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Effect of Cd^{2+} on the Electrochemical Activity and Spectroscopic Property of Horseradish Peroxidase in the Simulated Physiological Solution

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The effect of Cd^{2+} on horseradish peroxidase (HRP) electrochemical activity and spectroscopic property in the simulated physiological solution was investigated by using the electrochemical techniques and spectroscopic methods. In this work, Cd^{2+} could interact with the amide groups of the peptide of the horseradish peroxidase molecules, leading to the change in the conformation of horseradish peroxidase. In which the interaction between Cd^{2+} and horseradish peroxidase molecules would lead to the decrease in the contents of α-helix and β-sheet and the increase in the content of the β-turn and random coil as well as the decrease in the content of hydrogen bonds inter- and intra-peptide chain in horseradish peroxidase molecules. This change would increase the planarity of porphyrin cycle in heme group and decrease the exposure extent of heme active center, Fe(III). The decrease in the exposure extent of heme active center would significantly decrease the catalytic and electrochemical activities of horseradish peroxidase.

Key Words: Electrochemical activity, Horseradish peroxidase, Cd^{2+}, Spectroscopic property, Conformation change.

INTRODUCTION

Contamination of our environment with heavy metals is a significant health threat. Active heavy metal salts accumulated in soil and water are capable of efficiently entering the food chain using plants and animals as transit vehicles[1]. Cadmium, a non-essential element for plants and humans, has been identified as the most mobile and easily bio-accumulating heavy metal by green plants[2]. Anemia and pulmonary disease, edema, pneumonitis, animpaired endocrine system, defects in sensory function and bone damage have all been attributed to Cd ingestion[3]. Cadmium(II) affects animal, plant and microbial cells by forming either ionic or covalent complexes with electron donor atoms present in cellular compounds containing sulfur, nitrogen and oxygen, especially with sulfhydryl groups of proteins[4]. Up take of Cd^{2+} into cells of various types induces formation of abnormal proteins[5] or inhibition of various enzyme activities[6].

Horseradish peroxidase (HRP) (EC 1.11.1.7) a member of the plant peroxidase superfamily, has long been a representative system for investigating the structure, dynamic and thermodynamic properties of peroxidases, which catalyzes one electron oxidation of substrates by hydrogen peroxide[7]. The crystal structure of horseradish peroxidase has been solved, it includes 308 amino acid residues, two Ca^{2+} binding sites proximal and distal to the heme, four disulfide bridges (Cys11-Cys91, Cys44-Cys49, Cys97-Cys301, Cys177-Cys209), an extensive hydrogen-bonding network and contains one tryptophan (Trp) and five tyrosine (Tyr) presenting intrinsic fluorescence[8,9]. In this paper, the effects of Cd^{2+} on horseradish peroxidase electrochemical activity and spectroscopic property were investigated by using electrochemical techniques, ultraviolet-visible, FT-infrared and circular dichroism. We hope that our results can provide some references for better understanding the toxic mechanism of heavy metal ions on the organic body.

EXPERIMENTAL

Lyophilized powder of horseradish peroxidase ($R_Z = A_{403}/A_{275} \geq 2.5$) was purchased from Sigma Co. and was further purified before used. The concentration was determined spectrophotometrically using the extinction coefficient at 403 nm of 102 mM^{-1} cm^{-17}. Other chemicals were of analytical reagents.

The cyclic voltammetric measurements were performed with an EG & G PAR Model 273 Potentiostat/galvanostat with a traditional three-electrode electrochemical cell. A Pt wire and the saturated calomel electrode (SCE) were used as the auxiliary and reference electrodes, respectively. All potentials are quoted with respect to SCE. The glassy carbon electrode with 0.031 cm^2 apparent surface area was used as the substrate of the working electrode. It was polished with 1.0, 0.3 and 0.05 μm alumina slurries sequentially. Then, it was sonicated

for 1 min in the distilled water, washed and dried. Subsequently, 5 µL cysteine solution was dropped onto the surface of the electrode and then one thin layer of Au colloid was coated on the cysteine layer. After it was repeated three times, the working electrode was obtained and noted as Cys-Au/GC electrode. Finally, the Cys-Au/GC electrode was placed in the HRP solution with Cd^{2+} for 10h at 4 ºC. The electrolyte was the simulated physiological solution. The electrolyte solutions were purged with high purity nitrogen prior to the electrochemical experiments.

