SUBSTRATE SPECIFICITY OF A GLUCOSYLTRANSFERASE AND AN N-HYDROXYLASE INVOLVED IN THE BIOSYNTHESIS OF CYCLIC HYDROXAMIC ACIDS IN GRAMINEAE

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Key Word Index—Gramineae; glucosyltransferase; hydroxamic acids; 1,4-benzoxazin-3-ones; N-hydroxylase; DIMBOA; DIBOA; HBOA; HMBOA.

Abstract—Microsomal preparations from maize seedlings exhibited N-hydroxylase activity with 2-hydroxy-1,4-benzoxazin-3-one (HBOA) as substrate, but not with its 7-methoxy analogue (HMBOA), or their corresponding 2-O-β-D-glucosides. Extracts of the hydroxamic acid (Hx)-accumulating species rye, wheat and Hordeum lechleri, showed UDP-glucose:Hx-glucosyltransferase activity. The hydroxamic acid, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), and its 7-methoxy analogue, DIMBOA, were accepted as substrates, but not HBOA or HMBOA. The Hx-glucosyltransferase in the protein precipitate obtained between 30 and 60% ammonium sulphate saturation from either rye, wheat or H. lechleri had a higher $V_{max}$ value and lower $K_m$ value with DIMBOA as substrate. The Hx-glucosyltransferase from rye, which occurred in both roots and shoots throughout plant development, was purified 35-fold and characterized. The $M_r$ of the enzyme was 43 000 and the isoelectric point 4.4. The $K_m$ values for DIBOA and DIMBOA in the partly purified fraction were 73 and 82 μM, respectively, and the $V_{max}$ for DIMBOA twice that for DIBOA. The results indicate that the glucosides of HBOA and HMBOA are not intermediates in the pathways to Hx-glucosides, and that the Hx-glucosyltransferases from species with different patterns of Hx-accumulation, are similar.

INTRODUCTION

Seedlings of several Gramineae, including rye, maize and wheat, accumulate glucosylated cyclic hydroxamic acids (Hx-Glc) derived from 2,4-dihydroxy-1,4-benzoxazin-3-one (HBOA) [1]. These compounds are involved in the defence of the plant against a wide variety of organisms including bacteria [2], fungi [3, 4] and a range of insects [5–7].

The presence of Hx in maize was related to resistance to the first brood of the European corn borer. This resistance was enhanced by increasing Hx concentrations in the plant through breeding [8, 9]. In the case of aphids, Hx combine within the same molecule, the properties of a behavioral pest control agent and a physiological pest control agent, thus providing the possibility of an efficient way to aphid control [10]. Hx have been proposed as targets for wheat breeding aimed at aphid-resistant cultivars [11].

The possibility of using genetic engineering techniques for manipulating the accumulation of Hx in plants could be greatly increased by knowledge about their biosynthesis and degradation. The biosynthesis of Hx has not yet been fully explored; the present information comes only from work on maize. Biogenetic studies with radiolabelled precursors indicated that the aromatic ring is derived from an intermediate in the shikimic acid pathway, and that the two heterocyclic ring carbon atoms are derived from ribose [13]. Later work showed that radioactivity from anthranilic acid was recovered in Hx, and it was suggested, that in analogy with the biosynthesis of tryptophan, N-(5′-phosphoribosyl)anthranilate might be an intermediate in the pathway [14].

The immediate precursors of Hx are the corresponding lactams, which also accumulate in the plants, but in lower amounts than the Hx [15]. The proposed biosynthetic relationships between lactams and Hx are shown in Fig. 1. The conversion of HBOA (2-hydroxy-1,4-benzoxazin-3-one) to DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) was deduced from radiolabel incorporation experiments with [14C]HBOA [14], and was later demonstrated in vitro [16]. The other steps indicated in Fig. 1, i.e. the methoxylation of either HBOA to HMBOA (7-methoxy-2-hydroxy-1,4-benzoxazin-3-one) or DIBOA to DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), and the conversion of HMBOA to DIMBOA, as well as the reverse reaction, might all be deduced from radiolabel incorporation experiments [14], but none of them has as yet been confirmed in vitro. Figure 1 shows the compounds as aglucones, but both lactams and Hx accumulate in the intact plant as 2-O-β-D-glucosides. Hence, it is possible that the lactam glucosides rather than the aglucones are intermediates in the biosynthetic pathway.
In the present work, we have focussed on the role of the lactam glucosides, and on glucosylating enzymes in the pathway to Hx glucosides. Firstly, we examined whether the lactam glucosides are substrates of an N-hydroxylation reaction. Secondly, we investigated the substrate specificity of glucosyltransferases in the pathway to Hx-glucosides. Since the lactam glucosides were the substrates for the conversion of lactams into Hx, one would expect specificity of glucosyltransferases in the pathway to Hx-glucosyltransferase activity. Furthermore, since maize accumulates both DIBOA and lactams are found in the intact plant as 2-O-/1-D-glucopyranosides. Hx-glucosyltransferases of Hx-accumulating plants might concentrate on rye, a species which accumulates Hx-glucosides in its leaves.

