



Conjugation of Peroxidase from *Brassica oleracea gongylodes* for Use as a Label- Prospect of a Novel Enzyme Tag for Immunoassay Systems

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Abstract

Peroxidase tagged proteins are being used successfully as immune-histological probes for the demonstration of tissue antigens, and in enzyme amplified immunoassay systems for the quantitative determination of soluble and insoluble antigens. The glycoprotein nature of peroxidases can be exploited for conjugation to proteins of interest. Peroxidase extracted from the bulbs of *Brassica oleracea gongylodes* was salted out at 40-80% ammonium sulfate saturation and activated by treatment with 1-Fluoro-2,4-dinitro benzene (FDNB) and periodate. Treatment with 0.08% FDNB and 12.5mM periodate was optimized for activation of the enzyme. The treated enzyme was found to conjugate successfully to immunoglobulin fractions harvested from egg yolk (IgY), human plasma and goat serum. Enzyme conjugated to IgY fraction showed improvement in its pH stability and temperature stability. The affinity of the enzyme for its substrate phenol did not alter to a significant extent upon activation and conjugation. The conjugates exhibited high affinity towards phenol, bromocresol purple and bromothymol blue in comparison to HRP conjugates prepared using the same protocol.

Keywords: Peroxidase; *Brassica oleracea gongylodes*; Immunoglobulins; Periodate treatment; Conjugation.

Introduction

Peroxidases (EC 1.11.1.7/ phenolic donor:hydrogen-peroxide oxidoreductase) are heme containing enzymes distributed widely in plant kingdom in a variety of tissues (Duarte-Vazquez *et al*, 2007). From the commercial point of view, peroxidases are enzymes of great significance as they have varied applications (Yu *et al*, 1994; Bholay *et al*, 2012; Dorantes *et al*, 2012, Chandrasekaran *et al*, 2014). These enzymes catalyze the oxidation by hydrogen peroxide of a broad range of hydrogen donor substrates such as phenols, ascorbic acid, aromatic amines and their derivatives (Ye *et al.*, 2006; Vernawal *et al.*, 2006). Alteration in the color of many of the organic substrates due to enzymatic action has allowed the use of this enzyme as a reporter enzyme. Simplicity of peroxidase assays, availability of various substrates and small size of peroxidase makes it ideal to be used as a label in immunoassay probes (Belcarz *et al*, 2007).

Peroxidase extracted from horse radish (HRP) roots in conjugation with antibodies and other protein tools is being

used extensively in immunoassay probes for the detection of tissue antigens and measurement of antigens in enzyme amplified immunoassays.. Horse radish (*Armoracia rusticana*) is cultivated in temperate regions of the world (Canada and northern regions of USA) mainly for the culinary value of its roots. Although a rich source of peroxidase, the enzyme extracted from horse radish exerts low stability in its hydrated state. Increasing demand for HRP in recent years has propelled the researchers to search for an alternative source of peroxidase with commercially viable properties. Peroxidase substrates being toxic, an enzyme which can function efficiently at lower concentrations of these phenolic substrates may provide an economic and environmentally beneficial alternative to the existing system. Many plants belonging to *Brassica* sp are documented as good sources of peroxidase. The bulb of *Brassica oleracea gongylodes* which is used as a vegetable in some parts of India is a reservoir of peroxidase. The enzyme is reported to possess high affinity for organic substrates (Shetty *et al*, 2012). The storage stability of the enzyme was also reportedly satisfactory.

In the present investigation, we aim to conjugate the peroxidase from *Brassica oleracea gongylodes* to immunoglobulins. The glycoprotein nature of the enzyme can be exploited for conjugation and the conjugate will be characterized with regards to its stability and affinity for the substrates.

Materials and Methods

Brassica oleracea gongylodes bulbs were purchased from local central market. 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.

Extraction of the Enzyme

Fresh vegetable was washed, peeled and sliced into small pieces. The slices were macerated in liquid nitrogen to a pasty consistency. The extract (10g tissue in 50ml distilled water) was centrifuged at 6000 rpm for 15min. The supernatant was stored at -20 °C until use.

