

Original Article

Decolorization of synthetic dyes using partially purified peroxidase from green cabbage (*Brassica oleracea*)

Cliff C. Victor¹, Kingsley Ozioma Omeje^{1*}, Arinze Linus Ezugwu¹,
Sabinus Oscar Onyebuchi Eze¹, Ferdinand Chiemeka Chilaka¹,
Ikechukwu Noel Emmanuel Onwurah¹, and Pius Oziri Ukoha²

¹ *Department of Biochemistry, Faculty of Biological Sciences,
University of Nigeria, Nsukka, 410001 Nigeria*

² *Department of Chemistry, Faculty of Physical Sciences,
University of Nigeria, Nsukka, 410001 Nigeria*

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Abstract

Peroxidase was extracted from cabbage and was purified 13.14 fold by a three-step process of ammonium sulphate precipitation, dialysis, and gel filtration with a yield of 9.44. The partially purified peroxidase had maximal activity at pH 5.0 and temperature 45°C. Michaelis constant (K_m) and Maximum velocity (V_{max}) obtained from Lineweaver-Burk plot of initial velocity data at different concentrations of H_2O_2 and O-dianisidine were 3.68mM/ 37.04U and 9.89mM/ 28.57U, respectively. The partially purified peroxidase from cabbage decolorized many synthetic Azo and Vat dyes, with Azo tryptan blue having highest decolorization of 88.62% while vat orange II showed 12% decolorization, after contact time of one hour. This suggests that peroxidase from cabbage has the potential to decolorize synthetic dyes.

Keywords: decolorization, green cabbage, peroxidase, synthetic dyes

1. Introduction

Peroxidases (EC 1.11.1.7) are oxido-reductases that catalyze the reduction of peroxide, such as hydrogen peroxide (H_2O_2), and the oxidation of a variety of organic and inorganic compounds (Shamsher, Neelam, & Lata, 2012). Textile dyes represent a major class of organic pollutants that are found in the waste effluents released by various industries (Kalsoom, Ashraf, Meetani, Rauf, & Bhatti, 2013). These chemicals can be toxic, carcinogenic, mutagenic, or teratogenic to various aquatic species and humans (Celebi, Altikatoglu, Akdeste, & Yildirim, 2012). Among the textile dyes, Azo dyes account for 60-70% of all textile dyestuffs (Bae & Freeman, 2007). The

treatment of wastewater containing dyes involves chemical and physical methods and these methods have different disadvantages in decolorization capability and operating speed, and have proven to be costly and to produce large amounts of sludge (Leelakriangsak & Borisut, 2012). Biological processes have received increasing interest as a viable alternative owing to their cost effectiveness, ability to produce less sludge and environmental friendliness (Banat, Nigam, Singh, & Marchant, 1996). Enzymatic approach has gained considerable interest in the decolorization of textile and other industrially important dyes present in wastewater. This strategy is eco-friendly and effective in comparison to conventional methods. Peroxidases have been reported as excellent oxidant agents to degrade dyes (Kirby, Mullan, & Marchant, 1995). Fungal and bacterial peroxidases have been utilized in dye removal processes (Bholay, Borkhataria, Jadhav, Palekar, Dhalkari, & Nalawade, 2012; Ghasemi, Tabandeh, & Bambai, 2010). Among the plant peroxidases,

*Corresponding author

Email address: kingsley.omeje@unn.edu.ng

the most studied are native or recombinant horseradish peroxidases, (HRP) (Tirola *et al.*, 2006). Plant peroxidases have been extracted from African oil bean seeds (Eze, 2012), sorghum (Eze, Chilaka, & Nwanguma, 2000), *Ipomoea batatas*, *Raphanus sativus*, *Sorghum bicolor*, soybean (Al-Ansari, Modaressi, Taylor, Bewtra, & Biswas, 2010; Diao *et al.*, 2011), and from red cabbage Ghahfarrokhi, Garmakhany, and Mousavi (2013), but not from green cabbages. This research is therefore focused on the isolation, characterization and partial purification of peroxidase from green cabbage and its application on decolorization of industrial synthetic dyes.

