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Inhibition of Soybean Cell Growth by the Adsorption of *Rhizobium japonicum*

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ABSTRACT

Soybean cells in suspension culture were inhibited in their growth by mixed culture with *Rhizobium japonicum* 5033. *Rhizobium* cells had the ability to adsorb on the surface of soybean cells. Cell envelope prepared from *Rhizobium* by sonic oscillation inhibited the growth of soybean cells. The growth-inhibiting activity of the cell envelope was depressed by β-glucosidase, KIO_4, urea, sodium cholate, and Triton X-100, but was stable on heating at 120 C for 15 minutes. Adsorption of the cell envelope on soybean cells was depressed by only β-glucosidase. The sodium cholate-soluble fraction of the cell envelope had the growth-inhibiting activity. Results in this paper suggest that components of the *Rhizobium* cell surface cause the inhibition of soybean cell growth after the adsorption of the *Rhizobium* cell to the soybean cell.

Under certain conditions, when cultured soybean cells are mixed with *Rhizobium* root nodule bacteria nitrogenase activity as determined by an acetylene reduction assay (1, 5, 8) is observed. We also reported a similar phenomenon (7), but in our experiment the reproducibility was very poor. The growth of soybean cells when mixed with *Rhizobium* was often rapidly inhibited and in that case, nitrogenase activity did not appear. Growth of soybean cells in the presence of *Rhizobium* seems to be a prerequisite for a development of a nitrogen fixation system (1, 7). To establish a nitrogen fixation system in the mixed culture of Rhizobia and soybean cells, it is important to know how and in what case the growth of soybean cells is inhibited by *Rhizobium*.

Animal cells in culture normally cease their movements and divisions in spite of being rich in nutrients if they are in contact with other cells (6). The cell surface seems to play an important role. Adsorption of a *Rhizobium* cell to a soybean cell (9) may also result in a series of intracellular reactions leading to growth inhibition of the soybean cell.

This paper shows that *Rhizobium japonicum* cells are able to adsorb on soybean cells and inhibit their growth, and that both cell envelope prepared from *Rhizobium* and the Na-cholate-solubilized fraction of the cell envelope are also able to inhibit the growth of soybean cells.

MATERIALS AND METHODS

Organisms and Cultivation. Soybean cell culture was derived from root explants of soybean (*Glycine max* cv. Kingen No. 1) after the manner described by Gamborg (4). The cells were maintained in 500-ml flasks containing 100 ml of Gamborg B-5 medium on a reciprocal shaker (130 oscillation/min, horizontal excursion of 7 cm) at 30 C in the dark. Aliquots of the culture (10 ml) were transferred to 100 ml of fresh medium every 10 days. We used cells which had been cultured for over 3 years and did not require any kinetin for growth. The number of soybean cells was estimated by direct cell count under a microscope. To determine a dry weight, cells were collected on a filter paper, washed with water, and dried at 90 C for 20 h. *R. japonicum* 5033 used for our experiments was obtained from the National Institute of Agricultural Sciences, Japan. This strain can nodulate soybean and fix atmospheric nitrogen. *Rhizobium* was maintained on yeast extract-mannitol agar slants (5) and then transferred to a liquid medium prior to use.

Examination for Ability of *Rhizobium* cells to Adsorb on Soybean Cells. *Rhizobium* cells exponentially growing in a yeast extract-mannitol liquid medium at 30 C were harvested by centrifugation (10,000*g*, 10 min) and washed three times with 0.15 M NaCl. Soybean cells harvested at various periods were washed gently with 0.15 M NaCl. The washed cells of soybean and *Rhizobium* were suspended in the same flask containing 40 ml of 50 mM K-phosphate buffer (pH 5.5) at a concentration of 1×10^6/ml and 5×10^7/ml, respectively. The mixed suspensions were incubated on the reciprocal shaker described above at 30 C. At regular intervals, soybean cells in the suspension were filtered off through a filter paper (Toyo Filter Co., No. 5A), and the O.D. of the filtrate was read at 550 nm.

