Additional Evidence for the Identity of *Scytlidium lignicolum* Acid Proteinases with the Carboxyl Proteinase Group: The Interaction between Angiotensin I and S-PI-Insensitive Acid Proteinases by Means of a Zinc(II)-Dye Complex as a Probe†

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*S. lignicolum* acid proteinases A-1, A-2, and B (S-PI-insensitive) were examined for the structure of the active sites by the method of Nakatani using PAD as a probe. We attempted to screen for substances that release zinc(II)-PAD from the complex of zinc(II)-PAD–S-PI-insensitive acid proteinases. Angiotensin I released zinc(II)-PAD from the complex as well as from the complex of S-PI-sensitive acid proteinases. Angiotensin I is a good substrate for these enzymes regardless of S-PI-sensitivity. These results suggest that the two carboxyl groups capable of binding to zinc(II)-PAD are at the active site regions of *S. lignicolum* enzymes and that they are involved in their catalytic actions.

Acid proteinase has two indispensable carboxyl residues of aspartic acid in the active sites, one of which is modified with DAN and the other with EPNP. Therefore, acid proteinases are now called aspartic proteinases.¹

*Scytlidium lignicolum* ATCC 24568, which we isolated in 1972, produces acid proteinases A-1, A-2, B, and C, all of which are a new type insensitive to such acid proteinase inhibitors as DAN, EPNP, S-PI (Pepstatin Ac), and Pepstatin, except for the sensitivity of B to EPNP.³⁻¹³ Such unique behavior against inhibitors suggest that no carboxyl residue participates in their action. On the other hand, the zinc(II)-PAD experiments¹⁴ suggested the presence of two carboxyl groups very close to each other in these enzymes, like those in pepsin and other acid proteinases.

To explain the discrepancy, we did two experiments; one with kinetics¹³ and the other by the zinc(II)-PAD method as a catalytic site probe.

In this paper, the results of the latter is described.

*¹* Studies on New Acid Proteases from *Scytlidium lignicolum* ATCC 24568. Part IX. For previous papers, see refs. 2⁻¹³.

Abbreviations: S-PI, *Streptomyces*-pepsin inhibitor; Pepstatin Ac, N-acetyl pepstatin; DAN, Diazoacetyl-DL-norleucine methyl ester; EPNP, 1,2-epoxy-3-(p-nitrophenoxy) propane; PAD, Pyridine-2-azo-p-dimethyl aniline.
MATERIALS AND METHODS

1) Enzymes. Acid proteinases A-2 and B of Scytalidium lignicolum ATCC 24568 were purified from culture filtrates as reported previously, and electrophoretically homogeneous preparations were used for this study. We also purified carboxyl proteinases of Rhodotorula glutinis and Cladosporium sp. in this laboratory. Porcine pepsin was purchased from Sigma Chemical Co. Carboxyl proteinases Type A and B from Aspergillus niger was from Seikagaku Kogyo, Co., Ltd.

2) Substrates. Ac-Phe-Tyr (F) was purchased from the Sigma Chemical Co. Z-Phe-Leu-Ala-Ala and angiotensin I were kindly donated by Dr. K. Morihara of the Shionogi Research Laboratory, Shionogi & Co., Ltd. and Dr. K. Tomoda of the Central Research Division, Takeda Chemical Industries Ltd., respectively.

3) Inhibitors. Crystalline S-PI (acetyl-valyl-valyl-4-amino-3-hydroxy-6-methylheptanoyl-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid) was prepared by the method of Murao and Sato.

4) Other chemicals. Zinc sulfate and PAD were obtained from Nakarai Co., Ltd.

5) Zinc(II)-PAD experiment. All sample solutions were prepared in 0.05 M acetate buffer, pH 5.0. Absorption spectra at visible wavelengths were measured with a Hitachi 320 spectrophotometer at 30°C.

6) Chemical modification with EPNP. Chemical modification of acid proteinase B with EPNP and the EPNP contents of EPNP-acid proteinase B were calculated by the method of Tang.

7) Assay for proteinase activity. Proteinase activity of acid proteinase B was assayed at pH 2.0 with casein as the substrate.

