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Enhancing Alkaline Protease Stability through Enzyme-Catalyzed Crosslinking and Its Application in Detergents

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Abstract: Enzymatic additives, particularly alkaline proteases, play a crucial role in enhancing detergent effectiveness against protein-based stains. Despite advancements in enzyme stabilization techniques, there is a need for innovative strategies to further improve protease stability in laundry detergents. However, research exploring the utilization of substrate imprinting technology to achieve this objective remains limited. Therefore, this study aims to enhance the stability of alkaline proteases in laundry detergents by employing casein as an imprinting substrate and utilizing transglutaminase-mediated (TGase) crosslinking to modify proteases 102 and 306. The optimal temperature, pH, and thermal stability of the modified alkaline proteases 102 and 306 showed no significant changes. However, these two modified alkaline proteases exhibited varying degrees of improvement in stability among the 14 detergent additives tested. Under 40 °C incubation for 24 h, the relative enzyme activity of modified alkaline protease 102 increased approximately 1.4–15-fold in AEO-9, BS-12, CMI, APG, FMEE, and SOE, while the relative enzyme activity of modified alkaline protease 306 increased approximately 1.2–3.7-fold across different additives (FMEE, AEO-9, BS-12, SOE, FAA, and AEC-9Na). These modified proteases demonstrated improved stability and wider applicability across commercial detergent formulations available. Integrated into standard laundry detergent at a 1:7 ratio before and after modification, they effectively removed protein stains from the cotton fabric after 24 h of 40 °C incubation. These findings provide insights into more effective stain-removal techniques.

Keywords: laundry detergent; alkaline protease; TG enzyme; stability

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1. Introduction

Proteases dominate over 60% of the global enzyme preparation market and are pivotal in laundry detergents owing to their capacity to hydrolyze various protein stains. They effectively target and remove stains bound to proteins and work synergistically with surfactants [1–3]. As the market for laundry detergents continues to expand, the addition of enzymes to laundry detergents emerges as a trend in detergent technology development. The presence of free water in detergent formulations facilitates enzyme migration but poses stability challenges [4]. Additionally, diverse detergent additives such as surfactants, chelating agents, and bleaching agents can influence proteinase activity and application efficacy. High-temperature washing further exacerbates enzyme instability [5–7].

Adjusting detergent formulations can enhance enzyme stability to a certain extent. Russell et al. reported that 15% anionic surfactant sodium dodecylbenzenesulfonate (LAS) could lead to the complete inactivation of proteases. However, the incorporation of 10% nonionic surfactant, fatty alcohol polyoxyethylene ether (AE), resulted in a residual enzyme activity of 33% under identical conditions [8]. While the combination of anionic and

nonionic surfactants can attenuate the adverse impact of anionic surfactants on protease activity, additional measures are necessary to further enhance enzyme stability. These measures encompass the utilization of stabilizers (primarily reversible protease inhibitors), protein modifications, and chemical alterations. Among these strategies, the direct addition of stabilizers emerges as the most straightforward approach [9–12]. Initially employed as a stabilizer, boric acid was subsequently substituted by more efficient stabilizers such as 4-formylphenylboronic acid (4-FPBA) and a peptide aldehyde. Despite their effectiveness, these stabilizers are characterized by their high cost, limited diversity, and challenges associated with large-scale implementation [13,14]. The encapsulation of proteases can also enhance their stability under certain conditions. Zhang employed a self-assembly method, with β-cyclodextrin serving as the main molecule and a triblock copolymer PEO133-PPO39-PEO133 (F88) as the guest molecule to encapsulate alkaline protease. Under 40 °C incubation in a commercially available enzyme-free laundry detergent, the stability of encapsulated alkaline protease was found to be inferior [15]. Wu et al. immobilized proteases using sodium alginate and chitosan as carrier materials. Unimmobilized protease lost all activity following 24 h of storage at 20 °C in 5–25% AEO-9, while the residual enzyme activity of immobilized protease was highest at 23.49% under the same conditions [16].

Although these methods enhance protease stability under specific conditions, each comes with advantages and disadvantages. Therefore, exploring different approaches for improving protease stability from various perspectives is essential. Substrate imprinting technology—based on molecular imprinting technology (MIT)—relies on the conformational memory of enzymes. It leverages the interaction between enzymes and substrates to alter the enzyme conformation. It is a direct enzyme modification technique developed using the flexible characteristics of enzyme molecules in aqueous solutions [17–19]. Once the substrate binds to the active center of the protease, the enzyme undergoes conformational changes, allowing it to swiftly recognize and locate the corresponding sites of stains. Transglutaminase (TGase) can crosslink proteases, facilitating the formation of covalent bonds between protease molecules, while also mediating crosslinking reactions within protease molecules [20–22]. Despite advancements in enzyme stabilization techniques, such as the use of stabilizers and encapsulation, there is still a lack of better methods to improve the stability of proteases in laundry detergents. Moreover, research on the application of substrate imprinting technology remains limited, particularly concerning the utilization of casein as the substrate followed by crosslinking with TGase to enhance protease stability. Therefore, this study aims to imprint proteases initially using casein as the substrate, followed by crosslinking them using the protein-crosslinking enzyme TGase. Casein, being a major component of milk and considering that protein stains on clothing often involve casein, was selected as the imprinting substrate. Preliminary experiments identified two alkaline proteases, and a subsequent evaluation confirmed that the washing performance of the composite proteases (at a ratio of 1:7) was superior to that of the individual proteases. Furthermore, different proteases exhibit varying tolerances to detergent additives. Therefore, in this study, TGase was employed to modify the two alkaline proteases separately using the substrate-imprinting method. The washing performance of the modified composite proteases was subsequently evaluated.

