PERGAMON

Phytochemistry 53 (2000) 187–193

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Hydroquinone:*O*-glucosyltransferase from cultivated *Rauvolfia* cells: enrichment and partial amino acid sequences

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Received 26 July 1999; received in revised form 4 October 1999; accepted 6 October 1999

Abstract

Plant cell suspension cultures of *Rauvolfia* are able to produce a high amount of arbutin by glucosylation of exogenously added hydroquinone. A four step purification procedure using anion exchange, hydrophobic interaction, hydroxyapatite-chromatography and chromatofocusing delivered in a yield of 0.5%, an approximately 390 fold enrichment of the involved glucosyltransferase. SDS-PAGE showed a M_r for the enzyme of 52 kDa. Proteolysis of the pure enzyme with endoproteinase LysC revealed six peptide fragments with 9–23 amino acids which were sequenced. Sequence alignment of the six peptides showed high homologies to glycosyltransferases from other higher plants.

Keywords: *Rauvolfia serpentina* cell culture; Apocynaceae; Hydroquinone; Uridine-diphosphate-glucose transferase; Arbutin; Partial purification; Partial amino acid sequence

1. Introduction

The *O*-ß-D-glucoside of hydroquinone (HQ), also named arbutin, is a widely distributed compound in various higher plants. Especially in species such as *Vaccinium vitis-idaea* (L.) and *Arctostaphylos uva-ursi* (L.) SPRENG of the plant family Ericaceae (Steglich, Fugmann & Lang-Fugmann, 1997) and in the plant *Bergenia crassifolia* (L.) FRITSCH of the family Saxifragaceae, arbutin is accumulated in leaves in significant amounts of up to 20% of the dry weight (Tschitschibabin, Kirssanow & Rudenko, 1930).

From the pharmacological point of view, arbutin has attracted much interest for two therapeutical applications. As it exhibits an antibacterial effect, the leaves of *A. uva-ursi* are used as tea preparations for the treatment of infections of the urogenital tract (Weiss & Fintelmann, 1997). Furthermore, arbutin is known to be an inhibitor of the biosynthesis of the human pigment melanin (Akiu, Suzuki, Fujinuma, Asahara & Fukada, 1988) and is used by the cosmetic company, Shiseido, as a lightener in cosmetics. Because of this application various attempts have been made in the past to generate arbutin in a biotechnological way instead of using a chemical synthesis. One of the most promising procedures for biological arbutin production was the biotransformation in plant cell suspension cultures of exogenously added aglycone hydroquinone with resultant detoxification by glucosylation. Earlier experiments with cell systems clearly indicated such biotransformations but the yields were relatively low (Suzuki, Yoshioka, Tabata & Fujita, 1987; Mizukami, Hirano & Ohashi, 1987). However, cultivated cells of the alkaloid-delivering Apocynaceous species *Catharanthus roseus* (L.) G. DON were proven to be able to produce up to 9 g of pure arbutin per litre cell suspension (Inomata, Yokoyama, Seto & Yanagi, 1991) which was the highest production of a natural product by cultivated plant cells at that time. Commercial production of the glucoside by biotransformation has therefore tentatively been suggested (Yokoyama & Yanagi, 1991). When a similar process was developed with a high density *Rauvolfia* cell sus-

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0031-9422/00/$ - see front matter
PII: S0031-9422(99)00539-7

