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Preparation and characterization of immobilized lysozyme and evaluation of its application in edible coatings

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ABSTRACT

In this manuscript, we describe the process of immobilizing lysozyme onto a chitosan powder surface using different methods, and we assess the antimicrobial characteristics of the immobilized lysozyme when acting as an edible coating. The immobilized enzyme retained 72.50% of the specific activity of free enzyme when the covalent attachment procedure was utilized. The properties of the immobilized enzyme, including optimum pH and temperature, reusability, storage stability and kinetic parameters, were also examined. Compared to free and immobilized enzyme, the covalently immobilized lysozyme exhibited remarkable characteristics on optimum pH (6.0) and temperature (65 °C), and storage stability. The covalently bound enzyme maintained approximately 80% of its initial activity after 14 cycles, and the K_m and V_{max} were found to be 28.17 μg/ml and 1.41×10^4 U/mg, respectively. In addition, the antimicrobial effect of the immobilized lysozyme was monitored when applied as part of a whey protein concentrate and chitosan coating. The data indicated that the active coating was effective in inhibiting the growth of *Escherichia coli* O157: H7 (CICC 21530) and *Staphylococcus aureus* (CICC 21600) for a week. The study demonstrates the potential for the use of immobilized lysozyme as an antimicrobial component for antimicrobial packaging.

1. Introduction

Over the past decade, considerable research has been conducted to investigate and develop biodegradable or edible films and coatings. This is mainly due to the demands for environmentally friendly and renewable replacements of petroleum-based polymeric materials and plastic [1–3]. Particularly, increasing attention has been focused onto incorporating antimicrobial agents in edible packaging, which is expected to inhibit food spoilage caused by microbial growth and improve the shelf of food products [4]. Different antimicrobial agents such as organic or inorganic acids [5,6], essential oils [7] and functionalized nanoparticles [8] have been tested for their effects on the edible packaging. However, considering the environmental issue and the health and safety for both consumers and manufacturers, researchers are getting interested in applying natural biopreservatives such as bacteriocins [9] and antimicrobial enzymes [10] in edible packaging.

Lysozyme (muramidase, EC 3.2.1.17) as a naturally antimicrobial enzyme has been frequently incorporated into packaging materials. It is wildly found in several mammalian secretions and in hen egg whites [11]. Particularly, this enzyme shows effective antimicrobial activity mainly on Gram-positive bacteria. Because of the protection of lipopolysaccharide layer surrounding their outer membrane, lysozyme is not susceptible to Gram-negative bacteria. It damages bacterial cell walls by catalyzing the hydrolysis of 1, 4-beta-linkages between N-acetyl-D-glucosamine and N-acetylmuramic acid residues in peptidoglycans and between N-acetyl-D-glucosamine residues in chitodextrins, which increases the permeability of bacteria and causes bacteria to burst [12].

However, the practical application of the free enzyme may be limited due to its relatively low stability and activity. Therefore, most research has centered on the conduct of molecular proteins that are immobilized on a solid support [13,14]. In contrast to the free enzyme, immobilized enzymes show improved stability to temperature and pH values, and more robust resistance to environmental changes [15,16]. In addition, the heterogeneity of the immobilized enzyme system provides easy recovery of both the enzyme and product, allowing for reuse of the enzyme, can provide continuous operation of the enzymatic process, and can facilitate rapid termination of the reaction [17]. Those techniques used for immobilization of enzymes can be divided into six different categories: adsorption, ionic binding, covalent attachment, cross-linking, entrapment and