2. Materials and Methods

2.1. Reagents and Materials

Alkaline protease 102 (2.0×10^5 U/g), alkaline protease 306 (3.3×10^5 U/g) and TGase (6617 U/g) were supplied by Shandong Longkote Enzyme Preparations Co., Ltd. (Linyi, China). Various chemicals including sodium dodecylbenzenesulfonate (LAS), sodium α-olefin sulfonate (AEC-9Na), sodium alkyl ether sulfate (AES), nonylphenol polyoxyethylene ether (AEO-9), and fatty alcohol amide (FAA) were purchased from Tianjin Tianzhi Fine Chemical Co., Ltd. (Tianjin, China). Dodecyl dimethyl betaine (BS-12) and carboxymethyl imidazoline (CMI) were purchased from Guangzhou Churen Chemical Co., Ltd. (Guangzhou, China). Sodium methyl ester sulfonate (MES) was purchased

from Beijing Coolaborate Technology Co., Ltd. (Beijing, China). Fatty acid methyl ester ethoxylate (FMEE) was purchased from Shandong Yousuo Chemical Technology Co., Ltd. (Linyi, China). Modified fatty alcohol ethoxylate (SOE), sodium alkyl ether sulfate (SNS-80), alkyl polyglucoside (APG), triethanolamine (TEA), and cotton cloth were purchased from Guangzhou Junxin Chemical Technology Co., Ltd. (Guangzhou, China). Analytical grade reagents, including anhydrous sodium citrate (ANP), sodium hydroxide, furfuryl alcohol, L-tyrosine, and trichloroacetic acid, were obtained from China National Pharmaceutical Group Chemical Reagent Co., Ltd. (Shanghai, China). The protein stain solution was obtained from Zhongqing Daily Chemical Testing and Certification Co., Ltd. (Shanghai, China), and standard casein was sourced from the China National Institutes for Food and Drug Control (Beijing, China).

2.1.1. Preparation of Stained Fabric

Cut cotton fabric was cut into circular pieces with diameters of 6 cm. An appropriate quantity of protein stain solution was filtered through double-layer gauze. Following that, 200 μL of the filtrate was collected and dropped in the center of the fabric. It was then circulated to a suitable stain size using a circular instrument. Subsequently, it was air-dried naturally and stored at 4 °C for subsequent use.

2.1.2. Preparation of Laundry Detergents

Laundry Detergent A: Standard laundry detergent comprising 4% AEO-9, 2% AES, 8% LAS, 0.5% TEA, and 0.5% ANP was dissolved in water, with pH adjusted to 8.0–9.0 using sodium hydroxide solution.

Laundry Detergent B: Standard laundry detergent was added to pre-modified composite alkaline protease.

Laundry Detergent C: Standard laundry detergent was added to post-modified composite alkaline protease.

Based on the preliminary experimental findings, the ratios of composite alkaline proteases 102 and 306 before and after modification were 1:7, respectively, resulting in a total enzyme activity of 400 U/mL.

2.2. Alkaline Protease Activity Determination

Enzyme Activity Determination:

A solution of alkaline protease at 10 U/mL underwent the following procedures: It was incubated at 40 °C for 2 min, followed by the addition of 1 mL of casein solution with thorough mixing. After further incubation at 40 °C for 10 min, 2 mL of 65.4 g/L trichloroacetic acid was added with thorough mixing, and the mixture was allowed to stand for 10 min. Subsequently, filtration was performed using slow qualitative filter paper, and 1 mL of the filtrate was collected. This filtrate was then combined with 5 mL of 42.4 g/L sodium carbonate solution and 1 mL of diluted furfuryl alcohol. The mixture was uniformly blended and incubated at 40 °C for 20 min. The absorbance was measured at 680 nm. The blank group underwent the same process as previously described, with the addition of an enzyme solution, a 2 min incubation at 40 °C, followed by the sequential addition of trichloroacetic acid and subsequent addition of standard casein solution [23].

L-Tyrosine Standard Curve Construction:

To create the L-tyrosine standard curve, the 100 μg/mL L-tyrosine standard stock solution was serially diluted to concentrations of 0, 10, 20, 30, 40, and 50 μg/mL. Each dilution (1 mL) was mixed with 5 mL of 42.4 g/L sodium carbonate solution and 1 mL of diluted furfuryl alcohol. The mixtures were uniformly blended and then incubated at 40 °C for 20 min. The absorbance was measured at 680 nm using a microplate reader (Synergy2, Shanghai, China). Subsequently, a standard curve was generated with L-tyrosine concentration plotted on the *x*-axis and absorbance on the *y*-axis, as shown in Figure A1. One unit of enzyme activity was defined as the enzyme quantity capable of hydrolyzing the substrate to produce 1 μg of tyrosine per minute under the specified experimental conditions.

The calculation methods for enzyme activity and relative enzyme activity are detailed as follows:

$$\text{Enzyme Activity} = A \times K \times \frac{4}{10} \times n \quad (1)$$

A: Absorbance.
K: Slope of the standard curve.
n: Dilution factor.
4: The overall volume of the reaction reagents.
10: A reaction time of 10 min.

$$\text{Relative Enzyme Activity}(\%) = \frac{X_1}{X} \times 100\% \quad (2)$$

X_1: Enzyme activity after different treatment conditions in U/mL.
X: Original enzyme activity in U/mL.

2.3. Modification of Alkaline Protease

The modification method underwent optimization in the initial phases of this study. An 8% alkaline protease solution and a 4% casein solution were prepared. Equal volumes of both solutions were mixed and placed in a shaking water bath at 40 °C with a shaking speed of 150 rpm for 10 min. The TG enzyme was then added, and the incubation continued for an additional 2 h at 40 °C, with the TG enzyme exhibiting an activity of 5 U/mL in the entire system. Upon completion of the reaction, the mixture was precooled at −20 °C for 24 h and subsequently subjected to freeze-drying.

