Occurrence of 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and a β-Glucosidase Specific for Its Glucoside in Maize Seedlings

Kenkichi Ebisui, Atsushi Ishihara, Nobuhiro Hirai and Hajime Iwamura

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606–8502, Japan, and CREST, Japan Science and Technology Corporation (JST)

2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its glucoside, DIMBOA-G, appeared concurrently with germination in maize (*Zea mays* L.), and their concentrations per fresh weight reached a maximum 24–36 hr after germination. The aglycone then decreased to disappear as the plants began autotrophic growth, and the glucoside to a concentration half that of the maximum. The level of DIMBOA-G was always higher than that of the aglycone. A β-glucosidase activity which hydrolyzes DIMBOA-G to DIMBOA and glucose was detected in crude enzyme extracts from the seedlings. Its activity per fresh weight varied concurrently with the occurrence of DIMBOA. The purification was performed to an apparent homogeneity by cryoprecipitation followed by cation exchange chromatography and gel filtration. The K_m value for DIMBOA-G was 0.07 mM, whereas that for DIBOA-G, that was substantially absent in the seedlings of the cultivar studied, was 0.52 mM. The activity on salicin and esculin was too low to be detected. The set of results suggested that free DIMBOA occurs constitutively or in a programmed manner as defense compound at a seedling stage of growth, and a specific glucosidase is involved in this process.

Introduction

The cyclic hydroxamic acids, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its methoxy analogue, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA; see Fig. 1), are known to occur as glucosides (DIBOA-G and DIMBOA-G) in gram-
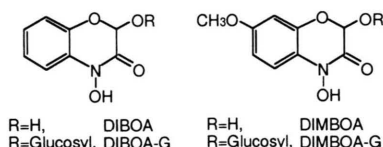

Fig. 1. Structures of hydroxamic acids and their glucosides.

ineae plants, including maize, wheat and rye (Niemeyer, 1988). They share their biosynthetic precursors with tryptophan, indole being identified as an intermediate in maize (Desai *et al.*, 1996), and involvement of cytochrome P-450's has been suggested in biosynthetic pathway form indole to DIBOA (Frey *et al.*, 1997; Glawischnig *et al.*, 1997; Leighton *et al.*, 1994). Identification of enzymes

that catalyze 7-hydroxylation and successive methylation to produce DIMBOA from DIBOA has not been reported so far, but a glucosyltransferase to produce glucosides has been found in maize seedlings (Bailey and Larson, 1989).

The hydroxamic acids have been implicated in the resistance of plants to pathogens and insects, and in the tolerance of maize to triazine herbicides (Niemeyer, 1988). Biologically active species, free DIBOA and DIMBOA, are not found in autotrophic, adult plants. They are thought to be liberated from the glucosides through the action of cellular β-glucosidase (EC 3.2.1.21) when plants are damaged by microbial or insect attack (Niemeyer, 1988). In wheat seedlings, however, we have found that free DIMBOA and DIBOA occur with germination with their glucosides, reach a maximum amount after 24–48 hr and then decrease to disappear (Nakagawa *et al.*, 1995). [^{14}C]Anthranilic acid administered to germinating seeds was effectively incorporated into DIMBOA-G and DIBOA-G to show that the occurrence of these compounds is due to *de novo* synthesis. This transient occurrence of hydroxamic acids was little affected by infection with pathogens and wounding with a razor blade, and has been supposed to be a programmed defense mechanism against pathogens and insects at

---

Reprint requests to Dr. H. Iwamura.
Fax: +81–75–753–6408.
E-mail: iwamura@kais.kyoto-u.ac.jp.

a vulnerable, juvenile stage of growth (Iwamura *et al.*, 1996; Nakagawa *et al.*, 1995). In this study, we examined their occurrence in maize seedlings, with the isolation of a specific glucosidase.

Materials and Methods

Seedling culture

Maize seeds (*Zea mays* L. cv. W79A x CM37) were immersed in concentrated sulfuric acid for 3 min to facilitate germination, and washed several times with sterilized water. Seeds were then placed on a sheet of wet paper and incubated at 25 °C with a 12-hr period of illumination from fluorescent lamps (60 W m^{-2}). Germination occurred 42–48 hr after seeding, greening started to occur at the top of the seedlings about 24–36 hr after germination, and the first leaf with a yellowish green color began to emerge from the sheath 72–86 hr after germination to reach a height of about 7 cm at the end of the experimental period (120 hr after germination).