Concentration of the Enzyme

The crude extract was subjected to precipitation by fractional ammonium sulfate saturation (ASS) at 0-40%, 40-60% and 60-80% saturation levels. The salted out fractions were dissolved and dialyzed against 10mM phosphate buffer (pH 6.4).

Activation of the Enzyme

The salted out fraction (40-80% ASS) containing 6-7 units of activity was treated with 0.05%-0.12% of FDNB in 0.9 ml of 25mM carbonate buffer, pH 8.5 for 30min at 25°C. Sodium meta periodate (PI) was then added to a final concentration of 5-22.5mM in one ml reaction mixture and was allowed to react for 30 min at 25°C. Ethylene glycol at a concentration of 40mM was used for neutralization. Reaction mixture was dialysed overnight at 4-5°C. The dialysate was used for conjugation.

Harvesting of Immunoglobulins (Igs)

Igs from eggs: Immunoglobulins from egg yolk (IgY) were harvested by precipitating with polyethylene glycol 6000 (Polson et al, 1980).

Igs from Human plasma (IgHu): Plasma, 0.5 ml was added to 5.5 ml water. Sodium Sulphate, 1.5g was added and the tube was incubated over night at 40°C. The contents were mixed and filtered through Whatman filter paper 1. The residue was dissolved in phosphate buffered saline (PBS) and dialysed against ten times diluted PBS.

Igs from goat serum (IgGo): To 1.0ml of serum, 10ml of 19.0% ammonium sulfate solution containing 0.4g NaCl was added and left overnight at 4-5°C. The suspension was centrifuged at 12000rpm for 15min. The precipitate was dissolved in PBS and globulins were re-precipitated with 10% PEG 6000. The precipitate was dissolved in PBS and dialysed.

Conjugation of the Activated Enzyme

The activated enzyme was incubated with Ig fraction in 40mM carbonate buffer, pH 8.5. The reaction mixture was kept for 30 min at 25°C and then incubated in refrigerator at 4-5°C for completion of conjugation.

Gel Filtration using Sephadex G-75

The activated sample conjugated with IgY for 72h, was loaded onto a 25ml Sephadex G-75 column equilibrated with 25mM carbonate buffer, pH 8.6. The flow rate was adjusted to 012ml/min. Fractions were manually collected every 8min (\approx 0.96ml). Total of 45 fractions were collected and 25 μ l from each fraction was dispensed into micro-well plates and incubated with H₂O₂ (2mM) and Phenol/Aminoanipyrene mixture (85mM/1.25mM respectively) in 50mM buffer (phosphate buffer, pH 7) and incubated at ambient temperature (30°C) for 15-20 min. Fractions showing pink coloration were noted.

Analytical Methods

Enzyme assay

Appropriately diluted enzyme was incubated with H₂O₂ (2mM) and Phenol/Aminoanipyrene mixture (85mM/1.25mM respectively) in 50mM phosphate buffer, pH 7 and incubated at 37 \pm 0.5°C for 10min. The Absorbance was immediately read at 500nm.

One unit of activity is the amount of enzyme which produces one micromole of the quinoneimine dye (Molar extinction co-efficient=13600) in one minute under the defined conditions (Khuchareontaworn *et al*, 2010).

Estimation of proteins

Total protein in the enzyme/protein preparations were quantified by Biuret method.

Estimation of carbohydrate content: To 0.5ml of the sample to be analysed, 0.5ml of 5% phenol was added and mixed, followed by gentle addition of 2ml Sulphuric acid followed by incubation for 10min. The contents were mixed and absorbance was read at 490 min after 20min.

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 – 8% T, 3.33% C). The gel was removed, rinsed with D/W and equilibrated in 50mM PO₄ buffer, pH 6.4 for 10min, followed by immersion in Phenol/AAP mixture with 50mM H₂O₂ till pink bands were observed.