2.4. Optimal Temperature and Thermal Stability of Modified Alkaline Protease

The enzyme activities of the alkaline protease, before and after modification, were assessed at 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C [24]. Relative enzyme activities were calculated at each temperature, with the enzyme activity before modification serving as the reference (set to 100%). For the thermal stability analysis, both the modified and unmodified alkaline proteases were subjected to incubation at temperatures of 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C for 2 h. Subsequently, the relative enzyme activities were determined, with the enzyme activity measured after incubation at 40 °C for 2 h set as the baseline (100%).

2.5. Optimal pH of Modified Alkaline Protease

Buffer solutions were prepared with pH values ranging from 7.0 to 9.0 using 0.2% sodium dihydrogen phosphate solution and from pH 9.0 to 12.0 using 1% sodium borate solution. A diluted solution of the modified alkaline protease was employed to measure enzyme activity at 40 °C across pH conditions ranging from 7.0 to 12.0 [25]. Relative enzyme activities were determined using the same method employed for measuring alkaline protease activity.

2.6. Stability of Modified Alkaline Protease in Washing Aids and Commercial Laundry Detergents

To assess the stability of the modified alkaline protease in washing aids, a 2000 U/mL enzyme sample was mixed with a 20% aqueous solution of the washing aid and enzyme activity was immediately measured. Following storage at 40 °C for 24 h, the enzyme activity was reassessed [26]. Enzyme activity in the absence of a washing aid served as the baseline (considered 100%).

To explore the stability of the modified alkaline proteases in commercially available laundry detergents, five top-selling laundry detergents with added enzymes were chosen. The detergents were used to deactivate the enzymes, then diluted to a 10% aqueous solution, and alkaline protease was introduced. The enzyme activity of the system was standardized to 2000 U/mL. The detergents containing the added alkaline protease were subjected to temperatures of 30 °C, 40 °C, and 50 °C for 2 h, and enzyme activity was subsequently measured. Enzyme activity in the absence of laundry detergent was considered to be 100%.

2.7. Evaluation of Washing Performance of Modified Alkaline Protease

2.7.1. Stain Removal Efficiency of Modified Alkaline Protease

An appropriate amount of laundry detergents A, B, and C was taken. They were then incubated at 40 °C for 24 h. Subsequently, 100 mL of hard water was placed in a conical flask and incubated at 50 °C for 10 min. Following that, 0.2 g of the laundry detergents was added before and after incubation. Protein-stained fabric pieces were then placed in the flask and washed at 50 °C with a shaking speed of 150 rpm for 50 min. Following washing, the fabric pieces were rinsed three times with water and air-dried at room temperature. The whiteness reflectance of the fabric surface at 457 nm was measured using a whiteness meter (INESA WSB-L, Electrophysical Optical Instrument, Shanghai, China) before and after washing.

Whiteness values on each side of the fabric pieces before and after washing were measured at two points, and the average of four measurements represented the whiteness value of the fabric piece. The stain removal value (R) and stain removal ratio (P) were then calculated to reflect the change in whiteness before and after washing as follows:

$$R = \mathrm{F}_2 - \mathrm{F}_1 \quad (3)$$

F_1: Whiteness value before washing the stained fabric.
F_2: Whiteness value after washing the stained fabric.

$$P = \mathrm{R_i} \div \mathrm{R}_0 \quad (4)$$

$\mathrm{R_i}$: Stain removal value for the sample.
R_0: Stain removal value for the standard laundry detergent.

2.7.2. Stain Removal Efficacy Characterization

To evaluate stain removal efficacy, the fiber status and residual stains between the fibers were observed using a Scanning Electron Microscope (SEM, Phenom Pure Plus, Shanghai, China) operating at a voltage of 10 kV. The observation was conducted at a magnification of 400×.

2.7.3. Residual Element Analysis

The residual elements in the fabric samples were analyzed using X-ray photoelectron spectroscopy (XPS, ESCALABXi+, Waltham, MA, USA).

2.8. Statistical Analysis

Each experiment was replicated three times, and the average values were used for statistical analysis. Experimental data are presented as mean ± standard deviation. Statistical analyses and graphical representations were conducted using IBM SPSS Statistics 26 and Origin 2018 software.

3. Results and Discussion

3.1. Optimal Temperature, Heat Stability, and Optimal pH of Modified Alkaline Protease

The TGase induces crosslinking reactions between and within molecules of alkaline protease, thus resulting in a structurally more stable, modified alkaline protease. In the preliminary stage of the study, the protein molecular weights of unmodified alkaline proteases 102 and 306 were determined to be 30 kDa and 15 kDa, respectively, through SDS-PAGE analysis, as illustrated in Figure A2. Following modification, the protein molecular weights of alkaline proteases 102 and 306 were observed to be 40 kDa and 20 kDa, respectively. In the preliminary stages of the study, the half-life of modified alkaline proteases 102 and 306 in Tris-HCl buffer (pH 8.0, 50 mM) at 40 °C was determined before and after modification. It was found that the half-life of modified alkaline proteases 102 and 306 increased by 2.1-fold and 2.4-fold, respectively, compared to the original values. The initial enzymatic activities of alkaline proteases 102 and 306 were 200,000 and 330,000 U/g, respectively.

Following modification, both enzymes experienced a reduction in activity, with reductions of 33.62% and 34.76%, respectively, compared to their unmodified counterparts. Under incubation at 40 °C for 24 h, the unmodified alkaline protease 102 practically lost its activity, while the modified alkaline protease 102 retained a residual activity of 154,280 U/g despite experiencing a decline in enzymatic activity. Conversely, the unmodified alkaline protease 306 retained an activity of 41,250 U/g. In contrast, modified alkaline protease 306 essentially lost its activity after the same incubation period.

