ENZYMIC STUDY ON PECTINASE EXTRACTED FROM RED DRAGON FRUIT PEELS

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Abstract

The extraction of pectinase enzyme from red dragon fruit peels was done by using ammonium sulphate precipitation (20–80 %) method, and then the purification of the enzyme was done by using the gel filtration chromatographic method on Sephadex G-100. The enzyme activity was measured by utilizing DNS method to detect the release of the reducing sugar group. Specific activity, protein recovery, and degree of purification were measured in each purification step. The pectinase enzyme was purified 6.49-fold over crude extract, and protein recovery was found to be 8.43 %. The K_m values of the crude and partially purified pectinase enzymes were found to be 0.1992×10^{-2} g mL^{-1} and 0.358×10^{-2} g mL^{-1}, respectively. The reaction order (n) of the pectinase-catalyzed reactions was found to be first order. The activation energy (E_a) of the partially purified pectinase-catalyzed reaction was lower than that of the crude pectinase-catalyzed reaction. Both crude and partially purified pectinase enzymes were used in the clarification of apple juice, and good results were observed.

Keywords: pectinase, *gel filtration chromatography, ammonium sulphate precipitation method, DNS method, clarification,* apple juice

Introduction

The primary source of industrial enzymes is microorganisms, out of which 50 % originate from fungi and yeast, 35 % from bacteria, and the remaining 15 % are either of plant or animal origin (Anisa and Girish, 2014). Today, pectinases are one of the most important enzymes in the commercial sector. Pectinases are responsible for the degradation of the long and complex molecules called pectin. Pectin occurs as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells (Kashyap *et al.,* 2001). Pectinases are classified into three groups according to the following criteria: hydrolytic (hydrolases) or trans-eliminative (lyases) cleavage of the glycosidic bonds; endo (randomic) or exo (from the molecule end) mechanism of action; and preference for substrate, pectic acid, or pectin. The three main types of pectinases are pectinesterase, depolymerizing enzymes, and protopectinases (Kumar, 2015). Pectinases have crucial industrial significance in improving juice yields, scouring of cotton, degumming of plant fibres, wastewater treatment, vegetable oil extraction, and so on. It plays an important role in the food and wine industry in the processing of fruit juices (Pauldas and Jain, 2018). This study aims to extract pectinase from red dragon peels and study its kinetic properties and efficiency in apple juice clarification.

Materials and Methods

Red dragon fruit (*Selenicereus costaricensis* (F. A. C. Weber) S. Arias & N. Korotova) samples were collected from Hledan Market, Kamayut Township, 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

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Laboratory, Department of Chemistry, University of Yangon. 3,5-Dinitrosalicyclic acid (DNS) and all other chemicals used in this work were of analytical grade and were products of BDH Chemical Limited (England).

Sample Preparation and Extraction of Pectinase

Red dragon fruit samples were washed with distilled water and dried at room temperature. Then the red dragon fruits were peeled and cut into small pieces. 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 about 20 min at 6000 rpm to obtain the extract. Solid ammonium sulphate was added to this extract to obtain 20-80 % saturation and stored at 4 ºC. After standing overnight, the protein precipitate containing the pectinase was collected by centrifugation for 20 min at 6000 rpm.

Enzyme Purification

The crude pectinase enzyme was dissolved in 0.1 M sodium acetate buffer (pH 5.0). Then it was put into the column filled with Sephadex G-100 equilibrated with sodium acetate buffer. The flow rate was adjusted to 1.5 mL per 5 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 3,5-dinitrosalicyclic acid (DNS) method. The fraction with the highest enzyme activity was pooled to obtain a partially purified enzyme and stored at 4 °C.

