immunogen and coating antigen

The immunogen of Rac-BSA was prepared as described previously with some modifications [11]. Briefly, Rac (85 mg) and glutaric anhydride (32 mg) were added to 2 mL pyridine. The mixture was stirred overnight at room temperature and the mixture was dried under a nitrogen atmosphere. A total of 5 mL of a solution of dimethylformamide:dioxane:triethylamine (v/v/v, 40:40:1) was added and stirred for 15 min at 0℃. 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℃ in refrigerator.

The coating antigen of Rac-OVA was synthesized according to the Li et al. [12].

D. Immunization

Ten BALB/c female mice were immunized with Rac-BSA conjugates by subcutaneously injections. The first dose consisted of 60 μg of immunogen as an emulsion of PBS and FCA. Three subsequent injections were given at 3-week intervals with the same dosage of immunogen emulsified in FIA. After a resting period of at least 3 weeks from the last injection, Mice were tail-bled and screened for anti-Rac activity. The mouse showing the highest anti-Rac activity received a final soluble intraperitoneal (ip) injection of 100 μg of conjugate in PBS, 3-4 days prior to cell fusion.

E. Cell fusion and mAbs production

The mouse (that received the booster injection) was sacrificed by cervical dislocation, and the splenocytes were isolated and fused with myeloma cells at a 10:1 ratio using PEG 1500. The fused cells were then distributed onto 96-well culture plates, in which mouse peritoneal macrophages were prepared on the day before the fusion and were grown with the selective HAT medium. Supernatants of hybridoma colonies were screened using indirect competitive ELISA, and positive hybridomas cells were cultured using limiting dilution method. To produce anti-Rac mAbs, female BALB/c mice were injected intraperitoneally (i.p.) with 0.5 mL of paraffin 10 days before receiving an i.p. injection of the positive hybridoma cells suspended in RPMI 1640 medium. Ascites fluid was collected 10-14 days after the injection and then stored at –20 °C until use.

F. Characterization of mAbs

Purification of mAb was performed according to the modified caprylic acid ammonium sulphate precipitation (CAASP) method described before [13]. The protein content was determined according to the following formula: protein concentration (mg/mL) $= 1.45 OD_{280nm} - 1.74 OD_{260nm}$, where OD value is the optical density. Measurement of monoclonal antibody affinity (Ka) was carried out according to the procedure described by Wang et al. [14]. The class and subclass of the isotypes of the purified antibody were determined by using a mouse monoclonal antibody isotyping kit. The IC_{50} values were calculated to determine the sensitivity, which represent the concentration of Rac that produced 50% inhibition of antibody binding to the hapten conjugate. Specificity was defined as the ability of structurally related chemicals to bind to the specific antibody and cross-reactivity (CR) was calculated as: (IC_{50} of Rac)/ (IC_{50} of chemicals) ×100.

G. LC-MS determination in real cattle muscles

The detection conditions were followed the reference [15]. A finely chopped cattle sample (10 g) was weighed into a 50 mL centrifuge tube. Then 20 mL of ethyl acetate and 1 mL of potassium carbonate solution were added, and the sample was homogenized for 2 min and centrifuged for 10 min at 3000 rpm. The resulting ethyl acetate solution was evaporated to dryness, and the residue was dissolved in 30 mL of acetonitrile. The acetonitrile solution was vigorously shaken twice with 20 mL of n-hexane. The resulting acetonitrile solution was evaporated to dryness, and the residue was redissolved in 1.0 mL of methanol.

The LC-MS analysis were performed on a SURVEYOR liquid chromatograph (Thermo company, USA), equipped with a PDA plus Detector and a Thermo ODS-HYPERSIL column (3 μm, 2.1 ×150 mm). The mobile solution was 100% methanol at a constant temperature of 25℃ and a flow rate of 0.4 mL/min. Mass spectrometry was performed using a mass spectrometer equipped with a TurboIonSpray ESI source (LCQ Deca XP MAX, Finnigan Company, USA). A pseudo-molecular ion $[M+H]^+$ was selected as the parent ion, and the injection volume was 10 μL.

III. RESULTS AND DISCUSSIONS.

A. 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 [16]. 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 **Fig.1 and 2**.
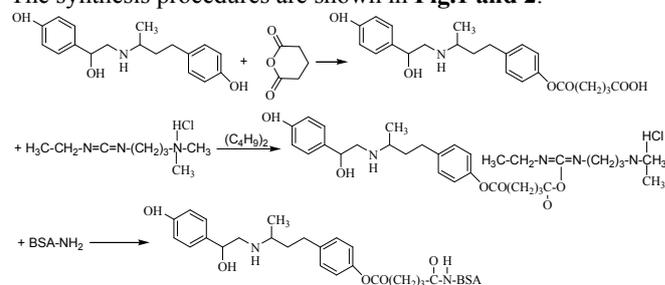

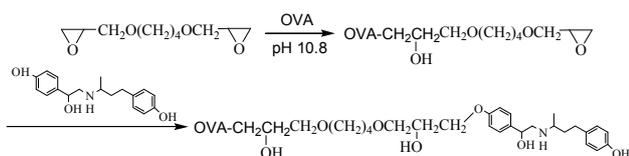Fig. 1. Synthesis procedure for Rac immunogen through mixed anhydride method.