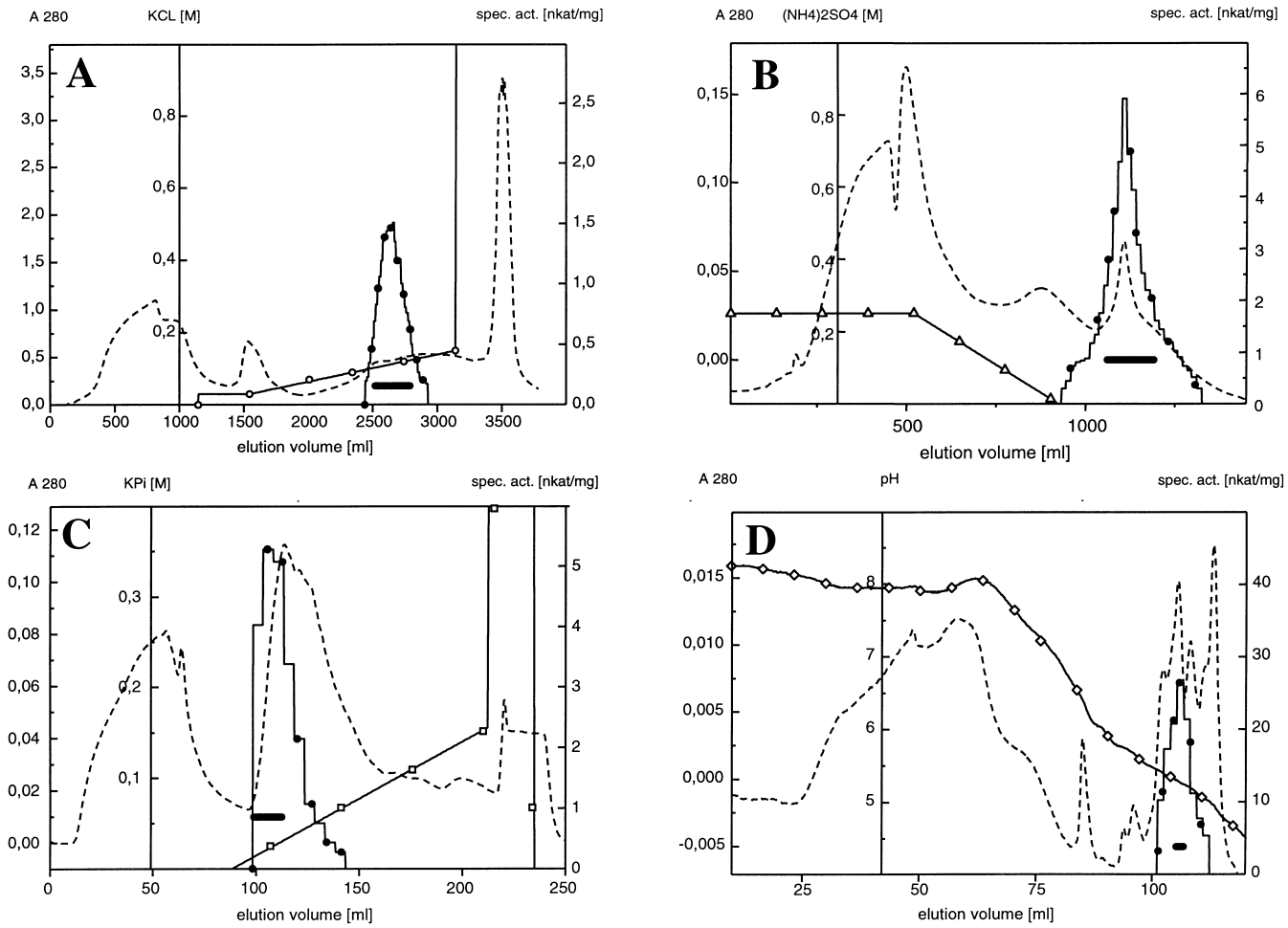

Fig. 1. Purification of hydroquinone:*O*-glucosyltransferase. (A) elution profile of DEAE-sepharose anion exchanger with linear gradient 0.03–0.15 M KCl; (B) enrichment of the enzyme by the fast flow Phenyl-sepharose CL-4b with a linear gradient of 250 to 0 mM $(NH_4)_2SO_4$; (C) seperation on a ceramic hydroxyapatite column with a linear gradient of 0.01–0.4 M KPi; (D) Chromatofocusing on a Mono P column starting at pH 7.5 followed by elution with 5% polybuffer 74 pH 4.0 (- - - -), UV absorption; (-●-), specific activity; (-○-), KCl gradient; (–△–) $(NH_4)_2SO_4$ gradient (–□–), KPi gradient; –◇–, pH value; (——), pooled fraction.

pension instead of the *C. roseus* cells, the amounts of formed arbutin could be considerably enhanced with an optimum accumulation of 18 g/l nutrition medium (Lutterbach & Stöckigt, 1992), demonstrating the exceptional glucosylation potential of these cells. In order to understand this glucosylation process more precisely, a prerequisite is the investigation of the involved enzyme system.

Here, we report the enrichment and the partial amino acid sequences of the hydroquinone:*O*-glucosyltransferase (E.C. 2.4.1...), including sequence comparison to other glycosyltransferases from higher plants.

2. Results and discussion

The structure of the enzyme product was determined after acetylation by EI–MS, showing identical fragmentation pattern with a reference sample. A short HPLC assay which takes 5 min only was developed to monitor the activity of the enzyme hydroquinone:*O*-glucosyltransferase during formation of arbutin. A good separation of the cofactor uridine diphosphate glucose (UDPG), the substrate hydroquinone and the enzyme product arbutin with retention times of 1 min 6 s, 2 min 42 s and 3 min 35 s, respectively, allowed exact quantitation of the catalysed reaction. Based on this assay, kinetics could be established to compare the cell culture growth of *R. serpentina* suspensions and enzyme production (data not shown). Because of the excellent growth characteristics of the *Rauvolfia* cells in the standard LS nutrition medium described by Linsmaier and Skoog (1965) and in 1 l Erlenmeyer flasks, these conditions were used to generate kilograms of cell material. These cultures showed optimum

