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## Hydrolysis of sterol esters by an esterase from *Ophiostoma piceae*: application to pitch control in pulping of *Eucalyptus globulus* wood

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**Abstract:** A sterol esterase purified from cultures of the sapstain fungus *Ophiostoma piceae* was able to hydrolyse sterol esters and glycerides. The kinetics of sterol esters and triglyceride hydrolysis by this new esterase, estimated using a pH-stat, showed a  $K_m^{app}$  and a  $k_{cat}^{app}$  in the range of 0.9–1.1 mM and 70–300 s<sup>-1</sup>, respectively. Its ability to hydrolyse both pure sterol esters and natural mixtures of saponifiable lipids from eucalypt wood was compared with those of commercial sterol esterases from other microbial sources. Its specific activity on sterol esters was higher than that found with all the commercial esterases assayed, and the highest hydrolysis of eucalypt sterol esters was also attained using the *O. piceae* esterase. This sterol esterase could be of biotechnological interest for the hydrolysis of sterol esters that form pitch deposits in paper pulp manufacturing.

**Keywords:** sterol esterase; wood extractives; biocontrol; fungus; ascomycete.

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## 1 Introduction

Lipophilic wood extractives cause pitch deposition problems during pulp and paper manufacturing that decrease pulp quality and produce important economic losses in this industrial sector (Hillis and Sumimoto, 1989). The lipophilic extractives include fatty and resin acids, waxes, fatty alcohols, sterols, sterol esters, glycerides, ketones and other oxidised compounds (Hillis, 1962; Fengel and Wegener, 1983). The composition of extractives is different in hardwoods and softwoods and include important variations in different parts of the tree (Rowe, 1989; Gutiérrez et al., 1999; Back, 2000).

Lipases (EC 3.1.1.3), whose function in nature is to hydrolyse glycerides, have received growing attention industrially. Moreover, it has been demonstrated that hydrolytic enzymes can catalyse also reactions of synthesis in organic solvents (Plou et al., 2003). Some microbial lipases are being studied for their application in pulping processes to decrease pitch problems. The treatment of pine pulp with *Candida cylindracea* lipase was successfully used in Japanese mills to reduce pitch deposition (Irie, 1990). In the same way, a recombinant lipase expressed in *Aspergillus oryzae* is commercialised by Novozymes as Resinase<sup>®</sup>, and successfully used for pitch biocontrol in mechanical pulping of pine wood (Fujita et al., 1992; Sharyo, 1993; Fischer et al., 1993). These enzymes decrease pitch problems in softwood mechanical pulping, where triglycerides are the main problematic compounds, but they are not effective on pulps from hardwoods (as *Populus tremula* and *Eucalyptus globulus*) and some softwoods (as *Picea abies*) with a high content of esterified sterols.

Eucalypt wood is a major raw material for paper pulp manufacturing in Spain, Portugal, and Brazil. Pitch troubles have increased significantly by the use of TCF (totally chlorine free) bleaching sequences in the manufacture of eucalypt kraft pulps, which are substituting sequences using chlorine and chlorine dioxide. Free and esterified sterols, the major compounds in the lipophilic extractives of *E. globulus* wood (Gutiérrez et al., 1999), have been detected in pitch deposits from eucalypt kraft pulp bleached using a TCF sequence (Del Río et al., 1999, 2000). While triglycerides are completely hydrolysed and the fatty acids dissolved during kraft pulping of eucalypt wood, free and esterified sterols show high resistance to kraft cooking and bleaching processes, and consequently are involved in the formation of deposits. Sterol esterases (E.C. 3.1.1.13) are being studied for pitch control of pulps with high levels of sterol esters, and a sterol esterase able to hydrolyse both sterol esters and triglycerides has been isolated from cultures of the sapstain ascomycete *Ophiostoma piceae* (Calero-Rueda et al., 2002). In the present work we have studied the ability of this enzyme to hydrolyse sterol esters from eucalypt wood, compared with commercial sterol esterases. In addition, its possible application to decrease pitch deposition problem in hardwood and softwood pulping processes is discussed.

