UNIVERSITI TEKNOLOGI MARA

CONFERENCE PROCEEDING

ICITSBE 2012

1ST INTERNATIONAL CONFERENCE ON INNOVATION AND TECHNOLOGY FOR SUSTAINABLE BUILT ENVIRONMENT

16 -17 April 2012

Organized by:
Office of Research and Industrial
Community And Alumni Networking
Universiti Teknologi MARA (Perak) Malaysia
www.perak.uitm.edu.my

PAPER CODE: GT 37

PREPARATION, CHARACTERIZATION AND IMMOBILIZATION OF SILVER NANOPARTICLES WITH HORSERADISH PEROXIDASE: ITS FUTURE ASPECTS IN BIOSENSOR APPLICATIONS

Zoheb Karim and Rohana Adnan
School of Chemical Sciences, Universiti Sains Malaysia, 11800 Minden Penang, Malaysia,
zoheb.karim@gmail.com

Abstract
Several new types of carrier and technology have been implemented in the recent past to improve traditional enzyme immobilization which aim to enhance enzyme loading, activity and stability in order to reduce the cost of enzyme in industrial processes and in sensing technology. Thus, the present study aims to work out a simple and high yield procedure for the immobilization of horseradish peroxidase on silver nanoparticles. FTIR, UV-Vis and TEM were used to characterize the silver nanoparticles. The single cell gel electrophoresis (comet assay) was also performed to confirm the genotoxicity of silver nanoparticles. To decrease the toxicity of silver nanoparticles, β-cyclodextrin was used as support. A comparative stability study of soluble and immobilized enzyme preparations was investigated against pH, temperature and chaotropic agent; urea. The results show that cross-linked peroxidase was significantly more stable as compared to soluble counterparts. The reusability study of immobilized enzyme preparation was also performed to use the immobilized preparation up to the industrial level.

Keywords: Silver Nanoparticles (AgNPs), Horseradish Peroxidase (HRP), Characterization, Statistical Analysis

1. Introduction

Peroxidases (E.C. 1.11.1.7) are ubiquitous heme-proteins, which utilize hydrogen peroxide to catalyze the oxidation of a wide variety of organic and inorganic substrates (Everse and Everse, 1999). Plant peroxidases are receiving increasing attention due to their extensive bioactivation property and potential applications in clinical, biochemical, biotechnological and related areas (Azevedo et al., 2003). Nanosized particles have attracted worldwide attention in recent times due to their unusual optical, chemical, photoelectrochemical and electronic properties (Krolikowska et al., 2003). These particles have had a wide application in a range of areas, including catalysis, optics, mechanics, magnetics, energetics, and biomedical sciences. It seems that nanoscience might take center stage in many key future technologies because of recent efforts in fabricating these nanosized structures into predefined superstructures. The interaction of enzymes with ligands offered stimulating opportunities for a wide variety of applications in the field of biotechnology and medicine (Karim and Adnan, 2012). Generally proteins undergo structural changes when interacting with ligands. Adsorption as well as the crosslinking of proteins on solid surfaces strongly depends on the nature of the protein and surface geometry and physicochemical characteristics of the solid surface. Various specific and nonspecific interactions such as electrostatic, hydrogen bonding and hydrophobic interactions are involved in the adsorption/crosslinking of protein on nanoparticles surfaces which affect the structure and stability of proteins (Karim et al., 2012).

The present study is focused on preparation, characterization and immobilization of silver nanoparticles (AgNPs) with horseradish peroxidase (HRP). The characterization of AgNPs was performed by Fourier transform infrared (FT-IR), UV-vis and Transmission electron microscopy (TEM). Comet assay was also performed to measure the genotoxicity of AgNPs. β-cyclodextrin (β-CD) was used as a agent to reduce the toxicity of AgNPs. A comparative stability study of soluble and surface immobilized peroxidase on nanoparticles has been carried out against various physical and chemical denaturants. Immobilized HRP preparations have also been studied for their reusability towards development of biosensor in future.

2. Materials and Methods

2.1 Materials

Silver nitrate, ethanol, sodium borohydride, trisodium citrate dehydrate, dimethylformamide, bovine serum albumin, *o*-dianisidine HCl and HRP were obtained from Sigma Chem. Co. (St. Louis, MO USA). Other chemicals and reagents employed were of analytical grade.

