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Extraction and purification of peroxidase enzyme from sweet potato

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Abstract
This study focuses on extraction and partial purification of peroxidase enzyme from *Ipomoea batatas* the sweet potato, using ammonium sulphate precipitation coupled by dialysis tubing. The activity of the extracted enzyme was determined spectrophotometrically at different stages of purification. The assay was done using Ortho-Phenylenediamine Dihydrochloride (OPD) as substrate at room temperature. OPD, in the presence of peroxidase enzyme, reacts with hydrogen peroxide, to produce 2, 3-diaminophenazine, yellow-orange compound which has absorption maxima of 437 nanometer. The concentration of the total enzyme was calculated using the Lowry's method followed by the determination of the specific activity of the protein.

Keywords: Peroxidase enzyme assay, Spectrophotometer, OPD

Introduction
Peroxidase is a biotechnologically important and ubiquitous enzyme belonging to the oxidoreductase class of enzyme. This enzyme generally catalyses a reaction between hydrogen peroxide as electron acceptor and many kinds of substrates by means of oxygen liberation (Zia *et al*., 2011) [10]. It is present in plant kingdom, micro-organisms and animals where it catalyses the reduction of hydrogen peroxide to water, rendering it harmless. The enzyme is present naturally in plants like potato tuber, horse radish, beet, soybean, tomato, banana, papaya, carrot, turnip, wheat, dates, beats and strawberry (Reed 1974) (Ambreen *et al* 2000) [8, 1].

The peroxidases enzyme that finds its use in different biotechnological, biomedical and other applications. An example is in preparing enzyme conjugated antibodies due to its high specificity towards certain substances that involve change in colour and has a wide range of applications as diagnostic kits for enzyme immunoassays and as an important component of ELISA. Other uses include as biocatalysts and bio-electro catalysts.

At present horse radish is used as the major source of commercially available peroxidase. However, research continues for the finding of new peroxidases of elevated stability and suitable characteistics e.g., spring cabbage peroxidase was suggested as a potential tool in biocatalysis and bio-electro catalysis (Belcarz *et al*. 2007) [2]. Peroxidases have possibilities for applications clinically, in addition to in diagnostic kits and antibody labelling in waste-water treatment and in food and paper industries. Isoenzymes exist that have stability towards external factors (example, enzyme activity for substrates, pH and temperature). Enzyme stability towards external factors plays an essential role in biocatalysis and bioremediation. An acidic horse radish peroxidase isoenzyme A2 has been found more stable towards H_2O_2 inactivation than an isoenzyme C. However, an isoenzyme E showed higher specific activity in oxalacetate oxidation than the isoenzymes A2 and C. Taken to consideration these different properties of these isoenzymes may allow the finding of enzymes more suited to a particular application.

The peels of *Ipomoea batatas* are known to be rich in peroxidase enzyme. The aim of this study was to find the specific activity of this enzyme. This is the *activity* of an *enzyme* per milligram of total protein (expressed in μmol min^{-1} mg^{-1}). *Specific activity* gives a measurement of *enzyme* purity in the mixture. The first focus of the experiment was the extraction of the crude enzyme. This involved briefly, addition of a buffer followed by blending and centrifugation to retain the supernatant. Then followed was the storing of this supernatant at -20. Determining activity of this crude enzyme was carried out with a spectrophotometer.

The oxidation product of o-phenylenediamine produced by *peroxidase* is 2, 3-diaminophenazine, yellow orange compound which has absorption maxima of 437 nanometer. This was followed by partial purification of the peroxidase enzyme, using ammonium sulphate precipitation and centrifugation to give both the supernatant and the pellet and this was later coupled by dialysis tubing to purify enzyme further. The activity of the extracted enzyme was determined spectrophotometrically at different stages of purification. The concentration of the total enzyme was calculated using the Lowry's method with BSA as standard to then determine the specific activity of the protein. Also calculated were the fold purifications and the percentage yield for the extract, when possible, at each of the stages. The Beer-Lambert Law of absorbance was used to calculate enzyme activity and the Lowry with a BSA standard, for specific activity.

Experimental Method

Source collection and preparation of crude enzyme extract were carried out as follows:

Sweet potato was peeled, and the peelings were used as the sample. 10 ml of phosphate buffer of pH 7.0 was added to 1g of the sample and blended thoroughly using a mortar and pestle. This extract was centrifuged at 10,000 rpm for 15 minutes and the supernatant was retained. To selectively inactivate the traces of catalase moieties, the supernatant was heated at 65 °C for 3 minutes followed by placing it in ice bucket for 15 minutes. This crude extract was stored at -20 °C for future use.

