Conjugation of genetically-engineered protein phosphatases to magnetic particles for okadaic acid detection

Diana Garibo[1], Eric Devic[2], Jean-Louis Marty[3], Jorge Diogène[1], Idoia Unzueta[4], María Blázquez[4], Mònica Campàs[1]

[1] IRTA, Carretera de Poble Nou, km 5.5, 43540 Sant Carles de la Ràpita, Spain

[2] GTP Technology, Rue Pierre et Marie Curie, 31681 Labège Cedex, France

[3] IMAGES EA4218, Université de Perpignan-Via Domitia (UPVD), Building S, 52 Av. Paul Alduy, 66860 Perpignan Cedex, France

[4] INKOA Sistemas S.L., Ribera de Axpe 11, Edificio D-1, Local 208, 48950 Erandio, Spain

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Corresponding author: Mònica Campàs

IRTA

Carretera de Poble Nou, km 5.5

43540 Sant Carles de la Ràpita, Spain

Tel.: +34 902 789 449 (ext. 1842); Fax: +34 977 744 138

E-mail address: monica.campas@irta.cat

Abstract

This work presents the functional characterisation of a protein phosphatase 2A (PP2A) catalytic subunit obtained by genetic engineering and its conjugation to magnetic particles (MPs) via metal coordination chemistry for the subsequent development of assays for diarrheic lipophilic marine toxins. Colorimetric assays with free enzyme have allowed the determination of the best enzyme activity stabiliser, which is glycerol at 10%. They have also demonstrated that the recombinant enzyme can be as sensitive towards okadaic acid (OA) (LOD = 2.3 µg/L) and dinophysistoxin-1 (DTX-1) (LOD = 15.2 µg/L) as a commercial PP2A and, moreover, it has a higher operational stability, which makes possible to perform the protein phosphatase inhibition assay (PPIA) with a lower enzyme amount. Once conjugated to MPs, the PP2A catalytic subunit still retains its enzyme activity and it can also be inhibited by OA (LOD = 30.1 µg/L).

Keywords: Genetically-engineered protein phosphatase 2A (PP2A); protein phosphatase inhibition assay (PPIA); magnetic particles (MPs); Ni-His affinity; marine toxin okadaic acid (OA); dinophysistoxin-1 (DTX-1).

1. Introduction

Okadaic acid (OA) and dynophysistoxins (DTXs), OA derivatives, are marine toxins produced by dinoflagellates of the genera *Dinophysis* and *Prorocentrum* (Steidinger, 1993). These phycotoxins are accumulated in the digestive glands of shellfish with no evidence of toxic effects on them. However, the consumption of contaminated seafood by humans may result in diarrheic shellfish poisoning (DSP), responsible for gastrointestinal disturbances such as diarrhea, nausea, vomiting and abdominal pain (Yasumoto and Murata, 1993). The action mechanism of OA and DTX-1 is based on the inhibition of protein phosphatases (PPs), enzymes that play an important role in protein desphosphorylation in cells. These toxins bind to the receptor site of the enzyme, blocking its activity. As a consequence, hyperphosphorylation of proteins that control sodium secretion by intestinal cells and of cytoskeletal or junctional moieties that regulate solute permeability is favoured, causing sodium release and a subsequent passive loss of fluids, responsible for the diarrheic symptoms (Aune and Yndestad, 1993).

The Commission Regulation (EC) No. 853/2004 of the European Community has established a maximum permitted level of 160 µg of OA equivalents/kg in bivalve molluscs. Numerous methods are reported in the literature for the detection of OA alone or in combination with DTXs. Until this year, the official control method was the mouse bioassay (MBA) (Yasumoto et al., 1978). This method is based on the administration of shellfish samples to mice and the evaluation of the lethal doses. Despite the low specificity and the controversial ethical implications, the assay is useful because it gives an indication of the total toxicity of a sample. However, very recently, the Commission Regulation (EC) No. 15/2011 has established that liquid chromatography-tandem mass spectrometry (LC-MS/MS) should be applied as the reference method. This new regulation has applied from 1st July 2011, being possible to use MBA until 31st December 2014. Nevertheless, this Commission Regulation allows a series of methods, such as other chromatographic techniques with appropriate detection, immunoassays and functional (e.g. phosphatase inhibition) assays, as alternatives or supplementary to LC-MS/MS, provided that either alone or combined they can detect the required analogues, that they fulfil the corresponding method performance criteria, and that their implementation provides an equivalent level of public health protection. In the development of these alternative methods, the use of toxicity equivalent factors for different compounds of a marine toxin group will be very important to

protect consumers and guarantee that toxins levels are below values established in the legislation (Botana et al., 2010).

Cell-based assays (CBAs) have also been used as toxicological models for the evaluation of OA (Cañete and Diogène, 2008; Cañete et al., 2010). The use of that simple and widespread tool may be advantageous in comparison to more sensitive but complex methods. Chromatographic techniques, such as high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD) or to tandem mass spectrometry (LC-MS/MS), allow the separation of toxins and their sensitive quantification (de la Iglesia et al., 2008). However, they are laborious, time-consuming and require trained personnel. Immunoassays, based on the affinity interaction between monoclonal or polyclonal antibodies and OA and the use of enzymes as labels, have also been developed (Kreuzer et al., 1999; Campàs et al., 2008). Although enzyme-linked immunosorbent assays (ELISAs) may present the limitation of cross-reactivity, which makes impossible the discrimination of individual toxins, this feature may also be considered as an advantage since all the toxins within the same structural family could be detected. Like CBAs, immunoassays are a promising tool for routine detection and quantification, due to the simplicity, high sample throughput and relative low cost. Finally, another biochemical method for OA determination is the PP inhibition assay (PPIA) (Tubaro et al., 1996; Càmpas and Marty, 2007). The PPIA detects DSP lipophilic toxins and it is interesting as a simple, cost-effective and rapid screening tool.

