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ORIGINAL PAPER

Interaction of Cu^{2+}, Pb^{2+}, Zn^{2+} with Trypsin: What is the Key Factor of their Toxicity?

Tong Zhang · Hao Zhang · Guiliang Liu · Canzhu Gao · Rutao Liu

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Abstract Heavy metals possess great endangerment to environment even human health because of their indissolubility and bioaccumulation. The toxicity of heavy metal ions (Cu^{2+}, Pb^{2+}, Zn^{2+}) to trypsin was investigated by fluorescence, synchronous fluorescence, UV–vis absorption, circular dichroism (CD) spectroscopy, isothermal titration calorimetry (ITC), and enzyme activity assay. The experimental results showed that toxic effect of heavy metal ions was due to their own characteristic, rather than the electric charges of the ion. Zn^{2+} could not show the obvious toxicity to trypsin, while the structure and function of trypsin was damaged when the enzyme explored to Cu^{2+} and Pb^{2+}. From the spectra results, we found that Cu^{2+} would bind with trypsin, which lead to the fluorescence quenched and hydrophobicity increased. Pb^{2+} could also change the structure and reduce the activity of trypsin in high concentration. In vitro measurement, the toxicity order of heavy metal ions to trypsin is: $Cu^{2+} > Pb^{2+} > Zn^{2+}$. In addition, isothermal titration calorimetry analysis proved that the interactions between Cu^{2+}, Pb^{2+}, Zn^{2+} and trypsin were all spontaneous and exothermic, which indicated the adverse effect of these heavy metal ions to trypsin.

Keyword Heavy metal ions · Trypsin · Spectroscopy · Isothermal titration calorimetry

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T. Zhang · H. Zhang · C. Gao (✉) · R. Liu (✉)
School of Environmental Science and Engineering, China -America CRC for Environment & Health, Shandong Province, Shandong University, 27# Shanda South Road, Jinan 250100, People's Republic of China
e-mail: gaocz@sdu.edu.cn
e-mail: rutaoliu@sdu.edu.cn

G. Liu
Shandong Institute for Food and Drug Control, Jinan 250101, China

Introduction

The pollution of heavy metal is a witness of fast-developing economy of our world [1–7]. Consequently, the toxicity of heavy metal to human's health has been a concentration in public [8,9]. When the heavy metal enters into the body, they are not easily excreted, but accumulated gradually. If the load of heavy metal exceeds the physiology of human body, it will cause the changes in physiological function, and lead to acute, chronic or long-term harms such as chronic toxicity, carcinogenic, teratogenic effects, allergy [10] and so on. the detrimental effects of low dose heavy metal to humans will be accumulated dramatically by the food chain [11].

Trypsin, a kind of water-soluble globular protein, is a serine protease which plays an important role in digestion and other biological processes. Trypsin is made up of 223 amino acid residues with a molecular weight of 23,300 Dalton. And the active sites of trypsin are the catalytic triad – His 57, Asp 102 and Ser 195, Ca^{2+}, locating between the two domains [12].

Since 1990s, an increasing number of researchers investigated the effects in molecular level on the basis of acute toxicity experiments. Yajie Liu et al. studied the acute toxicity of Zn^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+} to bay scallop [13]. A. Fargasova et al. learned the phytotoxic effects of Cd, Zn, Pb, Cu and Fe on sinapis alba L. seedings and their accumulation in roots and shoots [14]. In above papers, the researchers both found the sequence of acute toxicity is Cu^{2+}, Zn^{2+}, Pb^{2+}. What's more, Hao Zhang et al. researched toxic effects of different charged metal ions (Na^{+}, Cu^{2+}, Al^{3+}) on the target—Bovine serum albumin, and they inquired that electric charge of ion was not the main factor affecting the structure of BSA [15]. But no scholars have discussed the interactions between metal ions with the same electric charge and enzyme which in human body at the molecular level up till now. Therefore, our group selected Cu^{2+}, Zn^{2+}, Pb^{2+} as the metal ions and trypsin as the target molecule by spectroscopic

techniques, isothermal titration calorimetry experiments and enzyme activity experiments.

Materials and Methods

Reagents

Trypsin was purchased from Beijing BioDee biotechnology co., LTD (Beijing, China) and was dissolved in ultra pure water to form a 1×10^{-5} mol/L solution for each experiment. Use trypsin right after it was ready, to avoid it foaming during the process of dispensing.

BAEE (N-R-benzoyl-$_L$-arginine ethyl ester) was purchased from Shanghai Ruji biotechnology co., LTD (Shanghai, China) and was dissolved in ultra pure water to form a 1×10^{-3} mol/L solution and preserved at 0–4 °C.