The peroxidase enzyme assay

The peroxidase enzyme assay was carried out by adding 0.01 ml 3% of H_2O_2, 0.09ml of distilled water, 2mg of OPD (0.4mg/ml) and 4.8ml of phosphate buffer in a sample cuvette in a spectrophotometer. The spectrophotometer was switched on at the same time when 0.1 ml of enzyme was added to the sample cuvette and the reading of OD is taken at 437nm at absorption noted down for every 10 seconds up to 2 minutes. The blank cuvette contained all the components of the sample cuvette except the enzyme extract and the volume was made up to 5ml by adding 4.9ml of phosphate buffer. The activity of the enzyme was found by drawing a graph of time against and OD at 437nm on the y axis where absorbance is calculated as the slope of the tangent to the curve obtained with Beer-Lambert law, A= Ɛcl, and indicated as the concentration of the enzyme.

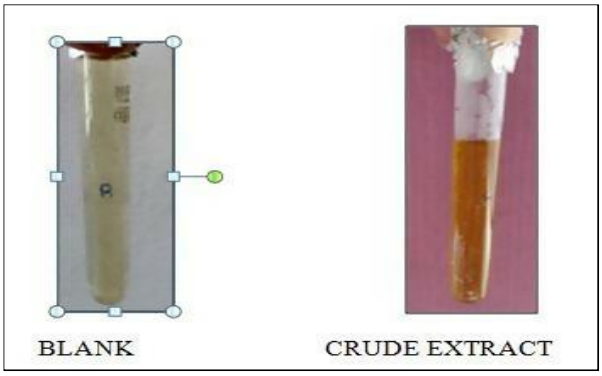


Fig 1: Blank and crude. Flip-cap centrifugation tubes, blank on the left, and with crude extract, on the right.

Ammonium sulphate precipitation

Ammonium sulphate precipitation included the crude extract precipitated using 80% ammonium sulphate. The precipitate was subjected to centrifugation at 10,000 rpm for 15 minutes. The supernatant and pellet were separated, and the pellet was suspended in 4ml of phosphate buffer. The peroxidase assay was performed on both the supernatant and the suspended pellet and the absorbance, and hence the activity, is determined the same way as that of the crude.

Dialysis and the repeated peroxidase assay

Dialysis of the enzyme included first the activation of dialysis membrane. The membrane was boiled for 10min in 2% in sodium bicarbonate solution with 2ml EDTA. The partially purified enzyme was loaded in the activated dialysis membrane tube and was let to undergo dialysis for 18 hours at 4 ^{0}C with citrate buffer. The dialysed sample was collected, and the peroxidase assay repeated to calculate the activity.

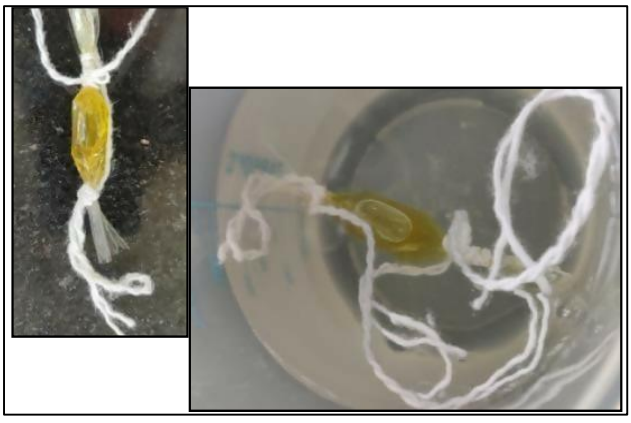


Fig 2: The dialysis. The figure shows the dialysis tubing to carry out the dialysis of the supernatant from Ammonium sulphate precipitation.

Lowry's method for specific activity, fold purification, and % yield are calculated

Total protein and specific activity determination of the amount of total protein was calculated by Lowry's method. This protein assay is a biochemical assay for determining the total level of enzyme in a solution. The total enzyme concentration is exhibited by a colour change of the sample solution in proportion to enzyme concentration, which can then be measured using colorimetric techniques. The method combines the reactions of copper ions with the peptide bonds under alkaline conditions (the Biuret test) with the oxidation of peptide bonds.

0.2ml, 0.4ml, 0.8ml and 1.0ml of 0.2mg/ml of BSA was added to the test tubes. Another test tube was included and to this was added 1 ml of the enzyme extract. The volumes in these tubes were made up to 1 ml using distilled water followed by the addition of 5ml of alkaline copper sulphate to every test tube including the test tube that contained 1 ml of the enzyme extract. After incubation of 10 minutes at RT, 0.5 ml of FC reagent was added to each test tube followed by a 30-minute incubation at RT. The OD was measured at 720nm. A graph was plotted with amount of protein in mg on the x-axis and OD at 720nm on the y-axis and hence the total amount of the enzyme extract, specific activity, fold purification (Fold purification refers to the number of times a protein preparation is enriched for the protein being purified**)**, and % yield are calculated. Absorbance reading for the 1ml crude sample was 0.67.

