Green Production of Anionic Surfactant Obtained from Pea Protein

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Abstract A pea protein isolate was hydrolyzed by a double enzyme treatment method in order to obtain short peptide sequences used as raw materials to produce lipopeptides-based surfactants. Pea protein hydrolysates were prepared using the combination of Alcalase and Flavourzyme. The influence of the process variables was studied to optimize the proteolytic degradation to high degrees of hydrolysis. The average peptide chain lengths were obtained at 3–5 amino acid units after a hydrolysis of 30 min with the mixture of enzymes. Then, *N*-acylation in water, in presence of acid chloride (C12 and C16), carried out with a conversion rate of amine functions of 90%, allowed to obtain anionic surfactant mixtures (lipopeptides and sodium fatty acids). These two steps were performed in water, in continuous and did not generate any waste. This process was therefore in line with green chemistry principles. The surface activities (CMC, foaming and emulsifying properties) of these mixtures were also studied. These formulations obtained from natural renewable resources and the reactions done under environmental respect, could replace petrochemical based surfactants for some applications,.

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Introduction

Surfactant molecules based on the design of molecular mimics of natural compounds are an alternative to synthetic surfactants obtained from petrochemical sources [1]. Given their high biodegradability and low toxicity, they are one of the preferred choices for pharmaceutical or cosmetic applications. Several classes of biosurfactants have been identified by their non-conventional structure derived from amino acids/peptides, oligosaccharides and glycerol [2–4]. Lipopeptides have become increasingly important because of their functional properties. Conventional surfactants contain one or more apolar tails linked to a polar head group that may be charged (cationic, anionic or zwitterionic) or uncharged. Thus, the combination of polar amino acids/peptides (hydrophilic moiety) and non-polar chain compounds (hydrophobic moiety) produces an amphiphilic structure with high surface activity. An approach to obtain the hydrophilic moiety may involve the digesting of native protein into a complex mixture of short peptide sequences [2]. The hydrolysis of native proteins can be carried out either by chemical or enzymatic way. The latter, using selective proteases, provides milder process conditions and little or no undesirable side reactions or products. Enzymatic hydrolysis can produce hydrolysates with well defined peptide profiles and extensive reviews or publications of enzymatic protein hydrolysates were reported in the literature [5–7].

Proteins used for this goal are generally low cost food proteins, including casein [8], whey [9, 10], soy [11], wheat gluten [11], and pea [12–16] etc. Until now, the major application of peas is rather limited; they are mainly used for

animal feed. Thus, the development of new applications of major components (such as proteins) could enhance their cultivation. Besides, the pea protein is not listed among the major allergens that could lead to specific labeling. This point is interesting for cosmetic application. The three major proteins found in the isolate of pea protein are albumins (21%, 4–53 kDa), globulins (66%, 150–400 kDa) and glutelins (12%). As pea protein contains a large amount of reactive amino groups and basic amino acid residues, controlled enzymatic hydrolysis can provide access to these high reactive functions. Chemical modification such as acylation could effectively be carried out in order to produce anionic lipopeptides-based surfactants. For this industrial application, the protein hydrolysates should be rich in low molecular weight peptides, especially tripeptides, to reach a high solubility in water and a high reactivity for acylation onto fatty acids to obtain lipopeptides [2]. By controlling the reaction conditions during enzymatic hydrolysis, hydrolysates of various characteristics can be obtained and affect the degree of hydrolysis (DH) [17]. The DH is defined as the percentage of peptide bonds cleaved per gram of protein compound with the total amount of peptide bonds per gram of protein. DH is a key parameter that needs to be controlled to obtain reproducible results and optimum peptide size. A lot of proteases have been extensively used for pea protein hydrolysis as Alcalase [12, 13], Trypsin, Papain, Chymotrypsin [13, 14, 16], Flavourzyme [13] and Protamex [12]. The mixture of endo-peptidase and exo-peptidase has been used for other proteins like soybean, canola or wheat gluten, and showed that the DH increased [18]. In previous researches, the combination of Alcalase (an endo-peptidase) and Flavourzyme (an endo- and exo-peptidase) gave high yields of soluble protein and the average peptide chain length was estimated at 3–5 amino acids units in hydrolysis of defatted soy bean flour [18]. Both of these enzymes meet the specifications recommended by the FAO-OMS for food applications. Alcalase is an endo-peptidase produced by *Bacillus licheniformis* (2.4 AU/g) with an optimum pH of 6–8. The activity is expressed in Anson Unit [19]. An Anson Unit (A.U.) is defined as the amount of enzyme which digests urea-denatured hemoglobin under specified conditions. The initial rate of enzyme must be such that an amount of TCA-soluble product is liberated per minute, amount corresponding to the same color with Folin-Ciocalteu Phenol reagent as one milliequivalent of Tyrosine (25 °C, PH = 7.50).

