Covalent Immobilization of β-Galactosidase on Carrageenan Coated with Chitosan

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ABSTRACT: β-Galactosidase was covalently immobilized to carrageenan coated with chitosan for the hydrolysis of lactose. The chitosan-carrageenan polyelectrolyte interaction was found to be dependent on the chitosan pH. At pH 4, the chitosan reached its maximum binding of 28.5% (w/w) where the chitosan surface density was 4.8 mg chitosan/cm^2 g of carrageenan gel disks, using Muzzarelli method. Glutaraldehyde was used as a mediator to incorporate new functionality, aldehydic carbonyl group, to the bio-polymers for covalent attachment of β-galactosidase. The enzyme was covalently immobilized to the biopolymer at a concentration of 2.73 mg protein per g of wet gel. FTIR proved the incorporation of the aldehydic carbonyl group to the carrageenan coated with chitosan at 1720 cm^{-1}. The optimum time for enzyme immobilization was found to be 16 h, after which a plateau was reached. The enzyme loading increased from 2.65 U/g (control gel) to 10.92 U/g gel using the covalent technique. The gel's modification has shown to improve the carrageenan gel thermal stability as well as the immobilized enzyme. For example, the carrageenan gel treated with chitosan showed an outstanding thermal stability at 95°C compared with 35°C for the untreated carrageenan gel. Similarly, the immobilization process shifted the enzyme's optimum temperature from 50°C for the free enzyme towards a wider temperature range 45–55 °C indicating that the enzyme structure is strengthened by immobilization. In brief, the newly developed immobilization method is simple; the carrier is cheap, yet effective and can be used for the immobilization of other enzymes. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 17–24, 2009

Key words: biopolymers; hydrogels; covalent immobilization; β-galactosidase; biotechnology

INTRODUCTION

Efficient commercial carriers suitable for the immobilization of enzymes are relatively expensive.[1] The immobilization technique would enable the reusability of enzymes for tens of times, reducing the enzyme and product cost significantly. To prepare a new carrier for enzyme immobilization it is naturally an advantage if substances that are already permitted for use in the pharmaceutical or food industries can be utilized. Carrageenans and chitosans are two polysaccharide families belonging to this category that are commercially available, have diverse features and are available at a reasonable cost.[2]

κ-Carrageenan gel is a naturally abundant biopolymer isolated from red seaweed polysaccharides (carrageenose 4′-sulphate).[3] The polyanion contains one sulfate group $-SO_4^-$ per molecule [Fig. 1(a)]. κ-Carrageenan is one of the main supports used for cell and enzyme immobilization via entrapment.[4–6] However, a drawback of unmodified carrageenan is its poor mechanical and thermal stability,[7] in addition, it has lack of active functional groups to immobilize enzymes covalently.

Chitosan is a cationic naturally occurring polymer obtained from the deacetylation of chitin, which is the second most abundant polymer in nature after cellulose.[8,9] The degree of deacetylation (DD) of typical commercial chitosan is usually between 70% and 95% [Fig. 1(b)], revealing an abundance of amino groups (70–95%). The chitosan possesses primary amine groups (NH_2) that become protonated (NH_3^+) in acidic environments. According to the study made by Chao et al.,[10] to harden the carrageenan gels using different amine compounds, they concluded that only polyamines substantially improved the carrageenan gels thermal stability.

Unfortunately, the carrageenan-polyelectrolyte systems were limited to the entrapment of enzymes,[4–6] which have the major problem of enzyme leakage. For example, Boadi and Neufeld[11] used alginate and carrageenan to entrap tannase, then crosslinked the gel beads with chitosan followed by glutaraldehyde. The entrapment technique limits their industrial use as supports for enzyme immobilization due to enzyme leakage. So, efforts to immobilize enzymes

