ISOLATION, PARTIAL PURIFICATION, AND CHARACTERIZATION OF ALKALINE PROTEASE ENZYME FROM SOYBEAN AND ITS APPLICATION

Ei Pa Pa Zaw[1], Jue Jue Khin[2], Ye Myint Aung[3]

Abstract

This study focuses on the isolation, purification, and characterization of alkaline protease enzyme (EC 3.4.21.63) from soybean. The enzyme was extracted using a Tris base buffer solution and purified through 30–60% ammonium sulfate precipitation followed by gel filtration chromatography on a Sephadex G-100 column. The purification process achieved a 3-fold increase in purity with a protein recovery rate of 5.8%. Qualitative confirmation of the enzyme was demonstrated using a ninhydrin test, while the enzyme activity was quantified at 2734.96 EU/g. Characterization of the enzyme revealed an optimum pH of 9.0 and an optimum temperature of 55 °C. Enzyme kinetics were studied, yielding an activation energy (E_a) of 4.553 kcal/mol, a Michaelis constant (K_m) of 0.67×10^{-2} g/mL, and a maximum velocity (V_{max}) of 60.97×10^{-3} mM/min using Lineweaver-Burk plot. The reaction order was determined to be first-order. The enzyme exhibited pH stability and thermostability under tested conditions. Finally, the potential application of the crude alkaline protease enzyme in the detergent industry was evaluated, showing its effectiveness as a laundry detergent additive. These findings highlight its practical utility in industrial applications, particularly in environmentally friendly cleaning formulations.

Keywords: Soybean, alkaline protease enzyme, *gel filtration chromatography,* K_m, V_{max}, laundry detergent additive

Introduction

Enzymes are proteins that help speed up metabolism, or the chemical reactions in our bodies. They build some substances and break others down. All living things have enzymes. They can also be extracted from cells and then used to catalyze a wide range of commercially important processes. Proteases, also known as proteinases or proteolytic enzymes, are a large group of enzymes that catalyze the hydrolysis of peptide bonds in proteins and polypeptides (Rao *et al*., 1998). Proteases may be classified into acidic, neutral, and alkaline (basic) proteases according to the optimal pH. The proteases with pH optima in the range of 2.0-5.0 are called acid proteases; proteases having pH optima around 7.0 are neutral proteases, and alkaline proteases have pH optima in the range of 8.0-11.0. Neutral and alkaline proteases have a great application potential in the enzyme detergent and leather industry because of the increasing trend of development of ecofriendly technologies (Sawant and Nagendran, 2014). Proteases are usually produced in small amounts with high purity: they thus require large-scale purification processes. The reactants in an enzymatic chemical reaction are called substrates. Casein has a variety of uses, from those used as the main ingredient in cheese to those used as food additives (Lucey, 2002). A milk protein called casein can cause unnecessary distress in people with dairy allergies. Casein can used in paints, inks, coatings, emulsion stability, cement-based applications (Dyson and Wright, 2002).

Soybean, a rich source of protein, similar to beef in its amino acid composition, has been used as an inexpensive source of high-quality protein for humans and animals (Whitehurst and Oort, 2010). It is well reported that the consumption of soy protein in place of animal protein

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lowers blood cholesterol levels and may provide other cardiovascular benefits. The present study aimed to study the kinetic behaviour of the alkaline protease enzyme extracted from *soybeans.*

Materials and Methods

Sample Collection

Soybean (*Glycine max* (L.) Merr.) samples–were collected from local market, Yangon Region. Then, identification of the sample was done at the Department of Botany, University of Yangon. Sample extraction and purification were performed at the Analytical Chemistry Research Laboratory, Department of Chemistry, University of Yangon.

Materials

Casein and all other chemicals used in this work were of analytical grade and were products of BDH Chemical Limited (England).

Sample Isolation

Soybean (20 g) were washed and placed in distilled water, allowed to stand overnight and then homogenized for 3 min (as appropriate) using a blender (Philips, China) with 100 mL of 0.5 M tris base buffer pH 9.0 and stirring for 1 h. The homogenate was filtered through cheesecloth. The filtrate was centrifuged for about 30 min at 3000 rpm to obtain 75 mL of extract. Solid ammonium sulphate (13.18 g mL^{-1}) was added to this extract to obtain 30% saturation and stirred and allowed to stand for 2 h at 4°C. After standing for 2 h, the suspension was centrifuged (3000 rpm, 30 min), the precipitates were discarded, and supernatant liquid 66 mL was brought to 60% saturation with (12.87 g mL^{-1}) of solid ammonium sulphate and stored at 4°C (Adulyatham and Owusu-Apenten, 2005). After standing overnight, the precipitated protein containing alkaline protease enzyme was collected by centrifugation for 30 min at 3000 rpm (Ahmad *et al*., 2014).

