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Full Paper

Production of monoclonal antibodies for ractopamine residue detection in pork

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Abstract: Ractopamine (RAC) is used as a feed additive to deposit fat and improve the growth rate of food-producing animals. Because RAC residues in meat are unhealthy for consumers, RAC has been banned in many countries and its routine screening is now essential. We aim to generate highly sensitive and specific anti-RAC monoclonal antibodies (mAb) for detecting RAC residues in pork. By means of the hybridoma technique, BALB/c mice were immunised with RAC conjugated with bovine serum albumin. Indirect and competitive enzyme-linked immunosorbent assay (ELISA) identified five hybridoma clones that secrete anti-RAC antibodies. The mAb 10A4 which shows the highest sensitivity was used in an indirect competitive ELISA in a biotin-streptavidin system, which demonstrates 50% inhibition at 0.3 ng/mL and shows detection and quantitation limits at 0.07 ng/mL and 0.13 ng/mL respectively. To evaluate this method, the pork was fortified with RAC at different concentrations. The recovery and coefficient of variation ranges in the intra-variation assay were 91-97% and 3.2-6.7% respectively, whereas those in the inter-variation assay were 90-99% and 6.4-9.9% respectively. The quantification based on the linear range of RAC standard was 0.05-5.0 ng/mL. The anti-RAC mAb generated could be potentially useful for RAC residue detection in pork using the indirect competitive ELISA with the biotin-streptavidin system.

Keywords: ractopamine, beta agonist, monoclonal antibody, veterinary drug residue, pork

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INTRODUCTION

Ractopamine (RAC), 4-[3-{2-hydroxy-2-(4-hydroxyphenyl)ethylamino}butyl]phenol (Figure 1) is a synthetic beta agonist. It is well known as a 'red meat promoter' or 'lean meat agent' because it increases muscle mass and promotes weight gain in livestock [1, 2]. RAC has been approved as a feed additive in many countries including the US, Canada, Japan and Mexico [1]. However, human consumption of meat or by-products from animals fed with RAC may result in undesirable side effects such as headache, muscle spasms and high arterial blood pressure [1]. Therefore, the European Union, China and Thailand prohibit RAC use in food-producing animals except for therapeutic use under direct veterinary supervision [1]. Because of human safety concerns, the Codex Alimentarius Commission on food additives has set the maximum residue limits (MRLs) at 10 ppb in pork and beef, 40 ppb in liver and 90 ppb in kidney [3]. Moreover, a heavy financial loss may result if RAC residue is detected in food intended for export.

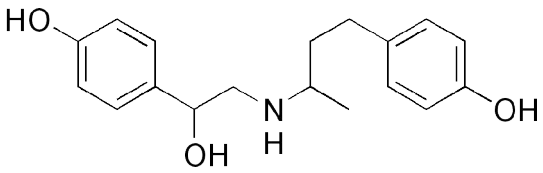

Figure 1. Structure of RAC

As a result, various methods for RAC residue detection have been investigated. Chemical-based methods, such as high performance liquid chromatography, gas chromatography - mass spectrometry and liquid chromatography - mass spectrometry/mass spectrometry, are conventionally used as confirmation methods as they have high accuracy and precision of detection [4-6]. However, these tests are time-consuming and require instruments that are expensive to maintain as well as well-trained personnel to operate. They are not suitable for screening a large number of samples at once. Alternative detection methods based on immunoassay have also been studied. Many of these studies have shown that the enzyme-linked immunosorbent assay (ELISA) is suitable as a screening method for drug residue in food because of its high specificity and sensitivity, low cost and short analysis time. Importantly, it requires only an ELISA reader. A polyclonal antibody (pAb) has been produced to detect RAC in pig liver by an indirect competitive ELISA [7, 8] and in pig muscle by ELISA [9]. A monoclonal antibody (mAb) has also been developed for detecting RAC in animal feed [10], swine urine [11] and cattle, sheep and duck tissues by ELISA [12-13].

This study investigates the preparation of a highly specific mAb against RAC and the development of an indirect competitive ELISA using the biotin-streptavidin system. Stable hybridomas have been successfully generated that produce mAb suitable for RAC residue detection in pork.