UV-visible absorption spectra were obtained using a Shimadzu UV265 recording spectrophotometer with 0.5 cm path length cell. The changes in FTIR spectra for the solutions of HRP, cast on TlIBr disks and dried in vacuum, were determined by a Nexus 670 FT-IR spectrometer (Nicolet, USA). Circular dichroism spectra were measured on a JASCO J-715 spectropolarimeter using quartz cell of 0.1cm path length. The final spectrum was obtained by averaging over four consecutive scans. The background absorption of the solvent is subtracted for each spectrum. The secondary structure content were analyzed using the SSE-338 program provide by JASCO Co. in terms of Chen *et al.*[10,11].

RESULTS AND DISCUSSION

Effect of Cd^{2+} on the electrochemical activity of horseradish peroxidase: Fig. 1A shows the cyclic voltammograms of the 4 µM HRP in the simulated physiological solution with 8 µM Cd^{2+} at Cys-Au/GC electrode. In the absence of Cd^{2+}, a pair of redox peaks is located at -0.33 and -0.39 V, which is attributed to the direct electrochemical reaction of the heme group of HRP(a)[12]. The difference between the cathodic and anodic peak potentials, ΔE_p is 60 mV. The anodic peak current (I_{pa}) is 0.104 µA, which is almost equal to the cathodic peak current (I_{pc}), indicating that HRP can undergo a direct and quasi-reversible electrochemical reaction at the Cys-Au/GC electrode[13]. The formal redox potential, E°' is -0.36 V, it is in the good agreement with former reports[14]. In the presence of 8 µM Cd^{2+}, a pair of redox peaks is at -0.32 and -0.38 V and ΔE_p is 60 mV. I_{pa} is almost equal to I_{pc}, indicating that HRP also undergoes a direct and quasi-reversible electrochemical reaction at the Cys-Au/GC electrode in the simulated physiological solution with Cd^{2+}. E°' is -0.35 V, which is more positive than that in the absence of Cd^{2+}. The positive shift of E°' illustrated that the increase in the hydrophobicity of the microenvironment of the heme group of HRP. Furthermore, I_{pa} of HRP is 0.072 µA, which is decreased by 30.8 % comparing with that for HRP in the absence of Cd^{2+}. The above results demonstrated that the electrochemical reaction activity of HRP in the presence of 8 µM Cd^{2+} is lower than that for HRP without Cd^{2+}.

Fig. 1B shows the cyclic voltammograms of 4 µM HRP in the simulated physiological solution without (a) and with 8 µM Cd^{2+} (b) in the presence of 0.42 M H_2O_2 at the Cys-Au/GC electrode. In the absence of Cd^{2+}, a marked reduction peak of H_2O_2 appears and its I_{cat} is 1.4 µA (a). In the presence of 8 µM Cd^{2+}, the peak current of H_2O_2 reduction is significantly decreased to 0.98 µA (b). The phenomenon indicated that Cd^{2+} can indeed inhibit the electrocatalytic activity of HRP for the reduction of H_2O_2. The results indicated that Cd^{2+} can inhibit the electrocatalytic activity of HRP for the H_2O_2 reduction.