Biosensors have also been developed for marine toxin detection. Most biosensors for OA are immunosensors, i.e. based on immunoassays. The Quartz Crystal Microbalance (QCM) has been applied to the construction of a label-free immunosensor (Tang et al., 2002), which has been able to detect 3.6 µg/L. Lower concentrations have been detected by Marquette et al. (1999) with a semi-automatic chemiluminescent immunosensor integrated into a flow-injection analysis (FIA) system. Their device had a limit of detection (LOD) of 2.5 µg/L, corresponding to 2 ng/g, with an overall measurement time of 20 min. Surface Plasmon Resonance (SPR)-based immunosensors have also been developed and applied to the analysis of shellfish extracts (Llamas et al., 2007; Stewart et al., 2009). The immunosensor was able to detect 2 µg/L of OA. Originally, the immunosensor was not able to detect DTX-1 and DTX-2 (Llamas et al., 2007). Nevertheless, the authors improved its performance using a new monoclonal antibody, able to detect not only DTX-1 and DTX-2 but also DTX-3 (Stewart et al., 2009). The use of electrochemical methods involves important advantages in

terms of sensitivity, cost effectiveness, ease of handling and possibility of miniaturisation. Therefore, electrochemical immunosensors for OA and other phycotoxins have also been developed attaining LODs of 1.5 µg/L for OA (Tang et al., 2003; Kreuzer et al., 2002). Campàs et al., (2008) improved the LOD by combining the immunosensor with a diaphorase-based recycling system. This signal amplification allowed the immunosensor to detect as low as 0.03 µg/L of OA, more than one order of magnitude less than the same system without amplification (1.07 µg/L). Moreover, the working range was enlarged by approximately two orders of magnitude.

Only one enzyme sensor for OA detection, based on the inhibition of immobilised PP, has been reported (Campàs and Marty, 2007). Although the authors demonstrated the feasibility of that electrochemical biosensor and applied it to the detection of OA in microalgae extracts, the LOD was not low enough for specific applications, such as the determination of production of trace amounts of OA by dinoflagellates or the detection of this toxin in shellfish matrices. The authors entrapped PP2A into a photopolymeric matrix and on a screen-printed graphite electrode. Although this immobilisation method maintains the biomolecules in a flexible conformation and substantially retains the stability of the enzyme activity, critical limitation of PPs, it also creates a barrier, which limits accessibility to the enzyme by both the substrate and the toxin. This suggests that the method for enzyme immobilisation is crucial to the performance of the biosensor. Other immobilisation methods are available, such as adsorption, covalent attachment, inclusion into composites, self-assembling and those based on affinity interactions. The affinity interaction between metals and amino acid residues has been exploited to develop acetylcholinesterase (AChE)-based biosensors for pesticides detection (Adreescu et al., 2001). The authors immobilised AChE to Ni-modified electrodes through a hexa-histidine (hexa-His) tag introduced to the enzyme by genetic engineering. The same research group combined this approach with the use of magnetic particles for biosensor development (Istamboulie et al., 2007). The authors attained LODs lower than those reported for a sensor based on entrapment of the enzyme into a poly (vinylalcohol) matrix. In fact, by carefully locating the hexa-His tail far away from active and inhibitory sites, it seems possible to immobilise the enzyme in such a way that access to those sites is not impaired. These oriented immobilisations together with the absence of immobilisation barriers are likely to produce assays with lower LODs.

In this work we propose the use of genetically-engineered PPs with hexa-His tails allowing conjugation to Ni-modified magnetic particles (MPs) and the development of colorimetric assays for

OA detection. First, genetically-engineered PPs have been characterised in terms of activity, stability and inhibition by diarrheic lipophilic toxins and compared with the commercially available enzyme (Upstate Biotechnology). Then, recombinant PPs have been conjugated to MPs. The conjugation has been characterised and the stability of the PP-MP conjugates assessed. Finally, inhibition of the conjugates by OA has been tested. With the use of this immobilisation technique and when the electrochemical biosensors will be set up, we expect to attain lower LODs and to improve the currently available enzyme sensors for OA and derivatives.

2. Materials and methods

2.1 Reagents and materials

Okadaic acid (OA) (14300 µg/L methanolic solution) was purchased from the National Research Council (Halifax, Canada). Dinophysistoxin-1 (DTX-1), from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan), was dissolved in ethanol (0.1 g/L) and subsequently diluted in a buffer solution at pH 8.4 containing 30 mM Tris–HCl, 20 mM $MgCl_2$ (buffer 1) prior to use. Commercial protein phosphatase 2A (PP2A), isolated as the heterodimer of 60 kDa and 36 kDa subunits from human red blood cells, was obtained from Upstate Biotechnology (NewYork, USA). The genetically-engineered PP2A catalytic subunit was produced by Gene to Protein (GTP) Technology (Toulouse, France) and contains a hexa-His tail at the C-terminus. The activity of the stock solutions was of 10,800 U/mL for Upstate Biotechnology and between 520 U/mL and 1,545 U/mL for GTP Technology, 1 U being defined as the amount of enzyme required to hydrolyse of 1 nmol p-nitrophenyl phosphate (p-NPP) in one min at 22 °C. Components of buffers and p-NPP were purchased from Sigma (St. Quentin Fallavier, France). All solutions were prepared using Milli-Q water. The Histidine Adem-kit was provided by Ademtech (Pessac, France).

