

Detergent formulations for wool domestic washings containing immobilized enzymes

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Abstract The stability of immobilized and native Esperase, a commercial serine protease, was studied by incubating the enzymes in four formulations containing the same amount of anionic and non-ionic surfactants. The results show that the activity of the immobilized enzyme is not affected by the presence of detergents while the native enzyme lost 50% of activity after 20 min of incubation in these four formulations. The washing performance of the detergents prepared with the immobilized Esperase was studied on cotton and wool fabric samples stained with human blood and egg yolk, using as control the detergent containing native Esperase. The best stain removal for cotton samples stained with human blood was achieved using the detergent with immobilized Esperase. Several physical tests confirmed that wool keratin was not degraded by the immobilized Esperase, validating the ability to use formulated detergents containing this immobilized enzyme for safe wool domestic washing.

Keywords Detergents · Eudragit · Immobilization · Serine proteases · Stain removal · Wool

Introduction

Proteases constitute the most important group of industrial enzymes and their major application is the detergent industry. Biological detergents are commonly used in domestic laundry because the enzymes they contain provide the added benefit of low temperature washes with improved cleaning performance. Addition of proteases to detergents considerably increases the cleaning effect by removing protein stains such as blood and egg, and increases the consumption of surface-active substances thereby decreasing the pollution load (Moreira et al. 2002; Grebeshova 1999). However, in addition to their ability to remove protein stains, proteases can also hydrolyse natural protein fibres such as wool keratins and silk causing severe and irreversible damage to the garments (Cortez et al. 2005; Shen et al. 1999). For this reason, the immobilization of proteases in soluble matrices could be a way of controlling the enzyme hydrolysis process (Heine and Höcker 1995) and overcome common problems of heterogeneous reaction systems like the poor contact between the insoluble substrate (wool) and the immobilized

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enzyme as well as the incomplete separation of the immobilized enzyme from unreacted solid substrates (Fujimura et al. 1987; Taniguchi et al. 1989).

Available important proteases in commercial detergents are Subtilisin Carlsberg, Subtilisin BPN, Alcalase, Esperase and Savinase. All these enzymes are stable in the presence of various components of detergents and are active at normal washing temperatures and pH (Moreira et al. 2002; Gupta et al. 1999). Proteases should also be stable in the presence of oxidizing agents, surfactants, bleachers and other additives that might be present in the detergent formulation (Moreira et al. 2002; Greene et al. 1996).

Incorporation of enzymes into detergent formulations can lead to several difficulties. Proteases are susceptible to autolytic degradation, oxidation and denaturation. These processes are often enhanced by surfactants, bleachers and water-softening builders which must be included in any laundry detergent (Stoner et al. 2004; Crutzen and Douglas 1999). Proteases also catalyse the degradation of other additives present in the formulation. In powder formulation this problem can be resolved by isolating the enzyme in separate particles. In liquid formulations the physical isolation of enzymes is more difficult and the presence of water amplifies the detrimental effect of surfactants and enhances the rate of undesirable reactions (Stoner et al. 2004; Lalonde et al. 1995). To overcome this problem, chemical additives are added to the formulations. The combination of carboxylic acid salts and calcium chloride can protect against protease degradation (Stoner et al. 2004; Crossin 1989). Boron compounds (boric acid, borate salts) in conjunction with polyols (propylene glycol, glycerol) also have been shown to stabilize enzymes in liquid detergent formulations (Stoner et al. 2004).

In the present work, the stability and washing performance of different detergent formulations containing enzymes was studied. A commercial protease, Esperase, was added to the formulations, either in the native or immobilized form. This immobilized enzyme can be used in the liquid state covalently linked to a soluble–insoluble polymer.

Material and methods

Enzyme, reagents and textile materials

The enzyme used in this study was the alkaline serine protease Esperase, a commercial subtilisin (E.C.3.4.21.62) purchased from Sigma. Eudragit S-100 (MW 135,000 composed by 1:2 copolymer of methacrylic acid and methyl methacrylate) a commercial product from Rhöm Pharma was a generous gift from Degussa-Hüls, S.A., Barcelona. Carbodiimide hydrochloride (EDC) and ethanolamine were purchased from Sigma (St. Louis, USA). The surfactants used, Lutensit A-LBN 50 and Lutensol ON 70, anionic and non-ionic respectively, were provided by BASF. All other chemicals used were of analytical grade.