Extraction and analysis of hydroxamic acids

The extraction of plant material was performed according to the method developed in the previous report (Nakagawa *et al.*, 1995), based on the stability of DIMBOA and DIBOA in aqueous solution as well as recovery efficiencies of both the aglycones and glucosides. Briefly, seedlings were divided into parts which were immediately ultrasonicated with methanol containing 2% acetic acid at 50 °C for 10 min. The solution was filtered through a disposable membrane filter (LCR13-LH, Millipore) and injected into a 6 x 100 mm metal-free Wakosil II 5C18 HG column (Wako, Osaka) which was eluted with methanol-water (22:78, v/v) containing 0.1% acetic acid at 40 °C at a flow rate of 1 ml min^{-1}. The monitoring was made at 280 nm. Dry seeds were ground in a mortar with a pestle, and extracted and analyzed as above.

Preparation of hydroxamic acids

DIMBOA and DIMBOA-G were extracted from maize seedlings of 3–4 days old as described above, and purified by HPLC as described previously (Nakagawa *et al.*, 1995).

Extraction of β-glucosidase

Shoots and roots were cut out from the seedlings, and each part (300 mg) was ground with 5 vol. 100 mM citrate-200 mM phosphate buffer (pH 5.5) containing 2% Polyclar AT and sea sand (300 mg) in a mortar with a pestle. After centrifugation at 3,000 x g for 12 min and 12,000 x g for 30 min, 1 ml aliquot of the supernatant was subjected twice to ultrafiltration using Centricon-10 (5,000 x g, 1 hr, Amicon) for removal of endogenous hydroxamic acids. The residue was filled up to 1.5 ml with 100 mM citrate-200 mM phosphate buffer (pH 5.5) followed by 400-fold dilution with the same buffer and used as crude enzyme preparation. All operations were carried out at 4 °C. The enzyme solution could be preserved for a month without detectable loss of activity when stored at −30 °C.

β-Glucosidase assay for crude enzyme preparation

The reaction mixture consisted of 50 μl of the enzyme solution, 50 μl of 1.3 mM DIMBOA-G and 400 μl of 100 mM citrate-200 mM phosphate buffer (pH 5.5). After an incubation for 15 min at 30 °C, the reaction was stopped by adding 50 μl of 1N HCl. The reaction products were analyzed by HPLC as described above. The amount of DIMBOA in the reaction mixture increased linearly up to 60 min under this condition and rate of cleavage was in proportion to the amount of proteins added.

Purification of β-glucosidase

Shoots (2.5 g) of 3-day-old seedlings were frozen in liquid N$_2$ and extracted with 2 vol. 50 mM sodium acetate, pH 5. The extract was centrifuged at 17,000 x g for 30 min. The pH of the supernatant was adjusted to 4.6 by adding acetic acid, left in ice water for 24 hr, and then centrifuged at 12,000 x g for 30 min. The supernatant was adjusted to pH 4 by acetic acid and loaded on a column of Accell Plus CM (Sep-Pak Vac 20 cc, Waters) that had been equilibrated with 50 mM sodium acetate, pH 4. The column was washed with the same buffer. The bound proteins were eluted stepwise with 20 ml each of 50 mM sodium acetate, pH 4.8, 5.5, 6 and 7. The pH 5.5 fraction that had DIMBOA-G glucosidase activity was collected, and

concentrated to 1 ml by using a column of Accell Plus CM (Sep-Pak Vac 1 cc, Waters). The concentrated enzyme solution was loaded on a column of Superdex 200 pg (2 x 60 cm, Pharmacia) that had been equilibrated with 50 mM sodium acetate (pH 5.5) containing 200 mM sodium chloride, and eluted with the same buffer at a flow rate of 0.8 ml min^{-1}. The fractions having DIMBOA-G β-glucosidase activity were used for characterization. Protein contents were determined colorimetrically with BSA as standard (Bradford, 1976). All operations were carried out at 4 °C.

Electrophoresis

Crude enzyme preparations and chromatographic fractions were subjected to SDS-PAGE analyses. The samples were electrophoresed through 0.75 mm thick gel slabs (10% resolving gels), and stained with silver staining kit (Sil-Best Stain, Nakarai, Kyoto).