Assay of the Crude and Partially Purified Enzymes

The enzyme activity was determined by measuring the release of sugar groups using the 3,5-dinitrosalicyclic acid (DNS) method. In brief, 0.5 mL of enzyme solution was incubated with 0.5 mL of 1 % pectin substrate in 0.1 M acetate buffer (pH 5.0) at 45 ℃ for 10 min. Then, 1 mL of DNS solution was added, and this mixture was kept at 90 ℃ in a boiling water bath for 5 min. 1 mL of 1 M sodium-potassium tartrate was added in order to stop the reaction, and 2 mL of distilled water was added to the mixture. The absorbance of the mixture was measured at 591 nm by using a UV-visible spectrophotometer. One unit of enzyme was defined as one micromole of glucose that was liberated per minute.

Proten content in each purification step was determined by the Biuret method at 550 nm

Kinetic Studies of the Crude and Partially Purified Pectinase Enzymes

The Michaelis-Menten constant (K_m), maximum velocity (V_{max}), and the reaction order of crude and partially purified enzymes were determined using pectin as a substrate in the range of 2 mg mL^{-1} to 16 mg mL^{-1}. The activation energy of the pectinase-catalyzed reaction was also evaluated by using different temperatures of 20 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC, and 45 ºC.

Application of the Crude and Partially Purified Pectinase Enzymes in Apple Juice Clarification

Apple fruits purchased from the local market were washed carefully and chopped into small pieces with a sharp knife. Firstly, 40 g of chopped samples were mixed with 40 mL of distilled water and blended for 2 min in a blender. After that, apple fruit juice was filtered through cheesecloth to remove insoluble materials. Next, 8 mL of apple fruit juice was taken in a

test tube and warmed in a water bath at 45 ℃ for 10 min to inactivate any natural fruit enzymes or bacteria present. Then, crude and partially purified pectinase solutions (2 mL each) was added to 8 mL of apple fruit juice. After a 4-h incubation period, the sample was heated for 3 min at 45 ℃. The juice was centrifuged for 20 min at 3000 rpm, and the supernatant was filtered out with filter paper. The clarity of the apple juice was calculated by measuring the absorbance at 660 nm (Maznila *et al*., 2008) with a UV-Vis spectrophotometer. Similarly, the above procedure was carried out by using (i) 10 mL of apple juice without enzyme and (ii) 8 mL of apple juice and 2 mL of distilled water. The clarity was expressed in percentages.

Results and Discussion

Purification of Pectinase from Red Dragon Fruit Peels

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 (Pawar, 2023). Figure 1 shows the chromatogram of pectinase on Sephadex G-100 gel. The protein content of the eluate was checked spectrophotometrically at 280 nm, and the enzyme activity was determined at 591 nm. The fractions with the highest activity (47–61 fractions) were pooled. The partially purified pectinase enzyme was purified up to 6.49 folds with a specific activity of 0.851 U/mg over crude extract. The pectinase activity, specific activities of the enzyme solutions, and purity of the enzyme were described in Table 1.

Effect of Substrate Concentration on Pectinase-catalyzed Reaction

The effect of substrate concentration on the velocity of the pectinase-catalyzed reaction was studied. It was found that velocity increases with an increase in substrate concentration from 0.2×10^{-2} to 1.6×10^{-2} g mL^{-1}. However, at higher concentrations of pectin, the enzyme becomes saturated with substrate and reaches V_{max}, the enzyme's maximum velocity. Further increasing the concentration of substrate does not increase the velocity significantly. The results are shown in Tables 2 and 3 for crude and partially purified pectinase, respectively. Michaelis-Menten, Lineweaver-Burk, and Eadie-Hofstee plots for crude and partially purified pectinase enzymes are depicted in Figures 2, 3, 4, 5, 6, and 7, respectively. In this study, linear regression method was used to obtain V_{max} and K_m from experimental results. These values are shown in Table 4 in comparison with the V_{max} and K_m values obtained by Lineweaver-Burk and Eadie-Hofstee plots, which are not much different from each other, implying that they are comparable on the basis of quantitative aspects. The K_m and V_{max} values of the pectinase were found to be 0.1992×10^{-2} g mL^{-1} and 0.5839×10^{-2} mM min^{-1}, respectively, for the crude enzyme, and 0.358×10^{-2} g mL^{-1} and 28.389×10^{-2} mM min^{-1} for the purified enzyme.

