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Enzymatic methods for the determination of pollution in seawater using salt resistant alkaline phosphatase from eggs of the sea urchin *Strongylocentrotus intermedius*

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ABSTRACT

A new salt resistant alkaline phosphatase from eggs of the sea urchin *Strongylocentrotus intermedius* (StAP) has been shown to have a unique property to hydrolyze substrate in seawater without loss of enzymatic activity. The enzyme has pH optimum at 8.0–8.5. Model experiments showed various concentrations of copper, zinc, cadmium and lead added to seawater or a standard buffer mixture to inhibit completely the enzyme activity at the concentrations of 15–150 μg/l. StAP sensitivity to the presence in seawater of metals, pesticides, detergents and oil products appears to be considerably less. Samples of seawater taken from aquatic areas of the Troitsy Bay of the Peter the Great Bay, Japan Sea have been shown to inhibit the enzyme activity; the same was shown for the samples of fresh waters. The phosphatase inhibition assay developed proved to be highly sensitive, technically easy-to use allowing to test a great number of samples.

1. Introduction

Increasing pollution of the environment and growing anthropogenic pressure on aquatic ecosystems have urged active searching and developing easy-to use and highly sensitive methods to test seawater quality. At present monitoring of marine aquatic environment under anthropogenic pollution is carried out into practice by a complex of integrated methods among which test-systems applying various enzymes are becoming of wider use. Enzyme analyzing methods seem to be most suitable to solve the problems of pollution express diagnostics for such a complicated system as seawater. The rate of enzymatic reactions occurring in aquatic environment is known to be affected by the presence of natural and anthropogenic polluting inhibitor substances. That is why adding the enzyme and its substrate to a water sample studied with following registration of the enzyme activity inhibition allows to estimate the probability of such a reaction occuring in the environment under study. The advantages of the enzymatic methods are as follows: high sensitivity, selectivity, the equipment and experimental techniques are easy-to use, economically efficient and allow to get integrated information on environment polluting.

Up to date test systems using certain enzymes (cholinesterase, luciferase, DNase, phosphatases, trypsin, amilase) are actively applied to obtain integrated information on toxicity level of various substances (Ugarova and Lebedeva, 1987; Nikolskaya et al., 1994; Korneeva, 1996; Menzorova and Rasskazov, 1999; Zhavoronkova et al., 2003). Acetylcholinesterases and cholinesterases are widely used to determine residual quantities of organophosphorous and carbamate pesticides in water, air, soil, plants and food products (Nikolskaya et al., 1994; Guilhermino et al., 2000; Choi et al, 2011). Tests basing on immobilized cholinesterase preparations are used to estimate the environment heavy metal pollution (Tingfa et al., 1989; Evtyugin et al., 2002). Particularly high sensibility is characteristic of chemiluminiscent methods involving luciferases (Ugarova and Lebedeva, 1987). Methods have been described to test various substances using both monoenzyme and bienzyme systems of luminous bacteria (Kratasyuk et al., 2001; Esimbekova et al., 2007). Hydrolytic enzymes including alkaline phosphatases from different sources were applied to identify transition and heavy metal ions in fresh waters and soils (Zhavoronkova et al., 2003; Shekhovtsova et al., 2007).

The reasons making the enzyme methods difficult to be practically applied to environment monitoring result from specific features of the natural objects under control compared to the samples under laboratory studies. Practical application of most enzyme tests for analysing sea and waste waters as well as sludge deposit extracts appears to be considerably limited due to mineralization of the samples studied. So the test device basing on cholinesterase could be used only if water mineralization in a sample were no more than 0.10 mol/l (Ristori et al., 1996). Enzyme

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test-systems using trypsin and amylase were applied to estimate pollution of the Black Sea waters (Korneeva, 1996). However while testing seawater (0.50 mol/l of NaCl) by trypsin inhibiting method high salt concentration fell back the enzyme activity twice as much even with no toxicants present (Korneeva et al., 1990, 2002).

Up to date a test has been developed to assess seawater pollution basing on the unique property of a salt resistant DNase from the eggs of sea urchin *S. intermedius* to retain activity in water media with high salt concentration, seawater included (Menzorova and Rasskazov, 1999). The test system proved to be of great help while monitoring water samples from the Japan and Okhotsk Seas both under natural conditions and anthropogenic pressure (Menzorova and Rasskazov, 2007). However, with all advantages of the above test system the technique used presents certain difficulties under field conditions. Further studies of alkaline phosphatase from sea urchin *S. intermedius* eggs (StAP) made it possible to classify it as a hydrolase (EC 3.1.3.1) catalyzing the hydrolysis of various phosphomonoesters (Coleman, 1992; Millan, 2006). It proved to be a salt resistant enzyme retaining its activity in seawater (Menzorova et al., 2010, Patent). The above property was used to develop a more simple enzyme test for estimation of natural seawater pollution.

The purpose of the present study is to develop a new simple enzymatic method to evaluate marine and fresh water pollution basing on the assay of the level of inhibition of the activity of the alkaline phosphatase from sea urchin *S. intermedius* eggs (StAP). The study was also aimed at working out a phosphatase test to broaden a range of enzyme methods for assessment of natural waters pollution.

2. Materials and methods

2.1. Reagents and test solutions

Phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), dithiothreitol (DTT), coomassie R-250, *p*-nitrophenylphosphate (*p*-NPP), Tris, N-ethylmaleimide, *p*-chloromercuribenzoate were obtained by Sigma Chemical Co. (USA). Sodium lauryl sulfate (SDS) (Serva, Germany). The other reagents used were analytical grade and ultra pure grade substances obtained from Helicon and Reakhim (Russia).

