

Biosorption properties of Cd(II), Pb(II), and Cu(II) of extracellular polymeric substances (EPS) extracted from *Aspergillus fumigatus* and determined by polarographic method

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Received: 3 December 2011 / Accepted: 2 January 2013 / Published online: 12 January 2013
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Abstract Extracellular polymeric substances (EPS) were extracted from *Aspergillus fumigatus* using cationic exchange resin technique. The EPS were mainly composed of polysaccharide and low quantities of protein and nucleic acid. Biosorption of Cd(II), Pb(II), and Cu(II) of EPS was investigated as a function of pH using differential pulse polarography and the Ruzic model. Results showed that the EPS biosorption capacity determined using either the direct titration curves $i=f(C_M)$ or the method proposed by Ruzic (Analytica Chimica Acta 140:99–113, 1982) were coincident. Cu(II) had the highest affinity with EPS followed by Pb(II) and Cd(II). The total number of binding sites for Cu(II) and Cd(II) increased with pH in the range of 4.0–7.0. Similar trend was observed for Pb(II) at pH 4.0–5.0, while precipitates were observed at pH 6.0 and 7.0. The conditional binding constants of these three metals displayed low levels of fluctuation with pH and ranged from 4.02 ± 0.02 to 5.54 ± 0.05 .

Keywords *Aspergillus fumigatus* · Extracellular polymeric substances (EPS) · Biosorption · Heavy metals · Polarography

Introduction

Extracellular polymeric substances (EPS), secreted by microorganisms during growth, are mainly composed of polysaccharides, proteins, humic-like substances, uronic acid, nucleic acids, and lipids (Wingender et al. 1999; Liu and Fang 2002) and contain functional groups such as carboxyl, phosphoric, amine, and hydroxyl groups (Liu and Fang 2002). EPS may be defined as “soluble” or “bound” EPS according to its relative proximity to the cell surface. EPS that are tightly linked to the cell surface via a covalent or non-covalent interactions are known as capsular EPS (or bound EPS), while EPS that are not directly attached to the cell surface are known as slime (or free EPS or soluble EPS) (Burdman et al. 2000). The soluble fraction is extractable with water, whereas the bound fraction requires a chemical or physical extraction method.

EPS play a crucial role in the biosorption of heavy metals (Wingender et al. 1999; Liu and Fang 2002; Comte et al. 2008). These polymers may have a significant effect on the metal adsorption characteristics of bacterial cells due to the presence of active functional binding sites. In general, metal biosorption by EPS involves physicochemical interactions between

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the metal and the functional groups on the cell surface. It involves complicated mechanisms, including physical adsorption, ion exchange, complexation, and precipitation (Ledin 2000).

In most cases, determination of the extent of metal binding to sorbent has relied upon the use of traditional analytical techniques which need to separate sorbent from medium and are time consuming. However, several simple and rapid alternative methods such as anodic stripping voltammetric and differential pulse polarography (DPP) (Morlay et al. 2000; Savvaidis et al. 2003; Guibaud et al. 2004; Comte et al. 2008) have also been used to directly monitor the biosorption of metal ions by a macromolecular ligand without separating the EPS-bound metal from the bulk solution.

The biosorption mechanisms of EPS extracted from activated sludge (Liu and Fang 2002; Comte et al. 2008) and pure bacterial strains (Loaec et al. 1997; Lau et al. 2005) have been well characterized. But the biosorption properties of EPS from fungi remain less explored. In the present study, an isolated fungus, *Aspergillus fumigatus* (abbr. *A. fumigatus*) was used for EPS extraction. The biosorption capacity of the EPS for Cd(II), Cu(II), and Pb(II) at different pH values was investigated using DPP. The results contribute to a better understanding of the adsorption capacities of the EPS affected by pH.

Materials and methods

A. fumigatus biomass preparation

The *A. fumigatus* strain used in this study, which was previously isolated by Cheng and Hu (2004), was grown for 48 h in a liquid medium in shake culture (150 rpm) at 30 °C. The composition (in gram per liter) of the medium was: glucose (15), KH₂PO₄ (0.2), Na₂HPO₄·12H₂O (2.9), (NH₄)₂SO₄ (1), MgSO₄·7H₂O (0.5), NaCl (0.5), and beef extract (1.0). *A. fumigatus* pellets were then harvested by filtering the growth medium through a 150-μm sieve and washing three times with deionized water.

