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CHARACTERIZATION OF CYANOGENIC GLUCOSIDES AND β-GLUCOSIDASES IN *TRIGLOCHIN MARITIMA* SEEDLINGS

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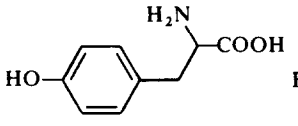
Key Word Index—*Triglochin maritima*; Juncaginaceae; cyanogenic glucosides; triglochinin; taxiphyllin; NMR; seedling development; specific β-glucosidases.

Abstract—In addition to triglochinin, taxiphyllin has been detected as a cyanogenic glucoside in seedlings of *Triglochin maritima*. Taxiphyllin at first increases during seedling development and then decreases, whereas triglochinin increases to a level higher than that ever reached by taxiphyllin and remains there during further seedling development. Two β-glucosidases have also been characterized in these seedlings. One of these shows a distinct specificity for triglochinin, whereas taxiphyllin appears to be the preferred substrate of the other.

INTRODUCTION

Previous studies [1, 2] on the cyanogenesis of *Triglochin maritima* have indicated triglochinin (**3**) as the only cyanogenic glucoside in this plant. This compound has been identified in a number of other plant species [3–12]. Sharples *et al.* observed the incorporation of tyrosine (**1**) into triglochinin in *Thalictrum aquilegifolium*. This suggests that during biosynthesis of triglochinin cleavage of an aromatic ring occurs. Some authors have suggested that *p*-hydroxylated mandelonitrile glucosides such as dhurrin (**2a**) or taxiphyllin (**2b**) might be precursors for triglochinin [13, 14].



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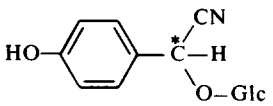
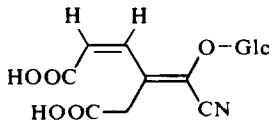


2a Dhurrin,* = (*S*)
2b Taxiphyllin,* = (*R*)



3 Triglochinin

Thus it is of interest that the flower pedicels of *Platanus orientalis* contain both dhurrin and triglochinin [9] and that *Thalictrum aquilegifolium* contains triglochinin as well as proteacin and *p*-glucosyloxymandelonitrile in the shoots [18]. While looking for a suitable system to study the biosynthesis of triglochinin, we found taxiphyllin as well as triglochinin in *T. maritima*. In addition, the characterization of β-glucosidases from *T. maritima* which hydrolyse each of these compounds is described.

RESULTS

Isolation and structure elucidation of cyanogenic glucosides

The residue from a cold methanol extract of lyophilised seven-day-old seedlings released an equivalent of 550 ppm of cyanide when dissolved in H_2O and treated with β-glucosidases. TLC of an aliquot of the methanol extract on silica gel revealed only two areas on the chromatogram with cyanogenic activity. The major one (85% of the total cyanide) was triglochinin while the other one (15%) had a chromatographic behaviour like dhurrin/taxiphyllin. Chromatography of the extract on a PVP column according to the method of Reay [15] yielded two cyanogenic fractions. TLC of the first fraction against a standard showed that it contained triglochinin plus other substances and this fraction was not further investigated. The second fraction contained only a single compound which migrated as dhurrin/taxiphyllin in the TLC system. This fraction showed a λ_{max} at 232 nm (MeOH) with a bathochromic shift of 26 nm after adding NaOMe, indicating a phenol. The TMS-derivative had a greater retention time than dhurrin during GLC and coincided with the retention time of a taxiphyllin standard. That the compound was taxiphyllin was confirmed by recording the ^1H NMR (100 MHz) spectrum of the underivatized compound in acetone-d_6. The spectrum, not yet published in this solvent, exhibited the typical AA'BB' pattern (δ 7.44 and 6.94, 4 H) of a *p*-substituted aromatic ring, a sharp singlet at δ 5.84 (1 H, methine-H), a poorly resolved [16] doublet of the anomeric glucose proton (δ 4.36, 1 H, J = 7.5 Hz, β-configuration) and the remaining sugar protons between δ 3.2 and 3.9. The spectrum of dhurrin isolated from *Sorghum bicolor* was recorded under the same conditions and the spectra were identical with exception of the position of the methine proton (δ 5.97)

