Cysteine-conjugate β-Lyase from *Mucof javanicus*

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Studies on the metabolism of dichloronitrobenzenes as model compounds of certain pesticides led to the suggestion of a possible pathway in *Mucof javanicus* as shown in Scheme I and purification of *M. javanicus* glutathione transferase (step I). Cysteine conjugates are enzymatically converted to N-acetates (mercapturic acids), N-malonates, and methylthio compounds. The first step of the conversion to methylthio compounds is the C-S cleaving reaction (step 3) by cysteine-conjugate β-lyase [EC 4.4.1.13], a pyridoxal 5'-phosphate dependent enzyme which is found in mammalian tissues and intestinal microorganisms. It has been reported that the rat liver enzyme had kynureninase activity and that the rat kidney enzyme is identical to glutamine transaminase K. The enzymes from the intestinal microorganisms act on a wider range of substrates including S-alkyl-L-cysteines and S-aryl-L-cysteines. We purified a cysteine-conjugate β-lyase from *Mucor javanicus* that had different properties from the enzymes mentioned above.

Substrates. S-(2-Chloro-6-nitrophenyl)-, S-(2-chloro-4-nitrophenyl)-, and S-(3-chloro-6-nitrophenyl)-L-cysteines were prepared by substitution reaction of 2.3, 3.4, and 2.4-dichloronitrobenzenes with L-cysteine in alkaline solution. S-Phenyl- and S-ethyl-L-cysteines were prepared by the methods of Greenstein and Winitz. Other substrates were available commercially.

**Assay of enzyme activity.** Cysteine-conjugate β-lyase activity was assayed by measurement of pyruvate formed from 1.33 mm S-(2-chloro-6-nitrophenyl)-L-cysteine by the following procedure. The substrate was dissolved in 0.05 N HCl to become a 4 mm solution and stored. The substrate stock solution was mixed with an equal volume of 0.2 m potassium phosphate, pH 7.5, containing 0.3 mm pyridoxal 5'-phosphate and 4 nmol of NaOH to neutralize the HCl. The enzyme solution, pH 7.5, (100 µl) was incubated with 200 µl of the fresh substrate solution at 30°C for 15 min. The enzyme reaction was halted by the addition of 1.7 ml of 0.59 N HCl. The resulting mixture was incubated with 0.3 ml of 2,4-dinitrophenyldihydrazine in 2 N HCl for 15 min at 30°C, alkylized with 2 ml of 2 N NaOH, and used to measure absorbance at 540 nm. Linearity of the enzyme reaction was not kept during the incubation time of 15 min. The activity was tentatively defined as value (µmol/min) calculated from 15 min reaction with 0.1 mm pyridoxal 5'-phosphate in this paper. In an experiment to identify the effects of α-keto acids, enzyme activity was expressed by the amount of ammonia formed under the same conditions as mentioned above. The enzyme reaction was halted with 500 ml of 0.8 N HCl. After neutralization with 200 µl of 1.8 N NaOH, ammonia was measured by the method of Nagatsu and Yagi. Activities of kynureninase and glutamine transaminase were assayed by the methods of Stevens and Cooper and Meister, respectively.

**Purification of cysteine-conjugate β-lyase from *Mucor javanicus*.** The fungus strain, *Mucor javanicus* AHUB8010, was donated by the Laboratory of Applied Microbiology, Hokkaido University. The buffer solution used in the purification process was 0.05 M potassium phosphate, pH 7.5, containing 10 µM pyridoxal 5'-phosphate, 1 mM EDTA, 1 mM Mg-cacemtoethanol, and 10% glycerol. The fungus was cultured as described in previous paper and the collected mycelia were stored at -40°C. The frozen mycelia (50 g) were mixed with 400 ml of cold acetone with a Waring blender, collected by suction filter, and suspended in 500 ml of the buffer. The suspension was gently stirred for an hour in a cold room and centrifugation followed to isolate the extract. The extract from 200 g of the frozen mycelia was collected (2030 ml) and put on a DEAE-cellulose column (46.2 × 25 cm) equilibrated with the buffer containing 0.05 M KCl. The column was washed with 800 ml of the same buffer and eluted with a linear gradient of one liter of 0.05 to 0.5 M KCl in the buffer. Active fractions from the DEAE-cellulose column were collected, saturated with ammonium sulfate by 35%, and put on a column (1.5 × 11 cm) of butyl-Toyopearl (Tosho) equilibrated with 35% saturated ammonium sulfate. The column was washed with 500 ml of 0.4 M ammonium sulfate solution and protein was eluted with a linear gradient of 35 to 0% saturated ammonium sulfate in 200 ml of the buffer. An enzyme fraction was dialyzed against the buffer containing 0.1 M KCl and concentrated with polyethylene-glycol 20,000 to about 2 ml. The concentrated enzyme solution was put on an aminopropyl-Sepharose column (1 × 30 cm). The column was washed with 200 ml of the buffer containing 0.1 M KCl and protein was eluted with a linear gradient 0.1 to 0.5 M KCl in 140 ml of the buffer. The enzyme fraction was concentrated with polyethylene glycol 20,000 to a few milliliters and passed through a Bio-Gel P-150 column (1.5 × 40 cm) washed with the buffer. The enzyme fraction from the Bio-Gel P-150 column was put on a Mono Q HR-5/5 (Pharmacia) column with 0.05 M potassium phosphate, pH 7.5, containing 10% glycerol and a gradient of 0.05 to 0.275 M KCl. Active fractions were collected and rechromatographed with Mono Q.