Tris–HCl buffer prepared by mixing Tris (0.2 mol/L) with HCl (0.2 mol/L) was used to control pH at 8.0 and preserved at 0–4 °C.

$CuCl_2$ (2×10^{-3} mol/L) solution was prepared by dissolving 0.0341 g $CuCl_2\cdot2H_2O$ (Tianjin Kermel Chemical Reagent Research Institute) in 100 mL using ultra pure water.

$PbCl_2$ (2×10^{-3} mol/L) solution was prepared by dissolving 0.0556 g $PbCl_2$ (Tianjin Kermel Chemical Reagent Research Institute) in 100 mL using ultra pure water.

$ZnCl_2$ (2×10^{-3} mol/L) solution was prepared by dissolving 0.0273 g $ZnCl_2$ (Tianjin Baodi Chemical Reagent) in 100 mL using ultra pure water.

Apparatus and Methods

An F-4600 fluorescence spectrophotometer (Hitachi, Japan) was used to measure the fluorescence intensity, synchronous fluorescence. UV–vis absorption spectra and the activity of the enzyme were measured by UV-2450 spectrophotometer (Shimadzu UV2450, Tokyo, Japan) A Jasco-810 circular dichroism spectrometer (Jasco, Japan) was applied to investigate the changes of the secondary structure of the enzyme. A iTC200 (Microcal) was used to directly measure the binding of free energies, enthalpies, entropies, affinities, and other related thermodynamic data of biomoleculars.

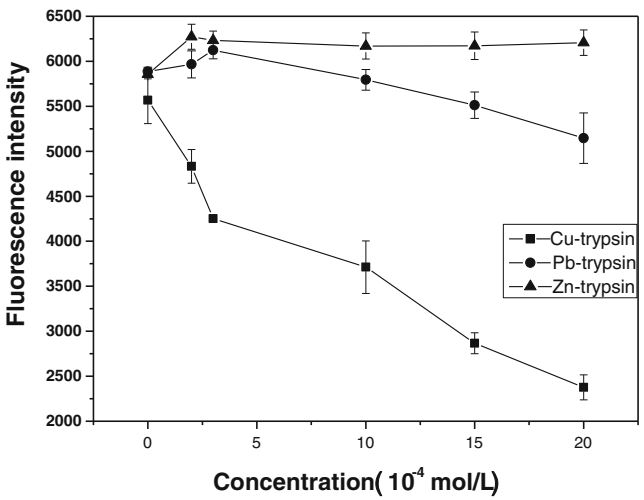

Fig. 1 Fluorescence intensity of trypsin with different concentrations of Cu^{2+}, Pb^{2+}, Zn^{2+}. Conditions: c (trypsin)=1×10^{-5} mol/L, c (Cu^{2+}, Pb^{2+}, Zn^{2+})/(1×10^{-4} mol/L):0, 2, 3, 10, 15, 20 respectively; pH 8.0

Fluorescence Measurements

The excitation and emission slit widths of all fluorescence spectra were set at 5.0 nm. The scan speed was 1200 nm/min, and the PMT (photo multiplier tube) voltage was 700 V. The solution system of measurements was carried out as follows: 1.0 mL Tris–HCl buffer (pH=8), 1.0 mL 1×10^{-5} mol/L trypsin, and different concentration

