MARINE BIOTECHNOLOGY

A Highly Active Alkaline Phosphatase from the Marine Bacterium *Cobetia*

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Received: 5 June 2003 / Accepted: 30 April 2004 / Online publication: 26 May 2005

Abstract

An alkaline phosphatase with unusually high specific activity has been found to be produced by the marine bacterium Cobetia marina (strain KMM MC-296) isolated from coelomic liquid of the mussel Crenomytilus grayanus. The properties of enzyme, such as a very high specific activity (15000 DE U/1 mg of protein), no activation with divalent cations, resistance to high concentrations of inorganic phosphorus, as well as substrate specificity toward 5′ nucleotides suggest that the enzyme falls in an intermediate position between unspecific alkaline phosphatases (EC 3.1.3.1) and 5′ nucleotidases (EC 3.1.3.5).

Key words: alkaline phosphatase — purification — properties — specificity — marine bacterium — *Cobetia marina*

Introduction

Alkaline phosphatases (APs) (orthophosphoric–monoesterphosphohydrolases, EC 3.1.3.1) are widely found in various organisms, indicating their important role in metabolism of different phosphorus-containing organic compounds (McComb et al., 1979; Bjorkman and Karl, 1994). The decay of nucleic acids and some other organic compounds by the microorganisms associated with marine invertebrates seems to be one of the possible pathways to by which mollusks produce inorganic phosphate. To gain insight into the types of APs that symbiotic microorganisms produce, we screened microorganisms associated with mollusks for phosphatase activity (Ivanova et al., 1992). From (about 200) strains able to produce AP, the strain *Cobetia marina*, isolated from the coelomic liquid of mussel *Crenomytilus grayanus*, has been selected for further work because of the unusually high specific activity of its AP.

Reasons for interest in this AP are its possible application as a tool for structural and functional studies of nucleic acids, as well as for preparation of Ig-enzyme conjugates for immunologic assays. Although many APs have been isolated and characterized, only commercial *Escherichia coli* AP and calf intestinal AP are routinely used in molecular biology. These commercially available enzymes have some limitations such as rather low specific activity in the case of *E. coli* AP of 60 U/1 mg of protein (Olsen et al., 1991). Unlike *E. coli* AP, the specific activity of AP isolated from calf intestine may approach values of about 6000 U/1 mg of protein (Hawrylak and Stinson, 1988). However, its tight binding with unspecific phosphodiesterase has restricted its application for structural investigations of nucleic acids.

Marine bacteria have been shown to be a valuable source for the isolation of novel APs with potential practical application. Heat-labile AP from Antarctic bacteria was shown to be more suitable for rapid 5′-end labeling of nucleic acids in comparison with the commercial APs (Kobori et al., 1984; Rina et al., 2000). Some advantages for practical applications have been revealed for APs from the marine bacterium *Alteromonas macleodii* (Fedosov et al., 1991) and from the Euryarchaeon *Pyrococcus abyssi* (Zappa et al., 2001).

In this report we describe the isolation of a novel AP from the marine bacterium *C. marina.* Some properties and the substrate specificity of this AP make it advantageous as a tool for practical application in comparison with the commercially available APs.

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Materials and Methods

Organism and Cultivation Conditions. The strain *C. marina* (KMM 296, Collection of Marine Microorganisms PIBOC FEB RAS) used as a source for AP purification was isolated from the mussel *C. grayanus.* Samples of the mussel were collected from a depth of 7 m in Troitza Bay, Japanese Sea. Strain identification based on phenotypic and biochemical properties was published earlier (Ivanova et al., 1994). Later the strain was reidentified as *C. marina* (formerly *Halomonas marina)* (Arahal et al., 2002). The strain was cultivated by the shake-flask method in a 1000-ml Erlenmeyer flask on a rotary shaker (160 rpm) for 36 hours. This microorganism was grown in a culture medium containing 5 g peptone, 2.5 g yeast extract, 1 g glucose, 0.05 g Mg_2SO_4, 0.2 g K_2HPO_4 per liter and 750 ml seawater, 250 ml distilled water.

