Effect of Partially Purified Components of Zoospores and Mycelia of *Phytophthora infestans* on Uptake of ^3H -Leucine by Potato Tuber Disks

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西村範夫*・富山宏平*・道家紀志*：ジャガイモ塊茎細胞の ^3H -ロイシン
吸収能を阻害する遊走子および菌糸成分について

Abstract

The zoosporial component of *Phytophthora infestans*, which was previously reported to cause reduction of ^3H -leucine uptake by potato tuber disks, was partially purified. Precipitate (A-fraction) was obtained by homogenizing zoospores with acetate buffer at pH 4.5 and centrifuging at $20,000 \times g$, and the A-fraction was suspended in borate buffer at pH 8.8, boiled for 1 hr and then centrifuged at $20,000 \times g$, giving the precipitate (B-fraction) and supernatant (C-fraction). Ten ml of 10 mM tris-HCl buffer containing 1 mM CaCl_2 at pH 7.4 was used to suspend A and B-fraction. The buffer was used as a control. A, B and C fractions obtained from $5-6 \times 10^6$ zoospores reduced uptake of ^3H -leucine by the tuber disks of potato cv. Rishiri, but the inhibition rates caused by these fractions differed markedly. However, very high correlation was found between inhibition rates of ^3H -leucine uptake and sugar contents of these fractions. There was no difference in the inhibition rates between the zoosporial components of incompatible and compatible races, when the activities were expressed in terms of the sugar contents. The mycelial components of *P. infestans* extracted by the modified method of Lisker and Kuć which was used to extract phytoalexin elicitor from that of *P. infestans*, also had the same effect as the zoosporial components (A, B, and C-fraction) on ^3H -leucine uptake by the disks. C-fraction containing 15 μg of sugar per ml sufficed to inhibit ^3H -leucine uptake at the maximum rate, and the maximum rate of inhibition was attained within 2 hr after the zoosporial component (C-fraction containing 30 μg sugar/ml) was administered to the disks.

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Introduction

It has been reported that changes in the permeability of the host cell membrane occur in the course of plant infection by a parasite^{3,5,10,11}. In previous experiments^{6,7} measuring ^3H -leucine uptake by potato tuber disks in an early stage of infection by *Phytophthora infestans*, it was presumed that the alteration in the physiological activity of the plasma membrane and/or the reduction of host metabolism is caused by inoculation with incompatible and compatible races of *P. infestans* before penetration of the host cell wall occurs. After penetration, the uptake of ^3H -leucine was more markedly inhibited by infection with the incompatible race of *P. infestans* than compatible one. Furthermore, it was reported that some substances in insoluble components of zoosporial homogenate of *P. infestans* might

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be at least one cause of the reduction in ^3H -leucine uptake.

In the present experiments, further purification of the factors in zoosporial components, which inhibit ^3H -leucine uptake by the disks, and characterization of the partially purified factors were performed.

Materials and Methods

Preparation of zoosporial and mycelial components. Zoosporial and mycelial components were extracted by a modified method of Lisker and Kuć⁴⁾. Zoospores ($5-6 \times 10^6$) were homogenized for 5 min with 20 mM acetate buffer at pH 4.5 using a teflon homogenizer, and centrifuged at $20,000 \times g$ for 30 min. The precipitate (A-fraction) was suspended in 50 mM borate buffer (pH 8.8), boiled for 1 hr, and centrifuged at $20,000 \times g$ for 30 min. The supernatant was dialyzed against ten liters of distilled water and finally against tris-HCl buffer (C-fraction) three times. The precipitate was resuspended in borate buffer and centrifuged giving washed precipitate (B-fraction). To each of these supernatant and precipitate fractions was added 10 mM tris-HCl buffer containing 1 mM CaCl_2 (final conc.) at pH 7.4 (final 10 ml). In the case of mycelial components, C-fraction was extracted with an equal volume of ether. The ether fraction was evaporated to syrup, and added with 80 % acetone (final conc.). The resultant precipitate was lyophilized. Sugar content was measured by the method of Dubois *et al.*⁵⁾. Concentrations of the fractions were expressed by sugar contents. As control, 10 mM tris-HCl buffer containing 1 mM CaCl_2 at pH 7.4 was used.

Treatment of potato tuber disks with zoosporial and mycelial components.

