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Difference in enzyme activity and conformation of glucose oxidase before and after purification

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Abstract

Enzyme activity of commercial glucose oxidase was enhanced after purification through a strong anionic exchange resin. In order to get a better insight into this phenomenon, surface pressure–area (π–A) isotherms and surface pressure–time (π–t) isotherms was used to study the interaction and the absorption at different pH values of the subphases between octadecylamine and glucose oxidase purified by a styrene system quaternary ammonium type strongly basic anionic exchange resin. Circular dichroism (CD), electrophoresis and enzyme activity measurements were conducted to study these phenomena. A preliminary hypothesis has been suggested to explain why the enzyme activity of purified glucose oxidase was higher than that of the commercial one.

Keywords: GOD; Purification; Surface pressure–area isotherms; Surface pressure–time isotherms; Octadecylamine

1. Introduction

Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, EC1.1.3.4) is an enzyme of much commercial importance [1–3], which can catalyze the oxidation of β-D-glucose to D-glucono-1,4-lactone and the concomitant reduction of molecular oxygen to hydrogen peroxide. Commercial GOD is purified from different fungi including *Aspergillus niger* and *Penicillium*. Kalisz et al. [4,5] had reported the method of purifying commercial GOD, but there was little in literature about the physical chemistry properties of purified GOD. In this study we investigated the enzyme activity and conformation of GOD before and after purification. The interaction between GOD and the cation surfactant octadecylamine monolayer at the air–water interface [6] was investigated as a modeling system to understand the reaction mechanism of anionic exchange resin and GOD. The interaction kinetics between octadecylamine and GOD at different surface pressures has been studied at different pH values of the subphase to find a possible explanation.

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2. Materials and methods

2.1. Method of purification

We have purified commercial GOD using FPLC and a Mono Q column [7] according to Refs. [4,5]. Then, a cheaper strongly basic anionic exchange resin was used to replace the Mono Q column and the same result was obtained. Commercial GOD (purchased from Sigma, type II, from *A. niger*) was dissolved in bidistilled water. The cheaper anionic exchanger was of the Styrene-DVB D380 styrene system quaternary ammonium type strongly basic anionic exchange resin (made in NanKai University, China). The starting buffer was 20 mmol l^{-1} phosphate buffer (pH 8.5) and the elution buffer was 50 mmol l^{-1} acetate buffer (pH 3.6) with 0.2 mol l^{-1} NaCl. The ultraviolet monitor was a 751G spectrophotometer (made in China) and the concentration of GOD was monitored at $\lambda = 280$ nm.

2.2. π–A and π–t isotherm

We have used two methods to measure the π–A and π–t isotherms. One method was the Langmuir type film balance, which we called the dynamic method, and the other was named as the static (injection) method.

2.3. Dynamic method

An LB trough (70 cm long, 14 cm wide) from Face (Japan) was used for this work. The subphase was GOD bidistilled water solution with $CdCl_2$ or KCl (the content of GOD was 0.001 mg l^{-1}, the concentration of $CdCl_2$ or KCl was 2.5 mmol l^{-1}). The process of compression was conducted at room temperature with a compression speed of 20 $cm^2\,min^{-1}$. Octadecylamine chloroform solution (240 μl, 1 mmol l^{-1}) was spread on the subphase by using a pipette and compression was started after waiting for 20 min. GOD solution was injected into the subphase by a pipette. The π–A isotherm was measured after 6 h of interaction between GOD in the subphase and the octadecylamine monolayer at high or low surface [6]. The high surface pressure was $\pi = 20$ mN m^{-1}; the low surface pressure was $\pi = 0$ while the barrier was kept at the same position.

2.4. Static method

The π–t isotherm using the static method was measured in a small glass cup with a side arm of 3 cm length. The cup was 4.2 cm high and the surface area of the cup was 15.5 cm^2. The subphase was bidistilled water or $CdCl_2$, KCl bidistilled water solution (2.5 mmol l^{-1}). The different amounts of injected octadecylamine caused the different initial surface pressures. Octadecylamine chloroform solution was spread on the subphase by a pipette and GOD was injected into the subphase by a syringe from the side arm after volatilizing chloroform for 20 min. The content of GOD in the subphase was 1 μg ml^{-1}. The advantage of the static method was that it could be experimented with very small amounts of GOD. The data obtained by the injection method could not be compared with that obtained by the dynamic method, because the method to control surface pressure was realized through the different concentrations of the spreading matter. However, data obtained by these two methods could complement and confirm each other.