Flavourzyme is a fungal complex of exo-peptidases and endo-proteases produced by *Aspergillus oryzae* (500 LAPU/g) with an optimum pH of 7. The activity is expressed in LAPU [20]. The LAPU is the amount of enzyme causing the hydrolysis of 1 µmol L-Leucine-*p*-nitroanilide to L-Leucine and *p*-nitroaniline (pH 7.2, 37 °C). Thus the purpose of this work is to study the effect of the mixture of Alcalase and Flavourzyme on the hydrolysis of a pea protein isolate and to optimize the experimental conditions (enzyme/substrate ratio, reaction time and enzyme addition) to obtain low molecular weight peptides that will be used as raw materials to produce lipopeptide-based surfactants. Protein hydrolysates were characterized by SDS–PAGE and Gel Permeation Chromatography (GPC) to provide the molecular size distribution of the corresponding peptides. In a second time, lipopeptide was obtained with Schotten–Baumann reaction, an environment friendly and "green" synthesis, to form anionic surfactant formulations. These surfactant formulations, containing a mixture of lipopeptides and sodium fatty acids, were obtained from pea proteins and fatty acids (under acid chloride form), each of them originated from vegetal raw material, by only using water as a solvent and by avoiding any rejection of by-products. Moreover, the energy supply for this synthesis was limited as well. The manufacturing process of the surfactants described here is therefore in adequacy with the green chemistry concepts [21]. Finally, the influence of the formulation of surfactant structure on surface active properties was studied.

Materials and Methods

Materials

Pea (*Pisum sativum* L.) protein isolate was purchased from BioSerae (Bram, France). These three protein families have high molecular weights ranging from 140,000 to 180,000 Da. This isolate contains 4% moisture, 3.2% ash, 2.3% lipids, 11.9% carbohydrates, and 82.6% protein. Alcalase 2.4L (EC 3.4.21.14) and Flavourzyme 500L (EC 232.752.2) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).

Sodium dodecyl sulphate (SDS), *o*-phthaldialdehyde (OPA), sodium tetraborate, L-Leucine, β-mercaptoethanol, dodecanoyl chloride, acrylamide, bis-acrylamide, tris, coomassie brilliant blue, tetramethyl ethylene diamine, sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), Cytochrome C, Aprotinine, $Glycine_6$ and Glycine were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Seeblue® pre-stained protein standards (LC5625) were purchased from Invitrogen.

Methods

Enzymatic Hydrolysis

General Preparation of Pea Protein Hydrolysates

Hydrolysis was performed using Alcalase and/or Flavourzyme under magnetic stirring at 500 rpm. A 10%

(w/v) aqueous dispersion of pea protein isolate was incubated in a water bath at 50 °C during 10 min and then adjusted to appropriate pH with 4 N NaOH solution depending on the used enzymes (pH 7 for Flavourzyme, pH 8 for Alcalase), then Alcalase (0.1–0.6 AU/g of protein) or Flavourzyme (40–340 LAPU/g of protein) was added. Hydrolysis was carried out from 30 min until 3 h by stirring continuously. The pH of the mixture was kept constant by continuously adding a 4 N solution of sodium hydroxide (NaOH). After the reaction period, enzyme inactivation was achieved by adding 4 N solution of hydrochloric acid (HCl) until pH 4.0. The slurry was cooled down to room temperature, adjusted to pH 7 with 4 N NaOH solution prior to freeze-drying. All the experiments were carried out in triplicate.

Specific Preparation of Pea Protein Hydrolysates in Case of Double Enzyme Treatment

Two experimental conditions were tested. According to the method of Megias et al. [22] with some modifications, sample 1 was hydrolysed by Alcalase at 50 °C and pH 8.0 for 30 min, followed by Flavourzyme at pH 7.0 for an additional time of 30 min. The conditions (pH 8.0 then 7.0 and 50 °C) were constantly monitored and maintained throughout the process. Finally, sample 2 was produced using a combination of the two enzymes [23] which were simultaneously introduced in the medium at pH 7.5 and 50 °C for 30 min.

N-acylation of Pea Protein Hydrolyzates

The protocol of Schotten–Baumann *N*-acylation was optimized on amino acid-like models [24] before to be extended to protein hydrolyzate. A 200 mL volume of the aqueous solution of hydrolysed proteins (20 g of peptide hydrolysate) is introduced into a 500-mL two-necked flask equipped with a cooling agent and mechanical stirring. The mixture was stirred at room temperature. Dodecanoyl chloride or hexadecanoyl chloride (1.5 eq/NH_2) was added dropwise for 1 h. The reaction temperature was thus maintained at 50 °C and the pH at 10 with 4 N sodium hydroxide solution. The total time of the *N*-acylation is 3 h. After the reaction, the pH was adjusted to 7 with 4 N hydrochloric acid solution and then the water was eliminated by freeze-drying.

