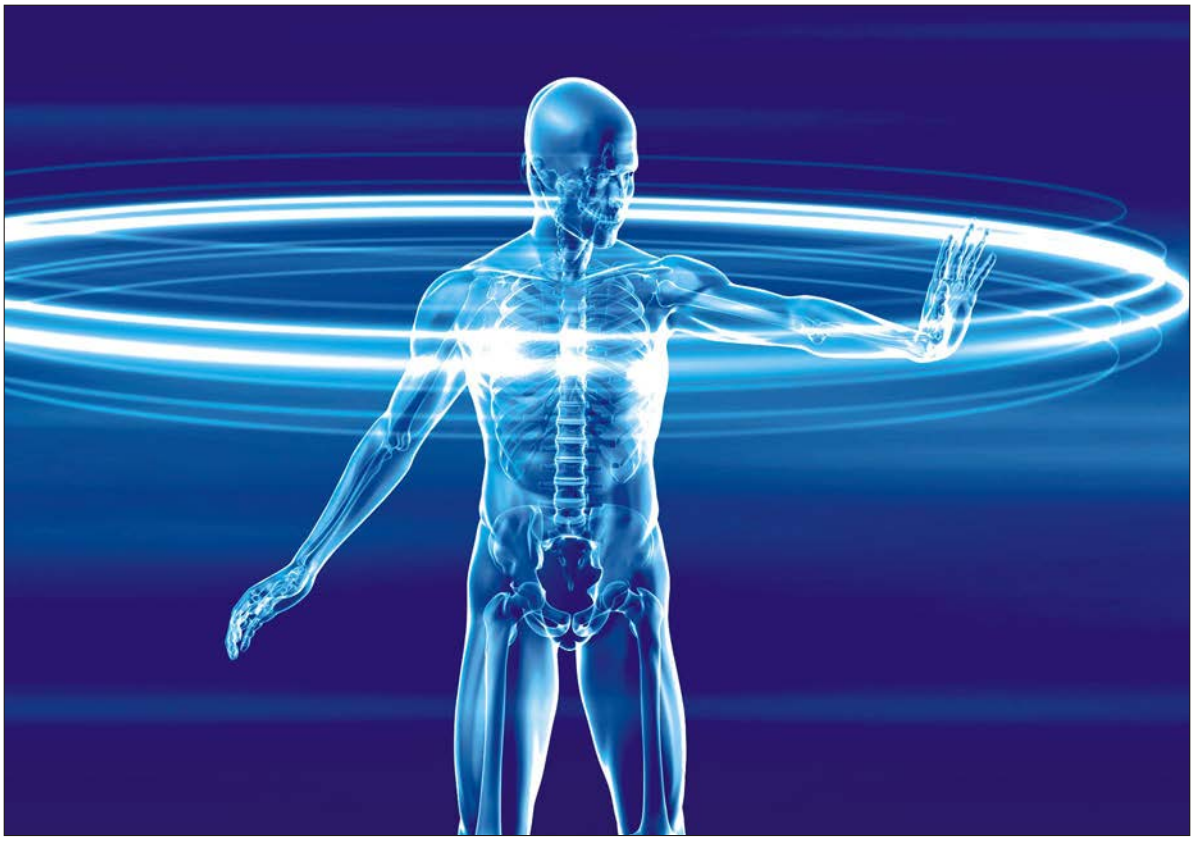

Research Journal of Immunology

ISSN 1994-7909

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Research Journal of Immunology 5 (1): 1-16, 2012
ISSN 1994-7909 / DOI: 10.3923/rji.2012.1.16

Development of a Simple Method for a New Immunoconjugate Utilizing Laccase

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ABSTRACT

Enzyme immunoassays utilize immunoglobulins conjugated to different enzyme labels or marker enzymes. In the current study, an immunoconjugate is being developed utilising the fungal enzyme laccase. The efficacy of this newly developed immunolaccase conjugate, prepared by standardized conjugation methods (glutaraldehyde and periodate based), has been demonstrated in indirect enzyme-immunoassay, for detection of purified rabbit IgG. The results of the assay indicated this newly developed immunolaccase conjugate to be potent for the detection of purified rabbit IgG when compared with commercially available peroxidase conjugated immunoglobulin or immunoperoxidase conjugate.

Key words: EIA, ELISA, enzyme antibody conjugate, immunoglobulin

INTRODUCTION

Enzyme immunoassay (EIA) or more specifically Enzyme Linked Immunosorbent Assay (ELISA) procedures are widely used for detection of many antigens and antibodies (Engvall and Perlmann, 1971; Suzuki *et al*., 2009). These assay systems are quite similar to Radio immunoassay (RIA) procedures but the difference is that the detector antibody is labeled with an enzyme instead of an heavy isotope. Almost any antigen can be detected by the application of ELISA. The two main methods, forming basis to all ELISA procedures are-Direct ELISA (Ahmed *et al*., 2003; Crowther, 2001) and Indirect ELISA (Ghazy *et al*., 2007; Kolhe *et al*., 2011). Various enzymes which are commonly used as marker enzymes for EIA are-glucose oxidase (Ekins and Chu, 1997). Alkaline phosphatase (Ishikawa *et al*., 1983) peroxidase (Johnston and Thorpe, 1987) and β galactosidase (Oellerich, 1984). These enzymes can be linked covalently, to antibody. HRP or Horseradish Peroxidase is one of the most popular enzyme labels, used for enzyme-immunoassay (El-Kenawy and El-Tholoth, 2011). Laccase (benzenediol oxygen oxidoreductase; EC.1.10.3.2) is an enzyme that has K_m values similar to HRP, for a number of chromogenic substrates. This suggests that Laccase may be a potential enzyme-label for enzyme-immunoassay. However, in the development of immunoassays, laccase has only rarely been tried as an enzyme-label.

Laccases are glycosylated multicopper oxidases. The enzyme was discovered a long time ago, from the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1883). Laccases have been found to be present among plants, insects, bacteria. They are widely scattered in basidiomycetous and ascomycetous fungi, where they perform a number of physiological functions (Das *et al*., 2011;

Desai and Nityanand, 2011; Khammuang and Sarnthima, 2009). The oxidation of a range of inorganic and aromatic substances (particularly phenols) are catalysed by laccase, with the concomitant reduction of O_2 to water. The oxidation of a reducing substrate by laccase typically involves the loss of a single electron and the formation of a free (cation) radical. The radical is usually unstable and may undergo further laccase-catalyzed oxidation (e.g., to form quinone from phenol) or non enzymatic reactions (e.g., hydration, disproportion, or polymerization) (Xu, 1996; Gilaki, 2010).

