

# Peroxidase assisted biosynthesis of silver and gold nanoparticles: Characterization and computational study

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## ABSTRACT

In this paper, we described a simple and single step procedure for the synthesis of horseradish peroxidase enzyme (HRP) capped silver and gold nanoparticles. HRP, a heme-containing enzyme utilizes hydrogen peroxide to oxidise a wide variety of organic and inorganic compounds. The biosynthesized nanoparticles were characterized by means of UV-VIS spectroscopy, Dynamic light scattering (DLS), Transmission electron microscopy (TEM), X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR). FTIR study confirms the presence of peroxidase enzyme on the nanoparticles. Computational studies reveal that exposed amino acids (*viz* serine, threonine, arginine and glycine) play key role in reduction and as well as stabilization of nanoparticles. The HRP assisted silver and gold nanoparticles retained its biological activity in the nanoparticles. The study indicates that Peroxidase which is found in almost all the plants can be used for the large scale synthesis of nanoparticles. Moreover additional attraction is the retention of the enzymatic activity on the nanoparticles. In a single step reaction enzyme is catalysing and in doing so it gets immobilized on it. The integration of biomolecules to nanoparticles is a tedious method mainly due to the surface of nanoparticles. Functionalization of noble metal nanoparticles with biomolecules (e.g., protein and DNA) is in demand because such systems possess numerous applications in catalysis, delivery, therapy, and imaging, sensing and controlling the structure of biomolecules. Computational study highlighted the amino acids which are interacting with the metal ions, thus synthetic peptides can also be designed to synthesize the metal nanoparticles. Copyright © 2015 VBRI press.

**Keywords:** Peroxidase; silver nanoparticles; gold nanoparticles; computational study.



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## Introduction

Metal nanoparticles such as gold and silver are a focus of interest because of their huge potential in nanotechnology. Today, these materials can be synthesized and modified by various approaches [1, 2]. The biosynthetic approach of

metal nanoparticles synthesis has an edge over the commonly used physical and chemicals methods as it is considered environmentally safe and less expensive as compared to these methods [3]. Moreover, increased use of these nanoparticles in biomedicine requires that they should be free from any toxic chemicals. Biosynthetic process involves the use of plant / plant extract and microorganisms [1, 3]. The nanoparticles can be synthesized either extracellularly or intracellularly. It has been suggested that the biomolecules present in the plants /microorganisms play important role in catalysing the synthesis. Purified biomolecules like-Proteins, amino acids, sugars, DNA, have been employed for the synthesis of metal nanoparticles but still the mechanism of synthesis is not yet fully understood [4-7]. Recent advancement to this technique is the use of enzymes as they are commercially available and have diverse biological functions. Further, enzymes/proteins consist of a number of amino acids that can act as reducing as well as stabilizing agent [8]. By using purified enzymes as reducing agents the interaction of the metal ions with the enzyme can be clearly understood. Broadly, protein-nanoparticle interactions can occur either through free amine group or cysteine residues in proteins and via the electrostatic attraction of negatively charged carboxylate groups in enzymes [9]. To study the interaction and role of different amino acids, a well characterized protein bovine serum albumin (BSA) was taken as a model protein for the synthesis of gold nanoparticles [10]. It was observed that BSA could synthesize Au nanoplates under acidic conditions at physiological temperature [10]. Furthermore, BSA can also synthesize very small gold nanoparticles or nanoclusters (<1 nm) under alkaline conditions [11]. Several other enzymes have been used successfully for the synthesis of gold nanoparticles like  $\beta$ -glucosidase [12], trypsin [13], pepsin [14] and laccase [15]. Silver nanoparticle synthesis has also been reported using pure enzymes like lysozyme [16] and a fibrinolytic enzyme produced by *Bacillus cereus* NK1 [17]. Rangnekar and their colleagues [18] investigated different enzymes and claimed that the enzymes which have free and exposed thiol groups were able to catalyze the synthesis of gold nanoparticles. Das et al. [19] used lysozyme enzyme for synthesis of nanoparticles of silver, gold and their alloy. They also depict that amino acids like phenylalanine, tyrosine, tryptophan, and histidine are the major players during synthesis as well as stabilization [19]. Ravindra, 2009, synthesized gold nanoparticles utilizing serrapeptase that serves as both reducing and stabilizing agent; they also reported that lysine is involved in reduction and stabilization of gold nanoparticles [20]. Gupta et al. [21] describes the synthesis of BSA capped silver nanoparticles with exposed thiol group of BSA and also with the modified thiol with DNTB (5-5'-Dithiobis (2-nitrobenzoic acid). They have found that modified BSA and unmodified BSA are equally good as the reducing agent [21]. Earlier in our lab we described the biosynthesis of silver nanoparticles using alpha amylase enzyme from *A. oryzae* which has one free exposed thiol group [6] and from neem leaf extract containing alpha amylase activity [22]. The nanoparticles obtained from neem leaf extract retained 85% enzyme activity. Thus, the enzyme catalysis the synthesis of silver nanoparticles and during this synthesis it gets

