Isolation of Peroxidase Enzyme from Various Vegetables and Coupling to IgG

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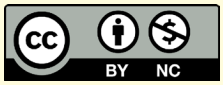

ABSTRACT

Coupling of IgG with peroxidase enzyme finds various applications in determining the antigen-antibody interactions, in diagnostics or for research purpose. In the present study, Peroxidase enzyme was isolated from various vegetables like tomato (*Solanum lycopersicum),* cabbage *(Brassica oleracea)* and Radish *(Raphanus sativus) a*nd partial purification of enzymes was done with ammonium sulphate fractionation to 80% saturation. The active peroxidase enzyme was coupled to Goat anti-human IgG and the efficiency of coupling procedure was tested against Protein A sepharose purified human IgG. The coupling procedure was done by using sodium periodate and coupling was confirmed by dot blot and ELISA procedures. The present study provides an indigenous method to isolate an active peroxidase enzyme and couple to various IgG and use in immuno detection methods at very cost effective manner.

Key words: Antibody coupling, Dot Blot, ELISA, Human IgG, Periodate, Peroxidase

1. Introduction

Peroxidases (EC.1.11.1.7) are heme-containing enzymes belonging to oxidoreductases. They are hydrogen peroxide decomposing enzymes associated with oxidation of the broad range of phenolic and non-phenolic substrates. Plant peroxidases have an essential physiological role in the growth and development of plant throughout its life cycle. Due to the versatility of peroxidases during reaction and their ubiquitous nature, they have potential applications in various immunological, medicinal, biotechnological, and industrial sectors (Pandey *et al.* 2017; Pandey & Dwivedi 2015; de Oliveira 2021). Plant peroxidases from various sources such as Ficus, lettuce, citrus, broccoli, royal palm, soybean, papaya, wheat grass, Solanum melongena, lemon etc. have been isolated, purified and characterized (Pandey *et al.* 2017. Chandrasekaran *et al.* 2014). Peroxidase enzyme isolated from various sources have been used in immunoassays, diagnostic test kits, wastewater treatment and soil remediation (Chukwudi *et al.* 2021). Immobilization of peroxidase enzyme has gained much attention in the construction of biosensors due to economical and viability in enzyme immunoassays (Shivakumar *et al.* 2017). The proficiency

of peroxidases to yield chromogenic products at low concentrations makes them compatible enzymes for the preparation of enzyme-linked immunosorbent assay (ELISA) kits, which are used in the diagnosis of several diseases (Singh 2019). Due to the peroxidase ability to yield chromogenic products at low concentrations and its relatively good stability, it is well-suited for the preparation of enzyme conjugated antibodies and application in diagnostic kits and as well peroxidases are widely used in clinical biochemistry and enzyme immunoassays (Krell 1991; Vamos-Vigyazo 1981; Lin 1996). Among all peroxidases isolated, horse radish peroxidases are most generally used for the analytical purposes (Idesa & Getachew 2018). However, other plant peroxidases having extensive pH and temperature stability are emerging as choice for HRP (Tijseen 1985).

Human Immunoglobulin G (IgG) can be purified from different human serum samples by Protein A sepharose column chromatography (Klaus Huse 2002). Conjugation of IgG – Peroxidase enzyme involves formation of stable, covalent linkages between the enzyme and antigen-specific monoclonal or polyclonal antibody (IgG). This involves various steps to produce the IgG-Peroxidase conjugate. The coupling reaction which produces a conjugate that resembles the native protein include, Sodium periodate oxidation method, Maleimide-sulfhydryl method, Cyanuric chloride methods (Weinryb 1968; Nakane & Kawaoi 1974; Jackson *et al.* 1987; Hosoda *et al.* 1985; Presentini & Terrana 1995).

2. Materials & Methods

Protein-A sepharose affinity column (2 mL) kit, Human IgG, Goat anti-human IgG, Goat anti-human IgG- HRP were purchased from Bangalore Genei, India. All other chemicals were from Qualigens, India.