Laccase reduces oxygen to water and this reduction is accompanied with the oxidation of a substrate. Oxidation of several substances are catalysed by laccase, particularly phenols and aromatic amines (D'Acunzo *et al.*, 2002; Prabu and Udayasoorian, 2005). Among the substrates for laccase, the three most commonly used ones are-Guaiacol, o-dianisidine and ABTS (Li *et al.*, 2008). These are also used as substrates for Horseradish peroxidase but in presence of hydrogen peroxide. So, in the present study, a comparison was done between laccase conjugated immunoglobulins (conjugated in lab.) and commercially available HRP conjugated immunoglobulins, in ELISA format, using the above mentioned substrates.

Several cross linking reagents are available for coupling of enzymes to proteins. These include-Glutaraldehyde (Avrameas, 1969; Avrameas and Ternynck, 1971), Toluene diisocyanate (Schick and Singer, 1961), p,p'-Difluoro-m,m'-dinitrophenyl sulfone (Tawde and Ram, 1962), N,N'-o-phenylenedimaleimide (Kato *et al.*, 1976) m-periodate (Nakane and Kawaoi, 1974) etc. Based upon these reagents, different methods for coupling of enzymes to antibodies have been described (Kennedy *et al.*, 1976). Among the direct conjugation methods used for coupling the HRP and antibody, the most popular ones are the periodate method (Kennedy *et al.*, 1976; Wilson and Nakane, 1978) and the glutaraldehyde method (Avrameas and Ternynck, 1971).

The aim of the current study was to develop an efficient method (based on Glutaraldehyde or Periodate) for conjugation of laccase to immunoglobulin and to demonstrate the efficiency of the immunolaccase conjugate in enzyme immunoassay in comparison with immunoperoxidase conjugates of similar composition.

MATERIALS AND METHODS

Laccase: The purified laccase used for preparing immunolaccase conjugates for detection of rabbit IgG by ELISA was purchased (Source: *Trametes versicolor*) from Sigma Chemical Co.

Chemicals: L-lysine monohydrate, sodium borohydride, sodium metaperiodate, tween 20 were bought from Sisco Research Laboratories (Mumbai, India). Bovine serum albumin, o-dianisidine, Guaiacol and ABTS were bought from Sigma Chemical Co. (USA). Glutaraldehyde (25%) was bought from Spectrochem Pvt. Ltd. (Mumbai). Purified Rabbit IgG, Goat anti-rabbit IgG, Rabbit anti-goat IgG and HRP-conjugated Rabbit anti-goat IgG was bought from Bangalore Genei (Bangalore, India). All other reagents were of analytical grade.

Assay for laccase activity: Laccase activity was assayed spectrophotometrically with o-dianisidine, guaiacol, ABTS as substrates. Enzyme activity was expressed in International Unit (Mazumder *et al.*, 2009).

Conjugation of laccase to immunoglobulin: Purified laccase was conjugated to commercially obtained, purified rabbit anti-goat IgG (secondary antibody) by two methods-glutaraldehyde conjugation method and periodate conjugation method.

Glutaraldehyde conjugation method: Glutaraldehyde conjugation method (Avrameas, 1969) was used for conjugating Laccase to immunoglobulin, with slight modifications. Ten milligram of purified laccase was added to 975 µL of double distilled water. Twenty five microliter 25% glutaraldehyde was added to the solution which was then incubated overnight at room temperature. The solution was dialysed against PBS at 4°C, after the overnight incubation. After dialysis, 93 µL of antibody solution i.e., rabbit anti-goat IgG, (21.7 mg mL^{-1}), 107 µL double distilled water and 200 µL of carbonate bicarbonate buffer (1 M, pH 9.5) were added to the dialysed solution. It was then incubated at 4°C overnight. After the overnight incubation, 20 µL of 0.2 M lysine was added to the solution and it was then incubated at 4°C for 2 h. After incubation, it was again dialysed against PBS at 4°C. To separate the unreacted enzyme from the mixture by salt precipitation, an equal volume of saturated ammonium sulfate was added to the solution, after dialysis, and was incubated at 4°C for 30 min. After incubation, it was centrifuged for 20 min at 4000×g and the supernatant was discarded. The precipitate was dissolved in 1 mL saline and dialysed extensively against several changes of PBS. It was next ultrafiltered using an ultrafiltration membrane (Pall Life Sciences) with an MWCO of 100 kD for separating the conjugate from free unreacted enzyme. The conjugate thus obtained was then preserved at -20°C.

Periodate conjugation method: Periodate conjugation method (Nakane and Kawaoi, 1974) was used for conjugating laccase to immunoglobulin, with slight modifications. Ten milligram of purified laccase was added to 1 mL double distilled water. Then 0.2 mL of freshly prepared 0.1 M Sodium metaperiodate was added to the laccase solution and was stirred for 20 min at room temperature. After stirring, the solution was dialysed against acetate buffer (1 mM, pH 4.4) for overnight at 4°C. After dialysis, the pH of the activated enzyme solution was adjusted to 9.0 by addition of 20 µL of 0.2 M carbonate-bicarbonate buffer (pH 9.5). After that, 138 µL of antibody solution i.e., rabbit anti-goat IgG, (27.5 mg mL^{-1}) and 862 µL of 10 mM carbonate-bicarbonate buffer (pH 9.5) were added to the solution immediately. It was then stirred at room temperature for 2 h. After stirring, to separate the unreacted enzyme from the mixture by salt precipitation, an equal volume of saturated ammonium sulfate was added to the solution and was incubated at 4°C for 30 min. Then it was centrifuged for 20 min at 4000 x g and the supernatant was discarded. The precipitate was dissolved in 1 mL saline and dialysed extensively against several changes of PBS at 4°C. It was next ultrafiltered using an ultrafiltration membrane (Pall Life Sciences) with an MWCO of 100 kD for separating the conjugate from free unreacted enzyme. The conjugate thus obtained was then preserved at -20°C.

Characterization of laccase-immunoglobulin conjugate by indirect ELISA and optimization of the assay: The laccase-immunoglobulin conjugate was characterized in indirect ELISA format for detection of rabbit IgG. The assay optimization was done, firstly, by using different concentrations of the laccase-immunoglobulin conjugate to detect a very low concentration of rabbit IgG. The optimal conjugate concentration was defined as that concentration of the conjugate giving the best absorbance signal. Secondly, the optimum concentration of laccase-immunoglobulin conjugate was used for detecting several lower concentrations of rabbit IgG. The optimal concentration of rabbit IgG was defined as that concentration giving the best absorbance signal. In both stages of optimization laccase-IgG conjugates were compared to their corresponding HRP-IgG conjugates. The assay was repeated using different chromogenic substrates. The assay was also repeated by using laccase-immunoglobulin conjugates prepared through different conjugation methods, viz. glutaraldehyde and periodate methods of conjugation.

