J Ind Microbiol Biotechnol (2008) 35:923–930
DOI 10.1007/s10295-008-0366-1

ORIGINAL PAPER

Utility of enzymes from *Fibrobacter succinogenes* and *Prevotella ruminicola* as detergent additives

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Received: 18 March 2008 / Accepted: 25 April 2008 / Published online: 8 May 2008

Abstract In this study, we investigated the application of cellulase and protease purified from rumen bacteria as detergent additives. Cellulase and protease were purified from the rumen cellulytic bacteria *Fibrobacter succinogenes* S85, and *Prevotella ruminicola* 23, respectively. An inhibitor test indicated that the purified protease belongs to the category of serine proteases and metalloproteases. Both the enzymes were effective at a high temperature (50 °C) and neutral pH (pH 7–8), but the protease activity increased with the increase in temperature and pH. The purified protease was treated with ten types of surfactants/detergents; it was found to retain over 60% of its activity in the presence of anionic and nonionic detergents. The cellulose plus protease combination was still effective after treatment with Triton X-100 and Tween 80, but the residual activity was low after treatment with Tween 20 than that after treatment with other nonionic detergents. Washing tests indicated that enzyme addition produced no significant improvement in the removal of grass stains, but individual enzyme addition in surfactants/detergents, especially in nonionic detergents, could improve the washing performance of the detergents by improving its ability to remove blood stains. This suggested that the surfactant/detergent class, enzyme properties, and the mixing ratio of ingredients should be considered simultaneously to enhance the washing performance.

Keywords Rumen bacteria · Cellulase · Protease · Detergent · Wash test

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Introduction

The first large-scale application of microbial enzymes was in detergents. Bacterial proteases are still the most important detergent enzymes because detergent enzymes account for approximately 90% of the total protease sales worldwide [11]. Cellulases have been a part of detergents since the early 1990s. In textile washing, cellulases remove cellulose microfibrils, which are formed during washing and during the manufacture of cotton-based cloth [15].

Cellulases can also be applied as color, brightening and softening agents. Taking into account the current detergent market trend, incorporating cellulases with proteases in detergent formulations would be advantageous for enhancing the washing performance of detergents and for reducing water consumption. However, detergent cellulases should have a good stability against proteases [32, 33]. The currently used detergent cellulases are often derived from fungal sources and bacterial sources. Fungal enzymes typically exhibit varying glucanase activities at an optimum pH, in the acidic to neutral range; this limits their application as common detergent additives [18, 23]. Only few bacteria secrete large amounts of cellulase and a multienzyme complex called cellulosome; other cellulytic bacteria produce relatively low amounts of cellulase, and lack cellobiase activity [5, 7, 8].

The rumen is a highly specialized, prepeptic adaptation of the ruminant digestive tract. It facilitates the storage and microbial processing of large quantities of plant material, including structural carbohydrates and proteins [14]. Lee et al. [4] indicated that the cellulose degradation ability of the rumen ecosystem was higher than that of any other known fermentation systems. *Fibrobacter succinogenes* is one of the major cellulytic bacteria in the rumen, and its endoglucanase is stable in Triton X-100 medium

and resistant to trypsin treatment [10]. Besides, the coculture of *Prevotella ruminicola* and *F. succinogenes* showed higher cellulytic activity than the culture of individual bacteria [6]. *P. ruminicola* is a predominant rumen bacterium that plays a significant role in the metabolism of proteins and peptides in the rumen [37]. Previous studies indicate that most of the protease activity of *P. ruminicola* occurs extracellularly or on the cell surface, that is, regions rich in serine, cysteine, and metalloproteases [9, 12, 38]. The defaunation experiment in the sheep carried out by Hsu et al. [13] indicated that an anion surfactant did not limit the protease activity and growth performance of bacteria in the rumen. The cellulase and protease derived from the rumen bacteria exhibit high activity even at mild temperatures (approximately 40 °C) and are stable in many detergent solutions.

The detergent market is expected to focus upon lower (cooler) washing temperatures (30–40 °C) and reduction in water consumption [32]. Lower washing temperatures are expected to decrease the efficiency of traditional ingredients in the detergent [19], and the ratio of fabric and soil to the wash liquor is higher when reducing water consumption is intended [25]. Although extensive research has been conducted on the use of protease as a detergent additive, there has been limited research on the combined use of a suitable cellulase and protease in detergents. Based on detergent tolerance and enzyme interaction, the properties of cellulase and protease from rumen bacteria might render their combination as a promising detergent additive.