The textile materials used were 100% wool fabric and scoured 100% white cotton fabric. Wool was pre-treated with Lutensol ON 30 (non-ionic surfactant) 1 g/l in a bath ratio 1:20, at pH 9.0 (Na_2CO_3 0.1 M and NaHCO_3 0.1 M buffer), for 30 min, at 40°C, on Rota-wash. After the washing procedure, the surfactant was removed from the fabric first with tap water, followed by distilled water. Thereafter, wool was immersed in a bath (same bath ratio) with 1% H_2O_2 (equivalent to 0.52 ml of peroxide 34% (1.131 g/ml) per 20 g wool), at pH 9.0 (Na_2CO_3 0.1 M and NaHCO_3 0.1 M buffer), for 1 h at 55°C, on Rota-wash machine.

Enzyme assay and gel electrophoresis

The activity of proteases was measured according to Silva et al. (2004) using casein as substrate. One unit of activity is defined as the amount of enzyme that hydrolyses casein to produce equivalent colour to 1 μmol of tyrosine, per minute, at pH 7.5 and 37°C.

SDS-PAGE was carried out using the Hoefer miniVe system from Amersham Pharmacia Biotech. The resolving gels (10% acrylamide of about 1.5 mm thickness) were run at a constant voltage (120 V) and prepared according to the method described by Laemmli (1970). Proteins were visualized by silver staining using Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic Anhydrase (30 kDa), Trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa) for calibration.

Immobilization and operational stability

The protease was covalently linked to Eudragit S-100 by the carbodiimide coupling by following a protocol described in Silva et al. (2006). For the determination of stability the native and immobilized enzymes were placed in a water bath at 40 or 60°C and 100 rpm of stirring and the remaining activity was measured after 3 h of incubation at this temperature.

Detergent formulation

The detergents formulations were prepared according to Table 1. The blended surfactants comprised 34% of the total formulation weight. The surfactants were then dissolved in 0.3 M Tris–HCl, pH 7.6, corresponding to 45% of the total formulation weight. The 21% remaining formulation space available is for the other additives as shown in Table 1. The experiments were done with 30 U of native and immobilized esperase, which were added prior to the experiments.

The stability of the native and immobilized enzyme in the presence of detergents was determined by incubating the enzyme (30 U) with a 0.5% solution of the different detergent formulations solutions, in a water bath at 40°C and 80 rpm of stirring for 3 h.

Washing tests

The performance of native and immobilized protease as a detergent additive was studied with wool and white cotton fabrics ($4 \times 4 \text{ cm}^2$) stained with human blood and egg. The stains on the samples were made by placing the same amount

of blood and egg yolk (100 μl) on the surface of the sample. The samples were then left at room temperature for 6 h in order to dry the stains. The stained samples were then washed with a 0.5% solution of detergent, detergent and 30 U of immobilized enzyme and detergent and 30 U of native enzyme, for 30 min in a water bath at 40°C and 80 rpm of stirring.

Tensile strength resistance and dimensional stability

Wool fibres were subjected to tensile strength tests according to the ASTM method D50035-s90. Wool fabric shrinkage was determined after one 7A and five 5A wash cycles according to Woolmark method TM31 in the Electrolux-Wascator machine.

Colour measurements

The colour of the dyed fabrics was evaluated using a reflectance measuring Datacolour apparatus at standard illuminant D65 (LAV/Spec. Incl., d/8, D65/10°). Data were evaluated according to the Kubelka–Munk relationship K/S where K is an adsorption coefficient and S is a scattering coefficient. Three areas on each sample were measured in various positions, and the results represent average values with up to 1% variation.

Weight loss

Wool fabrics were conditioned at 100°C for 2 h, desiccated and weighted until constant weight (considered as differences between successive weights inferior to 1 mg). Assays were performed in duplicate.