2.1. Isolation and characterization of peroxidase from vegetable sources

2.1.1. Sample collection and preparation of enzyme extract

Vegetables (Tomato, Cabbage, Radish) used for this study were intentionally selected and purchased from local market. After the preliminary procedures like washing, peeling and slicing, 50 g of each vegetable slices were crushed with pestle and mortar to obtain homogenous sample. 200 mL of distilled water was added separately to all the crushed substances and homogenized in a mechanical blender for 15 minutes and centrifuged at 6000 rpm for 15 minutes in a high speed cooling centrifuge. The supernatant was filtered through cheese cloth and the sediment was discarded. To selectively inactivate the contaminating traces of the catalase moieties, the supernatant was heated to 65 °C for 3 minutes in a water bath and cooled immediately by placing on ice bath for 30 minutes. After thermal inactivation, the samples containing crude enzyme were preserved at -20 °C until further use.

2.2. Partial purification of peroxidase enzyme by ammonium sulphate fractionation

The crude enzyme preparation was subjected to ammonium sulphate fractionation to 80% saturation under constant ice cold conditions. The precipitated protein was collected by centrifugation at 12000 rpm for 30 minutes. The precipitate was dissolved in small volume of 100 mM potassium phosphate buffer, pH 6 and dialyzed against same buffer for overnight under low temperature. The dialyzed fractions were analysed for protein content by Lowry's method (Lowry *et al.* 1955).

2.3. Peroxidase enzyme assay

To a clean, glass calorimeter tube, the following solutions were added one after other and as follows-1.7 mL of glass distilled water, 0.32 mL of 0.1 M potassium phosphate buffer (pH 6.0), 0.16 ml of 0.50% (v/v) hydrogen peroxide solution and 0.32 mL of 5% (w/v) pyrogallol solution. The reaction mixture was mixed by inversion and equilibrated at 20 °C in an incubator for 10 minutes. Suitable aliquots (0.4 mL) of enzyme solution was added to the reaction mixture and the solution was mixed thoroughly by inversion and the increase in A_{420} was recorded for 3 minutes. The $\Delta A_{420/30\ secs}$ were obtained using the maximum linear rate for all the tests and blanks (Shannon *et al*. 1966).

The Pyrogallol which was used as the substrate in the assay when combined with hydrogen peroxide gets converted to purpurogallin in the presence of peroxidase. The chromogenic reaction development of the enzyme activity was determined calorimetrically.

Unit – One unit of peroxidase will form 1 mg of purpurogallin from pyrogallol in 30 seconds at pH 6 at 20 °C.

$$\text{Units/ml enzyme} = \frac{(A_{420/30sec}\ \text{Test sample} - A_{420/30sec}\ \text{Blank})\ (3)\ (df)}{(12)\ (0.4)}$$

Where

- df = Dilution factor (4)
- 3 = Volume (in mL) of assay
- 12 = Extinction coefficient of 1mg/ml of purpurogallin at 420 nm.
- 0.4 = Volume (in mL) of enzyme used.

2.4. Effect of temperature on peroxidase:

To study the effect of temperature on peroxidase enzyme, the assay conditions were maintained under the study as described earlier, except that the temperatures were altered. The various temperatures used in the study were 20 °C, 40 °C, 60 °C and 80 °C.

2.5. Effect of pH on peroxidase

To study the effect of pH on peroxidase, the assay conditions were maintained under the study as described earlier, except that the pH of the buffer was altered. The phosphate buffer of 100 mM with various pH (pH-3,4,5,6,7,8) was used for the assay and temperature was maintained at 20 °C for all the tubes.

2.6. Effect of substrate on peroxidase

To study the effect of substrate on peroxidase, the assay conditions were maintained under the study as described earlier, except that the substrate concentration was altered. The various pyrogallol substrate concentrations used were (0.1%,0.2%,0.3%,0.4%,0.5%) at 20 °C and 100 mM potassium phosphate buffer, pH 6.0.

2.7. Preparation of Human serum

Human blood samples were procured from students of age group (21-22 years) with prior consent after explaining them for the reason to be used in the research project.