This study was conducted to determine whether the protease and cellulase derived from rumen bacteria could be used simultaneously as detergent additives to improve the washing performance of the detergent and to determine the optimum conditions for enzyme activity.

Materials and methods

Organisms and culture medium

P. ruminicola 23 (ATCC 19189) and *F. succinogenes* subsp. *succinogenes* S85 (ATCC 19169) were purchased from ATCC. *F. succinogenes* S85 was cultured in the Scott and Dehority medium [31] with the following modifications: cellobiose (0.4% w/v) was used as a carbon source in the first stage of incubation (24 h), and amorphous cellulose (acid-swollen cellulose; 0.2% w/v) was added as the carbon source in the second stage to stimulate cellulase production for another 12 h. *P. ruminicola* 23 was also cultured in the Scott and Dehority medium for 24 h as described previously [38]. All cultures were grown in an anaerobic chamber in 97% CO_2/3% H_2 atmosphere.

Cellulase purification

In this study, partially purified endoglucanase was used as a source of cellulase. The cell-free supernatant was harvested by centrifugation at 8,000×*g* for 20 min and then concentrated to approximately 20-fold by ultrafiltration, as described by McGavin and Forsberg [24]. A HiTrap Q column (GE Healthcare, USA) was preequilibrated with 50 mM sodium phosphate buffer (pH 6.8). After applying the sample, the column was washed with 2 times the bed volume (10 ml) of the starting buffer and then eluted in a linear gradient with five times the bed volume (25 mL) of 0–1 M NaCl in 50 mM sodium phosphate buffer (pH 6.8). The buffer gradient profile was determined by measuring the conductivity of the fractions and relating the value to a standard curve. The fractions showing endoglucanase activity were collected and concentrated by ultrafiltration through an Amicon PM-10 membrane and stored as aliquots at −70 °C for further use.

Protease purification

The cell-free supernatant was harvested as the cellulase sample; the supernatant fluids were decanted and stored in ice until use. The cell pellet was washed twice by centrifugation with 50 mM Tris–HCl buffer (pH 8.0). To obtain the crude enzyme extract, all cell-free supernatants were concentrated to approximately 50-fold at 4 °C by using an ultrafiltration membrane (BioMax 10; Millipore Corp., Beverly, MA). The crude enzyme extract was applied to a diethylaminoethanol (DEAE) column (5 mL; GE Healthcare) that was preequilibrated with 50 mM sodium Tris–HCl buffer (pH 8.0). The column was washed with two times the bed volume (10 mL) of starting buffer and then eluted in a linear gradient with ten times the bed volume (50 mL) of 0–1 M NaCl in 50 mM Tris–HCl buffer (pH 8.0). Finally, the fractions showing protease activity were collected and concentrated by ultrafiltration through an Amicon PM-10 membrane and stored as aliquots at −70 °C for further use.

Polyacrylamide gel electrophoresis

In order to determine the purity and molecular mass of the enzymes, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a polyacrylamide slab gel by using the Laemmli method [17]. The molecular masses were calibrated using a BlueRanger™ prestained protein molecular weight marker mix (Pierce, USA).

Enzyme assay methods

Cellulase activity was assayed by using carboxymethyl cellulose (CMC) as a substrate. The assays were carried out by

adding 100 µL of the sample to a tube containing 100 µL of 50 mM sodium phosphate buffer (pH 6.8) with 1% CMC. The mixture was incubated at 40 °C for 20 min, the reaction was stopped by adding the the Somogyi reagent, and reducing sugars thus produced were measured by the Nelson–Somogyi method [39]. One unit of cellulase activity is defined as the release of glucose at the rate of 1 µg/min at 40 °C under standard assay conditions. For the protease activity assay, the azocasein method was used [3]. One unit of protease activity is defined as azocasein digestion at the rate of 1 µg/h at 40 °C under standard assay conditions. Proteins were determined by the Bradford method [2] using bovine serum albumin as the standard.

