Food Chemistry 112 (2009) 582–586

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Identification of tetrodotoxin antigens and a monoclonal antibody

Y. Zhou [a], Y.S. Li [b], F.G. Pan [b], Z.S. Liu [a], Z. Wang [c,*]

[a] Key Laboratory of Zoonoses, Ministry of Education, Institute of Zoonoses, Jilin University, 5333 Xian Road, Changchun, Jilin 130062, PR China
[b] College of Light Industry and Economics and Management of Jilin University, Changchun, Jilin 130062, PR China
[c] Jilin University Changchun, Institute of Farming and Veterinary, 5333 Xian Road, Changchun, Jilin 130062, PR China

ARTICLE INFO

Article history:
Received 4 March 2008
Received in revised form 1 May 2008
Accepted 4 June 2008

Keywords:
Tetrodotoxin (TTX)
Carrier protein
Molecular coupling ratio
Monoclonal antibody (mAb)

ABSTRACT

Two kinds of antigens for tetrodotoxin (TTX) were made, using carrier protein ovalbumin (OVA) and bovine serum albumin (BSA), by the formaldehyde method. Nondenaturing gel electrophoresis, gel filtration and ultraviolet spectrophotometry were employed to analyze the coupling of TTX to carrier proteins. The results indicate that nondenaturing gel electrophoresis and gel filtration can be employed to analyze the coupling of TTX to BSA and OVA qualitatively, and ultraviolet spectrophotometry can be employed to analyze the molecular coupling ratio of TTX to BSA and OVA quantitatively. Spleen cells from Balb/C mice immunized with an artificial TTX-formaldehyde-ovalbumin conjugate were fused with SP2/0 myeloma cells. A hybridoma cell line (4D5), which secreted IgG_1 subclass monoclonal antibody against TTX, was produced by "limiting dilution" cloning. The molecular weight, the affinity constant and the titre of the monoclonal antibody (mAb) secreted by 4D5 were 183.69 kDa, 0.98×10^8 M^{-1} and 3.6×10^5, respectively. The number of the hybridoma chromosome was 88–104.

1. Introduction

Tetrodotoxin is one of the most potent marine neurotoxins, which blocks the sodium channels of the neuron cell membrane of animals. It was originally isolated from puffer fish (Yokoo, 1950). It has also been detected from widely differing invertebrate species, such as starfish *Astropecten* spp. (Maruyama, Nogguchi, Jeon, Haranda, & Hashimoto, 1984), blue-ringed octopus *Octopus maculosus* (Sheumack, Howden, Spence, & Quinn, 1978), crab *Atergatis floridus* (Noguchi et al., 1983), gastropod *Zeuxis samiplicutus* (Li, Kun, Hwang, & Hwang, 2002) and flatworm *Planocera multitentaculata* (Miyazawa et al., 1986), and vertebrate species inclusive of frog, *Atelopus* (Kim, Brown, & Mosher, 1975), goby *Gobius criniger* (Noguchi & Hashimoto, 1973), goby *Yongeichthys nebulosis* (Chen, Chou, Chen, & Lee, 2002), and newt *Taricha* (Brown & Mosher, 1963). Human ingestion of improperly cooked toxic fish causes paralysis, numbness and death. TTX poisoning often occurs in Japan (Fukiya & Matsumura, 1992) and the coastal areas of China.

However, TTX, is a low molecular weight (319) hapten with no substructure to induce immunoreaction, which makes it necessary to couple TTX to a carrier protein in order to produce antibodies against TTX. Generally, an indirect method is used to determine whether the hapten has been successfully conjugated to the carrier protein. (The putative conjugate was injected into laboratory animals. Evidence that the hapten was coupled to the carrier protein was obtained after antibodies that recognize TTX were detected). But this method is problematic, since several valuable weeks of research may be lost and laboratory animals may be wasted while waiting to test the presence of antibodies against TTX. Therefore, it is necessary to develop a technique for detecting the successful conjugation of TTX to carrier proteins. The conjugates of hygromycin B, fumonisin B_1, zearalanone carboxymethol oxime and furosemide to BSA and OVA were analyzed by nondenaturing agarose gel qualitatively (Kamps, Carlin, & Shefield, 1993). The conjugations of ciprofloxacin (CPFX) to carrier proteins were successfully detected (Zhou, Li, Wang, Tan, & Liu, 2006). There is no report concerning detection of conjugates of TTX to carrier proteins. Herein, a nondenaturing gel electrophoresis (including agarose gel electrophoresis and polyacrylamide gel electrophoresis) and gel filtration have been developed to analyze the conjugation of TTX to BSA and OVA qualitatively and an ultraviolet spectrophotometer has been developed to measure the conjugation quantitatively. One hybridoma cell line secreting IgG_1 monoclonal antibody against TTX was produced by "limiting dilution" cloning, and the specialties of the monoclonal antibody were studied.

