ENZYMIC STUDY ON PECTINASE EXTRACTED FROM WHITE DRAGON FRUIT PEELS

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Abstract

Isolation and purification of pectinase enzyme from the peels of white dragon fruit (*Selenicereus undatus*) were performed by 20-80 % ammonium sulphate precipitation followed by gel filtration chromatography on Sephadex G-100. Pectinase enzyme activity was determined using glucose as a standard and pectin as a substrate by a UV-Vis spectrophotometer at 591 nm. The optimum pH and temperature of crude and partially purified pectinase enzymes extracted from the white dragon fruit peels were found as 5 and 30 °C, respectively. The activation energies (E_a) of the crude and purified pectinase-catalyzed reactions were observed as 4.1185 kcal mol^{-1} and 3.4018 kcal mol^{-1}, respectively. The K_m and V_{max} values were found as 0.300×10^{-2} g mL^{-1} and 25.380×10^{-2} mM min^{-1} for crude pectinase enzyme and 0.509×10^{-2} g mL^{-1} and 26.281×10^{-2} mM min^{-1} for partially purified pectinase enzyme, using the Lineweaver-Burk plot. The reaction order (n) of the crude and purified pectinase-catalyzed reactions was calculated using the linear regression method, and it was found to be first order. Both crude and partially purified pectinase enzymes were used in the clarification of grape juice and the transmittance percents were 75.49 % and 86.29 %, respectively. The clarity of grape juice can be improved by enzymatic treatment using pectinase.

Keywords: pectinase, white dragon fruit, *Selenicereus undatus*, pectin, grape juice

Introduction

Pectinase alone accounts for about one-quarter of the world's food enzyme production and is among the most important industrial enzymes. Pectinases are enzymes that are responsible for the biological degradation of pectin, a polysaccharide with large molecular weight found in the cell walls of plants. This enzyme splits polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages (Swarnalatha *et al.,* 2021). In plants, pectinases play a diverse role, such as cell-cell adhesion, a source of signaling molecules, and the ripening of fruits (Okonji *et al.,* 2019). Pectinases are used in several conventional industrial processes. They are one of the most widely distributed enzymes in bacteria, fungi, insects, nematodes, and protozoa (Adeyefa and Ebuehi, 2020). They are of great significance, with a wide range of applications in the fruit, beverage, and textile processing industries, in the treatment of wastewater, degumming of plant fibres, pulp and papermaking, and coffee and tea fermentation (Ubani *et al.,* 2015). The current research aims to extract pectinase from white dragon fruit peel, and study its kinetic properties and its efficiency in juice clarification.

Materials and Methods

White dragon fruit (*Selenicereus undatus* (Haw) D. R. Hunt) was collected from Hledan Market, Kamayut Township, Yangon Region. 3,5-Dinitrosalicyclic acid (DNS) and all other chemicals were used from Sigma- Aldrich, England. All the chemicals used in this work

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were analytical grade. In all procedures, the recommended standard methods and techniques involving both conventional and modern methods were applied.

Isolation of Pectinase Enzyme from White Dragon Fruit Peels

White dragon fruits (*Selenicereus undatus*) was peeled and cut into small pieces. The peels (20 g) were homogenized with 160 mL of 0.1 M sodium acetate buffer (pH 5.0) for 5 min using a blender. After homogenization, the resulting slurry was filtered through cheesecloth. The filtrate was centrifuged for 20 min at 6000 rpm to obtain 82 mL of extract. Solid ammonium sulphate (9.3 g) was added to this extract to obtain 20 % saturation and stirred for 2 h at 4 ºC. After standing for 2 h, the suspension was centrifuged for 20 min at 6000 rpm. The supernatant liquid was decanted and brought to 80 % saturation with 23.6 g of solid ammonium sulphate, and stored at 4 ºC. After standing overnight, the precipitated protein containing pectinase enzyme was collected by centrifugation for 20 min at 6000 rpm, and the crude pectinase was obtained.

Purification of Pectinase Enzyme by Gel Filtration Chromatography

Gel filtration was carried out in a column (2.0 × 25 cm) packed with pre-swollen Sephadex G-100 using 0.1 M sodium acetate buffer (pH 5.0) and equilibrated with the same buffer (500 mL). The enzyme was eluted from the column with the same equilibrated buffer. The Sephadex column was flushed with the eluent for several hours prior to the addition of the sample.