Protease inhibitor test

The protease inhibitors used and their target proteases are listed in Table 1. In each assay tube, 0.02 mL of inhibitor solution was mixed with 0.18 mL of protease sample (dissolved in 50 mM sodium phosphate buffer, pH 6.8) at a preset inhibitor concentration. To initiate the reaction, all reaction samples were equilibrated with inhibitors at 39 °C for 15 min, and subsequently, 0.2 mL of 0.8% azocasein solution was added to the reaction samples and incubated in a water bath at 39 °C for 3 h. The inhibition of protease activity was calculated as the difference in the activity between the samples untreated and treated with the inhibitor.

Stability of enzymes in surfactants and commercial detergents

To determine the working temperature and pH range of the source enzymes, the reactions were carried out in the following buffer solutions (concentration, 50 mM) with 150 mM NaCl for 3 h: sodium phosphate (pH 7 and pH 8) and glycine–NaOH (pH 9 and pH 10). The stability of the enzymes in the presence of seven surfactants and in three commercial detergents (Table 2) was investigated by incubating cellulase (0.5 U/mL) and protease (0.4 U/mL) with these agents (5% w/v) at 40 °C under neutral conditions. After a 3-h incubation, the residual activity of cellulase and protease in the samples treated with different detergents was measured. The detergents used in this study are listed in Table 2.

Table 1 Effect of protease inhibitor on partially purification protease activity of *P. ruminocola* 23

Inhibitor	Fraction	
	Peak 1	Peak 2
Total activity[a], U	3,799	9,321
Residual activity[b] (%)		
PMSF	75.5	54.3
Pepstatin A	87.3	91.5
EDTA	42.7	66.1
Leupeptin	84.9	79.9

[a] Sample was collected from 1 L broth which incubation at 39 °C for 16 h

[b] Reaction at 39 °C and pH 6.8 for 3 h. Values are the means of four replicates

Wash tests

In order to evaluate the application of cellulase and protease as detergent additives, we used pieces of white cotton cloth (4.5 × 2.5 cm, 6 layers) that were stained with 200 µL grass sap or chicken blood and dried overnight at room temperature. The soiled pieces of cloth were washed at 40 °C at 80 rpm for 30 min with 50 mL water containing a detergent (0.045%) to which cellulase (0.26 U/mL) and/or protease (2.08 U/mL) was added. Before washing, all the stained pieces of cloth were soaked in the wash solution at 40 °C for 30 min. After washing, the test cloth pieces were rinsed with water twice for 5 min and then dried for determining the washing performance. The washing performance was measured by using a reflectometer (Photovolt™ Model 577, USA). The mean of the differences in the reflectance (ΔR) of each set was determined and compared with that of a set of control cloth pieces for each experiment; the control cloth pieces were washed with detergent only.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the statistical analysis system (SAS) for windows (SAS 8.0, SAS Institute). Differences among the treatment means were determined by using Duncan's test, and the differences were considered significant at $P < 0.05$.

Results

Enzyme purification

When the cellulase present in the extracellular culture fluid (ECF) of *F. succinogenes* S85 was separated on the HiTrap Q column, majority of the activity was noted at peak 2 and the fraction was eluted with 0.5 M NaCl (Fig. 1a). The specific activity of the partially purified cellulase was 314.3 U/mg protein and the yield was 13.2%. Although the purification result was similar to that of a previous study on *F. succinogenes* S85, endoglucanase showed a higher specific activity after ion-exchange purification [21] and a slightly

Table 2 Stability of enzymes in surfactants and commercial detergents

Surfactants/Detergents	Class	Residual activity (%)			
		Cellulase	Protease	Mixed enzyme	
				Cellulase	Protease
Sodium taurocholate	Anionic	18.10^{c}	58.56^{d}	66.68^{bc}	80.56^{c}
Saponin	Anionic	33.84^{a}	12.73^{d}	58.75^{bc}	191.67^{b}
Aerosol®	Anionic	24.67^{abc}	132.41^{a}	49.17^{bcd}	412.50^{a}
Sodium cholate	Anionic	22.93^{bc}	117.13^{ab}	ND	20.83^{c}
Triton X-100	Non-ionic	24.67^{abc}	89.12^{bc}	37.50^{bcd}	58.33^{c}
Tween 20	Non-ionic	24.17^{abc}	68.75^{cd}	25.00^{cd}	50.00^{c}
Tween 80	Non-ionic	22.31^{abc}	89.12^{cd}	79.17^{b}	52.78^{c}
Laundry detergent[e]	Mixed	19.34^{c}	ND	27.08^{cd}	ND
Dishwashing detergents[f]	Mixed	24.05^{ab}	ND	144.58^{a}	ND
Decon® Neutracon	Mixed	16.98^{bc}	119.68^{ab}	46.25^{bcd}	38.89^{c}

