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Microbial Growth Suppression in Food Using Calcinated Calcium

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Abstract
We observed that oyster-shell calcium treated electrically with ohmic heating delayed the spoilage of some foods. We carried out anti-microbial tests and application tests in foods. The calcium preparation and its major component, calcium hydroxide, showed almost the same minimum inhibitory concentrations (MICs) in the range of 0.07 to 0.1%, and no significant difference was observed between Gram-positive and negative bacteria. In the case of sodium and potassium hydroxide tested for comparison, MICs ranged from 0.09 to 0.15% and from 0.15 to 0.24%, respectively. Yeasts were inhibited at lower concentrations than bacteria. Viable bacterial cell number in food decreased by the addition of the calcium preparation. The growth of *Bacillus, Escherichia, Salomonella* and *Staphylococcus* inoculated to food was suppressed by the calcium preparation. Although this phenomenon was observed also for calcium hydroxide, the latter compound was not as effective as oyster-shell calcium. It was shown that the calcium preparation, used as a nutritional supplement could be applied to control the growth of microorganisms in food. Also, it was suggested that the preparation could be used in the alkaline range to keep the quality of food.

Discipline: Food/ Postharvest technology
Additional key words: antimicrobial, food poisoning, food preservation, food spoilage

Introduction

Food poisoning still occurs worldwide, and Japan also is concerned about the occurrence of large-scale food poisoning and increase of outbreaks of *Salmonella* diseases. Many countries experience infection with food-poisoning bacteria as well as other bacteria and microorganisms. Furthermore, in advanced countries as well as in developing countries, a large amount of food is discarded due to microorganism contamination[1,5,7,8]. On the other hand, the National Nutrition Survey in Japan reports every year that the calcium-intake is too low. Increase of calcium-intake is required for the prevention of osteoporosis and adult diseases[4]. For pregnant women, particularly who need calcium, various drugs and nutritious food have been recommended. However, consumers require foods that contain a large amount of calcium due to the awareness of the deficiency in calcium-intake[6].

In our studies on the preservation and improvement of food quality, we observed that oyster-shell calcium that had been sold for many years mainly as nutrition supplement could be used for food preservation purposes. We describe in this report a new method for suppressing the growth of microorganisms in foods.

Materials and methods

1) Reagents and samples

Calcium preparation: We used natural oyster-shells that were ground and whose pearl layer alone was treated electrically with ohmic heating (220 V, 60-100 A, 10-60 min). Then the shells were crushed into approximately 320 meshes (Kaiho Co., Ltd.). Calcium hydroxide, sodium hydroxide, potassium

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hydroxide (reagent grade) were purchased from Wako Pure Chemicals, Ltd.

Sample foods: We purchased the foods sold in Tsukuba city.

2) Chemical analysis in the calcium preparation

Ca content was measured by the potassium permanganate volumetric method. Fe content was measured by phenanthroline absorption spectroscopy. P content was measured by vanadium molybdate absorption spectroscopy, and the contents of other elements were determined by atomic absorption spectroscopy. The existing form of Ca in the calcium preparation was observed by X-ray diffractometry (Nihon Zetoc Co., Ltd.) under a voltage of 45 kV and current of 40 mA.

3) Examination of antimicrobial effects

(1) Microorganisms tested

Bacteria: *Bacillus subtilis* IFO 13722, *Bacillus subtilis* PCI 219, *Staphylococcus aureus* IFO 13276, *Staphylococcus aureus* JCM 2413, *Escherichia coli* JCM 1649, *Salmonella enteritidis* ATCC 1891, *Salmonella typhimurium* ATCC 14028.

Yeasts: *Debaryomyces hansenii* NFRI-67-40A, *Hansenula anomala* NFRI-150-40A, *Saccharomyces cerevisiae* NFRI-89-25A, *Torulaspora delbrueckii* NFRI-154-CA, *Candida tropicalis* NFRI-118-40A.