After collecting the whole blood, the blood was allowed to get clotted by leaving undisturbed at room temperature for 30 min-1 hour. Clots were removed by centrifuging at 3000 rpm for 10 minutes in a clinical centrifuge. The resulting serum (supernatant) was transferred into clean Eppendorf tubes and stored at –20 °C until further use.

2.8. Purification of IgG from Human serum by Protein A-Sepharose Column

Protein-A affinity Sepharose column Kit was procured from GeNei, Bangalore, India. The column was equilibrated with 10 bed volumes of 1X equilibration buffer and it was allowed to drain through the column. Serum was diluted with (1:10) in 1X Equilibration Buffer. Diluted Serum samples were loaded to the equilibrated Protein A Column. The column was washed with 25 bed volumes of 1X Equilibration Buffer and eluted with 5 bed volumes of 1X elution buffer as 1 ml fractions into eppendorf tubes, each tube containing 25 µl of neutralizing buffer. The absorbance of the eluted fractions was measured at 280 nm in a UV-VIS spectrophotometer. Graph was plotted with fraction number versus absorbance values. Fractions with highest absorbance were pooled and protein content was determined by Lowry's method (Lowry *et al.* 1955).

2.9. Determination of Purity of IgG by SDS-PAGE

Purity of IgG was checked on SDS-PAGE (Lamelli 1970) by mixing the fractions having highest absorbance with 2 X sample buffer and boiled for 5 minutes on a boiling water bath. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 (Brunelle Julie and Green Rachel 2014) and by Silver staining method Chevallet *et al*. (2006).

2.10. Molecular weight determination

The R_f values were calculated for the standard proteins and for heavy and light chains of IgG and molecular weight was determined by plotting the graph with log (MW) as a function of R_f. The equation $y = mx + b$ was used to determine the MW of the unknown proteins.

2.11. Confirmation of IgG by Western Blot & Dot blot

The purified IgG from Protein A Sepharose affinity column was confirmed by western blotting following the method developed by Towbin *et al.* (1979). After blotting onto the nitrocellulose membrane by electro transfer method, the membrane was blocked with 5% casein and finally incubated with commercial Goat anti-human IgG – HRP (1:100 dilution) and bands were developed by staining TMB/H_2O_2 substrate

For dot blot procedure, 20 µl of purified human IgG (1 mg/mL) was spotted on nitrocellulose membrane strips along with controls. The membranes were air dried and incubated with goat anti-human IgG – HRP (1:500 dilution) solution for 1 hour. Substrate (TMB/H_2O_2) was added to all the membranes and the spots were developed (Faoro *et al.* 2011; Oprandy *et al.* 1988).

2.12. Coupling of IgG with Peroxidase enzyme

Conjugation of Goat anti-human IgG with purified peroxidase was performed by periodate method developed by Pavliuchenko *et al.* with some modifications (Pavliuchenko *et al.* 2019). Freshly prepared 300 µl of 0.1 M Sodium periodate was added to 1500 µl of Peroxidase enzyme (1 mg/mL). The mixture was stirred gently on shaker for 20 minutes at room temperature in dark. 500 µl of 1 mM Sodium acetate buffer, pH 4.4 was added to the above mixture and pH was adjusted to 9.5 by adding 0.2 M Sodium carbonate buffer, pH 9.5. Goat anti-human IgG solution (250 µl) was added to the activated peroxidase solution. Mixture was stirred gently on shaker for 2 hrs at room temperature. 150 µl of freshly prepared 0.1 M Sodium borohydride solution was added to peroxidase - IgG mixture. The solution was incubated with Sodium borohydride for 90 mins at room temperature with gentle shaking. The sample was dialyzed against 1 X PBS (pH 7.2) under cold conditions for 7-8 hours. The goat anti-human IgG –peroxidase conjugate collected after dialysis was subjected to confirmatory tests by dot blot and ELISA tests.

