Seed Coat Soybean Peroxidase: Extraction and Biocatalytic Properties Determination

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Abstract

Plants and/or plant food wastes have been given much less attention or even disregarded. In some instances, however, oxidative enzymes from residual plant tissues have been shown to effectively degrade recalcitrant pollutants. Soybean seed coat peroxidase (SBP) is an inexpensive oxidoreductive enzyme and could be potentially used to oxidize/polymerize various organic pollutants of the industrial and petrochemical wastes. The catalytic properties of SBP are retained under a wide range of pH and at elevated temperatures. In the present study, the biocatalytic properties of SBP were estimated. The enzyme exhibited the highest activity and stability at pH 6.0 and retained over 75% of the maximum activity for 12 hours. The activity of SBP was found to be 2.5 times higher at an elevated temperature of 65°C compared to the activity at room temperature. The activity is retained over 95% for 30 min at 75°C. The pH and temperature of the reaction mixture showed significant influence on SBP activity. SBP is fairly active in organic solvents and exhibited the optimal activity in the presence of 20% (v/v) acetone. Increasing the organic solvent content resulted in a reduction in SBP activity.

Keywords: *Soybean Seed Coat Peroxidase, Oxidoreductive, Catalytic Properties, Enzyme Activity, Organic Solvents*

1- Introduction

1.1- Peroxidase sources

Peroxidases (POD, E.C. 1.11.1.x) are widespread in plants, microbes, and animal tissues, and the peroxidase superfamily is classified into three groups on the basis of amino acid homology and metal-binding capabilities. Class I comprises intracellular peroxidases, including cytochrome c peroxidase, ascorbate peroxidase and catalase-peroxidase. Class II contains the secretory fungal enzymes, such as manganese peroxidase and lignin peroxidase. Class III consists of the secretory plant peroxidase [1]. The seed coat soybean peroxidase (SBP) belongs to class III of the plant peroxidase super family, which includes horseradish (HRP), barley (BP1), and peanut (PNP) peroxidases [2]. Among the plant PODs, extensive researches were previously carried out on the application and characterization of HRP. The high

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commercializing costs for applying HRP led to the search for alternative cheaper sources of plant PODs to substitute HRP in various applications [3].

1.2. Peroxidase applications

Peroxidases have been used for various analytical applications in diagnostic kits, such as quantification of uric acid, glucose, cholesterol and lactose [4]. Some novel applications of peroxidases suggested include treatment of waste water containing phenolic compounds (that are present in widely varying concentrations in the wastewaters of oil refineries and numerous other industries, including the plastics, resins, textiles, iron, steel and forestry industries [5]), synthesis of various aromatic chemicals and removal of peroxide from materials such as food stuffs and industrial waste [6]. More recently some investigators have reported the decolorization and removal of textile dyes from polluted water and dyeing effluents by using soluble and immobilized peroxidases [4].

Plant peroxidases are receiving increasing attention due to their extensive bioactivation properties and potential applications in clinical, biochemical, biotechnological and related areas. Advances have recently been made in using them to synthesize, under mild and controlled conditions, chiral organic molecules, which are highly valuable compounds. They have also been successfully employed in the development of new bioanalytical tests, improved biosensors and in polymer synthesis [4].

Among the other plant PODs, soybean peroxidase (SBP) is an inexpensive by-product of soybean seed hulls. It can be detected in the root, leaf and seed hulls of the soybean. The POD obtained from seed hulls contains the highest activity compared to the POD obtained from other parts of the soybean plant [7]. SBP's marketed usages range from medical diagnostic tests to removal of chlorine-containing pollutants from industrial wastewater. An enzyme from soybean hulls is applied as a replacement for formaldehyde in adhesives, abrasives, protective coatings, and other products [8]. Applying organic solvents for enzymatic reactions has several advantages including increased solubility of organic substrates, and shifting the hydrodynamic equilibria to favor synthesis over hydrolysis [9]. However, quite a few reports are available on the biocatalytic characterization of SBP. Regarding its low price and potential applications, much more attention should be paid to SBP's biocatalytic efficacy in aqueous as well as in organic solvents, which is the aim of this investigation.

