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Binding Sites for Lead Ion in *Staphylococcus epidermidis*

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As *S. epidermidis* cell was fractionated into cell wall, cell membrane, and cytoplasm, the cell membrane proved to be the most efficient adsorbent for lead ion. Ultrasonication was effective, when the cells were treated during their exponential growth. The amount of the lead ion adsorbed in cell membrane decreased as hydrogen ion concentration of solution increased. Protein purified from the cell membrane showed higher adsorption capacity for the lead ion than peptidoglycan, teichoic acid from cell wall, or cell membrane lipid. Modification of carboxyl groups in the membrane protein with ethylenediamine and 1-ethyl-3-carbodiimide hydrochloride resulted in a considerable decrease of lead ion adsorption capability, suggesting that the main binding site for lead ion was the carboxyl groups of protein in cell membrane.

Key words: *Staphylococcus epidermidis*, lead ion, binding sites, functional group

Rapid and localized increase of heavy metal content in the hydrosphere and atmosphere has became a crucial issue. Chronic lead toxicity produces renal malfunction, anemia and nervous system disorders (20). Since Polikarpov (23) reported in 1966 that marine microorganisms accumulate radioactive materials from seawater, investigations on microorganisms that accumulate heavy metals from aqueous solution in their cells have been advanced actively in order to find ways to dilute, concentrate, or reuse the heavy metals. The uptake of heavy metal ions occurs through the interaction of functional groups existing on the surface of the cell wall or the cell membrane with the heavy metal ions, which are adsorbed on the cell wall and the cell membrane (26) or accumulated within the cell (5). The amount of adsorbed heavy metal ions has a close relationship with the number of functional groups that interact with metal ions. Heavy metal ions can also be deposited within the cell in a form of inorganic precipitates (3). This can be explained by the fact that the amount of adsorbed heavy metal ions in the cell is greater than that of the heavy metal ions bound stoichiometrically to the functional groups in the cell (3).

It was reported that heavy metal cations were bound mainly to functional groups like carboxyl, phosphoryl, and/or sulfhydryl groups (4, 12). Carboxyl groups exist in protein and muramic acid of bacterial cell wall. Phosphoryl groups are contained in cell membrane lipid, backbone of nucleic acid, cell wall teichoic acid, lipoprotein, and lipopolysaccharides. Sulfhydryl groups mainly exist in polysaccharides of algal cell wall. It was reported that anionic forms of heavy metals could bind with amine groups, imidazole groups and guanidino groups present in protein, amine groups of purine and pyrimidine rings and heterocyclic nitrogen (12). Binding capacity of the functional groups with heavy metals was shown to be affected by electric charge and ionic radii of the heavy metal ions as well as the degree of hydration of the metal ions and the pH of the solution (6, 11, 26). Solution pH determines essentially the formation of metal hydroxides (13, 14, 26). Moreover, hydrogen ion competes with cationic heavy metal ions for negatively charged functional groups so that the hydrogen ion concentration is the most important influencing factor for the uptake of metal ions (11)

To examine the binding sites for lead ion adsorption, we fractionated *S. epidermidis* cells into cell wall, cell membrane, and cytoplasm. Peptidoglycan and teichoic acid were separated from the cell wall fraction, while protein and lipid were obtained from the cell membrane. In addition, the negatively charged functional group (i.e. carboxyl group) was modified to have a positive charge and the positively charged functional group (i.e. amine group) was chemically treated to exhibit a negative charge so as to investigate the principal binding functional groups for lead ion uptake in the cell.

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Materials and Methods

Strain and growth conditions

Nutrient agar was used for stock culture of *Staphylococcus epidermidis* EVANS ATCC 12228, while nutrient broth was employed for liquid medium. 5 ml of sterile distilled water was added to the stock culture and cells were suspended uniformly with the aid of a vortex mixer (Scientific Industries K550G). The cell suspension thus obtained was inoculated into 200 ml of the liquid medium and cultured for 12 hours at 37℃ while being shaken at 180 rpm with a Rotary Shaker (Lab-Line 3579).

Ultrasonication

Cells were harvested after 3, 5, 7, 10, 15, 35, and 48 hours of liquid culture. 100 mg (dry weight) of the cells from each fraction was subjected to ultrasonication for 50 minutes at intervals of 5 minutes. Cells unsmashed by the ultrasonication procedure were separated by centrifugation (6000×*g*, 15 min).