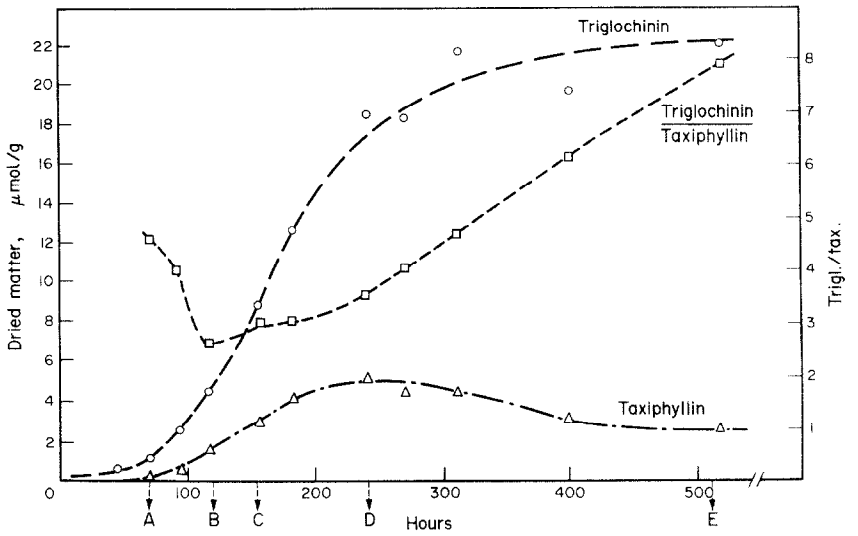


Fig. 1. Content of taxiphyllin and triglochinin in continuously illuminated seedlings and triglochinin:taxiphyllin ratio during seedling development. A: shoots (first leaf) emerge; B: shoots 5–8 mm long; C: roots emerge; D: shoots 15 mm long, roots 5 mm long, second leaf emerges; E: shoots 20 mm long, roots 10 mm long, third leaf emerges.

and the well resolved [16] doublet of the anomeric proton (δ 4.70), which are shifted upfield (0.13 and 0.36 ppm, respectively) in the substance isolated from *T. maritima*. This indicates a (*R*)-configuration for the chiral centre of the aglucone and thus the cyanogenic glucoside is taxiphyllin.

Content of triglochinin and taxiphyllin during seedling development

Quantitative estimation of taxiphyllin and triglochinin during the first three weeks of seedling development was accomplished by enzymatically liberating cyanide from triglochin and taxiphyllin after separating the compounds by TLC. Triglochinin is present in small amounts in the seeds and during germination and development of the young plants, its content increases from 0.4 to nearly 22 µmol/g dry wt by 500 hr (Fig. 1). Taxiphyllin was not detectable in ungerminated seeds, but reached a concentration of 5 µmol/g dry wt by 220 hr and then declined continuously reaching a concentration of 2.7 µmol by 500 hr. The ratio of triglochinin:taxiphyllin during development of the seedlings decreases in the first 100 hr from 4.6:1 to 2.6:1 and then increases continuously to 8:1.