2. Materials and methods

2.1. Reagents and animals

TTX was purchased from Hebei province aquatic institute, China. Tris, polyacrylamide and glycin were obtained from Biotechs Co. Ltd. Dialysis pocket, agarose, complete and incomplete Freunds adjuvant, polythylene glycol-4000 (PEG), RPMI 1640, fetal bovine serum (FBS), horseradish peroxidase-conjugated goat anti-mice

* Corresponding author. Tel.: +86 0431 6986005.
E-mail address: zhouyu69@sina.com (Z. Wang).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.06.022

antisera, bovine serum albumin (BSA), ovalbumin (OVA), HT, HAT, and *o*-phenylenediamine (OPD) were purchased from Sigma Chemical Co. Myeloma cells SP2/0 were conserved by our laboratory. Female Balb/C mice, 6- to 7-weeks old and 9- to 10-weeks old, immunized and used to produce ascites, respectively, were obtained from Changchun Institute of Biological Products, Jilin province China.

2.2. Preparation of TTX-protein conjugates

TTX–BSA and TTX–OVA were prepared using a modified formaldehyde method (Raybould, Bignami, & Inouye, 1992). 700 μl of TTX (1 mg/ml in 0.05 M PBS, phosphoric acid buffer, pH 7.4), 300 μl of acetate buffer (1 M, pH 7.4) and 179 μl of BSA (33.6 mg/ml in 0.05 M PBS), 41 μL 37% formaldehyde were added dropwise, in that order, to an amber glass vial. Then the reaction mixture was librated by a HZQ-C libration bed for 72 h at 37 °C. The conjugation was dialyzed (MwCO, 10 kDa) against 100 ml of 0.01 M PBS (pH 7.4) at 4 °C for 24 h. The PBS was collected for the measurement of molecule coupling ratio of TTX to carrier protein. Then the conjugation was dialyzed against 1 l of 0.01 M PBS (pH 7.4) at 4 °C for 72 h with four changes of PBS to remove residual free TTX. The same method was employed to prepare TTX–OVA. The amount of OVA was 200 μl (50 mg/ml). The dosages of other reagents were the same as that of the TTX–BSA preparation.

2.3. Analysis of conjugates by nondenaturing agarose gel electrophoresis and nondenaturing polyacrylamide gel electrophoresis

The conjugates were analyzed using a modification of the methods of Kamps et al. (1993). Briefly, TAE was employed for electrophoresis buffer and the mixture solution of 0.04% bromophenoland blue and 6.67% sucrose was used as loading buffer. 1% agarose gel was 3–5 mm thick. Each sample (5 μg/μl) of 1 μl, mixed with an equal volume of loading buffer, was applied to the gel and samples were separated at 230 V for 30 to 40 min. The gel was fixed with 20% trichloroacetic acid for 30 min, stained with Coomassie blue R-250 for more than 2 h and destained with ethanol-acetic acid (250 ml 95% ethanol + 80 ml acetic acid, distilled water to 1000 ml) with several changes until clear. The pictures of the gels were taken by a UVI gel auto imaging system and by nondenaturing polyacrylamide gel electrophoresis, using an adaptation of the method of Joseph and David (2001). The mixture solution of 0.04% bromophenoland blue and 6.67% sucrose was used as loading buffer. Each sample (5 μg/μl) of 2 μl, mixed with an equal volume of loading buffer, was applied to the 10% concentrated gel and samples were separated at 60 V in concentrated gel and 120 V in separated gel until the indicators were removed from the gel. The gel was fixed with 20% trichloroacetic acid for 30 min, stained with Coomassie blue R-250 for more than 4 h and destained with ethanol–acetic acid, with several changes until clear. The pictures of the gels were taken by a UVI gel auto imaging system.

2.4. Analysis of conjugates by filtration gel

0.4 ml of conjugates (the concentration of carrier protein was 1.98 mg/ml) were diluted to 1 ml by PBS. The samples were separated through Sephadex G-50 (column size: 5 cm diameter and 40 cm length) and were monitored at 280 nm by a ZHD-3 protein monitor instrument. Sample flow velocity and paper speed velocity were 1 drop/50 s and 3 cm/h, respectively.

