FOOD-CT-2004-514055

DetecTox

Development of an SPR-based biosensor for the detection of lipophilic phycotoxins in shellfish residues

Specific Targeted Research Project

Sixth Framework Programme, Priority 5; Food Quality and Safety

Publishable Final activity report

Period covered: from 01/01/2005 to 30/06/2008
Date of preparation:

Start date of project: 01/01/2005 Duration: 42 Months

Project co-ordinator name: Dr. Luis M Botana
Project co-ordinator organisation name: USDC.

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1. PROJECT EXECUTION

The overal objective of DETECTOX (contract VIth FP FOOD-CT-2004-514055) project was to develop a Surface Plasmon Resonance (SPR) biosensor to detect lipophilic toxins: diarrheic shellfish toxins (DSP), yessotoxins (YTXs), pectenotoxins (PTXs), azaspiracids (AZAs) and gymnodimine (GYM). Most of deliverables and milestones proposed in this project have been reached.
The contractors involved in DETECTOX project were: Queen´s University Belfast (QUB) (UK), Xenosense (XENOSENSE) (UK), Universidade de Santiago de Compostela (USC) (E) and Laboratoire de Neurobiologie Cellulaire et Moléculaire (CNRS) (F), (these 4 contractors were involved in the scientific project development), Community Reference Laboratory for Marine Biotoxins (CRLMB) (E) and ANFACO (E) (involved in collection of contaminated samples and comparison of biosensor development with current state of the art), National Reference Laboratory for Marine Biotoxins (NRLMB) (HE) and Ege University (EGE) (TR) (assesment and sample supplies)
Project co-ordinator name: Dr. Luis M Botana
Project co-ordinator organisation name: Universidade de Santiago de Compostela (USC) (E).

WORK PERFORMED and RESULTS ACHIEVED:

Work Package 2 – Production of reagents
Task 2.1.- The first objective of the project was the acquisition and purification of toxins to be used as reference materials to study the mechanism of action and to develop the biosensor assay. Even though some of these toxins were commercially available, due to the large amount required and to their high price, in DETECTOX purification protocols of each toxin from contaminated material were developed by USC. QUB provided contaminated mussels collected in the northwest cost of Ireland, and pure AZA-1 to 5 were obtained, but only AZA1 and AZA2 were abundant enough for antibody induction. For these toxins a specifically design purification protocol included organic extractions and separation by preparative HPLC was designed. YTX was obtained after development of new extraction and purification procedures from dinoflagellate cultures grown at USC. This protocol included organic extraction and partitions, two new cleaning steps and finally the separation through preparative HPLC. 1 mg PTX-2 was obtained from a kind gift from BIOTOX project (VIth FP contract 514074), and USC collected algae from a toxic episode and the purification of a small amount of this toxin was successfully done. This material was contaminated with okadaic acid (OA), dinophysistoxin-2 (DTX-2) and PTX-2. The purification included an organic extraction and the separation through preparative HPLC and later stabilization. With this protocol PTX-2 was purified but also OA and DTX-2. GYM was purchased from Tunisia (Institute Pasteur), as this toxin is rare in European coasts. All these purification protocols that were developed for the project and the toxin stability studies have been published in peper review journals. In summary several sources of toxins were explored in the project and interesting scientific results were

obtained, mostly including self developed purification methods; at least 1 mg of each toxin was supplied for antibody production.

Task 2.2.- The second task in WP2 was the identification of molecular targets of phycotoxins to be used as antibody alternatives to develop sensor chips. DSP, YTX and PTX molecular targets had been already described, but no information was available with regard to AZA. Several experiments were done at USC to clarify the mechanism of action of AZAs. Early and long-term responses in the presence of AZAs in different cellular models (human lymphocytes, human BE(2)-M17 neuroblastoma cells, human Caco-2 intestinal cells, human NCI-H460 lung carcinoma cells, human mast cells HMC-1 and primary cultures of cerebellar granule cells) were checked. An important effect in actin cytoskeleton disorganization was evidenced in the presence of AZA-1. In addition AZA-1 inhibited cellular proliferation and induced caspases activation after 48 hours cellular treatment. This effect was related with the molecular structure since fifteen different molecules (kindly provided by Dr. K.C. Nicolaou, Scripps Institute) were tested and only ABCD,C20-epi-AZA-1 had a similar effect to AZA-1. These effects were reported as irreversible and activated after a short incubation period in the presence of the toxin. There was a minor difference in potency between AZA-1 and AZA-2 and it was studied the kinetic on cell viability, actin cytoskeleton and caspase activation. The potency of both molecules for cytoskeleton alterations was the same even in experiments where a short exposure to the toxin was followed by 48 h incubation. Further structural studies with two modifications of the AZA-2 molecule where the carboxylic acid moiety at C1 being replaced by a methyl ester (AZA-2-ME) or by a biotinylated amide moiety (biotin-AZA-2); these changes suggest that the carboxylic group at C1 is relevant to AZA effect. In fact, the in vitro effects of AZA-2-ME and biotin-AZA-2 on cell cultures follow their rate of transformation into AZA-2. The effects of AZAs in non-tumoral cells, cerebellar granule cells and lymphocytes, reported an increase in cytosolic calcium levels and a cytotoxic effect depending on time as well as concentration. The cytotoxicity of AZA-1 and AZA-2 was not dependent on calcium effect, however was completely blocked when c-jun kinases (JNK) pathway was inhibited. From these studies it can be concluded a relationship between azaspiracid-induced cytotoxicity and specific modifications in cellular transduction signals, specifically JNK activation is associated with the cytotoxic effect of AZAs.