Preparation of *Rhizobium* Cell Envelope. The washed *Rhizobium* cells were suspended in 0.15 M NaCl at a concentration of 1×10^{10}/ml and broken by sonic oscillation (19.5 kHz, 200 w, 5 min) in an ice bath. The suspension was centrifuged at 500*g* for 25 min to remove unbroken cells as a precipitate. Cell envelopes were collected from the supernatant by centrifugation (10,000*g*, 10 min), followed by washing four times with 0.15 M NaCl. The quantity of cell envelope was expressed as a cell number of *Rhizobium* used for preparation.

Extraction of Growth-inhibiting Factor(s). Cell envelopes were extracted with 2 mM Na-cholate for 60 min at 23 C followed by centrifugation at 10,000*g* for 10 min. The supernatant was dialyzed against 0.15 M NaCl overnight at 4 C. The precipitate was washed four times with 0.15 M NaCl.

RESULTS AND DISCUSSION

In a pure culture, soybean cells grew exponentially for about 7 days after transfer to a fresh medium, with doubling time of about 50 h and final cell density of 5×10^6/ml (Fig. 1). When mixed with *Rhizobium* cells at the concentration of 10^{-1} cells per soybean cell, soybean cells ceased their growth, and changed in color from bright yellow to reddish brown. The older the soybean culture when *Rhizobium* cells were added, the earlier the soybean cells entered a stationary phase. *Rhizobium* growth was not inhibited in the presence of soybean cells. The growth rate of *Rhizobium* cells in the mixed culture was lower than that in the yeast extract-mannitol medium: doubling time of 40 h and 15 h, respectively.

The growth inhibition of soybean cells by *Rhizobium* is thought to be ascribed to some of the following three causes: (*a*) competition for essential nutrients in a medium; (*b*) diffusive products of

Rhizobium; or (*c*) nondiffusive products localized on the cell surface of *Rhizobium*. The inhibition of growth happened within 1 or 2 days after mixing. Since the nutrient pool in soybean cells seems to be large and the growth rate of *Rhizobium* in the mixed culture was very low, growth inhibition by the first cause is not likely to occur.

To examine whether the medium had been changed to be toxic against soybean cells during the mixed culture, soybean cells were transferred to a medium in which a mixed culture had been grown for 5 days. Soybean cells grew in the conditioned medium as fast as in normal medium. This indicates that *Rhizobium* does not excrete the diffusive growth-inhibiting substance into the medium.

When *Rhizobium* cells were mixed with soybean cells in buffered solution, turbidity of the filtrate of the mixture decreased without any lag time (Fig. 2). This decreased turbidity is thought to be due to the adsorption of *Rhizobium* cells on the surface of soybean cells, since neither autolysis nor aggregation of *Rhizobium* cells was observed. Furthermore, microscopic observation revealed the adsorption. The pH of the medium of soybean cell culture varied within the range of 5 to 6. In the mixture buffered at pH 5 to 6 with K-phosphate, the degree of adsorption increased with the culture stage of soybean cells used. *Rhizobium* cells scarcely adsorbed on the surface of 3-day-old soybean cells. With 8-day-old cells, O.D. at 550 nm of the filtrate increased markedly after 30 min. The cells at stationary phase gave the same pattern of increase in O.D. The changes or modifications in soybean cell wall constituents with the culture stage (3, 13), though not detected yet, may cause the differences in *Rhizobium* cell adsorption properties of soybean cells. In subsequent experiments, soybean cell culture at the 6th day was used.

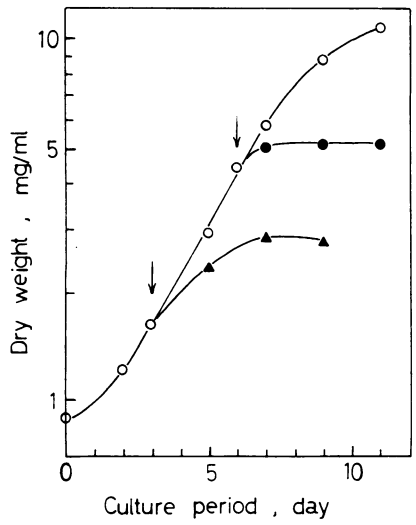

FIG. 1. Inhibition of growth of soybean cells by *Rhizobium* cells. Soybean cells in suspension culture were mixed with *R. japonicum* 5033 on the 3rd day (▲——▲) or the 6th day (●——●). Arrows indicate these mixing times respectively. Concentration of *Rhizobium* cells at mixing was 0.1 per soybean cell. (○——○): Growth curve of soybean cells in pure culture. Each point is mean of duplicate experiments.