(2) Measurement of minimum inhibitory concentration (MIC)

As culture media, we used nutrient broth for bacteria and YM broth for yeasts. For preventing a reaction of the calcium preparations with the culture media ingredients during the process of sterilization by autoclave, we separately sterilized them and prepared a broth containing the calcium preparations at various concentrations by mixing aseptically. We inoculated 0.02 ml of the microorganism suspension (approximately 1,000 cells) into 5 ml of the broth and cultured it with shaking at 30°C for 48 h. We measured the changes in turbidity at 660 nm to observe the growth of microorganisms. We determined the minimum concentration, at which the turbidity did not change, as the MIC. For comparison we also observed the antibacterial effect of calcium hydroxide, sodium hydroxide and potassium hydroxide.

(3) Treatment and storage test of foods

We added the calcium preparation directly into sampled foods, with thorough mixing. In other cases, we dissolved or suspended the calcium preparation in tap water, and then immersed the foods for a certain period of time. We compared the changes in viable microbial cell number, etc. with those when foods were treated with calcium hydroxide. We measured the increase in viable bacterial cell number in sample foods from time to time. The storage tests were performed at 4, 10, 25 and 30°C.

(4) Measurement of viable bacterial cell number

The samples were pretreated as follows: the samples were aseptically homogenized for inspection by using a mixer, 10 g was put into a plastic bag with a filter for stomaching blender (P-type, Gunze Industry), 90 ml of sterilized physiological saline was added, treatment with a stomaching blender (Labblender 400, Gunze Industry) was applied for 1 min, and the suspension was prepared after filtration as a sample for bacterial examination. We diluted the solutions 10 times with sterilized physiological saline and determined the viable bacterial cell number by the plating method using ordinary agar media.

Results

1) Analytical results of the calcium preparation

As shown in Table 1, Ca content of the calcium preparation was 60%. Mg content was about 0.5%. The amount of other elements such as Fe, P, Sr, Ni, K, Na, Mn, Zn, Ag, Cu was very small. Although metal ions like Ag ion or Cu ion exhibit antimicrobial effects, the contents of Ag and Cu were so small, 6.3 and 5.9 mg/kg, respectively that they could not account for the antimicrobial effects. The existing form of Ca in the calcium preparation was observed by X-ray diffractometry. Fig. 1 shows the analytical results. The spectrum of the calcium preparation showed strong peaks of calcium oxide, calcium hydroxide, and low peaks of calcium carbonate, suggesting that most of the calcium in the calcium preparation was present in the form of calcium oxide or calcium hydroxide. The amount of calcium carbonate, the main component of oyster-shell before electrical treatment, was very small.

Table 1. Elementary analysis of calcium preparation

Element	Content (%)	Element	Content (mg/kg)
Ca	60	Ni	28
Mg	0.48	K	19
Fe	0.038	Na	17
P	0.024	Mn	17
Sr	0.020	Zn	6.8
		Ag	6.3
		Cu	5.9