2- Materials and methods
2.1- Materials and reagents
Defatted soybean seed coats were obtained from the Maxsoy factory. All chemicals used in this study were of analytical grade and were obtained from commercial sources. ACS grade conjugate acids and bases were obtained from MERCK Chemical Corporation (Darmstadt, Germany) and were used to prepare buffers in accordance with the methods of Gomori [10]. Hydrogen peroxide (30% w/v), phenol (purity 99%), 4-aminoantipyrine (4-AAP, 98%), acetone, ethanol and methanol were also purchased from MERCK.

2.2- Crude peroxidase extraction from soybean seed coats

Two methods were used for extraction of peroxidase from soybean seed hulls in this study:

2.2.1- First method for peroxidase extraction

25 g of soybean hulls were soaked in 200 ml phosphate buffer (0.1M NaH_2PO_4/ Na_2HPO_4, pH 6.0) in 4°C for 24 h. The extract was then filtered through four layers of cheese cloth gauze; the filtrate was clarified by centrifugation at 6000 rpm for 15 min in 4°C to remove cell debris [11]. The final supernatant was collected and stored in 4°C and used as a source of crude SBP enzyme. The enzyme solution was warmed to room temperature immediately prior to use.

2.2.2- Second method for peroxidase extraction

1 g of defatted soybean flakes was mixed with 0.1M phosphate buffer, pH 8.0, for 2h in 4°C (1:25, w/w) and then centrifuged at 5000 rpm for 30 min. The supernatant was maintained in 4°C [12].

2.3- SBP assay and estimation of total protein and specific activity

Peroxidase activity in the enzyme solution was measured by Worthington colorimetric assay at 25°C using 4-AAP and H_2O_2 as substrates. 1 ml enzyme solution was diluted by buffer to 100 ml. The assay mixture contained 0.40 ml enzyme solution, 1.5 ml of 40 mM phenol, 0.75 ml of 24 mM 4 – AAP, 1.5 ml of 1 mM H_2O_2 and 1.85 ml buffer. All assay reactants were prepared in the phosphate buffer and were stored at 4°C. Hydrogen peroxide solution was prepared on

a daily basis. The volume of this mixture was 6 ml, and was immediately mixed by inversion and the increase in absorbance was recorded for 2 minutes. The SBP active concentration is proportional to the color development rate measured at 510 nm, during a period of time in which the substrate concentration is not significantly reduced. The color development rate during this period was converted to activity using an extinction coefficient of 6.280 $M^{-1}cm^{-1}$ based on hydrogen peroxide. One unit of enzymatic activity is defined as the amount of enzyme which transforms 1.0 μmol of hydrogen peroxide per minute at 25°C and pH 7.4 [13]. The activity of SBP was calculated based on Eq. (5) [14]:

$$\frac{Units}{ml\ enzyme} = \frac{dA/dt \times Volume\ of\ assay\ (in\ milliliters) \times Dilution\ factor}{Extinction\ coefficient\ (\varepsilon) \times Volume\ of\ enzyme\ used}$$

(1)

where dA/dt is the slope of the absorption intensity versus time in minutes. The activity of the obtained peroxidases from the two described methods was determined and compared.

Protein concentration was measured by the modified Lowry method for the protein determination using standard BSA solution for calibration [15].

Specific peroxidase activity was defined as the ratio of enzyme activity (U/ml) to total protein concentration (mg/ml) and is expressed in U/mg of protein [12]:

$$SA_{POD} = \frac{Enzyme\ activity}{Protein\ concentration}$$

(2)

2.4- Optimum pH for the activity and stability

The optimum pH value for SBP activity was found by assaying enzyme activity at different pH levels. The assay was carried out by taking buffers of different pH (pH 2.6–3.6, citric acid/Na_2HPO_4 buffer; pH 4.0–5.0, sodium acetate/acetic acid buffer; pH 6.0–8.0, Na_2HPO_4/NaH_2PO_4 buffer; pH 9.2–10.0, borax/NaOH buffer) [16] which were formulated according to Gomori [10]. The samples of crude SBP extract were diluted using the mentioned buffers and their activities were then measured. In order to evaluate the stability of the enzyme over a range of pH, each of the different buffers and enzyme mixtures were kept in 4°C overnight (ca. 12 h). The activity was then measured at pH 7.4 [16].