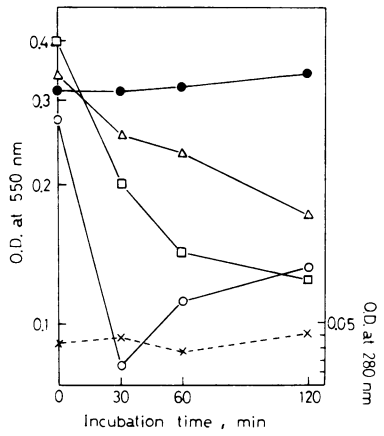

FIG. 2. Adsorption of *Rhizobium* cells on the surface of soybean cells. O.D. at 550 nm of the filtrate described under "Materials and Methods" was regarded as a quantity of *Rhizobium* cells which were not adsorbed on the surface of soybean cells. Soybean cells of 3 (●——●), 6 (△——△), 7 (□——□), and 8 (○——○) days old were suspended in 40 ml of 50 mM K-phosphate (pH 5.5), respectively. *Rhizobium* cells were added to these soybean cell suspensions at zero time. After centrifugation (5,000*g*, 10 min) of the filtrate of mixed suspension in 8-day-old soybean cells was used, O.D. of the supernatant was read at 280 nm (×---×). Each point is mean of duplicate experiments.

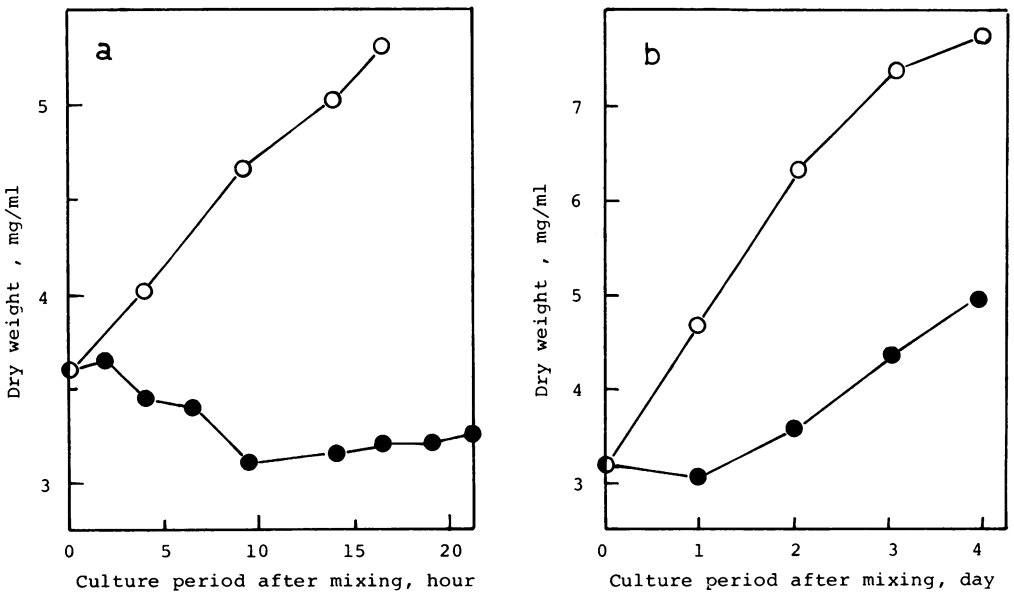

FIG. 3. Effect of *Rhizobium* cell envelopes on the growth of soybean cells. Cell envelopes prepared from *R. japonicum* 5033 were added to a soybean cell culture at the 6th day in concentration of 1×10^2/soybean cell. Results in (a) and (b) were obtained from separate flasks. Each point is average of four separate experiments. (○——○): Growth curve of soybean cells in a pure culture; (●——●): growth curve of soybean cells in a mixed culture.

