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Factors Influencing Activity of Enzymes and Their Kinetics

Bioprocessing of Skin

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Abstract

The conventional chemically based method of dehairing and fiber-opening discharges an enormous amount of pollutants in the processing of skins. Hence, bioprocessing of skin through a two-step process, dehairing using protease and fiber opening using α-amylase, has been developed. However, because this process involves two steps, we characterized commercial protease and α-amylase for their optimum activity and determine the influence of one enzyme on the activity of the other, in order to develop an integrated enzymatic dehairing and fiber-opening process. The influence of various factors, substrate concentration, time, pH, and temperature, on the activity of both protease and α-amylase was determined. Furthermore, the activity of protease on mixing with α-amylase and vice versa was investigated. It was found that there was no significant change in the activity of one enzyme in the presence of the other. Lineweaver-Burk plots showed K_m and V_{max} values of 31.6 mg/mL and 0.0106 mg/(mL@min) for protease and 8.79 mg/mL and 0.0912 mg/(mL@min) for α-amylase. This study provides substantial evidence for integrating the enzyme-based dehairing and fiber-opening processes using both the selected protease and α-amylase in one step.

Index Entries: Protease; α-amylase; dehairing; fiber opening; pollution; leather.

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Introduction

The conventional method of dehairing and fiber opening of skins and hides involves the use of chemicals such as sodium sulfide and lime. These processes discharge an enormous amount of pollutants, which account for nearly 60–70% of the total pollution from tannery wastewater apart from enormous amounts of lime sludge *(1)*. In the twentieth century, several lime and sulfide-free liming methods were introduced *(2)*. However, only partial replacement of sulfide has been possible in such applications. There has been a paradigmatic shift from chemical to bioprocessing in leather manufacturing in order to meet the growing challenges of the leather industry.

Enzymes are biomolecules that act as catalysts. Their specificity offers much finer product control, and the fact that they require low energy inputs and mild conditions, making them highly efficient, has distinct environmental advantages. They are biodegradable, being readily absorbed back into nature. Enzymes have been widely used in the manufacture of leather in the soaking, dehairing, bating, and degreasing processes *(3)*. The global application of enzymes for dehairing, nevertheless, requires lime and sodium sulfide. Furthermore, the opening of fiber bundles and fibers is conventionally carried out using lime. Thanikaivelan et al. *(4)* have recently established an environmentally friendly two-step process for dehairing using protease and fiber opening using α-amylase for cow hides. Saravanabhavan et al. *(5)* and Aravindhan et al. *(6)* have developed similar two-step processes for goat and sheep skins. There is a need to develop a single-step process for achieving dehairing and fiber opening in which protease and amylase are combined, in order to make the process commercially practicable. However, the addition of two enzymes in a process may cause the activity of one enzyme to affect the activity of the other. Hence, it is necessary to study the activity and reactivity of these enzymes under different process conditions.

In the present work, the optimal activity of commercially available protease and α-amylase at different conditions, time, temperature, pH, and substrate concentration, was investigated. The influence of protease on the activity of α-amylase and α-amylase on the activity of protease was scrutinized for developing an integrated dehairing and fiber-opening process. A kinetic study for both protease and α-amylase on casein and starch, respectively, was also conducted to obtain the reactivity of the enzymes.

Materials and Methods

Chemicals

Commercial-grade protease (bacterial) and α-amylase (bacterial) were obtained from Southern Petrochemical Industries Corporation Limited (Chennai, India). All chemicals used for the preparation of assay and analysis were of analytical grade.

Protease Assay

Protease activity was assayed by the method of Kunitz *(7)* with some modifications. Enzyme solution (0.1 mL) was incubated with 1 mL of the substrate (2% casein in Tris buffer, pH 8.0) for 2 h at 30°C. The addition of 2 mL of 5% trichloroacetic acid precipitated the residual protein. The precipitate was allowed to settle for 30 min. The contents of the tubes were centrifuged at 10,000 rpm for 20 min (7840*g*) After centrifugation, a 1-mL aliquot of supernatant was mixed with 2 mL of 1 *N* NaOH to alkalinize the contents of the tube. After 10 min, 0.5 mL of Folin and Ciocalteu phenol reagent was added and the contents were mixed. The absorbance of the blue color produced was measured at 750 nm after 30 min *(8)*. The tyrosine released by the enzyme was calculated from a tyrosine standard graph. One unit of protease activity was defined as the amount of enzyme required to release 1 μmol of tyrosine under standard assay conditions.

