1 **Encapsulation of lactase (β -galactosidase) into**
2 **κ -carrageenan-based hydrogel beads: Impact of**
3 **environmental conditions on enzyme activity**

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Abstract

Encapsulation of enzymes in hydrogel beads may improve their utilization and activity in foods. In this study, the potential of carrageenan hydrogel beads for encapsulating β -galactosidase was investigated. Hydrogel beads were fabricated by injecting an aqueous solution, containing β -galactosidase (26 U) and carrageenan (1 wt%), into a hardening solution (5% potassium chloride). Around 63% of the β -galactosidase was initially encapsulated in the hydrogel beads. Encapsulated β -galactosidase had a higher activity than that of the free enzyme over a range of pH and thermal conditions, which was attributed to the stabilization of the enzyme structure by K^+ ions within the carrageenan beads. Release of the enzyme from the beads was observed during storage in aqueous solutions, which was attributed to the relatively large pore size of the hydrogel matrix. Our results suggest that carrageenan hydrogel beads may be useful encapsulation systems, but further work is needed to inhibit enzyme leakage.

Keywords: carrageenan; hydrogel beads; microgels; enzyme activity; encapsulation

1. Introduction

Lactase, an enzyme within the β -galactosidase family, is found in a wide variety of natural sources, including microorganisms, plants and animals. This enzyme can catalyze the hydrolysis of terminal β -glycosidic bonds in polysaccharides, but its primary function is believed to be the hydrolysis of β -glycosidic bonds in lactose to produce glucose and galactose (Nussinovitch, Chapnik, Gal, & Froy, 2012). A deficiency of lactase in the human body may lead to lactose intolerance, which can cause a range of health problems, such as bloating, abdominal discomfort, flatulence, diarrhea and nausea (Lomer, Parkes, & Sanderson, 2008). Lactose intolerance may be combatted by creating lactose-free foods, by hydrolyzing lactose within foods prior to consumption, or by ingesting lactase supplements that promote lactose hydrolysis within the gastrointestinal tract (Harju, Kallioinen, & Tossavainen, 2012). Enzymatic hydrolysis of lactose is often more desirable than chemical hydrolysis, because it does not lead to undesirable changes in food quality or nutritional profile (Rodriguez-Nogales & Delgadillo, 2005). The enzyme β -galactosidase is commonly used in the food industry for its lactase activity, particularly in dairy products (Husain, 2010; Kim, Jeon, Ahn, & Kwak, 2006; Klein, Nunes, Rodrigues, Benvenuti, Costa, Hertz, et al., 2012). Nevertheless, there are some important factors limiting the utilization of β -galactosidase in food applications, for example, difficulties in recovering the enzyme from the hydrolysis products, and the sensitivity of the enzyme to changes in environmental conditions (Kim, Jeon, Ahn, & Kwak, 2006; Santos, & Garcia-Ochoa, 2006).

Immobilization of enzymes within porous matrices have been utilized for many years to improve their stability and recovery (Coviello, Matricardi, Marianecchi, & Alhaique, 2007; K. G. H. Desai & Park, 2005). In these techniques, the enzymes are trapped within a porous matrix that isolates them from direct contact with the surrounding medium (Es, Vieira, & Amaral, 2015). On the other hand, small

molecules (such as co-factors, substrates and products) can easily diffuse into and out of the porous matrix, which enables the enzyme to maintain its activity. Enzymes can be trapped within porous matrices using several approaches, including physical entrapment, physical bonding, and/or covalent bonding (Betancor, Luckarift, Seo, Brand, & Spain, 2008; Büyükbayram, Kıralp, Toppare, & Yağcı, 2006; Malhotra, Singhal, Chaubey, Sharma, & Kumar, 2005). Porous matrices are often fabricated by assembling hydrogels from synthetic or natural polymers, since these materials are easy to prepare, have a wide range of structural and functional properties, and may be biocompatible and biodegradable (Bhatia, Brinker, Gupta, & Singh, 2000; Smith, Silvernail, Rodgers, Elgren, Castro, & Parker, 2002). If the pore sizes of the hydrogel are sufficiently small compared to the dimensions of the enzyme molecules, then the enzymes will be physically trapped within the hydrogel network that surrounds them (Fu, Pacheco, & Prud'homme, 2009). If there is a strong attraction between the enzyme molecules and the hydrogel network, then the enzyme molecules may be immobilized through physical bonding, such as hydrogen bonding, electrostatic attraction, or hydrophobic attraction (Lin & Metters, 2006). The main advantage of these physical approaches to encapsulation is that no chemical modification of the enzyme is required to form a covalent linkage with the hydrogel network. Furthermore, these physical approaches do not typically cause pronounced changes in enzyme structure and activity, while still enabling the enzymes to interact with the substrate molecules.

