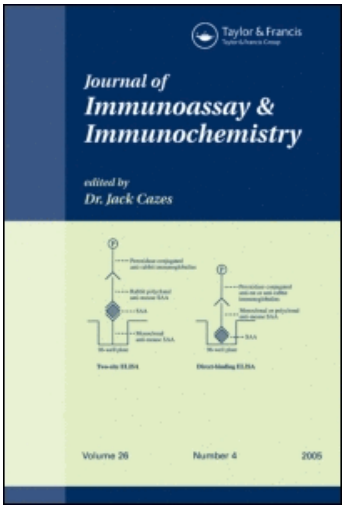

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DEVELOPMENT OF AN EFFICIENT AND SIMPLE METHOD FOR CONJUGATION OF LACCASE TO IMMUNOGLOBULIN AND ITS CHARACTERIZATION BY ENZYME IMMUNOASSAY

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DEVELOPMENT OF AN EFFICIENT AND SIMPLE METHOD FOR CONJUGATION OF LACCASE TO IMMUNOGLOBULIN AND ITS CHARACTERIZATION BY ENZYME IMMUNOASSAY

Swarnadeep Ray,[1] Protik Chowdhury,[2] Nirmalendu Das,[2] and Biswadev Bishayi[1]

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□ *A new laccase-conjugated antibody is developed, containing laccase as an enzyme marker, obtained from culture supernatant of the basidiomycetous fungi* Pleurotus ostreatus. *The efficacy of the laccase-conjugated antibody was demonstrated in indirect and direct enzyme immunoassay after using periodate and glutaraldehyde conjugation methods. The assay based on laccase-conjugated antibody is potent to detect antigens when compared with peroxidase conjugated antibody.*

Keywords EIA, ELISA enzyme antibody conjugate, immunoglobulin, laccase, *Pleurotus*

INTRODUCTION

In the field of *in vitro* diagnostics, there is an extensive use of reagents consisting of binding agents chemically linked to substances that provide signals detectable by instruments. Several methods have been devised to create this linkage without damaging the desired properties of the linked materials.[1]

In cases where both the binding and signaling components are proteins, such as linkage of an antibody to an enzyme, conventional coupling methods usually yield a mixture ranging from nonconjugated starting materials to large aggregates. The use of a solid-phase process to allow improved control in conjugation of macromolecules for use in immunodiagnostic reagents has been explored.[2] Reagents used for such

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protein–protein coupling are generally nonspecific, as they react with functional groups that are common to all proteins. The extent of intermolecular as opposed to intramolecular cross-linking will depend on the relative number and availability of such functional groups in the two proteins that are to be conjugated. The number of total functional groups present in any given protein can be determined, but whether these are available for intermolecular cross-linking to another protein cannot be tested easily. Therefore, the optimal conditions for conjugating two proteins to each other are determined by trial and error. There are several cross-linking reagents used for coupling of enzymes to proteins. These include glutaraldehyde,[3,4] toluene diisocyanate,[5,6] *p,p′*-difluoro-*m,ḿ*-dinitrophenyl sulfone,[7] carbodiimides,[8,9] *N,N′-o*-phenylenedimaleimide,[10] *m*-periodate,[11,12] and others. Depending upon these reagents, several methods for coupling of enzymes to antibodies have been described.[13] One of the most widely used is the glutaraldehyde conjugation method, described for the conjugation of alkaline phosphatase (ALP) to antibody by Avrameas.[4] Currently, the immunohistochemical technique using horseradish peroxidase (HRP)-labeled antibodies is widely used in pathology, cell biology, and immunology. Horseradish peroxidase when coupled with immunoglobulin (IgG) has been proved to be a useful marker for immunohistochemistry.[9] These studies have mostly used one of two direct conjugation methods for coupling the HRP and antibody, either the periodate method[11,14] or the glutaraldehyde method.[3] The most common enzymes used in enzyme immunoassay (EIA) as labels are glucose oxidase,[15] alkaline phosphatase,[16] peroxidase,[17] and β galactosidase.[18] These enzymes can be covalently coupled to antigen or antibody. Immunoassay based on competitive scheme has been described for enzyme labels of peroxidase.[19]

Laccases (benzenediol oxygen oxidoreductase; EC.1.10.3.2) are glycosylated multicopper oxidases. The enzyme was discovered more than one century ago from the Japanese lacquer tree *Rhus venicifera.*[20] Laccases have been found to be distributed among plants and subsequently they were discovered to be present in insects, bacteria, and widely scattered in basidiomycetous and ascomycetous fungi; they have performed a number of physiological functions like pathogenesis, lignin degradation, initiation of primordial, etc. in the producer organisms.[21–23] Laccases belong to the polyphenol oxidase group of enzymes. Oxygen is reduced to water by this enzyme, and this reduction is accompanied with the oxidation of a substrate. However, in the development of immunoassays, an enzyme like laccase has never been used for labels.