Qualitative Test for Alkaline Protease Enzyme by Ninhydrin Test

Test tube A containing 1 mL of enzyme solution and 3 mL of ninhydrin solution was swirled for 30 min. Test tube B containing 1 mL of enzyme solution and 1 mL of 1% casein solution was incubated at 37°C for 15 min. After that, 1 mL of 5% TCA was added to stop the reaction and was centrifuged for 10 min at 3000 rpm. After centrifugation, 1 mL of supernatant was mixed with 3 mL of ninhydrin solution and incubated for 5 min. The colour of test tubes A and B after the reaction with ninhydrin was compared.

Enzyme Purification

The crude alkaline protease enzyme was dissolved in tris base buffer (0.5 M, pH 9.0) and then put into the column filled with Sephadex G-100 equilibrated with tris base buffer. The flow rate was adjusted to 1.5 mL per 3 min. A 1.5 mL fraction was collected per tube using a fraction collector. After collection, each fraction was checked for protein content by measuring the absorbance at 280 nm and enzyme activity by measuring the amount of tyrosine released from casein at 275 nm. The fraction with the highest enzyme activity was pooled to obtain a partially purified enzyme and stored at 4°C.

Assay of Partially Purified Enzyme

The enzyme activity was determined by measuring the release of sugar groups. In brief, 0.1 mL of enzyme solution and 0.4 mL of pH 9.0 Tris base buffer solution were mixed and kept

at room temperature for 30 min. Then 1 mL of 1% casein solution was added to the mixture and incubated at 37°C for 30 min. After that, 2 mL of 5% TCA solution was added to stop the reaction. The absorbance of the mixture was measured at 275 nm by using a UV-visible spectrophotometer. The enzyme unit (EU) of the alkaline protease enzyme from soybean was determined at 2734.96 EU/g.

Effects of pH, Temperature, and Enzyme Concentration of Alkaline Protease

To find out the optimum pH of the alkaline protease produced by soybean, the activity of the partially purified enzyme was measured at different pH values. The Tris base buffer solution (pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0) was used to determine the activity of alkaline protease as described above. Effect of temperature on protease activity was studied by incubating the reaction mixture at different temperatures ranging from 30°C to 70°C with a 5°C interval. The reactions were carried out at the optimum pH of 9, and the activity of the enzyme was measured. Different enzyme concentrations (1.0, 1.5, 2.0, 2.5, and 3.0%) were used to study the effect of enzyme concentration. The reactions were carried out at pH 9 and 37°C.

Kinetic Studies of Alkaline Protease Enzyme

The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of partially purified enzymes were determined using casein as a substrate in the range of 2 mg $mL^{-1}$to 14 mg mL^{-1}. The reaction order and activation energy of the alkaline protease-catalyzed reaction were also evaluated.

Application of the Alkaline Protease Enzyme by Wash Performance Test

The three pieces of white cotton (5 × 5 cm) were stained by soaking in a tea-mix solution and left overnight to ensure uniform absorption of the stain. Then, they were washed with an alkaline protease enzyme solution (0.1 mL and 0.3 mL) and a 1% detergent solution. The stain removal of the enzyme was studied along with the same amount of detergent solution. The washing process was performed for 0, 30, 60, and 90 min, after which the residual absorbance was measured by the UV-visible spectrophotometric method at 280 nm for protein detection. The effectiveness of stain removal was quantified by measuring the change in absorbance.

Results and Discussion

Purification of Alkaline Protease Enzyme from Soybean

Enzyme purification is essential for various industrial applications of enzymes. The impurities present in the enzyme solution can affect the enzyme's stability, activity, and specificity, which can, in turn, affect the final product's quality and yield. Purified enzymes are more stable and active, and their specificity is higher, making them more efficient and effective in industrial processes (Liu *et al*., 2012). Figure 1 shows the chromatogram of alkaline protease on Sephadex G-100 gel. The protein content of the eluate was checked spectrophotometrically at 280 nm, and the enzyme activity was determined at 275 nm. The fractions with the highest activity (13-26 fraction number) were pooled. The partially purified alkaline protease enzyme was purified up to 3 folds with a specific activity of 0.00110 unit/mg over crude extract. The enzyme activity, specific activities of the enzyme solutions, and purity of the enzyme were described in Figure 1 and Table 1.