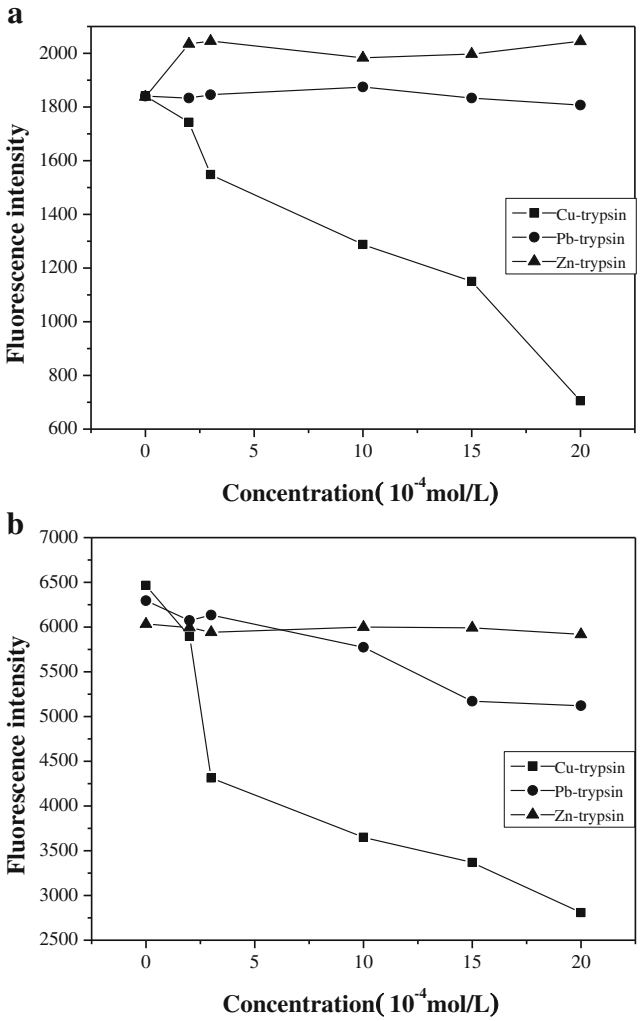

Fig. 2 Synchronous fluorescence spectra of trypsin with different concentrations of Cu^{2+}, Pb^{2+}, Zn^{2+}, (**a**) $\Delta\lambda$=15 nm, (**b**) $\Delta\lambda$ =60 nm. Conditions: c (trypsin) =1×10^{-5} mol/L, c (Cu^{2+}, Pb^{2+}, Zn^{2+})/(1×10^{-4} mol/L):0, 2, 3, 10, 15, 20 respectively; pH 8.0

gradients of Cu^{2+}, Pb^{2+}, Zn^{2+} were added to each of a series of 10 mL colorimeter tubes.

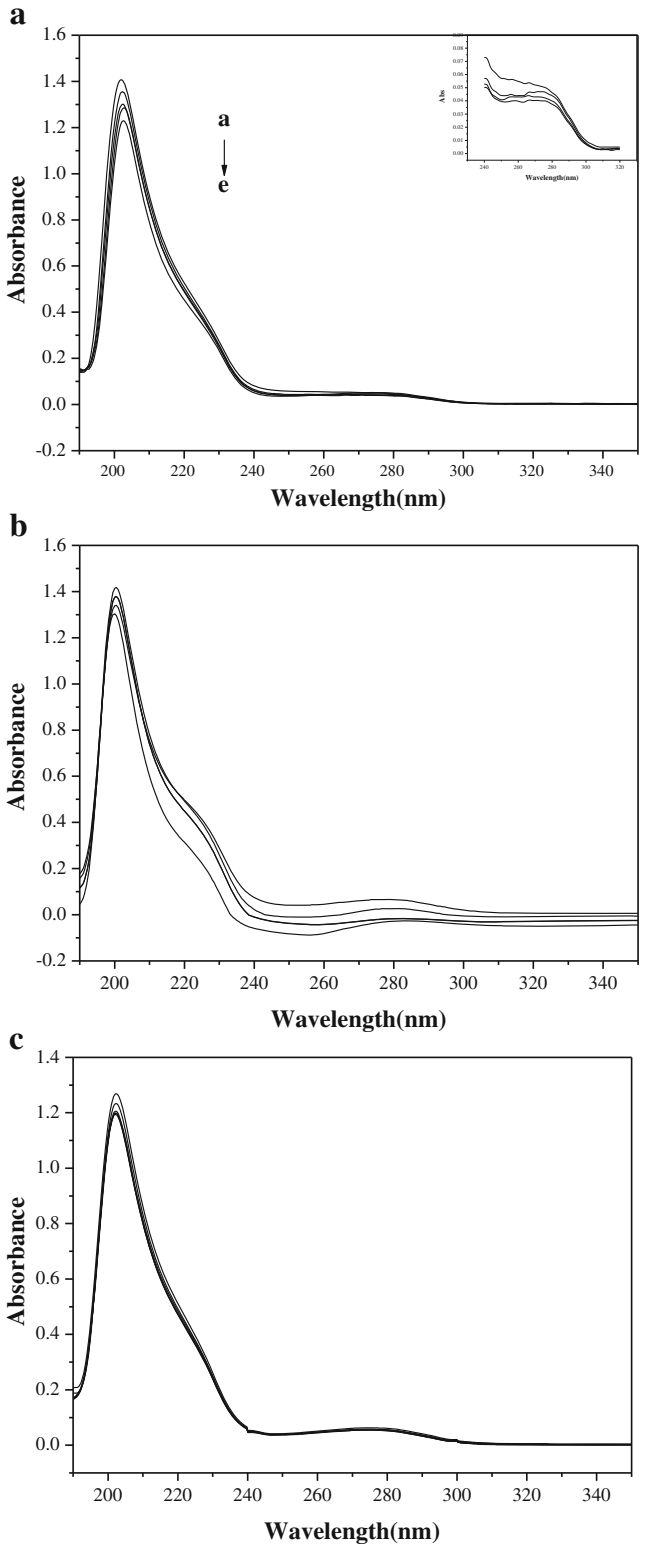

Fig. 3 UV–vis absorption of trypsin with different concentrations of ions (**a**) Cu^{2+}, (**b**) Pb^{2+}, (**c**) Zn^{2+}. Conditions: c (trypsin) $=1\times10^{-5}$ mol/L, c (Cu^{2+}, Pb^{2+}, Zn^{2+})/(1×10^{-4} mol/L):0, 2, 3, 10, 15, 20 respectively; pH 8.0