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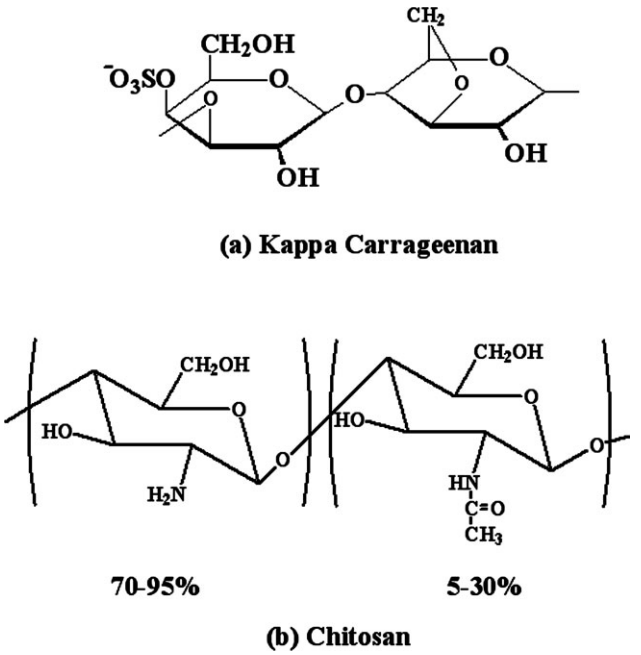


Figure 1 Chemical structures of (a) κ-carrageenan and (b) chitosan.

on newer type of carriers, especially with covalent bonds are still underway in many laboratories.[12–15] To our knowledge, previous reports have not any greater extent that dealt with carrageenan-chitosan polyelectrolyte as a use for the covalent immobilization of enzymes, with an exception of our recent work.[16] Among the few enzymes used in industries, β-galactosidase is used to hydrolyze the disaccharide lactose into glucose and galactose.[17]

The consumption of foods with a high content of lactose is problematic for almost 70% of the world population suffering with lactose intolerance).[18] Unfortunately, there is "no cure to lactose intolerance,"[19] this fact together with the relatively low solubility and sweetness of lactose, have lead to an increasing interest in the development of industrial processes to hydrolyze the lactose contained in dairy products. As an advantage, the products of hydrolysis are sweeter, more soluble, and more digestible than lactose.[20]

In this research,[21] the polyanionic κ-carrageenan gel was strengthened via the formation of an ionic complex (network) between the sulfate groups of the carrageenan gel (anions) and the protonated amino groups (cations) of chitosan at different pHs and concentrations. Knowing that the hardening of hydrogels by polyelectrolyte complexation is an interesting alternative to covalently crosslinked hydrogels.[22] The polyeclectrolytes complex was further modified with glutaraldehyde to create free aldehydic terminals to immobilize covalently the industrial enzyme, β-galactosidase. The chemical and physical modifications of the modified gel were elucidated by FTIR and the gel's thermal stability. Although, the enzyme's immobilization capacity and efficiency were proved by the enzyme's activity and loading efficiency.

MATERIALS

κ-Carrageenan (Mw: 154,000; sulphate ester ~ 25 %) and chitosan were supplied by Fluka. β-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae*, 11.8 U/mg and Cibacron brilliant red 3B-A (Mw 995.23) were purchased from Sigma-Aldrich. Other chemicals were of Analar or equivalent quality. Parallel plate equipment was made in our laboratory for uniform gel sheets preparation. The gel disks dimensions were measured using a micrometer (Micro 2000, 0–25 mm).

EXPERIMENTAL TECHNIQUES

As a general rule, all experiments were carried out in triplicate and data are means ± SD ($n = 3$).

Preparation of κ-carrageenan gel disks

κ-Carrageenan gels were prepared as previously reported by Elnashar et al.[16] Carrageenan at a concentration of 2% (w/v) was dissolved in distilled water at 70°C using an overhead mechanical stirrer until complete dissolution occurred. Glass parallel plates equipment designed by Elnashar et al.[23] with 10 mm gaps were then immersed into the hot molten gel to produce uniform gel sheets. The 10-mm thick gel-sheets were cut into disks using cork borers for enzyme immobilization. Typically, 4-mm diameter gel disks, of an average weight of 145 mg, were produced for immobilization.