Cell fractionations

Separation of cell wall was performed as described by Dolye and Birdsell (7) except that 0.05 M Tris-maleate buffer (pH 7.0) was used in place of distilled water.

Biomass of liquid culture was harvested by centrifugation (6000×*g*, 15 min), washed with buffer (pH 7.0) 3 times, and ultrasonicated after storage at room temperature for more than 18 hours (Ultrasonicator: Sonics & Inc. VC 600). After ultrasonication, unsmashed biomass was separated by centrifugation (6,000×*g*, 15 min). The resulting supernatant was centrifuged (39,000×*g*, 30 min, 4℃) to obtain cell wall as sediment. The cell wall thus obtained was confirmed by the method proposed by Beveridge and Murray (2).

The supernatant after centrifugal separation of the cell wall was subjected to ultra-centrifugation (100,000×*g*, 40 min, 4℃; Beckman, XL-90). The resulting supernatant was considered as part of cytoplasm and the precipitate as crude cell membrane. Separation of cell membrane was performed as described by Lee *et al.* (16). The solution containing cell membrane was ultracentrifuged (100,000×*g*, 2.5 hours, 4℃) to obtain a precipitated cell membrane. The supernatant was considered to be cytoplasm. The separated cell membrane was confirmed by Schnaitman (24). Dry weight of each fraction was measured after drying at 105℃ for 18 hours.

Separation of cell components

Separation of peptidoglycan from cell wall was performed as described by Hoyle and Beveridge (12), while separation of teichoic acid from cell wall was followed as indicated by Slabyi and Panos (25). Dry weight was measured under the same conditions as previously cited. Separation of lipid from cell membrane was carried out using the method of Beaven *et al.* (1) with slight modification. Cell membrane (dry weight 200 mg) was dissolved in 4 ml of methanol and subsequently received 8 ml of chloroform. Lipid was extracted from the solution after shaking (180 rpm) at room temperature for 2 hours. Cell membrane debris was filtered off. Impurities were precipitated out by the addition of 3 ml of KCl solution (0.88%). The solution was centrifuged (120×*g*, 5 min) and then the chloroform layer containing lipid was separated. Dry weight of the extracted lipid was measured after evaporating the solvent.

Extraction of protein from cell membrane was carried out as described by Mizuno and Kageyama (21) with some modification. The separated cell membrane was suspended in 0.1 M citrate-phosphate buffer (pH 7.0) containing 0.03 M 2-mercaptoethanol, 10 mM EDTA, 0.5 % lysozyme and 0.5% Triton X-100. The mixture was warmed up to 30℃ for a few minutes and kept at room temperature for 1 hour, and then incubated at 37℃ for 2 hours. Protein from cell membrane was obtained as the supernatant after centrifugation of the incubated solution. To remove the remaining EDTA, separated protein was dialyzed against 50 mM Tris-HCl buffer (pH 7.5) at 4℃, and Triton X-100 was removed by ion exchange chromatography using DEAE-sepharose gel (Sigma, CL-6B). Protein was analyzed quantitatively by UV/VIS spectrophotometer (Shimadzu, UV-120-02) after Lowry *et al.* (18) using bovine serum albumin as the standard protein.

Pretreatment of cell membrane and modification of functional groups

As was done by Nakajima *et al.* (22), the cell membrane was pretreated with hot water, chloroform, methanol, and an alkaline solution (0.2% NaOH). The resulting solution was pretreated with chloroform and methanol and was treated again with a concentrated alkaline solution (24% KOH).

Modification of amine and carboxyl groups of protein from cell membrane was carried out according to the method of Beveridge and Murray (3).

Determination of lead ion adsorption

To remove the buffer solution used for cell fractionation, cell wall, cell membrane, and peptidoglycan were washed with distilled deionized water in the centrifuge. Cytoplasm, teichoic acid, and protein were dialyzed twice against distilled deionized water for 24 hours. To 4.8

ml of distilled deionized water was added 0.1 ml of Pb^{2+} ($Pb(NO_3)_2$; Sigma) solution and 0.1 ml of the adsorbent suspended solution (dry weight 2 mg). The uptake of the lead ion was monitored for 1 hour at 25℃ with shaking.

