# Evaluation of Two Food Grade Proliposomes To Encapsulate an Extract of a Commercial Enzyme Preparation by Microfluidization

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The entrapment by microfluidization of a commercial enzyme extract (Debitrase DBP20) in liposomes using two food grade proliposome (C and S) preparations was studied. Liposomes obtained at a low microfluidization pressure (4000 psi) were distributed in a bimodal population of small (30−40 nm) and large vesicles (300−700 nm). The composition of the proliposome influenced entrapment efficiency and the repartition of the enzyme between the core and the surface of the liposome. More enzyme was associated with the liposomal surface and greater entrapment efficiencies (64%) were obtained for liposomes with the highest negative zeta potential (proliposome C). Increasing microfluidization pressure and increasing the number of passes through the microfluidizer resulted in losses in entrapment efficiency and enzyme activity, due to decreasing liposome size and enzyme denaturation. Entrapment efficiency was not influenced by external pH and enzyme activity was not adversely affected over storage for 18 days under the conditions evaluated.

KEYWORDS: Liposomes; entrapment; microfluidization; enzymes

## INTRODUCTION

Liposomes are defined as lipid vesicles made of one or more bilayers. These vesicles are composed of amphiphile bilayers containing an aqueous core (*1, 2*), and their size generally ranges from 20 nm to a few hundred micrometers (*3−5*). These vesicles primarily consist of phospholipids (*5*). The phospholipidic bilayer is organized to minimize the interactions between the phospholipid hydrophobic tails and the aqueous medium. Therefore, phospholipids have their hydrophilic heads orientated toward the outside of the bilayer and their hydrophobic tails toward the inside of the bilayer (*1*).

Liposomes are widely used as encapsulating agents in the pharmaceutical, medical and cosmetic industries and are also finding potential applications in the food and beverage industries (encapsulation of flavor compounds, enzymes and vitamins) (*6*). Different reviews have been dedicated to the utilization of liposomes in food science and agriculture (*6*) or more specifically to different liposome applications relative to enzyme encapsulation (*2*). More recently, the different methods that are relevant to analyze liposomes have also been extensively discussed (*7*), (*8*). As the active agents are typically water soluble, once encapsulated they are generally located inside the aqueous core of the liposome but a portion may also interact with the phospholipidic bilayers (via hydrophobic and electrostatic interactions) and remain at the surface of the vesicle.

Studies have been carried out on the encapsulation of active ingredients in liposomes and their use in food products (*9−13*), many of which are related to dairy applications, with most specifically addressed to the acceleration of cheese ripening: encapsulation of proteases (*11, 14, 15*), lipases (*12*), enzyme cocktails (*9*) or cell free extracts (*16*). The antibacterial agent nisin Z and vitamin D have also been effectively encapsulated in liposomes and utilized in cheese production (*10, 17*).

The formation of liposomes is generally not a spontaneous process and therefore requires energy (*1, 2*). This energy is generally a form of mechanical energy (high pressure homogenization, high intensity homogenization, membrane extrusion or ultrasonication) or a nonmechanical energy (reverse phase evaporation, freeze-drying and rehydration, thin film rehydration or detergent depletion) (*6*). Most methods lead to the formation of particles of more than 100 nm, called multilamellar vesicles (MLV), which contain layers of phospholipids around an aqueous core. Microfluidization is a homogenization method based on the use of relatively high pressures to form liposomes. The liposomes obtained with microfluidization mostly consist of small unilamellar vesicles (SUV) with a size ranging from 20 to less than 100 nm (*5, 18*). A major advantage of microfluidization is that organic solvents often used in other liposome preparation methods are not required and therefore

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such a technique is suitable for foods applications and for large scale production (*1, 14, 18*).

In this study we investigated proliposomes, which are commercially available semiprepared bilayers of vesicle-forming amphiphiles (phospholipids) in ethanol and water from Lucas Meyer (Chelles, France). Active agents to be encapsulated are added to the aqueous solution prior to contact with the proliposome (*2*).

Entrapment of an active ingredient in liposomes can be carried out for different purposes (*5*):

• Protect the active ingredient against possible denaturation during processing and storage.

• Improve the distribution of the active ingredient by increasing the surface area with the medium.

• Provide gradual release for the active ingredients in a spatiotemporal pattern.

Owing to these different properties, it is therefore possible to add the active ingredients in less concentrated forms to food systems and to target defined phases in foods, to have a more specific and local action for the active ingredient. Nevertheless, a limited number of liposomal encapsulation applications are found in the food industry, but this is predominantly due to the high costs of phospholipids rather than limited potential.

This study was carried out to assess the potential of two food grade proliposome preparations differing in hydrophobicity to encapsulate a water soluble extract of a commercial proteinase/peptidase preparation, Debitrase DBP20 (Danisco, Copenhagen, Denmark) using microfluidization. The parameters evaluated included the effect of pressure and the number of passes through the microfluidizer on the encapsulation efficiency and on the charge and size of liposomes. The influence of pH on encapsulation efficiency and the stability of the liposomal encapsulated enzymes over time were also studied.