The trends in optimal temperature and temperature stability of these two alkaline proteases remained similar before and after modification, with both exhibiting an optimal temperature of 60 °C. Following modification, there was a slight improvement in the heat stability of the enzymes. Specifically, the stability of modified alkaline protease 102 increased by 22% compared to its unmodified form after incubation at 40 °C for 2 h. Figure 1 provides further details.

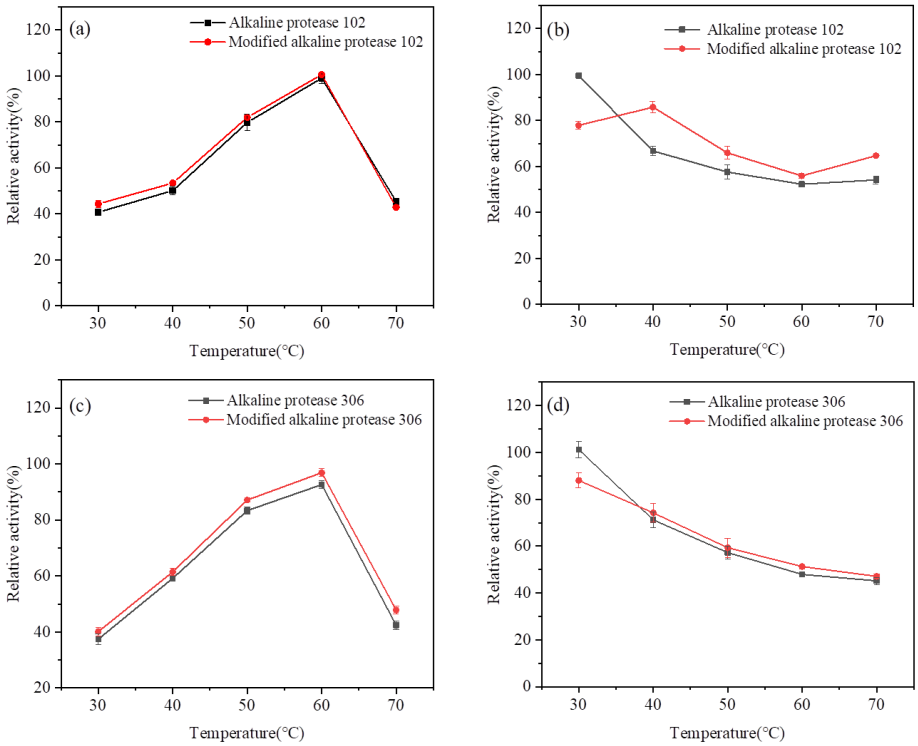

Figure 1. Optimal reaction temperature (**a**) and heat stability (**b**) of alkaline protease 102; optimal reaction temperature (**c**) and heat stability (**d**) of alkaline protease 306.

According to reports, O. Herrera-Márquez et al. found that the protease Bioproteasa L-800 was almost inactivated after 60 min at 60 °C [27]. Similarly, Chen, K. found that the alkaline protease 2709 had a relative enzyme activity of only about 35% after 2 h at 60 °C [28]. This suggests that the conditions for its use as a detergent additive involve cleaning processes conducted at shorter durations or lower temperatures. Conversely, modified alkaline protease 102 retains over 60% relative enzyme activity at 50–60 °C, with 60 °C being the optimal temperature.

The modified alkaline proteases 102 and 306 both demonstrated robust enzymatic activity within the pH range of 8.0–10.0, with relative enzyme activity surpassing 80%. The optimum pH for modified alkaline proteases 102 and 306 was found to be 10.0 and 8.0, respectively, both before and after modification (Figure 2).

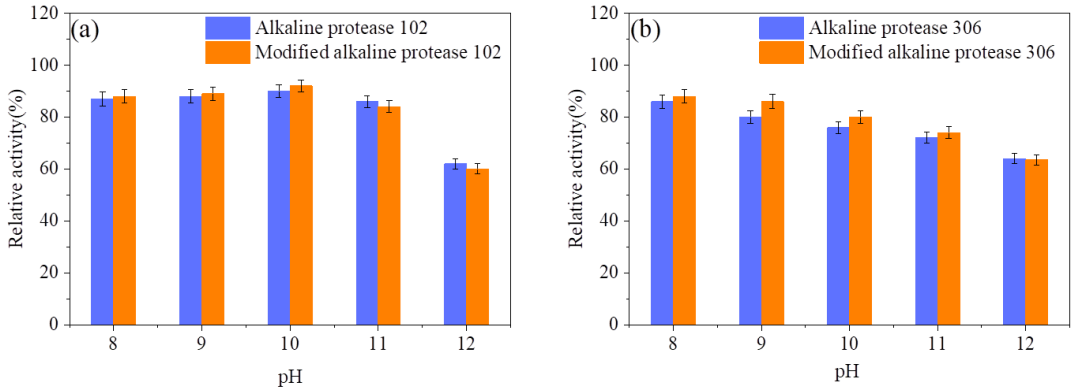

Figure 2. Optimal pH of alkaline proteases. Note: (**a**) The optimal pH for alkaline protease 102 before and after modification. (**b**) The optimal pH for alkaline protease 306 before and after modification.

3.2. Stability of Modified Alkaline Protease in Laundry Detergent Additives

Temperature and optimal pH are critical enzymatic properties that significantly influence the practical application performance of enzymes, particularly in laundry detergents. In this study, 14 laundry detergent additives commonly employed in standard and commercial laundry products were selected to evaluate the stability of modified alkaline proteases 102 and 306 under equivalent enzyme activities. Following a 40 °C incubation for 24 h, modified alkaline protease 102 demonstrated varying degrees of enhanced tolerance to 10 out of the 14 tested detergent additives (Figure 3).

