THE DEFLOCCULATING ENZYME PRODUCED BY A FLOC-FORMING BACTERIUM

YOSHITAKA TAGO AND KÔ AIDA

Institute of Applied Microbiology, The University of Tokyo, Tokyo

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The bacterium strain No. 12 isolated from a phenol-adapted activated sludge as a floc-forming bacterium was found to produce an exoenzyme which could deflocculate its own floc. As the floc of this bacterium was not deflocculated with EDTA and various enzymes such as cellulase, protease, and lysozyme, this exoenzyme was isolated and purified. The optimal pH of the enzyme for deflocculation was 3.8 to 5.0, and the optimal temperature 30°. The enzyme was unstable at a high temperature, and heating at 45° for 5 min resulted in complete loss of the activity. The activity of the enzyme was inhibited by a relatively low concentration of salts, acidic and basic amino acids, uronic acids, and amino sugars. Purified preparation of this enzyme released sugars from the No. 12 floc during deflocculation, though it could not hydrolyze cellulose, pectin, and ethylene glycol chitin. These results indicate that the No. 12 floc is composed of some exopolysaccharides other than those so far reported and deflocculated only by the enzyme produced by the bacterium itself.

Bacteria in activated sludge usually aggregate with each other (1) and this process is called floc-formation. The floc-formation is an integral part of activated sludge process. Sometimes normal activated sludge appears to break up into dispersed growth and the supernatant becomes extremely turbid (2). This phenomenon is called deflocculation. The deflocculation problems are closely related to the floc-formation mechanisms. Many investigators have attempted to elucidate the mechanisms of floc-formation in activated sludge process. A hypothesis that exopolymeric materials are responsible for the floc-forming properties of activated sludge was broadly supported (3-5).

Recently, FRIEDMAN *et al.* (6) and DEINEMA and ZEVENHUIZEN (7) reported that many bacteria were found to give flocculent growth due to the formation of cellulose fibrils and these flocs were deflocculated by the treatment with cellulase. KATO *et al.* (8) also reported the formation of flocs by certain bacteria and their deflocculation by cellulase, pectinase, or protease with no effect on bacterial growth.

Natural floc of activated sludge, however, could not entirely be deflocculated by treatment with the above enzymes or EDTA (9). Therefore, some other exopolymer unsusceptible to these enzymes may contribute to the floc-formation in natural activated sludge.

The present paper deals with a cellulase- and protease-unsusceptible floc-forming bacterium isolated from natural activated sludge and its production of an enzyme which shows the activity of deflocculating its own floc.

MATERIALS AND METHODS

Organism. The bacterium strain No. 12 isolated from a phenol-adapted activated sludge (10) was used throughout this work. This strain is a good floc-former, gram-negative, rods with rounded ends, $0.8\text{--}0.9 \times 1.0\text{--}2.5\text{ }\mu\text{m}$, occurring singly or in pairs, motile with a polar flagellum, and does not form endospores. Deflocculating enzyme was isolated from the culture fluid of this organism.

Cultivation conditions. The medium containing 15 g peptone, 1.0 g K_2HPO_4 , 0.5 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.02 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ per liter, pH 7.0, was used for the formation of the No. 12 floc and deflocculating enzyme. Ten ml of the 3-day culture of this strain No. 12 was inoculated into 1 liter of the same medium in a 5-liter flask and incubated on a rotary shaker at 20° for 5 days.

Preparation of No. 12 floc. The 5-day culture of this strain No. 12 was kept still for 30 min and the settled mass was washed several times with deionized cold water by decantation. This "native" floc was lyophilized and stocked in a desiccator.

Assay of deflocculating activity. The lyophilized floc was suspended in water, heated at 50° for 10 min, washed twice with deionized cold water by decantation, and resuspended in water to the concentration of 4 mg/ml. Deflocculation reactions were carried out with 0.5 ml of this suspension and 1 μg of the purified enzyme protein in 1.0 ml of acetate buffer (final concentration of 0.01 M), at 30° for 30 min. The reaction was stopped by the addition of 4.0 ml of 1 M NaCl solution and the residual floc was removed with a filter paper (Toppan Shoji Co., No. 2). Increase of turbidity in the filtrate was measured at 660 nm by a Shimadzu QR-50 photometer. A "unit" of deflocculating activity expresses the amount of the enzyme for a complete deflocculation of 0.2 mg of floc in 30 min.

