Synthesis and Apparent Turnover of Acid Invertase in Relation to Invertase Inhibitor in Wounded Sweet Potato Root Tissue

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ABSTRACT

Previously we showed that acid invertase activity increased and then decreased rapidly in wounded sweet potato (Ipomoea batatas L.) root tissue, and that the tissue contained a heat-stable, proteinaceous inhibitor with a molecular weight of about 19,500 daltons.

In response to wounding of sweet potato root tissue, inhibitor activity decreased during the increase in invertase activity and later increased slightly when invertase activity declined. Cycloheximide treatment did not affect the decrease in inhibitor activity that occurred during the early incubation stage, but did inhibit the increase in inhibitor activity that occurred during the late incubation stage. Intrinsic invertase activity, which was assayed after removing the inhibitor, increased and then decreased after wounding, as apparent activity did.

The degradative rate of acid invertase in root discs, when assayed by intrinsic activity, was roughly the same during both the early and late incubation stages after wounding, and the degradative rate of the enzyme during the late incubation stage was unaffected by cycloheximide treatment. These results suggest that in sweet potato root discs, enzyme synthesis occurs during the early incubation stage, and ceases during the late incubation stage; however, the enzyme undergoes constant degradation.

The change in acid invertase activity after wounding seems to be controlled in root tissue by the interactions of inhibitor-binding and turnover of the enzyme protein.

Enzyme concentration in living systems appears to be controlled by the combination of synthesis and inactivation. In higher plants, inactivation of enzyme may be caused by degradation, modification, or inhibitor-binding (2, 4, 7). Activity of acid invertase may be regulated by inhibitors in potato (11), maize (5), and sweet potato (10). Further, rapid turnover of acid invertase occurs in sugarcane (3).

In wounded sweet potato root tissue, acid invertase activity increased, reached a maximum, and subsequently decreased rapidly (8). Such observations support the notion that there exists an invertase-inactivating system involving proteolysis of the enzyme or combination with an inhibitor substance.

During the search for an enzyme-inactivating system in sweet potato root tissue, we found a heat-stable protein with a mol wt of about 19,500 daltons that inhibited acid invertase. We purified the inhibitor and showed that it could bind reversibly to acid invertase (10). Such results suggested that the change in acid invertase activity might be regulated in vivo by the fluctuation in concentration of the inhibitor.

In this paper, we describe results of studies on synthesis and degradation of acid invertase and fluctuation of inhibitor concentration as possible mechanisms regulating acid invertase activity in wounded sweet potato.

MATERIALS AND METHODS

Plant Materials. Roots of sweet potato (Ipomoea batatas Lam. cv. Norin No. 1) were harvested in the autumn and stored at 10 to 14°C until used. Roots were washed with tap water, surface-sterilized with 0.1% sodium hypochlorite, and washed thoroughly with tap water. The roots were sliced and discs (3 x 19 mm) of parenchymatous tissue were prepared from tissue slices, as previously reported (8). Procedures for incubating the tissue discs, and treatments with ethylene and cycloheximide were described previously (9).

Assay of Acid Invertase Activity. Reaction mixture consisted of 20 μmol of K-acetate buffer (pH 4.6), 40 μmol of sucrose, and 100 μl of enzyme solution in a final volume of 400 μl. Other details of the enzyme assay were described previously (8). The enzyme activity thus assayed is apparent, but not intrinsic. When necessary, the activity is called apparent enzyme activity.

Assay of Acid Invertase Inhibitor. One ml of crude extract of sweet potato root discs was heated at 70°C for 2 or 3 min to denature acid invertase and then cooled in ice. The heat-treated extract was used as the inhibitor solution. The inhibitor solution at each of four different dilutions was combined with 5 μl (approximately 55–60 units) of a purified acid invertase sample (10) containing 0.4% BSA and 20 μmol of K-acetate buffer (pH 4.6) in a final reaction mixture of 300 μl. The mixture was preincubated at 30°C for 15 min, mixed with 100 μl of 40 μmol of sucrose, and incubated at 30°C for 60 min to determine the remaining enzyme activity. The reciprocals of the remaining enzyme activity assayed were plotted against inhibitor concentrations. From this Dixon plot (1), the amount of the inhibitor required to inhibit acid invertase activity 50% was calculated and defined as 1 unit.