ND non detected

[abcd] Means with different superscript with in a column are significantly different ($P < 0.05$) ($n = 4$)

[e] Contain protease. Main cleaning ingredients: sodium Linear alkylbenzene sulfonate, non-ionic surfactant (7EO), protease, water softener, preventing soil redeposition agent (PAA), bleaching germicide agent (Oxygen-type), fabric softener and floral fragrance. (Compact powder UV laundry detergent with protease, Formosa Plastics Group, Taiwan)

[f] Main cleaning ingredients: sodium linear alkylbenzene sulfonate, nonionic surfactant and floral fragrance. (Anti-bacterial Dish Washing Detergent without protease, Mao Bao Inc., Taiwan)

lower yield. The peak two fractions were collected and concentrated for subsequent tests. The concentrated *P. ruminocola* P23 ECF sample was applied to the DEAE column with the Tris buffer system. The fractions eluted with 0.3 M NaCl (peak 3 in Fig. 1b) showed higher protease activity than the other peaks. Major protease activity peaks (peaks 2 and 3 of Fig. 1b) were collected separately and then concentrated for the protease inhibitor test. Based on the total enzyme activity, the protease from peak 3 (Fig. 1b) was used for the subsequent treatments for testing its stability and washing performance. The SDS-PAGE results of the partially purified enzyme are shown in Fig. 1c and d.

Enzyme properties

The specific activity of the partially purified cellulase from *F. succinogenes* S85 showed maximum activity at 50 °C and pH 7–8. There was a steep decline in the activity when the temperature was lowered to 25 °C or when the pH was raised above 9 (Fig. 2a). The protease purified from *P. ruminocola* P23 was highly stable at 50 °C and had a broad range of pH stability. Significantly high protease activity was observed at a high temperature and pH; however, the enzyme activity showed a sudden dip at pH 9 but slightly increased as the pH increased further (Fig. 2b). The protease inhibitor test results (Table 1) indicated that phenyl methyl sulfonyl fluoride (PMSF; serine protease inhibitor) and ethylene diamine tetra-acetic acid (EDTA; metalloprotease inhibitor) inhibited the protease activity to the greatest extent, whereas pepstatin A and leupeptin inhibited it to a lesser extent.

Stability of the enzymes with surfactants and in commercial detergents

The effect of detergents on the enzyme activity is shown in Table 2. After the 3-h treatment, the residual activity of cellulase alone decreased to 20–30%. When the enzyme combination was tested in surfactants/detergents, the residual activity of cellulase increased in all detergents except sodium cholate. Protease alone showed a high stability in many test detergents and retained about 60% of its activity after the 3-h treatment; however, saponin may strongly affect the activity of protease when protease is added alone. Interestingly, in the present case, protease derived from *P. ruminocola* P23 showed higher activity in the presence of Aerosol®, sodium cholate, and Decon® Neutracon. When the enzyme combination was tested in surfactants/detergents, the protease activity showed a steep increase in saponin and Aerosol®, but it decreased slightly in nonionic detergents. One of the commercial laundry detergents used in this study contained a cold and active protease from *Bacillus* sp. The residual activity of our protease was not detected when the purified protease was tested in this detergent; this indicated that the proteases contained in laundry detergents may counteract the protease from *P. ruminocola* P23.

