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Studies on Purification of Methamidophos Monoclonal Antibodies and Comparative Immunoactivity of Purified Antibodies[1]

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Objective To purify Methamidophos (Met) monoclonal antibodies with two methods and compare immune activity of purified antibodies. **Method** Caprylic acid ammonium sulphate precipition (CAASP) method and Sepharose protein-A (SPA) affinity chromatography method were used to purify Met monoclonal antibodies, UV spectrum scanning was used to determine protein content and recovery of purified antibodies, sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the purity of purified antibodies, and enzyme-linked immunosorbent assay (ELISA) was used to determine immune activity of purified antibodies. **Results** Antibody protein content and recovery rate with CAASP method were 7.62 mg/mL and 8.05% respectively, antibody protein content and recovery rate with SPA method were 6.45 mg/mL and 5.52% respectively. Purity of antibodies purified by SPA method was higher than that by CAASP method. The half-maximal inhibition concentration (IC_{50}) of antibodies purified by SPA to Met was 181.26 μg/mL, and the linear working range and the limit of quantification (LOD) were 2.43-3896.01 μg/mL and 1.03 μg/mL, respectively. The IC_{50} of antibodies purified by CAASP to Met was 352.82 μg/mL, and the linear working range and LOD were 10.91-11412.29 μg/mL and 3.42 μg/mL, respectively. **Conclusion** Antibodies purified by SPA method are better than those by CAASP method, and Met monoclonal antibodies purified by SPA method can be used to prepare gold-labelled testing paper for analyzing Met residue in vegetable and drink water.

Key words: Methamidophos; Monoclonal antibody; Purification; Immunoactivity

INTRODUCTION

Methamidophos [O,S-Dimethylphosphor-amidothiolate](Met), a broad spectrum organophosphorus insecticide and acaricide used all over the world, is the largest tonnage pesticides in the 19th-20th century in China[1]. Because of its use for fruits, vegetables and tea plants in violation of a ban or out of control, many cases of methamidophos poisoning were reported[2-4]. From beginning in 2001, Ministry of Agriculture and many provincial

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governments, such as Zhejiang, Shanghai, Guangdong in China have published documents to forbid the use of methamidophos pesticide, and therefore it is necessary to detect methamidophos residuel in vegetable, food, soil and drink water. The most commonly used methods for analysis of methamidpphos are GC, HPLC, and thin-layer chromato-graphy (TLC) *et al.*[5,6]. But these methods are laborious and time-consuming and require sophisticated equipment only available in well-equipped laboratories. Immunoassay is an alternative method, which is rapid, sensitive, and cost-effective[7-10]. In the past, we synthesized successfully Met artificial antigen, and prepared monoclonal antibodies of anti-methamidiphos[11,12]. Here we report two methods for purifying monoclonal antibodies of anti-methamidiphos, and comparing immunoactivity of these purified antibodies.

MATERIALS AND METHODS

Materials

Methamidophos(O,S-Dimethylphosphor-amidothiolate), ≥99%, was purchased from Institute of Chemistry and Industry (Shenyang, China), Sepharose protein-A (SPA) gel media and columns were purchased from Pharmacia Co. (Sweden). Horse radish peroxidase (HRPO)-goat anti-mouse IgG was purchased from Sigma Chemical Company. Ninety-six well-polystyrene microtitration plates were purchased from Haimei Co. (Jiangsu, China). Phosphate-buffered saline pH 7.4 (PBS): 8 g/L of NaCl, 0.2 g/L of $NaH_2PO_4 \cdot 2H_2O$, 2.9 g/L $Na_2HPO_4 \cdot 12H_2O$. PBS containing 0.05% tween 20 (PBST) for washing steps, substrate buffer: citric acid 0.94 g, $Na_2HPO_4 \cdot 12H_2O$ 3.68 g in 200 mL distilled water. Other reagents were all of analytical grades, made in China. Absorbance value obtained in ELISA was read and recorded with a Dynatech MR-700 microplate reader (Sussex, U. K.). UV spectra and visible-region absorbance was measured by a spectrophotometer (Ultraspec 4000, Pharmacia Biotech Co.).

Monoclonal Antibodies

Anti-methamidophos monoclonal antibody cell line used in this study was described in a previous publication[12]. Ascites tumors were induced by injection of 5.5×10^6 hybridoma cells into immunosuppressed four BALB/c mice. Forty days after, ascite fluids were collected in sterile conditions.