Hydrogel beads have been used widely for the encapsulation, protection, and delivery of active components in the food, personal care and pharmaceutical industries (Onwulata, 2012; Shewan & Stokes, 2013; Zhang, Decker, & McClements, 2014). Hydrogel beads can be fabricated using a variety of different approaches, including injection, coacervation, thermodynamic incompatibility, templating, and molding methods (Matalanis, Jones, & McClements, 2011). The injection-gelation

method is one of the simplest and inexpensive to implement, and does not typically require the use of organic solvents, surfactants, or lipids. In this method, a polymer solution containing the active component is injected into another “hardening” solution, under conditions that promote gelation of the polymer. This procedure results in the formation of a hydrogel bead with the active components trapped inside. Numerous synthetic and natural polymers can be used to form hydrogel beads suitable for enzyme immobilization (Di Serio, Maturo, De Alteriis, Parascandola, Tesser, & Santacesaria, 2003). However, for many commercial applications it is important that the components used to fabricate the hydrogel matrix are non-toxic, biocompatible, and biodegradable.

For applications in the food industry, it is advantageous to utilize natural polymers, such as proteins and polysaccharides, to construct hydrogel beads. In the current study, the potential for using κ -carrageenan as a food-grade biopolymer to fabricate hydrogel beads suitable for encapsulating enzymes was investigated. κ -carrageenan is a polysaccharide isolated from seaweed that is commonly used in the food and pharmaceutical industries as a gelling agent. Anionic κ -carrageenan molecules can form hydrogel matrices in the presence of cationic cross-linking agents, such as metal ions, peptides, and proteins, and can, therefore, be used to fabricate hydrogel beads by the injection method (Zhang, Zhang, Chen, Tong, & McClements, 2015).

In this study, the possibility of immobilizing enzymes within carrageenan hydrogel beads formed by injecting a β -galactosidase/ κ -carrageenan solution into a potassium chloride hardening solution was investigated. The cationic potassium ions (K^+) promote gelation of the anionic κ -carrageenan molecules by acting as salt bridges. The β -galactosidase should be trapped within the hydrogel beads, provided that the rate of hydrogel formation exceeds the rate of enzyme diffusion into the surrounding medium. The effect of hydrogel bead encapsulation on the catalytic

activity of β -galactosidase was studied using *o*-nitrophenyl- β -d-galactoside (*o*-NPG) as a substrate, since this molecule undergoes a measureable change in colour when its glycosidic linkage is cleaved. In particular, the impact of pH and temperature on the catalytic activity of the free and encapsulated enzyme was compared, as well as measuring changes in the retention of the enzyme during storage. Some insights into the physicochemical basis for the differences in the activities of the free and immobilized β -galactosidase were obtained by measuring the influence of solution composition. The results of this study should provide some valuable information for the rational design and fabrication of hydrogel beads for encapsulation of enzymes in the food and pharmaceutical industries.

2. Materials and methods

2.1. Materials

β -galactosidase (Lactozyme[®] 2600 L) produced by fermentation of a selected strain of the yeast *Kluyveromyces lactis*. (specific activity: 2600 U/g) was obtained from Sigma Chemical Co (St. Louis, MO, USA). Carrageenan was kindly donated by FMC Biopolymer (Viscarin SD 389, Philadelphia, PA). The reagents *o*-nitrophenol (*o*-NP) and *o*-nitrophenyl- β -d-galactosidase (*o*-NPG) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Double distilled water was used to make all solutions.