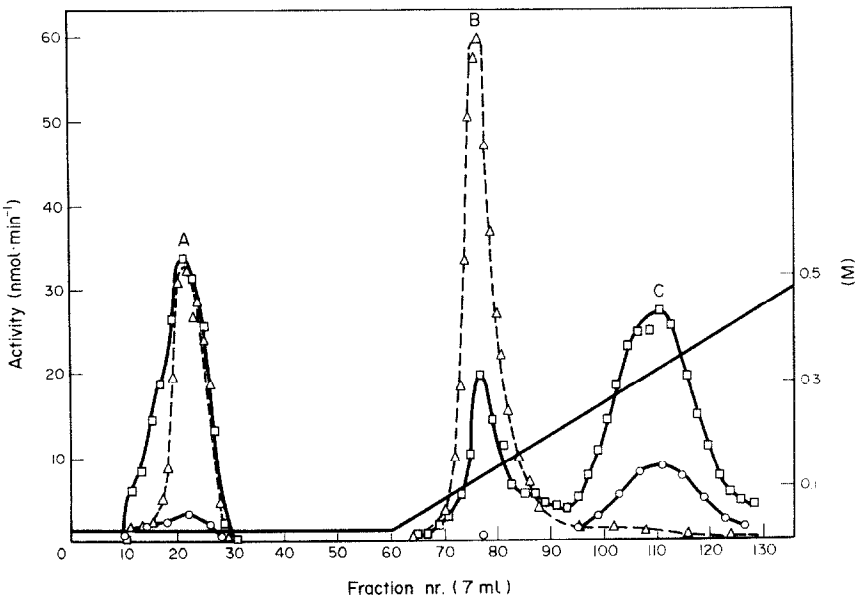


Fig. 2. Separation of *Triglochin maritima* β-glucosidases by DEAE 'cellex' column chromatography. △---△, triglochinin; ○——○, taxiphyllin; □——□, 4-nitrophenyl-β-glucoside; ——, Tris–HCl, pH 7, buffer gradient.

Table 1. Purification sequence for the β-glucosidases of *T. maritima* seedlings

Step	Glucosidase B: Triglochinin activity total	Triglochinin activity specific	4-NP-glc activity total	4-NP-glc activity specific	Protein mg	Ratio of Trigl./4-NP-glc activity	Glucosidase C: Taxiphyllin activity total	Taxiphyllin activity specific	4-NP-glc activity total	4-NP-glc activity specific	Protein mg	Ratio of Taxiph./4-NP-glc activity
Crude protein extract	7.5	0.02	13.4	0.038	350	0.56	3.2	0.01	13.4	0.038	350	0.24
70% $(NH_4)_2SO_4$ precipitation and Sephadex G 25 chromatography	13.2	n.d.	15.7	n.d.	n.d.	0.85	3.7	n.d.	15.7	n.d.	n.d.	0.24
	peak B (cf. Fig. 1)						peak C (cf. Fig. 1)					
DEAE 'cellex'	5.8	0.59	1.3	0.12	9.9	4.4	1.4	0.23	4.2	0.7	6	0.33
CM Sephadex C 50	2.2	1.1	0.5	0.25	2	4.4	1.3	n.d.	3.7	n.d.	n.d.	0.35
Sephadex G 200	0.7	1	0.17	0.24	0.71	4.1	0.8	1.1	1.7	2.3	0.74	0.47

Hydrolytic activity of the substrates indicated have been measured as described under methods at the following substrate concentrations: triglochinin: 0.04 mM; taxiphyllin: 0.2 mM; 4-nitrophenyl-β-glucosidase: 2 mM. Total activity = μmol. min^{-1}; specific activity = μmol. min^{-1}. mg^{-1}. n.d. = Not determined.

In one experiment, samples were taken at 1032 hr and the contents were 22 μmol of triglochinin and 1.5 μmol of taxiphyllin per g dry wt with a ratio of 15:1. In another experiment, the plants were illuminated for 48 hr and then grown a further nine days in the dark. These seedlings contained 34 μmol triglochinin and 2.5 μmol taxiphyllin per g dry wt (ratio 13.6:1). When the seedlings were grown under standard conditions and the roots and shoots were analysed separately, taxiphyllin was only detected in shoots. Thus, shoots contain both triglochinin and taxiphyllin, whereas roots seem to contain only triglochinin in an appreciable amount.

