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Encapsulation of chlorophyllase in hydrophobically modified hydrogel

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Abstract

Amphipathic chlorophyllase, in the form of a ternary micellar (TMS) or reverse micellar system (RMS) medium, was encapsulated in alginate hydrogels with yields of 100 and 60% respectively. In comparison, chlorophyllase in aqueous buffer was retained in the hydrogel to low levels (30%). Specific activities of chlorophyllase in TMS/alginate and RMS/alginate were 0.008 and 0.007 μM chlorophyll/$mg_{protein}$ min, respectively. A novel approach to encapsulating chlorophyllase involved emulsification/internal gelation of the chlorophyllase–TMS or –RMS system. Higher specific activity was observed (0.04 μM chlorophyll/$mg_{protein}$ min) in TMS/alginate microbeads. An examination of the partition behavior of chlorophyll substrate between hydrogel and hexane shows that chlorophyll demonstrates a 7–10-fold higher partition coefficient when in the TMS or RMS hydrophobic gel than in pure alginate hydrogel. With the same amount of organic solvent introduced in the form of a micellar medium into alginate sol, the RMS shows a higher capability to enhance the hydrophobicity of alginate beads, as measured through the partition coefficient of chlorophyll, than the TMS. The extent of enhancement of the partition coefficient depends on the amount and hydrophobicity of the components introduced into alginate, affecting the hydrophobic–hydrophilic balance of the gel.

Keywords: Alginate; Chlorophyllase; Ternary micellar system; Reverse micellar system; Entrapment; Partition coefficient

1. Introduction

Chlorophyllase is a membrane glycoprotein that catalyzes the bioconversion of chlorophyll and related pheophytin into chlorophyllides and pheophobides in vitro [1], as well as the synthesis of chlorophylls and pheophytins from chlorophyllides and pheophobides in vivo [2]. The problem associated with the hydrolysis of chlorophyll pigments in vitro results from the water-insolubility of both enzyme and substrate.

Alginate has been widely investigated as an entrapment matrix for enzymes and cells, dissolved or suspended in aqueous media. The attractiveness of alginate is related to its biocompatibility, and its ability to form gels under mild conditions using simple formulation procedures. There are relatively few reports on the use of alginate entrapped enzymes in non-aqueous media with the aim of hydrolyzing water-insoluble substrates. For application of entrapped biocatalysts in organic medium, the high water content in the alginate matrix as well as

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multi-point interactions between the matrix and enzyme could provide a stabilizing effect against the denaturing influence of the organic solvent. Alginate unlike most other hydrogels, was found to be stable in a range of organic solvents, with high enzyme retention [3] explained by the low solubility of proteins in organic solvents. The major disadvantage of applying alginate entrapped enzymes with water-insoluble substrates stems from external and internal diffusional restrictions imposed by the entrapment matrix.

The hydrophilic-lipophilic balance (HLB) of gels can affect the activity of entrapped enzyme in non-aqueous media [4,5]. The HLB can alter the partition coefficient of water-insoluble substrates between the non-aqueous external phase and the hydrophilic gel internal matrix, hence the mass transport of hydrophobic substrate from the hydrophobic external solution, to within the hydrophilic matrix containing the immobile enzyme. Thus, it could be expected that water-insoluble substrate would partition more effectively (higher partition coefficient) into a more hydrophobic gel matrix. In the present study, hydrophobic components were introduced into gel sol before gelation with the aim of changing its HLB. The additional potential advantage of introducing hydrophobic components into alginate matrix is that it may result in higher enzyme retention. In aqueous medium, it is known that the pores of the alginate matrix are too large to retain enzyme with low molecular weight [6]. However, when the alginate beads are placed in organic solvent, no leakage of enzyme was observed [3].

Hydrophobic components that have been introduced into alginate include organic [4,5,7] or colloidal silicate [8], and polymeric surfactant [9]. Alternatively, acetylation of alginate mannuronic acid residues has been used to lower the matrix HLB [10]. In the present study, hydrophobic organic solvent was stabilized in alginate matrix via a surfactant, as an alternative to the use of a hydrophobic copolymer to lower the gel HLB.