2.1 Apparatus

HulaMixer™ Sample Mixer from Invitrogen (Leek, The Netherlands) was used in the PP-MP conjugation. A Titramax 1000 Vibrating Platform Shaker from Heidolph (Schwabach, Germany) was used for agitation of microtiter plates. Z5342 MagneSphere® Technology Magnetic Separation Stand (for twelve 1.5-mL tubes) and Z5410 PolyATtract® System 1000 Magnetic Separation Stand (for one 15- or 50-mL tube) from Promega Corporation (Madison USA) were used for magnetic separations in

158 the PP-MP conjugation. Magnetic disks (4 mm diameter x 2 mm height) from Ademtech (Pessac,
159 France) were used for magnetic separations in microtiter plates. Colorimetric measurements were
160 performed with a U-2001 UV/Vis spectrophotometer from Hitachi High-Tech (Krefeld, Germany) and
161 an automated microplate reader KC4 from Bio-Tek Instruments, Inc. (Bad Friedrichshall, Germany).
162
163 ***2.2 Enzyme activity***
164 The stock buffer solution for PP2A from Upstate Biotechnology contains 20 mM 3-(N, morpholino)
165 propanesulfonic acid (MOPS), 500 mM NaCl, 60 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 1 mM ethylene
166 glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM $MnCl_2$, 1mM DTT, 10% glycerol,
167 0.1 mg/mL BSA, pH 7.4. The initial lot 1 of PP2A from GTP Technology was produced in the initial
168 stock solution containing 18 mM Tris-HCl, 150 mM NaCl, 54 mM 2-mercaptoethanol, 0.9 mM $MgCl_2$,
169 1mM EGTA, pH 7.5, therefore in order to find the best enzyme activity stabiliser, 10% glycerol (lots 2
170 and 3), 1 mg/mL BSA (lot 4), 5% sucrose and 0.1% dextran sulphate (lot 5), and 5% sucrose and 2%
171 phosphoglycerate (PGA) (lot 6) were added to the initial stock solution and the activity of the enzyme
172 solutions was evaluated. The PP2A enzyme activity was measured spectrophotometrically in a 1-mL
173 cuvette by adding 10 µL of enzyme to 890 µL of buffer 2. Composition of buffer 2 was 30 mM Tris–
174 HCl, 2 mM EDTA, 20 mM $MgCl_2$, 2 mM 1,4-dithiothreitol (DTT), 0.2 mg/mL bovine serum albumin
175 (BSA), pH 8.4. After 5-min incubation, 100 µL of p-NPP solution at 100 mM in buffer 2 were added to
176 the cuvette and the absorbance at 405 nm was measured for 1 min. Assays were performed in
177 triplicate.
178 To evaluate the operational stability of PP2A enzymes, the activity was measured
179 spectrophotometrically in a microtiter plate. In this case, 50 µL of buffer 1 were added into microtiter
180 wells containing 100 µL of enzyme solution at different concentrations (between 30 and 100 U/mL)
181 depending on the assay. 50 µL of 25 mM p-NPP solution were added and absorbance at 405 nm was
182 measured every 5-10 min during 1-hour incubation at 22 or 37 °C in the dark. Enzyme and substrate
183 solutions were prepared in buffer 2. Assays were performed in triplicate.
184
185 ***2.3 Colorimetric PPIA with free enzyme***
186 The colorimetric PPIA was similar to that described by Tubaro et al. Brifely, 50 µL of OA standard
187 solutions at different concentrations ranging from 0.2 to 100 µg/L were added into microtiter wells

containing 100 µL of enzyme solution at different concentrations (between 3 and 1.25 U/mL) depending on the assay. 50 µL of 25 mM p-NPP solution were added and after 1-hour incubation at 22 °C in the dark, absorbance at 405 nm was measured. Enzyme and substrate solutions were prepared in buffer 2 and OA solutions in buffer 1. Assays were performed in triplicate.

2.4 Conjugation of recombinant PP2A to MPs

The conjugation protocol was: (1) 15 µL of a MP suspension was added to a 1.5-mL microtube containing 150 µL of binding buffer (provided in the Histidine Adem-Kit); (2) the tube was placed on the magnetic device until supernatant clearing and the supernatant was then removed by pipetting; (3) 150 µL of binding buffer were added to the microtube and mixed by vortexing; (4) step (2) was repeated; (5) 500 µL of recombinant PP2A solution prepared in a buffer containing 30 mM Tris–HCl, 20 mM $MgCl_2$, 0.2 mg/mL BSA, pH 8.4 (buffer 3) were added and incubated for 15 min at 22 °C while shaking (800 rpm): (6) step (2) was repeated and free enzyme excess supernatant (SN) was removed from the microtube; (7) 220 µL of binding buffer were added and mixed with vortexing; (8) steps (2) and (7) were repeated thrice and three washing solutions were removed from the microtube (W1, W2 and W3). The resulting product consists on the PP-MP conjugate suspension.