Degree of Hydrolysis (DH)

The *o*-phthaldialdehyde (OPA) method is used for the determination of amino groups [25]. The hydrolysates were solubilized at 125 mg/L in a 12.5 mM sodium tetraborate buffer (pH 8.5) and 2% (w/v) SDS. This solution (1 mL) was mixed with 2 mL of the reagents. The reagent prepared in a 50 mL flask was composed of 25 mL of 0.1 M borate buffer (pH 9.2), 2.5 mL 20% (w, v) SDS solution, 100 µL of β-mercaptoethanol and 40 mg of OPA dissolved in 1 mL methanol. The flask was completed up to 50 mL with demineralized water. The mixture was allowed to stand for 2 min before measurement of the absorbance at 340 nm. The number of amino groups was determined with reference to the L-leucine standard curve (between 0.1 and 0.6 mM). The increase in amino groups between native pea isolate and hydrolysates was attributed to proteolysis and degree of hydrolysis was calculated by the following equation:

$$\mathrm{DH}(\%) = [(\alpha - \mathrm{n_i})/(n_T - n_i)] \times 100$$

n_T was the total number of amino groups in pea protein isolate after complete acid hydrolysis with 6 N hydrochloric acid (HCl) at 110 °C for 24 h and n_i was the number of amino groups in native pea protein isolate, while, α was the number of free amino groups measured in the pea protein hydrolysate. The average peptide chain length (APCL) was estimated from the DH, APCL = (100/DH) [26].

Molecular Weight Distribution

Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS–PAGE)

Molecular weight distribution in the protein hydrolysates was determined by SDS–PAGE, according to the procedure of Laemmli [27], as modified by Schägger and Von Jagow [28], using 4–12% Bis–Tris gels. The electrophoresis was run at 100 mA in 1.00 mm thick gels. The gels were stained with coomassie brilliant blue. The approximate molecular weight of the hydrolysates was determined according to the relative mobility of protein bands from standards sampled in the same gel (9 polypeptides in the range of 4–250 kDa).

Gel Permeation Chromatography (GPC)

The molecular size distributions of the samples were determined by gel permeation chromatography using a Dionex TCC-100 oven, Dionex ASI 100 sampler, Dionex P680 pump, Dionex UVD 340U detector and a Superdex™ Peptide HR 10/30 column, in the range of 100–7,000 Da, (No. 9611011, Pharmacia, Biotech, Uppsala, Sweden). The eluent was a 0.02 N phosphate buffer solution containing 0.25 M NaCl (pH 7.2), and the flow rate was 0.5 mL/min. The absorbance was measured at 214 nm. In order to estimate the molecular size, Cytochrome C (MW 12.5 kDa), Aprotinine (MW 6.5 kDa), $\mathrm{Glycine_6}$ (MW

360 Da), Glycine (MW 75 Da) were used as standard proteins to calibrate the column. A relationship between the elution volume and the log of the molecular mass of peptides used as standards has been established. The freeze-dried peptidic hydrolysates are solubilized in a 0.2 g/L buffer solution eluting from the column. 1 mL of each solution obtained is filtered on a 0.45 µm micropore filter and then injected on the column.

Analysis of Amino Groups and Determination of the Acylation Rate

Free amino groups were quantified using the reliable OPA method. The acylation rate (AR) was determined using the following formula:

$$\mathrm{AR} = (Ni - Nf)/Ni * 100$$

where *Ni* is the number of free amino groups in the peptidic hydrolysate before acylation, and *Nf* is the number of free amino groups in the final product determined by OPA.

Measurements of the Critical Micelle Concentration (CMC)

Surface tension was measured using a GBX-TEN 089 tensiometer equipped with a Wilhelmy plate. Mixtures consisting of lipopeptides and soaps (2.5 g/L) were dissolved in a 0.1 M sodium phosphate buffer solution at pH 7.0. This solution was added to 50 mL of the same phosphate buffer solution. Surface tension was continuously measured and recorded at 20 °C until a constant surface tension value was observed. The CMC was obtained at the breaking point of the surface tension curve in relation to the log of the mixture concentration.

Measurements of Foaming Properties

Foaming capacities were measured using the method described by Padmashree et al. [29]. A 3-g amount of surfactants is mixed with 300 mL of water in a graduated 1-L cylinder. The solution was stirred at 1,600 rpm. The volume was measured 30 s after stirring. The foaming capacity (FC) was expressed as the percentage of volume according to the following formula:

$$\mathrm{FC} = \frac{\text{Volume after stirring} - \text{Volume before stirring}}{\text{Volume before stirring}} \times 100$$

Foaming stability is measured by following the foam volume vs time. The foam volume was recorded at 5, 30 60, 120 and 300 min after stirring. Foaming stability (FS) was calculated using the following formula:

$$\mathrm{FS} = \frac{\text{Foam volume after a time ``}t\text{''}}{\text{Initial foam volume}} \times 100$$

