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# Characterization of peroxidase from *Brassica oleracea gongylodes* gives a lead for use of bromocresol purple as a novel substrate for peroxidase assay

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## Abstract

The study reports the novel results of characterization of peroxidase enzyme from *Brassica oleracea gongylodes* known as Navilkosu in Karnataka, India. The crude extract was concentrated by salting out at 80% ammonium sulfate saturation. Localization of activity on electropherogram revealed the presence of a major band of peroxidase activity in the dialysate. Navilkosu peroxidase (NKP) showed optimal activity at pH 6-6.5 and 35-40°C. The enzyme exhibited stability in the acidic range of pH and retained around 85% of activity after 1h of incubation at 50°C. NKP could decolorize bromocresol purple (BCP) and bromothymol blue (BTB) in presence of $H_2O_2$. Km values for the organic substrates namely phenol, Guaiacol, Ortho phenylenediamine, 3,3',5,5' tetramethyl benzidine, BCP and BTB were found to be significantly lower than horseradish peroxidase (HRP). Extracts of sweet potato, cabbage, radish and HRP were also found to decolorize BCP and BTB using $H_2O_2$ as the oxidising agent. Combined with its stability and high absorptivity, use of BCP as a substrate offers a novel and sensitive assay system for peroxidases.

**Key words:** *Brassica oleracea gongylodes,* peroxidase, characterization, Michaelis constant, Bromocresol purple

## Introduction

Peroxidases (EC 1.11.1.7/ phenolic donor:hydrogen- peroxide oxidoreductase) are heme containing enzymes that catalyse the oxidation by hydrogen peroxide of a range of organic compounds such as phenols, aromatic amines, ascorbic acid, indole etc (Veitch, 2004). Plant peroxidasesare implicated in a wide array of cellular processes such as lignin biogenesis, formation of ethylene, detoxification of $H_2O_2$ and cell growth. Commercial uses of peroxidases are numerous. The ability of peroxidase from *Armoracia rusticana* (horseradish) to act on a broad range of substrates yielding chromogenic products has been exploited extensively in the field of biotechnology. Peroxidase tagged immunoglobulins have been used successfully as immunohistological probes for the demonstration of tissue antigens, and in enzyme amplified immunoassay systems for the quantitative determination of soluble and insoluble antigens (Belcarz et al. 2007). Currently peroxidase extracted from horse radish roots is being used as the enzyme label in these applications. Several researchers have reported potential use of peroxidases in detoxification of aromatic hydrocarbons and textile dyes (da Silva et al. 2010, Dorantes and Zuniga, 2012). Peroxidases are used as model systems for studies on redox catalysis (Amjadi et al. 2007).

Increasing demand for horse radish peroxidase (HRP) in recent years has propelled the researchers to search for peroxidases with commercially viable properties from other sources. An indigenous alternative to HRP, which is equivalent or with better characteristics such as lower Km and higher thermostability will be of advantage to the research community.

Peroxidases are present throughout the plant kingdom and many such sources are yet to be explored. Many plants belonging to *Brassica sp* are documented as good sources of peroxidases. *Brassica oleracea gongylodes* known as Navilkosu in Karnataka, India is a source which remains unexplored. In the present investigation we undertook a study to characterize NKP. We also report the effect of enzyme action on various laboratory dyes.

## Materials and Methods

Vegetables were procured from Local central market places. HRP was procured from SRL, India. All the reagents used were of analytical grade. Thermoscientific Genesys 10 UV-Visible spectrophotometer was used for reading the absorbance in activity measurements. The absorption spectra were recorded using Variance Cary60 spectrophotometer.

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*Extraction of the enzyme*

Fresh vegetables (Table 1) were procured from the market, washed and the outer covering/layer was peeled off. The slices were cut and macerated in liquid nitrogen to a pasty consistency. Leaves obtained from Navilkosu (NK) plantlets were also ground. The extracts were prepared in distilled water (2g tissue in 10ml) and centrifuged at 6000 rpm for 15min. The supernatants were analysed for enzyme yield and stored at -20°C.