We suggested laccase as an efficient marker enzyme compared to peroxidase, since it is not inactivated during the reaction, is less sensitive to the presence of metal ions, has varying oxidation state in the reaction

medium, and enzyme-linked immunosorbent assay (ELISA) can be carried out with standard reagent and equipment used in peroxidase based analysis. The present study was attempted to develop a method for conjugation of laccase to immunoglobulin and to demonstrate the feasibility of laccases in enzyme immunoassay in comparison with immunoperoxidase conjugates of similar composition.

EXPERIMENTAL

Chemicals

L-Lysine monohydrate, sodium borohydride, sodium metaperiodate, and Tween 20 were bought from Sisco Research Laboratories (Mumbai, India). Bovine serum albumin (BSA) and Freund's complete reagent were bought from Sigma Chemical Co. (USA). Glutaraldehyde (25%) was bought from Spectrochem Pvt Ltd. (Mumbai). Purified human immunoglobulin (IgG), rabbit anti-human IgG, goat anti-rabbit IgG, and HRP-conjugated goat anti-rabbit IgG were bought from Bangalore Genei (Bangalore, India). All other reagents were of analytical grade.

Fungal Strain

Pleurotus ostreatus (Jacq.:Fr.) Kumm. (ITCC 3308) (previously reported as *P. florida*) was collected from the Society for Rural Industrialization, Ranchi, India, and was maintained on potato–dextrose agar (PDA) containing 20% potato extract, 2% dextrose, and 2% agar, pH 7.0.[24]

Inoculum Source

The fully covered slant (PDA) with fungus was gently rubbed (about 10 cm^2) and the mycelial mass was inoculated in one 250-mL Erlenmeyer flask containing 50 mL of medium. Liquid inoculum was developed by growing the *P. ostreatus* culture in liquid MYG (malt 1.0%, yeast extract 0.4%, glucose 2.0%, pH 7.0) medium in shake conditions for 5 days, followed by thorough homogenization of mycelial pellets for about 1 hr. Then 1% inoculum was inoculated in the medium.

Culture Conditions

The production of laccase was studied in PD medium containing 20% potato extract and 2% dextrose, pH 7.0, either in shaking (150 cycles/min) or in stationary condition at $28 \pm 1^{\circ}C$.

Laccase Purification

Purification of laccase was done according to Das et al. with slight modifications.[24] *Pleurotus ostreatus* was grown in yeast extract (0.5%) containing PD medium in shaking condition for 5 days. About 1000 mL of culture supernatant was obtained by centrifugation (21,000 × g, 30 min). The material was concentrated to 10 mL by amicon ultrafiltration using a 30-kD membrane. This material (in 100 m*M* sodium acetate buffer, pH 5.0) was used as the source of partially purified enzyme; 5 mL of this solution was dialysed against 10 m*M* sodium acetate buffer, pH 5.0, and applied to a DEAE-Sephadex (A-50) column (22 × 220 mm) equilibrated with same buffer. The enzyme was eluted with a linear gradient of 0–0.6 m*M* NaCl. Within the two laccase peaks, the fractions of the second peak were concentrated and dialyesd against sodium acetate buffer (100 m*M*, pH 5.0). This sample (2 mL) was then applied to a Bio Gel P-200 column (16 × 650 mm). Active fractions were pooled together and dialysed against sodium acetate buffer, 100 m*M*, pH 5.0, and used as the purified enzyme for further studies.

Assay for Laccase Activity

Laccase activity was assayed spectrophotometrically with *o*-dianisidine and guaiacol as the substrate.[25] Enzyme activity was expressed in international units (IU).

Raising Antibody Against BSA in Rabbit

Blood was collected from the rabbit before immunization to procure pre-immune serum. The rabbit was immunized subcutaneously, with emulsified BSA in Freund's complete adjuvant. At the end of the immunization period, blood was drawn from the marginal ear vein of the rabbit. The antiserum was then spun to remove red blood cells. Serum samples thus obtained were then stored in aliquots at −20°C and were used for conjugation experiments.

