4.2 Applications in Japan

4.2.1 Application of radiation degraded CM-chitosan for preservation of fresh fruits (Japan-Part 1)

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Abstract

CM-chitosan was irradiated with various doses in powder state and solution using Co^{60} gamma source. The changes of viscosity in solution as well as molecular weight were also measured. The molecular weight reduced with increasing of radiation dose. The antimicrobial activity of CM-chitosan and irradiated CM-chitosan in solution against *E.coli* was investigated. In this studies, the sensitivity of *E.coli* depended on the concentration of CM-chitosan supplemented into medium and the antimicrobial activity of irradiated CM-chitosan was found to increase with radiation dose and reached to maximum with dose of 100 kGy. The 2% aqueous solutions prepared from CM-chitosan and 100 kGy irradiated CM-chitosan as mentioned above were applied for apple preservation. All coating fruits have significantly reduced the weight loss, spoilage ratio compared with control. Chemical and sensory quality of coated fruits were evaluated and compared, the best results were achieved with fruit coated using irradiated CM-chitosan.

Introduction

Chitin, chitosan and their derivatives have attracted much attention in recent years not only because they are available and renewable but also because of their excellent properties such as biocompatibility, biodegradability, non-toxicity [1, 2]. In agricultural field, chitosan applications related to flocculating agents, coating property and some other potential properties. However, only limited attention has reported involved in food application of these natural polymers [3]. Chitosan can form a semi-permeable coating which can modify the internal atmosphere, thereby delaying ripening and decreasing weight loss in fresh fruits and vegetables [4]. Moreover, many various kinds of active substances can be incorporated into the coating materials to improve its functionality and create new extra function [5, 6]. Coating film is useful when they are used as carrier for

prosthetic substances with the aims of preservation, anti-oxidation or flavoring [7, 8].

Nowadays, the growing consumer demand of safety foods without any chemical preservatives has focused efforts in the discovery of new natural antimicrobial agents [3]. Recently, the antimicrobial activity of chitosan and derivatives against of bacteria, yeast and fungi has received consideration attention. Carboxymethylchitosan (CM-chitosan) has three types of reactive functional groups: an amino, a hydroxyl and a carboxyl group at the C_2, C_3 and C_6 positions, respectively. These functional groups have much related to its antimicrobial activity [2, 9]. The recently studies on chitosan and derivatives were proved that their antimicrobial activity is attributed to their molecular weight, degree of deacetylation, degree of substitution and their concentration [9, 10].

Molecular weight of biopolymer such as chitosan can easily controlled by irradiation in solution and solid state. Irradiated chitosan with dose of 100 kGy can inhibit the growth of E.coli completely at a concentration of 3 μg/ml [11]. In our previously studies on chitosan, results that we are received from fractionation using centrifugal filter devices showed that the molecular weight fraction of $3\text{-}5 \times 10^4$ has strongest inhibition of microbial growth [8]. Application of chitosan coating has been investigated on food application, especially is fruits preservation. Chitosan coating of pre-harvested and harvested strawberries protected them from infection by *B.cinerea* and improved their quality [7, 12]. The coating mango with irradiated chitosan has prolonged the shelf-life from 7 to 15 days [13].

CM-chitosan, the water-soluble derivative of chitosan was expected much potential in food application. In this article, we investigated antimicrobial activity, the changes of intrinsic viscosity of CM-chitosan and irradiated CM-chitosan with various doses in powder state and solution. The preservation of apple using native and irradiated CM-chitosan coating was also evaluated.

Experimental

Irradiation treatment

Carboxymethylated chitosan (CM-chitosan) with degree of substitution and degree of deacetylation are 0.91 and 84%, respectively was purchased from Koyou Chemical Industrial Co. Ltd, Japan. CM-chitosan solution of 5% was prepared by dissolving CM-chitosan in distilled water. The glass bottles of samples containing this CM-chitosan solution were irradiated with gamma Co-60 source (TRCRE) at dose ranging from 20 to 100 kGy with dose rate of 10 kGy/h. Irradiated CM-chitosan solution was filtered by Millipore with pore size of 0.22 μm (MILLEX-GX, Tokyo, Japan) to remove any microbial contamination.

Assessment of antimicrobial activity

Antibacterial activity of CM-chitosan against of *E.coli* was evaluated by using the optical density (OD) method [8, 9].

A loopful of culture of *E.coli* was spread to give single colonies on Nutrient Agar and incubated at 37°C for 48 h. The representative colony was picked off with a wire loop and placed in Nutrient Broth, which was then incubated overnight at 37°C for two times to adapt with culture medium. A culture where bacteria grew in a logarithmic growth phase was prepared for antimicrobial tests. Two percent of an overnight incubated culture of target microorganisms was transferred to L-shaped tubes containing cultural medium supplemented with different concentrations of original and irradiated CM-chitosan. All of these were inoculated under shaken cultivation at 37°C for 48 h. The turbidity of medium was measured at 650 nm by using a BioScanner (Tokyo, Japan) every 3 h during incubation.