Chemical analysis. Protein content was determined by the method of LOWRY *et al.* (11) or by measuring the absorbancy at 280 nm with a Shimadzu QR-50 photometer. Total sugar was determined by the method of DUBOIS *et al.* (12).

Chemicals. CM-Sephadex C-50 and Sephadex G-100 were the products of Pharmacia, Uppsala. Meicelase P was obtained from Meiji Seika Co., Kawasaki, Takamine cellulase from Miles Lab., Inc., Indiana, cellulase A-12-C from

Takeda Chem. Ind., Ltd., Tokyo, pronase E from Kaken Kagaku Co., Tokyo, trypsin from Sigma Co., Mo, and lysozyme from Seikagaku Kogyo Co., Tokyo. Other chemicals were of the purest grade commercially available.

RESULTS

Deflocculating tests of No. 12 floc by various enzymes

Susceptibility of the floc of the strain No. 12 to various commercial cellulases, proteases, lysozyme, EDTA, and the culture fluid of the strain No. 12 was examined. Cellulases used here were crude and showed the activities of cellulase, pectinase, hemicellulase, protease, *etc.* As shown in Table 1, all of the commercial enzymes

Table 1. Deflocculation tests on No. 12 floc.

Enzyme	Concn. (mg/ml)	Buffer	Reaction period (hr)	Reaction temperature (°C)	Defloc- culation
Meicelase P	10	0.1 M acetate pH 5.0	2	37	—
Takamine cellulase	10	"	2	37	—
Cellulase A-12-C	10	"	2	37	—
Pronase E	6	0.01 M Tris-HCl pH 8.0	1	35	—
Trypsin	6	"	1	35	—
Lysozyme	6	" (+0.01 M EDTA)	1	30	—
EDTA	5	deionized water	1	30	—
Dialyzed culture fluid of strain No. 12		0.01 M acetate pH 4.6	1	30	++

Deflocculation is expressed as complete deflocculation (++) and no deflocculation (—).

did not deflocculate the No. 12 floc, but the dialyzed culture fluid of strain No. 12 did. This fact indicates that the No. 12 floc is different in its hydrolase susceptibility from the flocs so far reported and that the strain No.12 produces the enzyme(s) capable of deflocculating its own floc.

Purification of the deflocculating enzyme

The culture fluid (10 liters) of the bacterium strain No. 12 was centrifugated at $10,000 \times g$ for 15 min to remove floc and free cells. To the supernatant fluid, $(\text{NH}_4)_2\text{SO}_4$ was added to 100% saturation with mechanical stirring and the mixture was allowed to stand at 4° overnight. The precipitate formed was collected by centrifugation at $10,000 \times g$ for 15 min, dissolved in a small amount of 0.1 M acetate buffer (pH 4.8), and dialyzed against the same buffer overnight at 4°. The dialyzed fluid was centrifuged at $10,000 \times g$ for 15 min to remove insoluble materials and the supernatant (157 ml) was then applied to a column (2.2×30 cm) of CM-Sephadex C-50 previously equilibrated with 0.1 M acetate buffer (pH 4.6).

The column was washed with 5 volumes of the acetate buffer and then elution began with a linear gradient of 0 to 0.5 M of NaCl in the acetate buffer. Fractions

Fig. 1. CM-Sephadex C-50 chromatography of the deflocculating enzyme of strain No. 12.

—○—, Deflocculating activity; ---x---, Absorbance at 280 nm.

Fig. 2. Sephadex G-100 chromatography of the deflocculating enzyme of strain No. 12.

