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Monoclonal Antibodies Production for Quantification of Ochratoxin A in Feedstuffs by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

Ochratoxin A (OTA), a mycotoxin, is produced from *Aspergillus* and *Penicillium* species. It is a nephrotoxin which is very toxic for the kidneys, and normally found in feedstuffs and other feeds. In this study, the monoclonal antibody (MAb) against OTA was produced to determine OTA in feedstuffs. It was found that 3 monoclones (5E7-3F2, 5E7-3G6 and 5E7-G10) reacted with OTA. MAb developed a specific and highly sensitive sandwich ELISA. No difference in quantification in samples was found when compared with a commercial test kit ($P>0.05$) and 50% binding of standard curve was found at 35 pg/assay. In this study OTA in feedstuff could be detected by MAb, which was self-producing by the ELISA method.

Key words: ochratoxin A, monoclonal antibody, ELISA, mycotoxin, immunoassay

INTRODUCTION

In 2000, the Codex committee on food additives and contaminants or CCFAC, a committee consisting of FAO and WHO considered the regulation of the maximal level of ochratoxin A which is a mycotoxin in feedstuffs or foods at 5 ppb for consumption and 20 ppb for commercial use by mailing a questionnaire to the membership nations and the members will be notified of the maximum level in the future. That is the main reason for food commodity countries like Thailand to be aware and try to keep ochratoxin A at a safe level as according to the standard of CCFAC. The contamination of ochratoxin A in feedstuffs in Thailand is at a high level in grains and oil plants. Nevertheless, other mycotoxins like aflatoxin, T-2 toxin, fumonisin, zearalenone, and deoxynivalenol were also found. Furthermore, the mycotoxins other than mentioned above can be found because imported feedstuffs are contaminated with a variety of fungi. Thailand is a tropical country which has a wide range of temperature from 0°C to 40°C and high humidity which is suitable for the growth of fungi (Pongpiachan, 2000).

Ochratoxin A is a mycotoxin produced from *Aspergillus ochraceus* and *Penicillum verucosum*. It is a nephrotoxin, which is highly toxic for the kidneys and is also a carcinogenic toxin (Marquardt and Frohlich, 1992). This toxin is found in feedstuffs, which has been stored for a long time and seriously affects animal performance. This may also have an effect on human referred to as Balkan disease. At present, The ELISA test kit is used for the quantification of ochratoxin A in feedstuffs which is easy, quick and accurate but it is expires in a short time. It is also very expensive to use.

The ELISA test kit of ochratoxin A is

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produced from antibodies against ochratoxin A. So, the objective of this study was to produce antibodies against ochratoxin A and to use the antibody produced to determine the amount in feedstuffs.

MATERIALS AND METHODS

Conjugated ochratoxin A (OTA) with human serum albumin (HSA).

OTA-HSA conjugates were prepared by diluting OTA (Sigma O-1877) 1 mg in absolute ethanol 0.5 ml. Next, mixed it with 7.5 mg of carboxymethylamin HCl (Aldrich C 1,340-2) in 4 ml of phosphate buffer saline (PBS). Then, added 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide (Sigma H-4633) 5 mg in 1 ml of PBS and stirred at 4°C for 20 minutes. Mixed 2 mg of HSA in 100 µl of PBS and stirred at 4°C for 3 days. The solution was dialyzed in 0.05 M of sodium bicarbonate for 5 days and the ratio of OTA conjugate to protein was estimated by measuring at 332 nm by UV-spectrophotometer.

Immunized antigen

100 µg of OTA-HSA was emulsified with an equal volume of complete adjuvant Freund, and 6-8 weeks old female BALB/C mice were immunized subcutaneous at two sites of the mixture at day 0, 14, and 44. The mice with high anti-sera against OTA were finally boosted inter peritoneally before 3 days of fusion.

