5836 *J. Agric. Food Chem.* **2004**, *52*, 5836–5842

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enzyme-Linked Immunosorbent Assay for the Detection of Yessotoxin and Its Analogues

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Polyclonal antibodies were produced for the development of competitive enzyme-linked immunoassays for use in quantifying yessotoxins in shellfish, algal cells, and culture supernatants. Immunizing and plate coating antigens were prepared by derivatization of yessotoxin either by ozonolysis or bromination and conjugation to proteins. Two assays that were the most sensitive for yessotoxin were optimized and characterized. Cross-reactivity studies indicated that the antibodies raised have broad specificity and that binding to analogues was strongly affected by changes to the A-ring and, to a lesser extent, the K-ring regions of the toxin molecule. ELISA provides a sensitive and rapid analytical method that is suitable for screening large numbers of samples and detects all the yessotoxin analogues that the European Commission currently requires shellfish to be tested for. The assay limit of quantitation for yessotoxin in whole shellfish flesh is 75 μg/kg; therefore, assay sensitivity is sufficient to measure toxin levels well below the maximum permitted level set by the European Commission. The antibodies produced can be used in additional applications such as the immunolocalization of yessotoxins in shellfish and preparation of immunoaffinity columns.

KEYWORDS: **Yessotoxin; ELISA; immunoassay; antibodies; shellfish toxin; DSP**

INTRODUCTION

The yessotoxins are lipophilic disulfated polyethers (*1*) that, together with the pectenotoxins, were until recently included with okadaic acid and the dinophysistoxins as diarrhetic shellfish poisoning toxins. Yessotoxin (**1**) (**Figure 1**) was first isolated from scallops (*1*). More recently, 45-hydroxyyessotoxin (**2**) and 45,46,47-trinoryessotoxin (**3**) (*2*) were also isolated from scallops. 45-Hydroxyyessotoxin (*3*, *4*) was isolated from mussels, as were homoyessotoxin (**4**), 45-hydroxyhomoyessotoxin (**5**) (*5*), 1-desulfoyessotoxin (**6**) (*6*), adriatoxin (**8**) (**Figure 2**) (*7*), carboxyyessotoxin (**9**) (*8*), carboxyhomoyessotoxin (**10**) (*9*), and 42,43,44,45,46,47,55-heptanor-41-oxohomoyessotoxin (ketohomoyessotoxin) (**11**) (*10*).

Yessotoxins are produced by the dinoflagellates *Protoceratium reticulatum* (*11*) and possibly *Lingulodinium polyedrum* (*12*). The structures of the yessotoxins differ from those of toxins in the diarrhetic shellfish poisoning toxins group and the pectenotoxins but are similar to the brevetoxins and ciguatoxins in having a ladder-shaped polycyclic ether skeleton (*2*).

Yessotoxin is toxic to mice when injected ip. Toxicological studies give a wide range of values for yessotoxin LD_{50} (90−1000 μg/kg) (*13*−*17*), and yessotoxin is considered to be less toxic than okadaic acid, which has in comparison LD_{50} values reported at 192 μg/kg (*18*) and 225 μg/kg (*17*). The yessotoxins are no longer included in the diarrhetic shellfish poisoning toxins because the mechanism of action for yessotoxin is different from that of okadaic acid and the dinophysistoxins because yessotoxin and its derivatives do not have a diarrhetic effect and do not inhibit protein phosphatase 2A activity (*14*). Effects on the cardiac muscles were observed after dosing yessotoxin to mice (*13*), although myocardial alterations are only observable by electron microscopy and were considered to be only moderate (*16*, *17*). Oral administration of yessotoxin to mice, even with dosing levels as high as 54 mg/kg (*15*), does not have a lethal effect (*13*, *14*, *16*, *17*). The analogues 45-hydroxyyessotoxin and 1-desulfoyessotoxin have lower toxicity than yessotoxin (*6*), while homoyessotoxin is slightly more toxic than yessotoxin (*17*). A short-term study of daily administration to mice of yessotoxin (2 mg/kg/day), homoyessotoxin (1 mg/kg/day), or 45-hydroxyhomoyessotoxin (1 mg/kg/day) for 7 days indicated that none of the three toxins was lethal or induced signs of toxicity, and only myocardial cells near capillaries displayed ultrastructural changes (*19*).