Reaction Order for Pectinase-catalyzed Reaction

Depending on the substrate concentrations, the kinetics of an enzyme-catalyzed reaction may be described by the first-order rate equation (Bergmeyer, 1983). In this research, the reaction order (n) value was determined from the plot of log $V/(V_{max} - V)$ vs. log [S] using the linear regression method (Table 5 and Figure 8). The reaction order (n) for the crude and purified pectinase-catalyzed reactions was calculated to be first order.

Activation Energy for Pectinase-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 favourable, thereby accelerating the rate at which the reaction will proceed but not fundamentally changing the energy levels of either the reactant or the product (Robinson, 2015). Table 6 shows the relationship between the velocity of the pectinase-catalyzed reaction and temperature. Figure 9 shows a plot of Log V as a function of 1/T for the determination of activation energy and Arrhenius constants. The Arrhenius constants of the crude and purified pectinase-catalyzed reactions were found to be 3.211×10^6 and 0.136×10^6, respectively. The activation energy (E_a) values of crude and partially purified pectinase-catalyzed reactions were determined to be 5.339 kcal mol^{-1} and 4.121 kcal mol^{-1} from linear regression and 5.491 kcal mol^{-1} and 4.290 kcal mol^{-1} from the graph, respectively, for temperatures between 20 and 45 °C.

Application of Crude and Partially Purified Pectinase Enzymes in Apple Juice Clarification

Pectinase plays an important role in the process of extracting and purifying the juice (Berutu *et al.*, 2017). In this research, the effect of pectinase on the clarification of apple juice was studied. The clarity of the apple juice was determined by measuring the absorbance at 660 nm with a spectrophotometer and expressed in terms of percent transmittance. A lower absorbance value i.e., a higher transmittance value indicated clearer juice. The clarity of treated apple juice improved because of the removal of colloidal and suspended particles in the juice, especially pectinaceous material (Joshi *et al.*, 2011). The juice became clearer, as did the increase in colour and appearance. The percents transmittance of crude and purified enzymes in apple juice were 74.47 % and 96.38 %, respectively, compared to 50.69 % and 96.30 % for apple juice without enzymes (Table 7).

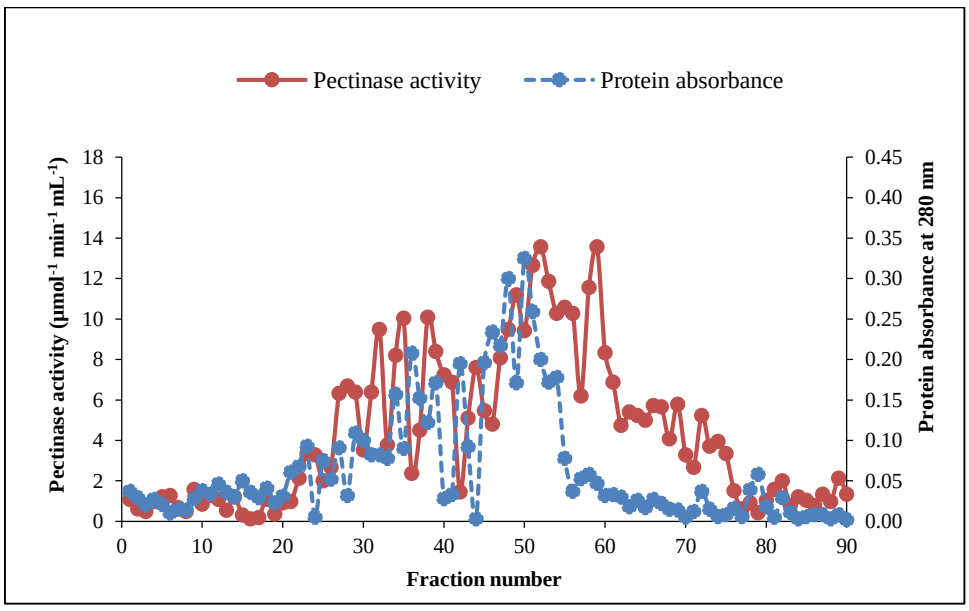

Figure 1. Chromatogram of crude pectinase enzyme on Sephadex G-100 column

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	140	42.00	320.60	0.13	100.00	1.00
20 % $(NH_4)_2SO_4$ precipitation	81.0	44.55	277.83	0.16	86.82	1.22
80 % $(NH_4)_2SO_4$ precipitation	14.5	16.40	41.11	0.39	12.82	2.97
after passing the Sephadex G-100	21.0	22.99	26.99	0.85	8.43	6.49