Carrageenan coated with chitosan and Schiff's base formation

In this experiment, the carrageenan gel was hardened using 0.3 M KCl for 3 h as a control,[5] and with a series of 0.5% (w/v) chitosan at pH 2–6 for 3 h. The gels were filtered and the supernatant was left to evaluate the optimum chitosan pH using Cibacron Brilliant Red reagent. Then, the pH at which the maximum amount of bound chitosan was achieved, was used at concentrations of 0.25–1.0% (w/v) for 3 h.

The modified gel disks were thoroughly washed with distilled water and soaked in 2% (v/v) glutaraldehyde solution for 3 h. In this step, glutaraldehyde acted as a chitosan crosslinker, improving the gels thermal stability[10] and as a spacer arm offering free aldehyde groups to react with the enzyme's amino groups via Schiff's base formation as shown in

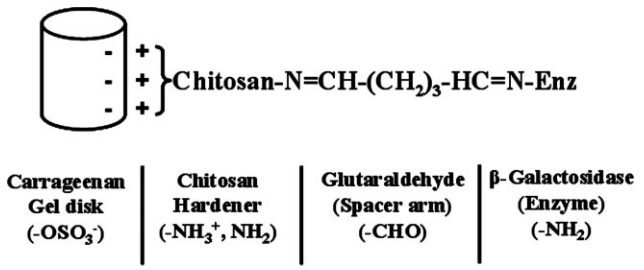


Figure 2 Carrageenan coated with chitosan followed by glutaraldehyde (GA) as a spacer arm to immobilize covalently β-galactosidase (Enz) to the chitosans amino groups via Schiff's base formation.

Figure 2. The modified gel was thoroughly washed with distilled water to get rid of any unbound glutaraldehyde molecules.

Colorimetric determination of chitosan

A colorimetric method for the determination of chitosan in an aqueous solution was described by Muzzarelli.[24] A solution of cibacron brilliant red 3B-A dye was prepared by dissolving 150 mg of the powder in 100 mL bi-distilled water. Five milliliter of the stock solution was diluted to 100 mL with 0.1M glycine hydrochloride buffer. The final concentration of the dye solution was 75 μg/mL. To prepare the standard curve of chitosan, 15, 30, 45, 60, 80, 100, 150, 200, and 250 μl of 0.5 g/L chitosan were filled into test tubes, followed by the addition of different volumes of buffer to reach 300 μl. Then, 3 mL aliquots of dye solution were added to each tube. The absorbance values were measured spectrophotometrically at 575 nm. The aqueous chitosan solutions were tested before and after the incubation of the gel disks. The difference in the amount of chitosan before and after incubation was the amount of chitosan bound to the carrageenan gel disks. The chitosan surface density coat per one wet gel disk was also calculated knowing that the surface area of one gel disk is equal to 1.256 cm^2 using the following equation:

$$\text{Surface area of a disk} = \pi dl = 3.14 \times 0.4 \times 1 \qquad (1)$$

Thermal stability of carrageenan gel formulations

Two formulations of gel disks were used for this test: the control gel and the modified gel of 2% (w/v) κ-carrageenan/KCl soaked in 0.75% (w/v) chitosan at pH 4.0 for 3 h followed by 2% (v/v) glutaraldehyde for 3 h. The gel disks were incubated for 1 h in distilled water at 25–95 °C. The appearance of the gels was then inspected visually to check whether the gel disks remained solid or dissolved.

Elucidation of the modified gel using FTIR

The FTIR spectrophotometer (FTIR-8300, Shimadzu, Japan) was used to prove the presence of the new functional group, aldehydic carbonyl group, in the modified gel. Three samples were used for this test, the carrageenan and chitosan powders and the carrageenan coated with chitosan followed by glutaraldehyde (modified gel). A total of 2% (w/w) of the sample, with respect to the potassium bromide (KBr; S. D. Fine Chem Ltd) disk, was mixed with dry KBr. The mixture was ground into a fine powder using an agate mortar before it was compressed into a KBr disk under a hydraulic press at 10,000 psi. Each KBr disk was scanned 16 times at 4 mm/s at a resolution of 2/cm over a wavenumber range of 400–4000/cm, using Happ–Genzel apodization. The characteristic peaks were recorded.