Table 1
Purification of hydroquinone:*O*-glucosyltransferase

Purification step	Volume (ml)	Total protein (mg)	Total activity (nkat)	Recovery (%)	Specific Activity (nkat/mg)	Enrichment (*x*-fold)
Crude extract	7100	7650	510	100	0.067	1
$(NH_4)_2SO_4$ (30–70%)	750	5520	443	86.6	0.08	1.2
DEAE-sepharose	300	340	365	71.6	1.07	16
Phenyl-sepharose CL-4b	140	34	120	23.5	3.53	52.7
Hydroxyapatite	15	2.9	13.8	2.7	4.75	70.1
Mono P	1.5	0.095	2.5	0.5	26.32	390

transferase activities. About 5.5 kg fresh *Rauvolfia* cells were grown and harvested for enzyme isolation and purification. After $(NH_4)_2SO_4$ fractionation about 5.5 g soluble protein was obtained which was further purified by four steps of column chromatography (see Fig. 1). As illustrated in Table 1, the separation with DEAE ion exchange chromatography of crude protein mixture from $(NH_4)_2SO_4$ precipitation resulted in a 16 fold enrichment with an acceptable yield of 70%. A further 3 fold enrichment was achieved, when the remaining protein from DEAE chromatography was subjected to fast flow Phenyl-sepharose Cl-4b chromatography which gave a relatively broad protein and activity peak; therefore only the fractions at the peak maximum, similar to highest enzyme activity, were pooled (Fig. 1(B)). The final yield of active enzyme in this step was rather low, amounting to 23%. When these prepurified protein fractions were tested by SDS-PAGE, the enrichment of the enzyme, which should appear at 52 kDa (determined by gel chromatography with a Sephacryl S-100 column), was not visible. When Hydroxyapatite chromatography was employed the active enzyme was eluted as a mixture with many other remaining proteins forming a sharp peak, but clearly showing pronounced tailing. As the activity maximum of the enzyme appeared in the ascending flank of the peak, only fractions of the first half of the peak were combined leading to a 70 fold enrichment and a low yield of 3% compared to the original enzyme activity (Table 1). In contrast to this step the chromatofocusing on a Mono P column was more effective. More than 50% of the proteins did not bind during the sample loading and enzyme activity eluted as a sharp peak from which only one fraction at the peak maximum was used for further investigations (Fig. 1(D)). As 3 mg of protein has been applied to this step of chromatography, sufficient enzyme for partial sequencing should remain at the end. In fact ca. 0.1 mg of the enzyme remained, which on SDS-PAGE was not homogeneous but showed a very strong enrichment remaining as the strongest protein band which could be assigned by its molecular mass of ~52 kDa (Fig. 2(A)). The identification of the correct protein band was confirmed by the fact that with higher amounts of this protein band the enzyme activity increased at the corresponding rate. Furthermore, we used native PAGE, followed by detection of enzyme activity and SDS-PAGE as a second dimension, which confirmed the identification of the correct protein band (Fig. 2(B)). By digestion of this band (Fig. 2(C)) with proteinase LysC, six peptide fragments were separated by HPLC and then sequenced (see Table 2).

The comparison of all six peptide sequences against databases (FASTA) (Pearson & Lipman, 1988) showed homologies (up to 100%) to other glycosyltransferases.