immobilized on the silver nanoparticles. This study prompted us to investigate other enzymes also for the synthesis of metal-biomolecule hybrids. The peroxidase enzymes are useful in biosensors, diagnostic kits and waste water treatment. In the present study, HRP a well characterized enzyme (devoid of any free thiol group) in terms of its three dimensional structure was used for biosynthesis of silver and gold nanoparticles; and their computational study was carried out to understand the possible amino acids involved for the biosynthetic process. This study will give some insight to design synthetic peptides for the synthesis of silver and gold nanoparticles and this is purely a green approach.

## Experimental

### Materials

Horseradish Peroxidase C (HRP), Tetramethylbenzidine (TMB) and Auric chloride were procured from Sisco Research Laboratories (SRL), Mumbai, India. Silver nitrate and Tris buffer were purchased from Merck India Ltd. All other chemicals and solvents used were of analytical grade and used without further purification.

### Methods

#### Synthesis of silver and gold nanoparticles by HRP

Synthesis of the silver and gold nanoparticles was carried out by incubating 1 ml of HRP enzyme (1 mg/ml for silver and 4mg/ml for gold in Tris-HCl buffer, pH 8.0) and 9 ml of freshly prepared aqueous solution of silver nitrate (1mM) / auric chloride. Solutions were kept at 25°C and the synthesis of nanoparticles was monitored by UV-VIS spectroscopy. The synthesized nanoparticles were purified as described earlier [6].

#### Determination of enzyme activity

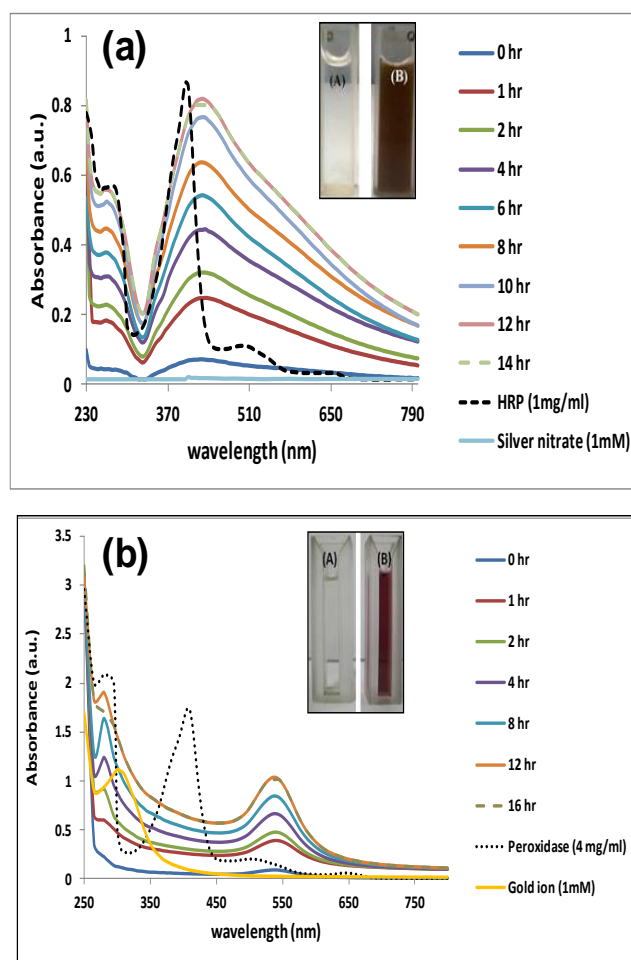
Peroxidase activity in free enzyme as well as in nanoparticles was measured by using spectrophotometric assay with TMB (Tetramethylbenzidine) as a substrate [23]. The nanoparticles were continuously shaken for the entire duration of assay. One unit of enzyme is defined as the amount of enzyme that catalyzes the oxidation of 1  $\mu$ mol of TMB per min at 25 °C into coloured product ( $\epsilon=5.9 \times 10^4$  /mole/cm at 450 nm).