In view of the rather high apparent $K_m$ values reported for DIBOA, we speculated that lactams might be better substrates for one or for both of the Hx-glucosyltransferases. Furthermore, since maize accumulates both DIBOA and HMBOA-glucoside in its leaves, it is possible, but that the rate of the reaction under the conditions employed in our assays, is too low to be detected.

RESULTS AND DISCUSSION

Substrate specificity of microsomal lactam N-hydroxylase

Microsomal preparations isolated from wheat, maize and rye, were incubated with the putative substrates, HBOA, HMBOA, HBOA-glucoside and HMBOA-glucoside. Lactam N-hydroxylation activity could only be detected in maize microsomes, but only with HBOA as the substrate. With HBOA as substrate, an activity of 74 pkat mg$^{-1}$ protein was found after 7 h of incubation. No product was found with any of the other substrates, even when incubations were carried out overnight, at pH 6.5, 7.5 or 8.5. Microsomal preparations from wheat (Triticum aestivum cv Kadett) or from three cultivars of rye (Secale cereale cvs Tetra Baer, Petkus II and Rogo) seedlings, did not exhibit lactam N-hydroxylating activity with any of the substrates. The possible presence of inhibitory substances in the preparation was tested by adding rye microsomes to incubations with maize microsomes. This addition, however, had no effect on the HBOA N-hydroxylating activity of maize. Therefore, at present, we cannot explain the negative results from the experiments with rye and wheat.

The results from the experiments with maize indicate that the lactam aglucones and not the lactam glucosides, are the intermediates in the pathway to Hx and, furthermore, that HBOA is a much better substrate than HMBOA. In our assays, HMBOA was not a substrate for the N-hydroxylase reaction. However, since $[14C]$HMBOA was to some extent converted into $[14C]$HMBOA in incorporation experiments [14], we presume that a transformation of HMBOA to DIMBOA is possible, but that the rate of the reaction under the conditions employed in our assays, is too low to be detected.

Hx-glucosyltransferase in rye: assay conditions and expression during development

For the work on glucosyltransferases, the conditions established for enzyme assays with rye extracts insured that no $\beta$-glucosidase activity was present. Enzyme extracts from rye exhibited Hx-glucosyltransferase activity, which was completely recovered in the supernatant after centrifugation at 100 000 g, and was precipitated between 30 and 60% ammonium sulphate saturation (data not shown).

The Hx-glucosyltransferase in rye seedlings, similarly to the maize enzyme(s) showed the highest activity at pH 8.5 [17], and 45°C; half-maximal activity was obtained at ca pH 6.7. Our incubations were carried out at 37°C, which produced 92% of the activity found in incubations at 45°C.

By analogy with the maize enzymes [17], the rye Hx-glucosyltransferase activity was stimulated by addition of $\beta$-mercaptoethanol and MgCl$_2$. With 5 mM $\beta$-mercaptoethanol, activity was 400% that of controls, and with 1 mM MgCl$_2$, 145% of the controls. These compounds were, therefore, included in the assay.

Enzyme activity was detected both in roots and shoots of the seedlings (Fig. 2). Samples extracted at different times during plant development, between days 4 and 20 after sowing, gave different specific activities. In the shoots, but not in the roots, a tendency to an increase in specific activity was observed at later stages. Hx content
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Fig. 2. Hx-glucosyltransferase activity (---), and Hx-content (-----) in rye during the development of the plant. A, shoots; B, roots. Bars represent standard deviations of the mean.

also showed variations during plant development, with a tendency to decrease with age in the leaves with no well defined pattern in the roots.