2.2. Methods

2.2.1 Encapsulation of β -galactosidase in carrageenan beads

The immobilization of β -galactosidase in κ -carrageenan beads was carried out using a method similar to that described previously for encapsulation of lipid droplets in alginate beads (Li, Hu, Du, Xiao, & McClements, 2011). Firstly, the β -galactosidase was diluted with a phosphate buffer (pH 7.0) to obtain a concentration equivalent to 52 units of activity. An aqueous κ -carrageenan (2% w/v) solution was

prepared by dissolving the powdered ingredient in distilled water by stirring at 60 °C for an hour, and then reducing the temperature to 35 °C. The enzyme and polysaccharide solutions were then mixed together (1:1 v/v) for 2 h, with continuous stirring, to form a solution that contained β -galactosidase (26 U) and κ -carrageenan (1% w/v). Enzyme-loaded hydrogel beads were prepared using a syringe to drip the β -galactosidase/ κ -carrageenan solution into 10 ml of 5% potassium chloride solution, with continuous stirring. The beads were allowed to crosslink with K^+ for 1 h at ambient temperature to form hydrogel beads. The beads were then collected by filtration and subsequently washed, with distilled water and phosphate buffer, to remove any excess K^+ from their surfaces.

2.2.2. Measurement of catalytic activity of β -galactosidase

The activity of β -galactosidase was assayed, by means of a colorimetric test, using o-NPG as substrate. A solution of o-NPG in phosphate buffer (3 mg/ml) was added to 0.5 ml diluted enzyme solution (26 U): one unit of β -galactosidase is defined as the amount of enzyme hydrolyzing 1 μ mol of substrate in 1 min, at operational conditions. The rate of formation of free o-NP was recorded spectrophotometrically ($\lambda = 420$ nm), using a 1-cm path length cuvette. The activity was then defined as the conversion rate of the substrate (o-NPG) into the reaction products per unit time and per unit volume (μ mol min⁻¹ ml⁻¹).

2.2.3. Effects of pH and temperature

The effect of pH on enzyme activity was established by incubating the enzymes in buffer solutions of pH 2.0–7.0 for 10 min at 25 °C. The effect of temperature on enzyme activity was determined by incubating the enzymes in buffer solutions at 25–60 °C for 10 min at pH 7. The activity of the β -galactosidase was then evaluated

using the procedures described in section 2.2.2, while the particle dimensions and microstructure were measured using the procedures described in section 2.2.5.

2.2.4. Effects of solution composition

The influence of immobilization on the activity of the β -galactosidase was established by comparing three samples: free enzyme, enzyme trapped within hydrogel beads and free enzyme mixed with preformed hydrogel beads. The final concentration of these three systems was kept constant so that direct comparisons could be made. In addition, the influence of mineral ions (1% potassium chloride, 5% potassium chloride or 5% calcium chloride) on the activity of the β -galactosidase was measured. The catalytic activity of the β -galactosidase in all these systems was determined by the methods described in section 2.2.2.

2.2.5. Measurement of bead dimensions

The diameter of the hydrogel beads after incubation at different pH values and temperatures was measured using a digital micrometer (0–300 mm, EC10, High Precision Digital Caliper, Tresna Instruments, Guilin, China). The bead diameter of at least 5 individual beads was measured, and the mean and standard deviation was calculated.

2.2.6. Enzyme retention and release

The ability of the hydrogel beads to retain the enzyme was determined by immersing them in PBS buffer (2 mM, pH 7) at room temperature, and then measuring the concentration of β -galactosidase in the surrounding aqueous phase at various time intervals (0–8h). The enzyme leakage was determined by withdrawing the surrounding phosphate buffer and recording the absorbance at 280 nm, using a UV-visible spectrophotometer. The protein content was quantified using a standard

curve, prepared by measuring the absorbance of a series of protein solutions with different BSA (bovine serum albumin) concentrations. The enzyme release rate is defined as the ratio of free protein concentration after storage to their initial concentration.

