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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

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Restricting detergent protease action to surface of protein fibres by chemical modification

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Abstract Due to their excellent properties, such as thermostability, activity over a broad range of pH and efficient stain removal, proteases from *Bacillus* sp. are commonly used in the textile industry including industrial processes and laundry and represent one of the most important groups of enzymes. However, due to the action of proteases, severe damage on natural protein fibres such as silk and wool result after washing with detergents containing proteases. To include the benefits of proteases in a wool fibre friendly detergent formulation, the soluble polymer polyethylene glycol (PEG) was covalently attached to a protease from *Bacillus licheniformis*. In contrast to activation of PEG with cyanuric chloride (50%) activation with 1,1′-carbonyldiimidazole (CDI) lead to activity recovery above 90%. With these modified enzymes, hydrolytic attack on wool fibres could be successfully prevented up to 95% compared to the native enzymes. Colour difference (ΔE) measured in the three dimensional colour space showed good stain removal properties for the modified enzymes. Furthermore, half-life of the modified enzymes in buffers and commercial detergents solutions was nearly twice as high as those of the non-modified enzymes with values of up to 63 min. Out of the different modified proteases especially the *B. licheniformis* protease with the 2.0-kDa polymer attached both retained stain removal properties and did not hydrolyse/damage wool fibres.

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Introduction

Highly efficient stain removal at low temperatures and low water consumption are the main ecological benefits of enzymes in detergent formulations. Enhancing the action of surfactants and improving the performance for shorter cycle times increased the applications of enzymes in the textile industry since the late sixties (Galante and Formantici 2003). Among the different enzymes used in detergency such as amylases, lipases, cellulases and mannanases, proteases are one of the most important groups of industrial enzymes. Removing a variety of stains based on proteins including egg, milk, sauces, grass, blood, proteases are present in nearly all kinds of laundry and automatic dishwashing detergents (Maurer 2004; Egmont 2005).

Alkaline proteases from *Bacillus licheniformis* are one of the first representatives for detergent proteases, used for 40 years and are still of industrial relevance. Bacterial proteases are mostly easily produced, thermo stable and active over a broad range of pH. Due to these excellent properties and the successful genetic engineering approaches in the 1980s, subtilisins from *Bacillus* sp., wild types and recombinant strains, provide all the proteases used in the detergent industry nowadays (Maurer 2004).

A great challenge for proteolytic detergent formulations is the stain removal on natural fibres such as wool and silk. In contrast to other proteases, detergent proteases act on a wide range of proteins (different stains). This low substrate specificity is also responsible for their main disadvantage,

causing severe damage on protein fibres. For obvious reasons, hydrolysis of wool fibres by proteases resulted in high strength and weight losses (Heine and Höcker 1995; Shen et al. 1999). Subtilisins usually used in detergents have a size of approximately 27 kDa (Maurer 2004) and are able to penetrate into the fibre easily and destroy the cortex involving a reduced tensile strength. Therefore, a restriction of the enzyme activity onto the wool surface and diffusion control is required. It has been previously shown that increasing the molecular weight by attaching synthetic polymers onto the enzyme could restrain the action range of protease to the outer part of wool fibres providing an anti-shrinking effect (Schroeder et al. 2004).

Chemical modification of proteins with polyethylene glycol (PEG) is widely used due to a plenitude of useful properties: very low toxicity, excellent solubility in aqueous and organic media and extremely low immunogenic behaviour (Dosio et al. 2001). Major applications of PEG conjugates are the stabilisation of protein in aqueous media leading to increased half-life (Besson et al. 1995; Yang et al. 1996; Kodera et al. 1998), improvement of solubility and stability in organic solvent (Hernáiz et al. 1997; Kwon et al. 1999; Koops et al. 1999) and improvement of characteristics of therapeutic proteins (Veronese 2001; Roberts et al. 2002)

In the following study, the potential of PEG-modified proteases with different molecular weights as an additive for wool detergent is demonstrated for the first time. Although we have previously reported on the chemical modification of a protease from *Bacillus lentus* for wool anti-shrinking treatment (Schroeder et al. 2004), this enzyme was not suitable for stain removal. Thus, proteases from *B. licheniformis* known for a wide substrate range were modified by attaching the enzyme covalently to methoxy polyethylene glycol to design a proteolytic enzyme for stain removal from wool fabrics which is not damaging for wool fibres. For this purpose, two different modification strategies were compared.

