PERGAMON

Phytochemistry 53 (2000) 201–207

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Indoxyl-UDPG-glucosyltransferase from *Baphicacanthus cusia*

Herbert Marcinek[a,✠], Walter Weyler[b], Brigitte Deus-Neumann[a], Meinhart H. Zenk[a,*]

[a]*Institute for Pharmaceutical Biology, Ludwig-Maximilians University of Munich, Karlstrasse 29, D-80333 Munich, Germany*
[b]*Genencor International Inc., 925 Page Mill Road, Palo Alto, CA 94304, USA*

Received 14 May 1999; accepted 11 August 1999

Abstract

The enzyme catalyzing the transfer of glucose from uridine diphosphate glucose to indoxyl yielding the indoxyl glucoside indican was isolated from *Baphicacanthus cusia* Bremek (Acanthaceae). The indoxyl-uridine diphosphate glucose (UDPG)-glucosyltransferase was purified to homogeneity in six chromatographic steps. The decisive step for the recovery of a homogeneous enzyme was the application of immobilized metal affinity chromatography yielding an 863-fold purified enzyme. From a total of 60 substances tested, in addition to the natural substrate 3-OH-indole (indoxyl), only 4-OH-, 5-OH-, 6-OH-, and 7-OH-indole were accepted as substrates by the glucosyltransferase. However, the latter substrates were metabolized to varying extent. The optimum pH of the enzyme was 8.5, the optimum temperature was 30°C and the isoelectric point was pH 6.5. The M_r of the enzyme was determined to be $60 \pm 2 \times 10^3$. Indoxyl as substrate yielded a K_m of 1.2 mM, while a K_m of 1.7 mM was found for UDPG.

Keywords: *Baphicacanthus cusia*; Acanthaceae; Indoxyl-UDPG-glucosyltransferase; Enzyme purification; Enzyme characterization

1. Introduction

Indican is the β-D-glucoside of 3-hydroxyindole (indoxyl). Indican is present in an exploitable amount in a surprising variety of plant families, the most important being Acanthaceae, Asclepiadaceae, Apocynaceae, Fabaceae, and Polygonaceae (Balfour-Paul, 1998) and in trace amounts in a considerable number of other families (Molisch, 1893, 1898). The ecochemical function of this compound in plants is unknown. After enzymic or chemical hydrolysis, indican forms indoxyl which in turn is spontaneously converted in the presence of oxygen to indigo. Indigo was one of the most important dye compounds in the past (Balfour-Paul, 1998) and is still an esteemed dye, but derived only to a minor degree from plant sources. Surprisingly little is known about the biosynthesis of indican. We have previously shown by ^{13}C-NMR and mass spectroscopy that labelled indole and not tryptophan is the biosynthetic precursor to the indoxyl derivatives indican and isatan B found in plants (Xia & Zenk, 1992). Indole is most likely formed in these dye plants by the action of tryptophan synthase α, the product of the *Bx1* gene (Frey et al., 1997), on indole glycerol phosphate. The remaining questions in the biosynthesis of indican concern two enzymic steps: the hydroxylation of indole in the 3 position and the transfer of the glucose moiety of an activated glucose, such as uridine diphosphate glucose (UDPG), onto the 3-hydroxyl group of indole thus furnishing the indican molecule. The hydroxylation of indole to indoxyl has proven, in this laboratory, to be experimentally difficult in that even under attempted anaerobic conditions the traces of indoxyl formed were oxidized further to analytically problematic derivatives. We, therefore, decided to first search for and purify a hypothetical

* Corresponding author. Present address: Biocentrum, Martin–Luther–Universität Halle–Wittenberg, Weinbergweg 22, D-06120, Halle, Germany. Tel.: +49-345-5525-064; fax: +49-345-5527-301.