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

B. Production and characterization of mAbs

Ten days following the fusion, growing hybridoma cell clones could be observed in many wells of the seeded 96-well plates, and the fusion rate was about 75%. Supernatants of all wells were screened by simultaneous noncompetitive and competitive assays, and the positive well rate was 22%. Selection from these positive clones by limiting dilution led to four stable hybridoma cell lines, which were named R1-B5, R2-B3, R2-C6, and R4-C8. Using a mouse monoclonal antibody isotyping kit, all four antibodies were of the IgG_1 isotype with k light chain (**Fig. 3**).
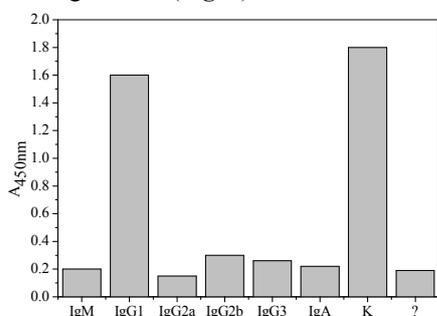

Fig. 3. Identification of Ig class and subclass of Rac mAbs.

The mAbs from ascite liquids were purified and characterized. After calculation, the protein concentrations of all mAbs were between 2.1–3.6 g/mL. The affinity constant produced by each of the 4 selected hybridomas could be measured by ELISA using serial dilutions of both coated antigen and Rac mAb. As in **Fig. 4**, the Kafs of R1-B5, R2-B3, R2-C6 and R4-C8 mAb were 4.8×10^9, 4.1×10^9, 3.8×10^9 and 2.7×10^9 L/mol respectively.
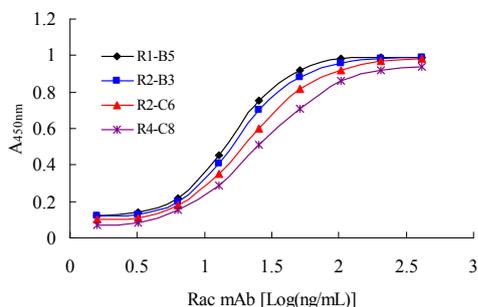

Fig. 4. Affinity constant (Kaf) curves of Rac mAbs.

The affinity constant (Kaf) plays an important effect upon the quality of mAb. It demonstrates the conjugating ability between antibody (Ab) and hapten, or one antigen

determinant, and is a significant signal of antibody stability. According to James [17], the four hybridomas produced in this study all have high-affinity antibodies (between 10^7 and 10^{12} L/mol).

C. Characterization of heterologous icELISA curve

Based on the R1-B5 mAb, a heterologous icELISA standard curve was developed. The results are shown in **Fig. 5**. From the checkerboard assays (data not shown), the optimum concentration of coating antigen was 1 μg/mL and mAb was 1:50,000 dilutions. The IC_{50} value, which represented the concentration of Rac that produced 50% inhibition of antiserum binding to the hapten conjugate, was 0.67 ng/mL; the working range for the icELISA (calculated as the concentration of the analyte providing a 20–80% inhibition rate) was from 0.013 to 33.7 ng/mL; the detection of limit (LOD), which defined as the lowest concentration that exhibits a signal of 15% inhibition, was 0.007 ng/mL.
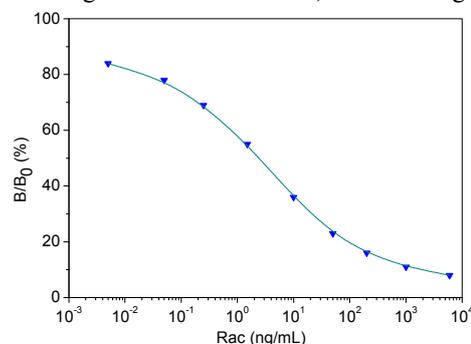

Fig. 5. Optimized heterologous icELISA standard curve for Rac. Data were obtained by averaging three independent curves, each run in triplicate. Rac-OVA (1 μg/mL) as coating antigen was prepared in CBS (pH 9.6), mAb originated from R1-B5 was diluted 1:50,000 in PBS (pH 7.4), Rac was prepared in PBS, containing 10% methanol, GaMIgG-HRP was diluted 1:1000 in incubation buffer. y = -7.65Ln (x) + 53.098 R2=0.9608.

D. Specificity

Specificity was evaluated by determination of the cross-reactivity based on the IC_{50} values. In this work, the study was undertaken by adding various functional related analogues. The cross-reactivity rate for each compound is presented in **Table 1**.