## 2 Materials and methods

### 2.1 Fungal strain and culture conditions

*Ophiostoma piceae* IJFM A667 (=CECT 20416) was maintained in malt extract (2%)-glucose (1%)-agar and cultivated in 1-l Erlenmeyer flasks with 200 ml of glucose-peptone medium supplemented with 0.5% olive oil (Calero-Rueda et al., 2002).

Mycelium from 3-day-old cultures was used as inoculum (1 g dry weight per liter of medium) and the cultures were maintained at 26°C and 160 rpm.

## 2.2 Enzyme preparations

The crude enzyme used to purify the *O. piceae* sterol esterase was obtained by ultrafiltration (Filtron 5 kDa cut-off membrane) from 12-day-old cultures, when maximal activity levels were attained. The esterase was purified using a HiTrap Octyl Sepharose FF cartridge (Pharmacia) equilibrated with 2M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 25 mM Tris-HCl buffer, pH 7.0 (Calero-Rueda et al., 2002).

Commercial cholesterol esterases from *Pseudomonas fluorescens* (Roche), *Candida rugosa* (Roche) and *Pseudomonas* sp. (Sigma) were used in comparative kinetic studies.

## 2.3 Analytical assays

Activity on *p*-nitrophenol esters was assayed by release of *p*-nitrophenol from *p*-nitrophenyl butyrate (pNPB) and *p*-nitrophenyl palmitate (pNPP) ( $\epsilon_{410} = 15200 \text{ M}^{-1} \text{ cm}^{-1}$ ) in a spectrophotometer Kontron 930 with magnetic stirring. The assay mixture (3 ml) contained the substrate in 100 mM Tris-HCl buffer, pH 7, with 0.15M NaCl.

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 the presence of 0.15 M NaCl and 5% (v/v) Genapol X-100, an effective non-ionic detergent to solubilise long-chain fatty-acid cholesterol esters. All assays were performed keeping the same stirring speed. One activity unit (U) was defined as that catalysing the release of 1  $\mu\text{mol}$  of free fatty acid per min.

For the curve fitting of experimental data, hyperbolic kinetics curves were analysed using the MMFIT iterative programme of the SIMFIT package. This programme performs weighted non-linear regression to the Michaelis-Menten equation and gives the corresponding table with the parameter values and their statistical confidence limits.

Protein concentration was determined by the method of Bradford (Bio-Rad protein assay) using albumin as standard. Molecular mass of the denatured enzyme was determined by SDS-PAGE using 7.5% polyacrylamide gels, and the content of *N*-linked carbohydrates was estimated from the molecular mass of the *O. piceae* esterase before and after deglycosylation with endo- $\beta$ -*N*-acetylglucosaminidase (Endo-H from Boehringer).

## 2.4 Extractive analyses

Lipophilic extractives were obtained from *E. globulus* wood chips (from ENCE, Pontevedra, Spain). The chips were dried in an aerated oven at 60°C, milled using a knife mill, and Soxhlet extracted with acetone (Tappi, 1993). The extracts were evaporated to dryness, and 500  $\mu\text{g}$  were suspended in 20 mM phosphate buffer (pH 6) with 0.02% Na-deoxycholate, and treated with 100 mU of esterase from *O. piceae* (activity measured using pNPB as substrate). The same treatment was carried out with 100 mU of

cholesterol esterase from *Pseudomonas* sp. to compare the efficiency of both esterases in the hydrolysis of sterols esters present in wood extractives.

The reactions were incubated for 3 hours at 37°C, and controls with boiled enzymes were carried out. After the enzymatic treatment, the chloroform-soluble compounds, obtained from three successive chloroform extractions, were analysed by gas chromatography (GC). The analyses of chloroform-soluble compounds were performed on a Hewlett Packard HP 5890 gas chromatograph equipped with a flame ionisation detector (FID), using a high temperature, polyimide coated fused silica 5 m × 0.25 mm DB5-5HT (0.1 µm film thickness, J&W Scientific) capillary column (Martínez et al., 1999). The injector and the detector temperatures were set at 300°C and 350°C respectively, and the oven was programmed from 100°C (1 minute) to 350°C (3 minutes) at 15°C/min. The different compounds were quantified by peak area using the corresponding calibration curves.