Table 1. Kinetic properties of Hx-glucosyltransferase from different Gramineae species

<table>
<thead>
<tr>
<th>Species</th>
<th>Main Hx in seedling</th>
<th>App. ( V_{\text{max}} ) (pkat)</th>
<th>App. ( K_m ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Triticum aestivum</em> cv. Millaleu</td>
<td>DIMBOA</td>
<td>33</td>
<td>239, 121</td>
</tr>
<tr>
<td><em>Secale cereale</em> cv Tetra Baer</td>
<td>DIBOA</td>
<td>72</td>
<td>233, 221</td>
</tr>
<tr>
<td><em>Hordeum lechleri</em> PI H1310</td>
<td>DIBOA</td>
<td>22</td>
<td>264, 87</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em> cv Libra</td>
<td>none</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>Avena sativa</em> cv Nahuen</td>
<td>none</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d.: Activity not detected (detection limit = 0.2 pkat).

Concentrations used were 0.0625, 0.125, 0.25, 0.5 and 1 mM Hx and 1 mM UDPG. \( K_m \) and \( V_{\text{max}} \) values were determined from Lineweaver-Burk plots. Correlation coefficients were 0.96 for DIMBOA and 0.98 for DIBOA in *T. aestivum*; 0.99 for DIMBOA and 0.998 for DIBOA for *S. cereale*; and 0.96 for DIMBOA and 0.97 for DIBOA for *H. lechleri*.

Extracts from the three Hx-containing species, wheat, rye and the wild barley *Hordeum lechleri*, exhibited glucosyltransferase activity, whereas extracts from cultivated barley (*H. vulgare*) or oats (*Avena sativa*), which do not accumulate Hx, did not. This indicates that the measured activity is specific for the Hx pathway. The Hx-glucosyltransferase activity from the species which accumulate Hx, either DIMBOA (wheat) or DIBOA (rye and wild barley) as the main Hx, all showed higher maximum activity with DIMBOA than with DIBOA as substrate, and a lower apparent \( K_m \) value for DIMBOA than for DIBOA.

The results obtained, therefore, suggested that the different species contained the same type(s) of Hx-glucosyltransferase(s). Our \( K_m \) values for DIBOA of 0.23, 0.24 and 0.26 mM for rye, wheat and wild barley, respectively, were lower than the value reported for maize of 0.64 mM [17]. The difference in relative activity of our enzyme between DIBOA and DIMBOA (ca 150%) was however, in accordance with one of the enzymes from maize, which showed with DIMBOA as substrate ca 175% of the activity with DIBOA as substrate.

Crude extracts and ammonium sulphate precipitates from maize, wheat and rye were tested for lactam glucosylating activity, with both HBOA and HMBOA as the substrate. No lactam glucosylating activity was detected even after prolonged incubations (60 min for HBOA and 120 min for HMBOA) or at different pH values (6.5, 7.5 and 8.5). These results support the conclusion that the lactam aglucones are the intermediates in the pathway to Hx and, thus, the glucosylation of Hx would be the last step of the pathway. However, the question of how the lactam glucosides are formed is left open. Possibly, a reduction of Hx to lactam, as was indicated in incorporation experiments [14], is operating under certain conditions.

Partial purification of Hx-glucosyltransferase from rye and molecular properties of purified enzyme

The protein precipitate obtained between 30 and 60% ammonium sulphate saturation may contain several Hx-glucosyltransferases, and, in order to detect a possibly
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Table 2. Purification of Hx-glucosyltransferase from rye seedlings using DIBOA as substrate

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (nkat)</th>
<th>Protein (mg)</th>
<th>Specific activity (pkat mg⁻¹ protein)</th>
<th>Yield (%)</th>
<th>Enrichment (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulphate 60/30%</td>
<td>83.0</td>
<td>1245</td>
<td>67</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>62.4</td>
<td>464</td>
<td>135</td>
<td>75</td>
<td>2</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>29.9</td>
<td>40</td>
<td>748</td>
<td>36</td>
<td>12</td>
</tr>
<tr>
<td>Mono Q</td>
<td>17.6</td>
<td>15</td>
<td>1174</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>Superdex 75</td>
<td>1.3</td>
<td>0.34</td>
<td>2375</td>
<td>1.6</td>
<td>35</td>
</tr>
</tbody>
</table>

Starting material: 288 g fr. wt of leaves of 14-day-old rye seedlings (S. cereale cv Tetra Baer).

species-specific one, the preparation from rye was subjected to several purification steps, resulting in a 35-fold purification. At the degree of purity achieved, the enzyme was stable. The purified preparation retained 70% of its initial activity for as long as three months, when kept at −20 °C in 20 mM Tris–HCl buffer pH 7, containing 10% glycerol. The results from the partial purification are shown in Table 2. Throughout the whole procedure only one peak of Hx-glucosyltransferase was observed.

