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Potential Use of Cutinase in Enzymatic Scouring of Cotton Fiber Cuticle

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Abstract

The present study characterized the ability of a bacterial cutinase to improve the wettability of raw cotton fabrics by specific hydrolysis of the cutin structure of the cuticle. The effect of cutinase was studied alone and in coreaction with pectin lyase. The changes in both the fabric and the reaction fluid were measured and compared to enzymatic hydrolysis with polygalacturonase, and to chemical hydrolysis with boiling NaOH. Water absorbancy, specific staining, fabric weight loss, and evaporative light-scattering reverse-phase high-performance liquid chromatography analysis of chloroform extract of the reaction fluid were measured to assess the enzymatic hydrolysis of the cuticle waxy layer. The pattern and extent of hydrolysis of the major cuticle constituents depended on the enzyme type and titers employed and paralleled the degree of wettability obtained. The combination of cutinase and pectin lyase resulted in a synergistic effect. The use of detergents improved enzymatic scouring. The major products released to the reaction medium by the cutinase treatment were identified by gas chromatography/mass spectrometry analysis as C:16 and C:18 saturated fatty acid chains.

Index Entries: Cotton fiber; plant cuticle; cutinase; pectin lyase; polygalacturonase; bioscouring; textile industry.

Introduction

The structure of the primary cell wall of the cotton fiber has a particular influence on textile manufacturing processes, and especially the outermost

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surface layer, the cuticle *(1,2)*. The hydrophobic noncellulosic components of the cuticle protect the cell from environmental damage and pathogenic invasion during growth and development, and impart the nonabsorbent nature of raw cotton. The cuticle comprises approx (% of the fiber dry wt) 0.6 waxes, 0.9 pectins, 1.3 proteins, 2 noncellulosic polysaccharides, ash, and miscellaneous constituents *(1,3)*. The cuticle is crosslinked to the primary cell wall by esterified pectin substances. Cutin and suberin are the major structural components of the waxy layer of the cuticle. Cutin is an insoluble polyester composed mainly of saturated C:16 and C:18 hydroxy-fatty acids *(4,5)*. The practice of scouring in the textile industry is done to remove the hydrophobic cuticle constituents to improve wettability of the fibers, which facilitates uniform dyeing and finishing. Conventionally, scouring is performed by hot hydrolysis with NaOH, which involves large quantities of water and energy and requires special handling of the strong alkaline effluents *(6)*. The pursuit for environmentally friendly alternatives for scouring gave rise to the use of enzymes for this purpose *(1–3,5–13)*.

Extracellular lytic enzymes involved in the degradation of the cell outer layer during invasion of plants by phytopathogenic fungi and bacteria have been considered as candidates for bioscouring *(4,5)*. These enzymes comprise lipases, pectinases, cellulases, hemicellulases, and proteases. Scouring with pectin-hydrolyzing enzymes, including endo- and exopolygalacturonase, pectin and pectate lyases, and pectin esterase, was extensively studied to increase the wettability of textile fibers *(7–10,13,14)*. Lipases, in most cases, were found to be less effective in fulfilling this task *(8,15)*. Proteases were found to be efficient for bleaching rather than scouring *(8)*. Cellulases were the only enzymes reported to improve wettability efficiently when applied alone; however, they also caused a decrease in fiber strength and hence fabric quality *(6,7,13,16,17)*. The most effective treatment for scouring fiber cuticle is the combination of several enzymes such as cellulases and pectinases or proteases *(2,6,9,10,13)* or lipases and pectinases *(13)*. In most cases, pretreatment with boiling water was needed to achieve a significant increase in wettability.

Most of the studies examined the changes in fabric properties as a result of the enzymatic treatment. Little information exists about the change in the reaction fluid and the products that are released from the fabric. The use of cutinase for bioscouring was not reported. Cutinase is an α/β hydrolase, belonging to the group of serine esterases, secreted by phytopathogenic fungi, bacteria, and actinomycetes *(18)*. Different isoenzymes are secreted by different organisms. Optimal pH of most isoenzymes ranges between 8.0 and 10.0. Cutinase is specific toward primary saturated esters composing the cutin polymer; however, most cutinases also display triacylglyceride-hydrolyzing activity.