Effect of Time, pH, Temperature, and Substrate Concentration on Protease Activity

The effect of time on the protease activity was studied by using varying incubation times of 0, 15, 30, 45, 60, 120, 180, 240, 300, and 360 min for different enzyme concentrations, such as 0.005, 0.0075, and 0.01 g/mL, in the previously described protease assay procedure. The effect of pH was studied using casein substrates made in Tris buffer of different pH values from 4.0 to 10.0 adjusted using 0.1 *N* HCl and 0.1 *N* NaOH for different incubation times such as 1, 3, and 5 h at an enzyme concentration of 0.005 g/mL in the protease assay. The effect of temperature was studied by using varying incubation temperatures of 40, 45, 50, 55, and 60°C at an enzyme concentration of 0.01 g/mL in the protease assay. The effect of substrate concentration was studied by using varying concentrations of casein of 0.2, 0.4, 0.6, 0.8, and 1% for different incubation times (1, 2, 3, 4, and 5 h), at an enzyme concentration of 0.01 g/mL in the protease assay.

α-Amylase Assay

α-Amylase activity was measured by the method of Bernfeld *(9)* with some modifications. The reaction mixture contained 0.5 mL of 1% starch in 20 m*M* sodium phosphate buffer (pH 7.0) and 0.5 mL of enzyme solution. The mixture was incubated at 30°C for 1 h. The reaction was stopped by adding 1 mL of dinitrosalicylic acid solution (100 mL of solution contained 1 g of 3,5-dinitrosalicylic acid, 30 g of sodium potassium tartarate, and 20 mL of 2 *N* NaOH). The reaction mixture was then heated in boiling water for 15 min, and the absorbance at 540 nm was measured after cooling in ice and diluting with 10 mL of distilled water *(10)*. The maltose released by the enzyme was calculated from a maltose standard graph. One unit of α-amylase activity was defined as the amount of enzyme required to release 1 μmol of maltose under standard assay conditions.

Effect of Time, pH, Temperature, and Substrate Concentration on α-Amylase Activity

The effect of time on α-amylase activity was studied by using varying incubation times of 0, 15, 30, 45, 60, 120, 180, 240, 300, and 360 min for different enzyme concentrations such as 0.005 and 0.01 g/mL in the previously described α-amylase assay procedure. The effect of pH was studied using casein substrates made in sodium phosphate buffer of different pH values from 4.0 to 10.0 adjusted using 0.1 *N* HCl and 0.1 *N* NaOH, for different incubation times such as 1 and 4 h at an enzyme concentration of 0.005 g/mL in the α-amylase assay. The effect of temperature was studied by using varying incubation temperatures of 35, 40, 45, 50, 55, and 60°C at an enzyme concentration of 0.01 g/mL in the α-amylase assay. The effect of substrate concentration was studied by using varying concentrations of starch such as 1, 2, 3, 4, and 5% for different incubation times such as 1, 2, 3, 4, 5, and 6 h at an enzyme concentration of 0.01 g/mL in the α-amylase assay.

Effect of α-Amylase on Protease Activity

The effect of α-amylase on protease activity was studied by varying the concentration of both α-amylase and protease. Protease (0.05 mL of 0.005, 0.01, 0.015, and 0.02 g/mL concentrations) was mixed with 0.05 mL of 0.015 g/mL α-amylase and analyzed using the protease assay procedure with an incubation time of 4 h. To determine the activity of protease without the presence of α-amylase, similar experiments were conducted by mixing 0.05 mL of 0.005, 0.01, 0.015, and 0.02 g/mL concentrations of protease with 0.05 mL of Tris buffer and analyzed using the protease assay procedure with an incubation time of 4 h. To determine the effect of α-amylase concentration on protease activity, 0.05 mL of 0.005, 0.01, 0.015, and 0.02 g/mL concentrations of α-amylase was mixed with 0.05 mL of 0.015 g/mL protease and analyzed using the protease assay procedure with an incubation time of 4 h.