Intrinsic viscosity of radiation degraded CM-chitosan

The viscosity measurement was carried out with Ubbelohde viscometer at 25°C. (UNI-THERMO BATH VISCOSITY, Yamoto Ltd.Co, Japan). The solvent was 0.1N sodium chloride aqueous solution. The irradiated CM-chitosan solutions with different concentration were used to determine viscosity. The relative viscosity η_r was approximated the time take by solution to flow in viscometer divide to the time take by solvent to flow in viscometer. Relative viscosity value was converted to specific viscosity ($\eta_{sp} = \eta_r - 1$). The reduced viscosity (η_{sp}/C) was independent on the concentration and the intrinsic viscosity is defined as the limit of the reduced viscosity as concentration approaches zero ($\eta_i = \lim_{C \to 0} \frac{\eta_{sp} - 1}{C}$) [14].

Fruit preservation of CM-chitosan

The 2% solutions of original and 100 kGy irradiated CM-chitosan were prepared to apply for coating preservation of apple. For comparison, the apples have just harvested in Gunma Experimental Garden were selected by size, color without any infection and mechanical injury. After cleaning and air drying, the apples were classified and dipped in various coating formulations 2 times, 1 minute for each. Coated apples were stored on carton box at ambient conditions (23 ± 0.5°C and 55 ± 5%RH). During storage, some sensory and quality criteria including weight loss, spoilage rate, and total acidity and vitamin C contents were evaluated and recorded every week.

Results and discussions

Dependence of intrinsic viscosity of CM-chitosan by radiation dose

For evaluation the changes of CM-chitosan by irradiation treatment, the intrinsic viscosity of CM-chitosan that irradiated in powder state and solution of 5% was calculated and exhibited in Fig. 1. This figure indicated that viscosity of irradiated CM-chitosan was inverted related with radiation dose. Similarly to chitosan, irradiation treatment was affected on chemical properties of CM-chitosan and lead to reduction of its viscosity. The viscosity reduction has suggested molecular weight also reduce with increase of radiation dose [15]. Moreover, the reduction of viscosity of CM-chitosan irradiated at solution state is higher than that at powder state. These results shown that gamma radiation was steeply decreased the viscosity of CM-chitosan at dose lower than 40 kGy, then slowly decrease when irradiation dose continuously increase. The figure also indicated that the radiation degradation yield of CM-chitosan in solution is higher than that in solid state. It may caused by a synergistic effectiveness of radiolysis products of water when irradiated in solution. Matsuhashi and Kume reported that the chemical properties of chitosan were not remarkably change by irradiation up to 100 kGy under dry condition [16]. Based on the results, CM-chitosan that irradiated at solution state with dose varying from 20 to 100 kGy were chosen for next experiments.

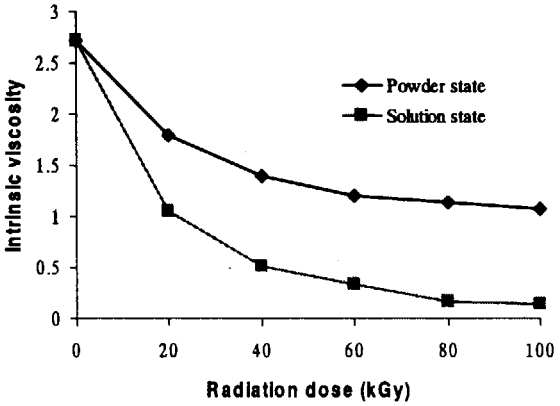
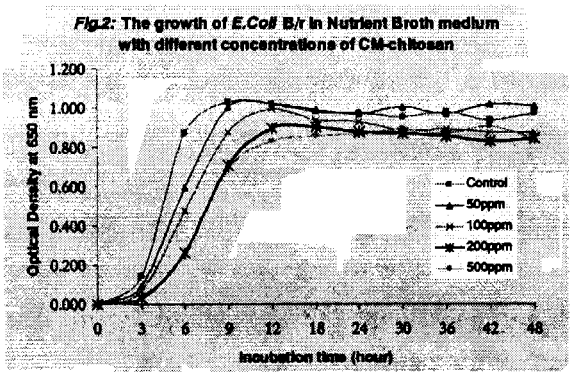
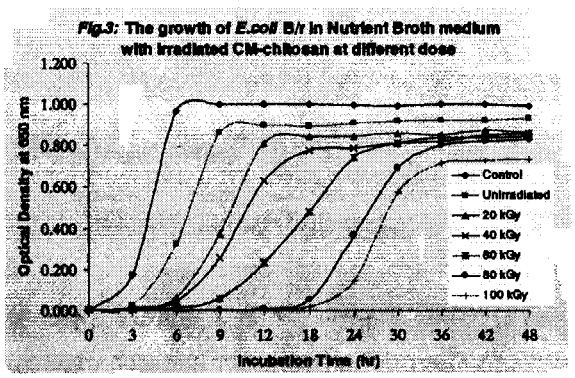