2. Materials and Methods

2.1 Materials

Azo Trypan Blue, Azo Blue 5, Azo Yellow 6, Citrus Red 2, Brilliant Black, Azo Pink, Azo Purple, Vat Orange 11 and Vat Green 9 were products of BASF Chemical Company Germany and were gotten from the Clothing and Textile Unit, Fine and Applied Arts Department, Faculty of Arts, University of Nigeria Nsukka, Enugu State Nigeria. Fresh cabbage was bought from the Ogige market, Nsukka, Enugu State, Nigeria.

2.2 Methods

2.2.1 Extraction of peroxidase

Green cabbage was washed and homogenized using an electrical bench-top laboratory high pressure homogenizer. 250ml of the extract was mixed with 500ml of phosphate buffer (0.05M) at pH 6.0. The mixture was left for 24 hr with frequent stirring using a magnetic stirrer, filtered with double-layered cheesecloth and the filtrate was centrifuged using a Cole-Palmer VS-13000 micro-centrifuge at 4000rpm for 30min. The supernatant was collected and stored at a temperature below 5°C as crude enzyme.

1) Protein determination

Protein content of the crude enzyme extract was determined by the method of Lowry, Rosebrough, Farr and Randall (1951), using Bovine serum albumin (BSA) as the standard.

2) Peroxidase assay

Peroxidase activity was assayed as in Eze *et al.* (2000) with the following modifications. The assay mixture contained 2.7ml of sodium phosphate buffer at pH 6.0 (0.05M), 0.1ml of 0.8% H₂O₂ (0.025M), 0.1ml of 1% o-dianisidine and 0.1ml of the enzyme extract. The change in absorbance due to oxidation of o-dianisidine in the presence of H₂O₂ was monitored using JENWAY 6405 UV/VIS spectrophotometer (Beckman/instruments Inc., Houston, Texas) at 460nm. The readings were taken every 30sec. for 5min. The mixture without the enzyme served as the blank. One unit of enzyme activity was defined as the amount of enzyme that gave an absorbance change of 0.1/min at 30°C.

2.2.2 Peroxidase purification

1) (NH₄)₂SO₄ precipitation

The crude enzyme was precipitated using ammonium sulphate. A known quantity (106g) of the salt was added to the crude enzyme solution before stirring for 4hr. The solution was centrifuged at 10,000 ×g for 30 min and the supernatant was brought to 80% (NH₄)₂SO₄ saturation, after which the solution was centrifuged at 10,000 ×g for 30 min. The pellet was dissolved in the sodium phosphate buffer at pH 6.0 (0.05M) and dialyzed against 0.05M sodium phosphate buffer at pH 6.0 for 12 hr with change of buffer every 6hr. The dialysate was also assayed for peroxidase activity and protein concentration as earlier described. 10ml of the dialyzed protein was introduced into a (50 x 2.5cm) gel chromatographic column and subjected to gel filtration. The gel was pre-equilibrated with 0.05M sodium phosphate buffer at pH 6.0. Fractions were collected at a flow rate of 1ml/1.8min. The protein concentration of each fraction was monitored at 280nm using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Houston, Texas). The peroxidase activity of each fraction was assayed as earlier described and the active fractions were pooled and stored at -10°C using Thermo-cool refrigerator.

2) Optimum pH

Peroxidase activity was determined using 50mM sodium acetate buffer at pH 3.5-5.5, 50 mM sodium phosphate buffer at pH 6.0-7.5 and 50mM Tris-HCl buffer at pH 8.0-9.0 using o-dianisidine as substrate in the presence of hydrogen peroxide.

3) Optimum temperature

The optimum temperature was determined by assaying for peroxidase activity at different temperatures in the range 30 - 70°C using o-dianisidine as substrate in the presence of hydrogen peroxide.

4) Effect of substrate concentration on peroxidase activity

Different concentrations of H₂O₂ (1-24mM) and o-dianisidine (1-10mM) were prepared and used to assay for the peroxidase activity using 50mM acetate buffer at pH 5 and 45°C. The Km (H₂O₂), Km (o-dianisidine); Vmax (H₂O₂) and that of o-dianisidine were calculated from Lineweaver-Burk plots.

