Kinetic Parameters of Peroxidase from Castor plant to substrate

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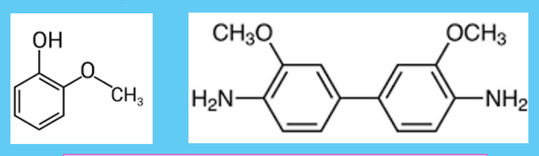
Introduction

Kinetic Values

This research focused on the kinetic values of peroxidase from castor leaves when three different substrates including guaiacol, ortho-dianisidine and hydrogen peroxide were used.Determination was carried out from kinetic constants such as the K*m* and Vmax values of peroxidase for each substrate from the Lineweaver-Burk plots curve.



Structures : Guaiacol and Ortho-dianisidine

Castor Plant

It is a plant in the rubber family Euphorbiaceae and the only plant in the genus Ricinus. It is native to the Mediterranean and East Africa. It is a shrub and It can be used as a source of oil from cosmetic products.

Raw Materials: Castor plant leaves

Materials and Methods

Determination of Activity and Kinetics for Different Substrates at Various Concentrations

Preparation of Castor Leaves for Extraction

- The stems are removed, and the leaves are washed and air-dried for 30 minutes.
- The leaves are wiped dry, weighed into 50–70 g portions, chopped into small pieces, and stored in plastic bags.
- They were frozen at -20°C for about two weeks to reduce enzyme activity to a suitable level before extraction.

Preparation of Castor Leaves and Crude Enzyme Extract

- The leaves are washed and left for 30 minutes.
- 25 g of leaves were weighed and blended with sodium acetate buffer (0.1 M, pH 6.0) containing:
- 1% (w/v) Polyvinylpyrrolidone (PVP)
- 1 mM Phenylmethylsulfonyl fluoride (PMSF) (dissolved in ethanol)
- The homogenate was filtered using cheesecloth and centrifuged at 10,000 rpm, 4°C, for 20 minutes.
- The supernatant was collected for enzymatic activity and protein constant analysis.

Enzyme Separation by Aqueous Two-Phase System

- 1.4 g Polyethylene glycol (PEG) 1500 and 1.2 g Sodium sulfate are dissolve in 7 mL crude extract.
- The solution was adjusted to 10 mL total volume, sealed with parafilm, and left for 3 hours for phase separation.
- The volumes of the upper and lower phases were recorded, and enzymatic activity and protein content were analyzed.

Determination of Peroxidase Specific Activity

- Three substrates were tested: Guaiacol, o-Dianisidine, and Hydrogen Peroxide, each at five different concentrations.
- Activity was measured at 470 nm using a UV-Vis spectrometer. The peroxidase unit is defined as the amount of enzyme catalyzing the formation of 1 µmol of tetraguaiacol per minute at 25°C.
- The activity is calculated using the equation:

$$\text{peroxidase activity (unit/ml)} = \frac{(A/t) \times V_t \times D_f \times 1000}{\varepsilon \times S_V \times S_f \times P}$$

where:

- A/t = slope of absorbance and time
- V_t = total reaction volume (mL)
- D_f = dilution factor
- ε = extinction coefficient of tetraguaiacol and ortho-dianisidine absorbance 470 nm and 460 nm (26.6 $mM^{-1}cm^{-1}$ and 11.3 $mM^{-1}cm^{-1}$)
- S_v = sample volume (µL)
- S_f = stoichiometric factor (1)
- P = cuvette path length (1 cm)

Protein Quantification by Bradford Assay

- Bradford reagent was prepared using Coomassie Brilliant Blue G-250, ethanol (95%), and phosphoric acid (85%).
- A standard curve was generated using Bovine Serum Albumin (BSA) at 0.01–0.05 mg/mL.
- The protein content in enzyme extract was measured by mixing 50 µL of the sample with 450 µL sodium phosphate buffer (0.1 M, pH 6.0) and 1 mL Bradford reagent.
- Absorbance was measured at 595 nm, and protein concentration was calculated using the standard curve.

