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The Oxidation of Phenylhydrazine by Tyrosinase

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Abstract Tyrosinase was found to catalyze the oxidation of phenylhydrazine to phenol in a reaction that did not resemble those typically performed by tyrosinase. The kinetics of this reaction was investigated by measuring the initial velocity of the formation of phenol (25 °C). The values of k_{cat} and K_M for the oxidation of phenylhydrazine were obtained as 11.0 s^{-1} and 0.30 mM, respectively. The generation of superoxides during the oxidation of phenylhydrazine by tyrosinase was monitored by nitroblue tetrazolium (NBT) assay. In the phenylhydrazine-tyrosinase reaction, 1 mol O_2 was required for the production of 1 mol phenol and 1/6 mol superoxide. The decomposition of superoxide by superoxide dismutase enhanced the rate constant of the oxidation of phenylhydrazine. Phenol formed in the oxidation of phenylhydrazine by tyrosinase was further oxidized by tyrosinase to an *o*-quinone, after the oxidation of phenylhydrazine by tyrosinase was almost completed.

Keywords Tyrosinase · Phenylhydrazine · Phenol · Superoxide

Introduction

Tyrosinase belongs to the family of type-3 copper proteins that contain a di-copper center [1, 2]. The structure of *Streptomyces castaneoglobiporus* tyrosinase was determined recently [3] and its di-copper center was shown to be similar to that of other members of this family: catechol oxidases [4] and hemocyanins [5]. The di-copper center in tyrosinase exists in three forms: *met*, *deoxy* and *oxy*. The *oxy* form binds di-oxygen as a peroxide in the side-on bridging (μ-η^2:η^2) manner and the *met* form contains two Cu(II) ions with a hydroxyl bridge. The *deoxy* form contains two Cu(I) ions and is converted into the *oxy* form by binding one molecular oxygen. The *oxy* form is capable of oxidizing L-tyrosine to produce L-dopa and subsequent dopaquinone, which leads to melanins [6, 7].

Tyrosinase has been used in the design of an electrochemical biosensor for the detection of hydrazine derivatives [8]. The formation of *o*-quinone from phenols by tyrosinase catalysis can be inhibited by hydrazine derivatives. This sensor detects hydrazine derivatives according to a decrease in *o*-quinone formation. However hydrazine derivatives may also

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react with tyrosinase, rather than inhibiting the enzyme. In addition, tyrosinase was been applied for the deprotection of amino acid phenylhydrazides used in peptide synthesis [9]. However, the reaction required large amounts of this enzyme (up to 2.5 mass equivalents with respect to the substrate) and the removal of the phenylhydrazide protecting group was shown to be time consuming (5 to 24 h) [10].

The oxidation of phenylhydrazine by tyrosinase has not been studied, since phenylhydrazine [11–14] is considered to be an inhibitor of tyrosinase and does not resemble the monophenolic or *o*-diphenolic moieties that serve as natural substrates for tyrosinase. Here we have investigated the oxidation of phenylhydrazine in the presence of tyrosinase. Spectrophotometric measurements showed gradual disappearance of phenylhydrazine and appearance of phenol during enzymatic oxidation. The reaction rate of the oxidation of phenylhydrazine was greatly enhanced by tyrosinase, compared to the autoxidation of phenylhydrazine.

Materials and Methods

Chemicals

1-Acetyl-2-phenylhydrazine was obtained from ICN Biomedicals Inc. 2,4-Dimethylphenylhydrazine hydrochloride was purchased from Alfa Aesar. 2,4-Dimethylphenol, 2,4-dinitrophenylhydrazine and phenylhydrazine were obtained from TCI. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Bio Basic Inc. H_2O_2 was obtained from Riedel-de Haën. 2-(*N*-Morpholine)-ethane sulphonic acid (MES) was from Amresco. Nitroblue tetrazolium (NBT), L-dopa, tyrosinase (*Agaricus bisporus*) and superoxide dismutate (horseradish) were obtained from Sigma-Aldrich. Urea was purchased from Mallinckrodt.