2.2.7. Microstructure analysis

It was not possible to measure the microstructure of the hydrogel beads formed using the simple syringe, since they were too large. We, therefore, prepared small carrageenan hydrogel beads loaded with β -galactosidase using a commercial encapsulation unit (Encapsulator B-390, BUCHI, Switzerland) and the same solution conditions described in section 2.2.1. The microstructure of all systems were examined using a confocal scanning laser microscope with a 20 $\times$ objective lens (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, U.S.). For the confocal microscopy, samples were dyed prior to particle formation. The β -galactosidase was dyed with fluorescein thiocyanate isomer I (FITC) solution (1 mg/ml dimethyl sulfoxide) by adding 0.1 ml of FITC dye solution to 2 ml of sample and storing at 5 °C overnight. The excitation and emission spectrum for FITC were 488 nm and 515 nm, respectively. The microstructure images for confocal microscopy were taken and analyzed using image analysis software (NIS-Elements, Nikon, Melville, NY).

2.3. Statistical analysis

All experiments were carried out in triplicate using freshly prepared samples. Means and standard deviations were calculated from a minimum of three measurements using Excel (Microsoft, Redmond, VA, USA).

3. Results and discussion

3.1. Immobilization of β -galactosidase in hydrogel beads

Previous studies have reported that various parameters may influence the size and shape of the carrageenan hydrogel beads, including biopolymer concentration,

potassium chloride concentration, needle diameter, injection flow rate, and hardening time (Popa, Gomes, & Reis, 2011). Preliminary experiments were therefore carried out to identify suitable conditions for forming hydrogel beads that maintained good physical stability after formation. It was found that hydrogel beads could be successfully formed by manually injecting a 1% carrageenan solution into a 5% potassium chloride solution, using a syringe with a needle diameter of 0.5 mm at a rate of one drop per 2 s. The beads formed were allowed to crosslink in the hardening solution containing potassium ions for 30 min at an ambient temperature. The resulting hydrogel beads had a spherical shape with smooth surfaces, and diameters ranging between 2 and 3 mm.

A suitable enzyme level to use in this study was established by measuring the enzymatic activity of different concentrations of β -galactosidase (13 to 260 U). A β -galactosidase level of 26 U was found to give a rate for the enzyme-catalyzed hydrolysis reaction that could conveniently be measured on the experimental time scale.

The encapsulation efficiency has been defined as the percentage of enzyme trapped within the hydrogel beads in relation to the total amount of enzyme present during bead formation, and is an important parameter affecting the performance of the immobilization methods. In the current study, the amount of β -galactosidase trapped inside the hydrogel beads immediately after formation was around 63%. This suggests that a considerable amount of the enzyme was able to diffuse out of the beads before they had time to harden during the preparation procedure.

3.2. Influence of temperature and pH on enzyme activity

3.2.1. General

One of the major limitations of utilizing enzymes to catalyze chemical reactions, in many commercial applications, is their sensitivity to changes in environmental conditions. Changes in pH or temperature during storage or during passage through the gastrointestinal tract may alter the conformation or aggregation state of enzymes,

thereby altering their catalytic activity. For this reason, we evaluated the influence of pH and temperature on the stability of β -galactosidase.

3.2.2. pH Stability

The activity of free and encapsulated β -galactosidase as a function of pH was determined (Fig. 1a). In this study, pH values from 2 to 7 were selected to cover the range of solution conditions that might be found in commercial food products or within the human gastrointestinal tract. Both the free and encapsulated β -galactosidase had a maximum catalytic activity around pH 7, which indicated that immobilization within the hydrogel beads did not alter the optimal pH of this enzyme. The enzyme activity decreased markedly when the pH was reduced, and was almost completely lost within the pH range from 2 to 4. This effect can be attributed to changes in the three-dimensional structure of the enzyme when the pH is reduced, which leads to alterations in the morphology and surface chemistry of the active site. These results indicate that the hydrogel beads could not prevent acid-induced loss of β -galactosidase activity. We postulate that the small H^+ ions could readily diffuse into the enzyme-loaded beads through the pores within the hydrogel matrix and were, therefore, still able to alter the properties of the active site.