5) Decolorization of dyes

Seven (7) azo dyes (Trypan Blue, Azo blue 5, Azo Brilliant Black, Azo Yellow 6, Azo Citrus Red 2, Azo Pink and Azo Purple) and two (2) vat dyes (Vat Green 9 and Vat Orange 11) were used. Each of reaction mixture with a dye contained 2.7ml acetate buffer (0.05M) at pH 5.0, 0.1ml of the dye solution, 0.1ml of H₂O₂, and 0.1ml of the peroxidase, and was incubated for 60 min. The absorbance was read at

wavelength 224nm for Azo tryotan blue, and at 614nm for Azo brilliant black. The percentage decolorization of each dye was calculated as

$$\text{Percentage Decolorization} = \frac{A_i - A_f}{A_i} \times 100$$

where A_i is the initial absorbance before incubation and A_f is the final absorbance after incubation.

3. Results and Discussion

Peroxidase was purified 13.14 fold with an activity yield of 9.44%. The protein concentration and specific activity of the crude were found to be 0.94mg/ml and 4.23U/mg. After ammonium sulphate precipitation and dialysis the protein concentration was found to decrease from 0.80mg/ml to 0.289, with the specific activity increasing from 19.91U/mg to 71.66U/mg (Table 1). After gel filtration, the specific activity of the enzyme decreased to 55.58U/mg. Osuji, Eze, Osayi and Chilaka (2014) reported protein concentrations of 3.981 and 5.669 mg for peroxidase partially purified from garlic. Also, Khatun *et al.* (2012) reported protein concentrations of 325 and 50.34 mg for crude and ammonium sulphate precipitated peroxidase from *M. oleifera* leaf. After gel filtration step, 48-fold purification was achieved for Tartary buckwheat shoot peroxidase (Mikami, Kurihara, Takahashi, & Suzuki, 2013).

The chromatogram showing the peroxidase activity after gel filtration is shown in Figure 1. Gel filtration is both a purification step as well as a protein polishing method. The fractions with peroxidase activity were from tubes 10-15. They were pooled for enzyme characterization.

Figure 2 shows that increase in pH from 3.5 was accompanied by an increase in enzyme activity up to pH 5.5, after which the enzyme activity decreased steadily making 5.5 the optimum pH for peroxidase activity. The decrease in enzyme activity at higher pH may be due to the amphoteric nature of protein molecules, which contain a large number of acidic and basic groups, mainly situated on the surfaces (Chaplin, 2014). This result is in accordance with the report of Hu, Wu, Luo and Mo (2012) on lettuce peroxidase. Increase in temperature from 25°C was accompanied by an increase in peroxidase activity. The pH 5.5 of this study is slightly above pH 5.00 that was reported by Osuji *et al.* (2014).

The optimum temperature observed in this study was 45°C. Further incubation above 45°C yielded a sharp decline of peroxidase activity as shown in Figure 3. The decrease in enzyme activity may be as a result of enzyme inactivation. Hu *et al.* (2012) reported the optimum temperature as 45°C for lettuce peroxidase. Bania and Mahanta (2012) reported the optimum temperature range 40-50°C for

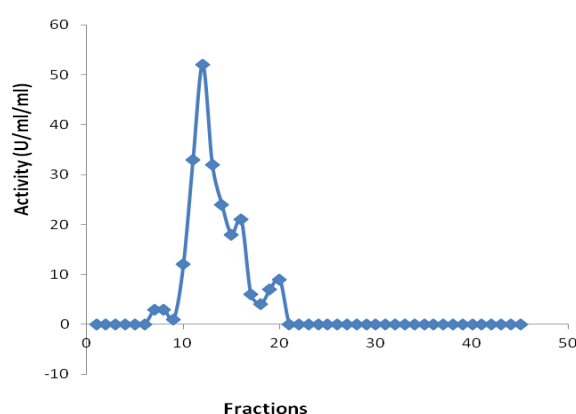


Figure 1. Gel filtration chromatography profile for peroxidase activity.

