The effect of detergent ingredients on stability of thermostable alkaline protease 50a in formulation of liquid stain remover

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The Effect of Detergent Ingredients on Stability of Thermostable Alkaline Protease 50a in Formulation of Liquid Stain Remover

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Abstract. New detergents approach such as pre-dosed liquid and powder as pre-treatment to enhance cleaning efficiency has received great interest among consumer and is becoming a trend in laundry industry. Formulation of detergents is continuously developed to improve cleaning efficiency and to adapt and fulfill market demands. In this study, three liquid stain remover formulations containing thermostable alkaline protease 50a as additive were developed. The developed stain remover formulations were specifically designed to remove proteinaceous stains on fabrics. Azo-casein assay was performed to evaluate the stability of the thermostable alkaline protease 50a in each formulation. Wash performance analysis was also carried out to determine the stain removal efficiency of the developed formulations on selected fabrics namely satin, crepe and koshibo. The results obtained shows that the thermostable alkaline protease 50a lost more than 89% of its activity after one week of incubation in these three formulations. The developed stain remover formulation exhibited good stain removal efficiency (84.8-96.46%) in comparison to tap water (74.6-86.28%) and commercial stain remover (88.85-95.44%). The outcomes of this study could provide additional insights on the effect of detergent ingredients on the thermostable alkaline protease 50a activity.

INTRODUCTION

Formulation of detergents is continuously developed to improve cleaning efficiency and to adapt and fulfill market demands. In recent times, new detergents approach such as pre-dosed liquid and powder pouches have been emerging and received substantial interest among consumers [1]. Hence, this trend has initiated the development of laundry detergent products of high cleaning efficiency with ease of handling and minimal washing.

Organic stains such as blood and sebum are primarily consist of protein and lipid which are often insoluble in water and highly adhesive on the surface of fabrics which makes it difficult to remove [2]. Protein residues may be oxidised and denatured due to the presence of oxygen, therefore the blood-stain becomes permanent on cloth [3]. Surfactants are the crucial ingredients in detergent formulation as it lowers the surface tension of a liquid allowing the liquid to disperse evenly over a surface easily [4]. The absence of surfactants would likely results in most of the cleavage products would retain onto the textile surface.

However, the use of cleaning enzymes in laundry detergents leads to a much better performance than the surfactant system alone. The addition of protease in stain remover formulation may enhance the washing efficiency due to its capability to hydrolyse peptide bond [5]. Most detergent enzymes cleave complex biological

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molecules, which could not be solubilised by surfactants. The application of enzymes in detergent formulation could help to reduce the amount of surfactant needed [6, 7]. The application of enzymes could also promote the development of environmentally friendly stain remover formulation [2, 8].

Recently, optimisation of enzymes particularly in terms of temperature, pH and stability in detergent has been initiated. Advanced technology such as granulated and immobilised enzymes in solid laundry detergents results in great stability over humidity, temperature and detergents elements such as bleach and alkalinity. Nonetheles, challenges concerning enzyme stability over time and temperature are the major issue in liquid laundry detergents [6]. However, studies reported on the interaction of enzymes with detergent ingredients are scarce. Therefore, in this work the interaction of thermostable alkaline protease 50a (TAP50a) with stain remover ingredients was assessed to determine the effects of the individual formulated stain remover on the stability of TAP50a. This study also aimed to determine the best stain remover formulation for proteinaceous stains. The TAP50a stability at room temperature was studied and used as comparison to elucidate the influence of detergents ingredients on the enzymatic activity of TAP50a. The findings of this study would be able to provide better understanding on the interaction and effects of detergents ingredients on the stability of the TAP50a.

MATERIALS AND METHODS

Standards, Reagents and Materials

All chemicals used in this study were of analytical grade (AR). PEG 4000, sodium carbonate, Tween 80, sodium citrate, sodium tetraborate, sodium perborate and trichloroacetic acid and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Sodium hydroxide and azocasein were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate was from Vivantis (US). Ethylenediaminetetraacetic acid and Tris were from Calbiochem (Germany). Thermostable alkaline protease 50a produced by *Bacillus subtilis* was obtained from the Postgraduate Laboratory UMK, Jeli Campus which is previously isolated from Lojing Highlands, Kelantan, Malaysia [9]. Commercial stain remover (Dwany Hijab Stain Remover) was obtained from a local supermarket.

