Enzymic Studies of Papain from Green Papaya Fruit (*Carica papaya* L.)

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Abstract

The research work is aimed to study the extraction of papain enzyme (EC 3.4.22.2) from green papaya fruit. The extracted enzyme was characterized by enzymic properties and enzyme kinetic parameters. Papain enzyme has the proteolytic activity and it hydrolyzed casein into amino acid gave tyrosine as product. In the preparation of papain from green papaya fruit, ammonium sulphate precipitation method was used in two successive steps (20 %) and (70 %) saturation. Finally, partially purified enzyme (1.2 g) was obtained from 200 g of green papaya fruit. Qualitative determination of liberated tyrosine from enzyme-catalyzed reaction was carried out by using thin-layer chromatographic method. The optimum pH and optimum temperature of green papaya fruit were found to be pH 6.8 and 60 °C, respectively. The reaction time of papain enzyme extracted from green papaya fruit was found to be 30 min. According to data analysis, the value of maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) were calculated by using statistical and graphical methods such as Michaelis-Menten, Lineweaver Burk and Eadie-Hofstee methods. The V_{max} and K_m values determined by Lineweaver-Burk were 0.2852 mM $min^{-1}$and 0.0069 g mL^{-1}, respectively.

Keywords*:* Papaya, papain, casein, tyrosine

Introduction

This paper involved the extraction and characterization of papain enzyme from green papaya fruit (*Carica papaya* L.). Papain is a proteolytic enzyme extracted from raw fruit of papaya plant. It is found in the leaves, roots and fruit of the papaya plant. Proteolytic enzymes are very important in digestion as they break down the peptide bonds in the protein foods to liberate the amino acids needed by the body (Ghosh, 2005). The greener the fruit, more active is the papain. Papain is a cysteine hydrolase that is stable and active under a wide range of conditions (Edwin *et al*., 2000).

The enzyme number of papain is EC 3.4.22.2 (Karlson, 1979). The number 3 indicates the hydrolases group, 3.4 indicates acting on peptide bonds, 3.4.22 indicates the thiol proteinases and 3.4.22.2 indicates papain enzyme. It is used as a digestive aid and for treating parasitic worms, inflammation of the throat and pharynx, shingles symptom, ongoing diarrhoea, hay fever, runny nose and skin condition called psoriasis.

Materials and Methods

Papain used in this study is purchased from Sanpya Market, Thingangyun Township, Yangon Region. The chemicals used are trichloroacetic acid (TCA), Na_2CO_3, NaOH (pellet), Na_2HPO_4, KH_2PO_4, casein, tyrosine and Folin-Ciocalteu (Phenol) reagent from BDH, *n*-butanol, acetic acid, 0.2 % ninhydrin reagent and acetone. Enzyme Activity was determined by the spectroscopic method. The liberated

tyrosine from enzyme-catalyzed was identified by thin layer chromatography using standard tyrosine.

Extraction of Papain from Green papaya fruit

Green papaya fruit (200 g) was weighed and placed in a blender. Then (100 mL) of phosphate buffer (pH 7.0) solution was added and the mixture was blended. It was allowed to respond for 20 minutes and then filtered through a cheese cloth. The solution was centrifuged at (3000 rpm) for 30 min. Supernatant obtained is called a crude enzyme. Then, the crude enzyme was partially purified by ammonium sulphate precipitation method. By adding ammonium sulphate into liquid crude enzyme in two successive steps (20%) and (70%) saturation, the partially purified enzyme (papain) was obtained.

Activity Assay of Papain

Enzyme solution (0.5 mL) was added to 1 mL of 1 % casein solution. They were mixed well. After 20 min incubation time, 1 mL of 5 % trichloroacetic acid solution was added to the reaction mixture in order to terminate the enzyme reaction.

After 20 min, 1 mL of supernatant was added into a test tube. A 5 mL of 0.5 M sodium carbonate solution and 1 mL of 1 M sodium hydroxide solution were added to the test tube. After 10 min, a 0.5 mL of Folin-Ciocalteu reagent was added to the test tube and mixed. The mixture was allowed to stand for 30 min and it was measured for absorbance at 600 nm by using a UV-visible spectrophotometer (Mukhtar and Ikram, 2008). A blank solution was prepared by carrying out the procedure as described above except that 0.5 mL of phosphate buffer was used instead of 0.5 mL of enzyme solution.