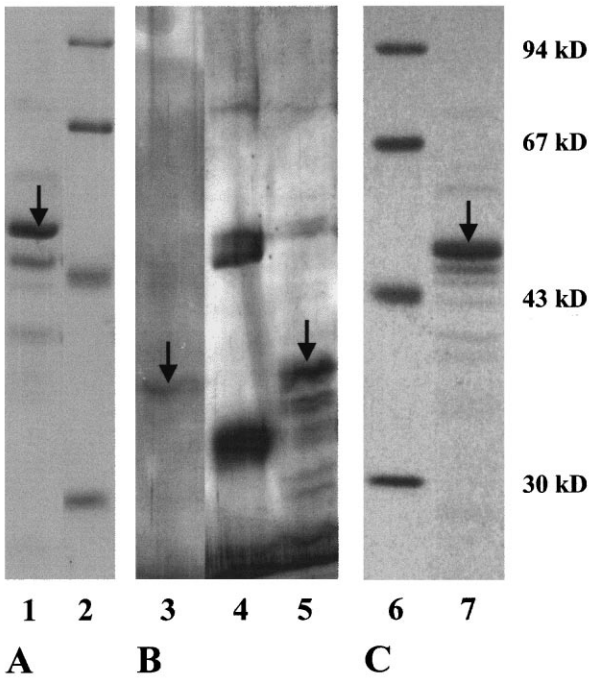

Fig. 2. SDS-PAGE of hydroquinone:*O*-glucosyltransferase after complete purification procedure. (A) SDS-PAGE (11%); lane 1: fraction showing highest enzyme activity, eluted from Mono P column; lane 2: molecular weight marker mixture. (B) SDS-PAGE (8%) as second dimension after native PAGE; lane 3: protein, eluted from native PAGE, showing transferase activity; lane 4: molecular weight marker mixture; lane 5: fraction with highest enyme activity after chromatofocusing (see gel A, lane 1). (C) SDS-PAGE (11%) that was used for sequencing after digestion of transferase band with endoproteinase LysC; lane 6: molecular weight marker mixture; lane 7: fraction with highest enzyme activity after chromatofocusing (see gel A, lane 1). The arrow indicates protein band of hydroquinone:*O*-glucosyltransferase.

Table 2
Sequence of 6 peptide fragments after hydroquinone:*O*-glucosyltransferase digestion with endoproteinase LysC, separated by HPLC

Peptide fragment	Amino acid sequence
Peptide 7a	Asp-Ala-Ala-Ser-Arg-Ala-Leu-Ser-Asp-Asp-Gly-Ser-Ser-Thr-Lys
Peptide 7b	Ile-Ala-Asn-Ala-Thr-Tyr-Phe-Ser-Ile-Gln-Asn-Glu-Asn-Asp-Ala-Leu-Ala-Tyr-Leu-Pro-Glu-Gly-Phe
Peptide 13	Ala-Leu-Ala-Ala-Glu-Leu-Ala-Glu-Lys
Peptide 18	Asp-Phe-Leu-Asp-Pro-Ala-Gln-Asp-Arg-Lys
Peptide 26	Ala-Gly-Glu-Asn-Gly-Leu-Ile-Gly-Arg-Val-Glu-Ile-Ala-Asn-Ala-Val-Lys
Peptide 30	Arg-Tyr-Arg-Leu-Ala-Glu-Gly-Ile-Met-Val-Asn-Thr-Phe-Asn-Asp-Leu-Glu-Pro

Fig. 3 displays the regions of different glycosyltransferases from various plants with homologies to the transferase isolated from *Rauvolfia*. Some of the enzymes exhibit homologies to all six peptides. As the putative glucosyltransferases from *Arabidopsis* and *Manihot* (Hughes & Hughes, 1994), whose functions are not published yet, all other transferases seem to be involved in secondary metabolism. Based on sequence comparison, most of them, such as the transferases isolated from *Perilla*, *Verbena*, *Ipomoea* and *Vigna*, are expected to be involved in the biosynthesis of flavonol or anthocyanin glycosides, compounds involved in pigment formation. A few other transferases are known to glucosylate plant hormones (Bandurski, Cohen, Slovin & Reinecke, 1995).

As far as we know, none of these enzymes is described as accepting hydroquinone as a substrate. Several glucosyltransferases, acting on phenolic substrates, have been described in detail (Gross, 1983; Tabata, Umetani, Ooya & Tanaka, 1988; Parry & Edwards, 1994; Politycka, 1997), but no sequence data are available till now. In addition, the substrate prefer-

Enzymes	Sequences with homologies to PF 18	Identity [%]	Sequences with homologies to PF 30	Identity [%]	Sequences with homologies to PF 7b	Identity [%]
PEPTIDE	**DFLDPAQDRK**		**RYRLAEGIMVNTFNDLEP**		**IANATYFSIQNQNDAL---AYLPEGF**	
AT	163 DFLDPAQDRK 172	**100**	185 RYKEAEGILVNTFFELEP 202	**72**	292 IANSSYFDSHSQTDPL---TFLPPGF 314	**52**
PF	174 TFLLPETPER 183	**30**	197 GEEKAK-VLVNTFDALEP 213	**44**	311 DDGEE--------------EEEELSC 322	**0**
GT	187 EELIMEDSQS 196	**10**	209 LHKA-TAVAVNSFEEIDP 225	**22**	311 KH-------------------LPENF 317	**17**
VH	179 FLLPSTHERF 208	**20**	201 GEEKPK-VLVNSFDALEP 217	**33**	314 ---EG--------------EEVLISC 322	**0**
ME	183 EVVDPMLDRT 192	**40**	205 EIPTADGILMNTWEALEP 222	**44**	313 KTGDAAFFTQGDGAD-DMSGYFEEGF 337	**30**
SB	185 VFLKFKEDDP 194	**30**	205 ANGRSFGSIFNTFEALDS 222	**22**	315 QQEEQGFGS------------VPKGF 328	**17**
PS	69 SFIRTTQPND 78	**20**	90 RAQKASAIILNTFDDLEH 107	**50**	205 A-GEN--------------SVLPQEF 215	**13**
IP	148 GEILAKDLQE 157	**0**	170 LPGA-NAVVINSFQNLEP 186	**28**	279 KH-------------------LPEGF 285	**22**
VM	83 GTAGTHLGQA 92	**0**	105 APPEARGVIVNSFEELEP 123	**44**	218 EEGAKHEEAAKPGDEFDLASMLPDGF 243	**26**

