GLUCOSIDASES INVOLVED IN INDOLE ALKALOID BIOSYNTHESIS OF *CATHARANTHUS* CELL CULTURES

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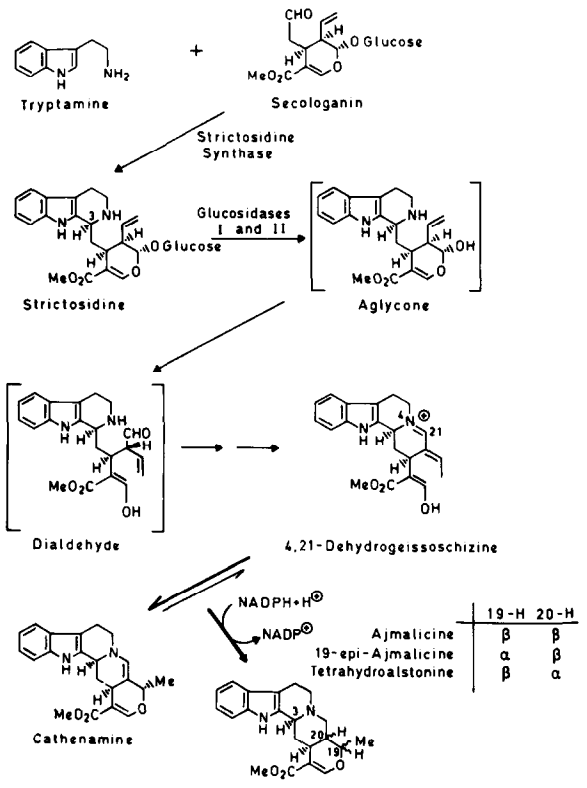
1. Introduction

Strictosidine, an alkaloidal glucoside [1–3], is a firmly established intermediate in monoterpenoid indole alkaloid biosynthesis. The enzyme catalyzing the condensation of tryptamine with the monoterpenoid glucoside secologanin was discovered in several plant cell cultures [2,4] and has been characterized from cultured *Catharanthus roseus* cells [5]. The step subsequent to strictosidine formation involves the removal of the glucose moiety of strictosidine yielding an unstable aglycone which in turn opens to form a highly reactive dialdehyde [6]. This intermediate undergoes further rearrangements and in our system finally yields ajmalicine and its two isomers [7] (see scheme 1). One crucial step, the removal of the glucose molecule from strictosidine, is most likely catalyzed by a β-D-glucosidase(s). The involvement of non-specific glucosidases in this reaction has been claimed using *C. roseus* [8]. Non-specific glucosidases hydrolyzed *p*-NPG as well as catalyzed the formation of indole alkaloids [8]. Both of these enzymes had mol. wt 55 000 and optimum pH 5.0–5.5, and were activated by tryptamine. It was furthermore claimed that these glucosidases are part of an enzyme aggregate termed 'ajmalicine synthetase'.

Here the involvement of glucosidases in indole alkaloid biosynthesis is studied using a specific assay system with strictosidine as substrate. We report the discovery and characterization in *C. roseus* cell cultures of two highly glucoalkaloid-specific β-glucosidases, which disprove the claims in [8].

Abbreviation: *p*-NPG, *p*-Nitrophenyl-β-D-glucoside



Scheme 1

2. Materials and methods

2.1. *Cell cultures*

C. roseus cell cultures were routinely grown in an alkaloid production medium [9]. All other cell cultures were used as in [5]. Cells were grown in 300 ml batches at 23°C or else in a 30 l airlift fermenter. The tissue was harvested and immediately frozen

with liquid nitrogen, used directly, or stored at −20°C. The tissue was processed for the enzyme preparations as in [4].

2.2. *Assay system*

p-Nitrophenyl-β-D-glucoside (Sigma, Munich) was used as substrate for unspecific glucosidases [8]. The specific strictosidine–glucosidase assay was performed as follows: [3-^{3}H]strictosidine (spec. act. 34 mCi/mmol) was prepared from tryptamine and [7-^{3}H]secologanin [10] using purified strictosidine synthase [5] as catalyst. [3-^{3}H]Strictosidine, 300 pmol (~8000 cpm), was incubated with 5 μmol phosphate buffer (pH 6.3) and 20–500 ng protein in total vol. 50 μl. The mixture was incubated for 30 min (unless indicated otherwise) at 30°C in a 2 ml Eppendorf reaction vessel. The reaction was stopped by the addition of 0.5 ml ice-cold 1 M phosphate buffer (pH 7.0). Subsequently 0.5 ml mixture of benzene:*n*-hexane (3:1) was added and the aqueous phase exhaustively extracted. An aliquot (0.2 ml) of the 'aglyca'-containing organic phase was transferred to a scintillation vial, 5 ml scintillation fluid added and the sample counted. The carry over of strictosidine was ~5% and was subtracted. One kat of strictosidine glucosidase is defined as that amount of enzyme which hydrolyses 1 mol strictosidine/s at pH 6.3 and 30°C at 6 μM substrate. Buffers used were: pH 4–7, citrate-phosphate; pH 5.5–7.5, phosphate; pH 8–9, borate; pH 8–9.5, glycine–NaOH.

