α-Linolenate Releasing Enzyme, Phospholipase A₂ of Rice Blade Infected by Blast Fungus*, **

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Key words: phospholipase A₂, α-linolenate releasing, rice blast.

As described in earlier reports1-3), the releasing of α-linolenic acid and formation of 13-hydroxy-9Z, 11E, 15Z-octadecatrienoic and 9-hydroxy-10E, 12Z, 15Z-octadecatrienoic acid in rice blades infected with blast fungus has been confirmed. The chemical structure of these compounds strongly suggested the participation of lipoxygenase (LOX)4) and of a subsequent reduction of the hydroperoxides in the formation of these unsaturated hydroxy C₁₈ fatty acids. The unsaturated hydroxy C₁₈ fatty acids exhibited antibacterial and antifungal activities1). Li et al.5) recently reported that a needle application of these unsaturated hydroxy C₁₈ fatty acids onto rice blades induced the formation of rice blade phytoalexins including momilactones6). The lines of evidence bore out the suggestion that these unsaturated hydroxy C₁₈ fatty acids participated in both antimicrobial and antcellular activity of infected cells and a signalling from the infected cell to surrounding cells to induce rice leaf phytoalexins. In this paper, it is described that the type of a phospholipase (PL) activated in situ linking with a transmembrane signalling in infected rice blade cells7) was surmised from lines of analytical evidence.

Rice cultivar (Jukkoku, resistant genotype assumed: P-ia) and races of blast fungus (incompatible: race 031, TH 68-85 and compatible: race 007, Hoku 373) were used as previous reports4). Both blast fungus conidia were separately inoculated to rice seedlings of 4th leaf stage by spraying, and inoculated seedlings were maintained at 25°C in a phytotron after overnight treatment in an inoculation chamber4). As it is a general rule of lipid chemistry that most part of saturated fatty acids are bound at sn 1 position, the action site of PL A₁, and most part of unsaturated fatty acids are bound at Sn 2 position, the action site of PL A₂, in plasma membrane phospholipids. The time-course surveys on contents of either saturated or unsaturated fatty acids in the infected rice blades were performed to determine the type of phospholipase activated in situ at the early phase of the infection. From the total lipid fraction8), free fatty acid fraction was obtained by Bond Elut-NH₂ microcolumn (Analytichem International Inc., Harbor, CA.)

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using 2% acetic-diethylether as an eluant after washing with chloroform-2-propanol (2:1). The methylation was performed by 0.2 M phenyl trimethyl ammonium hydroxide/methanol (Gausu-kuro K.K., Tokyo). The quantitative determination of fatty acids was done by a capillary gas chromatography (HP 5890 A, Hewlett-Packard) conjuncted with a integrator (HP 3392A, Hewlett-Packard) using a fused silica column DB-225 (30 m × 0.25 mm. I.D., 0.25 μm film, J.& W.). Carrier gas: helium (30 cm/sec). Injection port temp.: 230 C. Column temp.: 140 C for 1 min—rate=8° min—170 C for 2 min—rate=2°/min—200 C. FID: 170 C (air, 40 cm/ sec; H2, 30 cm/sec). The preparation of crude phospholipase A2 source from either infected or healthy leaves was done homogenizing 0.4 g of infected or healthy 4th blades in 10 ml of 0.5 M Tris-HCl buffer (pH 8.0) under ice cooling and centrifuged at 15,000×g for 40 min (Kokusano H-251 with roter B). The resultant supernatant was used for the enzyme source. For the substrate, 1-palmitoyl-2-linolenoyl-Sn-glycero-3-phosphocholine (Sigma Chemical Co., St. Louis, MO.) was used. The reaction mixture contained 2.5 ml of the crude enzyme source extracted from rice blades and 2.5 ml of 0.05 M Tris-HCl buffer (pH 8.0) and the hydrolytic reaction was performed at 25 C for 15 min under shaking. The substrate concentration was 10 mM with or without 10 mM CaCl2. The hydrolysis was stopped at pH 2.0 by the addition of 1 N HCl and fatty acids released were extracted with n-hexane. Free fatty acids in healthy leaves were subtracted as the blank. For the substrate of Naja naja venom partially purified phospholipase A2 (Sigma Chemical Co., St. Louis, MO.), 0.4 g of rice blades was immersed in boiling water for 3 min, and homogenized in 2 ml of 0.05 M Tris-HCl buffer (pH 8.0). The reaction mixture contained 2 ml of rice blade homogenate and 10 mM Ca2+ in total volume of 4.4 ml with 0.05 M Tris-HCl buffer (pH 8.0). The enzymic reaction was started at 25 C by the addition of 5 units of phospholipase A2 in 0.1 ml of the same buffer.

