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Antibacterial Activity of Calcined Shell Calcium Prepared from Wild Surf Clam

Kikuo Oikawa,*[,a] Takashi Asada,[a] Kazuo Yamamoto,[a] Hiroyuki Wakabayashi,[a] Manabu Sasaki,[b] Makoto Sato,[c] and Junji Matsuda[d]

[a]Niigata College of Pharmacy, 5–13–2 Kamishinei-cho, Niigata, Niigata 950–2081, Japan, [b]Ion Corporation, Ltd., 2–6–14 Omachi, Aoba-ku, Sendai, Miyagi 980–0804, Japan, [c]Yamagata Three Top, Ltd., 1624–5 Kunugizuka, Nanyo, Yamagata 999–2221, Japan and, [d]Denka Seiken Co.,Ltd., 1–2–2 Minami Honcho, Gosen, Niigata 959–1695, Japan

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Recently, the calcined powder of oyster shells has been found to possess antibacterial activity, and has been considered for use as a bactericidal agent. In this study, calcined shell preparations were made from oysters, scallops, clams and roll shells, and their antibacterial activities were compared using total aerobic counts and *E. coli*. It was found that the calcined shell calcium from surf clams had the highest activity. Further comparison of activity was made between the calcined surf oyster preparation which was known to have antibacterial activity and the calcined surf clam preparation, and calcined surf clam calcium was found to have higher antibacterial activities against pathogenic *Escherichia coli* O157 : H7, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The antibacterial activity was due to the strong alkalinity of aqueous solutions of this calcined calcium preparation.

Key word —— calcined shell calcium, surf clam, *Escherichia coli* O157 : H7, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

INTRODUCTION

Preparations made by incinerating shells of surf clams, clams, oysters and scallops are called "calcined shell calcium" and it is permitted as a food additive in the United States as well as in Japan. Calcined shell calcium has been long used as a process aid in food manufacturing or as a nutritional supplement for calcium. Lately, Calcined shell calcium from oysters has been reported to have antibacterial activity, and its application in food is being studied.[1–5] In this study, we prepared calcined shell calcium preparations from shells of the shellfish for which the confirmation of their bactericidal activities have not been reported, and compared their antibacterial activities. Some preparations that had higher antibacterial activities than others were studied for their antibacterial activities against enterohaemorrhagic *Escherichia coli* O157 : H7, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Furthermore, we studied the practical applications of these products which have higher antibacterial activity. It was found that the calcined shell calcium prepared from surf clam (Scientific name: *Spisula sacchalinesis*) has strong antibacterial activities against *E. coli* O157 : H7 and *P. aeruginosa*.

*To whom correspondence should be addressed: Niigata College of Pharmacy, 5–13–2 Kamishinei-cho, Niigata 950–2081, Japan. Tel.: +81-25-268-1207 Fax: +81-25-268-1281; E-Mail: oikawa@niigata-pharm.ac.jp

MATERIALS AND METHODS

Preparation of Calcined Shell Calcium —— Shells of oysters, surf clams, scallops, turbos, clams and roll shells were baked separately for 6 h in a kiln at a final temperature of 1000℃ while stirring occasionally, then cooled in an inert gas atmosphere. The baked shells were then crushed in a mill to obtain particle diameter with a mean value of 5nm with most of the diameters of the particles falling into a relatively narrow range. This was called "calcined shell calcium".

Evaluation of Antibacterial Activities (N=5) —— Although there were some variations between experiments, the common methods for all the experiments are described below. Test organisms used were *E. coli* ATCC8739, *P. aeruginosa* ATCC9027, *S. aureus* ATCC6538 and *E. coli* O157 : H7. Each species of the

organisms was suspended in 0.9% physiological saline to obtain 10^6 cfus/ ml. A half ml to 1.0 ml of a cell suspension described above was spread on an agar plate (9 cm), cultured for 24 or 48 h at 37℃, and their bacterial colonies were counted. The test method for antibacterial activity was as follows:

1. A 10 ml sample was prepared in a 15 ml test tube.
2. One hundred μl of a 10^8 cfu/ml bacterial suspension was added to 10 ml of the sample to make 10^6 cfu/ml test suspension.
3. The test suspension was stirred well and incubated at room temperature for a certain time, then 200 μl of this test suspension was transferred into 1.8 ml physiological saline, then serial dilutions were made with physiological saline. Five hundred μl of each of the undiluted test solution and the serial dilutions were plated and incubated.