Method for detection of purified rabbit IgG (10^{-7} M) using laccase conjugated IgG and commercially available HRP conjugated IgG (in the range of 0.00002 to 2 μg mL^{-1}) as secondary antibody in indirect ELISA: Commercially available purified rabbit IgG at a fixed molar concentration (10^{-7} M) were added to the wells of ELISA strips and incubated overnight at 4°C. After overnight binding of antigen, the wells were washed with phosphate buffered saline containing 0.05% Tween-20 (PBST). After washing, BSA (1%) was added to the wells to block non specific binding. Wells were washed with PBST and then primary antibody i.e., goat anti-rabbit IgG, at a dilution of 1:1000, was added to the wells and were incubated at room temperature for 2 h. After incubation, the wells were washed with PBST followed by addition of varying concentrations (0.00002 to 2 μg mL^{-1}) of secondary antibody i.e., rabbit anti-goat IgG, conjugated with either laccase or HRP enzyme and were further incubated at room temperature for 2 h. After incubation, the wells were washed with PBST. After washing, chromogenic substrate (o-dianisidine, 0.9 mM or Guaiacol, 10 mM or ABTS, 0.5 mM) for the enzyme Laccase or HRP, was added and incubated at room temperature for 2 h. After incubation, the absorbance of the reaction product was measured by ELISA Plate Reader (Biorad, USA, Model No.680).

Method for detection of rabbit IgG (in the range of 10^{-12} M to 10^{-7} M) using fixed concentration of laccase conjugated IgG and commercially available HRP conjugated IgG as secondary antibody in indirect ELISA: Commercially available purified Rabbit IgG at varying molar concentrations (10^{-12} M to 10^{-7} M) was added to the wells of ELISA strips and incubated overnight at 4°C. After overnight binding of antigen, the wells were washed with phosphate buffered saline containing 0.05% Tween-20 (PBST). After washing, BSA (1%) was added to the wells to block non specific binding. Wells were washed with PBST and then primary antibody i.e., Goat anti-rabbit IgG, at a dilution of 1:1000, was added to the wells and were incubated at room temperature for 2 h. After incubation, the wells were washed with PBST followed by addition of secondary antibody i.e., Rabbit anti-goat IgG, labeled with either laccase or HRP enzyme (Enzyme-antibody conjugate at 0.2 μg mL^{-1}) and were further incubated at room temperature for 2 h. After incubation, the wells were washed with PBST. After washing, chromogenic substrate (o-dianisidine, 0.9 mM or Guaiacol, 10 mM or ABTS, 0.5 mM) for the enzyme Laccase or HRP, was added and incubated at room temperature for 2 h. After incubation, the absorbance of the reaction product was measured by ELISA Plate Reader (Biorad, USA, Model No.680).

Statistical analysis: All the ELISA experiments were performed in duplicate and results were represented as mean+SE. One way ANOVA was performed between the groups. A level of $p<0.05$ was considered significant (Das and Das, 2005).

RESULTS

Detection of purified rabbit IgG (10^{-7} M) using laccase conjugated IgG (prepared by periodate conjugation method) and commercially available HRP conjugated IgG (in the range of 0.00002 to 2 μg mL^{-1}) as secondary antibody in indirect ELISA: A certain minimum molar concentration of rabbit IgG (10^{-7} M) was detected by using varying concentrations (0.00002 to 2 μg mL^{-1}) of laccase conjugated IgG (prepared by periodate conjugation method) and commercially available HRP conjugated IgG (commercially procured) in indirect ELISA. The results obtained with IgG conjugated to both laccase and HRP were compared (Fig. 1). The assay was repeated using three chromogenic substrates, viz., ABTS (Fig. 1a), o-dianisidine (Fig. 1b), Guaiacol (Fig. 1c).

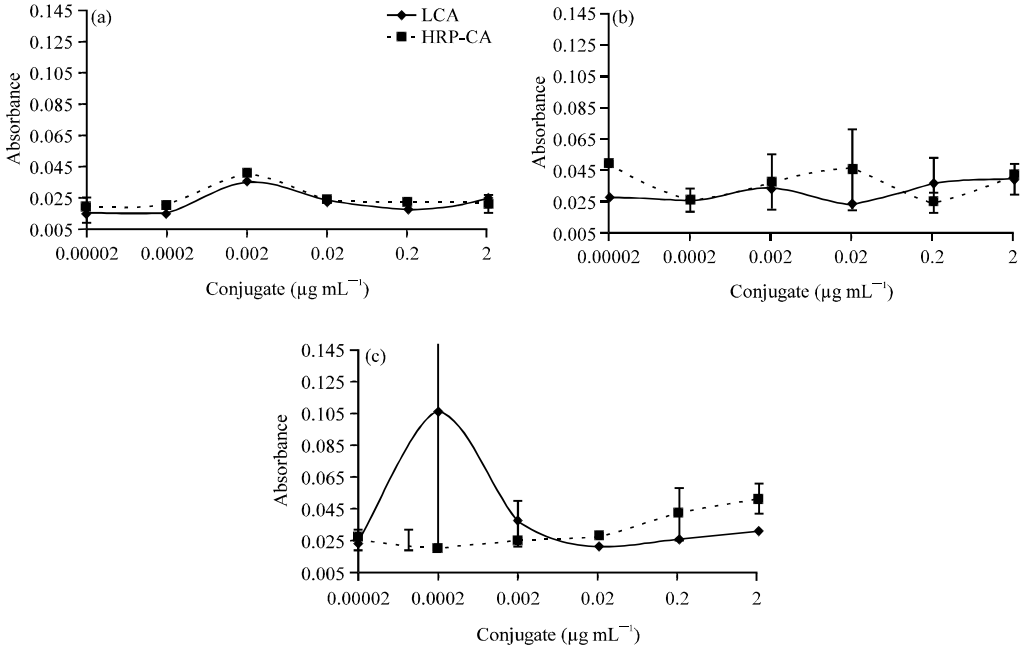

Fig. 1(a-c): (a)Detection of purified Rabbit IgG (10^{-7} M) using laccase conjugated IgG (prepared by periodate conjugation method) and HRP conjugated IgG (0.00002 to 2 µg mL) by indirect ELISA. Substrate: ABTS, (b) Detection of purified Rabbit IgG (10^{-7} M) using laccase conjugated IgG (prepared by periodate conjugation method) and HRP conjugated IgG (0.00002 to 2 µg mL^{-1}) by indirect ELISA. Substrate: o-dianisidine and (c) Detection of purified Rabbit IgG (10^{-7} M) using laccase conjugated IgG (prepared by periodate conjugation method) and HRP conjugated IgG (0.00002 to 2 µg mL^{-1}) by indirect ELISA. Substrate: Guaiacol. Results represented as Mean±SE

Detection of purified rabbit IgG (in the range of 10^{-12} M to 10^{-7} M) using a fixed concentration of laccase conjugated IgG (prepared by periodate conjugation method) and commercially available HRP conjugated IgG as secondary antibody in indirect ELISA: Purified rabbit IgG of varying molar concentrations (10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M) was detected using fixed concentrations (2 µg mL^{-1}) of laccase conjugated IgG (prepared by periodate conjugation method) and HRP conjugated IgG (commercially procured) by indirect ELISA. The results obtained with IgG conjugated to both laccase and HRP were compared (Fig. 2). The assay was repeated using three chromogenic substrates, viz., ABTS (Fig. 2a), o-dianisidine (Fig. 2b), Guaiacol (Fig. 2c).

