Conformational Changes of Phenylalanine Dehydrogenase in the Presence of Ionic Gold and Alkaline pH

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ABSTRACT

Phenylalanine dehydrogenase (PheDH) is an important enzyme for determining the serum L-phenylalanine levels to diagnose phenylketonuria (PKU) disease. PheDH enzyme catalyzes the reversible oxidative deamination of L-phenylalanine to phenylpyruvate in the presence of NAD^+ as a cofactor. In this study, recombinant histidine-tailed *Bacillus badius* PheDH was expressed and purified by Ni-Sepharose affinity chromatography column. The kinetic properties of the native enzyme such as K_m, k_{cat}, V_{max} and k_{cat}/K_m values for L-phenylalanine and NAD^+ substrates in the oxidative deamination reaction were determined. Then the effects of the gold salt and pH on the enzyme tertiary structure and enzyme activity was assayed using UV-Vis and fluorescence spectroscopy. The activity was decreased in the presence of certain concentrations of gold compared to the native enzyme. Also the results showed that gold or high pH (~12.5) affects the tertiary structure of the PheDH enzyme, because intrinsic fluorescence emission at 340 nm decreased for native enzyme in their presence.

INTRODUCTION

Phenylalanine dehydrogenase (PheDH; EC1.4.1.20) catalyzes the reversible oxidative deamination of L-phenylalanine (L-phe) to phenylpyruvate in the presence of NAD^+. Researchers have shown an interest in study of PheDH because it plays a key role in determining the blood L-phe levels in the diagnosis of phenylketonuria (PKU) disease [1-3], so it may be used in diagnostic biosensors and neonatal screening kit. It is also very useful in the asymmetric synthesis of L-phenylalanine for pharmaceutical and food industries [4,5]. The autosomal genetic disorder phenylketonuria is a disease caused by deficiency of the phenylalanine hydroxylase (PAH; EC 1.14.16.1) enzyme; deficiency of PAH causes phenylalanine accumulation, which can be toxic in the blood and brain and affects brain function. Therefore, PKU should be diagnosed and treated

in the first days of life [6-8]. PheDH occurs in various sources, it was first found in *Brevibacterium* sp. and then was isolated from several microorganisms. Among the various microorganisms used, Wild-type *Bacillus badius* PheDH shows greater specificity for L-phenylalanine; so it is more useful in monitoring PKU [5,9]. In fact, the *B. badius* PheDH as a strain with suitable activity has attracted much attention in enzymatic and diagnostic studies [1,5]. There are several types of PheDH in terms of structures and molecular weights. They may be octameric (PheDHs from *Sporosarcina* [10], *Bacillus* [10] and *Microbacterium* [11]), tetrameric (*Rhodococcus sp* [12]) dimeric (*R. Maris* [13]), and monomeric (*Nocardia* [14]) enzyme. The class of the PheDH enzymes also differ in kinetic properties and substrate specificity [15]. The molecular weights of the subunits are 36,000 to 42,000 [12]

Researchers have shown great interest in designing diagnostic biosensors for PKU disease [16-18]. As well as, gold nanomaterials have attracted much attention in many

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studies in recent decades due to their unique properties. Gold nanoparticles and gold nanoclusters have been used extensively in the development and optimization of nanobiosensors [19-22]. Recently, some researchers have introduced biosensors both PheDH enzyme and gold nanomaterials [23-25]. Therefore, examining the effects of gold on PheDH enzyme can be helpful for subsequent studies.

In the present study, after expression, purification and examination of the Phenylalanine dehydrogenase enzyme of *Bacillus badius* kinetic properties, we focused on the gold and pH effects on PheDH activity. Then the effects of the gold and pH on the tertiary structure was measured using fluorescence spectroscopy.

MATERIALS AND METHODS

B. badius PheDH in *E. coli* BL21 containing the expression plasmid pET28a histidine-tailed was provided by Nora Gene Pishro Company (Tehran, Iran). L-phenylalanine (free acid) were purchased from Merck (Germany), Nicotinamide adenine dinucleotide (NAD^+), Isopropyl-D-thiogalactopyranoside (IPTG), kanamycin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (USA). Ni-NTA Sepharose column from Novagene. All assays were performed in three replicates.

