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Determination of okadaic acid in shellfish by using a novel chemiluminescent enzyme-linked immunosorbent assay method

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ABSTRACT

A direct competitive chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) was developed to determine okadaic acid (OA). Concentrations of the capture monoclonal anti-OA antibodies, conjugate of OA-HRP and a composition of blocking buffers were varied to optimize the assay condition. The values of IC_{10}, IC_{50} and working range (IC_{20}–IC_{80}) for CL-ELISA were 0.01, 0.07, and 0.03–0.2 ng/mL, respectively. Additionally, the analytical recovery values of CL-ELISA from 3 shellfish spiked samples with OA concentrations of 0.03, 0.1 and 0.2 ng/mL ranged from 86.7% to 111.2%. Closely examining the OA concentrations in 19 various shellfish products performed by CL-ELISA revealed that OA concentrations in 6 of the 19 examined samples was undetected, whereas the 13 samples were contaminated with low levels of OA ranging from 1.2 to 8.0 ng/g.

1. Introduction

As a lipophilic marine biotoxin produced by *Dinophysis* and *Prorocentrum dinoflagellates* [1], the toxin okadaic acid (OA) easily contaminates various species of shellfish. Also, human consumption of the contaminated shellfish induces diarrhetic shellfish poisoning (DSP) and gastroabdominal disturbances in the first few hours, although the toxin does not harm bivalves after ingestion. DSP toxins, including okadaic acid, dinophysistoxin-1 (DTX-1) and DTX-2, are among the most widely occurring groups of toxins in shellfish worldwide [2,3]. Animal studies have also demonstrated the carcinogenic, mutagenic and immunotoxic effects of OA. Contaminated bivalves pose a global concern for the shellfish industry owing to their devastating economic impact. The European Union has thus specified the maximum permitted level of 160 ng/g of mussels (EC no. 2074/2005) [4].

Various analytical approaches have been developed to minimize the risk of human exposure by OA and control the contents of OA in shellfish. Conventionally, mouse bioassay has been used as the reference method for OA analysis [5]. However, the European Union prohibited this method on January, 2011, owing to its insufficient selectivity and accuracy as well as ethical problems [4] and recommend to apply other methods, including liquid chromatography (LC) coupled to fluorescence or mass spectrometry [6–9]. Unfortunately, chromatographic methods are limited by the requirement for expensive equipment and highly trained personnel.

A more promising method for OA detection is enzyme immunoassay. As a highly specific and sensitive assay, enzyme-linked immunosorbent assay (ELISA) facilitates the analysis of a massive number of samples, and does not require time-consuming procedures and complex equipment as chromatographic methods [10–13].

An additional dilution of samples is an effective means of preventing the matrix effect. In this case, a more sensitive immunoassay method should be adopted to determine the analytes. As is well known, replacing the chromogenic substrates oxidation, used to measure the enzymatic activity of peroxidase conjugates, with enhanced chemuliminescence reaction (ECR) may significantly increase the sensitivity of the immunoassay [14,15]. According to [16], the highest CL intensity was obtained in ECR catalyzed by using 3-(10′-phenothiazinyl)-propane-1-sulfonate (SPTZ) in combination with 4-morpholinopyridine (MORPH) as enhancers. A recent study described the enhanced mechanism by using SPTZ/MORPH [17]. The development of ultrasensitive ELISAs to determine ochratoxin A and aflatoxin B1

Abbreviations: OA, Okadaic acid; HRP, Horseradish peroxidase; ELISA, Enzyme-linked immunosorbent assay; CL, Chemiluminescence; SPTZ, 3-(10′-phenothiazinyl) propane-1-sulfonate; MORPH, 4-morpholinopyridine; BSA, Bovine serum albumin; ECR, Enhanced chemuliminescence reaction.

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has demonstrated the advantages of using the SPTZ/MORPH system [18,19].

