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PARTIAL PURIFICATION AND CHARACTERIZATION OF A HYDROXAMIC ACID GLUCOSIDE β-D-GLUCOSIDASE FROM MAIZE

LILIANA CUEVAS, HERMANN M. NIEMEYER and LISBETH M. V. JONSSON*

Departamento de Química, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile; *Department of Plant Physiology, Swedish University of Agricultural Sciences, Box 7047, S-750 07 Uppsala, Sweden

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Abstract—β-Glucosidase activities measured in extracts from maize leaves during development gave different curves when *p*-nitrophenyl β-D-glucopyranoside (PNP-Glc) or hydroxamic acid glucoside (Hx-Glc) were the substrates. The PNP-Glc glucosidase had a M_r of 60 000 and an isoelectric point of 6.4, whereas the Hx-Glc glucosidase had a M_r of 158 000 and an isoelectric point of 4.8. This latter enzyme had an optimum pH of activity at 6.0, was inhibited by castanospermine and, when partially purified, accepted PNP-Glc as well as Hx-Glc as substrates, albeit with a lower activity for the former.

INTRODUCTION

Hydroxamic acids derived from 4-hydroxy-1,4-benzoxazin-3-one are secondary compounds produced in cereals such as maize, wheat and rye [1], and in other Gramineae [2–4]. They are present in the intact plant as 2-*O*-β-D-glucopyranosides [5, 6]. Upon disruption of the tissue, for example by phytophagous or sucking insects, aglucones are liberated which are chemically labile [7–10] and of higher toxicity than the parent glucosides [11, 12].

Hydroxamic acids play a major role in the defence of the plant against insects such as the European corn borer *Ostrinia nubilalis* [13, 14], the western corn rootworm *Diabrotica virgifera virgifera* [15] and cereal aphids [16–20]. The concentrations of hydroxamic acids in a given tissue of the plant and in the plant as a whole decrease as the tissue or plant age, thereby rendering the plant more susceptible to aphid attack [17]. Accumulation of hydroxamic acids is a dynamic process resulting from their biosynthesis as well as from their degradation. The lability of the aglucones suggests that the initial step in the degradation process is the hydrolysis of the naturally-occurring glucoside. Thus, the β-D-glucohydrolysis may be a decisive factor in regulating the concentration of hydroxamic acids. We therefore investigated, in maize, the β-D-glucosidase activity during the development of the plant. One β-D-glucosidase was partially purified and shown to be specific for hydroxamic acid glucosides.

RESULTS AND DISCUSSION

β-Glucosidase activities in maize leaves during early developmental stages

Earlier work on wheat showed that the concentration of hydroxamic acids differed between the different leaves in a given plant, and that the oldest leaf always had the lowest concentration [19, 21]. The β-glucosidase activity of separate extracts from the first two leaves of plants up to the three-leaf stage was determined using both the artificial substrate *p*-nitrophenyl β-glucopyranoside (PNP-Glc) and 2-*O*-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc), which is the main hydroxamic acid in maize [22].

The results from determinations of specific activities are shown in Fig. 1. Using PNP-Glc as substrate, the β-D-glucosidase activity was much higher in the samples from the first leaf taken at days 6 and 7, than in any samples

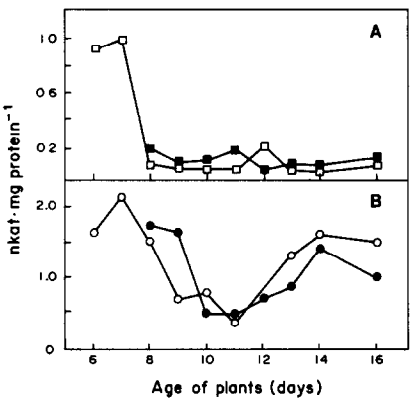

Fig. 1. β-D-Glucosidase activities in enzyme extracts from leaves of maize during development of the plant. Each value represents the average of two to three leaves which were taken from different plants of the same age. (A) PNP-Glc glucosidase activity; (B) DIMBOA-Glc glucosidase activity; open symbols: first (oldest) leaves; closed symbols: second leaves. Plant age is given with sowing as day 0.

from the following days. Otherwise there was neither a tendency towards an increase or decrease in β-D-glucosidase activity, nor a difference between the leaves which depended on their order of appearance.

When DIMBOA-Glc was used as substrate, the activities in first-leaf samples taken at day 6 and day 7 were not as different from leaves of other ages as in the case with the artificial substrate. Activity in both leaves exhibited similar behaviour with plant age. During the period of investigation, the specific activities first decreased and then again increased.

Our measurements of β-D-glucosidase activity in maize leaves covered the very early stages of plant development, when the concentration of hydroxamic acids is still high [21]. If the level of β-D-glucosidase activity was a limiting factor in the degradation process, one would expect no activity or very low activity during the first days, and an increase of activity with time. Our results did not support this concept, since DIMBOA-Glc glucosidase activity was found in all samples, and with no clear tendency towards an increase with time.