UV–vis Absorption Spectra

UV–vis absorption spectra were recorded on a spectrophotometer (Agilent 8453, USA). A cuvette with a path length of 1.0 cm was used for measurements.

Purification and Activity Assay of Tyrosinase

Mushroom tyrosinase (5,370 units/mg) purchased from Sigma was purified by the procedure of Duckworth and Coleman [1]. The activity of tyrosinase was assayed by using L-dopa as a substrate. To a solution containing 2.21×10^{-7}M tyrosinase in 20 mM HEPES (pH 6.8), a different amount (0.1 to 0.7 mM) of L-dopa was added for the reaction. The absorbance increase at 475 nm due to the formation of dopachrome ($\varepsilon_{475\ nm}$=3,600 $M^{-1}cm^{-1}$) was monitored as a function of time [15].

Enzymatic Oxidation of Phenylhydrazine by Tyrosinase

To a solution containing 2.21×10^{-7}M tyrosinase in 20 mM HEPES (pH 6.8), different amount (0.1 to 1.6 mM) of phenylhydrazine was added for the reaction. The reaction was carried out at 298 K. The reaction mixture was incubated for 5 min and separated by HPLC (Purospher STAR RP-18e). The product collected was determined as phenol by ESI-Mass and Gibbs reagent [16]. The wavelength of 270 nm was used to monitor the formation of phenol. The initial rate was obtained by a linear fitting for the first minute. The difference of absorbance was divided by the difference of extinction coefficient between phenol and

phenylhydrazine in order to obtain the concentration. The extinction coefficients of phenol and phenylhydrazine at pH 6.8 are 1,657±5 and 1,256±4 $cm^{-1}M^{-1}$, respectively. The difference of extinction coefficient between phenol and phenylhydrazine is 401 $cm^{-1}M^{-1}$.

The nitroblue tetrazolium assay for monitoring the existence of superoxide during the oxidation of phenylhydrazine by tyrosinase

To a solution containing phenylhydrazine (88.4 μM) and tyrosinase (2.21×10^{-7}M) in 20 mM HEPES (pH 6.8), NBT (33 μM) was added for the reaction. The reaction was carried out at 298 K and the absorbance increase at 580 nm due to the formation of formazan was monitored as a function of time. This assay based on the reduction of NBT by superoxide is detected by the change of colorless NBT to the blue formazan ($\varepsilon_{580\ nm}$= 30,000 $M^{-1}cm^{-1}$) [17].

The Oxidation of Phenylhydrazine by Tyrosinase in the Presence of Superoxide Dismutase

To a solution containing phenylhydrazine (88.4 μM) and tyrosinase (2.21×10^{-7}M) in 20 mM HEPES (pH 6.8), superoxide dismutase (SOD; 0.5 μM) was added for the reaction. The reaction was carried out at 298 K and the increase in absorption at 270 nm due to the formation of phenol was monitored as a function of time.

Oxygen Consumption of the Oxidation of Phenylhydrazine by Tyrosinase

Oxygen consumption of the oxidation of phenylhydrazine by tyrosinase was measured with a Clark-type electrode (YSI 5331A) in a biological oxygen monitor (YSI 5300A).

Results and Discussion

The Oxidation of Phenylhydrazine by Tyrosinase

Oxidation of phenylhydrazine in the presence of tyrosinase was first monitored by UV–vis spectra (Fig. 1). Phenylhydrazine has two absorption bands at 230 and 280 nm (reaction