Effect of Protease on α-Amylase Activity

The effect of protease on α-amylase activity was studied by varying the concentration of both protease and α-amylase. α-Amylase (0.25 mL of 0.005, 0.01, 0.015, and 0.02 g/mL concentrations) was mixed with 0.25 mL of 0.015 g/mL protease and analyzed using the α-amylase assay procedure with an incubation time of 4 h. To determine the activity of α-amylase without the presence of protease, similar experiments were conducted by mixing 0.25 mL of 0.005, 0.01, 0.015, and 0.02 g/mL concentrations of α-amylase with 0.25 mL of 20 m*M* sodium phosphate buffer and analyzed using the α-amylase assay procedure with an incubation time of 4 h. To determine the effect of protease concentration on α-amylase activity, 0.25 mL of 0.005, 0.01, 0.015, and 0.02 g/mL concentrations of protease was mixed with 0.25 mL of 0.015 g/mL α-amylase and analyzed using the α-amylase assay procedure with an incubation time of 4 h.

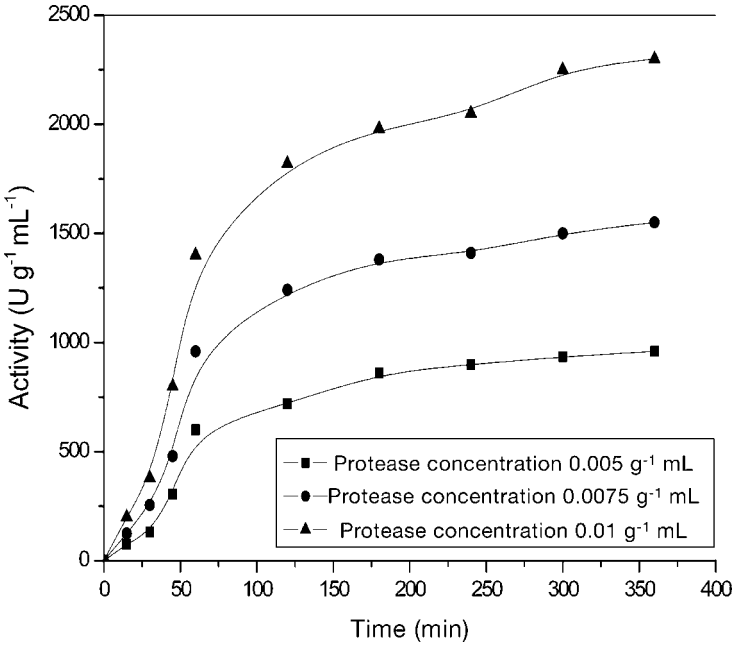

Fig. 1. Activity of various concentrations of protease at various incubation times.

Kinetic Study for Protease

The kinetics for protease were studied by using varying concentrations of casein of 0.2, 0.4, 0.6, and 0.8% for different incubation times such as 60, 120, and 180 min at an enzyme concentration of 0.01 g/mL in the protease assay.

Kinetic Study for α-Amylase

The kinetics for α-amylase were studied by using varying concentrations of starch such as 1, 2, and 3% for different incubation times such as 60, 120, 180, 240, 300, and 360 min at an enzyme concentration of 0.01 g/mL in the α-amylase assay.

Results and Discussion

Effect of Time and Substrate Concentration on Protease Activity

The activity of protease at different incubation times was studied to determine the optimum time for maximum activity. Figure 1 presents the effect of increasing time intervals on the activity of protease at varying concentrations. The activity of protease increased steeply from the time of onset and reached equilibrium at 120 min for all the concentrations of protease. Subsequently, there was no significant change in the activity of protease from 120 to 360 min. In addition, the activity increased when the concentration of protease increased from 0.005 to 0.01 g/mL. The results

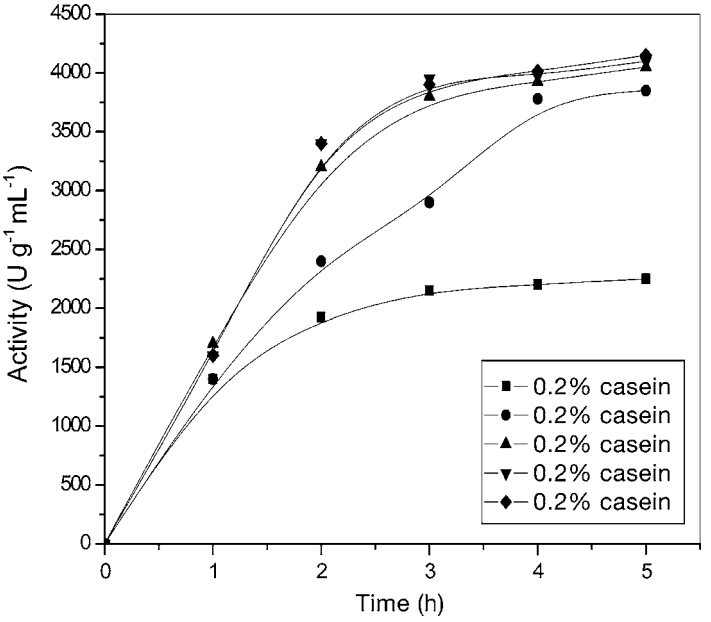

Fig. 2. Activity of protease at various substrate concentrations and incubation times. Protease concentration was 0.01 g/mL.

indicate that the time and the concentration of protease would influence the enzyme reaction with the substrate.