Immobilization of β-galactosidase and soluble protein determination

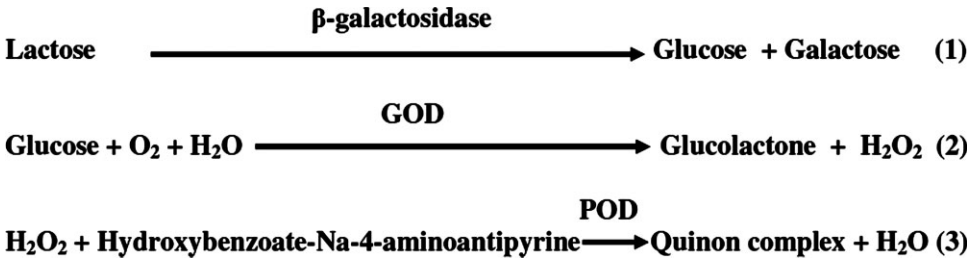
β-Galactosidase was immobilized onto the control and modified gel. Six disks of control and modified gel (10 mm height × 4 mm diameter) were washed thoroughly with distilled water and were incubated into 10 mL of enzyme solution (3.4 U/mL) prepared in 100 m*M* citrate-phosphate buffer at pH 4.5 for 16 h. The immobilized enzyme was washed thoroughly with the buffer solution containing Tris-HCl to block any free aldehyde group and to remove any unbound enzyme. The immobilized enzyme was stored at 4°C for further measurements. The supernatant and the wash were kept for soluble protein assay via bovine serum albumin (BSA) as a standard protein. The amount of protein immobilized onto and into the gel carrier P_g (mg/g) was calculated using the following equation:

$$P_g = \frac{C_o V_o - C_f V_f}{w} \qquad (2)$$

where C_o is the initial protein concentration (mg/mL), C_f is the protein concentration of the filtrate (mg/mL), v_o is the initial volume of the enzyme solution (mL), v_f is the volume of filtrate (mL), and w is the weight of gel carrier used (g).

β-Galactosidase activity assay

Activity of β-galactosidase was determined by the rate of glucose formation in the reaction medium. Known amounts of immobilized or free enzyme were incubated into 10 mL of 200 m*M* lactose solution in citrate-phosphate buffer (100 m*M*, pH 4.5) for 3 h at 37°C and 100 rpm. At the end of which 50 μl of reaction mixture was added to 950 μl buffer, boiled for 10 min to inactivate the enzyme and



Scheme 1 Hydrolysis of lactose by β-galactosidase and glucose determination using a mixture of enzymes, glucose oxidase (GOD), and peroxidase (POD).

analyzed for glucose content using the glucose test. One enzyme unit (IU) was defined as the amount of enzyme that catalyzes the formation of 1 μmol of glucose per minute under the specified conditions.

Glucose concentration was measured spectrophotometrically with a glucose test based on the Trinder reagent.[25] Glucose is transformed into gluconic acid and hydrogen peroxide by glucose oxidase (GOD). The hydrogen peroxide formed reacts in the presence of peroxidase (POD) with 4-aminoantipyrine and p-hydroxybenzene sulfonate to form a quinoneimine dye, as shown in Scheme 1.

The intensity of the color produced is directly proportional to the glucose concentration in the sample. The assay was performed by mixing 30 μl of a sample of unknown concentration and 3 mL of Trinder reagent, the reaction was left to proceed for 20 min at room temperature, and the absorbance at 510 nm was read. The absorbance was related to the concentration of glucose with a standard calibration curve.

Enzyme incubation period

In all above experiments, six disks of gels were incubated for 16 h in an enzyme solution of 34 U (10 mL of 3.4 U/mL). In this experiment, an incubation time of 0.5 h to 32 h was used to determine the optimum time for immobilization to reach the maximum enzyme loading capacity (ELC).

$$\%ELC = \frac{M_o - M_f}{M_o} \times 100 \quad (3)$$

where M_o is the initial enzyme activity (U), M_f is the enzyme activity of the filtrate (U) after immobilization.