✠ Deceased in a motorcycle accident on 30 August 1997

0031-9422/00/$ - see front matter
PII: S0031-9422(99)00430-6

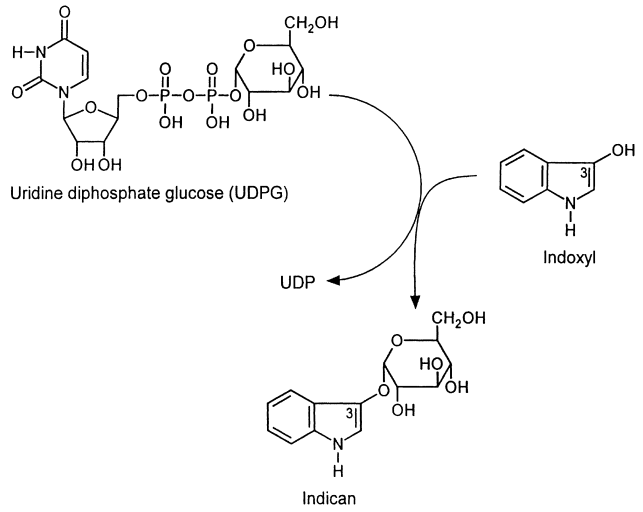


Fig. 1. Reaction catalyzed by action of indoxyl-UDPG-glucosyltransferase. The glucose moiety of UDPG is transferred to the 3-OH group of indoxyl yielding the 3-β-glucoside: indican.

glucosyltransferase, thus making it possible in future to stabilize enzymically formed indoxyl by converting it to indican.

This paper demonstrates the existence of a substrate specific hydroxyindole glucosyltransferase in *Baphicacanthus cusia* Bremek (Acanthaceae). This enzyme was purified to homogeneity, and its substrate and kinetic properties were characterized.

2. Results and discussion

2.1. Enzyme reaction and enzyme assay

The enzyme, indoxyl-UDPG-glucosyltransferase from *B. cusia*, catalyzes the transfer of glucose from UDPG to indoxyl yielding the indoxyl glucoside indican and uridine diphosphate (Fig. 1). Since under aerobic conditions, indoxyl dimerizes spontaneously to yield indigo, the enzyme assay had to be conducted under anaerobic conditions. [^{14}C]UDPG (uridine diphospho-D-[U-^{14}C]glucose) and indoxyl were synthesized and were used as substrates for the development of an enzyme assay. The assay mixture containing the enzyme and [^{14}C]UDPG was incubated in airtight vials under oxygen-free atmosphere prior to the addition of the indoxyl substrate. Radio TLC was