TABLE 1. CROSS-REACTIVITIES OF RELATED FUNCTIONAL ANALOGUES IN THE RAC IMMUNOASSAY.

Analogues	IC_{50} (ng/mL)	CR (%)
Ractopamine	0.67	100
Dobutamine	1.57	42.7
Clenbuterol	＞1340	＜0.05
Terbutaline	＞1340	＜0.05
Isoproterenol	＞6700	＜0.01
Salbutamol	＞6700	＜0.01

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 higher cross-reactivity (42.7%) to Dobutamine.

E. Recovery studies in cattle sample

To determine the accuracy, cattle samples (muscle, liver, kidney) containing 2, 5, and 10 μg /kg of Rac were tested. After extraction, the spiked concentrations corresponded to the concentration in the assay solutions of 0.1, 0.25, and 0.5 ng/mL, respectively. The test was carried out in triplicate, and the values were calculated from the standard curve. **Table 2** shows the recovery results under different conditions. The recoveries of Rac were in the range of 98.2-109.5%, 85.7-110.5% and 97.4-101.8% for cattle muscle, liver and kidney, respectively. The coefficients of variation (CV) values were all ＜10%, which also demonstrate the high accuracy of the ELISA method.

TABLE 2. RECOVERIES OF CATTLE SAMPLES FORTIFIED WITH DIFFERENT CONCENTRATIONS OF RAC (N = 6).

Cattle samples	Fortified levels (μg/kg)	Theoretical levels (ng/mL)	Recovery (%)	CV (%)
	2	0.1	98.2	8.3
Muscle	5	0.25	109.5	9.8
	10	0.5	102.3	7.8
	2	0.1	110.5	7.6
Liver	5	0.25	85.7	6.5
	10	0.5	98.2	8.2
	2	0.1	97.4	9.2
Kidney	5	0.25	101.8	9.6
	10	0.5	100.9	8.2

F. Correlation between ELISA and LC-MS analysis

To further evaluate the validity of the established icELISA method, all real cattle samples were analyzed using both the ELISA and LC-MS to obtain a direct comparison. **Fig. 6** displays the performance of the ELISA in comparison with the LC-MS method, and the correlation between the two group data. We can find that the data obtained from these two methods are very similar and with no significant differences. The correlation coefficient (R^2) of the ELISA and LC-MS data was 0.9373 in muscle. The results demonstrate this ELISA can be used as a screening method for detecting veterinary Rac residues in foodstuffs.
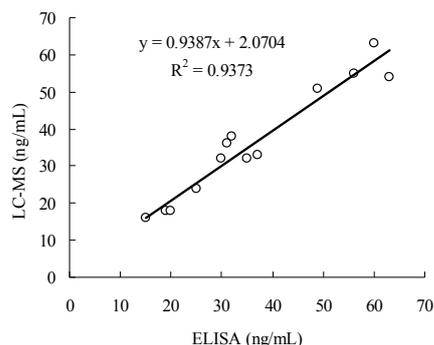

Fig. 6. Correlation between icELISA and the confirmatory LC-MS method in real cattle muscle samples.

REFERENCES

[1] J. P. Wang, S. X. Zhang and J. Z. Shen, Technical note: a monoclonal antibody-based immunoassay for determination of ractopamine in swine feeds. J Anim Sci, 2006, 84(5):1248-1251.

[2] FDA, "New animal drugs for use in animal feeds; ractopamine hydrochloride," Federal Register, 2000, 65:4111-4112.

[3] 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.

[4] 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.

[5] 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.

[6] 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.

[7] 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.

[8] 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.

[9] 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.

[10] 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.

[11] 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.

[12] M. Li, Z. Chen and Q. Zhu, Development and identification of monoclonal antibodies against ractopamine. Hybridoma (Larchmt), 2007, 26(3):148-154.

[13] Z. Wang, Y. Zhu, S. Ding, F. He, R. C. Beier, J. Li, et al., Development of a monoclonal antibody-based broad-specificity ELISA for fluoroquinolone antibiotics in foods and molecular modeling studies of cross-reactive compounds. Anal Chem, 2007, 79(12):4471-4483.

[14] Y. C. Wang, Z. Q. Guo, Y. Z. Li and W. B. Chang, Production and characterization of anti-estrone monoclonal antibody. Biomed Environ Sci, 2002, 15(2):103-112.

[15] 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.

[16] Y. Liang, X. J. Liu, Y. Liu, X. Y. Yu, and M. T. Fan, Synthesis of three haptens for the class-specific immunoassay of O,O-dimethyl organophosphorus pesticides and effect of hapten heterology on immunoassay sensitivity. Anal Chim Acta, 2008, 615(2):174-183.

[17] W. G. James, Monoclonal antibodies: Principles and Practice. New York: Academic Press, 1983, pp. 142-147.