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PURIFICATION OF UDP-GLUCOSE: 4-HYDROXYBENZOATE GLUCOSYLTRANSFERASE FROM CELL CULTURES OF *LITHOSPERMUM ERYTHRORHIZON*

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; cell cultures; enzyme purification; UDP-glucose: 4-hydroxybenzoate glucosyltransferase; affinity chromatography.

Abstract—UDP-glucose: 4-hydroxybenzoate glucosyltransferase (4HB glucosyltransferase, EC 2.4.1.194) was purified to near homogeneity from cell suspension cultures of *Lithospermum erythrorhizon*, using ammonium sulphate precipitation, and column chromatography on DEAE Sephacel, Superdex 200, hydroxyapatite, HiTrap blue, Mono P and Mono Q. Affinity chromatography on HiTrap blue was the crucial step for the success of this scheme. After removal of NAD-dependent enzymes from the HiTrap blue column with NAD (5 mM), the 4HB glucosyltransferase was eluted specifically with UDP-glucose (0–20 mM). The above purification procedure resulted in an enzyme fraction with 27 300-fold higher specific 4HB glucosyltransferase activity than the crude enzyme extract. The enzyme is a monomer of about M_r 51 k, determined by SDS-PAGE. © 1997 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Glucosyltransferases are often of prime importance for secondary metabolism in plants. Many plant secondary metabolites, such as flavonoids, triterpenoids, anthraquinones, etc., are converted into glycosides, which are mainly stored in the vacuole. Glucosylation may present an important detoxification mechanism for secondary products [1].

Glucosyltransferases can be involved in the regulation of secondary metabolism. This is the case in the biosynthesis of indole alkaloids in cell cultures of *Rauwolfia serpentina* [2, 3], where a specific glucosyltransferase converts the intermediate vomilenine into a glucoside and thereby prevents its conversion into the pharmaceutical ajmaline. A similar situation is encountered in the biosynthesis of the naphthoquinone pigment shikonin in cell cultures of *Lithospermum erythrorhizon* (Boraginaceae) [4]. 4-Hydroxybenzoate (4 HB) is a central intermediate of shikonin formation [5–7]. Under illumination with white light [8–10] or in the presence of ammonium ions [9, 11, 12], however, 4HB is converted into its glucoside, which is stored in the vacuole [13], while shikonin production is strongly reduced.

Since *Lithospermum erythrorhizon* cell cultures are used for the commercial production of shikonin [4], the study of this glucosylation is of biotechnological importance. It has been shown that the glucosyltransferase involved is highly specific for 4HB and UDP-Glc [14], that it is localized in the cytosol [13] and that it is regulated by light [9, 10]. The biochemical properties of this enzyme have been determined, using a partially purified enzyme fraction [14].

A detailed understanding of the regulation of glucosyltransferases in plant secondary metabolism would require the cloning of their genes. This would also allow experiments aimed at a genetic engineering of these pathways by sense or antisense expression of the glucosyltransferase cDNA. Only very few glucosyltransferases of plant secondary metabolism have been cloned so far [15, 16], and no significant homology can be established. For cloning, it is therefore necessary to purify the enzyme. We now describe the purification of 4HB glucosyltransferase from *Lithospermum erythrorhizon* cell cultures to near homogeneity, using as the crucial step affinity chromatography on a HiTrap blue column with UDP-Glc as a specific eluent. This procedure may be of interest also for the purification of other glucosyltransferases.

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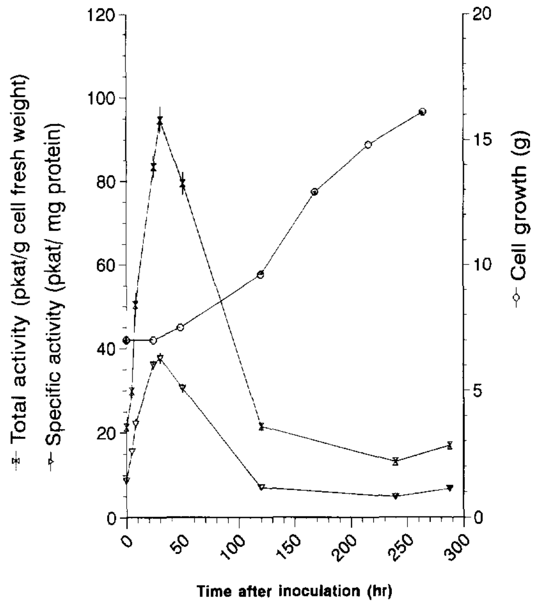

Fig. 1. Induction of 4HB glucosyltransferase activity in cultured cells of *Lithospermum erythrorhizon* by transfer of cells into fresh medium. Cells of the logarithmic growth phase (six days after inoculation) were used for this experiment. Vertical bars show the range of two independent enzyme assays from the same culture flask.