Firstly, the fluorescence spectra were measured. The excitation wavelength was set at 278 nm and the emission

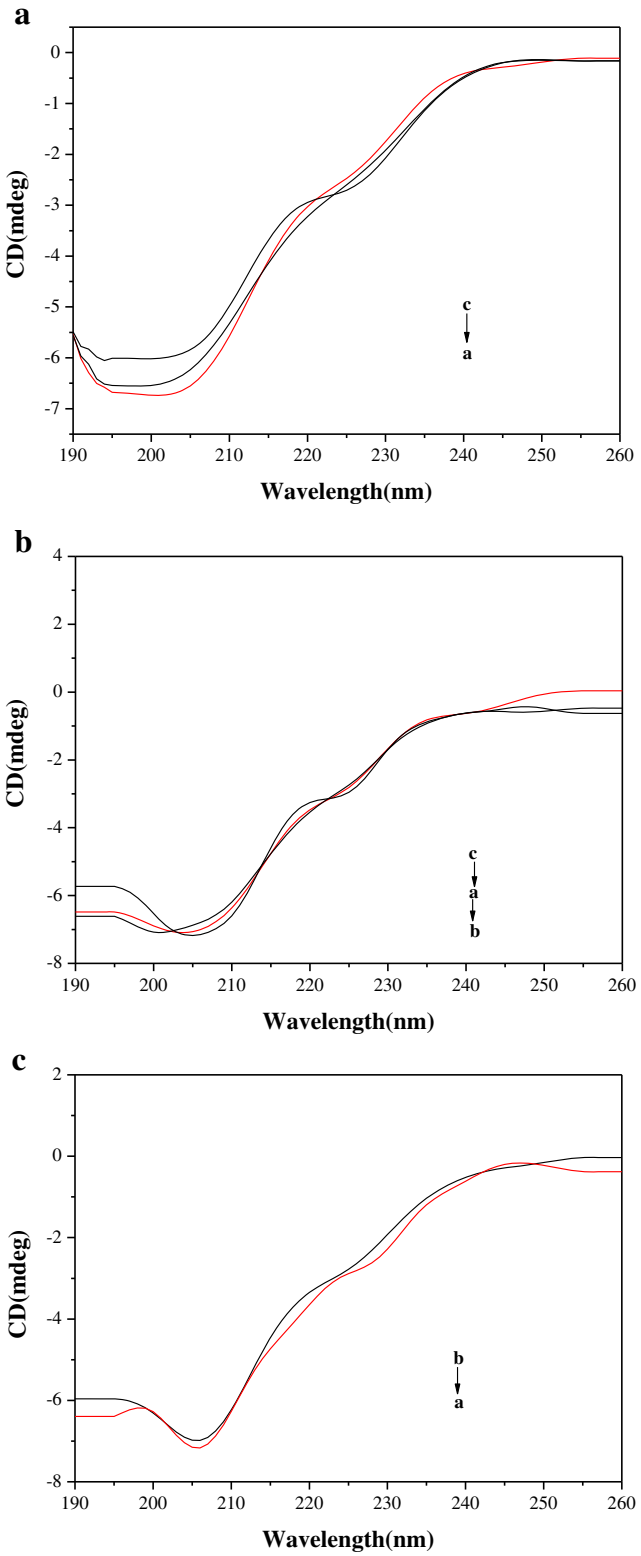

Fig. 4 CD spectra of trypsin with different concentrations of Cu^{2+}, Pb^{2+}, Zn^{2+}. Conditions: c (trypsin)$=1\times10^{-5}$ mol/L, pH 8.0; (**a**) c (Cu^{2+})/(1×10^{-4} mol/L):0, 10, 20 respectively; (**b**) c (Pb^{2+})/(1×10^{-4} mol/L):0, 5, 20 respectively; (**c**) c (Zn^{2+})/(1×10^{-4} mol/L):0, 20 respectively

wavelengths were 290–450 nm. Then, Synchronous fluorescence spectra of trypsin was measured (λ_{ex}=200–350 nm, $\Delta\lambda$=15 nm, and $\Delta\lambda$=60 nm, respectively).

UV–Visible Absorption Measurements

The slit width was set at 5.0 nm with the same scan speed of fluorescence measurements. And wavelengths were put from 190 nm to 350 nm. The solution system was the same with fluorescence measurements, but reference solutions had no trypsin.