Introducing organic solvent in the form of a ternary micellar medium or reverse micellar medium to alginate sol has not been previously reported. The present study is a part of an ongoing work with the aim of applying immobilized chlorophyllase to remove excess chlorophyll in Canola oil. The objective of this study was to investigate the feasibility of introducing organic solvent into alginate hydrogel, to optimize the protocol to entrap chlorophyllase into the new hydrophobically modified alginate gel matrix, and study the possibility of applying alginate entrapped enzyme to hydrolyse the hydrophobic substrate in organic solvent.

2. Materials and methods

2.1. Materials

Span 85, Aerosol OT (AOT), and isooctane were obtained from Sigma (St. Louis, MO). Hexane, tris(hydroxymethyl aminomethane crystalline), and acetone were purchased from Fisher (Nepean, Canada). Calcium chloride was provided by BDH Inc. (Toronto, Canada). Bovine serum albumin (BSA) was supplied by Boehringer Mannheim GmbH, (Germany). All reagents were used as received.

Alginate SG-150 was kindly donated by SKW Biosystems (France). Chlorophyll was extracted from spinach leaf according to Khalyfa et al. [11] and chlorophyllase was extracted from *Phaedactylum tricornutum* (algae) according to the protocol outlined in Kermasha et al. [12].

2.2. Preparation of encapsulant media containing chlorophyllase

Two encapsulant media containing hydrophobic components and chlorophyllase were introduced into alginate sol before gelation. A ternary micellar system (TMS) consisted of a mixture (70:30, v/v) of Tris–HCl buffer and hexane containing 75 μM Span 85. Alternatively, a reverse micellar system (RMS) consisted of a mixture (14:3, v/v) of Tris–HCl buffer and isooctane containing 200 mM AOT. Chlorophyllase was also introduced to alginate sol in Tris–HCl buffer (20 mM, pH 8.0) as an alternative to the hydrophobic encapsulant media described above. Volume ratios of chlorophyllase in the forms of TMS, RMS and Tris–HCl buffer to alginate sol are: 1:4, 1:5 and 1:9, respectively. The final enzyme concentration in alginate sol was 10 μg chlorophyllase/ml.

2.3. Immobilization of chlorophyllase

Sodium alginate solution of different concentrations was mixed with encapsulant media to yield a final

alginate concentration of 2% (w/v). Two methodologies were then followed to formulate alginate beads; that of external gelation (conventional approach) and emulsification/internal gelation (novel approach) [13,14]. Alginate beads were formed by dropwise addition of alginate sol containing chlorophyllase encapsulant into a calcium (0.2 M $CaCl_2$) gelation bath (external gelation). A coaxial stream of air around the extrusion needle was used to control the size of the beads. Alternatively, alginate/encapsulant mixture (20 ml) was mixed with 1.0 ml of calcium carbonate suspension (10%, w/v). The resulting alginate preparation was emulsified within 100 ml Canola oil by mixing with a marine type impeller at 200–500 rpm for 15 min, followed by the addition of 20 ml Canola oil containing 1.0 ml glacial acetic acid, reducing the alginate pH to approximately 6.5, releasing calcium ion from insoluble carbonate complex, and initiating gelation in situ (internal gelation).

2.4. Encapsulation yield

The encapsulation yield was evaluated in terms of the difference between the total protein added into alginate sol before gelation, and the protein released to the supernatant $CaCl_2$ bath after gelation. Protein in the $CaCl_2$ bath was quantified spectrophotometrically at 280 nm using BSA as standard.

2.5. Partition of substrate between organic and alginate phase

The compositions of each reagent added to make various mixtures of alginate sol are listed in Table 1. Following gelation, the beads were collected and dried with filter paper. Bead volume was determined by difference before and after adding alginate beads to water in a graduated cylinder.

Alginate beads were added to an equal volume of hexane containing 15 μg/ml chlorophyll, mixed and incubated 24 h at room temperature in an amber test tube with solvent proof cap. The chlorophyll concentration before and after incubation was determined spectrophotometrically at 661 nm. The partition coefficients were calculated by the ratio of chlorophyll concentrations in the organic phase to those in the gel phase, determined by mass balance.

2.6. Enzyme assay

2.6.1. Protein quantification

Protein content of chlorophyllase extract was determined by the modified Lowry method [15], using BSA as standard.

