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Original article

Encapsulation of protease from *Aspergillus oryzae* and lipase from *Thermomyces lanuginoseus* using alginate and different copolymer types

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ABSTRACT

Although the application of enzymes in food as a food processing aid and enzyme supplement is of interest and widely used, the enzymes can be easily deactivated or lose their activity due to many causes such as pH and moisture as well as through the introduction of incompatible ingredients during food processing and storage. These problems can be solved by the encapsulation technique, especially in a gel matrix. The influences were studied of the alginate concentration, types of copolymer and their concentrations on the bead size, encapsulation yield (EY), encapsulation efficiency (EE), leakage and the retention of enzyme activity during storage period of encapsulated protease from *Aspergillus oryzae* and lipase from *Thermomyces lanuginosus* beads. A solution of purified protease or lipase was encapsulated in calcium alginate-chitosan beads (CACB), calcium alginate-xanthan gum beads (CAXB) and calcium alginate-maltodextrin beads (CAMB) using the extrusion method. Increasing the alginate and copolymer concentrations in the solution increased the bead size, EY, EE and the retention of enzyme activity during the storage period and reduced leakage of both the encapsulated protease and lipase. In addition, different types of copolymer significantly ($p \leq 0.05$) affected these properties of both encapsulated enzymes. Furthermore, protease encapsulated using 2.0% alginate and 0.2% chitosan provided the highest EY (81.7%) and EE (77.2%) with a bead size of 1.85 mm and 8.1% leakage. The retention of encapsulated protease activity and the shelf-life of encapsulated enzyme which was expressed as half-life, the time required for the enzyme activity to decrease by half ($t_{half\ life}$) were 75.8% and 27.2 wk, respectively after storage at 4 °C for 10 wk. For lipase, encapsulation using 2.0% alginate and 0.4% xanthan gum provided the highest EY (42.5%) and EE (43.9%) and the bead size and leakage were 1.81 mm and 6.2%, respectively. The retention of encapsulated lipase activity and the $t_{half\ life}$ were 77.9% and 27.8 wk, respectively after storage at 4 °C for 10 wk. CACB was a suitable complex polymer for encapsulating protease while CAXB was suitable for lipase. EY and EE values of CACB-protease were higher than those of CAXB-lipase. Therefore, the encapsulation method, gelling conditions and interactions between carriers and lipase should be further studied.

Introduction

The global market for industrial enzymes was estimated at 3.3 billion dollars in 2010 and this market was expected to reach more than 4 billion dollars by 2015 (Gurung et al., 2013). Hydrolases (predominantly proteases and amylases followed by lipases) made up at least 75% of all such enzymes with 90% of them being produced from microorganisms by fermentation (Li et al., 2012). Proteases are enzymes capable of hydrolyzing the peptide bonds between amino acids of proteins and occur in plants, animals and microbes (Rao et al., 1998). Among all microbes, *Aspergillus* and *Bacillus* are normally generally recognized as safe strains with *Aspergillus oryzae* being the predominant fungal source of enzyme (Ou and Zhu, 2012). The neutral protease II (NpII) from *A. oryzae* is a zinc-containing metalloprotease, which is essential for catalytic activity and possesses the highest casein hydrolyzing activity (Ou and Zhu, 2012). Proteases remain the dominant enzyme type due to their extensive use in the dairy and detergent industries (Ou and Zhu, 2012). Lipase from *Thermomyces lanuginosus* catalyzes not only

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the hydrolysis of a given triglyceride, but also its synthesis from glycerol and fatty acids, with the main application fields for lipases including dairy products, detergents and oil processing (Cruz-Ortiz et al., 2011).

Beside their unquestionable benefits, there are also many practical problems in the use of enzymes: 1) it is expensive to isolate and purify enzymes; 2) they are unstable structures and sensitive to process conditions; and 3) most enzymes dissolve in water in homogeneous catalytic systems, which is why they contaminate the product and cannot be recycled in the active form from reaction mixtures (Krajewska, 2004). Among many methods suggested, the encapsulation technique has been found to be the most successful to overcome these limitations because compared to free enzymes in solution, encapsulated enzymes are easily separated and reusable several cycles with little loss of activity (Ertan et al., 2007). Encapsulation can also improve the storage stability (Vikartovská et al., 2007) and control enzyme release (Blandino et al., 2000). There are various techniques used for encapsulation of enzymes, including emulsion and extrusion (Bhandari, 2009). Among the methods, the extrusion technique is a simple method which can be performed using a syringe with a needle and carried out at the room temperature with the absence of organic solvents, making it suitable for enzyme encapsulation purposes (Anjani et al., 2007).

Alginate is widely used as the supporting material for the encapsulation of enzymes (Elçin, 1995; Ertan et al., 2007; Cruz-Ortiz et al., 2011). Encapsulation within calcium alginate gel is rapid, nontoxic, highly biocompatible, inexpensive and stable in an acidic pH (Azarnia et al., 2008). Therefore, it is used in many applications as the supporting material for releasing and encapsulating cells and enzymes in the pharmaceutical and food industries (Blandino et al., 2000). However, the weakness of this encapsulation procedure is the unstable enzyme and low encapsulation efficiency, especially with water-soluble substances like peptides. Anjani et al. (2007) discovered that about 80% of total flavourzyme was lost through the macroporous structure alginate gel into the calcium chloride solution during gelling, resulting in a poor encapsulation efficiency of around 16–22%.