RESULTS

1) Screening for substances that release zinc(II)-PAD from the zinc(II)-PAD-enzyme complex
As shown in Fig. 1, the zinc(II) complex of pyridine-2-azo-p-dimethyl aniline (PAD) is bound to such S-PI (Pepstatin Ac)-sensitive acid proteinases as pepsin and other microbial acid proteinases, at pH 5.0, accompanied by an increase of its absorption at about 530 nm. Zinc(II)-PAD bound to acid proteinase is released from the enzyme by the addition of a competitive inhibitor, S-PI (Pepstatin Ac). We speculated that zinc(II)-PAD is bound to two catalytic carboxylate groups in the active site of acid proteinases and the inhibitor is bound mainly to the substrate-binding site of the enzyme. In contrast, acid proteinases A-1,
A-2, and B isolated from *Scytalidium lignicolum* ATCC 24568 bound to the zinc(II)–PAD and formed zinc(II)–PAD–enzyme complexes, as did pepsin and microbial acid proteinases. The zinc(II)–PAD bound to the enzymes, however, was not released at all by the addition of S-PI [the result is quite reasonable since these acid proteinases are not inhibited at all by S-PI]. Accordingly, it is suggested that *Scytalidium* acid proteinases have two carboxyl groups very close to each other. We were unable to find whether they are involved in the action of the enzyme, although such is the case in S-PI-sensitive acid proteinases. To answer this question, we attempted to screen for substances that release zinc(II)–PAD from the complex of zinc(II)–PAD–S-PI-insensitive acid proteinases. As shown in

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Release of zinc(II)–PAD</th>
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<tr>
<td></td>
<td>Pepsin</td>
</tr>
<tr>
<td>S-PI (Ac-Val-Val-AHMHA-Ala-AHMHA)</td>
<td>+</td>
</tr>
<tr>
<td>Peptatin A (Isovaleryl-Val-Val-AHMHA-Ala-AHMHA)</td>
<td>+</td>
</tr>
<tr>
<td>Val-AHMHA-Ala-AHMHA</td>
<td>+</td>
</tr>
<tr>
<td>Benzoil-Val-AHMHA-Ala-AHMHA</td>
<td>+</td>
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<tr>
<td>Phenoxycetlyl-Val-AHMHA-Ala-AHMHA</td>
<td>+</td>
</tr>
<tr>
<td>2-Phenoxypropionyl-Val-AHMHA-Ala-AHMHA</td>
<td>+</td>
</tr>
<tr>
<td>Ac-Phe-Tyr (I₂)</td>
<td>+</td>
</tr>
<tr>
<td>Z-Phe-Leu-Ala-Ala</td>
<td>+</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>+</td>
</tr>
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</table>

AHMHA, 4-amino-3-hydroxy-6-methylheptanoic acid; +, Zinc(II)–PAD bound to enzyme was released; −: Zinc(II)–PAD bound to enzyme was not released.

**Fig 2.** Absorption Spectra of Zinc(II)–PAD–Acid Proteinase–Substrate (Angiotensin I) System. The meanings of a, b, and c are the same as those in Fig. 1, and d is the absorption spectrum of Zinc(II)–PAD–enzyme–Angiotensin I [S/E=5 (m/m)].
Table I, Pepstatin A, Pepstatin derivatives, synthetic substances, angiotensin I, etc. were examined. Among them, angiotensin I [Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu] alone released zinc(II)-PAD from the complex. Angiotensin I was effective also on S-PI-sensitive acid proteinases. Figures 2 and 3 show the spectral change of such S-PI-sensitive acid proteinases as pepsin, the acid proteinase from Cladosporium sp., Cladosporium macrosporus acid proteinase Type B, Aspergillus niger var. macrosporus acid proteinase Type B, Scytalidium lignicolom acid proteinase A-2, and Scytalidium lignicolom acid proteinase B from S. lignicolom. Angiotensin I is a good substrate for these enzymes regardless of the S-PI-sensitivity. From these results, the two carboxyl groups capable of binding to zinc(II)-PAD were considered to be located at the active site regions of S. lignicolom enzymes and involved in their catalytic actions.

2) Binding of zinc(II)-PAD to acid proteinase B modified with EPNP

As reported previously, acid proteinase B is modified with EPNP, and one mole of EPNP per molecule is incorporated with concomitant loss of its activity. This result indicates that acid proteinase B has at least one active carboxyl residue in its active site. With EPNP-modified enzymes, we searched for the active site of acid proteinase B. As shown in Fig. 4, EPNP-modified enzymes lost their binding ability in accordance with the incorporation of EPNP. These results also demonstrate that acid proteinase B has two carboxyl groups very close to each other and one of them can be modified by EPNP.