RESULTS

Optimization of the starting material

In order to obtain starting material with high 4HB glucosyltransferase activity, various methods were tested to induced the activity of this enzyme in *Lithospermum erythrorhizon* cell cultures. External feeding of the substrate, 4HB, at concentrations between 10 and 1000 μM was not successful for this purpose, and higher concentrations resulted in cell death. Likewise, no induction could be achieved by the addition of methyl jasmonate, an important signal transducer in plant secondary metabolism [22] (data not shown). Significant induction of the activity could be achieved, however, by a simple transfer of the cells in the logarithmic growth phase into fresh medium. Upon this transfer, cells reproducibly showed a transient four-fold increase of the activity with a maximum after about 35 hr (Fig. 1). This material was used for the enzyme extraction.

Early purification steps

Crude enzyme extracts were fractionated by ammonium sulfate precipitation. After desalting, the enzyme was further purified by column chromatography on DEAE Sephacel [Fig. 2(a)]. This step removed more than 90% of the total protein. The active fractions were combined, concentrated and purified on a Superdex 200 column. About 84% of the proteins were removed in this step [Fig. 2(b)]. After exchange of the buffer, the active fractions were applied to a hydroxyapatite column and eluted with a linear K–Pi gradient (10–150 mM) [Fig. 2(c)]. After these purification steps, the specific activity of the enzyme had increased about 130-fold in comparison with the crude extract (Table 1), and SDS-PAGE showed a very dominant band at 40 kDa. Chromatography of this fraction on HiTrap blue, using a 0–2 M NaCl gradient for elution, yielded this protein in near homogeneity (Fig. 3, lane b).

Removal of alcohol dehydrogenase

Since the 40 kDa protein was visible already in the SDS-PAGE of the crude extract (data not shown), it was unlikely to be the 4HB glucosyltransferase. However, all efforts to separate the 4HB glucosyltransferase activity from this protein using Mono P or Mono Q columns were unsuccessful. Finally, the 40 kDa protein was subjected to tryptic digestion and partial amino acid sequencing in order to establish its identity. The sequences obtained showed 90% identity to alcohol dehydrogenase (ADH) from higher plants (EC 1.1.1.1), and indeed massive ADH activity could be confirmed in this fraction [23]. A literature study showed that ADH has been successfully separated from other proteins using affinity chromatography on a HiTrap blue column, with its cofactor NAD as an eluent. Therefore, the active fractions from the hydroxyapatite column were applied to a HiTrap blue column. Indeed the 40 kDa protein, containing all ADH activity, could be specifically eluted with 5 mM NAD [23]. Subsequent elution with 0–2 M NaCl gave a fraction of high 4HB glucosyltransferase activity, which was nearly free of the 40 kDa band on SDS-PAGE (Fig. 3, lane c).

Removal of a zeta-crystallin-related protein

The active fractions eluted with 0–2 M NaCl from the HiTrap blue column, showed a dominant band at *ca* 35 kDa on SDS-PAGE (Fig. 3, lane c). This value contrasted to the apparent M_r of 47 500, determined for 4HB glucosyltransferase in an earlier study [14] by gel exclusion chromatography, which has been confirmed by the same method in the present work (data not shown). However, attempts to separate the 4HB glucosyltransferase activity from this 35 kDa protein by Mono P and Mono Q columns were unsuccessful. Therefore, the 35 kDa band was subjected to amino terminal sequencing. The 16 amino acid sequence obtained showed a high homology (60–75% identity) to zeta-crystallin (EC 1.6.5.5) from various organisms [24, 25]. This confirmed that the 35 kDa protein is not identical with the 4HB glucosyltransferase. Unfortunately, however, no specific purification procedure is described for zeta-crystallin. In order to remove this protein from 4HB glucosyltransferase activity, it was therefore attempted to refine the HiTrap blue chromatography step. After elution of ADH with NAD, a gradient of 0–20 mM UDP-Glc was applied. This