This work presents a sensitive direct competitive CL-ELISA method for determining OA. An attempt is also made to increase the sensitivity of the proposed assay, method by applying the chemiluminescent method to evaluate of HRP activity by using SPTZ and MORPH as enhancers. Moreover, OA concentrations in some commercially available shellfish samples are determined using the proposed assay method.

2. Material and methods

2.1. Materials

Okadaic acid (OA) (Fig. 1) was purchased from Taiwan Algal Science (Tauyuan, Taiwan). A analytical standard solution of OA (25 μg/mL), bovine serum albumin (BSA), Tween 20, luminol, pristane, dimethyl sulfoxide (DMSO), 1-ethyl-3-(3′-dimethylaminopropyl) carbodimide (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbeco Modified Eagle's Medium (DMEM) was obtained from GIBCO Laboratories (Grand Island, NY). Sodium 3-(10′-phenothiazinyl) propane-1-sulfonate (SPTZ) was prepared as described in [20]. 4-Morpholinopyridine (MORPH) was obtained from Aldrich (USA); Tris and H_2O_2 (30%) were from J. T. Baker (USA). Methanol was obtained from Merck (Germany). Black polystyrene plates (MaxiSorb) were obtained from Nunc (Demark).

2.2. Monoclonal antibody specific to OA

The monoclonal antibodies specific to OA (anti-OA-mAb OA) was generated in our laboratory which was from a stable hybridoma cell line, 6B1A3, generated by the fusion of P3/NS1/1-AG4-1 myeloma cells with spleen cells isolated from a BALB/c mouse immunized with OA-γ-globulin. The 6B1A3 mAb belongs to the immunoglobulin G1 (K chain) isotype. For production of the monoclonal antibody, female BALB/c mice (10 weeks old), were injected intraperitoneally with 0.5 mL pristane 7 days before receiving an intraperitoneal injection of 2×10^6 hybridoma cells suspended in DMEM. Ascites fluid developed 2–3 weeks after the injection of the cells and was collected every other day for 3 days. The ascites fluid was centrifuged at 7000 rpm (5900*g*) for 5 min to remove the cell debris. The IgG from the cleared ascites fluid was purified by ammonium sulfate precipitation (50% saturation for the final solution) twice and dialyzed against 2 L of phosphate buffered saline (PBS) for 72 h at 4 °C with two changes of buffer and then stored at −70 °C.

2.3. Conjugation of OA and horseradish peroxidase

Conjugation of OA and HRP was carried out by using the EDC/NHS method. Briefly, 0.2 mg of OA in 0.1 mL of DMSO was mixed with 0.6 mg of EDC and 0.4 mg of NHS, and then a HRP solution (0.8 mg of HRP in 0.3 mL of 0.1 M carbonate buffer, pH 9.6) was added. After being stirred at room temperature for 2 h, the OA-HRP conjugates without further purification was dialyzed against 0.01 M PBS for 72 h and then lyophilized for future use.

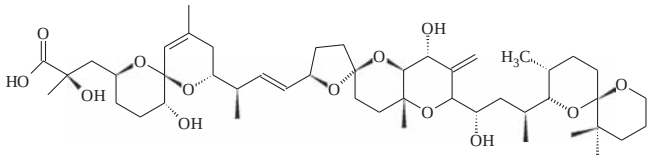

Fig. 1. Chemical structure of okadaic acid.

2.4. Determination of OA by CL-ELISA

CL-ELISA for determination of OA was carried out using 96-wells black polystyrene plates (MaxiSorb). The diagram of direct competitive CL-ELISA is shown in Fig. 2. The capture antibodies were coated by adding 100 μL of the solution of the monoclonal anti-OA antibody (dilution 1:5000–1:20,000) in the PBS to each plate well and incubated at 4 °C overnight. The plate was then washed by PBST four times using ELx 50 ELISA washer (Bio-Tek instruments, USA) and blocked by adding 170 μL of PBS containing 0.1% BSA for 30 min at 37 °C. The plate was washed four times with PBST. Subsequently, 50 μL of the OA standard in PBS concentrations from 1.0 pg/mL to 2.0 ng/mL or samples simultaneously with 50 μl of the OA-HRP conjugate (1:15,000–45000) in PBS were added and incubated at 37 °C for 1 h. After washing the plate as described above, 100 μL of freshly prepared substrate solution (80 mM Tris, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM MORP, and 1.75 mM H_2O_2 [16] were added to each well and stirred. Chemiluminescence intensity was monitored after 5 min at room temperature on a luminescence reader (FlexStation 3, Molecular Devices, USA).

