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Studies on Marine Bacteria Producing Lytic Enzymes-XI
Effect of Pressure Treatment on the Lytic and Autolytic Activities

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The effect of pressure treatment on lytic activity against *Micrococcus luteus* and autolytic activity was investigated using strain V 37 capable of producing lytic enzyme(s).

When the activity was assayed at ambient pressure at 40℃, prepressurization at 15,000 psi or less at 10 to 40℃ in the presence of substrate did not inactivate the lytic enzyme(s) of strain V 37.

No remarkable pressure effect at 15,000 psi or less was also observed on the autolytic activity of strain V 37 cells at 10, 20, 30 and 40℃, respectively.

Key words: lytic enzyme, autolysis, pressure effect, marine bacteria

In the previous papers (SUGAHARA *et al.* 1980, 1982, 1983, 1984), strain V 37 cells were shown to exhibit a considerable autolytic activity during growth from the early log to the middle log phases in the medium containing NaCl. However, no remarkable autolysis was detected in the stationary-phase cells. Strain V 37 also produced a significant amout of extracellular lytic enzyme(s) during the active growth from the middle log to the early stationary phases in the presence of NaCl. It is not yet clear about the properties of the purified lytic and autolytic enzymes from strain V 37 cells.

It was interesting to look for pressure effects in the enzyme reaction between *Micrococcus luteus* cells and lytic enzyme(s) of strain V 37, because *Micrococcus luteus* cells, as the substrate of the lytic enzyme(s), are very large particles and are also complicated.

However, little work has been reported on enzyme reactions using *Micrococcus luteus* cells as the substrate under moderate hydrostatic pressures (HOLYOKE and JOHNSON 1951).

The present study was designated to obtain preliminary information concerning mechanisms of autolysis of strain V 37 cells as well as lysis of *Micrococcus luteus* cells by lytic enzyme(s) of strain V 37. This paper describes the effect of pressure treatment at various pressures

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and temperatures on the lytic and autolytic activities at ambient pressure. The enzyme reaction under pressurization at different temperatures will be published elsewhere (in preparation).

Methods

Culture of strain V 37 capable of producing lytic enzyme(s)

The medium used in this study was composed of polypeptone (BBL), 10.0 g: Bacto-yeast extract (DIFCO), 5.0 g: NaCl, 0.5 mol (29.3 g) and 1,000 ml distilled water. The pH of the medium was adjusted to 7.0. The liquid medium was dispensed in 30 ml portions into 125 ml Erlenmeyer flasks. Sterilization was done by means of autoclaving at 121℃ for 15 min. The flask was inoculated with a loopful of strain V 37, and incubated at 30℃ on reciprocating shaker.

Assay of lytic activity

After 15—24 hours' cultivation of strain V 37, the culture fluids were centrifuged in order to remove the cells. The culture supernatant obtained was dialysed against distilled water at about 3℃ for 3 hr. The dialysate of the culture supernatant was used for the assay of bacteriolytic activity. Lysis of the bacterial suspension was estimated by following the change in optical density at 570 nm (OD_{570}) in a Bausch & Lomb Spectronic 20 spectrophotometer. The reaction mixture for the assay of lytic activity was as follows: heat-killed cell suspension of *Micrococcus luteus* (0.2 g dry cells/100 ml distilled water) 1.0 ml, 0.05 M Tris-HCl buffer (pH 7.0) 1.0 ml and dialysate of the culture supernatant 1.0 ml. The reaction mixture was subjected to various pressures and temperatures, and then incubated at different temperatures at ambient pressure for 10—80 min. Lytic activity (1 unit) was defined as a decrease of 0.01 in absorbance at 570 nm in 10 min. The decrease in turbidity of the cell suspension without enzyme solution was used as a blank.

Assay of autolytic activity

Afer 4—5.5 hours' cultivation (OD_{570}; about 0.30—0.35) of strain V 37 at 30℃, the cells were collected at about 3℃ by centrifugation. The harvested cells of strain V 37 were washed once in 0.05 M Tris-HCl buffer (pH 7.0) and suspended in the same solution to produce an absorbance of about 1.00 at 570 nm. The cell suspension (3.0 ml) was subjected to various pressures and temperatures for 1—120 min, and then incubated at different temperatures at ambient pressure for 5—75 min. The autolytic activity (1 unit) was defined as a decrease in absorbance of 0.01 at 570 nm in 5 min. The lysis of cells heated at 100℃ for 10 min was also determined as a blank.

