Note

Occurrence of a Novel Lyase Catalyzing β-Elimination Reaction toward threo-3-Chloro-L-aspartate in Pseudomonas putida TPU 7151

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A bacterium, Pseudomonas putida TPU 7151, which degrades threo-3-chloro-l-aspartate, was isolated from soil and the enzyme responsible for the degradation of the amino acid was partially purified from the cell-free extract of the strain. The enzyme, which required PLP for its reaction, catalyzed a stoichiometric β-elimination reaction of threo-3-chloro-L-aspartate to form oxaloacetate, Cl⁻, and NH₄⁺. The enzyme was active toward only threo-3-chloro-L-aspartate and l-cysteine, but did not catalyze a β-replacement reaction. The enzyme can be classified in a new group of PLP-dependent amino acid-lyases [EC 4.2.1.-].

Key words: lyase; screening; enzyme; β-elimination reaction; Pseudomonas

β-Chloro-L-alanine (BCA) is a bacteriostatic amino acid analog and it inhibits several pyridoxal 5'-phosphate (PLP)-dependent enzymes, such as threonine deaminase,¹ branching chain amino acid transaminase,¹ l-aspartate-β-carboxylase,² and alanine racemase² by its suicidal action. However, some enzymes such as tryptophan synthase, tyrosine phenol-lyase, cysteine desulphydrase, O-acetylserylne desulphydrase, and S-carboxymethyl-L-cysteine synthase, are insensitive to the suicidal function and degrade BCA by β-elimination or β-replacement reactions.⁵,⁶ Despite a number of studies on BCA, there had been no report on biochemical properties of other halogen-containing amino acids due to their unavailability. During the course of our studies on β-methylaspartase in enteric bacteria, we discovered an efficient enzymatic method for the synthesis of threo-3-chloro-L-aspartate (CLA) from chlorofumarate.⁷⁻¹¹ In this report, we screened for CLA-degrading bacteria from soil and discovered a novel enzyme catalyzing a β-elimination reaction on CLA in the cell-free extract of the strain (Fig. 1).

The standard reaction mixture (1 ml) consisted of 100 μmol of potassium phosphate buffer (pH 7.0), 5 μmol of CLA, 1 μmol of PLP, and the enzyme solution. The enzyme reaction was done at 30°C and was stopped by an addition of 1 ml of 0.2 N HCl. The concentration of α-keto acid was measured by the 2,4-dinitrophenylhydrazine method as described by Friedman.¹² Oxaloacetate and pyruvate were enzymatically analyzed by using malate¹³ and alanine dehydrogenases¹⁴, respectively. The concentrations of NH₄⁺ and Cl⁻ were measured by the phenol-sodium nitroprusside-NaClO₃ method¹⁵ and the Hg(SCN)₂ method,¹⁶ respectively. Amino acids were measured by a Hitachi L-8500 amino acid analyzer (Tokyo, Japan). One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of oxaloacetate or NH₄⁺ from CLA per min. Protein was measured with a Bio-Rad protein assay kit (Bio-Rad, USA) with bovine serum albumin as a standard or by measuring the absorbance at 280 nm. The substrate, CLA, was enzymatically prepared as described previously.⁷

CLA-degrading bacteria were isolated from soil collected in Toyama Prefecture using an enrichment culture technique.¹⁷ The screening medium (pH 7.0) was composed of 0.4% of glucose, 0.2% of CLA, 0.2% of K₂HPO₄, 0.1% of NaCl, 0.02% of MgSO₄·7H₂O, 0.05% of yeast extract (Nippon Sakeyaku, Tokyo), and 1.0% of vitamin mixture.¹⁸ Soil samples were added to 5 ml of screening medium in a test tube and incubated with shaking at 30°C. A loopful of the culture broth was transferred to a new medium once every other day. After 7 transfers, the culture broths were spread onto plates with the same medium containing 1.5% agar and colonies that formed were iso-

Fig. 1. Enzymatic β-Elimination Reaction of threo-3-Chloro-L-aspartate by Pseudomonas putida TPU 7151.

Abbreviations: BCA, β-chloro-L-alanine; CLA, threo-3-chloro-L-aspartate

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lated in the usual manner. A total of 10 strains that
degraded CLA as the sole of nitrogen source, were
isolated. The strain TPU 7151 was chosen for further
studies since it had the highest CLA-degrading activi-
ity. The taxonomical characteristics of the strain are
as follows. Gram-negative non fermentative rod.
Non spore-forming. Motile with a polar flagellum.
Strictly aerobic. Fluorescent pigment was produced in
King's A medium. Catalase and oxidase: positive.
Grew at 4°C but not at 41°C. Indole production:
negative. Acid produced from glucose and xylose but
not from maltose and sucrose. Nitrate and nitrite
were not reduced. Denitrification: negative. Arginine
dihydrolase: positive. Lysine and ornithine decarbox-
ylases: negative. Citrate use (Simmons and Christen-
son): positive. Urease: negative. Gelatin liquefaction:
positive. Casein and starch hydrolysis: negative. Ac-
cording to “Bergey’s Manual of Systematic Bacteri-
ology”9, the strain was identified as Pseudomonas
putida.