Table 2. Relationship between Velocity of Crude Pectinase-catalyzed Reaction and Substrate Concentration

$[S]x10^2$ (g mL^{-1})	$-[S] \times 10^2$ (g mL^{-1})	1/[S] ($10^{-2}g^{-1}mL$)	$V \times 10^2$ (mM min^{-1})	1/V ($10^{-2}mM^{-1}min$)	V/[S] (mM $min^{-1}g^{-1}mL$)	[S]/V (g $mL^{-1}mM^{-1}min$)
0.2	-0.2	5.00	0.292	3.425	1.460	0.685
0.4	-0.4	2.50	0.397	2.519	0.992	1.007
0.6	-0.6	1.67	0.429	2.331	0.715	1.399
0.8	-0.8	1.25	0.46	2.146	0.582	1.659
1.0	-1.0	1.00	0.482	2.075	0.482	1.988
1.2	-1.2	0.83	0.503	1.988	0.419	2.386
1.4	-1.4	0.71	0.515	1.942	0.368	2.718
1.6	-1.6	0.63	0.523	1.912	0.327	3.059

Table 3. Relationship between Velocity of Partially Purified Pectinase-catalyzed Reaction and Substrate Concentration

$[S] \times 10^2$ (g mL^{-1})	$-[S] \times 10^2$ (g mL^{-1})	1/[S] ($10^{-2}g^{-1}$ mL)	$V \times 10^2$ (mM min^{-1})	1/V (10^{-2} 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	10.138	0.099	50.693	0.019
0.4	-0.4	2.50	15.005	0.067	37.512	0.027
0.6	-0.6	1.67	17.844	0.056	29.740	0.034
0.8	-0.8	1.25	19.871	0.050	24.839	0.040
1.0	-1.0	1.00	21.088	0.047	21.088	0.047
1.2	-1.2	0.83	21.899	0.046	18.249	0.055
1.4	-1.4	0.71	22.305	0.045	15.932	0.063
1.6	-1.6	0.63	22.710	0.044	14.194	0.070

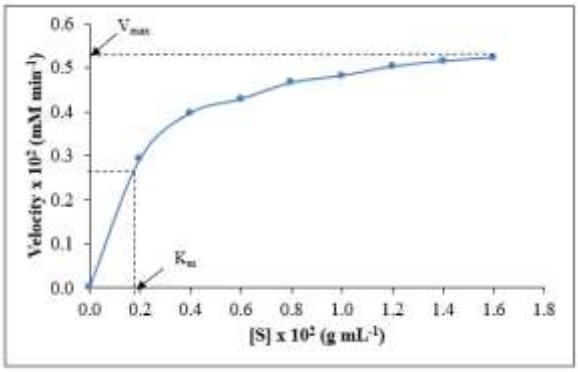

Figure 2. Michaelis-Menten plot of crude pectinase enzyme-catalyzed reaction

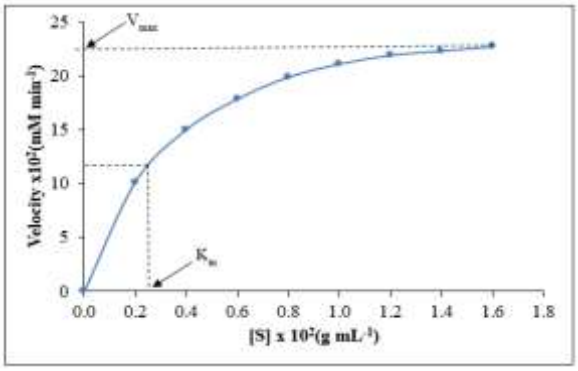

Figure 3. Michaelis-Menten plot of partially purified pectinase enzyme-catalyzed reaction

