

## A Sensitive and Reproducible Spectrophotometric Assay for Glucose Oxidase Activity and Its Application in Conformational Lock

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**Abstract**—Glucose-oxidase-horseradish peroxidase-guaiacol coupled system was applied for glucose oxidase assay with high sensitivity and perfect reproducibility. The optimum pH value was 5.8. The Michaelis-Menten constant ( $K_m$ ) and  $V_{max}$  were determined to be  $30.0 \pm 1.0$  mM and  $2.49 \pm 0.08$   $\mu$ M/s, respectively, for the assay system at 25 °C. The relative standard deviation was 2.8% for 31 times measurements of GOD activity. The GOD assay could be applied in conformational lock study on GOD. Results of bio-thermodynamics suggested that the number of contact area of GOD is two, which is consistent with the computation results by using Ligplot and Pymol.

**Keywords**—spectrophotometric assay; glucose oxidase; guaiacol; horseradish peroxidase; coupled system; conformational lock

### I. INTRODUCTION

Glucose oxidase (GOD) is an identical dimer and rigid glycoprotein, which catalyzes glucose in the presence of oxygen to gluconic acid and hydrogen peroxide [1]. The measurement of glucose oxidase activity is the basis study of its performance, with various methods. In a hydrochloric acid titration method [1], NaOH was added to glucose oxidase and  $\beta$ -D-glucose reaction system to terminate the reaction process, and then, a certain amount of hydrochloric acid was titrated in the system to calculate the consumption of hydrochloric acid solution, and concluded that the activity of glucose oxidase. In a spectroscopic method [2-3], glucose oxidase catalyzes the  $\beta$ -D-glucose to hydrogen peroxide. In the presence of peroxidase, the activity of glucose oxidase

can be measured by determination of colored product formation rate.

Phenol and o-dianisidine are two common chromogens. Unfortunately, phenol is volatile and harmful to the human body, the solubility of o-dianisidine in aqueous solution is low and the colored product has the maximum absorption peak at 400nm, which is unstable and easily disturbed by some background material in the sample [4].

The conformational lock is a bio-thermodynamic theory, which was suggested by Poltorak for the first time in 1998, to explain the properties of interfaces in oligomeric enzymes and their influence on catalytic activity through two independent methods, involving the use of the structural and kinetics data. From then on, various dimer enzymes from different sources have been investigated that in both methods are in reasonable agreement. Conformational lock theory may provide a special explanation about the process of thermal dissociation and denaturation of oligomeric proteins [5-10].

In this report, a new spectrophotometric method was proposed for the determination of GOD activity using a guaiacol-horseradish peroxidase-glucose oxidase coupled system. The suggested method has good sensitivity, reproducibility, and measurement accuracy. The GOD assay could be applied successfully in research on the conformational lock of the GOD.

## II. EXPERIMENTAL

### A. Reagents

Glucose oxidase from *Aspergillus niger* (GOD),  $\beta$ -D-glucose, o-dianisidine dihydrochloride, guaiacol and horseradish peroxidase (HRP, type II) were from Sigma. All chemicals were analytical grade without further purification. Double distilled water was used throughout these studies.

### B. The Kinetic Measurements

The activity of the glucose oxidase was determined using a TU-1901 spectrophotometer equipped with a temperature controller at 25 °C. HRP, GOD and guaiacol were added to phosphate buffer solution (PBS) and the reaction was initiated by adding  $\beta$ -D-glucose. The initial guaiacol oxidation rate was determined by the rate of colored product (tetraguaiacol,  $\epsilon_{470\text{nm}}=26.6\text{mM}^{-1}\text{cm}^{-1}$ ) [11-14]. Then initial rate of the reaction of glucose can be converted and the activity of GOD could be obtained. The relative reaction could be expressed as follows (Eq. 1 and 2):



The activity of GOD was calculated based on Eq. 3:

$$\text{Units/mg} = \frac{4 \times \Delta A_{470\text{nm}} / \text{min}}{\epsilon_{470\text{nm}} \times \text{mg(enzyme)/ml(reaction mixture)}} \quad (3)$$

### C. Research on Thermal Dissociation and Optimum Temperature ( $T_{\text{opt}}$ ) of GOD

The GOD samples (in various vials) were incubated separately at different temperatures in 50mM PBS at pH 5.8 for 30 minutes, then the samples was removed and cooled immediately in an ice-water bath and assayed for residual activity of GOD.