#### Characterization of nanoparticles

Dynamic light scattering (DLS) measurements were carried out using the Spectroscatter RiNA, GmbH class3B at 20°C for 10 cycles. Samples for transmission electron microscopy (TEM) were prepared by drop coating purified silver nanoparticles on to carbon-coated copper TEM grids. TEM measurements were performed on a JEOL, F2100 instrument operated at an accelerating voltage at 200kV. XRD patterns were recorded by X'Pert Pro X-ray diffractometer (PANalytical BV) by operating X-ray tube at 45 kV and 35 mA and radiation used was Cu- K $\alpha$ . Fourier-transform infrared (FTIR) spectra were recorded with a Shimadzu, FTIR spectrophotometer between 4000 and 400  $\text{cm}^{-1}$ , with a resolution of 4  $\text{cm}^{-1}$ .

## Computational studies

Crystal structure of horseradish peroxidase C at 2.15Å resolution (1ATJ) was obtained from protein data bank (PDB) source being *Armoracia rusticana*. Important information like metal binding, active site, binding sites, sequence conservation and relative solvent accessibility were extracted from uniprot database link: <http://www.uniprot.org/uniprot/P00433>, PDBSum, Findsitemetal program, ConSurf and ASAView. Structural study was carried out on the peroxidase enzyme [pdb id: 1ATJ (chain A)] as shown in **Fig. 6 (a)**. Solvent accessibility analysis was carried by ASAView [24]. Absolute surface area (ASA) of each residue is provided by DSSP has been transformed to relative values of ASA in this program. By using visualization program called PyMOL, we have represented all exposed residues like Ser, Thr, Arg, Gly, Lys and Tyr in the HRP enzyme and further it is also supported by some already reported studies which reflect the importance of these types of exposed amino acids in the function of enzyme. To infer conservation and secondary structure of these exposed residues we did ConSurf analysis also [25].



**Fig. 1.** (a) UV-VIS spectra for silver nanoparticles as a function of reaction time. Inset showing visual observation of the silver nanoparticles formation. (A) beginning of the reaction and (B) after 12 hr. (b) UV-VIS spectra of gold nanoparticles as a function of reaction time. Inset showing visual observation of the gold nanoparticles formation. (A) beginning of the reaction and (B) after 12 hr.

