# Catalytic and Thermodynamic Properties of *Cucumis melo* rind Peroxidase

**A presentation at the KU8 Consortium International Conference 2024 held at the University of Ilorin, Ilorin, Nigeria.**

**by**

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**August 2024**

# Introduction

- Peroxidases are oxidoreductases produced by plants, microorganisms and animals (Akbar, 2018). They catalyze the oxidation of several important substrates and play important roles in various physiological processes (Khalil-ur-Rehman, 2009). Hence they have potential applications in diagnosis, pharmaceutical and food industry (El-Khonezy, 2020).

- Peroxidases (EC 1.11.1.7) are heme-containing proteins and are involved in the oxidation of broad range of substrates using hydrogen peroxide as the oxidant (Adam *et al.*, 1999). The molecular weight of peroxidases ranges from 30 to 150 kDa (Bansan, 2013).

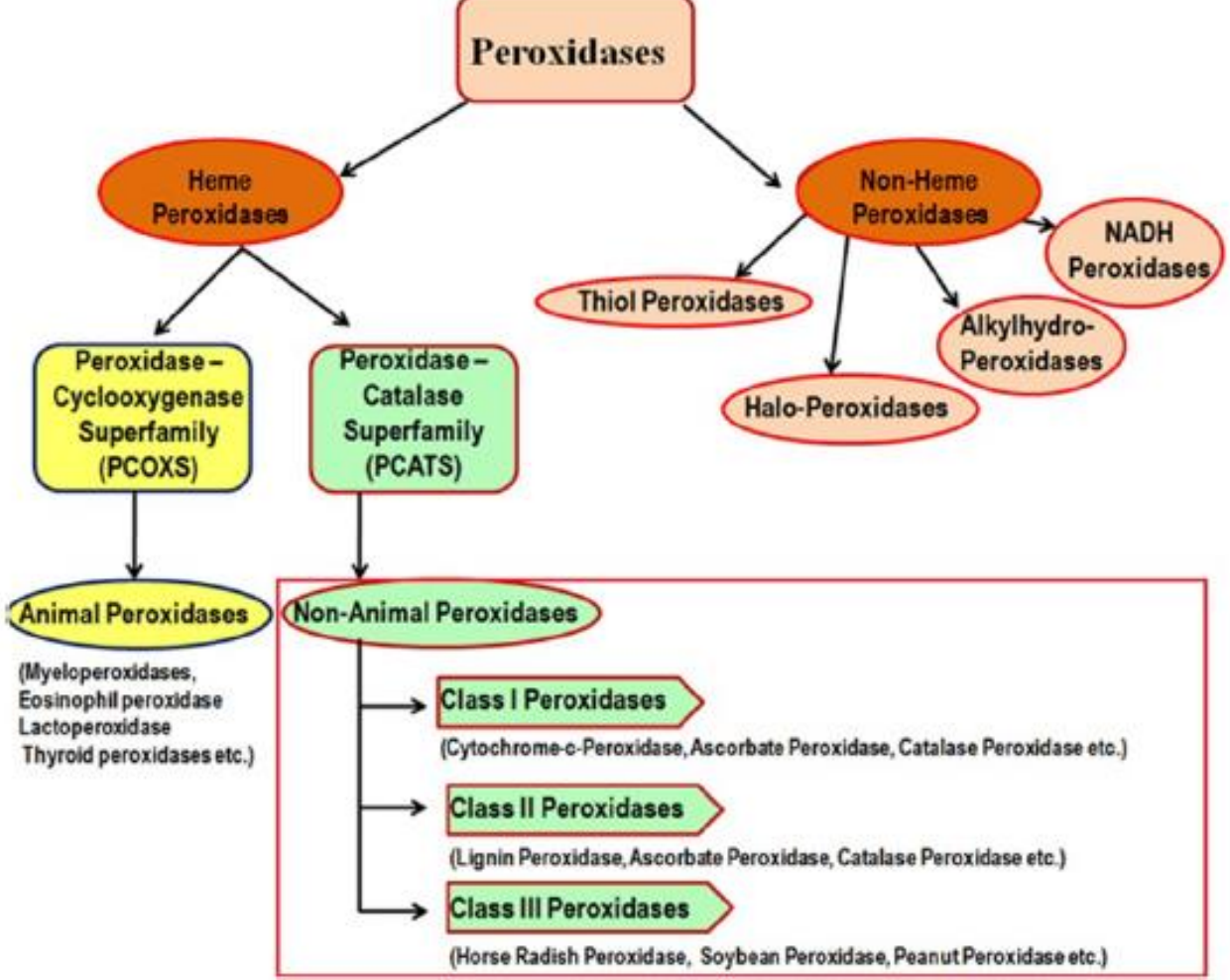


**Figure 1: Schematic representation of the classification of peroxidases**
Source: Pandey (2007)

## Introduction (Cont'd)

- Horseradish peroxidase (HRP) from horseradish roots is the most common, commercially available source used in large scale production of peroxidases owing to its high thermal stability and easy accessibility, however search for peroxidases with improved properties have led to the discovery of soybean peroxidase (Henriksen, 2001,), peroxidase from royal palm tree leaves, *Eichhornia crassipes, Pistia stratioses,* spring cabbage peroxidase among others with higher thermostable properties (Anna *et al.*, 2007; Arise *et al.*,2016, 2018).
- Plant peroxidases (belonging to Class III peroxidase)are involved in various vital processes of plant growth and development throughout the plant life cycle including cell wall metabolism, lignification, suberization, reactive oxygen species (ROS) metabolism, auxin metabolism, fruit growth and ripening, defense against pathogens, etc (Pandey, 2017).

