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# THE OCCURRENCE OF STEROL-ESTER HYDROLASE ACTIVITY IN ROOTS OF WHITE MUSTARD SEEDLINGS

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**Key Word Index**—*Sinapis alba*, Cruciferae, steryl esters, sterol-ester hydrolase

**Abstract**—Roots of white mustard (*Sinapis alba*) seedlings contain sterol-ester hydrolase activity Enzyme assays with [4-$^{14}$C]cholesteryl palmitate as the substrate show that the hydrolytic activity is located in cell membrane structures but can be easily solubilized with 0 1 % Triton X-100 During gel filtration on Sepharose 6B in the presence of Triton X-100 and sodium chloride the hydrolytic activity is eluted as a single peak This peak also contains hydrolytic activities toward some other esters such as *p*-nitrophenyl palmitate, tripalmitoylglycerol or *n*-hexadecanyl palmitate However, the rate of hydrolysis of [4-$^{14}$C]cholesteryl palmitate is not significantly affected by a large excess of these esters in the incubation mixtures, suggesting that different enzyme proteins are involved in the hydrolysis of steryl ester and the other esters tested Sterol-ester hydrolase from white mustard seedlings exhibits a marked specificity with respect to the length of the acyl chain bound to sterol For a series of steryl esters containing saturated fatty acids (from $C_2$ to $C_{22}$) the rate of hydrolysis is the highest for esters of $C_{14}$–$C_{18}$ acids Steryl acetate ($C_2$), butyrate ($C_4$) and behenate ($C_{22}$) are very poor substrates

## INTRODUCTION

Esters of sterols with fatty acids are commonly present in tissues of higher plants It seems that at least a part of these compounds is involved in cell membrane structures together with free sterols and their glycoside derivatives [1] Enzymatic synthesis of steryl esters was recently studied in mature spinach leaves [2–5] and in roots of white mustard seedlings [6–8] Solubilized, partially purified acyltransferases which catalysed acyl transfer from di- [3–5] or triacylglycerols [6,7] to sterols were obtained from these plants Subcellular distribution, specificity and some other catalytic properties of these acyltransferases were described [3–8]

In comparison with steryl ester biosynthesis very little is known about the breakdown of these compounds in plant tissues Most of our knowledge in this area comes from *in vivo* studies Net loss of steryl esters was observed under certain physiological conditions, particularly during the early stages of seed germination [9–11] Examples are germinating seeds of *Raphanus sativus* [9] and *Calendula officinalis* [10] or germinating spores of the moss *Polytrichum commune* [11] Grunwald [12] has reported that intact tobacco seedlings can hydrolyse [4-$^{14}$C]cholesteryl palmitate to yield free [4-$^{14}$C]cholesterol, implying the presence of a sterol-ester hydrolase in these plants Sterol-ester hydrolase (EC 3 1 1 13) is widely distributed in mammalian tissues [13] During the last decade several papers have been published reporting the occurrence of similar enzymes in a number of microorganisms such as bacteria [14, 15] or fungi [16, 17] So far there are no reports on enzymatic hydrolysis of steryl esters by cell-free preparations from higher plants

Our previous unpublished studies have shown that during germination of white mustard (*Sinapis alba*) a marked decrease occurs in the content of steryl esters with a concomitant increase of the free sterol content In an extension of these studies a search has been made for sterol-ester hydrolase activity in cell-free preparations from young white mustard seedlings

## RESULTS AND DISCUSSION

### *Hydrolysis of steryl esters by crude cell-free preparations*

Preliminary experiments with crude homogenates of white mustard seedlings showed the ability of these homogenates to catalyse the release of labelled cholesterol from [4-$^{14}$C]cholesteryl palmitate or labelled palmitate from sitosteryl [1-$^{14}$C]palmitate The rates of hydrolysis measured with both of these substrates were almost identical The hydrolytic activity, calculated per mg protein, changed with the age of the plants It was hardly detectable up to the fifth day of germination but then increased markedly The best results were obtained with 8–11-day-old plants The enzyme activity in roots was always much higher than in cotyledons For 11-day-old seedlings the homogenate prepared from roots had *ca* 17-times higher activity than the homogenate obtained from cotyledons (0 35 and 0 02 nmol/mg protein/hr, respectively) Thus, in subsequent experiments roots of 11-day-old seedlings were used

