Novel microbial-mediated modifications of wool

A. Catarina Queiroga, M. Manuela Pintado, F. Xavier Malcata *

Escola Superior de Biotecnologia, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal

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Abstract

A total of 158 microbial strains, previously isolated from raw wool samples of Portuguese *Merino* breed of sheep, were screened for extracellular protease activity. The 12 isolates with the highest overall activity were further tested via incubation in nutrient broth, and assaying of cell-free supernatants using casein as substrate protein. The cell-free supernatants of the three isolates exhibiting the best performance were finally tested on knitted wool using bursting strength and area shrinkage as quantitative parameters, and microstructure using scanning electron microscopy as qualitative parameter, to conclude on their putative role upon the fiber features. The aforementioned three isolates produced lower weight loss and area shrinkage than those brought about by a commercial reference enzyme under similar operating conditions, without significantly loosing bursting strength.

Introduction

Most biotechnological tools currently in use for textile processing are still inspired on approaches that have been applied for more than 2000 years [3]; in fact, few innovations have actually been meanwhile developed that feature mainly enzymes. Enzymes can indeed be applied to virtually all manufacturing steps in the textile industry, from fibre and fabric processing, to laundry detergents and effluent treatment. The most widely employed enzymes are hydrolases (e.g. cellulases and amylases) and oxidoreductases (e.g. laccases, peroxidases and catalases).

Recall that enzymes catalyze chemical reactions, and typically possess a great specificity and lead to major rate enhancements; this realisation provides paramount opportunities for industrial applications thereof, aimed at more efficient and economic conversions [1]. Furthermore, advances in biotechnology at large, and particularly in such areas as protein and genetic engineering, have made available enzymes especially tailored for specific applications [4]. So far, research on enzyme applications in textile processing has aimed at a balance between beneficial effects (mainly on texturization) and potential mechanical losses (mainly of strength) [3]. Furthermore, additional contributions to sustainability encompassing energy and raw material consumption, waste production and stability/safety of product [6], have also played a role as driving forces for said research efforts.

Wool is a complex proteinaceous matrix,which exhibits several unique properties among the natural fiber world. The surface scales of that fiber account for the distinctive felting and shrinking properties of wool upon wetting. Since consumers have placed increasingly higher demands on machine washability and sustained soft handle touch, the market value of wool has been steadily decreasing. In attempts to regain its once leading position within the European textile and clothing industry, innovation is urged as a basis for competitiveness, via alternatives both in quality of product and sophistication of process. Applications of enzymes to wool may thus contain the potential to bring about a greater added value; since wool fibres consist mainly of proteins and lipids, proteases and lipases will likely account for a major opportunity in processing of that fibre.

At present, applications of the aforementioned enzymes in wool processing are rather limited, not only because of technological difficulties in handling and control, but also due to poor knowledge of these enzymes when acting on such a substrate fibre. Note, in particular, that proteases are degradative enzymes that catalyze hydrolysis of proteins [5]; they have been employed for over 80 years in industrial treatment of wool, in attempts to impart such desirable properties as better handle features and higher shrink-resistance [2]. However, current

* Corresponding author. Tel.: +351 225580004; fax: +351 225090351.
E-mail addresses: acqueiroga@mail.esb.ucp.pt (A.C. Queiroga), mepintado@esb.ucp.pt (M.M. Pintado), fxmalcata@esb.ucp.pt (F.X. Malcata).

enzymatic processes are difficult to implement and control on the industrial level, given that such preset specifications as penetration of enzyme into the fiber can hardly be controlled, thus causing excessive damage to the fiber cuticle, with consequent high degrees of weight and strength losses [2]. For this reason, application of proteases in wool processing remains a challenging task; there are a number of commercial proteases available, but their performance has not yet been found fully satisfactory in inducing low shrinkage at low levels of strength loss.

The goal of this research effort was thus to screen for (wild) bacterial sources of extracellular proteases, that are able to act specifically on wool. To said purpose, a total of 158 strains previously isolated from raw wool samples of the Portuguese *Merino* breed of sheep, were screened for protease activity, via the spot technique on agar plates; the 12 best performers were then subject to refined characterization of their enzymatic activities. Their potential applicability in wool finishing was finally demonstrated for the very best in terms of such parameters as bursting strength and area shrinkage, as well as surface micromorphology.

