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Analytical Methods

Monoclonal antibody production and indirect competitive enzyme-linked immunosorbent assay development of 3-methyl-quinoxaline-2-carboxylic acid based on novel haptens

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ABSTRACT

Two novel immunizing haptens of 3-methyl-quinoxaline-2-carboxylic acid (MQCA) were synthesized and conjugated with cationized bovine serum albumin. Female BALB/c mice were immunized with above conjugates, splenocytes were fused with Sp2/0 cells to produce monoclonal antibody. Compared with previous studies, antibodies raised in this work showed higher sensitivity. Meantime, a novel heterologous coating hapten was also prepared. The indirect competitive enzyme-linked immunosorbent assay (icELISA) based on the optimum condition showed an IC_{50} of 3.1 μg/kg (ppb), and the linear range of 0.46–10.5 ppb for MQCA. The limit of detect (LOD) of MQCA in swine muscle, swine liver and chicken was 0.32, 0.54, and 0.28 ppb, respectively. The LOD of this assay can satisfy the minimum required performance levels (4 ppb) for MQCA. These results indicated that the proposed ELISA, with high sensitivity and specificity, as well as good reproducibility and accuracy, is suitable for determination of MQCA residues in food samples.

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1. Introduction

Olaquindox (OLA, Fig. 1) is well known antimicrobial and growth-promoting agents in aquaculture and animal husbandry. However, due to the potential adverse effects on human health, it has been banned by the European Union (Council Regulation, 1998). OLA can rapidly and extensively metabolize into different kinds of metabolites *in vivo*. 3-Methyl-quinoxaline-2-carboxylic acid (MQCA, Fig. 1), among the various metabolites of OLA, was proposed by the Joint FAO/WHO Expert Committee on Food Additives as the marker compound for residues of OLA in muscle and liver tissue (FAO & WHO., 1995). Meanwhile, it is one of main metabolites of mequindox (MEQ) (Liu et al., 2012) and quinocetone (QCT) (Huang et al., 2005). MQCA is toxic to L-02 and Chang liver cell (Zhang et al., 2012). In recent years, many countries have paid more attention to the control and detection of MQCA (Jiang, Beier, Wang, Wu, & Shen, 2013). The Joint FAO/WHO Expert Committee on Food Additives noted that 4 μg/kg (ppb) of MQCA in swine muscle is consistent with Good Veterinary Practice (FAO & WHO., 1995). In China, the limit of detection (LOD) of MQCA in swine liver is 10 ppb (China, 2015).

Many methods have been developed for the determination of MQCA, such as liquid chromatography (Duan, Yi, Fang, Fan, & Wang, 2013; Wu et al., 2007) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Boison, Lee, & Gedir, 2009; Hurtaud-Pessel, Pirotais, Blot, & Sanders, 2006; Merou, Kaklamanos, & Theodoridis, 2012; Zhang, Zheng, Zhang, Chen, & Mei, 2011), which provide solid evidence to confirm both the identity and the quantity of detected residue. Although being sensitive and specific, these methods are time-consuming and expensive. Enzyme-linked immunosorbent assay (ELISA) based on the binding reaction of antibody and antigen is a portable and rapid method for the detection of MQCA. Antibody is at the very heart of ELISA, and hapten is vital to the preparation of antibody (Song, Wang, Wang, Tang, & Deng, 2010). However, until now, few ELISA methods have been developed for the rapid monitoring of MQCA (Cheng, Shen,

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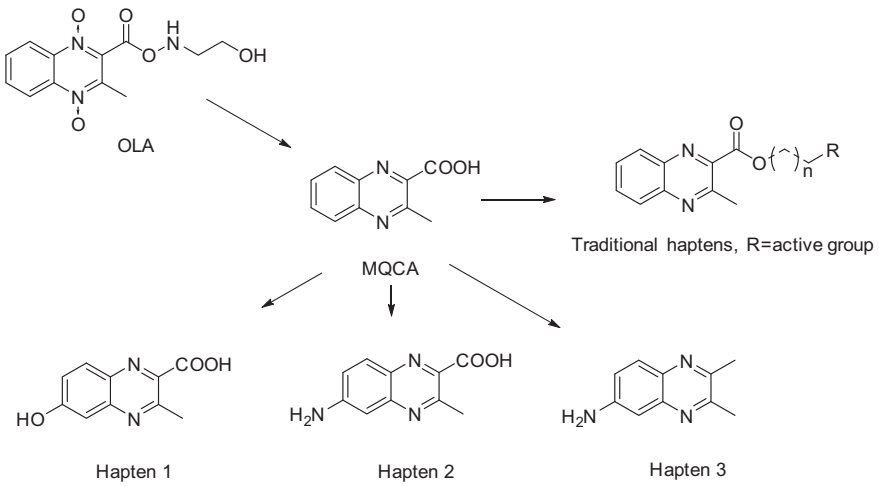

Fig. 1. Chemical structures of OLA, MQCA, and haptens.

Wang, Jiang, & Zhang, 2013; Jiang et al., 2013; Le, Wei, Niu, & Liu, 2014; Yue et al., 2009). In these studies, haptens were MQCA or the derivatives of the carboxyl of MQCA (Fig. 1). Though these haptens may elicit antibodies with sensitivity and specificity, due to the deficiency of integrity of the carboxyl in immunogens, the ability to produce ideal antibody may be weakened.