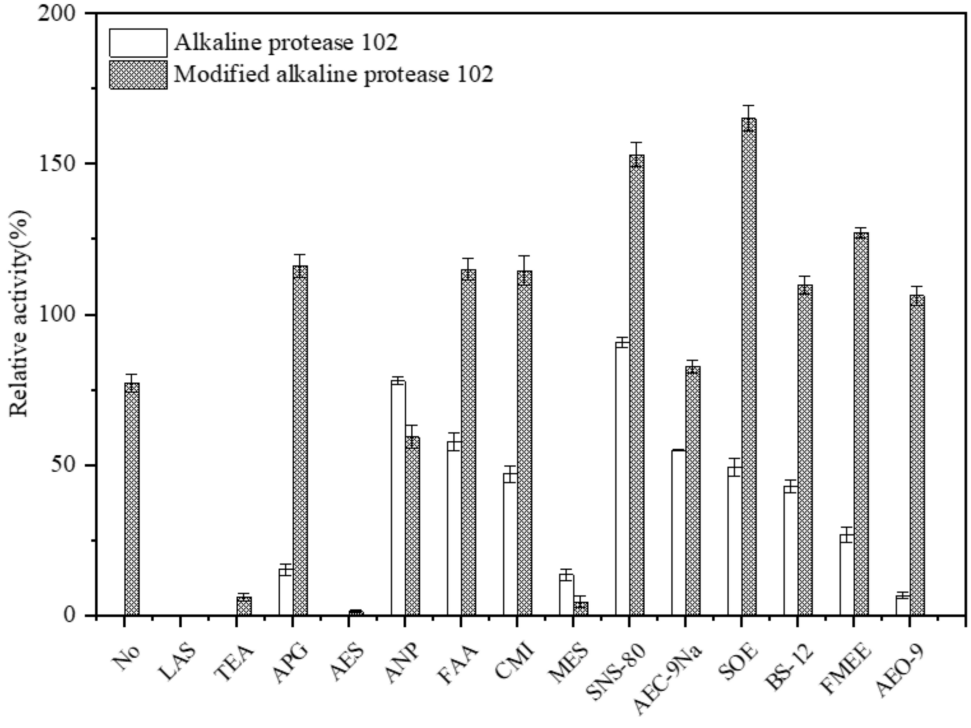

Figure 3. Relative enzyme activity of alkaline protease 102 in different laundry detergent additives.

Modified alkaline protease 102 maintained enzyme activity above 80% in nine types of laundry detergent additives. They exhibited a remarkable increase in activity by 1488.33% in AEO-9 compared to the unmodified enzyme. However, enzyme cross-linking modification methods failed to enhance the stability of alkaline protease 102 for all additives. For instance, in ANP, the enzyme could retain >50% of its activity, but its stability decreased compared to that of the original enzyme. In anionic surfactants such as AES, LAS, and

MES, the stability remained similar to that of the original enzyme, highlighting the need for more suitable methods to improve proteinase stability.

Before modification, alkaline protease 306 completely lost its activity in the MES solution, whereas, after modification, the relative enzyme activity was 165.28%. For the nonionic surfactants FMEE, AEO-9, BS-12, SOE, and FAA, and the anionic surfactant AEC-9Na, the relative enzyme activities increased by 117.08% to 373.66%. Among the essential components of standard laundry detergents MES, AEO-9, and the globally predominant anionic surfactant AES, the relative enzyme activities reached 165.28%, 265.28%, and 86.05%, respectively (Figure 4).

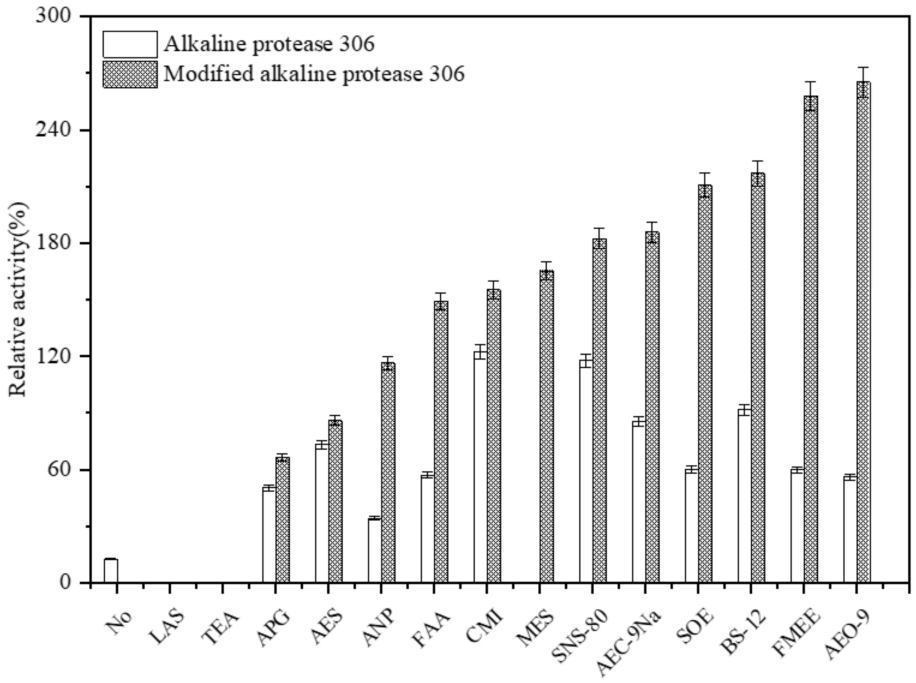

Figure 4. Relative enzyme activity of alkaline protease 306 in different laundry detergents.

