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Importance of extracellular proteins in maintaining structural integrity of aerobic granules

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ABSTRACT

Aerobic granules developed through self-immobilization of microorganisms are compact and structured microbial consortia embedded in a matrix of extracellular polymeric substances (EPS). This study investigated the contribution of extracellular proteins (PN) to maintaining the structural integrity of aerobic granule. It was found that hydrolysis of PN induced by Proteinase K led to significant disintegration of aerobic granules, whereas a substantial reduction of extracellular polysaccharides (PS) was also observed. It was proposed that hydrolysis of extracellular proteins present in the EPS matrix of aerobic granules led to collapse of the EPS matrix, and subsequent disintegration of aerobic granule. These suggested that extracellular proteins would be essential for maintaining structural stability of EPS matrix of aerobic granules. In addition, it was revealed that production of signaling molecules, such as autoinducer-2 (AI-2) and N-acyl homoserine lactones (AHLs) was also inhibited probably due to hydrolysis of quorum sensing receptor proteins by Proteinase K. This in turn provided an additional explanation for the observed Proteinase K-triggered dispersal of aerobic granules.

1. Introduction

Extracellular polymeric substances (EPS) produced by microorganisms can form complex matrix through inter-cross linkage of extracellular polysaccharides (PS) and proteins (PN) in which microbial cells are embedded. Evidence clearly shows that such EPS matrix is essential for development and maintenance of spatial structure of biofilms and aerobic granules [1,2], while it is highly debatable with regard to the specific roles of PS and PN. Some studies showed that PS contributed more than PN to the formation and structural stability of microbial aggregates [3,4]. On the contrary, Liu and Sun [5] found that the PN content in biomass increased with denitrifying granulation, whereas the PS content remained nearly unchanged at a lower level, whereas McSwain et al. [6] thought that formation and stability of aerobic granules were more dependent on PN than PS. In addition, Flint et al. [7] reported that reduced cell surface proteins after treating cells with trypsin or sodium dodecyl sulphate led to a 100-fold reduction in the number of attached bacteria, whereas reduced cell surface polysaccharides by treating the cells with sodium metaperiodate, lysozyme or trichloroacetic acid did not trigger significant biofilm detachment. In the study of adhesion of *Azospirillum brasilense* to glass and polystyrene surface, Dufrene et al. [8] also found that protein concentration at the cell surface or at the support surface was highly correlated with adhesion density under different experimental conditions.

Although the composition and quantity of components in EPS affect the structural integrity of biofilms and biogranules, there are still many contradictory reports on the specific role of PN versus that of PS in developing and further strengthening structure of aggregates as presented above. It has been thought that the interaction of components in matrix would be more significant than the composition for the structure stability of matrix [9]. Similar situation can be expected in aerobic granules where the EPS matrix would likely be arranged through non-specific interaction of extracellular proteins and polysaccharides. Therefore, this study was specifically designed to look into the essential role of extracellular proteins as well as possible extracellular polysaccharides–proteins interaction in maintaining the structural integrity of aerobic granules.

2. Materials and methods

2.1. Granules treated experiments

Aerobic granules precultured in SBR with a synthetic wastewater composed of 320 mg l^{-1} sodium acetate and 360 mg l^{-1} ethanol as carbon source, 200 mg l^{-1} NH_4Cl, 60 mg l^{-1} K_2HPO_4, 25 mg l^{-1}

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$MgSO_4{\cdot}7H_2O$, 20 mg l^{-1} $FeSO_4{\cdot}7H_2O$ and 30 mg l^{-1} $CaCl_2{\cdot}2H_2O$ were harvested by centrifugation (KUBATO, Tokyo, Japan) at 5000 rpm for 5 min. Harvested granules were reinoculated in the above fresh synthetic wastewater. Two series of batch experiments were conducted: one was served as control free of enzymes; while the other was supplemented with 1 mg ml^{-1} Proteinase K (Promega, Madison, USA). The concentration of aerobic granules was 0.5 g l^{-1} in the batch experiments. Granules sample in the above two series of batch experiments was incubated on a rotary shaker at 150 rpm and 37 °C. After incubation for one and two days, granules samples were taken from each incubator to do further assay, such as size, turbidity, AI-2, AHLs, etc.

2.2. Biofilm detachment assay

For microbial attachment assay, activated sludge microorganisms taken from a local wastewater treatment plant were acclimated with the above synthetic wastewater for 1 month. The acclimated microorganisms were then harvested through centrifugation (KUBATO, Tokyo, Japan) at 5000 rpm for 5 min. 0.5 g l^{-1} of harvested biomass was resuspended in fresh synthetic medium. 200 μl of this microbial suspension per well was added into 96-well plate, and further cultured on a rotary shaker at 150 rpm and 37 °C for 16-h. To study the effect of Proteinase K on dispersal of biofilms, the precultured 16-h old biofilms were rinsed twice with PBS solution (1×, Invitrogen, Carlsbad, CA, USA) followed by being subjected to 200 μl per well of buffer solution (50 mM Tris–HCl with 10 mM $CaCl_2$) supplemented with 0.5–2.0 mg ml^{-1} Proteinase K, whereas biofilms without exposure to Proteinase K were served as control. After 2-h and 4-h contact with Proteinase K at 37 °C, remaining biomass in the plate was quantified by crystal violet staining method, as described below.