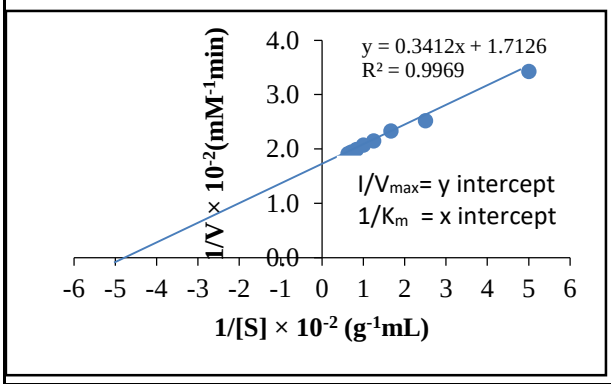

Figure 4. Lineweaver-Burk plot of 1/V *vs.* 1/[S] for crude pectinase enzyme-catalyzed reaction

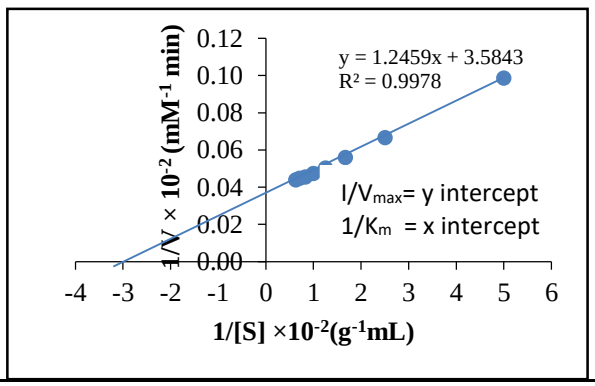

Figure 5. Lineweaver-Burk plot of 1/V *vs.* 1/[S] for partially purified pectinase enzyme-catalyzed reaction

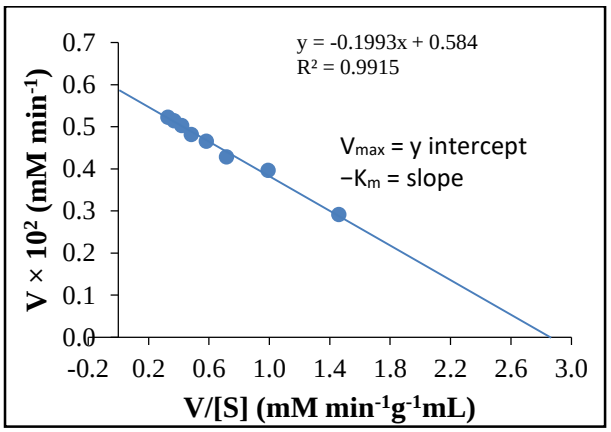

Figure 6. Eadie-Hofstee plot of crude pectinase enzyme-catalyzed reaction

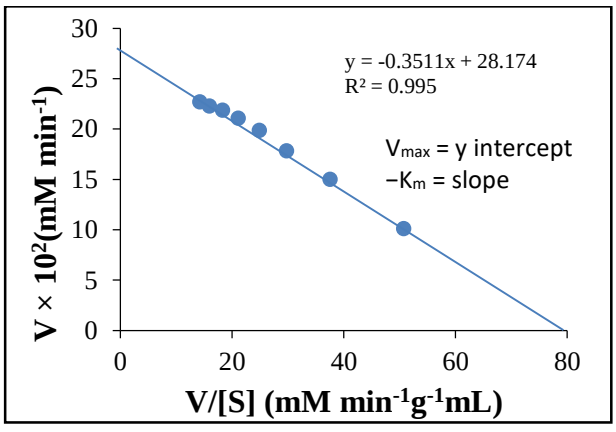

Figure 7. Eadie-Hofstee plot of partially purified pectinase enzyme-catalyzed reaction