2.2 Preparation of B-Cd Capped Silver Nanoparticles

$AgNO_3$ aqueous solution (1 mL, 0.01 M) and trisodium citrate dehydrate aqueous solution (1 mL, 0.03 M) were added to ultrapure water (97 mL), and sodium borohydride aqueous solution (1 mL, 1.79 mg/mL) was dropped into the solution with stirring. The formation of the AgNPs was confirmed by the solution changing to a yellow color after about 20 min, and this AgNP aqueous dispersion was used without further purification in the following procedure. β-CD and AgNPs were mixed in a 1:2 molar ratio, and the reaction mixture developed a buff color immediately, but we allowed the reaction to proceed for 24 h with continuous stirring. At this point, a precipitate was collected by centrifugation and washed with dimethylformamide (DMF) (4 x 50 mL) to remove free thiolated β-CD. The solid product was further washed (4 x 50 mL) with ethanol/water (90:10 v/v), collected by centrifugation, and dried at 60 °C under vacuum for 24 h. After this, the resulting powder was resuspended in distilled water to achieve a concentration of 9.85 mg/L (Chen et al., 2010).

3. Characterization of Silver Nanoparticles

3.1 TEM

TEM image were recorded on a JEOL-2000 microscope operated at 200 kV. For the TEM observation, samples were obtained by dropping 5 µl of solution onto carbon-coated copper grids. All the TEM images were visualized without staining.

3.2 UV-VIS Spectra

U.V-visible spectra were recorded by Perkin Elmer, Lambda 25 spectrophotometer. The measurements were done in a 10 mm quartz cell.

3.3 FT-IR SPECTRA

The FT-IR spectral studies were performed using KBr pelleting technique with Perkin Elmer System 2000 instrument in range of 400-4000 cm^{-1}. FT-IR analysis was carried out to determine the variation of the functional groups present in the native compounds and in the prepared complex.

3.4 Comet Assay

The comet assay was performed according to Singh et al. (1988) with minor modification (Duarte-Davidson et al., 2002). Briefly, treated lymphocytes with same concentration of AgNPs as well as the β-CD capped AgNPs were mixed with 50 µl of 1% low melting point agarose (Ameresco, LMA) at 37 °C and then on a fully frosted slides which were already covered with thin layer of 1% normal melting point agarose (Ameresco, NMA) to promote even and firm attachment of second layer. The slides were covered and then kept at 4 °C for 5 min to allow solidification of the agarose. After gently removing the coverglass, the slides were covered with a third layer of LMA and placed in the refrigerator for 5 min. The slides were submersed in the lysing solution, pH 10 were added fresh) for 1 h. The slides were then placed in unwinding buffer for 20 min and electrophoresis was carried out using the same solution and were stained with ethidium bromide.

3.5 Immobilization of HRP With β-CD Capped Silver Nanopartivles And Crosslinked By Glutaraldehyde

Finally, the obtained β-CD capped AgNPs were dissolved in sodium phosphate pH, 8.0 at 5 ^oC with different units of HRP to achieve best nanoparticles peroxidase ratio for the immobilization. The mixture was homogenized at 150 rpm for 20 min at 5 ^oC. The resulting solution was then centrifuged at 3000 rpm for 15 min. The filtrate was freeze-dried for 24 hours. This immobilized preparation was stored at 5 ^oC for further used. The immobilized preparation was crosslinked by 0.5% glutaraldehyde for 1 h at 5 ^oC as described by Karim and Adnan (2012). Finally crosslinked preparation was suspended in 100 mM sodium phosphate buffer, pH 8.0 and stored at 5 ^oC for further use.

3.6 Effect of PH on Soluble And AgNPs-HRP Preparation

Appropriate and equal amounts of soluble and crosslinked AgNPs- HRP preparation (2.0 U each) were taken to determine the activity of peroxidase in the buffers of different pH. The buffers used were glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0) and Tris-HCl (pH 8.0-10.0). The activity at pH-optimum was considered as control (100%) for the calculation of percent activity at other pH.

3.7 Effect Of Temperature On Soluble And AgNPs-HRP Preparation

The activity of soluble and crosslinked AgNPs-HRP preparation (2.0 U) was determined at various temperatures (20–80 °C) in 100 mM sodium phosphate buffer, pH 8.0. The activity at temperature-optimum was considered as control (100%) for the calculation of percent activity at other temperatures.

3.8 Effect of Urea On Soluble And AgNPs-HRP Preparation

Soluble and AgNPs-HRP preparation (2.0 U) were incubated with 4.0 M urea for varying times in 100 mM sodium phosphate buffer, pH 8.0 at 40 °C. Peroxidase activity was determined at the indicated time intervals. The activity of enzyme without incubation with urea was taken as control (100%) for the calculation of remaining percent activity.