To quantify the biomass attached on the 96-well plate, fixed biomass was rinsed three times with PBS solution (1×, Invitrogen, Carlsbad, CA, USA), and then stained with 200 μl per well of 0.1% crystal violet (Sigma–Aldrich, USA). Virgin wells were used as blank. After 10-min incubation at room temperature, the stained sample was rinsed three times with PBS solution, and then dried in a fume hood at room temperature. 200 μl per well of 95% ethanol solution was added into the dried plate. After 10 min contact with ethanol at room temperature, solution collected from the wells was transferred into a micro cuvette with a volume of 1.5 ml. Absorbance of the collected solution was finally determined at 595 nm with a UV–vis spectrophotometers (UV mini-1240, Kyoto, Japan) which represented the amount of fixed biomass.

2.3. Analytic methods

2.3.1. Autoinducer-2 (AI-2) and N-Acyl homoserine lactones (AHLs)

The protocol of extraction and determination of AI-2 was similar to that by Xiong and Liu [10]. The method for extraction of AHLs was similar to that for AI-2 except for the medium. For AHLs extraction, minimal medium (MM) was used instead of AB medium for AI-2. The MM was prepared by dissolving 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 10 mg $FeSO_4{\cdot}7H_2O$, 15 mg $CaCl_2{\cdot}2H_2O$, 3 mg $MnSO_4{\cdot}H_2O$, 2.0 g $(NH_4)_2SO_4$ and 2 g glucose into one liter milli-Q water. The extracted AHLs were determined as described below. Agrobacterium tumefaciensNT1 (traR, tra::lacZ749) was used as indicator and cultured in MM media, supplemented with 50 mg l^{-1} spectinomycin (Sigma–Aldrich, USA) and 5 mg l^{-1} tetrocycline (Sigma–Aldrich, USA). After the AHLs indicator being inoculated in a rotary shaker at 150 rpm and 30 °C for 24 h, the culture was harvested, and diluted with fresh MM medium to an optical density (OD) of 0.1. 35 μl of diluted AHLs indicator was then added into 96-well plate containing 65 μl of extracted sample in each well. The plate with lid was wrapped with aluminum foil and incubated in a rotary shaker at 150 rpm and 30 °C for another 24 h, followed by addition of 100 μl of Beta-Glo® assay reagent (Promega, Madison, USA) into each well. The mixture in the plate was incubated for 90 min. Finally, the inoculated sample was assayed using a microplate reader (BioTek, sygnergy 2,

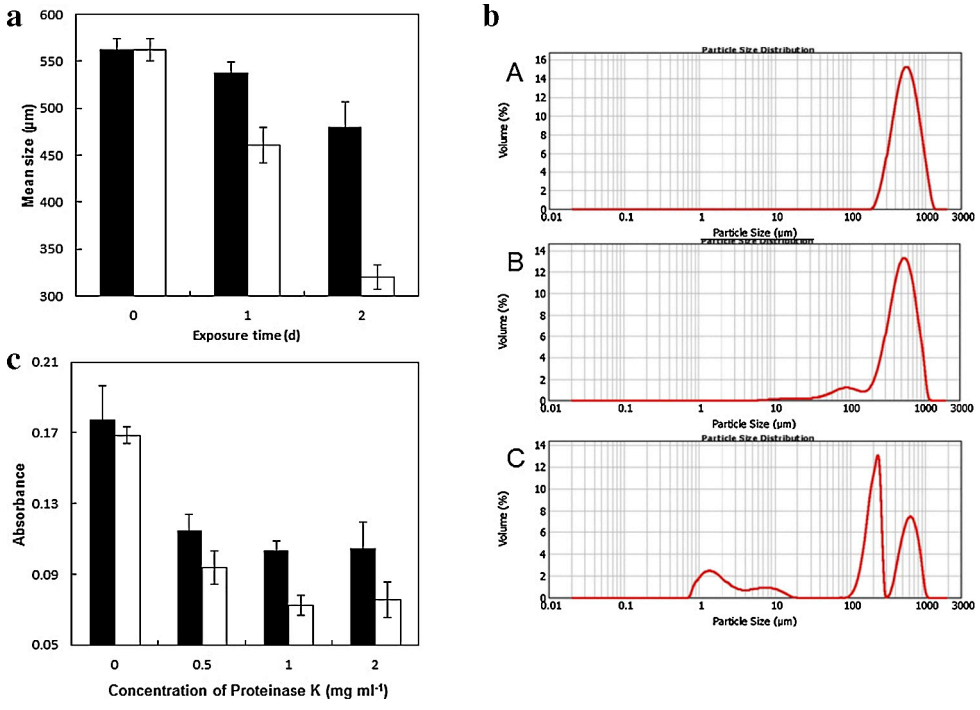

Fig. 1. Changes in mean size of aerobic granules after exposed to 1 mg ml^{-1} Proteinase K at different exposure times (■: control; □: 1 mg ml^{-1} Proteinase K) (a); size distribution of aerobic granules before and after treated by 1 mg ml^{-1} Proteinase K. A: Day 0; B: Day 2 without Proteinase K; C: Day 2 with Proteinase K (b); Detachment of 16-h precultured biofilm on 96-well plate after exposure to Proteinase K at different dosages and exposure times (■: 2-h exposure; □: 4-h exposure) (c).