The measurements of sterol-ester hydrolase activity of the crude subcellular fractions obtained by differential centrifugation of the homogenate demonstrated (Table 1) that *ca* 95% of the total activity was associated with cellular structures sedimenting at 15 000 *g* (crude mitochondria) and 105 000 *g* (crude microsomes) Quite different results were obtained when a synthetic detergent, Triton X-100, was present in the buffer used for homogenization at a final concentration of 0 1 %. In this case only *ca* 40% of the total enzyme activity was found in the sedimenting fractions The remaining part of the activity

Table 1 Sterol-ester hydrolase activity in the crude subcellular fractions from roots of white mustard seedlings

| Fraction | Enzyme activity (pmol/g fr roots/hr) | |
|---|---|---|
| | − Triton X-100 | + Triton X-100* |
| Crude mitochondria | 372 | 167 |
| Crude microsomes | 239 | 70 |
| Supernatant 105000 *g* | 34 | 335 |

* Triton X-100 (final concn 0 1%) was present in the buffer used for homogenization For other details see the Experimental

was recovered in the 105000 *g* supernatant fraction suggesting that Triton X-100 could be used for solubilization of sterol-ester hydrolase from the membrane fractions

*Solubilization and partial purification of the enzyme*

Based upon the above experiments the following procedure was developed for solubilization and partial purification of sterol-ester hydrolase from roots of white mustard seedlings The particulate fractions obtained from the homogenate were treated with 0 1 M Tris-HCl buffer, pH 7 3 containing 0 1% Triton X-100 After centrifugation at 105000 *g* for 1 hr the solubilized enzyme present in the supernatant was precipitated by acetone treatment as described in the Experimental Subsequently, acetone precipitated enzyme preparation was applied to a Sepharose 6B column When buffer containing no Triton X-100 was used for elution the hydrolytic activity, measured with [4-$^{14}$C]cholesteryl palmitate as the substrate, could be separated into two peaks (Fig 1) The first peak emerged in the void volume of the column indicating that this activity is associated with a protein (or rather a protein aggregate) with a MW of $4 \times 10^5$ or more Essentially different results were obtained when 0 1% Triton X-100 and 0 5 M sodium chloride were present in the buffer used for elution (Fig 2) In this case only a single peak of sterol-ester hydrolase activity was obtained which corresponded to the second peak observed in the above experiment This suggests that the peak with a high MW obtained in the absence of Triton X-100 represents an aggregated form of the enzyme Therefore, buffer containing Triton X-100 was used in all further experiments with gel permeation chromatography

*Effect of some factors on the rate of steryl ester hydrolysis*

Hydrolysis of [4-$^{14}$C]cholesteryl palmitate occurred within a fairly wide pH range of 5 6–9 2 (in 0 1 M sodium phosphate, pH 5 6–7 2 or in 0 1 M Tris–HCl, pH 7 2–9 2) At higher or lower pH values the activity sharply decreased No pronounced activity maximum was observed Triton X-100 stimulated the reaction at concentrations up to 0 5% The highest stimulation (2 5 ×) was found for 0 1% Triton X-100 Some other detergents, such as Tween 20, Tween 60 or sodium deoxycholate, exerted a strong inhibitory effect within the same concentration range For sodium deoxycholate, 50% inhibition was observed at a 0 25% concentration The hydrolysis rate was not affected by sulphydryl reagents such as 2-mercaptoethanol, dithiothreitol or reduced glutathione at concentrations up