2.5. CL-ELISA of real shellfish samples

Nineteen shellfish samples purchased from Taiwanese stores were used to determine the OA levels. The preparation of sample was performed in accordance with a protocol described in [18]. Briefly, each sample (20 g) was homogenized with 80 mL of extraction solvent (100% methanol) and incubated for 30 min with shaking (200 rpm) at 37 °C. After centrifugation at 14,000*g* for 10 min the extract was passed through a 0.45 μm syringe filter. The obtained extract was diluted in 5 times with PBS, and then directly subjected to CL-ELISA.

2.6. Data analysis

Standards and samples were run in triplicates, and the mean values were processed. Standard curves were obtained by plotting the light intensity against the logarithm of the analyte concentration and fitted to a four-parameter logistic equation using the Origin 6.0 Professional software (OriginLab Corp., United States):

$$Y = \{(A-D)/(1 + (x/C)^B)\} + D,$$

where A is the asymptotic maximum (intensity in the absence of an analyte, A_{max}), B is the curve slope at the inflection point, C is the x value at the inflection point, and D is the asymptotic minimum (A_{min}, background signal).

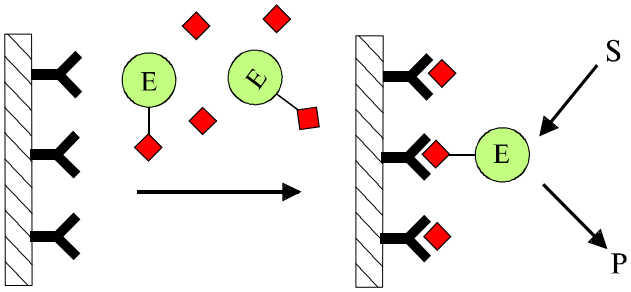

Fig. 2. Scheme of direct competitive CL-ELISA.

3. Results and discussion

3.1. Optimization of OA CL-ELISA.

Favorable conditions for the performance of OA CL-ELISA were estimated. Concentrations of the coating anti-OA antibody (anti-OA-mAb) and the conjugate of OA-horseradish peroxidase (OA-HRP) were varied to optimize the conditions of the chemiluminescent assay for generating a set of calibration curves. All curves had a typical calibration curve obtained in the performance of competitive ELISA (Fig. 3). The values of IC_{10}, IC_{50}, working range (IC_{20}–IC_{80}) and a ratio of A_{max}–A_{min} (A_{max}/A_{min}) were selected as the parameters used to calculate the assay efficiency.

Table 1 reveals that increasing the dilution of anti-OA antibody from 1:5000 to 1:20,000, significantly decreased the values of IC_{10} and IC_{50}. It subsequently shifted the working range in the area of lower concentrations of OA. The highest A_{max}/A_{min} ratio was obtained at an antibody dilution of 1:10,000. Additional dilution of the antibody worsened all analytical parameters of the assay.

Comparing the analytical parameters of the calibration curves obtained at the fixing anti-OA-mAb (1:10,000) and a variation of OA-HRP conjugate (1:30,000–90,000) dilutions revealed that the values of IC_{10}, IC_{50} and IC_{20}–IC_{80} resembled each other, whereas using the conjugate with a dilution of 1:60,000 yielded the highest ratio of A_{max}/A_{min}. Therefore, the solutions of anti-OA-mAb and OA-HRP conjugate with a dilution of 1:10,000 and 1:60,000 respectively were selected as optimal ones for CL-ELISA.