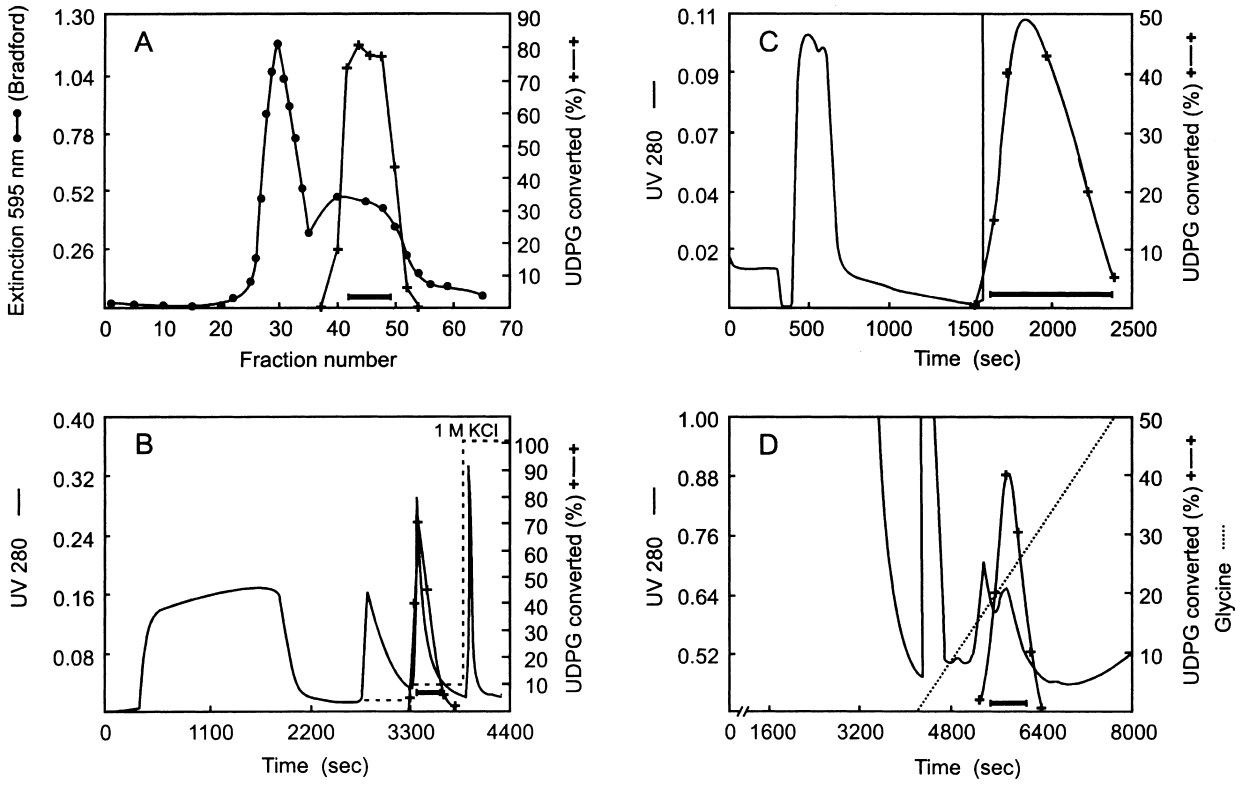


Fig. 2. (A) Ultrogel ACA-34 gel filtration of a pre-purified indoxyl-UDPG-glucosyltransferase preparation obtained after DEAE-Sephacel chromatography with a subsequent membrane filtration (10 kD) step. (B) Bio-Scale Q2 ion exchange chromatography of pooled active fractions of step (A). (C) Hi-Trap Blue fractionation of the pooled active fractions from a PD10 gel filtration step. (D) Immobilized metal affinity chromatography on a Hi Trap Chelating matrix using the combined active fractions from step (C) with a subsequent PD10 gel filtration. The bars indicate the pooled fractions, which were used for subsequent purification steps.

Table 1
Summary of an average purification of indoxyl-UDPG-glucosyltransferase from 200 g (fr. wt.) fresh, green leaves of *B. cusia*

Purification step	Volume (ml)	Protein (mg)	Total activity (nkat)	Specific activity (pkat mg^{-1})	Yield (%)	Purification (-fold)
Crude extract after						
XAD-2	300	429	22	51	100	1
DEAE	150	77	17	221	77	4
ACA-34	49	8.4	6.5	785	30	15
Q2	7.5	1.65	1.8	1090	8	21
Hi-Trap Blue	9	0.14	0.476	3400	2	67
IMAC	6	0.01	0.440	44,000	2	863

used for the determination and quantitation of the ^{14}C-labelled product indican.