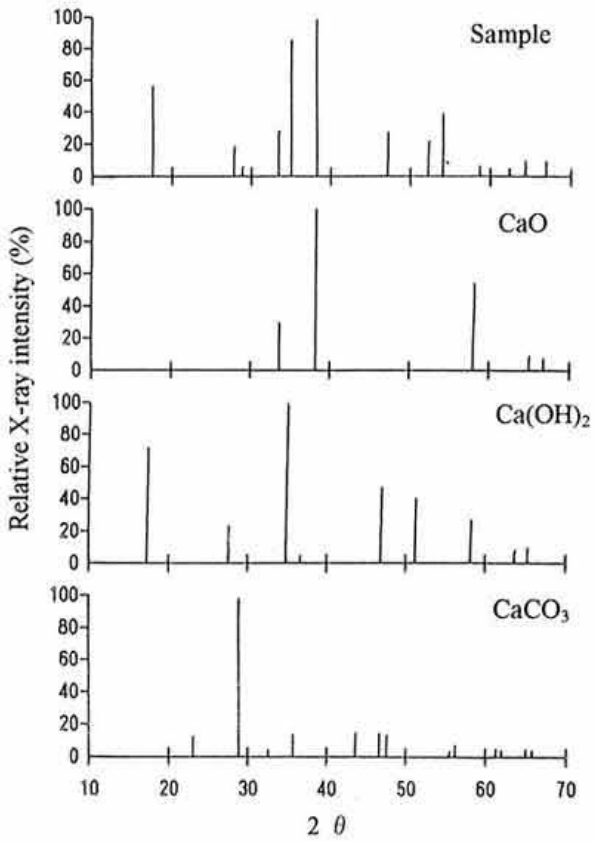

Fig. 1. X-ray diffractometry of calcium preparation

2) Antimicrobial effects of the calcium preparation

The antibacterial effects of the calcium preparation in liquid media are shown in Table 2. The MICs of the calcium preparation ranged from 0.07 to 0.1% with pH 9.3–9.8. There was no significant difference in the antibacterial effect between Gram-positive and negative bacteria, as in the case of calcium hydroxide. On the other hand, the MICs of sodium hydroxide and potassium hydroxide were in the range of 0.09–0.15% and 0.15–0.24%, respectively, with pH 9.8–10.7, which required higher concentrations and pHs. Table 3 shows the antimicrobial effect on yeasts. The MICs of the calcium preparation were in the range of 0.05–0.08%, values slightly lower than those of bacteria. For yeasts also, the MICs of the calcium preparation and calcium hydroxide were almost the same, and the values were slightly lower than those of sodium and potassium hydroxide. Then, in the measurement of the viable microbial cell number at the MICs, the values decreased more by the addition of the calcium preparation than calcium hydroxide. Therefore, although most of the antimicrobial effect of the calcium preparation was attributed to the major component, calcium hydroxide, minor minerals, were also more or less involved in the antibacterial effect. As a result, it was assumed that the antimicrobial effect was due to combined effects.

3) Effects of the calcium preparation on food preservation

(1) Direct addition tests

Fig. 2 shows the viable bacterial cell number in mashed potatoes to which the calcium preparation was added. Although the increase in the viable

Table 2. Minimum inhibitory concentration of calcium preparation for bacteria

Bacteria	Minimum inhibitory concentration (%)			
	Sample	$Ca(OH)_2$	NaOH	KOH
Bacillus subtilis IFO 13722	0.07 (9.3)	0.07 (9.1)	0.12 (10.3)	0.18 (10.0)
Bacillus subtilis PCI 219	0.07 (9.3)	0.07 (9.1)	0.09 (9.8)	0.15 (9.8)
Staphylococcus aureus IFO 13276	0.10 (9.8)	0.10 (9.8)	0.15 (10.7)	0.24 (10.7)
Staphylococcis aureus JCM 2413	0.08 (9.5)	0.10 (9.8)	0.15 (10.7)	0.24 (10.7)
Escherichia coli JCM 1649	0.08 (9.5)	0.09 (9.8)	0.12 (10.3)	0.18 (10.0)
Salmonella enteritidis ATCC 1891	0.10 (9.8)	0.10 (9.8)	0.15 (10.7)	0.24 (10.7)
Salmonella typhimurium ATCC 14028	0.10 (9.8)	0.10 (9.8)	0.15 (10.7)	0.18 (10.0)

( ): pH.

Table 3. Minimum inhibitory concentration of calcium preparation for yeasts

Yeasts	Minimum inhibitory concentration (%)			
	Sample	$Ca(OH)_2$	NaOH	KOH
Debaryomayces hansenii 67-40A	0.05 (9.0)	0.06 (8.9)	0.07 (9.4)	0.10 (9.3)
Hansenula anamala 150-40A	0.07 (9.3)	0.07 (9.1)	0.09 (9.8)	0.15 (9.8)
Saccharomyces cerevisiae 89-25A	0.05 (9.0)	0.05 (8.8)	0.06 (9.2)	0.10 (9.3)
Torulaspora delbrueckii 254-CA	0.05 (9.0)	0.05 (8.9)	0.06 (9.4)	0.10 (9.3)
Candida tropicalis 119-40A	0.08 (9.5)	0.09 (9.5)	0.15 (10.4)	0.24 (10.7)

( ): pH.