MATERIALS AND METHODS

Chemicals, Reagents, Materials and Animals

RAC hydrochloride, bovine serum albumin (BSA), ovalbumin (OVA), succinic anhydride, pyridine, N,N-dimethylformamide, dioxane, tributylamine, isobutylchloroformate, sodium borate decahydrate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium bicarbonate, sodium chloride, skim milk, Tween 20, potassium citrate tribasic monohydrate, citric acid, Trizma^{R}

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hydrochloride, complete and incomplete Freund's adjuvant, polyethylene glycol (MW 3,000-3,700 Da), 3,3′,5,5′-tetramethylbenzidine, 99.5% dimethyl fulfoxide, 30% hydrogen peroxide, 95-98% sulfuric acid, 35-37% hydrochloric acid, sodium hydroxide, aminohexanoylbiotin N-hydroxysuccinimide, hypoxanthine, aminopterin, thymidine and an isotyping kit were purchased from Sigma (USA). A tested beta-agonist was provided by the National Food Institute, Thailand. Rosewell Park Memorial Institute medium (RPMI 1640) and fetal bovine serum (FBS) were obtained from Biochrom AG (Germany). Goat anti-mouse IgG-horseradish peroxidase was purchased from Jackson Immuno Research Laboratories (USA). Streptavidin-horseradish peroxidase was obtained from Invitrogen (USA). A Protein G Sepharose was purchased from GE Healthcare Life Sciences. A BCA^{TM} protein assay kit was purchased from Pierce (USA), and 96-well ELISA culture plates and culture dishes were purchased from Corning (USA). Dialysis membrane (MW cut-off, 12,000-14,000 Da) was obtained from Membrane Filtration Products (USA).

Female BALB/c mice (8 weeks old) were purchased from National Laboratory Animal Centre (Mahidol University, Salaya, Thailand).

Methods

Synthesis of RAC conjugates

RAC hydrochloride was conjugated to BSA for immunisation and to OVA for ELISA. RAC-hemisuccinate was first synthesised by mixing RAC (0.1 mmol) and succinic anhydride (0.1 mmol) in pyridine (2 mL). The reaction was stirred overnight at room temperature. Pyridine was then evaporated under a stream of nitrogen. The obtained RAC-hemisuccinate was dissolved in N,N-dimethylformamide: 1,4-dioxane (1:1, 4 mL) and tributylamine (26.2 µL, 0.11 mmol) was added. The solution was stirred on ice for 10 min. and isobutylchloroformate (0.11 mmol) was added. The mixture was stirred for 1 hr at room temperature and added dropwise into an ice-cold BSA solution (100 mg BSA in 0.1M sodium borate, pH 8.5). The resulting solution (RAC-BSA) was brought to room temperature and allowed to react overnight. It was then dialysed in 0.1M phosphate-buffered saline (PBS), pH 7.4. OVA was conjugated to RAC using the same method as the RAC-BSA conjugate procedure [14]. The conjugated ratio was checked using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) at the Bioservice Unit of the National Centre for Genetic Engineering and Biotechnology, Thailand.

Immunisation

Female 8-week-old BALB/c mice were immunised intraperitoneally with RAC-BSA conjugate (100 µg) in sterile PBS (100 µL) emulsified with an equal volume of complete Freund's adjuvant. The mice were subsequently immunised at 2-week intervals with incomplete Freund's adjuvant. After 3-4 boosts, antibody titers were determined by indirect ELISA using RAC-OVA as the coating antigen and the ability of the antibody to bind to the free form of RAC was tested using indirect competitive ELISA as described below. The mice were finally boosted 3-4 days before hybridoma generation. All procedures involving laboratory animals were approved (approval no. 0961008) and conducted according to the guidelines of the Institutional Animal Care and Use Committees of the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University.