Pre-treatment of Ascites Fluids

Ascites fluids were centrifuged at 20 000 × g for 30 min at 4℃ prior purification.

Caprylic Acid Ammonium Sulphate Precipition (CAASP) Method for Antibodies Purification

CAASP method was performed according to Ogden[13], with the following minor modification. One point five mL ascites fluids above was diluted with 4 mL 60 mmol/L acetate buffer (NaAc • $3H_2O$, 8.2 g, glacial acetic acid 18.2 mL, with distilled water to 100 mL, pH 5.0), and the pH was adjusted to 4.8 with 0.1 mol/L hydrochloric acid at room temperature, then 33 μL caprylic acid was added slowly, after laying up for 2 h at 4℃, centrifuged 30 min (10 000 r/min) to remove precipitate; the supernatant was filtered through 0.2 μm millipore film, 1/10 volume 0.1 mol/L PBS was added to the filtered fluids, the pH was adjusted to 7.4 with 1 mol/L NaOH, then at 0℃, saturation ammonium sulfate was

added to the above filtered fluids, making its concentration to 45%. Laying up for 1 h and centrifuged 30 min (10 000 r/min), the precipitation was dissolved with 0.1 mol/L PBS containing 137 mmol/L NaCL, 2.6 mmol/L KCL, 0.2 mmol/L EDTA and dialyzed overnight in the above PBS, centrifuged 10 min (10 000 r/min), and 1.85 mL supernatant was collected.

Sepharose Protein-A (SPA) Affinity Chromatography Method for Ascites Fluids Purifying

The gel media used were preswollen protein A-sepharose, with high binding capacity to IgG. SPA affinity chromatography was carried out with a 2/10 column containing 5 mL protein A-sepharose, the gel had been equilibrated with PBS (NaCL 0.15 mmol/L, KCL 2.7 mmol/L, KH_2PO_4 5 mmol/L, Na_2HPO_4 8 mmol/L, pH 8.0). Before application to the column, 1.6 mL ascites fluids was diluted with 3.4 mL distilled water, then filtered through a 0.2 μm millipore film and put on the column. The column was washed with PBS after the absorbance of UV 280 nm had been dropped to baseline level. Bound IgG protein fluid was eluted with 0.05 mol/L glycine (pH 2.5). When absorbance value of UV 280 nm excessed 0.10, every 1 mL elution accepted to one 1.5mL eppendorf tube containing 50 μL antacid fluid (1.0 mol/L Tris, pH 10.5); however, when absorbance value of UV 280 nm lowered 0.10, the elution was stopped to accept. All elution was added up to 11 mL, and condensed with PEG6000, its total volume being 1.6 mL.

Determination of Protein Content and Recovery of Purified Ascites Antibodies

The method was suggested by Zhao[14]. Fourteen μL ascites fluid was diluted to 3 mL, 80 μL antibodies purified by CAASP method was diluted to 3 mL, 120 μL antibodies purified by SPA method was diluted to 3.12 mL, and then the three diluted fluids were scanned in 200-400 nm. Protein content and recovery were calculated according to the formulae below.

Protein content=(1.45×OD280-0.74×OD260 nm)×multiple dilution

Recovery=(protein content of antibodies purified/total protein content of ascites)×100%

Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

No purified ascites fluids or purified ascites as analyzed by SDS-PAGE in 10% separating gel, 5% concentrating gel according to Liu[15]. Electrophoresis was performed under a steady voltage of 150 V, the electrode buffer was tris-glycine SDS, pH 8.3. Protein was stained with Coomassie brilliant blue R-250, and then the gel was decolored and scanned with Ziguang scanner.