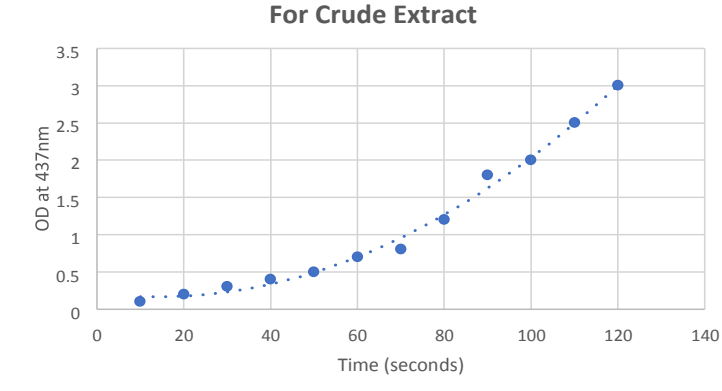
Calculations and Results

The crude extract obtained after centrifugation by collecting the supernatant only was taken to find its enzyme activity. Here the method used was to check for the amount of chromogenic diaminophenazine to be formed at 437nm. The molar extinction coefficient, **ε** of 2,3 DAP at 437nm is $4.14x10^4$ M^{-1} cm^{-1}.

The OD after every 10 seconds was noted down for a period of 5 minutes. In the cuvette, 0.01 ml 3% H_2O_2, 0.4mg/ml OPD, 0.09 ml distilled water, 0.1ml enzyme extract along with 4.8ml phosphate buffer was taken. The results of this experiment were shown in Table 1 below.

Table 1: OD at time intervals for crude. The table below the OD obtained at time internals for crude extract

Time (Seconds)	OD (absorbance at 437nm)
10	0.1
20	0.2
30	0.3
40	0.4
50	0.5
60	0.7
70	0.8
80	1.2
90	1.8
100	2
110	2.5
120	3

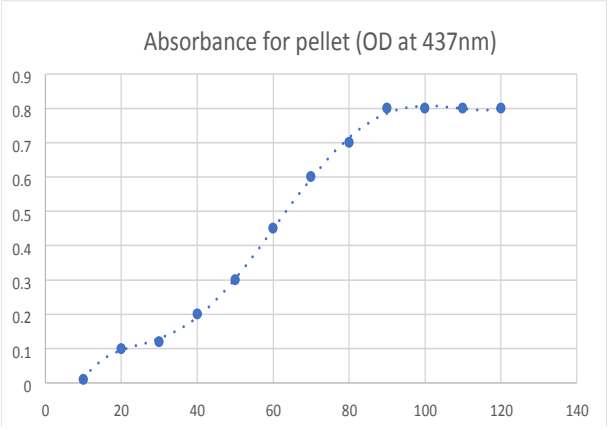


Graph 1: Graph for OD versus time for the crude.

The slope of the tangent to curve was calculated as Δ A/min and the enzyme activity calculated with the Beer-Lambert Law A= Ɛcl.

Table 2: Table 1. OD at time intervals. The table shows results of OD and time for the pellet after ammonium sulphate precipitation followed by centrifugation.

Time (s)	Absorbance (OD at 437nm)
10	0.01
20	0.1
30	0.12
40	0.2
50	0.3
60	0.45
70	0.6
80	0.7
90	0.8
100	0.8
110	0.8
120	0.8



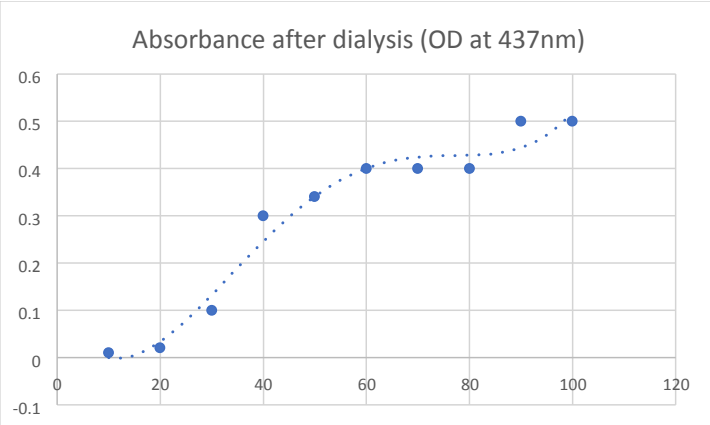
Graph 2: Graph for absorbance against time for the pellet.