Rhizobium cell envelopes temporarily inhibited the growth of soybean cells (Fig. 3b). They also adsorbed on soybean cell surface to the same degree as *Rhizobium* cells (data not shown). Heating at 120 C for 15 min had no effect on the growth-inhibiting activity of *Rhizobium* cell envelopes. Dry weight of soybean cells decreased rapidly with the lag of 2 h after cell envelopes were added, and then increased again. The color of soybean cells changed to reddish brown with the inhibition of growth. Soybean cells at 3 days old were not inhibited in their growth by *Rhizobium* cell envelopes. These results suggest that certain constituents of cell surface cause the growth inhibition after the adsorption of the *Rhizobium* cell to the soybean cell.

We use the following equation to express a degree of growth inhibition of soybean cells by the material added

$$GI = (Tm - Tp)/Tm$$

where GI is a degree of growth inhibition, Tm or Tp is a doubling time of soybean cells in mixed or pure culture, respectively. The doubling time of soybean cells was estimated by an increase in dry weight after 24 h.

Figure 4 shows a relation between concentration of cell envelope and growth-inhibiting activity. When a concentration of cell envelope was 1 to 1.3 per soybean cell, GI was 0.5.

The cell envelope of Gram-negative bacteria consists of "outer membrane," "inner (cytoplasmic) membrane," and a rigid layer of peptidoglycan interposed between the former two membranes (10). The outer membrane of cell envelope consists of lipopolysaccharides, proteins, and phospholipids (2, 11). To estimate the material or structure having a growth-inhibiting activity, *Rhizobium* cell envelopes were treated with various compounds. The growth-inhibiting activity of the *Rhizobium* cell envelope was depressed by β-glucosidase, 1 mM KIO_4, 0.5 M urea, 2 mM Na-cholate, and 1% (v,v) Triton X-100 (Table I). In contrast, pronase, trypsin, 1 N HCl, and 2% (v,v) mercaptoethanol had little effect on the growth-inhibiting activity under these conditions. Cell envelopes of *Rhizobium* also adsorbed onto soybean cells in a similar manner to *Rhizobium* cells. The adsorption of the cell envelope, however, was only depressed by β-glucosidase when treated as above. Therefore, the material or structure causing the growth inhibition of soybean cells may be different from those participating in the adsorption on soybean cells.

As shown in Table II, Na-cholate, a nontoxic detergent to soybean cells in a low concentration, solubilized the growth-inhibiting factor(s) from *Rhizobium* cell envelopes. The cholate supernatant was dialyzed overnight against 50 mM K-phosphate (pH 7.2) at 4 C. The dialyzed solution was applied to a Sephadex G-200 column (2 × 40 cm) which had been equilibrated with the same buffer as above. One peak of carbohydrate-containing material which had the growth-inhibiting activity was eluted from the column in the void volume. The fractions from the void peak were inhibited in their growth-inhibiting activity by 1 mM KIO_4, 0.5 M urea, and β-glucosidase. Heating at 120 C for 15 min had little effect on the activity. These results suggest that the growth-inhibiting factor(s) in this study is one of the macromolecular carbohydrate-containing constituents of the outer membrane. It is fully possible that the cell envelopes may be slightly contaminated with cytoplasmic materials during the process of preparation. Further experiments on the nature and location of the growth-inhibiting factor(s) are needed to explain an essential role of the cell surface in the inhibition of soybean cell growth by *Rhizobium*.