Isolation and purification of the β-glucosidases

A buffer extract of the acetone dry powder of ten-day-old *T. maritima* seedlings hydrolysed triglochinin, taxiphyllin and 4-nitrophenyl-β-glucoside (4-NP-glc) (Table 1). The β-glucosidase activity was separated into three main fractions when chromatographed on DEAE-cellulose, with fraction B preferentially hydrolysing triglochinin and fraction C demonstrating highest activity with taxiphyllin (Fig. 2). Fraction A (non-binding protein) demonstrated no clear preference for either of the substrates. Each fraction was further purified according to the scheme outlined in Table 1.

The triglochinin hydrolytic activity from fraction B eluted from a CM Sephadex C 50 column at a buffer concentration of 0.45 M when a linear gradient from 0.05 to 0.5 M was employed. Two minor peaks of β-glucosidase activities, which did not hydrolyse triglochinin but did hydrolyse 4-NP-glc, were separated from the main peak of β-glucosidase activity. One of these was eluted with the non-binding protein, the other one with a buffer concentration of 0.36 M. The β-glucosidase fraction, which was active on triglochinin, was further purified by column chromatography on Sephadex G 200 where it eluted as a single symmetrical peak without further separation of other glycosidic activities. Alkaline disc gel electrophoresis of this material demonstrated only one band of β-glucosidase activity when incubated with 4-methylumbelliferyl-β-glucoside. Staining with Coomassie blue revealed one additional protein band without β-glucosidase activity.

All the hydrolytic activity for taxiphyllin in fraction C eluted with the non-binding protein when chromatographed on CM Sephadex C 50 as described above. Additional β-glucosidases were not detected. Subsequent chromatography on Sephadex G 200 revealed a main β-glucosidase fraction active on taxiphyllin at an elution volume which corresponded to a MW of 290 000 and a small peak of β-glucosidase activity corresponding to a MW of 100 000. This latter β-glucosidase was not further investigated. Alkaline disc electrophoresis of the main peak showed one β-glucosidase band when incubated with 4-methylumbelliferyl glucoside and at least two protein bands when stained with Coomassie blue.

The non-binding β-glucosidase fraction A from the DEAE-cellulose chromatography was also chromatographed on CM Sephadex C 50 and at least six different peaks of β-glucosidase activities could be detected when assayed with 4-NP-glc. Only one of these peaks rapidly hydrolysed triglochinin. However, it demonstrated the same elution behaviour as the 'triglochinin-specific' glucosidase in fraction B described above, eluting at a

Table 2. Relative activities, K_m and V_{max} values of the purified β-glucosidases B and C

Substrate	β-Glucosidase B: K_m (mM)	V_{max} (μmol. min^{-1}. mg^{-1})	V_{max}/K_m	β-Glucosidase C: K_m (mM)	V_{max} (μmol. min^{-1}. mg^{-1})	V_{max}/K_m
4-Nitrophenyl-β-glucoside	2	1.1	0.55	0.13	4	31
Triglochinin	0.6	43	72	2	20	10
Taxiphyllin	Activity too low for determination			0.4	3.5	9
Dhurrin	2	1	0.5	0.4	0.7	1.75
4-Methylumbelliferyl-β-glucoside	2	1.4	0.7	0.1	6	60
	Relative activity* (4-Nitrophenyl-β-glucoside = 100)			Relative activity* (4-Nitrophenyl-β-glucoside = 100)		
4-Nitrophenyl-α-glucoside	1			0.5		
2-Nitrophenyl-β-glucoside	16			107		
4-Nitrophenyl-β-galactoside	15			12		
Salicin	18			69		

* 2 mM substrate concentration.

buffer concentration of 0.45 M. It therefore seems likely that this glucosidase is the same as that purified from fraction B and that the separation observed on DEAE (Fig. 2) might be caused by overloading the column. None of the six peaks obtained from fraction A was as preferentially active on taxiphyllin as that obtained from fraction C (Fig. 2) and most had very low activity on this substrate. Hence it can be assumed that no 'taxiphyllin-specific' β-glucosidase is contained in these peaks of β-glucosidase activity and they were therefore not further investigated. The overall purification procedures and the data obtained for the β-glucosidases B and C are given in Table 1.

