Preparation and characterization of gellan gum microspheres containing a cold-adapted β -galactosidase from *Rahnella* sp. R3

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Abstract

R- β -Gal is a cold-adapted β -galactosidase that is able to hydrolyze lactose and has the potential to produce low-lactose or lactose-free dairy products at low temperatures (4°C). Cold-adapted enzymes unfold at moderate temperatures due to the lower intramolecular stabilizing interactions necessary for flexibility at low temperatures. To increase stability and usage-performance, R- β -Gal was encapsulated in gellan gum by injecting an aqueous solution into two different hardening solutions (10 mM CaCl₂ or 10 mM MgCl₂). Enzyme characteristics of both free and encapsulated R- β -Gal were carried out, and the different effects of two cations were investigated. R- β -Gal showed better thermal and pH stability after encapsulation. Ca²⁺ gels had higher encapsulation efficiency (71.4%) than Mg²⁺ (66.7%) gels, and Ca²⁺ formed larger inner and surface pores. R- β -Gal was released from the Ca²⁺ hydrogel beads more rapidly than the Mg²⁺ hydrogels during storage in aqueous solution due to the larger inner/surface pores of the matrix.

Keywords: gellan gum; hydrogel beads; cold-adapted R- β -Gal; encapsulation

1. Introduction

Lactase, a β -galactosidase (β -gal, EC 3.2.1.23) necessary for the hydrolysis of lactose (Fan et al., 2015; Harju, Kallioinen, & Tossavainen, 2012), is an important industrial enzyme in the dairy industry and also for the production of functional saccharides such as lactulose and galacto-oligosaccharides (Asraf & Gunasekaran, 2010; Wu et al., 2013) (GOS). β -gals hydrolyze the β (1-3) and β (1-4) linkage in oligo- and disaccharides (such as lactose) and simultaneously transfer a galactosyl residue to other acceptors. β -gals are widely used to remove lactose from dairy products, to improve the taste of ice cream and to convert whey into other valuable products. (Escobar, Bernal, & Mesa, 2013) Currently, the major commercial β -gals include Lactozym Pure® (Novozymes) and Maxilact® (DSM Food Specialties). They are optimally active at moderate temperatures ($\sim 37^\circ\text{C}$) but are rarely active at 4°C . Cold-adapted enzymes are functional at 4°C , however, they have higher sensitivity to temperature (even moderate temperatures) and mechanical shear and lose activity. Thus the dairy industry is in need of an enzyme with optimal activity at the conditions compatible with dairy processing, i.e. at temperatures close 4°C , and pH near 6.5 but also with stability at moderate temperatures and mechanical shear.

There has been considerable interest in the development of novel enzyme immobilization systems because immobilized enzymes are more stable and can be easily separated during industrial processing. There are two main types of immobilization methods: carrier-bound and carrier-free. (Wang et al., 2011) Cross-linked enzyme aggregates are a form of carrier-free immobilization that usually have relative high immobilization efficiencies, however, a reagent (glutaraldehyde, genipin) to cross-link the proteins is required and these reagents are often carcinogenic. (Li et al., 2015; Wang et al., 2011) Encapsulation is a carrier-bound method that is

simple and mild and protects bioactive proteins from unfolding and aggregation.(Bhatia, Brinker, Gupta, & Singh, 2000) Recently interest in encapsulating proteins within polymer particles of different environments has increased.(Grosová, Rosenberg, Rebroš, Šipocz, & Sedláčková, 2008; Shen et al., 2011; Zhang, Zhang, Zou, & McClements, 2016) Hydrogels carrier particles are often used because they are inexpensive and simple to prepare. Hydrogels are porous and accessible to aqueous fluids containing enzyme substrates. They can be formed by molding, injection, coacervation, templating, and thermodynamic incompatibility.(Matalanis, Jones, & McClements, 2011) The injection method involves the injection of a gellable biopolymer solution containing bioactive components into a second solution containing an agent that causes the biopolymer to gel. It is relatively easy to control and economical, thus it is realistic to apply on an industrial scale. For safety consideration, it is advantageous to use natural polymers, proteins (e.g. egg white, soybean, and whey proteins)(Bryant & McClements, 1998; Clark, Kavanagh, & Ross-Murphy, 2001) and polysaccharides (chitosan,(Klein et al., 2016) alginate,(Zhang et al., 2016) k-carrageenan(Selvakumaran, Muhamad, & Razak, 2016) and gellan gum(Chakraborty et al., 2014)) to construct hydrogels.