Table 3: The OD at time intervals. The supernatant after centrifugation showed the following OD values at their corresponding times.

Time (s)	Absorbance (OD at 437nm)
10	0
20	0
30	0.01
40	0.01
50	0.012
60	0.014
70	0.02
80	0.022
90	0.023
100	0.024
110	0.024
120	0.024

Table 4: Table of results of dialysis. The table shows the results of OD at time intervals for the dialysed sample collected.

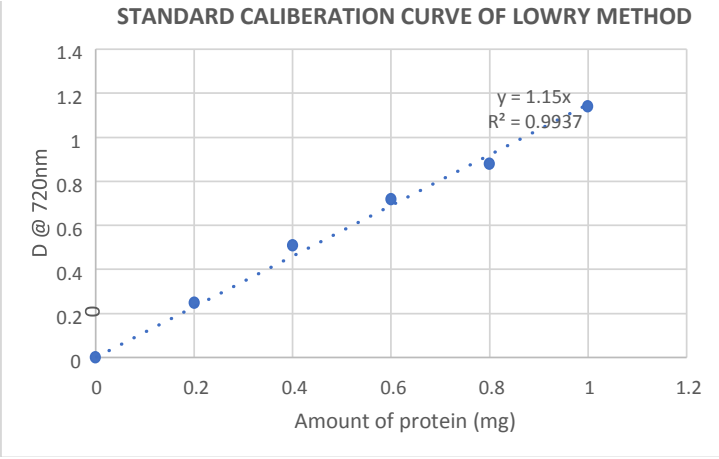
Time (s)	Absorbance (OD at 437nm)
10	0.01
20	0.02
30	0.1
40	0.3
50	0.34
60	0.4
70	0.4
80	0.4
90	0.5
100	0.5
110	0.5
120	0.5



Graph 3: Graph for the dialysis. Graph of absorbance against time for the dialysis.

Table 5: Specific Activity Determination. The table below shows the results for the BSA standard determined to calculate the specific activity calculation.

Vol. of Std BSA (ml)	Conc. of Protein (µg/ml)	Volume of DW (ml)	Volume of alkaline Cu reagent	Incubate	Vol. of FC reagent (ml)	Incubate	OD at 720nm
Blank	-	1	5	at	0.5	at	0
0.2	40	0.8	5	room	0.5	room	0.25
0.4	80	0.6	5	temperature	0.5	temperature	0.51
0.6	120	0.4	5	for	0.5	for	0.72
0.8	160	0.2	5	10	0.5	30	0.88
1	200	0	5	minutes	0.5	minutes	1.14
Crude Pellet Dialysis			5		0.5		0.67 0.3 0.41



Graph 4: The graph shows the standard calibration curve of Lowry Method for calculation of specific enzyme activity.

The fold purification and percentage yield of each purification step is also calculated.

Fraction	Activity (u/ml)	Specific Activity (u/mg)	Fold Purification	% Yield
Crude	0.432	0.074	1	100
Pellet after ammonium sulfate precipitation	0.181	0.078	1.05	41.8
Dialysis	0.0096	0.096	1.23	2.22

Purification is final specific activity / initial specific activity.
Percentage yield is final total units / initial total units x 100.

Conclusion

Peroxide formed as a toxic metabolite is reduced to non-toxic products by the action of the enzyme peroxidase. This study was carried out to find out the enzyme activity, the specific activity, the fold purification and the percentage yield of the enzyme peroxidase extracted sweet potato the from *Ipomoea batatas*. Plant peroxidase was extracted from peels from sweet potato and its activity was found. The crude extract activity obtained was 0.432U/ml. The fold increase after ammonium sulphate precipitation and dialysis increased only very marginally to 1.5 and 1.23 for the pellet after ammonium sulphate precipitation and the dialysis respectively. This shows that most of the protein was lost. Hence a better purification strategy has to be planned. It was also shown that after precipitation and centrifugation the enzyme was contained more in the pellet than in the supernatant. The activity of the supernatant was 0.00027 U/ml while the activity of the 'pellet' suspended in the 4ml solution was 1.81 U/ml almost 1000 times the increase.

An improvement to this study could have been the addition of enzyme standardisation for pH and temperature. The optimum and the working pH and temperatures could have been found, determining the specific activity of the enzyme at different pHs and temperatures.

The extraction and purification of plant peroxidases will help in the detection technique of ELISA or as biochemical catalysts where the horse radish peroxides is used majorly. Hence sweet potato peroxidase is a good alternative. Further having all these different isoenzymes with differing properties can also allow for recombinant technology that may enable us to perhaps exploit and combine such features for novel and improved biocatalysts when necessary.

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