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CHARACTERIZATION OF WAX-ESTER HYDROLASE FROM ROOTS OF WHITE MUSTARD (Sinapis alba L.) SEEDLINGS

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The activity of wax-ester hydrolase in roots of white mustard (Sinapis alba L.) seedlings is located in a membranous fraction sedimenting at 15000 g. The enzyme which shows a high degree of hydrophobicity was solubilized with a synthetic detergent Triton X-100 and purified about 70-fold by acetone precipitation and gel permeation chromatography on Sepharose 6B. The purified enzyme preparation was active within a broad pH range of 5.8-8.5. Hydrolase activity with hexadecanyl palmitate as the substrate was stimulated by Triton X-100 and dithioerythritol. Of wax esters containing saturated fatty acids C_{14}-C_{22} and saturated, primary alcohols C_{14}-C_{24} the highest rate of hydrolysis was found with the esters containing palmitic acid (C_{16}) and tetradecanol (C_{14}). Data presented suggest that wax esters and steryl esters are either hydrolyzed by different specific enzymes or that two enzymes are present of different specificity towards the two substrates.

Esters of long-chain primary alcohols and fatty acids (wax esters) are common constituents of plant surface lipids (Kolattukudy, 1975, 1976), nevertheless small amounts of these compounds are present in various intracellular structures. In a few cases wax esters may serve as a food reserve (Moreau & Huang, 1979; Yermanos, 1975; Yermanos & Duncan, 1976). For example in jojoba (Simmondsia chinensis) seeds wax esters constitute up to 60% of seed fresh weight (Yermanos & Duncan, 1976) and it was established that during germination these esters are metabolized to sugars and used by growing seedlings (Moreau & Huang, 1977). Huang et al. (1978) have reported that crude cell-free preparations from the cotyledons
Koch-Light (Colnbrook, England), Sepharose 6B, Sephacryl S-200, DEAE-
Sephacel, CM-Sepharose Cl-6B and Octyl-Sepharose Cl-4B from Pharmacia
(Uppsala, Sweden), Tris and dithioerythritol (DTE) from Serva (Heidelberg,
F.R.G.), Kieselgel 60 G and Triton X-100 from Merek (Darmstadt, F.R.G.),
tripalmitoylglycerol from Fluka A. G. (Basel, Switzerland), palmitic acid and
maleic acid from Reachim (U.S.S.R.) and sodium deoxycholate from P.O.Ch.
(Gliwice, Poland).

Enzyme preparations. Fresh roots of 11-day-old white mustard seedlings
(30 g), grown as described earlier (Wojciechowski et al., 1976), were homo-
genized with 180 ml of 0.1 M-Tris/HCl, pH 7.3, containing 0.25 M-sucrose.
The preparation of crude 3000-15000 g fraction, solubilization of the hydro-
lytic activity with 0.1% Triton X-100 and preparation of acetone precipitated
enzyme fraction have been described in detail earlier (Kalinowska & Wojcie-
chowski, 1983). For further purification the acetone precipitate was dissolved
in 0.1 M-Tris/HCl, pH 7.3, containing 0.1% Triton X-100 and 0.5 M-sodium
chloride (4 ml) and applied to a Sepharose 6B column (4.5 × 18 cm) equilibrated
with the above buffer. Elution was carried out, at 4°C, with the same
buffer and fractions of 18 ml each were collected. Fractions 11 and 12
containing most of hydrolytic activity, both with cholesteryl palmitate and
hexadecanyl palmitate as substrates, are referred as "Sepharose 6B fraction".

Enzyme assays. The standard incubation mixture of 1.05 ml contained
the enzyme preparation (usually Sepharose 6B fraction, 5-10 µg protein),
100 µmol Tris/HCl, pH 7.3, 1 mg Triton X-100, 0.05 ml ethanol and an
appropriate substrate: an ester of [1-14C]hexadecanol (5.5 × 10^4 dpm, 2.29
nmol), [1-14C]palmitate of primary alcohol (8.0 × 10^4 dpm, 5.2 nmol) or
[4-14C]cholesteryl palmitate (5.5 × 10^4 dpm, 0.5 nmol). Labelled substrates as
well as other lipid compounds used in some experiments (free palmitic
acid, tripalmitoylglycerol) were added as solutions in ethanol. Incubations
were carried out at 30°C. All enzyme activity determinations were made
at several time intervals (usually 10, 20 and 30 min) to ensure the proper
kinetic data.