Materials and methods

Materials

The alkaline protease from *B. licheniformis* and *B. lentus* were supplied by Novozymes (Bagsvaerd, Denmark) and Genencor (Leiden, The Netherlands), respectively. The protease was purified by gel filtration, using a Superdex 200 HiLoad 16/60 column (Amersham Pharmacia, Uppsala, Sweden), detection at 280 nm and a phosphate buffer (0.1 M, pH 7.0) as eluent.

Wool fibres (approximately 23 μm in diameter) and yarns of wool fibres were supplied by Drummond Parkland of England. These yarns have been used for the construction of knitted fabric using a Stoll CMS 330 knitting machine (right–right knitting [1×1] with two yarns; number of needles/in.: seven; stitch: 9.5). Yellow coloured woollen carpet was kindly donated by James (the Netherlands). All other chemicals and solvents were of analytical grade.

Synthesis of the *B. licheniformis* protease conjugates

In brief, 0.8 mmol of methoxypolyethylene glycol [molecular weight (M.W.) 5,000] from Fluka was dissolved in 100 ml of dried toluene and activated with 2.4 mmol cyanuric chloride (CC) from Sigma in the presence of 60 mmol sodium carbonate anhydrous and 6.0 g molecular sieves A4. After stirring for 40 h at 40°C, the mixture was centrifuged to discard the precipitate and precipitated in 300 ml of petroleum ether. The reaction was monitored by thin-layer chromatography (TLC) (silica gel, eluent: methanol/chloroform 1:4) (Abuchowski et al. 1977).

Furthermore, 0.8 mmol of methoxypolyethylene glycol (M.W. 5,000) was dissolved in 100 ml of dried toluene and activated with 0.9 mmol 1,1′-carbonyldiimidazole (CDI) from Sigma in the presence of 6.0 g molecular sieves A4 for 20 h at room temperature. After the desired reaction time, the mixture was centrifuged to discard the precipitate and precipitated in 300 ml of *n*-hexane. The activation was monitored by TLC (silica gel, eluent: methanol/chloroform 1:6) (Beauchamp 1983; Tondelli 1985). Activations of PEG 1,100 and PEG 2,000 (Fluka) were similarly prepared using the same molar ratio.

B. licheniformis protease solution, 2 ml (1 mg ml^{-1} protein), was mixed in 100 ml sodium borate buffer (0.1 M; pH 9.3) containing 3.0 g of 2-*O*-methoxypolyethylene glycol-4,6-dichloro-*s*-triazine and methoxypolyethylene glycol imidazolyl carbonyl, respectively. The reaction mixture was shaken at room temperature for 2 and 8 h, respectively. After the desired reaction time, the unbound polymer was removed by gel filtration, using a Superdex 200 HiLoad 16/60 column (Amersham Pharmacia, Uppsala, Sweden), and detection at 280 nm using a phosphate buffer (0.1 M, pH 7.0) as eluent. Modification with PEG 1,100 and PEG 2,000 was similarly prepared using the same molar ratio.

Determination of molecular weight

Analytical gel filtration was performed on a Superdex 75 HR 10/30 column (Amersham Pharmacia, Uppsala, Sweden), detection at 280 nm and using a phosphate buffer (0.1 M, pH 7.0) as eluent. Carbonic anhydrase from bovine erythrocytes (M.W. 29,000), albumin, bovine serum (M.W. 66,000), alcohol dehydrogenase from yeast (M.W. 150,000), β-amylase from sweat potato (M.W. 200,000) and apoferin from horse spleen (M.W. 443,000), all obtained from Sigma, were used for calibration.