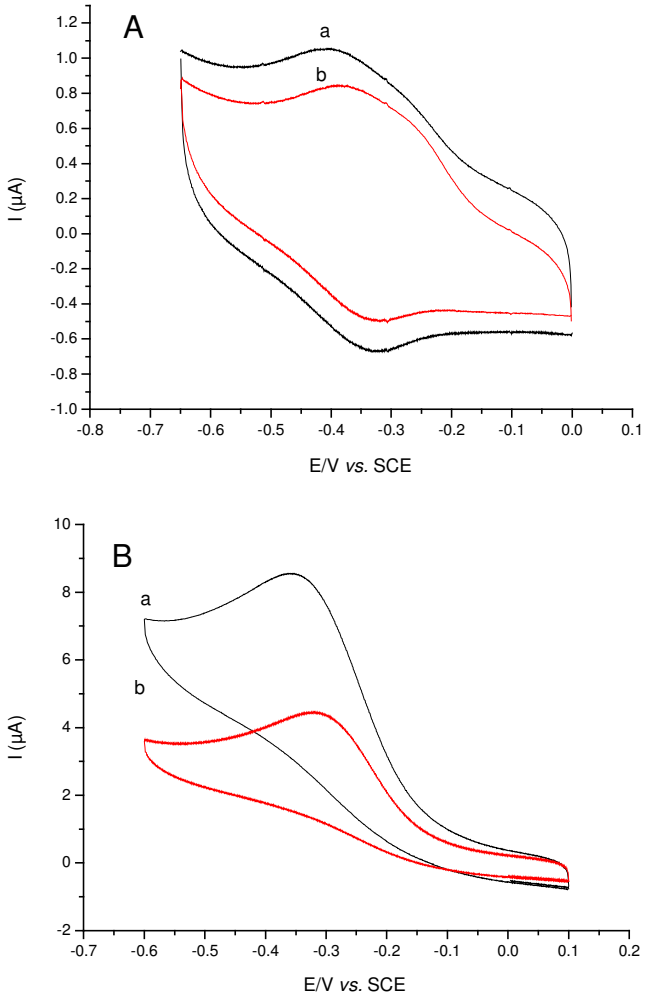


Fig. 1. (A) CV of 4 µM HRP at the Cys-Au/GC electrode in the simulated physiological solution without (a) and with 8 µM Cd^{2+} (b). (B) CV of 4 µM HRP in the presence 0.42 mM H_2O_2 at the Cys-Au/GC electrode in the simulated physiological solution without (a) and with 8 µM Cd^{2+} (b)

UV-visible spectra: Fig. 2 shows the UV-visible spectra of 4 µM HRP without (a) and with 8 µM Cd^{2+} (b). The peak near 205 nm is due to π-π* transition of amide in peptide chain and the band around 275 nm is corresponding to the buried tyrosine (Tyr) and sensitive tryptophan (Trp) residues, the Soret band around 403 nm (γ-band) is attributed to heme porphyrin cycle. It was clearly showed that after the addition of Cd^{2+} ion (curve b), the peak near 205 nm was decreased about 13.4 %, while the band near 275 nm, the γ-band was increased *ca.* 28.7 and 6.8 % comparing to curve a, respectively. The results indicated that there was strong interaction between Cd^{2+} ion and HRP and Cd^{2+} ion would mainly have an influence on the amino acid residues of HRP. In fact, the active site of HRP, *i.e.*, the heme group, would be buried below the molecular surface and accessible by one (or possible two) channel, which is long and lined with hydrophobic residues[15]. So it was clear that Cd^{2+} ion was unlikely bound to the porphyrin directly, but bound to the amino acid residues in the polypeptide of HRP firstly and then affected the heme porphyrin correspondingly.

FTIR spectra: Fig. 3 shows FTIR absorption spectra of the 4 µM HRP solution without (a) and with 8 µM Cd^{2+} ion (b). It could be seen that the C=O absorption of amide I was

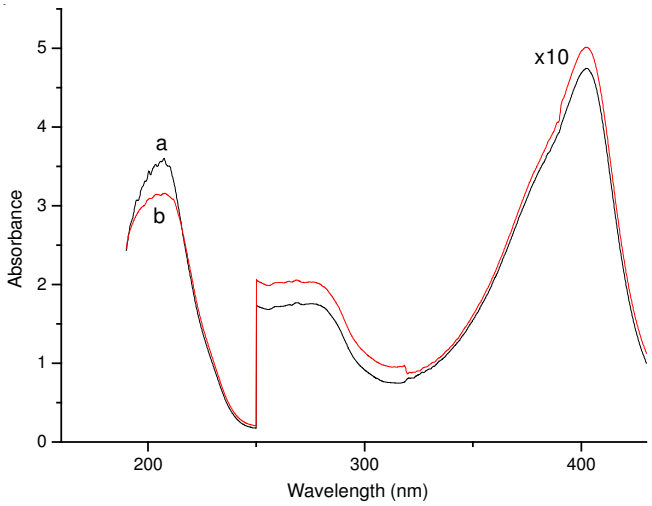


Fig. 2. UV-VIS spectra of 4 μM HRP without (a) and with 8 μM Cd^{2+} (b)