2.13. Confirmation of IgG-peroxidase conjugation: In order to determine the efficiency of coupling, the following procedures are were used

2.13.1. Dot blot

Purified human IgG samples of 20 µl were spotted on nitrocellulose membrane strips. After air drying the membranes for 5 minutes, the membranes were incubated with freshly coupled goat anti-human IgG-peroxidase for 1 hour. The blots were developed with TMB/H_2O_2 and observed for the coloured spots. In the control spot, the IgG was incubated with commercial goat anti-human IgG-HRP and the blank spot was incubated with distilled water.

2.13.2. ELISA

Sandwitch ELISA kit from GeNei, Bangalore was used for all the buffers to use in the current procedure. Test wells were loaded with 200 µl of purified human IgG which was mixed with coating buffer in 1:2 ratio along with control wells having the same antigen. The plate was incubated for 1 hr at 37 °C in a temperature regulated incubator. 200 µl of blocking buffer was added to all the wells and further incubated at room temperature for 1 hr. The wells were rinsed for 3 times with distilled water and the water was discarded completely. 200 µl of freshly coupled goat anti-human IgG-peroxidase conjugate was added to all test wells and 200 µl of commercial goat anti-human IgG-HRP conjugate was added to all control wells. The plates were incubated at room temperature for 1 hr. Unbound antibodies were discarded by plate inversion and wells were rinsed for 3 times with 1X wash buffer. 200 µl of Substrate TMB/H_2O_2 was added to all wells, test and control and incubated for 10 mins at room temperature for colour development. Stop solution of 100 µl was added to all the wells. The contents of each well were transferred completely to individual test tubes containing 2 mL of 1X stop solution. The substrate blank was prepared by adding 200 µl of 1X TMB/H_2O_2 to 2.1 mL of 1X stop solution. Absorbance was read at 450 nm in a UV-VIS spectrophotometer (Voller *et al.* 1978). The experiment was repeated twice to confirm the results.

3. Results

3.1. Protein content and enzyme activity

The peroxidase enzyme isolated from various vegetable sources of Tomato, Cabbage and Radish have shown good protein yield after ammonium sulphate fractionation and exhibited high enzyme activity with substrate pyrogallol. The protein fraction containing peroxidase enzyme isolated from Tomato is having protein concentration of 10 mg/mL as determined by Lowry's method and enzyme activity of 2.275 U/ml. The peroxidise isolated from Cabbage has shown 20 mg/mL protein and 2.675 U/mL enzyme activity, whereas from Raddish it was found as 8 mg/mL protein and 2.125 U/mL enzyme activity respectively. The intensity of the yellow-orange colour obtained during the assay reaction further matches with the concentration of enzyme in the preparations.

3.2. Effect of temperature on enzyme activity

The optimum temperature for enzyme extracted from sources cabbage and tomato is at 60 °C whereas radish has showed burst of activity at 22 ^{0}C and again at 60 ^{0}C showed varied nature of enzyme (Fig. 1)

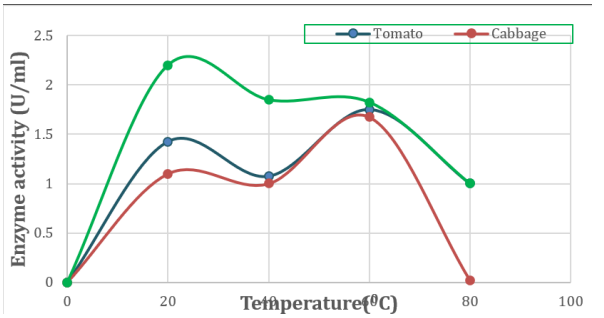

Fig. 1: Effect of temperature on Peroxidase activity

3.3. Effect of pH on enzyme activity

All the enzymes from sources tomato, cabbage and radish have shown broad range activity from pH 4-8. Radish has shown consistently high activity compared to other two (Fig. 2).