2.5 Characterisation of the PP-MP conjugation

To characterise the PP-MP conjugation, a colorimetric assay was performed in microtiter plates. 110 µL of PP-MP conjugate suspension and 50 µL of buffer 3 were added into microtiter wells. Then, 50 µL of 25 mM p-NPP solution were added and incubated for 30 min while shaking (800 rpm) at 22 °C in the dark. After substrate incubation, PP-MPs were separated with magnets and 200 µL of clear solution were taken for absorbance reading at 405 nm. Assays were performed in duplicate. To evaluate the effect of the number of washing steps, the absorbance values reached by the supernatant and washing solutions were also recorded.

Because the interaction of His with MPs is reversible and imidazole can compete with His residues for metal coordination positions, the PP-MP conjugation was also confirmed by removal of the bound PP2A by an excess of imidazole. In this case, after PP-MP conjugation the following steps were performed: (1) the tube containing the PP-MP conjugate suspension was placed on the magnetic device until supernatant clearing and the supernatant was then removed by pipetting; (2) 220 µL of

elution buffer 1 (with 100 mM imidazole) provided in the kit were added and mixed while shaking (800 rpm) at 22 °C for 1 hour; (3) step (1) was repeated; (4) 220 µL of elution buffer 2 (with 500 mM imidazole) provided in the kit were added and mixed while shaking (800 rpm) at 22 °C for 1 hour; (5) step (1) was then repeated; (6) and finally, 220 µL of binding buffer were added. The colorimetric assay protocol for the "theoretically" imidazole-MP conjugate was the same as the previously described for PP-MP.

2.6 Storage stability of PP-MP conjugates

The storage stability of the PP-MP conjugates was tested at 4 °C and -20 °C. PP-MP suspensions were kept at these temperatures and the activity of the conjugates was measured at storage times of 0 (reference activity value), 24, 48 and 72 hours using the protocol described above for the characterisation of the PP-MP conjugation.

2.7 Colorimetric PPIA with PP-MP conjugates

50 µL of OA standard solutions at different concentrations ranging from 1.6 to 100 µg/L or shellfish (mussel, wedge clam, flat oyster and Pacific oyster) extracts at 25 mg/mL spiked with OA at 3200 µg/kg were added into microtiter wells containing 110 µL of PP-MPs at 50 U/mL in the conjugation (or 55 µL of PP-MPs at 100 U/mL in the conjugation). Then, 50 µL of 25 mM p-NPP solution were added and incubated for 30 min while shaking (800 rpm) at 22 °C in the dark. After substrate incubation, PP-MPs were separated with magnets and 200 µL of clear solution were taken for absorbance reading at 405 nm. Assays were performed in duplicate.

3. Results and discussion

3.1 PP2A activity characterisation

The performance of recombinant PP2A catalytic subunit from GTP Technology was compared to that of the commercial PP2A from Upstate Biotechnology, used as an enzyme model. The inherent PP instability is one of the critical limitations of this enzyme and the derived assays. Several compounds were tested as possible enzyme activity stabilisers for stock solutions. Lyophilisation was also investigated as a possible procedure to maintain enzyme activity during storage. Table 1 reports the enzyme activity values of these stock solutions with different supposed stabilisers, determined

248 immediately after synthesis and one week later. The best enzyme activity stabiliser was 10% glycerol
249 (lots 2 and 3), as used by Upstate Technology in their stock solutions. The difference in activity
250 between lots 2 and 3 could be explained by the production process (different insect cell lines were
251 used) leading to protein batches with different enzyme purity and specific activity. The other
252 compounds were not appropriate stabilisers, probably due to enzyme inactivation during transport and
253 storage. Nevertheless, since lot 5 was also lyophilised, it is not possible to determine if its low enzyme
254 activity was due to the stabiliser or to the lyophilisation procedure. In the case of lot 6, it was not
255 possible to redissolve it and enzyme activity could not be quantified.
256 Compared with PP2A from Upstate, the GTP enzyme was less active. Nevertheless, when the
257 operational stability (ability to retain the enzyme activity during the PPIA) of both enzymes was
258 compared at 22 and 37 °C, results showed that PP2A from GTP was more stable during the hour
259 required for the assay (attaining higher absorbance values). In order to quantify this operational
260 stability, the enzyme activity at the last 5 min was compared to the enzyme activity at the first 5 min
261 and expressed in percentage. Whereas PP2A from Upstate retained 52 and 3% of the activity after 1
262 hour working at 22 and 37 °C, respectively, PP2A from GTP retained 100 and 24% of the activity at
263 the same temperatures. This higher operational stability of PP2A from GTP implies that a lower
264 enzyme amount can be used to reach an appropriate absorbance value and thus, the PPIA could be
265 more sensitive than that performed with PP2A from Upstate. For the rest of the study, only lots 2, 3
266 and 5 of PP2A from GTP and PP2A from Upstate were used.
267
268 ***3.2 Colorimetric PPIA with free enzyme***
269 The PPIA demonstrated the inhibitory effect of OA on the PP2A activity. The calibration curves were
270 described by the sigmoidal logistic four-parameter equation (SigmaPlot software package 9.0):

$$y = y_0 + \frac{a}{1+(x/x_0)^b}$$

271
272 Where a and y_0 are the asymptotic maximum and minimum values, respectively, x_0 is the x value at
273 the inflection point and b is the slope at the inflection point. In Table 2, the concentration of enzyme
274 used in the assay, the absorbance value reached by the controls without toxin at the end point, the
275 50% inhibition coefficient (IC_{50}) values and the working ranges (defined between IC_{20} and IC_{80}) are
276 presented together with the equations and the corresponding R values.