Enzymes	Sequences with homologies to PF 26	Identity [%]	Sequences with homologies to PF 7a	Identity [%]	Sequences with homologies to PF 13	Identity [%]
PEPTIDE	**A-GENGLIGRVEIANAVK**		**DAASRAL-SDDGSSTK**		**ALAELAEK**	
AT	390 A-GDDGLVRREEVARVVK 406	**53**	426 EAACRVLK-DDGTSTK 440	**67**	441 ALSLVALK 448	**50**
PF	397 MNEGGGV-DGSEIERCVE 413	**24**	434 TLAREAM-GEDGSSLK 448	**47**	449 NLNAF--- 453	**13**
GT	392 ----GGVFTEDETTRVLE 405	**12**	425 EKAKDAVK-ANGSSTR 439	**40**	440 NFESLL-- 445	**13**
VH	397 ANEEGSVVDGDEIRRCIE 414	**24**	435 DLARKAM-EEDGSSVN 449	**47**	450 NLKVF--- 454	**13**
ME	413 NLPAKEVVKREEIERMIR 430	**18**	450 DSGEKALN-EGGSSFN 464	**40**	465 YMSALGNE 472	**13**
SB	403 CEGSNSVPDPIELGRKIN 420	**18**	437 VEALEAVK-IGGSSKK 451	**40**	452 DLDSIVKE 459	**13**
PS	290 DAKRDKI-------ESLV 300	**0**	321 KLAPNAASGPNGSSFM 336	**33**	337 NLEKM--- 341	**13**
IP	360 ----GGKFTKDETLKAIN 373	**18**	393 GEAMEAVK-PHGSSTK 407	**47**	408 EFQELV-- 413	**25**
VM	319 ERVEDGFVSAEEVEKRVR 336	**24**	353 QMAMAAVA-EFGSSTT 367	**40**	368 AIAHLLHS 375	**38**

Fig. 3. Sequence comparision of hydroquinone:*O*-glucosyltransferase fragments against Swiss Prot data base using FASTA. Enzymes are: (AT) Putative glucosyltransferase from *Arabidopsis thaliana* (AAB61023). (PF) Anthocyanin 5-*O*-glucosyltransferase *Perilla frutescens* (BAA36421). (6T) Flavonol 3-*O*-glucosyltranferase from *Gentiana trifolia* (Q96493). (VH) Anthocyanin 5-*O*-glucosyltransferase from *Verbena x hybrida* (BAA36423). (ME) Flavonol 3-*O*-glucosyltransferase from *Manihot esculenta* (Q40287). (SB) UDPG glucosyltransferase from *Solanum berthaultii* (AAB62270). (PS) UDP-glucuronosyltransferase from *Pisum sativum* (AAB99950). (IP) Flavonoid 3-*O*-glucosyltransferase from *Ipomoea purpurea* (AAB86473). (VM) Flavonoid glycosyltransferase from *Vigna mungo* (BAA36412).

ences of most of the plant glucosyltransferases, which sequences are known, have not been determined. The most important reasons for this have been undoubtedly the difficulties experienced in obtaining plant glucosyltransferases in a homogeneous state as discussed by Ford, Boss and Høj (1998), although few examples of successful work in this field were recently reported (Vogt, Zimmermann, Grimm & Strack, 1997, Ford et al., 1998).

Because hydroquinone and arbutin seem not to be naturally occurring compounds in *Rauvolfia* plants or cell cultures, the enzyme must have another substrate specificity. However, the homologies of the peptide fragments to other plant glycosyltransferases indicate that, most probably, the *Rauvolfia* transferase described here is also involved in secondary metabolism. To settle this question additional testing of the enzyme specificity is necessary, but the need for higher quantities of pure protein for future investigations could only be satisfied by heterologous expression of active enzyme.

The sequence information discussed herein will be very useful because it can be a tool for the isolation of the appropriate cDNA or genomic clone encoding for this particular enzyme.