Extraction and analysis of EPS

The pellets were used to extract bound EPS using cationic exchange resin technique (Jahn and Nielsen 1995; Frolund et al. 1996; Liu and Fang 2002). This

exchange mechanism mainly occurs between divalent cations bound with EPS and resin-Na⁺. Pellets (100 g (wet weight)) were resuspended in 500 mL deionized water and added with 100 g cationic exchange resin (Guangzhou Reagent Co., Ltd, Guangzhou, China). The suspension was put on an oscillator at 220 rpm for 4 h at 4 °C and then centrifuged at 4,000×g for 20 min at 4 °C. The EPS-rich supernatant was filtered through a 0.45-μm membrane and dialyzed in 3,500 Da dialysis bag to remove low molecular weight constituents. The dialysate was finally lyophilized at -50 °C for 48 h and stored under -18 °C before use.

Characteristics of the EPS were determined by the following methods. The dry weight and the volatile dry weight content (VDW) were measured at 105 and 600 °C, respectively. The Coomassie procedure (Bradford 1976) was used to measure protein; the phenol-sulfuric acid method (Gerhardt et al. 1994) for polysaccharide content; and the phosphorus analysis method for determining the nucleic acid content (Skidmore et al. 1964). Total organic carbon (TOC) was determined using a TOC meter (Liqui TOC, Elementar Analysensysteme GmbH). The tests were repeated three times and the results given were the average values.

Determination of pK_a

Potentiometric titration according to Comte et al. (2008) was used for the determination of the pK_a of EPS. The acid-base surface reactions are described by the law of mass action based on the protonation (in the form: S-OH and S-OH₂⁺) of the surface functional groups (S-O-) and determined by analogy with amphoteric compounds with:

$$K_{a1} = [\text{H}^+] \times [\text{S} - \text{OH}] / [\text{S} - \text{OH}_2^+] \quad (1)$$

$$K_{a2} = [\text{H}^+] \times [\text{SO}^-] / [\text{S} - \text{OH}] \quad (2)$$

The values pK_{a1} and pK_{a2} can be determined after the determination of surface charge (*Q*) and the protonic exchange capacity by potentiometric titration. A solution of EPS (25 ml) was placed in a 25 °C cell with a thermostat. Titration was carried out by adding NaOH (0.01 M) into an automatic titrator (Metrohm 751GPD) equipped with a pH electrode (pH0-14/0-80 °C; KCl 3 M).

Biosorption study

Theoretical considerations

According to the Ruzic (1982) method and Morlay et al. (2000), the conditional stability constant (K') and the concentration of the total available complexing sites of the ligand (C_c) were calculated using the following equation:

$$\frac{m}{C_M - m} = \frac{m}{C_c} + \frac{1}{K' \times C_c} \tag{3}$$

where C_M represents the total concentration of metal ion introduced, and m represents the free metal ion concentration. The value of C_c determined in this way will be denoted “ C_{ccalc} .”

Chemicals and reagents

All the reagents were of analytical grade. Metal ion stock solutions (0.02 M) were prepared from copper nitrate, cadmium nitrate, and lead nitrate, acidified to pH3 with 0.1 M HNO_3 , and standardized using ICP-OES (5300DV, Perkin-Elmer Optima). $NaNO_3$ (1 M) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma-Aldrich, USA) were used as supporting electrolyte and buffer. HEPES is often used in electrochemical methods for the determination of complexation constants because it does not complex metals (Loaec et al. 1997).