Results so far suggest that health risks from yessotoxin and its analogues may be much less important than those from okadaic acid or dinophysistoxins (*17*, *20*). A maximum permitted level (MPL) for yessotoxin, 45-hydroxyyessotoxin, homoyessotoxin, and 45-hydroxyhomoyessotoxin has been set at 1 mg/kg of yessotoxin equivalents in shellfish meat by the

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10.1021/jf049395m CCC: $27.50 © 2004 American Chemical Society
Published on Web 08/25/2004

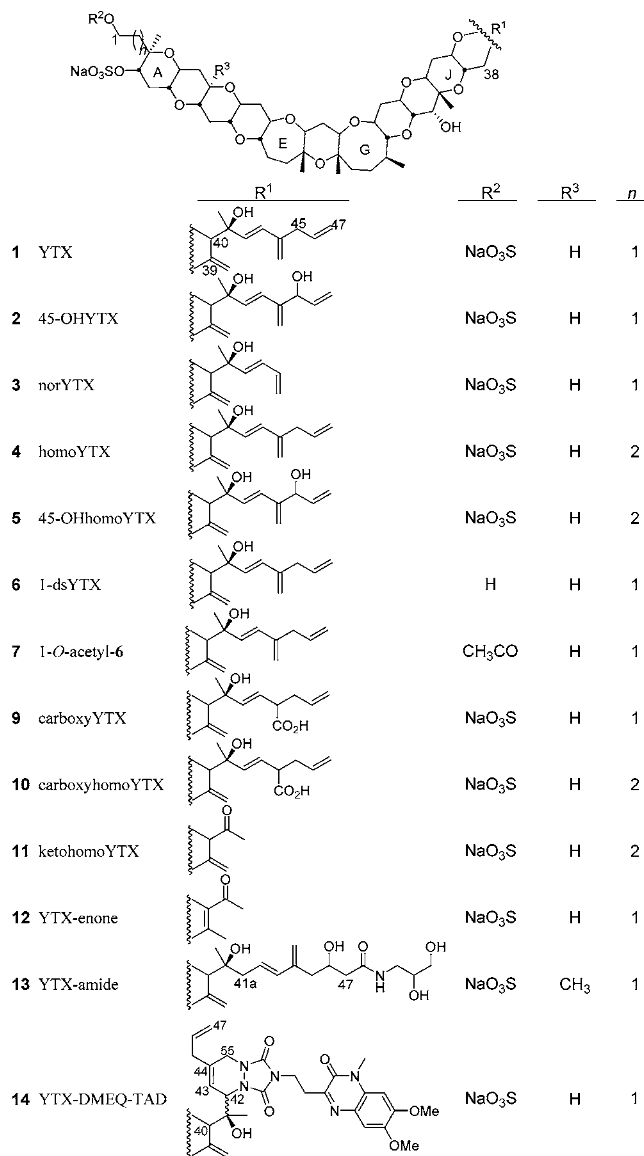

Figure 1. Structures of yessotoxin (YTX) and analogues.

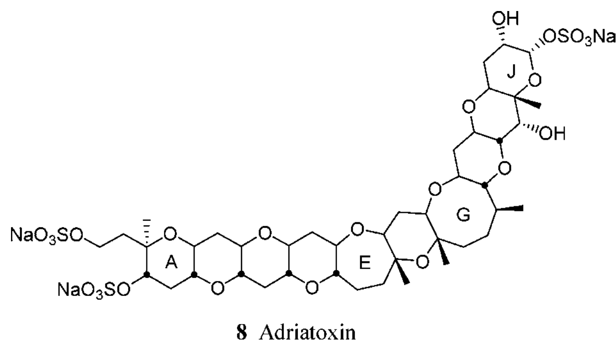

Figure 2. Structure of adriatoxin (**8**).

European Commission (*21*), although further toxicity studies are needed before the risk to human health can be fully evaluated.

The regulatory level set for the four yessotoxin analogues is higher than that for okadaic acid, dinophysistoxins, and pectenotoxins, which is set at 160 μg/kg of okadaic acid equivalents (*21*). The yessotoxins are coextracted with diarrhetic shellfish poisoning toxins from contaminated shellfish by methanol and acetone, where they contribute to the total toxicity detected in the mouse bioassay for diarrhetic shellfish poisoning toxins. Therefore, testing shellfish extracts with the mouse bioassay when yessotoxin is present gives an overestimation of diarrhetic shellfish poisoning toxin content and the risk to human health, with consequent unnecessary closure of shellfish harvesting and interference in the international trading of shellfish. Specific assays for yessotoxins and for the diarrhetic shellfish poisoning toxins are therefore required to avoid these problems.