Temperature effect on the enzyme activity

The effect of temperature on the activity of the free and immobilized enzyme was examined. The immobilized β-galactosidase was incubated into 10 mL of 250 m*M* lactose at pH 4.5 using temperatures ranging from 30 to 70°C for 3 h. The maximum activity was taken as 100% activity. The relative activity at each temperature is expressed as a percentage of the maximum activity.

RESULTS AND DISCUSSION

Carrageenan coated with chitosan elucidation structure

The carrageenan gel was prepared in uniform sheets using the parallel plates apparatus,[23] cut into disks and its surface was modified using partially protonated chitosan. The protonated chitosan amino groups ($-NH_3^+$) formed a polyelectrolyte complex with $-OSO_3^-$ of the carrageenan gel, to increase the gel's thermal stability[10] and to incorporate free amino groups (chitosan) as a new functionality.[16] The free chitosan amino groups ($-NH_2$) were used to form Schiff's base and covalently immobilize β-galactosidase via glutaraldehyde as a mediator, as shown in Figure 2. The Schiff's base color showed visually an increase from pale yellow to brown red by varying the chitosan pH from 2 to 6.

The FTIR bands of the carrageenan, chitosan, and the modified gel (carrageenan/chitosan/glutaraldehyde) were shown in Figure 3. By comparing the

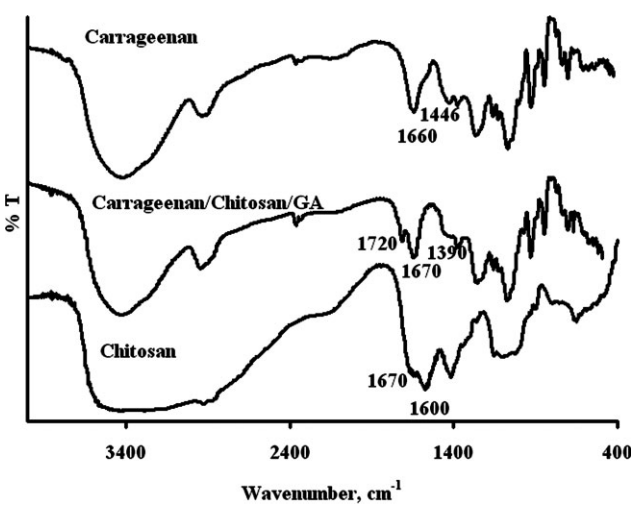


Figure 3 FTIR of carrageenan, chitosan, and the carrageenan coated with chitosan followed by glutaraldehyde (GA).

TABLE I
Thermal Stability of Control Carrageenan Gel and Carrageenan Coated with Chitosan

Chitosan concentration % (w/v)	0	0.25	0.5	0.75	1.0
Thermal stability at 30°C	+	+	+	+	+
Thermal stability at 35°C	−	+	+	+	+
Thermal stability at 95°C	−	+	+	+	+

The appearance of the gel was inspected visually, where (−) means turbid solution (gel dissolved), and (+) means clear solution (gel remained solid).

spectrum of the three compounds, a new band at 1720 cm^{-1} appears only for the modified gel spectrum. This band proved the presence of a new gel functional group, aldehydic carbonyl group, which is in agreement with the authors previous work.[16] The FTIR bands also revealed a decrease in intensity and a shift of the carrageenan $-OSO_3^-$ absorption band from 1446 cm^{-1} to 1390 cm^{-1} after reaction with the chitosan. This ionic interaction between the carrageenan and the chitosan evidenced the formation of strong polyelectrolyte complexes.[26]

Thermal stability of carrageenan and carrageenan coated with chitosan

It is well known that a disadvantage of carrageenan gel is its thermal stability.[7] The thermal stability of the carrageenan coated with chitosan revealed greater stability over the control gel, as revealed in Table I, where the control gel dissolved at 35°C and the modified gel remained intact at 95°C. This fact was supported by Chao et al.,[10] who proved that only polyamines substantially improved the carrageenan gels thermal stability. This outstanding improvement in the modified gels thermal stability could be the result of the strong polyelectrolyte complexation between the polyanion, carrageenan gel, and the chitosan polycation,[27] and to the reinforcement of the carrageenan gel's network using chitosan through many points,[28] as shown in Figure 2. This result could also be supported by the fact that hardening of hydrogels by polyelectrolyte complexation is an interesting alternative to covalently crosslinked hydrogels.[22]