Apparatus

DPP measurements were performed using a Metrohm 797 VA Computrace fitted to a three-electrode arrangement. The working and the auxiliary electrodes were a static mercury drop electrode and a platinum electrode, respectively. The reference electrode was a saturated electrode (Ag/AgCl–KCl). The instrumental parameters were kept constant and listed as follows: voltage step time, 1 s; sweep rate, 0.006 V/s; cathodic pulse amplitude, 0.05 V; pulse time, 0.04 s; voltage step, 0.006 V; and potential range scanned, Cu(II) +0.20 to –0.20 V, Pb (II) –0.20 to –0.60 V, and Cd(II) –0.40 to –0.70 V.

Analytical procedure

All samples were treated in exactly the same way. The solutions to be analyzed contained 1 ml of $NaNO_3$

(1.0 M), 10 ml of HEPES (1.0 M), and 10 ml of ultrapure water or EPS. The measurements were performed at 25 ± 1 °C under a nitrogen atmosphere and with a total volume of 21 ml in the analysis cell.

The pH of the sample was adjusted to the desired value. Micro-additions (10, 20, 30, or 100 μ l) of the metal ion stock solution were then performed, after which the pH was readjusted to the desired value. The response of the system (i) was recorded after 20 min nitrogen purge. The calibration experiment was carried out in the exactly same manner in the absence of EPS to calibrate the response of the polarographic system in DPP mode. All the experiments were carried out in triplicate.

Results and discussion

EPS characteristic

The characteristics of the EPS are summarized in Table 1. Polysaccharide is the most abundant component of the EPS. The contents of nucleic acid and protein are very low due to the difficulty of breaking the cell wall of the *A. fumigatus*.

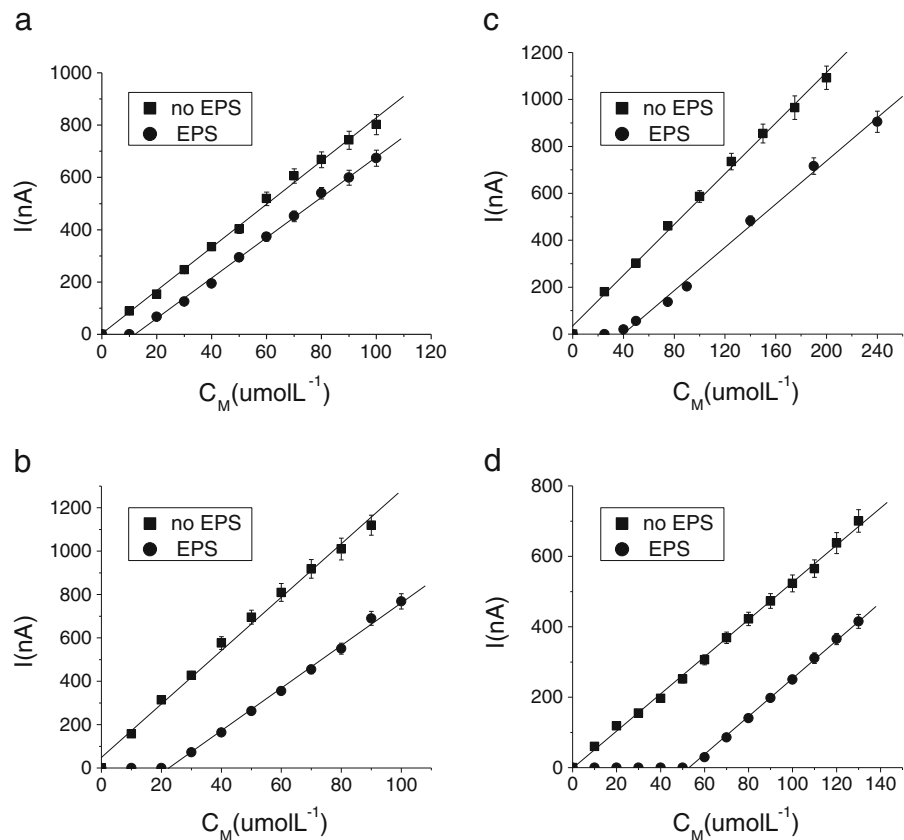
Polarographic titration curves of Cu(II), Cd(II), and Pb (II) at different pH

Figure 1 shows the polarographic titration curves $i=f(C_M)$ of copper(II) both in the absence and presence of EPS at pH4.0, 5.0, 6.0, and 7.0 (Fig. 1a–d). In the presence of EPS, similar curves having two portions were observed at different pH values. The first portion