2.5- Effect of temperature on enzyme activity and thermal inactivation study

In order to investigate the effect of temperature on SBP activity, the glass cuvette containing diluted enzyme solution, phenol and 4 – AAP were heated by water bath to a pre-set temperature. H_2O_2 was immediately added to the cuvette and the enzyme activity was measured [11]. Heat inactivation of SBP was performed over a temperature range of 45 to 95°C by varying the heating time in the range of 0 to 30 min. The solution was then rapidly cooled by immersing the tubes in an iced water bath and the samples were assayed immediately [17]. The percent of remained peroxidase activity after the heat treatment was calculated.

2.6- Effect of organic solvent on enzyme activity

For the experiments involving organic solvents, the same procedure as described above was followed, except the enzyme was pre-diluted with a different ratio of water and organic solvent mixture in the range of 0–100% (v/v) with 20% spans [11].

2.7- Determination of the enzymatic kinetic parameters

The initial experiment was carried out to determine the K_m and V_{max} values for H_2O_2 by assaying the activity at different concentrations of H_2O_2 ranging from 0.25 to 2.5 mM at a constant phenol concentration (40 mM). Based on this experiment a constant concentration of 1 mM H_2O_2 was selected to determine the K_m and V_{max} values for the other substrate (phenol). The K_m and V_{max} values were determined by using the reciprocal plot of Lineweaver–Burk graphic method [18] for the two substrate ping-pong mechanism followed by SBP. Assuming initial rates (v_0), a general equation for this mechanism in the forward direction given by Whitaker is as follows [19]:

$$1/v_0 = 1/V_{max} + K_a/A_0V_{max} + K_b/B_0V_{max} \quad (3)$$

where V_{max} = maximum velocity (µmol/ml.min), $K_a = K_m$ (mM) for substrate A (H_2O_2), A_0 = concentration of substrate A (mM), $K_b = K_m$ (mM) for substrate B (˙H donor), and B_0 = concentration of substrate B (phenol) (mM). By plotting Eq. (3), the lines for enzyme systems, which follow sequential mechanism (random or ordered), intercept the y-axis, whereas the systems that follow a

ping-pong mechanism represent parallel lines [19]. When A_0 is constant, Eq. (3) yield a slope given by

$$\text{Slope} = K_b/Vmax \qquad (4)$$

Therefore, the constants K_m and V_{max} are determined from Eq. (4).

3- Results and discussion
The activity and protein concentration of obtained SBP from the two described methods of extraction were determined and compared.

The major biocatalytic properties of SBP were affected by some parameters such as pH, temperature of the reaction mixture, and the nature of organic solvent. Influences of these parameters on the activity of SBP were investigated.

3.1- Effect of extraction method on activity
The values for activity, protein concentration, and specific activity of two obtained peroxidases from two different methods of extraction are listed in Table 1. As seen, the activity and specific activity of SBP1 are about 3 times higher than those for SBP2. Thus, the crude enzyme obtained from the first method was used in further studies.

3.2- Effect of pH on activity and stability
The relative activities of SBP as a function of pH are presented in Fig. 1. Relative activity is defined as the rate of H_2O_2 consumption at a particular pH normalized with respect to the highest rate [20]. Experiments were performed using different buffer solutions to maintain the identical pH conditions in the pH ranging from 2.6 to 10.0. Fig. 1 indicates that SBP is active over a wide range of pH with a maximum activity between pH 5.0 and 6.5. More than 90% of enzyme activity was observed at pH 6.5, more than 80% at pH 4.5 and 7.0, and >59% between pH 2.6 and 10. Similar results were reported with little shift in pH for SBP activity in aqueous guaiacol by Geng et al [11].

The enzyme was stable over a wide range from pH 2.6 to pH 10.0 with the highest stability between 5.0 and 6.5 (Fig. 2). These results indicate that SBP from seed hulls retains significant activity over a wide range of pH conditions and thus demonstrates its ability to oxidize different substrates in a broader range of pH.

Table 1. Comparison between two different extraction methods

Extraction method	Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)
SBP1[a]	52.6	0.735	71.56
SBP2[b]	17.3	0.723	23.93

[a] Obtained SBP through the first method,
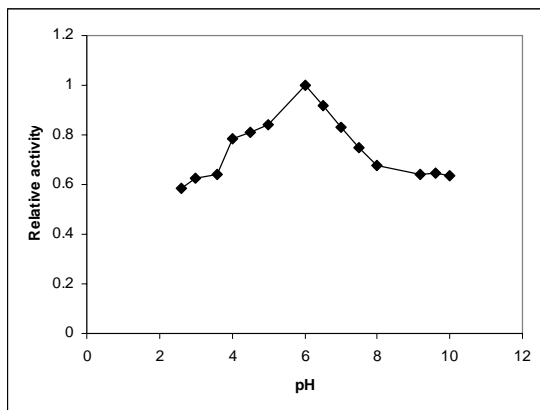[b] Obtained SBP through the second method