Measurements of Emulsifying Stabilities

Emulsifying stabilities were measured using the method described by Yasumata et al. [30] with adjustments. The surfactants (1.25 g) are homogenized with 50 mL of water during 30 s, with a homogenizer Polyton at 11,000 rpm. The sunflower oil (25 mL) is added to each sample, and the mixture is homogenized for 90 s. The emulsions obtained are divided in an equal way in 3 tubes. The emulsion volume was recorded at 5, 30 60, 120 and 300 min after stirring. Emulsifying stability (ES) was calculated using the following formula:

$$\mathrm{ES} = \frac{\text{Emulsion volume after a time ``}t\text{''}}{\text{Initial emulsion volume}} \times 100$$

Results and Discussion

The Hydrolysis of Pea Protein

The control of enzymatic hydrolysis was related to the mechanism of proteolytic reactions involving a soluble enzyme and an insoluble and soluble substrate (pea protein isolate). Degrees of hydrolysis of the hydrolysates were determined and the kinetics of the reaction were considered of relation to enzyme concentration, reaction time, enzyme addition and the introducing enzyme order in case of double enzyme treatment.

Effect of Enzyme Concentration on the Degree of Hydrolysis (DH)

Figure 1a shows the hydrolysis curve obtained for different enzyme (Alcalase)/substrate ratio. The DH increased with increasing enzyme concentration. A DH up to 14% was observed with a concentration of 0.4 AU/g and no further significant increase was found above this concentration. It is important to note that when the enzyme quantity used is doubled (from 0.2 to 0.4 AU/g), the DH increases only by 2% (from 12 to 14%). When the pea protein was hydrolysed by Flavourzyme, a similar curve was observed and the highest DH was 32% with a concentration of 200 LAPU/g (Fig. 1b). Concerning the achievement of the peptidic hydrolysate with the treatment both enzymes, the values of 0.2 AU/g for Alcalase and 63 LAPU/g for Flavourzyme are selected (Fig. 2) because these ratios E/S represent the minimum quantities of enzymes which allow to obtain a DH near 30%. Indeed, the addition of more Alcalase would have very few effects on the DH. Contrariwise, this addition of a greater quantity of Flavourzyme

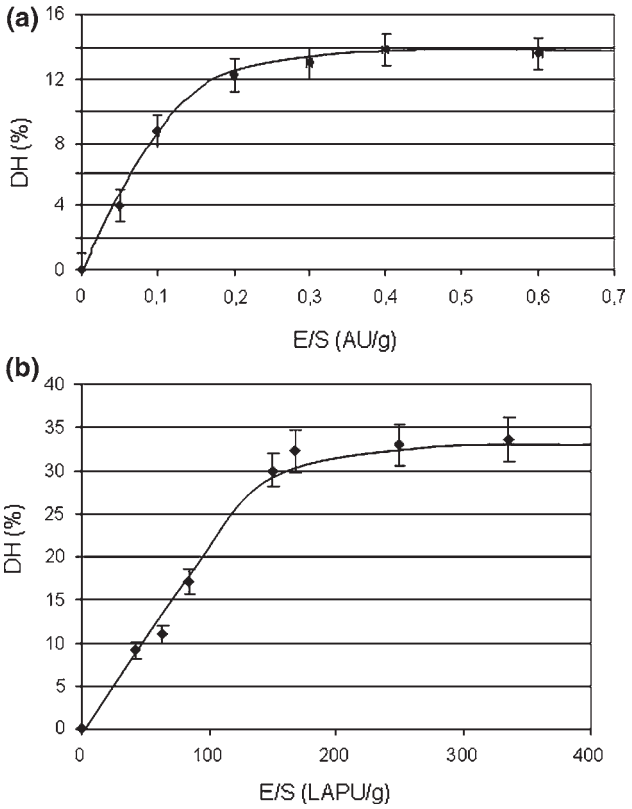

Fig. 1 Hydrolysis of 1 g pea protein isolate **a** by Alcalase at different enzyme/substrate ratios (E/S) over a 30-min period at pH 8 and 50 °C; **b** by Flavourzyme at different enzyme/substrate ratios (E/S) over a 30-min period at pH 7 and 50 °C

