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Studies on the Lytic Action of Extracellular Enzymes Produced by Marine Bacteria on Aquatic-Food-Poisoning Microorganisms— II Lytic Activity on the Cells and Cell Walls of Food-Putrefying Bacteria

Isao SUGAHARA, Koichiro HAYASHI, Toshio KIMURA and Nobuhiro TAKAYAMA

Faculty of Fisheries, Mie University

The study on the lytic action of extracellular enzyme(s) produced by strain V37 was carried out to obtain fundamental information with a final object of applying lytic enzymes to the preservation of food.

In the present study, strain V 37 exhibited a remarkable lytic action on the cell walls as well as on the living cells and heat-killed cells of strain HK 14 capable of putrefying kamaboko. However, the cell walls and living cells of *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Staphylococcus aureus* showed low or no susceptibility to lytic enzyme(s) of strain V 37.

Key words ; marine bacteria, lytic enzyme, putrefying bacteria, cell walls

A number of studies have been made on the application of egg white lysozyme as a preservative for food stuffs, such as cooked sausage, vienna sausage, salami sausage, non-packaged kamaboko, sake and milk (AKASHI 1969, 1970, 1971, AKASHI and OONO 1972, KATAOKA and NAKAE 1972, YAJIMA *et al.* 1968, 1971). However, little information concerning the application of bacteriolytic enzymes except egg white lysozyme is available.

In the previous papers (SUGAHARA *et al.* 1975, 1976), among bacterial strains isolated from coastal waters and capable of lysing *Micrococcus luteus* or *Vibrio parahaemolyticus*, strain V 37 exhibited high lytic activity on the heat-killed cells of strain HK 14 capable of putrefying kamaboko, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli*.

The present study was designated to obtain fundamental information concerning the application of lytic enzymes to food preservation.

Methods

Preparation of cell walls

The cells were collected at the end of the exponential phase or in the stationary phase of growth (OD_{570} of about 1.0) and washed once in 0.067 M phosphate buffer (pH 7.8). The washed cells were sonically treated for 30 minutes at 0° C with a sonicator (Ultrasonics W-375). The cell walls were isolated by differential centrifugation and treated with trypsin, as shown in Fig. 1. They were then washed twice in 0.067 M phosphate buffer (pH 7.8) and once in distilled water.

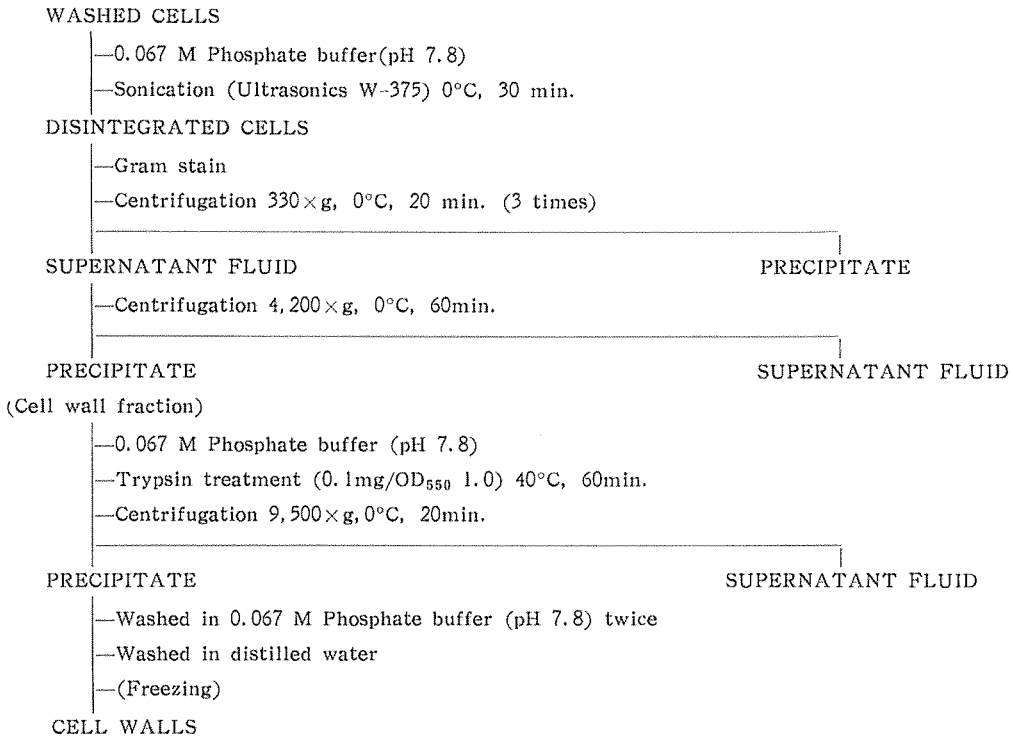

Fig. 1. Flow chart for the preparation of cell walls.

