Application of the encapsulated aminopeptidase of *Lactobacillus rhamnosus* to accelerate Cheddar cheese ripening

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Introduction

Cheese ripening is a complex microbial and biochemical process required for the development of the flavour, texture and aroma through proteolysis, lipolysis and glycolysis. Proteolysis is believed to be the main reaction in flavour and aroma formation in Cheddar cheese. As maturation of cheese is an expensive and slow process, any reduction of this period has economic and industrial benefits. Addition of enzymes into milk homogeneously while elevating their retention in Cheddar cheese matrix appears to be the simplest and cheapest method for uniform delivery of enzymes into the cheese matrix. However, the major concern of this method is the enzyme lost into the whey during cheesemaking.

Lactic acid bacterial enzymes have an important role in the ripening and development of flavour in cheese (Lee *et al.,* 1990; Arora and Lee, 1994), and thus we were interested in the use of lactic enzymes to speed up cheese maturation. Because the yield of enzymes from wild type strain is very low, we genetically over-expressed (about 1000-fold) an aminopeptidase from *Lactobacillus rhamnosus* produced in *E. coli* (Lee and Robert, 1997).

It is believed that the encapsulated enzymes could be a way for uniform delivery of enzymes into the cheese matrix (Kailasapathy and Lam, 2005; Azarnia *et al*., 2006). For this purpose, natural polysaccharides such as Na-alginate can be used as encapsulating materials. This polysaccharide is a polymer of mannuronic and guluronic acid residues and has the capacity to bind divalent cations such as calcium resulting in a 3-dimentional gel network (Roberts, 1992). However, the problem of alginate gels for this application is the large pores of the gel leading to the release of enzymes from the capsules into the whey during the coagulation, drainage and cheddaring stages. This problem could be reduced by formation of a polyelectrolyte complex between alginate as an anionic polymer and chitosan as a cationic polymer (Roberts, 1992). The two polymers have already been used as an immobilization matrix for biomolecules and microorganisms. However, to our knowledge, very little works have been carried out in using them for cheese ripening purpose.

In the present work, the purified recombinant aminopeptidase (PepN) from *Lactobacillus rhamnosus* S93 was immobilized in alginate-chitosan beads and applied to shorten the duration of Cheddar cheese ripening.

Materials and Methods

Purification of the PepN and enzyme assay**:** All the chemicals were of analytical grade. The recombinant PepN was purified from the crude supernatant in a single step on a preparative ion exchange chromatography column using a FPLC system (Waters™ 650E Advanced Protein Purification System). Active fractions were pooled and the activity of enzyme was measured spectrophotometerically at 405 nm using a final 16.4 mM of leucine-ρ-nitroanilide (leu-ρNA) dissolved in methanol (Arora and Lee, 1994).

Preparation of freeze dried alginate or chitosan-coated alginate beads: A portion (1g) of crab shell chitosan (85% deacetylated; Sigma Chem. Co.) were dissolved in 700 mL of 0.1 M glacial acetic acid and stirred overnight. After complete dissolution, the pH was adjusted to 5.4 with NaOH. A $CaCl_2$ solution was added to the chitosan solution and the volume adjusted to 1L with deionized distilled water, yielding a cationic solution of 1g chitosan L^{-1} in (0.1 M) $CaCl_2$ (Zhou *et al*., 1998). The enzyme and the Na-alginate solution (1.6 %) were mixed gently for 2 min at a ratio of 1:3. The mixture was extruded through a 300µm nozzle of an encapsulator (Inotech Labor, IE-50 R, Eulerstrasse, Switzerland) into a cold (4°C) gelling solution containing $CaCl_2$ (0.1M) or chitosan (0.1%)- $CaCl_2$ (0.1M). All capsules were allowed to harden in the gelling solution for 10 min in agitation (70 rpm) resulting in the formation of alginate beads. The capsules were harvested by filtration, washed with deionized distilled water to remove the untrapped enzyme and excess calcium chloride from the surface of the beads. After washing, 1 g of coated or uncoated capsules (wet weight) was suspended in the release buffer (20 g L^{-1} trisodium citrate) at room temperature and gently stirred (70 rpm) (Champagne *et al*., 1992). The enzyme activity having leaked into the release buffer was measured after complete dissolution of the beads in the release buffer.