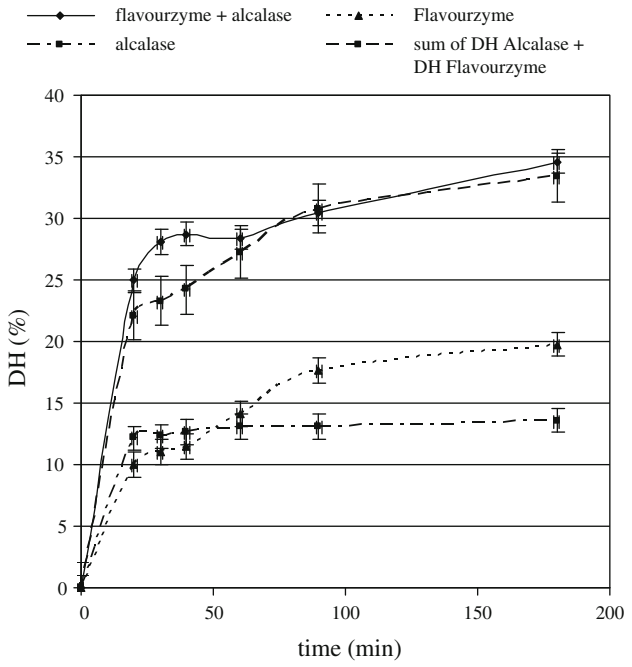

Fig. 2 Hydrolysis of 1 g pea protein isolate with Alcalase (0.2 AU/g) and/or Flavourzyme (63 LAPU/g)

would lead to higher DH, which is not sought after. Indeed, during hydrolysis, Alcalase, which is an endo-peptidase, leads to peptides [26], whereas Flavourzyme leads to peptides by its endo-peptidase activity and to amino acids by its exo-peptidase activity [26, 31]. The aim of this work is to produce peptides with a mean length of 3–4 amino acids (DH $\approx$ 30%). It is better to limit the Flavourzyme quantity used in order to limit the free amino acids quantity present in the hydrolysate, although their presence is not annoying as they exhibit excellent surfactant properties when acylated [24].

Effect of Reaction Time on Protein Hydrolysis

With Alcalase alone, the DH of the pea protein rapidly increases during the 20 first minutes to reach 12% (Fig. 2). The activity is then hugely slowed down: it reaches a maximum DH of 14%. The use of Flavourzyme also permits the rapid increase of the DH during the 20 first minutes: it reaches 10%. From 20 to 100 min, the reaction speed is slowed down but the enzyme activity is maintained all along the experiment: a DH of 20% is reached for an incubation time of 200 min. After 20 min, the exo-peptidase activity of the Flavourzyme is preserved, allowing the DH to keep on increasing [20]. Our results are consistent with previous reports that have shown that Flavourzyme produces protein hydrolysates with DH greater than similar hydrolysates (amino acids) produced with endo-peptidases [13]. Nevertheless, the sample hydrolysed by Flavourzyme alone had the lowest DH (20%), whereas the double enzyme method of protein hydrolysis yielded to 35% degree of hydrolysis after 200 min. Thus, the use of the combination of Alcalase and Flavourzyme yielded to a higher efficiency to hydrolyse pea protein isolate than the use of a single enzyme. Moreover, a synergy is observed during the first hour of hydrolysis (Fig. 2): starting with Alcalase, pre-digestion of the proteins is achieved increasing the number of terminal sites (NH_2 and COOH functions) for the action of Flavourzyme which contains exo-peptidases activities. These results have already been obtained in the case of the hydrolysis of rapeseed protein [32] and defatted soybean flour [18]. In order to obtain a peptidic hydrolysate with a DH close to 30%, a reaction time of 30 min with 63 LAPU/g of Flavourzyme and 0.2 AU/g of Alcalase seems to be convenient.

Effect of the Addition of the Two Enzymes on the Degree of Hydrolysis (DH)

Previous researches have been conducted to examine the effect of the combination of Alcalase and Flavourzyme on various food proteins and two kinds of experimental protein hydrolysis were described [22, 23, 32]. In the first

preparation, the hydrolysates were obtained by treatment with Alcalase followed by Flavourzyme, yielding to DH of 30 ± 2%. In the case of the second hydrolysis process, the samples were simultaneously treated by the two enzymes, the DH is then of 28 ± 2. The hydrolysates gave similar DH values. However, the hydrolysis process with the simultaneous introduction of the two enzymes was preferred due to its shorter reaction time (30 min instead of 60 min) and its easier implementation.

Characterization of Pea Protein Hydrolysates

To study the effect of the combination of Alcalase and Flavourzyme on protein breakdown, SDS–PAGE and GPC of pea protein hydrolysates were performed to obtain information on their molecular weight [7].

SDS–PAGE

Figure 3, lane "h" shows the profile of a pea protein hydrolysate obtained after a treatment by the combination of Alcalase (0.2 AU/g) and Flavourzyme (63 LAPU/g), Lane "m" shows the molecular weight marker. The hydrolysate profile obtained after the double enzyme treatment was mainly composed of low molecular weight proteins in the range of 60–10 kDa. There is no peptide with mass between 10 and 3 kDa. It should be also noted that no native protein hydrolysate which mass varies from 140 to 180 kDa is left. The peptides of mass less than 3 kDa, main components of the hydrolysate studied, are not detected visually on the gel because this technique of separation and coloration of the gel is not adapted to the study of peptides with short chain and weak mass [33]. The peptidic hydrolysate studied is thus devoid of native protein. It is constituted of several polypeptides of mass between 60 and 10 kDa and of a large quantity of small polypeptides of mass less than 3 kDa, hence with a chain length less than 30 amino acids. The mass distribution of these small polyamino acids is studied by size exclusion chromatography.

