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Accelerating ripening of Iranian white brined cheesesusing liposome-encapsulated and free proteinases

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ABSTRACT

The role of liposome encapsulated flavourzymes on the ripening acceleration of Iranian white brined cheese has been studied. Liposomal enzymes (made by heating method) had a better performance than free enzymes, resulting in cheeses with lower total solid content and a higher concentration of soluble nitrogen (pH 4.6 and 12.5% trichloroacetic acid) through the ripening period (30 days at 12 oC). The ripening time could be shortened for 10 to 20 days. The entrapment efficiency and diameter of the liposomal enzyme were 26.5% and 189 nm, respectively. The proposed technology could be a potential treatment for the acceleration of Iranian white brined cheeses ripening.

Keywords: *Heating method; liposome, proteinase; Iranian white brined cheese; ripening acceleration.*

1. INTRODUCTION

Ripening is a multifactorial and complex biological process, and it is required for the development of flavor, body and texture in cheeses [1]. Ripening time differs according to the type of produced cheese from a few days in soft cheeses to two years in very hard cheeses [2]. Cheese ripening is a slow, cost-demanding and not fully controllable stage in the processing [3, 4, 5] of ripened cheeses. Therefore, there is a demand for technologies to accelerate cheese ripening [6]. Various strategies have been described such as increasing the ripening temperature [7], high pressure processing [3], the addition of attenuated starters and adjunct cultures [2], use of genetically modified LAB as a starter [8], and addition of exogenous enzymes such as lipase and proteases [9].

The addition of enzymes is considered the most specific and simple of all methods used to accelerate cheese ripening. This method is scientifically advanced and easily scalable, which allows the development of new technologies at industrial scales [10, 11]. Furthermore, the addition of enzymes to cheese milk in combination with starter cultures and rennet is practical. However, the direct addition of proteinases to milk results in a large loss (at a rate of nearly 95%) of the enzyme in whey, which increases the cost of production. Furthermore, it causes early proteolysis, by hydrolyzing caseins to soluble peptides. Typically, up to 90% of the water-soluble peptides are removed during cheese production as proteinaceous “retentate” fractions. Early breakdowns of caseins disrupt the enzyme orderly structures, prevent gel formations and render curds that are soft and unusable at later stages of the curd acidification [11, 12].

To solve these problems, enzyme encapsulation techniques are used to protect enzymes from the environmental effects or to control the release of enzymes. The liposome microencapsulation of enzymes has multiple benefits compared to other methods used for the enzyme immobilization via encapsulation, such as 1) liposomes are made from natural ingredients (lipid and/or phospholipid molecules), 2) they can protect the casein from early hydrolysis, and 3) they are well distributed in the curd matrix [13-16] Furthermore, the use of enzyme-loaded nanoliposomes in cheese production can improve texture and flavor of cheeses in only half of the time normally used for this process. Moreover, the overall quantity of the necessary enzymes decreases to nearly one percent. Finally, nanoliposomes are generated from compounds with potential health benefits, which reveals additional advantages of these systems compared to alternative methods [10, 16-19]. However, for solubilizing the phospholipids, the liposome preparation techniques typically use detergents and solvents, which do not fit in food-grade claim. Furthermore, they contribute to limit the encapsulation efficiencies, are not suitable for continuous processes, and present difficulties in scaling up at acceptable levels and costs [16].

The heating method (HM) is a technology that can solve many of the stated problems. It enables the preparation of liposomes, nanoliposomes and other carrier systems in a single step operation, using a single apparatus and in less than one hour, without needing potentially toxic solvents [16, 20-21]. Liposomal encapsulated Flavourzyme® (LEF) was produced[10], modified [15] and applied in cheeses in a study using HM [22]. The Flavourzyme® includes a combination of fungal enzymes, consisting of exopeptidases and endoproteases, that can be used in the production of cheeses and acceleration of the cheese ripening

processes, and is particularly efficient for debittering and formation of cheese flavor [15]

As far as the authors know, the use of LEF in cheeses has not been compared to the use of free enzymes, and its efficiency in accelerating the cheese ripening has not been assessed. Therefore, the aim of the present study was to investigate the potential of HM liposomes as carriers for the encapsulation of Flavourzyme (LEF) aiming the acceleration of Iranian white brined cheese ripening. Proteolysis was assessed in encapsulated enzyme and free enzyme cheeses as well as those with no added enzymes (control).

