Affinity isolation of algal organic matters able to form complex with aluminium coagulant

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Abstract Mass propagation of algae in drinking water source affects the coagulation process in water treatment systems. Many researchers indicated that some kinds of algal organic matters (AOMs) interacted with coagulants by coordinate bonds and brought about several disorders due to the increase in both the coagulant demand and the residual coagulant in treated water. However, the inhibitory mechanism on the coagulation by AOMs has not been fully elucidated. In this study, AOMs able to form complex with aluminium coagulant were isolated and analyzed. The results of the gel chromatography showed that extracellular organic matters (EOMs) from Microcystis aeruginosa (NIES-91) have molecular weight of between 10 and 20 kDa, around 40 kDa and more than 600 kDa. The amount and diversity of EOMs increased as the algal growth. AOMs able to form complex with aluminium coagulant were successfully isolated with the affinity chromatography. SDS-PAGE analysis revealed that these AOMs included proteins that have molecular weight between 43 and 67 kDa. Since several kinds of proteins such as metallothionein are known for strongly adsorbing multivalent cations, the isolated algal proteins able to form complex with aluminium might have a high capacity of capturing coagulants and inhibit the coagulation in the drinking water treatment.

Keywords Affinity chromatography; algal organic matters (AOMs); aluminium coagulant; coordinate bond; extracellular organic matters (EOMs); SDS-PAGE

Introduction
Semi-closed water areas such as reservoirs supplies about 40% of the total amount of drinking water source in Japan. One of the crucial problems in the semi-closed water area is the seasonal mass propagation of algae, which brings about several disorders in water treatment processes (Chow et al., 1999). Especially, the coagulation process is the most sensitive to the excess growth of algae (Alam et al., 2001). In general, pre-chlorination is employed to decrease the algal activity, but the chlorination results in the increase of dissolved organic matters from destroyed algal cells. It is known that the organic matters from algae (algal organic matters: AOMs) can form complexes with coagulant species (e.g., multivalent cations and hydrates of the coagulant) by coordinate bond, which directly links to the excess consumption of the coagulant (Bernhardt et al., 1991). Furthermore, problems about the production of trihalomethane owing to the reaction of AOMs with chlorine and the remaining of aluminium coagulants in treated water are also generated (Benschoten et al., 1990). The inhibition of the coagulation by AOMs has to be immediately resolved in order to ensure the safe utilization of tap water.

However, there has been a difficulty in making the fundamental review for the coagulation process without understanding of the detail mechanism on the inhibition of the coagulation by AOMs. Therefore, it is of great interest in elucidating the inhibition mechanism by AOMs. Some researchers reported that AOMs inhibit the forming of chelate...
hydrate of cations and the neutralizing charges of suspended substances that cause the
decrease of the coagulation efficiency (Bernhardt et al., 1986). It has been also reported that
the difference in inhibitory effect on the coagulation was observed among the AOMs
obtained from the different algal species or different growth phases. For example, Micro-
cystis species excrete some kinds of extracellular organic matters (EOMs), and these EOMs
in logarithmic growth phase bring about the inhibition of the coagulation (Magara et al.,
1986). Other researchers reported that the inhibitory effect on coagulation by AOMs of
Dictyosphaerium and Melosira depends on the growth phase in which the AOMs are
produced (Bernhardt et al., 1991). These things imply that the inhibitory effect on the
coagulation is determined by the constituents of AOMs.

The variety of AOMs makes it difficult to determine the species of AOMs responsible for
the inhibition of the coagulation. In this study, the affinity chromatography was used for
screening AOMs that have the ability to adsorb to aluminium coagulant with the coordinate
bond. The algal biomass can be used for metal-absorbent (Gin et al., 2002; Malik et al.,
2002), which means some kinds of AOMs have a high affinity with cations, and they might
cause the excess consumption of coagulants and the decrease in the coagulation efficiency.
The affinity chromatography using a coagulant as a ligand could be a powerful tool for
determining AOMs relating to the inhibition of the coagulation.

The purpose of this study is to elucidate the mechanism of the inhibition of the coagu-
lation by AOMs. As the first step, AOMs able to form complex with aluminium coagulant
were isolated and analyzed. The aluminium coagulant is widely employed in water treatment
plants in Japan. Microcystis aeruginosa was cultivated, and organic matters were recovered
from the culture. Then, recovered organic matters were fractionated according to their
molecular weight by the gel filtration chromatography. At the same time, AOMs that have an
ability to capture aluminium coagulant with the coordinate bond were recovered by the
affinity chromatography, and recovered proteins were analyzed by SDS-PAGE. Then, the
contribution of the complex-forming AOMs to the inhibition of the coagulation is discussed.

Materials and methods

Cultivation of algae

M. aeruginosa (NIES-91) was used in this study, which was supplied by the National
Institute for Environmental Studies (NIES), Japan. This algae was grown in MA media at
25°C with a shaking apparatus (S-102D, Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at
70 rpm. The 12h-light/12h-dark cycle was employed. The light intensity was 5,000 lux.
Algae was harvested on days 4, 12, 17 and 25 of the cultivation period. Chlorophyll-a was
measured according to the method for tap water examination (Japan Water Works Asso-
ciation, 2001) in order to depict the growth curve of M. aeruginosa.

