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Full Length Research Paper

Generation and characterization of a monoclonal antibody to penicillic acid from *Penicillium cyclopium*

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Penicillic acid is one of the main mycotoxins in moldy feedstuff and has toxic effect on livestock and poultry and probably humans due to food chain transmission. The objective of this study was to generate and characterize a monoclonal antibody to penicillic acid for the efficient detection of penicillic acid from *Penicillium cyclopium* by immunological methods. To this end, penicillic acid was conjugated to bovine serum album (BSA) using the Mannich reaction and coupled with ovalbumin (OVA) by the method of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) to generate artificial antigens penicillic acid-BSA and penicillic acid-OVA. A hybridoma cell line was obtained after fusion of mouse SP2/0 myeloma cells with spleen cells of BALB/c mice immunized with artificial antigen penicillic acid-BSA. A monoclonal antibody specific against penicillic acid was produced *in vivo* by this hybridoma cell line. Further analysis revealed that the monoclonal antibody to penicillic acid was of the IgG1 subtype, with a titer of 1: 2.05 × 10^5. The antibody to penicillic acid had no or less cross-reaction with mycotoxins, including aflatoxin B1, zearalenone, T-2 toxin and fumonisins and more importantly, it assumed an affinity of about 1.54 × 10^8 liters per mol. Our ability to produce a monoclonal antibody to penicillic acid provides necessary groundwork for the effective detection of penicillic acid in various tissues of animals and human, using the immunocytochemistry, Western blots and enzyme-linked immunosorbent assay (ELISA).

Key words: Penicillic acid, cell fusion, monoclonal antibody, affinity.

INTRODUCTION

Penicillic acid is one of the main poisonous metabolites generated from *Penicillium cyclopium*. In 1931, Alsbrg and colleagues first separated penicillic acid from corn contaminated with *Penicillium puberulum*. It has been reported that a total of 28 types of fungi including *Aspergillus, Penicillium* and *Phialide Penicillium,* could produce penicillic acid. Many feedstuff raw materials, such as sorghum, barley, oats, wheat, corn and rice, are able to produce penicillic acid with a high output and penicillic acid is regarded as the main mycotoxin in moldy feedstuff. Penicillic acid is a colorless and needle-like crystal with a melting point at 83°C. As lactones toxin, it can be easily dissolved in hot water, ethanol, ether and chloroform but not in pentane or hexane. Recent studies demonstrated that penicillic acid has toxic effect on pigs, chickens, dogs, rabbits, frogs, rodents and other species of animals and can cause damage to heart, liver, kidney and other organs (Sorenson and Simpson, 1986; Pandiyan et al., 1990; Bernhoft et al., 2004; Keblys et al., 2004). Penicillic acid is also considered as a potential carcinogen (Mori et al., 1984). Notably, the interaction of penicillic acid and

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Abbreviations: EDC, 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride; **BSA,** bovine serum albumin; **OVA,** ovalbumin; **PEG,** polyethylene glycol; **FCS,** fetal calf serum; **PBS,** phosphate buffer saline; **UV,** ultraviolet; **ELISA,** enzyme-linked immunosorbent assay; **HRP,** horseradish peroxidase; **HT,** hypoxanthine-thymidine; **HAT,** hypoxanthine, aminopterin, and thymidine; **DMF,** dimethylformamide; **NHS,** N-hydroxysuccinimide; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis.

other mycotoxins, including citrinin, cyclopiazonic acid, ochratoxin A and patulin, on animals contributes to cumulative toxicity and enhance its toxicity (Stoev et al., 2001; Bernhoft et al., 2004; Keblys et al., 2004). Recently, the pollution by penicillic acid has aroused considerable attention and thus, a rapid and effective detection of penicillic acid is essential for identifying and quantifying penicillic acid in food and feeds.

There are many analytical techniques for measuring mycotoxins. Among these techniques, immunogenic assays that have been extensively developed due to its higher specificity and sensitivity and can be applied to samples with little or no clean-up, such as enzyme- linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and immunoaffinity chromatography. All of these methods are antibody-based assays; therefore, the specificity and sensitivity of the antibodies used are of essence. Since penicillic acid is a low-molecular-weight hapten, which is not immunogenic, we conjugated penicillic acid with a protein carrier to gain antibody. Monoclonal antibodies are widely used in the development of diagnostic and research reagents of mycotoxins due to its high homogeneity and can be produced in potentially unlimited amounts. With this in mind, we set out to develop the monoclonal antibody to penicillic acid for the development of immunodiagnostic assays. In the study reported here, we produced and characterized a mouse monoclonal antibody to penicillic acid by the hybridoma technology and compared its affinity and specificity for penicillic acid from *P. cyclopium*.