Figure 1. Relative activity of SBP under different pH buffers at 25°C
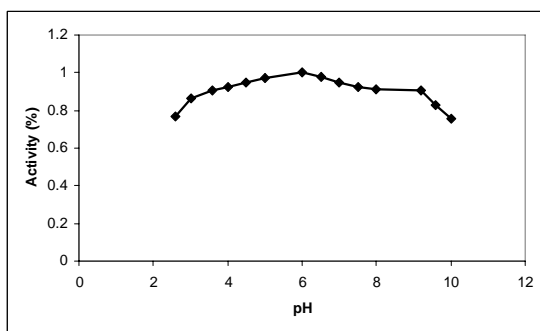

Figure 2. Effect of pH on the stability of SBP

Nicell et al. reported that HRP experienced peak activity at pH 7.4, with >90% of the maximum rate observed between pH 5.7 and 8.0, and >10% between pH 3.5 to 10.0 [19]. HRP is stable at a narrow range of pH between 4.0 and 6.0 and shows the highest stability at pH 9.0 [16]. Therefore, the versatility of SBP is an advantage allowing wider applications than HRP.

A comparison of these results shows that the activity of SBP appears to be slightly more sensitive to pH than HRP; however, both peroxidases retain significant activity over a wide range of pH conditions frequently encountered in wastewater treatment situations.

3.3- Combined effect of pH and temperature and thermo-stability of the SBP

The combined effect of pH and temperature on SBP activity was studied by varying the reaction temperature between 25 and 95°C. Initially, the pH of the reaction mixture was maintained at 6.0, as it resulted in the highest enzymatic activity (Fig. 1). Fig. 3 demonstrates the temperature profiles of SBP activity at different pHs. Interestingly, at pH 6.0, the highest enzymatic activity is observed at the temperature of 65°C and is about 2.5 times higher than the activity obtained at room temperature. More than 95% of the highest enzyme activity is observed between 55 and 75°C, and >70% between 45 and 85°C. In similar studies Geng et al. [11] found that the optimum temperature for SBP activity is 80°C and is about three times higher than the activity at room temperature. In comparison, the native HRP presented a typical bell-shaped curve, with maximum activity at 45°C followed by significant reduction of activity as the temperature increases [21]. Most of the organic pollutants are soluble in aqueous phase at higher temperatures [11]. Hence increase in reaction temperature would result in an increase in the rate of reaction of SBP against these substrates, as SBP is stable and active at elevated temperatures. From these observations, it can be postulated that SBP can be perceived to polymerise/oxidise most of the aromatic organic compounds presented in the industrial wastewater at a relatively higher rate compared to many plant/microbial peroxidases.

Most of the organic pollutants (such as chlorophenol, etc.) are soluble at neutral or alkaline pH. Their solubility reduces in

acidic pH [11]. In order to find out the influence of temperature and reaction pH on SBP activity at neutral and towards alkaline pH side, experiments were conducted at pH 7.0 and 8.0 by incubating the reaction mixture at different temperatures ranging from 25 to 95°C. The highest SBP activity was observed at a temperature of 65°C in both pH 7.0 and 8.0, but the relative activity is just only 1.9 and 1.77 times its original activity at room temperature, respectively (Fig. 3). The SBP activity is significantly lowered in the case of pH 8.0, when compared to the activity at pH 6.0 and 7.0. These results indicate that the combined effect of temperature and reaction pH greatly influences the activity of SBP.
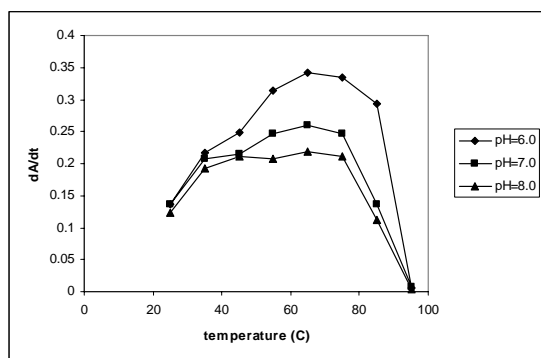

