Effects of Bay m 1099, an α-Glucosidase Inhibitor, on Starch Metabolism in Germinating Wheat Seeds

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To examine the significance of α-glucosidase in starch metabolism in vivo, wheat seedlings and seeds were treated with Bay m 1099 (Miglitol, N-hydroxyethyl-1-deoxyxynojirimycin), an α-glucosidase inhibitor. Bay m 1099 did not affect germination, but it inhibited growth of seedlings at the high dosage (100 µg/ml medium). Treatment of normally grown seedlings with Bay m 1099 (10 and 100 µg/ml) decreased α-glucosidase activity with a dose response and caused accumulation of maltose in tissues with decreases in glucose. The decrease in glucose formation would inhibit plant growth, which was observed particularly at higher dosages of the inhibitor. When wheat seeds were treated with Bay m 1099 at 10 µg/ml for 4 days, under which the growth of the plants after germination was minimized, the induction of α-glucosidase, but not amylose, in kernels was considerably suppressed during germination and maltose metabolism to glucose was disturbed. In addition, the Bay m 1099 treatment decreased the initial rate of starch degradation by 48%, compared with the control. These results suggest that wheat α-glucosidase participates in maltose hydrolysis as well as in the onset of starch degradation with collaboration of amylose.

Starch breakdown in germinating cereal seeds is initiated by α-amylases [EC 3.2.1.1] that are synthesized de novo in response to gibberelic acid, since α-amylases are only capable of binding to and attacking starch granules.1–4 The solubilized malto-dextrins formed by α-amylases have been considered to undergo hydrolytic degradation to form maltose by carboxydrases including β-amylase [EC 3.2.1.2], debranching enzyme [EC 3.2.1.41], and α-glucosidase [EC 3.2.1.20]. Phosphorylation is less important, since maltose and glucose are the almost exclusive products,5–6 and there was little or no activity of phosphorylase upon germination.6–9

For years it has been recognized that the rate of starch granule degradation in vitro by α-amylase alone was significantly lower than that by crude extracts from cereal seeds,7–11 suggesting that activators of α-amylolysis are present in the crude extracts. α-Glucosidase has been suggested to be a more relevant factor than β-amylase or debranching enzyme. Indeed, Sun and Henson12 have reported that α-glucosidases purified from germinated barley seeds could hydrolyze native barley starch granules and the enzymes showed a marked synergistic effect on granule hydrolysis by α-amylases. Using path coefficient analysis, Sun and Henson13 found that α-glucosidase contributed primarily to multistep starch degradation with the collaboration of α-amylase.

Thus, the physiological significance of α-glucosidase in starch degradation upon germination has been paid much attention, but its assessment in vivo is still unclear. This may be the time to try an approach with an α-glucosidase inhibitor.

Bay m 1099 (Miglitol, N-hydroxyethyl-1-deoxyxynojirimycin, see Fig. 1) and acarbose are potent α-glucosidase inhibitors from microbes. Blocking the breakdown of oligosaccharides by intestinal α-glucosidase, these sugar analogs are expected to be anti-hyperglycemic drugs for diabetes and obesity.14–16 These analogs also inhibit lysosomal acid α-glucosidase to accumulate glycogen in lysosomes, mimicking the cytological and biochemical feature of type II glycogenesis (Pompe’s disease).17–19 Bay m 1099 and acarbose also strongly inhibit plant tissue α-glucosidases in vitro.20

In this paper, we describe for the first time the effects of Bay m 1099 on starch metabolism in germinating wheat seeds. We observed that this drug significantly inhibited α-glucosidase activity and delayed the initial starch degradation upon germination. We discuss the physiological significance of the enzyme in vivo.

Materials and Methods

Reagents. Bay m 1099 was generously supplied from Bayer AG (Wuppertal, Germany). Maltose phosphorylase (720 U/ml) was a product of Oriental Yeast Co., Ltd., Osaka, Japan. 4-Methylumbelliferyl α-D-glucoside (4MUαG) was purchased from Sigma. Other reagents were of the highest grade available.

Plant material. Wheat (Triticum aestivum cv Norin 61) seeds were the generous gift of Dr. K. Komae (National Agriculture Research Center, Tsukuba, Japan). The seeds were soaked in 3% antifomarin for 30 min to remove surface contamination, and rinsed with distilled water before use. Twelve seeds were placed on a filter paper in a Petri dish (6 cm in diameter) containing sterile water (2 ml) with or without Bay m 1099 (0.1–100 µg/ml).