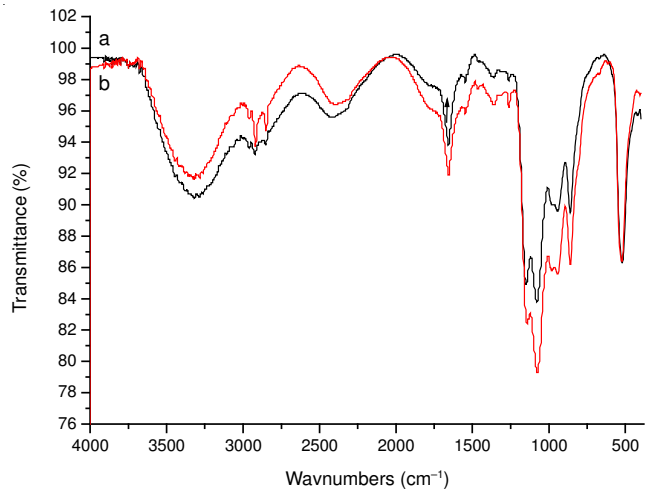
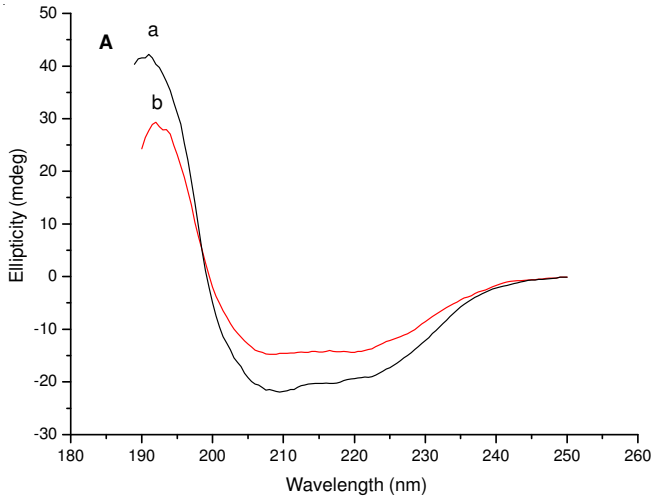


Fig. 3. FTIR spectra of 4 μM HRP without (a) and with 8 μM Cd^{2+} (b)



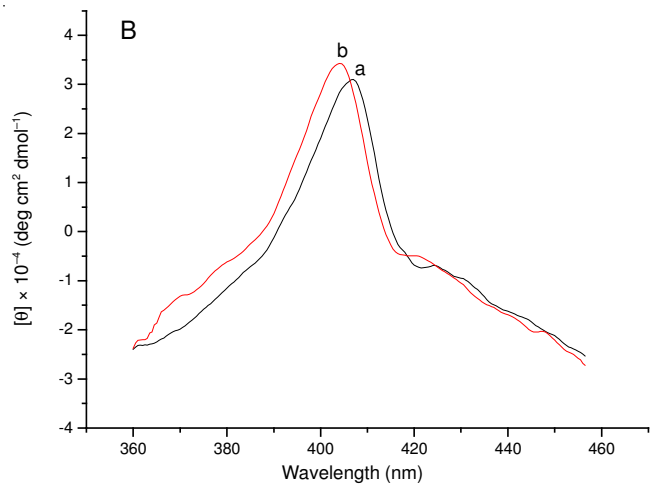


Fig. 4. (A) Far-UV CD spectra of 4 μM HRP without (a) and with 8 μM Cd^{2+} (b) wavelength region: 190-250 nm. (B) CD spectra of 4 mM HRP in Soret region without (a) and with 8 μM Cd^{2+} (b). wavelength region: 360-460 nm

shifted from 1651 to 1657 cm^{-1}, the peak at 1075 cm^{-1} was shifted to 1081 cm^{-1} due to C-O stretching vibration and the C-N absorption of amide III was also transferred from 1144 to 1151 cm^{-1}, but the N-H stretching band was changed from 3320-3297 cm^{-1} by adding Cd^{2+} ion, respectively. The above results indicated that the absorption peak of ν(C=O), ν(C-O) and ν(C-N) was changed, which indicated that Cd^{2+} ion may interact with the S, O and/or N containing groups in the polypeptide of the HRP molecule and then altered the conformation of HRP[16].