The first colorimetric PPIA was performed at 3 U/mL concentration for PP2A from Upstate and for PP2A from lots 2 and 5 of GTP. Fig. 1 shows the normalised calibration plots. The calibration curves showed that lot 2 from GTP was more sensitive than lot 5: $LOD_{GTP\ lot\ 2}$ = 14.8 µg/L in front of $LOD_{GTP\ lot\ 5}$ = 29.0 µg/L (LOD was defined as the 20% inhibition coefficient (IC_{20}) value). This difference may be due to the specific activity of each one of the lots. Although both PPIAs were performed at 3 U/mL, this concentration may represent a lower number of enzyme molecules when using the stock solution with a 6.7-fold higher activity (lot 2) and thus, a higher sensitivity towards the toxin. However, both lots were less sensitive than PP2A from Upstate, which provided a $LOD_{Upstate}$ of 1.8 µg/L. Despite the higher LODs attained with PP2A from GTP, it was observed again that GTP enzymes were more stable during the 1-hour experiment (whereas an absorbance value of 0.4 is reached at the end point in the control wells without toxin with PP2A from Upstate, 0.8-0.9 is reached with PP2A enzymes from GTP).

With the aim of optimising the sensibility of the assay and taking into account the higher operational stability of PP2A from GTP, the PPIA was performed with lot 2 at 1.25 U/mL. Nevertheless, no significant differences were observed and the assay was still less sensitive than that performed with PP2A from Upstate. It seems that the 2.4-fold lower enzyme concentration in the assay was not enough to appreciate a significant improvement of the LOD.

The inhibition of lot 3 from GTP by OA was tested together with the inhibition of DTX-1 in order to compare both toxins (Fig. 2). As expected due to the higher activity of the stock solution, this lot resulted to be more sensitive to OA than lots 2 and 5, and practically as sensitive as enzyme from Upstate. Moreover, it was more stable during 1 hour of experimentation. DTX-1 showed a lower inhibitory power than OA (5.1-fold lower IC_{50}).

3.3 Conjugation of PP2A to MPs

The conjugation of PP2A to MPs is based on the Ni^{2+} ability to bind strongly by chemical coordination to proteins containing His (or Cys) residues. On the one hand, the PP2A catalytic subunit produced by GTP Technology contains six consecutive His residues added to the C-terminus. On the other hand, MPs from Ademtech have iminodiacetic acid (IDA) groups covalently bound to their surface and they are precharged with Ni. Three of the six sites of the Ni coordination sphere are occupied by the

306 tridentate chelating group of IDA and the other three sites are occupied by three of the six His
307 residues of the genetically-engineered enzyme.
308 In order to determine the optimum enzyme concentration to use in the conjugation of PP2A from GTP
309 to MPs, several enzyme concentrations were tested. Fig. 3 shows the absorbance values attained by
310 the PP-MP conjugates as well as their corresponding supernatants from the free enzyme excess (SN)
311 and the three washing steps (W1, W2 and W3). The colour development observed in the colorimetric
312 assays with PP-MP demonstrates that conjugations were successful. The absorbance attained in the
313 assays increased proportionally to the enzyme concentration used in the conjugation protocol. No
314 saturation plateau was observed, although it was not our purpose to fully coat the MPs with enzyme
315 (as previously mentioned, too much enzyme could result in higher LODs). Looking at the absorbance
316 from the SNs, free enzyme was started to be detected at 37.5 U/mL. Nevertheless, the absorbance
317 attained with the PP-MP conjugate at this concentration was not enough to perform a PPIA. The PP-
318 MP conjugation at 75 U/mL produced conjugates with appropriate catalytic responses but it was not
319 economically efficient, since a high amount of enzyme was detected in the SN. A concentration of 50
320 U/mL was chosen in further conjugations.
321 The effect of the number of washing steps on the PP-MP conjugates was characterised, since each
322 washing could be compromising their enzyme activity. To this purpose, an experiment was performed
323 stopping the conjugation protocol at different washing steps. Fig. 4 shows the absorbance values
324 reached with the PP-MP conjugates, the first supernatant (SN) and the corresponding washings (W1,
325 W2 and W3). The PP-MP bars show that the absorbance of the conjugates decreases with the
326 number of washing steps. In order to know if the decrease is because of the removal of unbound PP
327 (desired) or to the inactivation of the bound PP (undesirable), it is necessary to consider the sum of all
328 the absorbance values for each set with washing steps with respect to the same value for the set
329 without washing steps (in percentage values). Whereas the absorbance of the PP-MP conjugates
330 decreases with the number of washing steps (96 ± 2, 80 ± 6 and 71 ± 3 %, for the conjugates washed
331 1, 2 and 3 times, respectively), in the global balances (∑PP-MP+SN+W), absorbance values do not
332 significantly vary in the first (102 ± 4 %) or the second (98 ± 6 %) washing step. In the third washing
333 step the sum starts to decrease (84 ± 6 %), may be due to the inactivation of the conjugated enzyme.
334 Nevertheless, in order to fully remove adsorbed enzyme and taking into account that the absorbance