2.2. Purification of glucosyltransferase from B. cusia

Purification of the enzyme was carried out using fresh, green leaves of *B. cusia*. The crude extract was immediately subjected to a Servachrom-XAD-2 column to remove indican. Otherwise, indican would have been converted to indoxyl by action of the glucosidases present in the crude extract and the subsequent spontaneous formation of indigo would interfere with the further chromatographic procedures. Therefore, the aim of XAD-2 chromatography was to stabilize the crude extract for the following purification steps. The XAD-2 column was eluted with standard buffer A, and the eluate was collected and subjected to DEAE-Sephacel chromatography. Elution of the enzyme activity was achieved with KCl in standard buffer A. The active fractions were pooled yielding an enriched enzyme extract. For the following gel filtration step, the volume of the DEAE-Sephacel eluate had to be reduced and the concentrate was then applied in two portions to gel filtration on Ultrogel ACA-34 (Fig. 2A). In addition to the separation of proteins, a desalting of the enzyme solution was achieved by this step. The enzyme was eluted with standard buffer A, the active fractions were combined and then subjected to ion exchange chromatography by FPLC employing a Bio-Scale Q2 matrix (Fig. 2B). Elution was done with a KCl-step gradient in standard buffer A. The enzyme activity eluting with the 100 mM KCl step was collected and subsequently subjected to PD10 gel filtration. With this purification step, GSH and MeSH were removed from the enzyme solution and an exchange to standard buffer B was achieved. In the next purification step, the PD10 filtrate was applied to an FPLC Hi-Trap Blue column (Fig. 2C) and the enzyme activity eluted with elution buffer containing UDPG. For a following enzyme analysis of the collected fractions, the eluate had to be purified from UDPG by a PD10 gel filtration step. Finally, the enzyme preparation was subjected to Immobilized Metal Affinity Chromatography (IMAC) on a Hi Trap Chelating matrix (Fig. 2D). Prior to this step, the Hi-Trap Blue eluate was mixed with NaCl to avoid ionic effects within the gel matrix. Elution was done with a linear gradient of elution buffer and the active fractions were combined. Final analysis by SDS-PAGE and the staining of the gel with silver nitrate showed the purified indoxyl-UDPG-glucosyltransferase to be homogeneous. Within six chromatographic steps, 0.002% of the initial protein and 2% of the enzyme activity could be recovered yielding a 863-fold purified enzyme (Table 1). The decisive step for the recovery of a homogeneous enzyme was the application of IMAC, which allowed an extraordinarily high purification factor in combination with a minimum loss of total enzyme activity.

2.3. Properties of indoxyl-UDPG-glucosyltransferase

The purified enzyme eluted in a Bio-Scale Q2 purification step was used to characterize the catalytical properties of the glucosyltransferase. The pH profile of the enzyme activity extended between pH 6.0 and 10.5 with the optimum at pH 8.5 and half-maximal activity at pH 6.8 and 10.2. The optimum temperature of glucosyltransferase was determined using Tris–HCl buffer, pH 8.0, and was shown to be 30°C (Fig. 3). SDS-PAGE under denaturating conditions was used to

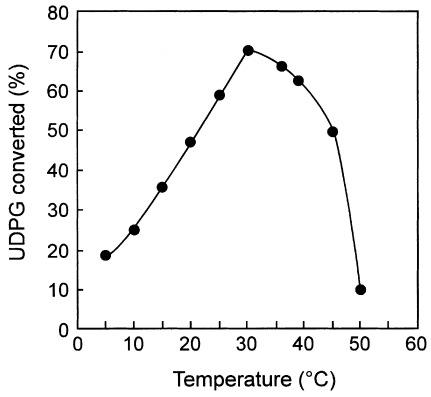
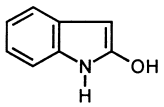
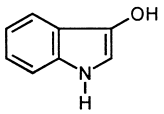
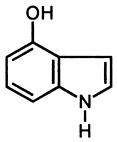
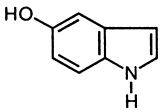
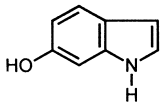
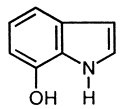


Fig. 3. The effect of temperature on the catalytic activity of indoxyl-UDPG-glucosyltransferase. Buffer used: 300 mM Tris–HCl, pH 8.0. Incubation was for 2.5 h.

Table 2
Substrate specificity of the homogeneous indoxyl-UDPG-glucosyltransferase (IMAC eluate)[a]

Substrate	Relative activity (%) 3-OH-Indole=100%	K_m Value (mM)	Relative V_{max} (%)
 2-OH-Indole	0	no conversion	no conversion
 3-OH-Indole (Indoxyl)	100	1.2	100
 4-OH-Indole	3	1.9	16
 5-OH-Indole	98	0.5	30
 6-OH-Indole	12	3.3	20
 7-OH-Indole	68	1.4	26

[a] The substrate (2 μmol) was incubated together with 50 μmol Tris–HCl buffer, pH 8.0, 60 nmol UDPG, 45 nmol [^{14}C]UDPG, and 6 pkat of the homogeneous enzyme under standard assay conditions. Incubation was for 2.5 h (3-, 5-, 7-OH-indole) and for 7 h (2-, 4-, 6-OH-indole).

determine the M_r of the enzyme. By comparison with appropriate standard proteins and silver staining of the gel, it was determined to be $60 \pm 2 \times 10^3$. The isoelectric point of the enzyme under investigation was measured by chromatofocusing as described in Section 3 to be at pH 6.5.