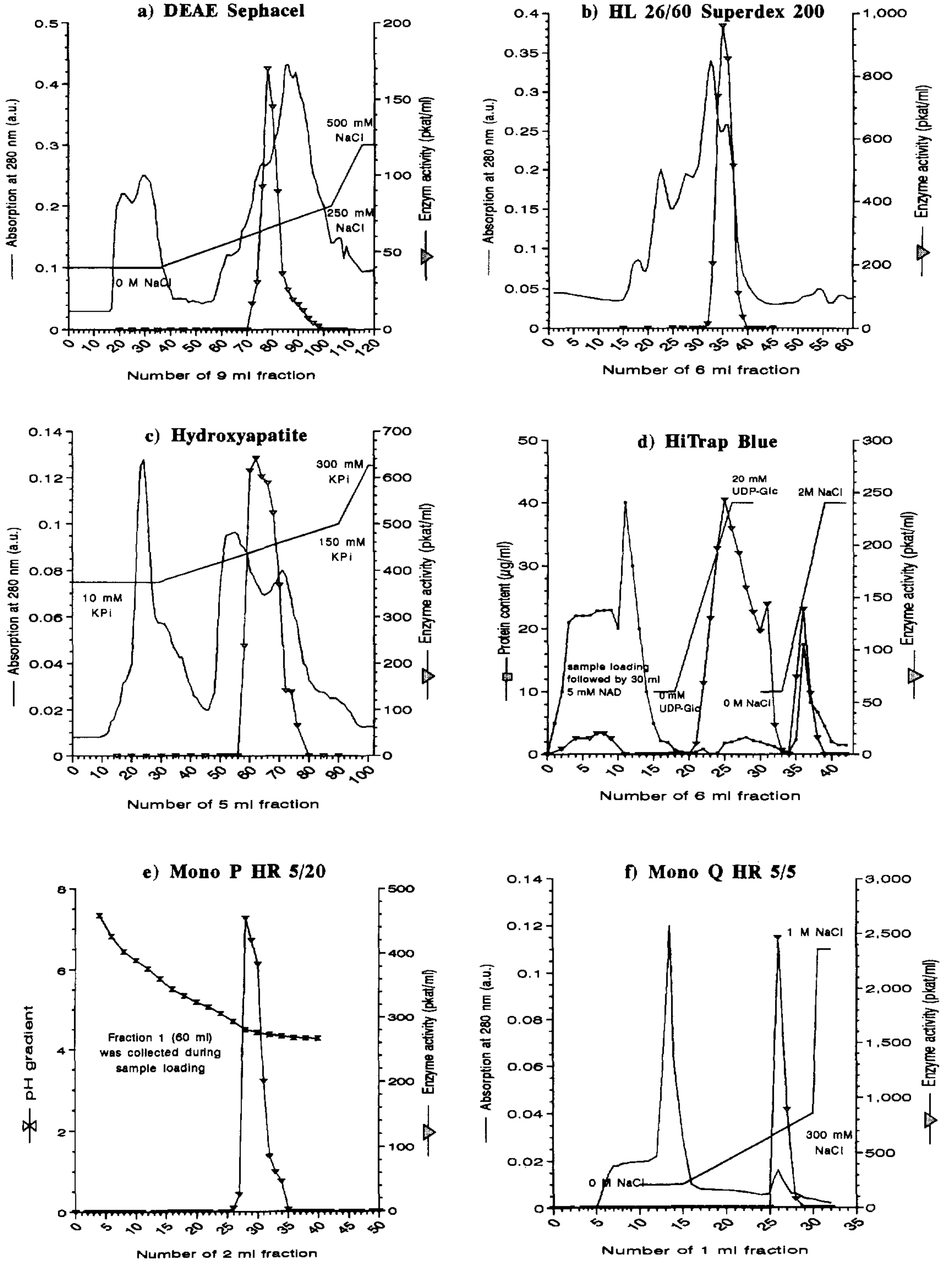

Fig. 2. Elution profiles of 4HB glucosyltransferase from six subsequent chromatographic purification steps. a.u. = arbitrary units. For details see Experimental.

allowed the specific desorption of 4HB glucosyltransferase activity. Most of other proteins, including the 35 kDa band, were retained on the column and could be eluted subsequently with 0–2 M NaCl [Fig. 2(d)]. The active fractions eluted with UDP-Glc nearly free of the 35 kDa band (Fig. 3, lane d).

