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STRICTOSIDINE GLUCOSIDASE FROM SUSPENSION CULTURED CELLS OF *TABERNAEMONTANA DIVARICATA*

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Key Word Index—*Tabernaemontana divaricata*; Apocynaceae; cell culture; indole alkaloids; strictosidine; strictosidine glucosidase.

Abstract—Strictosidine β-D-glucosidase (E.C. 3.2.1.105) was partially purified from suspension cultured cells of *Tabernaemontana divaricata*. Three forms of different M_r were found, which all specifically catalysed the deglucosylation of strictosidine (K_m 0.3—1 mM). Some enzyme characteristics resembled those described before for the *Catharanthus roseus* SG enzyme. Several products were shown to be formed after strictosidine hydrolysis, of which cathenamine, 21-hydroxyajmalicine and iso-vallesiachotamine were identified.

INTRODUCTION

The plant genus *Tabernaemontana* (Apocynaceae) is known for the production of a wide variety of alkaloids. Several classes of monoterpene indole alkaloids are involved, e.g. corynanthean, aspidospermatan, plumeran, ibogan and dimeric type [1]. At least 16 different indole alkaloids have been reported from cell suspension cultures [2, 3].

Little is known about the biosynthesis of these compounds. For the early stages, generally the same biosynthetic route as in *Catharanthus roseus* is assumed, in which the principal step in secondary metabolism is made by coupling tryptamine and secologanin in a Pictet–Spengler type reaction, to form the glucoalkaloid strictosidine. This is regarded as the general intermediate in monoterpenoid indole alkaloid biosynthesis [4, 5]. The reaction in which this compound is formed, is catalysed by the vacuolar enzyme strictosidine synthase, which has also been reported from suspension cultured cells of *Tabernaemontana divaricata* [6]. Generally, the routes beyond strictosidine are not known, but the converting enzyme known from *C. roseus* [7], strictosidine β-D-glucosidase (SG; EC 3.2.1.105), may be involved in the next biosynthetic step.

In *C. roseus*, SG catalysed strictosidine hydrolysis results in the formation of epi-cathenamine via 21-hydroxyajmalicine (see Fig. 1). The former can be reduced by a reductase eventually to yield ajmalicine and isomers [8]. In *Tabernaemontana*, strictosidine hydrolysis products have not yet been described. Possibly the enzyme induces a different type of rearrangement of the aglycone; SG might thus play a decisive role, steering the biosynthesis in the direction of a specific type of alkaloid. In this paper, we report the partial purification and characterization of SG from suspension cultured cells of *T. divaricata* and present some results on product identification.

RESULTS AND DISCUSSION

SG activity of cell cultures of the different *Tabernaemontana* species and cell lines available in our laboratory varied considerably; two *T. divaricata* lines (59 and 6B) were selected for their relatively high and constant SG activity levels (up to 1 nkat g^{-1} fr. wt). These lines produced 10–15 mg alkaloid l^{-1} of cell culture, mainly vallesamine and its *O*-acetyl derivative [9].

SG was partially purified from the two selected cell lines by ammonium sulphate precipitation (interval 25–45% saturation), size-exclusion chromatography (SEC) over Sephacryl S-500 HR, anion-exchange chromatography (AEC) over Q-Sepharose FF, and size-exclusion FPLC using a Superose 6 column (Tables 1 and 2). Concentration of enzyme activity after Sephacryl S-500 HR was achieved using an Amicon unit, equipped with a 30 kDa filter. Concentration of AEC pools was carried out using Centricon units (10 kDa) to a final activity of *ca* 5 nkat ml^{-1}. Using this procedure it was shown that at least three SG forms were present. These were numbered I, II and III, in order of elution from the Superose 6 column. Although already in an early stage of the purification three active bands could be seen on a native PAGE gel after SG activity staining, the three SG forms