MATERIALS AND METHODS

Chemicals and antibodies

Penicillic acid was purchased from Iris Biotech GmbH Co. and then stored in 100% ethanol at -20℃. Imject® Pharmalink™ Immunogen Kit was purchased from Pierce. Water-soluble carbodiimide (EDC), Ovalbumin (OVA), coomassie brilliant blue, horseradish peroxidase (HRP) goat anti-mouse immunoglobulin G (IgG), hypoxanthine, aminopterin, and thymidine (HAT) medium, hypoxanthine-thymidine (HT) medium, 50% polyethylene glycol (PEG) and mouse monoclonal antibody isotyping reagent (IgG1, IgG2a, IgG2b, IgG3, IgM, IgA) were obtained from Sigma. PRMI1640 medium, newborn calf serum and fetal calf serum (FCS) were obtained from Hyclone Chemical Co. Freund's complete adjuvant and Freund's incomplete adjuvants were purchased from Gibco Chemical Co. The Sp2/0 myeloma cells were purchased from the Preservation Center of Typical Culture in China and BALB/c mice were from Laboratory Animal Center in Central South University in China. The use and care of animals were in accordance with the guideline of Hunan Agricultural University.

Generation and identification of artificial antigens

Penicillic acid was coupled to bovine serum albumin (BSA) by the Imject® Pharmalink™ Immunogen Kit (77158). 500 ug of penicillic acid per 2 mg of protein was used in the test. The reaction was incubated at 40℃ for 20 h and the immunogen was separated by D-Salt™ Dextran Plastic Desalting columns. Penicillic acid was conjugated with OVA by the carbodiimide method. Two milligrams of penicillic acid, 17 mg OVA, 23 mg EDC and 9.2 mg N-hydroxysuccinimide (NHS) were fully dissolved into 1 ml of dimethylformamide (DMF) and kept for 6 h at 4℃. 6.5 mg BSA were dissolved into 2.0 ml of 0.01 M phosphate buffer saline (PBS, PH7.4) and then reaction liquid was added slowly while shaking and kept at room temperature for 12 h. The resultant products were dialyzed with 0.01 M PBS (pH 7.4) at 4℃ for 72 h to remove the un-conjugated penicillic acid. The dialysis fluid was changed once every four hours on the first day and once every 12 h. The immunogen was saved at -20℃, respectively. Ultraviolet (UV) full-length scanning of penicillic acid-BSA and penicillic acid-OVA was performed and the coupling ratio and concentration of the artificial antigen were calculated.

Immunizations

Each of twelve 8-week-old BALB/c mice was administered by intraperitoneal injection of 100 µg of penicillic acid-BSA conjugate in 0.1 ml PBS emulsified with an equal volume of Freund's adjuvant Emulsion at 2-week intervals. The complete Freund's adjuvant Emulsion antigen and booster adopted incomplete Freund's adjuvant were used. Blood was taken from the tail vein at the 7th day after being immunized. The serum was screened by an indirect ELISA for specific serum antibody induction and the mice with the best specificity were given an intravenous boost of conjugate without adding adjuvant three days before spleens were removed.

Hybridization

Spleen cells from immunized mice were fused with Sp2/0 myeloma cell line as described by Galfre and Milstein (1981) with some modifications. Peritoneal macrophages of the mice served as feeder cells using the conventional method at 1 - 2 days before the fusion. About 10^9 spleen cells of the immunization-strengthened mice and 10^8 Sp2/0 cells were mixed in the presence of 50% PEG 1450 (pH 8.0) in 1640 medium and then the cells were cultivated with HAT selective medium on 96-well microtiter plates with feeder cells and incubated in 5% CO_2 at 37℃. HAT medium was changed with HT medium 7 - 10 days later and the growth of hybridoma was observed. The supernatants of culture medium were measured by an indirect ELISA for detecting the secreted antibody when the cells became 1/10 of confluence. The positive hybridoma cells were subcloned by limiting dilution for three successive times until the positive percentage reached 100% and they were cultured for expansion and subsequently cryopreservated.