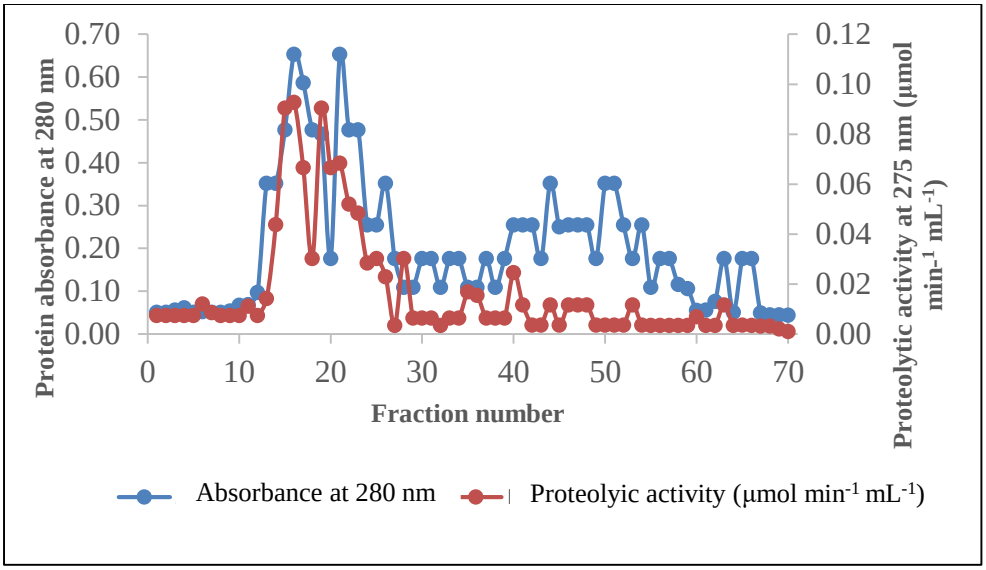

Figure 1. Purification of crude alkaline protease enzyme by Sephadex G-100 gel filtration chromatograph

Table 1. Alkaline Protease Enzyme Activities, Protein Contents and Specific Activities of the Enzyme Solution at Different Purification Steps

Fraction	Total volume (mL)	Total enzyme activity (unit)*	Total protein content (mg)	Specific activity (unit/mg)**	Protein recovery (%)	Degree of Purity (fold)
crude	85	13916.2	5.185	0.00037	100.00	1.00
30 % $(NH_4)_2SO_4$ filtrate	75	7708.5	4.050	0.00050	55.4	1.35
60 % $(NH_4)_2SO_4$ precipitate	66	4047.1	2.574	0.00063	29.1	1.70
after passing the Sephadex G-100	34	804.4	0.850	0.00110	5.80	3.00

* µmol min^{-1} mL^{-1} ** µmol min^{-1} mg^{-1}

Proteolytic Activity of Alkaline Protease Enzyme by Ninhydrin Test

The ninhydrin test assesses proteolytic activity by detecting free amino acids and amines, products of protease enzyme action on proteins. A positive result is indicated by deep blue or purple colour, reflecting successful hydrolysis. Conversely, a lack of colour change signals that no significant protein breakdown occurred, suggesting either insufficient enzyme activity or low enzyme concentration (Figure 2).

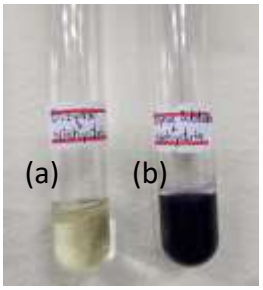

Figure 2. Ninhydrin test to detect presence of alkaline protease enzyme

(a) enzyme + ninhydrin: no colour change

(b) supernatant from enzyme substrate reaction mixture + ninhydrin: colour change to purple

Optimum pH for Alkaline Protease Enzyme Activity

In the present research, the tris base buffer solution (pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0) was used to determine the activity of alkaline protease. The nature of the activity vs pH curve of the enzyme (Table 2 and Figure 3) was found to be symmetrical, and it was found that the optimum pH for maximum alkaline protease enzyme activity was pH 9. This value is in agreement with the literature (Sawhney, 2000) which ranges between pH 7 and 11 for the alkaline protease enzyme. From lower to optimum pH, the activities increased with pH gradually, and beyond optimum pH, the activities decreased rapidly due to the denaturing of enzyme proteins (Bhaskar *et al*., 2006).

