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# Characterization of the enzymatic property of thermostable carboxypeptidase *Taq* by addition of metal ions and replacement of active center metal

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## Abstract

We analyzed improvement on the enzyme activity of CPase *Taq* by addition of various metal ions. The enzyme activity was increased more then four times by 1 mM cobalt ion and almost three times by 1 mM calcium ion. However, the active center metal zinc ion did not affect the enzyme activity. In order to investigate whether the active center metal affects the enzyme activity, zinc ion which is occupied the active center of the enzyme was replaced by cobalt ion which activates the enzyme activity very effectively. Since the cobalt ion in the active center of the cobalt-substituted CPase *Taq* did not affect the enzyme activity, it could act as the native metal ion in the active center of the enzyme.

**Key words** – carboxypeptidase, zinc-dependent, thermostable, cobalt-substituted

## Introduction

Carboxypeptidase (CPase) hydrolyzes the peptide bond at the C-terminus of peptides and proteins. CPases A and B which are zinc-dependent enzymes have been well investigated as to their structure and function [2,7,13]. Previously, we reported the purification and properties of CPase *Taq* from *Thermus aquaticus* YT-1, an extremely thermophilic bacterium [9]. The enzyme is a thermostable zinc-dependent metallo-carboxypeptidase and it has broad substrate specificity. The optimum temperature for its reaction is 80℃. The enzyme is a molecular weight of 56,000. We also described the cloning, sequencing and expression in *E. coli* cells of the CPase *Taq* gene [10]. The deduced amino acid sequence of CPase *Taq* exhibits no any similarity to any protein sequences [10], including those of other metallo-carboxypeptidases like CPases A and B, suggesting that it is a novel type of metallo-carboxypeptidase. On the other hand, a conserved active-site motif (His-Glu-X-X-His) of zinc-dependent endopeptidases and aminopeptidases was found in the deduced amino acid sequence of the enzyme [10,11].

Since the optimum temperature of the enzyme for its reaction is 80℃, CPase *Taq* is a thermostable carboxypeptidase [9]. To investigate thermostable mechanisms of thermostable enzymes is helpful in order to make a functional enzyme more heat-stable. Analysis for metal ions bound to the enzyme found that one molecule of the enzyme contains one tightly bound zinc ion [10]. If the active center zinc ion is replaced by transition metal, cobalt ion, it is possible to get the structural information of the active center by electron spin resonance, ESR [6].

In this paper, we describe improvement on the enzymatic activity of thermostable CPase *Taq* by addition of metal ions. We also investigated whether the active center metal affects the enzyme activity. Active center zinc ion of the enzyme is replaced by transition metal,

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cobalt ion and the cobalt-substituted CPase *Taq* was subjected to characterize the enzyme activity and bound metals.

## Materials and Methods

### Expression of the CPase *Taq* gene in *E. coli* and purification of the enzyme

*E. coli* MV1184 cells harboring the expression plasmid for the CPase *Taq* gene which is controlled by *tac* promoter [10] were grown in 1 L LB medium containing ampicillin (100 mg/ml) at 37℃ for 9 h in the presence (1 mM) and absence of isopropyl β-D-thiogalactopyranoside (IPTG). After incubation, cells were collected by centrifugation and then washed with 0.05 M Tris-HCl buffer (pH 7.2). The washed cells were suspended in a 10-fold volume of the same buffer and then disrupted by sonication. The sonicated sample was centrifuged at 20,000×g for 20 min, and then the supernatant obtained was treated at 70℃ for 1 h and centrifuged again at 20,000×g for 20 min to remove denatured proteins. The supernatant was brought to 30% saturation with ammonium sulfate and then stirred at 4℃ for 30 min. After the precipitate had been removed by centrifugation at 20,000×g for 20 min, the supernatant was subjected into a column (1.6×8 cm) of butyl-Toyopearl 650M (Tosoh, Tokyo, Japan) equilibrated with 50 mM Tris-HCl buffer (pH 7.2) 30% saturated with ammonium sulfate. The column was washed with the same buffer 20% saturated with ammonium sulfate. Proteins were eluted with the same buffer 10% saturated with ammonium sulfate, in a total volume of 50 ml, at the flow rate of 3 ml per min. The eluate was dialyzed against 50 mM Tris-HCl buffer (pH 7.2), and then put on a column (1.6 ×8 cm) of DEAE-Toyopearl 650S (Tosoh) equilibrated with 50 mM Tris-HCl buffer (pH 7.2). The column was washed with the same buffer containing 50 mM NaCl. Proteins were eluted with the same buffer containing 100 mM NaCl, in a total volume of 50 ml, at the flow rate of 3 ml per min. The protein concentrations were measured with the BCA protein assay reagent (Pierce Chemical Co. USA), with bovine serum albumin as the standard protein.