The homogeneous enzyme recovered after IMAC was used for the calculation of the enzyme turnover. For this purpose, the enzyme concentration of the IMAC effluent was determined by SDS-PAGE and comparison with bovine serum albumin standards to be 1 ng μl^{-1}. Based on the M_r of the enzyme, a concentration of 1.7 pmol in the standard incubation mixture was calculated. A kinetic investigation revealed an enzyme activity of 2.3 pkat and allowed to calculate the turnover of the homogeneous enzyme to be 1.4 kat mol^{-1}.

2.4. Substrate specificity

The substrate specificity of the indoxyl-UDPG-glucosyltransferase was analyzed by incubating 2 μmol of various potential substrates with 6 pkat of the homogeneous enzyme under standard assay conditions.

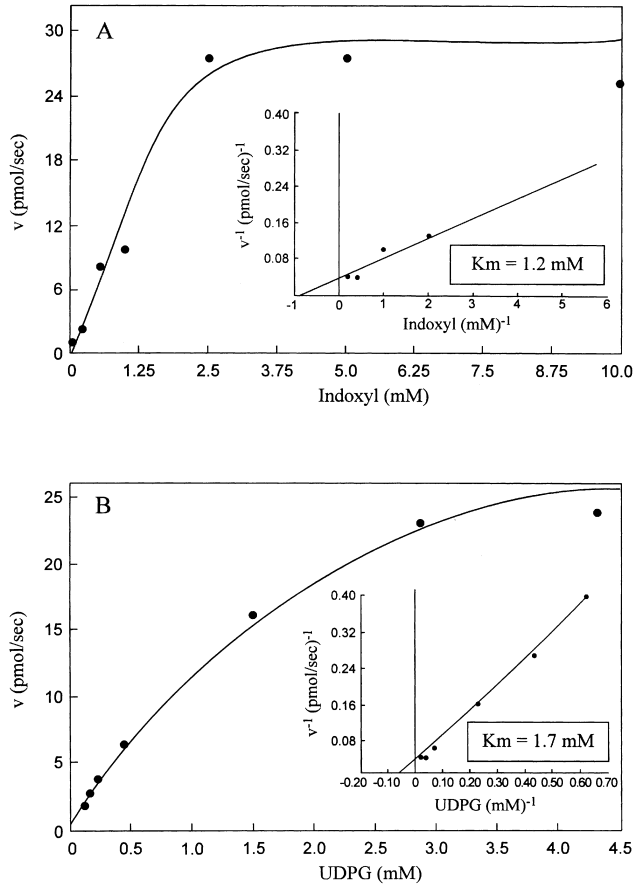


Fig. 4. The effect of substrate concentration on the activity of indoxyl-UDPG-glucosyltransferase. (A) Indoxyl: standard incubation mixture with 1.8 mM UDPG and 18 μg protein. (B) UDPG: standard incubation mixture with 8.5 mM indoxyl and 18 μg protein. Incubation was for 2.5 h at 30°C and pH 8.0. The insert show the respective Lineweaver–Burk plots.

Among these potential substrates were 2-OH-, 4-OH-, 5-OH-, 6-OH-, and 7-OH-indole as well as differently substituted indole carboxylic acids, methylindoles, and phenols. To allow the determination of minute amounts of substrate conversion, the concentration of unlabelled UDPG in the incubation mixture was reduced to 60 nmol, which made a higher assay sensitivity possible. TLC analysis of the reaction mixtures after incubation for 2.5–7 h demonstrated that from 60 substances tested only five were converted to their respective glucosides. The results of this investigation are partly shown in Table 2. While 2-OH-indole was not accepted as substrate by the glucosyltransferase, all other hydroxylated indoles were glucosylated by this enzyme. Minor activity was found with 4-OH- and 6-OH-indole, while 5-OH-indole was converted to almost the same extent as the natural substrate, the 3-OH-indole indoxyl. Since HPLC analysis and GC–MS proved that the tested substrates were free of impurities, the presence of indoxyl in these substrates can be excluded. The reason for the almost identical conversion of 5-OH- and 3-OH-indole may be the similarity of their three-dimensional structures. Obviously, 5-OH-indole fits equally well into the active center of the enzyme, while the structures of the other indoles differ from indoxyl more clearly and were converted by the indoxyl-UDPG-glucosyltransferase to only a lower extent.