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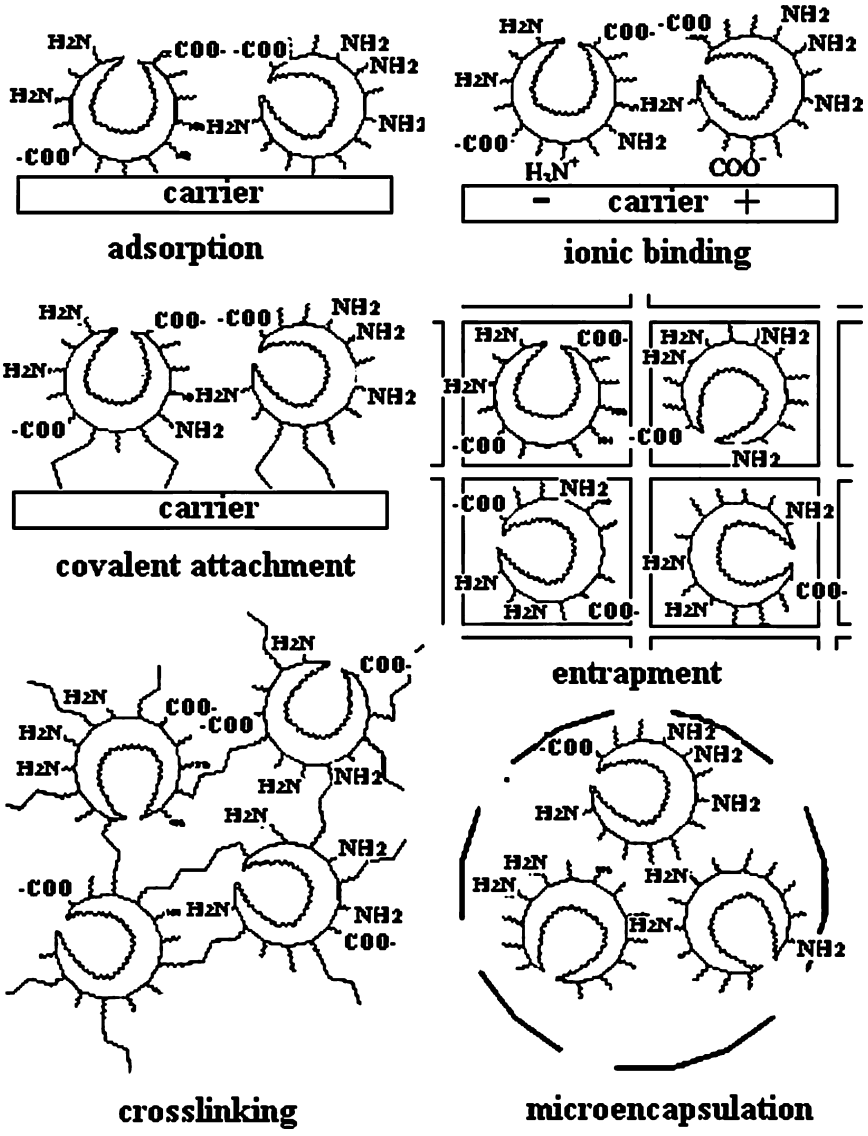

Fig. 1. Schematic of enzyme immobilization methods.

microencapsulation [18,19], as illustrated in Fig. 1. The lysozyme has been immobilized on many different supports by a variety of methods [20,21], and many studies have also reported the use of edible films and coatings containing free lysozyme as an antimicrobial agent [22]. However, to the best of our knowledge, the use of the immobilized lysozyme as an antimicrobial agent in edible coatings has not been systematically reported yet.

The present work aims to examine the effect of edible coating containing immobilized lysozyme as an antimicrobial agent. Lysozyme was immobilized on chitosan powder surfaces to investigate its antimicrobial effect in whey protein concentrate-chitosan coatings. The antimicrobial experiments of the immobilized lysozyme with low activity were also studied. First, the immobilized lysozyme was pretreated at extreme conditions; next, antimicrobial experiments were conducted. To investigate immobilized lysozyme, two immobilization methods, including physical adsorption and covalent attachment, were compared. The characteristics of the immobilized enzyme and the factors that affect the activity of immobilized enzyme were also conducted.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation was 90%, molecular weight of 40 kDa) produced from the deacetylation of shrimp shells was purchased from Sinopharm Chemical Reagent Co., Ltd. It served as the carrier of the immobilized enzyme and was the chief material of the edible coating. Lysozyme obtained from the protein of chicken egg whites, with a specific activity of 40,000 U/mg, was supplied by Sigma Chemical Co., Ltd. and was used without further purification. *Micrococcus lysodeikticus* was used to assay lysozyme activity and was supplied by Shijiazhuang Huarui Innovative Biotechnology Development Center. Whey protein concentrate (80%) was obtained from Glanbia PLC. All other chemicals involved were of analytical grade.

2.2. Assay of lysozyme activity

The activity of free and immobilized lysozyme was determined by the hydrolysis of *M. lysodeikticus* according to the method described by Shugar [23]. The process included monitoring the degradation rate of *M. lysodeikticus* through the reduction in absorbance at 450 nm. In a 1 cm quartz cuvette, 2.5 ml of the *M. lysodeikticus* solution (OD_{450} = 1.3) in 0.1 M phosphate buffer (pH 6.24) and 0.5 ml of the enzyme sample were mixed quickly, and the reduction in the absorbance at 450 nm was recorded using a TU-1810 UV spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) for 5 min. The difference in absorbance was recorded

each minute. All measurements were carried out at 25 °C and pH 6.24. The hydrolytic activity of (immobilized) lysozyme can be calculated using formula (1) below:

$$\text{activity(U/mg)} = \frac{\Delta \text{OD}_{450}}{0.001m} \quad (1)$$

where ΔOD_{450} is the difference in absorbance of *M. lysodeikticus* solution for the wavelength of 450 nm every minute; *m* refers to the mass (mg) of lysozyme in 0.5 ml of free enzyme solution or immobilized enzyme solution.

2.3. Methods of immobilization

Lysozyme solutions of various concentrations were dissolved in a phosphate buffer (0.1 M, pH 6.24) that ranged from 1.0 to 2.4 mg/ml. These lysozyme solutions were used to investigate the immobilization method.