2. MATERIALS AND METHODS

2.1. Preparing proteinase loaded liposomes.

Chemical compounds used for the preparation of liposomes were supplied by Acros Company, Belgium. Milli Q water (Millipore, USA) was used for the preparation of solutions. Liposomes were prepared using the modified method proposed by Mozafari [14, 15] and HM. First, 0.225% w/v of cholesterol was dissolved in 3% v/v of glycerolat 120˚ C. Solutions of 4.5% w/v lecithin and cholesterol in glycerol were added to a 0.675% w/v) solution of Flavourzyme® in tris buffer (0.01 M) at pH 6.0 and then preheated to 40 °C (kindly donated by Novozyme, Tehran, Iran) under continuous stirring (nearly 900 rpm) for 30 min using hotplate stirrer (HCR2, Germany). Encapsulation efficiency (EE) of the LEF was calculated by measuring the activity of Flavourzyme® as (introduced as leucine aminopeptidase units per millilitre) LAPU mL^{-1} in encapsulated and free forms using the following equation [15]

$$EE\ (\%) = \frac{\text{Encapsulated } \left(\text{LAPU ml}^{-1}\right)}{\text{Uncapsulated } \left(\text{LAPU ml}^{-1}\right) + \text{encapsulated } \left(\text{LAPU ml}^{-1}\right)}$$

Brined cheese preparation

White brined cheese was prepared using bovine raw milk based on the methodology proposed by Azarnia et al. (1997). The milk contained 2.5% fat, 3.2% protein, 12.6% total solids and had pH 6.7. Three formulations of cheese were prepared, being A) LEF (encapsulated enzyme-added cheese), B) FF (free enzyme-added cheese), and C) control (no added enzyme). The raw milk was pasteurized at 65 °C for 30 min and cooled down to 32–35 °C. Then, 0.15 g kg-1 of CaCl2 was added to the milk. Starter culture R704 was added to the milk according to recommendations of Chr. Hansen, Denmark. After decreasing the pH to 6.2 (60 min), 0.25 g of a commercially available microbial rennet enzyme powder (Rennilase®; DSM, French) with clotting activity of 1 g 100-1 (for control cheese) and was added per kg of the milk. After one hour (coagulation time), 0.3 % w/v of LEF and FF were added to the milk. The resulting curd was mixed, molded and the whey was discarded. For shaping, curd was pressed into the containers for 90–120 min using 20-kg weights. Then, curd was cut into sizes of 10 ×7 × 4 cm and soaked in 10% w/v of saturated brine at 12 ±2 °C for 30 days for the ripening [23, 24]. On Days 1, 10, 20 and 30, samples were collected for the analysis. All analyses were carried out in duplicates and the experiment was repeated twice.

2.2. Gross analysis.

The total cheese solids were analyzed by drying cheeses at 105°C until a mass constancy was achieved [25]. The cheese salt contents were analyzed using AgNO3 titration [25].

2.3. Proteolysis assessment.

Assessment of nitrogen content: The Kjeldahl method was used to assess cheese total nitrogen (TN) according to AOAC (2002).The water-soluble nitrogen (WSN) content was assessed as a proteolysis index using the methodology described by AOAC (2002) and expressed as the percentage WSN/TN. Non-protein nitrogen (NPN) was assessed according to the methodology by AOAC (2002) and expressed as the percentage of TN (TCA/TN) [22]

2.4. Sample preparation for electrophoresis.

Briefly, 0.4 g for the cheese sample was grated, dissolved in 5 mL of urea buffer (containing 7.5 g L^{-1} of 60-mM tris-HCl, 8 M of urea and 20 mL L^{-1} of 2-mercaptoethanol and adjusted to pH 7.6 using HCl), and incubated at 40 °C for 30 min, then, samples were instantly cooled down under running tap water and centrifuged at 2590 × g, for 10 min at 4 °C to remove fats and suspended particles. Then, 100 mL of clear supernatants were mixed with 300 mL of urea buffer and 6 mL of prepared casein and added to a well [26]