Characterization of β-glucosidase

Solutions of substrates and enzyme were prepared in 100 mM citrate-200 mM phosphate buffer (pH 5.5). *p*-Nitrophenyl-D-glycosides were incubated with 2.5 ng of purified enzyme for 15 min at 30 °C. The reaction was stopped by adding 50 μl of 6N sodium hydroxide solution, and the absorbance of the *p*-nitrophenol liberated was measured at 410 nm. For other substrates, 2.5 ng of purified enzyme was used. After incubation at 30 °C for 5 or 15 min the reaction was stopped by adding 50 μl of 1N HCl, and the reaction mixture was subjected to HPLC analyses. For determination of pH optimum of the purified enzyme, 100 mM citrate-200 mM phosphate buffers adjusted to pH 4–7 were used. Kinetic constants were obtained from Lineweaver-Burk plots.

Results

Occurrence of hydroxamic acids in seedlings

In both shoots and roots, DIMBOA appeared about 12 hr after germination (Fig. 2). DIMBOA-G was already at a significant level, but barely detectable in dry and imbibed seeds. In shoots, the contents of DIMBOA and DIMBOA-G reached a maximum 36 hr after germination. In roots, they attained a maximum 24 hr after germination. The
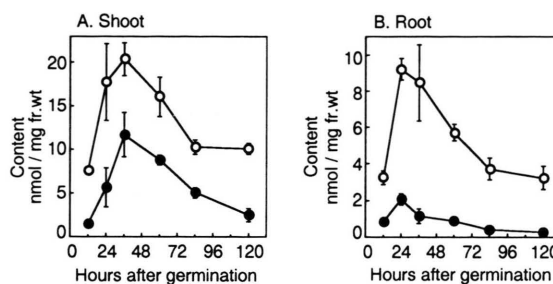

Fig. 2. Changes in shoots (**A**) and in roots (**B**) of DIMBOA-G (○) and DIMBOA (●) contents after germination. Vertical lines at data points show standard errors.

aglycone became barely detectable as the plants grew to an autotrophic stage (first leaf stage); it was 120 hr after germination in roots, and 150 hr after germination in shoots (data not shown). The glucoside in shoots decreased gradually to a level about 1/2 that of the maximum about 86 hr after germination and remained at this level thereafter (up to 150 hr after germination). The situation was similar in roots as well.

The maximum concentration in shoots of DIMBOA was 12 mM (12 nmol/mg fr. wt) and that in roots 2 mM (2 nmol/mg fr. wt.), when calculated assuming that the compound is uniformly distributed and the density of the plant tissues to be uniform and unity (Fig. 2). Through the most of the experimental period (12–120 hr after germination), it occurred more than 1 and 0.3 mM levels in shoots and roots, respectively. The maximum level of DIMBOA in shoots was more than 10 times higher than that in wheat, and that in roots was more than 2 times higher (Nakagawa *et al.*, 1995). The level of glucoside was always higher than that of aglycone, DIMBOA/DIMBOA-G ratio at the maximum being 0.6 in shoots and 0.2 in roots. DIBOA-G was barely detectable in the extracts through the experimental period, and DIBOA was detected only when the extracts were concentrated, the cultivar used in this study being the one that does not substantially produce DIBOA species (Zúñiga *et al.*, 1983).

The apices of shoot and radicle of 30-hr-old seedlings were cut off by razor blade as a model of wounding caused by insect feeding, and then the plants were grown for appropriate periods up to 120 hr after germination and extracted for HPLC analyses. Little indication of induced occur-

rence of DIMBOA was observed (data not shown).

β-*Glucosidase activity*

In view of the possibility that a specific glucosidase may occur in relevance to the sequential occurrence of DIMBOA, we investigated the enzyme activity in crude extracts prepared from the seedlings. A glucosidase activity that hydrolyzes DIMBOA-G to DIMBOA and glucose was detected in both shoots and roots, the maximum response being observed at pH 5.5. As shown in Fig. 3, the activity appeared with germination, reached the maximum (0.95 and 0.38 nkat/mg fr. wt in shoots and roots, respectively) 24 hr after its occurrence, and declined to a constant level (0.2 nkat/mg fr. wt). The extractable β-glucosidase activity in shoots was about 2.5 times higher than that in roots.
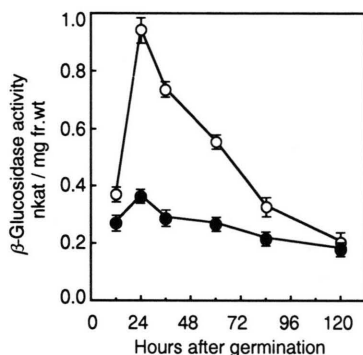

Fig. 3. Changes in shoots (○) and roots (●) of DIM-BOA-G β-glucosidase activity after germination. Vertical lines at data points show standard errors.

