Research Article

Encapsulation of Mulberry leaf extract in chitosan nanoparticle to develop eco-friendly edible coating for fresh strawberry reservation

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ABSTRACT

In this research, *Morus alba* Mulberry leaf extract was encapsulated in chitosan nanoparticles by ionic gelation method to develop coating for fresh strawberries. We aimed to study encapsulation of mulberry leaf extract in chitosan nanoparticle for coating on the fresh strawberry. The obtained nanoparticles exhibited a regular distribution and spherical shape with the average size range of 87.23 to 139.5 nm as observed by using a laser light scattering equipment and SEM technique. The results of FTIR studies were clearly indicated that the nanochitosan gets effectively bonds with the molecules of secondary metabolites in *Morus alba* Mulberry leaf extract. The FTIR spectra showed the present of peak at 1163.38 cm^{-1} which represented the interaction between NH_3^+ of chitosan and PO_4^{3-} of tripolyphosphate crosslinker and the peaks at 3402, 1078, 1023 and 699 cm^{-1} which indicated the presence of C-N, C-OH, -C-O-C- and N-H bonds of protein molecules of secondary metabolites in *Morus alba* Mulberry leaf extract. The XRD pattern showed the change in crystallinity of chitosan to the amorphous polymer region of chitosan nanoparticles. The effect of mulberry leaf extract loaded chitosan nanoparticle coating on the shelf life of strawberry showed slower deterioration compared to uncoated strawberry at 12 days while the uncoated strawberry started to decay at day 4. According to the result, the 40 mg/mL mulberry leaf extract loaded chitosan nanoparticle inhibited the growth of *Bacillus subtillis* with the mean inhibition zone diameter of 7.87±0.20 mm. The hGF cell line viability after treating with 1–10,000 g/mL sample concentration ranged from 98.26±10.25% to 88.37±7.54%cell viability. Therefore, it showed that the effectiveness against *B. subtillis* was significantly higher due to encapsulation process which could serve as a promising strategy to replace other coated materials for higher post-harvest quality.

Keywords: encapsulation, chitosan nanoparticle, ionic gelation, mulberry leaf

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Introduction

Nowadays, the use of an active edible coating that has the potential to prolong storage life and control fruit rots may represent a valid and alternative way for preservation. Chitosan coating can control plant diseases due to its ability against infection caused by bacteria, mold and other pathogens. It has been proved to be applicable for preventing the loss of weight, titratable acidity, total soluble solid and bioactive compounds in fruits and vegetables during storage [1, 2]. Moreover, one of the possible strategies is to focus on plants rich in bioactive compounds that are well known for their antimicrobial properties of isolated phytochemicals with safety, effectiveness and multiple pharmacological properties. However, there is still a limitation due to low bioavailability, owing to their rapid elimination, poor adsorption, aqueous solubility and stability [3, 4]. Combining different fields' approaches including nanotechnology, the optimization of natural products' features and wider use is more feasible than ever. Encapsulation is a technique which active agents are entrapped into a biodegradable matrix or "wall" material, forming micro/nano-systems. Encapsulation of bioactive natural compounds, is widely used in food, agriculture, pharmaceutical and cosmetic industries and has proved to be a very useful method for the protection of unstable bioactive compounds from harsh processing conditions, the construction of targeted delivery systems and controlled-release of the encapsulated compound products and the increase in aqueous solubility etc. [3]. Among all the encapsulation techniques, ionic gelation is a mild, simple and organic solvent-free approach for the formation of stable nanoparticles. This approach is based on the interaction between oppositely charged macromolecules and a nontoxic and multivalent material in order to provide charge density. Chitosan, a unique cationic polysaccharide, is the most widely used according to well-known antioxidant, lipid-lowering and antimicrobial activities, film-forming and gelling properties, encapsulation potential, etc. This biopolymer is considered as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA). The cationic nature of chitosan under acidic conditions leads to the development of various forms, therefore it has been extensively used as a matrix for the encapsulation of extracts, essential oils and bioactive compounds. Regarding ionic gelation, it occurs due to the inter- and intra-molecular cross-linking of the polycationic chitosan by an anionic cross-linker such as the most commonly used tripolyphosphate (TPP). Mulberry (*Morus alba*) leaf extraction have been reported their antimicrobial activities against for *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. [5]. The antimicrobial activities are principally attributed to soluble protein and phenolic compounds including mulberrin, albanol A (mulberrofurna G), albanol B and quercetin. [6]. So far, there is no report on the synthesis of mulberry leaf extract loaded in chitosan nanoparticle for fresh fruit coating. Strawberry (*Fragaria ananassa*) is a widely consumed as fresh fruit and used as an ingredient in processed products. However, it is one of the most delicate fruits with an extremely short postharvest life. A major problem often occurred during its storage is gray mold infection [7]. In order to prevent fungal and insect attack and prolong postharvest shelf life, many chemical treatments have been used, despite that it reduces food safety.