Michaelis–Menten kinetics were recorded for those substrates that were glucosylated by the enzyme under investigation as well as for UDPG. The plots of turnover rate versus substrate concentration for indoxyl and UDPG, respectively, are shown in Fig. 4. The inserts are showing the respective Lineweaver–Burk transformations for the determination of K_m values. For indoxyl, an apparent K_m value of 1.2 mM was detected with UDPG at a concentration of 1.8 mM. With 8.5 mM indoxyl as second substrate, UDPG showed an apparent K_m value of 1.7 mM. Kinetic investigations prior to the determination of K_m values confirmed that the glucosyltransferase reaction was linear up to 3.5 h of incubation. The enzyme reaction did not slow down due to UDPG exhaustion during the relatively long times of incubation used for K_m value determination. The K_m values of all further substances accepted as substrates by the indoxyl-UDPG-glucosyltransferase are summarized in Table 2. For these determinations, the concentration of unlabelled UDPG in the enzyme assay mixture (155 μl) had to be reduced to 60 nmol to allow the radiodetection of even very small substrate conversions. Since the enzyme reaction thus could not proceed with UDPG saturation, the results could only be calculated by Lineweaver–Burk transformation. The double reciprocal Lineweaver–Burk plot yields, however, linear kinetics even at low substrate concentrations thus allowing the graphical determination of K_m values. At low substrate concentrations, the UDPG concentration is not limiting and the enzyme kinetic is still linear. Furthermore, the relative V_{max} was calculated for every substrate tested. A comparison of the results (Table 2) showed that the natural substrate of the glucosyltransferase, the 3-OH-indole indoxyl, was glucosylated most rapidly by the enzyme showing the highest V_{max} value among all the substrates tested.

Moreover, the kinetic optimum of the enzyme was calculated, taking into account the K_m value for indoxyl and was determined to be $k_{cat}/K_m = 1166.7$ s^{-1} M^{-1}.

3. Experimental

3.1. Plant material

Baphicacanthus cusia was collected as cuttings in

Thailand, and the plants were rooted and cultivated under greenhouse conditions. Intact green leaves without obvious injuries were used for enzyme purification.

3.2. Chemicals

Indoxyl was enzymically synthesized from indoxyl phosphate under anaerobic conditions according to Heymann and Seligmann (1967). Alkaline phosphatase (5 mg, Sigma P 5521) was suspended in 0.5 ml Tris–HCl buffer (50 mM, pH 7.95) in 0.8 ml airtight vials and the solution gassed with 5% H_2/95% N_2 for 3 min. Indoxyl phosphate (27.5 mg) was separately dissolved in 1 ml of the above Tris–HCl buffer and distributed to three airtight vials under oxygen-free conditions. Thereafter, 1 mg (0.1 ml) of phosphatase in solution (see above) was injected with a 1 ml syringe into every vial containing the indoxyl phosphate solution (100 mM), and the reaction mixture was incubated for 5 h at 37°C. Subsequently, the mixture was incubated for 10 min at 96°C to denature the phosphatase and enzyme impurities. With this procedure, a 77 mM indoxyl solution was obtained that was stored at −20°C until use.