A fluorometric HPLC method for measuring yessotoxins was developed (*3*) on the basis of the labeling of yessotoxin with the fluorogenic reagent 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD). Although this method is used extensively (*22*), it has limitations when included in shellfish monitoring programs where high sample throughput is required, because sample derivatization and cleanup procedures are required before HPLC analysis. Yessotoxin can also be measured by liquid chromatography coupled with mass spectrometry (*23*). Excellent sensitivities can be achieved using this technology, but the expense of equipment and the requirement for highly skilled personnel is often outside the scope of many screening laboratories. ELISAs offer an alternative methodology ideally suited to monitoring programs. Sample extract preparation usually only involves dilution of extracts, and the assays are sensitive, rapid, and cheap to perform, provide high sample throughput, and require relatively low-cost equipment. ELISAs have been developed for the quantification of many shellfish toxins (*24*).

Here we describe the production of polyclonal antibodies to yessotoxin and their use in an ELISA for detection of yessotoxins in shellfish and algal samples. The antibodies have broad specificity for many of the known yessotoxin analogues and are suitable for use in the development of commercial ELISA kits.

MATERIALS AND METHODS

Materials. Yessotoxin for immunization and yessotoxin analogues for cross-reactivity studies were obtained from Tohoku University (Sendai, Japan). Homoyessotoxin and 45-hydroxyhomoyessotoxin standards were supplied by University of Trieste (Trieste, Italy). Yessotoxin for use in plate-coater synthesis and as an analytical standard was obtained from ESR Ltd. (Wellington, New Zealand). More recently yessotoxin was extracted and purified in our laboratories (*25*). Green-lipped mussels (*Perna canaliculus*), Bluff oysters (*Tiostrea lutaria*), and scallops (*Pecten novaezelandiae*) were obtained from shellfish suppliers when the shellfish were known to be free of toxin (negative mouse bioassays and absence of toxic phytoplankton). Bovine serum albumin (BSA) (ELISA grade), ovalbumin (OVA), and Freund's complete (containing heat-killed *Mycobacterium tuberculosis*) and incomplete adjuvants (without *M. tuberculosis*) were from Sigma Chemical Co. (St. Louis, MO). Cationized BSA (cBSA) was prepared by the method of Hermanson (*26*). Donkey anti-sheep immunoglobulin-horseradish peroxidase conjugate (anti-sheep-HRP) was from Silenus Laboratories Pty. Ltd. (Victoria, Australia). Poly(vinylpyrrolidone) 25 (PVP) was from Serva Electrophoresis. 3,3′,5,5′-Tetramethylbenzidine (TMB) was from Roche Diagnostics NZ Ltd. (Auckland, New Zealand). Maxisorp immunoplates were from Nunc (Roskilde, Denmark).

All inorganic chemicals and organic solvents were of reagent grade or better. Coating buffer was carbonate buffer (50 mM, pH 9.6). Phosphate-buffered saline (PBS) contained NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (8 mM), and KH_2PO_4 (1.5 mM), pH 7.4). ELISA washing buffer (PBST) consisted of 0.05% Tween 20 in PBS. Sample buffer was 10% methanol (v/v) in PBST and the antibody buffer consisted of 1% BSA or 1% PVP (w/v) in PBST. The substrate solution for HRP (*27*) was prepared by addition of 600 μL of TMB (1 mM) in acetone−methanol (1:9) to citrate buffer (12 mL, 210 mM, pH 3.95), to which 4 μL of 30% H_2O_2 was added immediately prior to use.

Hapten Synthesis and Conjugation. *Ozonized Yessotoxin* (**Figure 3**). To yessotoxin (0.5 mg) in deuteriomethanol (ca. 0.5 mL) was added

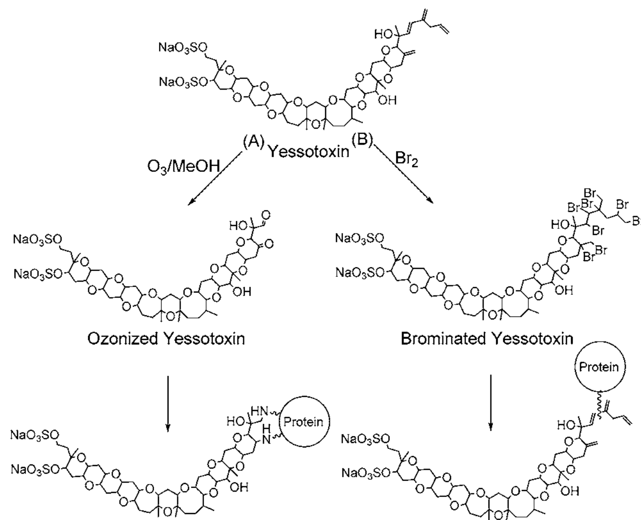

Figure 3. Preparation of immunizing and coating conjugates by derivatization of yessotoxin by ozonolysis (A) and by bromination (B) and conjugation with proteins.