*Concentration of the enzyme*

The crude extract prepared from NK bulbs was subjected to 0-80% ammonium sulfate precipitation. The precipitate was dissolved and dialysed against 10mM phosphate buffer (pH 6.4) containing 1mM CaCl2. Dialysate was further used for characterization.

*Analytical methods*

*Enzyme assay*

Appropriately diluted enzyme was added to the reaction mixture containing $H_2O_2$ (2mM) and Phenol/Aminoanitpyrine mixture (85mM/1.25mM respectively) in 50mM buffer (acetate buffer, pH 4.8/ phosphate buffer, pH 7 or 6.4/ NaOH-glycine buffer, pH 9.2) and incubated at 27±0.5°C. The $A_{500nm}$ was read at every 3-5 min intervals for 20min. Blank was set by adding heat killed enzyme to the reaction mixture and absorbance resultant of auto-oxidation was recorded for correction of the readings.

One unit of activity is the amount of enzyme which produces one micromole of the quinoneimine dye (ε=13600 – Khuchareontaworn et al. 2010) in one minute under the defined conditions.

*Localization of enzyme activity on polyacrylamide gel*

Disc electrophoresis was carried out under non-denaturing conditions (Stacking gel – 5% T, 3.33% C; Resolving gel – 10% T, 3.33% C). NK extracts, the dialysate and HRP preparations (0.1U of each) were loaded and electrophoresis was performed at 20mA. The gel was removed, rinsed with D/W and equilibrated in 50mM PO4 buffer, pH 6.4 for 10min. The gel was then immersed in Phenol-AAP mixture with 50mM $H_2O2$ till pink bands were observed.

*Characterization of the enzymes*

*Effect of pH and temperature:* Enzyme activity and stability studies were performed at various pHs ranging from 3.6-9. Effect of temperature on the activity and stability were studied at pH 6.4 from 25-70°C. For pH stability studies the enzyme was incubated in 25mM buffers of various pHs for 30min. Thermostabilty studies were performed by incubating the enzyme at various temperatures for 1h followed by assay of residual activity.

*Km for various substrates*

The effect of varying concentrations of different organic substrates namely phenol, Guaiacol, Ortho phenylenediamine (OPD) and 3,3',5,5' tetramethyl benzidine dihydrochloride (TMB-HCl) was studied in presence of 3mM $H_2O_2$ in 50mM buffer, pH 6.4 at 27±0.5°C at wavelengths of 500, 470, 410 and 650nm respectively. Effect of $H_2O_2$ was studied using phenol as the second substrate. Km was determined using Michaelis-Menten and Lineweaver-Burke (LB) plots. TMB however, could not be used at saturating concentrations as concentrations above 0.6 mM resulted in appearance of precipitate in the reaction mixture. The study was therefore performed in the range of 0.02 – 0.6mM TMB and the Km was determined by LB plot. Action of the enzyme on all these substrates resulted in formation of coloured products. The molar absorptivities of the respective products were considered for calculating the Vmax. The products tetraguaiacol (ε=25500) and diimine derivative (ε=39000) resulted from the oxidation of guaiacol and TMB respectively (Sakuragawa et al. 1998, Metelitza et al. 2004)

The color disappeared in case of BCP and BTB due to enzymatic action and hence the effect of these substrates was studied in the first order kinetics. Decolorization of BCP at 590nm was studied in the range of 1-40µM. Effect of BTB concentration on enzyme activity was monitored at 430nm over a range of 15-150µM. The Km values were extrapolated by plotting LB graphs. Molar absorptivity of BCP and BTB was also determined at pH 6.4 in 25mM buffer for calculation of Vmax values.

*Action of the enzyme on various dyes*

One mg each of various dyes was dissolved in 1ml of D/W with the exception of methyl red which was dissolved in ethanol. Each was diluted to a translucent intensity with D/W containing 50mM $H_2O_2$. To 1ml of each solution, 0.055U of NKP was added and the color change was observed over a period of 6h (27±0.5°C) in comparison to the respective blank to which inactivated enzyme had been added.