CD Measurements

CD spectra were collected from 190 nm to 260 nm at 293 K. Bandwidth was 1 nm.

Scanning speed was 100 nm/min.

Isothermal Titration Calorimetry Experiments

For the sake of inquiring the binding affinity constant, enthalpy changes, entropy changes and binding stoichiometry of interactions between trypsin and Cu^{2+}, Pb^{2+}, Zn^{2+}, isothermal titration calorimetry (ITC) experiments were carried on ITC200 microcalorimeter (Microcal Inc., Northampton, MA) at 298 K. Both of trypsin and Cu^{2+} should be dissolved in 0.02 M Tris–HCl buffer (pH 8.0), and filtrated by 0.22 μm filter membrane. About 40 μL of Cu^{2+} (2.5 mM) were titrated into 200 μL trypsin (0.0125 mM), and the stirring speed was set at 1000 rpm. The first drop was set to 0.4 μL followed by 13 subsequent 3 μL. In order to achieve complete thermodynamic equilibration, the spacing time between each injection was set to 120 s. The methods of the Pb-trypsin and Zn-trypsin systems were the same with Cu-trypsin system.

Trypsin Activity Measurement

Because trypsin could catalyze BAEE into N-α-benzoyl-$_L$-arginine (BA) which has a stronger UV absorption than that of BAEE at 253 nm [16], BAEE was used as the substrate. The measurement of trypsin activity was tested with UV-2450 spectrophotometer. The inhibition rate of trypsin activity was calculated by the following equation:

$$\text{Inhibition rate} = \Delta A_1 / \Delta A_0 \quad (1)$$

ΔA_1 and ΔA_0 are the absorption value at 253 nm in 30 s before and after the addition of trypsin with Cu^{2+}, Pb^{2+}, Zn^{2+}, respectively.

Table 1 Fractions of secondary structure of trypsin in the absence and presence of Cu^{2+}

Concentration of Cu^{2+}(mol/L)	Secondary structural elements in trypsin			
	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
0	2.8	49.0	22.9	25.2
1×10^{-3}	2.1	50.3	24.1	23.5
2×10^{-3}	1.2	51.1	23.0	24.6

Results and Discussion

Fluorescence Spectra

The intrinsic fluorescence of trypsin is contributed to tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues, primarily [17]. Whereby, fluorescence of Trp and Tyr is learned frequently to reveal changes of protein structure by examining the micro-environment of the amino acids [18]. Fluorescence emission spectra of trypsin in addition to different concentrations of Cu^{2+}, Pb^{2+}, Zn^{2+} at room temperature were recorded (Fig. 1). From the fluorescence intensity, we found that the strong peak signal is 330 nm [19] (Supporting Information), and the peak position did not shift almost when the concentration changes. As is shown in Fig. 1, the fluorescence intensity of trypsin decreased regularly with increasing Cu^{2+} concentration. The phenomenon indicated that Cu^{2+} was reacted with residues of trypsin which could emit fluorescence, such as tryptophan (Trp) and tyrosine (Tyr). Trypsin was quenched in this system through the static quenching [20]. In Pb-trypsin system, when the concentration of Pb^{2+} was more than 1×10^{-3} mol/L, the fluorescence intensity decreased, but was not as sharp as Cu-trypsin system. This appearence suggested that the conformation of trypsin was changed by the high concentration of Pb^{2+}[21,22]. Electro negativity by Pauling scale of Cu (II), Zn (II) and Pb (II) are: 1.92, 1.65 and 2.20. Because of the biggest constant, Pb^{2+} could be more inclined to react with the amino nitrogen and carboxyl oxygen that on the amino acid residues of polypeptide chain by coordination reaction [21]. In Zn-trypsin system, Zn^{2+}did not quenched the fluorescence of

Table 2 Fractions of secondary structure of trypsin in the absence and presence of Pb^{2+}

Concentration of Pb^{2+}(mol/L)	Secondary structural elements in trypsin			
	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
0	2.8	49.0	22.9	25.2
5×10^{-4}	3.4	41.3	21.3	34.0
2×10^{-3}	1.3	50.8	23.2	24.5

Table 3 Fractions of secondary structure of trypsin in the absence and presence of Zn^{2+}

Concentration of Zn^{2+}(mol/L)	Secondary structural elements in trypsin			
	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
0	2.8	49.0	22.9	25.2
2×10^{-3}	2.6	49.1	23.1	23.4

trypsin but increase it, which indicated that Zn^{2+} made the structure of trypsin tighter and hydrophobicity enhanced. It is indicated that the toxicity to trypsin of Zn^{2+} is not fiercer as Pb^{2+} and Cu^{2+}.

