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ISOLATION, PURIFICATION AND CHARACTERIZATION OF UDP-GLUCOSE: *CIS-p*-COUMARIC ACID-β-D-GLUCOSYLTRANSFERASE FROM *SPHAGNUM FALLAX*

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Abstract—A stereospecific glucosyltransferase, catalysing the transfer of glucose from UDP-glucose to the 4′-hydroxy-group of *cis-p*-coumaric acid, has been isolated and partially purified from *Sphagnum fallax* Klinggr. (Klinggr. clone 1) by ion exchange chromatography, gel filtration, and hydrophobic interaction chromatography. The enzyme was purified 600-fold with a yield of 0.4%. The M_r was estimated by gel filtration on a Superdex 200 column to be about 56 000. Optimal enzyme activity was observed in glycine-KOH at pH 9.3. The transferase activity was not influenced by divalent cations (1 mM) or EDTA (5 mM), but it was inhibited by NaCl (0.4 M) to 40% and by 2-mercaptoethanol (14 mM) to 30%. The enzyme showed a pronounced substrate specificity towards *cis-p*-coumaric acid with a K_m value of 44 μM, *trans-p*-coumaric acid was not transformed. The apparent K_m value for the cosubstrate UDP-glucose was 110 μM. The only reaction product was 4′-*O*-β-D-glucosyl-*cis-p*-coumaric acid. The substrate *cis-p*-coumaric acid inhibited the reaction at a concentration higher than 1 mM. The only other phenolic substrate transformed by the purified glucosyltransferase was *cis*-caffeic acid, with an enzyme activity of 10% compared with that using *cis-p*-coumaric acid as substrate. © 1997 Elsevier Science Ltd

INTRODUCTION

The secondary metabolism of *Sphagnum* spp. is distinguished by several phenolics, which have been described as being unique in peat mosses. The most important cinnamic acid derivative is *trans*-sphagnum acid (*p*-hydroxy-β-[carboxymethyl]-cinnamic acid) [1, 2], forming up to 0.1% of the plantlets dry weight [3]. Tracer experiments gave evidence for the formation of the carboxymethyl side chain from acetate [4, 5]. In some *Sphagnum* species *trans*-sphagnum acid is predominantly stored as buffer-soluble free acid within the cells, and furthermore excreted to a great extent into the external medium [6]. Exogenously supplied precursors like L-phenylalanine, *trans*-cinnamic acid and *p*-coumaric acid were rapidly taken up by *S. fallax* and metabolized to *trans*-sphagnum acid [7].

However, application of *trans-p*-coumaric acid under the light conditions used for cultivation of *S. fallax* bioreactor cultures stimulated not only the formation of *trans*-sphagnum acid but, above all, the endogenous accumulation and excretion of a *p*-coumaric acid conjugate. Recently, we isolated this conjugate and elucidated its structure by NMR- and IR-spectroscopy as 4′-*O*-β-D-glucosyl-*cis-p*-coumaric acid. An *in vitro* glucosyltransferase (GTase) assay, catalysing the transfer of glucose from UDP-glucose on the 4′-hydroxy group of *cis-p*-coumaric acid, was developed. More detailed *in vivo* studies on the biosynthesis of this glucoside demonstrated, that *trans-p*-coumaric acid, supplied to *S. fallax* batch cultures, was isomerized photochemically in the external medium, and taken up by the plantlets preferentially as *cis-p*-coumaric acid, which was then conjugated with glucose [8].

Glucosyltransferases, catalysing the transfer of glucose from sugar nucleotides to phenolic compounds, are of widespread occurrence in plants [9]. Both, ester formation [10–13] and glucoside formation [14, 15], is possible with hydroxycinnamic acids as glucosyltransferase substrates, although more often ester formation was observed [16]. However, there is little information concerning the occurrence and biosynthesis of *cis*-hydroxycinnamic acid sugar esters or glycosides. According to refs [17–19], these phenolic conjugates are formed by photochemical means from their *trans*-isomers after the glucosylating reaction. To our knowledge, the only case of the glucosylation

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of a phenolic *cis*-isomer by a stereospecific glucosyltransferase is the biosynthesis of *cis*-coniferin from *cis*-coniferyl alcohol and UDP-glucose in American beech (*Fagus grandifolia* Ehrh) bark [20].