Alkaline Phosphatase Assay and Protein Determination. The standard assay for AP activity was carried out at 37°C for 30 minutes using 2 mM *p*-nitrophenylphosphate (pNPP) (Sigma Chemical Co.) as a substrate in 0.1 M Tris-HCl buffer, pH 9.5, containing 0.2 M NaCl. The activity in diethanolamine (DEA) units was measured in 1 M DEA buffer, pH 10.3, containing 15 mM pNPP as a substrate. The volume of the reaction mixture was 0.5 ml. The reaction was terminated by adding 2 ml of cold 0.5 M NaOH, and the absorbance of *p*-nitrophenol formed was measured at 400 nm as the difference between the assay and control samples. One unit of AP activity was defined as the quantity of the enzyme required to release 1.0 μmol of *p*-nitrophenyl from pNPP in 1 minute. The specific activity is given as units per 1 mg of protein. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Enzyme Purification. After harvesting the microbial cells were resuspended in a buffer containing 0.01 M Tris-HCl buffer, pH 8.6, and 0.01% NaN_3 (buffer A) and disintegrated by ultrasonic treatment. The intracellular AP was eluted with buffer A containing 0.4 M NaCl from a DEAE-cellulose column (Sigma Chemical Co.). The fractions with enzyme activity were collected and after addition of ammonium sulfate up to a concentration of 1.5 M were loaded onto a Butyl Toyopearl-650 M column (Tosoh Corp.). The enzyme was eluted with a gradient concentration of sulfate ammonium (1.5 M–0 M) with buffer A. The fractions with enzymatic activity were desalted with G-75 column (Pharmacia).

Molecular Weight Determination. Molecular weight of the AP was determined by gel filtration on a Sephadex G-150 calibrated column and by sodium dodecylsulfate polyacrylamide gel (7.5%) electrophoresis (SDS-PAGE) under denaturing conditions according to Laemmli (1970).

Isoelectric Point Determination. The isoelectric point of the protein was estimated by chromatofocusing with PBE 74 polybuffer (Pharmacia). After electrophoresis the gel strip was cut into 2-mm slices and minced. The protein concentration and activity of eluted enzyme were determined by standard procedures.

Substrate Specificity. Substrate specificity of AP was tested by measuring phosphate release in a reaction mixture containing 2 mM of each substrate in 0.1 M Tris-HCl buffer, pH 9.5, with 0.2 M NaCl at 37°C after 30 minutes of hydrolysis. The inorganic phosphorus concentration was determined by the method of P. S. Chen et al. (1956).

Labeling of 5′ Ends of* Eco*RI-Fragments of Phage λ DNA. The 5′ ends of *Eco*RI fragments of phage λ DNA (0.5 mg) were labeled by a previously described standard method using (dephosphorylation, phenol treatment, and T_4 polynucleotide kinase treatment (Fedosov et al., 1991), with some modification: that is, AP KMM 296 was used instead of AP KMM 162 (0.04 U).

Preparation of Enzymatic Conjugates. The enzymatic conjugates were prepared by a standard method (Gate et al., 1991).

Results and Discussion

Characteristics of the Enzyme. The strain *C. marina*, isolated from the coelomic liquid of a mussel, produced intracellular AP with high specific activity, about 20 to 40 U/1 mg of protein, even at the initial stage of fractionation (in bacterial lysate). At the final purification stage, the enzyme preparation was practically homogenous, and its specific activity reached about 14000 to 15000 DEA U/1 mg of protein (Table 1). The specific activity of our AP seems to be one of the highest among all available commercial APs. For instance, the specific activity of AP isolated from the hepatopancreas of shrimp *Pandalus borealis* (SAP) approaches values of about 6000 to 7000 DEA U/1 mg of protein (http://www.biotec.as), whereas the specific activity of the commercial preparations of AP isolated from bovine calf intestinal mucosa (CAP) is in the range of 3000 DEA U/mg