Materials and methods

Microrganism sources

Raw wool samples were collected from three distinct parts (viz. head, flank and rear) of Portuguese *Merino* sheep. Solutions of each sample (10 g wool + 95 mL sterile water) were homogenised for 15 min at 260 rpm, using a Stomacher® Lab Blender (Seward, UK), and cultured in quadruplicate on Plate Count Agar (Merck, Germany) and *Bacillus cereus* Medium Agar (LabM, UK), at 37 and 50 °C, for eventual isolation of strains, which were tentatively pinpointed as those colonies bearing distinct morphological characteristics. After purification via sequential steps of plating on Nutrient agar plates and culturing on Nutrient broth, strains were kept at −80 °C with glycerol (30%, w/v) until further use.

Wool source

Knitted, 100% *Merino* wool fabrics (with an average fiber diameter of 19.5 μm) were kindly supplied by Orfama, Organização Fabril de Malhas (Portugal). Pieces of 12 cm × 12 cm were cut therefrom and duly cast off, and a square of 10 cm × 10 cm was marked on the fabrics using a pen. All fabrics were pre-treated with 2.5% (v/v) chlorine, in order to modify the scale surface, and thus make the fibres more susceptible to enzyme attack [8].

Protein content assay

Throughout the period of protease activity monitoring (as described below in detail), aliquots were withdrawn for total protein determination (by absorbance at 660 nm), using the Total Protein Kit, Micro-Lowry, with modifications by Onishi and Barr (Sigma–Aldrich, USA).

Proteolytic activity assays

Qualitative assays

All strains isolated from *Merino* wool samples (as described above) were screened for protease activity, via the spot technique, on Calcium Caseinate Agar (Merck) containing 1% (w/v) skim milk (Oxoid, UK) [7]; results were recorded every day for a 5 day-period.

The isolates exhibiting the highest protease activity, detected as largest clearance diameter (at least 5 mm by 1 day, and at least 20 mm by 5 days), were selected for further (refined) estimation of enzymatic activity.

Qualitative assays

Upon isolation as described above, the selected isolates were further cultured in Nutrient Broth E (Lab M, UK), for alternative estimation of protease activity, using casein as substrate. The activity of each strain was evaluated for 5 days, via monitoring the proteolytic activity of cell-free supernatants (sterilized by filtration through a 0.45 μm-filter) using colorimetric determination (at 660 nm, with the Folin–Ciocalteu's reagent) of the extent of casein breakdown, according to the instructions of the supplier (Sigma–Aldrich, USA). One unit (U) of proteolytic activity was defined as the amount of enzyme able to hydrolyze casein, so as to produce an absorbance equivalent to that produced by 1.0 μmol of tyrosine per min, at pH 7.5 at 37 °C.

Wool finishing assays

Enzyme source

The neutral alkaline protease for textiles, Protex Multiplus L™, from *Bacillus lentus*, was kindly supplied by Genencor International (Rochester, NY, USA). Before enzymatic treatment of wool fabrics was in order, its activity in solutions of 0.75 and 1 g/L (in 10 mM sodium carbonate buffer, pH 9) was determined, using also casein as substrate.

Those strains exhibiting proteolytic activities on casein larger than that exhibited by the higher concentration of the commercial protease used as reference were selected for supplementary assaying directly on wool fabrics. Hence, the culture supernatants of those strains were filtered through 0.45 μm sterile membranes, and applied as (crude) enzyme solutions on knitted wool fabrics (as described below).

Protease processing

Each pre-treated fabric was subject (in triplicate) to processing by eight different enzyme combinations, for two reaction times (15 and 30 min), and plain air-dried (for treatments with odd numbers) or tumble-dryied (for treatments with even numbers) after home laundering, as depicted in Table 1; a total of 32 independent experiments were thus carried out. In addition, the fabrics were also subject to two control treatments, using only 10 mM phosphate buffer (pH 7) or 10 mM sodium carbonate buffer (pH 9) for the same two reaction times, and two drying methods described above, thus totalling eight control experiments. All those 40 different treatments were performed at 60 °C, using a Washtester WT (Werner Mathis, Germany), at a weight ratio of ca. 1:20. Quenching of the enzyme was via rapidly raising the temperature to 75 °C, and washing for 5 min. The fabrics were afterwards cooled, drained and rinsed.

Qualitative assays

The bursting strength and shrinkage extent of enzyme-treated wool fabrics were monitored during three subsequent household wash and dry cycles, using a model WFL 1300 washing machine (Bosch, Germany); the program for wool (35 min wash, at 30 °C) was used, with one measure of Woolite® fabric wash, and a total wash load of 1 kg (dry mass). After each wash cycle, the fabrics were air- or tumble-dried using a WTL 4310 tumble-drying machine (Bosch), with the program F (60 °C) for 50 min.