The crude pectinase enzyme (2 g) was dissolved in 4 mL of sodium acetate buffer (pH 5.0). The solution was applied to a Sephadex G-100 gel filtration column with the flow rate of 1.5 mL per 5 min. A 1.5 mL fraction was collected per tube using a fraction collector. After collection, the protein content of each tube was checked by measuring the absorbance at 280 nm wavelength using a UV-Vis spectrophotometer. Each tube was also measured for pectinase activity at 591 nm using a UV-Vis spectrophotometer. The fractions that had the highest pectinase activity were pooled.

Pectinase Enzyme Assay

For the enzyme assay, 0.5 mL of enzyme solution was incubated with 0.5 mL of 1 % pectin substrate (in 0.1 M sodium acetate buffer of pH 5.0) at 30 ℃ for 10 min. Then, 1 mL of 1 M of DNS solution was added, and this mixture was boiled at 90 ℃ for 5 min. Next, 1mL of 1 M sodium potassium tartrate solution was added to stop the reaction, and then 2 mL of distilled water was added to the mixture. The absorbance of the diluted mixture was measured at 591 nm by a UV-Vis spectrophotometer. For a blank solution, 0.5 mL of distilled water was used instead of 0.5 mL of enzyme solution.

Protein Determination

Protein content in each purification step was determined by Biuret method at 550 nm using bovine serum albumin (BSA) as standard.

Effects of pH, Temperature, and Substrate Concentration on Pectinase Activity

The effect of pH on pectinase activity was determined by varying the pH from 4.4 to 5.8 at intervals of 0.2. The effect of temperature on pectinase activity was determined by varying the temperature from 20 to 50 °C at intervals of 5 °C. The effect of substrate concentration on the pectinase-catalyzed reaction was studied by varying the concentration of substrate (pectin) from 2-16 (mg mL^{-1}). The pectinase activity was assayed as described above using 1% pectin as the substrate. Reaction order of the pectinase-catalyzed reaction was also calculated.

Determination of Activation Energy of the Pectinase-catalyzed Reaction

The velocities of pectinase-catalyzed reaction were measured at the temperature range of 20-50 ℃ at 5 unit intervals. An Arrhenius plot of the initial velocity data was performed to determine the activation energy.

Application of Pectinase Enzyme in Grape Juice Clarification

In the experiment, 4 mL of grape juice was added to two separate test tubes. Then, 1 mL each of crude and partially purified enzymes was added to the grape juice. The contents of the test tubes were agitated to mix the enzyme throughout the juice and kept for 4 h. After that, the test tubes were heated for 3 min at 40℃ to inactivate the enzyme reaction. Subsequently, the samples were centrifuged for 20 min at 3000 rpm, and the supernatant was filtered. Similarly, the above procedure was carried out without enzymes by using (i) 5 mL of grape juice, and (ii) a mixture of 4 mL of grape juice and 1 mL of distilled water. The clarity of the grape juice obtained was determined by measuring the absorbance of the solution at 660 nm (Ahmed and Sohail, 2020) with a UV-Vis spectrophotometer. The clarity was expressed in percent transmittance.

Results and Discussion

Purification of Pectinase Extracted from White Dragon Fruit Peels

The pectinase enzyme was isolated from a white dragon fruit peel sample and partially purified by the solid ammonium sulphate precipitation method. Figure 1 shows the chromatogram of pectinase on Sephadex G-100 gel. The fraction numbers (30-43) showing the highest pectinase activity were pooled. Table 1 shows the pectinase activities, protein contents, specific activities of the enzyme solutions, and purity of the enzyme. The specific activity of the pectinase increased 3.50 folds over that of crude extract, and the protein recovery was found to be 2.50 %. One unit of enzyme was defined as one micromole of glucose that was liberated per minute.

Optimum pH and Temperature, and Activation Energy of Pectinase-catalyzed Reaction

The optimum pH for pectinase-catalyzed reaction was found to be 5.0 for both crude and partially purified pectinase-catalyzed reactions. It was obvious that the activity of pectinase increased from 4.4 to 5.0 and then decreased from 5.0 to 5.8 as shown in Figure 2. The optimum temperature for pectinase-catalyzed reaction was found to be 30 ℃ in sodium acetate buffer (pH 5.0) for both enzymes. It was observed that the activity of pectinase increased from 20 to 30 ℃ and then decreased from 30 to 50 ℃ as shown in Figure 3.

The activation energy of the pectinase-catalyzed reaction was calculated using the Arrhenius equation. Table 2 shows the relationship between temperature and velocity of pectinase–catalyzed reaction and Figure 4 shows the graph for the determination of activation energy. The partially purified pectinase-catalyzed reaction was found to have a lower activation energy (3.4018 kcal mol^{-1}) compared to crude pectinase-catalyzed reaction (4.1185 kcal mol^{-1}).