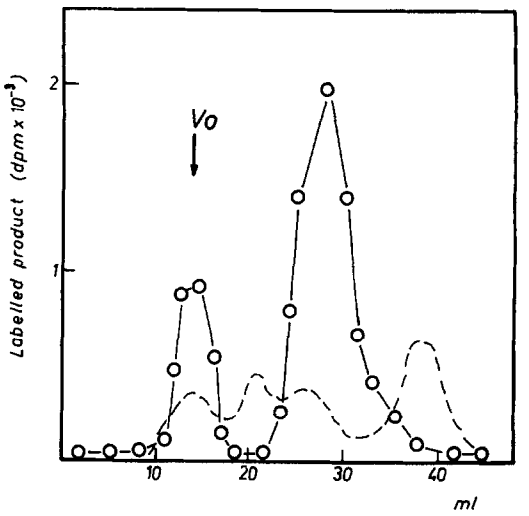


Fig 1 Gel permeation chromatography of solubilized sterol-ester hydrolase from roots of white mustard seedlings on Sepharose 6B Tris–HCl buffer (0 1 M, pH 7 3) without Triton X-100 was used for equilibration of the column and elution Fractions of 1 5 ml were collected and assayed for protein content (----) and sterol-ester hydrolase activity with [4-$^{14}$C]cholesteryl palmitate as the substrate (O) Void volume ($V_0$) was determined with the use of Blue Dextran For other details see the Experimental

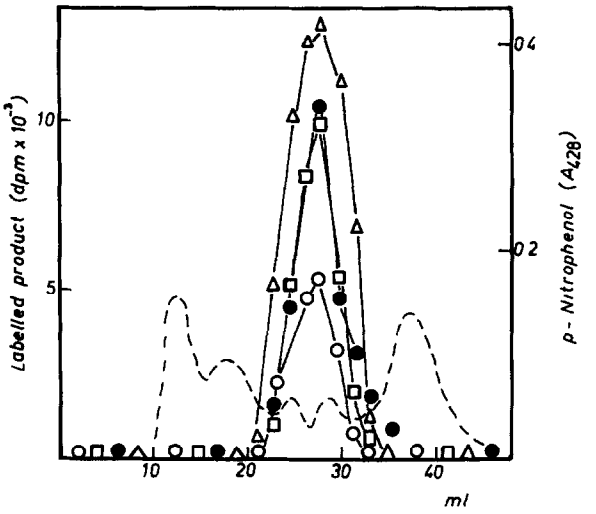


Fig 2 Chromatography of sterol-ester hydrolase on Sepharose 6B in the presence of 0 1% Triton X-100 and 0 5 M NaCl Fractions were collected as described in the legend to Fig 1 and assayed for protein content (----) and hydrolytic activities toward [4-$^{14}$C]cholesteryl palmitate (O), [1-$^{14}$C]*n*-hexadecanyl palmitate (△), tri-[1-$^{14}$C]palmitoylglycerol (□) or *p*-nitrophenyl palmitate (●, $A_{428}$) For other details see the Experimental

to $10^{-2}$ M Divalent metal ions such as $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ exerted a pronounced inhibitory effect (*ca* 70% at $10^{-3}$ M concentration) whereas monovalent ions such as $NH_4^+$ or $Na^+$ were inactive Free palmitic acid had no effect on the rate of hydrolysis of cholesteryl palmitate at concentrations up to $3 \times 10^{-5}$ M but, rather

unexpectedly, we have found that free cholesterol stimulated the hydrolysis within the same concentration range The highest stimulation, reaching *ca* 100%, was observed at a cholesterol concentration of $1.5 \times 10^{-5}$ M

*Fatty acid specificity*

Specificity of the hydrolase for the acyl moiety of the steryl ester was determined by incubation of the partially purified enzyme with a series of [4-$^{14}$C]cholesteryl esters containing saturated fatty acids of different chain length ranging from $C_2$ to $C_{22}$ The results of these experiments, shown in Table 2, indicate that the enzyme is markedly specific with respect to the length of the acyl chain present in the substrate Cholesteryl palmitate was hydrolysed at the highest rate High reaction rates were observed also for cholesteryl myristate and stearate For esters containing shorter or longer acyl chains the rates of hydrolysis were much reduced Cholesteryl acetate ($C_2$), butyrate ($C_4$) and behenate ($C_{22}$) were hydrolysed at very low rates amounting to only 4–6% of the hydrolysis rate with cholesteryl palmitate as the substrate