We report here, for the first time, the isolation, partial purification and characterization of a stereospecific UDP-glucose: *cis*-*p*-coumaric acid glucosyltransferase from axenically cultivated *S. fallax* plantlets.

RESULTS AND DISCUSSION

Purification of the Glucosyltransferase

A glucosyltransferase, which catalyses the glucosylation of the 4′-OH-position of *cis*-*p*-coumarate in the presence of UDP-glucose as the glucosyl donor, was extracted as soluble enzyme from axenically cultured *S. fallax*. The specific GTase activity of the crude enzyme was *ca.* 30 pkat mg^{-1} protein. The purification protocol developed is presented in Table 1. The supernatant of the centrifuged homogenate (crude enzyme) was layered onto a Q Sepharose column, GTase activity eluted as one single peak at 0.23 M KCl [Fig. 1(A)]. The enzyme was further purified by gel filtration (Superdex 200 I) [Fig. 1(B)], which resulted in a 42-fold purification with a recovery of 19% of the total activity. Successive chromatography on Phenyl Sepharose [Fig. 1(C)], Resource Q [Fig. 1(D)], and, finally, on Superdex 200 II, resulted in an increased specific activity of 600-fold. Although a further separation of 88% of the protein was achieved in the last purification step on the Superdex 200 II column, no enhancement of the specific activity was achieved, due to a loss in enzyme activity. The small yield and the instability of GTase prevented therefore a further purification of the enzyme.

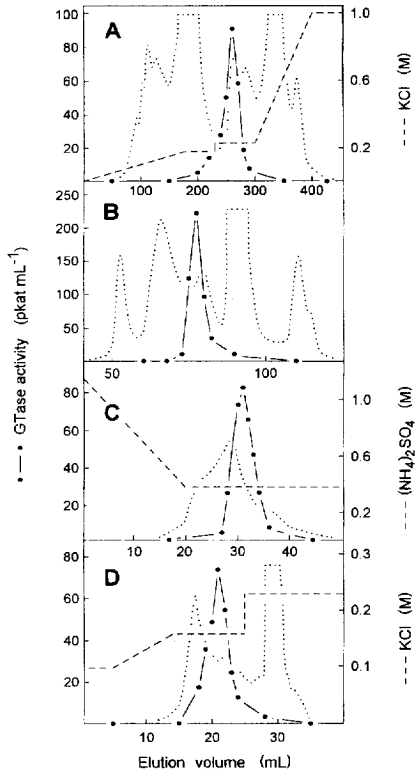

Fig. 1. Chromatographic purification of UDP-glucose: *cis*-*p*-coumaric acid glucosyltransferase from *S. fallax*. Elution profiles of GTase activity on A: Q-Sepharose, B: Superdex 200 I, C: Phenyl Sepharose, and D: Resource Q. ●—●, GTase activity (pkat ml^{-1}); – – –, Gradient (A and D = KCl; C = $(NH_4)_2SO_4$); . . . , Protein (A_{280}).

General properties

The optimum pH range, as determined in different buffers at 30°, was very broad (8.5 to 9.3). Maximum glucosyltransferase activity was measured in 100 mM glycine-KOH at a pH of 9.3. Below pH 7.8 and above 9.5, there was a rapid drop in enzyme activity. This pH optimum is relatively high compared with that of UDP-glucose:hydroxycinnamate glucosyltransferases isolated from other plants, which has been determined to be pH 7.5 or lower [13, 15, 21]. On the other hand it is in the same range as found for *O*-glucosyltransferases isolated from alfalfa with activities towards flavonoids and isoflavonoids as substrates [22].