Size Exclusion Chromatography

The Fig. 4a shows the elution profile of standard peptides which are Cytochrome C (12,500 Da), Aprotinine (6,500 Da), $Glycine_6$ (360 Da), Glycine (75 Da) and respectively represented by 1, 2, 3, 4. From Fig. 4b, it is clear that the pea protein isolate is composed of high molecular weight proteins which might be eluted in the void volume. This corresponds to a material totally excluded from the gel and hence a molecular weight larger than 7,000 Da. The elution profile of peptidic hydrolysate confirms the absence of polypeptides of mass between 10

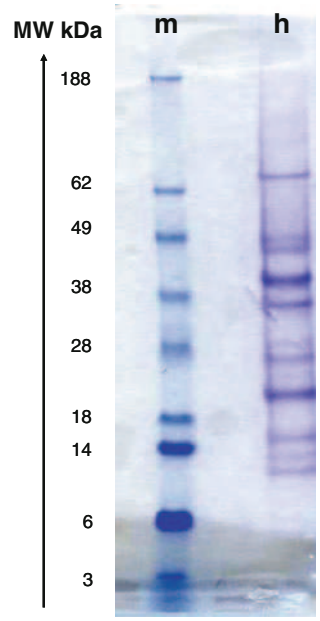

Fig. 3 SDS–PAGE profile of standard (lane 'm') and of a pea protein hydrolysate (lane 'h') after treatment by 0.2 AU/g (Alcalase) and 63 LAPU/g (Flavourzyme)

and 3 kDa. Material coming from the column observed at 7.5 mL of elution (void volume of the column) indicates the presence of several polypeptides of high molecular mass as it is already observed on the electrophoresis gel. The major peptides of the hydrolysate show masses between 800 and 100 Da, with a maximum at 355 Da, this corresponding to small peptides containing 1–7 amino acids, the most abundant being for 3 amino acids. For the masses less than 75 Da, the elution profile of the proteic isolate and the hydrolysate merged. Indeed, the proteic isolate used for this study is constituted with 82% of protein; the by-products being essentially lipids and sugars that do not exhibit absorbance at the wavelength used for this experiment. But, it has been shown that the pea seeds contain significant quantities of polyphenol [34]. These products are likely to be present in the hydrolysate and isolate, and to strongly absorb at 214 nm (wavelength used for the CPG detector). This analysis permitted to show that the peptidic hydrolysate generated by the simultaneous use of Flavourzyme (63 LAPU/g) and Alcalase (0.2 AU/g) is mainly composed of tri-amino acids.

N-acylation of Pea Protein Hydrolysates: Obtention of Anionic Surfactants

From the hydrophilic part previously obtained by hydrolysis, an alkyl chain has to be grafted to form lipopeptide surfactant mixtures. Indeed, if we consider the chemical structure of an amino acid, the fatty chain can be introduced via the amine or carboxylic function. However, the