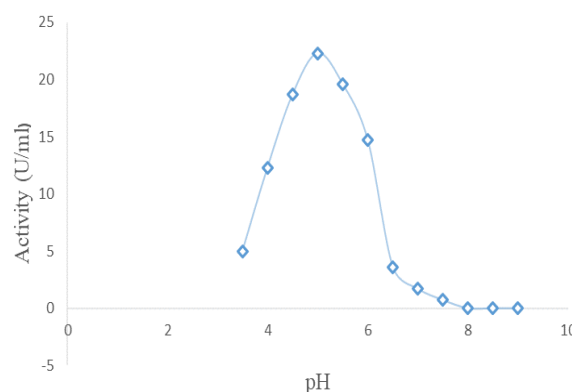


Figure 2. Effect of pH on peroxidase activity.

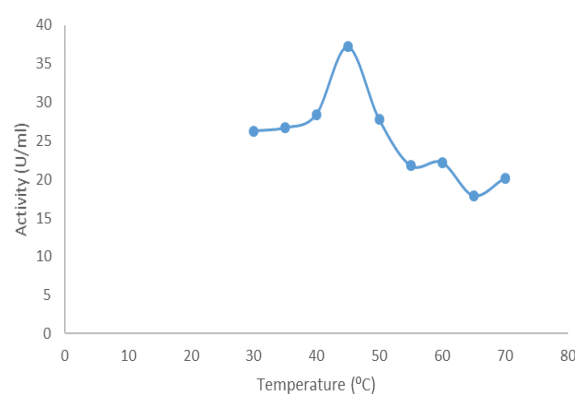


Figure 3. Effect of temperature on peroxidase activity.

Table 1. Partial purification steps of peroxidase from green cabbage.

Purification step	Volume of Enzyme (ml)	Protein Conc. (mg/ml)	Peroxidase Activity (U/ml)	Specific Activity (U/mg)	Total Activity(U)	Purification Fold	% yield
Crude Enzyme	1500	0.94	3.98	4.23	5970	1	100
80% (NH ₄) ₂ SO ₄ precipitation	300	0.80	15.93	19.91	4779	4.71	80.05
Dialyzed Enzyme	30	0.289	20.71	71.66	621.3	16.94	10.41
Gel filtrated Enzyme	15	0.676	37.57	55.58	563.55	13.14	9.44

cabbage peroxidase. The K_m and V_{max} values obtained from Lineweaver-Burk plot of initial velocities at different concentrations of H_2O_2 and o-dianisidine (Figures 4 and 5) were found to be 3.68mM, 37.04U/ml and 9.89mM 28.57U/ml, respectively. The K_m of H_2O_2 obtained in this work was two times the 1.93mM /ml reported by Eze (2012) for peroxidase extracted from African oil bean seed.

The green cabbage peroxidase was used to decolorize seven (7) azo dyes (Trypan Blue, Azo blue 5, Azo Brilliant Black, Azo Yellow 6, Azo Citrus Red 2, Azo Pink and Azo Purple) and two (2) Vat dyes (Vat Green 9 and Vat Orange 11). Of the nine dyes, Azo Trypan Blue had the highest decolorization (88.62%) followed by Azo Blue 5 and Brilliant Black with decolorizations of 72 and 63%, respectively, after contact time of 1 hour (Figure 6). The Azo Citrus Red 2 showed 32% decolorization and Azo yellow 6 showed 33.87%. Chanwun, Muhamad, Chirapongsatunkul and Churngchow (2012) reported 83 and 88% decolorizations for aniline blue and water blue, respectively, using peroxidase from rubber trees and contact time of 1 hr. Maddhinni *et al.* (2006) reported 70% decolorization for Azo yellow 12 at pH 4 after contact time of 1 hr, and 20.4% and 22.6% decolorizations for Azo Pink and Azo Purple. The Vat dyes (Vat Green 9 and Vat Orange 11) had 18 and 12% decolorization, respectively. Chanwun *et al.* (2012) reported 68% decolorization for Brilliant Green. It may be inferred that peroxidase is more effective in decolorizing Azo dyes than Vat dyes. Garlic peroxidase was reported to decolorize Vat Yellow 2, Vat Orange II and Vat Black 27 better (Osuji *et al.*, 2014).