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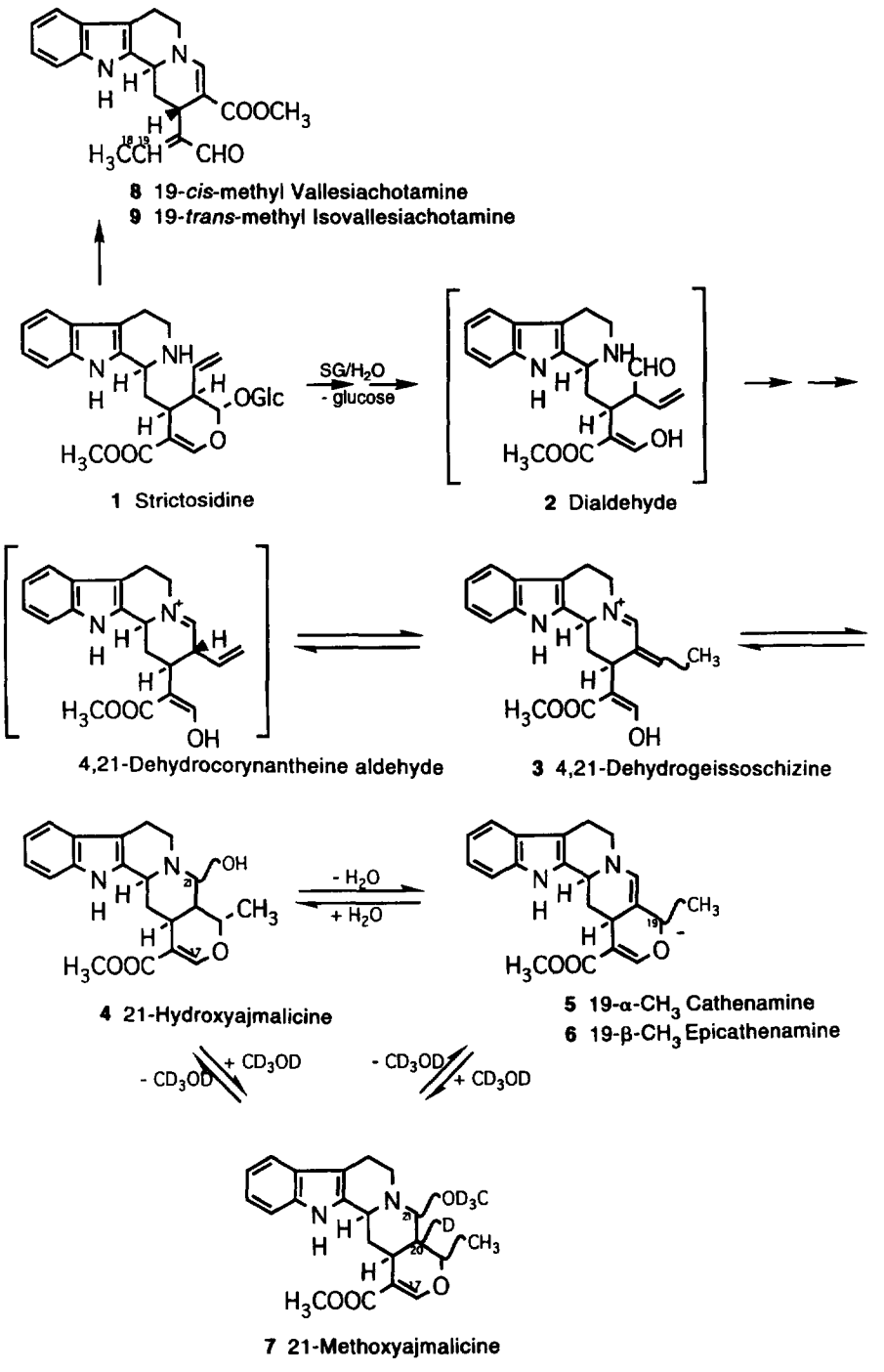

Fig. 1. Scheme showing the conversion of strictosidine and subsequent steps in monoterpenoid indole alkaloid biosynthesis.

were only obtained separate after the Superose 6 run (see Fig. 2). Apparently, during the first SEC step (over Sephacryl S-500 HR), they are still present in a high M_r complex, eluting shortly after the void volume peak. For both cell lines, purification was most effective for SG I, with purification factors of 262 and 41.9. SG II and III showed lower purification factors, due to a less effective separation from other proteins. SDS–PAGE gels (not shown) of all three purified fraction revealed several bands, indicating purification had not yet been complete.