Table 2. Relationship between Alkaline Protease Activity and pH of the Tris Base Buffer Solution

No.	pH	Absorbance at 275 nm	Protease activity (μmol $min^{-1}mL^{-1}$)
1	7.00	0.053	0.009
2	7.50	0.087	0.016
3	8.00	0.124	0.022
4	8.50	0.172	0.031
5	9.00	0.202	0.037
6	9.50	0.165	0.030
7	10.0	0.105	0.019
8	10.5	0.055	0.010
9	11.0	0.018	0.003

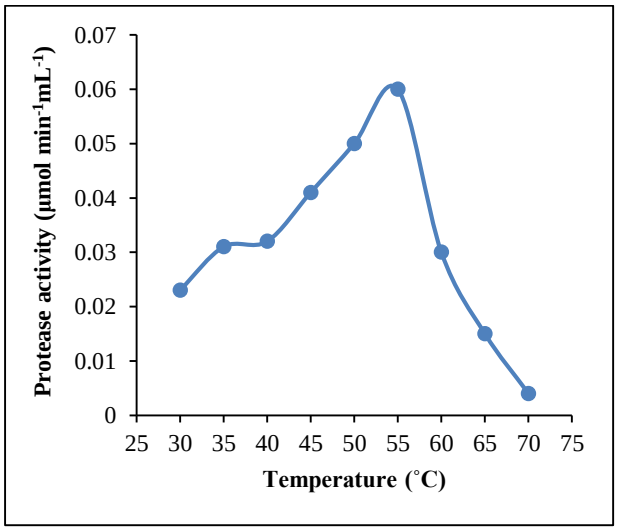

Figure 3. Plot of alkaline protease activity as a function of pH of the solution

Optimum Temperature for Alkaline Protease Enzyme Activity

In this study, the effect of temperature on the alkaline protease activity was investigated in the temperature range of 30 to 70°C. The optimum temperature for alkaline protease was found to be 55 °C in Tris base buffer pH 9.0 (Table 3 and Figure 4). It was found that the activity of alkaline protease increased from 30 to 55°C and then decreased from 55 to 70°C.

Table 3. Relationship between Protease Activity and Temperature of the Solution at pH 9.0

No.	Temperature (°C)	Absorbance at 275 nm	Protease activity (µmol $min^{-1}mL^{-1}$)
1	30	0.125	0.023
2	35	0.168	0.031
3	40	0.177	0.032
4	45	0.220	0.041
5	50	0.263	0.050
6	55	0.306	0.060
7	60	0.143	0.030
8	65	0.080	0.015
9	70	0.020	0.004

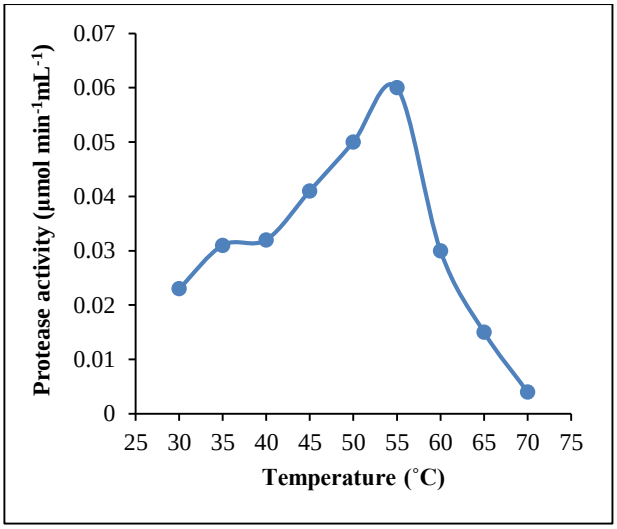

Figure 4. Plot of alkaline protease activity as a function of temperature of the solution at pH 9.0

Activation Energy for Alkaline Protease-Catalyzed Reaction

Enzymes are regarded as lowering the activation energy of a system by making it energetically easier for the transition state to form. In the presence of an enzyme catalyst, the formation of the transition state is energetically more favorable, thereby accelerating the rate at which the reaction will proceed but not fundamentally changing the energy levels of either the reactant or the product (Atkins, 1994). Table 4 shows the relationship between velocity of the protease-catalyzed reaction and temperature. Figure 5 shows a plot of Log V as a function of 1/T for the determination of activation energy and Arrhenius constants. Arrhenius constants of alkaline protease-catalyzed reactions were found to be 3.211×10^6. The activation energy (E_a) values of partially purified alkaline protease-catalyzed reaction were determined to be 4.553 kcal mol^{-1} from linear regression method.