Table 1 Composition of the detergent formulations used in this study

Component	Formulation 1	Formulation 2	Formulation 3	Formulation 4
Lutensit A-LBN50 (g)	10.5	10.5	10.5	10.5
Lutensol ON70 (g)	6.5	6.5	6.5	6.5
0.3 M Tris–HCl, pH 7.6 (ml)	22.5	22.5	22.5	22.5
0.5 M Sucrose (ml)	0	10.5	0	0
0.01 M EDTA (ml)	0	0	1.5	0
0.1% CaCl_2 (g)	0	0	0	0.5
3% Borax (g)	0	0	0	2.0
5% Sorbitol (g)	0	0	0	3.5
H_2O (ml)	10.5	0	9.0	4.5

SEM pictures

Scanning electron microscopy (SEM) pictures were obtained in the model Leica S360 with a backscattered and secondary electron detector.

Results and discussion

The major drawback of wool domestic washing is the irreversible damage caused by the hydrolytic degradation of wool keratin by proteases. In this work wool degradation by native and immobilized enzymes was compared under severe conditions. After treatment of 72 h with 100 U of both enzymes, at 40°C, with 90 rpm of stirring wool fibres were completely degraded (Figs. 1 and 2).

Wool samples treated with immobilized enzyme retained 76% of the initial tensile strength while those treated with native enzyme only retained 37%. These results are in accordance with data previously reported (Silva et al. in press). Wool samples treated with immobilized Esperase achieved around 8% of shrinkage whereas the samples treated with native enzyme showed approximately 7% of shrinkage. Immobilized Esperase is unable to penetrate the fibre; it only removes partially the cuticle from the fibre surface which is responsible for the shrinkage effect (Heine and Höcker 1995; Feughelman 1997). On the other hand, the native enzyme can penetrate into the fibre, which lead to the total degradation

and therefore to a drastic decrease of the tensile strength.

These results were confirmed with SEM pictures (Fig. 2) where the degradation of wool fibres caused by the native enzyme can be clearly seen (Fig. 2b). The results of Table 2 related to the weight loss of samples are also in accordance with the previous results. It was observed that the samples treated with native enzyme showed a higher weight loss than the samples treated with immobilized Esperase.

The immobilized enzyme retained all of its activity after 3 h of incubation at 40°C, both in solution without the detergent and in the detergent formulations (Table 3). The native enzyme lost 50% of its activity after approximately 20 min of incubation, when present in the detergent formulations. The control (native enzyme alone) lost 50% of activity only after 131 min of incubation. For the immobilized enzyme there was no difference between the four detergent formulations. The same was true for the native enzyme, although, for the formulation 4 (F4) the half-life time was slightly higher (around 30 min). This could mean that the additives used in this formulation increased the stability of the native Esperase in the presence of detergents.

Previous studies demonstrated a direct relationship between measured protease activity within liquid detergent formulations and the observed protease degradation rate (Stoner et al. 2004; Lalonde et al. 1995). These authors concluded that autolysis was the primary mechanism of protease activity loss over time. To establish if this was the case in our detergent formulations, a detergent sample containing native and immobilized enzyme (30 U) was incubated at 40°C for 3 h in a water bath with 80 rpm of stirring. The samples were then analysed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

As shown in Fig. 3, the bands corresponding to the immobilized enzymes are diffuse and appear in the beginning of the resolving gel, confirming their high molecular weight. Since no bands of smaller molecular weight appeared in the gel, we can assume that there was no autolysis of these samples.

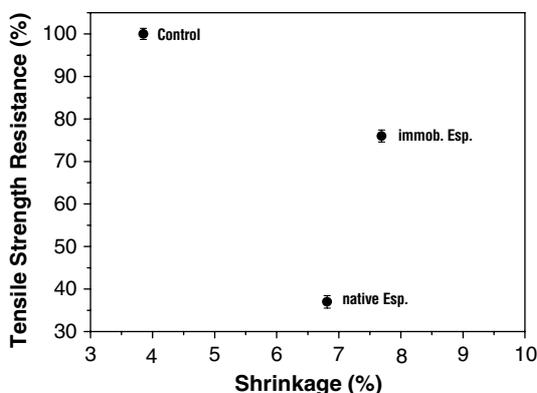


Fig. 1 Tensile strength resistance (%) and shrinkage (%) obtained for the wool fabrics treated with immobilized and native enzyme and without enzymatic treatment (control)

