Isolation and characterization of an extracellular polymer closely related to flocculation of activated sludge

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Abstract

The present study was conducted to obtain a bioflocculant from activated sludge by a simple procedure. An extracellular polymer mainly composed of protein was extracted by sonication from an activated sludge suspension. The polymer exhibited flocculating activity for kaolin clay, activated charcoal, and muddy water (when cations were present), and stimulated reflocculation of deflocculated sludge. Flocculating activity for kaolin clay was related to a concentration of the polymer below 10 ppm. The activity was significantly affected by pH since in basic buffer the activity was inhibited completely while in acidic buffer it was increased. When the polymer was digested with pronase, flocculating activity significantly decreased, although the activity was not affected by heat treatment at 100°C for 5 min. These results suggest that flocculating activity is mainly due to the portion of heat stable protein in the polymer. The results obtained in the present study suggest that the isolation of new biodegradable and economical flocculants from activated sludge is feasible.

Key words:

activated sludge, extracellular polymer, flocculating activity

Introduction

In the activated sludge process, extracellular polymers produced by floc-forming microorganisms in sludge play an important role in floc formation and in the adsorption of organic or inorganic pollutants. The extracellular polymers, which are extremely

viscous in solution, are mainly composed of polysaccharides, although proteins and nucleic acids from cell autolysis may be present (Stoveland and Lester, 1980; Lester, 1983).

Various flocculating agents involving organic and inorganic compounds are widely used in wastewater treatment, dredging, and fermentation industries. However, some of these substances such as inorganic compounds (e.g. heavy metals) or synthetic high molecular weight organic compounds (e.g. polyacrylamide) might cause secondary environmental pollution. From this viewpoint, biodegradable and nonhazardous flocculating agents obtained from natural sources are needed. Takagi and Kadowaki (1985a, b) obtained a flocculant that aggregated several suspended solids in aqueous solution from the culture filtrate of *Paecilomyces* sp.-1, and explained that this material was a polysaccharide composed of galactosamine. Kurane *et al.* (1986) extracted a flocculant from the culture filtrate of *Rhodococcus erythropolis*, and its aggregation activity was significantly increased by cations such as Ca^{2+} . Dermilim *et al.* (1999) also showed that an acidic polysaccharide produced by *Klebsiella* sp. possessed the ability to flocculate kaolin suspension in the presence of CaCl_2 . Bar-or and Shilo (1987) showed that a flocculant produced by *Phormidium* was a sulfated heteropolysaccharide to which fatty acids and proteins were bound.

Although many previous studies about bioflocculants have been conducted with pure cultures of microorganisms as previously mentioned, flocculants obtained from natural activated sludge have not been well investigated. If a strong flocculant could be obtained from natural activated sludge, it would be economical and it might bring about the effective use of excess sludge. The present study was conducted to obtain a bioflocculant from natural activated sludge. An extracellular polymer was extracted from an activated sludge suspension and was characterized because an extracellular polymer closely related to flocculation of activated sludge would be useful as a flocculating agent.

Materials and Methods

Activated sludge samples.

Activated sludge samples were collected from the aeration tank of Obihiro-south local domestic sewage treatment plant. Samples were stored at 4°C for subsequent experiments.

Extraction of extracellular polymer from activated sludge floc.

Six hundred milliliters of activated sludge was washed 5 times with distilled water, and

suspended in 900 ml of water. This activated sludge suspension was sonicated for 10 min with BRANSONIC 92 sonicator (45 kHz, 425 W) and was centrifuged at 8,500 rpm for 30 min. A crude extracellular polymer was obtained by dialysis of the supernatant against distilled water at 4°C for 72 h. The dialysate was then lyophilized and stored in a freezer. The lyophilized powder was subjected to gel filtration.

Gel filtration of extracellular polymer.

Gel filtration of the extracellular polymer was performed through a column (26×300 mm) of Bio-Gel P-100 previously equilibrated with 10 mM Tris (hydroxymethyl) aminomethane-hydrochloride (Tris-HCl) buffer of pH 8.0. Elution was done with the same buffer at a flow rate of 9 ml/h and 5 ml fractions were collected. The void volume of the column was determined by the elution volume of Blue Dextran (Pharmacia). The partially purified polymer obtained from Bio-Gel P-100 column chromatography was used throughout the present study.

Analysis of constituent neutral sugar in extracellular polymer.