## Results and discussion

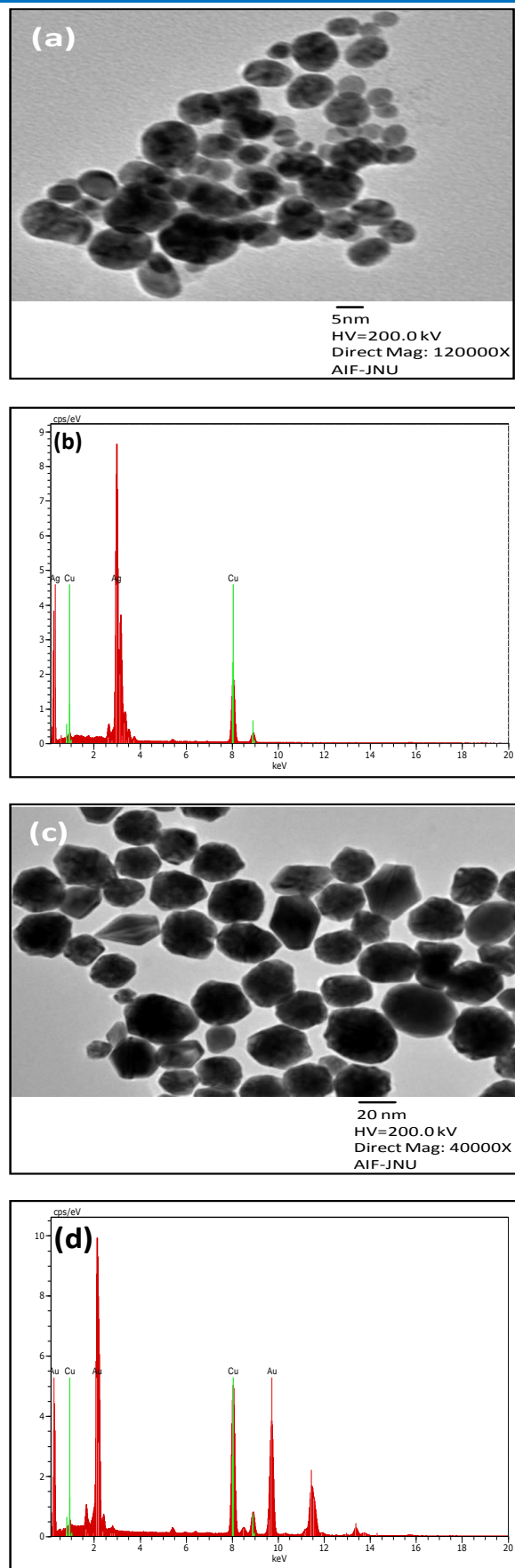
The horseradish (*Armoracia rusticana*) is a hardy perennial herb cultivated in temperate regions of the world for its roots. These roots are also a rich source of peroxidase, a heme-containing enzyme. Production of peroxidase from horseradish roots occurs on a large scale because of the commercial uses of the enzyme, for example as a component of clinical diagnostic kits and for immunoassays. In this study horseradish peroxidase enzyme was used for the in vitro synthesis of silver and gold nanoparticles. When the horseradish peroxidase enzyme solutions were mixed separately with freshly prepared aqueous solution of silver nitrate and auric chloride solution, the colour of mixture turned yellowish brown in case of silver; and pink in case of gold. **Fig. 1a** shows the UV-VIS spectra of silver nanoparticles with effect of time. The absorption centred at 430 nm surface plasmon resonance (SPR) of silver nanoparticles increased in intensity up to 12 hrs (**Fig. 1 a**) and after that it remains same. In the case of gold nanoparticles the absorption peak is around 540 nm which is known to arise due to surface plasmon oscillations specific to gold nanoparticles; it also increases up to 12 hrs (**Fig. 1b**). The increase in surface Plasmon resonance with increase in time has been reported earlier also [6, 27]. Control experiments were carried out in which the spectra of silver nitrate and auric chloride solutions were taken at different time interval; they did not show any peak indicating that peroxidase enzyme is responsible for the synthesis of silver as well as gold nanoparticles. Thus, the concentration of bioreactants and time of reaction are crucial parameters in the biosynthesis process.

Further, the UV-VIS spectra of only HRP shows an intense peak around 280 nm characteristic of proteins and with two major peaks at around 405 nm (the Soret band) and 515 nm [26]. The 280 nm region peak is also observed during the synthesis of silver and gold nanoparticles (**Fig. 1 a and b**) which also increases as the synthesis time is increased. Similar observations i.e., increase in absorbance at 280 nm with increase in time of synthesis have been reported earlier also [6, 27]. The data suggests that protein concentration on nanoparticles increases with increase in time of synthesis.

The sizes of the silver and gold nanoparticles were observed by TEM. TEM images **Fig. 2(a)** showed that the silver nanoparticles are quasi-spherical in shape and are in the range of 5–40 nm. Energy dispersive X-ray confirmed that these nanoparticles are composed of only silver (**Fig. 2b**). The size of gold nanoparticles was estimated around 20–80 nm having anisotropic shapes (**Fig. 2c**). Silver nanoparticles formed are smaller than the gold nanoparticles indicate that the size depends upon the interaction of the protein/enzyme with the metal ions. In order to know the elemental composition, EDX was carried out for gold nanoparticles. Elemental gold signal confirms presence of gold (**Fig. 2d**).