Reference

- Ilesanmi, O.S., & Adedugbe, O.F. (2023). Novel Peroxidase from Bitter leaf (Vernonia amygdalina): Purification, Biochemical Characterization and Biotechnological applications. Biocatalysis and Agricultural Biotechnology 49:102662. https://doi.org/10.1016/j.bcab.2023.102662.
- Bekavac, N.; Benkovi´c, M.;Jurina, T.; Valinger, D.; Gajdoš Kljusuri´c, J.; Jurinjak Tušek, A.; Šali´c, Advancements in Aqueous Two-phase Systems for Enzyme Extraction, Purification and Biotransformation (Faculty of Food Technology and Biotechnology, University of Zagreb, 2024

Abstract

This research aimed to study the kinetic values of peroxidase extracted from castor leaves. Three types of substrates including guaiacol, ortho- dianisidine and hydrogen peroxide were examined.The experiment was designed to investigate the effect of substrate concentration on the activity of the enzyme. Five concentrations for each type of substrate were used while peroxidase activity from castor was at 2.14 kU for each test. The kinetic constants including the Michaelis- Menten constant (Km) and the maximum velocity (Vmax) were calculated. The experimental results indicated that when the concentration of hydrogen peroxide was held constant, peroxidase from castor beans exhibited specificity as follows. Km was 1.8339 mM and Vmax was 15.6740 units/mL for guaiacol, while for ortho- dianisidine, Km was 0.0937 mM and Vmax was 13.9860 units/mL. The Km and Vmax values suggest that the enzyme showed higher affinity for the ortho- dianisidine compared to that of guaiacol, but the enzymatic efficiency in releasing the product was relatively lower than that of guaiacol. For hydrogen peroxide, Km was 4.9603 mM and Vmax was 21.2314 units/mL, when guaiacol concentration was held constant. This study provided the understanding of the kinetic values of peroxidase from castor leaves demonstrating the enzymatic behavior towards different types of substrates. These data are useful in research and development of peroxidase application in industrial processes related to oxidation reactions, wastewater treatment and synthesis of important organic compounds via enzymatic approach.

Results

Table 1 : Crude extract preparation from castor leaves and ATPS

Number of experiments	1	2	3
Weight of castor leave (g)	50	25	25
Volume of extract buffer (mL)	200	100	100
Volume of crude extract (mL)	190	73.5	75.0
Volume of crude extract in ATPS (mL)	10	10	10
volume of Top phase (mL)	4.0	3.6	3.8
volume of Bottom phase (mL)	6.0	6.4	6.2

Table 2 : BSA Calibration curve

protein content (mg)	Number	A_{595}	A_{595} (avg)
0.005	1	0.1160	0.1200
	2	0.1210	
	3	0.1230	
0.010	1	0.2100	0.2297
	2	0.2170	
	3	0.2620	
0.015	1	0.2890	0.3143
	2	0.3220	
	3	0.3320	
0.020	1	0.3520	0.3977
	2	0.4060	
	3	0.4350	
0.025	1	0.4330	0.4753
	2	0.4740	
	3	0.5190	

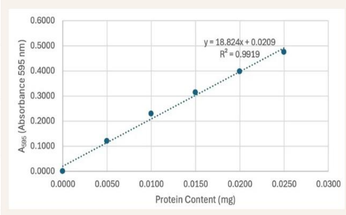


Figure 1 : BSA Calibration curve

Table 3 : Enzymatic activity assay and protein content determination for peroxidase extracted from castor leaves

