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PURIFICATION AND SOME PROPERTIES OF STERYL β-D-GLUCOSIDE HYDROLASE FROM *SINAPIS ALBA* SEEDLINGS

MAŁGORZATA KALINOWSKA and ZDZISŁAW A. WOJCIECHOWSKI
Department of Biochemistry, Warsaw University, 02-089 Warsaw, al. Żwirki i Wigury, Poland

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Key Word Index—*Sinapis alba*; Cruciferae; steryl glucosides; hydrolysis by specific β-glucosidase; intracellular distribution.

Abstract—Homogenates of 7-day-old *S. alba* seedlings hydrolysed cholesteryl[4-H^{14}C] β-D-glucoside or sitosteryl β-D-glucoside-[6-^{3}H]. Activity was located predominantly in the cell membrane structures sedimenting at 1000–15000 *g* and was solubilized by acetone treatment. Partially purified enzyme preparation, with an about 1500 times higher specific activity with respect to the crude homogenate, was obtained by repeated acetone precipitation and subsequent chromatography on DEAE-Sephadex and Sephadex G-100. During this procedure a considerable separation from other enzymes with β-glucosidase activity was achieved. The enzyme had MW 65000 daltons, pH optimum at 5.2–5.6. Two observations suggested that the enzyme was a specific steryl β-D-glucoside hydrolase. Firstly, there was no substrate competition between steryl glucosides and several other β-D-glucosides. Secondly, enzyme activity was strongly inhibited by low concentrations of various 3β-OH sterols with a planar ring system and an intact side chain.

INTRODUCTION

The accumulating evidence [1–5] indicates that steryl β-D-monoglucosides are common components of plant membrane structures together with free sterols. It has been suggested [6] that reversible glucosylation–deglucosylation of sterols present in membranes may have a regulatory effect on membrane organization and function due to changes in lipid–lipid and/or lipid–protein interactions. In fact, diverse effects of free sterols and steryl glucosides on plant membrane permeability were reported [7–8]. It seems that studies of the various enzymatic activities participating in the biosynthesis and metabolism of steryl glucosides would contribute to a better understanding of the biological role of these compounds.

It is well known that various plant tissues contain a specific UDPG:sterol glucosyltransferase [6, 9–12]. This enzyme is membrane-bound and occurs predominantly in Golgi complex [13–14] and plasmalemma [1]. However, so far, there has been no evidence that a similarly specific steryl glucoside hydrolase is present in plants. Our previous *in vivo* studies on the labelling dynamics of steryl glucosides in *S. alba* seedlings have demonstrated that in this plant rapid sterol glucosylation is accompanied by rapid deglucosylation [15]. In an extension of these studies, a search has been made for a specific steryl glucoside hydrolase in cell-free preparations.

RESULTS AND DISCUSSION

Hydrolysis of steryl β-D-glucosides by crude cell-free preparations

Preliminary experiments were made with a crude homogenate of 7-day-old *Sinapis alba* seedlings or crude subcellular fractions obtained by differential centrifugation. Cholesteryl β-D-glucoside or sitosteryl β-D-glucoside labelled respectively in the sterol or sugar moiety were used as specific substrates. The hydrolytic activity was measured on the basis of the radioactivity of free sterol or glucose released from the glucoside by enzyme action (see Experimental). β-Glucosidase activity toward *p*-nitrophenyl-β-D-glucoside was also assayed. This artificial substrate is easily hydrolysed by a majority of β-glucosidases and is often used for 'total' β-glucosidase activity determination [16]. As it was found that preparation of acetone powders from crude subcellular fractions increased their hydrolytic activity 6–8 times toward steryl glucosides such acetone precipitated preparations were used in further studies. Table 1 summarizes the effects of incubation conditions and some reagents on the hydrolytic activities of the acetone powder prepared from the crude homogenate toward cholesteryl-[4-^{14}C] glucoside and *p*-nitrophenyl glucoside. Both activities were similarly affected by pH and gluconolactone which is known as a specific competitive inhibitor of many β-glucosidases of plant and animal