A native PAGE (4–15% gradient) gel of SG obtained after the different purification steps is shown in Fig. 3. A significant change in SG activity pattern can be seen after AEC (lane 3): a very strong band in the SEC pool (lane 2) that had not reached the separation gel had disappeared almost completely, with a concomitant

Table 1. Purification scheme for strictosidine glucosidase from *T. divaricata* suspension cells (600 g cells, line 59). Since SEC over Superose 6 only allows application of 200 μl, the results for this step were recalculated to fit the scale of the rest of the scheme

Step	Volume (ml)	Protein (mg)	Activity (nkat)	Spec. activity (pkat mg^{-1})	Yield (%)	Purification factor
Crude extract	1420	960	36.1	38	100	1
25–45% $(NH_4)_2SO_4$ ppt	20	250	37.0	150	102	3.9
SEC (Sephacryl S-500 HR), concd and desalted	23.5	43	24.0	560	66	14.8
AEC (Q-Sepharose FF), concd	12	7.3	22.2	3000	61	80
SEC (Superose 6), SG I	33.8*	1.1	10.4	9900	29	262
SEC (Superose 6), SG II	20.3*	4.2	3.85	920	11	24
SEC (Superose 6), SG III	13.5*	4.7	1.05	220	2.9	5.9

*Including 15% (v/v) glycerol.

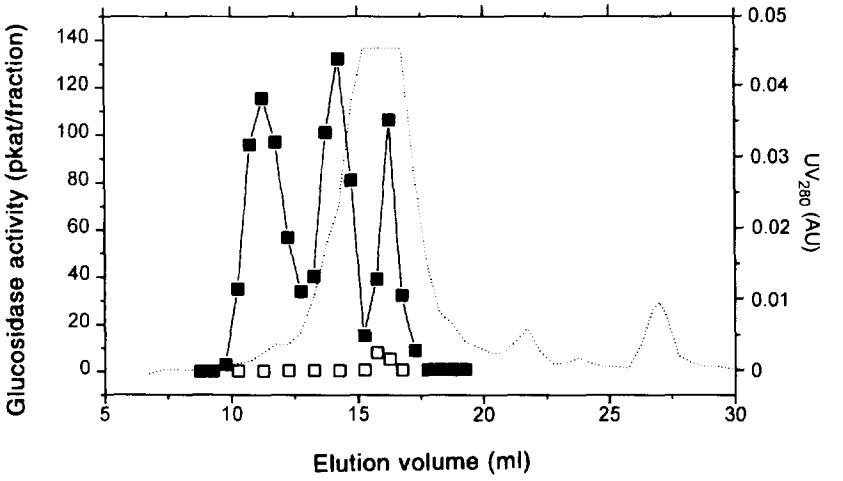

Fig. 2. Superose 6 elution profile for *Tabernaemontana divaricata* SG I, II and III (■) and non-specific glucosidase activities (□) from both cell lines 6B and 59. The dotted line represents the UV_{280} absorption.

increase in intensity of the SG II band and the appearance of the SG I band. This effect could be simulated by treatment of the SEC pool with 0.5 M NaCl. Apparently, a high M_r complex, in which the SG activity is initially present, is broken down under influence of high salt concentration, e.g. as during the AEC step. However, separation of the SG forms only took place during the final SEC run over Superose 6 (lanes 4, 5 and 6).

SG proved to be highly stable, as the activity was not affected when kept for 24 hr at 4°. No loss of activity was observed after six months of storage of the AEC pool at − 80°. In contrast, after separation over Superose 6, the three SG forms showed a highly increased sensitivity for freezing and thawing. The stability could be maintained by addition of glycerol (final concentration 15%) immediately after the column run.

In Fig. 4, the pH dependence of activities of the SG forms (line 6B) is shown. A more or less constant activity level can be observed in the pH interval 4.5–8.0. SG I however, showed two optima, at pH 4.5–5.0 and 7.0. SG III showed a decreasing activity at pH levels over 7.0. Similar pH optima have also been reported for isoflavone 7-*O*-glucoside specific β-glucosidases [10, 11], for the avenacosidases [12] and for the SG from *C. roseus* [13]. However, the latter enzyme has also been reported to have a sharp pH optimum of 6.3 with only 50% of activity at pH 6.5 [7]. Other, non-specific, plant β-D-glucosidases generally have optimum pH values of *ca* 4.5 [14, 15].

Assays using the three separate SG forms at different temperatures yielded activation energy values of 200, 163 and 122 J mol^{-1}, for SG I, II and III, respectively. The SG forms did not accept secologanin, strictosamide (strictosidine lactam) or *p*-nitrophenyl-β-D-glucopyranoside as substrate, and were thus found to behave similar to the *C. roseus* enzyme [7, 13]. Strictosidine did not act as substrate for the non-specific glucosidases present in the cell extracts. Therefore, it is not likely that these play a role in this part of the indole alkaloid biosynthesis.

