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Production of antibodies and development of highly sensitive formats of enzyme immunoassay for saxitoxin analysis

Abstract In this paper the production of antibodies against saxitoxin (STX) is described, as is the optimization and comparison of two competitive ELISA formats (direct and indirect) for the detection of this toxin. Tests were performed in a 96-well microplate using the toxin-specific polyclonal antibodies produced in our laboratory, obtained from rabbits immunized with saxitoxin-keyhole limpet hemocyanin (STX–KLH). In indirect ELISA format saxitoxin, conjugated to bovine serum albumin (STX–BSA) was coated onto the microtitre plate and incubated with standard toxin and anti-STX antibody. A goat anti-rabbit IgG Peroxidase conjugate was used to enable detection. In the direct ELISA format, STX standard, STX conjugate to horseradish peroxidase (STX–HRP), and enzyme substrate/chromogen solution were sequentially added to the microplate after antibody coating.

Results showed the saxitoxin detection limit to be 3 and 10 pg mL^{-1} for direct and indirect ELISA formats, respectively.

The suitability of the assay for quantification of saxitoxin in mussels was also studied. Samples were spiked with saxitoxin before and after sample treatment to study the extraction efficiency and matrix effect, respectively. After treatment, samples were analysed at 1:1000 v/v dilution in PBS to minimize the matrix effect and to detect the regulatory limit of 40–80 μg saxitoxin per 100 g mussels as stipulated by the Food and Drug Administration. The efficiency of extraction of saxitoxin was from 72 to 102%. These data were confirmed by liquid chromatography coupled with fluorimetric detection, the technique currently used for quantitative determination of toxins in seafood.

Keywords Enzyme immunoassay · Saxitoxin · Paralytic shellfish poisoning

Introduction

Saxitoxin is one of the most lethal non-protein toxins (LD$_{50}$ 9 μg kg^{-1}) [1]; it is one of the "paralytic shellfish poisons" (PSP), produced by several marine dinoflagellates and fresh water algae. Contamination of shellfish with saxitoxin has been associated with harmful algal blooms throughout the world.

The anthropogenic enrichment of nitrogen and phosphorus in coastal waters, the increase in aquaculture, the dispersal of marine plankton by ships' ballast water, and the transfer of shellfish stocks are considered to be among the main factors involved in the increase in cases of contamination and poisoning related to saxitoxin [2].

In humans, paralytic shellfish poisoning causes dose-dependent perioral numbness or tingling sensations and progressive muscular paralysis, which can result in death through respiratory arrest [3]. The maximum tolerance levels established by the European Union and the Food and Drug Administration are 40–80 μg PSP per 100-g edible portion of fresh, frozen, or tinned shellfish [4].

The Official AOAC method for these algal toxins is the "mouse bioassay" (MBA) [5]. The limitations of this procedure are the high variability of the results and the low sensitivity. It also requires a continuous supply of mice and results are affected by test conditions such as animal strain and sample extract preparations. Other methods include fluorimetry [6] and liquid chromatography [7, 8]. The latter requires expensive equipment and pre- or post-column analyte oxidation [7, 8]. Samples must also be analysed singly, so the method is unsuitable for routine on-site testing.

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Immunochemical methods have advantages of both sensitivity and speed, and are therefore of increasing importance in food control as rapid screening tests. Because of the highly specific antigen–antibody interaction, several laboratories have attempted to develop an immunoassay for PSP [9, 10]. Although an ELISA kit [11] for saxitoxin is now on the market, free antibodies against this toxin are not commercially available, despite this toxin having been included in the list of chemical weapon compounds (Paris Convention 13/01/1993, Low 496 25/11/1995 1A07)

In this paper the production of specific antibodies against saxitoxin is described. Among several approaches tested we found that the antibodies produced in rabbits after treatment with saxitoxin conjugated to keyhole limpet hemocyanin (KLH) were useful for the toxin assay. These antibodies were used to develop both indirect and direct enzyme-linked immunosorbent assays (ELISA). Details of the production, characterization, and test procedures for the determination of saxitoxin in mussels are reported. A comparison with the commercially available ELISA test kit is also reported.