Expression and Purification of Phenylalanine Dehydrogenase

For the purpose of expression histidine-tailed PheDH, 10 ml of Luria-Bertani (LB) medium containing 50 μg ml^{-1} kanamycin and 10 μl of relevant bacterial stock was prepared as a preculture; and incubated overnight at 37 °C, 180 rpm. Then, 1 ml of this seeding were cultured in 250 ml of Terrific Broth (TB) medium containing kanamycin (50 μg ml^{-1}) at 37 °C at 180 rpm for about 4 h until an OD600 ~0.6-0.8 was reached. Subsequently, the promoter was induced by addition IPTG to the final concentration 0.8 mM; and the solution was incubated at 18 °C for 16 h with 180 rpm shaking. Then the cells were collected by centrifugation (6000 g for 10 min at 4 °C). The pellets can be kept at -20 °C for further uses. Finally, the resulting cell pellets were resuspended in lysis buffer (Tris 50 mM, NaCl 300 mM and Imidazole 10 mM, pH 7.8) on ice. The

bacterial cells disrupted by sonication on ice for 15 min. Following this, for the purpose of supernatant collection, cell debris was removed by centrifugation (15000 g for 20 min, at 4 °C) in two steps. The collected supernatant was used for the enzyme purification. Finally, *B. badius* PheDH enzyme was purified by Ni-Sepharose affinity chromatography column. For this purpose, the supernatant was applied to Ni-Sepharose column on ice, and then washed with a washing buffer (Tris 50 mM, NaCl 300 mM and Imidazole 40 mM, pH 7.8). In the end, the histidine-tagged PheDH was eluted from the column by increasing the imidazole concentration to 250 mM. Purified PheDH was dialyzed in 100 mM glycine/KCl/KOH buffer (pH ~ 10.5) at 4 °C, and then sucrose was added to a final concentration 0.5 M, followed by storage at 4 °C [1]. These steps are shown in Fig. 1.

### Protein Purity and Concentration

The Purity of the fractions containing purified PheDH was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12%). Proteins were detected by coomassie blue staining. The concentration of purified recombinant PheDH was estimated by Bradford assay using bovine serum albumin as standards and coomassie blue at 595 nm.

Enzyme Assay

The reaction was assayed at room temperature spectrophotometrically by monitoring the formation of NADH at 340 nm in a 1 cm cuvette with a WPA Spectrophotometer. The reaction mixture (250 μl) was containing 100 mM glycine/KCl/KOH buffer (pH ~ 10.5), 25 μl NAD^+ (25 mM) and 25 μl L-phenylalanine (10 mM) and the enzyme solution [2,9]. The absorbance change for the initial 1 min was used for the enzyme activity because reaction gives a linear change in absorbance during this time.

