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In Vitro Improved Production of Monoclonal Antibody against Zearalenone in Supplemented Cell Culture Media

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Abstract
Zearalenone (ZEN) is a phenolic resorcylic acid lactone, produced by several Fusarium species causing reproductive disorders such as abortion and stillbirth in livestock resulting from hyperestrogenism. A monoclonal antibody (mcAb) specific to zearalenone was produced from hybridoma cell line 5A8. It was generated by the fusion of Sp2/0 myeloma cells with spleen cells isolated from Balb/c mice with highest-titer (1: 64,000, v/v). The mice were immunized intraperitoneally with zearalenone–cationic bovine serum albumin (ZEN-cBSA). ZEN-cOVA conjugate was used as coating antigen to test titer of mice serum. Hybridoma cells were grown on cell culture media supplemented with carbohydrates (CHO) respectively. Monoclonal antibody produced by hybridoma in vitro belongs to the immunoglobulin subtype IgG1 with specific antibody titer 0.70. The most effective culture medium for hybridoma clones was supplemented with maltose that achieved best titer at 1.473 as determined by icELISA. The use of supplemented cell culture media is an effective approach to improve the production of monoclonal-antibody, better hybridoma growth and viability.
Keywords: Monoclonal antibody, zearalenone, hybridoma technology.

INTRODUCTION

Mycotoxins are known as poisonous metabolites of fungi that exist on farmland or in the process of producing, transporting, handling or storing farm commodities and foodstuff (Zhang *et al.*, 2014), contaminating up to 25% grain crops every year (Brenn-Struckhofova *et al.*, 2007). Considering the substantial risks of public health and economic, Zearalenone (ZEN), has received much attention among scientists who attempt to detect mycotoxins in foodstuff and fodder in recent years (Bucheli *et al.*, 2008). ZEN contamination of cereal, feed, and the environment are recognized as a worldwide problem. ZEN can be identified with the systematic name 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7(8H)-dione or 6-(10-hydroxy-6- oxo-*trans*-1-un-decenyl)-β-resorcylic-acid-lactone. Together with other compounds, it belongs to the group of resorcylic acid lactone (RALs) carrying a keto group and an olefinic double bond (Figure S1) (Budavari *et al.*, 1989). It is a nonsteroidal mycotoxin possessing the estrogen-like activity and has been associated with pubertas praecox, endometrial neoplasms, endometrial hyperplasia as well as carcinoma of the uterine cervix (Shim *et al.*, 2009).

Hybridoma technology was first invented for the production of monoclonal antibodies in 1975 (Bretton *et al.*, 1994) (Figure S2). Cell culture comprises isolation of cells from (in vivo) and their growth under a controlled environment (in vitro) in the sufficient supply of culture media and nutrients (Cooke *et al.*, 2008). The mAbs are clinically significant homogeneous and mono-specific scientific biomolecules produced from hybridoma cells by hybridoma technology (Zhang, 2012). Since their discovery, these molecules have been used as research tools and have revolutionized the fields of biotechnology, immunology, diagnostics, and medicine (Priyabrat *et al.*, 2014). Improvements in hybridoma technology are based on research demand, cost effectiveness, human labor, and reduced development time. Similarly, the production of mAbs requires multiple phases, long duration, and high cost. CHO is provided largely in the form of glucose as a carbon source for the hybridoma cells. In some cases, glucose is substituted with other sugars to enhance cell growth, viability and protein production (Altamirano *et al.*, 2004; Wilkens *et al.*, 2011). The aim of this study was the production of mcAb against ZEN by CHO supplemented culture media for the establishment of immunoassay purposes.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were carried out according to the rules by the Animal Welfare Committee of Fujian Agriculture and Forestry University, Fuzhou, China.