—○—, Deflocculating activity; ---x---, Absorbance at 280 nm.

of 5 ml each were collected. As shown in Fig. 1, the deflocculating enzyme was separated from the greater part of other proteins mixed. The active fractions were combined, concentrated to 0.5 ml in a collodion bag, and applied to a column (1.0×100 cm) of Sephadex G-100 previously equilibrated with 0.02 M acetate buffer (pH 4.6). The deflocculating activity flowed out from the column a little after void volume, as shown in Fig. 2. The active fractions were combined, dialyzed against 0.1 M acetate buffer (pH 4.6), and then applied to a column (1.0×20 cm) of CM-Sephadex C-50. Elution was made with 100 ml of a linear gradient of 0 to 0.5 M NaCl in the acetate buffer and fractions of 2 ml each were collected. Figure 3 shows the elution pattern, where a single symmetrical peak of the deflocculating enzyme is observed. Table 2 summarizes these results. Specific activity of the deflocculating enzyme increased about 2,000-fold over the culture fluid.

Fig. 3. Second CM-Sephadex C-50 chromatography of the deflocculating enzyme of strain No. 12.

—○—, Deflocculating activity; ---×---, Absorbance at 280 nm.

Deflocculation of the No. 12 floc

Deflocculation of the No. 12 floc was examined with the purified enzyme preparation. Figure 4 shows the deflocculation of the lyophilized floc. The rate of deflocculation was proportional to the amount of the enzyme in the range of 1 to 6 units. Native floc was also deflocculated in a similar manner, without any damage on the growth activity. Release of cells from the lyophilized floc began after 10-min incubation and then proceeded linearly with incubation time. This delay of cell release may be explained by the hypothesis that the floc was first broken up by the enzyme to pieces, from which cells were released gradually.

Table 2. Purification of deflocculating enzyme from strain No. 12.

Procedure	Protein (mg)	Deflocculating (U)	activity (U/mg)	Yield (%)	Purification ratio
Culture fluid	9,024	22,500	2.4	100	1
CM-Sephadex C-50 (1st)	15.4	11,600	753.2	52	299
Sephadex G-100	1.8	6,800	3,777.8	30	1,496
CM-Sephadex C-50 (2nd)	1.1	5,500	5,000.0	24	2,071

Fig. 4. Deflocculation of No. 12 floc and release of sugar from the floc by the purified deflocculating enzyme.

Reaction mixture contained 36 mg of the lyophilized No. 12 floc and 40 units of deflocculating enzyme activity in 2 ml of 0.01 M acetate buffer (pH 4.6). —○—, OD₆₆₀ of the supernatant; —●—, Total sugar in the supernatant (as glucose).

Fig. 5. Effect of pH on the deflocculating enzyme activity.

Each assay mixture containing 5 units of deflocculating enzyme activity. —○—, Formate buffer, $\mu=0.01$; —●—, Acetate buffer, $\mu=0.01$; —△—, Phosphate buffer, $\mu=0.01$; —▲—, Tris buffer, $\mu=0.01$.

Concurrently with this cell release, an increase of sugar in the supernatant of the reaction mixture was observed. The amount of total sugar in the supernatant was proportional to the incubation time. Because of the lack of the activity to hydrolyze cellulose, pectin, and ethylene glycol chitin in the enzyme preparation used, the sugar released from the floc will not be the product of the hydrolysis of these compounds.

Properties of the purified deflocculating enzyme

Properties of the purified enzyme preparation were investigated with regard to the activity of deflocculating the lyophilized No. 12 floc. The optimal pH for deflocculation was found to be 3.8 to 5.0 (Fig. 5). The enzyme was unstable at temperatures higher than 35°, and heating at 45° for 5 min resulted in the complete loss of the activity (Fig. 6). As shown in Fig. 7, the optimal temperature was

Fig. 6. Effect of temperature on the deflocculating enzyme activity. Each assay mixture contained 5 units of deflocculating enzyme activity.

Fig. 7. Thermal stability of the purified deflocculating enzyme. Each enzyme preparation contained 5 units of deflocculating activity was heated for 5 min, cooled, and the remaining activity was measured.

Table 3. Effect of various salts and EDTA on deflocculating enzyme activity.

Salt added	Concentration (mM)	Deflocculating activity (%)
None	—	100
NaCl	5	15
KCl	5	44
MgCl ₂	2	4
CaCl ₂	2	7
(NH ₄) ₂ SO ₄	2	45
EDTA	2	20

Each assay mixture contained 5 units of deflocculating enzyme activity.

Table 4. Effect of amino acids on deflocculating enzyme activity.

