Self-assembly of soybean peroxidase nanohybrid for activity enhancement and dye decolorization: experimental and computational studies

Sunil Bhapkar, Navanath Kumbhar, Praful Sharma, Shweta Jagtap, Rajesh Gacche, Vitthal T. Barvkar, Dnyaneshwar Sonunee, Kailas D. Sonawane and Umesh Jadhav

Department of Microbiology, Savitribai Phule Pune University, Pune, Maharashtra, India; Department of Biotechnology, Savitribai Phule Pune University, Pune, Maharashtra, India; Department of Instrumentation Science, Savitribai Phule Pune University, Pune, Maharashtra, India; Department of Botany, Savitribai Phule Pune University, Pune, Maharashtra, India; CHROMEIN, Pune, Maharashtra, India; Department of Biochemistry, Shivaji University, Kolhapur, Maharashtra, India

Communicated by Ramaswamy H. Sarma

ABSTRACT

The soybean peroxidase (SBP) mediated nanohybrid [SBP-Cu₃(PO₄)₂·3H₂O] synthesis was carried out in the present study. The scanning electron microscopy (SEM) analysis showed a characteristic flower-like hierarchical structure of the SBP-nanohybrid. The mechanism of SBP-nanohybrid formation was elucidated using computational approaches. The predicted Cu²⁺ binding sites followed by molecular docking studies showed the two lowest energy (−4.4 kcal/mol and −3.56 kcal/mol) Cu²⁺ binding sites. These two binding sites are located at the opposite position and might be involved in the formation of SBP-nanohybrid assemblies. Further, these sites are different than the catalytic active site pocket of SBP, and may facilitate more substrate catalysis. Obtained computational results were confirmed by in-vitro guaiacol oxidations studies using SBP-nanohybrid. The effect of various parameters on SBP-nanohybrid activity was studied. The pH 7.2 was found optimum for SBP-nanohybrid activity. The enzyme activity increased with an increase in temperature up to 50 °C temperature and then decreased with an increase in temperature. Around ~138% enhanced activity was recorded using SBP-nanohybrid compared to crude SBP. Also, the SBP-nanohybrid showed around 95% decolorization of methylene blue (MB) in 1 h and the MB degradation was confirmed by high-pressure liquid chromatography analysis (HPLC).

KEYWORDS

Soybean peroxidase; nanohybrid; molecular docking; binding sites; dye decolorization

Introduction

Enzymes are excellent biocatalysts. They have high catalytic activity, substrate specificity, low toxicity, and water-solubility, etc. (Altinkaynak et al., 2016; Lee et al., 2015). Due to these properties, enzymes offer promising applications in chemistry, biochemistry, medicine, pharmaceutical science, food, and textile (Altinkaynak et al., 2016; Mateo et al., 2007). Despite these unique properties of enzymes, their use at the industrial level is limited due to certain drawbacks. The extreme temperature, pH, and the presence of organic solvents hamper the enzyme activity and sometimes denature the enzyme (Y. Zhang et al., 2015). They cannot be recovered from reaction mixtures which makes their reuse highly impossible (Sardar & Ahmad, 2015). The low reproducibility and the requirement for complex, expensive purification processes are other inherent drawbacks of free enzymes (Mateo et al., 2007). Several researchers used immobilization techniques to overcome these issues and various carriers are available for enzyme immobilization. Enzyme immobilization offers several advantages (Sheldon & van Pelt, 2013). It enhances enzyme stability, improves tolerance to adverse environmental conditions, and gives a longer working cycle (Köhler et al., 2013). It also simplifies the removal and recovery of enzymes and facilitates continuous and repeated use. It offers higher catalytic turnover, prevention of interactions with interfaces, rigidification via multipoint covalent attachment, prevention of subunit dissociation, resistance to inhibitions, and even an improved purity (Valencia et al., 2018). However, enzyme immobilization is a highly critical process. It is difficult to achieve the desirable extent of enzyme immobilization using the conventional processes (Gonçalves et al., 2019). These processes suffer from loss of catalytic activity due to the use of organic solvents during immobilization, the blocking of the active site of the enzyme, and an unfavorable change in enzyme conformations. They exhibit restricted flexibility and mass transfer limitations between the enzyme and substrate (Bilal et al., 2019; Hanefeld et al., 2013). Therefore, the development of an alternate immobilization approach is essential to overcome the deficiencies of conventional immobilization (Altinkaynak et al., 2016; Yu et al., 2015). Recently, Ge et al. (2012) developed a novel, one-step enzyme immobilization technique. In this study, phosphate-buffered saline (PBS), copper sulphate (CuSO₄), and
protein were mixed to form an organic-inorganic hybrid nanoflower/nanohybrid (Ge et al., 2012). Hence, characteristic hierarchical porous nanostructure provided a high surface-to-volume ratio and decreased mass transfer limitation (Cui & Jia, 2017; Liu et al., 2019). This helped to increase the loading capacity. This unique structure gave more stability to the enzyme and also increased the catalytic activity (Liu et al., 2019). Considering these benefits several researchers developed nanohybrids using various enzymes and metals (Altinkaynak et al., 2016, 2017; Nadar et al., 2016; Somturk et al., 2019). This helped to increase the loading volume ratio and decreased mass transfer limitation (Cui & Jia, 2017; Liu et al., 2019). This approach is often referred to as nanohybridization, where the protein and metal are covalently or non-covalently linked to form a composite material.

**Materials and methods**

**Materials**

All the chemicals were purchased from Sigma-Aldrich. The soybeans were purchased from the local market.

**Extraction of crude SBP**

In the present study, the soybean seeds were used as a source of SBP and SBP was used for the synthesis of nanohybrid. A 100 g of soy bean seeds were soaked in 400 ml of distilled water and were kept overnight. The seeds were then homogenized in a mixer grinder. Further, the homogenate was centrifuged at 4226 xg for 20 min at 4°C followed by filtration. The filtrate was then heated in a water bath at 60°C for 3 min to inactivate catalase present in the extract and cooled by placing it in an ice bucket for 10 min (Ambreen et al., 2000). Finally, this supernatant was considered as crude SBP extract.