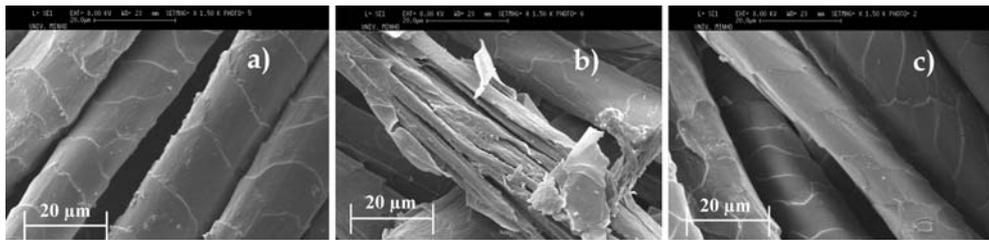


Fig. 2 SEM pictures of wool samples (original magnification: $\times 1500$). All samples were pre-treated with surfactant (a) and afterwards treated with native (b) and immobilized

(c) Esperase for 72 h with 100 U of enzyme, at 40°C and 90 rpm of stirring

Table 2 Weight loss (%) of wool samples treated with native and immobilized Esperase

Enzyme	Weight loss (%)
Control (no enzyme)	1.40 \pm 0.05
Native Esperase	17.2 \pm 0.5
Immobilized Esperase	4.4 \pm 0.5

Table 3 Half-life times $t_{1/2}$ (h) for the native Esperase in the presence of detergents. The immobilized Esperase did not loose any activity was lost during 3 h incubation

Control	2.2 \pm 0.30
F1 + Enzyme	0.36 \pm 0.08
F2 + Enzyme	0.34 \pm 0.09
F3 + Enzyme	0.32 \pm 0.09
F4 + Enzyme	0.40 \pm 0.10

These results correlate with the half-life time results from Table 3. The same result was obtained for the native enzyme (data not shown), which is not in accordance with the results from Table 3. Therefore, the activity loss observed for the native enzyme in the presence of detergents is not due to autolysis mechanisms but might be due to enzyme denaturation with temperature and also due to the presence of detergents.

Previous results indicate that the optimum temperature for both native and immobilized Esperase was around 70°C (Silva et al. in press). A normal domestic washing temperature is 60°C for cotton and 40°C for wool. As evident from the half-life times listed in Table 4 the immobilized enzyme would easily withstand a common washing cycle at these temperatures without significant loss of activity. Both the native and immobilized enzymes were more stable at 40°C (Table 4).

To determine the efficacy of the enzyme for use as a detergent additive, wash performance analysis of cotton and wool samples was carried out with human blood and egg at 40°C for 30 min. For the blood stained cotton sample it was observed that the best removal of stain was with the detergent formulation 2 (F2) in the presence of immobilized enzyme (Fig. 4). This formulation contains sucrose, a protein stabilizer, which protects proteins against oxidation, aggregation and damage during lyophilization (Stoner et al. 2004; DePaz et al. 2000; Krishnan et al. 2002; Kendrick et al. 1998; Kreilgaard et al. 1998). For the cotton sample stained with egg the best removal was obtained with the detergent formulation 3 (F3) which contains EDTA in the presence of immobilized enzyme (Fig. 4). Stability of the enzyme in presence of EDTA is essential since an enzyme to be used as a detergent additive should not require a metal cofactor. Detergents contain high amounts of chelating to remove divalent cations responsible for water hardness and enhancing stain removal (Oberoi et al. 2001). Interestingly, blood stains were removed better than eggs stains from cotton fabric although blood penetrates into the fabric more easily. However, the higher protein content of blood might explain these results.

For the wool samples stained with human blood the best stain removal was obtained when the immobilized enzyme was combined with formulation 2 (F2) which contains sucrose (Fig. 5). For the wool samples stained with egg yolk the best removal was achieved in the washing done with detergent formulation 1 (F1) in the presence of immobilized enzyme (Fig. 5). Like with cotton, human blood stains were removed more easily than egg yolk. Nevertheless, both stained wool