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Production of hybridomas

To generate hybridomas, a selected mouse was euthanised. The spleen was removed and splenocytes were harvested. The P3/NS-1/1-Ag4-1 (NS-1) myeloma cells were grown in RPMI 1640 supplemented with 20% FBS and collected. NS-1 myelomas were then fused with splenocytes at 1:2 ratio of pellet cells using polyethylene glycol as the fusogen. The fused cells were cultured in hypoxanthine-aminopterin-thymidine medium (RPMI 1640 supplemented with 20% FBS containing 10μM hypoxanthine, 0.4μM aminopterine and 16μM thymidine) and placed into 96-well culture plates (10 plates, 200 μL/well). The plates were incubated at 37°C and 5% carbon dioxide. After culturing in hypoxanthine-aminopterin-thymidine medium for 7 days, the medium was replaced with hypoxanthine-thymidine medium without aminopterine (200 μL/well). After 10-14 days, only the splenocyte-fused myeloma cells (hybridomas) had grown. Culture supernatants from each well were then screened for antibody against RAC by indirect ELISA and competitive ELISA as described below. The hybridomas that produced the antibody against RAC were selected and subsequently subcloned at least twice by a limiting dilution method until monoclones were obtained and expanded for storage in liquid nitrogen.

Screening for hybridomas by ELISA

Indirect ELISA was used in the first screening for antibody-producing hybridomas. Plates were coated with 100 μL/well of RAC-OVA (10 μg/mL dissolved in PBS overnight at 4°C). They were washed 3 times with 0.05% Tween 20 in PBS and blocked in a blocking solution (5% skim milk in PBS, 300 μL/well) for 1 hr at room temperature. After washing, the culture supernatant or serum (100 μL/well) was added, and the plates were incubated at 37°C for 2 hr. After washing three times, Goat anti-mouse IgG-horseradish peroxidase (1:10,000 in PBS, 100 μL/well) was added and the plates were incubated at 37°C for 1 hr. After washing, the substrate solution (prepared by dissolving 2.5 mg of 3,3,5,5-tetramethylbenzidine in 250 μL of dimethyl sulfoxide and adding the resulting solution to 10 mL of 205mM potassium citrate buffer, pH 4.0, containing 3 μL of 30% hydrogen peroxide) was added (100 μL/well). The reaction was allowed to develop in the dark for 10 min. and stopped with 100 μL of 1M sulfuric acid. The absorbance was measured at 450 nm using a microplate reader. Positive clones were further screened by indirect competitive ELISA.

For indirect competitive ELISA, plates were coated, blocked and washed in the same manner as for indirect ELISA procedure except that differing concentrations of RAC (50 μL as competitor) and subsequent culture supernatant or serum (50 μL) were added. The assays were carried out as stated previously. The isotype of the selected mAb was identified by sandwich ELISA using an isotyping kit.

Characterisation of mAbs

The sensitivity of the mAb was defined in terms of inhibition concentration at 50% (IC_{50}) and limit of detection (LOD). The IC_{50} was calculated using the following formula:

$$IC_{50} = 50\% (B/B_0)$$

where B and B_0 are the average absorbances obtained from the indirect competitive ELISA with and without various concentrations of RAC respectively.

The LOD was defined as the concentration corresponding to 3 standard deviations above the mean, and the limit of quantitation was defined as the concentration corresponding to 10 standard deviations above the mean [15].

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The specificity of mAb was reported in terms of per cent cross-reactivity calculated as the ratio of IC_{50} using the following formula:

$$\% \text{ Cross reactivity} = (IC_{50} \text{ of RAC}/ IC_{50} \text{ of tested compound}) \times 100$$

Purification of mAb

The selected clone was cultured in RPMI 1640 containing 10% FBS in a 2-L spinner flask at 37°C, 5% CO_2. After cell separation by centrifugation, the mAb in the culture medium was partially purified using a Protein G sepharose affinity column. The mAb was eluted from the column with 0.1M citrate buffer (pH 3.0) at a flow rate of 1 mL/min. Each collected fraction (1 mL) was added with 1M Tris-HCl buffer (pH 9.0) to adjust the pH to 7.0. The absorbance of each fraction was measured at 280 nm. Fractions with absorbance values higher than 0.5 were pooled and the protein concentration was determined using the BCA^{TM} protein assay kit.