2.6.2. Enzyme preparation

Enzyme suspension was freshly prepared in Tris–HCl buffer (20 mM, pH 8.0) containing 4 mM $MgCl_2$ and 5 mM dithiothreitol, then homogenized in a tissue grinder at 4 °C [16].

2.6.3. Substrate preparation

Stock chlorophyll solution (1 mg/ml) was prepared by dissolving chlorophyll in cold (−20 °C) hexane:acetone (75:25, v/v) according to Khamessan et al. [16]. The stock solution and subsequent dilution (by cold hexane, −20 °C) were freshly prepared before use.

2.6.4. Activity assay

Enzymatic reactions were carried out in triplicate in a ternary micellar system, which consists of a

Table 1
Compositions of starting alginate sol for partition experiment

Number	Na–alginate (mg)	Tris–HCl buffer (ml)	Reverse micellar system (ml)	Ternary micellar system (ml)
1	0.2	10		
2	0.22	10	1	
3	0.24	10	2	
4	0.22	10		1
5	0.24	10		2
6	0.28	10		4
7	0.32	10		6
8	0.36	10		8
9	0.40	10		10

mixture (70:30, v/v) of Tris–HCl buffer and hexane containing 75 μM Span 85 [17]. Alginate beads loaded with 0.5 μg protein, and 70 μl of chlorophyll (0.12 μM) substrate solution were added to 880 μl of the ternary micellar system in a test tube with solvent proof cap. The enzyme reaction was carried out at 35 °C for 60 min with continuous shaking (300 rpm/min). The reactions were stopped by adding 1 ml of 0.5 M NaCl, 3 ml cold acetone (−20 °C), and 2 ml hexane (−20 °C) to the reaction medium successively. The reaction mixture was vortexed for 15 s and the residual hydrolyzed chlorophyll located in the hexane upper layer was separated and measured spectrophotometrically at 661 nm. The specific activity of chlorophyllase was referred to as μmol hydrolyzed chlorophyll per mg of protein per min.

3. Results and discussion

3.1. Immobilization of chlorophyllase

Fig. 1 summarizes the encapsulation yield of alginate beads formed via external gelation, containing chlorophyllase in various encapsulant media. When using Tris–HCl buffer as encapsulant media, gel beads retained only 30% of the chlorophyllase. The high permeability of alginate gel will permit the relatively low molecular weight chlorophyllase (43 kDa [18]) to be released, when immobilized in aqueous media. However, when chlorophyllase was immobilized in the form of a ternary micellar system (TMS), chlorophyllase was fully retained. It is hypothesized that the hydrophobic microenvironment provided by the TMS system was more effective in retaining the enzyme with its amphipathic character, in the hydrogel environment of the beads. When the chlorophyllase was introduced into alginate matrix in the form of RMS, only 60% of the chlorophyllase was retained in the gel matrix. Release to the extent of 40% of the initial enzyme can be explained by the interference of sodium ion from AOT on the Ca–alginate gel structure, and repulsion between anionic alginate and anionic surfactant AOT. This hypothesis is partially proven by the fact that beads could not be formed when increasing the RMS:alginate sol volume ratio to 2:5. Also, the bulk organic solvent in RMS might interfere with the gelation of alginate.

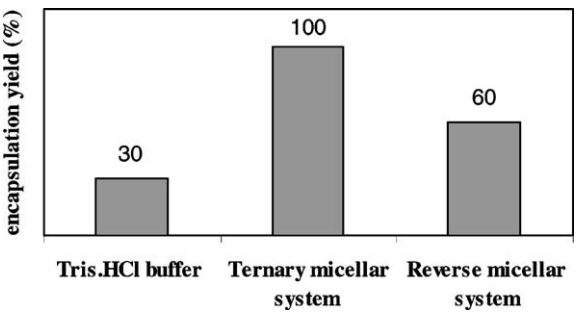

Fig. 1. Yield of chlorophyllase in the form of Tris–HCl buffer, ternary micellar system and reverse micellar system, encapsulated within alginate by external gelation (conventional approach).