Final purification steps

The previously established procedures of chromatofocusing on Mono P and ion exchange chromatography on Mono Q, which did not successfully remove the 40 kDa and the 35 kDa proteins, could now conveniently be used for a final purification of the

Table 1. Purification scheme for 4HB glucosyltransferase from *Lithospermum erythrorhizon* cell cultures

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (pkat mg^{-1})	Yield (%)	Purification factor
Crude extract	18 200	326	18.0	100	1.0
$(NH_4)_2SO_4$ (33–55%)	4350	189	43.5	58.0	2.42
Sephadex G-25	3790	132	34.8	40.5	1.94
DEAE Sephacel	312	62.8	201	19.3	11.2
Superdex 200	49.5	37.7	762	11.6	42.5
Hydroxyapatite	11.6	27.0	2328	8.3	130
HiTrap blue	0.39	12.5	32 000	3.8	1783
Mono P	n.d.	5.6		1.7	
Mono Q	0.007	3.4	490 000	1.1	27 300

n.d.: not determined.

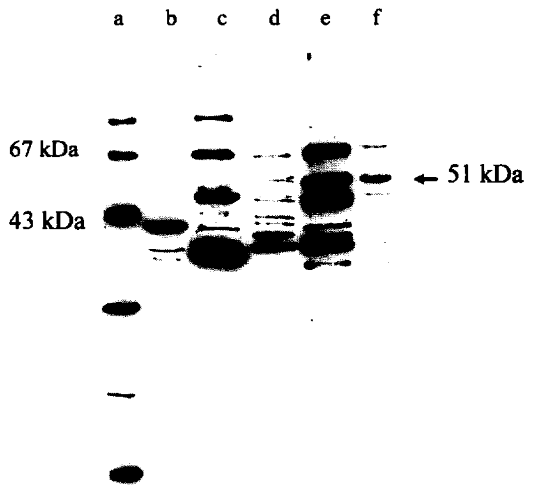

Fig. 3. SDS-PAGE of enzyme fractions during the purification of 4HB glucosyltransferase. Lane a: M_r standards: 94 000; 67 000; 43 000; 30 000; 20 100; 14 400; Lanes b–d: pooled active fractions eluted from HiTrap blue with the following alternative methods: b, 0–2 M NaCl after washing with buffer A; c, 0–2 M NaCl after washing with buffer A and 5 mM NAD; d, 0–20 mM UDP-Glc after washing with buffer A and 5 mM NAD; Lane e, pooled active fractions from Mono P column [see Fig. 2(e)]; Lane f, pooled active fractions from Mono Q column [see Fig. 2(f)]. The gel was stained with silver nitrate. Protein fractions were suitably concentrated before sample loading.

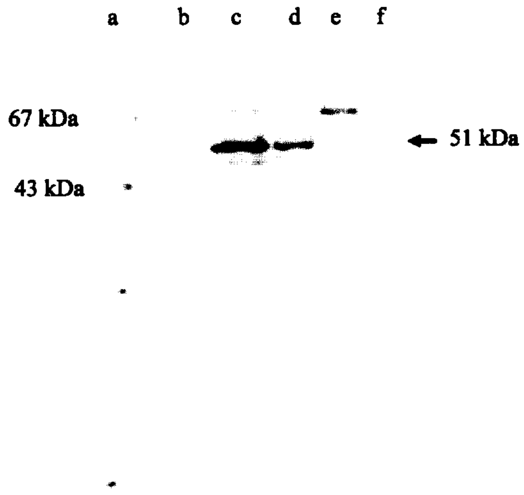

Fig. 4. SDS-PAGE of fractions from Mono Q column [see Fig. 2(f)]. Lane a: M_r standards: 94 000; 67 000; 43 000; 30 000; 20 100; 14 400; Lane b: fraction 25 (<7 pkat); lane c, fraction 26 (820 pkat); lane d, fraction 27 (296 pkat); lane e, fraction 28 (26 pkat); lane f, fraction 29 (<7 pkat). Enzyme activity was determined after buffer exchange to 50 mM Tris, pH 7.6. The gel was stained with silver nitrate.

protein [Fig. 2(e) and (f)]. The activity of the resulting enzyme fraction exceeded that of the crude extract by a factor of *ca* 27 300 (Table 1), and showed a clearly dominant band at 51 kDa on SDS-PAGE after staining with silver nitrate (Fig. 3, lane f). SDS-PAGE showed that the intensity of the 51 kDa band correlated well with the enzyme activity of fractions from Mono Q column [Fig. 2(f)], whereas the intensity of the minor bands did not (Fig. 4). The 51 kDa band was eluted from the SDS gel and subjected to digestion with endoproteinase Lys-C and to microsequencing. The resulting amino acid sequences (9, 12, and 20 amino acids) did not show significant homology to known proteins in the database.

