

Direct enzymatic esterification of cotton and Avicel with wild-type and engineered cutinases

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Abstract In this work, the surface of cellulose, either Avicel or cotton fabric, was modified using cutinases without any previous treatment to swell or to solubilise the polymer. Aiming further improvement of cutinase ester synthase activity on cellulose, an engineered cutinase was investigated. Wild-type cutinase from *Fusarium solani* and its fusion with the carbohydrate-binding module N1 from *Cellulomonas fimi* were able to esterify the hydroxyl groups of cellulose with distinct efficiencies depending on the acid substrate/solvent system used, as shown by titration and by ATR-FTIR. The carbonyl stretching peak area increased significantly after enzymatic treatment during 72 h at 30 °C. Cutinase treatment resulted in relative increases of 31 and 9 % when octanoic acid and vegetable oil were used as substrates, respectively. Cutinase-N1 treatment resulted in relative increases of 11 and 29 % in the peak area when octanoic acid and vegetable oil were used as substrates, respectively. The production

and application of cutinase fused with the domain N1 as a cellulose ester synthase, here reported for the first time, is therefore an interesting strategy to pursuit.

Keywords Cellulose ester · Reverse esterase · Transesterification · Carbohydrate-binding module · Caprylic acid

Introduction

Cellulose is the most abundant natural polymer and it is a valuable natural resource (Steinmann 1998). The more traditional industrial applications of cellulose are paper and textiles but the development on cellulose chemistry allowed new processes and a continuous growth of its derivatives. As the expected consequence of cellulose molecular structure, esterification is a well explored route to modify either homogeneous or heterogeneously this polysaccharide. Several approaches aiming at cellulose esterification are described in the literature (Vaca-Garcia et al. 1998; Freire et al. 2006; Braun and Dorgan 2009; Uschanov et al. 2011). The low molecular weight cellulose esters have been used as a raw material for plastics, textiles, filter tows, films or membranes (Edgar et al. 2001; Rustemeyer 2004). More recently, a growing interest in cellulose nanostructures has boost research on surface esterification of cellulose with long fatty acids in order to overcome its incompatibility with nonpolar matrices while preserving the desired mechanical properties (Braun and Dorgan 2009; Uschanov et al. 2011).

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In a textile context, cellulose esterification is the basis of durable chemical finishing methods. Through textile finishing, the surface of fibres can be modified to add useful qualities to the fabric, ranging from attractive appearance and fashion aspects to high performance properties for particular industrial needs (Schindler and Hauser 2004). Almost all apparel and home furnishing textiles are treated with softeners because, during their processing, natural oils and waxes are often removed (Schindler and Hauser 2004). Another important cotton treatment based on esterification is the durable press finish. There are recent publications concerned with the easy-care properties improvement of cotton by ester crosslinking of cellulose without resorting to formaldehyde-releasing compounds (Ford et al. 2011; Hashem et al. 2011; Fouda and Fahmy 2011; Peng et al. 2012).

The chemical surface modification of textile polymers, in addition to the environmental issues, is not always easy to control and in some cases severe fibre yellowing and weight lost occur (Gübitz and Cavaco-Paulo 2008). Enzymes are a good alternative to chemical surface modification due to several reasons. As macromolecules, they are well-suited for targeting surface functionalization of polymers since their mode of action is normally restricted to the most superficial layers of polymer fibres. They are considered green catalysts not just because they are biodegradable themselves but also because of their mild operating conditions (Rozzell 1999). Besides, many enzymes can accept unnatural substrates. The specificity, the stability and the specific activity can be further improved through genetic modifications, and reaction medium engineering can modulate their catalytic properties (Koeller and Wong 2001).