Fig. 1. Structure of Bay m 1099 (Miglitol, N-Hydroxyethyl-1-deoxyxynojirimycin).

Abbreviations: 4MUαG, 4-methylumbelliferyl-α-D-glucoside; 4MU, 4-methylumbelliferone.
solution, followed by germination at 25°C in the dark. Normally grown wheat seedlings were also treated with the inhibitor (see Figs. 3 and 4). Whole seedlings or the separated organs (coleoptile, roots, and kernel) were frozen in liquid nitrogen after weighing and stored at -80°C until use.

**Enzyme assay.** Tissues were homogenized with 20 mM Hepes buffer (pH 7.0) (10 ml/g) using an Ultra Turrax homogenizer. The supernatant (crude extract) obtained by centrifugation (20,000 × g, 20 min) was used as the source of the enzyme. β-Glucosidase activity was measured fluorimetrically with 4MUβG (0.1 mM) as a substrate, as described previously.18,20 The incubation mixture (1 ml) contained 0.6 ml of 0.1 mM citrate-phosphate buffer (pH 4.5), 0.1 ml of enzyme, and 0.3 ml of 0.33 mM substrate. The reaction was started by adding the substrate at 37°C and stopped by 3 ml of 0.5 M glycine–NaOH buffer (pH 10.3). Fluorescence of 4MU was measured with a Shimadzu RF-540 spectrophotometer at an emission wavelength of 450 nm and an excitation wavelength of 366 nm. One unit was defined as the amount of enzyme forming 1 μmol of 4MU/min. Total amylase activity was measured as deextrinizing power using amylose as a substrate, as described by Fuwa21 with slight modifications. The incubation mixture (0.1 ml) contained 25 μl of 0.1 M acetate buffer (pH 5.5), 50 μl of 0.2% amylose, and 25 μl of the diluted enzyme. The reaction was started by the addition of the substrate, left for 10 min at 30°C, and stopped by the addition of 1 M perchloric acid (0.1 ml), followed by 10-fold dilution with water. Absorbance at 660 nm was measured after the addition of 1% 1,10-phenanthroline (0.1 ml). One unit was defined as the amount of enzyme that decreased the color intensity by 10%/min.

**Extraction and measurements of sugars and starch.** Extraction of glucose, maltose, and starch was done as follows. Tissues were homogenized with 80% ethanol (2 ml/g tissue) and the supernatant (ethanol-soluble fraction) by centrifugation (20,000 × g, 10 min) was collected. The resulting sediment, after it was washed again with 80% ethanol, was treated with dimethylsulfoxide (90°C, 60 min) to solubilize starch. Glucose in the ethanol-soluble fraction was measured by the glucose oxidase-peroxidase method. Maltose was measured similarly after its conversion to glucose by maltose phosphorylase (0.3 U/ml reaction mixture) in the presence of arsenate at pH 5.2.20 Starch was measured by the phenol-sulfuric acid method.21

**Results**

**Inhibition of β-glucosidase activity in vitro**

Bay m 1099 strongly inhibited acid β-glucosidase activity in crude extracts obtained from normal wheat seedlings. The concentration of the inhibitor giving 50% inhibition was about 0.1 μg/ml (Fig. 2). Total amylase activity was not inhibited by Bay m 1099.

**Effects of Bay m 1099 on germination**

The germination rate (%) of wheat seeds after 24 h was 83–94% in the absence or presence of Bay m 1099 (0–100 μg/ml) (see Table I). However, exposure to Bay m 1099 at 100 μg/ml considerably inhibited the growth of seedlings (data not shown). We therefore examined the direct effects of Bay m 1099 on growth of seedlings, as described below.

**In vivo effects of Bay m 1099 on the growth, β-glucosidase activity, and glucose and maltose levels in seedlings**

The 2-day wheat seedlings grown in the absence of Bay m 1099 were treated with water (control) and Bay m 1099 (1, 10, 100 μg/ml) for 4 days. The growth of coleoptile, leaf, and seminal and crown roots were completely inhibited by Bay m 1099 at 100 μg/ml, although not at 1 μg/ml (Table II). At 10 μg/ml of Bay m 1099, the effect was intermediate and varied among organs.