DISCUSSION

For the purification of the 4HB glucosyltransferase to homogeneity, a starting material with a high specific activity was desirable. External feeding of the aglycone did not yield an increased enzyme activity, nor did the addition of the signal transducer methyl jasmonate. However, the unspecific stress caused by the inoculation of cells (of the logarithmic growth phase) into fresh medium gave a reproducible, four-fold induction. Similar induction effects by fresh or diluted medium have been described for other secondary metabolic enzymes [26–30].

The crucial step in our purification scheme was the affinity chromatography on HiTrap blue. This material has been used for the purification of a wide range of proteins [31]. After removal of NAD-dependent enzymes with their cofactor NAD, 4HB glucosyltransferase could be specifically released by application of a gradient of UDP-Glc, the cosubstrate of this enzyme, while a large amount of protein was eluted subsequently with 0–2 m NaCl [Fig. 3(d)]. The finding that 4HB glucosyltransferase can be eluted with UDP-Glc is in accordance with the established reaction mechanism of this enzyme, in which UDP-Glc is the first substrate to bind in an ordered sequential reaction mechanism; i.e. UDP-Glc will bind to the active site of the enzyme even in the absence of 4HB, whereas 4HB would not bind in the absence of UDP-Glc [14]. Purification of a glucosyltransferase by specific elution from a HiTrap blue column with UDP-Glc has not been reported before, and it will be interesting to test whether this procedure is effective for other glucosyltransferases as well.

The finally obtained 4HB glucosyltransferase fraction showed a dominant band at 51 kDa, which is in good agreement with the apparent M_r of 47 500 previously determined by gel exclusion chromatography [14]. The native enzyme is therefore a monomer. Since the intensity of the 51 kDa band clearly correlated with the enzyme activity of various enzyme fractions, and microsequencing of this band did not reveal homology to known proteins, it appears very likely that this band corresponds to 4HB glucosyltransferase. Final proof will have to be provided by cloning and active expression of the responsible gene.

EXPERIMENTAL

Cell cultures. Cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. were derived from germinating seeds [8]. Cell suspension cultures were initiated and maintained as described in ref. [17]. For enzyme purification, cells derived from strain M18 were cultured in 1000 ml conical, containing 300 ml of liquid Linsmaier–Skoog medium [18] supplemented with 1 μM indole-3-acetic acid and 10 μM kinetin, in the dark at 25°.

Enzyme assay. 4HB glucosyltransferase activity was measured by a modification of the method of ref. [14]. The assay (final vol. 100 μl) contained 2.5 mM 4HB, 5 mM UDP-Glc, 15 μM bovine serum albumin (BSA) and 70 μl enzyme fr. or enzyme fr. diluted with 50 mM Tris buffer, pH 7.6. After incubation at 37° for 60 min, the reaction was terminated by addition of 10 μl TCA (1.5 M). After addition of 50 nmol 3,5-dihydroxybenzoic acid as int. standard, the enzymatic product was quantified by HPLC [14]. For rapid determination of 4HB glucosyltransferase activity in the frs from column chromatographic sepns, a radioactive assay was used. UDP-[U-^{14}C]Glc (0.5 mM, 25 000 dpm) was incubated with 2.5 mM 4HB, 15 μM BSA and enzyme fr. After termination of the reaction with TCA, 4HB-[U-^{14}C]glucoside was extracted with *n*-BuOH (350 μl). The radioactivity in 300 μl supernatant was counted using a liquid scintillation counter.

Protein determination. The protein content was determined according to the method of ref. [19] using BSA as a standard. Protein concn in frs from columns was either continuously monitored by UV at 280 nm or using the method of ref. [19].

Protein extraction and enzyme purification. All procedures were carried out between 4 and 8°. Buffers for column chromatography contained 1 mM dithiothreitol (DTT), 40 μM phenylmethylsulfonylfluoride (PMSF) and 15% glycerol and were filtrated through 0.22 μm Millipore filters. PMSF (50 mM stock soln in iso-PrOH) and DTT were added to the buffers immediately before use. Desalting and buffer exchanges of the enzyme soln were carried out using Sephadex G-25 columns. All columns were equilibrated with starting buffer before sample loading.