The GYM target was studied at CNRS. The pharmacological characterisation of the interaction of GYM-A with its putative membrane receptors was achieved. Data shown that the molecular target for GYM-A is the nicotine acetylcholine membrane receptor. GYM-A, is a powerful ligand interacting with subnanomolar affinities on muscular and, homopentameric and heteropentameric neuronal receptors of various animal species. To confirm GYM effect, these receptors have been purified from the electric organ of *Torpedo marmorata* and microtransplanted and expressed in xenopus oocytes. Receptor expression in membrane oocytes was confirmed by electrophysiological measurements in the presence of the natural agonist. After successfully receptor expression, the effect of GYM was checked. A significantly inhibition on agonist response was found in the presence of toxin. These results confirm that the nicotine acetylcholine receptor is the molecular target for GYM. Highly purified samples of GYM-A and GYM-B from clams were used to assess their toxicology activity. After intraperitoneal (i.v.), intracerebroventricular (i.c.v.) and subcutaneous (s.c.) injections, the LD_{50} for GYM A was: 100 µg/kg (s.c.), 80 µg/kg (i.p.) and 3 µg/kg (i.c.v.). GYM B was also toxic to mice, but was much less toxic than gymnodimine A. The LD_{50} for GYM B was 800 µg/kg (i.p.).

Task 2.3.- The last objective in WP2 was the binding reagent production. The technical specifications and possible synthetic routes for reagent production have been researched, and the assay development discussed between QUB and XENOSENSE. Immunogens for all the toxins have been successfully studied and prepared by XENOSENSE. These immunogens were inoculated for the subsequent production of antibodies by QUB. In these conditions polyclonal and monoclonal antibodies for OA have been produced and characterized. However, after six immunizations with an YTX conjugate no polyclonal antibodies were obtained. Two polyclonal antibodies to an AZA-1 were produced and purified. In the case of GYM, several reactive molecule fragments were used to prepare antibodies and very good titres were obtained. No antibodies or reactive molecules were done in the case of PTX.

Work Package 3 – ASSAY DEVELOPERS

The antibodies obtained were used to develop the inhibition test (WP3). For this matter, other key step in the project was the development of sensor chip surfaces with immobilized toxins (task 3.1) to then develop the inhibition assay (task 3.2). Different strategies have been used depending on the toxin studied. The OA-N-hydroxy succinimide ester was immobilized onto an amine sensor chip and after some modifications and using the polyclonal antibody before described, a rapid and specific biosensor immunoassay has been developed and published. From this assay an OA prototype kit was produced. This assay showed cross reactivity with DTX1. Later on when the monoclonal antibody was ready other assay was developed. In this case the cross reactivity with DTX-1 was 100% and 60% with DTX-2, and no cross reactivity with DTX-3. These data correlate exactly with the intrinsic toxic potency of the OA-group of toxins and point to the monoclonal antibody as an excellent candidate for full biosensor.

Different approaches have been done to develop YTX sensor chips surfaces. No success with YTX chips has been obtained even though different strategies have been tried. The YTX antibody did not show binding in the biosensor even when it reacted with YTX-conjugate. This means that the original designed chemistry had been successful and that the toxin orientation on the chip surface may have been the problem. An alternative to detect YTX by means of using their receptor, the phosphodiesterases, was developed at USC. These enzymes are the YTX intracellular target and had been used to develop an activated surface in a resonant mirror optical biosensor to detect YTX. This idea was transferred to the Biacore biosensor to develop a direct assay (hence with no inhibition). In this assay phosphodiesterases were immobilized in a CM5 activated sensor surface and YTX interaction was studied. The results showed that phosphodiesterases were stable in the chip surface and also a good correlation between response and toxin concentration. These data were published and the assay is going to be converted to Biacore Q format when enough toxin becomes available.