Comparison of results obtained using different chromogenic substrates (o-dianisidine, guaiacol, ABTS) for detection of rabbit IgG (10^{-7} M) by laccase conjugated IgG (prepared by periodate conjugation method) and commercially purchased HRP conjugated IgG (in the range of 0.00002 to 2 µg mL^{-1}) as secondary antibody in indirect ELISA: A certain minimum molar concentration of rabbit IgG (10^{-7} M) was detected by using varying conc. (0.00002 to 2 µg mL^{-1}) of laccase conjugated IgG (prepared by periodate conjugation method) and commercially purchased HRP conjugated IgG using ABTS, o-dianisidine and Guaiacol

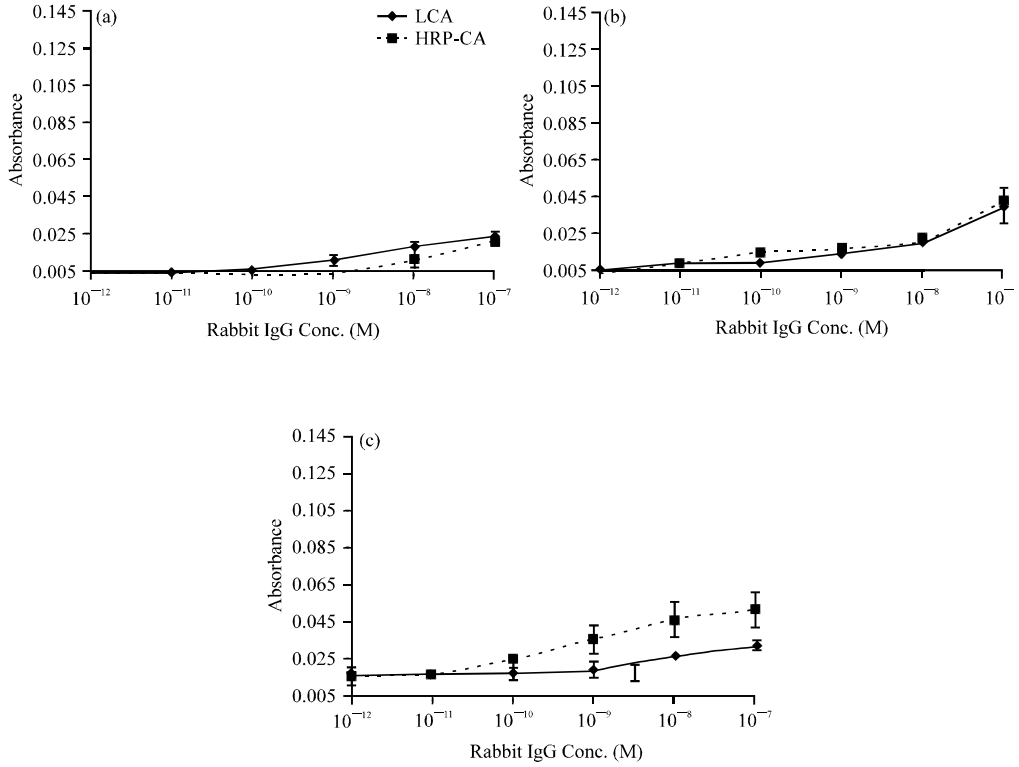

Fig. 2(a-c): (a) Detection of purified Rabbit IgG (10^{-12} M to 10^{-7} M) by a fixed concentration (2 μg mL^{-1}) of laccase conjugated IgG (prepared by periodate conjugation method) and HRP conjugated IgG by indirect ELISA. Substrate: ABTS, (b) Detection of purified Rabbit IgG (10^{-12} M to 10^{-7} M) by a fixed concentration (2 μg mL^{-1}) of laccase conjugated IgG (prepared by periodate conjugation method) and HRP conjugated IgG by indirect ELISA. Substrate : o-dianisidine and (c) Detection of purified Rabbit IgG (10^{-12} M to 10^{-7} M) by a fixed concentration (2 μg mL^{-1}) of laccase conjugated IgG (prepared by periodate conjugation method) and HRP conjugated IgG by indirect ELISA. Substrate: Guaiacol. Results represented as Mean±SE

as chromogenic substrates in indirect ELISA. The results obtained with ABTS, o-dianisidine and guaiacol were compared (Fig. 3).

Comparison of results obtained using different chromogenic substrates (o-dianisidine, guaiacol, ABTS) for detection of rabbit IgG (in the range of 10^{-12} M to 10^{-7} M) by fixed concentrations of laccase conjugated IgG (prepared by periodate conjugation method) and commercially purchased HRP conjugated IgG as secondary antibody in indirect ELISA: Purified rabbit IgG of varying molar concentrations (10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M) were detected by using fixed concentrations (2 μg mL^{-1}) of laccase conjugated IgG and commercially purchased HRP conjugated IgG by indirect ELISA., using ABTS, o-dianisidine and Guaiacol as chromogenic substrates. The results obtained with ABTS, o-dianisidine and guaiacol were compared (Fig. 4).

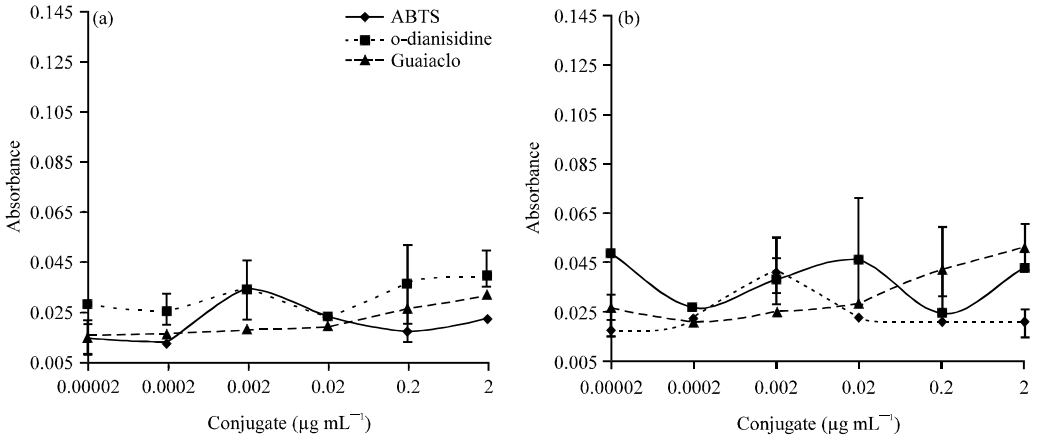

Fig. 3(a-b): Comparison of different chromogenic substrates (ABTS, o-dianisidine, Guaiacol) in detection of purified rabbit IgG (10^{-7} M) by laccase conjugated IgG (prepared by periodate method) and commercially purchased HRP conjugated IgG (0.00002 to 2 µg mL^{-1}), in indirect ELISA. (a) LCA and (b) HRP-CA. Results represented as Mean±SE