To study the effect of substrate concentration on protease activity, the concentration of casein was varied from 0.2 to 1%; Figure 2 presents the results for increasing incubation time. The activity of protease increased with an increase in the concentration of casein up to 0.6%. For a further increase in the concentration of casein up to 1%, the activity of protease was almost similar, indicating the nonavailability of enzyme for reacting with the substrate. These differences are explicit only when the reaction is carried out for a longer duration from 1 to 5 h. In other words, during the initial course of the reaction up to 1 h, there was no significant change in the activity of protease for all the concentrations of casein.

Effect of pH and Temperature on Protease Activity

The protease solution was treated with casein at various pHs in the range of 4.0–10.0 in order to determine the optimal pH for maximum activity. Figure 3 presents the influence of pH on the activity of the enzyme for increasing incubation time. The activity was low in the acidic region and increased significantly on increase in pH, especially in the alkaline region. The selected protease had maximum activity at pH 9.0 for all the incubation times. A further increase in the pH did not increase the activity of the selected protease.

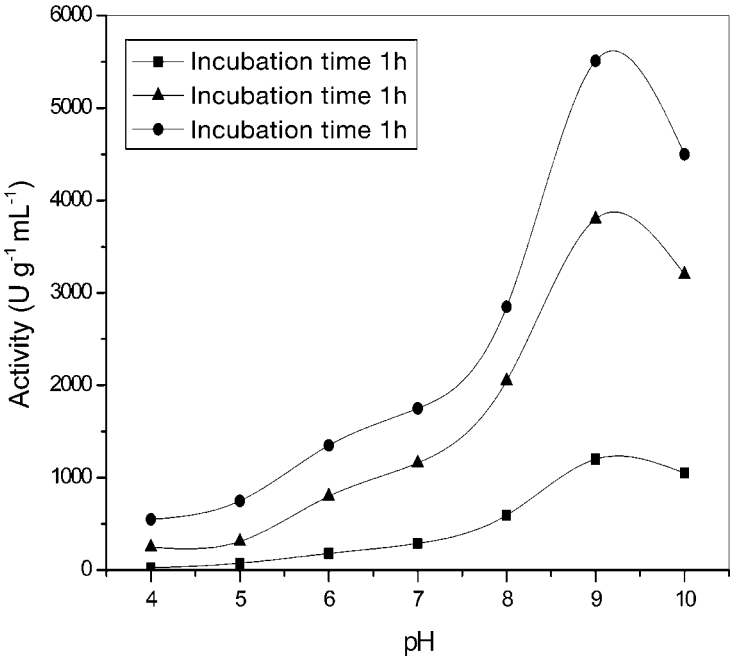

Fig. 3. Activity of protease at various pHs and incubation times. Protease concentration was 0.005 g/mL.

The effect of temperature on the protease activity was studied to determine the optimum temperature for maximum activity. Protease-casein samples were incubated at various temperatures from 40 to 60°C; Figure 4 presents the results. The activity increased with an increase in temperature up to 55°C. In principle, the increase in temperature increased the reaction rate. This is primarily owing to the increase in the rate of collision (faster Brownian motion) between the enzyme and the substrate on increase in temperature *(11)*. The activity of the protease fell sharply on a further increase in temperature from 55 to 60°C. Hence, the selected protease can be effectively used up to 55°C.

Effect of Time and Substrate Concentration on α-Amylase Activity

The activity of the α-amylase at different time intervals was studied to determine the optimum time for maximum activity. Figure 5 shows the activity of the α-amylase on increasing incubation time with varying concentrations of α-amylase. The activity of α-amylase increased rapidly from the time of onset and reached equilibrium at 300 min. Subsequently, there was no significant change in the activity of α-amylase from 300 to 360 min. For both concentrations of α-amylase, equilibrium was reached at 300 min. It seems that the activity was slightly higher for the higher concentration of α-amylase. The results indicate that the incubation time had more influence on the reaction of enzyme with the substrate.