2.3.1. Immobilization by physical adsorption

A total of 1.0 g of chitosan powder was incubated with 2.5 ml of the enzyme solution at 4 °C for 12 h. Next, the unbound enzyme was removed from the carrier by centrifugation (4000 rpm) for 5 min (or slightly longer) using distilled water until the protein could not be detected in washing [24]. The separated supernatant was collected and used to detect the content of unbound lysozyme and calculate the activity of immobilized enzyme.

2.3.2. Immobilization by covalent attachment

In separate experiments, a total of 1.0 g chitosan powder was treated with 2.5 ml of glutaraldehyde and stored at 4 °C for 5 h (cross-linking incubation). After treatment with glutaraldehyde, all carriers were collected by filtration and washed five times with distilled water by centrifugation (4000 rpm) for 5 min to remove excess glutaraldehyde. The wet chitosan was mixed with 2.5 ml of lysozyme solution and then stored at 25 °C for 4 h (immobilization time) in a water bath. The method described above was used to release the enzyme. To determine the optimal parameters for immobilization of lysozyme, the glutaraldehyde concentration, cross-linking and immobilization time were optimized. The activity of the immobilized enzyme was expressed as percent relative activity with respect to the specific activity of free enzyme (40,000 U/mg protein), which designated as 100%.

2.4. Immobilized lysozyme estimation

The amount of lysozyme bound to the chitosan powder was calculated by measuring the difference between the protein concentrations of the lysozyme in the supernatant before and after immobilization. The amount of protein in the initial solution and in the supernatant was measured according to UV absorbance assays at 450 nm using the *M. lysodeikticus* solution as a substrate [25].

2.5. Property characterization studies

2.5.1. Effect of pH and temperature on lysozyme activity

The effect of pH on the activity of the free and immobilized lysozyme was determined at 25 °C in a pH range varying from 3.5 to 8.0 in acetate buffer (0.1 M, pH 3.5–6.0) and phosphate buffer (0.1 M, pH 6.5–8.0), respectively. The effect of temperature on the free and immobilized lysozyme activity was studied in the temperature range between 20 °C and 90 °C with increasing intervals of 10 °C at pH 6.24. The *M. lysodeikticus* solution served as the substrate.

2.5.2. Reusability of immobilization lysozyme

Reactions were performed at 25 °C with 10 ml of the *M. lysodeikticus* solution in phosphate buffer (0.1 M, pH 6.24). All reactions were initiated by the addition of 1.0 g immobilized lysozyme, and each reaction was performed for 5 min. At the end of each batch, the immobilized lysozyme was collected from the reaction medium and washed with 0.1 M phosphate buffer (pH 6.24) to remove any substrate or product retained in the support material. The immobilized lysozyme was used again for the subsequent reaction cycle, which utilized fresh *M. lysodeikticus* solution.

2.5.3. Storage stability

Storage stability of free and immobilized enzyme was investigated by determining the residual activity after storing the enzyme at intervals of up to 60 days. Samples (1.0 g immobilized lysozyme) were stored in 0.1 M phosphate buffer (pH 6.24) at 4 °C. Immobilized lysozyme was separated from the buffer solution and was used for assay activity at intervals of five days.

2.5.4. Determination of kinetic constants

The kinetic constants, Michaelis constants K_m and V_{max}, for free and immobilized lysozyme were determined using *M. lysodeikticus* as substrate at concentrations between 0 and 400 μg/ml. The experiments were conducted under the above-determined optimized assay conditions. The apparent K_m and V_{max} values for the free and immobilized lysozyme were calculated using Lineweaver–Burk plots.

2.6. Preparation of edible coating

The edible coating was synthesized from a whey protein concentrate–chitosan coating. A plasticized blend of coating made from whey protein concentrate and chitosan was prepared under acidic conditions. The chitosan solution (CS) was prepared in 100 ml of 2% (v/v) acetic acid by dispersing the chitosan powder overnight with agitation by magnetic stirrer until the chitosan dissolved completely. The whey protein concentrate (WPC) solution was prepared in a 3% (w/v) glycerol (as plasticizer) water solution to obtain 100 ml 2% (w/v) suspension. The WPC solution was denatured in a water bath at 90 °C for 30 min under agitation to complete the protein denaturation. Before applying heat, the WPC solution was adjusted to pH 10 by the drop-wise addition of 1 M NaOH to prevent gelling of the protein. Immediately after the denaturation, the WPC solution was cooled in an ice bath to room temperature (25 °C). The two solutions were mixed using a magnetic stirrer to form the WPC–CS coating. The pH of the coating solution was 3.8–4.0 after mixing, and afterwards adjusted to 4.5 with 1 M NaOH solutions before each use.

2.7. Culture preparation

Two strains of *Escherichia coli* O157: H7 (CICC 21530, Gram-negative bacteria) and *Staphylococcus aureus* (CICC 21600, Gram-positive bacteria) were used in this study. Each strain was pre-cultured using nutrient broth slants stored at 4 °C. Cultures of each strain were transferred to fresh nutrient broth agar before use and were grown separately at 37 °C for 24 h.