Table 2 Specificity of sterol-ester hydrolase from roots of white mustard seedlings for cholesteryl esters containing saturated fatty acids of different chain length

| Ester of [4-$^{14}$C]cholesterol | Rate of hydrolysis | |
|---|---|---|
| | dpm $\times 10^{-3}$ | % |
| Acetate ($C_2$) | 0 52 | 6 |
| Butyrate ($C_4$) | 0 31 | 4 |
| Caprylate ($C_8$) | 1 70 | 21 |
| Laurate ($C_{12}$) | 1 41 | 18 |
| Myristate ($C_{14}$) | 4 86 | 61 |
| Palmitate ($C_{16}$) | 7 96 | 100 |
| Stearate ($C_{18}$) | 4 01 | 50 |
| Arachidate ($C_{20}$) | 0 80 | 10 |
| Behenate ($C_{22}$) | 0 34 | 4 |

All esters had sp act = 47 Ci/mol Incubations were carried out for 30 min, the enzyme concentration was 0 5 mg protein/sample

*Specificity of the enzyme toward steryl esters*

As shown in Fig 2, after chromatography of the solubilized enzyme preparation on Sepharose 6B, fractions which catalysed hydrolysis of cholesteryl palmitate also contained hydrolytic activities toward some other esters of palmitic acid such as *n*-hexadecanyl palmitate, tripalmitoylglycerol or *p*-nitrophenyl palmitate Elution profiles of these activities were almost identical This may suggest that the hydrolysis of all the above-mentioned esters, including steryl ester, is catalysed by one, relatively unspecific enzyme of the esterase type For the most active fractions obtained from the column the activity ratio with cholesteryl palmitate, *n*-hexadecanyl palmitate and tripalmitoylglycerol as substrates was *ca* 1 0 2 0 1.6 This indicates that the affinity of *n*-hexadecanyl palmitate or tripalmitoylglycerol for the enzyme is somewhat higher than that of cholesteryl palmitate

However, some results obtained in the course of this work (see below) suggest the possibility that hydrolysis of steryl esters and other esters tested may be catalysed by different hydrolases, i e that two or more hydrolases with different specificity patterns are present in the purified enzyme preparation Experiments with an enzyme preparation purified by preparative gel permeation chromatography on Sepharose 6B (see Experimental) demonstrated that there was no pronounced substrate competition between cholesteryl palmitate and several other non-steryl esters of palmitic acid (Fig 3) As expected the release of labelled cholesterol from [4-$^{14}$C]cholesteryl palmitate sharply decreased when increasing amounts of unlabelled cholesteryl palmitate (3–30 nmol/sample, i e 6–60-fold molar excess) were present in the incubation mixture Under the conditions used in this experiment the release of labelled cholesterol was almost completely restrained at a concentration of unlabelled cholesteryl palmitate as low as 15 nmol/sample (unlabelled cholesteryl ester–labelled cholesteryl ester molar ratio, 30 1) The rate of hydrolysis of [4-$^{14}$C]cholesteryl palmitate in the presence of increasing amounts of unlabelled tripalmitoylglycerol or *n*-hexadecanyl palmitate was affected to a much lower degree Even in the presence of a 60-fold molar excess of these esters (30 nmol/sample) only a partial inhibition of hydrolysis of [4-$^{14}$C]cholesteryl palmitate was observed (31 or 51%, respectively) Similar results were obtained with unlabelled 1,2-dipalmitoylglycerol, 1-monopalmitoylglycerol and *p*-nitrophenyl palmitate (data not shown) The results of the above experiments suggest that all non-steryl esters used in these experiments must have much lower affinity for the enzyme responsible for hydrolysis of steryl esters

