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Polydopamine-coated chitosan hydrogels for enzyme immobilization

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Abstract To address inherent weaknesses such as low mechanical strength and limited enzyme loading capacity in conventional chitosan or alginate beads, an additional step involving the exchange of anionic surfactants with hydroxide ions was employed to prepare porous chitosan hydrogel capsules for enzyme immobilization. Consequently, excellent thermal stability and long-term storage stability were confirmed. Furthermore, coating the porous chitosan hydrogel capsules with polydopamine not only improved mechanical stability but also exhibited remarkable enzyme immobilization efficiency (97.6% for M1-D0.5). Additionally, it was demonstrated that the scope of application for chitosan hydrogel beads, prepared using conventional methods, could be further expanded by introducing an additional step of polydopamine coating. The enzyme immobilization matrix developed in this study can be selectively applied to suit specific purposes and is expected to be utilized as a support for the adsorption or covalent binding of various substances.

Keywords Chitosan hydrogel · Enzyme immobilization · Hydrogel capsule · Polydopamine-coated · Stability

Introduction

Chitosan is a polycationic linear polymer generated through various degrees of *N*-deacetylation from chitin, a natural polymer [1,2]. Chitosan not only has many amino groups with positive charges (-$NH3^+$) but also other functional groups (hydroxyl-, acetamido-), so its surface is hydrophilic and has diverse chemical reactivity. Furthermore, chitosan is inexpensive, non-toxic, biodegradable, and exhibits excellent biocompatibility, leading to its wide usage in food, the environment, and medicine. While research on utilizing chitosan hydrogels as enzyme immobilization matrices has been actively conducted, challenges like weak mechanical strength have limited its applications, resulting in issues such as enzyme desorption from chitosan matrices. Consequently, ongoing research aims to address these challenges and enhance the practical applications of chitosan hydrogels in enzyme immobilization [3,4].

To address the issues associated with chitosan hydrogel beads produced by the conventional method using an alkaline solution (OH^-) as a gelling agent, our research group employed sodium dodecyl sulfate (SDS), an anionic surfactant, instead of an alkaline solution to produce a novel form of chitosan hydrogel capsule with enhanced mechanical strength and adsorption capacity per unit dry weight [5] (Fig. 1). The studies initially focused on utilizing it as an adsorbent for water treatment to eliminate contaminants like heavy metals and dyes. Subsequently, this research group applied the process of replacing SDS with hydroxide ions by adding NaOH as a gelling solution to chitosan hydrogel capsules obtained using an SDS solution, thereby creating porous chitosan hydrogel capsules with further improved adsorption capacity. Furthermore, our research group applied an additional process to enhance adsorption capacity by introducing a procedure where NaOH, as a gelation solution, was added to chitosan hydrogel capsules obtained using an SDS solution. This process replaced SDS with hydroxide ions in the shell of the capsules, resulting in the production of porous chitosan hydrogel capsules with improved adsorption capabilities [6]. Additionally, the author's research group developed a novel enzyme immobilization method by applying modified procedures to immobilize the model enzyme *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (TeSADH; thermostable alcohol dehydrogenase) using porous chitosan hydrogel capsules as carriers [7].

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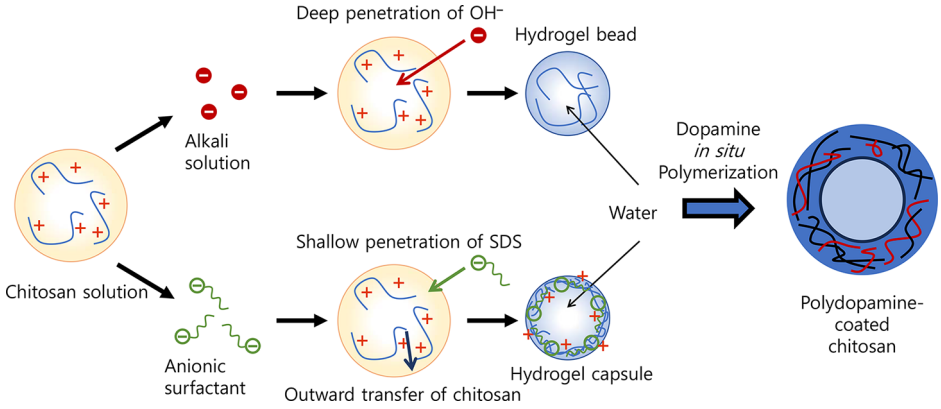