At the end of the adsorption, the adsorbents were separated from the solution either with a centrifuge (cell wall, cell membrane, peptidoglycan) or with dialysis against sterilized deionized water for 72 hours (cytoplasm, teichoic acid, protein and lipid from cell membrane). Amount of the adsorbed lead ion in the adsorbents is determined by comparing the lead ion concentration in the solution before and after adsorption using Atomic Absorption Spectrophotometer (AAS: Shimadzu, AA-670).

Results and Discussion

Utrasonication yield of *S. epidermidis* cell

Cells were subjected to ultrasonication to fractionate them into cell wall, cell membrane and cytoplasm. Effect of phase of growth of the cells on the ultrasonication efficiency was investigated as shown in Fig. 1. Cells cultivated for less than 7 hours, corresponding to the exponential phase of growth, were shown to be more susceptible to ultrasonication than those grown for a longer period of time. Table. 1 represents the content of the cell fractions after ultrasonication.

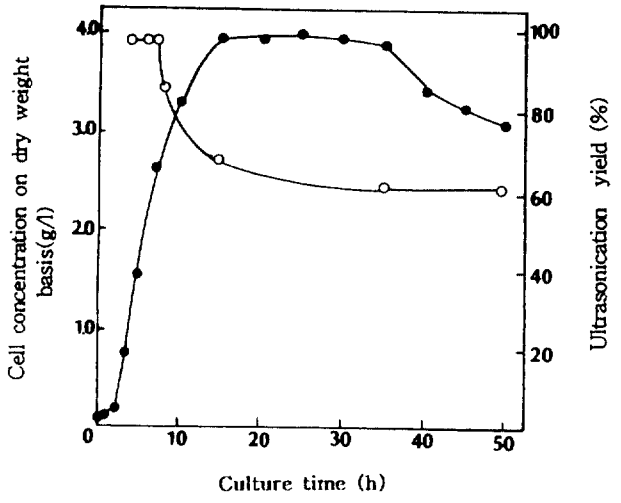

Fig. 1. Ultrasonicaton yield as a function of culture time of *S. epidermidis*. Cells were disrupted by ultrasonication for four intervals of 15 min. ○—○; ultrasonication yield, ●—●; growth curve.

Table 1. Content of cell fractionation.

Fraction	Content (wt %)
Cell wall	15
Cell membrane	8
Cytoplasm	56
Others	21

Lead ion adsorption after cell fractionations

S. epidermidis cell was fractionated into cell wall, cell membrane, and cytoplasm. The specific uptake of the lead ion is shown in Fig. 2. It can be seen that the cell membrane fraction adsorbed the highest amount of the lead ion among the three fractions. Pb^{2+} at 10^{-3} M was adsorbed in an amount larger than at 10^{-4} M for all the cell fractions tested.

Fig. 3 shows the specific uptake of lead ion in peptido-

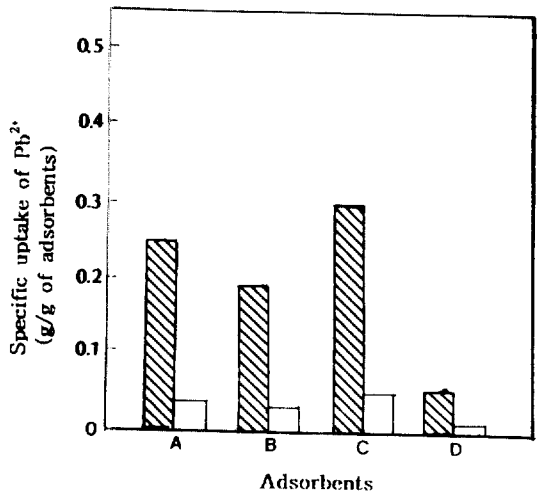

Fig. 2. Adsorption of Pb^{2+} in different cell fractions of *S. epidermidis*. [Pb^{2+}]; 1.0×10^{-3} M (▨), 1.0×10^{-4} M (□), pH; 6.0, A; whole cell, B; cell wall, C; cell membrane, D; cytoplasm.