335 reached by the PP-MP conjugate is still appropriate, further experiments were performed with 3
336 washing steps.
337 In order to verify the PP-PM conjugation, imidazole was added to the conjugate for the competition
338 with His residues for metal coordination positions. After imidazole addition, the MPs did not show any
339 significant absorbance value compared to the PP-MP conjugate, indicating that most of the enzyme
340 was effectively conjugated and not adsorbed.
341
342 ***3.4 Storage stability of the PP-MP conjugates***
343 In order to investigate if it is possible to store the PP-MP conjugates until use or if, on the contrary,
344 they should be freshly prepared, their storage stability was determined at 4 °C and -20 °C. In Fig. 5,
345 the activity percentages of the PP-MP conjugates after several times at different temperatures are
346 presented.
347 It is clearly observed that PP-MP was more stable when stored at -20 °C. Whereas after 72 hours at
348 that temperature the activity was retained by 72%, at 4 °C only 56% of the activity was maintained.
349 Nevertheless, it is interesting to mention that free PP2A can be only stored at -20 °C, its enzyme
350 activity drastically decreasing when storing it at 4 °C. Therefore, the immobilisation of PP2A on MPs is
351 increasing the stability of the enzyme activity, as others authors have also reported when using this
352 [22] or other immobilisation techniques [12].
353
354 ***3.5 Colorimetric PPIA with PP-MP conjugates***
355 The inhibitory effect of OA on the enzyme activity of PP-MP conjugates was investigated. Since no
356 significant differences were observed between 1-hour and 30-min incubation (data not shown), the
357 shortest time was chosen for the colorimetric assay. Calibration curves with free and conjugated
358 enzyme are shown in Fig. 6. Again the calibration curves were described by the sigmoidal logistic
359 four-parameter equation. Table 3 reports the absorbance value reached by the controls without toxin
360 at the end point, the IC_{50} values and the working ranges together with the sigmoidal logistic equations
361 and the *R* values. The PPIA demonstrated that the PP-MP conjugates were inhibited by OA, although
362 in a lower extent than free enzyme ($LOD_{PP\text{-}MP}$ was of 30.1 µg/L).
363 The PPIA with PP-MP conjugates was applied to the determination of OA in spiked shellfish extracts.
364 Recovery results were 83 ± 10%, 82 ± 5%, 83 ± 11% and 96 ± 7% for mussel, wedge clam, flat oyster

and Pacific oyster, respectively. Although the toxin contents used for the spiking were very high (3200 µg OA eq/kg shellfish), results demonstrate the applicability of the approach. It is expected that the electrochemical detection to be used in the development of the biosensor will improve the sensitivity of the analysis and thus, shellfish samples with toxin contents near the maximum permitted levels (160 µg OA eq/kg shellfish) will be reliably detected.

4. Conclusions

This work characterises a recombinant PP2A (GTP) catalytic subunit in terms of activity, stability and inhibition by diarrheic lipophilic toxins, and compares it with a commercial PP2A (Upstate). Due to the instability inherent to PP, the effect of several enzyme activity stabilisers has been evaluated, glycerol being the most appropriate one. Moreover, the operational stability of the recombinant enzyme is higher than that provided by the commercial one. One of the lots of genetically-engineered PP2A has resulted to be as sensitive towards OA as the commercial enzyme. Another advantage of the genetically-engineered PP2A is the His tail, which has allowed the conjugation of the enzyme to Ni-modified magnetic particles via coordination chemistry. Colorimetric assays have demonstrated that the PP-MP conjugation is successful and that PP2A retains the enzyme activity. Removal of conjugated PP2A by imidazole has also demonstrated that the enzyme was effectively conjugated and not simply adsorbed. A colorimetric inhibition assay with PP-MP conjugates has been developed, resulting in a detection limit of 30.1 µg/L of OA. Although the LOD is lower than those attained with free PP2A in solution, the electrochemical detection will probably provide with better sensitivities. Work is in progress to develop the corresponding electrochemical biosensor. It is expected that the high sensitivity inherent to the electrochemical detection together with the immobilisation through MPs will decrease the LODs compared to those achieved by colorimetric assays and previous electrochemical biosensors.

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Figure legends

Fig. 1. Colorimetric calibration curves for the inhibition of PP2As (from Upstate Biotechnology and from lots 2 and 5 of GTP Technology at 3 U/mL) in solution by OA. Inhibition is expressed as percentage of the control (no OA). *x* values refer to initial OA concentrations.

Fig. 2. Colorimetric calibration curves for the inhibition of PP2A (from lot 3 of GTP Technology at 1.25 U/mL) in solution by OA and DTX-1. Inhibition is expressed as percentage of the control (no toxin). *x* values refer to initial toxin concentrations.

Fig. 3. Absorbance values attained in the colorimetric assay from PP-MP conjugates obtained using several initial enzyme concentrations (with lot 3 of GTP Technology) and their respective supernatants from the free enzyme excess (SN) and the three washing steps (W1, W2 and W3). *indicates absorbance values beyond the spectrophotometer interval range.

Fig. 4. Absorbance values attained in the colorimetric assay from PP-MP conjugates (synthesised at 50 U/mL with lot 2 of GTP Technology), from the free enzyme excess (SN) and from the washing steps (W1, W2 and W3) for non-washed, 1-washed, 2-washed and 3-washed PP-MP conjugates.