Denaturing PAGE

SDS PAGE was carried out in 12% gel. The gel was stained with 0.125% Coomassie Brilliant Blue R-250. MW markers in the range of 14–97.4 KDa were used for localization of heavy and light chains of Igs.

Characterization of the Enzymes

Effect of pH and temperature: Stability studies were performed at various pH ranging from 3.6-11. The enzyme was incubated in 25mM buffer at respective pH for 30min and then assayed. Effect of temperature on the stability was studied at pH 7 from 25-70°C. The enzyme was incubated at respective temperature for 1h and assayed for residual activity.

Determination of Km

The effect of varying concentrations of phenol, BCP and BTB was studied in presence of 3mM H₂O₂ (Shetty et al, 2012). Km was determined using Lineweaver-Burke (LB) plots.

Results and Discussion

Extract prepared from the bulb of *Brassica oleracea gongylodes* was assayed for peroxidase activity. The yield of the enzyme was found to be around 2.9±0.23 U/g. The extract was subjected to fractional ASS. Negligible activity of around 5% was recovered in the fraction salted out at 0-40% saturation. The fractions salted out at 40-60% and 60-80% comprised of 39% and 21% of activity respectively. Therefore, peroxidase salted out at 40-80% ASS was employed for further studies. The enzyme was concentrated 6-8 times with a recovery to an extent of 67.5% with a specific activity of 3.5 and an overall fold purity of 6.2.

Peroxidases of plant origin are glycosylated in general. Extensive work is been reported in literature on the glycosylation sites of HRP (Gazaryan et al, 1998; Veitch, 2003). The glycosyl residues of proteins are extensively exploited in immobilization and conjugation processes. PI is often employed to introduce oxidative cleavage in the carbohydrate moieties of glycoproteins. The hydroxyl groups on the adjacent carbon atoms are oxidized to aldehyde groups, facilitating fission of the intervening C-C bonds (Wong and Wong, 1992, Shetty and Jaffar, 2017). The aldehyde groups generated tends to interact with amino groups resulting in Schiff bond formation between the interacting proteins. Bonding via glycosyl residues is therefore, a frequently used approach in preparation of conjugates. Several other approaches are employed for enzyme-antibody conjugation. Linker agents such as N-succinimidyl 3-(2-pyridyldithio) pro-pionate, glutaraldehyde, meta-maleimidobenzoyl-N-hydroxysuccinimide ester etc. have found applications in conjugation procedures. (O'Sullivan et al, 1978; Nygren & Hansson, 1981; Jeanson et al, 1988; Ray et al, 2012, Ramesh Kumar et al, 2014). The enzyme salted out at 40-80% ASS was treated with 5, 10 and 15 mM PI and the glycosyl content was estimated. The carbohydrate content in mg/100mg of protein in the salted out fraction was 21.5±1.8 and, in the dialysates subjected to 5, 10 and 15mM PI treatment, it was around 13.4, 12.4 and 11.3 respectively accounting for around 37.5-47.4% deglycosylation. The sample treated at 10mM concentration was loaded onto non

denaturing PAGE. As the migration rate of the enzyme was very low in poly acrylamide gel at 10% T, electrophoresis was carried out at 8% T for localization of activity. ASS fraction showed two bands of activity, while in the PI treated sample, both these bands were found to move as diffused patches of activity which moved faster than their respective native forms (Fig. 1A). For conjugation, Igs from chicken eggs, human plasma and goat serum were harvested. As presented in Fig. 2, precipitation with sodium sulfate as per the method of Howe (1921) was able to remove the highly abundant albumin fraction efficiently from human plasma (Howe, 1921). The IgHu fraction showed major bands at around 50-55 and 25 KDa corresponding to the molecular masses of heavy and light chains respectively. Molecular masses of the bands representing the heavy chain of IgY was found to be around 66 KDa and of light chain fraction was around 24-27KDa. An intermediate band with the mass in the range of 35-40 KDa was also observed. Goldring and Coetzer (2003) and Pauly et al (2011) have reported similar results with IgY fraction. This fraction reportedly interacted with the anti IgY antibodies proving it to be comprising of perhaps incompletely processed IgY chains. Combination of ammonium sulfate and sodium chloride was shown to precipitate globulin efficiently (Levin et al, 1950). Fractionation of goat serum using ammonium sulfate and NaCl mixture likewise showed presence of heavy and light chains exhibiting molecular masses of around 50 and 25 KDa respectively. Thus, all the three Ig fractions showed major bands representing Ig chains.

