Isolation and Physiological Activity of Sclerin, a Metabolite of Sclerotinia Fungus

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Sclerin, a colorless crystalline, C_{13}H_{14}O_{4} compound melting at 123°C was at first isolated as a lipase formation stimulating constituent of Sclerotinia libertiana, from its own mycelial extract, and infrared spectrum showed the presence a hydroxyl group and a lactone ring. Sclerin was found to promote also the enzyme formation and growth of various plant seedlings such as those of castor bean-, mung bean-, and rice seedlings. In the growth of the sclerin-treated plant, promotion of root formation and increase of dry weight per unit shoot length were noticed, and the combined use of sclerin and gibberellin brought about a synergistic effect on the growth of rice seedlings. The relationship between sclerin and some other plant growth regulators in the enzyme formation of germinating seeds was also described.

INTRODUCTION

It was previously found that the intracellular lipase of Sclerotinia libertiana increases with the fat consumption of the mycelium, and in the experiment by washed mycelium, the mycelial extract of the fungus has a promoting effect on the lipase formation.\(^1\)\(^,\)\(^2\) The lipase formation stimulating factor in the mycelium was isolated and a pure active substance obtained. The name, sclerin (SCL) was proposed to this substance. Since an analogy was present in the mechanism of lipase formation between the fungus and germinating fatty seeds such as castor beans\(^3\) the effect of SCL was also investigated with castor beans. As a result, the lipase formation accompanied by the germination of castor beans was remarkably increased by SCL, and at the same time, with the seedlings, the growth promoting effect of SCL was found. Since SCL may have a hormone-like activity on plants, the growth and enzyme formation of the other plants, such as those of rice and mung beans which develope α-amylase during germination, was examined. Interaction of SCL, and gibberellin (GLB), auxin (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) or blasticidin S (BCS) on growth and enzyme formation of young plants was also studied in this paper.

EXPERIMENTAL AND RESULTS

Isolation of Sclerin

A strain of Sclerotinia libertiana was cultured on bran for nearly 2 weeks at 23–25°C, till sclerotium was sufficiently formed. The bran culture, after drying in air and pulverizing with a glinder, was immersed in about ten volumes of 50% alcohol, and the supernatant concentrated to a small volume under reduced pressure. During the concentration insoluble precipitate formed was removed by filtration and the concentrate extracted with ethylacetate. The extract, after removing ethyl-
acetate by evaporation in vacuo, was dissolved in ether. After ether solution had been washed with a small volume of water and mixed with a dilute (3%) ammonia water, the lower layer was separated. The separated ammoniac solution was combined and concentrated under reduced pressure. The concentrated solution, after removing oily impurities by filtration, was acidified with hydrochloric acid solution, and kept in a refrigerator over night. Then, the crude SCL was precipitated.

The precipitate, after drying in vacuo and dissolving in chloroform, was further purified by silicic acid chromatography. For about 50 mg of the precipitate, the column containing 7 g of silicic acid mixed with chloroform was used, and elution was carried out with chloroform. Since SCL gave a blue color with ferric chloride solution, the chromatograms were followed by the reaction with the use of a spectrophotometer. The ferric chloride test positive fraction was collected and after removing chloroform by evaporation, crystalline substance remained was recrystallized from methanol and water in a refrigerator. A colorless rectangular crystals, melting at 123°C was obtained, about 0.01%. The formula \( \text{C}_{13}\text{H}_{14}\text{O}_4 \) was assigned to SCL by elemental analysis and Rast molecular weight determination.

Anal. Found: C, 66.34; H, 6.00%. Calcd. for \( \text{C}_{13}\text{H}_{14}\text{O}_4 \): C, 66.66; H, 5.98%. Rast mole wt. 244 (±20) Infrared bands exhibited were \( \nu_{\text{max}} \) 3200, 1795, 1695, 1600, 1570 cm\(^{-1}\). The bands at 3200 and 1795 cm\(^{-1}\) indicated the presence of a hydroxyl group and a five membered lactone ring, respectively.

Further structural characteristics will be reported in another paper. A sodium bicarbonate solution of SCL was used in the following physiological experiments.