Purification of β-*glucosidase*

The crude extracts prepared from shoots of 3-day-old seedlings (those grown for 24 hr from germination) were subjected to a selective cryopre-

cipitation at pH 4.6 (Esen, 1991), and this increased the activity two times when DIMBOA-G was used as substrate. Most of the activity of the pH 4.6 supernatant bound to the Accell CM column at pH 4. The column was eluted stepwise between pH 4 and 7, and DIMBOA-G glucosidase activity was found in pH 5.5 fraction. The active fraction was loaded on a Superdex 200 pg column, and elution with 50 mM sodium acetate (pH 5.5) containing 200 mM sodium chloride gave a single active peak, the degree of purification being 340 fold (Table I). The peak fraction approximately corresponded to a molecular mass of 60 kDa. The fraction gave a single band on a SDS-PAGE gel after silver staining (data not shown), and again showed a correspondence of an approximate mass of 60 kDa. The data of gel filtration and SDS-PAGE analysis indicate that the active form of the enzyme is a monomer of about 60 kDa, resembling to carrot β-glucosidase consisting of an about 46 kDa single polypeptide (Konno *et al.*, 1996). A detectable DIMBOA-G glucosidase activity other than this one was not observed in the fractions obtained during the purification.

Properties of the β-*glucosidase*

The maximum activity of the β-glucosidase was observed at pH 5.5. The enzyme activity was effectively inhibited by 0.1 mM castanospermine, an inhibitor of glucosidase (Saul *et al.*, 1983).

Substrate specificity of purified β-glucosidase was examined with naturally occurring glucosides and *p*-nitrophenyl-D-glycopyranosides. The lowest K_m value was observed for DIMBOA-G (0.07 mM) (Table II). DIBOA-G also acted as a substrate but the K_m value was much higher (0.52 mM). In terms of relative V_{max}/K_m, DIBOA-G was a poorer substrate than synthetic *p*-nitrophenyl-β-D-fucopyranoside. *p*-Nitrophenyl-β-D-glucopyranoside as a

Table I. Purification of DIMBOA-G β-glucosidase.

Purification step	Protein [mg]	Total activity [nkat]	Yield (%)	Specific activity [nkat/mg protein]	Purification factor (fold)
Crude extract	19.95	670	100	33.6	1
Cryoprecipitation	7.98	537	80	67.3	2.0
Cation exchange	0.164	332	50	2024	60.2
Gel filtration	0.015	171	26	11400	340

Table II. Michaelis-Menten constants for the hydrolysis of glycosides by maize DIMBOA-G glucosidase.

Substrate	K_m [mM]	Relative V_{max}	Relative V_{max}/K_m
DIMBOA-G	0.07	100	100
DIBOA-G	0.52	37	5.0
Salicin	n.d.[a]	n.d.	–
Esculin	n.d.	n.d.	–
p-Nitrophenyl-β-D-glucopyranoside	0.36	13	2.6
p-Nitrophenyl-β-D-fucopyranoside	0.28	72	18.0
p-Nitrophenyl-β-D-galactopyranoside	n.d.	n.d.	–
p-Nitrophenyl-β-D-cellobioside	n.d.	n.d.	–
p-Nitrophenyl-β-D-mannopyranoside	n.d.	n.d.	–

[a] n.d.: Not detected.

substrate was poorer than DIBOA-G, and other p-nitrophenyl-D-glycopyranosides including α-derivatives and naturally occurring glucosides, salicin and esculin, were barely hydrolyzed. These results suggested that the β-glucosidase isolated in this study is highly specific to DIMBOA-G.

Discussion

In maize seedlings, free DIMBOA occurred transiently with its glucoside. The fashion of appearance and disappearance was similar to that observed for benzoxazinones in juvenile wheat (Nakagawa et al., 1995), suggesting that the process is common to gramineae plants that produce hydroxamic acids.

The effective concentration of DIMBOA against deleterious microbes has been reported to be around 0.3 mM (Nakagawa et al., 1995), and thus the DIMBOA concentration detected in this study is thought to be enough for acting as a defense during the seedling stage of growth. Moreover, the appearance in the high level of hydroxamic acids possibly endows the seedlings with the ability to detoxify triazine herbicides.