### 2.5 Chemicals

Triolein, tributyrin, *p*-nitrophenyl palmitate and butyrate, cholesterol esters and Genapol X-100 were all purchased from Sigma. Sodium deoxycholate was from DIFCO. All others reagents were of the purest grade available.

## 3 Results and discussion

### 3.1 Production and characterisation of *O. piceae* sterol esterase

After a previous screening on ascomycetes, basidiomycetes and conidial fungi, *O. piceae* (synonym *Ceratocystis piceae*) was selected as the best enzyme producer, exhibiting both lipase and sterol esterase activities (Calero-Rueda, 2001).

The crude enzyme obtained from ultrafiltered 12 day-old cultures, when maximal esterase activity was produced, was applied to an Octyl Sepharose column equilibrated with 2 M ammonium sulphate in 25 mM HCl-Tris, pH 7. A homogeneous protein with activity on *p*-nitrophenol and cholesterol esters was eluted after the end of the decreasing ammonium sulphate gradient, when 0.1% reduced Triton X-100 was applied to the column. According to previous results, both activities corresponded to a single enzyme showing a molecular mass ( $M_r$ ) around 56.5 kDa by SDS/PAGE, 8% N-linked carbohydrate, and the N-terminal sequence TTVNVKYPEGEVVG (Calero-Rueda et al., 2002). A different extracellular esterase with lowest  $M_r$  (around 35 kDa) and different N-terminal sequence has been isolated from *O. piceae* and described as a lipase (Gao and Breuil, 1995). The enzyme studied in the present work shows similar  $M_r$  and N-terminal sequence to that reported in *Ophiostoma piliferum* (Brush et al., 1999), although they showed different catalytic properties (as discussed below).

The kinetic study showed the broad substrate specificity of the *O. piceae* esterase, which is able to hydrolyse *p*-nitrophenol esters, triglycerides and cholesterol esters (Table 1). The hydrolysis efficiency increased in parallel with the length of the fatty acid esterifying glycerol, and the presence of double bonds in the acyl chain affect  $k_{cat}$  more than  $K_m$  (Calero-Rueda et al., 2002; Brush et al., 1999). A low activity on cholesterol esters has been also reported for the other esterase isolated from *O. piceae* (Gao and Breuil, 1998). Sterol esterases are produced by fungi and bacteria but only a small

number of them have been fully characterised (Rahim and Sih, 1969; Ghosh et al., 1995). Some microbial sterol esterases from *P. fluorescens* and *C. rugosa* are being commercialised for their medical application in the determination of serum cholesterol (Allain et al., 1974). Since sterol esters are problematic compounds forming pitch deposits during pulping of eucalypt and other hardwoods (Del Río et al., 1999), they could have high interest also for pitch biocontrol in paper pulp manufacturing. Table 2 shows the activities of these commercial enzymes on sterol esters compared with the *O. piceae* esterase. Only the sterol esterases from *Pseudomonas* sp. and *O. piceae* could hydrolyse *p*-nitrophenyl butyrate, in the presence of 1% Genapol X-100, the former being more efficient at 0.3 mM substrate concentration. It has been reported that the specific activity of the enzyme *O. piceae* esterase decreased with the concentration of Genapol X-100 probably due to a competitive inhibition effect (Calero-Rueda et al., 2002). Then, the negative result on the hydrolysis of pNPB by some commercial enzymes could be probably due to the presence of this non-ionic detergent in the reactions. On the other hand, although all the sterol esterases checked hydrolysed the cholesterol esters assayed in the presence of 10% Genapol X-100, the highest specific activity was obtained in all cases with the enzyme from *O. piceae*, followed by the sterol esterase from *Pseudomonas* sp. In an attempt to check the possibility to use sterol esterases for pitch biocontrol during manufacturing of hardwood paper pulp, the ability of the two latter enzymes to hydrolyse those sterol esters present in lipophilic wood extractives was analysed.