**Effect on the lipase formation of Sclerotinia fungus**

Shaking growing culture of the fungus in 100 ml of a medium (5% decoction of wheat bran containing 0.3% of CaCO\(_3\)) at 25°C for about 3 days was diluted by distilled water to 200 ml and divided into quarters in respective flasks. SCL was added in the medium of each flask varying in concentration (5 p.p.m.~20 p.p.m.), and the culture was continuously shaken at 25°C. Lipase activity was measured at intervals of 24 hr with 3 ml of the mycelial suspension containing about 30 mg of dry weight. Measurement of the lipase activity was as follows: 1 ml of olive oil, 2 ml of McIlvane buffer solution (pH 7.0), 3 ml of the enzyme preparation and 4 ml of \( \text{M}/20 \) CaCl\(_2\) were mixed and shaken at 37°C for 20 hr. Then, the contents were taken out and after addition of 30 ml of alcohol titrated by \( \text{N}/20 \) NaOH at about 55°C. The titration value subtracted by blank test with heat inactivated enzyme solution represents the lipase activity. As shown in Fig. 1. within 48 hr, the lipase forming activity of the fungus was steadily developed by SCL at a fairly lower concentration. Whereas, at a higher concentration of SCL, it was promptly developed at first, till 24th hr, but not extended.

![](image.png)

**FIG. 1.** Effect of SCL on the Lipase Formation of *Sclerotinia* Fungus.
afterwards and gradually fell down.

**Effect on the lipase formation of germinating castor beans**

Seeds were soaked in 50% alcohol for 1 min., sterilized with mercuric chloride solution for 10 min. and washed with water. They were then, after steeping in sterilized water for 4 hr., laid on absorbent cotton containing 20 ml of test solution (pH adjusted to 4.0) in Petri dish and illuminated at 1000 lux by fluorescent lamp in an incubator kept at 30°C. A chymous solution containing 100 mg of endosperms was prepared by glinding the hulled seeds with a small volume of water in a motor, and its lipase activity was measured as described above.

SCL was found to promote also the lipase formation of castor beans, at a concentration of the same level with the fungus, and, as presented in Fig. 2, the effect was constantly progressed during germination, till the 5th day. On the other hand, the promoting effect of GIB in the same concentration suddenly appeared on the 4th day, but it did not continue and the lipase activity sharply fell down before extending to the same level with the SCL-treated seeds. Also, the combination of SCL and GIB produced an antagonistic effect and rather a lower lipase activity than with GIB alone.

**Effect on the α-amylase formation of germinating rice seeds**

The variety of the rice seeds used were "Yamabuki" and "Tokaiasahi". Seeds were soaked in 75% alcohol for 5 min, sterilized with mercuric chloride solution for 15 min, and washed with water. They were then, after steeping in sterilized water for 2 hr, laid on absorbent cotton containing 20 ml of test solution (pH 4.0) in Petri dish and allowed to germinate under the same condition with castor beans. α-Amylase activity was measured as follows: Every 10 endosperms removed from the seeds were glinded with a small amount of sea-sand in a motor, and extracted with 5 ml of 0.5% CaCl₂ solution for 1 hr at 40°C. The supernatant was centrifuged, diluted by distilled water to an appropriate concentration and used as the enzyme solution. The activity was determined according to Blue Value method.⁵)

Within 6 or 7 days, the \( \alpha \)-amylase activity of the seedlings reached the maximum. The promoting effect of SCL (5 p.p.m.) on the \( \alpha \)-amylase formation was remarkable with either varieties of the rice seeds, Fig. 3. The effect of GIB was also recognized, but not so markedly in the same concentration, especially with "Yamabuki". The combined effect of SCL and GIB in the concentration of 4 p.p.m. and 1 p.p.m. was, to some extent, lower than that of SCL alone, but still higher than that of GIB alone. The optimum concentration of SCL on the \( \alpha \)-amylase formation ranged from 0.5 p.p.m. to 5.0 p.p.m. and the activity in this range was almost constant, as shown in Table I.