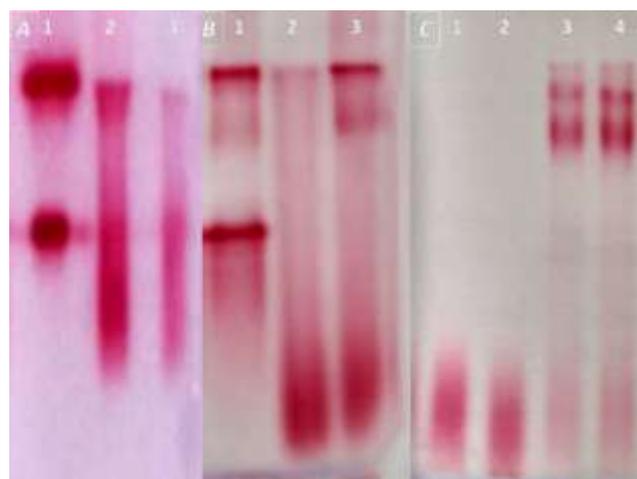


Fig. 1: Activation of the enzyme and conjugation

[A: Lanes 1, 2 & 3- Salted out enzyme, Enzyme treated with 10mM PI, PI treated enzyme incubated with IgHu for 72h respectively;
 B: Lanes 1, 2 & 3- Salted out enzyme, enzyme activated by 0.1% FDNB and 10mM PI followed by incubation with IgHu for 0h and 72h respectively;
 C: Lanes 1, 2, 3 & 4: Activated enzyme incubated with IgY for 0h, IgGo for 0h, IgY for 120h and IgGo for 120h respectively.]

Peroxidase treated with 10mM PI was incubated with IgHu fraction (protein concentration of treated extract to that of Ig fraction was maintained at 1:2) for 3 days. As seen in Fig. 1, absence of an additional band of activity indicated that

the PI treated enzyme was not able to conjugate effectively with the Igs. One of the major concern in using periodate as a tool in conjugation experiments is that the aldehyde groups generated may interact with the amino groups of the basic amino acids in the molecule during PI treatment process itself. Pretreatment of the glycoprotein with FDNB alleviates the problem by interacting with the amino groups of the basic amino acids. The enzyme was therefore treated with 0.1% FDNB followed by oxidation with 10mM PI. In the PI treated sample not exposed to FDNB, both the the activity bands moved faster than their respective native forms, the migration was slower in comparison to FDNB treated PI oxidized enzyme. As shown in Fig. 1B, FDNB treatment followed by oxidation by PI resulted in disappearance of both the native bands of activity and appearance of a patch of activity which moved at a much faster rate indicating decreased mass consequent to PI treatment in addition to possibility of reduction the positive charges on the molecule in response to FDNB treatment. Thus, PI treatment without prior treatment with FDNB appears to be less efficient in conjugating with IgY, while the enzyme pretreated with FDNB showed appearance of a new activity band. Appearance of this band with a concurrent decrease in the intensity of the treated (activated) form as seen in the electrophoregram indicates conjugation (Fig. 1B). The activated enzyme could also conjugate to both IgY and IgGo (Fig. 1C). Thus, treatment with FDNB was carried out prior to activation with PI in subsequent protocols. One egg yolk yielded around 72-76 mg of protein in the Ig fraction. Easy availability of egg yolk and abundance of Ig in the yolk prompted us to employ IgY for optimizing the conjugation protocol.