2.5. Analysis of conjugates by ultraviolet spectrophotometry

The ultraviolet absorbance of any substance is constant at a particular wavelength, and the absorbance of mixed solution equals the summation of all substances, separately, according to the formulas $A_T\lambda1 = K_T\lambda1C_TL$, $A_T\lambda2 = K_T\lambda2C_TL$, $A_F\lambda1 = K_F\lambda1C_FL$, $A_F\lambda2 = K_F\lambda2C_FL$ *(A: absorption value, K: absorption coefficient, C: the concentration of the substance being analyzed, L: the path length of the light through the sample,* λ: the wavelength, T: tetrodotoxin, F: formaldehyde). The absorption coefficients ($K_T\lambda1$, $K_T\lambda2$, $K_F\lambda1$, $K_F\lambda2$) and the absorbance values ($A_T\lambda1$, $A_T\lambda2$, $A_F\lambda1$, $A_F\lambda2$) of formaldehyde and TTX at the wavelength of λ_1 and λ_2 can be obtained.Formula (3) can be obtained from formula (1) and formula (2).

$$A_{(T+F)}\lambda1 = K_T\lambda1C_{TX}L + K_F\lambda1C_{FX}L \quad (1)$$

$$A_{(T+F)}\lambda2 = K_T\lambda2C_{TX}L + K_F\lambda2C_{FX}L \quad (2)$$

$$C_{TX} = C_T[(A_F\lambda1 * A_{(T+F)}\lambda2 - A_F\lambda2 * A_{(T+F)}\lambda1)] / A_T\lambda2 * A_F\lambda1 - A_T\lambda1 * A_F\lambda2. \quad (3)$$

$A_{(T+F)}\lambda1$ and $A_{(T+F)}\lambda2$ are the absorbance values of dialysis buffer at the wavelengths $\lambda1$ and $\lambda2$, respectively. C_{TX} and C_{FX} are the concentrations of TTX and formaldehyde in the dialysis buffer, respectively.

2.6. Animal immunization

A mixture of TTX–OVA conjugate (100 μg, 0.5 ml) and Freunds complete adjuvant (0.5 ml) was injected (female Balb/C mice, 6- to 7-weeks old) at multiple s.c. (subcutaneously) sites as the first immunization. The subsequent injections were i.p. (intraperitoneally) with 50 μg of TTX–OVA conjugate (0.5 ml) and the same volume Freunds incomplete adjuvant at the 3rd, 5th, 7th, and 9th weeks, respectively. After the third immunization, at the 10th day of each injection, the animals were bled by tail–tip cut method, and the serum was tested for its ability to bind to TTX–BSA or the carrier proteins OVA and BSA, in an enzyme-linked assay (ELISA): TTX–BSA, OVA and BSA were diluted to 1.0 μg/ml and dropped into a microtitre plate at 100 μl per well (four wells/each concentration) overnight at 4 °C. The plate was then washed three times with PBS-Tween for 3–5 s each time. The serum was diluted (from 1:500 to 1:160,000) and dropped into coated wells. Following a 1 h incubation at 37 °C, the plate was washed again. 100 μl of goat anti-mice IgG peroxidase conjugate (1:4000 dilution in PBS containing 0.1% BSA) were added to each well, and the plate was incubated for a further hour at 37 °C. The plate was washed again. The substrate solution (OPD), plus hydrogen peroxide in phosphate citrate buffer of 100 μl was finally added to each well. After developing the colour for about 20 min at 37 °C, the reaction was terminated with 50 μl of 2 M H_2SO_4 and the absorbance values at 492 nm were read in a spectrophotometer. The animals, whose sera bind to TTX–BSA only (no ability to bind BSA and OVA), and whose titres were 10^4 or higher, were selected to be spleen donors for hybridoma production, and received i.v. boosts of 20 μg TTX–OVA conjugate in PBS (pH 7.0). Four days after the final boost, spleens were removed from immunized mice.