Materials

Zearalenone, bovine serum albumin (BSA), ovalbumin (OVA), Tween 20, dimethyl sulfoxide (DMSO), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and N-hydroxysuccinimide (NHS), Goat anti-mouse-peroxidase conjugate, substrate solution 3, 3′,5,5′-tetramethylbenzidine (TMB), Polyethylene glycol (PEG 1500), hypoxanthine (H), aminopterin (A), and thymidine (T) were purchased from Sigma Chemical Co. (St. Louis, MO). 96-well immunoassay plates were purchased from (Maxisorp; Nunc). Freund's complete and incomplete adjuvant, Roswell Park Memorial Institute medium (RPMI-1640), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco Laboratories (Grand Island, NY). All other chemicals and organic solvents used were of reagent grade or better.

Mice and Cell Lines

Virus-free, 6-8 weeks old, three female BALB/c mice were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (SLAC) China. The murine myeloma cell line Sp2/0 was obtained from Shanghai cell culture bank (SIBS), China. All cell lines were confirmed to be *Mycoplasma*-free by RT-PCR analysis.

Preparation of Conjugates

The hapten of ZEN was conjugated to the modified carrier proteins, (cationic BSA; immunogen) and (cationic OVA; coating antigen) according to the method described previously (Figure S3 and S4).

Immunizations and serum characterization

Three female Balb/c mice were immunized intraperitoneally, at two-week intervals, with 100 µg of ZEN-cBSA in 0.01 M PBS in a total volume of 200µl. After 4 times of injection, the titer and sensitivity of antibody in the serum was determined by improved icELISA.

Optimization of CHO supplemented cell culture media

The four test media contained RPMI-1640, 10% FBS, and penicillin/streptomycin, supplemented with glucose (control), fructose, galactose and maltose at a concentration of 15 mg/mL each as carbon sources. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C to optimize media components.

Fusion and Screening

Spleen cells were isolated from the immunized mice and mixed with Sp2/0 murine myeloma cells at a ratio of 1:10 in the presence of 1mL 50% PEG. Cell fusion and hybridoma culture procedures were carried out essentially

following previous reports by limiting dilution (Ling *et al.*, 2014; Ling *et al.*, 2018; Ling, 2015).

Cell density

Static cultures using several 25 cm^2 flasks (Corning, Sigma-Aldrich) were inoculated with an initial cell density of 10^5 cells/ml following incubation at 37°C in 5% CO_2. One flask was used every time a cell count was accomplished after 3 d, 6 d, and 9 d respectively to determine the cell density by the trypan blue exclusion method (Louis, 2011) with some modifications. The cells were successfully passaged each time into new flasks with fresh media.

Statistical analysis

Data are presented as mean SD. Appropriate statistical tests were performed using GraphPad Prism. One-way ANOVA, followed by Dunnett's multiple comparisons test was used for experiments with more than 3 groups. P values of less than 0.05 were considered significant.

RESULTS

Preparation of Immunogen and Coating Antigen

The results of the ZEN-carrier proteins conjugation were confirmed by gel electrophoresis. The gel results indicated that ZEN-cBSA and ZEN-cOVA conjugates were successfully conjugated as they traveled faster in the gel compared to slower movements of standard proteins i.e., BSA and OVA respectively due to the difference in charges (Figure 1). The protein concentration in the conjugates was determined by bicinchoninic acid assay (BCA).

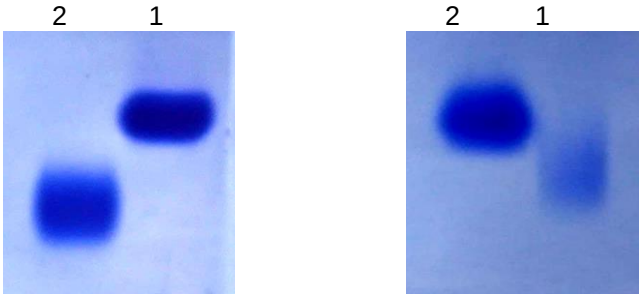

Fig. 1. Coomassie blue-stained gel electrophoresis. The analysis revealed successful cross-linking of ZEN molecules with cBSA and cOVA. (a). lane 1, BSA standard; lane 2, ZEN-cBSA; (b). lane 1, ZEN-cOVA; lane 2, OVA standard.

