Letter to the Editor

Detection of Atrazine Residue in Food Samples by a Monoclonal Antibody-based Enzyme-linked Immunosorbent Assay*

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Atrazine (AT, 2-chloro-4-ethylamino-6-isopropyl-amino-*s*-triazine) has been detected in ground water in several areas of the United States for many years, as well as in China, wherein the growth rate of its gross usage is 20% per year. AT can easily enter the human body through the biological cycle as a risk to human health[1]. AT is one of the dangerous pollutants in groundwater in many countries[2]. Development of simple and rapid methods for monitoring AT is thus quite urgent. Several conventional methods have been reported for monitoring AT residue in crops and environment, including HPLC, GC, and GC-MS[3]. These methods are very sensitive and reliable; however, they are time-consuming and expensive, and require professional technicians and complicated pretreatment of samples. ELISA is one of the most common immunochemical techniques for detecting low-molecule weight haptens for food and environment safety[4]. Specific antibodies against haptens are of great importance; however, it is difficult to obtain them for immunoassay. The present study is primarily aimed to prepare an immunogen conjugate for inducing immune response in mice, select the best anti-AT mAb from cell fusion, and eventually establish IC-ELISA with high specificity, sensitivity and reproducibility for quantitatively detecting AT in actual samples.

The preparation of carboxylated AT followed the described method in Ref. 5[5], with some modifications. Structure confirmation of carboxylated AT was performed by ^{1}HNMR (^{1}H Nuclear Magnetic Resonance Spectrum), was also used to confirm the molecular mass of carboxylated AT under the positive ion mode.

Carboxylated AT was coupled to BSA as an immunogen, and simultaneously coupled to OVA by using the active ester method to obtain a coating antigen[6].

Three female *BALB/C* mice (8-10 wk old) were immunized with AT-BSA. Twelve days after each booster injection, the mice were tail-bled, and their antisera were tested by using noncompetitive indirect ELISA after the third injection. Three days before the fusion, the mouse with the highest titer was given another 160 μg of antigen in physiological saline without any Freund's adjuvant.

SP 2/0 murine myeloma cells were cultured in RPMI 1640 media supplemented with 2% fetal bovine serum and 1‰ penicillin-streptomycin. Cell fusion procedures were carried out as described by Kishiro et al.[7], with some modifications. The selected hybridomas were subcloned by using the limiting dilution assay, and these stable antibody-producing clones were expanded. IC-ELISA was then employed to determine whether the antibodies from the expanded clones could specifically recognize AT. The anti-AT mAb was obtained from ascites of mice injected with the suspensions of subclonal cells.

AT and its analogues (i.e., melamine, simazine) were used for the cross-reactivity (CR) study by IC-ELISA. CR rate from them was expressed as the ratio by comparing the analyte concentration giving half-maximal inhibition (IC_{50}, ng/mL). Inhibition rate and CR values were calculated according to equations (1) and (2), respectively.

$$Inhibition\ rate\ (\%) = \frac{A - A_{ex}}{A_0 - A_{ex}} \times 100 \quad (1)$$

Where A is the 450 nm absorbance of hapten at standard concentration, A_0 is the 450 nm

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absorbance at zero hapten concentration, and A_{ex} is the 450 nm absorbance at excessive hapten concentration.

$$CR(\%) = \frac{IC_{50}\ of\ AT}{IC_{50}\ of\ AT\ analogue} \times 100 \quad (2)$$

The affinity of anti-AT mAbs was evaluated by SPR (Auto Lab, Netherlands). The results were analyzed with Autolab ESPRIT Data Acquisition 4.3 and Kinetic Evaluation 5.0 software. The affinity of the selected mAb was demonstrated with the dissociation constant (K_D).

The noncompetitive indirect ELISA was conducted as described previously[8], with some modifications. The microtiter plates were first coated with the optimal concentration of AT-OVA in coating buffer (CBS, pH 9.6) and incubated at 4 °C overnight. On the following day, the wells were washed and then blocked with 1% (W/V) OVA in PBS (120 μL/well) for 45 min at 37 °C. After washing three times with PBST, antiserum, culture supernatants, or mAbs diluted with PBS were added to the plates, which were then incubated for 1.5 h at 37 °C. After the plates were washed thoroughly with PBST, Sec-Ab at the dilution of 1:3000 in PBS was added into each well. After a second incubation for 45 min, the plates were washed. Afterward, TMB substrate system was added to each well. The wells were then incubated away from light for 7 min at 37 °C. After the color development, the reaction was halted by 50 μL/well of 2 mol/L sulfuric acid (H_2SO_4). The absorbances at 450 and 630 nm were then determined with an ELISA reader (Multiskan MK3, Thermo, China).