For determination of the apparent K_m values for hexadecanyl palmitate
or cholesteryl palmitate the labelled substrates (1.5-22 nmol) were added
to the incubation mixtures in the form of mixed micelles: phosphatidylcholine
(from hen egg)-labelled ester (molar ratio 5:1) in 0.1 ml 1% Triton X-100,
as described by Garcia & Mudd (1978).

For determination of pH dependence of enzymatic hydrolysis of hexadecanyl
palmitate the following 0.1 M buffers were used within various pH ranges:
sodium acetate (pH 3.6-5.6), Tris/maleate (pH 5.6-8.8), sodium phosphate
(pH 6.2-7.7), Tris/HCl (pH 7.2-9.2) and glycine/NaOH (pH 8.6-10.6).

Isolation of radioactive reaction products, i.e. labelled hexadecanol,
cholesterol or palmitic acid, by thin layer chromatography on Kieselgel
60 G, has been described earlier (Kalinowska & Wojciechowski, 1983).
Other methods. Protein was estimated by the method of Lowry et al. (1951). Fractions containing Triton X-100 were dialyzed against water for 72 h before protein determination. The radioactivity was measured as described previously (Zimowski & Wojciechowski, 1981).

RESULTS

Purification of wax-ester hydrolase. In the purification procedure methods similar to those elaborated earlier (Kalinowska & Wojciechowski, 1983) were used, i.e. isolation of the membranous fraction sedimenting between 3000 and 15000 g, solubilization of this fraction with 0.1% non-ionic detergent Triton X-100, precipitation with acetone and, finally, preparative chromatography on Sepharose 6B (see Fig. 1). Hydrolytic activities of all fractions, at different stages of purification, were assayed both with cholesteryl palmitate and hexadecanyl palmitate as substrates. A highly sensitive and accurate microassay employing [1-14C]hexadecanyl palmitate (A) or with [4-14C]cholesteryl palmitate (B). Protein concentration in the eluate was continuously monitored at 280 nm (C).

Fig. 1. Preparative gel permeation chromatography of wax-ester hydrolase from Sinapis alba seedlings on Sepharose 6B. Hydrolytic activity was assayed with [1-14C]hexadecanyl palmitate (A) or with [4-14C]cholesteryl palmitate (B). Protein concentration in the eluate was continuously monitored at 280 nm (C).
hexadecanyl palmitate and cholesteryl palmitate increased gradually during purification from about 1:2 in the crude homogenate up to about 1:1 in the Sepharose 6B fraction. The shift was particularly noticeable at the two last steps of the purification procedure, i.e. during acetone precipitation and Sepharose 6B chromatography suggesting that a partial separation of the hydrolytic activities towards cholesteryl ester and hexadecanyl ester could be achieved. However, all our further attempts to obtain a more complete separation of these two hydrolytic activities by an additional chromatography of the Sepharose 6B fraction on Sephacryl S-200, DEAE-Sepharal, CM-Sepharose Cl-6B, Octyl-Sepharose Cl-4B etc, were unsuccessful. A serious problem in all these attempts was a low stability of the Sepharose 6B fraction.

### Table 1

**Purification of wax-ester hydrolase activity from roots of 11-day-old white mustard seedlings**

Hydrolytic activity was measured with [1-14C]hexadecanyl palmitate (HP) or with [4-14C]cholesteryl palmitate (ChP) added in the form of mixed micelles (see Materials and Methods). Total activity refers to 30 g fresh roots.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Substrate</th>
<th>Total activity (nmol/min)</th>
<th>Specific activity (nmol/mg protein/min)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>HP</td>
<td>62.8</td>
<td>1.02</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>ChP</td>
<td>121.0</td>
<td>1.98</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Fraction</td>
<td>HP</td>
<td>48.6</td>
<td>1.65</td>
<td>77</td>
<td>1.6</td>
</tr>
<tr>
<td>15 000 g</td>
<td>ChP</td>
<td>78.1</td>
<td>2.66</td>
<td>65</td>
<td>1.3</td>
</tr>
<tr>
<td>Extract with</td>
<td>HP</td>
<td>26.1</td>
<td>5.96</td>
<td>41</td>
<td>5.8</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>ChP</td>
<td>39.6</td>
<td>9.05</td>
<td>33</td>
<td>4.6</td>
</tr>
<tr>
<td>Acetone precipitate</td>
<td>HP</td>
<td>15.0</td>
<td>15.07</td>
<td>24</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>ChP</td>
<td>18.5</td>
<td>18.61</td>
<td>15</td>
<td>9.4</td>
</tr>
<tr>
<td>Sepharose 6B</td>
<td>HP</td>
<td>7.4</td>
<td>74.80</td>
<td>12</td>
<td>73.1</td>
</tr>
<tr>
<td></td>
<td>ChP</td>
<td>7.4</td>
<td>74.58</td>
<td>6</td>
<td>37.8</td>
</tr>
</tbody>
</table>