Comparative Immunoactivity of Purified Antibodies by ELISA

Six lines 10-well polystyrene microtitration plates were used as follows: in well 1100 μL PBS was added; in well 2-9, 100 μL, 0.1 μg/mL, 1.0 μg/mL, 10.0 μg/mL, 50.0 μg/mL, 100.0 μg/mL, 500.0 μg/mL, 1000.0 μg/mL, 10000.0 μg/mL, Met in PBS was added respectively, in well 10, 200 μL PBS was added. Then 100 μL 0.06 μg/μL antibodies purified by CAASP method was added to well 1-9 of three lines plates above, 100 μL 0.06 μg/μL antibodies purified by SPA method was added to well 1-9 of another three lines plates above, then the six lines plates were incubated for 1 h at 37℃, 100 μL/well was added to six lines 2 μg/mL OVAM pre-coated and blocked plates[12], incubated for 1 h at 37℃, washed with PBST for four times, 100 μL/well diluted (1:3 000, PBS) HRPO-goat anti-mouse IgG was added, incubated at 37℃ for 1 h, washed; then 100 μL of 1,2-phenylendiamine (0.4 mg/mL in substrate buffer) containing 0.4 μL/mL hydrogen per- oxide (30%,v/v) was added to each well, incubated for 15 min. Color development was terminated by adding of 50 μL/well of

10% H_2SO_4, and the absorbance was read at 492 nm.

Met inhibition standard curves were drawn with logarithm value of Met concentration as abscissa (lg[Met(μg/mL)]), B/B_0% as vertical coordinate.

$B/B_0\%=(OD_n$ of average value of three wells - OD_{CK} of average value of three wells)/ (OD_1 of average value of three wells- OD_{CK} of average value of three wells) × 100%

n= 2, 3, 4, 5, 6, 7, 8, 9

SAS statistics method was used to obtain Met inhibition recursive equation, and the half-maximal inhibition concentration (IC_{50}) was the concentration of Met when B/B_0% was 50%. The linear working range was the inhibition concentration at 80%-20% of B/B_0%, and the limit of quantification (LOD) was the inhibition concentration at 90% of B/B_0% according to the suggestion of Hennion[16].

RESULTS

Results of Protein Content and Recovery of Purified Ascites Antibodies

UV absorbance spectra figures of ascites fluids and antibodies purified by two methods are as follows:

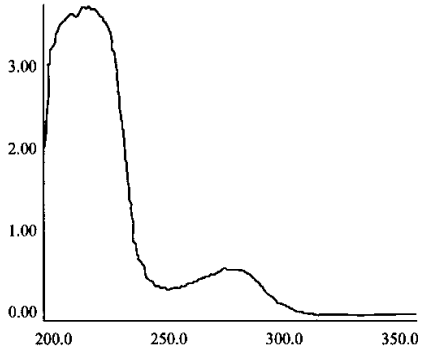

FIG. 1. UV spectrum of no purified antibodies in ascites fluids.

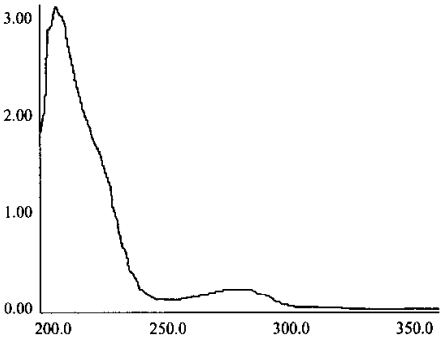

FIG. 2. UV spectrum of antibodies purified with CAASP method.

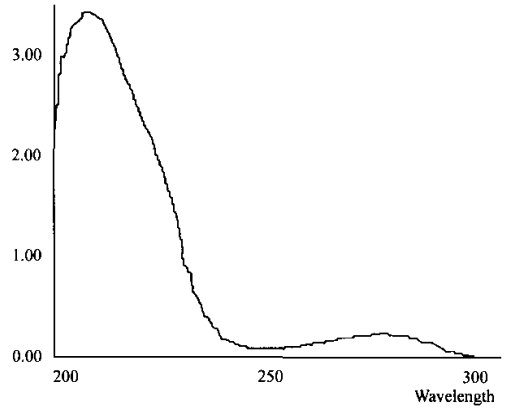

FIG. 3. UV spectrum of antibodies purified with SPA method.