Determination of Reaction Time, pH, Temperature and Substrate Concentration

Papain from green papaya fruit enzyme action was studied for the reaction time ranging from 10 - 60 min using phosphate buffer pH 7.0. The optimum pH of the enzyme was determined by using the enzyme solution of different pH value ranging from pH 5.6 to 8.0. For the determination of the optimum temperature, the enzyme reaction was conducted at temperatures between 30 °C and 80 °C. The various substrate concentrations ranging from 0.01 - 0.06 gml^{-1} were determined in this research.

Results and Discussion

The enzyme activity was determined by calculating the micro mole of tyrosine liberated per milliliter per minute of enzyme solution.

Identification of Tyrosine Liberated from Papain-catalyzed Reaction

In the present work, casein was used as a substrate in enzyme-catalyzed reactions. The protein, casein, is decomposed by protease enzyme to give degraded stages of protein products which includes among them, tyrosine. Tyrosine liberated from enzyme-catalyzed reaction was identified qualitatively by one dimensional ascending thin-layer chromatography method using precoated silica gel on aluminum sheets. Ninhydrin reagent was used for color developing. The solvent system used was *n*-butanol: acetic acid: water (11: 6: 1) v/v and the chromatogram was shown in Figure 1. Tyrosine from BDH was used as authentic marker and tyrosine liberated from enzyme-catalyzed reaction was used as compare marker.

Sample and authentic tyrosine were appeared as spots with equal distance on chromatogram. The distance travelled by the solvent front, standard tyrosine and sample tyrosine were 3.8 cm, 2.15 cm and 2.1 cm respectively from the base line. The R_f value of standard tyrosine was 0.5789 and sample tyrosine was 0.5736. Therefore, R_f value of standard and sample were almost nearly the same as shown in Figure 1.

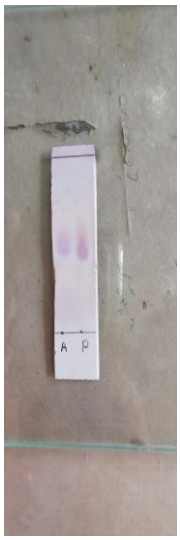

Figure 1. Identification of standard tyrosine and tyrosine liberated from enzyme-catalyzed reaction by thin-layer chromatogram

Effect of Reaction Time on Papain-catalyzed Reaction

Reaction time is also a major factor in the determination of enzyme activity. The significant of the reaction time is created by the different enzymes to record for the starting time (Das, 2010).

In this research, green papaya fruit enzyme action was studied for reaction time ranging from 10 - 60 min using phosphate buffer pH 7.0. At the beginning of the reaction, tyrosine concentration increased very rapidly, after 30 min tyrosine concentration increased very slowly (Table 1 and Figure 2). The plot of velocity *vs.* reaction time (Figure 3.) shows that the velocity decreased from 10 min - 30 min and not significantly changes from 30 - 60 min. Therefore, the reaction time of 30 min was chosen for this enzyme reaction.

Table 1. Variation of Velocity of Papain-catalyzed Reaction with Various Reaction Time

Time(min)	**Concentration of tyrosine liberated (mM)**	**Velocity (mM min^{-1})**
10	5.499	0.549
20	6.416	0.320
30	6.796	0.226
40	6.979	0.174
50	7.044	0.140
60	7.044	0.140

Optimum pH of Papain Enzyme

One of the most striking features of enzyme activity is its very marked dependence on the pH of the mixture in which the reaction is going on (Moss, 1968). Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. Many enzymes have their optimum activity at pH values close to neutrality. Each enzyme has optimum pH at which it works fastest.

In this study, different pH values of phosphate buffer solution ranging from 5.6 - 8.0 were used to determine the activity of the prepared protease sample. Figure 4 shows unsymmetrical nature of the curve and the optimum was pH 6.8. Before the optimum pH, the activities increased with pH gradually increase and after the optimum pH, the activities decreased due to the denaturing of enzyme.

Optimum Temperature of Papain Enzyme

In common with other proteins, enzymes are sensitive to temperature change (Campbell and Smith, 2000). Optimum temperature is the temperature at which the enzyme activity is at its maximum. For each enzyme, there is a certain temperature at which the enzyme activity is maximum and the activity progressively declines both above and below this temperature (Oser, 1965).