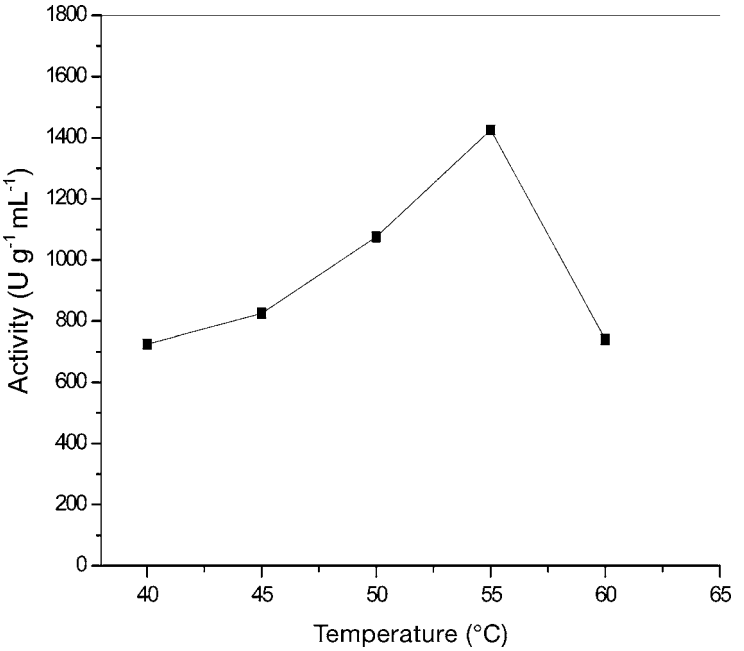

Fig. 4. Activity of protease at various incubation temperatures. Protease concentration was 0.01 g/mL.

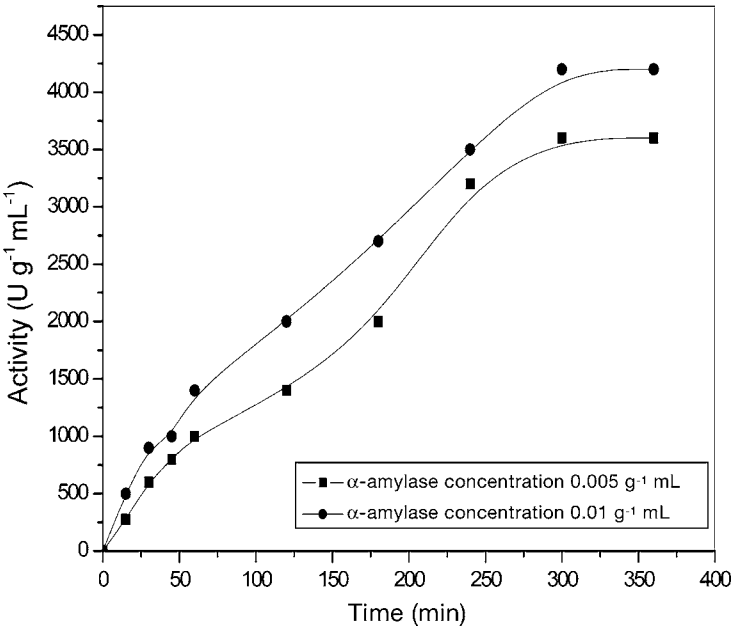

Fig. 5. Activity of various concentrations of α-amylase at various incubation times.

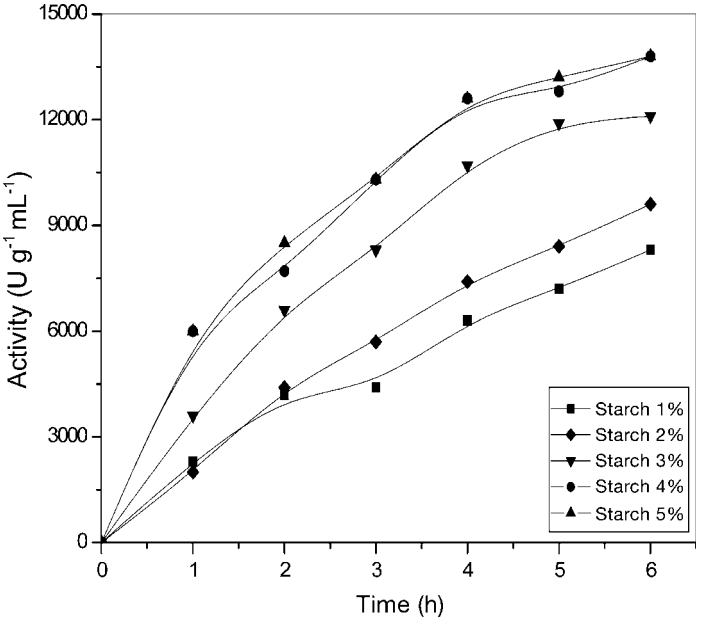

Fig. 6. Activity of α-amylase at various substrate concentrations and incubation times. α-Amylase concentration was 0.01 g/mL.