Protease activity

The proteolytic activity of the native enzyme and conjugates was determined using azocasein as substrate. Of the sample, 150 μl was mixed with 250 μl of a 2% azocasein solution in phosphate buffer (25 mM, pH 7.5). After an incubation time of 30 min at 37°C, the reaction was stopped by adding 1.2 ml 10% trichloroacetic acid and

then centrifuged (4 min, 3,000×*g*). Of the supernatant, 600 μl was added to 700 μl of a 1 M NaOH solution and mixed. The absorbance at 440 nm was monitored using a Kontron UVIKON 940 spectrometer. One unit of protease activity is defined as the amount of enzyme required to produce an absorbance change of 1.0 in a 1-cm cuvette within a minute. Protein concentration was determined according to Bradford 1976 using bovine serum albumin as standard.

Stability of protease from *B. licheniformis* and conjugates

After different incubation times at 50°C, the proteolytic activity of the different protease conjugates was assayed to determine the effects of PEG modification on the thermal stability of the enzyme. The compatibilities of the native protease from *B. licheniformis* and its PEG conjugates were tested by incubating in a common detergent formulation for laundry and a commercial preparation for stain removal on woollen carpets, respectively. Therefore, an aqueous solution of 5 mg ml^{-1} detergent was prepared and the stain removal agent was diluted with a 1:4 ratio with water.

Staining of wool carpet and knitted fabrics

Chocolate milk (10 ml, temperature 60°C) from a beverage machine was brought on a piece of yellow coloured carpet (12×6 cm) with the aid of a pipette. Knitted wool fabrics were prewashed in a household machine using wash program 7A and IEC standard detergent. The fabrics (15×15 cm) were stained with spaghetti paste sauce (60°C) using a spoon to create circular stains with a diameter of approximately 8 cm.

Stains on carpets and fabrics were air dried and aged for another 24 h at room temperature. The stained fabrics were cut prior to the cleaning procedure. One half was used for cleaning without enzyme, the other, for the cleaning in the presence of PEG conjugated enzyme.

Stain removal

Stained carpet pieces were first incubated in a Linitester (one piece of carpet/beaker) using 300 ml, 25 mM borate buffer pH 8.5 containing 5 mM $CaCl_2$ and in the presence and absence of PEG conjugated protease from *B. licheniformis* for 30 min at 40°C. Subsequently, the carpets were cleaned using a spray-extraction procedure and water containing 0.5 g l^{-1} tergitol 15-S-12 non-ionic surfactant after which, the carpet was air dried.

Stained knitted fabrics were washed in a Linitester for 30 min at 40°C using 300 ml standard detergent solution IEC (3 g l^{-1}) in the presence and absence of PEG-conjugated protease from *B. licheniformis* (0.6 mU against azocasein). After subsequent rinsing in tap water, the fabric was air dried and analysed.

Stain removal was determined by measuring light reflectance of the remaining stains after the cleaning procedure using an X-rite 968 spectrophotometer in combination with QA-Lite software. The impact of enzyme dosage on the cleaning efficiency was determined by calculating the ΔE value from the obtained L, a and b values from the stains cleaned in the presence and absence of the enzyme system (Harold 1987).

Determination of tensile strength

Tensile strength of yarns from washed fabrics has been measured according to ISO 2062 using a Hounsfield tensile tester.

Treatment of the wool fibres

The optimum incubation conditions to determine the enzymatic effect were determined by using an experimental design with four factors (agitation speed, component ratio, enzyme activity and incubation time) at four different levels, varying from 0 to 5.5 (agitation), from 0 to 4 (ratio), from 0 to 4 (activity) and from 0 to 5.5 (time) based on a Central Composite statistical design. The effects and interactions of the various factors studied and the combinations thereof which showed the best results for the weight loss were calculated using the Design Expert 6.0 computer software programme (StateEase, Minnesota, USA).

Wool fibres, pre-tempered for 10 min at 50°C, were incubated in sodium borate buffer (25 mM; pH 8.5) at 50°C at different ratio, enzyme concentration, incubation time and agitation level according to the experimental design. The enzyme in the incubation mixture was inactivated by raising the temperature to 70°C for a further 20 min without agitation.

After rinsing the sample three times with 30 ml of distilled water the fibres were air dried. The weight loss of enzyme-treated wool fabrics was elaborated by using an electronic balance after conditioning for 24 h in a desiccator.