This study investigated the encapsulation of mulberry leaf extract into chitosan nanoparticles by using ionic gelation technique to develop edible coating for fresh strawberries. We focused on the

effect of active edible coatings on postharvest quality. The antimicrobial activity of a chitosan powder, chitosan nanoparticles, mulberry leaf extract and mulberry leaf extract encapsulated in chitosan nanoparticles were determined by agar disc diffusion assay. Furthermore, the possible cytotoxicity of active edible coating was also analyzed.

Materials and Methods

Plant materials and Extraction

Mulberry (*Morus alba* L.) leaves were obtained from Bandu district, Chiang Rai, Thailand. Plant materials were washed thoroughly in water, cut into small pieces, dried in an oven at 50°C for 24 h. and ground in stone mortar. After that, the plant powder was soaked in 95% ethanol at a ratio of 1:4 for 48 h. at room temperature. After extraction, supernatant was filtered through Whatman No. 1 filter paper. The extract filtrate was then evaporated using a rotary evaporator at 50°C under reduced pressure of 650 mmHg and dried through lyophilization.

Preparation of chitosan nanoparticles and plant extract loaded nanoparticles

The ionic gelation method was followed for the preparation of chitosan nanoparticles. Chitosan, MW=50,000-190,000 Da, was purchased from Sigma-Aldrich. The 0.5, 1.0 and 1.5% w/v chitosan samples were dissolved in 100 mL of 1.0% acetic acid and homogenized using a magnetic stirrer at room temperature for 60 min. After that, the chitosan solution was added drop wise by 50 mL of (1mg/mL) sodium tripolyphosphate (TPP) crosslinking agent under stirring at room temperature [1, 2]. Opalescent color was observed and stirring was continued for overnight. Then, it was centrifuged for 30 min at 8,000 rpm to separate any large aggregates and to leave the nanoparticles suspended in the supernatant. The pellet was carefully collected and washed with deionized water approximately 5 times. The precipitated mass was freeze-dried and kept at 4°C.

Loading of plant extract to chitosan nanoparticles was performed by dissolving 1.0 %w/v of mulberry leave extract in distilled water (50 mL) and was added drop wise to 100 mL of 0.5, 1.0 and 1.5% w/v chitosan solution in magnetic stirring and further stirred for 60 min. After that, 50 mL of (1mg/mL) sodium tripolyphosphate (TPP) was added drop wise into the mixed solution under stirring for overnight. Then, it was centrifuged for 30 min at 8,000 rpm, and the pellet was carefully collected and washed with deionized water approximately 5 times. The precipitated mass was then freeze dried and kept at 4°C.

Characterization

Morphology of the samples was observed using a scanning electron microscope (SEM) while the chemical elements and functional groups were observed using SEM-EDS and Fourier Transformed Infrared Spectroscopy (FTIR). The crystalline phase identification of the samples was carried out using X-ray diffraction (XRD). To determine the average particle size and size distribution with dynamic light scattering, Nano Particle Size Analyzer, Horiba, Partica LA-950V2 and Nanopartica SZ-100 was applied.

Determination of antimicrobial activity against *B.subtillis* was determined by agar disc diffusion assay using Gentamycin (0.01 mg, Oxide Ltd., UK) as a reference standard. The

sulforhodomine B (SRB) colorimetric assay was used to evaluate the cytotoxic effect of sample on human gingival fibroblasts.

Results and Discussion

Characterization of chitosan nanoparticles and Mulberry leave extract loaded chitosan nanoparticles

Pictures of freeze-dried chitosan nanoparticles and freeze-dried Mulberry leave extract loaded chitosan nanoparticles at 0.5, 1.0 and 1.5%w/v are shown in Figure 1. The optimum encapsulation process was obtained by using a mixture of 0.5%w/v chitosan solution which generated small, uniform and well dispersed chitosan nanoparticles, as shown in Figure 1(d).