Preparation of extracellular organic matters (EOMs) and algal organic matters (AOMs)

AOMs are classified into three categories including EOMs, inner organic matters (IOMs)
and surface-retained organic matters (SOMs) as indicated in Figure 1. Two types of sample
(EOMs and AOMs) were prepared as follows:

Preparation of EOMs. Harvested growth media including algal cells on days 4, 12, 17 and 25
were filtered with 0.22-μm membrane filter (GSWPO4700, Millipore). The filtrate was
concentrated tenfold with a rotary evaporator (Rotavator, Shibata, Tokyo, Japan), and stored
as EOMs from M. aeruginosa at −80°C.

Preparation of AOMs. AOM samples were prepared as follows. Harvested algal cells on days
4, 12, 17 and 25 were destroyed with ultrasonic treatment (20 kHz, 60 W, 10 min, six times).
The destruction of algal cells was confirmed by an optical microscopy (Axiovert 25, Carl Zeiss Japan, Tokyo, Japan). Then, residual solids were removed by the 0.22-μm membrane filter, and the filtrate was stored as AOMs from *M. aeruginosa* at −80°C.

**Molecular weight fractionation**

EOMs and AOMs were dialyzed against 0.05 M phosphate buffer including 0.15 M NaCl (pH 7.0) with a dialysis membrane (UC 8-32-500, Viskase Companie, Inc., Illinois, USA, molecular weight cut off: 14,000). The dialyzed samples were applied to the gel filtration chromatography for the molecular weight fractionation. The gel filtration chromatography was performed by the AKTA FPLC system (Amersham Bioscience Corp., NJ, USA) at room temperature (the room temperature is controlled at 23°C). Superdex 200 HR10/30 (molecular weight range: 10,000–600,000) was used as the gel filtration column. The buffer for this chromatography was 0.05 M phosphate buffer including 0.15 M NaCl (pH 7.0), and the flow rate was 0.5 ml/min. The absorbance at 280 nm was measured at the outlet port of the chromatography system.

**Affinity isolation of organic matters able to form complex with aluminium hydrate**

Figure 2 indicates the procedure for the affinity isolation of organic matters by the affinity chromatography. EOMs and AOMs were applied to the affinity column (HiTrap chelating, Amersham Bioscience Corp., NJ, USA) in which aluminium ion was immobilized as a ligand. Aluminium hydrate would be formed in the prepared affinity column, because the phosphate buffer of pH 7.2 was used for the equilibration of the affinity column. Affinity chromatography was performed by the AKTA FPLC as well as the gel filtration chromatography. The start buffer of the affinity chromatography was 20 mM phosphate buffer including 0.5 M NaCl (pH 7.2). The elution buffer was the same with the start buffer, but its pH was 3.5. The flow rate was set at 0.4 ml/min, and 1 ml each of affinity chromatographic

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**Figure 1** Classification of algal organic matters (AOMs)

**Figure 2** Procedure of the affinity chromatography for the isolation of AOMs able to form complex with aluminium hydrate
fraction was collected by a fraction collector (Frac-900, Amersham Bioscience Corp., NJ, USA). Affinity chromatographic fractions were then desalted by a dialysis against 10 mM NH$_4$HCO$_3$ (pH: 8.0) for at least 12 hours and EOMs and AOMs in these fractions were concentrated tenfold with a vacuum and centrifugal dehydrator (CVE-100, Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The samples concentrated were preserved at –80°C until further analysis.

**SDS-PAGE analysis**

Molecular weights of proteins in EOMs and AOMs were estimated with SDS-PAGE. A portion of sample was suspended in an equal volume of SDS buffer (50 mM Tris-HCl (pH: 8.0), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) and heated for 5 min at 95°C. Proteins were separated with a 10% polyacrylamide gel (3FE304, Biocraft Co., Ltd, Tokyo, Japan) in an electrophoretic cell (Amersham Bioscience Corp., NJ, USA) for 1.5 hours in a running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Then, the gels were stained by silver (Amersham Bioscience Corp., NJ, USA).

**Results and discussion**

**Molecular fractionation by gel filtration chromatography**

The growth curve of *M. aeruginosa* was depicted with the measurement of chlorophyll-a (Figure 3). Algae was harvested on days 4, 12, 17 and 25 of the cultivation. Figure 4 shows the profiles of the gel filtration chromatography for EOMs. EOMs included organic matters of around 10–20 kDa as shown in Figures 4(a) and (b). Large substances of around 40 kDa and more than 600 kDa appeared on days 17 and 25 (Figure 4(b)). These high-molecular
weight constituents of EOMs (more than 600 kDa) might be combined substances such as peptideglycan. The comparison of peak area and shape between Figures 4(a) and (b) implies that the amount and diversity of EOMs increased as the algal growth.