According to UV absorbance value of OD280 nm and OD260 nm, protein content was calculated as follows:

Antibody protein content in no purified ascites (mg/mL)=(1.45×0.584−0.74×0.408)×(3000/14)=116.76

Antibody protein content with CAASP method (mg/mL)=(1.45×0.209−0.74×0.135)×(3000/80)=7.62

Antibody protein content with SPA method (mg/mL)=(1.45×0.245−0.74×0.145)×(3120/120)=6.45

Recovery of antibody protein with CAASP method=(1.85×7.62)/(1.5×116.78)×100%=8.05%

Recovery of antibody protein with SPA method=(1.60×6.45)/(1.6×116.78)×100%=5.52%

SDS-PAGE of Antibodies

Fig. 4 shows that antibody purified by SPA affinity chromatography method displayed two bands in SDS-PAGE, one was light chain of IgG molecule, and the other was heavy chain of IgG molecule, without impurity protein band. Antibodies purified by method of CAASP displayed three bands, one was light chain, the other was heavy chain, and the middle one was impurity protein band.

Immunoactive Comparison of Purified Antibodies by ELISA

Working concentration of two kinds of antibodies was determined as 0.06 μg/μL. The results of ELISA of purified antibodies are showed in Fig. 5. Met inhibition recursive equation was obtained with SAS statistics method. The recursive equation for antibodies purified by SPA was y=100.85-22.517x (R=-0.95, n=3). On the basis of reports by Krotzky *et al.* and Hennion *et al.*[16,17], the half-maximal inhibition concentration (IC_{50}) for antibodies purified by SPA to Met was 181.26 μg/mL, and the linear working range and the limit of quantification (LOD) were 2.43-3896.01 μg/mL, 1.03 μg/mL, respectively. The recursive equation for antibodies purified by CAASP was y=100.62-19.87x (R = -0.98, n=3).

The IC_{50} of inhibition standard curve for antibodies purified by CAASP to Met was 352.82 μg/mL, and the linear working range and LOD were 10.91-11412.29 μg/mL, 3.42 μg/mL, respectively.

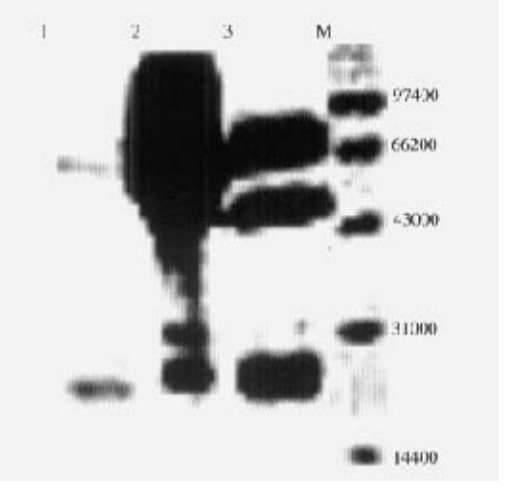

FIG. 4. SDS-PA7GE analysis of Met antibodies.
Lane 1 antibodies purified with SPA method, Lane 2 no purified antibodies of ascites fluids, Lane 3 antibodies purified with CAASP method, Lane 4 protein markers.

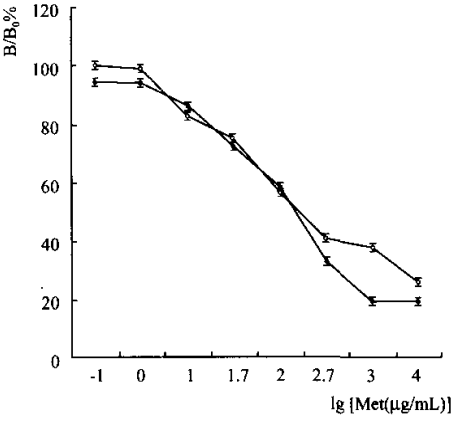

FIG. 5. Inhibition ELISA curve of purified antibodies.
○ antibodies purified by CAASP, ● antibodies purified by SPA.

DISCUSSION

CAASP method for purifying antibodies is widely used but its purity is low. SPA method for purifying antibodies is highly selective for the IgG class, by which nearly 100%

pure IgG can be obtained by one single step. Our results are similar to other reports[18]. Because the purity of antibody by CAASP is lower than that by SPA, so LOD and IC_{50} of antibody purified by CAASP are higher than those by SPA. Antibody purified by CAASP can not achieve testing criterion of Met residue in our country, so it can not be directly used to prepare gold-labelled testing paper, and has to be further purified for application. Antibody purified by SPA method can be used to prepare gold-labelled testing paper for analysis of Met residue. Therefore, the studies have laid an important basis for the preparation of gold-labelled testing paper for analyzing Met residue in vegetable and drink water.

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