Addition of the SH-group protector 2-mercaptoethanol (14 mM), which is described by many authors as a stabilizer of GTase activity [23, 24], to the homogenization buffer caused an enzyme inhibition of up to 30%. A concentration of 0.5 mM PMSF in the homogenization buffer did not stabilize the enzyme activity. Treatment of the homogenate with 1% insol-

Table 1. Purification protocol of UDP-glucose: *cis*-*p*-coumaric acid glucosyltransferase from *S. fallax* bioreactor cultures

Purification step	Protein	Specific activity	Purification	Recovery
	mg	pkat mg^{-1}	-fold	%
Crude enzyme	195.0	29	1	100
Q Sepharose	13.9	123	4	30
Superdex 200 I	0.86	1220	42	19
Phenyl	0.12	4000	138	9
Resource Q	0.01	17300	567	3
Superdex 200 II	0.0012*	17500	603	0.4

* protein content was estimated from the peak in the elution profile ($A_{280\,nm}$).

uble PVP (Polyclar AT) or 0.2% Dowex (1X-8, 200–400 mesh, counter ion $HCOO^-$) also had no positive effect on the GTase activity.

The enzyme lost more than 25% of its activity at 4° within 24 hr. Freezing at −18° resulted in a loss of activity of more than 50%. Glucose (1%) had no stabilizing effect and 5% glycerol caused the total loss of activity after 48 hr at 4°.

Divalent cations as Mg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+} at 1 mM concentration, as well as EDTA at 5 mM, had no effect on glucosylation, indicating that the reaction had no requirement for divalent cations. NaCl at a concentration of 0.4 M caused a loss of 40% of the enzyme activity, whereas KCl had no inhibitory effect. At optimum pH and 30°, the reaction rate was linear with time up to 4 hr.

Kinetic data

The purified GTase displayed Michaelis–Menten kinetic. The apparent K_m value of the enzyme, calculated from Eadie–Hofstee-plots, for the cosubstrate UDP-glucose was 110 μM at a *cis*-*p*-coumaric acid assay concentration of 1 mM. The K_m value for *cis*-*p*-coumaric acid was 44 μM at a UDP-glucose assay concentration of 7.5 mM. An assay concentration of *cis*-*p*-coumaric acid higher than 1 mM resulted in a strong inhibition of enzyme activity. At 10 mM *cis*-*p*-coumaric acid, 90% of the activity were lost. Such a substrate inhibition was also observed in UDP-glucose:coniferyl alcohol GTase preparations from Paul's Scarlet Rose cell suspension cultures [25].

Molecular weight

The apparent native M_r of the purified glucosyltransferase was estimated to be 56000, based on gel filtration on a calibrated Superdex 200 column. The M_r is within the range of these reported for the enzyme that catalyses the glucosylation of coniferyl alcohol [23, 26]. The M_r of a UDP-glucose:*trans*-cinnamic acid glucosyltransferase from *Ipomoea batatas* was estimated to be 45000 [12].

However, SDS-PAGE analysis and silver staining of the UDP-glucose:*cis*-*p*-coumaric acid GTase activity containing fractions eluting from the Superdex 200 II column revealed that this highly purified glucosyltransferase preparation was not homogeneous (data not shown). After treatment of the GTase containing fractions with 5 M urea (95°, 5 min), two major protein bands at 56000 and 44000 (compared with small M_r markers), were very weakly visible. A further concentration of the protein did not improve the stainability of the protein bands, hence, a satisfying SDS-gel could not be provided.

Substrate specificity

The substrate specificity of the glucosyltransferase was tested with cinnamic, *o*-and *p*-coumaric, sphagnum, ferulic, and caffeic acid as acceptors of UDP-glucose. A transformation of the *trans*-isomers of these cinnamic acids, as is described, e.g. for the cinnamic acid metabolism of *Cestrum poeppigii* [11, 27] or suspension cultures of *Phaseolus vulgaris* [24], was in no case observed. The enzyme isolated from *S. fallax* had a pronounced substrate specificity for *cis*-*p*-OH-substituted cinnamic acids. The *cis*-isomer of *p*-coumaric acid was by far the best acceptor. The only other case of GTase activity was found with *cis*-caffeic acid, its activity was estimated by the decrease of *cis*-caffeic acid in the assay to be 10% of that compared with the enzyme activity with *cis*-*p*-coumaric acid as substrate.

