Inhibition of Photosystem II of Spinach by Lichen-derived Depsides

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Received June 8, 1998

Barbatic acid, a lichen-derived depside, inhibited oxygen evolution in spinach thylakoid membranes. It also affected parameters of Chl fluorescence, (Fm’-F)/F and Fv/Fm. Using specific donors and acceptors of electrons, we found two sites of inhibition in the PS II complex. The primary site, which is responsible for the inhibition of oxygen evolution, is at the reducing side of QA, possibly at Qb. The other site is at the oxidizing side of P680 but not in the oxygen evolving complex, suggesting Yz as the target. At both sites, irreversible binding of the depside to the targets seems to be responsible for the inhibitions. Among the 8 lichen acids compared, barbatic acid was the most potent inhibitor for both the reducing site and oxidizing site.

Key words: barbatic acid; depside; lichen; photosystem II; spinach

Lichens have been used as folk medicines and poisons. Secondary metabolites, such as depsides and dibenzofurans have been reported to be responsible for some of these biological activities in lichens. Depsides and dibenzofurans peculiar to lichens, so called lichen acids, are synthesized via the acetyl-palmitonolyl pathway, and composed of two or three orcinol or β-orcinol-type phenolic units by ester, ether, and C-C bonds. Nishitoba et al. isolated several plant growth inhibitors from Usnea longissima. The sample contained a large amount of usnic acid, a dibenzofuran, although its growth inhibitory activity against lettuce seedlings was very low even at high concentrations. Eight active compounds identified were all depside derivatives. From these experiments, an allelopathic function of the depsides was suggested, but mechanism of the inhibition was not clarified.

In this study, to identify the sites of inhibition by lichen-derived depsides in photosynthesis of higher plants, effects of barbatic acid (BA), one of the most prevalent lichen depsides, on chlorophyll (Chl) fluorescence and oxygen evolution in the thylakoid membranes of spinach were examined.

Materials and Methods

Preparation of thylakoid membranes. Spinach leaves were obtained from local markets. Intact chloroplasts were prepared from mature leaves. They were osmotically ruptured in buffer A containing 2 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 30 mM KCl, 0.25 mM KH₂PO₄, and 50 mM HEPES at pH 7.6. The thylakoids were sedimented with a centrifugation (10,000 × g, 10 min) and resuspended in the buffer A with 300 mM sorbitol.

PS II membranes and Tris treatment. PS II membranes were prepared from the thylakoid membranes with Triton X-100 treatment. The PS II membranes (1 mg Chl ml⁻¹) suspended in buffer B (10 mM CaCl₂, 0.4 mM sucrose, 50 mM 2-morpholinolclohexane-sulfonate-NaOH, pH 6.5) were incubated in 0.8 μl Tris-HCl, pH 9.0, on ice for 30 min. Pellet obtained after a centrifugation (1,000 × g, 10 min) was resuspended in buffer B.

Measurements. Chl fluorescence was measured with a PAM-2000 fluorometer (Walz, Effeltrich, Germany). Thylakoid suspension (5 μg Chl ml⁻¹) put in a photometer cell was gently stirred during measurements. A fiberoptics connected with the fluorometer was attached to the side of the cell. The nomenclature of van Kooten and Snel was used for parameters of Chl fluorescence. For reviews on theoretical basis of quenching analysis of Chl fluorescence, see references 14 and 15. Oxygen evolution was measured with a Clake-type oxygen electrode (Hansatech, Kings Lynn, England) under a saturating white light (1,000 μE m⁻² s⁻¹).

Chemicals. Usnic acid obtained from Wakojunyaku Co., Ltd. (Tokyo, Japan) was recrystallized before use. Barbatic acid from Cladia aggregata, evernic acid and diffractatic acid from Usnea longissima, and sphearofohn from Sphaerophorus melanocarpus were isolated as described in reference 7. Atranorin from Hypogymnia physodes and nephroactin from Nephroma arcticum were provided by Prof. Takahashi, Meiji College of
Pharmacy, Japan. Methyl gyprophore from *Solorina crocea* was provided by Prof. Yamazaki, Chiba University, Japan.

**Results and Discussion**

**Inhibition of photosynthetic electron transport by barbatic acid**

Effects of barbatic acid (BA) on the photosynthetic electron transport from H₂O to ferricyanide was examined using Chl fluorescence as a probe (Fig. 1). When a low concentration (10 μM) of barbatic acid was added to a suspension of the thylakoid membranes isolated from spinach, the level of steady state Chl fluorescence (F) under illumination increased. Because of this increase in F, the parameter (Fm'–F)/Fm', which represents the photochemical quantum yield of PS II, decreased. When a high concentration of BA was present in the suspension of the thylakoids, Fm in the dark (and consequently Fv/Fm also), was drastically decreased. This type of change is often observed when electron transport at the oxidizing side of P680 is inhibited.