*Decolonization of the dyes*

Dye solution containing 1mg of BCP/BTB in 10ml of 25mM phosphate buffer, pH 6.4 was incubated with 0.15U of NKP in presence of 50mM $H_2O_2$. The incubation was continued for 5h at 27±0.5°C with periodic addition of 50% $H_2O_2$ (5µl) every 1h. Absorption spectrum (240-800nm) was recorded at 0h, 3h and 5h. For BCP, aliquots were taken and diluted 7folds with the buffer and scanned.

*Effect of pH on the peroxidase treated BCP and BTB*

BCP/BTB solutions were subjected to decolonization as per the procedure given above. The preparations (7 times diluted BCP / undiluted in case of BTB) were diluted with equal volume of 3 different buffers namely, 0.2M at pH 3.2 (acetate buffer), 6.4 (phosphate) and 9.0 (NaOH-glycine). The reaction mixtures were scanned from 240-800nm. Dye treated in a similar manner with heat inactivated enzyme was used as control.

*Action of peroxidases from various sources on BCP and BTB*

BCP (0.05mg) and BTB (0.2mg) in 1ml of PO4 buffer, pH 6.4 were treated with radish, sweet potato and cabbage extracts, and HRP solution containing 0.5U of activity in presence of 100mM H2O2 for 4h. The color was compared with the respective controls.

*Assessment of cytotoxicity by Trypan blue exclusion assay*

Blood collected from normal volunteers was collected in tubes containing NaF. The blood was immediately processed by Hypaque-Ficoll method for isolation of peripheral blood mononuclear cells (PBMNs).

Freshly collected PBMNs were incubated for 90min at $37^0$C with minimal essential media ± 10% FBS. PBMNs were then treated with the filter sterilized native and peroxidase treated decolorized forms of BCP at various concentrations ranging from 10ng to 1mg/ml and

incubated for 120 min. The cell suspensions were then mixed with 0.05% trypan blue solution (1:9) and observed microscopically and counted within 3min at 400x magnification. The number of viable (unstained) and nonviable (stained) cells were counted in a Hemocytometer and the percentage of viable cells was calculated from these counts.

## Results and Discussion

Roots/tubers of many plants such as potato, sweet potato, horse radish etc are reported to be rich sources of peroxidases. Study of peroxidases from plants belonging to *Brassica oleracea* have been extensively reported in the literature. As mentioned earlier, literature on *Brassica oleracea gongylodes* is scarce. Manzoori et al. 2006 and Amjadi et al. 2007 have reported application of peroxidase extracted from Kohlrabi (purple bulb of *Brassica oleracea gongylodes*) in estimation of $H_2O_2$ and Thiamine. However, its characteristics is not been reported. We selected NK (*Brassica oleracea gongylodes* which produces green bulb) for our study and compared the peroxidase content of this vegetable with that from various other sources. Neutral, acidic and basic isoforms of peroxidases are known to exist in various plant sources (Thongsook and Barrett, 2005, Triplett and Mellon, 1992). The isoforms may differ with respect to their pH optima and often one isoform may be more pronounced in a particular source. HRP C which is the most abundant isoform present in horse radish roots is highly cationic (Veitch, 2004, Ryan and O Fagain, 2007).

Table 1: Comparative yield of peroxidase from various sources

| | U/g at various pHs* | | |
|---|---|---|---|
| Source | Acidic | Neutral | Basic |
| Cabbage | 1.55 ± 0.18 | 1.72 ± 0.14 | 0.58 ± 0.06 |
| Navilkosu | 3.12 ± 0.12 | 3.03 ± 0.17 | 0.83 ± 0.03 |
| Spring Onion | 0.04 ± 0.002 | Neg | Neg |
| Sweet potato | 3.7 ± 0.33 | 3.9 ± 0.08 | 0.7 ± 0.03 |
| Radish | 0.77 ± 0.08 | 0.88 ± 0.1 | 1.1 ± 0.09 |
| Potato | 0.05 ± 0.002 | 0.08 ± 0.01 | Neg$^{\Psi}$ |
| Yam | Neg$^{\Psi}$ | Neg$^{\Psi}$ | Neg$^{\Psi}$ |