Total counts of microorganisms were determined using the standard agar medium of Japan Food Hygiene Act, *i.e.*, peptone – yeast extract agar (peptone 5.0 g, yeast extract 2.5 g, glucose 1.0 g, agar 15.0 g, and water 1 l; pH 6.8—7.7). Coliform counts were determined using deoxycholate agar.

Fluorescence X-Ray Analysis and X-Ray Analysis —— Fluorescence X-ray reflection analysis: Component analysis of calcined shell calcium was done using fluorescence X-ray analysis:

Equipment: Rigaku Industrial Corporation, Fully automated sequential X-ray spectrometer,
Model: RIX300; Rh-Tube: 3.0 kw
Current: 50 kv, 50 mA
Atmosphere: *vacuo*
Detector: PC/SC
Quantitative determination: FP method

X-ray diffraction analysis:

Equipment: Rigaku Corporation, X-ray Diffractometer Geigerflex,
Model: RAD-IIB; Cu-tube: emission 2 kw; Graphite monochrometer
Current: 40 kv, 30 mA
Detecter: SC
Filter: none
X-ray diffraction analysis: Rigaku Corporation, Data Analyzer for X-ray diffraction analysis

RESULTS AND DISCUSSION

Screening for Antibacterial Activities of Shell Powder and Calcined Shell Powder

In this experiment, we first prepared crushed samples of calcined and uncalcined shells of scallops, turbos, surf clams, clams and roll shells. Crushed preparation each of calcined and uncalcined shells were added to river water to make 0.1% w/v suspension and the total counts and coliform counts were determined after incubating the suspension for 10 min at room temperature, so that the bactericidal effect of different shell preparations were compared. The results of this experiment are shown in Table 1. Coliform counts obtained after incubating uncalcined shell preparations with river water were similar to each other, and showed no decrease from the

Table 1. Bactericidal Activities of Calcined and Uncalcined Shell Preparations of Several Shellfish Species

Sample (10 min after shell preparation was added to river water)	Total aerobic count (per ml; plate count agar)	Coliform (per ml; deoxycholate agar)
Calcined surf clam calcium	6	0
Uncalcined surf clam	3900	55
Calcined oyster calcium	620	0
Uncalcined oyster	3700	62
Calcined clam calcium	630	0
Uncalcined clam	3700	48
Calcined scallop calcium	720	0
Uncalcined scallop	4100	62
Calcined turbo calcium	720	0
Uncalcined turbo	3700	54
Calcined roll shell calcium	650	0
Uncalcined roll shell	3600	47
Control (river water without addition)	3100	56

original counts before the incubation or some samples showed slight increases. However, coliform counts in river water incubated with calcined shell preparations were not all detectable, thus calcined shell preparations showed bacterial effect. Total plate counts in river water samples included with uncalcined shell preparations showed some increase, whereas those incubated calcined shell preparations showed decreases, that is, the bactericidal effect of calcined shell preparations. Among different shell preparations, the calcined shell of surf clams showed the strongest bactericidal effect reducing the total count to less than 1/100 of the original.

A Comparison between the Antibacterial Activities of Calcined Surf Clam Calcium and Calcined Oyster Calcium

The calcined shell preparation of surf clams which showed the strongest bactericidal activity in the above preliminary experiment and that of oyster which had been previously reported to have bactericidal activity[4,5] were compared for their activities. Their antibacterial activities against *E. coli* were compared (Table 2). The *E. coli* count in the suspension that was incubated for one hour with 0.025% surf clam preparation was 4 cfu/ml, whereas that with 0.5% oyster preparation was 6.4×10^2 cfu/ml, thus the activity of the latter was much weaker. After 24 h incubation, the *E. coli* count in suspension included with 0.025% surf clam preparation was less than 2 cfu/ml, but the count in a 0.1% oyster preparation was 2.0×10^2 cfu/ml. Their antibacterial activities against *P. aeruginosa* and *S. aureus* were also tested (Table 3). After 1 h and 24 h incubations, the surf clam preparation showed quite strong bactericidal activities, but the oyster preparation decreased bacterial counts only a little and the activities were not comparable to those of surf clams. Activities against *S. aureus* were weaker than those against *E. coli* or *P. aeruginosa*, and while 1% surf clam preparation showed strong bactericidal activity, 0.1% preparation showed only weak activity. Oyster preparation was slightly active at 1% concentration, but the activity was weaker than that of the surf clams.