With the Sepharose 6B fraction the apparent $K_m$ values with hexadecanyl palmitate and cholesteryl palmitate were $2.8 \times 10^{-5}$ M and $4.5 \times 10^{-6}$ M, respectively. In these experiments, in order to enable activity measurements within a wide range of substrate concentrations, the labelled substrates were added to the incubation mixture in the form of mixed micelles: phosphatidylcholine:labelled ester:Triton X-100 (see Material and Methods).

**Specificity for fatty acid and alcohol moiety.** The specificity of the enzyme for the acyl moiety of the ester was determined by incubation of the Sepharose 6B fraction with a number of [1-14C]hexadecanyl esters containing...
saturated fatty acids of chain length ranging from C₂ to C₂₂. The results of these experiments (see Table 2) indicate high specificity with respect to the acyl group in the ester. Hexadecanyl palmitate was hydrolyzed at the highest rate. For esters containing shorter or longer acyl chains the rates of enzymatic hydrolysis decreased gradually with shortening or elongation of fatty acid chain. For hexadecanyl acetate the hydrolysis rate was only 3% of that of hexadecanyl palmitate. The above results are very similar to that reported earlier for the hydrolysis of steryl esters containing fatty acids of different chain length by the enzyme preparation from the same plant (Kalinowska & Wojciechowski, 1983).

Table 2

Hydrolysis of n-hexadecanyl esters containing saturated fatty acids of different chain length

All esters prepared from [1-¹⁴C]hexadecanol and corresponding fatty acid chlorides or anhydrides (see Materials and Methods) had sp. act. = 407 MBq/mmol. Incubations were carried out for 10, 20 and 30 min in order to ensure that proper reaction rates were obtained. Data for 10-min-long incubations are given.

<table>
<thead>
<tr>
<th>Fatty acid esterifying hexadecanol</th>
<th>Hydrolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic (C₂)</td>
<td>0.34 dpm × 10⁻³</td>
</tr>
<tr>
<td>Butyric (C₄)</td>
<td>1.16 dpm × 10⁻³</td>
</tr>
<tr>
<td>Caprylic (C₆)</td>
<td>3.09 dpm × 10⁻³</td>
</tr>
<tr>
<td>Lauric (C₁₂)</td>
<td>3.93 dpm × 10⁻³</td>
</tr>
<tr>
<td>Myristic (C₁₄)</td>
<td>4.34 dpm × 10⁻³</td>
</tr>
<tr>
<td>Palmitic (C₁₆)</td>
<td>12.20 dpm × 10⁻³</td>
</tr>
<tr>
<td>Stearic (C₁₈)</td>
<td>5.86 dpm × 10⁻³</td>
</tr>
<tr>
<td>Arachidic (C₂₀)</td>
<td>3.29 dpm × 10⁻³</td>
</tr>
<tr>
<td>Behenic (C₂₂)</td>
<td>2.60 dpm × 10⁻³</td>
</tr>
</tbody>
</table>

Specificity of the hydrolase for the alcohol moiety was studied using a series of synthetic [1-¹⁴C]palmitates of primary, saturated, straight-chain alcohols with chain length ranging from C₂ to C₂₄ (see Table 3). In this case the highest rate of the hydrolysis was found with the ester of C₁₄ alcohol, i.e. with tetradeacyl palmitate. A gradual reduction of the hydrolysis rate with palmitates of alcohols containing shorter or longer carbon chains was observed, however, not as dramatic as in the case of hexadecanyl esters containing fatty acids of different chain length. It seems, therefore, that specificity for the fatty acid moiety is higher than for the alcohol moiety of the ester.