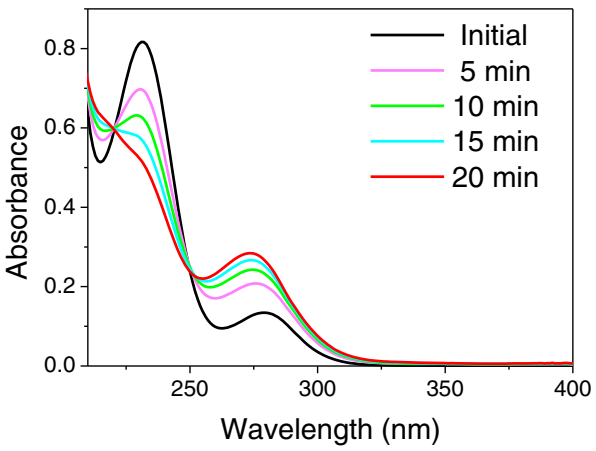

Fig. 1 The changes in UV–vis spectra during the reaction of phenylhydrazine with tyrosinase. [phenylhydrazine]=88.4 μM, [tyrosinase]=0.221 μM, pH=6.8, 20 mM HEPES buffer

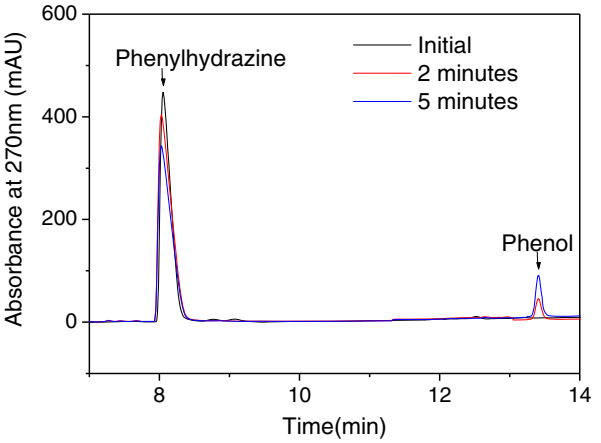

Fig. 2 The HPLC profile of the reaction of phenylhydrazine with tyrosinase. [phenylhydrazine]= 88.4 μM, [tyrosinase]=0.221 μM, pH=6.8, 20 mM HEPES buffer

time: 0 min). After adding tyrosinase, the band at 230 nm decreased and the maximum absorption of the second band gradually shifted from 280 to 270 nm (Fig. 1). The new absorption band centered at 270 nm was due to the formation of phenol. Figure 2 shows the analyses of the reaction mixture by HPLC. Five minutes into the reaction, a new peak was observed in the HPLC profile at an elution time of 13.5 min. This was indicative of phenol, which was the product of oxidation of phenylhydrazine and was confirmed by ESI Mass and Gibbs reagent.

The initial velocity of oxidation of phenylhydrazine by tyrosinase catalysis was obtained by a linear fitting of absorbance change at the wavelength 270 nm during the first minute. Kinetic parameters (k_{cat} and K_M) were obtained by Lineweaver–Burk plots (Fig. 3) and are listed in Table 1. The values of k_{cat} and K_M for the oxidation of phenylhydrazine were obtained as 11.0 s^{-1} and 0.30 mM, respectively. Compared to L-dopa [18], the K_M values of both molecules were similar, but the k_{cat} value of L-dopa was higher than that of phenylhydrazine. On the other hand, the k_{cat}/K_M value of L-dopa was 4-fold higher than that of phenylhydrazine. Unlike L-dopa, phenylhydrazine is not a natural substrate for tyrosinase; therefore, it has a lower k_{cat}/K_M value.

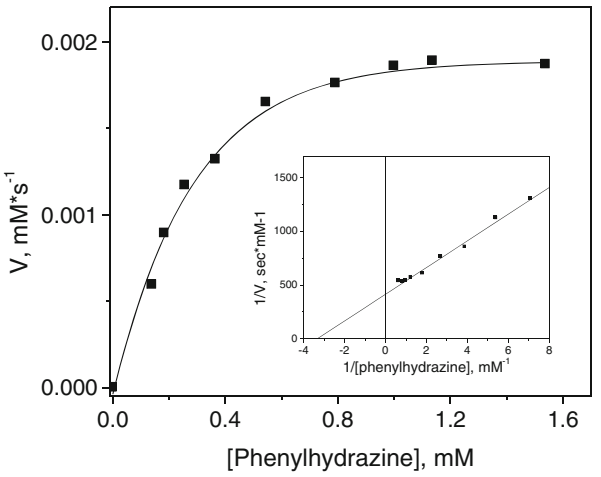

Fig. 3 Kinetics of the oxidation of phenylhydrazine by tyrosinase. *Inset*: the data re-plotted with $1/V$ as a function of 1/[phenylhydrazine]. [tyrosinase]=0.221 μM, 20 mM HEPES at pH 6.8