As described previously [8], glucose was only conjugated to the hydroxy-group of the phenolic ring and not to the carboxylic group, so, no ester formation was observed. Fleuriet and Macheix [21] isolated a glucosyltransferase from tomato fruits which catalysed the transfer of glucose from UDP-glucose either on the phenolic hydroxy group or on the carboxylic group from *p*-coumaric and ferulic acid. No ester but only glucoside formation was observed with caffeic acid in this enzyme preparation. Although the authors did not explicitly mention, if the *cis*-or *trans*-cinnamic acids were transformed, it can be assumed that the synthetic *trans*-isomers were used as substrates; no indication of photochemical reactions was given by the authors.

To our knowledge, the only glucosyltransferase with a stereospecificity towards a *cis*-isomer was isolated from *Fagus grandifolia* [20], catalysing the transfer of glucose from UDP-glucose to the 4′-OH-group of *cis*-coniferyl alcohol. Hence, our presented data are the first report on the purification and characterization of a stereospecific UDP-glucose:*cis*-*p*-coumaric acid glucosyltransferase.

Our results show that in the formation of 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid in *S. fallax*, the isolated UDP-glucose:*p*-coumaric acid glucosyltransferase exhibits a strong substrate affinity for *cis*-and not *trans*-*p*-coumaric acid. It needs to be determined whether other plant species, which produce hydroxycinnamic acid glucosides, exhibit a similar affinity for the *cis*-cinnamic acids or if this enzymatic reaction is specific for *Sphagnum*. The accumulation of the glucoside of *cis*-*p*-coumaric acid in *Sphagnum* plantlets gives rise to questions concerning the function and regulation of the isolated GTase in the secondary metabolism of *Sphagna*. In ref. [28] a key role of glucosyltransferases in the detoxification of salicylic acid in oat roots is discussed, whereas in ref. [20] further support to the hypothesis is provided that glucosides of coniferyl alcohol are involved in the lignification process in beech bark and may be used for either transport or storage purposes. The isolated *cis*-*p*-coumaric acid glucoside is a natural constituent of *S. fallax* and also detectable in plantlets cultivated in continuous feed bioreactors without exogenously supplied *p*-coumaric acid.

EXPERIMENTAL

Plant material. Sphagnum fallax Klinggr. (Klinggr., clone 1) was cultivated axenically in continuous feed bioreactors [29, 30] with a 14 hr light period (photon fluence rate: 105 μmol m^{-2} s^{-1}). The 4-fold conc standard nutrient soln was applied at a flow rate of 100 ml hr^{-1}.

Preparation of substrates. The *trans*-isomers of cinnamic, *p*-coumaric, ferulic, and caffeic acid, and coniferyl alcohol were purchased from Serva. Sphagnum acid was synthesized as described in ref. [31]. Each *trans*-isomer was dissolved (10 mM) in buffer A (100 mM glycine-KOH, pH 9.3). Mixtures of *cis-/trans*-isomers were prepared by irradiation of these solns with UV light (254 nm, 1.5 mW cm^{-2}, 5 hr). Pure *cis-p*-coumaric acid was isolated from an irradiated MeOH *p*-coumaric acid soln by semi-preparative RP-HPLC. UDP-glucose was purchased from Sigma and dissolved (30 mM) in buffer A.

Extraction and assay of transferase activity. S. fallax plantlets were homogenized in buffer B (20 mM tricine-KOH, pH 8.5; 1:5), using a cell homogenizer with CO_2 cooling (MSK, Braun). The homogenate was filtered through gauze (80 μm) and the filtrate centrifuged (14000 *g*, 30 min). 2.5 ml of the supernatant (crude enzyme) were loaded on a Sephadex G 25 column (1.6 × 5 cm, Pharmacia), pre-equilibrated with buffer A, and eluted with 3.5 ml buffer A (desalted crude enzyme). All steps were carried out at 4°.

The transferase assay contained 70 μl enzyme soln, 15 μl UDP-glucose (30 mM) and 15 μl *cis-p*-coumaric acid (7 mM). After 30 min incubation at 30°, the reaction was stopped by boiling (3 min, 100°). Proteins were pptd (10000 *g*, 10 min) and the supernatant analysed by RP-HPLC [8]. Quantification of the GTase activity was based on the formation of 4′-*O*-β-D-glucosyl-*cis-p*-coumaric acid and expressed as pkat.