The procedure of IC-ELISA is similar to noncompetitive indirect ELISA, except some differences as described below. After the microtiter plates were coated and blocked as described above, a series of concentrations of free AT (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, and 0 μg/mL) in 10% methanol-PBS (50 μL/well) were added to each well. Another 50 μL of antisera, culture supernatants, or mAbs diluted with PBS were added shortly thereafter. The subsequent steps were the same as those in noncompetitive indirect ELISA.

First, 5 g pretreated food samples (except tap water sample) were spiked with standard AT in methanol at 5, 15, and 45 ng/g. After being shaken for 1 min, chloroform ($CHCl_3$, 5 mL) was accurately added. Then, the mixtures were extracted ultrasonically for 15 min, and centrifuged at 4000 rpm for 5 min.

The upper impurity of samples was removed to obtain the lower liquid. After the lower liquid had been dried (through vacuum or mild N_2 drying), 10% methanol-PBS (5 mL) was added. The samples were continuously shaken until they were completely dissolved. Blank samples without AT were prepared as described above. All liquids for AT detection by ELISA were used directly, with each sample detected three times by ELISA.

In the current study, we chose two representative samples: tomato and pear. The pretreatment was the same as described above, except that the samples were spiked with standard AT in methanol at 0, 6, 30, and 80 ng/g. After the same pretreatment, all the liquids were filtered, and then underwent direct detection of AT by both IC-ELISA and HPLC (Alliance 2695, Japan) for five replicates. The optimized chromatographic conditions were as follows: column: C_{18}; column temperature: 25 °C; mobile phase: methanol-water (70:30, V/V); flow rate: 1.0 mL/min; and detection wavelength: 224 nm.

In the present study, hapten AT has a chlorine atom in the triazine ring that is active and can easily be replaced by some active compounds with a bifunctional group, such as 3-mercaptopropionic acid. The carboxylated AT was then characterized by nuclear magnetic resonance (NMR). The MW of carboxylated AT was in accordance with the theoretical value (285.37), which indicated that carboxylated AT was modified successfully.

After the successful identification of carboxylated AT, carrier proteins (BSA or OVA) were used to react with the modified hapten by the active ester method to form AT-protein conjugates. The conjugations were dialyzed in PBS for 3 d to remove nonconjugated carboxylated AT, and then examined by both UV-Vis spectrophotometry and MS.

After the second immunization, the titer of serum from each mouse was tested by noncompetitive indirect ELISA. The results showed that the titer values were 0.532 to 0.867 at 1:100 000 dilution after the fourth immunization. Clearly, the immune response after consecutive immunizations achieved good results. IC-ELISA was then performed to test the serum from these three mice. The Mouse #3 was the most sensitive because of its lowest IC_{50} value and was chosen for cell fusion.

Four monoclonal cell lines (named $C_7B_1A_{12}$, $C_7B_1C_9$, $C_7B_1F_3$, and $C_{12}A_4D_{10}$) were obtained. Table 1 summarizes the characteristics of these four antibodies against AT in terms of class, subclass, IC_{50}, and limit of detection (LOD, equal to IC_{10}) values. The results of these four mAbs show that the $C_{12}A_4D_{10}$

line had the calibration curve with the lowest LOD (0.35 ng/mL) and IC_{50} (5.62 ng/mL) values.

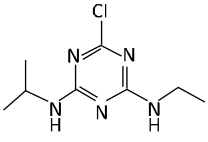
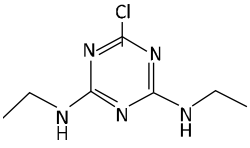
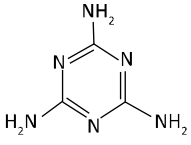
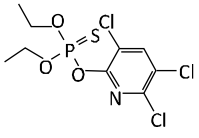
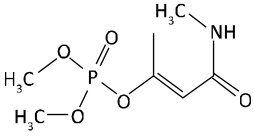
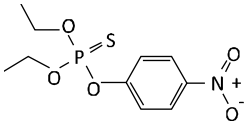
The results summarized in Table 2 show that $C_7B_1A_{12}$, $C_{12}A_4D_{10}$, $C_7B_1C_9$, and $C_7B_1F_3$ had CRs of 100% to AT, 21.1%, 3.5%, 17.5%, and 86.5% to simazine and negligible to other analogues, respectively. Clearly, CRs with simazine were higher than other analogues. The reason may be that the chemical structure of simazine is similar to AT, but has an additional methyl group. In particular, $C_{12}A_4D_{10}$ had the highest specificity among all mAbs. So the follow-up study was carried on based-$C_{12}A_4D_{10}$.