The aim of the present study was to characterize the ability of a bacterial cutinase to enable the improvement of the wettability of raw cotton fabrics by specific hydrolysis of the cutin structure of the cuticle. The effect of cutinase was studied alone and in coreaction with pectin lyase. The changes

Table 1
Specification of Enzymes Employed for Scouring Trials[a]

	Cutinase[b]	Pectin lyase[c]	Polygalacturonase[c]
Microbial source	*Pseudomonas mandocino*	Unknown	*Aspergillus niger*
Commercial name	Cutinase ISC-02-BE1	Pectazym HF	Rapidase C80L
Supplier	InterSpex Products	Rakuto Kasei (Israel)	Gist-brocades
Nominal activity	1200–1600 U/g	24,500 U/mL	135,000 U/mL
pH	8.0	8.0	5.0
Temperature (°C)	37	45	45

[a]According to the supplier's specifications in the respective product information sheet.
[b]Solid preparation.
[c]Aqueous solution.

in both the fabric and the reaction fluid were measured and compared to enzymatic hydrolysis with polygalacturonase and to chemical treatment with NaOH.

Materials and Methods

Enzymatic Scouring

Raw cotton fabrics were kindly supplied by AvcoChem (Beit Shemesh, Israel). Prior to treatment, the fabrics were cut into square swatches of approx 1 g, dried at 105°C for 2 h, and weighed to determine precise weight. Enzymatic treatments were performed by incubating one swatch of fabric in 10 mL of reaction mixture contained in a 50-mL polycarbonate screw-capped test tube placed diagonally in a rotary shaker at 175 rpm and 37°C for 20 h. Unless otherwise indicated, the reaction mixture contained 0.1% (w/v) nonionic detergent composed of a blend of polyoxyethylene fatty alcohols (AvcoPal-AWS; AvcoChem), appropriate buffer (100 m*M* phosphate buffer, pH 8.0, for cutinase and pectin lyase; and 50 m*M* acetate buffer, pH 5.0, for polygalacturonase), and the indicated enzyme. The enzymes used and their properties are summarized in Table 1.

Controls of the respective enzymatic treatments were incubated at the same conditions but without enzyme. Each treatment point consisted of at least three replicates. Enzyme activity was as indicated in each case. Cutinase activity was measured spectrophotometrically (λ = 405 nm) according to Kolattukady et al. *(19)* with *p*-nitrophenyl butyrate (Sigma) as substrate, with one unit representing 1 µmol of *p*-nitrophenol released/min at 37°C and pH 8.0. Pectin lyase activity was measured spectrophotometrically (λ = 235 nm) according to Spagna et al. *(20)* with polygalacturonic acid (Fluka) as substrate, with one unit representing 1 µmol of unsaturated galacturonic acid released/min at 50°C and pH 7.0. Polygalacturonase activity was measured using the neocuproine reagent according to Dygert et al. *(21)* with polygalacturonic acid (Fluka) as substrate, with one unit

representing 1 µmol of D-galacturonic acid released/min at 37°C and pH 5.0.

Chemical scouring was carried out by digesting dried and weighed fabric samples (approx 1 g) in 20 mL of boiling NaOH solution, at the indicated concentration, for 30 min. Controls of chemical scouring were done with boiling double distilled water (DDW) for 30 min.

Following incubation or digestion, the fabric samples were separated from the reaction fluid and then exhaustively rinsed with DDW and squeezed. Rinsing and squeezing were repeated three times. The rinsed samples were then dried at 105°C, weighed for calculation of weight loss, and further used for water absorbancy determination and specific staining tests. The collected reaction fluid was extracted and analyzed for chemical composition as described next.

