SPRUCE PULP TREATMENT WITH AN ESTERASE FROM *Ophiostoma piceae*
SIGNIFICANTLY DECREASES THE CONTENT OF BOTH TRIGLYCERIDES AND
STEROL ESTERS RESPONSIBLE FOR PITCH DEPOSITS

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ABSTRACT
An esterase has been isolated from the ascomycete *Ophiostoma piceae* showing high affinity (Kₘ around 1 mM) and activity on both glycerol and sterol esters of long chain fatty acids (kₐₜ around 290 s⁻¹ for triolein and 138 s⁻¹ for cholesteryl oleate). This enzyme could have high potential for the enzymatic control of pitch in paper pulp manufacturing from different raw materials. Northern spruce (*Picea abies*) pulp containing triglycerides and sterol esters as the main lipophilic compounds, was treated with the *O. piceae* esterase (a control with boiled enzyme was included). The treated pulps were lyophilized, Soxhlet extracted with acetone, and the chloroform soluble compounds analyzed by gas chromatography-mass spectrometry. After 3-h treatment, 87% of triglycerides and 46% of sterols esters in the spruce pulp were degraded by the *O. piceae* esterase. These results suggest that *O. piceae* esterase could be used for pitch biocontrol in both hardwood and softwood paper pulp manufacturing

I INTRODUCTION
Lipophilic wood extractives, principally triglycerides, resin acids and free and esterified sterols, are involved in pitch deposit formation during manufacturing of both mechanical and chemical paper pulps (8,14,7). These resinous compounds cause production and quality problems which can be reduced by adding lipases to pulp in papermaking. These enzymes hydrolyze glycerides and decrease pitch problems in softwood mechanical pulps. However, they are not effective on pulps from woods with high levels of esterified sterols, such as birch, aspen or eucalypt wood. Recently we have isolated an esterase from the ascomycete *Ophiostoma piceae* with activity on triglycerides and sterol esters (5). In order to verify the potential of *O. piceae* esterase for pitch biocontrol in paper pulp manufacturing: i) the kinetic constant of the enzyme on triglycerides and cholesterol esters has been studied; and ii) *Picea abies* pulps, as a model pulp with high concentration of both triglycerides and sterol esters. (15), was treated with this esterase and the chloroform soluble fraction analyzed by gas chromatography-mass spectrometry (GC-MS).

II EXPERIMENTAL

Fungal strain and culture conditions. *Ophiostoma piceae* IJFM A667 (= CECT 20416) was maintained in 2% malt extract-glucose-agar and cultivated in 1-l Erlenmeyer flasks with 200 ml of medium, at 26 °C and 160 rpm (4). Mycelium from 3-day-old cultures was used as inoculum (1 g dry weight per liter of medium).

Enzyme purification and characterization. The *O. piceae* esterase was purified from 12-day-old cultures, when maximal activity levels were attained. The culture filtrate was concentrated by ultrafiltration (Filtron 5 kDa cut-off membrane), equilibrated with 2 M (NH₄)₂SO₄ in 25 mM Tris-HCl buffer, pH 7.0, and applied to a HiTrap Octyl Sepharose FF cartridge (Pharmacia). Proteins were eluted with a linear gradient (2-0 M ammonium sulfate in the same buffer) and the protein bound to the gel was released with 0.1% reduced Triton X-100 (4). Homogeneity, molecular mass of the denatured enzyme and N-linked carbohydrate content were determined by SDS-PAGE using 7.5% polyacrylamide gels. The N-terminal sequences were obtained by automated Edman degradation.

Kinetic studies. The hydrolysis of tributyrin, triolein and cholesterol esters was assayed titrimetrically at pH 7.0 and 25°C in a pH-stat (Mettler, model DL50) using 0.1 M NaOH as titrant. The reaction mixture (20 ml) contained the substrate in presence of 0.15 M NaCl and 10% (v/v) Genapol X-100. All assays were performed keeping the same stirring speed. One unit (U) of activity was defined as the amount of enzyme that releases 1 µmol of free fatty acid per min.
Enzymatic pulp treatments. Unbleached pulp from thermomechanical pulping (TMP) of Northern spruce (Picea abies) was obtained from UPM (Valkeakoski mill, Finland). TMP pulp (10 g, dry weight) was treated with 10 U of O. piceae esterase in presence of 200 ml of 10 mM sodium phosphate buffer, pH 6 and 0.05% sodium deoxycholate (as dispersant agent). The treatment was carried out at room temperature and 160 rpm during 3h. A control with boiled enzyme was included. The pulps were lyophilized, extracted in a Soxhlet with acetone for 6 h and the chloroform soluble compounds analyzed by GC-MS using a DB-5HT capillary column (11).