Experimental

Reagents and materials

Saxitoxin (STX), used as standard and for conjugation, was purchased from Biomol (USA). KLH (keyhole limpets hemocyanin) was bought from Calbiochem (USA). New Zealand female three-week-old rabbits were used for the immunization. Antibody purification was conducted using Protein A Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden). Bicinchonic acid (BCA) (BCA Protein Assay Kit; Pierce, Rockford, USA) was used to determine protein concentration. Blank control serum was obtained from rabbits not yet immunized with the toxin. Maxisorp surface, 96-well polystyrene microtitre plates were purchased from Nunc (Roskilde, Denmark). Affinity purified anti-rabbit IgG (H+L, from goat) horseradish peroxidase (HRP) conjugate, and purified mouse IgG were purchased from Vector Laboratories (Berlingame, CA, USA). Non-fat dry milk, blotting grade, came from Bio-Rad Laboratories (Hercules, CA, USA). 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB), bovine serum albumin (BSA), ABTS (2,2'-azino bis (3-ethylbenzthiazoline-6-sulfonic acid) ammonium salt 98%) and all other reagents were from Sigma (St Louis, MO, USA). The Ridascreen saxitoxin kit was from R-Biopharm (Germany). All solvents were of LC or analytical reagent grade (Farmitalia, Carlo Erba-MI, Italy). Mussel samples were obtained from local supermarkets. The BCR reference material containing saxitoxin (BCR-543 mussel) was purchased from the Institute for Reference Materials and Measurements (IRMM, Belgium).

Apparatus

A model 550 microplate reader (Bio-Rad) was used to read the absorbance on ELISA plates at 405 nm. A model 238 UV cord S II, instrument from LKB (Bromma, Sweden), at 280 nm, was used for detection of proteins during collection of the antibodies in the purification process [12].

The LC system consisted of a Perkin–Elmer (Beaconsfield, UK) 410 LC pump and an injection port (Rheodyne 7125; Cotati, CA, USA) with 20-μL loop. The column used was a Supelcosil LC-18 (25 cm×4.6 mm id, 5 μm; Supelco). The fluorimetric detector was a dual-monochromator Perkin–Elmer LC 240 fluorescence detector set at 330 (excitation) and 400 nm (emission). The integrating recorder was a PE Nelson model 1020 from Perkin Elmer.

Procedures for preparation of Saxitoxin conjugates

Preparation of immunogen – conjugation of saxitoxin with KLH

The immunogen was prepared according to Renz and Terplan [13]. In a typical experiment KLH (2 mg) in sodium acetate buffer (0.1 mol L^{-1}, pH 4.4, 0.5 mL) was mixed with saxitoxin-acetate (150 μg) in the presence of formaldehyde (37% w/w, 40 μL). The reaction was left to proceed at room temperature for 72 h, then at 4 °C for another 12 h.

The reaction mixture was dialysed against acetic acid solution 1 mmol L^{-1} for 6 h, then against deionized water for 18 h. After the dialysis the STX–KLH was precipitated with aluminium potassium sulfate (10%, 800 μL) and NaOH (1 mol L^{-1}, 500 μL) overnight at 4 °C. The resulting precipitate was washed six times with cold phosphate-buffered saline (PBS), pH 7.2. The conjugate was prepared freshly for each immunization, because freezing can increase the hapten-carrier toxicity [14]. Animals were immunized and boosted according to the procedure reported below.

Preparation of coating antigen (STX–BSA)

The conjugation of saxitoxin to BSA involved the one-step formaldehyde method [15]. STX acetate salt (200 μg) in acetic acid (0.1 mol L^{-1}) was mixed with BSA (2 mg; 20 mg mL^{-1} in H$_2$O) and formaldehyde solution (37%, 12 μL) was added. The reaction was left to proceed at room temperature for 3 days.

The reaction mixture was dialysed against PBS buffer at 4 °C for 3 days to remove any free toxin. The average final concentration of STX–BSA, calculated by use of the BCA method, was 1.2 mg mL^{-1}. The conjugate solution was stored at –20 °C.

Conjugation of STX with horseradish peroxidase

Saxitoxin was coupled to HRP by the periodate reaction [11, 16]. HRP (4 mg) was added to sodium periodate solution (0.1 mol L^{-1}, 0.2 mL) and the solution was gently mixed for 20 min at room temperature. The mixture was dialysed against sodium acetate buffer, pH 4.4, overnight at 4 °C and the pH of the final solution was adjusted with carbonate buffer (0.2 mol L^{-1}, pH 9.6, 20 μL).