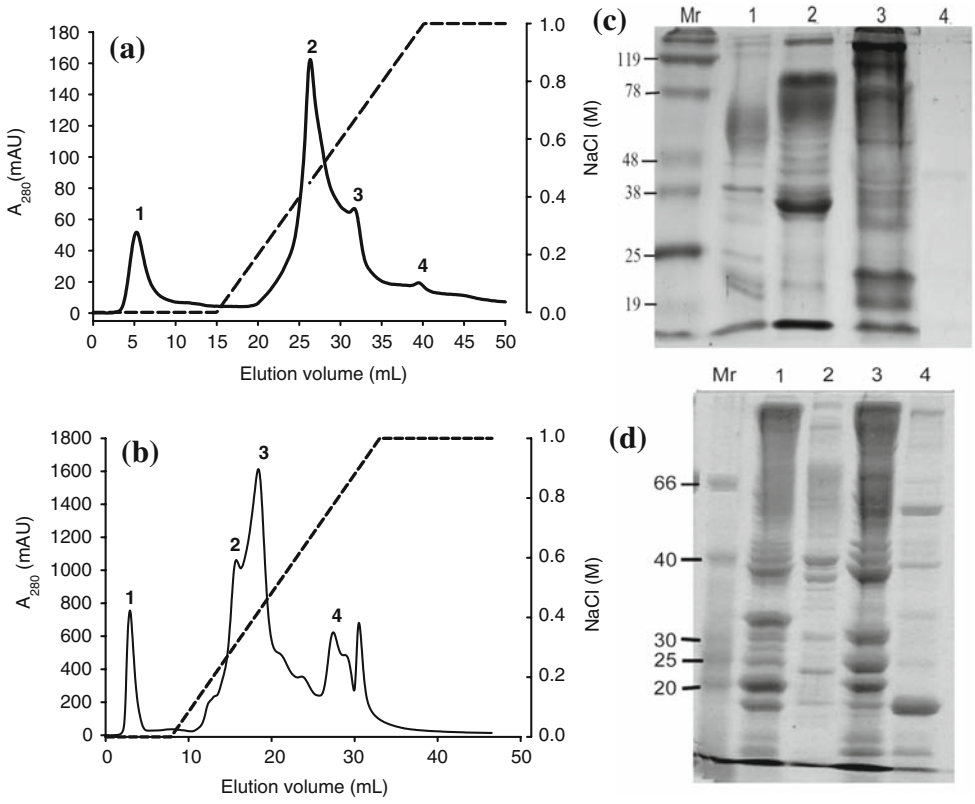

Fig. 1 The ion exchange chromatography of **a** cellulase from extra cellular fluid of *F. succinogenes* S85. The fractions of peak 2 were collected and concentrated for tests. **b** Protease from extra cellular fluid of *P. ruminocola* P23. The fractions of peak 3 were collected and concentrated for tests. **c** 10% SDS-PAGE of samples from cellulase purification peak fractions. **d** 7.5% SDS-PAGE of samples from protease purification peak fractions. *Lanes 1–4* indicated the fraction peak number in (**a**) and (**b**). All gels stained with Coomassie blue

Washing performance analysis

To determine the efficacy of the two enzymes for their use as detergent additives, the washing performance analysis using cotton fabric stained with grass sap and chicken blood was carried out at 40 °C. The washing performance (Fig. 3a) with respect to the removal of grass stains was improved by 10% when individual enzymes were added to Triton X-100, Tween 20, sodium cholate, and commercial laundry detergent. Surfactants and detergents containing both protease and cellulase had a better washing performance than those containing individual enzymes, except in the case of Triton X-100 and Tween 20. Tween 80 and dishwashing detergents (without protease) had a notably better washing performance after the addition of the enzyme combination. In this study, the washing performance with respect to grass stain removal did not show a significant improvement after any treatments (lesser than 20% improvement). The washing performance with respect to blood-stain removal indicated that individual enzyme addition had a positive effect on the cleaning ability, but the use of the enzyme combination with Tween 80, sodium cholate, and Decon® Neutracon did not improve the cleaning ability (Fig. 3b). In this study, the washing tests revealed that enzyme addition produced a greater improvement in removing blood stains than grass sap stains. It also showed that addition of enzymes to nonionic detergents could improve their washing ability (Fig. 3b).

Discussion

The criteria for using an enzyme as an additive in detergents are as follows: (1) it should be active at an alkaline pH and (2) efficacious at wash temperatures of 20–40 °C [15]. The optimal working conditions of the cellulase derived from *F. succinogenes* S85 were similar to the pH and temperature conditions of the rumen described in previous studies [20, 24]. The data on the variation in and inhibition of protease activity suggested that the partially purified protease derived from *P. ruminocola* P23 may contain more than one type of protease.

Alkaline proteases are suitable for laundry application. Many alkaline proteases either have serine in the center (serine protease) or belong to the category of metalloproteases [11, 22]. The subtilisin-like serine proteases from bacilli have been used in various industrial fields worldwide, particularly in the production of detergents used for laundry and in dishwashers [29, 30]. The partially purified protease derived from *P. ruminicola* 23 contains both the types of proteases (Table 1), and this bacterial species secretes large amounts of extracellular proteases [9, 38]. These data show that the protease from *P. ruminocola* P23 probably has a high potential for industrial applications.