Figure 3. Combined effect of temperature and pH on SBP activity

The rate of heat inactivation was investigated in order to understand the relationship between the peroxidase and heat treatment. As shown in Fig 4, peroxidase activity remained heat-stable at 75°C. This suggests that SBP remains stable at this temperature. SBP exhibits non-linear patterns of heat inactivation due to the probable presence of other iso-enzymes and a wide range of potential substrates [22].
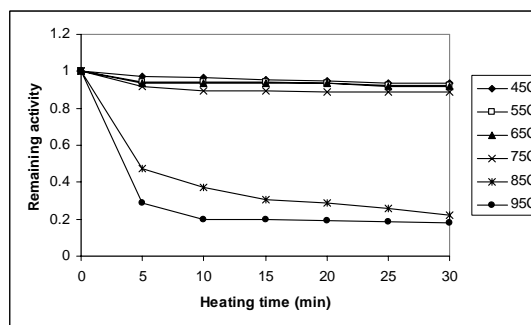

Figure 4. Thermostability and heat inactivation of SBP

3.4- SBP activity in organic solvents

Enzymes ubiquitously exhibit their activity predominantly in the aqueous phase. However, some enzymes are active in the presence of organic solvents in the aqueous phase [11]. In order to find out whether or not the SBP is active in organic solvents, experiments were carried out using different organic solvents such as acetone, ethanol, and methanol. The organic solvent affects the activity of the soybean enzyme. Some organic solvents seem to be more suitable than others for application with enzymatic reactions. Fig. 5 shows the effect of different organic solvents on an average relative activity of SBP at pH 6.0. From these results, it can be concluded that the enzyme exhibits maximum activity in acetone compared to the other tested organic solvents. The next suitable solvents are ethanol and methanol, respectively.

Similar experiments were performed by Geng et al. in order to determine the solvent effect on SBP activity [11]. The enzyme exhibited its highest activity in the presence of 16.67% (w/v) ethanol followed by acetone, methanol and acetonitrile.
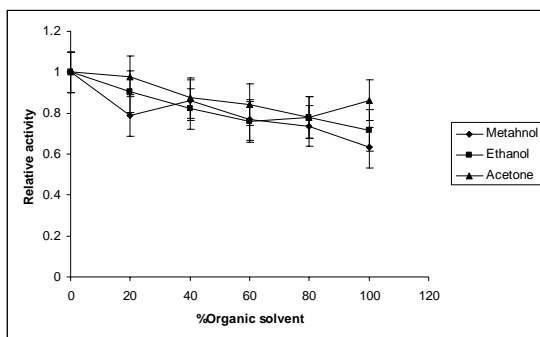
Figure 5. Effect of different organic solvents on the relative SBP activity

The ratio of water to organic solvent affects the activity of the enzyme. As the percentage of the organic solvent in the sample increases, the relative activity of the enzyme reduces. This result was expected since the enzyme activity under low water conditions tends to be restricted, and therefore reduced. Applying organic solvent is advantageous over the aqueous phase alone as it renders the enzyme to act against a variety of organic pollutants, which are highly soluble in organic solvent and hydro-organic mixtures. Similar to other enzymes, the best medium for enzymatic activity of SBP is water. However, depending on the applications and utilization of SBP, observing enzyme activity in a system made up of mostly organic solvents may be more beneficial than a system containing mostly water. Different organic solvent systems affect the activity of the SBP enzyme, while the enzyme is still found to be fairly active. The activity of the enzyme also depends on the organic solvent being used as a medium and on the ratio of water to organic solvent. As the amount of water in the system is decreased, the activity of the enzyme is also reduced.

3.5- Kinetic studies

In general, peroxidases are specific for H_2O_2 as substrate. However, they can also use a number of $^\bullet H$ donors, such as phenol [23]. Hence, the experiments were carried out to test the substrate specificity of SBP. The SBP was assayed at various concentrations of phenol and H_2O_2. Eqs. (3) and (4) were used to determine the K_m and V_{max} values from the Lineweaver–Burk (Fig. 6). Fig. 6a is the Lineweaver–Burk plot for H_2O_2 at 40 mM phenol, which shows a higher K_m value of 1.092 mM rather than phenol. This means that the enzyme shows more affinity towards phenol as indicated by the lower K_m value. The value of K_m for phenol is 0.765 mM (Fig. 6b), while this value is lower than those found for oxidation of guaiacol by POD from turnip roots (3.7 mM) [23] and Korean radish roots (6.7–13.8 mM) [24]. These results suggest that SBP has a higher specificity to phenol than to hydrogen peroxide in this study. The obtained kinetic results are summarized in Table 2.