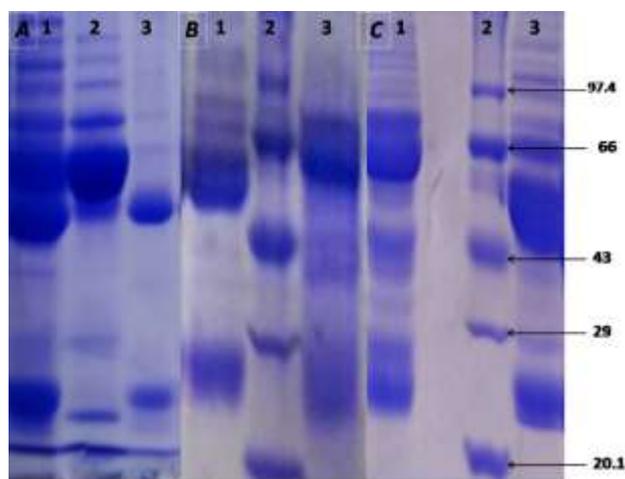


Fig. 2: Denaturing PAGE of Ig fractions

[A: Lanes 1, 2 & 3- Human serum, sodium sulfate filtrate (albumin fraction) and residue containing IgHu (globulin fraction) respectively;
 B: Lanes 1, 2 & 3- IgHu, molecular weight markers (20.1-97.4 kDa) and IgY fraction obtained by PEG precipitation respectively;
 C: Lanes 1, 2 & 3- IgY fraction, molecular weight markers and Ig fraction from goat serum respectively.]

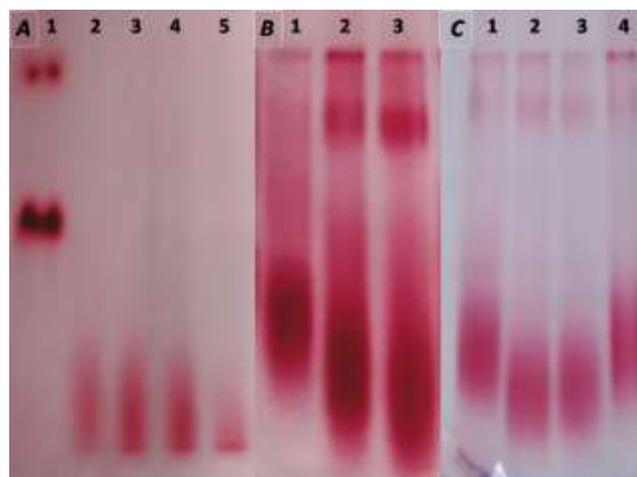


Fig. 3: Optimization of activation protocol

[A: Lane 1- Salted out fraction, Lanes 2, 3& 4- Enzyme treated with 0.1% FDNB followed by 9, 12, 15 and 22.5mM PI respectively. s B and C: Conjugated enzymes

B: Lanes 1, 2 & 3- treated with 0.1% FDNB and oxidation with 5, 10 and 15 mM PI respectively.

C: Lanes 1, 2 & 3- Treatment with 0.05%, 0.08% and 0.12% FDNB and activated with 12mM PI. Lane 4: treated with 0.08% FDNB and 9mM PI.]