Procedure for Assay of Intrinsic Activity of Acid Invertase. Crude extract (9 or 10 ml) was applied to a DEAE-cellulose (Whatman DE52) column (1.5 x 4 cm) pre-equilibrated with 10 mM K-phosphate buffer (pH 6.0). The column was washed with 30 ml of the same buffer and then acid invertase free of inhibitor was eluted with 30 ml of the above buffer containing 0.1 M KCl. This preparation was used as assay of intrinsic activity of acid invertase.

Purification of Acid Invertase and the Inhibitor. Acid invertase and the inhibitor were purified by the previous methods (10) unless otherwise stated.

Protein Content. Protein content was determined from 10% trichloroacetic acid-insoluble material of crude extract by the Lowry method (6) using BSA as the standard.

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RESULTS

Time Course of Activities of Acid Invertase and the Inhibitor.
Inhibitor activity cannot be assayed in crude extracts unless ac in invertase is removed. Since we demonstrated that the inhibitor is heat-stable (10), we tried to inactivate acid invertase preferentially by heating the tissue extracts. We observed that inhibitor activity increased concomitantly with the inactivation of acid invertase when preparations were heated at 70 C. After a 2-min heat treatment, acid invertase activity was completely lost and inhibitor activity could be assayed in such heat-treated preparations without the influence of the enzyme (Fig. 1). When a mixture of the purified inhibitor and the purified enzyme was preincubated, 87% of the inhibitor activity was recovered after heating (Table I). Therefore, by the heat treatment, inhibitor activity in crude extracts is recovered by its release from an enzyme-inhibitor complex with the enzyme denaturation.

In confirmation of our earlier findings (8), acid invertase activity rapidly increased, reached a maximum in 18 hr, and then decreased in response to wounding (Fig. 2). In contrast, the inhibitor activity initially decreased rapidly and then increased. However, the amount of increase in inhibitor activity in tissue discs during the late incubation stage varied according to the conditions of cultivation (for example, see Figs 2 and 5) and the variety of sweet potato (cv. Kokei No. 14 to cv. Norin No. 1, data not shown).

The properties of the inhibitors in the tissue discs during the early and late stages of incubations were similar in DEAE-cellulose column elution profiles, heat stability, and invertase-binding character, suggesting that the decrease and subsequent increase of the inhibitor activity by wounding represent changes in concentration of the same inhibitor.

Time Course of Apparent and Intrinsic Acid Invertase Activities. To assay intrinsic enzyme activity, we separated the enzyme from its inhibitor by stepwise elution from a DEAE-cellulose column with 10 mM K-phosphate buffer (pH 6) containing 0.1 M KCl and 0.15 M KCl. As shown in Table II, the enzyme was eluted mainly in 0.1 M KCl fraction and the inhibitor eluted in 0.15 M KCl fraction together with small amounts of the enzyme.

Using the method described above, we assayed the change in intrinsic enzyme activity. As shown in Figure 3, intrinsic enzyme activity was found even in fresh tissue. The time course pattern of intrinsic enzyme activity in response to wounding was similar to the change in apparent enzyme activity (Fig. 3). The inhibition of enzyme activity by the inhibitor could not account for the decrease in enzyme activity in tissue during the late stage, but it did appear to contribute significantly to the low activity as

### Table I. The Effect of Heating on Inhibitor Activity in the Mixture of the Enzyme and the Inhibitor

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Remaining Activity</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor before heating</td>
<td>18.9</td>
<td>100</td>
</tr>
<tr>
<td>Inhibitor after heating</td>
<td>17.7</td>
<td>93.7</td>
</tr>
<tr>
<td>Inhibitor-enzyme after heating</td>
<td>16.4</td>
<td>86.0</td>
</tr>
</tbody>
</table>

1 Purified inhibitor (12µg) was diluted in 85 µl of 0.118 M K-phosphate buffer (pH 7) and the inhibitor activity assayed.
2 Purified inhibitor (12µg) in 85 µl of 0.118 M K-phosphate buffer (pH 7) was heated at 70 C for 2 min and then cooled.
3 Purified inhibitor (12µg) was mixed with purified enzyme (0.87µg) in 35 µl of 29 mM K-acetate buffer (pH 4.6) and incubated at 30 C for 15 min. This incubated mixture was mixed with 50 µl of 0.2 M K-phosphate buffer (pH 7) to a final concentration of 0.118 M, heated at 70 C for 2 min and then cooled.

