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Effects of heavy metals and albumin on lysozyme activity

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Abstract Lysozyme is an antibacterial enzyme that is found in most of body fluids. Lysozyme in tears plays a primary role in protecting eye from harmful environments; if lysozyme is degraded or inhibited, eyes are likely to be more vulnerable to bacterial infection. In this study, lysozyme activity was evaluated according to varying concentrations of heavy metals, copper, zinc, cobalt and manganese and light metal, calcium that are frequently found in airborne particulate matters and was assayed using a dye-quenching lysozyme substrate, *Micrococcus lysodeikticus*. Less fluorescence intensity was observed with increasing amounts of copper, zinc, manganese and cobalt but not with calcium suggesting that these metals have some affinity with lysozyme and inhibit lysozyme activity. When albumin, the second most common protein in tears, was added on the reaction of lysozyme and metals, lysozyme activity was partially restored. This finding suggests that the albumin might protect damage caused by metals on lysozyme. To identify whether the decrease in enzymatic activity was related to structural changes of lysozyme, SDS-PAGE was conducted and only with copper did lysozyme show marked smearing bands on the SDS-gel, meaning that copper degraded lysozyme consistent with the sharpest activity decrease.

Keywords Albumin · Heavy Metals · Lysozyme activity

Introduction

Industrial development has resulted in serious environmental problems and heavy metals in particulate matter (PM), are considered one of the main causative factors for environmental problems; heavy metals are defined as metals with specific density above 4.0. Some such as copper, calcium and zinc are essential for biological metabolism, but they can be severely toxic above the required amounts when they accumulate in the body. Cadmium, lead, mercury in air, for example, bind to and modify a number of cellular factors, inhibiting cell growth [1]. Eyes can also be severely affected by heavy metals; corneal or conjunctival damage by heavy metals causes conjunctivitis and dry eyes and the metals' accumulation, especially copper in choroid and retinas causes blindness and sunflower cataract [2].

In addition to eye diseases, heavy metals also affect proteins in tears including lysozyme. Lysozyme is the first physiological enzyme that kills bacteria by hydrolyzing glycosidic bonds in the peptidoglycan of bacterial cell walls [3]. Several types of lysozyme exist in various bodily fluids and serve as the primary protection layer, especially for eyes [3]. Human lysozyme consists of 130 residues and belongs to the c-type class of lysozymes [4]. Functional degradation in lysozyme due to heavy metals makes eyes susceptible to infection.

Heavy metals accumulate after thousands of exposures even when only a small amount of airborne metals is directly exposed to eyes at a given time because there is a study that lead and cadmium accumulate in human ocular tissues when heavy metal concentration was measured in human eyes [5]. For this study four heavy metals and one light metal usually found in PM; copper, zinc, calcium, cobalt and manganese [6,7] were selected to

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examine effects of heavy metals on lysozyme activity. Based on the proteins in tears, various concentrations of all metals were added to chicken egg white lysozyme consisting of 129 amino acids with molecular weight 14.3kDa and classified as c-type, the same as human lysozyme [8]. Albumin, another main protein in tears, has been added to see how it affects lysozyme activity in the presence of metals. This is the first description to reveal how heavy metals influence lysozyme in the presence of albumin. It is described here that all heavy metals used for the study except for light metal, calcium, reduced lysozyme activity whereas albumin exhibited protective effects on heavy metal-treated lysozymes.

Material and Methods

Lysozyme activity assay using fluorescence

Copper (II) sulfate (Sigma Aldrich, St. Louis, MO, USA), zinc sulfate (Sigma Aldrich), calcium chloride dehydrate (Sigma Aldrich), manganese sulfate (Sigma Aldrich), cobalt (II) chloride hexahydrate (Sigma Aldrich) were dissolved in deionized water. All metals were added in concentrations of 50, 130, 260, and 390 μM to 130 μM of chicken egg white lysozyme (Sigma Aldrich) in phosphate buffer saline at pH 7.4 which is consistent with the normal concentration in tears. After the mixture were incubated at 36 °C for 30 min, the dye-quenching lysozyme substrate, *Micrococcus lysodeikticus* (EnzCheck Lysozyme Assay Kit from Thermo Fisher Scientific, Hampton, NH, USA) was added to start the reaction. Digestion products from the lysozyme substrate have absorption maxima at 494 nm and fluorescence emission maxima at 518 nm. The fluorescence intensity was measured at 518 nm using spectral scanning multimode reader (Varioskan, Thermo Fisher Sceintific, Vantaa, Finland). The same set of experiments was also performed in the presence of 3 μM of albumin which is consistent with the normal concentration in tears. Aliquots of the above reactions were assessed by 12% SDS-PAGE at 100 V for 100 min.

All experiments were repeated three times. Each fluorescence intensity from metal-treated lysozyme activity was normalized against no metal-treated lysozyme activity. Three replicate values were entered in side by side sub-columns and plot mean and error.