In this work we present a new approach for the simple and direct biomodification of hydroxyl groups on the surface of cellulose, either as Avicel or as plain woven cotton fabric, a heterogeneous reaction where the players are not all in the same physical state. Using a well known cutinase, from *Fusarium solani pisi* (Araújo et al. 2007; Purdy and Kolattukudy 1975a, b) and a chimeric cutinase fused with the carbohydrate-binding module (CBM) N1 of the Endoglucanase C (CenC) from *Cellulomonas fimi* (Matamá et al. 2010), the accessible hydroxyl groups of cellulose were esterified with octanoic acid in isooctane and with edible vegetable oil. Cutinase is a very versatile enzyme: it can use a broad range of substrates, it is

rather stable and at low water activities cutinase catalyses the reverse reaction—transesterification of fats and selective esterification of alcohols (Badenes et al. 2010; de Barros et al. 2009, 2010, 2011). Very recently, a commercial immobilized cutinase from *F. solani* has been used to acylate cellulose with long fatty acids in non conventional media with the ultimate purpose of conferring a thermoplastic behaviour to the polymer (Gremos et al. 2011a, b). In water, the fusion of cutinase with N1 domain of CenC resulted in an increase of esterase activity yield on cellulose acetates when compared to the wild-type cutinase (Matamá et al. 2010). A major outcome of the present study was the insight that the fusion with CBM N1 also presents particular advantages in organic media to the reverse esterase activity of cutinase on cellulosic substrates.

Experimental

Reagents and enzymes

The properties of the cotton fabric used in this work were: 50/66 ends/picks per cm and 131 g m^{-2} . The microcrystalline cellulose powder Avicel PH-101 was purchased from Sigma-Aldrich (Sigma-Aldrich Quimica SA, Madrid, Spain). The free fatty acid used to esterify cellulose was octanoic acid (purity $\geq 98 \%$) from Sigma. The brand of a commercial edible-grade vegetable oil used in this work was Fula Alimentar (Sovena Group, Algés, Portugal). All other reagents were laboratory grade reagents from Sigma-Aldrich.

Expression and purification of recombinant cutinases

The DNA coding for *F. solani pisi* cutinase (EC 3.1.1.74) and for cutinase fused in frame with the carbohydrate binding module N1 (cutinase-N1) from *C. fimi* CenC, were cloned, and the respective proteins expressed and purified as previously reported by Matamá et al. (2010). Briefly, clones of *Escherichia coli* strain BL21(DE3), harbouring the constructs pCWT and pCWT::CBM_{N1}, were grown overnight, at 30 °C and 200 rpm, in 1 L of culture medium supplemented with $100 \mu\text{g mL}^{-1}$ ampicillin and 0.2 % α -lactose. The cells were harvested by centrifugation and frozen at $-80 \text{ }^\circ\text{C}$. The ultrasonic

disruption of the bacterial cells was accomplished on ice with a 25.4 mm probe in an Ultrasonic Processor VCX-400 watt (Cole-Parmer Instrument Company, Illinois, USA). The lysate was centrifuged for 30 min at 30,000g and 4 °C. The supernatant was collected and cutinase purification was done by affinity chromatography using HiTrap Chelating HP system (GE Healthcare Bio-Sciences Europe GmbH, Munich, Germany) coupled to a peristaltic pump. The cutinase-N1 was purified using its selective adsorption to Avicel PH-101 (Coutinho et al. 1992). The fractions obtained were monitored by SDS-PAGE with silver staining. The fractions of interest were frozen at -80 °C and lyophilised (Christ Alpha 2-4 freeze dryer, B. Braun Biotech International, Melsungen, Germany).

Quantification of total protein concentration

Total protein in solution was quantified following Bradford methodology (Bradford 1976). Bovine serum albumin was used as standard. All samples were measured in triplicate.

Esterase activity assay

Esterase activity was determined following the *p*-nitrophenol release at 400 nm during 3 min at 30 °C (SepctraMax 340 PC spectrophotometer, Molecular devices, Sunnyvale, CA, United States). The final reaction volume was 300 µL consisting in 275 µL of 50 mM phosphate buffer pH 7, 15 µL of 1.5 mM *p*-nitrophenol butyrate in ethanol (ϵ 1.84×10^4 M⁻¹ cm⁻¹, de Barros et al. 2010) and 10 µL of lyophilised enzymes, previously rehydrated in ultra-pure water. All the assays were performed at least in triplicate. One unit (U) of esterase activity was defined as one µmol of *p*-nitrophenol released per minute.