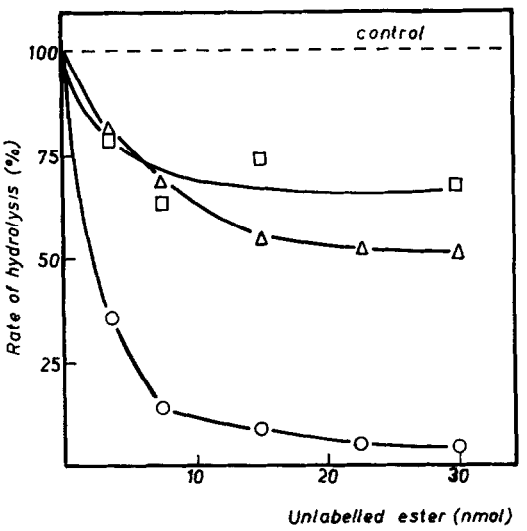


Fig 3 Effect of some esters of palmitic acid on the rate of hydrolysis of [4-$^{14}$C]cholesteryl palmitate Enzyme preparation purified by chromatography on Sepharose 6B (0 02 mg protein/sample) was incubated with [4-$^{14}$C]cholesteryl palmitate (0 5 nmol/sample) in the presence of 0–30 nmol of unlabelled cholesteryl palmitate (O), *n*-hexadecanyl palmitate (△) or tripalmitoylglycerol (□)

The results of another experiment (Fig 4) indicate that at least in the case of *n*-hexadecanyl- and steryl ester the hydrolysis is probably catalysed by different hydrolytic enzymes During the development of white mustard

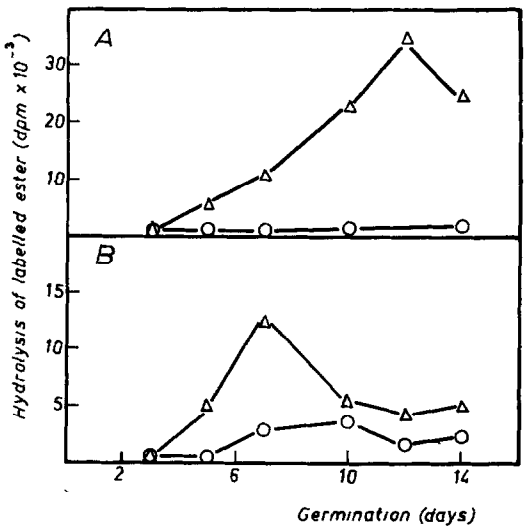


Fig 4 Hydrolysis of cholesteryl palmitate (O) or *n*-hexadecanyl palmitate (△) by crude homogenates from cotyledons (A) or roots (B) of germinating white mustard plants Homogenates were prepared as described in the Experimental using 1 g fresh plant material/3 ml buffer

seedlings changes of hydrolytic activities toward cholesteryl palmitate and *n*-hexadecanyl palmitate are not parallel The activity ratio with both substrates is not constant but differs considerably in plants of different age and in different parts of the plant This is particularly evident when hydrolytic activities in roots and cotyledons are compared The activity towards *n*-hexadecanyl palmitate is several-fold higher in homogenates prepared from cotyledons than that in homogenates prepared from roots By contrast, the hydrolytic activity measured with cholesteryl palmitate as the substrate was much higher in roots than in cotyledons

## EXPERIMENTAL

*Enzyme preparations* Roots of 11-day-old white mustard plants (35 g) were homogenized with 0 1 M Tris–HCl buffer, pH 7 3, containing 0 25 M sucrose (105 ml) Unbroken cells and cell debris were removed by filtration through cheesecloth The resulting filtrate was centrifuged at 15 000 *g* for 20 min The pellet was suspended in 40 ml buffer (without sucrose) containing 0 1 % Triton X-100 The suspension was stirred at 4 ° for 30 min and then centrifuged at 105 000 *g* for 1 hr The supernatant was added dropwise to a 10-fold amount of cold (4 °) $Me_2CO$, the ppt was collected by centrifugation at 6000 *g* for 10 min, washed twice with cold $Me_2CO$ and dried *in vacuo* All operations were carried out at 4 ° This preparation, stored at − 20 °, retained almost all sterol-ester hydrolase activity for several months