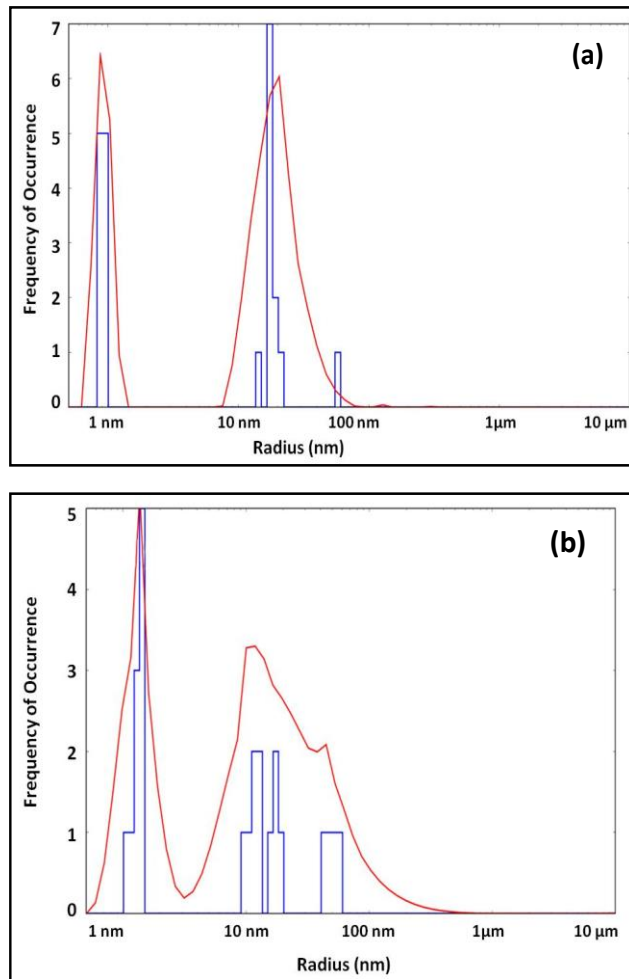
Particle size estimation and distribution in the aqueous solution of the silver and gold nanoparticles were determined by DLS as shown in **Fig. 3 (a)** and **(b)** which is in close agreement with TEM studies.





**Fig. 2.** Transmission Electron Microscopy (TEM) images of purified nanoparticles. (a) TEM image of silver nanoparticles (b) EDX spectrum of silver nanoparticles (c) TEM image of gold nanoparticles and (d) EDX spectrum of gold nanoparticles.

FTIR spectra of native HRP enzyme along with HRP assisted silver and gold nanoparticles are shown in **Fig. 4**. The intense peak around  $1650\text{ cm}^{-1}$  (Amide I peak) [28, 29] is observed in all the FTIR spectra, HRP only (**Fig. 4a**), silver nanoparticles (**Fig. 4b**) and gold nanoparticles (**Fig. 4c**). The broad absorption band appears in the range of  $3000\text{--}3400\text{ cm}^{-1}$  is the attestation of association intermolecular hydrogen bonds arising from  $\text{-NH}_2$  and  $\text{-OH}$  groups in peroxidase enzymes [29].



**Fig. 3.** Dynamic Light Spectrophotometer (DLS) result showing mean particle size (a) silver nanoparticles and (b) gold nanoparticles.

This further confirms that enzyme HRP is serving as a reducing and capping agent during the synthesis. The crystalline nature and phase purity of the silver and gold nanoparticles were analysed and confirmed by XRD (**Fig. 5 a and b**). XRD of powdered silver nanoparticles reveals peaks at (111), (200), and (220) are in agreement with the standard value of joint committee for powder diffraction set (JCPDS) data card no. 04-0783. Whereas, the XRD spectra of gold nanoparticles give peaks at (111), (200), (220) and (311) matched with the standard joint committee for powder diffraction set (JCPDS) data card no. 04-0784.