The relative enzyme activity of modified alkaline protease 306 in AES, MES, FMEE, AEO-9, and AEC-9Na surpasses that of modified alkaline protease 102 by 102.77% to 6783.72%, where AES serves as an important anionic surfactant. However, in the case of APG, the relative enzyme activity of modified alkaline protease 102 was 74.82% higher than that of modified alkaline protease 306. Both alkaline proteases exhibited variations in stability across different laundry detergents after modification, with modified alkaline protease 306 demonstrating higher overall stability. This distinction holds significant importance for further applications in laundry detergents.

Previous research has highlighted the significant impact of anionic surfactant LAS on enzyme activity. Ethoxy segments in polyethylene oxide nonionic surfactants, such as AEO-9 and FAA, have been shown to mitigate damage to enzymatic activity, which is consistent with the findings of this study. These nonionic surfactants can be incorporated into composite systems to counteract the inhibitory effects of anionic surfactants on enzyme activity [29,30]. Surfactants possessing longer hydrophobic alkyl chains, such as AEO-9, FAA, BS-12, FMEE, and SOE, are associated with lesser damage to enzyme activity [31]. This is attributed to the interaction between the hydrophobic regions of the enzyme molecules and the hydrophobic alkyl chains of the surfactants. This results in a protective effect. Therefore, in formulating enzyme-containing laundry detergents, prioritizing surfactants with longer hydrophobic alkyl chains will enhance compatibility among detergent components.

3.3. Stability of Modified Alkaline Protease in Commercial Laundry Detergents

To assess the stability of the modified alkaline proteases in commercial laundry detergents, we added the alkaline proteases 102 and 306, both before and after modification, to commercially available laundry detergents that had undergone enzyme inactivation (Figure 5).

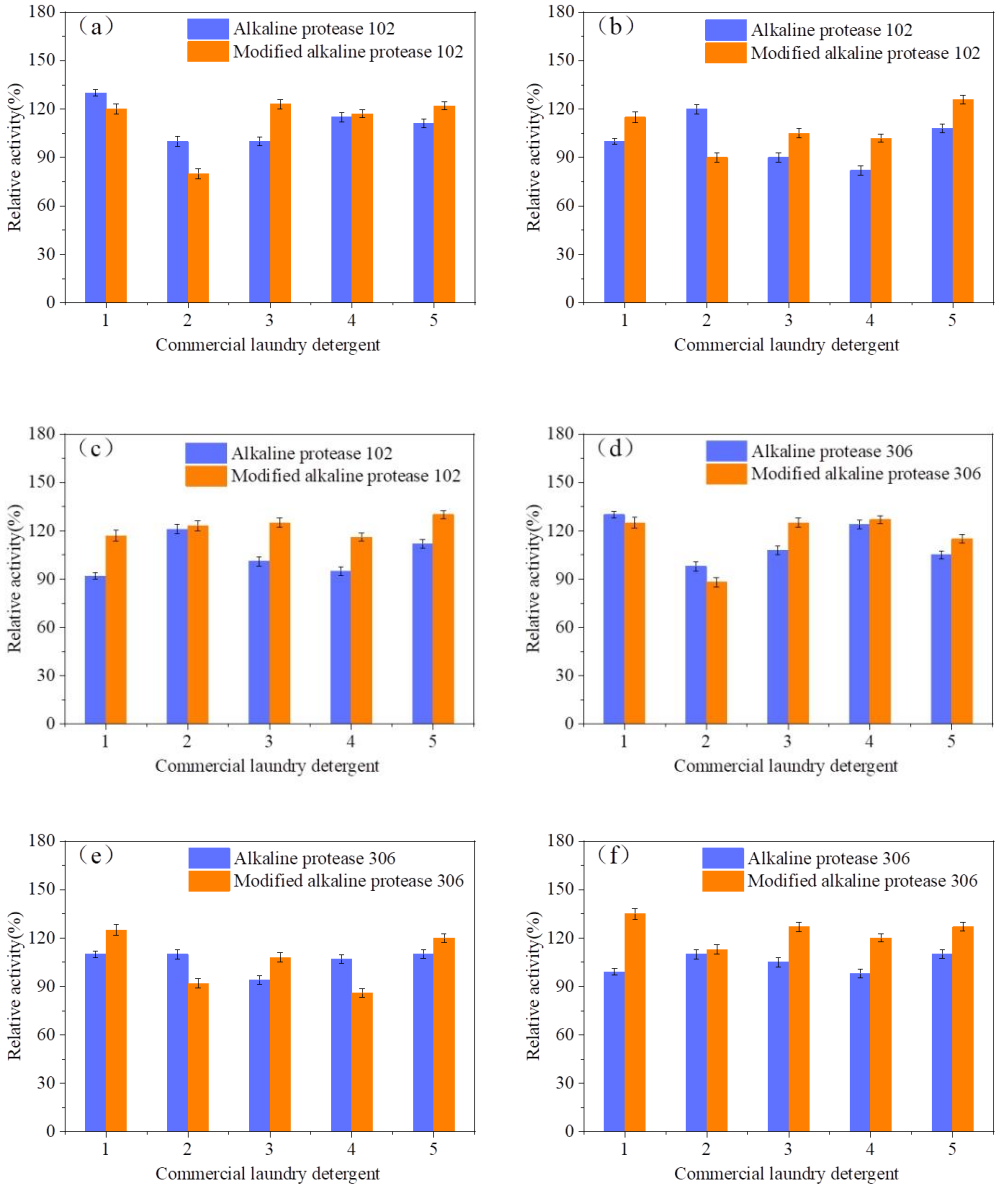

Figure 5. Depicts the relative enzyme activity of alkaline proteases 102 and 306 in commercial laundry detergents. Note: (**a**–**c**) correspond to 30 °C, 40 °C, and 50 °C, and (**d**–**f**) correspond to 30 °C, 40 °C, and 50 °C.