Anionic and cation exchangers were used to change the pH of the subphase. The anionic or cation exchange resin was put into the KCl bidistilled water solution (2.5 mmol l^{-1}) to change the pH of the subphase based on the following reaction:

$$KCl + R\text{–}H = R\text{–}K + HCl$$

$$KCl + R'\text{–}OH = R'\text{–}Cl + KOH$$

2.5. Circular dichroism spectrum

The data of circular dichroism (CD) were obtained from JASCO J-70 Spectropolarimeter, made in Japan. The CD spectrum of the GOD solution was obtained by putting a quartz cuvette that is to be measured in the light path as compared to the reference water solution. A computer calculated the content of GOD conformation [8,9].

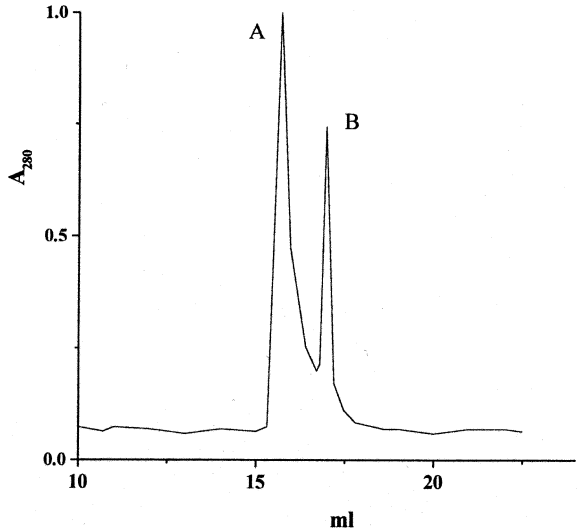

Fig. 1. Separation of *A. niger* GOD (peak A represents GOD A, peak B represents GOD B).

2.6. ζ Potential

The ζ potential was conducted from Zeta Plus (purchased from Brookhaven Instruments Corp., USA). The entire sample dissolved in 20 mM PO_4^{3-} buffer (pH 8.5) and their concentrations were the same.

2.7. Enzyme activity [10]

The flavoprotein glucose oxidase catalyses the oxidation of β-D-glucose by molecular oxygen to D-glucono-1,4-lactone and hydrogen peroxide. GOD is specific for β-D-glucose. We used *o*-dianisidine to react with hydrogen peroxide and detected the absorbance at 460 nm by a UV detector. The enzyme activity can be calculated by the following formula:

Table 2
The value of ζ potential of GOD molecules

	Commercial GOD	GOD A	GOD B
ζ Potential (mV)	−6.1	−4.3	−6.4

$$\text{unit (mg or ml)} = \frac{\Delta A_{460\ \text{nm min}^{-1}}}{11.3 \times \text{mg enzyme ml}^{-1}} \times 50$$

3. Results

It has been found in the UV spectrum that two peaks appeared after purification (Fig. 1). The first peak we obtained, denoted as GOD A, accounted for about 75% of the total amount of GOD; the second peak (denoted as GOD B) accounted for 25% of the total amounts of GOD. Enzyme activity measurements showed that change of enzyme activity and conformation of GOD had occurred after purification. It has been found that GOD B had almost the same enzyme activity as that of the commercial one whereas GOD A had a much higher enzyme activity (Table 1). The CD spectrum results showed that the ratio of α-helix to β-sheet of GOD A had increased from 0.23 to 0.35.

The ζ potential of commercial GOD and purified GOD molecules was examined as a proof of our hypothesis. The amount of surface charge of protein molecules could be analogized from the ζ potential quantitatively. The larger the value of ζ potential, the more the amount of surface charge of the protein molecules. Table 2 shows the values of ζ potential of different GOD molecules.

In the monolayer experiments, we could not obtain the π–A isotherms of the purified GOD. Fig. 2 shows the π–A isotherms of GOD before and after purification in the $CdCl_2$ subphase.

Table 1
Enzyme activity and conformation of commercial and purified GOD

	Enzyme activity (unit)	α-Helix (%)	β-Sheet (%)	α/β
GOD A	86.9	17.9	51.4	0.35
GOD B	27.3	13.1	47.7	0.27
Commercial GOD	25.2	11.5	49.3	0.23

In order to know the mechanism of the above phenomena, we have studied the influence of octadecylamine, a cation surfactant, to mimic the interaction of GOD with the anionic exchange resin. We could obtain the π–A isotherms at different pH values of the subphases using an ion exchanger to adjust the pH in the KCl solution (2.5 mmol l^{-1}) as the subphase.

The interaction kinetics between octadecylamine and GOD in the subphase has been studied at different initial surface pressures. In the dynamic method, the injection of the GOD solution by a pipette could not make the concentration in subphase homogeneous owing to its large surface area. The static method was used in this experiment.

The interaction between octadecylamine with GOD at different pH values (pH 5.2 and 7.4) is shown in Fig. 4.

Fig. 5 shows the π–t isotherms of GOD under the octadecylamine monolayer. It shows that the interaction between RNH_2 and commercial GOD was stronger than that between RNH_2 and GOD A.