The activated enzyme was added to saxitoxin, as the acetate salt, (80 μg) in acetic acid (0.1 mol L^{-1}, 250 μL) and the pH was adjusted to 7.5 with sodium carbonate buffer (0.5 mol L^{-1}, pH 9.6). After 45 min at room temperature, NaBH$_4$ (4 mg mL^{-1}, 0.1 mL) was added and the mixture was incubated for 15 min at 4 °C and finally dialysed against PBS (0.15 mol L^{-1}).

The concentration of enzyme in the conjugate was determined spectrophotometrically at 403 nm (80 μg mL^{-1} protein) and the residual activity was also determined with ABTS by use of a spectrophotometer (1.73 U mL^{-1} residual activity against 5.02 U mL^{-1} of native activity).

The analysed fractions were stocked and stored at –20 °C.

The conjugation ratios (mol toxin bound per mol^{-1} HRP) could not be determined, because the PSP toxins did not sufficiently absorb UV.

Antibody production

Immunization procedure

Three rabbits were immunized using saxitoxin conjugated with KLH. Animals were immunized once every 3–4 weeks for a total of 20 times using 250 μg of hapten-carrier dissolved in PBS and emulsion with complete Freud's adjuvant emulsion (CFA). The second immunization (3 weeks later) was performed using the same amount of antigen after emulsification with incomplete Freud's adjuvant (IFA). Subsequent immunizations were performed by injecting STX–KLH, after precipitation in aluminium potassium sulfate, plus 3 μg free toxin. Blood was harvested from the rabbit's ear vein 7–10 days after the last immunization. The blood was cen-

trifuged at 3000 r.p.m. for 1 h at 4 °C; sera were then collected, stocked, and stored at –20 °C until use.

Antibody purification

Antibodies were purified with 10 mL protein A by affinity chromatography, the whole system consisting of the chromatographic column connected to a UV detector. The column was subjected to sequential pre-washing with: phosphate buffered saline pH 7.4 (PBS), NaCl 0.7%, $(NH_4)_2SO_4$ 1 mol L^{-1} pH 9.0, $MgCl_2$ 3.5 mol L^{-1}, and $(NH_4)_2SO_4$ again. Serum diluted in $(NH_4)_2SO_4$ (1 mL serum in 9 mL $(NH_4)_2SO_4$) was left to run overnight continuously with a flow system. Next day antibodies were eluted with $MgCl_2$ and the protein fraction was collected. Antibodies were dialysed in deionized water overnight and their titre determined spectrophotometrically [17].

Antiserum titration

After purification the binding specificity of the antibody against saxitoxin was measured by indirect enzyme-linked immunosorbent assay (ELISA). Polyclonal antibody screening was performed by coating the microplate with STX–BSA conjugate (3 μg mL^{-1}, 100 μL) in carbonate buffer, pH 9.6, overnight at 4 °C. After a washing step with PBS-T solution (PBS+0.01% Tween 20), the blocking buffer (1% skimmed milk+10 μg mL^{-1} mouse-IgG in PBS) was added to the wells and left for 1 h at 37 °C. The wells were then washed again and different dilutions of antibody against saxitoxin in PBLI (0.1% skimmed milk+1 μg mL^{-1} IgG in PBS) were added and the wells were incubated for 2 h at 4 °C. Plates were washed again and the secondary antibody, labelled with HRP (1:1500 v/v) was incubated in the wells for 1 h. After another washing step the colorimetric reaction was performed by addition of chromogen/substrate solution (100 μL) to each well. After 10 min incubation at room temperature the enzymatic reaction was blocked with HCl (2 mol L^{-1}, 100 μL) and the absorbance at 405 nm was read.

To obtain polyclonal antibodies with a higher affinity for saxitoxin and titre, additional rounds of immunization were performed.

Western immunoblot [18] with purified antibodies was performed to check purity.

Procedure for ELISA spectrophotometric assay

Indirect competitive assay

For this assay the antibody titration procedure was modified in the competition step only. Fixed concentration PAb (5 μg mL^{-1}, 50 μL) were added to each well and incubated for 5 min. Different concentrations of saxitoxin in PBLI (50 μL) were added and the competition reaction was performed for 1 h at 4 °C.