Protein was measured by the micro-biuret method in the crude extract and by measurement of the absorbance at 280 nm in all other preparations. The process of the purification is summarized in Table I. Activity in the crude extract was assayed after removal of low molecular mass substances, especially acetone, with a small column of Sephadex G-25 and centrifugation to avoid the interference of the enzyme assay. The preparation from Mono Q rechromatography showed one band in polyacrylamide gel disc electrophoresis at pH 8.0 and SDS polyacrylamide gel electrophoresis, (M, 47,000). In the other hand, gel filtration with

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**Fig. 1.** Effects of Pyridoxal 5'-Phosphate on *Mucor* Cysteine-conjugate β-Lyase Reaction.

Enzyme (0.011 µmol/min) and 1.33 mm S-(2-chloro-6-nitrophenyl)-L-cysteine were incubated with 0 (△), 10⁻⁴ (□), 5 × 10⁻⁵ (△), 10⁻⁵ (△), 5 × 10⁻⁶ (□), and 10⁻⁶ (○) m pyridoxal 5'-phosphate at pH 7.5 at 30°C.
Table 1. Purification of Cysteine-conjugate $\beta$-Lyase

<table>
<thead>
<tr>
<th></th>
<th>Protein (mg)</th>
<th>Activity (µmol/min)</th>
<th>Specific activity (µmol/min/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>15,000</td>
<td>150</td>
<td>0.010</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>1,100</td>
<td>71</td>
<td>0.066</td>
<td>47</td>
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<tr>
<td>Butyl-Toyopearl</td>
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<td>52</td>
<td>2.2</td>
<td>35</td>
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<tr>
<td>Aminohexyl-Sepharose</td>
<td>6.4</td>
<td>31</td>
<td>4.8</td>
<td>20</td>
</tr>
<tr>
<td>Bio-Gel P-150</td>
<td>3.7</td>
<td>29</td>
<td>7.8</td>
<td>19</td>
</tr>
<tr>
<td>Mono Q (rechromatography)</td>
<td>0.500</td>
<td>11</td>
<td>22</td>
<td>7.3</td>
</tr>
</tbody>
</table>

$\beta$-lyase purified from rat liver had kynureninase activity three-fold over $\beta$-lyase activity with S-2-benzothiazolyl-$l$-cysteine. The *Mucor* cysteine-conjugate $\beta$-lyase had a minor kynureninase activity, in which the reaction ratio was $5 \times 10^{-4}$.

Glutamine transaminase activity. Purified cysteine-conjugate $\beta$-lyase from rat kidney was described as being identical to glutamine transaminase K. The reported ratio of the transaminase activity to the $\beta$-lyase activity with S-(1,2-dichlorovinyl)-$l$-cysteine is about 5. No activity of transamination was detected in *Mucor* cysteine-conjugate $\beta$-lyase with two pairs of substrates: $l$-glutamine-phenylpyruvate and $l$-methionine-phenylpyruvate.

Effects of $\alpha$-keto acids. Several $\alpha$-keto acids stimulate rat kidney cysteine-conjugate $\beta$-lyase activity. This essential function of $\alpha$-keto acids is presumably explained by the transamination activity of the enzyme.

The *Mucor* enzyme was not stimulated by the addition of 0.1 mm or 1 mm $\alpha$-keto acids; glyoxylate, pyruvate, $\beta$-hydroxypyruvate, $\alpha$-ketobutyrate, $\alpha$-ketovalerate, $\alpha$-ketoisovalerate, $\alpha$-ketoacaproate, $\alpha$-ketoisocaproate, phenylpyruvate, or $\alpha$-ketoglutarate. This result is consistent with the description in a preceding section.

References