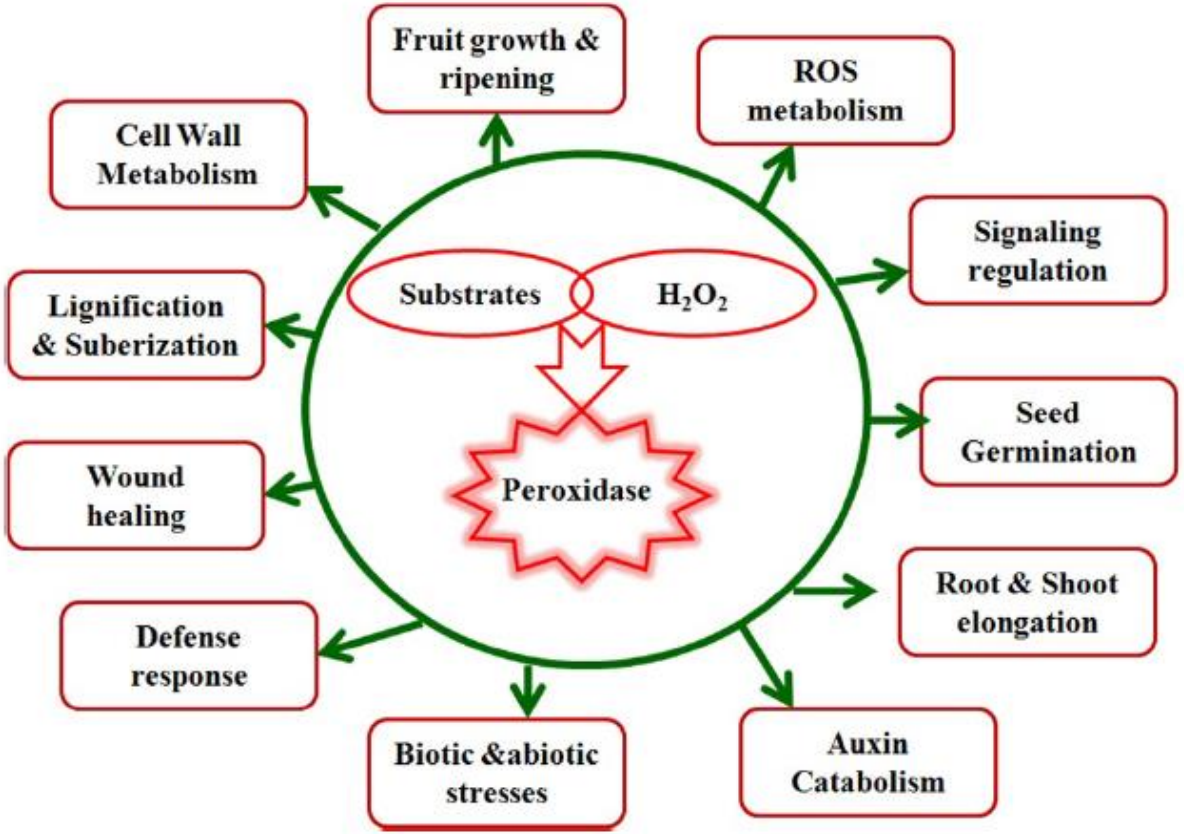


**Figure 2: Reaction catalyzed by peroxidase in plants**
Source: Pandey (2007)

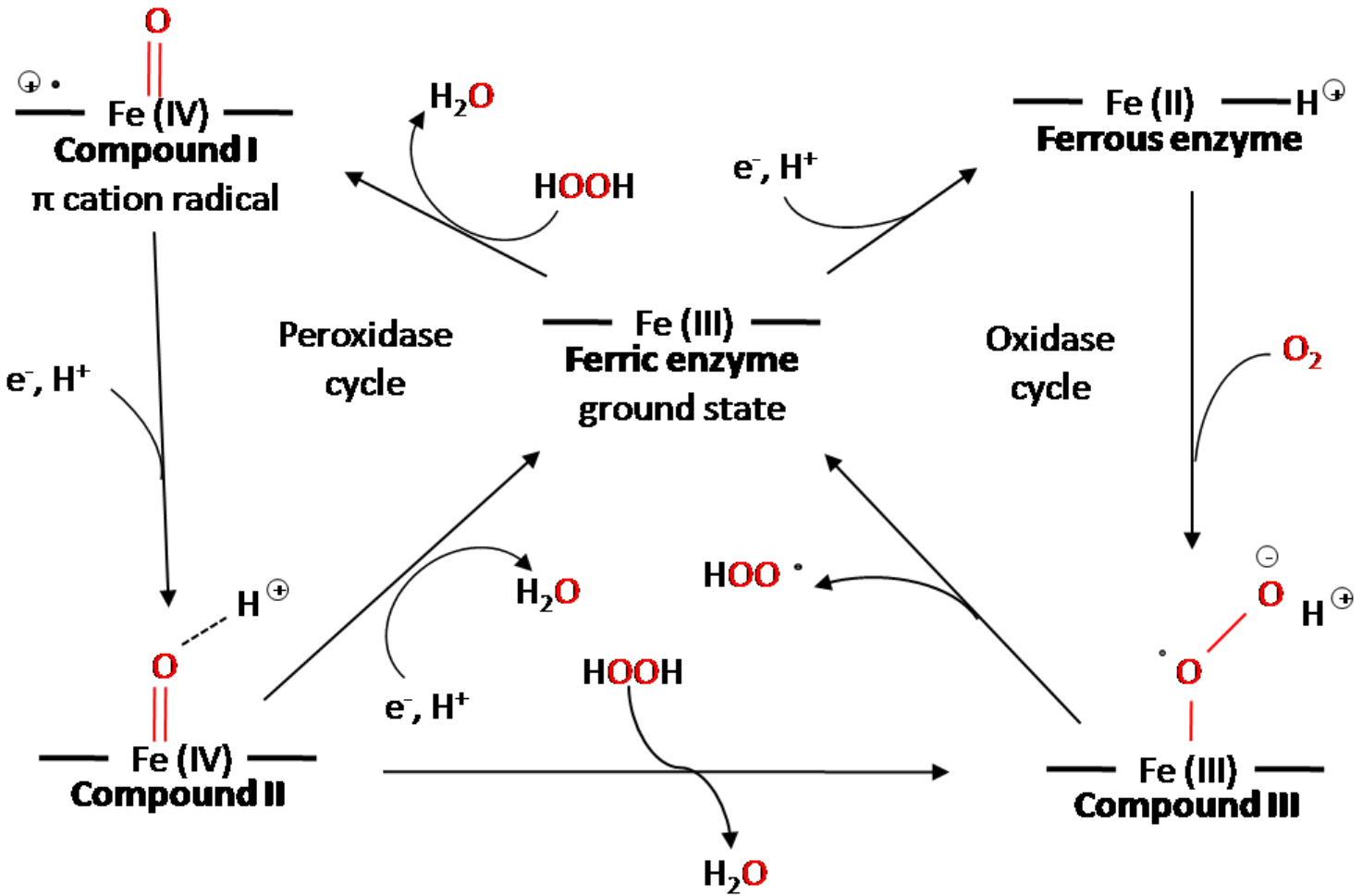


**Figure 3: Redox reaction catalyzed by peroxidase**
Source: Veitch and Nigel (2004)

## Industrial Applications of Peroxidases

- Peroxidases have been employed in biotechnology, biomedicine, pharmacy and agriculture because they possess thermal stability, pH stability and resistance to chemical denaturation (Veitch, 2001).