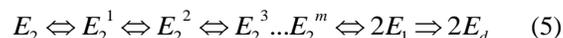
Optimum temperature ( $T_{\text{opt}}$ ) has been defined as the maximum temperature at which the activity of the enzyme does not change during the incubation time [15]. GOD samples at a concentration of were incubated in the thermostat at different temperatures in 50mM PBS at pH 5.8 for a certain minutes, cooled rapidly in an ice-water bath, and assayed for residual activity of GOD. The final concentrations of GOD, glucose, guaiacol and HRP, were  $9 \times 10^{-6}$  mM, 50mM, 3mM, and  $2.5 \times 10^{-5}$  mM, respectively.

### D. The Mechanism of Thermal Dissociation and Conformational Lock

According to the theory of Poltorak, a simple thermal dissociation could be expressed as Eq. 4:



$E_2$  is an active dimer form of enzyme,  $E_1$  and  $E_d$  are the monomers for the reversible transformation of initial structure and for denatured form of irreversible transformation of  $E_1$ , respectively. The  $E_d$  could not reunite and form an active enzyme ( $E_2$ ). Generally, there are several intermediate active forms of dimer enzyme (Eq.5):



It was supposed that the real number (m) of intermediates of catalytically active protein is always greater than a certain quantity (n). Here, n represents different active forms of the dimer enzyme, and can be obtained from an empirical Eq. 6 and 7 [5-10]:

$$n = \frac{0.13 + \delta}{0.13 - 0.05\delta} \quad (6)$$

$$\delta = R - 1 \quad (7)$$

Where,  $\delta$  is obtained from the kinetic plot of residue active versus time and depending only on the number of steps (n) before loss of activity of enzyme. These make it possible to estimate the minimal number of steps for a dimer enzyme in the process of thermal dissociation into inactive monomers. These phenomenons of interprotein contacts with possible partial breaks were also supposed as “conformational lock”.

### E. Biochemical Computation by Using Ligplot and Pymol

Ligplot [16] is a computer program for plotting interactions of protein-ligand. This program can generate schematic diagrams automatically of protein-ligand interactions from a given PDB file. The operating manual is located at the site:

<http://www.biochem.ucl.ac.uk/bsm/ligplot/manual/>.

Pymol is an open source based molecular visualization system initiated by user, and used to view 3D molecular structures of a protein's PDB file.

## III. RESULTS AND DISCUSSION

### A. Time Dependence of UV-Vis Spectra During GOD Assay

UV-vis spectra of the guaiacol oxidation process were shown in Fig.1. A colored product increased gradually at 470nm with increasing time, it is supposed that GOD catalyzes glucose to produce hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in the presence of oxygen, then guaiacol react with  $\text{H}_2\text{O}_2$  in the presence of HRP, and generates a colored product (tetraguaiacol). At last, the activity of GOD could be converted easily (see also Eqs. 1, 2 and 3).

