2011 International Conference on Asia Agriculture and Animal
IPCBEE vol.13(2011) © (2011)IACSIT Press, Singapore

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST 1-AMINOHYDANTOIN

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Abstract. Nitrofurans are antibiotic drug which have been widely and effectively used for the prevention and treatment of gastrointestinal infections in animals. However, they have been banned from use in animals for consumption because of their carcinogenicity and mutagenicity. Since they are rapidly metabolized upon intake, the effective monitoring of nitrofuran drugs in animal tissues must be achieved by measuring tissue concentrations of bound nitrofuran metabolites. Enzyme-linked immunosorbent assay (ELISA) has been proved to be a suitable method for screening detection of many substances. Therefore, the aim of this study is to produce monoclonal antibodies with suitable properties for AHD detection based on ELISA. A carboxyphenyl derivative of AHD was prepared and conjugated to immunogenic carrier protein and used in mice immunization. After conventional cell hybridization technique, a monoclonal antibody (clone number 28/10E/8B/1E) was generated. Characterized by indirect competitive ELISA, the obtained antibody was highly specific for nitrophenylbenzal-1-aminohydantoin (NPAHD) and possed excellent sensitivity with the 50% inhibition concentration (IC_{50}) of 3.816 ppb and the limit of detection (LOD) of 0.194 ppb, respectively. These results indicated that this antibody is suitable for the development of screening ELISA test kit to detect AHD residues.

Keywords: 1-aminohydantoin, monoclonal antibody, indirect competitive ELISA

1. Introduction

Nitrofurans are antibiotics which have been banned from use in animals destined for human consumption in the European Union (EU) and other countries because of concerns over their carcinogenicity and mutagenicity [1]. Despite the potentially harmful effects on human health, they are still illegally used in many countries. Because of the concerns over their adverse effect, these nitrofurans were placed in Annex IV of regulation 2377/90/ECC, which prohibits the use of certain chemicals in food-producing animals in the EU, and in products from third countries intended for the EU market. The EU Commission Decision of 13 March 2003 had set a MRPL at 1 μg/kg (for each nitrofuran metabolite) for any methods dealing with the analysis of nitrofurans in poutry meat and aquaculture products [2].

Nitrofurans have been used both therapeutically [3] and prophylactically in a number of food-producing animal species, including pigs, sheep, goats, cattle, chickens and turkeys [4]. It is recognized that methods for detecting nitrofuran residues by measuring the parent species (furazolidone, furaltadone, nitrofurazone and nitrofurantoin) are inappropriate as the drugs are rapidly metabolized *in vivo* (between 4 and 9 days

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half-life time) and do not persist in edible tissues [5]. However, their metabolites (3-amino-2-oxazolidone (AOZ), 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD)) bind to tissue proteins and persist for considerable periods in animal tissues after treatment. Because of their covalently binding to proteins, they must be released from the tissues by derivatization with *o*-nitrobenzaldehyde (*o*-NBA) to form nitrophynyl (NP) derivatives prior to the detection [6].

Analytical methods reported for the detection of the nitrofuran metabolites in animal tissues have been based on UV-HPLC, LC-MS and LC-MS/MS which usually requires time-consuming sample preparation steps and expensive instruments [7]. In contrast, enzyme-linked immunosorbent assay (ELISA) provides an alternative inexpensive, sensitive and fast screening assay for detection of AHD. Therefore, the objective of this research is to prepare a suitable monoclonal antibody for the ELISA development in order to detect AHD.

2. Objectives

The objective of this study is to produce and charaterize a monoclonal antibody against AHD.

3. Methods

3.1. Synthesis of CPAHD derivative

An excess of AHD over carboxybenzaldehyde (CBA) was stirred overnight in pyridine. The desired product in the reaction mixture was detected by thin-layer chromatography (stationary phase: aluminum silica, solvent system: 5% methanol in chloroform). CBA was visualized under UV light while AHD was visualized by ninhydrin staining. The presence of carboxyphenyl-1-aminohydantoin (CPAHD) was also confirmed by MALDI-TOF MS.

3.2. Preparation of antigen

CPAHD was conjugated to an immunogenic carrier protein bovine serum albumin (BSA) via an active ester method. The carboxylic acid groups on the hapten molecule was activated with 1-ethy-3-(3-dimethylaminopropyl) carbodimide (EDC) and N-hydroxysuccinimide (NHS) to produce an active ester, which then reacted with amine group of BSA to form an amide bond.