Apparatus for increased hydrostatic pressure

The apparatus used in this study was described by ZOBELL and OPPENHEIMER (1950). A pressure cylinder completely filled with water was connected to a hand-operated hydraulic pump with 50% aqueous ethylene glycol as the hydraulic fluid. The tubes containing the

reaction mixture (enzyme and substrate in Tris-HCl buffer) were closed with No. 000 rubber stoppers, and kept in an ice bath until used. They were then dropped into the pressure cylinders previously equilibrated in the water bath. The cylinders were closed, pumped to pressure and replaced in the water bath. Less than 1 min is required for the entire operation. The pressure cylinders maintaining different hydrostatic pressures were incubated in a large water bath at different temperatures. After different periods of pressure treatment, the pressure was released, and then the reaction mixture in each tube was measured for enzyme activity at 1 atm. The reaction mixture was reincubated at different temperatures (usually at 40℃) at ambient pressure for an additional period of time. In the case of a 1 min pressure treatment, the tubes containing the reaction mixture were compressed to various pressures at different temperatures, and immediately the pressure was released for the assay of enzyme activity.

Results

Effect of pressure treatment on the lytic activity

The lytic enzyme(s) of strain V 37 was subjected to various pressures and temperatures in the presence of substrate. The decrease in OD_{570} of *Micrococcus luteus* cell suspension without lytic enzyme(s) (blank test value) was very small in the pressure-treated samples.

As shown in Fig. 1, the tubes containing lytic enzyme(s) and *Micrococcus luteus* cells were compressed to 12,500 psi (lb/in^2) or 15,000 psi at different temperatures. After pressure treatment for 1, 120 or 180 min, the tubes were decompressed for further incubation at 40℃. The optical density (OD_{570}) of the reaction mixture was measured at intervals. The rate of lysis of *Micrococcus luteus* cells exhibited no remarkable difference between the pressure-treated enzyme and the untreated enzyme (control).

After pressure treatment at 15 and 20℃ at 15,000 psi for 1 min, the tubes containing lytic enzyme(s) and substrate were incubated at 30℃ for 30 min at ambient pressure. As shown in Fig. 2—B, the decrease in turbidity was almost the same as control. No inactivation of the enzyme(s) was also observed by pressure treatment for 60, 120 or 180 min, when lytic activity was assayed at 40℃ (Fig. 2).

After pressure treatment at 10 and 20℃ at 15,000 psi for 1 min, the tubes containing the enzyme(s) and substrate were incubated at the same temperature as that used in pressure treatment for 30 min at ambient pressure. Although no significant change in OD_{570} was observed, lytic activity of the pressure-treated enzyme(s) decreased, as compared with the control (Fig.3, Table 2). However, no inhibitory effect was detected by pressure treatment at 15,000 psi at 10 and 20℃, respectively, when lytic activity was assayed at 40℃ at ambient pressure (Fig.3, Table 1).

Table 1 summarizes lytic activity at 40℃ after pressure treatment at various pressures and temperatures. Judging from the activity of lytic enzyme(s) after pressure treatment, which is expressed as the % of the activity of the untreated lytic enzyme(s), no significant

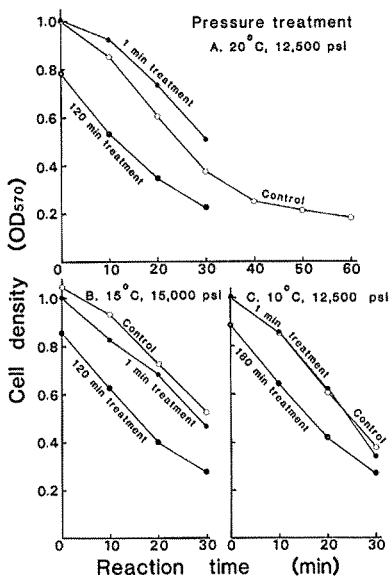

Fig. 1. Lysis of *Micrococcus luteus* cells by pressure-treated lytic enzyme(s) of strain V 37.
Open circle; Control (No pressure treatment): Pressure treatment; A. 20℃, 12,500 psi for 1 and 120 min. B. 15℃, 15,000 psi for 1 and 120 min. C. 10℃, 12,500 psi for 1 and 180 min: Reaction was carried out at 40 ℃ at ambient pressure after pressure treatment.