Fig. 5. Activity percentage from PP-MP conjugates (synthesised at 50 U/mL with lot 2 of GTP Technology) after several storage times at 4 °C and -20 °C.

Fig. 6. Colorimetric calibration curves for the inhibition of PP2A (from lot 2 of GTP Technology at 1.25 U/mL) and PP-MP conjugates (synthesised at 50 U/mL with lot 2 of GTP Technology) in solution by OA. Inhibition is expressed as percentage of the control (no OA). *x* values refer to initial OA concentrations.

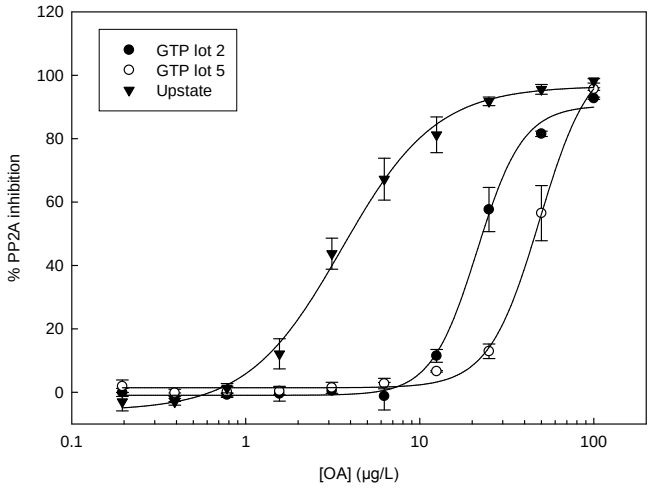
Table legends

Table 1. Enzyme activities (U/mL) of the stock solutions of lots of PP2A from GTP Technology with different stabilisers, as reported by GTP Technology after synthesis and determined experimentally at IRTA approximately one week later. Relative standard deviation values (%) are shown in parentheses. *Indicates lyophilised stock enzymes.

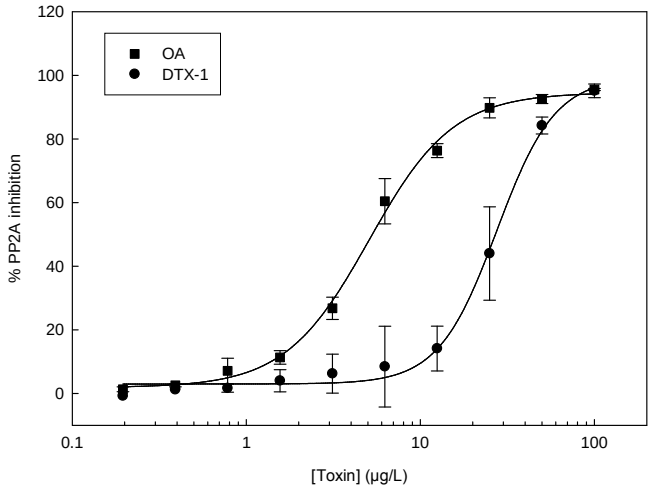
Table 2. Curve parameters derived from the sigmoidal logistic four-parameters fitting for the inhibition of PP2As by the marine toxins OA and DTX-1 in colorimetric assays.

480 **Table 3.** Curve parameters derived from the sigmoidal logistic four-parameters fitting for the inhibition
481 of PP2A and PP-MP conjugates (both with lot 2 of GTP Technology) in solution by OA in colorimetric
482 assays.
483

Figure 1


GTP lot 2
GTP lot 5
Upstate
% PP2A inhibition
[OA] (µg/L)
120
100
80
60
40
20
0
0.1
1
10
100

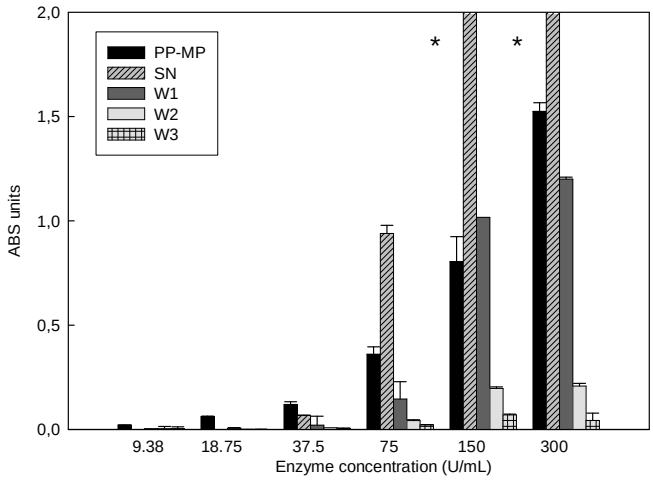
487 **Figure 2**



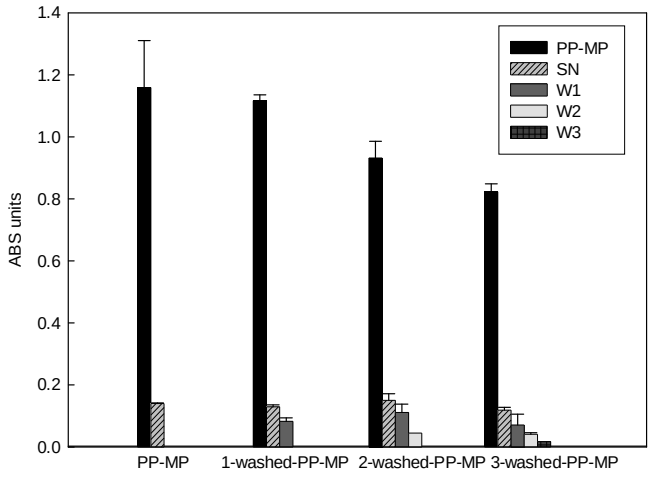
488

489

Figure 3


PP-MP
SN
W1
W2
W3
ABS units
2,0
1,5
1,0
0,5
0,0
*
*
9.38
18.75
37.5
75
150
300
Enzyme concentration (U/mL)