**Synthesis of SBP-nanohybrid**

The synthesis of nanohybrid was carried out with some modification using the method described by Ge et al. (2012). The crude SBP extract was used for nanohybrid synthesis. In our previous study, Acidiphilium acidophilum (NCIM 5344) (A. acidophilum) was used for bioleaching of copper (Cu) from waste printed circuit board. A. acidophilum was grown in 9 K medium with sulfur as substrate. During its growth A. acidophilum oxidized sulfur to sulfuric acid. The A. acidophilum spent medium containing sulfuric acid was collected and used for bioleaching experiments. During the process Cu was leached from the surface of waste printed circuit board and solubilized (Chandane et al., 2020). This bioleached Cu solution was used for the synthesis of the SBP nanohybrid. A 100 mM CuSO4 stock solution was prepared. Then SBP nanohybrid was synthesized by mixing 1 ml CuSO4 stock solution with 50 ml of PBS (pH 7.4, 5 mM). This resulted in 2 mM CuSO4 concentration in reaction mixture. To this reaction mixture, a crude SBP enzyme solution (200 µl) was added and the reaction mixture was then incubated at 4°C for three days. At the end of three days, the blue color precipitate was observed at the bottom of each reaction tube. This precipitate (nanohybrid) was collected via centrifuge at 6797 xg for 15 min, washed with buffer for three times, and then dried at room temperature (Altinkaynak et al., 2018; Yu et al., 2015). In another experiment, the effect of varying CuSO4 concentration (1–4 mM) on SBP nanohybrid structure was studied.

**Characterization of SBP-nanohybrid**

The scanning electron microscopy (Nova NanoSEM 450, Resolution 1 nm at 15 KV and 30pa)-energy dispersive spectroscopy (Bruker XFlash 6i30, Resolution 123 eV at Mn Kα)(SEM-EDS) was used for the analysis of SBP-nanohybrid.

**Study of interactions of Cu3(PO4)2 with SBP using a computational approach**

Prompted by the formation of Cu3(PO4)2 mediated hybrid nano-flowers of SBP, we explored the nanohybrid assembly formation mechanism by predicting the binding interactions of Cu3(PO4)2 to SBP. The possible Cu2+ binding sites on the
surface of SBP were predicted using fragment transformation method available on public MIB server (http://bioinfo.cmu.edu.tw/MIB/introduction). The crystal structure coordinates of SBP (1FHF.pdb) were downloaded from the protein databank (rcsb.org) and used for the metal ion binding site prediction and molecular docking studies. Predicted Cu^{2+} binding sites were validated using molecular docking studies. Initially, blind docking was performed and two different lowest energy binding sites were selected for induced fit docking. Further, the mechanism of catalytic oxidation of guaiacol by SBP was investigated by performing a molecular docking study.

**Molecular docking studies of Cu_3(PO_4)_2 and guaiacol with SBP**

Molecular docking studies of Cu_3(PO_4)_2 and guaiacol against the SBP were performed using the Autodock 4.2 software (Morris et al., 2009). The structure of receptor (SBP) was prepared using Autodock wizard. The molecular structures of Cu_3(PO_4)_2 and guaiacol were generated and geometrically optimized by employing the density functional theory (DFT) method (B3LYP/6-31G** basis set) using Guassianto99 software (Frisch et al., 2016). Receptor structure (SBP) was refined by substituting non-polar hydrogen atoms with polar hydrogen atoms and adding Kollman united atom charges in Autodock wizard. Similarly, the gasteiger charges and hydrogen atoms were added to guaiacol. The parameters for the copper atom were added from the earlier studies (Corona-Motolinia et al., 2020; Hu et al., 2016). The sum of VDW radii of two like atoms (3.50 Å), the VDW well depth (0.005 kcal/mol), the atomic solvation volume (120 Å³), the atomic solvation parameter (−0.00110). The H-bond radius of the heteroatom in contact with hydrogen (0.0 Å), the well depth of the H-bond (0.0 kcal/mol), and different integers show the type of H-bonding atom and indexes for the generation of the auto grid map (0, −1, −1, 1, respectively). The grid maps for Cu_3(PO_4)_2 and guaiacol over SBP were calculated by assuming that it would cover the ligand and active site pocket of the receptor molecule. The grid size was set to 48 Å × 48 Å × 48 Å for Cu_3(PO_4)_2 and 40 Å × 40 Å × 40 Å for guaiacol with a grid spacing of 0.375 Å. The step size of 1 Å for translation and the maximum number of energy evaluations was set to 2,500,000. The 100 runs were performed for receptor-ligand molecules with a maximum number of 2,700,000 LGA operations that were generated on a single population of 150 individuals. The operator weights for a crossover (0.80), mutation (0.02), and elitism (1) were maintained as default parameters. Docked complexes yielding the lowest energy stable complexes were analyzed for molecular interactions using Autodock wizard and the pictorial presentation was made using Chimera software (Pettersen et al., 2004).

**Determination of SBP enzyme activity and optimization of process parameters**

The SBP enzyme assay was carried out using guaiacol (5 mM) as a substrate. The guaiacol solution was prepared in 50 ml of PBS buffer. The 10 mg of either crude SBP extract or SBP nanohybrid was added to the 1 ml substrate (5 mM). To this 30% hydrogen peroxide (H_2O_2) (0.019 ml) was added. The solution was mixed rapidly, and its absorbance was determined after 15 min at 470 nm by using a spectrophotometer. Here, the solution without crude SBP extract or SBP nanohybrid was used as a blank. One unit of peroxidase activity (U) was defined as the amount of enzyme required to change the absorbance value within 1 min. The protein concentration of crude SBP extract or SBP-nanohybrid was determined via Folin-Lowry protein assay.