Determination of Scouring Efficiency

Water Absorbancy

The treated fabric swatches, after washing and drying, were tested for wettability using the AATCC Test Method No. 27-1977 *(22)*. The time (in seconds) between the contact of a 20-µL water drop, carefully deposited onto the fabric surface, and its disappearance into the fabric matrix (i.e., the time required for the specular reflection of the water) was recorded as the fabric-wetting time. Each fabric swatch was tested in at least nine different areas and the mean time was calculated.

Staining

The efficiency of removal of the cuticle constituents by each specific treatment was qualitatively estimated by staining the treated fabric swatches with Congo red (10 mg/mL in a 20% ethanolic solution), which assesses for cellulose accessibility. Specific removal of pectin substances was confirmed by staining with ruthenium red (10 mg/mL in DDW) *(2)*. Staining (1 g of fabric swatch/10 mL of staining solution) was performed for 10 min at 35°C through shaking (200 rpm). Subsequently, samples were destained with DDW several times until no color was released.

Analysis and Identification of Products Released to Reaction Fluid

Reverse-Phase High-Performance Liquid Chromatography Analysis

The collected reaction fluid was extracted with chloroform (1:1 [v/v]) in a separation funnel. The fractionated organic fraction was then evaporated to dryness in a rotary evaporator and kept overnight in a vacuum desiccator. The dried samples were redissolved in 1 mL of tetrahydrofuran (THF), filtered in a 0.45-µm GHP syringe filter (Acrodisc; Gelman), and stored at –70°C in nitrogen gas until use. High-performance liquid chromatography (HPLC) analysis was conducted on a Hewlett-Packard HPLC (HP1100 series) equipped with an UV detector and an evaporative light-scattering detectors (ELSD) connected in series, using a Luna C18 column

(25 cm × 4.60 mm id, 5 µm; Phenomenex). The UV detector was set at 210 nm. The ELSD was set to 38°C and 2 atm with gain at 7/8. Elution was performed using a gradient system consisting of solvent A (acetonitrile), solvent B (1 m*M* trifluoroacetic acid solution), and solvent C (THF) in the following program (% [v/v]): initially 50A:25B:25C; linear gradient over 30 min to 60A:20B:20C; linear gradient over 5 min to 60A:5B:35C, then held isocratically for a further 15 min. The flow rate was maintained at 0.85 mL/min. All solvents were of far-UV-quality HPLC-grade purity.

Gas Chromatography/Mass Spectrometry Analysis

The dried samples after chloroform extraction were silylated in 50 µL of dioxane with 50 µL of bis-(trimethylsilyl)-trifluoroacetamide for 30 min at 60°C. Trimethylsilylated (TMS) derivatives were separated using a 0.25 mm × 30 m HP5 Phe Me Silicone column on a Hewlett-Packard 5972 series gas chromatograph with He as the carrier gas and detected with a Hewlett-Packard 5972 mass selective detector. The column was ramped at 10°C/min from 150 to 300°C and held for 20 min. The injector and detector were set at 300°C. Gas chromatography/mass spectrometry (GC/MS) analysis of the raw fabric extracts were performed under identical conditions as just mentioned after overnight extraction of the fabrics with dichloromethane (DCM) in a Soxhlet apparatus.

Results

The ability of cutinase to improve the wettability characteristics of cotton fiber was assessed by measuring the change in weight loss and wetting time as a function of enzyme concentration and incubation time (Fig. 1). Enzyme concentration was tested in the range of 0–34 U/mL after an incubation period of 20 h (Fig. 1A), whereas incubation time was tested in the range of 1–20 h on incubation with 12.8 U/mL (Fig. 1B). In both cases, the decrease in wetting time displayed a decreasing saturation pattern reaching an asymptotic value of approx 30 s, whereas the increase in fabric weight loss displayed a linear dependency. Note that the loss in weight of the control treatment without enzyme (axis origin in graphs) represented almost 70% of the total weight lost, indicating removal of adhered impurities from the raw fabrics.