2.3. *Enzyme separation*

The protein fraction precipitating between 0–45% $(NH_4)_2SO_4$ saturation was, after dialysis, layered onto a 2.3 × 10.5 cm Whatman DE-52 cellulose column. Proteins were eluted at +4°C with a non-linear 0–200 mM gradient of KCl in 20 mM K-phosphate (pH 7.8). Fractions (10 ml) were collected at a speed of 1 ml/min.

Molecular weight determination was carried out by gel filtration on a calibrated Sephadex G-150 superfine column according to [12], using cytochrome *c* (12 500), bovine serum albumin (67 000), aldolase (158 000), catalase (230 000), and ferritin (450 000) as markers.

2.4. *Protein determination*

The amount of protein in the enzyme fraction was measured according to [13].

2.5. *Chemicals*

Secologanin was a gift of Dr F. Kaiser, Boehringer (Mannheim) and [14-^{3}H]vincoside (1.02 mCi/mmol) a gift of Dr Hutchinson, Madison. [1-^{3}H]Desacetyl-ipeco- and isoipecoside (16 mCi/mmol) [14], [7-^{3}H]secologanol (94 mCi/mmol) and [7-^{3}H]secologanin (34 mCi/mmol) were prepared as in [10]. [5-^{14}C]Vincoside lactam (1 mCi/mmol) and [5-^{14}C]-strictosidine lactam (10 mCi/mmol) were prepared by standard methods [15].

3. Results

3.1. *Limited survey of enzyme distribution*

Cell cultures of a number of different species were assayed for unspecific (substrate: *p*-NPG) and specific (substrate: strictosidine) glucosidases as above. Representatives of the indole alkaloid containing family *Apocynaceae* as well as 3 indole alkaloid free families were tested. The results are summarized in table 1. Unspecific activity was present in every single species investigated, while the strictosidine-specific glucosidase activity was confined exclusively to the members of the indole alkaloid containing *Apocynaceae*. Because of the general interest in *C. roseus* [16] and the availability of optimized cell culture lines [9] this plant was selected for more detailed examination of the glucosidases. As can be judged from the results in table 1, it was likely already at this stage that strictosidine is hydrolysed by specific β-glucosidases.

3.2. *Separation of glucosidases*

In a preliminary experiment it was found that all of the specific as well as most of the unspecific glucosidase activities were present in the 0–45% $(NH_4)_2SO_4$ fraction of the crude homogenate. DEAE-column chromatography of this fraction resulted clearly in the separation of ⩾3 unspecific glucosidases acting on *p*-NPG and 2 distinct fractions containing enzymes hydrolysing strictosidine (fig.1). The unspecific glucosidases did not hydrolyze strictosidine under our experimental conditions. The 2 strictosidine-specific glucosidase fractions will subsequently be referred to as enzyme I and II. This experiment proved the existence of strictosidine-specific glucosidases, and both enzyme I and II were purified each 120-fold (procedure not shown here).

Table 1
Survey of strictosidine-specific and unspecific glucosidase activities in plant cell cultures

Species	Family	pkat *p*-NPG glucosidase/mg protein	pkat Strictosidine glucosidase/mg protein
Amsonia salicifolia	Apocynaceae	27	32
Catharanthus ovalis	Apocynaceae	12	29
C. pusilus	Apocynaceae	75	228
C. roseus	Apocynaceae	12	230
C. trichophyllus	Apocynaceae	87	99
Rauwolfia vomitoria	Apocynaceae	173	4
Rhazya orientalis	Apocynaceae	78	8
Vinca major	Apocynaceae	772	10
Lonicera tartarica	Caprifoliaceae	700	0
Melilotus albus	Fabaceae	153	0
Nicotiana tabacum	Solanaceae	31	0