Despite being unable to protect the enzyme from acid-induced loss of activity, encapsulation of the β -galactosidase in the hydrogel beads did lead to an appreciable increase in its performance under moderate pH conditions, for example, the activity of the free and encapsulated enzymes was around 63 and 266 $\mu\text{mol min}^{-1} \text{ml}^{-1}$ at pH 7, respectively. This increased activity of the encapsulated form may be due to the presence of potassium ions (K^+) in the hydrogel beads. It has been reported that potassium ions are required for the proper functioning of this enzyme (Wheatley, Juers, Lev, Huber, & Noskov, 2015). Potassium ions were used to cross-link the carrageenan molecules during the preparation of the hydrogel beads, and there are likely to have been some free K^+ ions that were able to interact with the enzyme in this system. In contrast, potassium ions were not added to the system containing the

free enzyme. Studies have also shown that the activity of β -galactosidase may be altered by the presence of divalent cations, such as Mn^{2+} and Mg^{2+} (Wheatley, Juers, Lev, Huber, & Noskov, 2015). Further insights into the influence of the ionic environment on the activity of the β -galactosidase was therefore obtained by measuring its catalytic activity in different solutions, which is discussed in section 3.3.

3.2.3. Temperature stability

The thermal stability of β -galactosidase was investigated by measuring its residual activity after incubation at temperatures ranging from 25 to 60 °C (Fig. 1b). Encapsulation of β -galactosidase in the hydrogel beads led to a noticeable increase in its thermal stability compared to the free enzyme. For example, the free enzyme was totally deactivated after 10 min incubation at 37 °C, while the encapsulated one still showed high catalytic activity. This result may again have been due to differences in the local ionic environment of the enzyme: the solution containing the encapsulated enzyme contained potassium ions, whereas the solution containing free enzyme only contained sodium ions. Studies have reported that K^+ is capable of increasing the thermal stability of β -galactosidase by reducing the flexibility of the polypeptide backbone (Wheatley, Juers, Lev, Huber, & Noskov, 2015). Contrarily, the activity of this enzyme declined rapidly with temperature and time when the reaction medium contained Na^+ ions (Jurado, Camacho, Luzón, & Vicaria, 2004). Therefore, knowledge of the effects of different ionic environments on enzyme activity would be helpful in understanding the impact of encapsulation *versus* specific ion effects on β -galactosidase performance.

3.3. Influence of an ionic environment on β -galactosidase activity

The activity of β -galactosidase was highly sensitive to the nature of the ionic environment (Fig. 2). The free enzymes in buffer solution containing no added K^+ ions was much lower ($19 \mu\text{mol min}^{-1} \text{ml}^{-1}$) than the free enzymes in buffer solution containing 1% K^+ ions ($59 \mu\text{mol min}^{-1} \text{ml}^{-1}$) or 5% K^+ ions ($73 \mu\text{mol min}^{-1} \text{ml}^{-1}$),

which confirms the ability of potassium ions to enhance the activity of β -galactosidase. Encapsulated β -galactosidase had a higher hydrolytic activity ($53 \mu\text{mol min}^{-1} \text{ml}^{-1}$) than the non-encapsulated enzyme in the presence of the hydrogel beads ($41 \mu\text{mol min}^{-1} \text{ml}^{-1}$). These results suggest that encapsulation may have contributed to the improved activity of the enzyme, since both of these systems had the same overall composition. The origin of this effect may be due to the fact that there was a higher local concentration of potassium ions surrounding the enzyme in the hydrogel beads. The lower activity of the enzymes trapped in the hydrogel beads ($53 \mu\text{mol min}^{-1} \text{ml}^{-1}$) compared to the free enzymes in 5% K^+ solution ($73 \mu\text{mol min}^{-1} \text{ml}^{-1}$) can be attributed to the fact that some of the potassium ions were held within the electrostatic salt bridges between the carrageenan molecules in the beads.