Effect of Enzyme Concentration on Alkaline Protease-Catalyzed Reaction

In this present work, the validity of the enzyme assay method was tested using different concentrations of enzyme: 1.0, 1.5, 2.0, 2.5, and 3.0%. The enzyme activity was found to have a linear relationship with different enzyme volumes (Table 5 and Figure 6). It was found that the higher the enzyme concentration, the higher the alkaline protease enzyme activity in accordance with the literature (Walsh, 1968).

Table 4. Relationship between Alkaline Protease Enzyme Velocity and Temperature of the Solution at pH 9

No.	Temperature (°C)	Temperature (K)	1/T × 10^3 (K^{-1})	V (mM min^{-1})	Log V
1	30	303	3.300	0.0085	-2.0705
2	35	308	3.246	0.0094	-2.0268
3	40	313	3.194	0.0106	-1.9746
4	45	318	3.144	0.0119	-1.9244
5	50	323	3.095	0.0137	-1.8632
6	55	328	3.048	0.0147	-1.8326

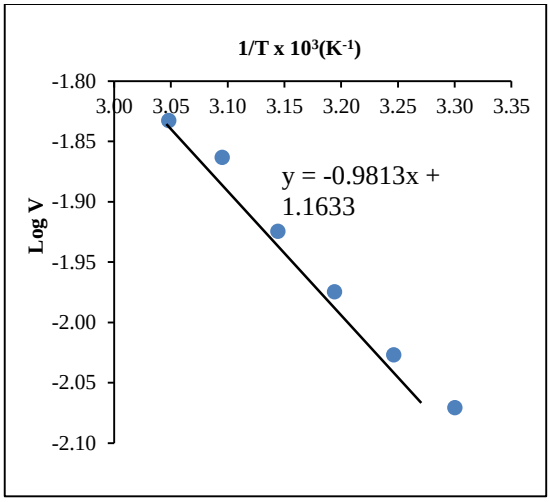

Figure 5. Plot of Log V as a function of 1/T for the determination of E_a

Table 5. Relationship between Enzyme Concentration and Activity of Alkaline Protease-Catalyzed Reaction

No.	Enzyme concentration (%)	Protease activity (µmol min^{-1} mL^{-1})
1	1.0	0.014
2	1.5	0.019
3	2.0	0.028
4	2.5	0.034
5	3.0	0.041

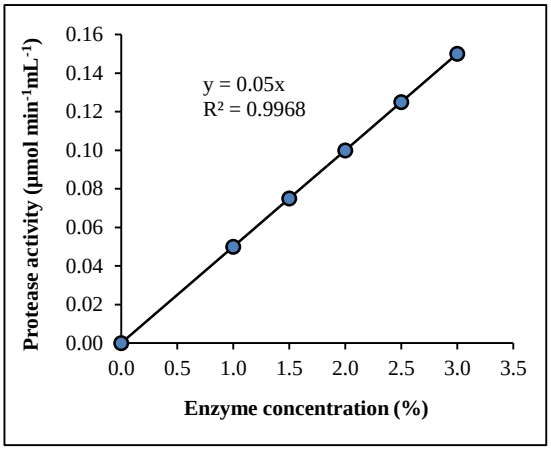

Figure 6. Plot of alkaline protease activity as a function of enzyme concentration of the solution

Effect of Substrate Concentration on Alkaline Protease-Catalyzed Reaction

In the present study, the velocities of enzyme reactions measured at varying levels of casein concentration and their reciprocal values are shown in Table 6. The Michaelis-Menten plot of V vs. [S] is shown in Figure 7. The alkaline protease enzyme reaction is followed by a hyperbolic curve. As the concentration of casein was increased, the rate of reaction also increased until a point was reached where the enzyme was working as fast as it could; after that, it was transforming its maximum number of casein molecules each minute (White *et al.*, 1968). At this point, the enzyme was said to be saturated with substrate, and further increases in the concentration of the casein would not increase the rate of reaction. The enzyme could have worked no faster. For the more accurate estimation, K_m and V_{max} values were computed using statistical and various graphical methods (Figures 8 and 9), and the values are presented in Table 7.