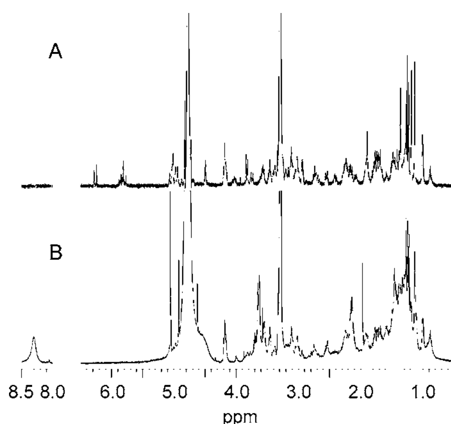

Figure 4. ^{1}H NMR spectra of yessotoxin (A) before and (B) after ozonolysis, indicating loss of olefinic resonances (5.8–6.3 ppm) during ozonolysis.

MeOH (10 mL). The solution was cooled to −76 °C and treated with ozone (50 L/h) using a glass diffuser. After 10 min the reaction became pale blue, indicating completion of ozonolysis, and the flask was purged with oxygen to remove ozone. The reaction was warmed to ambient temperature and water (50 mL) was added. After keeping for 6 h, the contents were concentrated in vacuo and the residue lyophilized. This residue was dissolved in deuteriomethanol, examined by ^{1}H NMR (**Figure 4**), and then treated with triphenylphosphine (ca. 1 mg) to reduce any residual ozonide. The solvent was removed in vacuo, the solid taken up in dimethylformamide (1 mL), and 330 μL of this solution was added to solutions of cBSA, BSA, and OVA (10 mg each, in 1 mL PBS) with stirring. A solution of sodium cyanoborohydride (ca. 5 mg) in PBS (ca. 100 μL) was then added to each protein solution, which was kept at 4 °C in the dark for 18 h. The resulting conjugates were clarified by centrifugation, and the supernatant was repeatedly concentrated by centrifugal ultrafiltration (Filtron, 30 kDa cutoff) and rediluted on the membrane with PBS, until the dilution of low molecular weight contaminants, including unconjugated yessotoxin, was calculated to be ca. 2×10^{5}-fold. The conjugates were freeze-dried and stored at −20 °C. When required for use as immunogens or coating antigens, aliquots of this material were reconstituted to 1 mg/mL in water.

*Brominated Yessotoxin (**Figure 3**).* Bromine (5 μL) was dissolved in methanol (10 mL), and 5 μL of this solution was added to a solution of yessotoxin (100 μg) in methanol (50 μL). The reaction was allowed to proceed in the dark at ambient temperature for 3 h. The methanol and excess bromine were removed under a stream of dry nitrogen to afford brominated yessotoxin as a colorless solid. This material was dissolved in methanol (100 μL) and added to a solution of ovalbumin (5.8 mg) in carbonate buffer (500 μL, 50 mM, pH 9.6). The conjugation reaction was allowed to proceed in the dark at 4 °C for 36 h. Conjugation was terminated by addition of cysteine (10 mg) in PBS (1 mL). The conjugate was purified by centrifugal ultrafiltration, and aliquots were freeze-dried and stored as above.

Immunization. Three groups of three sheep were each immunized with BSA-ozonized yessotoxin, cBSA-ozonized yessotoxin, or OVA-ozonized yessotoxin in PBS (250 μL). For primary immunizations, immunogens were prepared as water-in-oil emulsions by injecting the conjugate (1 mg) in PBS (1 mL) into Freund's complete adjuvant (2.5 mL), followed by vortex mixing. Immunogens for secondary and subsequent immunizations were prepared as above but as emulsions in Freund's incomplete adjuvant. Immunogens (0.5 mL of emulsion containing 140 μg of immunogen) were administered intramuscularly (0.25 mL per hind leg) at 4-weekly intervals. Test-bleeds (10 mL) were taken from the jugular vein of each sheep 1 week after the third immunization and the sera screened by ELISA for antibodies suitable for use in competitive ELISAs for yessotoxin. Within 1 week after screening, 200 mL of blood was collected from selected sheep into blood bags under negative pressure, and the antisera were stored at −20 °C. Animal experiments were approved by the AgResearch Ruakura Animal Ethics Committee.