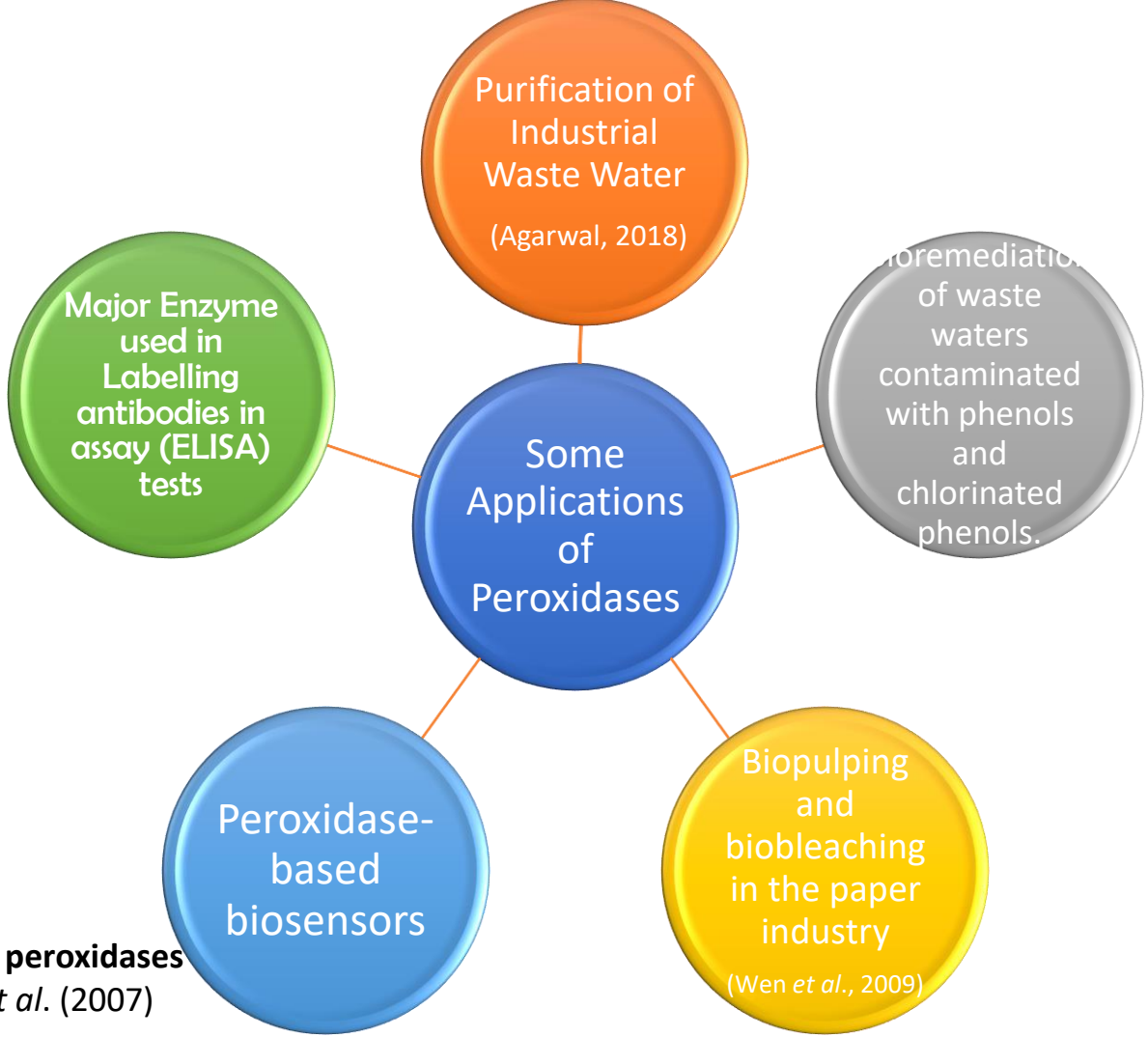


**Figure 4: Application of peroxidases**
Adapted from Pandey *et al*. (2007)

## Table 1: Some Peroxidases isolated and purified from plant sources

| S/N | Source | Mol.Weight | Optimum pH | Optimum Temp | Km/affinity for substrates | Thermostability | Effect of metal/organic solvent | References |
|---|---|---|---|---|---|---|---|---|
| 1 | ***Eichhornia crassipes*** | - | **4-9.0** | 40°C | **ABTS:31.1mM; Guaiacol:21.91mM; Pyrogallol:6.45nM** | **Stable up to 60°C activation energy - 122.29 kj $mol^{-1}$** | **Inhibited by $Pb^{2+}$, Hg2+ and EDTA** | Arise *et al.* (2016) |
| 2 | ***Pistia Stratiotes*** | - | **6-6.5** | 40°C | - | **Stable up to 60°C** | **Inhibited by $Pb^{2+}$, $Hg^{2+}$ and Ni,$^{2+}$, $Co^{2+}$, $Mn^{2+}$ and EDTA** | Arise *et al.* (2018) |
| 3 | ***Carica papaya*** | 240 kDa | **7.0** | 40°C | **O-diansidine: 0.125mM; Guaiacol: 0.8mM; ascorbic acid: 5.2mM $H_2O_2$: 0.25mM** | **Retained 80% activity at 60°C up to 1hr** | **$Mn^{2+}$ as a potent activator and $Ca^{2+}$ as a weak activator** | Pandey *et al.* (2012) |
| 4 | ***Ficus sycomorus*** | 43 kDa | **5.5-7.0** | 5°C-40°C | **O-diansidine: 16.6mM; Guaiacol: 9.5mM; Pyrogallol:6.45mM $H_2O_2$: 1.2mM** | - | **Activated by $Ca^{2+}$, $Na^{+}$ and $Mg^{2+}$, Inhibited by $Mn^{2+}$, $Fe^{3+}$, $Zn^{2+}$ and $Hg^{2+}$** | Mohamed *et al.* (2011) |
| 5 | ***Leucaena leucocephala*** | 200 kDa | **5.0** | 55°C | **Guaiacol:2.9mM; $H_2O_2$: 5.6mM** | **Fully active at 65°C for 20 min** | **Activated by $Ca^{2+}$, $Mn^{2+}$ $Na^{+}$ azide insensitive** | Pandey and Dwivedi (2011) |
| 6 | ***Eruca vesicariasb sp. Sativa*** | 34 kDa | **6.0** | 50°C | **Guaiacol:375.74mM; Pyrogallol:510.14mM** | **Retained 50% activity at 80°C after 30min incubation** | **Activated by $Ca^{2+}$, $Na^{+}$ and $Mg^{2+}$, Inhibited by $Mn^{2+}$, $Fe^{3+}$, $Zn^{2+}$ and $Hg^{2+}$** | Nadaroglu *et al.* (2013) |
| 7 | ***Citrus medica*** | 32 kDa | **6.0** | 50°C | **Guaiacol:8mM; O-diansidine: 1.8mM $H_2O_2$: 0.66mM** | **Stable at 60°C - 65°C** | **Activated by $Cu^{2+}$,$Co^{2+}$ and $Mg^{2+}$, Inhibited by $Mn^{2+}$, $Fe^{3+}$, $K^{+}$ $Zn^{2+}$ and $Hg^{2+}$** | Mall *et al.* (2013) |