Determination of the Specificity of the Antiserum by Ouchterlony Double Diffusion Technique

The test was performed by pouring molten agarose (0.8% in 0.9% sterile saline) into a plastic petri dish (35 mm in diameter) where it was allowed to harden. Small wells were punched out equidistantly in the agarose, a few millimeters apart. At the central well, 30 µl antiserum (to BSA) was added.

At the other three peripheral wells, 10 µl, 20 µl, and 30 µl BSA antigens, from 1 mg/mL stock, were added, respectively. In another peripheral well, 30 µl saline was added as control. It was kept in a moist chamber and placed into a 37°C incubator for 24 hr. The line of precipitation was observed.

Conjugation of Laccase to Antibody

Conjugation of laccase to antibody raised against BSA was performed by the glutaraldehyde conjugation method and periodate oxidation conjugation method with slight modifications.

Glutaraldehyde Conjugation Method

First, 0.5 mL of partially purified laccase (7 mg/mL) solution was added to 25 µL of 25% glutaraldehyde solution and was incubated overnight at room temperature. After incubation, the solution was dialysed against PBS at 4°C. After dialysis, 500 µL of antiserum against BSA (5 mg/mL) and 50 µL of carbonate bicarbonate buffer (1 *M*, pH 9.5) were added to the dialysed solution. It was then allowed to stand at 4°C overnight. After the overnight incubation, 25 µL of 0.2 *M* lysine was added to the solution and it was then incubated at room temperature for 2 hr. After incubation, it was again dialysed against PBS at 4°C. After dialysis, 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. 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. The conjugate thus obtained was then preserved at −20°C.

Periodate Oxidation Conjugation Method

First, 300 µL of partially purified laccase (7 mg/mL) was added to 700 µL double-distilled water. Then 0.2 mL of freshly prepared 0.1 *M* sodium metaperiodate was added to the laccase solution and this was stirred for 20 min at room temperature. After stirring, the solution was dialysed against acetate buffer (1 m*M*, pH 4.4) 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, 800 µL of antiserum (5 mg/mL) and 200 µL of 10 m*M* carbonate–bicarbonate buffer (pH 9.5) were added to the solution immediately. It was then stirred at room temperature for 2 hr. 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 × 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. The conjugate thus obtained was then preserved at −20°C.

Indirect ELISA for Characterization of Laccase-Conjugated Anti-BSA Antibody

Antigen at different concentrations from the stock (10 mg/mL BSA) 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, casein (1%) was added to the wells to block nonspecific binding. Wells were washed with PBST and then antisera containing primary antibody (5 mg/mL) against BSA was added to the wells and they were incubated at room temperature for 2 hr. After incubation, the wells were washed with PBST followed by addition of enzyme-labeled antibody (laccase–antibody conjugate at 0.04 μg/mL) and were further incubated at room temperature for 2 hr. After incubation, the wells were washed with PBST. After washing, chromogenic substrate (*ortho*-dianisidine, 0.9 m*M*) for the enzyme laccase was added and incubated at room temperature for 2 hr. After incubation the absorbance of the reaction product was measured by an ELISA plate reader (Biorad, USA, model 680).

Direct ELISA for Characterization of Laccase-Conjugated Anti-BSA Antibody

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Assay Optimization

The optimization was done, first, by titration of varying concentrations of BSA in the presence of a fixed concentration of laccase–antibody

conjugate. The optimal BSA concentration was defined as the lowest concentration of BSA giving the maximum absorbance signal. Second, different concentrations of the primary antibody were used for the detection of optimum concentration of the antigen (BSA). Again, different concentrations of the laccase–antibody conjugate were also used to detect the optimum concentration of the antigen (BSA).

The optimal conjugate concentration was defined as the lowest concentration of the conjugate giving the maximum absorbance signal.

Detection of Unreacted Enzyme Separated From the Enzyme–Antibody Conjugate by Comparison of Mean Absorbance of the Reaction Product in the Presence of Substrate Alone and that of Substrate along with the Supernatant Recovered after Ammonium Sulfate Precipitation of the Conjugate

The unreacted enzyme was separated from the enzyme–antibody conjugate using ammonium sulfate (35%) precipitation method. In the case of successful separation of the unreacted enzyme from the conjugate, the supernatant should contain the unreacted enzyme. To investigate this, the supernatant (204 μg/mL) was reacted directly with guaiacol (10 m*M*), a chromogenic substrate. This was done by adding chromogenic substrate to the wells of an ELISA strip containing supernatant. Another strip contained only the chromogenic substrate but no enzyme. The strips were incubated for 2 hr. The absorbance was then measured using an ELISA plate reader (Biorad, USA, model 680). The absorbance signals obtained from the first strip were then compared to that obtained from the second strip.