Effect of Substrate Concentration on Pectinase-Catalyzed Reaction

The velocities of crude pectinase-catalyzed enzyme reaction measured at different concentrations of substrate and their reciprocal values are shown in Table 3 and the Michaelis-Menten, Lineweaver-Burk, and Eadie-Hofstee plots are depicted in Figures 5, 6, and 7, respectively. In Michaelis-Menten plot, it was found that as the concentration of the substrate was increased while all other factors are kept constant, the velocity increased to a maximum

value, V_{max} until there was no further increase. The velocity of the reaction at this high substrate concentration is termed as the maximum velocity. The substrate concentration corresponding to half of the maximum velocity is called the Michaelis-Menten constant and is termed as K_m. The Michaelis-Menten plot gives only apparent K_m and V_{max} values. Table 4 shows the velocities of partially purified pectinase-catalyzed enzyme reaction as a function of concentrations of substrate. In this work, linear regression method was used to obtain K_m and V_{max} from the experimental results. These values are shown in Table 5 in comparison with K_m and V_{max} values obtained by Lineweaver-Burk and Eadie-Hofstee plots. K_m for crude pectinase was found to be 0.300×10^{-2} g mL^{-1} and that of partially purified pectinase was 0.509×10^{-2} g mL^{-1} from Lineweaver-Burk plot. V_{max} values were 25.380×10^{-2} mM min^{-1} and 26.281×10^{-2}mM min^{-1} for crude and partially purified pectinase enzyme, respectively. K_m and V_{max} values are not much different between Lineweaver-Burk and Eadie-Hofstee plots. However, the K_m and V_{max} values of the Michaelis-Menten plot are totally different from those of other plots.

Reaction Order for Pectinase-catalyzed Reaction

Reaction order (n) refers to the number of molecules involved in forming a reaction complex that is required to proceed to the product (s) (Giese, 2004). Depending on the substrate concentrations, the kinetics of an enzyme-catalyzed reaction may be described by a first-order rate equation (Bergmeyer, 1983). In the present work, reaction order (n) values were determined from the plot of log $V/(V_{max}-V)$ *vs* log [S] using the linear regression method as shown in Table 6 and Figure 11. The reaction order (n) for pectinase was calculated to be ~1, showing that the reaction order is first order.

Application of Pectinase Enzyme in Grape Fruit Juice Clarification

The cloudiness in the juices is due to the presence of polysaccharides such as pectin, cellulose, and starch (Sandri *et al*., 2011).

In this present work, Table 7 described the clarification of grape juice using pectinase enzyme from white dragon fruit peels. The test tube (A) is only grape juice, the test tube (B) is grape juice and water, and the test tube (C) is the mixture of grape juice and enzyme. The crude and partially purified pectinase showed good activity by clarifying the juices as compared to the control. The pectinase degrades the pectin and increases the clarity of the juice, enhancing its appearance. The clarity in terms of transmittance percent of the grape juice treated with crude pectinase was 75.49 % whereas that of grape juice treated with purified pectinase was 86.29 %. Thus, the partially purified pectinase enzyme has a higher efficiency for clarifying grape juice than crude pectinase enzyme. Thus, pectinase enzyme can be used for the clarification of fruit juice.