# *Cucumis melo*

- *Cucumis melo* is an apple shaped melon which belongs to the Cucurbitaceae family. It is often called 'Sweet melon' and similar to others like Musk melon, squash, cucumber, water melon, gourds, and pumpkin Cucurbitaceae family (MFO, 2013)
- They are juicy, delicious fruit famous for their nutritional and medicinal uses. Cucumis in Latin means "cucumber", which is the English common name of another species of the genus. (Mahoney, 2013).
- The cantaloupes, or Cantalupensis Group, are similar but have a smooth or warted rind and are popular in parts of Europe (Paris *et al.,* 2012).
- *Cucumis* species and *C. melo* have been reportedly used to manage ulcer, inflammation, cardiovascular disorders, and various disease conditions (Uphadhay *et al., 2018).*

**Plate 1:** ***Cucumis melo*** **fruit**

**Statement of the problem**

- The multifaceted applications of peroxidase has made it a highly sought after enzyme, hence researchers have tried to isolate and purify peroxidase from cheaper sources other than horseradish (the major source for the commercially available source (Arise *et al.*, 2016, 2018; Akbar, 2018 and Askari, 2022).

- Efforts have also been made to explore recombinant technology in production of horseradish peroxidase (HRP) but recombinant production causes hyperglycosylation of HRP that complicates the conjugation of HRP to antibodies and lectins (Capone *et al.*, 2015). The major focus of previous research efforts have been to purify a thermostable and cheaply available, alternative sources of peroxidases however most reported purifications have been from plants parts (Belcarz *et al.*, 2008; Gill *et al.*, 2011; Zia *et al.*, 2011; Sonkar *et al.*, 2023) that are not readily available, and the stages of purification end up to be costly.

**Statement of the problem**

- Despite the medicinal benefits of *Cucumis melo* fruits and seeds, there is dearth of literature reports on the exploration of its rind (an agro waste) as a potential cheap source of peroxidase thus a study to isolate, purify and characterize peroxidase from *Cucumis melo* rind is imperative.