## MATERIALS AND METHODS

**Preparation of the Enzyme Solution.** Debitrase DBP20 is a granulated enzyme preparation derived from *Lactococcus lactis* and *Aspergillus oryzae,* which is predominantly used to prevent/alleviate bitterness development in protein hydrolysates, cheese and enzyme-modified cheese. This enzyme has high levels of post-proline dipeptidyl aminopeptidase (Pep X) activity (*19*), and the measurement of this activity was used in this study to assess entrapment efficiencies and liposomal stability. Pep X activity was quantified using H-Gly-Pro-pNA (Bachem, Switzerland) as a substrate which was diluted in water at a concentration of 5 mM. The release of pNA (*p*-nitroanilide) is proportional to the Pep X activity of Debitrase DBP20. The product pNa absorbs light at 410 nm. The release of pNA was followed over 60 min, at 37 °C, using a Cary 100 Bio UV/visible spectrophotometer (Varian, Walnut Creek, CA) with a multicell peltier. A sample volume of 50 $\mu$L was vortexed with 50 $\mu$L of substrate and 1400 $\mu$L of 25 mM Tris-HCl buffer at pH 7.4 in a glass tube. A 1.5 mL aliquot was transferred to a semimicro cuvette before being placed in the spectrophotometer. Each measurement was performed in triplicate. The enzyme activity was measured using the slope of the curve (Abs/min) between 20 and 60 min. We previously checked that within this time frame, the absorbance of the samples increased proportionally with time. The slope was converted in $\mu$mol.min$^{-1}$, using a molar extinction coefficient of 8800 M$^{-1}$·cm$^{-1}$ for pNA taken from the literature (*20*).

It was necessary to prepare a water soluble extract of Debitrase DBP20 to aid encapsulation. This involved grinding the preparation using a mortar and a pestle until a fine powder was obtained, this was suspended in 25 mM Tris-HCl buffer at pH 7.4 to a final concentration of 2.5% (w/w). The insoluble part of the enzyme preparation was removed by centrifugation at 3500$g$, 15 min at 21 °C. The supernatant was recovered, and the enzyme activity accounted for 19.9 ± 0.8% of the initial enzyme activity present in the original commercial preparation.

**Preparation of the Liposomes.** Two different food grade proliposome preparations were studied: Prolipo C and Prolipo S (Lucas Meyer, Chelles, France). Prolipo C contains 40% (w/w) of unsaturated soybean phospholipids (phospholipid composition: 23% phosphatidylcholine, 14% phosphatidylinositol and 63% other phospholipids) and 60% (w/w) of aqueous media. Prolipo S contains 30% (w/w) of unsaturated soybean phospholipids (phospholipid composition: 80% phosphatidylcholine, 1.5% phosphatidylinositol and 18.5% other phospholipids) and 70% (w/w) of aqueous media (*17*). The liposomes were prepared according to the procedure of Dufour et al. (*15*). The following components were first mixed: 20 g of Prolipo C or S and 100 g of the water soluble extract of Debitrase DBP20. This mixture was agitated using an overhead stirrer, RW 20DZM (Janke and Kunkel, Staufen, Germany), operated at 300 rpm for 15 min at 21 °C, after which 400 mL of 25 mM Tris-HCl at pH 7.4 was added under agitation at 500 rpm at 21 °C for 15 min. The mix was recovered and processed through a microfluidizer, M-110-EH-30 (Microfluidics, Chesham, U.K.), at different pressures (4000, 5000, 6000, 8000 and 10000 psi). The microfluidizer was equipped with a Y interaction chamber and operated with a refrigerant, which allowed the temperature of the mix to be controlled at 16 °C after one pass through the interaction chamber.

To recover the liposomes, the mixture was ultracentrifuged (Discovery 90 SE, rotor T1270, Thermo Scientific, USA) at 85000$g$, at 4 °C over 1 h. The supernatant was removed, and the vesicles were resuspended in 20 mL of 25 mM Tris-HCl buffer at pH 7.4.

In order to determine entrapment efficiencies it was necessary to disrupt the liposomes: 2 mL of the resuspended vesicles was mixed with 2 mL of a 2% aqueous solution of Triton X-100 (Fisons Scientific, Loughborough, England) and vortexed for 5 s (*11*).

**Characterization of the Liposomes.** *Zeta Potential and Size Distribution of Liposomes.* The zeta potential and average hydrodynamic diameter of the liposomes were measured at 21 °C, with photon correlation spectrometry using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). The refractive index of liposomes was taken as 1.45 for the liposomes and 1.34 for the water (*21*). Each sample measurement was carried out four times for the hydrodynamic diameter and ten times for the zeta potential measurement. The measurement was conducted on the liposome mixture immediately after microfluidization. The zeta potential measurements were carried out on liposomes with and without encapsulated enzyme extract.

*Confocal Scanning Laser Microscopy and Atomic Force Microscopy.* The size and shape of the liposomes were determined with two microscopic methods: confocal scanning laser microscopy (CSLM) (Leica DM 6000B, Leica Systems, Wetzler, Germany) and atomic force microscopy (AFM) (MFP-3D-AFM, Asylum Research, Oxford, U.K.). The two techniques were used to observe the two populations of particles present in our samples. The particles under 100 nm could be observed with the AFM technique, whereas bigger size particles could be observed using CSLM.