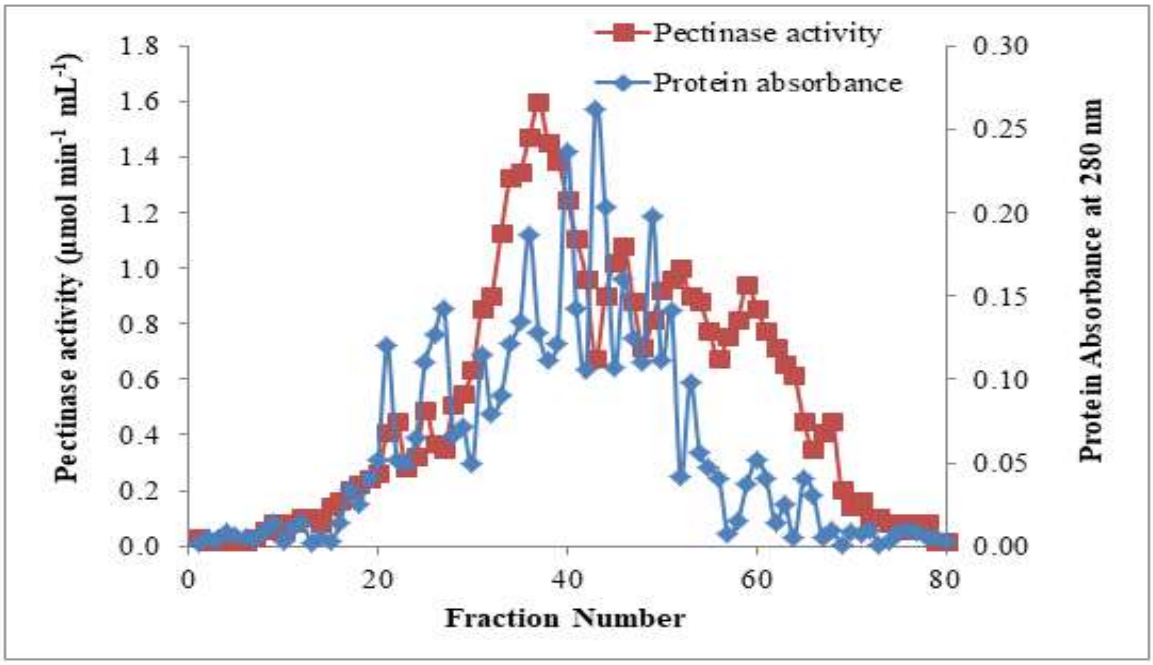

Figure 1. Sephadex G-100 gel filtration chromatogram of crude pectinase enzyme

Table 1. Pectinase 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	145	165.3	749.51	0.22	100	1.00
20 % $(NH_4)_2SO_4$ filtrate	90.0	45.00	179.55	0.25	24.0	1.14
80 % $(NH_4)_2SO_4$ precipitate	20.0	20.00	60.50	0.33	8.07	1.50
after passing the Sephadex G -100	19.5	14.63	19.11	0.77	2.50	3.50