Bursting strength was assessed on 7.3 cm^2 pieces of wool fabrics, after handling (washing and drying) as described above, according to ISO 13938-2 except in number of replicates (which was only three instead of five, owing to lack of sample size), using a *Tru-Burst 610*.

Shrinkage extent was calculated based on the original size (length and width) of the fabric, prior to enzyme treatment [9]. Measurements in each direction were made on the specimens after handling (washing and drying) as described above, and the average dimensional change (% DC) was calculated via $\%\ DC = (A - B)/B \times 100$, where A and B denote the area of fabric after and before, respectively, the three subsequent household wash and dry cycles [10].

Qualitativeassays

The putative fiber damage caused by the experimental enzymatic treatment was ascertained via scanning electron microscopy. Toward this purpose, 2.5 cm × 2.5 cm pieces were cut off from the 12 cm × 12 cm knitted wool fabrics, glued to aluminium specimen studs (with double-sided tape), and coated with a thin layer of gold in a sputter coater (for 60 s, at 2 kV and 22 mA). Imag-

Table 1
Experimental design encompassing treatment conditions

Processing conditions	Experimental runs																				
	1–2	3–4	5–6	7–8	9–10	11–12	13–14	15–16	17–18	19–20	21–22	23–24	25–26	27–28	29–30	31–32	33–34	35–36	37–38	39–40
Buffer at pH	7	7	9	9																
Protex Multiplus L (g/L)					0.75	0.75	1	1												
Crude extract of isolate 102 at pH									7.9	7.9	9	9								
Crude extract of isolate 151 at pH													7.7	7.7	9	9				
Crude extract of isolate 152 at pH																	7.7	7.7	9	9
Reaction time (min)	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30

ing was obtained in a JSM-5600LV scanning electron microscope (Jeol, Japan), operating at 15 kV.

Statistical analyses

All results shown (unless otherwise stated) are the arithmetic means of at least three specimens of each sample, with the corresponding standard deviations. The means of enzyme-treated samples were compared with the corresponding controls, using one-way ANOVA at a significance level of 5%, with the supplementary multiple comparison tests LSD and Tukey-HSD, to identify which pairs of means were statistically different at that significance level.

Results and discussion

Bacterial enzymes, employed by the textile industry for some time, include amylases that can act at boiling temperatures, and proteases that can stand the alkaline range; both are obtained from extremophiles, and account for ca. 70% of the overall market in our area of interest. Enzymes from non-extremophiles are not used so often; hence, enzymes from wild strains that populate natural environments (as is actual wool on the living animal) are in principle interesting, and were thus considered.

The 158 bacterial isolates, screened for protease activity, produced the results tabulated in Table 2. Of the isolates tested, 125 out of 158 (i.e. 79.1%) exhibited some degree of protease activity on agar plates, as apparent by the presence of a clear zone surrounding the corresponding colonies. Furthermore, 58 out of 158 (i.e. 36.7%) showed a considerable degree of protease activity, as indicated by a diameter of the clearance zone around the colony greater than 20 mm by 5 days. Only 12 out of 158 isolates (i.e. 7.6%) exhibited halos greater than 5 mm by 1 day, and greater than 20 mm by 5 days.

The latter 12 isolates were further monitored for production of protease, using 5 days of incubation, as plotted in Fig. 1; solutions of 0.75 and 1.0 g/L of the commercial reference protease exhibited 36.4 and 54.5 U/mL, respectively. To quantitatively assay for protease activity of those isolates, the substrate chosen was casein, because it is considered as a standard, commercially available substrate that is suitable for accurate analysis and wide

Fig. 1. Quantitative estimation of protease activity by the best performant 12 isolates from Portuguese raw *Merino* wool samples (white bars) and by commercial protease (grey bars), on casein by 5 days of incubation (average ± S.D.).