Table 1 The characteristics of the EPS extracted from *Aspergillus fumigatus*

Parameters	Value
General characterization of EPS solution ($g l^{-1}$)	
Dry weight (DW)	0.81±0.1
Volatile dry weight (VDW)	0.72±0.1
Total organic carbon ($mg l^{-1}$)	375±10
Biochemical composition of EPS ($mg g^{-1}$ EPS VDW)	
Proteins	17.6±2
Polysaccharides	392±8
Nucleic acid	18.3±2
pK_{a1}	4.4±0.1
pK_{a2}	6.7±0.1

Fig. 1 Polarographic titration curves for copper (II) at pH4.0 (a), 5.0 (b), 6.0 (c), and 7.0 (d)



shows the complexation phenomenon before saturation of the binding sites on EPS with all Cu(II) added bound by EPS. When all the EPS binding sites are occupied, the Cu(II) added remains in solution and the second portion of the curve shows almost identical slope to that of the corresponding curve in the absence of EPS. The bound Cu(II) concentration increased from 9.0 to 50.0 μM when pH increased from 4.0 to 7.0. This indicates that the biosorption capacity of EPS towards Cu(II) increases with increasing pH within our experimental pH range.

The polarographic titration curves $i=f(C_M)$ of Cd(II) at pH4.0, 5.0, 6.0, and 7.0 are similar with those of Cu(II). The curves present the same shape having two linear portions in the presence of EPS. In the first one, a part of Cd(II) added is bound by EPS. When all the EPS binding sites are occupied, the curves turn to the second linear portion. The change in the slope of the representation $i=f(C_M)$ obtained in the presence of EPS is less obvious compared to that obtained with Cu(II) which indicates a lower EPS biosorption capacity for Cd(II).

With Pb(II), gel-like precipitate was observed at pH6.0 and 7.0 in the presence of EPS during the polarographic titration experiments in our operative conditions. This precipitate could stem from the interaction of Pb(II) and components of EPS, such as polysaccharide and protein. Similar results have been obtained by other researchers. Santamaria et al. (2003) found that in the presence of Fe(III), Al(III), and Th(IV), EPS produced by *Bradyrhizobium* strain BGA-1 can form a gel-like precipitate composed of polysaccharide, protein, lipopolysaccharide, and the added metal. Morillo et al. (2008) found that the EPS produced by *Paenibacillus jamilae* was able to precipitate Fe(III). Therefore, the formation of EPS–metal precipitate mainly depends on the components of the EPS.

At pH4.0 and 5.0, polarographic titration curves of $i=f(C_M)$ of Pb(II) in the presence of EPS present similar shape as those of the EPS/Cd(II) system. The change in the slope of the curves in the presence of EPS was a little more obvious than that obtained with

Cd(II), which indicates a higher biosorption capacity of EPS for Pb(II) than for Cd(II).

Conditional stability constant and total available binding sites

Table 2 presents the values of C_{ccalc} , the conditional stability constant (expressed as $\log k'$), the total available binding sites, and r^2 of EPS with metal at different pHs which were calculated from the slope and intercept of the plots of $m/(C_M - m)$ versus m . The high correlation coefficient (r^2) of the lines at all pH values tested suggests that the method proposed by Ruzic is valid for the system of EPS/Cu(II), EPS/Cd(II), and EPS/Pb(II).

The C_{ccalc} varied from 18.8 to 178.6 μM for copper and 7.3 to 15.7 μM for cadmium. The C_{ccalc} for lead, however, varied from 16.9 to 37.3 μM at pH4.0 and 5.0. The $\log k'$ for Cu(II), Cd(II), and Pb(II) increased a little with increasing pH within our experimental pH range and varied from 4.31 ± 0.02 to 5.54 ± 0.05 , 4.02 ± 0.02 to 4.89 ± 0.04 , and 4.17 ± 0.02 to 4.91 ± 0.02 , respectively.