These experiments were carried out on the liposomes S prepared at the pressure of 4000 psi as described previously. After the ultracentrifugation step, the liposomes were recovered and resuspended in 20 mL of 25 mM Tris-HCl buffer at pH 7.4. These samples were further diluted in the Tris-HCl buffer at the ratio of 1:200. The AFM was carried out in dry contact mode where 10 $\mu$L of the diluted sample was pipetted onto freshly cleaved mica and subsequently dried in a desiccator. A cantilever AC 240 with the spring constant of 1.8 N/m (Olympus Optical CO., Japan) was applied, driving at a frequency of 79.58 kHz, and scan rate at 1 Hz, and the sample was imaged using a TR 800 tip. For the CSLM, the vesicles were stained with Nile red, a hydrophobic dye that specifically targeted the liposomes which were observed with differential interference contrast (DCI) mode. Determination of the size and shape of the liposomes was carried out on preparations without any encapsulated enzyme extract.

**Determination of the Entrapment Efficiency.** The entrapment efficiency is defined as the percentage of enzyme entrapped in the liposome relative to the total amount of enzyme initially present in the mixture (*2, 15*). The entrapment efficiency was determined using the Pep X assay previously described. The enzyme activity was measured in the following samples:
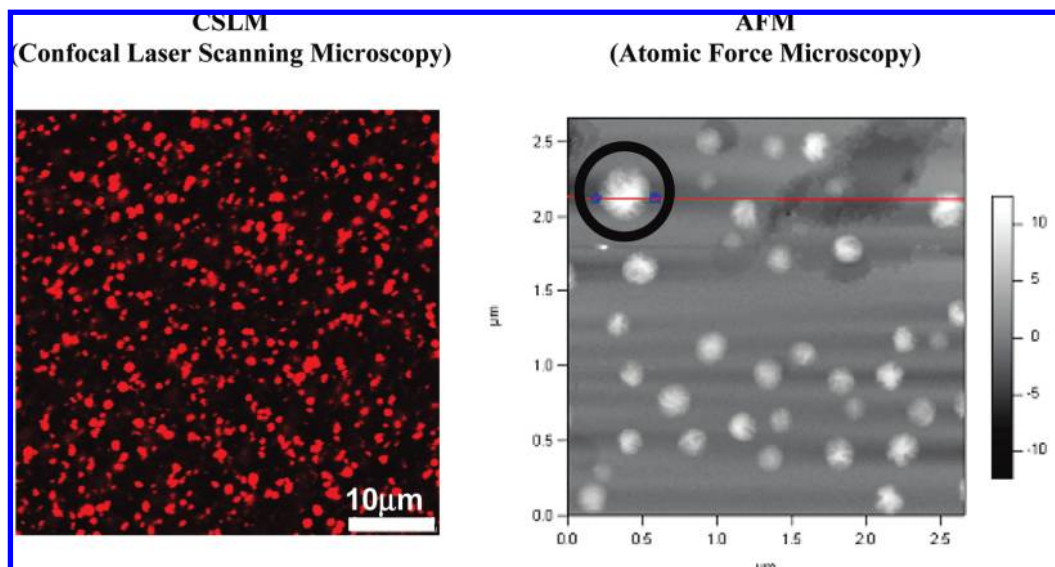
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**Figure 1.** CSLM (confocal laser scanning microscopy) of the liposomes (Prolipo S, 4000 psi, dilution 1:200) colored with nile red, zoom ×5 and AFM (atomic force microscopy, dry contact mode, cantilever AC 240; spring constant, 1.8 N/m; frequency, 79.58 KHz; scan rate, 1 Hz) of the liposomes (Prolipo S, 4000 psi, dilution 1:200). The liposome highlighted has the following dimensions: 390 nm diameter and 17 nm height.
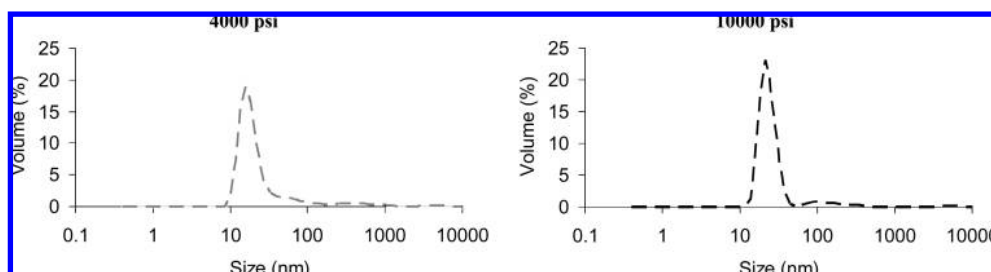


**Figure 2.** Influence of the microfluidization pressure on the size distribution of the liposomes, Prolipo C, 1 pass: (**a**, left) 400 psi; (**b**, right) 10000 psi.

• the supernatant, to determine the unencapsulated enzyme activity;
• the pellet, to determine the enzyme activity at the liposome surface;
• the disrupted pellet (in 2% Triton X-100 solution), to determine the encapsulated enzyme (both at the surface and the core of the liposome). Subtraction of the activity of the pellet from the disrupted pellet provides the enzyme activity in the core.

The enzyme activity in each phase was then expressed as a percentage of the total enzyme activity determined in the mix obtained just after microfluidization.