Table 1. Purification of Highly Active Alkaline Phosphatase from Marine Bacterium *Cobetia marina*

Step of purification	*Protein concentration (mg)*	*Specific activity (U/mg)*	*Total activity (U)*	*Purification (fold)*	*Yield (%)*
Bacterial lysate	1900	45	85,000	1	100
Chromatography on DEAE-cellulose column	24	270	65,150	6	79
Chromatography on butyl-Toyopearl column	13	4860	62,900	108	74
Gel filtration on Sephadex G-100 column	4	14580	57,800	324	68

(http://www.worthington-biochem.com/) to 7500 DEA U/mg (http://www.biozyme.com). The preparation of AP from *C. marina* did not contain any admixture of phosphodiesterase or other nucleases and did not lose the initial specific activity in the storage buffer of 0.1 M Tris-HCl, 0.02 M NaCl, 0.02% NaN_3, 30% glycerol, pH 8.5, for 6 to 12 months.

The molecular weight of the AP was determined by gel filtration to be 62 kDa. Analysis of the heat-denatured AP by SDS-PAGE revealed a main protein band migrating with an apparent molecular weight of 55 kDa. These results suggest that the enzyme consists of one subunit of about 55 to 62 kDa. APs are classically described as homodimeric, which is the case with *E. coli* AP (94 kDa) and mammalian APs (range, 130–170 kDa). However, many monomeric forms have been described, including APs from marine bacteria. So AP from Antarctic bacteria with a molecular weight of 68 kDa and extracellular AP from a marine *Vibrio* sp. of 55 ± 6 kDa have been purified (Kobori et al., 1984; Hauksson et al., 2000).

The isoelectric point of the AP was at pH 4.5, similar to that of most mammalian and microbial alkaline phosphatases (McComb et al., 1979).

Optimum pH, Thermostability, and Metal Requirements. The optimum pH of this AP was 10.3 in 1.0 M DEA buffer with pNPP substrate (Figure 1). The enzyme has high stability to pH changes and retains its activity after preincubation for 15 minutes at pH values from 6.0 to 11.0 (Figure 2). The AP was stable when incubated at temperatures from 20° to 55°C, whereas this enzyme lost more than 90% of initial activity at 60°C (Figure 3).

As shown in Table 2, the enzyme does not require the presence of divalent cations for activity. The presence of 5 mM EDTA in the incubation mixture does not appreciably inhibit AP. However, the addition of cations Cd^{2+}, Cu^{2+}, Co^{2+}, or Zn^{2+} up to a 5 mM concentration inhibited activity by 30%, 40%, 60%, and 100%, respectively (Table 2), whereas decreasing concentrations of these ions reduced their inhibitory effect. It is noted worthy that the AP from the marine bacterium *Pseudomona* sp. did not change its activity in the presence of the majority of cations, but was inhibited by Zn^{2+} and Hg^{2+} ions (Kobori and Taga, 1980). As shown in Figure 4, monovalent cations Na^+ and K^+ at 0.1 M concentrations stimulated activity of AP from *C. marina.* These results suggest that increasing ionic strength weakens electrostatic interactions owing to more rapid dissociation of the phosphate noncovalently bound with the active center (Poe et al., 1993).

Treatment of AP from *C. marina* with sulfhydrilic reagents such as dithiothreitol or 2-mercaptoethanol at 10 mM concentration decreased the initial activity by 95% and 70%, respectively. The sensitivity of the enzyme to sulfhydrilic reagents suggests that the presence of SH-groups is essential for enzyme activity. The ability of the enzyme to be inactivated completely by heated at 55° to 60°C in the presence 2-mercaptoethanol or dithiothreitol for 15 minutes appears to be one of this enzyme's advantageous features. The complete inactivation of AP after removing the end phosphates of DNA fragments has been shown to be one of the main conditions for effective labeling of 5′ ends of DMA by polynucleotide kinase. The inhibitors of thiol-