(a) 0.5% (b) 1.0% (c) 1.5%

Chitosan nanoparticles

(d) 0.5% (e) 1.0% (f) 1.5%

Mulberry leaf extract loaded chitosan nanoparticles

Figure 1 Freeze-dried chitosan nanoparticles and freeze-dried Mulberry leave extract loaded chitosan nanoparticles

The XRD pattern (Figure 2) showed the change in crystallinity of chitosan to the amorphous polymer region of chitosan nanoparticles. The crystalline structure have been fully destroyed after crosslinking with TPP during formation of chitosan nanoparticle. The amorphous polymer of chitosan nanoparticles had network linkage structure made up of chitosan polymer chains and TPP crosslinkers, as shown in the board peak of chitosan nanoparticle, this in agreement with a previous work of Abdelfattah Ali [8].

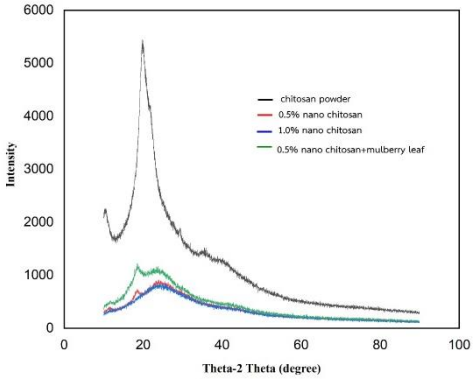


Figure 2 The XRD pattern

FTIR spectroscopy was used to investigate the interaction between inter- and intra-molecular cross-linking of polycationic chitosan by an anionic cross-linker of sodium tripolyphosphate (TPP) by measuring the samples in a range of 4000-500 cm^{-1} at a resolution of 1 cm^{-1}, as shown in Figure 3. Chitosan nanoparticle represented the peak at 3385.92 cm^{-1} which was attributed to a broader N-H, indicating that ionic crosslinking had occurred between the protonated amine groups of chitosan and TPP. The strong bands observed at 2920.57, 2908.57, 1635.23, 1510.05, 1376.80 and 1219.00 cm^{-1} were asymmetry and symmetry stretches of $-CH_2$, $-NH_3^+$, C=O of amide and N-H bending, -OH of alcohol and P=O stretching [2]. The peak at 1163.38 cm^{-1} was attributed to the overlap of C-O stretching of chitosan nanoparticle due to the interaction of $-NH_3^+$ (ammonium ion) and $-PO_4^{3+}$ (phosphate ion) [2]. The peaks at 1636 and 1385 cm^{-1} indicated the presence of amide I and C-N stretching of protein, respectively. The bands observed at 1078, 1023 and 669 cm^{-1} were the characteristics of C-OH vibrations of protein, -C-O-C- bending mode and might be the plane bending vibrations of N-H groups of proteins, respectively [9]. Secondary metabolites such as flavonoids, phenols, amino acids and anthocyanin was reported to present in *Morus alba* leaf extract [9].

The average particle size of the obtained nanoparticles was analyzed by dynamic light scattering (DLS), as shown in Table 1. The average size of chitosan powder was 585.6 nm with a polydispersity index (PI) of 4.82. The average size of 0.5%w/v chitosan nanoparticles, 1.0%w/v chitosan nanoparticles and 0.5%w/v chitosan loaded with 1.0%w/v mulberry leaf extract nanoparticles were found to be 87.23 nm (PI 2.47), 125.40 nm (PI 2.66) and 139.5 nm (PI 2.74), respectively.

The average size of chitosan nanoparticles increased when the concentration of chitosan solution increased and mulberry leaf extract was added. The lower polydispersity index of chitosan nanoparticles confirmed the uniformity of nanoparticles diameter.