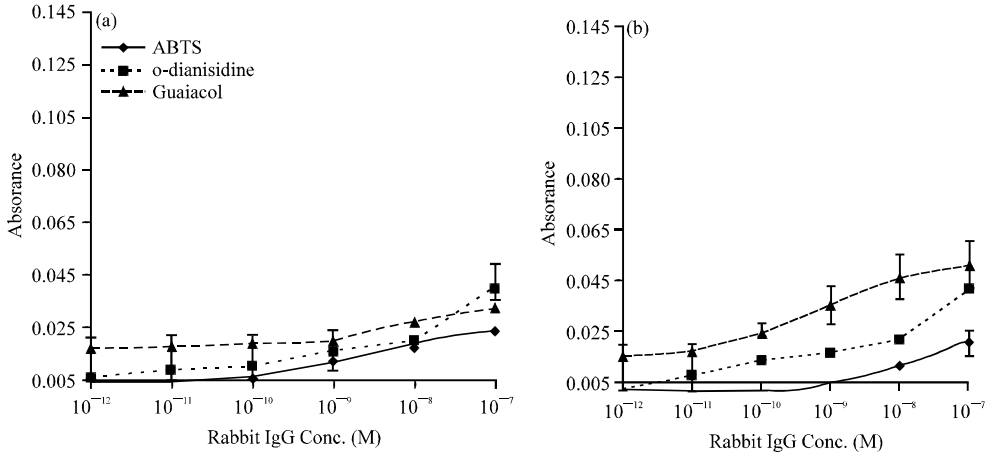

Fig. 4(a-b): Comparison of different chromogenic substrates (ABTS, o-dianisidine, Guaiacol) in detection of purified rabbit IgG (10^{-12} M to 10^{-7} M) by a fixed concentration (2 µg mL^{-1}) of laccase conjugated IgG (prepared by periodate method) and commercially purchased HRP conjugated IgG in indirect ELISA. (a) LCA and (b) HRP-CA. Results represented as Mean±SE

Detection of purified rabbit IgG (10^{-7} M) using laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and commercially available HRP conjugated IgG (in the range of 0.00002 to 2 µg mL^{-1}) as secondary antibody in indirect ELISA: A certain minimum molar concentration of rabbit IgG (10^{-7} M) was detected by using varying concentrations (0.00002 to 2 µg mL^{-1}) of laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and commercially available HRP conjugated IgG in indirect ELISA. The results obtained with IgG conjugated to both laccase and HRP were compared (Fig. 5). The assay was repeated using three chromogenic substrates, viz., ABTS (Fig. 5a), o-dianisidine (Fig. 5b), Guaiacol (Fig. 5c).

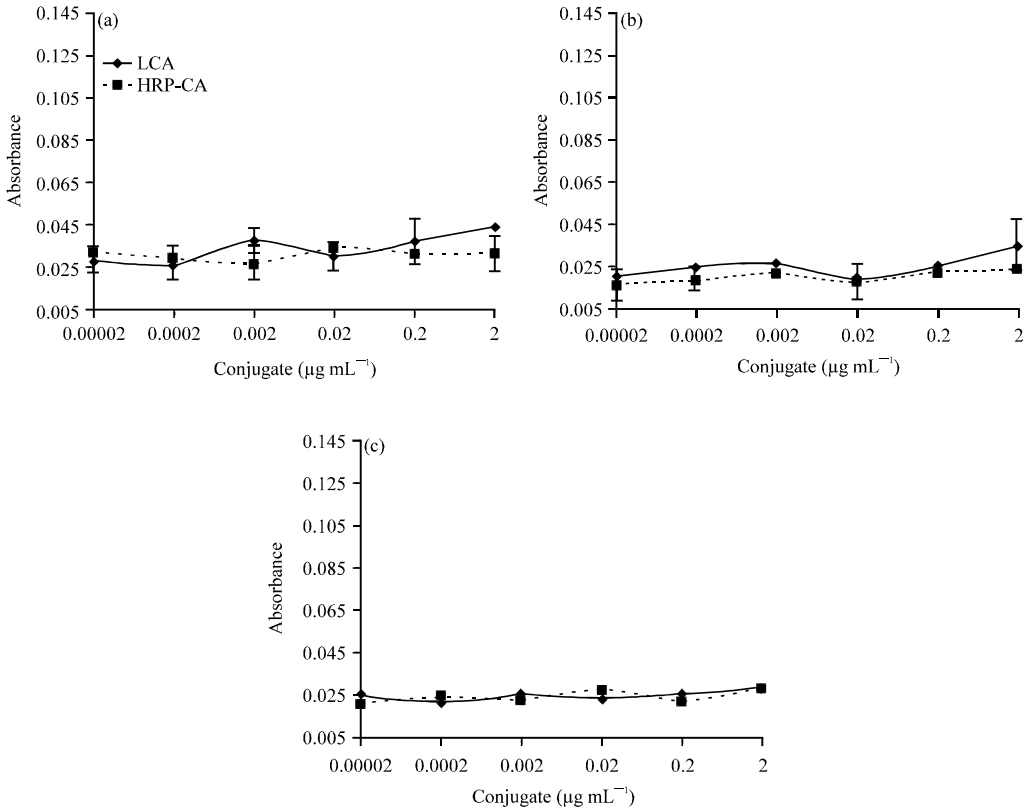

Fig. 5(a-c): (a) Detection of purified Rabbit IgG (10^{-7} M) using laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and HRP conjugated IgG (0.00002 to 2 µg mL^{-1}) by indirect ELISA. Substrate: ABTS, (b) Detection of purified Rabbit IgG (10^{-7} M) using laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and HRP conjugated IgG (0.00002 to 2 µg mL^{-1}) by indirect ELISA. Substrate: o-dianisidine and (c) Detection of purified Rabbit IgG (10^{-7} M) using laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and HRP conjugated IgG (0.00002 to 2 µg mL^{-1}) by indirect ELISA. Substrate: Guaiacol. Results represented as Mean±SE

Detection of purified rabbit IgG (in the range of 10^{-12} M to 10^{-7} M) using a fixed concentration of laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and commercially available HRP conjugated IgG as secondary antibody in indirect ELISA: Purified rabbit IgG of varying molar concentrations (10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M) was detected using fixed concentrations (2 µg mL^{-1}) of laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and HRP conjugated IgG by indirect ELISA. The results obtained with IgG conjugated to both laccase and HRP were compared (Fig. 6). The assay was repeated using three chromogenic substrates, *viz.*, ABTS (Fig. 6a), o-dianisidine (Fig. 6b), Guaiacol (Fig. 6c).

