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# Controlled release of casein-derived peptides in the gastrointestinal environment by encapsulation in water-in-oil-in-water double emulsions

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ABSTRACT

Dye-labeled peptides were produced by hydrolysis of azocasein and encapsulated in water-in-oil-in-water double emulsions. The effect of degree of hydrolysis (0%, 3.5%, or 7%) and oil phase composition (butter oil, linseed oil, or mineral oil) on the emulsion characteristics and peptide release kinetics during simulated gastrointestinal digestion was investigated. The double emulsions made from linseed oil and butter oil had smaller oil droplets, lower viscosity, and greater creaming stability than the double emulsion made from mineral oil did. The encapsulation efficiency was higher than 93%. The percentage of peptide release at the end of the gastric phase varied from 4% to 42%. Butter oil and linseed oil were attacked by the lipases during the intestinal phase of the digestion, and the peptide release increased progressively to more than 80%. The release of hydrolyzed azocasein was faster than the release of unhydrolyzed azocasein in both phases of digestion. Mineral oil was indigestible and strongly inhibited peptide release, which remained lower than 18%. The release behavior was strongly influenced by the composition as well as the hydrophobicity of the oil phase. Depending on peptide molecular weight, the kinetics of release can be controlled by adjusting oil phase composition.



## 1. Introduction

In recent years, there has been growing interest in using bioactive peptides in the formulation of functional foods because of the peptides' physiological activities and health benefits. Milk proteins are known to be precursors of bioactive peptides (Dziuba, Dziuba, & Iwaniak, 2009). Active fragments usually contain 2 to 25 amino acid residues, and the activity of those fragments depends on their amino acid sequence and location in the protein chain (Dziuba et al., 2009). Among milk proteins, caseins are an important source of bioactive peptides with antithrombotic, antihypertensive, opioid, immunomodulatory, and antimicrobial activities (Silva & Malcata, 2005). To exert physiological effects in vivo, bioactive peptides must survive gastrointestinal digestion and reach their target sites after absorption (Chen & Li, 2012). However, the digestive proteases and pH conditions in the gastrointestinal tract are likely to alter the activity of bioactive peptides. Therefore, one of the greatest challenges in developing functional foods with casein peptides as ingredients is proving the in vivo efficacy of the bioactive components (Ao & Li, 2013). The impact of the molecular weight (Chen & Li, 2012), charge properties (Ao & Li, 2013), and chain length (Roufik, Gauthier, & Turgeon, 2006) of milk-derived peptides on resistance to simulated gastrointestinal digestion in vitro has been studied. Peptides with high basic amino acid content and long-chain bioactive peptides, especially ones with more than five amino acid residues, have lower tolerance to gastrointestinal digestion and need to be protected against gastrointestinal degradation in order to be able to exert their physiological effects in the organism.

Water-in-oil-in-water ($W_1/O/W_2$) double emulsions have been proposed in pharmaceutical and food applications for the encapsulation, protection, and delivery of probiotic bacteria, sensitive water-soluble nutrients (minerals or vitamins), or bioactive compounds (Dickinson, 2011; Giroux et al., 2013; McClements, 2015; Pimentel-González, Campos-Montiel, Lobato-Calleros, Pedroza-Islas, & Vernon-Carter, 2009; Prichapan & Klinkesorn, 2014; van der Graaf, Schroën, & Boom, 2005). In $W_1/O/W_2$ double emulsions, water droplets containing the bioactive material are finely dispersed within the larger oil droplets of an oil-in-water emulsion.

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The ability of a double emulsion to serve as an encapsulation tool is dependent on the emulsion's stability, which is determined by the nature of the oil phase, the type of emulsifiers, and the nature of the entrapped materials (Su, Flanagan, Hemar, & Singh, 2006; Su, Flanagan, & Singh, 2008). Numerous studies have been conducted to optimize the preparation and the stability of double emulsions (Garti, 1997).

The objective of the present study was to encapsulate azocasein hydrolysates in $W_1/O/W_2$ double emulsions and to determine the effect of degree of hydrolysis and oil phase composition (linseed oil, mineral oil, or butter oil) on the characteristics of emulsions and the kinetics of azocasein peptide release during simulated gastrointestinal digestion. The monitoring of peptide release during gastrointestinal digestion is a challenge because of the complexity of the digestive juices. Azocasein is typically used as a substrate to quantify proteolytic activity by spectrophotometric measurement of the released azopeptides at 440 nm (Bendicho, Martí, Hernández, & Martín, 2002; Conesa & FitzGerald, 2013). In the present study, azocasein was chosen to produce dye-labeled peptides and facilitate the evaluation of the encapsulation efficiency and stability of double emulsions during digestion.

## 2. Materials and methods

### 2.1. Materials

Azocasein, trypsin from bovine pancreas (type I), α-amylase (type VI-B) and pancreatin from porcine pancreas, pepsin from porcine gastric mucosa, and bile extract porcine were purchased from Sigma–Aldrich (Oakville, ON, Canada). Mineral oil was obtained from Fisher Scientific (Ottawa, ON, Canada), and linseed oil was obtained from La Maison Orphée inc. (Quebec City, QC, Canada). Butter oil was prepared from commercial unsalted butter by removing water and nonfat milk solids by decantation at 50 °C. Mineral oil is composed mainly of saturated hydrocarbons (984 g/kg), whereas linseed oil and butter oil are composed of saturated, monounsaturated, and polyunsaturated fatty acids in different proportions (Table 1). Polyglycerol polyricinoleate (Grindsted PGPR 90) was provided by Danisco Canada Inc. (Scarborough, ON, Canada). Sodium caseinate (Alanate 180) was obtained from Nealanders International Inc. (Dorval, QC, Canada). All other reagents were of analytical grade.