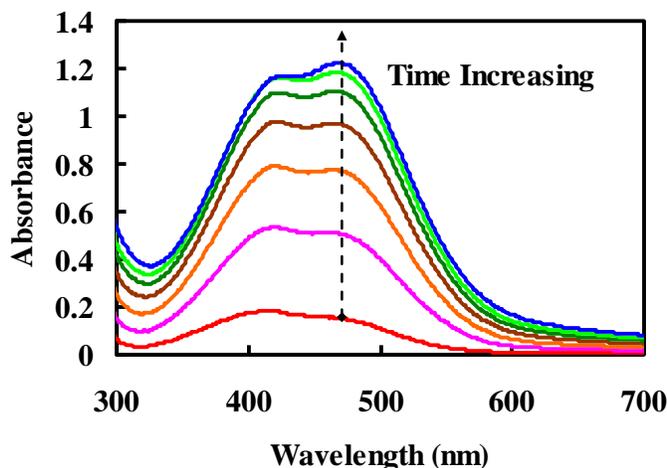


Figure. 1. Time dependence of UV-vis spectra of GOD in the presence of glucose, guaiacol and HRP in 50mM, pH5.8 PBS. The spectra were collected at 100s intervals.

### B. The Sensitivity Comparison between Guaiacol and O-Dianisidine Methods in GOD Assay

The guaiacol and o-dianisidine in the presence of HRP, GOD and glucose were used as chromogens for GOD assay. It could be seen that the guaiacol shows a higher sensitivity (Fig.2A and B, curve a) respect to o-dianisidine (Fig.2A and B, curve b). The Fig.2B was generated from Fig.2A. It could be seen that the two methods have very good linearity in the beginning of enzymatic reaction. The o-dianisidine method was suggested by sigma .

(<http://www.sigmaaldrich.com/china-mainland/zh/technical-documents/protocols/biology/enzymatic-assay-of-glucose-oxidase.html>).

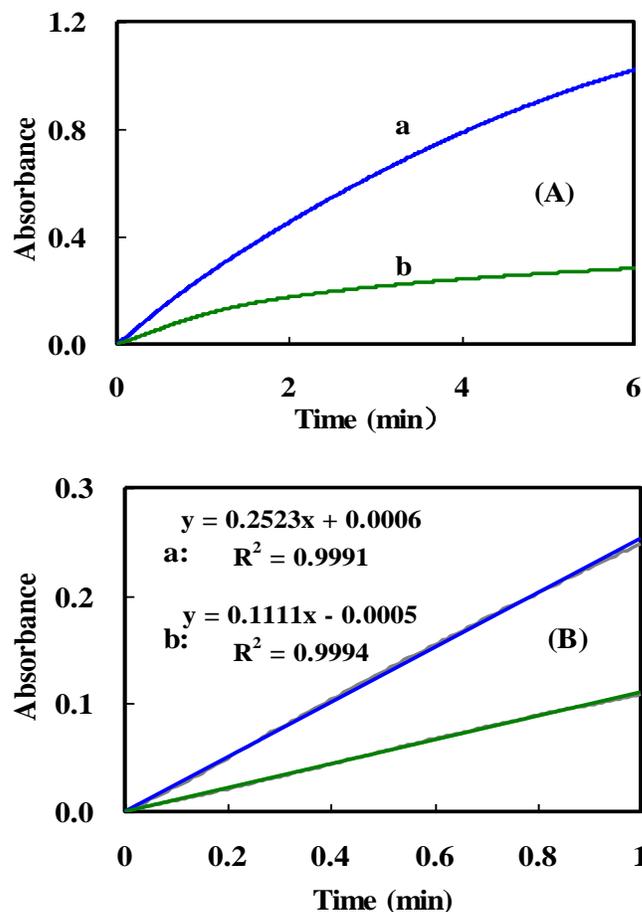


Figure. 2. (A) Relationship between absorbance and time for guaiacol (curve a) and o-dianisidine (curve b) in GOD assay. (B) Sensitivity comparison between guaiacol (curve a) and dianisidine (curve b) methods. The experiments were operated at 470nm for 3mM guaiacol (curve a), and 400nm for 0.16mM dianisidine (curve b) in 50mM, pH5.8 PBS at 25 °C containing  $2.5 \times 10^{-5}$  mM HRP,  $9 \times 10^{-6}$  mM GOD and 50mM glucose, respectively