Quantification of Protease Activity

Protease activity was assessed using sulphanilamide azocasein substrate with slight modification [10, 11]. A 0.5% azocasein solution was prepared by dissolving 0.05 g azocasein in 10 mL of 0.1 M (pH 9). 1 mL azocasein solution was spiked with 100 µL of sample and was incubated at 80 ºC (± 1ºC) for 30 min. After incubation, 1 mL of 10 % (w/v) trichloroacetic acid (TCA) was added into the mixture to inactivate the reaction and left at room temperature for 30 minutes. Next, the sample was then centrifuged at 10, 000 rpm for 10 min. 1 mL of the supernatant was neutralised by the addition of 1 mL of 1 M sodium hydroxide (NaOH) solution. The absorbance of the sample was measured using a Thermo Scientific Evolution 160 UV-Vis spectrophotometer (USA) at 450 nm. Similar assay procedure was followed for control sample. However, the control sample was inactivated with 1 mL of 10 % (w/v) TCA prior to incubation. All samples were prepared in triplicates (n=3). One unit (U) of azocaseinase activity is defined as the amount of enzyme activity that produces a change of absorbance (0.001 per min) at 450 nm under the standard assay conditions.

Compatibility of Thermostable Alkaline Protease 50a with Detergent Ingredients

The activity of purified TAP50a was assessed by mixing it with 1% (w/w) of 1 mM solutions of different detergent ingredients individually namely PEG 400, Tween 80, sodium carbonate, sodium citrate, sodium dodecyl sulfate, water, lavender oil, sodium perborate, sodium tetraborate and ethylenediaminetetraacetic acid at room temperature for one week [12]. Next, protease activity of each mixtures were determined to assess the compatibility of the detergent ingredients with TAP50a.

Formulations of Liquid Stain Remover

The stain remover solution was prepared by placing PEG 4000 in a beaker and heated at 60 ºC until melted. Water was then added into the beaker containing the melted PEG 4000 followed by the addition of sodium carbonate. Next, Tween 80, TAP50a and lavender oil were added into the solution and were stirred at 400 rpm for five minutes. Similar procedure was used for the preparation of the other two formulations. The materials used and their composition of each formulations is shown in Table 1.

TABLE 1. Formulations of liquid stain remover containing TAP50a

Ingredients	Formulation 1	Formulation 2	Formulation 3
PEG 4000	/	/	/
Tween 80	/	/	/
Sodium carbonate	/	/	/
Deionised water	/	/	/
50a protease	/	/	/
Lavender oil	/	/	/
Sodium citrate		/	
Sodium perborate			/
Sodium tetraborate			/
EDTA		/	
Sodium dodecyl sulfate		/	

Determination of Stain Removal Efficiency

Clean fabric square pieces (5 cm × 5 cm) were stained with 100 µL of chicken blood collected from local abattoir. Three different types of fabrics were used namely crepe, satin and koshibo. Blood was selected as stain since it was the commonly used protein stain for washing performance analysis in previous study [12, 13]. The fabrics were oven-dried at 60 ºC for 12 hours and kept at room temperature for one month. The blood-stained fabrics were sprayed with 300 µL of the formulated stain remover followed by gentle rubbing for several times before rinsing with 1 mL of tap water [14]. Next, the fabrics were then air-dried for 3 hours before measuring the stains intensity on cloth using colorimeter (CR-400 Head, Konica Minolta, NJ, USA). The stain removal efficiency of the formulated stain remover was determined by chroma meter using CIE L*a*b* colour space by which L indicates lightness [15]. Similar procedure was followed for tap water and commercial stain remover which were performed for comparison purposes. The stain removal efficiency was calculated using the following equation [16]:

$$\text{Stain Removal (\%)} = \left(\frac{\text{Lightness of the stained and washed cloth}}{\text{Lightness of the clean and unstained cloth}}\right) \times 100\ \% \quad (1)$$

RESULTS AND DISCUSSION

Determination of Protease Stability of the Purified Protease 50a

The enzyme activity of purified stock TAP50a at room temperature for four consecutive weeks is shown in Fig. 1. Results indicated that the TAP50a activity gradually decrease over weeks. However, the enzyme is capable to retain its activity at 81.45% (Table 2) by week 4. This indicates that the enzyme is stable at room temperature. This is because the TAP50a used in this study is a thermostable enzyme by which it could maintain its activity at high temperature (80 ºC). However, the activity of protease in stain remover formulation should be studied in order to determine its interaction with detergent ingredients. This is crucial as free enzyme is vulnerable when exposed to harsh conditions (i.e detergent ingredients). Therefore, the compatibility of protease with detergent ingredients should be assessed. This is crucial as the enzyme must retain its activity when incorporated into stain remover formulation to ensure best washing efficiency.