If this is successful, the UDPG dependent transferase might be expressed heterologously as demonstrated for a number of other enzymes of the secondary metabolism from plant cell suspension cultures (Kutchan, 1989; Dittrich & Kutchan, 1991; Kutchan, 1998) in order to obtain enough protein to perform a detailed analysis of this enzyme for the first time. This could, however, also offer the possibility of generation of the required commercially employed arbutin by an enzyme mediated synthesis or by biotransformation of hydroquinone with genetically engineered organisms like *E. coli* or yeast systems. The appropriate investigations are currently in progress.

3. Experimental

3.1. Plant cell material

Cell suspension cultures of *R. serpentina* were grown in 1 l Erlenmeyer flasks in Linsmaier and Skoog medium (1965) for 10 days under continuous light (600 lux) and shaking (100 rpm) at 24 ± 2°C. For an optimum enzyme isolation, transferase activities and cell growth were determined every day after inoculation. The cell material was harvested by suction filtration, frozen with liquid nitrogen and stored at −26°C.

3.2. HPLC and transferase assay

HPLC was performed on a Merck-Hitachi system connected to a LiChrospher® 60 RP — select B column (125×4 mm, 5 μm). Product separation was achieved in an isocratic mode with 5% MeOH in H_2O adjusted with H_3PO_4 to pH 2.5 with a flow rate of 1 ml/min and UV detection at 284 nm; R_t UDPG 1 min 06 s, HQ 2 min 42 s, arbutin 3 min 35 s.

The enzyme incubation mixture (total volume 125 μl) contained 100 mM Tris–HCl buffer (pH 7.5), 2 mM UDPG, 1 mM HQ and various amounts of protein. The incubation time varied between 10 and 60 min, depending on enzyme activity. The standard assay temperature was 50°C.

3.3. Protein determination

Protein concentrations were determined by the method of Bradford (1976) mixing 0.1 ml protein solution, 0.9 ml Bradford reagent and measuring the absorption at 595 nm. For calibration bovine serum albumin was used. For estimating protein amounts during enzyme enrichment by column chromatography, the UV curves were integrated with the ÄKTA-Explorer Software.

3.4. SDS-PAGE protein staining

Staining of proteins in SDS gels was carried out with Coomassie solution (0.25% Coomassie Brilliant Blue R-250, 45% MeOH and 9% HOAc in water); destaining of background was performed with a solution of 30% MeOH and 9% HOAc in water. Silver staining was performed as published previously (Heukeshoven & Dernick, 1985). Enzyme enrichment during the purification of the glucosyltransferase was checked by SDS-PAGE under denaturing conditions using acrylamide amounts ranging between 8 and 11%. Marker protein used was the LMW marker mixture (Amersham Pharmacia Biotech, Freiburg); for native PAGE CleanGel 25S (Pharmacia) was applied.

3.5. Buffers

Buffer A: 0.15 M Tris–HCl, 20 mM ß-mercaptoethanol (EtSH), pH 7.5. Buffer B: 20 mM Tris–HCl, 10 mM EtSH, pH 7.5. Buffer C: as buffer B and 1 M KCl. Buffer D: as buffer B and 250 mM $(NH_4)_2SO_4$. Buffer E: 10 mM KPi, 10 mM EtSH, pH 7.5. Buffer F: 0.4 M KPi, 10 mM EtSH, pH 7.5. Buffer G: 5% Polybuffer 74 (Pharmacia), 10 mM EtSH, pH 4.0. Buffer H: 20 mM Tris–HCl, 10 mM EtSH, pH 7.5, 20% polyethylen glycol 20,000. Buffer I: as Buffer B and 150 mM NaCl.

3.6. MS measurements

For MS investigation of the enzyme product arbu-

tin, a Finnigan MAT 44 S quadrupole instrument was used under direct inlet conditions (70 eV) at an ion source temperature of 200°C; arbutin was acetylated by a mixture of acetic anhydride and dried pyridine (1:1) in the MS tube for 12 h and dried under vacuum for the same time before measurement.

3.7. Preparation of crude protein extracts

Cells of *R. serpentina* cell suspensions (5.5 kg, fresh weight) were frozen with liquid nitrogen, added to 2.5 l buffer A and mixture-aliquots were homogenized with an ultraturrax for 1 min each, then filtered through cheese cloth. The resulting solution was centrifuged at 10.000 × g for 30 min and the supernatant (7.1 l) was used for the next purification step. All purification procedures were carried out at 4°C.