Table 1. Key Parameters of the Four Anti-AT Single Cell Lines

Cell Lines	Class and Subclass	Linear Equation	R^2	Working Range (ng/mL)	IC_{50} (ng/mL)	LOD (ng/mL)
$C_7B_1A_{12}$	IgG1	IC=37.28lgC+120.39	0.9847	2.03-82.52	12.94	1.09
$C_7B_1C_9$	IgG1	IC=36.26lgC+120.77	0.9856	1.66-75.10	11.18	0.88
$C_7B_1F_3$	IgG1	IC=36.07lgC+119.13	0.9857	1.78-82.26	12.12	0.94
$C_{12}A_4D_{10}$	IgG2b	IC=33.29lgC+124.90	0.9926	0.71-44.80	5.62	0.35

Note. C, Concentration of AT.

Table 2. Cross-reactivity of the Monoclonal Antibodies against Selected Compounds (*n*=3)

Inhibitors	Structure	Name of Cell Line							
		$C_7B_1A_{12}$		$C_{12}A_4D_{10}$		$C_7B_1C_9$		$C_7B_1F_3$	
		IC_{50} (μg/mL)	CR (%)	IC_{50} (μg/mL)	CR (%)	IC_{50} (μg/mL)	CR (%)	IC_{50} (μg/mL)	CR (%)
Atrazine		0.0129	100	0.0056	100	0.0112	100	0.0121	100
Simazine		0.061	21.1	0.16	3.5	0.064	17.5	0.014	86.4
Melazine		30.3	0.04	21.8	0.03	28.4	0.04	24.71	0.05
Chlorpyrifos		ND	≤0.01	ND	≤0.01	ND	≤0.01	ND	≤0.01
Monocrotophos		ND	≤0.01	ND	≤0.01	ND	≤0.01	ND	≤0.01
Parathion		165	≤0.01	97.2	0.011	999	≤0.01	933	≤0.01

Note. ND, presented infinite IC_{50} values that could not be fitted with four-parameter logistic equation.

In order to calculate the affinity constant, the anti-AT mAb ($C_{12}A_4D_{10}$) was appropriately diluted with PBS, and then analyzed at five different antibody concentrations. The equilibrium dissociation constant (K_D) determined independently with Kinetic Evaluation 5.0 software, was also calculated based on the fitting curve. The K_D of $C_{12}A_4D_{10}$ was measured as 889 nmol/L.

The parameters from chequer-board titration assay guided the final conditions towards a coating antigen concentration of 1.6 μg/mL and a 1:16 000 dilution of anti-AT mAb. The result of calibration curve for AT based on IC-ELISA is shown in Figure 1. The correlation coefficient (R^2=0.9958) of the detection standard curve indicates a good linear relationship. According to this standard curve, LOD of 1.97 ng/mL and working range of 3.20 ng/mL to 95.69 ng/mL were obtained. From the screening of cell lines, the sensitivity was clearly much lower than that in Table 1. This finding suggests that the matrix effect (the active conditions of mAbs) may affect the optimal activity of the immunoassay[9].

Seven common samples were chosen to evaluate and correct the matrix interference. The results in Table 3 indicate that the recoveries of the seven actual samples ranged from 80% to 120%, and that the coefficient of variation (CV, %) of the samples' recoveries ranged from 0.4% to 7.9%, demonstrating good accuracy and reproducibility of IC-ELISA with the sample pretreatment method. Therefore, the established IC-ELISA is a potential screening tool for quantitative determination of AT residue in food or environmental monitoring studies.

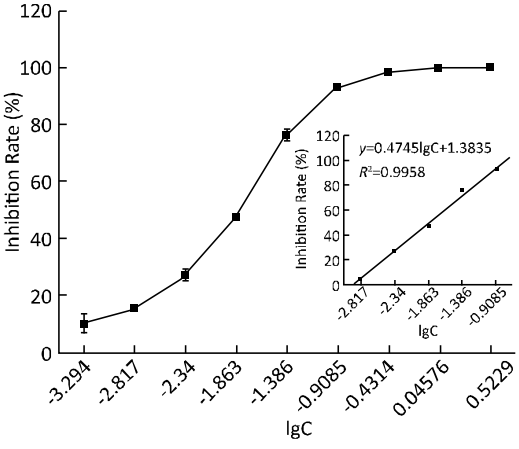


Figure 1. Standard curve of IC-ELISA for detecting AT (*n*=5).