Table 1. The influence of some factors on the β-glucosidase activity of the acetone precipitated enzyme fraction prepared from homogenate of *S. alba* seedlings

Factor investigated	Glucosidase activity toward Cholesteryl-[4-^{14}C] β-D-glucoside	*p*-Nitrophenyl β-D-glucoside
Optimal pH	broad, 5.2–5.6	broad, 5.2–5.6
Optimal temperature	30°	60°
Effect of EtOH		
at 20% concn	stimulation (64%)	stimulation (56%)
at 50% concn	no activity	inhibition (39%)
Effect of dithiothreitol (10^{-5}–10^{-2} M)	45% stimulation at 10^{-4} M	no effect
Effect of gluconolactone (10^{-5}–10^{-2} M)	50% inhibition at 4×10^{-3} M	50% inhibition at 2×10^{-3} M

Table 2. Subcellular distribution of steryl glucoside hydrolase and UDPG: sterol glucosyltransferase in crude subcellular fractions obtained by differential centrifugation of the crude homogenate of *S alba* seedlings

Fraction	Steryl glucoside hydrolase (nmol/mg protein/hr)	UDPG sterol glucosyltransferase (pmol/mg protein/hr)
Crude homogenate	0 26	5.02
1000 *g*	0 55	33 30
3000 *g*	0 88	28 30
8000 *g*	1 52	39 90
15000 *g*	1.55	7 79
15000 *g* Supernatant	0.03	0 97

Acetone precipitated preparations obtained from individual fractions were used in this experiment. The hydrolytic activity was assayed with cholesteryl-[4-^{14}C] β-D-glucoside in succinate buffer, pH 5.2 as described in the Experimental. Glucosyltransferase activity was measured in Tris–HCl, pH 7.5 using UDP-glucose-[6-^{3}H] as the sugar donor and sitosterol as the acceptor [10].

origin [17]. However, striking differences were also found. Temperature optimum for hydrolysis of *p*-nitrophenyl glucoside was at 60°. At this temperature hydrolysis of cholesteryl glucoside did not occur. Ethanol stimulated both activities at concentrations up to 25% but exerted an inhibitory effect at higher concentrations. The inhibition of cholesteryl glucoside hydrolysis was much more pronounced. At 50% ethanol there was no hydrolysis of cholesteryl glucoside but more than 60% of the activity toward *p*-nitrophenyl glucoside was still present. In contrast to the hydrolysis of *p*-nitrophenyl glucoside the hydrolysis of cholesteryl glucoside was stimulated by dithiothrectol. These results indicate clearly that different enzymes are engaged in the hydrolysis of both substrates.

The determination of steryl glucoside hydrolase activity of the crude subcellular fractions obtained by differential centrifugation of the homogenate demonstrated (Table 2) that almost all hydrolytic activity was associated with cellular structures sedimenting between 1000 and 15000 *g*. For comparison, the distribution of another enzyme involved in sterol glucoside metabolism, UDPG:sterol glycosyltransferase, was determined. The subcellular distribution of this enzyme in some other plants was studied in detail by several workers [1, 10, 13, 14] who showed that it is predominantly localized in dictyosomes [13, 14] and plasmalemma [1]. The localization of steryl glucoside hydrolase and UDPG:sterol glucosyltransferase is similar although not identical (Table 2). In both cases only traces of the activity were found in the 15000 *g* supernatant indicating the membranous nature of both enzymes.

In the above experiments, as well as in some experiments described below, almost identical results were obtained using sitosteryl β-D-glucoside-[6-^{3}H] instead of cholesteryl-[4-^{14}C] β-D-glucoside. Therefore only data obtained with cholesteryl glucoside are given.

Table 3. Solubilization and purification of steryl glucoside hydrolase from *S. alba*

Enzyme preparation	Steryl glucoside hydrolase: Total activity (nmol/10g fresh plants hr)	Steryl glucoside hydrolase: Specific activity (nmol/mg protein/hr)	Activity ratio Cholesteryl glucoside / *p*-Nitrophenyl glucoside
Homogenate	0 56	0 024	
Fraction 1000–15000 *g*	0 88	0 092	
Me_2CO precipitated enzyme I	2 60	0 36	1 0
Extract from I	5 04	1 66	8 5
Me_2CO precipitated enzyme II	17 64	9 53	31 0
DEAE-Sephadex	16.52	14 97	47 9
Sephadex G-100	16.24	36 21	104 6

The hydrolytic activity was assayed with cholesteryl-[4-^{14}C] β-D-glucoside. For other details see Experimental.