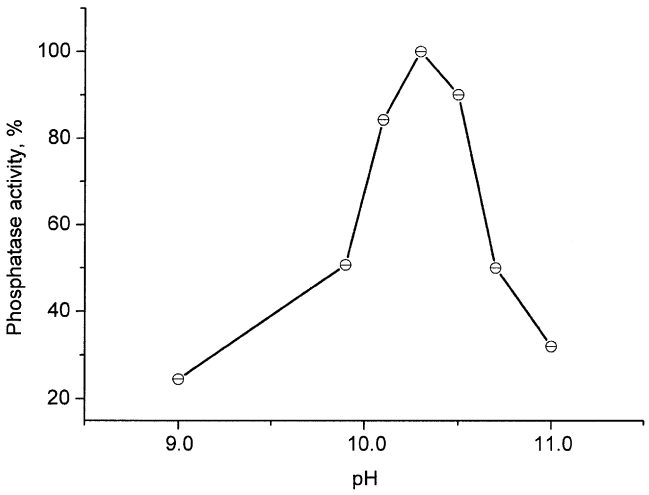

Fig. 1. Optimum pH curve for AP from marine bacterium *Cobetia marina* with *p*-NPP as substrate.

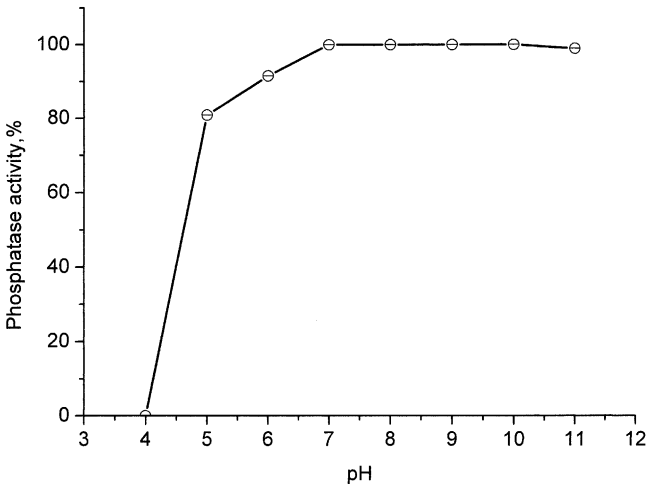

Fig. 2. The pH stability curve for AP from marine bacterium *Cobetia marina*. Three buffer systems: 0.1 M citrate buffer (pH 4.0–6.0); 0.1 M Tris-HCl buffer (pH 7.0–9.5); 0.1 M carbonate buffer (pH 10.0–11.0).

Table 2. Effect of Metal Ions on Alkaline Phosphatase from Marine Bacterium *Cobetia marina*[a]

Metal cations at 5 mM	*Remaining activity of AP (%)*
Li	120
Mg	115
Mn	110
Ca	102
Ba	100
Sr	100
Fe	88
Ni	73
Cu	60
Co	40
Cd	20
Zn	0
EDTA	90–100

[a]Various metal ions were added to the reaction mixture at 5 mM concentration. The phosphatase activity was measured using the standard method described in "Materials and Methods." Activity is expressed as specific activity relative to a control incubated in the absence of any metal ions.

groups, namely, *p*-chlormercuribenzoate (*p*HMB) at 0.1 to 0.2 mM concentration inhibited AP activity by 85% to 100%, whereas iodacetamide and NaN_3 at 2 to 10 mM concentrations had no effect on the enzyme activity. The addition of glycerol at concentrations up to 10%, 20%, and 40% into the incubation mixture inhibited AP activity by 35%, 60%, and 90%, respectively.

Substrate Specificity. All phosphorylated compounds tested were good substrates for our enzyme (Table 3). In fact the *C. marina* AP cleaves effectively at equal rates such substrates as pNPP, 5′-AMP, 5′-CMP, and 5′-dCMP. At the same time, the rate at which the enzyme hydrolyzed 5′-GMP 5′-UMP, 3′-AMP, ADP, ATP, NADP, glucose-6-phosphate, and 2-glycerophosphate was 3 to 8 times slower.