Serum anti-ZEN titer

Serum titers of the immunized BALB/c mice were subsequently determined by icELISA. The results showed that mouse 2 achieved the highest titer (1: 64,000, v/v) of anti-ZEN (Figure 2), and selected for cell fusion with Sp2/0 cells.

Production and characterization of mAb in selected CHO media

Supernatants of hybridoma cells were determined after 9 d post fusion for the reactivity with ZEN by icELISA, which resulted in 10 positive clones. One clone was selected using limiting dilutions method, and was characterized as 5A8G1 (Figure S5) with highest-titer (OD: 1.473) in maltose supplemented media, other CHO showed lower antibody titers i.e. galactose (OD: 1.21), fructose (OD: 1.20), sucrose (OD: 0.60) and lowest in glucose as a control (OD: 0.35) respectively (Figure 3), with statistically significant differences (P<0.05). The isotype determination showed that 5A8G1 mAb was IgG1 (Figure 4).

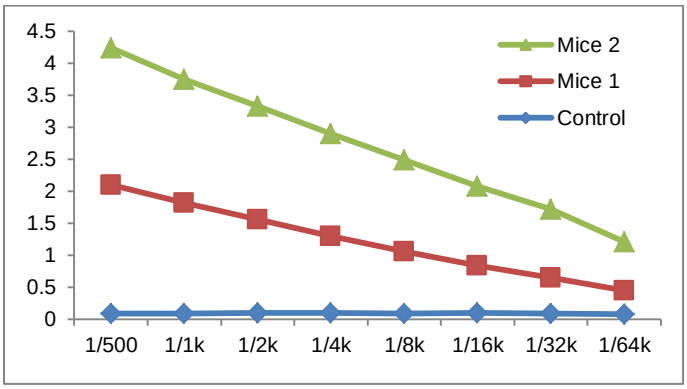

Fig. 2. Mouse anti-zearalenone antibody titer assayed by icELISA demonstrated Mice 2 showed the highest antibody titer (1: 64,000, v/v) as compared to control.

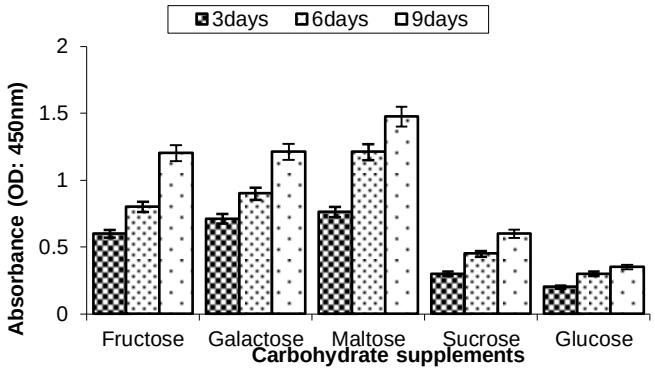

Fig. 3. Effect of CHO supplemented cell culture media in vitro on the production of mAb. After 9 d of 5A8G1 growth, the highest titer of 1.47 was obtained in maltose media. Glucose media was used as a control (P < 0.05).

Cell density

Viable cell densities were calculated by harvesting hybridoma cells after 9 d culture *in vitro*. Maltose supplementation increased cell densities up to 1.23 million/mL compared to the control of 0.45 million/mL, the difference was statistically significant (P<0.05) (Figure 5).

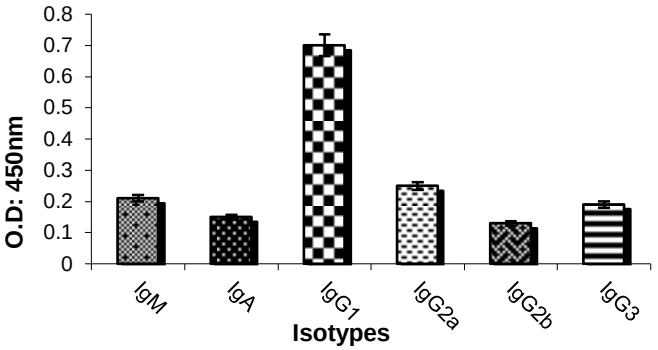

Fig. 4. The isotyping of 5A8G1 as determined by icELISA.