### C. Kinetic Parameters of GOD

The Michaelis-Menten plot and relative Lineweaver-Burk plot of GOD are represented in Fig.4A and B, respectively. The Michaelis-Menten constant ( $K_m$ ) and maximum reaction rate of glucose ( $V_{max}$ ) could be obtained from Lineweaver-Burk plot using Eq.8:

$$\frac{1}{V_0} = \frac{K_m}{V_{max} [S]} + \frac{1}{V_{max}} \quad (8)$$

Where,  $V_0$  represents the initial glucose reaction rate in GOD assay. From the regression equation (Fig.3B):  $y=12.03x+0.4011$ ,  $V_{max}$  and  $K_m$  were calculated to be  $2.49 \pm 0.08 \mu\text{M/s}$  and  $30.0 \pm 1.0 \text{mM}$ , respectively. The  $K_m$  value is smaller than data provided by Sigma [16-17].

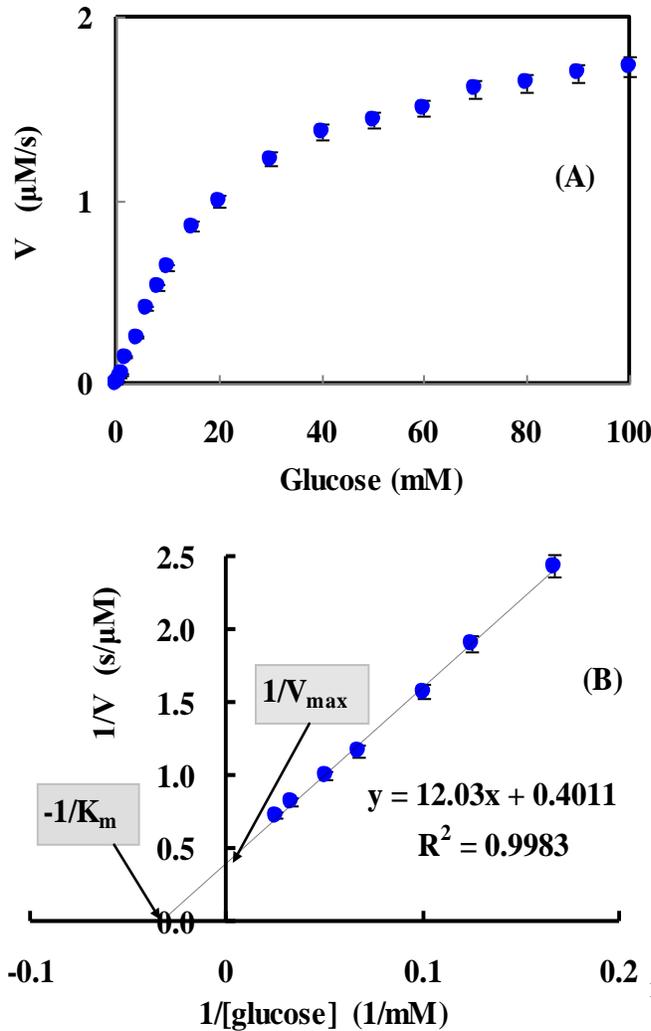


Figure. 3. (A) Michaelis-Menten and (B) Lineweaver-Burk plots of GOD in PBS (50mM, pH5.8, 25 °C) containing HRP ( $2.5 \times 10^{-5}$  mM), GOD ( $9 \times 10^{-6}$  mM), guaiacol (3mM), and glucose (ranging from 0.05 to 100mM).

#### D. The Ph Effects and Repeatability of GOD Assay

Fig.4A represents the effects of pH values on the GOD activity in the presence of glucose, guaiacol and HRP. The GOD activity increased with increasing pH value, and reached a maximum value at pH 5.8, which is close to the optimum pH value (pH5.5) for the native GOD from *Aspergillus niger* [18-19], indicates that the present GOD assay method does not change the optimum pH value of GOD.