Table 6. Relationship between Substrate Concentration and Velocity of Alkaline Protease-catalyzed Reaction

No.	[S]*×10^2 (g mL^{-1})	-[S] ×10^2 (g mL^{-1})	1/[S] ×10^{-2} (g^{-1} mL)	V×10^3 (mM min^{-1})	1/V×10^{-3} (mM^{-1} min)	V/[S] ×10^{-1} (mM min^{-1} g^{-1}mL)	[S]/V×10^1 (g mL^{-1} mM^{-1} min)
1	0.2	-0.2	5.00	13.644	0.073	68.220	0.014
2	0.4	-0.4	2.50	22.532	0.044	56.333	0.017
3	0.6	-0.6	1.67	28.925	0.034	48.208	0.020
4	0.8	-0.8	1.25	33.214	0.030	41.517	0.024
5	1.0	-1.0	1.00	35.942	0.027	35.942	0.027
6	1.2	-1.2	0.83	38.125	0.026	31.771	0.031
7	1.4	-1.4	0.71	39.763	0.025	28.402	0.035

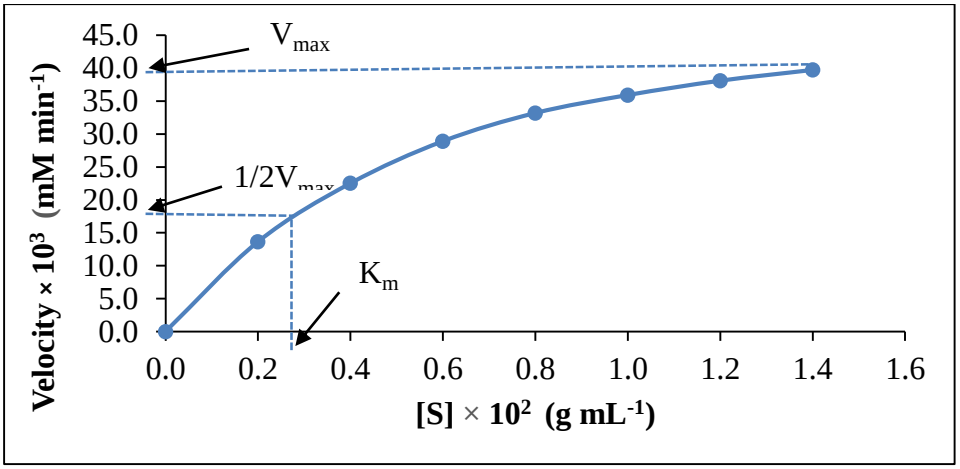

Figure 7. Michaelis-Menten plot of alkaline protease-catalyzed reaction

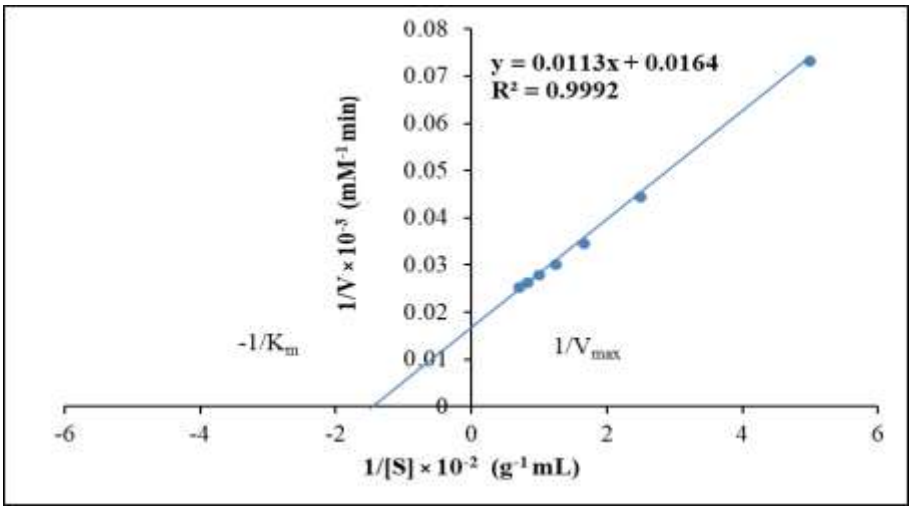

Figure 8. Lineweaver-Burk plot of 1/V vs. 1/[S] for alkaline protease-catalyzed reaction