Fig. 1 Schematic representation illustrating the distinctions between conventional chitosan hydrogel beads and the chitosan hydrogel capsules prepared in this study, accompanied by the concept of polydopamine coating

To develop enzyme immobilization matrices capable of maintaining operational stability even in some enzymatic reaction processes where it is desirable to perform under reaction conditions requiring enhanced mechanical stability (e.g., with high shear stress or using organic solvent reaction media), this study adopted a polydopamine surface coating process onto porous chitosan hydrogel capsules and chitosan hydrogel beads by *in situ* polymerization of dopamine.

Materials and Methods

Reagents

Chitosan (>85% deacetylated) and SDS were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Expression and purification of TeSADH enzyme

The double mutant (C295A/I86A) TeSADH enzyme, designated as DM TeSADH, used as a model enzyme for immobilization, was expressed in *E. coli* DH5α cells and purified following the method previously reported by the author's research group [8,9].

Preparation of porous chitosan hydrogel capsules using a 0.5% (w/v) chitosan solution (M1 capsules)

Chitosan powder (0.5 g) was added to 20 mL of a 5% (v/v) acetic acid solution and initially dissolved for about 2 h. Distilled water was then added to reach a final volume of 100 mL. A 0.5% (w/v) chitosan solution dissolved in a final 1% (v/v) acetic acid solution was prepared by continuously shaking at 150 rpm in a shaking incubator at 30 ºC for 24 h. For the gelation process, 10 mL of the chitosan solution (equivalent to 0.05 g of chitosan) was slowly injected dropwise using a syringe at a flow rate of 0.1 mL/min (roughly 20 µL volume per drop) into 200 mL of a 5 g/L SDS solution, while stirred at 150 rpm at room temperature for 3 h. The resulting chitosan hydrogel capsules were filtered and washed three times with distilled water to remove any residual SDS on the gel surface, and then stored in distilled water.

The prepared chitosan hydrogel capsules were subjected to additional gelation replacement in NaOH as follows. The hydrogel capsules were stirred slowly in a 0.05 M NaOH solution at room temperature for 3 h. During this process, SDS in the shell of the capsules was extracted, simultaneously replacing it with hydroxide ions. The porous chitosan hydrogel capsules were filtered and washed several times with distilled water to remove residual sodium hydroxide and SDS. The resulting porous chitosan hydrogel capsules were stored in either a storage buffer solution (50 mM Tris-HCl buffer containing 10 µM $ZnCl_2$; pH 8.0 at 25 ºC) when used as a carrier for enzyme immobilization with DM TeSADH or in distilled water when proceeding to the additional polydopamine coating step.

Preparation of porous chitosan hydrogel capsules using a 1% (w/v) chitosan solution (M2 capsules)

To further increase the strength of chitosan hydrogel capsules, a commonly used 1% (w/v) chitosan solution was employed in the preparation of chitosan hydrogel capsules. A 1% (w/v) chitosan solution dissolved in a final 2% (v/v) acetic acid solution was used according to the preparation M1 method with some modifications to the conditions as follows. 1 gram of chitosan powder was added to 40 mL of 5% (v/v) acetic acid solution. Additional shaking was performed at 35 ºC for an additional 6 h. For gelation, 10 mL of the 1% (w/v) chitosan solution (equivalent to 0.1 g of chitosan) was injected dropwise into 200 mL of 5 g/L SDS solution, following the steps of gelation and subsequent replacement in NaOH.

Preparation of alkaline chitosan hydrogel beads using an alkaline gelation solution

Similar to the M2 method, a 1% (w/v) chitosan solution dissolved in a final concentration of 2% (v/v) acetic acid solution was prepared and used. Unlike the M2 method, an alkaline gelation

solution was used instead of an SDS solution. The preparation of alkaline chitosan beads was carried out as follows. A 10 mL portion of the previously prepared 1% (w/v) chitosan solution (equivalent to 0.1 g of chitosan) was slowly dropped, as mentioned in the M1 method, but instead into 200 mL of slowly stirred alkaline gelation solution, composed of 10% sodium hydroxide by weight, 50% methanol, and 40% distilled water. After being left at room temperature for 3 h with slow stirring, the resulting chitosan particles were filtered and washed several times with distilled water to remove residual sodium hydroxide and methanol. The prepared alkaline chitosan beads were stored in distilled water to further proceed with the polydopamine coating step.