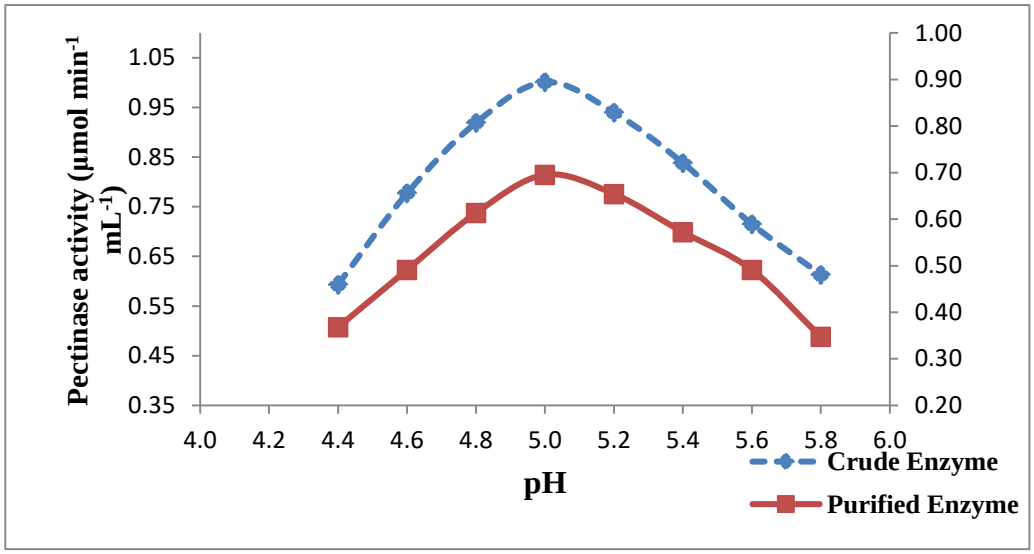

Figure 2. Plot of pectinase activity as a function of pH of the solution

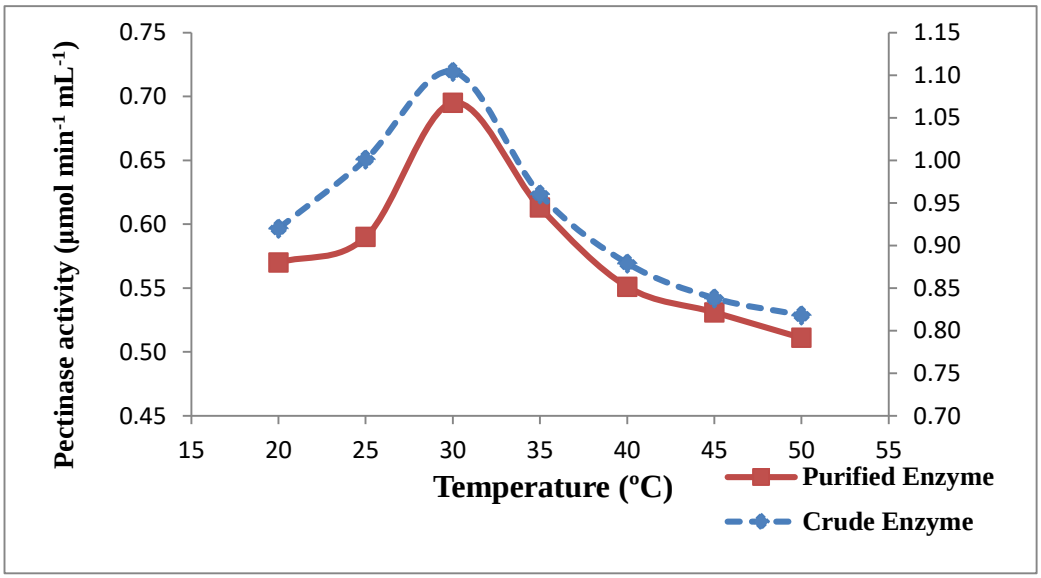

Figure 3. Plot of pectinase activity as a function of temperature of the solution at pH 5

Table 2. Relationship between Temperature and Velocity of Crude and Partially Purified Pectinase-catalyzed Reactions

Temperature (C)	Temperature (K)	1/T × 10^3 (K^{-1})	Crude enzyme		Partially purified enzyme	
			Velocity × 10^3 (mM min^{-1})	Log V	Velocity × 10^3 (mM min^{-1})	Log V
20	293	3.413	174.4	2.241	113.6	2.055
25	298	3.356	198.7	2.298	125.8	2.099
30	303	3.300	214.9	2.340	137.9	2.139
35	308	3.247	190.6	2.280	122.7	2.120
40	313	3.195	174.4	2.241	109.5	1.969
45	318	3.145	166.3	2.220	105.4	1.887
50	323	3.096	162.2	2.210	101.4	1.209

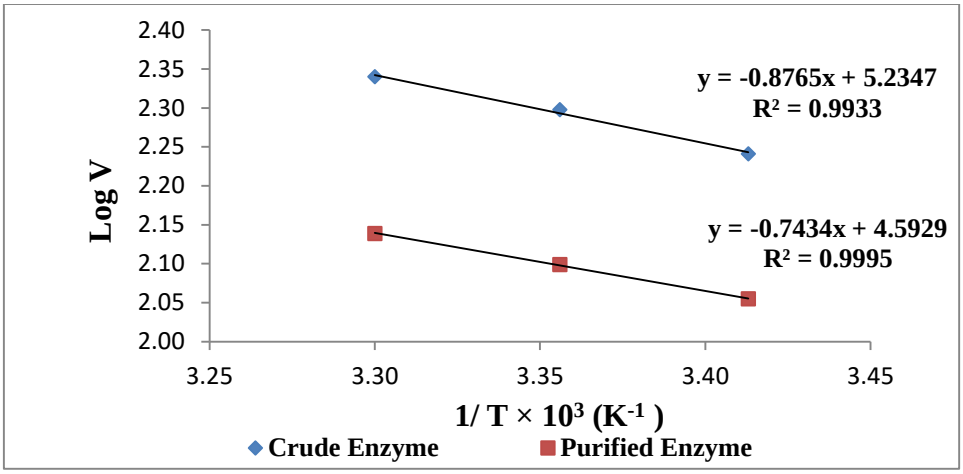

Figure 4. Plot of Log V as a function of 1/T for crude and purified pectinase-catalyzed reactions

Table 3. Relationship between Velocity of Crude Pectinase-Catalyzed Reaction and Substrate Concentration

$[S]\times10^2$ (g mL^{-1})	$-[S]\times10^2$ (g mL^{-1})	1/[S] (10^{-2}g $^{-1}$mL)	$V \times 10^2$ (mM min^{-1})	1/V ($10^{-1}$$mM^{-1}$ min)	V/[S] (mM $min^{-1}g^{-1}$ mL)	[S]/V (g mL^{-1} mM^{-1}min)
0.2	-0.2	5.00	10.10	0.99	50.5	0.019
0.4	-0.4	2.50	14.59	0.685	36.48	0.027
0.6	-0.6	1.67	17.03	0.587	28.38	0.035
0.8	-0.8	1.25	18.65	0.536	23.31	0.043
1	-1	1.00	19.47	0.514	19.47	0.051
1.2	-1.2	0.83	20.28	0.493	16.9	0.059
1.4	-1.4	0.71	20.68	0.484	14.77	0.068
1.6	-1.6	0.63	21.09	0.474	13.18	0.076