**Table 1** Apparent kinetic constants of *O. piceae* esterase on commercial cholesterol esters and triolein

	$K_m^{app}$ (mM)	$k_{cat}^{app}$ (s <sup>-1</sup> )	Efficiency ( $k_{cat}^{app} / K_m^{app}$ ) (mM <sup>-1</sup> s <sup>-1</sup> )
Cholesteryl butyrate	3.0	47	16
Cholesteryl palmitate	0.9	67	74
Cholesteryl stearate	1.1	70	64
Cholesteryl oleate	1.0	138	138
Cholesteryl linoleate	1.0	150	150
Tributylin	9.9	180	181
Triolein	1.0	290	290

Reactions were carried out in the presence of 10% Genapol X-100 at Calero-Rueda et al., 2002.

**Table 2** Specific activities (U/mg protein) of *O. piceae* sterol esterase, and commercial esterases from three microbial sources, on cholesterol esters and pNPB

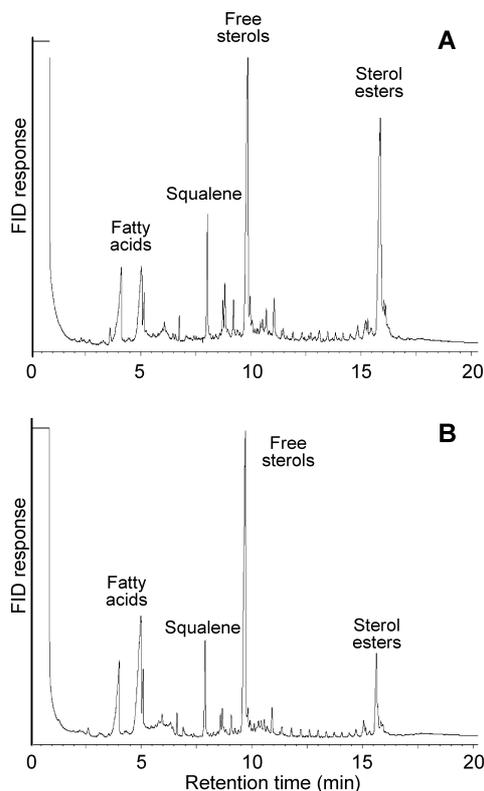
	<i>pNPB</i>	<i>Cholesteryl butyrate</i>	<i>Cholesteryl stearate</i>	<i>Cholesteryl oleate</i>
<i>O. piceae</i>	23	17.0	15.3	53.4
<i>P. fluorescens</i>	0	8.1	9.2	7.2
<i>C. rugosa</i>	0	0.4	0.4	10.1
<i>Pseudomonas</i> sp.	72.1	1.5	3.2	26.1

Reactions were carried out using 0.3 mM substrate concentration, in the presence of Genapol X-100 (1% and 10% for hydrolysis of pNPB and cholesterol esters, respectively).

### 3.2 Degradation of lipophilic extractives from eucalypt wood involved in pitch deposition

Previous studies showed that the extractives in pulp and process waters from *E. globulus* kraft pulping and TCF bleaching have similar composition to those in the raw material (Gutiérrez et al., 2001a, 2001b). To investigate the potential of sterol esterases to hydrolyse sterol esters in eucalypt pulping, an aqueous suspension of eucalypt wood acetone extract containing the lipophilic compounds involved in pitch deposition, was treated with the sterol esterase from *O. piceae* and with the commercial sterol esterase from *Pseudomonas* sp. Both enzyme preparations were able to hydrolyse the sterol esters increasing the ratio between free sterols or fatty acids and sterol esters in the treated lipophilic fraction, although the best results were obtained with the esterase from *O. piceae* (Figure 1). The *O. piceae* sterol esterase decreased around 70% sterol esters, increasing the percentage of free sterols and fatty acids accordingly, whereas the enzyme from *Pseudomonas* sp. only produce a decrease around 50% under the same conditions. These results suggest that process waters from eucalypt pulping could be treated with sterol esterases to remove some of the compounds responsible for pitch deposit formation.