Preparation of polydopamine-coated chitosan hydrogel capsules and beads

Polydopamine coating was additionally performed on the chitosan hydrogel capsules prepared according to the above M1 and M2 methods, respectively. The porous chitosan hydrogel capsules produced using each method (referred to as M1 capsules and M2 capsules, respectively) were placed in a beaker containing distilled water. Ammonia water was added to adjust the pH to approximately 8.5-9, while gently stirred at room temperature. Then, 1 g of dopamine dissolved in 10 mL of distilled water was injected dropwise using a syringe at a flow rate of 0.1 mL/min. After stirring for an additional 4 h, the mixture was left undisturbed for 24 h. The polydopamine-coated chitosan hydrogels were retrieved, excess dopamine was removed, and they were washed several times with distilled water to achieve a pH of 7. The coated hydrogel capsules were then placed in a storage buffer solution for enzyme immobilization.

The prepared polydopamine-coated hydrogel capsules were designated as M1-D1.0 (weight ratio, chitosan:dopamine =1:20) and M2-D1.0 (weight ratio, chitosan:dopamine =1:10), respectively. In the case of the chitosan hydrogel capsule prepared using the method M1, the polydopamine coating was additionally performed using the above method with a solution of 0.5 or 1.5 g of dopamine dissolved in 10 mL of distilled water, respectively, resulting in M1-D0.5 (1:10) and M1-D1.5 (1:30).

Polydopamine coating for alkaline chitosan hydrogel beads (equivalent to 0.1 g of chitosan) was carried out under the same conditions as the M2-D1.0 capsules. Polydopamine coating was performed on alkaline chitosan hydrogel beads using a solution of 1 g of dopamine dissolved in 10 mL of distilled water, resulting in a weight ratio of chitosan to dopamine of 1:10.

Enzyme immobilization by adsorption method

DM TeSADH enzyme was immobilized onto chitosan hydrogel capsules and beads using the enzyme adsorption method as described below. In the enzyme adsorption process, 20 chitosan hydrogel capsules or beads were placed in a 15-mL conical tube containing 4 mL of storage buffer. Subsequently, 200 μL of the enzyme (1.2-2 mg) was added, and the mixture was gently shaken at 25 ºC, 100 rpm for 24 h. The chitosan hydrogel capsules and beads immobilized with the enzyme were washed about 7 times with a storage buffer solution and then stored in the storage buffer solution.

Measurement of the activity of free and immobilized DM TeSADH enzymes

The enzyme activity of free and immobilized DM TeSADH enzymes was determined by measuring the spectrophotometric absorbance of NADPH production at 340 nm (ε_{340}=6.2 $mM^{-1}cm^{-1}$) for 2 min at 60 ºC to determine the slope. Values were expressed as the average of triplicates. For the activity analysis of immobilized enzymes, three different hydrogel capsules were used. The reaction mixture consisted of a total of 1.2 mL of 50 mM Tris-HCl (pH 8.9 at 60 ºC) containing 0.5 mM $NADP^+$, 10 μM $ZnCl_2$, 3.0 mM DTT, and 4.0 mM isopropanol as the substrate.

Thermal stability and long-term storage stability of DM TeSADH enzyme immobilized on chitosan hydrogel capsules with polydopamine coating

The thermal stability analysis of DM TeSADH enzyme immobilized on chitosan hydrogel capsules with polydopamine coating was conducted at two different temperatures. The residual enzyme activity was expressed as a percentage relative to the initial activity value.

In the first thermal stability experiment, three different hydrogel capsules were taken out every 30 min while remaining in a constant-temperature water bath at 75 ºC for 150 min. TeSADH enzyme activity was measured at 60 ºC according to the method described above. In the second experiment, the thermal stability of the immobilized enzyme on hydrogel capsules was evaluated at the temperature of 60 ºC, which is the reaction temperature of the thermostable TeSADH enzyme. Three hydrogel capsules were initially placed in the 60 ºC constant-temperature bath for 120 min, and all three capsules were taken out every 30 min, and the residual activity of the TeSADH enzyme was measured at 60 ºC according to the method described above.