ELISA. *Noncompetitive ELISA.* All assay procedures were carried out at 20 °C. Microtiter plates were coated with either BSA-ozonized yessotoxin or OVA-ozonized yessotoxin (4 μg/mL) in coating buffer (100 μL/well). After incubation for 16 h, plates were washed four times with PBST and blocked for 1 h with 1% BSA in PBST (200 μL/well). This was followed by four washes with PBST. To each well was added 50 μL of sample buffer followed by 50 μL of various dilutions of antiserum in antibody buffer and the plate was incubated for 1 h. After four washes (PBST), 100 μL of anti-sheep-HRP (diluted 1:5000 in antibody buffer) was added. Plates were incubated for 2 h and washed four times with PBST, and freshly prepared substrate solution (100 μL/well) was added. The reaction was stopped after 15 min by addition of sulfuric acid (0.3 M, 100 μL/well). The absorbance of wells was determined at 450 nm using a Versa$_{max}$ microplate reader (Molecular Devices Corp., CA).

Competitive ELISA (cELISA). Plates were coated and then blocked (1% BSA or 1% PVP) as above. When OVA-ozonized yessotoxin was not available for coating plates, OVA-brominated yessotoxin was used. A standard of yessotoxin in methanol (1 μg/mL) was diluted in washing buffer to give a methanol concentration of 10%, and further dilutions were made in sample buffer. To each well was added 50 μL of standard or diluted sample, followed by 50 μL of antibody at a dilution such that the maximum absorbance (in the absence of analyte) in the assay was approximately 1.0 absorbance. All samples were analyzed in duplicate. The assay was completed as described for the noncompetitive ELISA. Data analysis was performed using SOFTmax PRO data analysis software (Molecular Devices Corp.). Curve fits of mean absorbance versus the logarithm of the analyte concentration were performed by four-parameter curve fit.

Shellfish Extraction. Ten grams of fresh or thawed whole shellfish was homogenized in a Waring blender for 1 min. Two grams of the homogenate was weighed into a Falcon tube, 90% methanol (18 mL) was added, and the material homogenized (Ultra-Turrax T25) for 1 min at 19 000 rpm. The homogenate was centrifuged at 3000*g* for 10 min at 15 °C, and the supernatant was removed and stored at −20 °C until analysis. The final methanol concentration in the supernatant was approximately 80%.

Assay Validation. ELISA matrix effects were detected by comparing the response of the assay to toxin standards in buffer and in extracts of toxin-free shellfish. When curves were not coincidental, it was concluded that further sample treatment or assay modification was required. The optimized method was validated by spiking standard yessotoxin in methanol (20 μL) into homogenized green mussel (2 g). Samples were each spiked with four different concentrations of analyte. Three replicate samples were spiked at each concentration, and each sample was assayed in six replicates duplicated each day on two plates. Recoveries were determined by comparing the cELISA results with the calculated yessotoxin concentrations. The intra-assay (intraplate)

Table 1. Immune Response of Sheep Immunized with Yessotoxin Immunogens after Three Immunizations

animal	immunogen	titer[a]	% competition with yessotoxin[b]
501	BSA–hapten 1[c]	1000	51
502	BSA–hapten 1	700	55
503	BSA–hapten 1	600	32
504	cBSA–hapten 1	2000	77
505	cBSA–hapten 1	5000	84
506	cBSA–hapten 1	2000	65
507	OVA–hapten 1	1000	41
508	OVA–hapten 1	3000	6
509	OVA–hapten 1	4000	34

[a] Antiserum dilution required to reduce ELISA absorbance to 50% of the maximum absorbance. [b] Percent reduction in maximum absorbance (no yessotoxin present) caused by addition of yessotoxin (600 ng/mL) in the ELISA. Antiserum used at a dilution to give approximately 1.0 absorbance in the absence of toxin. [c] Hapten 1 represents ozonized yessotoxin.

variation was determined from the variation of the mean yessotoxin concentration of each of the three spiked samples for the four different concentrations on one plate. The inter-assay (interplate) variation was determined from the variation of the mean yessotoxin concentrations determined on each plate over 4 days.

RESULTS

Hapten Synthesis and Conjugation. Yessotoxin (**1**), as demonstrated by ^{1}H NMR (**Figure 4**), was readily oxidized by ozone and the resulting ozonized yessotoxin was conjugated with proteins by reductive amination. Yessotoxin was also readily oxidized with bromine, and the resulting bromo derivative was conjugated to OVA via nucleophilic amination in basic solution. Ozonized yessotoxin and brominated yessotoxin conjugated to OVA provided coating antigens suitable for use in the ELISA. These appeared to be interchangeable, in that coincidental standard curves could be obtained using either coating conjugate at an appropriate concentration (data not presented), but the brominated yessotoxin conjugates are more convenient in that they do not require any special equipment, such as an ozone generator, to prepare.