Based on the stability of enzymes in surfactants and commercial detergents, the cellulase derived from *F. succinogenes* S85 may be damaged by detergents, probably by

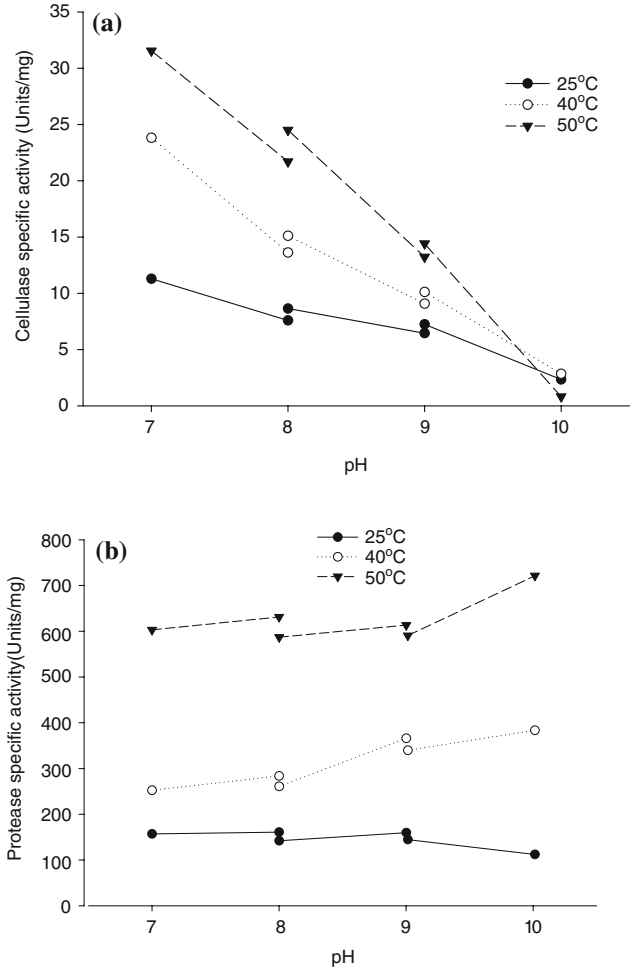

Fig. 2 Effect of pH and temperature on the stability of **a** partially purified cellulase from *F. succinogenes* S85. **b** Partially purified protease from *P. ruminocola* P23. (pH 7 and 8: sodium phosphate; pH 8 and 9: Tris–HCl; pH 9 and 10: glycine–NaOH)