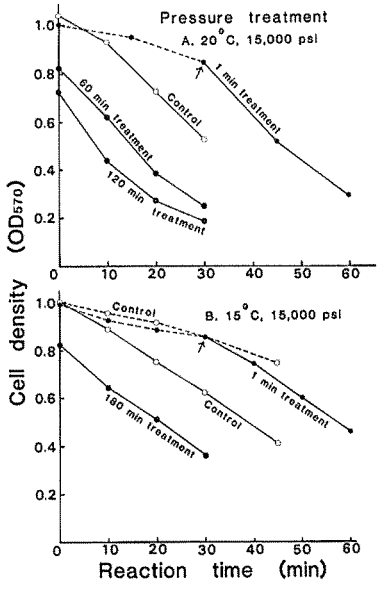

Fig. 2. Lysis of *Micrococcus luteus* cells by pressure-treated lytic enzyme(s) of strain V 37.
Open circle; Control (No pressure treatment): Pressure treatment; A. 20℃, 15,000 psi for 1, 60 and 120 min. B. 15℃, 15,000 psi for 1 and 180 min: Reaction was carried out at 40℃(solid lines) and 30℃(broken lines) at ambient pressure after pressure treatment. Each arrow indicates a change in reaction temperature from 30℃ to 40℃.

pressure effect was observed on the lytic activity of strain V 37 in the presence of substrate.

Effect of pressure treatment on the autolytic activity

Strain V 37 cells capable of producing lytic enzyme(s) were subjected to various pressures and temperatures. The tubes containing strain V 37 cells (cell density; OD_{570}, about 1.00) were compressed to 5,000, 10,000 and 15,000 psi at 10, 20, 30, and 40℃, respectively. After pressure treatment for 1 to 120 min, the tubes were decompressed for further incubation at different temperatures. Fig.4 shows the time course of the autolysis of strain V 37 cells after pressure treatment at 15,000 psi at 20, 30 and 40℃, respectively. As indicated in Tables 3—4 and Fig. 4, there was no remarkable pressure effect on the autolytic activity of strain V 37 cells at 10 to 40℃ tested.

Table 1. Effect of pressure treatment on the lytic activity of strain V 37.

Pressure treatment			Lytic activity (40℃, 1 atm)		
			No treatment	Pressure treatment	Percentage
10℃	15,000 psi	30 min	27.3	34.0	124.5
		1 min	28.6	(33.1)	(115.7)
	12,500 psi	180 min	27.0	28.0	103.7
		1 min	24.4	26.2	107.4
	10,000 psi	30 min	28.7	33.7	117.4
		1 min	28.0	(32.9)	(117.5)
	7,500 psi	180 min	27.6	27.6	100.0
		1 min	24.5	24.4	99.6
	5,000 psi	30 min	28.5	31.9	111.9
		1 min	28.9	(33.1)	(114.5)
15℃	15,000 psi	180 min	25.7	29.7	115.6
		120 min	29.9	34.0	113.7
		1 min	27.4	26.7	97.4
		1 min	25.9	(24.9)	(96.1)
	10,000 psi	180 min	25.7	31.2	121.4
		120 min	29.9	29.9	100.0
		1 min	29.8	(30.4)	(102.0)
	5,000 psi	180 min	25.7	30.8	119.8
		120 min	29.9	30.7	102.7
		1 min	29.9	(25.3)	(84.6)
20℃	15,000 psi	120 min	29.6	43.1	135.0
		60 min	28.8	32.8	113.9
		30 min	27.5	30.7	111.6
		1 min	27.5	(31.0)	(112.7)
		1 min	28.8	(32.8)	(113.9)
	12,500 psi	120 min	26.1	26.0	99.6
		1 min	24.4	19.6	80.3
	10,000 psi	120 min	29.8	39.0	130.9
		60 min	29.4	34.7	118.0
		1 min	25.6	(29.9)	(116.8)
	7,500 psi	120 min	23.9	23.9	100.0
		1 min	24.5	18.8	76.7
40℃	15,000 psi	1 min	25.7	23.4	91.1

Values in parentheses show lytic activity of the reaction mixture reincubated at 40℃ for an additional period of time after the preceding incubation at 10, 20 or 30℃ at ambient pressure for 30 min. The activity of the enzyme after pressure treatment is also expressed as the % of the activity of the untreated enzyme.

Pressure ; psi : pounds per square inch. 1 atm = 14.696 psi (lb/in^2). 15,000 psi (about 1,021 atm), 12,500 psi (about 851 atm), 10,000 psi (about 680 atm), 7,500 psi (about 510 atm) and 5,000 psi (about 340 atm).