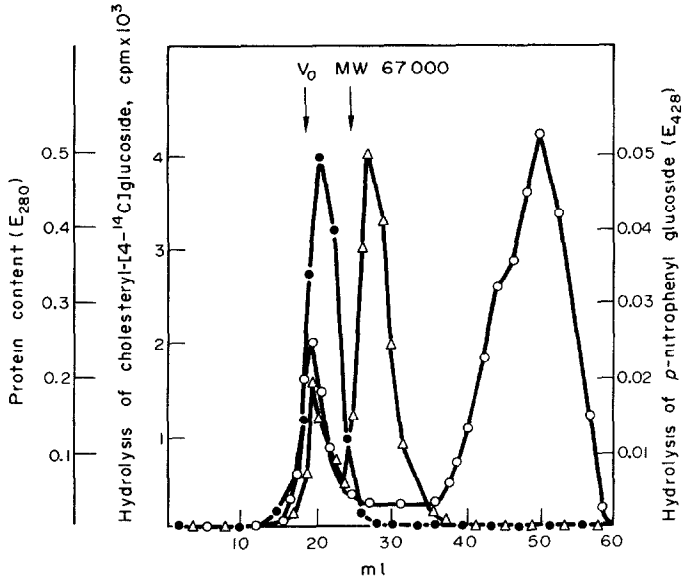

Fig. 1. Gel filtration of the solubilized steryl glucoside hydrolase from *S. alba* on Sephadex G-100. Fractions of 1 ml were collected and assayed for protein content (E_{280}, ○) and their hydrolytic activity toward *p*-nitrophenyl β-D-glucoside (E_{428}, ●) or cholesteryl-[4-^{14}C] β-D-glucoside (cpm, △). Arrows show the elution volumes of blue dextrane (V_0) and bovine albumin (MW 67000). For other details see Experimental.

Solubilization and particle purification of steryl glucoside hydrolase

Based upon the above experiments the particulate fraction (1000–15000 *g*) was used for further enzyme purification. The enzyme was solubilized by cold acetone treatment as described in Experimental and partially purified by subsequent ion-exchange chromatography on DEAE-Sephadex and gel filtration on Sephadex G-100. The procedure used, as shown in Table 3, gave an enzyme preparation with about 1500 times higher specific activity (per mg protein) as compared to the activity of the crude homogenate. It is, however, evident that this activity increase was not only due to the true purification (i.e. separation from other proteins) but was, in part, the result of enzyme activation during cell fractionation and acetone treatment. The total activity of the enzyme (per 10 g plant material) increased about 30 times during these steps. This activation can be explained by the release of the enzyme from the membrane structures. It seems also possible that the partial removal of endogenous sterols during subsequent steps of cell organellae fractionation and acetone precipitation may be a reason for the increase of steryl glucoside hydrolase activity. Free sterols, as it will be shown later, are potent inhibitors of the enzyme. During the subsequent steps of enzyme purification a considerable separation of the steryl glucoside hydrolase from other proteins with β-glucosidase activity occurred as indicated by an about 105-fold increase of the ratio of hydrolytic activities toward cholesteryl glucoside and *p*-nitrophenyl glucoside. Fig. 1 illustrates the final step of the purification procedure, i.e. gel filtration on Sephadex G-100. The hydrolytic activity measured with cholesteryl glucoside was separated into 2 peaks. The major part of the activity eluted from the column in fractions 24–36 was almost completely separated from the hydrolytic activity toward *p*-nitrophenyl glucoside. For this peak a MW of *ca* 65000 was obtained by extrapolation after calibration of the column with standard proteins [11]. Some hydrolytic activity toward cholesteryl glucoside was eluted in fractions 17–23 together with the activity toward *p*-nitrophenyl glucoside. It can not be ruled out that this smaller peak with a higher MW ($>10^5$) represents an aggregated form of the enzyme with MW 65000. It is also possible that the same β-glucosidase fraction which catalysed hydrolysis of *p*-nitrophenyl glucoside has some activity toward steryl glucoside.