Two coating conditions were compared to optimize the CL-ELISA method. In the first method the monoclonal antibodies were coated at 4 °C overnight or at 37 °C for 1 h to run the CL-ELISA. The obtained results demonstrated that the analytical parameters such as IC_{10}, IC_{50} and IC_{20}–IC_{80} of CL-ELISA were similar (data not shown). At the same time, the CV value in the working range of the assay at coating antibody at 4 °C overnight was 5-fold lower than that at 37 °C for 1 h. Moreover, A_{max}/A_{min} ratio at coating antibody at 4 °C overnight was higher than that at 37 °C for 1 h (Table 2). Therefore, the coating of the monoclonal antibodies at 4 °C overnight was applied in all subsequent CL-ELISA. Following optimization of the CL-ELISA conditions values of IC_{10}, IC_{50} and IC_{20}–IC_{80} were 0.01, 0.07, and 0.03–0.2 ng/mL, respectively.

Some immunochemical methods have been developed for OA determinations [21–26]. The most sensitive of these methods was CL-ELISA [23]. However, this work failed to describe the composition of the used substrate solution. The linear range of their CL-ELISA method ranged from 0.08125 to 20 ng/mL. Therefore, for the CL-ELISA method with use of SPTZ and MORPH developed in this work, its working range was 0.03–0.2 ng/mL, which was more sensitive CL-ELISA method for OA detection.

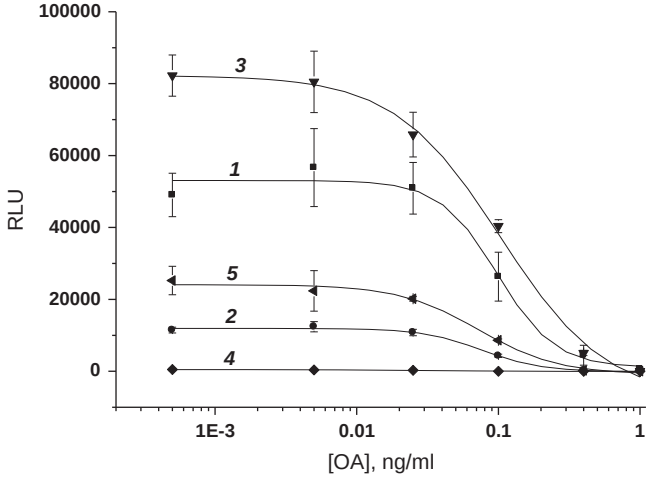

Fig. 3. Determination of okadaic acid in buffered solutions by CL-ELISA. At the competitive step the reaction solution contained monoclonal anti-OA-antibody and the conjugate OA-HRP in the following dilutions: (1) 1:10,000/1:30,000, (2) 1:10,000/1:90,000, (3) 1:5000/1:60,000, (4) 1:20,000/1:60,000, and (5) 1:10,000/1:60,000, respectively.

Table 1
Determination of the optimal concentrations of coating anti-OA-mAb antibody and conjugate OA-HRP in CL-ELISA.

Anti-OA-mAb	OA-HRP	A_{max}/A_{min}	IC_{10} (ng/mL)	IC_{50} (ng/mL)	IC_{20}–IC_{80} (ng/mL)
1:10,000	1:30,000	83	0.02	0.1	0.05–0.22
1:10,000	1:90,000	11,500	0.02	0.08	0.04–0.16
1:5000	1:60,000	5100	0.015	0.1	0.04–0.29
1:10,000	1:60,000	25,200	0.01	0.07	0.03–0.20
1:20,000	1:60,000	500	0.004	0.03	0.01–0.08