Gellan gum is a water soluble linear tetrasaccharide composed of repeating units of β -1,3-D-glucose, β -1,4-D-glucuronic acid and α -1,4-L-rhamnose residues in a molar ratio of 2:1:1.(Jansson, Lindberg, & Sandford, 1983) Gelation is the result of the formation of double helical junction zones by different polymer strands followed by the aggregation of the hydrophobic double helical segments to form a three-dimensional network by complexation with cations and hydrogen bonding with water.(Agnihotri, Jawalkar, & Aminabhavi, 2006) Compared to other gelling agents, gellan gum is functional at very low use levels and capable of forming gels with all ions, including sodium, calcium, magnesium, and hydrogen ions which are

frequently used in food processing. The physical or ionic gelation, lack of toxicity, and resistance to heat and acid stress during fabricating make gellan gum suitable as a structuring and gelling agent in food and biomedical fields.(Posadowska, Brzychczy-Wloch, & Pamula, 2016; Wang et al., 2007) It is used in a wide range of food products including confectionery products, jams, fabricated foods, water-based gels, dairy products such as ice cream, yogurt, milkshakes, and gelled milk.(Bayarri, Costell, & Duran, 2002; Sworn, Sanderson, & Gibson, 1995)

Previously, we isolated a cold-adapted β -gal from *Rahnella* sp. R3 (R- β -Gal). The enzyme was cloned and expressed in *E. coli*, and the enzymatic properties of the purified recombinant protein were systematically studied.(Fan et al., 2015) At 4°C, R- β -Gal (free enzyme) showed a K_m value of 6.5 mM and 2.2 mM toward *ortho*-nitrophenyl- β -galactoside (ONPG) and lactose, respectively. R- β -Gal which specifically hydrolyzes lactose showed relative high activity toward lactose at 4°C and neutral pH (6.5) without side reactions, indicating that R- β -Gal can be potentially used in dairy industry to remove lactose in dairy products at 4°C to avoid spoilage and flavor changes. In this study in order to improve the stability of R- β -Gal against temperature and mechanical stresses, we used an ionotropic technique to encapsulate R- β -Gal into gellan gum microspheres. Two different cations, calcium and magnesium, were used to induce cationic gelation of gellan gum and their effects on encapsulation and catalytic activities were investigated.

2. Material & Methods

2.1 Materials

Ortho-Nitrophenyl- β -galactoside (ONPG), *o*-nitrophenol (ONP), low acyl gellan gum, and glucose (GO) assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nutrient media were supplied by Oxoid (Basingstoke, UK). NuPAGE 4-12% Bis-Tris Gel was purchased

from Life Technologies (NY, USA). All other reagents were analytical grade and used without further purification.

2.2 Protein purification

The expression and purification of R- β -Gal was previously reported.(Fan et al., 2015; Fan et al., 2016) The same protocols were used in this study. Briefly the enzyme was cloned into *E. coli* by transformation of a plasmid containing the R- β -Gal sequence. After lysing the harvested bacteria on ice the protein was purified by column chromatography. All of the chromatographic steps were carried out at room temperature (20°C) using an FPLC system (GE Healthcare, USA). The protein purity was examined on NuPAGE 4-12% Bis-Tris Gel and the protein concentration was determined by UV absorption at 280 nm. The NaCl concentration of the final purified R- β -Gal sample was reduced to less than 1 mM by repeated dilution and concentration with a buffer containing 10 mM Tris (pH 7.0) using an Ultracel-30k filter device (Millipore, MA, USA).

2.3 Activity assay of R- β -Gal

The hydrolytic activity of the encapsulated R- β -Gal was determined with both ONPG (10 mM, pH 7.0) and lactose (14.6 mM, pH 7.0). One unit of enzyme activity is defined as 1 μ mol of product (ONP or glucose) released per minute under given condition. The activity was determined by combining 100 μ L enzyme, 400 μ L of 10 mM ONPG or 400 μ L of 14.6 mM lactose in 10 mM potassium phosphate buffer (pH 7.0) for 10 min at 4°C. The ONPG reaction was stopped by adding 500 μ L of 10% (w/v) sodium carbonate. The lactose reaction was stopped by heating the mixture to 90°C for 2 min. The released D-glucose was determined by absorbance at 540 nm using a glucose (GO) assay kit, and the released ONPG was determined at 420 nm.