Some other properties of the hydrolase. Enzymatic hydrolysis, as measured with hexadecanyl palmitate, showed a broad pH optimum within pH range of 5.8-8.5 without a definite maximum peak. Below pH 5.5 and over pH 9.0
Table 3

Specificity of white mustard enzyme for palmitic acid esters of various saturated, straight-chain, primary alcohols

All esters prepared from [1-14C]palmitoyl chloride and corresponding alcohols had sp. act. = 78.1 MBq/mmol. For other details see the legend to Table 2.

<table>
<thead>
<tr>
<th>Alcohol component</th>
<th>Hydrolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm × 10⁻³</td>
</tr>
<tr>
<td>Ethanol (C₂)</td>
<td>6.7</td>
</tr>
<tr>
<td>Butanol (C₄)</td>
<td>5.13</td>
</tr>
<tr>
<td>Octanol (C₈)</td>
<td>6.21</td>
</tr>
<tr>
<td>Decanol (C₁₀)</td>
<td>5.67</td>
</tr>
<tr>
<td>Dodecanol (C₁₂)</td>
<td>5.78</td>
</tr>
<tr>
<td>Tetradecanol (C₁₄)</td>
<td>6.74</td>
</tr>
<tr>
<td>Hexadecanol (C₁₆)</td>
<td>5.17</td>
</tr>
<tr>
<td>Octadecanol (C₁₈)</td>
<td>4.90</td>
</tr>
<tr>
<td>Eicosanol (C₂₀)</td>
<td>4.85</td>
</tr>
<tr>
<td>Docosanol (C₂₂)</td>
<td>4.69</td>
</tr>
<tr>
<td>Tetracosanol (C₂₄)</td>
<td>4.09</td>
</tr>
</tbody>
</table>

the activity sharply decreased. Very similar results were obtained for hydrolysis of cholesteryl palmitate (Kalinowska & Wojciechowski, 1983).

Similarly as in the case of sterol-ester hydrolase the hydrolytic activity towards hexadecanyl palmitate is membrane-bound which indicates high hydrophobicity of the enzyme. The presence of Triton X-100 is necessary for solubilization of both hydrolytic activities (see Table 1). Furthermore, gel permeation chromatography on Sepharose 6B column provided evidence that the presence of detergent is necessary to keep both activities in solution. Thus, in the presence of 0.1% Triton X-100 the hydrolytic activity towards hexadecanyl palmitate is always maintained within the same fraction whereas in the absence of Triton X-100 a substantial amount of the activity was eluted in the void volume, similarly as we observed earlier (Kalinowska & Wojciechowski, 1983) for sterol-ester hydrolase activity.

Triton X-100 stimulated hydrolysis of hexadecanyl palmitate and cholesteryl palmitate to a different extent (see Fig. 2). At 0.1% concentration hydrolysis of cholesteryl palmitate was stimulated about 2.5-fold whereas that of hexadecanyl palmitate only about 1.5-fold. Also hydrolysis of hexadecanyl palmitate was much more abolished by Triton X-100 at high concentrations than the hydrolysis of cholesteryl palmitate. In contrast to the action of non-ionic Triton X-100, an ionic detergent — deoxycholate exerted an inhibitory effect in both cases.

Some differences were found in the effect of dithioerythritol and free palmitic acid on the two hydrolytic activities (see Fig. 3). Dithioerythritol had a marked stimulatory effect (about 40% at 5 mM concentration) on the
Fig. 2. Hydrolysis of hexadecanoyl palmitate (O, □) or cholesteryl palmitate (△, ▲) in the presence of Triton X-100 (open symbols) or sodium deoxycholate (closed symbols). Final concentration of detergent in incubation mixtures is given.

Fig. 3. Effect of dithioerythritol (DTE; A) and free palmitic acid (B) on the hydrolysis rate of hexadecanoyl palmitate (O) or cholesteryl palmitate (△).

hydrolysis of hexadecanoyl palmitate but not on the hydrolysis of cholesteryl palmitate (Fig. 3A). Free palmitate at low concentration of 0.02-0.1 mM unexpectedly stimulated the hydrolytic reaction with hexadecanoyl palmitate as the substrate. The reason for this stimulation is not clear but it may be due to detergent properties of free palmitic acid.
In our previous paper (Kalinowska & Wojciechowski, 1983) on sterol-ester hydrolase from white mustard we have shown that the rate of hydrolysis of [4-\(^{14}\)C]cholesteryl palmitate was not significantly affected by a large excess of some other unlabelled esters, among them hexadecanyl palmitate and tripalmitoylglycerol. The results of a similar but reverse experiment are shown in Fig. 4. As expected, the release of labelled hexadecanol from its ester rapidly decreased on increasing concentration of unlabelled hexadecanyl palmitate (6-50-fold molar excess) due to dilution of labelled substrate. At a 50-fold molar excess of unlabelled hexadecanyl palmitate less than 10\% of labelled hexadecanol was released. On the other hand, in the presence of unlabelled cholesteryl palmitate or tripalmitoylglycerol even at 50-fold excess inhibition of [1-\(^{14}\)C]hexadecanyl palmitate hydrolysis did not exceed 30\%. The results of this experiment confirmed limited competition between hexadecanyl palmitate and cholesteryl palmitate (or tripalmitoylglycerol) for an active centre of the wax-ester hydrolase.