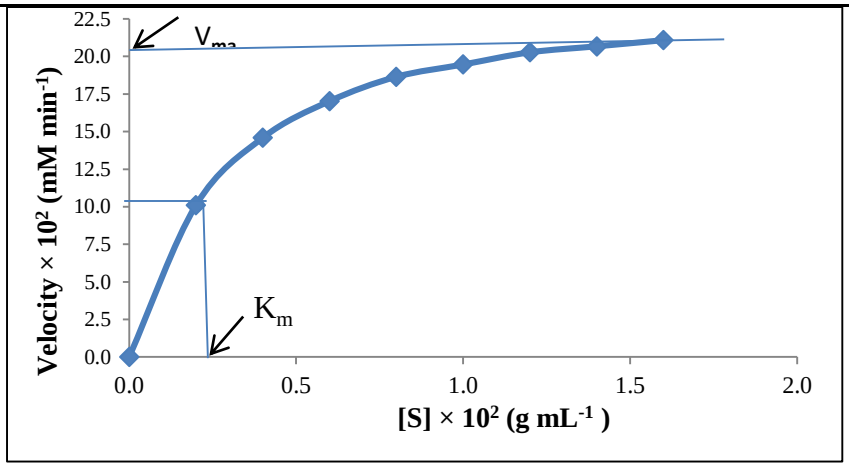

Figure 5. Michaelis-Menten plot of the velocity of crude pectinase-catalyzed reaction as a function of substrate concentration

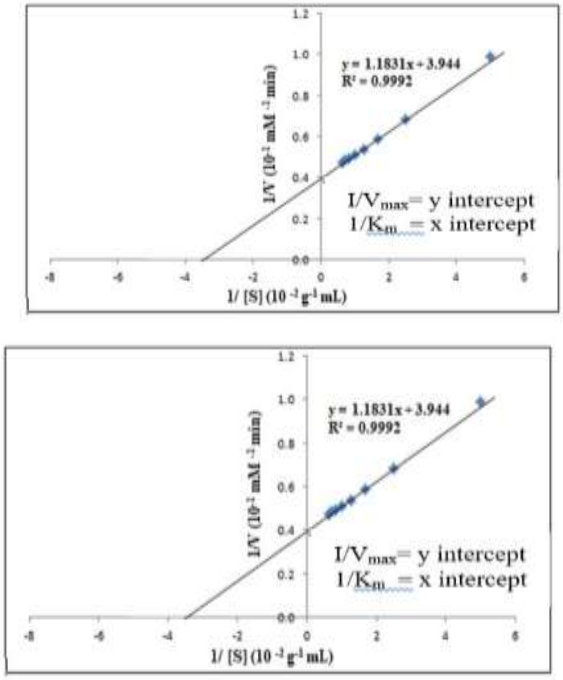

Figure 6. Lineweaver-Burk plot of 1/V *vs* 1/[S] used for graphic evaluation of V_{max} and K_m for crude pectinase enzyme

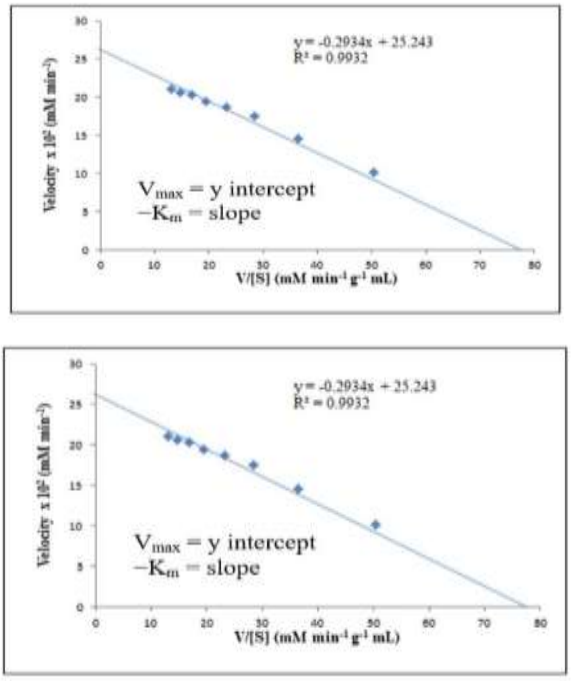

Figure 7. Eadie-Hofstee plot of V *vs* V/[S] Used for graphic evaluation of V_{max} and K_m for crude pectinase enzyme