3.9 Reusability of Nanoparticles Bound HRP

Immobilized preparation was taken in triplicates for assaying the peroxidase activity. After each assay the enzyme preparations were taken out, washed and stored in 100 mM sodium phosphate buffer, pH 8.0 overnight at 5 °C. The activity was assayed for 5 successive days. The activity determined for the first time was considered as control (100%) for the calculation of remaining percent activity after each use.

4. Measurement of Peroxidase Activity

Peroxidase activity was estimated from the change in optical density (A_{460} nm) at 40 °C by measuring initial rate of oxidation of *o*-dianisidine HCl (18 mM) by H_2O_2 (6.0 mM). The assay mixture with immobilized HRP was continuously stirred for the entire duration of assay (Karim and Adnan, 2012). One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 (mol of *o*-dianisidine HCl per min at 37 °C.

5. Statistical Analysis

Each value represents mean of three independent experiments performed in duplicates, with average deviations, < 5%. Data expressed in various studies was plotted using Sigma Plot-10.0 and Microsoft Excel 2003. *P*-values < 0.05 were considered statically significant.

6. Results and Discussion

6.1 Characterization of Silver Nanoparticles

In order to prove the above idea, β-CD-modified AgNPs were synthesized in analogy to the previous procedure. The preparation of β-CD, and β-CD modified AgNPs was monitored by infrared spectrum which demonstrated the successful formation of β-CD-modified AgNPs. As shown in Figure 1, the stretching vibration peak of S-H for β-CD was located at 2569 cm^{-1} and the stretching vibration peak of S-H disappeared when β-CD modified with AgNPs, which showed the apparent evidence of the successful formation of β-CD and anchoring β-SH-CD on AgNPs. This is consistent with the results reported in the literature (Liu et al., 1999). The FT-IR Fourier transform infrared spectroscopy measurements were carried out to identify the biomolecules and capping reagents in the reaction extract that were potentially responsible for the reduction of ions and the stability of the nanoparticles, respectively. AgNPs were prepared by sodium borohydride reduction of silver nitrate with a citrate stabilizer. The method ensured that the nanoparticles provided an intense surface plasmon band that could be used in the colorimetric assay. The negative citrate ions surrounding the AgNPs (-10 nm) provided enough electrostatic repulsion to overcome the attractive hydrophobic and van der Waals forces and, in doing so, caused the AgNPs to remain stable and dispersive in an aqueous solution (Ono et al., 2004).

Taking into account the fact that the optical properties of the aqueous suspension of metal nanoparticles are closely associated with their shape (Wiley at al., 2006), we recorded ultraviolet-visible absorption spectra for the synthesized AgNPs in aqueous suspension and the absorption peak of the formed nanoparticles was recorded at 400 nm (Figure 2). The ultravioletvisible absorption spectra of the silver nanoparticles harvested after 24 hours of reaction showed the perfect patterns with an increase in the concentration of the extract in the reaction mixture as reported previously by some earlier workers (Chen et al., 2010). Figure 3 represents a transmission electron micrograph of silver nanoparticles synthesized by the reaction of aqueous ions. This is in concordance with the shift in the ultraviolet spectra of the silver nanoparticles. Our data was in the support of recently published paper related to the application of the synthesized silver nanoparticles in the determination of isomers (Chen et al., 2010).

6.2 DNA Breakage In Comet Assay

The AgNPs as well as the β-CD-capped AgNPs were tested for DNA breakage in intact lymphocytes (comet assay). Photographs of comets exhibited no breakage in case of β-CD-capped AgNPs treated DNA (Figure 4a). The results of the other set of experiment conforms the toxicity AgNPs as shown in Figure 4b. Recently Chen et al. (2010) shows the toxicity of chemically synthesized AgNPs same as reported in this manuscript.

6.3 Preparation Of β-CD-HRP Complex

The β-CD has been exploited for immobilization of peroxidase from horseradish. Binding of HRP on β-CD-capped AgNPs. Enzyme was maximally adsorbed at pH 8.0 and retained higher U of HRP g^{-1} of β-CD with an very high effectiveness factor (η). Crosslinking of adsorbed HRP by glutaraldehyde resulted in a loss of only 1% activity. There are various supports used for the immobilization of enzymes but all these supports have some demerits such as low stability, less effective and more expensive immobilized reparation, leaching of enzymes from support etc (Bhunia et al.., 2001). In this case, the yield of immobilization was quite superior over other methods used for the immobilization of (Levy, et al., 2003).