Figure 5 shows the comparison of gel filtration chromatographic profiles for EOMs and AOMs. AOMs consist of EOMs, IOMs and SOMs (Figure 1), because algal cells were destroyed by ultrasonic treatment. AOM constituents contained a large amount of high molecular weight compound (more than 600 kDa). The peak height for the organic matters of more than 600 kDa was about 18 times larger than that of EOMs (Figure 5(a)). In contrast, the chromatographic profiles of EOMs and AOMs at day 25 were very similar with each other (Figure 5(b)). These results indicated that IOMs and SOMs were already released by the cell death during the algal cultivation, and recovered as EOMs in the sample preparation.

Affinity isolation of AOMs and EOMs able to form complex with aluminium coagulant

Figure 6 shows the affinity chromatographic profiles of EOMs and AOMs. Organic matters which don’t have the affinity with aluminium were flown out from the column at the washing step. Then, organic matters able to form complex with aluminium were obtained at the elution step. Figure 6(a) shows the affinity chromatographic profiles for EOMs on days 5 and 12. The peaks at the elution step mean that organic matters able to form complex with aluminium were successfully obtained. In case of the EOMs on day 7, two peaks were observed at the elution step, which indicates that there is difference in the ability of forming complex between EOMs in these peaks.

On the other hand, Figure 6(b) shows the affinity chromatographic profiles for EOMs on days 17 and 25. The isolation of the organic matters able to form complex with aluminium hydrate was succeeded as well as the EOM samples. The peak height at the elution step in Figure 6(b) was apparently larger than that in Figure 6(a). It is considered that the amount of complex-forming organic matters increased as the algal growth.

Figure 6(c) shows the comparison of affinity chromatographic profiles between EOMs and AOMs on day 17. The profile for the EOMs is totally the same with that in the Figure 6(a). The apparent distinction between the peak heights at the elution step in Figure 6(c) means that the released organic matters by ultrasonic treatment contained a large amount of complex-forming organic matters. A similar result was obtained in the affinity chromatographic profiles for the EOMs and AOMs on day 25 (Figure 6(d)). As indicated in the result of gel chromatography (Figure 6(a)), AOMs on day 17 included a larger amount of high molecular weight organic matters than EOMs. Therefore, it is considered that these high
molecular substances might exhibit the ability to form complex with the aluminium hydrate in the affinity column.

**SDS-PAGE analysis**

Figure 7 shows the result of SDS-PAGE for the EOMs and the isolated substances by the affinity chromatography. It was confirmed that EOMs included a variety of proteins (lanes B to E). In contrast, the isolated proteins by the affinity with the aluminium hydrate had molecular weight of between 43 and 67 kDa (lanes F to I). These proteins were also detected in the complex-forming organic matters from AOMs (lanes J and K). These results indicate that some kinds of algal protein have the ability of adsorbing aluminium hydrate by the

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**Figure 6** Affinity chromatographic profiles for EOMs and AOMs from *M. aeruginosa* (NIES-91). (a) Profiles for EOMs on days 5 and 12, (b) profiles for EOMs on days 17 and 25, (c) the comparison between profiles for EOMs and AOMs on days 17, (d) The comparison between profiles for EOMs and AOMs on day 25

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**Figure 7** The result of SDS-PAGE for the EOMs and the complex-forming proteins in EOMs and AOMs from *M. aeruginosa* (NIES-91)
coordinate bond. If these AOMs were produced by the mass propagation of *M. aeruginosa* in the semi-closed water area, they might strongly bind coagulant and bring about the excess consumption of coagulant in the water treatment processes. The analysis of these algal proteins such as the determination of the N-terminus amino acid sequences will be conducted in the further study.

The pale color of bands for the complex-forming proteins (Lanes F to K in Figure 7) means that the concentrations of the complex-forming proteins in both EOMs and AOMs were low in this study. On the other hand, the results of the affinity chromatography showed that the amount of the complex-forming substances in AOMs were apparently larger than those in EOMs. These results imply that not only proteins but also other organic matters such as combined polysaccharides have the ability to form complex with aluminium coagulant, and might contribute to the coagulation inhibition in the drinking water treatment.

**Conclusions**

The conclusions derived from this study are as follows.

1) Extracellular organic matters (EOMs) from *Microcystis aeruginosa* (NIES-91) have molecular weight of between 10 and 20 kDa, around 40 kDa and more than 600 kDa. The amount and diversity of EOMs increased as the algal growth. Furthermore, AOMs from *M. aeruginosa* contained a large amount of high molecular weight compound (more than 600 kDa).

2) EOMs and AOMs able to form complex with aluminium coagulant were successfully isolated by the affinity chromatography. The complex-forming organic matters included proteins of 43–67 kDa.

3) The isolated algal proteins able to form complex with aluminium might have a high capacity of capturing coagulants and inhibit the coagulation in the drinking water treatment.

These findings suggested that there are specific AOMs inhibiting the coagulation in the water treatment processes. If the specific AOMs are determined, it will be possible to elucidate the inhibition mechanism on coagulation by AOMs and develop an innovative coagulant that can be used without any inhibitions of the coagulation.

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