The changes in the fabric surface were accompanied by the appearance in the reaction fluid of specific hydrolysis products of the cutin structure. The most distinctive products found in the residual reaction fluid on cutinase hydrolysis (19 U/mL for 23 h) were identified by GC/MS analysis as TMS derivatives of saturated fatty acid chains of 16 and 18 carbons, which corresponded to the two main DCM-extractable constituents of raw cutin, hexadecanoic (retention time [RT] = 9.7 min) and octadecanoic acids (RT = 11.6 min). For hexadecanoic acid, the main ion peaks at *m/z* 73, 117, and 313 in the mass spectrum corresponded to the loss of the TMS, OTMS-CO, and CH_3 fragments, respectively, confirming the mol weight of the TMS derivative of hexadecanoic acid to be 328 (256 for the free acid).

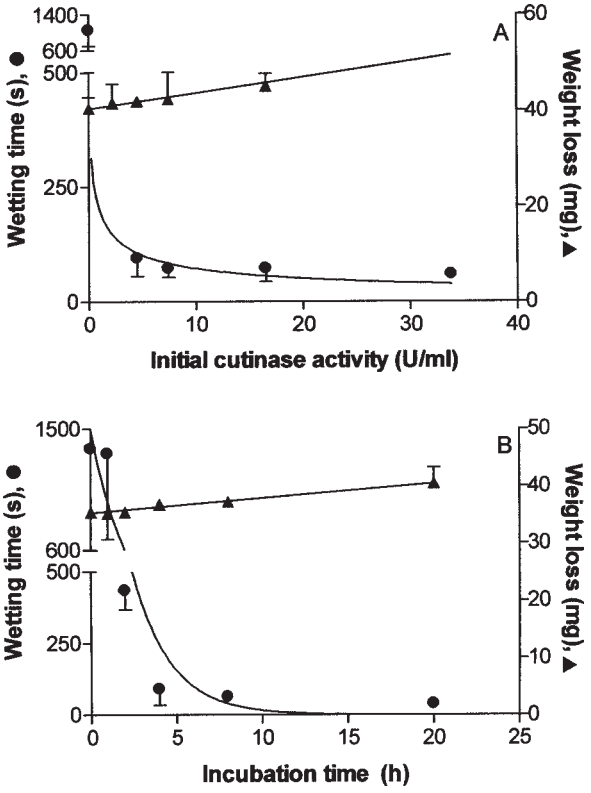

Fig. 1. Changes in weight loss and wetting time during incubation of cotton fabrics with cutinase. **(A)** Effect of initial enzyme activity. Incubation time was 20 h. **(B)** Effect of incubation time. Initial cutinase activity was 12.8 U/mL. Incubation conditions were 1 g of fabric swatches, 10-mL reaction mixture containing 100 m*M* phosphate buffer (pH 8.0) and 0.1% AvcoPal-AWS nonionic detergent, 37°C. Control treatments (axis origin) were incubated at the same conditions but without enzyme. Values represent the average of three independent replicates. Bars indicate SD.

Similarly, for octadecanoic acid, the ion peaks at *m/z* 73, 117, and 341 corresponded to the loss of the TMS, OTMS-CO, and CH_3 fragments, respectively, confirming the mol weight of the TMS-derivative to be 356 (284 for the free acid). The mass spectra of both acids were compared with the corresponding mass spectra of authentic standards and were found to be identical. The intensity of the respective peaks increased with the severity of the treatment within the range of time and concentrations tested. The major constituents released from the cotton fabric as a result of the enzymatic attack were also detected by ELSD-reverse-phase (RP) HPLC (see Fig. 2). The extent of degradation of the fiber cuticle increased respectively with the severity of the treatment, as suggested by the increase in the major peaks corresponding to hexadecanoic and octadecanoic acids, as further identified by GC/MS on analysis of isolated fractions.