Results and Discussion

Normal concentration of metal in atmospheric PM was <2 μM. When the concentration of 2 to 30 μM of metals was treated to lysozyme, no significant effect was observed. It should be considered that eyes are directly exposed to atmospheric PM at a given time. Therefore, all metals in concentrations of 50, 130, 260, and 390 μM were added to 130 μM of chicken egg white lysozyme which is consistent with the normal concentration in tears.

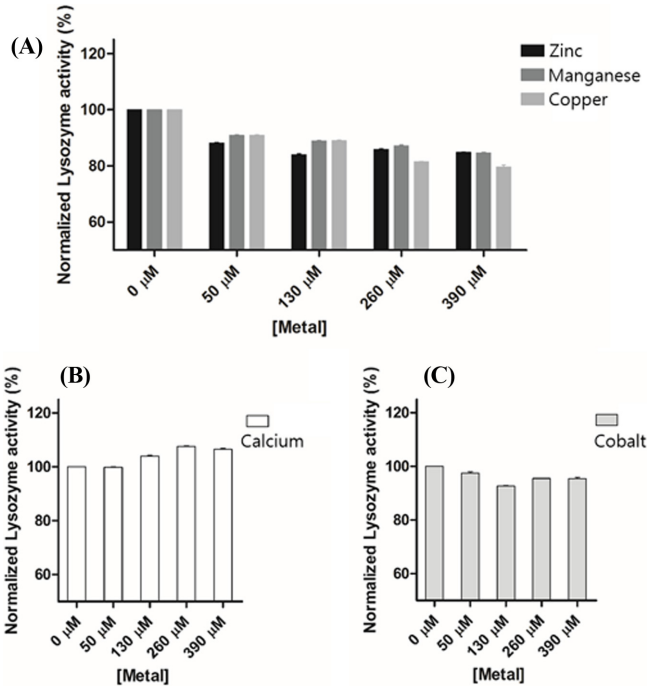

Fig. 1 Lysozyme activity with metals. Catalytic lysozyme activity was measured at different concentrations of zinc, manganese and copper (A), calcium (B) and cobalt (C)

Lysozyme activity decreased sequentially with increasing copper concentration until 390 μM (Fig. 1A). The presence of albumin maintained lysozyme activity (Fig. 2A). Severe SDS-PAGE smearing bands copper-added lysozyme demonstrated reduced enzymatic activity caused by structural degradation (Fig. 3). In the presence of albumin, relatively decreased smearing was observed on the SDS gel at higher concentrations of copper (Fig. 3).

The irreversible effect of copper on lysozyme has been studied in a variety of contexts [9], and finds are consistent that copper reduces catalytic lysozyme function by approximately 80% at 390 μM and that structural lysozyme stability decreases as well (Fig. 1A). Cu^{2+} binds to residues Glu35 and Asp52 located at the catalytic site [9] and degrades overall construction as seen in the severe smearing on SDS gel (Fig. 3). However, fluorescence, the marker for cell wall degradation used as a substrate, in contrast increased with copper concentrations above 400 μM. It assumed that this reaction takes place because of the direct toxicity of copper on cell walls. When copper forms a complex with cell wall, polysaccharides and hydroxyl ions occur and causes polysaccharide solubilization [10]. To determine the exact relationship between heavy metals and lysozyme, in this study the effects were observed metal concentrations under 400 μM. It should be noted that there was no effect on substrates' cell wall destruction by divalent metal ions with <400 μM because increases of fluorescence intensity were not detected at the points under 400 μM.

The other metals, except for calcium (Fig. 1B) also inhibited lysozyme activity but not by as much as the reduction with added

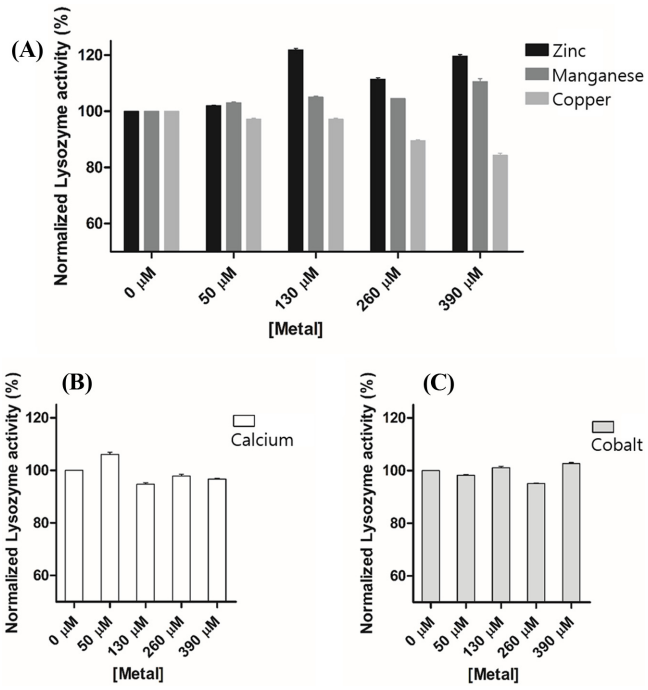

Fig. 2 Lysozyme activity at varying concentrations of metals in the presence of albumin. Catalytic lysozyme activity was measured at different concentrations of zinc, manganese and copper (A), calcium (B) and cobalt (C)