Determination of the pH-optimum. Plants were homogenised in 50 mM K-Pi buffer (pH-range 5.5–8.0), 50 mM tricine–KOH buffer (pH-range 7.0–9.5), or 100 mM glycine–KOH buffer (pH range 7.5–10.5) and the glucosyltransferase assayed as described above.

Purification of the glucosyltransferase. S. fallax plantlets (30 g fr. wt) were harvested from the bioreactor and homogenized in 150 ml buffer B. All subsequent purification steps were performed at 4°, if not stated otherwise.

The crude enzyme was loaded onto a Q Sepharose column (1 × 12 cm, Pharmacia), equilibrated with buffer B. After removing unbound proteins by washing the column with 150 ml buffer B at a flow rate of 0.8 ml min^{-1}, bound proteins were eluted with an increasing concn of KCl. The discontinuous gradient was as follows: From 0 to 180 ml KCl was linear increased from 0 to 0.18 M, held from 180 to 230 ml and at 230 ml increased to 0.23 M. From 300 ml to 400 ml KCl was linear increased to 1 M and held from 400 to 450 ml.

Frs with GTase activity were pooled, conc to 3 ml (Centriprep-10, Amicon) and applied to a Superdex 200 column (Superdex 200 I, 1.6 × 60 cm, Pharmacia). The column was equilibrated with buffer A and the enzyme eluted at 10° with 130 ml buffer A at a flow rate of 0.2 ml min^{-1}.

Active frs were brought to 30% $(NH_4)_2SO_4$ satn, stirred for 30 min, and centrifuged (12000 *g*, 20 min). The supernatant was applied to a Phenyl Sepharose column (0.5 × 4 cm, Pharmacia), pre-equilibrated with buffer C. Unbound proteins were washed from the column with 20 ml buffer C (50 mM glycine–KOH, 1.15 M $(NH_4)_2SO_4$, pH 8.5). $(NH_4)_2SO_4$ was decreased to 0.39 M in a total vol. of 20 ml and held for 30 ml.

Frs exhibiting GTase activity were pooled, desalted on a Sephadex G 25 column, pre-equilibrated with buffer B. The desalted enzyme soln was applied to a Resource Q column (gel vol. 1 ml, Pharmacia), previously equilibrated with buffer B. Unbound proteins were washed from the column with 15 ml buffer B containing 0.1 M KCl. Bound proteins were eluted at a flow rate of 0.8 ml min^{-1} with the following gradient of KCl: From 5 to 15 ml KCl was increased from 0.1 to 0.16 M, than held at 0.16 M for 10 ml, increased at 25 ml to 0.23 M, and held for 15 ml.

Frs containing GTase activity were pooled, conc (Nanosep 10 K, Filtron) to a vol. of 1 ml, and loaded onto a Superdex 200 column (Superdex 200 II, 1.6 × 60 cm, Pharmacia), equilibrated with buffer A containing 50 mM KCl. Elution was performed at 10° with 130 ml of the equilibration buffer at a flow rate of 0.2 ml min^{-1}.

The general properties of the glucosyltransferase were investigated with the pooled active frs eluting from the Superdex 200 I column.

Determination of the M_r. The M_r of native purified GTase was determined by gel filtration on a calibrated Superdex 200 column. Calibration proteins were eluted with buffer A containing 50 mM KCl at a flow rate of 0.2 ml min^{-1}. The following reference proteins (Boehringer) were used as standards for calibration: thyroglobulin (M_r 669000), ferritin (M_r 450000), catalase (M_r 240000), bovine serum albumin (M_r 68000), ovalbumin (M_r 45000), chymotrypsinogen a (M_r 25000), and cytochrome c (M_r 12500). The void volume (V_0) was determined with Blue Dextran (M_r of 2 million).

Protein concentrations. Estimated by using the Bio-Rad protein dye reagent with BSA as a standard [32].

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