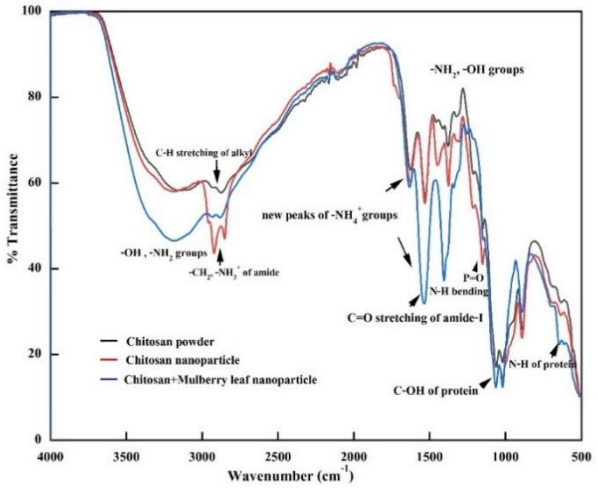
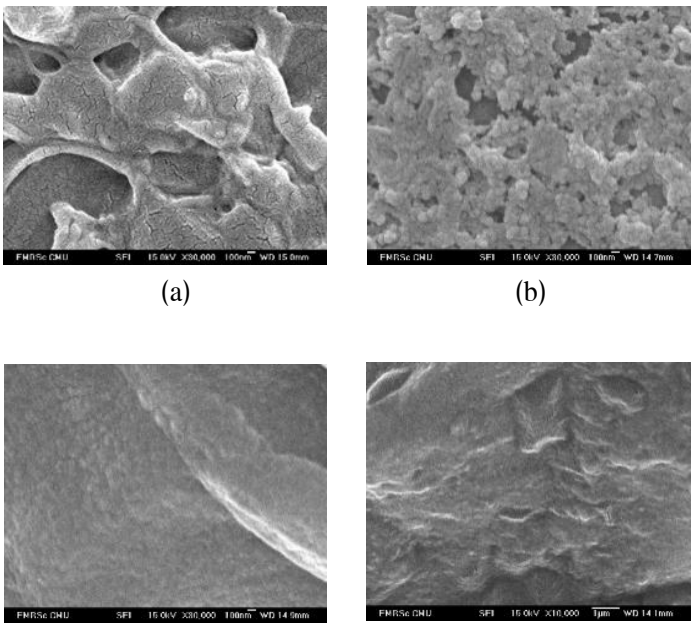


Figure 3 FTIR spectrum of chitosan powder, chitosan nanoparticle and mulberry leaf extract loaded chitosan nanoparticle

Table 1 Average size and polydispersity index of the nanoparticles obtained.

Chitosan particles	Diameter (nm)±S.D.	PI
Chitosan powder	585.6±10.50	4.82
0.5%Chitosan nanoparticles	87.23±14.17	2.27
1.0%Chitosan nanoparticles	125.40±32.23	2.66
0.5%Chitosan loaded with 1.0%mulberry leaf extract nanoparticles	139.50±23.83	2.74

Scanning electron microscope (SEM) image of the chitosan nanoparticle (Figure 4b) showed that the nanoparticles had a homogeneous size distribution, spherical morphology. The average size obtained by SEM was around 80 nm which was in accordance with the particle size from DLS.



(a) (b)

(c) (d)

Figure 4 SEM images of chitosan samples (chitosan powder (a), 0.5%chitosan nanoparticle (b), 1.0%chitosan nanoparticle (c), and 0.5%Chitosan loaded with 1.0%mulberry leaf extract nanoparticles (d)

Analysis of the micrograph in Figure 4c and Figure 4d showed that it was possible that the obtained particles were agglomerated, compacted and had smooth surface. The surfaces of mulberry leaf extract loaded chitosan nanoparticles became rougher and studded with dense granules than chitosan nanoparticles. Energy dispersive analysis (EDS) was used to identify the sample element contents as shown in Table 2. It was confirmed that the chitosan characteristic elements were composed of carbon, oxygen and nitrogen. Detection of sodium and phosphorus elements was attributed to TPP for both of

chitosan nanoparticles and mulberry leaf extract loaded chitosan nanoparticles . Chitosan nanoparticles prepared by ionic gelation showed the presence of sodium and phosphorus from the sodium tripolyphosphate crosslinking, corresponded to the FTIR analysis which showed the ammonium functional group, the P=O stretching and P-O bending in chitosan molecules. [1, 2]. It was possible that polyphosphoric molecules of sodium polyphosphate interacted with ammonium groups of chitosan molecules.

Effects on shelf-life of fresh strawberries

The edible films were prepared by dissolving 1.0 g of chitosan particles in 100 ml of 1% acetic acid solution with continuous stirring at room temperature. Strawberries were washed with portable water and left to dry for 2 h, then immersed for 3 min in the coating solutions of 1%acetic acid, 0.5%chitosan, 0.5%chitosan nanoparticle, 1%mulberry leaf extract and 0.5%chitosan loaded with 1%mulberry leaf extract nanoparticle. For each treatment, 20 strawberries were used and stored for 12 days at room temperature (25°C). The appearance of strawberry sample was observed for 12 days, as shown in Figure 5. The uncoated strawberries (control) showed the black spot caused by mold infection on the fourth day and juice leakage happened on the sixth day of storage. The strawberries coated with mulberry leaf extract loaded chitosan nanoparticle solution showed a slower deterioration compared to control and another groups. The shelf life of strawberries coated with mulberry leaf extract loaded chitosan nanoparticle solution was 12 days, as shown in Figure 5. According to the results of antimicrobial activity obtained by the disc diffusion method, there only the 40 mg/mL mulberry leaf extract loaded chitosan nanoparticle inhibited the growth of *Bacillus subtillis* with the mean inhibition zone diameter of 7.87±0.20 mm (the inhibition zone diameter of 0.01 mg/ml Gentamycin was 20.01±0.86), as shown in Figure 6. *Bacillus* spp are bacteria that can digest proteins and fruit pectin, causing the fruits to spoil.