**Table I. Effect of Concentration of SCL on \( \alpha \)-Amylase Formation of Rice Seedlings ("Tokai-asahi")**

<table>
<thead>
<tr>
<th>SCL p.p.m.</th>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.5</th>
<th>1.0</th>
<th>5.0</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase Activity</td>
<td>63</td>
<td>84</td>
<td>110</td>
<td>119</td>
<td>120</td>
<td>119</td>
<td>105</td>
<td>97</td>
<td>69</td>
<td>55</td>
</tr>
</tbody>
</table>

(With 6 days old seedlings)

**Effect on the \( \alpha \)-amylase formation in ungerminated rice seeds**

The variety "Tokai-asahi" was used, and after sterilizing and steeping in water as described above, the embryo was removed from the seed by using the razor blade. The embryo free endosperms thus obtained were incubated at 30°C with 5 ml of test solution (pH 4.0) in Petri dish. The enzyme activity with the extract of the endosperms and the incubating medium was measured together in the same way as the above and summed up.

Contrary with the germinating seeds, \( \alpha \)-amylase activation in ungerminated seeds was remarkably stimulated by GIB, but only slightly by SCL as presented in Fig. 4.

**Relation to other plant growth-regulators in the \( \alpha \)-amylase formation of rice seedlings**

Seeds were taken out after incubation with each solution of SCL (5 p.p.m.), GIB (5 p.p.m.), and SCL (4 p.p.m.) plus GIB (1 p.p.m.) under the same condition as described above for 2 days, and treated by IAA, 2,4-D, and BeS for 24 hr in Petri dish. They were washed several times with water, and again incubated with newly prepared solutions of SCL-, GIB-, or SCL and GIB. Two controls, treated and
untreated by the reagents, incubating with water, were also performed.

Though the effects of the treatment by IAA (1 p.p.m.), 2,4-D (1 p.p.m.), and BcS (0.2 p.p.m.) were all inhibitive on the α-amylase formation, more or less reversed by SCL in any case. Also, with the IAA-treatment, GIB showed a tolerable reversing effect which appears earlier than by SCL, and SCL only by combination with GIB could exhibit its original promoting effect on the α-amylase formation, as shown in Fig. 5. With 2,4-D, such a characteristic additive effect between SCL and GIB did not occur, and the α-amylase activity was restored to only about the same level with the control, Fig. 6. On the other hand, with the BcS-treated seedlings, SCL almost perfectly restored the α-amylase activity to its original level, but GIB had not such effect, as presented in Fig. 7.

Growth responses of mung bean-, castor bean-, and rice seedlings

Effect of light. Mung bean seeds (Phaseolus aureus Roxb) was sterilized with a 3 per cent sodium hypochlorite solution for 10 min., soaked in sterilized water for 1 hr, washed with water and allowed to germinate under water at 30°C in darkness. Then, the uniform seedlings grown about 24 hr were selected and, respectively, planted in sea-sand containing each 10 ml of test solution (pH 4.0) in glass tube (3 x 20 cm) kept at 30°C with or without exposing to 1000 lux of the fluorescent light. Till the 7th day of the incubation, SCL (5 p.p.m.) similarly stimulated the growth of the seedlings in either light or darkness, whereas GIB (5 p.p.m.) did not, unless in light as shown in Fig. 8. Under illumination, there was not difference in the promoting effect between these, and in combination, a rater lower effect observed.

With castor beans, the uniform seedlings grown for 2 days were selected, and respectively, placed in a glass tube containing each 20 ml of test solution (pH 4.0), covering with a thin layer of glass wool for settling the seedlings at the bottom of the tube. Also, to the test solution, nutrients were dissolved in the following KNO₃, 0.85; NH₄H₂PO₄,
FIG. 8. Effect of Light on the Growth Response of Mung Bean Seedlings to SCL and GIB.

FIG. 9. Effect of Light on the Growth Response of Castor Bean Seedlings to SCL and GIB.
0.15; Ca(NO₃)₂·4H₂O, 0.47; MgSO₄·7H₂O, 0.72%. The tube was plugged with a cotton wool and incubated at 30°C for 7 days with or without illumination. In this case (SCL 2.5 p.p.m., GIB 2.5 p.p.m.), the result was the same with mung beans, as shown in Fig. 9. The effect of SCL had no relation to the light. However, effect of the nutrient in the medium was not so obvious as shown in the following experiment.