Esterification of cotton fabric and Avicel

Several experiments were carried out varying the enzyme and the acid substrate/organic solvent. For all experiments, the treatment of cellulose substrates was performed in the Rotawash MKIII (vertical agitation simulating European washing machines, from SDL International Ltd.) operating at 30 °C and 20 rpm for 72 h. Samples (100.2 ± 0.1 mg) of Avicel PH-101 and cotton fabric were incubated either in 10 mL of

isooctane containing 185 mM octanoic acid or in 10 mL of edible vegetable oil. The maximum enzyme load was 1.4 mg g⁻¹ of cellulose. As controls, the cellulose was subjected to the same treatment but without the enzymes.

After treatment, all fabric samples and Avicel cellulose were washed twice with methanol and twice with *n*-hexane (Gremos et al. 2011b). The cellulose samples were let at room temperature inside an extractor hood during 48 h till complete solvent evaporation.

Relative ester content determination

The esterified cellulose samples (Avicel and cotton) were saponified according to Gremos et al. (2011b). Ten millilitres of ethanol (75 %) were added to samples and the mixture was heated at 55 °C for 30 min. Afterwards, 6 mL of 0.5 M NaOH were added; the mixture was heated at 70 °C for 30 min more and left to settle at room temperature for 3 days. The excess NaOH was measured by titration with 0.5 M HCl in the presence of phenolphthalein as indicator. An excess of 1 mL of acid was added and the mixture was left overnight. The excess of added acid was then titrated back with 0.5 M NaOH solution.

The ester content (%) was determined as the relative molar percentage of the ester link regarding the total hydroxyl groups present according to the following equation:

$$\text{Ester content (\%)} = \left\{ \left[V_{\text{NaOH}}(\text{sample}) - V_{\text{NaOH}}(\text{control}) \right] * C_{\text{NaOH}} - \left[V_{\text{HCl}}(\text{sample}) - V_{\text{HCl}}(\text{control}) \right] * C_{\text{HCl}} \right\} / \left[3 * W_{\text{sample}} / M(\text{C}_6\text{H}_9\text{O}_5) \right]$$

where *V* is the respective volume (L), *C* is the respective molar concentration, *W* is the weight of the sample (g) and *M* is the molar mass of the anhydro-glucose repeating unit of cellulose.

Fourier transform infrared spectroscopy (FTIR)

The attenuated total reflectance (ATR) technique was used to collect the infrared spectra of cotton fabric controls and samples treated during 72 h with cutinase and cutinase-N1. The spectra were recorded in a Jasco FT/IR-4100 type A spectrometer (Jasco Inc., Maryland, USA) with a Golden Gate ATR accessory with a

single reflection monolithic diamond (Specac Ltd., Slough, UK). All the absorbance spectra were acquired in the range 4,000–600 cm^{-1} at 8 cm^{-1} resolution and as result of the accumulation of 64 scans. Six different measurements were performed per sample.

Results and discussion

Without previous swelling or solubilisation, cellulose bioesterification was accomplished in a heterogeneous reaction with wild-type as well as with the genetically modified cutinase from *F. solani pisi*. Wild-type (wt) cutinase and cutinase fused with the cellulose-binding domain N1 (cutinase-N1) were produced in *E. coli*, purified and lyophilised. After rehydration and using *p*-nitrophenol butyrate as substrate at 30 °C, the esterase activities for wt cutinase and for cutinase-N1 were 14 and 21 kU mg^{-1} , respectively. The ability of these enzymes to esterify cellulose was investigated using Avicel powder and cotton fabric. One of the acid substrates used was a high purity fatty acid, octanoic acid, diluted in isoctane so that its concentration was equimolar to the concentration of the alcohol substrate (cellulose hydroxyl groups). According to the literature, when using cutinase from *F. solani*, the highest ester yields were obtained for an acid to alcohol molar ratio of one (de Barros et al. 2009, 2011). The solvent isoctane was chosen due to its good biocompatibility and to the fact of being recognized as safe in food and beverages industrial processing by FDA (de Barros et al. 2009). In addition, the good performance of cutinase in isoctane for ester synthesis/transesterification is known (Badenes et al. 2010; de Barros et al. 2009). The other acid substrate tested in this study was common edible oil, a mixture of triglycerols and free fatty acids used without any dilution. Based on other enzymatic treatments and acquired experience with these enzymes, the esterification reaction was allowed for 72 h at 30 °C, under a continuous vertical agitation.