Table 1 Kinetic parameters of the oxidation of phenylhydrazine by tyrosinase

Substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}s^{-1}$)
L-Dopa	107.4±3.1[a]	0.8±0.03[a]	134±9[a]
Phenylhydrazine	11.0±0.3	0.30±0.01	36.7±1.1
1-Acetyl-2-phenylhydrazine	5.0±0.2	3.60±0.11	1.40±0.05
2,4-Dimethylphenylhydrazine	2.5±0.4	0.29±0.01	8.62±0.05

[a] Data are obtained from Beleski et al. [23]

The reaction of acetylphenylhydrazine with tyrosinase was found to be similar to that of phenylhydrazine. The product of oxidation of acetylphenylhydrazine by tyrosinase was also phenol, but the rate of oxidation of acetylphenylhydrazine by tyrosinase was slower. The k_{cat} value of acetylphenylhydrazine was 5.0 s^{-1}, lower than that of phenylhydrazine (11.0 s^{-1}). The K_M value of acetylphenylhydrazine was 3.60 mM, 12-fold higher than that of phenylhydrazine (0.30 mM). This indicates that tyrosinase has low binding affinity toward acetylphenylhydrazine. In addition, the k_{cat}/K_M value of phenylhydrazine was 26-fold higher than that of acetylphenylhydrazine. This indicates that the oxidation of phenylhydrazine by tyrosinase was more efficient than that of acetylphenylhydrazine. This accounts for low efficiency of the deprotection of amino acid phenylhydrazides, an acetylphenylhydrazine analog, requiring large amounts of tyrosinase and a time-consuming (5–24 h) deprotection process. 2,4-Dimethylphenylhydrazine and 2,4-dinitrophenylhydrazine were also tested in the reaction with tyrosinase. 2,4-Dimethylphenylhydrazine was oxidized by tyrosinase, but no oxidation was observed for 2,4-dinitrophenylhydrazine. Both compounds have substitutions in the 2 and 4 positions of benzene, but have different reactivity. The oxidation potentials of phenylhydrazine and 2,4-dinitrophenylhydrazine are 0.41 and 0.58 V, respectively [19]. The higher oxidation potential of 2,4-dinitrophenylhydrazine accounts for its non-reactivity with tyrosinase.

Generation of Superoxide During the Oxidation of Phenylhydrazine by Tyrosinase

Superoxide was found to be an intermediate during the oxidation of phenylhydrazine by Cu(II) [20, 21]. In addition, superoxide was also found in the oxidation of L-dopa by tyrosinase [22]. In order to explore the existence of superoxide during the oxidation of phenylhydrazine by tyrosinase, the NBT assay was used. This assay is based on the reduction of NBT by superoxide and is detected by the change of colorless NBT to the blue formazan (Fig. 4). Each NBT can be reduced by four superoxide ions [23]. Figure 5 shows the results of addition of NBT to the oxidation of phenylhydrazine by tyrosinase. The absorbance at 580 nm increased with time during the oxidation of phenylhydrazine by tyrosinase. In addition, phenylhydrazine and tyrosinase did not react with NBT. These