Table 3. Recoveries of Different Spiked Samples by ELISA

Food Samples	Blank Sample (ng/g)[a]	AT Added (ng/g)	Measured Value[b] (ng/g)[a]	Recovery (%)[a]	CV (%)
Chinese cabbage	1.16±0.05	5	5.62±0.15	89.20±0.03	2.7
		15	13.34±0.22	81.20±0.01	1.7
		45	40.91±0.84	88.33±0.02	2.1
Tomato	4.00±0.11	5	8.11±0.15	82.22±2.92	1.9
		15	20.54±1.28	110.27±8.55	6.2
		45	54.06±1.74	111.24±3.88	3.2
Carrot	1.57±0.22	5	6.00±0.20	88.60±3.93	3.3
		15	14.19±0.70	84.13±4.64	4.9
		45	52.00±0.23	112.07±0.51	0.4
Pear	1.04±0.06	5	5.55±0.05	90.20±0.01	0.9
		15	13.38±1.06	82.27±0.07	7.9
		45	37.87±0.80	81.84±0.02	2.1
Cane	4.17±0.58	5	9.28±0.12	102.20±2.46	1.3
		15	18.65±0.35	96.53±2.35	1.9
		45	49.22±3.36	100.11±7.46	6.8
Lean meat	0.76±0.06	5	4.81±0.15	81.00±2.98	3.1
		15	17.62±1.26	112.40±8.37	7.2
		45	52.96±1.04	116.00±2.32	2.0
Tap water of Tianjin	1.81±0.03	5	5.83±0.24	80.40±4.87	4.1
		15	16.23±1.12	96.13±7.44	6.9
		45	52.13±0.45	111.82±0.99	0.9

Note. a: Mean±SD (standard deviation); b: ELISA measured value = (measured value of IC-ELISA) - (mean of sample background value).

As shown in Table 4, the average recoveries by HPLC ranged from 88.48% to 126.7%, which were similar to those of IC-ELISA ranging from 88.16% to 107.8%. Thus, IC-ELISA can be used for detecting AT residue in actual food with high accuracy.

Table 4. Results for AT Analysis in Spiked Samples by both IC-ELISA and HPLC

Food Samples	Atrazine Added (ng/g)	ELISA[a]		HPLC	
		Measured Value (ng/g)	Recovery (%)	Measured Value (ng/g)	Recovery (%)
Pear	0	1.36	—	N	—
	6	7.83	107.83	N	—
	30	28.06	89.00	38.01	126.70
	80	74.85	91.86	70.78	88.48
Tomato	0	1.55	—	N	—
	6	6.97	90.33	N	—
	30	28.00	88.17	36.29	121.00
	80	72.75	89.00	74.73	93.41

Note. N: Not determined by this method. —: Not calculated; a: ELISA measured value = (measured value of IC-ELISA) - (mean of sample background value).

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REFERENCES

1. Aharonson N, Cohen SZ, Drescher N. Potential contamination of ground water by pesticides. Pure Appl Chem, 1987; 59, 1419-46.
2. Lima DLD, Silva CP, Schneider RJ, et al. Development of an ELISA procedure to study sorption of atrazine onto a sewage sludge-amended luvisol soil. Talanta, 2011; 85, 1494-9.
3. Rocha C, Pappas EA, Huang CH. Determination of trace triazine and chloroacetamide herbicides in tile-fed drainage ditch water using solid-phase microextraction coupled with GC-MS. Environ Pollut, 2008; 152, 239-44.
4. Feng Y, Zhou YX, Zou Q, et al. Preparation and characterization of bisphenol A-cationized bovine serum albumin. J Immunol Methods, 2009; 340, 138-43.
5. Lei HT, Shen YD, Song LJ, et al. Hapten synthesis and antibody production for the development of a melamine immunoassay. Anal Chim Acta, 2010; 665, 84-90.
6. Liu XJ, Yan CR, Liu Y, et al. Application of current hapten in the production of broad specificity antibodies against organophosphorus pesticides. Agr Sci China, 2008; 7, 1341-7.
7. Kishiro Y, Kagawa M, Naito I, et al. Novel method of preparing rat-monoclonal antibody-producing hybridomas by using rat medial iliac lymph node cells. Cell Struct Funct, 1995; 20, 151-6.
8. Sai N, Chen YP, Liu N, et al. A sensitive immunoassay based on direct hapten coated format and biotin-streptavidin system for the detection of chloramphenicol. Talanta, 2010; 82, 1113-21.
9. Feng Y, Ning BA, Su P, et al. An immunoassay for bisphenol A based on direct hapten conjugation to the polystyrene surface of microtiter plates. Talanta, 2009; 80, 803-8.