Cell fusion and cloning

The myeloma cell line X63 Ag 8.653 was used for fusion. After three days of the last boosted, the spleen was removed and collected the spleenocyte. The spleen cells were fused with myeloma cell at a ratio of 5:1 by 50% polyethylene glycol (PEG, Sigma P-3640) in dimethyl sulfoxide (DMSO, Merck, Art. 802912), and then the hybrid cells were distributed into 96-well culture plate. After selection in HAT medium (200 ml of Iscove's Modified Dulbecco's medium, 20 ml of fetal bovine serum and 2 ml of 100x HAT), the hybridomas were cultured for 10 days. Next, supernatants from individual wells were screened for specific immunoglobulins to OTA by indirect ELISA. Positive clones were subsequently grown in HT medium (200 ml of Iscove's Modified Dulbecco's medium, 20 ml of fetal bovine serum and 2 ml of 100x HT). The hybridomas producing OTA antibodies were cloned by the limiting dilution method.

Purified monoclonal antibodies from culture media

This method was adapted from Arvieux and Williams (1988), the media from the positive clones were collected at 50 ml and centrifuged at 10000x, 30 minutes. The supernatant was mixed with sodium sulfate (Na_2SO_4) 18 g (%w/v) and incubated for 30 minutes at room temperature. Centrifuged the mixture at 5000x, 15 minutes and added Na_2SO_4 16 % (%w/v) 33 ml, incubated at room temperature for 30 minutes, centrifuged again at 5000x, 15 minutes and the precipitate was collected. Dissolved the precipitate with 25 ml of deionized water and dialyzed in 50 mM of sodium chloride for 3 days. The solution was purified by the thiophilic column chromatography.

Isotype monoclonal antibodies

The culture supernatants of hybridomas producing the anti-OTA were coated in 96-well culture plate and incubated at 4°C over night. Next, the plate was washed and dried, blocked plate with 1% gelatin and incubated at 37°C for 1 hour. Then, washed and dried again, added horseradish peroxidase conjugate goat anti-mouse IgG or IgM to compare the type of antibodies, incubated at 37°C for 1 hour. Washed and dried, added substrate and incubated in dark room for 30 minutes. Read the absorbance at 492 nm by ELISA reader.

Indirect ELISA for determination antibody titers and screening hybridomas

OTA-HSA 10 µg/ml were coated in the 96-well microplate 100 µl/wells and incubated at 4°C over night. After washing and drying, 1% gelatin was added at 100 ml/wells, incubated at 37°C for 1 hour. After washing and drying serum at 1:1000 dilution or supernatant of culture media was added 100 µl/wells, incubated at 37°C for 1 hour. After washing and drying, horseradish peroxidase conjugate anti-mouse IgG at 1:500 was added 10 µl/wells, incubated at 37°C for 1 hour. Washed and dried again and added substrate 100 µl/wells, incubated in dark room for 30 minutes. Stopped with 4 N H_2SO_4 and read the absorbance at 492 nm.

Sandwich ELISA for standard curve and determination of OTA in sample

Coated 96-well plate with 1:10 of monoclonal antibody (MAb) 100 µl/wells, incubated at 4°C over night. After washing and drying, 1% of gelatin was added 100 µl/wells, incubated at 37°C for 1 hour. After washing and drying, extracted sample solution and dilution of OTA standard was added 100 µl/wells, incubated at 37°C for 1 hour. After washing and drying MAb conjugated with horseradish peroxidase at ratio 1:5 was added 20 µl/wells, incubated at 37°C for 1 hour. Washed and dried again, added substrate 100 µl/wells, incubated in dark room for 30 minutes and read the absorbance at 492 nm.

Extracted sample for determination of OTA

50 g of sample which was grind passed through a 20 mesh sieve were added with 70 % methanol and blended for 2 minutes at a high speed blender. Filtered the extract by Whatman No. 1 and collected the filtrate as a sample.

RESULTS

Antigen conjugation

The ratio of OTA conjugate with protein was 21:1 and the concentration of protein in solution was 2.67 mg.

Immunization

After 14 days the mice in the immunized group showed OTA antibody titer and mouse number I2 was collected for fusion because of the highest antibody titer. The results are shown in Figure 1.