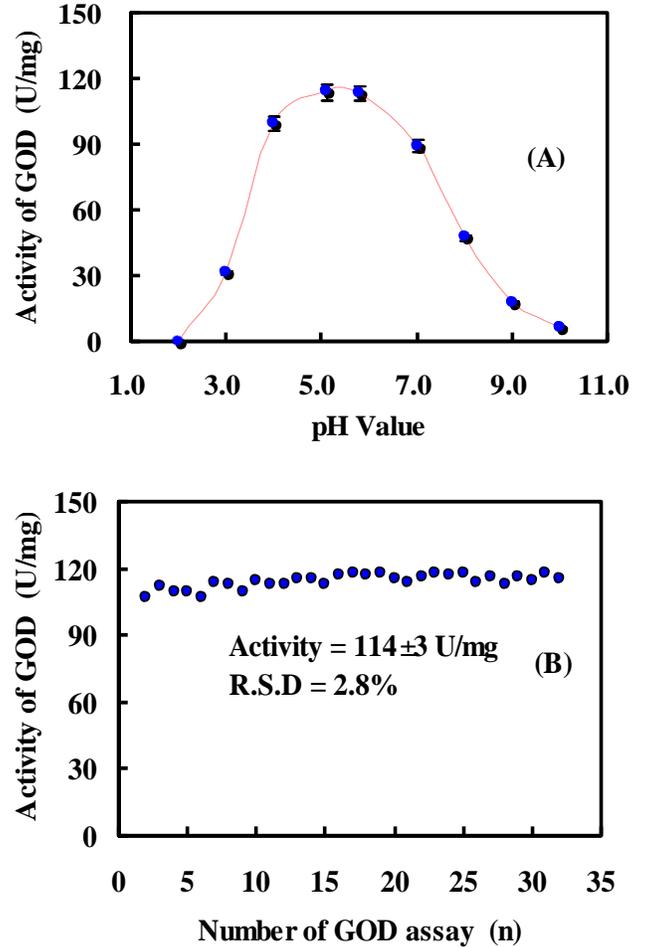


Figure.4 (A) Effects of pH values on the activity of GOD in 50mM PBS in the presence of GOD ( $9 \times 10^{-6}$  mM), glucose (50mM), guaiacol (3mM) and HRP ( $2.5 \times 10^{-5}$  mM). (B) Repeatability of the assay of GOD, the experiments was operated in PBS (50mM, pH 5.8, 25 °C) containing HRP ( $2.5 \times 10^{-5}$  mM), GOD ( $9 \times 10^{-6}$  mM), guaiacol (3mM), and glucose (50mM) at 470nm.

The repeatability of the assay was checked by determining the activity of GOD sample repeatedly, and the mean value of the GOD activity was calculated to be  $114 \pm 3 \text{ U/mg}$  using Eq.3 (see also Fig.4B), with a relative standard deviation (R.S.D) of 2.8% for 31 measurements. The determination GOD activity value is consistent with the data provided by sigma (Type VII from *Aspergillus niger*, Activity  $\geq 100 \text{ U/mg}$ ), indicates perfect repeatability of this method.

#### E. Linear Range and Detection Limit

To evaluate the linear range and detection limit for the concentration of GOD in this method, reaction rate ( $\Delta A/\text{min}$ ) of the enzymatic samples was measured when the concentration of GOD was increased gradually. It could be seen that the linear range for measurement of GOD activity was from  $4.3 \times 10^{-5}$  mM to  $9.3 \times 10^{-3}$  mM (Fig.5A). The detection limit was  $4.3 \times 10^{-5}$  mM (Fig.5B) [20-22].