**DISCUSSION**

Many properties of wax-ester hydrolase from white mustard roots described in this paper closely resemble those of sterol-ester hydrolase from the same plant (Kalinowska & Wojciechowski, 1983). Both enzyme activities are membrane-bound, can be solubilized with Triton X-100 and seem to be...
connected with a protein(s) showing high degree of hydrophobicity. Both activities show similar pH dependence and are stimulated by a non-ionic detergent Triton X-100 whereas deoxycholate, an ionic detergent, exerts an inhibitory effect. Our results (Kalinowska & Wojciechowski, 1983) suggested that these two activities represent two enzymes of different specificity since: (i) enzyme preparation from white mustard seedlings of different age or from different seedling parts (roots or cotyledons) showed different ratios of the activities with sterol esters and wax esters as substrates, and (ii) the rate of steryl ester hydrolysis was only slightly affected by a large excess of hexadecanoyl palmitate.

The results described in the present paper give additional information confirming the possibility of the occurrence of two different hydrolases catalyzing the hydrolysis of sterol and wax esters in white mustard seedlings. The two activities can be partly separated by the method employed for enzyme purification. Our procedure allows to purify wax-ester hydrolase activity to a much higher extent than sterol-ester hydrolase. We have found that some effectors such as dithioerythritol, free palmitate and, in some respect, also detergents showed a different effect on either of the two activities. However, the strongest indication that sterol-ester and wax-ester hydrolases may be different enzyme proteins comes from the experiment in which we found but limited competition between labelled hexadecanoyl palmitate and unlabelled cholesteryl palmitate or tripalmitinoylglycerol for the active site of the enzyme (see Fig. 4). The results of this experiment suggest that both cholesteryl palmitate and tripalmitinoylglycerol have a distinctly lower affinity for the enzyme hydrolyzing hexadecanoyl palmitate. On the other hand the apparent $K_m$ values obtained for the Sepharose 6B fraction from white mustard roots indicate clearly a higher affinity for cholesteryl palmitate than for hexadecanoyl palmitate. The above discrepancy can be easily explained on the assumption that there are two different hydrolases in white mustard showing different specificities for steryl esters and wax esters, respectively.

As it has been pointed out in the introduction, some properties of a crude wax-ester hydrolase preparation from jojoba cotyledons were reported by Huang et al. (1978). Jojoba is a very special plant which stores a large amount of wax esters in the seeds as the main energy reserve. White mustard seedlings contain much less wax esters (approximately 0.15% of plant dry weight) but a relatively rapid metabolism of these compounds must take place since the alcohol and fatty acid composition of this fraction undergoes fast changes with seedling growth (Kalinowska & Wojciechowski, unpublished results). It is interesting that the white mustard enzyme shares several features in common with the jojoba enzyme. Both enzymes are membrane-bound. The jojoba enzyme was suggested to be associated with membranes of so-called wax bodies—specific organelles in
which wax esters are stored. Dry seeds of both plants show very small activity and develop it after a few days of germination (see also Kalinowska & Wojciechowski, 1983). Although the activity of jojoba enzyme is not directly stimulated by -SH reagents, the inhibition of wax-ester hydrolase by p-chloromercuribenzoate can be reversed by dithiothreitol (DTT) (Huang et al., 1978), this indicating that -SH groups are necessary for the enzyme activity. It is difficult to compare data on specificity of jojoba wax-ester hydrolase with the white mustard enzyme since only a few esters of the same chain length of acyl and alcohol moieties were used by Huang et al., (1978). Anyway, the highest hydrolysis rate with tetradeceyl myristate reported for the jojoba enzyme is in a good agreement with our results.

REFERENCES


Received 11 April, 1985