III RESULTS AND DISCUSSION

Esterase was obtained from the culture medium supplemented with 0.5% olive (4). The enzyme was purified from 12-day-old cultures, when maximal activity levels were attained. An unique electrophoretically-homogeneous esterase was obtained, with a high yield (around 70%), using ultrafiltration followed by a single chromatographic step on a hydrophobic interaction column. The enzyme studied here (8% N-linked carbohydrate content and a molecular mass around 56.5 kDa) is different to the lipase characterized from another O. piceae strain, which has lower molecular mass (around 35 kDa) and a different N-terminal sequence (9). This enzyme has not been detected in our O. piceae strain under the culture conditions mentioned above. The new esterase described in O. piceae shows molecular mass and N-terminal sequence similar to those found in two O. piliferum isoenzymes, which were only partially characterized (1).

The kinetic study with different triglycerides and cholesterol esters was performed to analyze the substrate specificity of O. piceae esterase (Table I). Due to the low solubility in water of most of the substrates tested, the study was carried out in presence of a tensioactive agent. Among the surfactants assayed Genapol X-100 was found the most effective to solubilize long-chain fatty-acid cholesterol esters, preserving enzyme activity. The O. piceae esterase showed notable lipase (triacylglycerol esterase) and sterol esterase activities and the affinity increased with the length of the fatty acid esterifying glycerol or cholesterol. In the case of sterol esters, the affinity for cholesteryl palmitate was more than 3-fold higher compared with the obtained for cholesteryl butyrate. The presence of double bonds in the acyl chain caused the main differences in the kcat rather than in Km.

Table I.- Apparent kinetic constants of O. piceae esterase on p-nitrophenol esters, triglycerides and cholesterol esters. Reactions were carried out in presence of the non-ionic detergent Genapol X-100 (conditions described in the Materials and Methods section)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Kapp (mM)</th>
<th>kapp (s⁻¹)</th>
<th>kcat / Kapp (s⁻¹ mM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tributyrin C4:0</td>
<td>9.90 ± 0.80</td>
<td>179 ± 4</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>Triolein C18:0</td>
<td>0.98 ± 0.08</td>
<td>290 ± 7</td>
<td>296 ± 18</td>
</tr>
<tr>
<td>Cholesteryl butyrate C4:0</td>
<td>3.00 ± 0.50</td>
<td>47 ± 2</td>
<td>15.6 ± 1.8</td>
</tr>
<tr>
<td>Cholesteryl palmitate C16:0</td>
<td>0.87 ± 0.09</td>
<td>67 ± 3</td>
<td>77 ± 5</td>
</tr>
<tr>
<td>Cholesteryl stearate C18:0</td>
<td>1.10 ± 0.30</td>
<td>71 ± 6</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>Cholesteryl oleate C18:1nc</td>
<td>1.00 ± 0.10</td>
<td>138 ± 4</td>
<td>138 ± 9</td>
</tr>
<tr>
<td>Cholesteryl linoleate C18:2nc,12c</td>
<td>0.99 ± 0.06</td>
<td>150 ± 3</td>
<td>152 ± 6</td>
</tr>
</tbody>
</table>

Commercial lipases are being successfully used for pitch biocontrol in softwood mechanical pulps (6). These enzymes hydrolyze triglycerides but they do not degrade other lipophilic compound, as sterols and sterol esters. The enzymatic control of pitch in eucalypt and other hardwood and softwood, with high levels of free and esterified sterols (10,12), require the use the enzymes able to hydrolyze sterols. Previous report showed the hydrolysis of sterol esters from Eucalyptus globulus synthetic process liquid after O. piceae esterase treatment of a water suspension (2). In this case, the results of spruce TMP pulp treatment with the O. piceae esterase showed that the problematic compounds responsible for pitch deposition decrease after 3h (Fig. 1). The quantification of the lipophilic wood extractives, using standards compounds, showed that both triglycerides and sterols esters were degraded after the enzymatic treatment (around 87% and 46%, respectively). These results
suggest that *O. piceae* esterase could be used to remove compounds responsible for pitch deposit formation in soft and hardwood pulps, paper and mill equipment. These results are included in a Spanish patent (3). Other enzymatic preparations with sterol esterase activity for controlling pitch during paper manufacture have been reported but the enzymes are not fully characterized (13). The advantage of the esterase from *O. piceae* is its ability to hydrolyze both triglycerides and sterol esters, decreasing these problematic compounds in pulps and process waters and preventing pitch deposit formation in pulp and paper manufacturing.

![Fig. 1.- GC analysis of the lipophilic compounds present in TMP pulp from Northern spruce (*P. abies*) after 3 h of treatment with *O. piceae* esterase: A) Control using denatured enzyme; B) problem using native enzyme.](image)

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V. REFERENCES