Gas-liquid chromatographic (GLC) analysis of constituent neutral sugar monomers in the extracellular polymer was performed after conversion into their corresponding alditol acetates, using a glass column (0.3×200 cm) packed with 3% ECNSS-M at 180°C in a gas-liquid chromatograph (Hitachi Model 163). The detection and injection port temperatures were 270°C and 300°C, respectively. One milligram of the polymer was completely hydrolyzed with 0.5 ml of 0.5 N H₂SO₄ in 90% acetic acid at 80°C. After 16 h, 0.5 ml of distilled water was added and further allowed to stand at 80°C for 5 h. The alditol acetates were prepared by the reduction of the hydrolyzed sugars with sodium borohydride followed by acetylation with pyridine/acetic anhydride 1:1 (v/v) at room temperature overnight in a sealed test tube under N₂ gas.

Evaluation of flocculating activity of extracellular polymer.

Kaolin clay, activated charcoal, muddy water, silica gel (Wakogel B-5) and deflocculated sludge were used for the flocculating test. Muddy water was prepared as follows: 500 g of soil was washed 3 times with distilled water and suspended in 1,000 ml of water. The mixture was allowed to stand for 10 min and the supernatant was collected and used for the flocculating test. Deflocculated sludge was prepared by letting 75 ml of activated sludge to be suspended in 225 ml of distilled water followed by sonication for 3 min. The sludge suspension was centrifuged at 1,000 rpm for 5 min to remove the remaining floc. The supernatant was further centrifuged at 8,500 rpm for 30 min to collect deflocculated cells. The deflocculated cells were washed 3 times with distilled water. The polymer solution was added to these suspensions prepared in distilled water or the water

containing 1 μM MgCl_2 and 1 μM FeCl_2 at 2.5 ppm final concentration in a test tube. After standing the mixtures for 3 min, flocculating activity was estimated visually. To estimate the concentration of the polymer, 2 ml of kaolin clay suspension (5,000 ppm) was mixed with the polymer solution at final concentrations of zero to 12.5 ppm. After sufficient shaking, absorbance at 660 nm (A_{660}) was measured. The activity was expressed as the extent of flocculation calculated by the following formula.

$$\text{Extent of flocculation (\%)} = (A-B) \times 100/A$$

A: A_{660} of the total mixed liquor

B: A_{660} of the upper layer

Effect of pH on flocculating activity.

Kaolin clay and activated charcoal suspensions (5,000 ppm) prepared in buffers adjusted to pH 2.5~10.8 (pH 2.5~8.2, 0.1 M citrate-0.2 M Na_2PO_4 ; pH 9.3~10.8, 50 mM $\text{Na}_2\text{B}_4\text{O}_7$ -0.1M NaOH) were mixed with the polymer solution at a final concentration of 5 ppm to estimate the effect of pH. After 3 min, A_{660} was measured and the extent of flocculation was calculated.

Pronase digestion of extracellular polymer.

Forty milligrams of the extracellular polymer was dissolved in 40 ml of 10 mM Tris-HCl (pH 8.0), containing 0.03% NaN_3 and 0.0025% chloramphenicol. The mixture was treated with 2 mg pronase at 37°C for 72 h. The pronase-digested extracellular polymer was obtained by purification of this reaction mixture using Bio-Gel P-100 column chromatography. The effect of pronase digestion on flocculating activity was estimated with kaolin clay suspension as previously described.

Heat treatment of extracellular polymer.

The extracellular polymer was dissolved in distilled water at a concentration of 0.5 mg/ml and heated at 100°C for 5 min. The estimation of flocculating activity for kaolin clay was performed using the procedure described above.

Other analyses.

Total saccharide was determined using the anthrone- H_2SO_4 method (Ough, 1964) with a glucose/galactose 1:1 standard. Uronic acid was determined using the method of Blumenkrantz and Hansen (1973) with a glucuronic acid/galacturonic acid 1:1 standard. Hexosamine was determined by the method of Blix (1948) with a glucosamine standard. Phosphorus was determined using the molybdenum- H_2SO_4 method (Schinttger *et al.*, 1959). Protein was determined according to the method of Lowry *et al.* (1951) using a standard of bovine serum albumin. Nucleic acid and protein content in the gel filtration

eluate were determined by measuring the absorbance at 260 nm and 280 nm, respectively. All analyses were performed in triplicate.

