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

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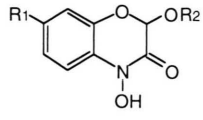
Occurrence and properties of hydroxamic acid glucoside glucosidase were investigated in 10-day-old, autotrophic maize (*Zea mays* L.) in which 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-G) is a major benzoxazinone component. Crude extracts of both leaves and roots showed glucosidase activity for both DIMBOA-G and 2,4-dihydroxy-1,4-benzoxazin-3-one glucoside (DIBOA-G). A cation-exchange chromatography after cryoprecipitation of the extract from leaves gave a peak with both activities, and further purification by ion-exchange and hydroxyapatite chromatography gave a fraction with an apparent homogeneity, the purification being 560 fold. The K_m values (mM) of the purified glucosidase were 0.16 for DIMBOA-G, 0.68 for DIBOA-G and 2.96 for *p*-nitrophenyl-β-D-glucopyranoside. The activity on salicin and esculin was too low to be detected. The data indicate that a glucosidase specific for DIMBOA-G comes into contact with constitutive benzoxazinone glucosides producing defensive aglycone when plants are damaged by microbial or insect attacks.

Introduction

Gramineae plants, including rye, maize and wheat, are known to accumulate hydroxamic acids, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its 7-methoxy analogue (DIMBOA), as glucosides (Niemeyer, 1988). The glucosides (DIBOA-G and DIMBOA-G) are readily hydrolyzed when plant tissues are destroyed by microbial or insect attack. Free hydroxamic acids exhibit fungistatic and bacteriostatic activity as well as antifeeding activity, and they have been implicated in the resistance of plants to deleterious microbes and insects. The benzoxazinones are also known to catalytically degrade *s*-triazine herbicides and thus responsible for their detoxification (Willard and Penner, 1976).



R1=H, R2=H	DIBOA
R1=H, R2=Glucosyl	DIBOA-G
R1=OMe, R2=H	DIMBOA
R1=OMe, R2=Glucosyl	DIMBOA-G

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

Recently, we have found in both wheat and maize that benzoxazinone glucosides occur in a high amount during the stage from germination to autotrophic growth, together with a lesser amount of free aglycones (Nakagawa *et al.*, 1995; Ebisui *et al.*, 1998). As the plants enter into the autotrophic growth stage, the glucosides decrease to a constant, lower level, and the aglycones become barely detectable. In maize, we have found that a DIMBOA-G specific glucosidase also occurs concurrently with the transient occurrence of benzoxazinones, suggesting that the specific glucosidase plays a role in microbial and insect attacks to produce defensive aglycones in a high amount during the vulnerable, juvenile stage of growth. In this study, we examined whether the glucosidase that comes into contact with the glucosides when autotrophic tissue is disintegrated is a specific one.

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Materials and Methods

Preparation of hydroxamic acids

DIMBOA-G was extracted from maize by methanol containing 2% acetic acid and DIBOA-G from rye. The purification was made by HPLC as described previously (Nakagawa *et al.*, 1995).

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 sowed in vermiculite and incubated at 25 °C with a 12-hr period of illumination with fluorescent lamps (60 W m^{-2}). Germination occurred 42–48 hr after seeding, the first leaf began to emerge from the sheath 72–86 hr after germination, and the plant height was 20–25 cm after 10 days from seeding.

Analysis of hydroxamic acids in plant material by HPLC

Leaves were macerated under liquid nitrogen and extracted with methanol containing 2% acetic acid. The solution was filtered through a disposable membrane filter (LCR13-LH, Millipore) and injected into a 4.6 x 150 mm metal-free Wakosil II 5C18 HG column (Wako) which was eluted with methanol-water (22:78, v/v) containing 0.1% acetic acid at 40 °C at a flow rate of 0.8 ml min^{-1}. The monitoring was made at 280 nm.