2.5. Urea polyacrylamide gel electrophoresis (urea-PAGE).

Proteolysis casein samples were used in urea polyacrylamide gel electrophoresis (urea-PAGE) according to the modified method proposed by Andrews [27]. Mini Protean III (BioRad, USA) working unit with PAC 300 power supply (BioRad, USA) was used. The separating gel buffer was prepared by merging 46 gL^{-1} of 380-mM Tris-HCl and 4.5 M of urea, pH 8.9. The separating gel (12% T, 3.8% C) was made by mixing 3mL of 40% acrylamide and 1.6% bisacrylamide solutions. Then, 7mL ofN,N,N,Ntetramethyl-ethylene diamine (TEMED), 7 µL of separating gel buffer and 0.1mL of 100 gL^{-1} ammonium persulfate solution were added to the mixture. The stacking gel buffer was prepared by mixing 7.5 g L^{-1} of 60-mM tris-HCl and 8 M of urea, pH 7.6. Then, 0.5 mL of acrylamide/bisacrylamide solution, 3 mL of stacking gel buffer, 5 µL of TEMED and 70 µL of 10 0 gL^{-1} ammonium persulfate were mixed to prepare the stacking gel buffer (5.7% T, 3.8% C). Three grams of tris and 14.6 g of glycine were dissolved in 1000 mL of distilled water (D.W.) to prepare solution buffer of electrophoresis; pH was adjusted to 8.2. The running time was 70 min at a constant current of 30 mA. Coomassie blue G-250 was used for 12 h for gel staining. Gels were de-stained using overnight immersion in 80 mL L^{-1} of acetic acid solution [26].

2.6. Statistical analysis.

Data from total solid, salt, WSN/TN and TCA/TN assessments were analyzed using split plot design. Treatments (control cheese and cheeses with LEF and FF) were selected as the main plot and ripening time as subplot factors. The general linear model (GLM) of statistical analysis system (SAS, 2004) was used for the analysis of variance to assess the effects of treatment for the response variables ($P \leq 0.05$). Statistically significant differences between the treatments were analyzed using Fisher's least significant difference test approach (F-value). Furthermore, Duncan's multiple range test was used to compare mean values of the total solids, salt contents and proteolysis indices of the cheeses.

3. RESULTS

3.1. Liposome characterization.

The EE of Flavourzyme encapsulation in HM liposomes was 26.5%. Size distribution clearly showed that the average diameter of Flavourzyme-loaded liposomes was 189 nm [15].

3.2. Total solid content of cheeses.

According to Figure 1, the TS decrease in cheese containing LEF was not slight and it was statistically significant compared to control (C) and samples containing free enzymes (B) ($P \leq 0.05$).

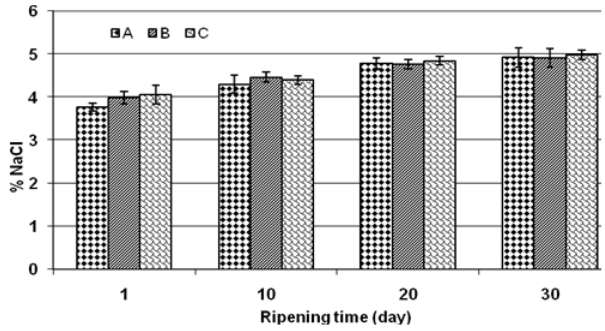

Figure 1. Total solid of Iranian white brined cheese during ripening (1, 10, 20 and 30 days). Formulations: A (encapsulated enzyme-added cheese), B (enzyme-added cheese), and C (control). The error bars represent the standard deviation (n=4). Different lowercase letters indicate a significant difference ($p \leq 0.05$).

3.3. Salt contents of cheeses.

Figure 2 presents the changes in salt contents of the Iranian white brined cheeses during ripening. Cheeses prepared in this study presented salt contents of approximately 4–5% during ripening; similar to the average salt contents of Iranian white brined cheeses evaluated in previous studies[28-30]. No significant differences were observed in the salt contents of the samples containing FF and LEF and the control cheese in different day of ripening 1, 10, 20 and 30 days ($P>0.05$).