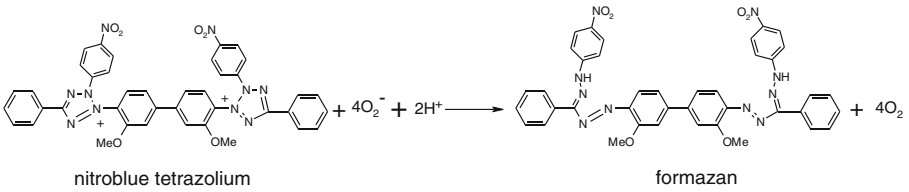

Fig. 4 The reaction of nitroblue tetraolium (NBT) with superoxide

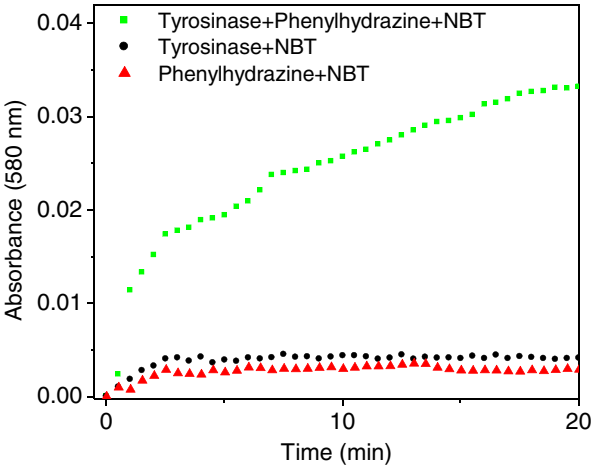

Fig. 5 The changes in UV–vis spectra during the reaction of phenylhydrazine with tyrosinase in the presence of NBT. [phenylhydrazine]=88.4 μM, [tyrosinase]=2.21×10^{-7} M, [NBT]=33 μM, 20 mM HEPES, pH 6.8

observations clearly indicated the formation of superoxide in the oxidation of phenylhydrazine by tyrosinase.

With a fixed concentration of phenylhydrazine, superoxide formation was a linear function of tyrosinase concentration (Fig. 6). The relationship between O_2 consumption and product (phenol and superoxide) formation is listed in Table 2. Under the same reaction conditions (with 0.221 μM tyrosinase), 1 mol O_2 is required for the production of 1 mol phenol and 1/6 mol superoxide. Superoxide can be generated by the reduction of O_2 by phenylhydrazyl radical and by Cu(I) in tyrosinase.

In order to see the influence of superoxide on the oxidation of phenylhydrazine by tyrosinase, SOD was added into the reaction mixture. Figure 7 shows the influence of SOD on the oxidation of phenylhydrazine by tyrosinase. In the presence of SOD, the overall reaction of phenylhydrazine by tyrosinase was faster than that without SOD. Superoxides can be self-converted to H_2O_2 and O_2, but the conversion rate is slow. SOD can quickly decompose superoxide to H_2O_2 and O_2. The reaction rate enhancement by SOD is due to H_2O_2, which can accelerate the oxidation of phenylhydrazine. In addition, the oxidation reactions of phenylhydrazine in the presence or absence of SOD were almost identical; SOD did not alter the auto-oxidation rate of phenylhydrazine.

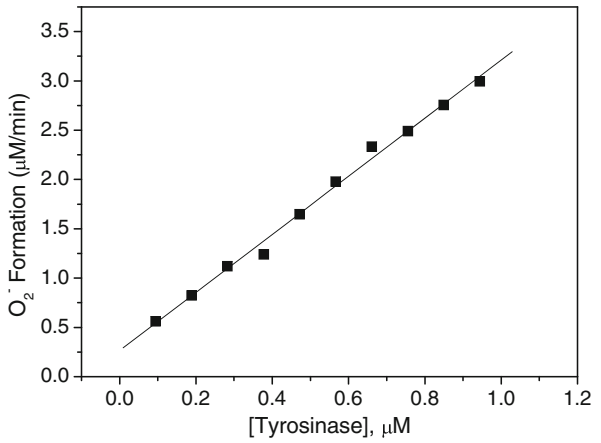

Fig. 6 Relationship between superoxide formation and the concentration of tyrosinase. The reaction mixture contains: [phenylhydrazine]=88.4 μM, [NBT]=30 μM, 20 mM HEPES, pH 6.8

Table 2 O_2 consumption and phenol, superoxide formation in the oxidation process of phenylhydrazine by tyrosinase[a]

Substance	μM/min[b]
O_2^- formation	0.96±0.03
O_2 consumption	5.97±0.08
Phenol formation	6.20±0.09