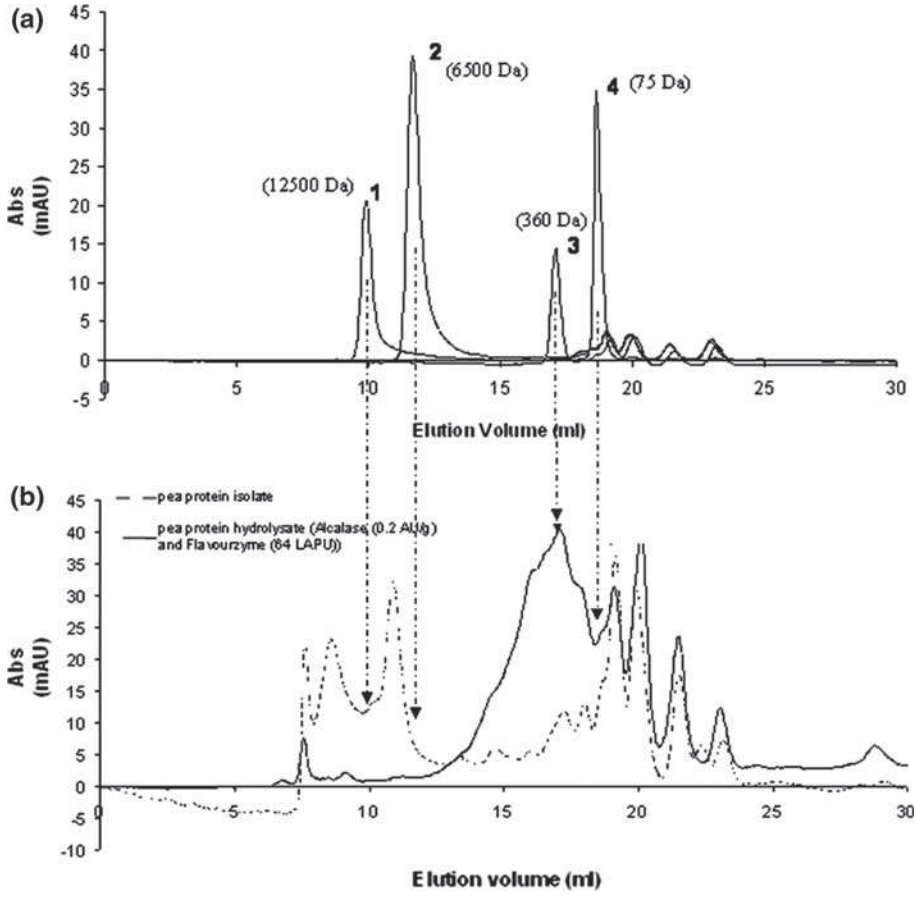

Fig. 4 Gel Permeation Chromatography by size exclusion. **a** Elution profile of standard peptides. **b** Elution profile of pea protein isolate and pea hydrolysate after treatment by Alcalase (0.2 AU/g) and Flavourzyme (63 LAPU/g)

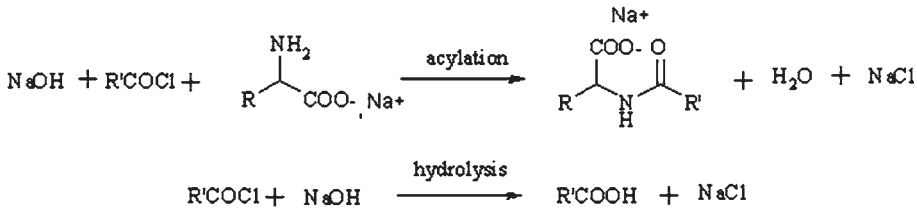

Fig. 5 The hydrolysis of acid chloride in sodium alkanoate

reactivity of the amine function in aqueous medium is widely higher than that of carboxylic acid. The synthesis by *N*-acylation using an acid chloride in water, following the Schotten–Baumann reaction method was chosen because it respects the green chemistry principles (no use of organic solvent, no production of organic waste, no or little heating…). Chains with 12 and 16 carbons were grafted on amino acid and peptide amine functions. The reaction was optimized on amino acids as model molecule [24]. The degree of *N*-acylation of amine functions is determined with amine function assay by OPA method before and after *N*-acylation. An acylation rate of the NH_2 functions of 91 ± 2% is obtained with the dodecanoyl chloride, this rate being at 84 ± 2% with the hexadecanoyl chloride. The slightly higher acylation rate observed with the dodecanoyl chloride can be explained by the higher reactivity of the acid chloride carbon compared to the hexadecanoyl chloride one. This is due to inductive effects of the alkyl chain more important for an aliphatic chain of 15 carbons than for one of 11 carbons. In parallel, it is important to note that the dodecanoyl or hexadecanoyl chlorides introduced in excess were totally hydrolyzed in sodium dodecanaote or sodium hexadecanoate following the reaction Fig. 5. The proportion of acid chloride which was hydrolyzed in fatty acid salt can be estimated from acylation rates mentioned above. In the case of acylation by the dodecanoyl chloride, the surfactant mixtures obtained is constituted from 57% of lipopeptides, 6% of no acylated peptides and 37% of sodium dodecanoate (molar fraction).

Concerning the acylation with the hexadecanoyl chloride, the composition of the mixture is: 51% of lipopeptides, 9% of no acylated peptides and 40% of sodium hexanoate (molar fraction). These fatty acid salts represent

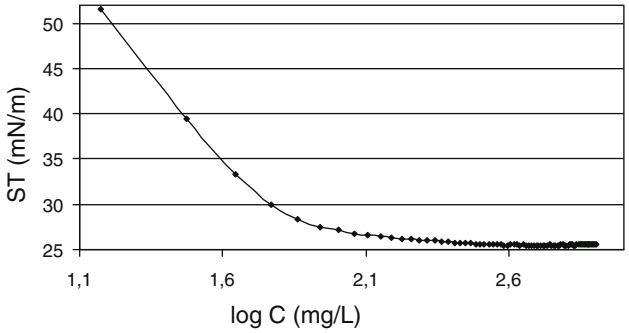

Fig. 6 Surface tension curves of acylated peptides by the dodecanoyl chloride at room temperature

Table 1 Critical micellar concentration and foaming capacities of surfactant mixtures obtained, sodium dodecanoate and sodium hexadecanoate

	CMC (mg/L)	FC (%)
Peptides acylated with C12	75 ± 10	230 ± 5
Peptides acylated with C16	40 ± 10	60 ± 5
Sodium dodecanoate	240 ± 10	230 ± 5
Sodium hexadecanoate	115 ± 10	70 ± 5

anionic co-surfactants. They are maintained in the mixtures and contribute to their surfactant properties.