2.8. Antimicrobial effects of edible coating

Free or immobilized lysozyme was added into the WPC–CS coating to form edible antimicrobial coatings. Briefly, 1.0 g immobilized lysozyme sample or 5.0 mg free lysozyme was dispersed into the WPC–CS coating under continuous stirring. The immobilization of lysozyme was obtained by loading 1.0 g chitosan powder with 5.0 mg lysozyme using the covalent attachment method or 4.5 mg of lysozyme using physical adsorption. The antimicrobial effect of immobilized lysozyme with lower activity was also investigated. Those immobilized enzyme samples were pre-treated at low pH (3.5) or high temperature (90 °C) as mentioned in Section 2.5.1. Another set of edible coatings without the addition of enzyme was used as a control.

The antimicrobial activity of the immobilized lysozyme was examined against *E. coli* and *S. aureus* according to the inhibition zone assay [26]. Fifteen milliliters of molten nutrient broth agar were inoculated with a 100 μl bacterial culture (colony count of 10^5 cfu/ml). One sterile 6 mm filter paper disc was placed at the center of the bacterial lawn, and the required amount of antibacterial coating (0.01 ml) with different enzyme samples was carefully pipetted onto it. The agar plates were then incubated at 37 °C for one week in an appropriate incubation chamber. All experiments were carried out under sterile conditions. The diameter of the inhibition zone (mm) surrounding the test coating was measured each day and was used as an indicator of antimicrobial effectiveness of the immobilization lysozyme.

3. Results and discussion

3.1. Immobilization studies

Studies have varied the immobilization method, cross-linker dose, enzyme concentration, and cross-linking and immobilization time within feasible parameter ranges to determine the optimal conditions for the immobilization of lysozyme.

3.1.1. Immobilization by covalent and physical methods

The enzyme dosage–activity profile for the lysozyme immobilized by covalent attachment is shown in Fig. 2, where it is compared with the profile obtained for the physically adsorbed enzyme in the same conditions. The relative activity of immobilized lysozyme prepared by physical adsorption reached the equilibrium (62.50% of the specific activity of free enzyme) when the concentration of free lysozyme was 1.8 mg/ml and over. Specifically, 1.0 g of chitosan powder could load 4.5 mg of lysozyme through the physical method, and the immobilized enzyme activity was 112,500 U/g chitosan. Compared with physical adsorption, the covalent attachment process led to increased loading of lysozyme and to higher relative activity. The maximum enzyme loading of 1.0 g chitosan onto 5.0 mg lysozyme was achieved using a 2.0 mg/ml solution of lysozyme. The maximum relative activity and immobilized enzyme activity for the enzyme loaded through the covalent process was 72.5% of the specific activity of free enzyme and 145,000 U/g chitosan.

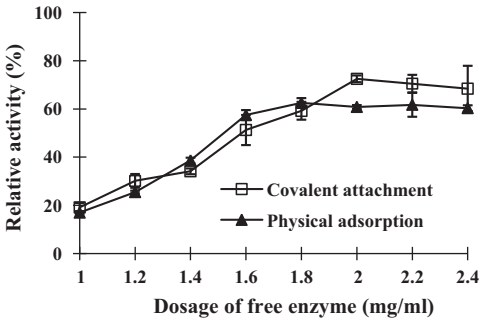

Fig. 2. The effects of enzyme doses on the activity of the immobilized enzyme. The experimental conditions were as follows: pH 6.24 and a concentration of glutaraldehyde 5% (v/v), the enzyme solution was incubated with *M. lysodeikticus* solution for 5 min at 25 °C. Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

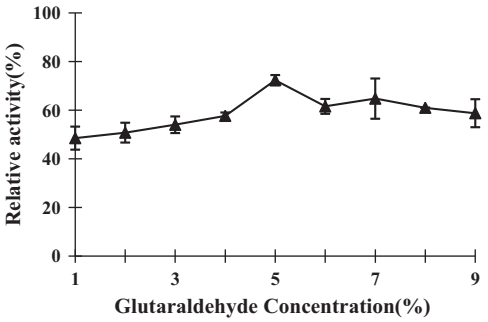

Fig. 3. Effects of the glutaraldehyde concentration on the activity of the immobilized enzyme. Experimental conditions were as follows: the enzyme solution (pH 6.24 and density 2.0 mg/ml) was incubated with 1.0 g chitosan that was prepared with different concentrations of glutaraldehyde for 5 h at 25 °C. Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

The reduction in specific activity after immobilization is a common phenomenon and may be attributed to the diffusional limitations resulting from the diminished molecular flexibility of the enzyme. In addition, during the immobilization process, the enzyme may be hidden and some active sites were damaged in the immobilized form of the enzyme [27,28]. Moreover, the activity of the physically immobilized lysozyme was lower than that of covalently bound lysozyme because it was fixed through physical adsorption, largely through intermolecular forces. The adsorption mechanism using intermolecular forces resulted in random orientations that were initiated by weak electrostatic interactions. There interactions may have facilitated easy desorption of the protein form the surface, eventually leaching to the sample solution during measurement and storage. The adsorption was also affected by pH and the temperature of the buffer. Moreover, the physical method displayed a low heat of adsorption, which makes the lysozyme immobilized on chitosan surface less stable than the enzyme immobilized by the covalent method. In contrast, the covalent attachment procedure was mediated by strong interactions with the carrier. It was less prone to protein detachment, and therefore, the stability of the linkage has been shown to be stronger with protein denaturation being less likely [29].