A mixture of CPAHD, NHS and EDC in dry 1, 4-dioxane was stirred overnight at room temperature. After centrifugation, this solution was added very slowly while stirring to a mixture solution of dimethylformamide (DMF) and phosphate buffer saline (PBS) containing BSA and stirred overnight at 4^{o}C. The mixture was dialyzed against PBS (0.1 mol·L^{-1}, pH 7.4) for 3 days.

3.3. Immunization of mice

BALB/c female mice, 6-8 weeks old (National Laboratory Animal Centre, Mahidol University, Thailand), were immunized by subcutaneous injections with 50 µg CPAHD-BSA in 100 µL of sterile PBS emulsified with 100 µL of Freund's complete adjuvant. After three booster doses of CPAHD-BSA (50 µg) in Freund's incomplete adjuvant at 2 week intervals, the sera were collected and assayed for anti-CPAHD antibodies by an indirect ELISA. Mice were immunized with a final dose of CPAHD-BSA (50 µg) in sterilized normal saline. All procedure involved animals have been approved by IACUC of Institute of Biotechnology and Genetic Engineering (IBGE) and conducted according to the IACUC guideline.

3.4. Production of hybridoma

Anti-CPAHD monoclonal antibody was produced using somatic cell fusion technique. The immunized mice were sacrificed and their spleens were removed. The splenocytes were fused with myeloma cells. The fused cells (hybridomas) were seeded in 96-well culture plates supplemented with hypoxanthine aminopterin thymidine (HAT) medium containing 20% fetal calf serum and cultured at 37^{o}C with 5% CO_2.

3.5. Screening of hybridoma

First round of screening by indirect ELISA

Plates were coated with CPAHD-BSA and incubated at 4°C overnight. Plates were washed three times with PBS containing 0.05% Tween20 (PBST) and blocked with 5% skimmed milk in PBS. After washing, culture supernatants or antisera were added, and the plates were incubated at 37°C for 2 hr. After washing, secondary antibody (goat anti-mouse IgG antibodies conjugated with horse radish peroxidase (GAM-HRP)) was added and the mixture was incubated at 37°C for 1 hr. Plates were washed and then substrate solution (tetramethylbenzidine (TMB) and H_2O_2 in 0.15 M citrate buffer pH 5.0 in 1 M sodium acetate buffer pH 6 with 0.34% H_2O_2) was added. The reaction was stopped after 10 min with 1 M H_2SO_4, and the absorption was measured at 450 nm using microtiter plate reader. Then the positive samples were further selected by secondary screening.

Second round of screening by indirect competitive ELISA

An indirect competitive ELISA was used to screen for antibodies specific for free nitrophenyl-1-aminohydantoin (NPAHD) and to evaluate the sensitivity and cross reactivity. Plates were coated and blocked as described in procedures of indirect ELISA. After washing, competitors (different concentrations) and antibodies were added, and the mixtures were incubated at 37°C for 2 hr. The plates were washed and assayed as described in indirect ELISA.

3.6. Evaluation of Sensitivity

The sensitivity of monoclonal antibody was justified based on 50% maximal inhibition concentration (IC_{50}). The IC_{50} was defined as the concentration at which 50% B/B_0 was obtained, where B_0 and B are the average of the absorbance obtained from the indirect competitive ELISA without and with different concentrations of NPAHD, respectively.

The multiple measurements of B_0 of the standard curve were used for calculating the limit of detection (LOD). The LOD was calculated by subtracting the concentration at B_0 with three times of its standard deviation (SD) obtained from indirect competitive ELISA without competitor of 9 replicates.

3.7. Cross-reactivity of evaluation

The cross-reactivity of monoclonal antibody was calculated by the ratio of the IC_{50} of standard NPAHD to IC_{50} of the competitors using the following formula:

$$\%\ \text{Cross reactivity} = \frac{IC_{50}\text{of NPAHD}}{IC_{50}\text{of competitor}} \times 100$$

4. Results and discussion

4.1. Preparation of CPAHD and its conjugate

Mass of the conjugated product was compared with that of BSA by MALDI-TOF-MS as shown in Figure 1. The result indicated that mass of the conjugate was higher than that of BSA with the difference of about 3579.26 dalton. Using the mass of CPAHD (283.68 dalton), the molecule ratio of CPAHD to BSA was calculated to be at 12.61:1.