Aged tuber disks of potato (cv. Rishiri having R_1 -gene) were prepared as described in a previous paper⁷⁾. Five disks were immersed and shaken for 1 or 2 hr in 10 ml of each fraction of zoosporial homogenate in a beaker at 24 C. Disks were washed three times with 10 ml of distilled water for each 3 min and excess water was blotted off and used for treatment with ^3H -leucine. The tuber disks were immersed in 10 ml of solution containing L-leucine-4, 5- ^3H ($0.2 \mu\text{Ci/ml}$, specific activity 32 Ci/mmol) and shaken (120 strokes/min) for 10 min at 24 C. After the treatment the disks were washed three times, blotted off and then frozen at -20 C . The frozen disks were ground in a mortar and pestle for 2 min with 4 volumes of cold 50 mM phosphate buffer at pH 7.7 containing 8 mM 2-mercaptoethanol. The homogenate was centrifuged at $20,000 \times g$ for 20 min. Aliquots of the supernatant were added with 10 ml of scintillator and the radioactivity was measured using Tri-Carb Liquid Scintillation Spectrometer Model 3320 (Packard Inc.). Sample oxidizer (Packard Model 306 Tri-Carb.) was used for the experiments shown in Fig. 6. In a previous paper⁶⁾, it was suggested that total uptake of ^3H -leucine into $20,000 \times g$ supernatant can be taken as approximately the total amount of radioactivity absorbed by the tuber disks.

Results

Effect of various fractions of zoosporial components on ^3H -leucine uptake

Potato tuber disks were treated for 1 hr with A, B and C fractions of zoosporial components extracted from $5-6 \times 10^6$ zoospores and the buffer as control (tris buffer), and then uptake of radioactivity of ^3H -leucine into $20,000 \times g$ supernatant of the treated disks was

Fig. 1. Effect of isolated fractions of zoosporeal homogenates on ^3H -leucine uptake by potato tuber disks. The zoospores were homogenized with 10 mM acetate buffer at pH 4.5 and then centrifuged at $20,000 \times g$. The precipitates (A) were suspended in 10 mM borate buffer at pH 8.8, boiled for 1 hr and then centrifuged at $20,000 \times g$, giving supernatant (C) and precipitate (B). Isolated fractions to which 10 mM tris-HCl buffer at pH 7.4 (final conc.) was added were used for treatment. The buffer was used for a control treatment. Treatments were given at 24 C for 1 hr. a: race 0 and b: race 1.

Fig. 3. Effect of mycelial components of *P. infestans* isolated by the modified method of Lisker and Kuć on uptake of ^3H -leucine by potato tuber disks. Cont.; treated with 10 mM tris-HCl buffer at pH 7.4, r-0: mycelial component of race 0 and r-1: that of race 1.

Fig. 2. Relation between concentration of zoosporeal component (A, B, C and buffer fraction) expressed as sugar content and their inhibitory effect on ^3H -leucine uptake by potato tuber disks. ●: race 0 and x: race 1.

investigated (Fig. 1). The uptake rate was reduced in different degrees by the treatment with the fractions A, B and C as compared with that of control. A high correlation, however, was observed between the reduction rate of the radioactivity uptake and sugar contents of the fractions A, B and C regardless of the origins (race 0 or race 1) from which the fractions were extracted (Fig. 2). The correlation coefficient was $r=0.987$. Mycelial components (containing $30 \mu\text{g}$ sugar/ml), which were extracted according to the modified method of Lisker and Kuć used to extract phytoalexin elicitors, also had an effect similar to that caused by A, B and C fractions of zoosporeal component on ^3H -leucine uptake (Fig. 3).

Effect of zoosporeal components on time course of ^3H -leucine uptake by disks.

The aged tuber disks were treated for 2 hr with the C-fraction of zoosporeal component (containing $30 \mu\text{g}$ sugar/ml) or the buffer (control), and then the treated disks were immersed in ^3H -leucine solution. The time course of ^3H -leucine uptake was investigated

(Fig. 4), and results showed that the radioactivity absorbed by the disks increased rapidly until about 10 min after immersion in ^3H -leucine solution, but thereafter the uptake was decelerated and increased linearly with time. By comparing the linear increasing rate of the radioactivity absorbed by the disks treated with C-fraction and that of control disks, it was concluded that ^3H -leucine uptake by the cells in treated disks was reduced to about 33 % of the control.

Effect of zoosporial components at different concentrations on ^3H -leucine uptake

The aged disks were treated with different concentrations of C-fraction of zoospores for 2 hr at 24 C (Fig. 5). The reduction rate of ^3H -leucine caused by the treatment increased rapidly as the concentration (expressed as sugar content) of C-fraction increased up to 15 $\mu\text{g}/\text{ml}$, and thereafter it increased slowly up to about 50 $\mu\text{g}/\text{ml}$ and reached almost the maximum rate of reduction, that is, about 70 % inhibition.

Fig. 4. Time course of absorption of ^3H -leucine by potato tuber disks treated with zoosporial component (C-fraction) or 10mM tris-HCl buffer at pH 7.4 as a control. ●: cont., ○: race 0 and ×: race 1.