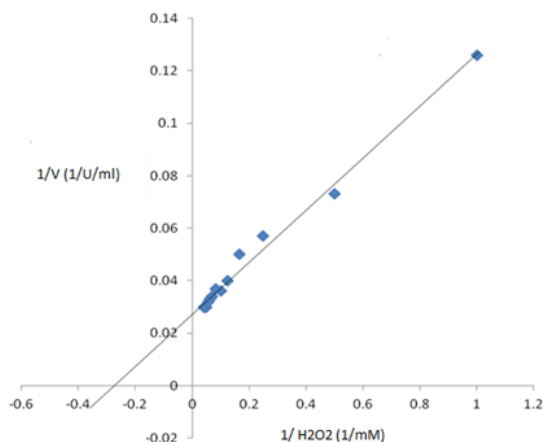


Figure 4. Lineweaver-Burk plot showing 1/Activity against 1/[H_2O_2].

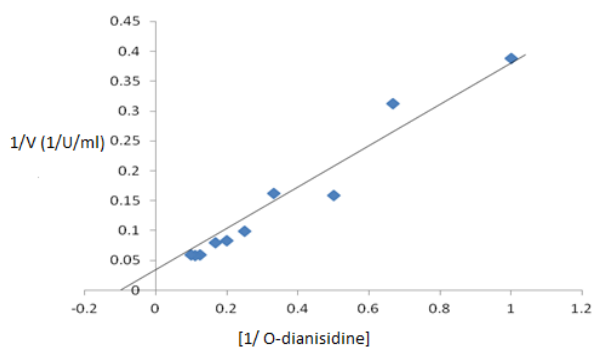


Figure 5. Lineweaver-Burk plot for o-dianisidine.

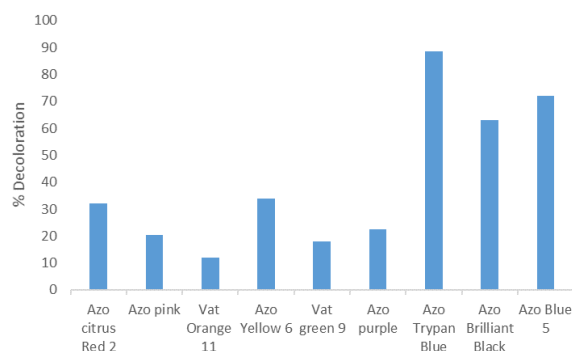


Figure 6. The decolorization of select dyes when treated with cabbage peroxidase for 1 hour.

4. Conclusions

Green cabbage peroxidase can decolorize synthetic dyes and is more effective for Azo dyes than for Vat dyes. This enzymatic decoloration of Azo dyes could be applied in the detoxification and remediation of wastewater effluents, especially from textile industry. Indeed, such novel process might be ecologically and economically friendly, since the enzyme by-products may be less toxic than those from other chemical methods, and further the source of these enzymes is relatively cheap.

References

- Al-Ansari, M. M., Modaressi, K., Taylor, K. E., Bewtra, J. K., & Biswas, N. (2010). Soybean peroxidase-catalyzed oxidative polymerization of phenols in coal-tar wastewater: comparison of additives. *Environmental Engineering Science*, 27, 967–975.
- Bae, J. S., & Freeman, H. S. (2007). Aquatic toxicity evaluation of new direct dyes to the *Daphnia magna*. *Dyes and Pigments*, 73(1), 81–85.
- Banat, I. M., Nigam, P., Singh, D., & Marchant, R. (1996). Microbial decolorization of textile-dye-containing effluents: A review. *Bioresources and Technology*, 58, 217–227.
- Bania, I., & Mahanta, R. (2012). Evaluation of peroxidase from various plant sources. *International Journal of Scientific and Research Publication*, 2(5), 2250–3153.
- Bholay, A. D., Borkhataria, B. V., Jadhav, P. U., Palekar, K. S., Dhalkari, M. V., & Nalawade, P. M. (2012). Bacterial lignin peroxidase: A tool for biobleaching and biodegradation of industrial effluents. *Universal Journal of Environmental Research and Technology*, 2(1), 58–64.
- Celebi, M., Altikatoglu, M., Akdeste, Z. M., & Yildirim, H. (2012). Determination of decolorization properties of reactive Blue 19 dye using Horseradish Peroxidase enzyme. *Turkish Journal of Biochemistry*, 37(2), 200–206.
- Chanwun, T., Muhamad, N., Chirapongsatunkul, N., & Churngchow, N. (2013). *Hevea brasiliensis* cell suspension peroxidase: purification, characterization and application for dye decolorization. *AMB Express*, 3, 1–9.