3.1.2. Effect of glutaraldehyde concentration

The concentration of the cross-linking agent had a significant effect on the activity of immobilized lysozyme. Fig. 3 shows the relative activity of immobilized lysozyme in different concentrations of glutaraldehyde (1–9%, v/v). Also, the values of enzyme activity, relative activity of immobilized enzyme and loaded proteins are shown in Table 1. In the range of concentrations that were investigated, the relative activities of the immobilized enzyme reached their maxima at a glutaraldehyde concentration of 5% (v/v). There was a decrease after the maximum point and a plateau was reached near 60% relative activity as the concentration increased further. The phenomenon mentioned above was attributed to the modification of critical residues and the quantity of binding sites in the protein that were essential for structure and activity. Because the amount of immobilized lysozyme was constant, excess of aldehyde groups for coupling lysozyme molecules were available. Consequently, the excess aldehyde groups may have resulted in unfolding, deformation or modification of the lysozyme molecules [27,30]. Similar results were reported by Martino *et al.* [31], whose research utilized glutaraldehyde as a cross-linking agent and immobilized β-glucosidase on chitosan. Alternatively, the random surface attachment could reduce protein function by steric hindrance of available bioactive sites. When the concentration of glutaraldehyde increased, the steric hindrance may have been revealed.

3.1.3. Effects of the cross-linking and immobilization time

The effects of the cross-linking time and the immobilization time (1–9 h) are shown in Fig. 4. Between 1 and 9 h, the relative activity gradually increased as the cross-linking period increased, and the maximum activity was observed at 5 h of cross-linking. The relative activity slightly decreased with cross-linking time beyond 5 h, but the decrease was fairly minimal. In some cases, an increase in cross-linking time made the cross-linking reaction complete, resulting in the enhancement of the overall cross-linking.

The effect of immobilization time on relative activity was distinct from the effects seen in the changes of the cross-linking time. The relative activity increased with the immobilization time and a significant increase took place when the immobilization time was increased from 3 to 4 h. Beyond 4 h, the relative activity decreased with immobilization time with only slight fluctuation. This result indicated that longer immobilization times led to denaturation and inactivation of the enzyme.

Table 1
The effects of the glutaraldehyde concentration on activity of the immobilized enzyme. Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

Concentration of gutaraldehyde (%)	Activity (U/mg protein)	Relative activity (%)	Loaded protein (mg)
1.0	19,402 ± 154.60	48.50 ± 0.30	3.35 ± 0.03
2.0	20,302 ± 153.00	50.76 ± 0.29	3.51 ± 0.03
3.0	21,605 ± 27.82	50.01 ± 0.01	3.73 ± 0.01
4.0	23,069 ± 50.00	57.67 ± 0.03	3.99 ± 0.01
5.0	28,940 ± 60.00	72.35 ± 0.05	5.00 ± 0.01
6.0	24,625 ± 40.73	61.56 ± 0.02	4.25 ± 0.01
7.0	25,899 ± 550.00	64.75 ± 3.78	4.47 ± 0.10
8.0	24,400 ± 0.00	61.00 ± 0.00	4.22 ± 0.00
9.0	23,501 ± 384.00	58.75 ± 1.84	4.06 ± 0.07

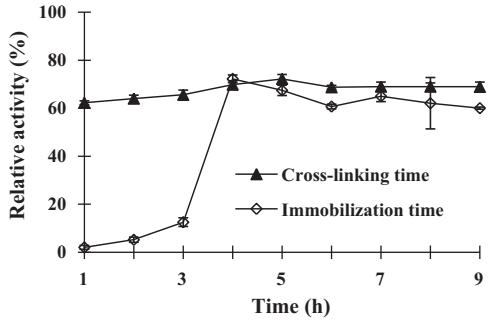

Fig. 4. The effects of the cross-linking and immobilization periods on the activity of the immobilized enzyme. Experimental conditions for cross-linking time were as follows: 1.0 g chitosan incubated with 5% glutaraldehyde (v/v) for varying durations at 4 °C. Experimental conditions for immobilization time were as follows: 1.0 g of the chitosan sample was incubated with the enzyme solution (pH 6.24 and density 2.0 mg/ml) for varying durations at 25 °C. Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

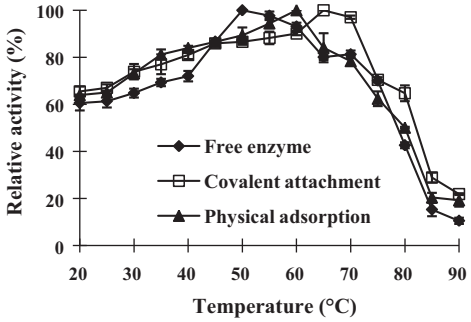

Fig. 6. The effects of temperature on the activity of free and immobilized lysozyme. The free and immobilized enzyme activity was assayed at varying temperatures between 20 and 90 °C, and at pH 6.24 for 5 min. The relative activities at the optimum temperature were designated as 100%. Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