A DIMBOA-G glucosidase activity occurred correlatively with the appearance and disappearance of DIMBOA. The activity/mg fr. wt in shoots was higher than in roots, being compatible with the higher concentration of DIMBOA in shoots than in roots. The purified β-glucosidase exhibited a high specificity for DIMBOA-G, and no other DIMBOA-G glucosidase activity was detected in the extracts. These results suggest that the occurrence of DIMBOA during the seedling stage is regulated by the expression of a single, specific β-glucosidase.

Previously, Esen (1991) has purified a β-glucosidase from 5- to 6-day-old seedlings grown in the dark, but relevant K_m values for benzoxazinone glucosides have not been determined. Cuevas et al. (1992) have detected a DIMBOA-G glucosidase in leaves of 6- to 16-day-old maize, but no clear correlation was observed between the accumulation of hydroxamic acids and the enzyme activity. The enzyme was not fully purified, but reportedly reacted with antibodies raised against that of Esen et al. (Babcock and Esen, 1994). The K_m values reported for the partially purified enzyme were 0.11 mM for DIMBOA-G and 0.17 mM for DIBOA-G. These values are apparently different from those determined in this study. However, the comparison of the kinetic data may be not feasible enough for knowing whether the present enzyme occurs only in the seedlings or the same enzyme as that in adult tissues is specifically activated, since they were determined under different buffer and temperature conditions with enzymes of different purification state. The problem remains to be elucidated in future.

Babcock G. D. and Esen A. (1994), Substrate specificity of maize glucosidase. Plant Sci. **101**, 31–39.

Bailey, B. A. and Larson, R. (1989), Hydroxamic acid glucosyltransferase from maize seedlings. Plant Physiol. **90**, 1071–1076.

Bradford M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Cuevas L., Niemeyer H. M. and Jonsson L. M. V. (1992), Partial purification of a hydroxamic acid glucoside β-D-glucosidase from maize. Phytochemistry **31**, 2609–2612.

Desai S. R., Kumar P. and Chilton W. S. (1996), Indole is an intermediate in the biosynthesis of cyclic hydroxamic acids in maize. Chem. Commun. 1321.

Esen A. (1991), Purification and partial characterization of maize (*Zea mays* L.) β-glucosidase. Plant Physiol. **98**, 174–182.

Frey M., Chomet P., Glawischnig E., Stettner C., Grün S., Winklmair A., Eisenreich W., Bacher A., Meeley R. B., Briggs S. P., Simcox K. and Gierl A. (1997), Analysis of a chemical plant defense mechanism in grasses. Science **177**, 696–699.

Glawischnig E., Eisenreich W., Bacher A., Frey M. and Gierl A. (1997), Biosynthetic origin of oxygen atoms in DIMBOA from maize: NMR studies with $^{18}O_2$. Phytochemistry **45**, 715–718.

Iwamura H., Nakagawa E. and Hirai N. (1996), Localization of benzoxazinones that occur constitutively in wheat seedlings. Z. Naturforsch. **51c**, 807–812.

Konno H., Yamasaki Y. and Katoh K. (1996), A β-glucosidase associated with cell walls from cell suspension cultures of carrot. Phytochemistry **43**, 1157–1161.

Leighton V., Niemeyer H. M. and Jonsson L. M. V. (1994), Substrate specificity of a glucosyltransferase and a N-hydroxylase involved in the biosynthesis of cyclic hydroxamic acids in Gramineae. Phytochemistry **36**, 887–892.

Nakagawa E., Amano T., Hirai N. and Iwamura H. (1995), Non-induced occurrence of cyclic hydroxamic acids in wheat during juvenile stage of growth. Phytochemistry **38**, 1349–1354.

Niemeyer H. M. (1988), Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defense chemicals in the Gramineae. Phytochemistry **27**, 3349–3358.

Saul R., Chambers J. P., Molyneux R. J. and Elbein A. D. (1983), Castanospermine, a tetrahydroxylated alkaloid that inhibits β-glucocerebroside. Arch. Biochem. Biophys. **221**, 593–597.

Zúñiga G. E., Argandoña V. H., Niemeyer H. M. and Corcuera L. J. (1983), Hydroxamic acid content in wild and cultivated Gramineae. Phytochemistry **22**, 2665–2668.