The scouring efficiency of cutinase relied on the addition of detergents to the reaction mixture (Fig. 2). The effect of the polyoxyethylene, nonionic

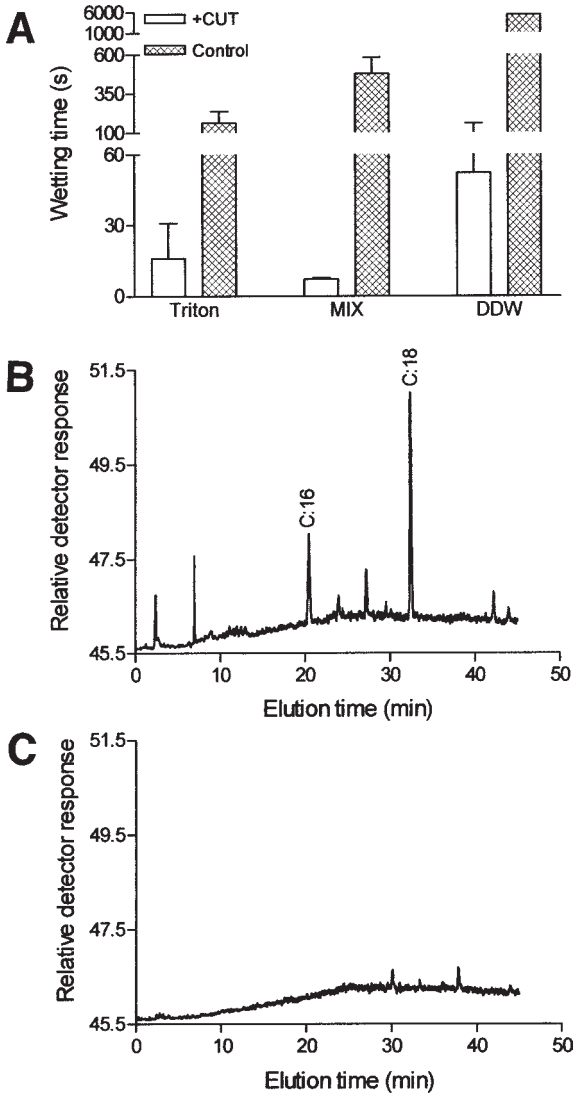

Fig. 2. Influence of detergents on scouring efficiency of cutinase. **(A)** Effect on wetting time. Triton, 0.1% Triton X-100; MIX, mixture of AvcoPal-AWS nonionic and AvcoBlank-AW anionic detergents (0.05% each); DDW, without detergent. Control indicates incubation with the respective detergent at the same conditions but without enzyme. Values represent the average of three independent replicates. Bars indicate SD. **(B)** ELSD-RP HPLC analysis of a chloroform extract of residual reaction fluid after incubation with Avco-Pal-AWS nonionic detergent. **(C)** ELSD-RP HPLC analysis of a chloroform extract of residual reaction fluid after incubation without detergent. Samples were incubated with 19 U/mL of cutinase for 23 h.

detergent Triton X-100 was compared with the effect of a commercial mixture of nonionic (AvcoPal-AWS; AvcoChem) and anionic (blend of mono and dialkyl potassium phosphate esters; AvcoBlank-AW, AvcoChem) detergents used in the textile industry. The results clearly indicated the