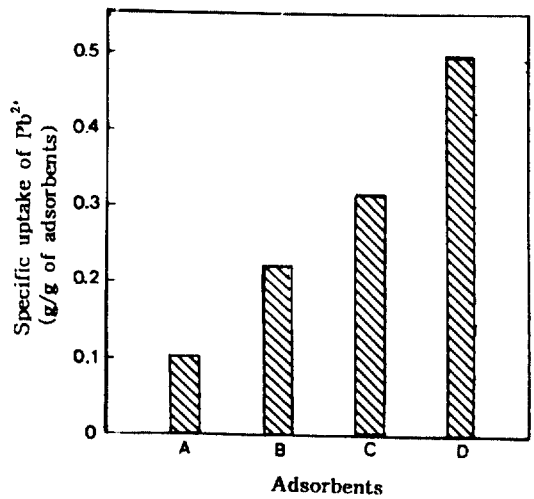

Fig. 3. Adsorption of Pb^{2+} by cell components. [Pb^{2+}]; 1.0×10^{-3} M, pH; 6.0, A; peptidoglycan in cell wall, B; teichoic acid in cell wall, C; lipid in cell membrane, D; protein in cell membrane.

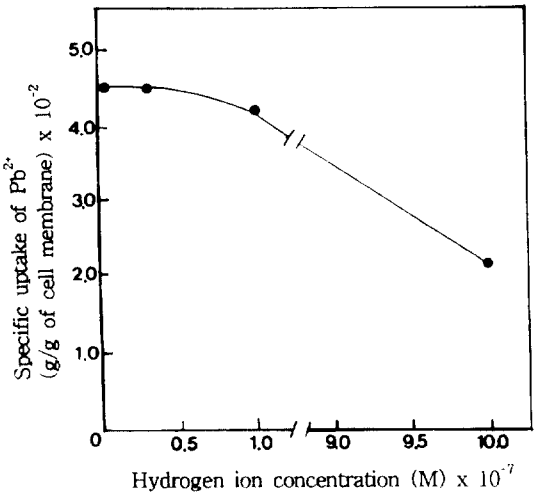

Fig. 4. Effect of hydrogen ion concentration on the adsorption of Pb^{2+} by cell membrane of *S. epidermidis*. $[Pb^{2+}]$; 1.0×10^{-4} M.

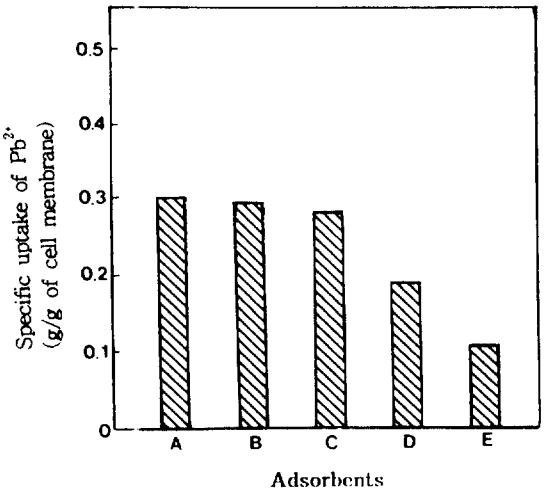

Fig. 5. Adsorption of Pb^{2+} by pretreated cell membrane of *S. epidermidis*. $[Pb^{2+}]$; 1.0×10^{-3} M, pH 6.0, A; unpretreated cell membrane, B; hot water treated cell membrane, C; chloroform-methanol treated cell membrane, D; alkali treated cell membrane, E; chloroform-methanol/concentrated alkali treated cell membrane.

glycan and teichoic acid, obtained from the cell wall, and that in the protein and lipid extracted from the cell membrane of *S. epidermidis*. Protein from cell membrane showed the highest adsorption capacity of the absorbents tested.

pH effect

Hydrogen ion is an important factor influencing the adsorption behavior of heavy metal ions. Fig. 4 demonstrates that hydrogen ion concentration of the solution decreased the specific uptake of lead into cell membrane. This result is not contradictory to those of others reported previously. Doyle *et al.* (8) reported that hydrogen ion competed with the metal ions for the binding sites, and the higher the concentration of the hydrogen ion, the fewer binding sites in *Bacillus subtilis* cell wall were available to the heavy metal ions. Affinity of the hydrogen ion for the binding sites was reported to be greater than that of heavy metal ions (19). Thus the hydrogen ion made the bound heavy metal ions desorbed from the binding sites. Therefore the adsorption of heavy metal ions becomes disturbed (10) as the concentration of the hydrogen ion gets higher. The significant decrease in the specific uptake of lead ion at hydrogen ion concentrations higher than 1.0×10^{-4} M was thought to be due to the loss of negative charge of the carboxyl groups in the cell at low pH (26). In addition, only a minor fraction of phosphoryl groups have negative charges at hydrogen ion concentration higher than 1.0×10^{-4} M (15).