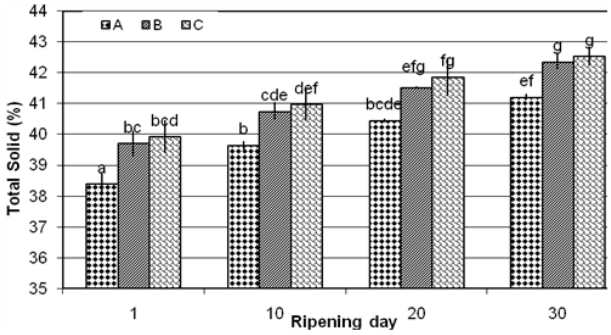

Figure 2. NaCl% concentration of Iranian white brined cheese during ripening (1, 10, 20 and 30 days). Formulations: A (encapsulated enzyme-added cheese), B (enzyme-added cheese), and C (control). The error bars represent the standard deviation (n=2).

3.4. Proteolysis of cheeses.

Proteolysis in control (C), LEF and FF cheeses were monitored through assessments of WSN/TN and TCA-SN/TN. The WSN/TN (Fig. 3) and TCA/TN (Fig. 4) fractions increased significantly in all samples during ripening ($P \leq 0.05$). At the beginning of ripening, WSN/TN increased by nearly 30% in control (C) and almost two-folds in LEF cheeses (A). After 20 days of ripening, WSN accounted for more than 20% of TN in control and experimental cheeses. Electrophoretic profile of the Iranian white brined cheeses was shown in Figure 5. No significant differences were observed in electrophoretic patterns of the three samples at Day 1, while degradations of αs1-casein and β-casein were noticeable at Day 30 of ripening in all cheeses.

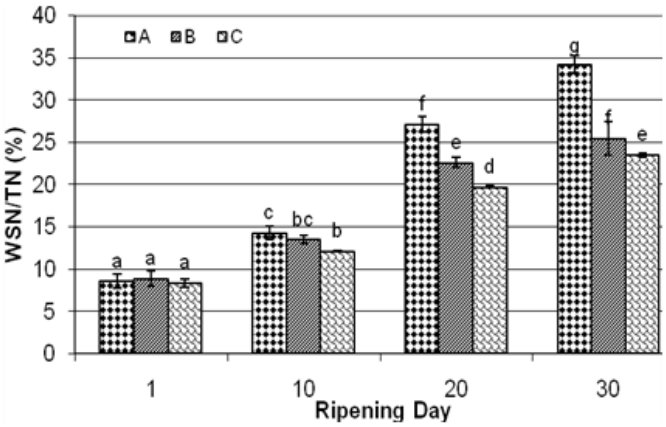

Figure 3. Changes in WSN/TN contents of Iranian white brined cheese during ripening (1, 10, 20 and 30 days). Formulations: A (encapsulated enzyme-added cheese), B (enzyme-added cheese), and C (control). The error bars represent the standard deviation (n=4). Different lowercase letters indicate significant difference ($p \leq 0.05$).

3.5. Discussion

3.5.1. Liposome characterization.

The EE of Flavourzyme encapsulation in HM liposomes was calculated as percentage of the active enzyme quantity entrapped in liposomes to total quantity of active enzyme present in liposome formation and entrapment. The percentage of EE in the present study (26.5%) was similar to the ones previously reported (21–35%) [22, 31, 32], demonstrating the efficiency of the process. The size distribution of the present study (189 nm) was similar to the size distribution of nisin-loaded HM liposomes of the study of Colas et al (190–284 nm based on the chemical compositions) [14].

Total solid content of the cheeses.