The effect of varying pH and temperature on SBP-nanohybrid enzyme activity was studied and was compared with that of crude SBP enzyme activity. The substrate (guaiacol 0.028 ml) was mixed in 50 ml of PBS buffer of varying pH (4.2–7.2). Then, 10 mg of either crude SBP extract or SBP-nanohybrid was added to the 1 ml substrate, followed by addition of 30% H_2O_2 (0.019 ml). The solution was mixed rapidly, and its absorbance was determined after 15 min at 470 nm by using a spectrophotometer (Schimadzu UV-1800). Similarly, the effect of varying temperatures was studied by incubating the reaction mixture at 4–50 °C temperature. An experiment was carried out to determine the K_m and V_max values. The enzyme activity of SBP-nanohybrid was determined at increasing guaiacol concentration to get line weaver Burker plot. The K_m and V_max values were estimated from the line weaver Burker plot.

**Decolorization of MB using SBP-nanohybrid**

In this experimentation, the MB (5 mg/L) solution was incubated with nanohybrid (10 mg) and H_2O_2 (1 ml). Three controls were used in this experiment. The first control contained only MB (5 mg/L) solution. In the second control MB (5 mg/L) solution was mixed with nanohybrid (10 mg) only. In the third control MB (5 mg/L) solution was mixed with H_2O_2 (1 ml) only. After 1 h of incubation period, a spectrum scan (400–800 nm) of all the reaction mixtures was recorded using a spectrophotometer. Increasing concentrations of MB (5–20 mg/L) were separately incubated with nanohybrid (10 mg) and H_2O_2 (1 ml) and decolorization were monitored by taking absorbance at λ_max.

The degradation process of MB was analyzed using HPLC (Waters 2695). The degraded products were enriched. The samples were then filtered through millipore discs of 0.22 µm before analysis. HPLC monitoring was carried out using a UV absorbance detector (2996 PDA) operated at 293 nm coupled to a Zorbax SB C18 5 µ (4.6×150) mm column. Mobile phase A consists of water, acetonitrile, formic acid (95:05:0.1). Mobile phase B consists of water, acetonitrile, formic acid (10:90:0.08). The flow rate of 1 ml/minute was maintained for 30 min. The diluent used was methanol, acetonitrile/water (7:3). All the experiments were carried out in triplicate.

**Results and discussion**

**Analysis of SBP-nanohybrid**

In the present study, the synthesis of the SBP-Cu_3(PO_4)_2·3H_2O nanohybrid was carried out.
The SEM micrograph showed a uniform characteristic hierarchical structure of nanohybrids (Figure 1A). Further, the properties of individual nanohybrid particles were studied. Figure 1B shows a flower-like hierarchical structure of individual nanohybrid. Similar flower-like SBP-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanohybrid was also observed by Yu et al. (2015). The EDS analysis of the nanohybrid was also carried out. It showed the presence of carbon (C), nitrogen (N), oxygen (O), phosphorous (P), and copper (Cu) in the nanohybrid (Figure 1C). The presence of C, N, and O may be due to SBP enzyme. The presence of P and Cu may be due to PBS buffer and CuSO$_4$, respectively. The effect of CuSO$_4$ concentration on nanohybrid structure was studied. It was observed that the 2 mM CuSO$_4$ concentration gave ideal hierarchal characteristic flower like structure. The flower like structure could not be completed by using 1 mM CuSO$_4$ concentration. Also, with an increase in CuSO$_4$ concentration beyond 2 mM deteriorated the flower like morphology of SBP nanohybrid (Figure S1 Supporting Data file). Several researchers predicted the mechanism behind the nanohybrid formation. It was suggested that nanohybrid formation is possible in three steps. He et al. (2015) suggested that the PBS buffer provide PO$_4^{3-}$ (Reaction 1).

$$\text{HPO}_4^{2-} \leftrightarrow \text{H}^+ + \text{PO}_4^{3-} \quad (1)$$

In their study they used Cu sheet for nanohybrid formation. The dissolved oxygen converted metallic Cu to Cu$^{2+}$. This oxidized Cu took part in nanohybrid formation by reacting with PO$_4^{3-}$ (He et al., 2015). Similarly, in the present study CuSO$_4$ is mixed with PBS buffer to initiate the early growth stage. The Cu$^{2+}$ from CuSO$_4$ reacted with PO$_4^{3-}$ to form crystals of Cu$_3$(PO$_4$)$_2$ (Reaction 2).

$$\text{PO}_4^{3-} + \text{Cu}^{2+} + 3\text{H}_2\text{O} \rightarrow \text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O} \quad (2)$$

In the second growth step, the Cu$_3$(PO$_4$)$_2$ crystals interact with protein/enzyme. The amide groups of protein/enzyme provide binding sites for Cu$_3$(PO$_4$)$_2$ and form flower petal-like structures. In the final step, the anisotropic growth results in flower-like nanohybrid formation (Altinkaynak et al., 2018; Bilal et al., 2019; Escobar et al., 2017; Yu et al., 2015). Our primary in-Vitro study showed that the formation of SBP-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanohybrid is possible. Therefore, in the present study an attempt was made to identify the nanohybrid formation mechanism using molecular docking studies.

**Prediction of Cu$^{2+}$ binding sites towards SBP and molecular docking studies with CuP and guaiacol substrate**

The amino acid residues having high binding towards Cu$^{2+}$ are highlighted on the surface of SBP (Figure 2), and the binding score is given in Table 1.

A predicted higher binding score of residues resembles more binding propensity towards Cu$^{2+}$. The residues having a binding score greater than 2.2 showed 95% prediction accuracies for Cu$^{2+}$ binding sites. These predicted binding sites were further validated by performing molecular docking studies. Initially, blind docking was performed to predict the possible CuP binding sites on the surface of SBP.