Fusion and cloning

After 10 days of fusion, 12 wells were

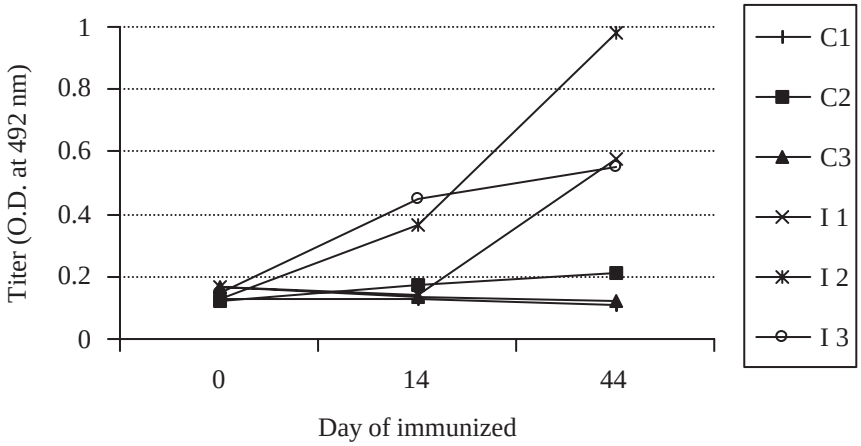

Figure 1 Antibody titer of the OTA-HSA immunized mice (C= Control, I= Immunize).

grown from 528 wells of microplates or 2.27 %, 4 of the wells or 33.33 % were produced antibodies against OTA, Well number 5E7 was selected to limit dilution, after limiting 19 positive clones were found. However 3 clones (5E7-3F2, 5E7-3G6, and 5E7-3G10) were selected which had high titer for using in this study. The results of the fusion are shown in Table 1.

Purification monoclonal antibody from the culture medium

From the purification by using thiophilic column chromatography, monoclonal antibodies from culture medium were about 1-2 mg/50 ml of culture medium. The data are shown in Table 2.

Isotype of monoclonal antibodies

Monoclonal antibodies from clones 5E7-3F2, 5E7-3G6, and 5E7-3G10 were immunoglobulin G (IgG). The results are shown in Table 3.

Standard curve for OTA with monoclonal antibodies

For use in the quantitative sandwich ELISA, IgG from 5E7-3F2 at 1:10 was coated plate and IgG from 5E7-3G6 was conjugated with horseradish peroxidase for secondary antibodies. The standard curve is shown in Figure 2. The 50 % binding was 35 pg/assay.

Quantification of OTA in sample

The samples were cereal grains and agar media for fungi growth. The results of the comparative test with ELISA test kit from VERATOX showed no different ($P<0.05$) (Table 5) and the recovery percentage is shown in Table 4. The correlation of comparative study is shown in Figure 3.

DISCUSSION

The ratio of OTA conjugated to HSA was 21:1, showing that HSA 1 mole bound with 21 mole of OTA, calculated from the method of Dean *et al.* (1971). When comparing the ratio with the results of Chu *et al*. (1976) and Breithotz-Emanuelsson *et al.* (1992) the ratios were 16.7:1 and 13:1 respectively. The results were close with this study and 5 and 10 mg of OTA were used respectively, whereas 1 mg of OTA was used in this study because OTA is very expensive. When the concentration of protein in conjugate solution was calculated, the concentration was 2.64 mg, which from OTA 1 mg and HSA 2 mg, 0.36 mg showed protein loss. The absorbance at 332 nm of OTA-HSA was 0.477 and HSA was 0.127 (data not shown), the wave length at 332 nm was OTA wave length, this result shows that OTA was conjugate to HSA and OTA had phenylalanine in the structure which can be bound easily with another protein. In the immunization, the mice could produce antibodies against OTA-HSA. The mice No. I1 and I3 had more antibody titer against HSA than OTA-HSA because their antibodies could be bound with HSA, but less with OTA. However, the mouse number I2 had more antibody titer against OTA-HSA than HSA, so it was selected to collect spleen cells for fusion.

From the fusion, 12 wells were grown from 528 wells (2.27%) and 4 wells tested positive for anti-OTA (33.33%); it was found that the opportunity for the fusion of spleen cells with myeloma cells was very low, and not every hybridoma clone could produce antibodies because the fusion was a random method. When calculating the percentage of positive clones per well which used in fusion method, it was 0.75%. Because a few opportunities, 6 of 96-well plates were used to increase the chance of fusion cells.