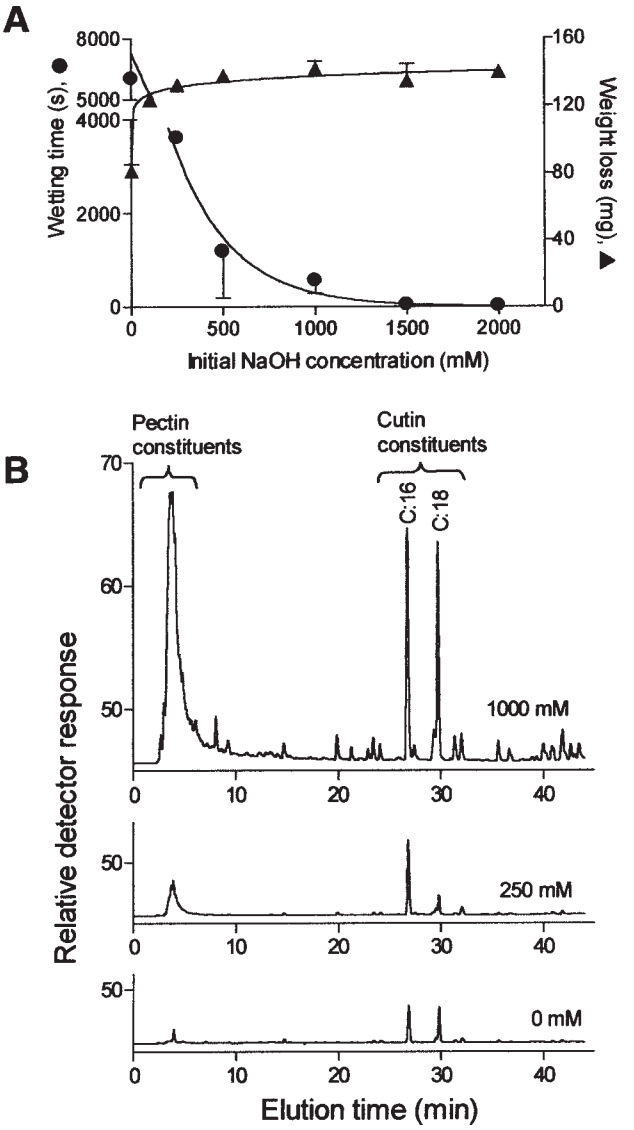

Fig. 3. Chemical scouring of cotton fabrics with NaOH. **(A)** Effect on weight loss and wetting time. Values represent the average of three independent replicates. Bars indicate SD. **(B)** ELSD-RP HPLC analysis of a chloroform extract of residual reaction fluid of treatments without (control) or with 250 and 1000 m*M* NaOH. Reactions were carried out for 30 min at 100°C in an NaOH solution at the indicated concentration. Control treatments (axis origin) were done with boiling DDW for 30 min.

enhanced effect of cutinase, approx a 70-fold increase, in the presence of the detergents (Fig. 2A). The optimal concentration was close to 0.1% (w/v). The increase in wettability corresponded to a parallel appearance in the reaction fluid of the hydrolysis products of the cutin layer, C:16 and C:18, detected by ELSD-RP HPLC and further identified by GC/MS on analysis of isolated fractions (Fig. 2B).

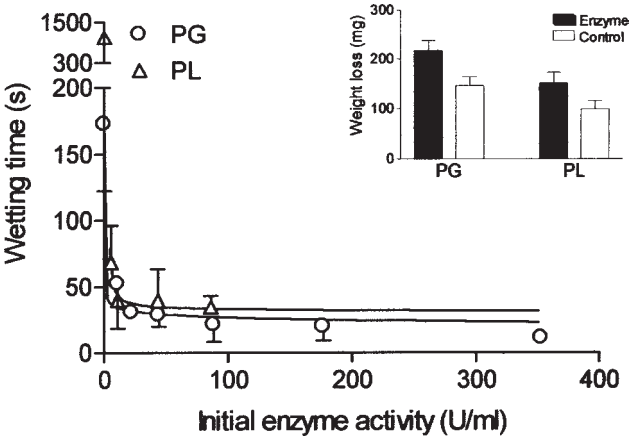

Fig. 4. Enzymatic scouring of cotton fabrics with polygalacturonase (PG) and pectin lyase (PL). Inset shows weight loss after enzymatic treatment with 85 and 350 U/mL of PL and PG, respectively. Values represent the average of three independent replicates. Bars indicate SD. Incubations were carried out at 37°C: 20 h and pH 8.0 (100 m*M* phosphate buffer) for PL or 16 h and pH 5.0 (50 m*M* acetate buffer) for PG. All other reaction conditions were as indicated in Fig. 1. Controls of the respective enzymatic treatments (axis origin) were incubated at the same conditions but without enzyme.