2.7. Hybridoma generation

The immunized mice spleen cells were mixed with myeloma cells (SP2/0) at a 10:1 (spleen:myeloma) ratio in the presence of polyethylene glycol (PEG), mol. wt 4000, and plated into 96-well dishes in RPMI + 20% FBS/HAT medium at 37 °C in an atmosphere of 5% CO_2. Hybridomas were screened by ELISA for antibodies against TTX. Hybridomas showing significant TTX-specific inhibition were cloned three times by limiting dilution. One clone was chosen for further study. Hybridomas (2×10^6 cells for each mice) were injected into abdomens of the 9- to 10-week old Balb/C mice for seven days after liquid olefin was injected. The ascites can be obtained through the needle of a 20 ml injector about seven days later. The mAb was purified from ascites using the method of Zhou

et al. (2006). Classes and subclasses of monoclonal antibody were identified by the "mouse monoclonal antibody isotyping reagents" (Sigma), and the operational procedure based on its instruction manual.

2.8. Measurement of mAb affinity

The affinity of mAb for TTX was measured by a noncompetitive enzyme immunoassay according to the method of Dong and Wang (2002). Hybridoma chromosome was identified by the colchicine inhibition method. The number of the chromosome was counted and the photos were taken under oil lens by an Olympus microscope.

3. Results

3.1. Qualitative analysis of molecular conjugate ratio

The results of nondenaturing agarose gel electrophoresis are shown in Fig. 1. The conjugate band migrations were different from those of formaldehyde-treated proteins and carrier proteins alone. The migration of TTX–BSA and formaldehyde-treated BSA became decentralized, and the migration velocity of TTX–BSA was faster than that of BSA alone and slower than that of formaldehyde-treated BSA. The migration velocities of TTX–OVA, formaldehyde-treated OVA and OVA alone were the same as those of TTX–BSA, formaldehyde-treated BSA and BSA alone; the decentralized trend of the migration of TTX–OVA was not the same as those of formaldehyde-treated OVA and OVA alone.

The results of nondenaturing polyacrylamide gel electrophoresis are shown in Fig. 2. The resolving power of nondenaturing polyacrylamide gel electrophoresis is higher than that of nondenaturing agarose gel electrophoresis; therefore, each component being analyzed can be clearly separated. The changed trends of band migration velocities and the decentralizations of TTX-carrier proteins, formaldehyde-treated carrier proteins and carrier proteins alone were consistent with the results of nondenaturing agarose gel electrophoresis.

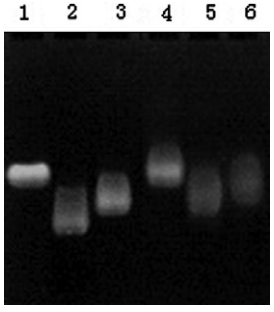

Fig. 1. Analysis of TTX–BSA conjugations and TTX–OVA conjugations by nondenaturing agarose gel electrophoresis. Lane 1. BSA, Lane 2. formaldehyde-treated BSA, Lane 3. TTX–BSA, Lane 4. OVA, Lane 5. formaldehyde-treated OVA, lane 6. TTX–OVA.

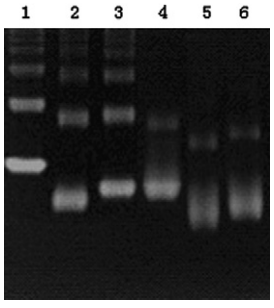

Fig. 2. Analysis of TTX–BSA conjugations and TTX–OVA conjugations by nondenaturing polyacrylamide gel electrophoresis. Lane 1. BSA, lane 2. formaldehyde-treated BSA, lane 3. TTX–BSA, Lane 4. OVA, lane 5. formaldehyde-treated OVA, lane 6. TTX–OVA.

3.2. Gel filtration

Not only was the apex form of the conjugations obviously different from those of the coupling reagent-treated protein and untreated carrier protein, but also the retention times of the conjugates were different from those of reagent-treated protein and untreated protein. The larger the molecule of the sample, the longer is the retention time of the sample. The retention time of the conjugates was shorter than those of the treated protein and untreated protein (Fig. 3). This indicates that TTX was successfully conjugated to the carrier proteins.

3.3. Quantitative analysis of molecular conjugation ratio

Different disposal methods from ciprofloxacin (Zhou et al., 2006) were employed to analyze the molecular conjugate ratio of TTX to BSA and OVA.

The concentrations of TTX in the first dialysis buffer of TTX–BSA and TTX–OVA were 2.44 μg/ml and 2.15 μg/ml, respectively, calculated by formulas (1) and (2). The TTX quantity that coupled to BSA and OVA were 453 μg and 283 μg. According to formula (3) and the molecular weight of TTX, BSA and OVA, the molecular conjugate ratio of TTX to BSA and OVA were 16:1 and 4:1, respectively.