The first evaluation of ester synthase activity was done by a simple titration after complete ester saponification (Gremos et al. 2011a, b; Sereti et al. 2001). Figure 1 shows the ester content determined by this method using 100 mg of cellulosic substrates. It was possible to detect a difference between enzymatic treated cellulose and control cellulose. Though this

approach may present some restrictions (Freire et al. 2005) along with a low analytical sensitivity considering the amounts used, it uncovered the ester formation for cotton treated with wt cutinase using octanoic acid as the acid substrate (Fig. 1a) and for both cotton and Avicel treated with cutinase-N1 in vegetable oil (Fig. 1b).

ATR-FTIR was performed to obtain further evidence of esterification by the two cutinases on cotton samples. Spectral data were normalized with a common baseline; no smoothing functions were used. The spectral zone of interest corresponding to the ester carbonyl stretching was further analysed (1,730–1,750 cm^{-1}) (Braun and Dorgan 2009; Uschanov et al. 2011; Freire et al. 2005). Figure 2 shows the average individual spectra for the control and enzymatic treated cotton samples (curves A–C) using octanoic acid (Fig. 2a) and vegetable oil (Fig. 2b) as the acid substrates. In these spectra there are two main peaks (1,734 and 1,746 cm^{-1}). The peak position depends on several factors like acyl chain length and the position of the esterified carbon in the anhydroglucose ring. After subtracting the control spectrum, the resulting spectra for wt cutinase and cutinase-N1 treated samples, curves D and E, respectively, show two less prominent peaks. Table 1 summarises the area values obtained by peak fitting with Origin software. The esterification was verified by ATR-FTIR on cotton surface for both enzymes and for both acid substrates. There is an unambiguous increase in the area of both peaks on enzyme treated samples regarding the respective control. The difference between the two enzymes is significant at 1,746 cm^{-1} . A closer look to the area of this peak reveals a good agreement between the spectrometric and titration results; cutinase-N1 has a better performance than wt cutinase in vegetable oil and the opposite stands true in octanoic acid. Wild-type cutinase treatment leads to a relative increase in the 1,746 cm^{-1} peak area of 31 % when octanoic acid is used as substrate and of 9 % when vegetable oil is used. The cutinase-N1 treatment leads to relative increases of 11 and 29 % when octanoic acid and vegetable oil are used as substrates, respectively.

Directed mutagenesis and structure determination of the CBM N1 in complex with an oligosaccharide elucidated the interaction of this CBM with a single chain of cellulose revealing the most important residues (Kormos et al. 2000; Boraston et al. 2002).

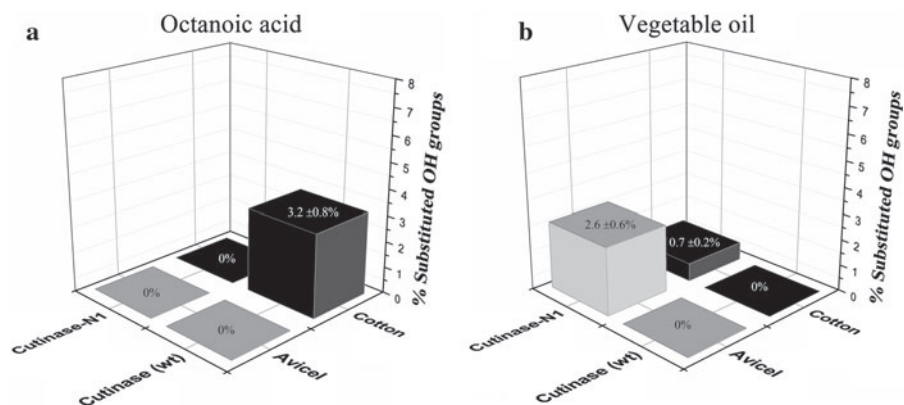


Fig. 1 Ester content (%) calculated as the relative molar percentage of the ester link, obtained by titration after ester saponification on cellulosic samples and controls. Avicel and

cotton were treated during 72 h at 30 °C with wild-type (wt) and genetically engineered (cutinase-N1) cutinases, using either octanoic acid (a) or edible vegetable oil (b) as the acid substrates