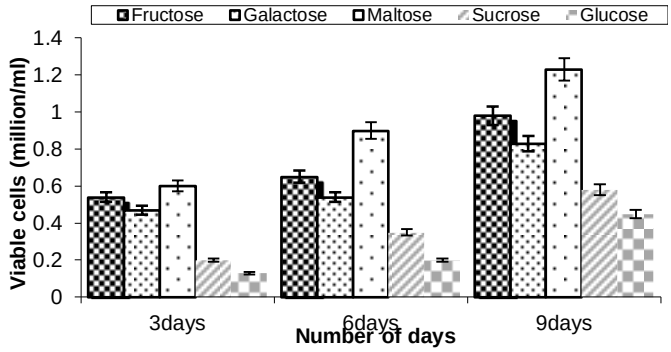

Fig. 5. Effect of CHO supplemented media on 5A8G1 cell viability in vitro. The cells were harvested after 9 days. Glucose was used as a control ($P<0.05$).

DISCUSSION

Hybridoma technology has been a significant and essential platform for producing high-quality mAbs (Zhang, 2012). It permits the generation of therapeutic antibodies in a native form being a classical and established route of generating specific antibodies all around the globe (Glukhova, 2016). The mAbs are clinically significant homogeneous and mono-specific scientific biomolecules produced from hybridoma cells by hybridoma technology (Zhang, 2012). Since their discovery, these molecules have been used as research tools and have revolutionized the fields of biotechnology, immunology, diagnostics, and medicine.

In this study, we produced and characterized mAb against Zearalenone (ZEN). ZEN being a hapten was conjugated with larger carrier proteins (cationic proteins) such as cOVA and cBSA that induce a strong immune response in mouse. The conjugation was confirmed with Coomassie blue-stained non-denaturing agarose gel electrophoresis analysis (Tsai and Lee, 2011). The analysis showed that the migration speed of the conjugates was faster than that of carrier proteins, indicating that the conjugated products carried more negative charges, and the net charge of the conjugates became more negative than that of the carrier proteins (Wang *et al.*, 2014).

ZEN-cBSA was used for immunization and ZEN-cOVA was applied as ELISA coating antigen. ZEN-specific antibody-secreting hybridomas were obtained. Since the mAb reacted with ZEN in icELISA assay, the antibodies were non-conformational (Kindt *et al.*, 2007). Monoclonal antibodies produced by the hybridomas were in the IgG1. Since secondary antibodies were specific for mouse immunoglobulins, non-immune mouse serum was included in the assay in order to provide a positive control for mouse IgG. The isotype of mcAb produced by this hybridoma cell line was IgG1 subclass (Ling *et al.*, 2014).

Cell culture media have advanced significantly during the past few decades, primarily by advancements in media supplementation. Various media supplements were tested and maltose proved to be the best CHO substitute in cell medium which raised mAb titer to a significant level. Glucose was used as a control, in general, metabolizes to form lactic acid resulting in the hindrance of the cell growth or decelerates growth. The substitution of glucose with fructose, galactose and maltose favored the growth of 5A8G1 hybridoma. Maltose supplementation gave the best antibody titer in agreement with previous studies (Anand *et al.*, 2010). The cell-culture medium provides an artificial environment conducive to survival and proliferation of cultured cells. Our results showed that maltose supplemented media improved cell density and viability (Sathya *et al.*, 2008; Trummer *et al.*, 2006). It illustrates that, in addition to having the optimal cell line and process, it is crucial to have the optimal cell culture medium and feed to maximize performance potential.

CONCLUSION

Progress in cell-culture technology has led to substantial enhancement in the production of recombinant protein products; however, media development continues to remain a key activity that is not completely understood. Highest titers of anti-ZEN mAb were obtained with maltose supplemented media. So, it is concluded that specific monoclonal antibody production, hybridoma density, and viability could be enhanced by adding superior nutrients to the basal cell culture media in clinical, biological and pharmacological applications. The synergistic approach of using a selective combination of supplements at specific times of addition holds potential to improve antibody productivity.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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