*Gel permeation chromatography* Crude $Me_2CO$ pptated enzyme (2 mg protein) was dissolved in 0 1 M Tris–HCl buffer, pH 7 3 containing 0 1 % Triton X-100 and 0 5 M NaCl (0 5 ml) and applied to a Sepharose 6B column, 1 × 60 cm, equilibrated with buffer as above Elution was carried out (0 15 ml/min) with the same buffer For large scale isolation the crude enzyme preparation (25 mg protein) was chromatographed on a Sepharose 6B column (4 5 × 18 cm) The major part of sterol-ester hydrolase activity was present in fractions eluting between 185 and 215 ml

*Sterol-ester hydrolase assay* The standard reaction mixture contained 1 0 ml enzyme preparation (0 02–0 5 mg protein depending on the activity of the fraction used) in Tris–HCl, pH 7 3 and 0 05 ml EtOH soln of [4-$^{14}$C]cholesteryl palmitate (55 000 dpm, 0 5 nmol) Usually Triton X-100 was also present (final concn 0 1 %) The reaction was run at 35 °, usually for 30 min, stopped by addition of MeOH (2 ml) and boiling for 5 min Subsequently, 0 05 mg unlabelled cholesterol was added to each sample as the carrier and lipids were extracted with $CHCl_3$ ( × 3, 4 ml) Free sterol was separated from its ester by TLC on Si gel with $CHCl_3$–MeOH (97 3) as the solvent Rhodamine 6G in $Me_2CO$ was used for localization of sterol on the plate The radioactivity was measured after elution from Si gel with $Et_2O$

*Hydrolase assays with other substrates* Enzymatic hydrolysis of *n*-hexadecanyl palmitate was determined as described above but labelled cholesteryl palmitate was replaced by [1-$^{14}$C]*n*-hexadecanyl palmitate (55 000 dpm, 2 29 nmol) Unlabelled *n*-hexadecanol (0 05 mg) was added as the carrier at the end of the incubation period Assays of 'true lipase' activity were performed using tri-[1-$^{14}$C]palmitoylglycerol (55 000 dpm, 1 18 nmol) as the substrate Other components of the incubation mixtures were identical to those for sterol-ester hydrolase assays After incubation, unlabelled palmitic acid (0 1 mg) was added as the carrier Samples were then acidified with 0 1 N HCl to pH 2–3 and extracted with $CHCl_3$ Free palmitic acid was separated by TLC on Si gel using $CHCl_3$–MeOH–Me COOH (97 3 1) as the solvent Enzymatic hydrolysis of *p*-nitrophenyl palmitate was assayed spectrophotometrically Incubation mixtures contained 1 ml enzyme preparation in 0 1 M Tris–HCl, pH 7 3 and 0 1 ml EtOH soln of *p*-nitrophenyl palmitate (0 158 mmol) Samples were incubated at 35 ° for 30 min The reaction was terminated by an addition of 0 4 ml 0 1 N NaOH and samples were read immediately at 428 nm

*Other methods* Labelled cholesteryl or *n*-hexadecanyl esters were obtained by refluxing [4-$^{14}$C]cholesterol (0 002 mmol, sp act 47 Ci/mol) or [1-$^{14}$C]*n*-hexadecanol (0 01 mmol, sp act 11 Ci/mol) with a large excess (*ca* 100 ×) of an appropriate acyl chloride in 1 ml dry $C_6H_6$–$C_5H_5N$ (95 5, by vol) for 1 hr Reaction products were purified by prep TLC on Si gel in *n*-hexane–$C_6H_6$ (1 1) Sitosteryl [1-$^{14}$C]palmitate was prepared as above using [1-$^{14}$C]palmitoyl chloride (0 014 mmol, sp act 7 1 Ci/mol) and a 100-fold excess of unlabelled sitosterol Protein concns were determined by the method of ref [18] with bovine serum albumin as standard Radioactivity was measured by liquid scintillation counting as described earlier [19]

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