**Inhibition curves**

Inhibitions of (Fm'–F)/Fm', Fv/Fm and oxygen evolution by varying concentrations of BA are shown in Fig. 2. Half-inhibition concentrations (IC₅₀) for (Fm'–F)/Fm' and oxygen evolution are 2.2 μM and 1.2 μM, respectively. The two inhibition curves are in the same range. By contrast, inhibition of Fv/Fm in the dark requires much higher concentration of BA (IC₅₀, 25 μM). Therefore, inhibition at the reducing side of P680, which causes an increase in F, is the most sensitive site to BA, and limits the rate of electron transport.

**Inhibition at reducing side of P680**

To examine whether the inhibition at the reducing side of P680 caused by a low concentration of BA is within the PS II complex, dimethylbenzquinone (DMBOQ), which accept electrons from the secondary quinone acceptor in PS II (Qₐ), was added to the thylakoids as an electron acceptor instead of ferricyanide (Fig. 3). A rapid increase of Chl fluorescence was observed upon the addition of 10 μM BA, showing that the inhibition site was within the PS II complex.

![Fig. 1. Effects of Barbatic Acid (BA) on Induction of Chl Fluorescence in the Thylakoid Suspensions.](image1)

Reaction mixture contained thylakoid membranes (10 μg Chl), 1 μM nigericin, 0.5 mM ferricyanide in 2 m buffer A. AL, actinic light (650 nm, 100 μE m⁻² s⁻¹). At the downward arrow, a pulse of saturating white light (2000 μE m⁻² s⁻¹) was applied to obtain Fm (Fm').

![Fig. 2. Inhibition by Barbatic Acid (BA) of the Quantum Yields Estimated from Chl Fluorescence and Oxygen Evolution.](image2)

Reaction mixture was the same as in Fig. 1. For Chl fluorescence, the same procedure as in Fig. 1 was employed. For oxygen evolution, a sample was illuminated with saturating white light. Values are expressed relative to the control (no addition of BA); 100% (Fm'–F)/F = 0.430, 100% (Fv/Fm) = 0.625, 100% oxygen evolution = 620 μmol O₂ (mg Chl)⁻¹ h⁻¹.

![Fig. 3. Effects of Barbatic Acid (BA, 10 μM) on Induction of Chl Fluorescence in PS II Complex.](image3)

The experimental setup was the same as in Fig. 1 except that 0.5 mm DMBQ instead of ferricyanide was used as electron acceptor. AL, actinic light (650 nm, 100 μE m⁻² s⁻¹). At the downward arrow, a pulse of saturating white light (2000 μE m⁻² s⁻¹) was applied to obtain Fm (Fm').
Table 1. Siliconolybdate-dependent Electron Flow by Spinach Thylakoids in the Presence of 10 µM Barbitric Acid (BA) or 0.5 µM DCMU.

<table>
<thead>
<tr>
<th></th>
<th>-Siliconolybdate (A)</th>
<th>+Siliconolybdate (B)</th>
<th>(B)-(A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±10.5(^{a})</td>
<td>n.d.(^{b})</td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>7.7±2.19</td>
<td>33±3.8</td>
<td>25</td>
</tr>
<tr>
<td>DCMU</td>
<td>5.1±1.68</td>
<td>43±2.3</td>
<td>38</td>
</tr>
</tbody>
</table>

\(^{a}\) Values are the averages ± standard deviations of 3 measurements, expressed relative to the control value; 100%(c)=450 µmol O₂ (mg Chl)\(^{-1}\) h\(^{-1}\).

\(^{b}\) n.d., not determined.

To examine whether the inhibition at the reducing side is up or downstream of QA, siliconolybdate, which accepts electrons only from QA,\(^{19}\) was used for the measurements of oxygen evolution (Table 1). Electrons from QA are catalytically transferred to ferricyanide through siliconolybdate.\(^{19}\) As a reference, a typical inhibitor at QB, DCMU is used. By 10 µM BA, oxygen evolution associated with electron transport from H\(_2\)O to ferricyanide was mostly but not completely inhibited. A similar level of leakage through QB was found when 0.5 µM DCMU was present. In the presence of 10 µM BA, considerable level of the siliconolybdate-dependent electron flow (represented by the value in the right column of Table 1) was observed, suggesting that the main inhibition site is downstream of QA. However, the siliconolybdate-dependent flow in the presence of DCMU is greater than that in the presence of BA, showing minor inhibition before or at QA. The QB site is known to be the primary target site of many herbicides that act by inhibiting photosynthesis. A hydrophobic area and a single electron pair from nitrogen bound with an electron-deficient sp\(_3\) atom have been reported to be essential structural elements of herbicides acting on QB such as triazines, substituted ureas, triazinones, and uracils.\(^{20}\) However, barbitric acid does not have these structural elements, suggesting that the mode of inhibition of barbitric acid is different from that of the known herbicides.