From the limited dilution, 23 monoclones were produced and 19 of 23 monoclones were positive. However 3 monoclones (5E7-3F2, 5E7-3G6, 5E7-3G1) were selected for culture and to produce monoclonal antibodies because the selected monoclones had a high level of antibody

Table 1 Antibody titer of media from fusion cells (*O.D. at 492 nm).

Well No.	Titer/ OTA-HSA*	Titer/HSA*	Difference
1C9	0.814	1.195	-0.381
1D3	0.864	1.068	-0.204
1F5	0.919	1.190	-0.271
2H2	1.514	1.813	-0.299
2H7	1.054	1.198	-0.144
3C5	0.906	0.916	-0.010
3C12	1.307	0.937	0.370
3E7	1.118	1.077	0.103
4H11	0.965	0.978	-0.013
5D3	1.010	1.133	-0.123
5E7	1.300	0.682	0.618
6G4	1.644	1.106	0.538

Table 2 Quantity of monoclonal antibodies from the culture medium.

Clone number	Antibody (mg)
5E7-3F2	2.057
5E7-3G6	0.921
5E7-3G10	1.931

Table 3 Isotype of monoclonal antibodies (*O.D. at 492 nm).

Clone No.	Antimouse IgG-HRP*	Antimouse IgM-HRP*	Difference
5E7-3F2	0.922	0.245	0.677
5E7-3G6	0.983	0.313	0.670
5E7-3G10	0.863	0.236	0.627

Table 4 Recovery of ochratoxin A from peanut meal.

Ochratoxin A added (ng/ml)	% Recovery
5	98.38
10	91.96
25	91.52
50	96.04
100	96.38

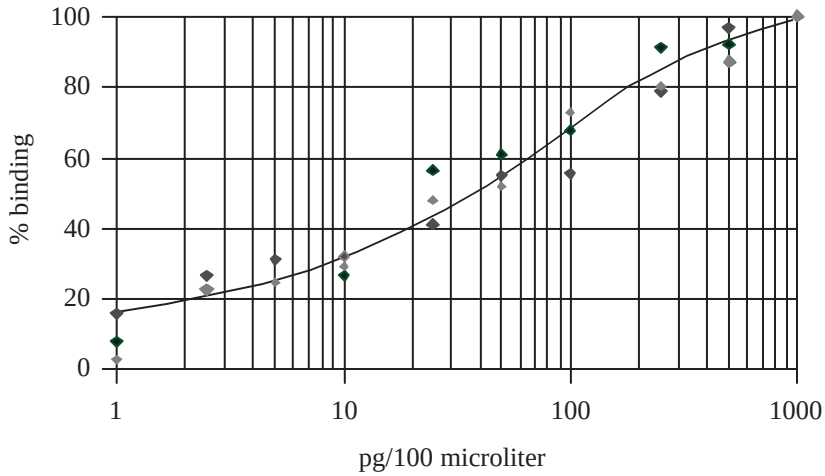

Figure 2 Standard curve of ochratoxin A.

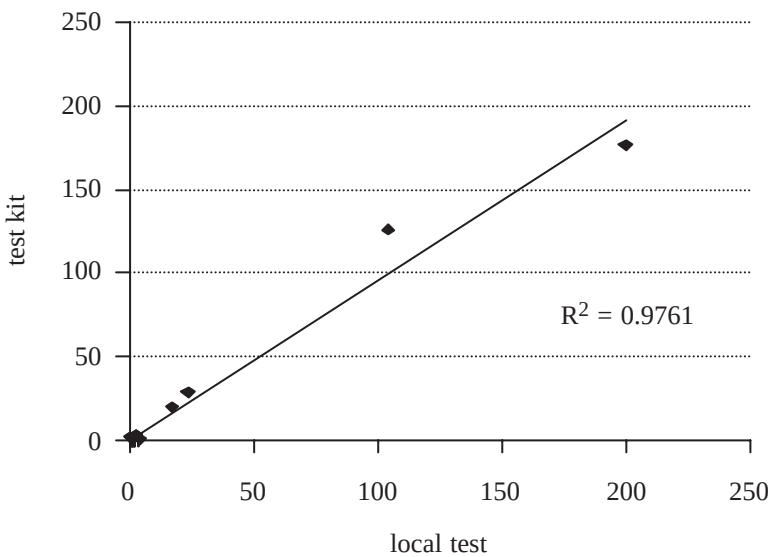

Figure 3 Correlation of ELISA test kit and Sandwich ELISA (ppb).