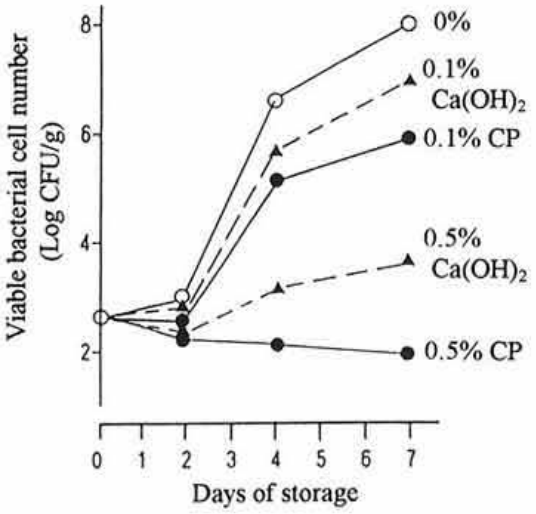

Fig. 2. Effect of calcium preparation (CP) on the number of bacteria in mashed potatoes
Storage temperature: 10°C.

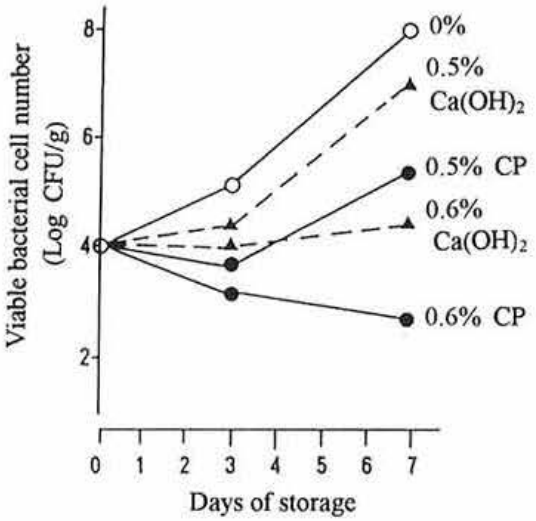

Fig. 3. Effect of calcium preparation (CP) on the number of bacteria in potato salad
Storage temperature: 10°C.

bacterial cell number in the sample without addition was observed, as shown in Fig. 2, the addition of 0.1% calcium preparation delayed the increase in the bacterial cell number. Addition of 0.5% calcium preparation completely inhibited bacterial growth. The calcium preparation showed a more pronounced antibacterial effect than calcium hydroxide. Then, we investigated the antibacterial effect of the calcium preparation on potato salad obtained from the market as a sample (Fig. 3). The components of potato salad we purchased were potato, carrot, onion, cucumber, mayonnaise, seasonings and pH preparations. The pH was adjusted to 5.0. The increase of the viable bacterial cell number observed for a 0.5% addition unlike in mashed potatoes was attributed to the pH preparations. Therefore we assumed that a quality-keeping effect could be achieved with a small amount of calcium preparation unless pH preparations were used.

We inoculated bacteria to mashed potatoes, and observed their growth and effects of the calcium preparation. Tables 4 and 5 show the results. In these experiments, we used *Bacillus* as a Gram-positive bacterium, *Escherichia* as a Gram-negative bacterium, *Staphylococcus* and *Salmonella* as food-poisoning bacteria. Mashed potatoes were prepared from normally boiled potatoes. Viable bacterial cell number increased in every sample without addition of the calcium preparation. Addition of more than 0.3% calcium preparation suppressed the increase in the viable bacterial cell number. In some cases, even a decrease in the viable bacterial cell number was

Table 4. Suppression of bacterial growth in mashed potatoes by calcium preparation (CP)

Bacteria and treatment	Viable bacterial cell number (CFU/g)		
	Initial	1 day	2 day
*Bacillus subtilis**			
Control	2.0×10^5	5.4×10^7	7.7×10^9
0.3% CP addition		$< 10^4$	$< 10^4$
0.3% $Ca(OH)_2$ addition		1.5×10^4	3.4×10^9
*Escherichia coli***			
Control	3.3×10^4	4.1×10^6	6.2×10^8
0.3% CP addition		2.2×10^4	2.0×10^4
0.3% $Ca(OH)_2$ addition		7.5×10^4	1.0×10^6

Storage temperature: 30°C. *IFO 13722, **JCM 1649.