To reduce the limitation of the use of alginate, the addition of a copolymer is required such as chitosan, xanthan gum, or maltodextrin. Chitosan, a β-1,4 linked linear polymer of 2-acetamide-2-deoxy-β-D-glucose, is a nontoxic, biodegradable and biocompatible natural polymer, which can be used in a wide range of applications such as biomedicine, membranes, drug delivery systems, hydrogels, water treatment and food packaging (Gåserød et al., 1998). Xanthan gum is a heteropolysacharide with a primary structure consisting of a cellulose backbone (β-(1 → 4)-D-glucose) with a trisacharide side chain on every other glucose at C-3, containing a glucuronic acid residue linked (1 → 4) to a terminal mannose unit and (1 → 2) to a second mannose that connects to the backbone (Pongjanyakul and Puttipipatkhachorn, 2007). Even at low concentrations, xanthan gum solutions show high viscosity compared with other polysaccharides and this property makes it as very effective thickener and stabilizer (Elçin, 1995). Suggested by Anjani et al. (2007), it is an alternative way of boosting the encapsulation efficiency of calcium alginate by changing the composition of the cationic solution as a chitosan-alginate cross-linking interaction is formed that leads to a more stable and microporous structure of alginate gel by effectively blocking alginate pores and they reported that it enhanced the encapsulation efficiency to as high as 84% when 0.3% of chitosan was used. In addition, from the limited available literature, enhanced encapsulation efficiency has been reported of encapsulated enzyme based on intermolecular hydrogen bonding between polysaccharides. For example, Pongjanyakul and Puttipipatkhachorn (2007) reported the use of sodium alginate and xanthan gum in composite beads; the intermolecular hydrogen binding between these polysaccharides suggested that xanthan gum could modulate the physicochemical properties of alginate beads.

A number of enzymes have been encapsulated in calcium alginate chitosan beads such as urease (DeGroot and Neufeld, 2001), flavourzyme (Anjani et al., 2007) and aminopeptidase (Azarnia et al., 2008). In addition, urease and *T. lanuginosus* lipase has been encapsulated in calcium xanthan-alginate beads (Elçin, 1995) and PVA-alginate beads (Cruz-Ortiz et al., 2011), respectively. However, there little has been reported in the literature on the comparison of encapsulated enzymes with different complex polymers such as alginate-chitosan, alginate-xanthan and alginate-maltodextrin or comparison of different enzymes. Therefore, determined suitable types and concentrations of different copolymers for the encapsulation of protease from *A. oryzae* and lipase from *T. lanuginosus* in alginate (as the main supporting material). Various parameters were also investigated—the encapsulation yield, encapsulation efficiency, bead size, storage stability and leakage of the encapsulated enzyme beads.

Materials and methods

Materials and chemicals

The molecular weight and degree of deacetylation of low molecular weight chitosan were not given in the specification sheet of the company (see http://www.sigmaaldrich.com/catalog/product/aldrich/448869?lang=en&region=TH). Maltodextrin DE 20 was used. Xanthan gum is a nature-derived, high molecular weight polysaccharide which has molecular weight varying with molecular weight distribution ranges from 2×10^6 to 20×10^6 Da.

Preparation of complex polymers and cationic solutions

The preparation of complex polymers and cationic solutions was derived from that of (Sivakumar et al., 2011) with some modifications. For preparation of calcium alginate-xanthan beads (CAXB) and calcium alginate-maltodextrin beads (CAMB), alginate (Srichem, India) and copolymer either xanthan gum (Shandong, China) or maltodextrin (Corn Product International, USA) were dissolved in 50–60 °C deionized water and maintained at this temperature for 1 h to obtain the complex polymer solutions of 1.5% and 2.0% (weight per volume; w/v) alginate; and 0.2% and 0.4% (w/v) of either xanthan gum or maltodextrin. The cationic solution for CAXB and CAMB was 0.1 M $CaCl_2$ solution containing 0.1% Tween 80 was used for surface tension reduction of the gelling water in order to form spherical beads.

The preparation of calcium alginate-chitosan beads (CACB) was derived from Anjani et al. (2007). Low-molecular-weight chitosan (0.2 g; specification: low viscosity 14 mPa in 1% w/v solution; Sigma, USA) was dissolved in 10 mL of 1% (w/v) acetic acid using a magnetic stirrer at high speed. The volume was then made up to 90 mL using deionized water and the pH was adjusted to 6.2 using NaOH. $CaCl_2$ solution (1 M) and Tween 80 (0.1%) were added to the chitosan solution. The final volume was made up to 100 mL to give a chitosan solution of 0.2% and 0.4% (w/v) in 0.1 M $CaCl_2$ containing 0.1% Tween 80.