6.4 PH Activity Profiles Of Soluble AgNPs-HRP Preparation

Nanoparticle bound HRP and native enzyme showed broadening in pH optimum (Figure 5). Immobilized enzyme retained significantly higher enzyme activity on both sides of pH optimum in comparison to free enzyme. The pH optimum of immobilized enzyme had slight difference from pH 7.0 to 9.0, although soluble enzyme showed pH optimum at pH 8.0. Recently, Karim and Husain (2011) performed a study for the immobilization of bitter gourd peroxidase (BGP) on fly ash. The broadening of pH profile was observed in the case of immobilized peroxidase.

6.5 Temperature Activity Profiles Of Soluble And Immobilized HRP

The AgNPs bound HRP preparation had no change in temperature-optima as compared to its soluble counterpart. Both the preparations exhibited temperature optima at 40 ^{o}C. However, AgNPs bound HRP preparation retained significantly greater fractions of catalytic activity at high temperatures (Figure 6). Some earlier workers also reported the same finding in the case of BGP immobilization on fly ash (Karim and Husain, 2011).

6.6 Urea Mediated Denaturation of Soluble and Immobilized HRP

Nanoparticles bound HRP was more resistant to inactivation induced by 4.0 M urea compared to its soluble counterpart. Exposure of soluble enzyme with 4.0 M urea for 2 h resulted in the loss of 70% activity whereas the immobilized enzyme retained more than 80% of the initial enzyme activity (Figure 7). Urea (4.0 M) is a strong denaturant of some proteins and it irreversibly denatures enzymes (Fatimah and Husain, 2007). However, action mechanism of urea on protein structure has not yet been completely understood, several earlier findings have suggested that protein could be unfolded by direct interaction of urea molecule with a peptide backbone via hydrogen bonding and/or hydrophobic interaction, which contributes to maintenance of protein conformation.

6.7 Reusability of Immobilized BGP

Reusability of immobilized preparations of HRP has been shown in Figure 8. After 5th repeated use, the silver nanoparticles bound HRP retained 97% of the original activity. Enzyme reuse provides a number of cost effective advantages that are often an essential prerequisite for establishing an economically viable enzyme-

catalyzed process. The activity loss during repeated use might be due to the inhibition of enzyme by product or by leaching of enzyme from the gel bead or due to damage of the beads.

7. Conclusion

The results presented in the present work showed that β-CD- capped-AgNPs with HRP was more stable as compared to soluble HRP against various types of denaturants. Thus this preparation retained more activity in its immobilized form and this perpetration has no problem of substrate and product diffusion. Thus, such immobilized enzyme preparation would be used for the preparation of biosensors for the detection/monitoring of pollutants present in wastewater. These immobilized complex used in a reactor would not affect the flow rate of the column. In view of these advantages offered by surface immobilized HRP preparation, we can suggest that this immobilized preparation would be most suitable for the treatment of huge volume of effluents/aromatic pollutants.

Acknowledgments

The authors would like to thank Universiti Sains Malaysia for financial support through USM RU grant (Grant no. 1001/PKIMIA/815160) to Dr. Zoheb Karim.