Results

Synthesis of the *B. licheniformis* protease–PEG conjugates

PEG has to be "activated" prior to the coupling to proteins and two different strategies for PEG activation were compared in this study. The first used cyanuric chloride for PEG activation resulting in 2-*O*-methoxypolyethylene glycol-4,6-dichloro-*s*-triazine and in the second, 1,1′-carbonyldiimidazole was used resulting in an imidazole carbamate derivative.

For both activation strategies, protease activity in the fractions collected after gel filtration of the modified

proteases shifted towards shorter retention times confirming thereby an increased molecular weight compared to the native enzyme. Thus, the increase of molecular weight due to attachment of the PEG was successful. Furthermore, the absence of native enzyme in the gel filtration pattern after the modification reaction indicated a quantitative coupling of the enzyme to the activated PEG polymer. Whereas the 2-(*O*-methoxypolyethylene glycol)-4,6-dichloro-*s*-triazine of modification procedure led to serious deactivation of the *B. licheniformis* protease, most of the activity on azocasein was recovered using methoxypolyethylene glycol imidazolyl carbonyl (Table 1). The size of the PEG molecules used for the modification correlated with the apparent molecular size of the resulting PEG-modified proteases. The molecular weight of the protease could be doubled and even increased ten times using 1.1 and 5 kDa methoxypolyethylene glycol, respectively.

Stability of PEG modified proteases

The compatibilities of the native protease from *B. licheniformis* and its PEG conjugates with different commercial detergent formulations were tested by incubation in aqueous detergent solutions at 50°C. Azocasein was used as a model substrate for stain removal activity due to enzymatic hydrolysis. The half-life times of the 2.0 kDa and the 5.0 kDa protease conjugates from *B. licheniformis* in this detergent mixture were above 1 h while the remaining activity of the native enzyme after 1-h incubation was only 25% of its initial activity (Fig. 1).

Stain removal

Different enzymes and their conjugates from *B. licheniformis* and *B. lentus* were tested, respectively. In contrast to the PEG conjugate from *B. lentus* which was not suitable for stain removal (data not shown), the modified protease from *B. licheniformis* showed a significant contribution to the stain removal from woolen fabrics and carpet. Destaining of spaghetti stains from fabrics with 5 and 2 kDa modified proteases resulted in ΔE values of 9 and 15, respectively. Enzymatic destaining of chocolate milk stains from carpets had best results with the 2-kDa modified proteases and gave ΔE values 3. Thereby, a higher activity towards the chocolate stains was observed for the protease modified with 2-kDa PEG compared to the 5-kDa PEG (the dosage of 5-kDa PEG modified enzyme required to obtain comparable performance level is approximately twice that of 2-kDa PEG modified enzyme). No difference of the tensile strength of fibres from both, enzyme treated and non-enzyme treated fabrics, after the stain removal experiments could be detected.

Table 1 Synthesis of *B. licheniformis* protease conjugates with 2-*O*-methoxypolyethylene glycol-4,6-dichloro-*s*-triazine (CC) and methoxypolyethylene glycol imidazolyl carbonyl, respectively (CDI) (M.W. 5000) using different molar ratios of activated polymer to enzyme

Molar ratio	Activity recovery (%) using CC activated PEG	Activity recovery (%) using CDI activated PEG
32	75	95
108	70	90
216	50	90

The activity recovery indicates the difference of activity before and after the modification reaction

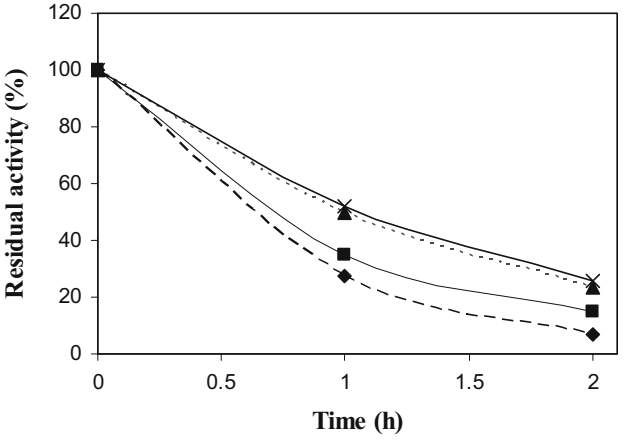

Fig. 1 Proteolytic activity of native and modified protease from *B. licheniformis* in the presence of detergent. The enzyme solutions were incubated at 50°C in a detergent formulation (5 mg ml^{-1}) at equal amounts of native enzyme (◆), conjugate with PEG 1.1 kDa (▪), 2.0 kDa (▲) and 5.0 kDa (×)