Stock standard solutions in a concentration of 10 mM were prepared by adding each metal salt ($CdCl_2{\cdot}2\ ½\ H_2O$, $ZnCl_2$, $Pb(CH_3COO)_2{\cdot}3H_2O$, $Cu(CH_3COO)_2{\cdot}H_2O$, $NiCl_2{\cdot}6H_2O$, $CoCl_2$, $FeSO_4$, $MnCl_2$) to deionized water. For the following experiment stock solutions were dissolved with pre-filtered seawater and buffer 10 and 100 times.

Stock solutions of commercial preparation of pesticides hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT), tetramethylthiuramdisulfide (TMTD), γ-hexachlorocyclohexane (lindane), o,o-dimethyl-o-4-nitrophenyltiophosphate (metaphos) were obtained by dissolving 5 mg of a substance in 5 ml of acetone. This solution (0.1 ml) was then transferred into an Erlenmeyer flask and the acetone was evaporated to dryness. The resulting thin film was dissolved in 10 ml of seawater (buffer) by vigorous shaking for a few hours at 37 °C. Aqueous solutions contained 10 μg/ml of pesticides. For the following experiment stock solutions were dissolved with seawater and buffer 10 times.

Water-soluble fraction hydrocarbons (WFH) of fuel oil and of L grade diesel fuel were prepared by mixing one part of an oil product with nine parts of seawater (Anderson et al. 1974). The mixture was allowed to stay for one day, and then the lower layer was removed. The highest concentration of hydrocarbons was observed one day after the addition of oil products to seawater (Vaschenko and Naidenko, 1989). Hydrocarbons concentration in the original WFH was measured by method infrared spectroscopy within the wave number length range of 2700–3100 cm^{-1} on the spectrophotometer Equinox 55 "Bruker" (USA). The WFH concentration in fuel oil agreed with 5.1 ± 0.3 mg/l, a diesel fuel – 5.8 ± 0.3 mg/l. Detergents (SDS), synthetic washing agents (SWA) "Tide" and "Fairy" in the concentration of 5 mg/ml were used in the experiments.

Artificial seawater contains 530 mM NaCl, 27 mM $MgCl_2$, 29 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KCl, 2 mM $NaHCO_3$ (pH 8.1) (Girard et al., 1982). All seawater samples were filtered on a Whatman GF/C filter (pore size 0.45 μm) and stored at 4 °C before analysis. Buffer solutions, stock solutions of salts, and pesticides were prepared using deionized or bidistilled water.

2.2. Isolation of cell extract StAP

The enzyme was isolated from sea urchin *S. intermedius* eggs, whose samples were prepared at the Marine Experimental Station (MES), Pacific Institute of Bioorganic Chemistry, Far East Branch of Russian Academy of Sciences, frozen to −20°C, delivered to the laboratory and stored there until the experiment (Vacquier, 2011).

Sea urchin eggs (1.0 g) were homogenized in a Downs glass homogenizer on ice bath in a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM FMSF, 1 mM EDTA, 1.5 mM DTT with 1:8 preparation to buffer proportion. The homogenate was kept at the temperature of 4 °C for 60 min and centrifuged at 14000*g* for 20 min. The enzyme preparation with 0.03–0.05 m/min/mg of protein specific activity was kept at −20 °C for a month without loss of activity.

2.3. Alkaline phosphatase activity

2.3.1. Alkaline phosphatase activity in a standard buffer mixture

The standard buffer mixture for assay of AP activity (0.5 ml) contained 25 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 150 mM NaCl, 3 mM *p*-NPP and 5-15 ml of the enzyme preparation (1.5–2.0 mg/ml protein).The samples were incubated at 25 or 37 °C for 60 min, and the reaction was terminated by adding 0.20 ml of 0.5 M NaOH. Digestion products were measured spectrometrically at 400 nm (Cecil CE 2021, England) as the difference between the assay and control samples. As optimal concentrations of AP those were chosen at which maximal rate of the enzyme reaction appeared most suitable to be registrated. One unit of phosphatase activity (U) was defined as the quantity of the enzyme required to release 1.0 μmol of *p*-nitrophenol from *p*-NPP in 1 min under standard conditions (ε = 18,300 M^{-1} cm^{-1}). The specific activity is given as a unit of activity per 1 mg of protein (Menzorova et al., 2008).

2.3.2. Alkaline phosphatase activity in seawater

The standard buffer mixture (0.5 ml) contained 0.48 ml of seawater (pH 7.9–8.1), 3 mM *p*-NPP and 5–15 μl of the enzyme preparation. The mixture was incubated at 25 or 37 °C for 60 min, and the reaction was terminated by adding 0.20 ml SDS (3.5 mg/ml) in 0.1 M Tris, pH 9.5. Digestion products were measured spectrometrically.

2.3.3. Alkaline phosphatase activity in 96 multiwell plates

AP activity was measured in "Dynatech microelisa" 96 multiwell plates (350 μl) using a microplate reader was taken according to the methods detailed in (Galgani et al., 1992). Each well contained 280 μl of seawater or standard buffer mixture, 2 mM *p*-NPP, 5 μl of enzyme preparation and then incubated at 25 °C for 30–60 min. Absorbance was recorded immediately at 400 nm in a microplate reader μQuant ("Fluoroscan Accent", Finland). Four replicates measurement were carried out per sample.

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