3.4. Cell fusion, screening and selection

A hybridoma cell line designated 4D5, was established after being subcloned for 3 cycles by "limiting dilution".

The hybridoma cells 4D5 were expanded and injected into Balb/C mice abdomen. The mAb was obtained from ascites and purified by the caprylic/ammonium sulfate precipitation (CA–AS) method. The protein concentration of ascites was 26.1 mg/ml. The ELISA titres (the diluted multiple of ascites and purified mAb) were 3.2×10^5 and 3.6×10^5, respectively.

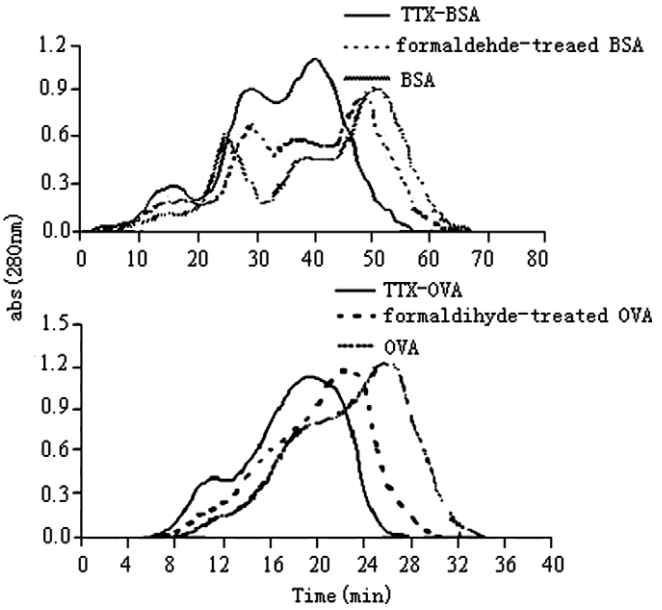

Fig. 3. Analysis of TTX–BSA and TTX–OVA conjugations by gel filtration (at 280 nm).

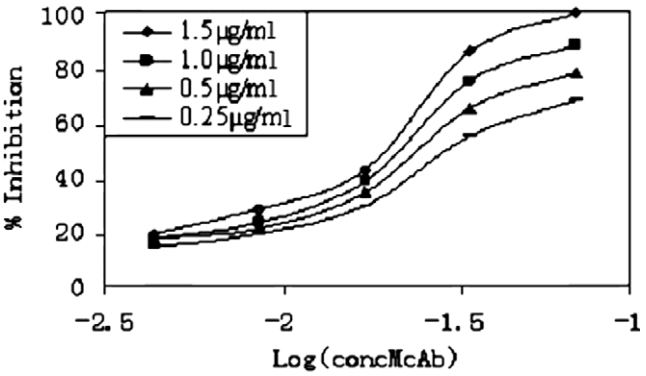

Fig. 4. The ELISA curves of different antigen concentrations.

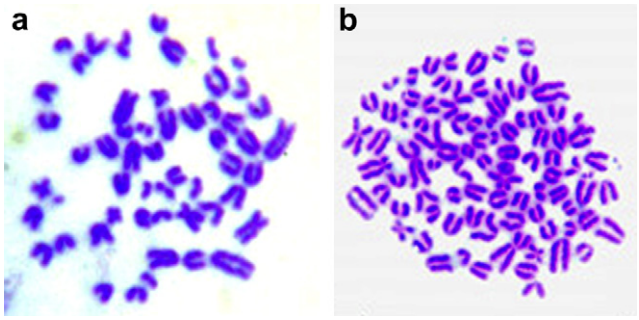

Fig. 5. The chromosome picture of SP2/0 cell and hybridoma cell: (a) SP2/0 cell and (b) hybridoma cell.

3.5. The subclasses, the affinity and the molecular weight of mAb

The mAb secreted by 4D5 belongs to the IgG_1 class. The measurement of mAb affinity is shown in Fig. 4. The average affinity is 0.98×10^8 M^{-1}. The molecular weight of mAb is 183.69 kDa, calculated by regression analysis.

3.6. Chromosome identification of hybridomas

The average chromosome numbers of SP2/0 and spleen cell were 62–70 and 38–40, respectively. The numbers of hybridomas were 88–104 (Fig. 5). The results indicated that the chromosomes of hybridomas were from SP2/0 and spleen cells of immunized mice. But some chromosomes were lost.