Protein precipitated by $(NH_4)_2SO_4$ between 0–70% saturation in crude homogenates were used as enzyme source throughout. Assay time for *p*-NPG glucosidases was 100 min, all other parameters were as in section 2

3.3. *Properties of specific glucosidases*

Using ~140 fkat purified enzyme in the standard assay, the reaction rate was linear for ~20 min and 100% conversion of substrate was reached after ~80 min. The reaction with both enzyme I and II was found to have optimum pH 6.0–6.4 (fig.2) a surprisingly high pH optimum when compared with other β-D-glucosidases. This optimum matches quite well the pH optimum for the overall reaction from tryptamine and secologanin to ajmalicin as determined by radioimmunoassay [17] which was found to be pH 6.5. It is interesting to note that the unspecific glucosidases, with *p*-NPG as substrate, showed a distinct pH optimum shifted towards the more acid region [8].

K_m-values were 0.2 mM for enzyme I and 0.1 mM for enzyme II and the respective V_{max}-values were 0.23 nmol/min and 0.12 nmol/min. Enzyme I shows

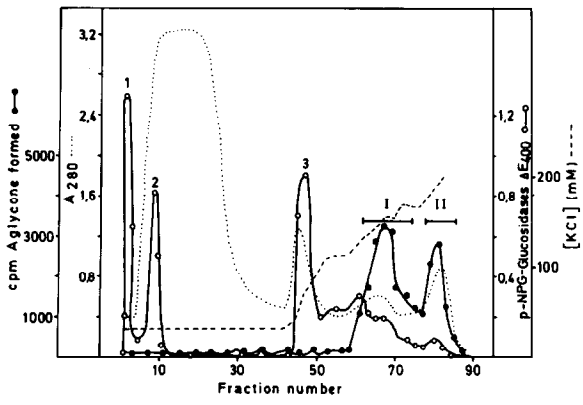


Fig.1. Elution profile of specific (I, II) and unspecific (1–3) glucosidases from DEAE-cellulose. DEAE-cellulose chromatography of a crude *C. roseus* enzyme preparation was performed by a KCl gradient (- - -) as in section 2. Fractions (10 ml) were collected, assays for specific (5 μl eluate diluted 1:30 (●——●)) and non-specific glucosidases (200 μl, A_{400} (○——○)) were done as in section 2. Protein was measured by following A_{280} (· · ·).

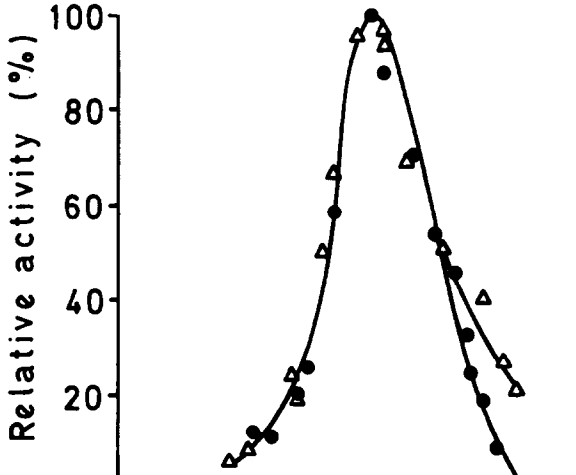


Fig.2. pH Optima of strictosidine hydrolysis catalyzed by strictosidine glucosidase I (●——●) and II (△——△).

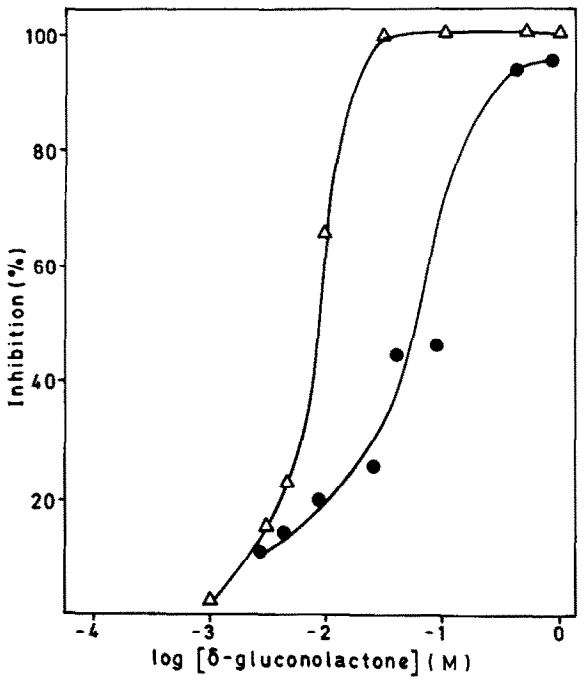


Fig.3. Differential inhibition of strictosidine glucosidase I (●——●) and II (△——△) by varying concentrations of the β-glucosidase inhibitor δ-gluconolactone.