A grid box of size (126 Å × 126 Å × 126 Å) was used to cover the entire protein surface area of SBP. The resultant two lowest energy (−4.4 kcal/mol and −3.56 kcal/mol) stable complexes were selected which showed similar interacting residues as observed in MIB server predictions. Using these two binding sites, molecular docking studies were performed once again by keeping interacting residues flexible during the docking runs. Figure 3A depicted two favorable binding sites for CuP on the surface of SBP. The 1st binding site showed the lowest energy (−6.36 kcal/mol) stable docking complex between CuP and SBP (Figure 3B).

This favorable complex was stabilized by co-ordinate and intermolecular hydrogen bonding interactions (Table 2). On the surface of SBP, the one end of CuP was bound to Asn284 from the N-terminal region by forming a co-ordinate bond having a 2.45 Å bond distance (Table 2). Further, the phosphate oxygen of CuP participated in strong hydrogen bonding interactions with Gln1 and Thr289 residues. In docked complex, the other end of CuP remained unbound to SBP. Thus, it might be involved in establishing the coordination link between first and second units of SBP during CuP mediated nanohybrid assembly formation. The second favorable docked complex between CuP and SBP having high binding affinity and lowest energy (−6.32 kcal/mol) stable conformation is depicted in Figure 3C. In this complex, the CuP was forming a co-ordinate bond with Asn188 of SBP by maintaining a 2.72 Å bond distance (Table 2). The phosphate oxygen of CuP participated in intermolecular hydrogen bonding interactions with Asn181, Asn185, Ser187, and Asn188 residues on the surface of SBP. Table S1 and S2 of supporting document show the Gaussian coordinates for the optimized geometry. These interactions were mainly involved in the stabilization of the docked complex (Figure 3C). Here also, the second end of the CuP remained unbound and might be involved in co-ordination with another unit of SBP during the formation of nanohybrid assembly. The Asn188 was coordinated with Cu$^{2+}$ of CuP which is similar to results predicted by the MIB server (Table 1). Both the predicted CuP binding sites are located at two different ends of SBP, and one end of each CuP remained unbound to the SBP which suggested their possible role in the formation of CuP mediated nanohybrid assembly of SBP. Moreover, these CuP binding sites are far from the catalytic site residues of SBP. Henceforth, we hypothesized that the catalytic site of SBP might be available for substrate catalysis. Similar results were reported by Yazawa et al. (2016). They carried out a heavy metal derivatization of enzyme by simply mixing praseodymium (Pr) ions into proteinase K solution. They used HATADAS database to identify an appropriate heavy metal for derivatization. The amino-acid sequence of proteinase K, played a crucial role in derivatization process. They found that Pr ions do not bind at the catalytic site of proteinase K. The mode, strength, and position of Pr-ion binding with proteinase K helped to enhance the thermal stability and activity of proteinase K.
Many factors govern the enzyme activity. The activity depends on enzyme structure, its active-site dynamics, and the physical properties of the medium. It is observed that the compact structural conformation and flexibility of the active site of bromelain enhanced its proteolytic activity. Also, the medium properties affected bromelain activity (Das et al., 2021). Considering these facts, the molecular docking study was performed using SBP along with guaiacol following the same docking protocol to validate the hypothesis, that the CuP binding sites are far from the catalytic site residues of SBP. Figure 4A depicted the energetically (~3.57 kcal/mol) stable docked complex of guaiacol with SBP. In docked complex, the guaiacol was positioned deep in the catalytic active site pocket of SBP by forming intermolecular hydrogen bonding interactions (Figure 4B and Table 2). The phenolic oxygen (O1) of guaiacol was involved in hydrogen bonding interaction with NH2 of Arg38 through 1.85 Å bond distance (Figure 4B). Similarly, the methoxy oxygen (O2) of guaiacol was hydrogen-bonded with NH2 of the same Arg38 (2.42 Å). A hydrogen bond was found between the phenolic oxygen (O1) and active site water (W1) molecule (1.88 Å). This water was also involved in hydrogen bonding interaction with NE2 of His42 (2.06 Å). Further, the second water molecule (W2) from the active site pocket participated in hydrogen bonding interaction with phenolic oxygen (O1) and active site water (W1) molecule (1.88 Å). This water was also involved in hydrogen bonding interaction with NE2 of His42 (2.06 Å). Further, the second water molecule (W2) from the active site pocket participated in hydrogen bonding interaction with phenolic oxygen (O1) of guaiacol and backbone oxygen of Pro139 by maintaining 2.64 Å and 2.2 Å distances, respectively (Figure 4B). The additional stabilization of the favored dock complex was expected from the hydrophobic interactions from Pro69, Ala140, and Pro141 active site residues (Table 2). Hydrophobic interactions may help in the proper positioning
of guaiacol in the active site pocket of SBP. The aromatic ring of guaiacol was almost coplanar with heme and situated between Pro69 residue and C18, C18-methyl, and C20 atoms of heme (Figure 3B). Similar hydrogen bonding and hydrophobic interactions network were reported in earlier crystallographic studies of horseradish peroxide in the presence of guaiacol in the active site pocket of SBP.