[^{14}C]UDPG was enzymically synthesized as follows: 170 μl [U-^{14}C]glucose-1-phosphate (11 μmol, 16.6 μCi, Boehringer–Mannheim, dissolved in 50 mM Tris–HCl buffer, pH 7.4), 300 μl UTP (30 μmol, Sigma, dissolved in 50 mM Tris–HCl buffer, pH 7.4), 10 μl pyrophosphatase (10 U, Sigma, aqueous solution) and 66 μl UDPG-pyrophosphorylase (33 U, Sigma, aqueous solution) were incubated for 12 h at 30°C. Thereafter, the reaction mixture was separated by PC in solvent system H_2O–HOAc–EtOH (3:2:5) and the product, [^{14}C]UDPG (R_f 0.5), eluted with 1 ml H_2O in 85% yield (14.2 μCi).

Materials for chromatography were purchased from Serva (Servachrom-XAD-2), Pharmacia (DEAE-Sephacel, Hi-Trap Blue, Hi Trap Chelating), and from Bio-Rad (Bio-Scale Q2). All other solvents and reagents were of the highest purity commercially available.

3.3. Enzyme assay

The enzyme assay was conducted under anaerobic conditions in airtight vials by treatment with 5% H_2/95% N_2. The standard assay mixture contained in a total volume of 155 μl: 300 nmol UDPG (20 μl, 10 mg ml^{-1}), 45 nmol [^{14}C]UDPG (5 μl, 67 nCi), 50 μmol (50 μl) Tris–HCl buffer, pH 8.0 and 80 μl enzyme solution (150 μg protein). The reaction was started by the injection of 770 nmol (10 μl) of the above prepared indoxyl solution, and the mixture was incubated for 4 h at 30°C. Thereafter, 8 μl of the reaction mixture was applied to TLC (Polygram SIL G/UV254, Macherey and Nagel, EtOAc–2-BuOH–HCO_2H–H_2O, 5:3:1:1) and the product, [^{14}C]indican (R_f 0.6), quantified by radio scanning (Berthold linear analyser, Tracemaster 20).

3.4. Enzyme purification

Fresh intact green leaves (200 g) of *B. cusia* were mixed with 250 ml ice cold standard buffer A (50 mM Tris–HCl, pH 8.0, containing 20 mM MeSH, 20% glycerol and 2 mg ml^{-1} GSH) and macerated in a mixer until a homogeneous brei was obtained, which was pressed through four layers of cheesecloth and subsequently centrifuged at 20,000 *g*. The supernatant was applied to a Servachrom-XAD-2 column (5 × 25 cm), pre-equilibrated with the above buffer and elution was performed with the same buffer at a flow rate of 50 ml min^{-1}. The crude extract was then applied to a DEAE-Sephacel column (6 × 20 cm), pre-equilibrated with standard buffer A. After washing with 400 ml of the buffer (2 ml min^{-1}), elution was performed with 100 mM KCl in the same buffer (400 ml; 10 ml frs) at a flow rate of 2 ml min^{-1}. The enzyme eluted between fr. 87 and 132. Frs showing the highest enzyme activity were pooled (150 ml) and concentrated by membrane filtration (10 kD). The concentrate (6 ml) was subsequently subjected in two portions to gel filtration on Ultrogel ACA-34. For each run, 3 ml of the concentrate was applied to an ACA-34 column (2.4 × 95 cm), pre-equilibrated with 400 ml standard buffer A at a flow rate of 20 ml h^{-1}. The enzyme was eluted with the same buffer at a flow rate of 18 ml h^{-1} and frs of 6 ml were collected. The active frs were combined (46 ml) and subsequently loaded onto an FPLC Bio-Scale Q2 column (0.7 × 5.2 cm; flow rate: 2 ml min^{-1}), pre-equilibrated with standard buffer A. After washing with 20 ml of the same buffer, the bound enzyme was eluted with a KCl-step gradient (40 mM KCl, 20 ml; 100 mM KCl, 20 ml; 1 M KCl, 15 ml) in standard buffer A at a flow rate of 2 ml min^{-1} and frs of 3 ml were collected. The enzyme was eluted with the 100 mM KCl step. The active frs were pooled and subjected to PD10 gel filtration for buffer exchange and to remove GSH and MeSH. The enzyme was eluted with standard buffer B (50 mM Tris–HCl, pH 8.0, containing 20% glycerol) and 7 ml of the eluate subjected to an FPLC Hi-Trap Blue column (2 × 1 ml; flow rate: 1 ml min^{-1}), pre-equilibrated with standard buffer B. The enzyme was eluted with 10 ml elution buffer (50 mM Tris–HCl, pH 8.0, containing 20% glycerol and 20 mM UDPG) and frs of 2.5 ml were collected. For enzyme analysis, UDPG had to be removed by a subsequent PD10 gel filtration step. The pooled active frs of the Hi-Trap Blue chromatography (9 ml) were mixed with 0.5 M NaCl and then subjected to IMAC