The long-term storage stability analysis of the TeSADH enzyme immobilized on chitosan hydrogel capsules with polydopamine coating was conducted over 16 days at 25 ºC. The residual enzyme activity was expressed as a percentage relative to the initial activity value. Three hydrogel capsules were placed in the constant-temperature chamber at 25 ºC from the start, and at each measurement point over the 16 days, all three capsules were taken out to measure TeSADH enzyme activity at 25 ºC as described above.

Table 1 Comparison of enzyme immobilization efficiency on chitosan hydrogel capsules under different polydopamine coating conditions

	M1	M1-D0.5	M1-D1.0	M1-D1.5	M2	M2-1.0	Free Enzyme
Bound activity (μmole/min)	0.818	2.364	1.513	1.492	1.515	0.476	2.754
Unbound activity (μmole/min)	0.650	0.066	0.195	0.519	0.727	0.750	
Immobilization Efficiency (%)	76.4	97.6	92.9	81.1	73.6	72.8	

The abbreviations are as follows: M1 and M2; double mutant (C295A/I86A) TeSADH enzyme immobilized on chitosan hydrogel capsules prepared using 0.5% (w/v) and 1% (w/v) chitosan solutions, respectively, without additional polydopamine coating. M1-D0.5, M1-D1.0, and M1-D1.5; double mutant (C295A/I86A) TeSADH enzyme immobilized on chitosan hydrogel capsules prepared using a 0.5% (w/v) chitosan solution, with additional polydopamine coating utilizing 0.5, 1, and 1.5 g of dopamine solutions at weight ratios of chitosan to dopamine of 1:10, 1:20, and 1:30, respectively. M2-D1.0; double mutant (C295A/I86A) TeSADH enzyme immobilized on chitosan hydrogel capsules prepared using a 1% (w/v) chitosan solution, with additional polydopamine coating utilizing 1 g of dopamine solution, at a weight ratio of chitosan to dopamine of 1:10

Results

Comparison of enzyme immobilization efficiency on chitosan hydrogel capsules under different polydopamine coating conditions

The enzyme immobilization efficiency according to different enzyme immobilization methods was calculated using Equation (1) below, and the comparison results are shown in Table 1.

Enzyme immobilization efficiency (%) = [(Free enzyme activity – Unbound enzyme activity) / Free enzyme activity]×100 (1)

In the case of porous chitosan hydrogel M1 capsules prepared using a 0.5% (w/v) chitosan solution by sequentially replacing the anionic surfactants SDS (5 g/L) and NaOH (0.05 M) with a gelling solution, the immobilization efficiency significantly improved with polydopamine coating, increasing from 76.4% for M1 capsules to 97.6% for M1-D0.5 capsules. However, as the content of treated dopamine increased, the enzyme immobilization efficiency dropped to levels similar to those without polydopamine coating. The additional polydopamine coating on chitosan hydrogels may allow a greater amount of enzyme to be loaded on the surface generated by the polydopamine-chitosan composite, but it may not affect the specific activity of the enzyme. More efficient enzyme adsorption is possibly due to the interactions between the enzyme and the polydopamine-chitosan composite, such as electrostatic interactions, van der Waals forces, and so on [10]. However, chitosan hydrogels may be potentially over-coated with polydopamine as the amount of added dopamine increases. Under the conditions of this study, it was observed that coating with 0.5-1 g of dopamine (chitosan to dopamine weight ratio = 1:10-20) efficiently enhanced enzyme immobilization.

Additionally, the enzyme activity of immobilized DM TeSADH enzymes at 60 ºC, prepared under various polydopamine coating conditions, is compared and presented in Fig. 2. For M1 capsules, the activity of the immobilized DM TeSADH enzyme increased with polydopamine coating, similar to the pattern of enzyme immobilization efficiency shown in Table 1, and the highest enzyme activity level was also achieved at a weight ratio of chitosan to dopamine of 1:20. In the case of M2 capsules, the activity of the immobilized DM TeSADH enzyme was also significantly improved by polydopamine coating.