The Superose 6 elution profile was used for estimation of the SG forms' M_rs, assuming globular structures. Both lines 59 and 6B showed similar M_r values of >670000,

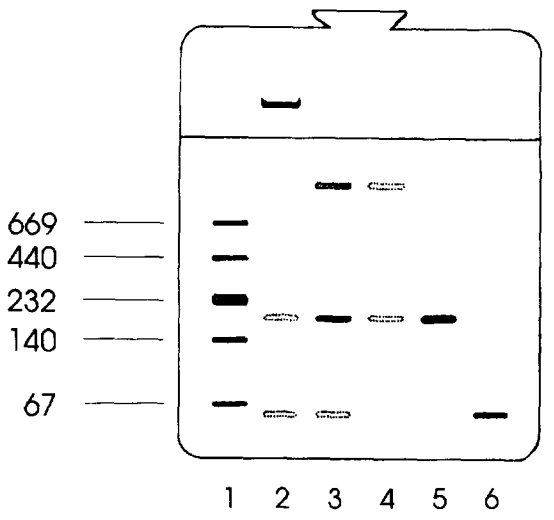

Fig. 3. Representation of native PAGE (4–15% gradient) after SG activity staining showing subsequent purification steps: lane 1, M_r markers; lane 2, SEC pool; lane 3, AEC pool; lane 4, SG I; lane 5, SG II; lane 6, SG III. Migration from top (cathode) to bottom (anode). Solid bars: high intensity stained bands; outlined bars: lower intensity stained bands.

187000 and 31000 for SG I, II and III, respectively. Native PAGE resulted in similar M_r values, *ca* 190000 for SG II, and 22000 for SG III. The SG I band remained outside the calibrated region making a reliable M_r estimation in this way impossible.

The occurrence of three SG forms was also found in *C. roseus* (M_rs corresponding to 240, 650 and 930 kDa), although these could only be separated on a native PAGE gel (Luijendijk, in preparation).

Kinetic studies were carried out using the separate SG forms, isolated from both cell lines. The substrate concentration range was 0.2–0.8 mM in all cases. Values of K_m and V_m were calculated using Hanes plots ($[S]/v$ vs. $[S]$) and the direct linear plot method [16], assuming that Michaelis–Menten kinetics were applicable. Both lines 59 and 6B gave similar results. At substrate concentrations higher than 0.5 mM, SG I displayed a significantly lower activity, presumably due to inhibition by the substrate. This effect was not observed for the two other SG forms. For this reason, Michaelis–Menten kinetics were considered not applicable for SG I. K_m values found for SG II and SG III were >1 and 0.25–0.33 mM, respectively. Values for V_m were 17.7–18.2 and 8.2–8.8 pkat, respectively. These parameters were different to those of the *C. roseus* enzyme; the latter was shown to have a much higher affinity for the substrate (K_m 10–18 μM) and a higher maximum velocity of turnover (Luijendijk, in preparation).

The data show that the three SG forms in *T. divaricata* not only display different characteristics regarding migration on native polyacrylamide gels, but also differ in their affinity for the substrate. They may, therefore, be distinctly different forms, instead of just variable-sized parts of one large protein.

SG activity was not much affected by the specific β-D-glucosidase inhibitor D(+)-gluconic acid δ-lactone [15, 17]; only at concentrations of 100 mM and higher was some inhibition observed. The highest concentration tested (316 mM) showed 55% inhibition of SG II and 32% inhibition of SG III (results not shown). In contrast, the *C. roseus* enzyme II was reported to be already inhibited by less than 10 mM of D(+)-gluconic acid δ-lactone [7, 18]. One of the few other reported specific β-D-glucosidases in higher plants, the raucaffricine β-D-glucosidase from cell cultures of *Rauwolfia serpentina* Benth., also showed this lack of inhibition [19]. Inhibitory activity by D(+)-gluconic acid δ-lactone may, therefore, be more specific than assumed.

^{1}H NMR analysis of strictosidine incubation in D_2O in the presence of partially purified SG showed a rapid decrease of most signals, while the glucose signals remained constant, with a new peak at δ5.2 (t), assigned to the proton at position 1 of the free glucose molecule [20]. The formation of a precipitate during this incubation was observed. Apparently, the glucose moiety is released and remains in solution, while the aglycone or its turnover product precipitates.