Encapsulation of enzymes

The encapsulated enzymes were produced using the inotropic gelation method (Anjani et al., 2007) with some modifications. For producing enzyme-CAXB and enzyme-CAMB, 10 mL of 2% (w/v)

protease solution (Sigma, Japan) or 2% (w/v) lipase solution (Novozymes, Denmark) were mixed with 40 mL of alginate-xanthan gum or alginate-maltodextrin solutions. These mixtures were then extruded using a disposable syringe with a needle of 0.30 × 13 mm inner diameter. The distance above the gelling solution (100 mL of 0.1 M $CaCl_2$ solution containing 0.1% Tween 80) was controlled to 3 cm. Enzyme-CACB was produced by mixing 10 mL of 2% (w/v) protease or 2% (w/v) lipase solutions with 40 mL alginate solution and then dropping it into a gently agitated gelling solution of 100 mL 0.1 M $CaCl_2$ containing chitosan and 0.1% Tween 80 at room temperature. After 30 min of gelation, the beads were separated by filtration, rinsed twice with deionized water, dried at room temperature for 30 min and stored at 4 °C.

Determination of protease activity

The protease activity was analyzed using the method described by Cupp-Enyard (2008). Casein was used as the substrate and the protease activity was expressed as units per milliliter of enzyme or units per milligram of solid. One unit hydrolyzes casein to produce color equivalents to 1.0 μmol tyrosine/min at 37 °C and pH 7.5 (colored by Folin-Ciocalteu reagent). Two vials containing 5 mL of 0.65% (w/v) casein solution were equilibrated in a water bath at 37 °C for about 5 min. Then, 1.0 mL of enzyme solution (prepared in enzyme diluents at 37 °C, pH 7.5) was added, mixed and incubated at 37 °C for 10 min. Trichloroacetic acid (TCA) reagent (5 mL) was then added into each tube to stop the reaction. The samples were incubated at 37 °C for 30 min and then filtered. The filtrate was used in color development. Deionized water was used as a blank to replace the sample.

A standard curve was constructed by adding 1.1 mM L-tyrosine standard stock solutions into six tubes at 0.05, 0.10, 0.20, 0.40, 0.50 and 0.80 mL, respectively. An appropriate volume of deionized water was then added to make up the final volume of 2 mL. For standard blank, 2 mL of the deionized water was used instead of L-tyrosine. An amount of 5 mL of 500 mM sodium carbonate solution and 1 mL of Folin and Ciocalteu's phenol reagent (25 mL original solution mixed with 75 mL deionized water) were added into filtrate (2 mL) and the standard. The tubes were then incubated at 37 °C for 30 min. The absorbance was carried out at 660 nm using ultraviolet spectrophotometry. The standard curve was then constructed.

Determination of lipase activity

The lipase activity was measured using soybean oil/gum arabic emulsion as a substrate (Pinsirodom and Parkin, 2003) and then expressed as units equivalent to millimoles per minute. For samples, 50 mL of 5% (w/v) soybean oil/gum arabic emulsion substrate were added into a 100 mL Erlenmeyer flask with stopper and preincubated at 37 °C for 15 min. An appropriate amount of enzyme was then added to initiate lipolysis. At seven suitable reaction intervals (0, 5, 10, 15, 20, 25 and 30 min), 5 mL subsamples were transferred to a flask containing a titration cocktail (10 mL of 95% (volume per volume; v/v) ethanol and 3 drops of 1% (w/v) thymol blue indicator). These mixtures were mixed immediately and then allowed to stand for 2 h at room temperature for later titrimetric analysis. These solutions were then titrated with 0.05 N NaOH until a light blue color appeared. The blank consisted of 5 mL phosphate-buffered soybean oil/gum arabic emulsion substrate and titration cocktail, instead of the sample. The quantity of fatty acids liberated in each subsample was calculated, based on the equivalent NaOH used to reach the titration end point. Lipase activity was determined from the slope of the linear portion of the curve by plotting the quantity of fatty acids liberated vs the time of reaction.

Encapsulation yield

The total weight of beads (g) was used for the determination of encapsulation yield (EY). The analysis was performed in triplicate in each analysis and the EY was calculated using Equation (1):

$$EY(\%) = M'M \times 100 \quad (1)$$

where M' is the total weight of beads (grams), M is the total weight of the complex polymer and enzyme solution (grams).

Encapsulation efficiency

For enzyme-CACB, 1 g of beads was disrupted using 25 mL of trisodium citrate (2% w/v) (Anjani et al., 2007; Azarnia et al., 2008). Phosphate buffer pH 6.8 (Sivakumar et al., 2011) was used for enzyme-CAXB and enzyme-CAMB at room temperature with gentle stirring at 70 rpm for 30 min. After 30 min, these mixtures were filtered and the filtrates were analyzed for the enzyme activity as previously described and the enzyme activity was used to determine the percentage of encapsulation efficiency (EE) based on the method of Anjani et al. (2007). The encapsulation efficiency was determined using Equation (2):

$$EE(\%) = \frac{\text{Enzyme activity recovered from the beads}}{\text{Initial enzyme activity}} \times 100 \quad (2)$$

where the initial enzyme activity is that in the alginate and copolymer-enzyme solution.

Bead size

The diameters of 120 randomly selected beads of each treatment were measured using a set of vernier calipers. The average values ± SD were presented.