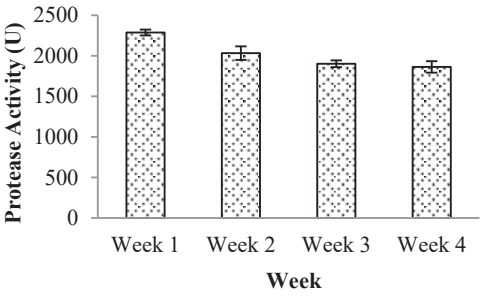

FIGURE 1. Enzyme activity of purified stock TAP50a at room temperature for four consecutive weeks

TABLE 2. Enzyme activity, declination rate and recovery of purified TAP50a at room temperature for four consecutive weeks

Enzyme	Week	Enzyme activity (U/mL)	Total enzyme activity (U)	Declination rate (%)	Recovery (%)
50a protease (Stock)	Week 1	228.78	2287.77	0	100
	Week 2	203.33	2033.33	11.12	88.88
	Week 3	190.22	1902.22	6.45	83.15
	Week 4	186.33	1863.33	2.04	81.45

Determination of Protease Stability in Detergent Ingredients

The stability of protease in detergents components is crucial to ensure efficient cleaning performance. Therefore, the interaction of TAP50a with stain remover ingredients was assessed to determine the effects of the individual formulated stain remover on the stability of TAP50a. The TAP50a stability at room temperature was studied and used as comparison to elucidate the influence of detergents ingredients on the enzymatic activity of TAP50a.

The selection of detergent ingredients must be carefully selected in order to ensure efficient cleaning efficiency. In addition, the ingredients should also compatible with the TAP50a incorporated into the formulation. The limits of detergent ingredient composition were based on the literature [17]. The key ingredient of a detergent is surfactant. Surfactant lowers the surface tension of a liquid which allows the liquid to spread evenly over a surface easily. Surfactant adsorbs onto the soil allowing them to remove the soil from the surface into the bulk liquid. In this study, anionic and non-ionic surfactants such as sodium dodecyl sulfate (SDS) and Tween 80 were studied to evaluate their effects on 50a protease activity. The activity of TAP50a in the presence of detergent ingredients is depicted in Fig. 2.

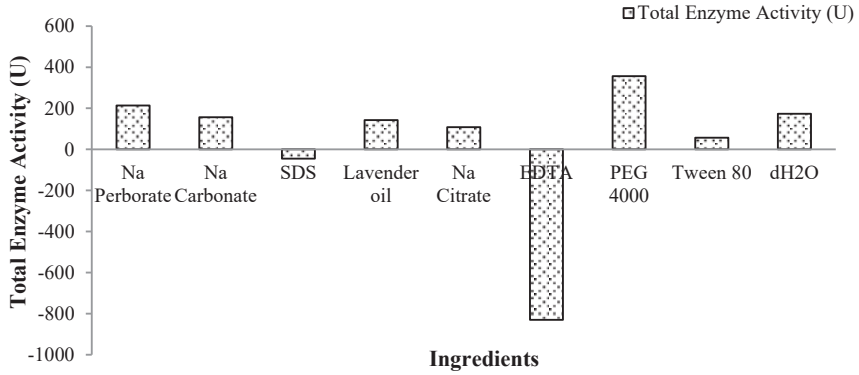

FIGURE 2. Activity of TAP50a in the presence of detergent ingredients

Results showed that the enzyme retained 2.98 % of its initial activity in the presence of 1% (w/v) Tween 80. This could probably be due to non-ionic surfactant could not bind to globular proteins surface. Hence, it does not