**Figure 1** Degradation of eucalypt wood lipophilic extractives by *O. piceae* sterol esterase analysed by GC (FID detector) after 3 hours treatment: A) control treated with denatured enzyme; B) problem treated with native enzyme



Commercial lipases are being used to control pitch problems caused by triglycerides in pine mechanical pulping (Fujita et al., 1992; Sharyo, 1993) but they are not effective during pulping of other softwoods, such as *P. abies* (Leone and Breuil, 1999), or hardwoods with high levels of sterol esters. Enzymatic preparations containing sterol esterase activity, where the enzymes are not fully characterised, in combination with commercial lipases have been reported for controlling pitch during paper manufacture (Buchert et al., 2000). The advantage of the *O. piceae* esterase is its ability to hydrolyse both triglycerides and sterol esters to pitch biocontrol. These results have been included in a patent (Calero-Rueda et al., 2002). Studies to confirm the effectiveness of the *O. piceae* esterase to control pitch problems are currently in course including treatment of both hardwoods and softwoods process waters, as well as optimisation of different enzyme doses and time of treatment.

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### References

- Allain, C.C., Poon, L.-S., Chan, C.S.G., Richmond, W. and Fu, P.C. (1974) 'Cholesterol oxidase is widely used in clinical medicine for determination of serum cholesterol', *Clin. Chem.*, Vol. 20, pp.470–475.
- Back, E.L. (2000) 'The location and morphology of resin components in the wood', in Back, E.L. and Allen, L.H. (Eds.): *Pitch Control, Wood Resin and Deresination*, Tappi Press, Atlanta, pp.1–35.
- Brush, T.S., Chapman, R., Kurzman, R. and Williams, D.P. (1999) 'Purification and characterization of extracellular lipases from *Ophiostoma piliferum*', *Bioorgan. Med. Chem.*, Vol. 7, pp.2131–2138.
- Buchert, J., Mustranta, A., Spetz, P., Ekman, R. and Holmbom, B. (2000) 'Enzymatic control of wood extractives', *Proc. Appita Conf. 2000*, APPITA, Melbourne, pp.571–573.
- Calero-Rueda, O. (2001) *Búsqueda de Enzimas Fúngicas con Actividad Esterasa para el Control Biológico del 'Pitch'*, Master Degree Thesis, Univ. Complutense de Madrid.
- Calero-Rueda, O., Gutiérrez, A., Del Río, J.C., Muñoz, C., Plou, F.J., Martínez, A.T. and Martínez, M.J. (2002) 'Method for the enzymatic control of pitch deposits formed during paper pulp production using an esterase that hydrolyses triglycerides and sterol esters', *Patent (International) No. WO 02/075045 A1* (PCT/ES02/00120; application 14th March 2002).
- Calero-Rueda, O., Plou, F.J., Ballesteros, A., Martínez, A.T. and Martínez, M.J. (2002) 'Production, isolation and characterization of a Sterol Esterase from *Ophiostoma piceae*', *Biochim. Biophys. Acta Proteins Proteomics*, Vol. 1599, pp.28–35.
- Del Río, J.C., Gutiérrez, A. and González-Vila, F.J. (1999) 'Analysis of impurities occurring in a totally-chlorine free-bleached kraft pulp', *J. Chromatogr.*, Vol. 830, pp.227–232.
- Del Río, J.C., Romero, J. and Gutiérrez, A. (2000) 'Analysis of pitch deposits produced in Kraft pulp mills using a totally chlorine free bleaching sequence', *J. Chromatogr. A*, Vol. 874, pp.235–245.