Process of purification	Total volume (mL)	Activity (units/mL)	Concentration Protein (mg/mL)	Total Activity (units)	Total protein content (mg)	Specific Activity ($units/mg_{protein}$)
Determination of activity and protein content from peroxidase extract from castor leaves, 1st						
Crude Extract	190	8.1441	6.8349	1,547.1790	1,298.6310	1.1914
Top phase	4.0	0.7284	3.5391	2.9136	14.1564	0.2044
Bottom phase	6.0	12.8308	3.4187	76.9848	20.5122	3.7528
Determination of activity and protein content from peroxidase extract from castor leaves, 2nd						
Crude Extract	73.5	11.3223	4.8906	832.1891	359.4591	2.3154
Top phase	3.6	0.0244	2.7953	0.0878	10.0631	0.0087
Bottom phase	6.4	16.8797	2.8449	108.0301	18.2074	5.9331
Determination of activity and protein content from peroxidase extract from castor leaves, 3rd						
Crude Extract	75.0	9.7538	5.7937	731.5350	434.5275	1.6837
Top phase	3.8	0.0282	2.6360	0.1072	10.0168	0.0107
Bottom phase	6.2	14.2594	2.8591	88.4083	17.7264	4.9875

Table 4 : Purification of peroxidase extracted from castor leaves.

Process of purification	Specific Activity ($units/mg_{protein}$)	Quantity equal to purity	Recovery percentage
Purification of peroxidase extracted from castor leaves, 1st			
Crude Extract	11.9137	1.0000	100
Top phase	2.0438	0.1715	0.1883
Bottom phase	37.5279	3.1500	4.9758
Purification of peroxidase extracted from castor leaves, 2nd			
Crude Extract	23.1540	1.0000	100
Top phase	0.0873	0.0038	0.0106
Bottom phase	59.3311	2.5625	12.9814
Purification of peroxidase extracted from castor leaves, 3rd			
Crude Extract	16.8372	1.0000	100
Top phase	0.1070	0.0064	0.0147
Bottom phase	49.8749	2.9622	12.0853

Table 5 : Enzymatic activity using guaiacol as a substrate while keeping hydrogen peroxide concentration constant.

Guaiacol concentration (mM)	Reciprocal of guaiacol concentration (mM^{-1})	Reciprocal of peroxidase activity for 1st extraction (mL/units)	Reciprocal of peroxidase activity for 2nd extraction (mL/units)	Reciprocal of peroxidase activity for 3rd extraction (mL/units)	Reciprocal of average peroxidase activity (mL/units)
4 mM	0.2500	0.1148	0.0949	0.0681	0.0926
7 mM	0.1429	0.1005	0.0810	0.0626	0.0814
12.5 mM	0.0800	0.0907	0.0707	0.0575	0.0730
18.5 mM	0.0541	0.0879	0.0681	0.0552	0.0704
25 mM	0.0400	0.0816	0.0677	0.0542	0.0678

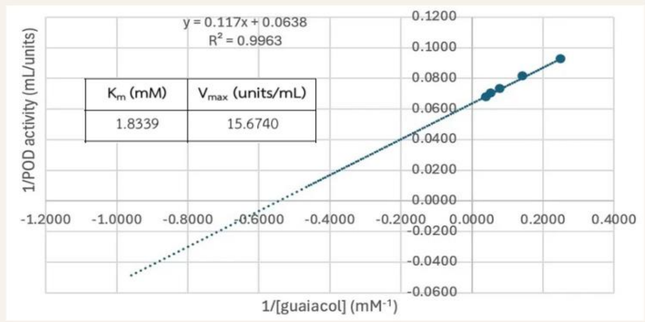


Figure 2 : Lineweaver Bulk Plot of Guaiacol

Table 6 : Determining of enzymatic activity using Ortho-Dianisidine as substrate and when hydrogen peroxide concentration was held constant.