Biotin - indirect competitive ELISA

Biotin was conjugated to the partially purified mAb by the reaction between aminohexanoyl-biotin N-hydroxysuccinimide (100 µg) and antibodies (1 mg) in 0.1M carbonate buffer (pH 8.4) at room temperature for 4 hr. The biotinylated mAb was dialysed in PBS 4 times and kept at -20°C until use [15]. A 96-well plate was coated with RAC-BSA (0.25-1.0 µg/mL, 100 µL/well) in PBS and incubated at 4°C overnight. After washing with 0.05% Tween 20 in PBS 3 times, the plate was blocked with 5% skim milk powder in PBS (300 µL/well) at 37°C for 1 hr. After another washing step, the plate was incubated with a mixture of the biotinylated mAb (0.05-0.125 µg/mL) and RAC (0-10 ng/mL) at room temperature for 1 hr. The plate was washed again and further incubated with streptavidin-horseradish peroxidase (1:10,000 in PBS, 100 µL/well) at 37°C for 15 min. After another washing, the reaction was analysed as described previously. Optimisation of the assay was performed by the checkerboard procedure to determine the optimal concentrations of RAC-BSA and the biotinylated mAb to obtain the highest sensitivity. The optimal condition was then used to quantify RAC in pork samples.

Preparation of pork samples

One-gram samples of minced pork ('No lean meat agent' brand) were fortified with RAC (100 µL) at different concentrations in the range of 2-40 ng/mL. Each sample was then extracted with methanol (2 mL). The mixture was vortexed for 15 min., followed by centrifugation at 1,252 × *g* for 10 min. The supernatant was collected and evaporated under nitrogen at 45°C. The residue was thoroughly resuspended in 20 mL of PBS (final concentration of RAC: 0.1-2 ng/mL) and analysed for RAC. The recovery (%) of RAC spiked to the blank samples was calculated using the following equation:

$$\text{Recovery (\%)} = [(\text{concentration of measured RAC}) / (\text{concentration of fortified RAC})] \times 100$$

The inter-assay variation was based on the analysis of 12 replicates of each concentration conducted on four different days (N = 4). The intra-assay variation was conducted using 12 replicates of each concentration on a single day (n = 12) [8].

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RESULTS AND DISCUSSION

Conjugation of RAC

RAC is a non-immunogenic hapten with a low MW of 337.85 Da. Therefore, it was conjugated to the carrier protein BSA to increase the immune response. The MW of the conjugated RAC-BSA was found to be 69,422 Da as analysed by MALDI-TOF-MS (Figure 2). Based on the MW of BSA, the molar ratio of conjugation between RAC and BSA was calculated to be 8:1, indicating that RAC was linked to BSA.