Leakage of encapsulated enzymes from beads

Leakage of encapsulated enzymes (L) was determined by suspending 5 g of beads into 25 mL deionized water under shaking conditions using an orbital shaker at 150 rpm for 120 min. Five milliliters of liquid samples from the suspension were taken at different time intervals (0, 30, 60, 90 and 120 min), while simultaneously, 5 mL of deionized water was added to maintain the total volume of samples. The enzyme activity was determined as previously described and the percentage L was calculated using Equation (3):

$$L(\%) = \frac{\text{Enzyme activity in deionized water}}{\text{Enzyme activity in beads used in leakage assay}} \times 100 \quad (3)$$

Stability of encapsulated enzyme in the beads

Encapsulated enzymes were stored at 4 °C for 10 wk (Anjani et al., 2007). One gram of beads was disrupted using 25 mL of trisodium citrate (2% w/v) (Anjani et al., 2007; Azarnia et al., 2008) used for enzyme-CACB and phosphate buffer pH 6.8 was used for enzyme-CAXB and enzyme-CAMB at room temperature with 70 rpm to release the encapsulated enzymes (Sivakumar et al., 2011). The samples were analyzed every 2 wk for enzyme activity as previously described and the percentage of enzyme activity retained over the 10 wk storage period was determined.

Statistical analysis

A randomized block and a 2 × 3 × 2 factorial design with three replications were used in this experiment. The mean comparisons were performed using Duncan's Multiple Range Test at the 95% confidence level.

Results and discussion

Some properties of encapsulated enzyme beads

Some properties of encapsulated protease and lipase beads and the process efficiency such as EY, EE and bead size are presented in Table 1.

For protease, it was noticed that the alginate concentration, type of copolymer and copolymer concentrations significantly affected the EY, EE and bead size. As the concentration of alginate increased, the EY and EE increased. This might have been due to the alginate being the main supporting material and increasing the alginate concentration leads to the formation of a denser network structure of beads with cohesive pores that entrap more enzymes. These results were in agreement with the studies of Soliman et al. (2013).

Conversely, when the concentration of copolymer increased, the EY either significantly increased or decreased depending on the type of copolymer at the same alginate concentration. As the concentration of xanthan gum and maltodextrin increased, the EY and EE increased for all alginate concentration used. On the other hand, a reduction in the EY and EE was observed when the concentration of chitosan decreased at all alginate concentrations. This might have been due to the one-stage coating used for chitosan. The beads were obtained by dropping enzyme-alginate solution into a mixture of calcium chloride and chitosan solution, leading to competition between the enzyme and chitosan to bind with the alginate and less chitosan was bound so that more chitosan remained in the calcium chloride solution. In contrast, in two-stage coating with alginate beads soaked in chitosan solution, more chitosan can bind onto the surface of the beads and into the interior gel network, resulting in higher a EE and EY. On the other hand, a higher concentration of xanthan gum and maltodextrin produced more viscous dispersion due to these polymers being mixed with alginate solution before dropping in the calcium chloride solution, resulting in the formation of larger droplets and consequently larger microspheres.

Encapsulation using CACB7 (2% alginate and 0.2% chitosan) provided the highest EY (81.7%), followed by CACB8 (78.1%) and CACB1 (64.1%), respectively. The lowest EY was found in CAMB5 (28.2%). Similar results were found for the EE. CACB7 gave the highest EE (77.2%), followed by CACB8 (73.1%) and CACB1 (65.6%), respectively, whereas the lowest was CAMB5 (11.5%). A similar trend in the bead size was found for the effect of alginate concentration. On the other hand, an increase in the bead size with copolymer concentration was noticed only at 2.0% alginate.

However, among the three types of copolymer used in this research, it was noticed that chitosan provided better results than the others for protease encapsulation, which was consistent with Gåserød et al. (1999) and Chávarri et al. (2010). This was probably caused by the low molecular weight chitosan used, leading to rapid diffusion into the calcium alginate gel. As a result, a denser structure at the surface was formed (Krasaekoopt et al., 2004), retarding the loss of enzymes and leading to a higher EY and EE. Furthermore, chitosan reacts with alginate as a cation (similar to $CaCl_2$) with anionic alginate (Azarnia et al., 2008). As chitosan is a large molecule compared with calcium ions, it is more efficient in blocking the pores in the alginate matrix when combined with $CaCl_2$, compared with alginate alone. Therefore, the alginate gel structure changes from macroporous to microporous, reducing any loss of enzyme. On the other hand, incorporation of alginate gel with maltodextrin and xanthan gum may result in less structural improvement or modification to the alginate gel compared to chitosan. As a result, the remaining macroporous structure could lead to a higher loss of protease and a lower EY and EE.

For lipase encapsulation, the EY, EE and bead size were significantly influenced by the alginate concentration and the types of copolymer and their concentrations. The concentrations of alginate and copolymer had positive effect on the EY, EE and bead size, which were similar to those of protease except for the chitosan

Table 1
Encapsulation yield (EY), encapsulation efficiency (EE) and bead size of encapsulated enzymes.