The encapsulation efficiency, *EE,* expressed as a percentage, was calculated as:
$EE = 100 \times (EA_b / EA_i)$. The degree of enzyme release*, ER,* during bead suspension in the release buffer was defined as: $ER = 100 \times [EA_{rb} / (EA_b + EA_{rb})]$, where, EA_b, EA_i and EA_{rb} were the enzyme activity (units) in the beads, the initial enzyme activity (units) in the polymer/enzyme mixture and the enzyme activity (units) in the release buffer, respectively (Huguet and Dellacherie, 1996). The rate of encapsulated enzyme entrapment was estimated from the quantity of the enzyme lost into the whey.

The encapsulated enzyme was frozen at -80°C, and was subjected to freeze-drying (FTS System, Inc. Biopharm Division, NY, USA) for 48 h at 22°C under a 90 millitor vacuum.

The images of alginate and chitosan-coated alginate beads were prepared using a scanning electron microscope (SEM) (Hitachi S-3000N, Japan) at 5 kV.

***Cheddar cheese production*:** Control and experimental cheeses were made in vats containing 200 L milk at pilot plant of Agriculture and Agri-Food Canada, Food Research and Development Centre (St-Hyacinthe, QC, Canada). Immobilized enzymes were added at renneting step. The cheese blocks were vacuum packed and stored at 4 °C for 6 months.

***Physicochemical and sensory analyses*:** The total nitrogen soluble in 5% phosphotungstic acid (PTA-N) and total free amino acids (TFAA) were determined in control and experimental cheeses. The sensory properties of the cheeses were evaluated according to the Cheddar cheese characteristics at the Agriculture and Agri-Food Canada, Food Research and development Centre (St-Hyacinthe, QC, Canada). The panel was asked to evaluate individually the acceptability of the cheeses considering three attributes (texture, flavor or aroma).

Results and discussion

Enzyme encapsulation: Addition of chitosan to the gelling solution led to an increase in *EE* from 10% to 90% and a reduction in the *ER* from Ca-alginate beads during dissolution in the citrate buffer. After one hour, almost 100% of the enzyme encapsulated in chitosan-free beads had leaked into the release buffer, whereas around 51% of the enzyme released from the beads hardened in a gelling solution containing 0.1% chitosan (Figure 1). The very spongy inner structure of the uncoated beads (Figure 2a) changed to one with small pores when the beads were coated (Figure 2b). The porous structure of uncoated alginate beads could result in the faster release of

recombinant enzyme from capsules. The entrapment rate of the immobilized enzymes into the cheese matrix was about 99% indicating the stability of the capsules during cheesemaking.