Kinetic Properties of Phenylalanine Dehydrogenase Enzyme

In order to study kinetic Properties for L-phenylalanine, the NAD^+ concentration was fixed (2.5 mM) and various concentrations of L-phenylalanine (final concentrations of 0.039, 0.078, 0.15, 0.3, 0.6, 1.2, 2.5 and 5 mM) was used.
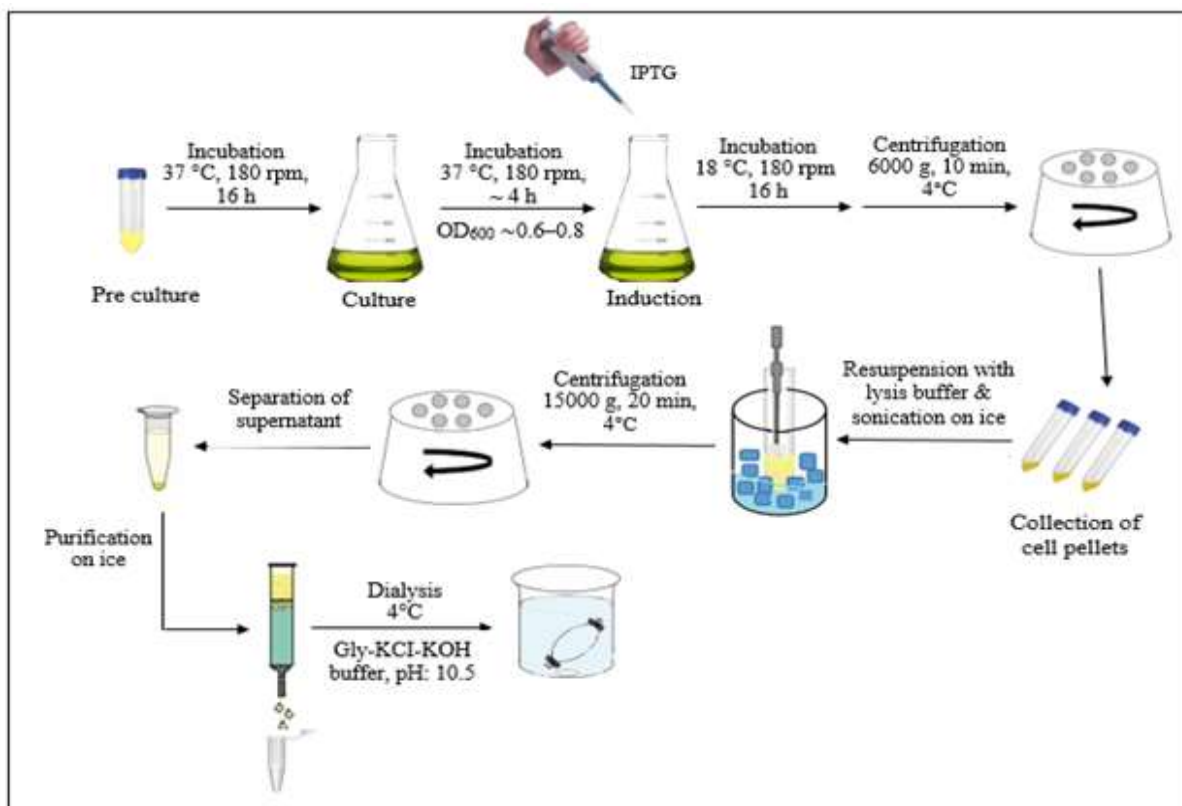
Fig. 1. Schematic representation of the expression and purification steps of the recombinant histidine-tailed PheDH.

NAD^+ kinetic properties was also determined in the presence of fixed concentration of L-phenylalanine (1 mM) and various concentrations of NAD^+ (final concentrations of 0.009, 0.019, 0.039, 0.078, 0.15, 0.3, 0.6, 1.2 and 2.5 mM). The enzyme assay was done under the described conditions. Each reaction was measured at room temperature during 1 min after injecting enzyme to cocktail solution at 340 nm [9].

Activity Studies of PheDH Enzyme in the Presence of Gold

For measurement of enzyme activity in the presence of gold, at first, 3 μl of enzyme (0.5 mg ml^{-1}) was incubated with 24 μl of glycine/KCl/KOH buffer (100 mM, pH ~ 10.5) and 3 μl $HAuCl_4$ solution to the final concentration 0.1, 1, 2.5, 5, 7, 10 and 100 μM for 4 min. After incubation, these solutions were added to the reaction mixture containing 170 μl of the same buffer, 25 μl NAD^+ (25 mM) and 25 μl L-phenylalanine (10 mM) and enzyme

activities were measured spectrophotometrically at room temperature during 1 min at 340 nm. The same conditions were applied to measure enzyme activity in the absence of Au.

Activity Studies of PheDH Enzyme at Different pH

Enzyme activities were evaluated at different pHs (5.0, 7.4, 10.6, 11.5 and 12.5; pHs of 11.5 and 12.5 were prepared using NaOH solution from the buffer at pH ~ 10.5). In each experiment, the solutions were used as blanks before enzyme addition. Finally, enzyme activities were assayed at room temperature by monitoring the absorption at the wavelength of 340 nm during 1 min.

Intrinsic Fluorescence of PheDH Enzyme in the Presence of Gold

The intrinsic fluorescence emission spectra of native PheDH in the Absence and Presence of gold were measured using a PerkinElmer spectrofluorimeter (LS55). For each

experiment, a specific concentration of HAuCl$_4$ (final concentrations of 0.1, 2.5, 7 and 100 µM) was added to PheDH enzyme (0.35 mg ml^{-1}), and was incubated at 25 °C for 5 min. Then the solution was excited at 280 nm and emission spectra were recorded between 300 nm and 450 nm. The same conditions were applied to measure the intrinsic fluorescence of native enzyme in the absence of gold.