Table 2. Antibacterial Activity of Calcined Shell Preparations against *Escherichia coli*

Sample	Sample concentration	*E.coli* (cfu/ml)	
		After 1 h	After 24 h
Calcined oyster calcium	1 %	<20	<20
	0.5 %	6.4×10^2	<20
	0.1 %	2.5×10^5	2.0×10^2
	0.05%	2.5×10^5	1.2×10^5
Calcined surf clam calcium	1 %	<20	<20
	0.5 %	<20	<20
	0.1 %	<20	<20
	0.05 %	<20	<20
	0.025%	4	<2
	0.005%	1.6×10^5	4.0×10^4

Antibacterial Activity against *E. coli* O157 : H7

Antibacterial activities of different shell preparations are presented in Table 4. The calcined surf clam calcium preparation demonstrated strong bactericidal activities at 0.025% in either 1 h or 24 h, reducing *E. coli* counts to 200—250 cfu/ml in 1 h incubation, and to almost undetectable levels at 24 h incubation. Oyster preparation at 0.5%, on the other hand, showed little effect on bacterial count at 1 h, but at 24 h the count was less than 2.5 cfu/ml, showing bacter-

Table 3. Antibacterial Effects of Calcined Oyster Calcium and Calcined Surf Clam Calcium against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Sample	Sample concentration	*Staphylococcus aureus* (cfu/ml)		*Pseudomonas aeruginosa* (cfu/ml)	
		After 1 h	After 48 h	After 1 h	After 48 h
Calcined oyster calcium	1 %	40	2	<20	2
	0.1 %	4.0×10^5	5.2×10^3	2.0×10^2	<1
Calcined surf clam calcium	1 %	<2	<2	<2	<2
	0.1 %	1.9×10^2	60	<2	<2
	0.025%			20	<2
	0.005%			2.4×10^4	2.0×10^2

icidal activity. A 0.1%, oyster preparation showed only weak bactericidal activities.

Effect of Incubation Time on the Bactericidal Activities of 0.15% Calcined Surf Clam Calcium Preparation

Bactericidal activities with only short time exposure such as 5 min or 10 min are required in washing precut vegetables. We tested the bactericidal activities against time of a 0.15% preparation of calcined surf clam calcium against *E. coli* O157 : H7, at exposure periods of 0 (control), 1, 2, 3, 4, 5 and 10 min. The results are shown in Table 5. The *E. coli* counts decreased with time, and with 3 minutes or longer exposure time periods, the counts became lower than the detection limited of <10 cfu/ml, thus, quite a rapid bactericidal action was observed at this concentration. Against *E. coli* ATCC8739 and *P. aeruginosa* also, 0.15% preparation of calcined surf clam calcium showed very strong activities with their counts becoming less than 10 cfu/ml after 5 min exposure, respectively.

Bactericidal Activities of 0.1% Calcined Surf Clam Calcium Preparation in the Precut Vegetable Wash

In many supermarkets, bactericidal treatments for precut lettuce and other vegetables are done in the following manner: first, lettuce and other vegetables are washed with water, then soaked in sodium hypochlorite solution of approximately 100—200 ppm for 5 to 10 min and washed again with water, then cut to appropriate sizes. In this experiment, 100 ppm calcined surf clam calcium solution and 90 ppm sodium hypochlorite solution was used as the soaking solutions in cut lettuce preparation. The lettuce was soaked in these solutions and total bacterial counts and coliform counts were determined after 4, 7 and 24 h. The results are shown in Table 6. The surf clam calcium showed an equal activity in reducing total counts to sodium hypochlorite. Sodium hypochlorite was more effective at first in reducing coliform counts, but surf clam calcium showed longer lasting activity in suppressing coliforms.

Table 4. Antibacterial Effects of Calcined Oyster Calcium and Calcined Surf Clam Calcium against *Escherichia coli* O157 : H7

Sample	Sample concentration	*E. coli* O157 : H7 (cfu/ml)	
		After 1 h	After 24 h
Calcined	0.5 %	50	<2.5
oyster calcium	0.1 %	$>1.0\times10^5$	5.0×10^2
Calcined	0.05 %	<2.5	<2.5
surf clam calcium	0.025%	2.0×10^2	<2.5

Table 5. Effect of Incubation Time on Killing of *Escherichia coli* O157 : H7 in Calcined Surf Clam Calcium Solution

	E. coli O157 : H7 (cfu/ml)						
Calcined surf clam calcium 0.15%	0 time	After 1 min	After 2 min	After 3 min	After 4 min	After 5 min	After 10 min
	3.5×10^6	4.2×10^5	4.2×10^4	<10	<10	<10	<10

Table 6. Bacterial Counts in Cut Lettuce Soaked in Solutions of Antibacterial Agents