Table 2. Purification scheme for strictosidine glucosidase from *T. divaricata* suspension cells (150 g cells, line 6B). Since SEC over Superose 6 only allows application of 200 μl, the results for this step were recalculated to fit the scale of the rest of the scheme

Step	Volume (ml)	Protein (mg)	Activity (nkat)	Spec. activity (nkat mg^{-1})	Yield (%)	Purification factor
Crude extract	381	530	150	290	100	1.0
25–45% $(NH_4)_2SO_4$ ppt	16.5	90	136	1500	91	5.3
SEC (Sephacryl S-500 HR), pool 2, concd	15.5	36	45.3	1300	30	4.4
AEC (Q-Sepharose FF), concd	9	5.8	19.1	3300	13	11.6
SEC (Superose 6), SG I	20.3*	0.42	5.02	12000	3.3	41.9
SEC (Superose 6), SG II	20.3*	2.0	5.47	2700	3.6	9.5
SEC (Superose 6), SG III	20.3*	2.7	4.29	1600	2.9	5.6

*Including 15% (v/v) glycerol.

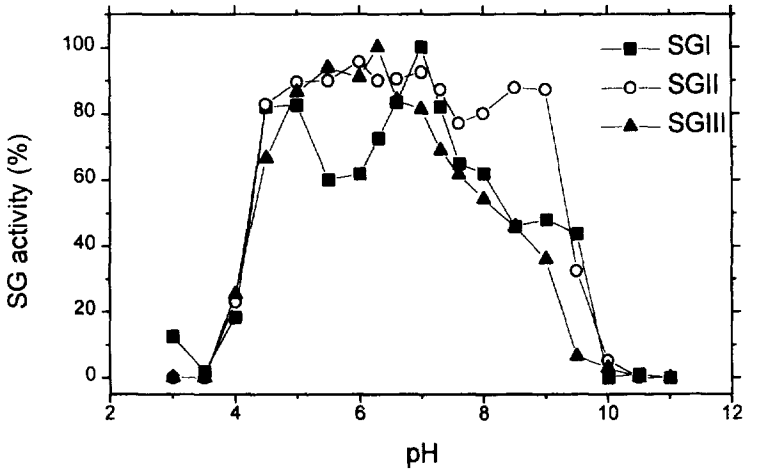

Fig. 4. Influence of pH on the activity of *Tabernaemontana divaricata* SG forms (line 6B). Buffers (0.1 M) used: citrate–phosphate (pH 3–6), sodium phosphate (pH 6–8), borate (pH 8–9), glycine–NaOH (pH 9.5–11). Data derived from duplicate assays. SG I: 100% = 0.25 nkat ml^{-1}; SG II: 100% = 0.27 nkat ml^{-1}; SG III: 100% = 0.21 nkat ml^{-1}.

A substantial amount of this product was prepared by incubation of 10 mg of strictosidine in 5 ml of sodium phosphate buffer (0.1 M) at 30° for 5 hr in the presence of *ca* 1.5 nkatal of *T. divaricata* SG (cell line 59, AEC pool). The incubation was continued overnight at 25° after addition of an extra 0.75 nkat of SG. The mixture was then centrifuged, and the precipitate was washed with incubation buffer and lyophilized. The dry product was dissolved in 500 μl of methanol-d_4, and analysed by ^{1}H NMR. The presence of at least eight compounds was demonstrated, as judged from the presence of eight relatively large singlets in the range δ3.50–3.75, probably corresponding with the methyl-carboxyl groups of the products. Heights of these peaks were used to calculate the ratio of abundance. Two compounds were found to be present in a relative quantity of *ca* 28% each, while the others contributed to *ca* 3–12%. Four compounds could be identified from the spectrum and their identity could be confirmed by HPLC with photodiode array detection. Cathenamine (**5**) and epicathenamine (**6**) were identified by their characteristics 21-H signals (δ6.17, 1H, *dd* and δ6.20, 1H, *dd*, respectively). These data are in accordance with Kan *et al.* [21], and Stevens [13], who reported similar shifts for the *C. roseus* products. Vallesiachotamine (**8**) and isovallesiachotamine (**9**) were identified by their 18-H$_3$ signals (δ2.01 and 2.11 (both *d*, 3H, $J_{18,19}$ = 8.0 Hz, respectively) and the 19-H signals (δ6.48 and 6.69, *q*, 1H, respectively) [22]. The latter two compounds were found to be formed non-specifically from strictosidine, when incubated at 30° in aqueous environment for a longer time (data not shown). Some of the signals in the NMR spectrum seemed to be indicative for the formation of 21-hydroxyajmalicine (**4**); it was found that after addition of one drop of 0.1 M NaOD to the sample, cathenamine signals decreased, with a corresponding increase of singlet signals at δ7.55 and 4.35. These signals were assigned to 17-H and 21-H, respectively, of 21-methoxyajmalicine (**7**), generated by addition of deuterated methanol to epicathenamine, in a similar fashion as described for *C. roseus* incubations [13]; the singlet for 21-H indicates a lacking proton at C-20, which therefore is probably deuterated. The peaks at δ1.41 (18-H$_3$) and 4.29 (19-H) could be assigned using a ^{1}H–^{1}H COSY spectrum, in which these showed a strong coupling. No signals were found to suggest the formation of the alleged intermediate 4,21-dehydrogeissoschizine (**3**) [23, 24].