Intrinsic Fluorescence of PheDH Enzyme at Different pHs

Intrinsic fluorescence investigation for PheDH purified enzyme at different pHs were performed by PerkinElmer spectrofluorimeter (LS55) apparatus at 25 °C. A volume of 4 µl from purified enzyme was added to 100 mM glycine/KCl/KOH buffer with different pH (5, 7.4, 10.6, 11.5 and 12.5; PHs of 11.5 and 12.5 were prepared using NaOH solution from the buffer at pH ~ 10.5) in a total volume of 60 µl. Emission spectra were recorded between 300 and 450 nm with an excitation wavelength of 280 nm.

RESULTS

### Expression and Purification of Phenylalanine Dehydrogenase

Expressed proteins were purified by affinity (Ni-NTA-Sepharose) chromatography. Figure 2 shows, obtained proteins were identical as reported earlier [1] from the SDS-PAGE analysis. The results indicated that the enzyme with molecular weight of 42 kDa was efficiently purified. The concentration of protein was estimated 3.5 mg ml^{-1} by Bradford assay.

Determination of Kinetic Properties of PheDH Enzyme

Kinetic parameters were calculated for the native *B. badius* PheDH after drawing Line weaver-Burk plot. These parameters such as K_m, k_{cat}, V_{max} and specificity constant (k_{cat}/K_m) for L-phenylalanine and NAD$^+$ substrates in the oxidative deamination reaction are summarized in Table 1. Effect of substrates concentration on the initial velocity are also presented in Fig. 3. Velocity (*V*) is defined as the amount of µmol NADH formed/min [2].
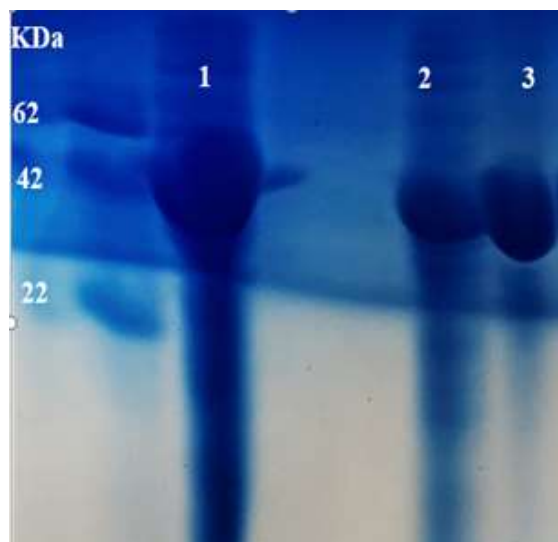
Fig. 2. SDS-PAGE analysis of *B. badius* PheDH enzyme. Protein marker, (1) bacterial flow soup, (2) washing step and (3) final eluted fractions were analyzed, then detected by staining with coomassie blue.
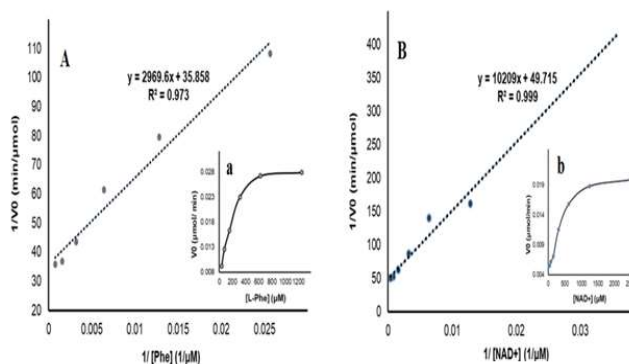
Fig. 3. Effect of substrates concentration on the initial velocity (V$_0$). (A) Lineweaver-Burk and (a) Michaelis-Menten plot in the presence of fixed concentration of NAD$^+$ (2.5 mM) and various concentrations of L- Phe. (B) Lineweaver-Burk and (b) Michaelis-Menten plot in the presence of fixed concentration of L-Phe (1 mM) and various concentrations of NAD$^+$.

Activity of PheDH Enzyme in the Presence of Gold

The activity analysis of native and PheDH-gold during 1 min at 25 °C showed a decrease in activity for enzyme in

Table 1. Kinetic Parameters of Native *B. Badius* PheDH for L-Phenylalanine and NAD$^+$ Substrates

Substrate	K_m (mM)	V_{max} (μmol min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
L- Phe	0.08	0.027	28	350
NAD$^+$	0.2	0.020	20.2	101

the Presence of gold ions compared to the free enzyme (Fig. 4).