Screening ELISA

An indirect ELISA was used to select specific antibody-producing clones. ELISA plates were coated with 100 µl penicillic acid-OVA conjugate by incubation for 1 h at 37℃ and overnight at 4℃ and washed 3 times with PBS. Each well was blocked with 100 µl 1% BSA at 37℃ for 1 h and washed 3 times with PBS. 100 µl serum or culture supernatants was added to each well and incubated at 37℃ for 1 h. After washes for 3 times with PBS, 100 µl HRP goat anti-mouse IgG was added and incubated at 37℃ for 1 h. The 3,3',5,5'-tetramethyl benzidine liquid was used as the substrate and the absorbance was measured at 490 nm using a UV scanning analysis.

Generation and purification of monoclonal antibody to pecinillic acid

BALB/c mice were injected intraperitoneally with 0.5 ml sterilized

liquid paraffin and then with 0.5 ml hybridoma (1 ~ 2 × 10^6 cells/ml) 1 - 2 weeks later. Ascites were gathered when the abdomens of mice were augmented apparently (usually 7 - 12 days after hybridoma injection). Centrifuge was performed to remove oil and supernatant was saved at -20℃. Saturated ammonium sulfate and caprylic acid - ammonium sulfate precipitation were used for the purification of ascites. Ascites, after purification for many times were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the purity of the purified antibodies and the purified ascites were saved at -20℃.

Characterization of monoclonal antibody to pecinillic acid

Analysis of hybridoma chromosome

The hybridoma were treated with 0.1 μg/ml colchicine and cultivated for 4 h at 37℃. The cells were harvested and resuspended in 5 ml of 0.075 mol/L potassium chloride hypotonic solution at 37℃ for 20 min. After centrifuging, the hybridoma was suspended three times with methanol: acetic acid stationary liquid and cytospin of the cells to glass slides was conducted. Giemsa staining was performed and the cells with good dispersion, no duplication and no dissipation of chromosomes were counted.

Determination of isotype of the monoclonal antibody to pecinillic acid

An indirect ELISA method was employed for determining the isotype of monoclonal antibody to pecinillic acid. ELISA plates were coated with monoclonal antibody (100 μl per well) and incubated overnight at 4℃. After 3 times of washes with washing buffer, the isotype specific reagents were added to different wells, respectively, and incubated for 30 min at room temperature. Goat anti-mouse IgG labeled with HRP was then added and incubated for 30 min at room temperature. After extensive washes, freshly prepared substrate was added to each well and incubated at room temperature. The reaction was terminated after 10 - 15 min and absorbance was measured using an ELISA reader.

Analysis of monoclonal antibody specificity

An indirect competitive ELISA method was used to identify the specificity of the monoclonal antibody to pecinillic acid. BSA, OVA and standard penicillium acid, aflatoxin B1 (AFB1), zearalenone (ZON), T-2 toxin and fumonisins (FB) with different concentration (1 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml and 1 μg/ml) were mixed with the diluted monoclonal antibody to pecinillic acid with equal volume, respectively, and incubated overnight at 4℃. Then the mixture was added into plates coated with pecinillic acid-OVA for reaction. After goat anti-mouse HRP conjugate and substrate were added, the absorbance of each well was measured for analysis. The rate of competitive inhibition was calculated using the formula

$$\text{Rate} = (A_1 - A_2)/A_1 \times 100\%$$

Where, A_1 represent the value of positive well and A_2 represent the value of blocking well. The rate of cross-reaction was calculated using the formula

$$\text{Rate} = C_1/C_2$$

Where, C_1 represent the concentration of pecinillic acid for 50% inhibition and C_2 represent the concentration of pecinillic acid's competitor for 50% inhibition.