Figure 1. **The dependence of intrinsic viscosity of CM-chitosan on irradiation dose**

Antimicrobial activity





Because the cultural medium become turbid as the bacteria grown then optical

density can be used as a criterion measuring the antimicrobial activity of CM-chitosan. The dependence of optical density of cultural medium on incubation time was plotted as curves. The growth of *E.coli* at different concentrations of CM-chitosan was presented in Fig.2. The growth was inhibited at least one log cycle for 6 hours at low concentration of CM-chitosan. This result is completely appropriated with our previous study of antimicrobial activity of chitosan [4]. The antimicrobial activity of chitosan depends on its concentration in cultural medium, namely chemical properties of chitosan. The exact mechanism of antimicrobial activity of chitosan and its derivatives is still unknown; several mechanisms were proposed in most studies on chitosan. Chitosan can block description to RNA from DNA [17, 18]. It also activates several defense processes in the host tissue [19]. Our results were proved the antibacterial activity of CM-chitosan. This activity is lower than chitosan at same concentration, however, in several study on chitosan and derivatives, the authors concluded that the activity can be increased when the -OH in chitosan molecule was substituted with $-CH_2COOH$ in carboxymethylated chitosan [9]. From these data, the concentration of 0.02 mg/ml that could clearly inhibit the growth of *E.coli* was chosen for next experiments.

In this study, the antimicrobial activity of irradiated CM-chitosan was investigated with radiation dose. As presented in Fig. 3, with the same concentration, the antimicrobial activity of irradiated CM-chitosan is higher than original CM-chitosan. There is insignificant difference of the growth of *E.coli* among media have been supplemented with native CM-chitosan and irradiated CM-chitosan at low dose. But, the supplementation with irradiated CM-chitosan at higher dose (more than 40 kGy) was inhibited its growth stronger than supplementation with CM-chitosan. This result also suggested that antimicrobial activity of CM-chitosan is depended on its chemical properties. Study on degraded chitosan and oligo-chitosan, some authors were proved that the antimicrobial activity is depended on their chemical properties [8, 9]. It has been reported that O-CM-chitosan produced from degraded chitosan is higher activity than that produced from native chitosan [9, 20, 21]. The study on antibacterial effect of irradiated chitosan on *E.coli*, Masuhashi and Kume indicated that irradiated chitosan at 100 kGy could inhibit completely the growth of *E.coli* even at a low concentration [16].

In this study, we only investigated activity of native and irradiated CM-chitosan with dose from 20 to 100 kGy and it also proved that the radiation treatment of CM-chitosan induced the same effect to chitosan. Its antimicrobial activity was increased along with irradiation dose and reached to maximum with dose of 100 kGy. In order to explain the improvement of antimicrobial activity of irradiated CM-chitosan, it is necessary to look up its functional properties. It may caused by irradiated CM-chitosan molecules with smaller chain can easily attached to microbial cell membranes leads to

shrinkage and cell death. In addition, the stronger inhibition of microbial growth of irradiated CM-chitosan than that of unirradiated one may be due to the irradiated degradation CM-chitosan can be penetrate into cell membrane and inhibit the description of RNA [3, 16].