substrate inhibition at ~1 mM while enzyme II does not. The temperature optimum for both enzymes was found to be 30°C. Assuming a globular shape of both enzymes, glucosidase I had mol. wt 230 000 and glucosidase II a mol. wt >450 000 as judged from gel filtration on Sephadex G-150. Tryptamine at ≤10 mM did not activate either of the two enzymes, contradicting [8]. At the tryptamine concentration (2.5 mM) reported as optimal (75% enhancement of activity) in [8], enzymes I and II were inhibited ~15%. Activity of the unspecific glucosidases was likewise not enhanced by tryptamine at any of the concentrations tested (0–10 mM). The strictosidine-specific glucosidases I and II can be distinguished by their differential sensitivity to δ-D-gluconolactone used to inhibit the β-glucosidases of *C. roseus* cell cultures [2,17]. As shown in fig.3, enzyme I is much less sensitive to this β-glucosidase inhibitor than enzyme II, a property shown also for several other glucosidase inhibitors [18]. Finally, the substrate specificities of glucosidase I and II were determined using several labelled substrates resembling the strictosidine molecule. The 3β (*R*) epimer of strictosidine, vincoside, which does not naturally occur in plants, is hydrolysed at <10% of the rate of strictosidine with 3α (*S*) configuration. However, neither strictosidine- or vincoside lactam, desacetylipeco- or desacetylisoipecoside, nor secologanin or secologanol were hydrolysed by either of the two glucosidases. Apparently the strictosidine glucosidases I and II are highly specific for their endogenous substrate and cannot be assayed by using *p*-NPG as attempted in [8].

4. Discussion

Two new enzymes have been discovered which are involved in monoterpenoid indole alkaloid biosynthesis. They catalyze the removal of the glucose moiety of strictosidine (see scheme 1). β-Glucosidase has been postulated to be involved in these reactions for a considerable time and biomimetic experiments under non-physiological conditions have given some indications of the chemistry involved [6]. The enzymes described here for the first time are highly specific for the hydrolysis of strictosidine. None of a range of closely related compounds served as substrate for the strictosidine-β-D-glucosidases I and II. The two enzymes were easily separated from each other, as well as from the unspecific glucosidases, by DEAE-cellulose chromatography. They proved to be distinctly different by their molecular weight, K_m- and V_{max}-values, substrate inhibition and differential inhibition by a glucosidase inhibitor (fig.3). Strictosidine-specific glucosidase(s) is present in cell cultures of all tested indole alkaloid containing species of the family *Apocynaceae*, while it is absent from cells of unrelated families devoid of these alkaloids. Strictosidine glucosidases I and II represent another example supporting the emerging picture of highly substrate-specific plant glucosidases [19]. Our insufficient knowledge of the true metabolic function of plant glucosidases is attributable to the fact that synthetic substrates (i.e., nitrophenylglucosides) instead of the endogenous glycosidic compounds were used in most studies [19]. β-D-Glucosidases capable of hydrolysing the glucosides of secondary plant products have usually been understood to act mainly on metabolic end-products. The strictosidine glucosidase is involved in an essential and initial step of a rather complicated biosynthetic sequence; this function obviously requires a high degree of specificity of these enzymes towards their

substrate, and the properties of the two glucosidases from *Catharanthus* described here are in full accord with these criteria.

Our results clearly demonstrate, that *p*-NPG cannot be used as a substrate for glucosidases specifically involved in strictosidine metabolism. The coelution of an 'ajmalicine synthetase' and of *p*-NPG-glucosidase activity during gel-filtration of a crude homogenate reported by [8] is to be considered fortuitous, and the statement that the 'glucosidases were capable of synthesizing ajmalicine from tryptamine and secologanin' [8] is an unjustified conjecture. In our hands, strictosidine synthase [4,5] and the specific strictosidine glucosidases can clearly be separated from each other by gel-filtration [18]. Therefore, there is no basis for the claim of an 'ajmalicine synthase' [18]. The biosynthesis of heteroyohimbine alkaloids in *Catharanthus roseus* is catalyzed by separable, non-aggregated and highly specific enzymes.

Acknowledgements

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