2.4 Preparation of hydrogel beads

Gellan gum beads were prepared by the cation-induced ionotropic gelation method.(Agnihotri et al., 2006) An aqueous gellan gum (1.2%) solution was prepared by dissolving the gellan gum polymer powder in distilled water by gentle stirring and kept at 90°C for 30 min to achieve complete hydration, and then the temperature was reduced to 40°C with further mixing. The enzyme and gellan gum solutions were combined (1:1 v/v) at 40°C to form a solution that contained R-β-Gal and gellan gum (0.6%). Then 200 μL gellan gum-enzyme solutions were added to 25 mL CaCl₂ (10 mM, pH 7.0, GG-Ca) or MgCl₂ (10 mM, pH 7.0, GG-Mg) solutions using a syringe with a 23 G hypodermic needle under gentle stirring. The hydrogel beads were allowed to harden for 30 min at room temperature (20°C). Then the hydrogel beads were collected by filtration and washed with distilled water to remove excess cations. The beads were used immediately after preparation.

2.5 Effect of pH, temperature, and metal ions

The influence of pH on the relative activity of both free and encapsulated enzyme was established by incubating the enzyme in a pH range of 5.5- 9.0 (pH 5.5- 8.0: 10 mM potassium phosphate buffer; pH 8.0- 9.0, 10 mM tris-HCl) for 10 min at 4°C. The effect of temperature on enzyme activity was determined by incubating enzyme at 4- 65°C for 10 min.

The effects of metal ions on enzyme activity, free/ encapsulated R-β-Gal was assayed with 10 mM ONPG in 10 mM Tris-HCl (pH 7.0) in the presence of 5 mM of various metal ions, Na⁺, K⁺, Ca²⁺, Zn²⁺, Mg²⁺, Co²⁺, and Mn²⁺, at 4°C for 10 min. Free/encapsulated enzyme in distilled water without any reagents was used as a control.

2.6 Kinetic parameters of free and encapsulated R-β-Gal

The kinetic parameters of free and encapsulated enzymes in the presence of ONPG or lactose substrates (ONPG: 1-35 mM, lactose: 1-100 mM) were measured at 4°C. The observed data were fitted to the Michaelis-Menten equation by GraphPad Prism (GraphPad Software, Inc., CA, USA) and Lineweaver-Burk plots were used to calculate the kinetic parameters.

2.7 Enzyme encapsulation efficiency, retention and release

The encapsulation efficiency was determined indirectly by measuring the protein concentration in CaCl₂ or MgCl₂ buffer and washing solution by UV absorption at 280 nm.(Yang, Xia, Tan, & Zhang, 2013) The encapsulation efficiency was calculated by the following equation:

$$\text{Encapsulation efficiency (\%)} = \{1 - [\text{enzyme}]_b / [\text{enzyme}]_a\} * 100\%$$

Where [enzyme]_a and [enzyme]_b are the concentration of protein added into the system and free protein, respectively.

The release characteristics were determined by immersing encapsulated enzyme in 10 mM potassium phosphate buffer (pH 7.0) at 4°C with 100 r/min shaking. The concentration of R-β-Gal was monitored at specified time intervals (0-8 h) by measuring the absorbance at A280. The percent enzyme release is defined as the ratio of free enzyme concentration to the initial concentration times 100 measured at different incubation times.

2.8 Surface morphology analysis

Freeze dried gellan hydrogel beads were mounted on an aluminum stub using double-side carbon tape (Ted Pella, Redding, CA) and coated with gold in a Denton Desk II Sputter coating unit for 45 s. The surface morphology of the hydrogel beads was observed and photographed by a Hitachi S-4700 field emission scanning electron microscope (Hitachi, Japan) at an accelerating voltage of 2 kV.

3. Results and discussion

3.1 Encapsulation of R- β -Gal in hydrogel beads

Experiments were carried out to investigate suitable conditions for fabricating R- β -Gal-loaded gellan gum hydrogel beads that had good enzyme performance and physical stability. It was found that hydrogel beads, formed by injecting 0.6% R- β -Gal-loaded gellan gum solution into 10 mM magnesium chloride/ calcium chloride solution at pH 7.0 with a 23G hypodermic needle, had good enzyme performance. The resulting beads had a spherical shape with smooth and hard surface. The enzyme load level was studied by assaying the activity of different concentrations of R- β -Gal. A R- β -Gal level of ~ 0.005 mg/ mL was found to give a rate for enzyme-catalyzed hydrolysis reaction that could be measured on the experimental scale.