3.2. Properties of the immobilized enzyme

3.2.1. The optimum pH and temperature

The effect of pH on the activity of both free and immobilized lysozyme was determined. A range of pH, between 3.5 and 8.0, was tested and the optimum pH values for the free and immobilized enzyme were 5.5 and 6.0, respectively (Fig. 5). The immobilized lysozyme showed a broadening in the pH activity profile on the chitosan powder. At the optimum pH, the maximum activities were 153,500 U/g chitosan for covalently bound enzymes and 120,500 U/g chitosan for physically adsorbed enzymes, but the activity of the free enzyme was only 35,070 U/mg protein at pH 5.5. At pH 4.5, which was the pH value for the WPC–CS coating, the relative activity was 57.9% for the covalently immobilized enzyme and was higher than that of both the physically adsorbed enzyme (53.6%) and the free enzyme (42.9%). The pH-activity profiles for the immobilized enzyme shifted towards more alkaline conditions and may be influenced by the electrostatic potential of the carrier microenvironment in the reaction [32]. Chitosan is a positively charged support, possessing both positive charge and electrostatic potential. The microenvironment of chitosan immobilized lysozyme consisted of an ion concentration that was lower than the bulk environment ($pH_{local} > pH_{bulk}$). Thus the pH-active profiles of the immobilized lysozymes moved to more alkaline pH values.

The optimum reaction temperatures were 65 °C for the covalently bound enzyme, and 60 °C for the physically adsorbed enzyme. Both of these temperatures were higher than that (50 °C) for the free enzyme as indicted in Fig. 6. At the optimum reaction temperature, the activities were 160,400 U/g chitosan (covalent attachment), 136,000 U/g chitosan (physical adsorption) and 36,490 U/mg protein (free enzyme), respectively. Compared with the free enzyme, the immobilized lysozyme displayed a broadened temperature profile. The multipoint covalent bond formation between chitosan powder and lysozyme could reduce the conformational flexibility and result in higher activation energy for the enzyme molecule to reorganize the proper conformation of binding to the substrate. Moreover, the increase in optimum temperature could be a result of the changing physical and chemical properties of the enzyme [33,34]. The higher values of the optimum reaction temperature for the immobilized enzyme also indicate that the applied immobilization procedure contributes to greater stability.

3.2.2. Reusability of the immobilized lysozyme

The reusability of the immobilized lysozyme was tested by measuring the hydrolysis of *M. lysodeikticus* over 14 cycles (Fig. 7). The covalently bound enzyme reached its equilibrium state at the fifth cycle and preserved approximately 80% of its original activity after 14 cycles. However, the residual activity of immobilized enzyme obtained through physical adsorption continued to decrease and retained only 54.3% of its catalytic activity at the end of the cycles. These results suggest that the strategy to immobilize the enzyme may help to preserve the enzymatic activity. However, the reusability of the covalently immobilized lysozyme on the chitosan powder appeared to be more stable than that of the physically adsorbed lysozyme. This was attributed to the considerable stability of the covalent attachment system over the stability of the physical adsorption system. Through fixation, the covalent attachments prevented conformational changes, and they maintained the

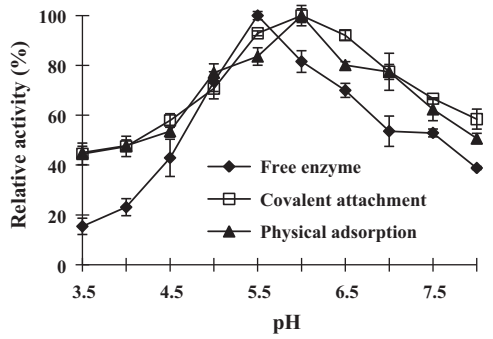

Fig. 5. The effects of pH on the free and immobilized lysozyme activity. The free and immobilized lysozyme activity was assayed at varying pH values between 3.5 and 8.0, and at 25 °C for 5 min. The relative activities at the optimum pH were designated to be 100%. Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

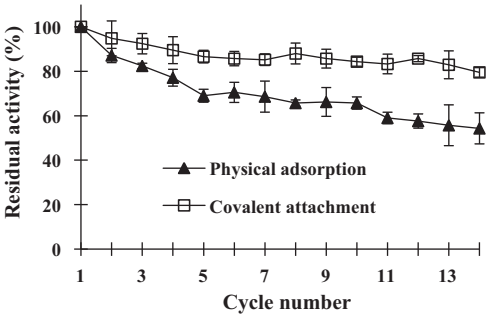

Fig. 7. The reusability of the immobilized lysozyme. The sample was incubated in a phosphate buffer solution 0.1 M (pH 6.24) for 5 min at 25 °C. The residual activity was calculated by designating the initial activity of enzyme as 100%. Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