- Fengel, D. and Wegener, G. (1983) *Extractives. Wood: Chemistry, Ultrastructure, Reactions*, De Gruyter, Berlin.
- Fischer, K., Puchinger, L. and Schloffer, K. (1993) 'Enzymatic pitch control of sulfite pulp on pilot scale', *J. Biotechnol.*, Vol. 27, pp.341–348.
- Fujita, Y., Awaji, H., Taneda, H., Matsukura, M., Hata, K., Shimoto, H., Sharyo, M., Abo, M. and Sakaguchi, H. (1992) 'Enzymatic pitch control in the papermaking process', in Kuwahara, M. and Shimada, M. (Eds.): *Biotechnology in Pulp and Paper Industry*, UNI Publishers Co., Ltd. Kyoto, pp.163–168.
- Gao, Y. and Breuil, C. (1995) 'Extracellular lipase production by a sapwood-staining fungus, *Ophiostoma piceae*', *World J. Microbiol. Biotechnol.*, Vol. 11, pp.638–642.
- Gao, Y. and Breuil, C. (1998) 'Properties and substrate specificities of an extracellular lipase purified from *Ophiostoma piceae*', *World J. Microbiol. Biotechnol.*, Vol. 14, pp.421–429.
- Ghosh, D., Wawrzak, Z., Pletnev, V.Z., Li, N., Kaiser, R., Pangborn, W., Jörnvall, H., Erman, M. and Duax, W.L. (1995) 'Structure of uncomplexed and linoleate-bound *Candida cylindracea* cholesterol esterase', *Structure*, Vol. 3, pp.279–288.
- Gutiérrez, A., Del Río, J.C., González-Vila, F.J. and Martín, F. (1999) 'Chemical composition of lipophilic extractives from *Eucalyptus globulus* Labill wood', *Holzforschung*, Vol. 53, pp.481–486.
- Gutiérrez, A., Romero, J. and Del Río, J.C. (2001a) 'Lipophilic extractives from *Eucalyptus globulus* pulp during kraft cooking followed by TCF and ECF bleaching', *Holzforschung*, Vol. 55, pp.260–264.
- Gutiérrez, A., Romero, J. and Del Río, J.C. (2001b) 'Lipophilic extractives in process waters during manufacturing of totally chlorine free kraft pulp from eucalypt wood', *Chemosphere*, Vol. 44, pp.1237–1242.
- Hillis, W.E. (1962) *Wood Extractives*, Academic Press, London.
- Hillis, W.E. and Sumimoto, M. (1989) 'Effect of extractives on pulping', in Rowe, J.W. (Ed.): *Natural Products of Woody Plants*, II Springer-Verlag Berlin, pp.880–920.
- Irie, Y. (1990) 'Enzymatic pitch control in papermaking system', *Abs. 1990 Papermakers Confer.*, April, Atlanta, pp.23–25.
- Leone, R. and Breuil, C. (1999) 'Biodegradation of aspen steryl esters and waxes by two filamentous fungi with or without other carbon sources', *World J. Microbiol. Biotechnol.*, Vol. 15, pp.723–727.
- Martínez, M.J., Barrasa, J.M., Gutiérrez, A., del Río, J.C. and Martínez, A.T. (1999) 'Fungal screening for biological removal of extractives from *Eucalyptus globulus* Labill', *Wood Can. J. Bot.*, Vol. 77, pp.1513–1522.
- Plou, F.J., Ferrer, M. and Ballesteros, A. (2003) 'Transesterification with biological catalysts', in Horvath, I.T. (Ed.): *Encyclopedia of Catalysis*, Vol. 6, Wiley Interscience New York, pp.483–506.
- Rahim, M.A. and Sih, C.J. (1969) 'Microbial steroid esterases', *Methods Enzymol.*, Vol. 15, pp.675–684.
- Rowe, J.W. (1989) *Natural Products of Woody Plants. I and II, Chemicals Extraneous to the Lignocellulosic Cell Wall*, Springer-Verlag, Berlin.
- Sharyo, M.H.H.M.Y.H. (1993) 'The recent progress and general status of the lipase pitch control technology in Japan', *Jpn.Tappi J.*, Vol. 47, pp.41–51.
- Tappi (1993) *Test Methods 1992–1993*, TAPPI, Atlanta.