## Justification of the study

- Many enzymes have been used as a great tool in several industrial applications (Khalil-ur-Rehman, 2009). Peroxidase is one of such enzyme of great economic importance.
- Agro wastes such as fruit rind and husks have been explored as sources of bioactive peptides and enzymes (Arise *et al.*, 2020, 2021) and nanoparticles (Bala *et al.,2023).*
- *C. melo*. seed and extracts have been explored for its antiulcer (*Vishwakarma et al.,* 2017), anticancer (Debnatch *et al., 2015),* anthelmintic and antimicrobial, anti-diabetic (Decorde *et al.,* 2010) activities. These activities have drawn attention of researchers to further investigation of its ethno-botanical advantages.

## Justification of the study

- The choice of cheaply available *Cucumis melo* is strategic as its abundant availability and adaptability to the high temperature of Northern Nigeria suggest it may possess peroxidase with high thermal stability.
- There is therefore a need to explore the catalytic potentials of peroxidase from *Cucumis melo* rind for its industrial application.

# Objective of the study

The overall objective of this study was to evaluate the catalytic and thermodynamic properties of *Cucumis melo* rind for industrial applications.

# MATERIALS AND METHODS

## Materials

Fresh fruits of *Cucumis melo* was obtained from Sweet melon Plantation at the College of Forestry, Jos, Plateau State. Plant authentication was carried out at the Herbarium of Plant Biology Department, University of Ilorin, Ilorin, Nigeria where a voucher number U.I.H.001/786 was assigned. Ammonium sulphate and Sephadex G-75 were obtained from BDH Chemicals, England, Guaiacol from Santa Cruz Biotech, USA. 2,2'-azino-bis-(3-ethylbenzthiazolin)-6-sulfonate (ABTS) and Pyrogallol were obtained from Sigma-Aldrich, USA. All chemicals used were of analytical grade were used without further purification.

# Methods

***Proximate composition of Cucumis melo rind***

- The fresh samples of *Cucumis melo rind were* analyzed for moisture, ash, fibre, protein, fat and carbohydrate by the methods of Association of Official Analytical Chemist (AOAC, 2005).

# Methods

***Preparation of Crude Extract of Cucumis melo* rind**

•Crude enzyme extraction was carried out according to the methods of Kornberg (1990) described by Arise *et al.* (2018). The extracts were obtained by homogenizing 100 g of *Cucumis melo* rind with cold 100 mM Tris HCl buffer, pH 7.5 in a blender for 5 min. The homogenate was filtered using cheese cloth arranged into 4 folds. The clear filtrate was centrifuged at speed of 10, 000 g for 10 min at 4°C. The supernatant obtained was used as the enzyme source.

# Methods

***Preparation of Crude Extract (cont'd)***

- To selectively inactivate the contaminating traces of catalase moieties, crude enzyme extract was heated at 65°C for 3 min (as described by Wang *et al.*(1999) in a water bath and cooled promptly by placing it in ice bucket for 30 min. After thermal inactivation of catalase, peroxidase activity, protein content and specific enzyme activity of final extract were determined before preservation at -20°C for further purification.

# Stepwise purification of the isolated enzyme

***1. Ammonium sulphate precipitation***

- Ammonium sulphate precipitation is a common method for salting out proteins because the solubility of proteins reduces at high salt concentration which leads to precipitation of proteins. Ammonium sulphate precipitation was carried out as described by Zia *et al.* (2011).
- Ammonium sulphate crystals (37.32g, 20.71g and 62.14g for 0-60%, 60-90% and 0-90% saturations, respectively) were weighed and gently added to the crude extract by constant stirring to ensure uniform and complete solubilisation of the salt. The solution was then allowed to stand overnight at 4°C for complete precipitation. The process was carried out step wisely to achieve between 0-60%, 60-90% and 0-90% ammonium sulphate saturations of the crude enzyme extract.

***Ammonium sulphate precipitation (cont'd)***

- Precipitate was collected by centrifuging the solution at 10 000g for 15 min at 4 ˚C in a cold centrifuge.

- The fraction obtained by 0-90% saturation with highest peroxidase activity was selected for further purification.

## *Dialysis*

• Samples obtained after precipitation was subjected to dialysis as described by El-khonezy (2020). Dialysis is a procedure that separates proteins from solvents by taking advantage of the proteins' larger sizes. Dialysis was carried out for 24 hours with 100 mM Tris HCl buffer, pH 7.5 using dialysis tube. The dialysis tube was tied with thread at one open end. The buffer was added and stirring was carried out intermittently using magnetic stirrer.

## *2. Ion exchange chromatography*

- Further purification of partially purified peroxidase was carried out by ion exchange chromatography using diethyl amino ethyl (DEAE) cellulose column (Cooper, 1977).
- Ion exchange chromatography separates proteins by taking advantage of migration of proteins (mobile phase) in a charged mobile phase. Proteins with a net positive charge migrates through the matrix more slowly than those with negative charge because the migration of positively charged proteins will be retarded by more interaction with the stationary phase.
- The column was packed to the height of 25 cm in a glass column with an internal diameter of 2.0 cm and equilibrated with phosphate buffer (pH 6.5) for 24 h. A total of 40 fractions of 2 ml each was collected at constant drop rate (15.2ml/sec) while the enzyme activity as well as the protein content was determined for each separate fraction.

### *3. Sephadex G-75 gel filtration chromatography*

- Gel filtration chromatography was carried out as described by Dunn and Corbett *et al.*(1996).
- Gel filtration chromatography separates proteins based on their size and shape. It uses small polymeric beads (e.g sepharose)with fixed pore sizes. The pore size is such that the molecules which are smaller or nearly equal can enter. Larger proteins cannot enter the beads. Thus, the larger proteins are eluted out first.
- Purified peroxidase enzyme (0.5 ml) obtained from ion exchange chromatography was subjected to gel filtration chromatography using sephadex-G-75 column by loading onto the column (1cm × 40 cm) previously equilibrated with 100 mM Tris HCl buffer, pH 7.5 and then washed with 50 ml of the eluting buffer (same as the equilibrating buffer). The eluates were collected as fractions of 5 ml each (at a flow rate of 22 ml/sec) and then assayed for the enzyme activity.