Determination of monoclonal antibody affinity

The affinity of monoclonal antibody to pecinillic acid was measured according to the procedure described as follows. Briefly, after coating plates with different concentration of complete antigen (1.25 μg, 2.5 μg and 5 μg /ml), the monoclonal antibody to pecinillic acid with various dilutions was added to each well and then the goat anti-mouse HRP conjugate and substrate were added subsequently. After termination of reaction, the absorbance value (A) of each well was measured. A curve diagram was made to show the relationship between the concentration of antibodies as the abscissa and the value of absorption as the ordinate and get the concentration of relevant antibodies when the absorption value was 50% (50% of A value) by taking advantage of 100% of A value at the flat section of the upper part of each curve. The K value was calculated according to the formula

$$K = (n-1)/2(nAb' - Ab)$$

Where, n = Ag/Ag′, Ag and Ag′ represented different concentration of antigen and corresponding Ab and Ab′ represent the concentration of antibody. The average of three K values was the affinity constant. Averages and standard errors between replicate samples were calculated using Excel 2003.

RESULTS

Identification of artificial antigen

UV scan showed that penicillic acid reached its maximum absorption peak at 237 nm, BSA at 279 nm and OVA at 278 nm, while artificial antigen PA-BSA reached its highest absorption peak in the peptide chain at 216 nm and PA-OVA at 210 nm (Figure 1A). The original characteristic absorption peak of the carrier protein BSA and OVA was shifted to the characteristic absorption peak of PA and the peak value decreased apparently, suggesting that PA was linked with the carrier protein BSA. The artificial antigen synthesized in SDS-PAGE trailed slightly but there was no significant difference from the carrier protein (Figure 1B, right panel), indicating that the molecular weight of artificial synthetic antigen was slightly heavier than the molecular weight of its relevant carrier (Figure 1B).

Cell fusion and selection of positive clones

The fusion rate was 80.1% (385 wells of clone cells of five 96-well microtiter plates) and the rate of positive clones was 12.5% (48 holes of positive clones). We got a positive clone cell line named 4C2-F8-D2 in response to penicillic acid, after indirect competitive ELISA screening and cloning for three times.

Purification of monoclonal antibody to penicillic acid

According to SDS-PAGE analysis, monoclonal antibody of the purified monoclonal antibody ascites had two distinct