Enzymatic treatment of wool fibres

To allow comparison of the destructive effect of modified and native *B. licheniformis* protease towards protein fibres, optimum conditions for the hydrolysis of wool by this protease were determined (worst-case scenario). To determine these optimum incubation conditions, an experimental design with four factors (agitation speed, liquid ratio component, enzyme activity and incubation time) at four different levels, varying from 0 to 300 rpm (agitation), from 1:100 to 1:500 (ratio), from 0 to 20 U (activity), and from 0 to 330 min (time) based on a Central Composite statistical design was followed. The plot of the predicted vs the laboratory values is represented in Fig. 2a. The suggested linear model fits well for obtained values and the Model F-value of 15.00 implies that the model is significant. In this case component ratio, enzyme activity and incubation time are significant model terms, and no interaction between the different factors is present.

One might attribute from the model, two different effects for weight loss during the incubation, on one hand the mechanical abrasion and on the other the enzymatic hydrolysis. In Fig. 2b, the impact of mechanical force is illustrated by the agitation and ratio. Whereas increasing the input of energy (high agitation) caused an increased weight loss, high ratio showed a depressant effect of the water. The factors, incubation time and activity dosed, played a major role.

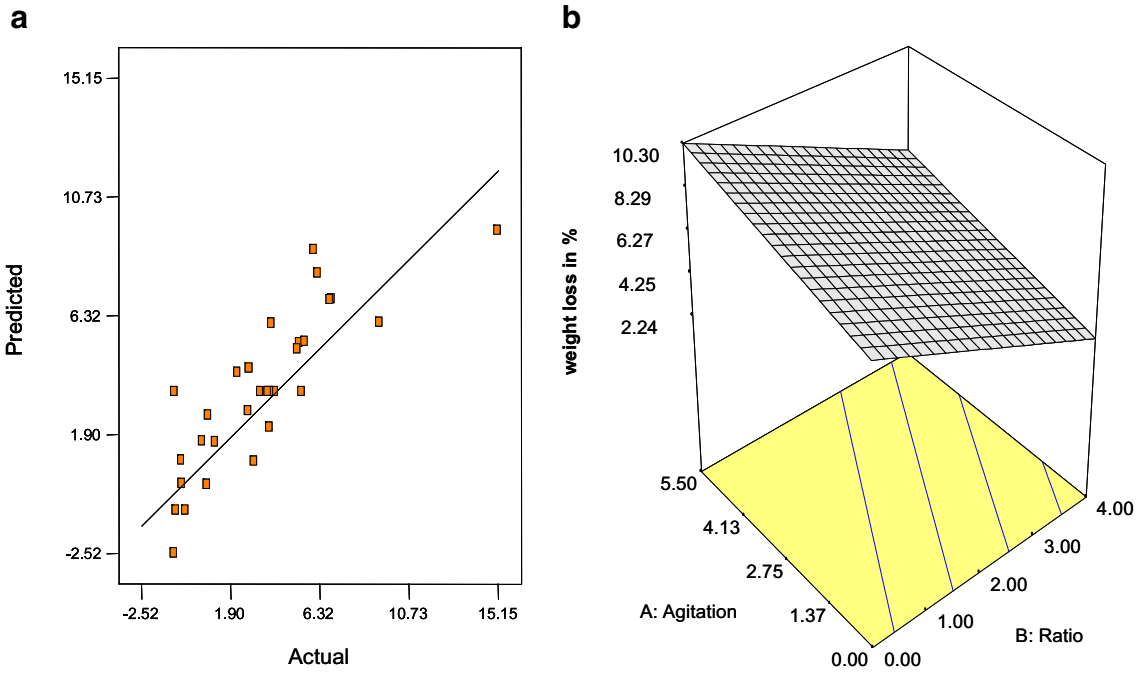

Fig. 2 Optimization of wool treatment with a protease from *B. licheniformis***a** Predicted vs actual data, **b** Two dimensional plot weight loss in percent vs agitation level and ratio (the values in the *x* and *y* axes are coded values)

At these optimum conditions for wool hydrolysis, fabrics were incubated with native and modified protease from *B. licheniformis* at the same level of activity against azocasein. Hydrolysis of wool with native enzyme resulted in severe damage of the fibres and high weight losses (16%) while covalent attachment of polyethylene glycol onto the enzyme resulted in protection of fibres against damage (Figs. 3 and 4).