Measurements of lysis of cells and cell walls

Measurements of lysis of cells and cell walls were made turbidimetrically. For lysis of bacterial cells, the cells were grown for various intervals at 30 °C, harvested and washed twice in Tris-HCl buffer (pH 7.0) at 0 °C by centrifugation. The cell suspension was transferred to the incubation mixture containing culture filtrate of strain V 37 and Tris-HCl buffer (pH 7.0) so that the starting turbidity was at an optical density of about 1.0, and then incubated at 40 °C. At appropriate intervals, the turbidity of the reaction mixture was read at 570 nm in a Spectronic 20 spectrophotometer. The reaction mixture for the measurement of cell wall lysis was as follows :

cell wall suspension 1 ml, 0.05 M Tris-HCl buffer (pH 7.0) 1 ml and culture filtrate of strain V 37 1 ml. The assay was also done starting with an optical density (OD_{570}) of about 1.0 and carried out for 30 minutes at 40 °C.

Results and Discussion

Lytic action of the culture filtrate of strain V 37 on the heat-killed cells of food-contaminating bacteria

As shown in Table 1, the heat-killed cells of strain HK 14, *Salmonella enteritidis*, *Pseudomonas aeruginosa* and *Micrococcus luteus* were susceptible to lytic enzyme(s) of strain V 37, whereas lytic action on the heat-treated cells of *Vibrio parahaemolyticus* and *Staphylococcus aureus* was not considerable. The susceptibility to lysis by strain V 37 seemed to depend on the age of the culture.

Lytic action of the culture filtrate of strain V 37 on the living cells of food-contaminating bacteria

Strain V 37 exhibited a remarkable lytic action on the living cells of strain HK 14, *Vibrio parahaemolyticus* and *Micrococcus luteus* (Table 1). However, no lytic action was observed on the living cells of *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Staphylococcus aureus*.

Autolytic activity was low in the living cells of *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Salmonella enteritidis*, *Staphylococcus aureus* and *Micrococcus luteus* except strain HK 14.

AKASHI (1965, 1968a, 1968b) found that egg white lysozyme exhibited a high lytic activity on the several strains of food-contaminating or food-poisoning bacteria, such as *Bacillus subtilis*, *Vibrio parahaemolyticus*, *Escherichia coli* and *Proteus vulgaris*.

High susceptibility to lytic enzyme(s) of strain V 37 was observed in the heat-killed cells than in the living cells of *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Staphylococcus aureus* and *Micrococcus luteus*. This may be partly due to the denaturation of cell surface by heat-treatment.

Lytic action of the culture filtrate of strain V 37 on the cell walls of food-contaminating bacteria

Cell walls prepared from seven strains showed marked difference in their sensitivity to lytic action of strain V 37, as shown in Table 2. Strain V 37 exhibited high lytic activity on the cell walls of strain HK 14 and *Micrococcus luteus*, while the cell walls of *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Salmonella enteritidis* and *Staphylococcus aureus* showed low or no susceptibility to lytic enzyme(s) of strain V 37.

Strain V 37 showed no lytic activity on the cell walls of *Salmonella enteritidis* and *Staphylococcus aureus*, whereas the heat-killed cells were sensitive to lytic enzyme(s) produced by strain V 37. However, it is not obvious whether the

Table 1. Lytic activity of culture filtrate of strain V 37 on the living cells and heat-killed cells of food-contaminating bacteria.

	Culture age of harvested cells (OD_{570})	Autolytic activity	Lytic activity of culture filtrate of strain V 37	
			Living cells	Heat-killed cells
Pseudomonas aeruginosa ATCC 10145	0.41	0.084	0	0.536
	0.60	0.104	0	0.657
	0.795	0.123	0.090	0.395
	1.00	0.182	0	0.352
Vibrio parahaemolyticus	0.40	0	0.631	0.166
	0.60	0.167	0.438	0.232
	0.85	0.276	0.100	0.225
	0.95	0.567	0	0.308
	1.00	0.116	0.277	0.158
Salmonella enteritidis	0.39	0.193	0	0.643
	0.60	0.146	0	0.766
	0.80	0.139	0	0.732
	1.00	0.086	0.055	0.456
Staphylococcus aureus ATCC 6538	0.405	0.227	0	0.164
	0.60	0.207	0	0.230
	0.81	0.155	0	0.260
	0.98	0.175	0	0.309
Micrococcus luteus IFO 3333	0.398	0.222	0.899	1.048
	0.60	0.096	0.607	0.974
	0.83	0	0.017	0.546
	0.83	0	0.066	0.510
	1.00	0	0	0.401
	1.00	0	0	——
	1.00	0	0.246	0.616
	1.00	0.033	0.200	0.636
Strain HK 14 (*Bacillus* sp.)	0.45	0.626	0.110	0.184
	0.62	0.554	0.100	0.239
	0.68	0.610	0.270	0.391
	0.80	0.604	0.136	0.212
	0.82	0.699	0.240	0.393
	1.05	0.284	0.506	0.464
	1.06	0.192	0.497	0.497
Strain V 37	0.395	0.520	0.618	0.330
	0.60	0.464	0.394	0.276
	0.79	0.126	0.174	0.014
	0.96	0.426	0.193	0.150
	1.50	0.012	0.115	0.114