Substrate specificity

The above experiments demonstrated clearly that among β-glucosidases occurring in *S. alba* a fraction with relatively high affinity toward steryl glucosides is present. The following evidence suggests that the enzyme with MW 65000 can be regarded as a highly specific steryl glucoside hydrolase.

The rate of hydrolysis of cholesteryl-[4-^{14}C] glucoside was not affected much by 200-fold or even a 20000-fold excess (0.064 or 6.4 mM concentration in the incubation mixture respectively) of several other non-steryl β-D-glucosides of alcohols or phenols, or of disaccharides containing a β-D-glucosidic bond (Table 4). These β-D-glucosides must have much lower (if any) affinity for the enzyme involved in the hydrolysis of cholesteryl-[4-^{14}C] glucoside. However, substrate competition could be clearly demonstrated in the case of another steryl glucoside, i.e. sitosteryl glucoside. The decrease of the cholesteryl-[4-^{14}C] glucoside hydrolysis in the presence of androstenolone (androst-5-en-3β-ol-17-one) glucoside was much lower than in the case of sitosteryl glucoside. This suggests the importance of the hydrocarbon side chain of the sterol molecule for the enzyme–substrate interaction.

Table 4. Effect of some β-D-glucosides on the rate of hydrolysis of cholesteryl-[4-^{14}C] β-D-glucoside by partially purified enzyme from *S. alba*

Glucoside added	Hydrolysis rate (%) At 0.064 mM concn	At 6.4 mM concn
None	100	100
Methyl glucoside	101	95
Cellobiose	90	79
p-Nitrophenyl glucoside	102	95
Phlorizin	78	63
Arbutin	67	53
Amygdalin	73	25
Salicin	100	65
Androstenolone glucoside	88	0
Sitosteryl glucoside	4	0

Incubation mixtures contained partially purified enzyme (0.08 mg protein), cholesteryl-[4-^{14}C] β-D-glucoside (0.15 nmol) and 200-fold or 20000-fold excess of unlabelled glucoside listed in the table (0.064 or 6.4 mM concn respectively).

It was also found that hydrolysis of cholesteryl glucoside was strongly inhibited by a number of natural free sterols, i.e. cholesterol, 5α-cholestanol, campesterol and sitosterol (Fig. 2). This effect seemed to be highly specific. A pronounced inhibition was observed at a concentration of free sterol as low as 1.5 μM (free sterol/cholesteryl glucoside molar ratio = 5). The solubility of free sterols in the incubation mixture containing less than 5 % EtOH is very limited. It is not clear whether the varying degree of inhibition by different sterols was due to the differences in their solubility or depended on different affinity

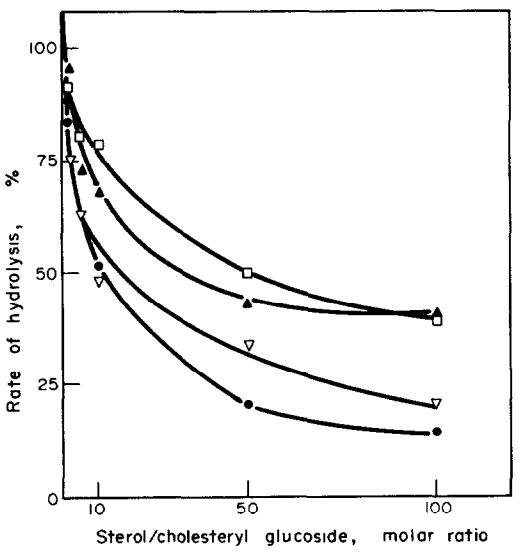

Fig. 2. Effect of some 3β-OH sterols on the hydrolysis rate of cholesteryl-[4-^{14}C] glucoside. Partially purified enzyme preparation (0.08 mg protein) was incubated with cholesteryl-[4-^{14}C] β-D-glucoside (0.15 nmol) in the presence of 0.15–15 nmol of cholesterol (▲), 5α-cholestanol (□), campesterol (▽), or sitosterol (●).