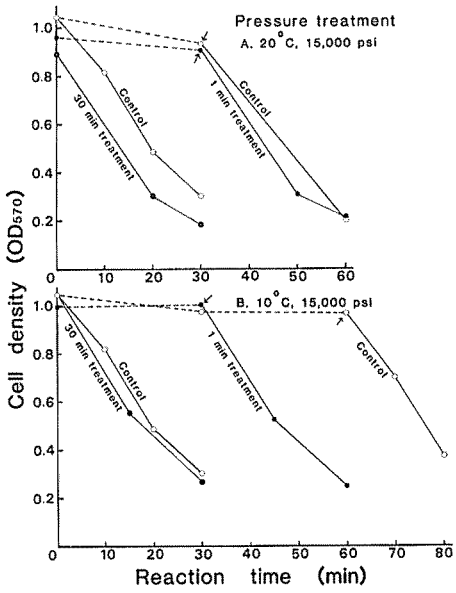

Fig. 3. Lysis of *Micrococcus luteus* cells by pressure-treated lytic enzyme(s) of strain V 37.
Open circle; Control (No pressure treatment): Pressure treatment; A. 20℃, 15,000 psi for 1 and 30 min. B. 10℃, 15,000 psi for 1 and 30 min: Reaction was carried out at 20℃ (broken lines for A), 10℃ (broken lines for B) and 40℃ (solid lines) at ambient pressure after pressure treatment. Each arrow indicates a change in reaction temperature from 20℃ (for A) or 10℃ (for B) to 40℃, respectively.

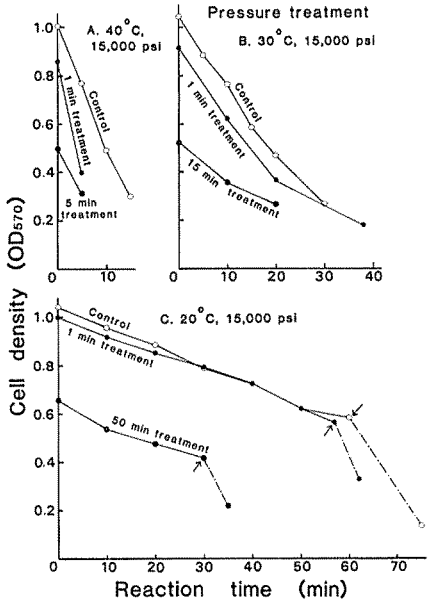

Fig. 4. Autolysis of pressure-treated cells of strain V 37.
Open circle; Control (No pressure treatment): Pressure treatment; A. 40℃, 15,000 psi for 1 and 5 min. B. 30℃, 15,000 psi for 1 and 15 min. C. 20℃, 15,000 psi for 1 and 50 min: Reaction was carried out at 40℃ (for A), 30℃ (for B) and 20℃ (for C), respectively, at ambient pressure after pressure treatment. Each arrow indicates a change in reaction temperature from 20℃ to 40℃.

Table 2. Effect of pressure treatment on the lytic activity of strain V 37.

Pressure treatment			Lytic activity (10, 20, 30 or 40℃, 1 atm)		
			No treatment	Pressure treatment	Percentage
10℃	10,000 psi	1 min	2.57	0.937	36.5
	5,000 psi	1 min	2.57	0.278	10.8
20℃	15,000 psi	1 min	3.85	2.01	52.2
	10,000 psi	1 min	3.85	2.81	73.0
	5,000 psi	1 min	3.85	4.92	127.8
30℃	15,000 psi	1 min	10.7	6.70	62.6
	10,000 psi	1 min	10.7	7.50	70.1
	5,000 psi	1 min	10.7	8.23	76.9
40℃	15,000 psi	1 min	25.7	23.4	91.1

Lytic activity was assayed at the same temperature as that used in pressure treatment, at ambient pressure.

Table 3. Effect of pressure treatment on the autolytic activity of strain V 37 cells.

Pressure treatment			Autolytic activity (40℃, 1 atm)		
			No treatment	Pressure treatment	Percentage
10℃	15,000 psi	120 min	40.1	53.1	132.4
		1 min	42.2	48.7	115.4
	10,000 psi	120 min	42.1	64.7	153.7
		1 min	40.6	51.8	127.6
	5,000 psi	120 min	39.8	49.1	123.4
		1 min	42.5	49.4	116.2
30℃	15,000 psi	30 min	34.2	32.5	95.0
		1 min	39.1	49.5	126.6
		1 min	43.1	42.8	99.3
	10,000 psi	30 min	34.0	30.2	88.8
		1 min	35.7	52.2	146.2
		1 min	44.2	48.0	108.6
	5,000 psi	30 min	33.7	37.2	110.4
		1 min	38.9	50.3	129.3
		1 min	44.2	49.6	112.2
40℃	15,000 psi	10 min	34.2	32.1	93.9
		5 min	21.4	12.3	57.5
		1 min	33.9	41.3	121.8
		1 min	39.6	44.4	112.1
		1 min	40.5	43.9	108.4
	10,000 psi	10 min	33.7	33.0	97.9
		5 min	18.7	17.2	92.0
		1 min	33.2	39.6	119.3
	5,000 psi	10 min	33.9	28.8	85.0
		5 min	18.3	18.6	101.6
		1 min	32.6	36.9	113.2

Table 4. Effect of pressure treatment on the autolytic activity of strain V 37 cells.