Pretreatment of cell membrane

To clarify principal component materials of cell membrane binding to the lead ion, each fraction obtained from *S. epidermidis* cell membrane was modified by chemical means. Oligosaccharides, polysaccharides, protein, and small molecular weight materials were extracted from cell membrane by hot water pretreatment. Most of the protein remained intact but lipids were easily extracted by pretreatment with chloroform and methanol. An alkaline solution dissolved protein and polysaccharides. From the cell membrane pretreated with chloroform and methanol, concentrated alkaline solution extracted protein and lipid (22).

Fig. 5 shows that the amount of the lead ion adsorbed in cell membrane pretreated with various means was lower than that of the untreated. Capability of the lead ion adsorption decreased as the content of protein in the cell membrane decreased due to the pretreatments. Therefore, it is suggested that the cytoplasmic membrane protein is the main binding site for the lead ion adsorption. On the contrary, Feofilora *et al.* (9) reported that lipid was the main binding site of the lead ion in *Aspergillus phoenics*, suggesting that the main binding site for lead ion is species-specific

Lead ion binding to modified functional groups in the cell membrane

All protein molecules have free carboxyl and amine groups at their terminal units while the polarity of a protein depends on it's amino acid composition.

Amino acids such as aspartic acid and glutamic acid have negative charges conferred from carboxyl groups at neutral pH. On the contrary, amino acids such as

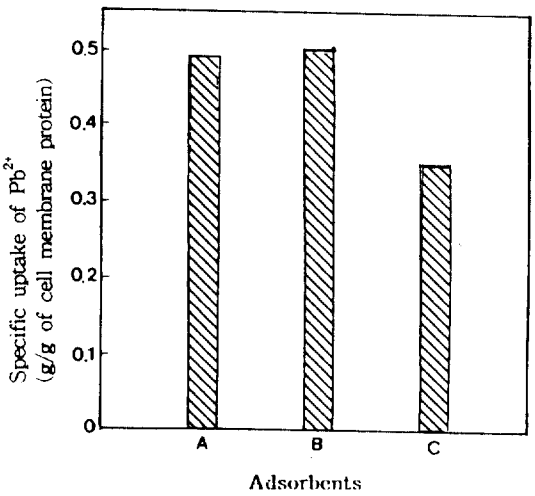

Fig. 6. Adsorption of Pb^{2+} by modified cell membrane protein. $[Pb^{2+}]$; 1.0×10^{-3} M, pH; 6.0, A; cell membrane protein with unmodified functional groups, B; cell membrane protein with modified amine group, C; cell membrane protein with modified carboxyl group.

lysine, arginine, and histidine have positive charges at neutral pH due to their amine groups. Some other amino acids appear to be uncharged but slightly polar at pH 7.0. They generally contain hydroxyl or sulfhydryl groups (17). The lead ion uptake was carried out at pH 6.0 in this study, and the carboxyl group would be negatively charged while the amine group would be positively charged. However the hydroxyl and sulfhydryl groups did not seem to affect the lead ion uptake behavior because they have only a slight polarity at this solution pH.

Hence only the carboxyl and amine groups were chemically modified and the changes in the capability of the lead ion uptake were examined. Amine groups having positive charges were modified to exhibit negative charges using S-acetyl-mercapto-succinic anhydride. The negative charges of carboxyl groups were transformed into positive charges using ethylenediamine and 1-ethyl-3-carbodiimide hydrochloride (3). The protein of cell membranes having modified amine groups showed somewhat larger amounts of lead ion adsorption than proteins with unmodified amine groups. This is thought to be due to the fact that the modified amine groups exhibit negative charges. Beveridge and Murray (3) reported that the lead ion could exist in anionic complexes, such as $Pb(OH)_3^-$ and $Pb(OH)_4^{2-}$ in an aqueous solution at neutral pH, and it could be bound to positively charged amine groups. However it is inferred from our data that the amine group was not operative for the lead ion binding. On the other hand, proteins having modified carboxyl groups showed less lead ion adsorption than unmodified proteins (Fig. 6), indicating that the carboxyl group was the principal binding site for lead ion adsorption. It was concluded that the adsorption of the lead ion by cell membrane protein at pH 6.0 was achieved by the binding of the lead ion to the negatively charged carboxyl group in the protein.

Acknowledgement

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