Detection of the Presence of Enzyme–Antibody Conjugate in the Ammonium Sulfate Supernatant (Recovered after Ammonium Sulfate Precipitation of the Conjugate) by Direct ELISA

The unreacted enzyme was separated from the enzyme–antibody conjugate using the ammonium sulfate precipitation method. In the case of successful separation of the unreacted enzyme from the conjugate, the supernatant should contain the free enzyme. The amount of conjugate present in the supernatant should be very little, if not nil. The presence of the conjugate in the supernatant was investigated by direct ELISA.

Antigen (10 mg/mL BSA) 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 Tween-20 (PBST). Casein was added to the wells to block nonspecific binding. After washing

with PBST, enzyme-labeled antibody (laccase–antibody conjugate at 0.04 μg/mL) was added to the wells of one strip, while supernatant (obtained after ammonium sulfate precipitation) was added to the wells of the other strip. Both were incubated at room temperature for 2 hr. After washing with PBST, chromogenic substrate (0.9 m*M* *ortho*-dianisidine) for the enzyme laccase was added and incubated at room temperature for 2 hr. After incubation the absorbance signal of the reaction was measured by an ELISA plate reader (Biorad, USA, model 680). The absorbance signals obtained from the strip containing the laccase–antibody conjugate were then compared with that containing the supernatant.

Comparison of Absorbance Signals of the Reaction Product in the Presence of Laccase-Conjugated Anti-BSA Antibody Obtained by Glutaraldehyde and Periodate Methods with Direct ELISA

Laccase was conjugated with antibody using two of the most popular methods used for conjugating proteins, i.e., the glutaraldehyde conjugation method and periodate oxidation conjugation method. The signal intensities of the conjugates produced by each of these methods were investigated following a direct ELISA procedure.

Conjugation of Purified Laccase with Purified IgG and its Characterization by ELISA

Purified laccase was conjugated to commercially obtained purified anti-rabbit immunoglobulin raised in goat using the glutaraldehyde conjugation method. This laccase–antibody conjugate was then used to detect purified human IgG (1 μg/mL), using anti-human IgG raised in rabbit as the primary antibody (1:500 dilution), by indirect ELISA format. The signal intensities of reaction product obtained, using different concentrations (0.0002, 0.002, 0.02, 0.2, and 2 μg/mL) of the laccase–antibody conjugate against a certain concentration of human IgG (1 μg/mL), were estimated. This was then compared with the signal intensities obtained using HRP-conjugated anti-rabbit IgG.

RESULTS AND DISCUSSION

Pleurotus ostreatus produced at least two laccase isozymes (L_1 and L_2) in PD media.[22] The time required for optimum laccase production was much less in the shaking condition than in the stationary condition (Figure 1). The L_2 laccase was purified to homogeneity and characterized.[24] The purified laccase used here contained the L_2 isozyme, whereas the partially

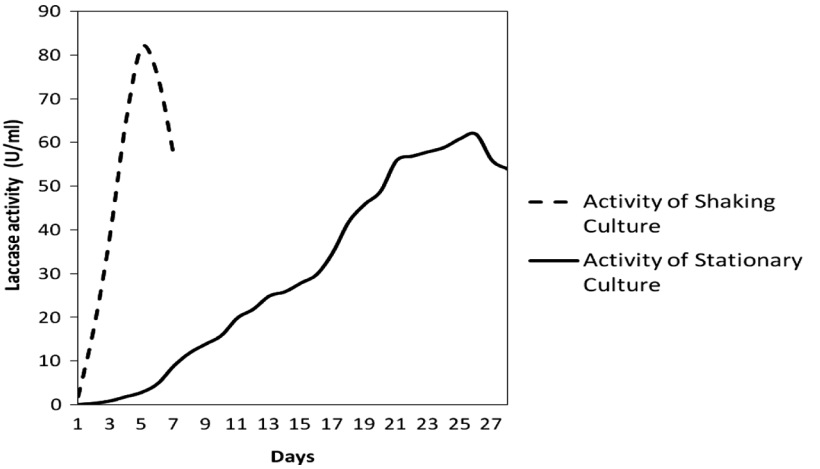

FIGURE 1 Day-wise laccase production in *P. osteatus* in shaking and stationary culture.

purified laccase contained both the isozymes. Laccase was purified from a number of fungal species. This enzyme was used for different biotechnological purposes in the food and beverage industry, pulp and paper industry, etc.[23] Laccase was also used as an immunosensor. Ghindilis et al. designed a potentiometric immunosensor using laccase without the presence of an electrochemically active mediator.[26] A highly sensitive immunoassay system based on laccase was also reported.[27]

The antiserum raised in rabbit demonstrated a single precipitin line against purified BSA in a double immunodiffusion plate of Ouchterlony. This shows that the antisera contained antibodies specific to the BSA.