4. Discussion

GOD had negative charge in bidistilled water due to the isoelectric point of GOD being 4.2 [11]

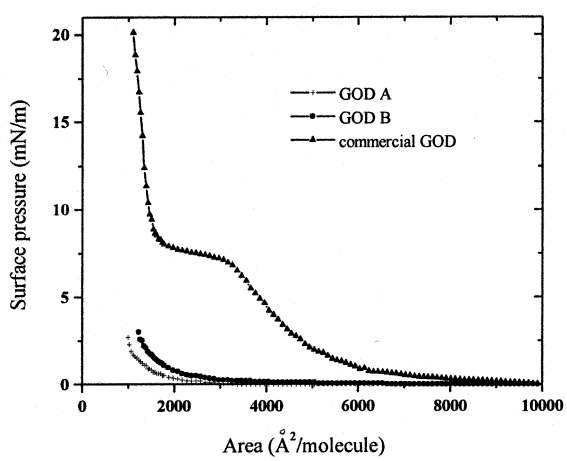

Fig. 2. Surface pressure (π–A) isotherms of GOD before and after purification. The subphase is $CDCl_2$ (2.5 mM) solution.

and could be absorbed at the beginning of the column. On eluting with the elution buffer (pH 3.6), GOD became a positive charge carrier and was desorbed from the anionic exchange resin. The different eluted portions of GOD carried different amounts of negative charge and this separated them from each other. The first separated part (GOD A) must have less charge than GOD B in the purification process, so the influence of charge impulse to the conformation of GOD A was small and GOD A had a higher enzyme activity. GOD B had more charge so it could only be separated after GOD A and it carried more negative charge than GOD A and tended to extend which destroyed the native conformation and decreased the enzyme activity [12] as shown in our previous work. This work proved this postulation again from another approach.

The values of the ζ potential of commercial GOD and GOD B were almost equal as shown in Table 2. They were less than that of GOD A, so, the amount of surface charge of commercial GOD and GOD B was less than that of GOD A [13]. This measurement showed the correctness of our hypothesis experimentally.

Fig. 2 showed that GOD A had more hydrophilicity than the commercial one. According to Krebs et al. [14], the surface pressure at the air–water interface correlated with the product ($\mu_H \times F$), where μ_H was the mean helical hydrophobic moment averaged over all helices in the molecule, and F was the fraction of the α-helix in the protein. Although the fraction of the α-helix in the purified GOD was higher than that in the commercial one, the value of F was very low. So, the surface pressure could not be high. As seen from Table 1, the enzyme activity became higher after purification; the α-helix in the conformation increased and the β-sheet decreased.

From Fig. 3 and Table 3, octadecylamine changed from a gas monolayer to a solid monolayer and the molecule area of octadecylamine decreased when the pH value in the subphase increased. This has proved that the polar group of octadecylamine RNH_2 has the following equilibrium in the subphase:

$$RNH_3^+ = RNH_2 + H^+$$

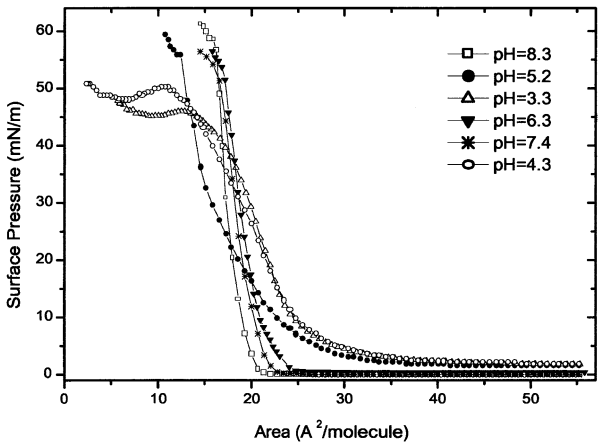

Fig. 3. Surface pressure–area (π–A) isotherms of octadecylamine with different subphase pH values. The subphase pH was adjusted by ion exchange resin.

When the pH decreased, i.e. $[H^+]$ increased, the equilibrium moved towards the left, and the polar group existed mainly as RNH_3^+. The π–A isotherm showed that the monolayer was a gas-like one because of the repulsion between charged groups. When the pH increased, i.e. $[H^+]$ decreased, the polar group existed mainly as RNH_2 and the repulsion between groups decreased and the π–A isotherms became a solid-like monolayer (Fig. 4).