3.2. Recovery of the spiked shellfish samples

The ability to evaluate samples using the proposed CL-ELISA method was estimated using OA-spiked shellfish samples. The standard OA toxin was added into OA-free shellfish samples and, then, extracted by methanol. The OA concentrations in the spiked samples were 0.03, 0.1 and 0.2 ng/mL. Table 3 summarizes the results of the determination of OA concentration in shellfish samples. The 2.5-fold dilution of the spiked samples led to a low recovery (54.4%) for the samples with an OA concentration of 0.03 ng/mL and high CV values (15–24%) for all spiked samples (Table 3A). This finding implies that 2.5-fold dilution of the samples does not prevent the matrix effect. Conversely, the recovery values from the 5-fold diluted samples within and between assays were 86.7–111.2% and 95.1–103.7%, respectively (Table 3B). Moreover, the CV within and between assays were 1.4–14% and 4.6–12.3%, respectively. Based on the latter results, we can infer that the proposed CL-ELISA method permits the precise measurement of OA in shellfish samples.

3.3. Analysis of real shellfish samples

The content of OA in 19 shellfish samples purchased in Taiwanese stores was determined using the proposed CL-ELISA method. All samples were extracted with 100% methanol and diluted with 10 times with 0.01 M PBS before subjecting to the CL-ELISA analysis. Table 4 summarizes the OA concentration in the studied shellfish samples. Analytical results indicated that 6 of the 19 examined samples were OA-free, whereas the other 13 samples had an OA level ranging from 1.2 to 8.0 ng/g. Since the Maximum Acceptable Level of OA in European Union and Russia is 160 ng/g, we can conclude that all studied samples of shellfish products analyzed with the developed method had at a level below the maximum acceptable level.

Table 2
Analytical parameters of CL-ELISA for OA determination by using different antibody coating conditions.

Conditions for coating of the anti-OA-mAb	A_{max}/A_{min}	CV in the working range (%)
1 h, 37 °C	25,200	15
Overnight, 4 °C	139,200	2.7

Table 3
Recovery and CV values of OA in spiked shellfish samples using CL-ELISA.

(A) Recovery and CVs at OA determination in the samples with different dilutions

Spiked OA (ng/mL)	Dilution of the shellfish sample			
	1:2.5		1:5	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
0.03	54.4 ± 8.2	15.1	90 ± 7.3	8.2
0.1	72.5 ± 17.4	24.0	86.7 ± 8.4	9.7
0.2	104.9 ± 15.7	15.0	111.2 ± 5.6	5.0

(B) Recovery and CVs of OA for 5-fold diluted samples in within assay and between assay

Expected conc. (ng/mL)	Within assay ($n=3$)			Between assay (n=3)		
	Found conc. (ng/mL)	Recovery (%)	CV (%)	Found conc. (ng/mL)	Recovery (%)	CV (%)
0.03	0.0270 ± 0.0022; 0.0293 ± 0.0004; 0.0293 ± 0.0006	90.0 ± 7.3 97.7 ± 1.3 97.7 ± 2	8.2 1.4 2.1	0.0285 ± 0.0013	95.1 ± 4.4	4.6
0.1	0.0867 ± 0.0084; 0.1110 ± 0.0052; 0.1001 ± 0.0140	86.7 ± 8.4 111.0 ± 5.2 100.1 ± 14	9.7 4.7 14.0	0.0993 ± 0.0122	99.3 ± 12.2	12.3
0.2	0.2224 ± 0.0111; 0.2068 ± 0.0097; 0.1930 ± 0.0172	111.2 ± 5.6 103.4 ± 4.9 96.5 ± 8.6	5.0 4.7 8.9	0.2074 ± 0.0147	103.7 ± 7.4	7.1

Table 4
Determination of OA in shellfish samples by CL-ELISA.

No. of shellfish sample	Concentration of the OA in shellfish sample (ng/g)	No shellfish sample	Concentration of the OA in shellfish sample (ng/g)
1	1.8	**11**	NF
2	8.0	**12**	NF
3	NF[a]	**13**	NF
4	5.4	**14**	3.8
5	2.5	**15**	NF
6	1.2	**16**	NF
7	3.4	**17**	1.5
8	2.7	**18**	1.3
9	3.6	**19**	1.4
10	2.6		

[a] NF–no found.