**Entrapment Efficiency as a Function of Different Parameters.** *Microfluidization Pressure and Number of Passes through the Microfluidizer.* The pressure of the microfluidizer was set at five different pressures (4000, 5000, 6000, 8000 and 10000 psi) for both liposome preparations (C and S). The number of passes was assessed on the Prolipo C mixture only, and the effect on the entrapment efficiency was studied over 5 passes at 20000 psi. These settings were chosen based on preliminary investigations. The effect of the microfluidization pressure and the number of passes on the entrapment efficiency of the Debitrase DBP20 extract and on the liposome characteristics (size and zeta potential) were determined.

*Influence of Changes in External pH on the Liposome Characteristics and Enzyme Activity.* The influence of pH on the zeta potential and enzyme activity of the Debitrase DBP20 extract encapsulated in Prolipo S was studied. A pH range between 4.0 and 7.0 was used. The pH was measured with a pH meter (PHM 82 standard, Radiometer Copenhagen) at 21 °C. A mixture of Prolipo S in 25 mM Tris-HCl buffer at pH 7.4 was prepared as previously described. The pH was adjusted with 10% (w/w) HCl aqueous solution to a range of pH values between 4.0 and 7.0, immediately prior to microfluidization where a pressure of 4000 psi for 1 pass was used. The microfluidized liposomes were then ultracentrifuged as before and the enzyme activities measured as previously described (pH 7.4) for the pellet and disrupted pellet.

*Stability of the Entrapped Enzyme over Time.* The enzyme stability in the liposomes (C and S) was measured over a period of 18 days. The liposomes as ultracentrifuged pellets were stored at 4 °C. Pellets were disrupted using Triton X-100 over the 18 day period, and Pep X activity was measured as previously described.

**Statistical Analyses.** ANOVA was carried out with SAS (version 9.1, Statistical Analysis System, Cary, USA). A Student−Newman−Keuls test was performed for mean multi comparison test at a significance level of 5%.

## RESULTS

**Liposome Characteristics: Size, Shape and Zeta Potential.** The liposomes can be seen as discrete spherical particles on the micrographs obtained by CSLM and AFM (**Figure 1**). The liposome size is of the order of several hundred nanometers, which is consistent with the values found with the zetasizer (liposome hydrodynamic diameter between 10 and 1000 nm; **Figure 1**). The use of CSLM and AFM enabled the 3D size of the liposomes to be determined. The highlighted liposome in **Figure 1** has a diameter of 390 nm diameter and a height of 17 nm, identifying it as a rodlike shaped vesicle.

The size repartition in volume for Prolipo C, at 4000 and 10000 psi, is illustrated in **Figure 2**. For 4000 psi, the graph shows a main peak between 10 and 100 nm with a tailing that goes up to 1000 nm (**Figure 2a**). Most of the liposome sizes lie between 10 and 100 nm. A similar pattern is obtained at 10000 psi (**Figure 2b**), except that the first peak for the smaller sized particles is clearly separated from the second peak relative to the larger liposomes. Furthermore, larger particles are
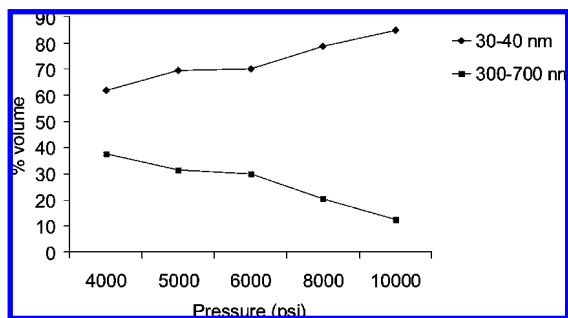
**Figure 3.** Influence of the microfluidizer pressure on the size distribution of Prolipo S, 1 pass.

**Table 1.** Influence of the Number of Passes on the Liposome Size[a]

| no. of passes | mean diameter (nm) | percentage of particles vol (%) |
|---|---|---|
| 1 | 18.4 (0.2)[b] | 92 |
| | 114.0 (7.0) | 8 |
| 2 | 19.1 (0.9) | 92 |
| | 108.0 (5.3) | 8 |
| 3 | 18.9 (0.6) | 93 |
| | 95.2 (1.3) | 7 |
| 4 | 23.0 (1.7) | 100 |
| 5 | 23.8 (1.1) | 100 |

[a] Prolipo C, microfluidization pressure 20000 psi. [b] Figures in parentheses are the standard deviation.

**Table 2.** Effect of the Microfluidization Pressure and the Presence of the Debitrase DBP20 Enzyme Extract on the Zeta Potential (mV) of Liposomes Made with Two Different Prolipo (C and S)[a]

| microfluidization pressure (psi) | Prolipo C | | Prolipo S | |
|---|---|---|---|---|
| | free[b] | +enzyme[c] | free[b] | +enzyme[c] |
| 4000 | −57.4 a | −34.5 e | −17.9 a | −17.0 a,b |
| 5000 | −51.7 b,c | −32.3 e,f | −14.3 d,e | −16.0 b,c |
| 6000 | −49.9 c | −32.1 e,f | −16.1 b,c | −15.3 c,d |
| 8000 | −53.1 b | −25.6 g | −16.6 a,b,c | −15.7 b,c |
| 10000 | −44.8 d | −30.8 f | −13.1 e | −14.2 e,d |

[a] For each liposome type (Prolipo C or S), figures with different letters are significantly different at 5%. [b] Free liposomes, without enzyme. [c] Liposomes containing Debitrase.

obtained at 4000 psi compared to 10000 psi. The influence of the microfluidization pressure on the size repartition of the liposomes for Prolipo S is illustrated in **Figure 3**. The same trend as observed previously with Prolipo C was seen: the volumetric proportion of the SUV increases and that of the larger vesicles decreases as the microfluidization pressure increases.