Since not enough amount of PTX was available to produce antibodies, several immobilisation experiments with the actin, the toxin target, were done. Different strategies to immobilize the protein to the sensor surface were checked at the USC but no binding protein-toxin was found. This approach points at actin as a weak binder for SPR technology.

Two polyclonal antibodies to AZAs were produced by QUB and several approaches have been done to develop AZAs sensor chips surfaces. Two strategies for activation of AZA were done by XENOSENSE finally a chip surface for AZA was produced. In these conditions the inhibition assay was developed and one of the polyclonal antibodies to AZA recognized the toxin. The assay is not fully developed yet but it is currently underway and it can be improved with a monoclonal antibody.

Several polyclonal antibodies to GYM fragment were produced by QUB and even though very good titres of antibodies against the fragments were obtained, no inhibition with the parent toxin was observed. The immunogens and the sensor surface chips were developed by XENOSENSE after they got the natural toxin. However, several rabbits and mice were immunised but no antibodies were produced. The cellular GYM receptor produced by CNRS was checked as an alternative to develop the chip surface at the USC. These experiments started by the study of toxin-receptor interactions in solution. After accurate results were obtained an inhibition assay was developed by immobilization of receptor in the sensor surface, however this assay design cannot be used for GYM detection probably due to the size of receptor.

Work Package 4 – VALIDATION AND COMPARISON

Prevalidation and comparison of biosensor with current state of art was also included in the project (WP4). The DSP detection kit developed in this project showed very good sensitivity and an excellent correlation with other analytical methods. Four kits were supplied to four laboratories and a pre-validation study has been done. The biosensor assay was determined to be accurate, reliable, fast and more cost effective in terms of toxin usage than the commonly used competitive and antigen coated ELISA techniques. These international laboratories found the assay simple and straight forward to perform. The results were reproducible between laboratories.

Work Package 5 – DEMOSTRATION AND DISEMINATION

Demonstration and dissemination activities were carried out in WP5. A webpage (www.detectox.eu) has been opened and updated along the project. The exchange of information through this space between partners was very fluent. A workshop to show biosensor methods for the detection of lipophilic toxins was organized by the CRLMB in Cyprus, 19Th October 2007. The workshop was a tutorial session on the "Principles of SPR" followed by a tutorial illustrating schematically the extraction procedures and sample preparation for lipophilic shellfish toxins. A questionnaire designed to obtain feedback from the workshop indicates that it was an extremely successful event. The workshop was concluded with a question and answer session. The workshop provided a forum for discussion of the projects' developments and their application between the scientists performing the research and the regulators for marine biotoxins within the EU. A second workshop is going to be organized at Vigo, in October 2008.

A virtual workshop was done. In addition 10 papers (6 already published) are directly related with results from this project, and several meeting presentations have been done.

www.detectox.eu

2. DISSEMINATION AND USE

Section 1 - Exploitable knowledge and its Use

Overview table

Exploitable Knowledge (description)	**Exploitable product(s) or measure(s)**	**Sector(s)** of application	Timetable for commercial use	Patents or other IPR protection	**Owner** & Other Partner(s) involved
Toxin binding reagents available for assay production	Biosensors for shellfish toxins	Public health monitoring	2010	Patent protection might be sought; if not, strict confidentiality will be maintained	QUB
Sensor chips for DSPs, YTX and GYM assay development	Biosensors for shellfish toxins	Public health monitoring	2010	Patent protection likely	XENOSENSE / USDC
Validated prototype assay method available (DSP)	Biosensors for shellfish toxins	Public health monitoring	2010	Patent protection likely	All partners

Section 2 – Dissemination of knowledge

Overview table

Planned/actual Dates	**Type**	**Type of audience**	**Countries addressed**	**Size of audience**	**Partner responsible /involved**
September 2008	16th European Section meeting of The International Society on Toxinology. Leuven, Belgium	Analytical community	All	100s	USDC
October 2008	Combined demonstration and dissemination workshop. Vigo, Spain	Stakeholders and producers and research institutions	Europe	100s	ANFACO/ all Detectox members, other cluster members
October 2008	Inform about final DETECTOX progress. Ljuljana, Slovenia	National reference Laboratories (NRL) meeting	Europe	30s	CRLMB/all Detectox members, other cluster members