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References

[1] P. Vale, M.A. Sampayo, Toxicon 37 (1999) 1565.
[2] P. Vale, V. Veloso, A. Amorim, Toxicon 54 (2009) 145.
[3] G.A. Codd, C.J. Ward, S.G. Bell, Arch. Toxicol. Suppl. 19 (1997) 399.
[4] European Commission, Official J. European Union 15 (2011) 0003.
[5] R. Draisci, L. Croci, L. Giannetti, L. Cozzi, L. Lucentini, D. De Medici, A. Stacchini, Toxicon 32 (1994) 1379.
[6] A. Gerssen, P.P. Mulder, M.A. McElhinney, J. de Boer, J. Chromatogr. A 1216 (2009) 1421.
[7] J.C. Gonzalez, F. Leira, M.R. Vieytes, J.M. Vieites, A.M. Botana, L.M. Botana, J. Chromatogr. A 876 (2000) 117.
[8] A.P. Louppis, A.V. Badeka, P. Katikou, E.K. Paleologos, M.G. Kontominas, Toxicon 55 (2010) 724.
[9] L.A. Stobo, J.P. Lacaze, A.C. Scott, S. Gallacher, E.A. Smith, M.A. Quilliam, J. AOAC Int. 88 (2005) 1371.
[10] Y. Hokama, Food Addit. Contaminants 10 (1993) 71.
[11] T. Tsumuraya, I. Fujii, M. Hirama, Toxicon 56 (2010) 797.
[12] F.Y. Yu, T.F. Chi, B.H. Liu, C.C. Su, J. Agric. Food Chem. 53 (2005) 6947.
[13] D. Knopp, Anal. Bioanal. Chem. 385 (2006) 425.
[14] C.A. Marquette, L.J. Blum, Anal. Bioanal. Chem. 385 (2006) 546.
[15] A. Roda, P. Pasini, M. Mirasoli, E. Michelini, M. Guardigli, Trends Biotechnol. 22 (2004) 295.
[16] M.M. Vdovenko, A.S. Demiyanova, T.A. Chemleva, I.Yu. Sakharov, Talanta 94 (2012) 223.
[17] I.Yu. Sakharov, M.M. Vdovenko, Anal. Biochem. 434 (2013) 12.
[18] F.Y. Yu, M.M. Vdovenko, J.J. Wang, I.Yu. Sakharov, J. Agric. Food Chem. 59 (2011) 809.
[19] F.Y. Yu, A.V. Gribas, M.M. Vdovenko, I.Yu. Sakharov, Talanta 107 (2013) 25.
[20] E. Marzocchi, S. Grilli, L. Della Ciana, L. Prodi, M. Mirasoli, A. Roda, Anal. Biochem. 377 (2008) 189.
[21] A. Hayat, L. Barthelmebs, J.L. Marty, Anal. Chim. Acta 690 (2011) 248.
[22] S.Y. Lu, C. Lin, Y.S. Li, Y. Zhou, X.M. Meng, S.Y. Yu, Z.H. Li, L. Li, H.L. Ren, Z.S. Liu, Anal. Biochem. 422 (2012) 59.
[23] Q. Wang, Y.C. Liu, J. Li, W. Jiang, Y.J. Chen, N.N. Song, J. Food Qual. 35 (2012) 76.
[24] L. Wang, Y.-X. Sang, X.-H. Wang, J. AOAC Int. 94 (2011) 1531.
[25] S.-Y. Lu, Y. Zhou, Y.-S. Li, C. Lin, X.-M. Meng, D.-M. Yan, Z.-H. Li, S.-Y. Yu, Z.-S. Liu, H.-L. Ren, Environ. Sci. Pollut. Res. Int. 19 (2011) 2619.
[26] C.A. Marquette, P.R. Coulet, L.J. Blum, Anal. Chim Acta 398 (1999) 173.