Characterization of Yessotoxin-Specific Antisera. Antisera titers were determined by noncompetitive ELISA, and antibody binding to yessotoxin was measured by cELISA with and without addition of yessotoxin (600 ng/mL). Antiserum from sheep 505, which had been immunized with cBSA-ozonized yessotoxin, gave the highest antibody titer and cELISA sensitivity (**Table 1**). Binding of antibodies to free yessotoxin occurred with all of the antisera obtained; however, inhibition of antibody binding to coating antigen by analyte was greatest when the immunogen used was cBSA-ozonized yessotoxin, less with BSA-ozonized yessotoxin, and least with OVA-ozonized yessotoxin; i.e., cBSA-ozonized yessotoxin > BSA-ozonized yessotoxin > OVA-ozonized yessotoxin (**Table 1**).

Antisera from sheep 504 and 505 were selected for use in cELISAs. Cross-reactivity studies indicated that binding of analogues by antibodies in these ELISAs (**Table 2**) was strongly affected by changes to the A-ring (C-1) and, to a lesser extent, the K-ring regions (C-40) of the toxin molecule (**Figure 1**).

Assay Optimization. Assay optimization was carried out using the bulk collection of antiserum taken from sheep 505 after three immunizations, as this gave the most sensitive assay for yessotoxin (**Table 3**). Optimal concentrations of plate-coating antigen, antiserum, and anti-sheep-HRP were determined by checkerboard assays. Assay sensitivity was increased (i.e., the I_{20} was decreased) by using 1% PVP, rather than 1% BSA, as the blocker (**Table 3**). Furthermore, use of PVP as the blocker resulted in a 4-fold reduction in the amount of antiserum required for the assay (**Table 3**). On the basis of the means from 10 assays, the optimized assay had a working range (I_{20}−I_{80}) of 0.05−1.11 ng/mL and the mean I_{50} was 0.23 ng/mL.

Table 2. Cross-Reactivities of Antisera[a] with Yessotoxin Analogues

structure[b]	compound	% CR[c] with 504	% CR[c] with 505
1	yessotoxin[d]	100	100
2	45-hydroxyyessotoxin[d]	159	116
3	trinoryessotoxin	59	40
4	homoyessotoxin[d]	39	15
5	45-hydroxyhomoyessotoxin[d]	51	23
6	1-desulfoyessotoxin	2	2
7	1-*O*-acetyl-**6**	0.8	0.8
9	carboxyyessotoxin	160	173
12	yessotoxinenone	168	174
13	yessotoxinamide	34	18

[a] From bulk bleed collected after three immunizations. [b] **Figure 1.** [c] Cross-reactivity = (I_{50} yessotoxin/I_{50} analogue) × 100, where I_{50} is the molar concentration of compound giving 50% inhibition of antibody binding to the coating antigen (OVA-brominated yessotoxin). [d] Cross-reactivities of yessotoxin analogues determined in ELISAs performed on three separate occasions. Interassay variation (CV) ranged from 1 to 12%.

Table 3. Performance of Two Sheep Antisera[a] in the Yessotoxin cELISA

animal	immunogen	ELISA blocker	antiserum dilution in assay	I_{50}[b] (ng/mL)	assay range (ng/mL) I_{20}	assay range (ng/mL) I_{80}
504	cBSA–hapten 1[c]	1% BSA	1:4000	0.83	0.35	1.91
		1% PVP	1:30000	0.85	0.18	4.00
505	cBSA–hapten 1	1% BSA	1:12000	0.40	0.17	0.98
		1% PVP	1:50000	0.35	0.04	1.18

[a] From test-bleed collected after three immunizations. [b] I_{20}, I_{50}, and I_{80} are the concentrations of yessotoxin giving 20, 50, and 80% inhibition, respectively, of binding of antibody to the coating antigen (OVA-ozonized yessotoxin). [c] Hapten 1 represents ozonized yessotoxin.

Analysis of Shellfish Extracts. The presence of toxin-free shellfish extracts at less than 100-fold dilution in PBS caused matrix effects in the ELISA for yessotoxin (data not shown). However, dilution of shellfish extracts 150-fold in sample buffer overcame this problem, and ELISA response curves for standards prepared in sample buffer and curves for standards in diluted extract were coincidental (**Figure 5**).

Assay Validation. Recoveries of yessotoxin extracted with approximately 80% methanol from mussel homogenates spiked with yessotoxin at 0.1, 0.2, 0.5, and 1 μg/g (**Table 4**) were 102.9, 111.9, 116.1, and 118.1%, respectively, with a mean recovery of 112.3%. The mean intra-assay coefficient of variation was 7.2%, while the mean inter-assay coefficient of variation was 7.1%.