In the present work, the effect of the temperature on papain enzyme activity was investigated in the temperature range from 30 - 80 $^{\circ}$C in phosphate buffer pH 6.8. It was found that the activity of enzyme increased from 30 to 60 $^{\circ}$C and then decreased from 60 - 80 $^{\circ}$C. Therefore, the optimum temperature for papain enzyme obtained from green papaya fruit was found to be 60 $^{\circ}$C (Figure 5). At higher temperatures, more molecules collide, increasing the chance that an enzyme will collide with its substrate. However, if the temperature is too high, an enzyme will denature, which causes the shape of the enzyme to change. If the enzyme's shape changes, it cannot bind to the substrate (Bruice *et al*., 2000).

Effect of Substrate Concentration on Papain-catalyzed Reaction

The maximum rate at infinite substrate concentration is called V_{max} and the substrate concentration that gives a rate of half V_{max} is called K_m. These quantities are useful for characterizing an enzyme (Moss, 1968). A good enzyme has a high V_{max} and a low K_m. A low K_m value indicates strong substrate binding and a high K_m value indicates weak substrate binding (Brown and Mc Clariin, 1981). In this research, the velocity of enzyme reaction measure at different levels of substrate concentration and their reciprocal values are shown in Table 2. The Michaelis-Menten plot of velocity, V *vs* substrate concentration [S] is shown in Figure 6. The Lineweaver-Burk plot of 1/V *vs* 1/[S] (Figure 7) and the Eadie-Hofstee plot of V *vs* V/[S] (Figure 8) were drawn using linear regression method to attain the accurate V_{max} and K_m values (Jain, 2003). Statistical methods were used to calculate V_{max} and K_m from the experimental results. These values are shown in Table 3. In comparison with V_{max} and K_m values obtained by Lineweaver-Burk and Eadie-Hofstee are not much different from each other.

Table 2. Variation of Substrate Concentration and Velocity of Papain-catalyzed Reaction

[S] (g mL^{-1})	**1/[S] ($g^{-1}mL$)**	**V (mM min^{-1})**	**1/V ($mM^{-1}min$)**	**V/[S] (mM $min^{-1}g^{-1}mL$)**
0.01	100	0.1680	5.9353	16.80
0.02	50	0.2156	4.6377	10.78
0.03	33	0.2322	4.3060	7.74
0.04	25	0.24268	4.1205	6.05
0.05	20	0.2496	4.0050	4.98
0.06	17	0.2549	3.9230	4.25

Figure 8. Graphical evaluation of V_{max} and K_m for papain-catalyzed reaction by Eadie-Hofstee plot

Table 3. Comparison of V_{max} and K_m values of Papain-catalyzed Reaction from Different Methods

No.	Methods	Statistical Representation		Graphical Representation	
		V_{max} (mM min^{-1})	**K_m (g mL^{-1})**	**V_{max} (mM min^{-1})**	**K_m (g mL^{-1})**
1	Michaelis-Menten	-	-	0.27	0.007
2	Lineweaver-Burk	0.2852	0.0069	0.2857	0.0069
3	Eadie-Hofstee	0.2845	0.0068	0.2841	0.0068

Conclusion

Papain is the name of a group of powerful protein digesting or proteolytic enzymes that are found in papaya plant. The proteolytic activity of papain enzyme was followed by determining the tyrosine products. Identification of tyrosine liberated from papain -catalyzed reaction was examined by using thin-layer chromatography method with standard tyrosine. Tyrosine sample and authentic tyrosine were appeared as spots with equal distance on chromatogram. The R_f value of standard tyrosine was 0.5789 and sample tyrosine was 0.5736. Therefore, R_f value of standard and sample were almost nearly the same.

The optimum pH and optimum temperature of papain enzyme were found to be pH 6.8 and 60 ˚C, respectively. The reaction time of papain enzyme was found to be 30 min. The plot of velocity against the reaction time shows that the velocity decreased from 10 min to 30 min and not significantly changes from 30 - 60 min. Therefore, the reaction time of 30 min was chosen for this enzyme reaction.

Graphical and statistical methods were used to calculate V_{max} and K_m from the experimental results. The V_{max} and K_m values of papain were found to be 0.2845 mM min^{-1} and 0.0068 g mL^{-1}, respectively for Eadie-Hofstee plot. A good enzyme has a

high V_{max} and a low K_m. Thus papain enzyme from green papaya fruit is a good enzyme.

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