Direct competitive assay

PAb against saxitoxin (10 μg mL^{-1}, 50 μL) prepared in PGG–NaCl buffer [10] (0.01 mol L^{-1} piperazine–glycylglycine pH 10.0+NaCl 150 mmol L^{-1}) were added to a microplate and left overnight at room temperature. After immobilization, the wells were washed three times with a solution of PBS-T (PBS+0.05% Tween 20).

A solution of 1% skimmed milk+10 μg mL^{-1} IgG (mouse) prepared in coating buffer was used in the blocking step (1 h at 37 °C).

After washing, the competition was performed as follows. Different dilutions of saxitoxin were added in triplicate to wells with a fixed (1:30 v/v) concentration of STX–HRP in PBS. The reaction was left to proceed for 2 h at room temperature then the microtitre plate was rinsed with PBS-T. Finally the chromogen/substrate solution was added to the wells and the enzymatic reaction was stopped after 10 min at room temperature by addition of H_2SO_4 (2 mol L^{-1}, 100 μL). Absorbance was read at 405 nm.

LC analysis

Separation and chromatographic analyses were performed as reported by Lawrence et al. [7]. This analytical method was used to analyse mussel samples for comparison of results with those from with ELISA assay.

Preparation of samples

Sample extraction

Sample extraction was performed according to the AOAC method [5]. Mussels were removed from their shells, washed in water, and homogenized. Aliquots of mussel (1 g) were weighed in a 4-mL glass vial and HCl (0.1 mol L^{-1}, 1 mL) was added. After mixing the vials were kept for 5 min at 85 °C. After cooling to room temperature, the solutions were adjusted to pH<4 and water was added to a final volume of 2 mL. The mixtures were then centrifuged at 3000 rpm for 5 min to remove the tissue. For the ELISA test the supernatant (5 μL) was diluted to 5 mL with buffer and used directly in the assay.

For LC analysis, the sample was purified by a solid-phase extraction (SPE) column cleanup. An aliquot (1 mL) of shellfish extract was passed through a 3-mL SPE reversed-phase C_{18} cartridge (Supelco) previously conditioned with methanol (6 mL) then HCl (0.1 mol L^{-1}, 6 mL). The effluent, and water (2 mL) used to elute the remaining toxin, were collected. The volume of the solution was adjusted to 4 mL with water and to pH 8 before the oxidation reaction.

To perform the peroxide oxidation aqueous H_2O_2 (10% (v/v), 25 μL) was added to NaOH (1 mol L^{-1}, 250 μL) in a 0.5-mL plastic microcentrifuge tube. Sample extract (100 μL) was then added, mixed, and left to react for 2 min at room temperature. Concentrated acetic acid (20 μL) was then mixed with the reactant solution and 20 μL of this final solution were injected into LC system.

Artificial contamination of mussel tissue

Several dilutions of saxitoxin standard were added to each gram of homogenized tissue to furnish the desired concentration. For example, 0.4 ng mL^{-1} STX corresponds to 800 ng g^{-1} tissue, when a 1:1000 (v/v) dilution of tissue extract is used, and also to 80 μg STX/100 g of mussel tissue, which is currently the highest permitted level for commercial mussels. Contaminated homogenates were stored at –20 °C or used directly in the test.

Calibration plots and analysis of samples

Standard curves were obtained by use of STX standard solutions prepared in PBS (3×10^{-4}–3 ng mL^{-1} for direct assay and 3×10^{-4}–3×10^2 ng mL^{-1} for indirect assay) for ELISA and in 0.03 mol L^{-1} acetic acid (0–100 ng mL^{-1}) for liquid chromatography.

Calibration plots (dependence of absorbance at 450 nm on competitor concentration) were fitted using "non-linear four-parameter logistic calibration plots" [20].

The four-parameters logistic function is:

$$f_{(x)} = \left\{ \frac{a - d}{1 + (x/c)^b} \right\} + d$$

in which a and d are the asymptotic maximum and minimum values, respectively; c is the value at the inflection point (IC$_{50}$), and b is the slope.

To enable direct comparison of some calibration plots absorbance values were converted into their corresponding test inhibition values (% A/A$_0$) as follows:

$$\% \frac{A}{A_0} = \frac{100 \times (A - A_{sat})}{(A_0 - A_{sat})}$$

where A is the absorbance value of competitors, and A_{sat} and A_0 are the absorbance values corresponding to the saturating analyte

and to the non-competition concentration, respectively (as evaluated by the four-parameter logistic function).