* Acidic- 4.8; Neutral -7.0; Basic-9.2. $^{\Psi}$Negligible - Values < 0.04; Cabbage-*Brassica oleracea var capitata,* Navilkosu-*Brassica oleracea gongylodes*, Spring onion- *Allium wakegi,* Sweet potato-*Ipomoea batatas,* Radish-*Raphanus sativu,* Potato-*Solanum tuberosum,* Yam-*Amorphophallus paeoniifolius*

Enzyme yield in various tubers and bulbs was determined at different pHs, the result for which is presented in Table 1. Sweet potato and NK were found to be the best amongst these various sources. The peroxidases present in both these sources were found to be prominently active at both the acidic and neutral pH employed. We continued our studies with NKP and compared its properties with HRP. The enzyme yield in the bulb and leaf extract was found be around 3.4 and 2.9U/g respectively at pH 6.4. NKP bulb extract was salted out at 80% saturation of ammonium sulfate (ASS) with a yield of around 75-80%. Crude extracts from bulb and leaf, ASS dialysate and HRP were subjected to PAGE and the activity was localized in the electropherogram (figure 1).

True to its cationic nature, HRP migrated at a very slow rate and remained at the cathodic end of the gel. Both the leaf and bulb extracts showed the presence of a major band of NKP which moved a little faster than HRP. Overall, the mobility of peroxidases in all the lanes was found to be slow and they remained in the stacking gel at the end of the run indicating the cationic nature of these enzymes.

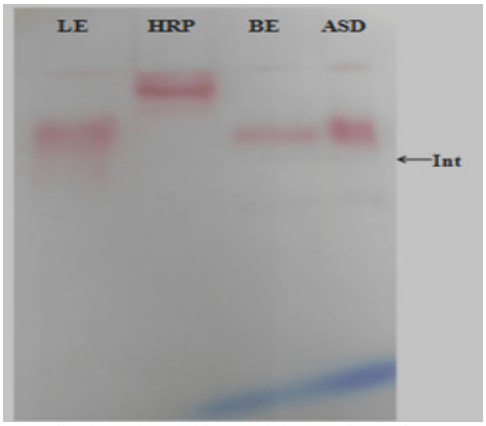


Figure 1: Localization of peroxidase activity on electropherogram LE- NK Leaf extract; BE- NK bulb extract; ASD- Dialysate obtained by salting out of bulb extract; Int- Interface between stacking and separating gels

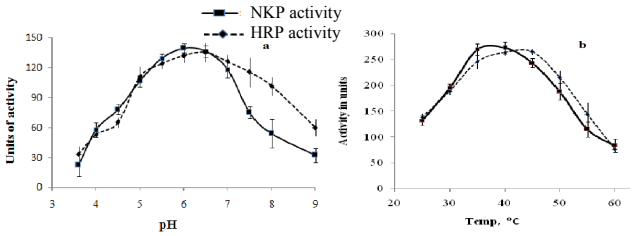


Figure 2: Effect of pH and temperature on the activity of NKP and HRP a) NKP activity (U/50g of the tissue extracted and subjected to salting out); HRP activity (U/mg). The pH-activity profile at 27±0.5°C; b) Temperature-activity profile at pH 6.4

For use of an enzyme as a commercial tool it is essential that it has high affinity for the substrate which is depicted by its Km. Thermostability of an enzyme influences the shelf life and, pH stability determines the range of pH at which it can be effectively employed and stored. The pH optima of both HRP and NKP are shown in Figure 2.