Characterization of Laccase-Conjugated Anti-BSA Antibody by Indirect ELISA

To investigate whether a conjugation reaction had actually taken place between the enzyme laccase and the anti-BSA antibody, an indirect ELISA was performed. The absorbance signals obtained from wells containing a primary anti-BSA antibody were found to be greater than those without it. This indicates a successful conjugation between the enzyme laccase and the anti-BSA antibody. Moreover, in this experiment, titration of varying concentrations of BSA was done in the presence of a fixed concentration of laccase–antibody conjugate. The minimum concentration of BSA that was found to give a maximum absorbance signal was 600 μg (Figure 2). This value might be much lower if fully purified samples of laccase and antibody could be used.

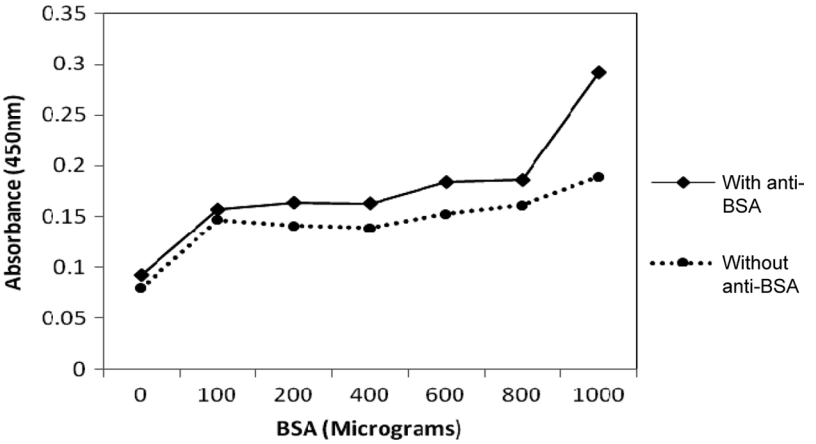

FIGURE 2 Characterization of laccase-conjugated anti-BSA antibody by using a fixed concentration (0.04 μg/mL) of it to detect different amounts of BSA in indirect ELISA. To investigate whether conjugation reaction has actually taken place between the enzyme laccase and the anti-BSA antibody, an indirect ELISA was performed. The intensity of absorbance signals from the wells was determined in the presence and absence of primary anti-BSA antibody using *o*-dianisidine as the chromogenic substrate after 2 hr.

Characterization of Laccase-Conjugated Anti-BSA Antibody by Direct ELISA

A direct ELISA procedure was also followed to investigate the success of the conjugation reaction. The absorbance signals obtained from the wells containing laccase–antibody conjugates were found to be greater than those not containing the conjugate (Figure 3). The results definitely

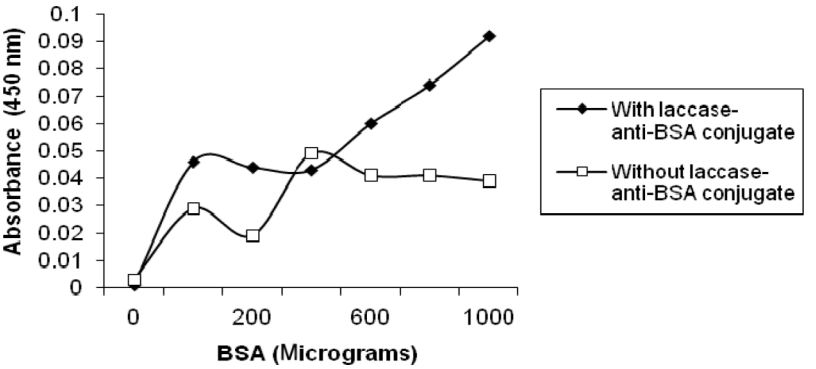

FIGURE 3 Characterization of laccase-conjugated anti-BSA antibody by using a fixed concentration (0.04 μg/mL) of it to detect different amounts of BSA in direct ELISA. A direct ELISA procedure was followed to investigate the success of the conjugation reaction. The intensity of absorbance signals from the wells was determined in the presence and absence of laccase–antibody conjugate using *o*-dianisidine as the chromogenic substrate after 2 hr.

suggest that a conjugation reaction has indeed taken place between the enzyme laccase and the anti-BSA antibody.