Type of enzyme	Alginate concentration (%)	Types of copolymer	Copolymer concentration (%)	EY (%)	EE (%)	Bead size (mm)	Code
Protease	1.5	Chitosan	0.2	$64.1 \pm 1.19^{c*}$	65.6 ± 1.70^{c}	1.73 ± 0.04^{de}	CACB1
Protease	1.5	Chitosan	0.4	58.2 ± 1.52^{d}	62.2 ± 2.55^{d}	1.74 ± 0.03^{d}	CACB2
Protease	1.5	Xanthan gum	0.2	34.7 ± 1.10^{hi}	15.5 ± 1.56^{fg}	1.62 ± 0.04^{f}	CAXB3
Protease	1.5	Xanthan gum	0.4	39.1 ± 0.61^{fg}	13.1 ± 0.63^{gh}	1.69 ± 0.03^{de}	CAXB4
Protease	1.5	Maltodextrin	0.2	28.2 ± 0.73^{j}	11.5 ± 0.23^{h}	1.36 ± 0.05^{g}	CAMB5
Protease	1.5	Maltodextrin	0.4	32.8 ± 1.27^{i}	14.3 ± 1.22^{gh}	1.35 ± 0.06^{g}	CAMB6
Protease	2.0	Chitosan	0.2	81.7 ± 2.42^{a}	77.2 ± 1.18^{a}	1.85 ± 0.05^{bc}	CACB7
Protease	2.0	Chitosan	0.4	78.1 ± 1.66^{b}	73.1 ± 2.12^{b}	1.94 ± 0.03^{a}	CACB8
Protease	2.0	Xanthan gum	0.2	41.5 ± 1.83^{f}	18.0 ± 1.49^{ef}	1.82 ± 0.05^{c}	CAXB9
Protease	2.0	Xanthan gum	0.4	51.2 ± 1.23^{e}	20.4 ± 2.36^{e}	1.89 ± 0.02^{ab}	CAXB10
Protease	2.0	Maltodextrin	0.2	37.4 ± 1.65^{gh}	13.2 ± 2.30^{gh}	1.66 ± 0.03^{ef}	CAMB11
Protease	2.0	Maltodextrin	0.4	40.0 ± 1.67^{fg}	15.7 ± 1.05^{fg}	1.68 ± 0.01^{def}	CAMB12
Lipase	1.5	Chitosan	0.2	31.0 ± 0.96^{f}	14.1 ± 1.48^{e}	1.01 ± 0.02^{h}	CACB13
Lipase	1.5	Chitosan	0.4	32.3 ± 1.23^{ef}	16.8 ± 1.50^{de}	1.08 ± 0.02^{g}	CACB14
Lipase	1.5	Xanthan gum	0.2	40.4 ± 0.57^{a}	30.7 ± 2.24^{c}	1.44 ± 0.05^{d}	CAXB15
Lipase	1.5	Xanthan gum	0.4	40.8 ± 1.39^{a}	35.1 ± 3.59^{bc}	1.50 ± 0.05^{c}	CAXB16
Lipase	1.5	Maltodextrin	0.2	34.3 ± 0.70^{cd}	19.5 ± 2.09^{de}	1.15 ± 0.03^{f}	CAMB17
Lipase	1.5	Maltodextrin	0.4	34.4 ± 1.02^{cd}	22.3 ± 1.93^{d}	1.17 ± 0.03^{f}	CAMB18
Lipase	2.0	Chitosan	0.2	33.2 ± 1.00^{de}	19.0 ± 2.24^{de}	1.28 ± 0.05^{e}	CACB19
Lipase	2.0	Chitosan	0.4	35.6 ± 1.13^{bc}	21.3 ± 1.99^{d}	1.41 ± 0.05^{d}	CACB20
Lipase	2.0	Xanthan gum	0.2	42.3 ± 0.23^{a}	40.2 ± 4.20^{ab}	1.67 ± 0.04^{b}	CAXB21
Lipase	2.0	Xanthan gum	0.4	42.5 ± 1.09^{a}	43.9 ± 2.67^{a}	1.81 ± 0.03^{a}	CAXB22
Lipase	2.0	Maltodextrin	0.2	36.8 ± 1.44^{b}	33.5 ± 1.73^{c}	1.41 ± 0.05^{d}	CAMB23
Lipase	2.0	Maltodextrin	0.4	40.9 ± 1.25^{a}	36.4 ± 1.71^{bc}	1.41 ± 0.04^{d}	CAMB24

Values expressed as mean ± SD (n = 3).
* = Means with different lowercase superscript letters in each column are significantly different ($p < 0.05$).The statistical analysis was done separately for each enzyme.

concentration. CAXB22 (2.0% alginate and 0.4% xanthan gum) provided the highest EY, EE and bead size (42.5%, 43.9% and 1.81 mm, respectively), followed by CAXB21 and CAMB24. At the same time, the lowest value was found in CACB13. These results were consistent with Sivakumar et al. (2011). The increase in viscosity might have been due to the higher concentration of alginate, leading to a stiffer structure and larger droplets formation. Moreover, the higher alginate concentration provides a large number of binding sites for Ca^{2+} ions, resulting in a denser gel structure and thereby a greater EY and EE.

The converse result was obtained when different types of copolymer were used compared to protease. The presence of xanthan gum provided a higher EE and EY than the others. This might have been caused by the interaction between lipase and chitosan or maltodextrin leading to lower activity after encapsulation. The possibility of interaction of chitosan or maltodextrin with the active lipase site cannot be ruled out (Betigeri and Neau, 2002). In addition, this might have been due to the different molecule weights of enzymes leading to different sizes. The molecular weight of the lipase used in this research might have been less than that of the protease, resulting in a loss of enzyme during the hardening period. Therefore, the EY and EE in the encapsulated protease were higher than those of lipase. In addition, Betigeri and Neau (2002) reported that it is necessary to evaluate the enzyme structure after encapsulation. Its correlation with enzyme bioactivity is available because enzyme structure alteration may lead to no biological activity.