Soybean cells cultured for periods exceeding 1 year were invariably inhibited in their growth by *Rhizobium* cells as described in this report, and the acetylene reducing activity did not appear. However, soybean cells which had been cultured for 6 months were not necessarily inhibited in their growth by *Rhizobium* cells, in spite of adsorbing them. The uninhibited mixed culture occasionally showed acetylene-reducing activity (7). Various traits of cultured soybean cells are altered by conditions of the culture (13). But the conditions under which the susceptibility to *Rhizobium*

Table I. Effects of Various Treatments and Compounds on the Growth-inhibiting Activity of Rhizobium Cell Envelopes and Cholate-Sup

Each treatment was performed with 10^{10} cell envelopes per 1.0 ml, or with 1.0 ml Cholate-Sup prepared from 10^{10} cell envelopes. Pronase and trypsin were incubated in 50 mM phosphate buffer (pH 7.1), whereas β-glucosidase in 0.2 M acetate buffer (pH 5.0). These enzymes were obtained from Sigma Chemical Co. All enzyme treatments were done at 37 C for 1 hr, and others at 23 C for 1 hr. After treatments, cell envelopes were washed four times with 0.15 M NaCl, and then added to soybean cell culture at the concentration of 2 X 10^2 cell envelopes per soybean cell. One ml of Cholate-Sup was added to 100 ml soybean cell culture. The degree of growth inhibition of soybean cells was expressed as descrived in "Results and Discussion". Each value is the average of duplicate experiments.

Treatments	GI[1] of	
	CE[2]	Cholate-Sup[3]
Untreated	1.0	1.0
Heating, 120 C, 15 min	1.0	1.0
1 N HCl	0.9	0.8
1 N NaOH	0.6	0.6
2% Mercaptoethanol	0.8	0.7
1 mM KIO_4	0.5	0.4
Pronase (1 mg/10^{10}CE)	1.0	0.9
Trypsin (1 mg/10^{10}CE)	0.7	0.8
β-glucosidase (560 g/10^{10}CE)	0.4	0.3
1% Triton X-100	0.2	n.a.[4]
2 mM Na-cholate	0.1	n.a.
0.5 M Urea	0.4	0.3

[1] GI : Degree of growth inhibition
[2] CE : Rhizobium cell envelope
[3] Cholate-Sup ; See Table II
[4] n.a : not ascertained

Table II. Growth-inhibiting Activities of Na-cholate Soluble and Insoluble Fractions of Rhizobium Cell Envelopes

Additions	Conc. of CE (per soybean cell)	GI[3]
Untreated CE	3 X 10^2	1.0
Cholate-CE[1]	5 X 10^2	0.1
Cholate-Sup[2]	2 X 10^2	0.7
Sodium cholate	90 μM	0.0

[1] Cell envelope after treatment with 2 mM Na-cholate at 23 C for 1 hr.
[2] Supernatant obtained by centrifugation and dialysis from the suspension of cell envelope (1 X 10^{10}) treated with 1.0 ml Na-cholate at 23 C for 1 hr.
[3] Each value is the average of duplicate experiments.

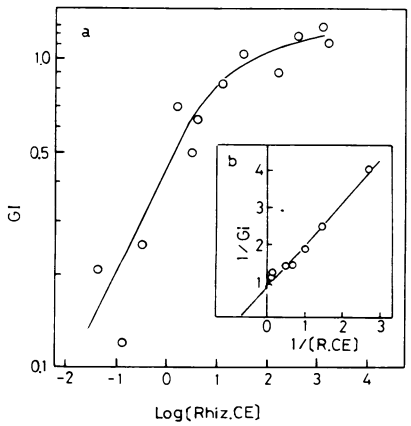

FIG. 4. Effect of concentration of cell envelopes on growth-inhibiting activity. [R.CE] indicates a quantity of *Rhizobium* cell envelopes. From the double reciprocal plot, the cell envelope concentration at which GI is half-maximal is about 1.3 per soybean cell.

cell disappears are entirely unclear. It is our ultimate purpose to obtain consistently a "healthy symbiotic" mixed culture with acetylene-reducing activity. For this purpose, it is necessary to elucidate the mechanism of the growth inhibition by *Rhizobium* cell. This paper indicates that the adsorption of *Rhizobium* cells onto soybean cells brought about the inhibition of soybean cell growth. It remains to be shown what reactions are raised by the adsorption before the growth inhibition occurs. It is of interest to know what components of soybean cells bind *Rhizobium* cells and how they transmit the signal of the binding into the soybean cells.

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