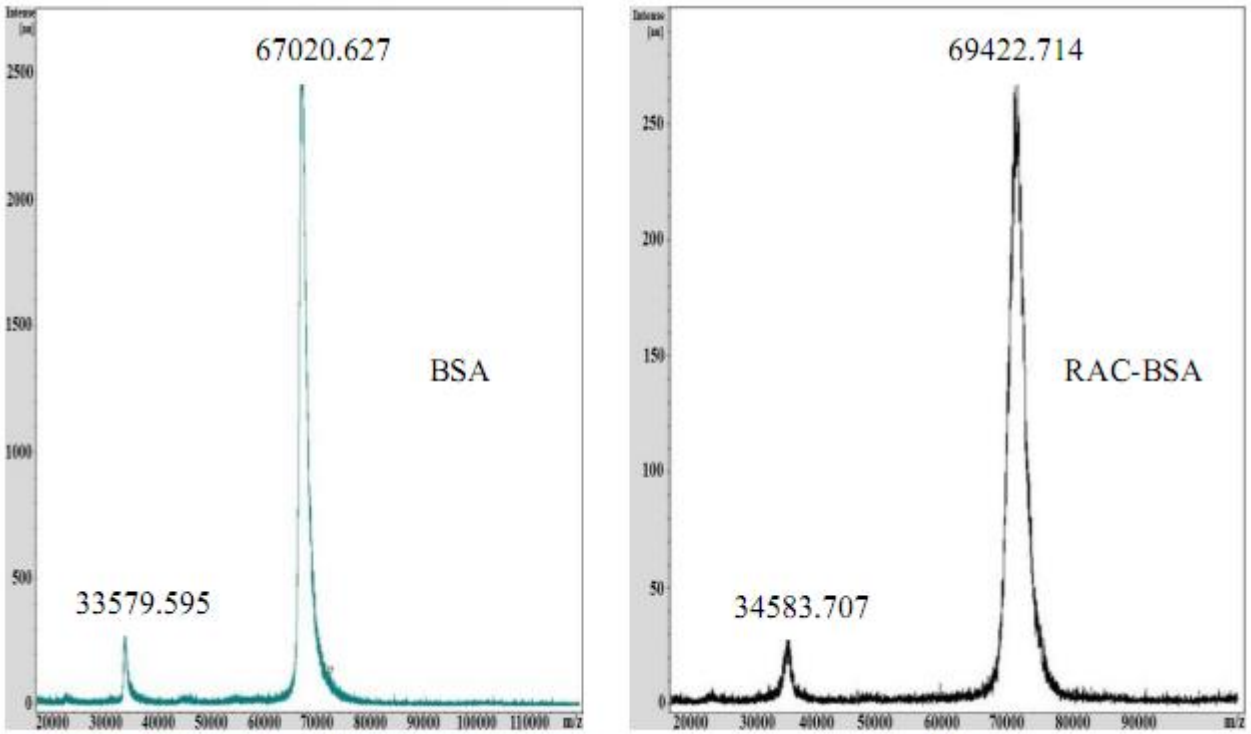

Figure 2. Results of MALDI-TOF-MS analysis: (a) MW of BSA = 67,020 Da ; (b) MW of RAC-BSA = 69,422 Da

Antibody Production and Characterisation

After immunisation, the antiserum was collected from the tail vein of each mouse and tested for antibodies by indirect ELISA. The antibody titer ranged from 32,000 to 128,000 (Table 1).

Table 1. Antibody titer and per cent competition of antisera analysed by indirect competitive ELISA

Antiserum from mouse no.	Antibody titer	Absorbance at 450 nm		Per cent competition
		without RAC	with RAC (5 µg/mL)	
1	32,000	0.872	0.296	66
33579.595	128,000	0.725	34583.707 :0	20
3	64,000	0.767	0.202	72

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Hapten density is an important parameter for the success of immunisation, including the quality as well as quantity of the antibody. A moderate to high antibody titer against the hapten has been generated with a hapten density of 15-30 molecules per carrier protein [16]. However, the lower molar ratio of conjugation (8:1) obtained in this study was sufficient for inducing a significant immune response. The ability of the antibody to bind to the conjugated RAC was also tested by indirect competitive ELISA. In this competition assay the binding of antibody to the free RAC results in a decrease in the amount of free antibody available to bind to the RAC conjugate coated onto the ELISA plate. The per cent competition is calculated as shown in Table 1. These results indicate that the antisera has antibodies specific to the free RAC.

Splenocytes were next fused with NS-1 myeloma cells to generate hybridomas that produce mAb against RAC. Hybridomas in cultures with positive test results on indirect ELISA and indirect competitive ELISA were subcloned to get monoclones by limiting dilution. Three fusions generated 5 stable mAb-producing hybridomas, designated 10A4, 2H3, 4B12, 3A6 and 10G3. The isotype of all mAbs was identified as IgG_1.