An aliquot of the Mono Q fraction was used to estimate the isoelectric point by means of chromatofocussing in a Mono P column. The analysis indicated an isoelectric point at pH 4.4. Gel filtration through Superdex 75 allowed the estimation of the M₉ of the protein as 43,000.

The purified Hx-glucosyltransferase was characterized with regard to kinetic properties (Table 3). The Hx-glucosyltransferase did not glucosylate the lactams HBOA or HMBOA, nor the structurally related hydroxycoumarin, esculetin.

The apparent Kₘ values obtained with the partially purified enzyme were lower than those obtained using the protein precipitate obtained between 30 and 60% ammonium sulphate saturation, indicating that the purification procedure had released interfering substances. The purified fraction showed a slightly lower apparent Kₘ value for DIBOA than for DIMBOA as substrate, but still had twice as high activity with DIMBOA than with DIBOA as substrate.

In conclusion, our results show that the Hx-glucosyltransferase is specific for Hx. They do not support the intermediacy of lactam glucosides in the biosynthesis of Hx glucosides. Furthermore, they do not support the concept that Hx-accumulating species contain glucosyltransferases specific for the type of Hx accumulated by the plant. The differences in apparent Kₘ values might be due to the interference of other compounds in the preparations, since neither previous workers [17] nor we, have purified any Hx-glucosyltransferase to homogeneity.

EXPERIMENTAL

Plant material. The following species were used in this study: Z. mays L. cv T 555; S. cereale L. cvs Tetra Baer, Rogo and Petkus II; T. aestivum L. cvs Millaleu and Kadett; H. vulgare L. cv Libru; H. lechleri L. PI H1310 and A. sativa cv. Nahuel. For the studies of N-hydroxylase activity, seedlings were grown at 21°C in Petri dishes on wet paper in the dark. Otherwise, seedlings were grown in soil at 25°C–30°C in a growth chamber with a photoperiod of 12 hr light and 12 hr darkness, at a light intensity of 7.2 klux.

Chemicals. All chemicals, with the exception of benzoxazinones, were from Sigma. DIBOA and DIMBOA were purified from Et₂O extracts of 7-day-old seedlings of rye and maize, respectively, as described in ref. [18]. The corresponding glucosides were obtained from MeOH extracts of 7-day-old seedlings of rye and maize, as described in ref. [8] and purified by chromatography on DEAE Sephadex A 25 followed by chromatography on Fractogel TSK HW 40-S. Up to 10 g of the aq. extract was loaded on a 44 ml DEAE Sephadex G 25 column equilibrated and eluted with distilled H₂O, with a flow of 1 ml min⁻¹. The frs showing a deep purple colouration with FeCl₃ reagent [10% (w/v) FeCl₃ in 1% MeOH HCl] were pooled and lyophilized. Up to 1 g of

Table 3. Kinetic properties of partly purified Hx-glucosyltransferase from rye

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Vₘₐₓ (nkat)</th>
<th>Kₘ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIBOA</td>
<td>8.2</td>
<td>73</td>
</tr>
<tr>
<td>DIMBOA</td>
<td>1.6</td>
<td>82</td>
</tr>
<tr>
<td>HMBOA</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>HBOA</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Esculetin</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d.: Activity not detected (detection limit = 0.2 pkat).

* The Superdex protein fraction (5 μg) was assayed with 0.0625, 0.125, 0.25, 0.5 and 1 mM of benzoxazinone and 1 mM UDPG.

† The Superdex protein fraction (5 μg) was assayed for 30 min at 17°C as described, or 60 μg of the 60/30% ammonium sulphate fraction were assayed for 60 min at 37°C, and at pH 6.5, 7.5 and 8.5.

The apparent Kₘ and Vₘₐₓ values were calculated from Lineweaver–Burk plots. Correlation coefficients for fits were 0.91 for DIMBOA, 0.99 for DIBOA and 0.95 for UDPG.
the lyophilized powder was then solubilized in H$_2$O and
loaded on a 5 x 25 cm Fractogel TSK HW-40 S column,
equilibrated and eluted with distilled H$_2$O with a flow of
1 ml min$^{-1}$. The FeCl$_3$-positive frs were pooled and
lyophilized.