### Assay of enzyme activity

The enzyme activity was measured by the ninhydrin method of Rosen [12] with 0.5 mM Cbz-Phe-Tyr (Protein Research Foundation, Osaka, Japan) as the substrate, at 70℃ for 30 min in 50 mM N-2-hydroxyethylpiperazine-N-3-propanesulfonic acid (HEPPS)-NaOH buffer (pH 8.5). One unit of enzyme activity was defined as the amount of enzyme that produced a ninhydrin-positive substance corresponding to 1 mmol of tyrosine per minute. The specific activity was expressed as units (U) per milligram of protein.

### SDS-PAGE

SDS-PAGE was performed by the method of Laemmli [8] with an 11% polyacrylamide gel. The protein was mixed with the sample buffer and incubated at 37℃ for 30 min before being put on the gel.

### Improvement on the enzyme activity of CPase *Taq* by addition of metal ions

The purified enzyme (0.32 nM concentration) from *E. coli* cells was incubated in the presence of 1 mM metal ions the at 25℃ for 30 min. The enzyme activity was measured in the presence of the same metal ion. The relative activities are shown.

### Replacement of active center zinc ion of CPase *Taq* by cobalt ion

About 2 mg of purified enzyme from *E. coli* cells was dialyzed against 500 ml of 100 mM 2-(N-morpholino) ethane sulfonic acid, monohydrate (MES)-NaOH buffer (pH 7.2) containing 10 mM 1,10-phenanthroline and 1 M NaCl at 4℃ for three days to remove zinc ions from the active center of CPase *Taq*. The enzyme was dialyzed

again against 500 ml of 100 mM Tris-HCl buffer (pH 7.2) containing 1 mM $CoCl_2$ at 4℃ for one day to add cobalt ions to the active center of CPase *Taq*. And then the enzyme was dialyzed 4 times against 300 ml each of 100 mM Tris-HCl buffers (pH 7.2) to remove non-bound cobalt ions from the enzyme solution. The treated enzyme was analyzed for bound metals by inductively coupled plasma atomic emission spectrometry (ICP-AES) (model SPS1200VR; Seiko Instruments Inc., Tokyo, Japan). The amount of zinc or cobalt was calculated using a zinc and a cobalt standard solutions. The enzyme activity of the treated enzyme was analyzed and compared with that of the native enzyme.

## Results and Discussion

### Expression of the CPase *Taq* gene in *E. coli* and purification of the enzyme.

*E. coli* cells grown in the absence of IPTG produced a high amount of CPase *Taq* [10]. On the other hand, *E. coli* cells cultured in the presence of 1 mM IPTG showed too slow cell growth to get an enough amount of cells (data not shown). The promoter used for the expression of CPase *Taq* gene in *E. coli* is *tac* promoter which can be induced by IPTG for over-production. But CPase *Taq* is proteolytic enzyme, the induced production of the enzyme in *E. coli* cells by IPTG might be harmful to bacterial growth (it may lead cells to lysis). This result suggests that the non-induced production of a harmful protein such as CPase *Taq* in *E. coli* cells is effective for over-production. Although various IPTG concentrations were also tested for the effective production of the enzyme, no sufficient amount of the enzyme protein was observed (data not shown). We also have tested the expression system that directed by strong T7 promoter to express CPase *Taq*, however, no any expression of the enzyme was observed (data not shown).

CPase *Taq* was effectively purified by heat treatment with a great yield, and successive butyl-Toyopearl and DEAE-Toyopearl chromatographic separation gave a purified preparation of the enzyme protein (Table 1 and Fig. 1). The mobility of the purified CPase *Taq* produced in *E. coli* (Fig. 1, lane 4) was identical with that of the CPase *Taq* purified from *T. aquaticus* YT-1 (Fig. 1, lane 5). The specific activities of the two purified enzymes were almost identical (Table 1 and reference 9).

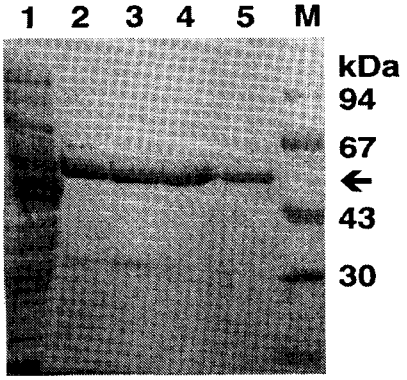


Fig. 1. SDS-PAGE of CPase *Taq* produced in *E. coli* cells harboring the CPase *Taq* expression plasmid.
Lane M. size standard marker: lane 1. the supernatant obtained on centrifugation after disruption of the cells by sonication: lane 2. soluble fraction obtained on centrifugation after heat treatment at 70℃ of the lane 1 material: lane 3. sample after butyl-Toyopearl chromatography; lane 4. sample after DEAE-Toyopearl chromatography: lane 5. purified CPase Taq from *T. aquaticus* TY-1. The arrow indicates the position of CPase *Taq*.