In all tested commercial laundry detergents except for commercial laundry detergent 2, both modified alkaline proteases 102 and 306 demonstrated exceptional compatibility and stability. These modified alkaline proteases exhibited higher activity at 50 °C across all tested commercial laundry detergents, with increases of 35% and 28% in the relative enzyme activity for modified alkaline protease 306 in commercial laundry detergents 1 and 3, respectively. Under the same temperature and detergent conditions, modified alkaline protease 306 displayed better compatibility and stability.

Zhang, J. improved enzyme stability by adding stabilizers to commercially available inactivated laundry detergents or by employing a self-assembly method to encapsulate

alkaline protease [15]. Although this significantly enhanced enzyme stability at 20 °C, stability notably decreased under 40 °C conditions. Tanksale et al. found that alkaline protease immobilized on polyamide using glutaraldehyde retained over 50% of its activity in the presence of Ariel and Surf Excel detergents after 1 h incubation at 28 °C [32]. Compared to previously reported methods, these two modified alkaline proteases exhibited improved compatibility and stability in commercial laundry detergents, particularly at high temperatures, where they maintained higher activity levels. Variations in activity observed in different laundry detergents may be attributed to discrepancies in the chemical formulations of foam regulators, fluorescent brighteners, fillers, water softeners, surfactants, fragrances, preservatives, and other components present. When formulating enzyme-containing laundry detergents, it is advisable to minimize the addition of the anionic surfactant LAS. We should instead prioritize nonionic surfactants as the primary active ingredient [33,34].

3.4. Washing Performance Test of Modified Alkaline Protease

3.4.1. Stain Removal Performance of Modified Alkaline Protease

Laundry detergent A served as the standard laundry detergent, while laundry detergent B included the standard laundry detergent with the incorporation of unmodified alkaline protease. However, laundry detergent C contained the standard laundry detergent with a 1:7 mixture of modified alkaline proteases 102 and 306. Table 1 presents the stain removal performance before and after 40 °C incubation for 24 h.

Table 1. Stain removal performance of different laundry detergents on protein-stained fabric.

Laundry Detergent	Stain Removal Value	Stain Removal Ratio
Laundry detergent A before incubation	17.66 ± 0.69 d	1.00 d
Laundry detergent A after incubation	17.75 ± 0.80 d	1.01 ± 0.05 d
Laundry detergent B before incubation	25.40 ± 1.08 a	1.44 ± 0.07 a
Laundry detergent B after incubation	19. 27 ± 0.64 c	1.09 ± 0.04 c
Laundry detergent C before incubation	25.38 ± 0.71 a	1.44 ± 0.07 a
Laundry detergent C after incubation	23.10 ± 0.88 b	1.31 ± 0.05 b

Note: In intergroup comparisons, lowercase letters denote significant differences between groups ($p < 0.05$).

Owing to the comparable enzyme activities of detergents B and C before incubation, a minimal disparity was observed in the stain removal efficacy on the protein-stained cloth. However, following 40 °C incubation for 24 h, detergent C, the standard detergent augmented with composite-modified alkaline protease, exhibited the most effective stain removal. Both modified alkaline proteases demonstrated commendable compatibility and stability with the laundry aids and commercial laundry detergents examined. Therefore, incorporating composite-modified alkaline protease into laundry detergents has the potential to elevate washing efficiency and enhance the applicability of detergent enzymes.

3.4.2. Characterization of Stain Removal Effect

The condition of the fibers and residual stains between them after washing the protein-stained cloth with various treatments was examined using scanning electron microscopy (Figure 6). Before washing, particles of stain adhering to the fiber surface were observed. While detergents A and B managed to eliminate some of the stain particles from the fiber surface without causing damage after incubation, detergent C, the standard detergent enriched with composite-modified alkaline protease, demonstrated the most effective washing outcome, with no visible stain particles observed on the fiber surface.

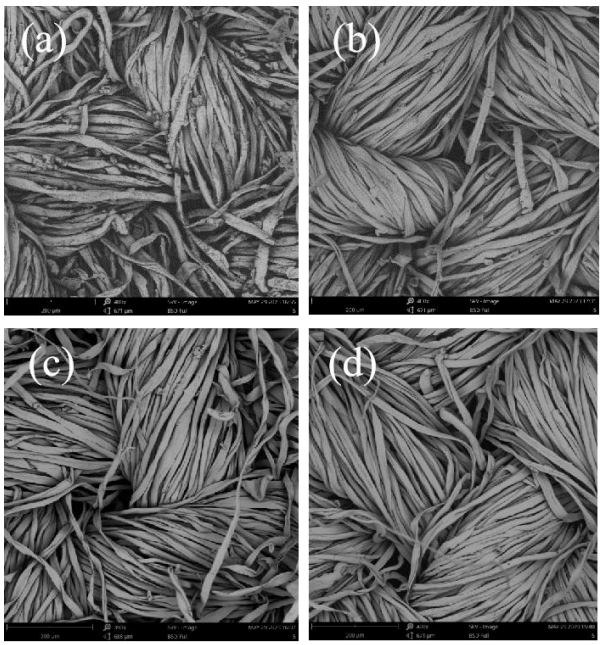

Figure 6. Microscopic characterization after washing protein-stained cloth. (**a**) Unwashed soiled cloth; (**b**) cloth washed with laundry detergent A after incubation; (**c**) cloth washed with laundry detergent B after incubation; (**d**) cloth washed with laundry detergent C after incubation.

3.4.3. Residual Element Analysis

To provide a more precise valuation of the cleaning efficacy of the modified alkaline protease on the protein-stained fabric and ascertain its capacity to resist stain residues, XPS was employed to analyze the residual nitrogen elements in the fabric samples. Figure 7 presents the results.