3.2. Activity assay of immobilized chlorophyllase

The specific activity of entrapped chlorophyllase in the form of ternary micellar medium and reverse micellar medium, in alginate beads prepared by external gelation, is shown in Fig. 2. Chlorophyllase showed similar specific activities in both micellar systems. However, the chlorophyllase in the form of a TMS in alginate beads prepared by emulsification/internal gelation demonstrated much higher activity based on the assumption that no chlorophyllase was lost during the gelation process. This could be rationalized by the fact that the emulsification method yields smaller diameter beads, giving rise to a lower diffusion limitation imposed by the alginate matrix. Alginate beads with diameter 2–3 mm were obtained by external gelation while sub-millimeter diameter beads were achieved by the emulsification/internal gelation technique. Previous work has shown that enhanced bioconversion rates are achieved with smaller diameter beads [19]. The results suggest that alginate beads prepared by emulsification/internal gelation demonstrate higher specific activity due to the lower mass transfer limitation encountered.

3.3. Partition coefficient of chlorophyll between alginate gel phase and hexane

The partition coefficient (K_p), defined as the ratio of chlorophyll in alginate gel phase to that in the hexane phase, was measured. The equilibrium partitioning of chlorophyll between alginate and hexane phases after

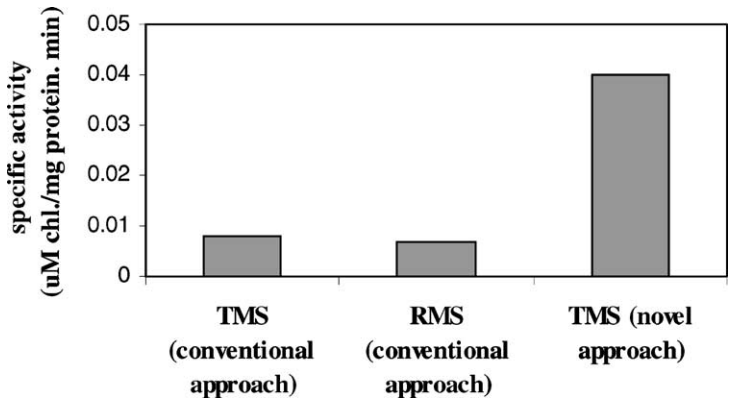

Fig. 2. Specific activity of entrapped chlorophyllase in the form of ternary micellar medium and reverse medium in alginate beads prepared by external gelation (conventional approach) and by emulsification/internal gelation (novel approach).

24 h incubation at room temperature is shown in Fig. 3. The results indicate that chlorophyll has a preference for the hexane over the alginate phase, with a $K_p < 1$. The results also demonstrate that higher partition coefficients can be obtained as more hydrophobic components are introduced into the alginate gel. The same trend was observed with both encapsulant media. This preference may be due to a combination of affects involving the hydrophilic nature of the hydrogel as well as the hydrophobic property of chlorophyll.

Results in Fig. 4 indicate that the reverse micellar system showed a stronger capability to enhance the partition coefficient of chlorophyll than that of the ternary micellar medium with the same organic solvent ratio in the hydrophobic alginate gel phase. This is at least partially due to the different hydrophobic components used in these two media.

Since the hydrophobicity of organic solvent is characterized by the log P value [20], the isooctane used in the reverse micellar system has higher log P (4.8) than that of hexane (3.5) used in the ternary micellar system. In addition, the surfactant AOT, which was used as a component in the reverse micellar encapsulant medium, is widely used in protein extraction by reversed micelles. AOT unlike most surfactants, possesses a wedge-shaped molecular geometry and