Table 1. Cu$^{2+}$ binding site prediction on soybean peroxide using MIB server.

| Residue number | Amino acid | MIB binding score | Residue number | Amino acid | MIB binding score |
|----------------|------------|-------------------|----------------|------------|-------------------|
| 28             | THR        | 3.432             | 187            | SER        | 2.339             |
| 29             | ASP        | 3.432             | 188            | ASN        | 2.339             |
| 38             | ARG        | 2.154             | 190            | GLY        | 2.293             |
| 40             | HIS        | 2.888             | 191            | ASN        | 2.293             |
| 41             | PHE        | 1.950             | 192            | PRO        | 2.366             |
| 42             | HIS        | 2.154             | 193            | ASP        | 2.366             |
| 43             | ASP        | 2.888             | 226            | ASP        | 2.779             |
| 49             | CYX        | 2.888             | 227            | GLN        | 2.779             |
| 54             | LEU        | 2.464             | 234            | SER        | 2.305             |
| 65             | GLN        | 2.484             | 235            | ASN        | 2.305             |
| 66             | ASP        | 2.484             | 266            | SER        | 1.996             |
| 81             | ASN        | 2.446             | 267            | ASN        | 1.996             |
| 84             | LYS        | 2.446             | 283            | GLN        | 2.738             |
| 97             | CYX        | 2.446             | 284            | ASN        | 2.738             |
| 98             | ALA        | 2.888             | 285            | ILE        | 2.292             |
| 102            | ALA        | 2.243             | 290            | GLY        | 2.275             |
| 123            | ARG        | 1.902             | 291            | ASP        | 2.275             |
| 124            | ARG        | 2.364             | 299            | CYS        | 2.446             |
| 125            | ASP        | 2.364             |                |            |                   |

Table 2. Geometrical parameters for hydrogen bonding interactions and coordinate bonding between the stable docked complexes of copper phosphate and guaiacol with Soybean peroxidase.

| Complex                      | Atoms involved in H-bond 1-2-3 | Distance 1-2 Å | Angle 1-2-3 (Degree) | Ref. Fig. |
|------------------------------|--------------------------------|----------------|----------------------|-----------|
| Copper phosphate and SBP     | CuP-Cu-O (Asn284)              | 2.45           | –                    | 2         |
|                              | CuP-O-H-O(Thr289)              | 2.64           | 136.09               |           |
|                              | CuP-O-H-N(Gln1)                | 1.99           | 140.08               |           |
|                              | CuP-Cu-O(Gln1)                 | 2.92           | –                    |           |
|                              | CuP-Cu-O(Asn188)               | 2.72           | –                    |           |
|                              | CuP-O-H-O(Ser187)              | 2.10           | 127.67               |           |
|                              | CuP-O-H-O(Ser187)              | 2.46           | 152.27               |           |
|                              | CuP-O-H-O(Asn188)              | 2.17           | 95.28                |           |
|                              | CuP-O-H-O(Asn185)              | 2.25           | 154.53               |           |
|                              | CuP-O-H-O(Asn181)              | 2.08           | 93.06                |           |
| Guaiacol and SBP             | Guaiacol-O-H-N(Arg38)          | 1.85           | 138.41               | 3         |
|                              | Guaiacol-O2-H-N(Arg38)         | 2.42           | 124.96               |           |
|                              | Guaiacol-O2-H-O(Ser73)         | 2.29           | 176.76               |           |
|                              | (W1)-O-H-O(Guaiacol)           | 1.88           | 147.22               |           |
|                              | (His42)-NE2-H-O(W1)            | 2.06           | 153.53               |           |
|                              | (W1)-O-H-N(Arg38)              | 2.54           | 155.23               |           |
|                              | (W1)-O-H-N(Arg38)              | 2.62           | 150.17               |           |
|                              | (HEM)-Fe$^{2+}$-O-O(W1)        | 3.64           | –                    |           |
|                              | (W2)-O-H-O(Guaiacol)           | 2.64           | 149.96               |           |
|                              | (Pro139)-O-H-O(W2)             | 2.20           | 95.71                |           |

Figure 3. Molecular docking results of Cu$_3$(PO$_4$)$_2$ and SBP. (A) Depicted the binding sites for Cu$_3$(PO$_4$)$_2$ on the surface of SBP, (B) Showed hydrogen bonding interactions between Cu$_3$(PO$_4$)$_2$ and SBP from 1st binding site and (C) Showed hydrogen bonding interactions between Cu$_3$(PO$_4$)$_2$ and SBP from 2nd binding site.

Figure 4. (A) Depicted lowest energy stable docking complex of guaiacol and SBP and (B) Intermolecular hydrogen bonding interactions between favored docking complex of guaiacol and SBP.
Benzhydroxamic acid (BHA) and Ferulic acid (FA) substrates (Berglund et al., 2002; Henriksen et al., 1998, 1999; Meno et al., 2002).

Based on the observed interaction patterns, a general mechanism of enzymatic oxidation of guaiacol by SBP through a hydrogen bonding interaction network was proposed. It was proposed that the general electron transfer is likely to take place from the highest occupied $\pi$-orbital of the substrate to heme $\pi$-orbital. Similar to the earlier proposed oxidation mechanism of FA and BHA by horseradish peroxides, the oxidation of guaiacol may take place in two steps (Henriksen et al., 1998, 1999). The first oxidation step of guaiacol was initiated by the hydrogen bonding interaction between Arg38 and phenolic oxygen (O1) of guaiacol. This hydrogen bond will assist the proton transfer from the phenol to the distal His42 through an active site water (W1) molecule. This water molecule is playing a significant role in the oxidation of guaiacol. In this step, the final destination of proton transfer is to the distal His42 and the electron is transferred from the phenolate to the C-18 methyl and C-20 edge of the heme group. Hence, this study may pave the way to explore the mechanism of CuP mediated SBP-nanohybrid assembly formation and enzymatic oxidation of guaiacol.

**Determination of SBP enzyme activity and optimization of process parameters**

The molecular docking studies showed that the binding sites for Cu$_3$(PO$_4$)$_2$ on SBP were different than the active site of SBP. Hence these results suggest that the immobilization of SBP in nanohybrid form should not hamper the enzyme activity. This was further confirmed by reacting SBP-nanohybrid and free SBP enzyme separately with its substrate guaiacol.