3.8. Fractional precipitation with $(NH_4)_2SO_4$

The protein in the resulting supernatant was subjected to $(NH_4)_2SO_4$ precipitation. The precipitated protein between 30 and 70% $(NH_4)_2SO_4$ was collected after centrifugation (30 min, 10.000 × g), solubilised in 500 ml buffer A and centrifuged. The supernatant was dialyzed twice against 10 l buffer B for 8 h each time. Centrifugation (20 min, 10,000 × g) and resolution of the precipitate yielded the soluble protein fraction (750 ml, 7.4 mg/ml).

3.9. Anion exchange chromatography on fast-flow DEAE-sepharose

The obtained protein solution was added at a flow rate of 10 ml/min to a fast-flow DEAE-sepharose column (20.3 × 5 cm, vol 400 ml, XK50/30-column, Pharmacia) which had been equilibrated with buffer B. After washing the column with one column volume (CV, 400 ml) of buffer B, one CV 0.03 M KCl in buffer B, the enzyme was eluted with a linear KCl-gradient (4 CV, 0.03–0.15 M KCl) prepared from buffers B and C. Enzyme activity appeared at 0.1 M KCl. Fractions containing transferase activity were pooled and prepared for the next purification step by adding $(NH_4)_2SO_4$ to a final concentration of 250 mM (300 ml).

3.10. Hydrophobic interaction chromatography on fast-flow Phenyl-sepharose Cl-4b

The resulting protein solution from anion exchange chromatography was pumped with a flow rate of 5 ml/min through a fast-flow Phenyl-sepharose column (38 × 2.6 cm, CV 200 ml, XK26/40-column, Pharmacia) which had been equilibrated with buffer D. After washing the column with 1 CV of buffer D, the enzyme was eluted with a gradient from 250–0 mM $(NH_4)_2SO_4$ prepared from buffers D and B. Fractions containing >0.86 nkat/mg enzyme activity were combined (140 ml) and concentrated by dialysis overnight against 3 l of buffer H to a remaining volume of around 1 ml.

3.11. Hydroxyapatite chromatography

This concentrated protein solution was diluted to a volume of 56 ml with buffer E and was added to a Macro-Prep® Ceramic Hydroxyapatite (Bio-Rad, Krefeld)) column (1.5 × 1.6 cm, CV 3 ml, XK16/20-column, Pharmacia), equilibrated before with buffer E at a flow rate of 2 ml/min. After washing the column with 7 CV of buffer E proteins were eluted with a KPi-gradient (10–400 mM, 35 CV) prepared with buffers E and F. 3 Fractions (15 ml) containing >4 nkat/mg enzyme activity were combined and dialyzed overnight against 2 l of buffer B.

3.12. Chromatofocusing with mono P

The dialyzed enzyme solution (55 ml) was added with a flow rate of 0.5 ml/min to a Mono P HR 5/20 column (Pharmacia) which had been equilibrated with buffer B. Proteins were fractionated with 30 ml buffer G at a flow rate of 0.5 ml/min generating an almost linear pH-gradient from pH 7.5 to 4.0. Transferase activity was eluted at a pH around 5.3.

3.13. M_r determination by sephacryl S-100 chromatography

An enzyme solution (0.5 ml) obtained by concentration with buffer H after chromatography on Phenyl-sepharose was applied to a calibrated HiPrep 16/60 Sephacryl S-100 HR column (Pharmacia) and eluted with buffer I (18 ml/h, FPLC, Pharmacia). Fractions of 1 ml were collected and in each fraction the enzyme activity was determined. Highest activities were monitored after 48 ml, corresponding to a relative molecular weight of the enzyme of 52 kDa (± 8%).

3.14. Sequencing of the enzyme and peptide sequence alignment

The enzyme was digested with endoproteinase LysC in the gel. After separation and fractionation of formed peptide fragments by HPLC on a Supersphere 60 RP select B column (Merck, Darmstadt), the peptides were sequenced.

Acknowledgements

Our thanks are due to Professor Dr. F. Lottspeich, Max Planck Institut für Biochemie (Martinsried, Germany) for performing the amino acid sequencing of the enzyme. We are also indebted to Professor Dr. W.E. Court (Mold, Wales) for correcting the English version of the manuscript, to the Fonds der Chemischen Industrie (Frankfurt/Main, Germany) and to the Deutsche Forschungsgemeinschaft (Bonn, Germany) for financial support.

References

Akiu, S., Suzuki, Y., Fujinuma, Y., Asahara, T., & Fukada, M. (1988). Inhibitory effect of arbutin on melanogenesis: biochemical study in cultured B16 melanoma cells and effect on the UV-induced pigmentation in human skin. *Proc. Jpn. Soc. Invest. Dermatol*, *12*, 138–139.

Bandurski, R. S., Cohen, J. D., Slovin, J., & Reinecke, D. M. (1995). In P. J. Davies, *Plant hormones: physiology, biochemistry, and molecular biology* (2nd ed) (pp. 39–65). Dordrecht, Netherlands: Kluwer Academic Publishers.

Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*, *72*, 248–254.

Dittrich, H., & Kutchan, T. M. (1991). Molecular cloning, expression, and induction of berberine bridge enzyme, an enzyme essential to the formation of benzophenanthridine alkaloids in the response of plant pathogenic attack. *Proc. Natl. Acad. Sci. U.S.A*, *88*, 9969–9973.

Ford, C. M., Boss, P. K., & Høj, P. B. (1998). Cloning and characterization of *vitis vinifera* UDP-glucose:flavonoid 3-*O*-glucosyltransferase, a homologue of the enzyme encoded by the maize Bronze-1 locus that may primarily serve to glucosylate anthocyanidins in vivo. *J. Biol. Chem*, *273*, 9224–9233.

Gross, G. G. (1983). Partial purification and properties of UDP-glucose:vanillate 1-*O*-glucosyltransferase from oak leaves. *Phytochemistry*, *22*, 2179–2182.

Heukeshoven, J., & Dernick, R. (1985). Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis*, *6*, 103–112.

Hughes, J., & Hughes, M. A. (1994). Multiple secondary plant product UDP-glucose glucosyltransferase genes expressed in cassava (*Manihot esculenta* Crantz) cotyledons. *DNA-Seq*, *5*, 41–49.

Inomata, S., Yokoyama, M., Seto, S., & Yanagi, M. (1991). High-level production of arbutin from hydroquinone in suspension cultures of *Catharanthus roseus* plant cells. *Appl. Microbiol. Biotechnol*, *36*, 315–319.

Kutchan, T. M. (1989). Expression of enzymatically active cloned strictosidine synthase from the higher plant *Rauvolfia serpentina*. *FEBS Lett*, *257*, 127–130.

Kutchan, T.M. (1998). Molecular genetics of plant alkaloid biosynthesis. In G.A. Cordell (Ed.), *The alkaloids* (Vol. 50, pp. 257–316).

Linsmaier, E. M., & Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant*, *18*, 100–127.

Lutterbach, R., & Stöckigt, J. (1992). High-yield formation of arbutin from hydroquinone by cell-suspension cultures of *Rauwolfia serpentina*. *Helv. Chim. Acta*, *75*, 2009–2011.

Mizukami, H., Hirano, A., & Ohashi, H. (1987). Effect of substituent groups on the glucosylation of xenobiotic phenols by cultured cells of *Gardenia jasminoides*. *Plant Sci*, *48*, 11–15.

Parry, A. D., & Edwards, R. (1994). Characterization of *O*-glucosyltransferases with activities toward phenolic substrates in alfalfa. *Phytochemistry*, *37*, 655–661.

Pearson, W. R., & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. U.S.A*, *85*, 2444–2448.

Politycka, B. (1997). Free and glucosylated phenolics, phenolbeta-glucosyltransferase activity and membrane permeability in cucumber roots affected by derivatives of cinnamic and benzoic acids. *Acta Physiol. Plant*, *19*, 311–317.

Steglich, W., Fugmann, B., & Lang-Fugmann, S. (1997). *RÖMPP Lexikon-Naturstoffe* (p. 53). Stuttgart, New York: Georg Thieme Verlag.

Suzuki, T., Yoshioka, T., Tabata, M., & Fujita, Y. (1987). Potential of *Datura innoxia* cell suspension cultures for glycosylating hydroquinone. *Plant Cell Rep*, *6*, 275–278.

Tabata, M., Umetani, Y., Ooya, M., & Tanaka, S. (1988). Glucosylation of phenolic compounds by plant cell cultures. *Phytochemistry*, *27*, 809–813.

Tschitschibabin, A. E., Kirssanow, A. W., & Rudenko, M. G. (1930). Über nicht gerbende Substanzen aus Badan (*Saxifraga crassifolia*) Part II: Arbutin. *Liebigs Ann. Chem*, *479*, 303–306.

Vogt, T., Zimmermann, E., Grimm, R., & Strack, D. (1997). Are the characteristics of betanidin glucosyltransferases from cell-suspension cultures of *Dorotheanthus bellidiformis* indicative of their phylogenetic relationship with flavonoid glucosyltransferases? *Planta*, *203*, 349–361.

Weiss, R. F., & Fintelmann, V. (1997). *Lehrbuch der Phytotherapie* (p. 47). Stuttgart: Hippokrates Verlag.

Yokoyama, M., & Yanagi, M. (1991). High level production of arbutin by biotransformation. In A. Komamine, M. Misawa, & F. DiCosmo, *Plant cell culture in Japan* (pp. 79–91). Tokyo: CMC Co.