Titration of Different Concentrations of Primary Antibody (Anti-BSA) against a Fixed Concentration of Antigen (BSA) by Indirect ELISA

An indirect ELISA procedure was again followed, by titrating a fixed concentration of antigen with the different graded concentrations of the primary antibody. The optimum signal intensity was obtained with a volume of 60 µl of primary antibody. Since the concentration of the antibody solution was 4.5 mg/mL, the optimum signal could be said to have been obtained with a concentration of 270 µg (Figure 4).

Comparison of Mean Absorbance of the Reaction Product in the Presence of Substrate Alone and that of Substrate along with the Supernatant Recovered after Ammonium Sulfate Precipitation of the Conjugate

To investigate the extent of separation of nonreacted laccase enzyme from laccase–antibody conjugate, the ammonium sulfate supernatant was directly reacted with guaiacol. The absorbance signal thus obtained was compared with that obtained from the substrate blank (containing only guaiacol but no enzyme). The intensity of the absorbance signal obtained from the former was much greater than the latter (Figure 5), indicating the presence of free or nonreacted laccase enzyme in the supernatant.

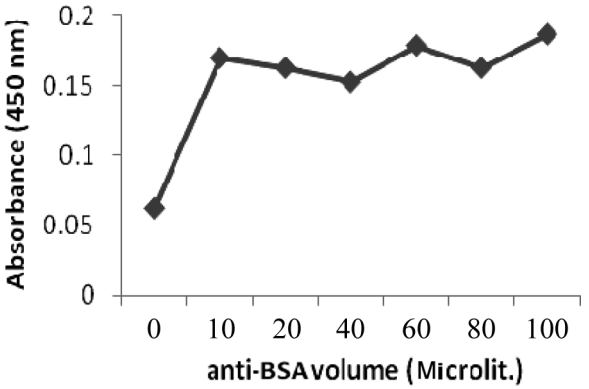

FIGURE 4 Detection of a fixed amount of BSA antigen (600 µg) by using different concentrations of the primary anti-BSA antibody with a fixed concentration (0.04 µg/mL) of the laccase-conjugated anti-BSA (secondary antibody). An indirect ELISA procedure was followed, by titrating a fixed concentration of antigen with the different graded concentrations of the primary anti-BSA antibody using laccase-conjugated anti-BSA (secondary antibody) and *o*-dianisidine as the chromogenic substrate.

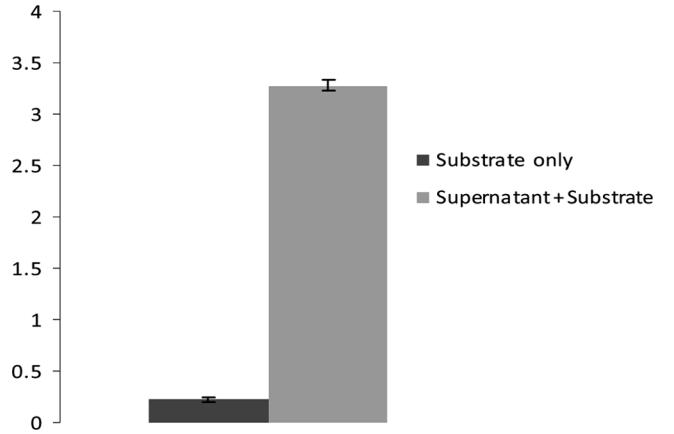

FIGURE 5 Comparison of the mean absorbance of the reaction product in the presence of the substrate alone and that of the substrate along with the supernatant recovered from the ammonium sulfate precipitation of the laccase–antibody conjugate. The absorbance signal obtained with substrate along with the supernatant (recovered after ammonium sulfate precipitation of the conjugate) was compared with that obtained from substrate blank (containing only guaiacol but no enzyme). Results are expressed as mean ± SD of three independent experiments.

Detection of the Presence of Enzyme–Antibody Conjugate in the Ammonium Sulfate Supernatant (Recovered after Ammonium Sulfate Precipitation of the Conjugate) by Direct ELISA

In the case of a proper separation of the unreacted enzyme from the conjugate, the presence of the latter in the supernatant should be negligible, if not nil. This was investigated by a direct ELISA procedure. In one set, the laccase–antibody conjugate was used, while in the other set, supernatant was used, against BSA. After incubation for a certain period, the strips were washed with PBST. Then *ortho*-dianisidine was added to each set. It was found that the absorbance signal from the set containing laccase–antibody conjugate was far greater than that containing the supernatant. This definitely shows that presence of the conjugate in supernatant is negligible (Figure 6).