Effect of pH on Enzyme Activity

Enzyme activity at different pHs was investigated. The results obtained from the optimum pH are presented in Fig. 5. The results showed a significant decrease in enzyme activity with increasing pH (~12.5).

### Intrinsic Fluorescence Analysis in the Presence of Gold

The fluorescence emission spectra are a key approach for the investigation of three-dimensional changes in protein structure [26]. The fluorescence spectroscopy results obtained from the free native enzyme alone and in the presence various concentrations of gold salt are presented in Fig. 6. From the data in this figure, it is apparent that PheDH in the presence of gold ions demonstrated the lower fluorescence emission at 340 nm comparison with native protein.

### Intrinsic Fluorescence Analysis at Different pHs

Intrinsic fluorescence for the native enzyme at its optimum pH (~10.5) and other pHs were investigated. As shown in Fig. 7, the intrinsic fluorescence intensity decreased sharply at 340 nm with increasing pH (~12.5). This indicates that at this pH, the structure of the enzyme changed.

DISCUSSION

Phenylalanine dehydrogenase is an important and key enzyme in the diagnosis of phenylketonuria in neonates [27-29]. Different forms of gold were used to design biosensors to detect phenylketonuria. [24,25,30]. In this study, in addition to kinetic studies, the tertiary structure and
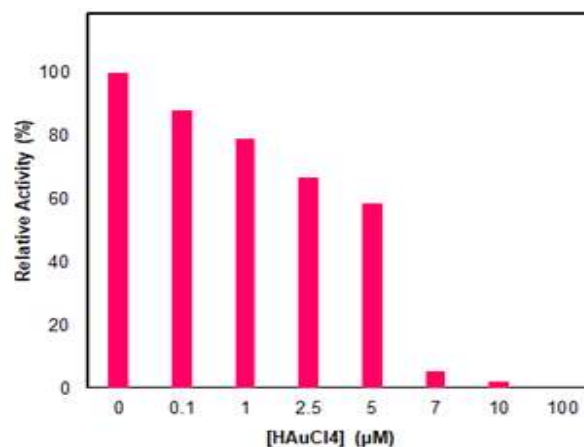

**Fig. 4.** Effect of various concentrations of gold on the PheDH activity during 1 min at 25 °C.
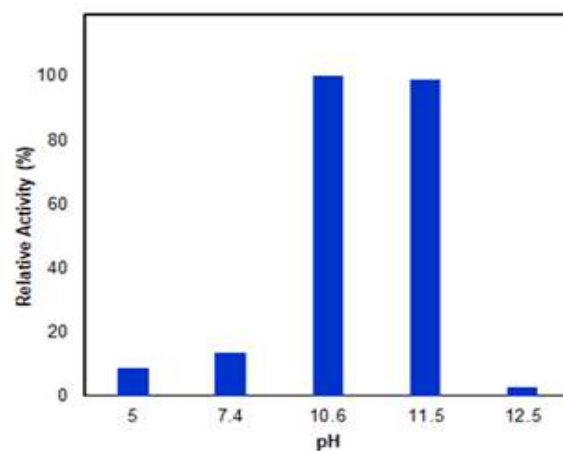
Fig. 5. Effect of different pHs on the PheDH activity during 1 min at 25 °C.

activity of the PheDH enzyme in the presence of various concentrations of gold salt and different pHs were investigated.