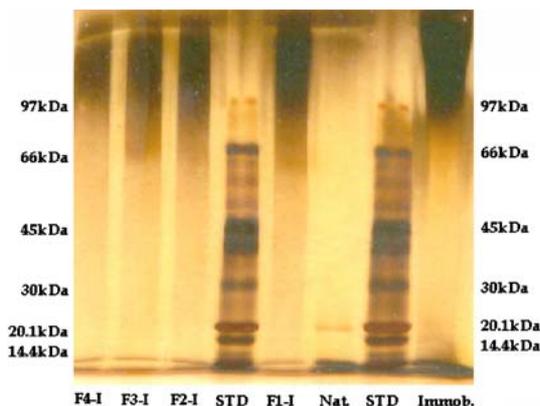


Fig. 3 SDS-PAGE of the immobilized enzyme after the incubation with different detergent solutions for 3 h in water bath at 40°C and 80 rpm of stirring

Table 4 Half-life times $t_{1/2}$ (h) for the native and immobilized Esperase at 40 and 60°C

Temperature (°C)	Native	Immobilized
40	2.2 ± 0.30	14.6 ± 4.9
60	0.58 ± 0.04	6.9 ± 0.9

samples show worse results than the cotton samples. Thus, there was no additional stain removal effect due to limited surface hydrolysis of the protein fibres.

Conclusions

The use of proteases as additives in detergents is a common practice. However, the domestic washing

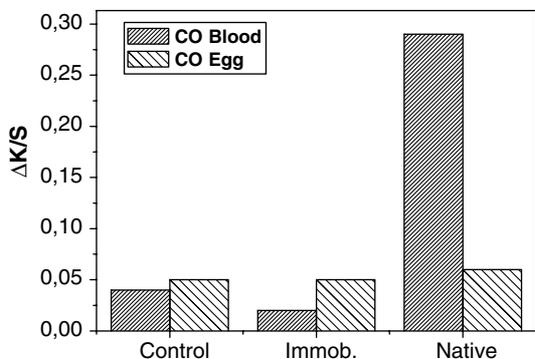


Fig. 4 $\Delta K/S$ values of cotton samples stained with human blood (CO Blood) and egg yolk (CO Egg) after washing, respectively, with detergent formulations F2 and F3 in the presence of immobilized and native enzyme

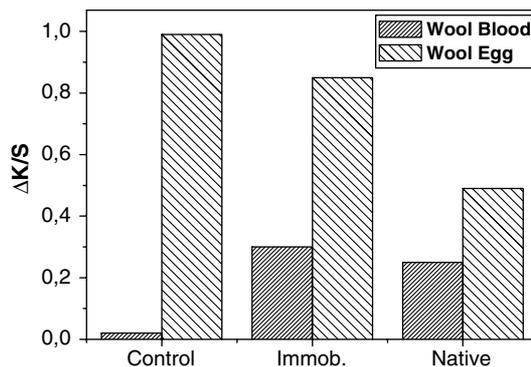


Fig. 5 $\Delta K/S$ values of wool samples stained with human blood (Wool Blood) and egg yolk (Wool Egg) after washing, respectively, with detergent formulations F2 and F1 in the presence of immobilized and native enzyme

of wool or silk goods with these detergents is not safe due to the irreversible damages caused by partial enzymatic hydrolysis of these protein fibres.

In this study, we demonstrated that immobilization of proteases leads to acceptable degrees of weight loss, tensile strength loss and shrinkage of wool fabrics treated under extreme conditions (72 h). On the other hand, the fabric treated with native enzyme showed severe damages on the wool fibres which was confirmed by the SEM pictures. Therefore, the immobilized protease can be considered to be used as additive in formulations of wool domestic detergents. It was also necessary to verify the efficacy of immobilized Esperase on the removal of proteic stains. Thus, wool and cotton fabrics stained with human blood and egg yolk were subjected to simulated washing with several detergents formulations containing immobilized and native enzyme. The immobilized Esperase was more stable in the presence of detergents than the native enzyme. The activity of the immobilized enzyme remained constant after the incubation while the native Esperase lost 50% of activity after approximately 30 min of incubation with detergents at 40°C. It was observed that the best removal was obtained on cotton samples stained with human blood. On wool samples the washing results were worst due to the hydrophobic character of wool fibres.

The stability of immobilized Esperase in the presence of detergents at 40°C and the ability to remove stains, demonstrate that this enzyme can

be used as a detergent additive to improve their washing performance.

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