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Inhibition of Alkaline Phosphatase from Pearl Oyster *Pinctada fucata* by *o*-Phthalaldehyde: Involvement of Lysine and Histidine Residues at the Active Site*

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Abstract: Alkaline phosphatase from *Pinctada fucata* was inactivated by *o*-phthalaldehyde (OPA). The inactivation followed pseudo first-order kinetics with a second rate constant of 0.167 $(mmol/L)^{-1}\cdot min^{-1}$ at pH 7.5 and 25°C. A Tsou's plot analysis showed that inactivation occurred upon formation of one isoindole group. The OPA-modified enzyme lost the ability to bind with the specific affinity column and the presence of substrates or competitive inhibitors protected the enzyme from inactivation. The results revealed that the OPA-reaction site was at the enzyme substrate binding site. Prior modification of the enzyme by lysine or histidine specific reagent abolished formation of the isoindole derivatives, suggesting that lysine and histidine residues were involved in the OPA-induced inactivation. Taken together, OPA inactivated the alkaline phosphatase from *Pinctada fucata* by cross-linking lysine and histidine residues at the active site and formed an isoindole group at the substrate binding site of the enzyme.

Key words: alkaline phosphatase; *Pinctada fucata*; chemical modification; kinetics

Introduction

Alkaline phosphatase (ALP, EC 3.1.3.1) is a substrate nonspecific phosphomonoesterase that catalyzes hydrolysis of a wide variety of phosphomonoesters under alkaline conditions. In the presence of a phosphate acceptor such as Tris or ethanolamine, ALP also acts as a transphosphorylase[1]. A detailed catalytic mechanism of the enzyme has been proposed, based on kinetic and structural studies of the native and a number of site-directed mutant *Escherichia coli* (*E. coli*) ALP. The reaction follows ping-pong replacement kinetics and proceeds through a phosphoseryl intermediate to yield inorganic phosphate or to transfer the phosphoryl group to another alcohol[2,3]. Although its detailed physiological functions have not been fully elucidated, the wide distribution of ALP in almost all living beings indicates its essential role in cell metabolism. In marine organisms, ALP is believed to be involved in the absorption of phosphate and calcium from seawater and the biomineralization process. The ALP activity has been used as a stress marker to evaluate the effects of environmental exotics, such as heavy metals and organic chlorides, on marine organisms[4,5].

The crystal structures of ALP from *E. coli*, *Pandalus borealis* and human placenta have been solved[1,6,7]. They are all homodimers with very similar overall topologies and their active site architectures all had a

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metal triad (usually two zinc ions and one magnesium ion). Comparative studies of the primary sequence of ALPs from *E. coli*, *Saccharomyces cerevisiae*, *P. borealis*, chicken, and humans showed 25%-45% sequence identity among ALPs from different species[8]. Most of the important functional residues, including the serine nucleophile (Ser-112 in *E. coli* ALP) and an arginine residue (Arg-166 in *E. coli* ALP) that is involved in substrate binding, are highly conserved, which implies that similar catalytic mechanisms are shared among ALPs from different species.

Although ALPs from bacteria and mammals have been extensively studied, there are few reports about ALPs from mollusca, the second largest phylum of the animal kingdom. *Pinctada fucata* (*P. fucata*), which belongs to the family Pteriidae of bivalve mollusca, is a key source of marine pearl production. ALP is believed to be involved in phosphate and calcium absorption in oysters and in the biomineralization process. A better understanding of oyster ALP would help identify the mechanism of shell and pearl formation. A previous paper illustrated the enzymatic properties of the tissue-nonspecific ALP from *P. fucata*[9]. This paper reports on the OPA modification of this ALP.

1 Materials and Methods

1.1 Animal and chemicals

Adult *P. fucata* were harvested from the Beihai Oyster Culture Centre, Guangxi Zhuang Autonomous Region, China. *p*-Nitrophenyl phosphate (pNPP) and *o*-phthalaldehyde (OPA) were from Amresco. 2,4,6-Ttrinitrobenzene-sulfonic acid (TNBS), diethylpyrocarbonate (DEPC), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and *L*-histidyldiazobenzylphosphonic acid agarose were Sigma products. All other chemicals were local products of analytical grade.

1.2 Enzyme preparation and activity assays

The ALP from *P. fucata* was prepared as previously described[9] to the step of Sephadex G-150 gel filtration. The active fractions were collected and evaluated using an affinity chromatography as described below. The active peaks were then combined and dialyzed for 72 h to remove the inorganic phosphate. All the purification procedures were carried out at 4℃. The final preparations, with specific activity of 1215 unit • mg^{-1}, showed homogeneity on polyacrylamide gel electrophoresis in both the absence and presence of SDS.

The standard enzyme activity assay was carried out as previously described[9]. The released *p*-nitrophenol was determined spectrometrically by measuring the absorbance increase at 405 nm using a molar extinction coefficient of 17.3 $(mmol/L)^{-1}$ • cm^{-1}. One unit of activity represents the amount of enzyme required to produce 1 μmol product per minute under the assay conditions.

1.3 Inactivation of ALP from *P. fucata* by OPA

All the reactions were carried out at (25±1)℃. The Tris that would interfere with the reaction was removed by first passing the enzyme through a Sephadex G-50 column previously equilibrated with 20 mmol/L Hepes buffer (pH 7.5). The OPA solutions were freshly made in 1% methanol. The modification procedure was carried out by incubating ALP with different concentrations of OPA in 50 mmol/L bicarbonate buffer, pH 9.0. The final concentration of the enzyme was 2.2 μmol/L. A control tube was maintained with the same amount of enzyme, but without any OPA. At various time intervals, aliquots were withdrawn and the reaction was terminated by the addition of an equal volume of stopping solution (5 mmol/L cysteine and 5 mmol/L 2-mercaptoethanol). The reaction mixture was ultra-centrifuged with a Centricon (millipore) apparatus to remove the excess OPA and then diluted 10 folds by 20 mmol/L Tris-HCl buffer. The residual enzyme activity was then assayed.

1.4 Stoichiometry of OPA inactivation

Various partially modified enzymes were obtained by mixing 2.3 μmol/L ALP with OPA at specific molar proportions ranging from 1:20 to 1:1000. After a period of time, the number of formed isoindole derivatives was determined from the absorbance increase at 337 nm using a molar extinction coefficient of 7.66 $(mmol/L)^{-1}$ • cm^{-1} (Ref. [10]). The mixture was then ultra-centrifuged, diluted, and assayed to determine the residual activity.

1.5 Protection experiments

The enzyme was preincubated with varying concen-