Ortho-Dianisidine concentration (mM)	Reciprocal of Ortho-Dianisidine concentration (mM^{-1})	Reciprocal of peroxidase activity for 1st extraction (mL/units)	Reciprocal of peroxidase activity for 2nd extraction (mL/units)	Reciprocal of peroxidase activity for 3rd extraction (mL/units)	Reciprocal of average peroxidase activity (mL/units)
0.125 mM	8.0000	0.1346	0.1187	0.1210	0.1248
0.2 mM	5.0000	0.1133	0.1004	0.1019	0.1052
0.325 mM	3.0769	0.0979	0.0901	0.0936	0.0939
0.5 mM	2.0000	0.0927	0.0821	0.0820	0.0856
0.7 mM	1.4286	0.0838	0.0795	0.0744	0.0792

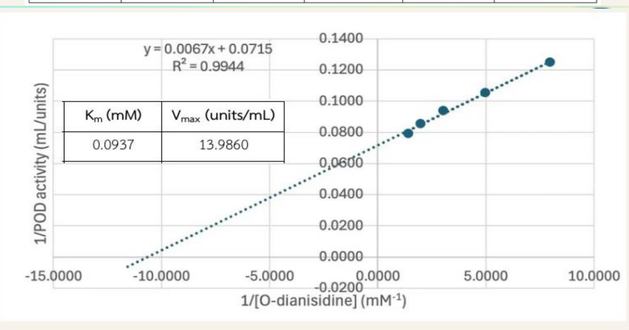


Figure 3 : Lineweaver Bulk Plot of Ortho-Dianisidine

Table 7 : Determining enzymatic activity using various hydrogen peroxide concentrations when guaiacol concentration was held constant.

Hydrogen Peroxide concentration (mM)	Reciprocal of Hydrogen peroxide concentration (mM^{-1})	Reciprocal of peroxidase activity for 1st extraction (mL/units)	Reciprocal of peroxidase activity for 2nd extraction (mL/units)	Reciprocal of peroxidase activity for 3rd extraction (mL/units)	Reciprocal of average peroxidase activity (mL/units)
1.25 mM	0.8000	0.2566	0.2289	0.2200	0.2352
2.5 mM	0.4000	0.1440	0.1406	0.1349	0.1398
5 mM	0.2000	0.0906	0.0874	0.0864	0.0881
10 mM	0.1000	0.0728	0.0716	0.0707	0.0717
15.5 mM	0.0645	0.0666	0.0660	0.0651	0.0659

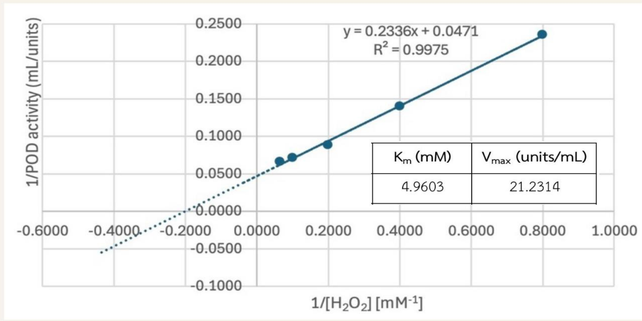


Figure 4 : Lineweaver Bulk Plot for Hydrogen Peroxide

Conclusion

The experimental results indicated that when the concentration of hydrogen peroxide was held constant, peroxidase from castor leaves exhibited specificity as follows. Km was found to be 1.8339 mM and Vmax was 15.6740 units/mL for guaiacol, while for ortho-dianisidine, Km was 0.0937 mM and Vmax was 13.9860 units/mL. The Km and Vmax values suggest that the enzyme has a higher affinity for the ortho-dianisidine compared to that of guaiacol, but the enzymatic efficiency in releasing the product was slightly lower than that of guaiacol. For hydrogen peroxide, Km was 4.9603 mM and Vmax was 21.2314 units/mL, when guaiacol concentration was held constant. This information is useful for choosing appropriate substrate for future application of peroxidase from this source.

Substrate	K_m (mM)	V_{max} (units/mL)
Guaiacol	1.8339	15.6740
Ortho-Dianisidine	0.0937	13.9860
Hydrogenperoxide	4.9603	21.2314