The influence of the number of passes through the microfluidizer at 20000 psi was studied for Prolipo C. At this pressure, an increase in the number of passes resulted in a reduction and then a disappearance of the bigger size liposomes (**Table 1**). Beyond 3 passes, no more particles with a mean diameter of around 100 nm were evident, thus, beyond 3 passes, the size repartition of the liposomes became monomodal with a mean diameter of around 20 nm.

The zeta potential of Prolipo C was more negative than Prolipo S. The zeta potential for both liposome preparations became less negative with increasing microfluidization pressures ($P < 0.05$) (**Table 2**). Very little difference in the zeta potential of Prolipo S was evident between liposomes with and without entrapped enzyme. However, regardless of the microfluidization pressure, the zeta potential of the Prolipo C preparations was less negative in liposomes with encapsulated enzyme extract than without.

**Table 3.** Influence of the Prolipo Type on the Enzyme Activity of the Debitrase DBP20 Extract in the Different Phases[a]

| microfluidization pressure (psi) | | Prolipo C % enzyme activity | Prolipo S % enzyme activity |
|---|---|---|---|
| 4000 | unencapsulated[b] | 37.3 (3.0) | 70.8 (1.8) |
| | encapsulated[c] | 62.7 | 29.2 |
| | surface[d] | 26.0 (3.9) | 7.9 (3.1) |
| | core[e] | 36.7 (0.0) | 21.3 (0.0) |
| 5000 | unencapsulated[b] | 38.3 (0.0) | 70.1 (1.7) |
| | encapsulated[c] | 61.7 | 29.9 |
| | surface[d] | 22.9 (3.9) | 7.8 (5.4) |
| | core[e] | 38.8 (3.9) | 22.1 (2.2) |
| 6000 | unencapsulated[b] | 39.0 (5.3) | 67.8 (0.0) |
| | encapsulated[c] | 61.0 | 32.2 |
| | surface[d] | 25.9 (3.9) | 0.0 (0.0) |
| | core[e] | 35.1 (3.9) | 32.2 (0.0) |
| 8000 | unencapsulated[b] | 36.1 (3.0) | 63.6 (1.7) |
| | encapsulated[c] | 63.9 | 36.4 |
| | surface[d] | 24.0 (4.89) | 7.8 (3.6) |
| | core[e] | 39.9 (3.9) | 28.6 (0.0) |
| 10000 | unencapsulated[b] | 38.3 (0.0) | 62.0 (0.0) |
| | encapsulated[c] | 61.7 | 38 |
| | surface[d] | 20.7 (3.9) | 2.6 (0.0) |
| | core[e] | 41.0 (3.9) | 35.4 (2.2) |

[a] Figures in parentheses are the standard deviation of the value. [b] Enzyme activity measured in the supernatant. [c] Entrapment efficiency = sum of the enzyme activity at the surface and in the core. [d] Enzyme activity measured in the liposome pellet. [e] Enzyme activity in the core = enzyme activity of the pellet − enzyme activity in the disrupted pellet.
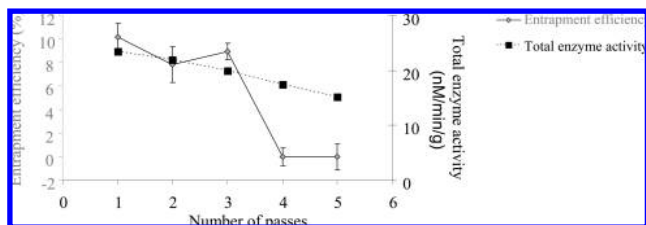


**Figure 4.** Influence of the number of passes through the microfluidizer on the entrapment efficiency and the total enzyme activity. Prolipo C, 20000 psi.

**Entrapment Efficiency of Debitrase DBP20 Extract in the Liposomes.** *Entrapment Efficiency as a Function of the Liposome Formulation.* The enzyme activity was measured in each phase (supernatant, liposome surface and in the liposome core) at 5 microfluidization pressures (4000, 5000, 6000, 8000 and 10000 psi) (**Table 3**). Some losses in activity were noted. On further investigation using microscopy it was found that Triton X-100 was not sufficiently disrupting the liposomes, as immediately after its addition larger vesicles were re-forming. Subsequently we found that the liposomes could be best disrupted using phospholipases and found that we were underestimating the entrapment efficiency in the core. To account for this, the "lost activity" values were added to the actual encapsulation efficiency measured in the core.

At 4000 psi, the entrapment efficiency (sum of the activity in the core and on the surface of the liposome) was 62.7% in Prolipo C and 29.2% in Prolipo S. The percentage of encapsulated enzyme in the core was highest in Prolipo C (36.7%). A large amount of enzyme was located at the surface of the liposomes, at 4000 psi the surface activity of the Prolipo C and Prolipo S accounted for 41% and 27% of the total encapsulated activity, respectively.