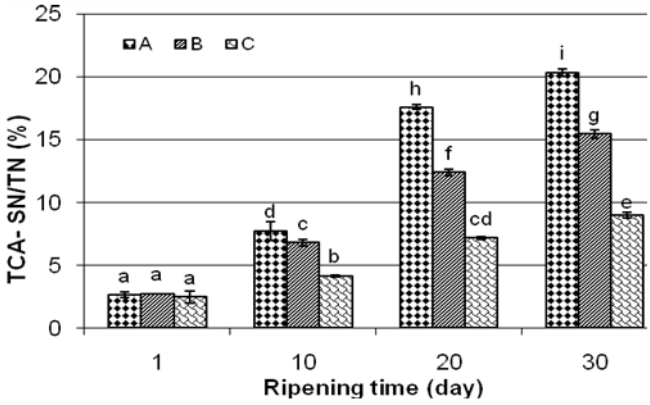

Figure 4. Changes in TCA/TN contents of Iranian white brined cheese during ripening (1, 10, 20 and 30 days). Formulations: A (encapsulated enzyme-added cheese), B (enzyme-added cheese), and C (control). The error bars represent the standard deviation (n=2). Different lowercase letters indicate significant difference ($p \leq 0.05$).

A statistically significant decrease in total solids was observed after addition of LEF (A) ($P \leq 0.05$), which have been previously reported by other researchers [33, 34]. This is mainly caused by water binding at the surface of liposome [33] and by the absorptions of water by the curd matrix, which result in the production of cheeses with increased water contents and decreased

total solids. Higher moisture contents contribute to production of cheeses with a softer texture. However, the growth of microorganisms could be favored, affecting the stability and shelf life of the products [35]. Similar moisture contents for Iranian white brined cheeses (58–62 %) have been reported by other researchers [28]. The total solid contents increased progressively during the ripening in control and experimental cheeses ($P \leq 0.05$). This increase was associated with the temperature of curd preservation (12°C) which affected casein hydration and increased salt concentration in curds due to the salt transference between the curd and brine [23].

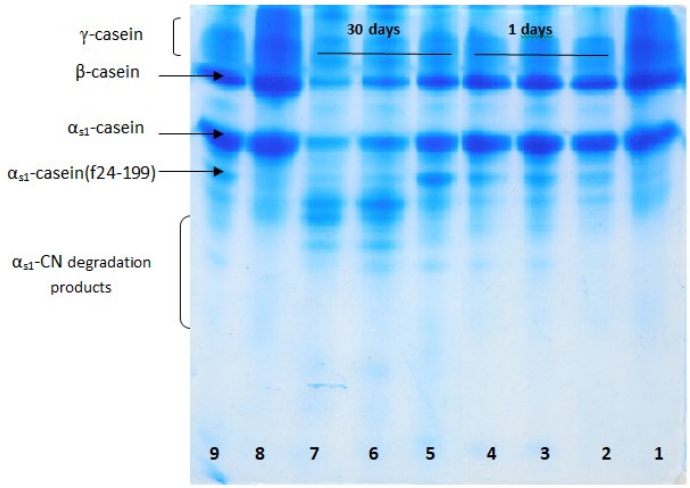

Figure 5. Urea-polyacrylamide gel electrophoretograms: experimental white brined cheese from Iran after 30 days of ripening. Lane 1,8 and 9: sodium caseinate; Lane 2, 5: control; Lane 3, 6: enzyme-added cheese; Lane 4, 7: encapsulated enzyme-added cheese.

3.5.2. Salt contents of cheeses.

No significant differences were observed in the salt contents of cheese samples containing FF and LEF and the control cheese in different day of ripening 1, 10, 20 and 30 days ($P > 0.05$) (Fig. 2), which indicate that the addition of encapsulated or free enzymes did not alter the salt contents of the products or the salt gradients during ripening. This result is important because it reveals that the use of the enzyme maintains the salt contents and hence does not require changes in salting processes. Salt is important because it improves flavor, texture and color of the cheeses, inhibits acid development by controlling the metabolism of the microorganisms and increases the shelf life by reducing water activity (a_w) and inhibiting germination of the microbial spores [30]. During ripening, the NaCl concentration of cheeses significantly increased in all the samples ($P \leq 0.05$). When soaking cheeses in brine, a dynamic mutual diffusion process begins as NaCl is transferred from brine into the cheese while water molecules diffuse out of the cheese matrix. This reduces the moisture contents of cheeses and increases the salt contents of the ripened cheeses [28]. This effect is considerably larger at the beginning of ripening. Therefore, rates of the salt transference into curd are higher at the beginning of ripening [23, 36].