We investigated the culture conditions for the for-
mation of the enzyme activity using the resting cells
of the strain. Although the enzyme activity was
formed constitutively, it was doubled by an addition
of CLA. Therefore, we added 0.2% of CLA as an in-
ducer to the medium. The strain grew well on sugars,
such as glucose, glycerol, and fructose, and organic
acids, such as acetate, fumarate, lactate, and pyru-
vate. Among of them, sucrose was the most suitable
carbon source for cell growth and formation of the
enzyme. Various nitrogen sources were added to the
screening medium containing sucrose and CLA.
Yeast extract, NZ-Amine (Wako, Osaka), corn steep
liquor (Sanei Touka, Aichi), beef extract (Difco,
Detroit, USA), and (NH₄)₂HPO₄ were effective in
promoting cell growth and the enzyme formation,
but the specific activity (units/mg cell) of the cells
grown with these nitrogen sources were low compared
to them grown on CLA as a sole nitrogen source. Bas-
ning on these results, the medium (pH 7.0) containing
0.5% sucrose and 0.2% CLA was optimal for the for-
mation of the enzyme activity.

To characterize the enzyme reaction, the enzyme
was partially purified 100-fold with a 67.6% yield
from the cell-free extracts of the strain grown under
the optimized conditions (Table 1). It was observed
that the activity was lost after dialysis against several
buffers at any pHs. Among various expected addi-
tives, PLP was shown to restore the lost activity.
Furthermore, the enzyme activity was strongly inacti-
vated by hydroxylamine or NaBH₄. These results
indicate that the enzyme activity depends on a loosely
bound PLP, therefore, 0.1 mM of PLP was present in
buffers throughout the purification.

The degradation of CLA was examined with the
partially purified enzyme. As shown in Fig. 2, one
mol each of oxaloacetate, NH₄⁺, and Cl⁻ was
stoichiometrically produced per mol of CLA con-

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific Activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
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<td>Cell-free extract</td>
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<td>100</td>
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<tr>
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<tr>
<td>Butyl-Toyopearl</td>
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<td>1.48</td>
<td>0.239</td>
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</tbody>
</table>

Table 1. Partial Purification of threo-3-Chloro-L-aspartate β-
Elimination Enzyme from Pseudomonas putida TPU 7151

Fig. 2. Course of threo-3-Chloro-L-aspartate β-Elimination by
the Partially Purified Enzyme from Pseudomonas putida TPU 7151.

The reaction mixture (1 ml) contained 100 μmol of potassium
phosphate buffer (pH 7.0), 1 μmol of PLP, 5 μmol of threo-3-
chloro-L-aspartate, and 0.25 units of the partially purified en-
zyme. Symbols: threo-3-chloro-L-aspartate (○), oxaloacetate
(●), Cl⁻ (▲), and NH₄⁺ (●).

sumed, suggesting that the enzyme catalyzed stoichiometric β-elimination reaction of CLA. The
enzyme reaction was done in several buffers at vari-
sous pHs. The enzyme showed maximal activity at
around pH 7.5–8.0 in potassium phosphate buffer.
Substrate specificity of the enzyme was examined by
an incubation of the enzyme (0.1 unit) with 5 mM of
various substrates in the standard reaction mixture for 4 hr. Among of L- and D-isomer of the standard amino acids found in proteins, L-cysteine was only accepted as substrate at a rate of 66% to that for CLA and was converted to pyruvate and NH$_4^+$. It is reported that the BCA-degrading enzymes, such as tryptophan synthase, tyrosine phenol-lyase, cysteine desulphhydrase, O-acetylserine desulphhydrase, and S-carboxymethyl-L-cysteine synthase, catalyze the $\beta$-elimination reaction of $\beta$-substituted L-alanine derivatives, e.g. L-serine, O-acetyl-L-serine, O-phospho-L-serine, and 2,3-diaminopropionate.\textsuperscript{5,6} However, the CLA-degrading enzyme did not act on these compounds under our experimental conditions. Aspartate derivatives, such as $\textit{threo}$-3-methyl-L-aspartate, $\textit{threo}$-3-ethyl-L-aspartate, and $\textit{threo}$-3-hydroxy-3-oxo-aspartate and various amines and amino alcohols were also inert as substrates. It is also shown that the BCA-degrading enzymes catalyze $\beta$-replacement reaction of BCA with indole, alkyl mercaptanes, and phenols to form tryptophan, S-alkyl-cysteine, and tyrosine derivatives, respectively.\textsuperscript{5,6} We examined the $\beta$-replacement reaction of 5 mm of CLA with 10–300 mmol of phenol, indole, thiophenol, and sodium hydrosulphite at pHs 6–10, but none of the reactions progressed. We conclude that the enzyme was different from the BCA degrading enzymes based on the following observations: 1) it was highly specific toward CLA and L-cysteine,\textsuperscript{14,9} it catalyzed only $\beta$-elimination reaction of CLA but not a $\beta$-replacement reaction. The enzyme could be categorized as a new group of PLP-dependent amino acid-lyases [EC 4.2.1.-].

These results provide good evidence for the occurrence of a novel $\beta$-elimination enzyme in the cell-free extract of \textit{P. putida} TPU 7151 isolated from soil.

References