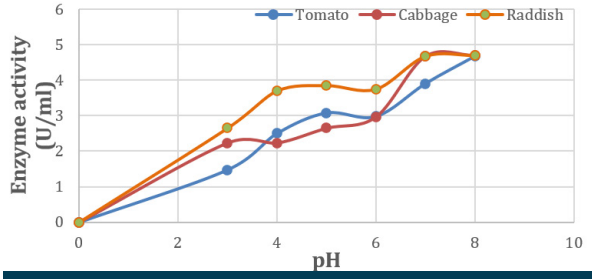

Fig. 2: Effect of pH on Peroxidase activity

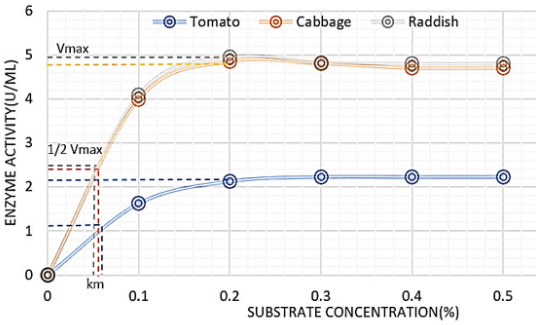

Fig. 3: Effect of substrate concentration on peroxidase activity

3.4. Effect of substrate concentration on enzyme activity

To study the effect of substrate on peroxidase, the assay conditions were maintained under the study as described earlier, except that the substrate concentration was altered. The various pyrogallol substrate concentrations used were (0.1%,0.2%,0.3%,0.4%,0.5%) at 20 °C and 100 mM potassium phosphate buffer, pH 6.0. V_{max} and K_M values were determined for all the enzymes and the values were found to be for Tomato 2.225 U/ml and 0.7 mg/ml; for Cabbage 4.85 U/mL and 0.6 mg/mL; for Radish 4.95 U/mL and 0.55 mg/mL respectively of V_{max} and K_M .

3.5. Purification of IgG from human serum by Protein A Column Chromatography

IgG from three different human serum samples were purified on protein-A sepharose column. The peak fraction number was different amongst all three samples as indicated in Fig. 4. The reason would be due to different affinities of IgG with protein A amongst different samples.

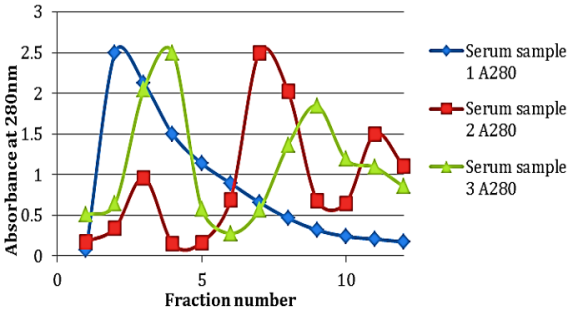

Fig. 4: Elution profile of IgG from serum samples on Protein A-Sepharaose column

3.6.SDS-PAGE

The purity of the IgG was checked on 12% gels under denaturing conditions by SDS-PAGE method and bands were visualized by both Coomassie Brilliant Blue R250 staining and silver staining procedures (Fig.s. 5 and 6). The heavy and light chains were appeared as pure bands. Little high molecular weight bands were visualized in silver stained gels, indicating aggregates of IgG.

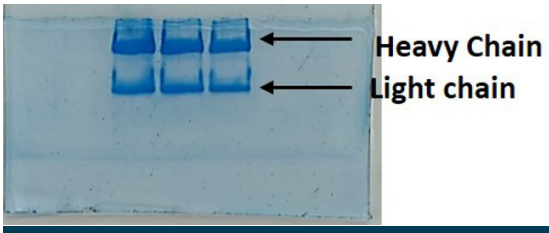

Fig. 5: Analysis of IgG by SDS-PAGE

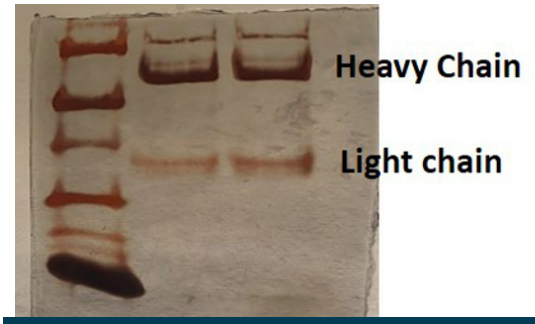

Fig. 6: Analysis of IgG by silver staining

3.7. Molecular weight determination

The molecular weight of heavy and light chains was determined and found to be 50 kDa and 23.5 kDa respectively (Fig. 7) and matching with the standard reports. R_f = migration distance of the protein