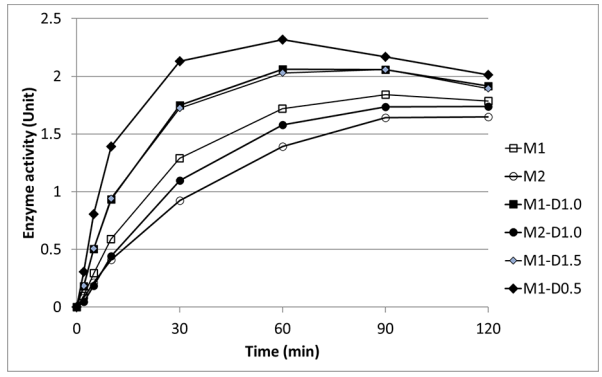

Fig. 2 Comparison of the NADPH production profile at 340 nm for DM TeSADH enzymes at 60 ºC when immobilized on chitosan hydrogel capsules under various polydopamine coating conditions. The abbreviations are explained in the notes section of Table 1

Thermal stability and long-term storage stability of DM TeSADH enzyme immobilized on chitosan hydrogel capsules with polydopamine coating

Thermal stability analysis of DM TeSADH enzyme immobilized on chitosan hydrogel capsules under polydopamine coating conditions was performed at two different temperatures by determining the residual enzyme activity of DM TeSADH relative to the initial value. The results of thermal stability at 75 ºC (Fig. 3A) revealed that the M2 capsules without polydopamine coating exhibited excellent thermal stability, maintaining approximately 93% relative enzyme activity up to 60 min and 68% up to 150 min. Similarly, even in the case of M1 capsules without polydopamine coating, 50% relative enzyme activity was maintained for up to 150 min. The residual activity of free and immobilized DM TeSADH enzyme on chitosan hydrogel M1 capsules at 80 ºC was previously shown by the author's research group elsewhere [7].

However, the thermal stability of both capsules with polydopamine coating decreased compared to the case without polydopamine coating. It was observed that the polydopamine-coated capsules gradually faded from dark brown to light brown over time, especially during the first 60 min. When considered together with

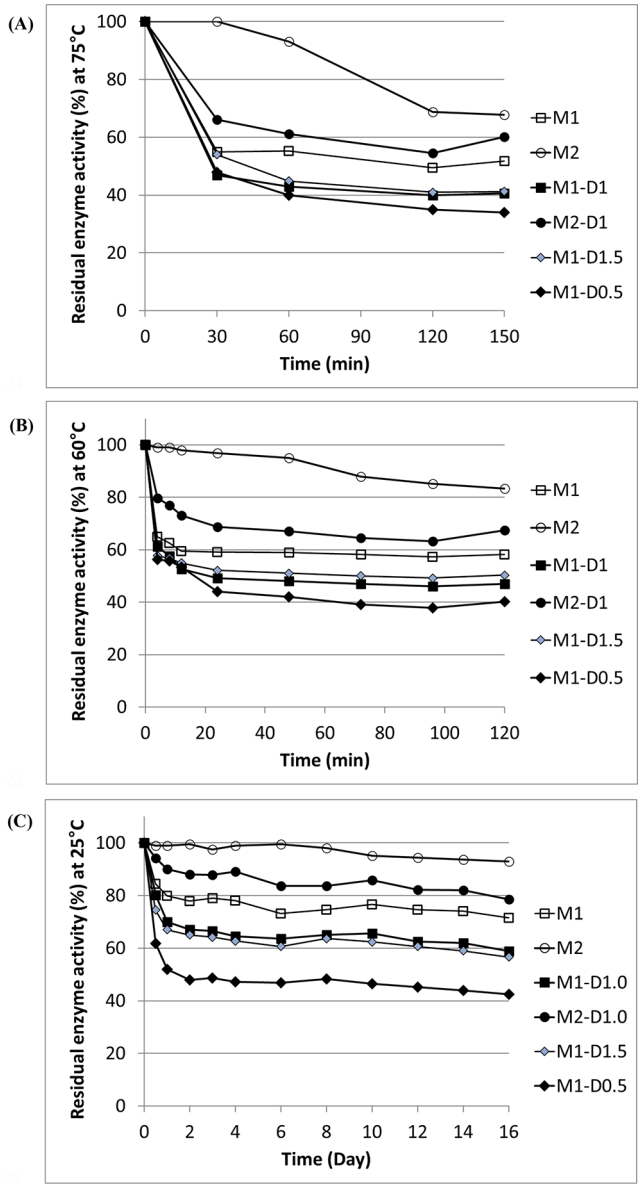

Fig. 3 Residual enzyme activity (%) at three different temperatures of DM TeSADH enzyme immobilized on chitosan hydrogel capsules with polydopamine coating. (A) Thermal stability at 75 ºC. The values represent the average of dual experimental data. (B) Thermal stability at 60 ºC. The values represent the average of triple experimental data. (C) Long-term storage stability at 25 ºC. The values represent the average of triple experimental data. The abbreviations are explained in the notes section of Table 1