Table 4. Relationship between Velocity of Partially Purified Polyphenol Oxidase-Catalyzed Reaction and Substrate Concentration

[S]×10² (g mL⁻¹)	-[S]×10² (g mL⁻¹)	1/[S] (10⁻² g⁻¹ mL)	V×10² (mM min⁻¹)	1/V (10⁻¹ mM⁻¹ min)	V/[S] (mM min⁻¹g⁻¹mL)	[S]/V (g mL⁻¹ mM⁻¹ min)
0.2	-0.2	5.00	7.40	1.351	37	0.027
0.4	-0.4	2.50	11.43	0.874	28.58	0.035
0.6	-0.6	1.67	14.19	0.709	23.65	0.042
0.8	-0.8	1.25	16.22	0.617	2.028	0.049
1.0	-1	1.00	17.84	0.562	17.84	0.056
1.2	-1.2	0.83	18.66	0.536	15.55	0.064
1.4	-1.4	0.71	19.10	0.524	13.64	0.073
1.6	-1.6	0.63	19.46	0.514	12.16	0.082

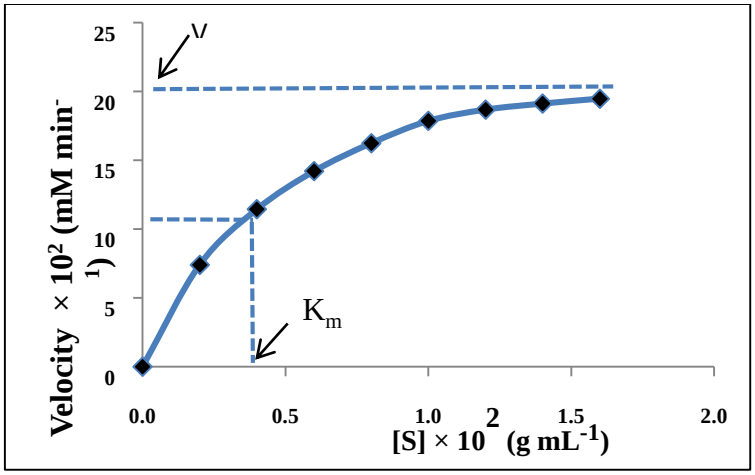

Figure 8. Michaelis-Menten plot of the velocity of partially purified pectinase-catalyzed reaction as a function of substrate concentration

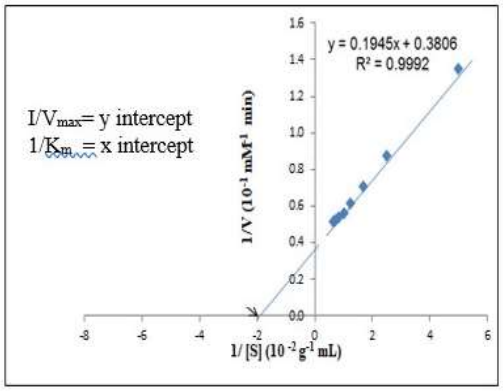

Figure 9. Lineweaver-Burk plot of 1/V *vs* 1/ [S] used for graphic evaluation of V_{max} and K_m for partially purified pectinase enzyme

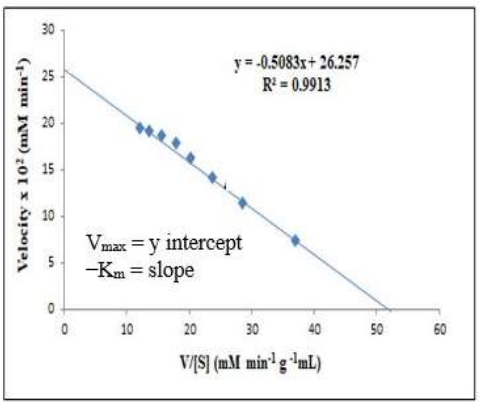

Figure 10. Eadie-Hofstee plot of V *vs* V/[S] used for graphic evaluation of V_{max} and K_m for partially purified pectinase enzyme

Table 5. Comparison of Kinetic Parameters of Crude and Partially Purified Pectinase from White Dragon Fruit Peels

Enzyme	Method	Linear regression method		Graphical method	
		$K_m \times 10^2$ (g mL^{-1})	$V_{max} \times 10^2$ (mM min^{-1})	$K_m \times 10^2$ (g mL^{-1})	$V_{max} \times 10^2$ (mM min^{-1})
crude enzyme	Michaelis-Menten	-	-	0.250	20.02
	Lineweaver-Burk	0.300	25.380	0.330	25.35
	Eadie-Hofstee	0.294	25.196	0.300	25.24
partially purified enzyme	Michaelis-Menten	-	-	0.045	20.05
	Lineweaver-Burk	0.509	26.281	0.500	26.32
	Eadie-Hofstee	0.520	26.503	0.508	26.26