*Entrapment Efficiencies at Different Microfluidization Pressures and Number of Passes.* Increasing microfluidization pressure had differing effects on the different preparations. For Prolipo C increased pressures appeared to have little or no

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**Table 4.** Effect of the pH on the Zeta Potential of Prolipo S with and without the Debitrase DBP20 Enzyme Extract[a]

| Prolipo S + enzyme | | Prolipo S free | |
|---|---|---|---|
| pH | zeta potential (mV) | pH | zeta potential (mV) |
| 4.15 | −8.5 a | 4.9 | −10.7 b,c |
| 4.64 | −8.8 a | 5.42 | −9.0 a |
| 5.13 | −10.5 b | 5.82 | −10.5 b,c |
| 5.69 | −8.8 a | 6.24 | −9.6 a,b |
| 6.21 | −11.2 c | 6.67 | −11.3 c |
| 6.86 | −10.1 b | 6.74 | −10.3 b,c |

[a] For each Prolipo type (Prolipo S + enzyme or Prolipo S free), figures with different letters are significantly different at 5%.

impact on entrapment efficiency. Nevertheless, the pressure affected the repartition of the enzyme between the core and the surface of the liposome for Prolipo C, with activity being displaced from the surface to the core (**Table 3**). For Prolipo S, increasing pressure from 4000 to 10000 psi moderately impacted on encapsulation efficiency. Similarly to Prolipo C, an increase in the pressure tended to displace the enzyme from the surface to the liposome core.

The effect of the number of passes upon the entrapment efficiency was studied at 20000 psi for Prolipo C (**Figure 4**). Increasing the number of passes reduced the entrapment efficiency of the Debitrase DBP20 extract, and no activity could be discerned beyond 3 passes at this pressure.

*Influence of External pH on the Liposome Characteristics and Enzyme Activity.* The effect of the pH value on the zeta potential was studied for Prolipo S (**Table 4**). Zeta potential values for liposomes with entrapped enzyme were marginally less negative than in liposomes without encapsulated enzyme for Prolipo S. For the liposomes with entrapped enzyme, the zeta potential was more negative as the pH increased, except at pH 5.69 ($P < 0.05$). However, within the pH range studied, the zeta potential values varied little.

Our results indicated that there was no significant effect of the pH on the encapsulated enzyme activity, but activity appeared to rise slightly as pH decreased (results not shown). Overall the encapsulated Debitrase DBP20 extract was not affected by changes of the external pH ($P < 0.05$).

*Stability of the Encapsulated Enzyme over Time.* Both liposomes (C and S) prepared at 4000 psi as an ultracentrifuged pellet containing encapsulated enzyme were kept at 4 °C for 18 days. Pep X activity was measured at different sampling times over this period. The percentage of enzyme activity remaining in the liposomes as a function of time is illustrated in **Figure 5**. For Prolipo C and S, the proportion of enzyme remaining in the liposomes was stable over time without loss in enzyme activity under the storage conditions evaluated.

## DISCUSSION

**Liposome Characteristics: Size, Shape and Zeta Potential.** The spherical rodlike shape of liposomes determined by AFM is in agreement with previous studies which reported a similar shape for liposomes made of milk fat globule membrane and soy phospholipids (*22*). This nonspherical shape is in part likely due to the preparation mode used during the AFM procedure as the drying process can alter the shape of the particles. The surface interactions between the mica and the liposomes could have caused spreading of the liposomes and thus exacerbated this shape. Other studies have attributed the nonspherical shape of the liposomes to unbalanced osmotic conditions in the medium surrounding the liposomes (*2*). The size of the liposome

was of the same order of magnitude as reported in other studies, where the average hydrodynamic diameter was found to be around 100 nm (*14, 21, 22*).

The liposome size distribution was affected by the microfluidization pressure (**Figure 2**). Increasing the microfluidization pressure from 4000 to 10000 psi resulted in a decrease in the proportion of the larger size liposomes (>100 nm, MLV and LUV (large unilamellar vesicles)) and in an increase in the proportion of smaller size liposomes (<100 nm, SUV). As a result, higher microfluidization pressures gave more homogeneous liposome dispersions in terms of size distribution. Similar trends were found by Thompson and Singh (*22*), who reported 40% decrease in the liposome hydrodynamic diameter when the microfluidization pressure was increased from 73 to 103 MPa (i.e., 10000 to 15000 psi).

Beyond 3 passes through the microfluidizer the population became monomodal with a mean diameter around 20 nm. Thompson and Singh (*22*) also reported a decrease in the mean hydrodynamic diameter of liposomes with the increasing number of passes through a microfluidizer. However, they found that the number of passes (up to 10 passes) had little impact on the polydispersisty of their liposomes. The maximum pressure used by Thompson and Singh (*19*) was only 15000 psi and may explain the difference in polydispersity observed between both studies.