These data suggest that our enzyme may be classified as an unspecific alkaline phosphohydrolase (orthophosphoric–monoester phosphohydrolase, EC 3.1.3.1).

One of the characteristics of unspecific APs is their high sensitivity to the final reaction product of the hydrolysis of orthophosphate monoester compounds, namely, to inorganic phosphate (P_1). Increasing the concentration of inorganic phosphate

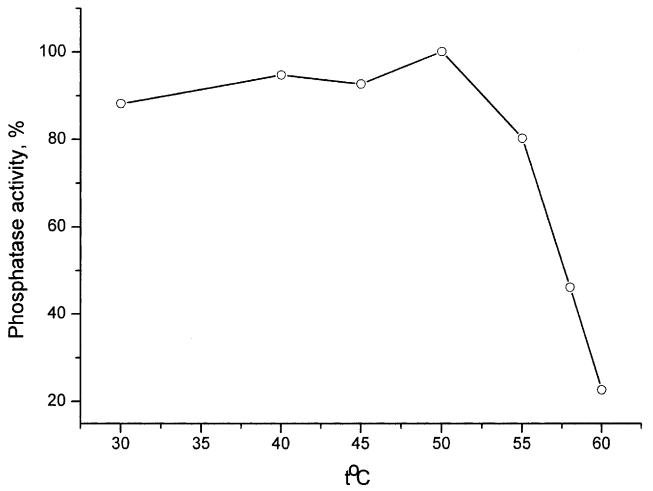

Fig. 3. Effect of temperature on stability of AP from marine bacterium *Cobetia marina*. After preincubation of the enzyme for 15 minutes at various temperatures in 0.01 M Tris-HCl buffer (pH 8.5), the samples were cooled and residual activities were determined.

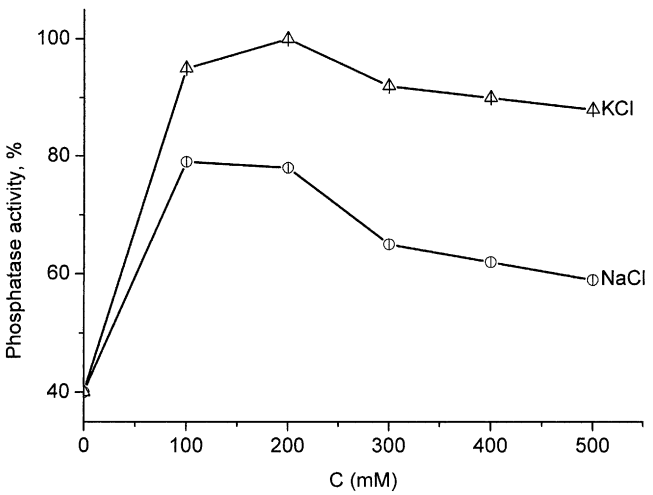

Fig. 4. Effect of NaCl and KCl on activity of AP from marine bacterium *Cobetia marina*. A value of 0.4 was assigned to the activity in 0.1 M Tris-HCl buffer (pH 9.5) without adding salts.

Table 3. Substrate Specificity of Alkaline Phosphatase from Marine Bacterium *Cobetia marina*[a]

Substrate at 2 mM concentration	*Rate of hydrolysis*
p-NPP	1.0
5′-AMP	0.9
(2′,3′)AMP	0.2
ADP	0.2
ATP	0.1
5′-GMP	0.4
5′-UMP	0.3
5′-CMP	0.8
5′-dCMP	0.8
NADP	0.2
(2′,3′) cAMP	0.0
(3′,5′) cAMP	0.0
UDPglc	0.2
α-Glycerophosphate	0.6
β-Glycerophosphate	0.2