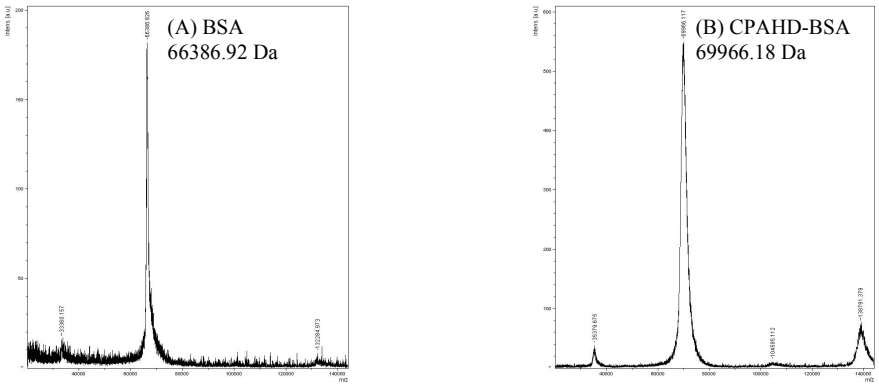

Figure1. Spectrums of (A) BSA and (B) CPAHD-BSA from MALDI-TOF-MS analysis.

4.2. Production of monoclonal antibody

After immunization, antiserum titer was monitored by indirect ELISA. The result shown in Figure 2 suggested that antibody against NPAHD was produced at the titer of 1,024,000. The fusion of splenocytes with myeloma was performed. Hybridomas were screened and selected until monoclone 28/10E/8B/1E was obtained.

4.3. Sensitivity of monoclonal antibody

The sensitivity of the obtained monoclonal antibody in this study was quantified as IC_{50} and LOD at 3.816 ng/ml and 0.194 ng/ml, respectively, by indirect competitive ELISA. These results indicated that the obtained monoclonal antibody is sensitive enough to detect AHD at the level lower than the MRPL currently enforced at 1 ng/ml.

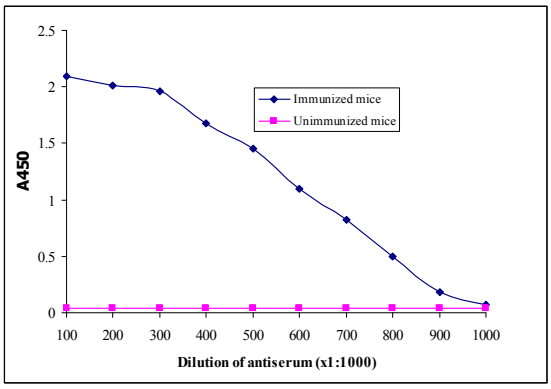

Figure2. Antiserum titers of unimmunized mice and immunized mice detected by indirect ELISA.

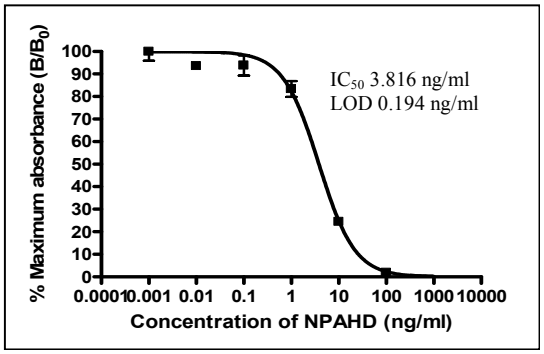

Figure3. Inhibition curves of indirect competitive ELISA of monoclonal antibody 28/10E/8B/1E against NPAHD.

Specificity of the monoclonal antibody was tested by the measurement of cross-reactivity using NPAHD and related compounds. The percentages of cross-reactivity of this monoclonal antibody, shown in Table1, demonstrated that monoclonal antibody 28/10E/8B/1E is very specific for NPAHD.

Table1. % Cross-reactivity of monoclonal antibody 28/10E/8B/1E with various drugs and related compounds of interest.

Competitor	% Cross-reactivity
NPAHD	100
NPSEM	<0.3
NPAMOZ	<0.3
NPAOZ	<0.3
Oxytetracycline	<0.03
Tetracycline	<0.03
Rolitetracycline	<0.03
Doxycycline	<0.03

5. Conclusion

The monoclonal antibody against AHD with promising properties was obtained. It can be further used for the screening of 1-aminohydantoin antibiotic by immunoassay-based method.

6. Acknowledgement

This work is partially supported by Thailand Graduated Institute of Science and Technology (TGIST), National Science and Technology Development Agency (NSTDA) and National Research University Development Project (AM1023A), Office of the Higher Education Commission, Thailand.

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