Pressure treatment			Autolytic activity (20, 30 or 40 ℃, 1 atm)		
			No treatment	Pressure treatment	Percentage
20℃	15,000 psi	50 min	7.25	6.77	93.4
		1 min	6.33	6.45	101.9
	10,000 psi	50 min	7.29	5.49	75.3
		1 min	7.23	6.64	91.8
	5,000 psi	50 min	7.29	5.29	72.6
		1 min	6.38	6.09	95.5
30℃	15,000 psi	15 min	12.7	10.4	81.9
		1 min	15.9	16.5	103.8
	10,000 psi	15 min	12.5	11.3	90.4
	5,000 psi	15 min	13.0	15.4	118.5
40℃	15,000 psi	10 min	34.2	32.1	93.9
		5 min	21.4	12.3	57.5
		1 min	33.9	41.3	121.8
		1 min	39.6	44.4	112.1
		1 min	40.5	43.9	108.4
	10,000 psi	10 min	33.7	33.0	97.9
		5 min	18.7	17.2	92.0
		1 min	33.2	39.6	119.3
	5,000 psi	10 min	33.9	28.8	85.0
		5 min	18.3	18.6	101.6
		1 min	32.6	36.9	113.2

Autolytic activity was assayed at the same temperature as that used in pressure treatment, at ambient pressure.

Discussion

Many reactions at moderate pressure (1 to about 1,000 atm) have been found to be frequently reversible when the pressure is released (JOHNSON *et al.* 1954).

However, MORITA and ZOBELL (1956) suggested that the succinic dehydrogenase system in *Escherichia coli* cells was inactivated by moderate pressures. According to them, the inactivating effects of pressure were most pronounced at temperatures either 40℃ or 8℃. The amount of inactivation increased progressively with the time of compression. Approximately half of the enzyme system was inactivated after 4 hr at 600 atm, and virtually all was irreversibly inactivated after 4 hr at 1,000 atm.

BERGER (1958) observed that no inactivation of phenylglycosidase of *Streptomyces qriseus* was detected at 35℃ at 1,000 atm for 20 min in the presence of substrate, while the enzyme activity decreased to the level of about 75% of the untreated enzyme activity by pressure treatment at 35℃ at 500 atm for 20 min in the absence of substrate. Pressure

treatment at 15℃ at 500 atm for 65 min in the absence of substrate did not cause a decrease in the enzyme activity. Increased pressure inhibited the rate of thermal denaturation. However, when substrate was absent, thermal (35−60℃) inactivation of the enzyme was accelerated by increased pressures.

Moderate pressures of 1,000 atm or less also retarded the heat-inactivation of α-amylase (SUZUKI and KITAMURA 1963, ZOBELL and HITTLE 1969).

ZOBELL and BUDGE (1965) described that subjecting washed cells of *Pseudomonas perfectomarinus* to pressure of 1,000 to 1,800 atm for a few minutes had no measurable effect on their ability to reduce nitrate after decompressing them to 1 atm, but holding such cells for several hours at high pressures inactivated their nitrate-reducing enzyme system. The rate of nitrate reduction by prepressurized cells at 10℃ at 1−1,800 atm for 24 hr decreased as the pressure increased. Enzyme inactivation by increased pressures was also more rapid at low temperature, 2℃, than at higher temperatures, 10, 21 and 30℃.

In this study, the lytic enzyme(s) of strain V 37 was pressurized to about 1,000 atm or less at 10−40℃ in the presence of substrate, *Micrococcus luteus* cells. When the pressure was released, the enzyme activity did not decrease (Figs.1−3, Table 1). A similar result was also obtained in the autolysis of strain V 37 cells (Fig.4, Table 3−4). The results of this study are consistent with the many reports to date (BERGER 1958, JOHNSON *et al.* 1954 etc.). However, the pressure effects on the thermal inactivation of lytic and autolytic enzymes in the absence or presence of substrate are not yet known. Further study will be necessary to demonstrate such pressure effects on the lytic and autolytic enzyme systems of strain V 37.

The present paper is a part of the studies carried out in the Department of Microbiology at the University of Hawaii.

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