Table 1. Purification of CPase *Taq* from *E. coli* cells harboring the CPase *Taq* expression plasmid

| Purification step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) |
|---|---|---|---|---|
| Cell-free extract | 720 | 55,440 | 77 | 100 |
| Heat treatment | 238 | 27,393 | 115 | 49 |
| Butyl-Toyopearl | 20 | 21,600 | 1,080 | 39 |
| DEAE-Toyopearl | 5 | 15,075 | 3,900 | 27 |

### Improvement on the enzyme activity of CPase *Taq* by addition of various metal ions.

Since the enzyme activity was inhibited by EDTA, this enzyme is metallo-carboxypeptidase [9]. Analysis of

netal-ions bound to the enzyme by ICP-AES revealed hat CPase *Taq* contained one mole of zinc ion per one nole of enzyme protein [10]. Treatment of enzyme with :obalt ions showed the four fold increase in enzyme ıctivity (Table 2). Activation by cobalt ion has also been )bserved in the cases of CPase E (enkephalin convertase) 4]. The enzyme was activated almost three times by :alcium ion. Moderate activation was observed by :opper or magnesium ion (Table 2). On the other hand, he enzyme was not activated by zinc, ferrous, or nickel on (Table 2). In contrast, the enzyme activity was nhibited by tin or mercury ion (Table 2). These results ndicated that the enzyme activity could be effectively ıctivated by addition of cobalt or calcium ion.

## Replacement of the active center zinc ion of CPase *Taq* by transition metal, cobalt ion.

To investigate thermostable mechanisms of CPase *Taq* hermostable enzymes is helpful in order to make a unctional enzyme more heat-stable. If the active center zinc ion is replaced by transition metal, cobalt ion, nformation of interaction between cobalt ions and it's surrounding was achieved by electron spin resonance, ESR [6]. The structures of the active sites of cobalt-substituted proteins such as carbonic anhydrase, superoxide dismutase, insect haemoglobin, and bovine serum amine oxidase were investigated by ESR and successfully determined [1,3,5,6]. Analysis for bound metals of the cobalt-substituted enzyme by ICP-AES revealed that the enzyme contained 6.1 mM of cobalt ion and 2.6 mM of zinc ion in 8.9 mM of enzyme protein (Table 3). This result indicates that approximately 70% of zinc ion in the enzyme was replaced by cobalt ion. We previously reported that CPase *Taq* contained one mole of zinc ion per one mole of enzyme protein (Table 3 and reference 10). Analysis of the enzyme activity of the metal-replaced enzyme showed no significant differences on the enzyme activity compared with that of the native enzyme (Table 3). These results indicated that the active center zinc ion of the enzyme was successfully replaced by cobalt ion. Structure of the active site of cobalt-substituted CPase *Taq* should be investigated by ESR.

Table 2. Improvement on the activity of CPase *Taq* by addition of metal ions

| Metal ion (1 mM) | Relative activity (%) |
|---|---|
| None | 100 |
| $Co^{2+}$ | 413 |
| $Ca^{2+}$ | 273 |
| $Cu^{2+}$ | 147 |
| $Mg^{2+}$ | 113 |
| $Zn^{2+}$ | 100 |
| $Fe^{2+}$ | 100 |
| $Ni^{2+}$ | 100 |
| $Sn^{2+}$ | 73 |
| $Hg^{2+}$ | 60 |

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Table 3. Comparison of bound metals between native and cobalt-substituted CPase *Taq*

| Metalion | Amount of metal per enzyme protein (mM) | Relative amount of metal (%) | Relative activity (%) |
|---|---|---|---|
| Native enzyme | | | 100 |
| $Zn^{2+}$ | 8.9 | 100 | |
| $Co^{2+}$ | 0.0 | 0 | |
| Cobalt-substituted enzyme | | | 94 |
| $Zn^{2+}$ | 2.6 | 30 | |
| $Co^{2+}$ | 6.1 | 70 | |

3.9 mM each of the native and cobalt-substituted enzyme proteins was subjected to analyze for bound metals.

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## 초록 : 금속이온 첨가와 활성중심 금속의 치환에 따른 내열성 카르복시펩 티다제 *Taq*의 효소적 특성 변화에 관한 연구

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다양한 금속이온의 첨가에 따른 CPase *Taq*의 효소활성의 개선에 관한 연구를 행하였다. 1 mA의 코발트이온의 첨가에 의해 효소활성이 4배 이상 증가했고, 1 mA의 칼슘이온의 첨가에 의해서는 효소활성이 거의 3배 정도로 증가했다. 하지만 활성중심에 존재하는 아연이온은 효소활성에 영향을 주지 않았다. 활성중심의 금속이온이 효소활성에 영향을 주는지를 알아보기 위해 활성중심을 차지하고 있는 아연이온을 본 효소를 효과적으로 활성화시키는 코발트이온으로 치환하였다. 그 결과, 코발트이온으로의 치환이 CPase *Taq*의 효소활성에 영향을 주지 않으므로, 코발트이온은 본 효소의 활성중심의 금속이온인 아연이온을 대신하여 CPase *Taq*가 효소활성을 가지는데 있어서 동일한 역할을 할 수 있는 금속이온이라 사료된다.