4. Discussion

There are many methods for analyzing molecular conjugation ratio of haptens to carrier proteins, such as the labelled antigen tracer method, nuclear magnetic resonance, and ultraviolet spectrophotometry. However, each method has it's own disadvantages (Zhou et al., 2006). In nondenaturing gel electrophoresis, a protein with a more negative charge migrates further in the gel towards the anode (+) than that with a less negative charge. In our study, the net charge of the conjugates becomes more negative than that of carrier protein and less than that of formaldehyde-treated protein. So the migration distances of conjugates were greater than that of carrier protein and the migration distances of formaldehyde-treated proteins were greatest (Figs. 1 and 2).

In gel filtration, the retention time of the protein is different because of the different molecular weight. The molecular weight of the conjugations became greater than that of the treated protein and untreated protein, so the retention times of conjugations were shorter. This further indicated that TTX was successfully coupled to carrier protein. For quantitative analysis, not all of the ultraviolet absorption values of carrier proteins which coupled with haptens will be changed; this depends on the characteristics of haptens. In our study, the ultraviolet absorption values of carrier proteins (BSA and OVA) which coupled with TTX, were not changed. But the ultraviolet absorption values of the same carrier proteins (BSA and OVA), which coupled with CPFX, were obviously changed (Zhou et al., 2006). Therefore, to analyze the molecular coupling ratio quantitatively, different methods should be employed according to the different haptens.

Not all carrier proteins can induce a strong immune response. The titration of antibody produced by BSA-TTX against TTX is lower (data not shown). So BSA is not an ideal carrier protein for producing immune antigen for TTX, but it is an ideal carrier protein for producing detection antigen for TTX (Wu, Wan, & Zhou, 1989). KLH is an ideal carrier protein for producing immune antigen for TTX. A higher titration of antiserum was obtained from immunized mice and rabbits by KLH–TTX (Schneid & Hammock, 1992) and a higher affinity mAb was also produced from KLH–TTX-immunized mice (Rong & Gong, 1980). Our study indicated that OVA is another ideal protein for producing immune antigen for TTX.

In our pre-studies, two kinds of immune schedules were used: one schedule involved lower dosages and shorter time periods. The immunization schedule was as follow: 50 μg of TTX–OVA conjugate (0.25 ml) were mixed with the same volume of Freunds complete adjuvant and injected at multiple s.c. sites as the first immunization. Two weeks later, the next injections were i.p. with 25 μg of TTX–OVA conjugate (0.25 ml) and the same volume of Freunds incomplete adjuvant at 2, 3, 4 and 5 weeks after the first immunization. After the third immunization, at the 7th day of each injection, the serum was tested. The other schedule is as in this paper, higher dosage and longer periods. The results showed that the titrations of the antisera were different from the two immune schedules. Higher dosage and longer periods are better than are lower dosage and shorter periods (the data will be published in another paper). This results are opposite to that of Xu, Huang, and Rong (2003). The difference may be caused by different molecular coupling ratios between TTX and carrier protein. Different molecular coupling ratios induce different immune results.

There are many factors that affect the cell fusion results, such as culture medium pH, the quality of FBS, the growth state of SP2/0, the ratio of spleen cell and SP2/0 numbers, and the experience of the operators. The PEG incubation period is one of the most important factors affecting cell fusion results. According to our pre-studies, the PEG incubation period should be kept within 4 min.

Many factors affect ascites output, such as the adjuvant, the strain, age and sex of the mice. Pristane and liquid olefin are often chosen as adjuvants; the ascites output by pristine is higher (3.2 ml/mouse) and the production time is shorter (9.5 d) than that by liquid olefin (Xu, Zhang, & Yu, 2002). For the same age mice, the ascites output by females is higher than that of males and, among the female mice, the ascites output is the highest by 9 to 10 week-old mice (Pan, Zeng, & Xu, 2003). In this study, liquid olefin was chosen as adjuvant and ascites inducing time and output were 7–10 days and 3–10 ml/mouse, respectively. Our study also found that the ascites output was different among different hybridoma cell lines.

Acknowledgements

This work was supported by Grants from the Ph.D priming Foundation of Jilin university (No. 430505010276) and the National Natural Science Foundation of China (No. 30771657, No. 30671762).