Purification of β-glucosidase

Leaves (13.5 g) of 10-day-old maize were frozen in liquid nitrogen and ground to powder with sea sand (40–80 mesh) and homogenized in 70 ml of 50 mM sodium acetate, pH 5.0. 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 for 24 hr at 4 °C, and then centrifuged at 12,000 x *g* for 15 min. The supernatant was loaded on an Accell Plus CM column (bed volume: 12 ml, Waters) that had been equilibrated with 50 mM sodium acetate, pH 4.8. The proteins were eluted stepwise with 100 ml of 50 mM sodium acetate, pH 5.5 and 7.0. The pH 5.5 fraction with DIMBOA-G glucosidase activity was loaded on a hydroxyapatite column (fast flow type, bed volume: 7 ml, Wako) that had been equilibrated with 50 mM potassium phosphate buffer, pH 6.8. The proteins were eluted stepwise with 100 ml of 300 and 500 mM potassium phosphate buffer, pH 6.8. The 300 mM fraction that had DIMBOA-G glucosidase activity was dialyzed against 10 mM tris(hydroxylmethyl)aminoethane/2-morpholinoethanesulfonic acid (Tris/MES) buffer (pH 6.5) for 18 hr and applied to a Mono-Q HR 5/5 column (Pharmacia) equilibrated with 10 mM Tris/MES buffer, pH 6.5. The proteins were eluted with a 0 to 300 mM NaCl linear gradient (200 ml) of 10 mM Tris/MES buffer (pH 6.5) at a flow rate of 1 ml/min. Active fractions were pooled, dialyzed against 10 mM potassium phosphate buffer (pH 6.8) for 18 hr, and loaded on a hydroxyapatite column (Bio-Scale CHT2-I, Bio-Rad). The proteins were eluted with potassium phosphate buffer (pH 6.8) in a gradient mode (10–60 mM, 0–4 ml; 60 mM, 4–24 ml; 60–500 mM, 24–60 ml). The fractions having DIMBOA-G glucosidase activity were used for characterization. Protein contents were estimated by the method of Bradford (1976) using bovine serum albumin as standard. All operations were carried out at 4 °C.

For assaying the glucosidase activity, a solution of appropriate amounts of enzyme preparation and DIMBOA-G in 500 µl of 100 mM citrate-200 mM phosphate buffer was incubated for 5 – 10 min at 30 °C. The reaction was stopped by adding 50 µl of 1N HCl, and the reaction products were analyzed by HPLC as described above.

Estimation of apparent molecular weight

The purified enzyme solution was loaded on a Superdex 200 HR 10/30 column (Pharmacia), and eluted with 50 mM sodium acetate buffer (pH 5.5) containing 100 mM NaCl at a flow rate of 0.5 ml/min. The following proteins were used for the calibration; human IgG (160 kDa), transferrin (81 kDa), ovalbumin (43 kDa), myoglobin (17.6 kDa).

Electrophoresis

Crude enzyme preparations and chromatographic fractions were subjected to SDS-PAGE analyses. Samples were electrophoresed through 0.75 mm thick gel slabs (10% resolving gels) according to the literature (Laemmli, 1970). The gel was stained with silver staining kit (Sil-Best Stain, Nakarai).

Characterization of β-glucosidase

Solutions of substrates and purified enzyme were prepared in 100 mM citrate-200 mM phosphate buffer (pH 5.5). 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 analysis. With *p*-nitrophenyl-β-D-glycopyranosides as substrates the reaction was stopped by adding 50 µl of 6N sodium hydroxide solution and the absorbance of the *p*-nitrophenol liberated was measured at 400 nm. Kinetic constants were obtained from Hanes-Woolf plots. For determination of pH optimum, 100 mM citrate-200 mM phosphate buffers adjusted to pH 4–7 were used.

Results

The 10-day-old maize used in this study was in a third leaf stage. The DIMBOA-G glucosidase activity and in a lesser extent DIBOA-G glucosidase activity were found in the extracts from both shoots and roots. For purification, the extracts from leaves were subjected to a selective cryoprecipitation at pH 4.6, and the activity of the pH 4.6 supernatnat mostly bound to an Accell CM column. The column was eluted at pH 5.5 and 7.0, the activity being found in pH 5.5 fraction. The active fraction was subjected to hydroxyapatite and Mono-Q column chromatographies, and finally again to hydroxyapatite column chromatography (Table I), giving a single active peak. The active fraction gave a single band of an approximate mass of 60 kDa on a SDS-PAGE gel after silver staining, and a gel filtration of the purified enzyme gave a single peak at 60–70 kDa, indicating that it is in a monomeric form. A detectable benzoxazinone glucoside glucosidase activity other than this one was not found in the fractions obtained during the purification.

The maximum activity of the glucosidase was observed at pH 5.5. Substrate specificity of the purified glucosidase was examined with the set of compounds shown in Table II. The lowest K_m (mM) and highest V_{max} (nkat/mg protein) values were observed for DIMBOA-G, 0.16 and 9480, respectively. DIBOA-G also acted as a substrate, but the K_m value (0.68 mM) was much higher and the V_{max} value (3460 nkat/mg protein) was much lower. Among the *p*-nitrophenyl-β-D-glycopyranosides tested, fucopyranoside gave a K_m value (0.44 mM) about the same as that of DIBOA-G but a V_{max} value (1110 nkat/mg protein) of about three times less. Glucopyranoside was far poorer substrate, and other glycosides including α-derivatives (data not shown) and naturally occurring salicin and esculin were barely hydrolyzed.

A careful examination of constitutive benzoxazinones revealed that on average the leaves contained 3.1 and 0.4 nmol/mg fr. wt of DIMBOA-G and DIMBOA, respectively. DIBOA-G and its aglycone were undetectable.