3.2 Influence of pH, temperature, and metal ions on enzyme activity

The effect of pH on the activity of encapsulated was compared to free R- β -Gal by measuring β -galactosidase activity in the pH range 5.5-9.0 with either ONPG or lactose as substrate (Figure 1). For both substrates, the optimum pH for the free enzyme was found to be about 6.5. After entrapment in gellan gum particles, the optimum pH shifted toward a more alkaline region, pH 7.0-7.5, for both GG-Mg and GG-Ca beads. Moreover, both immobilized enzymes had higher activity ($> 40\%$) in the pH range of 8.0-9.0 compared to the free enzyme ($< 30\%$), demonstrating interactions with the gellan matrix that stabilized the enzyme. However, at acidic pH, the enzyme activity decreased, with less than 40% and 50% remaining at pH 5.5, compared to the free form for ONPG (Figure 1a) and lactose (Figure 1b) as substrates, respectively. The lower activity may be due to displacement of the divalent metal ions by hydronium ions altering the gellan matrix and microenvironment surrounding the enzyme and changing the charged species around the enzyme catalytic sites in respect to the bulk solution.(Bayramoglu, Tunali, & Arica, 2007; De

Maio et al., 2003; Klein et al., 2013) As shown in Figure 1b, GG-Ca is more stable than GG-Mg in alkaline pH region when lactose is the substrate.

(a)

(b)

Figure 1. (a) Effect of pH on free and encapsulated R- β -Gal with 10 mM ONPG. (b) Effect of pH on free and encapsulated R- β -Gal with 0.5% lactose: GG-Mg (●); GG-Ca (■); free enzyme (□)

The activity of R- β -Gal after encapsulation in the range of 4°C to 65°C is shown in Figure 2. When ONPG was the substrate (Figure 2a), both free and encapsulated R- β -Gal had optimum activity at 35°C and the profile of activity between 4-35°C was similar for free and encapsulated enzyme. Above 45°C free R- β -Gal began to precipitate and lose activity. By contrast, the encapsulated R- β -Gal retained nearly 30% of the maximum activity at 65°C. When the substrate is lactose (Figure 2b), the optimum temperature of GG-Mg decreased 10°C from 25°C to 15°C, whereas the optimum temperature of GG-Ca was 25°C and the same as the free enzyme (Figure 2b). The temperature optimum for activity for *E. Coli* β -gal immobilized on glycophasse-coated glass was also reported to decrease, 10°C lower, than that of the free enzyme.(Manjon, Llorca, Bonete, Bastida, & Iborra, 1985) By contrast, the temperature optimum for β -gal from *Pseudoalteromonas* sp. 22b, another cold-adapted enzyme, immobilized on chitosan increased 10°C (from 40 to 50°C).(Makowski et al., 2007) At the highest temperature, 65°C, the encapsulated R- β -Gal, GG-Mg and GG-Ca, retained activity (>80% of maximum activity of free enzyme) while the free enzyme was completely inactivated at this temperature. The wider range and higher activity with temperature can be attributed to stabilizing interactions with the gellan matrix.

(b)

Figure 2. Effect of temperature on both free and encapsulated R-β-Gal with (a) 10 mM ONPG and (b) 0.5% lactose: GG-Mg (●); GG-Ca (■); free enzyme (□)

Some β-gals require metal ions, e.g. Mg^{2+} , Mn^{2+} and Na^{+} , as co-factors for their hydrolytic activity.(Bultema, Kuipers, & Dijkhuizen, 2014; Karasová-Lipovová et al., 2003) It has been reported that Na^{+} , K^{+} and Co^{2+} reduced free R-β-Gal activity slightly, whereas Ca^{2+} , Mg^{2+} , and Mn^{2+} increased the activity. Zn^{2+} precipitates and deactivates the free enzyme.¹ As shown in Figure 3, the effect of metal ions on encapsulated R-β-Gal is similar to that of the free enzyme. However, encapsulation strongly stabilized the R-β-Gal against Zn^{2+} precipitation, retaining approximately 25% of the free enzyme activity.

Figure 3. Effect of metal ions on free and encapsulated R-β-Gal. Free/ encapsulated enzyme in distilled water without any reagents was used as a control.

3.3 Kinetic parameters

The apparent enzyme kinetic parameters ($K_{m, app}$ and $V_{max, app}$) for free and encapsulated R-β-Gal with ONPG and lactose as substrates were determined. Compared to the free enzyme the $K_{m, app}$ of the encapsulated enzyme increased approximately 2-fold and 4.5-fold when ONPG and lactose were the substrate, respectively (Table 1). The higher $K_{m, app}$ of the encapsulated enzyme indicates lower affinity for the substrate and requires a higher substrate concentration to achieve the same enzymatic rate. The higher K_m may be due to diffusional limitation by the matrix, the repulsive electrical potential due to same charges on support and substrate, and matrix-enzyme interactions leading to less favorable conformational changes.(De Maio et al., 2003; Fischer et al., 2013) The observed $V_{max, app}$ for the free enzyme was slightly higher for ONPG than for lactose.