The sensitivity of mAb 10A4 in terms of IC_{50} and LOD as determined by indirect competitive ELISA was 3.0 and 0.4 ng/mL respectively (Figure 3, Table 2). As these values are lower than the other mAbs, we conclude that mAb 10A4 has the highest sensitivity. Compared to the sensitivity of other mAbs against RAC, mAb 10A4 had greater sensitivity than that reported by Li et al. [17] and Wang et al. [10] (IC_{50} values: 21.25 and 5.3 ng/mL respectively), but has lower sensitivity than that reported by Shelver et al.[12], Zhang et al.[11] and Chang et al.[18] (IC_{50} values: 2.69, 2.7 and 0.67 ng/mL respectively). Different preparations of pAb against RAC have been reported with IC_{50} values of 0.44-4.2 ng/mL [8, 19, 20]. However, mAb 10A4 is sensitive enough to be used for detection of RAC residue within the MRL value (10 ng/mL). The specificity of the mAbs was quantified in terms of per cent cross-reactivity using 7 structurally-related beta-agonists and 5 structurally non-related compounds (tested antibiotics), as presented in Table 3. The cross-reactivity of the mAbs is 100% against RAC but only 0.03-12% against the structurally-related beta-agonists. This is especially true for mAb 10A4 (<0.03%), indicating that mAb 10A4 is highly specific for RAC and has low cross-reactivity with other tested beta-agonists and unrelated antibiotics. The number of beta-agonists tested in previous reports differ, but the mAbs demonstrate similar cross-reactivity against RAC and low cross-reactivity against most beta-agonists tested [11, 18]. Therefore, mAb 10A4 was used for the subsequent experiments.

Development and Optimisation of ELISA

Of the different ELISA formats, the indirect competitive ELISA using biotin-streptavidin system was chosen to improve the sensitivity. In the competitive assay, the sensitivity is dependent on 2 factors: the concentration of the conjugated antigen coated or immobilised on the plate and the primary antibody. Thus, the optimisation of both concentrations was required in this study. The mAb 10A4 was purified and conjugated with biotin. The concentrations of the coated antigen (RAC-BSA) and biotinylated mAb were optimised using a checkerboard procedure to obtain the highest sensitivity at the absorbance value (~1.0) of the indirect competitive ELISA. In the assay three combinations of RAC-BSA as the coating antigen with biotinylated mAb concentration were selected and their competitive inhibition curves are shown in Figure 4. The concentrations of 0.5

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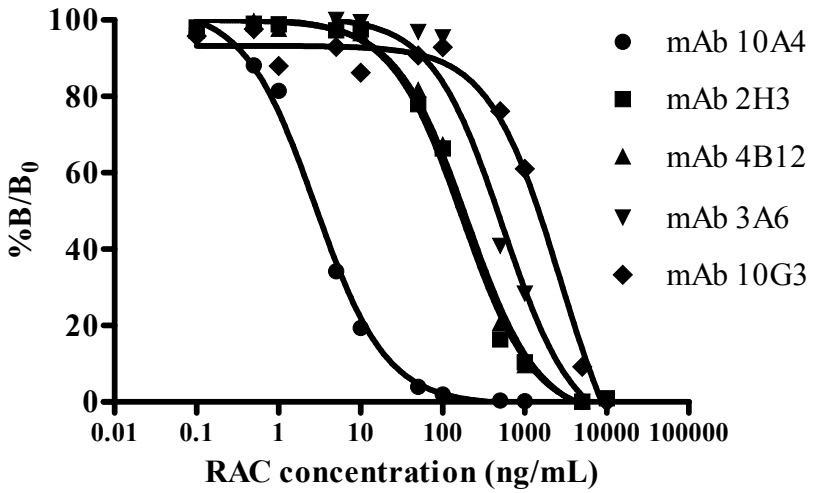

Figure 3. Competitive inhibition curve of RAC analysed by indirect competitive ELISA using mAb 10A4, 2H3, 4B12, 3A6 and 10G3. The ELISA plate was coated with RAC-BSA

Table 2. Sensitivity of five mAb

mAb	IC_{50} (ng/mL)	LOD (ng/mL)
10A4	3.0±0.15	0.4±0.10
2H3	155.4±1.65	29.2±0.93
4B12	172.4±1.59	15.4±0.70
3A6	451.1±1.35	92.2±1.01
10G3	121.6±1.15	19.8±0.85

Table 3. Per cent cross-reactivity of five mAbs with beta-agonists and tested antibiotics