Discussion

Two different strategies for the covalent attachment of PEG to a protease from *B. licheniformis* were compared to produce enzymes for protein stain removal from wool

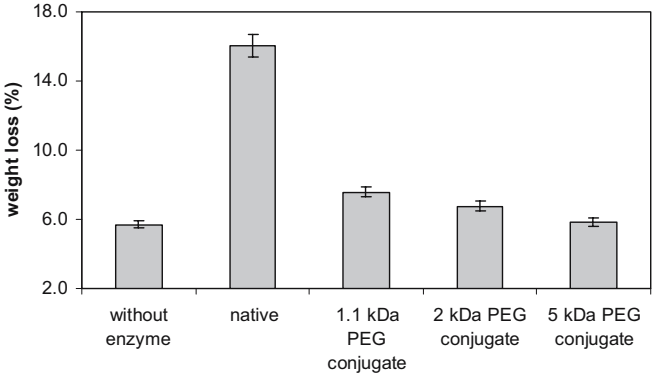

Fig. 3 Weight losses (percentage of its original weight) after treatment of wool fibres with protease from *Bacillus licheniformis* modified with different sized polyethylene glycol resulting in enzyme conjugates with different molecular weights

materials. First, the replacement of one chlorine atom of triazine with the terminal hydroxyl group of the PEG chain resulting in 2-(*O*-methoxypolyethylene glycol)-4,6-dichloro-*s*-triazine was studied. In general, due to the two remaining chlorine atoms, the activated polymer is enabled to react with the ε-amino group of lysine residues on the surface of the enzyme (Inada et al. 1995; Abuchowski et al. 1977).

The expected product in the second activation using 1,1′-carbonyldiimidazole is an imidazole carbamate derivative. This product was successfully purified by repeated precipitation in *n*-hexane instead of the dialysis described in the literature (Beauchamp 1983). In our study, only the second strategy leads to conjugates with high activity recovery of up to 95%.

The loss of enzyme activity after attachment to 2-*O*-methoxypolyethylene glycol-4,6-dichloro-*s*-triazine activation could be due to reaction of this compound with sulfhydryl groups of the enzyme. In contrast with CDI activation, a certain selectivity in the conjugation can be achieved due to a lower reaction rate of the acylating reaction which might explain the higher activity recovery found in this study (Veronese 2001; Yoshinga and Harris 1989). However, the modification strategy choice seems to depend strongly on the enzyme used. While a protease of *B. lentus* was successfully modified with 2-(*O*-methoxypolyethylene glycol)-4,6-dichloro-*s*-triazine and retained up to 80% of its initial activity (Schroeder et al. 2004), this was not the case for the protease of *B. licheniformis* investigated in this study.

A detergent enzyme should have good temperature stability and should be active over a longer period of time. Interestingly, after PEG modification, the stability of the *B.*

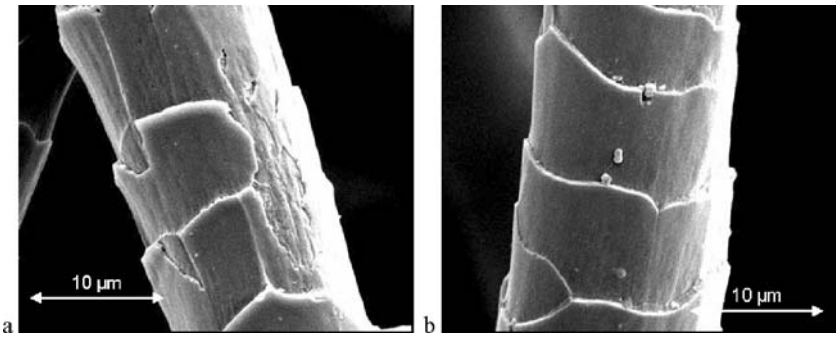

Fig. 4 SEM images after enzymatic treatment of wool fibres with native protease from *Bacillus licheniformis* [*left* (**a**)] and modified enzyme [*right*: (**b**)]