[a] The reaction mixture contains [phenylhydrazine]=88.4 μM, [tyrosinase]=0.221 μM, [NBT]=30 μM, 20 mM HEPES, pH 6.8

[b] Initial rates

H_2O_2 is an intermediate generated from the decomposition of superoxide and has been found to accelerate the oxidation of phenylhydrazine by Cu(II) [20]. Figure 8 shows the influence of H_2O_2 on the oxidation of phenylhydrazine by tyrosinase. Addition of H_2O_2 increased the overall oxidation velocity of phenylhydrazine by tyrosinase. Tyrosinase has been found to be a peroxygenase, which utilizes H_2O_2 to form oxy-tyrosinase [24]. The oxidation of phenylhydrazine by *oxy*-tyrosinase was faster than by *met*-tyrosinase. This accounts for faster oxidation of phenylhydrazine by tyrosinase in the presence of H_2O_2.

Further Oxidation of Phenol by Tyrosinase

Phenol, the product of the oxidation of phenylhydrazine by tyrosinase, was further oxidized by tyrosinase. Figure 9 shows the reaction profile of the oxidation of phenylhydrazine by tyrosinase. The absorbance at 270 nm (red dash line in Fig. 9) was used to monitor the formation of phenol. From the beginning to 1,800 s (30 min), the absorbance at 270 nm increased. This is due to the formation of phenol. From 2,100 to 2,500 s, the absorbance at 270 nm decreased and a new band at 415 nm was observed. The new absorption band at 415 nm (black dash line in Fig. 9) is a typical absorption of an *o*-quinone. This indicated that phenol, the product of the oxidation of phenylhydrazine by tyrosinase, was further oxidized by tyrosinase to be an *o*-quinone. The consumption of phenol resulted in a decrease in absorbance at 270 nm. After 2,500 s, a rapidly increasing absorbance at 270 nm was

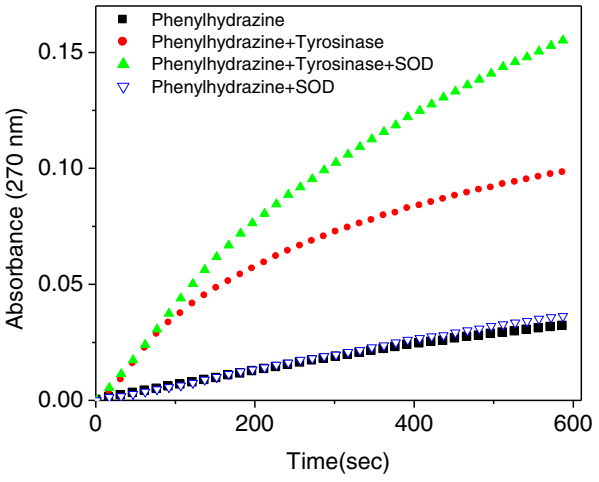

Fig. 7 The changes in UV–vis spectra during the reaction of phenylhydrazine with tyrosinase in the presence of SOD. [phenylhydrazine]=88.4 μM, [tyrosinase]=0.221 μM, [SOD]=0.5 μM, 20 mM HEPES, pH 6.8

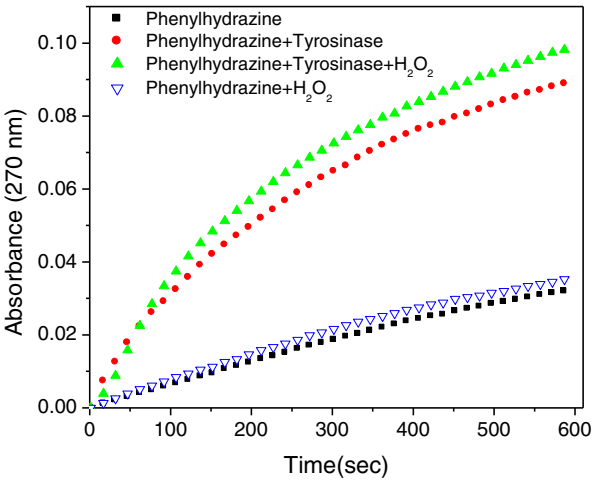

Fig. 8 The changes in UV–vis spectra during the reaction of phenylhydrazine with tyrosinase in the presence of H_2O_2. [tyrosinase]=0.221 μM, [H_2O_2]=5.37 mM, 20 mM HEPES at pH 6.8

observed. The final spectrum (46 min) was very broad, extending to 800 nm, suggesting the formation of melanoid polymers [25].