The stability of nanoparticles was checked after keeping them at  $4^\circ\text{C}$  for six months and then again the UV spectra were recorded. No change in the spectra was observed in both the nanoparticles (silver and gold). The biological activity also did not change after storage at  $4^\circ\text{C}$  for six

months. These results indicate that biosynthesized nanoparticles are quite stable and can be stored for a long period of time.

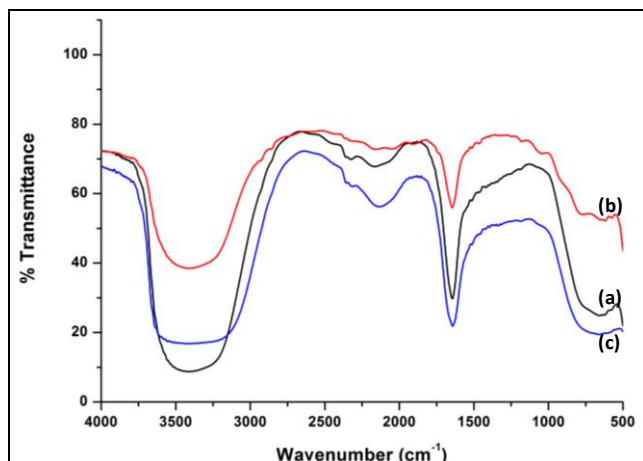


Fig. 4. FTIR spectra of (a) only HRP enzyme (b) silver and (c) gold nanoparticles.

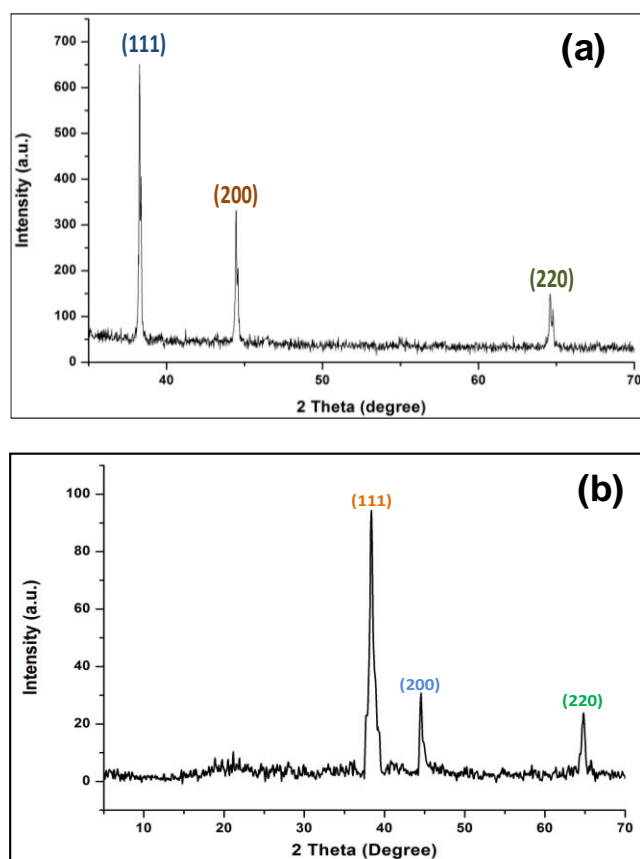


Fig. 5. XRD profile of purified powder of (a) silver and (b) gold nanoparticles synthesized by HRP enzyme.

Horseshoe peroxidase isoenzyme C comprises a single polypeptide of 308 amino acid residues and the sequence was determined by Welinder [30]. The N terminal residue is blocked by pyroglutamate and the C terminus is heterogenous. There are 4 disulphide bridges between cysteine residues 11–91, 44–49, 97–301 and 177–209; and a buried salt bridge between Asp99 and Arg123. To study amino acids involved in the biosynthesis process, we have

calculated and tabulated corresponding absolute surface area (ASA), secondary structure and amino acid conservation for all exposed residues like Ser (10, 28, 60, 89, 116, 306); Thr (59, 199, 200, 204, 227, 293); Arg (27, 62, 93, 153, 264); Gly (113, 191, 207, 213, 292, Lys (232, 174); His170 and Tyr185 in the enzyme (Fig. 6 a and b).