Carrageenan hydrogel beads will have pore sizes that are appreciably larger than the small ions, substrates, and products used in this study. This observation is consistent with the yellowish appearance of the hydrogel beads after the reaction had taken place, which suggests that o-NP formation occurred within the hydrogel matrix (Fig. 3). This result indicates that the reagent (o-NPG) could diffuse from the bulk solution into the beads. It is interesting to note that the β -galactosidase was totally inactive in the presence of Ca^{2+} ions, which suggests that calcium ions inhibited enzyme hydrolysis of the substrate. Previous studies have also shown that Ca^{2+} ions can inhibit β -galactosidase activity (Demirhan, Apar, & Ozbek, 2008). This effect might be due to the fact that Ca^{2+} induces changes in the conformation or aggregation state of the enzyme that lead to a loss of activity (Garman, Coolbear, & Smart, 1996). Consequently, biopolymers that are cross-linked by calcium ions may be unsuitable as encapsulating agents for this type of enzyme, for example, calcium alginate, pectate, or γ -carrageenan hydrogels. In general, different types of ions may have different influences on hydrogel formation and enzyme activity, and it will therefore be

important to ensure that any ionic cross-linking agent does not lead to an undesirable loss of enzyme activity.

3.4. Influence of temperature and pH on bead integrity

For commercial applications, it is important to understand the influence of environmental conditions on the integrity of the hydrogel beads, as this will influence their stability during the manufacturing process, within products, and in the gastrointestinal tract after ingestion. Therefore, the influence of pH and temperature on the integrity of the carrageenan hydrogel beads was measured. The beads were separated from the hardening solution and then placed into a series of continuously stirred buffer solutions, with different pH values or at different temperatures (25-60 °C) for 30 min before each measurement. The bead dimensions remained relatively constant when they were incubated in solutions ranging from pH 3 to 7, but decreased slightly when they were incubated at pH 2 (Fig. 4a). Previous studies have reported that the 1,3-glycosidic bonds of carrageenan may be dissociated by acidic hydrolysis, which could lead to partial degradation of the carrageenan hydrogels (Qiu & Park, 2012). This phenomenon would lead to the release of bioactive agents from carrageenan beads under the acidic environment of the stomach, and would limit the application of these beads for intestinal or colon delivery.

Measurements of the influence of temperature on the dimensions of the beads showed that they were relatively large after incubation at 30 to 45 °C, but that they shrank appreciably after incubation at 50 to 60 °C (Fig. 4b). The observed reduction in the size of the beads at high temperature may be due to several reasons. Firstly, some of the carrageenan molecules might have dissociated from the surfaces of the beads at high temperatures, *i.e.*, surface erosion (Makino, Idenuma, Murakami, & Ohshima, 2001). Secondly, high temperatures may have altered the conformation and/or bonding of the carrageenan molecules, leading to a more condensed hydrogel matrix.

368 3.5. Leakage of entrapped β -galactosidase

369 For many commercial applications, it is important for the enzyme molecules to
370 remain trapped within the hydrogel beads, since this will improve their stability and
371 recoverability. Consequently, the tendency for the enzyme to leak out of the
372 carrageenan-based beads during storage at 25 and 37 °C and pH 7 was measured (Fig.
373 5). Over 25% of the immobilized β -galactosidase was released from the carrageenan
374 beads into the surrounding solution after 8 h incubation at 25 °C, while nearly 45% of
375 the enzyme was released when the samples were incubated at 37 °C. The observed
376 substrate hydrolysis by the enzyme may therefore have partly occurred within the
377 hydrogel beads due to the encapsulated β -galactosidase, and partly outside the beads
378 due to the released β -galactosidase.