DISCUSSION

We report the production of polyclonal antibodies against yessotoxin and the development of the first ELISA method for quantification of yessotoxin. Most yessotoxins have very similar structures, with most of the major analogues differing only in the K-ring end of the molecule (**Figure 1**). The aim was to develop an ELISA with broad specificity for yessotoxins; therefore, a hapten linked through the K-ring end of the molecule

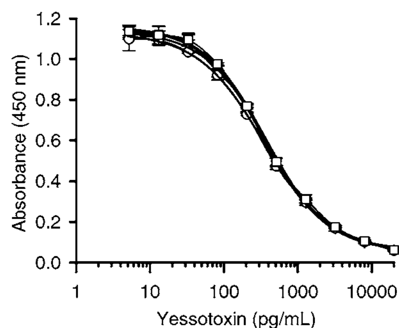

Figure 5. Yessotoxin ELISA standard curves in the absence (□) and presence of methanolic extracts of mussels (○), scallops (△), and oysters (▽). All samples were diluted 150-fold in PBS containing 10% methanol and 0.05% Tween 20. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

Table 4. Recovery of Yessotoxin Spiked into Green-Lipped Mussel Homogenate and Methanolic Extracts Diluted 150-Fold with Sample Buffer before Analysis by ELISA

yessotoxin added (μg/2 g)	MPL[a] equiv	yessotoxin in diluted extract (ng/mL)	% yessotoxin measured by ELISA[b]	*n*[c]	CV%
2	1	0.67	102.9	8	11.6
1	1/2	0.33	111.9	8	6.5
0.4	1/5	0.13	116.1	8	5.5
0.2	1/10	0.07	118.1	8	4.8

[a] Maximum permitted level for yessotoxin set by the European Commission for shellfish at 1 mg/kg. [b] Concentration measured in cELISA using OVA-brominated yessotoxin as the coating antigen, antiserum 505, and PVP as blocker. [c] Indicates number of separate assays run.

was produced so that the resulting immunogen would expose the left-hand polycyclic region of yessotoxin to the immune system during immunization. Ozonolysis of yessotoxin resulted in complete loss of the olefinic resonances (5.8−6.3 ppm) in the ^{1}H NMR spectrum of the product (**Figure 4**), indicating complete conversion of olefinic groups into carbonyl groups. Although no aldehyde resonances were observed in the NMR spectrum, this may be due to the reversible formation of acetal/ketal and enol derivatives of the oxidation product. The unpurified oxidation product was coupled to BSA, cBSA, and OVA via reductive amination. This procedure is highly efficient but requires an ozone generator. A method for bromination of yessotoxin, and coupling of the resulting bromide to OVA, was developed in order to provide a method that was suitable for use in laboratories lacking ozonolysis equipment. Material prepared by this route gave similar assay performance to plate-coater prepared by ozonolysis, although the latter reaction appears to result in more efficient coupling of toxin to protein.

The use of cationized proteins as carriers for hapten conjugation is known to give an increase in immune response compared to their native forms (*26*). This was also the case in the present study, where the highest titers of anti-yessotoxin antibodies and cELISA sensitivities were obtained with antisera from sheep immunized with cBSA-ozonized yessotoxin (**Table 1**). As anticipated, the resulting antibodies had high cross-reactivity (**Table 2**) to analogues of yessotoxin that differed in the polyene side chain at C-40−C-47 on the K-ring (**1**, **2**, **3**, **9**, **12**, and **14**), whereas cross-reactivity with analogues differing at the A/B-ring was less (**4**, **6**, and **13**) (**Figure 1**).

A study of yessotoxin from Norwegian blue mussels (*28*) showed that the levels of yessotoxins determined by ELISA were higher than those by LC−MS. There was good correlation between the two methods, which suggests that there are other analogues present in the extract that the ELISA detects but which are not detected by current LC−MS methods. Studies are underway to determine the nature of the yessotoxin analogues responsible for the increased response of the ELISA. Chromatography of extracts from *P. reticulatum* followed by ELISA analysis has provided ELISA-reactive fractions, and analysis of these fractions by NMR and LC−MS has confirmed the presence of a complex mixture of yessotoxin analogues (*29*). Some of these analogues have been characterized (*25, 30*). Similar studies on contaminated shellfish are also planned.

The sensitivity of the ELISA compares very well with methods previously reported for yessotoxin measurement, making this methodology suitable for the analysis of water samples and algal cell extracts (*30*). After 150-fold dilution of shellfish extracts, the limit of quantitation (I_{20}) for samples in the ELISA was determined (mean of 10 assays) to be 75 μg/kg in whole shellfish flesh, which is equivalent to 7.5% of the MPL. This means that the assay will detect all analogues present at 1 MPL or less as long as cross-reactivity with the analogue of interest is greater than 7.5% (**Table 2**). Shellfish were extracted with 90% methanol in water, which gives a final concentration of approximately 80% methanol, a procedure reported to give an average recovery of 94% of yessotoxin spiked into scallops at 0.2−20 μg/g (*3*). Hepatopancreas of shellfish is extracted in many countries to take advantage of the concentration of toxin in this tissue. The sensitivity of the ELISA permits extraction of whole shellfish for toxin and therefore avoids the need to separate the hepatopancreas from the rest of the shellfish before extraction and analysis.