The total binding sites of EPS for metals can be used to estimate the EPS biosorption capacity. The total binding sites varied from 55 to 461 $\mu\text{mol g}^{-1}$ VDW EPS for Cu(II) and showed an increased trend at pH4.0–7.0. It varied from 19 to 41 $\mu\text{mol g}^{-1}$ VDW EPS for Cd(II) and showed small changes with pH, revealing that the metal biosorption capacity of EPS for Cd(II) was very low. For

Pb(II), it varied from 44 to 109 $\mu\text{mol g}^{-1}$ VDW EPS in the pH range of 4.0–5.0.

For all the metals tested, the number of total EPS binding sites decreased with the decrease of the pH of the EPS/metal systems. Within our experimental pH range, the number of the total EPS binding sites was in the order of $\text{Cu(II)} > \text{Pb(II)} > \text{Cd(II)}$.

Possible mechanisms of metal biosorption for the EPS as a function of pH

The above results demonstrate that the metal biosorption is significantly affected by the pH of the aqueous medium. Deprotonation of functional groups and ion exchange equilibrium are popular mechanisms used to explain biosorption of metals by EPS.

It had been widely recognized that the deprotonated form of the reactive sites (i.e., carboxylate groups) is primarily responsible for the binding of metal ions by polymers of a carboxylic nature (Morlay et al. 2000). The reactive sites can be assigned according to their $\text{p}K_a$. The $\text{p}K_a$ of the EPS extracted from *A. fumigatus* is 4.4 and 6.7 (Table 1), and the results showed that the EPS contained carboxylic and phosphoric groups. The $\text{p}K_a$ mainly indicates the binding of metal ions by polymers at pH4, 5, 6, 7, and 8 because at very low pH, the binding of protons increases and leads to reduced metal ion binding (Liu and Fang 2002; Esparza-Soto and Westerhoff 2003). The pH of the medium affects the ionization state of the functional groups like carboxylate and phosphate of the EPS. The negatively charged carboxylate and phosphate allow the EPS to complex cations (Ozdemir et al. 2003).

Ion exchange equilibrium mechanism can also explain the influence of the medium's pH on the biosorption of metals. According to ion exchange equilibrium, the metal biosorption increases as pH increases in the pH range of 1–10 because ion exchange is more effective when less protons are available to compete with the metal for the negatively charged metal binding sites (Schiewer and Volesky 1995). In this study, the results of metal biosorption showed a trend of increased biosorption when the pH increased. Ion exchange equilibrium mechanisms might be involved in the metal biosorption of EPS.

Table 2 Values of C_{ccalc} , $\log k'$, total binding sites, and r^2 of EPS with metal at different pH values

	pH	C_{ccalc} ($\mu\text{mol l}^{-1}$)	Total binding sites ($\mu\text{mol g}^{-1}$ VDW EPS)	$\log k'$	r^2
Cu	4.0	18.8	55	4.31 ± 0.02	0.9614
	5.0	46.1	135	5.16 ± 0.04	0.9892
	6.0	166.7	432	5.20 ± 0.02	0.9953
	7.0	178.6	461	5.54 ± 0.05	0.9923
Cd	4.0	7.3	19	4.02 ± 0.02	0.9705
	5.0	8.8	23	4.78 ± 0.02	0.9904
	6.0	10.5	27	4.86 ± 0.02	0.9565
	7.0	15.7	41	4.89 ± 0.04	0.9867
Pb	4.0	16.9	44	4.17 ± 0.02	0.9763
	5.0	37.3	109	4.91 ± 0.02	0.9708

Conclusion

The biosorption capacity of *A. fumigatus* EPS determined using either direct titration curves $i=f(C_M)$ or the method proposed by Ruzic afforded similar results. The metal affinity order for EPS is $\text{Cu(II)} > \text{Pb(II)} > \text{Cd(II)}$. The number of EPS binding sites increased with pH in the range of pH4.0–7.0 for Cu(II) and Cd (II). As for Pb(II), the number of binding sites increased with pH in the range of pH4.0–5.0, and solid forms were observed at pH6.0–7.0. The conditional binding constant of these three metals showed only narrow fluctuations with pH.

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