- Chaplin, M. (2014). Effect of pH and ionic strength on enzyme catalysis, *Enzyme technology*. Retrieved from <http://www1.lsbu.ac.uk/water/enztech/ph.html>
- Diao, M., Ouédraogo, N., Baba-Moussa, L., Savadogo, P. W., N'Guessan, A. G., Bassolé, I. H. N., & Dicko, M. H. (2011). Bio depollution of wastewater containing phenolic compounds from leather industry by plant peroxidases. *Biodegradation*, 22, 389–396.
- Eze, S. O. O., Chilaka, F. C., & Nwanguma, B. C. (2000). Purification and characterization of sorghum (KSV8) peroxidase. *Plant Production Resource Journal*, 5, 51-59.
- Eze, S. O. O. (2012). Kinetic analysis of the thermostability of peroxidase from African oil bean (*Pentaclethra macrophylla Benth*) seeds. *Journal of Biochemical Technology*, 4(1), 459-463.
- Ghahfarrokhi, I. S., Garmakhany, A. D., & Mousavi, S. M. (2013). Estimation of peroxidase activity in red cabbage by Artificial Neutral Network (ANN). *International Association for Cereals Science and Technology*, 5(2), 163-167.
- Ghasemi, F., Tabandeh, F., & Bambah, B. (2010). Decolorization of different azo dyes by *phanerochaete chrysosporium* RP78 under optimal condition. *International journal of Environmental Science and Technology*, 7(3), 457-464.
- Hu, Y., Wu, J., Luo, P., & Mo, Y. (2012). Purification and partial characterization of peroxidase from lettuce stems. *African Journal of Biotechnology*, 11(11), 2752-2756.
- Kalsoom, U., Ashraf, S. S., Meetani, M. A., Rauf, M. A., & Bhatti, H. N. (2013). Mechanistic study of a diazo dye degradation by soybean peroxidase. *Chemistry Central Journal*, 7(93), 1-10.
- Khatun, S., Md, A., Md, R. K., Pervin, F., Absar, N., & Rosma, A. (2012). Peroxidase from *M. oleifera* leaves. *Bio Resources*, 7(3), 3237-3251.
- Kirby, N., Mullan, G., & Marchant, R. (1995). Decolorization of an artificial textile effluent by *Phanerochaete chrysosporium*. *Biotechnology Letters*, 17, 761-764.
- Leelakriangsak, M., & Borisut, S. (2012). Characterization of the decolorizing activity of azo dyes by *Bacillus subtilis* azo-reductase AzoR1. *Songklanakarinn Journal of Science and Technology*, 34(5), 509-516.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275.
- Mikami, D., Kurihara, H., Takahashi, K., & Suzuki, T. (2013). Effects of metal ions on the activity and stability of peroxidase in Tartary buckwheat shoots. *Journal of Agriculture, Chemistry and Environment*, 2(3), 59-64.
- Osuji, A. C., Eze, S. O. O., Osayi, E. E., & Chilaka, F. C. (2014). Bio-bleaching of industrial important dyes with peroxidase partially purified from garlic. *The Scientific World Journal*, 2014, 1-8.
- Shamsher, S. K., Neelam, B., & Lata, K. (2012). Peroxidase and its applications. *International Journal of Institutional Pharmacy and Life Sciences*, 2(2), 1-28.
- Tirola, T., Jaakkola, Bloigu, A., Paladanius, M., Sinisalo, M. S., Silvennoinen-Kassine, S., . . . Leinonem, M. (2006). Novel enzyme immunoassay utilizing lipopolysaccharide-binding protein as a capture molecule for the measurement of chlamydial lipopolysaccharide in serum. *Diagnostic Microbiology Infectious Diseases*, 54, 7-12.