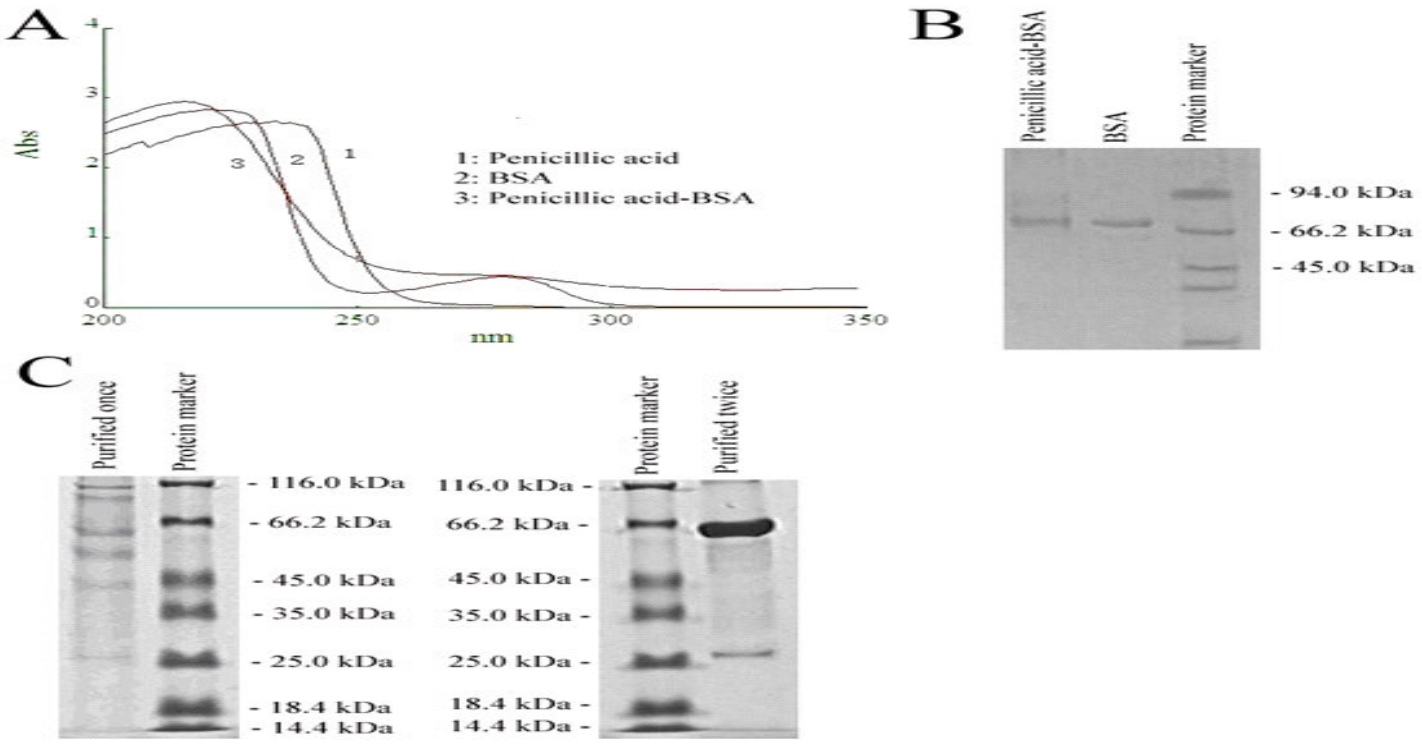

Figure 1. Biochemical analysis of penicillic acid-BSA, penicillic acid-PA and a monoclonal antibody to penicillic acid. (A) The ultraviolet scanning assay of penicillic acid (0.2 mg/ml), BSA (0.4 mg/ml) and penicillic acid-BSA (0.31 mg/ml). (B) SDS-PAGE analysis of BSA and penicillic acid-BSA. (C) SDS-PAGE analysis of a monoclonal antibody to penicillic acid after purification.

bands, one heavy chain band with about 57 kDa and the other light chain band with about 25 kDa. The molecular weight of the protein was 164 kDa (Figure 1C).

Characterization of monoclonal antibody to penicillic acid

Chromosome counts revealed that the chromosome number of SP2/0 myeloma cells was 60 - 68 (Figure 2A) and that the chromosome number of mice spleen cells was 40 and the chromosomes number of the hybridoma was 95 - 108 with an average of 98 (Figure 2B). The protein concentration of the monoclonal antibody to penicillic acid was 2.56 mg/ml according to the UV scanning result of purified ascites. The antibody titer of positive hybridoma cell line was 1: 400 and ascite titer was 1: 2.05 × 10^5 as measured by an indirect ELISA. There was no significant difference in the titer of ascite antibodies before and after purification (data not shown). Identification with an indirect ELISA displayed that the antibody type of the monoclonal antibody subclass to penicillic acid was IgG1.

For the identification of specific monoclonal antibody, we drew curves respectively based on the results of the ELISA competition between antibodies with different dilution and penicillic acid, BSA, OVA, aflatoxin B1, zearalenone, T-2 toxin and fumonisins and figured out half-inhibition rate according to these curves and cross reaction rate. Our data showed the better specificity of monoclonal anti-penicillic acid in response to penicillic acid and low cross-reaction with other antigens (Table 1). The determination of monoclonal anti-penicillic acid affinity is based on the relationship between absorbance and degree of dilution. Our results showed that the affinity constant of monoclonal antibody to penicillic acid was 1.54 × 10^8 L/mol (Figure 2C).

DISCUSSION

Penicillic acid belongs to a mycotoxin with low small molecular weight. Since penicillic acid has no immunogenicity, it can not stimulate the body to produce specific antibodies. However, penicillic acid can acquire its immunogenicity after it is coupled with carrier proteins. At present, the commonly used carrier proteins are bovine serum albumin, ovalbumin, human serum albumin, etc. In comparison to the difficulty of coupling between haptens and carriers, bovine serum albumin is rich in lysine and it facilitates its combination with haptens. In this study, we used the Minnch reaction to couple penicillic acid and protein carrier BSA. Certain sufficiently active hydrogens can be condensed with formaldehyde and be amino-