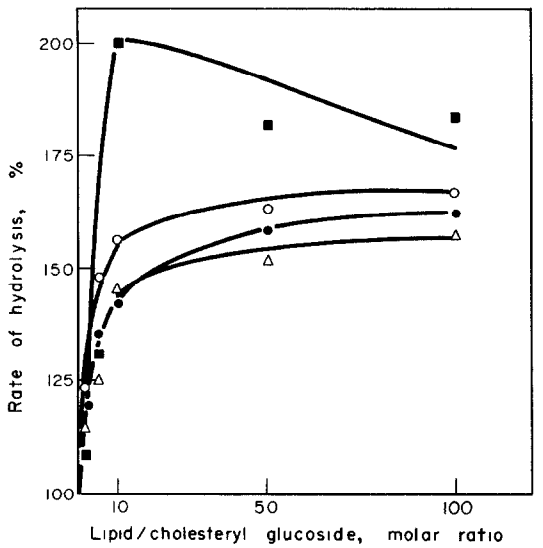

Fig. 3. Influence of some sterol analogues and neutral lipids on the hydrolysis rate of cholesteryl-[4-^{14}C] β-D-glucoside. Coprostanol (○), androstenolone (△), phytol (■) or methyl stearate (●) were present in the incubation mixture. For other details see Fig. 2.

of these sterols for the enzyme but the above effect can be explained as enzyme inhibition by the reaction product. It is possible that this effect has some importance for regulation of the enzyme activity *in vivo*. It is interesting that compounds structurally related to the natural sterol substrate, such as coprostanol (5β-cholestanol) or androstenolone (androst-5-en-3β-ol-17-one) exerted no inhibitory effect on the enzyme activity (Fig. 3). This proves that some structural features of the sterol molecule (intact side chain or planar ring system) are necessary for the interactions with the enzyme leading to enzyme inhibition. In fact both coprostanol and androstenolone stimulated hydrolysis of cholesteryl glucoside by the enzyme from *S. alba*. However, this stimulation seemed to be unspecific as it could be obtained with some neutral lipids structurally unrelated to sterols such as phytol or methyl stearate (Fig. 3). Stimulatory effects of various lipids on many enzymes of membranous origin are well known [6, 18, 19]. It is possible that this effect also has some importance for regulation of the steryl glucoside hydrolase *in vivo*.

EXPERIMENTAL

Labelled substrates. Steryl β-D-glucosides labelled in the sugar or sterol moiety were obtained enzymatically by modification of an analytical procedure described earlier [11]. Crude UDPG·sterol glucosyltransferase (Me_2CO precipitated enzyme fraction) from *Physarum polycephalum* microplasmodia was obtained according to ref. [11]. Cholesteryl-[4-^{14}C] glucoside was prepared as follows. The incubation mixture contained in a total vol. of 70 ml: enzyme preparation (500 mg); 5 mmol Tris–HCl, pH 7.2; cholesterol-[4-^{14}C] (200 μCi, sp. act. 47 mCi/mmol); UDPG, diNa salt (380 mg); EtOH (2.3 ml) and egg phosphatidylcholine (1.2 mg). Incubation was carried out at 30° for 2 hr Radioactive glucoside formed was extracted with $CHCl_3$–MeOH (2:1) and purified by repeated TLC on Si gel with $CHCl_3$–MeOH (9:1). Yield after TLC purification was 28% Radiochemical purity was checked by TLC in several solvent systems [10] and autoradiography. For preparation of sitosteryl glucoside-[6-^{3}H] the incubation mixture contained in a total vol. of 20 ml: enzyme preparation (200 mg); 2 mmol Tris–HCl, pH 7.2; UDPG-[6-^{3}H] (5 μCi, sp. act. 6.1 Ci/mmol); sitosterol (50 mg); egg phosphatidylcholine (50 mg) and EtOH (1 ml). Incubation was carried out at 30° for 30 min and radioactive product isolated and purified with 41 % yield as described for cholesterol-[4-^{14}C] glucoside. As the enzyme preparation did not contain appreciable amounts of endogenous sterols or UDPG it was assumed that sp. act. of glucosides obtained were identical to those of radioactive precursors used. The β-configuration of the glucosidic bond in the glucosides prepared by use of *Ph. polycephalum* enzyme has been proved earlier [11].