493 **Figure 4**



494

495

Figure 5

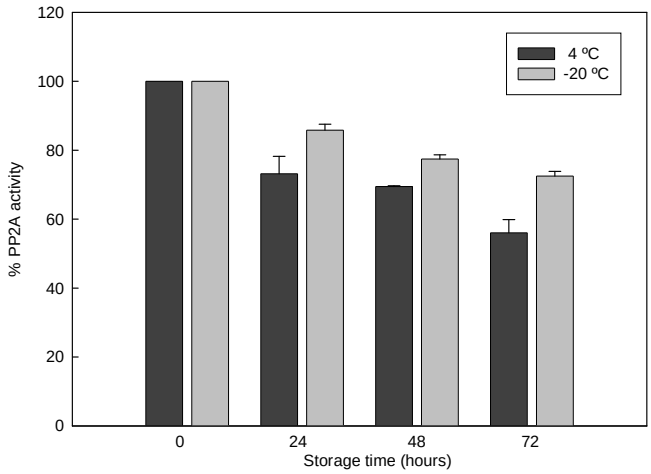
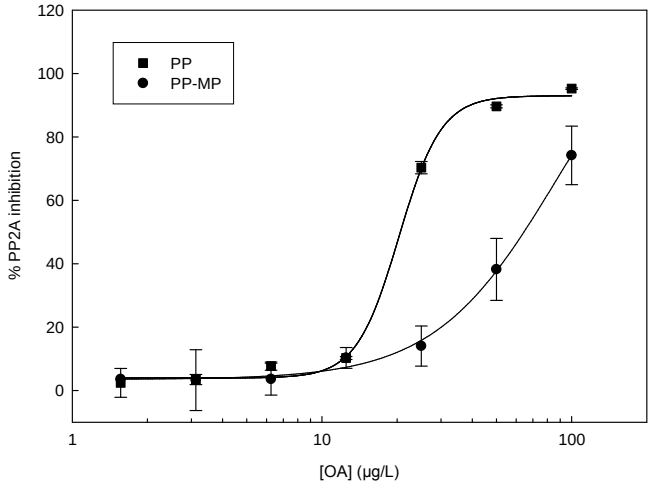

120
100
80
60
40
20
0
% PP2A activity
0
24
48
72
Storage time (hours)
4 ºC
-20 ºC

Figure 6



502 **Table 1.**

Lot number	Stabiliser	Enzyme activity (U/mL)	
		Determined at IRTA	Reported by GTP
Lot 1	-	5 (0.1)	120
Lot 2	10% glycerol	302 (13.7)	329
Lot 3	10% glycerol	1473 (6.0)	1400
Lot 4	0.1% BSA	8 (6.5)	153
Lot 5*	5% sucrose / 0.1% dextran sulphate	45 (7.7)	115
Lot 6*	5% sucrose / 2% PGA	-	32

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505 **Table 2.**

Toxin	Enzyme	$[PP2A]_0$ (U/mL)	ABS_f (units)	IC_{50} (µg/L)	Working range (µg/L)	Sigmoidal logistic equation	R
OA	Upstate	3	0.433	4.0	1.8-9.9	$y = -5.8 + \frac{102.4}{1+(x/3.5)^{-1.6}}$	0.9986
	GTP lot 5	3	0.799	46.2	29.0-69.9	$y = 1.4 + \frac{105.3}{1+(x/48.6)^{-2.9}}$	0.9990
	GTP lot 2	3	0.863	22.9	14.8-39.8	$y = -0.9 + \frac{91.4}{1+(x/21.3)^{-3.2}}$	0.9988
	GTP lot 2	1.25	0.441	20.4	14.9-29.0	$y = 2.4 + \frac{90.6}{1+(x/20.0)^{-4.8}}$	0.9989
	GTP lot 3	1.25	0.400	5.3	2.3-12.9	$y = 1.8 + \frac{92.7}{1+(x/5.0)^{-1.8}}$	0.9995
DTX-1	GTP lot 3	1.25	0.406	27.0	15.2-46.8	$y = 2.9 + \frac{96.3}{1+(x/27.5)^{-2.5}}$	0.9988

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507

508 **Table 3.**

Enzyme	ABS_f (units)	IC_{50} (µg/L)	Working range (µg/L)	Sigmoidal logistic equation	R
Free PP	0.441	20.4	14.9-29.0	$y = 2.4 + \frac{90.6}{1+(x/20.0)^{-4.8}}$	0.9989
PP-MP	0.407	64.4	30.1-111.2	$y = 3.4 + \frac{126.1}{1+(x/87.1)^{-1.7}}$	0.9991

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