Surfactant Properties of Amphiphilic Molecule Mixtures

Critical Micellar Concentration

A linear decrease in surface tension is observed when the concentration of the mixture is increased for all surfactants, up to the CMC, beyond which there is no observable change in surface tension. This behavior is common for surfactants in solution. The CMC values of our different mixtures were measured.

The protein isolate and proteins hydrolysates have not good surfactant properties (CMC > 2 g/L). Indeed, the peptides do not have amphiphilic structures, which are necessary for a compound to decrease the surface tension at interface. On the other hand, after acylation, a linear decrease in surface tension is observed when the concentration of the lipopeptide-based mixtures is increased. As an example, the behaviour of peptides acylated by the dodecanoyl chloride is described in Fig. 6. The CMC values of lipopeptide-based mixtures are given in Table 1. These CMC are low therefore showing that the mixtures of lipopeptides and fatty acid salts own excellent surfactant properties. Besides, the CMC of sodium dodecanoate and hexadecanoate are a bit more higher than the corresponding lipopeptides, but they also testify of a quite good surfactant

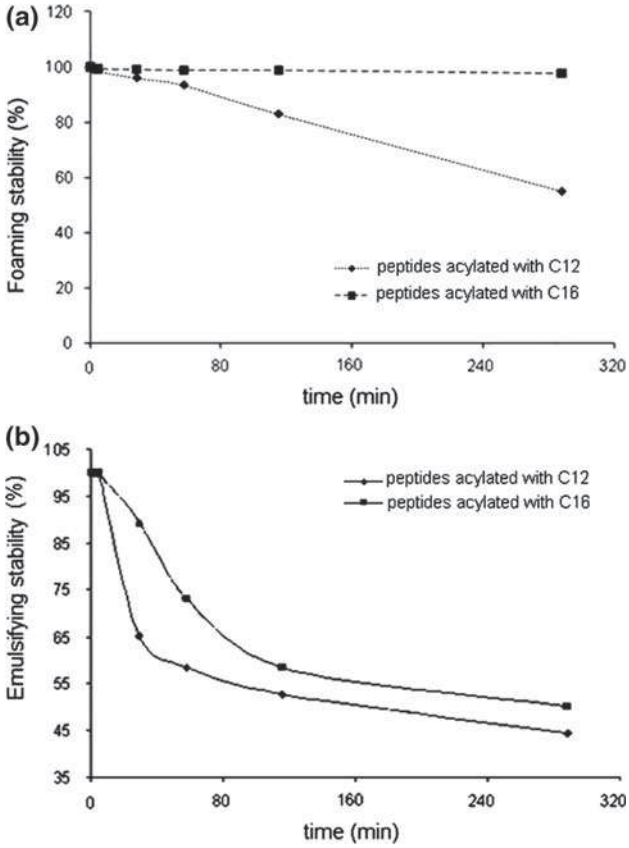

Fig. 7 Foaming and emulsifying properties: **a** foaming stability of peptide-based surfactant mixtures; **b** emulsifying stability of peptide-based surfactant mixtures

properties. This shows that the fatty acid salts are co-surfactants that contribute to the mixture properties [24]. The C16-lipopeptide-based mixtures exhibit a better CMC than their equivalent in C12. In fact, a longer, more hydrophobic chain results in a lower surface tension. This is attributed to the increase of the affinity of lipophilic molecules for interfaces [35].

Foaming Properties

The protein isolate has a weak foaming capacity (FC = 25%). After hydrolysis of pea proteins, peptides do not longer produce foam under stirring. Due to their small length, the hydrophobic and hydrophilic parts cannot be distinguished in the peptide as it happens in proteins [36]. As a consequence, they do not have an amphiphilic character and do not adsorb at the water/air interface. After acylation, the lipopeptides have a foaming capacity similar to commercial surfactants one [24]. The produced quantity of foam varies versus the alkyl chain length; shorter is the lipophilic chain, higher is the foaming capacity. Although possessing a low foaming capacity, the C16-alkyl chain mixtures form a foam presenting a high stability (Fig. 7a).

Indeed, it has been shown that the mixture possesses a better capacity in decreasing the surface tension in the interfaces compared to its equivalent in C12, thus permitting a better stabilization of this unstable thermodynamic system.

Emulsifying Stabilities

Emulsions were formed by *N*-acylated peptide-based formulation. The emulsifying stability against coalescence was followed by the phase separation after stirring and is given Fig. 7b. Concerning the *N*-acylated peptide formulations, the stability of emulsions was improved by a longer hydrophobic chain. These formulations were therefore very effective in generating stable emulsions. Indeed, after 2 h, only 25% of the emulsion were broken. As for the foams, this stabilization results from the low surface tension at the water/oil interface of the emulsion. These results are in agreement with the observations made by Sanchez-Vioque et al. on *N*-acylated rapeseed peptides [35].

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