Table 5. Suppression of the growth of food poisoning bacteria in mashed potatoes by calcium preparation (CP)

Bacterial and treatment	Viable bacterial cell number (CFU/g)		
	Initial	1 day	2 day
*Staphylococcus aureus**			
Control	1.7×10^4	7.4×10^7	8.9×10^9
0.3% CP addition		1.0×10^3	3.6×10^3
0.3% $Ca(OH)_2$ addition		2.7×10^4	1.3×10^7
*Salmonella enteritidis***			
Control	4.0×10^4	1.4×10^6	2.4×10^8
0.3% CP addition		1.3×10^4	2.2×10^4
0.3% $Ca(OH)_2$ addition		5.0×10^3	$< 10^3$

Storage temperature: 30°C. *IFO 13276, **ATCC 1891.

observed. As for calcium hydroxide, the same effect was obtained only by the addition of nearly 0.5%.

(2) Dipping test into suspension

We washed whole cucumbers with tap water and dipped them into the suspension of the calcium preparation in tap water. The viable bacterial cell number decreased as shown in Fig. 4. It was observed that the viable bacterial cell number began to decrease after a 10 min treatment and the effectiveness increased with the increase in the amount of the calcium preparation and duration of the dipping period. The negligible difference between concentrations of 0.25 and 1.0% was ascribed to the fact that the calcium preparation solubility was saturated at a concentration of 0.25%. Then, in the same way, we tested the antibacterial effect of radish sprouts (Kaiwaredaikon, Table 6), by dipping them into the suspension of the calcium preparation. Decrease of viable bacterial cell number was observed at a suspension of 1% of the calcium preparation. In some samples of radish sprouts, the viable bacterial cell number was above 10^9/g when purchased. However

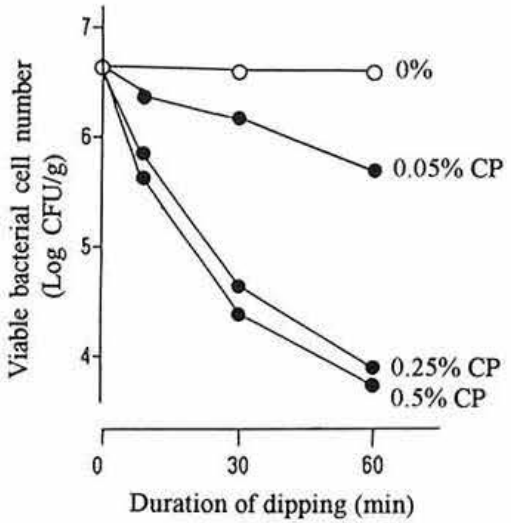

Fig. 4. Change in the number of bacteria in cucumber dipped in suspension of calcium preparation (CP)

this treatment enabled to decrease the number to about 10^5/g. Also, it was not necessary to wash

Table 6. Decrease in viable bacterial cell number in mini-radish sprouts (Kaiwaredaikon) by dipping into suspension of calcium preparation

Treatment	Dipping duration (min)	pH	Viable bacterial cell number (CFU/g)
Tap water	10	5.3	2.0×10^8
	60	5.3	2.2×10^8
1% Calcium suspension	10	5.2	8.0×10^5
	60	5.9	7.8×10^5

with tap water again and it appeared even that the taste was improved.

Discussion

The advantage of using the calcium preparation for the control of microorganisms in food are as follows: 1) decrease of disposal rate of food resources, 2) improvement and preservation of food quality, 3) prevention of calcium deficiency, and 4) supply of wholesome foods. The deficiency in calcium-intake in Japan is a major problem[4] and many people suffer from osteoporosis, adult diseases and other diseases which are related to the calcium-intake. We would like to promote the use of calcium preparations for food preservation, due to their contribution to nutrition supplement. Although food preservation has been achieved in the acid range by the addition of vinegar or organic acids, no method of food preservation has been developed in the alkaline range[2,3,9]. Our studies showed that preservation was possible in the alkaline range. There were some changes in the food characteristics such as color, taste and flavor depending on the amount of calcium preparation added. For example, excessive addition of the calcium preparation resulted in cooked rice that was hard or bitter, and in the yellowing of noodles (Udon). Studies will be carried out on the absorption as calcium and effect on nutrients such as vitamins, etc., and on the advantages and disadvantages of the calcium preparation. It is important to decrease the use of unnecessary microorganisms to produce good foods. As a result, we plan to decrease the number of viable microbial cells by treating foodstuffs with the calcium preparation, and to improve the color, taste or flavor until the final manufacturing stage, if necessary.

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