Chemicals.

Bio-Gel P-100 was the product of Bio Rad Laboratories. Blue Dextran was the product of Pharmacia Fine Chemicals. Pronase was obtained from E. Merck, Dramstadt. ECNSS-M was obtained from Gas-Chro Kogyo Co. Tokyo. Silica gel (Wakogel B-5) was obtained from Wako Pure Chemical Industries. Other chemicals used in this study were of reagent grade.

Results and Discussion

Extraction and partial purification of extracellular polymer.

The floc was satisfactorily deflocculated within 10 min by sonication of the activated sludge suspension. Accompanying deflocculation, light yellow and extremely viscous substances were released into the aqueous phase. A total of 274.8 mg of crude extracellular polymer was obtained from 600 ml of activated sludge by sonication for 10 min. Figure 1A shows the elution profile of the crude extracellular polymer obtained using a Bio-Gel P-100 column. Through gel filtration on this column, both protein and saccharide were eluted in the void volume (70 ml) at the same position as a single peak. Therefore, the molecular weight was higher than 1×10^5 Da. A total of 110 mg of partially purified extracellular polymer was obtained from 274.8 mg of the crude polymer by this procedure.

Fig. 1. Bio-Gel P-100 column chromatogram of extracellular polymer extracted from activated sludge.
A; 20 mg of the crude extracellular polymer, B; half of the reaction mixture of pronase digestion. Symbols; ○; saccharide, △; absorbance at 260 nm, ▲; absorbance at 280 nm.

Components of extracellular polymer.

Components of the extracellular polymer were analyzed and the results are summarized in Table 1. These results showed that the polymer was a protein containing polysaccharide. One milligram of the partially purified polymer contained 309.7 μg of protein, 160.2 μg of neutral sugar, 58.5 μg of hexosamine, 30.8 μg of uronic acid and 3.8 μg of phosphorus. Constituent neutral sugar analysis showed that the neutral sugar was composed of rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose, and their approximate molar ratios were 3:3:1:1:5:10:10, respectively.

Table 1. Components of extracellular polymer extracted from activated sludge.

Components	Contents
	$\mu\text{g}/\text{mg}$
Protein	309.7
Neutral Sugar	160.2
Hexosamine	58.5
Uronic acid	30.8
Phosphorus	3.8

	Molar ratio
Rhamnose	0.35
Fucose	0.32
Arabinose	0.09
Xylose	0.10
Mannose	0.51
Galactose	0.98
Glucose	1.00

Flocculating activities of the extracellular polymer.

Flocculating activities of the extracellular polymer are summarized in Table 2. The polymer showed flocculating activity for kaolin clay and activated charcoal suspension, and stimulated reflocculation of deflocculated sludge in distilled water at a polymer concentration of 2.5 ppm. Deflocculated sludge also reflocculated without the polymer, suggesting that polymers remained on the surface of microbial cells after sonication. This results to adsorption of polymers onto microbial cells or immediate production of new polymers. When cations were present in the suspension, the polymer showed flocculating activity for muddy water. This shows that the ionic characteristics of extracellular polymers might have important roles in flocculating activity. Our results proved that deflocculation by sonication in a short time was reversible and microbial cells were not injured. The results also agreed with other investigations carried out by Banks and Walker (1977) and Hall (1981). Therefore sonication is an effective method for the extraction of extracellular polymers.

Table 2. Flocculating activity of extracellular polymer extracted from activated sludge.

Suspended solids	Flocculating activity	
	Cations —	Cations +
Kaolin clay	+	+
Activated charcoal	+	+
Muddy water	—	+
Silicic acid	—	—
Deflocculated sludge	±	+

Symbols: +; flocculation was observed, ±; flocculation was stimulated, -; flocculation was not observed

Figure 2 shows the effect of the concentration of the polymer on the flocculation of kaolin clay. When the polymer concentration was changed from zero to 12.5 ppm, the extent of flocculation showed positive correlations to the concentration of the polymer solution added, although a distinct effect was not observed when the polymer concentration was above 7.5 ppm. This suggests that an optimum concentration of the bioflocculant for

flocculating activity exists. Dermim *et al.* (1999) also reported that an excess dosage of the polymer isolated from *Klebsiella* sp. might cause the resuspension of kaolin particles.