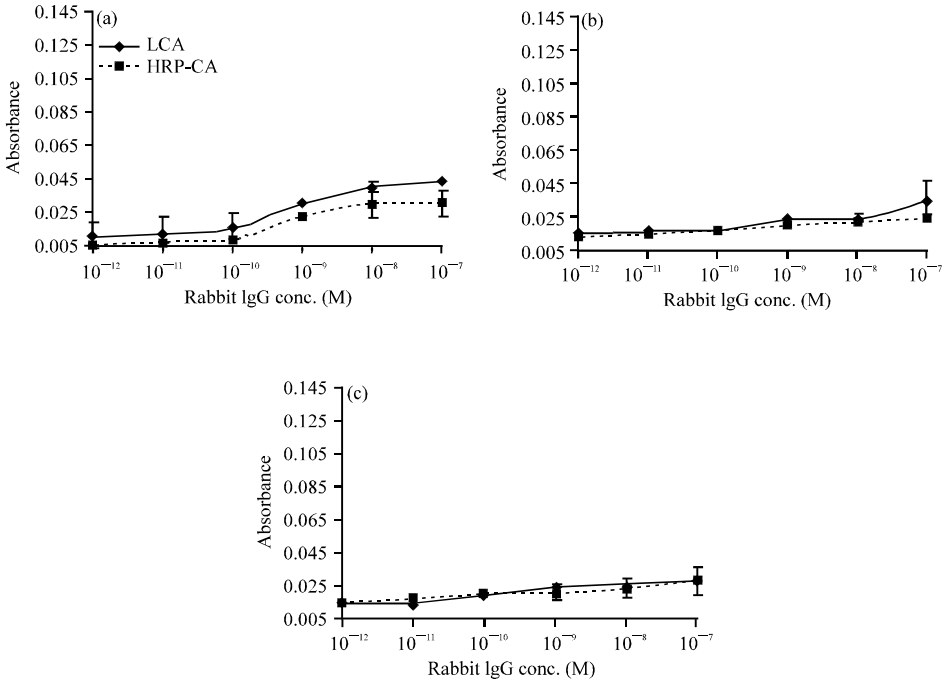

Fig. 6 (a-c): (a) Detection of purified Rabbit IgG (10^{-12} M to 10^{-7} M) by a fixed concentration (2 μg mL^{-1}) of laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and HRP conjugated IgG by indirect ELISA. Substrate: ABTS, (b) Detection of purified rabbit IgG (10^{-12} M to 10^{-7} M) by a fixed concentration (2 μg mL^{-1}) of laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and HRP conjugated IgG by indirect ELISA. Substrate: o-dianisidine and (c) Detection of purified Rabbit IgG (10^{-12} M to 10^{-7} M) by a fixed concentration (2 μg mL) of laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and HRP conjugated IgG by indirect ELISA. Substrate: Guaiacol. Results represented as Mean±SE

Comparison of results obtained using different chromogenic substrates (o-dianisidine, guaiacol, ABTS) for detection of rabbit IgG (10^{-7} M) by laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and commercially purchased HRP conjugated IgG (in the range of 0.00002 to 2 μg mL^{-1}) as secondary antibody in indirect ELISA: A certain minimum molar concentration of rabbit IgG (10^{-7} M) was detected by using varying conc. (0.00002 to 2 μg mL^{-1}) of laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and commercially purchased HRP conjugated IgG, using ABTS, o-dianisidine and Guaiacol as chromogenic substrates in indirect ELISA. The results obtained with ABTS, o-dianisidine and guaiacol were compared (Fig. 7).

Comparison of results obtained using different chromogenic substrates (o-dianisidine, guaiacol, ABTS) for detection of rabbit IgG (in the range of 10^{-12} M to 10^{-7} M) by fixed concentrations of laccase conjugated IgG (prepared by glutaraldehyde conjugation method) and commercially purchased HRP conjugated IgG, as secondary antibody in indirect ELISA: Purified rabbit IgG of varying molar concentrations

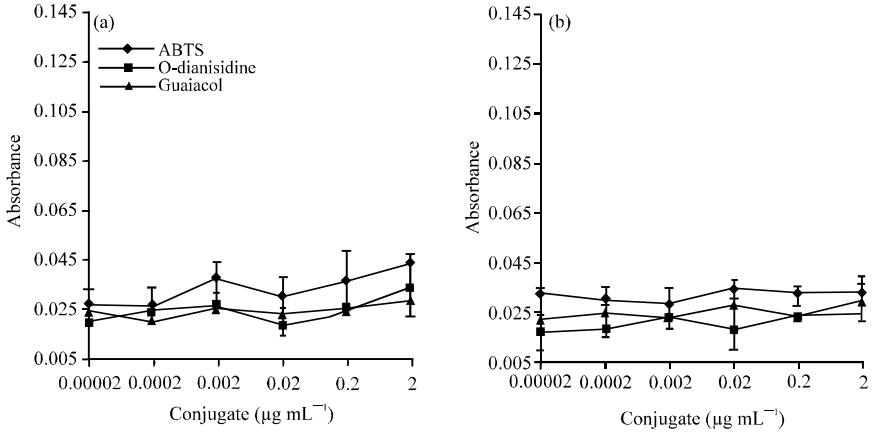

Fig. 7(a-b): Comparison of different chromogenic substrates (ABTS, o-dianisidine, Guaiacol) in detection of purified rabbit IgG (10^{-7} M) by laccase conjugated IgG (prepared by glutaraldehyde method) and commercially purchased HRP conjugated IgG (0.00002 to 2 µg mL^{-1}), in indirect ELISA, (a) LCA and (b) HRP-CA. Results represented as Mean±SE

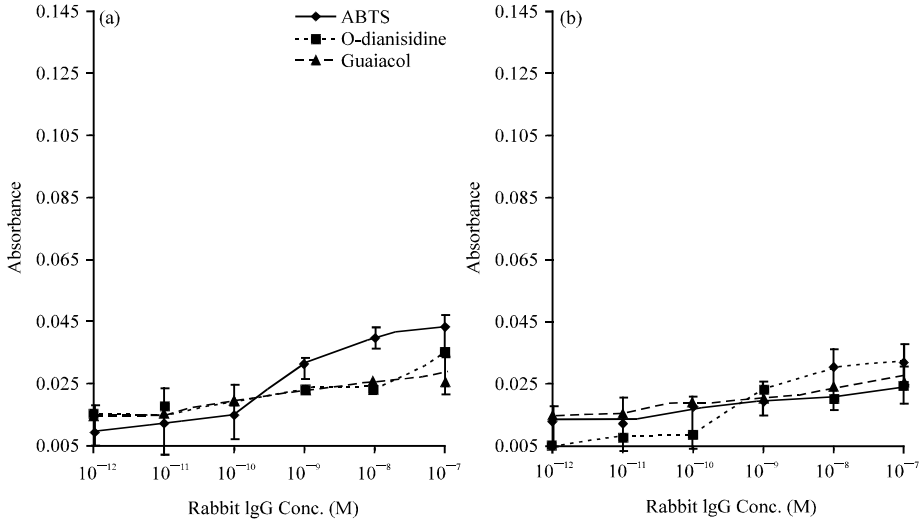

Fig. 8(a-b): Comparison of different chromogenic substrates (ABTS, o-dianisidine, Guaiacol) in detection of purified rabbit IgG (10^{-12} M to 10^{-7} M) by a fixed concentration (2 µg mL) of laccase conjugated IgG (prepared by glutaraldehyde method) and commercially purchased HRP conjugated IgG in indirect ELISA, (a) LCA and (b) HRP-CA. Results represented as Mean±SE