Oxidation of phenylhydrazine by tyrosinase was not completely unexpected as Cu(II) ions have been applied for the oxidation of hydrazine derivatives [26]. Since tyrosinase is a copper-containing enzyme, it can initiate the oxidation of phenylhydrazine. From what is known about tyrosinase's substrate specificity, the access to the active site of tyrosinase is critical for the oxidation of phenylhydrazine. Compared to natural substrates such as phenol and catechol, phenylhydrazine also has a phenyl group and it is proposed that this interacts with histidine before the reaction [3, 27]. In addition, aniline was also found to react with tyrosinase to form aminophenol [28]. Phenylhydrazine has an extra amine than aniline and is also able to approach the di-copper center.

In this work, the oxidation of phenylhydrazine was found to be initiated by tyrosinase, a copper protein with a di-copper center near the protein surface. The oxidation of phenylhydrazine was first initiated by *met*-tyrosinase; the major form after purification containing a di-copper(II) center. Phenylhydrazine binds to copper ions in the di-copper center and undergoes electron transfer from phenylhydrazine to Cu(II) to form a

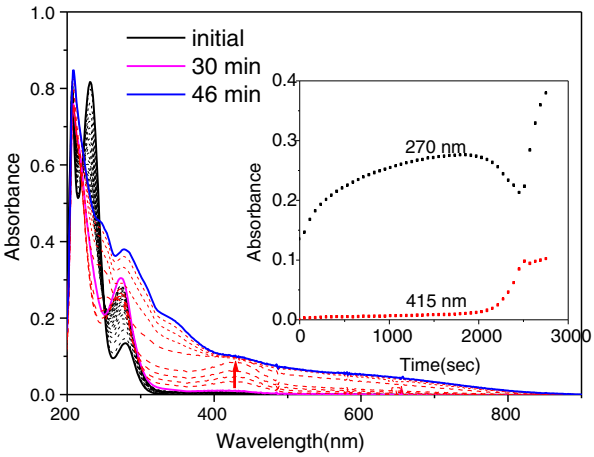

Fig. 9 The changes in UV–vis spectra during the reaction of phenylhydrazine with tyrosinase. [phenylhydrazine]=88.4 μM, [tyrosinase]=0.221 μM, pH=6.8, 20 mM HEPES buffer

benzenediazonium ion and Cu(I). Cu(I) further reacts with di-oxygen to form *oxy*-tyrosinase in which the oxidation of phenylhydrazine is able to continue. In addition, further reaction of benzenediazonium ions with water will form N_2 and phenol [29, 30].

Phenol was the product of the oxidation of phenylhydrazine by tyrosinase and was further oxidized by tyrosinase to be an *o*-quinone. Phenol and phenylhydrazine can both be oxidized by tyrosinase. Oxidation of phenol by tyrosinase was only observed after the oxidation of phenylhydrazine by tyrosinase was almost completed. This observation indicated that the oxidation of phenylhydrazine by tyrosinase was dominant. Phenylhydrazine, a reduction reagent, is more favored to be oxidized by tyrosinase than phenol.

In conclusion, we have demonstrated that tyrosinase can catalyze the oxidation of phenylhydrazine to phenol. Superoxide was generated in the reaction and retarded the oxidation of phenylhydrazine. Removal of superoxide by SOD enhanced the rate constants due to reducing tyrosinase degradation. Further oxidation of phenol to be an *o*-quinone was observed after the oxidation of phenylhydrazine by tyrosinase was almost completed.

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