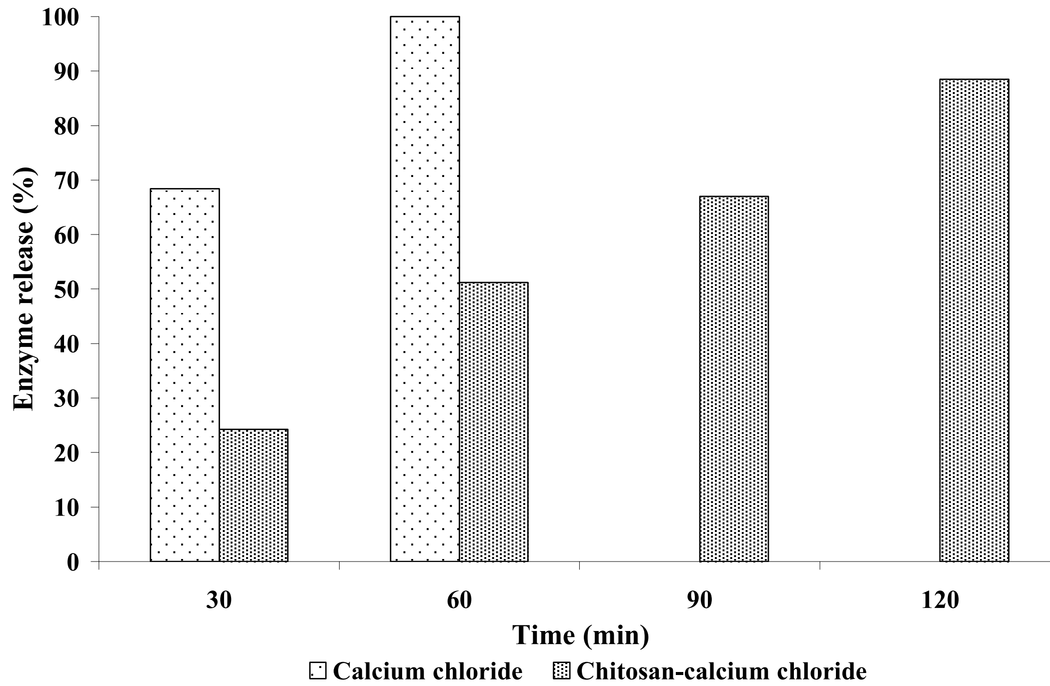

Figure 1. Effect of chitosan on the enzyme release (%)

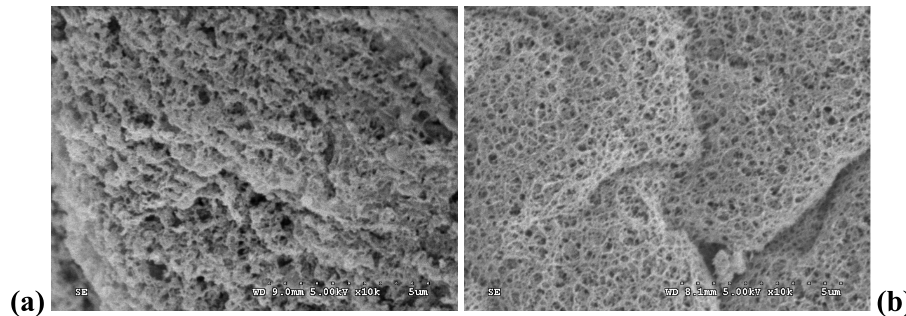

Figure 2. The SEM images of wet Ca-alginate beads. The inside view of Ca-alginate (a) and Ca-alginate coated with chitosan (b) (original magnification: ×10k); voltage (5.00 kV)

Cheddar cheese acceleration: In general, PTA-N and TFAA were increased in control and experimental cheeses during ripening. However, the amounts of PTA-SN and TFAAs were similar in the experimental cheese at 2 months of ripening compared with those of the control cheese at 6 months that suggests a possibility in acceleration of about 4 months in proteolytic indices (Table 1). When the cheeses were graded by the panel, the experimental cheese obtained the higher scores for sensory characteristics than those of the control (Table 2).

Parameters	Ripening time (Day)	Cheese	
		Control	Cheese with encapsulated enzymes
PTA-N/ TN (%)	1	0.74	0.85
	60	1.21	2.07
	120	1.64	2.87
	180	2.00	3.40
Total free amino acids (µg/g cheese)	1	1005	1227
	60	1962	4429
	120	3116	6587
	180	4456	8502

Table 1. Changes in proteolysis during ripening

Cheese	Texture	Flavour	Aroma
Control	8	2	10
Cheese with encapsulated enzymes	19	16	21

Table 2. Sensory evaluation scores

Conclusions

In conclusion, this is the first report of encapsulation of a recombinant aminopeptidase from *Lb. rhamnosus* in alginate based capsules with the 99% incorporation efficiency into the cheese matrix, and its application to speed up Cheddar cheese ripening. This also demonstrates a method to enhance the proteolysis at about 70% and sensory characteristics.

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