licheniformis protease conjugates increased with the molecular weight of the PEG chain attached onto the enzyme. The conjugates were generally more stable than the native unmodified enzyme when incubated in a detergent solution. Although only few is known in the literature about stabilities of PEG-modified enzymes in detergent formulations, enhanced stability (in buffer) of PEG-trypsin was reported previously by Gaertner and Puigserver (1992). These authors ascribed the stability increase to the formation of a highly hydrogen-bonded structure around the enzyme caused by the long PEG chains. A household machine washing program normally takes 60 to 90 min; therefore, the activity of the added enzyme should remain high over a substantial part of such period (Banik and Prakash 2004). The 2.0 kDa and the 5.0 kDa protease conjugates from *B. licheniformis* retained 50% of the initial proteolytic activity after 1 h, and hence, showed their suitability as potential additives in a detergent formulation. Stoner et al. 2004 reported recently that the loss of proteolytic activity in detergents is caused by autolysis. Protection towards auto-degradation can be achieved by chemical modification (Yang et al. 1996), whereas the impact increases with higher molecular weight of the attached polymer.

The application of the *B. licheniformis* protease conjugates as a detergent additive was studied using white wool fabrics stained with spaghetti sauce and yellow woollen carpet stained with chocolate milk, respectively. These protease sensitive stains were recommended by a carpet cleaner (James, Grubbenvorst, The Netherlands) specialising in wool fabrics and carpets. Stain removal was quantified with the ΔE value which represents the shift of the coordinates of the colour in the cylindrical colour space L^*, a^* and b^*, based on the theory that colour is perceived by black–white (L), red–green (a) and yellow–blue (b) sensations (Harold 1987). Based on the ΔE values, treatment with PEG modified protease from *B. licheniformis* showed a high potential for stain removal from both woolen fabrics and carpet. In contrast, we did not measure any destaining effect with PEG modified proteases from *B. lentus* although these enzymes were able to partially hydrolyse wool cuticles resulting in a wool anti-shrinking effect (Schroeder et al. 2004).

In general, PEG modification can restrict the access of the enzyme to substrates due to sterical hindrance or limitation of diffusion into fibrous materials. For this particular application in the destaining of protein materials, PEG modification should limit diffusion of the enzymes into wool fibres and prevent hydrolysis of these, while other macromolecular protein materials contained in stains should remain accessible to the enzyme. Thus, in the second phase, the effect of PEG modification of *B. licheniformis* protease on wool hydrolysis was studied in more detail.

Enzymatic hydrolysis of wool fibres is affected by different factors such as nature and ionic strength of the treatment buffer (Shen et al. 1999) and enzyme activity and incubation time (Stanescu et al. 2002). Furthermore, the process depends on adsorption and desorption of the enzymes which can result in a nonuniform treatment (Riva et al. 1993). In addition, for weight loss during incubation, mechanical abrasion has to be considered. To elaborate only the enzymatic effects for wool treatment, the incubation was performed only with gentle agitation.

Treating the wool fibres with native protease resulted in severe damage of the fibres and unacceptable high weight losses, conforming previous results (Riva et al. 1993) for high enzyme concentrations. Recently, a successful restriction of the hydrolysis of wool fibres to cuticles was shown, resulting in 90% decrease of the weight loss compared to non-modified enzymes (Schroeder et al. 2004). These moderate proteolytic reaction and low damages were in correlation with decreased enzyme diffusion into the fibres. Similar studies confirmed these results (Silva et al. 2005). However, the current study showed that an optimal modification of the protease can only be found in a balance between small polymer chain for good stain removal properties and long polymer chain restricting the damage of wool fibres and increasing half life. Therefore, a consensus could be reached modifying a protease from *B. licheniformis* with a 2-kDa methoxy polyethylene glycol. Such enzymes do not only have a high potential as constituents of detergents for woolen cloth but also for carpet cleaning agents improving hygienic aspects and lifetime of these materials.

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