In the presence of SBP and H$_2$O$_2$, guaiacol can be oxidized into a water-soluble, red product (3,3-dimethoxy-4,4-biphenoquinone) with an absorption maximum at approximately 470 nm (Tonami et al., 2004). Our results show that SBP-nanohybrid was able to oxidize guaiacol. The enzyme activities 9.55 ± 0.03 and 13.56 ± 0.5 U/mg of protein were recorded for crude SBP and SBP-nanohybrid respectively (Figure SII Supporting Data file). These results are following molecular docking studies. Further, the effect of various parameters on SBP-nanohybrid activity was studied. The effect of pH (4.2–8.2) on the activity of crude SBP and SBP-nanohybrid was studied. The pH 7.2 was found optimum for both types of enzymes. The enzyme activities 10.55 ± 0.04 and 14.36 ± 0.57 U/mg of protein were recorded for crude SBP and SBP-nanohybrid respectively (Figure 5A). Similarly, the effect of varying temperatures (30–60°C) on enzyme activity was studied. The enzyme activity increased with an increase in temperature for both the enzymes and the 50°C temperature was found to be optimum. The activity decreased with an increase in temperature beyond 50°C (Figure 5B). The enzyme activities 15.54 ± 0.13 and 21.45 ± 0.38 U/mg protein were recorded for crude SBP and SBP-nanohybrid respectively (Figure 5B). One interesting phenomenon observed here is that the activity of SBP-nanohybrid is more compared to crude SBP. Around ~138% enhanced activity was recorded using SBP-nanohybrid compared to crude SBP. Yu et al. (2015) reported similar results. They suggested three reasons for this type of enhanced catalytic activity. The characteristic hierarchical structure of nanohybrid stabilized SBP enzyme. The nanohybrid provided a large surface area and broad confinement which increased the accessibility of the guaiacol to the active molecule which is situated equidistant between His42 and ferryl oxygen. Here the final destination of the proton transfer process is the ferryl oxygen and the electron is transferred to the heme. The water (W1) molecule is significantly involved in proton transfer by hydrogen bonding to the phenolic oxygen of guaiacol. All the hydrogen bonding and hydrophobic interactions we observed in docking studies of SBP and guaiacol are in line with earlier crystallographic information (Henriksen et al., 1998, 1999). Owing to similar interaction patterns, here, we propose the mechanism of enzymatic oxidation of guaiacol by SBP which was mediated by the active site water molecules. Hence, this study may pave the way to explore the mechanism of CuP mediated SBP-nanohybrid assembly formation and enzymatic oxidation of guaiacol.
sites of SBP. At the same time, the nanohybrid formation purified the SBP. Further, the activity of SBP-nanohybrid at increasing guaiacol concentration was determined. The activity of SBP-nanohybrid increased with an increase in guaiacol concentration (Figure SIII Supporting Data file). The $K_m$ and $V_{max}$ values were estimated using line weaver burk plot. The 1.286 mM and 0.535 $\mu$M/min $K_m$ and $V_{max}$ were observed respectively for SBP-nanohybrid.

One more experiment was conducted to validate the molecular docking studies. The decolorization of MB was studied using SBP-nanohybrid. Around 95% MB decolorization was observed in 1 h using SBP-nanohybrid + $H_2$O$_2$. However, 2.5 and 2.3% MB decolorization was observed using only SBP-nanohybrid and only $H_2$O$_2$ respectively (Figure 6). Our results are comparable with Yao and Wang (2010) and Manghabati and Pazuki (2014). Yao and Wang (2010) used TiO$_2$ sol and UV radiation for MB decolorization (Yao & Wang, 2010). They showed 92.3% decolorization in 160 min. While Manghabati and Pazuki (2014) used Spirodela polyrrhiza for phytoremediation of MB (Manghabati & Pazuki, 2014). They showed 95% decolorization in 7 days. The ability of SBP-nanohybrid to decolorize increasing MB concentration was tested. The MB decolorization decreased with an increase in dye concentration.

The decolorization decreased from 95% to 41% when dye concentration was increased from 5 to 20 mg/l (Figure 7).

These results suggest that the immobilization of SBP in the form of nanohybrid do not affect the active site of SBP and it is still possible to explore its catalytic activity. The HPLC analysis was performed to study MB degradation.

Conclusions
The synthesis of $Cu_3(PO_4)_2$ mediated SBP-nanohybrid formation was carried out. The formed SBP-nanohybrid showed a characteristic flower-like structure. The MIB server and molecular docking studies showed two distinct binding sites for CuP located at different regions on the surface of SBP. Results anticipated that some SBP regions may act as specific nucleation sites for the formation of hierarchical flower-like nanohybrid structures. These sites might be potentially contributed to the formation of SBP-nanohybrid assembly. Also, these binding sites are different from the catalytic site of SBP. The in-silico hypothesized catalysis of guaiacol was validated through in-vitro studies. The Arg38, His42, and Pro139 residues from the active site pocket of SBP were mainly involved in the catalysis of guaiacol. Furthermore, the guaiacol oxidation process is mediated by active-site water
molecules that significantly contributed to the proton transfer mechanism. The in-Vitro catalysis of guaiacol is faster in presence of a nanohybrid assembly of SBP as compared to crude SBP. This study may help in the immobilization of SBP without hampering the active site. Hence, the combined approach involving computational modeling and in-Vitro studies will help to develop more efficient biocatalysts with different functional properties and applications.

Acknowledgement

Mr. Sunil Bhapkar acknowledges Council of Scientific & Industrial Research for the research fellowship. Navanath Kumbhar sincerely acknowledges to Savitribai Phule Pune University (SPPU) (Formerly Pune University), Pune for providing the SPPU post-doctoral fellowship (ST/BL/2018-2018/0203).

Author contributions

Mr. Sunil Bhapkar: Experiments for Soybean peroxidase-nanohybrid synthesis, characterization, optimization of enzyme activity, dye decolorization study.

Dr. Navanath Kumbhar: In-silico studies, designing the protocols and studied the nanohybrid formation mechanism using computational approaches.

Mr. Prafful Sharma: Helped for nanohybrid synthesis and dye decolorization experiments.