Comparison of Absorbance Signals of the Reaction Product in the Presence of Laccase-Conjugated Anti-BSA Antibody Obtained by Glutaraldehyde and Periodate Methods with Direct ELISA

The signal intensities obtained using the laccase-conjugated anti-BSA antibody procured by the glutaraldehyde and periodate methods were investigated following a direct ELISA procedure. The laccase-conjugated antibody obtained by the glutaraldehyde method (0.054 mg/mL) was

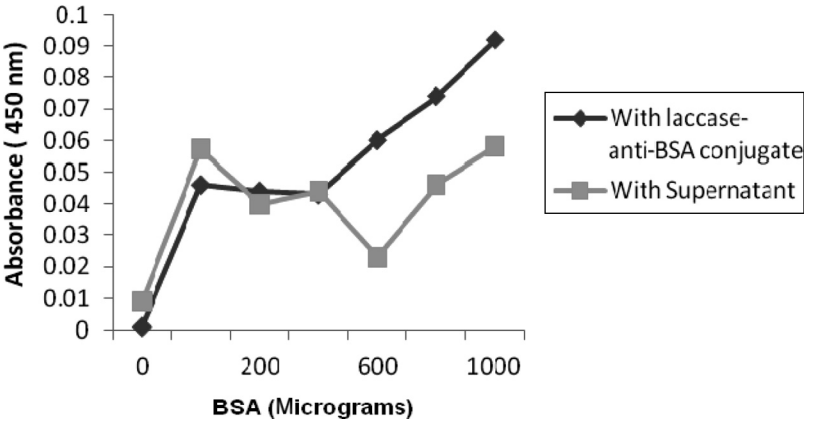

FIGURE 6 Detection of separation of laccase–antibody conjugate from free laccase by comparison of the absorbance of the reaction product in the presence of laccase-conjugated antibody and in presence of supernatant (recovered after ammonium sulfate precipitation) respectively by direct ELISA. A direct ELISA procedure was performed. In one set, the laccase–antibody conjugate was used, while in the other set, supernatant (recovered after ammonium sulfate precipitation of the conjugate) was used, against BSA, with *o*-dianisidine as the chromogenic substrate after 2 hr.

found to show greater signal intensity as compared to that obtained by the periodate method (0.171 mg/mL) (Figure 7). This suggests that the glutaraldehyde method may be a better method for conjugation of laccase with antibody.

This experiment also involved titrating different volumes (hence concentrations) of the laccase–antibody conjugate against a fixed concentration of BSA antigen. The minimum volume of conjugate that was found to give maximum absorbance signal was 80 µL (Figure 7). Hence, the minimum

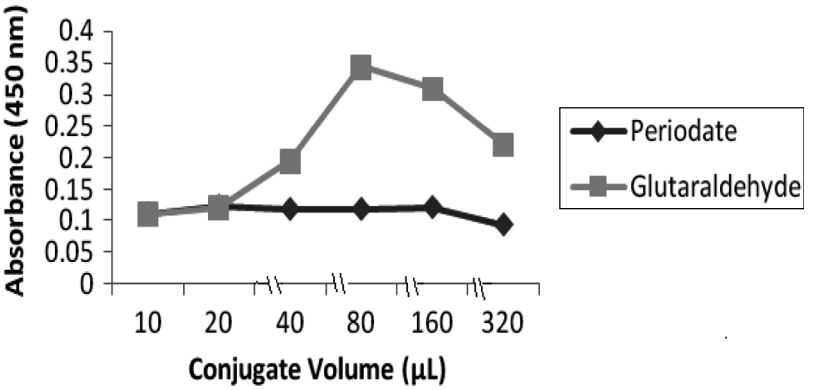

FIGURE 7 Comparison of absorbance signals of the reaction product in the presence of laccase-conjugated anti-BSA antibody obtained by glutaraldehyde and periodate methods with direct ELISA. The signal intensities obtained using the laccase conjugated anti-BSA antibody procured by the glutaraldehyde and periodate methods were investigated following a direct ELISA procedure.

concentrations of conjugate giving maximum absorbance signal were 13.68 μg (for conjugates prepared by periodate method) and 4.32 μg (for conjugates prepared by glutaraldehyde method). These values might be much lower if fully purified samples of laccase and antibody could be used.