The enrichment for both β-glucosidases in the final step (Sephadex G 200) is in the range of 100-fold. In the case of β-glucosidase B, the amount recovered with respect to the triglochinin activity in the crude protein extract was 9%, and the amount of β-glucosidase C recovered was 22% with respect to the taxiphyllin activity.

Properties of β-glucosidases B and C

Relative hydrolysis velocities for different substrates as well as the K_m and V_{max} values for both β-glucosidases are given in Table 2.

β-Glucosidase B showed optimal activity at pH 5.2 and β-glucosidase C at pH 5.0. The MW for glucosidase B as determined by Sephadex G 200 was 125000 (±10%) and 120000 (±10%) as determined by 5–20% linear sucrose gradient centrifugation. The MW of glucosidase C was 290000 (±10%) and 220000 (±10%) when measured by the same two techniques. Aldolase (MW 147000) and catalase (MW 240000) were used as marker proteins in the sucrose gradient centrifugations. Two independent runs were performed for each determination. Isoelectric points were between pH 4.5 and 5 for glucosidase B and between 4 and 4.5 for glucosidase C.

In contrast to the 'triglochinin-specific' β-glucosidase from *Alocasia macrorrhiza* [19], the β-glucosidase B from *T. maritima* was not inhibited by *p*-hydroxymercuribenzoate and methoxyethanol did not increase enzymatic activity. The β-glucosidases B and C could be stored at 4° for several months without loss of activity.

DISCUSSION

The co-occurrences of triglochinin and *p*-hydroxymandelonitrile glucosides in *T. maritima*, *Thalictrum aquilegifolium* [13] and *Platanus orientalis* [9] suggest a connection between the biosynthesis of triglochinin and the *p*-hydroxymandelonitrile glucosides. Because tyrosine is a precursor of dhurrin and taxiphyllin [17] as well as of triglochinin [13], there are two possibilities: biosynthesis involves part of a common pathway which branches to produce the different substances, or both glucosides are located in series in the same pathway. If the latter situation is true, then it would seem likely that the *p*-hydroxymandelonitrile glucosides would appear first as previously has been suggested [13, 14]. The observation that taxiphyllin decreases during development of *T. maritima* until it is not detectable in the adult plant whereas triglochinin increases to a high level may indicate that taxiphyllin is a precursor for triglochinin.

Our results also show that β-glucosidases with different specificities for cyanogenic glucosides are present in *T. maritima* seedlings. One of these shows a very distinct specificity for triglochinin (Table 2). The enzymatic specificity of this particular β-glucosidase and its behaviour during purification were similar to that of the earlier described β-glucosidase from *A. macrorrhiza* [19]. However, the influence of *p*-hydroxymercuribenzoate as well as of methoxyethanol on enzyme activity was different indicating that the enzymes are not identical.

It is interesting that in *T. maritima* there is another β-glucosidase which might be regarded as 'specific for taxiphyllin'. Further work will determine the relationship between taxiphyllin content and this particular β-glucosidase in *T. maritima* seedlings. It will also clarify whether the low, but remarkable hydrolysis of 4-nitrophenyl-α-glucoside (Table 2) is due to this β-glucosidase or to the presence of an α-glucosidase as an impurity.

EXPERIMENTAL

Growth conditions and extraction of plant material for cyanogenic glucosides. Seeds were obtained from fruits of *T. maritima* collected in September 1977 on the coast of the North Sea. The seeds were floated on H_2O at a temp. of 24 ± 1° and illuminated by two 40 W Osram Fluora lights. Seedlings were collected, grounded in liquid N_2 and freeze-dried. This material was extracted with cold MeOH using an Ultra-turrax, and quantitative estimates and purification of the cyanogenic glucosides were done on these extracts.