the residual enzyme activity profile, the polydopamine coating tends to detach slowly, along with the immobilized enzyme, from chitosan hydrogel capsules at elevated temperatures. Particularly, more enzyme desorption occurred for M1-D0.5 capsules, probably because a greater amount of enzyme was adsorbed on a smaller amount of dopamine.

Thermal stability of the enzyme immobilized on the hydrogel capsules at 60 ºC (Fig. 3B) revealed patterns in residual enzyme activity profiles, capsule size reduction, and color changes similar to those observed at 75 ºC. Even for the M1-D0.5 capsules, which showed the lowest residual enzyme activity, residual enzyme activity was maintained at around 40% after 60 min. Considering that the temperature for most enzymatic reaction processes is below 60 ºC, it is deemed that the M1-D0.5 capsules with polydopamine coating could still be effectively utilized.

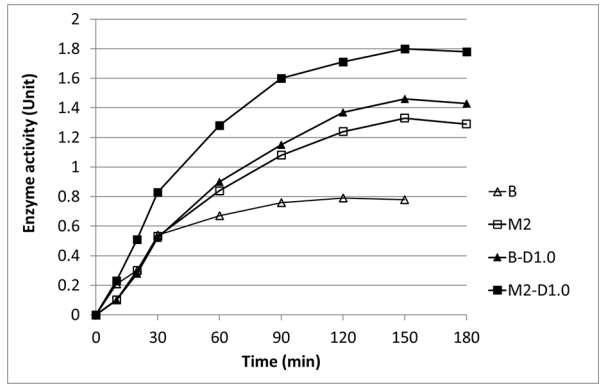

Fig. 4 Comparison of the NADPH production profile at 340 nm for DM TeSADH enzymes at 60 ºC when immobilized on chitosan hydrogel capsules and chitosan hydrogel beads, both with and without polydopamine coating. Polydopamine-coated capsules (M2-D1.0) and beads (B-D1.0) were prepared with the same chitosan to dopamine weight ratio (1:10). The abbreviations mean as follows: B; double mutant (C295A/I86A) TeSADH enzyme immobilized on chitosan hydrogel beads prepared using a 1% (w/v) chitosan solution without additional polydopamine coating. M2; double mutant (C295A/I86A) TeSADH enzyme immobilized on chitosan hydrogel capsules prepared using a 1% (w/v) chitosan solution without additional polydopamine coating. B-D1.0; double mutant (C295A/I86A) TeSADH enzyme immobilized on chitosan hydrogel beads prepared using a 1% (w/v) chitosan solution, with additional polydopamine coating utilizing 1 g of dopamine solution, at a weight ratio of chitosan to dopamine of 1:10. M2-D1.0; double mutant (C295A/I86A) TeSADH enzyme immobilized on chitosan hydrogel capsules prepared using a 1% (w/v) chitosan solution, with additional polydopamine coating utilizing 1 g of dopamine solution, at a weight ratio of chitosan to dopamine of 1:10

The results of long-term storage stability at 25 ºC (Fig. 3C) showed that the M2 capsules without polydopamine coating, in particular, exhibited exceptional stability, maintaining approximately 93% relative enzyme activity even after 16 days of storage. Although the M1-D0.5 capsules, coated with polydopamine at a weight ratio of chitosan to dopamine of 1:20, showed the lowest values, they still demonstrated 43% residual enzyme activity after 16 days. The polydopamine-coated capsules stored at 25 ºC faded slightly over time, but to a much lesser extent than observed at higher temperatures.