The detection limit was defined as the concentration of saxitoxin equivalent to the three times the value of the standard deviations (s) measured in the absence of saxitoxin (mean value −3 s). The midpoint value (IC_{50}) was evaluated as the concentration of saxitoxin at 50% A/A_0. The working range was evaluated as the toxin concentration that gives test inhibition values of 90% and 10% of A/A_0.

In a preliminary study, the matrix effect of blank samples on both ELISA formats was tested. Different dilutions (0, 1:10, 1:100, 1:1000, and 1:10,000 v/v) in PBS, pH 7.4, of blank mussel acid extracts were fortified with STX standard solutions.

To evaluate the extraction efficiency, calibration plots were constructed for blank mussel tissue spiked with STX standard solutions (0.01–0.8 µg g^{-1}) before extraction. The extraction was performed as reported in the sample-extraction procedure. Each experiment was performed in triplicate and mean values were used for curve fitting.

The data obtained for each curve were plotted and fitted by use of SigmaPlot software (SPSS). Regression analysis on the linear portion of the sigmoidal curves was also performed.

Validation samples (0.2, 0.4, 0.8 µg g^{-1} tissue), used to evaluate the accuracy and the precision of the methods, were prepared in the same way as for the calibration plots. These concentrations were chosen because they corresponded to double (0.8 µg g^{-1}), half (0.2 µg g^{-1}), and the critical level (0.4 µg g^{-1}) of saxitoxin stipulated by the Food and Drug Administration.
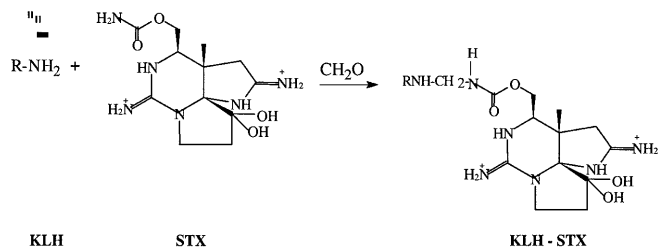
Results and discussion

Antibody production

Specific humoral response against small molecules (haptens) can usually be achieved by coupling the small compounds to high-molecular-weight protein (e.g. keyhole limpet hemocyanin, KLH). This conjugation improves the immunogenicity of the hapten by increasing the size of the antigen [12]. Because STX is a molecule with a low molecular weight, coupling of this hapten to carrier proteins is required to induce a better specific immune response.

The conjugate STX–KLH was produced (Scheme 1) and used to immunize the rabbits. Sera obtained from these rabbits were monitored with non-competitive ELISA for the antibody production. Rabbits started to elicit antibodies as early as 5–6 weeks after immunization.

During the development of experimental procedures several changes were made to the ELISA method, mainly in the coating, blocking, and antibody-incubation steps. Analysis conditions were optimized by means of a "checkerboard" titration. Antiserum production increased substantially after the first booster. Figure 1 shows that after approximately 9–10 weeks antibodies were produced, as confirmed by tests performed using both specific and pre-immune sera.
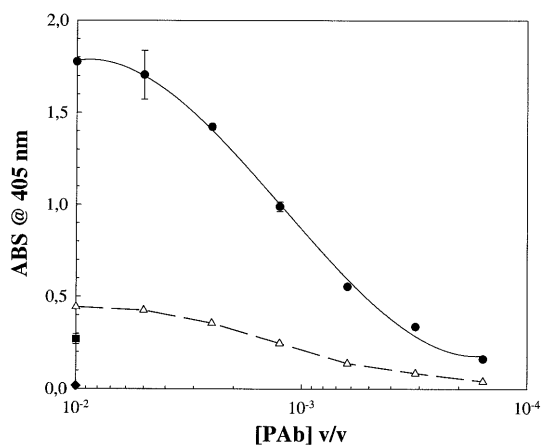
Fig. 1 Titration of antibody by indirect ELISA – antisera from rabbit immunized with STX–KLH (*circles*); pre-immune serum (*triangles*); no coating (*diamonds*); and no antibody (*square*). Antibody titre of immunoserum was 1:100 v/v

Polyclonal antibodies against saxitoxin were collected from those rabbits for which good titre and affinity for the antigen were achieved.

