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Biochimica et Biophysica Acta 1750 (2005) 103 – 111
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Characterization of a thermostable alkaline phosphatase from a novel species *Thermus yunnanensis* sp. nov. and investigation of its cobalt activation at high temperature

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Received 21 September 2004; received in revised form 10 April 2005; accepted 9 May 2005
Available online 23 May 2005

Abstract

A thermostable alkaline phosphatase with high specific activity and thermal resistance was purified from a novel species of *Thermus* sp. named as *Thermus yunnanensis* sp. nov. The enzyme contains a single peptide with a molecular mass of about 52 kDa on SDS-PAGE analysis and appears to be a homodimer in solution with the molecular mass of 104 kDa. The optimal pH and temperature for its activities are pH 8.0–10.0 and 70–80 °C, respectively. The catalytic activities of the enzyme are metal ion dependent, and Mg^{2+}, Zn^{2+} and Co^{2+} are the main activators. Among these, Co^{2+} is the most active stimulator and has unique activation effect at high temperature. Metal binding analysis showed the binding of Mg^{2+} at the metal binding site was easy to loss in the thermoinactivation, and Co^{2+} was apt to bind at that site and kept the favorable configuration of catalysis, which would result high activation in the incubation with Co^{2+} at high temperature. According to this study, a model was proposed for the explanation of the activation and the results of actual experiments demonstrated the validity of the model.

Keywords: Thermostable alkaline phosphatase; *Thermus yunnanensis* sp. nov.; Thermostability; Cobalt activation

1. Introduction

Alkaline phosphatase (AP, EC 3.1.3.1) is a non-specific phosphomonoesterase which hydrolyzes a wide variety of phosphate esters and is classified as alkaline phosphatase according to its optimum pH [1]. In a very wide variety of organisms, AP plays a vital role in phosphate transportation and metabolism and is a most crucial enzyme for the survival of organisms under phosphate starvation [2,3]. In practical applications, the enzyme has a wide use in the diagnostics, immunology and molecular biology, such as served as biochemical markers in quantitative measurements of disease, linked enzymes in ELISA and used in non-radioactive detection techniques, probing, blotting and sequencing systems [4–8]. Because of the important principle and practical values of the enzyme, more and more research interests have been attracted in the field. So far, the most commonly used APs are *Escherichia coli* AP and calf intestine AP, but their inherently low thermal resistance and shelf lives have restricted their further applications under some special circumstances, for instance, under high temperature. Therefore, thermostable alkaline phosphatase (TAP) has attracted many attentions because it has the advantage of being used under some extreme conditions.

Until now, TAPs have been purified and characterized from many thermophilic microorganisms, such as *Thermus*

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doi:10.1016/j.bbapap.2005.05.007

sp. [9–12], *Thermotoga* sp. [13,14], *Pyrococcus abyssi* [3], *Bacillus stearothermophilus* [15], *Meiothermus ruber* [16] and *Scytalidium thermophilum* [17]. Even though many advances of the study have been made, no TAPs have been recommended for practical application to address some major problems, for instance, inherently low specific activity and troubled culturing or low yield of production organisms. Screening of more specific and high efficient enzymes is still attractive.

In this study, we screened and identified a new thermophilic bacterial species with high production ability of thermostable alkaline phosphatase from a hot spring site, named as *Thermus yunnanensis* sp. nov., and characterized its thermostable alkaline phosphatase (Tyu TAP). Moreover, a significantly thermal activation was observed in the enzyme when incubated with Co^{2+} at high temperatures. The cobalt activation was firstly investigated in our enzyme through the binding metal ions at the active site, and we proposed a model for its kinetic reaction.

2. Materials and methods

2.1. Chemicals

DEAE-Sepharose A50 and Sephadex G150 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). L-histidyldiazobenzylphosphonic acid-agarose was purchased from Sigma Chemical Co. (St. Louis, MO, USA). *p*-nitrophenyl phosphate (*p*NPP) was from Amresco (Solon, OH, USA). BCA reagents and protein standards were obtained from Pierce (Rockford, IL, USA).

2.2. Screening and culturing of thermophilic bacteria

Samples of water and environmental deposits were collected from the hot spring locations of Tengchong, Yunnan province, People's Republic of China. The bacteria in the samples were grown on the surface of MD agar plates as described in references [18,19] at 65 °C for 4 days. Individual colonies of thermophilic bacteria were selected and stored for further identification and TAP production study. Cultures for TAP production analysis were grown in 50 ml MD medium at 55 °C for bacteria of *Meiothermus* sp. and *Bacillus* sp., and 65 °C for *Thermus* sp. on a rotary shaker for 24 h, then cells were collected by centrifugation, and the yields of TAP from each individual culture were assayed by using TAP assay standard procedure described in the next context.

2.3. Producing and purification of TAP

High yield of TAP strain was cultured in large volume of GTG media (modified MD medium, in which the amount of tryptone and yeast extract were all decreased from 1.0 g/l to 0.08 g/l, but adding 2 g/l glutamic acid with 0.5 g/l glucose) as the same procedure as analysis study; after a 24-h growth, the cells were harvested with centrifugation. Cell pellets were stored at −20 °C for enzyme purification.

The TAP enzyme in the collected was purified as following procedures: cell pellet was suspended in 2.5 (2.5 ml/g cells) volume of buffer A (50 mM Tris–HCl, pH 7.8) and treated with sonication. Cell debris was removed by centrifugation (12,000×*g*) and the supernatant containing the active enzyme (Fraction I) was brought to 70% saturation of ammonium sulfate to precipitate the protein and treated with 1:5 (v/v) n-butanol extraction to eliminate yellow pigment in the crude enzyme preparation. The ammonium sulfate precipitate was re-suspended in a small volume of bufferA (Fraction II), dialyzed and loaded onto DEAE-Sepharose A50 ion-exchange column equilibrated with buffer A and eluted with 0–0.5 M linear gradient of NaCl in the buffer A. The eluted fractions containing the TAP enzyme were pooled (Fraction III) and applied onto L-histidyldiazobenzylphosphonic acid-agarose affinity chromatography column equilibrated with buffer B (buffer A pulse 0.5% NaCl), washed with 30 mM KH_2PO_4 in buffer B and eluted out with buffer C (0.5 M NaCl in buffer A). The eluted enzyme was pooled, concentrated (Fraction IV) and stored under −80 °C for characterization studies. All the chromatography fractions throughout the above procedures were monitored by both activities assay and 280 nm spectrometer measurement.

2.4. Molecular mass determination

Native protein's molecular mass was determined by using gel filtration chromatography. Purified enzyme was loaded onto a Sephadex G150 column (1.6×100 cm) equilibrated and eluted with buffer B. The standard proteins were catalase (116 kDa), bovine serum albumin (66 kDa), albumin egg (42.7 kDa) and lysozyme (14.4 kDa). The flow rate was 4 ml h^{-1}. Elution of the standard proteins and Tyu TAP was determined by 280 nm UV detection and activity assay.

Protein samples were analyzed by 12% SDS-PAGE at 10 °C using the method of Laemmli [20], and the gel was visualized by stained with 0.1% Coomassie Brilliant blue R250.

2.5. Protein concentration determination

The protein concentration was determined using BCA protein assay reagents A and B (Pierce) with bovine serum albumin as the standard protein.

2.6. TAP assay

TAP activity was assayed by following the release of *p*-nitrophenol (*p*NP) from *p*NPP. The standard assay