Table 6. Relationship between Velocity of Pectinase-Catalyzed Reaction and Substate Concentration for the Determination of Reaction Order

[S] (10^{-2}g mL^{-1})	log [S]	Crude enzyme		Partially purified enzyme	
		V × 10^2 (mM min^{-1})	Log V/(V_{max}-V)	V × 10^2 (mM min^{-1})	Log V/(V_{max}-V)
0.2	-2.699	10.10	-0.179	7.40	-0.407
0.4	-2.398	14.59	0.131	11.93	-0.114
0.6	-2.222	17.03	0.309	14.19	0.070
0.8	-2.097	18.65	0.442	16.22	0.208
1.0	-2.000	19.47	0.518	17.84	0.324
1.2	-1.921	20.28	0.599	18.66	0.390
1.4	-1.854	20.68	0.643	19.10	0.426
1.6	-1.796	21.09	0.692	19.46	0.456

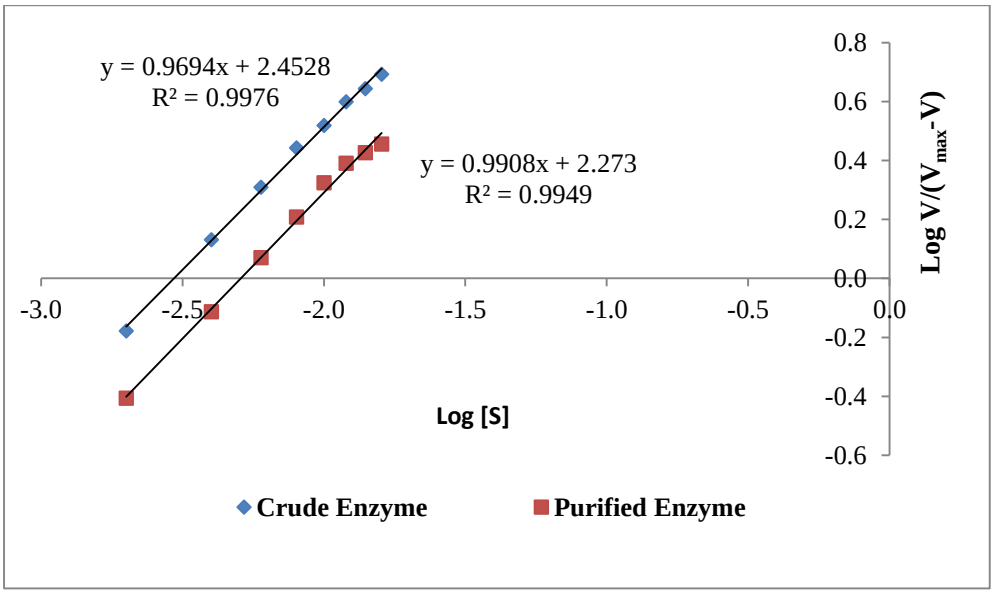

Figure 11. Plot of Log V/(V_{max}-V) as a function of log [S] for crude and purified pectinase-catalyzed reaction

Table 7. Grape Juice Clarification at 660 nm by Pectinase

Test	Absorbance	Transmittance (%)	Test	Absorbance	Transmittance (%)
A	0.186	65.16	A	0.185	65.31
B	0.179	66.22	B	0.178	66.37
C (crude)	0.122	75.49	C (purified)	0.064	86.29

A= control (juice only)
B= grape juice and water
C= grape juice and enzyme

Conclusion

In this research, the pectinase was isolated from white dragon fruit peel by extraction with sodium acetate buffer (pH 5). The specific activity of the enzyme increased about 3.50 folds from crude to the final purification step. The enzymic properties: optimum pH (5.0), optimum temperature (30 °C) and the K_m and V_{max} were determined by the spectrophotometric method. K_m and V_{max} values were calculated to be 0.509 x 10^{-2} g mL^{-1} and 26.281 x 10^{-2} mM min^{-1} for partially purified pectinase. The reaction order (n) for crude and purified pectinase was a first-order reaction. For the clarification of grape juice, the purified enzyme was found to have a higher efficiency than the crude enzyme. It indicated that the pectinase extracted from peel waste was effective in the clarification of fruit juice. This pectinase needs to be studied more to make sure it works better in the fruit industry.

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