Figure 3 shows the effects of Bay m 1099 on β-glucosidase activities, and maltose and glucose levels in whole seedlings. The β-glucosidase activity in the control group decreased naturally from near maximum level. The enzyme activities in the treated groups were lower than that of the control and responded to the dosage of the inhibitor (Fig. 3A).

**Table I. Effects of Bay m 1099 on Germination (%)**

<table>
<thead>
<tr>
<th>(μg/ml)</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bay m 1099</td>
<td>93.6 ± 3.3</td>
</tr>
<tr>
<td>0.1</td>
<td>90.0 ± 5.8</td>
</tr>
<tr>
<td>1</td>
<td>90.0 ± 5.8</td>
</tr>
<tr>
<td>10</td>
<td>83.3 ± 6.7</td>
</tr>
<tr>
<td>100</td>
<td>90.0 ± 0.0</td>
</tr>
</tbody>
</table>

Ten wheat seeds were germinated at 25°C in the dark in the absence and presence of Bay m 1099 (0–100 μg/ml). Germination rate (%) was measured 24 h after sowing. Values are mean ± SE of 3 different tests.

**Table II. Effects of Bay m 1099 on Growth of Wheat Seedlings**

<table>
<thead>
<tr>
<th>Bay m 1099 (μg/ml)</th>
<th>Days after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Coleoptile</td>
<td>6.3 ± 1.1</td>
</tr>
<tr>
<td>1</td>
<td>5.7 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>4.1 ± 0.6*</td>
</tr>
<tr>
<td>100</td>
<td>2.3 ± 0.2*</td>
</tr>
<tr>
<td>Leaf</td>
<td>5.0 ± 1.6</td>
</tr>
<tr>
<td>1</td>
<td>5.8 ± 1.6</td>
</tr>
<tr>
<td>10</td>
<td>3.1 ± 1.1</td>
</tr>
<tr>
<td>100</td>
<td>1.9 ± 0.8*</td>
</tr>
<tr>
<td>Seminal root</td>
<td>4.6 ± 1.4</td>
</tr>
<tr>
<td>1</td>
<td>4.8 ± 1.2</td>
</tr>
<tr>
<td>10</td>
<td>3.8 ± 0.8</td>
</tr>
<tr>
<td>100</td>
<td>3.2 ± 0.5*</td>
</tr>
<tr>
<td>Crown root</td>
<td>6.5 ± 1.4</td>
</tr>
<tr>
<td>1</td>
<td>7.3 ± 1.5</td>
</tr>
<tr>
<td>10</td>
<td>5.0 ± 1.4</td>
</tr>
<tr>
<td>100</td>
<td>1.8 ± 0.3*</td>
</tr>
</tbody>
</table>

Wheat seedlings grown with water only for 2 days were treated with the various concentrations of Bay m 1099 for 2-4 days. Lengths (mm) of coleoptile, leaf, and seminal and crown roots were measured. Values are mean ± SE (n = 12). Statistical significance of difference between control and the Bay m 1099-treated groups were calculated by Student's t-test. * p < 0.05, + p < 0.01, ′ p < 0.005, and ″ p < 0.001.

![Fig. 2. In Vitro Effects of Bay m 1099 on β-Glucosidase and Amylase Activities.](image-url)

The crude extract from the 3-day wheat seedlings was used as a source of enzyme. β-Glucosidase (C) and total amylase (■) activities were measured at pH 4.5 and at pH 5.5, respectively, in the presence of Bay m 1099 (0–100 μg/ml).
glucose level in the control group increased at about 3 mg/day/g tissue, while the maltose level was almost constant (7–8 mg/g) for 4 days (Figs. 3B and 3C). Excepting the case at 1 µg/ml, Bay m 1099 changed these sugar levels in tissues significantly. The high dosage of Bay m 1099 (100 µg/ml) prevented glucose formation by its complete inhibition of α-glucosidase activity, while maltose accumulated at high levels in tissues. The dosage of 10 µg/ml also decreased the glucose level and increased the maltose level, although the effect was moderate. These results suggest that α-glucosidase has responsibility for maltose hydrolysis, as characterized by other purified cereal α-glucosidases. It has been shown that maltose is formed in endosperm, but not in other organs. There was no difference in starch levels (about 10 mg/seedling) between control and all the treated groups, which was about 50% of the starch level seen in the ungerminated seeds. This suggests that Bay m 1099 had no effect on starch degradation which proceeded to some extent during germination. A similar result was also obtained, as described below (see Fig. 7C).