The effect of the enzymatic treatment was compared with that of chemical scouring with boiling NaOH in the concentration range of 100–2000 m*M* for 30 min (Fig. 3). The efficiency of the alkaline hydrolysis measured as a decrease in wetting time, which displayed an exponential pattern, increased with the severity of the treatment, reaching a saturation value between 1 and 1.5 *M* NaOH (Fig. 3A). The conventional industrial treatment is carried out at 250 m*M* NaOH. The change in weight loss was very close to saturation at the initial NaOH concentration of 100 m*M*. Again, note that control treatment with boiling DDW (axis origin in graph), caused a weight loss of approx 60%. The changes in the fabric surface were accompanied by the appearance in the reaction fluid of specific hydrolysis products of both the pectin and waxy layers, suggesting complete hydrolysis of the fiber cuticle (Fig. 3B). As depicted by the ELSD-RP HPLC chromatograms, the intensity of the peaks representing the pectin constituents (3–8 min) and wax constituents (26 and 30 min), quantitatively corresponded to the increase in wettability.

Enzymatic scouring was also evaluated with polygalacturonase and pectin lyase, within a range of concentrations of 10–350 and 5–85 U/mL, respectively (Fig. 4). Although incubation with polygalacturonase was more effective, resulting in an asymptotic wetting time close to 10 s vs approx 35 s for pectin lyase, both enzymes showed comparable efficiency with that of cutinase. A similar change was also observed for the weight loss along the treatment (see inset in Fig. 4). Removal of pectin was evidenced by a concomitant increase in color on staining with Congo red, and a decrease in color on staining with Ruthenium red. The changes in the

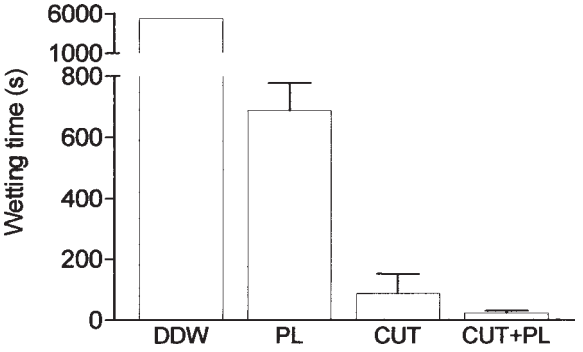

Fig. 5. Effect on wetting time after scouring of cotton fabrics with a mixture of pectin lyase and cutinase. DDW, control without enzymes; PL, pectin lyase alone; CUT, cutinase alone; CUT+PL, mixture of both enzymes. Incubations were carried out for 24 h at 37°C and pH 8.0 (100 m*M* phosphate buffer) with 200 U/mL of PL and 80 U/mL of CUT, in the absence of detergent. Values represent the average of three independent replicates. Bars indicate SD.

fabric surface paralleled the appearance in the reaction fluid of hydrolysis products of the pectin layer, as detected by the ELSD-RP HPLC analysis (data not shown).

Finally, the effect of a mixture of pectin lyase and cutinase on the cuticle hydrolysis, permissible because of their equivalent optimum pH of activity, was studied without detergent in the reaction mixture (Fig. 5). Incubated alone, cutinase resulted in a somewhat more efficient decrease in wetting time, perhaps owing to its better accessibility to the waxy layer in the absence of detergent, compared to pectin lyase. When incubated together, a synergistic effect between the two enzymes was evident, which led to a fourfold increase in water absorbancy (compared to treatment with cutinase alone). The amount of the corresponding degradation products released to the reaction fluid was again in accordance with the increase in wettability observed (data not shown).