It is important to emphasize that, because of the low antigenicity of saxitoxin, several boosters were needed to obtain a good antibody titre, which was determined after purification of the antiserum on a protein A column and subsequent lyophilization of the purified product.

The titre was first determined spectrophotometrically by the Bradford assay [17]. Indirect ELISA tests were performed on this lyophilized serum and the inflection point of the curve was taken as the titre value for the antiserum. This value was 5 µg mL^{-1}, confirming the Bradford results, and was taken as the optimum concentration of the polyclonal antibody obtained (Pab or primary antibody).

Antisera specificity and selectivity were determined by competitive ELISA in which other compounds, e.g. L-OVA (lactosylated ovalbumin) and KLH, competed with the STX–HRP conjugate for binding of antibody coated to the solid phase. KLH and L-OVA were chosen because the first was the carrier used for the conjugation and the second had the same cross-linker (bridge) of the KLH–STX conjugate, used for the immunization. These experiments were performed to detect bridge interferences usually observed for small haptens. After incubation with the STX–HRP conjugate and these competitors, the conjugate bound to the antibody immobilized on the solid phase was determined by reaction with the enzyme substrate. Figure 2 shows that KLH and L-OVA did not compete with STX–HRP conjugate for binding to the antibody. Our results showed that the antibodies obtained are specifically directed against the small haptens.

Studies of cross-reactivity with the STX homologs were not performed, because these products were not commercially available.
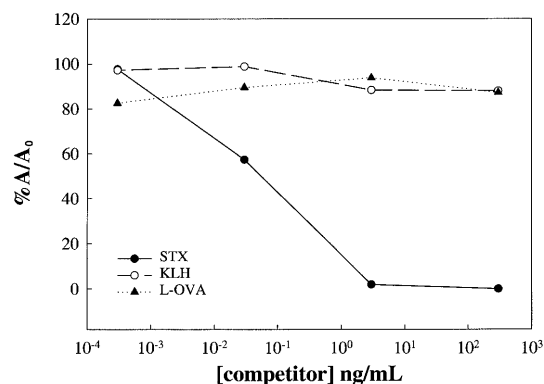
Scheme 1 The conjugation reaction between STX and KLH

Fig. 2 Direct competitive ELISA for STX (*filled circles*), KLH (*unfilled circles*), and L-OVA (*triangles*). Antibody against STX (10 µg mL^{-1}) was coated on the ELISA plate and STX–HRP (1:30 v/v) was used as competitor

## Optimization of ELISA procedures

Two ELISA formats were developed to compare sensitivity, working and linear ranges, and detection limit. Optimization of ELISA conditions, e.g. temperature, buffer, and amount of antibody, was initially performed by use of standard solutions of the analyte under investigation.

For the indirect format the STX–BSA conjugate was prepared to be used as the coating antigen, to increase the response and sensitivity of the indirect assay. The use of conjugated haptens is considered necessary for non-covalent immobilization, because of the tendency of proteins to stick to the plastic surface of microwells. The involvement of protein carriers resulted in different orientations and stereochemical accessibility of hapten molecules [22].

For both systems different amounts of specific antibodies and conjugates were then tested for the development of competitive ELISA. The best conditions were found to be a coating of 3 µg mL^{-1} STX–BSA for the indirect format and 10 µg mL^{-1} of the antibody for the direct assay with 1:30 (v/v) STX–HRP (prepared during this study).

The optimized competition curves for the direct and indirect formats of the assay are shown in Figs. 3a and 3b, respectively. The working range and detection limit for saxitoxin were determined from the "non-linear four-parameter logistic calibration plots" [20].