# Total protein and enzyme activity assay

- Protein concentration was estimated using bovine serum albumin (standard protein) as described by Lowry *et al*. (1951).

This method of protein concentration determination is based on the reactivity of the peptide nitrogen(s) with the copper [II] ions under alkaline conditions and the subsequent conversion of a mixture of phosphomolybdic acid and phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids such as tyrosine and tryptophan. The colour intensity is proportional to the protein content of the sample at wavelength of 650 nm.

- The specific enzyme activity was calculated and the purification yield was determined (Rudrappa *et al*. 2007).

Specific enzyme activity is calculated as

$$\frac{\text{Total enzyme activity (µ mole/min)}}{\text{Total amount of protein}}$$

# CHARACTERISATION OF PURIFIED PEROXIDASE FROM *Cucumis melo* rind

**Determination of purified *Cucumis melo rind* peroxidase activity**

- *Cucumis melo peroxidase* activity in enzyme extracts was assayed by monitoring changes in absorbance at 470 nm as described by Civello *et al.*(1995)
- Enzyme activity is a measure of the amount of product formed from substrate per unit time by an enzyme molecule. It can be expressed as

$$\frac{\text{Change in substrate concentration (µ mole)}}{\text{Time taken (min)}}$$

- The reacting mixtures consisted of 0.02 M $Na_2HPO_4$ and 0.08 M $NaH_2PO_4$, 20 mM guaiacol, 4mM $H_20_2$, enzyme extract (10 mL), pH 6, in a total volume of 3 mL (Civello *et al.*,1995). Enzyme activity unit represents the amount of enzyme that caused an increase in absorbance of 0.001 unit/min at 50 ˚ C.

## Determination of substrate specificity of *Cucumis melo* rind *peroxidase*

- The activity of the purified peroxidase using ABTS, guaiacol and pyrogallol as a reducing substrates were determined as described by Gray and Montgomery (2003).
- Briefly, 2.5 ml of 0.1 M phosphate buffer, pH 6.0 was added to a test tube followed by the addition of 0.1 ml of enzyme source after which 0.2 ml of 100 mM guaiacol was added to the reaction mixture. The reaction was initiated with 0.2 ml of freshly prepared 150 mM $H_2O_2$ solution and the change in absorbance of the mixture was read at 470 nm at 25°C over a period of 3 minutes at 1 minute interval. One unit of activity (U) is defined as the amount of enzyme that caused the oxidation of 1 µmole of substrate per min under standard conditions.
- Kinetics of inhibition of purified *C.melo* rind peroxidase by EDTA, salicyclic acid, Mercury ($Hg^{2+}$) and Lead ($Pb^{2+}$) were determined as described by Thomasz (2007). Double reciprocal plots of both inhibited and non-inhibited catalysis of purified *C.melo* rind peroxidase were obtained.

## DETERMINATION OF KINETIC AND CATALYTIC PARAMETERS OF *Cucumis melo* RIND PEROXIDASE

### Optimum temperature and thermal stability

- The optimum temperature of *Cucumis melo* peroxidase was determined by assaying peroxidase activity at temperatures between 20 and 80 ˚C at 10 ˚C intervals as described by Arise *et al.* (2018).
- Proteins (enzymes) can be denatured by high temperature. The activity of peroxidases increases with increasing temperature until the optimum temperature is attained.

- The optimum temperature was determined by incubating the enzyme sample at a particular temperature for 10 minutes and the enzyme activity immediately assayed at that temperature. The temperature ranges studied were between 20 and 80˚C.
- The thermal stability of the enzyme was determined at the range of 40-80˚C. Briefly, the enzyme sample was heated to a specific temperature over a period of 60 minutes using water-bath. Aliquots of the enzyme were withdrawn at 10 minutes intervals. The withdrawn samples were then cooled in ice-bath for 30 minutes and incubated for 10 minutes at 25 ˚C. Percentage residual enzyme activity was determined as previously described.

# Optimum pH

- Optimum pH of the purified *Cucumis melo* peroxidase *was* determined for three reducing substrates, ABTS, guaiacol and pyrogallol. Optimum pH for ABTS was determined as described by Dawson *et al*. (1986).
- pH stability of the enzyme was determined using guaiacol as a substrate and the same buffer range as previously described. The enzyme was incubated with specific buffers of known pH range for a period of 24 h at 4 ˚C, and the peroxidase activity was thereafter determined.

## DETERMINATION OF THERMODYNAMIC/BIOENERGETICS PARAMETERS OF *Cucumis melo* PEROXIDASE

- The thermodynamic and bioenergetics parameters of *Cucumis melo* peroxidase was determined as described by Maragoni (2003).
- The change in enthalpy, entropy, free energy and D-value were calculated from the following equations;

$$\Delta H = Ea - RT \quad (1)$$

$$\Delta G = - RT \ln K_h/K_b \quad (2)$$

$$\Delta S = \Delta H - \Delta G/T \quad (3)$$

$$D\text{-value} = 2.303/Ka \quad (4)$$

$$t_{1/2} = \ln 2/kd \quad (5)$$

**Kd -** first order rate constant**, D-value-** Time required for 90% reduction in activity**,** $t_{1/2}$ -half life **Ea-activation** energy of the reaction**,** ΔG – Gibb's Free Energy, ΔH- Enthalpy of reaction, **T-** temperature (K)**; R -** gas constant ($8.314 Jmol^{-1}K^{-1}$)**, Kh-** Planck's constant ($11.04x10^{-36} Jmin^{-1}$)**, Kb =** Boltzmann constant ($1.38x10^{-23} JK^{-1}$)

# Statistical analysis

• Data are presented as means of three separate determinations and were subjected to one-way analysis of variance using Statistical Analysis System Software (SPSS, version 20). Data obtained for enzyme studies were analyzed using Graph pad Prism version 7.0.