MEETING PRESENTATIONS

Title	Autors	Venue	Date	Presenter
The European Approach to Improving analysis of DSP and PSP marine toxins	C. Elliot, M. Kotterman, L.M. Botana	3rd International Symposium on Recent Advances in Food Analysis	November 2007	L.M. Botana
Azaspiracids: Mechanism of action and structure-activity studies	L.M. Botana	16th European Section meeting of The International Society on Toxinology. Leuven, Belgium	September 2008	L.M. Botana

SECTION 3 - PUBLISHABLE RESULTS

The following publications were obtained within this project:

A. Villar-González, M.L Rodríguez-Velasco, B. Ben-Gigirey, L.M. Botana. 2006. First evidence of spirolides in Spanish shellfish. Toxicon, 48, 1068-1074.

Llamas, N. M., Stewart, L.D., Fodey, T., Higgins, H.C., Velasco, M. R., Botana, L., Elliott, C.T. 2007 Development of a novel immunobiosensor method for the detection of okadaic acid contamination in shellfish extracts. Anal. Bioanal. Chem. 2007; 389, pp 581-587.

Eskiocak U, Ozkan-Ariksoysal D, Ozsoz M, Oktem HA. Label-free detection of telomerase activity using guanine electrochemical oxidation signal. Anal Chem. 2007;79(22):8807-11

Vilariño, N.; Nicolaou, K.C.; Frederick, M.O.; Vieytes, M.R.; Botana, L.M. Irreversible cytoskeletal disarrangement is independent of caspase activation during in vitro azaspiracid toxicity in human neuroblastoma cells. Biochem Pharmacol. 2007 Jul 15;74 (2):327-35.

Alfonso, C.; Alfonso, A.; Pazos, M.J.; Vieytes, M.R.; Yasumoto, T.; Milandri, A.; Poletti, R.; Botana, L.M. Extraction and cleaning methods to detect yessotoxins in contaminated mussels. Analytical Biochemistry. 2007, 363, 228-38.

Vale, C.; Gómez-Limia, B.; Nicolaou, K.C.; Frederick, M.O.; Vieytes, M.R.; Botana, L.M. The c-Jun-N-terminal kinase is involved in the neurotoxic effect of azaspiracid-1. Cell. Physiol. Biochem. 2007, 20 (6):957-66.

A. Villar-González, M.L Rodríguez-Velasco, B. Ben-Gigirey, L.M. Botana. 2007. Lipophilic toxin profile in Galicia (Spain): 2005 toxic episode. Toxicon, 49, 1129-1134.

Fonfría, E.S., Vilariño N, Vieytes MR, Yasumoto T, Botana LM. Feasibility of using a surface plasmon resonance-based biosensor to detect and quantify yessotoxin. Anal. Chim. Acta. 2008 Jun 9; 617(1-2):167-70.

Okzan-Arksoysal, D.; Tezcanli, B.; Kosova, B.; Ozsoz, M.. Design of Electrochemical Biosensor Systems for the Detection of Specific DNA Sequences in PCR-Amplified Nucleic Acids Related to the Catechol-O-methyltransferase Val108/158Met Polymorphism Based on Intrinsic Guanine Signal. Anal Chem. 2008; 80(3):588-96.

Yean CY, Kamarudin B, Ozkan DA, Yin LS, Lalitha P, Ismail A, Ozsoz M, Ravichandran M. Enzyme-linked amperometric electrochemical genosensor assay for the detection of PCR amplicons on a streptavidin-treated screen-printed carbon electrode. Anal Chem. 2008, 80 (8): 2774-9.

Alfonso, C.; Alfonso, A.; Otero, P.; Rodriguez, P.; Vieytes, M.R.; Elliot, C.; Higgins, C.; Botana, L.M. Purification of five azaspiracids from mussel samples contaminated with DSP toxins and azaspiracids. J Chromatogr B, 2008, 865, 133-40.

Vale C, Wandscheer C, Nicolaou KC, Frederick MO, Alfonso C, Vieytes MR, Botana LM. Cytotoxic effect of azaspiracid-2 and azaspiracid-2-methyl ester in cultured neurons: Involvement of the c-Jun N-terminal kinase. J Neurosci Res. 2008 May 23. In press

Chapela M.J., Reboreda A., Vieites J.M., Cabado A.G. Lipophilic toxins analysed by liquid chromatography-mass spectrometry and comparison with mouse bioassay in fresh, frozen and processed molluscs, Journal of Agricultural and Food Chemistry. 2008. In press.