To study the effect of substrate concentration on α-amylase activity, the concentration of substrate was varied from 1 to 5%; Figure 6 presents the results for increasing incubation time. The activity of α-amylase increased with an increase in the concentration of starch up to 4%. A further increase in the concentration of starch did not increase the activity of α-amylase. This is owing to the nonavailability of enzyme for reaction with the excess substrate in a 5% starch concentration.

Effect of pH and Temperature on α-Amylase Activity

α-Amylase was treated with starch at various pHs in the range of 4.0–10.0 in order to determine the optimal pH for maximum activity; Figure 7 presents the results for different incubation times. It is interesting to note that α-amylase was highly active at pH 5.0 as well as at 8.5, at which it had maximum activity. In addition, higher incubation time resulted in higher activity in the entire pH range.

The effect of temperature on α-amylase activity was studied to determine the optimum temperature. Hence, enzyme-substrate samples were incubated at various temperatures from 35 to 60°C; Figure 8 presents the results α-Amylase activity increased with an increase in temperature up to 55°C. The increase in temperature increased the reaction rate owing to faster Brownian motion, which results in increased activity. The activity of α-amylase fell sharply at temperatures above 55°C. Hence, the selected α-amylase can be effectively used up to 55°C, similar to the selected protease.

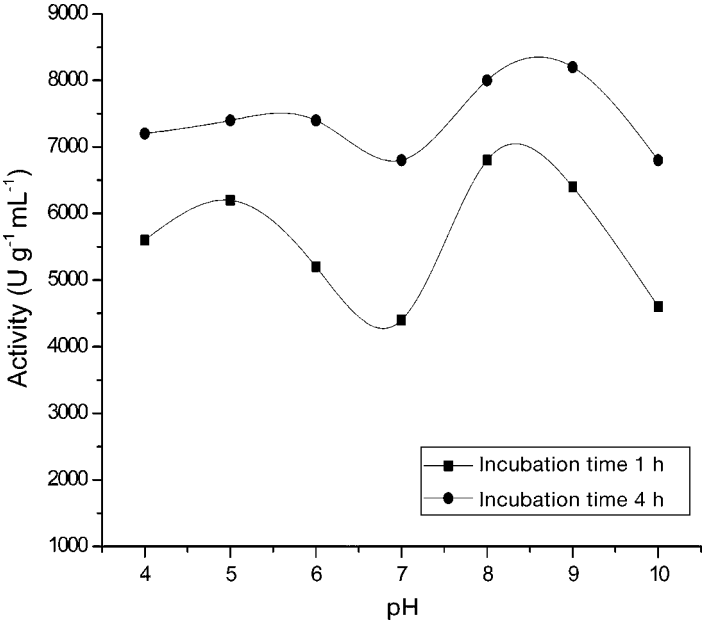

Fig. 7. Activity of α-amylase at various pHs and incubation times. α-Amylase concentration was 0.005 g/mL.