**DISCUSSION**

Most acid proteinases are inactivated upon esterification of carboxyl groups by DAN and EPNP, but the acid proteinases A-1, A-2, B, and C from S. lignicolom ATCC 24568 are not inactivated by these chemicals, except for the sensitivity of B to EPNP. In addition, these enzymes are not inactivated by such
microbial carboxyl proteinase inhibitors as S-PI (Pepstatin Ac), Pepstatin, and their derivatives.\textsuperscript{2,5,12} These results with \textit{S. lignicolum} acid proteinases suggest that no carboxyl residue participates in their actions. To confirm this, first of all, we examined the interaction between the acid proteinases and zinc(II)–PAD, which is a catalytic site probe for acid proteinases.\textsuperscript{14} Acid proteinases A-2 and B bound to zinc(II)–PAD, forming a zinc(II)–PAD–enzyme complex, as did pepsin. For S-PI-sensitive acid proteinases, we speculated that zinc(II)–PAD would bind to the two catalytic carboxyl groups in the active site, for zinc(II)–PAD binding to the enzyme was replaced by a competitive inhibitor, S-PI. On the other hand, with \textit{S. lignicolum} acid proteinases, unlike pepsin, the absorption characteristic for the zinc(II)–PAD–enzyme complex did not change by the addition of S-PI; the initial absorption spectrum of zinc(II)–PAD binding the \textit{Scytalidium} enzymes was not released by the addition of S-PI. It can, therefore, be said that acid proteinases A-1, A-2, and B each have two carboxyl groups very close to each other (about 3Å), like the active carboxyl groups of S-PI-sensitive acid proteinase. It is not clear whether the carboxyl groups are involved in their catalytic action.

This study was done to confirm this. We attempted to screen for substances that release zinc(II)–PAD from the complex of zinc(II)–PAD–S-PI-insensitive acid proteinases. Among various substances, angiotensin I [Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu] alone released zinc(II)–PAD from the complex (Table I). This substance was effective also on S-PI-sensitive ones (Table II and Fig. 2). Angiotensin I is a good substrate for these enzymes regardless of their pepstatin sensitivity.\textsuperscript{25}

The release of zinc(II)–PAD from the complex by the addition of angiotensin I was not in a stoichiometric manner, in contrast that observed by the addition of S-PI to zinc(II)–PAD–pepsin complex.\textsuperscript{14} An excess of angiotensin I was required to release zinc(II)–PAD from the complex; the rate of the release differed from enzyme to enzyme (Fig. 3).

By the addition of 2 molar excess of angiotensin I, about 35% of zinc(II)–PAD bound to \textit{S. lignicolum} acid proteinase A-2 was released. Additional angiotensin I gave no further effects without changing \(\Delta c/\Delta c_0\). Since angiotensin I is a good substrate, and hydrolysed by the enzyme during the experiments, its actual

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
\textbf{Acid proteinase} & \textbf{Molecular weight} & \textbf{Inhibition by} & \textbf{Zinc(II)–PAD binding} & \textbf{Release of zinc(II)–PAD by S-PI} & \textbf{Release of zinc(II)–PAD by angiotensin I} \\
& & S-PI & DAN & EPNP & & \\
\hline
\textit{Pepsin} & 34,500 & + & + & + & + & + \\
\textit{Rhizopus chinensis} & 35,000 & + & + & + & + & + \\
\textit{Tremetes sanguinea} & 34,000 & + & + & + & + & + \\
\textit{Aspergillus niger Type B} & 34,000 & + & + & + & + & + \\
\textit{Rhodotorula glutinis} & 30,000 & + & + & + & + & + \\
\textit{Cladosporium sp.} & 32,000 & + & + & + & + & + \\
\textit{Scytalidium lignicolum} & 40,000 & - & - & - & + & + \\
\textbf{A-1} & & & & & & \\
\textit{Scytalidium lignicolum} & 40,000 & - & - & - & + & + \\
\textbf{A-2} & & & & & & \\
\textit{Scytalidium lignicolum} & 21,000 & - & - & + & + & + \\
\textbf{B} & & & & & & \\
\textit{Scytalidium lignicolum} & 360,000 & - & - & - & n.d. & n.d. \\
\textbf{C} & & & & & & \\
\textit{Aspergillus niger Type A} & 19,000 & - & + & + & + & + \\
\hline
\end{tabular}
\caption{Summary of the Properties of Acid Proteinases from Various Sources toward Several Reagents}
\end{table}

Positive and negative effects are shown by the symbols + and −, respectively.

concentration in the assay mixture must have been below the initial one, or Angiotensin I (or its hydrolysates) may have been bound to the zinc(II)–PAD–enzyme complex, forming a quarternary zinc(II)–PAD–enzyme–angiotensin I (or its hydrolysates) complex.

In contrast, 75% of zinc(II)–PAD bound to S. lignicolum acid proteinase B was released by addition of a 5 molar excess of angiotensin I.

Table II lists our results together with some other characteristics of acid proteinases.

The two carboxyl groups capable of binding to zinc(II)–PAD were considered to be at the active site regions of S. lignicolum enzymes and involved in their catalytic actions. This was demonstrated clearly with acid proteinases B partially modified with EPNP (Fig. 4).

Thus, we concluded that Scytailidium enzymes have two active carboxyl residues participating in the action, as do S-PI-sensitive acid proteinases (Fig. 5). This conclusion is supported also by the results of the kinetic study reported previously.13) It remains unclear whether the catalytic residues are of aspartic or glutamic acid.

REFERENCES

S-PI-Insensitive Acid Proteinases from *S. lignicolum*