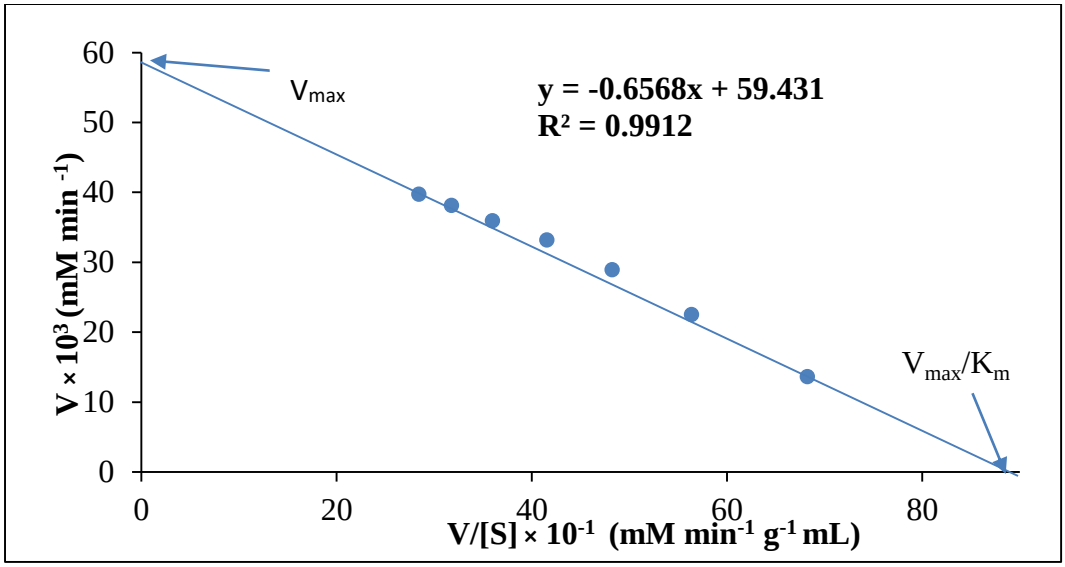

Figure 9. Eadie-Hofstee plot of alkaline protease-catalyzed reaction

Table 7. V_{max} and K_m Values of Alkaline Protease Enzyme using Statistical and Graphical Methods

Methods	Statistical		Graphical	
	$K_m \times 10^2$ (g mL^{-1})	$V_{max} \times 10^3$ (mM min^{-1})	$K_m \times 10^2$ (g mL^{-1})	$V_{max} \times 10^3$ (mM min^{-1})
Michaelis-Menten	-	-	0.33	39.76
Lineweaver-Burk	0.67	60.97	0.67	59.82
Eadie-Hofstee	0.66	59.43	0.66	59.62

Reaction Order on Alkaline Protease-catalyzed Reaction

A reaction specified by the transformation of one molecule of A to one molecule of B with no impact from any other reactant or solvent is a first-order reaction (Martin, 1993). In this study, the 'n' value was determined from the plot of Log V/ (V_{max} – V) *vs.* Log [S] for alkaline protease activity using the linear regression method (Table 8 and Figure 10). The reaction order (n) was observed to be a first-order reaction for the alkaline protease enzyme due to the evaluated value being 1.0437.

Washing Performance Test of Alkaline Protease Enzyme

In this present work, the detergent washing activity used 0.1 and 0.3 mL of enzyme solution and was compared with the control. In the case of removing protein stains from tea-mix-stained clothes, it was seen that the alkaline protease was able to remove tea-mix stains very easily. Tea-mix stains often contain tannins, proteins, and carbohydrates from tea or additives. The proteins in these stains bind to fabric fibers, making the stain difficult to remove with just water. The addition of protease in detergent formulation is effective in removing proteinaceous stained since it can breakdown the protein into smaller polypeptides and make it easier to be

removed (Indhumathi *et al*., 2023; Noor *et al*.,2020). The highest stain removal percentage (96.59%) was obtained from test sample-2 at 90 min. It was indicated that the higher the additive amount of enzyme, the higher the removal percentage of stains on the fabric (Table 9 Figure 11).