Preservation coating of apple

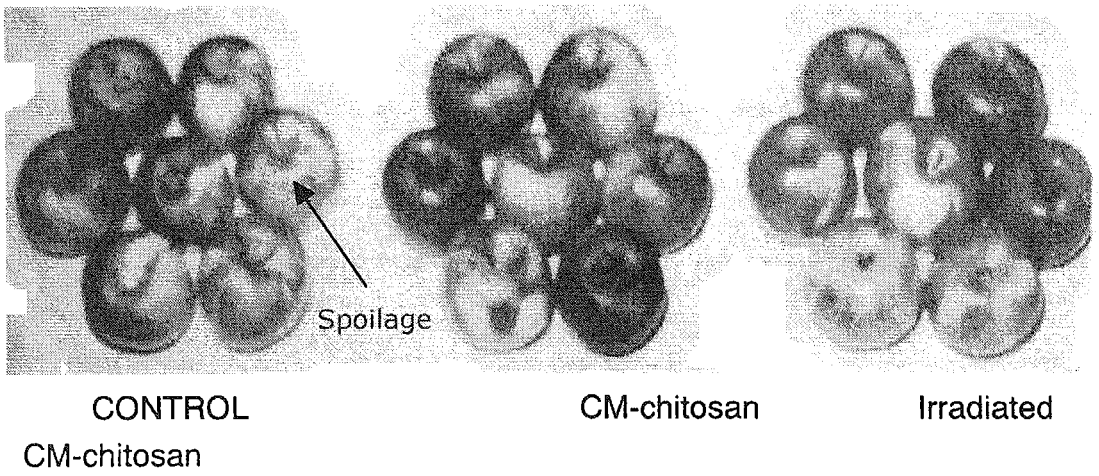


Figure 4: **The coated apples stored at ambient conditions**

From results as mentioned above, original CM-chitosan and 100 kGy irradiated CM-chitosan were chosen for coating preservation of apple. Fig. 4 showed the changes of appearance and spoilage of apple during storage. As seen from this figure, the differences in treatments were significant after 2 week storage. The spoilage has first occurred with control fruit, whereas coating apples were still remained marketability. The coating films were retarded the ripening and eliminated the spoilage rate of preserved apples. As results of antimicrobial activity, the coating films could prevent the infection and growth of microorganism and keep appearance and freshness of stored apples. These results suggested that coating fruits with CM-chitosan may have some positive effects for elongation of storage of apple. In addition, irradiated CM-chitosan is better than original CM-chitosan. The effect of CM-chitosan on spoilage was attributed to the combination of its antimicrobial properties and its ability to stimulate defense responses in the host tissue. Like chitosan, it can inhibit the growth of microorganisms, including some species causing spoilage on fruits and vegetables. During storage, quality of apple has been evaluated by weight loss, spoilage ratio, vitamin C content and total acidity. The results were presented in Table 1.

Table1. The quality properties of apples during storage

Samples	Periods of storage (days)				
		First day	1 week	2 week	3 week
Control (Water Coating)	Weight Loss (%)	0.00	3.23	29.33	100.00
	Spoilage Rate (%)	0.00	0.00	16.80	100.00
	Vitamin C (mg/100g)	20.84	22.63	22.73	31.90
	Acidity (mg/g)	2.62	2.55	2.53	2.02
CM-Chitosan Coating	Weight Loss (%)	0.00	3.39	28.47	62.60
	Spoilage Rate (%)	0.00	0.00	0.00	37.94
	Vitamin C (mg/100g)	20.84	19.80	19.80	29.50
	Acidity (mg/g)	2.62	24.20	3.12	2.30
Irradiated CM-Chitosan Coating	Weight Loss (%)	0.00	3.25	6.54	44.86
	Spoilage Rate (%)	0.00	0.00	0.00	33.12
	Vitamin C (mg/100g)	20.84	19.10	19.90	27.27
	Acidity (mg/g)	2.62	2.94	2.76	2.58

Theses results showed that along with ripening process of apple, content of vitamin C gently increased and total acidity decreased during storage. Total acidity and vitamin C are quite stable over 2 weeks. However, the changes of vitamin C content and acidity were significant at the third week. These data implied quality reduction of stored apples was occurred at that time. In addition, the increase of vitamin C contents of coating apples was lowest with irradiated CM-chitosan coating and highest with control fruits. It is attributed to the quality of control coating fruits was reduced faster than coating fruits and the apples coated with irradiated CM-chitosan are better than that with CM-chitosan. The decrease of acidity of coating apples was lower than control. This result shown that the ripening of irradiated CM-chitosan coating fruits is delayed longer than that of CM-chitosan coating apples [13]. The use of carboxymethylchitin film to preserve fruit over long periods has been approved in both Canada and USA [21]. From this study, it is expected to improve the antimicrobial activity of CM-chitosan using gamma radiation that could be applied on fruit preservation.

CONCLUSION

To evaluate the antimicrobial activity of CM-chitosan, *E.coli* was incubated in the Nutrient Broth medium supplemented with different concentrations of CM-chitosan. The study indicated that CM-chitosan have an antimicrobial activity at low concentration of 0.2 mg/ml. The improvement of antimicrobial activity can expand its application on food preservation. Radiation treatment reduced intrinsic viscosity of CM-chitosan and the reduction is reversal proportion with radiation dose. This study also indicates that CM-chitosan can be used to control the spoilage of apple. The CM-chitosan coating can delay ripening and keep the marketability of stored apple after 3 weeks at ambient conditions (23 ± 0.5^{O}C and 55 ± 5% RH). Irradiated CM-chitosan coating can keep the quality properties of apple better than original CM-chitosan. Since these properties, irradiated CM-chitosan could be applied to preserve the fresh fruits effectively.

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