Table 2. Lytic action of lytic enzyme(s) of strain V 37, lysozyme and trypsin on the cell walls of food-contaminating bacteria.

	Culture age of harvested cells (OD_{570})	Autolysis of cell walls	Lytic activity (Decrease in turbidity) Culture filtrate of strain V 37	Lysozyme (1.0mg/ml)	Trypsin (0.1mg/ml)
Pseudomonas aeruginosa	1.486	0.009	0.158	0	0.117
Vibrio parahaemolyticus	1.523	0	0.225	0.106	0.315
Salmonella enteritidis	0.964	0.046	0	0	0.419
Staphylococcus aureus	1.271	0	0.007	0	0.006
Micrococcus luteus	1.022	0.013	0.984 1.020 0.804	0 0 0.041	—— —— 0.184
Strain HK 14 (*Bacillus* sp.)	1.131	0.058	0.567 0.560	0 0	0.216 ——
Strain V 37	1.155	0.038	0.361 0.407	0.757 0.785	0.018 ——

denaturation of cell walls occurred by the heat-treatment or whether lytic enzyme(s) could act on the uncertain sites of cell surface other than peptidoglycan of cell walls.

Most of cell walls prepared were lysozyme-resistant (Table 2). Gram-positive bacteria such as *Micrococcus luteus*, *Bacillus megaterium* and *Bacillus subtilis* were sensitive to lysozyme, whereas gram-negative rods, *Escherichia coli* and *Pseudomonas aeruginosa*, and gram-positive cocci, *Staphylococcus aureus* and *Streptococcus lactis*, were not lysed by lysozyme. Lysozyme-resistant strains among *Micrococcus luteus* and *Bacillus subtilis* have been also reported. BRUMFITT *et al.* (1958) demonstrated that the cell walls of the resistant strain of *Micrococcus lysodeikticus* (*luteus*) contained more than one-hundred-fold greater content of O-acetyl groups than the cell walls of the sensitive strain. The decrease in O-acetyl content corresponded with increase in lysozyme sensitivity. The presence of O-acetyl groups in the cell walls of lysozyme-resistant *Bacillus megaterium* and *Staphylococcus aureus* also contributed to their resistance to egg white lysozyme (GHUYSEN and STROMINGER 1963, YOSHIMOTO and TSURU 1974).

Lytic enzyme(s) produced by strain V 37 exhibited high activity on the cell walls as well as on the living cells and heat-killed cells of strain HK 14 capable of putrefying kamaboko (Tables 1 and 2).

On the other hand, strain HK 14 cell walls prepared using a French pressure cell (OHTAKE model 5501M) at 0-2,000 lbs/inch^2 were resistant to lytic enzyme(s) of strain V 37, lysozyme, zymolyase-5000 and trypsin (Table 3). It is not confirmed why cell walls prepared with two methods had different susceptibilities to lytic enzyme(s) of strain V 37. However, further fundamental studies will be needed in order to obtain available information and to apply lytic enzyme(s) of strain V 37 as a food preservative.

Table 3. Lytic action of lytic enzyme(s) of strain V 37, lysozyme, zymolyase-5000 and trypsin on the food-putrefying bacteria (strain HK 14).

		pH of reaction mixture	Lytic activity (Decrease in turbidity) Lytic enzyme(s) of strain V 37	Lysozyme (1.0mg/ml)	Zymolyase-5000 (0.4mg/ml)	Trypsin (0.1mg/ml)
Micrococcus luteus	Heat-killed cells	7.0	0.920	0.165	0.033	0.109
		4.5	0.518	0.282	0	0
Strain HK 14	Heat-killed cells	7.0	0.815	0	0.041	0.322
		4.5	0.150	0.203	0	0.072
	Living cells	7.0	0.658	0	0.045	0.243
		4.5	0.126	0.126	0	0.113
	Cell walls	7.0	0	0	0	0.029
		4.5	0	0	0	0

Cells were harvested after 48 hours of cultivation. For the preparation of cell walls, cells were disrupted in a French pressure cell (OHTAKE model 5501M) at 0-2,000 lbs/inch^2.

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