	Compound	Cross-reactivity (%)				
		mAb 10A4	mAb 2H3	mAb 4B12	mAb 3A6	mAb 10G3
Beta-agonists	RAC	100	100	100	100	100
	Clenbuterol	< 0.03	< 1	< 2	< 5	< 12
	Clenproperol	< 0.03	< 1	< 2	< 5	< 12
	Brombuterol	< 0.03	< 1	< 2	< 5	< 12
	Bromchlorbuterol	< 0.03	< 1	< 2	< 5	< 12
	Cimbuterol	< 0.03	< 1	< 2	< 5	< 12
	Salbutamol	< 0.03	< 1	< 2	< 5	< 12
Antibiotics	Norfloxacin	< 0.03	< 1	< 2	< 5	< 12
	Penicilin G	< 0.03	< 1	< 2	< 5	< 12
	Sulfamethazine	< 0.03	< 1	< 2	< 5	< 12
	Chloramphenicol	< 0.03	< 1	< 2	< 5	< 12
	Tetracycline HCl	< 0.03	< 1	< 2	< 5	< 12

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µg/mL of RAC-BSA and 0.075 µg/mL of biotinylated mAb yielded the greatest inhibition (IC_{50} 0.3 ng/mL) and the highest sensitivity (LOD 0.1 ng/mL) in this ELISA assay (Table 4). Similarly, others reported that a suitable ratio of coating antigen to primary antibody can improve the sensitivity of the ELISA assay [21-22]. Using the optimised conditions, the standard curve revealed a limit of quantitation of 0.13 ng/mL. The linear relationship between RAC concentrations and the absorbance ratios was obtained at the RAC concentration range of 0.05-5 ng/mL (Figure 5). This result is similar to that reported by Zhang et al. [22], who observed improved sensitivity in biotin-streptavidin ELISA to RAC residues in muscular tissue using a pAb (IC_{50} 0.3 ng/mL, LOD 0.02 ng/mL).

Detection of RAC in Fortified Pork

To evaluate the optimised ELISA, samples of minced pork were fortified with RAC at different concentrations. After extraction, the amount of RAC was quantified by the biotin - indirect competitive ELISA under the optimised conditions described. The efficiency of detection was measured as % recovery and % coefficient of variation (CV), parameters widely used to represent the accuracy and precision of analysis as determined by inter- and intra-variation assay. Five concentrations of RAC (2-40 ng/mL) were used to spike the pork blank. The inter-variation assay was done by averaging 12 replicates at each concentration carried out over 4 days (N = 4). The

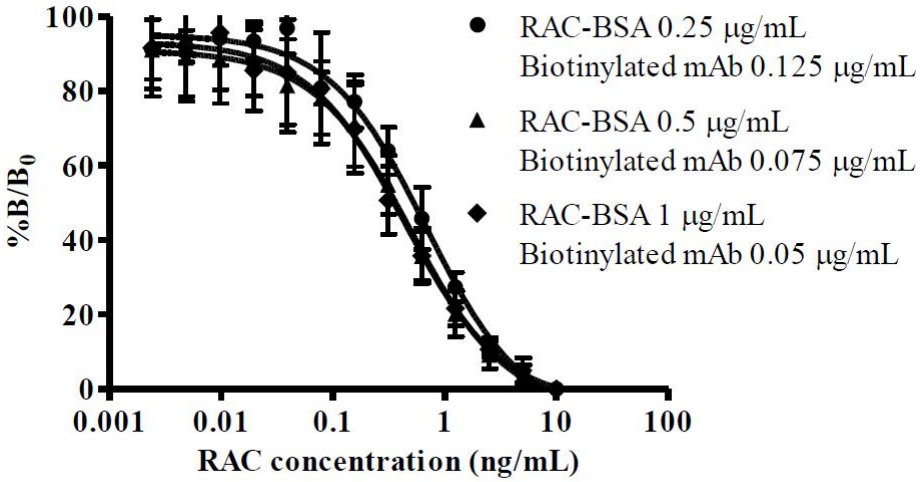

Figure 4. Competitive inhibition curves of RAC for optimisation of biotin - indirect competitive ELISA

Table 4. Results of biotin - indirect competitive ELISA optimisation

RAC-BSA concentration (µg/mL)	Biotinylated mAb concentration (µg/mL)	IC_{50} (ng/mL)	LOD (ng/mL)
0.25	0.125	0.5 ± 0.09	0.1 ± 0.02
0.5	0.075	0.3 ± 0.11	0.07 ± 0.05
1	0.05	0.3 ± 0.08	0.1 ± 0.06