Leakage of encapsulated enzymes

The leakage of encapsulated protease and lipase from the beads is shown in Fig. 1. The leakage of encapsulated enzymes was quite low—in the range 6.7–17.6% for protease (Fig. 1A and B) and in the range 6.2–20.6% (Fig. 1C and D) for lipase. The leakage was observed in the first 60 min; after that, it changed only slightly until the end of the test. This result might be beneficial in food applications especially for the acceleration of the cheese ripening process. The ripening process requires a high rate of enzyme activities at the beginning and then a lower rate during storage in the curing room (Anjani et al., 2007). The initial burst leakage of enzymes might have been caused by the leakage of enzymes near the bead surface (Sivakumar et al., 2011). Alginate at the surface has a very

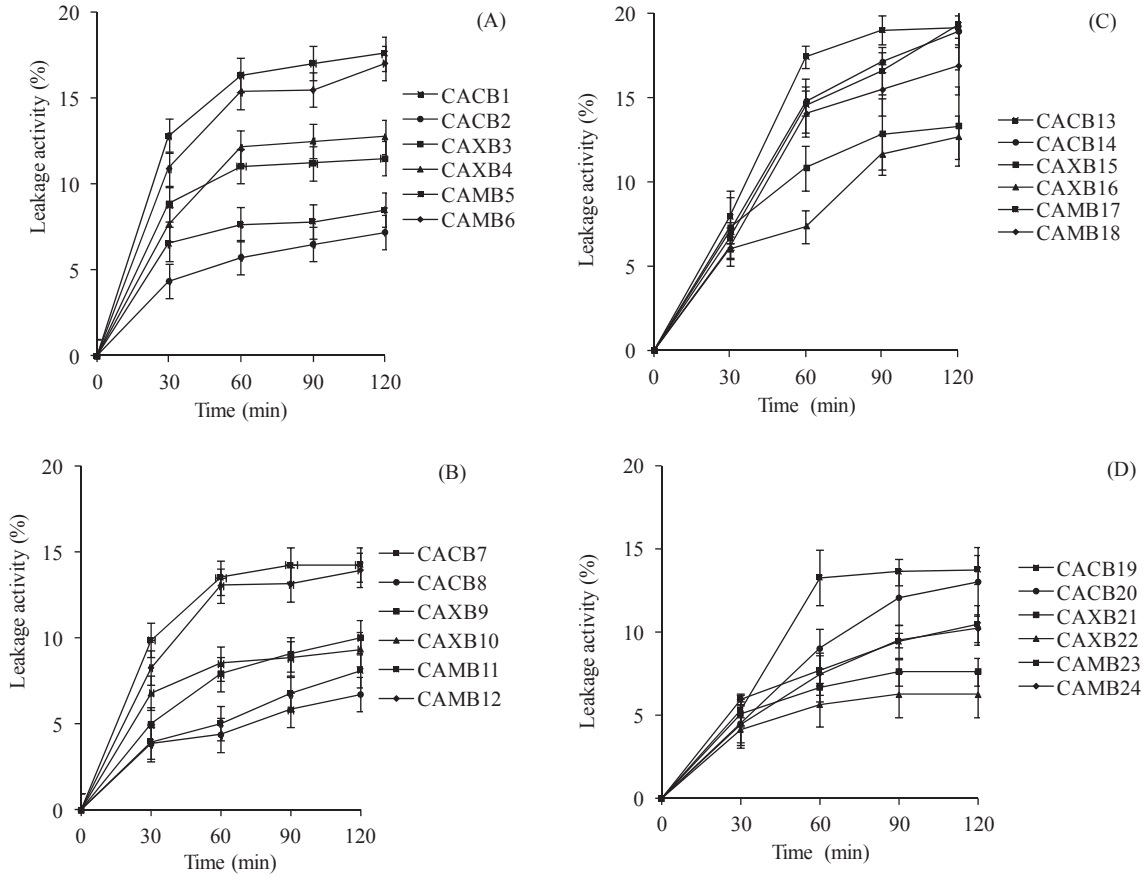

Fig. 1. Percentage of leakage activity of encapsulated enzymes at 150 rpm for 120 min: (A) encapsulated protease at 1.5% alginate; (B) encapsulated protease at 2.0% alginate; (C) encapsulated lipase at 1.5% alginate; (D) encapsulated lipase at 2.0% alginate. Error bars show ± SD. CACB1 = protease encapsulated in 1.5% alginate and 0.2% chitosan, CACB2 = protease encapsulated in 1.5% alginate and 0.4% chitosan, CAXB3 = protease encapsulated in 1.5% alginate and 0.2% xanthan gum, CAXB4 = protease encapsulated in 1.5% alginate and 0.4% xanthan gum, CAMB5 = protease encapsulated in 1.5% alginate and 0.2% maltodextrin, CAMB6 = protease encapsulated in 1.5% alginate and 0.4% maltodextrin, CACB7 = protease encapsulated in 2.0% alginate and 0.2% chitosan, CACB8 = protease encapsulated in 2.0% alginate and 0.4% chitosan, CAXB9 = protease encapsulated in 2.0% alginate and 0.2% xanthan gum, CAXB10 = protease encapsulated in 2.0% alginate and 0.4% xanthan gum, CAMB11 = protease encapsulated in 2.0% alginate and 0.2% maltodextrin, CAMB12 = protease encapsulated in 2.0% alginate and 0.4% maltodextrin, CACB13 = lipase encapsulated in 1.5% alginate and 0.2% chitosan, CACB14 = lipase encapsulated in 1.5% alginate and 0.4% chitosan, CAXB15 = lipase encapsulated in 1.5% alginate and 0.2% xanthan gum, CAXB16 = lipase encapsulated in 1.5% alginate and 0.4% xanthan gum, CAMB17 = lipase encapsulated in 1.5% alginate and 0.2% maltodextrin, CAMB18 = lipase encapsulated in 1.5% alginate and 0.4% maltodextrin, CACB19 = lipase encapsulated in 2.0% alginate and 0.2% chitosan, CACB20 = lipase encapsulated in 2.0% alginate and 0.4% chitosan, CAXB21 = lipase encapsulated in 2.0% alginate and 0.2% xanthan gum, CAXB22 = lipase encapsulated in 2.0% alginate and 0.4% xanthan gum, CAMB23 = lipase encapsulated in 2.0% alginate and 0.2% maltodextrin, CAMB24 = lipase encapsulated in 2.0% alginate and 0.4% maltodextrin.

low content of negative charges and cannot interact strongly either with calcium or positively charged chitosan (Blandino et al., 2000). There was a clear decline in the leakage of encapsulated enzyme as the alginate and copolymer concentrations increased, enhancing the exchange of sodium ions from glucuronic acid with the divalent cations during gelation (Sheela et al., 2011). This resulted in a denser matrix structure and a reduction in the bead porosity, resulting in a reduction of leakage.