Fig. 5. Effect of concentration of zoosporial component (C-fraction) expressed as sugar content on uptake of ^3H -leucine by tuber disks. Treatment with C-fraction was performed at 24 C for 2 hr followed by treatment with ^3H -leucine solution for 10 min.

^3H -leucine uptake by the disks after treatment with zoosporial component for different length of time

The disks were treated with C-fraction of zoosporial component containing 30 $\mu\text{g}/\text{ml}$ of sugar. At intervals, the disks were removed from the treatment solution and treated with ^3H -leucine. Thirty min after beginning of treatment with zoosporial component, the rate of radioactivity uptake into disks had already been reduced. The reduction continued up to 2 hr after the zoosporial component treatment, and then reached a plateau (Fig. 6). The

Fig. 6. Relation between length of time during which the potato tuber disks were treated with zoosprial component (C-fraction, concentration expressed as sugar content was 30 μ g / ml) and inhibition rate of ^3H -leucine uptake by the disks. ●: cont., ○: race 0 and ×: race 1.

method of Lisker and Kuć⁴⁾ used to extract phytoalexin elicitor from *P. infestans* also inhibited the ^3H -leucine uptake. A very high correlation was found between sugar contents of the ^3H -leucine uptake inhibitors isolated by various methods and their inhibition rates in ranges of low sugar content. Albersheim¹⁾ and Kuć⁴⁾ presented evidence that a physiologically active site of so-called elicitors of phytoalexins extracted by the similar method to that used in the present experiments may be cell wall components of mycelia. These findings suggest that the ^3H -leucine uptake inhibitor of *P. infestans* contains cell wall component as an active site. The time course of ^3H -leucine uptake in the tuber disks treated with C-fraction of zoosprial component showed that the absorbed radioactivity increased sharply within 10 min after immersion in ^3H -leucine solution and thereafter increased slowly with time (Fig. 4). The sharp increase within 10 min is considered to be related to uptake into apparent free space, and the steady increase thereafter may be due to the uptake by cells of the radioactivity through the cell membrane⁹⁾.

The present experimental results showed that treatment of the disks with the isolated fraction containing 15 μ g sugar/ml for 2 hr was enough to reduce the ^3H -leucine uptake to the maximum rate. Death of cells in tuber disks treated with the elicitor of phytoalexin extracted from *P. infestans* was found to occur 12 hr after the treatment⁸⁾. It is noted, nevertheless, that the inhibition of ^3H -leucine uptake by the zoosprial component occurs very soon after the treatment.

These results suggested that the zoosprial non-dialyzable component containing sugar isolated in the present experiments may play some role in the reduction of ^3H -leucine and

surface cells of the disks were still alive at this stage.

Discussion

In a previous paper⁶⁾, it was reported that uptake of ^3H -leucine by potato tuber disks was reduced to the same extent by infection with both incompatible and compatible races of *P. infestans* before their penetration through the host cell wall. The same tendency was observed with the uptake of $\text{H}_3^{32}\text{PO}_4$. However, after penetration, uptake of ^3H -leucine and $\text{H}_3^{32}\text{PO}_4$ was more markedly reduced by infection with the incompatible race than with the compatible one. It was also reported⁷⁾ that an insoluble fraction of zoosprial component extracted with 10mM phosphate buffer at pH 7.0 or 20 mM acetate buffer at pH 4.5 contained factors inhibiting ^3H -leucine uptake by the disks.

The present experimental results showed that fractions which were isolated from zoospores and mycelia of *P. infestans* by modified

$H_3^{32}PO_4$ uptake by the disks infected by *P. infestans*.

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和 文 摘 要

ジャガイモ塊茎細胞の 3H -ロイシン吸収能を阻害する
遊走子および菌糸成分について

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ジャガイモ塊茎細胞の 3H -ロイシン吸収能を阻害する成分がジャガイモ疫病菌の遊走子に存在することを前報で報告した。本論文では吸収能を阻害する成分を遊走子と菌糸から抽出し部分純化した。遊走子に酢酸緩衝液 (pH 4.5) を加えて磨砕し, 20,000×g で遠心分離した。沈殿分画 (A) をホウ酸緩衝液に懸濁し, 1 時間煮沸したのち, 20,000×g で遠心分離して沈殿分画 (B) と上清分画を得た。上清分画を蒸留水で透析した (C)。各分画を終濃度 1 mM $CaCl_2$ を含む 10 mM トリス緩衝液 (pH 7.4) に懸濁した。各分画に阻害効果があり, 阻害率と糖濃度の間に高い相関がみられた。C 分画で処理した場合, 糖濃度が 15 μg/ml のとき阻害率は 2 時間で最高に達した。また Lisker & Kuć がファイトアレキシンエリシターとして抽出した方法に従って, 菌糸から抽出した成分も遊走子成分と同じ吸収阻害活性を示した。