References

Brown, M. S., & Mosher, H. S. (1963). Tarichatoxin: Isolation and purification. *Science, 140*, 295–296.

Chen, C. Y., Chou, H. N., Chen, Y. M., & Lee, T. H. (2002). Detection of tetrodotoxin by HPLC in shellfishes and goby from south Taiwan. *Journal of Natural Toxins, 11*, 63–68.

Dong, Z. W., & Wang, Y. (2002). *Antibody Engineering*. Beijing: Beijing Medical University press (pp. 281).

Fukiya, S., & Matsumura, K. (1992). Active and passive immunization for tetrodotoxin in mice. *Toxicon, 30*, 1631–1634.

Joseph, S., & David, W. R. (2001). *Molecular Clone, A Laboratory Manual*. Cold Spring Harbor Laboratory Press (pp. 1713–1720).

Kamps, C., Carlin, R. G., & Shefield, L. (1993). Analysis of hapten-carrier protein conjugates by nondenaturing gel electrophoresis. *Journal of Immunological Methods, 164*, 245–253.

Kim, Y. H., Brown, G. B., & Mosher, H. S. (1975). Tetrodotoxin: Occurrence in atelopid frogs of Costa Rica. *Science, 189*, 151–152.

Li, M. S., Kun, C., Hwang, P. A., & Hwang, D. F. (2002). Identification of tetrodotoxin in marine gastropods implicated in food poisoning. *Journal of Natural Toxins, 11*, 213–219.

Maruyama, J., Nogguchi, T., Jeon, J. K., Haranda, T., & Hashimoto, K. (1984). Occurrence of tetrodotoxin in the starfish. *Astropecten latespinosus. Experientia, 40*, 1395–1396.

Miyazawa, K., Joen, J. K., Maruyama, J., Noguchi, T., Ito, K., & Hashimoto, K. (1986). Occurrence of tetrodotoxin in the flatworm Planocera multitentaculata. *Toxin, 24*, 645–650.

Noguchi, T., & Hashimoto, Y. (1973). Isolation of tetrodotoxin from a goby Gobius criniger. *Toxicon, 11*, 305–307.

Noguchi, T., Uzu, A., Koyama, K., Maruyama, J., Nagashima, Y., & Hashimoto, K. (1983). Occurrence of tetrodotoxin as the major toxin in a xanthid crab Atergatis floridus. *Buletin Japan Society Science Fish, 49*, 1887–1892.

Pan, X. W., Zeng, Y., & Xu, J. (2003). The comprehend of preparation and purification of hybridoma ascites monoclonal antibody with BALB/C mice. *Journal of Shenyang Medical College, 5*, 123.

Raybould, T. J. G., Bignami, G. S., & Inouye, L. K. (1992). A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples. *Journal of Clinical Lab Analysis, 6*, 65–72.

Rong, K. T., & Gong, X. Q. (1980). Antitoxic response of paraoxon–hemocyanin immunized rabbits. *Chinese Journal of Preventive Medicine, 14*, 301–304.

Schneid, P., & Hammock, B. D. (1992). Influence of the ELISA for *s*-triazine herbicide using monoclonal antibodies. *Journal of Agricultural Food Chemistry, 40*, 525–529.

Sheumack, D. K., Howden, M. E. H., Spence, I., & Quinn, R. J. (1978). Maculotoxin: A neurotoxin from the venom glands of the octopus Hapalochlaena maculosa identified as tetrodotoxin. *Science, 199*, 188–189.

Wu, S. R., Wan, Y. S., & Zhou, X. (1989). Immunoassay technic for plant small molecule substances. *Plant Physiology Communications, 5*, 68–72.

Xu, Q., Huang, K., & Rong, K. T. (2003). Comparison of immunologic effect between different immunization schedule of anti-tetrodotoxin vaccination in mice. *Journal of Medical Colleges of People Liberation Army, 28*, 449–451.

Xu, X., Zhang, J. F., & Yu, W. B. (2002). The effect factors of the productivity of hybridoma ascites. *Journal of Fourth Military Medical University, 23*, 1632.

Yokoo, A. (1950). Study on chemical purification of tetrodotoxin (3)—Purification of spheroidine. *Journal of the Chemical Society, 71*, 590–592.

Zhou, Y., Li, Y. S., Wang, Z., Tan, J. H., & Liu, Z. S. (2006). Synthesis and identification of the antigens for ciprofloxacin. *Chinese Journal of Veterinary Science, 26*, 200–203.