There is an interesting phenomenon in Fig. 5. When the initial surface pressure π_i was low, i.e. the concentration of octadecylamine on the surface of the subphase was low ($\pi = 0.7$ mN m^{-1}), the surface pressure increased as the time increased. However, when π_i increased to a higher value ($\pi_i = 3.5$, 6.4 mN m^{-1}), the surface pressure dramatically decreased first and then increased. This phenomenon also appeared in the case of the dynamic method and in the experiments using phosphate buffer as the subphase

Table 3
The molecule area of octadecylamine with different subphase pH

PH	3.3	4.3	5.2	6.3	7.4	8.3
Åre a (A^2/molecule)	26.5	27.0	24.3	21.8	20.3	18.6

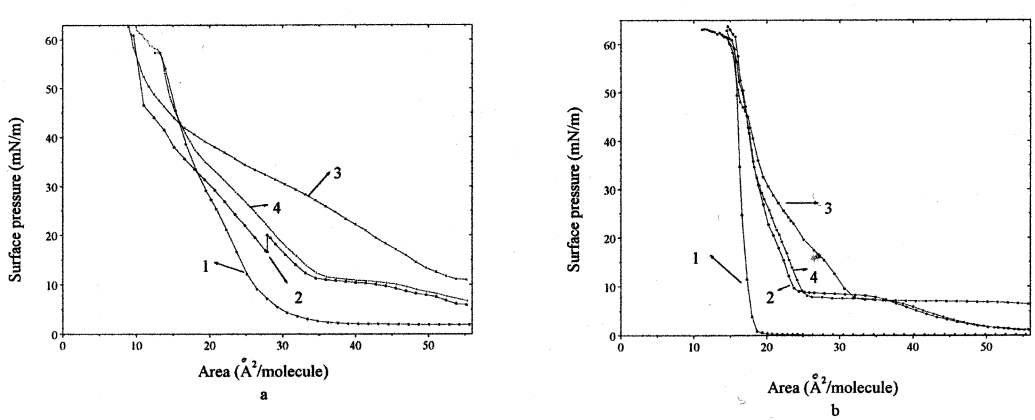

Fig. 4. Surface pressure–area (π–A) isotherms of octadecylamine at different subphases. a, pH 5.2; b, pH 7.4 (1, without commercial GOD in the subphase; 2, interaction with commercial GOD in the subphase at $\pi = 20$ mN m^{-1} for 6 h; 3, interaction with commercial GOD in the subphase at $\pi = 0$ for 6 h; 4, with commercial GOD in the subphase).

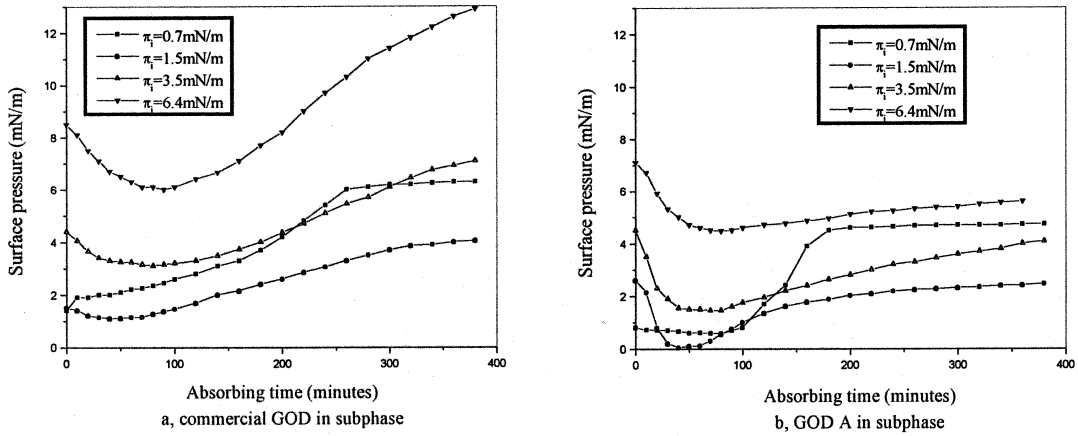

Fig. 5. Surface area–absorbing time (π–t) isotherms of the octadecylamine monolayer. The initial surface pressure was different and GOD in the subphase was injected by using the static method (a, commercial GOD in the subphase; b, GOD A in the subphase).

(pH 5–8) and might be explained by the different orientation of octadecylamine on the aqueous surface.

In summary, the reason for these phenomena could be explained as follows. Commercial GOD was a mixture of negatively charged GOD species, when they went through the anionic exchanger in the elution (pH 3.6) solution. GOD A, which eluted first, carried less charge than that of GOD B. The former was more hydrophilic than the latter. So, the interaction between the purified GOD molecules and the RNH_2 hydrocarbon chains would be weaker than the commercial ones. The CD spectra and the enzyme activity data evidenced our hypothesis [15] that the lesser charge of GOD molecules result in the increase in enzyme activity and the native conformation content became higher after the commercial GOD molecules were purified.

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