Detection of Purified Human IgG with Laccase-Conjugated Anti-Rabbit IgG and Its Comparison with HRP-Anti-Rabbit IgG Conjugate

By using different concentrations of laccase–antibody conjugate to detect a fixed concentration of human IgG, it was found that the optimum signal intensity is obtained with a concentration of 0.0002 μg/mL of laccase anti-rabbit IgG conjugate. For HRP–antibody conjugate, the signal intensity obtained for the same concentration was found to be less (Figure 8).

Determination of Optimum Time for Laccase-Conjugated Anti-Rabbit IgG and Its Comparison with HRP-Conjugated Anti-Rabbit IgG in the Detection of Purified Human IgG

The change of signal intensity with increasing time was also recorded for both the conjugates. The signal intensity of laccase–antibody conjugate was found to be greater than that of HRP–antibody conjugate irrespective of time (Figure 9).

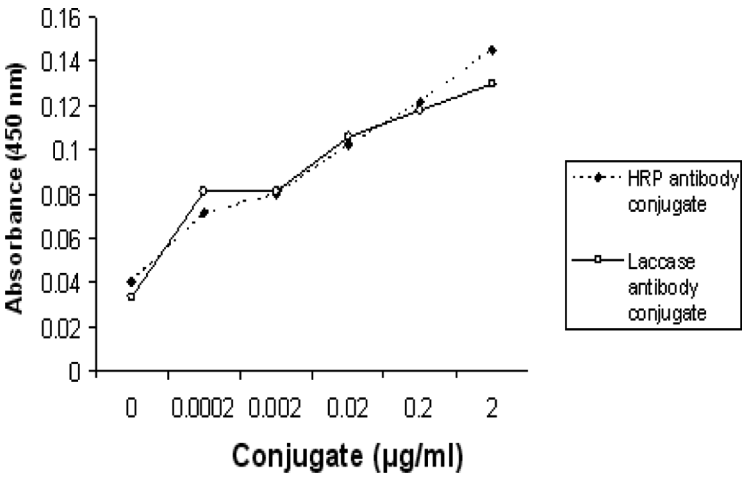

FIGURE 8 Detection of purified human IgG (1 μg/mL) by using laccase-conjugated goat anti-rabbit IgG (secondary antibody) and also HRP-conjugated goat anti-rabbit IgG (secondary antibody) by indirect ELISA. The results obtained using laccase-conjugated goat anti-rabbit IgG were compared to those of HRP-conjugated goat anti-rabbit IgG.

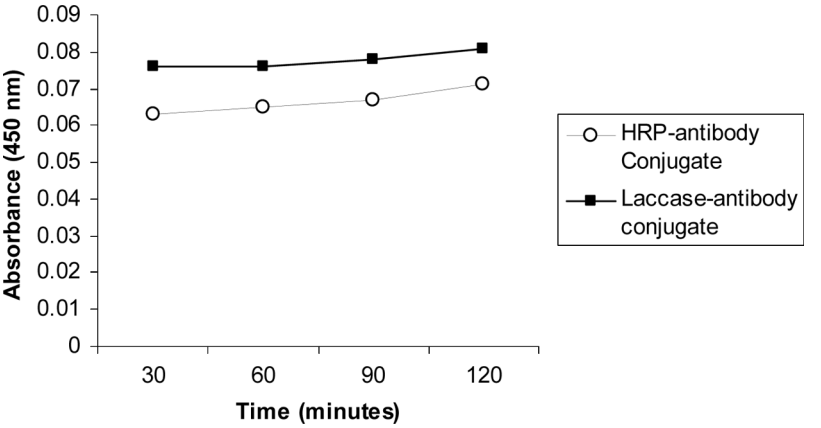

FIGURE 9 Time dependence of the accumulation of the chromogenic reaction product during incubation with laccase-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-rabbit IgG. Purified laccase was conjugated to commercially obtained purified anti-rabbit immunoglobulin raised in goat using the glutaraldehyde conjugation method. This laccase–antibody conjugate was then used to detect purified human IgG (1 μg/mL), using anti-human IgG raised in rabbit as the primary antibody (1:500 dilution), by indirect ELISA format. The change of signal intensity with increasing time was also recorded for both the conjugates. Conjugate concentration: 0.0002 μg/mL.

CONCLUSION

In this study a method for conjugation of laccase with antibody was developed. The laccase–antibody conjugate was then characterized using enzyme immunoassay. The result suggests that conjugation has actually taken place between laccase and antibody raised against BSA and also between laccase and purified anti-rabbit IgG raised in goat. This conjugate was also successfully used as the enzyme-labeled antibody in ELISA. Further investigations could be made in this direction by conjugating laccase with other purified immunoglobulin samples and characterizing this conjugate in enzyme immunoassay and immunoblot formats.

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