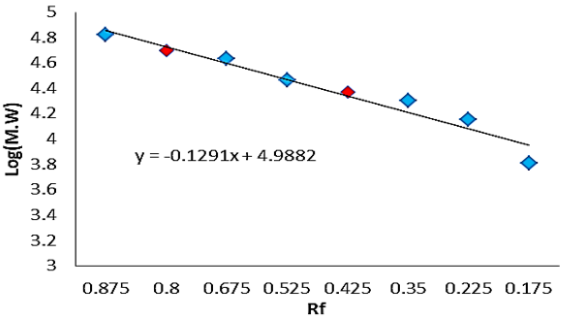

Fig. 7: Molecular Weight Determination by SDS-PAGE

3.8. Western blot Analysis of IgG

The purified human IgG was confirmed by western blot technique and found it was cross-reactive with commercial Goat anti- human IgG-HRP. Both heavy chain and light chain reacted with the antibodies and stained with substrate TMB/H_2O_2 (Fig. 8).

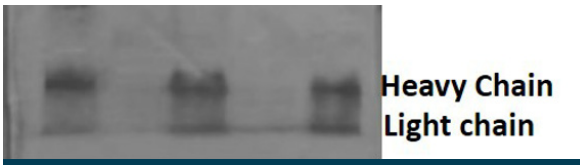

Fig. 8: Analysis of IgG by Western Blot

3.9. Dot blot

The isolated IgG was further subjected to dot blot analysis by using commercial Goat anti- human IgG- HRP antibodies and blue dots observed on the nitrocellulose membrane, further confirming the isolated protein was human IgG. Control antigen used was commercial human IgG from GeNei. Bangalore (Fig. 9).

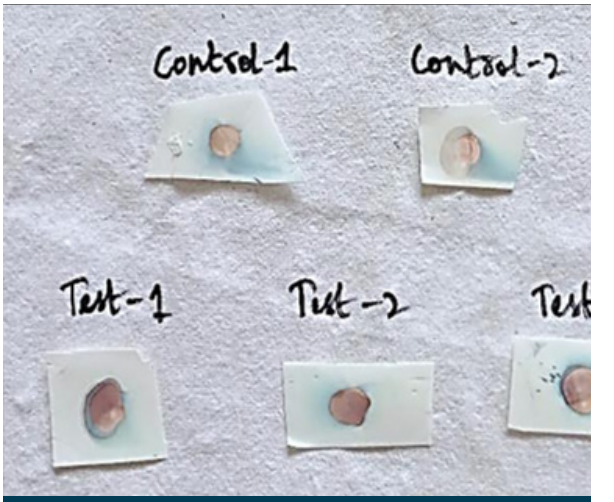

Fig. 9: Dot blot analysis of IgG

3.10. Confirmation of coupling of peroxidase to Goat anti -human IgG

3.10.1. Dot blot

The coupling efficiency of goat anti-human IgG-peroxidase was tested against purified human IgG by dot blot method and results clearly indicated the coupling procedure was efficient by the appearance of blue spots on nitrocellulose membrane (Fig. 10).