Table 1. Michealis-Menten constant

Substrate	Temperature (°C)	K_m , app (mM)	V_{max} , app (U/mg)
ONPG	Free R- β -Gal	6.5 $\pm$ 0.65	6.2 $\pm$ 0.16
	GG-Mg	15.7 $\pm$ 4.3	11.9 $\pm$ 1.39
	GG-Ca	11.4 $\pm$ 1.78	11.3 $\pm$ 0.64
Lactose	Free R- β -Gal	2.7 $\pm$ 0.55	1.9 $\pm$ 0.06
	GG-Mg	12.6 $\pm$ 2.31	3.0 $\pm$ 0.16
	GG-Ca	12.1 $\pm$ 1.83	3.2 $\pm$ 0.14

3.4 Encapsulation efficiency and leakage of R- β -Gal

The encapsulation efficiency and enzyme leakage are important production and processing parameters. The encapsulation efficiency was determined by measuring the protein concentration in the supernatant immediately after formation of the hydrogel beads. The encapsulation efficiencies for GG-Mg and GG-Ca were 66.7% and 71.4%, respectively, suggesting that a considerable amount of R- β -Gal escaped during the formation of the hydrogel. Furthermore, the leakage of R- β -Gal during storage at 4°C is shown in Figure 4. Less than 20% and about 30% of the encapsulated R- β -Gal was released from the GG-Mg and GG-Ca beads, respectively, after 7 h incubation. The difference in leakage rate between GG-Ca and GG-Mg may be attributed to different pore sizes.

Figure 4. The leakage of R- β -Gal from GG-Ca and GG-Mg as a function of incubation time. The hydrogel beads were immersed in potassium phosphate buffer (10 mM, pH7.0) at 4°C. GG-Mg (●); GG-Ca (■);

3.5 Microstructure of the hydrogel beads

The microstructure of the freeze-dried R- β -Gal-loaded Ca^{2+} and Mg^{2+} gellan gum beads was examined by scanning electron microscope (SEM) and are shown in Figure 5a and Figure 5b. The freeze-dried beads appeared spherical with approximate average diameters of 2.2 mm and 1.5 mm for the GG-Ca and GG-Mg beads, respectively. The smaller diameter and greater surface of the GG-Mg may have resulted in the lower encapsulation efficiency due to loss of enzyme at the interface during gelation. The surface of both hydrogel beads appeared relatively continuous. However, a close inspection identified several small cracks probably formed during the freeze-drying and pores that may explain the leakage of encapsulated enzymes (Figure 5c, Figure 5d). Figure 5e and Figure 5f images clearly show the appearance of a highly porous structure in the hydrogel beads. The inner surface of the GG-Ca hydrogel bead has larger pores than the GG-Mg bead. The larger pores may facilitate diffusion of the enzyme from the hydrogel bead.(Blandino,

Macías, & Cantero, 2000) The observed protein release rate of GG-Ca is slightly higher than that of GG-Mg (Figure 4).

(a)

(b)

(c)

(d)

(e)

(f)

Figure 5. Scanning electron microscope images of the freeze-dried GG-Ca and GG-Mg beads. (a), (c), (e) the surface image (x50), (x100), and cross-sectional image of GG-Ca beads. (b), (d), (f) the surface image (x50), (x100), and the cross-sectional image of GG-Mg beads.

Conclusion

The gellan gum hydrogel beads incorporating the cold-adapted β -galactosidase R- β -Gal were prepared by a mild ionotropic gelation which avoids high-temperature encapsulation, toxic cross-linking reagents and organic solvents or reagents. The GG-Mg and GG-Ca gellan gum encapsulated beads were shown to be more effective at low temperature (4°C) and moderate pH (7.0) compare to the free enzyme. Moreover, they had better thermal stability. Encapsulated GG-Ca and GG-Mg showed similar enzyme kinetics characteristics with higher K_m and V_{max} than the free enzyme. Encapsulation efficiency was about 70%. There was about 20-30% enzyme leakage after 7 h. SEM showed the beads were 1-2 mm spheres with a honeycomb interior after freeze drying. In conclusion, the GG-Ca and GG-Mg, gellan gum hydrogel beads, have potential for dairy food processing where enzyme activity at low temperatures and stability to mechanical shear are necessary.

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