The findings show that, by the action of SG from *T. divaricata*, a similar *in vitro* product formation takes place as in *C. roseus*. The major intermediate after rearrangement of the strictosidine aglycone is therefore thought to be 21-hydroxyajmalicine. On the other hand, more products are formed, of which at least four have not yet been identified. It is possible that these are intermediates towards indole alkaloids characteristic for the *Tabernaemontana* genus, specifically formed by one of the SG forms. Other possibilities include the hydrolysed alkaloid moiety *in vivo* being rapidly passed on to another—as yet unknown—enzyme for further transformation, or chemical conversion of the strictosidine aglycone taking place under plant specific *in vivo* conditions. Further NMR studies, e.g. using isolated SG forms, will therefore be necessary.

EXPERIMENTAL

Chemicals. The secologanin obtained was further purified on a silica gel column using a stepwise Me_2CO gradient (0–50%) in EtOAc. Strictosidine was prepd by means of enzymic coupling of secologanin and tryptamine, using purified strictosidine synthase from *C. roseus* linked to a CNBr activated Sepharose column [18, 25].

Cell cultures. Tabernaemontana divaricata (L.) R. Br. ex Roem. et Schult. cell suspension culture lines were derived from the one described in ref. [26] by subculturing

in an MS medium [27] containing 30 g/l^{-1} sucrose, and (for line 6b) 2,4-dichlorophenoxyacetic acid (1 mg/l^{-1}) and kinetin (1 mg/l^{-1}) or (for line 59) naphthaleneacetic acid (2 mg/l^{-1}) and benzylaminopurine (0.2 mg/l^{-1}) [9]. The cultures were routinely grown under constant white light (1500 lux) in conical flasks at 25° on a gyratory shaker (*ca* 120 rpm).

Protein preparation. Extraction was as described previously [28]. All protein chromatography steps were carried out at 4°; frs of 10 ml were collected, unless stated otherwise. SEC was performed using Sephacryl S-500 HR in a 2.6 (i.d.) × 90 cm glass column, or by FPLC using a Superose 6 HR column (1.0 i.d. × 30 cm). For the Sephacryl column, a 50 mM Bis–Tris (pH 6.1) elution buffer was used (flow rate 5.7 cm/hr^{-1}), the FPLC runs were performed using 50 mM Na-Pi (pH 6.5), with 150 mM NaCl, 3 mM EDTA and 6 mM DTT (flow rate 30 cm hr^{-1}). For Superose 6 chromatography, frs of 0.5 ml were collected. For AEC, a Q-Sepharose FF column (2.6i.d. × 17 cm) was used. Elution was initially with 150 ml 50 mM Bis–Tris (pH 6.1), followed by a linear 0–1 M NaCl gradient in 750 ml of same buffer. Flow rate was 41 cm hr^{-1}. Protein prepns were concd by membrane filtration. SDS- and native PAGE were carried out using pre-cast gels (usually 4–15% gradient). Staining of active SG in native gels as achieved by incubation of the gel for at least 48 hr under gentle swirling in a 1 mM soln of strictosidine in 0.1 M Na-Pi buffer (pH 6.3) [29]. Total visualization of proteins in native or SDS gels was done using silver staining [30].

Protein and enzyme determinations. SG activity in enzyme prepns was assayed as described previously [13]. Protein concns in crude enzyme extracts were determined by the method of ref. [31]; for purified prepns that of ref. [32] was used. Non-specific β-D-glucosidases were assayed by incubating 25 μl enzyme prepn in a total vol. of 500 μl 2 mM *p*-nitrophenyl-β-D-glucopyranoside in Na-Pi buffer (pH 6.3) at 30°. After 2 hr, the reaction was stopped by addition of 800 μl 1 M Na_2CO_3 and the *A* at 400 nm was measured against H_2O as reference. The enzyme activity was calculated from the amount of *p*-nitrophenol formed, using a molar absorption coefficient of 10 500 M^{-1} cm^{-1}, after correction for a blank assay, in which the substrate was incubated with buffer instead of enzyme soln.

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