For protease (Fig. 1A and B), the lowest leakage (8.1%) was found when chitosan was used, followed by xanthan gum (7.7%) and maltodextrin (12.6%) at the end of the test, which was in agreement with Sheela et al. (2011). This could be explained by differences in the pore size and the thickness of the complex polymer gel. Chitosan can reduce the leakage of encapsulated protease by reducing the pore size of the alginate gel matrix and improving the thickness of the gel membrane via cross-linking between the amine group of chitosan and the carboxyl group of alginate (Gåserød et al., 1998). In addition, low molecule weight chitosan diffused easily into the alginate gel matrix (Gåserød et al., 1999), which leads to a reduced pore size and increased membrane thickness in the calcium alginate-chitosan beads. Therefore, the pore size is smaller and the alginate-chitosan gel is thicker. For lipase (Fig. 1C and D), xanthan gum produced the lowest leakage (6.2%) at all concentrations of alginate, which was similar to the studies of Sheela et al. (2011). This could be explained by the different size between protease and lipase and by the interaction between lipase and the supporting materials used for encapsulation, leading to changes in the gel structure of the alginate gel (Betigeri and Neau, 2002).

Stability of encapsulated enzymes

The encapsulated enzyme activity retention during storage was determined periodically every 2 wk for 10 wk at 4 °C. A slow rate of enzyme loss was observed during the storage period for all samples (Fig. 2).

For protease (Fig. 2A and B), when the concentration of alginate or copolymer increased, the retention of encapsulated protease activity increased. Chitosan also provided better stability of encapsulated protease than for other polymers. CACB8 gave the highest protease stability (81.4%) when 2.0% alginate and 0.4% chitosan were used with the highest coefficient of determination (R^2) of 0.9941. This condition also provided the lowest rate of rate constant (k) of 0.0205, indicating a slow rate of reaction during the 10 wk storage at 4 °C. The highest shelf-life of encapsulated enzyme which was expressed as half-life, the time required for the enzyme activity to decrease by half ($t_{half\ life}$) was produced (33.7 wk), resulting in a longer shelf life or higher stability of encapsulated protease, followed by CACB7 (2% alginate and 0.2% chitosan) with protease stability, R^2, k and $t_{half\ life}$ of 75.8%, 0.9645, 0.0255 and 27.2 wk, respectively. These results were similar to the studies of Chávarri et al. (2010) who reported that chitosan (a positively charged polyamine) forms a semipermeable membrane around a negatively charged polymer such as alginate. This membrane does not dissolve in the presence of Ca^{2+}chelators or antigelling agents and thus enhances the stability of the gel and provides a barrier to enzyme release. The lowest protease stability during the storage was observed for maltodextrin with a $t_{halflife}$ of 7.8–13.4 wk. The