Optimization of chitosan pH and concentration

In this section, the optimum pH and concentration of chitosan were studied to find out the maximum ELC. Lowry assay showed that the enzyme activity did not decrease by the immobilization process, i.e., almost 100% retention of enzyme efficiency. The units of enzyme per milligram protein were found to be 4 U/mg and not 11.8 U/mg as stated by the manufacturer.

Optimization of chitosan pH

Chitosan was prepared at pH 2 to 6 to find out the best pH for maximum chitosan coating to the carrageenan gel by forming a polyelectrolyte complex between the chitosan ($-NH_3^+$) and the carrageenan ($-OSO_3^-$).

Because the pK of chitosan is ~ 6.3 and the sulphates groups of carrageenan have negative charges in pure water (~ pH 5.5),[29,30] we were expecting the chitosan and carrageenan to be ionized, reaching their maximum binding at low pHs, pH 2–3. Then by increasing the chitosan pHs, pH 4–6, carrageenan/chitosan polyelectrolyte complex formation was expected to decline gradually due to deprotonation of chitosan. Amazingly, as discerned in Table II, the maximum chitosan/carrageenan binding of 5.7 mg chitosan/g of wet gel disks was reached at pH 4 instead of pH 2 then it decreased afterward to 3.6 mg chitosan/g wet gel disk at pH 6. The low carrageenan/chitosan polyelectrolyte binding at pH 2–3 could be regarded as the fact that κ-carrageenan under pH ~ 2.6 is susceptible to hydrolytic degradation. Degradation of carrageenan resulting in the formation of low-molecular weight material provides an obstacle in the regulation of the polyelectrolyte complex process.[2]

In terms of chitosan surface density, it reached its maximum of 4.5 mg chitosan/cm^2 g wet gel disks, knowing that chitosan coat was almost on the carrageenan surface due to its high molecular weight and viscosity. The results in Figure 4 were in agreement with that of Table II, where the maximum enzyme loading of 9.6 U/g wet gel was achieved as well at pH 4. This could be attributed to the more bound chitosan to the carrageenan, the more free amino groups to bind enzyme to the gel via glutaraldehyde as a mediator.[16]

TABLE II
Optimization of Chitosan pH

Chitosan pH	mg Chitosan/g wet gel disk	mg Chitosan/cm^2 g wet gel disk	Chitosan/carrageenan % (w/w) dry gel
2	**4.0**	**3.2**	20.2
3	**4.0**	**3.2**	20.2
4	***5.7***	***4.5***	***28.5***
5	**4.2**	**3.3**	20.9
6	**3.6**	**2.9**	18.2

Carrageenan gels hardened with 0.3 *M* KCl for 3 h were soaked in a series of 0.5% (w/v) chitosan at pH 2–6 for 3 h. The chitosan concentration was determined using Cibacron reagent. The values in italics are Cibacron Brilliant Red 3B-A dye.