Table 8. Relationship between Log]S] and Log V/(V_{max}-V) for Alkaline Protease--Catalyzed Reaction

No.	Log [S]	Log V/(V_{max}-V)
1	-2.699	-0.5406
2	-2.398	-0.2319
3	-2.222	0.0498
4	-2.097	0.0779
5	-2.000	0.1571
6	-1.921	0.2221
7	-1.854	0.2729

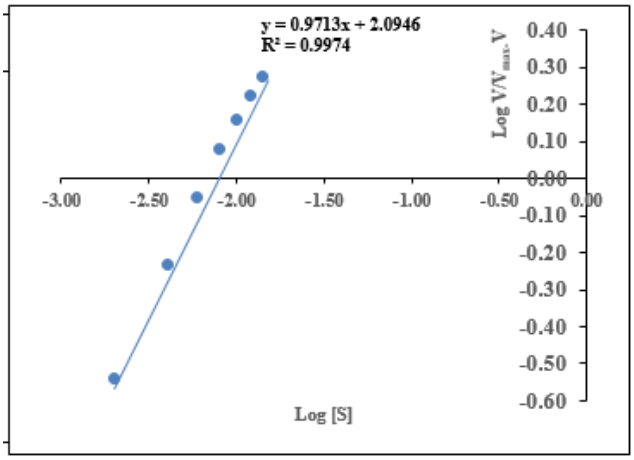

Figure 10. Plot of log V/V $_{max}$-V as a function of log [S] of alkaline protease-catalyzed reaction

Table 9. Stain Removal Percentage Using Alkaline Protease Enzyme in Wash Performance Test

Washing time (min)	Stain removal (%)		
	A	B	C
30	44.58	63.69	64.63
60	56.24	80.38	84.69
90	62.49	90.19	96.60

A = Control (without enzyme), B = Test sample-1 (with 0.1 mL of enzyme solution)

C = Test sample-2 (with 0.3 mL of enzyme solution)

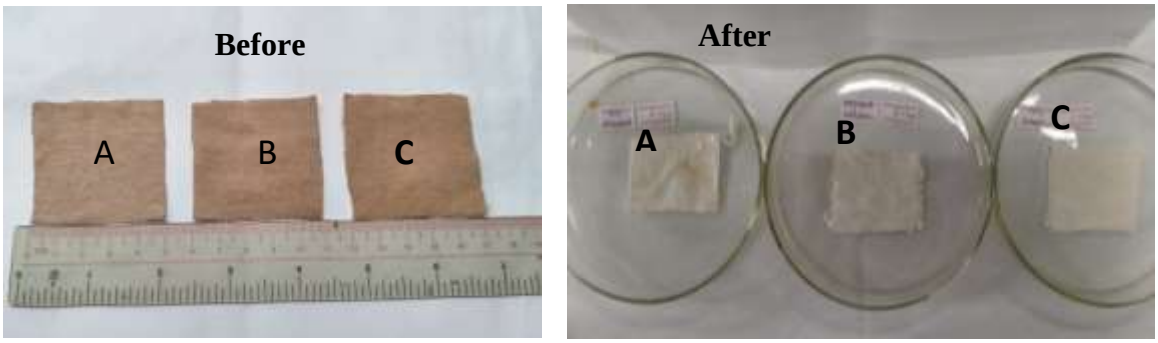

Figure 11. Washing performance on tea-stained fabric (5 × 5 cm) using alkaline protease enzyme; A = Control (without enzyme), B = Test sample-1 with 0.1 mL of enzyme solution), C = Test sample-2 (with 0.3 mL of enzyme solution)

Conclusion

In this research, alkaline protease enzyme was extracted from soybean. The alkaline protease enzyme was purified 3-fold over crude extract after gel filtration chromatography. The enzyme exhibited optimal activity at pH 9.0 and 55°C, with kinetic parameters K_m=0.67×10^{-2} g/mL and V_{max}=60.97×10^{-3} mM min^{-1}. The application of alkaline protease enzyme in laundry detergent was tested by using tea-stained fabric. The enzyme achieved a maximum stain removal efficiency of 96.60% within 90 min using 0.3 mL of the enzyme solution. These findings confirm that soybean-derived alkaline protease is an effective additive for laundry detergents, significantly enhancing their cleaning performance.

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