[a]Each substrate was assayed at the same concentration (2 mM) in 0.1 M Tris-HCl buffer (pH 9.5) at 37°C, 30 minutes. Orthophosphate released was determined by the method of P.S. Chen et al. (1956). Rates of phosphorolysis were expressed relative to *p*NPP (relative rate, 1.00).

in the course of the reaction up to 100 μM caused the repression of APs (Ammerman and Azam, 1985). Another representative of the group of enzymes capable of cleaving phosphomonoester bonds within nucleotide substrates 5′-nucleotidase (EC.3.1.3.5) has been shown to be insensitive to high concentrations of P_1 (up to 100 μM), suggesting their active participation in the regeneration of P_1 in aquatic ecosystems (Ammerman and Azam, 1985).

Alkaline phosphatase from *C. marina* was shown to be rather resistant towards increasing concentrations of P_1 up to limits from 100 to 1000 μM, suggesting that this enzyme falls in an intermediate position between unspecific APs (EC 3.1.3.1) and 5′-nucleotidases (EC.3.1.3.5).

Labeling 5′-Ends of* Eco*RI Fragments of DNA Phage λ. The capability of AP from *C. marina* to cleave 5′-terminal phosphate with high effectiveness allowed us to apply it to introduction of labeled ^{32}P into the 5′ ends of DNA fragments. Dephosphorylation of DNA fragments, the main step in this method, was carried out by adding the *C. marina* AP to the incubation mixture. Using this AP instead of AP of *E. coli* gave much more complete removal of phosphate 5′ ends of *Eco*RI-generated DNA fragments. This allowed very effective introduction of labeled ^{32}P into 5′ termini of DNA fragments. The electropherogram of the end-labeled (^{32}P) fragments of DNA phage λ followed by treatment of the KMM 296 AP appeared to be similar to that presented previously (Fedosov et al., 1991).

In contrast to the highly active, thermostable AP isolated previously from the marine bacterium *Pseudoalteromonas macleodii* (Fedosov et al., 1991) that was unsuitable for immunologic analysis because of its high sensitivity to chemical modification and loss of its activity during the storage, the KMM 296 AP is resistant to denaturation in the process of preparing Ig-enzyme conjugates (Gate et al., 1991).

Possible Biological Role of the Enzyme. In the bacteria associated with mollusks, a great number of strains are promising producers of AP (Ivanova et al., 1992). APs have been demonstrated both in a number of free-living marine bacteria (Hauksson et al., 2000; Rina et al., 2000), and in some marine invertebrates, including northern shrimp (Olsen et al., 1991) and green crab (Q.X. Chen et al., 2000). Previously, high phosphomonoesterase activity was found in the digestive system and mantle tissues of the oyster *C. angulata*, and it was suggested to be a result of intensive food metabolic activity (Gonzales De Canales and Martin Del Rio, 1985). The authors explained the high level of AP activity in mantle tissues by its participation in shell formation. Interestingly, AP activity does not depend on the stage of maturity of the animal. We speculate that the ability of *C. marina* to produce highly active AP is related to the possible symbiotic relationship between the bacteria and the mussel *C. grayanus* in which the KMM 296 AP may play an active role in processes of shell formation. It is also possible that, like APs of free-living marine bacteria, our bacterial AP is involved in the processes of organic-matter degradation in the eutrophic marine environment, where dissolved DNA, RNA, and protein appear to be the main sources of organic phosphorus for bacteria and phytoplankton (Gonzales De Canales and Martin Del Rio, 1985; Paul et al., 1990). Cotner and Wetzel (1991) suggested similar functions of APs in the processes of phosphorus regeneration in the eutrophic zone after analysis of levels and properties of nucleotidase and AP in the eutrophic and oligotrophic zones.

Acknowledgments

We are grateful to Mrs. Shepetova for helpful discussion and translation this article into English. This work was supported by grants from the Russian Foundation of Fundamental Research (02-04-49517), the Ministry of Industry, Science and Technologies of the Russian Federation (2-2-16), and the Russian Academy of Sciences Program of Fundamental Research Physico-chemical Biology (03-1-05-002).

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