Culture medium	Antibacterial agent	Bacterial count (cfu/g)			
		0 time	After 4 h	After 7 h	After 24 h
SA medium (Aerobic count)	Untreated (count in cut lettuce)	6.6×10^5	3.9×10^6	7.3×10^5	7.4×10^4
	100ppm calcined surf clam calcium	2.7×10^4	1.2×10^5	2.2×10^4	3.5×10^3
	90ppm sodium hypochlorite	1.8×10^4	1.5×10^5	5.5×10^4	3.6×10^4
	Control (tap water)	1.5×10^5	8.5×10^6	2.7×10^5	5.8×10^5
DA medium (Coliforms)	Untreated (count in cut lettuce)	1.9×10^5	9.9×10^5	6.2×10^5	1.4×10^5
	100ppm calcined surf clam calcium	2.1×10^3	8.7×10^3	1.4×10^4	3.5×10^4
	90ppm sodium hypochlorite	6.4×10^2	1.1×10^5	4.1×10^4	1.6×10^5
	Control (tap water)	1.2×10^5	2.2×10^5	1.9×10^5	9.8×10^5

Mechanism of Antibacterial Action of Calcined Calcium

The results of fluorescent X-ray analysis are shown in Table 7. The main components of both calcined surf clam calcium and calcined oyster calcium preparations were calcium, but the calcium content of surf clam preparation was approximately 99% in weight, whereas that of oyster preparation was a little less, 97.5%. The crystal structures were analyzed using X-ray diffraction. The main component of uncalcined shell was $CaCO_3$ (calcite) in all of the shells, and by baking, it is converted to CaO (lime). In calcined oyster preparation, however, some $CaCO_3$ was found also. CaO reacts with water to form $Ca(OH)_2$ with heat emission:

$CaO+H_2O=Ca(OH)_2+15.2$ kcal.

Thus, the aqueous solution of CaO shows strong alkaline property due to the hydrolysis of $Ca(OH)_2$. Solutions of calcined surf clam calcium and calcined oyster calcium were prepared at the same concentrations as those used in the experiment for antimicrobial activity determination, and their pH and oxidation-reduction potential were determined. Also, the concentrations of $Ca(OH)_2$ were determined. The results are shown in Table 8. The pH of 0.025% surf clam preparation was weaker. Even at 1.0% concentration, oyster calcium preparation contained less calcium than surf clam preparation as was evident from the result of the fluorescence X-ray analysis and it also contained $CaCO_3$ demonstrated by the X-ray diffraction analysis, hence it appeared that the concentration of OH-ion generated by hydrolysis of CaO was lower and thus showing lower pH than those with the surf clam preparation (Fig. 1). The amount of calcium ion in 0.025% and 0.5% surf clam preparation and oyster preparation was determined using ion chromatography. As shown in Fig. 2, the solutions of surf clam preparation had higher Ca ion concentrations,

Table 7. Elements Found in Calcined Shell Calcium Preparations by Fluorescence X-Ray Analysis

	Calcined surf clam calcium wt%	Calcined oyster calcium wt%
Ca	98.90	97.50
Na	0.41	0.85
Sr	0.40	0.27
S	0.17	0.32
Cl	0.05	0.34
Si	0.05	0.27
K	0.02	0.04
Fe	0.00	0.14
Al	0.00	0.05
P	0.00	0.06

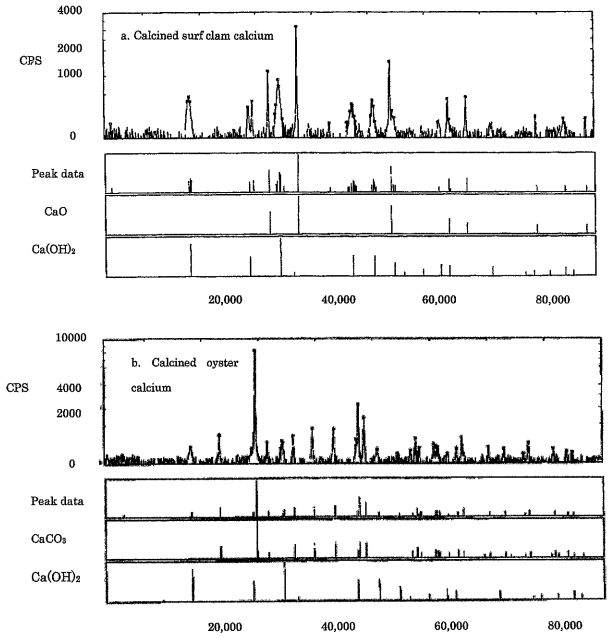

Fig. 1. X-Ray Diffraction Spectrum of Calcium and Calcined Surf Clam Calcium and Calcined Oyster Calcium