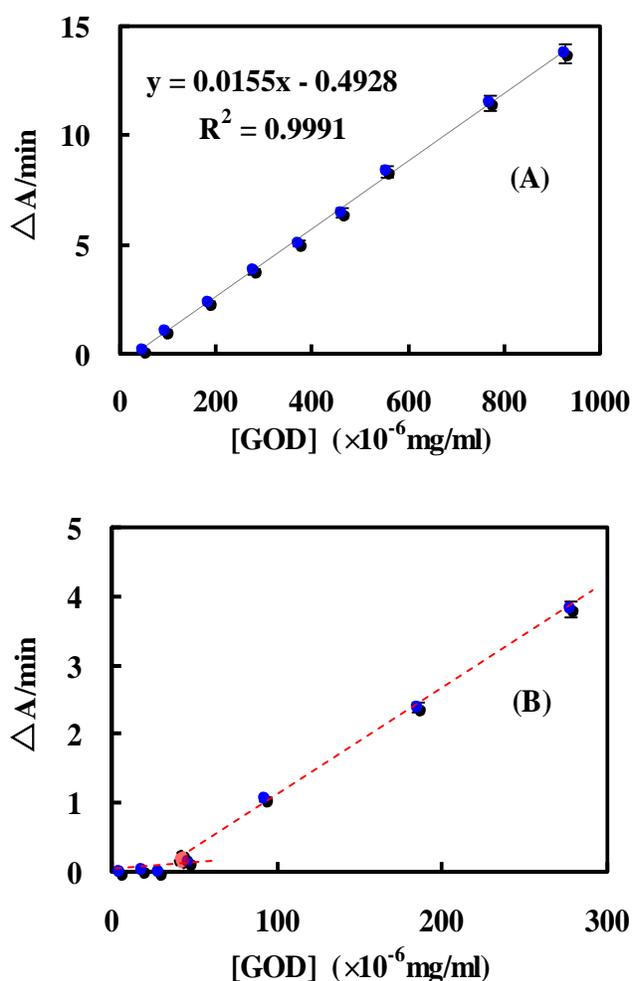


Figure.5 (A) Linear range and (B) Detection limit of the measurement for activity of GOD.

#### F. Optimum Temperature and *N* Value of GOD

There was less change for the residual activity of GOD at incubation temperatures below 55 °C. The activity of GOD will reduce rapidly at above 60 °C. Then,  $T_{opt}$  of GOD could be obtained to be 57 °C (Fig.6A). From Fig.6B, *R* value could be obtained to be 1.09 and 1.13, at 60 and 65 °C, respectively. Then  $\delta$  value ( $\delta=R-1$ ) could be conclude to be 0.09 and 0.13, and *n* could be calculated to be 1.77 ( $\approx 2$ ) and 2.1 ( $\approx 2$ ), at 60 and 65°C, respectively. From the results of the bio-thermodynamic research, there are two contact areas for the dimer GOD.