affect protein conformation or induce protein association [18]. Besides that, non-ionic surfactant can also shield the hydrophobic core of proteins from the solvent, thus increasing its solubility [19]. On the contrary, the enzyme activity is greatly inhibited after being incubated in the presence of 1.0 % (w/w) anionic surfactant which is SDS. Anionic surfactants are charged surfactants which could interact with the charged surface and hydrophobic core of proteins. Thus, they could affect protein solubility and conformation via the formation of small aggregates around their polypeptide backbone leading to enzyme denaturation [18, 20]. Therefore, Tween 80 is selected as the surfactant for further use as it provided the highest residual enzyme activity. Besides that, it is also selected because non-ionic surfactant provides good cleaning power, milder to human skin, highly soluble and is effective at stabilising emulsions [17, 21].

Next, the effect of builder on the TAP50a activity is studied. Several types of builder such as ethylenediaminetetraacetic acid (EDTA), sodium carbonate and sodium citrate were selected to identify the best builder for the protein stain remover. Sodium carbonate and sodium citrate were capable to retain enzyme activity with 8.23 % and 5.69 % of initial enzyme activity, respectively. EDTA is a chelating agent which is commonly used in liquid detergent as a builder to reduce the effects of water hardness (eliminate alkaline earth ions in water), thus maximising the surfactant performance [22]. However, results showed that the enzyme activity decreased substantially in the presence of 1.0 % (w/w) EDTA. This is because EDTA is an enzyme inhibitor. However, Ibrahim & Yusoff [9] reported that the TAP50a belongs to serine protease, hence EDTA did not greatly inhibited the enzyme (at concentration 10 mM). On the contrary, in this study EDTA inhibited 100% of the enzyme activity. This could probably due to high concentration of EDTA used, which is 1.0 % (w/w). Hence, EDTA was excluded from the stain remover formulation to retain enzyme activity. Sodium carbonate is selected as the builder for the stain remover formulation as it retained the highest residual enzyme activity.

Subsequently, polyethylene glycol (PEG 4000) exhibited good enzyme stability as it is capable to retain 18.74 % of the initial enzyme activity. PEG 4000 was selected as the binder and anti-redeposition agent as it is effective on synthetic and synthetic-cellulose garments which are commonly used in recent times. Sodium perborate is also capable to retain enzyme activity 11.24 %. It is selected because peroxygen bleaches are colour and fabric safe. It is also more compatible with most detergents' ingredients. Lavender oil is able to retain 7.47 % of enzyme activity. Therefore, it is included in the stain remover formulation to impart pleasant smell to fabrics and cover the chemical odour of the stain remover formulation [23].

Comparison of Residual Enzyme Activity in Three Different Formulations

Three different stain remover formulations were formulated using different ingredients and composition to determine the best formulation for proteinaceous stains removal as depicted in Table 3. The residual enzyme activity of each formulation was determined to identify the optimum stain removal formulation for proteinaceous stains. Formulation 1 showed the highest enzyme stability (10.86%) compared to other formulations. However, the solution was heterogenous by which an emulsion was formed due to partitioning of the lavender oil (results not shown). Hence, the formulation needs to be improved in terms of its selection of ingredients and concentration. The high residual enzyme activity of formulation 1 might probably due to high quantity of TAP50a incorporated into the formulation which is 50% (w/w) of the total composition.

Formulation 2 exhibited the lowest residual enzyme activity which is only 3.6%. This could probably due to the presence of 1.0% (w/w) SDS which is an enzyme denaturant. Besides that, the presence of 1.0% (w/w) EDTA also contributed to the substantial decrease in the enzyme activity as it is an enzyme inhibitor. Formulation 3 exhibited high enzyme residual activity (10.51%) in comparison to other formulation after being incubated for two weeks. Besides that, it also showed good stain removal efficiency. A slight improvement (6.84%) in the residual enzyme activity was observed when SDS and EDTA were excluded in formulation 3. This showed that SDS and EDTA is a strong anionic surfactant and enzyme inhibitor that leads to significant inactivation of enzyme activity. The results indicated that Formulation 3 showed good potential as a protein stain remover. Therefore, this formulation was selected for further use in washing performance analysis to determine the blood stain removal efficiency on selected fabrics.