Dr. Shweta Jagtap: Designed the experiments for nanohybrid synthesis and characterization.

Prof. Rajesh Gacche and Prof. Kailas D. Sonawane: Designed the experiments for molecular modelling.

Dr. Vitthal T. Barvkar: Designed the protocol for HPLC analysis of dye degradation.

Mr. Dnyaneshwar Sonune: Analyzed the dye degradation using HPLC.

Dr. Umesh Jadhav: Put forth the hypothesis that the molecular modeling/bioinformatics approaches can be used for identification of nanohybrid formation mechanism. He also designed the in-vitro studies. Collaborated with authors from various backgrounds and executed the whole study.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

Altinkaynak, C., Kocazorbaz, E., Özdemir, N., & Zihnioglu, F. (2018). Egg white hybrid nanoflower (EW-hNF) with biomimetic polyphenol oxidase reactivity: Synthesis, characterization and potential use in decolorization of synthetic dyes. International Journal of Biological Macromolecules, 109, 205–211. https://doi.org/10.1016/j.ijbiomac.2017.12.072

Altinkaynak, C., Tavlasoglu, S., Kalin, R., Sadeghian, N., Ozdemir, H., Ocsoy, I., & Özdemir, N. (2017). A hierarchical assembly of flower-like hybrid Turkish black radish peroxidase-Cu2+ nanobiocatalyst and its effective use in dye decolorization. Chemosphere, 182, 122–128. https://doi.org/10.1016/j.chemosphere.2017.05.012

Altinkaynak, C., Tavlasoglu, S., Özdemir, N., & Ocsoy, I. (2016). A new generation approach in enzyme immobilization: Organic-inorganic hybrid nanoflowers with enhanced catalytic activity and stability. Enzyme and Microbial Technology, 93-94, 105–112. https://doi.org/10.1016/j.enzmictec.2016.06.011

Ambreen, S., Rehman, K., Zia, M. A., & Habib, F. (2000). Kinetic studies and partial purification of peroxidase in soybean. Pakistan Journal of Agricultural Sciences, 37(3-4), 10–13.

Berglund, G. I., Carlsson, G. H., Smith, A. T., Szöke, H., Henrikson, A., & Hajdu, J. (2002). The catalytic pathway of horseradish peroxidase at high resolution. Nature, 417(6887), 463–468. https://doi.org/10.1038/417463a

Bilal, M., Asgher, M., Shah, S. Z. H., & Iqbal, H. M. N. (2019). Engineering enzyme-coupled hybrid nanoflowers: The quest for optimum performance to meet biocatalytic challenges and opportunities. International Journal of Biological Macromolecules, 135, 677–690. https://doi.org/10.1016/j.ijbiomac.2019.05.206

Chandane, P., Jori, C., Chaudhari, H., Bhapkar, S., Deshmukh, S., & Jadhav, U. (2020). Bioleaching of copper from large printed circuit boards for synthesis of organic-inorganic hybrid. Environmental Science and Pollution Research International, 27(6), 5797–5808. https://doi.org/10.1007/s11356-019-07244-x

Corona-Motolinia, N. D., Martinez-Valencia, B., Noriega, L., Sánchez-Gaytán, B. L., Méndez-Rojas, M. Á., Melendez, F. J., Castro, M. E., & González-Vergara, E. (2020). Synthesis, crystal structure, and computational methods of vanadium and copper compounds as potential drugs for cancer treatment. Molecules, 25(20), 1–23. https://doi.org/10.3390/molecules25204679

Cui, J., & Jia, S. (2017). Organic–inorganic hybrid nanoflowers: A novel host platform for immobilizing biomolecules. Coordination Chemistry Reviews, 352(29), 249–263. https://doi.org/10.1016/j.ccr.2017.09.008

Das, N., Khan, T., Subba, N., & Sen, P. (2021). Correlating Bromelain’s activity with its structure and active-site dynamics and the medium’s physical properties in a hydrated deep eutectic solvent. Physical Chemistry Chemical Physics: PCCP, 23(15), 9337–9346. https://doi.org/10.1039/d1cp00046b

Escobar, S., Velasco-Lozano, S., Lu, C. H., Lin, Y. F., Mesa, M., Bernal, C., & Lopez-Gallego, F. (2017). Understanding the functional properties of bio-inorganic nanoflowers as biocatalysts by deciphering the metal-
binding sites of enzymes. *Journal of Materials Chemistry*, B, 5(23), 4478–4486. https://doi.org/10.1039/cdbb03295h

Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Scalmani, G., Barone, V., Peterson, G. A., Nakatsuji, H., Li, X., Caricato, M., Marenich, A., Bloino, J., Janesko, B. G., Gomperts, R., Mennucci, B., Hratchian, H. P., Ortiz, J. V., … Fox, D. J. (2016). Gaussian 09, Revision A.02, Gaussian, Inc.

Ge, J., Lei, J., & Zare, R. N. (2012). Protein-inorganic hybrid nanoflowers. *Nature Nanotechnology*, 7(7), 428–432. https://doi.org/10.1038/nnano.2012.80

Godoy, C. A., Rivas, B. D. L., Grazi, V., Montes, T., Guisán, J. M., & López-Gallego, F. (2011). Glyoxyl-disulfide agarose: A tailor-made support for site-directed rigidification of proteins. *Biomacromolecules*, 12(5), 1800–1809. https://doi.org/10.1021/bm200161f

Gonçalves, M. C. P., Kieckbusch, T. G., Pema, R. F., Fujimoto, J. T., Morales, A. S. V., & Romanielli, J. P. (2019). Trends on enzyme immobilization research based on bibliometric analysis. *Process Biochemistry*, 76(2019), 95–110. https://doi.org/10.1016/j.procbio.2018.09.016

Hanefeld, U., Cao, L., & Magner, E. (2013). Enzyme immobilization: Fundamentals and application. *Chemical Society Reviews*, 42(15), 6211–6212. https://doi.org/10.1039/c3cs90042h