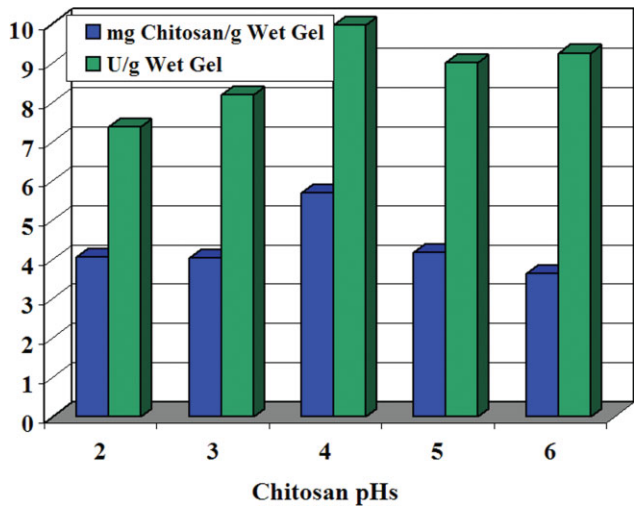


Figure 4 Effect of chitosan pHs on the amount of bound chitosan and the units of enzymes immobilized per gram wet gel. Carrageenan gel disks hardened with KCl were soaked in 0.5% (w/v) chitosan at pH 2–6 for 3 h afterward in 2% (v/v) glutaraldehyde for 3 h then in 34 U of β-galactosidase for 16 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

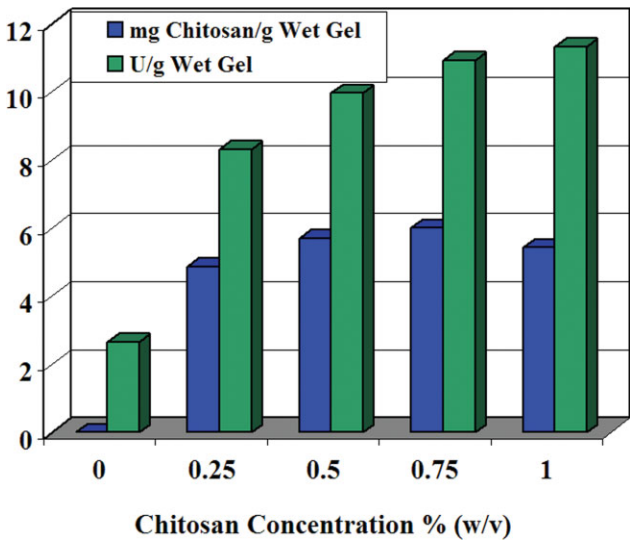


Figure 5 Effect of chitosan concentration on the amount of bound chitosan and the units of enzymes immobilized per gram of wet gel. Carrageenan gel disks hardened with KCl were soaked in 0.25–1.0% (w/v) chitosan at pH 4 for 3 h afterward in 2% (v/v) glutaraldehyde for 3 h then in 34 U of β-galactosidase for 16 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Optimization of chitosan concentration

The optimum formulation of Section 4.2.1 was chosen for this experiment, where chitosan formulations of 0.25–1.0% (w/v) at pH 4 were used to coat the carrageenan gel. The data in Table III revealed an increase in the maximum binding to 6.0 mg chitosan/g wet gel disks and the maximum surface density to 4.8 mg chitosan/cm^2 g wet gel disks using a chitosan solution of 0.75%. In terms of dry gel, the chitosan to carrageenan percentage (w/w) increased to 30%. By increasing the chitosan concentration to 1.0%, the binding efficiency decreased. This may be due to the high viscosity of chitosan solution, which caused difficulty for the chitosan molecules to penetrate the carrageenan gel pores.

TABLE III
Optimization of Chitosan Concentration

Chitosan conc.	mg Chitosan/ g wet gel disk	mg Chitosan/ cm^2 g wet gel disk	Chitosan/ carrageenan % (w/w) dry gel
0.25	**4.9**	**3.9**	24.3
0.50	**5.7**	**4.5**	28.5
0.75	***6.0***	***4.8***	***30.0***
1.0	**5.4**	**4.3**	27.1

Carrageenan gels hardened with 0.3 *M* KCl were soaked in a series of chitosan concentrations of 0.25–1.0% (w/v) at pH 4 for 3 h. The chitosan concentration was determined using Cibacron reagent. The values in italics are Cibacron Brilliant Red 3B-A dye.