The lactams, HBOA and HMBOA, were synthesized as
described in refs [20, 21]. The glycosylated lactams,
HBOA-Glc and HMBOA-Glc, were obtained by reduc-
tion of the corresponding Hx-Glc with Zn in HOAc. The
mixture was heated at 60° with vigorous stirring and the
reduction monitored with FeCl$_3$ reagent. Once the reduc-
tion was complete, the mixture was filtered, washed with
H$_2$O and lyophilized. The resulting powder was washed
with Me$_2$CO, and the glucosides further purified by gel
filtration on Fractogel TSK HW 40-S, as described
above. The chromatographic sepns were monitored by
HPLC. The identity of each compound was verified by
NMR spectra taken at 300 MHz.

**Extraction and analysis of Hx.** Root or shoot tissue
(0.2–0.4 g fr. wt) was macerated and extracted as described
in ref. [22]. The content of Hx was analysed using
HPLC as described below.

**Enzyme extraction.** All steps were carried out at 4°. In
the studies of enzyme activity during development, up to
1.5 g fr. wt of shoot or root tissue was macerated with
sand using a mortar and pestle, in up to 2.5 ml g$^{-1}$ fr.
tissue of homogenization buffer [0.1 M Tris–HCl pH 7.5,
containing 5 mM $\beta$-mercaptoethanol 1 mM MgCl$_2$, and
30% (v/v) PVPP (buffer A)] and then filtered through
cheesecloth. The homogenate was centrifuged at 10000 g
for 45 min and the supernatant filtered through Sephadex
G 25 columns equilibrated with 20 mM Tris–HCl pH 7,
containing 10% (v/v) glycerol (buffer B).

For studies of Hx-glucosyltransferase activities in differ-
cent species, shoot tissue was used at days 6–14 after
sowing, depending on plant development. Shoot tissue
(15 g fr. wt) was homogenized in up to 25 ml g$^{-1}$ fr.
tissue of buffer A, as described above. The homogenate
was filtered through cheesecloth and the filtrate centrifuged
at 40000 g for 20 min. The proteins present in the super-
natant were precipitated with a two-step (30 and 60%)
(NH$_4$)$_2$SO$_4$ procedure. The ppt. between 30 and 60% satn
was resuspended in a small vol. of buffer B and filtered
through Sephadex G 25 columns equilibrated with the
same buffer. The precipns were used immediately for
determination of enzymatic activity, or after storage
at $-20°$.

**Preparation of microsomes.** Microsomes were prep
from the radicles and epicotyls of 2.5- to 3.5-day-old
seedlings as described in ref. [17]. The final (washed)
pellet was resuspended in 20 mM Heps buffer, pH 8.5.

**Protein determinations.** In the micromolar prepns, pro-
tein content was determined using the bicinchoninic acid
protein assay according to the supplier's instruction
(Pierce), and in other samples, protein was determined
according to the Bradford procedure [23], using BSA as
standard.

**Enzyme assays.** The N-hydroxylase assay contained
0.5 mM substrate, 2 mM $\beta$-mercaptoethanol, 0.5 mM
NAD(P)H, 23 mM glucose-6-phosphate, 1.5 U glucose-6-
phosphate-dehydrogenase, 40 mM Hepes buffer pH 8.5
and 0.85–1.5 mg protein in a final vol. of 1 ml. The
substrates were suspended in 0.04% (v/v) orthophos-
phoric acid and 1 mg ml$^{-1}$ Tween 20, and diluted
10 times in the assay. Incubations were carried out at 37°
with vigorous agitation, usually for 2.5 hr. In some exps
(see Results), incubations were carried out overnight, and
with other buffers (75 mM Bis–Tris pH 6.5; 75 mM
Tris–HCl pH 7.5), and/or with the addition of NADH.
Incubations containing lactam glucosides were stopped by
the addition of 4 ml of CHCl$_3$–MeOH–HOAc
(200:100:3); after vigorous shaking, the mixt. was centri-
gufied at 13000 g for 3 min (Folch extraction). Part of the
upper (MeOH–H$_2$O) phase was analysed using HPLC as
described below. The incubations containing the lactam
aglucones were stopped with HCl (60 mM final concn)
and processed as described in ref [16].