TABLE 3. Total enzyme activity from four different formulations

Formulation	Enzyme activity (U/mL)	Total enzyme activity (U)	Declination rate (%)	Recovery (%) (Residual activity)
Initial activity	190.3	1903	-	100
F 1	20.67	206.70	89.14	10.86
F 2	6.78	67.80	96.33	3.67
F 3	20	200	89.49	10.51

Washing performance

The washing performance of the formulated stain remover on chicken blood stained cloths is shown in Fig. 3. The stain removal efficiency of chicken blood on three different types of clothes namely crepe, chiffon and koshibo using tap water, commercial stain remover (Brand Y) and the formulated stain remover is shown in Fig. 4. Results obtained showed that the developed stain remover showed the highest stain removal percentage for crepe (96.46 %) and koshibo (94.28%) in comparison to tap water (81.81-86.28%) and commercial Brand Y (88.85-95.44%). The results indicated that the presence of TAP50a in the stain remover formulation increased the stain removal efficiency. Nonetheless, Brand Y stain remover showed better stain removal efficiency (92.03%) for satin cloth in comparison to the formulated stain remover (84.8%) and tap water (74.6%). The good performance of the developed stain remover might probably due to degradation of protein by protease. The protease hydrolyses their peptide bonds, thus leading to the generation of shorter by-products [12]. Hence, this makes the removal of stains from the fabrics becomes easier.

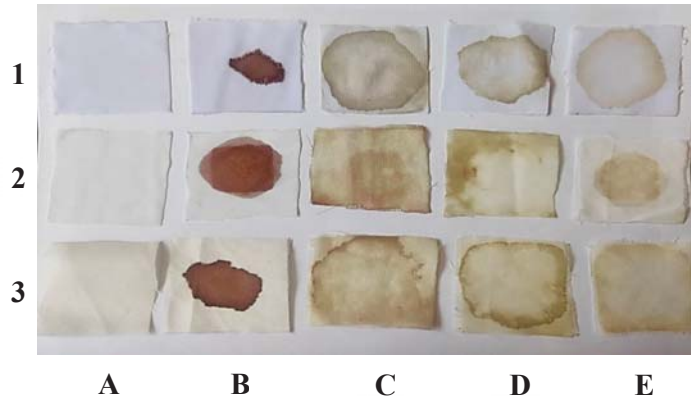

FIGURE 3. Washing performance of the formulated stain remover on chicken blood stained cloths

Note: (1) Koshibo, (2) Satin, (3) Crepe, (A) positive control (unstained), (B) negative control (stained), (C) tap water only, (D) Commercial stain remover (Brand Y), (E) Formulated stain remover

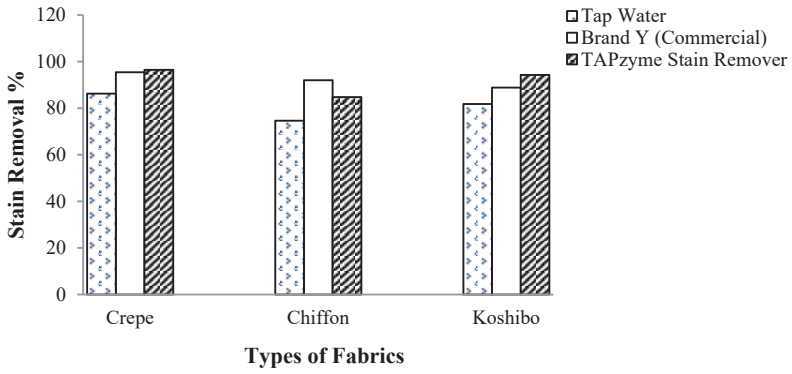

FIGURE 4. Stain removal efficiency of the formulated stain removers on chicken blood stained cloths

CONCLUSION

TAP50a protease exhibited good stability in majority of the studied detergent ingredients which indicates its potential as an additive in detergent formulation. Thus, the TAP50a was applied in stain remover formulation which is specially developed for the removal of proteinaceous stains. Good stain removal efficiency was obtained using the prepared formulation. The developed stain remover provides merits in terms of ease of use, effectiveness and environmentally friendly as only minimal washing is required. Therefore, the stain remover offers a good potential as an alternative to the commercial stain remover for proteinaceous stains.

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