In this work, we designed three original haptens (Fig. 1) to produce monoclonal antibody (mAb) against MQCA, and to develop highly sensitive and specific ELISA for the determination of MQCA in meat. Three haptens were designed and synthesized, which were further conjugated with cationized bovine serum albumin (cBSA) and ovalbumin (OVA) to prepare immunogens and coating antigens. Then mAb was produced through a normal procedure of mice immunization and cell fusion. In order to improve the sensitivity of the assays, heterogeneous antibody/coating antigen combinations were adopted. The established ELISA was characterized in terms of sensitivity, specificity, accuracy and precision. Under optimum conditions, the ELISA was used for the analysis of MQCA in meat samples as well as validated by high-performance lipid chromatography (HPLC) method.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade and without further purification unless particularly specified once. OLA, MEQ, QCT, MQCA, quinoxaline-2-carboxylic acid (QCA), carbadox (CBX), cyadox (CYA), BSA, OVA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide·HCl (EDC·HCl), complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA), polyethylene glycol 4500 (PEG 4500), 3,3′,5,5′-tetramethylbenzidine (TMB), hypoxanthine-aminopterin-thymidine (HAT), hypoxanthine-thymidine (HT) and glutaraldehyde were obtained from Sigma (St. Louis, MO, USA). OLA and other quinoxaline-1,4-dioxides were purchased from Dr. Ehrenstorfer (Augsburg, Germany). RPMI1640 and fetal bovine serum (FBS) were bought from Life Technologies (Gaithersburg, MD, USA).

BALB/c mice were obtained from Beijing Vital River Laboratory Animal center [Certificate No. SCXK (Beijing) 2012–0001]. Sp2/0 cells were obtained from the American Type Culture Collection (Manassas, VA, USA).

2.2. Buffers and solutions

0.1 M PBS, pH 7.4: 77 mM Na_2HPO_4, 137 mM NaCl and 23 mM NaH_2PO_4; 0.01 M PBS, pH 7.4: 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl and 1.8 mM KH_2PO_4; washing buffer: PBS with 0.05% (v/v) Tween 20 and 0.03% (v/v) proclin 300; coating buffer: 0.05 M carbonate-bicarbonate buffer consist of 15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6; blocking buffer: coating buffer with 0.5% (m/v) casein and 0.03% (v/v) proclin 300; antibody dilution solution: PBS with 0.2% (m/v) BSA and 0.03% (v/v) proclin 300; substrate solution: 1% H_2O_2 and 0.1% TMB in 0.05 M citrate-acetate buffer, pH 5.5; stop solution: 2 M H_2SO_4.

2.3. Haptens synthesis and identification

Three haptens of MQCA (Fig. 1) were designed, synthesized and confirmed by MS and nuclear magnetic resonance (NMR). Hapten 1 (6-hydroxy-3-methyl-quinoxaline-2-carboxylic acid) and Hapten 2 (6-amino-3-methyl-quinoxaline-2-carboxylic acid) were custom synthesized by Mashilabs Co., Ltd. (Shanghai, China). Hapten 1: MS (ESI) m/z 202.9 (M−H), 205 (M+H), 227.1 (M+Na); ^{1}H NMR (300 MHz, DMSO): δ 2.75 (t, CH_3, 3H), 7.28 (d, aromatic-H, 1H), 7.45 (q, aromatic-H, 1H), 7.89 (d, aromatic-H, 1H), 9.58 (s, aromatic-OH, 1H),12.25 (s, COOH, 1H). Hapten 2: MS (ESI) m/z 201.9 (M−H), 204 (M+H), 226 (M+Na); ^{1}H NMR (300 MHz, DMSO): δ 2.75 (t, CH_3, 3H), 5.38 (m, NH_2, 2H), 6.97 (d, aromatic-H, 1H), 7.45 (q, aromatic-H, 1H), 7.89 (d, aromatic-H, 1H), 12.25 (s, COOH, 1H).

Hapten 3 was synthesized according to the method mentioned by Hui et al. (2006). Briefly (Fig. 1), a mixture of 4-nitro-O-phenylenediamine (10 mmol) and 2,3-butanedione (14 mmol) in 50 mL ethanol (95%, v/v) was refluxed for 2 h under N_2. After cooling, the product was precipitated, filtered, washed by cold ethanol and dried. Then, this compound (1 equiv.) and $SnCl_2{\cdot}5H_2O$ (5 equiv.) in EtOAc was refluxed for 5 h under N_2. The reaction mixture was alkalined to pH 8 or 9 with saturated $NaHCO_3$, and the solution was filtered through Celite to remove the precipitate, and further the organic phase and the water phase was separated. The water phase was extracted by EtOAc for three times. The combined organic phase was washed by brine, dried over anhydrous Na_2SO_4 and concentrated under vacuum. MS (ESI) m/z: 174 (M+H). ^{1}H NMR (300 MHz, DMSO): δ 2.68 (m, CH_3, 6H), 4.10 (s, NH_2, 2H), 7.10 (q, aromatic-H, 2H), 7.76 (q, aromatic-H, 1H).

2.4. Antigens' preparation and determination

2.4.1. Preparation of cBSA

The protocol of preparation of cBSA was according to Hermanson (2013) with slight modification. In brief, a solution of 1.5 g of BSA (22.5 μmol) in 10 mL of PBS (0.1 M, pH 7.4) was added

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