Synchronous Fluorescence Spectra

Synchronous fluorescence spectroscopy is the method to give information about the molecular environment in the vicinity of a chromophore such as tryptophan (Trp) and tyrosine (Tyr). The shift of the emission maximum (λ_{em}) reflected the changes of polarity around the chromophore molecule. In synchronous fluorescence, the spectrum is scanned at the excitation (λ_{ex}) and emission (λ_{em}) wavelengths at the same time. When the wavelength interval ($\Delta\lambda$) are set at 15 nm, the synchronous fluorescence gives the characteristic information of Tyr residue, and when at 60 nm, Trp residue [23].

The synchronous fluorescence spectra of trypsin with three kinds of ions in Fig. 2a indicates that the Tyr of trypsin was quenched only if the system was added to the Cu^{2+} . In Fig. 2b, we can figure out that Cu^{2+} and Pb^{2+} could also affect the trypsin system, and the effect of Cu^{2+} was obvious than Pb^{2+}.

The synchronous fluorescence spectra of trypsin with Cu^{2+} (Supporting Information) showed that both Trp and Tyr were quenched, and the intensity of Trp was larger than Tyr, which suggested that the dominating fluorophore of trypsin is Trp, and the binding sites between Cu^{2+} and trypsin was closer to Trp [24]. Slight red shift happened on Tyr (from 293.4 to 294.8 nm) and Trp (from 276.6 to 279.2 nm) were with the concentrations increased gradually. The emission peaks of Trp and Tyr prove that molecular conformation of trypsin changed because of the toxic effect of Cu^{2+}, and the hydrophobicity of the Tyr and Trp decreased [25]. Due to this, we know both of two chromophores buried in the non-polar hydrophobic cavities were moved to a more hydrophilic environment [26,27]. Furthermore, the Trp residue of trypsin with Pb^{2+} also shows the slight red shift (from 277 to 278.4 nm), so it indicates that the coordination reaction between trypsin and Pb^{2+} leads to the Trp residue to exposure to a hydrophilic environment. However, the the Tyr residue of trypsin with Pb^{2+} show little change, so maybe it still stay the nonpolar hydrophobic cavities [28].

UV–Vis Absorption Spectra

UV–vis absorption spectroscopy technique can be able to explore the structural changes of protein and to investigate protein-ligand complex formation [29]. Trypsin has two absorption peaks at 206 and 276 nm alone. The strong peak at