**Table 2.** Determination of kinetic parameters for crude SBP

Substrate	Concentration range(mM)	K_m (mM)	V_{max} (μmol/ml.min)
phenol	5 – 40	0.765	51.02
H_2O_2	0.25 – 2.5	1.092	84.03

4- Conclusions

The application of soybean peroxidase to catalyze the polymerization and precipitation of aqueous phenols by hydrogen peroxide is potentially promising since this peroxidase is more convenient and less expensive than
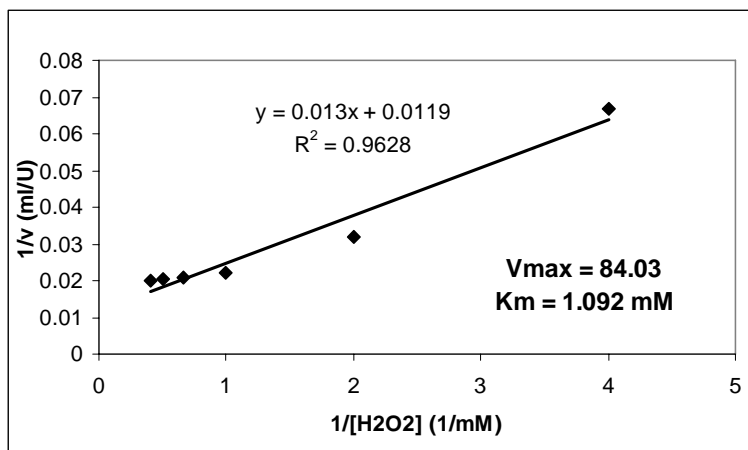
(a)
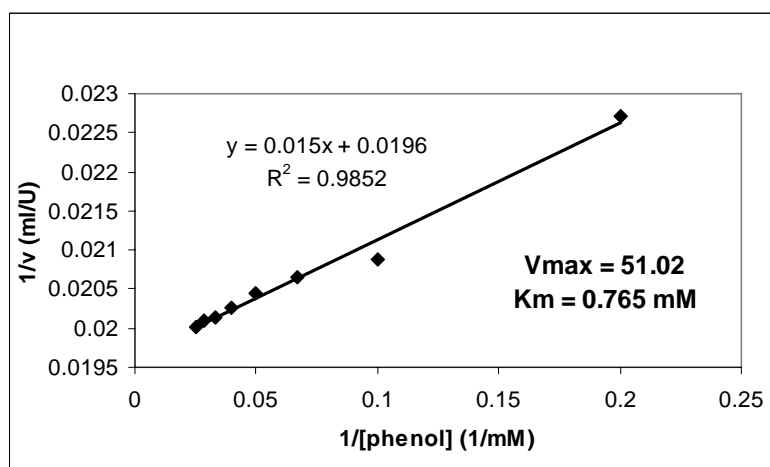
(b)

Figure 6. Lineweaver–Burk double reciprocal plots for SBP activity at various concentrations of H_2O_2 at 40 mM phenol (a); phenol at 1 mM H_2O_2 (b)

horseradish peroxidase (HRP), which has been the focus of most wastewater researches. From the findings of this study, it is then concluded that SBP extracted from soybean seed hulls is a highly robust enzyme and possesses higher stability and activity under a wide range of pH and at elevated temperatures. Also, this enzyme is fairly active in the presence of organic solvents such as acetone, methanol and ethanol, which widens the applicability of SBP for the treatment against a variety of organic pollutants present in industrial and petroleum waste waters, and its application may be advantageous as a biosensor or for lower cost industrial wastewater treatment compared to other peroxidases such as HRP.

5- Nomenclature

dA/dt   slope of adsorption intensity, min

ε extinction coefficient

υ_0 initial rates, μmol/ml.min

K_m Michaelis constant, mM

V_{max} maximum velocity, μmol/ml.min

K_a K_m for substrate A (H_2O_2), mM

A_0 concentration of substrate A, mM

K_b K_m for substrate B ($^\bullet$H donor), mM

B_0 concentration of substrate B (phenol), mM

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