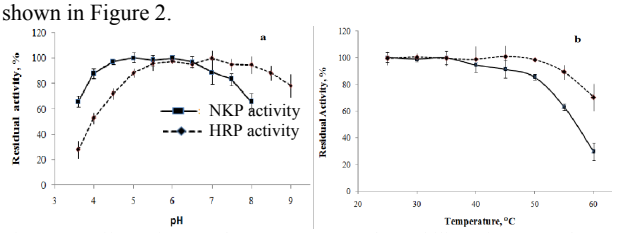


Figure 3: Effect of pH and temperature on the stability of NKP and HRP; a) The pH stability profile (100% for NKP - 2. 76 U of activity /g of the tissue subjected to salting out; 100% for HRP - 157.7 U/mg); b) Thermo-stability (100% for NKP - 2.67 U of activity / g of the tissue subjected to salting out; 100% of HRP - 143.9 U/mg)

The pH optima of both NKP and HRP lied in the range of 6 - 6.5. NKP was optimally active at 35-40°C and HRP performed optimally at 40-45°C. The results of stability studies are displayed in figure 3. NKP was relatively stable in the acidic range of pH from 4.5-6.5 and HRP was stable in the range of 5.5-8. HRP tagged immunoassay systems generally employ buffers at near neutral/neutral pH (Law et al. 2005). Use of neutral pH poses risk of contamination and hence it would be preferable to use a buffer which possesses slightly acidic pH. The stability of NKP in the near neutral region (pH 6-6.5) was comparable to HRP. Thus NKP may find use as an enzyme label in immunoassays provided it retains its stability upon conjugation to immunoglobulins. Thermostability

Table 2: Kinetic properties of NKP and HRP

| Substrate | Km, mM | | Vmax$^{\Psi}$ | | Specificity constant$^{*}$ | |
|---|---|---|---|---|---|---|
| | NKP | HRP | NKP, U/g | HRP, U/mg | NKP | HRP |
| Phenol | 0.43 ± 0.03 | 4.23 ± 0.4 | 2.82±0.06 | 137 ± 6.1 | 6.56 | 32.45 |
| Guaiacol | 0.81± 0.023 | 4.13 ± 0.23 | 1.99 ± 0.21 | 62 ± 4.8 | 2.46 | 15.1 |
| OPD | 0.89 ± 0.056 | 4.0 ± 0.36 | - | - | - | - |
| TMB | 0.62 ± 0.1 | 1.17 ± 0.17 | 1.86 ± 0.09 | 288 ± 37.9 | 3.01 | 246.5 |
| BCP | 0.38 ± 0.029 | 2.86 ± 0.22 | 5.58 ± 0.25 | 145 ± 8.1 | 14.67 | 50.69 |
| BTB | 0.45 ± 0.044 | 3.46 ± 0.29 | 3.72 ± 0.39 | 180±23.9 | 8.26 | 51.94 |
| $H_2O_2$ | 0.2 ± 0.015 | 0.19 ± 0.013 | 2.80 ± 0.077 | 146±10.9 | 13.99 | 767.8 |

$^{*}$Specificity constant = Vmax/Km

$^{\Psi}$Vmax for BCP and BTB - Amount of enzyme which converts one micromole of the substrate under the defined conditions; For rest of the substrates it is measured as the amount of enzyme which produces one micromole of the respective product

studies showed that NKP retained 86% of its activity at 50°C at the end of one hour incubation period. HRP was relatively more thermostable as it could retain 89% of its activity at 55°C (Figure 3b).

Affinity of the enzymes for various organic substrates namely phenol, Guaiacol, OPD and TMB was also studied. The results are presented in Table 2. The affinity of NKP for organic substrates was markedly higher in comparison to HRP. The comparison revealed that NKP exhibited almost 10 folds stronger affinity for phenol. With the exception of TMB, the Km values for the other substrates were also found to be significantly less than those of HRP. This is a valuable characteristic of NKP as it can function efficiently at low concentration of these substrates, most of which are reportedly toxic. Both the enzymes showed affinity towards $H_2O_2$ to the same extent.

Peroxidases are known to act on a broad range of organic substances and this potential is being assessed for their use in detoxification of textile dyes. We decided to test the effect of NKP on various laboratory dyes in presence of $H_2O_2$ (Table 3). Discoloration to a significant extent was observed in case of bromocresol purple (BCP) and bromothymol blue (BTB).