Fig. 2 Normalized ATR-FTIR absorbance spectra of cotton samples and controls in the carbonyl stretching vibrations range. Cotton samples were treated during 72 h at 30 °C with wild-type (wt) and genetically engineered (cutinase-N1) cutinases, using either octanoic acid (a) or edible vegetable oil (b) as the acid substrates. The curves E and D are the result of control spectra subtraction

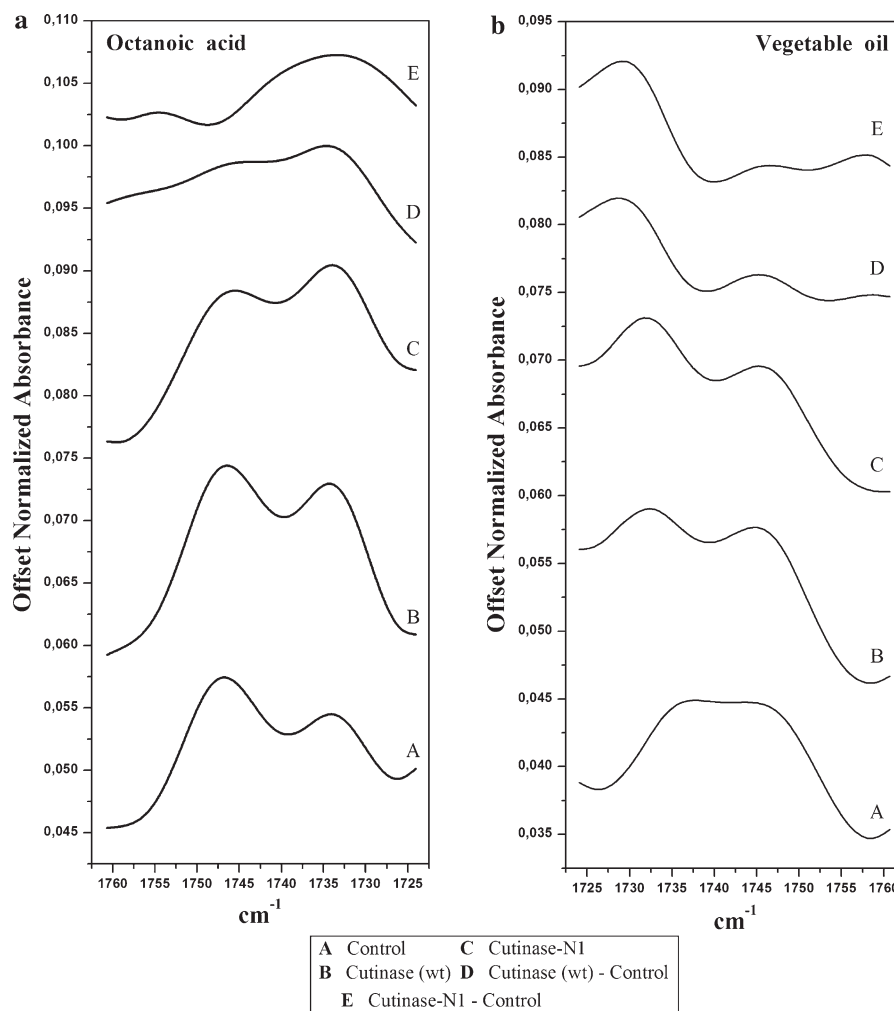


Table 1 Peak area values after Gaussian peak fitting of ATR-FTIR absorbance spectra in the range 1,724–1,760 cm^{-1} using Origin software