Amino acid	Concn. (mM)	Inhibition	Amino acid	Concn. (mM)	Inhibition
L-Glycine	10	—	L-Methionine	10	—
L-Alanine	10	—	L-Phenylalanine	10	—
L-Valine	10	—	L-Proline	10	—
L-Leucine	10	—	L-Tryptophan	10	—
L-Isoleucine	10	—	L-Asparagine	10	+
				1	—
D,L-Serine	10	—	L-Histidine	10	+
				1	—
L-Threonine	10	—	L-Lysine	10	+
				1	—
L-Cysteine	10	—	L-Arginine	10	+
				1	—

Each assay mixture contained 5 units of deflocculating enzyme activity.

Table 5. Effect of various sugars on deflocculating enzyme activity.

Sugar	Concn. (mM)	Inhibition	Sugar	Concn. (mM)	Inhibition
Arabinose	10	—	Lactose	10	—
Xylose	10	—	Saccharose	10	—
Glucose	10	—	Maltose	10	—
Galactose	10	—	Raffinose	10	—
Mannose	10	—	Cellobiose	10	—
Fructose	10	—	Trehalose	10	—
Sorbose	10	—	Melibiose	10	—
Rhamnose	10	—	Glucosamine	10	+
				1	+
Fucose	10	—	Galactosamine	10	+
				1	—
N-Acetyl-glucosamine	10	—	Mannosamine	10	+
				1	—
N-Acetyl-galactosamine	10	—	Glucuronic acid	10	+
				1	—
N-Acetyl-mannosamine	10	—	Galacturonic acid	10	+
				1	—

Each assay mixture contained 5 units of deflocculating enzyme activity.

at 30°. The activity was effectively inhibited with 5 mM of NaCl or KCl, or 2 mM of MgCl₂, CaCl₂, or (NH₄)₂SO₄, as shown in Table 3. Irrespective of the kind of the buffer used, the activity was inhibited at the ionic strength (μ) higher than 0.01. Basic or acidic amino acids such as L-Histidine, L-Arginine, L-Lysine, and L-Asparagine inhibited the enzyme activity at the concentration of 10 mM, while no inhibition was observed at the concentration of 1 mM, as shown in Table 4. The activity was also inhibited by 10 mM of amino sugars and uronic acids, as shown in Table 5. Glucosamine effectively inhibited the activity at the concentration of 1 mM, but others did not.

DISCUSSION

The bacterium strain No. 12 is a good floc-former, and its culture consists of more than 90% of flocculated cells and a little of free cells (10). The present paper shows that the No. 12 floc was different from the flocs so far reported (6-9), being not deflocculated by treatment with various cellulases, proteases, lysozyme, and EDTA. The present experiments also showed that the strain No. 12 produced an exoenzyme which powerfully degrades its own floc. Purified preparation of this enzyme released sugars from the No. 12 floc during deflocculation, though it could not hydrolyze cellulose, pectin, or ethylene glycol chitin. These results indicate that the No. 12 floc is composed of some exopolysaccharides other than those so far reported and is deflocculated only by the enzyme produced by itself. The isolation and properties of these exopolysaccharides will be reported later.

The life of the No. 12 floc is determined by the activity of the polysaccharide-synthesizing enzyme and its own deflocculating enzyme. The activity of the latter enzyme was the highest in the pH range of 3.8 to 5.0 and less than 10% was observed at pH 7.0 which was the usual condition for floc cultivation. The No. 12 deflocculating enzyme was inhibited by relatively low concentration of salts, acidic and basic amino acids, uronic acids, and amino sugars.

Concerning the mechanism of deflocculation of activated sludge, the balance of the exopolymer-synthesizing activity of floc-forming microorganisms and exopolymer-degrading activity must be considered. Deflocculation will be the result of the hydrolysis of the exopolymers exceeding their synthesis. The exopolymer-degrading enzymes may be produced by floc-formers themselves or by other microorganisms in the activated sludge. The present report will give one example of the possibility that in some cases the deflocculation might occur due to the action of an exopolymer-degrading enzyme produced by the floc-former itself. Environmental or internal conditions affecting the synthesis or activity of the deflocculating enzyme must be further clarified.

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