(10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M) were detected by using fixed concentrations (2 µg mL^{-1}) of laccase conjugated IgG (prepared by Glutaraldehyde method) and commercially purchased HRP conjugated IgG, by indirect ELISA., using ABTS, o-dianisidine

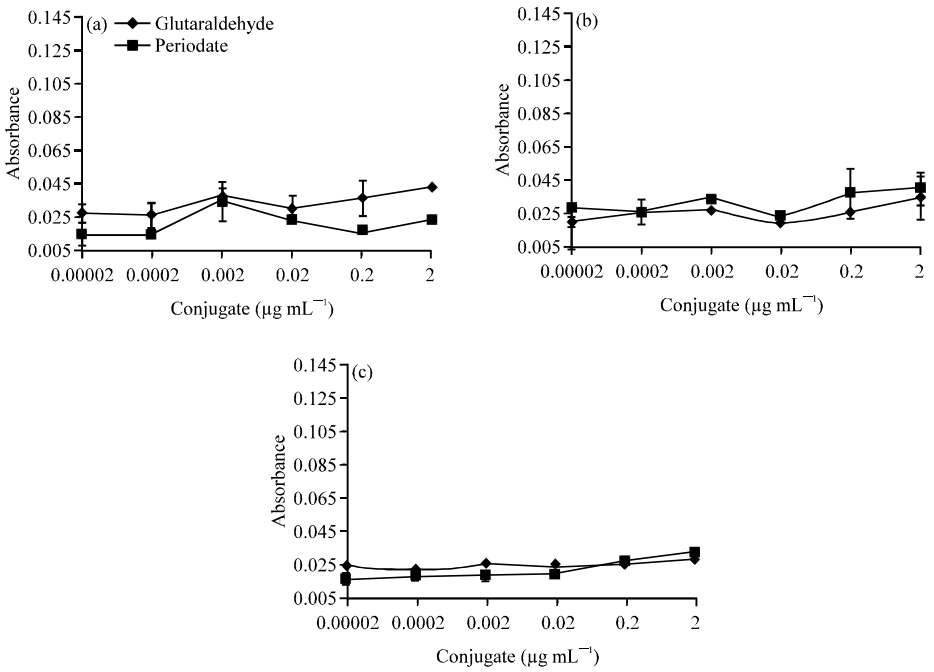

Fig. 9 (a-c): (a) Comparison of results obtained using laccase conjugated IgG (in the range of 0.00002 to 2 µg mL^{-1}) prepared through glutaraldehyde and periodate conjugation methods as secondary antibody, for detection of purified rabbit IgG (10^{-7} M) by indirect ELISA. Substrate: ABTS (b) Comparison of results obtained using laccase conjugated IgG (in the range of 0.00002 to 2 µg mL^{-1} prepared through glutaraldehyde and periodate conjugation methods as secondary antibody, for detection of purified rabbit IgG (10^{-7} M) by indirect ELISA. Substrate: o-dianisidine (c) Comparison of results obtained using laccase conjugated IgG (in the range of 0.00002 to 2 µg mL^{-1}) prepared through glutaraldehyde and periodate conjugation methods as secondary antibody, for detection of purified rabbit IgG (10^{-7} M) by indirect ELISA. Substrate: Guaiacol. Results represented as Mean±SE

and Guaiacol as chromogenic substrates. The results obtained with ABTS, o-dianisidine and guaiacol were compared (Fig. 8).

Comparison of results obtained using laccase conjugated IgG (in the range of 0.00002 to 2 µg mL^{-1}) prepared through glutaraldehyde and periodate conjugation methods as secondary antibody, for detection of purified rabbit IgG (10^{-7} M) by indirect ELISA: Purified rabbit IgG (10^{-7} M) was detected by using as low as 0.00002 µg mL^{-1} of laccase conjugated IgG made through glutaraldehyde and periodate conjugation methods by indirect ELISA. The results obtained with LCA conjugated by both Glutaraldehyde and Periodate methods were compared (Fig. 9). The assay was repeated using three chromogenic substrates, viz., ABTS (Fig. 9a), o-dianisidine (Fig. 9b), Guaiacol (Fig. 9c).

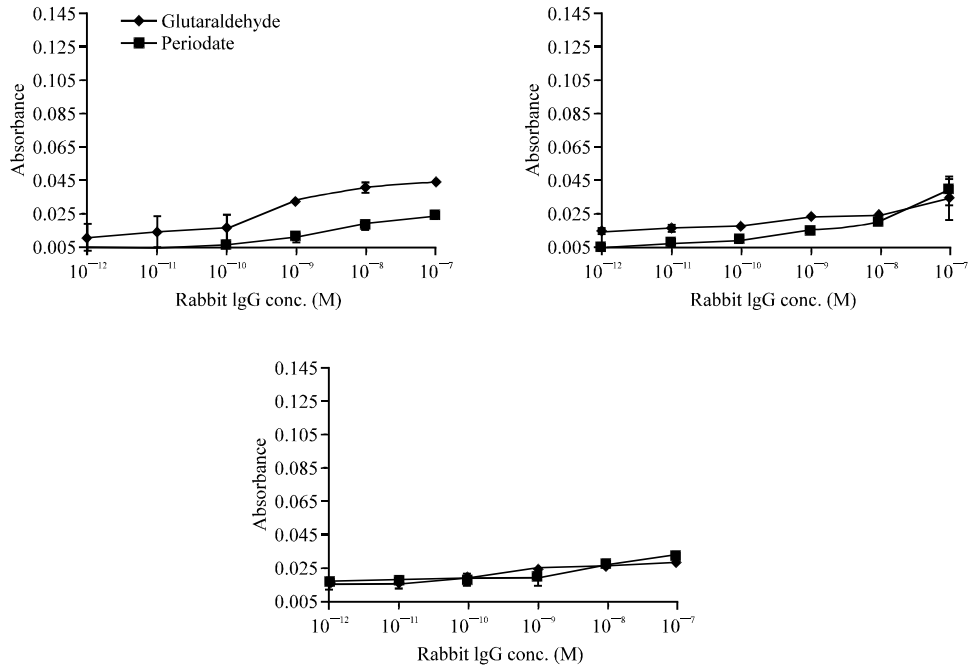

Fig. 10 (a-c): (a) Comparison of results obtained using fixed concentration of laccase conjugated IgG (2 μg mL^{-1}) prepared through Glutaraldehyde and Periodate conjugation methods, as secondary antibody, for detection of purified rabbit IgG (in the range of 10^{-12} M to 10^{-7} M) by indirect ELISA. Substrate: ABTS, (b) Comparison of results obtained using fixed concentration of laccase conjugated IgG (2 μg mL^{-1}) prepared through glutaraldehyde and periodate conjugation methods, as secondary antibody, for detection of purified rabbit IgG (in the range of 10^{-12} M to 10^{-7} M) by indirect ELISA. Substrate: o-dianisidine and (c) Comparison of results obtained using fixed concentration of laccase conjugated IgG (2 μg mL^{-1}) prepared through Glutaraldehyde and Periodate conjugation methods, as secondary antibody, for detection of purified rabbit IgG (in the range of 10^{-12} M to 10^{-7} M) by indirect ELISA. Substrate: Guaiacol. Results represented as Mean±SE