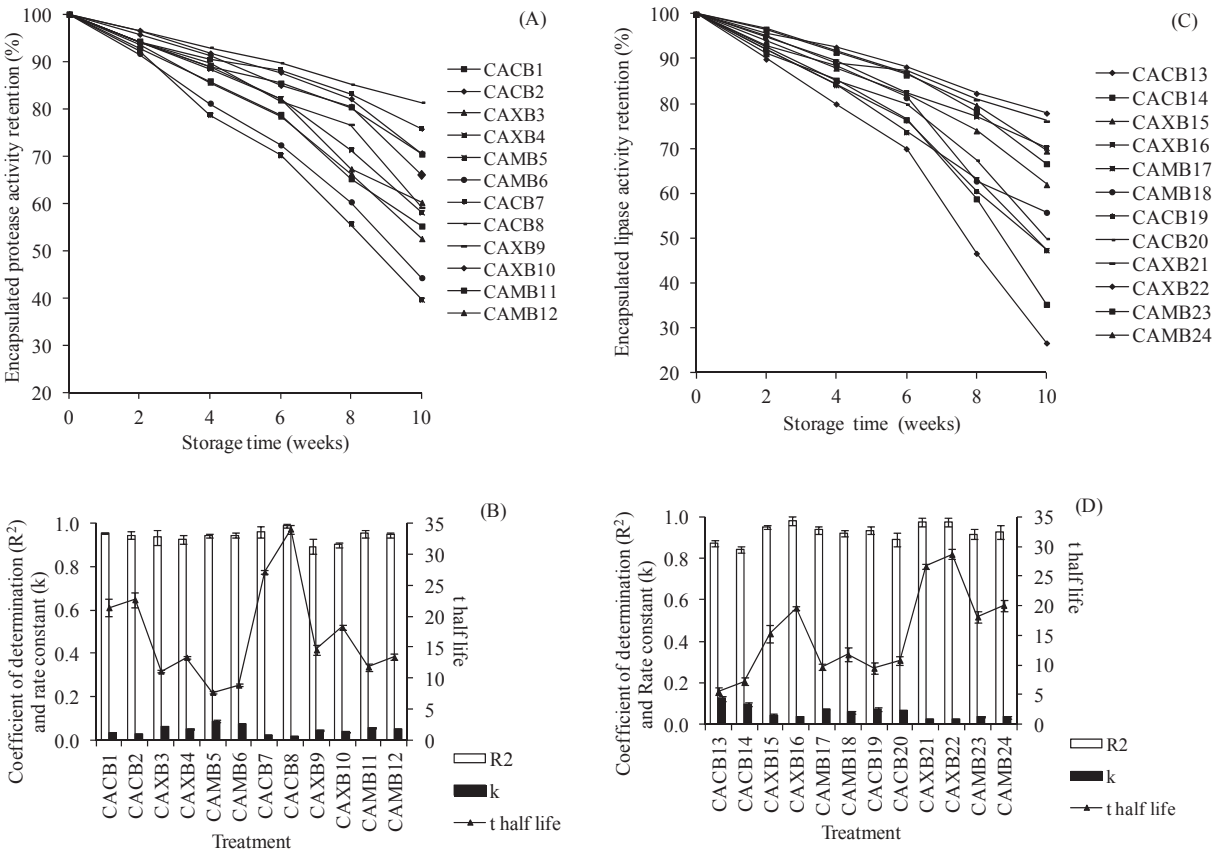

Fig. 2. Stability of encapsulated enzymes at 4 °C for 10 weeks: (A) encapsulated protease activity retention; (B) coefficient of determination (R^2) and $t_{half\text{-}life}$ of encapsulated protease; (C) encapsulated lipase activity retention; (D) R^2 and the shelf-life of encapsulated enzyme which was expressed as half-life, the time required for the enzyme activity to decrease by half (t half-life) of encapsulated lipase. Error bars show ± SD. See Fig. 1 caption for definitions of plotted data.

presence of starch or starch derivatives (maltodextrin) might lower the hardness of the alginate gel (Anjani et al., 2007).

For lipase (Fig. 2C and D), similar to protease, when the concentration of alginate or copolymer increased, the retention of encapsulated lipase also increased. Xanthan gum had the highest lipase retention (77.9%) with R^2 of 0.9855 and k of 0.0249, resulting in the highest $t_{half\ life}$ of 27.8 wk. At the same time, the lowest lipase stability was found in chitosan ($t_{half\ life}$ of 5.6 wk). This result was consistent with those of the EY and EE for the encapsulation of lipase when xanthan gum was used as the copolymer. There might have been an interaction between the lipase and chitosan that resulted in the negative changes in the alginate gel structure. The amount of chitosan might be have been sufficient to change macroporous to microporous structures in the alginate gel (Betigeri and Neau, 2002), therefore, the lowest EY, EE and stability were produced.

In addition, the highest EY, EE, bead size and retention activity and the lowest rate of leakage of encapsulated enzymes were found when protease was encapsulated in calcium alginate-chitosan beads and lipase was encapsulated in calcium alginate-xanthan gum beads.

Inotropic gelation and extrusion methods were used for the encapsulation of protease and lipase in the alginate and different copolymers. A higher EY, EE and retention activity and a bigger bead size with lower leakage of the encapsulated protease and lipase beads were obtained when the alginate and copolymer concentrations were increased. In addition, the type of polymer had an effect on the EY, EE, bead size, leakage activity and the encapsulated enzyme activity retention. Moreover, encapsulated protease provided a higher EY and EE than did encapsulated lipase. Therefore, the effect of the pH, gelling solution and time and rate of stirring during the gelling period on the EY, EE and bead size of lipase-encapsulated beads should be further studied as well as the application of these encapsulated enzymes in foods.

Conflict of interest

No conflict of interest.

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