titer against OTA and could be grow in 24 well plate but other clones could not grow and died because of change in the conditions. After increasing the amount of cells and collecting media for purified MAb, from 3 monoclones, 2.057, 0.921 and 1.931 mg/50ml of MAb were obtained, which had few antibodies. However, MAb production by *in vitro* method was better than *in vivo* because the antibodies were pure. This study did not detect the cross-reaction of MAb with another toxic analog (OTB, OTC, and OTa). However, OTA was found more than other types.

In a standard curve, 50% binding was found at 35 pg /assay which was close to Kawamura *et al.* (1998), at 40 pg/assay. When comparing the results of the test samples with ELISA test kit, no difference in quantification of samples ($P<0.05$) was found, so this MAb could be used to detect OTA in samples.

Table 5 Quantification of ochratoxin A in samples (ppb).

Samples	Test kit	Local test
1. Cassava chips	30.40	25.79
2. Cassava meal	0.00	1.05
3. Peanut meal	0.03	0.03
4. Broiler feed	1.18	4.42
5. Corn husk	0.00	1.99
6. Rice flour	2.33	1.77
7. Rice polishing	0.00	0.03
8. Broken rice	0.00	0.04
9. Bran	0.00	0.11
10. Corn	0.00	0.97
11. Full fat soybean	0.00	0.02
12. Mustard	0.39	0.04
13. Soybean meal 1	0.00	1.33
14. Soybean meal 2	0.00	1.32
15. Swine feed	0.42	0.57
16. Rice	19.70	17.58
17. TAN*	0.32	0.72
18. S1*	3.20	2.40
19. S2*	176.10	200.00
20. S3*	2.90	2.40
21. S4*	126.40	104.00
22. PDA*	0.00	2.00
23. PDA7178*	0.00	2.90
24. PIG*	1.80	0.20
25. PIG7178*	0.00	0.10

* Fungi agar sample.

CONCLUSION

In conclusion, the monoclonal antibodies against ochratoxin A from this production can be used to determine the amount of ochratoxin A in feedstuffs by the ELISA method.

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LITERATURE CITED

Arvieux, J. and A. F. Williams. 1988. Immunoaffinity Chromatography, pp. 113-136. *In* D. Catty (ed.). **Antibodies**, Volume I: A Practical Approach. IRL Press, Oxford.

Breithotz-Emanuelsson A., G. Dalhammar and K. Hult. 1992. Immunoassay of ochratoxin A, using antibodies developed against a new

ochratoxin-albumin conjugate. **J. AOAC Internat** 75(5) : 824-829.

Chu, F. S., F. C. Chang and D. R. Hinsdill. 1976. Production of antibody against ochratoxin A. **Appl and Environment Microb**. 31(6) : 831-835.

Dean, D.G., D. Exley and M. W. Johnson. 1971. Preparation of 17 beta-oestradiol-6-(O-caeboxymethyl)-oxime-bovine serum albumin conjugate. **Steroids**. 18(5) : 593-603.

Kawamura, O., S. Sato, H. Kajii, S. Nagayama, K. Ohtani, J. Chiba and Y. Ueno. 1998. A sensitive enzyme-linked immunosorbent assay of ochratoxin A based on monoclonal antibodies. **Toxicon**. 27(8) : 887-897.

Marquardt R. R. and A. A. Frohlich. 1992. A review of recent advance in understanding ochratoxicosis. **J. Anim. Sci**. 70 : 3968-3988.

Pongpiachan P. 2000. Molds and mycotoxins in feed raw material, p. 31. *In* Kemin Seminar. **The Food Quality Chain**. Miracle Grand Convention Hotel, Bangkok, Thailand.