on Hi Trap Chelating. For this purpose, 10 ml of a ZnCl solution (4 mg ml^{-1}) was loaded onto a Hi Trap Chelating column (1 ml) at a flow rate of 1 ml min^{-1} and subsequently washed with 20 ml H_2O. The column was equilibrated with standard buffer C (50 mM Tris–HCl, pH 7.7, containing 20% glycerol and 0.5 M NaCl), and 5 ml of the Hi-Trap Blue eluate was administered at a flow rate of 0.5 ml min^{-1}. After washing with 10 ml standard buffer C, the enzyme was eluted with a linear gradient (0–50%) of elution buffer (50 mM Tris–HCl, pH 7.7, containing 20% glycerol, 0.5 M NaCl and 200 mM glycine) within 30 ml at a flow rate of 0.5 ml min^{-1}. The active frs were combined, the homogeneity of the enzyme proved by SDS-PAGE (Laemmli, 1970) and the protein was detected by staining the gel with silver nitrate (Blum, Beier & Gross, 1989).

3.5. Analytical procedures

Relative protein values were analyzed according to Bradford (1976). The molecular weight of the enzyme was determined by SDS-PAGE (Laemmli, 1970) and comparison with molecular weight standards (rainbow marker, Amersham). The isoelectric point of the glucosyltransferase was measured by chromatofocusing on an FPLC Mono P column (HR 5/20, Pharmacia), equilibrated with 25 mM Tris–HCl buffer, pH 8.2. After applying 3 ml of a Bio-Scale Q2 eluate, the column was washed with 10 ml 25 mM Tris–HCl buffer, pH 8.2, and the enzyme eluted with a diluted (1:10 with H_2O) mixture of Polybuffer 96 and 74 (3:7). Frs of 2 ml were collected and tested for pH and enzyme activity. For the determination of enzyme activity, a reduced amount of unlabelled UDPG (145 nmol instead of 300 nmol) was used.

The pH profile of the enzyme activity was determined using 300 mM buffer (citrate pH 5–6.5; Tris–HCl pH 6.5–10.5; glycine pH 10–11).

Acknowledgements

Our thanks are due to Dr J. Page for his linguistic help in the preparation of this manuscript. This work was supported by SFB 369 of Deutsche Forschungsgemeinschaft, Bonn, and by Fonds der Chemischen Industrie, Frankfurt/Main.

References

Balfour-Paul, J. (1998). *Indigo*. UK: British Museum Press.
Blum, H., Beier, H., & Gross, H. J. (1989). *Electrophoresis*, *8*, 93–99.
Bradford, M. M. (1976). *Anal. Biochem*, *72*, 248–254.
Frey, M., Chomet, P., Glawischnig, E., Stettner, C., Grün, S., Winklmair, A., Eisenreich, W., Bacher, A., Meeley, R. B., Briggs, S. P., Simcox, K., & Gierl, A. (1997). *Science*, *277*, 696–699.
Heymann, H., & Seligmann, A. (1967). *J. Med. Chem*, *10*, 662–664.
Laemmli, U. K. (1970). *Nature*, *227*, 680–685.
Molisch, H. (1893). *Sitzungsber. Akad. Wissensch. Abt. Mineralogie*, *102*, 269–289.
Molisch, H. (1898). *Kaiserl. Akad. Wissensch. Wien. Math. Nat. Klasse. Sitzungsber. Abt. 1*, *107*, 747–779.
Xia, Z.-Q., & Zenk, M. H. (1992). *Phytochemistry*, *31*, 2695–2697.