Circular dichroism spectra: The circular dichroism remains the spectra probe most sensitive to polypeptide backbone conformations, especially secondary and tertiary structure[11]. Fig. 4A shows CD spectra of 4 μM HRP in the simulated physiological solution without (a) and with 8 μM Cd^{2+} (b) in the far-UV region. In the absence of Cd^{2+}, a positive band at 192 nm was observed and it corresponds to the π-π* transition of the amide groups in the peptide of HRP. Two distinct negative bands at about 208 and 222 nm are attributed to the π-π* and n-π* amide transition in the peptide of HRP, respectively and assigned to α-helix of enzyme[17]. When the concentration of Cd^{2+} is 8 μM, the positive band at 192 nm is evidently decreased *ca.* 18.0 % comparing with that of HRP without Cd^{2+}, indicating that Cd^{2+} can interact with the amide groups in the peptide of HRP. In addition, the intensities of two negative bands are decreased, suggesting that the -helix content in HRP was decreased[18]. The above results indicated that Cd^{2+} can interact with the amide groups in the peptide of HRP and change the conformation of HRP. The interaction between Cd^{2+} and HRP would increase the content of the disordered conformation of HRP. This may be the reason for that Cd^{2+} decreases the catalytic activity of HRP.

Data of the conformation of HRP with Cd^{2+} calculated from the CD spectra was listed in Table-1. It was found that for 4 μM HRP in the absence of Cd^{2+}, α-helix content is 38.2 %, it was a typical α-protein. When the concentration ratio of Cd^{2+} and HRP is 2, the contents of the α-helix and β-sheet conformation of HRP are decreased by 16.0 and 0.5 %, respectively, while the contents of the β-turn and random coil of HRP are increased by 6.2 and 10.3 % comparing with that without Cd^{2+}, respectively.

Heme is CD inactive in its free form, while in an asymmetric environment of a heme enzyme, the heme group would become CD-active[19]. Fig. 4B shows the CD spectra of 4 μM HRP in the simulated physiological solution without (a) and with 8 μM Cd^{2+} (b) in the Soret region. In Fig. 4B curve a, the

TABLE-1
SECONDARY STRUCTURE OF THE 4 μM HRP WITHOUT AND WITH 8 μM Cd^{2+} ION

Condition	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
HRP	38.2	19.1	2.6	40.1
$[Cd^{2+}]/[HRP] = 2$	22.2	18.6	8.8	50.4

Soret band is located at 407 nm. In the presence of 8 μM Cd^{2+}, the Soret band is blue-shifted to 404 nm and its intensity is increased by 6.3 % (b) comparing with that without Cd^{2+}. The blue shift and the increase in the intensity of the Soret band suggested that the planarity and π-π* transition energy of the porphyrin cycle in the heme group was increased[20]. The increase in the planarity of porphyrin cycle in heme group illustrates the decrease in the exposure extent of heme active center, Fe(III) is decreased[21]. Thus, the catalytic activity of HRP and the electrochemical activity are decreased as mentioned above.

Horseradish peroxidase has a very extensive hydrogen-bonding network in the proximal and distal regions of the heme group. The distal region is connected to the proximal region by a hydrogen-bonding network. It was known from the above results that Cd^{2+} can interact with some amide groups in HRP and then may destroy the hydrogen-bonding network in HRP. The results of the CD measurements indicated that after Cd^{2+} interacting with HRP, both the contents of α-helix and β-sheet are decreased and the content of the β-turn and random coil is increased, indicating that the content of hydrogen bonds inter- and intra-peptide chain in HRP molecules is decreased, causing the looseness of the structure of the HRP molecule and thus, the looseness HRP molecules would significantly decrease the catalytic and electrochemical activities of HRP.

Conclusion

In this work, it was found that when Cd^{2+} interacts with HRP. Cadmium(II) can interacts with the amide groups of the peptide of the HRP molecules, leading the change of HRP conformation. The interaction between Cd^{2+} and HRP molecules would lead the decrease in the contents of α-helix and β-sheet and the increase in the content of the β-turn and random coil as well as the decrease in the content of hydrogen bonds inter- and intra-peptide chain in HRP molecules. This change would increase the planarity of porphyrin cycle in heme group and decrease the exposure extent of heme active center, Fe(III). The decrease in the exposure extent of heme active center would significantly decrease the catalytic and electrochemical activities of HRP. This study could provide some references for understanding heavy metals on peroxidase toxicity in living organisms.

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