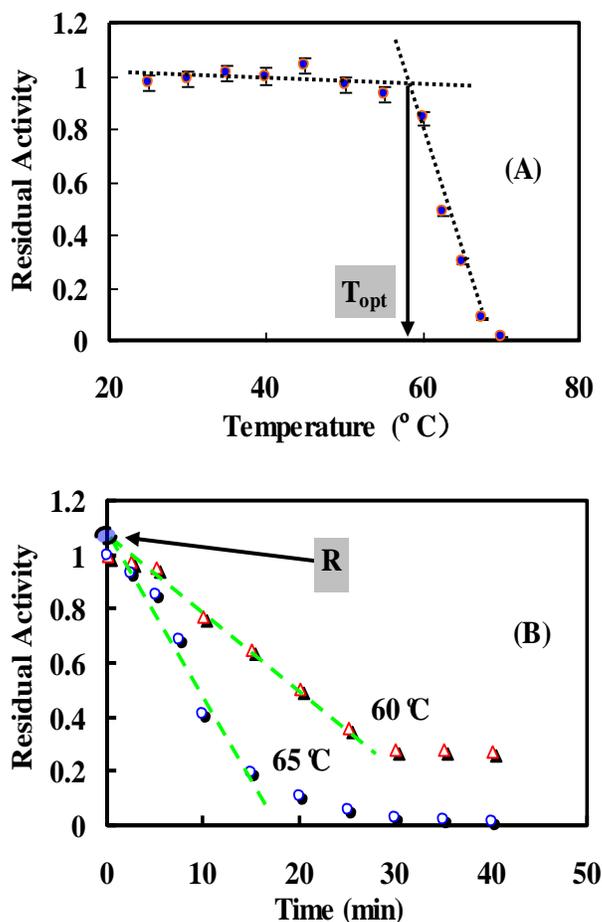


Figure.6 (A) Residual activity of GOD versus incubation temperature in 50mM PBS in the presence of guaiacol (3mM), HRP ( $2.5 \times 10^{-5}$ mM), GOD ( $9 \times 10^{-6}$ mM) and glucose (50mM). GOD was incubated at the indicated temperature for 30min followed by immediately cooling to 0 °C in an ice-water bath and assayed for residual activity. (B) Thermal dissociation of GOD. The GOD was incubated for a certain time at 60 (Δ) and 65°C(○), respectively, in PBS (50mM, pH5.8) containing HRP ( $2.5 \times 10^{-5}$ mM), GOD ( $9 \times 10^{-6}$ mM), guaiacol (3mM), and glucose (50mM), and quenched in the ice-water bath, and assayed for residual activity.

#### G. Biochemical Computation of The Conformational Lock of GOD

Fig. 7A and B are generated by using Pymol and Ligplot [16], respectively, to show the contact areas and main interactions involving the contact area. It could be seen that there are two identical contact areas between two subunits of GOD, The amino acid residues (e.g. Asn62, Asp63, Thr80, Lys 277, Leu449 and Tyr448), which are located at each contact area (Fig.7A), may play an important role in conformational lock of GOD.

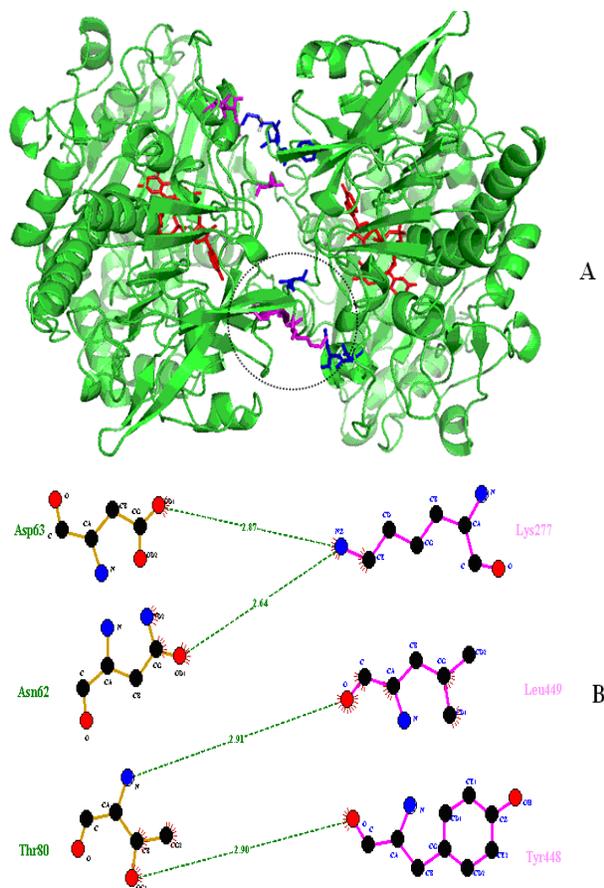


Figure.7 (A) Contact areas of glucose oxidase from *Aspergillus niger* (PDB ID: 1GPE). The figure was generated by using the Pymol. (B) Amino acids and interactions involving the contact area. The figure was generated by using Liglot. Green dashed lines represent hydrogen bonds, spiked residues show hydrophobic contacts..

#### IV. CONCLUSIONS

Glucose oxidase-horseradish peroxidase-guaiacol coupled system could be applied for glucose oxidase assay. The method has perfect repeatability and high sensitivity. The assay could be used for kinetic research about the conformation lock of glucose oxidase.

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