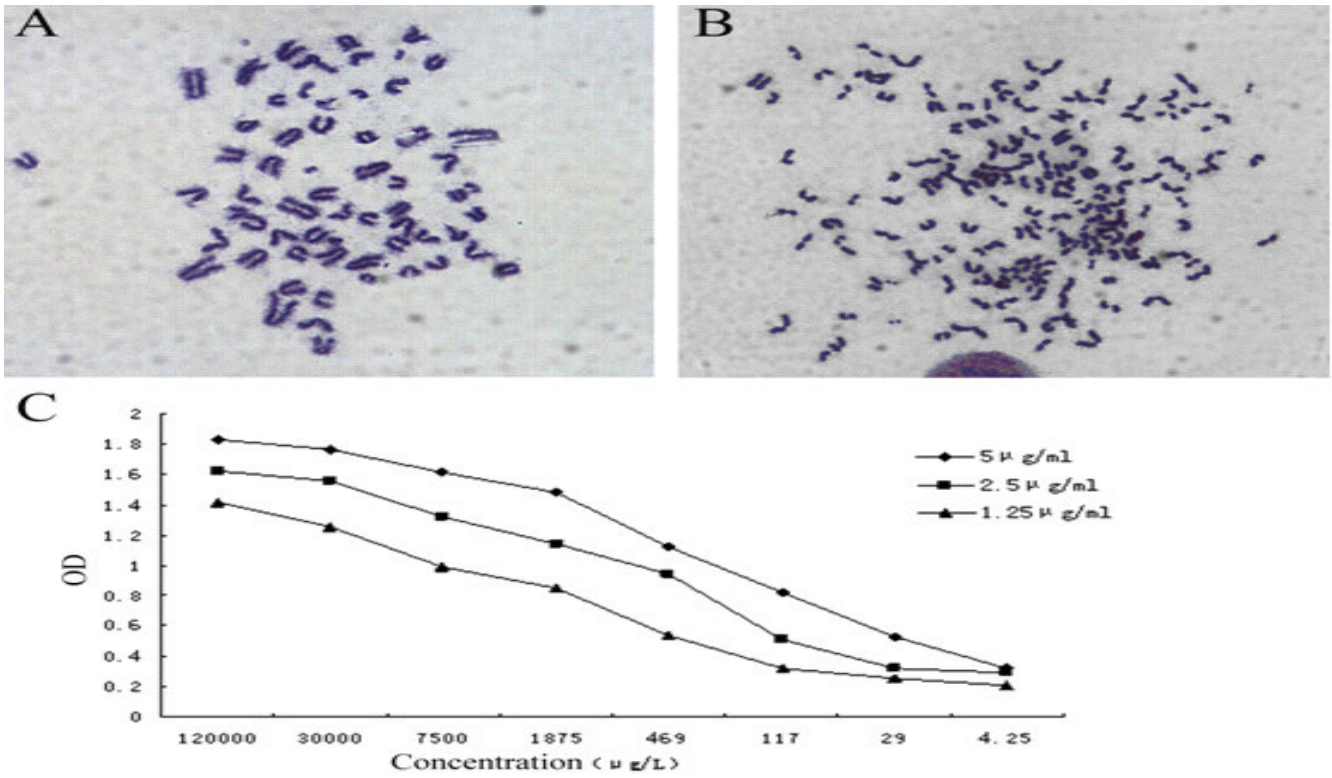

Figure 2. The karyotype analysis of hybridomas and SP2/0 myeloma cell and monoclone antibody dilution curves. (A) Representative karyotype photos of hybridomas (B) and SP2/0 myeloma cell (B) were shown (C) The dilution curves of monoclone antibody to penicillic acid at different coated antigen concentrations.

Table 1. Cross-reaction of monoclonal anti-penicillic acid with various antigens.

Antigens	Croos-reaction
Penicillic acid	100%
Aflatoxin B1	< 0.01%
Zearalenone	< 0.01%
T-2 toxin	< 0.01%
Fumonisins	< 0.01%
BSA	< 0.01%
OVA	< 0.01%

alkylated in the reaction (Figure 3). The result of UV scanning analysis and animal immunological test show that a strong immune response is produced after the mice are immunized, indicating that the synthetic artificial antigen of penicillic acid-BSA has a good immune effect.