	Octanoic acid		Vegetable oil	
	1,734 cm^{-1}	1,746 cm^{-1}	1,734 cm^{-1}	1,746 cm^{-1}
Control	0.078 \pm 0.001	0.127 \pm 0.001	0.056 \pm 0.002	0.076 \pm 0.002
Cutinase (wt)	0.097 \pm 0.002	0.167 \pm 0.003	0.073 \pm 0.002	0.083 \pm 0.002
Cutinase-N1	0.101 \pm 0.002	0.141 \pm 0.002	0.071 \pm 0.001	0.098 \pm 0.001

The side chains of polar residues form direct hydrogen bonds with the hydroxyl groups of the cellopentose and three tyrosines are involved in the interaction with the glucose rings of cellulose, mainly through van der Waals forces. The structure of CBM N1 is fairly rigid and cellulose binding is not associated with significant conformational changes. Furthermore, osmotic stress studies and the crystal structure demonstrated that the release of water from the CBM N1-ligand interface has an unusually small impact on binding (Boraston et al. 2002). Taken all together, it was reasonable to assume that the CBM N1 binding to cellulose could occur in a nonaqueous environment.

The DNA sequence and the affinity of CBM N1 from *C. fimi* were first described by Coutinho et al. (1993); it binds preferentially amorphous cellulose but it can also bind reversibly Avicel but not crystalline cellulose (Tomme et al. 1996). According to literature, the amorphous content, type of cellulose allomorphs, and crystallite size is very similar between Avicel and cotton linters (Mittal et al. 2011). The more pronounced esterification of Avicel than cotton by cutinase-N1 (Fig. 1b) could be related to surface area available for binding, but why this was verified only in vegetable oil and not in isooctane still remains unclear to the authors. Both isooctane and edible vegetable oil (soybean) are aprotic, on the other hand, viscosity and dielectric constants are different: 0.5 cP (22 °C) and 1.9 (25 °C) for isooctane and 54.3 cP (24 °C) and 3.1 (25 °C) for soybean oil, respectively (Noureddini et al. 1992; Lizhi et al. 2008). Different dielectric constants could explain different abilities to recognize cellulose, since solvent polarity affects the strength of noncovalent interactions either the ones involved in CBM N1 binding (Aquino et al. 2002) or in the structure stability of CBM N1, which has a relatively low maximum stability compared to other single-domain globular proteins (Creagh et al. 1998). The factor viscosity would work against the binding in vegetable

oil (Sitnitsky 2010) but given the duration of the experiment and the mechanical agitation, viscosity may play a minor role.

In *F. solani pisi* cutinase, the catalytic triad is located at one edge of the ellipsoid protein, in a hydrophobic cleft rather accessible to the solvent (Prompers et al. 1999). It was verified that a cutinase from *Thermobifida fusca* and its null mutant possess detergency properties. A simulation study revealed that the active sites of both cutinases have great affinity to aliphatic chains and due to their amphipathic nature, the proteins improved fat stain removal from cotton samples (Silva et al. 2012). Analogously, the reason for cutinase (wt) being more efficient in octanoic acid than in vegetable oil may be the fact that the active site of cutinase is stably entrapped in oil without a cellulose-binding domain to drive the enzyme to the hydroxyl substrate.

Conclusions

Here reported, the ability to esterify the surface of cellulose in a simple, controlled and eco-friendly reaction is important not only to the textile industry but also to other areas where this key natural resource has been applied to (Freire et al. 2005, 2006; Braun and Dorgan 2009; Uschanov et al. 2011; Edgar et al. 2001). Designing engineered enzymes that mimic strategies found in Nature has become a powerful tool in synthetic biology. The idea of using cutinase-N1 was devised to mimic other carbohydrate-active enzymes which by means of CBM domains display improved activity in aqueous media. The fact that cutinase and the N1 domain interact differently with their surroundings can explain the differences seen in the catalysis of cutinase (wt) and cutinase-N1 in octanoic acid/isooctane and vegetable oil. Moreover, new perspectives are open for the CBMs applicability

in nonaqueous systems especially whenever the catalytic partner is less efficient. In the particular case of cutinase fused with CBM N1 production and application as a cellulose ester synthase will be an interesting strategy to pursue and to optimize for textiles with biomedical applications.

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