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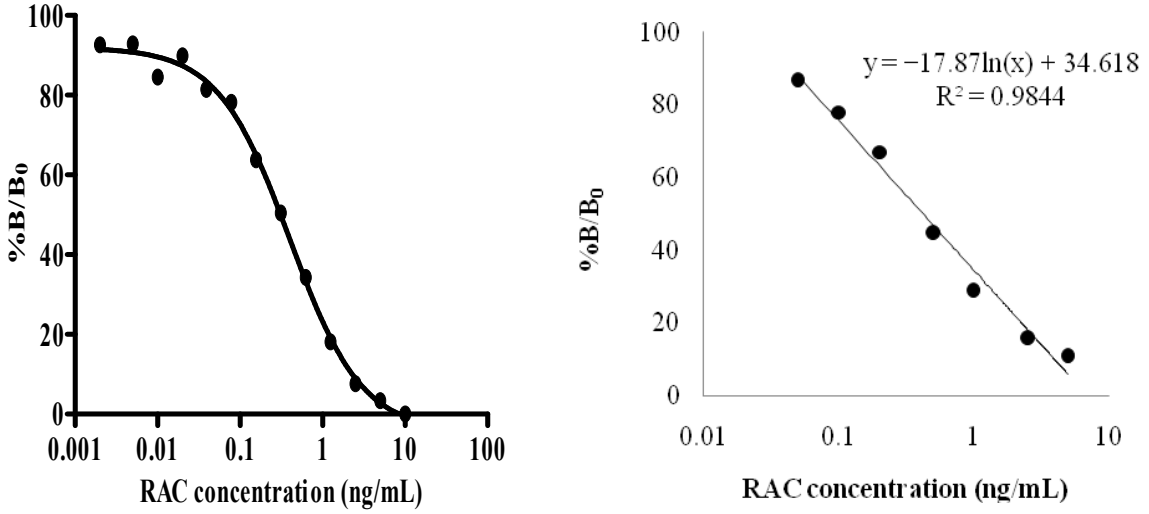

(a) (b)

Figure 5. Competitive inhibition curve (a) and linear range (b) of standard curve for RAC detection (0.05-5 ng/mL). Concentrations of RAC-BSA conjugate coating and biotinylated mAb are 0.5 and 0.075 µg/mL respectively.

intra-variation assay was performed using 12 replicates (n = 12) on a single day. The ranges of % recovery and % CV of the intra-variation assay are 91-97% and 3.2-6.7% respectively, while those of the inter-variation assay are 90-99% and 6.4-9.9% respectively (Table 5). Since the % recovery and % CV of the analysis are in the conventionally acceptable ranges of 80-120% and below 20% respectively [22], the optimised biotin - indirect competitive ELISA seems to be efficient for detecting RAC residue in pork at the currently enforced MRL of 10 ng/mL [3].

Table 5. Analysis of RAC in fortified pork muscle sample by biotin - indirect competitive ELISA

Spiked	Intra-variation assay (n=12)			Inter-variation assay (N=4)		
RAC (ng/mL)	Found (ng/mL)	Recovery (%)	CV (%)	Found (ng/mL)	Recovery (%)	CV (%)
2.0	1.9±0.1	97±5	5.5±0.5	1.9±0.2	97±4	7.9±2.5
5.0	4.5±0.2	90±4	4.3±0.2	3.9±0.3	90±4	6.4±4.8
15.0	14.0±0.8	93±5	5.4±0.3	14.0±1.4	94±5	9.9±4.6
25.0	24.6±1.7	91±7	6.7±0.4	24.6±1.7	99±7	6.8±1.6
40.0	39.0±1.3	97±3	3.2±0.1	39.0±2.8	97±3	7.2±2.5

CONCLUSIONS

The mAbs against RAC have been successfully generated in this study. Of the obtained mAbs, mAb 10A4 was selected for the development of an indirect competitive ELISA using a biotinylated antibody. The ELISA shows sufficient sensitivity and accuracy for the detection of RAC residue in pork under the current enforced MRL value and may be used as a screening tool for monitoring the residues of RAC in pork samples.

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