**Effect of nutrient.** The uniform 7 days old castor bean seedlings grown with water were, respectively, cultured on each 20 ml of the above test solution containing nutrients, under illumination at 30°C. Within 3 days of the cultivation, the shoot elongation of the seedling yielded a distinct difference on the growth promoting effect between SCL and GIB, as shown in Table II. With such the grown seedlings, the presence of the nutrient in medium greatly increased the sensitivity of plant to SCL, but not to GIB.

**TABLE II. EFFECT OF NUTRIENT ON GROWTH RESPONSE OF CASTOR BEAN SEEDLINGS TO SCL AND GIB.**

<table>
<thead>
<tr>
<th>Shoot Growth in cm for 10 days</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth in water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for 7 days</td>
<td>10.5</td>
<td>10.3</td>
<td>10.3</td>
</tr>
<tr>
<td>Control</td>
<td>11.7</td>
<td>12.5</td>
<td>15.3</td>
</tr>
<tr>
<td>with GIB</td>
<td>13.0</td>
<td>14.9</td>
<td>20.1</td>
</tr>
<tr>
<td>with SCL</td>
<td>14.2</td>
<td>16.8</td>
<td>24.3</td>
</tr>
</tbody>
</table>

**Synergism with GIB.** With mung beans and castor beans, no synergism was found between SCL and GIB. The rice seeds ("Tokai-asahi") was used in this experiment. Seeds were, after sterilizing and steeping in water, planted in sea-sand containing 40 ml of test solution (pH 4.0) in flask and allowed to germinate under the same condition as described above. The growth of the seedlings were measured on the 7th day.

As shown in Table III, the effect of SCL was specific in the promotion of root formation and the increase in dry weight per unit shoot length. The combination of SCL with GIB brought about a distinct synergistic effect on growth measured by either length and weight of whole, or each sections of the seedlings.

**DISCUSSION**

Various growth phenomena influenced by hormones may involve a change in the enzyme level of a special organ, and GIB is known to elevate the activity of certain enzymes in young plants. In the present work, it was shown that SCL promotes the enzyme formation of various seedlings, and the effect is much greater than that of GIB. Also, combination of SCL and GIB had an antagonistic relationship in the enzyme formation. While, with the rice seedlings previously treated by IAA, SCL needed the combined

**TABLE III. GROWTH RESPONSE OF RICE SEEDLINGS TO SCL AND GIB.**

<table>
<thead>
<tr>
<th>Seedlings grown for 7 days with</th>
<th>Shoot length in mm</th>
<th>Shoot weight</th>
<th>Root weight in mg</th>
<th>Whole weight in mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCL 5 p.p.m.</td>
<td>58</td>
<td>(4.1)</td>
<td>23.7</td>
<td>24.2</td>
</tr>
<tr>
<td>GIB 5 p.p.m.</td>
<td>102</td>
<td>(2.8)</td>
<td>28.4</td>
<td>6.0</td>
</tr>
<tr>
<td>SCL + GIB 4 p.p.m. 1 p.p.m.</td>
<td>125</td>
<td>(2.8)</td>
<td>34.4</td>
<td>14.4</td>
</tr>
<tr>
<td>no addition (control)</td>
<td>49</td>
<td>(4.3)</td>
<td>21.3</td>
<td>13.2</td>
</tr>
</tbody>
</table>

( ): Weight per 10 mm of length.

7) H. Yomo and H. Inuma, This Journal, 27, 76 (1963).
action with GIB for completely reversing the inhibited enzyme formation. On the other hand, the low enzyme forming activity of the seedlings pre-treated by BcS, could be reversed by SCL alone. There are several theories concerning IAA-GIB relationhip,8,9) and BcS is said to inhibit the protein synthesis in Piricularia oryzae10) and rice plant.11,12) The mechanism of action of SCL clearly differ from that of GIB and seems to have a close relation with the protein synthesis. With the α-amylase formation in embryo free endo-
sperms of the ungerminated rice seeds, the effect of GIB was very remarkable, but that of SCL slight. Accordingly, it is considered that embryonic control may be involved in the SCL action on the enzyme formation.

In the growth of the SCL-treated plant, shoot elongation and root formation were simultaneously stimulated, and increase in dry weight per unit shoot length was characteristic. Also, presence of the nutrient in medium significantly increased the sensitivity of plant to SCL. These responses of the plant seem to suggest that SCL may be a new plant growth-regulator specific for promoting the protein synthesis.

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