copper on lysozyme (Fig. 1C). Manganese and zinc degraded catalytic function similarly, approximately 85% at 390 μM (Fig. 1A). These metals are known as interacting lysozyme or lysozyme-like enzyme secreted from starfish [11-13]. The binding of manganese and zinc on the active site of lysozyme might prevent lysozyme activity. Cobalt exhibited weak binding affinity to lysozyme at the residue Asp52 [14-16] compared with manganese [17], resulting in relatively little activity reduction (Fig. 1C). No smearing bands on SDS gel were observed for zinc, calcium, cobalt or manganese (data not shown) in contrast with the bands for copper-treated in lysozyme. That is, there was little structural damage from those metals, and the decreases in observed activity were not related to structural damages [16].

The presence of albumin clearly helped to maintain lysozyme activity (Fig. 2). Albumin serves as a carrier for a variety of nutrients, metabolites and metals. Four different metal binding sites are described as ATCUN motif, site A, site B and reduced Cys34 [18]. Meanwhile, copper, zinc, calcium, and cobalt are known to bind with albumin [18,19]. Metals may compete to bind on lysozyme or albumin, and thus lysozyme activity was inhibited less in the presence of albumin than in its absence. When zinc and manganese reacted with lysozyme, activity increased with albumin suggesting something happened among the complexes of zinc and manganese and albumin plus lysozyme. It requires to prove in further.

Interestingly, lysozyme activity increased with calcium (Fig. 1B). Higher concentrations of calcium, increased the lysozyme activity. The presence of albumin in the calcium-treated lysozyme didn't make any remarkable difference in activity (Fig. 2B), even on SDS-PAGE analysis. Calcium appeared not to influence either lysozyme activity or structure. It is thought that fluorescence increase not because calcium interacts with lysozyme but it destabilizes cell walls. Calcium is in fact used to transforming *Micrococcus lysodeikticus* and destabilize cell walls [20]. When albumin is added, calcium binds to the albumin at site B [18], and only lysozyme catalyze the cell walls; as result, fluorescence

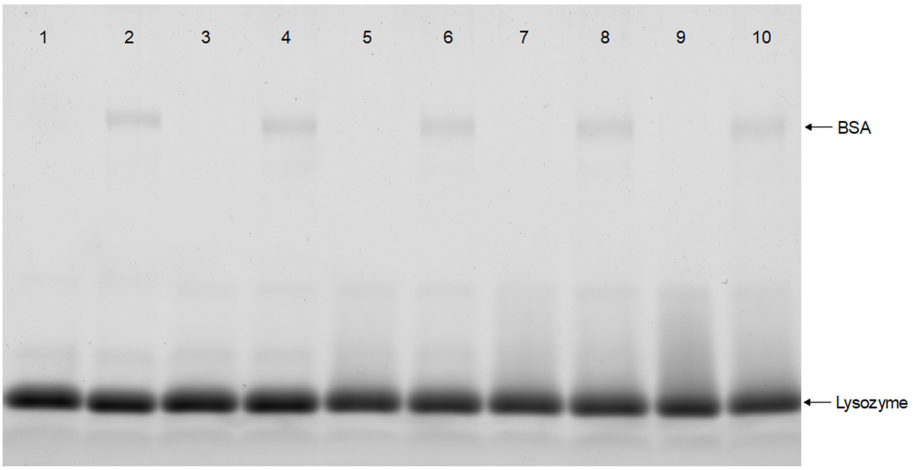

Fig. 3 SDS-PAGE of copper-treated lysozyme in the absence and presence of albumin. Lysozyme samples were subjected to 12% SDS-PAGE. Lane 1, 130 μM of Lysozyme; Lane 2, 130 μM of lysozyme with 3 μM of albumin; Lane 3, 130 μM of lysozyme with 50 μM of copper; Lane 4, 130 μM of lysozyme with 50 μM of copper and 3 μM of albumin; Lane 5, 130 μM of lysozyme with 130 μM of copper; Lane 6, 130 μM of lysozyme with 130 μM of copper and 3 μM of albumin; Lane 7, 130 μM of lysozyme with 260 μM of copper; Lane 8, 130 μM of lysozyme with 260 μM of copper and 3 μM of albumin; Lane 9, 130 μM of lysozyme with 390 μM of copper; Lane 10, 130 μM of lysozyme with 390 μM of copper and 3 μM of albumin

remains approximately 100% regardless of calcium concentration. PM has long been studied in a variety of fields and is closely related to daily life and health. The findings from this study reflect this trend and particularly regarding the effects of heavy metals on lysozyme. There have been many studies on the interaction between metals and proteins, but this is the first attempt to reveal how heavy metals influence lysozyme in the presence of albumin. The results in here indicated that albumin might primarily interact with heavy metals for the lysozyme activity *in situ*.

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