The comparative time-course survey on the releasing of α-linolenic, linoleic and palmitic acid from rice blades infected with the incompatible or compatible races was shown in Fig. 1. The first maximum accumulation of α-linolenic acid was found at 9 hr after the spray inoculation of incompatible race, whereas that of α-linolenic acid was found at 18 hr after the spray inoculation of compatible race. The earlier releasing of α-linolenic acid was generally observed by the incompatible combination as in other resistant reactions such as O2 generation and the sequential induction of enzymes participated4). The accumulation of linoleic acid was hardly observed by the cultivar and race combinations used in this study. The releasing of α-linolenic acid was thus confirmed as one of earlier phenomena suggesting that phospholipase A2 was activated by an intrinsic function of the plasma membrane as in the releasing of arachidonic acid from anti-IgE stimulated mast cells, a mammalian cell9). Other fatty acids including α-linolenic and palmitic acid released from rice blades by the infection with the incompatible or compatible races and by the addition of Naja naja venom phospholipase A2 to the homogenate of heat treated rice blades were summarized in Table 1. It was noted that per cent of α-linolenic acid in total fatty acids released was similar to those of incompatible, compatible combination and the hydrolysis by Naja naja venom phospholipase A2. Whereas per cent of palmitic acid in total fatty acid released was different in the each experimental run reflecting that the time of maximum accumulations of palmitic acid after the inoculation was different as at 13 hr by the case of compatible combination comparing with 18 hr of the corresponding case of linolenic acid releasing (Fig. 1). The difference of time at maximum accumulations of palmitic acid bores out the suggestion that phospholipase A2 was also independently activated beside the activation of type A2. When the synthetic substrate, 1-palmitoyl-2-linolenoyl-Sn-glycero-3-phosphocholine, was hydrolyzed by crude phospholipase A2 source extracted from the infected rice blades, the releasing of linolenic acid was merely determined in the presence of Ca2+ as shown in Table 2, whereas the dependence on Ca2+ of the releasing of palmitic acid was relatively lower.

Fig. 1. Comparative time-course survey on the releasing of $\alpha$-linolenic, linoleic and palmitic acid in rice blades infected by incompatible or compatible blast fungus race. Ordinate: net fatty acid contents in rice blades ($\mu$mol/g fresh weight). Abscissa: time after inoculation (hr). A: incompatible combination. B: compatible combination. ●: $\alpha$-linolenic, ○: linoleic, ▲: palmitic acid.

Table 1. Per cent fatty acid in total fatty acids released in rice blades infected by incompatible or compatible blast fungus and in heat treated rice blade homogenate hydrolysed by *Naja naja* venom phospholipase A$_2$

<table>
<thead>
<tr>
<th>Species of fatty acids</th>
<th>Incompatible$^{a)}$</th>
<th>Compatible$^{b)}$</th>
<th><em>Naja naja</em> PL A$_2$$^{c)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$mol/g fr. wt.</td>
<td>%</td>
<td>$\mu$mol/g fr. wt.</td>
</tr>
<tr>
<td>Palmitic</td>
<td>2.3</td>
<td>13.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Stearic</td>
<td>6.1</td>
<td>35.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Oleic</td>
<td>0</td>
<td>0</td>
<td>4.1</td>
</tr>
<tr>
<td>Linoleic</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$\alpha$-Linolenic</td>
<td>8.9</td>
<td>51.5</td>
<td>8.8</td>
</tr>
</tbody>
</table>

$^{a)}$ Incompatible combination, per cent at 9 hr after the spray inoculation.

$^{b)}$ Compatible combination, per cent at 18 hr after the spray inoculation.

$^{c)}$ *Naja naja* venom phospholipase A$_2$, per cent at 4 hr incubation of the reaction mixture at 25 C in the presence of 10 mM Ca$^{2+}$ (see text).
Table 2. Palmitic and linolenic acid released from the synthetic substrate, 1-palmitoyl-2-linolenoyl-
        sn-glycero-3-phosphocholine, by crude enzyme source extracted from rice blades infected
        with blast fungus

<table>
<thead>
<tr>
<th>Rice blade ( \text{a)} )</th>
<th>Molecular species of fatty acids released</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palmitic acid ( + \text{Ca}^{2+} )</td>
</tr>
<tr>
<td></td>
<td>( - \text{Ca}^{2+} )</td>
</tr>
<tr>
<td>Diseased</td>
<td>0.38</td>
</tr>
<tr>
<td>Healthy</td>
<td>0.27</td>
</tr>
</tbody>
</table>

\( \text{a)} \) The sample of rice blades inoculated by spray method with the compatible race was taken at 26 hr
        after the inoculation for palmitic acid releasing and at 40 hr after the inoculation for linolenic acid
        releasing as the releasing of both fatty acids became higher than the earlier time along an intermittent
        wise.

The lines of analytical evidence in this study totally bore out the suggestion that a type of phospholipase
        responsible to releasing of \( \alpha \)-linolenic acid in rice blades infected by blast fungus was
        phospholipase A\(_2\).

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和文摘要

関沢泰治・芳賀 実・加納大聖：イネいもち病菌に感染したイネ葉身の \( \alpha \)-リノレン酸放出酵素—ホスホ
        リパーゼ \( \text{A}_2 \).

感染葉身細胞の原形質膜リン脂質から \( \alpha \)-リノレン酸を切り出す酵素は、膜情報伝達系の作動によって活
        性化されるホスホリパーゼの一つの型であるので、本報では \( \text{in situ} \) で活性化されるこの酵素の型の同定を
        試みた。噴霧接種菌の葉身では \( \alpha \)-リノレン酸の蓄積は、非親和性細胞で接種後 9 時間、親和性細胞で
        接種後 18 時間で観察され、後者では接種後 13 時間にパルミチン酸の蓄積が認められた。総放出脂肪酸中の
        \( \alpha \)-リノレン酸および同時間でのパルミチン酸の百分率を合わせ考え、不飽和脂肪酸の大半は \( \text{Sn} 2 \) 位に結合す
        るとのリン脂質化学的一般則に照し、\( \alpha \)-リノレン酸を切り出すこの酵素は \( \text{A}_2 \) 型と推定された。このホスホ
        リパーゼ \( \text{A}_2 \) は 10 mM \( \text{Ca}^{2+} \) の存在で感染葉身でののみ、その酵素活性が認められた。

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