The development of an ELISA for yessotoxin provides a sensitive and rapid analytical method that uses inexpensive instrumentation and could easily be incorporated into a monitoring program or used as a tool for shellfish toxin research where large numbers of shellfish or algal extracts are to be analyzed. The broad specificity of the antibodies means that the ELISA cannot quantify specifically for yessotoxin in crude extracts, but it provides an estimate of the total content of yessotoxin and some analogues. The ELISA is able to detect all the yessotoxin analogues that the European Commission (*21*) currently requires shellfish to be tested for, i.e., yessotoxin, 45-hydroxyyessotoxin, homoyessotoxin, and 45-hydroxyhomoyessotoxin, as the cross-reactivities in the assay are 100, 116, 15 and 23%, respectively.

The ELISA will detect yessotoxins in algae because yessotoxin was reported to be the major analogue in *P. reticulatum* from the Adriatic Sea (*31*), and also extracts of a New Zealand isolate of *P. reticulatum* were reported to contain 30−50% yessotoxin as the single most abundant analogue together with at least 80−100 other yessotoxins (*32*). Similar results have been obtained with extracts of a Norwegian isolate (*33*). The ELISA will detect yessotoxins in contaminated mussels, as at most locations the predominant analogues observed are yessotoxin, 45-hydroxyyessotoxin, and carboxyyessotoxin (unpublished results), which have high cross-reactivity in the assay. Occasionally, up to 70% of yessotoxins in mussels from the Adriatic Sea are accounted for by homoyessotoxin and 45-hydroxyhomoyessotoxin (*5*). In this case, the ELISA signal will not be a true indication of toxicity relative to yessotoxin, although because the cross-reactivities of both analogues are above 7.5%, toxin concentration can be detected at levels below the MPL. The ELISA has since been reformatted into an assay suitable for commercialization using antiserum from sheep 504, which has greater cross-reactivity to the homoyessotoxins (**Table

2) and after further immunizations (data not presented) provided an assay with the sensitivity required.

The anti-yessotoxin antibodies have also been used to study the distribution of yessotoxins within the mussel *Mytilus galloprovincialis* by immunolocalization (*34*). It is anticipated that the antibodies could be used in additional applications, such as the preparation of immunoaffinity columns to provide cleanup and concentration of yessotoxins for research purposes such as characterization and identification of novel yessotoxin analogues. Because the antibodies bind yessotoxin analogues and derivatized yessotoxin analogues labeled with the fluorogenic reagent DMEQ-TAD (**Table 2**), such immunoaffinity columns could also provide a simple and efficient cleanup procedure for samples intended for either LC−MS or fluorometric HPLC analyses.

ABBREVIATIONS USED

cBSA, cationized bovine serum albumin; cELISA, competitive indirect enzyme-linked immunosorbent assay; CR, cross-reactivity; DMEQ-TAD, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione; ELISA, indirect enzyme-linked immunosorbent assay; MPL, maximum permitted level; LOQ, limit of quantitation; OVA, ovalbumin; PBS, phosphate-buffered saline; PVP, poly(vinylpyrrolidone) 25.

ACKNOWLEDGMENT

We gratefully acknowledge M. Satake and T. Yasumoto (Tohoku University, Sendai, Japan) for supplying yessotoxin used to make the immunogen and 45-hydroxyyessotoxin, 45,46,47-trinoryessotoxin, 1-desulfoyessotoxin, 1-*O*-acetyldesulfoyessotoxin, carboxyyessotoxin, and DMEQ-TAD-yessotoxin for use in cross-reactivity studies. We are grateful to A. Tubaro (University of Trieste, Trieste, Italy) for providing homoyessotoxin and 45-hydroxyhomoyessotoxin standards and to P. Truman and D. J. Stirling (ESR Ltd., Wellington, New Zealand), for yessotoxin used as a standard and to make the plate coater. We thank R. P. Hansen and R. A. Thompson (University of Waikato, Hamilton, New Zealand) for obtaining the NMR spectra, R. P. Hansen for assisting with the ozonolysis procedure, and the University of Auckland Chemistry Department for the use of the ozonolysis apparatus.

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Received for review April 15, 2004. Revised manuscript received July 15, 2004. Accepted July 16, 2004.

JF049395M