3.5.3. Proteolysis of cheeses.

Figures 3 and 4 show increased WSN/TN in control sample (C) and LEF cheeses (A) from Day 1 to Day 30 of ripening. The primary proteolysis is related to the quantities of the casein-originating peptides, which are predominantly resulted from the rest of coagulation activity. Products of this primary fraction contain large peptides and various peptides of medium molecular sizes. The reported increase during the later ripening stage was linked to bacterial proteinases that were heavily released due to the cell lysis [36]. The TCA-SN calculated as the percentage of TN (TCA-SN/TN) directly after salting was lower than 5%. During the early ripening stages (after 10 days), TCA-SN/TN increased by nearly two folds. At subsequent ripening stages, TCA-SN/TN increase accelerated until reached nearly 8% in control cheeses (C) and 20% in LEF cheeses after 30 days. Caseins and peptides of high and medium molecular sizes are enzymatically degraded to peptides of lower molecular sizes and monomeric amino acids that are soluble in 12% of TCA. Consequently, the quantity of TCA-soluble nitrogen increases with increased cheese ripening [37]. The TCA-soluble non-proteinaceous nitrogen compounds, containing peptides of molecular mass below 3 kDa, amino acids, urea, and ammonia, are indicators of the proteolysis progress [37].

In general, no effects of the enzyme were observed on the proteolysis indices in Day 1 ($P > 0.05$). Both free and encapsulated-enzyme cheeses improved the proteolysis; however, cheese containing LEF showed the highest increase in WSN/TN and TCA/TN, compared to the other cheese samples over the ripening time (Figs. 3 and 4). This indicates that the liposome treated cheese (A) promoted much more proteolysis compared to that of free enzyme-added (B) and control (C) cheeses. Based on the contents of WSN/TN and TCA/TN, addition of LEF could accelerate ripening time by 10 and 20 days, respectively. Furthermore, water-soluble fraction (TCA/TN) was previously described as an essential component for the flavor intensity in mild and ripened cheeses [31], indicating that LEF cheese could increase the flavor intensities. The encapsulation process possibly protected the enzyme from the environment and progressively released it during the ripening, resulting in higher enzymatic stabilities in the cheese matrix. As a consequence of the ripening process, a significant decrease in the quantities of intact casein was observed as well as a parallel increase in degraded products [26]. In cheese containing encapsulated enzymes, degradations of αs1-casein and β-casein were more extended compared to control and free enzyme-added cheeses. This indicates that initial casein proteolysis in Iranian white brined cheeses was intensified by the addition of Flavourzyme in free or encapsulated forms to cheese milk. However, upon ripening, LEF added cheeses showed further formation of casein degradation products. The results of this study showed that supplementation of cheese milk with LEF induced a noticeable progressive rate of proteolysis, and that higher stability of encapsulated enzymes in the cheese matrix could be observed, with a further controlled release of enzymes from liposomes during the ripening. Furthermore, the β-casein band was strongly hydrolyzed with LEF, which resulted from the action of Flavourzyme on β-casein, compared to that on α-casein.

4. CONCLUSIONS

The heating method (HM) of liposome encapsulated protease (LEF) technology could be a potential treatment for the acceleration of ripening in Iranian white brined cheeses, resulting in cheeses with significantly higher proteolysis rates (concentrations of the WSN/TN and TCA/TN) and moisture contents. Based on the contents of WSN/TN and TCA/TN, supplementation of LEF could accelerate the ripening time by 10 and 20 days, respectively. The results of the present study are important for the dairy industry and have the potential for scaling up, as the proposed methodologies are economic and have technological advantages. In fact, HM is an easily scalable one-step, non-toxic, robust and fast technique with an excellent entrapment efficiency. The encapsulation technology seems to overcome problems associated with direct addition of non-immobilized enzymes to milk or curds during the cheese making process, improving the ripening process time. Future studies should be carried out to assess the sensory acceptance of cheeses by consumers.

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