Steady state kinetic properties including enzyme substrate affinity (K_m), k_{cat}, V_{max} and specificity constant (k_{cat}/K_m) in the oxidative deamination reaction was calculated for both substrates using Line weaver-Burk plot. According to the kinetic studies, K_m values of PheDH enzyme were obtained for L-Phenylalanine and NAD$^+$ 0.08 and 0.2 mM respectively, which is almost consistent with previous studies [1,2,32]. K_m value for L-phenylalanine was lower than that for NAD$^+$. The PheDH specificity constant
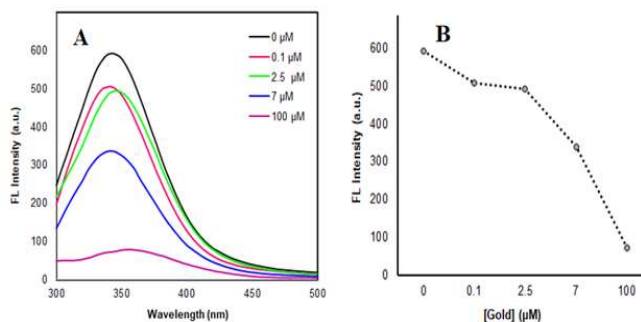
Fig. 6. (A) Effect of gold on the fluorescence intensity of PheDH, (B) Changes in the fluorescence intensity of PheDH in the absence and the presence of 0.1, 2.5, 7 and 100 μM HAuCl4 at 340 nm.
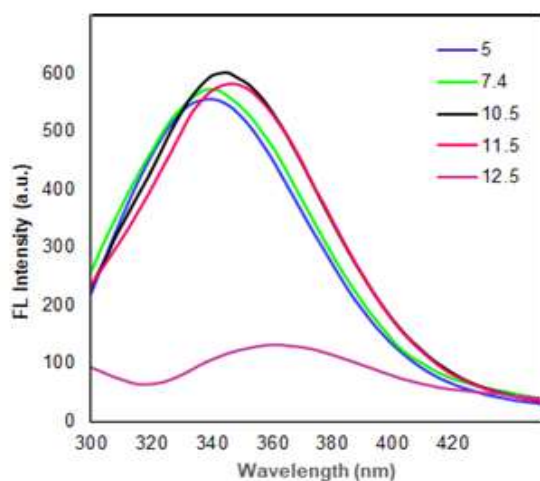
Fig. 7. Effect of different pHs on the intrinsic fluorescence intensity of PheDH.

(k_{cat}/K_m) for L-phenylalanine substrate was obtained about 3-fold relative to the NAD$^+$ substrate.

The activity assay showed a decrease in activity for enzyme in the presence of some concentrations of gold compared to the native. As well as the results showed a decrease in enzyme activity with increasing pH (~12.5). A comparison of the two results reveals that for the design of biosensors based on PheDH, the direct use of gold salt or changing pH may cause problems in the catalytic function of the PheDH. According to activity analysis, HAuCl$_4$ solution at final concentration of about 10 μM, caused a very sharp decrease in PheDH activity (2% compared to native enzyme).

The tertiary structural was investigated by the fluorescence analysis [31] for native PheDH and PheDH enzyme in the presence of gold. Phenylalanine dehydrogenase enzyme from *Bacillus badius* has several tyrosine residues, so was excited at 280 nm. Any changes in the enzyme conformation usually affect tyrosine fluorescence of protein. From the data we can see that intrinsic fluorescence spectra decreased for native enzyme in the presence of some concentrations of gold. In 2013, Ivana *et al.* published a paper in which they described Inactivation of cholinesterases by gold and silver ions. They reported that Inactivation of the enzyme may be due to a possible reaction of metal ions with protein amino acids such as Cys, Met, Trp and His [33]. In another major study, Berberich *et al.* (2005) reported that silver ions bind tightly to a cysteine residue and inhibit activity and normal dynamics of creatine amidinohydrolase enzyme[34]. Therefore, in this study, inactivation of the enzyme may be due to a possible reaction of gold ions with some amino acids. Also increasing pH (~12.5) caused a sharp decrease in fluorescence emission at 340 nm in comparison with native enzyme. Therefore, both of these factors, changed the conformation of PheDH enzyme.

CONCLUSIONS

Phenylalanine dehydrogenase is a vital enzyme in medical diagnostic for detection of phenylketonuria in newborn children. In summary, the purpose of the current study was to investigate the effect of gold ions on PheDH activity and its intrinsic fluorescence due to the widespread use of this metal in nanomaterials and biological applications. Enzyme activity and intrinsic fluorescence at different pHs were also investigated. Some concentrations of gold and high pH (~12.5) probably affected the tertiary structure of the PheDH enzyme, because intrinsic fluorescence decreased for native enzyme in their presence. Decrease of enzyme activity in the presence of these factors compared to the native enzyme was also observed. The correlations between PheDH and these parameters is interesting because this finding has important implications for designing and developing gold nanobiosensors using PheDH in the future.

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