Subcellular fractionation. 7-Day-old seedlings of *S. alba* were homogenized with a 3-fold amount of 0.1 M Tris–maleate, pH 5.2. After filtration through cheese-cloth, crude homogenate was successively centrifuged at 1000 *g* (10 min), 3000 *g* (10 min), 8000*g* (10 min) and 15000 *g* (20 min). Individual fractions were then suspended in the same buffer and added dropwise to a 20-fold amount of cold (−15°) Me_2CO. The ppts were collected by centrifugation, washed 2× with Me_2CO and dried in a vacuum. Such preparations, stored at −20°, retained almost all steryl glucoside hydrolase activity for several months.

Enzyme solubilization and purification. Me_2CO precipitated enzyme preparation from cellular structures sedimenting between 1000 *g* and 15000 *g* (Me_2CO precipitated enzyme I) was suspended in 0.1 M succinate, pH 5.2 (1 mg/ml) using a Potter–Elvehjem homogenizer and centrifuged at 15000 *g* (10 min). The supernatant was added dropwise to a 20-fold amount of cold Me_2CO and precipitating material collected as above (Me_2CO ppd enzyme II). This material was extracted again with succinate buffer as above and applied (10 mg protein, 10 ml) to a DEAE-Sephadex A-25 column (4 × 10 cm). The column was eluted with the same buffer. The first 20 ml of eluate were collected and protein pptd by cold Me_2CO treatment as above. The ppt was again dissolved in succinate buffer and subsequently applied (2 mg protein, 2 ml) on a Sephadex G-100 column (1 × 60 cm). The volumn was eluted with 0.1 succinate buffer, pH 5.2 containing 1.5 M NaCl. Fractions of 1 ml were collected. All operations were carried out at 0–4°.

Glucosidase assay with cholesteryl-[4-^{14}C]-glucoside. Standard reaction mixture contained 0.5 ml enzyme preparation (20–200 μg protein depending on the activity of the fraction used) in 0.1 M succinate, pH 5.2 and 0.02 ml steryl glucoside in EtOH (10^4 cpm, 0.152 mnol). The reaction was run at 30°, stopped after 20 min by the addition of 1 ml MeOH and the sample evapd to dryness in a hot air stream. Labelled cholesterol released from glucoside by the enzyme action was then extracted with Et_2O and after addition of about 20 μg unlabelled cholesterol as the carrier separated by TLC on Si gel with $CHCl_3$–MeOH (95:5). Rhodamine 6 G in Me_2CO was used for localization of sterol on the plate. The radioactivity was measured after elution from Si gel with Et_2O.

Glucosidase assay with sitosteryl glucoside-[6-^{3}H]. The incubation conditions were as above only cholesteryl-[4-^{14}C] glucoside was replaced by sitosteryl glucoside-[6-^{3}H] (10^4 cpm, 1.5 pmol). After boiling with MeOH 3 ml H_2O was added, pptd protein removed by filtration and unused sitosterol glucoside extracted with *n*-BuOH (5 × 4 ml). The aq. layer containing radioactive glucose was evapd to dryness, dissolved in 0.4 ml H_2O and taken for radioactivity measurement.

Other methods. Protein was estimated according to Lowry *et al.* [20]. Enzymatic hydrolysis of *p*-nitrophenyl β-D-glucoside was assayed spectrophotometrically at 428 nm [16]. Glucosyltransferase UDPG: sterol was measured as described previously [10] with sitosterol as an acceptor. Radioactivity was determined by liquid scintillation counting. The scintillator was PPO (3 g/l.) and POPOP /0.3 g/l.) in toluene for free sterols and SG or PPO (4 g/l.), POPOP (0.05 g/l.) and naphthalene (120 g/l.) in dioxane for glucose

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