The curves of β-glucosidase activity vs plant age had different shapes when PNP-Glc or DIMBOA-Glc was the substrate. This suggested that the two types of β-D-glucosidase activities were caused by different enzymes.

Separation of DIMBOA-Glc glucosidase and PNP-Glc glucosidase

Enzyme preparations from maize leaves were submitted to separation on chromatofocusing and gelfiltration columns in order to find out whether DIMBOA-Glc glucosidase activity could be separated from other β-glucosidases. β-Glucosidase activities were analysed in fractions eluted from the columns using PNP-Glc and DIMBOA-Glc as substrates.

The results from the gel filtration experiment are shown in Fig. 2. With the artificial substrate PNP-Glc, which was expected to reveal all β-glucosidases, only one peak of activity was found. The calibration curve from the column, obtained with reference proteins of known M_r indicated that the M_r of this enzyme was 60000. The measurements of DIMBOA-Glc glucosidase activity also gave one peak, but one that was clearly separated from the PNP-Glc glucosidase. The top fraction for DIMBOA-Glc glucosidase activity corresponded to a M_r of 158000. Likewise, the separation based on isoelectric points of proteins, resulted in two β-glucosidase peaks: one with DIMBOA-Glc glucosidase activity and one with PNP-Glc glucosidase activity (Fig. 3). The isoelectric points of the two proteins, as deduced from the pH gradient, were pH 4.8 and pH 6.4, respectively.

Many plant β-glucosidases are glycoproteins, and bind to lectins such as concanavalin A. Attempts to separate the maize β-glucosidases on a concanavalin A–Sepharose column were however not successful, since both PNP-Glc glucosidase and DIMBOA-Glc glucosidase activities passed through the column under our experimental conditions.

Properties of DIMBOA-Glc glucosidase

The fractions from the chromatofocusing column which exhibited DIMBOA-Glc glucosidase activity were pooled, desalted and concentrated. After this treatment, glucosidase activities were restored, such that the total

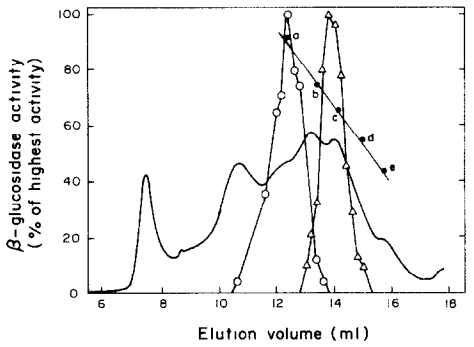

Fig. 2. β-Glucosidase activities from maize leaves separated by gel filtration. The enzyme sample contained 0.8 mg protein, 83 pkat DIMBOA-Glc glucosidase activity and 200 pkat PNP-Glc glucosidase activity in a total volume of 0.2 mg. (△—△) = PNP-Glc glucosidase activity, 100% = 22 pkat/fraction (total recovery 60%); (○—○) = DIMBOA-Glc glucosidase activity, 100% = 7 pkat/fraction (total recovery 40%); (——) = A_{280} The calibration (log/lin) graph indicates M_r and elution volumes of reference proteins (M_r): a = aldolase (158000); b = bovine serum albumine (67000); c = ovalbumin (43000); d = chymotrypsinogen (25000); e = ribonuclease a (13700).

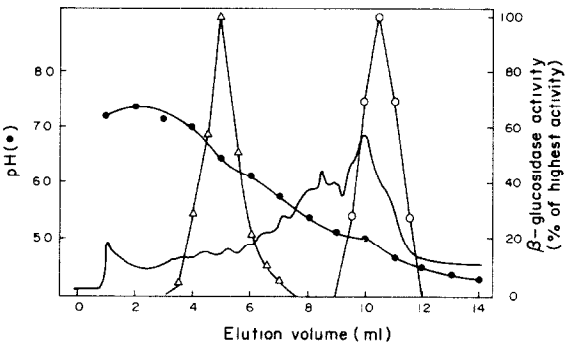

Fig. 3. β-Glucosidase activities from maize leaves separated by chromatofocusing. The enzyme sample contained 3.6 mg protein, with 767 pkat of either PNP-Glc glucosidase or DIMBOA-Glc glucosidase activity in 3.5 ml. (△--△) = PNP-Glc glucosidase; 100% = 275 pkat/fraction (total recovery 45%); (○ -○) = DIMBAO-Glc glucosidase activity, 100% = 2 pkat/fraction (total recovery 1%). (——) = A_{280}.

recovery of DIMBOA-Glc glucosidase activity increased from 1 to 5%. PNP-Glc glucosidase activity, which was not detectable in the original fractions, could now be detected. The preparation was used to further characterize the hydroxamic acid glucoside β-D-glucosidase.