Table 4. V_{max} and K_m Values of Crude and Partially Purified Pectinase using Linear Regression and Graphical Methods

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 pectinase	Michaelis-Menten	-	-	0.199	0.530
	Lineweaver-Burk	0.1992	0.5839	0.200	0.583
	Eadie-Hofstee	0.1993	0.5840	0.194	0.584
partially purified pectinase	Michaelis-Menten	-	-	0.270	22.50
	Lineweaver-Burk	0.3580	28.389	0.333	27.90
	Eadie-Hofstee	0.3510	28.173	0.352	28.20

Table 5. Relationship between log $V/(V_{max}-V)$ and Log [S] for the Determination of Reaction Order for Crude and Partially Purified Pectinase-catalyzed Reactions

No	Log [S]	Crude enzyme	Partially purified enzyme
		Log $V/(V_{max}-V)$	Log $V/(V_{max}-V)$
1	-2.699	0.0001	-0.246
2	-2.398	0.3272	0.066
3	-2.222	0.4424	0.246
4	-2.097	0.5969	0.396
5	-2.000	0.6749	0.484
6	-1.921	0.7936	0.527
7	-1.854	0.8736	0.563
8	-1.796	0.9339	0.601

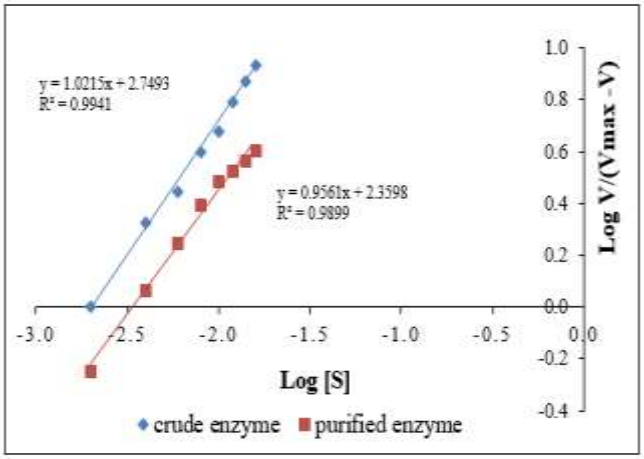

Figure 8. Plot of Log $V/(V_{max}-V)$ as a function of Log [S] of crude and partially purified pectinase-catalyzed reactions

Table 6. Relationship between Velocity of Crude and Partially Purified Pectinase-catalyzed Reactions and Temperature of the Solution at pH 5

Temperature (°C)	$1/T \times 10^3$ (K^{-1})	Crude pectinase		Partially purified pectinase	
		Velocity $\times 10^3$ (mM min^{-1})	Log V	Velocity$\times 10^3$ (mM min^{-1})	Log V
20	3.413	336.664	2.527	81.109	1.909
25	3.356	409.603	2.612	101.387	2.006
30	3.300	454.213	2.657	117.609	2.085
35	3.247	466.379	2.668	125.720	2.126
40	3.195	559.655	2.748	150.053	2.231
45	3.145	783.706	2.894	206.829	2.315

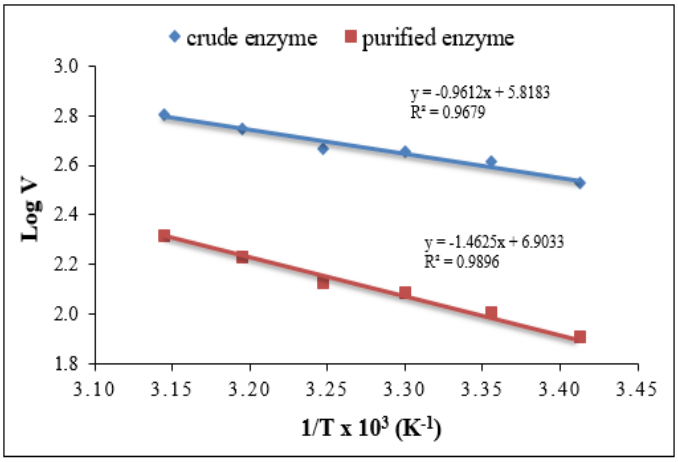

Figure 9. A plot of Log V as a function of 1/T for the determination of E_a

Table 7. Transmittance and Absorbance of Crude and Partially Purified Pectinase Enzyme on Apple Juice at 660 nm