Monoclonal antibody can be utilized to improve the sensitivity and specificity of detection of toxin antigens in view of its high purity and strong specificity. Mycotoxins including penicillic acid have serious toxicity to animals and probably human due to food chain transmission (Sorenson and Simpson, 1986; Pandiyan et al., 1990; Bernhoft et al., 2004; Keblys et al., 2004). Therefore, it is of great significance to generate monoclonal antibody for the clinical detection of mycotoxin (Yoshizawa et al., 2004). In this study, a monoclonal antibody specific against penicillic acid has been successfully produced by immunizing animals with synthetic artificial antigen penicillic acid -BSA and hybridoma technology using cell fusion, clone screening, expansion of cultivation, induction of antibodies in animals, purification, etc. Our results obtained from ELISA, SDS-PAGE and chromosome analyses reveal that the average number of chromosomes in hybridoma cells is about 98 and prepared monoclonal anti- penicillic acid can bind penicillic acid specifically but not the carrier proteins. Further analysis shows that the antibody titer is 1: 2.05×10^5 and antibody subtype is IgG1. The monoclonal antibody to penicillic acid has good specificity since it has no or very less cross reaction with other mycotoxins, including aflatoxin B1, zearalenone, T-2 toxin and fumonisins. The cultivated supernatant of hybridoma cells not only has specific antibodies, the ascites, but also contains non-antibody protein. Therefore, it is necessary to purify the proteins. The commonly adopted ways of separation and purification include salting-out, gel filtration, ion-exchange and affinity chromatography (Maragos et al., 2006). By purifying antibodies twice with the methods of caprylic ammonium sulfate and ammonium sulfate, the antibody concentration is much higher and purer than that produced with a single method. Besides, this purifi-

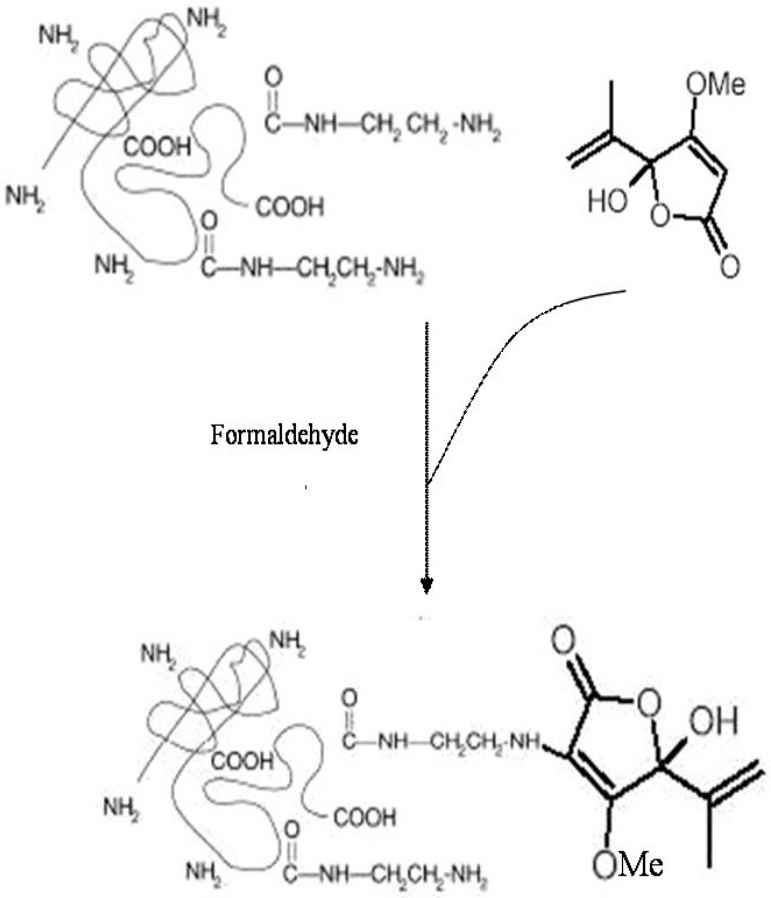

Figure 3. The synthesis path of PA-BSA by the Mannich reaction.

cation method has no apparent influence on the activity of monoclonal antibodies. Many studies show that the antibody with its affinity ranging from 10^5 to 10^{12} L/mol has better effect of application (Maragos et al., 2008). The affinity constant of our monoclonal antibody to penicillic acid is 1.54×10^8 L/mol, which would provide a necessary basis for the rapid and effective immunoassay detection of penicillic acid, such as the further development of *P. cyclopium* toxin – penicillic acid ELISA kit.

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