Enzyme extraction, protamine sulphate and $(NH_4)_2SO_4$ *fractionation.* Cells (6 kg) were harvested 25–35 hr after inoculation into fresh medium, filtrated, washed, mixed with 6 l K–Pi buffer (0.1 M, pH 6.5) containing 20 mM DTT, 40 μM PMSF and PVPP (600 g), and then ground with a mortar to a fine slurry. After treatment with a Branson sonifier (Danbury, Connecticut, U.S.A.), the homogenate was centrifuged at 17 000 *g* for 20 min. A soln of 2.0% (w/v) protamine sulphate in 0.5 M K–Pi buffer (pH 6.5) was added to the enzyme extract (1 ml soln/200 mg protein). After gentle stirring for 10 min and centrifugation at 17 000 *g* for 15 min, solid $(NH_4)_2SO_4$ was added to the supernatant under continuous stirring. The pH value of the enzyme soln was kept at 6.5 by addition of 5 M NH_3 soln during the pptn procedure. The ppt between 33 and 55% salt satn was collected by centrifugation at 17 000 *g* for 20 min.

DEAE Sephacel column chromatography. The protein pellet of the $(NH_4)_2SO_4$ ppt was dissolved in buffer A (50 mM Tris, pH 7.6) and then desalted. The desalted enzyme fr. (230 ml) was chromatographed on two parallel DEAE Sephacel columns (2.5 × 25 cm, 180 ml gel vol.). Proteins were eluted with a linear gradient from 0 to 0.25 M NaCl in buffer A at a flow rate of 1.5 ml min^{-1}.

Superdex 200 *column chromatography.* The active frs of the DEAE Sephacel column chromatography (140 ml) were pooled and concd with an Amicon cell using a membrane with an exclusion limit of 30 kDa. The concentrate (12 ml) was loaded onto a HiLoad 26/60 Superdex 200 column. Proteins were eluted with 150 mM NaCl in buffer A at a flow rate of 1.5 ml min^{-1}.

Hydroxyapatite column chromatography. The buffer of the active frs from Superdex 200 (36 ml) was changed to 10 mM K–Pi buffer, pH 7.6, and the obtained soln was applied to a hydroxyapatite column (2.5 × 15 cm, 100 ml of Bio-Gel HT). The proteins were eluted with a linear gradient of 10–150 mM K–Pi buffer (pH 7.6) at a flow rate of 2.0 ml min^{-1}.

HiTrap blue column chromatography. The active frs from the hydroxyapatite column (50 ml) were applied directly to a HiTrap blue column (10 ml bed vol.). After removal of unbound proteins by washing with 15 ml of buffer A and elution of NAD-dependent enzymes with 30 ml 5 mM NAD in buffer A, the 4HB glucosyltransferase was eluted with a linear gradient of 0–20 mM UDP-Glc in buffer A, and residual proteins with 0–2 M NaCl in buffer A. The elution was performed at a flow rate of 2 ml $\min^{-1}$.

Chromatofocusing on Mono P column (*HR* 5/20). After changing the buffer to buffer A, the active frs from the HiTrap blue column (60 ml) were applied to a Mono P column equilibrated with 25 mM Bis–Tris, pH 5.3. 4HB glucosyltransferase was eluted with polybuffer 74 using a dilution of 1 to 20, pH 4.5 at a flow rate of 0.5 ml $\min^{-1}$. The frs (2 ml) were collected in tubes containing 0.4 ml 200 mM Tris, pH 8.2. The protein content in the frs was so low that it could not be determined with the method of ref. [19].

Mono Q (*HR* 5/5) *column chromatography*. Active frs from Mono P (12 ml) were directly applied to a Mono Q column. After removal of unbound proteins and polybuffer with buffer A, 4HB glucosyltransferase was obtained with a linear gradient using 0–0.3 M NaCl in buffer A at a flow rate of 0.5 ml $\min^{-1}$. The protein content was established by comparison with a reference protein (43 kDa) on SDS-PAGE.

Gel electrophoresis. Purity and the M_r of proteins were analysed on SDS-PAGE using a discontinuous buffer system containing 0.1% SDS [20]. Gels (1.5 mm, 12%) were usually run at 30–40 mA constant current per gel at 6–10° and stained with $AgNO_3$ [21].

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