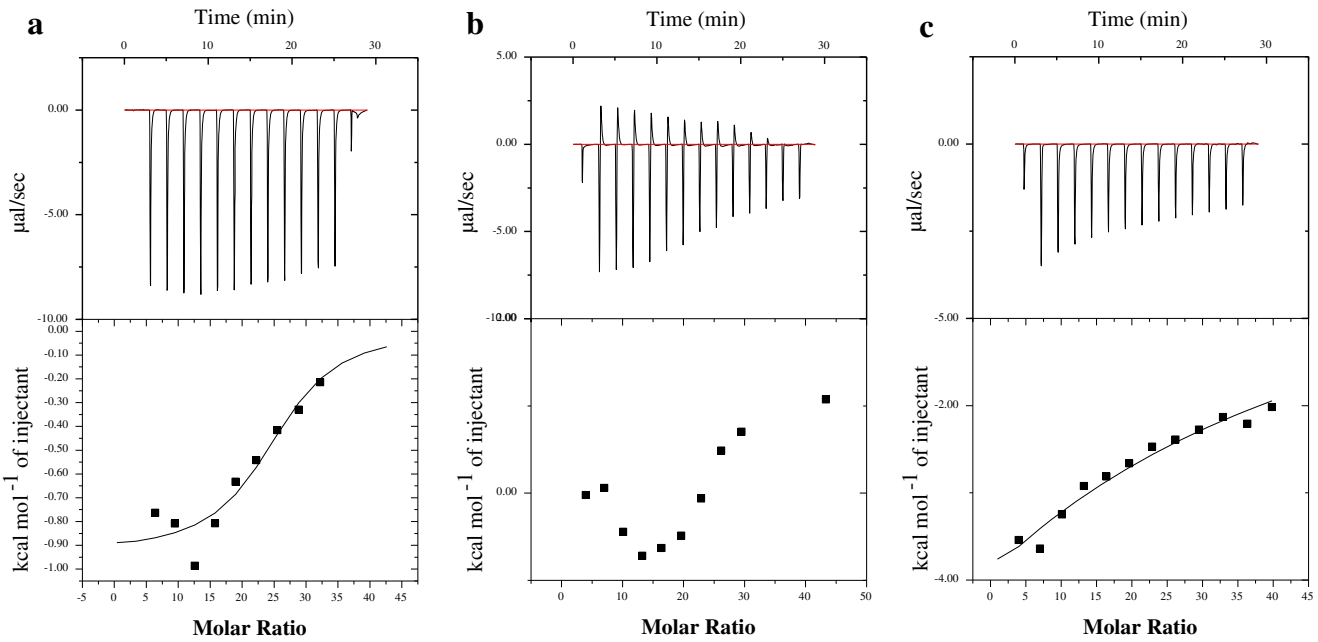

Fig. 5 ITC profiles for the binding of ions to trypsin at 298 K, pH 8.0. (**a**) Cu^{2+}, (**b**) Pb^{2+}, (**c**) Zn^{2+}. The top panel shows the raw data for heat flow of each injection of ions in addition to trypsin, and the bottom panel represents integrated heat data after corrections of dilution heat. The solid line shows the least-square fit to a single set of binding site model

Table 4 Thermodynamic parameters for interaction of Cu^{2+}, Pb^{2+}, Zn^{2+} with trypsin by ITC, T=298 K

	n	$K_a(10^3\ M^{-1})$	ΔH (cal/mol)	ΔS(cal/mol K)	ΔG(cal/mol)
Cu-trypsin	24.7±1.48	77.1±57.6	−927.0±95.49	19.2	−6648.6
Pb-trypsin	0.121±627	4.73±171	−5.309±2.758 (10^4)	−161	−5112.0
Zn-trypsin	o.262±306	0.407±1.64	−2.861±3.356 (10^6)	-9.59×10^3	−3180.0

206 nm reflects the framework conformation of the enzyme, so it stands for the strong absorption of peptide bond. Then, the weak peak is at 276 nm, on account of the aromatic amino acids residues (Trp, Tyr and Phe) in the enzyme.

The UV–vis absorption spectra of trypsin in the presence of Cu^{2+}, Pb^{2+}, Zn^{2+} were shown in Fig. 3a, b, c respectively. As is shown in Fig.3a, with the rising concentration of Cu^{2+} to trypsin, the intensity of the peak at 206 nm decreased with red shift and the intensity of the peak at 276 nm also decreases but without red shift. This experimental result suggested that the toxic effect of Cu^{2+} on trypsin would lead to the loosening and unfolding of the trypsin skeleton [30] and change the microenvironment of aromatic amino acids residues by decreasing the hydrophobicity [31] of them.

In Fig. 3c, we found that the peak shape and position of trypsin remained stable in 276 nm but a little increase in 206 nm, with the gradual addition of Zn^{2+} into trypsin system. This phenomenon indicated that only the secondary structure of trypsin was changed because of the Zn^{2+}.

Circular Dichroism Spectra

Circular dichroism (CD) spectroscopy is a technique that is able to detect secondary structure changes of enzyme caused by ligands [32]. The CD spectra of trypsin exhibited a negative bands at about 208 nm, the characteristic of the α-helix of proteins [33]. The CD spectra of trypsin with different concentrations of Cu^{2+}, Pb^{2+}, Zn^{2+} were shown in Fig. 4 respectively. By the calculation of CDpro, the contents of α-helix were obtained in Table 1, 2, 3 respectively.

The α-helix and β-sheet are the characteristic of the secondary structure of the trypsin [33]. As is shown in Fig. 4a, it was obvious that the α-helix of trypsin in addition to different Cu^{2+} decreases, which means the conformation of trypsin, was damaged by Cu^{2+}. And as is shown in Fig. 4a, when the concentration of Pb^{2+} was higher than 10^{-3} mol/L, the structure of trypsin was severely destroyed by higher concentration of Pb^{2+}. However, the Fig. 4c did not indicate that the harmful toxicity of Zn^{2+} to trypsin.

ITC Analysis

Gibbs free-energy change (ΔG) can be calculated by the thermodynamic equation: $\Delta G = -RT\ ln\ K_a = \Delta H - T\Delta S$ (R is the universal gas constant and T is the absolute temperature). ITC is a convenient and efficient method to describe molecular interactions by investigating the binding affinity constant (K_a), number of binding sites (n), enthalpy changes (ΔH) and entropy changes (ΔS) between metal ions and enzyme [34,35]. The raw data graph of heat flow of each titration (mcal/s) in time of minutes (min) was shown in Fig. 5 (top panel), which illustrated the titration of 2.5 mM Cu^{2+} into 0.0125 mM trypsin. The ITC titrations of trypsin with Cu^{2+} yielded negative heat peak, which suggested that the reaction was an exothermic process. The bottom part of the figure showed that integrated heat in terms of kcal/mol of injectant plotted against molar ratio of Cu^{2+} / trypsin, and the derived thermodynamic parameters such as K_a, ΔH, ΔS and ΔG were summarized in Table 4. Whereby, all the ITC results were corrected for dilution heat by subtracting the baseline data measured in identical series of injections into Tris–HCl buffer. Negative ΔG value revealed that the interaction between Cu^{2+} and trypsin was spontaneous. Furthermore, negative ΔH and positive ΔS implied that the electrostatic forces play the major role during the interaction.