Table 3: Action of NKP on various dyes

| Dye | Effect$^{\Psi}$ | Dye | Effect$^{\Psi}$ |
|---|---|---|---|
| Basic fuchsin | - | Eosin | - |
| Brilliant blue | - | Fast green | - |
| Bromocresol green | ++ | Methyl red | + |
| Bromocresol purple | - | Methyl orange | - |
| Bromophenol blue | - | Orcein | - |
| Bromothymol blue | ++ | Phenolphthalein | - |
| Crystal violet | - | Tartrazine | - |

$^{\Psi}$Change in color or intensity with respect to the corresponding blank tubes; ++ Significant discoloration; + Slight decolourization; - No change

The absorption spectra of BCP, BTB and their decolrised forms are shown in figure 4. Decrease in the intensity to an extent of 80% was observed in case of BCP at its λmax (590nm). Similarly, the absorbance of BTB decreased to an extent of around 60% at both its absorption maxima i.e. 425 and 615nm. As both these dyes are acid-base indicators, we studied the spectra of both native and decolorised forms at three different pHs namely, 3.4, 6.4 and 9. In accordance with its characteristics, BCP absorbed to a great extent at 590nm at both pH 6.4 and 9 (figure 5a). As the pH decreased to 3.4, the form of the dye which predominated displayed a λmax of 430nm. However, the decolorized form of the dye showed insignificant absorbance in the visible region at all the three pHs. Results of studies conducted to analyse the effect of pH on BTB and its decolorised form is given in figure 6.

As seen in figure 6a, native form of BTB showed its characteristic spectra. The basic form of the dye absorbed maximally at 620nm. As the pH decreased, the form of BTB which had a λmax of 430nm appeared. At pH 6.4, although the peak representing the basic form was present, the acidic form was found to be dominant. Spectrum at pH 3.4 showed the absence of basic form. The decolrised form of the BTB was also exposed to different pHs. It showed spectra characteristic of BTB. However due to decolorization, significant decrease in the intensity of the respective peaks was seen at different pHs (figure 6b). Peroxidase action did not result in formation of new peaks in the spectra of BCP and BTB, nor was shift observed in any of the λmax.

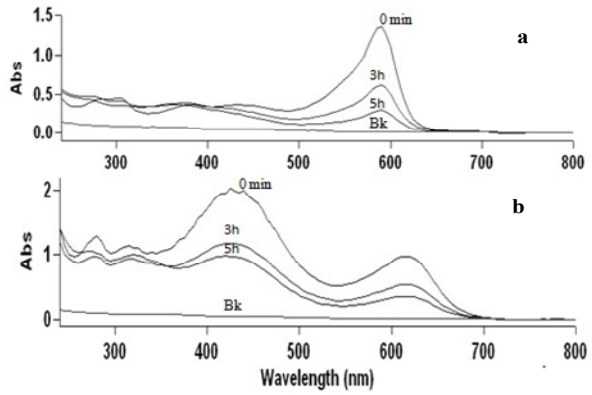


Figure 4: NKP action on BCP and BTB; a) Decolorization of BCP b) Decolorization of BTB- 0 min, 3h and 5h - Time intervals at which the absorption spectrum was acquired; Bk- absorption spectrum of the buffer, pH 6.4.

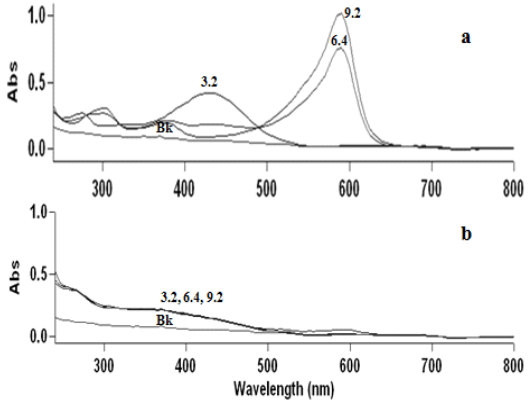


Figure 5: Absorption spectra of the native and decolorized forms of BCP at various pHs a) Absorption spectra of BCP; b) Absorption spectra of peroxidase treated BCP 3.2, 6.4 and 9.2 are the three pHs at which the spectral scanning was performed; Bk – Absorption spectrum of the buffer at pH 6.4 (absorption spectra of the other two buffer systems were also similar)

We found that HRP as well as peroxidases from radish, sweet potato and cabbage could efficiently decolorize BCP and BTB. Thus these two dyes appear to act as common substrates for plant peroxidases.