The sulforhodamin B (SRB) colorimetric assay was used to evaluate the cytotoxic effect of herb-loaded chitosan nanoparticles on cultured cells. The SRB assay is based on the ability of the SRB dye to bind basic amino acid residues of proteins. Human gingival fibroblast (hGF) (passage-8) cells were exposed to 1-10,000 μg/mL solutions of herb-loaded CS-TPP in a 96-cluster well culture plate for 24 h. Cell vitality after exposure to the studied sample was examined. The cytotoxic effects of mulberry leaf extract loaded chitosan solution and ascorbic acid standard solution on the viability of hGF cell lines were presented as the percentage of cell ability. The cytotoxic effect of the samples at concentration of 1–10,000 μg/mL was in a rage of 98.26±10.25% - 88.37±7.54% (%cell viability). There was no significant difference of cell viability between the sample and the ascorbic acid standard (85.15±4.79% - 72.67±6.92%) at concentration of 0.1–1,000 μg/mL.

Table 2 Energy dispersive analysis (EDS) of the sample element contents

Chitosan particles	Elements	Atomic%
1. Chitosan powder	C	60.14
	N	8.24
	O	31.62
	Na	-
	P	-
2. 0.5%Chitosan nanoparticles	C	38.25
	N	9.34
	O	49.67
	Na	1.76
	P	0.98
3. 1.0%Chitosan nanoparticles	C	35.06
	N	10.07
	O	52.10
	Na	1.81
	P	0.96
4. 0.5%Chitosan loaded with 1.0%mulberry leaf extract nanoparticles	C	42.10
	N	11.05
	O	45.11
	Na	0.96
	P	0.78

Day 1 Day 4 Day 12

(1) Control

Day 1 Day 4 Day 12

(2) 1%acetic acid

Day 1 Day 4 Day 12

(3) 0.5%chitosan solution

Day 1 Day 4 Day 12

(4) 0.5%chitosan nanoparticle solution

Day 1 Day 4 Day 12

(5) 1%mulberry leaf extract solution

Day 1 Day 4 Day 12

(6) 0.5%chitosan loaded with 1%mulberry leaf extract nanoparticle solution

Figure 5 Effect on shelf-life of fresh strawberries

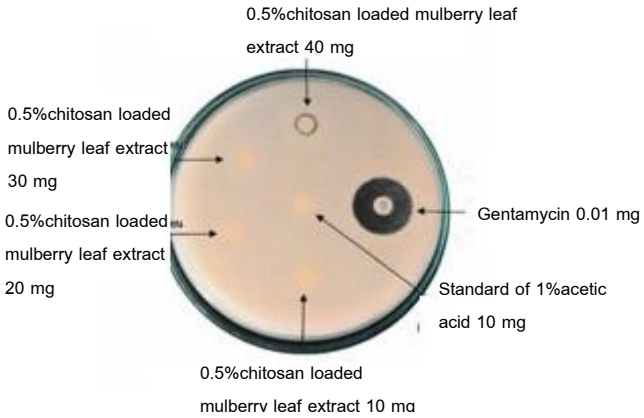


Figure 6 The antimicrobial activity of coating solution

Conclusions

The results of this study indicated that encapsulation of mulberry leaf extract in chitosan nanoparticle by ionic gelation showed the higher effectiveness of the antimicrobial activity of *B. subtillis* than the chitosan nanoparticles and mulberry leaf extract without encapsulation. The obtained nanoparticles exhibited a regular distribution and spherical shape with the average size range of 87.23 to 139.5 nm. The results of FTIR studies were clearly indicated that the nanochitosan gets effectively bonds with the molecules of secondary metabolites in mulberry leaf extract. The mulberry leaf extract loaded chitosan nanoparticles also effectively delayed the ripening and rotting of strawberries due to an inhibitory effect against pathogenic foodborne bacteria. Based on these results, edible coatings with mulberry leaf extract loaded chitosan nanoparticles can be a promising strategy to improve the post-harvest quality of fruits and substitute other coated materials currently in use.

Acknowledgements

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