Compared with Cu-trypsin system, the numbers of binding sites of Pb-trypsin and Zn-trypsin system were infinitesimal, which indicated the weak binding. The negative values of ΔH and ΔS in these systems meant that during the interaction, Van der Waals' force and hydrogen binding are the important intermolecular forces, and the randomness in Pb-trypsin and

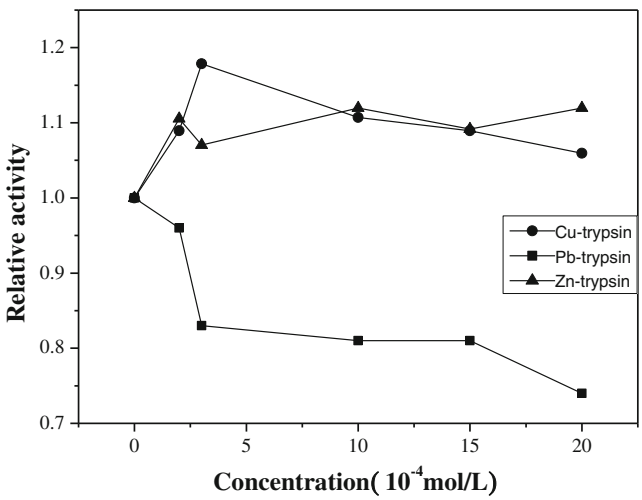

Fig. 6 Relative activity of trypsin with different concentrations of Cu^{2+}, Pb^{2+}, Zn^{2+}. Conditions: c (trypsin) =1×10^{-5} mol/L, c (Cu^{2+}, Pb^{2+}, Zn^{2+})/ (1×10^{-4} mol/L):0, 2, 3, 10, 15, 20 respectively; pH 8.0

Zn-trypsin systems decreases. Similarly, negative ΔG value reveals that the interaction between Pb^{2+}, Zn^{2+} and trypsin is spontaneous.

Effect of Metal Ions on Trypsin Activity

In order to further investigate the effect of trypsin physiological function by Cu^{2+}, Pb^{2+}, Zn^{2+} respectively, and to supply the results of fluorescence spectra, absorption spectra and circular dichroism spectra, we determined the activities of trypsin with different concentrations of these three metal ions. The consequence of the relative activity of trypsin is shown in Fig. 6. As is described in the picture, we found that the Cu^{2+} had the most obvious inhibiting effect to trypsin. The phenomenon indicated that the toxicity of Cu^{2+} to trypsin was the most severe. For Pb-trypsin system, the activity of trypsin was promoted by Pb^{2+} in low concentration($<1\times10^{-3}$ mol/L) but inhibited in high concentration($>1\times10^{-3}$ mol/L). That because a little amount of Pb^{2+} cannot replace the whole Ca^{2+}, which are in the active site of the trypsin. However, if the amount of the Pb^{2+} is large, all the Ca^{2+} will be substituted by Pb^{2+} and lead to trypsin inactivate [21]. And we also discovered that Zn^{2+} did not have tremendous impact on activity of trypsin. All these appearances of trypsin activity were consistent with fluorescence spectra and absorption spectra [36].

Conclusions

In this paper, we investigated the effect of the same outermost electrons (II) heavy metal ions including Cu^{2+}, Pb^{2+}, Zn^{2+} on trypsin in vitro by multiple spectroscopic techniques, isothermal titration calorimetry and enzyme activity experiments under physiological conditions. Through these experiments, we gained the conclusion that the relevance between outermost electrons of heavy metal ions and the destruction of the structure and activity of trypsin was slim, and the key factor may be their intrinsic characteristics. Trypsin was static quenched in that it could be integrated to a new larger particle by Cu^{2+}, which conformed to the acute toxicity test of copper. Neurovirulence as lead has, the toxicity of Pb^{2+} to trypsin was weaker than Cu^{2+}. The interactions between trypsin and Pb^{2+} was by the way of that the Ca^{2+} in active site of trypsin were replacement by Pb^{2+} only if high concentration (more than 10^{-3} mol/L). This was why neurovirulence as lead had, but the toxicity of Pb^{2+} to trypsin was weaker than Cu^{2+}. But for Zn^{2+}, there was not a huge impact on the activity and structure of trypsin. Therefore, it was beneficial for our humans to ingest appropriate zinc.

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