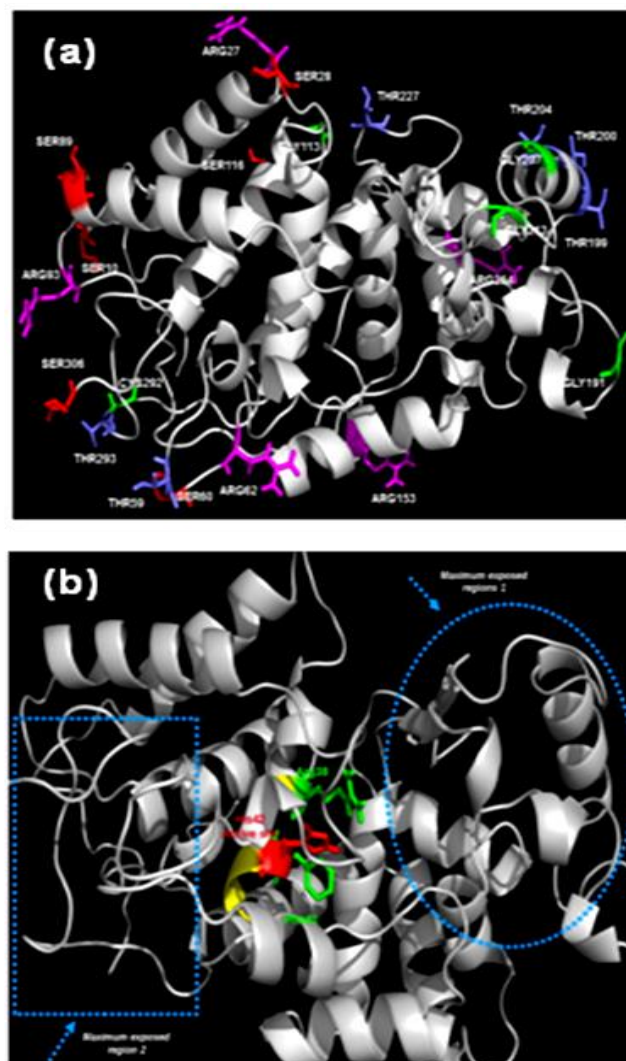


Fig. 6. (a) HRP showing exposed residues like Ser, Thr, Arg, Gly, Lys and Tyr and (b) Maximum exposed region of HRP which are far away from active site (in red).

All the above mentioned residues are away from the active site. Table 1 (a, b, c, d, e) shows the detailed information for each exposed residue. Out of all exposed residues, Ser306 and Thr59 showed the maximum exposure being 100% exposed and can be available to make possible important interactions with silver and gold ions. Ser306 lies in coil region and is not well conserved. Other Ser residues like at 89 and 116 position also are exposed but they are in alpha helix and poorly conserved (Table 1a). If we see at Arg, Thr, Gly residues some are exposed about 50% but these residues are also important to make interaction with Silver ions (Table 1. b, c, d). Lys232, 174, His170 and Tyr185 are partially exposed in comparison to other type of residues (Table 1e). There is also an important amino acid that is cysteine and there are 8 cysteine's in this enzyme but

they are not accessible for interaction which obviously decrease the chances for interaction with silver and gold ions. From the residue conservation study we have predicted that Thr293, Gly113, Gly292 and His170 are highly conserved. We can assume that these residues might be playing role in stability of enzyme rather taking part in enzyme function.