With either DIMBOA-Glc or PNP-Glc as substrate, the enzyme showed an activity optimum at pH 6.0, with half maximal activities at pH 4.3 and 7.7. The enzyme was efficiently inhibited by castanospermine, an alkaloid which has been shown to inhibit β-glucosidases in general, and among them β-glucosidases acting upon glucosinolates and cyanogenic glucosides [23–25]. A complete inhibition of DIMBOA-Glc glucosidase was obtained at 100 μM castanospermine, and 50% inhibition at a concentration *ca* 20 μM.

The substrate specificity of the enzyme was investigated using PNP-Glc and two different hydroxamic acid glucosides as substrates: DIMBOA-Glc and its demethoxylated analogue 2-*O*-β-D-glucopyranoxyl-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc). The latter compound is the main hydroxamic acid glucoside in rye [3, 26] and occurs at low concentrations in maize [22]. The relative maximal activity was determined with either substrate at saturating concentrations, and K_m values were obtained from V/S curves. The results are shown in Table 1. Among the substrates tested, both the lowest K_m value and the highest relative activity were obtained with DIMBOA-Glc.

A number of plant β-glucosidases acting upon secondary compounds have been characterized [27, 28]. Their substrates belong to different chemical families such as cyanogenic glucosides, glucosinolates, flavonoids, alkaloids, steroids and phenols. From a viewpoint of function, the β-glucosidic cleavage of hydroxamic acid glucosides resembles mostly that of cyanogenic glycosides and glucosinolates. These compounds, like the hydroxamic acid glucosides, are involved in plant defence, and after β-glucosidase action they give rise to more toxic compounds [11, 27, 29].

The β-glucosidases acting on cyanogenic glycosides and glucosinolates all possess acidic pH optima (pH 4.0–6.2). Most of them are glycoproteins, and many occur in multiple forms. The degree of substrate specificity varies. The β-glucosidase in *Hevea brasiliensis* [30], *Linum usitatissimum* [31] and *Lepidium sativum* [23], were found to hydrolyse an artificial substrate with similar efficiency as the natural substrate. Others, such as those from *Sorghum bicolor* and *Triglochin maritima* gave 3% or less activity with an artificial substrate as compared to that with the natural substrate [28].

At present we have no evidence that the hydroxamic acid glucoside glucosidase in maize is a glycoprotein or occurs in multiple forms, but other properties were similar to the above mentioned enzymes. The maize enzyme exhibited an optimum at pH 6.0 and was inhibited by castanospermine at very low concentrations, like the β-glucosidases from cress or black cherry [23–25].

With regard to the substrate specificity, the maize enzyme belongs to the group of β-D-glucosidases which accept several substrates with good efficiency: both hydroxamic acid glucosides DIMBOA-Glc and DIBOA-Glc, and PNP-Glc were accepted as substrates.

In conclusion, our results strongly indicate that maize contains a β-D-glucosidase specific for hydroxamic acid glucosides. Whether this is a common feature of plants which accumulate hydroxamic acid glucosides remains to be established. In maize, the level of β-D-glucosidase activity is high even at young developmental stages and therefore seems not to be important for the regulation of the accumulation of hydroxamic acid glucosides. The functional analogy with β-glucosidases hydrolysing cyanogenic glucosides and glucosinolates, suggests that the enzyme might rather be a part of a defence mechanism which is activated when the tissue is damaged and the enzyme comes into contact with the substrate. The question of the subcellular and tissue localization of this enzyme is therefore of special interest.

EXPERIMENTAL

Plant material. Maize plants (*Zea mays* L. cv. T 555) were cultivated at 25° in a growth chamber, with a photoperiod of 16 hr light and 8 hr darkness.

Chemicals. DIMBOA and DIMBOA-Glc were isolated from *Z. mays* and DIBOA and DIBOA-Glc from *Secale cereale* cv. Forrajero. The aglucones were isolated from ethereal extracts as described [32], and the glucosides from boiling MeOH extracts as described [33]. The purity of the compounds was verified by UV, IR, NMR, HPLC and TLC.

Enzyme assays. PNP-Glc glucosidase assay: for the developmental curve, the assay was carried out at 25°. The reaction mixture contained 0.1 M of succinic acid pH 5, 0.6 mM PNP-Glc and 0.05 ml enzyme in 0.5 ml. In the other experiments, incubations were at 30°. Unless otherwise indicated, they contained 0.1 M of Bis–Tris buffer pH 6, 0.6 mM PNP-Glc and 0.04 ml enzyme in 0.4 ml. Incubation times varied between 5 min and 3 hr. The incubations were stopped by addition of 1 M Na_2CO_3 to 1 ml, and the amount of liberated *p*-nitrophenol was determined from measurements of A_{405}.