Discussion

The present study characterized the capability of cutinase to bioscour natural textile fabrics. Although no pretreatment with boiling water was practiced in the present study, wetting times of 20–30 s were achieved by incubating raw cotton fibers with cutinase alone, which were comparable with those reported for other lytic enzymes *(2,3,6)*. Although lipases alone were reported, in most cases, to be less effective for bioscouring than other enzymes *(8,15,23)*, our findings suggest a high accessibility of the outermost waxy layer of the cuticle structure by cutinase. The increase in water absorbancy, with increasing cutinase concentration and incubation time and presence of nonionic detergent, is in line with previous reports for enzymatic scouring *(2,6,8–13,17)*. The addition of detergents may reduce

surface tension and facilitate the close contact of cutinase with the water-repellant cuticle. In contrast to anionic and cationic detergents, nonionic detergents, containing uncharged, hydrophilic head groups, interact less with proteins and, therefore, are considered nondenaturant *(24)*.

Pectin lyase and polygalacturonase, as well, led to an acceptable water absorbancy of the treated fabrics. Although cutinase hydrolyzes the ester linkages of cutin, whereas polygalacturonase and pectin lyase split the glycosidic bonds of the pectin chain, the final effects on scouring were comparable. The increase in wettability, with the intensity of specific enzymatic hydrolysis of cuticle components, resulted in weight loss and in the appearance of the respective hydrolysis products in the reaction fluid. Similar results were observed by the alkaline chemical scouring. The main hydrolysis products of cutinase found in the reaction fluid on extraction with chloroform consisted of C:16 and C:18 fatty acid chains, whose concentration increased with the severity of the treatment. n-C:16 and n-C:18 hydroxyfatty acids, with hydroxyl groups in positions 1 and 3, were identified as the major apple and lime cutin monomers *(25,26)*.

When applied in a mixture, cutinase and pectin lyase, which have an equivalent pH optimum (8.0), displayed a synergistic effect, achieving a wetting time of 5 s. This behavior complies with the best results reported for the combination of cellulase and pectinase or pectinase with lipase or proteases *(2,6,9,10,13)*. The addition of lipase to pectinase treatment was reported to enhance water absorbancy approx fourfold and was more efficient than the addition of cellulase or xylanase *(6)*. The combined treatment with pectinase and lipase also resulted in a minimal decrease in the fiber strength. By contrast, cellulases, which efficiently improve wettability even when applied alone, caused a decrease in fiber strength and, hence, diminished fabric quality *(2)*.

Based on scanning electron microscopy studies, Hardin et al. *(2)* reported that plant cell wall hydrolytic enzymes penetrate the cuticle in aqueous solutions through micropores and make contact with their substrates. Hartzell and Hsieh *(6)* and Krebs Lange *(23)* found a higher water contact angle for pectinase than for cellulase-treated cotton fabrics, which suggests that pectinase cannot adequately access the pectin layer in the primary cell wall of cotton. By contrast, cellulase is able to gain access to the cellulose, and in due process removes the noncellulosic components residing on the fabric's surface. Furthermore, the synergistic effect between the cellulase and pectinase, when applied in a mixture, suggests that some, if not all, pectins are located close to the secondary cell wall. The synergistic effect between cutinase and pectin lyase found in our work is in support of this hypothesis, suggesting a succession of processes. It appears that the digestion of the cuticle by cutinase results, first, in the enlargement of existing micropores in addition to formation of more apertures. This effect may subsequently enable a rapid penetration of pectinase to the inner pectineous layer, bringing about an enhanced fabric scouring.

Enzymatic scouring of cotton has several benefits in comparison to the conventional alkaline treatment at 100°C. These include low working temperatures (30–40°C); less quantity of water and energy involved; easy care of the wastewater generated, compared to the special handling required by the strong alkaline effluents; and the ability to scour only cotton in blends containing other components such as wool or polyester *(9)*. Our results presented a potential new biotechnology for improving water absorbancy of natural fibers in textile processing, based on the use of cutinase. The constituents of the fiber cuticle found in the reaction medium suggest that a specific degradation of the cutin layer, with minimal alteration of fiber strength, occurred. Nevertheless, much about enzymatic scouring is yet to be explored.

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