Oxidation of the enzyme with PI may affect the activity and/ stability of the enzyme. Therefore, it is essential that the PI treatment protocol be optimized. Activation with increasing concentrations of PI showed that, at 22.5 mM of PI, the enzyme activity was affected to a significant extent (Fig. 3A). The enzyme was therefore, treated at concentrations ranging from 5 to 15mM of PI and incubated with IgY (two times protein concentration) for 72h. The enzyme oxidized with 10 and 15mM of PI conjugated successfully to IgY (Fig. 3B). FDNB treatment at 0.08 and 0.12% followed by activation with 12mM PI showed initiation of conjugation (Fig. 3C). The activated enzyme was incubated with IgY for various time periods in refrigerator. Conjugation appeared to increase with increasing time of incubation. Incubation with 3-4 times IgY (protein concentration) was found to give satisfactory conjugation at 144h of incubation (Fig. 4A & 4B). Pre-treatment with 0.08% FDNB followed by PI treatment at 12.5mM was finalized for activation of the enzyme. The enzyme lost 18% of activity consequent to FDNB and PI treatment. Further loss in activity occurred upon conjugation. Overall, the enzyme retained 54% of its activity after conjugation. Enzyme incubated with IgY for 96h was loaded onto Sephadex G-75 column. Elution of activity began in the 6th fraction and continued till the 9th fraction and trailed beyond till 10th fraction. 8th fraction showed intense pink coloration. Activity was also found to elute in the fractions towards the end of bed volume initiating in the 21st fraction which continued till the 27th fraction. Fraction numbers from 6-8, 9-12, 21-23 and 24-27 were pooled and concentrated by dialysis against sucrose in dialysis bag. The concentrates were loaded onto native PAGE and electrophoresed. Sephadex G-75 has a fractionation range of 4- 80KDa for globular proteins. As seen in Fig. 4C, the void volume which contains high molecular mass proteins (>80 KDa) migrated slowly

corresponding to the position of conjugated band. Peroxidases are relatively small molecular weight proteins known to possess molecular weights in the approximate range of 35-45 KDa (Agostini et al, 2002; Thongsook and Barrett, 2005; Reeteshkumar et al, 2011). The enzyme is likely to have reduced mass subsequent to PI treatment due to deglycosylation. As seen in Fig. 4C, activated band was found to elute towards the end of the bed volume indicating its small size. Low molecular weight of the activated enzyme and increased molecular mass subsequent to incubation with IgY, implies conjugation. HRP treated with FDNB and PI was subsequently conjugated to the three Ig fractions. Activated HRP was also found to move faster when subjected to native PAGE. Conjugation of the activated HRP to all the three Ig fractions could be achieved successfully as shown in Fig. 5.

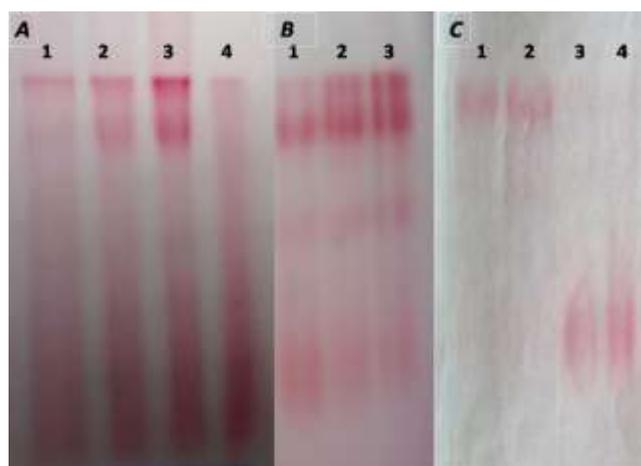


Fig. 4: Optimization of conjugation and, gel filtration of Conjugated sample

[A: Lanes 1, 2, 3 and 4- Activated enzyme incubated with IgY (2x protein concentration) for 24h, 48h, 96h and 0h respectively;
 B: Activated enzyme incubated with varying concentrations of IgY for 144h. Lanes 1, 2 and 3- Ratio of protein concentration in enzyme extract and IgY - 1:2, 1:3 and 1:4 respectively;
 C: Gel filtration on Sephadex G-75: Lane 1 & 2- Pooled concentrates of void volume V1 and V2; Lanes 3&4- Pooled concentrates B1 and B2 eluted at bed volume.]