### 2.2. Preparation of azocasein hydrolysates

Azocasein hydrolysates were prepared by the pH-stat technique using a TitraLab 856 titration workstation (Radiometer Analytical, Lyon, France) equipped with a Radiometer Analytical PHC2701-8 combined pH electrode. Azocasein consists of casein conjugated with an azo-dye. The dye is attached to the hydroxyl groups of tyrosine residues in the protein. Azocasein was solubilized in deionized water to a protein concentration of 55 g/kg and adjusted to pH 7.5 with 0.1 mol/L HCl. The azocasein solution (75 mL) was placed in a jacketed glass beaker (100 mL) connected to a water bath set at 37 °C. Trypsin was added under stirring to the azocasein solution maintained at 37 °C, at an enzyme:substrate ratio of 1:100 (Saini et al., 2014). During the hydrolysis, the pH was kept constant at 7.5 by automatic addition of the titrant (0.5 mol/L NaOH), and the base consumption curve was recorded as a function of time. The base consumption was used to calculate the degree of hydrolysis (DH) according to Adler-Nissen (1986) (chap. 5), as follows:

$$DH = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100\% \quad (1)$$

where $B$ is the base consumption in milliliters; $N_b$ is the normality of the base; $\alpha$ is the average degree of dissociation of the $\alpha$-amino groups at the pH and temperature of hydrolysis (0.549); $MP$ is the mass of protein in the reaction vessel in grams; and $h_{tot}$ is the total content of peptide bonds in the protein substrate in milliequivalents (meqv) per gram (8.2 for casein).

The azocasein was hydrolyzed in independent trials to a DH of 3.5% or 7%. The enzyme activity was stopped by adding the same volume of boiling water and heating at 80 °C for 5 min. Then, the hydrolysate was rapidly cooled to 4 °C in an ice bath, frozen at −20 °C, and freeze-dried. The dehydrated azocasein hydrolysates were stored at 4 °C until use.

**Table 1**
Fatty acid profile of the butter oil and linseed oil (g/kg).

| Fatty acid | Oil | |
|---|---|---|
| | Butter[a] | Linseed[b] |
| Saturated | 690 | 40 |
| Monounsaturated | 270 | 150 |
| Polyunsaturated | 30 | 810 |

[a] Data source: Walstra & Jenness, 1984.
[b] Data source: Product specifications, La Maison Orphée inc.

### 2.3. Characterization of azocasein hydrolysates

The molecular weight distribution of the azocasein hydrolysates was determined in triplicate by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10–20% Mini-Protean Tris-Tricine peptide analysis gels (Bio-Rad Laboratories Inc., Hercules, CA, USA). The samples were diluted in tricine sample buffer containing β-mercaptoethanol as reducing agent and heated at 95 °C for 5 min. The sample load was 25 μg. The gels were fixed in a mixture of methanol, acetic acid, and deionized water (40:10:50) for 30 min and stained with Bio-Safe Coomassie Blue G-250 (Bio-Rad Laboratories) for 1 h. The destaining step was performed by incubation in deionized water for 2 h. Molecular weights were determined using Bio-Rad Laboratories polypeptide SDS-PAGE standards with molecular weights ranging from 1.4 to 26.6 kDa. The digitalized gel images were obtained with an ImageScanner III LabScan 6.0 (GE Healthcare, Piscataway, NJ, USA) and analyzed by the ImageQuant TL 7.0 software (GE Healthcare). The total surface area corresponding to each band was integrated, separated into four ranges of molecular weight (>18 kDa, 18–10 kDa, 10–6 kDa, and <6 kDa), and expressed as percentages of the total area.

### 2.4. Preparation of $W_1/O$ primary emulsions

The water-in-oil ($W_1/O$) primary emulsions were prepared as described by Giroux et al. (2013) using butter oil, linseed oil, or mineral oil as the oil phase. Briefly, the internal aqueous phase ($W_1$) was composed of 0.001 mol/L phosphate buffer (pH 7) containing 0.1 mol/L NaCl. The addition of NaCl made it possible to equilibrate the osmotic pressure between the inner and outer aqueous phases and thus minimize water transfer between the two phases (Hemar, Cheng, Oliver, Sanguansri, & Augustin, 2010). Azocasein hydrolysates (DH = 0%, 3.5%, or 7%) were added to the internal aqueous phase at 50 g/kg. This concentration was chosen to facilitate the spectrophotometric determination of azocasein concentration released during gastrointestinal digestion. The oil phase (O) consisted of butter oil, linseed oil, or mineral oil containing 80 g/kg PGPR 90 as emulsifier (Giroux et al., 2013). The $W_1/O$ primary emulsions were prepared by adding the internal aqueous phases containing azocasein hydrolysates ($W_1$) (200 g/kg) to the linseed