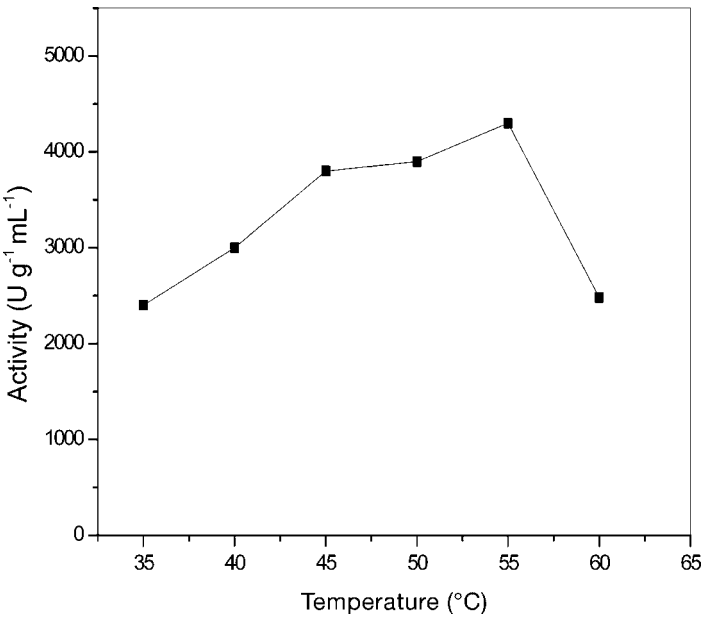

Fig. 8. Activity of α-amylase at various incubation temperatures. α-Amylase concentration was 0.01 g/mL.

Table 1
Activity of Various Concentrations of Protease in Presence and Absence of α-Amylase

Protease concentration (g/mL)	Protease activity (U/[g@mL])[a]	
	With α-amylase	Without α-amylase
0.005	3600	3540
0.01	1900	1800
0.015	900	950
0.02	850	925

[a]α-Amylase concentration was 0.015 g/mL, and. incubation time was 4 h.

Table 2
Activity of Protease in Presence of Various Concentrations of α-Amylase

α-Amylase concentration (g/mL)	Protease activity (U/[g@mL])[a]
0.000	870
0.005	820
0.01	840
0.015	875
0.02	820

[a]Protease concentration was 0.015 g/mL, and incubation time was 4 h.

Activity of Enzyme in Enzyme Cocktail

Effect of α-Amylase on Protease Activity

To determine the effect of α-amylase on protease activity, the concentrations of both protease and α-amylase were varied and the activity of protease was analyzed. Table 1 presents the activity of protease in the presence and absence of α-amylase with increasing concentrations of protease. There was no significant change in the activity of protease in the presence and absence of α-amylase. It is interesting to note that the activity of protease decreased with an increase in the concentration of protease. This is primarily owing to inadequate substrate for increasing concentrations of protease. Table 2 presents the activity of protease for increasing concentrations of α-amylase. The activity of protease almost remained constant in all the concentrations of α-amylase. Hence, it is clear that α-amylase did not interfere with the activity of protease.

Effect of Protease on α-Amylase Activity

To determine the effect of protease on α-amylase activity, the concentrations of both protease and α-amylase were varied and the activity of α-amylase was analyzed. Table 3 presents the activity of α-amylase in the presence and absence of protease with increasing concentrations of

Table 3
Activity of Various Concentrations of α-Amylase in Presence and Absence of Protease

	α-Amylase activity (U/[g@mL])[a]	
α-Amylase concentration (g/mL)	With protease	Without protease
0.005	5700	5600
0.01	4000	3800
0.015	2450	2530
0.02	2100	1900

[a]Protease concentration was 0.015 g/mL, and incubation time was 4 h.

Table 4
Activity of a-Amylase in Presence of Various Concentrations of Protease

Protease concentration (g/mL)	α-Amylase activity (U/[g@mL])[a]
0.000	2530
0.005	2600
0.01	2530
0.015	2600
0.02	2660

[a]α-Amylase concentration was 0.015 g/mL, and incubation time was 4 h.

α-amylase. There was no significant change in the activity of α-amylase in the presence and absence of protease. It is interesting to note that the activity of α-amylase decreased with an increase in the concentration of α-amylase. This is primarily owing to inadequate substrate available for reacting with increasing concentrations of α-amylase. Table 4 presents the activity of α-amylase for increasing concentrations of protease. The activity of α-amylase almost remained constant in all the concentrations of protease. Hence, it is clear that protease did not interfere with the activity of α-amylase.

Kinetics of Protease and α-Amylase

The changes of product concentrations, in terms of product formation, against time at different initial substrate concentrations, S (mg/mL), during the hydrolysis process of casein and starch were plotted and the slopes of straight lines were obtained, which provides the initial rate of reaction, V (mg/[mL@min]). The relation between substrate concentration and rate of reaction can be described by the Michaelis-Menten equation:

$$V = \frac{V_{\max} S}{K_m + S} \tag{1}$$

in which $V_{\max}$ is the maximum rate of reaction and K_m is the Michaelis constant (mg/mL). K_m is defined as the substrate concentration at which