Comparison of results obtained using laccase conjugated IgG (in the range of 0.00002 to 2 μg mL^{-1}) prepared through glutaraldehyde and periodate conjugation methods as secondary antibody, for detection of purified rabbit IgG (10^{-7} M) by indirect ELISA: Purified rabbit IgG (10^{-7} M) was detected by using as low as 0.00002 μg mL^{-1} of laccase conjugated IgG made through Glutaraldehyde and Periodate conjugation methods by indirect ELISA. The results obtained with LCA conjugated by both Glutaraldehyde and Periodate methods were compared (Fig. 9). The assay was repeated using three chromogenic substrates, viz., ABTS (Fig. 9a), o-dianisidine (Fig. 9b), Guaiacol (Fig. 9c).

Comparison of results obtained using fixed concentration of laccase conjugated IgG (2 μg mL^{-1}) prepared through glutaraldehyde and periodate conjugation methods, as secondary antibody, for detection of purified rabbit IgG (in the range of 10^{-12}M to 10^{-7} M) by indirect ELISA: Purified rabbit IgG of varying molar concentrations (10^{-12} M, 10^{-11} M,

10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M) was detected using IgG conjugated to laccase through Glutaraldehyde and Periodate conjugation methods respectively by indirect ELISA. The results obtained with LCA conjugated by both Glutaraldehyde and Periodate methods were compared (Fig. 10). The assay was repeated using three chromogenic substrates, viz., ABTS (Fig. 10a), o-dianisidine (Fig. 10b), Guaiacol (Fig. 10c).

DISCUSSION

In a previously reported study, Laccase has been conjugated successfully to anti-BSA antibody and was characterized by enzyme-immunoassay. The assay was suitably optimized. The conjugate was found to be successful in detection of BSA in enzyme-immunoassay (Ray *et al.*, 2010).

The current study emphasizes upon standardization of glutaraldehyde and periodate conjugation methods, for successful conjugation of purified laccase to commercially purchased purified immunoglobulins; application of this immunolaccase (Laccase-Ig) conjugate as a secondary antibody for the detection of purified rabbit IgG by indirect ELISA; comparison of this Laccase-IgG conjugate with corresponding HRP-IgG conjugate (commercially purchased) in ELISA.

When compared to commercially purchased HRP (Horseradish Peroxidase) conjugated immunoglobulin, as a secondary antibody, in a similar assay format (ELISA), the Laccase conjugated immunoglobulin (conjugated in our lab.) was found to produce comparable results. Like HRP conjugated IgG, Laccase conjugated IgG was also found to be successful in detection of purified rabbit IgG. One way ANOVA shows that mean absorbance obtained by using Laccase conjugated IgG was not significantly different with that obtained by using HRP conjugated IgG ($p<0.05$). Thus efficiency of Laccase conjugated IgG (conjugated in our lab) in detecting purified rabbit IgG is similar to that of HRP conjugated IgG (Commercially purchased). HRP being a standard enzyme-label used in ELISA, our findings suggest Laccase to be another potent enzyme-label for ELISA.

The minimum molar concentration of rabbit IgG that have been detected by using laccase conjugated secondary antibody is 10^{-12} M (Fig. 2, 6). Most consistent results were however, obtained with a concentration of 10^{-7} M. The laccase conjugated secondary antibodies in concentrations as low as 0.00002 μg mL^{-1} or 20 pg mL^{-1} have been found to detect different concentrations of purified rabbit IgG (Fig. 1, 5). However, most consistent results were obtained with a conjugate concentration of 2 μg mL^{-1}. This suggests that laccase could be used as an efficient marker enzyme in the detection of such minute levels (10^{-12} M to 10^{-7} M) of IgG.

Both glutaraldehyde and periodate conjugation methods were standardized and used for conjugating laccase to immunoglobulin. Conjugate prepared by both the methods were almost equally efficient for detecting lower concentrations (10^{-12} M, 10^{-11} M, 10^{-10} M) of purified rabbit IgG, however for relatively greater concentrations (10^{-9} M, 10^{-8} M, 10^{-7} M), the conjugate prepared by glutaraldehyde conjugation method was found to give better results than that prepared by periodate conjugation method (Fig. 9, 10). However, the storage life of the periodate conjugates was found to be longer.

Out of the three chromogenic substrates for laccase used in this study *viz.*, ABTS, o-dianisidine and Guaiacol, the first two was found to give better results (Fig. 3, 4, 7 and 8).

The current study finds Laccase to be a prospective marker enzyme for ELISA. Immunolaccase (Laccase-IgG) conjugates have been prepared successfully by conjugating Laccase to immunoglobulins by both Glutaraldehyde and Periodate conjugation methods, standardized in our

lab. These Immunolaccase (Laccase-IgG) conjugates have been used successfully, for the detection of very low concentrations of purified rabbit IgG. The results obtained were found to be comparable to that obtained with immunoperoxidase (HRP-IgG) conjugates. HRP being one of the most widely used marker enzymes for ELISA, the findings strongly suggest Laccase to be a potential marker enzyme. Among the different chromogenic substrates used, the laccase immunoconjugates were found to produce best results with substrates like ABTS and o-dianisidine.

To conclude, this study involved standardization of Glutaraldehyde and Periodate based methods (used for protein-protein cross linking) for preparation of efficient Laccase-IgG conjugate, which was found to be comparable to commercially available HRP-IgG conjugate, in enzyme-immunoassay format.

Further investigations could be made in this direction by testing the efficacy of the Laccase-IgG conjugate in other assay formats like Immunoblot.

ACKNOWLEDGMENT

The work was financially supported by Department of Biotechnology (DBT) under Ministry of Science and Technology, Govt. of India.

ABBREVIATIONS

ABTS = 2, 2'-azinobis [3-ethylbenzothiazoline-6 sulfonic acid]
HRP = Horseradish peroxidase
HRP-CA = HRP conjugated antibody
IgG = Immunoglobulin G
LCA = Laccase conjugated antibody
MWCO = Molecular weight cut off

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