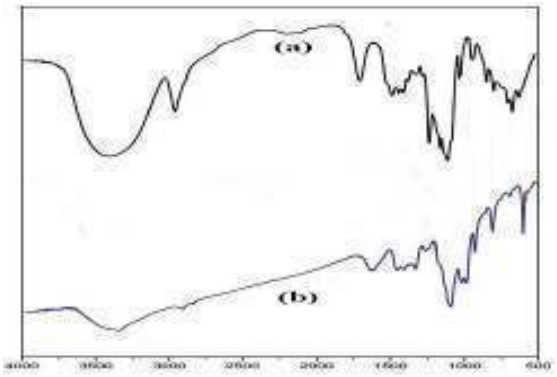

Figure 1 :FT-IR spectra of (a) β-CD, (b) β-CD modified AgNPs

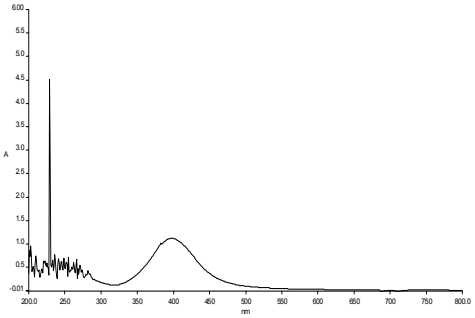

Figure 2: U.V-visible spectra of AgNPs

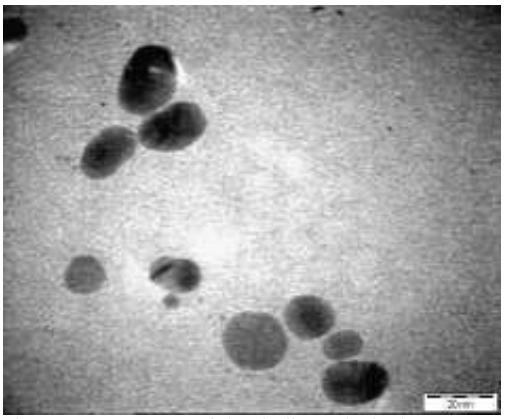

Figure 3 : TEM spectra of chemically synthesized AgNPs

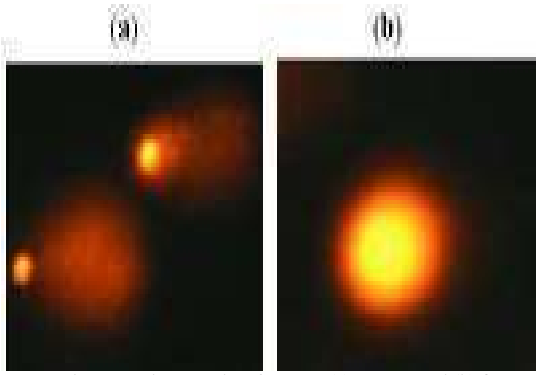

Figure 4: Photographs of comets (a) AgNPs and (b) β-CD capped AgNPs

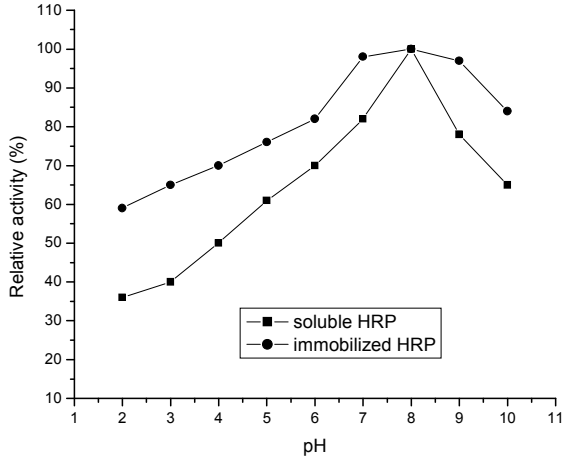

Figure 5 : pH activity profile of soluble and immobilized HRP preparation

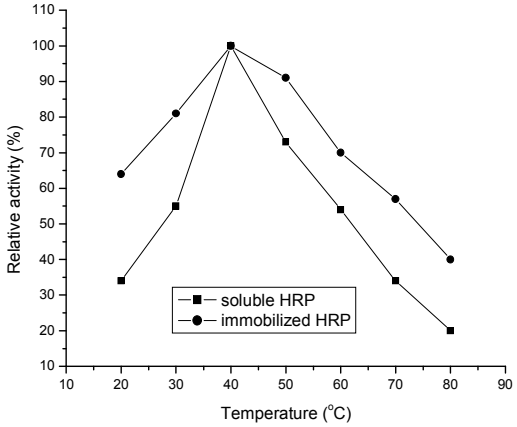

Figure 6: Temperature activity profile of soluble and immobilized HRP preparation

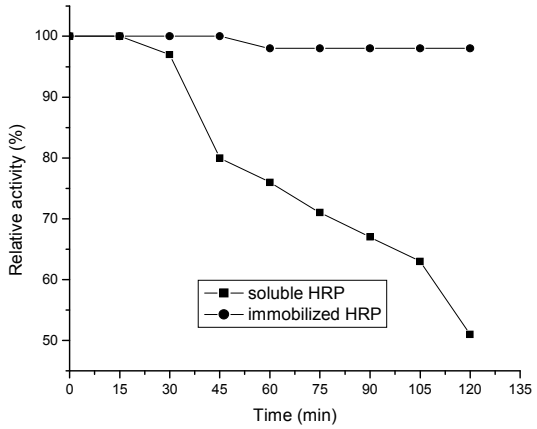

Figure 7: Inactivation of soluble and immobilized preparation by 4.0 M urea

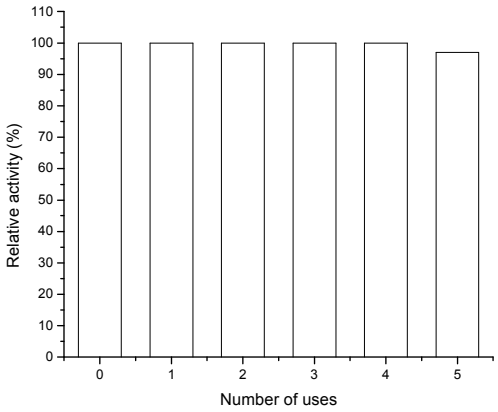

Figure 8: Reusability of immobilized HRP

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