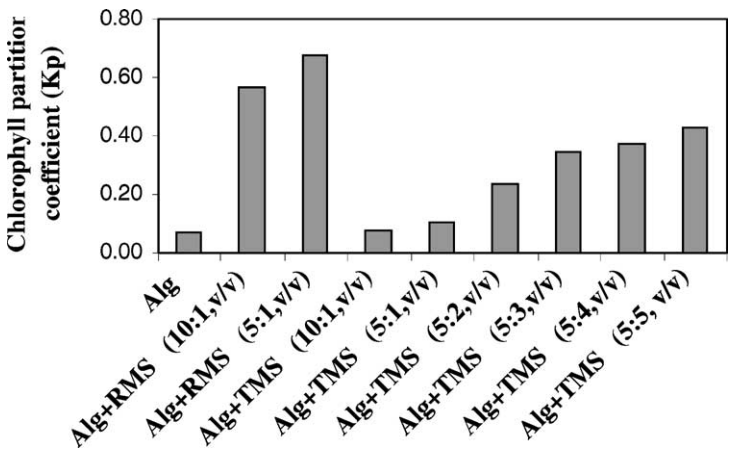

Fig. 3. Effect of compositions of hydrophobic alginate hydrogel on chlorophyll partition coefficient. Partition coefficient is defined as the ratio of chlorophyll concentration in gel phase to that in hexane phase after incubation at room temperature for 24 h.

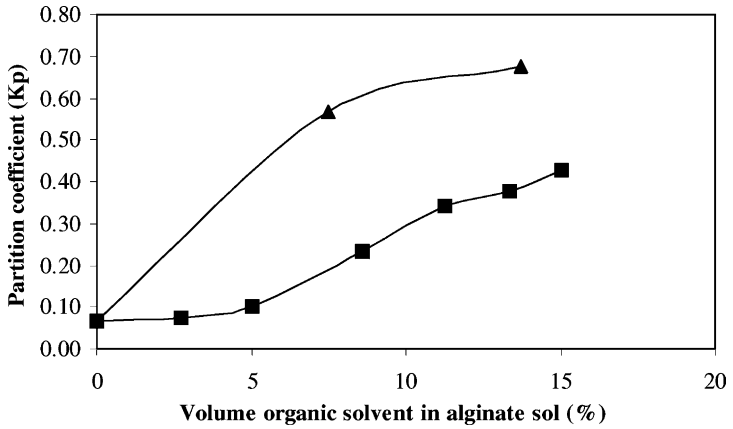

Fig. 4. Affect of volume percent of organic solvents in starting mixture on chlorophyll partition coefficient between hydrophobic alginate gel phase and hexane phase. (▲) Volume fraction of isooctane in starting alginate sol mixture; (■) volume fraction of hexane in starting alginate mixture.

can form reverse micelles without any additional amphiphile or co-surfactant [21]. The hydrophobic double tails on AOT endows it with high hydrophobicity and capability to reassemble itself in bulk organic solvent, forming reverse micelles. Span 85 (HLB 1.8 [22]) used in the ternary micellar system may also form reverse micelles, but requires a co-surfactant, and is likely not as hydrophobic as AOT.

The results indicate that the extent of enhancement of the partition coefficient depends on the amounts and hydrophobicity of the hydrophobic components that were introduced into alginate, affecting the hydrophilic-hydrophobic balance (HLB) of the gel. Similar findings were reported by Kawakami et al. [4,5]. However, these authors changed the HLB in the gels by introducing silicone prepolymer, in contrast to the use of organic solvents in the present study.

Although chlorophyll showed higher partition coefficient in alginate gel containing reverse micellar medium than that of the gel containing ternary micellar medium, the entrapped chlorophyllase showed slightly lower specific activity. These results may partially be explained by the denaturing affect of AOT. Although there are no reports on the interaction between chlorophyllase and AOT, stability of microencapsulated cutinase in AOT reverse micelles indicated very short half-lives [23–25]. Rapid deactivation was explained by a reversible denaturation process, probably caused by interaction between the enzyme and the surfactant interface of the reverse micelle. Deactivation of cutinase by anionic surfactants was confirmed by Pocalyko and Tallman using SDS [26]. A strategy to stabilize cutinase in AOT reverse micelles was developed by incorporating alcohol, such as hexanol into reverse micelles [27].

4. Conclusions

The experimental results indicated that hydrophobic micellar components in alginate matrix can not only enhance the encapsulation yield, but also increase the specific activity of gel-entrapped chlorophyllase. These findings may be explained by the higher retention of the low molecular weight amphipathic enzyme, and increased partition coefficient of chlorophyll into the gel, in comparison to pure alginate hydrogel.

Although chlorophyll showed a higher partition coefficient toward reverse micellar–alginate gel compared to ternary micellar–alginate gel, slightly lower specific activity of entrapped chlorophyllase was observed in the reverse micellar gel. This lower activity may be due to AOT-induced deactivation of chlorophyllase.

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