The detection limit, defined as the concentration of toxin standard equivalent to three standard deviations at A$_0$ (no competition), was 10 pg mL^{-1} for the indirect assay and 3 pg mL^{-1} for the direct format. The working ranges, defined as the standard toxin concentration range between 90% and 10% of the maximum signal (A$_0$) [23], were 3×10^{-3}–3×10^{-1} ng mL^{-1} for the direct assay and 3×10^{-3}–3 ng mL^{-1} for the indirect format. In both tests the linear ranges were 5×10^{-3}–4×10^{-1} ng mL^{-1}. These results indicated that the antibody had high affinity for saxitoxin. The concentrations causing 50% inhibition (IC$_{50}$) of antibody binding to the antigen (STX) were 60 and 25 pg mL^{-1}, respectively, for the indirect and direct assays.
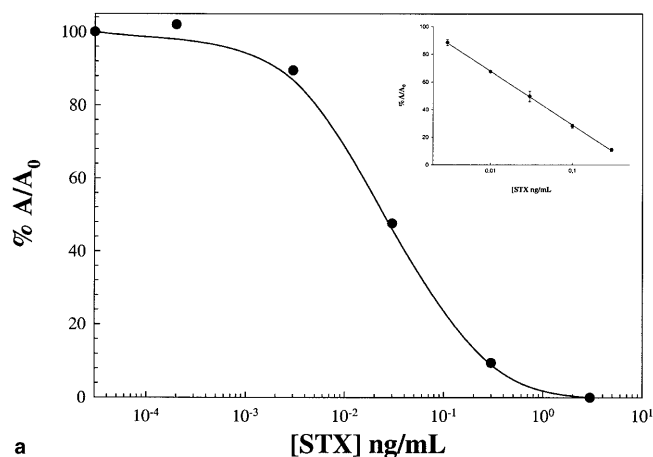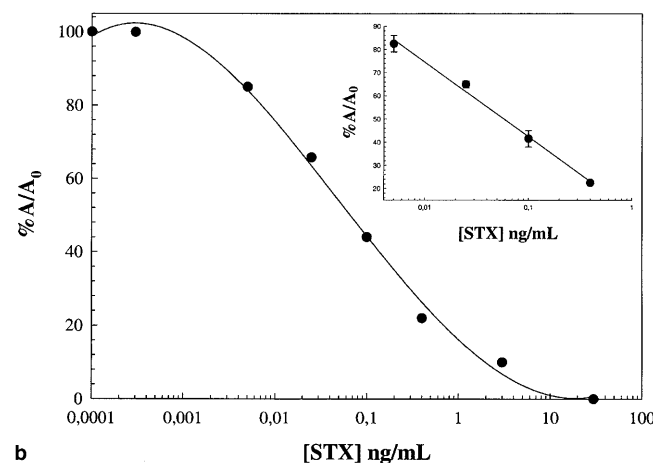
Fig. 3 (**a**) Direct competitive ELISA for saxitoxin. Antibody against STX (10 µg mL^{-1}) was coated on the ELISA plate and STX–HRP (1:30 v/v) was used as competitor. Linear regression shows a working range of 5–400 pg mL^{-1} (r^2=0.934) (*top right insert*). (**b**) Indirect competitive ELISA for saxitoxin. STX–BSA (3 µg mL^{-1}) was coated on the ELISA plate. Linear regression shows a working range of 5–400 pg mL^{-1} (r^2=0.990) (*top right insert*)

The optimized direct ELISA and the Ridascreen kit [11], a commercially available kit, were compared. The latter is based on a competitive enzyme immunoassay for quantitative analysis of STX in mussels. The microtiter plate with 48 wells is coated with antibodies directed against STX. The mean lower detection limit of the kit was approximately 10 pg mL^{-1}, compared with 3 pg mL^{-1} for our direct test; this was probably because of different antibody affinity for saxitoxin. The sensitivity, defined by means of the IC$_{50}$ value (parameter c of the logistic function), of our assay and of the commercial kit were shown to be comparable by use of the "Student t-test" (n=6) [24].

Stability studies

The stability of the coating reagents was evaluated by use of microplates coated with conjugated antigen or antibody; these were then blocked and stored at 4 °C. Assays were performed periodically by use of the assessed proce-

dures. Results showed that the plates coated with the anti-
bodies (direct test) could be used for up to 3 weeks after
the coating step whereas the antigen immobilized on the
wells was stable for 24 h only (indirect test). Better results
obtained from the direct format could, therefore, be be-
cause of higher antibody stability.

Measurement of saxitoxin in mussels

The spectrophotometric ELISA was then applied to mus-
sels. Mussel samples were collected and the extraction
procedure performed as described in the experimental sec-
tion to evaluate matrix effects and extraction efficiency.