**Table 1 (a).** ASA analysis of fully exposed Ser residues HRP and amino acid conservation.

| S.No. | Residue (Pdb:1ATJ) | ASA% (ASAview) | Secondary structure |
|-------|--------------------|----------------|---------------------|
| 1.    | Ser10              | 58.9           | Coil                |
| 2.    | Ser 28             | 85.3           | Coil                |
| 3.    | Ser 60             | 81.1           | Coil                |
| 4.    | Ser 89             | 86.2           | Alpha helix         |
| 5.    | Ser 116            | 81.9           | Alpha helix         |
| 6.    | Ser 306            | 100            | Coil                |

**Table1 (b).** ASA analysis of fully exposed Thr residues HRP and amino acid conservation.

| S.No. | Residue (Pdb:1ATJ) | ASA% (ASAview) | Secondary structure |
|-------|--------------------|----------------|---------------------|
| 1.    | Thr59              | 100            | Coil                |
| 2.    | Thr199             | 74.3           | Alpha helix         |
| 3.    | Thr200             | 72.1           | Alpha helix         |
| 4.    | Thr204             | 57.7           | Alpha helix         |
| 5.    | Thr227             | 65.6           | Coil                |
| 6.    | Thr293             | 74.3           | Coil                |

**Table 1 (c).** ASA analysis of fully exposed Arg residues HRP and amino acid conservation.

| S.No. | Residue (Pdb:1ATJ) | ASA% (ASAview) | Secondary structure |
|-------|--------------------|----------------|---------------------|
| 1.    | Arg27              | 79             | Coil                |
| 2.    | Arg62              | 62             | Coil                |
| 3.    | Arg93              | 56.8           | Coil                |
| 4.    | Arg153             | 55.9           | Alpha helix         |
| 5.    | Arg264             | 51.5           | Alpha helix         |

So, ASA analysis shows that the trend of exposed residues is like this: Ser (6 residues) > Thr (6) > Arg(5) > Gly (5) > Lys (2) > His (1) > Tyr (1).

Synthesis of nanoparticles using ser, arg, lys, thr and tyr has been reported earlier also [31-34]. Peroxidase attached to nanoparticles has retained the biological activity and shows that amino acids which were involved with the metals (silver and gold) during synthesis are not active site residues. It has been reported that enzymes immobilized on nanoparticles are stable as compared to their soluble counterparts and can reused over a longer period of time. Therefore, the enzyme HRP immobilized on metal nanoparticles can be used as biosensors, waste water treatment etc. To generalize this methodology for the synthesis of nanoparticles and simultaneous immobilization of enzymes on it one has to study the interaction of amino acid residues with metal. Further work by blocking the

exposed residues of the enzyme can give the exact mechanism and the kind of interaction between enzyme and the metal. The biological responses to nanoparticles are highly affected by the main forces at the bionano interface and also by the intrinsic characteristics of the nanoparticles. Therefore, a better understanding of the nanoparticle protein complex is essential to the development of functional and safe nanoparticles.

**Table 1 (d).** ASA analysis of fully exposed Gly residues HRP and amino acid conservation.

| S.No. | Residue (Pdb:1ATJ) | ASA% (ASAview) | Secondary structure |
|-------|--------------------|----------------|---------------------|
| 1.    | Gly113             | 62.3           | Coil                |
| 2.    | Gly191             | 57.2           | Coil                |
| 3.    | Gly207             | 73.7           | Alpha helix         |
| 4.    | Gly213             | 52.1           | Coil                |
| 5.    | Gly292             | 64.8           | Coil                |

**Table 1 (e).** ASA analysis of partially exposed other important residues HRP and amino acid conservation.

| S.No. | Residue (Pdb:1ATJ) | ASA% (ASAview) | Secondary structure |
|-------|--------------------|----------------|---------------------|
| 1.    | Lys232             | 37.4           | Alpha helix         |
| 2.    | Lys174             | 37.9           | Alpha helix         |
| 3.    | His170             | 29.7           | Alpha helix         |
| 4.    | Tyr185             | 22.9           | Coil                |

## Conclusion

Metal nanoparticles (silver and gold) were synthesized using an enzyme peroxidase with the retention of the enzymatic activity in the nanoparticles. The amino acids involved in the synthesis were studied by ASA analysis. The exposed amino acid residues like ser, thr, arg, gly and lys might be involved in the reduction of metal to nanoparticles. Based on the amino acid and metal interaction studies one can design multifunctional peptides, which can catalyse the synthesis of metal nanoparticles and also gets integrated to them. Such hybrid systems have dual advantage—properties of metal and as well as of biomolecule. Such systems attract substantial interest in the rapidly developing area of nanotechnology.

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