Hx-glucosyltransferase assays contained 1 mM
UDPG, 0.5 mM DIBOA, or other substrates as indicated
(solubilized in 0.04% orthophosphoric acid), 1 mM
MgCl$_2$, 5 mM $\beta$-mercaptoethanol, 50 mM Tris–HCl
pH 8.5 and 0.1 mg protein in a final vol. of 0.1 ml. For
assays at pH below 8.5, castanospermine at 0.1 mM final
conc was used to inhibit $\beta$-glucosidases. All
assays were carried out at 37° in duplicate and with
controls where UDPG or substrates were omitted.
Incubations with extracts from A. sativa and H. vulgare
contained 0.2 mg protein and were allowed to proceed for
105 min. Incubations with esculetin were carried out for
40 min with 0.6 mM substrate (solubilized in H$_2$O) and
0.6 mg of the ppt. obtained between 30 and 60%
(NH$_4$)$_2$SO$_4$ satn or 2.25 µg of the semi-purified protein
(fr. eluted from the Superdex 75 column, see Table 2) in
the assay. Incubations with lactams were carried out for
60 min with 0.5 mM substrate (solubilized in 0.04%
orthophosphoric acid) and with 0.1 mg protein in the
assay. All other incubations were carried out for
5–20 min (reaction was at initial velocity). The incuba-
tions were stopped by a Folch extraction and the aq.
phase analysed by HPLC as described below.

**Purification of Hx-glucosyltransferase from rye.** Up to
0.6 g of protein (NH$_4$)$_2$SO$_4$ ppt., obtained as described
above, was loaded on a 60 ml DEAE-cellulose column,
equilibrated in buffer B. Proteins were eluted with a
320 ml gradient of 0 to 0.3 M KCl in buffer B, with a flow
rate of 0.5 ml min$^{-1}$. In the hydroxylapatite step, up to
0.2 g of protein was loaded on a 21 ml column equili-
brated in 5 mM K-Pi pH 6.8, and the proteins eluted with a
200 ml gradient of 5 mM K-Pi, with a flow rate of
0.7 ml min$^{-1}$. For sepns on Mono Q HR 5/5 (Pharmacia),
up to 50 mg of protein was loaded on a column equili-
brated in buffer B and eluted with a 20 ml gradient of
0–0.35 M KCl, with a flow rate of 0.5 ml min$^{-1}$. Gel
filtration was carried out using a Superdex 75 prep. grade
column (600 x 16 mm) (Pharmacia), equilibrated with
20 mM Tris–HCl pH 7, containing 0.25 M NaCl and
10% (v/v) glycerol. Proteins (2 mg, 1 mg ml$^{-1}$) were
loaded and eluted at a rate of 0.4 ml min$^{-1}$ with frs of 2 ml
being collected. During all purification steps, protein
elution was monitored by measuring A at 280 nm.

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Chromatofocussing was carried out using a Mono P HR 5/5 column (Pharmacia), equilibrated with 25 mM Bis-Tris-iminodiacetic acid pH 7, containing 10% (v/v) glycerol. The proteins (up to 50 mg) were eluted with 20 ml of polybuffer 74-iminodiacetic acid pH 4, containing 10% glycerol (v/v) at a flow rate of 0.5 ml min \(^{-1}\). Each fr. (0.5 ml) was collected over 50 µl of 1 M Tris–HCl, pH 8.

**HPLC analysis.** Compounds were sepd on a C18 Nucleosil column (particle size 5 µM, dimensions 150 x 4.6 mm). Detection was carried out at 263 nm. After Hx-glucosyltransferase assays, peaks were resolved with linear gradients between MeOH and 0.04% (v/v) aq. orthophosphoric acid: 0-2 min: 25% MeOH; 2-2.5 min: 25-50% MeOH; 2.5-3 min: 50-25% MeOH; 3-7 min: 25% MeOH, with a flow of 1 ml min \(^{-1}\). The amounts of products were determined from calibration curves with standards. Detection of esculin and esculetin was at 340 nm, and elution under isocratic conditions with 1 ml min \(^{-1}\) of H2O–MeOH–HOAc (69:30:1). After hydroxylation assays, peaks were resolved by eluting with H2O–110Ac (9:1) at 1 ml min \(^{-1}\). Detection of Hx in roots and shoots was at 263 nm and linear gradients between MeOH and 0.04% aq. orthophosphoric acid: 0–3 min: 35–50% MeOH; 3–4 min: 50% MeOH; 4–6 min: 50–35% MeOH; 6–9 min: 35% MeOH.

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