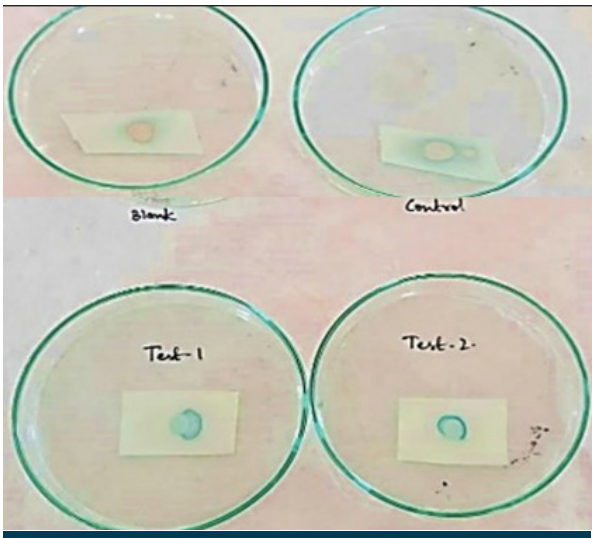

Fig. 10: Analysis of coupling reaction by Dot blot

3.10.2. ELISA

Another confirmatory test for the coupling procedure was by ELISA method. The ELISA plate wells were coated with antigen purified human IgG and incubated with coupled Goat anti- human IgG- peroxidase conjugate. Control also was run with commercial antibodies of Goat anti human IgG- HRP. The coloured wells of the test (B well) indicated comparatively high color intensity and clearly confirms efficient coupling between Goat anti-human IgG and peroxidase enzyme from vegetable sources (Fig. 11).

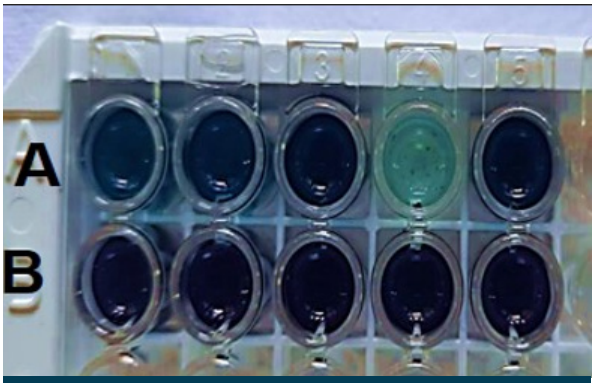

Fig. 11: Analysis of coupling reaction by ELISA

4. Discussion

Peroxidase enzyme has great ability to produce stable chromogenic product and is a suitable enzyme in various diagnostic kits based on enzyme-conjugated antibody technology (Pandey *et al*. 2017; Shivakumar *et al.* 2017). The present study is directed to prepare Peroxidase –IgG conjugate with high thermal stability, strong binding affinity for the substrate compared with high-cost commercial HRP-conjugate. Extraction and partial purification of peroxidase enzyme from tomato, cabbage and radish was done very cost-effectively with simple laboratory procedures. Thermal stability of the enzymes was observed as very high as the enzymes have shown good activity starting from temperature 40 ^{0}C and continued till 80 ^{0}C. This indicates that the enzymes are suitable for storage for long time usage. These results are similar to the reported ones of litchi peroxidase and asparagus (Mizobutsi *et al.* 2010; Rodrigo *et al.* 1996) suggesting that extensive heat treatments are needed to inactivate the peroxidases and suitable for coupling procedures.

Though several methods were reported for the purification of IgG from serum, a simple, one-step procedure by affinity chromatographic method has shown as a highly suitable and efficient method (Mariam *et al.* 2015). Also, large yield of affinity-purified IgG was reported from protein A Sepharose column (Blanc *et al*. 2009). During coupling process, the periodate solution provided a slightly acidic medium that prevents the self-coupling of peroxidase (Beyzavi *et al.* 1987). Conjugates of radish peroxidase with commercial goat anti-human polyclonal antibodies were produced by a modified process of the previously described periodate oxidation method [26]. Functionality of prepared conjugates was also tested by dot blot and ELISA methods and the results were satisfactory.

5. Conclusions

The present study explained the coupling procedure of Peroxidase enzyme, isolated from Tomato (*Solanum lycopersicum),* Cabbage *(Brassica oleracea)* and Radish *(Raphanus sativus)* with Goat anti-human IgG. The coupling method involved chemical reagents of sodium periodate and Sodium borohydride. The efficiency of coupling procedure was tested by dot blot and ELISA procedures using human IgG, purified by using Protein A-Sepharose affinity column. The present study provides an indigenous method to isolate an active peroxidase enzyme from various natural sources

and couple to IgG and use in immuno detection methods at very cost effective manner.

Competing Interests

None

Acknowledgments

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