Table 2
Semi-quantitative estimation of protease activity by isolates from Portuguese raw *Merino* wool samples, on caseinate agar plates incubated at two temperatures for two periods

Isolates tested (number)	Isolates within each clearance diameter range (number)							
	No proteolysis		≥5 mm halo (1 day)		<20 mm halo (5 days)		≥20 mm halo (5 days)	
	37 °C	50 °C	37 °C	50 °C	37 °C	50 °C	37 °C	50 °C
158	32	1	22	22	38	29	41	17

comparison. By 1 day of growth, all strains exhibited an activity of less than 36.4 U/mL, which is the activity equivalent to the minimum recommended by the supplier for application of the commercial protease; a special mention is deserved by strains 9 and 102. By 5 days of growth, 6 out of the 12 isolates (i.e. 33, 102, 123A, 140, 151 and 152) exhibited protease activity above 36.4 U/mL. On the other hand, 3 out of those 6 isolates (i.e. 102, 151 and 152) actually showed proteolytic activities greater than 54.5 U/mL. Furthermore, those three isolates yielded the greatest specific activities (4.4, 2.6 and 2.1 U/μg of protein). Hence, only isolates 102, 151 and 152 were selected for preliminary studies of action upon wool fabrics, including weight loss. This parameter provides information about the activity over the wool fibre, but not about where the enzyme acts in/on the fibre; therefore, data on the burst strength were generated as well.

Statistical analyses of our results unfolded statistically significant differences between the levels of area shrinkage among the samples, and between their levels of bursting strength. However, one-way ANOVA does not allow one to conclude which treatment(s) entail(s) bursting strengths and area shrinkages significantly different from the others; a multiple comparison test is further required in this particular, as was accordingly applied.

Data on dimensional changes, specifically on percent area shrinkage, are plotted in Fig. 2. The data pertaining to surface area measurements following wash-drying cycles suggest that shrinkage was most reduced by enzymatic treatment for 30 min, with the enzyme solution from isolate 102 (pH 9), followed by air-drying (see assay 23), with an area shrinkage of only 3.95 ± 0.27%. However, Tukey-HSD test indicated that the result from assay 23 did not statistically differ from those from

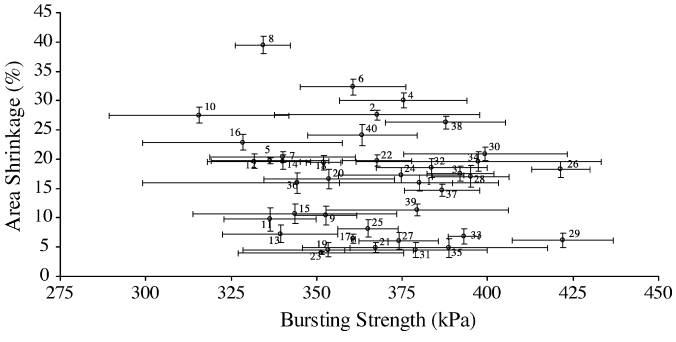

Fig. 2. Area shrinkage (average ± S.D.) and bursting strength (average ± S.D.) for each treatment condition combination of enzyme, pH, reaction time and drying method, after three wash cycles, of samples of knitted wool fabrics (as detailed in Table 1). The samples treated with buffer solutions (at pH 7 or 9, assays 1–8) instead of enzyme, whether commercial or newly isolated, were used as controls for this experiment.

assays 13, 17, 19, 21, 25, 29, 33 and 35, all encompassing treatments with novel enzymes except assay 13 that encompasses use of 1 g/L of commercial protease, for 15 min (p-value of 0.097).

Inspection of Fig. 2 further indicates that tumble-drying causes unacceptable dimensional changes on wool fabrics, even when proteases were employed at values ranging from 15.95 to 39.52%. For all types of treatment, one finds that the percent area shrinkage was smaller when samples were treated for longer reaction times (30 instead of 15 min), except when plain buffer solutions (pH 7 or pH 9) were considered (assays 1–9, Fig. 2). Treatment of knitted wool with plain buffer solutions produced in fact the greatest percent area shrinkage, thus indicating that presence of said solutions could not protect the fabric against shrinkage as much as enzymatic treatments did. Air dried samples treated with enzyme from isolate 102 exhibited smaller shrinkages at pH 9 (assays 21 and 23) than at pH 7.94 (assays 17 and 19); however, both treatments originated area shrinkages below 7%, which is acceptable for an "easy care"-labelled wool product.