379 The leakage of the enzymes may be attributed to the relatively large pore size of
380 the beads compared to the dimensions of the enzyme molecules. The faster leakage at
381 the higher temperature may have been due to the higher diffusion coefficient of the
382 enzyme, or due to an increase in bead pore size. As it has been highlighted in previous
383 studies, leakage is one of the major drawbacks of the hydrogel encapsulation
384 technique (Blandino, Macías, & Cantero, 2000). A number of approaches may be
385 utilized to overcome this limitation: (i) preparing hydrogel beads with smaller pore
386 sizes; (ii) utilization of attractive physical interactions between the enzyme and
387 hydrogel matrix, for example, hydrogen bonding, electrostatic, or hydrophobic
388 attraction; (iii) coating the hydrogel beads with a layer that inhibits molecular
389 diffusion, for example, by electrostatic deposition; (iv) utilization of beads with larger
390 dimensions to increase the diffusion path length. In future studies, we intend to
391 examine some of these strategies for improving the encapsulation and retention
392 efficiency of the beads.

3.6. Microstructure

The microstructure of the enzyme-loaded carrageenan beads was measured using confocal scanning laser microscopy (Fig. 6). In this case, smaller beads were prepared so that they could be examined under the microscope, and the β -galactosidase was stained green using a fluorescent dye (FITC). The enzyme was homogeneously distributed within the hydrogel beads immediately after they were fabricated (Fig. 6a). However, after storage, the outer continuous phase became a uniform green colour suggesting that some of the β -galactosidase molecules were released into the surrounding medium (Fig. 6b). To further confirm enzyme release, the medium surrounding the beads was routinely removed and replaced with a fresh buffer solution, and the change in appearance of the beads was measured. These experiments showed that the fluorescence intensity inside the beads was greatly reduced (Fig. 6c) compared with the initial beads (Fig. 6a), which, again, highlights that the enzyme molecules readily diffused out of the hydrogel beads. The release of enzymes from the hydrogel beads suspended in buffer solution is mainly due to diffusion through the water phase. We therefore used the fluorescent method to measure changes in the concentration of the enzyme within freeze-dried beads after a period of storage (Fig. 6d). In this case, the fluorescence intensity remained relatively constant, which can be attributed to the fact that the protein could not diffuse out of the beads in a dried state. It may, therefore, be advantageous to convert the encapsulated enzymes into a powdered form, and then rehydrate them immediately prior to utilization.

4. Conclusions

In this study, enzyme-loaded hydrogel beads were fabricated using an injection-gelation method: a β -galactosidase/ κ -carrageenan solution was injected into a hardening solution containing K^+ ions. The beads formed had a smooth spherical shape with diameters ranging from around 2 to 3 mm. Catalytic activity studies

indicated that encapsulation of the enzyme within the hydrogel beads could not prevent acid-induced or thermal-induced loss of activity, but it could increase β -galactosidase activity under moderate pH and temperature conditions. The physicochemical origin of this effect was mainly attributed to the ability of the K^+ ions used to cross-link the polysaccharide chains to increase the stability and activity of the β -galactosidase. One of the main limitations of the hydrogel beads identified in this study was the fact that the enzyme leaked out of the beads during storage in aqueous solutions. Consequently, further work is needed to develop hydrogel beads that are better able to retain the enzyme, while also maintaining its activity. The results of our study could provide useful information for the rational design and fabrication of hydrogel beads for enzyme encapsulation and delivery.

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Figure(s)

(a)

(b)

Figure 1. The catalytic activity of free and encapsulated β -Gal as a function of (a) pH and (b) temperature. The enzyme and beads were prepared in 5mM PBS buffer at pH 7 or 25 $^{\circ}\text{C}$.

Figure 2. The catalytic activity of β -Gal prepared in different solution (25°C, pH 7).

Figure 3. Visual appearance of the color change in different beads followed the formation of the yellow o-NP product (left to right: non-encapsulated bead, encapsulated bead, and free enzyme with bead after reaction).

(a)

Figure 4. The particle diameter of bead as a function of (a) pH and (b) temperature. An average was calculated out of 10 beads.

Figure 5. Leakage of β -Gal from hydrogel beads as a function of incubation time. The capsules were immersed into PBS buffer (5mM, pH 7) at room temperature and body temperature.

Figure 6. Confocal micrographs of enzymes-loaded beads (a) just after the fabrication (b) after storage (c) after storage and continually washed (d) after storage in dehydrated state. The enzyme (green) was stained with FITC.