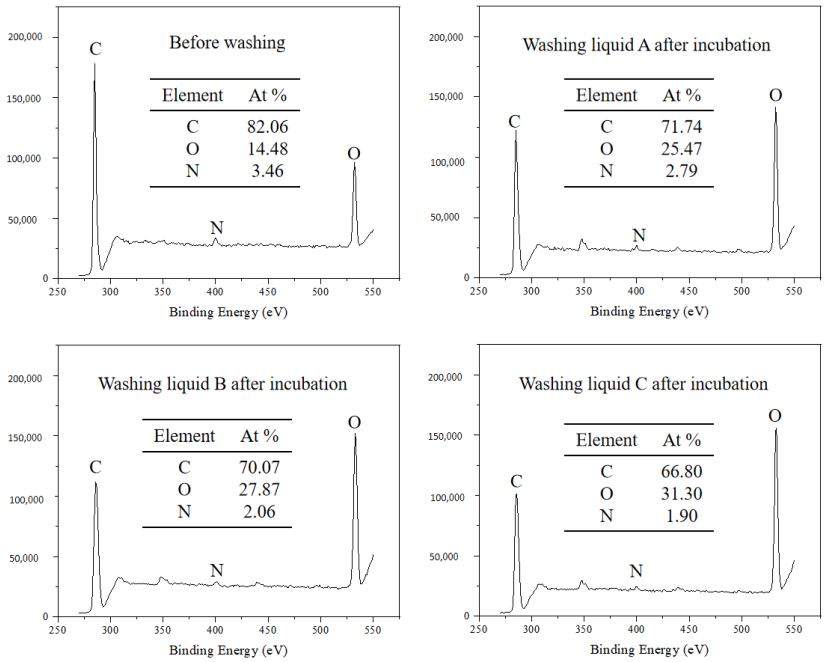

Figure 7. Surface element analysis after washing protein-stained fabric.

After washing the stained fabric with various laundry detergents, the elemental composition of the cotton fabric remained unchanged compared to the unwashed stained fabric. All samples contained the same chemical elements (C, O, and N). However, upon

comparing the atomic concentrations of these elements, it was observed that the cotton fabric washed with laundry detergent C, that is, the standard laundry detergent augmented with composite-modified alkaline protease, displayed the lowest atomic concentration of N. This suggests minimal residue of the protein stain, aligning with the findings of stain-removal value testing and stain-removal effectiveness characterization. The enzyme-assisted washing procedure resulted in deep cleaning.

4. Conclusions

This study employed substrate imprinting technology and the TG enzyme for the cross-linking modification of alkaline proteases 102 and 306. The results indicate that the modified alkaline proteases exhibited significantly improved tolerance to laundry detergents while maintaining their original optimal temperature, heat stability, and optimal pH. Among the 12 tested laundry detergent additives, excluding LAS and TEA, both modified alkaline proteases demonstrated notably enhanced stability. Specifically, the activity of modified alkaline protease 102 increased nearly 15-fold in AEO-9, while the activity of pre-modified alkaline protease 306 was completely lost in MES solution, and after modification, its relative enzyme activity reached 165.28%. Due to their enhanced stability post-modification, these alkaline proteases showed broader adaptability to various commercially available laundry detergents. Upon incorporation into a standard laundry detergent in a 1:7 ratio, the composite-modified alkaline proteases exhibited enhanced stability and efficacy in stain removal. In summary, the modified alkaline proteases investigated in this study hold promising prospects for application in the detergent industry and can provide valuable insights for its advancement.

Author Contributions: Conceptualization and Methodology: H.Y. and J.X.; Validation: H.Y. and X.R.; Data Analysis: Y.Z.; Investigation: T.X. and H.C.; Writing—Original Draft preparation: H.Y. and X.R.; Writing—Review and Editing: H.Y. and J.X.; Funding Acquisition: J.X. All authors have read and agreed to the published version of the manuscript.

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Appendix A

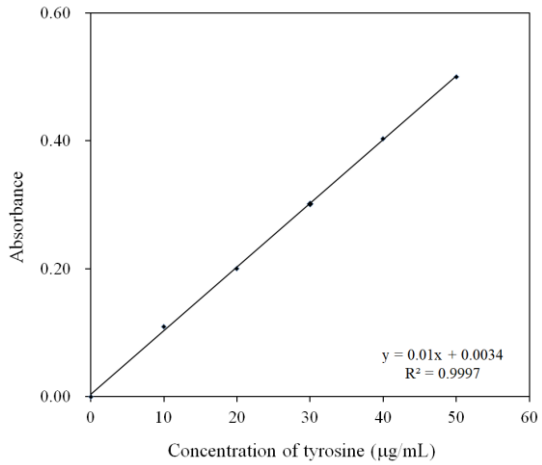

Figure A1. Tyrosine standard curve.

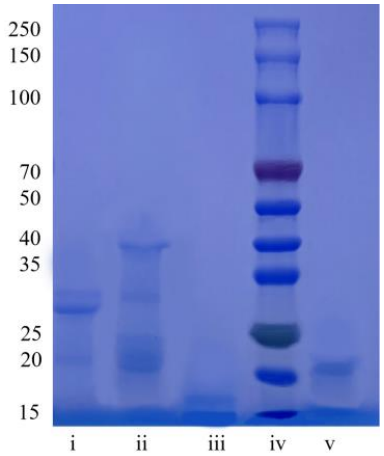

Figure A2. The molecular weights of the alkaline protease before and after modification. Note: (i) alkaline protease 102; (ii) modified alkaline protease 102; (iii) alkaline protease 306; (iv) molecular weight marker; (v) modified alkaline protease 306.

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