Test	Absorbance	Transmittance (%)	Test	Absorbance	Transmittance (%)
A	0.295	50.69	A	0.303	49.77
B	0.270	53.70	B	0.274	53.21
C (crude)	0.128	74.47	C (partially purified)	0.016	96.38

A = (juice only)
B = (distilled water and juice)
C = (enzyme and juice)

Conclusion

For the extraction and partial purification of pectinase from red dragon fruit peels, the ammonium sulphate precipitation method and gel filtration chromatographic method (Sephadex G-100) were used. The partially purified pectinase increased 6.49 folds compared to crude enzyme extracts. The Michaelis-Menten constant, K_m, of the crude pectinase enzyme was found to be 0.1992×10^{-2} g mL^{-1}, whereas the partially purified enzyme was 0.358×10^{-2} g mL^{-1}. The reaction order (n) of the pectinase-catalyzed reaction was found to be first order. The activation energy values of the crude and purified pectinase-catalyzed reactions were 5.339 kcal mol^{-1} and 4.12 kcal mol^{-1}, respectively. The crude and purified pectinase enzymes from red dragon fruit peels were used for the clarification of apple juice. The percent transmittance value of apple juice using purified pectinase was found to be higher (96.38 %) compared to crude pectinase (74.74 %). This study shows the efficiency of pectinase in fruit juice clarification.

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References

Anisa, S. K. and K. Girish. (2014). "Pectinolytic Activity of *Rhizopus sp.*, and *Trichoderma viridae*". *International Journal of Research in Pure and Applied Microbiology*, vol. 4 (2), pp. 28-31.

Bergmeyer, H. U. (1983). *Method of Enzymatic Analysis.* New York: Academic Press Inc., pp. 69-78.

Berutu, C. A. M., F. Fahrurrozi, and A. Meryandini. (2017). "Pectinase Production and Clarification Treatments of Apple (*Malus Domestica*) Juice". *Annales Bogorienses*, vol. 21 (2), pp. 63–68.

Joshi, V. K., M. Parmar, and N. Rana. (2011). "Purification and Characterization of Pectinase Produced from Apple Pomace and Evaluation of its Efficacy in Fruit Juice Extraction and Clarification". *Indian Journal of Natural Products and Resources*, vol. 2 (2), pp. 189-197.

Kashyap, D. R., P. K. Vohra, S. Chopra, and R. Tewari. (2001). "Application of Pectinase in the Commercial Sector: A Review". *Bioresource Technology*, vol. 77 (3), pp. 215-227.

Kumar, S. (2015). "Role of Enzyme in Fruit Juice Processing and its Quality Enhancement". *Advances in Applied Science Research*, vol. 6(6), pp. 114-124.

Mazlina, M. K. S., L. A. A. Ghani, A.R. N. Aliaa, H. S. Aslina, and O. Rozita. (2008). "Comparison on

Optimization of Star Fruit Juice Using RSM between Two Malaysian Star Fruit Varieties (B11 and B10)". *Pertanika J. Sci. & Technol*., vol.16 (1), pp.1-13.

Pauldas, K. and A. Jain. (2018). "Optimization of Pectinase Production Kinetics by *Candida tropicalis* and its Applications in Fruit Juice Clarification". *International Journal of Pharmacy and Biological Sciences*, vol. 8(3), pp. 946-958.

Pawar, H. (2023). "Role of Enzyme Purification Techniques and its Importance". *Enzyme Engineering*, vol. 12(1), p. 1.

Robinson, P.K. (2015)."Enzyme: Principles and Biotechnological Applications". *Essays in Biochemistry*, vol. 59, pp. 1-41.