Fig. 2. Effect of the concentration of extracellular polymer on flocculating activity.

—○— 0 ppm —●— 2.5 ppm —△— 5 ppm —▲— 7.5 ppm
 —□— 10 ppm —■— 12.5 ppm

Effect of pH on flocculating activity.

Table 3 shows the effect of pH on flocculating activities of the extracellular polymer for kaolin clay and activated charcoal. The buffer greatly affected the flocculating activity of the polymer, especially with kaolin clay. Compared with the control test, in which the polymer solution was not added, in the acidic region both kaolin clay and activated charcoal flocculated satisfactorily (the flocculation values at pH 2.5 of kaolin clay and activated charcoal were 79.6% and 67.1%, respectively). On the other hand, in the basic region flocculation did not occur. Flocculation of kaolin clay was also inhibited in the neutral region. This phenomenon is consistent with the results reported by Takagi and Kadowaki (1985a) for *Paecilomyces* sp.-1 and the results of Nam *et al.* (1996) for *Aspergillus* sp. JS-42. Sato and Ose (1980) also reported that viscous substances extracted from activated sludge by NaOH flocculated kaolin and *Escherichia coli* suspensions below pH 3. In the acidic region, both kaolin clay and activated charcoal precipitated slightly without the polymer.

Table 3. Effect of pH on the flocculating activity of extracellular polymer extracted from activated sludge.

pH	Extent of flocculation (%)			
	Kaolin clay		Activated charcoal	
	Control	With polymer	Control	With polymer
2.5	12.4	79.6	19.6	67.1
3.2	0.8	26.1	20.5	55.5
4.3	0.8	4.2	9.6	41.1
5.4	1.7	1.7	2.7	34.0
6.4	1.7	1.7	2.7	31.8
7.2	0.8	1.7	2.0	32.7
8.2	1.7	1.7	3.4	36.6
9.3	0.9	0.9	0.0	26.5
9.9	0.8	0.8	0.0	20.4
10.3	0.9	0.9	0.0	16.3
10.8	0.9	2.6	0.0	6.7

Pronase digestion and heat treatment of extracellular polymer.

Initially, 16.8 mg of pronase-digested extracellular polymer was obtained from 40 mg of the intact polymer. Figure 1B shows the elution profile of the pronase-digested polymer obtained using a Bio-Gel P-100 column. As compared with the intact polymer (Fig. 1A), the peak of the absorbance at 280 nm showing protein fell against that of saccharide. Components of the pronase-digested polymer are shown in Table 4. As compared with that of the intact polymer (Table 1), protein decreased from 309.7 µg/mg to 97.0 µg/mg. These results indicated that protein in the polymer was satisfactorily hydrolyzed by pronase digestion.

Table 4. Components of pronase-digested extracellular polymer extracted from activated sludge.

Components	Pronase-digested polymer
	µg/mg
Protein	97.0
Saccharide	225.9
Phosphorus	1.0

Figure 3 compares the flocculating activities of the pronase-digested polymer, the heat-treated polymer and the intact polymer for kaolin clay (each polymer concentration was 5 ppm). The flocculating activity of the pronase-digested polymer significantly decreased compared to the intact polymer. Heat treatment at 100°C for 5 min did not affect flocculating activity. Willén *et al.* (2003) reported that the protein had the biggest influence on the surface properties and flocculating ability of the sludge floc. Our results also support the idea that flocculating activity of the polymer is mainly brought about by

its protein portion and is not due to steric interactions, but to the electrostatic force of heat stable protein in the polymer.

Fig. 3. Effect of pronase-digestion and heat treatment of extracellular polymer on flocculating activity.

—○— Intact polymer —●— Pronase-digested polymer —△— Heated polymer —▲— Blank

The results obtained in the present study suggest that the isolation of new biodegradable and economical flocculants from activated sludge is feasible.

Conclusions

This study was conducted to obtain a new bioflocculant from activated sludge. The following conclusions were drawn from the results of the study.

1. An extracellular polymer closely related to flocculation of activated sludge was obtained from the sludge suspension by sonication. The polymer exhibited flocculating activity for several suspensions.
2. Pronase digestion and heat treatment of the polymer showed that the flocculating activity was mainly brought about by heat stable protein in the polymer.
3. The isolation of new biodegradable and economical flocculants from activated sludge is feasible based from the results obtained in the present study.

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