The zeta potential of liposomes is dependent on the phospholipid composition and reflects the overall surface charge. The composition of Prolipo S and C differs in terms of the amount of phosphatidylcholine and phosphatidylinositol present in each preparation. Prolipo S has a high content of phosphatidylcholine (89%) with small quantities of phosphatidylinositol (1.5%), whereas Prolipo C contains a lower amount of phosphatidylcholine (23%) and a higher level of phosphatidylinositol (14%) (*17*). As phosphatidylcholine consists of zwitterions, it is thus primarily neutral, therefore it is the negatively charged phosphatidylinositol that confers a negative zeta potential to the preparations (*23*). Therefore, Prolipo C has the highest negative zeta potential. High zeta potential values (in absolute values) are responsible for electrostatic repulsions, which are more likely to prevent destabilization processes, such as coalescence and aggregation of the liposomes (*22*). Therefore the most stable liposome preparation would appear to be Prolipo C.

The microfluidization pressure affected the zeta potential of the liposomes (**Table 2**). The difference was particularly obvious between the 2 extremes of pressure (4000 and 10000 psi), and showed a decrease in the zeta potential with an increase in pressure. The pressure might affect the zeta potential value by changing the arrangement of phospholipids at the liposomal surface, thus modifying the exposition of certain charged groups. The effect of the microfluidization pressure on the zeta potential value was more pronounced in liposomes without encapsulated enzyme extract. Entrapped enzymes can interact with the phospholipid groups of the liposome bilayer, creating bigger structures that are less easily displaced by microfluidization pressure.

For most of the pressures studied, the addition of the Debitrase DBP20 extract in the liposomes did not affect the zeta potential of Prolipo S. However the inclusion of the enzyme extract caused a significant decrease of the zeta potential value of Prolipo C. Thus, the addition of enzymes in Prolipo C may decrease the liposomal stability.

**Influence of the Liposome Formulation on the Entrapment Efficiency of the enzyme Debitrase DBP20 Extract.** The entrapment efficiencies (**Table 3**) measured for Prolipo S and
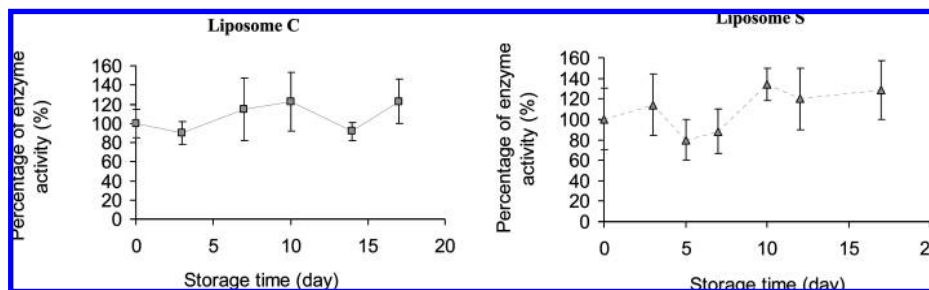
**Figure 5.** Percentage of enzyme activity remaining in the Prolipo C and S as a function of the storage time at 4 °C.

C were between 29 and 64%, respectively, and are of the same order as those determined in previous studies using prolipo-somes: 32−36% for bacterial and fungal proteinases in Prolipo VPF 012 (*11*), 35.9% for Palatase M and 40.3% for Lipase 50 in Prolipo VPF 012 (*12*) and between 56.8 and 58.4% for a cell free extract of *Lactobacillus casei* subsp. *pseudoplantarum* in Prolipo S (*16*). Much higher entrapment efficiencies 68 and 96% for chymotrypsin were achieved using Prolipo 3080 S and Prolipo 3045 S, respectively (*15*).

The distribution of the Debitrase DBP20 extract at the surface and core of the different proliposomes is in accordance with literature (*15*). In fact, Dufour et al. (*15*) found that the amount of chymotrypsin located at the liposomal surface varied from 24 to 100%, depending on the amount of enzyme used, the proliposome type and the ionic strength. The presence of the Debitrase DBP20 extract at the liposomal surface was linked to the physicochemical properties of the proliposomes used. A definite positive correlation existed between the association of the Debitrase DBP20 extract at the surface of the liposome and the increasing negative charge of the phospholipid. This is likely due to electrostatic interactions between the enzyme and the negative phosphatidylinositol groups (*17*). Furthermore, hydro-phobic interactions may also play a role in the attachment of the enzyme at the liposome surface. Prolipo C contains a higher proportion of phospholipids (40%) than Prolipo S (30%) and had a higher proportion of enzyme at its surface. The partitioning of the enzyme between the core and the liposome surface seems to be driven by the affinity of the enzyme for the phospholipids present in the liposome membrane. As the enzyme is a protein, it has an amphiphilic character and may interact with the hydrophobic regions of the liposomal membrane. Indeed, hydrophobic interactions or electrostatic interactions are most likely responsible for the attachment of the enzyme at the liposomal surface (*15, 24*). This suggests that the entrapment efficiency could be enhanced by increasing the number of hydrophobic bonds between the enzyme and the liposomes through the use of higher concentrations of proliposomes. In addition it is also probable that the hydrophilic parts of the enzymes are immobilized at the liposomal surface and orientated toward the external aqueous phase (*21*), making them readily available for reactions in food systems (*23*).

**Influence of the Microfluidization Pressure and the Number of Passes on the Entrapment Efficiency.** An increase in the microfluidization pressure resulted in less negative zeta potential values for the liposomes (**Table 2**). The changes in the microfluidization pressure might have induced a modification in the repartition of the phospholipids at the liposomes surface. These changes could be responsible for a different exposure of the hydrophobic groups and an easier access of the enzyme to the latter, which may cause a greater attachment of the enzyme at the liposome surface as seen for Prolipo S. In the case of Prolipo C, despite possibly better access of the hydrophobic groups to the enzyme, the less negative charges at higher

pressures might cause a slight reduction of the attachment of the enzyme. The overall effect (hydrophobic interactions and electrostatic interactions) resulted in no further improvement in entrapment efficiency with increased pressure.