Inhibition at oxidizing side of P680

To examine if the inhibition site that affects Fv/Fm in the dark is within oxygen evolution complex or in D1 protein, effects of DPC, an electron donor to the tyrosine-160 (Yz) residue in D1, in Tris-treated PS II membranes was tested (Fig. 4). Yz mediates electron transport between the oxygen evolution complex and P680.\(^{21,22}\) In the absence of DPC a very low Fv/Fm was observed because an electron was donated to P680\(^{+}\) in the Tris-treated PS II that lacks an oxygen evolution complex.\(^{12}\) The value of Fv/Fm increased by the addition of the DPC, which donated electron to P680\(^{+}\) via Yz. However, further addition of 100 µM BA lowered Fv/Fm again, demonstrating that electron donation to P680\(^{+}\) was stopped. These results showed that BA-dependent inhibition is downstream of or at Yz. The two sites of inhibition estimated here are illustrated in Fig. 5 with electron flows induced by artificial electron mediators in PS II used in this study.

The inhibitions of reducing and oxidizing sides shown here were not restored even after the BA-treated thylakoids were centrifuged and resuspended in fresh buffer (data not shown). A dark incubation of thylakoids with BA and dark washing resulted in similar levels of inhibition as in the light (data not shown). These results suggest that the inhibition by BA is due to irreversible binding to the target sites and that this binding process does not require illumination.
Table 2. $I_{50}$ (μM) of (Fm·F/Fm') and Fv/Fm', Which Respectively Represent the Inhibitions of the Reducing Side and Oxidizing Side, by Various Lichen Acids in Spinach Thylakoids (5 μg Chl ml⁻¹). Suspend- ed in Buffer A Supplemented with 1 μM Nigericin, 0.5 mM Ferricyanide, and 0.3 M Sorbitol.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(Fm·F/Fm')</th>
<th>Fv/Fm'</th>
</tr>
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<tbody>
<tr>
<td>Barbatic acid (R₁ = H)</td>
<td>2.2</td>
<td>25</td>
</tr>
<tr>
<td>Diffractaic acid (R₂ = CH₃)</td>
<td>29</td>
<td>82</td>
</tr>
<tr>
<td>Evenic acid (R₂ = CH₃)</td>
<td>5.7</td>
<td>210</td>
</tr>
<tr>
<td>Sphaerophorin (R₂ = C₇H₁₅)</td>
<td>28</td>
<td>31</td>
</tr>
<tr>
<td>Atranorin</td>
<td>&gt;800</td>
<td>&gt;800</td>
</tr>
<tr>
<td>Nephroarctin</td>
<td>&gt;400</td>
<td>&gt;400</td>
</tr>
<tr>
<td>Methyl gyprophorurate</td>
<td>&gt;700</td>
<td>&gt;700</td>
</tr>
<tr>
<td>Usnic acid</td>
<td>28</td>
<td>&gt;60</td>
</tr>
</tbody>
</table>

Values are the averages of at least 3 measurements.
Intensity of actinic light, 100 μE m⁻² s⁻¹

Inhibition by other lichen acids

Inhibitory effects of barbatic acid and other related compounds isolated from various lichens were compared using Chl fluorescence as a probe (Table 2). That is, $I_{50}$ of (Fm·F/Fm') representing the inhibition of the reducing side of P680, and $I_{50}$ of Fv/Fm' representing the inhibition at oxidizing side, were estimated from inhibition curves as shown in Fig. 2. Two depsides, atranorin and nephroarctin, and a tridepside, methyl gyprophorurate, did not show any inhibitory activities. Among the measured compounds, barbatic acid was the most potent inhibitor for both reducing and oxidizing sides of P680. Evenic acid showed a strong inhibition at the reducing side, but weaker inhibition at the oxidizing side. By contrast, sphaerophorin showed moderate inhibition at the reducing side and stronger inhibition at oxidizing side. Thus each compound showed different ratio of $I_{50}$ at the reducing side and that at the oxidizing side, suggesting that the modes of inhibition at the two sites are different. A substitution of a single-H at the 2 position in S ring (R₁ in Table 2) of barbatic acid by -CH₃ yields diffractaic acid, and this substitution changed the inhibitory activity at the reducing side drastically. The different lengths of alkyl side chain in A ring in evenic acid and sphaerophorin (at R₂ site in Table 2) affected especially at the oxidizing side inhibition, showing that the hydrophobicity of A ring was a critical factor for the inhibition. Diffractaic acid has been reported to be a stronger inhibitor of the growth of lettuce seedlings than barbatic acid and evenic acid,⁴ but in this study, it showed weaker inhibitory activities at both inhibition sites. Usnic acid showed a moderate level of inhibition at the reducing side, although it has been reported not to be a potent inhibitor of the growth of lettuce seedlings.⁴ These discrepancies might be derived from differences in the permeability of the cell membranes and chloroplast envelope to each compound. Alternatively, there might be an unidentified inhibition site beside PS II, which cannot be found by this study with the thylakoid membranes. The third possibility is the different responses to lichen acids by different plant species. To evaluate allelopathic effects of the depsides, inhibition experiments at the cellular and plant levels is important. Fortunately, the non-destructive measurements of Chl fluorescence established here can be applied to cell suspensions and plant leaves. In vivo studies with various algae and plants are now in progress.

Acknowledgments

We thank Prof. Takahashi, Meiji College of Pharmacy and Prof. Yamazaki, Chiba University for providing lichen-derived compounds. We also thank Dr. J. Mano, Kyoto University, for fruitful discussion.

References

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