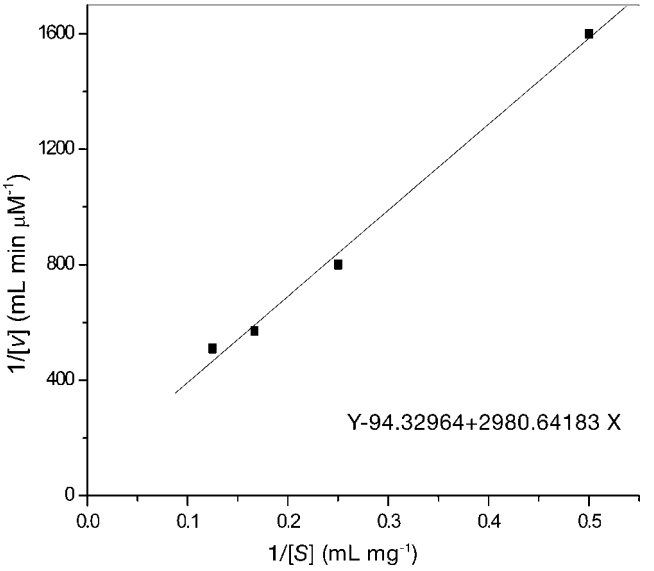

Fig. 9. Lineweaver-Burk plot for catalytic reaction of protease with casein.

enzyme shows half of the maximal velocity. V_{max} varies with the total concentration of enzyme present; however, K_m is independent of enzyme concentration and it is characteristic of the system being investigated. The reciprocal of the Michaelis-Menten equation provides the values of V_{max} and K_m, as shown in Eq.2, by plotting $1/V$ vs $1/S$, from a Lineweaver-Burk plot:

$$\frac{1}{V} = \frac{1}{V_{max}} + \left(\frac{K_m}{V_{max}}\right)\frac{1}{S} \quad (2)$$

Figures 9 and 10 show the Lineweaver-Burk plot for protease and α-amylase, respectively. The slope (K_m/V_{max}) and intercept ($1/V_{max}$) values were obtained from the plots, and the K_m and V_{max} values were calculated for protease and α-amylase. The values of K_m and V_{max} for protease were found to be 31.6 mg/mL and 0.0106 mg/(mL@min), whereas for α-amylase they were 8.79 mg/mL and 0.0912 mg/(mL@min). α-Amylase provided maximum catalytic reaction even with a low substrate concentration when compared with protease. This shows that the selected α-amylase can be used for processes in which low levels of substrate are present, which is typically the case in fiber opening during leather processing.

Conclusion

Commercial protease and α-amylase enzymes used in leather processing for dehairing and fiber opening, respectively, were assayed and characterized in order to develop a newer and simple enzymatic method of leather processing. The optimized time, substrate concentration, pH, and temperature for the chosen protease were 180 min, 0.6% casein, 9.0, and

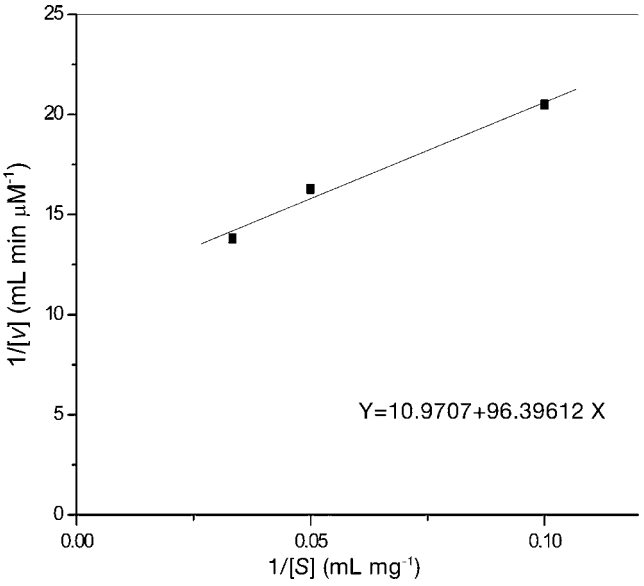

Fig. 10. Lineweaver-Burk plot for catalytic reaction of α-amylase with starch.

55°C, respectively, and the optimized conditions for α-amylase were 300 min, 4.0% starch, 8.5, and 55°C, respectively. Enzyme-blending studies showed that there was no significant change in the activity of one enzyme in the presence of the other. Kinetic studies revealed that the selected α-amylase was more sensitive in reaction with substrate than protease, which is more suitable for the skin fiber–opening process. This study paves the way for the integration of dehairing and fiber-opening processes using protease and α-amylase.

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