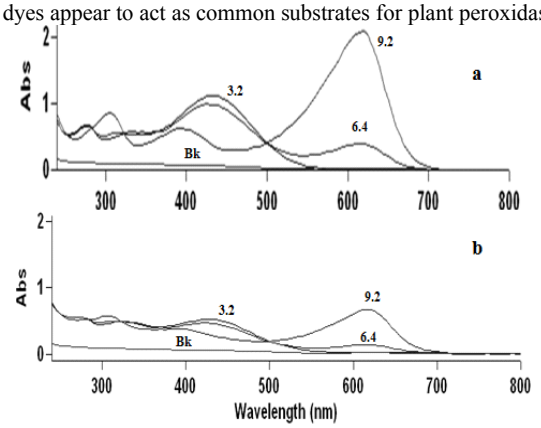


Figure 6: Absorption spectra of the native and decolorized forms of BTB at various pHs. a) Absorption spectra of BTB b) Absorption spectra of peroxidase treated BT 3.2, 6.4 and 9.2 are the three pHs at which the spectral scanning was performed; Bk – Absorption spectrum of the buffer at pH 6.4 (absorption spectra of the other two buffer systems were also similar)

From the studies performed to assess the effect of varying concentrations of the dyes on NKP, the Km values were estimated to be 0.38 and 0.45mM for BCP and BTB respectively (Table 2). The results suggested that in comparison to HRP, NKP had high affinity for these substrates which is comparable to its affinity for phenol. Molar extinction co-efficient of BCP and BTB were found to be 54000 and 14000 in phosphate buffer at pH 6.4. Vmax was calculated based on these values. Vmax for BCP was higher than the Vmax for BTB. Specificity constant was thus found to be around 1.8 times higher for BCP. Although, HRP exhibited higher affinity for BCP in comparison to BTB, the specificity constants for both the dyes were comparable. As seen in figure 4 and mentioned earlier, BCP was decolorized to a greater extent than BTB by same units of NKP. Thus BCP serves as a better substrate for peroxidase assay. Also as BCP displays high absorptivity, oxidation of a small amount of BCP by peroxidase will result in a measurable change in absorbance (1μM change - ΔAbs of 0.054 at pH 6.4). Thus use of BCP as a substrate offers a sensitive assay procedure for peroxidases. However, being an acid-base indicator, absorptivity of BCP is a function of pH and hence, it is essential to construct the calibration curve under the experimental conditions at λmax of 430nm at pH≤ 5.2, or 590nm at pH ≥ 5.5. Enzyme reaction can be studied by following first order kinetics. Zero order kinetics will be relatively laborious to follow.

Most of the organic substrates used in peroxidase assays are toxic. Material safety data sheets of these chemicals report the LD50 values of OPD, phenol, guaiacol and pyrogallol based on the toxicological studies performed on animal models. Toxicological data for TMB and BCP however is not yet reported in the MSDS. We decided to investigate the cytotoxic effect of BCP on PBMNs by trypan blue uptake assay. Simultaneously the enzyme treated BCP fraction was also analysed for its cytotoxicity. Serum albumin is known to bind to BCP (Clase et al. 2001) and hence the cytotoxicity tests were done on the cells suspended in the media ± FBS. As presented in figure 7, the toxicity of BCP was found to be not very significant. However, the product formed by peroxidase action exhibited significant toxicity.