Table 8. The pH and Oxidation - Reduction Potential Values of Calcined Surf Clam Calcium, Calcined Oyster Calcium and NaOH Solutions

Sample concentration	Calcined surf clam calcium		Calcined oyster calcium		NaOH	
	pH	ORP mV	pH	ORP mV	pH	ORP mV
0.025%	12.02	55	10.42	145	12.06	88
0.05 %	12.29	40	10.89	120	12.30	70
0.1 %	12.60	30	11.31	78	12.58	40
0.5 %	12.75	22	11.55	51	13.07	18
1.0 %	12.85	15	11.82	20	13.26	5

*Temperature: 18℃ ; ORP values are not adjusted

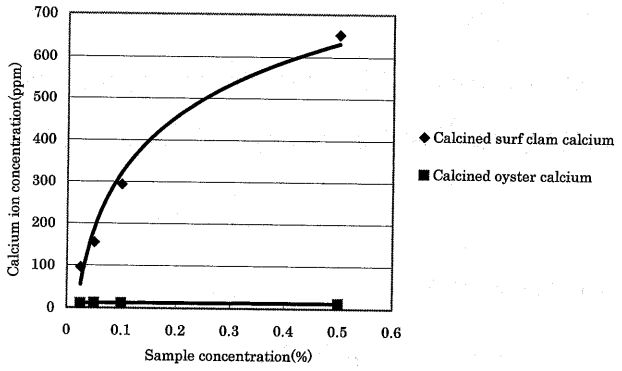

Fig. 2. Calcium Ion Concentration in Calcined Surf Clam Calcium and Calcined Oyster Calcium Solution of Different Strengths

Table 9. Bactericidal Effects of Calcined Surf Clam Calcium Preparation on *E. coli* O157 : H7 at Different pH Levels

pH	Survived *E. coli* (cfu/ml)
12.4[a)]	0
11.0[b)]	42
10.0[b)]	8.4×10^5
9.0[b)]	2.0×10^6
8.0[b)]	4.0×10^6
7.0[b)]	4.0×10^6
5.7[c)]	4.0×10^6

a) Calcined surf clam calcium 0.5%, pH was unadjusted. *b*) pH adjusted with HCl. *c*) Distilled water.

Table 10. Bactericidal Effects of Calcined Surf Clam Calcium Preparation and Sodium Hydroxide Solution on *E. coli* O157 : H7 and *Pseudomonas aeruginosa* at Different pH Levels

Sample	pH	*Escherichia coli* O157 : H7 (cfu/ml)	*Pseudomonas aeruginosa* (cfu/ml)
Calcined surf clam	6.5	1.0×10^5	8.0×10^4
calcium[a)]	11.8	<2.5	<2.5
Distilled water[b)]	6.3	1.0×10^5	8.0×10^4
NaOH	12.0	<1.25	<1.25

a) HCl was used to adjust pH of the calcined surf clam calcium solution to near neutral.
b) Distilled water was unadjusted for pH.

hence the oyster preparation appeared to generate lower OH-ion concentration in aqueous solution.

The Relationship between Bactericidal Efficacy and pH

To investigate the effect of pH on bactericidal activity of calcined surf clam calcium preparation, a 0.5% solution of surf clam preparation was made and its portions were adjusted to several different pH values between pH 7 and 12, then the enterohaemorrhagic *E. coli* O157 : H7 was added at a concentration of 10^6cfu/ml. The survived bacterial counts were determined after 20 h incubation (Table 9). At pH 11, the bactericidal property of alkali, suspensions of suenterohaemorrhagic *E. coli* O157 : H7 and *P. aeruginosa* were prepared and NaOH or calcined surf clam calcium preparation (0.5%) was added and pH adjusted to incubation (Table 10). Both NaOH solution that had been adjusted to pH 12 and the surf clam calcium preparation with pH 11.8 were found to have very strong bactericidal activities, and only less than 1.25 cfu/ml of either bacterium survived. Whereas, when the pH value was adjusted to near neutral, hardly any bactericidal activities were demonstrated by either NaOH solution or surf clam calcium solution.

As described above, the bactericidal effect of calcined surf clam calcium preparation appeared to be due to the strong alkalinity of its solutions. The effect is speculated to be exerted mainly by OH-ion, which penetrates the cell membrane and hydrolyzes the cytoplasm. This experiment demonstrated that the pathogenic *E. coli* O157 : H7 was killed at pH 12 or above.

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