He, G., Hu, W., & Li, C. M. (2015). Spontaneous interfacial reaction between metallic copper and PBS to form cupric phosphate nanoflower and its enzyme hybrid with enhanced activity. *Colloids and Surfaces, B: Biointerfaces*, 135, 613–618. https://doi.org/10.1016/j.colsurfb.2015.03.030

Henriksen, A., Schuller, D. J., Meno, K., Welinder, K. G., Smith, A. T., & Gajhede, M. (1999). The structures of the horseradish peroxidase catalytic residue variants H42E and R38S/H42E: Implications for the catalytic cycle. *Acta Crystallographica. Section D, Biological Crystallography*, 55( Pt 2), 1803–1812. https://doi.org/10.1107/s090744499201329X

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., & Olson, A. J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry*, 30(16), 2785–2791. https://doi.org/10.1002/jcc.20084

Nadar, S. S., Gawas, S. D., & Rathod, V. K. (2016). Self-assembled organic-inorganic hybrid glucoamylase nanoflowers with enhanced activity and stability. *International Journal of Biological Macromolecules*, 92, 660–669. https://doi.org/10.1016/j.ijbiomac.2016.06.071

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., & Ferrin, T. E. (2004). UCSF Chimera – A visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, 25(13), 1605–1612. https://doi.org/10.1002/jcc.20084

Sardar, M., & Ahmad, R. (2015). Enzyme immobilization: An overview on nanoparticles as immobilization matrices. *Biochemistry & Analytical Biotechnology*, 4(2), 1–8. https://doi.org/10.4172/2161-1099.1000178

Sheldon, R. A., & van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: Why, what and how. *Chemical Society Reviews*, 42(15), 6223–6235. https://doi.org/10.1039/c3cs60075k

Somturk, B., Hancer, M., Ocsoy, I., & Özdemir, N. (2015). Synthesis of copper ion incorporated horseradish peroxidase-based hybrid nanoflowers for enhanced catalytic activity and stability. *Dalton Transactions (Cambridge, England: 2003)*, 44(31), 13845–13852. https://doi.org/10.1039/c5dt01250c

Sun, J., Ge, J., Liu, W., Lan, M., Zhang, H., Wang, P., Wang, Y., & Niu, Z. (2014). Multi-enzyme co-embedded organic-inorganic hybrid nanoflowers: Synthesis and application as a colorimetric sensor. *Nanoscale*, 6(1), 255–262. https://doi.org/10.1039/c3nr04425d

Tonami, H., Uyama, H., Nagahata, R., & Kobayashi, S. (2004). Guaiacol oxidation products in the enzyme-activity assay reaction by horseradish peroxidase catalysis. *Chemistry Letters*, 33(7), 796–797. https://doi.org/10.1246/cl.2004.796

Varqa, G. (2018). An immobilized and highly stabilized self-sufficient monooxygenase as biocatalyst for oxidative biotransformations. *Journal of Chemical Technology & Biotechnology*, 93(4), 985–993. https://doi.org/10.1002/jctb.5450

Wang, L. B., Wang, Y. C., He, R., Zhuang, A., Wang, X., Zeng, J., & Hou, J. G. (2013). A new biocatalytic system based on allosteric effect with dramatically enhanced enzymatic performance. *Journal of the American Chemical Society*, 135(4), 1272–1275. https://doi.org/10.1021/ja3120136

Wu, Z., Li, H., Zhu, X., Li, S., Wang, Z., Wang, L., Li, Z., & Chen, G. (2017). Using laccases in the nanoflower to synthesize viniferin. *Catalysts*, 7(6), 188–112. https://doi.org/10.3390/catal7060188

Yao, J., & Wang, C. (2010). Decolorization of methylene blue with TiO2 sol via UV irradiation photocatalytic degradation. *International Journal of Photoenergy*, 2010, 1–6. https://doi.org/10.1155/2010/643182

Yashchenok, A. M., Borisova, D., Parakhonskiy, B. V., Masic, A., Pinhasik, B., Möhwald, H., & Skirtach, A. G. (2012). Nanoplasmonic smooth silica versus porous carbon bead biosensors for detection of biomarkers. *Annalen Der Physik*, 524(11), 723–732. https://doi.org/10.1002/andp.201200158

Yazawa, K., Sugahara, M., Yutani, K., Takehira, M., & Numata, K. (2010). Derivatization of proteinase K with heavy atoms enhances its thermal stability. *ACS Catalysis*, 6(5), 3036–3046. https://doi.org/10.1021/csca1060000

Yue, Y., Fei, X., Tian, J., Xu, L., Wang, X., & Wang, Y. (2015). Self-assembled enzyme-inorganic hybrid nanoflowers and their application to enzyme purification. *Colloids and Surfaces, B: Biointerfaces*, 130, 299–304. https://doi.org/10.1016/j.colsurfb.2015.04.033

Zhang, Y., Ge, J., & Liu, Z. (2015). Enhanced activity of immobilized or chemically modified enzymes. *ACS Catalysis*, 5(8), 4503–4513. https://doi.org/10.1021/acscatal.5b00996
Zhang, Z., Zhang, Y., He, L., Yang, Y., Liu, S., Wang, M., Fang, S., & Fu, G. (2015). A feasible synthesis of Mn3(PO4)2@BSA nanoflowers and its application as the support nanomaterial for Pt catalyst. *Journal of Power Sources, 284*, 170–177. https://doi.org/10.1016/j.jpowsour.2015.03.011

Zheng, J. N., He, L. L., Chen, C., Wang, A. J., Ma, K. F., & Feng, J. J. (2014). One-pot synthesis of platinum3cobalt nanoflowers with enhanced oxygen reduction and methanol oxidation. *Journal of Power Sources, 268*, 744–751. https://doi.org/10.1016/j.jpowsour.2014.06.109