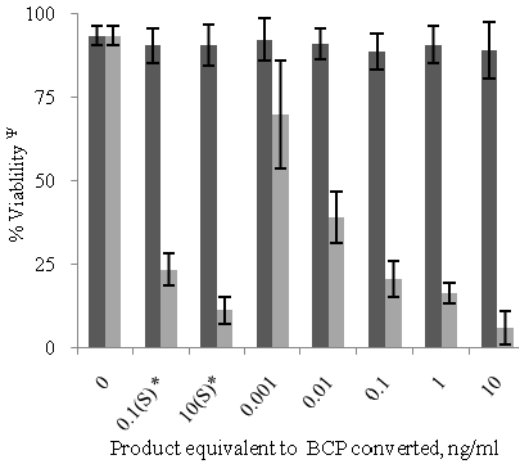


Figure 7: Cell viability assay by Trypan blue exclusion, (■) Cells treated with native form of BCP, (■) Cells treated with decolorized form of BCP, $^{\Psi}5x10^{5}$ cells in one ml of the suspension; ∗ Cells suspended in FBS containing medium

Decolorized form exerted cytotoxicity irrespective of the presence/absence of albumin. At a concentration of the decolorized form equivalent to 0.01ng/ml of the dye oxidized by the enzyme, more than 50% of the cells lost their viability. OPD is oxidized to diaminophenazine by the action of peroxidases. Researchers have reported toxic and mutagenic potential of diaminophenazines (Wagner et al. 1996, Fu et al.2005). Quinoneimine is generated due to peroxidase mediated oxidative coupling of phenol and aminoantipyrine. Reactivity and toxicity of quinoneimine derivatives have also been implicated (Powis et al. 1987, Martínez-Cabot et al. 2005). Our results indicated generation of toxic product/s as a consequence of peroxidase action on BCP. If used as a substrate, reduction in the absorbance of BCP needs to be monitored. Due to its high absorptivity, BCP can be employed as a substrate at a concentration as low as of 30-60μM. Hence, the amount of toxic product produced due to enzyme action will be in nM to μM range. Moreover, BCP is easily accessible in laboratories as it is used as a general purpose acid-base indicator. It is also included in few microbial media such as P-A Broth, BCP lactose agar etc. Use of this dye for estimation of serum albumin is well documented (Clase et al. 2001).

Often in *in-vivo* detoxification processes, toxic compounds are converted by oxidizing enzymes to more harmful intermediates prior to their degradation into non-toxic metabolites (Wu et al. 2004). Peroxidases are being extensively studied for their use in treatment of industrial effluents generated especially from textile and petrochemical industries which contain dyes and other phenolic substances. Also there are reports wherein discolorization of a dye is associated with detoxification. Decolorized form equivalent to 1ng/ml of BTB also exhibited cytotoxicity to a significant extent as only 17% of the PBMN cells survived. In presence of the native form of BTB at the same concentration, 84% of the cells retained their viability (results not shown). Our results indicated that peroxidase action on BCP and BTB generated colorless toxic products. Decolorization is often associated with degradation and hence, detoxification of the dyes (Contreras et al. 2011). Although the goal of the present communication is remotely connected to detoxification, the authors would like to stress on exercising caution while using peroxidases in bioremediation processes as discoloration of a dye may not necessarily make it less harmful.

## Conclusions

NKP was relatively less thermostable than HRP. However, NKP exhibited much higher affinity for the organic substrates and hence, it can function optimally at lower concentrations of these substrates. The crude extracts and the dialysate could be stored at -20°C for a prolonged period of 9 months without significant loss of NKP activity (<5% loss). Horseradish is cultivated in Canada and northern regions of USA. An alternative indigenous source of peroxidase will confer benefit to the community. NK is relatively a less expensive vegetable which grows in the southern regions of India. NK plants were successfully grown from seeds in green house conditions as well. The leaf can also serve as the source of enzyme.

The investigation also revealed the ability of peroxidases to decolorise BCP and BTB. Most of the organic substrates such as OPD and Guaiacol undergo oxidation and hence should be preferably used within an hour of preparation. BCP and BTB solutions however, did not exhibit any change in their absorbance even after 8h of preparation. Combined with its higher absorptivity, BCP can therefore serve as a substrate for peroxidase assay.

## References


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