Matrix effect were characterized by using mussels in
which the toxin was not present. To assess the effect of the
matrix on the performance of the optimized ELISA proce-
dures (direct and indirect formats), calibration curves were
constructed by adding saxitoxin standard solutions to the
pulp extract at different dilutions (0, 1:10, 1:100, 1:1000,
1:10000 v/v). Comparison of the results with the calibra-
tion curves obtained from use of PBS revealed the effect
of the matrix was lowest when the matrix dilution was
1:1000 v/v (data not shown). This dilution also enabled
detection of the regulatory limit of 40 µg saxitoxin/100 g
mussels. A linear range between 5×10^{-3} and 4×10^{-1} ng mL^{-1}
was obtained for both formats, although regression analy-
sis performed in this range showed that the direct format
($r^2=0.996$) gave better results than the indirect format
($r^2=0.986$). For this reason, and because of the shorter

time necessary for the analysis, the direct format was cho-
sen for further experiments.

The extraction efficiency was evaluated by comparison
of calibration curves (Fig. 4) constructed before and after
extraction of blank mussels spiked with known amounts
of saxitoxin. The recovery rate for artificial contamina-
tion mussel samples ranged from 72 to 102% (the value of
72% was observed for the lowest concentration of the
toxin). The repeatability and accuracy of ELISA assays
were evaluated by means of six replicate analyses of tissue
from mussels bought on different days and from different
stores. Blank control samples fortified with saxitoxin at
concentrations equal to twice (0.8 µg g^{-1}), to half (0.2 µg g^{-1}),
and to regulatory limit (0.4 µg g^{-1}) were prepared and ex-
tracted on three days for each concentration (n=18).

The precision was determined by the calculation of the
relative standard deviation (RSD%) for replicate measure-
ments and the accuracy (relative error, RE%) was calcu-
lated by assessing the agreement between measured and
nominal concentrations for the fortified samples. Results
obtained were confirmed by analysis of the same extracts
by use of a previously validated LC method [7]. A refer-
ence material from the IRMM was also analysed in tripli-
cate. Results showed that the accuracy (RE%) of the di-
rect ELISA method was approximately 7.5% (STX found=
0.51 µg g^{-1} instead of 0.48 µg g^{-1} as certified by the com-
pany). Values obtained for artificially contaminated mus-
sels by use of the spectrophotometric ELISA method and
the validated LC method were in good agreement (Table 1).

Conclusion

Results obtained from this study indicate that antibodies
obtained from rabbits immunized with STX–KLH are suit-
able for the determination of STX in foods by our ELISA
method.

ELISA assays were shown to be suitable screening
tools for routine determination of saxitoxin in mussels.
Compared with the LC method spectrophotometric direct
ELISA gave similar results but with the advantages of a
faster, simpler, and less expensive technique. In addition
this method does not require sample purification. Stability
studies showed that antibody-coated microplates could be
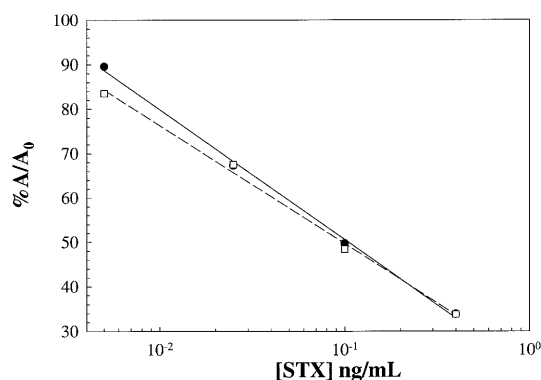stored for up to 3 weeks before the competition step.

Fig. 4 Effect of sample treatment and matrix on competitive direct
ELISA for saxitoxin. The calibration curves were obtained by
spiking mussel with known amounts of saxitoxin before (*circles*)
and after (*squares*) extraction. In both cases the extracts were di-
luted 1:1000 w/v and assayed

Table 1 Precision (RSD) and accuracy (RE%) for determination of saxitoxin in mussel by ELISA and LC

STX added (µg g^{-1})	Direct ELISA			LC		
	STX found (µg g^{-1})	RSD	RE%[a]	STX found (µg g^{-1})	RSD	RE%[a]
0.2	0.17	6	–15	0.18	4	–10
0.4	0.44	5	10	0.42	5	5
0.8	0.85	4	6	0.84	5	5

[a]RE% (relative error%)=[(measured value-true value)/true value]×100

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