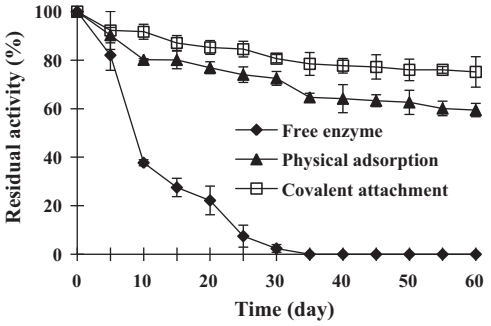

Fig. 8. Storage stability of the free and immobilized lysozyme. The enzyme sample was stored for 60 days in 0.1 M phosphate buffer (pH 6.24) at 4 °C. The residual activity was calculated by designating the initial activity of enzyme as 100%. Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

chemical structure of the enzyme better than the physical adsorption method. This result demonstrates that lysozyme immobilized on the chitosan powder may be used successfully for industrial applications requiring long-term reaction stability.

3.2.3. Storage stability

Stability is the most important requirement for immobilized enzymes in industrial applications, and an increased stability may render an immobilized lysozyme as more advantageous than its free form. The stability of immobilized lysozyme was evaluated over a period of 60 days at 4 °C. The enzymatic activity was determined for each sample (Fig. 8). The free enzyme lost 62.29% of its initial activity within 10 days and nearly 100% of its activity after a month. Under the same storage conditions, the activity losses of both physically and covalently immobilized enzyme were lower than that of the free enzyme. After 35 days, the activity loss of physically adsorbed enzyme was approximately 35%, and was 40.57% after 60 days. However, the covalently bound lysozyme exhibited the highest storage stability. After one month, it preserved more than 80% of its initial activity, and approximately 75% of the initial activity was maintained after 60 days. The multipoint immobilization may prevent the dissociation of enzyme from the carriers to prevent leaking of the enzyme into buffer solution. Furthermore, the multipoint covalent attachment system between the enzyme and the carrier conveyed a higher conformational stability.

3.2.4. Determination of the K_m and V_{max} of free and immobilized enzymes

The kinetic parameters, Michaelis constants K_m and V_{max}, for free and immobilized lysozyme were determined using the Lineweaver–Burk plot (Fig. 9). The regressions for the Lineweaver–Burk plots of free and immobilized lysozymes were $y_F = 0.0014x + 0.000057$ ($R^2 = 0.9938$), $y_P = 0.0020x + 0.000074$ ($R^2 = 0.9962$) and $y_C = 0.0020x + 0.000071$ ($R^2 = 0.9987$), respectively. The K_m of the free enzyme was 24.54 μg/ml, and the V_{max} was calculated to be 1.75×10^4 U/mg protein. The kinetic constants of the immobilized lysozyme were also determined in a batch system. The K_m values were 27.03 μg/ml and 28.17 μg/ml for the physical adsorption and covalent attachment systems, respectively. The V_{max} values of the immobilized lysozyme for the physical and covalent preparations were estimated from the data to be 1.35×10^4 and 1.41×10^4 U/mg, respectively. As expected, the K_m and V_{max} values were significantly affected after immobilization onto the chitosan powder. The slightly elevated K_m of the immobilized enzyme was attributed to the diffusional resistance of the carrier against the substrate and/or the product [35].

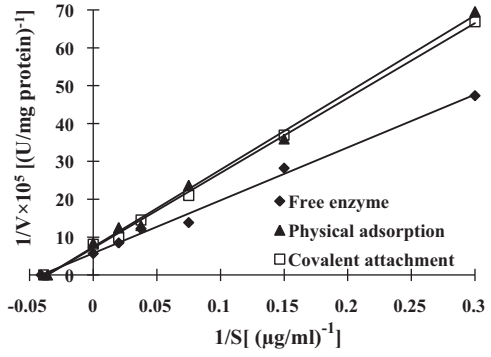

Fig. 9. Lineweaver–Burk plot for estimating of kinetic parameters.