Discussion

The β-glucosidase found in autotrophic, 10-day-old maize was a highly specific one for DIMBOA-G, the specificity being as high as that of the glucosidase found in 3-day-old, non-autotrophic plant (Ebisui *et al.*, 1998). This was the only DIMBOA-G glucosidase found in the fractions during the purification. Apparently the plant provides a highly specific glucosidase against deleterious microbes and insects, as well as non-autotrophic

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

Purification step	Protein [mg]	Total aktivity [nkat]	Yield (%)	Specific activity [nkat/mg protein]	Purification factor (fold)
Crude extract	19.8	87	100	4.4	1
Cryoprecipitation	12.4	55	63	4.4	1
Cation exchange (Accell CM)	1.15	30	35	26	6
Hydroxyapatite	0.71	30	34	41	10
Anion exchange (Mono Q)	–	7.5	8.7	–	–
Hydroxyapatite	0.0003	0.8	0.9	2450	560

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

Substrate	K_m [mM]	V_{max} [nkat/mg protein]	Relative V_{max}/K_m
DIMBOA-G	0.16	9480	100
DIBOA-G	0.68	3460	8.6
Salicin	n.d.[a]	n.d.	–
Esculin	n.d.	n.d.	–
p-Nitrophenyl-β-D-glucopyranoside	2.96	900	0.51
p-Nitrophenyl-β-D-fucopyranoside	0.44	1110	4.3
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 detectable.

seedlings (Ebisui *et al.*, 1998). The lower activity for DIBOA-G may be consistent with the fact that the predominant benzoxazinone species in maize is DIMBOA-G (Niemeyer, 1988).

Glucosidases with pronounced specificity have been increasingly postulated to be involved in plant's defense mechanism, e.g. conversion of biologically inactive avenacosides and isoflavone and cinnamoylalcohol glucosides to active analogues, and degradation of cyanogenic glucosides to release HCN, upon disintegration of the tissues (Hösel and Conn., 1982; Osbourn, 1996). The present enzyme is considered to basically fall into this category of enzymes.

Knowledge has also been accumulated that glucosidases having the specificity for aglycones rather than sugar moieties are involved in liberation of a certain aglycone with a physiological objective; e.g. releases of certain secondary metabolites and plant hormones like gibberellins and cytokinins from their stocks (Hösel and Todenhagen, 1980 and references therein; Hedden *et al.*, 1978; Jameson, 1994). In autotrophic Gramineae, benzoxazinone species present have been believed to be only glucosides (Niemeyer, 1988). Recently, however, Zúñiga and Massardo (1991) have reported that four cultivars of 7-day-old wheat contained in epicotyl tissue 1 – 3 nmol/mg fr. wt DIMBOA. A minute examination in this study revealed that the 10-day-old maize contained 0.4 nmol/mg fr. wt on average of DIMBOA. The level is one 30th that of 3-day-old, non-autotrophic plant (Ebisui *et al.*, 1998) but still fugistatic (Nakagawa *et al.*, 1995). Considering the approximate 8 times higher level of DIMBOA-G (3.1 nmol/mg fr. wt), the glucosidase is thought to be not in full contact with the glucoside in the intact tissue. It has been reported that in maize DIMBOA-G measured as DIMBOA equivalent after hydrolysis of the extracts is found in an extravacuolar compartment and vascular bundle tissues in a ratio of about 2:1, while DIMBOA-G glucosidase activity appears in vacuolar fraction and in one 7th extent in vascular bundles (Massardo *et al.*, 1994). Separate analyses of the glucoside and aglycone may give a clue to elucidate the mechanism of constitutive occurrence of the aglycone.

Previously, Esen (1992) has isolated a β-glucosidase from maize seedlings using *p*-nitrophenylglucoside as substrate. The enzyme was reportedly active on DIMBOA-G but a relevant K_m value has not been determined (Babcock and Esen., 1994). Cuevas *et al.* (1992) have also partially purified a DIMBOA-G glucosidase from maize leaves. The K_m value for DIMBOA-G (0.11 mM) was consistent with that of the present study, but those for DIBOA-G (0.17 mM) and *p*-nitrophenylglucoside (0.46 mM) were different. The buffer and temperature conditions, as well as the purification state, were different, and thus the comparison of the kinetic data for three substrates only may allow for a conclusion that the present enzyme has a different specificity performance. However, the apparent molecular mass of the previous enzyme has been reported to be 160 kDa in a native state (Cuevas *et al.*, 1992), while the present glucosidase is a 60 kDa monomeric protein. This difference cannot be explained at the moment.

The K_m values of the present enzyme for DIMBOA-G and *p*-nitrophenylglucoside were about twice and 6 times higher than those of the one isolated from a non-autotrophic plant, respectively (Ebisui *et al.*, 1998). Other properties such as specificity for the substrates listed in Table II and molecular mass were similar. Amino acid sequencing is under way to answer the question whether they are the same enzyme or different ones with the same physiological function.

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