DIMBOA-Glc glucosidase assay: the standard assay mixture consisted of 10 μl of 3 mM DIMBOA-Glc solubilized in 5 mM HCl, 40 μl of 0.1 M Bis–Tris buffer pH 6, and enzyme in a total vol of 100 μl. After incubation at 30° for various times between 5 min and 3 hr, 400 μl of $CHCl_3$–MeOH–HOAc (66:33:1) was added. The mixtures were vortexed and then centrifuged for 1 min at 10000 *g*. Part of the upper (MeOH–H_2O) phase was analysed using HPLC as described below. Glucosidase activity was evident from the decrease of the glucoside substrate and concomitant appearance of the corresponding aglucone. The latter was found in both the upper and lower phase after partition, whereas almost 100% of the glucoside was found in the upper phase. The conversion was therefore quantified by measuring the decrease of substrate in incubations containing enzyme, compared with incubations with buffer instead of enzyme. Conditions employed with DIBOA-Glc as substrate were similar to those with DIMBOA-Glc.

Table 1. Kinetic properties of partially purified hydroxamic acid glucoside glucosidase from maize

Substrate	Relative maximal activity (pkat mg^{-1} protein)	Concentration* (mM)	K_m (mM)
DIMBOA-Glc	360	0.3	0.11
DIBOA-Glc	120	0.4	0.17
PNP-Glc	180	1.8	0.46

*Concentration of saturated solution.

All determinations of enzyme activities were carried out in duplicates which varied in the time for incubation. For measurements of activities at different pH values, citrate buffer was used between pH 4 and 5.5 and K-Pi buffer between pH 6 and 8.

Protein determination. Protein contents was determined according to ref. [34], with bovine serum albumin as standard.

HPLC analysis. The compounds were separated on a C18 Nucleosil column (particle size 5 μM; dimensions 150 × 4.6 mm). Isocratic elution was carried out at room temp. with 1 ml min^{-1} of H_2O–MeOH–HOAc (61 : 38 : 1).Under these conditons, the elution times for DIMBOA-Glc and DIMBOA were 3.0 and 3.6 min, and for DIBOA-Glc and DIBOA, 2.7 and 3.4 min, respectively Detection was carried out at 262 nm. For quantification, molar absorptivities used were 7570 for DIBOA-Glc [35] and 10000 for DIMBOA-Glc.

Enzyme extraction. All steps were carried out at 4° or on ice. Maize leaves were homogenized using mortar and pestle, with 100 mM K-Pi buffer pH 7.5, 50 g l^{-1} PVPP, 16 mM β-mercaptoethanol as extraction buffer (5 ml buffer per g fr. wt of leaves). The homogenate was filtered through a single layer of cheese cloth, and the filtrate centrifuged at 20000 *g* for 20 min. The supernatant contained the major part of β-glucosidase activity. For the development curves, the supernatants were used as enzyme samples, after filtration through Sephadex G25 M columns eluted with 25 mM K-Pi buffer pH 7.5, 16 mM β-mercaptoethanol.

Enzyme purification. Chromatography with concanavalin A-Sepharose. part of a supernatant sample (in 0.1 M citrate buffer pH 6.5 containing 0.25 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$, and after gel filtration on a Sephadex G25 M column) was applied to a column of concanavalin A-Sepharose (5 × 1.6 cm) and eluted with the same buffer at a flow rate of 0.5 ml min^{-1}. Enzyme activities were assayed in the void vol fractions and in fractions eluted from the column with buffer containing 0.1 M methyl α-D-glucopyranoside.

Gel filtration: proteins were salted out from a supernatant sample by addition of $(NH_4)_2SO_4$. The fraction precipitating between 60 and 30% satn of $(NH_4)_2SO_4$ was collected by centrifugation at 20000 *g* for 15 min. Part of this fraction was desalted on a Sephadex G25 M column, eluted with 0.1 M K-Pi buffer pH 7.5 and then used for gel filtration on a Superose 12 HR 10/30 column. Elution was carried out with 0.1 M K-Pi buffer pH 7.5 at a flow rate of 0.4 ml min^{-1} Fractions of 0.2 ml were collected and assayed for enzyme activity.

Chromatofocusing: part of a 60 to 30% $(NH_4)_2SO_4$ fraction as described above was desalted on a Sephadex G25 M column, eluted with 25 mM Bis–Tris–iminodiacetic acid buffer pH 7.1. This preparation was applied to a Mono P HR 5/5 column. Proteins were eluted at a flow rate of 0.5 ml min^{-1} using polybuffer 74-imino diacetic acid pH 4. Fractions of 0.5 ml were collected in tubes to which 50 μl 1 M Tris buffer pH 8 had been added.

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