On the other hand, samples treated with enzyme from isolate 151 led to an area shrinkage, viz. 7.68%, larger than at pH 9 (assays 29 and 31), which were found to be unacceptable dimensional changes for a commercial woolen product. Another conclusion that can be withdrawn from Fig. 2 is that enzymes from isolates 102, 151 and 152 protected the samples from shrinkage better than the commercial enzyme tested did. All enzymatic treatments that caused a dimensional change below 8% (viz. assays 25 < 31 < 19 < 35 < 21 < 27 < 29 < 17 < 33) are not statistically different from each other, but are significantly lower than those produced by treatment with commercial enzyme except again with regard to run 13. Considering only the air dried samples, all treatments with enzymes from new isolates presented a great potential for anti-shrinkage of wool fabrics, as indicated by the associated values of area shrinkage below said 8%.

Data on bursting strength are also presented in Fig. 2. There were no significant changes, as given by Tukey-HSD test, in the bursting strengths of wool samples subject to the same enzymatic treatment, but to different drying methods. Hence, tumble-drying significantly promotes dimensional changes on wool samples, yet the drying method plays no significant role on bursting strength of said samples. When samples were treated for longer reaction times (30 instead of 15 min), no significant differences could be pinpointed in bursting strength; this observation might indicate that the enzyme acts only on the surface (as made apparent by the smaller percent area shrinkage), hence not damaging the fibre itself.

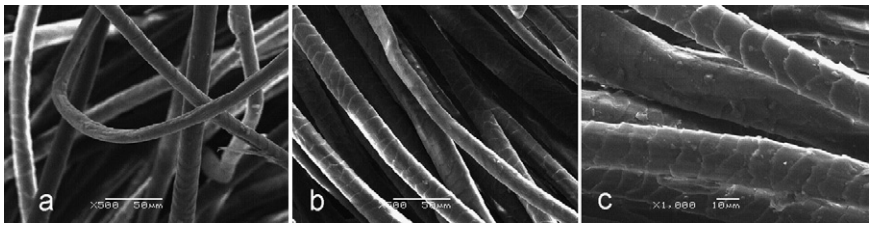

Fig. 3. Scanning electron micrographs of samples of knitted wool fabrics, treated with: (a) commercial protease (1 g/L) for 30 min and air dried, after pre-treatment with 2.5% (v/v) chlorine; (b) crude enzyme solution from isolate 151 for 15 min, at pH 9, after pre-treatment with 2.5% (v/v) chlorine; (c) none, after pre-treatment with 2.5% (v/v) chlorine.

Analysis of the results obtained following enzymatic treatments leading to dimensional changes below 8%, one finds somewhat unexpected results in terms of bursting strength: the smaller the percent area shrinkage, the smaller the bursting strength. Globally, the treatment leading to the highest bursting strength was assay 29 (422 ± 15 kPa), which entails the use of enzyme from isolate 151, at pH 9, for 15 min and subject to air-drying; however, the bursting strengths obtained in experimental runs 21, 27, 31 and 35 are not significantly different from that obtained in assay 29 (according to a Tukey-HSD test, which yielded a p-value of 0.117).

A good enzyme for wool finishing should induce low dimensional shrinkage and low strength loss (or, equivalently high bursting strength), i.e. one should focus on the lower right corner in Fig. 2. Since all those enzymatic treatments produced no significantly different dimensional changes, one may then choose that yielding the highest bursting strength coupled with economic and environment considerations. The choice would then be of process 29, which encompasses use of enzyme from isolate 151, at pH 9, for 15 min and air-drying, even tough isolate 102 yielded the highest protease activity. The rationale underlying this choice is a major issue, if industrial applications are sought.

Selected micrographs, obtained via scanning electron microscopy, are displayed in Fig. 3. No significant differences could be pinpointed, in terms of surface appearance, between all enzyme-treated and control samples, except regarding samples treated with commercial protease at 1 g/L for 30 min (assay 15), which caused fibres to break (as apparent in Fig. 3a). One can also conclude that treatment with enzyme from isolate 151 did not cause perceptible fibre damage, even though it greatly reduced the scales on the surface relative to the control sample, which encompassed only chlorine pre-treatment (see Fig. 3b and c).

Conclusions

Use of our novel microbial proteases in handling of clothes does not lead to increased damage of wool fabric, as compared to conventional industrial conditions, i.e. plain chlorination treatment; this realisation entails bursting strength, area shrinkage and microstructural parameters. However, statistically significant decreases in area shrinkage after laundering were observed, without significant losses of bursting strength. Although the best anti-shrinkage performance is obtained with the enzyme from isolate 151, at pH 9, for 15 min and subject to air-drying, employment of similar treatment conditions for smaller periods of time is a viable alternative, since it leads to a very good bursting strength with no significant difference in area shrinkage.

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