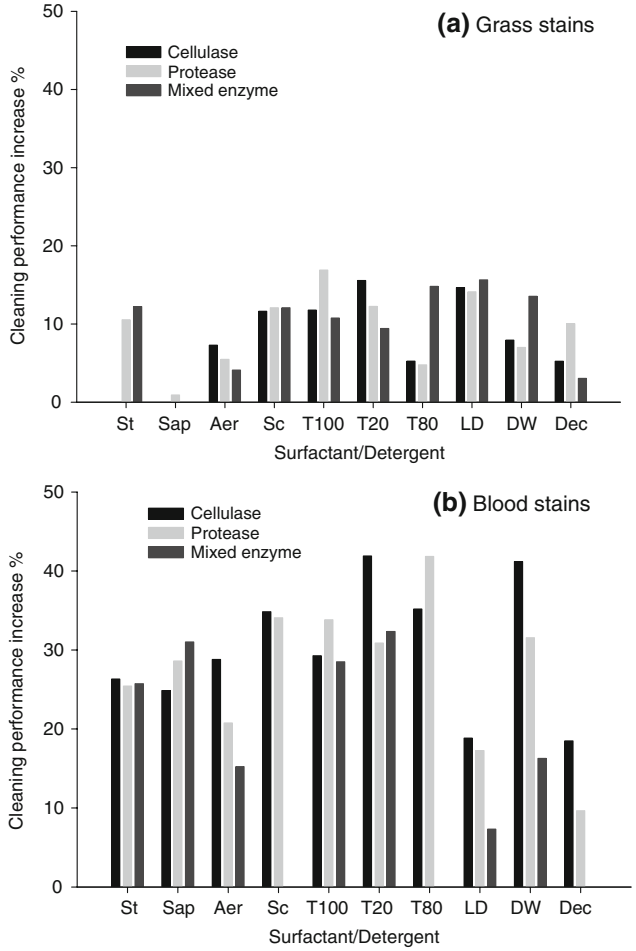

Fig. 3 The washing test result of **a** grass stains and **b** blood stains. *St* sodium taurocholate, *Sap* Saponin, *Aer* Aerosol®, *Sc* Sodium cholate, *T100* Triton X-100, *T20* Tween 20, *T80* Tween 80, *LD* Laundry detergent (compact powder UV laundry detergent with protease, Formosa Plastics Group, Taiwan), *DW* dishwashing detergent (Anti-bacterial Dish Washing Detergent without protease, Mao Bao Inc. Taiwan), *Dec* Decon® Neutracon. Washing condition: 0.045% surfactant/detergent, 40 °C, 30 min

proteolytic degradation and anionic surfactant-induced unfolding [27, 28]. Interactions between proteins and surfactant monomers are shown to play a key role in determining the kinetics of the unfolding process [34, 35]. Further studies have reported that protease was highly active against unfolded or partially unfolded proteins [21, 35]. This may explain the marked decrease in the activity of residual cellulase in sodium cholate.

The results of the stability test of the enzyme combination (Table 2) suggest the need for further studies on the complex interactions that occur when multiple enzymes are added to detergent formulations. A study of rumen microbial enzymes revealed that the addition of a nonionic detergent increased protease activity at lower detergent concentrations (0.05–0.4% v/v) and significantly protected the cysteine protease activity [16]. In the present study, the major types of proteases were serine and metalloproteases, and this suggested that the hydrophobic environment within the enzyme molecule created by the nonionic detergents could not significantly enhance the interaction of the enzyme with potential substrates [36].

The pigment in grass sap may have a significant staining effect, and a bleaching agent may be required to remove the colored non-washable soil stains that adhered to the fibers [33]. Our study showed that the enzymes had no ability to thoroughly remove the grass sap stains. A bleaching agent is probably required to improve the washing performance. In previous studies, the washing performance of detergents with respect to blood-stain removal from cotton fabrics was improved by approximately 25–34% after *Bacillus* sp. protease treatment of detergents [11, 26]. In this study, treatment of Tween 20 with cellulase and Tween 80 with protease increased the washing performance with respect to blood-stain removal to approximately 42%. The cellulase

hydrolyzes the cellulose in cotton and cotton blends to deliver cleaning benefits, whitening, and a dingy cleanup [32]. The released fiber structure provides greater surface area for the protease to remove insoluble proteins. The optimal temperature and concentration of detergent enzymes are important considerations while testing washing performance. In the previous study, the increase in the working temperature and concentration of the enzyme under suitable conditions was found to enhance the enzyme activity [1]. This suggested that an appropriate enzyme concentration in the detergent and appropriate washing conditions should be considered when an enzyme is used as a detergent additive. In this study, the washing performance indicated that individual enzymes, that is, cellulase or protease from rumen bacteria, could be good additives in detergent formulations, but their combined use may negatively affect the washing performance. The data obtained in this study suggest that individual enzyme addition leads to a better washing performance in cleaning blood stains, but the addition of both the enzymes may provide some benefit in removing grass sap stains. Among all the tested detergents/surfactants, Triton X-100 and Tween 20 were suitable surfactants for individual or combined enzymes, but Tween 80 with an individual enzyme exhibited the best washing performance with respect to blood-stain removal.

Hence, based on the properties of enzymes, cellulases and proteases from rumen bacteria were found to be suitable detergent additives. The cellulase from *F. succinogenes* was stable in the tested detergents, and its activity increased when used along with protease. The partially purified protease fraction derived from *P. ruminicola* showed a wide range of activity, included two types of proteases, and had high potential in industrial application. The enzymes used in this study were effective at mild temperatures and individual enzyme addition to nonionic detergents was highly beneficial in removing blood stains. The detergent class, enzymes properties, and mixing ratio of ingredients have to be considered when combining enzymes and detergents for achieving a better cleaning performance. Further research on the sequence of enzyme addition and enzyme-detergent combination in washing processes are needed not only to enhance the cleaning performance but also to reduce water consumption.

Acknowledgments The research was supported by a grant from National Science Council, Taiwan (ROC). (NSC94-2313-B-034-004).

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