### Table II. Separation of Acid Invertase and Inhibitor Activities by DEAE Column Chromatography

<table>
<thead>
<tr>
<th>Conditions for Elution</th>
<th>Acid Invertase activity</th>
<th>Inhibitor activity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/7-discs</td>
<td>units/7-discs</td>
<td>units/7-discs</td>
</tr>
<tr>
<td>Before elution</td>
<td>3483</td>
<td>590</td>
<td>-</td>
</tr>
<tr>
<td>Buffer without KCl</td>
<td>144</td>
<td>41</td>
<td>-</td>
</tr>
<tr>
<td>Buffer with 0.1 M KCl</td>
<td>5924</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>Buffer with 0.15 M KCl</td>
<td>604</td>
<td>425</td>
<td>452</td>
</tr>
</tbody>
</table>

Seven discs incubated for 43 hr were extracted according to the methods of the previous paper (8). Nine ml of crude extract were applied to DEAE-cellulose column (1.5 x 5 cm) preequilibrated with 100 mM K-phosphate buffer (pH 6). The column was washed with 30 ml portions of the above buffer containing KCl in the concentrations indicated in the first column. Effluent samples of 29.0, 28.0 and 29.4 ml were obtained, respectively.

*One ml of extract or effluent samples were heated at 70 C for 2 min.*
detected in the lag phase during the early incubation stage.

Effect of Ethylene on the Change in Inhibitor Activity. We reported that ethylene repressed the increase in acid invertase activity in root tissue following wounding (9). Therefore, we examined the effect of ethylene on the change in inhibitor activity in root discs. Figure 4 shows that ethylene prevented to some extent the decrease in inhibitor activity after wounding. However, Table III shows that ethylene prevented the increase in intrinsic enzyme activity as well as the decrease in inhibitor activity. This, the effect of ethylene on the increase in apparent acid invertase activity is not only due to this inhibitory effect on the decrease in the inhibitor concentration, but also due to that on the formation of acid invertase.

Effect of Cycloheximide on the Change in Acid Invertase and Inhibitor Activities. The previous report (9) showed that cycloheximide completely inhibited the increase in acid invertase activity during the early incubation stage. We examined the effect of cycloheximide on the change in acid invertase activity in relation to the effect on the change in the inhibitor activity. In a series of experiments (Fig. 5), cycloheximide inhibited the enzyme activity during the early incubation stage as previously (9). Its treatment at the 18 hr-incubation time slightly repressed the decline in apparent enzyme activity.

Addition of cycloheximide at zero time did not prevent the decrease in the inhibitor activity in root discs during the early incubation stage, but its addition at the 18-hr incubation time completely prevented the subsequent increase in the inhibitor activity.

Turnover of Acid Invertase. A semilogarithmic plot of decrease in apparent acid invertase activity in root tissue during the late incubation stage was not linear, perhaps due to the effect of changing inhibitor concentrations. However, the decrease in intrinsic acid invertase activity gave a linear plot showing first order kinetics for enzyme degradation with a half-life of 13 hr (Fig. 6).

We next attempted to elucidate the rate of turnover of acid invertase by comparing intrinsic acid invertase activities in root discs treated and not treated with cycloheximide. Figure 7 shows that intrinsic acid invertase activity during the early incubation stage increased in the untreated root discs but decreased in those treated with cycloheximide. In root discs during the late incubation stage, cycloheximide did not affect the decrease in intrinsic acid invertase activity.

From semilogarithmic plots of the experimental results involving the data in Figures 3 and 7, the calculated values for the half-life of acid invertase in cycloheximide-treated root discs during the early incubation stage were 8.5 and 12 hr and those in untreated and treated root discs during the late incubation stage were 12 to 13 hr (Fig. 6).

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**Fig. 3.** Apparent and intrinsic acid invertase activities in sweet potato root discs in relation to incubation time. ○-○, ●-●: activities of apparent, intrinsic acid invertase, and the inhibitor, respectively.

**Fig. 4.** Effect of ethylene on the change in acid invertase and inhibitor activities in sweet potato root discs. Each group of seven discs was incubated in an air-tight chamber (inside volume, 300 ml) with or without 10 μl/l ethylene and 5 ml of 20% KOH. Air or air-ethylene mixture in the chamber was exchanged every 9 hr. A: Enzyme activity; B: inhibitor activity; ○-○, ●-●: activity in absence and presence of ethylene, respectively.