The results in Figure 5 were in accordance with that of Table III, where the amount of immobilized enzyme was gradually increased by increasing the chitosan concentration from 0.25% to 0.75%, reaching its maximum of 10.92 U/g wet gel (2.73 mg protein/g wet gel) at 0.75% chitosan, which is more than four folds that of the control gel, 2.65 U/g wet gel. At 1.0% (w/w) chitosan, the amount of enzyme units immobilized slightly increased, but owing to the high viscosity of chitosan and the difficulty of preparation of 1.0% (w/w) chitosan, we have chosen

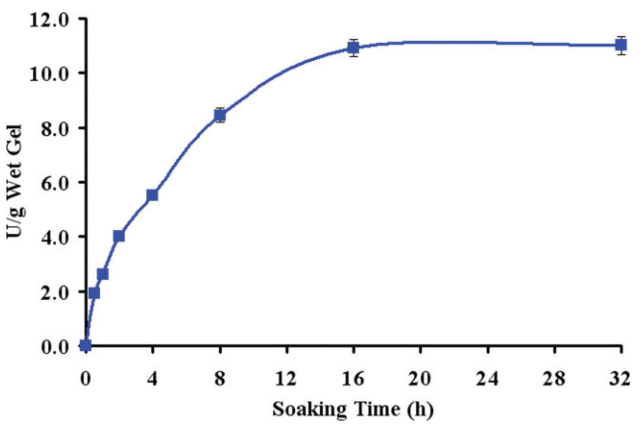


Figure 6 β-Galactosidase incubation period. Carrageenan gels disks (carrageenan/chitosan/glutaraldehyde) were soaked in 10 mL of 3.4 U/mL of enzyme solution at an incubation time of 0.5 h to 32 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

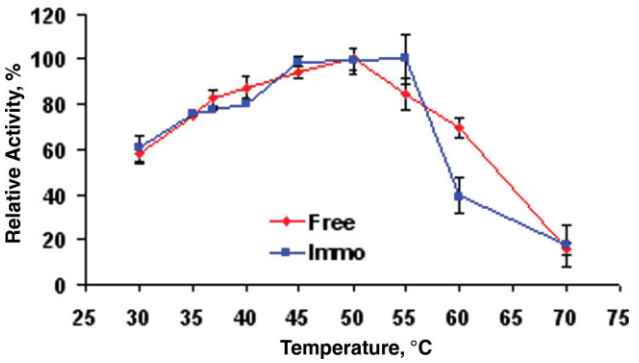


Figure 7 Temperature-activity profile of free and immobilized β-galactosidase. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the concentration of 0.75% (w/w) chitosan for future experiments.

Enzyme incubation period

The optimum immobilization time for β-galactosidase was found to be 16 h, at which 28% of the initial enzyme solution were immobilized, after which a plateau was attained as shown in Figure 6. By increasing the time of immobilization from 0.5 h to 16 h, the immobilized ELC increased gradually from 1.9 U/g to 10.92 U/g of wet gel, respectively. A longer enzyme incubation time has no effect on the immobilization efficiency, which could be regarded to all aldehyde groups have been engaged with the enzymes after 16 h.

Temperature-activity profile of free and immobilized β-galactosidase

In general, the enzymatic activity is dependent on the temperature in the same way as that of the chemical catalysts, except that there is an optimum temperature of the enzymatic reaction above which the activity decreases due to the denaturation of the enzyme protein. The results in Figure 7 demonstrate that the immobilization process increased the enzyme's temperature tolerance. The optimum temperature for the free β-galactosidase occurred at 50°C compared with 45–55°C for the immobilized enzyme.

The shift of the optimum temperature towards a wider temperature range when the biocatalyst is immobilized indicates that the enzyme structure is strengthened by the immobilization process. The formation of a molecular cage around the protein molecule (enzyme) was found to enhance the enzyme's thermal stability.[31] The immobilization process maintains the structure of the enzyme's catalytic site at temperatures higher than those deactivating the native form.

CONCLUSION

β-Galactosidase from *Aspergillus oryzae* was immobilized covalently on novel biopolymers of carrageenan coated with chitosan. The chitosan-carrageenan polyelectrolyte complex increased the thermal stability of the carrageenan gel from 35°C to 95°C providing evidence that the new carrier could be suitable for thermophilic enzymes. The modified natural biopolymers have the advantage of being already permitted for use in the pharmaceutical or food industries. In addition, the covalent immobilization and stabilization of the β-galactosidase occur simultaneously providing a simple and rapid method for preparation.

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