Effects of Bay m 1099 on starch metabolism in wheat seeds during germination

In the following experiment, wheat seeds were treated with Bay m 1099 to examine the effects on the initial stage of starch degradation upon germination. We chose the inhibitor concentration of 10 µg/ml for minimization of the plant growth after germination and because we expected fluctuations in starch metabolism. Under these conditions, no significant inhibition of growth of coleoptile was observed, which was different from the result using wheat seedlings (Table II), although the growth of roots was lower by 20%, compared with control. The following results were from kernels, unless otherwise stated.

i) α-Glucosidase activity. We detected α-glucosidase activity (0.6 mU/g kernel) in ungerminated seeds. It increased upon germination and reached the maximum level 4 days later. The treatment with Bay m 1099 suppressed the enzyme activity of kernels considerably (Fig. 4C). The effects of Bay m 1099 were partially reversible. The α-glucosidase activities of coleoptile and roots were also increased upon germination and they were inhibited by Bay m 1099 (Figs. 4A and B), but fewer reversible effects were observed. This probably was from an untimely medium exchange.

ii) Apparent concentration of Bay m 1099 in tissues. Since we found α-glucosidase-inhibitory activity in the heat-treated crude extract of the Bay m 1099-treated seeds, but not of control seeds, we could measure apparent content of Bay m 1099 in seeds. The inhibitory activity of Bay m 1099 was taken up by seeds (about 4 µg/g kernel) during 0–24 h and the tissue concentration increased gradually thereafter (Fig. 5). These amounts were enough to inhibit α-glucosidase activity, as observed in vitro (see Fig. 2). It is unknown whether Bay m 1099 is transformed in the cells, but it has been reported that Bay m 1099 is neither phosphorylated nor oxidized in rat hepatocytes.

iii) Total amylase activity. Total amylase activity was not detectable in ungerminated seeds, but it increased rapidly upon germination in both control and the Bay m 1099-treated groups (Fig. 6). Interestingly, the amylase activity
of the treated group was higher than that of control. The reason for this phenomenon is unknown, although it might be compensatory under the Bay m 1099-bearing conditions. No significant reversible effect was observed (data not illustrated).

iv) Starch, glucose, and maltose levels in kernels. In ungerminated wheat seeds, glucose and maltose levels were found to be 1 mg/g kernel and 17 mg/g kernel, respectively. In control group, glucose levels increased gradually upon germination and maltose level also increased after dropping for first 24 h (Fig. 7). This drop in maltose level would be due to hydrolysis of the preexisted maltose by z-glucosidase. In the Bay m 1099-treated group, the glucose and maltose levels were lower and higher, respectively, than those in the controls at any stage of germination. There were no significant reversible effects of Bay m 1099 on these sugars (data not illustrated).

Bay m 1099 delayed starch degradation. The initial rate of the degradation during 0–24 h was 5.7 mg/day/kernel in the treated group and was 10 mg/day/kernel in control group. However, starch degradation proceeded at a similar rate after 3 days of inhibition in both groups (Fig. 7).

Discussion
A possible mechanism by which Bay m 1099 inhibits z-glucosidase activity

It has been reported that in ungerminated seeds z-glucosidase has been found in barley germ,28 or in spherosome-like lytic bodies, together z- and β-amyloses, in water-insoluble form in maize, barley and sorghum endosperm.29,30 On the other hand, z-glucosidase has been considered to be synthesized de novo and secreted from the aleurone layer of barley and rice seeds in response to gibberellic acid upon germination,1,2,12,28,31,32 which is similar to the mechanism of z-amylose induction.