The number of passes affected the entrapment efficiency of the Debitrase DBP20 extract: beyond 3 passes at 20000 psi, enzyme activity was not detected. This might be due to denaturation of the enzyme resulting from the processing conditions (heating and high shearing rate in the interaction chamber of the microfluidizer). A coolant was used during processing, but the temperature in the interaction chamber increased up to 50 °C after 1 pass. Indeed, the number of passes affected the total enzyme activity (sum of the enzyme activity encapsulated and unencapsulated). In a previous study, Koide and Karel (*25*) found that the encapsulation efficiency was halved after 5 passes through the microfluidizer, compared to 1 pass. The enzyme activity was not totally lost, but the operating pressure was 10 times lower (2000 psi) than the one used in this study.

**Effect of External pH on the Liposome Characteristics and Enzyme Activity.** *Influence of External pH on the Zeta Potential.* The zeta potential values increased slightly for the liposomes with entrapped enzyme, but overall did not vary much in the pH range studied (4.0−7.0). This result is very similar to those of Thompson and Singh (*22*), who did not find great variations in the zeta potential of liposomes from milk fat globule membrane in the same pH range (4.0−7.0). However, they did find that the pH greatly influenced the zeta potential of liposomes below pH 4.0.

*Influence of External pH on Enzyme Activity.* The entrapment in the core slightly decreased when the pH increased, and the entrapment at the surface remained unaffected by external pH changes (data not shown). Previous studies conducted on the influence of pH on the entrapment efficiency found an improve-ment of the entrapment efficiency of pepsin and lysozyme at pH 3.0 and 5.88 respectively (*25*). They also attributed this effect of pH to a higher solubility of both enzymes and to the changes in the electrostatic repulsions between the enzymes and the phospholipids. As previously discussed, the Debitrase DBP20 extract is likely to interact with the phospholipids owing to electrostatic interactions. Nevertheless, in the pH range studied, the charges at the liposome surface did not change significantly, which may explain the fact that the enzyme did not further interact with the liposome surface.

**Influence of Storage Time on the Entrapment of Debitrase DBP20 Extract.** The enzyme activities were stable over an 18 day period at 4 °C. This result highlights potential practical uses for liposomal encapsulated enzymes. Obviously it would be of great benefit to have stable preparations as end users would be unlikely to have or invest in the technologies (microfluidization or ultracentrifugation) required for their preparation. Similarly, Koide and Karel (*25*) found that pepsin or lysozyme entrapped in liposomes were relatively stable as less than 5% of the

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enzyme was released during 12 days of storage in a buffer at 10 °C. In our study, there is a negligible amount of buffer outside the liposomes as they were kept as a pellet. Therefore, the gradient between the inner and outer parts of the liposome would be unlikely to be sufficient to drive the diffusion process of the Debitrase DBP20 extract outside of the liposome. The concentration differences between the outside and the inner part will quickly be negligible, and this explains the increased liposome stability in a pellet as opposed to a dispersion.

In conclusion, conditions were optimized to encapsulate an extract of a commercial enzyme (Debitrase DBP20) in liposomes by microfluidization using two different proliposome preparations (C and S). The liposomes produced consisted of two populations of small (30−40 nm) and large (300−700 nm) vesicles. Low microfluidization pressures, such as 4000 psi, with 1 pass through the Y chamber achieved optimal encapsulation efficiencies. Higher microfluidization pressures and increased number of passes reduced liposome size (from MLV/LUV to SUV type particles), lowered entrapment efficiency and also appeared to result in enzyme denaturation. Liposomal composition influenced encapsulation efficiency and the repartition of the enzyme at the core and surface. Liposomes with a higher content of phosphatidylinositol (Prolipo C) had a greater negative zeta potential and a greater entrapment efficiency and contained more than 40% of the entrapped enzyme at the surface of the particle. It appears that the enzyme interacts with the phospholipidic membrane by both electrostatic and hydrophobic interactions and that these interactions are greatest in proliposome preparations that contain more highly charged phospholipids. Increasing microfluidization pressure increased the negative surface zeta potential of the liposomes with higher contents of charged phospholipids compared to those containing less charged phospholipids. This effect was less pronounced in liposomes containing entrapped enzyme, presumably due to the presence of enzyme on the liposomal surface.

The activity of the entrapped Debitrase DBP20 extract in the liposomes was not influenced over an external pH range of 4.0 to 7.0, where no change in the zeta potential was noted. The enzyme activity of Debitrase DBP20 extract was stable in both types of liposomes for 18 days at 4 °C as a pellet.

## ABBREVIATIONS USED

AFM, atomic force microscopy; ANOVA, analysis of variance; CSLM, confocal scanning laser microscopy; DCI, differential interference contrast; EE, entrapment efficiency; Gly, glycine; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; Pep X, post-proline dipeptidyl aminopeptidase; pNA, *p*-nitroanilide; Pro, proline; SUV, small unilamellar vesicles.

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