Comparison of the activity of DM TeSADH enzyme immobilized on chitosan hydrogel capsules and beads with and without polydopamine coating

An experiment was performed to compare the activity of the TeSADH enzyme immobilized on four different forms of chitosan

hydrogels (Fig. 4): M2 capsules and beads were prepared using the same amount of chitosan, and polydopamine-coated capsules (M2-D1.0) and beads (B-D1.0) with the same chitosan to dopamine weight ratio (1:10). As evident from Table 1 and Fig. 2, comparing two types of chitosan hydrogel capsules and their polydopamine-coated forms reveals that M2 and M2-D1.0 capsules exhibited lower enzyme immobilization efficiency and lower enzyme activity compared to the M1 capsule series, particularly M1-D0.5. However, as indicated in Figs. 3, the M2 and M2-D1.0 capsules showed better thermal stability and long-term storage stability of the immobilized TeSADH enzyme, so they were selected and compared with the alkaline chitosan hydrogel beads.

The enzyme adsorption process was performed with some modifications as follows. The process was conducted at 25 ºC in 50 mM Tris-HCl buffer solution (pH 8.9 at 60 ºC) containing 10 μM $ZnCl_2$ with gentle shaking at 120 rpm for 5 h. In this case, the activity of the immobilized TeSADH enzyme was measured at 60 ºC using two types of hydrogels. The results showed that both M2 capsules and chitosan beads exhibited an increase in the activity of immobilized enzymes by introducing the polydopamine coating addition process (Fig. 4), and the M2 series capsules showed better results compared to chitosan beads.

Discussion

The conventional method of producing chitosan hydrogel beads involves using an alkaline solution as a gelling agent for gelation through neutralization, achieved by dropping chitosan solution dissolved in acid into an anionic alkaline solution. However, there is a limitation in controlling the density of the final beads because the hydroxide ions in the gelation solution rapidly penetrate the chitosan droplets, causing instant neutralization. Ultimately, this results in the formation of highly hydrated hydrogels with very large porosity, which can lead to various issues in practical industrial processes.

Dopamine has been reported to form a polydopamine coating layer through the oxidation of catechol on the surface of various materials, regardless of the chemical properties of the surface, when a substance intended for surface modification is placed in a dopamine solution under basic pH conditions (pH 8.5 or higher), similar to the marine environment, and taken out after a certain period of time [11]. The polydopamine coating layer formed on the surface retains catechol functional groups, which possess both oxidation and reduction capabilities. It enables immobilization on the surface through specific adhesion as well as metal ion reduction, allowing metal nanoparticles to be placed on the desired surface. Additionally, since the catechol functional group can form a covalent bond with the amine functional group, various molecules having an amine functional group can be introduced to the surface as a secondary surface modification.

The purpose of this study is to selectively utilize porous chitosan hydrogel capsules, prepared by anionic surfactant gelation and sequential alkaline substitution, along with additional polydopamine-coated chitosan hydrogel capsules, as versatile matrices for enzyme immobilization to suit the intended use. In more detail, the hierarchically porous structures developed in this study, combined with factors that can protect, maintain, and enhance enzyme activity, are envisioned to be utilized as composite enzyme (or protein) immobilization matrices in various industrial bioprocesses. Additionally, the results of this study could find valuable applications in industries and research and development related to the use of proteins, including enzymes, such as in the biosensor manufacturing process.

The chitosan-polydopamine composite developed in this study fundamentally addresses the major issue of weak mechanical strength inherent in conventional chitosan hydrogels used for enzyme immobilization. Thermal stability and long-term storage stability of enzymes immobilized on chitosan hydrogel capsules or beads with polydopamine coating could be improved through the possible formation of covalent bonds between the amine groups of chitosan and the quinone groups of oxidized forms of dopamine during the *in situ* polymerization process, with an additional step such as UV irradiation [10]. Before practical applications, especially for M2-D1.0 hydrogel capsules and B-D1.0 hydrogel beads, the weight ratio of chitosan to dopamine needs to be optimized for additional polydopamine coating.

This breakthrough is expected to impart remarkable performance and new characteristics in the immobilization of enzymes, proteins, cells, drugs, nanoparticles, and metal catalysts. Porous chitosan hydrogel capsules are particularly suitable for applications in food and pharmaceutical processes, while polydopamine-coated chitosan hydrogel beads and capsules are deemed suitable for applications in enzymatic reaction processes, biosensor fabrication, and other processes where greater mechanical stability is desired under reaction conditions.

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