Purification of taxiphyllin. The MeOH extract of 3.3 g dried matter, representing *ca* 4500 8–12-day-old seedlings, was evapd to dryness and dissolved in 2–3 ml H_2O. This was chromatographed on a PVP-column (2.5 × 50 cm) with H_2O containing 0.1% HOAc and fractions were tested for cyanogenic substances by the Feigl–Anger test [21]. Triglochinin eluted in the range of 110–180 ml, taxiphyllin in the range of 290–340 ml. Taxiphyllin-containing fractions were homogeneous by TLC and GLC and were used for GLC, UV and NMR spectroscopy.

Cyanide estimation. The MeOH was evapd from this extract and the residue was dissolved in 1–2 ml H_2O and 1 ml citrate buffer (0.5 M, pH 6) contained in a small vial. Cyanide was liberated by hydrolysing the glucosides with suitable amounts of β-glucosidase from almonds (Serva) and *Alocasia* [19] for 6 hr at 40°. A stream of N_2 was passed through the vials and then into 2 ml N NaOH. The cyanide in the NaOH was estimated by a modified Aldridge method using anthranilic acid [22]. Material from TLC plates was transferred directly into the vial and estimated for cyanide by the same procedure.

Chromatographic systems. TLC: Si gel, MeCOEt–EtOAc–H_2O–HCO_2H, 5:7:1:0.5; detection by UV 254 nm. GLC: OV-225, 3% on chromosorb AW-DMCS 80–100 mesh, 5m × 2 mm i.d., steel; 200–245, 1°/min; FID.

Isolation and purification of β-glucosidases. The Me_2CO dry powder (17.5 g) of 10-day-old *T. maritima* seedlings (corresponding to 150 g fr. wt) was extracted in a mortar for 30 min with 700 ml citrate–Na-phosphate buffer (0.2 M, pH 5). The extract was centrifuged at 25000 *g* for 30 min and the supernatant was subsequently purified by the following procedures: 70% $(NH_4)_2SO_4$ precipitation, Sephadex G 25 chromatography, DEAE 'cellex' chromatography, CM Sephadex C 50 and Sephadex G 200 chromatography. In each case the conditions were the same as described earlier [19, 20]. All enzyme purification procedures were carried out at 4° and 0.02% NaN_3 was present in the protein preparations throughout to prevent microbial growth. Alkaline disc electrophoresis, isoelectric focussing in polyacrylamide disc gels (ampholine range pH 3.5–10) and 5–20% linear sucrose gradient centrifugation were performed

according to published methods [cf. 19, 20]. Protein content was determined according to Lowry.

Measurement of enzyme activities. Hydrolysis of 4-nitrophenyl-glucosides, 4-methylumbelliferyl-glucoside, 2-nitrophenyl-glucoside and salicin was measured at 2 mM concn using the assay described elsewhere [19, 20]. Triglochinin hydrolysis was measured spectrophotometrically by following the decrease of absorption at 276 in a 30° thermostatically controlled cuvette. In the standard assay triglochinin concn was 40 μM and the vol. was 0.5 ml. After pre-incubating the substrate for 5 min, the reaction was started by addition of the β-glucosidase. The hydrolysis of 4.1 nmol triglochinin corresponds to a decrease of 0.1 at 276 nm (1 cm cuvette; 0.5 ml vol.; pH 5; $\varepsilon = 12.200$ ($M^{-1}.cm^{-1}$). Initial velocity was determined graphically from the linear part of the graphs. Taxiphillin and dhurrin hydrolyses were measured as follows: standard assays consisted of 0.2 mM substrate, 50 mM with respect to citrate–Na-phosphate buffer (pH 5) in a total vol. of 0.5 ml. After 30 min incubation at 30°, the reaction was stopped by addition of 0.5 ml Tris–HCl buffer 0.2 M, pH 8.8) which shifted the pH to 8.3. The absorption at 330 nm, which is due to liberated 4-hydroxybenzaldehyde, was immediately determined against an appropriate blank. The ε value of 4-hydroxybenzaldehyde was determined to be 21.000 ($M^{-1}.cm^{-1}$) under these conditions (pH 8.3). Lineweaver–Burk plots were used to calculate K_m and V_{max} values.

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