In this study, we also found z-glucosidase activity in ungerminated seeds and its rise upon germination. Although it is unknown how z-glucosidases different in localization share in starch degradation, they were inhibited in vitro and in vivo by Bay m 1099, an z-glucosidase inhibitor. Bay m 1099 was apparently taken up by seeds at imbibition. The effects of Bay m 1099 were partially reversible (see Fig. 4). That is, the recovery of the enzyme activity by removal of Bay m 1099 halfway would be due to dilution of the tissue concentration of the inhibitor, thereby biosynthesis of new enzyme would occur. Wisselaar et al.19 have reported in human fibroblasts that Bay m 1099 inhibited microsomal neutral z-glucosidase activity that is involved in the processing of the carbohydrate moiety of N-linked glycoproteins, resulting in limitation of the lysosomal acid z-glucosidase level. Whether a similar mechanism is present in wheat seedlings in our study requires further investigation. However, neutral z-glucosidases involved in the processing have also been purified from mung beans.33,34

Bay m 1099 also inhibited the z-glucosidase activity of normally grown wheat seedlings (see Figs. 3 and 4). In this case, Bay m 1099, after absorption by tissues, possibly via roots, would bind firmly to the preexisted enzyme and inactivate it. Indeed, Bay m 1099 showed high affinity to z-glucosidases and the Kᵣ has been reported as 7.4 × 10⁻⁸ M for the banana enzyme.28 A similar value was reported for rat intestinal disaccharidase.35,36

Physiological role of wheat z-glucosidase

We could estimate the physiological role of z-glucosidase in germinating seeds, using Bay m 1099. First, this enzyme is essential in the hydrolysis of maltose to supply glucose in wheat seedlings (Fig. 3). Unavailability of glucose caused by Bay m 1099, particularly at a high dosage (100 μg/ml), would affect the plant growth indirectly (Table II), since glucose formed in endosperm is secreted and used for sucrose synthesis in germ, from where sucrose is transported to other organs. Aoki and Hatanaka37 found that treatment of lucerne seedlings with 1,4-dideoxy-1,4-iminoarabinitol, a sucrose inhibitor, inhibited plant growth by unavailability and/or a defect of transport of sucrose.

The second significance is that z-glucosidase is obviously involved in the initial stage of starch granule degradation, possibly with concerted action with z-amylose. It was proven by the fact that Bay m 1099 inhibited z-glucosidase induction (Fig. 4C), but not amylose induction (Fig. 6), in germinating wheat seeds, by which the initial velocity of starch degradation was reduced by 48% (Fig. 7). The cooperative action in starch granule degradation observed in the normal germination process was, however, considerably lower than synergism observed in vitro. Indeed, Sun and Henson12 found that about 10-fold synergism in starch granule hydrolysis occurred in vitro by z-amylose and
z-glucosidase. These discrepancies would be due to characteristics of the in vivo system. One of the factors in vivo that controls the rate of starch degradation in cereal endosperm has been proposed to be a balanced biosynthesis and secretion of z-amylase and endogenous proteinaceous z-amylase inhibitor.5,38,39

The effects of Bay m 1099 on debranching enzyme activity were not examined in this study. However, Maeda et al.10,11 observed that barley debranching enzyme contributed insignificantly to synergism of starch granule hydrolysis by z-amylase, although Bollen et al.27 showed that Bay m 1099 inhibited the z-1,6-glucosidase activity, but not the transferase activity, of rat liver debranching enzyme.

The mechanism by which z-glucosidase enhanced z-amylolysis of starch granules has been traced to removal of maltose by z-glucosidase, since maltose binds to a non-catalytic site of z-amylase and decreases the rate of starch hydrolysis.2,4 The inhibition of maltase activity, as shown in Fig. 7, maltose levels in kernels of the Bay m 1099-treated group were roughly estimated to be 18–30 mg during 24–48 h after imbibition, which was twice as high as the control group (10–21 mg). The former concentration of maltase gave 22–30% inhibition and the latter 15–23% by in vitro assay we used (see Materials and Methods), with no significant difference. The maltose concentration giving 50% inhibition required 100 mg in the assay (data not shown). Thus, we cannot explain how the initial delay of starch degradation is attributable to maltose, if we assume that maltose is evenly distributed in tissues. Sun and Henson12 proposed that the marked synergism of starch granule degradation by z-amylase and z-glucosidase in vitro was not due to simple removal of maltose by z-glucosidase, but also to the ability of z-glucosidase to remove non-z 1,4-linkages, barriers against z-amylolysis, which exist in starch granules. However, the latter role of z-glucosidase in their proposal was not demonstrable under the z-glucosidase-deficient conditions we studied.

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