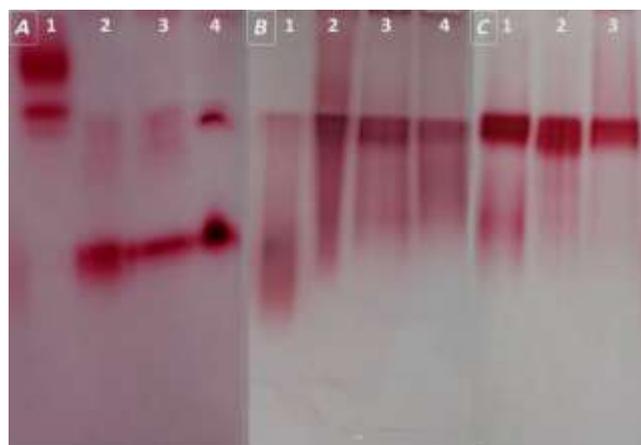


Fig. 5: Conjugation of Horseradish peroxidase in Ig fractions

[Lanes 1, 2, 3 & 4- HRP native form, HRP activated with FDNB and PI, Activated HRP conjugated with IgY for 0h, 24h, 48h respectively; B: Lanes 1, 2, 3 & 4: Activated HRP, Activated HRP conjugated with IgGo, IgHu and IgY for 96h respectively.]

For use of an enzyme as a commercial tool it is essential that the stability of molecule is not reduced due to chemical modifications. Activated peroxidase preparations conjugated with the Ig fractions for 7 days were subjected to characterization (Table 1). The native and the activated enzymes were stable in the range of pH from 5-7.2 and 5-8 respectively. The conjugated enzyme preparation was stable over a broad range of pH from 5-9.2. The native and the activated forms lost around 35-40% of activity at the end of 1h incubation period at 55°C. Interestingly, the conjugated enzyme was able to retain 94% of its activity for 1h at 55°C. Thus both temperature and pH stability of the enzyme improved upon conjugation (Table 1). The affinity of the enzymes for phenol was assessed. The native, activated and IgY conjugated forms exhibited Km of 0.35-0.39mM for phenol. IgHu and IgGo conjugates also exhibited Km in the range of 0.33-0.39mM for phenol. The Affinity of native, activated and the enzyme conjugate with IgY fraction was also studied for the dyes BCP and BTB, as both these dyes are known to be oxidized to decolorized forms by peroxidases. For both the dyes, Km values were calculated to be in the range around 0.5-0.6 mM. HRP and its IgY conjugate exhibited Km of around 5-6mM for these dyes. The native form of HRP, its activated form and all the three conjugates exhibited Km in the range of 4.5-5 mM for phenol as substrate. Km values were not significantly affected due to activation and conjugation. The peroxidase from *Brassica oleracea gongylodes* showed strong affinity for phenol and dyes in comparison to the HRP conjugates. The consumption of the organic substrates in enzyme assay can therefore be limited by using peroxidase elaborated by *Brassica oleracea gongylodes* enzyme labels in immunoassay probes. All the three conjugated fractions were found to retain 95% of their activity even after 2 months, when stored at -20°C.

Table 1: Characterization of peroxidase from *Brassica oleracea gongylodes* conjugated to IgY fraction

Parameters	Native form	Activated form	IgY conjugate
Temperature stability (100% retention of activity for 1h)	40°C	40°C	50°C
The pH stability	5-7.2	5-8	5-9.2
Km, mM			
Phenol	0.36±0.12	0.39±0.38	0.35±0.41
Bromocresol purple	0.5±0.35	0.54±0.61	0.61±0.29
Bromothymol blue	0.54±0.44	0.53±0.17	0.49±0.5

Conclusion

Isoforms of peroxidases have been observed in many plants expressing peroxidases. In our previous study, we had observed a major band of activity (slow moving in electrophoretic field). In the present study, we found two isoforms of peroxidase in the extract. Both the bands appear to respond to the activation treatment and the resulting

activity moved as a single patch of activity when subjected to native PAGE. On incubation with Ig fractions, the activated enzyme was found to conjugate successfully. The pH and temperature stability of the conjugates improved upon conjugation. The conjugated forms exhibited lower Km for phenol and the two dyes tested, in comparison to HRP conjugates. It is likely that the enzyme conjugated to purified Igs will retain their high affinity for the substrates while functioning as components of the immunoassays. Consequently, it may be feasible to perform the assays at 8-10 folds lower concentrations of these substrates. Many of the peroxidase substrates being hazardous, the study offers an environmentally safer option.

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