**Fig. 5.** Effect of cycloheximide on the change in apparent acid invertase and inhibitor activities in sweet potato root discs. Each group of seven discs was immersed in 20 ml of water or cycloheximide (2 μg/ml) and subjected to vacuum infiltration (40 mm Hg) for 1 min. Treated discs were blotted with filter paper and then incubated. The roots were harvested in November at the Agricultural Experiment Station, Toyo-hashi, Aichi. A: ○, ○: activities of apparent acid invertase and the inhibitor, respectively, in nontreated discs; B: ○-○, ●-●: apparent acid invertase activity in discs treated with water and cycloheximide, respectively; C: ○-○, ●-●: inhibitor activity in discs treated with water and cycloheximide, respectively; ↓: discs were given water or cycloheximide at the time shown by the symbol.

**Table III.** Effect of Ethylene Treatment on Apparent and Intrinsic Acid Invertase and Inhibitor Activities in Tissue Discs

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Enzyme Activity</th>
<th>Inhibitor Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apparent</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Control</td>
<td>60.4</td>
<td>69.7</td>
</tr>
<tr>
<td>Ethylene</td>
<td>34.8</td>
<td>40.6</td>
</tr>
</tbody>
</table>

Seven discs were incubated for 18 hr in an air-tight chamber with or without 10 μl/l ethylene.
DISCUSSION

The inhibitor activity changed inversely with respect to fluctuations in acid invertase activity in response to wounding, suggesting that acid invertase activity was controlled by the inhibitor content. Indeed, intrinsic acid invertase activity was inhibited owing to the presence of a large amount of the inhibitor, especially during the early incubation stage (Fig. 3). It is possible that inhibition by the inhibitor in the intact cells would be much greater than in the cell-free system, presumably because it is concentrated in a particular cell compartment. However, it is very likely that the increase in acid invertase activity after wounding is not only due to the decrease in inhibitor activity, but also to the synthesis of acid invertase. This is supported by the observation that the increase in acid invertase activity in root discs was completely inhibited by cycloheximide treatment although the decrease in inhibitor activity was not affected at all (Fig. 5), and further intrinsic acid invertase activity increased during the early incubation stage (Fig. 3).

On the other hand, during the late incubation stage, the decrease in acid invertase activity seems to be only partly dependent upon the increase in the inhibitor activity, because the decrease in acid invertase activity was only slightly suppressed by cycloheximide whereas the increase in inhibitor activity was completely inhibited (Fig. 5), and intrinsic acid invertase activity actually decreased (Fig. 3). Thus, the decrease in acid invertase activity during the late incubation stage may involve enzyme degradation, in addition to the increase in the inhibitor concentrations. However, the rate of degradation of acid invertase may be about the same during both the early and late incubation stages (Fig. 6) and the synthesis of acid invertase appears to cease during the late incubation stage because the half-life of acid invertase was the same in the absence and presence of cycloheximide (Fig. 6). Such results would suggest that activity of the acid invertase degradation system remains constant and the decrease in acid invertase during the late incubation stage is largely due to cessation of its synthesis.

In potato and maize, it seems likely that acid invertase activities are, at least partially, controlled by proteinaceous inhibitors. When potato tubers were stored at 18°C, high inhibitor and low invertase activities were observed while the reverse occurred at 4°C (12). A proteinaceous inhibitor accumulated in maize endosperm during development when invertase I activity decreased (5). In sugarcane, the rapid decrease in invertase activity with half-life of about 2 hr was observed in intact tissue and attributed to its turnover by an enzyme-degrading system (3). Recently, Sacher et al. (13) indicated that wound-induced RNase in turnip was controlled by the synthesis and degradation, and the decline in RNase activity was due to cessation of synthesis.

In wounded sweet potato, acid invertase activity may be controlled by both the reversible binding of the proteinaceous inhibitor and by the synthesis and degradation of the enzyme itself. It seems that the enzyme is synthesized and the inhibitor is inactivated following wounding, and during the late incubation stage enzyme synthesis ceases and the inhibitor is synthesized. Meanwhile, the degradation system of the enzyme operates at a constant level in the course of the whole period. Such simultaneous changes in rates of enzyme synthesis and inhibitor activity may account for the fluctuation in the apparent enzyme activity, which is associated with the changes in sucrose content in wounded sweet potato within the period between 12 and 24 hr following wounding (8).

At present, the following subjects remain to be solved: the mechanisms of the enzyme induction and the inhibitor inactivating response to wounding, and of the cessation of the enzyme synthesis and the synthesis of the inhibitor during the late incubation stage. In addition, it will be important to elucidate the enzyme-inactivating system that is apparently at a constant concentration during the entire incubation period. Elucidating the above questions may also help to define the biochemical principles of metabolic changes due to wounding.

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SWEET POTATO INVERTASE AND ITS INHIBITOR

LITERATURE CITED