# Results

Table 2: Proximate composition of *Cucumis melo* rind

| Parameter | **Moisture Content (%)** | **Ash Content (%)** | **Carbohydrate (%)** | **Protein (%)** | **Fat and Oil (%)** | **Crude Fibre (%)** |
|---|---|---|---|---|---|---|
| Values | 72.58±0.42 | 1.86±0.02 | 12.22 ± 0.35 | 8.30±.02 | 2.50±0.10 | 2.54±0.03 |

values are means of three samples ± standard deviation

**Table 3 :Ammonium sulphate saturations and *Cucumis melo* rind peroxidase activity**

| Ammonium sulphate saturations | Enzyme Activity (µM $min^{-1}$) |
|---|---|
| **Crude Extract** | 413.53 |
| **0-60%** | 412.90 |
| **0-90%** | 1353.38 |
| **60-90%** | 112.78 |

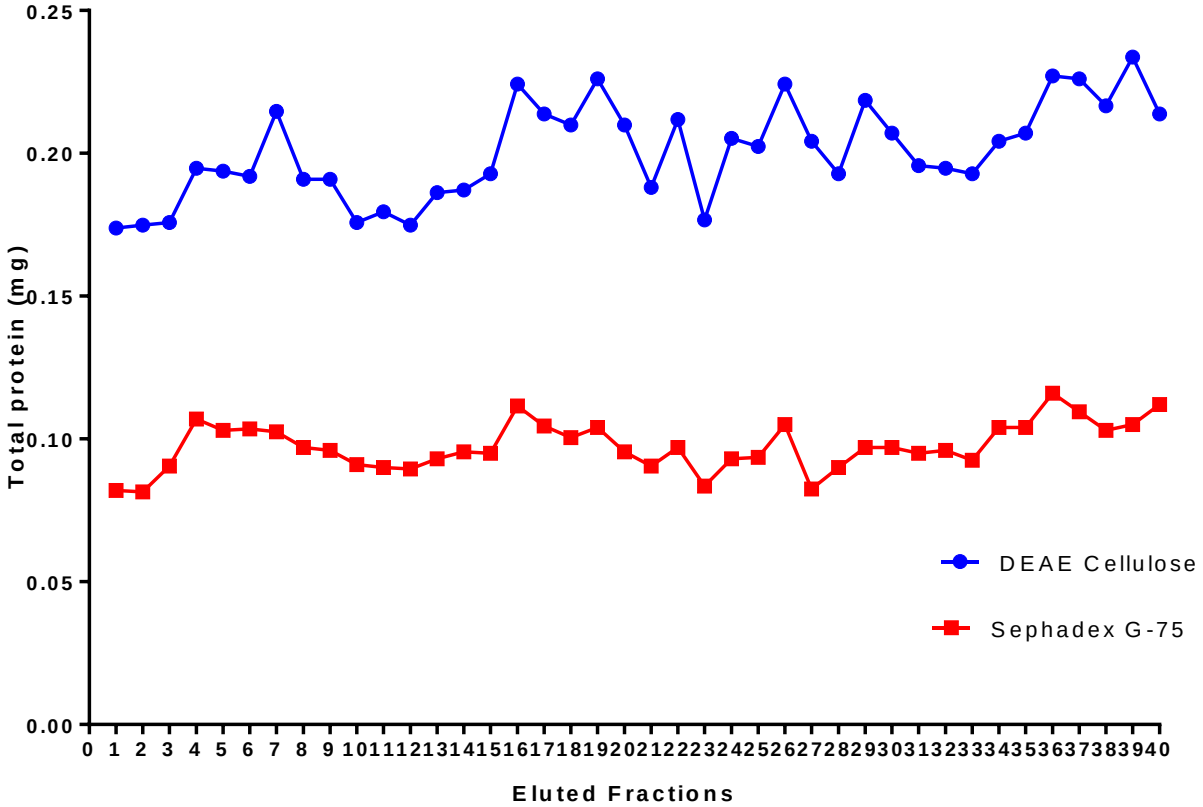


**Figure 7:Protein concentration profile of *Cucumis melo* rind peroxidase purified by DEAE cellulose ion exchange and Sephadex G-75 gel filtration chromatography.**