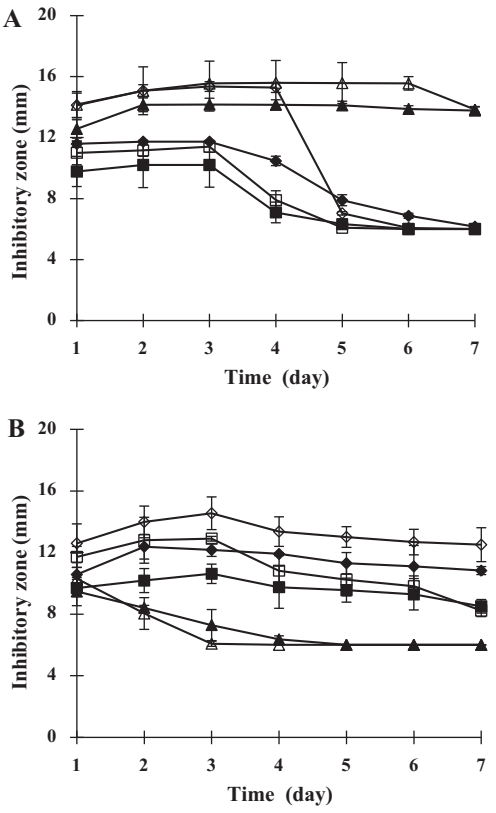

Fig. 10. Antimicrobial effects of the enzyme. The experiment was performed at 25 °C and pH 4.5. (A) The enzyme without any pretreatment. Symbols: (♦) free lysozyme inhibiting *E. coli*; (▲) physically adsorbed lysozyme inhibiting *E. coli*; (■) covalently bound lysozyme inhibiting *E. coli*; (◊) free lysozyme inhibiting *S. aureus*; (△) physically adsorbed lysozyme inhibiting *S. aureus*; (□) covalently bound lysozyme inhibiting *S. aureus*. (B) The enzyme was pretreated at low pH (3.5) or high temperature (90 °C). Symbols: (♦) covalently bound enzyme pretreated at low pH inhibiting *E. coli*; (■) covalently bound lysozyme pretreated at high temperature inhibiting *E. coli*; (▲) edible coating with enzyme inhibiting *E. coli*; (◊) covalently bound enzyme pretreated at low pH inhibiting *S. aureus*; (□) covalently bound lysozyme pretreated at high temperature inhibiting *S. aureus*; (△) edible coating with enzyme inhibiting *S. aureus*. That diminished inhibition zone (to 6 mm) suggests that the samples lost their antimicrobial activities. Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

3.3. Antimicrobial effect of immobilized enzyme

The antimicrobial effect of immobilized lysozyme is the most important property for the industrial application of antimicrobial agents. The antimicrobial efficiency of immobilized lysozyme in edible coatings has been investigated (Fig. 10). Each enzyme sample in the WPC–CS coating, including free lysozyme and immobilized lysozyme with and without pretreatment at low pH or high temperatures exhibited antimicrobial activity against microorganisms.

As expected, the significant antimicrobial activity against *E. coli* and *S. aureus* was observed when the coating contained covalently immobilized enzyme throughout the entire process, and the growth of the microorganism was inhibited for a week (Fig. 10A). Physically immobilized lysozyme expressed a clear inhibitory effect that lasted approximately six days, but the inhibition zone was small. Free enzyme had little antimicrobial effect against microorganisms. Starting on the third day, the inhibition zone began to shrink, and by the fourth day, the active coating lost its antimicrobial effect. To investigate the antimicrobial effects and the stability of the immobilized lysozyme with low activity, the pretreatment was conducted at low pH or high temperature (Fig. 10B). After pretreatment at low pH (3.5), the antimicrobial effects of the covalently immobilized enzyme were identical to the sample without pretreatment. Although the activity of covalently immobilized enzyme after treating at high temperatures was lower (48,120 U/g chitosan) than that without pretreatment, the antimicrobial effect was still demonstrated for a week. However, extreme conditions significantly affected the antimicrobial efficacy of the physically immobilized lysozyme, and its antimicrobial effect was weak and short (data not shown).

The results reveal that the covalently immobilized lysozyme was more effective at inhibiting the growth of microorganisms in WPC–CS coatings than the enzyme in other forms. Also, its antimicrobial effect could last as long as a week. Even treated at extreme conditions, it also displayed significant antimicrobial effects. This result is largely a result of the excellent stability of the enzyme immobilized by the covalent method. Therefore, this system has great potential as an active component in edible packaging.

4. Conclusion

One important aim of this study was to enhance the antimicrobial effects of immobilized lysozyme in edible coating relative to the free form. In this work, lysozyme was successfully immobilized onto the surface of chitosan powder through both physical adsorption and covalent attachment with a glutaraldehyde cross-linker. The immobilized lysozyme was also incorporated into a WPC–CS coating to investigate its antimicrobial effect as an active component. The results presented here demonstrated that the covalently immobilized lysozyme had the maximum activity (145,000 U/g chitosan) and its relative activity was 72.50%. The optimum conditions for the covalently immobilized lysozyme on chitosan powder were found to be 2.0 mg/ml for lysozyme solution, 5% (v/v) for glutaraldehyde concentration, 5 h for cross-linking time, and 4 h for immobilization time. The covalently immobilized enzyme displayed fairly remarkable stability. It also could be reused 14 cycles with approximately 80% of the original activity being retained. When applied as part of an edible coating, the covalently immobilized lysozyme could inhibit the growth of microorganism effectively even with low activity. Meanwhile, its antimicrobial effect could last for as long as a week. Therefore, the edible antimicrobial coating could be effectively used on preservation of foods. Future research will focus on quantifying immobilized enzymes in the WPC–CS coating or other edible coatings and on the synergistic effects between immobilized enzymes and their coatings. Moreover, the preservation of edible coating with immobilized lysozyme should be investigated on fruit, vegetables, meat, and other foods that require hygienic freshness.

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