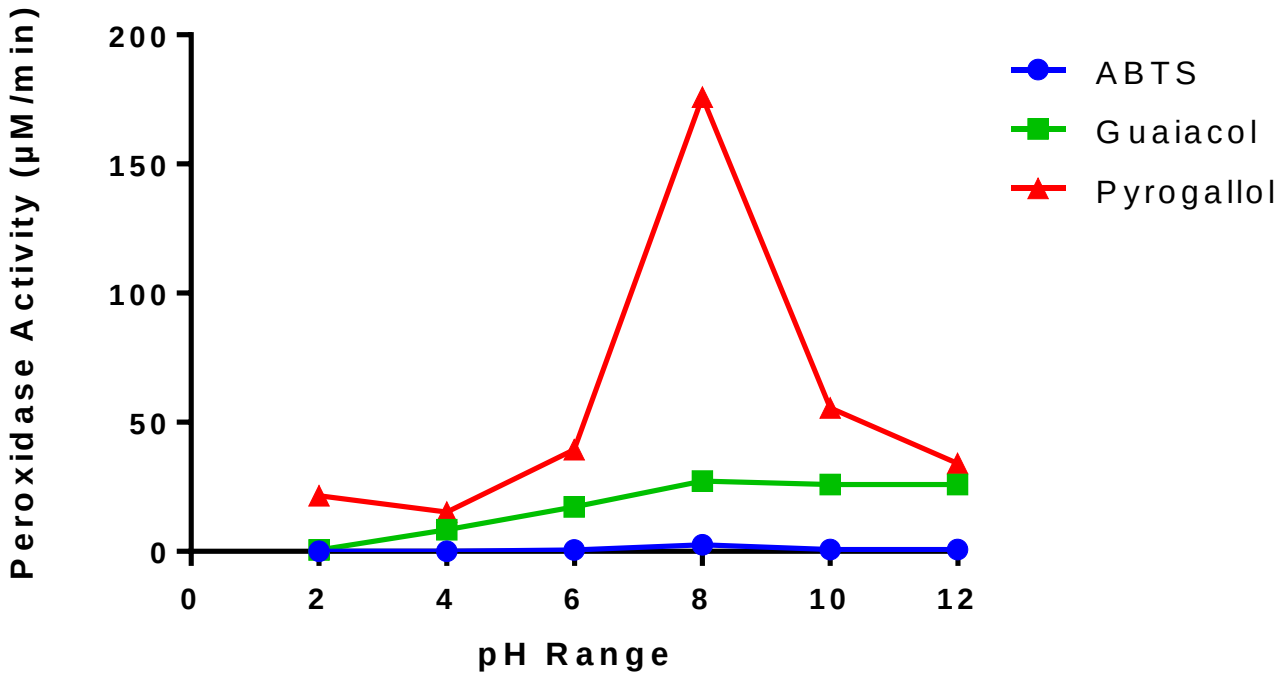


**Figure 13:Time dependent effect of pH on *Cucumis melo* rind peroxidase using ABTS, guaiacol and pyrogallol as substrates**

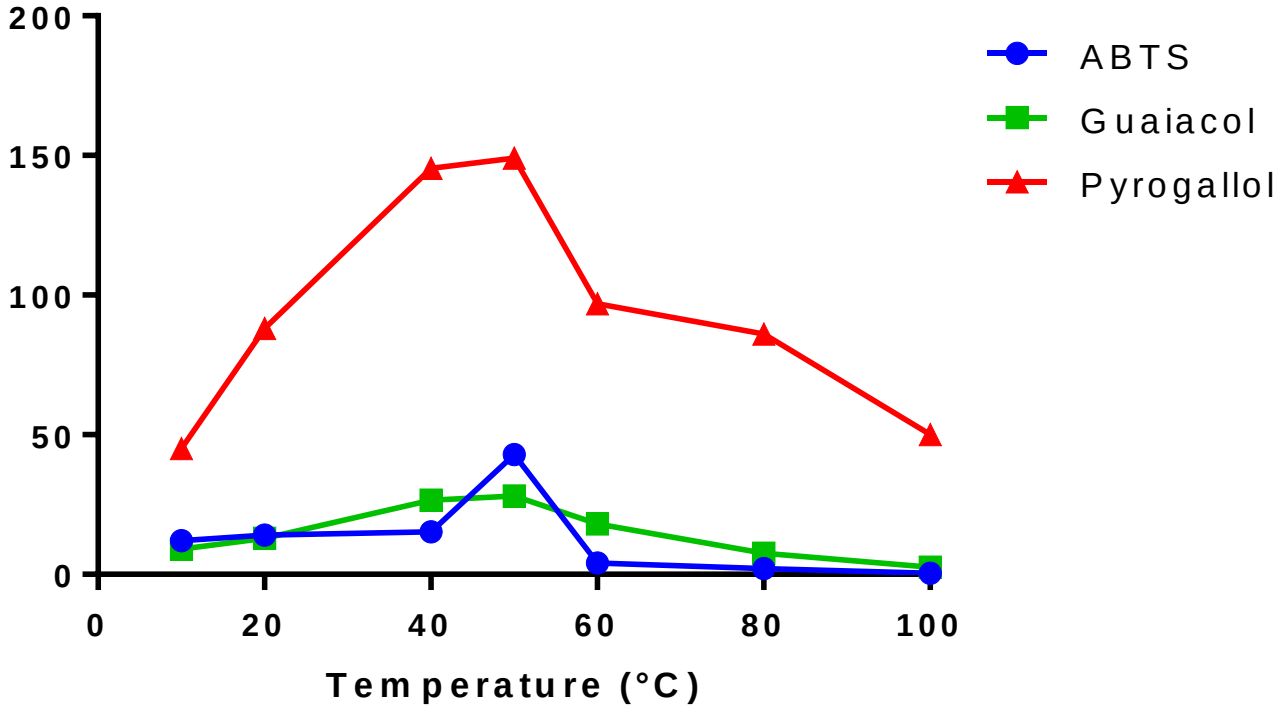


**Figure 17:Effect of temperature on the activity of *Cucumis melo* rind peroxidase using different substrates**

# Conclusion and recommendation (cont'd)

- *Cucumis melo* rind (CMR) has thermodynamics profiles with half-life of 64.18 mins observed at 40°C which decreased as the temperature increased. The free energy (ΔG) values of 72.42, 77.16 and 81.71Kj/mol and entropy (ΔS) values of – 0.23 were obtained for *Cucumis melo* rind peroxidase. It also displayed an activation energy (Ea) values of 33.3Kj/mol, 33.39Kj/mol and 33.29Kj/mol at 40°C, 60°C and 80°C respectively.

- *Cucumis melo* rind peroxidase is an ideal candidate for industrial and biotechnological applications.